



Uniwersytet
Gdański



Wydział Chemii Uniwersytetu Gdańskiego

mgr Ola Grabowska

**TERMODYNAMICZNE I STRUKTURALNE
ASPEKTY ODDZIAŁYWAŃ
ZWIĄZKÓW MAŁOCZĄSTECZKOWYCH
Z ALBUMINĄ I LIZOZYMEM**

Promotor:

dr hab. Dariusz Wyrzykowski, prof. UG

Praca doktorska wykonana w Katedrze Chemii Ogólnej i Nieorganicznej

Gdańsk 2026

*Pragnę serdecznie podziękować mojemu Promotorowi,
dr. hab. Dariuszowi Wyrzykowskiemu, prof. UG,
za nieustanne wsparcie, wyrozumiałość oraz nieocenioną
pomoc merytoryczną. Dziękuję za inspirację, zaangażowanie,
cenne wskazówki oraz poświęcony czas, które miały istotne
znaczenie na wszystkich etapach prowadzonych badań
i podczas przygotowywania niniejszej rozprawy.*

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Streszczenie

Oddziaływania między związkami małowcząsteczkowymi (ligandami) a białkami stanowią fundament praktycznie wszystkich procesów biologicznych, odgrywając kluczową rolę w prawidłowym funkcjonowaniu organizmów żywych. Zdolność liganda do rozpoznania i związania się z białkiem zależy zarówno od jego właściwości fizykochemicznych, wynikających ze specyficznej struktury, jak i od czynników środowiskowych, takich jak pH, temperatura czy obecność konkurencyjnych ligandów. Zrozumienie, jak i dlaczego dochodzi do tych interakcji, wymaga równoczesnego poznania ich aspektów termodynamicznych oraz wpływu, jaki wywierają na strukturę białka.

Wyjaśnienie mechanizmów oddziaływań ligand-białko na poziomie molekularnym wykracza zatem poza podstawowe badania naukowe. Dostarcza bowiem informacji niezbędnych do racjonalnego projektowania nowych związków o pożądanych właściwościach. Znaczenie tej wiedzy jest szczególnie widoczne w takich dziedzinach jak chemia bioorganiczna, farmacja czy biotechnologia, gdzie umożliwia przewidywanie zachowania cząsteczek w złożonych układach biologicznych, a w konsekwencji świadome projektowanie nowych związków biologicznie aktywnych.

Celem niniejszej rozprawy doktorskiej było określenie kluczowych cech strukturalnych i fizykochemicznych ligandów, które decydują o sile ich wiązania z białkiem, mechanizmie i lokalizacji tych oddziaływań. Jako obiekty badawcze wybrano dwa komplementarne, a zarazem kontrastujące układy modelowe: albuminy, pełniące kluczową rolę w transporcie związków endo- i egzogennych, oraz lizozym, enzym charakteryzujący się odmienną strukturą i ładunkiem powierzchniowym. Przeanalizowano kilka klas związków różniących się budową chemiczną, wielkością ładunku oraz właściwościami fizykochemicznymi: anionowe surfaktanty (1-alkilosiarczany i 1-alkilosulfoniany) o zróżnicowanej długości łańcucha węglowodorowego, jony tetrafenyloboranowe, polioksowanadanowe oraz jony heksacyjanożelazianowe(II)/(III). Wybrane ligandy, choć nie są lekami *per se*, swoją strukturą nawiązują do różnych motywów obecnych w związkach o istotnym znaczeniu biologicznym. Pozwoliło to na przeanalizowanie kluczowych cech decydujących o sposobie oddziaływania tych ligandów z białkami.

W badaniach zastosowano izotermiczne miareczkowanie kalorymetryczne oraz szereg wzajemnie uzupełniających się technik eksperymentalnych, spektroskopowych i elektrochemicznych, które umożliwiły opis badanych oddziaływań na poziomie molekularnym oraz dostarczyły informacji o zmianach strukturalnych białek wywołanych czynnikami fizycznymi i chemicznymi. Wyniki eksperymentalne uzupełniono metodami *in silico*, co pozwoliło na uzyskanie dodatkowego wglądu w charakter oddziaływań oraz identyfikację potencjalnych miejsc wiążących w białkach. Zastosowane w badaniach multidyscyplinarne podejście umożliwiło systematyczną analizę wpływu kluczowych parametrów strukturalnych ligandów na mechanizm i siłę ich oddziaływań z białkami, a także sformułowanie ogólnych zasad określających, w jaki sposób struktura i właściwości fizykochemiczne ligandów kształtują ich powinowactwo do białek.

W toku badań udokumentowano, że kluczową rolę w tym procesie odgrywają domenowa budowa oraz plastyczność białek. To właśnie te cechy pozwalają na selektywne wiązanie ligandów o zróżnicowanych właściwościach, co stanowi podstawę ich funkcji biologicznych. Przeprowadzone badania pokazują, że to nie pojedyncza cecha liganda, lecz synergiczne oddziaływanie jego rozmiaru, ładunku i charakteru hydrofilowo-hydrofobowego decyduje o mechanizmie wiązania: od interakcji elektrostatycznych, przez efekty mieszane (elektrostatyczne i hydrofobowe), aż po głównie hydrofobowe.

Takie kompleksowe spojrzenie pozwala nie tylko wyjaśnić obserwowane zjawiska na poziomie molekularnym, ale także stanowi podstawę do przewidywania zachowania związków w układach biologicznych oraz racjonalnego projektowania nowych substancji o pożądanych właściwościach.

Summary

Interactions between small-molecule compounds (ligands) and proteins constitute the foundation of nearly all biological processes and are essential for the proper functioning of living organisms. The ability of a ligand to recognize and bind to a protein depends not only on its physicochemical properties, derived from its specific structure, but also on environmental factors such as pH, temperature, and the presence of competing ligands. Therefore, a thorough understanding of how and why these interactions occur requires a comprehensive analysis of both their thermodynamic properties and their effects on protein structure.

Therefore, elucidating the mechanisms of ligand-protein interactions at the molecular level goes beyond fundamental research. It provides essential insights for the rational design of new compounds with desired properties. This knowledge is particularly valuable in fields such as bioorganic chemistry, pharmacy, and biotechnology. It enables the prediction of molecular behaviour in complex biological systems and supports the design of biologically active compounds.

This PhD thesis aimed to identify the key structural and physicochemical features of ligands that determine the strength of their binding to proteins, as well as the mechanism and location of these interactions. Two complementary yet contrasting model systems were selected: albumins, which play a key role in the transport of endogenous and exogenous compounds, and lysozyme, an enzyme characterized by a different structure and surface charge distribution. Several classes of compounds differing in chemical structure, charge, and physicochemical properties were analyzed, including anionic surfactants (1-alkylsulfates and 1-alkylsulfonates) with varying hydrocarbon chain lengths, tetraphenylborate ions, polyoxovanadates, and hexacyanoferrate(II)/(III) ions. Although the selected ligands are not drugs *per se*, their structures resemble motifs commonly found in biologically relevant compounds, enabling the identification of key features that govern their interactions with proteins.

The study employed isothermal titration calorimetry along with a range of complementary experimental, spectroscopic, and electrochemical techniques. This combination enabled the characterization of ligand-protein interactions at the molecular level and provided insight

into protein structural changes induced by physical and chemical factors. The experimental results were further supported by *in silico* methods, which offered additional understanding of the nature of these interactions and allowed for the identification of potential binding sites on the proteins. This multidisciplinary approach allowed for a systematic analysis of how key structural parameters of ligands influence the mechanism and strength of their interactions with proteins, as well as for the formulation of general principles describing how ligand structure and physicochemical properties govern protein affinity.

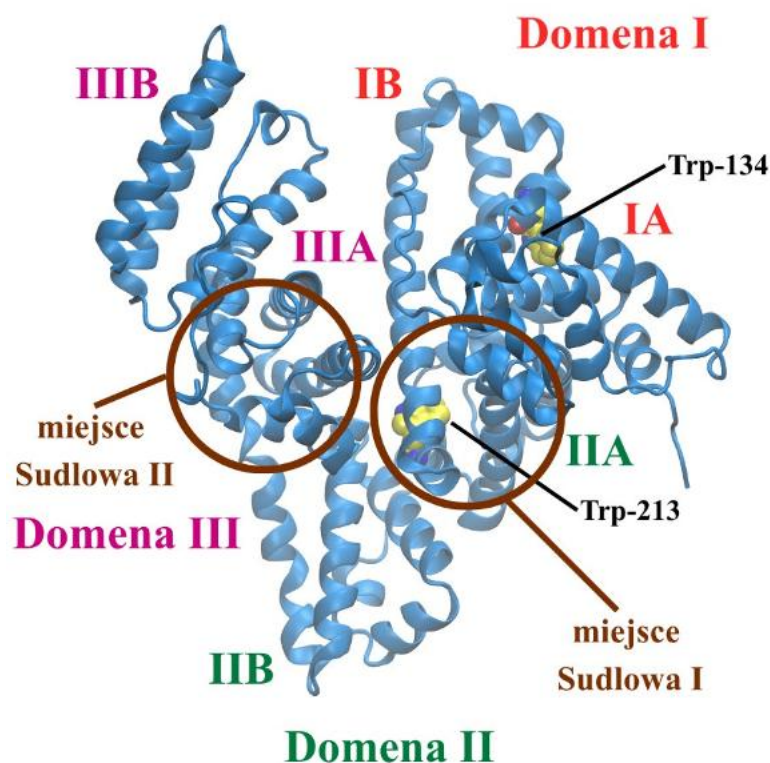
The results demonstrate that protein domain organization and structural flexibility play a key role in this process. These features enable the selective binding of ligands with diverse properties, forming the basis of protein biological functions. The results show that it is not a single ligand feature, but rather the synergistic interplay of size, charge, and hydrophilic-hydrophobic character that determines the binding mechanism, ranging from electrostatic interactions, through mixed (electrostatic and hydrophobic) effects, to predominantly hydrophobic interactions.

This comprehensive overview of the interactions between proteins and small-molecule ligands explains the observed phenomena at the molecular level and can serve as a basis for predicting the behavior of such compounds in biological systems, as well as for the rational design of new substances with desired properties.

1. Wprowadzenie

1.1 Albuminy (BSA, HSA) oraz lizozym – białka modelowe w badaniach reakcji z ligandami małowcząsteczkowymi

Albuminy, stanowiące największy odsetek białek transportowych w surowicy krwi, pełnią kluczową rolę w wiązaniu i dystrybucji różnorodnych substancji [1-10]. Do najszerzej badanych przedstawicieli tej grupy należą albumina surowicy bydlęcej (BSA) oraz albumina surowicy ludzkiej (HSA) [11, 12]. Oba białka wykazują wysoki stopień homologii sekwencyjnej (ok. 76%) oraz bardzo podobną budowę przestrzenną, charakteryzującą się podziałem na trzy homologiczne domeny (I, II, III), z których każda składa się z dwóch subdomen: A i B (Rysunek 1) [13-20]. Ta modułowa budowa determinuje powstawanie wyspecjalizowanych, hydrofobowych wnęk stanowiących miejsca wiązania ligandów. Głównymi miejscami odpowiedzialnymi za selektywne wiązanie i transport związków endo- i egzogennych są dwie hydrofobowe kieszenie zlokalizowane odpowiednio w subdomenach IIA (miejsce Sudlowa I) i IIIA (miejsce Sudlowa II) (Rysunek 1) [21-24]. Różnice w budowie przestrzennej i chemicznej tych miejsc decydują o ich selektywności (Tabela 1). Każde z tych miejsc wykazuje wyraźną selektywność wobec określonych klas ligandów, które służą jako swoiste markery. Miejsce Sudlowa I preferencyjnie wiąże takie związki jak warfaryna, fenylobutazon, salicylany czy diklofenak [25-29]. Z kolei w miejscu Sudlowa II wiążą się ligandy, takie jak ibuprofen, ketoprofen, diazepam czy benzodiazepiny [25-29]. Ta specyficzność umożliwia albuminom wiązanie ligandów o zróżnicowanych właściwościach fizykochemicznych oraz ich efektywny transport w krwioobiegu.



Rysunek 1. Struktura przestrzenna BSA z zaznaczonymi odpowiednio domenami (I, II, III), subdomenami (A, B), miejscami Sudlowa (I, II) oraz resztami tryptofanu (Trp-134, Trp-213).

Albuminy są powszechnie wykorzystywane jako białka modelowe w badaniach oddziaływań z ligandami małocząsteczkowymi [30-38]. Określenie ich powinowactwa do albumin ma fundamentalne znaczenie na etapie projektowania nowych związków terapeutycznych. Parametr ten decyduje o ich biodystrybucji, profilu farmakokinetycznym oraz ryzyku interakcji z innymi substancjami [39]. Badania te są również niezwykle istotne z praktycznego punktu widzenia, szczególnie w analizach *in vitro*. W testach biologicznych z wykorzystaniem linii komórkowych standardowe pożywki hodowlane zawierają zazwyczaj wysokie stężenie BSA, około 0,040 mM [40]. Substancja badana może konkurować z naturalnymi składnikami pożywki o miejsca wiązania na albuminie, co istotnie modyfikuje stężenie jej wolnej, biologicznie aktywnej frakcji w układzie. W efekcie, pominięcie tej interakcji może prowadzić do błędnej oceny wyników aktywności lub toksyczności.

Tabela 1. Różnice między subdomeną IIA (miejsce Sudlowa I) a subdomeną IIIA (miejsce Sudlowa II).

Kryterium	Subdomena IIA (miejsce Sudlowa I)	Subdomena IIIA (miejsce Sudlowa II)
Lokalizacja	▪ w domenie II (subdomena A) [41, 42]	▪ w domenie III (subdomena A) [41, 42]
Rozmiar i kształt wnęki	▪ większa, bardziej elastyczna [43, 44]	▪ mniejsza, węższa, bardziej sztywna [43, 44]
Charakter chemiczny	▪ hydrofobowa, z resztami naładowanymi dodatnio (np. Arg-257, Lys-199) [45-47]	▪ hydrofobowa, z resztami zdolnymi do tworzenia wiązań wodorowych (np. Tyr-411) [45-47]
Preferowana klasa ligandów	▪ duże, płaskie, często wielopierścieniowe cząsteczki o charakterze kwasowym [48, 49]	▪ mniejsze, zwarte cząsteczki, często z grupami karboksylowymi lub aromatycznymi [48, 49]
Wpływ wiązania na strukturę białka	▪ często wywołuje zmiany konformacyjne w całej cząsteczce [50]	▪ zazwyczaj powoduje mniejsze zmiany strukturalne [50]

Ponadto, badania oddziaływań wybranych ligandów małowcząsteczkowych zostały uzupełnione o ich interakcje z lizozymem. Włączenie lizozymu do badań tworzy komplementarny, kontrastujący układ modelowy. Pozwala on na weryfikację, czy obserwowane prawidłowości (np. wpływ długości łańcucha, wielkość ładunku) są uniwersalne, czy specyficzne dla typu białka. Umożliwia to pełniejszą charakterystykę oddziaływań liganda z białkiem, począwszy od specyficznych wiązań z białkami transportowymi, takimi jak albuminy, aż po niespecyficzne interakcje powierzchniowe, które w przypadku lizozymu są determinowane głównie siłami elektrostatycznymi (Tabela 2).

Tabela 2. Lizozym – komplementarne białko modelowe w badaniach oddziaływań z ligandami.

<i>Kryterium porównawcze</i>	<i>Albumina (BSA/HSA)</i>	<i>Lizozym</i>	<i>Charakter badań</i>
<i>Ładunek całkowity (pH = 7)</i>	▪ ujemny: BSA (−17), HSA (−15) [51, 52]	▪ dodatni (+8) [53, 54]	Pozwala na badanie czystego wpływu elektrostatyki na wiązanie anionowych ligandów. Ujawnia decydującą rolę ładunku liganda (np. $[\text{Fe}(\text{CN})_6]^{4-}$ wiąże się silniej niż $[\text{Fe}(\text{CN})_6]^{3-}$), co nie zawsze jest możliwe w przypadku albumin.
<i>Struktura i plastyczność</i>	▪ wysoko elastyczna, plastyczna [55, 56] ▪ rozległe, płytke wnęki [57]	▪ sztywna, zwarta [58] ▪ głębokie, specyficzne centrum aktywne [59]	Umożliwia ocenę wpływu sztywności konformacyjnej białka na zdolność wiązania i adaptację do ligandów o różnym rozmiarze/kształcie.
<i>Charakter miejsc wiązania</i>	▪ specyficzne, zdefiniowane wnęki o mieszanym charakterze (Sudlow I/II) [60, 61]	▪ powierzchniowe regiony bogate w reszty Arg/Lys [62] ▪ brak analogicznych głębokich wnęk	Pozwala rozróżnić wiązanie do specyficznej wnęki (albuminy) od niespecyficznego wiązania powierzchniowego sterowanego elektrostatyką (lizozym).
<i>Główna funkcja biologiczna</i>	▪ białko transportowe [63, 64]	▪ enzym katalityczny (hydroliza peptydoglikanu) [65, 66]	Umożliwia zbadanie funkcjonalnych konsekwencji wiązania – czy ligand jest inhibitorem, czy nie zaburza aktywności (testy biochemiczne).
<i>Wrażliwość na czynniki denaturujące</i>	▪ stosunkowo odporna na denaturację przez surfaktanty	▪ wysoko wrażliwy na substancje denaturujące (np. K12S)	Stanowi czuły model do badania mechanizmów denaturacji białek przez amfifilowe ligandy i skuteczności ochrony (np. przez β -cyklodekstrynę).

1.2 Badane ligandy małowcząsteczkowe

W kontekście roli albumin w transporcie oraz lizozymu stanowiącego komplementarny, kontrastujący układ modelowy, przebadane zostały wybrane klasy związków, różniących się budową chemiczną oraz właściwościami fizykochemicznymi (Tabela 3).

Tabela 3. Wybrane klasy związków pełniących rolę ligandów małowcząsteczkowych w oddziaływaniach z albuminą.

Klasa związków	Uzasadnienie badań w kontekście oddziaływań z albuminą
1-alkilosiarczany (SXS)/ 1-alkilosulfoniany (KXS) (o różnej długości łańcucha węglowodorowego, X = C8-, C10- oraz C12-) [Publikacja: P1, P4, P5]	1. Praktyczne znaczenie: Są to główne składniki detergentów, środków czystości i kosmetyków, z którymi organizm ma stały kontakt (naskórnice, inhalacyjnie) [67-69]. 2. Aktywność biologiczna: Mogą działać denaturująco na białka, zaburzając ich funkcję. Zbadanie, czy i jak wiążą się z albuminą jest istotne dla oceny ich potencjalnej toksyczności oraz zrozumienia ich wpływu na stabilność i funkcję białek osocza. 3. Mechanizm interakcji: Ich amfifilowy charakter (hydrofobowy łańcuch i część hydrofilowa) pozwala badać konkurencję z endogennymi ligandami (jak kwasy tłuszczowe) i określić, czy ich wiązanie jest specyficzne, czy tworzą niespecyficzne kompleksy.
Jony tetrafenyloboranowe, [B(Ph)₄]⁻ [Publikacja: P2]	1. Analogia i model: Ich stosunkowo duże ugrupowania fenyłowe, rozmieszczone w sposób tetraedryczny wokół atomu boru, czynią je doskonałym modelem hydrofobowych anionów organicznych. 2. Selektywność wiązania: Badanie ich oddziaływania z albuminą pozwala określić powinowactwo białka do dużych, hydrofobowych anionów, które nie są typowymi ligandami miejsc Sudlowa, a mogą konkurować o inne, słabiej scharakteryzowane miejsca wiązania.

Klasa związków	Uzasadnienie badań w kontekście oddziaływań z albuminą
<i>Polioksowanadany (tetramery, dekamery)</i> <i>[Publikacja: P3]</i>	1. Znaczenie biologiczne: Transport związków wanadu we krwi (głównie przez albuminę i transferynę) decyduje o dystrybucji, akumulacji i działaniu [70-72]. 2. Mechanizm interakcji: Związki wanadu wykazują tendencję do oligomeryzacji prowadzącej w zależności od pH środowiska oraz stężenia do powstania di-, tetra-, penta- oraz dekamerów różniących się między sobą zawartością wanadu(V) oraz ładunkiem. Te własności decydują o ich powinowactwie do białek [73, 74]. 3. Badania biologiczne: Wiązanie związków wanadu przez albuminę obecną w pokarmach stosowanych w hodowlach komórkowych to złożony proces, mogący wpływać na aktywność biologiczną testowanego związku.
<i>Jony heksacyjanożelazianowe(II)/(III), $[Fe(CN)_6]^{4-/3-}$</i> <i>[Publikacja: P9]</i>	1. Model anionów nieorganicznych: Reprezentują klasę trwałych termodynamicznie i kinetycznie jonów kompleksowych, obdarzonych wysokim ładunkiem ujemnym, o charakterze hydrofilowym oraz symetrycznej, oktaedrycznej strukturze [75, 76]. 2. Test dla modeli wiązania: Ich oddziaływanie z albuminą służy do weryfikacji modeli opisujących wiązanie jonów przez białko. Ich obecność w układzie może wpływać na powinowactwo albumin do innych, słabiej wiążących się ligandów (np. leków), co w konsekwencji może wpływać na biodostępność i skuteczność terapeutyczną.

1.3 Izotermiczna kalorymetria miareczkowa (ITC)

Większość reakcji zachodzących w przyrodzie to reakcje równowagowe, co czyni je przedmiotem intensywnych badań w dziedzinach takich jak chemia, biologia molekularna czy biotechnologia [77]. Zrozumienie tych procesów ma fundamentalne znaczenie zarówno dla poznania ich mechanizmów, jak i praktycznego zastosowania, na przykład w projektowaniu leków, rozwoju biotechnologii czy inżynierii procesowej [78]. Do badania reakcji równowagowych w roztworach dostępnych jest obecnie wiele technik pomiarowych, a wybór odpowiedniej metody zależy głównie od właściwości fizykochemicznych analizowanych substancji. Tabela 4 zestawia najczęściej stosowane metody analizy równowag reakcji chemicznych, wskazując dla każdej z nich charakterystyczną właściwość związku, która pełni rolę wskaźnika monitorującego postęp reakcji.

Tabela 4. Zestawienie metod pomiarowych stosowanych do badania reakcji równowagowych zachodzących w roztworach.

<i>Metoda pomiarowa</i>	<i>Wskaźnik postępu reakcji</i>	<i>Wymagana właściwość związku</i>
<i>Izotermiczna kalorymetria miareczkowa (ITC)</i>	zmiana ciepła układu [79, 80]	brak wymagań strukturalnych
<i>Spektroskopia fluorescencyjna (SF)</i>	zmiana intensywności fluorescencji [81, 82]	obecność ugrupowania fluoroforowego, znakowanie fluoroforem
<i>Spektrofotometria UV-Vis</i>	zmiana absorpcji światła [83, 84]	obecność ugrupowania chromoforowego
<i>Spektroskopia dichroizmu kołowego (CD)</i>	zmiana absorpcji światła spolaryzowanego kołowo [85, 86]	obecność chiralności, struktury drugorzędowej
<i>Potencjometria</i>	zmiana potencjału elektrody [87, 88]	zdolność do wymiany (dysocjacji/asocjacji) protonu
<i>Konduktometria</i>	zmiana przewodnictwa roztworu [89, 90]	dotyczy elektrolitów
<i>SwitchSense</i>	zmiana intensywności fluorescencji [91, 92]	zdolność do immobilizacji na powierzchni sensora
<i>Powierzchniowy rezonans plazmonowy (SPR)</i>	zmiana współczynnika załamania światła [93, 94]	zdolność do immobilizacji na powierzchni sensora
<i>Termoforeza mikroskalowa (MST)</i>	zmiana intensywności fluorescencji zależnej od temperatury [95, 96]	obecność ugrupowania fluoroforowego, znakowanie fluoroforem
<i>Spektroskopia magnetycznego rezonansu jądrowego (NMR)</i>	zmiana sygnałów chemicznych [97, 98]	obecność jąder magnetycznie aktywnych, rozpuszczalność w rozpuszczalniku deuterowanym

Do analizy badanych w niniejszej pracy równowag zastosowano jako główne narzędzie technikę miareczkowania kalorymetrycznego (z ang. *Isothermal Titration Calorimetry*, w skrócie ITC). ITC jest techniką eksperymentalną służącą do badania efektów energetycznych reakcji zachodzących w roztworach w stałych warunkach temperatury i ciśnienia [99-102]. W przeciwieństwie do wielu innych metod analitycznych, ITC nie wymaga od badanych związków specyficznych właściwości strukturalnych (np. obecności chromo- lub fluoroforów, chiralności, zdolności do dysocjacji), co czyni ją metodą uniwersalną [103]. Dzięki temu może być stosowana jako samodzielna technika lub uzupełnienie innych metod (np. spektrofotometrii, spektroskopii dichroizmu kołowego), szczególnie gdy związki nie spełniają wymagań narzucanych przez inne techniki (Tabela 4). ITC wyróżnia się brakiem takich ograniczeń, ponieważ miarą postępu reakcji jest zmiana ciepła układu, co pozwala na badanie niemal każdej reakcji, której towarzyszy efekt energetyczny.

Metoda ITC dostarcza bezpośrednich danych eksperymentalnych, takich jak:

- Stała trwałości kompleksu (K) – informuje o trwałości termodynamicznej.
- Zmiana entalpii (ΔH) – określa charakter energetyczny reakcji (egzo- lub endotermiczny).
- Stechiometria (N) – wskazuje stosunek molowy reagentów w powstającym kompleksie [104].

Znając wartości K oraz ΔH , można obliczyć pozostałe funkcje stanu, tj. zmianę energii swobodnej (ΔG) oraz zmianę entropii (ΔS), korzystając z podstawowej zależności termodynamicznej [104]:

$$\Delta G = -RT \ln K = \Delta H - T\Delta S$$

gdzie: R to stała gazowa; T to temperatura pomiaru.

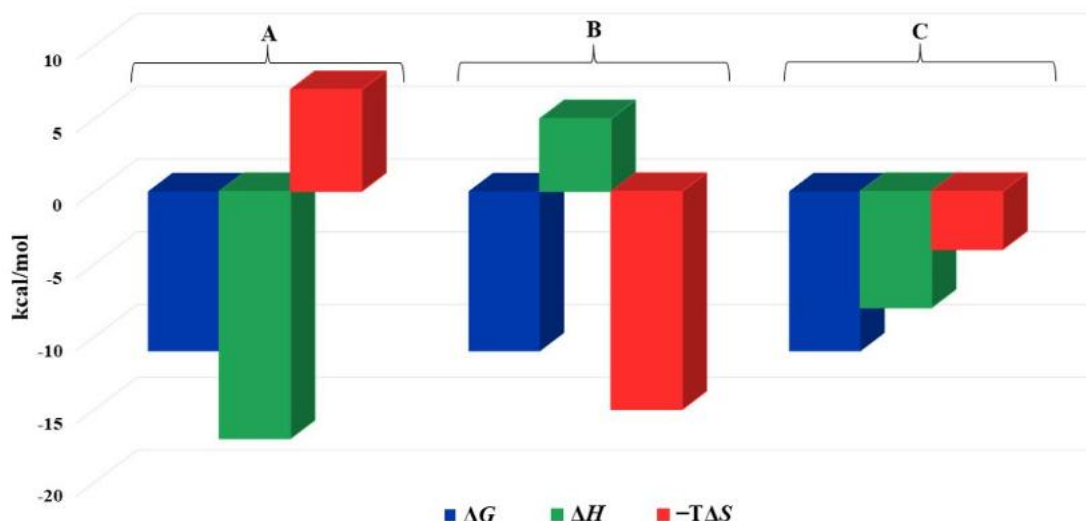
Wartość stałej trwałości (K) odzwierciedla termodynamiczną trwałość kompleksu. Pozwala ona na bezpośrednie porównanie stabilności różnych kompleksów. Stała K zależy od temperatury, ale nie zależy od stężeń reagentów. Analiza parametrów termodynamicznych, takich jak zmiana entalpii (ΔH) i zmiana entropii (ΔS), dostarcza natomiast informacji o charakterze procesu oraz o typie oddziaływań stabilizujących

powstający kompleks. Dzięki temu możliwe jest rozróżnienie, czy trwałość wynika głównie z efektów energetycznych (czynnik entalpowy), czy też ze wzrostu nieuporządkowania układu (czynnik entropowy) [105]. W Tabeli 5 zestawiono wpływ znaków ΔH i ΔS na samorzutność i kierunek przebiegu reakcji chemicznej.

Tabela 5. Wpływ wartości parametrów termodynamicznych na przebieg reakcji chemicznej.

<i>Wartość ΔH</i>	<i>Wartość ΔS</i>	<i>Mechanizm reakcji chemicznej</i>
ujemna (-)	dodatnia (+)	▪ proces samorzutny ($\Delta G < 0$) ▪ korzystny czynnik entalpowy ($\Delta H < 0$) ▪ korzystny czynnik entropowy ($\Delta S > 0$)
ujemna (-)	ujemna (-)	▪ proces samorzutny przy niskich temperaturach ▪ korzystny czynnik entalpowy ($\Delta H < 0$) ▪ niekorzystny czynnik entropowy ($\Delta S < 0$)
dodatnia (+)	dodatnia (+)	▪ proces samorzutny przy wysokich temperaturach ▪ niekorzystny czynnik entalpowy ($\Delta H > 0$) ▪ korzystny czynnik entropowy ($\Delta S > 0$)
dodatnia (+)	ujemna (-)	▪ proces wymuszony ($\Delta G > 0$) ▪ niekorzystny czynnik entalpowy ($\Delta H > 0$) ▪ niekorzystny czynnik entropowy ($\Delta S < 0$)

Analiza wartości funkcji termodynamicznych (ΔH , ΔS i ΔG) dostarcza informacji o mechanizmie reakcji kompleksowania, wykraczających poza samą trwałość termodynamiczną wyrażoną przez stałą równowagi (K) [105]. Można to zilustrować na przykładzie trzech hipotetycznych kompleksów (A, B i C) o identycznej wartości zmiany energii swobodnej (ΔG), a co za tym idzie, o tej samej stałej trwałości (Rysunek 2). Mimo jednakowego ΔG , mechanizmy powstawania tych kompleksów są zasadniczo różne, co ujawniają wartości ΔH i ΔS .



Rysunek 2. Wykres wartości funkcji termodynamicznych (ΔG , ΔH , $-T\Delta S$) trzech związków kompleksowych: A, B oraz C.

1. Kompleks A ($\Delta H < 0$, $\Delta S < 0$): Ujemna wartość ΔH wskazuje na reakcję egzotermiczną, której przebieg jest uwarunkowany wydzielaniem ciepła. Ujemna wartość ΔS świadczy natomiast o spadku entropii układu, co jest zazwyczaj niekorzystne dla samorzutności procesu. W tym przypadku korzystny (ujemny) czynnik entalpowy kompensuje niekorzystną zmianę entropii, prowadząc do ujemnego ΔG . W takim przypadku trwałość kompleksu A jest determinowana głównie przez czynnik entalpowy, co sugeruje dominującą rolę bezpośrednich oddziaływań międzycząsteczkowych, takich jak wiązania wodorowe, oddziaływania elektrostatyczne oraz oddziaływania van der Waalsa.
2. Kompleks B ($\Delta H > 0$, $\Delta S > 0$): Dodatnia wartość ΔH oznacza reakcję endotermiczną, której towarzyszy pochłonięcie energii z otoczenia. Proces ten staje się jednak samorzutny dzięki dużej, dodatniej zmianie entropii ($\Delta S > 0$). W tym przypadku korzystny czynnik entropowy kompensuje niekorzystny efekt endotermiczny. O trwałości kompleksu B decyduje przede wszystkim czynnik entropowy. Jest to charakterystyczne dla procesów, w których kluczową rolę odgrywają oddziaływania hydrofobowe. Powstanie kompleksu uwalnia uporządkowane cząsteczki wody z hydratacyjnych powłok reagentów, co prowadzi do znacznego wzrostu nieuporządkowania w układzie ($\Delta S > 0$).
3. Kompleks C ($\Delta H < 0$, $\Delta S > 0$): W tym przypadku zarówno zmiana entalpii, jak i entropii jest korzystna ($\Delta H < 0$ i $\Delta S > 0$). Oba czynniki synergistycznie przyczyniają się

do ujemnej wartości ΔG . Na trwałość kompleksu C wpływają zarówno oddziaływania bezpośrednie (entalpowe), jak i efekt entropowy, najczęściej związany z oddziaływaniami hydrofobowymi. Takie połączenie jest często obserwowane w wysoce specyficznych i stabilnych kompleksach.

Wartość ΔS dostarcza również informacji o zmianach konformacyjnych. Ujemna ΔS (jak w kompleksie A) wskazuje na zwiększenie uporządkowania, co może wynikać z usztywnienia ligandów lub ograniczenia ich swobody ruchu po związaniu. I odwrotnie, dodatnia ΔS może wiązać się z uwolnieniem cząsteczek rozpuszczalnika lub rozluźnieniem struktury [105].

Rozkład całkowitej energii swobodnej (ΔG) na składowe entalpowe (ΔH) i entropowe ($-T\Delta S$) pozwala zidentyfikować główną siłę napędową reakcji oraz rodzaj oddziaływań molekularnych odpowiedzialnych za tworzenie kompleksu (Tabela 6).

Tabela 6. Zależność między dominującymi czynnikami termodynamicznymi a oddziaływaniami stabilizującymi dane połączenia chemiczne.

<i>Czynnik dominujący</i>	<i>Warunek sprzyjający powstawaniu połączenia</i>	<i>Oddziaływania stabilizujące</i>
<i>Czynnik entalpowy (ΔH)</i>	$\Delta H < 0$	oddziaływania kowalencyjne/niekowalencyjne (wiązania wodorowe, oddziaływania elektrostatyczne oraz van der Waalsa)
<i>Czynnik entropowy ($-T\Delta S$)</i>	$\Delta S > 0$	oddziaływania hydrofobowe
<i>Czynnik entalpowy (ΔH) oraz czynnik entropowy ($-T\Delta S$)</i>	$\Delta H < 0$ oraz $\Delta S > 0$	oddziaływania kowalencyjne/niekowalencyjne oraz hydrofobowe

1.3.1 Wpływ warunków pomiarowych na wartości parametrów reakcji

Podczas pomiaru kalorymetrycznego rejestrowany jest całkowity efekt energetyczny (ΔH_{ITC}), który jest sumą trzech składowych:

1. $\Delta H_{reakcji}$ – zmiana entalpii właściwej reakcji kompleksowania.
2. $\Delta H_{miesz.}$ – efekt cieplny związany z mieszaniem roztworów.
3. $\Delta H_{rozc.}$ – efekt cieplny rozcieńczania titranta.

$$\Delta H_{ITC} = \Delta H_{reakcji} + \Delta H_{miesz.} + \Delta H_{rozc.}$$

Aby wyznaczyć wartość $\Delta H_{reakcji}$, konieczne jest wyeliminowanie obu efektów ubocznych ($\Delta H_{miesz.}$ oraz $\Delta H_{rozc.}$), niezwiązanych bezpośrednio z ciepłem tworzenia związku kompleksowego [80].

a) *Eliminacja efektu mieszania ($\Delta H_{miesz.}$)*

Efekt ten można zminimalizować poprzez rozpuszczenie reagentów w identycznym roztworze buforowym (o tym samym pH, składzie i stężeniu). Dzięki temu unika się dodatkowych efektów cieplnych, takich jak ciepło zobojętniania ($H_3O^+ + OH^- = 2H_2O$) [80].

b) *Eliminacja efektu rozcieńczania ($\Delta H_{rozc.}$)*

W tym celu przeprowadza się eksperyment kontrolny, polegający na miareczkowaniu roztworu buforu (bez analitu) tym samym titrantem. Zmierzony w ten sposób efekt $\Delta H_{rozc.}$ odejmuje się od całkowitego sygnału kalorymetrycznego uzyskanego w głównym pomiarze (miareczkowanie titranta do roztworu analitu) [80]. Ostatecznie entalpię reakcji oblicza się według wzoru:

$$\Delta H_{reakcji} = \Delta H_{ITC} - \Delta H_{rozc.}$$

c) *Wpływ warunków eksperymentu na wynik pomiaru ITC*

Dokładność i wiarygodność pomiarów ITC są w dużej mierze determinowane przez warunki eksperymentu, spośród których kluczową rolę odgrywa stabilność pH środowiska reakcji. Nawet niewielkie zmiany pH mogą znacząco wpływać na powinowactwo reagentów, szczególnie w przypadku ligandów zdolnych do przyłączania lub dysocjacji protonów.

Wielkość efektów ubocznych zależy od warunków pomiaru:

- $\Delta H_{\text{miesz.}}$ rośnie wraz ze wzrostem różnic w pH, stężeniu lub temperaturze między roztworami.
- $\Delta H_{\text{rozc.}}$ jest istotna, gdy składniki titranta mają tendencję do asocjacji (tworzenia dimerów lub agregatów) w roztworze [80].

1.3.2 Rola buforowania i potencjalne reakcje konkurencyjne

Utrzymanie stałego pH za pomocą roztworu buforowego jest niezbędne dla stabilności kompleksów. Należy jednak pamiętać o dwóch typach reakcji konkurencyjnych, które mogą zakłócać pomiar:

1. *Konkurencja między składnikiem roztworu buforowego a ligandem o jon metalu* – występuje, gdy bufor wykazuje powinowactwo do badanego indywiduum.
2. *Konkurencja między protonem a jonem metalu o ligand* – dotyczy ligandów z grupami kwasowymi [106].

1.3.3 Warunkowa stała trwałości

Metoda ITC dostarcza informacji na temat warunkowej stałej trwałości (K_{ITC}), zależnej od pH, rodzaju buforu i temperatury. Aby obliczyć bezwzględną stałą trwałości (K), wprowadza się współczynniki korekcyjne:

$$K = K_{ITC} \cdot \alpha_{\text{proton}} \cdot \alpha_{\text{bufor}}$$

gdzie:

- α_{proton} to współczynnik sprotonowania liganda, uwzględnia reakcje konkurencyjne liganda (koryguje wpływ protonowania liganda).
- α_{bufor} to współczynnik reakcji konkurencyjnych jonu metalu (uwzględnia oddziaływania badanego indywiduum np. jonu metalu ze składnikiem roztworu buforowego) [80].

Aby zminimalizować wpływ reakcji ubocznych i uprościć analizę, zaleca się:

- stosowanie buforów, których składniki nie wykazują powinowactwa do jonów metali (wówczas $\alpha_{\text{bufor}} = 1$), co eliminuje wpływ reakcji konkurencyjnej pomiędzy składnikiem buforowym a ligandem wobec jonu metalu.
- dobór takiego pH, przy którym ligand występuje w formie zdeprotonowanej, co eliminuje wpływ reakcji konkurencyjnej pomiędzy protonem a jonem metalu wobec liganda (wówczas $\alpha_{\text{proton}} = 1$) [106].

Reasumując, za wyborem izotermicznego miareczkowania kalorymetrycznego (ITC) jako głównego narzędzia do analizy oddziaływań albumin (BSA, HSA) i lizozymu z badanymi ligandami przemawiają następujące argumenty praktyczne:

1. ITC jako metoda typu „label-free” – nie wymaga od ligandów ani białek posiadania specyficznych grup chromoforowych, fluoroforowych lub zdolności do dysocjacji. Z tego powodu doskonale nadaje się do badania zróżnicowanego szeregu ligandów, obejmującego zarówno związki organiczne (SXS, KXS, $[B(Ph)_4]^-$), jak i nieorganiczne kompleksy jonów metali ($[Fe(CN)_6]^{4-/3-}$, polioksowanadany). Pozwala to na bezpośrednie porównanie siły wiązania wszystkich związków w identycznych warunkach.
2. ITC dostarcza jednocześnie podstawowych informacji opisujących równowagę, tj.: stała trwałości (K), stechiometria (N) i liczba miejsc wiążących (n) w białku, a także parametry termodynamiczne reakcji (ΔH , $T\Delta S$). Pozwalają one uzyskać kompletny obraz termodynamiczny badanych oddziaływań (mechanizm wiązania).
3. ITC doskonale nadaje się do prowadzenia eksperymentów konkurencyjnych (np. z użyciem SDS jako liganda konkurencyjnego), pozwalając na identyfikację miejsc wiążących w białku.
4. Możliwość prowadzenia pomiarów ITC w różnych temperaturach i pH pozwala na zbadanie wpływu środowiska reakcji na wiązanie ligandów oraz na analizę zjawiska kompensacji entalpowo-entropowej, kluczowego dla pełnego zrozumienia termodynamiki procesu.

2. Cel i zadania badawcze

2.1 Cel badawczy

Określenie cech strukturalnych oraz fizykochemicznych, które decydują o sile, mechanizmie i lokalizacji oddziaływań między białkami (BSA, HSA oraz lizozymem) a zróżnicowanymi klasami związków małocząsteczkowych.

2.2 Zadania badawcze

1. Scharakteryzowanie termodynamiki (ΔG , ΔH , $T\Delta S$) i stechiometrii wiązania serii modelowych ligandów (surfaktanty anionowe SXS/KXS, jony kompleksowe, hydrofobowe aniony) z albuminami (BSA, HSA) oraz lizozymem.
2. Określenie wpływu podstawowych parametrów fizykochemicznych ligandów (tj. długość łańcucha węglowodorowego, wartość i dystrybucja ładunku, równowaga hydrofilowo-hydrofobowa) na liczbę i lokalizację miejsc wiązania w badanych białkach.
3. Zbadanie wpływu warunków środowiskowych (pH, temperatura) oraz obecności ligandów konkurencyjnych na stabilność kompleksów ligand-białko.
4. Ocena strukturalnych konsekwencji oddziaływań (denaturacja/rozfałdowanie, stabilizacja) wywołanych przez ligandy oraz weryfikacja, w jaki sposób tworzenie kompleksów inkluzyjnych z cyklodekstryną modyfikuje te oddziaływania.
5. Wykorzystanie obliczeń teoretycznych do identyfikacji potencjalnych miejsc wiązania i wizualizacji oddziaływań na poziomie atomowym, w celu weryfikacji i interpretacji danych eksperymentalnych.

2.3 Uzasadnienie podjęcia badań

Podjęcie tego typu badań jest niezwykle istotne z kilku zasadniczych powodów, zarówno poznawczych, jak i być może ściśle aplikacyjnych:

1. Zrozumienie, jak konkretne cechy liganda (np. obecność dodatkowego atomu tlenu w strukturze surfaktantu – różnica SXS vs. KXS) przekładają się na siłę i mechanizm wiązania z białkami osocza. Pozwala to w sposób uzasadniony modyfikować strukturę syntetycznych związków wykazujących aktywność biologiczną, aby optymalizować parametry farmakokinetyczne, takie jak czas półtrwania we krwi czy ich dystrybucję.
2. Ponad 90% leków jest transportowanych przez albuminy. Badania konkurencyjne, jak te podjęte z SDS, warfaryną czy ibuprofenem, bezpośrednio modelują scenariusz, w którym dwa leki konkurują o to samo miejsce wiązania. Zrozumienie tych mechanizmów na poziomie molekularnym jest kluczowe dla przewidywania niebezpiecznych interakcji, które mogą prowadzić do wzrostu toksycznego, wolnego stężenia jednego z leków.
3. Praca eksperymentalnie potwierdza i bada zasygnalizowany w literaturze problem związany z obecnością wysokich stężeń albuminy w pożywkach hodowlanych, której obecność może mieć wpływ na wyniki testów toksyczności i aktywności biologicznej. Doświadczenia z metawanadanem(V) sodu i SDS jako blokerem miejsc wiązania dostarczają modelowego dowodu, że oddziaływanie z albuminą może w pewnych warunkach eksperymentalnych zmieniać pozorną siłę działania związku. To ma fundamentalne znaczenie dla wiarygodności badań przesiewowych nowych substancji czynnych.
4. Wykazanie, że cyklodekstryna może skutecznie "wylapywać" i neutralizować destrukcyjne działanie surfaktantów na białka (np. lizozym), może otworzyć drogę do projektowania zaawansowanych systemów dostarczania leków lub formulacji, w których niepożądane efekty składników pomocniczych są celowo minimalizowane.

5. Odpowiednio dobrane klasy ligandów o zróżnicowanej strukturze i cechach fizykochemicznych oraz białek o kontrastujących właściwościach, umożliwiając uzyskanie ważnych informacji na temat korelacji między strukturą badanych reagentów a ich wzajemnym powinowactwem.

3. Materiały i metodyka badań

Wykaz odczynników chemicznych użytych w badaniach przedstawiono w Tabeli 7. Zastosowane metody pomiarowe zostały zestawione w Tabeli 8, a szczegółowy opis procedur eksperymentalnych opisano w publikacjach (P1-P10).

Tabela 7. Stosowane odczynniki chemiczne.

<i>Nazwa związku</i>	<i>Skrót/wzór chemiczny</i>	<i>Czystość</i>	<i>Numer CAS</i>
albumina surowicy bydlęcej	BSA	≥ 96,0%	9048-46-8
albumina surowicy ludzkiej	HSA	≥ 96,0%	70024-90-7
lizozym	-	-	12650-88-3
oktylosiarczan sodu	S8S	≥ 95,0%	142-31-4
decylosiarczan sodu	S10S	98,0%	142-87-0
dodecylosiarczan sodu	SDS/S12S	≥ 99,0%	151-21-3
1-oktanosulfonian sodu	K8S	≥ 99,0%	5324-84-5
1-dekanosulfonian sodu	K10S	≥ 99,0%	13419-61-9
1-dodekanosulfonian sodu	K12S	≥ 99,0%	2386-53-0
tetrafenyloboran sodu	Na[B(Ph) ₄]	≥ 99,5%	143-66-8
metawanadan(V) sodu	NaVO ₃	≥ 98,0%	13718-26-8
heksacyjanożelazian(II) potasu (trihydrat)	K ₄ [Fe(CN) ₆]	≥ 98,5%	14459-95-1
heksacyjanożelazian(III) potasu	K ₃ [Fe(CN) ₆]	≥ 99,0%	13746-66-2
β-cyklodekstryna	β-CD	≥ 99,0%	7585-39-9
kakodylan sodu (trihydrat)	Caco	≥ 98,0%	6131-99-3
warfaryna (sól sodowa)	C ₁₉ H ₁₆ O ₄ Na	92,1%	129-06-6
ibuprofen (sól sodowa)	C ₁₃ H ₁₇ O ₂ Na	≥ 98,0%	31121-93-4

Wszystkie ww. odczynniki pochodziły z firmy Sigma-Aldrich (Polska).

Tabela 8. Stosowane metody pomiarowe.

<i>Metoda pomiarowa</i>	<i>Sprzęt pomiarowy</i>	<i>Producent</i>	<i>Uzyskane dane/ Wyznaczane wielkości</i>	<i>Efekt badań</i>
<i>Izotermiczna kalorymetria miareczkowa (ITC)</i>	kalorymetr AutoITC	MicroCal Inc. GE Healthcare, Northampton, USA	▪ liczba miejsc wiążących (n) ▪ stechiometria (N) ▪ stała trwałości (K) ▪ parametry termodynamiczne (ΔH, ΔG, $T\Delta S$) ▪ krytyczne stężenie micelarne (CMC)	▪ określenie trwałości kompleksów ligand-białko [Publikacja: P1-P5, P9, P10] ▪ określenie trwałości kompleksów typu gość-gospodarz [Publikacja: P7, P8] ▪ określenie sił odpowiedzialnych za proces wiązania ▪ określenie oddziaływań stabilizujących kompleks [Publikacja: P1-P5, P7-P10] ▪ ocena procesu agregacji surfaktantu [Publikacja: P1]

<i>Metoda pomiarowa</i>	<i>Sprzęt pomiarowy</i>	<i>Producent</i>	<i>Uzyskane dane/ Wyznaczane wielkości</i>	<i>Efekt badań</i>
<i>Spektroskopia fluorescencyjna (SF)</i>	spektrofluorymetr Cary Eclipse Varian	Agilent, Santa Clara, CA, USA	▪ stała Sterna-Volmera (K_{SV}) ▪ bimolekularna stała szybkości wygaszania fluorescencji (k_q) ▪ stała wiązania (K_{bind}) ▪ liczba miejsc wiążących (n) ▪ krytyczne stężenie micelarne (CMC) ▪ liczba agregacji (N_{agg})	▪ określenie efektywności oddziaływań ligand-białko ▪ określenie zmian mikrośrodowiska wokół reszt tryptofanu na skutek związania liganda [Publikacja: P1-P5, P9, P10] ▪ określenie mechanizmu wygaszania fluorescencji [Publikacja: P2, P4, P9, P10] ▪ ocena procesów agregacji surfaktantów [Publikacja: P6]
<i>Spektrofotometria UV-Vis</i>	spektrofotometr UV-Vis Perkin Elmer Lambda 650	PerkinElmer, Waltham, MA, USA	▪ absorbancja (A) (efekt filtra wewnętrznego)	▪ określenie efektywności oddziaływań ligand-białko ▪ korekta wyników fluorescencji [Publikacja: P1-P5, P9, P10]

<i>Metoda pomiarowa</i>	<i>Sprzęt pomiarowy</i>	<i>Producent</i>	<i>Uzyskane dane/ Wyznaczane wielkości</i>	<i>Efekt badań</i>
<i>Spektroskopia dichroizmu kołowego (CD)</i>	automatyczny spektropolarymetr Jasco-715	Jasco Inc. Easton, MD, USA	▪ skład procentowy struktury drugorzędowej białka	▪ określenie struktury drugorzędowej białka oraz jej zmian w obecności liganda [Publikacja: P1, P2, P4, P8-P10] ▪ określenie stabilności termicznej białka oraz jej zmian w obecności liganda [Publikacja: P1, P2, P4]
<i>Skaningowa kalorymetria różnicowa (DSC)</i>	mikrokalorymetr VP-DSC	MicroCal Inc. GE Healthcare, Northampton, USA	▪ temperatura przejścia fazowego (T_m)	▪ określenie stabilności termicznej wolnego białka oraz jej zmian w obecności liganda [Publikacja: P1, P2, P8]
<i>Konduktometria</i>	mikrotitrator Cerko Lab System	Cerko, Gdańsk, Polska	▪ krytyczne stężenie micelarne (CMC) ▪ zmiana energii swobodnej (ΔG) ▪ stopień wiązania przeciwnonu (β) ▪ stała trwałości (K) ▪ przewodnictwo molowe (Λ_c)	▪ ocena procesów agregacji surfaktantów [Publikacja: P6] ▪ określenie efektywności oddziaływań typu gość-gospodarz [Publikacja: P7, P8]

<i>Metoda pomiarowa</i>	<i>Sprzęt pomiarowy</i>	<i>Producent</i>	<i>Uzyskane dane/ Wyznaczane wielkości</i>	<i>Efekt badań</i>
<i>Woltamperometria cykliczna (CV)</i>	wielokanałowy potencjostat/ galwanostat Multi Autolab M204	Metrohm, Herisau, Szwajcaria	▪ różnica potencjałów (ΔE_p)	▪ określenie kinetyki przenoszenia elektronów związku oraz jej zmian w obecności białka [Publikacja: P10]
<i>Testy cytotoksyczności</i>	czytnik mikropłytek (EnSpire)	PerkinElmer, Waltham, MA, USA	▪ procent przeżywalności komórek nowotworowych	▪ określenie cytotoksyczności związku oraz jej zmian w obecności blokera miejsc wiążących [Publikacja: P3]
<i>Testy biochemiczne</i>	czytnik płytek (EnSpire Multimode)	PerkinElmer, Waltham, MA, USA	▪ aktywność enzymatyczna ▪ współczynniki kinetyczne (K_m, V_{max}, k_{cat})	▪ określenie aktywności enzymatycznej białka oraz jej zmian w obecności liganda ▪ określenie powinowactwa enzymu do substratu oraz jej zmian w obecności liganda [Publikacja: P10]
<i>Testy biologiczne</i>	czytnik płytek (EnSpire Multimode)	PerkinElmer, Waltham, MA, USA	▪ stopień lizy komórek bakteryjnych	▪ określenie aktywności litycznej enzymu oraz jej zmian w obecności liganda [Publikacja: P10]

<i>Metoda pomiarowa</i>	<i>Sprzęt pomiarowy</i>	<i>Producent</i>	<i>Uzyskane dane/ Wyznaczane wielkości</i>	<i>Efekt badań</i>
<i>Badania teoretyczne</i>	oprogramowanie: AMBER16-20, Autodock3, Gaussian 09, Gromacs 2020	-	▪ potencjalne miejsca wiązania ligandów na powierzchni białka ▪ prawdopodobny mechanizm wejścia w układy typu gość-gospodarz ▪ zmiana energii swobodnej (ΔG)	▪ określenie lokalizacji potencjalnych miejsc wiązania ligandów na powierzchni białka [Publikacja: P1, P2, P4, P5, P9, P10] ▪ określenie mechanizmów wejścia w układy typu gość-gospodarz [Publikacja: P7, P8] ▪ oszacowanie głównych oddziaływań odpowiedzialnych za proces wiązania [Publikacja: P1, P2, P4, P5, P7-P10] ▪ określenie zdolności jednego surfaktantu do współtworzenia miceli drugiego [Publikacja: P6] ▪ określenie stabilności miceli o składzie mieszanym [Publikacja: P6] ▪ wsparcie i interpretacja wyników badań eksperymentalnych [Publikacja: P1, P2, P4-P10]

4. Dyskusja wyników

4.1 Badania oddziaływań dodecylosiarczanu sodu (SDS) z albuminą surowicy bydlęcej (BSA) [Publikacja 1]

Celem badań było określenie wpływu parametrów eksperymentalnych: temperatury (288,15 K oraz 298,15 K) i pH (5 oraz 7), na mechanizm wiązania jonów dodecylosiarczanowych (SDS) z albuminą surowicy bydlęcej (BSA). Praca miała na celu dostarczenie szczegółowych informacji strukturalnych i termodynamicznych dla układu SDS-BSA oraz identyfikację potencjalnych miejsc wiązania tych ligandów na powierzchni białka.

Przylączenie się surfaktantu może istotnie zmieniać właściwości fizykochemiczne białka, prowadząc m.in. do zmiany jego konformacji, blokady miejsc wiążących lub zaburzenia stabilności. W konsekwencji, proces ten wpływa bezpośrednio na aktywność biologiczną albuminy. Zrozumienie szczegółów tych oddziaływań ma fundamentalne znaczenie dla przewidywania i kontroli wpływu surfaktantów na funkcjonowanie albuminy w układach biologicznych i jej właściwości transportujące.

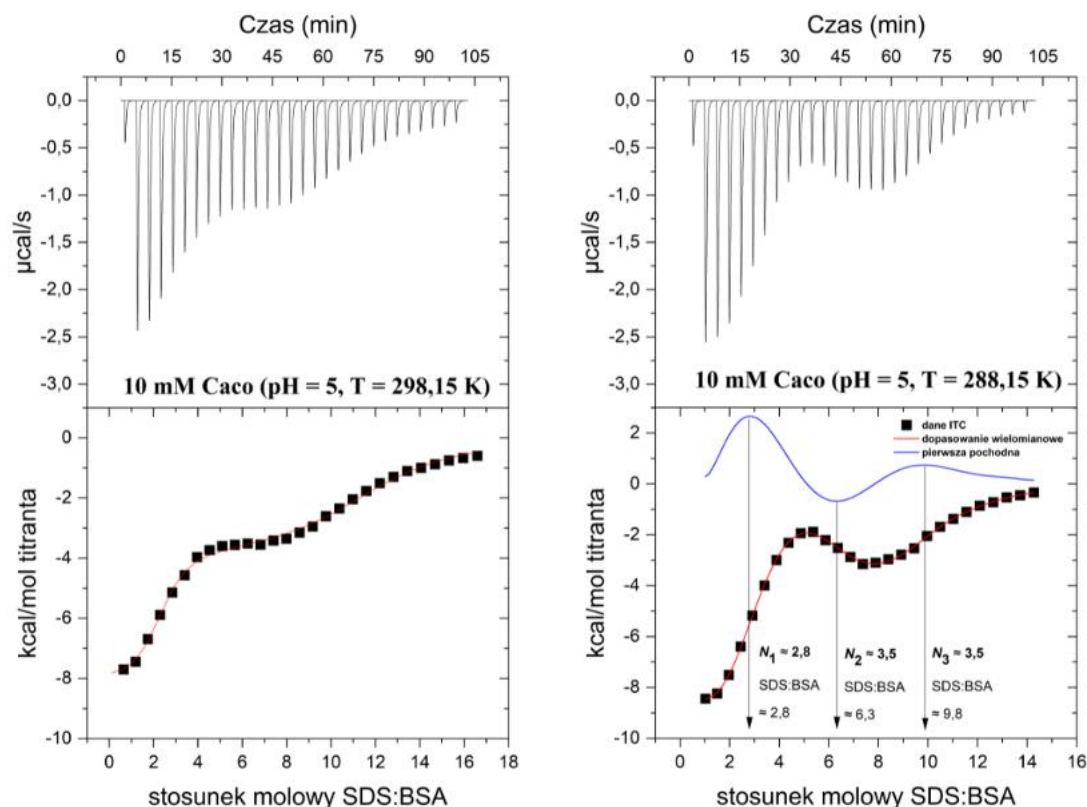
Wartości krytycznego stężenia micelnego (CMC, ang. *Critical Micelle Concentration*) SDS w warunkach eksperymentalnych (10 mM bufor Caco o pH = 5 i 7 w temperaturze 298,15 K) zostały wyznaczone na podstawie danych miareczkowania konduktometrycznego i pomiarów ITC (Figure 1, Publikacja 1). Obliczone wartości CMC (5,4-5,6 mM) są niższe od wartości podanych dla wody (CMC ok. 8 mM), co wskazuje, że zastosowany do badań 1 mM roztwór SDS zawiera jony dodecylosiarczanowe w postaci monomerów (pre-micelarny zakres stężeń), które biorą bezpośredni udział w oddziaływaniu z białkiem.

Badania kalorymetryczne (ITC) potwierdziły, że albumina surowicy bydlęcej (BSA) posiada co najmniej dwa specyficzne miejsca wiązania dla monomerów dodecylosiarczanu sodu (SDS). Analiza parametrów termodynamicznych wykazała, że stabilność powstających kompleksów jest sterowana entalpowo ($|\Delta H| > |T\Delta S|$), a ujemne wartości ΔH wskazują na dominującą rolę oddziaływań elektrostatycznych między anionowym SDS a dodatnio naładowanymi resztami białka (Lys, Arg, His), wspieranych przez oddziaływania van der

Waals i wiązania wodorowe. Zaobserwowano również istotny wpływ pH i temperatury na stechiometrię wiązania, szczególnie w drugim miejscu. Co ciekawe, w warunkach obniżonej temperatury i pH (288,15 K, pH = 5) pojawiły się przesłanki istnienia trzeciego miejsca wiążącego, prowadzącego do powstania kinetycznie stabilnego kompleksu o stechiometrii około 6,5:1 (SDS:BSA) (Tabela 9). Całkowita liczba związanych ligandów w tych warunkach była zbliżona do wartości uzyskanej w wyższej temperaturze (298,15 K), co sugeruje, że obniżenie temperatury nie zmienia ogólnej stechiometrii wiązania, lecz sprzyja innej ścieżce tworzenia kompleksu (Rysunek 3).

Tabela 9. Wpływ warunków doświadczalnych na wiązanie SDS przez albuminę surowicy bydlęcej (BSA) – podsumowanie wyników kalorymetrycznych [Publikacja 1].

Parametr/ Warunki	1. miejsce wiążące (wysokie powinowactwo)	2. miejsce wiążące (niższe powinowactwo)	Obserwacje dodatkowe/ 3. miejsce wiążące
Stała wiązania (logK)	$\log K_1$ (wyższa wartość)	$\log K_2$ (niższa wartość)	nie określono dla potencjalnego 3. miejsca
Stechiometria (SDS:BSA)	$N_1 \approx 2$	$N_2 \approx 4-5$ (przy pH = 7)	w T = 288,15 K i pH = 5 zaobserwowano istnienie 3. miejsca wiązącego: $N_1 \approx 2,8$, $N_2 \approx 3,5$, $N_3 \approx 3,5$
		$N_2 \approx 9$ (przy pH = 5)	
Schemat reakcji	$2 \text{ SDS} + \text{BSA} = (\text{SDS})_2\text{-BSA}$ (niezależnie od pH)	$\text{pH} = 7$ $4-5 \text{ SDS} + (\text{SDS})_2\text{-BSA} = (\text{SDS})_2\text{-BSA}-(\text{SDS})_{4-5}$	powstaje kinetycznie stabilny kompleks o stechiometrii całkowitej: $N \approx 9,8:1$ (SDS:BSA)
		$\text{pH} = 5$ $9 \text{ SDS} + (\text{SDS})_2\text{-BSA} = (\text{SDS})_2\text{-BSA}-(\text{SDS})_9$	
Stabilność kompleksu	sterowanie entalpowe ($ \Delta H_1 > T\Delta S_1 $)	sterowanie entalpowe ($ \Delta H_2 > T\Delta S_2 $)	-
Dominujące siły wiązania	głównie oddziaływania elektrostatyczne (ujemny ładunek SDS-dodatnie reszty białka) oraz van der Waalsa i wiązania wodorowe	głównie oddziaływania elektrostatyczne (ujemny ładunek SDS-dodatnie reszty białka) oraz van der Waalsa i wiązania wodorowe	-



Rysunek 3. Termogramy oraz krzywe miareczkowania kalorymetrycznego przedstawiające oddziaływanie SDS-BSA w 10 mM buforze Caco o $\text{pH} = 5$ i $T = 298,15 \text{ K}$ oraz $288,15 \text{ K}$.

Badania kalorymetryczne (ITC) dotyczące oddziaływania SDS z albuminą (BSA) zostały uzupełnione o analizy spektroskopowe i termiczne, które dostarczyły informacji o zmianach strukturalnych i stabilności białka.

Analiza fluorescencyjna reszt tryptofanu (Trp-134 i Trp-213) wykazała, że wiązanie SDS powoduje wygaszenie fluorescencji i przesunięcie hipsochromowe maksimum emisji, wywołane przeniesieniem reszt Trp do bardziej hydrofobowego środowiska. Dane sugerują, że pierwsze miejsce wiązania SDS znajduje się w pobliżu eksponowanej reszty Trp-134. Stałe wiązania wyznaczone tą metodą potwierdziły, że pH nie wpływa istotnie na powinowactwo do pierwszego miejsca, natomiast obniżenie temperatury zwiększa stabilność kompleksu (Tabela 10).

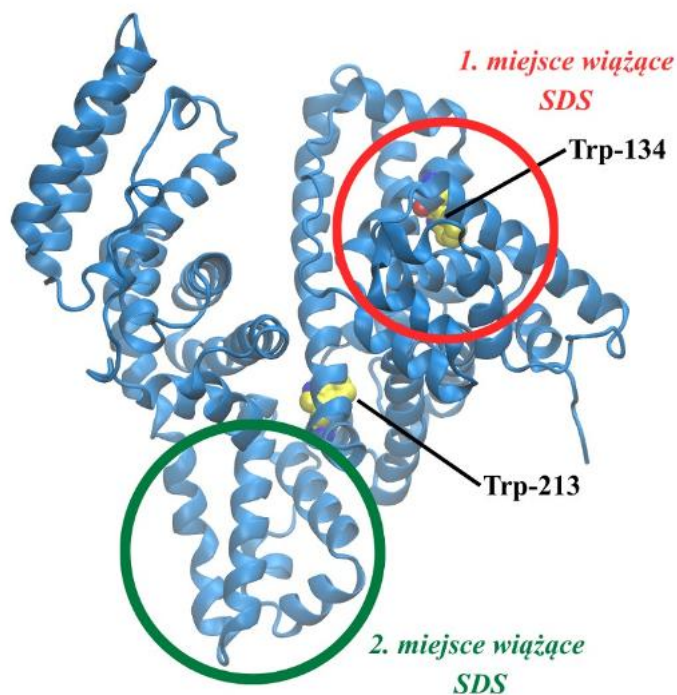
Badania skaningowej kalorymetrii różnicowej (DSC) oraz dichroizmu kołowego (CD) ujawniły wpływ SDS na stabilność termiczną i strukturę BSA. Nasycenie pierwszego miejsca wiązania SDS powoduje wyraźny wzrost stabilności termicznej białka (o ok. 10 K).

oraz jego struktury α -helikalnej. Wysycenie drugiego miejsca nie przynosi już istotnych zmian stabilizujących. Potwierdza to, że pierwsze miejsce wiązania ma kluczowe znaczenie dla modyfikacji właściwości fizykochemicznych albuminy przez surfaktant (Tabela 10).

Tabela 10. Wpływ wiązania SDS na właściwości strukturalne i termiczne BSA – podsumowanie badań uzupełniających [Publikacja 1].

<i>Metoda badawcza</i>	<i>Główne obserwacje</i>	<i>Wnioski dotyczące wiązania SDS</i>
<i>Spektroskopia fluorescencyjna (SF)</i> <i>(fluorescencja tryptofanu)</i>	- wygaszenie fluorescencji i przesunięcie hipsochromowe maksimum emisji (w kierunku krótszych długości fal) wraz ze wzrostem stężenia SDS - sugerowana lokalizacja 1. miejsca w pobliżu Trp-134 (eksponowane), a nie Trp-213	- SDS zmienia środowisko reszt Trp na bardziej hydrofobowe - powinowactwo do 1. miejsca nie jest wrażliwe na zmiany pH, ale rośnie przy obniżeniu temperatury
<i>Skaningowa kalorymetria różnicowa (DSC)</i> <i>(stabilność termiczna)</i>	- wysycenie 1. miejsca wiązania zwiększa temperaturę denaturacji (T_m) BSA o ok. 10 K - wysycenie 2. miejsca nie zmienia istotnie T_m - obniżenie pH nieznacznie zwiększa stabilność BSA	1. miejsce wiązania jest krytyczne dla stabilizacji termicznej konformacji BSA przez SDS - białko ulega nieodwracalnej denaturacji termicznej
<i>Spektroskopia dichroizmu kołowego (CD)</i> <i>(struktura drugorzędowa)</i>	- w obecności SDS (w 298,15 K) dominuje struktura α-helikalna - obecność SDS znacząco stabilizuje strukturę helikalną wobec wzrostu temperatury, szczególnie po wysyceniu 1. miejsca	- SDS nie zmienia struktury drugorzędowej BSA w warunkach badania - działa stabilizująco na konformację α-helisy, co koreluje z wynikami DSC

Symulacje dynamiki molekularnej (MD) przeprowadzono dla czterech układów, różniących się wartościami pH (5 i 7) oraz temperaturą (290 K i 300 K). Umożliwiło to zidentyfikowanie potencjalnych miejsc wiązania SDS przez albuminę (Rysunek 4). Dla tzw. „pierwszego” miejsca wiążącego wartości energii swobodnej wiązania wyniosły $-14,6 \pm 3,3$ kcal/mol (metoda MM-GBSA) oraz $-33,4 \pm 6,0$ kcal/mol (metoda LIE). Dla „drugiego” miejsca wartości te były istotnie niższe: $-8,7 \pm 4,6$ kcal/mol (MM-GBSA) i $-19,8 \pm 10,1$ kcal/mol (LIE). Różnica ta jednoznacznie wskazuje na wyższe powinowactwo SDS do BSA i większą stabilność kompleksu ligand-białko w pierwszym miejscu. Co istotne, symulacje wykazały, że całkowita energia swobodna wiązania w pierwszym miejscu jest niezależna od pH, co stanowi bezpośrednie potwierdzenie wcześniejszych obserwacji eksperymentalnych. Analiza składowych energii ujawniła, że decydującą rolę w tym miejscu odgrywa składowa energii oddziaływań van der Waalsa.



Rysunek 4. Struktura przestrzenna BSA z zaznaczonymi odpowiednio resztami tryptofanu (Trp-134, Trp-213). Pierwsze oraz drugie miejsce wiążące SDS oznaczono odpowiednio czerwonym i zielonym okręgiem.

4.2 Badania oddziaływań jonu tetrafenyloboranowego ($[B(Ph)_4]^-$) z albuminą surowicy bydlęcej (BSA) [Publikacja 2]

Celem badań było scharakteryzowanie fizykochemicznego charakteru oddziaływań między jonami tetrafenyloboranowymi ($[B(Ph)_4]^-$) a albuminą surowicy bydlęcej (BSA). Praca obejmowała ocenę wpływu tego wiązania na strukturę przestrzenną białka oraz identyfikację potencjalnych miejsc wiązania tych jonów na powierzchni albuminy (Tabela 11, Rysunek 5).

W badaniach wykorzystano wcześniej zdobytą wiedzę na temat oddziaływań SDS-BSA jako punkt odniesienia. Istotnym elementem metodologii było zastosowanie podejścia konkurencyjnego, w którym proces wiązania jonów $[B(Ph)_4]^-$ analizowano w obecności poprzednio badanego liganda – dodecylosiarczanu sodu (SDS). Ta strategia eksperymentalna umożliwiła bezpośrednie porównanie miejsc wiązania obu ligandów i tym samym identyfikację specyficznych miejsc na BSA zdolnych do wiązania jonów tetrafenyloboranowych.

Badania kalorymetryczne (ITC) potwierdziły, że zarówno jony $[B(Ph)_4]^-$, jak i SDS wiążą się z BSA w dwóch miejscach o porównywalnej stabilności termodynamicznej. Różnicę obserwuje się w mechanizmie wiązania: kompleksowanie $[B(Ph)_4]^-$ jest sterowane entropowo przez oddziaływania hydrofobowe, podczas gdy wiązanie SDS jest sterowane entalpowo przez siły elektrostatyczne (typu ładunek-ładunek) oraz oddziaływania van der Waalsa i wiązania wodorowe.

Różnica w mechanizmie wiązania, entalpowym dla SDS vs. entropowym dla $[B(Ph)_4]^-$, jest bezpośrednią konsekwencją ich chemicznej natury. SDS, jako klasyczny tenzyd z naładowaną częścią hydrofilową, wiąże się głównie poprzez specyficzne oddziaływania polarne. $[B(Ph)_4]^-$, jako zwarty, aromatyczny anion, zachowuje się jak typowy ligand hydrofobowy. Pomimo tej różnicy, oba ligandy osiągają porównywalną ogólną siłę wiązania (ΔG), co świadczy o uniwersalności i plastyczności miejsc wiążących albuminy.

Eksperymenty konkurencyjne i analizy spektroskopowe wykazały, że oba ligandy konkurują o to samo pierwsze miejsce wiązania. Jego zajęcie przez $[B(Ph)_4]^-$ wymusza wiązanie SDS w drugim miejscu, co skutkuje mniejszą całkowitą liczbą przyłączonych

jonów surfaktantu. Pomimo tej konkurencji, BSA zachowuje zdolność do wiązania SDS nawet po całkowitym wysyceniu obu miejsc jonami $[B(Ph)_4]^-$.

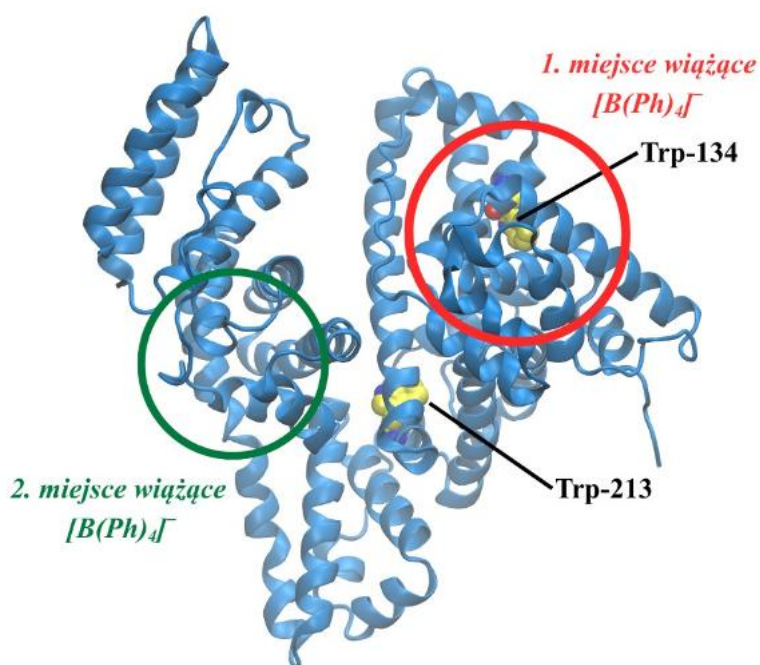
Badania strukturalne (CD, DSC) ujawniły, że kompleksowanie $[B(Ph)_4]^-$ nie zaburza struktury drugorzędowej BSA, a wręcz istotnie zwiększa jej stabilność termiczną, czyniąc białko bardziej odpornym na denaturację.

Tabela 11. Porównanie wiązania SDS i jonów $[B(Ph)_4]^-$ przez BSA – podsumowanie wyników [Publikacja 2].

<i>Cecha/ Parametr</i>	<i>Ligand: SDS</i>	<i>Ligand: $[B(Ph)_4]^-$</i>	<i>Wnioski i interpretacja</i>
<i>Liczba miejsc wiążących</i>	2 główne miejsca	2 główne miejsca	1. miejsce wspólne dla obu ligandów: subdomena IA 2. miejsce różne: SDS – subdomena IIB $[B(Ph)_4]^-$ – subdomena IIIA
<i>Stechiometria (1. miejsce)</i>	$N_1 \approx 2$	$N_1 \approx 2$	identyczna stechiometria wiązania w 1. miejscu
	2 SDS + BSA = (SDS) ₂ -BSA	2 $[B(Ph)_4]^-$ + BSA = ($[B(Ph)_4]^-$) ₂ -BSA	
<i>Stechiometria (2. miejsce)</i>	$N_2 \approx 5$	$N_2 \approx 4$	różna stechiometria wiązania w 2. miejscu
	5 SDS + (SDS) ₂ -BSA = (SDS) ₂ -BSA-(SDS) ₅	4 $[B(Ph)_4]^-$ + ($[B(Ph)_4]^-$) ₂ -BSA = ($[B(Ph)_4]^-$) ₂ -BSA- ($[B(Ph)_4]^-$) ₄	
<i>Stała wiązania (logK₁)</i>	7,61 (±0,38)	7,09 (±0,10)	porównywalne wartości – podobna stabilność termodynamiczna obu kompleksów w 1. miejscu
<i>Stała wiązania (logK₂)</i>	5,29 (±0,06)	5,39 (±0,06)	porównywalne wartości – podobna stabilność termodynamiczna obu kompleksów w 2. miejscu

<i>Cecha/ Parametr</i>	<i>Ligand: SDS</i>	<i>Ligand: [B(Ph)₄]⁻</i>	<i>Wnioski i interpretacja</i>
Mechanizm (1. miejsce)	sterowanie entalpowe ($ \Delta H_1 > T\Delta S_1 $) siły: oddz. elektrostatyczne (typu ładunek- ładunek) oraz van der Waalsa	sterowanie entropowe ($ \Delta H_1 < T\Delta S_1 $) siły: głównie oddziaływania hydrofobowe	zbliżona trwałość termodynamiczna (ΔG_1), ale uwarunkowana innym rodzajem oddz. międzycząsteczkowych
Mechanizm (2. miejsce)	sterowanie entalpowe ($ \Delta H_2 > T\Delta S_2 $) siły: oddz. elektrostatyczne (ładunek-ładunek) oraz van der Waalsa	sterowanie entropowe ($ \Delta H_2 > T\Delta S_2 $) (oddz. hydrofobowe)	różne mechanizmy wiązania: SDS – oddz. elektrostatyczne oraz van der Waalsa [B(Ph) ₄] ⁻ – oddz. hydrofobowe
Konkurencja	konkuruje z [B(Ph) ₄] ⁻ o 1. miejsce; po jego zajęciu SDS wiąże się w 2. miejscu	konkuruje z SDS o 1. miejsce; jego zajęcie blokuje dostęp SDS do tego miejsca	dowód na wspólne powinowactwo do 1. miejsca wiązania
Wpływ na strukturę BSA	stabilizuje strukturę α -helikalną, zwiększa temperaturę denaturacji (T_m)	nie zmienia struktury drugorzędowej, zwiększa stabilność termiczną (T_m)	oba ligandy działają stabilizująco, choć przez różne mechanizmy
Wnioski ogólne	klasyczny ligand polarny/amfifilowy, wiązany siłami elektrostatycznymi	modelowy ligand hydrofobowy/ anionowy, wiązany siłami hydrofobowymi	BSA wykazuje uniwersalność wiązania – może z podobną siłą kompleksować ligandy o skrajnie różnych cechach chemicznych

Aby lepiej zrozumieć wyniki eksperymentalne na poziomie atomowym, przeprowadzono symulacje dynamiki molekularnej (MD) dla układu $[B(Ph)_4]^-$ -BSA. Celem symulacji było przewidzenie potencjalnych miejsc wiązania jonów $[B(Ph)_4]^-$ na powierzchni albuminy oraz szczegółowa charakterystyka i porównanie ich powinowactw wiązania. Symulacje potwierdziły lokalizację dwóch głównych miejsc. Pierwsze miejsce wiązania zlokalizowano w pobliżu reszty Trp-134 w subdomenie IA. Jest to to samo miejsce, które wiąże jony SDS w pierwszej kolejności. Natomiast drugie miejsce wiązania okazało się różne dla obu ligandów. Dla jonów $[B(Ph)_4]^-$ znajduje się ono w subdomenie IIIA, podczas gdy jony SDS wiążą się w tym przypadku w subdomenie IIB (Rysunek 5).

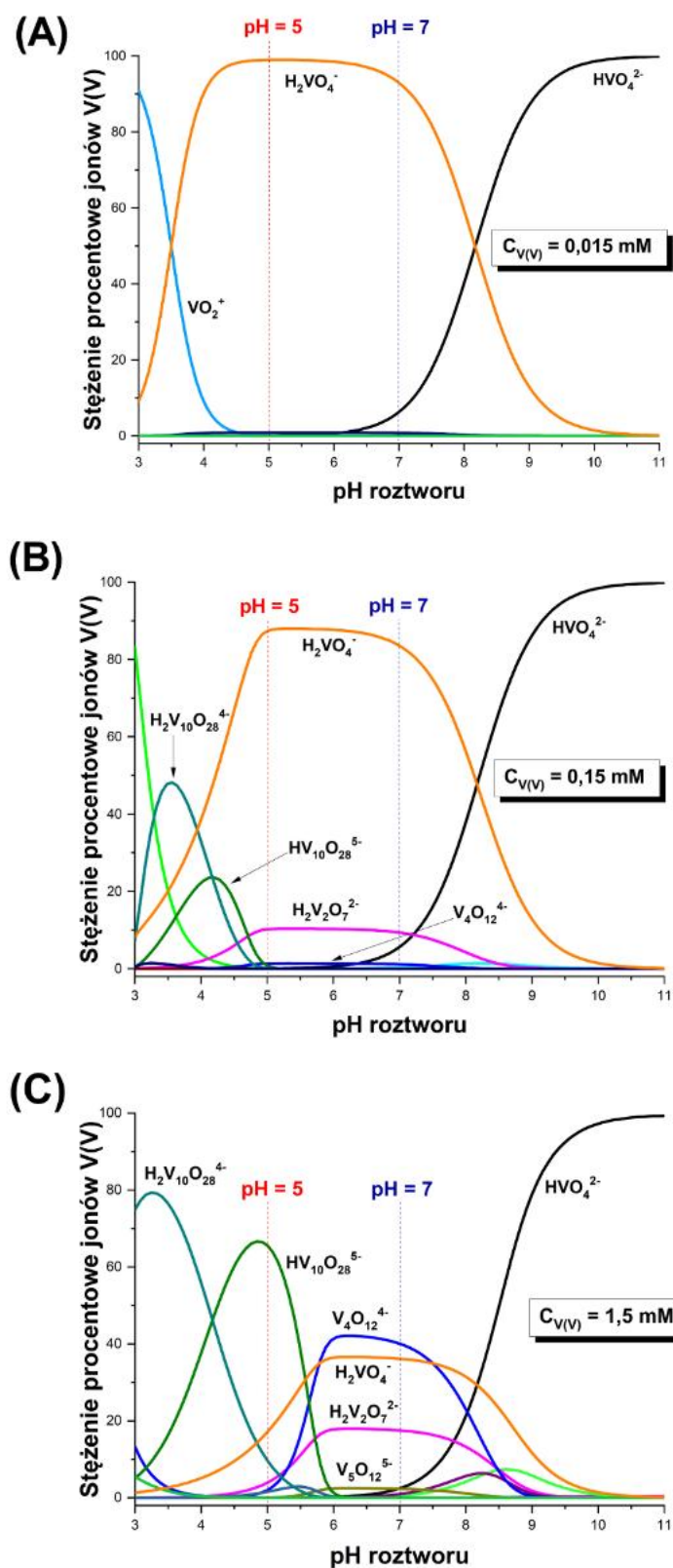


Rysunek 5. Struktura przestrzenna BSA z zaznaczonymi odpowiednio resztami tryptofanu (Trp-134, Trp-213). Pierwsze oraz drugie miejsce wiążące $[B(Ph)_4]^-$ oznaczono odpowiednio czerwonym i zielonym okręgiem.

4.3 Analiza oddziaływań konkurencyjnych polioksowanadanów i jonów dodecylosiarczanowych wobec albuminy surowicy bydlęcej (BSA)

[Publikacja 3]

Ostatnie doniesienia literaturowe zwracają uwagę na istotny problem związany z obecnością wysokich stężeń albuminy (sięgających do ok. 0,040 mM) w standardowych pożywkach hodowlanych. Prowadzi to do kluczowego pytania badawczego: Czy powinowactwo albuminy do badanych związków małych cząsteczkowych może wpływać na wyniki testów ich aktywności biologicznej? Uzasadnione jest podejrzenie, że silne wiązanie się substancji testowanej z albuminą obecną w pożywce zmienia stężenie jej wolnej, biologicznie aktywnej frakcji w układzie. Jest to szczególnie istotne w badaniach zależności stężenie-efekt, np. przy ocenie wpływu związku na przeżywalność komórek (testy cytotoksyczności). Aby zbadać ten problem, przeprowadzono kompleksowe badania modelowe z wykorzystaniem metawanadanu(V) sodu (NaVO_3) – dobrze rozpuszczalnego i stabilnego źródła jonów wanadanowych(V), V(V) , które w roztworach wodnych tworzą złożone mieszaniny równowagowe (Rysunek 6).



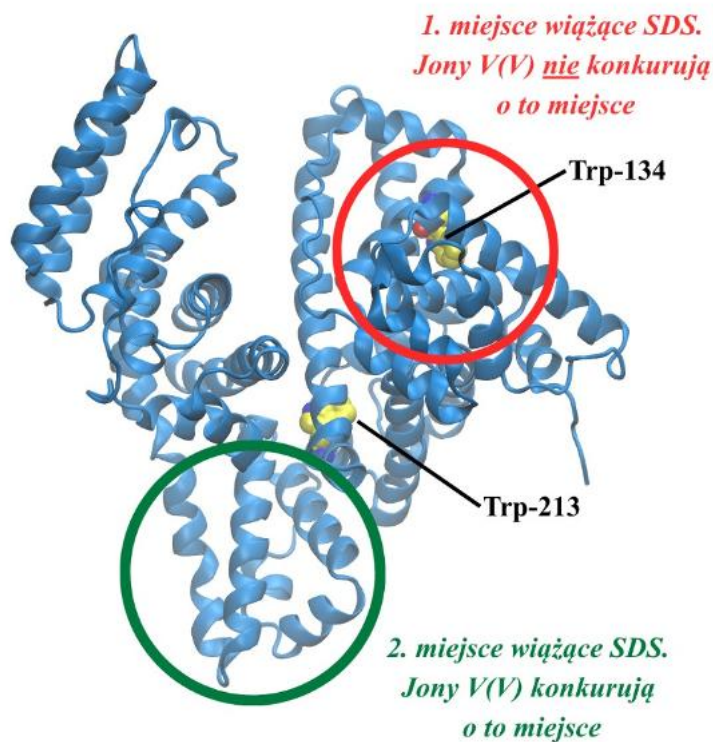
Rysunek 6. Wpływ pH oraz stężenia jonów wanadanowych(V) na udział procentowy poszczególnych ich form w roztworze wodnym: (A) 0,015 mM, (B) 0,15 mM, (C) 1,5 mM.

Aby zbadać, czy wiązanie z albuminą w pożywce hodowlanej może maskować prawdziwą aktywność biologiczną związków, przeprowadzono kompleksowe badanie modelowe. Prace podzielono na dwa uzupełniające się etapy:

1. *Etap chemiczny*: Jego celem było scharakteryzowanie bezpośrednich oddziaływań między jonami wanadanowymi(V) a albuminą (BSA). Dodatkowo, wykorzystując SDS jako bloker, zbadano konkurencyjny wpływ tego surfaktantu na wiązanie jonów V(V).
2. *Etap biologiczny*: Jego celem było praktyczne sprawdzenie, czy blokada miejsc wiązania na albuminie zmienia aktywność biologiczną jonów V(V). Przetestowano to na modelu komórek raka prostaty (PC3), bezpośrednio porównując cytotoksyczność jonów wanadanowych(V) w standardowej pożywce (z aktywną albuminą) i w pożywce z dodatkiem SDS (z zablokowaną albuminą).

Badania kalorymetryczne (ITC) ujawniły złożony i zależny od pH mechanizm wiązania jonów wanadanowych(V) przez albuminę (BSA). Podczas gdy przy pH 7 zidentyfikowano dwa miejsca wiązania o różnej stechiometrii i mechanizmie, przy pH 5 obserwowano tylko jedno miejsce (Tabela 12). Analiza parametrów termodynamicznych wskazała na udział różnego rodzaju oddziaływań międzycząsteczkowych odpowiedzialnych za trwałość powstających kompleksów: wiązanie przy pH 5 oraz w pierwszym miejscu przy pH 7 jest uwarunkowane głównie przez oddziaływania hydrofobowe (sterowanie entropowe: $(|\Delta H_1| < |T\Delta S_1|)$), natomiast w drugim miejscu przy pH 7 dominują siły elektrostatyczne (sterowanie entalpowe: $(|\Delta H_2| > |T\Delta S_2|)$).

Wyniki pomiarów ITC z wykorzystaniem SDS wykazały, że użyty surfaktant modyfikuje wiązanie V(V). Wysycenie BSA w pierwszym miejscu wiązania jonami SDS powoduje obniżenie stechiometrii i stabilności kompleksów V(V)-BSA, natomiast całkowite zablokowanie białka ligandem SDS hamuje wiązanie jonów V(V). Surfaktant nie jest wypierany przez jony wanadanowe(V), co jest zgodne z jego wyższym powinowactwem. Wyniki te sugerują, że jony V(V) konkurują z SDS o drugie miejsce wiązania (subdomena IIB), ale nie o pierwsze miejsce (subdomena IA) (Rysunek 7).



Rysunek 7. Struktura przestrzenna BSA z zaznaczonymi odpowiednio resztami tryptofanu (Trp-134, Trp-213). Pierwsze oraz drugie miejsce wiążące jony SDS oznaczono odpowiednio czerwonym i zielonym okręgiem; jony V(V) nie konkurują o pierwsze miejsce wiązania, natomiast konkurują o drugie.

Badania fluorescencyjne potwierdzają konkurencję między jonami wanadanowymi(V) a SDS o miejsca wiązania na albuminie (BSA). Obserwowany spadek intensywności fluorescencji BSA po dodaniu V(V) ulegał osłabieniu w obecności SDS, a zmiany te były mniej wyraźne dla układów zawierających większy stosunek molowy SDS:BSA. Ponadto w żadnym z analizowanych przypadków nie obserwowano przesunięcia maksimum emisji wraz ze wzrostem stężenia jonów V(V), co wskazuje, że jony te nie wpływają na mikrośrodowisko wokół reszt Trp. Wyznaczone stałe wiązania wykazały wyraźny trend: najwyższe wartości stałych odnotowano dla wolnej BSA, a najniższe – dla BSA całkowicie związanej z SDS. Ta systematyczna redukcja powinowactwa wskazuje na konkurencyjne oddziaływanie obu ligandów o dostępne miejsca na powierzchni białka.

Tabela 12. Wpływ pH i konkurencji z SDS na wiązanie jonów wanadanowych(V) przez BSA – podsumowanie kluczowych wyników [Publikacja 3].

Warunki/ Parametr	pH = 5	pH = 7 (1. miejsce wiążące)	pH = 7 (2. miejsce wiążące)	pH = 7 (po wysyceniu 1. miejsca przez SDS)
Liczba miejsc	1	1	1	2 (zmodyfikowane)
Stechiometria {V(V):białko}	$N_1 \approx 10$	$N_1 \approx 2$	$N_2 \approx 7$	$N_1 \approx 1$ (1. miejsce)
				$N_2 \approx 5$ (2. miejsce)
Schemat reakcji	$10 \text{ V(V)} + \text{BSA} = \{\text{V(V)}\}_{10}\text{-BSA}$	$2 \text{ V(V)} + \text{BSA} = \{\text{V(V)}\}_2\text{-BSA}$	$7 \text{ V(V)} + \{\text{V(V)}\}_2\text{-BSA} = \{\text{V(V)}\}_2\text{-BSA} - \{\text{V(V)}\}_7$	$\text{V(V)} + \text{BSA} = \{\text{V(V)}\}\text{-BSA}$
				$5 \text{ V(V)} + \{\text{V(V)}\}\text{-BSA} = \{\text{V(V)}\}\text{-BSA} - \{\text{V(V)}\}_5$
Stała wiązania (logK)	4,96 ($\pm 0,06$)	6,11 ($\pm 0,06$)	4,80 ($\pm 0,02$)	5,26 ($\pm 0,10$) (1. miejsce)
				3,78 ($\pm 0,03$) (2. miejsce)
Dominujące siły wiązania	oddziaływania hydrofobowe z niewielkim udziałem oddz. elektrostatycznych	głównie oddziaływania hydrofobowe	oddziaływania elektrostatyczne z niewielkim udziałem oddz. hydrofobowych	zmodyfikowane przez konkurencję z SDS
Wpływ SDS	nie badano	SDS nie konkuruje o to miejsce (subdomena IA)	SDS konkuruje o to miejsce (subdomena IIB)	redukcja stochiometrii i stabilności kompleksu V(V)

Badania biologiczne na komórkach raka prostaty (PC3) potwierdziły, że obecność albuminy w pożywce hodowlanej (dostarczanej z 10% FBS) może istotnie modyfikować pozorną aktywność cytotoksyczną jonów wanadanowych(V) (z NaVO_3). Zaobserwowano, że jony V(V) wykazują cytotoksyczność przy stężeniach $> 10 \mu\text{M}$ po 24 i 48 godzinach. Jednakże, gdy do pożywki dodano SDS – blokera miejsc wiązania na albuminie – zaobserwowano jedynie jeden istotny efekt: po 24 godzinach przy stężeniu $100 \mu\text{M}$ NaVO_3 żywotność komórek spadła dodatkowo z $\sim 64\%$ do $\sim 47\%$. W pozostałych warunkach SDS nie zmieniał stężeniowo-zależnej toksyczności jonów wanadanowych(V). Wynik ten wskazuje, że wiązanie V(V) z albuminą może, w specyficznych warunkach, wpływać na ich dostępność biologiczną, jednak efekt ten nie jest prosty i powszechny. Chociaż SDS może pełnić funkcję blokera miejsc wiązania V(V) na albuminie, jego zastosowanie w hodowlach komórkowych wymaga dalszych badań ze względu na złożoność układu.

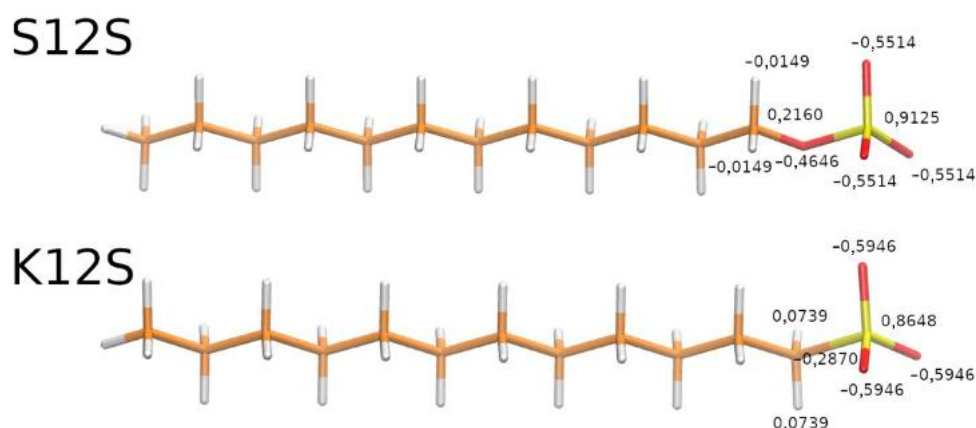
Przeprowadzone badania wskazują na konieczność uwzględniania obecności albumin w pożywkach podczas planowania i analizy testów biologicznych dla wszystkich związków wykazujących do nich powinowactwo, gdyż może to prowadzić do błędnej oceny wyników zależności przeżywalności komórek od dawki związku.

4.4 Analiza porównawcza właściwości kompleksotwórczych i fizykochemicznych 1-alkilosiarczanów (SXS) oraz 1-alkilosulfonianów (KXS) [Publikacja 4-6]

Przeprowadzono porównanie właściwości kompleksotwórczych dwóch strukturalnie podobnych grup anionowych surfaktantów: 1-alkilosiarczanów (SXS) i 1-alkilosulfonianów (KXS). W badaniach uwzględniono związki o różnej długości łańcucha węglowodorowego: oktylowy ($X = C_8$), decylowy ($X = C_{10}$) i dodecylowy ($X = C_{12}$). Dla grupy SXS były to odpowiednio: oktylosiarczan sodu (S8S), decylosiarczan sodu (S10S) i dodecylosiarczan sodu (S12S, czyli SDS). Dla grupy KXS badano analogiczne sulfoniany: 1-oktanosulfonian sodu (K8S), 1-dekanosulfonian sodu (K10S) i 1-dodekanosulfonian sodu (K12S).

Zasadnicza różnica między tymi grupami związków leży w sposobie połączenia części hydrofilowej z hydrofobową. W 1-alkilosulfonianach (KXS) grupa sulfonianowa ($-\text{SO}_3^-$) jest połączona bezpośrednio z łańcuchem alkilowym (C_x-SO_3^-). W 1-alkilosiarczanach (SXS) między łańcuchem węglowodorowym a atomem siarki znajduje się atom tlenu ($\text{C}_x-\text{O}-\text{SO}_3^-$) (Rysunek 8). Ta pozornie niewielka modyfikacja strukturalna ma fundamentalny wpływ na właściwości cząsteczek [Publikacja 5]:

1. *Rozkład ładunku elektrostatycznego*: Obecność atomu tlenu w SXS indukuje częściowy ładunek dodatni na sąsiadującej z nim grupie α -metylenowej ($-\text{CH}_2-$). W efekcie, sama grupa hydrofilowa $-\text{O}-\text{SO}_3^-$ staje się bardziej ujemna i polarna w porównaniu do grupy $-\text{SO}_3^-$ w KXS.
2. *Wzorce hydratacji*: Silniejsza polaryzacja części hydrofilowej SXS prowadzi do tworzenia silniej uporządkowanej i ściślej związanej otoczki hydratacyjnej. Część hydrofilowa KXS, będąca mniej polarną, charakteryzuje się słabszą i mniej uporządkowaną hydratacją.



Rysunek 8. Ładunki punktowe RESP atomów należących do grup hydrofilowych S12S i K12S [Publikacja 5].

4.4.1 Oddziaływania KXS i SXS z albuminami [Publikacja 4, 5]

Badania z wykorzystaniem szeregu wzajemnie się uzupełniających technik eksperymentalnych oraz obliczeniowych umożliwiły charakterystykę oddziaływań serii 1-alkilosulfonianów (KXS) i 1-alkilosiarczanów (SXS) z albuminą surowicy bydlęcej (BSA) i ludzkiej (HSA). Wyniki ujawniły istotne różnice w liczbie miejsc wiązania, termodynamice i lokalizacji, które wynikają zarówno z budowy liganda, jak i drobnych różnic strukturalnych między białkami.

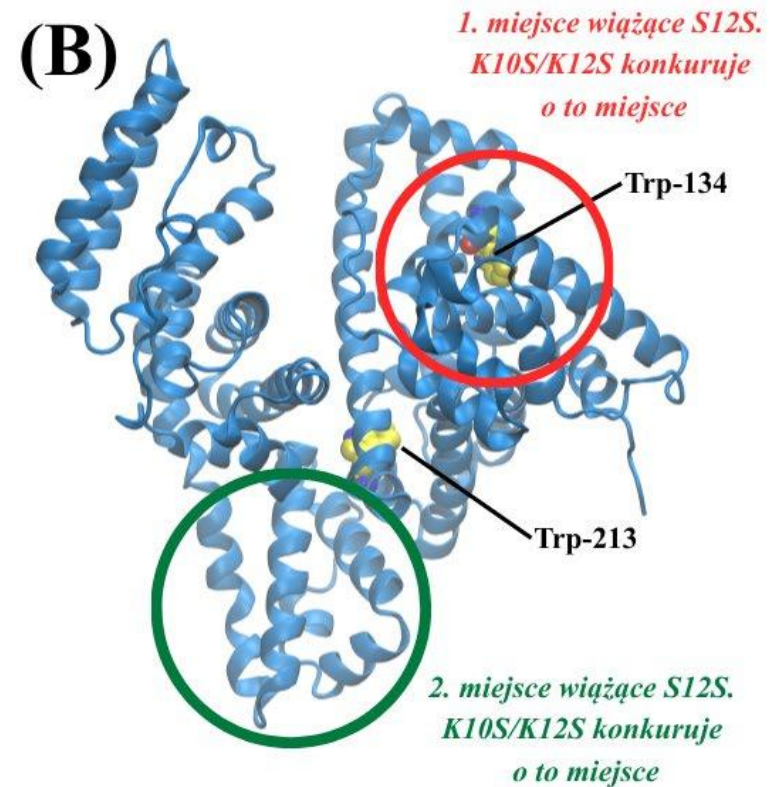
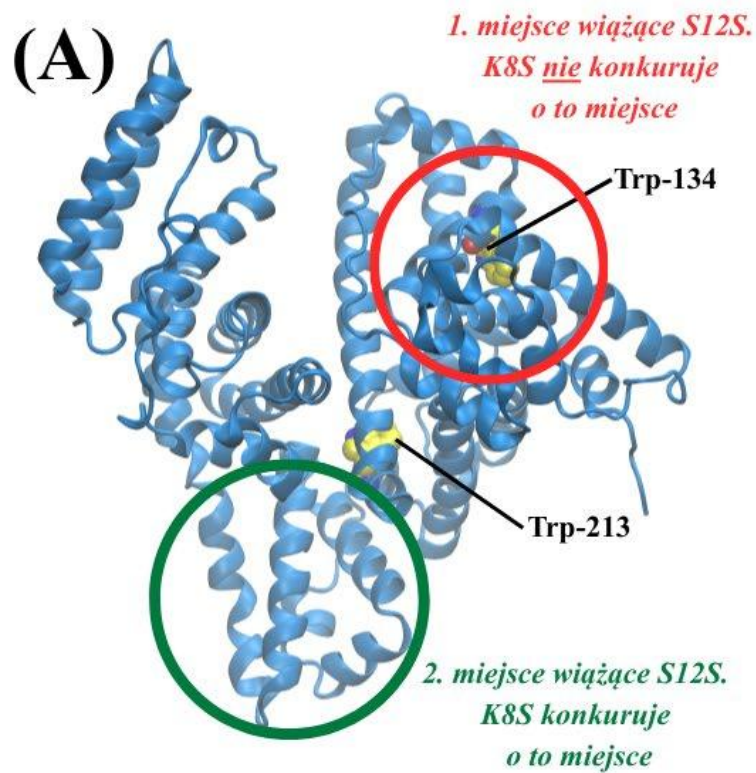
a) Oddziaływanie KXS z BSA [Publikacja 4]

Badania serii 1-alkilosulfonianów (KXS) z albuminą surowicy bydlęcej (BSA) ujawniły, że długości łańcuchów węglowodorowych (C8-, C10- oraz C12-) są elementami strukturalnymi decydującymi o liczbie miejsc wiązania, stechiometrii, termodynamice i lokalizacji kompleksowania (Tabela 13, Rysunek 9). Parametr ten zmienia główną siłę napędową procesu od wiązania sterowanego oddziaływaniami polarnymi dla krótkich łańcuchów (K8S) do wiązania, w którym coraz większe znaczenie zaczyna odgrywać efekt hydrofobowy dla długich łańcuchów (K12S).

Tabela 13. Główne różnice w oddziaływaniach 1-alkilosulfonianów (KXS) z albuminą surowicy bydlęcej (BSA).

Parametr/Cecha	K8S (C8)	K10S (C10)	K12S (C12)	Kluczowy wniosek
Liczba miejsc wiązania	1 (tylko jedno)	2 (pierwsze i drugie)	2 (pierwsze i drugie)	Dostępność drugiego miejsca zależy od długości łańcucha (min. C10).
Stechiometria (KXS:BSA) (1. miejsce wiążące)	$N_1 \approx 1$	$N_1 \approx 1$	$N_1 \approx 1$	1. miejsce charakteryzuje się niską stochiometrią.
Stechiometria (KXS:BSA) (2. miejsce wiążące)	brak wiązania	$N_2 \approx 5$	$N_2 \approx 5$	2. miejsce wykazuje wysoką stochiometrię (~5 ligandów).
Stabilność (ΔG_1 [kcal/mol])	-7,45 ($\pm 0,05$)	-8,34 ($\pm 0,18$)	-9,41 ($\pm 0,48$)	Siła wiązania rośnie z długością łańcucha: K8S < K10S < K12S.
Mechanizm termodynamiczny [kcal/mol]	silne sterowanie entalpowe $\Delta H_1 = -16,81$ ($\pm 0,22$) $T\Delta S_1 = -9,36$	sterowanie entalpowe $\Delta H_1 = -10,77$ ($\pm 0,74$) $T\Delta S_1 = -2,43$	sterowanie entalpowe z zauważalnym udziałem entropii $\Delta H_1 = -8,15$ ($\pm 0,21$) $T\Delta S_1 = +1,26$	Przejsie od dominacji oddziaływań polarnych (K8S) do rosnącego znaczenia efektu hydrofobowego (K12S).

<i>Parametr/Cecha</i>	<i>K8S (C8)</i>	<i>K10S (C10)</i>	<i>K12S (C12)</i>	<i>Kluczowy wniosek</i>
<i>Dominujące siły wiązania (wniosek)</i>	silne oddziaływania polarne: (elektrostatyczne oraz van der Waalsa i wiązania wodorowe)	oddziaływania polarne: (elektrostatyczne oraz van der Waalsa i wiązania wodorowe)	oddziaływania polarne z zauważalnym udziałem oddz. hydrofobowych	Wraz ze wzrostem długości łańcucha węglowodorowego rośnie udział oddz. hydrofobowych kosztem oddz. polarnych.
<i>Konkurencja z S12S (lokalizacja)</i>	wiąże się w 2. miejscu wiązania S12S (nie konkuruje o 1. miejsce)	konkuruje z S12S o 1. i prawdopodobnie 2. miejsce wiązania	konkuruje z S12S o 1. i prawdopodobnie 2. miejsce wiązania	K12S/K10S i S12S współdzielą 1. i prawdopodobnie 2. miejsce. K8S wiąże się w 2. miejscu S12S.
<i>Wpływ na fluorescencję BSA</i>	wygaszenie fluorescencji, przesunięcie hipsochromowe (relokacja reszty Trp w kierunku hydrofobowego wnętrza białka)	wygaszenie fluorescencji, przesunięcie hipsochromowe (relokacja reszty Trp w kierunku hydrofobowego wnętrza białka)	wygaszenie fluorescencji, przesunięcie hipsochromowe (relokacja reszty Trp w kierunku hydrofobowego wnętrza białka)	Wszystkie KXS zmieniają środowisko reszt tryptofanu na bardziej hydrofobowe.
<i>Wpływ na stabilność termiczną BSA</i>	znaczący wzrost stabilności termicznej struktury α -helisy (o ~ 10 K)	znaczący wzrost stabilności termicznej struktury α -helisy (o ~ 10 K)	znaczący wzrost stabilności termicznej struktury α -helisy (o ~ 10 K)	Wszystkie ligandy KXS działają stabilizująco na konformację BSA.
<i>Lokalizacja (symulacje MD)</i>	tylko w 1. miejscu (S1)	w 1. (S1) i 2. (S2A) miejscu	w 1. (S1) i 2. (S2B) miejscu	1. miejsce (S1) jest wspólne dla wszystkich KXS. 2. miejsce ma różną lokalizację dla K10S (S2A) i K12S (S2B).



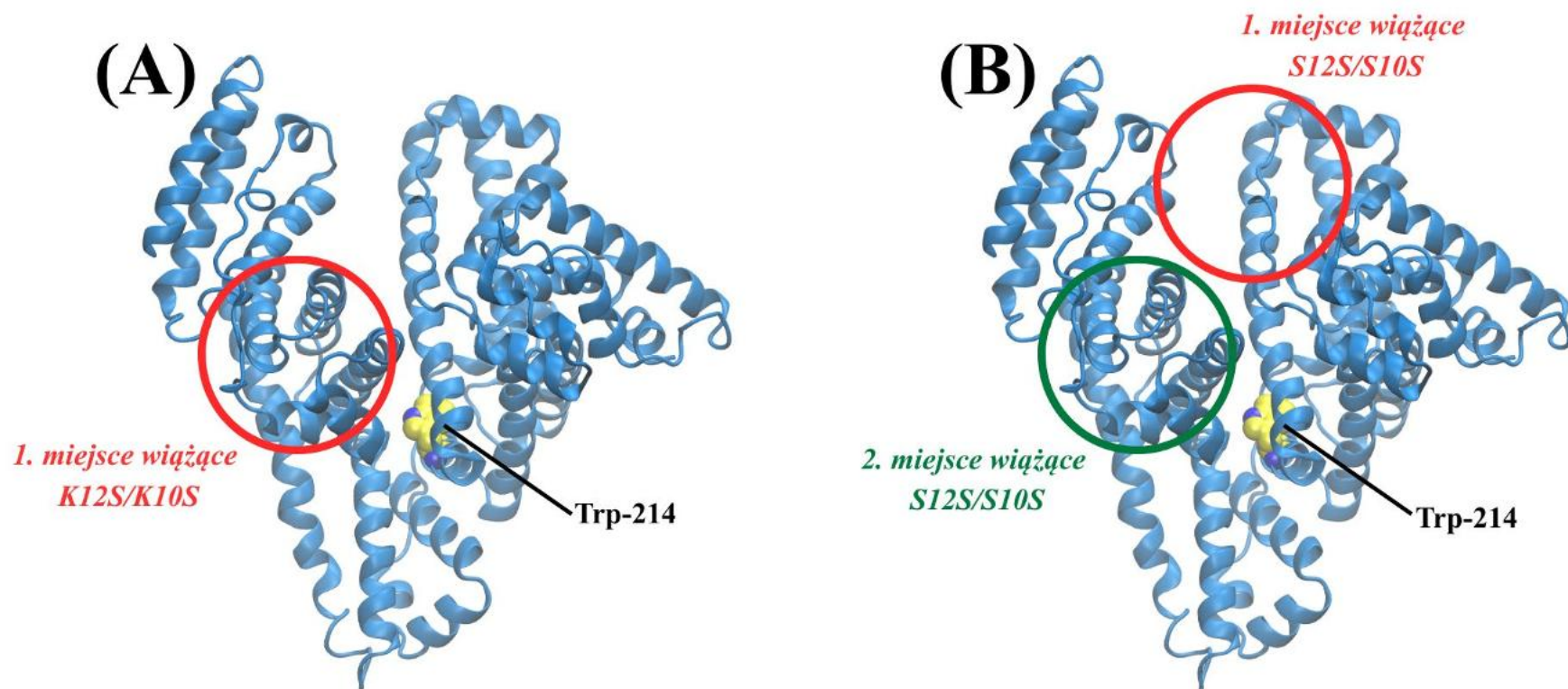
Rysunek 9. Struktura przestrzenna BSA z zaznaczonymi odpowiednio resztami tryptofanu (Trp-134, Trp-213). Pierwsze oraz drugie miejsce wiążące jony S12S oznaczono odpowiednio czerwonym i zielonym okręgiem; (A) K8S nie konkuruje o pierwsze miejsce wiązania, natomiast konkuruje o drugie, (B) K10S oraz K12S konkuruje o pierwsze i prawdopodobnie drugie miejsce wiązania.

b) *Oddziaływanie KXS i SXS z HSA [Publikacja 5]*

Drobna modyfikacja strukturalna ($-\text{SO}_3^-$ vs. $-\text{O}-\text{SO}_3^-$) prowadzi do istotnych różnic w polarności części hydrofilowej, które skutkują jakościowo i ilościowo odmiennym profilem wiązania z HSA (Tabela 14). SXS są silniejszymi ligandami, tworzącymi z białkiem kompleksy o wyższej stechiometrii. Obecność atomu tlenu w SXS ($-\text{O}-\text{SO}_3^-$) sprawia, że ich fragment jonowy jest bardziej polarny i ma większy efektywny ładunek ujemny niż część hydrofilowa KXS ($-\text{SO}_3^-$). Dzięki temu SXS tworzy silniejsze oddziaływania elektrostatyczne z dodatnimi centrami HSA, co tłumaczy ich wyższe powinowactwo ($\log K$) i mniej niekorzystny bilans entropowy. Silne, egzotermiczne oddziaływania polarne (ujemne ΔH) wiążą ligand w sztywnej pozycji, co ogranicza jego ruchy i zmniejsza entropię (ujemne $T\Delta S$). Jest to szczególnie widoczne dla KXS, których wiązanie jest silnie powiązane ze stratą entropii. Ponadto wyniki pokazują, że subdomena IIIA HSA jest uniwersalnym miejscem wiązania dla obu klas surfaktantów. Jednakże tylko SXS, dzięki swojej budowie, posiadają dodatkowe, specyficzne miejsce w subdomenie IB sąsiadującej z subdomeną IIIA, co przekłada się na ich wyższą stechiometrię wiązania i większe powinowactwo do albuminy (Rysunek 10).

Tabela 14. Główne różnice w oddziaływaniach KXS (K12S, K10S) oraz SXS (S12S, S10S) z albuminą surowicy ludzkiej (HSA).

Aspekt	1-alkilosulfoniany (KXS: K12S, K10S)	1-alkilosiarczany (SXS: S12S, S10S)
Liczba miejsc wiązania i stechiometria	Tylko jedno miejsce o stechiometrii ~ 1:1 (ligand:białko).	Dwa odrębne miejsca: ▪ Pierwsze: stechiometria ~ 1:1 (wysokie powinowactwo). ▪ Drugie: stechiometria ~ 4:1 (niższe powinowactwo).
Siła wiązania (powinowactwo)	Znacznie niższa. Stała wiązania dla 1. miejsca: $\log K_1 \sim 4,6$.	Znacznie wyższa. Stała wiązania dla 1. miejsca: $\log K_1 \sim 5,8-6,1$. Oznacza, że SXS wiążą się ok. 30-40 razy silniej niż KXS.
Mechanizm termodynamiczny	Silnie ujemna entalpia (ΔH) kompensuje wyraźnie niekorzystny (ujemny) wkład entropowy ($T\Delta S$). Wiązanie jest silnie sterowane entalpowo.	Także ujemna entalpia (ΔH), ale ze znacznie mniej niekorzystnym wkładem entropowym ($T\Delta S$). Wiązanie ma charakter mieszany (entalpowo-entropowy).
Wpływ na fluorescencję HSA	Powodują wzrost intensywności fluorescencji i przesunięcie hipsochromowe.	Wywołują silniejszy niż KXS efekt wzrostu intensywności fluorescencji i przesunięcie maksimum pasma emisji, a efekt rośnie z długością łańcucha.
Lokalizacja miejsc wiązania	Wiążą się wyłącznie w jednym miejscu w subdomenie IIIA.	Wiążą się w dwóch miejscach: ▪ 1. miejsce: charakterystyczne dla SXS, zlokalizowane w rejonie subdomeny IB graniczącym z subdomeną IIIA. ▪ 2. miejsce (wspólne z KXS): w subdomenie IIIA, w bezpośrednim sąsiedztwie miejsca KXS.



Rysunek 10. Struktura przestrzenna HSA z zaznaczoną odpowiednio resztą tryptofanu (Trp-214); (A) Pierwsze miejsce wiążące ligandy K12S oraz K10S oznaczono odpowiednio czerwonym okręgiem; (B) Pierwsze oraz drugie miejsce wiążące ligandy S12S oraz S10S oznaczono odpowiednio czerwonym i zielonym okręgiem.

4.4.2 Analiza procesów agregacji surfaktantów [Publikacja 6]

Badania podatności dodecylosiarczanu sodu (SDS) do agregacji w obecności serii 1-alkilosulfonianów (KXS: K8S, K10S, K12S) oraz NaCl ujawniły fundamentalną różnicę w zachowaniu związku o najdłuższym łańcuchu, tj. 1-dodekanosulfonianu sodu (K12S). Wykazano, że podczas gdy obecność NaCl, K8S i K10S wpływa na micelizację SDS w sposób porównywalny i niespecyficzny (głównie poprzez efekt siły jonowej, zmniejszający odpychanie elektrostatyczne między grupami jonowymi surfaktantów), K12S działa w sposób odmienny, specyficzny. Wskazują na to poniższe dowody eksperymentalne (Table 1, Publikacja 6):

- a) *Obniżenie krytycznego stężenia micelnego (CMC)*: K12S obniża wartość CMC SDS znacznie silniej niż pozostałe związki (K8S, K10S, NaCl) przy tym samym stężeniu. To wskazuje, że nie tylko zmniejsza odpychanie elektrostatyczne, ale aktywnie stabilizuje micelę.
- b) *Termodynamika micelizacji*: Proces micelizacji SDS w obecności K12S staje się wyraźnie bardziej korzystny termodynamicznie (ujemne ΔG^0_{mic}), jest sterowany zarówno czynnikiem entalpowym, jak i silnym, korzystnym przyrostem entropii.
- c) *Wzrost liczby agregacji (N_{agg})*: Obecność K12S, podobnie jak innych badanych ligandów, zwiększa rozmiar miceli, jednakże dla K12S efekt ten jest szczególnie wyraźny.

Symulacje dynamiki molekularnej dostarczyły bezpośredniego dowodu na specyficzne zachowanie K12S. Profil energii swobodnej dla włączenia anionu K12S do miceli SDS jest praktycznie identyczny z profilem dla samoorganizacji SDS (Publikacja 6, Figure 6). Dla K8S i K10S profile te wskazują na znacznie niższe powinowactwo. Ponadto, symulacje długoczasowe układów, w których część anionów SDS została zastąpiona anionami KXS, wykazały, że tylko K12S jest trwale włączany do struktury miceli, tworząc stabilne micidele o mieszanym składzie (SDS/K12S). K8S i K10S mają tendencję do wypierania (dysocjacji) z miceli.

Można zatem przypuszczać, że z uwagi na identyczną długość łańcucha hydrofobowego (C12-), ligand K12S jest nierozróżnialny dla procesu micelizacji SDS

pod względem oddziaływań hydrofobowych. W konsekwencji, SDS "nie rozpoznaje" K12S jako obcego indywiduum i pozwala mu pełnić funkcję mimetyka surfaktantu i współuczestniczyć w tworzeniu rdzenia micelnego.

4.5 Tworzenie kompleksów inkluzyjnych KXS i SXS z β -cyklodekstryną – konsekwencje dla ochrony struktury lizozymu [Publikacja 7, 8]

Badania opisane w publikacjach 7 i 8 koncentrowały się na analizie tworzenia kompleksów inkluzyjnych anionowych surfaktantów (1-alkilosulfonianów, KXS, oraz 1-alkilosiarczanów, SXS) z β -cyklodekstryną. Głównym celem była analiza, w jaki sposób wiązanie hydrofobowych łańcuchów KXS we wnęce cyklodekstryny wpływa na ich oddziaływanie z białkiem modelowym, lizozymem, oraz czy proces ten może skutkować ochroną struktury drugorzędowej białka przed denaturującym działaniem surfaktantów.

4.5.1 Powstawanie kompleksów inkluzyjnych [Publikacja 7, 8]

Badania kompleksów inkluzyjnych ligandów anionowych (KXS i SXS) z β -CD wykazały, że obie serie tworzą kompleksy o stechiometrii $\sim 1:1$, a stabilność termodynamiczna ($\log K$, $|\Delta G|$) rośnie wraz z długością łańcucha węglowodorowego w obu grupach: S8S/K8S < S10S/K10S < S12S/K12S. Główne różnice znajdują swoje odzwierciedlenie w mechanizmie termodynamicznym procesu kompleksowania i jego dynamice.

Dla SXS: Proces jest sterowany w porównywalnym stopniu zarówno entalpowo jak i entropowo. Wyraźniej większe ujemne wartości ΔH , w porównaniu z KXS, wskazują na istotną rolę bezpośrednich oddziaływań molekularnych: elektrostatycznych (naładowany fragment jonowy-grupy -OH β -CD) oraz van der Waalsa (łańcuch alkilowy-hydrofobowa wnęka). Wkład entropii, choć wyraźny, ma w tym przypadku mniejsze znaczenie.

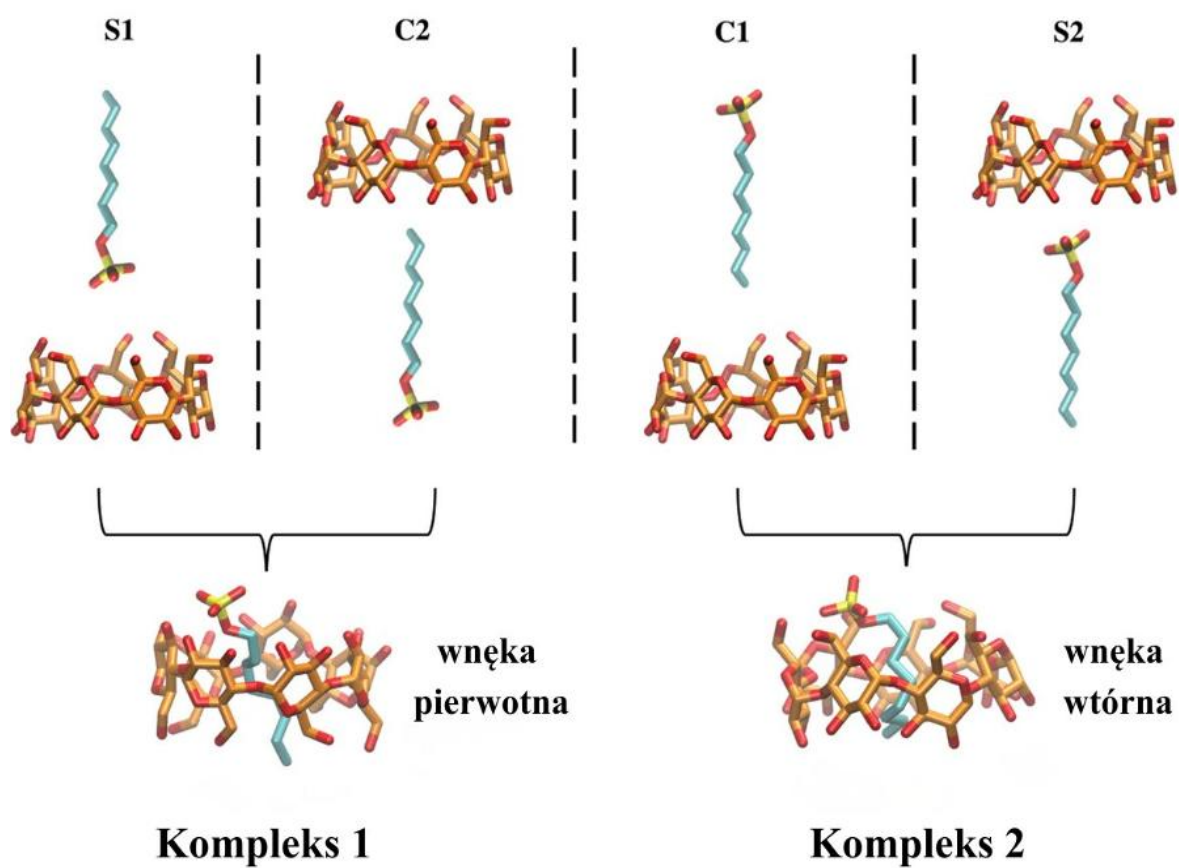
Dla KXS: Proces jest sterowany entropowo, mimo że towarzyszy mu uwalnianie ciepła ($|\Delta H| < |T\Delta S|$). Wskazuje to, że istotną rolę w stabilności powstających kompleksów inkluzyjnych odgrywają oddziaływania hydrofobowe: korzystny przyrost entropii układu związany z wypieraniem uporządkowanych cząsteczek wody z hydrofobowych powierzchni

podczas włączenia liganda do wnęki. Oddziaływania bezpośrednie (ujemne ΔH) są obecne, ale słabsze niż dla SXS.

Różnice w parametrach termodynamicznych oddziaływania KXS oraz SXS z β -cyklodekstryną można tłumaczyć odmienną budową grupy polarnej obu rodzajów ligandów:

- Ugrupowanie $-O-SO_3^-$ w SXS jest bardziej polarne i naładowane, co sprzyja silnym oddziaływaniom elektrostatycznym z β -CD, ale również silniej wiąże cząsteczki wody, czyniąc dehydratację mniej korzystną entropowo.
- Ugrupowanie $-SO_3^-$ w KXS jest mniej polarne, przez co oddziaływania elektrostatyczne są słabsze, ale jego sfera hydratacyjna jest mniej uporządkowana – uwolnienie wody na skutek tworzenia kompleksu daje większy przyrost entropii, stając się główną siłą wiązania.

Symulacje dynamiki molekularnej ujawniły, że sposób wnikania liganda do wnęki β -CD (czyli orientacja i koniec łańcucha, przez który wchodzi) zależy od długości łańcucha i rodzaju grupy polarnej (Rysunek 11). Uzyskane metodą symulacji dynamiki molekularnej potencjalne sposoby wejścia gościa do wnęki gospodarza zestawiono w Tabeli 15.



Rysunek 11. Cztery potencjalne mechanizmy wejścia ligandów SXS/KXS do wnęki β -CD: S1: S-końcem przez wnękę pierwotną (węższą), C2: C-końcem przez wnękę wtórną (szerszą), C1: C-końcem przez wnękę pierwotną, S2: S-końcem przez wnękę wtórną.

Tabela 15. Porównanie mechanizmów inkluzji ligandów do wnęki β -cyklodekstryny.

<i>Cecha/Ligand</i>	<i>S12S</i> (C12-O-SO ₃ ⁻)	<i>S10S</i> (C10-O-SO ₃ ⁻)	<i>S8S</i> (C8-O-SO ₃ ⁻)	<i>K12S</i> (C12-SO ₃ ⁻)	<i>K10S</i> (C10-SO ₃ ⁻)	<i>K8S</i> (C8-SO ₃ ⁻)
Preferowany mechanizm wnikania	S1 (S-końcem, tworzy kompleks wchodząc przez wnękę węższą)	C1/C2 (C-końcem przez obie wnęki)	elastyczny (C1, C2, S1)	C1 (C-końcem przez wnękę węższą)	C2 (C-końcem przez wnękę szerszą)	C1/C2 (C-końcem przez obie wnęki) oraz S2 (unikatowy w obrębie badanych ligandów mechanizm)
Stabilność kompleksu	stabilny; dobre dopasowanie łańcucha C12 do objętości wnęki	stabilny	stabilny	stabilny kompleks typu 2	stabilny kompleks typu 1	kompleks nietrwały w skali symulacji
Interpretacja	długie, sztywne ugrupowanie; część hydrofilowa zaangażowana w wejście	kompromis między długością łańcucha a oddziaływaniami części hydrofilowej	krótki łańcuch pozwala na wiele orientacji	preferuje wejście C-końcem	preferuje wejście C-końcem	za krótki łańcuch i zbyt słabe oddziaływania dla stabilnej inkluzji

4.5.2 Ochrona struktury lizozymu [Publikacja 8]

Badania wykazały, że wpływ 1-alkilosulfonianów (KXS) na strukturę drugorzędową lizozymu jest silnie zależny od długości ich łańcuchów hydrofobowych. Podczas gdy K8S nie wywołuje zmian, a K10S powoduje jedynie częściowe rozwinięcie białka, K12S prowadzi do poważnego zaburzenia struktury α -helikalnej lizozymu. Jest to bezpośredni skutek silnych oddziaływań hydrofobowych między długim łańcuchem liganda a hydrofobowymi regionami białka, które skutkują jego rozfałdowaniem.

Tendencja 1-alkilosulfonianów do tworzenia stabilnych kompleksów inkluzyjnych z β -CD sprawia, że cyklodekstryna może pełnić funkcję ochronną. Działając jako skuteczny ligand konkurencyjny, β -cyklodekstryna odwraca destrukcyjny wpływ surfaktantów na białka. Dowodzą tego następujące argumenty:

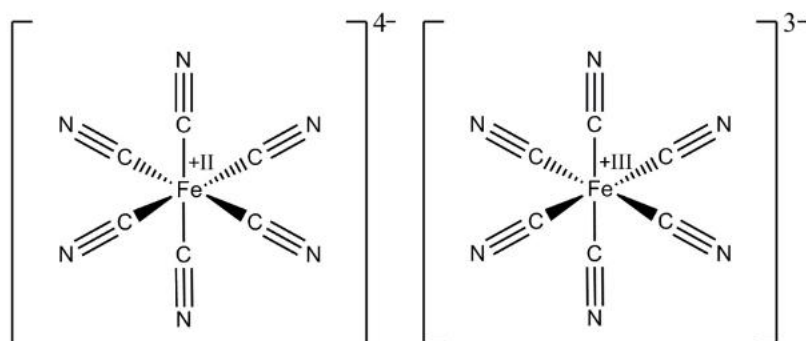
1. *Przywracanie struktury natywnej*: Dodatek β -CD do układów K12S-lizozym i K10S-lizozym powoduje stopniowy wzrost zawartości struktury α -helikalnej, aż do przywrócenia poziomu charakterystycznego dla natywnego, wolnego białka. Oznacza to, że β -CD usuwa ligandy KXS z białka, umożliwiając lizozymowi ponowne zwinięcie się w prawidłową konformację.
2. *Zależność stężenie-efekt*: W przypadku najsilniej działającego liganda K12S stopień odtworzenia struktury białka jest proporcjonalny do stężenia β -CD. Im więcej β -CD w układzie, tym pełniejsza regeneracja struktury lizozymu. Do zneutralizowania słabszego działania K10S wymagane jest niższe stężenie β -CD, co potwierdza mechanizm konkurencyjnego wiązania.
3. *Ochrona stabilności termicznej*: Badania kalorymetryczne (DSC) dostarczają bezpośredniego dowodu na ochronną rolę β -CD:
 - W układzie K12S-lizozym struktura białka jest na tyle zaburzona, że nie można wyznaczyć jego temperatury przejścia (T_m).

- W układzie K12S-lizozym- β -CD stabilność termiczna białka zostaje w pełni przywrócona: wartość T_m jest porównywalna z wartością dla wolnego lizozymu, a proces denaturacji pozostaje odwracalny bez procesów agregacji.

Przedstawione w publikacji 8 wyniki badań wskazują, że β -CD pełni funkcję ochronną, działając jako molekularna pułapka dla surfaktantów KXS. Wiążąc ich hydrofobowe łańcuchy w swojej wnęce, skutecznie konkuruje o nie z lizozymem. Ta konkurencja zapobiega destrukcyjnym oddziaływaniom hydrofobowym między K12S a białkiem, co bezpośrednio przekłada się na zachowanie integralności strukturalnej, odtworzenie prawidłowej konformacji drugorzędowej oraz naturalną stabilność termiczną lizozymu.

4.6 Badania oddziaływań jonów heksacyjanożelazianowych(II)/(III) z albuminami i lizozymem [Publikacja 9, 10]

Badania oddziaływań jonów heksacyjanożelazianowych(II)/(III) z białkami, takimi jak albuminy (BSA, HSA) i lizozym, są uzasadnione z kilku powodów. Po pierwsze, jony te stanowią idealny model indywiduów obdarzonych wysokim ładunkiem ujemnym, o dobrze zdefiniowanej geometrii (oktaedrycznej) i rozmiarze (Rysunek 12). Dzięki temu pozwalają na ilościowe badanie podstawowych sił wiązania, głównie oddziaływań elektrostatycznych, bez komplikacji związanych z efektem hydrofobowym lub specyficznym rozpoznaniem kształtu. Po drugie, porównanie ich powinowactwa do różnych białek (np. anionowej albuminy i kationowego lizozymu) służy jako test dla zrozumienia podstaw oddziaływań ligand-białko. Pozwala zweryfikować modele opisujące rolę ładunku powierzchniowego białka, potencjału elektrostatycznego i dostępu do miejsc wiązania.



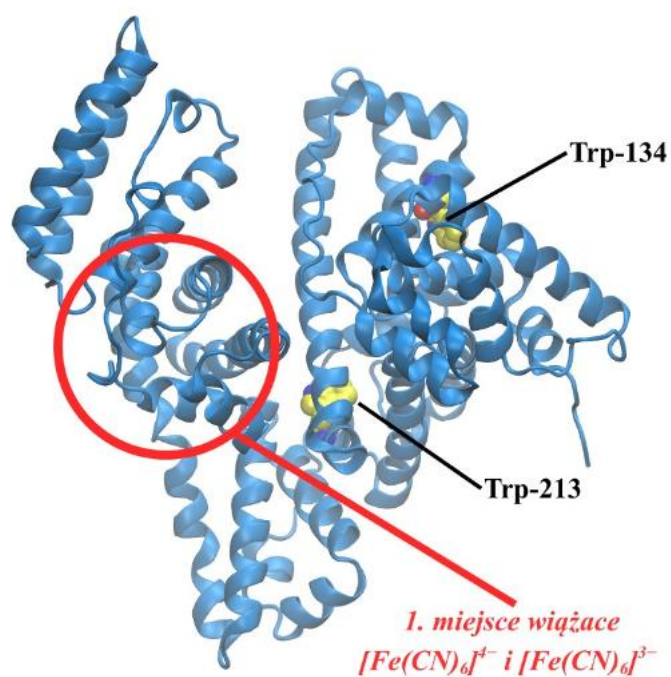
Rysunek 12. Wzór strukturalny jonu kompleksowego $[Fe(CN)_6]^{4-}$ oraz $[Fe(CN)_6]^{3-}$.

4.6.1 Oddziaływanie z albuminami [Publikacja 9]

Badania kalorymetryczne (ITC) wykazały, że zarówno BSA, jak i HSA posiadają jedno główne miejsce wiązania dla jonów heksacyjanożelazianowych(II)/(III), tworząc kompleksy o stechiometrii $\sim 1:1$. Siła wiązania jest porównywalna dla obu anionów ($[\text{Fe}(\text{CN})_6]^{4-}$ i $[\text{Fe}(\text{CN})_6]^{3-}$), co wskazuje, że ładunek liganda nie ma decydującego znaczenia. Zasadnicza różnica dotyczy rodzaju albuminy: wiązanie jest wyraźnie silniejsze z BSA niż z HSA. Mechanizm jest sterowany entalpowo, a stabilność zapewniają głównie oddziaływania elektrostatyczne (ładunek-ładunek) oraz van der Waalsa.

Badania fluorescencyjne wykazały, że wiązanie obu jonów powoduje statyczne wygaszanie fluorescencji reszt tryptofanu. Towarzyszy temu hipsochromowe przesunięcie pasma emisji, co świadczy o zmianie środowiska tych reszt na bardziej hydrofobowe. Eksperymenty konkurencyjne z markerami miejsc Sudlowa (warfaryna: miejsce I, ibuprofen; miejsce II) wykazały, że jony heksacyjanożelazianowe(II)/(III) konkurują o dostęp do tych regionów, ale nie wiążą się w jednym, ściśle specyficznym miejscu. Sugeruje to wiązanie w rozległych, dodatnio naładowanych regionach powierzchni albumin. Symulacje dynamiki molekularnej wskazały region Sudlowa II (subdomena IIIA) jako preferencyjne miejsce oddziaływań dla obu jonów z BSA (Rysunek 13). Analiza interakcji wykazała, że kluczowy wkład w proces wiązania mają dodatnio naładowane reszty aminokwasowe (przede wszystkim lizyny i argininy). Potwierdza to tym samym elektrostatyczny charakter obserwowanych oddziaływań. Jony $[\text{Fe}(\text{CN})_6]^{4-}$, dzięki wyższemu ładunkowi, zajmują bardziej zdefiniowane pozycje w tej okolicy. Obecność jonów $[\text{Fe}(\text{CN})_6]^{4-}$ i $[\text{Fe}(\text{CN})_6]^{3-}$ nie powoduje istotnych zmian w strukturze BSA ani HSA. Oznacza to, że oddziaływania te mają charakter powierzchniowy i niedestrukcyjny, nie wpływając bezpośrednio na stabilność rdzenia białka ani jego aktywność biologiczną związaną z konformacją.

W Tabeli 16 zestawiono najważniejsze informacje podsumowujące badane oddziaływania.



Rysunek 13. Struktura przestrzenna BSA z zaznaczonymi odpowiednio resztami tryptofanu (Trp-134, Trp-213). Preferencyjne pierwsze miejsce wiążące jony $[Fe(CN)_6]^{4-}$ i $[Fe(CN)_6]^{3-}$ oznaczono odpowiednio czerwonym okręgiem.

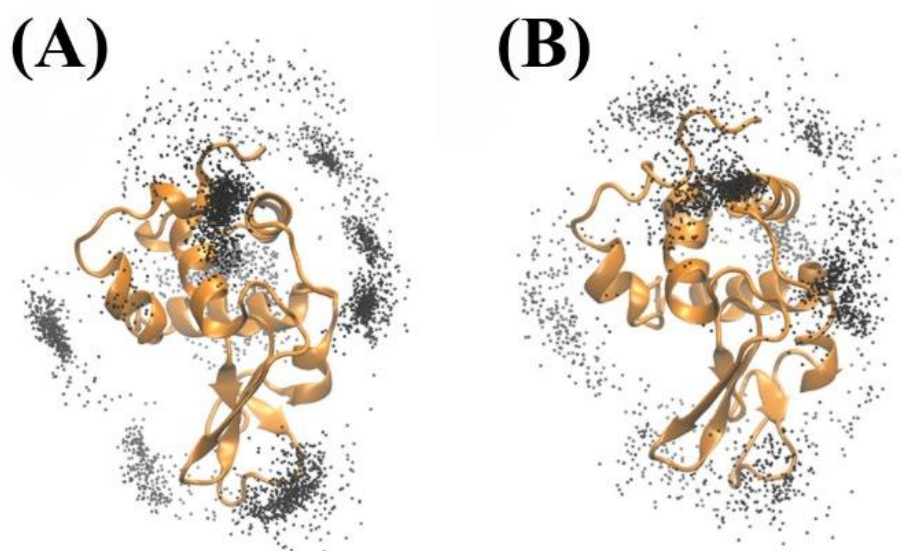
Tabela 16. Porównanie oddziaływań $[Fe(CN)_6]^{4-}$ i $[Fe(CN)_6]^{3-}$ z albuminą surowicy bydlęcej (BSA) i ludzkiej (HSA).

Kryterium/ Parametr	$[Fe(CN)_6]^{4-}$	$[Fe(CN)_6]^{3-}$	Porównanie BSA vs. HSA	Główne wnioski
Liczba miejsc wiązania i stechiometria	jedno miejsce o stechiometrii ~ 1:1 (ligand:białko)	jedno miejsce o stechiometrii ~ 1:1 (ligand:białko)	identyczna dla obu albumin	Oba jony zajmują pojedyncze miejsce na powierzchni albuminy.
Siła wiązania ($\log K$, ΔG)	BSA: $\log K = 5,31 (\pm 0,11)$ $\Delta G = -7,25 (\pm 0,03)$ [kcal/mol]	BSA: $\log K = 5,23 (\pm 0,05)$ $\Delta G = -7,14 (\pm 0,07)$ [kcal/mol]	wyraźnie silniejsze wiązanie do BSA niż HSA; różnica ładunku liganda (-4 vs. -3) ma pomijalny wpływ na ogólną siłę wiązania z tym samym białkiem	BSA ma większe powinowactwo do tych anionów. Ładunek liganda nie jest czynnikiem decydującym.
	HSA: $\log K = 4,78 (\pm 0,03)$ $\Delta G = -6,52 (\pm 0,04)$ [kcal/mol]	HSA: $\log K = 4,70 (\pm 0,02)$ $\Delta G = -6,41 (\pm 0,02)$ [kcal/mol]		
Mechanizm termodynamiczny	sterowanie entalpowe ($ \Delta H > T\Delta S $); duży, ujemny wkład ΔH	sterowanie entalpowe ($ \Delta H > T\Delta S $); duży, ujemny wkład ΔH	mechanizm identyczny; wartości ΔH są bardziej ujemne dla HSA, ale nie przekładają się na wyższe powinowactwo	Oddziaływania elektrostatyczne są główną siłą napędową wiązania.
Wpływ na fluorescencję	statyczne wygaszanie fluorescencji Trp; niewielkie przesunięcie hipsochromowe	statyczne wygaszanie fluorescencji Trp; niewielkie przesunięcie hipsochromowe	jakościowo identyczny efekt dla obu białek	Jony wiążą się w pobliżu reszt Trp, zmieniając ich środowisko na bardziej hydrofobowe.

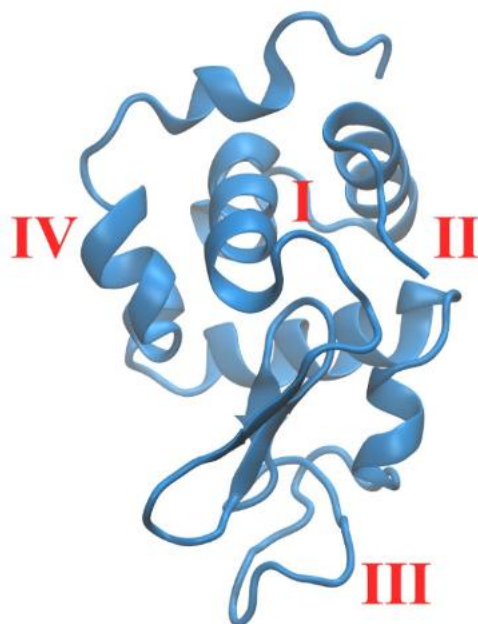
<i>Kryterium/ Parametr</i>	<i>$[Fe(CN)_6]^{4-}$</i>	<i>$[Fe(CN)_6]^{3-}$</i>	<i>Porównanie BSA vs. HSA</i>	<i>Główne wnioski</i>
<i>Lokalizacja (eksperymenty konkurencyjne)</i>	konkurencja z warfaryną (Sudlow I) i ibuprofenem (Sudlow II)	konkurencja z warfaryną (Sudlow I) i ibuprofenem (Sudlow II)	prawdopodobnie zbliżona lokalizacja w obu białkach	Nie wiążą się specyficznie w jednym punkcie, ale w rozległych regionach obejmujących miejsca Sudlowa.
<i>Lokalizacja (symulacje MD dla BSA)</i>	w regionie Sudlowa II (subdomena IIIA); oddziaływania z Arg-409, Arg-194, Lys-114 i innymi resztami dodatnimi	w regionie Sudlowa II (subdomena IIIA); oddziaływania z Lys-114, Lys-537, Arg-194 i innymi resztami dodatnimi	wyniki symulacji MD pozostają w zgodzie z danymi eksperymentalnymi dotyczącymi miejsc wiązania ligandów z BSA	Elektrostatyczne wiązanie w dodatnich obszarach ładunków na powierzchni białka. $[Fe(CN)_6]^{4-}$ zajmuje bardziej zdefiniowane pozycje.
<i>Wpływ na strukturę drugorzędową (widma CD)</i>	brak istotnego wpływu na zawartość α -helisy	brak istotnego wpływu na zawartość α -helisy	brak zmian w obu białkach	Oddziaływanie ma charakter powierzchniowy i niedestrukcyjny – nie denaturuje białka.
<i>Podsumowanie charakteru wiązań</i>	niespecyficzne wiązanie elektrostatyczne przez zlokalizowany ładunek ujemny	niespecyficzne wiązanie elektrostatyczne przez zlokalizowany ładunek ujemny	jony są modelowymi ligandami do badania podstawowych oddziaływań anion-białko	Kluczową rolę odgrywa gęstość ładunku dodatniego na powierzchni białka, a nie szczegóły struktury liganda.

4.6.2 Oddziaływanie z lizozymem [Publikacja 10]

Badania opisane w publikacji 10 ujawniły dominującą rolę oddziaływań elektrostatycznych w wiązaniu $[\text{Fe}(\text{CN})_6]^{4-}$ oraz $[\text{Fe}(\text{CN})_6]^{3-}$ do dodatnio naładowanego lizozymu, z wyraźną zależnością od ładunku liganda. Badane procesy są typowymi przykładami sterowanego oddziaływania elektrostatycznymi, wiązania jonów na powierzchni białka. Wykazano, że siła tego wiązania jest bezpośrednio proporcjonalna do ładunku liganda, co znajduje potwierdzenie w termodynamice, lokalizacji z symulacji MD (gęstsze skupiska dla $[\text{Fe}(\text{CN})_6]^{4-}$) (Rysunek 14) oraz w kontraście z zachowaniem tych ligandów wobec albumin (Tabela 17). Proces ten nie narusza jednak integralności strukturalnej białka. Na Rysunku 15 przedstawiono cztery regiony na powierzchni lizozymu zdolne do wiązania jonów $[\text{Fe}(\text{CN})_6]^{4-}$ oraz $[\text{Fe}(\text{CN})_6]^{3-}$.



Rysunek 14. Gęstość centrów mas ligandów w promieniu $10,0 \text{ \AA}$ od białka, wizualizowana z krokiem 1 ns z $0,5 \text{ \mu s}$ symulacji: (A) $[\text{Fe}(\text{CN})_6]^{4-}$, (B) $[\text{Fe}(\text{CN})_6]^{3-}$.



Rysunek 15. Cztery zidentyfikowane regiony (I-IV) na powierzchni lizozymu zdolne do wiązania jonów $[\text{Fe}(\text{CN})_6]^{4-}$ oraz $[\text{Fe}(\text{CN})_6]^{3-}$.

Badania biochemiczne jednoznacznie wykazały, że wiązanie jonów $[\text{Fe}(\text{CN})_6]^{4-}$ i $[\text{Fe}(\text{CN})_6]^{3-}$ z lizozymem nie wpływa istotnie na jego aktywność enzymatyczną. Pomimo tworzenia się stabilnych kompleksów, jony te nie działają jako inhibitory kompetencyjne, tj. nie blokują dostępu do centrum aktywnego enzymu. Aktywność katalityczna lizozymu wobec substratu (4-MUF-triNAG) po godzinnej inkubacji z jonami utrzymywała się na wysokim poziomie. Dla $[\text{Fe}(\text{CN})_6]^{3-}$ nie zaobserwowano istotnej zmiany, a dla $[\text{Fe}(\text{CN})_6]^{4-}$ aktywność względna spadła jedynie do $\sim 82\%$ w porównaniu z wolnym enzymem. Analiza kinetyki reakcji potwierdziła, że obecność któregośkolwiek z jonów nie zmienia ani powinowactwa enzymu do substratu (K_m), ani jego maksymalnej aktywności katalitycznej ($V_{\max}$). Oznacza to, że mechanizm katalizy pozostaje nienaruszony. Zaobserwowana, niewielka redukcja aktywności w obecności $[\text{Fe}(\text{CN})_6]^{4-}$ może wynikać z jego silniejszego wiązania elektrostatycznego z powierzchnią enzymu, które w minimalnym stopniu może wpływać na dynamikę lub dostępność regionu aktywnego. Co istotne, profil aktywności biologicznej kompleksów (jak i wolnego lizozymu) był przede wszystkim zdeterminowany przez budowę ściany komórkowej bakterii. Enzym skuteczniej działał na bakterie Gram-dodatnie (np. *B. subtilis*), podczas gdy bakterie Gram-ujemne

(np. *E. coli*, *P. aeruginosa*) były mniej podatne ze względu na ochronną obecność zewnętrznej błony lipidowej.

Podsumowując, wyniki badań wskazują, że jonów heksacyjanożelazianowych(II)/(III) nie można uznać za inhibitory lizozymu. Ich wiązanie, choć silne i zależne od ładunku, ma charakter powierzchniowy i nieingerujący w centrum katalityczne, co potwierdza, że oddziaływania czysto elektrostatyczne z regionami oddalonymi od miejsca aktywnego nie zakłócają funkcji enzymatycznej tego białka.

Tabela 17. Podsumowanie oddziaływań jonów $[Fe(CN)_6]^{4-}$ i $[Fe(CN)_6]^{3-}$ z lizozymem.

Kryterium/ Parametr	$[Fe(CN)_6]^{4-}$	$[Fe(CN)_6]^{3-}$	Wnioski ogólne
Liczba miejsc wiązania i stechiometria	jedno miejsce wiążące; stechiometria ~ 1:1 (ligand:białko)	jedno miejsce wiążące; stechiometria ~ 1:1 (ligand:białko)	Lizozym posiada jedno główne miejsce wiązania dla obu anionów.
Sila wiązania (logK)	$\log K = 4,56 (\pm 0,03)$ (silniejsze wiązanie)	$\log K = 3,90 (\pm 0,02)$ (słabsze wiązanie)	Ładunek liganda ma decydujące znaczenie. Jon o wyższym ładunku ujemnym ($[Fe(CN)_6]^{4-}$) wiąże się istotnie silniej.
Mechanizm termodynamiczny	silne sterowanie entalpowe ($ \Delta H > T\Delta S $)	silne sterowanie entalpowe ($ \Delta H > T\Delta S $)	Stabilność kompleksu jest napędzana głównie przez oddz. elektrostatyczne (ładunek-ładunek) i van der Waalsa.
Wpływ na strukturę drugorzędową (widma CD)	niewielkie, nieznaczące zmiany	niewielkie, nieznaczące zmiany	Wiązanie ma charakter powierzchniowy i niedestrukcyjny – nie zaburza struktury rdzenia białka.
Wpływ na fluorescencję lizozymu	statyczne wygaszanie fluorescencji bez przesunięcia maksimum pasma emisji	statyczne wygaszanie fluorescencji bez przesunięcia maksimum pasma emisji	Ligand wiąże się w obszarze reszt Trp, jednak nie następuje relokacja tych reszt do środowiska o innej polarności. Jest to bezpośredni dowód na to, że wiązanie liganda nie indukuje zmiany konformacji (fałdowania) białka.
Lokalizacja (symulacje MD)	wiąże się w 4 regionach bogatych w dodatnie reszty (Arg, Lys); punkty lokalizacji są gęsto skupione	wiąże się w 4 regionach bogatych w dodatnie reszty (Arg, Lys); punkty lokalizacji są bardziej rozproszone	Elektrostatyka steruje lokalizacją. Wyższy ładunek $[Fe(CN)_6]^{4-}$ prowadzi do bardziej zlokalizowanego i stabilnego przyłączenia.
Porównanie z albuminami (BSA/HSA)	wiązanie z lizozymem jest słabsze niż z albuminami	wiązanie z lizozymem jest słabsze niż z albuminami	Sila wiązania zależy od specyfiki powierzchni białka (rozmieszczenie ładunków, elastyczność), a nie tylko od jego ładunku.

5. Podsumowanie

W niniejszej pracy przedstawiono wyniki badań nad oddziaływaniem białek z serią ligandów, stanowiących przykłady związków o zróżnicowanej złożoności strukturalnej i właściwościach fizykochemicznych (Tabela 18). Badane ligandy, pomimo że nie są lekami *per se*, reprezentują kluczowe motywy strukturalne i fizykochemiczne powszechnie występujące w związkach wykazujących udokumentowane właściwości biologiczne (np. lipopeptydy, niesteroidowe leki przeciwzapalne, potencjalne leki przeciwcukrzycowe na bazie związków wanadu, kompleksy metaloorganiczne itp.). Zastosowane podejście umożliwiło systematyczną analizę wpływu kluczowych parametrów ligandów, takich jak rozmiar, ładunek oraz równowaga hydrofilowo-hydrofobowa badanego indywiduum, na mechanizm i siłę ich oddziaływań z białkami. Udokumentowano, że domenowa budowa i plastyczność białek pozwalają na selektywne wiązanie ligandów różniących się właściwościami, co leży u podstaw ich funkcji biologicznych, takich jak transport (albuminy) czy funkcja ochronna (lizozym). Wykorzystanie możliwie szerokiego spektrum technik badawczych pozwoliło na sformułowanie ogólnych zasad, które określają, w jaki sposób struktura i właściwości fizykochemiczne ligandów kształtują ich interakcje z białkami (Tabela 19).

5.1 Liczba miejsc wiązania

Wykazano, że liczba miejsc wiązania jest zwykle niewielka (1-2) i zależy od dostępności wnęk w białku, które są komplementarne pod względem rozmiaru i charakteru chemicznego. Przykładem są 1-alkilosiarczany (SXS) oraz 1-alkilosulfoniany (KXS), których amfifilowość umożliwia wiązanie w kilku miejscach o różnej specyficzności.

5.2 Lokalizacja liganda

Lokalizacja liganda w strukturze białka jest determinowana zasadą dopasowania chemicznego: ligandy o ładunku ujemnym przylłączają się do dodatnich regionów, hydrofobowe wnikają do hydrofobowych wnęk, a amfifilowe wiążą się na granicy takich obszarów (Tabela 20). Pomimo wysokiego stopnia podobieństwa strukturalnego i funkcjonalnego między HSA i BSA, białka te mogą wykazywać istotne różnice w specyficzności wiązania ligandów. Dowodem takiego zachowania są oddziaływania z S12S i K12S. Dla liganda S12S obserwuje się wyraźną różnicę w miejscach wiązania. W przypadku HSA, S12S tworzy kompleksy w dwóch lokalizacjach: w subdomenie IB (graniczącej z subdomeną IIIA) oraz bezpośrednio w subdomenie IIIA. Zupełnie inaczej wygląda sytuacja w BSA, gdzie ten sam ligand wiąże się w subdomenie IA oraz w subdomenie IIB. Równie znaczące różnice ujawniają się w oddziaływaniach z ligandem K12S. W HSA związek ten wiąże się wyłącznie w jednym miejscu, tj. w subdomenie IIIA. Tymczasem w BSA ligand K12S traci tę specyficzność i tworzy kompleksy w dwóch różnych miejscach białka. Jego pierwsze miejsce zlokalizowane jest w subdomenie IA, natomiast drugie prawdopodobnie w subdomenie IIB, podobnie jak w przypadku liganda S12S. Kluczową rolę w lokalizacji odgrywa ładunek powierzchniowy. Dla silnie naładowanych jonów ($[\text{Fe}(\text{CN})_6]^{4-3-}$) oraz w przypadku lizozymu, wiązanie jest dyktowane oddziaływaniami elektrostatycznymi i zachodzi na rozległych powierzchniach o wysokim zagęszczeniu ładunku przeciwnego, a nie poprzez dopasowanie do wąskiej wnęki. W przypadku surfaktantów istotny wpływ ma długość łańcucha hydrofobowego. Dłuższe łańcuchy (C12-) umożliwiają zajmowanie większej liczby miejsc wiązania (np. dwa miejsca dla K12S w BSA vs. jedno dla K8S) oraz zapewniają silniejsze oddziaływanie z hydrofobowymi wnękami białka.

5.3 Stabilność strukturalna białka

Wpływ wiązania liganda na strukturę białka zależy od charakteru liganda. Dowiedziono, że przy stężeniach reagentów stosowanych w niniejszych badaniach, długołańcuchowe surfaktanty mogą destabilizować rdzeń hydrofobowy lub wywoływać zmiany konformacyjne, podczas gdy małe, naładowane jony powodują jedynie minimalne perturbacje.

5.4 Mechanizm wiązania i stechiometria

Na podstawie analizy parametrów termodynamicznych wykazano, że mechanizm wiązania i stechiometria są bezpośrednio związane z naturą liganda. Ładunek danego indywiduum determinuje wiązanie sterowane entalpowo, hydrofobowość – sprzyja powstawaniu połączeń, których trwałość uwarunkowana jest czynnikiem entropowym, a amfifilowość może prowadzić do mechanizmu mieszanego. Podczas gdy stechiometria ~ 1:1 jest typowa dla specyficznych miejsc, ligandy amfifilowe mogą wiązać się w większych stosunkach molowych.

Ponadto, badania wykazały, że surfaktanty anionowe SXS i KXS tworzą stabilne kompleksy inkluzyjne z β -cyklodekstryną (β -CD), co istotnie modyfikuje ich oddziaływanie z białkami. Cyklodekstryna pełni w tym układzie rolę molekularnego modulatora oddziaływań i pełni funkcję ochronną. Kluczowy mechanizm to kompetencyjne "pułapkowanie": β -CD selektywnie wiąże hydrofobowy łańcuch surfaktantu, a to z kolei skutecznie chroni to białko (tu: lizozym) przed denaturacją wywołaną oddziaływaniem z łańcuchami hydrofobowymi K12S, pozwalając zachować strukturę i aktywność enzymu. Obecność β -CD wprowadza nową równowagę, która może spowalniać, hamować lub odwracać proces denaturacji białka. Wykazano tym samym, że zdolność tego typu ligandów do tworzenia kompleksów z β -cyklodekstryną stanowi skuteczne narzędzie do kontroli aktywności biologicznej surfaktantów i ochrony białek przed destrukcyjnym działaniem wywołanym zatłoczeniem molekularnym.

Tabela 18. Charakterystyczne cechy strukturalne badanych ligandów.

Właściwość	Charakterystyka		
Rozmiar	(od najmniejszego do największego) 1. $[\text{Fe}(\text{CN})_6]^{4-/3-}$ (mały: sztywne, oktaedryczne kompleksy) 2. SXS/KXS (średni: liniowe łańcuchy alkilowe) 3. $[\text{B}(\text{Ph})_4]^-$ (duży: kulisty anion aromatyczny) 4. Polioksowanadany (bardzo duży: oligomeryczne jednostki koordynacyjne)		
Ładunek (wpływa na siłę oddziaływań elektrostatycznych)	niski: ładunek -1 ▪ SXS ▪ KXS ▪ $[\text{B}(\text{Ph})_4]^-$	średni: ładunek -3 ▪ $[\text{Fe}(\text{CN})_6]^{3-}$	wysoki: ładunki $-4, -6$ ▪ $[\text{Fe}(\text{CN})_6]^{4-}$ ▪ polioksowanadany
Hydrofilowość/Hydrofobowość	hydrofilowe (aniony nieorganiczne) ▪ polioksowanadany ▪ $[\text{Fe}(\text{CN})_6]^{4-/3-}$	amfifilowe (część hydrofilowa + hydrofobowy łańcuch) ▪ SXS ▪ KXS	hydrofobowe (ładunek w hydrofobowej otoczce) ▪ $[\text{B}(\text{Ph})_4]^-$
Mechanizm oddziaływań z białkami {determinuje selektywność i lokalizację wiązania oraz wpływa na termodynamikę (udział entalpii vs. entropii)}	elektrostatyczne ▪ $[\text{Fe}(\text{CN})_6]^{4-/3-}$	mieszane (elektrostatyczne + hydrofobowe) ▪ SXS ▪ KXS (wiązanie przez część hydrofilową + efekt łańcucha) ▪ polioksowanadany	hydrofobowe (+ słabe elektrostatyczne) ▪ $[\text{B}(\text{Ph})_4]^-$ (głównie efekt hydrofobowy)

Tabela 19. Wpływ cech strukturalnych oraz fizykochemicznych badanych ligandów na ich oddziaływanie z białkami.

Cecha liganda	Wpływ na liczbę miejsc wiązania	Wpływ na lokalizację w BSA/HSA	Wpływ na lokalizację w lizozymie	Wpływ na zmiany strukturalne białka	Wpływ na mechanizm i stechiometrię wiązania
Rozmiar/Objętość	▪ duże ligandy ($[\text{B(Ph)}_4]^-$, polioksowanadany) zwykle wiążą się w 1-2 miejscach ze względu na ograniczoną dostępność dużych, komplementarnych wnęk ▪ średnie ligandy (SXS/KXS) mogą wiązać się w kilku miejscach o różnej specyficzności (np. 2 miejsca dla SDS w BSA) ▪ małe ligandy ($[\text{Fe(CN)}_6]^{4-/3-}$) często wiążą się w jednym głównym miejscu o wysokim powinowactwie	▪ duże/objętościowe ligandy (np. $[\text{B(Ph)}_4]^-$) lokalizują się w dużych hydrofobowych wnękach ▪ średnie/amfifilowe (SXS) – w specyficznych miejscach z dodatnimi resztami ▪ małe/naładowane ($[\text{Fe(CN)}_6]^{4-/3-}$) – w regionach o dużym zagęszczeniu ładunku dodatniego	▪ wszystkie jony lokalizują się na powierzchni bogatej w Arg/Lys, z dala od centrum aktywnego ▪ rozmiar wpływa na mobilność/rozłokowanie w obrębie tych regionów	▪ amfifile o długich łańcuchach, np. S12S, użyte w niskich stężeniach, mogą stabilizować strukturę albumin lub, jak w przypadku K12S, mogą destabilizować białko (np. lizozym) ▪ małe, naładowane ligandy i hydrofobowe aniony zazwyczaj nie zmieniają struktury (oddziaływania powierzchniowe)	▪ małe ligandy: stechiometria często $\sim 1:1$ ▪ średnie ligandy (SXS) mogą tworzyć kompleksy o zmiennej stechiometrii, przy czym 1. miejsce charakteryzuje się niską, a drugie wysoką stechiometrią ▪ mechanizm: – duże ligandy często wiążą się poprzez efekt hydrofobowy (entropia) – małe ligandy: elektrostatycznie (entalpia)

<i>Cecha liganda</i>	<i>Wpływ na liczbę miejsc wiązania</i>	<i>Wpływ na lokalizację w BSA/HSA</i>	<i>Wpływ na lokalizację w lizozymie</i>	<i>Wpływ na zmiany strukturalne białka</i>	<i>Wpływ na mechanizm i stechiometrię wiązania</i>
<i>Ładunek (wartość i dystrybucja)</i>	- wysoki ładunek zlokalizowany ($[\text{Fe}(\text{CN})_6]^{4-}$) promuje silne, ale niespecyficzne wiązanie, często do jednego regionu - niski/zdelokalizowany ładunek ($[\text{B}(\text{Ph})_4]^-$) umożliwia wiązanie w specyficznych, hydrofobowych miejscach bez silnej konkurencji elektrostacyjnej	- silnie naładowane aniony ($[\text{Fe}(\text{CN})_6]^{4-/3-}$) gromadzą się przy dodatnich rejonach białka - hydrofobowe aniony ($[\text{B}(\text{Ph})_4]^-$) wnikają do wnętrza hydrofobowych kieszeni - amfifile anionowe (SXS) – część hydrofilowa wiąże się elektrostacyjnie, łańcuch – hydrofobowo w sąsiedztwie	- liczba miejsc wiązania jest niska (~ 1), ale ładunek decyduje o sile wiązania: $[\text{Fe}(\text{CN})_6]^{4-} > [\text{Fe}(\text{CN})_6]^{3-}$ - lokalizacja w kilku dodatnich rejonach na powierzchni	- silne oddziaływania elektrostacyjne mogą wywoływać drobne zmiany konformacyjne bez denaturacji - słabe, hydrofobowe wiązanie zazwyczaj nie destabilizuje rdzenia białka	- wysoki ładunek: wiązanie głównie entalpowe, sterowane oddziaływaniami elektrostacyjnymi - niski ładunek + hydrofobowość: wiązanie sterowane entropowo (efekt hydrofobowy) - stechiometria często $\sim 1:1$ dla silnie naładowanych, może być wyższa np. dla surfaktantów

<i>Cecha liganda</i>	<i>Wpływ na liczbę miejsc wiązania</i>	<i>Wpływ na lokalizację w BSA/HSA</i>	<i>Wpływ na lokalizację w lizozymie</i>	<i>Wpływ na zmiany strukturalne białka</i>	<i>Wpływ na mechanizm i stechiometrię wiązania</i>
<i>Równowaga hydrofilowo-hydrofobowa</i>	▪ hydrofilowe ($[\text{Fe}(\text{CN})_6]^{4-/3-}$): 1 miejsce o wysokim powinowactwie elektrostatycznym; (polioksowanadany): 1-2 miejsca o wysokim powinowactwie ▪ amfifilowe (SXS/KXS): mogą wiązać się w wielu miejscach (specyficzne dla części hydrofilowej + niespecyficzne dla hydrofobowego łańcucha) ▪ hydrofobowe ($[\text{B}(\text{Ph})_4]^-$): 1-2 specyficzne miejsca w głębokich, hydrofobowych wnękach	▪ hydrofilowe/naładowane: powierzchnia białka, regiony polarne ▪ amfifilowe: granica regionów hydrofilowych/hydrofobowych ▪ hydrofobowe: wnętrze hydrofobowych kieszeni	▪ hydrofilowe/naładowane: powierzchniowe rejony dodatnie	▪ amfifile o długich łańcuchach mogą denaturować białko poprzez wiązanie hydrofobowe i zaburzenie struktury ▪ hydrofilowe ligandy: minimalny wpływ na strukturę	▪ hydrofilowość: wiązanie entalpowe (oddziaływania elektrostatyczne) ▪ hydrofobowość: wiązanie entropowe (efekt hydrofobowy) ▪ amfifilowość: mechanizm mieszany; stechiometria zależy od pojemności miejsca (np. 2:1 dla miejsca pierwszego "niezależnie od pH 5 lub 7", (5-4):1 lub 9:1 dla drugiego w SDS-BSA, odpowiednio w pH 5 oraz 7)

Tabela 20. Lokalizacja badanych ligandów w białkach: BSA, HSA i lizozymie.

<i>Ligand/Białko</i>	<i>Albumina surowicy bydlęcej (BSA)</i>	<i>Albumina surowicy ludzkiej (HSA)</i>	<i>Lizozym</i>
<i>1-alkilosiarczany (SXS)</i> <i>S12S (SDS), S10S, S8S</i>	▪ SDS: dwa miejsca – Subdomena IA: miejsce pierwsze (wysokie powinowactwo, stechiometria ~ 2:1). – Subdomena IIB: miejsce drugie (niższe powinowactwo, stechiometria ~ 5:1 w pH = 5 oraz ~ 9:1 w pH = 7).	▪ S12S, S10S: dwa miejsca – Subdomena IB/granica z IIIA: miejsce pierwsze (wysokie powinowactwo, ~ 1:1). Unikalne dla SXS. – Subdomena IIIA (Sudlow II): miejsce drugie (wspólne z KXS, ~ 4:1).	Nie badano.
<i>1-alkilosulfoniany (KXS)</i> <i>K12S, K10S, K8S</i>	▪ K12S, K10S: dwa miejsca (dla K8S tylko jedno) – Subdomena IA: miejsce pierwsze (wspólne z S12S, ~ 1:1). Potwierdzone konkurencją. – prawdopodobnie Subdomena IIB: drugie miejsce (wspólne z S12S ~ 5:1). ▪ K8S: tylko jedno miejsce (w 2. miejscu S12S)	▪ K12S, K10S: tylko jedno miejsce – Subdomena IIIA (Sudlow II). Nie stwierdzono wiązania w subdomenach, do których powinowactwo wykazują ligandy SXS. ▪ K8S: prawdopodobnie zbyt słabe wiązanie	Nie badano szczególnie w kontekście specyficznej lokalizacji domenowej. Wiązanie powoduje denaturację.

<i>Ligand/Białko</i>	<i>Albumina surowicy bydlęcej (BSA)</i>	<i>Albumina surowicy ludzkiej (HSA)</i>	<i>Lizozym</i>
Jon tetrafenyloboranowy $[B(Ph)_4]^-$	▪ dwa miejsca wiązania: – Subdomena IA: miejsce pierwsze (~2:1). – Subdomena IIIA (Sudlow II): miejsce drugie (~4:1). ▪ konkurencja z SDS o 1. miejsce	Nie badano szczegółowo (brak danych). Analogia do BSA jest prawdopodobna, ale niepotwierdzona.	Nie badano. Prawdopodobnie słabe wiązanie z hydrofilową, dodatnią powierzchnią.
Jony heksacyjanożelazianowe(II)/(III) $[Fe(CN)_6]^{4-/3-}$	Region Sudlowa II (Subdomena IIIA) oraz sąsiednie dodatnio naładowane powierzchnie. Nie wiążą się w jednym, punktowym miejscu, ale w rozległym regionie elektrostatycznym obejmującym wejście do tej wnęki (udział reszt: Arg-409, Arg-194, Lys-114 i inne). Konkuruja z markerami obu miejsc Sudlowa z dominującym współzawodnictwem z ibuprofenem (markerem miejsca Sudlowa II).	Prawdopodobnie region Sudlowa II (Subdomena IIIA) – analogicznie jak w BSA. Konkuruja z markerami obu miejsc Sudlowa z dominującym współzawodnictwem z ibuprofenem (markerem miejsca Sudlowa II).	Cztery główne regiony powierzchniowe bogate w dodatnie reszty argininy (Arg) i lizyny (Lys), z dala od centrum aktywnego. Symulacje MD wskazały regiony wokół reszt: I: Arg-5, Lys-33, Arg-125; II: Lys-1, Arg-14; III: Arg-45, Arg-68; IV: Arg-112, Arg-114, Lys-116.

6. Perspektywy dalszych badań

Dalsze badania powinny skupić się na czterech podstawowych kierunkach: rozszerzeniu bibliotek ligandów i białek, uzupełnieniu danych termodynamicznych o kinetykę wiązania, eksperymentalnej walidacji miejsc wiązania oraz testowaniu nowych blokerów miejsc wiążących albuminy w pożywkach hodowlanych. Taka wielotorowa strategia pozwoli na głębsze zrozumienie i poszerzenie wiedzy o oddziaływaniach molekularnych białek z ligandami małocząsteczkowymi.

6.1 Rozszerzenie biblioteki ligandów i białek

Ligandy: Wprowadzenie ligandów o innej strukturze i właściwościach (np. surfaktanty kationowe lub obojętne, lipopeptydy) pozwoliłoby uzupełnić i rozwinąć uzyskaną wiedzę na temat właściwości kompleksotwórczych testowanych w pracy białek.

Białka: Badanie innych białek osocza, np.:

- a) Zbadanie, czy i jak ligandy hydrofobowe ($[B(Ph)_4]^-$) lub amfifile (SXS, KXS) wiążą się i/lub włączają w strukturę lipoprotein (LDL, HDL), byłoby to kluczowe dla zrozumienia dystrybucji leków hydrofobowych.
- b) Badanie konkurencji ligandów (np. związków wanadu(IV/V)) o miejsca wiązania żelaza w transferynie) dostarczyłoby informacji o metabolizmie i toksyczności jonów metali.

6.2 Badania kinetyki wiązania

Wykorzystanie techniki Powierzchniowego Rezonansu Plazmatycznego (ang. *Surface Plasmon Resonance*, SPR) do pomiaru stałych szybkości asocjacji (k_a) i dysocjacji (k_d). To uzupełniłoby dane termodynamiczne (ITC) o informacje o trwałości kinetycznej kompleksów, kluczowej dla działania leków.

6.3 Walidacja eksperymentalna miejsc wiązania ligandów w białkach

Zastosowanie metod eksperymentalnych np. spektroskopii NMR w stanie stałym do wizualizacji kompleksów ligand-białko dostarczyłoby bezpośrednich dowodów na temat lokalizacji ligandów w białku. To z kolei pozwoliłoby potwierdzić lokalizacje miejsc wiążących w białkach uzyskane z symulacji dynamiki molekularnej (MD).

6.4 Optymalizacja blokowania miejsc wiązania

Systematyczne poszukiwanie i testowanie bezpiecznych (nietoksycznych), specyficznych blokerów miejsc wiążących w albuminie (alternatywnych dla SDS) do stosowania jako składnik w pożywkach hodowlanych, co zwiększyłoby wiarygodność badań *in vitro*.

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ANEKS

PUBLIKACJA 1

**Physicochemical nature of sodium dodecyl sulfate interactions
with bovine serum albumin revealed
by interdisciplinary approaches**



Physicochemical nature of sodium dodecyl sulfate interactions with bovine serum albumin revealed by interdisciplinary approaches

Aleksandra Tesmar¹, Małgorzata M. Kogut¹, Krzysztof Żamojć, Ola Grabowska, Katarzyna Chmur, Sergey A. Samsonov, Joanna Makowska, Dariusz Wyrzykowski*, Lech Chmurzyński

Faculty of Chemistry, University of Gdańsk, Wita Stwosza 63, 80-308 Gdańsk, Poland

ARTICLE INFO

Article history:

Received 4 May 2021

Revised 1 July 2021

Accepted 3 August 2021

Available online 5 August 2021

Keywords:

Sodium dodecyl sulfate

Bovine serum albumin

Thermodynamic parameters

Binding site

Molecular dynamics

ABSTRACT

To rigorously characterize the interactions of sodium dodecyl sulfate (SDS) with bovine serum albumin (BSA) a set of experimental methods, namely isothermal titration calorimetry, conductometric titration, steady-state fluorescence spectroscopy, differential scanning calorimetry and circular dichroism spectroscopy, supported by *in silico* analysis have been applied. The influence of pH and temperature on the binding mode has been revealed. At a low molar ratio of SDS to BSA up to ca. 16:1, there are at least two structurally distinct binding sites in BSA. The formation of SDS-BSA complexes is an enthalpy-driven process in which the van der Waals interactions play a crucial role. The first binding site, located close to the Trp-134 residue within the sub-domain 1A, is pH-independent and binds two molecules of SDS per one molecule of BSA whereas the total number of SDS molecules bound to the second site of albumin is affected by temperature and pH. The saturation of the first binding site of BSA (ca. 0.009 mg of SDS per 1 mg of BSA) is sufficient to thermally stabilize the helical conformation of BSA. The presented results have important structural and thermodynamic implications to understand the influence of a widely used anionic surfactant on globular protein functionality in modern branches of chemistry.

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1. Introduction

Surfactants are the subject of interest to many research groups since it has been found that they can be utilized in several industrial and technical applications, including detergent industry, food chemistry, drug delivery, and cosmetic preparation as well as developments in life sciences [1–4]. Much attention has been focused on studies of surfactant interactions with biologically relevant macromolecules such as proteins [5–11].

An excellent review on the protein – surfactant interactions provided by Otzen reveals the most important techniques for analysing these interactions and highlights the different issues related to this field, namely the impact of surfactant on protein denaturation, binding affinity and unfolding processes [12]. The macromolecule – surfactant interactions depend on many factors, including the topology of the investigated surfactant species (monomeric and micelle forms), a chemical structure of a surfactant (anionic, cationic, amphoteric or non-ionic) as well as experimental conditions (pH, temperature). A remarkable degree of specificity and high

affinity of surfactants towards proteins results in changes in their physicochemical properties. Consequently the conformational changes and the saturation of the binding sites of a macromolecule affect its biological activity upon a complex formation.

The understanding of the thermodynamic and thermal stability of the binding process and the structural changes of a protein in the presence of ubiquitous surfactants may be helpful for an in-depth interpretation of a surfactant role underlying biologically relevant protein's functions. Moreover, it is fundamental from the viewpoint of the surfactants application in environmental processes and the pharmaceutical industry [13]. In this paper, we report the influence of pH and temperature on the interactions of the sodium dodecyl sulfate (SDS), a common, anionic surfactant, with the protein bovine serum albumin (BSA). As far as we are concerned, there are few reports on the SDS – BSA interactions [14–19]. However, in contrast to these previous studies, we have focused our attention on a low SDS to BSA molar ratio range. Furthermore, the experimental and *in silico* data related to the structural impact of pH and temperature on the potential binding sites and poses of BSA have been discussed.

* Corresponding author.

E-mail address: dariusz.wyrzykowski@ug.edu.pl (D. Wyrzykowski).

¹ Authors contributed equally.

2. Experimental part

2.1. Materials

Bovine serum albumin (BSA, lyophilized powder, $\geq 96\%$, Sigma-Aldrich, Poland), sodium dodecyl sulfate (SDS, BioUltra, for molecular biology, $\geq 99\%$, Sigma-Aldrich, Poland), sodium cacodylate trihydrate (Caco, $\geq 99\%$, Sigma-Aldrich, Poland) were used as obtained without further purification. Double-distilled water with conductivity not exceeding $0.18 \mu\text{S cm}^{-1}$ was used for preparations of aqueous solutions.

2.2. Conductometric analysis

A microtitration unit (Cerkolab System, Poland) fitted with a 5 mL syringe (Hamilton, Poland) and a CD-201 conductometric cell (Hydromet, Poland) were used for conductometric measurements. A weight calibration method was used for the syringe. The conductometric electrode was standardized with conductivity standards (aqueous KCl solutions) of a conductivity of 84 and $200 \mu\text{S cm}^{-1}$ (Hamilton, Poland). The measurements were carried out at $298.15 \pm 0.10 \text{ K}$, controlled by the Lauda E100 circulation thermostat.

2.3. Isothermal titration calorimetry

All ITC experiments were performed at 288.15 K and 298.15 K using an AutoITC isothermal titration calorimeter (MicroCal Inc. GE Healthcare, Northampton, USA). The details of the measuring devices and experimental setup have been described previously [20]. The reagents (BSA, SDS) were dissolved directly in 10 mM Caco buffers of pH 5 and 7. The experiment consisted of injecting $10.02 \mu\text{L}$ (29 injections, $2 \mu\text{L}$ for the first injection only) of 1 mM buffered solution of SDS into the reaction cell which initially contained the 0.0125 mM buffered solution of BSA. A background titration, consisting of an identical titrant solution but with the buffer solution in the reaction cell only, was subtracted from each experimental titration on account of the heat of dilution. The titrant (SDS) was injected at 5 min intervals. Each injection lasted 20 s. The stirrer speed was kept constant at 300 rpm. The CaCl_2 - EDTA titration was performed to check the apparatus and the results (stoichiometry, K , ΔH) were compared with those obtained for the same samples (a test kit) at MicroCal Inc. GE Healthcare.

2.4. Steady-state fluorescence spectroscopy and UV spectrophotometry

Steady-state fluorescence experiments were carried out at 288.15 K and 298.15 K using a Cary Eclipse Varian (Agilent, Santa Clara, CA, USA) spectrofluorometer equipped with a temperature controller and a 1.0 cm quartz multicell holder. The initial concentration of the stock solution of BSA ($2 \mu\text{M}$) was confirmed spectrophotometrically ($\epsilon_{\text{BSA}}^{280} = 41180 \text{ M}^{-1} \text{ cm}^{-1}$) based on the absorbance and the values of the molar extinction coefficients of tryptophan, tyrosine, and cysteine determined at 280 nm ($\epsilon_{\text{W}}^{280} = 5690 \text{ M}^{-1} \text{ cm}^{-1}$; $\epsilon_{\text{Y}}^{280} = 1280 \text{ M}^{-1} \text{ cm}^{-1}$; $\epsilon_{\text{C}}^{280} = 120 \text{ M}^{-1} \text{ cm}^{-1}$) [21]. The absorption spectra were recorded at 288.15 K and 298.15 K on a Perkin Elmer Lambda 650 (Waltham, MA, USA) UV/Vis spectrophotometer. The fluorescence emission spectra ($\lambda_{\text{ex}} = 275 \text{ nm}$) of BSA ($2 \mu\text{M}$) were recorded from 280 to 500 nm in the absence and presence of increasing concentrations of SDS, up to $29.3 \mu\text{M}$. In the performed fluorescence titration experiments, 2 mL of BSA at $2 \mu\text{M}$ was titrated with thirty $1 \mu\text{L}$ aliquots of SDS solution at 1 mM and then with fifteen $1 \mu\text{L}$ aliquots of SDS solution at 2 mM . The intensity of the band at 348 nm (corresponding to the maximum emission of BSA) was used to calculate the binding constants (K) and other parameters.

2.5. Differential scanning calorimetry

Calorimetric (DSC) measurements were made with a VP-DSC microcalorimeter (MicroCal Inc. GE Healthcare, Northampton, USA) at a scanning rate 90 K h^{-1} . All scans were run at two different pH values: 5 and 7 in the Caco buffer, in a temperature range from 298.15 K to 363.15 K . At first, scans were obtained for pure BSA at a concentration of 0.015 mM . In the second step, scans were obtained for BSA/SDS mixtures in stoichiometric ratios: 1:4, 1:8, and 1:14, respectively. The cell volume was always 0.5 mL . The reversibility of the transition was checked by cooling and reheating the same sample. These measurements were recorded two times. Results from DSC measurements were analyzed using the software Origin 7.0 from MicroCal, employing routines included with the instrument [22]. The quantity measured by DSC is the difference between the heat capacity of the Caco buffer-protein BSA solution and that of a pure Caco buffer. To perform the DSC measurement of protein unfolding, the reference cell was filled with buffer and the sample cell with the appropriate protein solution. They were then heated at a constant scan rate (90 K h^{-1}). When a protein unfolds, during DSC measurements, the occurring absorption of heat causes a temperature difference (ΔT) between the cells. The reference (Caco buffer) and sample solutions (protein BSA) were properly equilibrated with dissolved air before being introduced into the cells. Five minutes of vacuum treatment was required to degas all samples. The first step of the calibration was to carry out the buffer/buffer scans with run parameters exactly the same as for the comparative scans when the protein is in the sample cell. A pre-scan thermostat period was 15 min as recommended.

2.6. Circular dichroism spectroscopy

Circular dichroism (CD) spectra were recorded in water on a Jasco-715 automatic recording spectropolarimeter (Jasco Inc., USA) for following systems: pure BSA and two BSA/SDS mixtures in stoichiometric ratios: 1:4 and 1:8, respectively. The experiments were performed in the 10 mM Caco buffer at two different pH: 5 and 7 in a temperature range from 298.15 K to 368.15 K , every 10 degrees. Measurements were made in 1 mm quartz cuvettes. The spectra were recorded in the 200 – 260 nm wavelength range, using a sensitivity of five millidegrees and a scan speed of 50 nm min^{-1} . The results were plotted as the mean residue ellipticity Θ [$\text{degree cm}^2 \text{ dmol}^{-1}$]. The CD measurements were made at the 0.015 mM protein concentration. The volume of samples was 0.3 cm^3 . The secondary structure content was estimated from all CD spectra using the CONTIN/LL program, a variant of the CONTIN method developed by Provencher and Glockner [23] provided within the CDPPro software package.

2.7. Theoretical studies

Structures. The structure of BSA was obtained from the Protein Data Bank (PDB ID: 1F5S, $2.47 \text{ \AA}$). The structure of SDS was built in xleap of AMBER 16 package [24].

Molecular docking. The Autodock3 [25] software was used to perform molecular docking. A maximum gridbox size was used ($126 \text{ \AA} \times 126 \text{ \AA} \times 126 \text{ \AA}$) that covered the entire protein surface, including the expected/predicted SDS “first” binding site with the default grid step of $0.375 \text{ \AA}$. The Lamarckian genetic algorithm was deployed for 1000 independent runs. The size of 300 for the initial population and 10^5 generations for termination conditions were chosen and 9995×10^5 energy evaluations were performed. For different protonation states, His residues with and without a proton covalently bound to the nitrogen atom were used.

Molecular dynamics simulations. All-atom molecular dynamics (MD) simulations were performed with the use of a AMBER16

software package [24]. The initial structures of the complexes were created by a random placement of 15 SDS molecules around the BSA protein. The truncated octahedron TIP3P periodic box of 8 Å water layer from box's border to solute was used to solvate complexes. The ff14SBonlysc force field for the protein [26] and gaff force field [27] with RESP charges [28] obtained in antechamber module of AMBER16 [24] for SDS molecule were used. Charge was neutralized with either Na⁺ or Cl⁻ counterions. An energy minimization was carried out in two steps: beginning with 500 steepest descent cycles and 10³ conjugate gradient cycles with 100 kcal/mol/Å² harmonic force restraints, continued with 3x10³ steepest descent cycles and 3x10³ conjugate gradient cycles without any restraints. Following minimization steps the system was heated up from 0 to 300 K for 10 ps with harmonic force restraints of 100 kcal/mol/Å². Then, the system was equilibrated at 300 K and 10⁵ Pa in an isothermal isobaric ensemble for 100 ps. Afterwards, the two independent prediction MD runs were carried out in the same isothermal isobaric ensemble for 100 ns. The particle mesh Ewald method for treating electrostatics and SHAKE algorithm for all the covalent bonds containing hydrogen atoms were implemented in the MD simulations.

Binding free energy calculations. Energetic post-processing of the trajectories and per-residue energy decomposition were performed for all systems with the use of the Linear Interaction Energy (LIE) with dielectric constant of 80 and Molecular Mechanics Generalized Born Surface Area (MM-GBSA) [29] using a model with the surface area and Born radii default parameters as implemented in the igb = 2 model in AMBER16 [30]. All frames from MD simulations were analysed.

3. Results

3.1. Critical micelle concentration (CMC) of SDS in cacodylate buffer

Surfactant molecules reveal tendency to self-association and formation of ordered structures (micelles). Thus, the investigations of systems in which surfactants are involved require determination of the critical micelle concentration (CMC) [31,32]. The presence of monomeric surfactants (below the CMC) and micelles modifies solution properties as well as has a considerable impact on their interaction's mode with various proteins [33–35]. The CMC value depends on many factors, among which the ionic strength of an aqueous solution as well as a type of electrolytes play the crucial role [36–38].

The CMC values of SDS under the experimental conditions (the 10 mM Caco buffers of pH 5 and 7, at 298.15 K) were determined from conductometric titration data and ITC measurements (Fig. 1). The specific conductivity–surfactant concentration plots comprise two straight lines with different slopes corresponding to the SDS concentration range below and above CMC, respectively. The CMC values were calculated from the intersection of these lines [36]. In turn, the CMC values from ITC data were determined from the maximum peak of the fitted curve (the Gauss model from Origin 8.5) to the heats associated with injections of the SDS buffered solution into the buffer [39].

The calculated CMC values (5.4–5.6 mM) are lower than that reported for water (CMC ca. 8 mM) [40]. It is worth noticing that the pH of the Caco buffers (5 and 7) does not affect the CMC of SDS. Thus, the ionic strength of the solution maintained by buffer components is a key factor which favours the SDS micelle formation. The experimentally determined CMC values (CT and ITC measurements) correlate very well with a theoretical one (5.5 mM) calculated through the empirical expression developed by Corrin and Harkin [41]:

$$\log(\text{CMC}) = a + b \log(c_i)$$

where c_i denotes the total counterion concentration (mol dm⁻³). The literature values of a and b variables for SDS are -3.23 and -0.486, respectively [36].

3.2. Isothermal titration calorimetry (ITC)

Representative binding isotherms for the SDS-BSA interactions in the 10 mM Caco buffers of pH 5 and 7 at 298.15 K and 288.15 K are shown in Fig. 2, whereas conditional parameters of the interactions are summarized in Table 1. The thermodynamic parameters (K_{ITC} and ΔH_{ITC}) were obtained directly from ITC measurements by fitting isotherms (using nonlinear least-squares procedures) to a model that assumes two sets of binding sites. The assumed model has given the best fitting of calculated data to the experimental ones for all systems under study except for the SDS-BSA interactions at pH 5, at 288.15 K. Thus, for the latter system only a stoichiometry (expressed with N denoting SDS:BSA molar ratio) of the interactions was calculated based on the first derivative of the fitting curve obtained by the polynomial fit (OriginPro 8.5). The standard thermodynamic relationships: $\Delta G_{\text{ITC}} = -RT \ln K_{\text{ITC}} = \Delta H_{\text{ITC}} - T \Delta S_{\text{ITC}}$ were used to calculate the free energy of binding, ΔG_{ITC} , and the entropy change, ΔS_{ITC} (Table 1).

According to the ITC and conductometric titration measurements, under the experimental conditions ($c_{\text{SDS}} < \text{CMC}$), monomer SDS species take part in the interactions with the protein. Calorimetric data revealed that there are at least two binding sites of BSA capable to bind SDS. The first site has a higher affinity towards SDS than the second one ($\log K_{\text{ITC}(1)} > \log K_{\text{ITC}(2)}$, Table 1) and binds two SDS molecules followed by the saturation of the second site. The second binding site is saturated, depending on the pH of a buffer solution, by ca. 4–5 (at pH 7) and 9 (at pH 5) SDS particles. On the other hand, it is worth noticing that the reduction of the negative net charge of the protein by lowering pH from 7 to 5 does not affect the number of SDS molecules bound to the first (high affinity) site but has an impact on the number of SDS molecules inserted into the second (low affinity) BSA site.

It is interesting to note that an additional energy effect appears in the ITC curve at pH 5 at 288.15 K which suggests the presence of a third binding site in the protein at lower temperature and pH (see Fig. 2, 10 mM Caco, pH 5, 288.15 K). Unfortunately, in this case the theoretical curve generated based on equilibrium models implemented in Origin 7 from MicroCal cannot be fitted to the experimental points. Under these conditions ca. 2.8, 3.5 and 3.5 molecules of SDS are bound to the first, the second and the third binding sites of BSA, respectively. However, a total number of SDS molecules bound to BSA at 288.15 K is equal (in the range of the experimental error) to the number of SDS molecules interacting with the albumin under the same conditions (pH 5) at 298.15 K. This phenomenon indicates that lowering the temperature (288.15 K) favours the formation of a kinetically stable complex of the stoichiometry SDS:BSA equal to ca. 6.5:1, at pH 5.

The stability of the investigated SDS-BSA complexes, both in the first and in the second protein binding sites, is governed by the enthalpy factor (Table 1). The negative values of ΔH_{ITC} point to the fact that mainly the charge – charge electrostatic interactions established between negatively charged SDS anions and positively charged functional groups of amino acids such as lysine, arginine, histidine as well as van der Waals interactions and/or hydrogen bonds [42,43] involved in SDS – protein binding events, play a pivotal role in the stabilization of the resulting complexes. The release of a greater amount of energy on account of SDS-BSA interactions in the first binding site can be explained by the participation of a larger number of bonds or the presence of stronger interactions compared to the second binding site. Moreover, the presence of a cavity in the BSA protein to which SDS molecules fit spatially (sterically) could be taken into considerations to explain the differences

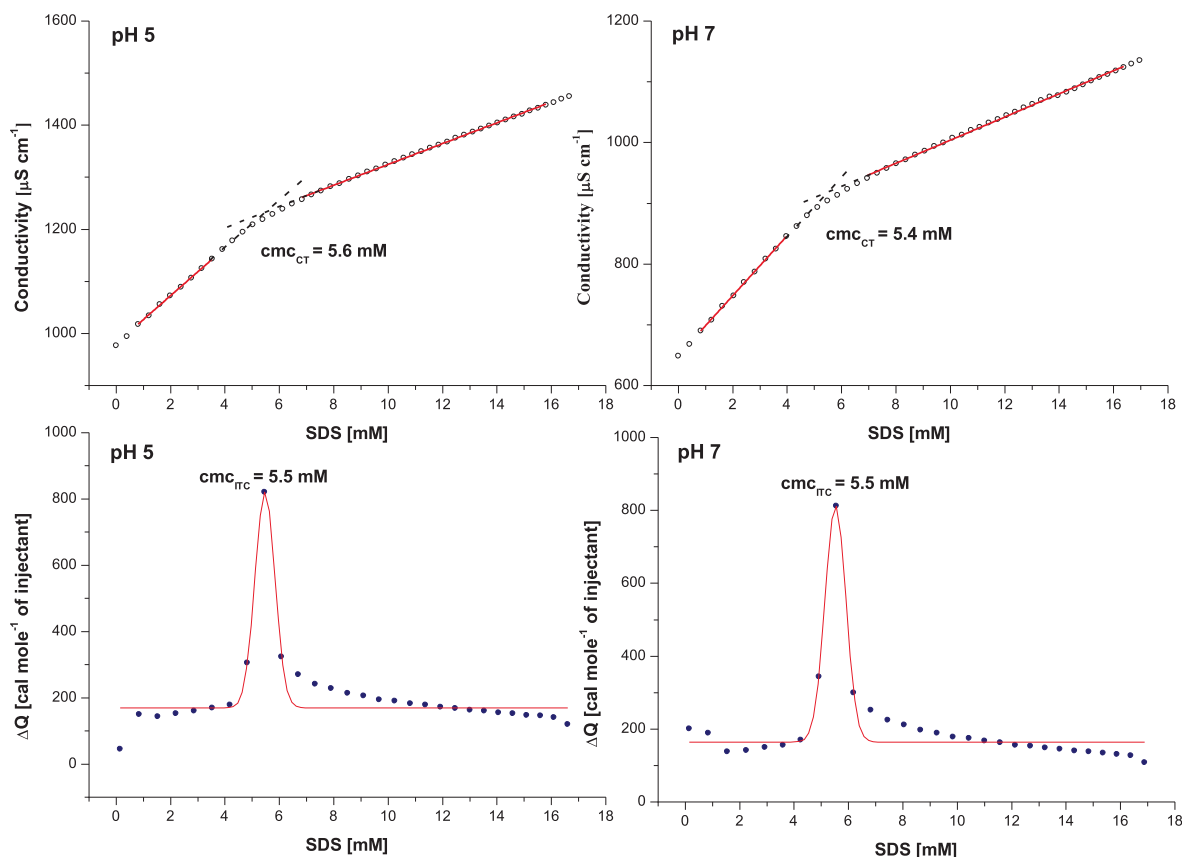


Fig. 1. The electrical conductivity (χ) and enthalpy change (ΔQ) per mole of SDS as a function of SDS concentration recorded in the 10 mM Caco buffers of pH 5 and 7, at 298.15 K. The CMC_{CT} and CMC_{ITC} denote CMC determined from conductometric titrations and ITC measurements, respectively.

in the enthalpy changes corresponding to the binding interactions in the first and second binding sites. Furthermore, the enthalpy – entropy compensation phenomenon is observed in the SDS-BSA interactions. The stronger interactions (the more negative value of ΔH) the lower mobility of the interacting molecules which is reflected by the low value of the entropic factor (Table 1) [44–46].

3.3. Steady-state fluorescence spectroscopy

The presence of two tryptophan residues in the BSA structure, namely Trp-134 and Trp-212 enables to exploit fluorescence spectroscopy for studying the BSA – ligand interactions [47–50]. The fluorescence emission spectra of BSA recorded in the presence of various SDS concentrations, using protein excitation at 275 nm, revealed that with the increasing concentration of the surfactant the fluorescence intensity decreases steadily with a blue shift of the maximum of emission wavelength. It indicates the presence of several different species in solution and is consistent with the fact that the change in the environment of tryptophan residues takes place and an increase of hydrophobicity in the vicinity of this residue occurs. The changes in the Trp fluorescence intensity are pronounced in the SDS concentration range corresponding to the binding of the substrate molecules in the first site of albumin (Figs. 3 and 4). After the first binding site is saturated only slight changes in the fluorescence intensity are observed with the increase of the SDS concentration. Thus, it can be assumed that the higher affinity BSA site is located close to the Trp-134 or Trp-212 residue which is sensitive to SDS binding to this site. Considering that Trp-134 (located in sub-domain IA) is exposed on the surface of albumin whereas a hydrophobic binding pocket com-

prises Trp-212 (located in sub-domain IIA), the Trp-134 containing binding site seems to be a potential first binding site.

The binding constants of the SDS-BSA complexes in the first binding site were calculated from spectrofluorimetric data using the Scatchard equation [51]:

$$\log\left(\frac{F_0-F}{F}\right) = \log K + n \log [Q]$$

where F_0 and F are the fluorescence intensities in the absence and presence of a quencher, n denotes the number of binding sites, while $[Q]$ is the quencher (SDS) concentration. The representative plots of $\log\left(\frac{F_0-F}{F}\right)$ vs. $\log[Q]$ for SDS/BSA systems under experimental conditions and the calculated parameters of the interactions ($\log K$ and n) are presented in Fig. 5. The binding constants obtained at the same temperature, but different pH values are comparable to one other (within the range of the experimental error). This proves that the change in pH of the system does not affect the affinity of SDS towards the first site of the protein. On the other hand, binding constants determined at 288.15 K are higher than those at 298.15 K. The increase of temperature favors the dissociation of weakly bound substrates leading to the reduction of the thermodynamic stability of SDS-BSA complexes. This, in turns, results in lower binding constant values [52].

3.4. Differential scanning calorimetry (DSC)

The BSA thermal stability in the presence and absence of SDS was assessed based on the transition midpoint T_m (Table 2). DSC measurements were carried out for the systems containing buffered solutions of pure BSA (control) and the SDS:BSA mixtures with the surfactant/protein molar ratios corresponding to the sat-

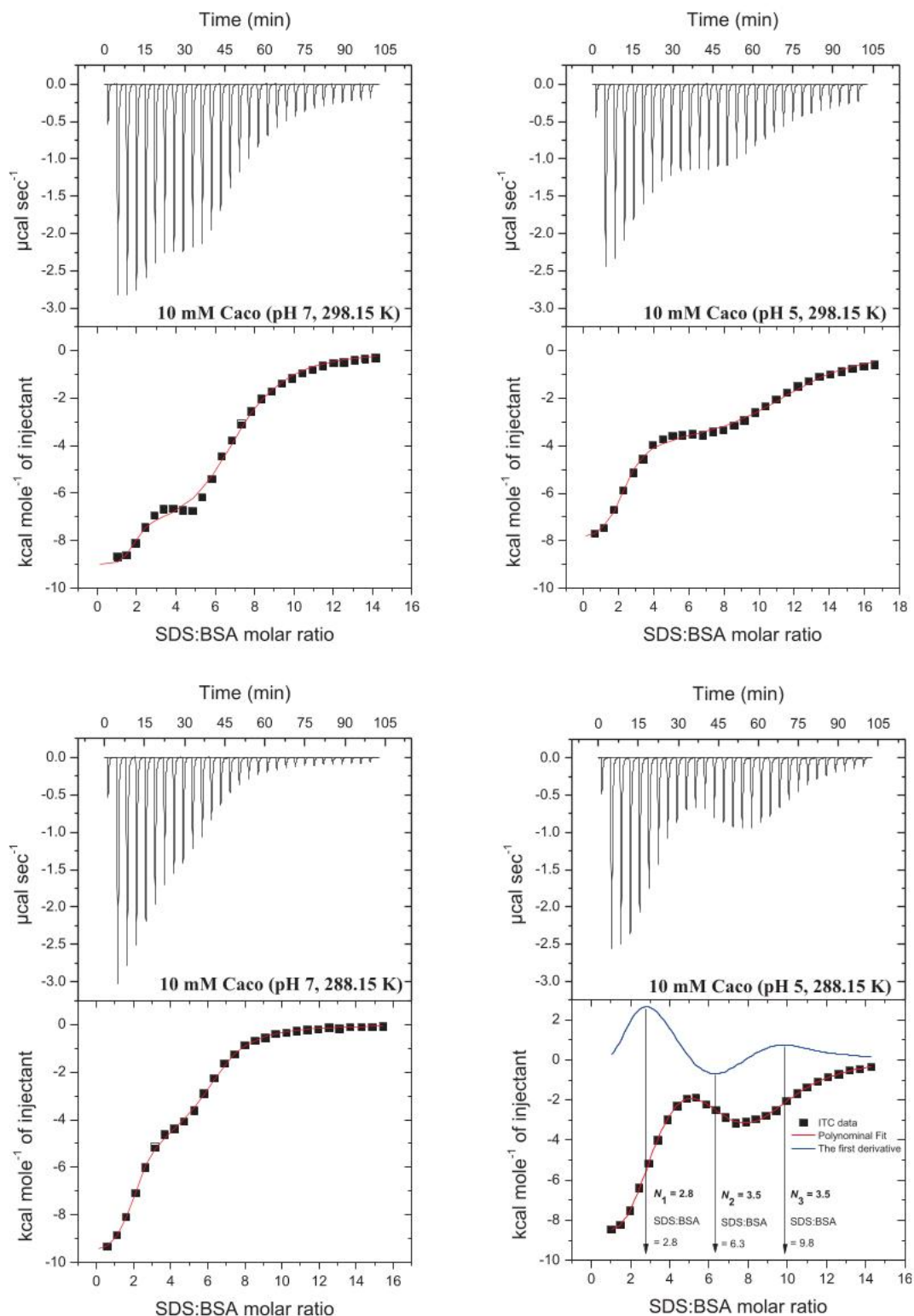


Fig. 2. Calorimetric titration isotherms of the binding interactions between SDS and BSA in the 10 mM Caco buffers of pH 5 and 7, at 288.15 K and 298.15 K.

uration of the first (4:1) and the second (8:1 and 14:1) binding sites of the albumin. The T_m values of BSA under experimental conditions were estimated by fitting the experimental C_p vs. T curves to a non-two state model using Marquardt non-linear least squares method.

For all investigated systems the irreversible thermal unfolding was observed. Furthermore, the lack of negative peaks in the

heat-capacity curves has indicated that all tested samples do not aggregate over the experimental temperature range [53]. The thermal stability of BSA at pH 5 which is close to the isoelectric point ($pI = 4.7$) [54] is slightly higher than at pH 7 (Table 2). The shift of T_m towards lower temperature with the increase of pH is due to the increase of the negative net charge of the protein on account of proton ionizations. In consequence,

Table 1

The conditional thermodynamic parameters of SDS monomers binding to BSA (standard deviation values in parentheses) in the 10 mM Caco buffers of pH 5 and 7, at 288.15 K and 298.15 K.

Parameter	Caco buffer (pH 7)		Caco buffer (pH 5)
	288.15 K	298.15 K	298.15 K
N_1	2.02 (± 0.04)	1.75 (± 0.14)	2.16 (± 0.07)
$\log K_{(ITC)1}$	6.87 (± 0.06)	7.61 (± 0.38)	6.96 (± 0.11)
$\Delta G_{(ITC)1}$ [kcal mol ⁻¹]	-9.08 (± 0.08)	-10.38 (± 0.67)	-9.49 (± 0.15)
$\Delta H_{(ITC)1}$ [kcal mol ⁻¹]	-9.75 (± 0.12)	-9.11 (± 0.26)	-8.17 (± 0.21)
$T\Delta S_{(ITC)1}$ [kcal mol ⁻¹]	-0.67	1.27	1.32
N_2	4.24 (± 0.05)	5.33 (± 0.29)	9.32 (± 0.18)
$\log K_2$	5.36 (± 0.03)	5.29 (± 0.06)	5.21 (± 0.07)
$\Delta G_{(ITC)2}$ [kcal mol ⁻¹]	-7.07 (± 0.04)	-7.22 (± 0.09)	-7.11 (± 0.09)
$\Delta H_{(ITC)2}$ [kcal mol ⁻¹]	-5.11 (± 0.16)	-7.98 (± 0.27)	-3.95 (± 0.13)
$T\Delta S_{(ITC)2}$ [kcal mol ⁻¹]	1.96	-0.76	3.16

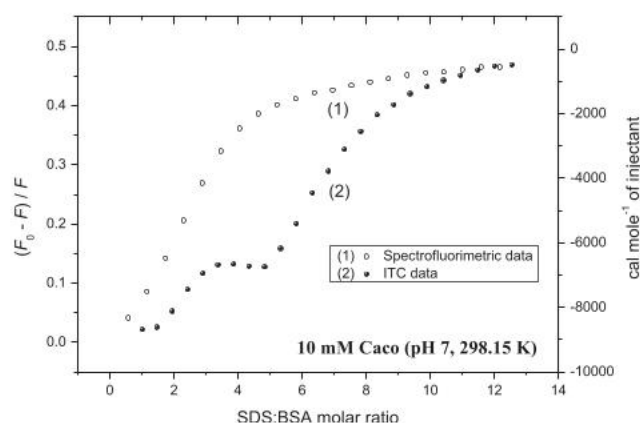


Fig. 3. The plots of $\frac{F_0-F}{F}$ (1) and integrated heats (2) vs. the SDS:BSA molar ratio in 10 mM Caco (pH 7), at 298.15 K.

the increase in the repulsive electrostatic interactions between the negatively charged functional groups of the side chains favours the BSA unfolding. The saturation of the first binding site (SDS:BSA = 4:1 M ratio system) improves the thermal stability of the system by ca. 10 K in comparison with the control (BSA buffered solution with no ligands). It should be highlighted that regardless of a pH of a system, the effect of SDS on a thermal unfolding of BSA after the saturation of the second binding site (SDS:BSA = 8:1 and 14:1 M ratio system) is not as pronounced as for the saturation of the first one. Thus, it can be supposed that the first binding site

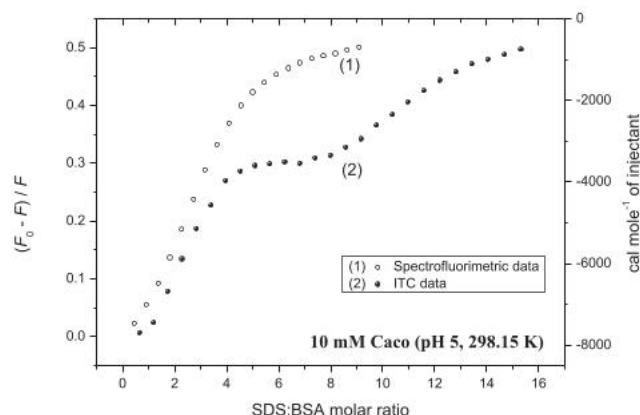


Fig. 4. The plots of $\frac{F_0-F}{F}$ (1) and integrated heats (2) vs. the SDS:BSA molar ratio in 10 mM Caco (pH 5), at 298.15 K.

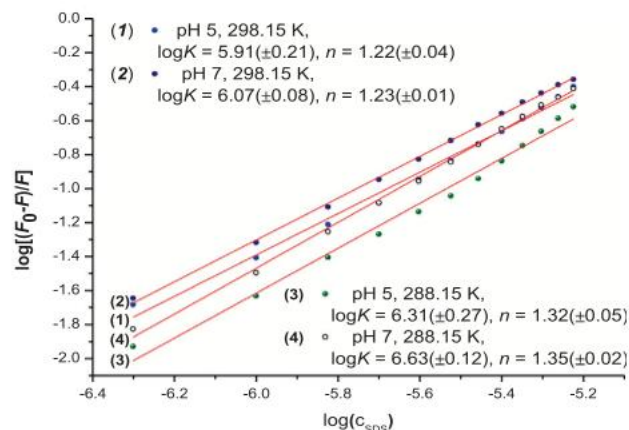


Fig. 5. The plots of $\log\left(\frac{F_0-F}{F}\right)$ vs. $\log(c_{SDS})$ for the BSA/SDS systems in the 10 mM Caco buffers of pH 5 and 7, at 288.15 K and 298.15 K (standard deviation values of $\log K$ and n in parentheses).

is more susceptible for the conformational changes upon the temperature increase.

3.5. Circular dichroism (CD)

CD spectra of the investigated protein in the absence and presence of SDS enabled to get some insight into the thermal stability of the BSA secondary structure (Supplementary Information Figs. S1 – S7). The percentage contents of α -helix, β -structure, β -turns and random coil were collected in Table 3.

It has been found that in the presence of SDS (the SDS:BSA molar ratio = 4:1, 8:1 and 14:1) the protein remains predominantly an α -helix structure at 298.15 K. Thus, under the experimental conditions the interactions of SDS with BSA do not alter the intermolecular forces maintaining the BSA structure. In CD spectra some conformational changes in the structure of BSA are observed due to the temperature increase. It is reflected in the reduction of the negative bands at 208 nm (the $\pi \rightarrow \pi^*$ transition) and 222 nm (the $n \rightarrow \pi^*$ transition) characteristic for the α -helix structure [55–57]. For all systems studied the content of α -helix decreases gradually with the increase of temperature. However, in the presence of SDS the thermal stability of the helical conformation is higher (Table 3). Above the temperature corresponding to the transition midpoint T_m (DSC results) a more prominent decrease in the α -helix content is observed, regardless the SDS:BSA molar ratio (Table 3). This is in lines with the results obtained from DSC measurements and confirms the fact that the saturation of the first binding site of the protein by SDS molecules is sufficient to noticeably increase its thermal stability.

3.6. Molecular modelling

To gain a better understanding of the experimental results at the atomistic level, docking simulations were performed. As a first step Autodock3 (AD3) was employed to localize potential binding

Table 2

The transition midpoints T_m for BSA (0.015 mM) in the presence of different concentrations of SDS in the 10 mM Caco buffers of pH 5 and 7.

SDS:BSA molar ratio	T_m [K] 10 mM Caco, pH 7	T_m [K] 10 mM Caco, pH 5
Control – no SDS	338.15	~342.15
4:1	349.15	351.15
8:1	352.15	353.15
14:1	–	~353.15

Table 3

The secondary structure content (in %) of BSA at different SDS:BSA molar ratios and different pH in 10 mM Caco buffer in the temperature range 298.15–368.15 K revealed from CD measurements.

Temperature	pH 7			pH 5			
	SDS:BSA 0:1	SDS:BSA 4:1	SDS:BSA 8:1	SDS:BSA 0:1	SDS:BSA 4:1	SDS:BSA 8:1	SDS:BSA 14:1
The percentage content of α -helix/ β -structure/ β -turn/random coil [%]							
298.15 K	62.3/3.5/ 11.1/20.4	62.2/7.2/ 10.3/19.2	62.6/8.2/ 10.3/18.9	57.3/8.0/ 12.1/21.9	60.8/8.3/ 11.9/19.0	62.5/7.4/ 10.0/21.5	61.8/6.9/ 10.4/21.0
308.15 K	61.1/4.6/ 12.4/22.2	62.1/8.6/ 9.8/19.7	61.8/6.9/ 12.0/19.7	57.9/9.4/ 11.2/22.0	61.8/6.8/ 10.7/21.5	61.9/5.3/ 11.0/21.9	62.1/5.8/ 10.6/21.4
318.15 K	58.7/5.7/ 14.0/24.1	61.6/7.3/ 10.2/20.7	61.2/7.4/ 10.9/23.0	55.8/9.6/ 13.1/21.9	59.0/14.0/ 10.9/15.4	61.0/5.7/ 11.5/22.1	61.4/5.1/ 11.4/21.9
328.15 K	52.1/7.9/ 16.6/26.2	59.2/8.9/ 10.4/21.9	60.6/7.5/ 10.8/21.5	52.3/10.3/ 14.1/23.5	58.8/10.2/ 11.9/19.9	61.0/5.6/ 11.5/22.6	61.1/5.4/ 11.8/22.0
338.15 K	42.6/12.6/ 17.9/27.9	53.8/7.4/ 13.6/25.5	58.4/6.5/ 12.1/23.7	46.2/10.8/ 15.8/27.6	55.1/16.2/ 12.4/16.5	58.8/6.6/ 12.0/23.2	60.5/5.4/ 11.5/22.8
348.15 K	37.1/14.4/ 19.4/30.4	40.8/12.1/ 17.7/29.5	40.9/13.0/ 17.3/29.2	22.7/24.5/ 22.4/30.5	37.7/17.5/ 17.7/27.3	45.3/11.2/ 16.8/26.9	50.7/8.9/ 14.8/25.5
358.15 K	27.0/18.8/ 22.5/33.4	36.5/14.7/ 20.5/28.0	34.9/15.5/ 19.5/29.6	9.5/34.1/ 24.8/31.5	15.2/30.4/ 22.7/30.9	10.2/34.5/ 24.1/30.0	7.9/36.7/ 23.5/30.9
368.15 K	23.7/23.2/ 22.9/31.4	27.3/21.5/ 21.6/28.7	31.8/17.3/ 22.3/28.3	6.8/35.2/ 25.2/31.5	7.9/32.5/ 25.5/33.2	9.0/34.8/ 23.2/32.0	6.7/36.5/ 24.6/31.6

sites at two different pH values (5 and 7). In each case, the top 50 structures were visualized together on the BSA protein (Fig. 6). AD3 did not reveal any differences between the two pH values and so did not help to localize any pH-specific binding site. For this reason, the structures obtained by molecular docking were not used for further analysis.

Therefore, molecular dynamics (MD) simulations were conducted for four systems (pH 5 and 7, 290 K and 300 K). This approach allowed us to identify the potential “second” binding site, which was most pronounced at lower pH and temperature (see Fig. 7).

The “first” binding site was not found in any of these computational experiments suggesting that the MD simulations were too short for the SDS molecule to enter any of the cavities close to the Trp residues. Therefore, based on steady-state fluorescence spectroscopy results of the SDS-BSA interactions as well as taking into account the known structure of HSA with myristic acid (PDB ID: 2XVW, 2.65 Å), the SDS molecule was inserted in a similar binding mode close to Trp-134. Then, a 100 ns MD simulation with additional 15 SDS molecules was run twice at two different pH values. Subsequently, binding free energy ΔG was calculated with the use of two commonly deployed methods: linear interaction energy

(LIE) and molecular mechanism Generalized Born surface area (MM-GBSA). Binding free energy (ΔG) computation can play a key role in prioritizing compounds to be evaluated experimentally on their affinity for target proteins.

The data showed that the “inserted” SDS molecule and a neighbouring one approaching it during the MD simulation had the lowest energies. Therefore, they were the most stable in terms of binding to the BSA protein (Supplementary Information Tables S1 and S2) when compared to the ΔG values for other SDS molecules including the ones corresponding to the putative “second” binding site.

The binding free energy values for the “first” binding sites were: -14.6 ± 3.3 and -33.4 ± 6.0 whereas for the “second” binding site they equalled: -8.7 ± 4.6 and -19.8 ± 10.1 (MM-GBSA and LIE respectively, values given in kcal/mol). Based on these results, not only the “first” binding site was localized but also it was shown that this site is pH-independent when the total binding free energy is considered (this does not apply to the free energy components though). Van der Waals free energy component plays decisive role for binding in this site. This finding supports the thermodynamic parameters (the negative value of ΔH_{ITC}) for the SDS-BSA interactions obtained from ITC experiments.

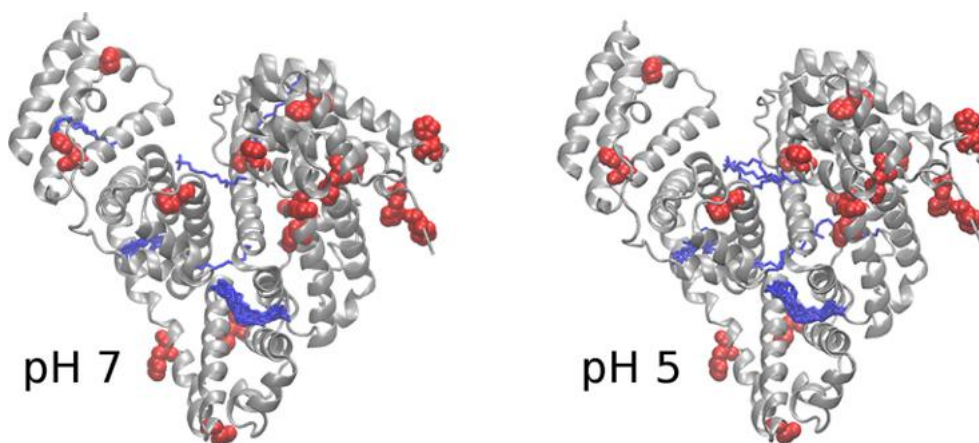


Fig. 6. Top 50 AD3 docking poses (in blue licorice) for docking SDS to BSA (in grey cartoon) under pH 7 (left) and pH 5 (right). HIS residues are represented with red VDW spheres.

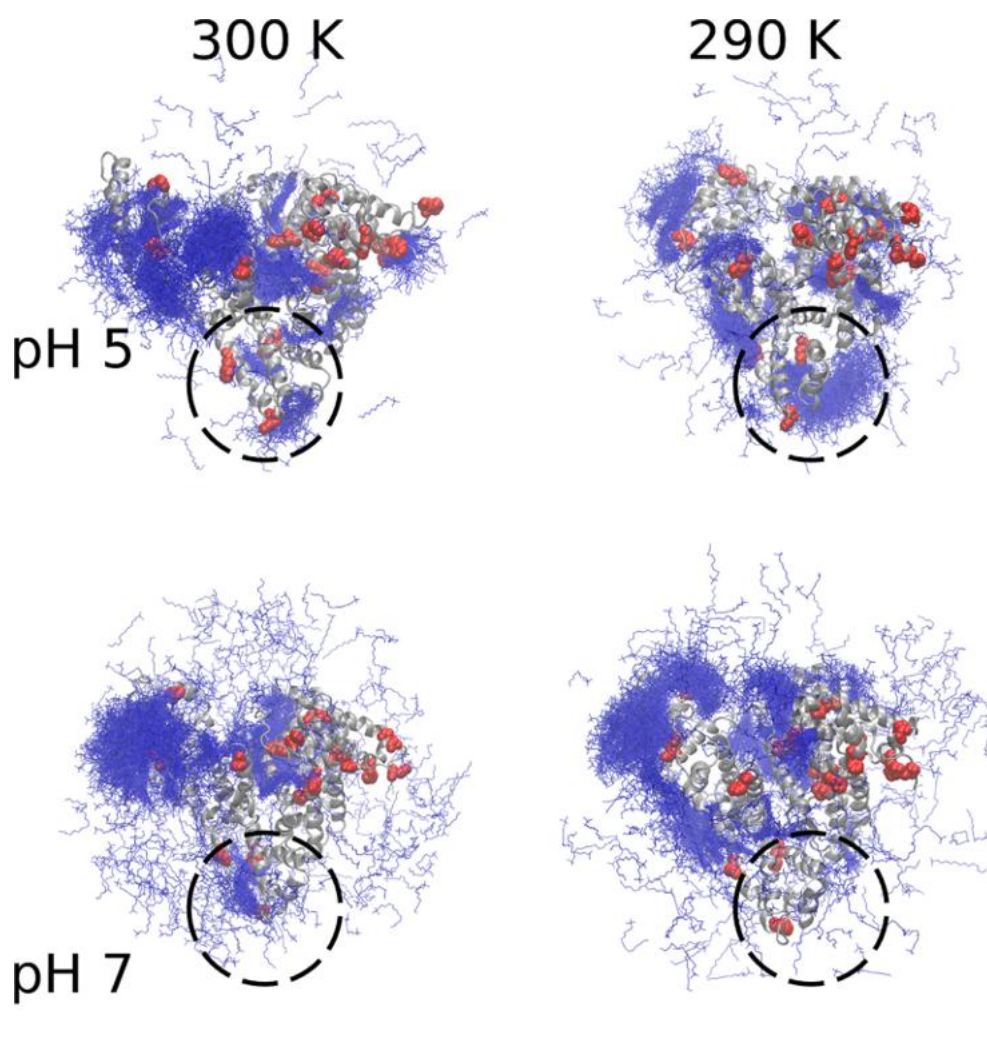


Fig. 7. MD-based analysis of SDS-BSA complexes at different pH and temperatures. BSA protein: grey cartoon; HIS residue: red VDW spheres; SDS molecules after the MD converged (frames are shown each 100 ps): blue licorice; the potential “second” binding site is shown with black circles. The data shown are obtained from two MD simulations.

4. Conclusions

In this study, we showed that a low concentration of SDS (0.009 mg/1 mg SDS/BSA) significantly affects the BSA structure, which may have an impact on its biological functions. It should be stressed that the binding constant and the stoichiometry of the resulting BSA complexes are so-called condition-dependent parameters as their values often depend on experimental conditions (pH and temperature). Furthermore, the determination of the binding properties of BSA is not always straightforward, especially when multiple SDS molecules are potentially bound. In such cases the effect of the SDS competition with other small ligands of BSA should be taken into consideration. At the same time, this phenomenon can be exploited to modulate the binding properties of BSA. Finally, the MD-based computational analysis corroborated the experimental findings and provided atomistic details explaining the differences on the binding of SDS molecules in the high-affinity pH-independent and the low-affinity pH-dependent binding sites.

CRediT authorship contribution statement

Aleksandra Tesmar: Conceptualization, Data curation. **Małgorzata M. Kogut:** Methodology, Investigation. **Krzysztof Żamojć:**

Methodology, Investigation. **Ola Grabowska:** Investigation, Resources. **Katarzyna Chmur:** Investigation, Resources. **Sergey A. Samsonov:** Methodology, Supervision. **Joanna Makowska:** Methodology, Investigation. **Dariusz Wyrzykowski:** Conceptualization, Project administration, Writing - review & editing. **Lech Chmurzyński:** Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the Polish National Science Centre under Grant No. 2016/23/D/ST4/01576. Theoretical research was funded by National Science Centre of Poland, grant number UMO-2018/30/E/ST4/00037. Computational resources were provided by the Polish Grid Infrastructure (PL-GRID, grants plgions-gpu, plggagstr2), ZIH at TU Dresden (grant p_gag) and the local cluster PIASEK at the Faculty of Chemistry, University of Gdansk.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.molliq.2021.117185>.

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PUBLIKACJA 2

**Effect of tetraphenylborate on physicochemical properties
of bovine serum albumin**

Article

Effect of Tetraphenylborate on Physicochemical Properties of Bovine Serum Albumin

Ola Grabowska [†], Małgorzata M. Kogut [†], Krzysztof Żamojć , Sergey A. Samsonov , Joanna Makowska , Aleksandra Tesmar , Katarzyna Chmur , Dariusz Wyrzykowski ^{*}  and Lech Chmurzyński

Faculty of Chemistry, University of Gdańsk, Wita Stwosza 63, 80-308 Gdańsk, Poland; ola.grabowska@phdstud.ug.edu.pl (O.G.); malgorzata.kogut@phdstud.ug.edu.pl (M.M.K.); krzysztof.zamoj@ug.edu.pl (K.Ż.); sergey.samsonov@ug.edu.pl (S.A.S.); joanna.makowska@ug.edu.pl (J.M.); aleksandra.tesmar@ug.edu.pl (A.T.); k.chmur.820@studms.ug.edu.pl (K.C.); lech.chmurzynski@ug.edu.pl (L.C.)

^{*} Correspondence: dariusz.wyrzykowski@ug.edu.pl

[†] Authors contributed equally.

Abstract: The binding interactions of bovine serum albumin (BSA) with tetraphenylborate ions ($[B(Ph)_4]^-$) have been investigated by a set of experimental methods (isothermal titration calorimetry, steady-state fluorescence spectroscopy, differential scanning calorimetry and circular dichroism spectroscopy) and molecular dynamics-based computational approaches. Two sets of structurally distinctive binding sites in BSA were found under the experimental conditions (10 mM cacodylate buffer, pH 7, 298.15 K). The obtained results, supported by the competitive interactions experiments of SDS with $[B(Ph)_4]^-$ for BSA, enabled us to find the potential binding sites in BSA. The first site is located in the subdomain I A of the protein and binds two $[B(Ph)_4]^-$ ions ($\log K_{(ITC)1} = 7.09 \pm 0.10$; $\Delta G_{(ITC)1} = -9.67 \pm 0.14$ kcal mol⁻¹; $\Delta H_{(ITC)1} = -3.14 \pm 0.12$ kcal mol⁻¹; $T\Delta S_{(ITC)1} = -6.53$ kcal mol⁻¹), whereas the second site is localized in the subdomain III A and binds five ions ($\log K_{(ITC)2} = 5.39 \pm 0.06$; $\Delta G_{(ITC)2} = -7.35 \pm 0.09$ kcal mol⁻¹; $\Delta H_{(ITC)2} = 4.00 \pm 0.14$ kcal mol⁻¹; $T\Delta S_{(ITC)2} = 11.3$ kcal mol⁻¹). The formation of the $\{[B(Ph)_4]^- \}$ -BSA complex results in an increase in the thermal stability of the alpha-helical content, correlating with the saturation of the particular BSA binding sites, thus hindering its thermal unfolding.

Keywords: bovine serum albumin; sodium tetraphenylborate; sodium dodecyl sulfate; binding site; thermodynamic parameters



Citation: Grabowska, O.; Kogut, M.M.; Żamojć, K.; Samsonov, S.A.; Makowska, J.; Tesmar, A.; Chmur, K.; Wyrzykowski, D.; Chmurzyński, L. Effect of Tetraphenylborate on Physicochemical Properties of Bovine Serum Albumin. *Molecules* **2021**, *26*, 6565. <https://doi.org/10.3390/molecules26216565>

Academic Editors: Danuta Witkowska and Simona Collina

Received: 24 September 2021

Accepted: 28 October 2021

Published: 29 October 2021

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1. Introduction

In aqueous media, proteins undergo many physicochemical changes such as conformational alterations, denaturation, folding/unfolding processes, and ligand exchange [1–6]. These phenomena can be invoked by variations in the temperature and/or the pH of a solution. Consequently, the environmental conditions may affect the binding properties of a biomolecule, such as the affinity of low-molecular weight compounds to a protein, the number of potential binding sites, and the stoichiometry of the resulting protein–ligand complexes [7]. As a result, the biological and pharmacological activity of the protein is often highly influenced by the presence of the different ligands in the system. Generally, the nature and the concentration of some potential ligands present in the system, which are capable to compete with other compounds (for example drugs) to a biomolecule, may affect the binding properties and functions of a protein. In the field of physicochemical studies, this phenomenon is applied to the studying of the potential binding sites of BSA by competitive displacement assays [8,9]. There are many techniques used for studying the albumin–ligand interactions. However, due to the presence of two tryptophan residues in BSA, namely Trp-134 (subdomain IA) and Trp-213 (subdomain IIB) [10], fluorescence spectroscopy is the most exploited. An excellent review on the application of direct fluorescence titrations for studying serum albumin–ligand interactions provided by Macii

and Biver reveals the aspects underlying the experimental challenges and recommends new experimental design to gain some additional information on the binding type and sites of a protein [11]. It has been reported that phenylbutazone and warfarin [12] can be successfully employed as probes for determining site I (located in the hydrophobic pocket of subdomain IIA) [13] whereas diazepam and flufenamic acid [14] for site II, located in the hydrophobic cavity of subdomain IIIA.

It is also worth highlighting that albumins are one of the main objects of study in the field of bioinorganic- and metallo-drug design [15–17]. However, in the frame of biological studies, cell culture media used in cytotoxicity assays of new compounds usually contain relatively high amount of BSA (ca. 0.04 mM). This may result in the binding interaction of the tested compound with the cell incubation media component leading to the lowering the concentration of the free (active) form of the investigated substance.

We have focused our attention on bovine serum albumin (BSA), as it represents one of the most common model molecular systems for binding studies [18]. Serum albumins are involved, among others, in transport and biodistribution of endogenous ligands such as fatty acids [19] as well as endogenous substances, for example ibuprofen, warfarin, penicillin or diazepam [11]. They also participate in the metabolism of low-molecular weight compounds. Recently, we have proven that the isothermal titration calorimetry technique supported by experimental and in silico methods can be used as an alternative to fluorescence spectroscopy for studying a stoichiometry of the resulting albumin–ligand complexes, the type of the binding interactions, and the number of the binding sites [20]. The studies performed on BSA and sodium dodecyl sulfate (SDS) have revealed that the investigated protein possesses two main binding sites: the first site is pH and temperature independent, and binds two moles of SDS and is located close to Trp-134 residue. In contrast, the total number of SDS bound to the second site of the protein depends on the pH of the solution (pH 5 and pH 7) and the temperature (290 K and 300 K) (Figure 1) [20].

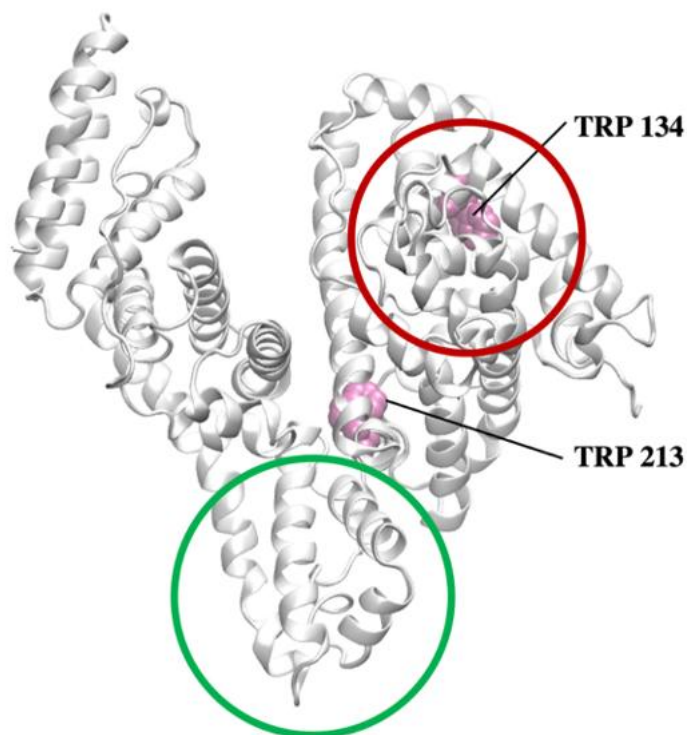


Figure 1. The secondary structure of BSA (grey cartoon) with marked Trp residues (magenta VDW representation). The first and second potential binding sites of SDS are shown with red and green rings, respectively.

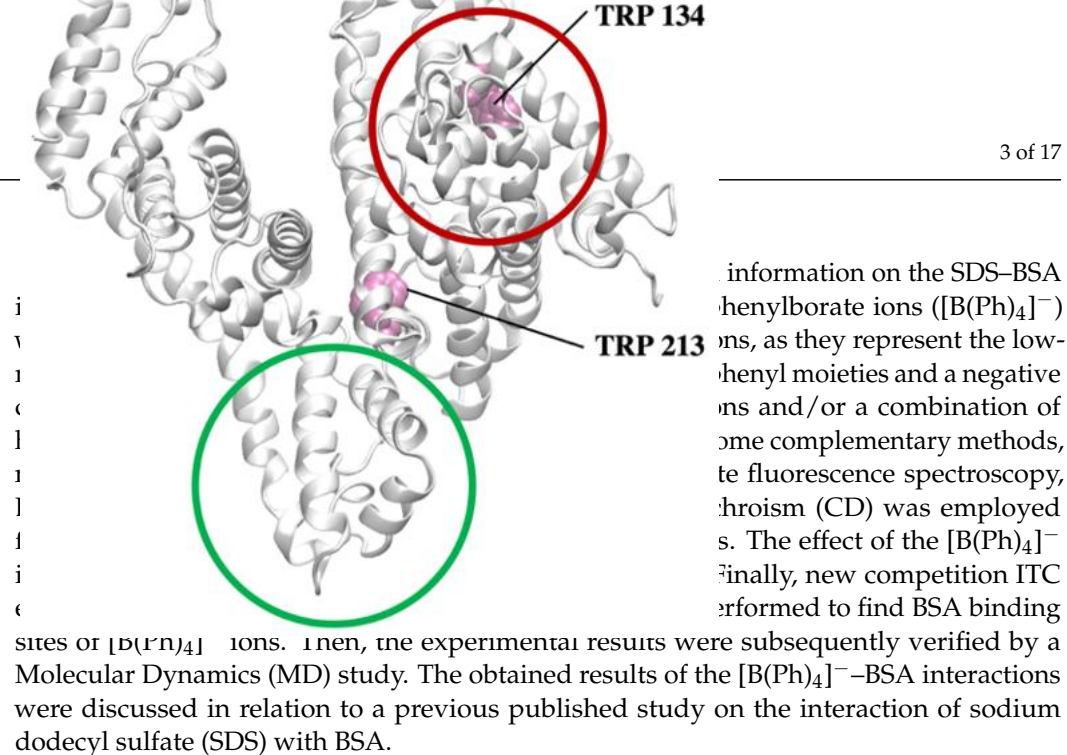


Figure 2. Chemical structure of sodium tetraphenylborate ($Na[B(Ph)_4]$) and sodium dodecyl sulfate (SDS).

2. Results and Discussion

2.1. Isothermal Titration Calorimetry (ITC)

Thermodynamic parameters of the BSA interaction with $[B(Ph)_4]^-$ ions determined by the ITC technique in the cacodylate buffer solution of a pH 7, at 298.15 K, were subsequently compared with the results obtained under the same experimental conditions for the BSA–SDS system [20]. In both investigated cases, namely $\{[B(Ph)_4]^-$ –BSA and SDS–BSA, nonlinear least-squares procedures were applied for fitting isotherms to a model that assumes two sets of binding sites. The stoichiometry of the ligand–albumin complexes ($N = [ligand]/[BSA]$), binding constants (K_{ITC}) and the enthalpy change (ΔH_{ITC}) were obtained directly from ITC measurements. The free energy of binding (ΔG_{ITC}) and entropy change (ΔS_{ITC}) were calculated using the standard thermodynamic relationships: $\Delta G_{ITC} = -RT \ln K_{ITC} = \Delta H_{ITC} - T \Delta S_{ITC}$. The obtained parameters are so-called condition-dependent parameters and can be compared with those obtained under the same pH, temperature and the type of a buffer solution [22]. Representative binding isotherms for

the investigated systems are shown in Figure 3, whereas conditional parameters of the interactions are summarized in Table 1.

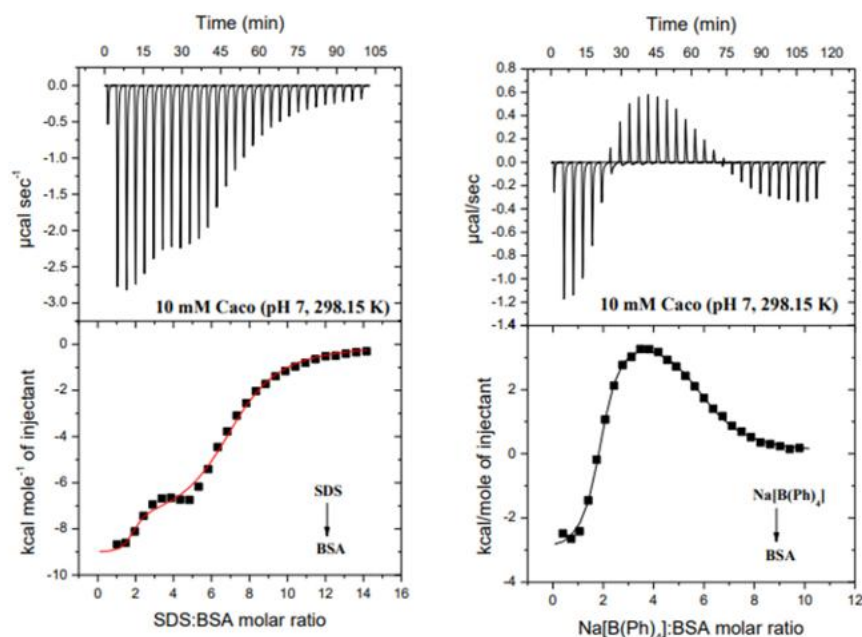


Figure 3. Calorimetric titration isotherms of the binding interactions between SDS and BSA (LEFT) and Na[B(Ph)₄] and BSA (RIGHT) in the 10 mM Caco buffer of pH 7, at 298.15 K.

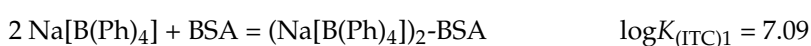
Table 1. The conditional thermodynamic parameters of SDS and Na[B(Ph)₄] binding to BSA (standard deviation values in parentheses) in the 10 mM Caco buffer of pH 7, at 298.15 K.

Parameter	SDS/BSA ⁽¹⁾	Na[B(Ph) ₄]/BSA
N_1	1.75 (±0.14)	1.78 (±0.02)
$\log K_{(ITC)1}$	7.61 (±0.38)	7.09 (±0.10)
$\Delta G_{(ITC)1}$ [kcal mol ^{−1}]	−10.38 (±0.67)	−9.67 (±0.14)
$\Delta H_{(ITC)1}$ [kcal mol ^{−1}]	−9.11 (±0.26)	−3.14 (±0.12)
$T\Delta S_{(ITC)1}$ [kcal mol ^{−1}]	1.27	6.53
N_2	5.33 (±0.29)	4.09 (±0.09)
$\log K_{(ITC)2}$	5.29 (±0.06)	5.39 (±0.06)
$\Delta G_{(ITC)2}$ [kcal mol ^{−1}]	−7.22 (±0.09)	−7.35 (±0.09)
$\Delta H_{(ITC)2}$ [kcal mol ^{−1}]	−7.98 (±0.27)	4.00 (±0.14)
$T\Delta S_{(ITC)2}$ [kcal mol ^{−1}]	−0.76	11.3

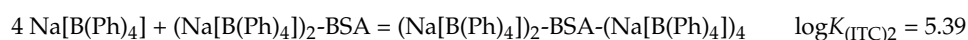
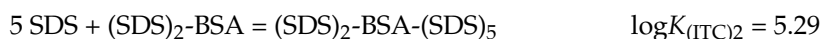
⁽¹⁾ Literature data [17].

ITC data revealed that BSA has two binding sites capable of binding tetraphenylborate ions, as has been previously observed for SDS monomers (Figure 3, Table 1). The simplified chemical equations of the ligand (SDS or [B(Ph)₄][−])–BSA complex formation which take into account the stoichiometry of the resulting species are given below:

The first binding site



The second binding site



The first site of albumin binds two $[\text{B(Ph)}_4]^-$ ions followed by the saturation of the second site by four tetraphenylborate ions. The binding constants ($K_{(\text{ITC})}$), and consequently the free energy of binding ($\Delta G_{(\text{ITC})}$), for both SDS–BSA and $[\text{B(Ph)}_4]^-$ –BSA complexes are comparable to one another (in the range of experimental error) indicating their similar thermodynamic stability (Table 1). In contrast to the SDS–BSA interactions in which the charge–charge type or/and van der Waals interactions are involved in the ligand–protein complex formation ($|\Delta H_{(\text{ITC})}| > |T\Delta S_{(\text{ITC})}|$), the binding of $[\text{B(Ph)}_4]^-$ ions in the first site of the albumin is both enthalpy- and entropy-driven. However, the hydrophobic interactions seem to prevail ($|\Delta H_{(\text{ITC})}| < |T\Delta S_{(\text{ITC})}|$) [23].

A different scenario is observed for binding $[\text{B(Ph)}_4]^-$ ions in the second site. The positive value of $\Delta H_{(\text{ITC})2}$ shows that the thermodynamic stability of the resulting complexes strongly depends on the entropy change, while the charge–charge type or/and van der Waals interactions are involved in the SDS binding to the second site (Table 1). Therefore, it is supposed that $[\text{B(Ph)}_4]^-$ –BSA (2nd site) binding occurs mainly via hydrophobic interactions which contribute to an increase in entropy on account of the dehydration of the reactants or as a result of ions exchange occurring in the $[\text{B(Ph)}_4]^-$ binding process. Subsequently, this supposition has been verified by MD simulations.

The obtained findings were employed for studying the potential $[\text{B(Ph)}_4]^-$ binding sites on the surface of the protein. To do this, the first and then the second binding site were saturated with $[\text{B(Ph)}_4]^-$ ions, and checked for whether the albumin is still able to bind SDS monomers (Figure 4). It turned out that BSA with the first site saturated by $[\text{B(Ph)}_4]^-$ ions is still able to bind SDS ($\log K_{(\text{ITC})} = 4.92 \pm 0.04$; $\Delta G_{(\text{ITC})} = -6.73 \pm 0.05 \text{ kcal mol}^{-1}$; $\Delta H_{(\text{ITC})} = -12.85 \pm 0.35 \text{ kcal mol}^{-1}$; $T\Delta S_{(\text{ITC})} = -6.12 \text{ kcal mol}^{-1}$), but in such a system only around four to five molecules of SDS are bound to one site, as opposed to around seven ($N_1 \sim 2$, $N_2 \sim 5$), as was observed in the absence of $[\text{B(Ph)}_4]^-$ ions in the solution (Table 1).

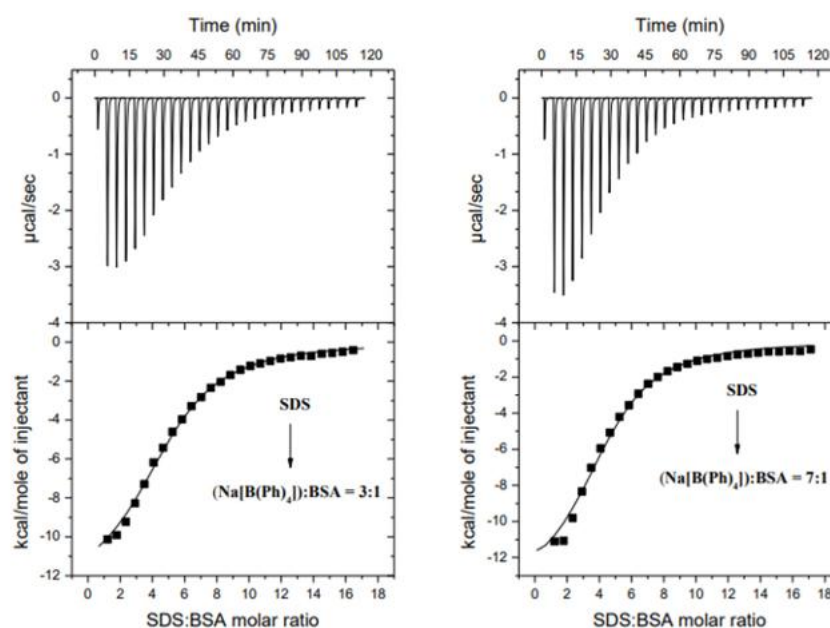


Figure 4. Calorimetric titration isotherms of the binding interactions between SDS and BSA in the presence of $\text{Na[B(Ph)}_4]$ in the $\text{Na[B(Ph)}_4]$:BSA molar ratio 3:1 (left) and 7:1 (right) in the 10 mM Caco buffer of pH 7, at 298.15 K.

Based on these observations, it can be assumed that both ligands ($[\text{B(Ph)}_4]^-$ and SDS) reveal an affinity to the same “first” binding site in BSA. After a saturation of the first site by $[\text{B(Ph)}_4]^-$ there is not enough space for binding of SDS monomers to the same surface of BSA. For this reason, negatively charged SDS anions are forced to bind to the second site of the protein. The above assumption confirms the following observations:

- (a) There are no significant differences in the thermodynamic stability of the ligand–albumin complexes formed in the first binding site. Thus, SDS is not a strong enough ligand to replace $[\text{B(Ph)}_4]^-$ in the first binding site.
- (b) Binding constants of the SDS–BSA complexes formed in the absence and in the presence of $[\text{B(Ph)}_4]^-$ have similar values:

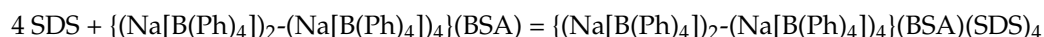
$$5 \text{ SDS} + (\text{SDS})_2\text{-BSA} = (\text{SDS})_2\text{-BSA}-(\text{SDS})_5 \quad \log K_{(\text{ITC})2} = 5.29 (\pm 0.06)$$

$$5 \text{ SDS} + (\text{Na}[\text{B(Ph)}_4])_2\text{-BSA} = (\text{Na}[\text{B(Ph)}_4])_2\text{-BSA}-(\text{SDS})_5 \quad \log K_{(\text{ITC})} = 4.92 (\pm 0.04)$$

In the absence of $[\text{B(Ph)}_4]^-$ in the system, the binding constant of SDS to the first binding site of BSA is expected to be $\log K_{(\text{ITC})}$ approximately 7 (Table 1). The ITC data showed that in cases where the $[\text{B(Ph)}_4]^-$ ions occupy the first site of the protein, the calculated binding constant ($\log K_{(\text{ITC})} \sim 5$) corresponds to the binding of SDS to the second binding site.

- (c) The repulsion interactions between the negatively charged ligands ($[\text{B(Ph)}_4]^-$ and SDS) disfavor occupation by them the same binding site in BSA.

A similar phenomenon has been observed after the saturation of the albumin by $[\text{B(Ph)}_4]^-$ in both binding sites (Figure 4). Under such experimental conditions, the albumin still possesses one binding site capable of binding four moles of SDS monomers ($\log K_{(\text{ITC})} = 4.97 \pm 0.07$; $\Delta G_{(\text{ITC})} = -6.78 \pm 0.09 \text{ kcal mol}^{-1}$; $\Delta H_{(\text{ITC})} = -13.95 \pm 0.71 \text{ kcal mol}^{-1}$; $T\Delta S_{(\text{ITC})} = -7.17 \text{ kcal mol}^{-1}$), according to the following general equation:



Moreover, it is worth noticing that the parameters of the interactions between SDS and BSA within the first as well as both sites being saturated by $[\text{B(Ph)}_4]^-$ are comparable (within the range of the experimental error). This confirms the fact that the investigated ligands show affinity only for the first site in BSA. Upon saturation of the same “first” site in BSA, $[\text{B(Ph)}_4]^-$ and SDS are bound at different sites.

2.2. Steady-State Fluorescence Spectroscopy

Figure 5 presents the fluorescence emission spectra of free BSA and the protein mixed with $\text{Na}[\text{B(Ph)}_4]$ in molar ratios equal to 1:3, 1:7, and 1:15 under the action of increasing concentrations of SDS. First of all, an increase in the amount of $[\text{B(Ph)}_4]^-$ in the system has no impact on the position of an initial strong emission band of tryptophan residues in BSA at approximately 348 nm. Secondly, it can be observed that the intrinsic fluorescence intensity of the albumin (free and saturated with $[\text{B(Ph)}_4]^-$) decreases regularly with the addition of SDS, accompanied by a significant blue shift from 348 nm to 332 nm, which may be a result of conformational changes in the BSA structure under the action of the surfactant (exposure of tryptophan residues to a more hydrophobic environment) [24]. Furthermore, the higher the amount of $[\text{B(Ph)}_4]^-$ ions in the mixture, the lower the changes in the fluorescence intensity of BSA under the action of SDS. These findings are in line with the ITC results, and confirm the fact that upon saturation of BSA with $[\text{B(Ph)}_4]^-$ the changes in the Trp fluorescence intensity are not as pronounced as for the $[\text{B(Ph)}_4]^-$ -free solution. This is related to the fact that under such experimental conditions, the total number of SDS monomers bound to the protein is lower, and the SDS monomers are bound to their second binding site, which is far from sensitive to SDS binding of the Trp-134 and Trp-213 residues [20].

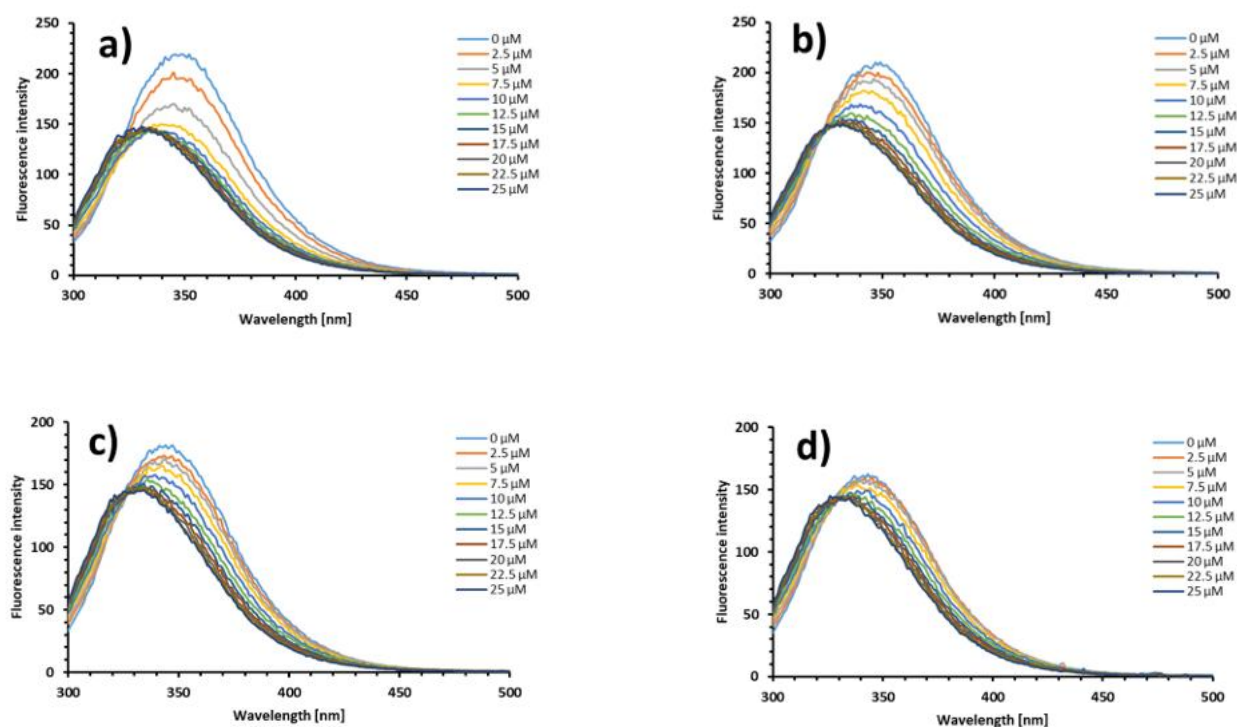


Figure 5. The fluorescence emission spectra of free BSA (a) and the solutions of BSA with Na[B(Ph)₄] mixed in molar ratios 1:3 (b); 1:7 (c); and 1:15 (d) in the presence of increasing concentrations of SDS (0–25 μM) in the 10 mM Caco buffer of pH 7.0 at 298.15 K.

To obtain a better insight into the mechanisms of the interactions between BSA (and its mixtures with [B(Ph)₄][−]) and SDS, the obtained results were analyzed according to a well-known Stern–Volmer equation: $\frac{F_0}{F} = 1 + K_{SV}[Q] = 1 + k_q\tau_0[Q]$, where F_0 and F denote the fluorescence intensities in the absence and presence of a quencher (SDS), $[Q]$ is the quencher concentration, K_{SV} is the Stern–Volmer quenching constant, k_q is the bimolecular quenching rate constant, while τ_0 is the lifetime of the fluorophore (BSA) in the absence of quencher [25,26]. The graphs of $\frac{F_0}{F}$ versus $[Q]$ plotted according to the Stern–Volmer equation are shown in Figure 6. Consequently, Table 2 presents the newly determined values of Stern–Volmer quenching constants along with linear correlation coefficients (R^2) and bimolecular quenching rate constants (k_q) recovered for the studied systems. The latter ones were calculated based on the value of the average fluorescence lifetime τ_0 of free BSA (in Tris-HCl, pH 7.0), which equals approximately 6.3 ns [27]. It can be observed that the quenching (binding) constants decrease with the increase in the [B(Ph)₄][−] concentration of the system, which can be assigned to the competition of SDS with [B(Ph)₄][−] in BSA [28,29]. Furthermore, the estimated values of k_q for all systems are of the order $10^{12} \text{ M}^{-1}\cdot\text{s}^{-1}$, which is approximately a few hundred times higher than the maximum value possible for the diffusion-controlled quenching-rate constant ($2.0 \times 10^{10} \text{ M}^{-1}\cdot\text{s}^{-1}$) [30]. Since the values of bimolecular quenching rate constants are considered to be definitive in differentiating between dynamic and static quenching mechanisms, the predominant role of ground-state complexation (static quenching) in the investigated systems was confirmed as albumin was measured using an extinction coefficient eqd.

2.3. Differential Scanning Calorimetry (DSC)

The influence of [B(Ph)₄][−] ions on a thermal unfolding transition of BSA was assessed based on DSC experiments. The representative, original DSC curves are shown in Figure 7. Two distinct peaks are observed in the heat-capacity curve for the sample of free BSA. The transition midpoints $T_{m1} = 328.9 \text{ K}$ and $T_{m2} = 346.7 \text{ K}$ were estimated by fitting the experimental C_p vs. T curves to a non-two-state model using Marquardt

nonlinear least-squares method (T_m denotes the temperature value at which a maximum endothermic effect appears). Previously, it has been reported that the appearance of these peaks corresponds to the low-temperature (T_{m1}) and the high-temperature (T_{m2}) transition resulting from the melting of structurally independent parts of the protein [31–34]. The less thermally stable domain comprises the subdomains IIB, IIIA, and IIIB, whereas the subdomains IA, IB, and IIA represent the region (the energetic domain) more resistant to thermal unfolding [35].

The formation of fairly stable Na[B(Ph)₄]-BSA complexes makes the BSA structure more compact and thermostable. This is reflected in the shift of the heat-induced transitions towards a higher range of temperatures with the increase in the Na[B(Ph)₄]:BSA molar ratios in the mixture, namely $T_{m1} = 341.8$ K and $T_{m2} = 351.9$ K for the saturation of the first binding sites (Na[B(Ph)₄]:BSA = 2.5:1) and $T_{m1} = 349.2$ K and $T_{m2} = 356.2$ K for BSA with both binding sites saturated (Na[B(Ph)₄]:BSA = 7:1).

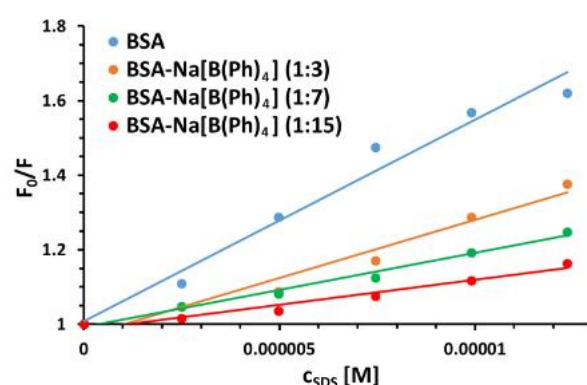


Figure 6. Stern–Volmer plots for the steady-state fluorescence quenching of BSA and its mixtures with Na[B(Ph)₄] (1:3; 1:7; and 1:15) by SDS in the 10 mM Caco buffer of pH 7.0 at 298 K.

Table 2. Stern–Volmer quenching constants, linear correlation coefficients (R^2) and bimolecular quenching rate constants (k_q) recovered for the steady-state fluorescence quenching of pure BSA and its mixtures with Na[B(Ph)₄] (1:3; 1:7; and 1:15) by SDS in the 10 mM Caco buffer of pH 7.0 at 298 K.

System	K_{SV} [10^4 M ^{−1}]	R^2	k_q [M ^{−1} ·s ^{−1}]
BSA	5.41	0.972	8.58×10^{12}
BSA-Na[B(Ph) ₄] (1:3)	3.12	0.965	4.94×10^{12}
BSA-Na[B(Ph) ₄] (1:7)	1.98	0.987	3.15×10^{12}
BSA-Na[B(Ph) ₄] (1:15)	1.35	0.962	2.14×10^{12}

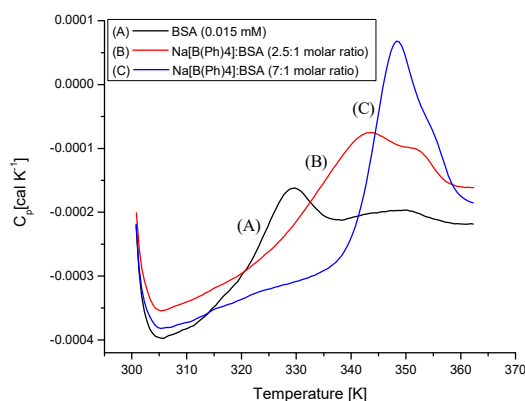


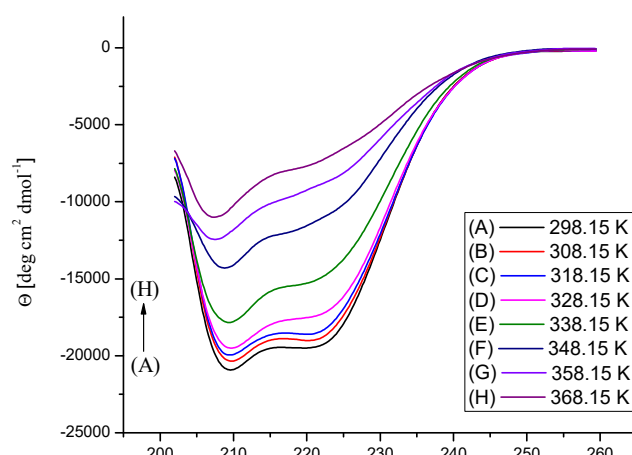
Figure 7. The raw heat capacity data for: (A) free BSA (0.015 mM) and the mixtures (B) with the Na[B(Ph)₄]:BSA molar ratios 2.5:1 (0.0375 mM Na[B(Ph)₄], 0.015 mM BSA) and (C) 7:1 (0.105 mM Na[B(Ph)₄], 0.015 mM BSA) in the 10 mM Caco buffer of pH 7.

2.4. Circular Dichroism (CD) Spectra Analysis

The effect of $\text{Na[B(Ph)}_4\text{]}$ ions' binding and temperature on the secondary structure of BSA was assessed by temperature-dependent CD measurements in the range of temperatures from 298.15 K to 368.15 K (Figures 8–10). The performed experiments showed that the saturation of BSA with $[\text{B(Ph)}_4]^-$ ions at 298.15 K does not contribute to noticeable changes in the secondary structure of the protein. On the other hand, the thermal stability of the α -helix structure increases on account of the formation of the $\text{Na[B(Ph)}_4\text{]}-\text{BSA}$ complexes (Table 3). These findings are in agreement with the DSC results.

Figure 8. CD spectra of BSA in 10 mM Caco buffer at pH 7, in the temperature range 298.15–368.15 K. The concentration of BSA was maintained at 0.0015 mM.

Figure 9. CD spectra of the $\text{Na[B(Ph)}_4\text{]}/\text{BSA}$ mixture ($\text{Na[B(Ph)}_4\text{]}:\text{BSA} = 2.5:1$ molar ratio) in 10 mM Caco buffer at pH 7, in the temperature range 298.15–368.15 K. The concentration of BSA was maintained at 0.0015 mM.



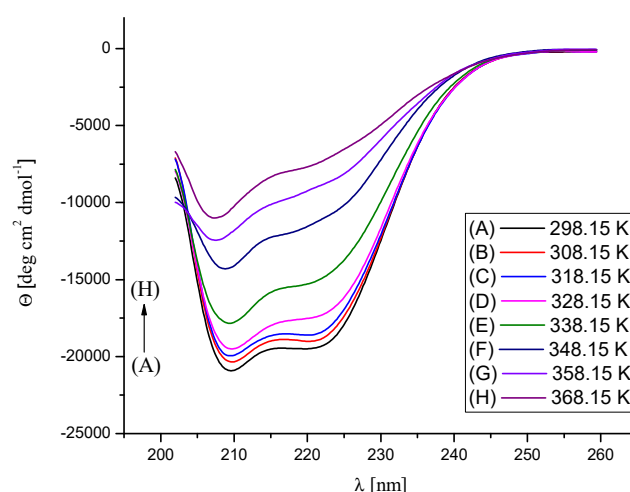


Figure 10. CD spectra of the Na[B(Ph)₄]/BSA mixture (Na[B(Ph)₄]:BSA = 7:1 molar ratio) in 10 mM Caco buffer at pH 7, in the temperature range 298.15–368.15 K. The concentration of BSA was maintained at 0.0015 mM.

Table 3. The secondary structure content (%) of BSA at different Na[B(Ph)₄]:BSA molar ratios in 10 mM Caco buffer at pH 7 in the temperature range 298.15–368.15 K, revealed from CD measurements.

T [K]	BSA	Na[B(Ph) ₄]:BSA	Na[B(Ph) ₄]:BSA
		2.5:1	7:1
		(Molar Ratio)	(Molar Ratio)
	The Percentage Content of α -Helix (H); β -Structure (S); β -Turn (Trn); Random Coil (Unrd) [%]		
298.15	59.8 (H); 6.7 (S) 11.1 (Trn); 22.3 (Unrd)	62.5 (H); 5.2 (S) 10.5 (Trn); 21.1 (Unrd)	59.2 (H); 6.1 (S) 11.5 (Trn); 23.1 (Unrd)
308.15	60.5 (H); 8.0 (S) 11.7 (Trn); 20.3 (Unrd)	63.9 (H); 4.5 (S) 10.3 (Trn); 21.3 (Unrd)	59.5 (H); 6.8 (S) 11.5 (Trn); 22.6 (Unrd)
318.15	56.0 (H); 7.3 (S) 13.4 (Trn); 23.6 (Unrd)	61.3 (H); 5.8 (S) 10.9 (Trn); 22.6 (Unrd)	58.2 (H); 7.8 (S) 12.1 (Trn); 22.4 (Unrd)
328.15	50.8 (H); 10.5 (S) 14.1 (Trn); 24.9 (Unrd)	58.0 (H); 7.0 (S) 12.5 (Trn); 22.6 (Unrd)	56.0 (H); 8.4 (S) 12.1 (Trn); 23.8 (Unrd)
338.15	44.4 (H); 11.5 (S) 16.7 (Trn); 27.5 (Unrd)	47.7 (H); 11.3 (S) 15.6 (Trn); 25.9 (Unrd)	50.2 (H); 8.4 (S) 15.8 (Trn); 25.9 (Unrd)
348.15	36.4 (H); 15.0 (S) 19.8 (Trn); 29.4 (Unrd)	39.1 (H); 14.4 (S) 18.6 (Trn); 28.3 (Unrd)	50.5 (H); 9.1 (S) 15.7 (Trn); 25.5 (Unrd)
358.15	25.4 (H); 21.2 (S) 24.1 (Trn); 29.8 (Unrd)	35.4 (H); 15.6 (S) 20.7 (Trn); 27.7 (Unrd)	23.7 (H); 22.4 (S) 22.9 (Trn); 31.0 (Unrd)
368.15	25.3 (H); 22.2 (S) 22.6 (Trn); 30.4 (Unrd)	22.0 (H); 24.9 (S) 22.3 (Trn); 31.0 (Unrd)	22.6 (H); 22.5 (S) 22.5 (Trn); 32.1 (Unrd)

2.5. Molecular Modelling

To gain a deeper understanding of the experimental results at the atomistic level, MD simulation was conducted for the [B(Ph)₄][−]–BSA system. In particular, the MD approach was applied to predict potential binding sites of [B(Ph)₄][−] on the BSA surface and to further characterize and compare them in terms of the binding affinities for these ions. The protein was surrounded by 15 randomly distributed [B(Ph)₄][−] ions and the simulation was performed for 100 ns at 300 K and a pH of 7. After the careful examination of the frames for each [B(Ph)₄][−] ligand, the last 40% of the simulation was used for the further LIE analysis (Table 4). Based on our results, two bindings sites were localized for three

molecules (Figure 11). Interestingly, one binding site is in the proximity to Trp-134, as was observed in the case of our previous SDS–BSA study [20]. Two ions bound to site I and one ion to site II after 40 and 60 ns, respectively, and remained there until the end of the simulation. Their RMSD as well as the RMSD of the protein are shown in Supplementary Figure S1 to demonstrate the convergence. LIE energy values for the ions bound in sites I and II are shown in Supplementary Figure S2, supporting the convergence achieved for the binding in these sites. In order to strictly define the binding event in sites I and II, a 5 Å cutoff criteria was applied for the distance between the residues of the sites and $[B(Ph)_4]^-$ ion atoms. According to LIE calculations, site I has slightly higher affinity towards $[B(Ph)_4]^-$ than site II due to the more favorable van der Waals component of the free binding energy (the electrostatic and van der Waals components calculated for each $[B(Ph)_4]^-$ ion are provided in Table 4). Site I comprises Lys116, Pro119, Glu140, Ile141, Arg144, His145, Leu178, Glu182, Arg185, and Val188, while site II is formed by Leu386, Asn390, Phe402, Leu406, Arg409, Thr410, Lys413, Thr448, Ser479, Arg484, and Phe487. Clearly, site I has a more charged nature and lower propensity for establishing pi–pi and cation–pi interactions than site II. Additionally, when looking at the right panel of Figure 11, it seems that theoretical calculations confirm that hydrophobic rings of phenyl groups contribute to the stabilization of interactions between BSA and $[B(Ph)_4]^-$.

Table 4. LIE free-energy decomposition values for $[B(Ph)_4]^-$ ligands in the $[B(Ph)_4]^-$ –BSA complex. Three $[B(Ph)_4]^-$ ions (no 2, 6 and 11) did not form stable complexes with BSA throughout the simulation. ΔG_{ele} , ΔG_{vdW} and ΔG_{tot} correspond to the electrostatic component, van der Waals component and total energy, respectively.

No of $[B(Ph)_4]^-$	ΔG_{ele} [kcal mol ^{−1}]	ΔG_{vdW} [kcal mol ^{−1}]	ΔG_{tot} [kcal mol ^{−1}]
1	−0.3 ± 0.1	−21.0 ± 2.5	−21.3 ± 2.6
2	N/A	N/A	N/A
3	−0.2 ± 0.1	−23.0 ± 3.2	−23.3 ± 3.2
4	−0.6 ± 0.2	−22.5 ± 2.9	−23.1 ± 2.9
5	−0.2 ± 0.1	−23.4 ± 3.4	−23.6 ± 3.4
6	N/A	N/A	N/A
7	−0.4 ± 0.2	−18.6 ± 5.7	−18.7 ± 5.9
8	−0.6 ± 0.1	−29.9 ± 2.5	−30.5 ± 2.5
9	−0.3 ± 0.1	−15.8 ± 2.5	−16.1 ± 2.5
10	0.7 ± 0.1	−32.2 ± 2.6	−32.8 ± 2.6
11	N/A	N/A	N/A
12	−0.3 ± 0.2	−21.2 ± 2.9	−21.5 ± 2.9
13	−0.2 ± 0.1	−15.2 ± 1.9	−15.4 ± 1.9
14	−0.6 ± 0.2	−28.5 ± 3.2	−29.0 ± 3.3
15	−0.3 ± 0.2	−20.8 ± 3.8	−21.1 ± 3.9

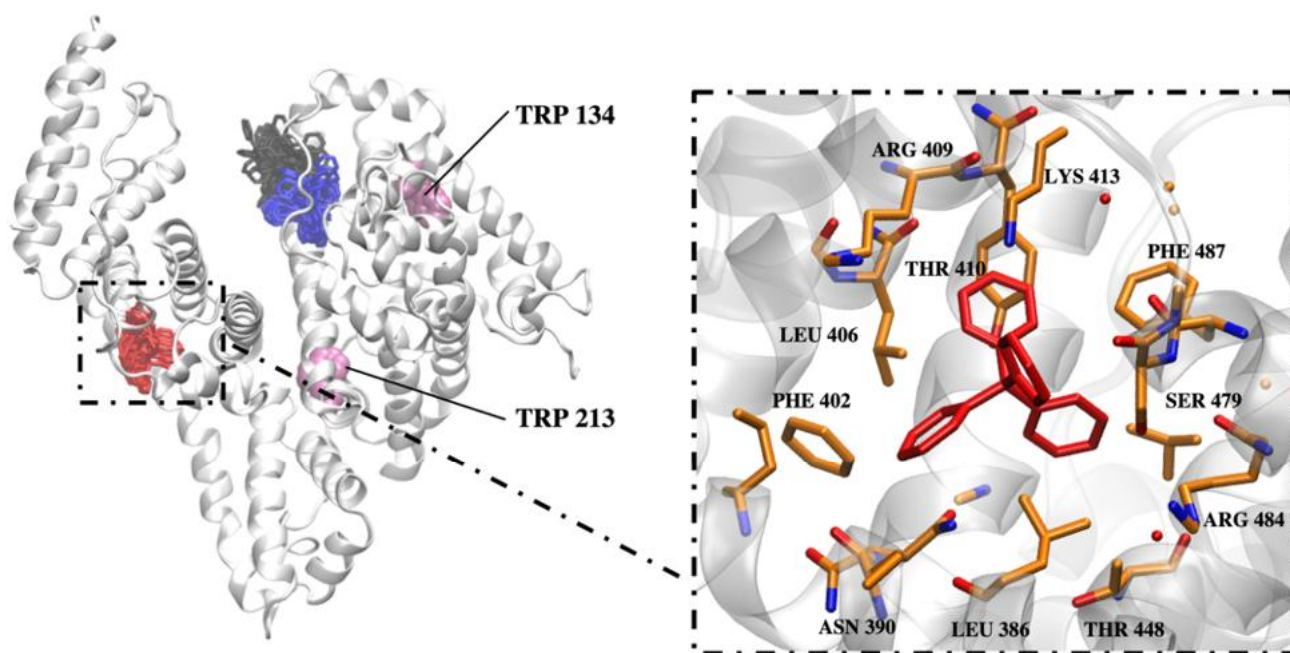


Figure 11. On the left, BSA protein (grey cartoon), Trp residues (magenta VDW representation) and three clusters (licorice) representing $[B(Ph)_4]^-$ ions that bound to the protein with the highest affinity. Colors of the clusters correspond to the color coding used in Table 4. On the right panel, a representative frame for one $[B(Ph)_4]^-$ ion and its protein surrounding.

3. Materials and Methods

3.1. Reagents

Bovine serum albumin (BSA, lyophilized powder, $\geq 96\%$), sodium dodecyl sulfate (SDS, for molecular biology, $\geq 99\%$), sodium tetraphenylborate ($Na[B(Ph)_4]$, $\geq 99.5\%$), sodium dodecyl sulfate (SDS, for molecular biology, $\geq 99\%$) and sodium cacodylate trihydrate (Caco, $\geq 98\%$) were obtained from Merck (Poland) and employed as received without further purification. Double-distilled water with conductivity not exceeding $0.18 \mu S cm^{-1}$ was used for preparations of buffer solutions.

3.2. Isothermal Titration Calorimetry (ITC)

All ITC experiments were performed at 298.15 K using an AutoITC isothermal titration calorimeter (MicroCal Inc. GE Healthcare, Northampton, MA, USA). The details of the measuring device and experimental setup have been described previously [20]. The reagents were dissolved directly in 10 mM Caco buffer of pH 7. The experiments consisted of injecting $10.02 \mu L$ (29 injections, $2 \mu L$ for the first injection only) of (a) 1 mM buffered solution of $Na[B(Ph)_4]$ into the reaction cell which initially contained the 0.02 mM buffered solution of BSA, and (b) 1 mM buffered solution of SDS into the reaction cell which initially contained the $Na[B(Ph)_4]$:BSA mixture of the 3:1 ($0.0375 mM Na[B(Ph)_4]$, $0.0125 mM BSA$) or 7:1 ($0.0875 mM Na[B(Ph)_4]$, $0.0125 mM BSA$) molar ratio. A background titration, consisting of an identical titrant solution but with the buffer solution in the reaction cell only, was subtracted from each experimental titration on account of the heat of dilution. The titrant was injected at 5 min intervals. Each injection lasted 20 s.

3.3. Steady-State Fluorescence Spectroscopy and UV Spectrophotometry

The stock solutions of BSA, SDS and $Na[B(Ph)_4]$ were prepared in 10 mM Caco buffer of pH 7 (all subsequent dilutions were made with this buffer). The concentration of bovine serum albumin was measured using an extinction coefficient equal to $\epsilon_{280}^{BSA} = 41,180 M^{-1} cm^{-1}$, calculated based on the content of tryptophan ($\epsilon_{280}^W = 5690 M^{-1} cm^{-1}$), tyrosine ($\epsilon_{280}^Y = 1280 M^{-1} cm^{-1}$), and cysteine ($\epsilon_{280}^C = 120 M^{-1} cm^{-1}$) [36]. A maximum ab-

sorbance value of approximately 0.08 at 280 nm (corresponding to a protein concentration of 2 μM) was used to avoid the inner-filter effect.

Steady-state fluorescence experiments were carried out with a Cary Eclipse Varian (Agilent, Santa Clara, CA, USA) spectrofluorometer, equipped with a temperature controller and a 1.0 cm multicell holder. The absorption spectra were recorded on Perkin Elmer Lambda 650 (Waltham, MA, USA) UV/Vis spectrophotometer. In the performed fluorescence titration experiments: (i) 2 mL of pure BSA (2 μM BSA); (ii) 2 mL of $\text{Na}[\text{B}(\text{Ph})_4]$ and BSA mixed in ratio 3:1 (6 μM $\text{Na}[\text{B}(\text{Ph})_4]$, 2 μM BSA); (iii) 2 mL of BSA and $\text{Na}[\text{B}(\text{Ph})_4]$ mixed in ratio 7:1 (14 μM $\text{Na}[\text{B}(\text{Ph})_4]$, 2 μM BSA); and (iv) 2 mL of BSA and $\text{Na}[\text{B}(\text{Ph})_4]$ mixed in ratio 15:1 (30 μM $\text{Na}[\text{B}(\text{Ph})_4]$, 2 μM BSA) were simultaneously titrated with ten 5 μL aliquots of sodium dodecyl sulphate (1 mM). The fluorescence intensity of the band at 348 nm—corresponding to the initial maximum emission of BSA—was used to calculate the quenching constants and then other parameters. Excitation wavelength was always set at 280 nm. All experiments were performed at 298.15 K.

3.4. Differential Scanning Calorimetry (DSC)

Calorimetric (DSC) measurements were made with a VP-DSC microcalorimeter (MicroCal Inc. GE Healthcare, Northampton, USA) at a scanning rate 90 K h^{-1} . All scans were run in 10 mM Caco buffer of pH 7 in the temperature range of 298.15–363.15 K at a scan rate of 1.5 K min^{-1} . The scans were obtained for pure BSA (0.015 mM) and for the solution containing $\text{Na}[\text{B}(\text{Ph})_4]$ and BSA mixed in ratio 2.5:1 (0.0375 mM $\text{Na}[\text{B}(\text{Ph})_4]$, 0.015 mM BSA) and 7:1 (0.105 mM $\text{Na}[\text{B}(\text{Ph})_4]$, 0.015 mM BSA). All the samples were degassed before measurements. These measurements were recorded two times. The details of the measuring device, experimental setup and data processing have been described previously [20].

3.5. Circular Dichroism Spectroscopy (CD)

Circular dichroism (CD) spectra were recorded in water on a Jasco-715 automatic recording spectropolarimeter (Jasco Inc., Easton, MD, USA) for the following systems: pure BSA (0.0015 mM) and two $\text{Na}[\text{B}(\text{Ph})_4]$ /BSA mixtures in stoichiometric molar ratios 2.5:1 (0.00375 mM $\text{Na}[\text{B}(\text{Ph})_4]$, 0.0015 mM BSA) and 7:1 (0.0105 mM $\text{Na}[\text{B}(\text{Ph})_4]$, 0.0015 mM BSA) in the 10 mM Caco buffer of pH 7, in a temperature range from 298.15 K to 368.15 K, every 10 degrees. The details of the measuring device, experimental setup and data processing have been described previously [20]. The secondary structure of BSA under experimental conditions in the absence and presence of $\text{Na}[\text{B}(\text{Ph})_4]$ was determined using the CONTIN/LL program, a variant of the CONTIN method developed by Provencher and Glockner [37] provided within the CDPro software package.

3.6. Theoretical Studies

3.6.1. Structures

The structure of Bovine Serum Albumin (BSA) was obtained from the Protein Data Bank (PDB ID: 4F5S, 2.47 Å). The structure of $[\text{B}(\text{Ph})_4]^-$ (referred to as BPH for simplicity) was parameterized as by Kurt and Temel [38,39] and later built in xleap of AMBER16 package [40].

3.6.2. Molecular Dynamics Simulations

The MD approach was applied to localize potential binding sites and to characterize them in terms of the binding affinities. There are several reasons why such an approach is superior to the classical molecular docking approach, and therefore was used instead of molecular docking in our study: (1) Docking small ligands as SDS in our previous work [20] and $[\text{B}(\text{Ph})_4]^-$ to BSA in this work did not allow for finding more than a single binding site. (2) MD allows for the complete flexibility of the protein residues, which could be crucial for the binding of a small ligand, while rigid receptor molecular docking does not allow for it. Taking into account a structural ensemble of receptors in molecular docking is a possible

alternative which is computationally more expensive than the MD approach. (3) In contrast to molecular docking, solvent is treated explicitly in the MD simulations. (4) Molecular docking scores are far less reliable than MD-derived binding affinities due to the fact that molecular docking scores single, static poses, while multiple frames corresponding to the statistical ensemble of the structures are considered in the MD-based calculations. (5) In most of the state-of-the-art modelling studies, the poses obtained by a molecular docking approach are further analyzed with the MD approach. Therefore, the MD approach, when applied for predicting binding sites, is already self-consistent in comparison to the two-step procedure that includes molecular docking followed by MD. Hence, the MD approach is preferable. (6) Furthermore, we previously demonstrated both convergence and high predictive power of the MD approach when applied to the protein-ion systems [41]. All all-atom molecular dynamics (MD) simulations were performed with the use of AMBER16 software package [40]. The initial structures of the complexes were created by random placement of 15 $[B(Ph)_4]^-$ ions around the BSA protein. A truncated, octahedron, TIP3P periodic box of an 8 Å water layer from the box's border to the solute was used to solvate complexes. The ff14SBonlysc force field for the protein [42] and gaff force field [43] with RESP charges [44] obtained in the antechamber module of AMBER16 [40] for $[B(Ph)_4]^-$ were used. The net negative charge of the system was neutralized with Na^+ counterions. Energy minimization was carried out in two steps: beginning with 500 steepest descent cycles and 10^3 conjugate gradient cycles with 100 kcal/mol/Å² harmonic force restraints on solute atoms, continued with 3×10^3 steepest descent cycles and 3×10^3 conjugate gradient cycles without any restraints. Following minimization steps, the system was heated up from 0 to 300 K for 10 ps with harmonic force restraints of 100 kcal/mol/Å² on solute atoms. Then, the system was equilibrated at 300 K and 10^5 Pa in isothermal isobaric ensemble for 100 ps. Afterwards, an independent prediction MD run was carried out in the same isothermal isobaric ensemble for 100 ns. The particle mesh Ewald method for treating electrostatics and the SHAKE algorithm for all the covalent bonds containing hydrogen atoms were implemented in the MD simulations. Such parameters within the MD simulation were shown to be sufficient for a system of ions with a protein, which was substantially bigger than BSA, allowing for proper prediction of the ion binding sites [41].

3.6.3. Binding Free-Energy Calculations

Energetic postprocessing of the trajectories and per-residue energy decomposition was performed for the system with the use of Linear Interaction Energy (LIE) with dielectric constant of 80. Frames from the MD simulation were carefully examined with VMD [45]. The last 40% of the simulation was chosen for the analysis, since in the corresponding frames no more events of ion dissociations or associations were observed. LIE takes into account only the electrostatic component (scaled by the dielectric constant) and the van der Waals component of the binding free energy. Although the binding free energies obtained by this method are not expected to represent absolute binding free energies, these calculations are useful to compare the binding strength in different binding sites.

4. Conclusions

A few complementary experimental methods supported by in silico analysis have successfully been applied to describe the physicochemical nature of tetraphenylborate ions' interactions with BSA. The obtained results were subsequently discussed in relation to a previously studied system, namely the interaction of sodium dodecyl sulphate (SDS) with BSA. The described approach enables us to find the BSA potential binding sites of $[B(Ph)_4]^-$.

In conclusion, two main binding sites of $[B(Ph)_4]^-$ were unveiled. They are located in the subdomain IA and IIIA, respectively, of the investigated protein. Both tetraphenylborate ions and SDS molecules reveal a similar affinity to the first specific binding site in BSA which is located close to the Trp-134 residue. In turn, the second binding site on the BSA surface

for investigated ligands are different. The binding of $[B(Ph)_4]^-$ ions in the subdomain IIIA is an entropy-driven process, whereas SDS in the subdomain IIB is enthalpy-driven.

It has been proven that $[B(Ph)_4]^-$ ions participate in the stabilization of the tertiary structure of BSA at higher temperatures on account of the formation of fairly stable $[B(Ph)_4]^-$ -BSA complexes of a different stoichiometry ($[B(Ph)_4]^-$:BSA = 2:1 and 5:1). This phenomenon underlying its biological activity could be responsible for the protection of the protein from thermal unfolding.

The results clearly show that the presence of competitive ligands may have some implications on the concentration of the compounds tested (as has been found for $[B(Ph)_4]^-$ ions or SDS) in the cell-culture media containing albumin. On the one hand, BSA, an important component of the cell culture media, binding to the compounds of a hydrophobic nature, as $[B(Ph)_4]^-$ or SDS, can lead to a decrease in the concentration of free, active species. In consequence, this can affect the cytotoxic action of the tested compound. On the other hand, the saturation of the potential binding sites of BSA by a nontoxic, low-molecular-weight ligand may prevent the interactions of albumin with other biologically active compounds, thus affecting their dose-dependent action.

The findings presented in this contribution are worth taking into consideration during the analysis of the cytotoxicity assays of the compounds being characterized.

Supplementary Materials: The following are available online. Figure S1: RMSD of protein (upper panel) and $[B(Ph)_4]^-$ ions in site I (middle panels) and in site II (bottom panel) in the course of the MD simulation, Figure S2: LIE free energy of binding for $[B(Ph)_4]^-$ ions in site I (upper and middle panels) and in site II (bottom panel) in the course of the MD simulation.

Author Contributions: Conceptualization, O.G., M.M.K., S.A.S. and D.W.; methodology, S.A.S., D.W.; formal analysis, M.M.K., K.Ž. and D.W.; investigation, O.G., M.M.K., K.Ž., J.M., A.T., K.C. and D.W.; data curation, O.G., M.M.K., A.T., K.C.; writing—original draft preparation, O.G., M.M.K., K.Ž., S.A.S., D.W. and L.C.; writing—review and editing, M.M.K., K.Ž., S.A.S., D.W. and L.C.; visualization, O.G., M.M.K., K.Ž. and D.W.; supervision, S.A.S., D.W. and L.C.; project administration, D.W.; funding acquisition, K.Ž. and S.A.S. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Polish National Science Centre under Grants No. UMO-2016/23/D/ST4/01576 (K.Ž.), UMO-2018/30/E/ST4/00037 (S.A.S.).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

Sample Availability: All compounds used in the study are commercially available (for provider, see Section 3).

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PUBLIKACJA 3

**Implications of albumin in cell culture media
on the biological action of vanadates(V)**



Implications of albumin in cell culture media on the biological action of vanadates(V)

Ola Grabowska^a, Magdalena Zdrowowicz^a, Dan Miłaś^b, Krzysztof Żamojć^a, Katarzyna Chmur^a, Aleksandra Tesmar^a, Martyna Kapica^a, Lech Chmurzyński^a, Dariusz Wyrzykowski^{a,*}

^a Faculty of Chemistry, University of Gdansk, Wita Stwosza 63, 80-308 Gdansk, Poland

^b Faculty of Chemistry, Biology, Geography, West University Timișoara, Strada Johann Heinrich Pestalozzi 16, Timișoara, Romania

ARTICLE INFO

Keywords:

Vanadate(V)
Bovine serum albumin
Prostate cancer cells

ABSTRACT

In this article, the implications of binding competition of vanadates(V) with dodecyl sulfates for bovine serum albumin on cytotoxicity of vanadium(V) species against prostate cancer cells have been investigated. The pH- and SDS-dependent vanadate(V)-BSA interactions were observed. At pH 5, there is only one site capable of binding ten vanadates(V) ions ($\log K_{(ITC)1} = 4.96 \pm 0.06$; $\Delta H_{(ITC)1} = -1.04 \pm 0.03 \text{ kcal mol}^{-1}$), whereas at pH 7 two distinctive binding sites on protein were found, saturated with two and seven V(V) ions, respectively ($\log K_{(ITC)1} = 6.11 \pm 0.06$; $\Delta H_{(ITC)1} = 0.78 \pm 0.12 \text{ kcal mol}^{-1}$; $\log K_{(ITC)2} = 4.80 \pm 0.02$; $\Delta H_{(ITC)2} = -4.95 \pm 0.14 \text{ kcal mol}^{-1}$). SDS influences the stoichiometry and the stability of the resulting V(V)-BSA complexes. Finally, the cytotoxicity of vanadates(V) against prostate cancer cells (PC3 line) was examined in the presence and absence of SDS in the culture medium. In the case of a 24-h incubation with 100 μM vanadate(V), a ca. 20 % reduction in viability of PC3 cells was observed in the presence of SDS. However, in other considered cases (various concentrations and time of incubation) SDS does not affect the dose-dependent action of vanadates(V) on the investigated prostate cancer cells.

1. Introduction

The interactions of proteins with low-molecular-weight compounds depend on many factors, among which the pH of a system plays a vital role [1,2]. The change in the pH of a solution invokes conformational changes in the protein's structure and consequently affects its biological activity. Moreover, the pH of an environment determines the overall charge of a protein which, in turn, influences ligand-protein interactions and molecular recognition. The situation is slightly more complicated when polyoxido-metalates are involved in the interactions [3,4]. Due to the concentration- and pH-dependent equilibria in aqueous solutions the investigations of their interaction with proteins are challenging and remain the subject of ongoing experimental and theoretical studies [5–9]. An excellent review provided by Aureliano et al. emphasizes the influence of experimental conditions on the vanadium species, and consequently their specific protein interactions [3]. Although there are many techniques used for studying the protein-ligand interactions, the choice of the proper one is not always a simple task and depends on the specific physicochemical features of the reagents under study.

In our studies, we have used the isothermal titration calorimetry (ITC) technique supported by steady-state fluorescence spectroscopy for studying the interactions of bovine serum albumin (BSA) with vanadates (V) at different pH values. According to our knowledge, the interactions of V(V) species with BSA, in contrast to many other proteins [10], have not so far been widely analyzed. The application of the ITC technique enabled for the first time the description of the investigated interactions at the molecular level. Furthermore, the stoichiometry of the resulting V(V)-protein complexes as well as number of the potential binding sites were found and discussed. However, it should be emphasized the obtained thermodynamic parameters are condition-dependent and can only be compared to the data acquired under the same experimental conditions by using other analytical techniques [11].

It is also worth highlighting that the biological activities of the V(V) species are well documented. Vanadates(V) have extensively been investigated ranging from their antidiabetic action, through a generation of free radicals leading to oxidation damages, lipid peroxidation to anticancer properties [12–19]. However, a relatively high amount of BSA (ca. 0.04 mM) in the cell culture media may contribute to the

* Corresponding author.

E-mail address: dariusz.wyrzykowski@ug.edu.pl (D. Wyrzykowski).

<https://doi.org/10.1016/j.ijbiomac.2023.127875>

Received 23 August 2023; Received in revised form 31 October 2023; Accepted 1 November 2023

Available online 2 November 2023

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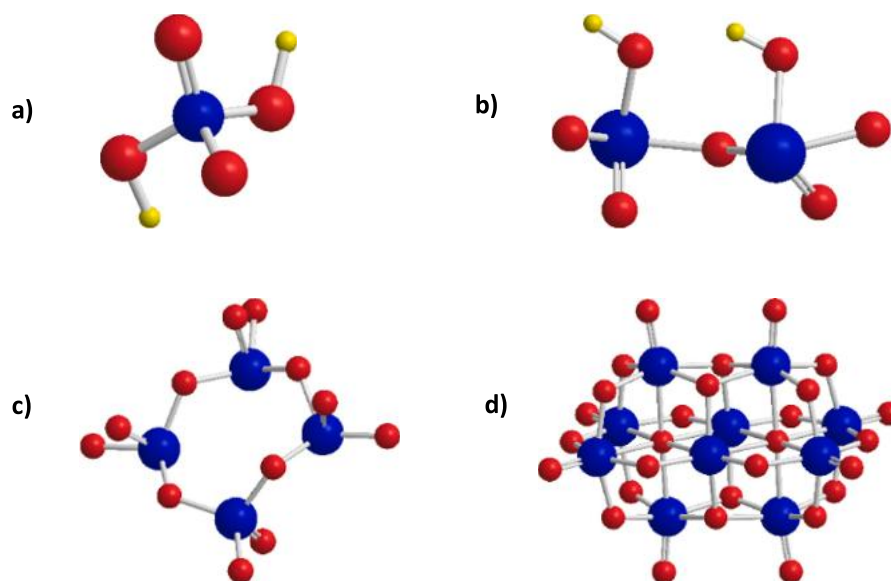


Fig. 1. Schematic structures of metavanadate species like (a) orthovanadate $[\text{H}_2\text{VO}_4]^-$ (V1), (b) dimeric vanadate species $[\text{H}_2\text{V}_2\text{O}_7]^{2-}$ (V2), (c) cyclic tetrameric vanadate species $[\text{V}_4\text{O}_{12}]^{4-}$ (V4) and (d) decavanadate anion $[\text{V}_{10}\text{O}_{28}]^{6-}$ (V10).

lowering of free vanadium species on account of their binding with albumin [20]. In consequence, these interactions may affect the dose-dependent activity of the investigated vanadium compounds evaluated by *in vitro* studies. Thus, to get some insight into this problem we have also assessed the effect of the saturation of the binding sites of the albumin with the blocker of the V(V) binding sites, namely sodium dodecyl sulfate (SDS), on the V(V)-BSA interactions. The obtained results enabled to draw some conclusions on the impact of SDS in the cell culture media on the dose-dependent cytotoxicity of metavanadates toward prostate cancer cells (PC3).

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA, $\geq 96\%$), sodium metavanadate (NaVO_3 , $\geq 98\%$), sodium dodecyl sulfate (SDS, $\geq 99\%$) and sodium cacodylate trihydrate (Caco, $\geq 99\%$) were purchased from Sigma-Aldrich (Poland). The 10 mM Caco buffers of pH 5 and 7 were used to prepare the solutions of BSA, NaVO_3 (a yellow solution at pH 5, a colourless solution at pH 7), and SDS. The concentration of BSA was determined spectrophotometrically using an extinction coefficient of $41,180 \text{ M}^{-1} \text{ cm}^{-1}$, which was calculated based on the amount of tryptophan ($\epsilon_{280}^{\text{W}} = 5690 \text{ M}^{-1} \text{ cm}^{-1}$), tyrosine ($\epsilon_{280}^{\text{Y}} = 1280 \text{ M}^{-1} \text{ cm}^{-1}$), and cysteine ($\epsilon_{280}^{\text{C}} = 120 \text{ M}^{-1} \text{ cm}^{-1}$) [21].

2.2. Isothermal titration calorimetry (ITC)

ITC experiments were accomplished on the AutoITC isothermal titration calorimeter (MicroCal Inc. GE Healthcare, Northampton, MA, USA). The ITC device details and experimental setup were described previously [22,23]. The 1.5 mM solution of NaVO_3 was injected into the reaction cell which contained a) the 0.0125 mM solution of free BSA (1) (pH 5 and 7), b) the mixture of SDS:BSA (pH 7) in the molar ratio of 2.6:1 (2) and 7.6:1 (3), respectively. ITC parameters: temperature 298.15 K; cell volume: 1.4491-mL; injection volume: 10.02 μL (29 injections, 2 μL for the first injection only); injection duration 20 s.; injection interval 240 s.; stirring speed 300 r.p.m.; reference power: 10 $\mu\text{cal/s}$. The CaCl_2 -EDTA titration was performed to check the apparatus and the results (stoichiometry, K , ΔH) were compared with those obtained for the same samples (a test kit) at MicroCal Inc. GE Healthcare. ITC data were

processed with Origin 7 (MicroCal). The ΔG_{ITC} and $T\Delta S_{\text{ITC}}$ parameters of the interactions were calculated using the relationships: $\Delta G_{\text{ITC}} = -RT \ln K_{\text{ITC}} = \Delta H_{\text{ITC}} - T\Delta S_{\text{ITC}}$.

2.3. Steady-state fluorescence spectroscopy

Steady-state fluorescence experiments were carried out on Cary Eclipse Varian (Agilent, Santa Clara, CA, USA) spectrofluorometer equipped with a 1.0 cm multicell holder. The inner filter effect has been compensated for all the fluorescence intensity values [24–26]. The widths of the excitation and emission slits were set at 5 nm. The excitation wavelength was tuned to 280 nm. In the fluorescence titration studies: (i) 2 mL of pure BSA (2 μM); (ii) 2 mL of BSA and SDS mixed in a molar ratio 1:2.5 (2 μM BSA; 5 μM SDS); (iii) 2 mL of BSA and SDS mixed in a molar ratio 1:7.5 (2 μM BSA; 15 μM SDS); and (iv) 2 mL of BSA and SDS mixed in a molar ratio 1:15 (2 μM BSA; 30 μM SDS) were concurrently titrated with ten 5 μL aliquots of sodium metavanadate (5 mM). The fluorescence intensities of the band at 348 nm (for pure BSA), 344 nm (for 1:2.5 mixture of BSA and SDS), 337 nm (for 1:7.5 mixture of BSA and SDS), and 330 nm (for 1:15 mixture of BSA and SDS) – matching the initial maximum emission of the protein in each amount of SDS – were used to obtain appropriate binding parameters. All spectrofluorometric measurements were performed in four separate experiments.

2.4. Cell culture

PC3 cells were purchased from ATCC (Manassas, Virginia) and were cultured in an F12k medium supplemented with 10 % FBS and 100 U mL^{-1} of antibiotics (penicillin-streptomycin), at 310.15 K in a humidified environment with 5 % CO_2 .

2.5. Cytotoxicity assays

The MTT test was used to determine the cytotoxicity of NaVO_3 and SDS toward PC3 (prostate cancer cells) and to evaluate the impact of SDS on the cytotoxicity of NaVO_3 . In the first step, the concentration of SDS used during the experiments has been optimized. Cells were seeded in 96-well plates at a density of $4 \cdot 10^3$ per well and left to incubate overnight at 37 °C and 5 % CO_2 . On the next day, the cells were treated with studied compounds or a vehicle (ultrapure water, negative control) and incubated for 24 or 48 h. Then, after the addition of tetrazolium dye

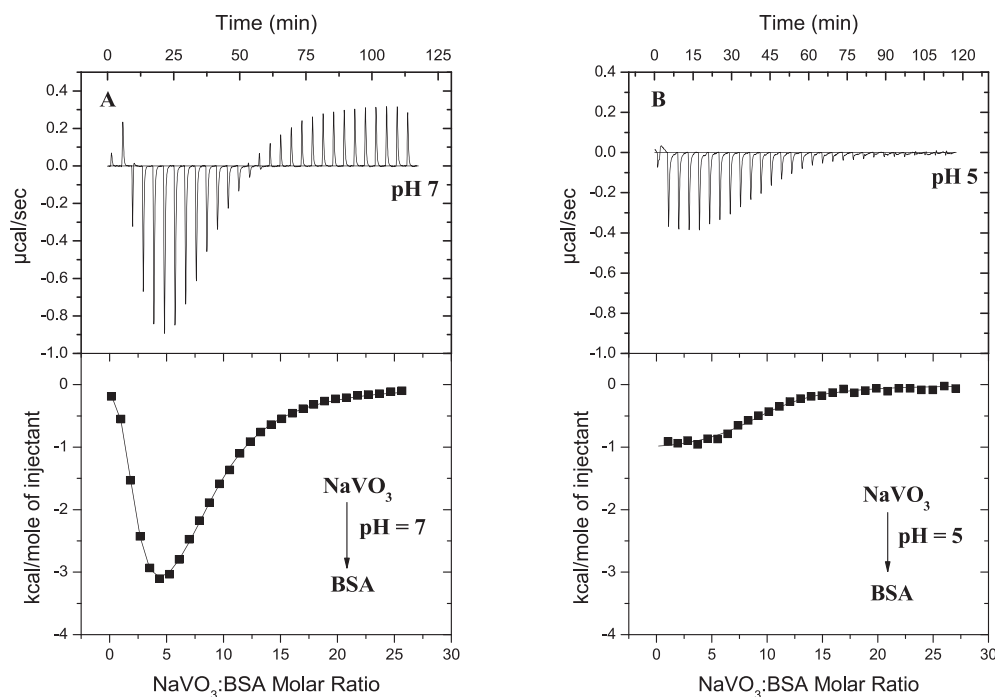


Fig. 2. ITC titrations of BSA (0.0125 mM) with NaVO_3 (1.5 mM), in the 10 mM Caco buffers of pH 7 (A) and 5 (B), at 298.15 K.

Table 1

ITC parameters of NaVO_3 - BSA interactions in the absence and the presence of SDS in the 10 mM Caco buffer of pH 7, and in the 10 mM Caco buffer of pH 5, at 298.15 K ($\pm$ standard deviation values).

Parameter	NaVO_3 /BSA pH 7	NaVO_3 /BSA:SDS ^a pH 7	NaVO_3 /BSA pH 5
N_1	1.73 ± 0.03	1.02 ± 0.07	10.28 ± 0.20
$\log K_{(\text{ITC})1}$	6.11 ± 0.06	5.26 ± 0.10	4.96 ± 0.06
$\Delta G_{(\text{ITC})1}$ [kcal mol ⁻¹]	-8.34 ± 0.08	-7.18 ± 0.14	-6.77 ± 0.08
$\Delta H_{(\text{ITC})1}$ [kcal mol ⁻¹]	0.78 ± 0.12	0.72 ± 0.40	-1.04 ± 0.03
$T\Delta S_{(\text{ITC})1}$ [kcal mol ⁻¹]	9.11	7.9	5.73
N_2	6.58 ± 0.13	4.87 ± 0.29	–
$\log K_2$	4.80 ± 0.02	3.78 ± 0.03	–
$\Delta G_{(\text{ITC})2}$ [kcal mol ⁻¹]	-6.54 ± 0.02	-5.15 ± 0.04	–
$\Delta H_{(\text{ITC})2}$ [kcal mol ⁻¹]	-4.95 ± 0.14	-6.19 ± 0.15	–
$T\Delta S_{(\text{ITC})2}$ [kcal mol ⁻¹]	1.59	-1.04	–

^a The BSA:SDS molar ratio = 1:2.6.

(MTT) aqueous solutions (4 mg mL⁻¹, 25 µL per well) and 3 h of incubation, the resulting formazan product was dissolved in DMSO. A microplate reader (EnSpire, PerkinElmer, Waltham, MA, USA) was used to measure the absorbance at a wavelength of 570 nm (660 nm was used as the reference wavelength). The viability of the control cells was taken to be 100 %. Three repetitions of each of three independent experiments were carried out. The results were statistically analyzed using GraphPad Prism 7 by one-way ANOVA followed by Tukey's multiple comparison test.

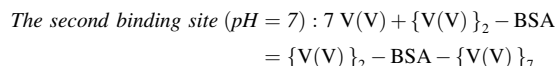
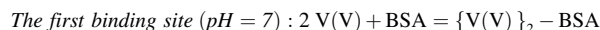
3. Results and discussion

3.1. pH-dependent interactions of NaVO_3 with BSA

The pH of a solution and the nature of potential ligands present in an

environment are essential factors that affect directly the concentration of particular vanadium species occurring simultaneously in equilibrium. Moreover, the mutual ratio of the particular species at a constant pH is also influenced by the total concentration of V(V) [20,27]. Thus, vanadium speciation is complicated and the equilibrium is highly sensitive to the change in experimental conditions. Taking into account ITC experiments with 1.5 mM NaVO_3 (in the 10 mM Caco buffer), in a neutral solution (pH 7) the percentage concentrations of V(V) species are lowering in the direction $[\text{H}_2\text{VO}_4]^-$ (V1) (ca. 50 %) > $[\text{V}_4\text{O}_{12}]^{4-}$ (V4) (ca. 30 %) > $[\text{H}_2\text{V}_2\text{O}_7]^{2-}$ (V2) (ca. 20 %). In turn, decavanadate ions (V10), namely $[\text{HV}_{10}\text{O}_{28}]^{5-}$ (ca. 55 %) and $[\text{H}_2\text{V}_{10}\text{O}_{28}]^{4-}$ (ca. 10 %) dominate at pH 5 together with a small amount of $[\text{H}_2\text{VO}_4]^-$ (V1) (ca. 20 %) and $[\text{H}_2\text{V}_2\text{O}_7]^{2-}$ (V2) (ca. 10 %) [3,20,28–31] (Fig. 1). These V(V) species exist not only in solution but were also isolated in the solid state [32]. Moreover, they reveal an affinity for various types of ligands, with which they can form quite stable complexes. This phenomenon manifests itself in the NaVO_3 -BSA interactions. Representative binding isotherms for these interactions in the 10 mM Caco buffers of pH 5 and 7 at 298.15 K are shown in Fig. 2, whereas appropriate thermodynamic parameters are summarized in Table 1.

At pH 7, BSA possesses two different binding sites capable to bind V(V) ions. The first site with a higher affinity toward vanadium(V) species binds ca. 2 mol of inorganic ions per one mole of BSA. Then, the second site of the protein is saturated with ca. 7 mol of the ions. The simplified chemical equations of the interactions under study are given below:



Different molecular mechanisms are involved in the interactions of V(V) ions with BSA at pH 7. The binding of the vanadium species in the first site of the protein is an entropy-driven process ($|\Delta H_{(\text{ITC})1}| < |T\Delta S_{(\text{ITC})1}|$), whereas in the second site, the formation of the V(V)-BSA complexes depends on the enthalpy change ($|\Delta H_{(\text{ITC})2}| > |T\Delta S_{(\text{ITC})2}|$). The observed exothermic effects suggest the important contribution of the electrostatic interactions to the stability of the complexes formed in

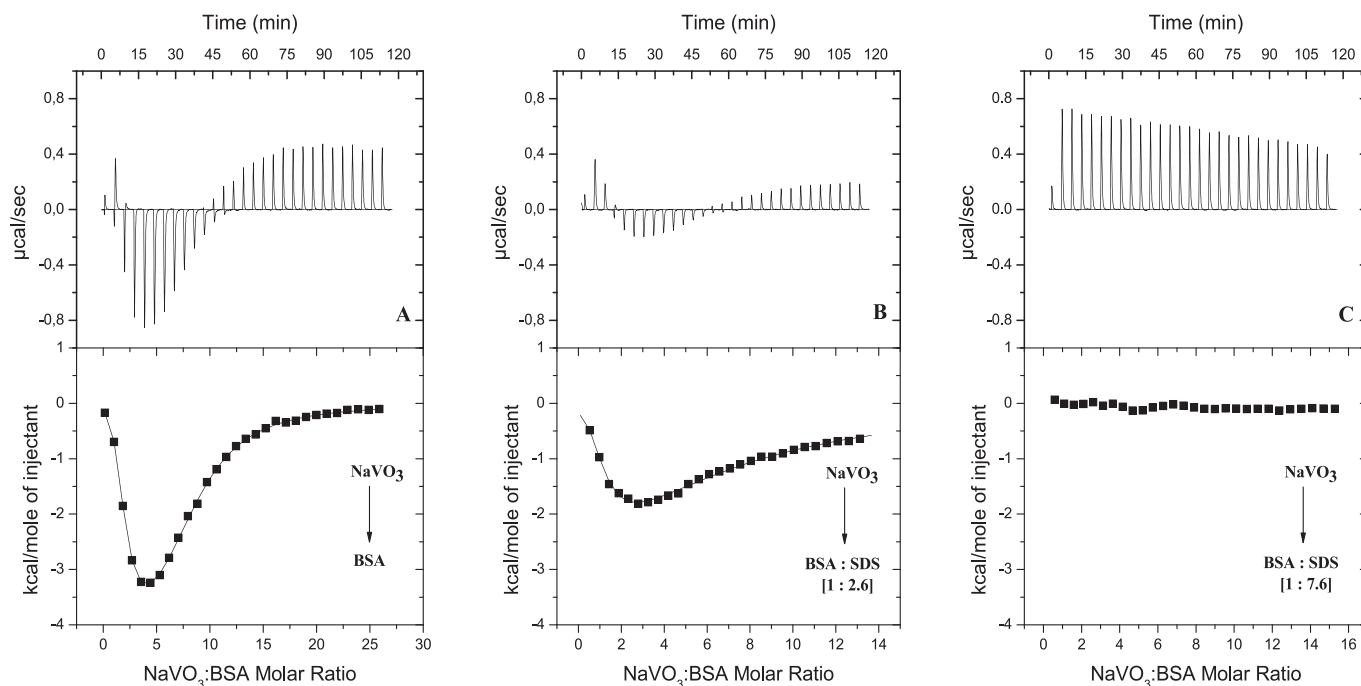
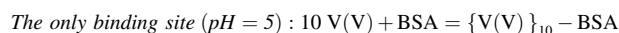


Fig. 3. ITC titrations with NaVO_3 (1.5 mM): (A) BSA (0.0125 mM), (B) BSA and SDS mixed in a molar ratio 1:2.6; (C) BSA and SDS mixed in a molar ratio 1:7.6, at pH 7 (298.15 K).

the second site of BSA.

Different mode of the V(V)-BSA interaction occurs at pH close to the isoelectric point of BSA (pI 4.7) [33,34]. It is related to the increase in the positive net charge of BSA [35,36] and the formation of poly-oxidovanadates at a lower pH range [3,20]. In contrast to the ITC data obtained at pH 7, there is only one binding site on the protein surface

that is saturated at pH 5 with 10 mol of vanadate(V) ions:



The stability of V(V)-BSA complexes formed at pH 5 is comparable to the stability of the complexes formed in the second binding site at pH 7,

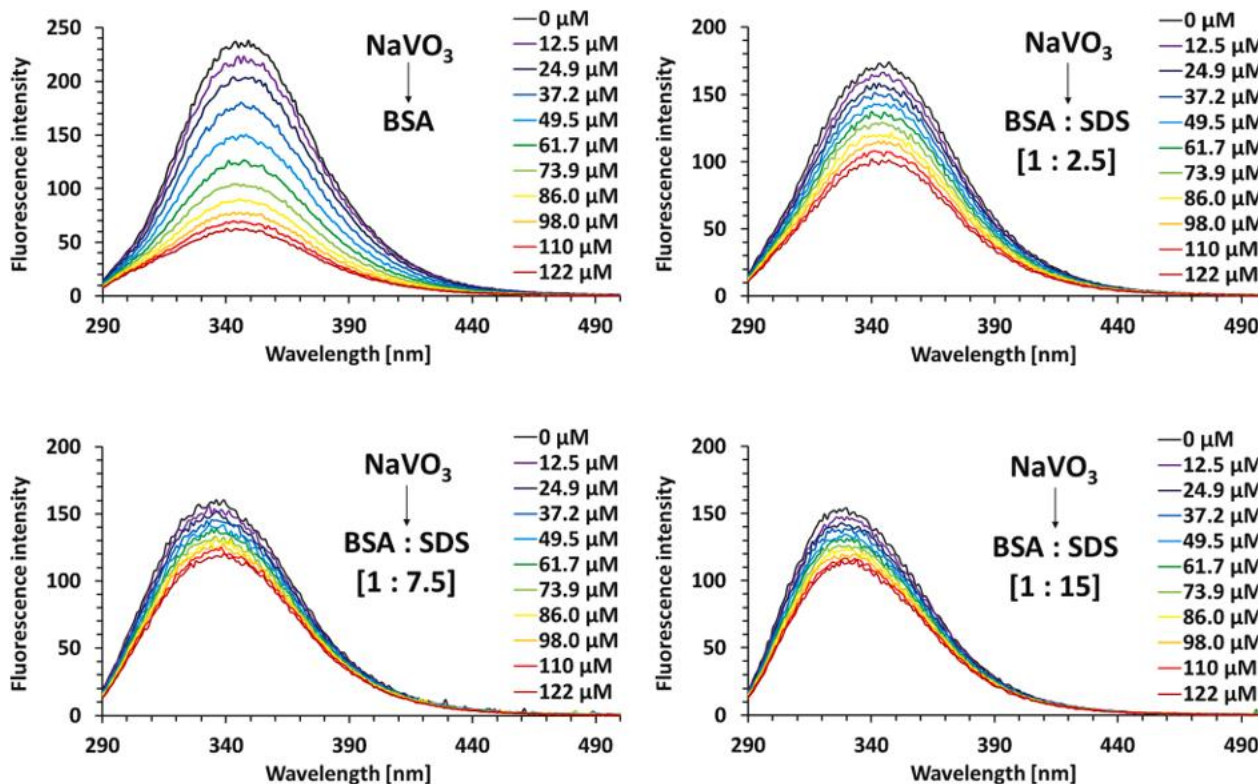


Fig. 4. Fluorescence emission spectra of free BSA and BSA solutions with SDS combined in molar ratios of 1:2.5, 1:7.5, and 1:15 in the presence of increasing concentrations of NaVO_3 (0–122 μM) in the 10 mM Caco buffer of pH 7, at 298.15 K.

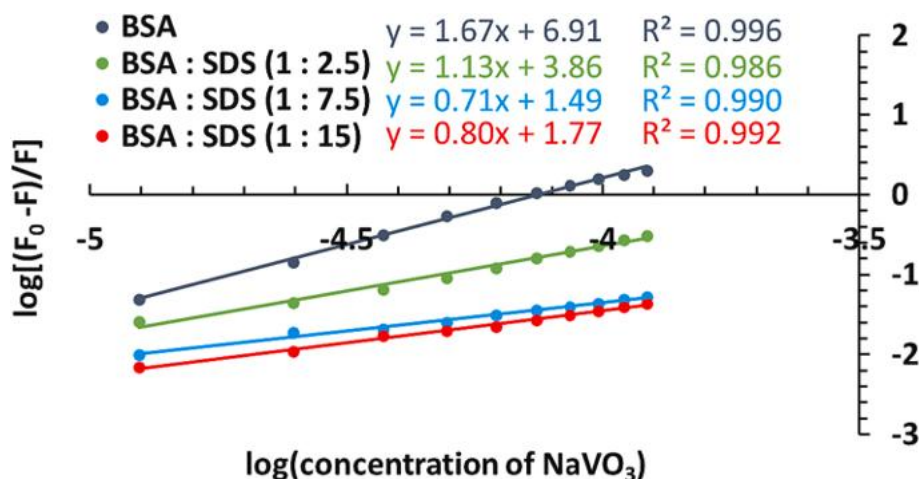


Fig. 5. The plots of $\log \frac{F_0-F}{F}$ as a function of $\log(\text{concentration of NaVO}_3)$ for the steady-state fluorescence quenching of BSA and its mixtures with SDS (1:2.5; 1:7.5; and 1:15) by NaVO_3 in the 10 mM Caco buffer of pH 7, at 298 K.

but the complexation process is less exothermic. The binding of the V(V) ions is both enthalpy- and entropy-driven, however, the entropic factor prevails ($|\Delta H_{\text{ITC}}| < |T\Delta S_{\text{ITC}}|$) [37,38].

3.2. SDS-dependent interactions of NaVO_3 with BSA

It has previously been reported that SDS acts as a strong binding ligand and forms fairly stable complexes with BSA. SDS molecules are bound to BSA in subdomain IA (the SDS:BSA molar ratio ca. 2:1, $\log K = 7.61$) and in subdomain IIB (the SDS:BSA molar ratio ca. 5:1, $\log K = 5.29$) [1]. On account of the proven affinity of SDS and NaVO_3 to BSA, the saturation of the investigated protein by SDS may prevent the binding of inorganic anions if both substrates compete for the same binding site of BSA. The ITC data revealed that BSA with saturated the first binding site by SDS molecules is still capable to bind V(V) ions (Table 1, Fig. 3). However, under such experimental conditions, the lowering in the thermodynamic stability ($\log K$, ΔG) and the stoichiometry (N) of the resulting V(V) complexes are observed (Table 1). SDS molecules are not displaced by V(V) ions that stay in line with the binding constants of SDS-BSA and V(V)-BSA complexes, $K_{\text{SDS-BSA}} > K_{\text{V(V)-BSA}}$ (Table 1). A different scenario is observed for BSA saturated by SDS in both binding sites. In such a case, there is a lack of evidence for

the V(V) - BSA interactions (Fig. 3). Thus, it can be expected that SDS is competitive for V(V) binding at site II (subdomain IIB), but not competitive for the vanadate binding at site I (subdomain IA).

The fluorescence emission spectra of the investigated systems are presented in Fig. 4. It has been found that the position of an initial strong emission band of tryptophan residues for free BSA at 348 nm is shifted to 330 nm for a mixture of BSA and SDS in a molar ratio of 1:15. Furthermore, the addition of NaVO_3 decreases the intrinsic fluorescence intensity of both free and SDS-saturated albumin. However, the observed changes on account of NaVO_3 action are less pronounced in the presence of a high amount of SDS in the system. The above phenomenon stays in line with the ITC results.

When small molecules bind independently to a set of equivalent sites of a protein, the equilibrium between free and bound molecules is given by the following equation [39–42]: $\log \frac{F_0-F}{F} = n \cdot \log [Q] + \log K_b$. Consequently, the apparent binding constant (K_b) have been determined from the intercepts of the linear plots of $\log \frac{F_0-F}{F}$ as a function of $\log(\text{concentration of NaVO}_3)$ (Fig. 5). It can be clearly observed that the highest K_b values were obtained for pure BSA, while the lowest binding is observed for the systems where all the binding sites in the BSA structure are fully saturated with SDS. The increase of the SDS concentration resulting in the decrease of the binding constants values can be assigned to the

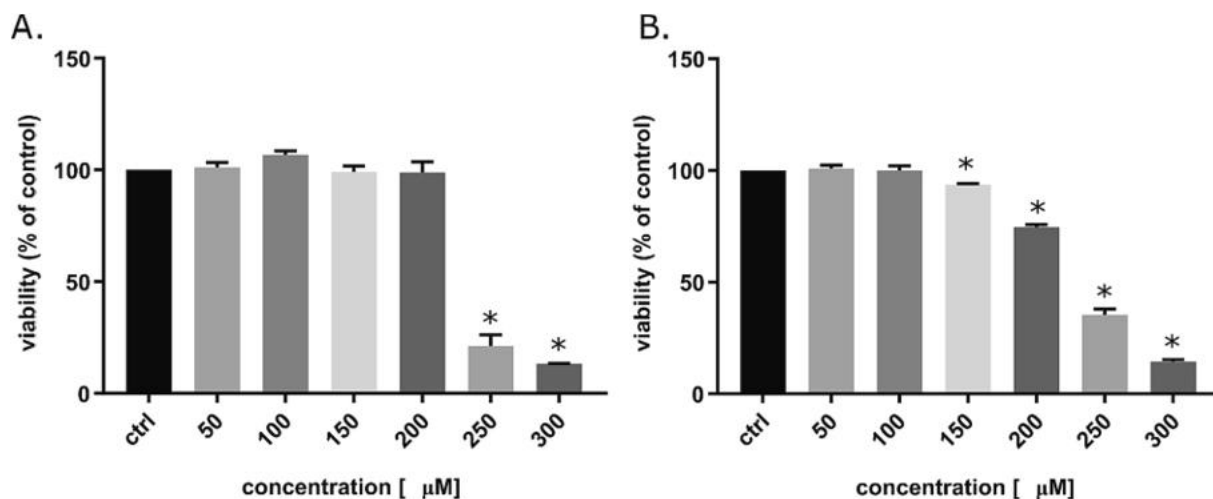


Fig. 6. Viability of PC3 cells after 24 h (A) and 48 h (B) of incubation with SDS in the concentration range from 50 μM to 300 μM . Results of three independent experiments carried out in triplicate are presented as mean $\pm$ standard deviation. *There is a statistically significant difference ($p < 0.05$) between the treated samples and an untreated sample (the control).

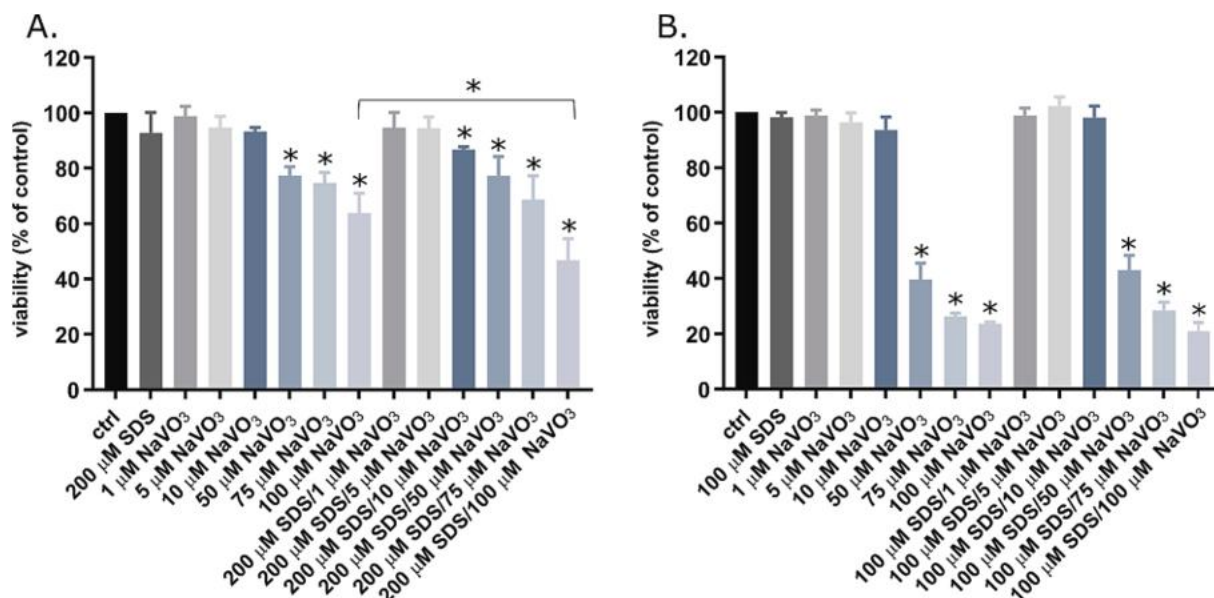


Fig. 7. Viability of PC3 cells after 24 h (A) and 48 h (B) of incubation with NaVO₃ or NaVO₃/SDS mixtures. Results of three independent experiments carried out in triplicate are presented as mean $\pm$ standard deviation. *There is a statistically significant difference between the treated samples and an untreated sample (the control, $p < 0.05$) or between compared groups ($p < 0.01$).

competition of SDS with NaVO₃ for BSA [43,44].

3.3. Biological studies

It has previously been reported that NaVO₃ inhibits the proliferation of human prostate cancer cell lines (PC3 and DU145) via G2/M arrest. The anticancer effect of the inorganic salt is dose-dependent and probably involves the increase of ROS, but the pathway of G2/M arrest is still unclear [18,45]. To assess the binding effect of V(V) to albumin on the biological properties of NaVO₃ we have compared the biological action of NaVO₃ on prostate cancer cells (PC3) with and without the use of SDS as a blocker of albumin binding sites. The MTT tests were performed in the presence and absence of SDS in the cell culture media supplemented with 10 % fetal bovine serum (FBS). The concentrations of SDS used in biological experiments equal to 200 μ M and 100 μ M, respectively for 24 h and 48 h of incubation, were set up based on the MTT tests (Fig. 6). These are the highest concentrations of SDS that do not affect the viability of the investigated cell line under experimental conditions.

The cytotoxic effect of NaVO₃ was found in its concentrations above 10 μ M after 24 h and 48 h incubation time. Then, we assessed the impact of SDS on the cytotoxicity of NaVO₃ toward the PC3 cell line. The MTT tests reveal that only in the case of 24 h incubation time, the mean viability of PC3 cells containing 100 μ M NaVO₃ decreased from 63.87 % to 46.73 % due to the presence of SDS in the culture medium (Fig. 7). In other tested variants, SDS does not affect the dose-dependent cytotoxicity of vanadates(V). Because V(V) ions bind to albumin, an important component of the cell culture media, and these interactions can affect their biological activity (at least under certain conditions), this effect should be taken into account during the planning and analysis of biological assays.

4. Conclusions

In the present paper, the pH- and SDS-dependent interactions of vanadates(V) with bovine serum albumin (BSA) have been successfully described. The protein-V(V) interactions were found to be sensitive to the experimental conditions (pH, the presence of SDS) which affect the stoichiometry and stability of the resulting complexes and the number of binding sites on the surface of BSA capable of binding vanadate(V)

species. The studies performed have proven that the saturation of the albumin with SDS effectively hinders the binding of V(V) to the protein. Thus, sodium dodecyl sulfate can be considered as a potential V(V) binding site blocker. However, the question of the use of SDS in cell culture media as a blocker of V(V) binding sites remains open. The research conducted does not provide a clear answer to this question due to the complexity of the studied processes. Nevertheless, the experiments performed emphasize the need to take into account the presence of relatively large amounts of albumin in cell culture media. This issue may be of great importance when analyzing data on the dose-dependent activity of compounds showing albumin affinity.

CRediT authorship contribution statement

Ola Grabowska: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Funding acquisition. **Magdalena Zdrawowicz:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Dan Milas:** Formal analysis, Investigation, Writing – review & editing. **Krzysztof Żamojć:** Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Katarzyna Chmur:** Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Funding acquisition. **Aleksandra Tesmar:** Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Funding acquisition. **Martyna Kapica:** Formal analysis, Investigation. **Lech Chmurzyński:** Formal analysis, Investigation. **Dariusz Wyrzykowski:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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PUBLIKACJA 4

**Elucidation of binding mechanisms of bovine serum albumin
and 1-alkylsulfonates with different hydrophobic chain lengths**



Elucidation of binding mechanisms of bovine serum albumin and 1-alkylsulfonates with different hydrophobic chain lengths

Ola Grabowska^a, Sergey A. Samsonov^a, Małgorzata M. Kogut-Günthel^{a,b}, Krzysztof Żamojć^a, Dariusz Wyrzykowski^{a,*}

^a Faculty of Chemistry, University of Gdańsk, Wita Stwosza 63, 80-308 Gdańsk, Poland

^b Leibniz-Institut für Lebensmittel-Systembiologie an der Technischen Universität München, 85354 Freising, Germany

ARTICLE INFO

Keywords:

Bovine serum albumin
1-Alkylsulfonates
Site marker

ABSTRACT

In this article, the binding interactions between bovine serum albumin (BSA) and three 1-alkylsulfonates, namely sodium 1-dodecanesulfonate, sodium 1-decanesulfonate, and sodium 1-octanesulfonate, have been thoroughly investigated. The study employed various experimental techniques such as isothermal titration calorimetry (ITC), steady-state fluorescence spectroscopy (SF), circular dichroism spectroscopy (CD), and molecular dynamics-based simulations. The objective was to understand the influence of the alkyl chain length of the investigated ligands on several aspects, including the strength of the interaction, the stoichiometry of the resulting complexes, the number of BSA binding sites, and the underlying mechanisms of binding. Notably, the study also demonstrated that sodium dodecyl sulfate (S12S) can serve as an effective site marker for BSA when studying ligands with similar structural and topological features. These findings may have significant implications for enhancing our understanding of the interactions between small amphiphilic molecules and proteins.

1. Introduction

Albumins play a crucial role in various physiological functions within the body, such as regulating blood osmotic pressure [1] and maintaining optimal blood pH [2,3]. The affinity of albumins to various types of low- and high-molecular-weight compounds enables the delivery of specific molecules to target sites through the bloodstream [4,5]. Consequently, the exploration of the binding capabilities between serum albumin and specific compounds remains an ongoing focus of research. Insight into the number and nature of potential binding sites on albumin, and correlating this information with ligand factors that influence the strength of protein-ligand interactions, provides a valuable opportunity to improve our understanding of drug distribution and pharmacokinetics in the body [6–8]. In this contribution, bovine serum albumin (BSA), a chemical analogue of human serum albumin, was used as a model globular protein [9–12] to describe the nature of the binding interactions with three 1-alkylsulfonates, namely sodium 1-dodecanesulfonate (K12S), sodium 1-decanesulfonate (K10S), sodium 1-octanesulfonate (K8S). The investigated 1-alkylsulfonates ligands, labeled as KXS, where X represents the number of carbon atoms in the alkyl chain, with X being either 12, 10, or 8 are the sodium salts of sulfonic acids,

which can be classified as surfactants based on their structure (Fig. 1). The distinctive feature of these ligands is their amphiphilic nature, which arises from the presence of a hydrophilic (head) component and a hydrophobic (tail) component in their structure [13–15]. It should also be noted that the 1-alkylsulfonates tested (KXS: $C_X-SO_3^-$) do not have an oxygen atom linking the hydrophobic and hydrophilic parts, which distinguishes them from the 1-alkylsulfates (SXS: $C_X-O-SO_3^-$) of which the anionic surfactant S12S (sodium dodecyl sulfate) is a well-known representative. These seemingly insignificant differences in the structure of both types of ligands are responsible for the different charge distribution between the negative head group and the α -methylene group directly attached to the head group. The 1-alkylsulfates ($C_X-O-SO_3^-$), unlike the 1-alkylsulfonates ($C_X-SO_3^-$), have a partial positive charge on the α -CH₂ group. As a result, the head group of the 1-alkylsulfates held a much more negative charge than the head group of the 1-alkylsulfonates. The structural differences mentioned above are reflected in their interactions with cationic [16] and nonionic surfactants [17]. Interestingly, recent findings have shown that K12S comprising a carbon chain length equal to that of S12S, unlike the 1-alkylsulfonates with shorter hydrophobic tails, can mimic a surfactant and be incorporated into the formation of S12S micelles [18].

* Corresponding author.

E-mail address: dariusz.wyrzykowski@ug.edu.pl (D. Wyrzykowski).

<https://doi.org/10.1016/j.ijbiomac.2024.131134>

Received 9 February 2024; Received in revised form 20 March 2024; Accepted 23 March 2024

Available online 25 March 2024

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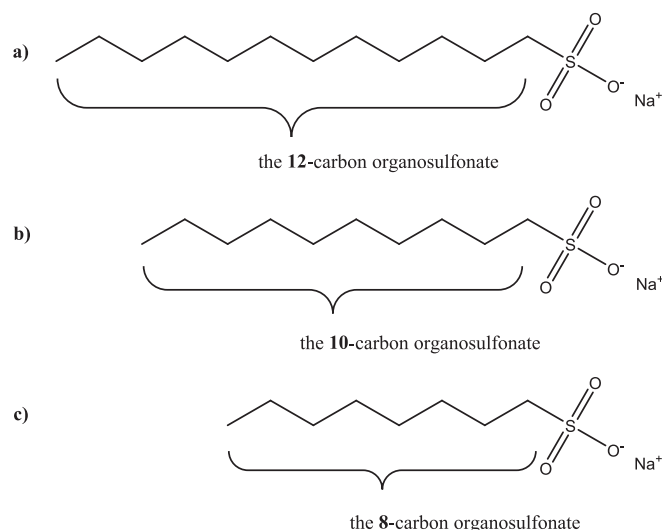


Fig. 1. Chemical structures of a) sodium 1-dodecanesulfonate (K12S), b) sodium 1-decanesulfonate (K10S), c) sodium 1-octanesulfonate (K8S).

When considering interactions between proteins and ligands, the primary factors that influence affinity are the topology of the surfactants (whether they are monomeric or micellar), the chemical structure of the ligands, and the experimental conditions (e.g. pH and temperature) [19–21]. However, as far as we are aware, in contrast to the well-documented S12S-albumin interactions [20], there is a lack of information on the physicochemical nature of albumin interactions with 1-alkylsulfonates, which show a similar chemical structure to the 1-alkylsulfates. This prompted us to embark on this research. This article presents experimental and *in silico* data regarding the influence of the hydrophobic carbon chain length of the studied ligands (KXS) on the stoichiometry, stability, and number of binding sites in the resulting KXS-protein complexes. In addition, the results provide valuable insights into the mechanisms of complex formation, and regarding the possibility of using the S12S ligand as a binding site marker for amphiphilic ligands with similar topological and structural features.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA, $\geq 96\%$), sodium 1-dodecanesulfonate (K12S, $\geq 99\%$), sodium 1-decanesulfonate (K10S, $\geq 99\%$), sodium 1-octanesulfonate (K8S, $\geq 99\%$), sodium dodecyl sulfate (S12S, $\geq 99\%$), sodium cacodylate trihydrate (Caco, $\geq 99\%$) were obtained from Sigma-Aldrich (Poland) and used without additional purification. All solutions were prepared in the 10 mM Caco buffer of pH 7. Based on the amount of tryptophan ($\epsilon_{280}^W = 5690 \text{ M}^{-1}\text{cm}^{-1}$), tyrosine ($\epsilon_{280}^Y = 1280 \text{ M}^{-1}\text{cm}^{-1}$) and cysteine ($\epsilon_{280}^C = 120 \text{ M}^{-1}\text{cm}^{-1}$) in BSA, the protein extinction coefficient ($\epsilon_{280}^{BSA} = 41180 \text{ M}^{-1}\text{cm}^{-1}$) was determined, and then the protein concentration was calculated from the UV absorption spectra recorded using a Perkin Elmer Lambda 650 (Waltham, MA, USA) UV/Vis spectrophotometer [22].

2.2. Isothermal titration calorimetry (ITC)

Experiments were conducted using AutoITC isothermal titration calorimeter (MicroCal Inc. GE Healthcare, Northampton, MA, USA). Previous works [23,24] provide detailed information about the ITC calorimeter and the experimental system. A 1 mM solution of KXS (where X = 12, 10, 8) was injected into the reaction cell, which contained: (a) a 0.02 mM solution of free BSA, (b) a mixture of BSA:S12S in molar ratios of 1:2.5 and 1:8.5, respectively. ITC experiments were

conducted under specific conditions: temperature 298.15 K, cell volume 1.4491 mL, injection volume 10.02 μL (29 injections, 2 μL for the first injection only), injection interval 240 s, stirring speed 300 r.p.m., reference power 10 $\mu\text{cal s}^{-1}$.

2.3. Steady-state fluorescence spectroscopy (SF)

The Cary Eclipse Varian (Agilent, Santa Clara, CA, USA) spectrofluorometer equipped with a 1.0 cm multicell holder and temperature controller was used to record emission spectra and measure fluorescence intensities. The experiments were performed in the 10 mM Caco buffer (pH 7). The excitation and emission slit widths were fixed at 5 nm. The excitation wavelength was always set at 280 nm. The fluorescence emission spectra of BSA (2 μM) were recorded from 290 to 500 nm in the absence and presence of increasing concentrations of K12S, K10S, and K8S (scan mode). In the fluorescence titration experiments (kinetics mode performed manually by using a micropipette), 2 mL of BSA at a fixed concentration equal to 2 μM was titrated by five successive additions of 2 μL stock solutions of K12S (2 mM), K10S (2 mM), and K8S (8 mM). The concentration of 1-alkylsulfonates was varied and dependent on the observed quenching efficiency. The fluorescence intensities of the band at 348 nm – corresponding to the initial maximum emission of BSA – were used to calculate the binding constants and other parameters. The inner filter effect has been compensated for all the fluorescence intensity values [25,26]. All experiments were conducted at 283.15 K, 288.15 K, 293.15 K, 298.15 K, 303.15 K and 308.15 K.

2.4. Circular dichroism spectroscopy (CD)

CD spectra were obtained using a Jasco-715 automated spectropolarimeter (Jasco Inc., USA) for (a) free BSA (3 μM) and (b) the mixture of BSA:KXS (where X = 12, 10, 8) in a molar ratio of 1:50. Quartz cuvettes (1 mm) were used for the measurements. The measurement conditions were as follows: wavelength range of recorded spectra 160–260 nm, temperature range 298.15 K–368.15 K, sensitivity 5 millidegrees, scan speed 50 nm min^{-1} , sample volume 0.3 mL. The results were plotted as mean residual ellipticity Θ (degrees $\text{cm}^2 \text{dmol}^{-1}$). The percentage change in the secondary structure of the protein was estimated using the Dichroweb online server [27] with the CSSR analysis algorithm [28] and reference dataset 7 [29].

2.5. Molecular dynamics (MD) simulations

2.5.1. Modelling BSA-KXS interactions

2.5.1.1. Structures. The structures of K12S, K10S and K8S were built in Xleap module of AMBER16 software package [30]. The structure of S12S was used from our previous work [20]. Modifications were made by removing an O atom (to obtain K12S), followed by the preparation of K10S and K8S structures through the removal of $-\text{C}_2\text{H}_5$ and $-\text{C}_4\text{H}_9$ groups from K12S, respectively. The structures of BSA (PDB ID: 4OR0) with K12S, K10S and K8S molecules were built by placing 15 KXS ligands randomly around the protein.

2.5.1.2. Molecular dynamics simulations. All-atom molecular dynamics (MD) simulation for each BSA-KXS system was performed with the use of AMBER16 software package [30]. Truncated octahedron TIP3P periodic box of 10 Å water layer from box's border to solute was used to solvate complexes. The charge was neutralized with Na^+ counterions. Gaff force field parameters [31] with RESP charges [32] obtained in an antechamber module of AMBER16 [30] for K12S, K10S, K8S were used. Energy minimization was carried out in two steps: beginning with 500 steepest descent cycles and 103 conjugate gradient cycles with 100 $\text{kcal mol}^{-1} \text{\AA}^{-2}$ harmonic force restraints, continued with 3×10^3 steepest descent cycles and 3×10^3 conjugate gradient cycles without

any restraints. Following minimization steps, the system was heated up from 0 to 300 K for 10 ps with harmonic force restraints of 100 kcal mol⁻¹ Å⁻². Subsequently, the system was equilibrated at 300 K and 10⁵ Pa in the isothermal isobaric ensemble for 500 ps. Afterward, the productive MD run was carried out in the same isothermal isobaric ensemble for 500 ns. The particle mesh Ewald method for treating electrostatics and SHAKE algorithm for all the covalent bonds containing hydrogen atoms were implemented in the MD simulations.

2.5.1.3. Binding free energy calculations. Energetic postprocessing of the trajectories was performed with the use of Linear Interaction Energy (LIE) with a dielectric constant of 80.

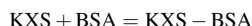
2.5.1.4. Data analysis and visualization. Data analysis and its visualization were performed with the use of the R-package [33] and VMD [34].

3. Results and discussion

3.1. Isothermal titration calorimetry (ITC)

ITC measurements revealed that BSA has two binding sites capable of binding K12S and K10S. The first site is saturated with 1 mol of KXS (X = 12,10), while the second site binds 5 mol of these ligands. However, when K8S interacts with BSA, only one binding site is observed, resulting in the formation K8S-BSA complex of a stoichiometry 1:1.

The first binding site of BSA (available for all the ligands, K12S, K10S and K8S)



The second binding site of BSA (available only for K12S and K10S)

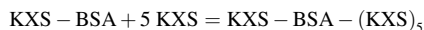


Fig. 2 shows representative binding isotherms, while Table 1 summarises the thermodynamic parameters of the interactions. It is interesting to note that the length of the hydrophobic carbon chain of KXS determines the strength of the interaction at the first site of the protein. The thermodynamic stability of the resulting KXS-protein complexes

increases in the following direction: K8S < K10S < K12S (Table 1). The enthalpy factor plays a greater role in stabilizing the resulting complexes when the hydrophobic moiety in KXS is shorter. The smaller volume of the ligand allows for closer contact with the protein surface, leading to a higher engagement of polar forces such as electrostatic interactions, hydrogen bonds, and/or van der Waals interactions in the complex formation. Simultaneously, shortening the hydrophobic part of the ligand weakens the hydrophobic effect of water release from the protein surface. This is reflected in the entropic factor values of the first binding site (Table 1). The second binding site of BSA is occupied by five moles of either K12S or K10S ligands, with both ligands exhibiting equal binding strength. The stability of the resulting complexes in both cases is primarily determined by the enthalpic factor, with K12S showing a stronger enthalpic contribution. This enthalpic contribution compensates for the unfavorable entropic contribution observed in the case of the K12S-BSA interaction (Table 1).

Our previous studies performed under the same experimental conditions (10 mM Caco buffer, pH 7) revealed that BSA binds two moles of sodium dodecyl sulfate (S12S) molecules close to Trp-134 residue (subdomain IA) and five moles of S12S in subdomain IIB [20]. To identify the potential binding sites for KXS-BSA interactions, we exploited the previously gathered information along with the structural resemblance between S12S and the ligands under investigation. Consequently, we employed S12S as a site-binding marker. Thus, we saturated the first binding site of BSA with the S12S marker (at an S12S:BSA molar ratio of ca. 2.5:1) and subsequently both binding sites (at an S12S:BSA molar ratio of ca. 8.5:1) to investigate its affinity towards KXS ligands. The representative ITC thermograms and the titration curves for the K12S system are shown in Fig. 3. It turned out that the protein saturated with S12S in the first site was still capable of binding KXS (Table 1). The affinity of the protein to K8S and the stoichiometry of the K8S-BSA complex remained unchanged, but its formation is less exothermic. A different scenario was noticed for K12S and K10S, for which only one binding site (instead of two) on the BSA surface was observed. Furthermore, under such conditions, the affinity of K12S and K10S to BSA saturated with S12S is lower in comparison to the free (unsaturated with S12S) protein (Table 1). It is important to note that when both binding sites in BSA are saturated with S12S, the protein no longer exhibits affinity for KXS (Fig. 3). This behavior is likely a result of K12S, K10S, and S12S binding to the same site on the protein, while K8S reveals an affinity for the second S12S binding site in BSA (Table 1).

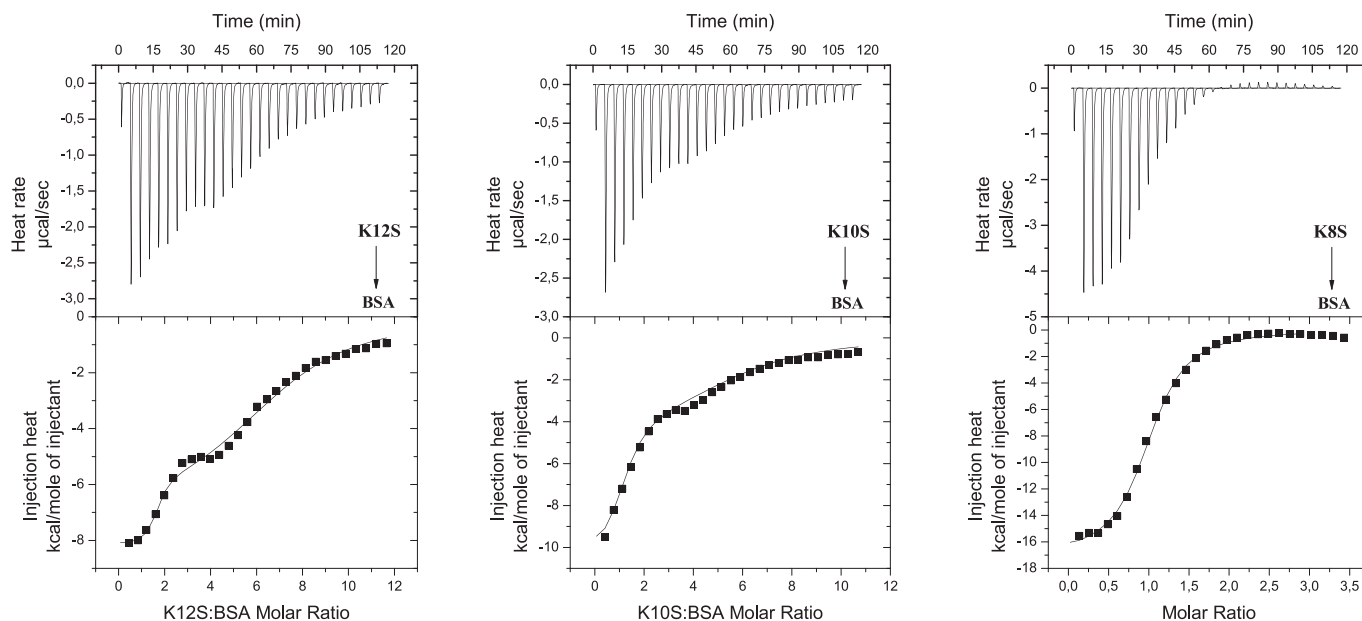


Fig. 2. Calorimetric titration isotherms of the binding interactions between BSA and K12S, K10S, K8S in the 10 mM Caco buffer of pH 7 at 298.15 K.

Table 1
Thermodynamic parameters of the interaction of KXS with BSA in the presence and absence of S12S in the system. Measurement conditions: temperature 298.15 K; the 10 mM Caco buffer of pH 7 (standard deviation values in parentheses).

Parameters	K12S → BSA	K12S → BSA:S12S ^a	K10S → BSA	K10S → BSA:S12S ^a	K8S → BSA	K8S → BSA:S12S ^a	S12S → BSA ^b
<i>The first KXS-binding site in BSA</i>							
N_1	1.50 (±0.10)	3.33 (±0.05)	1.13 (±0.06)	2.36 (±0.05)	0.99 (±0.01)	0.95 (±0.03)	1.75 (±0.14)
$\log K_{(ITC)1}$	6.90 (±0.35)	5.15 (±0.04)	6.11 (±0.13)	4.93 (±0.03)	5.46 (±0.04)	5.24 (±0.07)	7.61 (±0.38)
$\Delta G_{(ITC)1}$ [kcal mol ⁻¹]	-9.41 (±0.48)	-7.02 (±0.06)	-8.34 (±0.18)	-6.72 (±0.04)	-7.45 (±0.05)	-7.15 (±0.09)	-10.38 (±0.67)
$\Delta H_{(ITC)1}$ [kcal mol ⁻¹]	-8.15 (±0.21)	-6.39 (±0.14)	-10.77 (±0.74)	-6.88 (±0.20)	-16.81 (±0.22)	-6.68 (±0.26)	-9.11 (±0.26)
$T\Delta S_{(ITC)1}$ [kcal mol ⁻¹]	1.26	0.64	-2.43	-0.16	-9.36	0.47	1.27
<i>The second KXS-binding site in BSA</i>							
N_2	5.43 (±0.17)	—	4.77 (±0.41)	—	—	—	5.33 (±0.29)
$\log K_{(ITC)2}$	4.74 (±0.07)	—	4.74 (±0.11)	—	—	—	5.29 (±0.06)
$\Delta G_{(ITC)2}$ [kcal mol ⁻¹]	-6.47 (±0.10)	—	-6.46 (±0.16)	—	—	—	-7.22 (±0.09)
$\Delta H_{(ITC)2}$ [kcal mol ⁻¹]	-6.83 (±0.43)	—	-4.30 (±0.70)	—	—	—	-7.98 (±0.27)
$T\Delta S_{(ITC)2}$ [kcal mol ⁻¹]	-0.36	—	2.16	—	—	—	-0.76

^a BSA saturated with S12S in the first binding site. The BSA:S12S molar ratio = 1:2.6.
^b Thermodynamic parameters of S12S binding to BSA (measurement conditions: 298.15 K; the 10 mM Caco buffer of pH 7), taken from ref. [20].

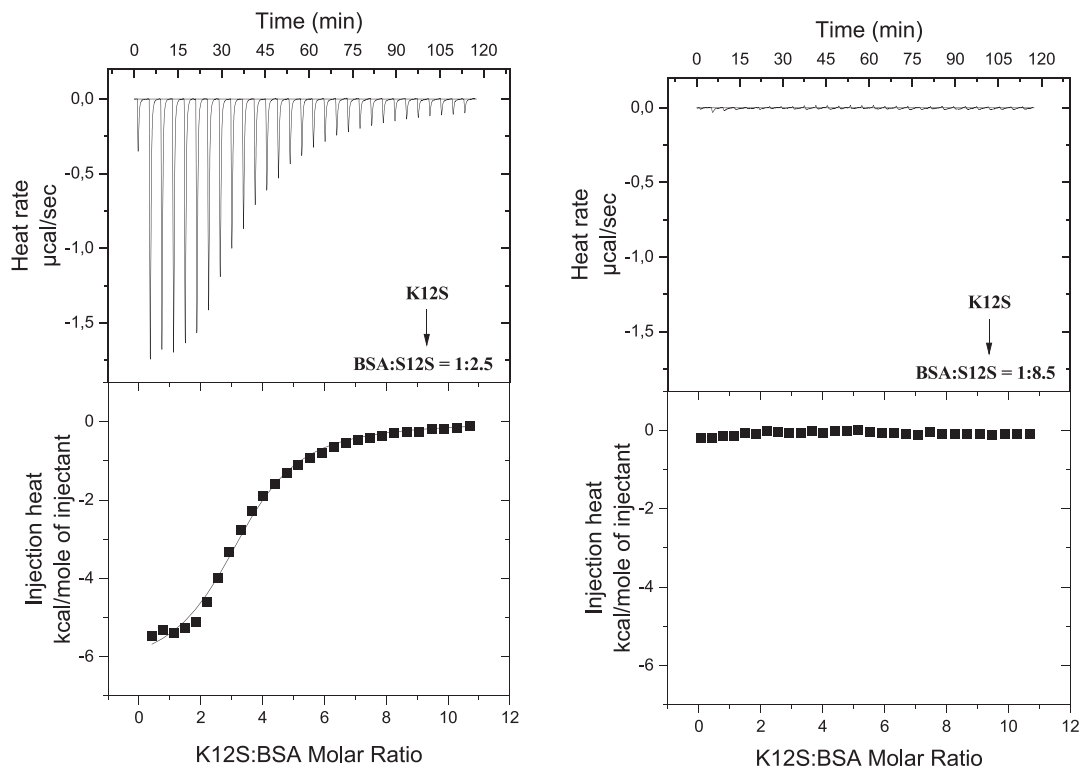


Fig. 3. Calorimetric titration isotherms of the binding interactions between BSA and K12S in the presence of S12S in the system, in the 10 mM Caco buffer of pH 7 at 298.15 K.

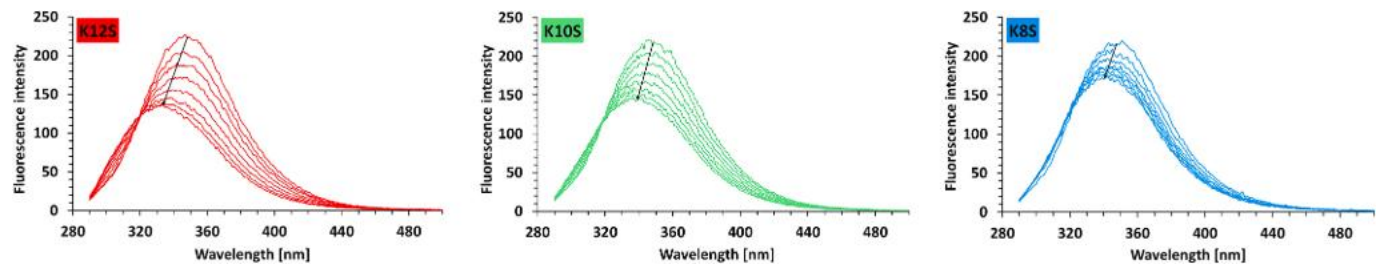


Fig. 4. Fluorescence emission spectra of BSA (2 µM) in the presence of increasing concentrations of K12S (0–17.2 µM), K10S (0–17.2 µM), and K8S (0–138 µM); λ_{ex} = 280 nm; in the 10 mM Caco buffer of pH 7 at 298.15 K.

3.2. Steady-state fluorescence spectroscopy (SF)

To understand the effect of 1-alkylsulfonates on the tryptophan environment, the intrinsic fluorescence changes of BSA in the presence of increasing concentrations of K12S, K10S and K8S have been studied. BSA dissolved in the 10 mM Caco buffer of pH 7 after excitation at 280 nm exhibits a single emission band with a maximum at 348 nm (due to tryptophan residues). Upon the continuous addition of KXS, a gradual decrease in protein fluorescence intensity is observed, accompanied by a distinct shift of the band towards shorter wavelengths (Fig. 4). It is consistent with our previous reports, where similar changes in protein emission spectra in the presence of surfactants and ionic liquids have been observed [35,36]. These observations confirm that the binding of the 1-alkylsulfonates to BSA results in the relocation of the protein's tryptophan residue to a more hydrophobic environment. Consequently, these interactions induce a change in the environment of the tryptophan and cause an increase in hydrophobicity in the vicinity of this residue [37,38].

Then, to gain a better insight into the mechanism of the interactions between BSA and all studied 1-alkylsulfonates, the results obtained from steady-state fluorescence quenching experiments performed at different temperatures were analyzed according to the Stern–Volmer equation:

$$\frac{F_0}{F} = K_{SV} \cdot [Q] + 1 = k_q \cdot \tau_0 \cdot [Q] + 1$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher Q (KXS), K_{SV} is the Stern–Volmer quenching constant, k_q is the bimolecular quenching rate constant, while τ_0 is the lifetime of the fluorophore in the absence of quencher (Fig. 5) [39]. Within the chosen range of concentrations straight lines have been obtained (at higher concentrations the curves exhibited downward curvature, indicating the existence of more than one tryptophan residues with intrinsic fluorescence in distinct environments [40]). The values of bimolecular quenching rate constants for all studied systems were calculated based on the value of the average fluorescence lifetime of free BSA equal to 6 ns at a phosphate buffer of pH 7 [41]. For all systems studied under experimental conditions, they were found to be significantly higher than the maximum scattering collision quenching rate constant, which for the interactions of various quenchers with biopolymer in aqueous media is equal to $2.0 \cdot 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ (Table 2), indicating some kind of binding interactions [42,43]. Since the values of bimolecular quenching rate constants are considered to be definitive in differentiating between dynamic and static quenching mechanisms, the predominant role of the formation of ground-state complexes in the investigated systems was affirmed [44]. On the other hand, it can be observed that with an increase in temperature the quenching efficiency increases. At first sight, the following observations are characteristic of dynamic quenching (the higher the temperature, the faster diffusion and more numerous collisions [45]). It has to be mentioned that such a dissimilarity with the classical theory has been observed previously for the binding of anticancer hydrazone to BSA [46] and can be explained by the Arrhenius theory. According to this theory, the higher the

temperature is, the bigger the rate constant k is. For the classical static quenching theory, the lower the temperature, the more stable the complex. If the increase caused by the higher temperature is more significant than the decrease led by the unstable tendency, the quenching constants will increase with temperature.

When small molecules bind independently to a set of equivalent sites of a protein, the equilibrium between free and bound molecules is given by the following modified Stern–Volmer equation [47]:

$$\log \frac{(F_0 - F)}{F} = n \cdot \log[Q] + \log K_{\text{bind}}$$

Consequently, the apparent binding constants (K_{bind}) and the binding numbers (n) have been determined from the intercepts and slopes of the linear plots of $\log \frac{(F_0 - F)}{F}$ as a function of $\log(c_{\text{KXS}})$ (Fig. 6, Table 2). The strength of the interaction in the resulting KXS-BSA complexes follows the order: $\text{K8S} < \text{K10S} < \text{K12S}$, which is consistent with the results from the ITC data. Generally, the obtained values of binding constants for the considered complexes are moderate when compared to other strong protein-ligand complexes with binding constants ranging from 10^7 to 10^8 M^{-1} . However, lower binding constants ($\sim 10^3$ – 10^4 M^{-1}) were also reported using fluorescence spectroscopic methods [48–50]. It can be observed that binding constants decrease with an increase in temperature (except for K8S where no clear trend is observed). It results possibly due to the reduction of the stability of 1-alkylsulfonate-BSA complexes [51].

The acting forces for binding between a small molecule and protein may include hydrophobic and electrostatic interactions, van der Waals interactions, hydrogen bonds, etc. [52]. The noncovalent interaction forces between various ligands and biomolecules can be described by thermodynamic parameters, which can be calculated from the van't Hoff equation:

$$\ln(K_{\text{bind}}) = -\frac{\Delta H}{RT} + \frac{\Delta S}{R}$$

where ΔH and ΔS are enthalpy change and entropy change, respectively, R is the gas constant, and T denotes the absolute temperature [53]. The plot of $\ln(K_{\text{bind}})$ (for K12S) measured at six different temperatures vs. T^{-1} is linear within the experimental error (Fig. 7), which allowed one to calculate ΔH and ΔS for the binding process. The negative ΔH ($-25.7 \text{ kcal mol}^{-1}$) and ΔS ($-67.8 \text{ cal mol}^{-1} \text{ K}^{-1}$) indicate that van der Waals interactions and hydrogen bonding play a major role with less dominating electrostatic forces or hydrophobic interactions. The negative sign for free energy change of binding ΔG indicates the spontaneity of the process [51]. These results (negative ΔH , ΔS and ΔG values) are in agreement with ITC experiments.

3.3. Circular dichroism spectroscopy (CD)

CD spectra of BSA in the absence and presence of the studied ligands allowed us to estimate the thermal stability of the protein's secondary structure. The percentage of secondary structure content for free BSA and the mixture of albumin-ligand is presented in Table 3. The collected

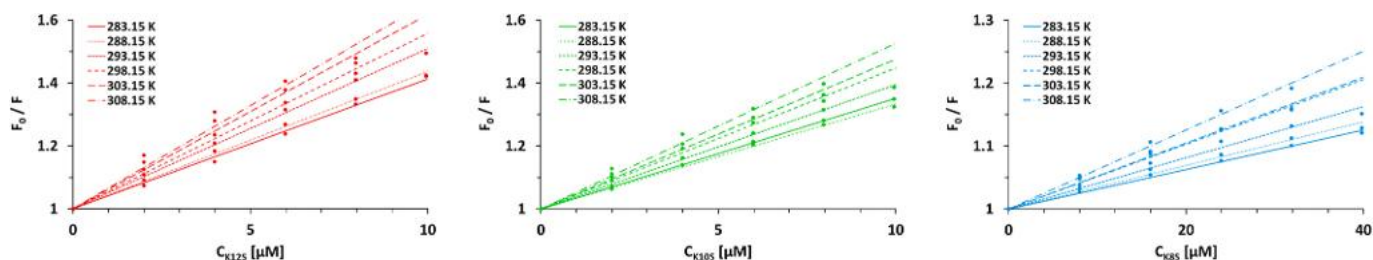


Fig. 5. Stern–Volmer plots for the steady-state fluorescence quenching of BSA (2 μM) by K12S, K10S and K8S; $\lambda_{\text{ex}} = 280 \text{ nm}$, $\lambda_{\text{em}} = 348 \text{ nm}$; in the 10 mM Caco buffer of pH 7 at 283.15 K, 288.15 K, 293.15 K, 298.15 K, 303.15 K and 308.15 K.

Table 2
Stern-Volmer quenching constants (K_{SV}), bimolecular quenching rate constants (k_q), apparent binding constants (K_{bind}), and numbers of binding sites (n) along with linear correlation coefficients (R^2) for the appropriate plots recovered for the steady-state fluorescence quenching of BSA by K12S, K10S and K8S; $\lambda_{ex} = 280$ nm, $\lambda_{em} = 348$ nm; in the 10 mM Caco buffer of pH 7 at 283.15 K, 288.15 K, 293.15 K, 298.15 K, 303.15 K and 308.15 K.

		$K_{SV} [M^{-1}]$	R^2 (Stern-Volmer plot)	$k_q [M^{-1} s^{-1}]$	$K_{bind} [M^{-1}]$	n	R^2 (modified Stern-Volmer plot)
K12S	283.15 K	$4.13 \cdot 10^4$	0.997	$6.88 \cdot 10^{12}$	$1.19 \cdot 10^5$	1.09	0.999
	288.15 K	$4.36 \cdot 10^4$	0.999	$7.27 \cdot 10^{12}$	$2.84 \cdot 10^4$	0.95	1.000
	293.15 K	$5.10 \cdot 10^4$	0.999	$8.50 \cdot 10^{12}$	$2.67 \cdot 10^4$	0.96	0.999
	298.15 K	$5.58 \cdot 10^4$	0.997	$9.30 \cdot 10^{12}$	$1.22 \cdot 10^4$	0.87	0.999
	303.15 K	$6.18 \cdot 10^4$	0.987	$1.03 \cdot 10^{13}$	$4.94 \cdot 10^3$	0.79	0.993
	308.15 K	$6.65 \cdot 10^4$	0.974	$1.11 \cdot 10^{13}$	$2.23 \cdot 10^3$	0.72	0.991
K10S	283.15 K	$3.52 \cdot 10^4$	0.999	$5.87 \cdot 10^{12}$	$8.03 \cdot 10^4$	1.07	0.998
	288.15 K	$3.34 \cdot 10^4$	0.999	$5.57 \cdot 10^{12}$	$1.53 \cdot 10^4$	0.92	1.000
	293.15 K	$3.95 \cdot 10^4$	0.999	$6.58 \cdot 10^{12}$	$1.45 \cdot 10^4$	0.93	1.000
	298.15 K	$4.48 \cdot 10^4$	0.995	$7.47 \cdot 10^{12}$	$9.69 \cdot 10^3$	0.87	0.999
	303.15 K	$4.76 \cdot 10^4$	0.993	$7.93 \cdot 10^{12}$	$6.71 \cdot 10^3$	0.84	0.998
	308.15 K	$5.27 \cdot 10^4$	0.988	$8.78 \cdot 10^{12}$	$4.31 \cdot 10^3$	0.79	0.999
K8S	283.15 K	$3.14 \cdot 10^3$	0.997	$5.23 \cdot 10^{11}$	$1.49 \cdot 10^3$	0.93	0.999
	288.15 K	$3.47 \cdot 10^3$	0.986	$5.78 \cdot 10^{11}$	$3.43 \cdot 10^2$	0.78	0.999
	293.15 K	$4.07 \cdot 10^3$	0.986	$6.78 \cdot 10^{11}$	$3.02 \cdot 10^3$	0.97	0.986
	298.15 K	$5.15 \cdot 10^3$	0.995	$8.58 \cdot 10^{11}$	$9.79 \cdot 10^2$	0.84	0.998
	303.15 K	$5.24 \cdot 10^3$	0.993	$8.73 \cdot 10^{11}$	$1.22 \cdot 10^3$	0.86	0.999
	308.15 K	$6.28 \cdot 10^3$	0.995	$1.05 \cdot 10^{12}$	$2.04 \cdot 10^3$	0.90	0.993

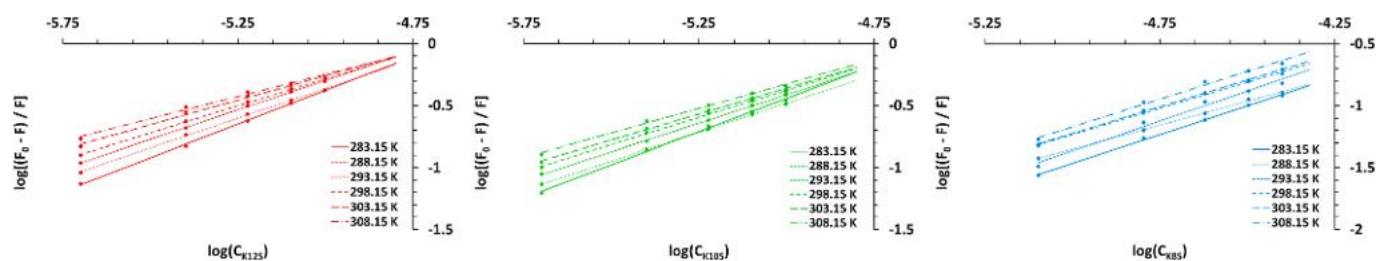


Fig. 6. Modified Stern-Volmer plots for the steady-state fluorescence quenching of BSA (2 μ M) by K12S, K10S and K8S; $\lambda_{ex} = 280$ nm, $\lambda_{em} = 348$ nm; in the 10 mM Caco buffer of pH 7 at 283.15 K, 288.15 K, 293.15 K, 298.15 K, 303.15 K and 308.15 K.

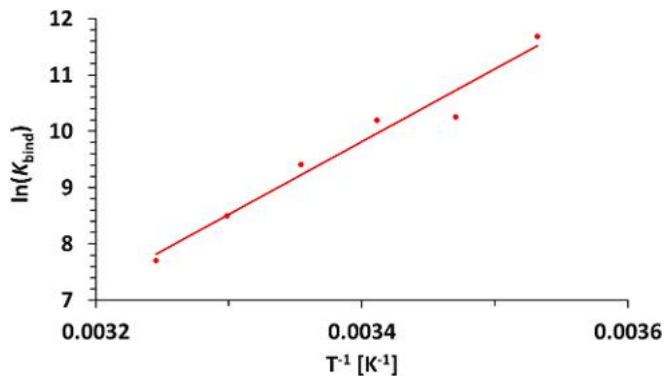


Fig. 7. Van't Hoff plot for the steady-state fluorescence quenching of BSA (2 μ M) by K12S; $\lambda_{ex} = 280$ nm, $\lambda_{em} = 348$ nm; in the 10 mM Caco buffer of pH 7 at 283.15 K, 288.15 K, 293.15 K, 298.15 K, 303.15 K and 308.15 K.

results show that the free protein mainly consists of an α -helix structure. On the other hand, BSA fully saturated with the KXS ligand significantly enhances the thermal stability of the α -helix. This effect is particularly noticeable in the temperature range of 338.15 K – 348.15 K.

3.4. Molecular dynamics (MD) simulations

MD simulations were performed to propose potential binding sites of KXS ligands on the BSA surface and to distinguish differences in KXS binding depending on their alkyl chain length. Based on the LIE-based analysis of binding free energies, longer KXS alkyl chains bind stronger. However, the differences obtained in these calculations are

Table 3
The secondary structure content (in %, revealed from CD measurements) of BSA (3 μ M) in the absence and presence of KXS ligands (X = 12, 10, 8) in the system; in the 10 mM Caco buffer of pH 7; 298.15 K - 368.15 K.

Temperature [K]	System (molar ratio)			
	BSA	BSA:K12S (1:50)	BSA:K10S (1:50)	BSA:K8S (1:50)
The percentage content of α -helix/ β -structure/ β -turn/random coil [%]				
298.15	49/21/11/19	46/20/15/18	46/25/13/16	47/23/12/18
308.15	49/21/10/19	47/21/13/19	46/22/15/17	47/20/14/18
318.15	48/22/13/17	46/19/13/21	45/20/15/20	48/19/12/20
328.15	45/25/15/16	47/22/12/20	46/18/15/21	48/22/16/15
338.15	45/19/15/21	46/20/15/18	46/21/14/19	44/19/16/20
348.15	24/25/20/31	45/20/16/19	35/18/19/29	48/22/16/15
358.15	22/18/19/42	20/21/21/39	20/19/18/43	21/21/18/41
368.15	18/21/18/43	18/22/18/43	18/23/18/42	18/21/17/44

statistically insignificant. The visualization of the MD trajectory frames (Fig. 8) also suggests that more KXS molecules bind during the simulation when X is higher. All three ligands were predicted to share one common primary binding site (labeled as S1 in Fig. 8), while for K10S and K12S two additional different secondary binding sites (S2A and S2B) were also highly populated, which provides a potential explanation of

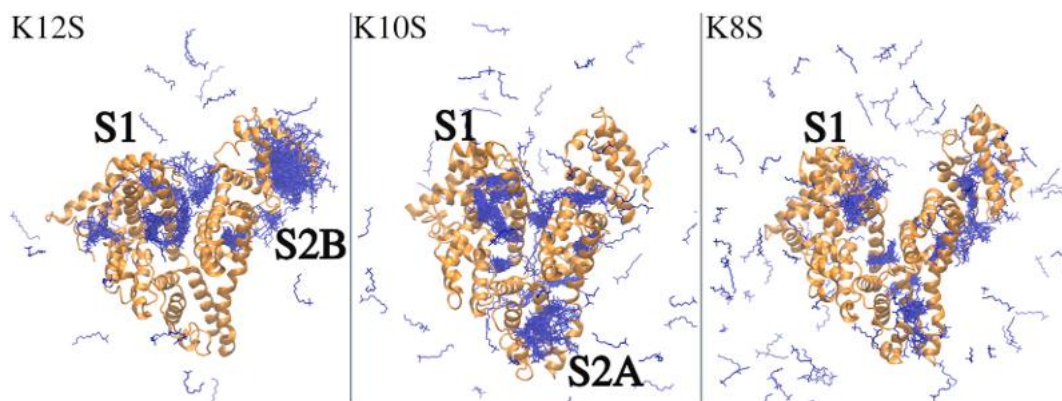


Fig. 8. MD trajectory snapshots (each 10th frame) from 500 ns simulation of BSA (in orange cartoon) with 15 KXS ligands (in blue licorice). Representative binding sites S1, S2A, S2B corresponding to the highest densities of the ligands are labeled.

the experimental results at the structural level.

4. Conclusions

In this article, we aim to elucidate the binding mechanisms of BSA interactions with three different 1-alkylsulfonate ligands, focusing on variations in the hydrophobic carbon chains. Our experimental results clearly show that these interactions are primarily driven by enthalpy, highlighting the crucial role of polar forces in complex formation. Furthermore, our investigation has shown that the strength of these interactions, as well as the number of BSA binding sites, is influenced by the alkyl chain length of the KXS ligands. In addition, the resulting complexes exhibit significantly improved thermal stability compared to free albumin. The experimental results, subsequently supported by MD simulations, showed that sodium dodecyl sulfate (S12S), which has a similar structure to the ligands studied, was a reliable marker for site binding. This allowed the identification of potential KXS binding sites on the protein surface. It is concluded that S12S can be effectively used as a BSA site marker when studying the interaction of albumin with ligands having similar topological and structural features. The outcomes presented in this study could have significant implications for understanding the interactions between small molecules of amphiphilic nature and proteins.

CRedit authorship contribution statement

Ola Grabowska: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Sergey A. Samsonov:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization. **Małgorzata M. Kogut-Günthel:** Methodology, Investigation, Formal analysis. **Krzysztof Zamojć:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Dariusz Wyrzykowski:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the grants for Young Scientists 2023 from University of Gdansk, Poland (539-T090-B989-23). Theoretical research was funded by National Science Centre, Poland (UMO-2018/

30/E/ST4/00037).

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PUBLIKACJA 5

Exploring the impact of subtle differences in the chemical structure of 1-alkylsulfates and 1-alkylsulfonates on their interactions with human serum albumin

Article

Exploring the Impact of Subtle Differences in the Chemical Structure of 1-Alkylsulfates and 1-Alkylsulfonates on Their Interactions with Human Serum Albumin

Ola Grabowska, Ankur Singh, Krzysztof Żamojć , Sergey A. Samsonov  and Dariusz Wyrzykowski 

Faculty of Chemistry, University of Gdańsk, Wita Stwosza 63, 80-308 Gdańsk, Poland; ola.grabowska@ug.edu.pl (O.G.); a.singh.706@studms.ug.edu.pl (A.S.); krzysztof.zamojc@ug.edu.pl (K.Ż.)

* Correspondence: sergey.samsonov@ug.edu.pl (S.A.S.); dariusz.wyrzykowski@ug.edu.pl (D.W.)

Abstract: The objective of this study was to examine the interactions between anionic surfactants, specifically 1-alkylsulfonates (KXS) and 1-alkylsulfates (SXS) ions, with human serum albumin (HSA). A combination of experimental techniques, including isothermal titration calorimetry (ITC), steady-state fluorescence spectroscopy (SF), and molecular dynamics-based approaches was employed to gain a comprehensive understanding of these processes. It has been demonstrated that the subtle variations in the charge distribution on the anionic surfactant headgroups have a significant impact on the number of binding sites, the stoichiometry of the resulting complexes, and the strength of the interactions between the surfactants and the protein. Additionally, we established that the affinity of the investigated ligands to specific regions on the protein surface is governed by both the charge of the surfactant headgroup and the length of the aliphatic hydrocarbon chain. In summary, the findings highlight the crucial role of charge distribution on surfactant functional groups in the binding mode and the thermodynamic stability of surfactant–protein complexes.

Keywords: 1-alkylsulfonates; 1-alkylsulfates; human serum albumin; protein–ligand interactions; isothermal titration calorimetry; fluorescence spectroscopy; molecular dynamics



Citation: Grabowska, O.; Singh, A.; Żamojć, K.; Samsonov, S.A.; Wyrzykowski, D. Exploring the Impact of Subtle Differences in the Chemical Structure of 1-Alkylsulfates and 1-Alkylsulfonates on Their Interactions with Human Serum Albumin. *Molecules* **2024**, *29*, 4598. <https://doi.org/10.3390/molecules29194598>

Academic Editor: Maofa Ge

Received: 28 August 2024

Revised: 16 September 2024

Accepted: 20 September 2024

Published: 27 September 2024



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1. Introduction

The amphiphilic structure of 1-alkylsulfonates (KXS) and 1-alkylsulfates (SXS), where X represents the number of carbon atoms in the hydrocarbon chain, classifies them as surface-active compounds (surfactants) with the capacity to reduce surface tension between liquids or between a liquid and a solid surface. The remarkable properties of 1-alkylsulfates and the related 1-alkylsulfonates make them incredibly versatile and applicable across numerous industrial and technological fields. These applications include the detergent industry, food chemistry, drug delivery, cosmetic preparation, and the life sciences [1–5].

1-Alkylsulfonates are characterized by the presence of a sulfonate group ($-\text{SO}_3^-$), whereas 1-alkylsulfates, closely related to their sulfonate counterparts, boast a sulfate group ($-\text{OSO}_3^-$) in their molecular structure. In contrast to SXS, sulfonates feature a direct linkage between the sulfur atom of a headgroup and a carbon chain of specific lengths (Figure 1).

These seemingly subtle differences in the structure of SXS and KXS have a significant impact on the overall charge of the headgroup of the sulfate and sulfonate ions [6]. This, in turn, is reflected in the processes determined by (de)hydration effects [7,8]. These differences, in turn, have far-reaching implications for the physicochemical properties of SXS and KXS, including critical micelle concentration (CMC), critical aggregation concentration (CAC), micellar micropolarity, and aggregation number.

One noticeable distinction lies in the critical micelle concentrations (CMCs) of specific examples like sodium dodecyl sulfate (S12S) and sodium 1-dodecanesulfonate (K12S). For S12S, the CMC is 8.3 mM, whereas for K12S, it is 10.8 mM [9]. Significant differences are

exhibited in the Krafft points of KXS, which are noticeably higher than those of SXS (the Krafft points of S12S and K12S are 282.15 K and 311.15 K, respectively) [10]. This disparity in Krafft points emphasizes the distinct thermal behaviour and solubility characteristics of 1-alkylsulfonates and 1-alkylsulfates. Interestingly, we have recently proven, in one specific case, that K12S molecules are not recognized by S12S as the ones with a different structure and consequently are allowed to participate in the formation of the S12S micelles [11]. However, it can be postulated that in this specific case, K12S participates in the formation of S12S micelles due to the hydrophobic chain length being identical to that of S12S, and the differences in charge distribution on the headgroups being of less importance.

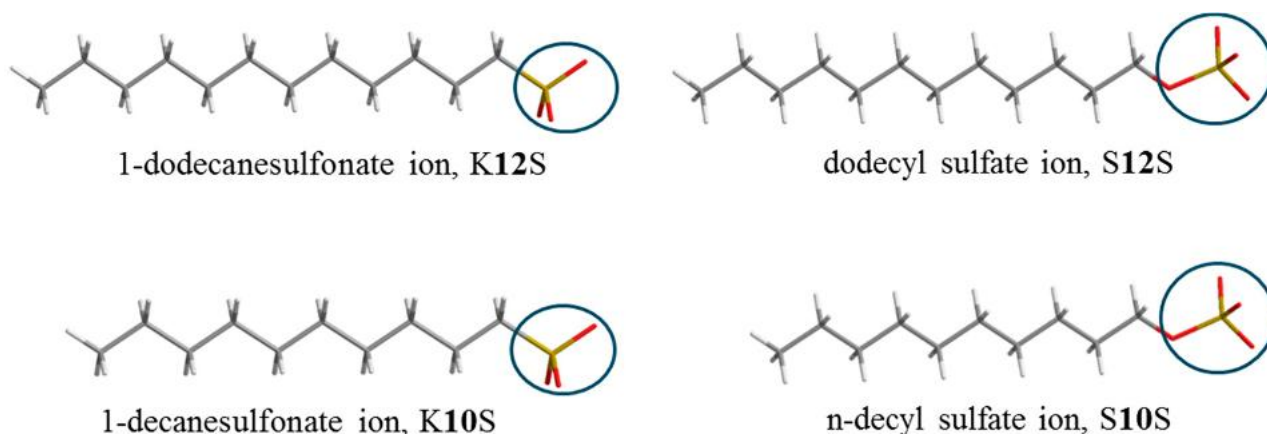


Figure 1. Chemical structure of the investigated ligands.

In the realm of surfactant–protein interactions, extensive research has been conducted on classical surfactants [12–19]. These studies have primarily focused on factors such as the surfactant topology (monomeric or micellar), chemical structure (cationic, anionic, nonionic, hydrocarbon chain length), and experimental conditions (pH, temperature) [12,20,21]. However, relatively little attention has been paid to the distinct headgroup structures of anionic surfactant components [6]. These headgroup structures have the potential to significantly influence ligand–protein interactions. Thus, the objective of this study is to delve into the impact of the differing headgroup charges between KXS and SXS (Figure 1) on their interactions with human serum albumin (HSA). By exploring this aspect, we aim to shed light on the intricate interplay between surfactant headgroup charge and protein interactions.

2. Results and Discussion

2.1. Interactions of 1-Alkylsulfonates (KXS) and 1-Alkylsulfates (SXS) Ions with Human Serum Albumin (HSA)

The binding of KXS and SXS ions to human serum albumin (HSA) has been analysed using isothermal titration calorimetry (ITC) and fluorescence spectroscopy. These methods offer essential insights into crucial information regarding ligand–protein complexes. This includes determining the stoichiometry of the investigated complexes, the number of binding sites on the protein surface and the strength of the interactions (known as binding affinity). In addition, thermodynamic parameters such as enthalpy and entropy changes due to the established interactions provide valuable information that contributes to a deeper understanding of the molecular interactions under study (Table 1).

2.1.1. The Stoichiometry and the Number of the Binding Sites

The relatively minor structural differences between KXS and SXS have a significant impact on their binding interactions with human serum albumin (HSA). These differences are manifested in the stoichiometry of the ligand–protein complexes and the number of binding sites. Conditional parameters for the interactions under study are summarized

in Table 1, whereas representative binding isotherms are shown in Figure 2. A significant difference in the interactions of KXS and SXS with the protein is reflected in the number of binding sites. In the case of the K12S and K10S ligands, HSA was found to have only one binding site, leading to the formation of complexes with a KXS:HSA molar ratio of approximately 1:1. In contrast to KXS, the S12S and S10S ligands bind to two separate sites on the HSA protein. The first site accommodates one mole of SXS per one mole of the protein, while the second site binds four moles of SXS (Scheme 1).

Table 1. The conditional thermodynamic parameters of KXS and SXS binding to HSA in the 10 mM Caco buffer of pH 7 at 298.15 K (standard deviation values in parentheses).

Parameters	K12S/HSA	K10S/HSA	S12S/HSA	S10S/HSA
N_1	1.47 (± 0.02)	0.89 (± 0.06)	0.99 (± 0.08)	0.77 (± 0.01)
$\log K_{(ITC)1}$	4.60 (± 0.02)	4.54 (± 0.04)	6.14 (± 0.20)	5.78 (± 0.07)
$\Delta G_{(ITC)1}$ [kcal mol ^{−1}]	−6.28 (± 0.03)	−6.20 (± 0.05)	−8.38 (± 0.28)	−7.89 (± 0.10)
$\Delta H_{(ITC)1}$ [kcal mol ^{−1}]	−11.29 (± 0.20)	−11.33 (± 0.86)	−9.03 (± 0.54)	−11.68 (± 0.77)
$T\Delta S_{(ITC)1}$ [kcal mol ^{−1}]	−5.01	−5.13	−0.65	−3.79
N_2	–	–	4.13 (± 0.17)	4.03 (± 0.76)
$\log K_{(ITC)2}$	–	–	4.72 (± 0.05)	4.29 (± 0.10)
$\Delta G_{(ITC)2}$ [kcal mol ^{−1}]	–	–	−6.44 (± 0.06)	−5.85 (± 0.14)
$\Delta H_{(ITC)2}$ [kcal mol ^{−1}]	–	–	−6.47 (± 0.51)	−5.10 (± 1.33)
$T\Delta S_{(ITC)2}$ [kcal mol ^{−1}]	–	–	−0.03	0.75

2.1.2. The Thermodynamic Parameters of the Interactions

In general, the interaction between surfactants and proteins is primarily driven by hydrophobic interactions between the ligands and the hydrophobic regions of the protein. In these cases, the stability of the resulting complexes is influenced by the length of the hydrophobic ligand; specifically, the stability of the ligand–protein complex tends to increase with a greater number of carbon atoms in the hydrocarbon chain. However, for the ligands under study, the charged headgroups of KXS and SXS can also participate in electrostatic interactions with the positively charged regions of HSA, influencing binding characteristics and conformational changes in the protein.

The $\log K$ and ΔH values indicating the affinity of K12S and K10S for human serum albumin are approximately equal (Table 1). A comparable situation in binding interactions also applies to S12S and S10S. This indicates that the difference in hydrocarbon chain length (C12 vs. C10) is insufficient to impact the binding strength of these ligands (Table 1).

It is noteworthy that, for all the systems studied, a negative value of ΔH indicates a significant contribution of electrostatic interactions, hydrogen bonds and/or Van der Waals interactions to the stabilization of the resulting ligand–protein complexes (Table 1).

The enthalpy–entropy compensation phenomenon is observed in the interactions studied. It is more pronounced for KXS–HSA interactions (Table 1). The more negative the value of ΔH , the lower the mobility of the interacting molecules, which is reflected in the low value of the entropy factor (Table 1). Furthermore, ab initio calculations showed that the α -methylene group of KXS had a partial negative charge, whereas the α -methylene group of SXS had a partial positive charge. Thus, the headgroup of KXS had a much smaller negative charge than SXS (for details see Section 2.3). Consequently, differences in the entropy change upon binding the ligands to HSA may be due to differences in the charge distribution of the headgroups of KXS and SXS.

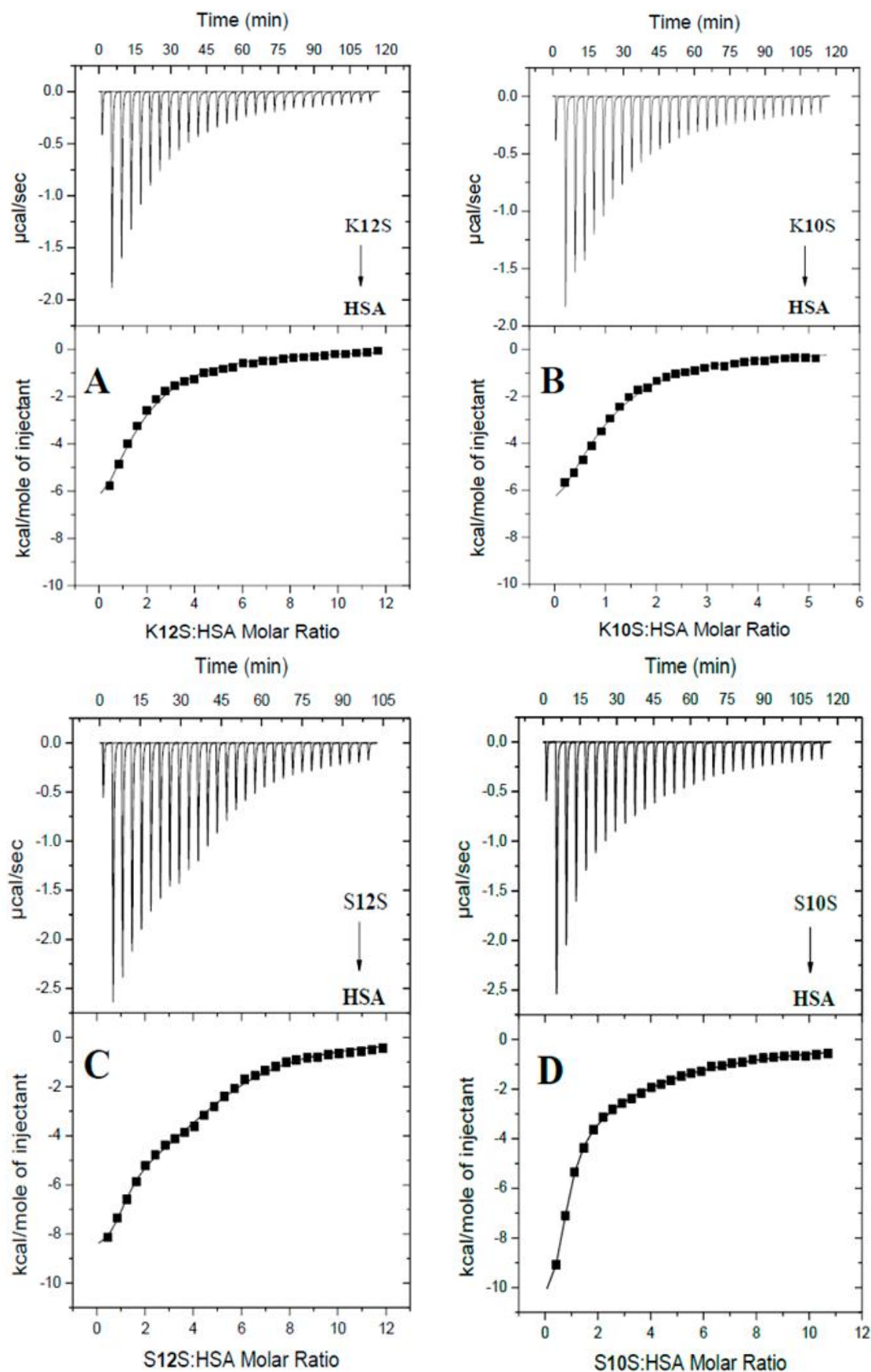


Figure 2. Calorimetric titration isotherms of the binding interactions between HSA and K12S (A), K10S (B), S12S (C), as well as S10S (D) in the 10 mM Cacodylate buffer of pH 7 at 298.15 K.

Scheme 1. The stoichiometry and number of binding sites of the investigated ligands with human serum albumin (HSA).

2.1.3. Effect of KXS and SXS on the Tryptophan Environment of Human Serum Albumin

To understand the effect of the studied compounds on the tryptophan environment of human serum albumin, the intrinsic fluorescence of the protein in the presence of increasing concentrations of K12S, K10S, S12S and S10S has been studied. HSA in 10 mM Caco buffer of pH 7 after excitation at 280 nm exhibits a single emission peak with a maximum at 343 nm (due to a tryptophan residue). On the addition of the ligands under study an enhancement in the protein fluorescence intensity can be observed (contrary to bovine serum albumin, where fluorescence quenching by anionic surfactants occurs [21–23]), which is consistent with the existence of a unique buried tryptophan residue in HSA with considerable static quenching in the native state [24]. Upon the binding, a removal of quenching occurs as a result of the interaction between the indole side chain of W214 residue with neighbouring quenching groups [22]. A very clear shift (up to 15 nm) of the band towards shorter wavelengths (Figure 3) additionally suggests that all selected for the studies compounds interact with HSA and induce a change of the microenvironment around the protein, with a tryptophan residue relocated to a more hydrophobic environment. The observed effect of the studied compounds on the fluorescence emission of HSA is similar to the interactions of the protein with—among others—syringin [25], salbutamol [26], and abiraterone [27]. It has been demonstrated that SXS has a more pronounced impact on emissions than KXS. Furthermore, the magnitude of the observed change is directly proportional to the length of the hydrocarbon chain, which is consistent with previous reports [28,29].

2.2. Potential Binding Sites for KXS and SXS on Human Serum Albumin

Different binding mechanisms determine the affinity of small molecules for HSA. Depending on the structural features of a ligand, several distinct regions with different binding mechanisms (involving hydrophobic interactions or polar forces) are available on the surface of albumin. To explore the potential binding sites of the studied ligands on the surface of the proteins and to gain insight into the differences in the binding of KXS and SXS, molecular dynamics simulations as well as ITC competitive experiments were performed.

2.2.1. A Molecular Dynamics Simulation Approach to Binding Site Determination

The computational simulation hypothesis was based on experimental data indicating the existence of multiple binding sites on human serum albumin (HSA), as well as observable differences in the binding affinity of KXS and SXS ligands to this protein. To further investigate the binding sites and ligand affinities on HSA, molecular dynamics simulations were performed using the AMBER software package and LIE (Linear Interaction Energy) methodology was used for binding free energy calculations.

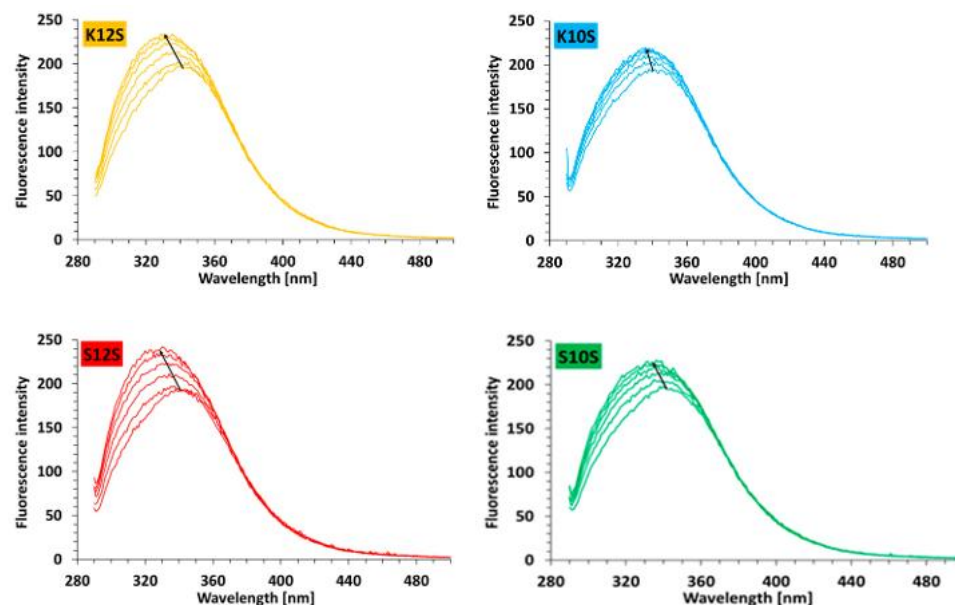


Figure 3. Fluorescence emission spectra of HSA in 10 mM Caco buffer of pH 7 in the presence of increasing concentrations (0–12.3 μ M) of K12S, K10S, S12S, and S10S; λ_{ex} = 280 nm; c_{HSA} = 2.2 μ M; 298.15 K.

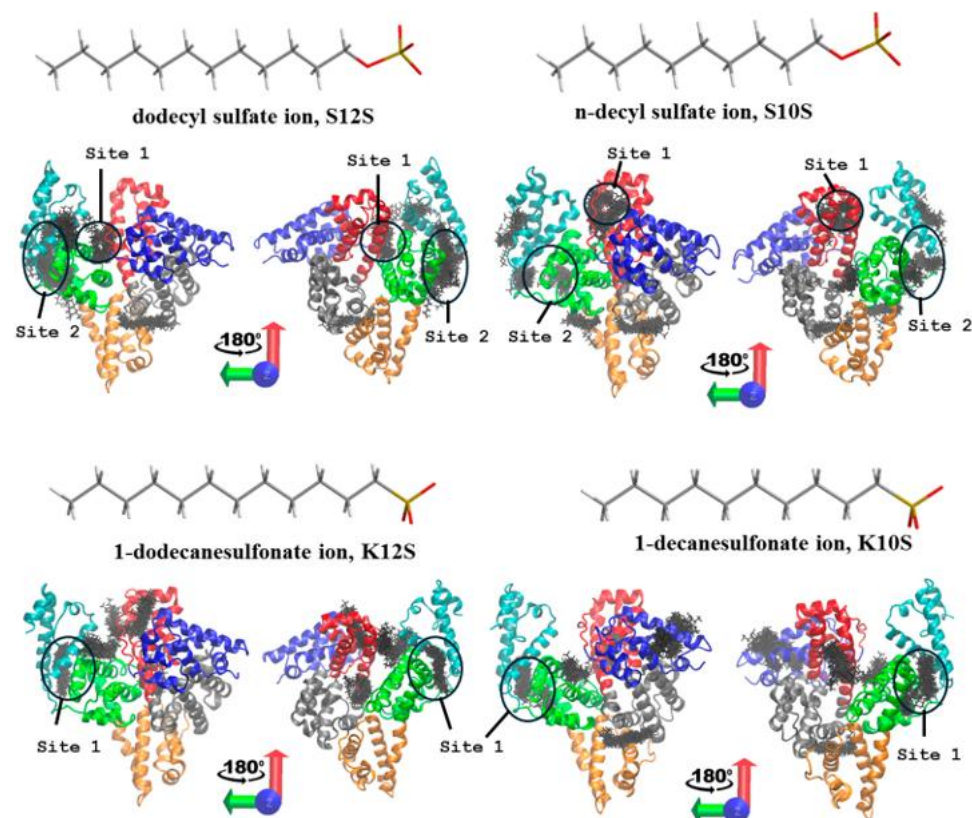


Figure 4. The potential binding sites of SXS and KXS on human serum albumin (HSA). The protein is represented as cartoon, while the bound surfactant molecules within the predicted binding sites are in sticks and correspond to multiple frames from the MD simulations.

The simulations confirmed the presence of multiple binding sites on human serum albumin (HSA) for the four ligands investigated: S12S, S10S, K12S, and K10S (Figure 4). These findings align with experimental data, which also indicated the existence of distinct binding sites for these ligands on the protein.

Ligands S10S and S12S revealed the ability to bind at two distinct sites on HSA, specifically within the IIIA, IIA, and IB domains. Free energy calculations showed that the binding affinity for S10S localized in the proximity of 5 Å from the analyzed putative binding sites was $-31.1 \pm 3.9 \text{ kcal mol}^{-1}$ at the first binding site, designated as “Site 1,” and $-32.3 \pm 4.3 \text{ kcal mol}^{-1}$ at the second binding site, referred to as “Site 2” (Figure 4). For S12S, the binding affinity was also slightly higher in “Site 2”, $-34.8 \pm 4.2 \text{ kcal mol}^{-1}$ compared with “Site 1” ($-32.1 \pm 5.0 \text{ kcal mol}^{-1}$). In contrast, ligands K10S and K12S were found to exhibit only one binding site, which was located in the IIIA domain of HSA.

The free energy analysis indicated that the IIIA domain has a high likelihood of serving as the binding site for ligands K10S and K12S. The binding affinities of K10S and K12S to HSA were found to be comparable, irrespective of the length of the hydrophobic moiety of the ligand, supporting our hypothesis that chain length does not significantly affect binding affinity in this instance. Notably, ligands S10S and S12S displayed a shared second binding site, which is situated close to the binding site for K10S and K12S within the IIIA domain. These findings suggest the presence of a shared binding site in this region for the ligands S10S, S12S, K10S, and K12S. However, the first binding sites for S10S and S12S were distinct from one another. For S12S, the binding site is located near the interface of the IB and IIIA domains, while for S10S, it is situated near the upper part of the IB domain. The favourable binding energy at the second binding site for S10S and S12S, along with the first binding site for K10S and K12S suggested potential interactions between these ligands and human serum albumin (HSA) in these predicted binding sites.

2.2.2. Competitive ITC Displacement Assays for Binding Site Determination

To verify the MD simulation findings, the ITC competitive experiments were conducted. In this approach, the S12S molecules have been employed as the HSA site markers, namely “Site 1” (located in the HSA cavity between the IIA and IB domains) and “Site 2” (located in the IIIA domain of HSA). Saturation of the HSA protein with the S12S ligand may hinder or even prevent the binding of other anions (i.e., S10S, K12S, K10S), provided that they compete with S12S for the same binding site in HSA.

ITC data revealed that HSA saturated with S12S at a molar ratio of S12S:HSA = 1.5:1 is still capable of binding K12S and K10S ligands (Figure 5, Table 2). In the case of S10S, its interactions with HSA are hindered. This is manifested by the presence of only one binding site for S10S (“Site 2” located in subdomain IIIA), and a reduced number of moles of S10S (ca. 2) bind to one mole of HSA under these specific conditions (Table 2).

Table 2. Thermodynamic parameters of the interaction of SXS and KXS with HSA in the presence and absence of S12S in the system. Measurement conditions: temperature 298.15 K; 10 mM Caco buffer of pH 7 (standard deviation values in parentheses).

Parameters	S10S → HSA:S12S ^a	S10S → HSA:S12S ^b	K12S → HSA:S12S ^a	K12S → HSA:S12S ^b	K10S → HSA:S12S ^a	K10S → HSA:S12S ^b
The first SXS/KXS-binding site in HSA						
N_1	1.94 (±0.05)	1.96 (±0.07)	1.99 (±0.02)	-	0.99 (±0.01)	-
$\log K_{(\text{ITC})1}$	4.51 (±0.01)	4.23 (±0.03)	4.45 (±0.02)	-	4.61 (±0.02)	-
$\Delta G_{(\text{ITC})1} [\text{kcal mol}^{-1}]$	-6.15 (±0.01)	-5.77 (±0.04)	-6.08 (±0.02)	-	-6.29 (±0.03)	-
$\Delta H_{(\text{ITC})1} [\text{kcal mol}^{-1}]$	-8.89 (±0.21)	-3.06 (±0.13)	-7.37 (±0.11)	-	-7.51 (±0.15)	-
$T\Delta S_{(\text{ITC})1} [\text{kcal mol}^{-1}]$	-2.74	2.71	-1.29	-	-1.22	-

^a HSA saturated with S12S in the first binding site. The HSA:S12S molar ratio = 1:1.5. ^b HSA saturated with S12S in the first and second binding sites. The HSA:S12S molar ratio = 1:6.

A different scenario is observed for HSA with fully saturated binding sites by S12S (S12S:HSA molar ratio = 6:1). In such a case, there is a lack of evidence for the interactions of HSA with K12S and K10S (Figure 6, Table 2). Thus, according to the MD simulation results, it can be expected that the potential binding sites of KXS will be located in subdomain IIIA.

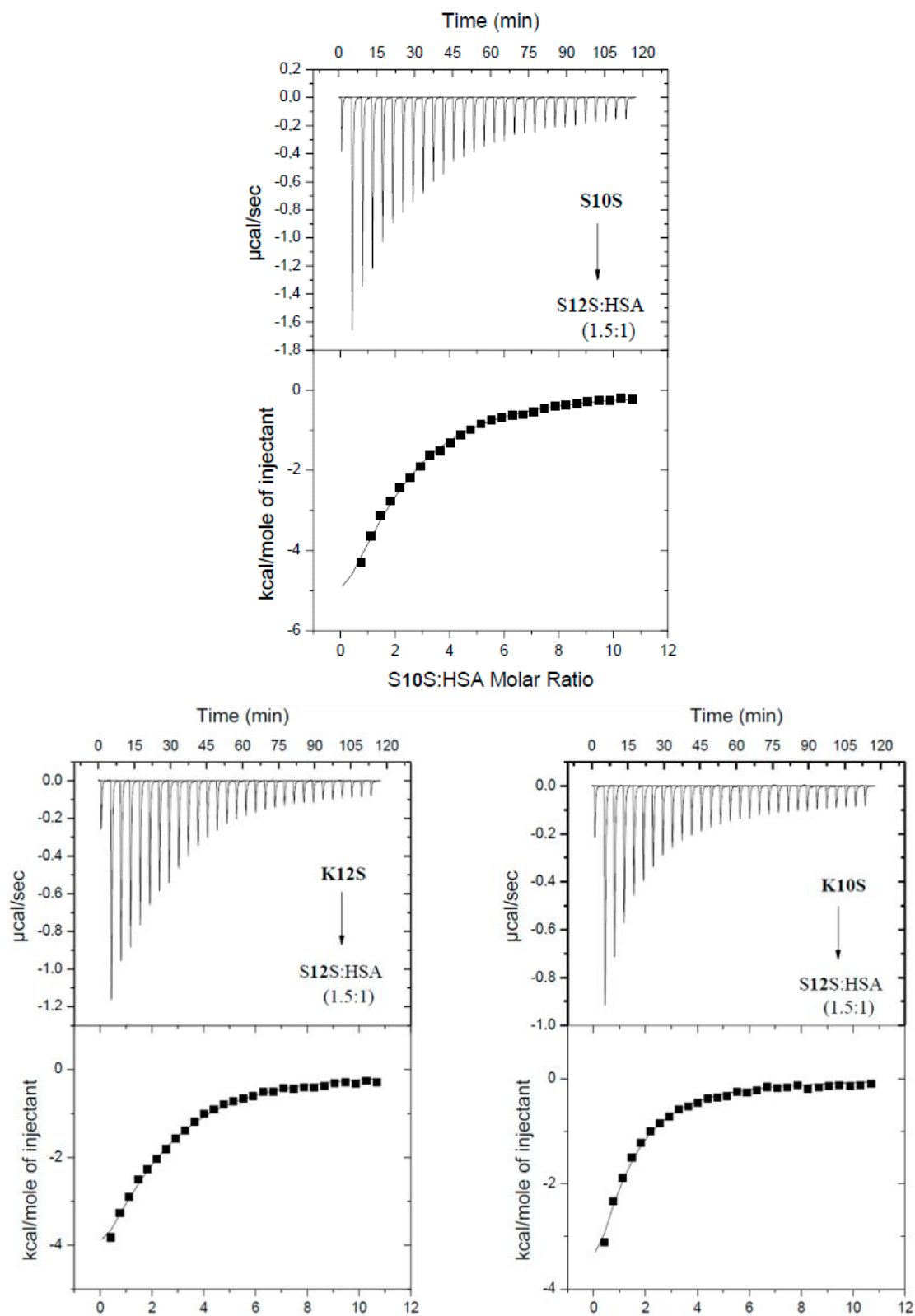


Figure 5. Calorimetric titration isotherms of the binding interactions between HSA and S10S, K12S, K10S in the presence of S12S in the system, in 10 mM Caco buffer of pH 7 at 298.15 K. The S12S:HSA molar ratio = 1.5:1.

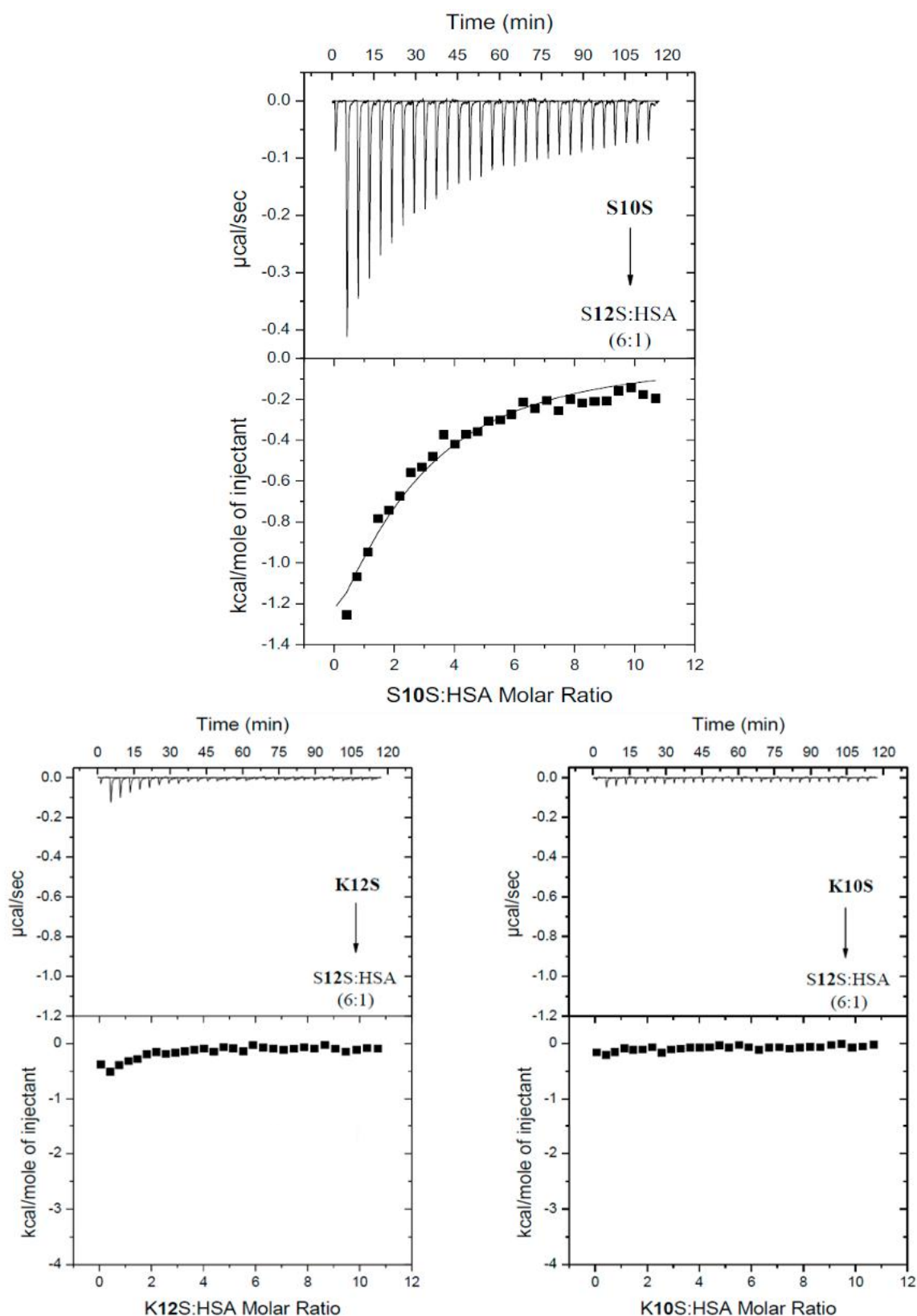


Figure 6. Calorimetric titration isotherms of the binding interactions between HSA and S10S, K12S and K10S in the presence of S12S in the system, in 10 mM Caco buffer of pH 7 at 298.15 K. The S12S:HSA molar ratio = 6:1.

It is noteworthy that despite the complete saturation of HSA with S12S molecules, S10S molecules, unlike 1-alkylsulfonates, are still able to interact with HSA (Figure 6, Table 2). However, compared to the interaction of S10S with free HSA, there is only one S10S binding site on the HSA surface under such specific conditions. This observation is consistent with theoretical results indicating that S10S and S12S have an affinity for one region of the protein (in the IIIA domain), while the other binding region of these ligands is different. For these reasons, complete saturation of HSA with the S12S ligand still allows S10S to bind near the interface of domains IB and IIIA.

2.3. The Hydration Patterns of 1-Alkylsulfonates (KXS) and 1-Alkylsulfates (SXS) Ions

To gain insight into the differences in physicochemical properties between KXS and SXS, the spatial distribution of water oxygen atoms in relation to the sulfate group was investigated using the radial distribution function (RDF). The obtained data indicate that significantly different affinities of KXS and SXS for HSA may be influenced by differences in the hydration patterns of the polar headgroups of the ligands (Figure 7). The observed differences in hydration properties originated from the charge distributions in the headgroups of SXS and KXS that are essentially different (Figure 8). While the charge differences of the atoms in SO_3^- are negligible, the main differences in the charge distribution of the headgroup correspond to the negatively charged oxygen atom present in SXS and absent in KXS. The presence of this oxygen atom strongly affects the charge of the adjacent carbon atom and hydrogen atoms bound to it. This effect is already very weak for the next carbon atom in the chain. The presence of an additional oxygen atom in SXS in comparison to KXS, therefore, leads to a more polar nature of the SXS headgroup. This, in turn, enhances the strength of the electrostatic interactions that this group can establish with its environment, affecting the organization of water molecules in its hydration shell. As a result, it can potentially influence both the enthalpy of binding to a protein and the entropy of dehydration when a direct contact with the protein is maintained.

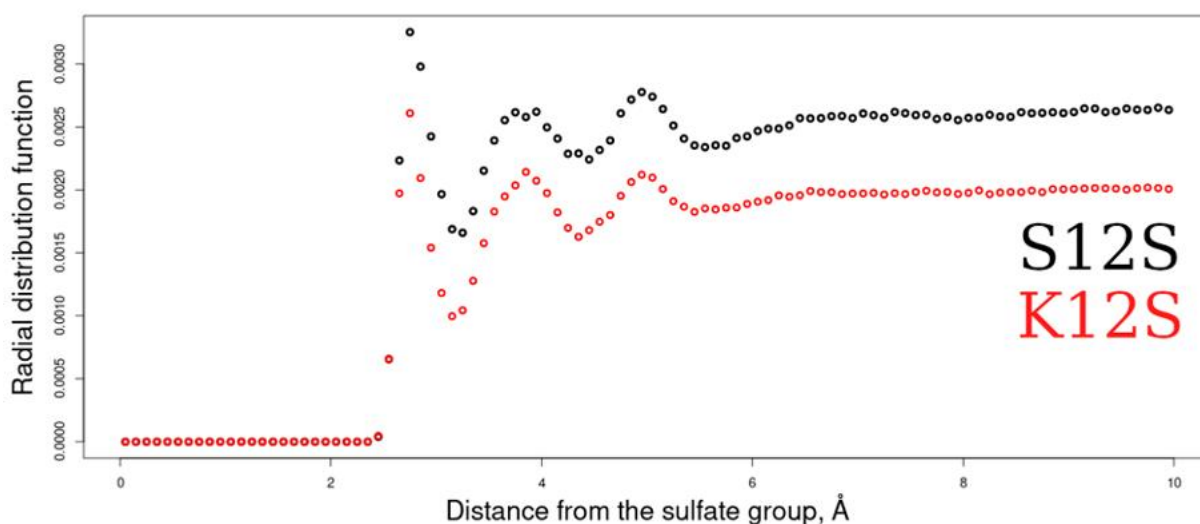


Figure 7. Radial distribution functions (RDFs) between the O atoms of the water molecules in relation to the charged headgroups of KXS and SXS.

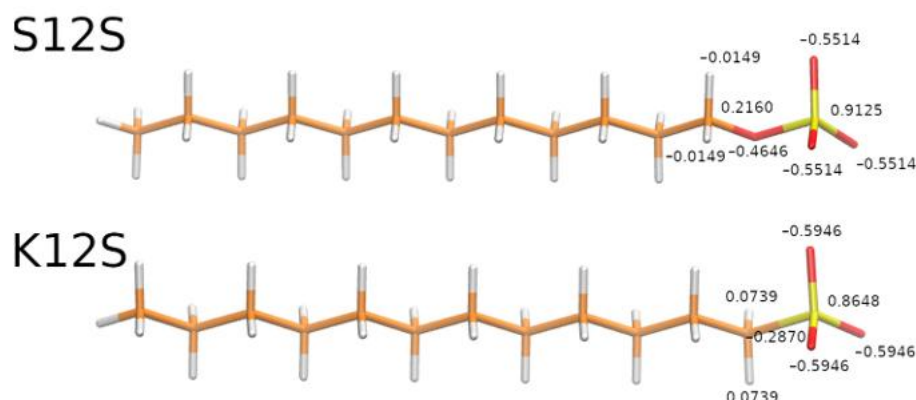


Figure 8. Point charges of the atoms belonging to the headgroups of S12S and K12S used in the corresponding libraries for MD simulations.

3. Materials and Methods

3.1. Materials

Human serum albumin (HSA, $\geq 96\%$), sodium dodecyl sulfate (S12S, $\geq 99.0\%$), sodium n-decyl sulfate (S10S, 98%), sodium 1-dodecanesulfonate (K12S, $\geq 99\%$), sodium 1-decanesulfonate (K10S, $\geq 99\%$), sodium cacodylate trihydrate (Caco, $\geq 99\%$) were obtained from Merck (Warsaw, Poland) and employed as received without further purification. All solutions were prepared in the 10 mM Caco buffer (pH 7).

3.2. Methods

3.2.1. Isothermal Titration Calorimetry (ITC)

Experiments were conducted using AutoITC isothermal titration calorimeter (MicroCal Inc. GE Healthcare, Northampton, MA, USA). A 1 mM solution of SXS or KXS (where X = 12, 10) was injected into the reaction cell, which contained: (a) a 0.04 mM solution of free HSA, (b) a mixture of HSA:S12S in molar ratios of 1:1.5 and 1:6, respectively. ITC experiments were conducted under specific conditions: temperature 298.15 K, cell volume 1.4491 mL, injection volume 10.02 μL (29 injections, 2 μL for the first injection only), injection interval 240 s, stirring speed 300 r.p.m., reference power 10 $\mu\text{cal s}^{-1}$. The thermodynamic parameters (K_{ITC} and ΔH_{ITC}) were obtained directly from ITC measurements by fitting isotherms (using nonlinear least-squares procedures) to a model that assumes a single set and two sets of binding sites, respectively for 1-alkylsulfonates (KXS) and 1-alkylsulfates (SXS).

3.2.2. Steady-State Fluorescence Spectroscopy (SF)

The concentration of the human serum albumin was measured spectrophotometrically using an extinction coefficient equal to $\epsilon_{280}^{\text{HSA}} = 32930 \text{ M}^{-1}\text{cm}^{-1}$. For this purpose, the UV absorption spectra were recorded on a Perkin Elmer Lambda 650 (Waltham, MA, USA) UV/Vis spectrophotometer. Fluorescence emission spectra were recorded on a Cary Eclipse Varian (Agilent, Santa Clara, CA, USA) spectrofluorometer equipped with a 1.0 cm multi-cell holder and temperature controller. Experiments were performed in 10 mM Caco buffer (pH 7). Excitation and emission slit widths were fixed at 5 nm. The excitation wavelength was always set at 280 nm. Fluorescence emission spectra of HSA (2.2 μM) were recorded in the range 290–500 nm in the absence and presence of S10S, K10S, S12S and K12S while increasing the concentration of each up to 12.3 μM . In the fluorescence titration experiments, 2 mL of HSA was titrated with 5 μL of S10S, K10S, S12S, and K12S (1 mM) stock solutions added in five consecutive additions. All experiments were performed at 298.15 K.

3.2.3. Molecular Dynamics (MD) Simulations

The structure of HSA was obtained from the PDB (PDB ID: 5 $\times$ 52, 3.00 $\text{\AA}$). The structures of SXS and KXS were built in xleap module of AMBER 20 package [30]. The

corresponding libraries for AMBER package have been created with Antechamber using a standard procedure for GAFF force field [31] and HF/6-31G* RESP charge derivation methodology [32]. Fifteen ligand molecules of each type for each simulation were placed randomly around HSA. GAFF and ff14SB [33] force field parameters were used for the ligands and the protein, respectively. These molecular systems were solvated in a truncated octahedron TIP3P water periodic box with a distance of 15 Å from the solute to the box and neutralized by counter-ions. MD simulations were preceded by two steps of energy-minimization: 1500 cycles of steepest descent and 1000 cycles of the conjugate gradient with harmonic force restraints of $10 \text{ kcal} \cdot (\text{mol} \cdot \text{\AA})^{-1}$ on protein and ligand atoms, then 3000 cycles of steepest descent and 3000 cycles of conjugate gradient without restraints. This was followed by heating of the system from 0 to 300 K for 10 ps, and a 100 ps MD equilibration run at 300 K and 105 Pa in NPT. After this equilibration procedure, 100 ns of productive MD runs were performed in periodic boundary conditions in NPT with an 8 Å cut-off for non-bonded interactions and the particle mesh Ewald method applied to treat long-range electrostatic interactions. Energetic post-processing of the trajectories was done using linear interaction energy (LIE) with a dielectric constant of 80. The obtained structures were visualized in VMD [34].

4. Conclusions

The objective of this study was to investigate the interactions between 1-alkylsulfonates (KXS) and 1-alkylsulfates (SXS) ions with human serum albumin (HSA). To gain insight into the binding characteristics of these two types of surfactants, a comparative analysis was conducted.

The results revealed that even small differences in the charge distribution on the anionic surfactant headgroups have a significant impact on the interactions between the surfactants and the protein. This influence is evident in the number of binding sites and the stoichiometry of the resulting complexes, as well as the strength of the interactions.

Furthermore, both single and shared regions were identified on the protein surface where KXS and SXS bind. The affinity of individual ligands for these sites depended on the surfactant headgroup's charge and the hydrocarbon chain length ($X = 12$ or 10). Understanding these binding regions provides insight into the specific interactions between surfactants and proteins.

It is important to note that the parameters obtained for these interactions depend on the pH of the system, the type of buffer used, and its concentration (ionic strength). Therefore, they can only be compared with parameters determined under identical experimental conditions. Nevertheless, the data obtained under the same experimental conditions confirmed previous findings regarding the significant impact of the hydrophilic moieties of KXS and SXS on their physicochemical properties. By gaining a deeper understanding of these subtle differences, we can improve our interpretation of processes involving 1-alkylsulfates and 1-alkylsulfonates, particularly in the context of protein–ligand interactions.

In conclusion, our study highlights the importance of charge distribution on surfactant functional groups and hydrophobic chain lengths in determining the binding characteristics and thermodynamic stability of surfactant–protein complexes.

Author Contributions: Conceptualization, O.G., D.W. and S.A.S.; methodology, D.W. and S.A.S.; formal analysis, A.S., K.Ż. and D.W.; investigation, O.G., A.S., K.Ż. and D.W.; data curation, O.G., A.S. and K.Ż.; writing—original draft preparation, O.G., A.S., K.Ż., D.W. and S.A.S.; writing—review and editing, K.Ż., D.W. and S.A.S.; visualization, O.G., A.S., K.Ż. and D.W.; supervision, D.W. and S.A.S.; project administration, D.W.; funding acquisition, O.G. and S.A.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by: (1) the grants for Young Scientists 2023 from University of Gdansk, Poland, grant number 539-T090-B989-23 (O.G.); (2) National Science Centre of Poland, grant number UMO-2023/49/B/ST4/00041 (S.A.S.).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding authors.

Conflicts of Interest: The authors declare no conflicts of interest.

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PUBLIKACJA 6

**Can sodium 1-alkylsulfonates participate
in the sodium dodecyl sulfate micelle formation?**



Can sodium 1-alkylsulfonates participate in the sodium dodecyl sulfate micelle formation?

Ola Grabowska^a, Krzysztof Żamojć^{a,*}, Michał Olewniczak^b, Lech Chmurzyński^a, Dariusz Wyrzykowski^a

^a Faculty of Chemistry, University of Gdansk, Wita Stwosza 63, 80-308 Gdansk, Poland

^b Faculty of Chemistry, Gdansk University of Technology, Narutowicza 11/12, 80-233 Gdansk, Poland

ARTICLE INFO

Article history:

Received 31 January 2023

Revised 21 February 2023

Accepted 1 March 2023

Available online 5 March 2023

Keywords:

Sodium dodecyl sulfate

Sodium 1-alkylsulfonates

Critical micelle concentration

Micellar aggregation number

Thermodynamic parameters

Fluorescence spectroscopy

Conductivity

Molecular dynamics

ABSTRACT

The aggregation behavior of sodium dodecyl sulfate (SDS) was studied in an aqueous solution in the presence of increasing concentrations of selected sodium 1-alkylsulfonates, namely sodium 1-octanesulfonate, sodium 1-decanesulfonate, and sodium 1-dodecanesulfonate. The critical micelle concentration (CMC) of SDS was determined by conductivity and fluorescence intensity measurements. The steady-state fluorescence quenching experiments with pyrene as a fluorescent probe were performed to obtain micellar aggregation numbers (N_{agg}) of the surfactant. The thermodynamic parameters of micellization (ΔG_{mic}^0 for all studied systems; ΔH_{mic}^0 and ΔS_{mic}^0 for sodium 1-dodecanesulfonate) have been determined and compared. The influence of alkyl chain length and concentration of the selected sodium 1-alkylsulfonates on the values of CMC, N_{agg} , ΔG_{mic}^0 , ΔH_{mic}^0 , and ΔS_{mic}^0 of SDS has been discussed. As it was found, all selected for the experiments electrolytes decrease significantly the CMC of the surfactant, while an opposite effect is observed for N_{agg} values. The obtained experimental results have been supported by theoretical calculations. Interestingly, it has been proven that solely the molecules of sodium 1-dodecanesulfonate (of the same carbon chain length) may act as the SDS mimetics – they are not recognized by SDS as the ones with a different structure and consequently are allowed to participate in the formation of the surfactant's micelles.

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1. Introduction

Surfactants, considering their structure and charge of the hydrophilic parts of the molecule, can be divided into cationic, anionic, amphoteric and nonionic ones [1]. The surface properties of these amphiphilic molecules, such as lipophilicity, viscosity, solubilization capacity, adsorption at the interface, self-assembly or micelle formation [2], are induced by the hydrophobic interaction between hydrocarbon parts of the surfactant molecules balanced by their hydration and electrostatic repulsive effects [3] and play an important role in many natural and industrial applications, *i.e.* detergent and pharmaceutical industries, food technology, and petroleum recovery processes [4]. The tendency of surfactants to self-aggregate is with no doubt one of the most fascinating aspects of these particles, while micelles remain one of the central topics of study within surface and colloid chemistry [5]. Important experimental characteristics describing micelles are the critical micelle concentration (CMC; the concentration where they are formed) [6], kinetic features (such as the micellar lifetime and reaction

rates) [7], their size, shape, dispersity in a solution [8], aggregation numbers (N_{agg} ; the amount of surfactant molecules making up a micelle) [9] as well as the thermodynamics [10] of the micellization process (entropy is believed to be the major driving force for micellization in aqueous solutions). All these parameters are sensitive to numerous internal (*e.g.*, hydrophobic structure and head group type [11]) and external (surfactant concentration [12], pressure [13], pH [14], temperature [15], ionic strength [16], solvent properties [17], presence of electrolyte [18] and non-electrolyte [19] additives, applied technique [20], *etc.*) factors and impact even on biological properties (it has been proven for example that the highest antimicrobial properties are observed at concentrations below CMC [21]). Therefore, it is of great importance from the practical point of view – particularly when the real systems are investigated – to consider the influence of additives and other factors on the features of surfactants [22].

Generally, added electrolytes are known to affect the aggregation behavior of the ionic surfactants, which is attributed entirely to the counter-ion effect [23]. However, there are reports indicating that a co-ion, depending upon its and surfactant's structures, can also affect the micellization phenomenon [24]. In this paper, we report the aggregation behavior of sodium dodecyl sulfate

* Corresponding author.

E-mail address: krzysztof.zamojc@ug.edu.pl (K. Żamojć).

(SDS) in the presence of selected sodium 1-alkylsulfonates of different chain lengths, namely sodium 1-octanesulfonate (K8S), sodium 1-decanesulfonate (K10S), and sodium 1-dodecanesulfonate (K12S) studied by steady-state fluorescence spectroscopy and conductivity measurements and supported by molecular dynamics simulations. All chosen sodium 1-alkylsulfonates are structurally very similar to the SDS surfactant (Fig. 1). In sodium dodecyl sulfate the sulfur of a headgroup is linked to the carbon atom via an oxygen atom, while in sulfonates it is linked directly to a carbon chain of an appropriate length (8, 10, and 12 carbon atoms in the case of K8S, K10S, and K12S, respectively). According to our knowledge, the determination of critical micelle concentrations, micellar aggregation numbers and thermodynamic parameters of micellization of SDS in the presence of the chosen sulfonates has been never performed, although such a need has been signalized previously [25]. Can molecules with a structure similar to SDS act as the surfactant's mimetics and be incorporated into its micelles? Or maybe SDS molecules recognize that other molecules have a slightly different structure and consequently do not allow them to participate in the formation of the surfactant's micelles? The search for the answers to questions raised has prompted us to embark on these studies.

2. Experimental

2.1. Materials

Sodium dodecyl sulfate (SDS, $\geq 99.0\%$), sodium 1-octanesulfonate (K8S, $\geq 99\%$), sodium 1-decanesulfonate (K10S, $\geq 99\%$), sodium 1-dodecanesulfonate (K12S, $\geq 99\%$), pyrene ($\geq 97\%$), cetylpyridinium chloride (CPC, $\geq 98\%$), and sodium chloride ($\geq 99\%$) were acquired from Merck (Poland) and used without additional purification as received. For all solutions preparation, double-distilled water with a conductivity of less than $0.18 \mu\text{S cm}^{-1}$ was employed. The studies of the aggregation behavior of SDS were performed in aqueous solutions of K8S, K10S, K12S,

and NaCl used at low concentrations (1–8 mM), because it is difficult to get reliable conductivity results in the high ionic strength [25,26]. Due to the limited solubility of K12S in water its highest concentration was set at 4 mM.

2.2. Methods

2.2.1. Steady-state fluorescence spectroscopy

The Cary Eclipse Varian (Agilent, Santa Clara, CA, USA) spectrofluorometer, equipped with a temperature controller and a 1.0 cm multicell holder, was used to measure the fluorescence intensities. All experiments were conducted at 298.15 K.

The fluorescence emission spectra of pyrene ($c = 2 \mu\text{M}$; $V = 2 \text{ mL}$; $\lambda_{\text{ex}} = 340 \text{ nm}$; the excitation and emission slit widths set at 5 nm) dissolved in: (i) 1, 2, 4, and 8 mM K8S; (ii) 1, 2, 4, and 8 mM K10S; (iii) 1, 2, and 4 mM K12S; (iv) water, 1, 2, 4, and 8 mM NaCl were recorded from 360 to 500 nm in both the absence and presence of increasing amounts of SDS (up to 11 mM) in order to determine the CMC values. The values obtained are the means of two separate experiments. All spectra were recorded in triplicate for each experiment.

In order to determine the N_{agg} values the fluorescence intensity of pyrene ($c = 2 \mu\text{M}$; $V = 2 \text{ mL}$; $\lambda_{\text{ex}} = 340 \text{ nm}$; $\lambda_{\text{em}} = 375 \text{ nm}$; the excitation and emission slit widths set at 5 nm) dissolved in a mixture of 10 mM SDS and: (i) 1, 2, 4, and 8 mM K8S; (ii) 1, 2, 4, and 8 mM K10S; (iii) 1, 2, and 4 mM K12S; (iv) water, 1, 2, 4, and 8 mM NaCl was measured in the absence and presence of increasing concentrations of the quencher, cetylpyridinium chloride, CPC (up to $19.5 \mu\text{M}$). The same titration was performed four times, thus all obtained values are means from four separate experiments.

2.2.2. Conductivity measurements

Conductometric measurements for all studied systems were performed at 298.15 K using a microtitration unit (Cerkolab System, Poland) fitted with a 5 mL syringe (Hamilton, Poland) and a CD-201 conductometric cell (Hydromet, Poland). The details of

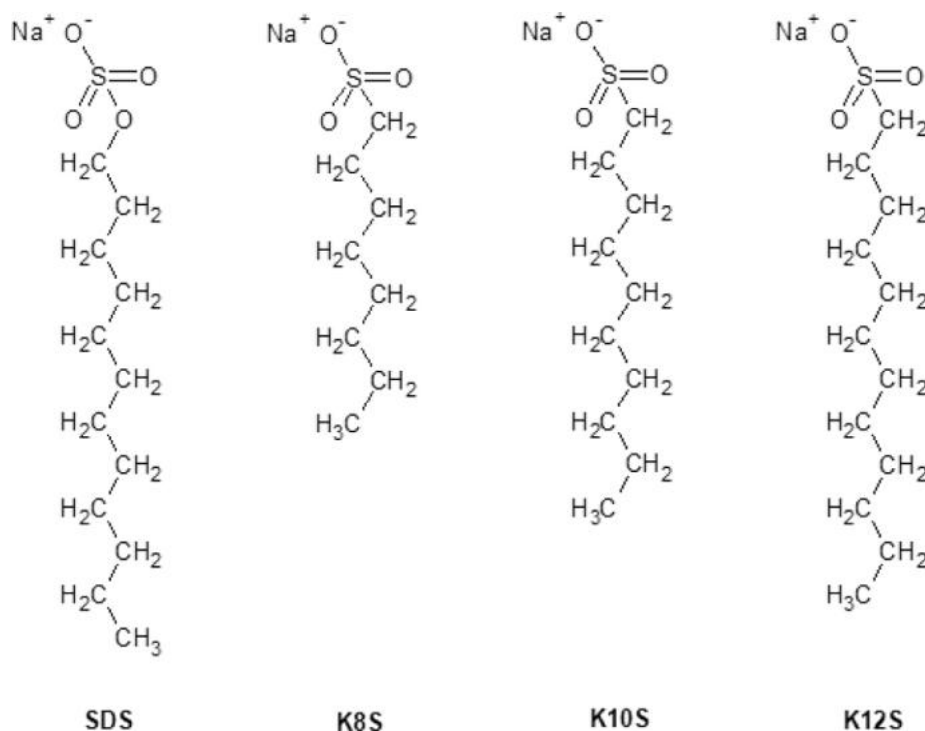


Fig. 1. Chemical structures of SDS, K8S, K10S, and K12S.

the measuring devices and experimental setup have been described previously [27]. The experiment consisted of injecting 0.01 mL of the titrant solution comprising SDS ($c = 60$ mM) and the appropriate ligand of different concentrations (1, 2, 4, and 8 mM K8S; 1, 2, 4, and 8 mM K10S; 1, 2, and 4 mM K12S; water, 1, 2, 4, and 8 mM NaCl), into the reaction cell containing the ligand solution only. In the case of 1, 2, and 4 mM K12S the conductometric titrations were additionally performed at 308.15 K and 318.15 K.

2.2.3. Molecular dynamics (MD) simulation protocol

All simulated systems were built using CHARMM-GUI server [28] and were performed using Gromacs 2020 [29] with Plumed 2.6 plugin [30] and applying CHARMM36m force field [31]. TIP3P model was used for water molecules. The simulations were carried out in the isothermal-isobaric (NPT) ensemble using periodic boundary conditions. Nose-Hoover algorithm [32] with a coupling constant of 1 ps was used to keep the temperature at 300.15 K, while the Parrinello-Rahman algorithm [33] with a coupling time of 5 ps was used to maintain the pressure of 1 bar. Electrostatic interactions were calculated with the Particle Mesh Ewald method [34] using a cut-off radius equal to 1.2 nm (with the switching function applied from the distance of 1 nm) and the Fourier grid spacing of 0.12 nm. Van der Waals interactions were calculated with Lennard-Jones potential with a cut-off radius of 1 nm. The Hydrogen Mass Repartitioning approach [35] was used, which enabled us to use the 4 fs time step of integration of the equations of motion. Minimization and equilibration of the systems were performed following the default CHARMM-GUI Membrane Builder protocol [36].

The initial micelle system was composed of 70 dodecyl sulfate anions placed in an $8.1 \times 8.1 \times 8.1$ nm rectangular box filled with 16,998 water molecules and 116 Na^+ and 46 Cl^- ions to provide 0.15 M ionic strength. Systems with mixed micelle compositions were prepared by exchanging the proper amount of dodecyl sulfate anions (7, 21 or 35) to 1-alkylsulfonate anions; for each system, 2.5 μs -long trajectories were obtained.

2.2.4. Free energy calculations

The free energy profiles for the sulfonates association to SDS micelle were determined with replica-exchange umbrella sampling method [37], using center-of-mass distance between SDS micelle and sodium sulfonate as the reaction coordinate. The initial configurations for US simulations were obtained with 100 ns-long steered-MD simulations, in which one SDS molecule was replaced by sodium sulfonate molecule and was pulled away from the SDS micelle with the harmonic constant of $500 \text{ kJ mol}^{-1} \text{ nm}^{-2}$.

9 equally spaced US windows were used, spanning a 1–5.0 nm range of the reaction coordinate. The spring constant of the harmonic biasing potential was set to $75 \text{ kJ mol}^{-1} \text{ nm}^{-2}$ in each US window. Each of the US windows were simulated for 1.5 μs and free energy profiles were determined using the weighted histogram analysis method [38]. The same procedure was used to calculate the referential free energy profile for SDS association to the micelle. Uncertainties were estimated using bootstrap error analysis taking into account the correlation in the analyzed time series.

3. Results and discussion

3.1. Critical micelle concentration

The CMC values of SDS in the presence of various concentrations of K8S, K10S, and K12S (and NaCl as a reference) were obtained – among others – from steady-state fluorescence spectroscopy measurements using pyrene, a well-known fluorescent

probe utilized for the micropolarity studies of its solubilization site in the micellar interior [39]. When pyrene is excited at approximately 335–340 nm, its fluorescence emission spectrum exhibits five typical vibronic peaks. The intensity ratio $\frac{F_{373}}{F_{384}}$ of the first (solvent-sensitive band at 373 nm) and the third (solvent-insensitive band at 384 nm) vibronic peaks [40] is very sensitive to solvent polarity and therefore has been widely used to measure the polarity of the microenvironment of the polycyclic aromatic hydrocarbon (high values indicate polar environment) and CMC of surfactants [41]. Thus, the ratio $\frac{F_{373}}{F_{384}}$ for pyrene was determined in the absence and presence of increasing amounts of SDS (at various concentrations of K8S, K10S, K12S, and NaCl). For all studied systems it decreased significantly with the increase of SDS concentration (Fig. 2). The inflection points of these curves indicate the CMC values of the micellization of SDS, which are presented in Table 1.

Furthermore, the CMC values of SDS were obtained for all studied systems from conductivity measurements. The plots of conductivity (κ) vs the concentration of SDS determined in the presence of different concentrations of K8S, K10S, K12S, and NaCl are shown in Fig. 3. It is known that the specific conductivity is linearly correlated to the surfactant concentration in both the premicellar and postmicellar regions, with the slope in the premicellar region greater than that in the postmicellar region [3], which is due to lower mobility of micelles when compared to monomers [26]. Indeed, the plots exhibit two straight lines (both increasing with the increase of the concentration of SDS in the solution) intersecting at the point indicating the formation of the micelle. The CMC values are gathered in Table 1.

The value of CMC of SDS in water (in the absence of any added electrolyte) is in a great agreement with the previous reports [25,42]. From the inspection of Fig. 2, Fig. 3, and Table 1 it can be clearly noticed that the CMC of SDS decreases very significantly with the increase of the concentration of the studied sodium 1-alkylsulfonates and sodium chloride. The lower values of CMC of SDS in the presence of K8S, K10S, K12S, and NaCl suggest its stronger tendency to self-assemble (SDS solutions containing sulfonates are more associated [43]) and form micelles under these conditions (micellization is favored in the occurrence of all studied salts in comparison to pure surfactant) [26,44]. In the case of sodium chloride the significant reduction of CMC values is related to the presence of additional ions from salt dissociation and their interactions with the SDS hydrophilic parts, monomers which are in the equilibrium with micelles or interactions with water molecules from the aqueous pseudophase [22,45]. The interactions of sodium cations with the negatively charged head of SDS micelles result in the reduction of electrostatic repulsions thereby increasing micelle stability and consequently, lowering the critical micelle concentration [22]. Such a trend has been observed previously for SDS/NaCl and SDS/KCl systems [22,25,46,47]. Furthermore, a comparison of CMC values of SDS determined in the presence of homologous series of the studied sodium sulfonates demonstrates that increasing the length of their hydrocarbon chain has the tendency of lowering the concentration of surfactant at which aggregation is initiated. However, NaCl exhibits very similar to K8S and K10S effect on the lowering the CMC of SDS – the values of critical micelle concentration in these two sulfonates are comparable and the co-ions seem not to have any other effect on the CMC of SDS than chloride ion. It means that the organic anions of K8S and K10S do not participate in the stabilization of micelles. It is in great accordance with previous reports concluding that there is indeed no co-ion effect on the micellization parameters of anionic surfactants by acetate, propionate and butyrate ions [25,48]. Interestingly, this is not the case for K12S – all studied solutions of that sodium 1-alkylsulfonate reduce CMC much stronger than K8S,

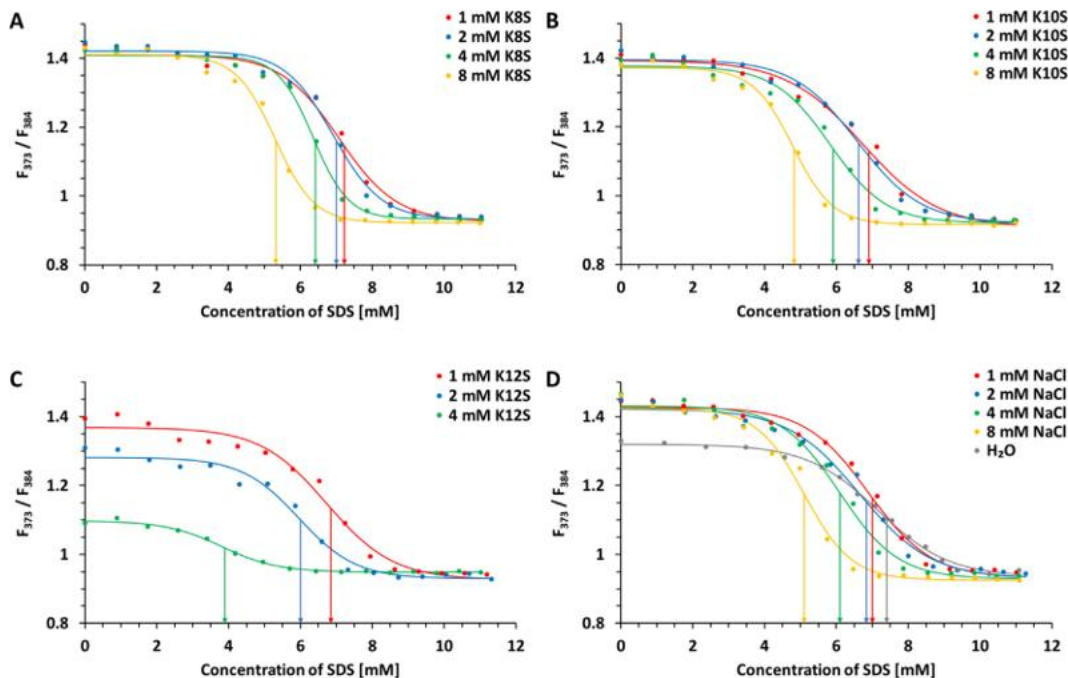


Fig. 2. Plots of fluorescence intensity ratio F_{373}/F_{384} for pyrene as a function of SDS concentration in the presence of various concentrations of K8S (A), K10S (B), K12S (C), and NaCl (D); $\lambda_{\text{ex}} = 340 \text{ nm}$; $c_{\text{pyrene}} = 2 \text{ }\mu\text{M}$; 298.15 K .

Table 1

Critical micelle concentration (CMC) obtained by both, conductometric titration (^C) and steady-state fluorescence spectroscopy (^F), degree of counter-ion binding (β), standard Gibbs's free energy change (ΔG_{mic}^0) and aggregation number (N_{agg}) values of the micellization of SDS in the presence of various concentrations of K8S, K10S, K12S, and NaCl.

System	CMC [mM]	β	$\Delta G_{\text{mic}}^0 [\text{kJ mol}^{-1}]$	N_{agg}	System	CMC [mM]	β	$\Delta G_{\text{mic}}^0 [\text{kJ mol}^{-1}]$	N_{agg}
1 mM K8S	7.8 ^C 7.2 ^F	0.622	−35.65	59	1 mM K12S	7.3 ^C 6.8 ^F	0.631	−36.12	66
2 mM K8S	7.4 ^C 7.0 ^F	0.626	−35.95	62	2 mM K12S	6.5 ^C 6.0 ^F	0.642	−36.83	68
4 mM K8S	6.7 ^C 6.4 ^F	0.626	−36.35	67	4 mM K12S	5.1 ^C 3.9 ^F	0.674	−38.55	76
8 mM K8S	5.8 ^C 5.3 ^F	0.628	−36.98	69	Water	8.1 ^C 7.4 ^F	0.643	−35.96	58
1 mM K10S	7.7 ^C 6.9 ^F	0.630	−35.88	64	1 mM NaCl	7.6 ^C 7.0 ^F	0.630	−35.93	60
2 mM K10S	7.3 ^C 6.6 ^F	0.631	−36.12	67	2 mM NaCl	7.5 ^C 6.8 ^F	0.622	−35.81	65
4 mM K10S	6.6 ^C 5.9 ^F	0.640	−36.72	68	4 mM NaCl	6.9 ^C 6.1 ^F	0.621	−36.12	68
8 mM K10S	5.5 ^C 4.8 ^F	0.657	−37.85	72	8 mM NaCl	6.0 ^C 5.1 ^F	0.622	−36.70	70

K10S, and NaCl at the same concentrations. Specifically, 2 mM K12S decreases the CMC similarly to K8S, K10S, and NaCl, but at concentrations twice higher, while 4 mM K12S exhibits significantly greater impact when compared to 8 mM K8S, 8 mM K10S, and 8 mM NaCl. Hence, it can be supposed that K12S participates in the formation of the SDS micelles on account of identical to SDS hydrophobic chain length.

3.2. Thermodynamics of micelle formation

The micellization behavior of surfactants can be influenced by structural/environmental modification which can be assessed by evaluating numerous thermodynamic parameters [26]. The standard Gibbs's free energy change of micellization (ΔG_{mic}^0) per mole of monomer of SDS was calculated for all the studied systems from conductivity measurements using the following equation:

$\Delta G_{\text{mic}}^0 = (1 + \beta)RT \ln X_{\text{CMC}}$, where X_{CMC} denotes CMC in mole fraction unit, R is the gas constant, T is the absolute temperature, and β ($=1 - \alpha$) is a degree of counter-ion binding (the values of the micelle ionization degree α were calculated as the ratio of the slopes of the two linear fragments of conductivity-concentration plot above and below CMC) [49]. The appropriate values of ΔG_{mic}^0 are listed in Table 1. It can be clearly observed that with increasing the alkyl chain length and concentration of the added sodium 1-alkylsulfonate the micellization process becomes more favorable (ΔG_{mic}^0 decreases). An opposite effect is observed for β – its values increase very slightly (the electrolyte anions are known to cause a much smaller change in the values of β for SDS than the cations [50]) with increasing the concentration and length of the sulfonates, which means that the binding of the counter-ions to the micelles increases [25]. The additional studies conducted at various temperatures for 1, 2, and 4 mM K12S (Fig. S1 in Electronic

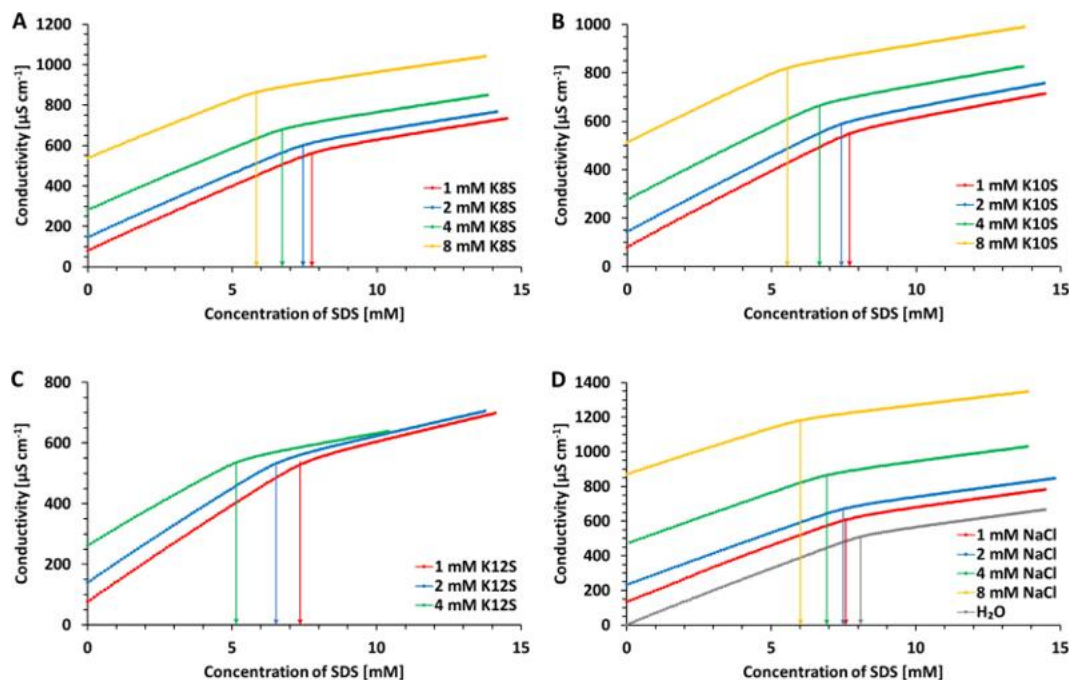


Fig. 3. Plots of conductivity (κ) as a function of SDS concentration in the presence of various amounts of K8S (A), K10S (B), K12S (C), and NaCl (D); 298.15 K.

Supplementary Information) enabled determination of the values of the standard enthalpy (ΔH_{mic}^0) and then the standard entropy (ΔS_{mic}^0) changes of micellization per mole of surfactant according to the following equations: $\Delta H_{\text{mic}}^0 = -(1 + \beta)RT^2 \frac{d \ln X_{\text{CMC}}}{dT}$ (where $\frac{d \ln X_{\text{CMC}}}{dT}$ corresponds to the slopes of the plots in Fig. S1) and $\Delta S_{\text{mic}}^0 = \frac{\Delta H_{\text{mic}}^0 - \Delta G_{\text{mic}}^0}{T}$ [49]. The standard enthalpy changes of micellization of SDS in 1, 2, and 4 mM K12S are -6.74 , -6.24 , and -4.68 kJ mol $^{-1}$, respectively. ΔH_{mic}^0 are rather small and negative indicating that micellization process is exothermic. The standard entropy changes of micellization of SDS are 99, 103, and 114 J mol $^{-1}$ K $^{-1}$ in 1, 2, and 4 mM K12S, respectively. ΔS_{mic}^0 is positive and increases with the increase of K12S concentration. The large entropy change values support the spontaneous micellization and hint that process is driven by an increase in entropy [42].

3.3. Aggregation number of SDS micelles

The mean aggregation numbers of SDS micelles were determined by fluorescence quenching method using pyrene as fluorescent probe and cetylpyridinium chloride (CPC) as quencher molecule. The most important advantage of this method is that the aggregation number can be determined also when the micelles interact in solution [51]. N_{agg} values of SDS in the presence of various concentrations of K8S, K10S, K12S, and NaCl were calculated from the slopes of the plots of $\ln(\frac{F_0}{F})$ versus $[Q]$ according to the equation: $\ln(\frac{F_0}{F}) = \frac{N_{\text{agg}}[Q]}{c - \text{CMC}}$, where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively, c is the total surfactant concentration (higher than CMC; here 10 mM), and $[Q]$ is quencher (CPC) concentration [52]. The highest CPC concentration (~ 20 μM) was much below its CMC (~ 100 μM), while the concentration of pyrene (2 μM) was much lower than the micelle concentration, what ensures quenching of the fluorescence of pyrene molecules that are solubilized in the micelles. This is indicated by the high values of the Stern–Volmer constants (K_{SV} from 11860 M $^{-1}$ to 41300 M $^{-1}$) obtained from the strictly linear (R^2 from 0.988 to 0.997) plots of Stern–Volmer equation,

$\frac{F_0}{F} = 1 + K_{\text{SV}}[Q]$ (Fig. 4). The plots of $\ln(\frac{F_0}{F})$ vs the concentration of CPC for 10 mM SDS are shown in Fig. 5. The N_{agg} values as obtained from the slopes of the straight lines are listed in Table 1. It can be observed that aggregation number of SDS increases in the presence of studied sodium 1-alkylsulfonates and sodium chloride, as is always observed for ionic surfactants [41]. Furthermore, the higher the length of the hydrocarbon chain of the present in the system sulfonates salts, the higher the average micellar aggregation number. This is an expected behavior because, in general, the value of the micelle aggregation number increases as the CMC value decreases [41,42].

3.4. Free energy of sulfonates association to SDS micelles

To investigate the possibility of sodium 1-alkylsulfonates participation in the SDS micelle formation, we firstly used umbrella sampling method to calculate the free energy profiles for sulfonates association to the SDS micelle. As can be seen on Fig. 6, our results clearly illustrate that elongating of the alkyl chain results in growing affinity of the sulfonate to the SDS micelle. Furthermore, the free energy profile for K12S matches exactly the referential profile for the SDS association to the micelle. As affinities to the micelle for K8S and K10S are lower than affinity for SDS self-association to the micelle, one can expect that in the presence of either K8S or K10S and SDS in solution, formed micelle will be composed of only SDS molecules. In the case of K12S however, the resemblance of the affinities for K12S to the micelle and SDS self-association hints that K12S molecules can form micelles along with the SDS molecules, which may explain the significant reduction of CMC observed in the experiments.

3.5. Stability of the mixed-composition micelles

In order to explore the stability of the mixed-composition micelles, we prepared systems with 10, 30 or 50% of dodecyl sulfate anions replaced by 1-alkylsulfonate anions, which were then simulated for 2.5 μs . During the simulations, the SDS core of the micelles remained intact, however, we observed events of sul-

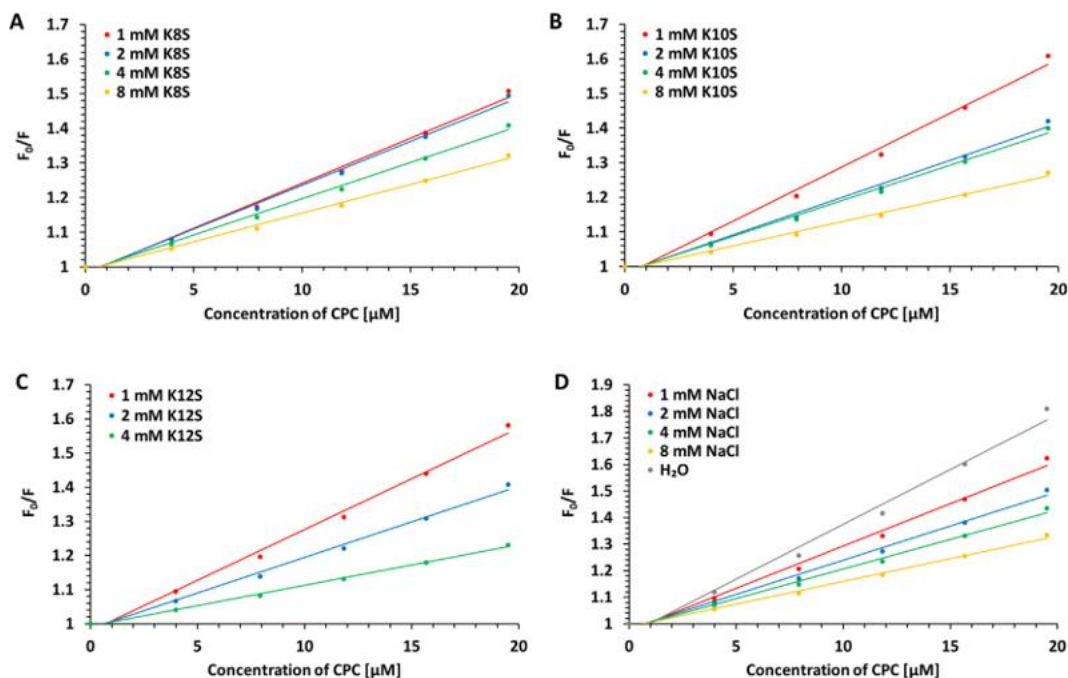


Fig. 4. Stern-Volmer plots for the quenching of pyrene as a function of CPC concentration (for 10 mM SDS; in the presence of various concentrations of K8S, K10S, K12S, and NaCl); $\lambda_{\text{ex}} = 340 \text{ nm}$; $\lambda_{\text{em}} = 375 \text{ nm}$; $c_{\text{pyrene}} = 2 \text{ } \mu\text{M}$; 298.15 K.

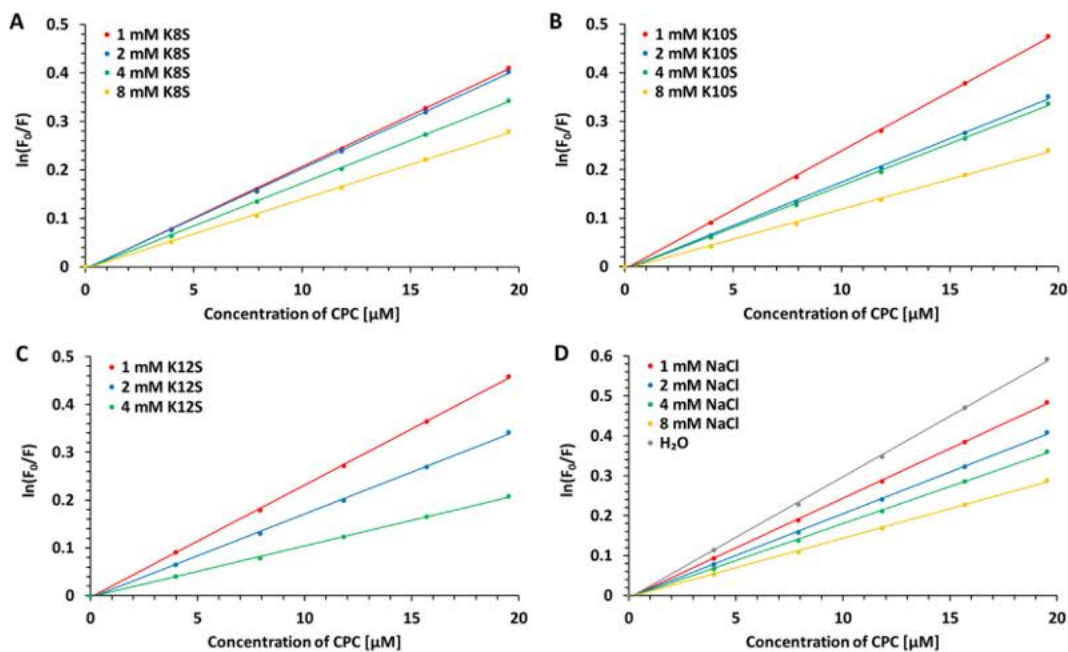


Fig. 5. Plots of $\ln(F_0/F)$ of pyrene as a function of CPC concentration (for 10 mM SDS; in the presence of various concentrations of K8S, K10S, K12S, and NaCl); $\lambda_{\text{ex}} = 340 \text{ nm}$; $\lambda_{\text{em}} = 375 \text{ nm}$; $c_{\text{pyrene}} = 2 \text{ } \mu\text{M}$; 298.15 K.

fonates dissociation from the micelle to the solvent. To compare this effect between simulated systems, we calculated the actual composition of micelles from the last microsecond of simulation (Table 2). The sulfonate molecules were treated as the part of micelle when their distance to the centre-of-mass of SDS molecules were smaller than 2.7 nm.

As, according to the calculated free energy profiles, K8S and K10S molecules are less preferred to participate in the micelle for-

mation than SDS, the K8S/SDS and K10S/SDS micelles can be treated as the referential states, stabilized mostly by the fact that the resultant number of SDS molecules in the systems is below their aggregation number. Although highly dynamic nature of the sulfonates exchange between the solvent and micelle, on average more K12S molecules participate in the micelle formation, in contrast to shorter sulfonates, what is particularly visible in the initial 50:50 investigated systems.

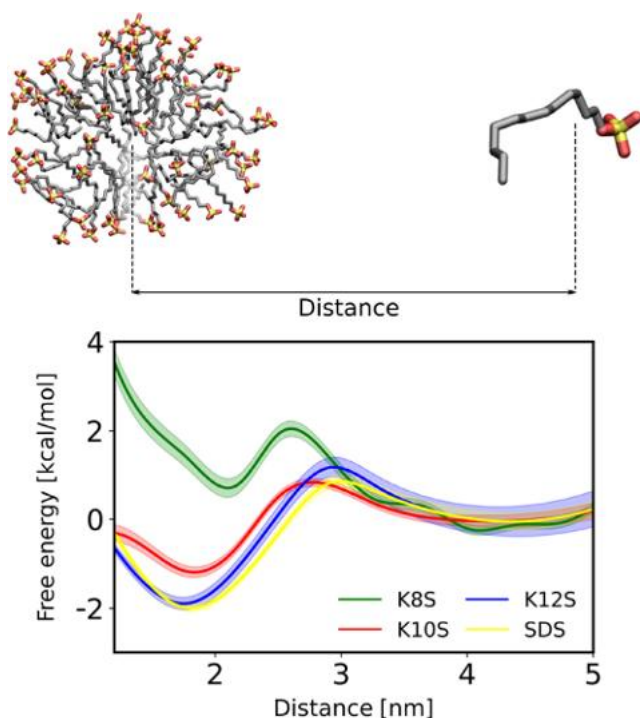


Fig. 6. (Top) Structural representation of the reaction coordinate used in the study. The image was created using VMD [53]. (Bottom) Free energy profiles for the sulfonates association to the SDS micelle.

Table 2

Average composition of the micelles during the last 1 μ s of simulations.

Initial composition of micelle (fraction of sulfonates [%])	Average fraction of sulfonates in the micelles [%]		
	K8S	K10S	K12S
10	8.1 (± 2.4)	3.7 (± 2.8)	8.7 (± 1.7)
30	17.3 (± 6.1)	19.4 (± 4.5)	21.0 (± 3.6)
50	28.1 (± 10.2)	22.7 (± 12.1)	34.4 (± 8.1)

4. Conclusions

The aggregation behavior of sodium dodecyl sulfate (SDS) surfactant was studied in aqueous solutions of selected 1-alkylsulfonates, namely sodium 1-octanesulfonate (K8S), sodium 1-decanesulfonate (K10S), and sodium 1-dodecanesulfonate (K12S). The present work establishes that co-ions like octanate and decanate (similarly to shorter acetate, propionate, and butyrate [25,48]) do not have any significant effect on the CMC and N_{agg} of SDS. In contrary, dodecanate ions show an influence on the micellization behavior of the surfactant. It seems that K12S molecules (of the same carbon chain length as SDS) may act as the surfactant's mimetics and be incorporated into its micelles. In other words, SDS does not recognize K12S molecules as the ones with a different structure and consequently allows them to participate in the formation of the surfactant's micelles. The presented results may have important implications to understand the nature of the interactions between surfactants and various electrolytes.

CRediT authorship contribution statement

Ola Grabowska: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review &

editing. **Krzysztof Żamojć:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Supervision. **Michał Olewniczak:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Lech Chmurzyński:** Project administration. **Dariusz Wyrzykowski:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Supervision.

Data availability

Data will be made available on request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This research was supported by PL-Grid Infrastructure and TASK computational center. We want to thank Dr. Łukasz Nierwicki for his scientific advice.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.molliq.2023.121568>.

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PUBLIKACJA 7

**Affinity and putative entrance mechanisms of alkyl sulfates
into the β -CD cavity**



Affinity and putative entrance mechanisms of alkyl sulfates into the β -CD cavity

Małgorzata M. Kogut¹, Ola Grabowska¹, Dariusz Wyrzykowski^{*}, Sergey A. Samsonov^{*}

Faculty of Chemistry, University of Gdańsk, Wita Stwosza 63, 80-308 Gdańsk, Poland

ARTICLE INFO

Article history:

Received 13 June 2022

Revised 26 July 2022

Accepted 28 July 2022

Available online 1 August 2022

ABSTRACT

Cyclodextrins (CDs) are novel pharmaceutical excipients and are widely used in the pharmaceutical industry and drug delivery due to their ability to form host–guest complexes. There are countless structural features of guest molecules responsible for the efficiency of guest–CD interactions. Among these factors, the hydrophobic moieties of a guest and their size are commonly considered to govern its binding affinity to CD molecules. Experimentally, it is often challenging to extract structural and dynamic information at the atomic scale about CD inclusion complexes, especially in the aqueous phase. Therefore, this study consisted of experimental (isothermal titration calorimetry and conductometric titration) and *in silico* analysis to rigorously characterize the interactions of β -CD with three alkyl sulfates of different hydrophobic chain lengths: sodium dodecyl sulfate (S12S), sodium decyl sulfate (S10S) and sodium octyl sulfate (S8S) in aqueous solutions. We find that the hydrophobic interactions are not always a key factor leading to the formation of stable inclusion complexes. Our results demonstrate that van der Waals interactions contribute more to complexation efficiency for sulfates with a longer hydrophobic tail, whereas electrostatic interactions are more pronounced for sulfates with shorter ones. Furthermore, we propose and discuss for the first time the putative mechanisms of the guest entering the host cavity conditioned by the lengths of hydrocarbon chains of alkyl sulfates. Finally, we prove that different types of inclusion complexes can exist in a conformational equilibrium depending on the size of a guest tail.

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1. Introduction

Cyclodextrins (CDs) are well-known excipients with applications ranging from life sciences to innovative technologies [1–10], and recently published reviews reflect their importance [11–17]. CDs belong to a class of cyclic oligosaccharides, the most common ones being α , β and γ -CDs formed by six, seven and eight α -(1–4) D-glucopyranoses units, respectively. The number of these units in the cyclic oligosaccharides determines the size of the cavity. The specific coupling regarding the glycosidic linkage type of these glucose monomers is responsible for a unique molecular structure of CDs with a hydrophilic exterior and hollow hydrophobic interior [3,18]. This distinctive structure of CDs is at the origin of their peculiar physicochemical properties, particularly their ability to form host–guest complexes. Due to the formation of these inclusion complexes, the guest molecules' biological, chemical, and physical properties can be altered. CDs have been used for pharmaceutical applications, mainly as solubilizers and stabilizers for hydrophobic

or biological drugs [19,20]. Therefore, investigations of the molecular recognition between CD (the host) and the rich diversity of guest species, namely low-molecular-weight compounds such as potential drugs, natural metabolites etc., are the subject of the ongoing studies. Of great interest are the structural factors governing the affinity of a guest to hosts.

Considering the specific structural features of CDs, the size and specific properties of the guests' hydrophobic and/or hydrophilic moiety play a crucial role in the stability of the resulting inclusion CD-complexes. Among guest molecules, surfactants are an important group of compounds for both a fundamental understanding of these systems and their practical applications [16]. In addition, they can be used as models for studying the relationship between the structure of a guest molecule and the stability of the resulting CD-complexes. To get better insights into the host–guest interactions numerous experimental and theoretical methods are employed. Much attention has been paid to the synthesis and structural characterization of CD complexes. Among these, there are 32, 112 and 6 inclusion complexes for α -CD, β -CD and γ -CD, respectively, which were identified by the X-ray diffraction technique (R-factor ≤ 0.075) [21]. According to the CCDC, the number of deposited β -CD-(guest) structures is much larger in comparison to the complexes with α -CD and γ -CD hosts due to the relatively low cost of the CD

^{*} Corresponding authors.

E-mail addresses: dariusz.wyrzykowski@ug.edu.pl (D. Wyrzykowski), sergey.samsonov@ug.edu.pl (S.A. Samsonov).

¹ Authors contributed equally.

production and the size of the β -CD cavity into which many guest fit. However, there are no reports on the crystal structures of CD-surfactant complexes. The examination of the nature and magnitude of the driving forces in a solution will provide useful information regarding the strategy of designing new, appropriate guests of desired properties. The characterization of CD inclusion complexes formation is not always a simple task and often requires different analytical techniques depending on the physical and chemical properties of the guest. The review by Mura portrays the most conventional analytical approaches for studying the CD-guest interactions in an aqueous solution, emphasizing their potential merits as well as disadvantages and limitations [22]. In the case of ionic surfactants as guest molecules, there are two main analytical tools to characterize the CD-surfactant interactions in solution [23]. The first one is conductometric titration successfully used to evaluate a binding constant and the critical micelles concentration (cmc) [24–26]. The second one is isothermal titration calorimetry (ITC) more accurate quantitative technique than electric conductivity. ITC allows the direct determination of stoichiometry, a binding constant and a binding enthalpy, and is considered a powerful tool.

Molecular dynamics (MD) simulations are becoming invaluable tools for studying biomolecular structure and dynamics [27,28]. They enable the determination of structural, energetic, and thermodynamical properties of molecular systems [29]. Since the early 2010 s, there has been a rapid increase in the number of works published concerning the substantial advantages of deploying MD simulations in the CD host-guest complexes, followed by post-MD calculations. Furthermore, the latest applications of umbrella sampling (US) proved that state-of-the-art methods of MD could be beneficial in studying and designing CD complexes. Mazurek et al. provided a comprehensive review of the current developments in the applications of MD simulations in the analysis of CDs [30]. Recently, we have demonstrated that MD simulations followed by binding free energy calculations are an effective way to predict, identify and rank the potential binding sites in protein-small molecule complexes [31,32].

In this paper, we report the results of calorimetric (ITC) and conductometric studies supported by *in silico* analysis on the interactions of β -CD with three anionic surfactants of different alkyl chain lengths, namely sodium dodecyl sulfate (S12S), sodium decyl sulfate (S10S) and sodium octyl sulfate (S8S) (Fig. 1.). The selected surfactants enabled to assess the effect of the length of the hydrophobic carbon chain of the guest on the stability of inclusion complexes and the binding mode of the guest molecules. The presented findings can broaden our knowledge of how the size of hydrophobic guest (drug) moiety influences the efficiency of complex formation, and thus the drug delivery potential of different lipophilicity as β -CD complexes.

2. Materials and methods

2.1. Materials

β -Cyclodextrin (β -CD, $\geq 97\%$, CAS: 7585-39-9), sodium dodecyl sulfate (S12S, CAS: 151-21-3, $\geq 99\%$), sodium decyl sulfate (S10S, CAS: 142-87-0, 98 %), sodium octyl sulfate (S8S, CAS: 142-31-4, $\geq 95\%$) were purchased from Sigma Aldrich (Poland) and used without further purification. Double-distilled water (purified by Hydrolab-system) with conductivity not exceeding $0.18\ \mu\text{S cm}^{-1}$ was used for preparations of aqueous solutions.

2.2. Conductometric measurements

Conductometric titration experiments were performed in water at $298.15 \pm 0.01\ \text{K}$, in a 30-mL thermostated cell, using the Cerko

Lab System microtitration unit fitted with the 5-mL Hamilton's syringe and the conductometric sensor (CD-201, Hydromet, Poland). The sensor was calibrated with conductivity standards (aqueous KCl solutions) of conductivity $84\ \mu\text{S/cm}$ and $200\ \mu\text{S/cm}$, purchased from Hamilton Company. The titrant (4 mM β -Cyclodextrin solution) was added to the titrand (0.2 mM surfactant solution: S12S, S10S or S8S) in increments of 0.02 mL and a pose 60 sec. Each titration was repeated a least twice in order to check the reproducibility of the data.

2.3. Isothermal titration calorimetry

All ITC experiments were performed at 298.15 K using the Auto-ITC isothermal titration calorimeter (MicroCal Inc. GE Healthcare, Northampton, USA). The details of the measuring devices and experimental setup were described previously [33]. The reagents were dissolved directly into H_2O . The experiment consisted of injecting $10.02\ \mu\text{L}$ (29 injections, $2\ \mu\text{L}$ for the first injection only, injection duration: 20 sec., injection interval: 240 sec.) of 4 mM β -cyclodextrin solution into the reaction cell which initially contained 0.2 mM surfactant solution (S12S, S10S or S8S). For each experiment, a blank was performed by injecting the titrant solution (β -CD) into the cell filled with water only. This blank was subtracted from the corresponding titration to account for the heat of a dilution.

2.4. Structures

The structures of β -CD-host with S12S, S10S and S8S guests were built in Xleap module of AMBER16 software package [34]. The structure of S12S was used from our previous work [31] and based on it S10S and S8S structures were prepared by removing $-\text{C}_2\text{H}_5$ and $-\text{C}_4\text{H}_9$ from S12S, respectively. The initial complexes were built by placing a guest molecule close but in a random orientation to the host molecule.

2.5. Electrostatic potential calculations

To calculate the electrostatic potential isosurfaces of β -CD, a Poisson Boltzmann surface area (PBSA) program from AmberTools [34] was used with a $1\ \text{\AA}$ grid spacing step. The obtained electrostatic potential maps were visualized with VMD software [35].

2.6. Molecular dynamics simulations

All-atom Molecular Dynamics (MD) simulation for each host-guest system was performed with the use of AMBER16 software package [34]. Truncated octahedron TIP3P periodic box of $10\ \text{\AA}$ water layer from box's border to solute was used to solvate complexes. Charge was neutralized with Na^+ counterions. GLYCAM06 force field for the β -CD-host [36] and gaff force field parameters [37] with RESP charges [38] obtained in antechamber module of AMBER16 [34] for the guests S8S, S10S and S12S were used, respectively. Energy minimization was carried out in two steps: beginning with 500 steepest descent cycles and 10^3 conjugate gradient cycles with $100\ \text{kcal/mol/\AA}^2$ harmonic force restraints, continued with 3×10^3 steepest descent cycles and 3×10^3 conjugate gradient cycles without any restraints. Following minimization steps, the system was heated up from 0 to 300 K for 10 ps with harmonic force restraints of $100\ \text{kcal/mol/\AA}^2$. Subsequently, the system was equilibrated at 300 K and $10^5\ \text{Pa}$ in the isothermal isobaric ensemble for 500 ps. Afterwards, the productive MD run was carried out in the same isothermal isobaric ensemble for 1 μs . The particle mesh Ewald method for treating electrostatics and SHAKE algorithm for all the covalent bonds containing hydrogen atoms were implemented in the MD simulations.

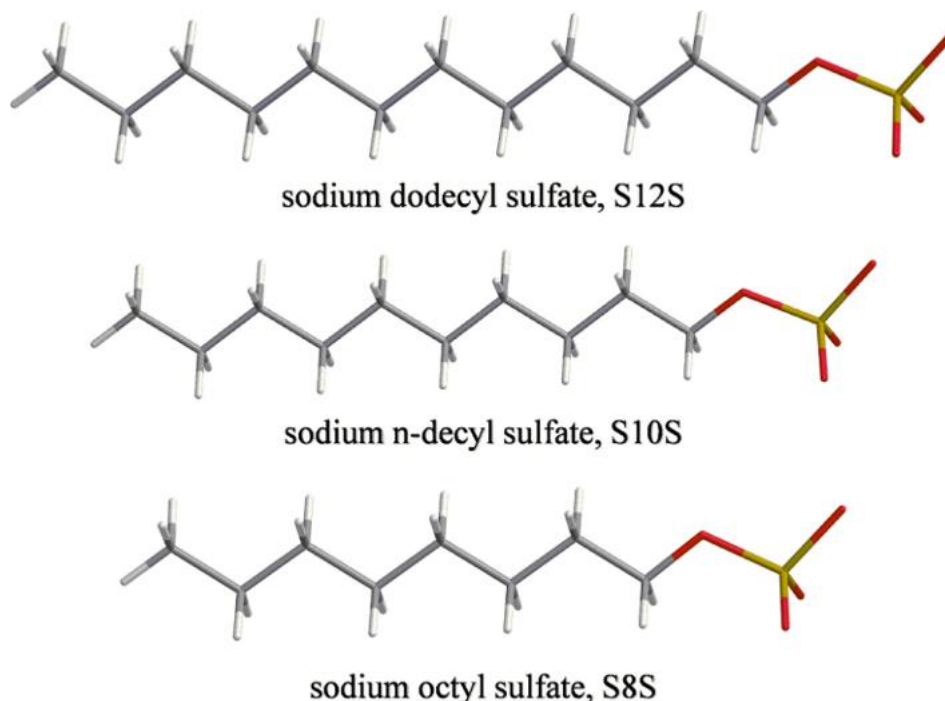


Fig. 1. Chemical structure of the investigated guest anions.

2.7. Binding free energy calculations

Energetic postprocessing of the trajectories and per-residue energy decomposition were performed for all systems with the use of Linear Interaction Energy (LIE) with a dielectric constant of 80 and Molecular Mechanics Generalized Born Surface Area (MM-GBSA) [39] using a model with surface area and Born radii default parameters as implemented in the $igb = 2$ model in AMBER16 [34]. MD frames corresponding to the complex in which structure reached convergence in terms of the RMSD were analysed.

2.8. Umbrella sampling and the potential of mean force

2.8.1. Preparation of US simulations

The initial structure of each complex for the US simulations was obtained from the MD trajectory frame, in which the lowest RMSD value to the average one of the guests throughout the simulation was observed. The distance between the C-end (carbon that is furthest from the S-atom, see Fig. 1) and the centre of mass of β -CD was measured and defined as a reaction coordinate. By using this bound conformation as a starting window, we gradually moved the guest along the positive (C-pull) and negative (S-pull) Y-axis with a 1.0 Å step for 50 Å in both directions, until the guest was unbound (was beyond the cut-off distance for non-bonded interactions). This way we defined the paths C-pull and S-pull for guest dissociation along the primary and secondary cavity of β -CD (Fig. 1).

2.8.2. Running US simulations

US was performed to compute the potential of mean force (PMF) along the dissociation pathway in AMBER 16 [40]. In US, a series of windows was evenly located along the reaction coordinate, and conformational sampling in these windows was achieved by enforcing an external biasing potential for 10 ns in each window. The sampling in each window overlapped with the ones in adjacent windows so that the unbiased PMF could be reproduced

by removing the biasing potential. The size of the periodic box was essentially increased in comparison to the previously described simulations to avoid potential artifacts in PMF calculations that could be caused by insufficient periodic box size in the course of the guest movement. Once all the US MD simulations were completed, the data collected from different simulation windows were combined along the reaction coordinate to calculate the PMF profile for the whole structural transition process with the Weighted Histogram Analysis Method (WHAM) [41] using the code developed by Alan Grossfield [42]. For each host–guest complex two US simulations were performed, along C-pull and S-pull pathways, making, in total, six US MD simulations. In WHAM, the tolerance of iteration was set to 0.001, and the temperature to 300 K. Finally, LIE was calculated for each window for each system for the energetically most favourable path.

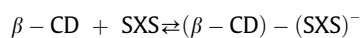
2.9. Data analysis and visualisation

Data analysis and its visualization were performed with the use of the R-package [43] and VMD [35].

3. Results and discussion

3.1. Equilibrium constants of complex formation – Conductometric studies

Equilibrium constants of the investigated (β -CD) – (SXS, X = 12, 10 and 8) complexes formation were calculated based on conductometric titration data. On the assumption a stoichiometry of the complexes under study equals 1:1 (host:guest), the binding constant K is expressed by the following equation:



$$K = \frac{[(\beta - \text{CD} - \text{SXS})^-]}{[\beta - \text{CD}][\text{SXS}^-]}$$

where $[(\beta\text{-CD})-(\text{SXS})]^-$, $[\beta\text{-CD}]$, $[\text{SXS}]^-$ are the concentrations of the complex, host and guest molecules in a solution in the equilibrium state, respectively. The binding constants K were refined from the equation [44]:

$$\Lambda_{\text{obs}} = [K \cdot (c_{\text{SXS}} - c_{\text{CD}}) - 1 + \sqrt{K^2 (c_{\beta} - c_{\text{SXS}})^2 + 2K(c_{\text{SXS}} + c_{\text{CD}}) + 1}] \left[\frac{\Lambda_f - \Lambda_c}{2K \cdot c_{\text{SXS}}} \right] + \Lambda_c$$

(where Λ_{obs} is the observed molar conductance of the titrated solution, Λ_f is the molar conductance of SXS in a pure solvent, Λ_c is the molar conductance of the $(\beta\text{-CD})-(\text{SXS})$ complex, c_{CD} and c_{SXS} are the total concentration of the host and the guest, respectively) by least-squares calculations from conductometric data using the Origin 8.5 program (ver. 8.5). The adjustable parameters, namely K and Λ_c were collected in Table 1. The free energy of binding (ΔG) was calculated using the standard thermodynamic relationship: $\Delta G = -RT \ln K$ (R is the gas constant, and T , in degrees Kelvin, is the absolute temperature at which the interaction takes place). The changes of molar conductance (Λ_{obs}) vs the $\beta\text{-CD}$ to SXS molar ratio ($[\beta\text{-CD}]/[\text{SXS}]$) together with the theoretical curves calculated based on the parameters (K and Λ_c) collected in Table 1 are presented in Fig. 2. The molar conductance decreases slightly with the increase of $\beta\text{-CD}$ in the reaction mixture on account of the formation of the $\beta\text{-CD}-(\text{SXS})$ complexes which are less effective as a charge carrier than the free (uncomplexed) SXS anions.

The conductometric data revealed that the thermodynamic stability of the resulting $\beta\text{-CD}-(\text{SXS})$ complexes increases with the increase of the hydrophobic chain length of the SXS surfactant (Table 1):

$$\log K_{\text{S12S}} > \log K_{\text{S10S}} > \log K_{\text{S8S}}$$

$$|\Delta G_{\text{S12S}}| > |\Delta G_{\text{S10S}}| > |\Delta G_{\text{S8S}}|$$

A similar phenomenon has already been observed for $\beta\text{-CD}$ complexes with cationic single chain surfactants and bolaforms (the surfactants with two water-soluble heads connected by a hydrophobic spacer) [23]. The changes in the binding parameter ν defined as the moles of bound SXS by each mole of $\beta\text{-CD}$ ($\nu = \frac{[(\beta\text{-CD})-(\text{SXS})]}{[\beta\text{-CD}] + [(\beta\text{-CD})-(\text{SXS})]}$) as a function of the free SXS ($[\text{SXS}]$) were compared for the investigated $\beta\text{-CD}/\text{SXS}$ systems. Graphic representations of the simulated data for the $\beta\text{-CD}/(\text{SXS})$ binding equilibrium with the experimentally determined binding constants (Table 1) are presented in Fig. 3.

The binding constant (K) and calculated indirectly the free energy of binding ($\Delta G = f(K)$) are well-known parameters describing the affinity of a macromolecule to a ligand. However, these parameters provide only general information about the stability of the complex. The complex formation is accompanied by a variety of processes, such as conformational changes of the reactants, breaking and formation of hydrogen bonds, electrostatic and hydrophobic interactions as well as fluctuations in the reaction environment. For these reasons, the direct determination of binding parameters (ΔH , and indirectly ΔS) becomes necessary for describing the energetic aspects of the interactions as well as the nature and magnitude of the forces responsible for the mutual affinity of the host to the guest.

3.2. Thermodynamic parameters of the interactions – Calorimetric studies

Binding parameters K and ΔG can be determined with a variety of methods [22]. However, the key to quantifying the binding thermodynamics is measuring the binding enthalpy (ΔH). Among the diverse analytical methodologies, isothermal titration calorimetry (ITC) is considered one of the most useful techniques enabling direct

Table 1

Logarithms of the equilibrium constants ($\log K$), the molar conductance (Λ_c) of the complex and free energies of binding (ΔG) of the $\beta\text{-CD}$ complexes with sodium dodecyl sulfate (S12S), sodium decyl sulfate (S10S) and sodium octyl sulfate (S8S) in aqueous solutions, at 298.15 K (standard deviation values in parentheses).

Parameter	S12S	S10S	S8S
$\log K$	4.11 (± 0.03)	3.69 (± 0.05)	3.38 (± 0.02)
Λ_c [$\text{S cm}^2 \text{mol}^{-1}$]	65.7 (± 0.2)	67.9 (± 0.6)	77.6 (± 0.2)
ΔG [kcal mol^{-1}]	-5.61 (± 0.05)	-5.04 (± 0.07)	-4.61 (± 0.03)

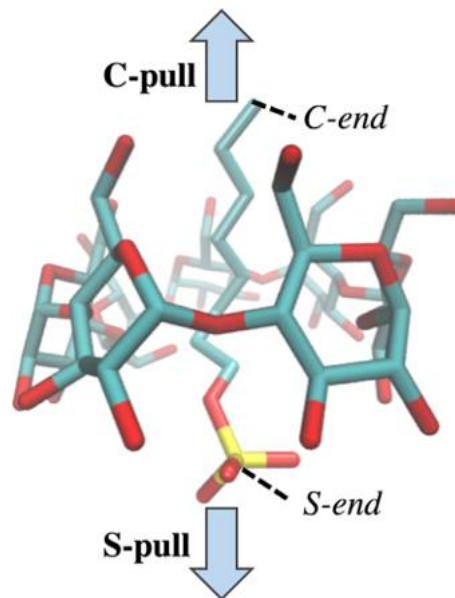


Fig. 2. Dissociation pathways of S8S from the $\beta\text{-CD}$ complex. The C-pull pathway is when the C-end of the guest is pulled and the S-pull pathway is when the S-end is pulled from the $\beta\text{-CD}$. For clarity, hydrogens are not shown.

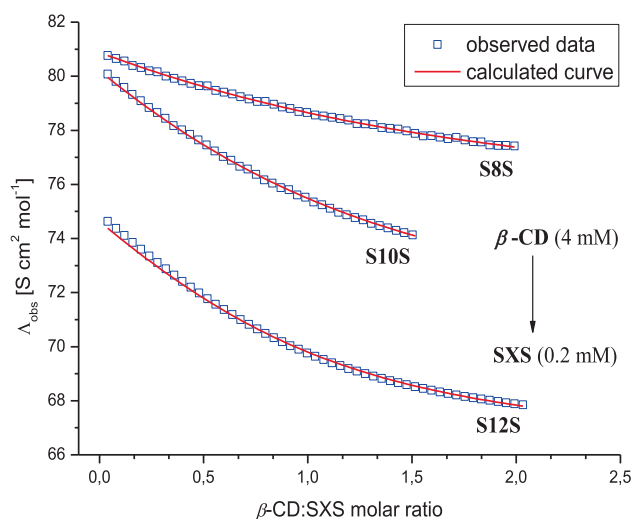


Fig. 3. Molar conductance (experimental data – blue squares) and (theoretical data – solid red lines) vs molar ratio $[\beta\text{-CD}]/[\text{SXS}]$ in an aqueous solution at 298.15 K.

calculation of ΔH_{ITC} based on the measurement of the heat released or absorbed upon a complex formation [45]. The knowledge of thermodynamic parameters of the reactions provides a complete thermodynamic characterization of the interactions under study, and thus enables a better understanding of the investigated processes than that of simple binding constants. Furthermore, the dependence of thermodynamic properties on structural detail is essential to comprehend how the interactions occur [46,47].

Representative binding isotherms for β -CD – SXS interactions in an aqueous solution are shown in Fig. 4, whereas thermodynamic parameters of the complex formation are summarized in Table 2. The stoichiometry of the inclusion complexes, the binding constant K_{ITC} and the binding enthalpy ΔH_{ITC} were obtained directly from the ITC experiments by fitting binding isotherms to the experimental data, using the non-linear fitting least-squares procedure and the equilibrium model that assumes a single set of identical binding sites. The free energy of binding ΔG_{ITC} and the entropy ΔS_{ITC} of the interaction were calculated using the relationships: $\Delta G_{ITC} = -RT \ln K_{ITC} = \Delta H_{ITC} - T \cdot \Delta S_{ITC}$.

The stoichiometry (N) of β -CD:SXS complexes determined directly from ITC data (Table 2) justifies the assumption taken during the analysis of conductometric data about the formation of 1:1 (the host to the guest) complexes. Furthermore, the binding constant K of the resulting β -CD complexes and thus the free energy of binding ΔG obtained based on conductometric and calorimetric data are equal, within the range of the experimental errors (Tables 1 and 2). The formation of the complexes is accompanied by the release of the heat. The binding enthalpy adopts slightly more negative values with the increase of the length of the hydrophobic tail of the guest. Simultaneously, the increase in the entropy change is observed. Among

Table 2

The thermodynamic parameters of sodium dodecyl sulfate (S12S), sodium decyl sulfate (S10S) and sodium octyl sulfate (S8S) binding to β -CD (standard deviation values in parentheses) in aqueous solutions, at 298.15 K.

ITC	S12S	S10S	S8S
N (the stoichiometry)	1.10 (± 0.03)	1.17 (± 0.07)	0.84 (± 0.06)
$\log K_{ITC}$	4.12 (± 0.03)	3.61 (± 0.04)	3.23 (± 0.03)
ΔG_{ITC} [kcal mol ⁻¹]	-5.62 (± 0.04)	-4.92 (± 0.05)	-4.41 (± 0.03)
ΔH_{ITC} [kcal mol ⁻¹]	-2.30 (± 0.07)	-2.13 (± 0.17)	-1.76 (± 0.16)
$T\Delta S_{ITC}$ [kcal mol ⁻¹]	3.32 (± 0.08)	2.79 (± 0.17)	2.65 (± 0.04)

different types of non-covalent forces, electrostatic and van der Waals interactions, as well as hydrophobic interactions, are involved in the interactions of β -CD with SXS. The former are enthalpy driven whereas the latter are entropy-driven. The inner volume of the β -CD cavity (270 Å³) fits best to accommodate the S12S chain with the methylene (–CH₂–) volume equal to 27 Å³. Consequently, due to the established essential contacts of the S12S tail with the CD cavity, van der Waals interactions seem to contribute significantly to the exothermic effect of the complex formation. Furthermore, a more rigid structure is expected for the shortest tail guest (S8S) on account of electrostatic interactions of a negatively charged head group (–OSO₃⁻) with primary or the secondary hydroxyl groups located on rims. It manifests itself in the decrease of translation and conformation freedom of SXS upon complexation that is reflected in less positive values of the entropic factors ($T\Delta S$) (Table 2). To get some insight into the factors underlying the stability of the investigated inclusion complexes and for a better understanding of the

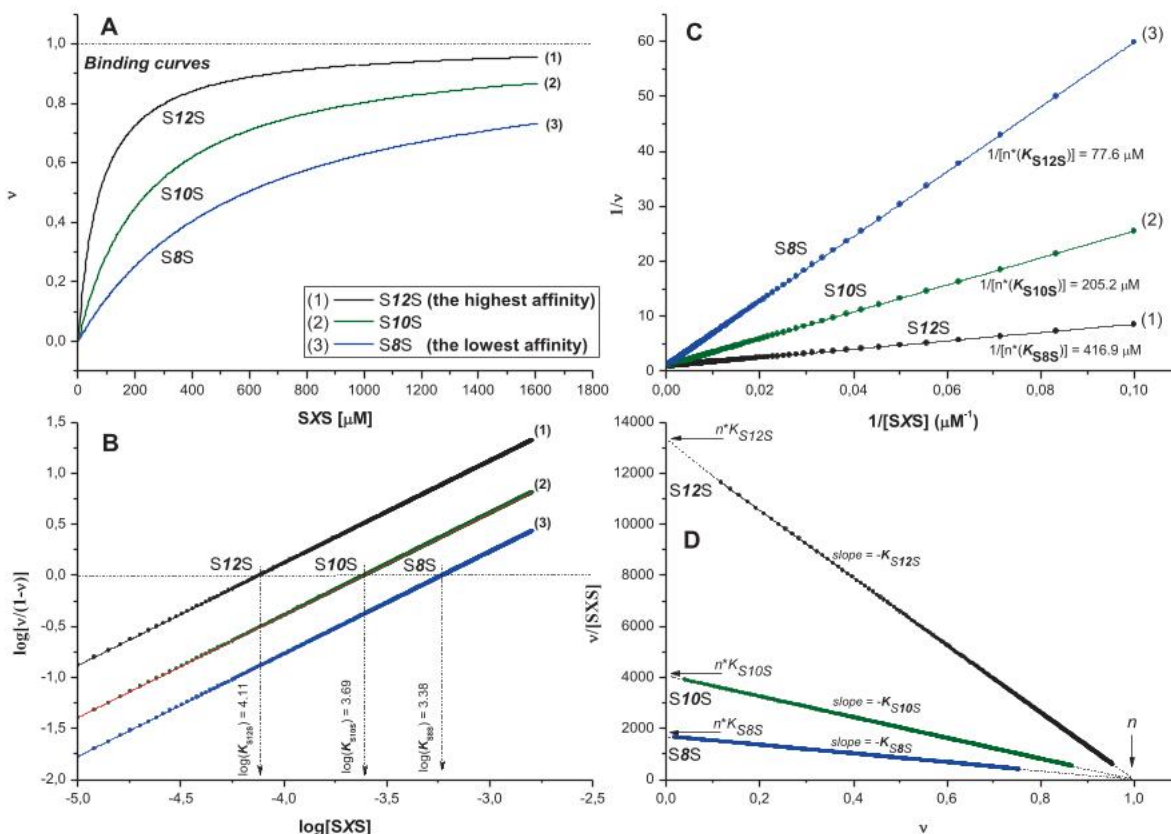


Fig. 4. Binding plots of the investigated systems: (1) β -CD/(S12S), (2) β -CD/(S10S) and β -CD/(S8S) simulated based on binding constants K_{SXS} (Table 1). (A) Binding curves: $v = \frac{K_{SXS}[SXS]}{1 + K_{SXS}[SXS]}$; (B) Double reciprocal representation: $\frac{1}{v} = 1 + \frac{1}{K_{SXS}[SXS]}$; (C) Hill representation: $\log\left(\frac{v}{1-v}\right) = \log(K_{SXS}[SXS])$; (D) Scatchard representation: $\frac{v}{[SXS]} = K_{SXS} - K_{SXS}v$.

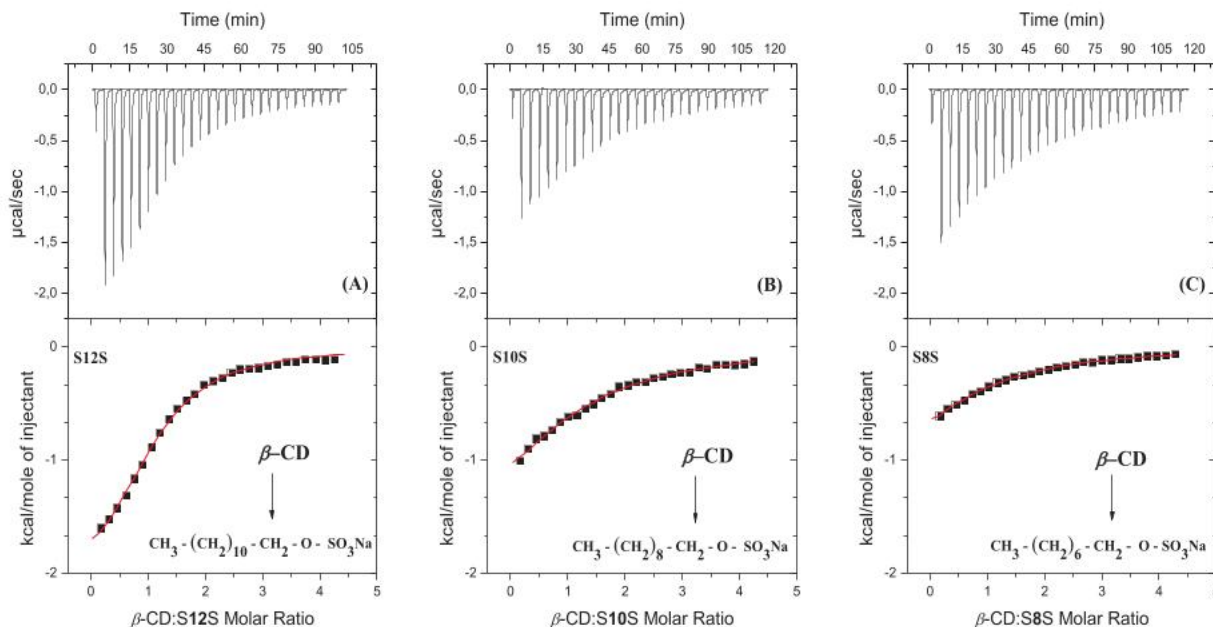


Fig. 5. Calorimetric titration isotherms of the binding interactions between β -CD and (A) sodium dodecyl sulfate (S12S), (B) sodium decyl sulfate (S10S) and (C) sodium octyl sulfate (S8S) in aqueous solutions, at 298.15 K.

mechanisms responsible for β -CD/SXS interactions computational methods have been employed.

3.3. Assessment of the electrostatic potential of β -CD cavities

The PBSA program from the AmberTools package was used to calculate the electrostatic potential isosurfaces corresponding to the primary and secondary cavity of β -CD. The obtained electrostatic potential maps are depicted in Fig. 5. The results revealed substantial differences between the analyzed cavities. It is clearly

visible that the potential binding site for the negatively charged $-\text{OSO}_3$ group is extended over a greater area in the case of the secondary cavity.

3.4. Putative entrance mechanisms - molecular dynamics (MD) simulations

Three 1 μs MD simulations were carried out to examine how various lengths of hydrocarbon chains of the guests might influence a host-guest complex formation. Mazurek et al. reported that

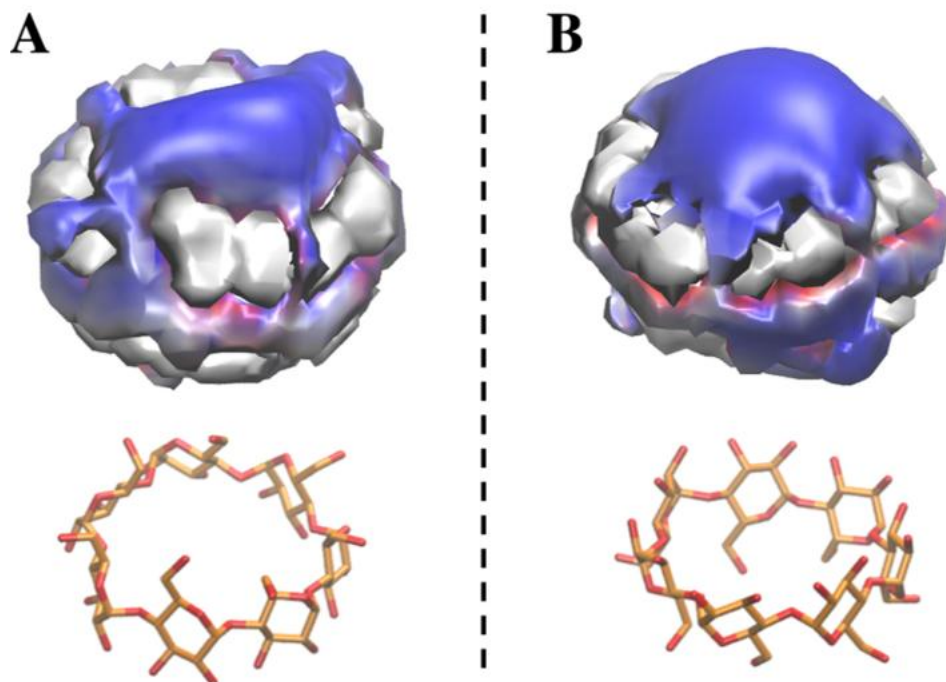


Fig. 6. Electrostatic potential isosurfaces for the primary (A) and secondary (B) cavities of β -CD (blue, 4 kcal mol⁻¹ e⁻¹).

Table 3

For each system five independent simulations were performed to inspect for the preferred mechanism of the entrance. The table summarizes whether the guest entered the host at any point of the simulation.

Guest	Mechanism of entrance							
	C1		C2		S1		S2	
S12S	1	No	1	No	1	Yes	1	No
	2	No	2	Yes	2	Yes	2	No
	3	No	3	Yes	3	Yes	3	No
	4	Yes	4	Yes	4	Yes	4	No
	5	No	5	Yes	5	Yes	5	No
S10S	1	Yes	1	No	1	Yes	1	No
	2	Yes	2	Yes	2	No	2	No
	3	Yes	3	Yes	3	No	3	No
	4	Yes	4	Yes	4	No	4	No
	5	Yes	5	Yes	5	No	5	No
S8S	1	No	1	Yes	1	Yes	1	No
	2	Yes	2	Yes	2	Yes	2	No
	3	Yes	3	Yes	3	Yes	3	No
	4	Yes	4	Yes	4	Yes	4	No
	5	Yes	5	Yes	5	Yes	5	No

Table 4

MMGBSA and LIE free energy decomposition values for dodecyl sulfate (S12S), decyl sulfate (S10S) and octyl sulfate (S8S) guests in the guest–host complexes (ΔG_{ele} = electrostatic component in vacuum, ΔG_{vdw} = van der Waals component, ΔG_{GB} = Generalized Born reaction field component, ΔG_{SURF} = non-polar solvation component, ΔG_{total} = total free energy of binding).

Guest	LIE [kcal/mol]			MMGBSA [kcal/mol]				
	ΔG_{ele}	ΔG_{vdw}	ΔG_{total}	ΔG_{ele}	ΔG_{vdw}	ΔG_{GB}	ΔG_{SURF}	ΔG_{total}
S12S	−0.2	−23.8	−23.9 ± 2.7	−12.5	−23.8	26.5	−3.5	−13.2 ± 2.3
S10S	−0.2	−22.4	−22.6 ± 2.7	−15.3	−22.4	28.9	−3.3	−12.1 ± 2.4
S8S	−0.2	−20.6	−20.8 ± 2.7	−16.7	−20.6	29.8	−3.1	−10.6 ± 2.3

Table 5

Free energy binding ($\Delta G_{\text{binding}}$) for dodecyl sulfate (S12S), decyl sulfate (S10S) and octyl sulfate (S8S) guests obtained from C-pull and S-pull dissociation pathways for Complex 1 and Complex 2.

Guest	$\Delta G_{\text{binding}}$ [kcal/mol]			
	Complex 1		Complex 2	
	C-pull path	S-pull path	C-pull path	S-pull path
S12S	−3.4	−1.3	−3.3	−2.0
S10S	−2.7	−1.8	−1.4	−2.2
S8S	−1.3	−1.5	−1.4	−3.4

there had not been any published attempt to simulate such systems other than through the molecular docking procedure [30]. In our work, we relied exclusively on the MD findings without performing a molecular docking procedure as a first step. Initially in each simulation, we placed the guest in proximity to β -CD. Subsequently, we performed a visual inspection of the generated trajectories. Although the guests successfully entered the cavity of the host, it did not happen in the same manner. We observed that S12S accessed the host through the primary cavity entering via S-end first, whereas S10S and S8S entered through the secondary cavity with the C-end first. Since the initial positions of guests were random, we decided to check whether any mechanism of how the guest enters the host would be preferable. We analyzed four ways of how it could happen: S-end first via the primary cavity (S1), C-end first via the secondary cavity (C2), C-end first via the primary cavity (C1) and S-end first via the secondary cavity (S2). In each

case, a guest was placed in proximity to either a primary or secondary cavity pointing its either C- or S-end at the center of β -CD (as shown in Fig. 3) and five short independent MD simulations were carried out. Subsequently, different entrance mode leads to different inclusion complex formation, if S1 or C2 were preferred Complex 1 was formed if C1 or S2 then Complex 2 (Fig. 6). Based on our findings that are summarized in Table 3 we see that:

- the mechanism S2 was never observed;
- for S12S, C2 and S1 were preferred, and Complex 2 was formed only once;
- for S10S, C1 and C2 were observed;
- S8S was the most flexible in terms of entering β -CD because C1, C2 and S1 entrance mechanisms were detected. To sum up, Complex 1 forms more preferentially.

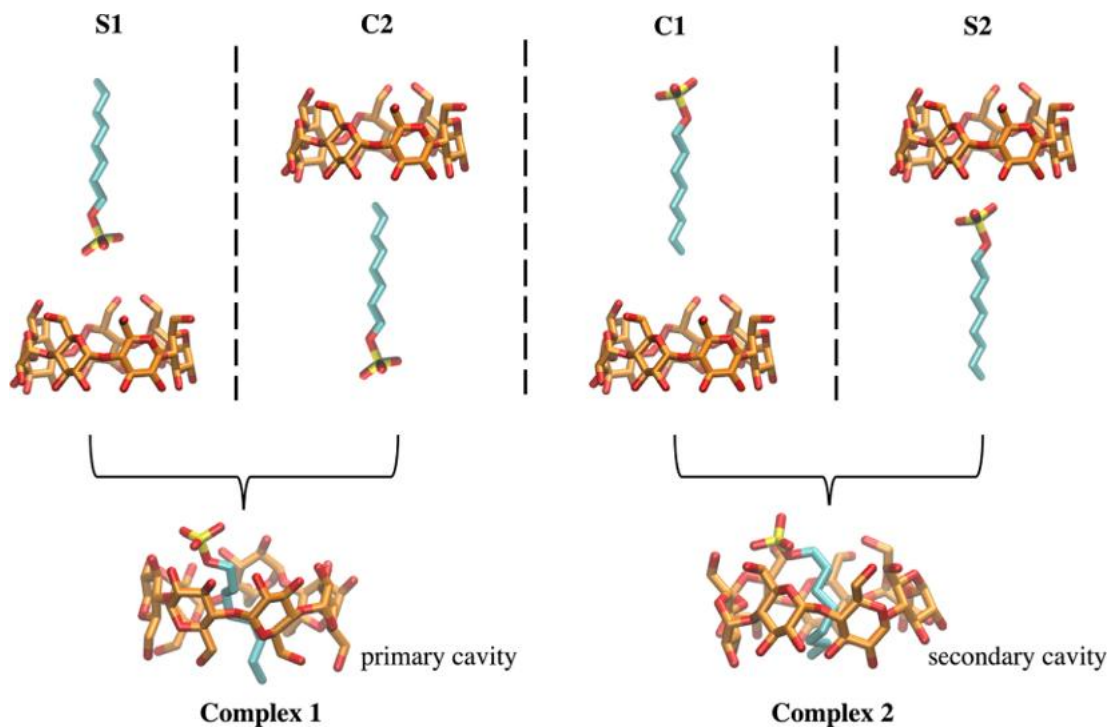


Fig. 7. Four potential mechanisms for how the guest could enter β -CD: S1 = S-end first via primary cavity, C2 = C-end first via secondary cavity, C1 = C-end first via primary cavity and S2 = S-end first via secondary cavity. For the clarity the colour of the guest molecule in Complex 1 and Complex 2 was changed in comparison to the unbound guest, and hydrogen atoms were omitted.

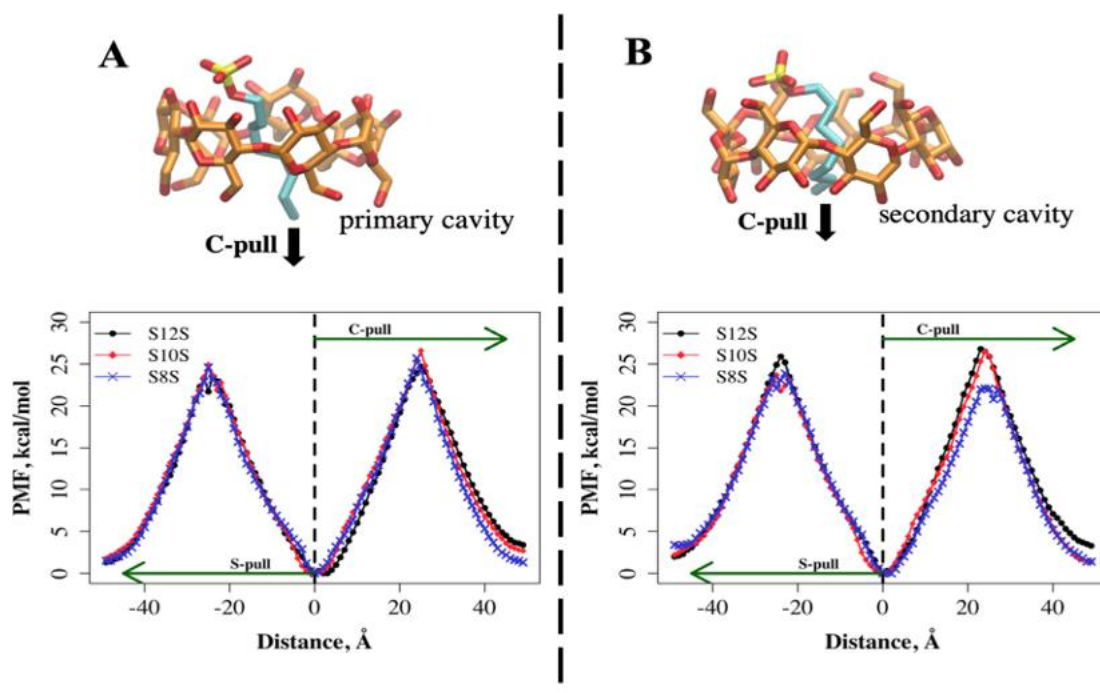


Fig. 8. PMF of dodecyl sulfate (S12S, black), decyl sulfate (S10S, red) and octyl sulfate (S8S, blue) dissociation from β -CD for Complex 1 (A) and Complex 2. Pathways C-pull and S-pull are combined on the same plot and the direction of each is marked by a green arrow.

3.5. Electrostatic vs Van der Waals interactions - binding free energy analysis

To estimate the free energy of binding in the β -CD-guest inclusion complexes, we used two popular endpoints approaches: LIE and MMGBSA (Table 4). Based on LIE results, we did not observe the change in the electrostatic component of the binding, which is calculated with the use of the isotropic dielectric constant. On the other hand, the MMGBSA technique showed that the shorter the hydrophobic chain, the stronger the electrostatic interactions in the host-guest complex (both *in vacuo* and with the consideration of GB implicit solvent model). At the same time, both methods revealed the same change in the van der Waals component, suggesting that the longer guests establish more contacts with the host. Based on both free energy calculation approaches, the total free energy of binding (ΔG_{total}) is the most favourable for S12S and least favourable for S8S, which agrees with the experimental

findings. Since we suggest that Complex 1 is more probable than Complex 2 this analysis was performed only for Complex 1.

3.6. Guest dissociation pathway analysis by umbrella sampling

To gather more detailed information about the binding interactions we conducted umbrella sampling simulations (US). The histograms used for WHAM analysis are presented in Supplementary Material Figs. S1 and S2. Deploying this method to build free energy profiles has already proved to be very effective in the case of β -CD and other small molecules [48–50]. As illustrated in Fig. 1, the S12S, S10S, and S8S ligands could potentially dissociate from the primary cavity (path C-pull) or the second cavity of β -CD in the case of Complex 1. Therefore, we constructed the PMF for both dissociation paths. According to the electrostatic potential analysis, the primary and secondary cavities of β -CD are not symmetric, and since the guest molecules are neither

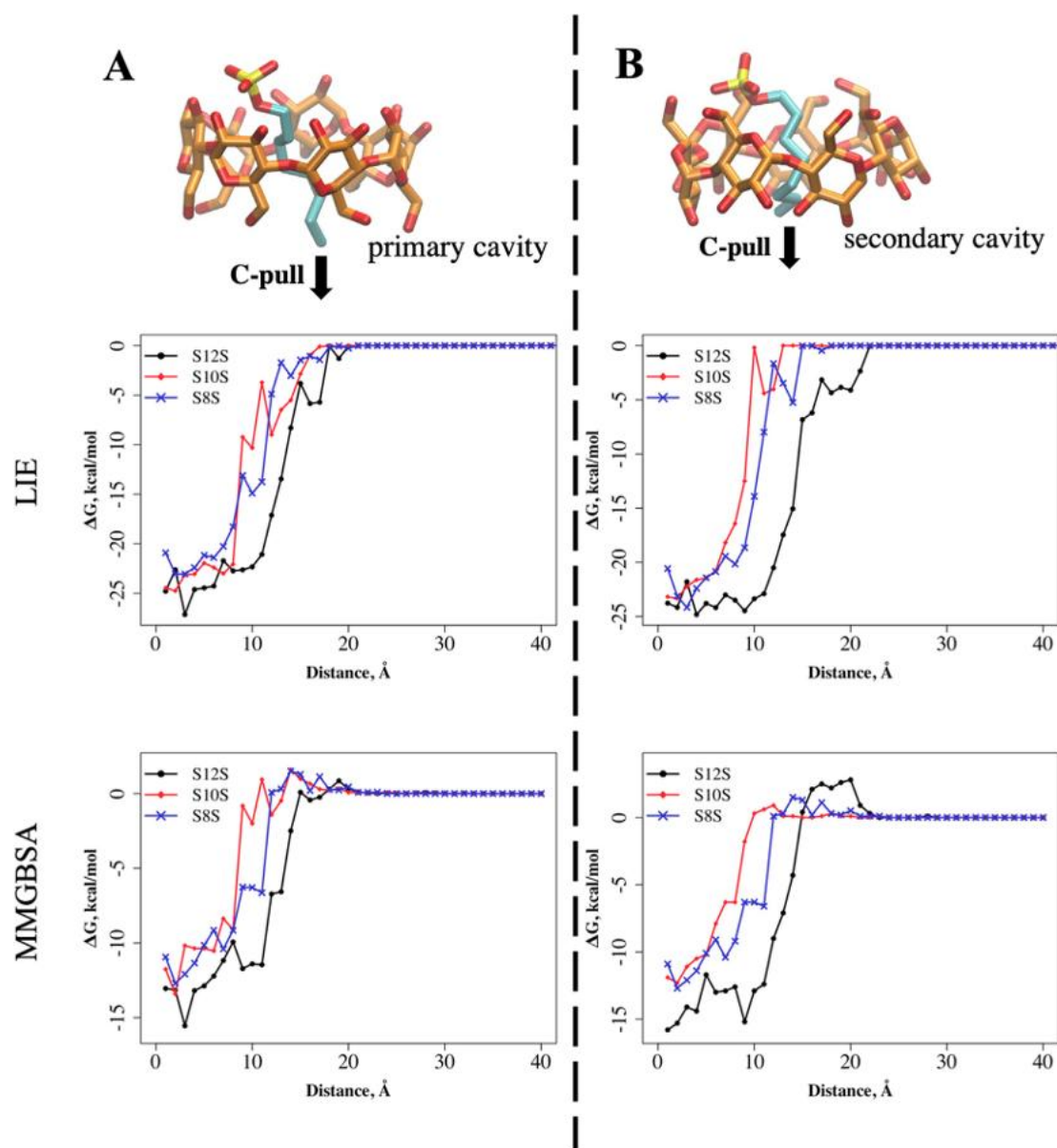


Fig. 9. Calculated LIE and MMGBSA in each US window for dodecyl sulfate (S12S), decyl sulfate (S10S) and octyl sulfate (S8S) for Complex 1 (A) and Complex 2 (B). Both LIE and MMGBSA were calculated for the C-pull pathway only.

symmetric, paths C-pull and S-pull may disclose the differences at least qualitatively. US calculations were performed for both Complex 1 and Complex 2 to check whether the different orientations of the guest could influence the dissociation pathway and resulting binding free energies. The $\Delta G_{\text{binding}}$ values are summarized in Table 5 and the constructed PMFs are depicted in Fig. 7. Small local minima visible on PMF plots for the S12S (Fig. 7A, S-pull) and S10S, S8S (Fig. 7B, S-pull) could be potentially explained in terms of the internal conformational preferences of the guest molecules appearing stochastically in the corresponding US windows.

3.6.1. Complex 1

For all guests, the energetic barrier is slightly higher in the case of the C-pull path, therefore this event could be less probable (Fig. 7). The data suggest that whereas the C-pull path shows distinct differences for $\Delta G_{\text{binding}}$ between guests, that is not the case for the S-pull path. This could be explained by the observation that during the S-pull path a part of the hydrocarbon chain is already outside the β -CD cavity together with the $-\text{OSO}_3$ group, therefore the remaining interactions are not that strong anymore. In the case of the C-pull path, the residual hydrocarbon chain needs to be pulled through the β -CD together with the $-\text{OSO}_3$ group.

3.6.2. Complex 2

Similarly to Complex 1, the energetic barrier is slightly higher for the guest in the case of the C-pull path except for S8S. The resulting $\Delta G_{\text{binding}}$ allows to rank the guest with the lowest binding free energy for S12S ($-3.3 \text{ kcal mol}^{-1}$) and then S10S and S8S (each $-1.4 \text{ kcal mol}^{-1}$). Interestingly, in the case of the S-pull path, S8S shows the lowest $\Delta G_{\text{binding}}$ value ($-3.4 \text{ kcal mol}^{-1}$), this might be due to the strong interactions between the hydrophobic interior of β -CD and shorter, compared to other guests, hydrocarbon tail of S8S.

In addition to the PMF profiles, we calculated $\Delta G_{\text{binding}}$ in each window, using LIE and MMGBSA, for the C-pull path only to check if these fundamentally different free energy calculation approaches would yield qualitatively the same results. In particular, the differences between the lowest and the highest binding free energy values calculated for trajectories in all US windows by LIE and MMGBSA define the probability of the dissociation of the guest (Fig. 8). Both methods suggest that the strongest binding between host and guest was in the β -CD/S12S complex, which agrees both with the PMF data and the experimental findings (see Fig. 9).

4. Conclusions

In this work, we characterized the structural features of three alkyl sulfates (S12S, S10S and S8S) governing the efficiency of the formation of inclusion complexes with β -cyclodextrin. We showed that the thermodynamic stability of the investigated complexes increased in proportion to the elongation of the guest alkyl chain. Both experimental and computational studies uncovered that besides the hydrophobicity, electrostatic and van der Waals interactions should be considered for investigating a structure-based host-guest complexes design. In fact, the obtained results revealed that the contribution of electrostatic interactions is more pronounced for the shorter alkyl chain sulfates. In contrast, van der Waals interactions were crucial for the complexation efficiency of the long tail guest. Additionally, four putative mechanisms of alkyl sulfates' entrance into the β -CD cavity were inspected for the first time by molecular dynamics-based computational approaches. It is worth noticing that a different entrance mode leads to the formation of different types of inclusion complexes. It can be supposed that the resulting complexes exist in a conformation equilibrium. Our results indicate that the length of guest

tails was vital for the complexation mode of β -CD. Among investigated mechanisms of the entrance of a guest through the secondary cavity with a hydrophilic head was never observed. S12S preferred to access the host predominantly through the primary cavity via the negatively charged $-\text{OSO}_3$ group (the S1 mechanism), whereas for S10S the C1 and C2 entrance mechanisms predominated. Oppositely, S8S demonstrated the highest variability in terms of entering the β -CD cavity. Finally, the observed phenomena were subsequently confirmed by a guest dissociation pathway analysis. To sum up, our work sheds more light on how the size of hydrophobic guest moiety impacts the host-guest complex formation, suggests potential entrance mechanisms and stresses the predictive power of computational approaches. All above could contribute to successful and faster drug design involving β -CD.

CRediT authorship contribution statement

Małgorzata M. Kogut: Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Ola Grabowska:** Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Dariusz Wyrzykowski:** Conceptualization, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Sergey A. Samsonov:** Conceptualization, Investigation, Funding acquisition, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Data availability

No data was used for the research described in the article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This research was funded by National Science Centre of Poland, grant number UMO-2018/30/E/ST4/00037. Computational resources were provided by the Polish Grid Infrastructure (PL-GRID, grants plggagstr3, plgionsgpu), and local cluster at the Faculty of Chemistry, University of Gdansk.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.molliq.2022.119978>.

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PUBLIKACJA 8

***β -cyclodextrin saves lysozyme from the clutches
of 1-alkylsulfonates***



β -cyclodextrin saves lysozyme from the clutches of 1-alkylsulfonates

Ola Grabowska^a, Małgorzata M. Kogut-Günthel^{a,b}, Sergey A. Samsonov^a,
Dariusz Wyrzykowski^a, Joanna Makowska^{a,*}

^a Faculty of Chemistry, University of Gdańsk, Wita Stwosza 63, 80-308 Gdańsk, Poland

^b Leibniz-Institut für Lebensmittel-Systembiologie an der Technischen Universität München, 85354 Freising, Germany

ARTICLE INFO

Keywords:

Lysozyme

β -CD

1-alkylsulfonates

Coordination properties

Molecular dynamics

ABSTRACT

Non-covalent interactions between proteins and small molecules are one of the factors, along with pH and temperature, that have a significant impact on protein function, stability and biological activity. Reversible protein folding induced by molecular crowding can be controlled by introducing ligands that compete with the protein for small molecules. One example is lysozyme (Lys), which unfolds in the presence of ligands that contain hydrophobic groups in their structure, such as 1-alkylsulfonates (KXS). In this paper, a series of experimental methods, namely circular dichroism spectroscopy, isothermal titration calorimetry, conductometric titration and differential scanning calorimetry, supported by *in silico* analysis, have been applied to characterise the process of reversibility changes of lysozyme structure in the presence of 1-alkylsulfonates with different hydrophobic chain lengths and β -cyclodextrin (β -CD) used as a strong competitive KXS-binding ligand. It has been shown that the observed structural changes can be reversed by introducing β -CD into the system, which, due to its higher affinity for KXS in comparison to lysozyme, effectively binds small ligands and so allows the protein to refold.

1. Introduction

The biological activity of proteins depends on their three-dimensional structure, which is referred to as the native state. The three-dimensional structure is essential for the proper functionality of proteins, including their interactions with other proteins or small molecules. The formation of a three-dimensional (3D) structure of proteins is a process known as protein folding. In the event of an error occurring during such a process, the protein fails to adopt its native conformation, resulting in the protein's inability to perform its function [1]. This misfolding can lead to the formation of intermediate or aberrant structures with altered functional properties or a propensity to aggregate. Protein misfolding plays a crucial role in several human diseases, as it has been closely linked to the development of various neurodegenerative diseases (NDs) such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS) [2,3]. In these diseases, specific proteins undergo misfolding and subsequently aggregate, leading to the formation of insoluble protein aggregates like amyloid fibrils. These aggregates are known to be toxic to cells and tissues, contributing to the progression of the diseases [4–9].

This article presents the findings of a study investigating the impact of agents that inhibit or prevent the unwanted unfolding of lysozyme, a

protein of significant importance to the immune system. Lysozyme is a ~ 14 kDa protein present in many mucosal secretions (tears, saliva, and mucus) and tissues of animals and plants. It plays a crucial role in the innate immune system, protecting against bacteria, viruses, and fungi. Three main different types of lysozymes are known: the c-type (chicken or conventional type), the g-type (goose type), and the i-type (invertebrate type). Lysozyme, also known as muramidase, serves as a natural antimicrobial agent within the body. It has a crucial role in the immune response by hydrolysing the glycosidic bonds in bacterial peptidoglycans. This enzymatic action disrupts the cell walls of bacteria, effectively inhibiting their growth and proliferation. The proper structure of lysozyme is essential for its antimicrobial activity and the correct functioning of the body's immune response. Any disturbances or abnormalities in the structure of lysozyme can impair its ability to carry out its antimicrobial functions effectively. In light of these considerations, much attention has been paid to exploring the energy landscape of lysozyme, focusing on its stability and denaturation processes across different temperature ranges. It has been established that the interaction between the solute (lysozyme) and the solvent (water) plays a crucial role in these processes. The hydrophilic and hydrophobic moieties of the amino acid residues within lysozyme exhibit distinct behaviours during the folding and unfolding processes [10].

* Corresponding author.

E-mail address: joanna.makowska@ug.edu.pl (J. Makowska).

<https://doi.org/10.1016/j.molliq.2025.127543>

Received 28 November 2024; Received in revised form 12 February 2025; Accepted 4 April 2025

Available online 5 April 2025

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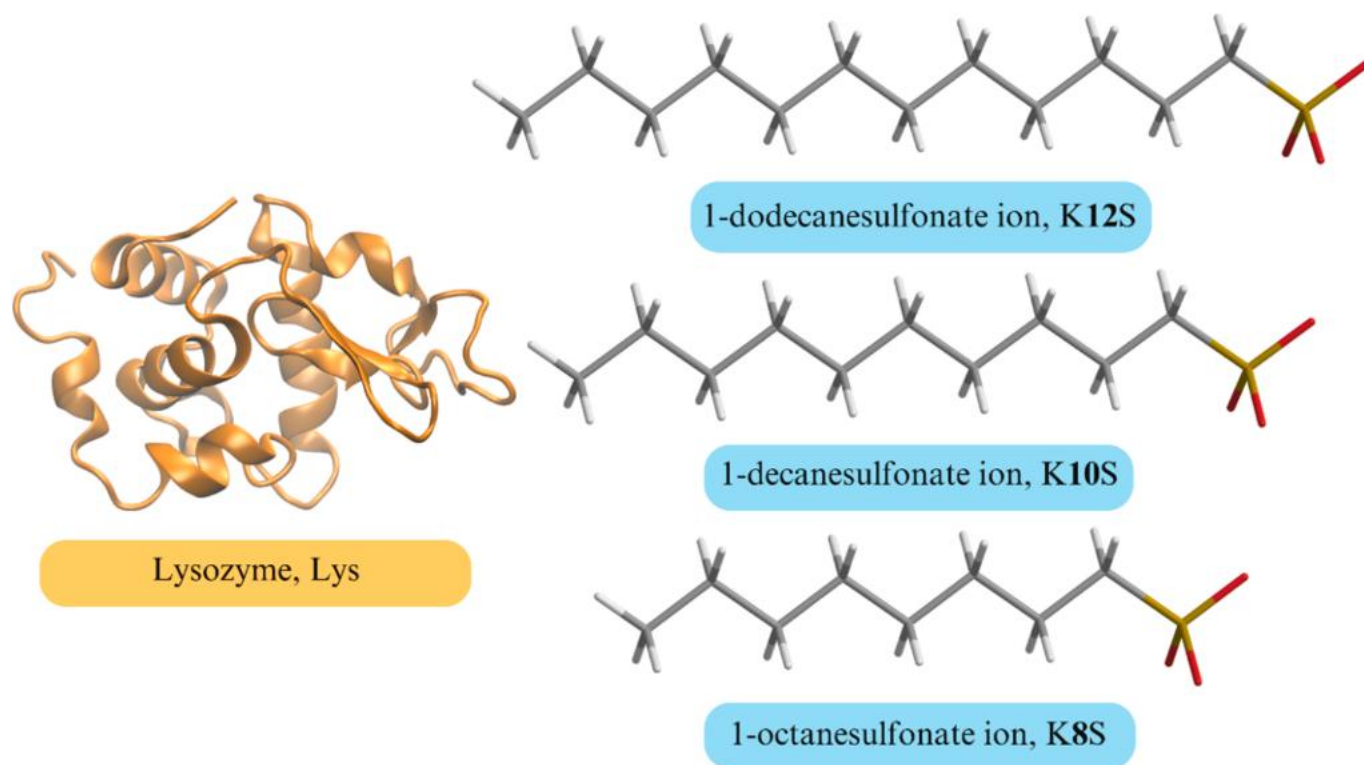


Fig. 1. Chemical structure of the lysozyme and KXS ligands.

Gaining a deeper understanding of the reversible folding/unfolding processes of lysozyme allows for exploring strategies to prevent or mitigate structural disturbances that may impair lysozyme's antimicrobial activity. This knowledge can ultimately contribute to the development of therapies or interventions that enhance lysozyme's effectiveness in the body's immune response. The impressive number of literature data, published in the last two decades, dealing with lysozyme antimicrobial activity proves the great interest provoked by this well-known biomolecule. This interest explains the extensive research of lysozyme modifications to improve the applications in medicine, veterinary, crop production, feed, and food preservation [11]. In biological systems, proteins often exist in crowded cellular environments where molecular crowding can significantly affect their structure and functionality. This research aims to improve our understanding of how reversible protein folding affected by molecular crowding can be manipulated by introducing ligands that compete with proteins for small molecules.

For this study, hen egg white lysozyme (HEWL) was chosen as a model protein. HEWL has a positive net charge under physiological conditions and a relatively rigid structure compared to other globular proteins such as albumins. The presence of ligands containing hydrophobic groups, such as 1-alkylsulfonates (KXS), can disrupt the secondary structure of HEWL by molecular crowding. Such perturbations have critical implications for enzyme activity, protein-protein interactions and various cellular processes. Understanding the factors that protect proteins from the negative effects of molecular crowding – such as misfolding or unfolding – remains a challenging endeavour. Understanding the protective mechanisms is essential for maintaining enzyme activity in crowded environments and controlling processes associated with aggregation and protein misfolding. Furthermore, this knowledge is also important for elucidating the underlying mechanisms of neurodegenerative diseases and developing potential therapeutics for these conditions. It is, therefore, of great importance to gain further insight into the potential for controlling the folding process of this protein. In this paper, we demonstrate how β -cyclodextrin saves hen egg white

lysozyme (HEWL) from the clutches of 1-alkylsulfonates (Fig. 1). The choice of cyclodextrin for this study was motivated by its widespread use as a common ligand that acts as a host for hydrophobic drug guests. Due to its unique properties, cyclodextrin has become a widely used agent in the biomedical and pharmaceutical fields, as well as in the development of supramolecular nanomedicines [12].

2. Methods

2.1. Reagents

Lysozyme from chicken egg white (Lys, dialyzed, lyophilized, powder, product no.: 62970), sodium 1-dodecanesulfonate (K12S, $\geq 99\%$, product no.: 106437), sodium 1-decanesulfonate (K10S, $\geq 99\%$, product no.: 30633), sodium 1-octanesulfonate (K8S, BioXtra, product no.: O0133), β -Cyclodextrin (β -CD, 99 %, product no.: 856088), sodium cacodylate trihydrate (Caco, $\geq 98\%$, product no.: C0250) were obtained from Sigma-Aldrich (Poland) and used without additional purification. Double-distilled water with conductivity not exceeding $0.18 \mu\text{S cm}^{-1}$ was used to prepare aqueous and buffer solutions.

2.2. Isothermal titration calorimetry (ITC)

All ITC experiments were performed at 298.15 K using the Auto-ITC isothermal titration calorimeter (MicroCal Inc. GE Healthcare, Northampton, USA). The details of the measuring devices and experimental setup were described previously [13,14]. All solutions were prepared directly into the water. The experiment consisted of injecting $10.02 \mu\text{L}$ (29 injections, $2 \mu\text{L}$ for the first injection only, injection duration: 20 sec., injection interval: 240 sec.) of 8 mM β -cyclodextrin solution into the reaction cell which initially contained 0.4 mM 1-alkylsulfonate solution (K12S, K10S or K8S). The stoichiometry, binding constant (K_{ITC}), and binding enthalpy (ΔH_{ITC}) of the inclusion complexes were determined through ITC experiments. The data was analyzed using a non-linear fitting least-squares procedure and an equilibrium model that

Table 1

The parameters of 1-dodecanesulfonate (K12S), 1-decanesulfonate (K10S) and 1-octanesulfonate (K8S) binding interactions with β -CD in aqueous solutions, at 298.15 K (standard deviation values in parentheses).

Parameter	K12S	K10S	K8S
ITC data			
The stoichiometry β -CD:KXS	1.13 (± 0.01)	1.22 (± 0.01)	1.00 (± 0.00)
$\log K_{ITC}$	4.15 (± 0.02)	3.71 (± 0.01)	2.90 (± 0.02)
ΔG_{ITC} [kcal mol ⁻¹]	-5.66 (± 0.03)	-5.06 (± 0.02)	-3.96 (± 0.03)
ΔH_{ITC} [kcal mol ⁻¹]	-1.65 (± 0.02)	-1.10 (± 0.01)	-0.89 (± 0.03)
$T\Delta S_{ITC}$ [kcal mol ⁻¹]	4.00 (± 0.04)	3.96 (± 0.02)	3.07 (± 0.04)
CT data			
$\log K_{CT}$	4.31 (± 0.01)	3.69 (± 0.02)	2.93 (± 0.02)
ΔG_{CT} [kcal mol ⁻¹]	-5.87 (± 0.02)	-5.04 (± 0.03)	-4.00 (± 0.03)
Λ_c [S cm ² mol ⁻¹]	78.62 (± 0.05)	65.31 (± 0.12)	62.58 (± 0.35)

assumes a single set of identical binding sites. By fitting the binding isotherms to the experimental data, the values of K_{ITC} and ΔH_{ITC} were directly obtained. To calculate the free energy of binding (ΔG_{ITC}) and the entropy factor ($T\Delta S_{ITC}$) of the interaction, the following relationships were used: $\Delta G_{ITC} = -RT \ln K_{ITC} = \Delta H_{ITC} - T\Delta S_{ITC}$. The obtained parameters are collected in Table 1.

2.3. Conductometric titration (CT)

Conductometric titrations were performed using Cerko Lab System microtitration unit. The details of the measuring devices and experimental setup have been described previously [13]. The titrant (8 mM β -CD solution) was added to the titrand (0.4 mM 1-alkylsulfonate: K10S or K8S) in increments of 0.01 mL and a pause 60 sec. The only exception was titration β -CD to K12S, where 4 mM titrant and 0.2 mM titrand were used. Each titration was repeated a least twice in order to check there reproducibility of the data. The binding constants (K) of the investigated β -CD-KXS complexes were calculated based on conductometric titration data, employing the following equation [15]:

$$\Lambda_{obs} = [K \cdot (c_{KXS} - c_{CD}) - 1] + \sqrt{K^2 (c_{\beta} - c_{KXS})^2 + 2K(c_{KXS} + c_{CD}) + 1} \left[\frac{\Lambda_f - \Lambda_c}{2K \cdot c_{KXS}} \right] + \Lambda_c$$

where: Λ_{obs} represents the observed molar conductance of the titrated solution, Λ_f denotes the molar conductance of KXS in pure water, Λ_c signifies the molar conductance of the β -CD-KXS complex, and c_{CD} and c_{KXS} are the total concentrations of the host and guest, respectively.

The data was analyzed using the Origin 8.5 program (ver. 8.5). The adjustable parameters, specifically K and Λ_c , are presented in Table 1. The free energy of binding (ΔG) was calculated following the standard thermodynamic relationship: $\Delta G = -RT \ln K$.

2.4. Circular dichroism spectroscopy (CD)

Circular dichroism spectra were obtained using a Jasco-715 automated spectropolarimeter (Jasco Inc., USA) for the following systems: (a) free lysozyme (14 μ M), (b) the mixtures of Lys-KXS (where X = 12, 10, 8) in a molar ratio of 1:100 (14 μ M:1.4 mM) and (c) the mixtures of Lys-KXS- β -CD (where X = 12, 10, 8) in a molar ratio of 1:100:200 (14 μ M:1.4 mM:2.8 mM). The only exception was the system Lys-K12S- β -CD, where a stoichiometric molar ratio of 1:100:400 (14 μ M:1.4 mM:5.6 mM) was additionally used. All solutions were prepared in the 10 mM Caco buffer of pH 5. The measurement conditions were as follows: wavelength range of recorded spectra 195–260 nm, temperature 298.15

K, sensitivity 5 millidegrees, scan speed 50 nm min⁻¹, sample volume 0.3 mL. The results were plotted as mean residual ellipticity Θ (degrees cm² dmol⁻¹). The details of the data processing have been described previously [16].

2.5. Differential scanning calorimetry (VP-DSC)

DSC measurements were made with a VP-DSC microcalorimeter (MicroCal Inc. GE Healthcare, Northampton, USA) at a scanning rate 90 K h⁻¹. All scans were run in water in the temperature range of 298.15–363.15 K at a scan rate of 1.5 K min⁻¹. The scans were obtained for pure lysozyme (0.25 mM) and the solution containing chosen molecules and lysozyme mixed in the Lys-K12S- β -CD molar ratio equal 1:5:20 (0.25 mM:1.25 mM:5 mM). All the samples were degassed before the measurements. These measurements were recorded two times. The details of the measuring device, experimental setup and data processing have been described previously [17].

2.6. Computational analysis

Structures. The structures of β -CD-host with K12S, K10S and K8S guests were built in Xleap module of the AMBER16 software package [18]. The structure of S12S was used from our previous work [19] and based on it were removed an O atom and then subsequently K10S and K8S structures were prepared by removing -C₂H₅ and -C₄H₉ from K12S, respectively. The initial complexes were built by placing a guest molecule in a random orientation close to the host molecule.

Molecular Dynamics Simulations. The Molecular Dynamics (MD) simulation protocols applied in this study were previously described in detail and applied to a similar system containing β -CD and alkyl sulfates of different length in our previous work [13]. In particular, all-atom MD simulation for each host-guest system was performed using the AMBER16 software package [18]. A truncated octahedron TIP3P periodic box of 10 Å water layer from the box's border to solute was used to solvate complexes. The charge was neutralised with Na⁺ counterions. GLYCAM06 force field for the β -CD-host [20] and gaff force field parameters [21] with RESP charges that are compatible with the applied

force field parameters [22] obtained in an antechamber module of AMBER16 [18] for the guests K8S, K10S, K12S and IBP were used, respectively. Energy minimization was carried out in two steps: beginning with 500 steepest descent cycles and 10³ conjugate gradient cycles with 100 kcal/mol/Å² harmonic force restraints, continued with 3x10³ steepest descent cycles and 3x10³ conjugate gradient cycles without any restraints. Following minimization steps, the system was heated up from 0 to 300 K for 10 ps with harmonic force restraints of 100 kcal/mol/Å². Subsequently, the system was equilibrated at 300 K and 10⁵ Pa in the isothermal isobaric ensemble for 500 ps. Afterwards, the productive MD run was carried out in the same isothermal isobaric ensemble for 1 μ s, which was sufficient to extensively sample the conformational space of the simulated complexes. The particle mesh Ewald method for treating electrostatics and the SHAKE algorithm for all the covalent bonds containing hydrogen atoms were implemented in the MD simulations.

Binding Free Energy Calculations. For all systems, an energetic post-processing of the trajectories was conducted, along with per-residue energy decomposition. This analysis was carried out using the Linear Interaction Energy (LIE) method with a dielectric constant of 80, as well as the Molecular Mechanics Generalized Born Surface Area (MM-GBSA) approach [23]. The MM-GBSA model utilized default parameters for

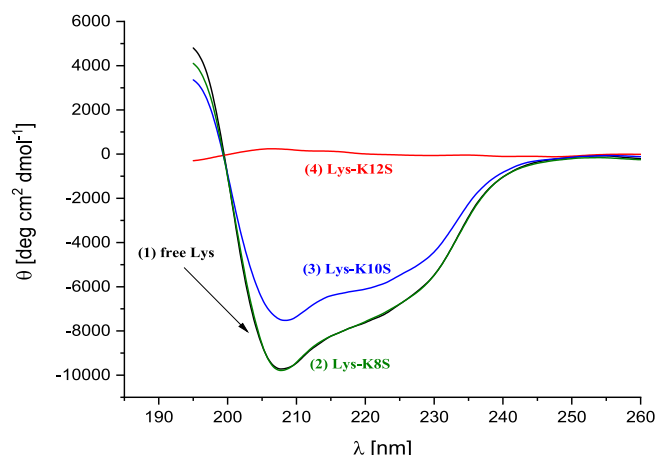


Fig. 2. CD spectra of free lysozyme (14 μM) and the mixtures of Lys-KXS in a molar ratio of 1:100, in the 10 mM Caco buffer of pH 5 at 298.15 K.

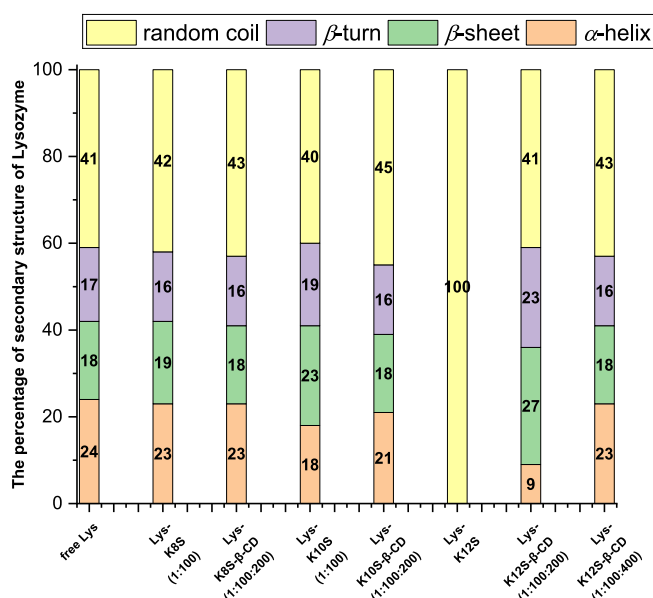


Fig. 3. The influence of 1-alkylsulfonates on the lysozyme secondary structure. Conditions: the 10 mM Caco buffer of pH 5 at 298.15 K.

surface area and Born radii, as implemented in the $\text{igb} = 2$ model within AMBER16 [18]. To ensure accurate analysis, only the MD frames corresponding to the complex structure that achieved convergence in terms of the root mean square deviation (RMSD) were considered for further examination. It is important to note that the binding free energies obtained by MM-GBSA rather reflect enthalpies while partially accounting for solvent entropy and, therefore, do not represent binding free energies in the strict sense.

Data Analysis and Visualisation. Data analysis and visualization were performed with the use of the R-package [24] and VMD [25].

3. Results

3.1. The effect of 1-alkylsulfonates on the lysozyme secondary structure

A common phenomenon is the alteration of the secondary structure of lysozyme, which can be induced by various external stimuli, including the binding of exogenous ligands. The circular dichroism (CD) method has been employed to investigate the alteration of the lysozyme secondary structures evoked by the presence of the 1-alkylsulfonates under

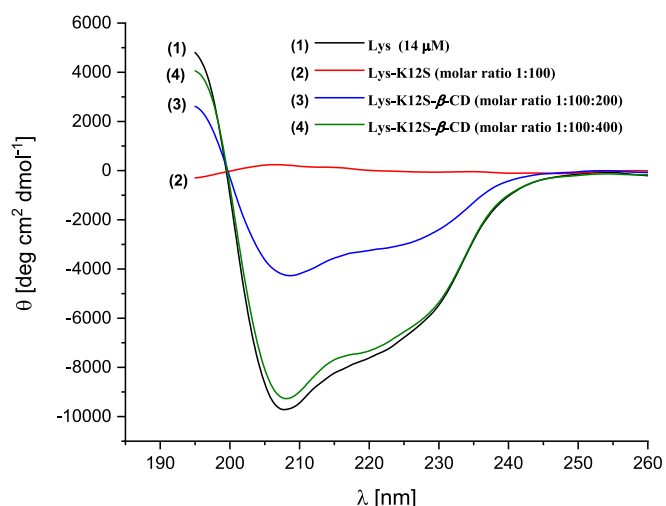


Fig. 4. CD spectra of free lysozyme (14 μM), the mixture of Lys-K12S and the mixture of Lys-K12S- β -CD in the 10 mM Caco buffer of pH 5 at 298.15 K.

study. The representative CD spectra of the investigated systems are shown in Fig. 2, whereas the calculated secondary structure contents of α -helix, β -sheet, β -turns and random coil were collected in Fig. 3. Two negative peaks were observed at 208 nm (the $\pi \rightarrow \pi^*$ transition) and 222 nm (the $n \rightarrow \pi^*$ transition), which are characteristic of the α -helix structure [26,27].

The data collected indicate that the length of the hydrophobic carbon chain of KXS influences the secondary structure of lysozyme in different ways. The disruption of the secondary structure is observed as a consequence of the presence of K12S, the 1-alkylsulfonate ligand with the longest hydrophobic moiety. In the presence of the K10S species, there is a slight decrease in the content of the α -helix, whereas the helical conformation remains unaltered when the K8S species are present in the system. Thus, it can be postulated that hydrophobic interactions are the driving forces responsible for the observed phenomenon. A similar influence of the attractive forces between non-polar regions of molecules was also observed during the hydrophobic interactions of lysozyme with two non-steroidal anti-inflammatory drugs (NSAIDs), namely paracetamol and ibuprofen [28], as well as azo food dyes [29].

The incorporation of the KXS into the hydrophobic cavity of the protein results in the unfolding of the biomolecule in proportion to the strength of the ligand–protein interactions. Consequently, considerable structural changes were observed in the case of lysozyme-K12S interactions. The interaction of the amino acid residues of the main polypeptide chain of lysozyme with K10S, which has a two-carbon atom shorter chain than K12S, results in only a partial unfolding of the protein. In contrast, the hydrophobic moiety of K8S is too short to affect the helical content of the investigated enzyme.

3.2. The refolding of lysozyme in the presence of 1-alkylsulfonates binding ligand (β -CD)

In the case of the lysozyme-K12S interactions, a significant alteration of the protein secondary structure is responsible for the overall loss of activity of the enzyme. Nevertheless, if the bound ligand is released, the lysozyme has the ability to refold back and return to its native structure. A phenomenon of this nature is observed when β -cyclodextrin (β -CD) is introduced to the system under study. In the presence of β -CD in the medium, the protein tends to refold back (Fig. 4). The observed weakening in the hydrophobic interactions of lysozyme with K12S can be attributed to the competition between lysozyme and β -CD for K12S, resulting in a reduction in the strength of these interactions with the increase in the concentration of β -CD in the system (Fig. 4). The same scenario was observed for the lysozyme-K10S system. In this case, the

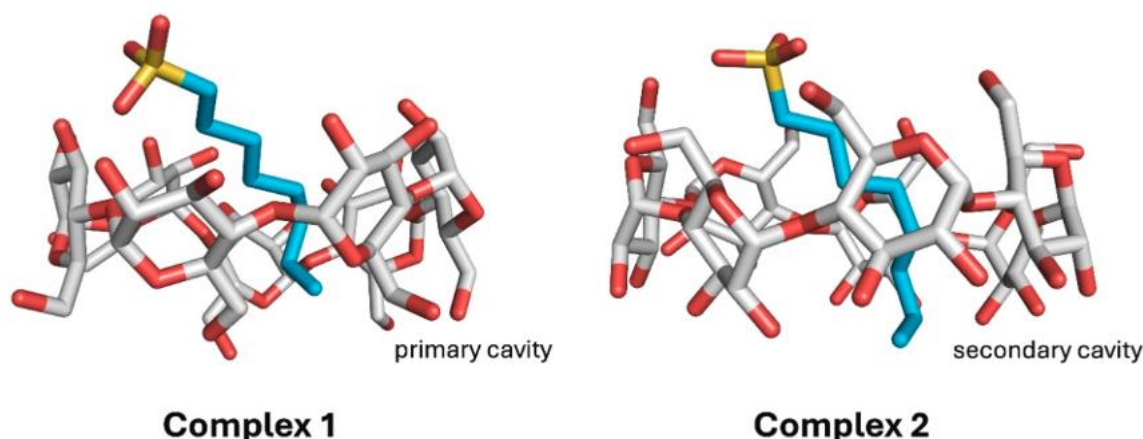


Fig. 5. The representative binding modes of KXS and β -CD in Complex 1 and Complex 2. Hydrogen atoms were omitted for clarity.

lower concentration of β -CD is sufficient to recover the change in the secondary structure of lysozyme due to the lysozyme-K10S interaction, in comparison to the β -CD concentration in the presence of K12S ligand.

3.3. Thermal stability of the lysozyme structure under experimental conditions

To evaluate the protective capabilities of β -CD against the deleterious impact of KXS on the lysozyme structure, differential scanning calorimetry (DSC) measurements were conducted. K12S was selected for this experiment as it exerted the most pronounced influence on the secondary structure of the protein. The DSC data presented for lysozyme and its mixture with K12S and β -CD are mean values from two independent measurements. For all systems under study, it was found that there are no significant negative peaks in the heat capacity curves, which demonstrates that none of tested samples aggregate under the experimental conditions [30]. The calorimetric measurements show that in both experiments the reversibility of the process was observed (Fig. 1 (A), (B) in SI). Analysis of the heat capacity curve of the pure lysozyme at pH 5 revealed the heat capacity peak at $T_m = 350.72 \pm 0.02$ K (Fig. 2 in SI). The transition point was estimated by fitting the experimental C_p vs. T curves to a non-two state model using the Marquardt non-linear least squares method (T_m denotes the temperature value of the peak, the temperature at which a maximum of the endothermic effect emerges). Subsequent DSC experiments conducted for a ternary system containing lysozyme, K12S and β -CD demonstrated that the stability of the protein under the experimental conditions was comparable to its thermal stability in a system devoid of K12S and β -CD. This observation corroborates the hypothesis that β -CD exerts a protective effect against the deleterious impact of K12S on the structural integrity of lysozyme (Fig. 4). In the case of the system containing lysozyme and K12S, it was not possible to determine the phase transition temperature of the protein due to the pronounced destructive effect of this small molecule ligand on the structure, which precluded further analysis. Upon heating the three-component sample to a temperature exceeding 303.15 K, the process of conformational transformation is initiated. The first low-energy conformational transition was observed at 327.72 K (Fig. 3 in the SI). Ultimately, at $T_m = 349.61 \pm 0.03$ K (Fig. 3 in the SI), the most energetically favourable phase transition occurs. The unfolding of lysozyme therefore occurs at a temperature approximately 1 K lower than that of the native protein.

3.4. The KXS- β -CD binding interactions

It has been revealed that the presence of a strong ligand capable of binding KXS in the system allows for the restoration of the native

Table 2

For each system, five independent MD simulations were performed to inspect for the preferred mechanism of the entrance. The table summarizes whether the guest entered the host at any point of the simulation with a particular mechanism.

Guest	Mechanism of entrance							
	C1		C2		S1		S2	
K12S	1	Yes	1	Yes	1	Yes	1	No
	2	Yes	2	No	2	Yes	2	→ C2
	3	Yes	3	Yes	3	No	3	No
	4	Yes	4	No	4	Yes	4	→ C2
	5	Yes	5	Yes	5	No	5	No
K10S	1	Yes	1	Yes	1	Yes	1	No
	2	No	2	Yes	2	No	2	No
	3	Yes	3	Yes	3	No	3	→ C2
	4	→ S1	4	Yes	4	No	4	→ C2
	5	Yes	5	Yes	5	No	5	No
K8S	1	Yes	1	Yes	1	Yes	1	Yes
	2	Yes	2	Yes	2	No	2	Yes
	3	Yes	3	Yes	3	No	3	→ C1
	4	Yes	4	Yes	4	Yes	4	→ C1
	5	Yes	5	Yes	5	No	5	Yes

structure of the protein, and consequently the enzyme activity. To assess the strength of the β -CD-KXS interactions two complementary experimental techniques have been employed, namely isothermal titration calorimetry (ITC) and conductometric titration (CT). The obtained binding parameters are collected in Table 1.

It has been found that the stoichiometry of the resulting host(β -CD)-guest(KXS) complexes equals 1:1 (β -CD:KXS). The thermodynamic stability of the complexes increases with the increase of the hydrophobic chain length of the 1-alkylsulfonates, $\log K_{K12S} > \log K_{K10S} > \log K_{K8S}$ ($|\Delta G_{K12S}| > |\Delta G_{K10S}| > |\Delta G_{K8S}|$) (Table 1). Although heat release accompanies the investigated interactions, the formation of the host-guest complexes is an entropy-driven process, $|\Delta H| < |T\Delta S|$. In such a case, hydrophobic interactions appear to play a more significant role in complexation efficiency than electrostatic interactions. On the other hand, van der Waals interactions (as indicated by the negative value of ΔH) are an additional factor contributing to the stability of the complexes. The electrostatic interactions are more pronounced for the KXS species with longer hydrophobic chain lengths (Table 1).

Table 3

MM-GBSA and LIE free energy decomposition for dodecyl sulfate (K12S), decyl sulfate (K10S) and octyl sulfate (K8S) guests in the host–guest complexes (ΔG_{ele} = electrostatic component in vacuum, ΔG_{vdw} = van der Waals component, ΔG_{GB} = Generalized Born reaction field component, ΔG_{SURF} = non-polar solvation component, ΔG_{total} = total free energy of binding).

Guest	LIE [kcal mol ⁻¹]			MMGBSA [kcal mol ⁻¹]				
	ΔG_{ele}	ΔG_{vdw}	ΔG_{total}	ΔG_{ele}	ΔG_{vdw}	ΔG_{GB}	ΔG_{SURF}	ΔG_{total}
K12S	−0.2	−23.1	−23.3	−14.2	−23.1	27.3	−3.4	−13.4
K10S	−0.2	−21.7	−21.9	−15.9	−21.7	28.5	−3.3	−12.3
K8S	−0.2	−19.6	−19.8	−17.3	−19.6	29.1	−3.0	−10.8

3.5. Entrance mechanisms of 1-alkylsulfonates into the β -CD cavity by molecular dynamics (MD) simulations

To gain insight into the factors underlying the formation of β -CD-KXS inclusion complexes, molecular dynamics (MD) simulations were employed. A particular focus was placed on the variation in the lengths of the hydrocarbon chains of KXS and their impact on the complexation mode of β -CD. A visual inspection of the generated trajectories revealed that 1-alkylsulfonates were entering the host cavity, although how this occurred differed, namely K12S accessed the host through the primary cavity entering via S-end first, whereas K10S and K8S entered through the secondary cavity with the C-end first. Furthermore, we systematically investigated the most probable mechanism of entrance, considering four potential scenarios, namely: S-end first via the primary cavity (S1), C-end first via the secondary cavity (C2), C-end first via the primary cavity (C1) and S-end first via the secondary cavity (S2). In each instance, a KXS was positioned in close proximity to either a primary or secondary cavity, with either its C- or S-end oriented towards the centre of β -CD. Subsequently, five short independent MD simulations were conducted. The choice of entrance mode determines the formation of different inclusion complexes. If S1, C2, or C1 and S2 are the preferred complexes, Complex 1 and Complex 2 would be formed, respectively (Fig. 5). The findings are summarised in Table 2 suggesting that:

- Both Complex 1 and Complex 2 are likely to be formed for K12S, however, the switch from S2 to C2 could indicate it is more favourable for K12S to enter β -CD via the C-end.
- Mechanism S2 was not observed for either K12S or K10S.
- Complex 1 is strongly preferred for the β -CD-K10S formation.
- For the first time, mechanism S2 was observed for K8S.
- β -CD-K8S was not stable within 100 ns of MD simulation (neither as a Complex 1 nor Complex 2).

3.6. β -CD-guest inclusion complexes: Electrostatic vs. van der Waals interactions by binding free energy analysis

To estimate the free energy of binding in the β -CD-guest inclusion complexes, two commonly used endpoint approaches were employed: Linear Interaction Energy (LIE) and Molecular Mechanics Generalized Born Surface Area (MM-GBSA) (Table 3). The LIE analysis, which employs isotropic dielectric constant, did not show any alteration in the electrostatic component of the binding. In contrast, the application of MM-GBSA technique affirmed that as the hydrophobic chain becomes shorter, the electrostatic interactions in the host–guest complex become stronger. This holds true whether considering the system *in vacuo* or with the GB (Generalized Born) implicit solvent model. Both methods indicated the same change in the van der Waals component, suggesting that longer guests establish more contacts with the host. By employing these two approaches to calculate the free energy, it was determined that the total free energy of binding (ΔG_{total}) is most favourable for K12S and least favourable for K8S.

4. Conclusions

1-alkylsulfonates (KXS) containing hydrophobic moieties induce

changes in the lysozyme structure. This effect is dependent on the length of the hydrocarbon chain of KXS (K12S > K10S > K8S). The obtained results showed that the presence of β -CD led to a weakening of the structural interactions between KXS and lysozyme that induce protein unfolding. This phenomenon can be attributed to the competition between lysozyme and β -CD for KXS. The resulting β -CD complexes were rigorously characterized in a solution by experimental approaches and the putative mechanisms of 1-alkylsulfonates entrance into the β -CD cavity were discussed based on *in silico* analysis. The relative stability of the host–guest (β -CD-KXS) complexes explains the effective protection effect of the investigated cyclodextrin against changes in protein secondary structure. These findings provide valuable insights into the interplay between lysozyme, KXS and β -CD, and their effects on the structural properties of lysozyme. Furthermore, it can be speculated that β -CD may also be considered as a potential protective factor against the unfolding processes of proteins exposed to the damaging effects of hydrophobic group-containing small molecules that bind β -CD.

CRedit authorship contribution statement

Ola Grabowska: Writing – original draft, Resources, Methodology, Funding acquisition, Conceptualization. **Małgorzata M. Kogut-Günthel:** Investigation, Formal analysis. **Sergey A. Samsonov:** Writing – review & editing, Writing – original draft, Supervision, Software, Funding acquisition. **Dariusz Wyrzykowski:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Formal analysis. **Joanna Makowska:** Writing – review & editing, Writing – original draft, Supervision, Methodology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the Grants for Young Scientists 2024 from University of Gdansk, Poland (539-T090-B124-24) and the National Science Centre of Poland (Narodowe Centrum Nauki, grant UMO-2023/49/B/ST4/00041).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.molliq.2025.127543>.

Data availability

Data will be made available on request.

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Investigation of hexacyanoferrate(II)/(III) charge-dependent interactions with bovine and human serum albumins



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Investigation of hexacyanoferrate(II)/(III) charge-dependent interactions with bovine and human serum albumins

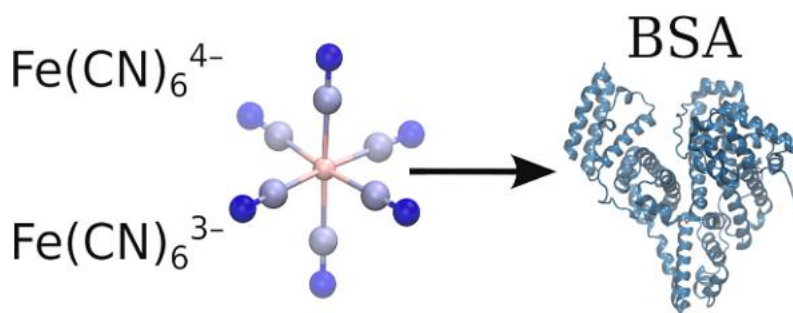
Ola Grabowska, Sergey A. Samsonov, Lech Chmurzyński, Dariusz Wyrzykowski, Krzysztof Żamojć*

Faculty of Chemistry, University of Gdańsk, Wita Stwosza 63, 80-308 Gdańsk, Poland

HIGHLIGHTS

- Hexacyanoferrates as model ligands endowed with a significant negative charge.
- Binding interactions of $[\text{Fe}(\text{CN})_6]^{4-}$ and $[\text{Fe}(\text{CN})_6]^{3-}$ with BSA and HSA.
- Hexacyanoferrates(II/III) can be considered as potential binding site markers.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:

Bovine and human serum albumins
Potassium hexacyanoferrates(II/III)
Binding properties
Steady-state fluorescence spectroscopy
Fluorescence quenching
Isothermal titration calorimetry
CD spectroscopy
Molecular dynamics simulations

ABSTRACT

In the present paper, the binding interactions of highly negative-charged ions, namely hexacyanoferrates(II/III), *i.e.* $[\text{Fe}(\text{CN})_6]^{4-}$ and $[\text{Fe}(\text{CN})_6]^{3-}$ with bovine and human serum albumins (BSA and HSA, respectively) have been studied for the first time in an aqueous solution (10 mM cacodylate buffer of pH 7.0) using steady-state fluorescence spectroscopy, isothermal titration calorimetry, and CD spectroscopy supported by molecular dynamics-based computational approaches. The Stern-Volmer equation as well as its modifications suggested that hexacyanoferrates(II/III) effectively quenched the intrinsic fluorescence of the albumins through a static mechanism. The proteins under study possess only one binding site on the surface capable of binding one mole of hexacyanoferrates(II/III) ions per one mole of albumin (HSA or BSA). The formation of albumin complexes is an enthalpy-driven process ($|\Delta H_{\text{ITC}}| > |T\Delta S_{\text{ITC}}|$). The strength of the interactions depends mainly on the type of albumin, and changes as follows: $\text{BSA-K}_3[\text{Fe}(\text{CN})_6] \sim \text{BSA-K}_4[\text{Fe}(\text{CN})_6] > \text{HSA-K}_3[\text{Fe}(\text{CN})_6] \sim \text{HSA-K}_4[\text{Fe}(\text{CN})_6]$. Finally, potential binding sites of bovine and human serum albumins have been investigated and discussed based on a competitive fluorescence displacement assay (with warfarin and ibuprofen as site markers) and molecular dynamics simulations.

1. Introduction

Serum albumins belong to the most abundant proteins which play a

very important role in the transport and delivery of a variety of exogenous and endogenous compounds (among others fatty acids, bilirubin, hormones, and xenobiotics) in the bloodstream to the target organs [1].

* Corresponding author.

<https://doi.org/10.1016/j.saa.2023.122505>

Received 21 September 2022; Received in revised form 21 January 2023; Accepted 13 February 2023

Available online 15 February 2023

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Furthermore, they are involved in the regulation of the osmotic pressure and pH of blood as well as act as antioxidants by the scavenging of radicals [2-4]. In general, the presence in the microenvironment of different types of high and low molecular-weight species can alter the functional activity, stability and transport properties of albumins. This phenomenon is especially enhanced in the presence of molecules such as drugs or metal ions capable to form fairly stable complexes with the proteins. For these reasons, many attempts have been made to understand the binding mode of natural and artificial molecules to serum albumins (which represent a primary target for the action of the medicine) with quantitative and detailed studies aimed at understanding the mechanism of its toxic and/or therapeutic activity [5-20]. Among the investigated molecules special effort has been devoted to different types of metal-based complexes, revealing interesting antitumor activity [21-23]. It has been previously reported that the presence of a relatively high amount of albumins in the cell culture media may contribute to a decrease in an effective concentration of examined complexes and consequently to their dose-dependent effect on the investigated cell lines. Thus, the biological results obtained under such specific experimental conditions should be always assessed very carefully. An excellent review presenting representative examples of complexes showing selective binding to bovine serum albumin has been provided by Macii and Biver [24].

Techniques used for these investigations include among others fluorescence and absorption spectroscopies [25-27], equilibrium dialysis [28,29], liquid chromatography [30], electrophoresis [31], circular dichroism spectroscopy [28,32], electrochemistry [33,34], ultrafiltration [35], isothermal titration calorimetry [36] and inductively coupled plasma-atomic emission spectroscopy [37]. Among them, fluorescence spectroscopy seems to be particularly promising since it enables obtaining essential information on the binding of small molecules to peptides/proteins, binding constants, binding sites, stoichiometry and intermolecular distances [38]. Our previous studies on the fluorescence quenching performed with the use of steady-state and transient methods have already provided a lot of valuable information about interactions between low-molecular-weight compounds with proteins [39] and DNA [40].

However, according to the literature survey, there are few reports on binding interactions of highly negative-charged ions with albumins. Thus, it was the reason that prompted us to embark on this study. The present study examines the binding of two hexacyanoferrates(II/III), i.e. $[\text{Fe}(\text{CN})_6]^{4-}$ and $[\text{Fe}(\text{CN})_6]^{3-}$, both considered here as important models of low-molecular-weight ions endowed with a significant negative charge, to bovine and human serum albumins (BSA and HSA) using steady-state fluorescence spectroscopy, isothermal titration calorimetry, and CD spectroscopy supported by theoretical calculations.

2. Materials and methods

2.1. Materials

Bovine and human serum albumins (BSA and HSA, lyophilized powders, $\geq 96\%$, PN: A2153 and A1653, respectively), potassium hexacyanoferrate(II) trihydrate ($\geq 98.5\%$, PN: P9387), potassium hexacyanoferrate(III) ($\geq 99\%$, PN: 244023), warfarin sodium (PN: PHR1435), ibuprofen sodium salt ($\geq 98\%$, PN: I1892), and sodium cacodylate trihydrate (CACO, $\geq 98\%$, PN: C0250) were obtained from Merck (Poland) and employed as received without further purification. Double-distilled water with conductivity not exceeding $0.18 \mu\text{S cm}^{-1}$ was used for the preparation of buffer solutions. The stock solutions of the proteins and potassium hexacyanoferrates were prepared in 10 mM CACO buffer of pH 7.0 (all subsequent dilutions were made with this buffer). The concentration of BSA and HSA was measured spectrophotometrically using extinction coefficients equal to $\epsilon_{280}^{\text{BSA}} = 41180 \text{ M}^{-1}\text{cm}^{-1}$ and $\epsilon_{280}^{\text{HSA}} = 32930 \text{ M}^{-1}\text{cm}^{-1}$, calculated based on the content

of tryptophan ($\epsilon_{280}^{\text{W}} = 5690 \text{ M}^{-1}\text{cm}^{-1}$), tyrosine ($\epsilon_{280}^{\text{Y}} = 1280 \text{ M}^{-1}\text{cm}^{-1}$), and cysteine ($\epsilon_{280}^{\text{C}} = 120 \text{ M}^{-1}\text{cm}^{-1}$) [41] – for this purpose the UV absorption spectra were recorded using a Perkin Elmer Lambda 650 (Waltham, MA, USA) UV/Vis spectrophotometer.

2.2. Methods

2.2.1. Steady-state fluorescence spectroscopy (SF)

The steady-state fluorescence emission spectra were registered and further emission measurements were carried out at Cary Eclipse Varian (Agilent, Santa Clara, CA, USA) spectrofluorometer equipped with a temperature controller and a 1.0 cm multicell holder. The excitation and emission slit widths were fixed at 5 nm. The excitation wavelength was always set at 275 nm. The concentration of both albumins was kept at 2 μM to avoid the inner filter effect - maximum absorbance values of BSA and HSA solutions at excitation wavelength were less than 0.09. Nevertheless, all fluorescence intensity values have been corrected for the inner filter effect based on the following equation:

$$F_{\text{corr}} = F_{\text{obs}} \times 10^{\frac{A_{\text{ex}} + A_{\text{em}}}{2}}$$

where F_{corr} and F_{obs} correspond to corrected and observed fluorescence intensity values, respectively; while A_{ex} and A_{em} correspond to absorbance values measured at the excitation and emission wavelength, respectively [42-44]. The fluorescence emission spectra of BSA and HSA (2 μM) were recorded from 280 to 500 nm in the absence and presence of increasing concentrations of $\text{K}_4[\text{Fe}(\text{CN})_6]$ and $\text{K}_3[\text{Fe}(\text{CN})_6]$, each up to 10 μM . In the performed fluorescence titration experiments, 2 mL of BSA and HSA at a fixed concentration equal to 2 μM were simultaneously titrated with ten 2 μL and then six 5 μL aliquots of $\text{K}_4[\text{Fe}(\text{CN})_6]$ and $\text{K}_3[\text{Fe}(\text{CN})_6]$ ($c = 0.4 \text{ mM}$). The fluorescence intensities of the band at 348 nm in the case of BSA and 340 nm in the case of HSA – corresponding to the initial maximum emission of each protein – were used to calculate the binding constants and other parameters. Generally, all experiments were performed at 298.15 K, but – in order to confirm the fluorescence quenching mechanism – additionally at 288.15 K, 303.15 K, and 318.15 K. In the site marker competitive experiments, $\text{K}_4[\text{Fe}(\text{CN})_6]$ and $\text{K}_3[\text{Fe}(\text{CN})_6]$ were gradually added to the solutions of BSA and HSA mixed in 1:12.5 molar ratio with appropriate site markers: warfarin for site I, ibuprofen for site II, as well as warfarin and ibuprofen used simultaneously for sites I and II at the same time. All spectrofluorometric experiments were performed twice, thus all obtained values are means from two independent experiments.

2.2.2. Isothermal titration calorimetry (ITC)

All ITC experiments were performed at 298.15 K using the AutoITC isothermal titration calorimeter (MicroCal Inc. GE Healthcare, Northampton, USA). The details of the measuring devices and experimental setup were described previously [40]. The reagents ($\text{K}_4[\text{Fe}(\text{CN})_6]$ and $\text{K}_3[\text{Fe}(\text{CN})_6]$, BSA and HSA) were dissolved directly in 10 mM CACO buffer of pH 7.0. The experiment consisted of injecting 10.02 μL (29 injections, 2 μL for the first injection only) of 1 mM buffered solution of $\text{K}_4[\text{Fe}(\text{CN})_6]$ and $\text{K}_3[\text{Fe}(\text{CN})_6]$ into the reaction cell which initially contained 0.05 mM buffered solution of albumin. A background titration, consisting of an identical titrant solution but with the buffer solution in the reaction cell only, was subtracted from each experimental titration on account of the heat of dilution. The titrant was injected at 4-min intervals. The stirrer speed was kept constant at 300 rpm.

2.2.3. Circular dichroism spectroscopy (CD)

Circular dichroism (CD) spectra were recorded on a Jasco-715 (Jasco Inc., USA) automatic recording spectropolarimeter for free HSA (3 μM) and BSA (3 μM) as well as the mixtures of albumin/hexacyanoferrate(II/III) in stoichiometric molar ratios 1:1, 1:2, and 1:4 in the 10 mM CACO buffer of pH 7.0, at 298.15 K. Measurements were made in 1 mm quartz cuvettes. The spectra were recorded in the 190–260 nm wavelength

range, using a sensitivity of five millidegrees and a scan speed of 50 nm min⁻¹. The results were plotted as the mean residue ellipticity Θ [degree cm² dmol⁻¹]. The volume of samples was 0.3 cm³. The change in the secondary structures of proteins was estimated from all CD spectra using the Dichroweb online server [45] with the CDSSTR analysis algorithm [46] and reference dataset 7 [47].

2.2.4. Molecular dynamics (MD) simulations

The structure of BSA was obtained from the PDB (PDB ID: 4F5S, 2.47 Å). Initial structures of hexacyanoferrates(II/III), i.e. [Fe(CN)₆]⁴⁻ and [Fe(CN)₆]³⁻ were built in the xleap module of AMBER20 [48]. These structures were optimized by B3LYP (6-31G*) in Gaussian 09 [49] with ReadRadii option (1.2 Å value was assigned for iron atom), and RESP procedure [50] was applied to calculate partial point charges compatible with the AMBER force fields. Nitrogen and carbon were assigned to n1 and c1 types from the gaff force field [51]. The missing bonded parameters for the equilibrium distances and angles were directly obtained from the geometry optimization in Gaussian 09, and the force constants were chosen to be characteristic for these types of bonds and angles to maintain the geometry in the molecular dynamics (MD) simulation in agreement with the one from quantum chemical optimization data (see [Supplementary Material](#) frmod files).

Molecular dynamics (MD) simulations of two molecular systems were performed, for the BSA molecule with 10 [Fe(CN)₆]⁴⁻ and [Fe(CN)₆]³⁻ molecules, respectively, in AMBER20 [48]. Ligands were placed randomly around the protein. Truncated octahedron TIP3P periodic box of 10 Å water layer from box's border to solute was used to solvate the molecular systems. 47 and 57 K⁺ counterions were added to the systems containing ferricyanide and ferrocyanide molecules, respectively. K⁺ was used instead of more commonly used Na⁺ for a better correspondence of the simulations to the experiments. ff14SB force field [52] for the protein and gaff force field [51] with the added

parameters for ferricyanide and ferrocyanide molecules were used, respectively. Two energy minimization steps were performed: first, 500 steepest descent cycles and 10³ conjugate gradient cycles with 100 kcal/(mol·Å²) harmonic force restraints on the solute and, second, 3x10³ steepest descent cycles and 3x10³ conjugate gradient cycles without any restraints. Then, the system was heated up from 0 to 300 K for 10 ps with harmonic force restraints of 100 kcal/(mol·Å²) solute. Finally, the system was equilibrated at a constant temperature of 300 K and a constant pressure of 10⁵ Pa for 100 ps. The productive MD run was performed in the same isothermal isobaric ensemble for 100 ns. The particle mesh Ewald method for treating electrostatics and the SHAKE algorithm for all the covalent bonds containing hydrogen atoms were applied. The analysis of the obtained trajectories was performed with the ccpraj module of AMBER 20, the visualization and statistical analysis were done in VMD [53] and R package [54], respectively. The Linear Interaction Energy approach with the dielectric constant of 80 was applied to characterize the energetics of the interactions within the system.

3. Results and discussion

3.1. Steady-state fluorescence spectroscopy (SF)

To understand the effect of the hexacyanoferrates on the tryptophan environment, the intrinsic fluorescence changes of bovine serum albumin (BSA) and human serum albumin (HSA) in the presence of increasing concentrations of iron complexes have been studied. BSA and HSA dissolved in 10 mM CACO buffer of pH 7.0 after excitation at 275 nm exhibit a single emission peak with a maximum at 348 nm and 340 nm, respectively (due to tryptophan residues). On the addition of both K₄[Fe(CN)₆] and K₃[Fe(CN)₆], a clear regular decrease in the fluorescence intensity of both proteins can be observed with a very slight shift (approximately 2–3 nm) of the band towards shorter wavelengths

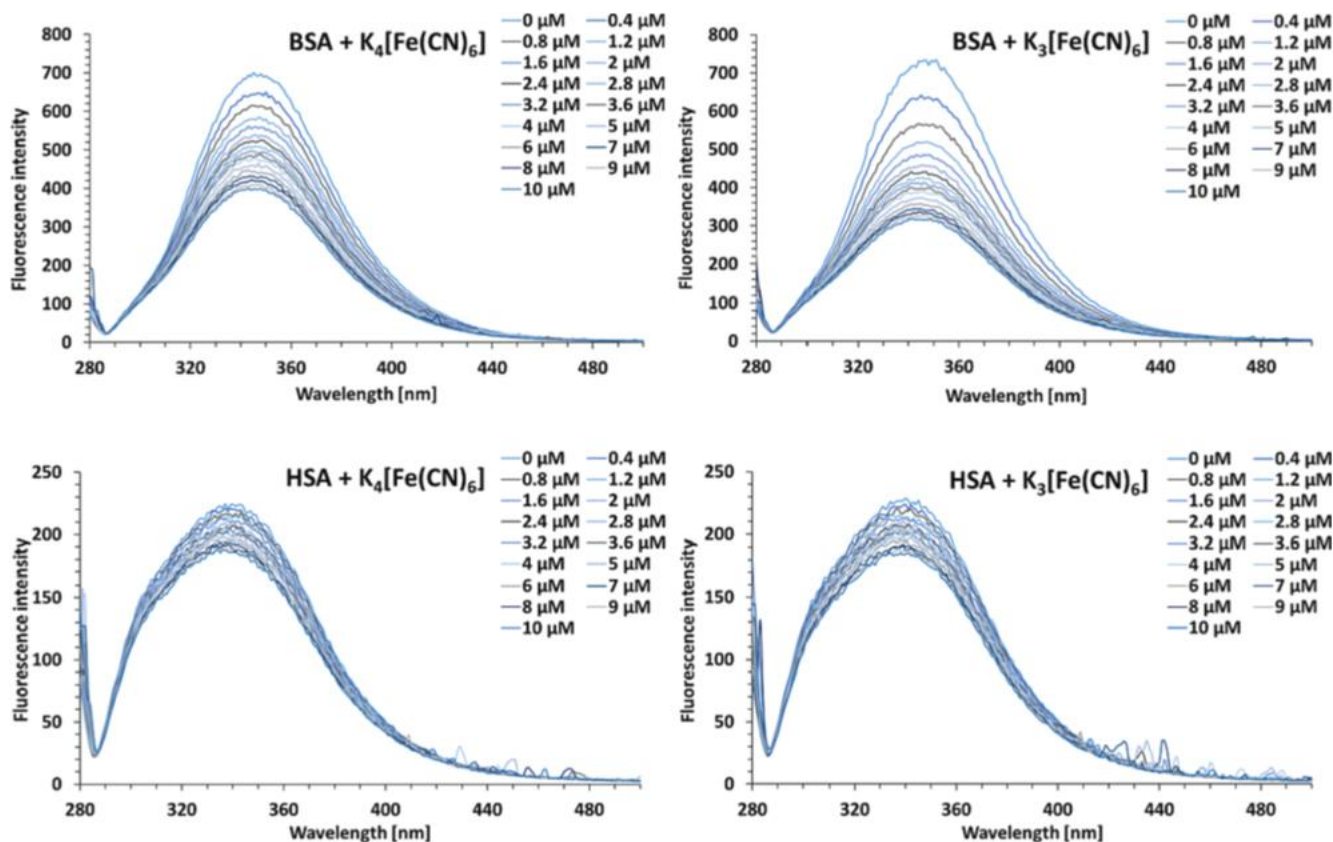


Fig. 1. Fluorescence emission spectra of BSA and HSA in 10 mM CACO buffer of pH 7.0 in the presence of increasing concentrations (0–10 μM) of K₄[Fe(CN)₆] and K₃[Fe(CN)₆]; $\lambda_{\text{exc}} = 275$ nm; $c_{\text{albumin}} = 2$ μM; 298.15 K.

(Fig. 1). These observations suggest that $K_4[\text{Fe}(\text{CN})_6]$ and $K_3[\text{Fe}(\text{CN})_6]$ can bind to BSA and HSA, with a tryptophan of both proteins placed in a more hydrophobic surrounding after the addition of hexacyanoferrates [55].

To get a deeper insight into the qualitative and quantitative mode of the interactions between the albumins and the hexacyanoferrates, the fluorescence quenching data were first analyzed with the Stern-Volmer equation:

$$\frac{F_0}{F} = 1 + K_{SV}[Q] = 1 + k_q\tau_0[Q]$$

where F_0 and F represent the steady-state fluorescence intensities in the absence and presence of a quencher ($K_4[\text{Fe}(\text{CN})_6]$ and $K_3[\text{Fe}(\text{CN})_6]$), $[Q]$ is the quencher concentration, K_{SV} is the Stern–Volmer quenching constant, k_q is the bimolecular quenching rate constant, while τ_0 is the average lifetime of the fluorophore (BSA, HSA) in the absence of a quencher. In the case of purely static or purely dynamic quenching the plots of $\frac{F_0}{F} = f([Q])$ are expected to be linearly dependent on the concentration of the quencher [56]. In our experiments for the quenching of both, BSA and HSA, the Stern–Volmer plots were found to be nonlinear in the initially chosen range of $K_4[\text{Fe}(\text{CN})_6]$ and $K_3[\text{Fe}(\text{CN})_6]$ concentrations (0–10 μM), displaying a downward curvature (towards x axis) which may indicate that only the fluorescent residues on the outer surface can be attained by the quencher (polar or charged quenchers are unable to penetrate in the hydrophobic interior of the protein) [57]. Thus, in the further part of the manuscript all results obtained for fluorescence quenching experiments are presented for the range of concentrations of $K_4[\text{Fe}(\text{CN})_6]$ and $K_3[\text{Fe}(\text{CN})_6]$ from 0 to 2 μM , corresponding to the final hexacyanoferrate/albumin molar ratio equal to 1.0. Fig. 2 presents the Stern–Volmer plots for the quenching of BSA and HSA tryptophan residues by $K_4[\text{Fe}(\text{CN})_6]$ and $K_3[\text{Fe}(\text{CN})_6]$. Consequently, Table 1 presents the newly determined values of Stern–Volmer quenching constants (K_{SV}) along with linear correlation coefficients (R^2) for Stern–Volmer plots and bimolecular quenching rate constants (k_q) recovered for the studied systems. The latter ones were calculated based on the values of the average fluorescence lifetimes τ_0 of free BSA and HSA equal to approximately 6 ns (at phosphate buffer of pH 7.0) [58] and 6.9 ns (at pH 7.5) [59], respectively.

In the studied narrowed concentration range strictly straight lines were obtained with no significant deviations from the observed linearity. The newly determined values of bimolecular quenching rate constants were compared with the largest possible value for collisional quenching, which for the interactions of various quenchers with biopolymer in aqueous media is equal to $2.0 \times 10^{10} \text{ M}^{-1}\text{s}^{-1}$ [60]. It can be clearly seen that the estimated values of k_q for all the studied systems are of the order 10^{12} – $10^{13} \text{ M}^{-1}\text{s}^{-1}$, which is significantly (more than two orders) higher than the limiting diffusion rate constant. Since the values of bimolecular quenching rate constants are considered to be definitive in differentiating between dynamic and static quenching mechanisms,

Table 1

Stern–Volmer quenching constants (K_{SV}), linear correlation coefficients (R^2) and bimolecular quenching rate constants (k_q) recovered for the steady-state fluorescence quenching of BSA and HSA by $K_4[\text{Fe}(\text{CN})_6]$ and $K_3[\text{Fe}(\text{CN})_6]$ in 10 mM CACO buffer of pH 7.0 at 298.15 K.

System		$K_{SV} [\text{M}^{-1}]$	R^2	$k_q [\text{M}^{-1} \text{s}^{-1}]$
BSA	$K_4[\text{Fe}(\text{CN})_6]$	1.51×10^5	0.987	2.52×10^{13}
	$K_3[\text{Fe}(\text{CN})_6]$	3.03×10^5	0.992	5.05×10^{13}
HSA	$K_4[\text{Fe}(\text{CN})_6]$	2.54×10^4	0.989	3.68×10^{12}
	$K_3[\text{Fe}(\text{CN})_6]$	2.80×10^4	0.993	4.06×10^{12}

the predominant role of the formation of ground-state complexes in the investigated systems was affirmed, while that from the dynamic collisions was found to be negligible [61–64].

Additionally, to confirm the static quenching mechanism, the effect of temperature on the values of Stern–Volmer constants was established. For this purpose, fluorescence quenching of BSA and HSA by $K_4[\text{Fe}(\text{CN})_6]$ and $K_3[\text{Fe}(\text{CN})_6]$ was performed at three different temperatures (288.15, 303.15, and 318.15 K) and the results of these experiments are presented as Stern–Volmer plots in Fig. 3. It can be clearly seen that for all studied albumin/hexacyanoferrate systems the slopes of Stern–Volmer plots decrease with an increase of temperature, what is characteristic for the static quenching (weakly bound complexes will typically dissociate at higher temperatures) [65,66], whereas for a dynamic quenching an opposite effect is observed (the higher is the temperature, the faster is diffusion and more numerous are collisions) [67].

Knowing that the fluorescence quenching of both BSA and HSA by $K_4[\text{Fe}(\text{CN})_6]$ and $K_3[\text{Fe}(\text{CN})_6]$ is a static quenching process, the binding (association) constants (K_{bind}) of the newly formed complexes can be determined by the following equation:

$$\frac{1}{F_0 - F} = \frac{1}{F_0} + \frac{K_d}{F_0[Q]}$$

where K_d denotes a dissociation constant ($K_{\text{bind}} = K_d^{-1}$) [68,69]. According to that appropriate plots were constructed for all investigated albumin/hexacyanoferrate systems based on the relationship of $\frac{F_0}{F_0 - F}$ vs. $\frac{1}{[Q]}$ (Fig. 4). From the regression equations applied for these curves, binding constant values were obtained (as the average of two independent experiments) and determined to be 2.24×10^5 for BSA- $K_4[\text{Fe}(\text{CN})_6]$, 3.54×10^5 for BSA- $K_3[\text{Fe}(\text{CN})_6]$, 3.41×10^4 for HSA- $K_4[\text{Fe}(\text{CN})_6]$, and 3.46×10^4 for HSA- $K_3[\text{Fe}(\text{CN})_6]$. The fact that K_{bind} values are very close to K_{SV} (see Table 1) confirms the sole static quenching mechanism [24]. By comparing the obtained binding constants it is clear that both $K_4[\text{Fe}(\text{CN})_6]$ and $K_3[\text{Fe}(\text{CN})_6]$ form more stable complexes with BSA than with HSA. Furthermore, the charge of the complex ions has negligible effect on the strength of the interactions with the albumin.

For a static quenching interaction, when small molecules bind independently to a set of equivalent sites of a protein, the equilibrium

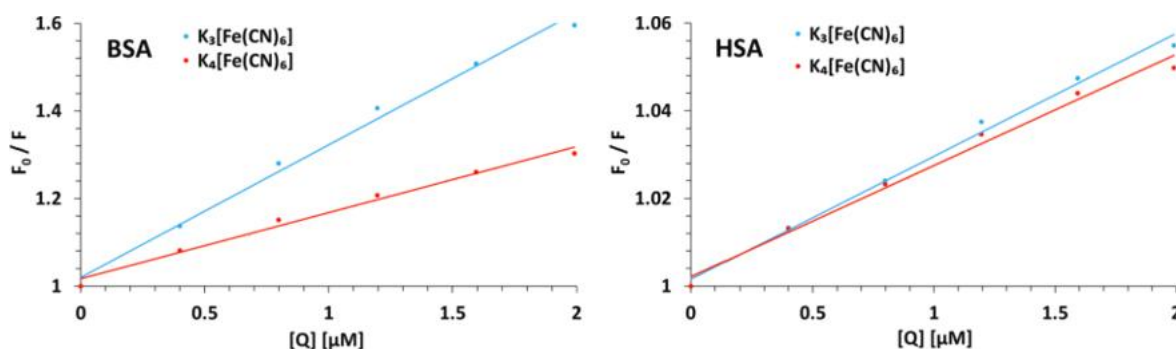


Fig. 2. Stern–Volmer plots for the steady-state fluorescence quenching of BSA and HSA by $K_4[\text{Fe}(\text{CN})_6]$ and $K_3[\text{Fe}(\text{CN})_6]$ in 10 mM CACO buffer of pH 7.0; $\lambda_{\text{ex}} = 275 \text{ nm}$; $\lambda_{\text{em}} = 348 \text{ nm}$ (BSA) and 340 nm (HSA); $c_{\text{albumin}} = 2 \mu\text{M}$; 298.15 K.

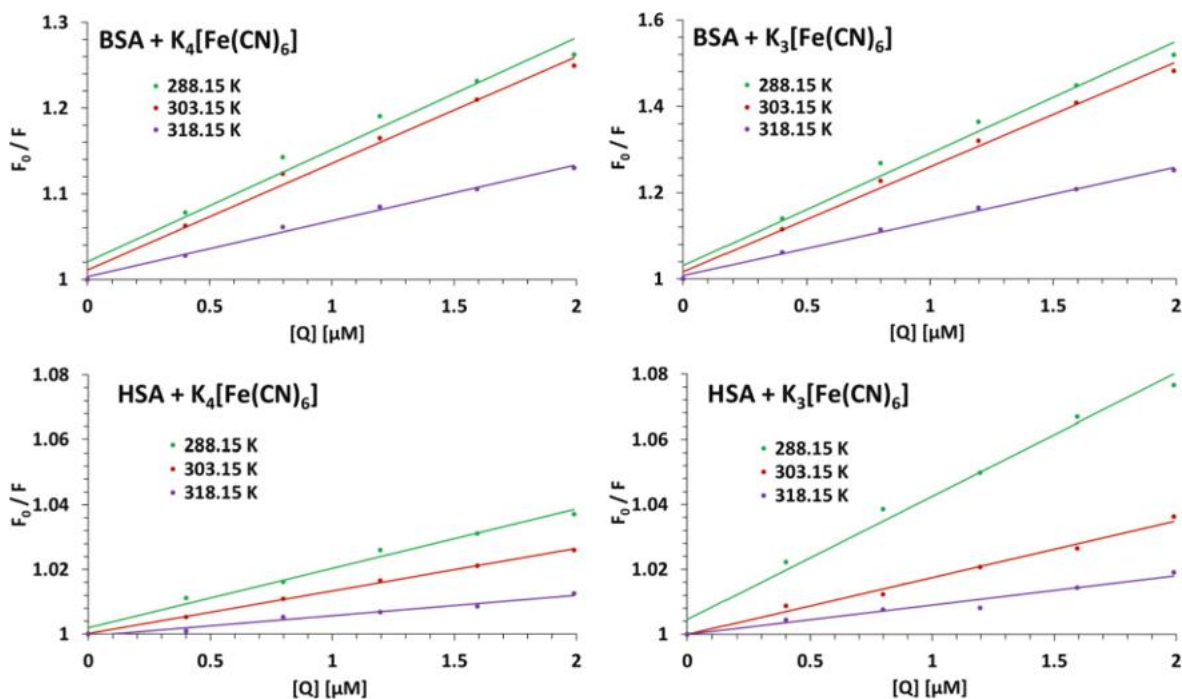


Fig. 3. Stern-Volmer plots for the steady-state fluorescence quenching of BSA and HSA by $K_4[Fe(CN)_6]$ and $K_3[Fe(CN)_6]$ in 10 mM CACO buffer of pH 7.0 at various temperatures (288.15, 303.15, and 318.15 K); $\lambda_{ex} = 275$ nm; $\lambda_{em} = 348$ nm (BSA) and 340 nm (HSA); $c_{albumin} = 2 \mu M$.

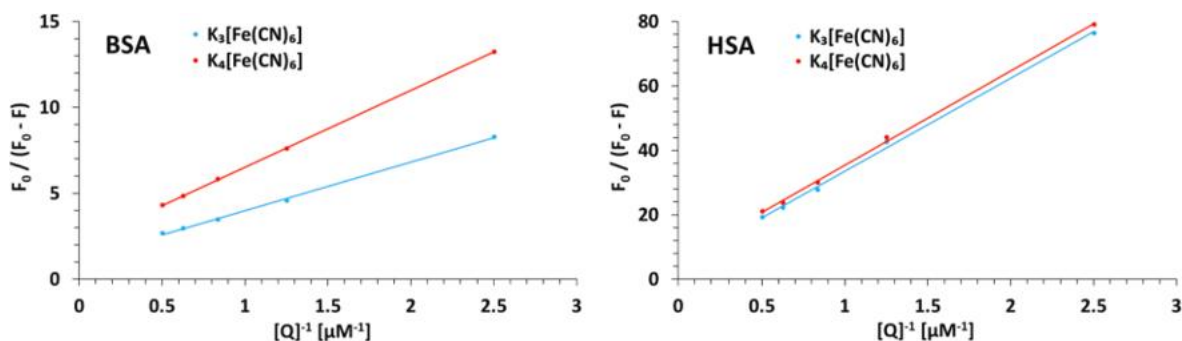


Fig. 4. The plots of $\frac{F_0}{F_0 - F}$ as a function of $[Q]^{-1}$ for the steady-state fluorescence quenching of BSA and HSA by $K_4[Fe(CN)_6]$ and $K_3[Fe(CN)_6]$ in 10 mM CACO buffer of pH 7.0; $\lambda_{ex} = 275$ nm; $\lambda_{em} = 348$ nm (BSA) and 340 nm (HSA); $c_{albumin} = 2 \mu M$; 298.15 K.

between free and bound molecules is given by the following modified version of the Stern-Volmer equation [70,71]:

$$\log \left[\frac{F_0 - F}{F} \right] = \log K_{bind} + n \cdot \log [Q]$$

Thus, the apparent binding constant (K_{bind}) and the number of binding sites (n) can be calculated from the intercepts and the slopes of the linear plots of $\log \left[\frac{F_0 - F}{F} \right]$ versus $\log [Q]$, respectively (Fig. 5). These values have been obtained (as the averages of two independent

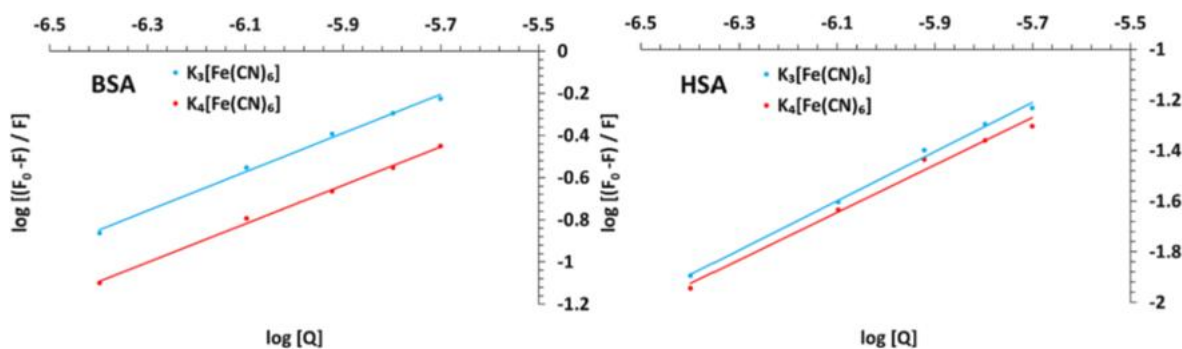


Fig. 5. Modified Stern-Volmer plots for the steady-state fluorescence quenching of BSA and HSA by $K_4[Fe(CN)_6]$ and $K_3[Fe(CN)_6]$ in 10 mM CACO buffer of pH 7.0; $\lambda_{ex} = 275$ nm; $\lambda_{em} = 348$ nm (BSA) and 340 nm (HSA); $c_{albumin} = 2 \mu M$; 298.15 K.

experiments) and are shown in Table 2, along with linear correlation coefficients (R^2) recovered for the appropriate plots.

The value of K_{bind} is particularly relevant for the understanding of the distribution of the drug in plasma. A weak binding, with binding constant values ranging from 10^2 to 10^4 M^{-1} [72–74], can lead to a short lifetime or a poor distribution, while a strong binding (K_{bind} values ranging up to 10^{10} M^{-1}) [26,75,76] is responsible for the reduction in the plasmatic distribution of free drug. The K_{bind} values obtained here (by different equations) for the studied albumin-hexacyanoferrate complexes are considered to lie in the range optimal for transport (the binding should be strong enough to ensure that a significant amount of adduct is actually formed, but at the same time weak enough to permit the ligand's release once reached the target [77]). For all protein-hexacyanoferrate complexes the value of n was approximately equal to one (see Table 2), indicating the existence of just a single binding site in BSA and HSA for the compounds studied. The values of binding constants estimated by this method are basically in accordance with those obtained by the previous equations ($K_{\text{bind}} \text{ BSA-K}_3[\text{Fe}(\text{CN})_6] > K_{\text{bind}} \text{ BSA-K}_4[\text{Fe}(\text{CN})_6] > K_{\text{bind}} \text{ HSA-K}_3[\text{Fe}(\text{CN})_6] > K_{\text{bind}} \text{ HSA-K}_4[\text{Fe}(\text{CN})_6]$). Furthermore, the values of K_{bind} and n obtained from fluorescence measurements are in a very good agreement with those from isothermal titration calorimetry experiments.

The performed spectrofluorometric experiments, apart from the evaluation of the fluorescence quenching mechanism and the determination of appropriate binding constants and numbers of binding sites, enabled to establish the stoichiometry of the resulting albumin-hexacyanoferrate complexes. The binding stoichiometry was evaluated on the basis of the plot of $\frac{F_0-F}{F_0}$ vs. the corresponding hexacyanoferrate/albumin molar concentration ratio [78,79]. The representative curves are presented in Fig. 6. Subsequently, the stoichiometry of the newly formed complexes was estimated from the points where these curves changed their slopes. It can be clearly observed that both BSA and HSA form 1:1 complexes with $\text{K}_4[\text{Fe}(\text{CN})_6]$ and $\text{K}_3[\text{Fe}(\text{CN})_6]$.

Generally, the principal regions of ligand binding to serum albumins are located in hydrophobic cavities in subdomains IIA and IIIA (called Sudlow's binding site I and II, respectively) [80]. The ligand selectivity is comparatively high for these two sites. For instance, warfarin (an anticoagulant drug) and ibuprofen (a non-steroidal anti-inflammatory agent) have been known as stereotypical ligands for Sudlow's site I and II, respectively [81]. On the other hand, there are in fact many distinct binding sites in the structures of both serum albumins [82]. Furthermore, it has been reported that the binding sites for some drug molecules are non-specific [83,84]. To identify the hexacyanoferrates binding sites on BSA and HSA, competitive binding experiments have been performed, using warfarin and ibuprofen as site markers (Fig. 7). It can be clearly observed that for all systems, i.e. BSA- $\text{K}_4[\text{Fe}(\text{CN})_6]$, BSA- $\text{K}_3[\text{Fe}(\text{CN})_6]$, HSA- $\text{K}_4[\text{Fe}(\text{CN})_6]$, and HSA- $\text{K}_3[\text{Fe}(\text{CN})_6]$, the quenching efficiency decreases significantly in the presence of both site markers. Interestingly, the saturation of both, BSA and HSA binding sites with warfarin and ibuprofen used simultaneously results in significantly lower quenching (binding) constants with comparison to the systems with markers added separately. Since significant variations in quenching for the free and the marked protein are observed, it indicates that both warfarin and ibuprofen compete with the hexacyanoferrates(II/III) for

the binding to BSA and HSA and the possible location is at the interface of the warfarin and ibuprofen binding sites [85]. This suggests that neither $\text{K}_4[\text{Fe}(\text{CN})_6]$ nor $\text{K}_3[\text{Fe}(\text{CN})_6]$ binds to the serum albumins in a specific manner [84,85].

3.2. Isothermal titration calorimetry (ITC)

The ITC parameters (the binding constants K_{ITC} and the enthalpy changes ΔH_{ITC}) for the BSA/ $\text{K}_4[\text{Fe}(\text{CN})_6]$, BSA/ $\text{K}_3[\text{Fe}(\text{CN})_6]$, HSA/ $\text{K}_4[\text{Fe}(\text{CN})_6]$, and HSA/ $\text{K}_3[\text{Fe}(\text{CN})_6]$ systems were obtained by fitting binding isotherms, using nonlinear least-squares procedures, to a model that assumes a single set of identical sites. This model gave the best fitting parameters to the experimental data (Fig. 8). ITC measurements revealed that in each albumin there is only one binding site capable of binding the investigated complex ions. This phenomenon stays in line with spectrofluorimetric results. The binding constants K_{ITC} , and consequently the free energies of binding ΔG_{ITC} for both BSA/ $\text{K}_4[\text{Fe}(\text{CN})_6]$ and BSA/ $\text{K}_3[\text{Fe}(\text{CN})_6]$ systems are comparable to one another indicating a similar affinity of both hexacyanoferrates to the bovine serum albumin (Table 3). The same observation has been made for HSA/ $\text{K}_4[\text{Fe}(\text{CN})_6]$ and HSA/ $\text{K}_3[\text{Fe}(\text{CN})_6]$ systems. On the other hand, K_{ITC} and ΔG_{ITC} values obtained for systems with BSA are clearly higher in comparison with these involving HSA, what is in a great accordance with spectrofluorometric experiments. Despite the fact that the exothermic effect of the interactions is more pronounced for the systems with HSA, the binding of hexacyanoferrates(II/III) to both albumins is enthalpy-driven ($|\Delta H_{\text{ITC}}| > |T\Delta S_{\text{ITC}}|$). Thus, the charge-charge type or/and van der Waals interactions seem to be involved in the ion-protein complex formation.

3.3. Circular dichroism spectroscopy (CD)

The CD spectra of the mixtures comprising the albumin/hexacyanoferrate(II/III) components in the molar ratios 1:1, 1:2 and 1:4 were recorded (data not shown), whereas the secondary structure content of albumin in the presence and absence of the coordination ions were collected in Table 4. No dramatic/substantial changes in the secondary structure of the examined albumins on account of hexacyanoferrates(II/III) binding were observed. Thus, the formation of fairly stable albumin complexes with the investigated ions should not influence the biological activity of the proteins.

3.4. Molecular dynamics-based analysis

Competitive fluorescence binding experiments with BSA site markers (warfarin and ibuprofen) indicate non-specific interactions of the protein with the investigated hexacyanoferrates. To support the experimental data and to propose potential binding regions for ferricyanide and ferrocyanide on the BSA surface, computational analysis was performed. The data obtained with molecular dynamics (MD)-based analysis suggest that both $[\text{Fe}(\text{CN})_6]^{3-}$ and $[\text{Fe}(\text{CN})_6]^{4-}$ were located in the proximity of Sudlow's site II of BSA (Fig. 9). This finding stays in line with the results of competitive bindings experiments. The hexacyanoferrate(II) ions occupied more defined areas than hexacyanoferrates(III) due to their higher negative charge. This means that BSA binding to both ligands is probably electrostatically driven. We applied the Linear Interaction Energy (LIE) approach to characterize the interactions in the system: the interactions of each protein residue with each ligand molecule were taken into account when the interaction energy was more favorable than -0.5 kcal/mol . The energies were then normalized through the whole MD trajectory. The following residues represented top 10 contributors according to the ranking based on the comparison of the obtained LIE values (listed starting from the residue contributing most favorably): Arg409, Arg194, Arg185, Asp1, Lys114, Lys413, Lys312, Lys187, Lys388, Arg217 and Lys114, Lys537, Lys499, Lys261, Lys409, Lys362, Lys350, Arg194, Lys275, Lys204 for

Table 2

Apparent binding constants (K_{bind}), linear correlation coefficients (R^2) and numbers of binding sites (n) recovered for the steady-state fluorescence quenching of BSA and HSA by $\text{K}_4[\text{Fe}(\text{CN})_6]$ and $\text{K}_3[\text{Fe}(\text{CN})_6]$ in 10 mM CACO buffer of pH 7.0 at 298.15 K.

System		$K_{\text{bind}} [\text{M}^{-1}]$	R^2	n
BSA	$\text{K}_4[\text{Fe}(\text{CN})_6]$	5.62×10^4	0.997	0.91
	$\text{K}_3[\text{Fe}(\text{CN})_6]$	1.10×10^5	0.995	0.92
HSA	$\text{K}_4[\text{Fe}(\text{CN})_6]$	1.20×10^4	0.988	0.94
	$\text{K}_3[\text{Fe}(\text{CN})_6]$	2.19×10^4	0.995	0.97

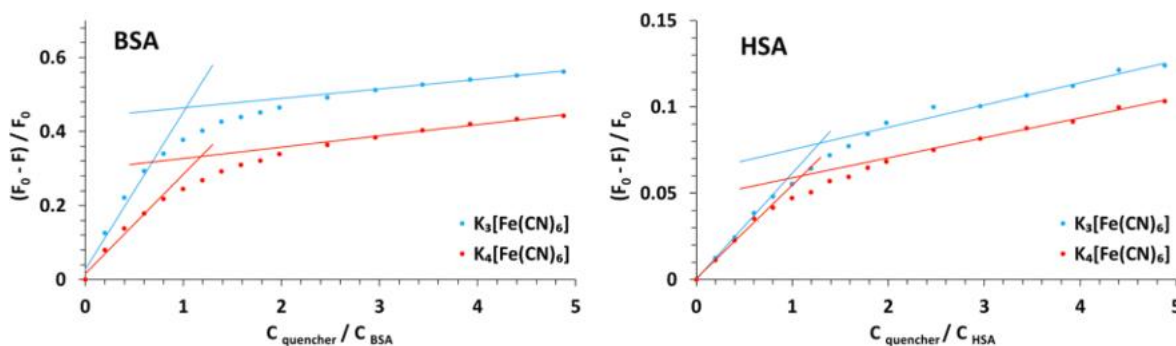


Fig. 6. The plots of $\frac{F_0 - F}{F_0}$ against the hexacyanoferrate/albumin molar concentration ratio for the steady-state fluorescence quenching of BSA and HSA by $K_4[Fe(CN)_6]$ and $K_3[Fe(CN)_6]$ in 10 mM CACO buffer of pH 7.0; $\lambda_{\text{ex}} = 275$ nm; $\lambda_{\text{em}} = 348$ nm (BSA) and 340 nm (HSA); $c_{\text{albumin}} = 2 \mu\text{M}$; 298.15 K. Line-line intersections represent the stoichiometry of the complexes under study.

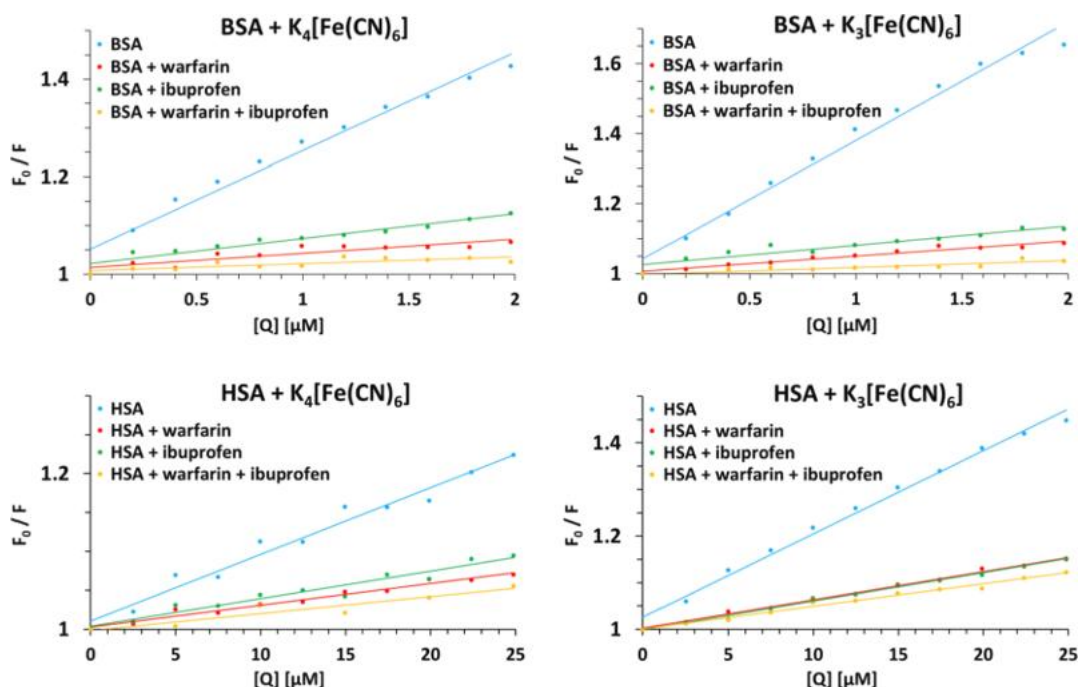


Fig. 7. Stern-Volmer plots of the site marker competitive experiments for BSA- $K_4[Fe(CN)_6]$, BSA- $K_3[Fe(CN)_6]$, HSA- $K_4[Fe(CN)_6]$, and HSA- $K_3[Fe(CN)_6]$ systems in 10 mM CACO buffer of pH 7.0; $\lambda_{\text{ex}} = 275$ nm; $\lambda_{\text{em}} = 348$ nm (BSA) and 340 nm (HSA); $c_{\text{albumin}} = 2 \mu\text{M}$; 298.15 K; the molar ratio of warfarin or/and ibuprofen to BSA and HSA was equal to 12.5:1.

ferricyanide and ferrocyanide, respectively. With the exception of Asp46, which interacted with the ligand by its positively charged N-terminal amino group, all the top contributing residues are positively charged suggesting that electrostatic forces/H-bonds represent the driving forces in the establishment of the protein-ligand interactions. The next top ranked residues had very similar scores and were also Arg and Lys suggesting rather unspecific electrostatic attraction in this system. Nitrogen atoms of the ligands establish H-bonds with the hydrogen atoms from the Arg and Lys side-chains. Furthermore, when comparing the LIE contributions of all protein residues, the corresponding Pearson and Spearman correlations for the complexes with ferricyanide and ferrocyanide are 0.63 and 0.55, respectively. All this suggests a similar binding pattern for both ligands supporting the findings visualized in Fig. 9.

When comparing the impact of ferricyanide and ferrocyanide binding on the dynamics of the BSA protein, the obtained data on RMSD (root mean squared deviation), RMSF (root mean squared fluctuation) and SASA (solvent accessible surface area) support slightly stronger binding effect of ferrocyanide in comparison to ferricyanide (Supplementary

Material; Figures S1-S3). In particular, RMSD in the case of the ferrocyanide binding is higher, meaning the stronger perturbation of the BSA structure (Supplementary Material; Figure S1), which corroborates the higher fluctuations for the residues ~ 450 –500 (Supplementary Material; Figure S2) corresponding to the site II where the binding is most probable according to our calculations. Apart from this, SASA of the protein in the presence of ferrocyanide is lower than in the presence of ferricyanide suggesting more stable contacts of the protein with the ligand in the former case (Supplementary Material; Figure S3). At the same time, no significant differences in terms of LIE energies for ferrocyanide and ferricyanide were obtained.

4. Conclusions

Steady-state fluorescence spectroscopy supported by isothermal titration calorimetry, circular dichroism spectroscopy and molecular dynamics-based computational analysis has been successfully applied to characterize the interactions of bovine and human serum albumins (BSA and HSA, respectively) with model highly negative-charged complexes,

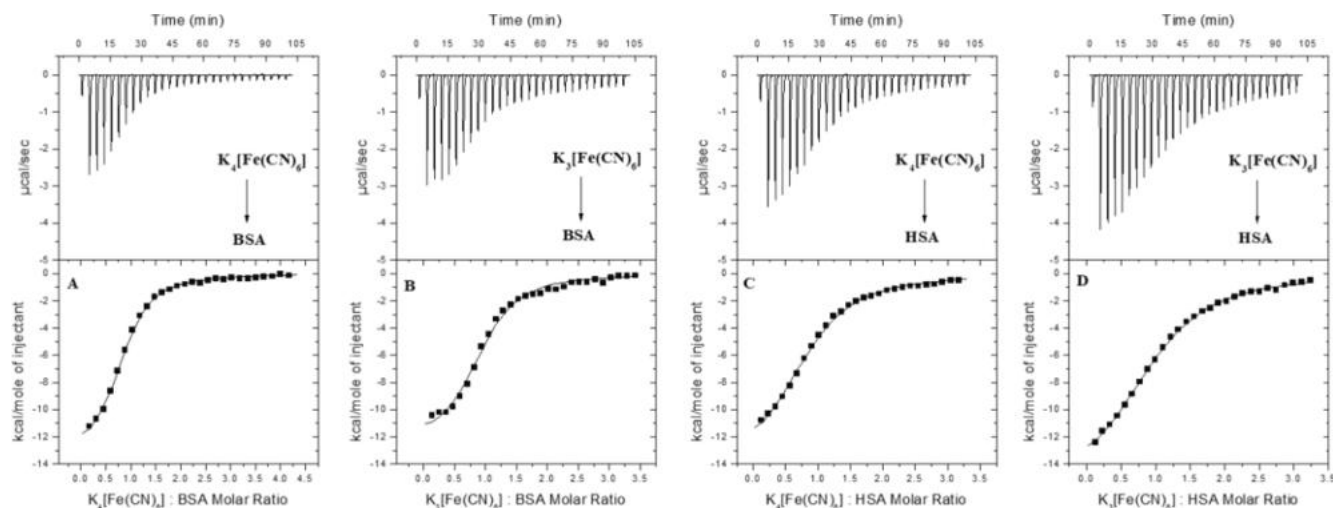


Fig. 8. Calorimetric titration isotherms of the binding interactions between $K_4[Fe(CN)_6]$ and $K_3[Fe(CN)_6]$ and the investigated albumins BSA (A, B) and HSA (C, D) in 10 mM CACO buffer of pH 7.0, at 298.15 K.

Table 3

The conditional thermodynamic parameters of $K_4[Fe(CN)_6]$ and $K_3[Fe(CN)_6]$ binding to BSA and HSA (standard deviation values in parentheses) in 10 mM CACO buffer of pH 7.0, at 298.15 K.

Parameter	BSA		HSA	
	$K_4[Fe(CN)_6]$	$K_3[Fe(CN)_6]$	$K_4[Fe(CN)_6]$	$K_3[Fe(CN)_6]$
Stoichiometry	0.84 (±0.01)	0.95 (±0.02)	0.87 (±0.01)	1.01 (±0.02)
$\log K_{ITC}$	5.31 (±0.11)	5.23 (±0.05)	4.78 (±0.03)	4.70 (±0.02)
ΔG_{ITC} [kcal mol ⁻¹]	-7.25 (±0.03)	-7.14 (±0.07)	-6.52 (±0.04)	-6.41 (±0.02)
ΔH_{ITC} [kcal mol ⁻¹]	-13.50 (±0.18)	-12.44 (±0.35)	-14.47 (±0.18)	-16.35 (±0.25)
$T\Delta S_{ITC}$ [kcal mol ⁻¹]	-6.25	-5.30	-7.95	-9.94

namely hexacyanoferrates(II/III), i.e. $[Fe(CN)_6]^{4-}$ and $[Fe(CN)_6]^{3-}$. The studies performed have proven that both hexacyanoferrates(II/III) form fairly stable complexes of a stoichiometry 1:1 (albumin to ligand), however, a higher affinity of hexacyanoferrates(II/III) to BSA was observed. Even though more exothermic binding was observed for $[Fe(CN)_6]^{4-}$ than $[Fe(CN)_6]^{3-}$ in case of BSA, the charge of the model complex ions does not determine the strength of their charge-charge binding interactions with the albumin. Furthermore, the lack of changes in albumins' secondary structure on account of the ligands binding suggests that the transport of these types of low-molecular-weight species, endowed with a significant negative charge, does not influence the biological activity of the investigated proteins. However, the presence in the systems of hexacyanoferrates(II/III) may affect the binding of weaker ligands (such as drugs) that reveal an affinity to Sudlow's site II of the albumins, as it is in the case of ligands under study. Furthermore, the hexacyanoferrates(II/III) can be considered as potential binding site markers for studying interactions of albumins with highly negative-charged metal complexes of comparable coordination geometry. Finally, the obtained results revealed that the concentration of hexacyanoferrates(II/III) in the systems is governed by their affinity to the protein. This finding highlights the need to take into account the presence of a relatively high amount of albumin in the cell culture media during the analysis of the dose-dependent activity data of the metal complexes evaluated by *in vitro* studies.

Table 4

The secondary structure content (in %, revealed from CD measurements) of BSA and HSA in 10 mM CACO buffer of pH 7.0 in the presence of increasing concentrations (0–12 µM) of $K_4[Fe(CN)_6]$ and $K_3[Fe(CN)_6]$; $c_{albumin} = 3 \mu M$; 298.15 K.

System (molar ratio)	α -helix [%]	strand [%]	turns [%]	unordered [%]
BSA	57	16	11	16
BSA : $K_3[Fe(CN)_6]$ (1:1)	57	20	12	11
BSA : $K_3[Fe(CN)_6]$ (1:2)	55	14	15	16
BSA : $K_3[Fe(CN)_6]$ (1:4)	55	20	12	13
BSA : $K_4[Fe(CN)_6]$ (1:1)	55	19	11	14
BSA : $K_4[Fe(CN)_6]$ (1:2)	55	19	12	14
BSA : $K_4[Fe(CN)_6]$ (1:4)	56	18	14	12
HSA	55	16	12	15
HSA : $K_3[Fe(CN)_6]$ (1:1)	56	16	13	14
HSA : $K_3[Fe(CN)_6]$ (1:2)	57	16	14	12
HSA : $K_3[Fe(CN)_6]$ (1:4)	56	16	15	13
HSA : $K_4[Fe(CN)_6]$ (1:1)	55	17	14	14
HSA : $K_4[Fe(CN)_6]$ (1:2)	55	13	15	17
HSA : $K_4[Fe(CN)_6]$ (1:4)	56	17	12	17

CRediT authorship contribution statement

Ola Grabowska: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing.
Sergey A. Samsonov: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing.
Lech Chmurzyński: Project administration. **Dariusz Wyrzykowski:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Supervision.
Krzysztof Żamojć: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Supervision.

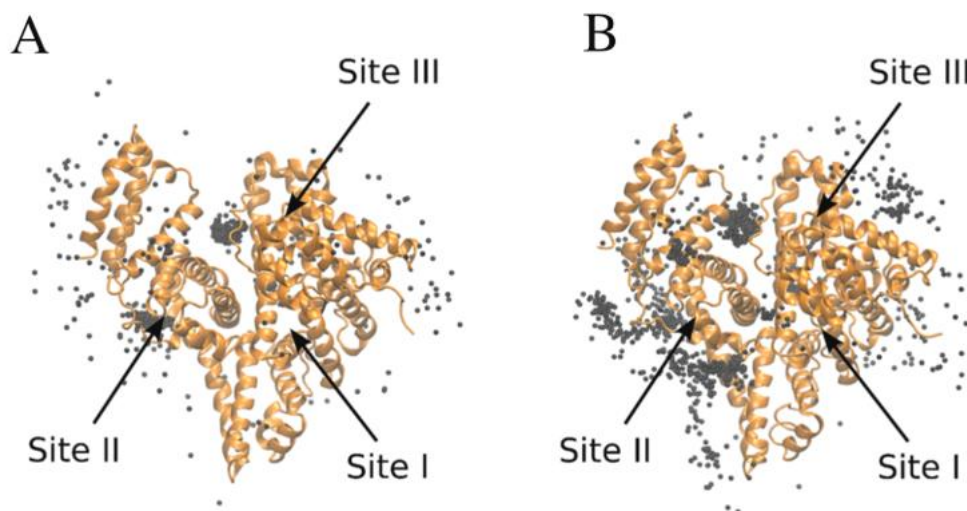


Fig. 9. Molecular dynamics-based analysis of BSA interactions with (A) $[\text{Fe}(\text{CN})_6]^{3-}$ and (B) $[\text{Fe}(\text{CN})_6]^{4-}$. The density of the centers of mass of ligands within 10.0 Å of protein visualized at a step of 0.5 ns as black spheres. The BSA ligand binding sites are labelled.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

This research was funded by National Science Centre of Poland under grant number UMO-2018/31/G/ST4/00246. The computational resources were provided at the local cluster *piasek* at the Faculty of Chemistry, University of Gdansk.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.saa.2023.122505>.

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PUBLIKACJA 10

**An interdisciplinary study of lysozyme interactions
with hexacyanoferrate(III)/(II) ions**



Article

An Interdisciplinary Study of Lysozyme Interactions with Hexacyanoferrate(III)/(II) Ions

Ola Grabowska ^{1,*}, Krzysztof Żamojć ¹, Anna Kloska ², Paweł Niedziałkowski ¹, Sergey A. Samsonov ¹ and Dariusz Wyrzykowski ^{1,*}

¹ Faculty of Chemistry, University of Gdańsk, Wita Stwosza 63, 80-308 Gdańsk, Poland; krzysztof.zamojc@ug.edu.pl (K.Ż.); pawel.niedzialkowski@ug.edu.pl (P.N.); sergey.samsonov@ug.edu.pl (S.A.S.)

² Faculty of Biology, University of Gdańsk, Wita Stwosza 59, 80-308 Gdańsk, Poland; anna.kloska@ug.edu.pl

* Correspondence: ola.grabowska@ug.edu.pl (O.G.); dariusz.wyrzykowski@ug.edu.pl (D.W.); Tel./Fax: +48-58-5235057 (O.G. & D.W.)

Abstract

In this article, the binding interactions of lysozyme with hexacyanoferrate(III)/(II), i.e., $[\text{Fe}(\text{CN})_6]^{3-}$ and $[\text{Fe}(\text{CN})_6]^{4-}$ ions, have been characterised using steady-state fluorescence spectroscopy (SF), isothermal titration calorimetry (ITC), circular dichroism spectroscopy (CD), cyclic voltammetry (CV), and molecular-dynamics-based computational approaches. Studies have shown that under experimental conditions (10 mM cacodylate buffer, pH 7, 298.15 K), complexes with a 1:1 stoichiometry are formed. Four distinct regions on the lysozyme surface patches with the potential to bind hexacyanoferrate(III)/(II) were identified and described. Thermodynamic parameters revealed that the interactions are predominantly governed by electrostatic and van der Waals forces. These interactions enhance the electron transfer kinetics of the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ system. The secondary structure of the protein is not affected by these interactions. Enzyme activity studies demonstrated that the affinity of lysozyme for the substrate remained unchanged regardless of whether free lysozyme or the lysozyme- $[\text{Fe}(\text{CN})_6]^{3-/4-}$ complex was present in the test sample. Finally, biological tests performed on both Gram-positive (*B. subtilis*, *S. aureus*) and Gram-negative (*E. coli*, *P. aeruginosa*) bacteria confirmed the results of the biochemical analysis, indicating that $[\text{Fe}(\text{CN})_6]^{3-/4-}$ ions do not block the active site of the enzyme and do not interfere with its activity.

Keywords: lysozyme; hexacyanoferrate(III)/(II) ions; binding interactions; enzymatic activity



Academic Editor: Piotr Paneth

Received: 13 August 2025

Revised: 29 August 2025

Accepted: 29 August 2025

Published: 2 September 2025

Citation: Grabowska, O.; Żamojć, K.; Kloska, A.; Niedziałkowski, P.; Samsonov, S.A.; Wyrzykowski, D. An Interdisciplinary Study of Lysozyme Interactions with Hexacyanoferrate (III)/(II) Ions. *Int. J. Mol. Sci.* **2025**, *26*, 8511. <https://doi.org/10.3390/ijms26178511>

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1. Introduction

Hexacyanoferrate(III) ($[\text{Fe}(\text{CN})_6]^{3-}$) and hexacyanoferrate(II) ($[\text{Fe}(\text{CN})_6]^{4-}$) ions are among the most thermodynamically stable cyanide complexes. They are characterised by their symmetrical octahedral geometry, the high negative charge of the complex anion and the ability to reversibly change the oxidation state of the coordination centre. From a kinetic point of view, these complexes can be classified as inert, exhibiting a slow exchange of CN^- ligands under certain conditions, namely high temperature and acidity. The specific physicochemical properties of hexacyanoferrate(III)/(II) ions make these coordination units useful in a wide range of chemical applications, including electrochemistry, the pigment industry and analytical and diagnostic chemistry [1–4]. As protons are not involved in the oxidation/reduction equation, the reduction potential of the $[\text{Fe}(\text{CN})_6]^{3-}/[\text{Fe}(\text{CN})_6]^{4-}$ redox couple (E^0 ca. 0.41 V) remains relatively stable across a broad pH range, specifically

from 4 to 13. The $[\text{Fe}(\text{CN})_6]^{3-}$ ion is a relatively weak oxidant with selective activity towards the most readily oxidisable substrates [5,6]. For this reason, it is often used as a scavenger and has the potential to remove contaminants from the system [7]. Hexacyanoferrate(III) ions are also present in some ecosystems as a result of anthropogenic human activities. Particularly high concentrations of these cyanocomplexes are found in wastewater from gold mining, which is associated with the use of cyanide ions in the leaching of gold from minerals [8]. The high affinity of hexacyanoferrate(III)/(II) ions for certain metals prevents their distribution in the natural environment [9,10]. It is interesting to note that hexacyanoferrate(II) in the form of Prussian blue, $\text{Fe}_4[\text{Fe}(\text{CN})_6]_3$, has also been used as an oral agent to increase the excretion of caesium-137 and thallium from the body. It has been shown that the accumulation of $\text{Fe}_4[\text{Fe}(\text{CN})_6]_3$ in the intestine and its interaction with caesium ions disrupt the intestinal-hepatic circulation of radioactive ions, thereby reducing the radioactive burden on the body [11]. However, it should be noted that the presence of exogenous oxidising compounds, such as hexacyanoferrate(III)/(II) ions, has important implications for the course of redox processes. The $[\text{Fe}(\text{CN})_6]^{3-}$ ion has been shown to act as a mild selective oxidant by reacting with glutathione, affecting its redox state, and thereby interfering with its function by impairing its activity in cellular defence against oxidative stress [12].

To the best of our knowledge, little attention has been given to the interactions between proteins and these common cyanocomplexes, which can serve as an excellent model for small molecules with a high negative charge. In recent studies, we have demonstrated that the affinity of hexacyanoferrate(III)/(II) ions for two globular proteins, namely human serum albumin (HSA) and bovine serum albumin (BSA), plays a key role in the bioaccumulation and transport of these species in biological systems. The formation of relatively stable albumin-hexacyanoferrate(III)/(II) complexes at the Sudlow II site effectively blocks this binding site [13]. Consequently, this inhibits the interaction of weaker small-molecule ligands in this region of the protein. To gain a deeper insight into the interactions between proteins and hexacyanoferrate(III)/(II) ions, we have extended our research in this area by focusing on the analysis of interactions with hen egg white lysozyme (HEWL), which contains six tryptophan residues (each can be differently accessible to quenchers—exposed or buried—because they are placed in distinct environments), several of which are known to be near the action site [14]. Understanding the physicochemical principles of these interactions is crucial to understanding the factors that influence the biological activity of lysozyme, which has been identified as a natural product with antiviral [15] and antibacterial [16] properties and is widely used in the life sciences [15]. In addition, HEWL is particularly interesting for the study of protein–ion interactions, especially with negatively charged ions. In contrast to previously studied proteins (HSA, BSA), it has a positive charge (at pH 7), a smaller size and a relatively rigid structure compared to albumins.

2. Results and Discussion

2.1. Binding Analysis of Hexacyanoferrate(III)/(II) Ions to Lysozyme

The intrinsic fluorescence changes in lysozyme in the presence of increasing concentrations of cyanocomplexes have been studied to understand the effect of the hexacyanoferrates (III)/(II) on the tryptophan environment. After being excited at 275 nm, lysozyme dissolved in 10 mM Caco buffer at pH 7 shows a single emission peak with a maximum at 345 nm. When $\text{K}_3[\text{Fe}(\text{CN})_6]$ and $\text{K}_4[\text{Fe}(\text{CN})_6]$ are added, the fluorescence intensity decreases with no significant shift in the observed band (Figure 1). These findings imply that $\text{K}_3[\text{Fe}(\text{CN})_6]$ and $\text{K}_4[\text{Fe}(\text{CN})_6]$ can bind to the protein, with a negligible change in the tryptophan's environment. This indicates that those tryptophan residues emitting at larger wavelengths are not quenched more readily than the shorter-wavelength tryptophans [14].

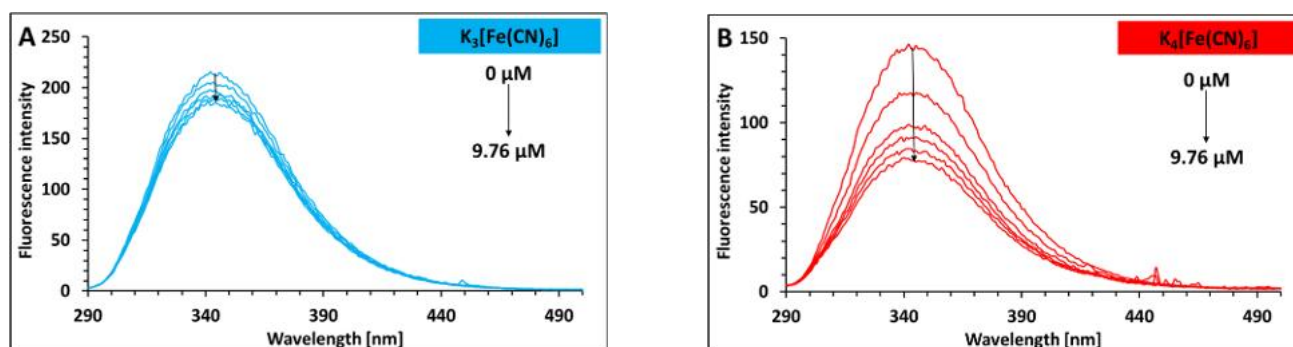


Figure 1. The fluorescence emission spectra of free lysozyme and its mixtures with $K_3[Fe(CN)_6]$ (A); The fluorescence emission spectra of free lysozyme and its mixtures with $K_4[Fe(CN)_6]$ (B). Concentration range: 0–9.76 μM ; in 10 mM Caco buffer of pH 7; λ_{ex} = 275 nm; c_{LYS} = 1.76 μM ; 298.15 K.

The Stern–Volmer equation was initially used to analyse the fluorescence quenching data in order to gain a better understanding of the qualitative and quantitative modes of the interactions between lysozyme and the hexacyanoferrates(III)/(II):

$$\frac{F_0}{F} = 1 + K_{SV}[Q] = 1 + k_q\tau_0[Q], \quad (1)$$

where $[Q]$ is the quencher concentration, K_{SV} is the Stern–Volmer quenching constant, k_q is the bimolecular quenching rate constant, τ_0 is the average lifetime of the lysozyme in the absence of a quencher, and F_0 and F represent the steady-state fluorescence intensities in the absence and presence of a quencher ($K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$), respectively [14]. Plots of $\frac{F_0}{F} = f([Q])$ should be linearly dependent on quencher concentration in the case of either purely static or purely dynamic quenching [17]. The Stern–Volmer plots in our experiments for the quenching of lysozyme by $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$ showed a downward curvature (towards the x-axis) and were non-linear in the initially selected concentration range of the quenchers (0–9.76 μM), which is a characteristic feature of two fluorophore populations, one of which is not accessible to the quencher [14]. This could mean that the quencher can only reach the fluorescent residues on the outer surface (polar or charged quenchers cannot penetrate the hydrophobic interior of the protein) [18]. Therefore, all of the fluorescence quenching experiment results are shown in the next section of the manuscript for the concentration range of $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$ from 0 to 3.96 μM , which corresponds to a final hexacyanoferrate/protein molar ratio of 2.25. The fluorescence quenching of tryptophan residue in lysozyme by $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$ is shown in Figure 2A (as Stern–Volmer plots) along with Stern–Volmer quenching constants (K_{SV}), linear correlation coefficients (R^2), and the newly found values of the bimolecular quenching rate constants (k_q). The latter were computed using the average fluorescence lifetime of free lysozyme, which is roughly 1.36 ns (at pH 8.0 phosphate buffer) [19]. Strictly straight lines with no appreciable departures from the observed linearity were obtained in the investigated narrow concentration range. The newly calculated bimolecular quenching rate constant values were contrasted with the maximum value for collisional quenching, which is $2.0 \times 10^{10} M^{-1} s^{-1}$ for interactions of different quenchers with biopolymers in aqueous media [20]. The estimated values of k_q for each system studied are much higher than the limiting diffusion rate constant (by about three orders of magnitude). Ground-state complex formation was confirmed to play a major role in the systems under investigation, whereas dynamic collisions were found to play a negligible role, as bimolecular quenching rate constant values are thought to be decisive in distinguishing between dynamic and static quenching mechanisms [21–24].

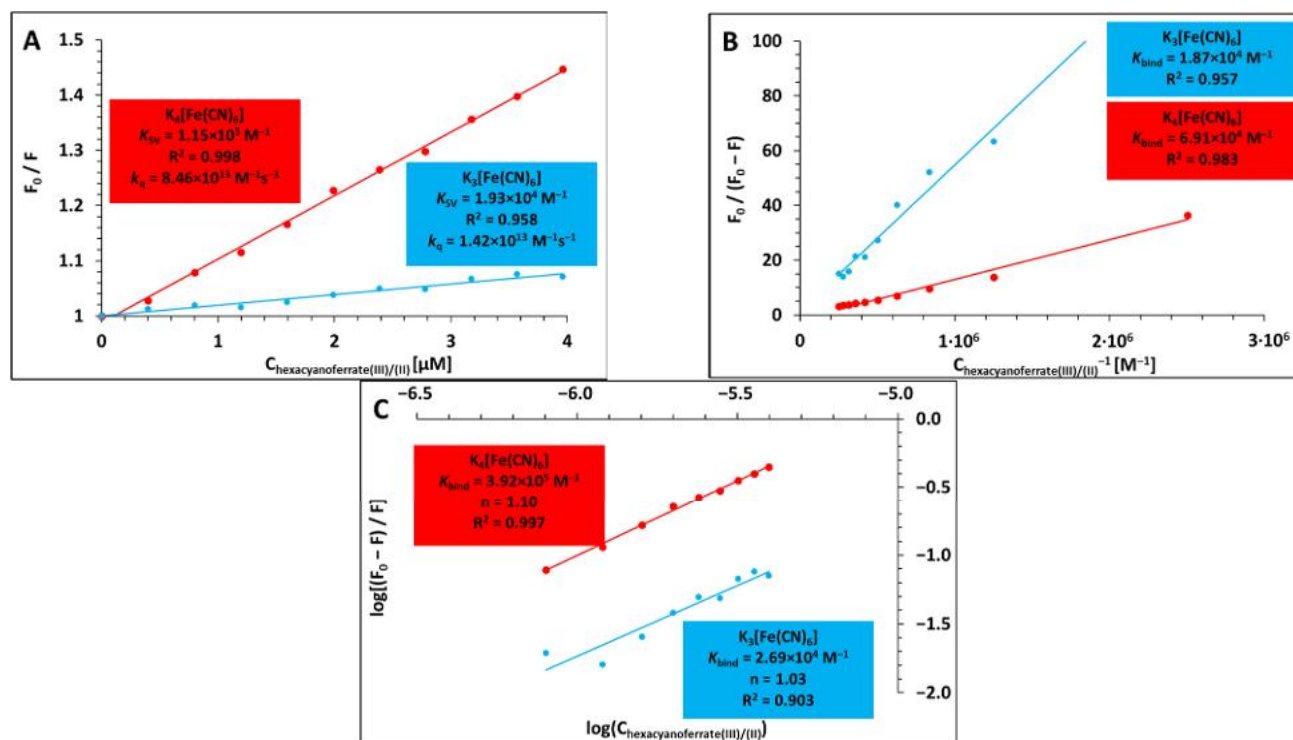


Figure 2. Stern–Volmer plots for the steady-state fluorescence quenching of lysozyme by $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$ (A); The plots of $\frac{F_0}{F_0-F}$ as a function of $[Q]^{-1}$ for the steady-state fluorescence quenching of lysozyme by $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$ (B); The plots of $\log\left[\frac{F_0-F}{F}\right]$ as a function of $\log[Q]$ for the steady-state fluorescence quenching of lysozyme by $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$ (C). All experiments were performed in 10 mM Caco buffer of pH 7; $\lambda_{ex} = 275$ nm; $\lambda_{em} = 345$ nm; $c_{LYS} = 1.76 \mu M$; 298.15 K.

Given that the fluorescence quenching of lysozyme by $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$ is a static quenching process, the following equations [25,26] were used to create appropriate plots (Figure 2B,C) and calculate the binding constants (K) of the newly formed complexes and the number of binding sites:

$$\frac{1}{F_0 - F} = \frac{1}{F_0} + \frac{1}{K \cdot F_0 [Q]}, \quad (2)$$

$$\log\left[\frac{F_0 - F}{F}\right] = \log K + n \cdot \log[Q]. \quad (3)$$

The binding constant values as well as the number of binding sites for both of the protein/hexacyanoferrate(III)/(II) systems under investigation were derived from the regression equations used for these curves and are gathered in Table 1.

Table 1. The conditional thermodynamic parameters of $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$ binding to lysozyme (standard deviation values in parentheses) in the 10 mM Caco buffer of pH 7, at 298.15 K.

Parameter	Lysozyme	
	$K_3[Fe(CN)_6]$	$K_4[Fe(CN)_6]$
n (number of binding sites)	1.03	1.10
N^{**} (stoichiometry)	1.01 (± 0.01)	0.86 (± 0.02)

Table 1. Cont.

Parameter	Lysozyme	
	$K_3[Fe(CN)_6]$	$K_4[Fe(CN)_6]$
$\log(K_{SV})^{*1}$	4.29	5.06
$\log(K)^{*2}$	4.27	4.84
$\log(K)^{*3}$	4.43	5.59
$\log(K)^{**}$	3.90 (± 0.02)	4.56 (± 0.03)
ΔG^{**} [kJ mol $^{-1}$]	−22.30 (± 0.13)	−26.02 (± 0.17)
ΔH^{**} [kJ mol $^{-1}$]	−16.61 (± 0.29)	−19.62 (± 0.59)
$T\Delta S^{**}$ [kJ mol $^{-1}$]	5.69	6.40

^{*1} SF data (Equation (1)); ^{*2} SF data (Equation (2)); ^{*3} SF data (Equation (3)); ^{**} ITC data.

Along with steady-state fluorescence spectroscopy, isothermal titration calorimetry (ITC) was used to provide a complete thermodynamic characterisation of the interactions studied. The parameters of the interactions, including the stoichiometry of the resulting protein–ligand complexes, binding constants, and thermodynamic parameters, are summarised in Table 1, while the representative binding isotherms for the lysozyme–hexacyanoferrate(III)/(II) system are shown in Figure 3.

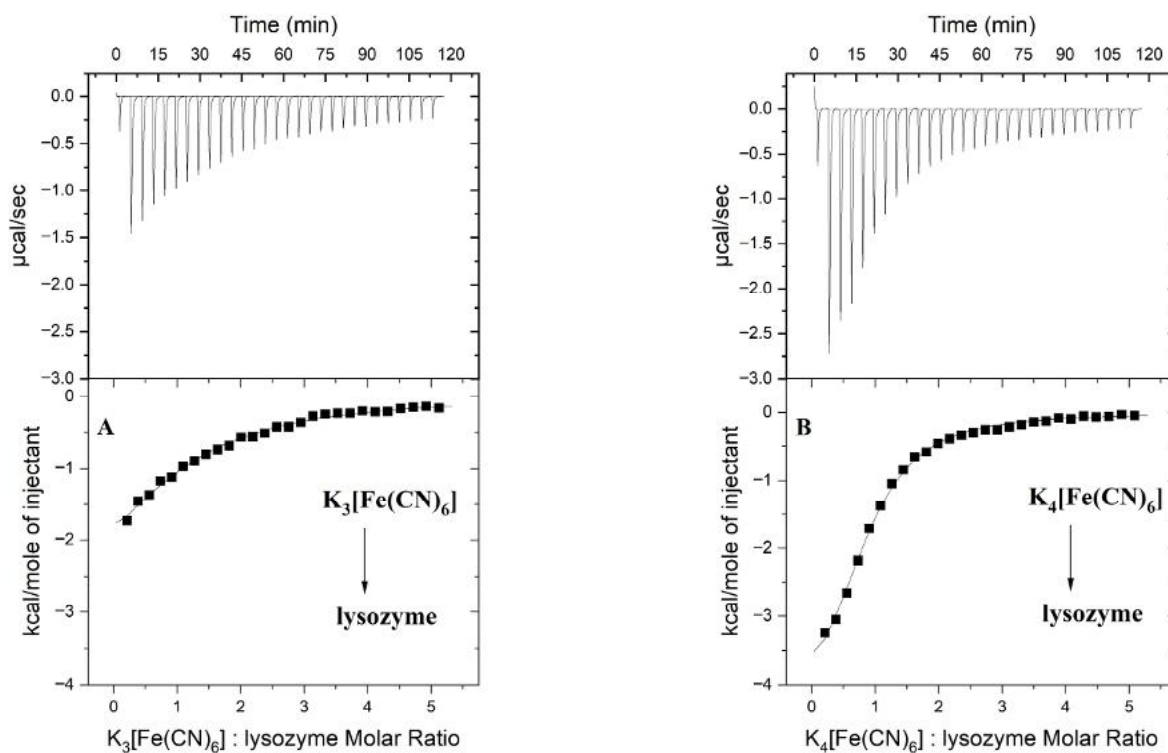


Figure 3. Calorimetric titration isotherm of the binding interactions of $K_3[Fe(CN)_6]$ with lysozyme (A); calorimetric titration isotherms of the binding interactions of $K_4[Fe(CN)_6]$ with lysozyme (B). Experiments were performed in the 10 mM Caco buffer of pH 7, at 298.15 K.

The ITC data were processed by fitting binding isotherms to the equilibrium model that assumes a single set of identical binding sites. The validity of the fitting model used was previously confirmed by the results of the SF, which revealed the presence of one site on the lysozyme surface capable of binding $[Fe(CN)_6]^{3-}$ and $[Fe(CN)_6]^{4-}$ complexes (Table 1).

The species distribution diagrams that represent the percentage of a free protein ([lysozyme]) and the fraction of a protein bound to a ligand ([lysozyme]-ligand) are shown in Figure 4. The saturation fraction, $\theta = \alpha_{\text{lysozyme-ligand}} = \frac{[\text{lysozyme-ligand}]}{c_{\text{lysozyme}}}$ (where $\alpha_{\text{lysozyme-ligand}}$ denotes the fraction of a protein bound to a ligand; c_{lysozyme} is the total concentration of the protein), quantifies the extent to which the binding site is occupied by $[\text{Fe}(\text{CN})_6]^{3-}$ and $[\text{Fe}(\text{CN})_6]^{4-}$ and reflects the dynamic equilibrium between the free and bound states of the protein–ligand complex. According to the SF and ITC results (Table 1), it has been proven that $[\text{Fe}(\text{CN})_6]^{4-}$, which carries a higher negative charge, exhibits stronger interactions with lysozyme compared to $[\text{Fe}(\text{CN})_6]^{3-}$. Consequently, this leads to more efficient protein saturation when bound to $[\text{Fe}(\text{CN})_6]^{4-}$, highlighting the crucial influence of charge in regulating binding affinity and saturation efficiency (Figure 4).

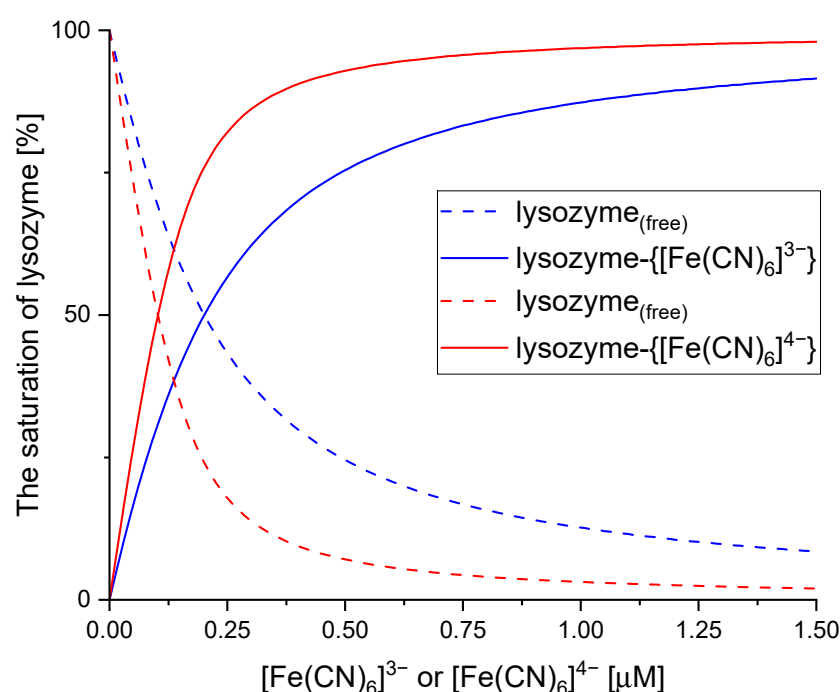


Figure 4. The relationship between the saturation fraction of lysozyme (θ) and the concentration of free ligands ($[\text{Fe}(\text{CN})_6]^{3-}$ or $[\text{Fe}(\text{CN})_6]^{4-}$).

Thermodynamic parameters of the resulting protein–ligand complexes indicate that the interactions are mainly governed by the enthalpy factors $|\Delta H| > |T\Delta S|$. The negative binding enthalpies confirm the pivotal role of electrostatic and van der Waals forces in the binding processes and simultaneously explain a slightly higher affinity of the positively charged protein for $[\text{Fe}(\text{CN})_6]^{4-}$ than for $[\text{Fe}(\text{CN})_6]^{3-}$. The observed slight increase in entropy values can be attributed to the dehydration of the reactants and/or ion exchange occurring at the binding site of the hexacyanoferrate(III)/(II) ions with the protein. However, circular dichroism studies showed that the observed affinity of $[\text{Fe}(\text{CN})_6]^{3-}$ and $[\text{Fe}(\text{CN})_6]^{4-}$ for lysozyme, although mainly governed by electrostatic interactions, had little effect on changes in the secondary structure of lysozyme, despite the different charges of the cyanocomplexes (Figure S1 and Table S1).

It is important to note that the total charge of a protein does not serve as a straightforward determinant of its affinity for negatively charged ligands. In addition to the charge of the ligand, factors such as the spatial arrangement of the ligand, the presence of hydrophobic regions within the ligand structure, and the ligand's size relative to the protein surface are also significant considerations. The observed differences in the hexacyanoferrate complexes are reflected in their binding affinities to lysozyme and albumin, namely

human serum albumin (HSA) and bovine serum albumin (BSA). Previous studies have shown that both albumins have a single binding site for the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ complexes, with a stoichiometric binding ratio of 1:1 [13]. Significantly, the binding strength of these complexes to albumins is higher even though albumin has a net negative charge under the same experimental conditions (pH 7). In contrast, the interactions between $[\text{Fe}(\text{CN})_6]^{3-/4-}$ and lysozyme revealed that the charge of the complexes affects the strength of the binding interactions with lysozyme. The situation is different for the interaction of $[\text{Fe}(\text{CN})_6]^{3-}$ and $[\text{Fe}(\text{CN})_6]^{4-}$ ions with BSA. Studies have shown a similar affinity of both ions for albumin, suggesting that the difference in charge between these ions does not affect their binding behaviour [13]. The possible explanation for this phenomenon is that albumin, as the primary transport protein in the bloodstream, has multiple binding sites that can accommodate a wide range of endogenous and exogenous compounds based on their shape, size or hydrophobicity. In addition, the structural flexibility of albumin facilitates conformational changes that enhance its ligand binding capacity. In contrast, the binding interactions of lysozyme tend to be more specific and less flexible. Since strong binding reduces the plasmatic distribution of free drug, while weak binding can result in a short lifetime or poor distribution, the value of the binding constant is especially important for understanding the drug's distribution in plasma [27,28]. When compared to other well-known strong biomolecule–ligand complexes with binding constants ranging from 10^5 to 10^8 M^{-1} [29–32], the binding constant values obtained for the investigated lysozyme complexes indicate a moderate binding affinity to the protein for both hexacyanoferrates.

2.2. The Electrochemical Behaviour of Hexacyanoferrate(III)/(II) and Its Lysozyme Complexes

The $[\text{Fe}(\text{CN})_6]^{3-/4-}$ ions are well known for their ability to reversibly accept or donate a single electron. It has been reported that the reduction potential of this redox couple depends on various environmental factors, including the type of solvent and the presence of metal ions in the system [33]. In this study, we have focused on the analysis of the effect of the experimental conditions (the type of buffer used) and the binding affinity of hexacyanoferrate(III)/(II) to lysozyme on the electrochemical behaviour of $[\text{Fe}(\text{CN})_6]^{3-/4-}$.

The cyclic voltammograms of the investigated samples (vs. Ag/AgCl reference electrode) are presented in Figure S2. The separation between the anodic (E_{pa}) and cathodic (E_{pc}) peak potentials ($\Delta E_p = E_{pa} - E_{pc}$) was studied in two solutions, namely 0.1 M KCl and 0.1 M Caco buffer (pH 7). The first system was used as a reference solution because 0.1 M KCl is the most commonly used electrolyte for electrochemical analysis. The results showed a strong influence of Caco buffer on the cyclic voltammetry response. In the case of 0.1 M KCl, the ΔE_p value was 82 mV, whereas in 0.1 M Caco buffer, the ΔE_p value increased to 168 mV, indicating a decrease in kinetic barriers resulting in less favourable electron transfer in the Caco buffer. At the same time, it is noteworthy that the components of the Caco buffer are not redox-active in the given experimental potential range (Figure S2).

Interestingly, quite different behaviour was observed in the case of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ bound with lysozyme. The peak-to-peak separation (ΔE_p) for the system containing $[\text{Fe}(\text{CN})_6]^{3-}$ saturated with protein was found to be 67 mV after correction for dilution. In particular, the observed oxidation and reduction current peaks were comparable to those observed in the voltammograms of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ in 0.1 M KCl. The observed electrochemical behaviour indicates that the interaction between $[\text{Fe}(\text{CN})_6]^{3-/4-}$ and the protein significantly enhances the electron transfer kinetics. This enhancement is evident when compared to the situation where hexacyanoferrate(III)/(II) ions are present in solution in an unbound (free) form.

2.3. Binding Sites of Hexacyanoferrate(III)/(II) Ions to Lysozyme

There are several binding sites on the lysozyme surface capable of binding small negatively charged species. Some of these sites are specific for lysozyme, while others are specific for the anion [34]. Lysozyme has been reported to bind polyanions such as DNA and RNA, both specifically and non-specifically [35]. Similar binding interactions were observed for small polyvalent anions, namely adenosine triphosphate (ATP) and triphosphate (TPP) [36].

To gain insights into the atomistic details that explain the differences in the binding of the $[\text{Fe}(\text{CN})_6]^{3-}$ and $[\text{Fe}(\text{CN})_6]^{4-}$ anions to the lysozyme, we employed molecular dynamics (MD) simulations. The MD simulations allowed the identification of four distinct regions that have the potential to bind the complexes (Figure 5). These regions are characterised by the presence of positively charged residues: Arg5, Lys33, Arg125 (region I); Lys1, Arg14 (region II); Arg45, Arg68 (region III); and Arg112, Arg114, Lys116 (region IV) (Figure 5A).

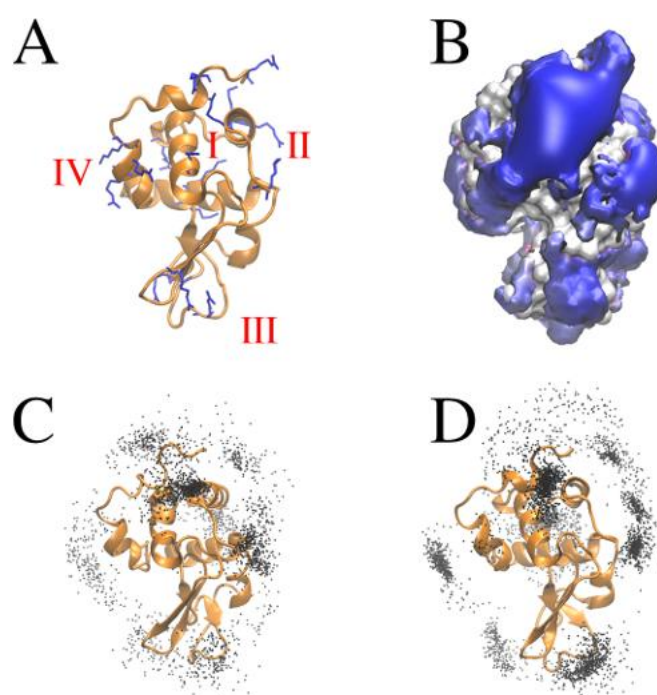


Figure 5. MD analysis of lysozyme interactions with the $[\text{Fe}(\text{CN})_6]^{3-}$ and $[\text{Fe}(\text{CN})_6]^{4-}$ anions. Experimental structure of chicken lysozyme (PDB ID: 193L). Positively charged residues are shown in blue sticks. Roman numbers (in red) correspond to the regions predicted to be involved in binding the $[\text{Fe}(\text{CN})_6]^{3-}$ and $[\text{Fe}(\text{CN})_6]^{4-}$ anions (A). An electrostatic potential isosurface of $2.5 \text{ kcal mol}^{-1} \text{ e}^{-1}$ calculated using the PBSA approach (B). The density of the centres of mass of ligands within $10.0 \text{ \AA}$ of protein visualised at a step of 1 ns: $[\text{Fe}(\text{CN})_6]^{3-}$ (C); $[\text{Fe}(\text{CN})_6]^{4-}$ (D).

The observation that these regions are localised similarly, regardless of the charge of $[\text{Fe}(\text{CN})_6]^{3-}$ or $[\text{Fe}(\text{CN})_6]^{4-}$ on the surface patches corresponding to the highest positive electrostatic potential (Figure 5B), indicates that the interactions between the anions and lysozyme are predominantly governed by electrostatic forces. Furthermore, the more pronounced density of the points in space corresponding to the position of the ligand centres of mass during the MD simulation for $[\text{Fe}(\text{CN})_6]^{4-}$ supports the crucial role of electrostatic interactions in this system (Figure 5C,D). These findings are consistent with the results of the calorimetric study. In the MD simulation, $[\text{Fe}(\text{CN})_6]^{4-}$ forms more contacts with lysozyme compared to $[\text{Fe}(\text{CN})_6]^{3-}$. These contacts are defined as those occurring within a cutoff distance of $10 \text{ \AA}$ between the centre of mass of a ligand molecule and any protein residue (Figure S3). Linear interaction energy (LIE) analysis also suggests that

the interactions between the $[\text{Fe}(\text{CN})_6]^{4-}$ and lysozyme are stronger than in the case of $[\text{Fe}(\text{CN})_6]^{3-}$: there are ~1.5 more frames where the LIE energies for a protein–ligand pair were lower than -1 and -2 kcal mol^{-1} . At the same time, the per residue contacts are very similar: the correlation between the time fractions of the contacts established by a residue for two different ligands is 0.79.

2.4. Lysozyme Activity

2.4.1. Biochemical Studies

The active site of lysozyme is located in a cleft on the enzyme's exterior. It is responsible for catalysing the hydrolysis of beta-(1,4)-glycosidic bonds between *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) in the peptidoglycan layer of the bacterial cell walls. Two critical amino acid residues within this active site, glutamic acid 35 (Glu35) and aspartate 52 (Asp52), are essential for catalytic activity [37]. To assess how the binding interactions between lysozyme and hexacyanoferrate(III)/(II) ions affect the enzymatic activity of lysozyme, a synthetic fluorogenic substrate, 4-methylumbelliferyl β -D-*N,N',N''*-triacetylchitotrioside (4-MUF-triNAG), was used. Lysozyme catalyses the hydrolysis of glycosidic bonds, resulting in the release of the fluorescent product, 4-methylumbelliferone (4-MUF). This is manifested by an increase in the fluorescence intensity, which directly correlates with the enzymatic activity of lysozyme. The enzymatic activities of the investigated systems are presented in Table 2.

Table 2. Enzymatic activity of the lysozyme systems investigated. The activity was assessed after 60 min of incubation with a synthetic substrate at 310.15 K. Each reaction contained 2 nmoles of enzyme and 10 nmoles of substrate (equivalent to 100 μM) in a final volume of 100 μL . *N*, number of replicates.

Reagent	<i>N</i>	Activity (Moles $\text{min}^{-1} \text{ mL}^{-1}$)	Activity (% Vs. Lysozyme)
lysozyme	5	21.9 (± 1.20) ^a	100 (± 5.5)
lysozyme- $[\text{Fe}(\text{CN})_6]^{3-}$	4	22.0 (± 1.59) ^b	101 (± 7.3)
lysozyme- $[\text{Fe}(\text{CN})_6]^{4-}$	4	17.8 (± 3.24) ^{ab}	82 (± 14.8)
$[\text{Fe}(\text{CN})_6]^{3-}$	3	0.3 (± 0.13)	1 (± 0.6)
$[\text{Fe}(\text{CN})_6]^{4-}$	3	0.2 (± 0.19)	1 (± 0.9)
Caco buffer	3	0.0 (± 0.00)	0 (± 0.0)

^{a,b}—significant difference between means with common superscript determined by ANOVA ($F(2,10) = 5.3067$, $p = 0.0269$) and Tukey's post hoc test ($p < 0.05$) performed for lysozyme-containing systems.

Lysozyme activity assessed after 60 min incubation with reaction substrate was maintained in the presence of $[\text{Fe}(\text{CN})_6]^{3-}$ but reduced in the presence of $[\text{Fe}(\text{CN})_6]^{4-}$ to $82 \pm 14.8\%$ relative activity compared to lysozyme alone. Controls containing only hexacyanoferrate(III)/(II) ions or buffer showed negligible activity in the assay, confirming the specificity of the enzyme–substrate reaction.

In addition, the experimental data were fitted to the Michaelis–Menten and Lineweaver–Burk equations to obtain the kinetic parameters, specifically the maximum reaction velocity (V_{max}) and Michaelis constant (K_{m}) characterising the studied systems (Table 3 and Figure S4).

The K_{m} values determined for the investigated systems were equal to the experimental error range. Moreover, a reaction rate equal to half V_{max} was reached at a substrate concentration of around 19 μM (Table 3). The rate of 4-MUF formation by hydrolysis of 4-MUF-triNAG was 5–6 nM min^{-1} for 20 μM enzyme, irrespective of the presence of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ ions in the system. These results indicate that the affinity of the enzyme

for the substrate remained constant, irrespective of whether free lysozyme or its complex (lysozyme- $[\text{Fe}(\text{CN})_6]^{3-/4-}$) was present in the test sample.

Table 3. Enzyme kinetic coefficients for the investigated systems. Values were calculated from data obtained from two independent experiments. [E], enzyme concentration; K_m , Michaelis constant; $V_{\max}$, maximal velocity (maximal reaction rate); k_{cat} , catalytic constant; k_{cat}/K_m , specificity constant (catalytic efficiency).

Coefficient	Unit	Lysozyme	Lysozyme- $[\text{Fe}(\text{CN})_6]^{3-}$	Lysozyme- $[\text{Fe}(\text{CN})_6]^{4-}$
[E]	μM	20	20	20
K_m	μM	19.12 (± 0.40)	18.57 (± 3.29)	19.04 (± 2.15)
$V_{\max}$	nM min^{-1}	5.39 (± 0.23)	5.89 (± 0.37)	5.28 (± 0.21)
k_{cat}	min^{-1}	0.270	0.295	0.264
k_{cat}/K_m	$\text{nM}^{-1} \text{ min}^{-1}$	0.014	0.016	0.014

No significant differences in K_m and $V_{\max}$ were detected by ANOVA, with $F(2,3) = 0.0341$ ($p = 0.9668$) and $F(2,3) = 2.7002$ ($p = 0.2134$), respectively.

2.4.2. Biological Studies

The results of biochemical studies on lysozyme activity were subsequently verified by studying the effects of a free enzyme and lysozyme- $[\text{Fe}(\text{CN})_6]^{3-/4-}$ complexes on selected bacterial strains with different morphologies. The biological tests were performed on both Gram-positive (*B. subtilis*, *S. aureus*) and Gram-negative (*E. coli*, *P. aeruginosa*) bacteria. Cacodylate buffer (used for studying binding interactions) and hexacyanoferrate(III)/(II) ions had negligible effects on bacterial strain viability. In turn, the activity of lysozyme and the lysozyme- $[\text{Fe}(\text{CN})_6]^{3-/4-}$ complexes depends mainly on the type of bacteria and the experimental conditions, namely the concentration of the lytic agents and the incubation time (Figure S5). Among Gram-positive bacteria, *S. aureus* was more resistant to lysis than *B. subtilis*, which is related to various adaptations exhibited by pathogenic *S. aureus*, including modifications to peptidoglycan structure (e.g., O-acetylation or presence of teichoic acid), secretion of lysozyme inhibitors, and the presence of surface-associated proteins that enhance resistance to enzymatic degradation which may contribute to its reduced susceptibility to lysozyme [38,39]. As a soil bacterium, *B. subtilis* is less exposed to lysozyme in its natural environment and may lack such robust defence mechanisms. On the other hand, Gram-negative *E. coli* and *P. aureus* were characterised by a significant resistance to lytic agents, primarily due to their outer membrane composed of lipopolysaccharides (LPS), phospholipids, and proteins, which is impermeable to lysozyme making them inherently more resistant to lysozyme compared to Gram-positive bacteria [38,39]. Interestingly, the bioassay results show that binding hexacyanoferrate(III)/(II) ions to the protein does not change the enzyme's activity. This finding aligns with the results from the chemical assays, indicating that $[\text{Fe}(\text{CN})_6]^{3-/4-}$ ions do not inhibit lysozyme by blocking access to its active site.

3. Materials and Methods

3.1. Materials

Lysozyme from chicken egg white (dialyzed, lyophilized, powder, CAS: 12650-88-3, Sigma-Aldrich, Poland), potassium hexacyanoferrate(III) ($\text{K}_3[\text{Fe}(\text{CN})_6]$, $\geq 99.0\%$, CAS: 13746-66-2, Sigma-Aldrich, Poznań, Poland), potassium hexacyanoferrate(II) trihydrate ($\text{K}_4[\text{Fe}(\text{CN})_6]$, $\geq 98.5\%$, CAS: 14459-95-1, Sigma-Aldrich, Poland), sodium cacodylate trihydrate (Caco, $\geq 98\%$, CAS: 6131-99-3, Sigma-Aldrich, Poland), sodium acetate (CH_3COONa , $\geq 99.0\%$, CAS: 127-09-3, Sigma-Aldrich, Poznań, Poland), sodium carbonate (Na_2CO_3 , $\geq 99.5\%$, CAS: 497-19-8, Sigma-Aldrich, Poznań, Poland), dimethyl sulfox-

ide (DMSO, $\geq 99.5\%$, CAS: 67-68-5, POCH, Poland), 4-methylumbelliferyl β -D- N,N',N'' -triacylchitotrioside (4-MUF-triNAG, $\geq 98.0\%$, CAS: 53643-13-3, Sigma-Aldrich, Poznań, Poland), 4-methylumbelliferone (4-MUF, $\geq 98.0\%$, CAS: 90-33-5, Sigma-Aldrich, Poznań, Poland), potassium chloride (KCl, p.a., CAS: 7447-40-7, Stanlab, Lublin, Poland) were used without additional purification.

3.2. Isothermal Titration Calorimetry (ITC)

ITC experiments were performed using an Auto-ITC isothermal titration calorimeter (MicroCal, GE Healthcare, Northampton, MA, USA). Details of the instrument and experimental setup have been described previously [13]. The experimental conditions were as follows: a 10 mM cacodylate buffer; pH 7; $T = 298.15$ K; the sample cell containing a 0.1 mM lysozyme solution; titrations involved 2.5 mM solutions of $K_3[Fe(CN)_6]$ or $K_4[Fe(CN)_6]$ as the titrant; injection duration = 20 s; injection spacing = 240 s.

3.3. Steady-State Fluorescence Spectroscopy (SF)

All spectrofluorometric experiments were performed at 298.15 K using the stock solutions of potassium hexacyanoferrates(III)/(II) and lysozyme, which were prepared in 10 mM Caco buffer of pH 7. A Perkin Elmer Lambda 650 (Waltham, MA, USA) UV/Vis spectrophotometer was used to record the UV absorption spectra in order to spectrophotometrically measure the lysozyme concentration using an extinction coefficient equal to $\epsilon_{280}^{LYS} = 38,460 \text{ M}^{-1} \text{ cm}^{-1}$ [40]. Emission spectra and fluorescence intensities were recorded using the Cary Eclipse Varian (Agilent, Santa Clara, CA, USA) spectrofluorometer, which has a temperature controller and a 1.0 cm multicell holder. Both devices were calibrated with certified reference materials for wavelength and absorbance to ensure the accuracy of the measurements. The widths of the excitation and emission slits were set at 5 nm. The constant excitation wavelength was 275 nm. The inner filter effect was adjusted for all fluorescence intensity values. In the absence and presence of increasing concentrations of $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$, each up to $9.76 \mu\text{M}$, the fluorescence emission spectra of lysozyme ($1.76 \mu\text{M}$) were recorded from 290 to 500 nm. Ten additions of $2 \mu\text{L}$ and then six additions of $5 \mu\text{L}$ stock solutions of $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$ ($c = 0.4 \text{ mM}$) were used to titrate 2 mL of the protein at a fixed concentration equal to $1.76 \mu\text{M}$ in the subsequent fluorescence titration experiments (conducted manually using a micropipette). The mixture was shaken after each addition, and at least ten readings of the fluorescence intensity were taken. The binding constants and other parameters were determined using the fluorescence intensities of the band at 345 nm, which corresponds to the initial maximum emission of lysozyme.

3.4. Circular Dichroism Spectroscopy (CD)

Circular dichroism (CD) spectra were recorded at 298.15 K using a Jasco-715 automated spectropolarimeter (Jasco Inc., USA). The analysed solutions contained $14 \mu\text{M}$ lysozyme and a ligand (either $[Fe(CN)_6]^{3-}$ or $[Fe(CN)_6]^{4-}$) at lysozyme-to-ligand molar ratios of 1:0, 1:1, 1:5 and 1:10. All solutions were prepared in the 10 mM Caco buffer of pH 7. The experimental data were processed according to the procedure described in [41].

3.5. Molecular Dynamics (MD) Simulations

The structure of the wild-type chicken lysozyme was obtained from the PDB (PDB ID: 193L, $1.33 \text{ \AA}$). Details of the structures and calculations involving $[Fe(CN)_6]^{3-}$ and $[Fe(CN)_6]^{4-}$ ions are described in [13]. Briefly, MD simulations were conducted using AMBER20 [42] for two systems: lysozyme with 10 hexacyanoferrate(III) and hexacyanoferrate(II) species. Ligands were randomly placed around the protein in a truncated octahedron TIP3P water box with a $10 \text{ \AA}$ water layer, and 22 and 32 K^+ ions were added to

the respective systems. The ff14SB force field [43] was used for the protein, and gaff [44] with custom parameters was used for the cyanide complexes. The systems underwent two energy minimization steps—first with restraints, then without—followed by heating from 0 to 300.15 K over 10 ps with restraints. Equilibration was performed at 300.15 K and 105 Pa for 100 ps, followed by a 0.5 μ s production run performed in the same isothermal isobaric ensemble. The particle mesh Ewald method for treating electrostatics and the SHAKE algorithm for all the covalent bonds containing hydrogen atoms were applied. The analysis of the obtained trajectories was performed with the ccpraj module of AMBER 20, the visualisation and statistical analysis were conducted in VMD [45] and R package [46], respectively.

3.6. Biochemical and Biological Assays

Lysozyme Activity Assay. The enzymatic activity of lysozyme was quantified using a fluorometric assay with a synthetic substrate for the enzyme. The protocol was developed based on previous studies [47,48]. The reagents and solutions included 20 mM sodium acetate at pH 5 (assay buffer), 0.2 M sodium carbonate at pH 10–11 (stop buffer), 10 mM 4-methylumbelliferyl β -D-N,N',N''-triacylchitotrioside (4-MUF-triNAG) hydrate prepared in dimethyl sulfoxide (substrate stock solution; stored in aliquots at 253.15 K protected from light), and 5 mM 4-methylumbelliferone (4-MUF) prepared in dimethyl sulfoxide (standard stock solution; stored in aliquots at 253.15 K protected from light).

Reactions were performed in 96-well black plates at a final volume of 100 μ L per well. Test samples (20 μ L) were mixed with 70 μ L of assay buffer and 10 μ L of substrate working solution (1 mM 4-MUF-triNAG prepared freshly by diluting the substrate stock solution in assay buffer immediately before use). Sample background controls (20 μ L sample and 80 μ L assay buffer) and reagent background controls (90 μ L assay buffer and 10 μ L substrate working solution) were included. Standards were prepared by serial dilution of 10 μ M 4-MUF working solution (freshly prepared in assay buffer from 4-MUF stock solution) to generate a standard curve (0–200 pmol/well). The reactions were incubated at 310.15 K for 60 min in the dark and terminated by adding 100 μ L of stop buffer. Fluorescence was measured immediately at excitation/emission wavelengths of 360/445 nm using a microplate reader.

The corrected fluorescence was determined by subtracting the blank readings from all the samples and standard readings. A standard curve was plotted, and the amount of 4-MUF generated in the test samples was calculated using the standard curve equation. The lysozyme activity ($\text{pmol min}^{-1} \text{mL}^{-1}$) was determined using the following formula:

$$\text{lysozyme activity} = \left(\frac{B}{\Delta T \times V} \right) \times D, \quad (4)$$

where B is the amount of 4-MUF (pmol) in the sample wells, ΔT is the reaction time (min), V is the sample volume (mL), and D is the dilution factor.

Lysozyme Kinetic Assay. The enzyme kinetic was assessed as described above, with reaction mixtures containing 4-MUF-triNAG substrate concentrations ranging from 1.5625 to 100 μ M, prepared by twofold serial dilutions, and a fixed enzyme concentration of 20 μ M. Reactions were terminated at 5 min intervals by the addition of stop buffer. The amount of 4-MUF generated was calculated using the standard curve described above, and these values were used to determine the kinetic parameters using the online tool ICEKAT [49,50].

Bacterial Lysis Assay. The effects of the tested compounds on bacterial lysis were evaluated using the broth dilution method. The bacterial strains used were *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. Overnight cultures of these strains were grown in Luria–Bertani (LB) broth at 310.15 K with shaking. For

inoculation, bacterial suspensions were prepared by diluting overnight cultures in LB broth to an optical density at 600 nm (OD₆₀₀) of 0.6.

In a 96-well plate, 100 µL of LB broth containing two-fold serial dilutions of the tested compounds (final concentration range: 3.13–25 µM) was combined with 100 µL of the prepared bacterial suspensions. Background controls consisted of LB broth with the tested compounds, but without bacterial inoculation. Plates were incubated at 310.15 K with shaking, and bacterial growth was monitored by measuring the OD₆₀₀ at 1 and 18 h using a microplate reader.

Bacterial lysis activity was calculated as the percentage of lysis relative to the growth of the control (bacteria in LB broth without compounds), based on OD₆₀₀ values corrected for background controls.

Statistical Analysis. One-way ANOVA followed by Tukey's post hoc test was performed to assess statistical significance, with a threshold of $p < 0.05$ considered significant.

3.7. Cyclic Voltammetry (CV)

The cyclic voltammetry (CV) was carried out using a Multi Autolab M204, by Metrohm. All measurements were performed in three electrode cells at room temperature, using a glassy carbon electrode as the working electrode, a platinum wire as the counter electrode and Ag/AgCl in 0.1 M NaCl as the reference electrode. The working electrode of diameter 3 mm before each experiment was polished on polishing pads (Buehler) using 3 µm alumina suspension. All cyclic voltammograms were obtained in degassed solutions by passing argon through them for 5 min, at a scan rate of 100 mV s^{−1}.

4. Conclusions

A few complementary experimental methods supported by *in silico* analysis have successfully been applied to describe the physicochemical nature of lysozyme interactions with a model highly charged species, namely [Fe(CN)₆]^{3−} and [Fe(CN)₆]^{4−}. Special attention was paid to the understanding of the physicochemical principles underlying these interactions, in particular the factors influencing the mutual affinity between the studied molecules and the nature of the intermolecular forces involved. In addition, the impact of these interactions on the biological activity of the enzyme and the redox properties of [Fe(CN)₆]^{3−/4−} ions has been assessed. Lysozyme–[Fe(CN)₆]^{3−/4−} complexes have been shown to exhibit moderate stability compared to other known protein–ligand complexes. In particular, their formation does not interfere with enzyme activity, as confirmed by biochemical assays and supported by molecular dynamics simulations. These findings suggest that hexacyanoferrate(III)/(II) ions do not hinder access to the active site of the enzyme, thus allowing its proper catalytic activity. This comprehensive approach is advancing our knowledge of the mechanisms governing enzyme–ligand interactions and their implications for both biochemical function and potential applications. Furthermore, the finding that [Fe(CN)₆]^{3−/4−} ions do not interfere with the active site of lysozyme suggests their potential role as cofactors in enzymatic reactions, which justifies further investigation of their mechanistic contributions in biological systems.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms26178511/s1>.

Author Contributions: Conceptualization, O.G. and D.W.; methodology, O.G., K.Ž., A.K., P.N. and S.A.S.; software, S.A.S.; formal analysis, O.G., K.Ž., A.K., P.N. and S.A.S.; investigation, O.G.; writing—original draft preparation, O.G., K.Ž., A.K., P.N., S.A.S. and D.W.; writing—review and editing, O.G., K.Ž., A.K., P.N., S.A.S. and D.W.; supervision, D.W.; funding acquisition, S.A.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by National Science Centre of Poland, grant number UMO-2023/49/B/ST4/00041. The computational resources were provided at the local cluster piasek at the Faculty of Chemistry, University of Gdansk.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article and Supplementary Materials.

Conflicts of Interest: The authors declare no conflicts of interest.

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**OŚWIADCZENIA AUTORÓW DOTYCZĄCE PUBLIKACJI
WCHODZĄCYCH W SKŁAD ROZPRAWY DOKTORSKIEJ**

mgr Ola Grabowska
Katedra Chemii Ogólnej i Nieorganicznej
Wydział Chemii Uniwersytetu Gdańskiego
ul. Wita Stwosza 63, 80-308 Gdańsk
e-mail: ola.grabowska@ug.edu.pl
tel: +48 58 523 50 57

Gdańsk, 22.04.2026 r.

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie poniższych publikacji polegał na opracowaniu wraz z promotorem koncepcji badań, projektowaniu oraz współprowadzeniu eksperymentów przy wykorzystaniu izotermicznego miareczkowania kalorymetrycznego, miareczkowania konduktometrycznego, spektroskopii dichroizmu kołowego. Współuczestniczyłam w analizie i interpretacji uzyskanych danych eksperymentalnych i teoretycznych. Zarządzałam danymi, współuczestniczyłam w przygotowywaniu i redakcji manuskryptów oraz odpowiadaniu na uwagi recenzentów.

Tytuł: Physicochemical nature of sodium dodecyl sulfate interactions with bovine serum albumin revealed by interdisciplinary approaches

Autorzy: A. Tesmar, M.M. Kogut, K. Żamojć, O. Grabowska, K. Chmur, S.A. Samsonov, J. Makowska, D. Wyrzykowski, L. Chmurzyński

Opublikowana w: Journal of Molecular Liquids, 2021, 340, 117185, liczba punktów MNiSW - 100 pkt, IF₂₀₂₁ = 6,6

Tytuł: Effect of tetraphenylborate on physicochemical properties of bovine serum albumin

Autorzy: O. Grabowska, M.M. Kogut, K. Żamojć, S.A. Samsonov, J. Makowska, A. Tesmar, K. Chmur, D. Wyrzykowski, L. Chmurzyński

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Ola Grabowska

Tytuł: Implications of albumin in cell culture media on the biological action of vanadates(V)

Autorzy: O. Grabowska, M. Zdrowowicz, D. Miłoś, K. Żamojć, K. Chmur, A. Tesmar, M. Kapica, L. Chmurzyński, D. Wyrzykowski

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Opublikowana w: International Journal of Biological Macromolecules, 2024, 266, 131134, liczba punktów MNiSW - 100 pkt, IF₂₀₂₄ = 8,5

Tytuł: Exploring the impact of subtle differences in the chemical structure of 1-alkylsulfates and 1-alkylsulfonates on their interactions with human serum albumin

Autorzy: O. Grabowska, A. Singh, K. Żamojć, S.A. Samsonov, D. Wyrzykowski

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Autorzy: O. Grabowska, K. Żamojć, M. Olewniczak, L. Chmurzyński, D. Wyrzykowski

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Autorzy: M.M. Kogut, O. Grabowska, D. Wyrzykowski, S.A. Samsonov

Opublikowana w: Journal of Molecular Liquids, 2022, 364, 119978, liczba punktów MNiSW - 100 pkt, IF₂₀₂₂ = 6,0

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Autorzy: O. Grabowska, M.M. Kogut-Günthel, S.A. Samsonov, D. Wyrzykowski, J. Makowska

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.....*Ola Grabowska*.....

podpis

dr hab. Dariusz Wyrzykowski, prof. UG
Katedra Chemii Ogólnej i Nieorganicznej
Wydział Chemii Uniwersytetu Gdańskiego
ul. Wita Stwosza 63, 80-308 Gdańsk
e-mail: dariusz.wyrzykowski@ug.edu.pl
tel: +48 58 523 50 62

Gdańsk, 24.03.2026 r.

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie poniższych publikacji polegał na opiece merytorycznej podczas planowania oraz realizacji prac badawczych, współudziale w przygotowaniu manuskryptów oraz odpowiadaniu na uwagi recenzentów.

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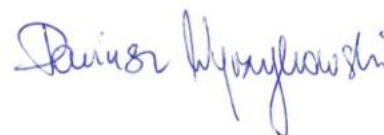
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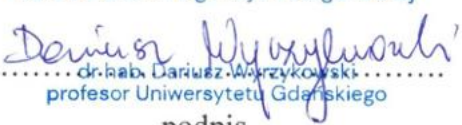
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KIEROWNIK
Katedra Chemii Ogólnej i Nieorganicznej

..... dr.hab. Dariusz Wyrzykowski
profesor Uniwersytetu Gdańskiego
podpis

dr inż. Krzysztof Żamojć
Katedra Chemii Ogólnej i Nieorganicznej
Wydział Chemii Uniwersytetu Gdańskiego
ul. Wita Stwosza 63, 80-308 Gdańsk
e-mail: krzysztof.zamojc@ug.edu.pl
tel: +48 58 523 50 57

Gdańsk, 16.03.2026 r.

OŚWIADCZENIE

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Autorzy: O. Grabowska, S.A. Samsonov, M.M. Kogut-Günthel, K. Żamojć, D. Wyrzykowski

Opublikowana w: International Journal of Biological Macromolecules, 2024, 266, 131134, liczba punktów MNiSW - 100 pkt, IF₂₀₂₄ = 8,5

Tytuł: Exploring the impact of subtle differences in the chemical structure of 1-alkylsulfates and 1-alkylsulfonates on their interactions with human serum albumin

Autorzy: O. Grabowska, A. Singh, K. Żamojć, S.A. Samsonov, D. Wyrzykowski

Opublikowana w: Molecules, 2024, 29, 4598, liczba punktów MNiSW - 140 pkt, IF₂₀₂₄ = 4,6

Tytuł: Can sodium 1-alkylsulfonates participate in the sodium dodecyl sulfate micelle formation?

Autorzy: O. Grabowska, K. Żamojć, M. Olewniczak, L. Chmurzyński, D. Wyrzykowski

Opublikowana w: Journal of Molecular Liquids, 2023, 377, 121568, liczba punktów MNiSW - 100 pkt, IF₂₀₂₃ = 5,3

Tytuł: Investigation of hexacyanoferrate (II)/(III) charge-dependent interactions with bovine and human serum albumins

Autorzy: O. Grabowska, S.A. Samsonov, L. Chmurzyński, D. Wyrzykowski, K. Żamojć

Opublikowana w: Spectrochimica Acta A: Molecular and Biomolecular Spectroscopy, 2023, 293, 122505, liczba punktów MNiSW - 140 pkt, IF₂₀₂₃ = 4,3



Tytuł: An interdisciplinary study of lysozyme interactions with hexacyanoferrate(III)/(II) ions

Autorzy: O. Grabowska, K. Żamojć, A. Kloska, P. Niedziałkowski, S.A. Samsonov, D. Wyrzykowski

Opublikowana w: International Journal of Molecular Sciences, 2025, 26, 8511,
liczba punktów MNiSW - 140 pkt, IF₂₀₂₄ = 4,9


dr inż. Krzysztof Żamojć
Uniwersytet Gdański
Wydział Chemii

.....
podpis

OŚWIADCZENIE

Oświadczam, że mój wkład w postanie poniższych publikacji polegał na przeprowadzeniu badań teoretycznych, współudziale w pisaniu manuskryptów, wizualizacji wyników oraz odpowiadaniu na uwagi recenzentów.

Tytuł: Physicochemical nature of sodium dodecyl sulfate interactions with bovine serum albumin revealed by interdisciplinary approaches

Autorzy: A. Tesmar, M.M. Kogut, K. Żamojć, O. Grabowska, K. Chmur, S.A. Samsonov, J. Makowska, D. Wyrzykowski, L. Chmurzyński

Opublikowana w: Journal of Molecular Liquids, 2021, 340, 117185, liczba punktów MNiSW - 100 pkt, IF₂₀₂₁ = 6,6

Tytuł: Effect of tetraphenylborate on physicochemical properties of bovine serum albumin

Autorzy: O. Grabowska, M.M. Kogut, K. Żamojć, S.A. Samsonov, J. Makowska, A. Tesmar, K. Chmur, D. Wyrzykowski, L. Chmurzyński

Opublikowana w: Molecules, 2021, 26, 6565, liczba punktów MNiSW - 140 pkt, IF₂₀₂₁ = 4,9

Tytuł: Elucidation of binding mechanisms of bovine serum albumin and 1-alkylsulfonates with different hydrophobic chain lengths

Autorzy: O. Grabowska, S.A. Samsonov, M.M. Kogut-Günthel, K. Żamojć, D. Wyrzykowski

Opublikowana w: International Journal of Biological Macromolecules, 2024, 266, 131134, liczba punktów MNiSW - 100 pkt, IF₂₀₂₄ = 8,5

Małgorzata Kogut - Günthel

Tytuł: Affinity and putative entrance mechanisms of alkyl sulfates into the β -CD cavity

Autorzy: M.M. Kogut, O. Grabowska, D. Wyrzykowski, S.A. Samsonov

Opublikowana w: Journal of Molecular Liquids, 2022, 364, 119978, liczba punktów
MNiSW - 100 pkt, IF₂₀₂₂ = 6,0

Tytuł: β -cyclodextrin saves lysozyme from the clutches of 1-alkylsulfonates

Autorzy: O. Grabowska, M.M. Kogut-Günthel, S.A. Samsonov, D. Wyrzykowski,
J. Makowska

Opublikowana w: Journal of Molecular Liquids, 2025, 428, 127543,
liczba punktów MNiSW - 100 pkt, IF₂₀₂₄ = 5,2



podpis

prof. dr hab. Sergey A. Samsonov
Katedra Chemii Teoretycznej
Wydział Chemii Uniwersytetu Gdańskiego
ul. Wita Stwosza 63, 80-308 Gdańsk
e-mail: sergey.samsonov@ug.edu.pl
tel: +48 58 523 51 66

Gdańsk, 23.04.2026 r.

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie poniższych publikacji polegał na opracowaniu metodologii badawczej, nadzorowaniu teoretycznej części badań, współudziale w pisaniu manuskryptów, ich edycji oraz odpowiadaniu na uwagi recenzentów.

Tytuł: Physicochemical nature of sodium dodecyl sulfate interactions with bovine serum albumin revealed by interdisciplinary approaches

Autorzy: A. Tesmar, M.M. Kogut, K. Żamojć, O. Grabowska, K. Chmur, S.A. Samsonov, J. Makowska, D. Wyrzykowski, L. Chmurzyński

Opublikowana w: Journal of Molecular Liquids, 2021, 340, 117185, liczba punktów MNiSW - 100 pkt, IF₂₀₂₁ = 6,6

Tytuł: Effect of tetraphenylborate on physicochemical properties of bovine serum albumin

Autorzy: O. Grabowska, M.M. Kogut, K. Żamojć, S.A. Samsonov, J. Makowska, A. Tesmar, K. Chmur, D. Wyrzykowski, L. Chmurzyński

Opublikowana w: Molecules, 2021, 26, 6565, liczba punktów MNiSW - 140 pkt, IF₂₀₂₁ = 4,9

Sergey Samsonov

Tytuł: Elucidation of binding mechanisms of bovine serum albumin and 1-alkylsulfonates with different hydrophobic chain lengths

Autorzy: O. Grabowska, S.A. Samsonov, M.M. Kogut-Günthel, K. Żamojć, D. Wyrzykowski

Opublikowana w: International Journal of Biological Macromolecules, 2024, 266, 131134, liczba punktów MNiSW - 100 pkt, IF₂₀₂₄ = 8,5

Tytuł: Exploring the impact of subtle differences in the chemical structure of 1-alkylsulfates and 1-alkylsulfonates on their interactions with human serum albumin

Autorzy: O. Grabowska, A. Singh, K. Żamojć, S.A. Samsonov, D. Wyrzykowski

Opublikowana w: Molecules, 2024, 29, 4598, liczba punktów MNiSW - 140 pkt, IF₂₀₂₄ = 4,6

Tytuł: Affinity and putative entrance mechanisms of alkyl sulfates into the β -CD cavity

Autorzy: M.M. Kogut, O. Grabowska, D. Wyrzykowski, S.A. Samsonov

Opublikowana w: Journal of Molecular Liquids, 2022, 364, 119978, liczba punktów MNiSW - 100 pkt, IF₂₀₂₂ = 6,0

Tytuł: β -cyclodextrin saves lysozyme from the clutches of 1-alkylsulfonates

Autorzy: O. Grabowska, M.M. Kogut-Günthel, S.A. Samsonov, D. Wyrzykowski, J. Makowska

Opublikowana w: Journal of Molecular Liquids, 2025, 428, 127543, liczba punktów MNiSW - 100 pkt, IF₂₀₂₄ = 5,2

Tytuł: Investigation of hexacyanoferrate (II)/(III) charge-dependent interactions with bovine and human serum albumins

Autorzy: O. Grabowska, S.A. Samsonov, L. Chmurzyński, D. Wyrzykowski, K. Żamojć

Opublikowana w: Spectrochimica Acta A: Molecular and Biomolecular Spectroscopy, 2023, 293, 122505, liczba punktów MNiSW - 140 pkt, IF₂₀₂₃ = 4,3

Sergiy
Samsonov

Tytuł: An interdisciplinary study of lysozyme interactions with hexacyanoferrate(III)/(II) ions

Autorzy: O. Grabowska, K. Żamojć, A. Kloska, P. Niedziałkowski, S.A. Samsonov, D. Wyrzykowski

Opublikowana w: International Journal of Molecular Sciences, 2025, 26, 8511, liczba punktów MNiSW - 140 pkt, IF₂₀₂₄ = 4,9


.....

podpis

dr Katarzyna Chmur-Wozińska
Katedra Oceanografii Chemicznej i Geologii Morza
Wydział Oceanografii i Geografii Uniwersytetu Gdańskiego
al. Marszałka Józefa Piłsudskiego 46, 81-378 Gdynia
e-mail: katarzyna.chmur@ug.edu.pl
tel: +48 58 523 68 31

Gdańsk, 17.03.2026 r.

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie poniższych publikacji polegał na współudziale w przeprowadzeniu pomiarów metodą izotermicznej kalorymetrii miareczkowej (ITC).

Tytuł: Physicochemical nature of sodium dodecyl sulfate interactions with bovine serum albumin revealed by interdisciplinary approaches

Autorzy: A. Tesmar, M.M. Kogut, K. Żamojć, O. Grabowska, K. Chmur, S.A. Samsonov, J. Makowska, D. Wyrzykowski, L. Chmurzyński

Opublikowana w: Journal of Molecular Liquids, 2021, 340, 117185, liczba punktów MNiSW - 100 pkt, IF₂₀₂₁ = 6,6

Tytuł: Effect of tetraphenylborate on physicochemical properties of bovine serum albumin

Autorzy: O. Grabowska, M.M. Kogut, K. Żamojć, S.A. Samsonov, J. Makowska, A. Tesmar, K. Chmur, D. Wyrzykowski, L. Chmurzyński

Opublikowana w: Molecules, 2021, 26, 6565, liczba punktów MNiSW - 140 pkt, IF₂₀₂₁ = 4,9

Katarzyna Chmur-Wozińska

Tytuł: Implications of albumin in cell culture media on the biological action of vanadates(V)

Autorzy: O. Grabowska, M. Zdrowowicz, D. Miłaś, K. Żamojć, K. Chmur, A. Tesmar,
M. Kapica, L. Chmurzyński, D. Wyrzykowski

Opublikowana w: International Journal of Biological Macromolecules, 2023, 253, 127875,
liczba punktów MNiSW - 100 pkt, IF₂₀₂₃ = 7,7

Katarzyna Chmur-Wojńska

podpis

dr hab. Paweł Niedziałkowski, prof. UG
Katedra Chemii Analitycznej
Wydział Chemii Uniwersytetu Gdańskiego
ul. Wita Stwosza 63, 80-308 Gdańsk
e-mail: pawel.niedzialkowski@ug.edu.pl
tel: +48 58 523 51 00

Gdańsk, 17.03.2026 r.

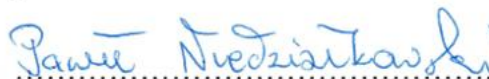
OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie poniższej publikacji polegał na przeprowadzeniu eksperymentów voltamperometrycznych, współudziale w pisaniu i przygotowaniu manuskryptu, jego edycji oraz odpowiadaniu na uwagi recenzentów.

Tytuł: *An interdisciplinary study of lysozyme interactions with hexacyanoferrate(III)/(II) ions*

Autorzy: O. Grabowska, K. Żamojć, A. Kloska, P. Niedziałkowski, S.A. Samsonov, D. Wyrzykowski

Opublikowana w: International Journal of Molecular Sciences, 2025, 26, 8511,
liczba punktów MNiSW - 140 pkt, IF₂₀₂₄ = 4,9



podpis
dr hab. Paweł Niedziałkowski
profesor Uniwersytetu Gdańskiego

Uniwersytet Gdański
Wydział Chemii
Katedra Chemii Analitycznej

dr Magdalena Zdrowowicz-Żamojć
Katedra Chemii Fizycznej
Wydział Chemii Uniwersytetu Gdańskiego
ul. Wita Stwosza 63, 80-308 Gdańsk
e-mail: magdalena.zdrowowicz@ug.edu.pl
tel: +48 58 523 51 17

Gdańsk, 16.03.2026 r.

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie poniższej publikacji polegał na przeprowadzeniu eksperymentów biologicznych (test MTT), współudziale w pisaniu i przygotowaniu manuskryptu, jego redakcji oraz odpowiadaniu na uwagi recenzentów.

Tytuł: Implications of albumin in cell culture media on the biological action of vanadates(V)

Autorzy: O. Grabowska, M. Zdrowowicz, D. Miłoś, K. Żamojć, K. Chmur, A. Tesmar, M. Kapica, L. Chmurzyński, D. Wyrzykowski

Opublikowana w: International Journal of Biological Macromolecules, 2023, 253, 127875,
liczba punktów MNiSW - 100 pkt, IF₂₀₂₃ = 7,7

Zdrowowicz-Żamojć.....

podpis

mgr Martyna Kapica
Katedra Chemii Ogólnej i Nieorganicznej
Wydział Chemii Uniwersytetu Gdańskiego
ul. Wita Stwosza 63, 80-308 Gdańsk
e-mail: martyna.kapica@phdstud.ug.edu.pl
tel: +48 58 523 50 56

Gdańsk, 17.03.2026 r.

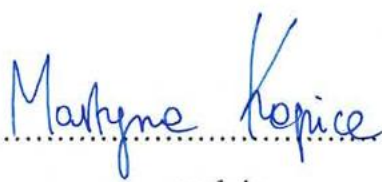
OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie poniższej publikacji polegał na współudziale w prowadzeniu eksperymentów chemicznych.

Tytuł: Implications of albumin in cell culture media on the biological action of vanadates(V)

Autorzy: O. Grabowska, M. Zdrowowicz, D. Miłaś, K. Żamojć, K. Chmur, A. Tesmar,
M. Kapica, L. Chmurzyński, D. Wyrzykowski

Opublikowana w: International Journal of Biological Macromolecules, 2023, 253, 127875,
liczba punktów MNiSW - 100 pkt, IF₂₀₂₃ = 7,7


.....
podpis

prof. dr hab. inż. Lech Chmurzyński
Katedra Chemii Ogólnej i Nieorganicznej
Wydział Chemii Uniwersytetu Gdańskiego
ul. Wita Stwosza 63, 80-308 Gdańsk
e-mail: lech.chmurzynski@ug.edu.pl

Gdańsk, 31.03.2026 r.

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie poniższych publikacji polegał na współredagowaniu manuskryptów.

Tytuł: Physicochemical nature of sodium dodecyl sulfate interactions with bovine serum albumin revealed by interdisciplinary approaches

Autorzy: A. Tesmar, M.M. Kogut, K. Żamojć, O. Grabowska, K. Chmur, S.A. Samsonov, J. Makowska, D. Wyrzykowski, L. Chmurzyński

Opublikowana w: Journal of Molecular Liquids, 2021, 340, 117185, liczba punktów MNiSW - 100 pkt, IF₂₀₂₁ = 6,6

Tytuł: Effect of tetraphenylborate on physicochemical properties of bovine serum albumin

Autorzy: O. Grabowska, M.M. Kogut, K. Żamojć, S.A. Samsonov, J. Makowska, A. Tesmar, K. Chmur, D. Wyrzykowski, L. Chmurzyński

Opublikowana w: Molecules, 2021, 26, 6565, liczba punktów MNiSW - 140 pkt, IF₂₀₂₁ = 4,9

Tytuł: Implications of albumin in cell culture media on the biological action of vanadates(V)

Autorzy: O. Grabowska, M. Zdrowowicz, D. Miłaś, K. Żamojć, K. Chmur, A. Tesmar, M. Kapica, L. Chmurzyński, D. Wyrzykowski

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Tytuł: Can sodium 1-alkylsulfonates participate in the sodium dodecyl sulfate micelle formation?

Autorzy: O. Grabowska, K. Żamojć, M. Olewniczak, L. Chmurzyński, D. Wyrzykowski

Opublikowana w: Journal of Molecular Liquids, 2023, 377, 121568, liczba punktów MNiSW - 100 pkt, IF₂₀₂₃ = 5,3

Tytuł: Investigation of hexacyanoferrate (II)/(III) charge-dependent interactions with bovine and human serum albumins

Autorzy: O. Grabowska, S.A. Samsonov, L. Chmurzyński, D. Wyrzykowski, K. Żamojć

Opublikowana w: Spectrochimica Acta A: Molecular and Biomolecular Spectroscopy, 2023, 293, 122505, liczba punktów MNiSW - 140 pkt, IF₂₀₂₃ = 4,3



.....
podpis

dr hab. Joanna Makowska, prof. UG
Katedra Chemii Ogólnej i Nieorganicznej
Wydział Chemii Uniwersytetu Gdańskiego
ul. Wita Stwosza 63, 80-308 Gdańsk
e-mail: joanna.makowska@ug.edu.pl
tel: +48 58 523 53 15

Gdańsk, 17.03.2026 r.

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie poniższych publikacji polegał na przeprowadzeniu pomiarów metodą skaningowej kalorymetrii różnicowej, współudziale w pisaniu i przygotowaniu manuskryptów, ich edycji oraz odpowiadaniu na uwagi recenzentów.

Tytuł: Physicochemical nature of sodium dodecyl sulfate interactions with bovine serum albumin revealed by interdisciplinary approaches

Autorzy: A. Tesmar, M.M. Kogut, K. Żamojć, O. Grabowska, K. Chmur, S.A. Samsonov, J. Makowska, D. Wyrzykowski, L. Chmurzyński

Opublikowana w: Journal of Molecular Liquids, 2021, 340, 117185, liczba punktów MNiSW - 100 pkt, IF₂₀₂₁ = 6,6

Tytuł: Effect of tetraphenylborate on physicochemical properties of bovine serum albumin

Autorzy: O. Grabowska, M.M. Kogut, K. Żamojć, S.A. Samsonov, J. Makowska, A. Tesmar, K. Chmur, D. Wyrzykowski, L. Chmurzyński

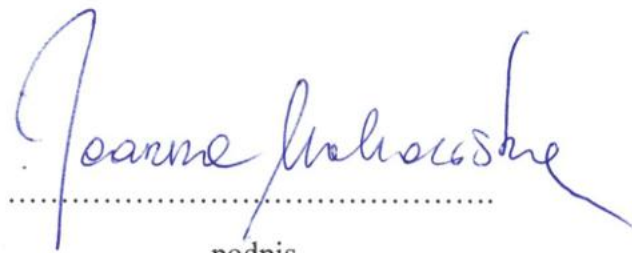
Opublikowana w: Molecules, 2021, 26, 6565, liczba punktów MNiSW - 140 pkt, IF₂₀₂₁ = 4,9



Tytuł: β -cyclodextrin saves lysozyme from the clutches of 1-alkylsulfonates

Autorzy: O. Grabowska, M.M. Kogut-Günthel, S.A. Samsonov, D. Wyrzykowski,
J. Makowska

Opublikowana w: Journal of Molecular Liquids, 2025, 428, 127543,
liczba punktów MNiSW - 100 pkt, IF₂₀₂₄ = 5,2



.....

podpis

dr Aleksandra Tesmar
Katedra Chemii Ogólnej i Nieorganicznej
Wydział Chemii Uniwersytetu Gdańskiego
ul. Wita Stwosza 63, 80-308 Gdańsk
e-mail: aleksandra.tesmar@ug.edu.pl
tel: +48 58 523 50 61

Gdańsk, 17.03.2026 r.

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie poniższej publikacji polegał na współudziale w przeprowadzeniu badań eksperymentalnych, a także współredagowaniu treści manuskryptu.

Tytuł: Physicochemical nature of sodium dodecyl sulfate interactions with bovine serum albumin revealed by interdisciplinary approaches

Autorzy: A. Tesmar, M.M. Kogut, K. Żamojć, O. Grabowska, K. Chmur, S.A. Samsonov, J. Makowska, D. Wyrzykowski, L. Chmurzyński

Opublikowana w: Journal of Molecular Liquids, 2021, 340, 117185, liczba punktów MNiSW - 100 pkt, IF₂₀₂₁ = 6,6



.....
podpis

dr Aleksandra Tesmar
Katedra Chemii Ogólnej i Nieorganicznej
Wydział Chemii Uniwersytetu Gdańskiego
ul. Wita Stwosza 63, 80-308 Gdańsk
e-mail: aleksandra.tesmar@ug.edu.pl
tel: +48 58 523 50 61

Gdańsk, 17.03.2026 r.

OŚWIADCZENIE

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Tytuł: Effect of tetraphenylborate on physicochemical properties of bovine serum albumin

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Opublikowana w: *Molecules*, 2021, 26, 6565, liczba punktów MNiSW - 140 pkt, $IF_{2021} = 4,9$

Tytuł: Implications of albumin in cell culture media on the biological action of vanadates(V)

Autorzy: O. Grabowska, M. Zdrowowicz, D. Miłaś, K. Żamojć, K. Chmur, A. Tesmar, M. Kapica, L. Chmurzyński, D. Wyrzykowski

Opublikowana w: *International Journal of Biological Macromolecules*, 2023, 127875, liczba punktów MNiSW - 100 pkt, $IF_{2023} = 7,7$

..........
podpis

dr hab. Dariusz Wyrzykowski, prof. UG
Katedra Chemii Ogólnej i Nieorganicznej
Wydział Chemii Uniwersytetu Gdańskiego
ul. Wita Stwosza 63, 80-308 Gdańsk
e-mail: dariusz.wyrzykowski@ug.edu.pl
tel: +48 58 523 50 62

Gdańsk, 22.04.2026 r.

OŚWIADCZENIE

W związku z brakiem możliwości kontaktu z Panem Ankur Singh, współautorem poniższej publikacji:

Tytuł: Exploring the impact of subtle differences in the chemical structure of 1-alkylsulfates and 1-alkylsulfonates on their interactions with human serum albumin

Autorzy: O. Grabowska, A. Singh, K. Żamojć, S.A. Samsonov, D. Wyrzykowski

Opublikowana w: *Molecules*, 2024, 29, 4598, liczba punktów MNiSW - 140 pkt, IF₂₀₂₄ = 4,6

oświadczam jako autor korespondencyjny tej pracy oraz promotor rozprawy doktorskiej Pani mgr Oli Grabowskiej, że udział Pana Ankur Singh w powstawaniu ww. publikacji polegał wyłącznie na współudziale w prowadzeniu obliczeń teoretycznych, które były bezpośrednio nadzorowane przez prof. Sergieja A. Samsonova.

KIEROWNIK
Katedra Chemii Ogólnej i Nieorganicznej



dr hab. Dariusz Wyrzykowski
profesor Uniwersytetu Gdańskiego

podpis

Dan Milas
e-mail: dan.milas@proton.me

Lille, 18.03.2026

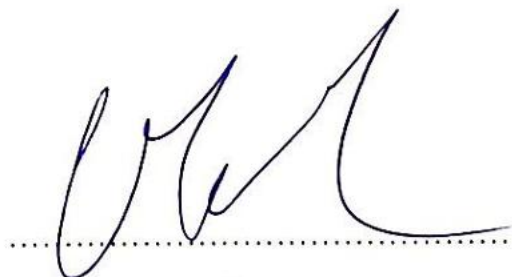
STATEMENT

I declare that my contribution to this work consisted of conducting spectroscopic and spectrofluorometric studies, writing, preparing, and editing the manuscript.

Title: Implications of Albumin in Cell Culture Media on the Biological Action of Vanadates(V)

Authors: O. Grabowska, M. Zdrowowicz, D. Milas, K. Żamojć, K. Chmur, A. Tesmar, M. Kapica, L. Chmurzyński, D. Wyrzykowski

Published in: International Journal of Biological Macromolecules, 2023, 253, 127875,
number of points MNiSW - 100 pts, IF₂₀₂₃ = 7.7



signature

dr Anna Kloska
Katedra Biologii i Genetyki Medycznej
Wydział Biologii Uniwersytetu Gdańskiego
ul. Wita Stwosza 59, 80-308 Gdańsk
e-mail: anna.kloska@ug.edu.pl
tel: +48 58 523 60 44

Gdańsk, 16.03.2026 r.

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie poniższej publikacji polegał na przeprowadzeniu eksperymentów biologicznych i biochemicznych, współudziale w pisaniu i przygotowaniu manuskryptu, jego edycji oraz odpowiadaniu na uwagi recenzentów.

Tytuł: An interdisciplinary study of lysozyme interactions with hexacyanoferrate(III)/(II) ions

Autorzy: O. Grabowska, K. Żamojć, A. Kloska, P. Niedziałkowski, S.A. Samsonov, D. Wyrzykowski

Opublikowana w: International Journal of Molecular Sciences, 2025, 26, 8511,
liczba punktów MNiSW - 140 pkt, IF₂₀₂₄ = 4,9

Kloska Anna

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podpis

mgr inż. Michał Olewniczak
Katedra Chemii Fizycznej
Wydział Chemiczny
Politechnika Gdańska
ul. Gabriela Narutowicza 11/12, 80-233 Gdańsk
e-mail: michalolewniczak@gmail.com
tel: +48 58 347 20 92

Gdańsk, 17.03.2026 r.

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie poniższej publikacji polegał na przeprowadzeniu badań teoretycznych, współudziale w pisaniu manuskryptu, wizualizacji danych oraz odpowiadaniu na uwagi recenzentów.

Tytuł: Can sodium 1-alkylsulfonates participate in the sodium dodecyl sulfate micelle formation?

Autorzy: O. Grabowska, K. Żamojć, M. Olewniczak, L. Chmurzyński, D. Wyrzykowski

Opublikowana w: Journal of Molecular Liquids, 2023, 377, 121568, liczba punktów MNiSW - 100 pkt, $IF_{2023} = 5,3$



podpis