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Referee report for a PhD thesis submitted by mgr inż. Maria Cristina Nevarez Martinez entitled: „Gold Nanorod – Affibody Conjugates for Targeted Photothermal Therapy on HER2-Positive Cancer Cells" prepared under the supervision of: prof. dr hab. inż. Adriana Zaleska-Medynska

The PhD thesis of Mrs Maria Cristina Nevarez Martinez is a 148 pages long document comprising "Abstracts", "Introduction", "Materials and methods", "Results and Discussion", and "Conclusions and Future Prospects" chapters followed by "References" list. Abstracts in English and Polish introduce the topic of the dissertation, which is a design of new platform combining Au nanorods (NRs) and Cys-modified HER2-affibody $Z_{HER2:2891}$ for photothermal therapy (PTT) of cancer cells under near infrared (NIR) light illumination. The scope of the research tasks is listed as: NR production and further PEGylation and affibody loading on nanoparticles, followed by microscopy studies of the internalization of PEG-Au NRs and Affi-Au NRs into HER2 positive cells. Finally, photothermal therapy with controlled light doses that confirm specific cytotoxicity of the therapy with SKOV-3 cells and constitute preliminary studies for further optimization.

The abstracts are followed by the "Introduction" with main concepts about HER2 positive cancers and their therapy. Current state of the art in nanotherapeutic agents for cancer treatment is well presented, with a focus on photothermal therapy performed with nanoparticles. This part gives very good overview about the functionalization of nanoparticles for specific targeting of cancer cells. Specifically, affibody molecules which are the targeting vectors chosen for further studies are discusses, with clear presentation of their advantages over previously described anti-

HER2 monoclonal antibodies (mAbs). The literature review is conveniently summarized in sub-chapter 1.8, with main points and remaining challenges clearly mentioned.

In chapter 2 Mrs. Martinez defines the aim of her PhD thesis as the design of a new and effective platform combining optical properties of NRs and targeting abilities of Affi to aim HER2 cancer cells with photothermal therapy. As the Affi has not been conjugated with NRs and studied in PTT, the approach proposed in the thesis is expected to broaden the knowledge on applicability of such systems as therapeutic agents in HER2 cancer cells, important in case of several types of cancer.

In chapter 3 Mrs. Martinez describes experimental procedures, materials and methods applied in the research work. The chapter provides precise protocols of the NRs synthesis, characterization methodology, synthesis of affibodies, preparation of cell cultures, three strategies of bioconjugation of Affi to NRs and physicochemical characterization of Au-based bioconjugates. Then, photothermal conversion system is described and techniques used for the verification of NRs functionalization (gel electrophoresis, flow cytometry) and internalization (confocal microscopy). Finally, protocol of photothermal therapy *in vitro* is presented with the setup specifically designed and constructed to illuminate cells in 96-well plate with 808 nm LEDs. The whole chapter is clearly written and provides sufficient information to recreate the experiments. What is more – legitimacy of each experiment is presented, what shows good understanding of the studied topic.

The next chapter "Results and discussion" starts with the analysis of seed-growth synthesis of Au NRs. Mrs. Martinez shows praiseworthy persistence in gaining the knowledge about NRs synthesis and systematically investigates which steps of the process are significant for the best reproducibility. Gold nanorods seed-growth synthesis protocols are the best studied and developed among noble metal nanoparticles. They routinely result in >95% yield of obtained shapes, with low heterogeneity of NRs sizes. It is visible in good prosperity of companies selling gold nanorods of selected NRs sizes with a few nm standard deviation. However, indeed some aspects of the NRs synthesis are still to be understood and Mrs. Martinez correctly identifies that seeds quality and homogeneity has crucial impact on the synthesis conditions. During investigation of seeds solution, Mrs. Martinez, after R. A Vaia and co-workers, correlates UV-Vis absorption peaks in the range 300-400 nm with Au nanoclusters

with 18-25 Au atoms (diameter <0.85 nm) and a band at 480 nm with Au nanoclusters consisting of 25-40 Au atoms (diameter ~ 1 nm). It is a great simplification as in case of gold nanoclusters there is no systematic red shift in the absorption bands with the size of nanoclusters (as shown in R. Jin's publication cited in R. A Vaia paper: Jin, R. Atomically Precise Metal Nanoclusters: Stable Sizes and Optical Properties Nanoscale 2015, 7, 1549– 1565), eg. Au18 clusters have absorption band at 562 and shoulder at 620 nm whereas Au20 has the lowest absorption band at ~485 nm. I recommend recent article reported in Nature Communications 14, 4408 (2023), where atomically-precise ultra-small gold clusters Au32 were confirmed to be the majority component in seed solution. I would suggest to stay with apparent sizes of seeds and not assign any number of Au atoms to them without further analysis. In this case methods such as MALDI-mass-spectrometry may be helpful in the detailed analysis of species present in the seed solution.

Mrs. Martinez performed statistical analyses of influence of various factors on the morphology and SPR resonance of nanorods. She concluded that non-statistically significant differences in the aspect ratio (AR) of NRs may still contribute to statistically significant differences in the L-LSPR band position. In this case, what may also be significant for the L-LSPR is the crystallinity of nanorods; also the full width at half maximum of the L-LSPR band (in energy units) may provide better insight into heterogeneity of the sample (in correlation with TEM images analysis). However, for most applications size differences of a few nm has no effect on the nanoparticles final performance in applications and a relevant question here is how the quality of NRs samples (size/shape homogeneity, LSPR position) affects their application in photothermal therapy.

Further steps of preparation of Affi-PEG-NR constructs are performed by Mrs. Martinez with similar systematic and precisely controlled manner as presented in case of NRs synthesis (comparison of Affi before and after refolding by dialysis or in Amicon tubes, analysis of conjugation effectiveness of Affi with DyLight 633 dye and further binding affinity with HER2 positive cells). With optimized Affi tag, functionalization of NRs was performed. Two-step procedure was required, as direct attachment of the peptide through thiol bonding turned out to be ineffective. Mrs. Martinez reports two methods: PEGylation followed by Affi loading and MUA attachment as a first step and clearly explains drawbacks of the latter method. Then Au

NRs bioconjugation applicability is evaluated. I have some concerns to the photoluminescence experiments described on page 88. Presented spectra do not show the photoluminescence of NRs, but rather scattering of the excitation beam (wavelengths between 360-510 nm) and the second order reflection from the diffraction grating of the monochromator in the spectrophotometer (wavelengths at ~twice the wavelength of the incident beam). It is a common artefact, which can be compensated in some spectrofluorometers. Photoluminescence band position should not depend on the excitation wavelength (only intensity will change). The fact that so called "luminescence" in NIR is just an artefact of the spectrometer is supported by the fact that it consistently shifts with the shift of the excitation wavelength and that it was not observed in further experiments performed in London.

Further experiments were performed with fluorescently labeled Affi-PEG-NRs, in order to image the constructs under fluorescence microscope. Mrs. Martinez presented the conjugation procedure. No indication of fluorescein is present in UV-Vis spectra of the constructs, but nevertheless it will be helpful to show the expected position of fluorescein absorption band in figure 41c, also to assess how excitation of the fluorophore may be affected by SPR bands. Moreover – was fluorescence of the fluorophore measured after conjugation with NRs? The authors attempted to image light scattered from the NRs, but the signal was not sufficient. It is understandable, as NRs used in this study are smaller than NRs usually imaged via scattering (usually NRs need to have >80 nm and >30 nm in length and width, respectively), what significantly influences the scattering cross-section. Imaging under confocal microscope showed internalization of NRs in case of HER2 positive cells when illuminated with 488 nm wavelength. Did the authors try to excite the cells with wavelengths that correspond to SPR band or set the collection channel to red wavelengths? I suppose that will provide reduced background and possibly more signal from nanoparticles.

Final paragraphs in the "Results and discussion" section present photothermal therapy tests. Although experiments performed in room temperature do not show any significant reduction in cell viability with or without illumination at 808 nm, introduction of nanoparticles not only inside, but also outside the cells results in a strong decrease of the number of cells, regardless of HER2-expressing levels. Change of the experimental conditions (starting temperature increase to 37 °C) and change of the control cell line (HEK293T) showed

significant photothermal effect with SKOV-3 cells, dependent on the NR conjugates concentration and light dose, whereas there was little effect observed for HEK293T cells.

The thesis ends with "Conclusions" section with a list of main conclusions drawn basing on the performed experiments. It is followed by "Future plans" section, where Mrs. Martinez provides interesting ideas how to proceed with the topic of HER2 targeting NR-constructs for efficient photothermal therapy. Indeed, the results obtained by the PhD candidate are encouraging and several systematic optimizations of experimental conditions presented in the thesis constitutes a solid base for further studies. At the end, I have one more question: is it possible to draw the conclusion which step of the preparation of Affi-PEG-NR-constructs is the limiting step for their final performance in PTT in cells?

Overall, the dissertation presents an original contribution to the knowledge on functionalization of plasmonic nanoparticles for the application in photothermal therapy of cancer cells. The presented research work as well as the editorial work on the dissertation are of high quality. Unfortunately, none of the parts of this thesis have been published yet in a scientific journal, but I have no concerns that the obtained results constitute good material for high quality publication.

I find the thesis interesting and communicating original findings in actively studied topic of plasmonic agents for photothermal therapy. Mrs. Martinez proved with the research described in the thesis that she can adopt appropriate research methods and draw meaningful conclusions to address scientific questions formulated at the beginning of his thesis. Hence, I conclude that the thesis presented by Mrs. Martinez meets all the requirements for doctoral dissertations and I recommend proceeding with further steps of her PhD degree procedure. I also recommend the distinction of the dissertation.

Joanna Olesiak-Bańska