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PhD thesis review

Candidate: *Anna Graczyk, MSc*

Thesis title: *Structural RNA conjugated with gold nanoparticles as a tool for gene expression regulation*

The doctoral dissertation submitted for the review was carried out under the supervision of Prof. Arkadiusz Chworoś and co-supervision of Dr. Róża Pawłowska at the Division of Bioorganic Chemistry, Laboratory of Biomaterials at the Centre of Molecular and Macromolecular Studies of the Polish Academy of Sciences.

According to the information indicated by the PhD candidate within the dissertation, the main aim of the studies was design, synthesis and application of a new generation of tectoRNA trimer conjugated with gold nanoparticles. Nevertheless, to be more precise, it was rather verification of potential applications, not necessarily application itself.

The main subject of dissertation concerns siRNA based nanotechnology making the study very interesting and in line with the latest global research trends on the application of nucleic acids as therapeutic, diagnostic or theranostic agents. To date, the nanotechnology as an emerging scientific field allowed to address limitation of imaging and therapeutics delivery simultaneously enabling the precise control of shape and size of nanoconstructs. Importantly, the dynamic development of nanomedicine allowed for design of a number of potential nanoscale drugs targeted towards various diseases giving a special attention to the conjugation of nanoparticles with diverse RNA molecules. Among those, siRNAs have ability to selectively knockdown target mRNA and, in consequence, to silence or control particular gene expression which is associated with human disease. Recently, many attempts to develop siRNA based nanoconstructs include basic siRNA duplexes conjugated with nanoparticles as well as siRNAs being a fragment of more complex RNA nanostructures *i.e.* trimers, hexamers or octahedrons. Therefore, the concept presented in the dissertation which assumes design and synthesis of spherical nucleic acids composed of gold nanoparticles and tectoRNA trimers fits perfectly into the modern trends observed in nanomedicine, nanotechnology and nucleic acid based therapeutic approaches.

The doctoral dissertation is written in English, has a total of 125 pages, 403 references, and is divided into three main parts, *i.e.* Introduction, Results and Discussion, and Materials and Methods. The doctoral thesis has a typical layout. The Abstract was placed at the beginning of the dissertation, preceded by the author's Achievements. Then the PhD candidate placed Table of contents and a list of abbreviations and symbols used in the work. In general, the dissertation gives the overall impression of being prepared with the highest care. The text contains only very minor typos therefore it was a pleasure to read it. However, the quality of some figures could be much better. The low quality of figures in the introduction part of the dissertation, prevented from deep analysis of the presented schemes and from facile understanding of some issues described in the text. Fortunately, the particular topics were described very clearly making the whole line of reasoning quite easy to follow even without support of the schemes.

The literature part provides a good overview of the current state of knowledge on the issues necessary to understand the research part of the work. In the following subsections, the PhD candidate presents the pivotal role of RNA molecules in the nature with comprehensive characteristics of RNA types, describes structural aspects of various RNA architectures and smoothly shifts to the RNA based nanostructures published so far. At the end of the Introduction section the characteristics of metal nanoparticles is also presented. Despite the poor quality of many drawings, it is indisputable that the literature part presented in this doctoral dissertation is a good introduction to the discussion of the results of own research, written in a factual manner. This part of the dissertation is undoubtedly facilitating the analysis and interpretation of the results contained in the further part of the dissertation.

The most important part of the dissertation is the Results and Discussion section. In this section, the PhD candidate comprehensively and substantively presents the results of her research. The way in which the obtained results were described indicates the great knowledge of Anna Graczyk in this subject. As a result of a series of rationally planned and consequently conducted experiments, the PhD student successfully:

- designed RNA trimer with thiol linker which is necessary for conjugation with gold nanoparticles,
- synthesized necessary monomers and performed RNA trimers association,
- synthesized spherical nucleic acids covered with siRNAs and RNA trimers,
- assessed the effectiveness of new conjugates to knock down gene expression within selected cell line.

The results of the above research clearly indicate the therapeutic potential of newly developed structuralized RNA conjugated with gold nanoparticles.

The last part of the doctoral dissertation is the experimental part. PhD candidate described in details the methods of preparation of the solutions used in the experiments, the source of oligonucleotides, the methods of their purification as well as all methodologies concerning conjugation and analysis of studied constructs. Even though the methodology descriptions contain a bit more spelling errors (still sparsely present) this part is written correctly and contains all necessary information.

It is the responsibility of the reviewer to find the weaknesses of the dissertation. Below, I list some of my doubts and comments. However, I would like to emphasize that they absolutely do not have any negative impact on my overall, very high assessment of the dissertation and do not decrease high quality of the presented research.

- Page 45, Figure 19, wouldn't an RNA ladder be a better option instead of G1 RNA monomer produced previously to confirm the length of transcriptionally synthesized RNAs? What method was used to confirm the length of G1 RNA monomer synthesized previously?
- Page 51-55, The cytotoxicity studies of metal nanoparticles were conducted for three various sized of gold (10, 50, and 100 nm), one size of silver (100 nm), and one size of platinum (50 nm). If we compare results presented in figures 27 and 28 (100 nm AuNP vs. AgNP cytotoxicity at 5µg/ml) for MDA-MB-231 it seems that silver nanoparticles are less toxic than gold. Thus, in my opinion we cannot exclude that 10 nm nanoparticles of silver would be also better choice than gold. Taking the above into account - why only gold was investigated in three different sizes?
- Page 51-55, why cytotoxicity studies of spherical nanoparticles were conducted on four different cell lines if the further studies focused only on MDA-MB-231? What was the purpose of this preliminary tests in the context of further investigations?
- Page 53, Figure 27 – is it not too far-reaching conclusion to assess treatment with 100 nm gold nanoparticles at 5 µg/ml as “non toxic” while observing 54% cell viability? Even if we assume that considerable toxicity is below 50%, then being aware of the measurement errors 54% is very close to the boundaries of toxicity.
- Page 63, the transmission electron microscopy was indicated as the technique which confirms diameter and provides information about shape and dispersion of nanoparticles. However, despite the presence of Figure 37, no observations or conclusions were described in this section.

- Page 67, according to information presented by PhD candidate, cell viability studies confirmed lack of cytotoxicity of the whole RNA-nanoparticle complex. Nevertheless, it would be convenient for the reader to add here also quite pivotal information about concentrations of complex and regulatory RNAs used in the MTT assay.
- Page 73, Figures 47 and 48, I would like to ask PhD candidate to shortly discuss during PhD defense the possible origin of (i) improved silencing efficiency of tecto-RNA-AuNPs in comparison with trimers and (ii) decreased silencing efficiency of RNA trimer in reference to siRNA
- In the final part of dissertation PhD candidate compared potency of novel complexes to siRNA efficiency and concluded that silencing efficiency of both nucleic acid based tools come up to the same level. Thus, I would like to ask PhD student to present potential assets of application of tecto-RNA-AuNPs over conventional siRNAs.

As a final point, I would like to highlight the very important aspect of the dissertation *i.e.* the interdisciplinary nature of the presented research. PhD candidate performed studies across boundaries of chemical and biological sciences and was forced to acquire knowledge from various sources, simultaneously deepening her learning experience. In consequence, the project covers topics more deeply and considers many various perspectives to be combined. In principle, interdisciplinary projects are not easy to be implemented, require greater involvement, but at the same time are extremely stimulating and provide not only valuable results but also priceless scientific experience for a young researcher.

Overall the dissertation of Anna Graczyk is very well written, interesting to read and concerns scientifically important aspects of RNA based nanotechnology, providing new and important results of the highest quality in the field of nanoengineering and nucleic acids therapeutic approaches. The experiments are well planned, performed and described, results are solid, and the conclusions are properly presented. Therefore, I assess the dissertation very high and fully support conferring a degree of doctor on Anna Graczyk. Additionally, I would like to request the Scientific Council of the Centre of Molecular and Macromolecular Studies Polish Academy of Sciences for distinction of the dissertation as a recognition of PhD candidate's excellent research.

Yours sincerely,

A handwritten signature in blue ink that reads "Anna Pasternak". The signature is written in a cursive, flowing style.