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Review Report of the Ph.D. thesis of Anna Graczyk

entitled

"Structural RNA conjugated with gold nanoparticles as a tool for gene expression regulation"

Supervisor Prof. Arkadiusz Chworos

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Anna Graczyk submitted the doctoral thesis dealing with the structural RNA conjugated with spherical gold nanoparticles as a tool for gene expression regulation.

Over the past decades, a new class of bioactive molecules, known as biological drugs, has played an increasingly important role in fighting disease in the human body. The Food and Drug Administration (FDA) has defined biological drugs as large, complex molecules derived from living cells or biological/chemical processes that are used to diagnose, prevent, treat, and cure a broad spectrum of diseases and medical conditions. Biological includes a wide variety of substances, including carbohydrates, proteins, nucleic acids, and developed composites of these substances. Unlike small molecules, biological drugs have sophisticated structures, ranging from several hundred to over a thousand times larger than classic drugs on the market. They are characterized by exceptional therapeutic properties with an excellent and unique specificity to target a precise biological process. Compared to small molecules, bioactive

molecules are superior in biomedical performance, off-target toxicity, and patient safety, making them ideal candidates for personalized medicine.

Among the biological molecules used as therapeutic agents, ribonucleic acids (RNA) are distinguished by their unique properties and various effects on the biological processes of the human body. RNA is a family of complex biological molecules made up of linear chains of monomeric nucleotides that play a fundamental role in various biochemical cellular mechanisms. Over the past decades, various roles of RNA have been discovered, suggesting their involvement in almost all biochemical pathways. These exciting discoveries have drawn the attention of many scientists to the testing of RNAs as therapeutic molecules, thereby triggering a steady increase in the results and scientific discoveries in the field. New technologies based on RNA, including production / synthesis, specific arrangement in three-dimensional, multi-member structures, and their application have been called RNA nanotechnology. In combination with the achievements of traditional nanotechnology, including the production, characterization and application of various types of controlled nanoforms that can be produced using a number of elements and chemical compounds, RNA-nanoparticle constructs can have additional properties. The use of safe delivery platforms can ensure effective delivery of therapeutic RNA and protect it from degradation, and compensate for its inherent hydrophilicity and negatively charged character as it crosses the cell membrane.

The reviewed doctoral dissertation concerns the above-presented subject and is part of the mainstream of interdisciplinary research. The aim of this dissertation is to design, synthesize, assemble, and investigate gold nanoparticles functionalized with rationally designed, structured RNA trimer. Despite the significant number of publications in this field, the dissertation should be considered valuable and contributing to the development of the scientific discipline.

The Ph.D. thesis is well structured and correctly presented. Its consists of 3 main chapters: introduction, results and discussion, materials and methods. At the beginning of dissertation list of abbreviations is introduced. Finally, summary, scientific activity of the PhD student, abstract in English and Polish, and appendix are presented. The thesis is written on 125 pages altogether and enriched by 51 figures. The theoretical introduction and the research part were validated with 403 valuable references.

In the theoretical part, the author efficiently guides the reader from basic information about various types of RNAs, their functions, forms, and processes to which they are subjected. Then the author goes on to discuss the so-called structural RNA, RNA 3D design methods,

triangular, square and polygons RNA structures and the concept called RNA nanotechnology. Next she focuses the reader's attention on metallic nanoparticles, in particular gold, silver and platinum. Discusses the variety of forms of available nanomaterials and points to their properties and application from a biological point of view. The methods of conjugation metallic nanoparticles with RNA were also presented, indicating the possibility of using nanoparticles as cargo for RNA. The theoretical part ends with a reference to commercial products based on RNA technologies, which indicates the author's mature thought - from basic information to commercial applications. Particularly worth emphasizing is the reference to up to 395 literature articles, which indicates the author's excellent understanding of the subject. In my opinion, this part of the work could be used successfully to prepare a review article. A certain difficulty for the reader in navigating the dissertation is the lack of chapter and subsection numbering. The arrangement of abbreviations in their list is also unclear, which also makes it difficult to find them; I would suggest an alphabetical arrangement.

In the experimental part, starting on page 40, the author describes the successive steps of the research leading to obtaining a stable structured RNA trimer each bearing regulatory sequence and examining the conjugate thus formed in terms of biological activity towards gene regulation functionality. The first part focused on the design and syntheses of thiol modified RNA trimer with each step controlled by gel electrophoresis. To build the trimer a synthetic complementary RNA fragment bearing thiol group and synthesized enzymatically by in vitro transcription with T7 RNA polymerase fragments were used. The next section is devoted to the selection of nanoparticles that will be used as cargo in further experiments. Gold, silver and platinum nanoparticles of various sizes were tested. Cytotoxicity tests were performed with the use of four cell lines. After analyzing the results, the author chose gold nanoparticles with a diameter of 10 nm for further experiments. The next step describes the procedure of creating a conjugate of siRNA and structural RNA with gold nanoparticles. An agarose gel electrophoresis, transmission electron microscopy, dynamic light scattering and zeta potential measurements were run to evaluate and characterize the complex formed. The transmission electron microscope was also used to visualize RNA-gold nanoparticles uptake in cells. In the last part effectivity of the regulatory RNA and obtain conjugates were tested by fluorescence microscopy imaging, flow cytometry, fluorescence plate reader and as a result up to 80% decrease of GFP fluorescence was observed after treatment with gold nanoparticles – RNA conjugates. The presented results clearly prove that the main goal of the study was achieved

and that time-stable gold-RNA conjugates with regulatory functionality were prepared and tested.

This thesis is very well written and documented. There are no typing errors, and the text is written in a clear and concise manner. Figures, schemes, and tables are shown properly. The arguments are well formulated with meritorious conclusions based on actual literature. The conclusions confirm that the assumed goal of the work has been successfully achieved.

There are, however, a few remarks that came to my mind that require a detailed explanation.

- Reports in the literature indicate that the toxicity of nanoparticles increases as their size decreases. How does this relate to the results presented in this dissertation?
- Was the presence of reducing agents and stabilizers the same for all colloids of metallic nanoparticles? In some parts of the work, one can find information on citrate-stabilized nanoparticles, and in others, on so-called naked nanoparticles.
- What was the criterion for selecting the sizes of Au nanoparticles 10 nm, 50 nm, 100 nm, Ag only 100 nm, Pt only 50 nm?
- How do you know the size / degree of aggregation of nanoparticles used in the research? Has it been tested?
- How do the results obtained with the DLS technique and the rough estimation of the size of siRNA and trimer-RNA compare to the distance between nanoparticles visible in the TEM images?

To sum up, the thesis demonstrates a solid understanding of the state-of-the-art in the research area and the knowledge of the most important and current literature. All experiments are well arranged and measurements techniques and methods are correctly applied. It is generally well presented and very interesting to read. Most importantly, thesis research generates significant new knowledge in the scientific field.

In my opinion, the thesis by Anna Graczyk fulfills all requirements for obtaining the Ph.D. degree and, from that point of view, is ready to be defended orally in front of the respective committee.