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Review of the doctoral dissertation of M.Sc. Dryhval Bohdan Oleksandrovych entitled:

"Biocompatibility and antibacterial properties of orthopedic implants from magnesium alloys modified using plasma electrolytic oxidation"

Academic supervisors:
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I was appointed to perform the review by decision of the Academic and Research Medical Institute, Sumy State University, no. 60/0020.

1 . Relevance of the dissertation topic.

Metal biomaterials for bones play a key role in modern tissue engineering. The new materials with good biocompatibility and antibacterial properties are looking for regenerative medicine. The most popular long-term material used as implant is titanium alloy (titanium grade 4). However titanium not degradable in physiological fluids. One of the key advantages of biodegradable implants is their ability to degrade in the patient's body over time. Biodegradable materials may fulfill the mechanical functions and stimulate bone tissue growing and mineralization process, deliver appropriate biochemicals and biophysical cue. This avoids the need

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for further surgical interventions to remove the implants after their main function has been completed. Magnesium alloys is one of the promising materials which may find application in medicine. This materials easily degrade in physiological body fluids, and its not toxic for the bone tissue. Magnesium alloys usually have lower Young's modulus compared to the titanium alloys, closer to the natural bone. Mechanical properties plays a crucial role in biomaterials, as well as cytocompatibility and antibacterial properties. Properties of the Mg alloys creates the opportunity to design a novel, biocompatible implants.

In the literature, we could found a lot techniques to modify surface of magnesium alloys to enhance their bioactivity. Plasma electrolytic oxidation (PEO) is one of the electrochemical techniques used for surface treatments. Cheap and simple techniques for formation a functional layer on complicated shapes of implants are desirable. Anodization process results in formation an oxide layers, when we anodize Mg mainly a magnesium oxide is form, and additional oxides based on the composition of the magnesium alloy. During this process the bioactive compounds like calcium, phosphorus could be incorporated in to the oxide layer, or antibacterial like silver-, copper-based particles. The porous ceramic layer is formed, and their thickness depends on electrochemical parameters applied during the process, chemical composition of the magnesium alloy and composition of the anodizing bath. When we increase the voltages and oxide layer breakdown, the spark discharges occurs, and the thick ceramic layer is formed. This process is called a plasma electrolytic oxidation (PEO) and has found application in surface engineering. Formation of the ceramic layer on the magnesium alloys is crucial to control the degradation time of the magnesium alloy into surrounding tissue.

2. Connection of the dissertation topic with state or branch scientific programs.

The PhD Student declare that dissertation work was carried in accordance with the scientific research plan of the Educational and Scientific Medical Institute of Sumy State University, projects entitled "*Biological effectiveness of the use of plasma electrolytic oxidation and sol-gel deposition to create a functional surface of implants*" (state registration number 0119U100823),

"*Development of a method for diagnosing and predicting the course of tumors using molecules of cell adhesion of cancer-embryonic antigen and cyclooxygenases*" (state registration number 0122U100111), "Determination of osteoconductive and osteoinductive properties of polymer-nano-hydroxyapatite biodegradable scaffolds" (state registration number 0122U000770), as well as of the Horizon-2020 MSCA-RISE project "*Nanostructural surface development for dental implant manufacturing*" (project number - 777926).

3. Scientific novelty of the research and the obtained results.

The dissertation presents a study on plasma electrolytic oxidation process for surface treatment of magnesium alloy to improve the quality of the alloy and enhance their biocompatibility. The scientific work was divided into three main parts: surface treatment and analysis, *in vitro* analysis and *in vivo* analysis. Application of the PEO is well-known, however the results of the surface treatment (especially magnesium alloys) in various electrolytes is still novel and should be analyzed in details. The PhD candidate has focused on the anodization process in silicates. In my opinion the composition of the anodization bath (Na_2SiO_3 + NH_4F + NaOH, or Na_2SiO_3 + NH_4F + $Ca(OH)_2$) was appropriate. Novelty of this dissertation is based on formation a biocompatible layer with antibacterial properties. The advanced biological analysis, including *in vivo* test, have proven that the main goals and objectives of the research work were achieved.

It should be mentioned that this research work is highly interdisciplinary because it presents aspects from material science, biology, chemistry and electrochemistry.

4. Structure of the dissertation, theoretical and practical significance of research results.

First off all, I should mention that this review was prepared based on the English version of the dissertation.

The presented dissertation contains literature review, description of materials and methods, results and discussion, analysis of the results and conclusions. The manuscript consists of 12 subsections and a list of 180 sources of cited literature. The dissertation is laid out on 124 pages. The dissertation is written in clear, concise and communicative language. The experiments were well-planed. In some part in this dissertation the methodology is presented without specific details, but the information were easily found on the scientific papers.

The experimental part includes:

- formation of the ceramic coatings on the Mg alloys,
- microstructural analysis, layer thickness and chemical composition,
- wettability analysis and surface roughness,
- analysis of photoluminescence of Mg samples,
- in vitro test in simulated body fluid,
- cytocompatibility test using human bone osteosarcoma (line U2OS) cell,
- microbial test using a Gram-positive Staphylococcus aureus bacteria,
- advanced in vivo test (histological examination, immunohistochemical study, analysis of cellular mechanisms).

The PhD student declare that he independently conducted all these experiments. It was declared that animal housing and experiments were carried out in accordance with the provisions of the "*European Convention for the Protection of Vertebrate Animals*" used for experiments and other scientific purposes (Strasbourg, 1986), "*General Ethical Principles of Animal Experiments*", adopted by the First National Congress on Bioethics (Kyiv, 2001) , "*Ethical principles and guidelines*

for experiments on animals", 3rd edition (Switzerland, 2005), by the Helsinki Declaration of the General Assembly of the World Medical Association (2000), standard provisions on ethics of the Ministry of Health of Ukraine No. 690 dated 23.09.2009, the Law of Ukraine "*On the Protection of Animals from Cruelty Treatment*" No. 3477-IV dated February 21, 2006, Directive 2010/63/EU of the European Parliament and the Council "*On the Protection of Animals Used for Scientific Purposes*" (2010). The in vivo results have been reported in paper entitled "*In vivo safety of new coating for biodegradable magnesium implants*" (Materials 2023, 16, 5807. https://doi.org/10.3390/ma1617580).

The progress of biomaterial science and technology is now very fast. There is a lot of ideas how to obtain the best, ideal implant to bone. The anodization process allows to create a porous oxide layers with various chemical composition, pore sizes, surface roughness, wettability, adhesion to the substrate etc. There properties strongly influence on the final cytocompatibility results, as well as on the biocompatibility. Thus, it is important to chose the most promising samples and provide complex analysis, including test under *in vivo* conditions. This dissertation shows how the PhD student has chosen the most promising Mg alloys with ceramic coatings and how the chemical composition of the coating influences on the cytocompatibility. *In vivo* analysis is a last step of the material investigation, only the selected biomaterial should be analyzed to decrease the number animals necessary to the experiments.

The methods used in this study are adequate for solving the tasks. They made it possible to scientifically investigate important aspects of the chosen research topic, providing reliable results.

In my opinion, the results presented in this dissertation influences the development of science and the discipline of medical science. I see practical applications of these results in the future in medicine.

6. Completeness of presentation of dissertation materials in published works

The number and level of published works meet the requirements in of the Resolution of the Cabinet of Ministers of Ukraine No. 44 dated January 12, 2022 (§8, no. 2). Dryhval Bohdan Oleksandrovych is a co-author in 14 scientific works were published on the topic of the dissertation, where 3 articles indexed by the Scopus scientometric database, 11 theses of reports in the materials of international scientific and practical conferences (page 6 of the dissertation). In one paper Dryhval Bohdan Oleksandrovych is a first co-author. Dryhval Bohdan Oleksandrovych conducted an experimental study, performed a literature review, and formulated the results of the study. Unfortunately, the declarations of the co-authors about their contributions were not attached to the dissertation, however their statement are presented in the published papers.

7. Questions, suggestions and comments.

This dissertation is interdisciplinary with interesting results. However I would like to discuss a few points:

- on page 38 it was mentioned that the PEO process was carried out for 10 minutes, however the graphs in the fig. 2.2 presents only the registered curves up to 5 min,
- please clarify the EDX analysis and cross-section analysis: how many samples were used out to analyze the chemical composition and thickness of the coatings formed on the Mg alloy?
- Figure 3.1. presents the results of the pore size measurement. When the voltage was increased, the pore sized decreased, probably the upper part of the coating was melt. Have you tried to analyze the pore distribution of the PEO coatings using a Brunauer-Emmett-Teller (BET) surface area analysis? This is my suggestion to analyze the most promising samples, maybe some relation between the pore size and good biocompatibility results could be find.
- Fig. 3.8., the presented value for C2 300B "0°" means that the zero, as a value, was determined. The images presents a water drop "inside" the sample. When the contact angle could

not be detected because the sample is highly hydrophilic, I suggest to mark it as "super hydrophilic" or "super-wetting" regime, where $r \geq 1/\cos\theta$,

- microbiological analysis was carried out using a Gram-positive *S. aureus* bacteria strains. Was it a reference or clinical bacteria? Have you analyzed the antibacterial properties of the coating using a Gram-negative bacteria?
- on page 64, it was reported that the bacteria biofilm formation increased over the time, how did you analyze formation of the biofilm ?
- on page 43, it was mentioned that the Mg samples were sterilized using ethanol. Ethanol reacts with the Mg alloys, thus please clarify the sterilization steps of the samples. In your opinion, what is the best sterilization technique for the implant made by Mg alloys?
- the methodology of the *in vivo* study should be better describe, it is not clear how the samples were prepared for the implantation (size, shape, sterilization process). Based on the presented results it could assume that the materials were implanted into rat bones (maybe I missed the point in the English version of this dissertation, please clarify),
- in the conclusions the PhD students wrote that the structural analysis was performed. I think the statement is related to the microstructural analysis of the ceramic coatings. In material science the structural analysis presents mechanical properties of the material and/or analysis of the unit cells of the chemical compounds (according to the XRD analysis). Usually the coatings formed on the Mg alloys via the PEO technique are composed of amorphous phase. Have you determined the phase composition of the ceramic coatings?

The biocompatibility analysis showed that the magnesium alloys may cause a necrosis or neovascularization. However, the anodized magnesium alloys could be more biocompatible. What is your opinion about the chemical composition of the PEO coating and how it could influence on the neovascularization process?

8. Conclusions.

The dissertation of Dryhval Bohdan Oleksandrovych meets the requirements of clauses 6, 7, 8, 9 of the Resolution of the Cabinet of Ministers of Ukraine No. 44 dated January 12, 2022 "*The procedure for awarding the degree of Doctor of Philosophy and canceling the decision of the one-time specialized academic council of a higher education institution, scientific institution on awarding degree of Doctor of Philosophy*", and the dissertation student deserves to be awarded the degree of Doctor of Philosophy in the field of knowledge 22 "Health Care" in the specialty 222 "Medicine".

Signed by /
Podpisano przez:

Alicja Maria
Kazek-Kęsik

Date / Data:
2024-02-01 11:48