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The biocompatibility and potential cytotoxicity of materials for joint replacement manufacturing and coating

PhD Thesis Summary

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Abstract

Currently used prostheses for total joint replacement still have numerous disadvantages: extreme stiffness or elastic modulus of the bulk metallic material; insufficient integration of the implant into the host bone; and a high wear and corrosion rate, which causes an accumulation of mostly metallic or polymeric wear debris. Because of these reasons, many patients experience increasing local pain, swelling, allergic reactions, and inflammation resulting in bone loss and the aseptic loosening of the implant leading to the need for painful and expensive revision surgery.

To address the mechanical issues of commonly used orthopaedic alloys, this thesis presents the development of the new β-type titanium alloy Ti-35Nb-7Zr-6Ta-2Fe-0.5Si with a relatively low elastic modulus (up to 85 GPa), increased tensile strength (880 MPa), and enhanced biocompatibility and osteoconductivity.

Considering the generally low osteoinductivity of metallic implants, various surface modifications and coatings have been developed to improve the cell-material interaction, e.g. carbon-based coatings. Among these coatings, C_{60} fullerene layers have emerged as a great candidate for coating orthopaedic implants due to their therapeutic potential in arthritis. The potential cytotoxicity and DNA damage response of fullerenes have been evaluated. Although the fresh C_{60} coating has attenuated the adhesion and proliferation of cells, no DNA damage or signs of cytotoxicity have been found. The biocompatibility of C_{60} films has improved with the increasing age of these films or by co-deposition of C_{60} molecules with Ti atoms, thanks to changes in their physicochemical properties (such as fragmentation, oxidation, polymerization, and graphitization).

In order to minimize the wear and corrosion of the Co-Cr-Mo alloy, the diamond-like-carbon (DLC) coating of this alloy with a titanium

gradient adhesive interlayer has been used. The wear analysis has revealed no visible wear or delamination of the DLC coating after 3 million cycles of increasing loading force of up to 2.5 kN. Moreover, no proof of any cytotoxicity of the potential wear debris has been found.

In the last project of this thesis, a biocompatible, fully optically transparent diamond-based planar biosensor for the non-invasive (label-free), real-time monitoring of cell cultivation has been successfully invented. The main advantage of this sensor is its transparency, which enables microscopic native cell observation and the wide frequency range of the sensor allowing the detailed study of different cellular processes.

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1 Introduction

The aging population together with the increasing demand for a longer, more active, and healthier lifestyle brings new challenges to regenerative medicine. Bone and joint degenerative or inflammatory problems (such as osteoarthritis, rheumatoid arthritis) affect several hundred million people worldwide. Furthermore, one in every three women over 50 years of age suffers a fracture caused by bone loss (osteoporosis). These large bone defects caused by diseases or different traumas (like bone fractures, infections, and tumours) often cannot be healed properly and require a surgical approach, including transplantation or total joint replacement (TJR, also called arthroplasty).

1.1 Requirements for orthopaedic prostheses

Orthopaedic implants are required to have high tensile and compressive strength to prevent prostheses fractures and improve their functional stability. In addition, sufficient elasticity (a low elastic modulus) is also needed to ensure the uniform distribution of stress on the implant and to minimize the relative movement at the implant-bone interface. If the elastic modulus of the prosthesis is too high, the load which is normally applied to the bone is carried by the stiff implant, and the bone tissue atrophies due to lack of mechanic stimulation (this process is called stress shielding). Consequent osteoporosis results in fractures of the surrounding bone or loosening of the implant [1]. Thus, a material with an excellent combination of a high strength and low but sufficient elastic modulus should be used for implantation to avoid loosening of the implant and the need for a revision surgery.

Apart from the mechanical properties, the main requirement for orthopaedic prostheses is their biocompatibility, which is described

as the ability of the material to support normal cellular activity without any local and systematic toxic effects to the host tissue [2].

Since the artificial bone implants are made of different, mostly metallic materials, high corrosion resistance is also required. In addition, materials for total joint replacement need to also be wear resistant to avoid debris formation from the friction of contact surfaces. The potential accumulation of metallic ions from corrosion or wear of the prosthesis causes allergic reactions, inflammation and bone resorption that may eventually lead to the implant loosening [3].

Another important criterion of a bone implant is its good integration into the host bone (process called osseointegration). A higher degree of osseointegration improves the mechanical stability of the implant, which decreases the probability of implant loosening.

1.2 Materials of orthopaedic prostheses

The stems of load-bearing orthopaedic implants like total hip replacement are mainly fabricated from metallic alloys due to their superior mechanical properties (hardness, stiffness, etc.). Despite a large number of metallic medical devices in use today, orthopaedic prostheses are manufactured of only a few metals such as 316L stainless steel, cobalt chromium alloys, and titanium-based alloys. However, all of these materials have several disadvantages such as inappropriate mechanical properties (a low strength or too high elastic modulus [4-6]) or insufficient biocompatibility, caused by the present of toxic, allergenic or tumorigenic elements (such as Ni, Co, Cr, V, Al, Mo, Pd and Sn) [7]. Therefore, the current research is focused on the development of new alloys, consisting entirely of biocompatible elements, which would have a sufficient strength but still enough elasticity (lower elastic modulus better matching the modulus of the host bone).

Apart from the inappropriate mechanical properties of currently used total joint replacement prostheses, the accumulation of metallic ions caused by the high corrosion and wear rate of bearing surfaces is the main reason for implant failure [3]. Therefore, also non-metallic materials such as ceramics and polymers have been employed for bearing surfaces manufacturing of the joint prostheses. However, later studies have revealed that not only metallic debris but also polymeric and ceramic debris cause adverse biological reactions [8-13]. Moreover, ceramic heads have been repeatedly reported for catastrophic failure and fracture as well as the production of unpleasant squeaking sound [14-18].

Therefore, different approaches for surface modification including various implant coatings have been investigated in order to reduce both the volume of wear and the corrosion of the prostheses. Moreover, specialized bioactive coatings of implanted stems have also been explored to improve the bonding of a metallic stem with the host bone. Various carbon-based coatings have recently received great attention due to their unique physicochemical properties including high chemical inertness, mechanical stability, excellent electrical conductivity as well as satisfactory biocompatibility [19, 20]. Diamonds and diamond-like coatings are one of these materials, which are used in a wide range of biomedical applications (from the wear and corrosion reduction of prosthesis bearing surfaces to the biosensors constructing; [21-26]). Another carbon-based material with great potential in biomedical applications is fullerene coating thanks to its strong antioxidant property as well as the therapeutic potential in arthritis prevention and treatment [27-30].

2 Objectives of the thesis

The general aim of this thesis was to evaluate the biocompatibility and potential cytotoxicity of promising materials for the manufacturing or coating of total joint replacement prostheses. The specific goals were focused on:

1. Developing a biocompatible beta titanium alloy with increased strength without an excessive increase in the elastic modulus and without a negative effect on the biocompatibility of this alloy. Verifying the biocompatibility and evaluating the osteogenic potential of the manufactured alloys with various Fe and Si additions were also included in our goals.

2. Evaluating the potential cytotoxicity and enhancing the biocompatibility (if needed) of fullerene layers for future use as a bioactive coating for orthopaedic implants.

3. Observing the wear of the Cr-Co-Mo alloy with a Ti gradient adhesive interlayer coated by DLC used for total trapeziometacarpal joint arthroplasty. Evaluating the potential cytotoxicity of wear debris generated by simulated loading was also included in our aims.

4. Developing a biocompatible, label-free, fully optically transparent diamond-based planar biosensor for real-time cell monitoring.

3 Materials and Methods

Author contribution:

All listed methods (chapters 3.2 – 3.8) were performed by the author of this thesis and are described in detail in each publication.

3.1 Materials

Material manufacturing and characterization of the physicochemical properties were performed by our collaborating partners.

3.2 Cells

Various bone cell types were used for different purposes: immortalized human osteosarcoma cell lines MG-63 or U-2 OS and human primary osteoblasts HOB-p.

3.3 Cell culture methods

Cell culture methods including cell freezing and thawing as well as passaging, seeding, harvesting and counting of cells were routinely performed. Treatment with osteogenic differentiation supplements and a DNA damage inducer was employed.

3.4 Evaluation of cell adhesion and proliferation

Different methods of cell counting were employed: using a Bürker haemocytometer, Vi-Cell XR analyser, or counting of fixed cells visualised by Texas Red/Hoechst staining. For indirect assessment, a Cell Proliferation Kit II (XTT assay) was used. xCELLigence system was utilized for real-time proliferation monitoring.

3.5 Evaluation of cell morphology

Cell morphology during the experiments was observed natively by using an inverted light microscope equipped with a digital camera.

Cells fixed in specific time intervals were visualised by Texas Red/Hoechst staining and detected by fluorescence microscopy.

3.6 Evaluation of membrane damage and cell viability

Cell viability and potential membrane damage of the cells were detected by trypan blue staining performed during cell counting in the Vi-Cell XR analyser or by using a LIVE/DEAD Viability/Cytotoxicity Kit.

3.7 Evaluation of DNA damage response

Potential DNA damage was assessed by immunofluorescence staining of the DNA damage response markers, which were analysed by fluorescence microscopy (53BP1) and flow cytometry (gamma-H2AX).

3.8 Evaluation of material's surface wettability

The wettability of the material's surface was estimated from the contact angle measured by a material-water droplet system using a reflection goniometer.

4 Results and Discussion

4.1 Objective No. 1 – Biocompatible beta titanium alloy

Publication I: Newly developed Ti-Nb-Zr-Ta-Si-Fe biomedical beta titanium alloys with increased strength and enhanced biocompatibility

The aim of this study was to develop a new biocompatible Ti-Nb-Zr-Ta alloy of increased strength without an excessive increase in the elastic modulus and without a negative effect on the biocompatibility of this alloy. In order to increase the relatively low strength (around 550 MPa) of this alloy, 5 combinations of small Fe and/or Si atoms additions to a benchmark Ti-35Nb-7Zr-6Ta alloy were used. To evaluate the biocompatibility and osteogenic potential of all manufactured alloys, adhesion, proliferation (under static as well as dynamic conditions), and differentiation of cells were performed in this study.

Indeed, the Fe and Si additions caused a significant increase in strength (from 550 MPa to 850 MPa) but also in the elastic modulus of the Ti-35Nb-7Zr-6Ta alloy. However, even the highest achieved elastic modulus (up to 85 GPa) was still much lower than that of the widely used Ti–6Al–4V alloy (around 115 GPa).

In vitro biological experiments proved that all six manufactured Ti-35Nb-7Zr-6Ta-based alloys are biocompatible and promote stronger adhesion of human osteoblast-like U-2 OS cells (especially the benchmark alloy and alloys with the addition of 1Si, 2Fe or 0.5Si + 1Fe). The metabolic activity of the U-2 OS cells (proportional to the cell number and proliferation) cultured on all manufactured alloys under static conditions was higher than the metabolic activity of cells grown on the reference Ti-6Al-4V alloy. Our observations are in accordance with the literature: When cultured with extracts of similar alloys (Ti–29Nb–13Ta–4.6Zr, Ti-10Zr-8Nb-2Ta-0.2Pd, and Ti-15Zr-4Nb-2Ta-0.2Pd) as opposed to

cultivation in the extract solution of a Ti-6Al-4V alloy, a higher proliferation of L929 and MC3T3-E1 cells was found [31, 32]. Correspondingly, the proliferation of various cell types was also higher on the Ti–15Zr–4Nb–4Ta as well as the Ti-35Nb-2Ta-3Zr alloy than on the Ti-6Al-4V alloy [33, 34].

Differentiation *in vitro* experiments revealed significantly higher population densities and collagen I production of the primary human osteoblast cells (HOB-p) grown on alloys with 2 wt. % of Fe and 0.5 wt.% of Si additions in comparison with the Ti–6Al–4V alloy. The beneficial effect of specific iron concentrations on the proliferation and collagen I synthesis of various cell types was also reported in the literature [35-38]. Similarly, Si-containing materials were found to promote the proliferation and osteogenic differentiation of various cell types [39-43].

In our first study, we developed the Ti-35Nb-7Zr-6Ta-2Fe-0.5Si alloy with significantly increased strength as well as overall biocompatibility. Thanks to its lower elastic modulus and better osteogenic potential, the better integration of the implant into the host bone should be achieved. This alloy is, therefore, promising material for the manufacture of load-bearing implants such as total joint replacement prostheses.

4.2 Objective No. 2 – Biocompatibility of fullerene layers

<u>Publication II:</u> Growth and potential damage of human bone-derived cells on fresh and aged fullerene C_{60} films
<u>Publication III:</u> Growth and potential damage of human bone-derived cells cultured on fresh and aged C_{60}/Ti films

Fullerene layer is a great candidate for coating orthopaedic implants due to the therapeutic potential of fullerenes in arthritis prevention and treatment [27-30]. The widely discussed potential cytotoxicity of fullerenes was investigated in these two publications. Because of the high reactivity of the fullerene molecules, the effect of the fullerene - C_{60} layers' age (from one week to one year) on the initial adhesion, proliferation, viability and metabolic activity of human osteoblast-like cells was evaluated. We also monitored potential membrane and DNA damage as well as morphological changes of the cells.

<u>Our first study</u>, performed on pure fullerene C_{60} films, revealed lower initial adhesion, cell numbers, metabolic activity and viability of the MG-63 cells cultured on fresh C_{60} layers. Moreover, the cells cultivated on the fresh C_{60} coating were poorly spread with a rounded morphology; however, no cytotoxic morphological changes, such as enlarged cells or cytosolic vacuole formation were observed. All investigated parameters (including cell adhesion, density, viability, and spreading) markedly improved with the aging of the C_{60} layers. Although the fullerene C_{60} coating, particularly the fresh ones, attenuated cell growth, experiments performed on human osteoblast-like U-2 OS cells did not reveal any DNA damage response, as evaluated by gamma-H2AX (phosphorylated histone H2AX) and 53BP1 (p53-binding protein).

In contrast to our results, numerous studies have described fullerenes and their derivatives as cytotoxic and genotoxic agents,

causing oxidative DNA damage, polyploidy, inflammation, and inhibition of the detoxificatory and antioxidant enzymes, as well as premature cell senescence and apoptosis [44-50]. However, recently tetrahydrofuran (THF), which was widely used as a solvent of fullerenes, was found to be cytotoxic. Thus, the toxic side products (γ-butyrolactone, 2-hydroxytetrahydrofuranol and formic acid) created during the preparation of C_{60} suspension are probably responsible for the cytotoxicity previously attributed to fullerenes, whereas fullerenes themselves have no harmful effect [51-53]. Likewise, Kepney has lately opposed that many studies are not observing data relevant to C_{60} exposure, but rather the data from a mixture of fullerenes plus solvent, which together make a completely new molecular entities with different cytotoxicity [54]. Accordingly, *in vivo* toxicity experiment in rodents revealed similar results: Not only did the aqueous C_{60} suspension prepared without using any polar organic solvent not cause any toxicity, but this treatment even had a protective effect on rodents' livers in a dose-dependent manner [55]. In addition, other *in vivo* studies have not observed any skin or eye irritation, allergic reaction, or tumour formation [56-58]. Furthermore, the beneficial effects of fullerene and their derivatives on cognitive behaviour and the lifespan of rodents have been reported [59, 60].

Aged C_{60} films provided a better support for the adhesion and growth of MG-63 cells, as all investigated parameters (including cell adhesion, density, viability, proliferation and cell spreading) improved markedly with the aging of C_{60} layers. The better cell performance can be attributed to changes in the physicochemical properties of the fullerene films during aging, such as fragmentation, oxidation, polymerization and graphitization (revealed by Raman spectroscopy and X-ray Photoelectron Spectroscopy - XPS). These changes probably decreased fullerene reactivity, modified the chemistry and enhanced the nanoscale

roughness of the C_{60} layers resulting in the facilitation of cell adhesion and proliferation [61, 62].

In our second study, fullerene C_{60} coating was enriched with titanium atoms in order to improve the mechanochemical stability and biocompatibility of the fullerene layers. The results, indeed, showed that the Ti addition improved the properties of the fullerene coatings (particularly the fresh ones), which became more suitable for cell cultivation. No significant differences in cell adhesion, viability, and growth between the fresh and aged layers were observed. In fact, the behaviour of cells cultured on both the fresh and aged coating was comparable to the behaviour of cells grown on reference glass coverslips and no DNA damage response or cytotoxic cellular morphology was found. The beneficial effect of the Ti addition on the biocompatibility and bioactivity of the coating has also been reported for DLC layers [63]. Similarly, the Ti addition to other carbon-related coatings, including amorphous carbon and the hydrocarbon plasma polymer, also enhanced the adhesion, spreading, and growth of osteoblast-like MG-63 cells [64, 65].

The difference between the pure C_{60} coating and Ti-enriched layers can be explained by the fact that the above-mentioned changes in the fullerene molecules (like fragmentation, polymerization, and oxidation) occurred not only with the aging of the coatings but even during C_{60} and Ti co-deposition by the mutual interaction of these molecules and atoms (confirmed by Raman spectroscopy and XPS). The Ti-enriched C_{60} coating can, therefore, be considered as a promising material for orthopaedic implant coating with osteoprotective potential [27- 30, 66-68].

4.3 Objective No. 3 – Wear and biocompatibility of DLC-coated Co-Cr-Mo alloy

Manuscript: The cytotoxicity and wear analysis of a DLC-coated Co-Cr-Mo alloy used for total trapeziometacarpal joint arthroplasty

Diamond-like-carbon (DLC) coating has been shown to have excellent hardness and to significantly reduce the wear and metal ion release as well as the corrosion rate of metallic bearing surfaces [24-26]. The purpose of this study was to observe the wear of a Cr-Co-Mo alloy with a titanium gradient adhesive interlayer coated by DLC used for total trapeziometacarpal joint arthroplasty and to analyse the potential cytotoxicity of wear particles generated by simulated loading.

Our results from simulated loading revealed the great wear resistance of the DLC coating with no visible wear or delamination after 3 million cycles of increasing loading force of up to 2.5 kN. These findings are in accordance with other studies, where the DLC coating significantly reduced the wear and enhanced the scratch resistance of the Cr-Co-Mo alloy [24, 25, 69, 70]. Moreover, the DLC-coated implant has been reported to stay undamaged even after aggressive wear tests employing third-body bone cement particles [71]. Additionally, the results of a simulated body fluid experiment performed for 2 years have shown a 100,000 times lower corrosion rate of the DLC-coated Cr-Co-Mo alloy in comparison with the uncoated alloy [72].

In order to investigate the potential cytotoxicity of the wear particles, the real-time monitoring xCELLigence system (measuring electrode impedance, displayed as cell index) was used to evaluate the proliferation of U-2 OS osteoblast-like cells cultivated in different wear particles solutions. After a 7 day long cultivation, no significant differences among the samples (number

of loading cycle) were found. Moreover, no correlation between increasing cycle numbers (with increasing loading force of up to 2.5 kN) and decreasing proliferation of the osteoblast-like cells incubated in the obtained wear suspensions was proven. Correspondingly, numerous studies have shown great biocompatibility of the DLC films, supporting the growth and viability of many cell types without any signs of cytotoxicity [73-76]. Furthermore, *in vivo* experiments have proven good acceptance of DLC-coated implants by surrounding tissues [77, 78]. Therefore, we can conclude that during the simulated loading of the DLC-coated Co-Cr-Mo alloy with the Ti gradient adhesive interlayer, no cytotoxic wear debris was formed.

4.4 Objective No. 4 – Biocompatible and transparent biosensor

<u>Publication IV:</u> H-terminated diamond as optically transparent impedance sensor for real-time monitoring of cell growth

Impedance sensors have attracted great attention as powerful characterization tools for real-time, non-invasive, label-free cell monitoring. However, current commercially available systems have several limitations including the restriction of the available area for microscopic native cell observation of the cultivated cells due to the use of non-transparent gold electrodes.

In this study, we successfully invented a biocompatible, fully optically transparent diamond-based planar biosensor with uniform intrinsic edges-free morphology for non-invasive (label-free), real-time monitoring of cell cultivation. The real-time proliferation as well as cell morphology of human osteoblast-like MG-63 cells seeded in various concentrations was evaluated. The commercially available xCELLigence sensor from Roche was used as a reference impedance system. Our diamond-based impedance sensor showed that the morphology and proliferation rate of MG-63 cells was comparable to that of the reference gold-based system. Time-dependent impedance measurements at 1 kHz exhibited similar profiles for the diamond and reference gold sensors.

Another big advantage of our sensor compared with the commercial alternatives is the wide frequency range of the impedance measurement (from 100 Hz up to 100 kHz) which allows detailed study of different cellular processes. For low-frequency measurements (<10 kHz), the current flows through gaps between the cells (paracellular flow), therefore the impedance is sensitive to cell density, growth, and movements. If the measurement frequency is high enough (>40 kHz), current can flow through the cell

membrane (transcellular flow), which means that the impedance is more sensitive to cell adhesion and less sensitive to cell population [79]. Moreover, our diamond-based impedance sensor may also be suitable for *in vitro* testing of various drugs and cytotoxic agents.

5 Conclusion

The issue of disadvantages of currently used total joint replacement prostheses was addressed in this thesis, consisting of four impacted publications and one already sent manuscript. Considering the insufficient mechanical properties of commonly used orthopaedic alloys, five new low-rigidity Ti-35Nb-7Zr-6Ta alloys, with various Fe and Si additions which significantly increase the tensile strength and enhance the biocompatibility and osteoconductivity of the benchmark alloy were developed. The alloy with final a composition of Ti-35Nb-7Zr-6Ta-2Fe-0.5Si exhibited the best combination of mechanical and biological properties and was therefore chosen as the most promising candidate for manufacturing load-bearing implants.

In order to improve the integration of the implant into the host bone, a fullerene coating with osteoprotective potential was investigated. Although no cytotoxicity or DNA damage was observed in osteoblast-like cells grown on fresh C_{60} layers, we improved the biocompatibility of the fresh fullerene coating by co-deposition of C_{60} molecules with Ti atoms.

Another carbon-related coating was used for the reduction of the Co-Cr-Mo wear rate. A DLC-coated Co-Cr-Mo with a Ti gradient adhesive interlayer exhibited no visible wear or delamination. Moreover, the safety of the DLC coating was proven by no observed correlation between the increasing cycles of simulated loading and the decreasing proliferation of the osteoblast-like cells incubated in the obtained wear suspensions.

Finally, a biocompatible, fully optically transparent diamond-based planar biosensor with uniform intrinsic edges-free morphology for non-invasive (label-free), real-time monitoring of cell cultivation with a wide range of applications was successfully invented.

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7 Curriculum vitae

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PERSONAL DETAILS

Date of Birth: 21. 03. 1985

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EDUCATION

2004 - 2009

Faculty of Science, Charles University, **Prague**

Master of Science in Cellular and Molecular Biology

WORKING EXPERIENCE

2009 - Present

Institute of Physiology Academy of Sciences of the Czech Republic / Ph.D. student

Department of Biomaterials and Tissue Engineering

2006 - 2009

Institute of Molecular Genetics Academy of Sciences of the Czech Republic / student

Department of Signal Transduction (2006-2007), Bachelor's degree earned

Department of Genome Integrity (2007-2009), Master's degree earned

COURSES AND ACHIEVEMENTS

2014

Genes and the Human Condition (From Behavior to Biotechnology), University of Maryland

Introduction to Forensic Science, Nanyang Technological University

2013

Epigenetic Control of Gene Expression, University of Melbourne

Programmed cell death, Ludwig Maximilians University Munich

Useful Genetics Part I and II, University of British Columbia

2011

Advances in Tissue Engineering, Rice University

SKILLS

Languages

Slovak: native speaker

Czech: proficient

English: advanced

Computer skills

MS Windows, MS Office, Adobe Photoshop

Mastered techniques

Cell culture techniques, immunofluorescence microscopy, immunoprecipitation, western blot, 2-D, SDS-PAGE, DNA electrophoresis, FASC

INTERESTS

Coursera courses, travelling, culture, literature, music, playing the piano, drawing

8 List of publications

8.1 Publications presented in this thesis

The results presented in this thesis are summarized in four impacted articles and in one already sent manuscript.

Kopova I., Strasky J., Harcuba P., Landa M., Janecek M., Bacakova L.: Newly developed Ti-Nb-Zr-Ta-Si-Fe biomedical beta titanium alloys with increased strength and enhanced biocompatibility. Mater Sci Eng C Mater Biol Appl. 2016 Mar 1;60:230-8.

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Izak T., Novotna K., Kopova I., Bacakova L., Rezek B., Kromka A.: H-terminated diamond as optically transparent impedance sensor for real-time monitoring of cell growth. Phys. Status Solidi B 2013;250(12):2741-2746.

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Kopova I. *, Kronek J. *, Bacakova L., Fencl J.: The cytotoxicity and wear analysis of a DLC-coated Co-Cr-Mo alloy used for total trapeziometacarpal joint arthroplasty. *Manuscript* (2016)

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8.2 Other publications

Bacakova L., Kopova I., Stankova L., Liskova J., Vacik J., Lavrentiev V., Kromka A., Potocky S., Stranska D.: Bone cells in cultures on nanocarbon-based materials for potential bone tissue engineering: A review. Phys. Status Solidi A 2014;211(12):2688–2702.

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Bacakova L., Kopova I., Vacik J., Lavrentiev V. Interaction of fullerenes and metal-fullerene composites with cells. In: Fullerenes: Chemistry, Natural Sources and Technological Applications. Edited by Ellis S.B., Nova Science Publishers, Inc., Hauppauge, New York, USA, 2014, pp 1-33. ISBN 978-1-63321-386-9.

Bacakova L., Filova E., Liskova J., Kopova I., Vandrovcova M., Havlikova J.: Nanostructured materials as substrates for the adhesion, growth and osteogenic differentiation of bone cells. In: Nanobiomaterials in hard tissue engineering; Applications of nanobiomaterials, Volume 4. Edited by Grumezescu A.M., Elsevier Inc., William Andrew Publishing, Oxford, Cambridge, 2016, Chapter 4, pp. 103-153, ISBN 978-0-323-42862-0.

9 List of conferences

Kopova I., Bacakova L., Vacik J., Lavrentiev V.: Micro-patterned layers of fullerenes (C_{60}) in bone tissue engineering. *PhD Student Workshop, Trest, Czech Republic,* **OP** (2011)

Kopova I., Bacakova L., Vacik J., Lavrentiev V.: Investigation of cytotoxicity and potential DNA damage of human osteosarcoma cells on fullerene C_{60} films. *Nanotechnology Conference, Xcaret, Mexico,* **PP** (2012)

Kopova I., Bacakova L., Strasky J., Harcuba P., Janecek M., Fencl J.: Investigation of advanced beta-type Ti-35Nb-7Zr-5Ta alloys with various Fe and/or Si additions. *PhD Student Workshop, Trest, Czech Republic,* **OP** (2013)

Kopova I., Strasky J., Harcuba P., Janecek M., Bacakova L.: Mechanical properties and biocompatibility of Ti-Nb-Ta-Zr alloy with varying Fe, Si and O content. *5th International Conference on Mechanics of Biomaterials and Tissues, Sitges, Spain,* **OP** (2013)

Kopova I., Bacakova L., Vacik J., Lavrentiev V.: Growth and potential damage of human bone-derived cells cultured on fresh and aged C_{60}/Ti films. *International Conference on Tissue Engineering and Regenerative Medicine, Pretoria, South Africa,* **OP** (2014)

Kopova I., Bacakova L., Strasky J., Harcuba P., Janecek M., Fencl J.: Proliferation and differentiation of osteoblast-like cells cultured on beta-Ti-35Nb-7Zr-6Ta alloys with various Si and/or Fe additions. *6th International Conference on Mechanics of Biomaterials and Tissues, Waikoloa, USA,* **PP** (2015)

Kopova I., Bacakova L., Strasky J., Janecek M.: The effect of different surface modifications of Ti-35.3Nb-7.3Zr-5.7Ta-0.7O alloy on differentiation of adipose tissue-derived stem cells. *9th Meeting of the Scandinavian Society for Biomaterials, Reykjavik, Iceland,* **PP** (2016)

OP - oral presentation

PP - poster presentation

Univerzita Karlova v Praze

Přírodovědecká fakulta

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Biokompatibilita a potenciálna cytotoxicita materiálov na výrobu a pokrývanie totálnych endoprotéz

Autoreferát dizertačnej práce

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Abstrakt

V súčasnosti používané totálne endoprotézy kĺbov majú niekoľko nedostatkov vrátane príliš veľkej tuhosti kovového materiálu, nedostatočnej integrácie implantátu do kosti a vysokej miery opotrebovania či korózie. To má za následok úbytok kostnej hmoty a následné aseptické uvoľnenie protézy s nutnosťou ďalšieho chirurgického zákroku.

Nepostačujúce mechanické vlastnosti súčasných ortopedických zliatin rieši táto práca vývojom novej beta-titánovej zliatiny s konečným zložením Ti-35Nb-7Zr-6Ta-2Fe-0.5Si, ktorá má relatívne nízky modul pružnosti (do 85 GPa), vysokú pevnosť v ťahu (850 MPa) a vylepšenú biokompatibilitu, ako aj osteokonduktivitu.

Vzhľadom k všeobecne nízkej osteoinduktivite kovových implantátov sa vyvíjajú rôzne povrchové úpravy a vrstvy pre zlepšenie interakcie buniek s materiálmi, ako napríklad vrstvy na báze uhlíka. Fullerénové vrstvy sa zdajú byť sľubným kandidátom na pokrývanie ortopedických implantátov vzhľadom k ich ochranným anti-oxidačným vlastnostiam. Hoci čerstvé C_{60} vrstvy tlmili adhéziu a rast buniek, žiadne známky bunkovej toxicity ani poškodenia DNA neboli nájdené. Sledovaná biokompatibilita fullerénových C_{60} filmov sa výrazne zlepšila s pribúdajúcim vekom vrstiev alebo s ko-depozíciou fullerénových molekúl s atómami titánu. Vysvetlenie spočíva v chemicko-fyzikálnych zmenách ako sú fragmentácia, oxidácia, polymerácia a grafitizácia fullerénových molekúl, ku ktorým dochádzalo v oboch spomínaných procesoch.

Na minimalizáciu opotrebovania a korózie Co-Cr-Mo zliatiny sme použili uhlíkovú DLC (diamond-like-carbon) vrstvu s medzivrstvou titánového gradientu na zlepšenie priľnutia DLC vrstvy ku kovovému substrátu. Analýza opotrebenia po 3 miliónoch záťažových cykloch so zvyšujúcou sa silou do 2,5 kN neodhalila žiadny viditeľný oter alebo

odlupovanie DLC vrstvy. Vyhodnotenie získanej oterovej suspenzie nepreukázalo cytotoxicitu prípadných vznikajúcich oterových častíc.

V poslednom projekte tejto práce sme skonštruovali biokompatibilný, plne priehľadný, diamantový biosenzor so širokým rozpätím použiteľných frekvencií na neinvazívne sledovanie kultivácie buniek v reálnom čase. Hlavnou výhodou tohto senzora je jeho optická priehľadnosť, ktorá umožňuje mikroskopické pozorovanie živých buniek a široký frekvenčný rozsah snímača umožňujúci sledovanie rôznych bunkových procesov.

Obsah

1 Úvod

Starnutie populácie spolu s narastajúcim dopytom po dlhšom a aktívnejšom živote prináša nové výzvy pre regeneratívnu medicínu. Degeneratívne či zápalové ochorenia kostí a kĺbov (napríklad osteoartritída a reumatoidná artritída) trápia niekoľko stoviek miliónov ľudí po celom svete. Okrem toho každú tretiu ženu nad 50 rokov postihne zlomenina spôsobená úbytkom kostnej hmoty v dôsledku osteoporózy. Kostné defekty veľkých rozmerov, spôsobené chorobou alebo úrazom (zlomeniny, infekcie a nádory), často vyžadujú chirurgický prístup vrátane transplantácie alebo použitia totálnych endoprotéz.

1.1 Požiadavky na totálne endoprotézy

Ortopedické implantáty musia mať veľkú pevnosť v ťahu aj tlaku, aby mali dobrú funkčnú stabilitu a nedochádzalo k ich fraktúram. Okrem pevnosti je tiež potrebná dostatočná pružnosť použitého materiálu (nízky modul pružnosti), aby bolo zabezpečené rovnomerné zaťaženie implantátu a bol minimalizovaný pohyb v mieste spojenia implantátu s kosťou. V prípade, že modul pružnosti endoprotézy je príliš vysoký, celá záťaž je nesená tuhým implantátom a okolité kostné tkanivo atrofuje v dôsledku nedostatočnej mechanickej stimulácie. Vznikajúca osteoporóza má za následok fraktúry okolitej kosti alebo uvoľnenie implantátu [1]. Preto je dôležité, aby bol na výrobu endoprotéz použitý materiál s vynikajúcou kombináciu vysokej pevnosti a relatívne nízkeho modulu pružnosti.

Ďalšou hlavnou požiadavkou je biokompatibilita použitých endoprotéz. Biokompatibilita je definovaná ako schopnosť materiálu podporovať prirodzenú bunkovú aktivitu bez akýchkoľvek škodlivých účinkov na okolité tkanivá [2].

Vzhľadom na to, že kostné implantáty sú vyrobené z rôznych, prevažne kovových materiálov, vysoká odolnosť proti korózii je dôležitou podmienkou. Materiály pre výrobu totálnych endoprotéz musia byť taktiež odolné proti opotrebeniu, aby sa zabránilo tvorbe oterových častíc z trenia styčných plôch protézy. Prípadné hromadenie kovových častíc v dôsledku korózie alebo opotrebenia endoprotézy vyvoláva alergické reakcie, zápal a resorpciu kosti, ktoré môžu nakoniec viesť k uvoľneniu samotného implantátu [3].

Ďalším dôležitým kritériom kostného implantátu je jeho dobrá integrácia do okolitej kosti, proces nazývaný osseointegrácia. Čím vyšší stupeň osseointegrácie, tým lepšia mechanická stabilita a ukotvenie endoprotézy, čo znižuje pravdepodobnosť uvoľnenia implantátu.

1.2 Materiály pre výrobu totálnych endoprotéz

Hlavným materiálom na výrobu totálnych endoprotéz sú kovové zliatiny vďaka ich vynikajúcim mechanickým vlastnostiam (tvrdosť, pevnosť atď.). Napriek veľkému počtu používaných kovových chirurgických pomôcok sú súčasné endoprotézy vyrábané z pár materiálov vrátane nerezovej ocele (316L), zliatin kobaltu s chrómom alebo zliatin na báze titánu. Avšak všetky tieto materiály majú niekoľko nevýhod, ako sú nevhodné mechanické vlastnosti (nízka pevnosť alebo príliš vysoký modul pružnosti) [4-6] alebo nedostatočná biokompatibilita spôsobená prítomnosťou toxických, alergénnych či karcinogénnych prvkov (ako sú Ni, Co, Cr , V, AI, Mo, Pd a Sn) [7]. Z tohto dôvodu je súčasný výskum zameraný na vývoj nových zliatin zložených výlučne z biokompatibilných prvkov, ktoré by mali dostatočnú pevnosť, ale zároveň aj dostatočnú pružnosť (nižší modul pružnosti blížiaci sa hodnotám okolitej kosti).

Hromadenie kovových častíc spôsobené koróziou a opotrebením styčných plôch endoprotéz je okrem nevhodných mechanických vlastností používaných endoprotéz hlavnou príčinou zlyhania týchto

implantátov [3]. Preto sa na výrobu styčných plôch endoprotéz začali používať nekovové materiály ako sú keramika a polyméry. Žiaľ, neskoršie štúdie ukázali, že nielen kovové, ale aj polymérne a keramické častice majú nežiadúce biologické účinky [8-13]. Okrem toho, keramické styčné plochy častejšie podliehajú fraktúram a pri pohybe trenie keramickej hlavice o keramickú jamku často vydáva nepríjemný škrípavý zvuk [14-18]

Z tohto dôvodu sú skúmané rôzne prístupy pre povrchovú úpravu endoprotéz vrátane nanášania rôznych vrstiev na elimináciu opotrebenia a korózie styčných plôch implantátov alebo na zlepšenie integrácie kovového implantátu do okolitej kosti (špeciálne bioaktívne vrstvy). Veľkú pozornosť v poslednej dobe získali vrstvy na báze uhlíka vďaka ich unikátnym fyzikálno-chemickým vlastnostiam ako sú vysoká chemická inertnosť, dobrá mechanická stabilita, vynikajúca elektrická vodivosť a dobrá biokompatibilita [19, 20]. Diamantové a diamantu podobné vrstvy sú jedným z týchto materiálov používaných v širokej škále biomedicínskych aplikáciách (od povlakov proti opotrebovaniu a korózii endoprotéz až po konštrukciu biosenzorov [21-26]). Ďalším materiálom na báze uhlíka s veľkým potenciálom v biomedicínskych aplikáciách sú vrstvy fullerénov, vďaka ich silným antioxidačným vlastnostiam, ako aj sľubnému terapeutickému potenciálu v prevencii a liečbe artritídy [27-30].

2 Ciele práce

Všeobecným cieľom tejto práce bolo zhodnotiť biokompatibilitu a potenciálnu cytotoxicitu perspektívnych materiálov na výrobu alebo poťahovanie totálnych endoprotéz. Konkrétnejšie ciele boli nasledovné:

1. Vývoj biokompatibilnej beta-titánovej zliatiny so zvýšenou pevnosťou, bez nadmerného zvýšenia modulu pružnosti a bez negatívneho efektu na biokompatibilitu tejto zliatiny. Overenie biokompatibility a zhodnotenie osteogénneho potenciálu vyrobených zliatin s rôznymi prídavkami atómov Fe a Si boli taktiež zahrnuté v našich cieľoch.

2. Stanovenie možnej cytotoxicity a v prípade potreby zlepšenie biokompatibility fullerénových vrstiev pre budúce použitie ako bioaktívne pokryvy ortopedických implantátov.

3. Pozorovanie opotrebenia zliatiny Cr-Co-Mo potiahnutej DLC vrstvou s použitím titánovej medzivrstvy s využitím pre totálnu náhradu trapeziometakarpálneho kĺbu. Vyhodnotenie potenciálnej cytotoxicity oterových častíc získaných simulovanými záťažovými testami bolo tiež zahrnuté v našich cieľoch.

4. Vývoj biokompatibilného, neinvazívneho, plne transparentného biosenzoru na báze diamantu pre sledovanie kultivácie buniek v reálnom čase.

3 Materiály a metódy

Všetky vymenované metódy (kapitoly 3.2 – 3.8) boli vykonané autorom tejto dizertačnej práce a sú detailne popísané v daných publikáciách.

3.1 Materiály

Výroba a charakterizácia fyzikálno-chemických vlastností použitých materiálov boli vykonané našimi spolupracovníkmi.

3.2 Typy buniek

Rôzne typy kostných buniek boli použité na rôzne účely: imortalizované bunkové línie ľudského osteosarkómu MG-63 alebo U-2 OS a ľudské primárne osteoblasty HOB-p.

3.3 Metódy kultivácie buniek

Metódy kultivácie buniek vrátane zmrazovania a rozmrazovania buniek, ako aj pasážovania, nasadzovania experimentov a počítania buniek boli rutinne vykonávané. Bunky boli ďalej kultivované s induktormi osteogénnej diferenciácie a induktormi poškodenia DNA.

3.4 Stanovenie adhézie a proliferácie buniek

Boli použité rôzne metódy počítania buniek s využitím Bürkerovej komôrky, Vi-Cell XR analyzátoru alebo počítanie fixovaných buniek vizualizovaných Texas Red / Hoechst farbením. Pre nepriame stanovenie bol použitý Cell proliferation Kit II (XTT test). Systém xCELLigence bol využitý pre sledovanie bunkovej proliferácie v reálnom čase.

3.5 Stanovenie morfológie buniek

Bunková morfológia v priebehu experimentov bola pozorovaná natívne pomocou invertovaného svetelného mikroskopu vybaveného digitálnym fotoaparátom. Bunky fixované v určitých časových intervaloch boli vizualizované Texas Red / Hoechst farbením a detegované fluorescenčnou mikroskopiou.

3.6 Stanovenie poškodenia bunkovej membrány a viability buniek

Životaschopnosť buniek a potenciálne poškodenie bunkovej membrány boli detegované farbením trypánovou modrou v priebehu počítania buniek Vi-Cell XR analyzátorom alebo pomocou komerčne dostupného LIVE / DEAD Kitu.

3.7 Stanovenie poškodenia DNA

Prípadné poškodenie DNA bolo sledované s použitím imunofluorescenčných markerov poškodenia DNA detegovaných pomocou fluorescenčnej mikroskopie (53BP1) alebo prietokovej cytometrie (gama-H2AX).

3.8 Stanovenie zmáčavosti materiálov

Zmáčavosť povrchu materiálov bola stanovená z merania kontaktného uhlu kvapiek vody za použitia goniometra.

4 Výsledky a diskusia

4.1 Cieľ č. 1 – Biokompatibilná beta-titanová zliatina

Publikácia č. I: Newly developed Ti-Nb-Zr-Ta-Si-Fe biomedical beta titanium alloys with increased strength and enhanced biocompatibility

Cieľom tejto štúdie bolo vyvinúť novú biokompatibilnú zliatinu s lepšou pevnosťou bez nadmerného zvýšenia modulu pružnosti a bez negatívneho vplyvu na biokompatibilitu tejto zliatiny. Na zvýšenie relatívne nízkej pevnosť (asi 550 MPa) základnej zliatiny so zložením Ti-35Nb-7Zr-6Ta bolo použitých 5 kombinácií malých prídavkov atómov Fe a / alebo Si. Pre stanovenie biokompatibility a osteogénneho potenciálu všetkých vyrobených zliatin bola sledovaná adhézia, proliferácia (za statických aj dynamických podmienok) a diferenciácia buniek.

Výsledky ukázali, že prídavky Fe a Si skutočne spôsobili výrazné zvýšenie pevnosti z 550 MPa na 850 MPa. Modul pružnosti bol taktiež vyšší, ale aj najvyššie dosiahnutá hodnota (do 85 GPa) bola výrazne nižšia ako modul pružnosti bežne používanej Ti-6Al-4V zliatiny (okolo 115 GPa).

Biologické *in vitro* pokusy potvrdili, že všetky vyrobené zliatiny sú biokompatibilné a podporujú silnú adhéziu ľudských osteoblastov U-2 OS (najmä zliatiny s prídavkami 1Si, 2Fe a 0,5Si + 1Fe). Metabolická aktivita U-2 OS buniek (priamo úmerná počtu buniek a bunkovej proliferácii) kultivovaných na všetkých vyrobených zliatinách za statických podmienok bola vyššia než metabolická aktivita buniek pestovaných na referenčnej zliatine Ti-6Al-4V. Naše pozorovania sú v súlade s literatúrou, kde bola pozorovaná vyššia proliferácia L929 a MC3T3-E1 buniek kultivovaných s extraktmi podobných zliatin (Ti-29Nb-13Ta-4.6Zr, Ti-10Zr-8Nb-2Ta-0.2Pd a Ti-15Zr-4Nb-2Ta-0.2Pd)

v porovnaní s kultiváciou buniek vo výluhu Ti-6Al-4V zliatiny [31, 32]. Podobne, proliferácia rôznych typov buniek bola lepšia na zliatinách Ti-15Zr-4Nb-4Ta a Ti-35Nb-2Ta-3Zr než na Ti-6Al-4V zliatine [33, 34].

Výsledky *in vitro* diferenciácie ukázali vyššiu hustotu ľudských primárnych osteoblastov, ako aj ich produkciu osteogénneho markeru kolagénu I po kultivácii na zliatinách s prídavkom 2 % Fe a 0,5 % Si než na Ti-6Al-4V zliatine. Priaznivý účinok špecifických koncentrácií železa na bunkovú proliferáciu a syntézu kolagénu I rôznych typov buniek bol taktiež popísaný v literatúre [35-38]. Podobne bolo zistené, že materiály obsahujúce kremík podporujú proliferáciu a osteogénnu diferenciáciu rôznych typov buniek [39-43].

V tomto projekte sme vyvinuli zliatinu s konečným zložením Ti-35Nb-7Zr-6Ta-2Fe-0.5Si s výrazne lepšou pevnosťou, ako aj celkovou biokompatibilitou. Vďaka jej relatívne nízkemu modulu pružnosti a zlepšenému osteogénnemu potenciálu by mala táto zliatina zabezpečiť lepšiu integráciu do hostiteľskej kosti. Táto zliatina je preto sľubný materiál na výrobu ortopedických implantátov, ako je totálna endoprotéza.

4.2 Cieľ č. 2 – Biokompatibilita fullerénových vrstiev

Publikácia č. II: Growth and potential damage of human bone-derived cells on fresh and aged fullerene C_{60} films
Publikácia č. III: Growth and potential damage of human bone-derived cells cultured on fresh and aged C_{60}/Ti films

Fullerénové vrstvy sú skvelým kandidátom na poťahovanie ortopedických implantátov vzhľadom k ich terapeutickému potenciálu v prevencii a liečbe artritídy [27-30]. Hojne diskutovaná cytotoxicita fullerénov bola skúmaná v týchto dvoch publikáciách. Vzhľadom k vysokej reaktivite fullerénových molekúl bola sledovaná závislosť medzi vekom fullerénových vrstiev – C_{60} (od jedného týždňa do

jedného roka) a adhéziou, proliferáciou, viabilitou a metabolickou aktivitou ľudských MG-63 buniek. Prípadné poškodenie bunkovej membrány a DNA, ako aj morfologické zmeny buniek boli tiež hodnotené.

Naša prvá štúdia zameraná na čisté fullerénové vrstvy odhalila nižšiu adhéziu, proliferáciu, metabolickú aktivitu a životaschopnosť buniek kultivovaných na čerstvých vrstvách. Hoci tieto bunky boli zaguľatené a slabo rozprestreté, neboli nájdené žiadne cytotoxické morfologické zmeny, ako sú zväčšené bunky alebo tvorba cytozolových vakuol. Všetky sledované parametre sa výrazne zlepšili so starnutím C_{60} vrstiev. Aj keď fullerénové vrstvy, obzvlášť v ich čerstvej forme, tlmili rast buniek, experimenty vykonané na ľudskej línii U-2 OS neodhalili zvýšenú mieru poškodenia DNA (hodnotené gama H2AX - fosforylovaný histón H2AX; a 53BP1 - proteín viažuci p53).

Tieto výsledky sú v rozpore so štúdiami popisujúcimi fullerény a ich deriváty ako cytotoxické a genotoxické látky spôsobujúce oxidatívne poškodenie DNA, polyploiditu, zápal, inhibíciu detoxifikačních a antioxidačných enzýmov, ako aj predčasnú senescenciu a apoptózu [44-50]. Pomerne nedávno bola zistená cytotoxicita tetrahydrofuránu (THF), ktorý sa bežne používa ako rozpúšťadlo fullerénov. To môže znamenať, že toxické vedľajšie produkty (γ-butyrolaktón, 2-hydroxytetrahydrofuranol a kyselina mravčia), vznikajúce počas prípravy suspenzie C_{60}, sú pravdepodobne zodpovedné za cytotoxicitu pôvodne prisudzovanú fullerénom, zatiaľ čo fullerény samotné nemajú žiadny škodlivý účinok [51-53]. Podobne Dr. Kepney oponoval, že mnoho štúdií pozoruje namiesto efektu fullerénov efekt zmesi fullerénov s rozpúšťadlami, ktoré spoločne tvoria úplne nové molekulárne entity s odlišnou cytotoxicitou [54]. V súlade s týmto tvrdením *in vivo* experimenty na hlodavcoch ukázali, že vodná suspenzia C_{60} molekúl pripravená bez použitia polárneho organického rozpúšťadla nielen že nespôsobila žiadnu toxicitu, ale dokonca mala ochranný účinok na pečeň hlodavcov v závislosti od použitej dávky

[55]. Podobne ďalšie *in vivo* štúdie nepozorovali žiadne podráždenie kože alebo očí, ani alergické reakcie či vznik nádorov [56-58]. Navyše bol preukázaný prospešný účinok fullerénov a ich derivátov na kognitívne správanie a životnosť hlodavcov [59, 60].

Staršie C_{60} vrstvy poskytovali lepšie podmienky pre adhéziu a rast MG-63 buniek vo všetkých sledovaných parametroch vrátane bunkovej adhézie, proliferácie, životaschopnosti aj morfológie. Tieto zmeny boli pripisované chemicko-fyzikálnym zmenám ako sú fragmentácia, oxidácia, polymerácia a grafitizácia fullerénových molekúl, ku ktorým dochádzalo počas „starnutia" vrstiev (potvrdené Ramanovou spektroskopiou a röntgenovou fotoelektrónovou spektroskopiou - XPS). Tieto zmeny pravdepodobne znížili reaktivitu fullerénových molekúl, viedli k ich chemickým modifikáciám a zvýšeniu nanodrsnosti fullerénových vrstiev, čo malo za následok uľahčenie adhézie a proliferácie buniek [61, 62].

V našej druhej štúdii bola fullerénová vrstva obohatená o atómy titánu za účelom zlepšenia stability a mechanochemickej biokompatibility fullerénových vrstiev. Výsledky potvrdili, že po prídavku Ti sa fullérenové filmy stali vhodnejšími pre kultiváciu buniek (hlavne čerstvé vrstvy). Neboli pozorované žiadne významné rozdiely v adhézii, viabilite a rastu buniek medzi čerstvými a starými vrstvami. Správanie buniek pestovaných na čerstvých aj starých C_{60}/Ti filmoch bolo zrovnateľné so správaním buniek pestovaných na referenčnom krycom sklíčku. Podobne ako v prípade čistých fullerénových vrstiev, C_{60}/Ti filmy nespôsobovali zvýšenú mieru poškodenia DNA ani cytotoxické morfologické zmeny buniek. Priaznivý účinok prídavku atómov Ti na biokompatibilitu a bioaktivitu bol tiež pozorovaný u DLC vrstvy [63]. Podobne, pridanie Ti k ďalším vrstvám na báze uhlíka vrátane amorfného uhlíka a uhľovodíkového plazmového polyméru tiež zvýšilo adhéziu a rast MG-63 buniek [64, 65].

Rozdiel medzi čistými C_{60} a Ti-obohatenými filmami môže byť vysvetlený skutočnosťou, že k uvedeným zmenám v molekulách

fullerénov (ako sú fragmentácia, polymerizácia a oxidácia) došlo nielen počas starnutia vrstiev, ale už v priebehu ko-depozície C_{60} molekúl s atómami titánu vzájomnou interakciou týchto molekúl a atómov (potvrdené Ramanovou spektroskopiou a röntgenovou fotoelektrónovou spektroskopiou - XPS). Titánom obohatené fullerénové filmy môžu byť preto považované za sľubný materiál s osteoprotektívnym potenciálom na pokrývanie ortopedických implantátov [27- 30, 66-68].

4.3 Cieľ č. 3 – Opotrebovanie a biokompatibila Cr-Co-Mo zliatiny potiahnutej DLC vrstvou

Rukopis: The Cytotoxicity and wear analysis of DLC-coated Co-Cr-Mo alloy used for total trapeziometacarpal joint arthroplasty

DLC (diamond-like-carbon) vrstva má vynikajúcu tvrdosť a výrazne znižuje opotrebovanie, koróziu a uvoľňovanie kovových iónov zo styčných plôch kovových implantátov [24- 26]. Cieľom tejto štúdie bolo sledovať opotrebenie Cr-Co-Mo zliatiny potiahnutej DLC vrstvou s použitím titánovej medzivrstvy pre celkovú náhradu trapeziometakarpálneho kĺbu a analyzovať potenciálnu cytotoxicitu oterových častíc generovaných simulovanými záťažovými testami.

Naše výsledky simulovaného zaťažovania odhalili vysokú odolnosť DLC vrstvy proti opotrebovaniu bez viditeľného oteru alebo odlupovania po 3 miliónoch cyklov zvyšujúcej sa sily až do 2,5 kN. Tieto zistenia sú v súlade s inými štúdiami, kde DLC povlak značne znížil opotrebenie a zvýšil odolnosť Cr-Co-Mo zliatiny proti poškriabaniu [24, 25, 69, 70]. Okrem toho, implantát potiahnutý DLC vrstvou ostal nepoškodený i po agresívnych oterových testoch využívajúcich cementové častice [71]. Navyše výsledky experimentu simulovaných telových tekutín vykonávané po dobu 2 rokov preukázali 100000 krát nižšiu rýchlosť korózie Cr-Co-Mo zliatiny s povlakom DLC v porovnaní s nepotiahnutou zliatinou [72].

Na sledovanie potenciálnej cytotoxicity oterových častíc bol použitý systém xCELLigence (merajúci impedanciu v reálnom čase) na hodnotenie proliferácie U-2 OS buniek pestovaných v rôznych suspenziách oterových častíc. Po 7 dňoch kultivácie neboli zistené žiadne významné rozdiely medzi vzorkami (počet záťažových cyklov). Okrem toho nebola pozorovaná žiadna korelácia medzi narastajúcim počtom cyklov (so zvyšujúcou sa silou do 2,5 kN) a znižujúcou sa proliferáciou buniek inkubovaných v oterových suspenziách. Podobne, početné štúdie preukázali výbornú biokompatibilitu DLC filmov, podporujúcu rast a životaschopnosť mnohých typov buniek bez známok cytotoxicity [73-76]. *In vivo* experimenty taktiež dokázali dobrú akceptáciu implantátov poťahovaných DLC vrstvou okolitými tkanivami [77, 78]. Preto môžeme konštatovať, že počas simulovaných záťažových testov zliatiny Cr-Co-Mo potiahnutej DLC vrstvou s použitím titánovej medzivrstvy nevznikali cytotoxické oterové častice.

4.4 Cieľ č. 4 – Biokompatibilný a priehľadný biosensor

<u>Publikácia č. IV:</u> H-terminated diamond as optically transparent impedance sensor for real-time monitoring of cell growth

Impedančné senzory vzbudili veľkú pozornosť ako skvelý nástroj pre neinvazívne monitorovanie buniek v reálnom čase. Avšak bežné, komerčne dostupné systémy majú radu limitácií vrátane obmedzeného priestoru pre mikroskopické pozorovanie živých buniek z dôvodu použitia nepriehľadných zlatých elektród.

V tejto štúdii sme úspešne skonštruovali neinvazívny, biokompatibilný, plne priehľadný diamantový biosenzor s jednotnou planárnou morfológiou pre monitorovanie buniek v reálnom čase. Bola hodnotená proliferácia v reálnom čase, ako aj morfológia ľudských MG-63 buniek nasadených v rôznych koncentráciách. Komerčne dostupný xCELLigence senzor od Roche bol použitý ako referenčný impedančný

systém. Náš diamantový biosenzor ukázal porovnateľnú morfológiu a proliferáciu MG-63 buniek ako referenčný systém. Časovo závislé meranie impedancie pri 1 kHz vykazovalo podobné profily pre diamantové aj zlaté (referenčné) elektródy.

Ďalšou veľkou výhodou nášho biosenzoru v porovnaní s komerčnými alternatívami je široký frekvenčný rozsah merania impedancie (od 100 Hz do 100 kHz), ktorý umožňuje detailné štúdium rôznych bunkových procesov. Pre nízkofrekvenčné merania (<10 kHz) prúd tečie cez medzery medzi bunkami (paracelulárne prúdenie), a preto je impedancia viac citlivá na hustotu, rast a pohyb buniek. V prípade, že je použitá frekvencia dostatočne vysoká (> 40 kHz), prúd môže pretekať cez bunkovú membránu (transcelulárne prúdenie), a preto je impedancia citlivejšia na adhéziu buniek [79]. Náš biosenzor môže byť taktiež použitý na *in vitro* testovanie rôznych liečiv, ako aj cytotoxických činidiel.

5 Záver

Problematika nevýhod bežne používaných totálnych endoprotéz bola riešená v tejto práci pozostávajúcej zo štyroch impaktovaných publikácií a jedného odoslaného rukopisu.

Vzhľadom k nedostatočným mechanickým vlastnostiam bežne používaných ortopedických zliatin sme vyvinuli päť Ti-35Nb-7Zr-6Ta zliatin s rôznymi prídavkami Fe a Si s výrazne vyššou pevnosťou v ťahu a lepšou biokompatibilitou, ako aj osteokonduktivitou v porovnaní so základnou Ti-35Nb-7Zr-6Ta zliatinou. Zliatina so zložením Ti-35Nb-7Zr-6Ta-2Fe-0,5Si vykazovala najlepšiu kombináciu mechanických a biologických vlastností, a preto je považovaná za sľubný materiál pre výrobu nosných implantátov – totálnych endoprotéz.

Pre zlepšenie integrácie implantátu do hostiteľskej kosti sme skúmali fullerénové (C_{60}) vrstvy s osteoprotektívnym potenciálom. Hoci *in vitro* experimenty neodhalili žiadnu cytotoxicitu ani zvýšenú mieru poškodenia DNA u buniek pestovaných na čerstvých C_{60} vrstvách, biokompatibilitu čerstvých filmov sme zlepšili ko-depozíciou C_{60} molekúl s atómami Ti.

Ďalšia vrstva na báze uhlíka bola použitá pre zníženie opotrebenia Co-Cr-Mo zliatiny. Analýza opotrebovania zliatiny Cr-Co-Mo potiahnutej DLC vrstvou s použitím titánovej medzivrstvy neodhalila žiadny viditeľný oter, odlupovanie a ani cytotoxicitu Co-Cr-Mo zliatiny s DLC vrstvou.

V neposlednom rade sme so spolupracovníkmi z Fyzikálneho ústavu skonštruovali biokompatibilný, plne priehľadný diamantový biosenzor na neinvazívne sledovanie kultivácie buniek v reálnom čase so širokou škálou aplikácií.

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with a multi-walled carbon nanotube-tyrosinase hybrid film. Biosensors and Bioelectronics 2015;74:830-835.
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7 Životopis

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OSOBNÉ ÚDAJE

Dátum narodenia: 21. 03. 1985

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Stav: Slobodná

Národnosť: Slovenská

VZDELANIE

2004 - 2009

Prírodovedecká fakulta Univerzity Karlovej v Prahe

Magisterský obor – Bunková a molekulárna biológia

ZAMESTNANIE

2009 - prítomnosť

Fyziologický ústav AV ČR / doktorand

Oddelenie biomateriálov a tkanivového inžinierstva

2006 - 2009

Ústav molekulárnej genetiky AV ČR / študent

Oddelenie signálnej transdukcie (2006-2007), bakalárske štúdium

Oddelenie genómovej integrity (2007-2009), magisterské štúdium

KURZY

2014

Genes and the Human Condition (From Behavior to Biotechnology), University of Maryland

Introduction to Forensic Science, Nanyang Technological University

2013

Epigenetic Control of Gene Expression, University of Melbourne

Programmed cell death, Ludwig Maximilians University Munich

Useful Genetics Part I and II, University of British Columbia

2011

Advances in Tissue Engineering, Rice University

SCHOPNOSTI

Jazyky

Slovenčina, angličtina, čeština

Počítačové schopnosti

MS Windows, MS Office, Adobe Photoshop

Osvojené techniky

Metódy kultivácie buniek, immunofluorescenčná mikroskopia, immunoprecipitácia, prietoková cytometria, western blot, SDS-PAGE, DNA elektroforéza

ZÁUJMY

Online kurzy - coursera, cestovanie, kultúra, literatúra, hra na klavíri, maľovanie

8 Zoznam publikácií

8.1 Publikácie, na ktorých je založená dizertačná práca

Výsledky prezentované v tejto práci sú zhrnuté v štyroch impaktovaných článkoch a v jednom odoslanom rukopise:

Kopova I., Strasky J., Harcuba P., Landa M., Janecek M., Bacakova L.: Newly developed Ti-Nb-Zr-Ta-Si-Fe biomedical beta titanium alloys with increased strength and enhanced biocompatibility. Mater Sci Eng C Mater Biol Appl. 2016 Mar 1;60:230-8.

$IF_{2014/2015}$ = 3.088

Kopova I., Bacakova L., Lavrentiev V., Vacik J.: Growth and potential damage of human bone-derived cells on fresh and aged fullerene C_{60} films. Int J Mol Sci. 2013 Apr 26;14(5):9182-204.

$IF_{2014/2015}$ = 2.862

Kopova I., Lavrentiev V., Vacik J., Bacakova L.: Growth and potential damage of human bone-derived cells cultured on fresh and aged C_{60}/Ti films. PLoS One. 2015 Apr 15;10(4):e0123680.

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Izak T., Novotna K., Kopova I., Bacakova L., Rezek B., Kromka A.: H-terminated diamond as optically transparent impedance sensor for real-time monitoring of cell growth. Phys. Status Solidi B 2013;250(12):2741-2746.

$IF_{2014/2015}$ = 1.489

Kopova I. *, Kronek J. *, Bacakova L., Fencl J.: The cytotoxicity and wear analysis of a DLC-coated Co-Cr-Mo alloy used for total trapeziometacarpal joint arthroplasty. *Rukopis* (2016)

* equal contribution

8.2 Ďalšie publikácie

Bacakova L., Kopova I., Stankova L., Liskova J., Vacik J., Lavrentiev V., Kromka A., Potocky S., Stranska D.: Bone cells in cultures on nanocarbon-based materials for potential bone tissue engineering: A review. Phys. Status Solidi A 2014;211(12):2688–2702.

$IF_{2014/2015}$ = 1.616

Bacakova L., Kopova I., Vacik J., Lavrentiev V. Interaction of fullerenes and metal-fullerene composites with cells. In: Fullerenes: Chemistry, Natural Sources and Technological Applications. Edited by Ellis S.B., Nova Science Publishers, Inc., Hauppauge, New York, USA, 2014, pp 1-33. ISBN 978-1-63321-386-9.

Bacakova L., Filova E., Liskova J., Kopova I., Vandrovcova M., Havlikova J.: Nanostructured materials as substrates for the adhesion, growth and osteogenic differentiation of bone cells. In: Nanobiomaterials in hard tissue engineering; Applications of nanobiomaterials, Volume 4. Edited by Grumezescu A.M., Elsevier Inc., William Andrew Publishing, Oxford, Cambridge, 2016, Chapter 4, pp. 103-153, ISBN 978-0-323-42862-0.

9 Zoznam konferencií

Kopova I., Bacakova L., Vacik J., Lavrentiev V.: Micro-patterned layers of fullerenes (C_{60}) in bone tissue engineering. *Výjazdné zasadanie PhD študentov, Třešť, Česká Republika,* **OP** (2011)

Kopova I., Bacakova L., Vacik J., Lavrentiev V.: Investigation of cytotoxicity and potential DNA damage of human osteosarcoma cells on fullerene C_{60} films. *Nanotechnology Conference, Xcaret, Mexiko,* **PP** (2012)

Kopova I., Bacakova L., Strasky J., Harcuba P., Janecek M., Fencl J.: Investigation of advanced beta-type Ti-35Nb-7Zr-5Ta alloys with various Fe and/or Si additions. *Výjazdné zasadanie PhD študentov, Třešť, Česká Republika,* **OP** (2013)

Kopova I., Strasky J., Harcuba P., Janecek M., Bacakova L.: Mechanical properties and biocompatibility of Ti-Nb-Ta-Zr alloy with varying Fe, Si and O content. *5th International Conference on Mechanics of Biomaterials and Tissues, Sitges, Španielsko,* **OP** (2013)

Kopova I., Bacakova L., Vacik J., Lavrentiev V.: Growth and potential damage of human bone-derived cells cultured on fresh and aged C_{60}/Ti films. *International Conference on Tissue Engineering and Regenerative Medicine, Pretoria, Juhoafrická Republika,* **OP** (2014)

Kopova I., Bacakova L., Strasky J., Harcuba P., Janecek M., Fencl J.: Proliferation and differentiation of osteoblast-like cells cultured on beta-Ti-35Nb-7Zr-6Ta alloys with various Si and/or Fe additions. *6th International Conference on Mechanics of Biomaterials and Tissues, Waikoloa, USA,* **PP** (2015)

Kopova I., Bacakova L., Strasky J., Janecek M.: The effect of different surface modifications of Ti-35.3Nb-7.3Zr-5.7Ta-0.7O alloy on differentiation of adipose tissue-derived stem cells. *9th Meeting of the Scandinavian Society for Biomaterials, Reykjavik, Island,* **PP** (2016)

OP – prednáška (oral presentation)

PP – plagát (poster presentation)