SHORT THESIS FOR THE DEGREE OF PHILOSOPHY (PHD)

Experimental studies with novel synthetic bone replacement materials, potentially usable in dentistry

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The Examination takes place at University of Debrecen, Faculty of Medicine, Department of Obstetrics and Gynaecology lecture hall on 16.12.2022. at 10:00.

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1. Introduction

Numerous descriptions and artifacts we have collected from the ancient centuries which demonstrated the early attempts for bone healing or bone replacement. With the development of sciences during the past decades, these initial efforts showed more and more improvements and success in this field thus, in addition to the general medicine, the osseointegration with bone substituent's grafting has now an important role in the dentistry as well. By using new innovative materials, like synthetic bone grafts and appropriate methods, the bone regeneration processes can be much faster and more powerful in cases of a tumor-related bone defect, bone fracture, large sized cyst, or during implantology and orthodontic treatments.

According to the classification of bone replacement materials, there are six groups in the terminology: autograft (same identity), isograft (genetically identical), allograft (same species), xenograft (different species), composite grafts, and alloplasts (synthetic materials). Regarding the latter groups, recently several research studies are going on to find out the most ideal synthetic bone graft materials. The successful bone replacement materials need to have special physical-chemical characteristics including to mobilize cells (eg, stem cells, bone marrow stromal cells, osteoblasts, fibroblasts, chondrocytes), to release signalling molecules (like cytokines, growth factors, molecules involved in cell adhesion), all mediated by the graft's appropriate structural properties (porosity and mechanical features) and the biological (osseoinductive and conductive) properties (1-4). The bioceramics aerogels from our previous research correspond to this description as a base.

The first aerogel (AE) was made by S. S. Kistler in 1931, when he dried out a wet gel (5). This mesoporous airy-light material, despite its good thermal insulation ability because of the sensitivity to moisture and weak mechanical properties, was not popular at the beginning. However, supplementing with functional groups the usability of the composite aerogel was much more functional (6-13).

Accordingly, we added β-TCP (β-tricalcium phosphate) granulates to the aerogel base. Although the calcium-phosphate substance of the bone was discovered in 1769 and calcium phosphate was then taken into account in bone healing and regeneration process significantly, the first synthetic forms have only been used in the medical practice since 1900 (14-17). The

calcium phosphate containing materials have three groups: the hydroxyapatite, the tricalcium phosphate and their combination, which can also be called as biphasic alloplastic materials (3). The β-TCP is a white, fragile, solid material, produced from calcium phosphate, or as a result of thermal treatment of hydroxyapatite. The specialty of the β-TCP is the three biological properties: osseoinductive, osseoconductive features, and the cell mediated resorption of β-TCP. Accordingly, during osseointegration of β-TCP, the immature pluripotent cells start differentiating via pre-osteoblasts into osteoblasts to induce osteogenesis - reflecting osteoinduction. On the other hand, the presence of β-TCP there is an osseoconductive growth along the bone surfaces to facilitate the repair.

Thirdly, during physiological conditions, there is a cell-mediated resorption of the β-TCP by activated macrophages and multinuclear giant cells (MNGC) which finally eliminate the grafting material.

As a result of these three biological properties, the graft is capable of integrating into the trabecular bone structure to facilitate repair while the unwanted remnant materials are being metabolized by the phagocytosing leukocytes (3, 18-34).

The in vivo investigations are essential for the examination of osseointegration, the osteogenesis and the bone healing process. In cases of traditional histological slide preparations, the decalcified specimens -after paraffin embedding- are being sectioned for the optimal thickness by a microtome. However, in cases of implants or bone replacement hard materials, these specimens cannot be sectioned by the traditional microtome because of the mechanical strength of these materials. This problem was solved by K. Donath for the first time with a special hard tissue microtome, who established a new non-decalcination-based histological slide preparation technique (35). For the optimal thickness of the slide, after using the hard tissue microtome, further grinding is necessary. However, during the grinding procedures, because of the mechanical forces, in many cases the whole sample or some parts may become detached from the histological glass surface. To overcome this problem, we introduced and used the chemical 10- MDP (methacryloyl-oxydecyl-dihydrogen-phosphate; the common dental bond) to prevent ground sections' detachment from glass slides. This monomer is able to bind firmly to the apatite, dentin, metal and zirconium as well, so all types of materials which our samples contained. In fact, the hard tissue slide preparation is a multi-step process, where the bonding has a major role to achieve the optimal (thin) slide thickness for appropriate microscopic evaluations.

2. Aim of the study

With these studies the following goals were wanted to achieve:

1. Mesoporous silica aerogel (AE) and β-tricalcium phosphate aerogel (β-TCP-AE) production, and their physico-chemical characterisation, in vitro examinations for the osteoblastic differentiation and the cytotoxicity on SAOS-2 cells, respectively.

2. Histopathological examinations for the in vivo effects of the fabricated AE and β-TCP-AE bone substituent compounds, focusing on the ossification processes in bone defects of the rat „calvaria critical size" model, using formalin-fixed paraffin embedded decalcified tissue sections.

In relation with the conventional histopathological evaluation of the in vivo studies on decalcified tissue slides (described above), originally (parallel to these), we wanted to prepare ground sections from non-decalcified native specimens as well to judge the real lime (mineral) content within the healing bone defects when grafted with AE or β-TCP-AE. However, the method for ground section preparation proved to be not reproducible due to occasional detachment from glass slide (therefore, the results on ground sections were omitted from my PhD thesis-based publication). The failure in this methodology inspired us to make an additional research goal to fulfil the reproducible ground sectioning method with the following specific aim:

3. Develop a new improved method for sectioning of those hard tissue samples (e.g., ceramics or titanium containing ones), which normally cannot be sliced with the traditional microtome but hard tissue microtome, only. In this respect, our major task was to find an appropriate bond-chemical to stick the hard thick tissue sections to the microscopic glass slide with firm adherence for a final thinning by means of grinding-polishing. In brief, optimalisation of adhesive system for better bonding must be achieved.

3. Material and methods

3.1.1. Aerogel fabrication procedures

To create the aerogel, named sample „A" was made from methanol, distilled water, diluted urea and ammonium solution at first. Sample „B" contained sample "A" completed with

methoxy-silane, β-TCP, and cellulose. Stirring was finished when the mixture became viscous, almost gelly consistency. This viscous material was left at room temperature in molds (66 x x28 mm), that were closed the tops with parafilm. After one night, the consistency changed to alkogel, whereafter the methanol was changed to acetone in multiple steps, followed by a supercritical drying. The aerogel calcification was finished in a thermal stove to keep their mechanical resistances. After burning, the following data were recorded: weight of the samples, the shape and density.

3.1.2. In vitro examinations

SAOS-2 undifferentiated osteosarcoma cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, low glucose; Sigma, USA) supplemented with bovine serum, antibiotic-antimycotic solution, and Glutamate (all from Gibco, USA) in a humidified incubator at 37°C. The cells were then trypsinized and seeded into culture plates (Nunc, Denmark). Following adherence, the culture medium (CM) was changed to new CM containing the aerogel or β-TCP- aerogel. These aerogel and β-TCP-aerogel particles sunk onto the cells to achieve direct contact between the material particles and the cells. The medium was then changed every second day, and examinations were terminated on the seventh day.

3.1.2.1. Viability and cytotoxicity

The viability of the cells and the cytotoxicity of the materials was examined using the alamarBlue Cell Viability Reagent (Invitrogen, USA). Briefly, the cells were incubated for 2 hours at 37 ^{o}C in the presence of 1/10 diluted alamarBlue reagent. Following treatments, the fluorescence of 200 µl samples was then measured using Hidex Sense microplate reader (Hidex, Finland).

3.1.2.2. Alkaline phosphatase (ALP) activity assay

After PBS washing, cells were lysed in lysis buffer. The cell debris were then removed from the lysed samples with centrifugation (Centrifuge 5810 R, Eppendorf, Germany). Supernatant was transferred to another microcentrifuge tube and used to determine the alkaline phosphatase activity by mixing the cell lysates with the enzyme substrate p-nitrophenyl phosphate (Sigma-Aldrich, USA). The absorbance of the samples was measured at 405 nm in a Hidex Sense microplate reader (Hidex, Finland). ALP activity was normalised to the protein concentration determined by using the BCA Protein Assay kit (Pierce, USA).

3.1.2.3. Gene expression

Total RNA was extracted with a standard method. Reverse RNA transcription was done with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA), after collecting the RNA from the cells by Quick RNA MiniPrep (Zymo Research, USA). Next, qPCR was performed with HOT FIREPol Probe qPCR Mix Plus (no ROX) (Solis Biodyne, Estonia) enzyme, and TaqMan probes (Applied Biosystems, USA) in LightCycler 480 machine (Roche, Switzerland). For gene expression measurements, the reference gene used for normalization was the GADPH, and the calculations were based on ΔCp technique.

3.1.3. In vivo examinations

For osseointegration studies, based on bone defect's repair by AE or β-TCP-AE, the „calvaria critical size defect model" was used on male Wistar rats (7-M/15/DEMÁB). The basis of this model is to create a full-thickness hole in the skull, with an 8 mm diameter trephine bur in the midline of the parietal bone region. In the first group the holes were left empty as a control group; in the second group the holes were filled with aerogel (AE); in the third group the holes were filled with β-TCP-aerogel, respectively. After one-, three-, and six months of the surgery, 3 samples of each group were collected to evaluate the bone defect's repair mediated by the grafting compounds focusing on the process of the osseointegration by means of histopathology techniques.

3.1.4. Histopathology and immunochemical examinations

One-, three-, (and six) months after the surgery, the control, the aerogel and the β-TCP aerogel samples were fixed in formalin and decalcified in 4% EDTA (ethylene- diamine-tetra-acetate) followed by embedding into paraffin and sectioning with the traditional microtome to 5-6 μm thin slices. Staining was made with haematoxylin-eosin (HE) and van Gieson. Immunohistochemical reactions were made as well in citrate buffer with antigen-retrieval using rabbit monoclonal primary Ki-67 antibodies (Abcam, UK). For slide digitalisation „Panoramic MIDI" digital slide-scanner (3D-Histech-Zeiss, Budapest) was used equipped with Hitachi 3CCD colour camera. Image analysis was carried out with HistoQuant „Panoramic Viewer software 1.15.2" (3D-Histech), and recorded by Mirax Viewer, or in some cases with Leica DM2500 microscope.

3.2.1. Sample preparations

To demonstrate the hard tissue section elements, three different kinds of materials were manufactured: epoxy from EpoFix resin plus Epofix hardener (EpoFix Kit, Struers, Ballerup, Dannmark), bone (from bovine) and the titanium samples in 5.3 mm diameter and 2 mm length shape. The histological slide glass (Thermo Scientific Menzel Gläser LOT#8501777) was cut to 13 x 25 mm pieces. Before cutting, the surface was treated with sandblasting (50 μm Al_2O_3) to increase the chemical-micromechanical adhesion.

3.2.2. Applied adhesives and resin matrix

In this study two different types of adhesives were used. One is the thermoplastic adhesive (Crystalbond 509 Mounting Adhesive, SPI Supplies, USA LOT#1180228) which melts at around 160°C and in turn solidifies as it cools down back to room temperature. The second adhesive has two parts: the first layer is a 10-MDP containing bond, and the second layer is a resin as a photo-initiator, which serves as a photopolymerizable resin. These two layers used as a sandwich technique between the glass surface and the samples: glass + 10-MDP bond + resin + resin + 10-MDP bond + sample (i.e., bone, epoxy, or the titanium containing samples).

3.2.3. Preliminary experiment: optimal time for etching

The optimal time for etching was determined in an earlier study. In this pre-study 7 pieces/group glass (13 x 25 mm) pieces were etched with 68% nitric acid (VWR, Debrecen, Hungary) from 0 hour (control group) to 5 hours (group 6). After the distilled water washing and drying of the glass pieces, the bonding way was the sandwich technique - mentioned above- between the glass and the epoxy sample. Finishing with the photopolymerization was in a light chamber unit (Scheu LC-6 Light Oven, Iserlohn, Germany), and shear bond strength was measured each of them.

3.2.4. Zeta potential measurements

For the zeta potential measurements, the glass slab pieces were ground with Analysette 3 Vibratory Sieve Shaker (Fritsch, Weimar, Germany) at 6 min 1.5 mm amplitude. The ground glass powder was washed three times by suspending and centrifuging in distilled water, dried at 90°C, then 0.1 g silica powder was placed into nitric acid for 3 hours. After decanting of

nitric acid supernatant, the powder was rinsed in ultra-pure water. The suspension was made in 0.001 M NaCl containing ultra-pure water from protonated silica powder. Unprotonated bare glass powder suspension was prepared by 0.1 g glass powder dispersed in 0.001 M NaCl background electrolyte. The pH of suspensions and background electrolyte was measured by Orion 2 Star (Thermo Scientific, Singapore). The zeta potential measurements of suspensions and electrolytes were performed with the help of Zetasizer Nano ZS (Malvern Instruments Ltd., Grovewood, Worcestershire, UK) based on electrophoretic mobility in folded capillary cells next to continuous 0.001 M NaCl as a background in ultrapure water. Each sample was measured five times (n = 5). Zeta potential was calculated using the Smoluchowski approximation.

3.2.5. XPS measurements

X-ray photoelectron spectra were obtained using an Al/Mg twin anode non-monochromatized radiation source and a Phoibos100 MCD-5 series hemispherical energy analyzer produced by SPECS (Berlin). The measurements were conducted with Al K-α (E = 1486 eV) rays on both acid-etched and untreated samples. Roughly 1 × 1 cm pieces were cut from a slide, their surfaces cleaned with a nitrogen jet, and were either mounted directly onto the XPS sample holder (untreated sample) or left to soak in the nitric acid for 3 h at room temperature, in the dark (etched sample). After the acid treatment, the samples were thoroughly washed with distilled water, dried by nitrogen jet, mounted onto the sample holder, and left to further dry and outgas in a vacuum (10-7 mbar) for 20 h before measurement. Core level spectra were recorded for Ca, Na, O, C, and Si and processed with CasaXPS (http://www.casaxps.com). Peak positions were normalized to that of carbon (284.5 eV), fitting mixed Gaussian/Lorentzian curves after Shirley baseline correction.

3.2.6. Contact angle measurements

Using a machine (Drop Shape Analyzer, Krüss GmbH, Hamburg, Germany) with a 0.5 mm diameter needle, a volume of 3 µl water has been dropped in an automatic way (n = 10) to glass, then protonated glass, then epoxy, then titanium surfaces, respectively. The same amount of water was dropped to adhesive surface manually, as well. Contact angles were measured based on the Young- Laplace equation.

3.2.7. Shear bond strength measurements

According to the adhesive type (thermoplastic or MDP containing) and the glass surfaces of non-treated, sand blasted, protonated ones, three different groups were created. First group: non treated glass surface with thermoplastic adhesive (1. group = TPA), second group: sand blasted glass with MDP containing bond (2. group = MDP), third group: sand blasted and protonated glass with MDP containing bond (3. group = PRO-MDP). In each group 15 samples epoxy, 15 samples of bone and 15 samples of titanium were bonded. The shear bond strength measurements were examined with an INSTRON 5544 (Norwood, USA) machine.

3.2.8. Detection of failure mode

At first shear bond strength measurements were done, then the surface of the samples examined with a microscope (Olympus SZ61, 45x) to see the fractures between the different materials. Based on this, the following classifications were named:

- Adhesive failure 1: fracture between the glass and adhesive
- Adhesive failure 2: failure between the adhesive and the samples (epoxy, bone or titanium)
- Cohesive failure 1: failure in adhesive
- Cohesive failure 2: failure in the glass

4. Results

4.1.1. Characterisation of the silica aerogel

Based on the adsorption-desorption porosimetry measurements, the average pore size was found 25 nm in the case of the silica aerogel, where both macropores and mesopores could be seen. For the pores that were found over 200 nm, scanning electron microscopy was used at 700x and 15x magnification, where 1–20-micron pores were seen for the β-TCP granulates' region.

4.1.2. Cytotoxicity of aerogels on SAOS-2 cells' viability

For the cytotoxicity of the aerogel, an alamarBlue Reagent-based measurements were done on SAOS-2 cells. For the control group, the cells were left in culture medium alone, while in the

second group the culture medium was supplemented with aerogel, and in the third group with β-TCP aerogel, respectively. Based on the results and as compared to control, AE did not show significant cytotoxicity, albeit some decrease in proliferation could be identified when co-cultured SAOS-2 cells with the β-TCP aerogel.

4.1.3. Alkaline phosphatase activity measurements

The osteoblastic differentiation-inducing ability of the aerogel and β-TCP- aerogel was tested with the alkaline phosphatase activity (ALP) assay. Both groups showed mild but significant increase in ALP under in vitro conditions. Because the increasing ALP activity is the marker of the osteoblastic differentiation, these results clearly confirm our assumption that the undifferentiated SAOS-2 cells undergo differentiation toward osteoblasts, induced by both the AE and the β-TCP-AE materials, respectively.

4.1.4. Gene expression

The BMP-2, BMP-7, Runx2 and OSX gene expressions were checked after seven days. Each of these osteoblast differentiation-induction genes was evaluated using the actual reference values normalized to GADPH during the examinations. Accordingly, the only significant difference was found in the β-TCP-AE samples compared to the control group in the case of the Runx2 gene.

4.1.5. Histopathological analysis

Following one month of the β-TCP-AE insertion into the bone defects, within the inflammatory granulation tissues there are incipient multifocal calcifications with an early sign of ossification inside the lesion. As opposed, in cases of the control bone defects (i.e., lesions were left unfilled with any bone substituent material), only inflammatory fibrous granulation tissues were detected inside the lesion by this time. Nevertheless, lateral reparative bone repair (arising from the internal surface of bony rim of the defect) could also be identified in both the control and the test sample types, but the intensity appeared more intensive with the presence of grafting materials, especially in β-TCP-AE treated samples. This reflects osteoconductive effects of the applied bone replacement materials. Remarkably, by the 3rd Month after surgery, the bone defects filled with AE but even more with β-TCP-AE all showed significantly progressive intralesional ossification resulting in large coalescent newly formed solid bone islets within mildly inflamed fibrous tissue, indicating powerful osteoinductive effects of the bone replacement compounds. However, it was noted that at this

time low grade foreign body giant cell-based inflammation could still be detected, in association with the presence of some non-metabolized AE and β-TCP-AE remnants, respectively.

4.2.1. Optimal timing for glass surface etching

Determining the optimal acid time for the glass surface the shear bond strength were measured between the glass- epoxy samples. The glass surfaces were etched 1-5 hours ago before the bonding. Based on the results the higher values were found in the case of the 3-hour group, so this is the most favourable acid time for glass surface protonation.

4.2.2. Zeta potential measurements results

The surface charging of the glass surface by protonation can be described by zeta potential measurements. Zeta potential was calculated using the Smoluchowski approximation. The most influential factors of zeta potential measuring are ionic strength and pH. In our measurements the ionic strength was constant. The pH of ground silica powder (d_{mean} = 1183 ± 230 nm) in the background electrolyte was 10.14. The alkaline pH was explained by the ion exchange between the protons/hydronium ions in the liquid layer outside the glass particles and sodium and calcium ions in the glass (39, 40). The glass surface is highly negatively charged in the alkaline region with zeta potential values down to −57.22 mV. The pH of the suspension of protonated glass particles was 1.82. The zeta potential of protonated ground glass particles shifted to smaller mV values, numerically +4.42 mV compared to unprotonated glass suspension. The acid etching with nitric acid provides a positively charged glass surface that can influence the adsorption of the next layer (10-MDP containing bond) to the silica.

4.2.3. XPS measurements results

The surface concentration of calcium and sodium are shown to decrease, while the Na 1s and Si 2p peaks undergo a slight shift towards higher binding energies (BE). This is most likely caused by dealkalisation, a type of glass corrosion caused by aqueous solutions wherein alkaline ions are leached into solution from a thin surface layer of the glass and replaced by other cations from the solution, most often H^+ and H_3O^+. This exchange is energetically favoured and accelerated in highly acidic solutions (such as cc. HNO_3) owing to the large excess of H^+ and H_3O^+. Binding energies usually increase with the number of bound oxygens,

thus, the BE shift shows that the leached alkaline ions are replaced with an oxygen-containing species, which in our case is H_3O^+.

4.2.4. Contact angle measurements results

For the optimal adhesion between the hydrophilic (glass surface) and hydrophobic (epoxi, titanium, bone) surfaces the moisturizer ability of the adhesion material has a major role. Our contact angle measurements indicated the MDP has excellent wetting ability on all the the surfaces, like titanium, bone, epoxy and glass, thus make it a perfect adhesion material between these.

4.2.5. Bond strength measurements results

The most prominent values were measured in the protonated glass case with MDP bond at titanium and bone samples. These values were significantly higher compared to the non-protonated glass surfaces.

4.2.6. Results of failure mode

According to the failure mode detection (%) of the tested specimens after shear bond strength measurements, in the first group the adhesive failure 1. values were the followings: epoxy samples 92%, bone samples 81%, titanium samples 83%. The cohesive failure 1: 8%, 19% and 17%.

In the second group the adhesive failure 1.: 75%, 65% and 55%, meanwhile the cohesive failure 1. was 13% in the bone samples.

In the third group adhesive failure 1. values: 83%, 54%, 61%. Adhesive failure 2. was 12% in titanium surfaces, and cohesive failure 2. 30% in bone samples.

In the first group the most prominent values showed by the adhesive failure 1., which represented the failure/fracture between the glass surface and the thermoplastic adhesive. Compared to the first group, in the second group the cohesive failure type 1. increased, which means the higher bond strength using the MDP bond compared to the thermoplastic adhesive. The adhesion was even better in case of the protonated glass surface.

5. Discussion, major results and conclusions

1. In our studies, silica aerogel (AE) and β-tricalcium phosphate aerogel (β-TCP-AE) could be produced successfully with optimal mesoporosous physico-chemical properties to be suitable as bone grafting materials, potentially useful in dentistry practices.

2. Based on the in vitro experiments, these materials are soluble in aqueous medium, non-cytotoxic, and they could induce the osteoblastic differentiation of immature osteosarcoma cell-lines, SAOS-2 as indicated by the increased alkaline phosphatase activity in conjunction with some genes' activations, typically involved in osteogenesis.

3. According to the in vivo experiments, the histopathological results proved that both the aerogel but even more the β-TCP aerogel could generate a reparative ossification in rat's calvaria bone defects by osteoconduction along the bone edges. In addition, the presence of these bioceramics, but most prominently the compound β-TCP aerogel could also induce intralesional multifocal new bone formations inside the bone defect, which appeared independent from the bony edges of the defect. This observation clearly indicates a powerful osteoinductive effects of the AE and the β-TCP-AE, respectively. Overall, it was concluded that when grafted the bone defect especially with the compound ceramics β-TCP-AE, 70% of the lesion became repaired via ossification by the 3rd month, while the majority of the bone substituent compound material became metabolized and eliminated by this time.

4. According to the morphology and histopathology, both the mesoporous AE and the β-TCP-AE grafts appear provide an optimal scaffold for bone defect's repair. Their silica components induce a foreign body giant cell mediated inflammation in the recipient tissue to resorb the exogenous materials, while in turn, an associated rapid fibrosis and new bone formation via ossification take place.

5. Based on the hard tissue section preparation, our results showed that the protonated glass surface with MDP adhesive could ensure the success in hard tissue preparation technique, even if contained other kinds of hard materials, like the titanium implant.

6. Summary

For bone substitutions, bioceramic-alloplasts maybe applied which preferably facilitate bone's repair by an osteo-induction, therefore, can be useful in reconstructive orthopaedics, jaw surgery or even in the process of dental implantology or parodontology, as well.

In the present study we constructed mesoporous silica aerogel (AE) and β-TCP-AE (β-tricalcium phosphate aerogel) composite materials for grafts to substitute bone defects. They were physicochemically characterized and proved to be non-toxic on cells applied. The materials have shown *in vitro* osteoinductive effects on a SAOS-2 cell line, indicating appropriate for grafting into a bone defect. Accordingly, *in vivo* studies with these materials on rat's calvaria bone confirmed that when 8 mm size bone defects were reconstructively substituted with either material, both but to a larger extent the β-TCP-AE showed an intense osteoconductive bone repair along the lateral edges of the defect, i.e., arising from the remaining bone-rim, manifested as early as the 1st Month which developed further by the 3rd Month, respectively. In addition, foci of intralesional calcifications with early *de novo* ossifications were also detected (i.e., independently from the lateral bony edges) characterized by osteoid matrix formation surrounded by osteoblasts in active cell cycle, reflecting osteoinduction. According to the histopathological results, the ossification processes are mediated by the presence of capillary-rich granulation tissue formation combined with the silica AE-induced foreign body giant cell granulomatous chronic fibrosing inflammation and, in turn, the activated macrophages and other cellular elements (most likely via autocrine and paracrine regulations) appear stimulate the osteoblastic differentiation toward new bone formations. Remarkably, however, by the 3rd Month of observational time a completed (100%) ossification of the defect could not be detected. Instead, within the remaining inflamed fibrous tissues small amounts of non-metabolized crystalloid silica remnants were found and an associated persistent low grade giant cell granulomatous inflammation while islets of additional new bone formations are being in progress. Originally, we wished to evaluate the real mineralized quantities of the osteoid matrix for the newly formed bone tissues within the defect using thin ground sections of the native (non-decalcified) hard tissue samples. However, we could not prepare such ground sections in a reproductive fashion, due to technical problems. Therefore, during the second part of our studies we established a modified method for preparing thin ground sections even from titanium-containing hard bony tissue samples. Accordingly, when using frosted slides with surface protonation followed by a

10-MDP adhesive bond's application, thin ground sections can readily be made in a reproductive fashion.

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List of publications related to the dissertation

1. Szalóki, M., **Hegedűs, V.**, Fodor, T., Martos, R., Radics, T., Hegedűs, C., Dezső, B.: Evaluation of the Effect of the Microscopic Glass Surface Protonation on the Hard Tissue Thin Section Preparation.
   Appl. Sci.-Basel. 10 (21), 1-21, 2020.
   DOI: http://dx.doi.org/10.3390/app10217742
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2. **Hegedűs, V.**, Kerényi, F., Boda, R., Horváth, D., Lázár, I., Győri, E., Dezső, B., Hegedűs, C.: β-Tricalcium phosphate silica aerogel as an alternative bioactive ceramic for the potential use in dentistry.
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List of other publications

3. Hajdú, P., Lampé, I., Rácz, R., Biri, S., Csik, A., Tóth, F., Szalóki, M., **Hegedűs, V.**, Dombrádi, Z. R., Varga, I., Csarnovics, I., Kökényesi, S., Beke, D. L., Hegedűs, C.: Optimized Size and Distribution of Silver Nanoparticles on the Surface of Titanium Implant Regarding Cell Viability.
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