HIGHER POROUS CERAMIC SCAFFOLDS FOR BONE TISSUE ENGINEERING

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- Kraków 2017 -
1 OUTLINE AND AIMS OF THE THESIS

Bone is the main supporting system in human body. Organic and mineral components of bone provide excellent tensile and loading strength. Bone plays key roles in human physiology such as protection, movement, support of organs, blood production, mineral storage and homeostasis, stem cells housing, blood pH regulation, and others. Bone possesses the intrinsic capacity for regeneration as part of the repair process in response to injury, as well as during skeletal development or continuous remodelling throughout adult life [1]. As such, the process of fracture healing recapitulates bone development and can be considered a form of tissue regeneration. Bone regeneration is comprised of a well-orchestrated series of biological events of bone induction and conduction. Those involve a number of cell types and intracellular and extracellular molecular signalling pathways, with a definable temporal and spatial sequence, in an effort to optimise skeletal repair and restore skeletal function [2]. When this complicated system ceases to work properly the quality of life of the individual degrades rapidly leading even to premature death. Metabolic diseases, bacterial infections along with traumatic injuries, orthopaedic surgeries and primary tumour resections lead to or induce bone defects. That, in the ageing societies, becomes a serious burden both socially and economically [3].

During the planning stage of the treatment for bone loss, several factors must be taken into account: the quality of the soft tissue envelope, the quality of vascular supply and the presence or absence of an infection [4]. Bone loss in certain anatomical locations has a more favourable prognosis due to better blood supply and corresponding osteogenic potential. In standard treatment protocols for bone defects the positive outcome rates vary significantly depending on affected site or overall patient’s health condition. The age of the patient, the presence of chronic disease (e.g. diabetes mellitus), use of medications, alcohol consumption and tobacco usage may hamper bone regenerative abilities [5]. Present therapeutic approaches include bone graft transplants (autologous or allogeneic grafts), implants of different biomaterials and distraction osteogenesis (Ilizarov technique), but none of them can be described as fully satisfactory.

For all above-mentioned reasons the focus of this thesis was to develop viable porous ceramic scaffolds as bone graft substitutes which at the same time will address the problem of common causes of the bone loss, such as microbial infections or poor condition of bone tissue resulting from metabolic diseases (such as osteoporosis) or bone tumours or bone metastasis.

Chapter 1 contains a systemic review of bone defects, their main causes, classification followed by brief summary of modern methods of bone loss treatment. A special focus is given to bone tissue engineering approach, what are the main advantages and disadvantages in comparison to conventional treatment methods, which are the most active areas of current research addressing those problems.

Chapter 2 of this thesis focuses on obtaining titanium dioxide (TiO$_2$) scaffolds with optimal mechanical properties, microstructure and architecture. Ceramic TiO$_2$ has been shown to have superb biocompatibility in contact with bone tissue. It also promotes
osseointegration. Scaffolds were fabricated by polymer sponge replication method. Proposed approach should combine optimal manufacturing method, appropriate architecture of the pores in the scaffolds, and biocompatibility of TiO$_2$ to obtain load-bearing constructs for bone tissue engineering.

In Chapter 3 the main aim was to improve manufacturing process of polymeric poly(L-lactide-co-glycolide) (PLGA) microparticles (MPs) loaded with four different drugs: gentamicin sulphate (Gent), vancomycin hydrochloride (Vanc), sodium alendronate (Aln) and salmon calcitonin (Calc). Parameters such as encapsulation efficiency, loading efficiency and particle size distribution were main indicators in fabrication process.

Post-surgical infections stand at the forefront of concern to the modern implantology. Therefore a viable systems ensuring constant local antibiotic delivery are in high demand. Chapter 4 covers my proposal for dealing with this problem by combining biocompatibility and osteoconductivity of TiO$_2$ scaffolds (Chapter 2) with controlled Gent and Vanc release from PLGA MPs described in Chapter 3. The main goal was to immobilize within the scaffolds Gent- or Vanc-loaded MPs while maintaining biocompatibility of the scaffolds, as well as an appropriate antibiotic release kinetics from MPs. To confirm utility of the systems drug release, cytocompatibility in contact with osteoblast-like cells and antimicrobial activity on Staphylococcus spp., both commercial and clinical strains isolated from infected bones and joints were tested.

Chapter 5 covers a slightly different approach to the bone remodelling after implantation, by addition of Aln (the main representative of bisphosphonates) or Calc to diminish osteoclasts’ activity at the implantation site. Similarly as in Chapter 4 the MPs on the scaffolds surface and pore walls to assure a suitable release dosage of the drugs were immobilised. Drug release kinetics was tested, followed by cytocompatibility assessment in contact with osteoblast-like cells and the influence of the systems on peripheral blood mononuclear cells differentiation towards osteoclasts.

In this short summary of the thesis only part of the research was presented, which was described in PhD dissertation. The selection of scaffolds’ optimal manufacturing process was exemplified by one-step sintering process (OSS) (thesis comprises also the results of the pre-experiment and two-step sintering (TSS)). The selection of microparticles’ optimal manufacturing process was presented by showing the effect of polymer and surfactant concentrations (in the thesis focus was placed also on drug content, mechanical stirring and drug type). As an example of the materials for prevention of bacterial infections only those with gentamycin were presented (dissertation focuses additionally on the vancomycin). As materials enhancing bone regeneration only systems with sodium alendronate were presented (dissertation contains also research on calcitonin).

2 OPTIMIZATION OF TITANIUM DIOXIDE SCAFFOLDS

2.1 Results – one-step sintering (OSS)

Figure 2-1 presents SEM microphotographs of the general morphologies, cross-sections of the struts as well as flat surfaces of the scaffolds manufactured by OSS
procedure at different temperatures. There was very little blockage of the pores in all cases, so the polymer template was well replicated. There is a clear difference in grain size between samples sintered at 1400 °C and higher temperature. Struts’ inward collapse is more visible in the case of samples sintered at 1600 °C. In the ceramics with the porosity over 90%, the accurate measurement of the porosity is very difficult, thus the observations of the cross-sections of the struts are very useful in assessing the densification within the material. All samples seemed to be fully densified except for the residual triangular void spaces in the struts, the leftover of polymer template burn-out.

Figure 2-1 SEM images of the architecture and microstructure of the scaffolds manufactured by OSS for temperatures: (A) 1400 °C, (B) 1450 °C, (C) 1500 °C, (D) 1550 °C, (E) 1600 °C. Pictures present: the general architecture of the scaffolds (left panels), cross-section of the struts (middle panels) and microstructure of the surface (right panels). Magnifications respectively 100x, 500x, and 1000x
Figure 2-2 A presents the grain distribution (lower part) and compressive strength (upper part) of the scaffolds sintered in OSS procedure. Median values range from 13.8 µm to 40.2 µm. There is a significant difference between samples sintered at 1600 °C and at other temperatures. The most visible drop in mechanical strength was in the case of samples sintered at 1400 °C. As opposed to compressive strength, the grain size distribution was less diversified in the case of 1400 °C scaffolds, the grains were also visibly smaller in comparison to those obtained at higher temperatures.

Additionally, µCT reconstructions were used to determine parameters characterising the morphology and architecture of produced scaffolds (Table 2-1). All the scaffolds had the same porosity (around 90%) and pore size (around 400 µm). Figure 2-3 and Table 2-1 present the summary of structural properties of all samples’ groups sintered at OSS procedure. There is a significant difference (p<0.01) between 1550 °C and 1600 °C groups and 1400 °C samples which concerns total porosity. Similar difference could be seen for strut thickness (p<0.01) and pore size (p<0.05). There was also a clear difference of pore size between 1450 °C and 1550 °C group. Samples sintered at 1600 °C shrank significantly more than other groups.
Figure 2-3 Comparison of the properties of scaffolds manufactured by OSS at different temperatures: (A) total porosity, (B) shrinkage, (C) pore size, (D) strut thickness; n=10, *p<0.05, **p<0.01

When it comes to other structural parameter such as structure linear density, intersection surface of surface-area to volume ratio (Table 2-1) there were no significant differences.

Table 2-1 Selected structural parameters of the TiO$_2$ scaffolds derived from the micro-CT data; mean ± S.D. (n=10)

<table>
<thead>
<tr>
<th>Sintering temperature (°C)</th>
<th>Structure linear density (1/mm)</th>
<th>Intersection surface (mm$^2$)</th>
<th>Surface-area to volume (1/mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1400</td>
<td>1.46 ± 0.13</td>
<td>5.5 ± 0.8</td>
<td>65.5 ± 4.3</td>
</tr>
<tr>
<td>1450</td>
<td>1.59 ± 0.11</td>
<td>6.7 ± 0.8</td>
<td>59.3 ± 3.7</td>
</tr>
<tr>
<td>1500</td>
<td>1.55 ± 0.10</td>
<td>6.3 ± 0.9</td>
<td>64.0 ± 4.6</td>
</tr>
<tr>
<td>1550</td>
<td>1.70 ± 0.23</td>
<td>7.4 ± 1.3</td>
<td>63.8 ± 3.9</td>
</tr>
<tr>
<td>1600</td>
<td>1.64 ± 0.11</td>
<td>7.1 ± 0.9</td>
<td>60.1 ± 4.5</td>
</tr>
</tbody>
</table>

In order to evaluate the correlations between compressive strength and the parameters of the scaffolds microstructure as well as sintering temperature calculations using the Spearman rank correlation coefficient were performed (Table 2-2). Strong correlation was found between compressive strength and: pore size and porosity (negative correlation), sintering temperature, strut thickness, linear density and intersection surface (positive correlations). Medium correlation was found between compressive strength and surface area-to-volume ratio (negative correlation).
2.2 Discussion

The experiment showed that polymer sponge replication method is a very effective technique for manufacturing of highly porous TiO$_2$ scaffolds for bone tissue engineering. The second experiment on the optimization of the scaffolds’ sintering profile was performed to investigate the influence of the first step sintering temperature on the properties of the scaffolds. Therefore samples were sintered by OSS and to be used in the future as a reference for TSS samples. There was a clear difference in grain size and compressive strength between the samples sintered at 1400 °C and higher temperatures. This is probably due to result of higher energy provided by the sintering, which allows on one hand faster grain growth but on the other hand better densification of the structure. Moreover, the grain size was more diverse in the case of higher temperatures. At higher temperatures, grains were growing at different rates and in the end, the microstructure of such materials became more irregular.

Additionally, the correlation studies were performed in OSS experiment. It is clear that there was significant positive correlation of sintering temperature for OSS and scaffolds mechanical properties. It is the strongest prevailing parameter in the correlation study. The highest compressive strength exceeding 1 MPa was obtained for the scaffolds sintered at 1600 °C. According to the correlation study the other effective way to strengthen mechanically the scaffold, was to increase the strut thickness, as previously reported by Min et al. or decrease the porosity as reported by Fostad et al. [6,7]. Since the scaffolds had porosity of over 90%, which is more than enough for a bone tissue engineering usage, therefore for future consideration multiple coating of the scaffolds is recommended. Multiple coating studies have been already performed on TiO$_2$ scaffolds by other researchers [6,8].

The compressive strength of the scaffolds described in this study was found in the range of 0.43 - 1.1 MPa and was at the lower limit of the strength of healthy human trabecular bone. For example compressive strength of trabecular bone originating from human mandible has been reported to be in the range of 0.2 - 10 MPa [9]. It was also similar to the value of TiO$_2$ scaffolds reported in the previous study obtained by OSS at 1500 °C for 20 h [10]. Since the mechanical properties of the scaffolds seem to be in the lower range of the limit of human bone it would be advisable as by OSS to further strengthen the
structure of the scaffolds by multiple coating procedure in which low-viscosity slurry would fill the hollow spaces inside the struts and therefore strengthen the material.

To sum up, polymer sponge replica method allows to obtain a highly-porous ceramics TiO$_2$ scaffolds with suitable architecture, microstructural and mechanical properties for trabecular bone tissue engineering. It was found that in OSS procedure the higher sintering temperature the better mechanical properties of the scaffolds. The structure of the scaffolds is more dense, their grains are bigger and less uniform. It is advisable to follow burn-out procedure with pre-coarsening, which not only stabilizes the scaffolds but also has positive impact on their microstructural properties. Hence, future optimization of the sintering procedure is recommended.

3 OPTIMIZATION OF PLGA DRUG CARRIES

3.1.1 Results - effect of PLGA and surfactant content

The PLGA MPs were manufactured by solid-in-oil-in-water emulsion solvent evaporation technique. First experiment was focused on the optimization of the surfactant and polymer concentration.

Figure 3-1 and Figure 3-2 depict the morphologies and size distributions of the MPs manufactured with 3.3% and 1.67% (w/v) PLGA concentrations, respectively. The investigated concentrations of PVA aqueous solutions were 8%, 6%, 4%, 2%, 1% and 0.5% (w/v). Table 3-1 presents more details on MPs size distribution parameters, such as 10th, 50th, 90th percentile and span. Moreover, the results of drug entrapment were presented with parameters such as %EE and %LE. The parameters chosen for future experiments were highlighted with grey colour in table. None of the samples presented normal distribution of the MPs. However, some of them had distributions resembling log-normal distribution, typical for particles manufactured this way. For both PLGA concentrations, the smallest fractions of the MPs were obtained for 8% and 6% PVA concentrations. In the case of 1.67% PLGA, 8% and 4% PVA, over 10% of the MPs had submicron size. 4% and 2% PVA concentrations for both PLGA solutions resulted in bigger MPs. However, the narrowest size distribution was obtained for 1.67% PLGA/4% PVA and 3.3% PLGA/2% PVA. For lower concentrations of the PVA over 50% of MPs were bigger than 15 µm.

Analysing the results of %EE and %LE it can be clearly observed that 8% and 6% PVA solutions lower significantly those values. Higher concentration of the PLGA resulted in significantly higher encapsulation efficiency of the drug (p<0.05), but in the case of loading efficiency only 8% and 6% PVA resulted in significantly lower values compared to 1.67% PLGA. The size of the MPs on one hand should be bigger than 6 µm in order to reduce immunological response of the organism but on the other hand the particles cannot be too big which could either block the pores of the scaffolds or reduce immobilization efficiency. Therefore, after taking those aspects into account in future experiments the focus was placed on the systems with 1.6% PLGA and 4% PVA, since MPs formulated under those parameters had small size distribution, their 95% of the MPs was in the 3 – 36 µm size
range and their loading efficiency was not significantly lower from the corresponding system with higher PLGA concentration.

Figure 3-1 Morphology under SEM microscope (left panels) and size distribution (right panels) of MPs loaded with Gent for 3.3% (w/v) PLGA concentration and different PVA concentrations: 8% (A), 6% (B), 4% (C), 2% (D), 1% (E) and 0.5% (w/v) (F)

Figure 3-2 Morphology under SEM microscope (left panels) and size distribution (right panels) of MPs loaded with Gent for 1.6% (w/v) PLGA concentration and different PVA concentrations: 8% (A), 6% (B), 4% (C), 2% (D), 1% (E) and 0.5% (w/v) (F)
Table 3-1 Formulation study of Gent-loaded PLGA MPs: effect of polymer and surfactant concentration on MPs size distribution, EE% and LE%. Mean ± S.E.M.; n=3, *p<0.05 between corresponding PVA concentrations. The best parameters chosen for future experiments were highlighted with grey colour

<table>
<thead>
<tr>
<th>PLGA concentration % w/v</th>
<th>PVA concentration % w/v</th>
<th>P10 µm</th>
<th>P50 µm</th>
<th>P90 µm</th>
<th>span</th>
<th>%EE</th>
<th>%LE</th>
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<tr>
<td>3.3</td>
<td>8</td>
<td>1.6</td>
<td>3.0</td>
<td>5.2</td>
<td>1.19</td>
<td>61 ± 3*</td>
<td>6.7 ± 0.3*</td>
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<tr>
<td></td>
<td>6</td>
<td>1.9</td>
<td>4.8</td>
<td>11.5</td>
<td>2.00</td>
<td>61 ± 4*</td>
<td>6.6 ± 0.3*</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>8.8</td>
<td>13.6</td>
<td>35</td>
<td>1.93</td>
<td>70 ± 3*</td>
<td>8.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>16.5</td>
<td>33</td>
<td>58</td>
<td>1.26</td>
<td>74 ± 5*</td>
<td>8.1 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>12.4</td>
<td>38.3</td>
<td>102</td>
<td>2.34</td>
<td>88 ± 4*</td>
<td>9.1 ± 0.4</td>
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<tr>
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<td>0.5</td>
<td>16.7</td>
<td>51.0</td>
<td>119.3</td>
<td>2.01</td>
<td>85 ± 2*</td>
<td>9.0 ± 0.2</td>
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<tr>
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<td>0.7</td>
<td>1.6</td>
<td>3.9</td>
<td>1.97</td>
<td>47 ± 3*</td>
<td>5.1 ± 0.2*</td>
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<tr>
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<td>6</td>
<td>0.7</td>
<td>1.3</td>
<td>4.4</td>
<td>2.80</td>
<td>48 ± 2*</td>
<td>5.2 ± 0.3*</td>
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<tr>
<td></td>
<td>4</td>
<td>4.9</td>
<td>10.5</td>
<td>22.5</td>
<td>1.66</td>
<td>65 ± 1*</td>
<td>8.4 ± 0.1</td>
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<tr>
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<td>2</td>
<td>4.4</td>
<td>10.9</td>
<td>47.5</td>
<td>3.95</td>
<td>61 ± 4*</td>
<td>8.0 ± 0.5</td>
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<tr>
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<td>7.2</td>
<td>18.1</td>
<td>40.7</td>
<td>1.85</td>
<td>63 ± 1*</td>
<td>8.7 ± 0.2</td>
</tr>
<tr>
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<td>0.5</td>
<td>7.9</td>
<td>30.7</td>
<td>79.8</td>
<td>2.34</td>
<td>64 ± 1*</td>
<td>8.8 ± 0.1</td>
</tr>
</tbody>
</table>

Figure 3-3 presents the morphology and size distribution of the 6.6% PLGA/4% PVA system. Table 3-2 presents the comparison of the size distribution parameters, %EE and %LE of three different PLGA concentrations for 4% PVA. MPs manufactured with 6.6% PLGA concentration were characterized by wide distribution (span=4.93 – the largest from all other systems). MPs were larger compared to lower PLGA concentrations, with 50% of them being of size lower than 25.5 µm. Neither %EE, nor %LE were not significantly different between those three systems.

Figure 3-3 Gent-loaded PLGA MPs’ morphology of and size distribution of the MPs manufactured from 6.6% (w/v) PLGA concentration and 4% (w/v) PVA concentration
Table 3-2 The effect of the polymer concentration on Gent-loaded PLGA MPs’ size distribution, EE% and LE%. Mean ± S.E.M.; \( n=3 \)

<table>
<thead>
<tr>
<th>PLGA concentration % w/v</th>
<th>PVA concentration % w/v</th>
<th>P10 µm</th>
<th>P50 µm</th>
<th>P90 µm</th>
<th>span</th>
<th>%EE</th>
<th>%LE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.6</td>
<td>4</td>
<td>4.9</td>
<td>10.5</td>
<td>22.5</td>
<td>1.66</td>
<td>65 ± 1</td>
<td>8.4 ± 0.1</td>
</tr>
<tr>
<td>3.3</td>
<td>4</td>
<td>8.8</td>
<td>13.6</td>
<td>35.0</td>
<td>1.93</td>
<td>70 ± 3</td>
<td>8.3 ± 0.2</td>
</tr>
<tr>
<td>6.6</td>
<td>4</td>
<td>10.6</td>
<td>25.5</td>
<td>136.5</td>
<td>4.93</td>
<td>71 ± 5</td>
<td>8.9 ± 0.4</td>
</tr>
</tbody>
</table>

3.2 Discussion

The aim of this study was to optimize the manufacturing parameters of drug loaded PLGA MPs for local treatment of bone defects and diseases, which will be optimal for future immobilization on TiO\(_2\) scaffolds.

PLGA copolymers are a family of polyesters composed of one or more of three different hydroxyl-acid monomers, D-lactic, L-lactic, and glycolic acids. They are biocompatible and biodegradable [11,12]. Those copolymers are approved by Food and Drug Administration as well as European Medicines Agency and their degradation rate can be finely adjusted (i.e. by changing lactide to glycolide ratio, molecular weight and chain structure) [12]. PLGA degrades by hydrolysis cleavage of its backbone ester linkages into oligomers, and finally monomers. It is a collective process of bulk diffusion, surface diffusion, bulk erosion and surface erosion [13]. PLGA of high molecular weight (\(M_n = 100\) kDa) and high ratio of polylactideto glycolide (PLGA 85:15) was chosen for manufacturing the MPs. Factors such as the copolymer molecular weight, the copolymer composition and the crystallinity play a significant role in polymer hydrolytic degradation. Longer polymer chains require a longer time to degrade and hence induce a longer release time of low molecular weight drugs. Crystalline PGA when co-polymerized with crystalline PLA result in PLGA which is amorphous and therefore increases the rate of hydration and hydrolysis of copolymer. Higher contents of PGA lead to quicker degradation, PLGA 50:50 exhibits the fastest degradation [11,12].

One of the main criteria for selecting optimal manufacturing parameters was the size of the MPs. Lower size threshold was established at 5-6 \(\mu\)m based on the other groups reports of the influence of MPs size on phagocytosis rate and immunological response of the organism. Upper threshold was set at 30 \(\mu\)m, as a size at which neither the further immobilization of the MPs on scaffolds will not be hindered nor the scaffolds pores will not be blocked by the MPs, thus allowing tissue ingrowth into the scaffold. Piskin et al. have conducted an experiment on polystyrene particles and their size influence on phagocytosis rate. They have stated that for smallest particles in their study (0.9 \(\mu\)m) the phagocytosis by leukocytes and peritoneal macrophages was the highest. The particle uptake dropped significantly with the particle size, still the cells were able to internalize microbeads of 4-6 \(\mu\)m [14]. Another study of Champion et al. supports the Piskin et al. findings, showing that 2-3 \(\mu\)m MPs were phagocytosed more readily than larger particles.
Another study on foreign body reaction in rats of Zandstra et al. showed that 5 µm PLGA microspheres caused more intense immunological reactions than the microspheres with 30 µm size, however only those two sizes were investigated, therefore it is not clear what is the impact of other size ranges [16].

Surfactants are used in external water phase during S/O/W emulsion manufacturing to reduce the surface tension and stabilize the droplet phase during emulsification process. Type and concentration of surfactant are important factors in stabilizing emulsions but also help to prevent aggregation of the microparticles. The concentration of the surfactant in the external water phase is an important factor in achieving high %EE of water soluble drugs, especially in water-in-oil-in-water (W/O/W) emulsion method [17,18]. PVA is non-ionic polymer widely used in polymeric micro- and nano-particles production due to its biocompatibility [19,20]. The results obtained during surfactant concentration optimization are in line with the findings of other groups. In general, the increase in the PVA concentration reduces the size of the MPs but it also reduces the encapsulation efficiency of the drug. The amount of surfactant plays an important role in microparticles formation, because it prevents the coalescence of the oil globules by lowering the free energy at the interface between two phases [20]. It is easy to understand that an insufficient amount of surfactant may lead to failure in stabilizing of all the microparticles and some of them would tend to aggregate. Therefore, microparticles with larger size would be manufactured [21]. Lowered %EE can be due to the increase in the solubility of drugs in the external water phase, thus leading to lower %EE [18]. Taking into account the size criteria established earlier, 4% PVA concentration was chosen for future experiments.

Polymer molecular weight as well as concentration are positively correlated to the organic phase’s viscosity and thus to the drug diffusion constant [22]. Therefore the use of higher concentrations of PLGA resulted in our case in higher %EE. Similar tendencies were presented by others [23]. However, the increase in polymer concentration did not affect %LE. But what is important it had a big impact on MPs size distribution. Especially for 6.6% (w/v) PLGA the particles were within the range of 10 to 160 µm. The differences between the %EE were not big in this case, and since %LE were similar therefore for next optimization steps, the 1.67% concentration was chosen.

To sum up, the solid-in-oil-in-water emulsion solvent evaporation technique is an adequate method to manufacture PLGA microparticles within the size range of 5 to 30 µm and good %EE and %LE. It is clear that the parameters of the MPs manufactured by this method can be modulated by numerous factors, such as surfactant concentration, polymer concentration, drug content, stirring rate, drug type, etc. In this work only some of those parameters were taken into consideration, however in the future optimization studies other factors can be evaluated, e.g. additives in external water phase, the volume of water phase, the volume of polymer phase, pH of the external phase.
4 MATERIALS FOR PREVENTION OF BACTERIAL INFECTIONS

4.1 Results

4.1.1 Optimization of the systems

Encapsulation efficiency for Gent-loaded MPs was 52.5 ± 5% and drug loading efficiency was 6.5 ± 0.6%. Different amounts of the Gent-loaded MPs were immobilized on the scaffolds surface with the use of different Alg concentrations (4, 2, 1, 0.2 and 0.02% (w/v)) to preliminary assess a suitable concentration for future experiments (Figure 4-1 and Figure 4-2). In the case of 4 and 2% (w/v) concentrations it was difficult to disperse MPs in the solutions homogenously which prevented from good penetration of the scaffolds with MPs or it was difficult to transfer them at all on the scaffolds. Moreover, the layer of the Alg that covered the MPs was likely to impede the future drug release, especially the first stage of the burst release (Figure 4-1 A-D). 1% (w/v) concentration allowed good homogenisation as well as lower concentration, but the layer of the Alg was still thick (Figure 4-1 E,F). 0.2 and 0.02% (w/v) concentrations proved to assure an even distribution of the MPs within the entire volume of the scaffold. SEM microphotographs revealed that MPs attached well to the surface of the scaffolds and the microstructure of both samples differing in concentration of Alg (0.2% and 0.02%) was similar. MPs attached mostly to the edges of pore walls, however some of them were found on struts' surfaces. Further studies of the stability of the coating showed that after 24 h incubation in PBS MPs remained on the surface of both types of scaffolds.

Figure 4-1 SEM images of TiO2 scaffolds coated with MPs loaded with Gent and different concentrations of Alg: 4% (A, B), 2% (C, D) and 1% (E, F) at magnifications 350x(A, C, E) and 3000x (B, D, F)
Figure 4-2 SEM images of TiO$_2$ scaffolds coated with MPs loaded with Gent and 0.2% Alg (A, C) and 0.02% Alg (B, D) at magnifications 350x and 1000x (inserts). Pictures were made on samples before (A, B) and after (C, D) 24 h incubation in PBS.

Figure 4-3 shows microstructure of the inner part of the scaffolds proving that MPs were successfully immobilized with the use of Alg not only on the outer part of the samples, but they were homogenously distributed within the entire volume of the scaffolds. It can be found that some of the MPs were totally embedded in Alg matrix (Figure 4-3, arrow 1), some were covered with a very thin hardly visible Alg layer, but sufficient to successfully attach the MPs (Figure 4-3, arrow 2); Alg thin film (partially destroyed due to cutting of the scaffold) is visible as well (Figure 4-3, arrow 3).

Gent release from the systems was studied in vitro immediately after fabrication. Several different batches of the scaffolds were tested in order to check the influence of drug’s and MPs’ amount per scaffold on the release kinetics. Table 4-1 shows
immobilization efficiency of MPs calculated from the total value of the released drug after 81 days. It was presented as a percentage of the initial fabrication drug amount. The results for Gent showed that in the case of immobilization of MPs providing 1 mg of the drug per scaffold almost 25% of the MPs was not attached. Samples with MPs providing 0.75 mg of the Gent lost around 15% of MPs whereas the ones with MPs providing 0.5 mg less than 10%. There was no significant difference between 0.2 and 0.02% Alg coating in any of the cases. Therefore in further studies only samples with 0.5 mg of the drug were tested.

Table 4-1 Immobilization efficiency of MPs on TiO₂ scaffolds (expressed as % of theoretical amount of drug introduced initially into the system) after MPs immobilization on the scaffolds for different drug amounts and Alg concentrations. Results presented as a percentage of the initial fabrication drug amount. Mean ± S.D.

<table>
<thead>
<tr>
<th>Drug amount</th>
<th>0.2% Alg</th>
<th>0.02% Alg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mg</td>
<td>77 ± 4%</td>
<td>72 ± 5%</td>
</tr>
<tr>
<td>0.75 mg</td>
<td>84 ± 4%</td>
<td>80 ± 6%</td>
</tr>
<tr>
<td>0.5 mg</td>
<td>92 ± 3%</td>
<td>95 ± 2%</td>
</tr>
</tbody>
</table>

Figure 4-4 Cumulative in vitro Gent release profiles from scaffolds with Gent-loaded MPs dispersed in Alg. Horizontal lines represent the designed initial amounts of the drug per scaffold: 1000, 750 and 500 mg, respectively. Mean ± S.E.M.

Figure 4-5 presents more detailed drug release profiles of Gent. This time the focus is on the samples only with 0.5 mg of the designed initial drug amount. The results showed that Gent was released slightly faster from the scaffolds containing MPs attached to pore walls with 0.02% Alg, although the results were not significantly different according to ANOVA (Figure 4-5). The initial burst release within 8 h was 125 μg from every single scaffold, which was around 25% of total encapsulated Gent. This first phase was followed by gradually decreasing release phase up to day 10. Afterwards up to day 50 a sustained release of around 3 μg/day was measured. In both cases total released amount of Gent was above 90% of initially introduced drug for each scaffold.
Figure 4-5 Cumulative *in vitro* Gent release profiles from scaffolds with Gent-loaded MPs dispersed in 0.2 and 0.02% Alg. Insert presents drug release up to 48 h. Mean ± S.E.M.

4.1.2 Antimicrobial activity

Figure 4-6 A shows the results of growth inhibition zones of *S. aureus* and *S. epidermidis*, both reference and clinical strains, in contact with scaffolds containing Gent-loaded MPs attached by 0.2% and 0.02% Alg. The zone of growth inhibition was observed with diameters of 24.0 – 34.6 mm depending on the pathogen type. The diameters of inhibition zones for scaffolds with 0.2% Alg were significantly lower in all the cases compared to systems with 0.02% Alg. The inhibition zone of 10 μg Gent standard was significantly higher than in scaffolds with 0.2% Alg except reference strain of *S. aureus*. Those results may correspond with previously described trend showed by drug release profiles, that 0.02% Alg scaffolds allowed slightly faster drug release during first couple of days. In the case of control (empty scaffolds) no inhibition zone was observed (data not presented).
Figure 4-6 Growth inhibition zones of *S. aureus* (DSM 24167, clinical isolate SA1-KC) and *S. epidermidis* (ATCC 700296 and clinical isolate SE1-KCR) on microphotographs (first row – TiO$_2$ scaffolds with drug-loaded MPs attached with 0.2% Alg, second row – TiO$_2$ scaffolds with drug-loaded MPs attached with 0.02% Alg) for systems with Gent. Precise values of growth inhibition zones are shown as diagrams below. Mean ± S.E.M.; * p<0.05

4.1.3 Biological test with osteoblast-like MG-63 cells

Figure 4-7 show metabolic activity of MG-63 cells evaluated by AlamarBlue, while Figure 4-8 present live-dead staining of the cells grown in contact with the extracts from studied scaffolds (with Gent and Vanc, respectively) diluted by factors 1:1 (undiluted), 1:2, 1:4 and 1:8. In general for both drugs cells cultured in extracts presented similar behaviour. The AlamarBlue assay results for both Gent and Vanc show that after 6 days cell viability was significantly compromised when the cells were cultured with undiluted extract from control TiO$_2$ scaffolds. On the other hand cell viability was the same when cultured in all the extracts from the scaffolds modified with MPs and Alg, irrespectively of dilution. The cells observed under fluorescence microscope after 1 day were viable and no dead cells were seen (Figure 4-8 A-F). After 6 days some red dead cells were observed (less than 2%), but whole culture showed no signs of lowered metabolic activity and viability (Figure 4-8 G-L).
Figure 4-7 Metabolic activity of MG-63 cells by reduction of AlamarBlue on day 1 and 6 for extracts diluted in MEM by the factors 1:1 (undiluted), 1:2, 1:4 and 1:8 from TiO₂ scaffolds, TiO₂ scaffolds containing Gent-loaded MPs dispersed in 0.2% Alg or 0.02% Alg; (amount of Gent in undiluted sample (1:1) was 42.6 ± 1.3 µg, for 1:2 dilution it was 21.03 ± 0.7 µg, for 1:4 dilution it was 10.6 ± 0.3 µg and for 1:8 dilution it was 5.3. ± 0.2 µg). As a reference, activity of cells cultured in pure MEM is presented. Mean ± S.D. *p<0.05

Figure 4-8 Live-dead staining of MG-63 cells on day 1 (A-F) or day 6 (G-L) in contact with extracts diluted in MEM by the factor 1:1 (amount of Gent 42.6 ± 1.3 µg) (A-C,G-I) and 1:2 (amount of Gent 21.03 ± 0.7 µg) (D-F,J-L) from TiO₂ scaffolds (A,D,G,J), from TiO₂ scaffolds containing Gent-loaded MPs dispersed in 0.2% Alg (B,E,H,K) or 0.02% Alg (C,F,I,L). Scale bar =100 µm (Carl Zeiss Axiocounter 40, Germany).

Figure 4-9 shows the results of metabolic activity test of MG-63 cells cultured on control scaffolds and those modified with Gent-loaded and Vanc-loaded MPs attached by 0.2% Alg and 0.02% Alg. Metabolic activity of the cells was the same on all the scaffolds.
regardless of the drug present in the system (no significant difference was found according to ANOVA).

Figure 4-10 depicts the MG-63 cells cultured on control scaffolds and those modified with Gent-loaded MPs. On day 1 on all the scaffolds well spread cells, polygonal in shape were visible (Figure 4-10 A-C). On day 3 the number of cells was higher and the cells were better spread and they were present on the surface as well in the outer parts of the scaffolds (Figure 4-10 D-F).

![Graph](image)

Figure 4-9 Metabolic activity by reduction of AlamarBlue on day 1 and 3 of MG-63 cells cultured on: control TiO$_2$ scaffolds, TiO$_2$ scaffolds containing Gent-loaded and Vanc-loaded MPs dispersed in 0.2% Alg or 0.02% Alg and on reference TCPS. Mean ± S.D. No statistical differences at *p<0.05

![Images](image)

Figure 4-10 Hematoxylin-eosin staining of MG-63 cells cultured on scaffolds: control TiO$_2$ (A, D) and TiO$_2$ scaffolds containing MPs dispersed in 0.2% Alg (B, E) or 0.02% Alg (C, F) on day 1 (A-C) or day 3 (D-F). Scale bar =100 µm (digital VHX-5000 Keynance optical microscope)
4.2 Discussion

The aim of this study was to modify TiO$_2$ scaffolds in order to provide them with antimicrobial properties. This goal was achieved by proposing a system containing Gent-loaded degradable MPs which were attached to the scaffolds’ pore walls by cross-linked Alg. The modified scaffolds were examined to assess their microstructure, drug release characteristics, antimicrobial properties and potential cytotoxicity.

MPs were manufactured from biodegradable PLGA considered safe for controlled release formulations [24]. MPs were characterized by sufficient encapsulation and drug loading efficiencies – accordingly 52.5 ± 5% and 6.5 ± 0.6%. Those numbers are similar to other types of MPs loaded with Gent manufactured by analogous emulsification techniques in other groups [25–27].

A simple attachment method of MPs within the TiO$_2$ scaffolds was developed. MPs were homogenously attached to the pore walls, both on the surface and inside the scaffolds. While other methods proposed for the efficient MPs immobilization are often combined with the use of potentially cytotoxic substances, our method did not present that threat [28–30]. For immobilization cross-linked Alg was used. Alginate gels have been investigated for the delivery of a variety of low molecular weight drugs [31]. They presented in various studies an excellent biocompatibility with different cell types [32–35]. The low concentrations of Alg were preferred to assure effective immobilization of MPs and to avoid presence of too thick layer of Alg which could hamper drug release (Figure 4-1, Figure 4-2 A, B). Crosslinking was sufficient to attach the MPs to the scaffolds surface and to assure drug delivery at the infection site. It was proved by SEM images that incubation in PBS for 24 h did not affect negatively MPs attachment, irrespectively of Alg concentration (Figure 4-2).

The first experimental objective of drug release study was screening of the optimal MPs amount on the scaffolds. It should allowed to design most efficient way of MPs immobilization. The results of drug release, especially the total amount of the released drug (Figure 4-4, Figure 4-5, Table 4-1) proved, that an optimal amount of the MPs was the one which contained 0.5 mg of the drug (7.7 mg/scaffold).

Drug release study showed that the systems released around 25% of initial drug amount during the first 8 h, which was followed by sustained drug release up to day 50. This number is important due to the so called “decisive period” of 6 h after surgery that is crucial for prevention of infection at the implantation site [36]. Long sustained release, that guarantees the task of negating microbial contamination of implant [37], was achieved by application of both MPs and Alg coating. Boontheekul et al. showed that release of drugs from alginate matrices is due to a hydrolytic chain scission at oxidised sugar residues [33]. In approach shown in this thesis combined release of drugs from PLGA with Alg anomalous transport mechanism, which will have a buffering effect on the drugs’ release profile was utilised [38,39]. Therefore, a long time sustained release of Gent from these systems was achieved. Interestingly Gent release was the same for both types of scaffolds, i.e. when MPs were immobilized with low- or more-concentrated Alg.
Antimicrobial susceptibility tests were performed on the systems by observation of growth inhibition of *Staphylococcus* spp. Both reference strains and clinical isolates of *S. aureus* and *S. epidermidis* susceptibility was tested since these strains are considered to be the main cause of bone infections [40]. It is indicated that reference strains are more susceptible to the Gent than clinical isolates [41]. Recent breakpoint tables for interpretation of MICs and zone diameter published by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) indicated that inhibition zone diameter for testing microbial sensitivity for *S. aureus* and coagulase-negative staphylococci, e.g. *S. epidermidis*, are 18 mm and 22 mm, respectively [42]. For all the systems the zones were bigger than suggested by EUCAST thus the growth of bacteria was successfully inhibited. Accordingly Alg coating and PLGA encapsulation did not hamper drugs’ bactericidal properties (Figure 4-6 A).

The extracts from scaffolds with Gent-loaded MPs attached by Alg of two concentrations (0.2 and 0.02%) did not present any cytotoxic behaviour irrespectively to their dilution, neither at the beginning nor at the end of the experiment (Figure 4-7 to Figure 4-10). This finding is coherent with previous experiments on TiO$_2$ scaffolds coated with cross-linked Alg of higher concentration (2%) containing simvastatin that were found cytocompatible with osteoblasts and mesenchymal stem cells [35,43,44]. Our results prove that the possible released substances did not affect cell adhesion and proliferation. The cells in contact with the extracts from control TiO$_2$ scaffolds diluted by the factor of 2, 4 and 8 behaved similarly. The only difference was found for the reference non-diluted samples after 6 days, for which the cells exhibited reduced viability. This may be explained by well documented phenomenon of reactive oxygen species (ROS) creation by TiO$_2$. However this hypothesis should be rather ruled out, because it has been reported for TiO$_2$ nanoparticles, which exhibit very high relative surface area [45]. Another reason might be release of silica, magnesium and aluminium ions from the TiO$_2$ scaffolds as shown in a recent paper published by our group [46]. However it was observed for media of low pH, but not in simulated body fluids (SBF) of pH 7.2. On the other hand there were several papers reporting that the TiO$_2$ scaffolds are fully biocompatible with bone tissue [10,47–49], which suggests that *in vitro* conditions not always resemble what is found *in vivo*. All in all, the most important finding from our study is that the TiO$_2$ scaffolds enriched with MPs attached by Alg with Gent are not cytotoxic and our method of manufacturing enhances biological performance of reference TiO$_2$ scaffolds by providing them antimicrobial properties.

Therefore, it was proved that it is possible to modify TiO$_2$ scaffolds to ensure their antimicrobial activity against common bone infection strains of *Staphylococcus* spp. It has been shown that: i) immobilization of Gent-loaded MPs with the use of low-concentrated cross-linked Alg is effective, ii) the systems provide required doses of drug to combat post-implantation infection, iii) the bactericidal drug activity is preserved and not affected by the whole manufacturing process, i.e. MPs manufacturing and their immobilization and iv) the systems are cytocompatible with osteoblast-like cells.
5 MATERIALS ENHANCING BONE REGENERATION

5.1 Results

5.1.1 Optimization of the systems

Encapsulation efficiency for Aln-loaded MPs was 71 ± 6% and drug loading efficiency was 7.7 ± 0.8%. Two different amounts of Aln-loaded MPs were immobilized on the scaffolds surface with the use of one concentration of collagen (40 µg/mL), in order to attach them by simple protein adsorption.

Figure 5-1 SEM image of the inner part of TiO$_2$ scaffold containing MPs loaded with Aln attached with collagen at magnification 3000x; MPs are covered with a very thin hardly visible collagen layer, but sufficient to successfully attach the MPs (arrows).

Figure 5-2 SEM images of TiO$_2$ scaffolds coated with MPs loaded with Aln (400 µg) at magnifications 350x (A, C, E) and 500x (B, D, F). Pictures were made on samples before (A, B), after 24 h (C, D) and after 3 days (E, F) incubation in PBS.
Figure 5-1 shows the inner part of the scaffolds with immobilized Aln-loaded MPs. A thin layer of collagen which attaches the MPs to the scaffolds surface is detectable (arrows). SEM microphotographs revealed that MPs attached well to the surface of the scaffolds (Figure 5-2). MPs attached to the edges of pore walls as well as on the surface of the struts (Figure 5-2 A, B). Further studies of the stability of the coating showed that incubation in PBS for 24 h (Figure 5-2 C, D) and 3 days (Figure 5-2 E, F) did not hamper MPs attachment to the scaffolds.

![Figure 5-2 SEM microphotographs](image)

**A**

![Drug release profile](image)

**B**

![Drug release profile](image)

Figure 5-3 Cumulative *in vitro* Aln release profiles from scaffolds with Aln-loaded MPs attached with collagen: (A) 400 µg and (B) 200 µg of the drug per scaffold. Inserts present drug release up to 48 h. Mean ± S.E.M.

Aln release from the systems was studied *in vitro* immediately after fabrication. Two different batches of the scaffolds were tested in order to check the influence of drug and MPs amount per scaffold on the release kinetics (Figure 5-3 A, B). No significant difference of relative drug loss after MPs immobilization on the scaffolds was observed between the samples, therefore both systems were evaluated further by biological tests on the cells. The results showed that Aln was released slightly faster from the scaffolds containing 400 µg of the drug. The initial burst release after 8 h of experiment was 28 ± 2% for the samples containing 400 µg Aln per scaffold whereas for samples with 200 µg Aln it was only 19 ± 1.5%. After 48 h in the case of the first 400 µg system 42 ± 3% of the
drug was released while for 200 µg systems it was 34 ± 3%. It was also visible that the dosage of the drug from one time point to another was stabilizing faster in the case of smaller amount of MPs. This first phase was followed by gradual stabilization of release dosage; this was especially visible for 200 µg systems where starting from day 3 up to day 18 the dose of Aln was in between 4.5 to 6 µg/day. In the case of 400 µg system the dosage was less stabilized. Afterwards up to day 44 the dosage was constantly decreasing in both cases. The released amount of Aln from both systems was above 90% of initially introduced drug for each scaffold.

5.1.2 Biological tests

Figure 5-4 shows metabolic activity of MG-63 cells evaluated by AlamarBlue, while Figure 5-5 presents live-dead staining of the cells grown in contact with the extracts from studied scaffolds with Aln diluted by factors 1:1 (undiluted), 1:2, 1:4 and 1:8. Additionally, also different concentrations of the pure drugs dissolved in medium were tested.

![AlamarBlue reduction graph](image)

**Figure 5-4** Metabolic activity of MG-63 cells by reduction of AlamarBlue on day 1, 3 and 6 for extracts diluted in MEM by the factors 1:1 (undiluted), 1:2, 1:4 and 1:8 from TiO₂ scaffolds (brown columns), TiO₂ scaffolds containing Aln-loaded MPs dispersed in collagen (two types of samples: with 400 mg and 200 mg of the drug, blue columns). Additionally, a pure Aln solutions in medium are shown (violet columns – 100, 50, 10, 5 and 1 µg/ml). As a reference, activity of cells cultured in pure MEM is presented (pink columns). Mean ± S.D.; *p<0.05, **p<0.01, ***p<0.001

The results show toxicity of the drug for concentrations higher than 10 µg/mL (Figure 5-4 and Figure 5-5 last column). Significantly lower cell activity is already visible after 3 days of cell culture (p<0.001). For all dilutions of 400 S Aln systems the cells viability was significantly lower both in comparison to cells cultured in extracts from to reference scaffolds as well as in pure medium - MEM (Figure 5-4 and Figure 5-5, first and second column). The same happened in the case of 10, 50 and 100 µg/mL Aln concentrations in medium - MEM (Figure 5-4 and Figure 5-5, last column). In the case of 200 S Aln samples (200 µg of Aln/sample) the decrease in cell activity was much lower than in the case of 400 S Aln samples (400 µg of Aln/sample). The cell activity was also
significantly lower in comparison to extracts from reference scaffolds and those cultured in pure medium but live-dead pictures showed that the cells were alive and even in the case of higher dilutions they formed uniform monolayer after day 6 of cell culture (Figure 5-5, third column). The extracts from reference scaffolds did not affect significantly cells activity in comparison to pure medium. After 6 days of the experiment cells formed a uniform monolayer, with some dead cells (less than 2%) (Figure 5-5, first column). Therefore for future experiments only 200 S Aln systems were used.

Figure 5-5 Live-dead staining of MG-63 cells on day 6 in contact with extracts diluted in MEM by the factors 1:1 (undiluted), 1:2, 1:4 and 1:8 from TiO2 scaffolds (first column), TiO2 scaffolds containing Aln-loaded MPs attached by collagen (two types of samples: with 400 mg and 200 mg of the drug) (second and third column, respectively). Additionally, a pure Aln solutions in medium are showed (last column – 100, 50, 10, 5 and 1 µg/ml). As a reference, activity of cells cultured in pure MEM is presented (lowest panel on the left). Scale bar =100 µm (Carl Zeiss Axiovert 40, Germany)

The aim of the next experiment was to assess if the drugs provided with the systems are able to prevent formation of the osteoclast-like cells from PBMCs and/or if they can lower the activity of the formed osteoclast-like cells. After 3, 8 and 15 days of cultivation samples for biochemical analysis were collected from the cell culture. In response to viability assessment from the experiment with MG-63 cells the applied concentrations of Aln in medium were smaller (5, 2.5, 1 and 0.5 µg/mL), as well as only 200 S Aln scaffolds were studied.

The differentiation of monocytes into osteoclasts was evaluated by the detection of TRAP 5b activity in culture with different extracts and drug concentrations. Figure 5-6 A presents the results of the study on the influence of different extracts and Aln concentrations on the total TRAP activity of differentiating PBMCs/macrophages/osteoclasts. TRAP has two different isoform secreted by different cells (5a isoform is
secreted only by macrophages and dendritic cell whereas 5b only by osteoclasts). In the case of measuring the levels of both isoforms as well as only 5b, the activity of the cells increases from day 3 to day 15 in all the samples. For both total TRAP activity the significant differences (p<0.05) in comparison to control are visible for day 8 and 15 for 200 S Aln dilutions 1:1 and 1:2 as well as for Aln 5 µg/mL concentration. That means that the extracts and the drug hinder the activity and probably the formation of osteoclasts and macrophages. Extracts from reference scaffolds did not influence the activity of the cells. Figure 5-6 B summarizes the results of the TRAP 5b isoform activity in the culture. Since TRAP 5b is not expressed by monocytes but mature osteoclasts, the assay is intended for use as an indicator of bone resorption and osteoclastic differentiation. Interestingly, for all the 200 S Aln extracts and Aln concentrations after 8 days of cell culture the TRAP 5b activity was significantly lower in comparison to control TCPS (p<0.05). After 16 days of culture only cells cultured in 5 µg/mL and in non-diluted extract from the scaffolds with Aln-loaded MPs showed significantly lower TRAP 5b activity compared to pure medium (p<0.01).

![Figure 5-6](image)

Figure 5-6 Total TRAP activity (A) and relative TRAP 5b (B) on day 3, 8 and 15 of cell culture for extracts diluted in αMEM by the factors 1:1 (undiluted), 1:2, 1:4 and 1:8 from TiO2 scaffolds (brown columns), TiO2 scaffolds containing Aln-loaded MPs attached with collagen (blue columns). Additionally, a pure Aln solutions in medium are showed (violet columns – 5, 2.5, 1 and 0.5 µg/ml). As a reference, activity of cells cultured in α-MEM medium is presented (pink columns). Mean ± S.D.; comparison with TCPS *p<0.05

Figure 5-7 and Figure 5-8 presents the results TRAP activity staining for Aln after 8 (Figure 5-7) and 15 (Figure 5-8) days of culture. Multinucleated cells positively stained for TRAP activity were formed after 8 and 15 days in all reference and control samples. On
day 8 the cells cultured in αMEM were round, well-spread. In some cases the cells had more than one nuclei, whereas for day 15 there are many multinucleated cells. Similar results were obtained for reference scaffolds. The addition of Aln to the medium resulted in the change of cells morphology, there was clearly more spindle-like cells which were not as closely packed as in the case of reference samples (this concerned higher concentrations). For 0.5 µg/mL Aln concentration the cells morphology was similar to that of reference samples. In the case of the systems with Aln it was clear that the morphology of the cells resembled the one of pure drug solutions, the higher dilution the less spindle-like cells and more well-spread, multinucleated cells. Similar differences can be found for 15 day culture. There is a clear difference both on day 8 and 15 for 200 S Aln dilutions 1:1 and 1:2 as well as for Aln 5 and 2.5 µg/mL concentration, the cells are many spindle-like cells, big (over 100 µm in diameter), well-spread multinucleated cells are rare in comparison to the reference samples. For bigger dilutions and smaller concentrations of Aln there is no clear difference between them and reference groups. Those observations correspond with TRAP and TRAP 5b activity measurements.

Figure 5-7 PBMC cultures visualised by TRAP staining after 8 days of culture in contact with extracts diluted in α-MEM by the factors 1:1 (undiluted), 1:2, 1:4 and 1:8 from reference TiO$_2$ scaffolds (first column - Ref), TiO$_2$ scaffolds containing Aln-loaded MPs attached with collagen (second column – 200 S Aln). Additionally, a pure Aln solutions in medium are showed (last column – 5, 2.5, 1 and 0.5 µg/ml). As a reference, activity of cells cultured in α-MEM medium is presented (lowest panel on the left). Scale bar = 50 µm (Carl Zeiss Axiovert 40 CFL, Germany)
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Figure 5-8 PBMC cultures visualised by TRAP staining after 15 days of culture in contact with extracts diluted in α-MEM by the factors 1:1 (undiluted), 1:2, 1:4 and 1:8 from TiO₂ scaffolds (first column), TiO₂ scaffolds containing Aln-loaded MPs dispersed in collagen (second column). Additionally, a pure Aln solutions in medium are showed (last column – 5, 2.5, 1 and 0.5 µg/ml). As a reference, activity of cells cultured in α-MEM medium is presented (lowest panel on the left). Scale bar = 50 μm (Carl Zeiss Axiovert 40 CFL, Germany)

5.2 Discussion

The aim of this study was to modify TiO₂ scaffolds in order to provide them with anti-resorptive properties which prevent bone loss. This goal was achieved by proposing systems containing either Aln-loaded degradable MPs which were attached to the scaffolds’ pore walls by a thin layer of collagen. The modified scaffolds were examined to assess their microstructure, drug release characteristics, potential cytotoxicity and influence on osteoclastogenesis.

MPs were manufactured from biodegradable PLGA considered safe for controlled release formulations [24]. PLGA 85:15 degradation rate is slower than polymers with lower ratio of lactide to glycolide, e.g. 75:15 or 50:50. Moreover, relatively high molecular weight of the polymer should allow to slow down hydrolytic degradation of the polymer matrix, which is responsible for further sustained release phase [50]. PLGA MPs were characterized by sufficient encapsulation and drug loading efficiencies – accordingly for
Aln 71 ± 6% and 7.7 ± 0.8%. The results correspond to other formulation of the polymeric MPs with those drugs reported in the literature [51–53].

Low concentration of the collagen proved to be sufficient to bind the MPs to the surface of the scaffolds by adsorption. Collagen is known to support osteoblast-like cells, as shown by the author previous work on similar collagen coatings on PLGA scaffolds [54]. Collagen provides cell adhesion domains, e.g. arginine-glycine-aspartic acid (RGD), which improve cell adhesion, proliferation and osteogenic differentiation [55–57]. In this study recombinant human collagen was used, which has several advantages over bovine or porcine collagen (no risk of pathogen transmission, the same sequence of aminoacids, better biocompatibility than collagens derived from animals).

Drug release study for Aln showed that from both systems initial burst release was observed. However in the case of systems with lower amount of drug (200 µg/scaffold) from day 3 up to day 18 the dose of the drug stabilized. 400 S Aln systems provided less stable dose in that period, which may be attributed to weaker bonding of the high amount of MPs on the surface of the scaffolds which might result in detachment of the MPs and thus exposing higher surface area of the MPs. The initial burst release may be associated with residual Aln adsorbed on the surface of microparticles [50].

During Aln release study the optimal MPs amount on the scaffolds was also searched. From previous work with Gent it was known that attempting to immobilize too high amount of MPs on the scaffolds would result in the lowered immobilization efficiency, thus lower initial amount of the drug on the scaffolds. Therefore the drug amount per scaffold was lowered to 400 µg for both drugs, and in the case of Aln the second system with half of that amount was proposed. Further in vitro cell studies proved that the Aln was cytotoxic above 10 µg/mL concentration toward MG-63 cells. Correia et al. investigated the cytotoxicity of the different concentrations of Aln in contact with human periodontal ligament fibroblasts. They found that concentration 10^{-5} M (which correspond to around 2.7 µg/mL) resulted in cells death after day 6 of culture [58]. Other study performed by Garcia-Moreno et al. showed that concentrations above 10^{-3} M did not affect the viability of the human osteoblasts [59].

Bisphosphonates are drugs used to suppress bone turnover, primarily through effects on osteoclasts. They are potent inhibitors of bone resorption by being selectively taken up and adsorbed to mineral surfaces in bone, from which they are released during resorption process and taken up by osteoclasts via endocytosis into intracellular vesicles. There are two mechanisms of the influence of the bisphosphonates on osteoclasts. In the cytosol bisphosphonates in their side chain can either be metabolised to toxic analogues of ATP and therefore induce apoptosis. In the second mechanism nitrogen-containing bisphosphonates inhibit the intracellular enzyme farnesyl pyrophosphate synthase, which prevents the prenylation of proteins essential for the function and survival of osteoclasts [60,61]. Most of the bisphosphonates are administrated either orally or intravenously which is usually associated with poor drug bioavailability (<1%), adverse effects like renal failure, osteonecrosis of the jaw (ONJ), etc. [62,63]. Therefore a local administration seems like a good alternative to minimise the side effects of conventional drug administration. Among
the bisphosphonates, sodium Aln is used for treatment of osteoporosis, primary hyperparathyroidism, malignant hypercalcemia and metastatic bone diseases. Additionally, several clinical trials have been already carried out which showed promising results for the treatment of periodontal disease. Aln increases bone formation and enhances osteoblasts proliferation and maturation and leads to inhibition of osteoblast apoptosis [52,59].

The most common cause of failure of the implants are due to the aseptic loosening due to periprosthetic osteolysis, which occurs in the bone supporting the implant. The sustained chronic inflammatory response initiated by particulate debris at the implant-bone interface is manifested by recruitment of a wide array of cell types. Among them are osteoclasts whose enhanced activity promotes osteolysis thus implant loosening [64].

The results of the influence of Aln on PBMCs differentiation toward osteoclasts and their activity showed that higher concentration of the drug inhibits the osteoclasts activity. This corresponds to the results obtained by other groups [65–67]. The results of TRAP 5b activity indicate the inhibitory effect of Aln and systems containing Aln on the differentiation and activity of the osteoclasts. Moreover, the initial significantly lower secretion of TRAP 5b by the cells indicate a relatively high dosage of the drug at the first week of incubation. The disappearance of those differences for bigger dilutions of extracts suggests the release of smaller Aln doses in further time points of the samples incubation which correspond to the in vitro release study results.

Therefore, it was proved that it is possible to successfully immobilize Aln- loaded MPs on the surface of the TiO$_2$ scaffolds with the use of collagen of very low concentration. The results of cytocompatibility and viability on osteoblast-like cells showed that higher concentrations of Aln are toxic, therefore the systems were optimized for lower concentrations of Aln. The systems with Aln provide required doses of the drug for inhibiting osteoclastogenesis and to reduce osteoclasts activity.

6 SUMMARY AND FINAL CONCLUSIONS

One of the most pressing issues in modern medicine is treatment of bone defects caused by various diseases or injuries. Although bones have a very good regenerative potential, critical-size defects often require a supporting structure for their healing process. Annually in the United States, more than half a million patients receive bone defect repairs, with a cost greater than $2.5 billion (data from 2012). The current golden standard treatment of critical-sized bone defect is autogenous bone grafting. To date, the dominant alternative for autologous grafting are donated allogenic bone grafts, which have several significant drawbacks such as potential infectious disease transmission, severe immunogenic responses etc. Bone defect repair using the tissue engineering approach is perceived as a potentially better option rather than natural bone grafts, since the repair process may be accompanied by the patient’s own tissue by the time the regeneration is complete.

In Chapter 1 of this dissertation the outline of the most common causes of the bone defects was presented. Second part of the chapter was concentrated on the current medical treatment options of such defects and accompanying diseases, which mostly focus on
autologous grafting and systemic drug delivery. In the chapter it was also presented the alternative approach to the problem with tissue engineering and the results of the clinical trials of those alternative systems. One thing is for certain, all future therapies of bone diseases and bone defects are directed into local delivery.

Scaffolds for osteogenesis should mimic bone morphology, structure and function in order to optimize integration with surrounding tissue. Therefore, the design of a scaffold is critical to its success as an implant material. For many decades, composites of HAp and other calcium phosphates have been mostly considered as osteoconductive bone graft alternatives. However, in recent years TiO₂ has been investigated as a potent substitute in bone tissue engineering. The results show that TiO₂ not only is biocompatible but also enhances bone and vascular ingrowth and has a certain degree of bacteriostatic effect. This dissertation focused on manufacturing such biocompatible scaffolds with appropriate mechanical properties. In Chapter 2 it was presented that by using polymer sponge replication method it is possible to manufacture highly-porous structures, with appropriate compressive strength for the use as a support system of human trabecular bone. Moreover, the scaffolds' architecture and microstructure were resembling the one of natural bone which provided a favourable microenvironment for bone ingrowth. This all was in accordance with other groups findings that the morphology and size of the pores are the key factors influencing cellular growth and tissue regeneration. The work focused additionally, on refining the scaffolds microstructure and enhancing their mechanical properties. However, the obtained results clearly indicated that further optimization of the manufacturing process can be performed.

As mentioned before, future perspective on combating various bone diseases and enhancing bone regeneration focuses on local drug delivery. In contrast to systemic delivery, the local drug delivery approach significantly improves the drug bioavailability at the site of infection/defect/impaired bone, and at the same time, reduces the adverse effects incidence. Many different approaches to this issue have been proposed in tissue engineering. The drugs can be incorporated directly into biomaterial forming scaffold/implant, they can be bound to the scaffolds surface, they can be a part of a layer formed from different material (mostly polymeric or bioceramic) on the scaffold/implant. But particularly interesting drug delivery systems are the micro- or nanoparticles (mostly polymeric) in which the drug is encapsulated. Polymeric MPs made of PLGA enable controlled drug-release kinetics, since their degradation rate can be regulated through the composition of the monomer units and by the microsphere size and morphology. Moreover PLGA MPs are biocompatible according to numerous studies. Therefore in Chapter 3 the optimization of manufacturing process of such systems was presented for two antibiotics (Gent and Vanc) as well as for Aln and Calc.

Post-implantation and post-traumatic osteomyelitis is currently one of the greatest challenges in orthopaedic surgery. Periprosthetic infection is the leading cause of revision total knee arthroplasty and the third most common cause for total hip arthroplasty. Bacteria of the genus Staphylococcus are the principal causative agents of osteomyelitis. Therefore, methods for prevention of perioperative infections are intensively studied. An early and appropriate antibiotic prophylaxis has been shown to dramatically reduce the incidence of
implant related infections. To this end in Chapter 4 a novel approach to adjust the TiO$_2$ scaffolds to minimize antimicrobial infection risk was described. The main aim was to immobilize within the scaffolds Gent- or Vanc-loaded PLGA MPs in order to achieve: i) burst release to combat immediate post-implantation infections and ii) a long-term drug release (up to 7 weeks) which addresses the problem of persistent *Staphylococci* infections. To this end sodium alginate of two concentrations was used to attach MPs to the surface of the scaffolds. To confirm utility of our system the drug release, cytocompatibility in contact with osteoblast-like cells and antimicrobial activity on *Staphylococcus* spp. both commercial and clinical strains isolated from infected bones and joints were tested. The results were promising, an effective immobilization of the Gent- and Vanc-loaded MPs on TiO$_2$ scaffolds with the low-concentrated cross-linked sodium alginate was performed. The systems provided required doses of the antibiotics to combat the post-implantation infections, drugs bactericidal activity was preserved and was not affected by the manufacturing process. Additionally, the proposed systems were cytocompatible with osteoblast-like cells.

Chapter 5 was oriented on the materials which may enhance bone regeneration and defect healing. During the bone remodelling after implantation, the osteoblast/osteoclast activity ratio may be imbalanced due to numerous diseases, among them most common osteoporosis. There are many drugs which are at present used in osteoporosis treatment. Among them Aln and Calc are ones of the most popular. The main aim of the Chapter 5 was to present the results of immobilization within the TiO$_2$ scaffolds Aln or Calc loaded PLGA MPs in order to achieve systems which may inhibit osteoclasts activity. To this end collagen was used to attach MPs to the surface of the scaffolds. To confirm utility of the systems’ drug release, cytocompatibility in contact with osteoblast-like cells was tested. Furthermore a study of their influence on differentiation towards bone resorbing cells was carried out. The immobilization of the Aln- and Calc-loaded MPs on the scaffolds was successful. The proposed systems released the drugs with initial burst release which might be a concern in the following cytocompatibility studies. After culturing the osteoblast-like cells in extracts from the systems, one of Aln systems had to be excluded from future experiments, because the released doses of the drug were toxic for the cells. Other systems with lower Aln content and the ones with Calc were proved to be cytocompatible. Final experiments focused on the influence of the systems with Aln and Calc on osteoclastogenesis and osteoclasts activity. Systems with Aln clearly inhibited the osteoclast formation and their activity, which in the view of possible use of the modified scaffolds in the defects of patients with osteoporosis proves them to be a viable option for future treatment. The systems with Calc did not affect neither osteoclasts formation nor their activity, but it is believed that further studies on the influence of the Calc on mature osteoclasts should be carried out.

To sum up, the presented evidence proved the main thesis of the dissertation that it is possible to manufacture biocompatible TiO$_2$ scaffolds and to modify them with PLGA microparticles containing various drugs to combat bacterial infections and to improve their effect on bone regeneration and healing processes.
REFERENCES