SUMMARY

Over the years, there has been a growing interest in tissue engineering products due to the high clinical demand in regenerative medicine. Biomaterials for bone tissue engineering applications should possess appropriate structural (e.g. surface topography, high porosity, good mechanical parameters) and biological properties (e.g. bioactivity, non-toxicity, biocompatibility).

The aim of the research carried out within the doctoral dissertation was to develop and optimize a novel production method of macroporous bone scaffold based on nanohydroxyapatite (nanoHA) and cryogel matrix composed of chitosan and agarose. The next step of the research was to comprehensively analyse structural, physicochemical, mechanical, and biological properties of the fabricated biomaterial, allowing reliable evaluation of its biomedical potential under *in vitro* conditions. Moreover, the research also aimed to modify the newly developed biomaterial with magnesium and zinc ions to increase its biocompatibility.

Developed novel production method of the bone scaffold was based on the simultaneous application of a foaming agent ($NaHCO_3$) and freeze-drying that allowed to obtain highly porous structure. In the first step of the study, the concentrations of a solvent (CH_3COOH) and a gas-foaming agent were optimized in order to select biomaterial with the best structural and mechanical properties. Selected biomaterial (containing 30% (w/v) nanoHA) was characterized by high total porosity (70% – based on microCT results), bioactivity, lack of cytotoxicity, and ability to promote adhesion and growth of osteoblasts. Nevertheless, it had relatively poor mechanical properties (Young's modulus and compressive strength). Thus, in the next step of the study, the content of nanoHA in biomaterial was increased to improve its mechanical parameters. Two types of bone scaffolds with different nanoHA content were produced: chit/aga/HA_L (containing 40% (w/v) nanoHA) and chit/aga/HA_H (containing 70% (w/v) nanoHA), and subjected to comprehensive analysis of their structural, physicochemical, mechanical and biological properties.

Assessment of structural properties of the developed biomaterials showed that both bone scaffolds had high specific surface area (approx. 30-31 m^2/g – based on the BET method) and total porosity in the range of 67-70% (based on MIP analysis). In addition, the macroporous structure of biomaterials was characterized by interconnected pores. In the next step of the

studies, mechanical tests were carried out, which showed that the developed biomaterials had compressive strength in the range of 1-1.4 MPa, which was comparable to spongy bone.

Physicochemical assessment (ATR-FTIR analysis, XPS) of chit/aga/HA_L and chit/aga/HA_H biomaterials revealed that hydrogen and covalent bonds were formed between chitosan and agarose and between the chitosan-agarose matrix and nanoHA, indicating that the developed biomaterials may be classified as class II hybrids, which are characterized by high stability. In addition, it was shown that both bone scaffolds had highly hydrophilic surface with numerous polar functional groups.

In the next step of the research, the liquid absorption ability of biomaterials was determined by measuring the weight increase with time of the samples after immersion in PBS solution and human blood plasma. The experiment showed that both scaffolds had high liquid absorption capacity, however, chit/aga/HA_L absorbed much more liquid than chit/aga/HA_H. In addition, the developed biomaterials were prone to enzymatic biodegradation (in the solution containing collagenase and lysozyme), biodegradation in an acidic environment (pH 3), as well as slow biodegradation under physiological conditions (pH 7.4).

In vitro bioactivity assessment (SEM-EDS analysis) showed that developed biomaterials had the ability to form apatite crystals on their surface, revealing Ca/P ratio equal to 2.23 and 1.68 for chit/aga/HA_L and chit/aga/HA_H, respectively. It is worth noting that apatite crystals formed on the surface of chit/aga/HA_H had similar ratio of Ca/P to hydroxyapatite naturally occurring in the bone tissue (Ca/P = 1.67).

In the next stage of the research, the ability of biomaterials to adsorb proteins was assessed. Quantitative (Lowry's method) and qualitative (immunofluorescent staining of adsorbed proteins) analysis showed that both biomaterials had the ability to adsorb high amount of plasma proteins (chit/aga/HA_L – 647.80 $\mu g/cm^2$, chit/aga/HA_H – 763.20 $\mu g/cm^2$), with the highest affinity to fibronectin (cell adhesive protein), which ensures good adhesion, spreading and cell proliferation on the surface of the implant.

Evaluation of the biological properties of the developed chit/aga/HA_L and chit/aga/HA_H biomaterials showed that the scaffolds were non-toxic to the osteoblast cell lines (MC3T3-E1 – mouse cells, hFOB 1.19 – human cells). Moreover, the surface of the biomaterials promoted adhesion, spreading and proliferation of osteoblasts. Additionally, evaluation of osteogenic differentiation of mesenchymal stem cells (BMDSC – cells derived from bone marrow, ADSC – cells derived from adipose tissue) cultured on the surface of

biomaterials was performed. It was shown that both biomaterials had ability to induce the differentiation of mesenchymal stem cells towards the osteoblast lineage, however, chit/aga/HA_L reveled better osteoinductivity compared to chit/aga/HA_H.

The final step of the research performed within the doctoral dissertation involved modification of the newly developed biomaterial with magnesium and/or zinc ions in order to increase its biocompatibility. The assessment of biological properties of modified biomaterials was carried out in direct contact of the cells with the surface of biomaterials and using liquid extracts of the bone scaffolds to evaluate the effect of released metal ions on cellular response. Produced biomaterials (with magnesium and/or zinc ions) were non-toxic to the MC3T3-E1 cells. In addition, stimulating effect of magnesium ions on adhesion and proliferation of MC3T3-E1 cells was demonstrated. In contrast, modification of the biomaterial with zinc ions showed lack of improvement in the biological properties of the scaffold. Moreover, the stimulating effect of magnesium and zinc ions on the process of osteogenic differentiation of mesenchymal stem cells (BMDSC, ADSC) was not demonstrated. Considering the obtained results, it was found that only the modification of biomaterials with magnesium ions had impact on the improvement of their biocompatibility.

To sum up, newly developed production method of biomaterial based on nanohydroxyapatite (nanoHA) and a cryogel matrix made of chitosan and agarose, allowed to obtain macroporous, hybrid, and biodegradable bone scaffolds. A comprehensive comparative analysis of two biomaterials (chit/aga/HA_L and chit/aga/HA_H) showed that both scaffolds are characterized by high biocompatibility, osteoconductivity, and osteoinductivity. Nevertheless, chit/aga/HA_L biomaterial possesses greater medical potential due to its better osteoinductive ability compared to chit/aga/HA_H material. Thus, chit/aga/HA_L may be potentially used in regenerative medicine as a bone scaffold designed for implantation in non-load bearing sites, where it will promote regenerative processes. The biomaterial may be also used under *in vitro* conditions as a scaffold for osteoblast expansion, serving as a three-dimensional model of bone tissue.