New approach in evaluation of ceramic-polymer composite bioactivity and biocompatibility

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Abstract Regeneration of bone defects was promoted by a novel β-glucan/carbonate hydroxyapatite composite and characterized by Raman spectroscopy, microCT and electron microscopy. The elastic biomaterial with an apatite-forming ability was developed for bone tissue engineering and implanted into the critical-size defects of rabbits' tibiae. The bone repair process was analyzed on non-decalcified bone/implant sections during a 6-month regeneration period. Using spectroscopic methods, we were able to determine the presence of amides, lipids and assign the areas of newly formed bone tissue. Raman spectroscopy was also used to assess the chemical changes in the composite before and after the implantation process. SEM analyses showed the mineralization degree in the defect area and that the gap size decreased significantly. Microscopic images revealed that the implant debris were interconnected to the poorly mineralized inner side of a new bone tissue. Our study demonstrated that the composite may serve as a biocompatible background for collagen ingrowth and exhibits the advantages of applying Raman spectroscopy, SEM and microCT in studying these samples.

Keywords Bioactivity · Biomaterials · Bone substitutes · Carbonate hydroxyapatite · Mineralization · Raman spectroscopy · SEM

Introduction

The increasing number of accidents, injuries and bone tumours along with developments in medical sciences result in growing demand for bone substitute materials. The global bone grafts

Electronic supplementary material The online version of this article (doi:10.1007/s00216-017-0518-0) contains supplementary material, which is available to authorized users.
and substitutes (BGS) market was valued at US$2358.3 million in 2014 and is expected to reach US$3482.0 million by 2023 according to a new report published by Transparency Market Research [1]. Plenty of scientific reports concerning novel materials appear every year and regard many different aspects such as material properties, scaffold design, host response, implant personalization, use of cells and signalling molecules [2, 3]. Ceramic-polymer composites containing synthetic hydroxyapatite have received much attention because of their advantageous properties such as biocompatibility, adaptation to the shape/size of bone defects, sufficient mechanical strength, non-toxicity and possibility of delivering drugs and macromolecules [4–10].

In vivo evaluation of a new bone substitute material in an animal model usually include radiographic examination [11], densitometry (BMC and BMD parameters) [12, 13], histological analysis [14], histomorphometry [15, 16], biochemistry [17], microhardness [18], computed tomography [19, 20], compressive test [21] and electron microscopy [22]. Recently, Raman spectroscopy (widely used in chemistry) has also found application in the assessment of bone quality. This technique has become a valuable tool in bone implant testing [23] and bone tissue characterization [24, 25]. It allows understanding how changes in bone composition and structure influence tissue-level mechanical properties. The information about the mineral and matrix collagen components, bone crystallinity, bone hardness, orientation of mineral crystallites, age of the tissue but also indirect information on collagen cross-links [26–28]. The technique can be used with fresh, as well as fixed and imbedded, specimens and in some limitation for non-invasive measurements on live animals [29]. The Raman spectroscopy has been used in various studies for the analysis of soft tissues without sample preparation and to obtain new and complementary information about biominerals [30].

In this study, we propose a new approach in evaluation of bone implants bioactivity and biocompatibility by the use of a combination of different techniques—Raman spectroscopy, high-resolution X-ray microtomography (microCT) and scanning electron microscopy (SEM). The combination of these non-invasive techniques was applied to study chemical changes in the material before and after implantation, bone implant integration, mineralization degree in different points within defect site (“bone maturation”), collagen ingrowth and samples surface in micro- and macro-scale. The subject of research was novel ceramic-polymer composite dedicated for bone tissue engineering consisting of carbonate-substituted hydroxyapatite (CHAP) and polysaccharide (β-1,3-glucan). Regenerative capability, bioactivity and osteoconductivity of the composite were tested in an animal model and examined qualitatively.

### Experimental

#### Sample preparation

CHAP granules were synthesized at the AGH-University of Science and Technology, according to patented procedures [31, 32]. β-1,3-Glucan (curdlan) from Alcaligenes faecalis (DP 450) was supplied by Wako Chemicals, Japan.

CHAP-glucan composite scaffolds were prepared according to the procedure described in European patent [33]. Briefly, samples were fabricated by mixing 3 g of CHAP granules with β-1,3-glucan aqueous suspension (0.625 g of glucan + 5 ml of distilled water), thus obtaining wt% proportion granules to β-1,3-glucan 83:17. The mixture of ceramic/glucan solution was put into a special glass mould to obtain cylindrical implants, 4 mm in diameter and 6 mm in length and heat treated at 90 °C for 15 min. Finally, the fabricated material was dried for 4 days at 37 °C, subjected to exsiccation for the next 3 days and sterilized. Table 1 summarizes properties of the CHAP-glucan composite.

#### Implantation procedure

With approval of the II Local Ethics Committee on Animal Research University of Life Sciences in Lublin, Poland (agreement no 16/2010), 24 New Zealand male white rabbits (6 months old, c.a. 3.5 kg body mass) were used in the experiment. Animals were kept separately in cages under standard conditions of housing, feeding and handling. The adaptation at the animal house of the Medical University of Lublin lasted 1 week. Prior to surgery, animals were premedicated with medetomidine (Domitor®, Orion Corp., Finland; dose 0.5 mg/kg) and anesthetized with ketamine (Bioketan®, Biowet, Poland; 0.5 mg/kg). Surgical procedure was described previously by Borkowski et al. [34, 35]. Briefly, a defect, 4 mm in diameter and 6 mm in depth, was drilled in the proximal tibial metaphysis and filled with CHAP-glucan composite material. The skin was sutured with Dexon 3–0 thread (Tyco Healthcare, UK). The implantation procedure has been shown in Fig. 1. After the operation, animals were allowed to move freely in cages.

### Table 1 Chemical compositions and physical parameters of the CHAP-glucan composite used in the study

| Characteristics | Composites CHAP/glucan |
|-----------------|------------------------|
| CHAP/glucan (wt% ratio) | 83:17 |
| Carbonate content (wt%) | 4.3 |
| Sorption index (%) | 119 |
| Compressive strength (MPa)* | 6.1 |
| Young’s modulus (GPa)* | 0.64 |

*Measured for dry composite samples
The spectra were baseline corrected in the range 2000–3000 cm$^{-1}$, not normalized and present relative intensity of bands. The autofocus at each measured point of sample was used in case of non-flat samples. The distance between five marked points in the Fig. 5d was 300 μm, and the total distance was about 1500 μm.

Raman spectroscopy

Raman spectra were collected using a DXR Raman Microscope (Thermo Scientific, Waltham, MA, USA), with a 780-nm laser with a maximum output power of 20 mW. The spectra were recorded over the spectral range of 2500–2000 cm$^{-1}$ using an operating spectral resolution of 4 cm$^{-1}$ of Raman shift. The 25 square aperture was used with the following settings: exposure time 6 s, number of exposures 8. The spectra were collected using both a ×10 and a ×100 objective. All data processing and image assembly were performed using OMNIC 8.2.0.387 software. The five spectra from the each area in the sample were collected then averaged. The spectra were baseline corrected in the range 2000–2000 cm$^{-1}$, not normalized and present relative intensity of the bands. The autofocus at each measured point of sample was used in case of non-flat samples. The distance between five marked points in the Fig. 5d was 300 μm, and the total distance was about 1500 μm.

MicroCT and SEM analysis

Bones with implanted composite were scanned in wet state using Skyscan 1172 X-ray computed tomography (Bruker microCT, Belgium). The sample, placed in tube made of polyethylene (PE) filled with distilled water, was rotated during scanning within the angular range of 0°–180° with a step of 0.2°. An averaging mode was used to obtain balanced exposure levels. In total, 900 photographs were made. An aluminium 0.5-mm-thick filter was used to reduce beam hardening effects. To eliminate possible artefacts, the sample was randomly moved for each projection. The set of images were then reconstructed into cross-sections using the NRecon software (Bruker microCT, Belgium). After the reconstruction, the isotropic voxel size was 3.94 μm in each axis. Imaging of results was made in DataViewer software (Bruker microCT, Belgium) using orthogonal sections and transforming grey scale into colours to easy recognize objects.

The bone implant sections were analysed using scanning electron microscopy (FE-SEM; Zeiss ULTRA plus).

Results and discussion

Figure 2a,b demonstrated irregular shapes of the synthesized CHAP granules and uniform density of the particles among fractions. Composite scaffolds were obtained after addition of β-1,3-glucan, which formed a compact mesh widely adhered to CHAP surface (Fig. 2c). Scaffold microarchitecture shown in Fig. 2d exhibited a high porosity and opened pore structure. SEM images also confirmed a homogenous distribution of granules in the scaffolds. Composite samples exhibited high flexibility, could be compressed or bent and adapted easily to appropriate shapes. The obtained material could be formed into different shapes at the preparation step (using different moulds) and after fabrication using scissors or lancet.

Raman spectroscopy

To investigate the chemical changes in the bone tissue regeneration process, we focused on sample that were implanted for 6 months in the area between the bone tissue and implanted composite. Raman spectroscopy was chosen as a non-destructive technique that requires minimal sample preparation [36] and that can be used to measure the chemical properties of the mineral and collagen parts. The Raman spectrometer was coupled with microscope optics for sample illumination and spectral acquisition, allowing investigation at the micron level [37].

A representative Raman spectrum of bone is shown in Fig. 3a. The band at 960 cm$^{-1}$ corresponds to the symmetric stretching vibration (ν1) of the phosphate ion PO$_4^{3-}$, the phosphate bending vibrations (ν2) 426 cm$^{-1}$, 450 cm$^{-1}$ and (ν4) appear at and 593 cm$^{-1}$ respectively, the non-symmetric stretch (ν3) at 1035 and 1069 cm$^{-1}$. There is a wide band at 1069–1073 cm$^{-1}$, indicating type B carbonate substitution in the bone specimen (carbonate substituting for phosphate in the apatite lattice). The band at 1003 cm$^{-1}$ corresponds to phenylalanine and HPO$_4^{2-}$ ion. The important Raman collagen bands are the amide III 1000–1260 cm$^{-1}$ and amide I at
1656 cm\(^{-1}\) bands, which arise largely from the collagen, and the CH\(_2\) peak at approximately 1450 cm\(^{-1}\) which is present in both collagenous and non-collagenous organic molecules.

Figure 3b, c shows pure basic components of implanted biomaterial. The 1-3-\(\beta\)-\(\alpha\)-d-glucan bands are as follows: 1465, 1371, and 1048 cm\(^{-1}\) are attributed to the presence of polysaccharides. Band at 891 cm\(^{-1}\) refers to the anomeric structure about the glycosidic bond and in particular the configuration of the main \(\beta\), \(\alpha\) and polysaccharides. Band at 428 cm\(^{-1}\) indicates the presence in the sample \(\beta\)-1,3-glu\(\zeta\)an; absence of a band at 950 cm\(^{-1}\) which is assigned to \(\alpha\)-1,3-glu\(\zeta\)an demonstrates the presence of only one form of glu\(\zeta\)an in the composite [38]. The glu\(\zeta\)an bands at 1107 and 1392 cm\(^{-1}\) can also be assigned to the \(\nu\) sym (COC) glycosidic and \(\delta\) (CH\(_2\)) bands, respectively. The bands of CHAP granules are described the same as the phosphorous bands.

The connection of optical microscope with Raman spectrometer enabled to distinguish several regions in bone samples with different spectra: compact bone, newly formed bone tissue, implant remains and region enriched with collagen and lipids (Fig. 4). In Fig. 4b, blue arrowhead indicates averaged Raman spectrum taken from the outer layer of a sample marked in Fig. 4c as blue area. This spectrum was assigned to a cortical bone as it exhibits the complementary bands as bone tissue in Fig. 3a. The spectrum of adjacent (green) area exhibits lower phosphorous bands and higher band at 1003 cm\(^{-1}\) and was attributed to newly formed bone tissue (Fig. 4b, c). The yellow area shows the implant remains with much lower phosphorous bands and high organic bands, which is especially characteristic for collagen and lipids. The comparison of the chemical changes in composite before (Fig. 3b, c) and after implantation is presented. It can be noted that the part between the newly formed bone and implant remains (which is marked with red arrow and red data set) do not have phosphorous bands, rather bands that are assigned for collagen and lipids at 1744 cm\(^{-1}\) (Fig. 4c). It was reported that the changes of lipid content in bone affect the metabolism of lipids, as the lipid content in articular cartilage increased with the age of the samples [39, 40]. Additional Raman images were presented on Fig. S1 the Electronic Supplementary Material (ESM) and exemplary differentiation between

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Fig. 2 MicroCT images of CHAP granules and SEM images presenting structure of CHAP/glucan biocomposite. (a) 0.2–0.3 mm fraction of CHAP granules, (b) 0.4–0.6 mm fraction of CHAP granules, (c) CHAP/glucan composite surface magnified 120× and (d) scaffolds microarchitecture magnified ×500

Fig. 3 The Raman spectra of representative bone tissue sample (a) and composite material components before implantation: CHAP granule (b) and \(\beta\)-1,3-glu\(\zeta\)an (c)
spectra within the selected area (red zone) were also presented on Fig. S2 in the ESM.

The optical microscope connected with Raman spectrometer enabled also to observe the bioresorption/dissolution of apatite. The composites were prepared using a mixture of particles with a size between 200 and 600 μm. As presented in Fig. 4d, the measured size of the largest remaining CHAP granules 6 months after composite implantation decreased to 500 μm. The smaller granules ca. 100 μm are visible and also smaller pieces, but due to the procedure of cutting, they may come from the large granules. Nevertheless, this result indicates degradation of granules localized inside the bone tissue.

Two important factors of bone quality are collagen crosslinking and the degree of mineralization [41]. The mineralization is a complex process involving bone cells (osteoblasts), growth factors (such as PDGF, TGF-β, IGF-I, IGF-II, FGF), endothelin (ET-1), bone morphogenetic proteins (BMPs) and others. As a result, crystals of calcium phosphate are produced and deposited within the bone's fibrous matrix. Generally, this process implies two successive steps: a primary mineral deposition on the calcification front and subsequent slow process of secondary mineralization of basic structure units (BSUs) [42, 43]. The degree of secondary mineralization depends on the lifespan regulated by rate of turnover [44]. Bone tissue may exhibit a heterogeneous degree of mineralization in different areas; therefore, the properties of newly formed bone may differ in tissue maturity from older bone [35, 42]. Our study of the relative intensity of PO$_4^{3-}$ at 960 cm$^{-1}$ in bone cross-sections revealed the differences in the phosphate content (Fig. 5b). Based on the obtained results, the scheme showing the direction of new bone formation and mineralization process was proposed (Fig. 5a). The spectra from marked points numbered 1–5 in the Fig. 5d revealed the highest phosphorus content in the point number 5 and the lowest in the point number 1. Other bands obtained from this point were illustrated in Fig. S3 in the ESM. The proportion between spectra of amides bands was the same as for phosphate bands. The distance between each point was 300 μm, and the total distance was about 1500 μm. The Raman intensity of phosphate bands confirms that this particular part of the sample is newly formed bone undergoing mineralization process. The difference of phosphorus content exhibit maturity of the bone tissue, the further away from the defect centre the higher mineralization/maturity of bone tissue. Therefore, the Raman spectroscopy may be a useful tool for estimating the degree of mineralization process and maturity of bone tissue.

Figure 5e presented part of sample marked with the green square on Fig. 5d. This image is equivalent with the SEM images presented in Fig. 6c, d. The spectrum obtained from fibrous connector visible within presented area exhibit similarity to collagen type I (bands at 1665, 1445 and 1070 cm$^{-1}$) together with phosphate bands at 960, 580 and 431 cm$^{-1}$. It is the same as the representative spectrum of bone tissue (Fig. 3a) and indicate mineralization process of bone matrix. The yellow square marked in Fig. 5d and its magnification (Fig. 5f) was taken at the border between the bone tissue and remains of the implanted composite. The chemical characterization of visible fibrous connector indicates area enriched with collagen without phosphate bands. This exposes the mineralization process that has not started yet in this area and confirms that the CHAP/β-1,3-glucan composite is a suitable background for collagen ingrowth, as it significantly aids an increase in its maturity with the time of bone growth process.
Figure 6 shows the SEM images presenting elaborately the defect 6 months after composite implantation. In the triangle cross-section of the tibia (Fig. 6a), there is visible ca. 100 μm gap within compact bone that remained after regeneration of 4 mm drilled bone void. Figure 6b–d demonstrates that the new bone tissue in the defect area consists of organic matrix and mineralized bone, thus suggesting that the regeneration/mineralization process has not finished yet. Implant debris visible in the proximity of bone defect were connected with new bone tissue, as presented in Fig. 6e, f. Some CHAP granules were tightly entwined by bone matrix, and some were encapsulated by the mineralized bone; however, the most granules are situated in the bone implant border. The bone implant integration indicates good biocompatibility of the composite material.

MicroCT images shown in Fig. 7 revealed that the new bone significantly recovered the defect area (in white circle) after 3 months. Implant debris were still visible around the bone defect site. Some composite particles were still embedded in compact bone; however, they appeared significantly smaller than others, indicating proceeding biore sorption (as presented also in Fig. 4d). The entire implants were highly radiopaque due to the dense structure of CHAP granules; thus, they were clearly visible in the area of implantation. Panel B exposed the penetration of newly formed osseous tissue into the 3-dimensional structure of the implant. Close contact between living bone and synthetic biomaterial and no signs of graft rejection were displayed. The results obtained by use of SEM (Fig. 6) and microCT (Fig. 7) are in accordance with the bone healing mechanism and compatible with the scheme presenting the direction of bone ingrowth/mineralization (Fig. 5a).

Conclusions

In this study, we investigated in vivo the bioactivity of β-1,3-glucan/CHAP composite using Raman spectroscopy and different imaging techniques (SEM, microCT). The imaging
tools such as optical microscope, SEM and high-resolution microCT showed relevant integration of ceramic-polymer composite material with bone tissue 6 months after implantation. Raman microspectroscopy has been employed to study chemical changes in the composite and material composition in bone. This analytical spectroscopic technique offers the possibility to obtain micro-level spatial resolution and permits the detection of local variations in composition. Based on this technique, bone regeneration in terms of (collagen rich) matrix formation and further mineralization was observed and described. Organic fibrils visible in the newly formed bone and at the bone implant border were assigned to collagen type I based on the spectrum of standard protein. It shows that the composite may serve as a biocompatible background for collagen ingrowth. The mineralization of newly formed bone reflected its maturation which progressed from the edge to the centre of defect area. Raman spectroscopy appeared to be very useful in estimating the degree of mineralization.
process and maturity of bone tissue. Implant debris were still visible after 6 months and remained inside the marrow cavity. This study leads to the conclusion that CHAP/glucan composite demonstrates bioactive and biocompatible properties for bone repair process.

The novel aspect of our approach is that we used complementary analytical and imaging techniques that provided a highly informative and reliable characterization of biological samples. We used Raman spectroscopy, microCT and SEM in the characterization of non-decalcified bone samples which requires minimal sample preparation process. This methodology illustrates the benefits of Raman spectroscopy in combination with microCT and high-resolution electron microscopy that allowed looking at the samples surface from a distance of different perspectives.

Acknowledgements The authors would like to acknowledge the financial assistance provided by the European Regional Development Fund within the Innovative Economy Operational Program, grant no. UDA-POIG 01.03.01-00-005/09-01, the DS2 of Medical University in Lublin, by the Medical University Student Grant Program awarded to Leszek Borkowski (MNsd 3) and by the National Science Centre grant no. ODW-5824/B/P01/2011/40. The experiments were conducted using the equipment purchased within the agreement no. PORPW.01.03.00-06-010/09-00 Operational Program Development of Eastern Poland 2007-2013. The authors would especially like to express their gratitude to mgr Tomasz Pieriśk for his assistance in histological preparations. Finally, KJ and ASB would like to acknowledge the Foundation for Polish Science (TEAM Programme 2009-4/5). The authors would like to acknowledge Grzegorz Kalisz for performing some of the Raman experiments.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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