PREPARATION AND PRELIMINARY *IN VIVO* STUDIES OF RESORBABLE POLYMER MODIFIED WITH ALLOGENIC BONE CHIPS FOR GUIDED BONE REGENERATION AND ORTHOPEDIC IMPLANTS

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Abstract

Composites made of resorbable polylactide modified with bone powder are part of the current search for implantable materials endowed with advantageous biomechanical functions, which make them suitable for orthopedics and traumatology applications. The bone additive containing active bone morphogenetic proteins (BMPs) and calcium phosphates introduced into the polymer matrix is to grant the implant with a biological activity. Subsequently, the resorbable matrix should get replaced with bone tissue. In order to avoid losing the osteoinductive properties of the designed material, it should be processed at low temperatures via physicochemical methods. This paper is devoted to the preparation and optimization of the composite production method suitable for biodegradable polymers and morphogenetic proteins along with the assessment of biocompatibility and biological properties of obtained materials. The tape-casting method was successfully applied. Resorbable polymer (medical poly-L-lactide, Purasorb PL38 by Purac) with 15 wt% of human bone powder (from tissue bank) were used to fabricate PLA-CP/BMPs composite implants. They were tested in in vivo studies that were performed in rabbit bone tissues. The results show a high biocompatibility of the material and good osteointegration with bone tissue.

Keywords: *bioactive composite, resorbable polymers, polylactide, GBR - guided bone regeneration, bone graft, BMP - bone morphogenetic proteins, osteofixation devices*

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Introduction

Tissue grafting has been a recognized therapeutic method for many years. Bone grafts are widely used in many fields of medicine, e.g. in maxillofacial surgery, orthopedics, neurosurgery, or plastic surgery. Transplants are usually used as fillers of bone defects that result from a resection of cancerous lesions or in dental implantations. The osteoinductive properties of the grafts, i.e. the ability to stimulate the proliferation and differentiation of the recipient's bone precursors, lead to complete reconstruction and replacement of the graft tissue with the patient's own bone tissue [1]. During the process, bone morphogenetic proteins (BMPs), which are growth factors, are released from the bone matrix and stimulate osteoblasts to produce new bone tissue. Due to their osteoinductive potential, BMPs play an extremely important role in the regeneration of bone tissue [2,3]. Therefore, ground bone containing BMPs is very often used to fill cavities created after bone fragments resection. In such procedure, small bone flakes or chips are packed to fill in the space of the cavity. Unfortunately, the size of the defect treated with a ground bone is limited, and the mechanical properties of such a filling are very poor, almost none. So far, there is no processing method to form a bulk material out of a ground bone, and thus, implants in the shape of rods, cubicles, or cylinders made of this material are not available.

The possible way to exploit the natural/biological advantages of the BMPs is the fabrication of a hybrid polymeric composite. For medical applications, the most popular are bioresorbable polymers and their composites, although these materials do not have osteoinductive properties and their biocompatibility is not sufficient. The degradation rate does not match the tissue regeneration processes, and quite often the regenerating tissue differs from the primary and surrounding one (fibrous instead of cancellous). Sometimes, after the biodegradation process is finished, a hollow space (cavity) may develop, reducing the mechanical strength of the patient's bone. This cavity can be filled with tissue fluid (fluid cyst), with a noticeable inflammation around the implant, having an adverse effect on the healing process [4].

Considering that, incorporation of bone chips containing BMPs into the biopolymer matrix should improve the characteristics of the material and appears to be a key to obtain malleable materials of desired shapes and sizes. Especially given the fact that polymer composites modified with bioceramic particles are well-known and have been successfully applied over the years.

The most popular polymer used for the resorbable implants fabrication is polylactide (PLA) and its copolymers [5-8]. In order to improve the response of bone tissue to foreign body, the polymer is modified with bioactive additives, such as hydroxyapatite (HAp) or tricalcium phosphate (TCP) [9-11]. These additives have an osteoconductive effect and improve osseointegration. This phenomenon is related to the chemical similarity of the phosphates present in the implant with the minerals that build the bones. A higher degree of osteoinductive bioactivity is possible when the material is enriched with a biological agent, and bone morphogenetic proteins (BMPs) seem to be a very good choice [12,13].

Because PLA is a thermoplastic polymer, simple and efficient methods, such as extrusion or injection molding, are usually applied to obtain various shapes of implants made of this thermoplast and its composites [14,15]. These processes need high temperatures to plasticize polymers (160-240ºC). Proteins like BMPs have low thermal resistance and they denature when heated over 40ºC; thus, in this case, high-temperature methods cannot be used.

In the field of low-temperature polymer processing, tape casting from the solution is widely used. This method is based on solvent solution preparation (liquid) which is poured and casted in a flat die, forming a layer. When the solvent evaporates, the material dries and solidifies. As a final result, a thin foil or sheet of the material is obtained. Unfortunately, because of that, this method cannot be applied if solid (bulk) three-dimensional shapes have to be formed. Another drawback of this method is related to the substrates that are used. The preparation of the polylactide solution involves the application of toxic solvents (e.g. dichloromethane, chloroform), thus the safe usage of the prepared material in medical applications requires absolute and complete solvent evaporation during the solidification process.

This paper focuses on the fabrication of a hybrid synthetic-natural composite bulk implant, using an adapted tape casting method. As a result, the composite composed of polylactide matrix, embedded with bone chips containing BMPs, was manufactured. The authors used a pile of stacked-up, thin composite foils, bonded with a solvent, and pressed together to form cylindrical samples. Unfortunately, this approach carried a risk of solvent trapping inside an implant what can reveal unwanted side effects. The main goal of the presented work was to verify a methodology of preparation of hybrid composite bulk samples, using a modified tape casting method, and to assess the biocompatibility of the prepared composite in *in vivo* tests.

Materials and Methods

Manufacturing composite material

The hybrid-composite material was obtained by physico-chemical methods. Bioresorbable polymer (homopolymer poly-L-lactide PL38 PURASORB® by Purac, The Netherlands) and human cortical bone chips (Central Tissue Bank in Warsaw, Poland) were used. The bone was ground (average particle size ~200 μm) and defatted at ambient temperature according to standard procedures. The tape casting method was applied to obtain thin composite films (FIG. 1a). Firstly, 1 g of PLA was dissolved in 15 ml of dichloromethane. Secondly, the solution was placed on the magnetic stirrer. After 24 h, 15 wt% of ground bone was added and mixed. The solution was poured and molded into Petri dishes (dia. = 14.5 cm) creating ~0.5 mm thick layer and left for 24 h at room temperature to solidify. Next, to achieve complete removal of possible residuals of solvent 12-hour vacuum drying was applied. When the foils were ready, round-shaped flakes were cut out (dia. 4 mm). Bulk cylinders (FIG. 1b) were formed by placing cut-out flakes in the metal tubular die. Each flake was covered on both sides with dichloromethane. Piled and staked up flakes created a multilayer cylinder. The die was then closed with the piston and the flakes pile was compressed (1 MPa). After 24 h, the bulk sample was removed from the die and then a two-stage drying process was followed. The samples were left in the open air at room temperature for 24 h and then for an additional 24 h, they were placed in the vacuum dryer. At the end, the samples were sterilized with plasma.

***In vivo* test**

The *in vivo* test surgical procedure was approved by the Local Ethics Committee on Animal Research by University of Life Sciences in Lublin (No 12/2014) and carried out in strict accordance with the guidelines for the care and use of experimental animals. In this study, the animals were ten healthy, skeletally mature (8-9 months of age) New Zealand White Rabbits, male, weighing 3.5-4 kg. The animals were housed in the vivarium of the University of Life Science in Lublin, under standard conditions of housing, feeding, and handling for laboratory animals. The rabbits were kept in individual cages and had free access to water and food. The surgery was performed after two weeks of quarantine. The animals were anesthetized with a mixture of xylazine (5 mg/kg *i.m.*) and ketamine (30 mg/kg *i.m.*) and subsequently by continuous infusion of the mixture of ketamine (30 mg/kg) in 5% glucose (40 mL/kg/h *i.v.*). Under aseptic conditions, a standard surgical approach to the proximal mataphyseal tibia was performed. After separating the periosteum and exposing the bone, the critical size defect (4 mm in diameter, 6 mm in depth) was drilled approximately 3 cm below the epiphyseal cartilage line. Before implantation, the defect was cleansed with a saline solution to remove any tissue debris from the cavity. The biomaterial was then inserted using the press-fit technique. Then, the skin was sutured and a postoperative radiological examination performed (Intemedical Basic 4003 device, exposure parameters were 4 mA, 50 kV). Butorphanol (0.2 mg/kg *s.c.*) for analgesia and Enrofloxacin (5 mg/kg) were administered for 3 days after the surgery to prevent probability of infection.

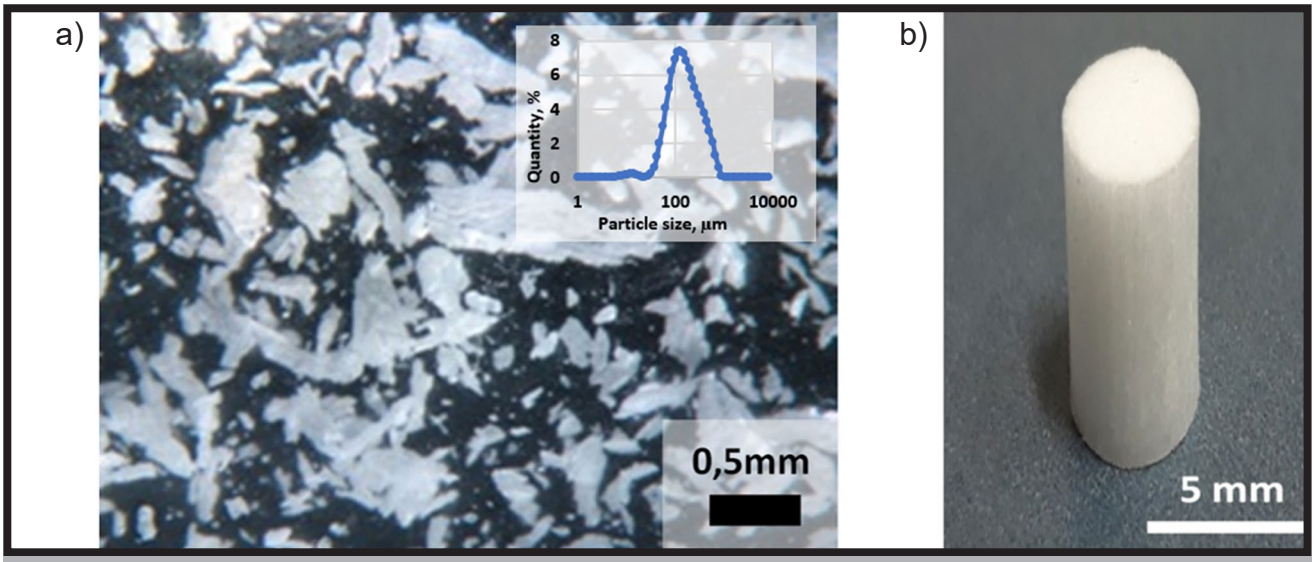

FIG. 1. Images of: a) PLA-bone composite foil with 15 wt% of bone additive (optical microscopy) with particle size analysis, b) implanted cylinder of PLA-bone.

The rabbits were sacrificed 12 weeks after surgery with an overdose of sodium pentobarbital (Morbital 1 ml/kg *i.v.*). Then a radiological examination was performed and specimens were collected for histological and SEM evaluation.

Microscopic observation

The biological preparations (FIG. 2a) were prepared using a low-speed diamond saw, then dried at room temperature and sputtered with carbon. The material was evaluated by means of SEM microscopy using a NOVA NANO SEM 200 microscope. An EDS analysis of the cross sections of the samples was also carried out.

Histopathological examination

Bone tissue was partially decalcified in the electrolytic decalcifying solution produced by Bio-Optica. Then it was placed in paraffin blocks (FIG. 2b) according to standard procedures [16] and cut into 4 μm sections using a microtome. Having been cut, the sections of the bone tissue were routinely stained with HE (hematoxylin and eosin). The preparations were scanned with the Hamamatsu NanoZoomer 2.0-HT scanner and evaluated using the NDP.view2 software.

Results and Discussion

Results

Evaluation of implantation healing process

There were no intraoperative complications either at the time of implantation or during the entire treatment period. Furthermore, no signs of inflammation or adverse tissue reactions around the implantation site were observed during the tests.

Macroscopic observation revealed completely filled bone defects with no empty spaces between implanted material and bone tissue. The small amount of biomaterial was still visible on the surface of the new bone tissue. There were no pathological macroscopic changes either in the soft tissue surrounding the implantation site or in the popliteal lymph nodes.

Radiographic examination

After 12 weeks from the operation, the bone defects were completely filled by the newly formed bone tissue and the small amount of residual biomaterial. The cortical layer of the bone defect was reconstructed. No radiological signs of any adverse reaction to the implant were observed (FIG. 3).

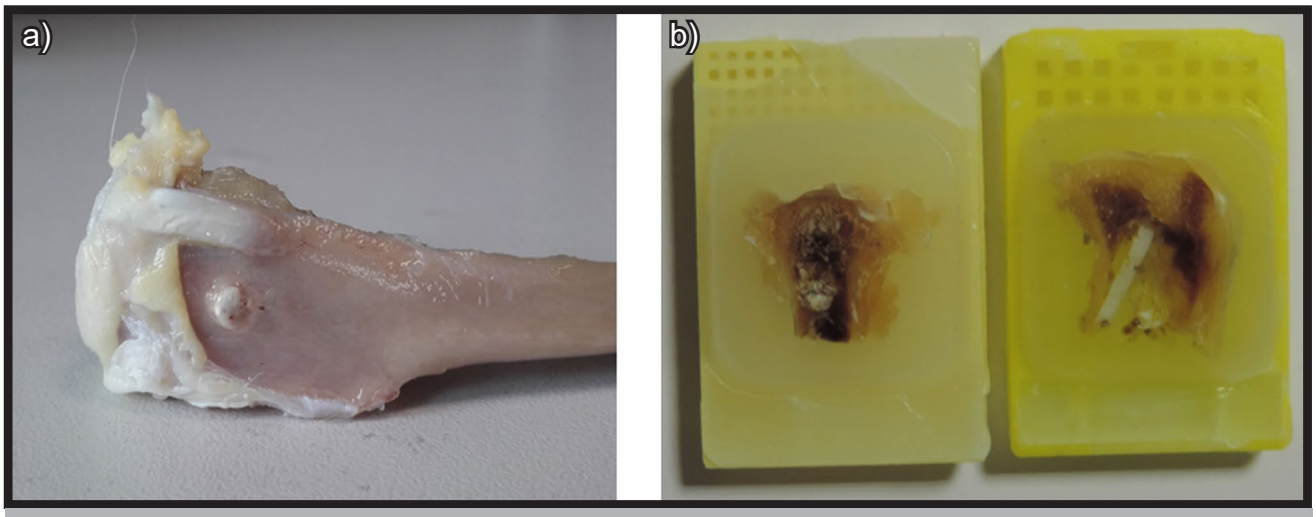

FIG. 2. a) The composite implant in the bone tissue – 12 weeks after surgery; b) Paraffin blocks containing bone tissue.

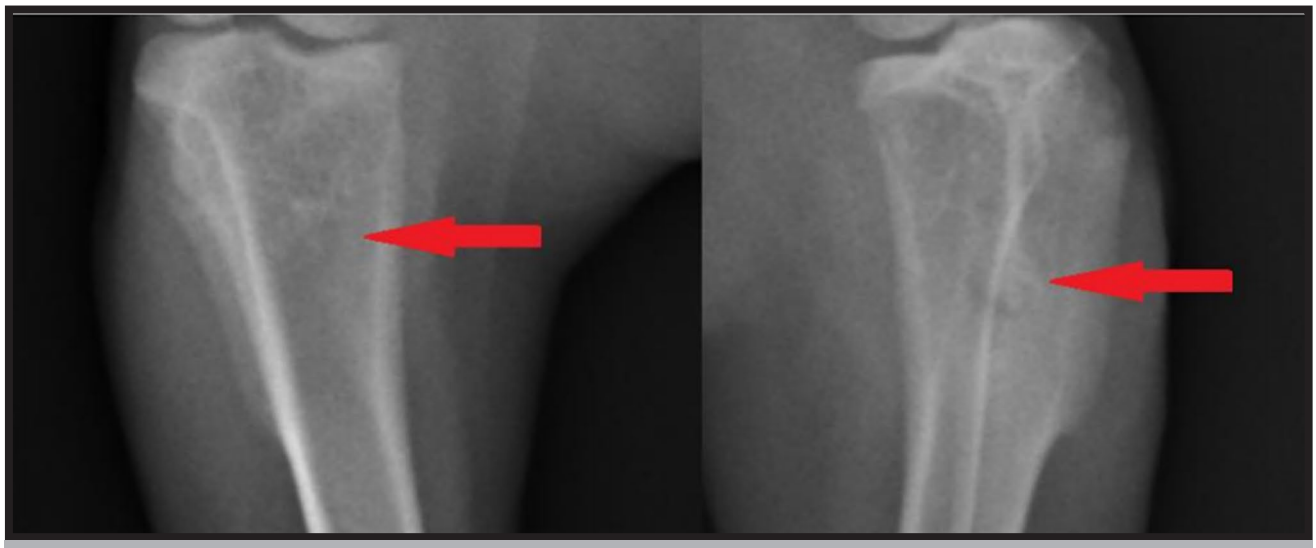

FIG. 3. Radiological images of bones with implants 12 weeks after implantation (implants are hardly visible due to their X-ray transparency and are marked with arrows).

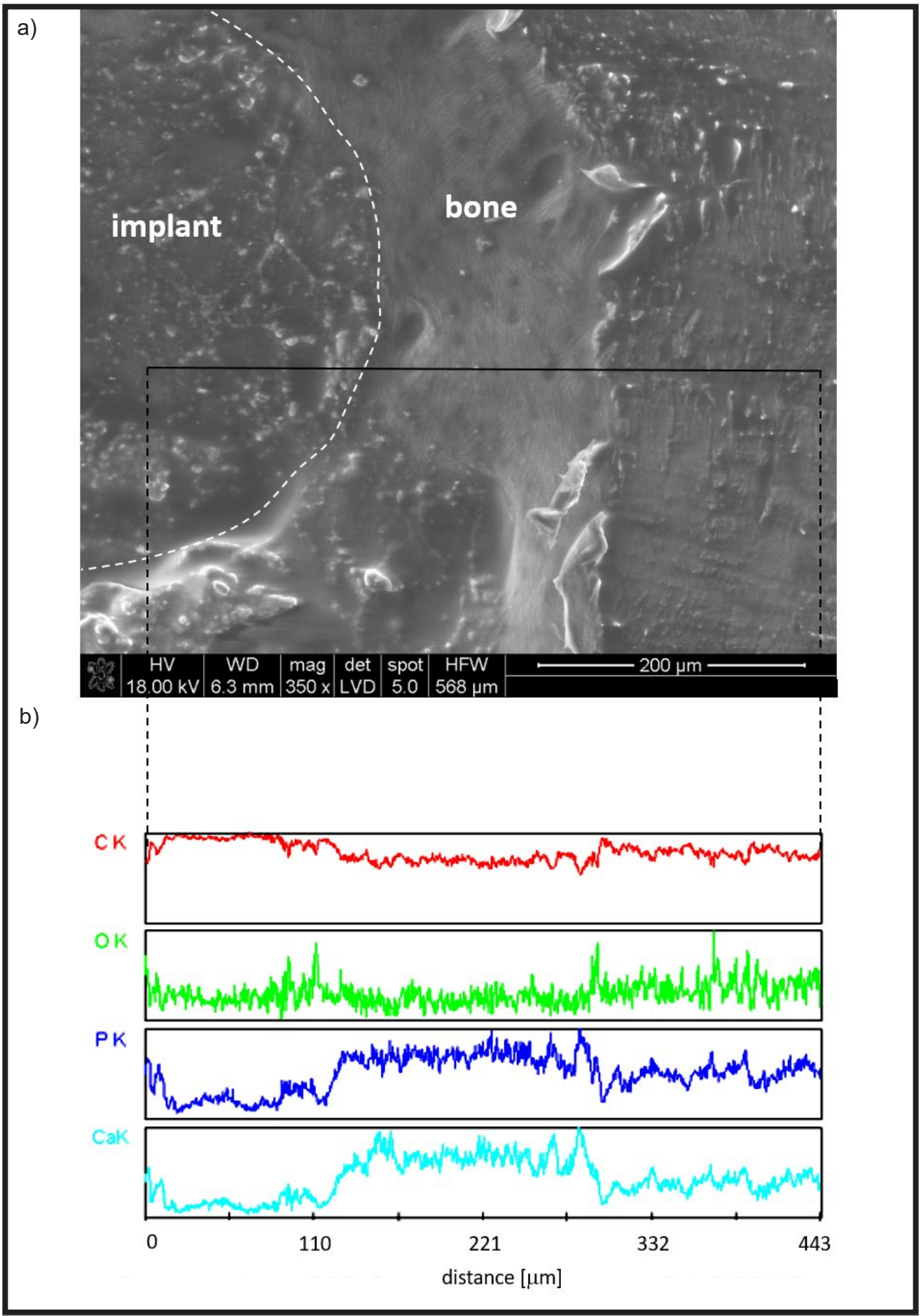

FIG. 4. a) SEM image of bone-implant section; b) EDS analysis of element composition (carbon, oxygen, phosphorus, calcium) along the line marked on the SEM image.

Microscopic observations of bone fractures (cross sections) performed 12 weeks after implantation confirmed the efficient connection of the implant with the bone (FIG. 4a). The absence of a fibrous envelope indicates the biocompatibility of the implant. The EDS analysis of the element composition along the line running from the implant to the bone (FIG. 4b) reveals the presence of calcium and phosphorus - mainly in the bone tissue, but also within the implant. At the same time, there is no noticeable change in the composition at the bone-implant connection site.

Histopathological evaluation was based on the tissue reaction around the examined material. In FIG. 5 the implanted material is visible (the spot marked with two circles), and FIGs 6 and 7 present the tissues directly adjacent to the implant. Several types of tissue changes were observed, none of them of undesirable character. In the vicinity of the implant, fibrous tissue was observed (FIG. 5) along with the new bone structure and increased osteogenic activity of neighboring bone trabeculas. A focal nonspecific granulomatous reaction of the "foreign body" type with visible multinucleated giant cells (FIG. 6) was not revealed in the immediate vicinity of the implant. None of the fields analyzed showed either an acute inflammatory response (without granulocytes) or a chronic inflammatory response (without lymphocytic infiltration). The samples did not reveal signs of necrosis, haemorrhage or other forms of tissue damage.

Discussion

The medical application of BMPs has been the subject of scientific works since the 1970s, raising both high hopes and various fears. Many authors indicate that morphogenetic proteins play an important role in bone tissue repair processes. An increase in the concentration of these proteins is observed in patients suffering from bone fractures [17]. *In vivo* studies prove the beneficial effect of proteins on the reconstruction of collagen, especially in cases of earlier estrogen deficiency and coexisting osteoporosis [18]. This is particularly important when treating patients with the pathologically changed bone tissue. At the same time, the presence of bio-based components in implants may raise recipients' concerns. Some patients may object to the application of human bone from the tissue bank, either for ethical reasons, the risk of disease transmission, or an unwanted biological response.

Bearing in mind all these considerations, in order to improve the biological response of bone tissue, especially in patients with reduced regenerative abilities, it seems reasonable to investigate the use of BMPs of natural origin (from native bone) as modifying factors of polymeric matrix, for example PLA.

An additional challenge is the fabrication of implants made of such composites, which would possess efficient biomechanical properties and could be formed into complex three-dimensional shapes (e.g. screws, nails, plates, or pins). On the one hand, there are forming methods such as extrusion or injection molding which would be appropriate for this task, but on the other hand, the temperatures applied in these processes (160-240°C) exclude incorporation of BMPs because of their low thermal resistance (up to 40°C).

As shown in this article, it is possible to prepare a hybrid composite material of PLA matrix, modified with embedded bone chips containing BMPs, which maintain their bioactivity. The authors adopted a tape casting method to form thin foils of composite material, and by piling them layer-on-layer, and pressing together, bulk cylindrical samples were made. It is presumed that bulk composite (green body) can be formed into screws or fixation plates by the machining process, but so far nothing can be said about the mechanical behavior and properties of such implants.

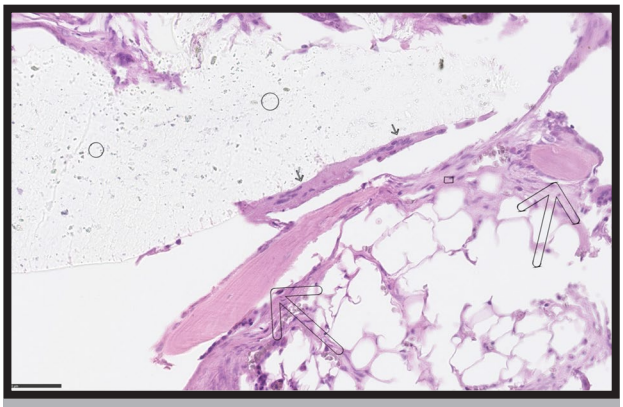

FIG. 5. Foci of bone formation, fibrosis and granulomatous reaction in the vicinity of the examined material (BM) - the implant marked with the circles (transparent area), the giant polynuclear cells with small arrows, the bone formation areas with large arrows.

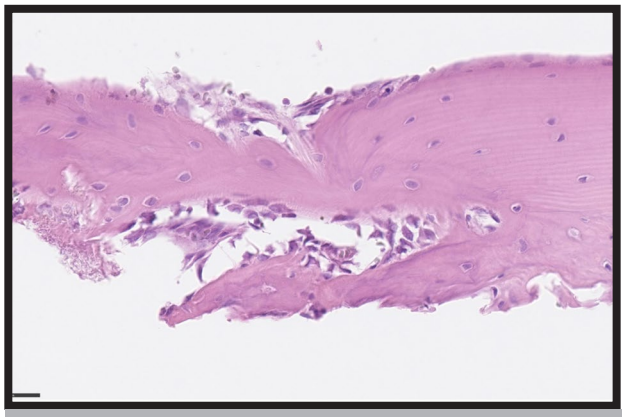

FIG. 6. Increased osteogenic activity in the bone trabeculas (on the edge of the osteoblast and newly-formed bone).

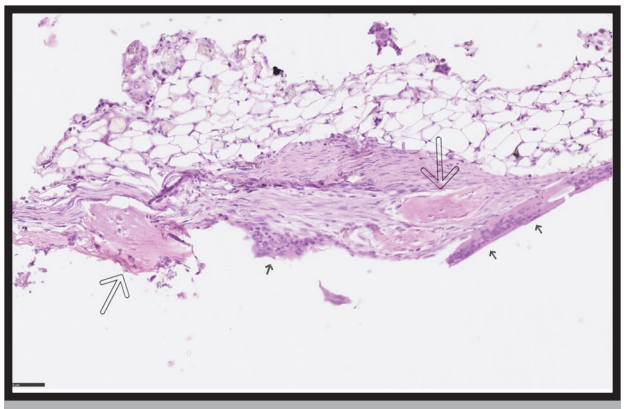

FIG. 7. Tissue in the immediate vicinity of the implant with foci of bone formation (large arrows), fibrosis and granulomatous reaction with the presence of cells around the foreign body (small arrows).

TABLE 1. Selected features of various materials [4,20-23].

Material	Osteoinduction	Bioactivity	Malleability	Formability
Cortical bone Cancellous bone	✓	*Biological* *Chemical*	x	x
BMP	✓	*Biological* *Chemical*	x	x
HAp TCP	✓	*Chemical*	x	x
PLA	x	*x*	✓	✓
PLA – CP (HAp, TCP)	✓	*Chemical*	✓	✓
PLA – CP/BMPs	✓	*Biological* *Chemical*	✓	x

The biological aspects of the obtained composite are considered by comparison with bone and other materials widely used for medical applications. TABLE 1 presents the characteristics of bone, BMPs, HAP and TCP, polylactide and composites with a polylactide matrix modified with HAP (PLA-CP/HAP), a polylactide matrix modified with TCP (PLA-CP/TCP), and a polylactide matrix modified with bone chips with osteoinductive supplements and BMPs (PLA-CP/BMPs).

When implanted, calcium phosphates (CP) like HAP and TCP (powder, granules, porous or bulk), reveal osteoconductivty. Because of their chemical similarity to bone tissue, they are very good bone-substitute materials. However, they are ceramics, and thus, their formability and malleability are limited. When HAP or/and TCP are introduced into the polymer matrix (PLA-CP/HAP, PLA-CP/TCP), the as-obtained composite can be easily formed and shaped by extruding or injection moulding. Such implants have rather sufficient mechanical properties, but their application is limited to non-load-bearing implantation sites [19]. They pose osteoconductivity with a controlled degradation rate of the polymer matrix. Judging by the comparison presented in TABLE 1, the best material among the listed seems to be the composite PLA-CP/BMPs. It is supposed to be chemically and, more importantly, biologically active. Having morphogenetic proteins inserted in the polymeric matrix, this composite inducts regeneration of natural tissue on various levels. That is why it is better than the other materials complied in TABLE 1.

The biological behavior of the prepared composite samples was tested in *in vivo* test. No empty spaces between the implanted material and the bone tissue were observed, and no pathological macroscopic changes, either in the soft tissue surrounding the implantation site, or in the popliteal lymph nodes were discovered. The biocompatibility of the implant was confirmed by the absence of a fibrous envelope and a complete filling of the bone defect by the newly formed bone tissue. The EDS analysis showed the presence of calcium and phosphorus within the implant what indicates the bone cell infiltration into the material structure. Because no unwanted reactions or side effects occurred, the authors claim that the multi-stage long-time drying applied after the tape casting preparation is an efficient method for removing all residuals of toxic solvent from the implant.

Conclusions

1. Bulk composite materials made of the resorbable polymer (medical poly-L-lactide, Purasorb PL38 by Purac), modified with 15 wt% human bone chips containing BMPs (from the tissue bank) were successfully obtained by tape casting, vacuum drying, foils piling, and pressing.
2. Composite PLA-CP/BMPs is modified with native ground bone, thus natural calcium phosphates and morphogenetic proteins are present. Combination of these factors gives the composite a high biological activity.
3. *In vivo* studies proved that the implants enriched with active morphogenetic factor (BMPs) derived from natural bone tissue possess high biocompatibility and stimulate good osteointegration.
4. Further research should focus on the fabrication of complex-shaped implants (nails, screws, plates) by machining of bulk pieces of PLA-BMPs composite, and their mechanical characterization. Such implants may be a promising alternative to traditional metal implants.

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