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Behavior of new hydroxyapatite/glucan composite in human serum

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Received 30 January 2017; revised 28 December 2017; accepted 20 January 2018
Published online 00 Month 2018 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jbm.b.34082

Abstract: Biomaterials for bone tissue regeneration, including polymer-based composites, are typically evaluated *in vitro* prior to the clinical trials. However, such composites tested *in vivo* may behave different due to the specific body conditions. For example, some composites implanted into the tissue acidified due to transient postoperative inflammation may unexpectedly swell which delays the wound healing. Such massive swelling in acidic medium was previously observed for new elastic hydroxyapatite (HAp)/β-glucan biomaterial. However, in further clinical cases concerning the composite implantation in patients without significant inflammation indicators, no side effects were observed. Therefore, it was reasonable to test the effect of human serum of neutral pH (typical for noninflamed tissues) on the composite parameters, in particular volume changes. Thus, this article shows the characterization of physicochemical parameters of the composite after incubation (5 days) in human serum of neutral pH by means of weight and volume measurement, scanning electron microscopy, X-ray diffraction, Fourier-transform infrared spectroscopy, microcomputed tomography, mercury intrusion, and biochemical techniques. Results showed that human serum collected from healthy people caused no uncontrolled changes in weight and volume, porosity and mechanical properties of the composite. Therefore, this suggests the lack of volume change-related side effects of HAp/glucan composite in bone defects treatment if postoperative inflammation is prevented. © 2018 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 00B: 000–000, 2018.

Key Words: bone substitutes, polymer-based composites, human serum, neutral pH, volume change

How to cite this article: Borkowski L, Lübek T, Jojczuk M, Nogalski A, Belcarz A, Palka K, Hajnos Mł, Ginalska G. 2018. Behavior of new hydroxyapatite/glucan composite in human serum. J Biomed Mater Res Part B 2015:00B:000–000.

INTRODUCTION

In recent years, an increasing attention has been paid to alternatives for bone auto- and allografts, including artificial polymer-ceramics composites. Polymers, acting as elasticity-increasing compounds in these constructs, are synthetic or natural in their nature and include polycaprolactone,[1] polylactic acid[2] and polylactic-glycolic acid,[3] gelatin,[4,5] collagen,[6,7] fibrin,[8,9] hyaluronic acid,[10] chitosan,[11] starch,[12,13] and curdlan.[14] Many polymers (e.g., chitosan, starch, hyaluronic acid, and curdlan) exhibit significant water-absorbing capacity[14–16] which is crucial for the circulation of oxygen, nutrients, cytotoxic degradation products, and metabolic wastes in polymer-based scaffolds and surrounding tissues. These phenomena are important for osteochondral and cartilage defect regeneration; thus, biomaterials based on water-absorbing polymers seem to be beneficial for reconstruction of tissue defects.

Water absorption by polymer-based biomaterials is often accompanied by their swelling (volume increase). This may be responsible for large deformation of biomaterials, failure of their mechanical properties, change of porosity, roughness, and surface geometry. These changes may affect the bone healing dynamics because cells attachment, orientation, and function strongly depend on surface topography.[17] Besides, swelling biomaterials may generate the excessive long-lasting pressure inside the implantation site, thus negatively affecting the processes of bone formation. Most probably this phenomenon is similar to that described as "compartment syndrome." Compartment syndrome is a well-known term used in trauma surgery, where increased pressure in a closed muscle compartment leads to disturbances in circulation and tissue function, resulting in hypoxia. This syndrome can be caused by the reduction of a given compartment (by surgical closure of fascia), or volumetric increase of the content of fascial compartment. This leads to the reduction of arterial blood supply to the compartment, release of vasoactive substances, increase of capillary permeability and impaired venous return. All the

Additional Supporting Information may be found in the online version of this article.
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Contract grant sponsor: Medical University in Lublin, Poland; contract grant number: DS2 grant
Contract grant sponsor: Operational Program Development of Eastern Poland 2007–2013, Priority Axis I: Modern Economy, Operations 1.3. Innovations Promotion; contract grant number: POPW.01.03.00–06-0109–00

factors mentioned above eventually lead to pressure increase in the compartment, ischemia, and consequent tissue necrosis.[18]

Appearance of tissue necrosis was also reported for periodontal ligaments subjected to occlusal forces. When pressure in periodontal ligaments is too great, the collagen fibers are compressed and become necrotic.[19] Rygh and Brudvik, in their study on histologic and histochemical reactions in periodontal ligament after the application of orthodontic forces, observed that beyond the certain level of stress, the vascular supply in this area decreases and dead cells appear between the stressed fibers.[20] Increased pressure in the localized region of periodontal ligament inhibited the differentiation of osteoclasts and induced a series of degenerative tissue reactions (hyalinization).[21]

We recently described the results of medical experiment concerning a novel elastic hydroxyapatite (HAp)/glucan composite which was implanted to alveolus extraction socket as a biomaterial for bone defects repair.[22] Clinical observations confirmed the appearance of significant composite swelling, assisted by stitches loosening and inflammation in the implantation site, within 5 days after the surgery. Inflammation causes the acidification of liquids in altered tissue; thus, the composite behavior in acidic medium was tested in 5 days long *in vitro* experiments. According to the results, acidic medium caused the significant composite remodeling and continuous massive swelling during the observation period.[22]

However, the results of another clinical case study concerning the composite implantation into long bone defects in five human patients were different. Within the observation period we observed high mineralization, hierarchical organization and radiological bony bonding features within the implant; moreover, lack of significant inflammation was stated (based on clinical observations and C-reactive protein [CRP] level in patients' serum). We suspected that such a positive bone healing process appeared due to the lack of local acidification of tissue liquids and minimization of composite remodeling and swelling. Of course, the examination of local pH of tissue liquids in the implantation site and evaluation of composite behavior *in vivo* was impossible, for medical reasons (to minimize the risk of perioperative complications). Therefore, we examined the behavior of HAp/glucan composite in blood serum collected from healthy people (of neutral pH) *in vitro,* including the evaluation of changes in composite volume and weight, mechanical parameters, porosity and calcium and phosphate ions absorption. This article summarizes all results obtained for performed clinical cases and subsequent *in vitro* study and tries to discuss the potential of HAp/glucan composite as biomaterial for bone defects repair in trauma cases.

MATERIALS AND METHODS

Preparation of samples (HAp/glucan composite and glucan)

HAp granules for the preparation of composite (porosity: 67%) were synthesized in laboratories of Faculty of Materials Science and Ceramics (AGH University of Science and Technology in Cracow, Poland) according to the procedure described in Ref. 23 for INNOAGH Ltd. (Cracow, Poland) and bought by Medical Inventi Inc. company (Lublin, Poland). β-1,3-glucan (curdlan) from *Alcaligenes faecalis* (DP 450) was supplied by Wako Chemicals (Japan).

Composite samples were synthesized as previously described[14,24] with a permission of Medical Inventi Ltd. (owner of intellectual property for HAp/glucan composite). Briefly, HAp granules (mixture of two fractions: 0.2–0.3 and 0.4–0.6 mm in the weight ratio of 25:75) were combined with aqueous suspension of β-1,3-glucan (dry weight proportion: 83 wt % granules and 17 wt % β-1,3-glucan). The resulting mixture was baked (90°C, 15 min) in special cylinder-shaped molds, cut into samples (ø 13, 15 mm length or ø 5, 10 mm length,), dried (48 h, 25°C) and finally sterilized in plastic/article peel pouch (ethylene oxide method, 1 h at 55°C, 20 h of aeration). Pure glucan samples of the same dimensions, prepared in the similar manner as the composites but without ceramic granules, were used as controls in swelling tests.

Overall, the following group of samples were prepared:

Group 1: HAp/glucan composite ø 5 mm ($n = 10$)
Group 2: HAp/glucan composite ø 13 mm ($n = 10$)
Group 3: Glucan ø 5 mm ($n = 10$)
Group 4: Glucan ø 13 mm ($n = 10$).

For *in vivo* experiments (implantation in patients), HAp/glucan composite in a form of cylinders (ø 13 or 5 mm; length individually selected for each patient) were prepared.

Clinical cases—Surgical procedure and evaluation of healing results

After obtaining the approval of the local Bioethics Committee (KE-0254/248/2011), HAp/glucan composite was used in patients with post-traumatic deficits of bone tissue in the site of the long bone fracture. The criterion of site selection was the defect size, exceeded the possibility to be filled with autogenous bone grafts. Five patients were selected for the treatment with HAp/glucan composite: two 59-year-old males, 25-year-old male, 60-year-old male, and 54-year-old female.

The patients were anesthetized by administration of 2–3 mL of 0.5% marcaine (for fractures of lower limb) or 40 mL of mixture of 0.1% bupivacaine and 2% lignocaine in 50/50 proportion (for fractures of upper limbs) to the brachial plexus. In the initial phase of the surgery, after skin disinfection with Skinsept Color, Ecolab (Germany; mixture of ethanol, isopropyl alcohol, benzyl alcohol, hydrogen peroxide, and purified water), access to the missing bone fragment was obtained. The operating field was rinsed with Octenisept, Schülke, and Mayr (Germany; mixture of octenidine dihydrochloride, phenoxyethanol, cocamidopropyl betaine 30% solution, sodium D-gluconate, 85% glycerol, sodium hydroxide, sodium chloride, purified water, hydrogen peroxide, and saline). Sequestra were removed, fracture ends

were renewed and size of the gap was assessed. A careful hemostasis was performed and appropriate sizes of HAp/glucan composite in a form of cylinders (ø 13 or 5 mm; length individually selected for each patient) were chosen. The composite samples were soaked for approximately 20–30 min with saline or blood collected from the operating field and cut longitudinally. After soaking, the composite samples obtained greater plasticity and were easier to form which allowed to accurately fill the bone gap and to adapt to the dimension of bone defect. Depending on the size of the gap, stabilization was done using metal implants because the composite alone does not provide security and physical scaffolding that would eliminate displacement of the bone fragments from the fracture site. After careful filling of all the gaps with HAp/glucan composite, the site of implantation was covered with muscles and fascia. Subcutaneous tissue and skin were sutured. Absorbable surgical polyglycolic acid threads (PGA multifilament) 2.0 or 3.0 for muscle and fascia suturing and nonabsorbable Dafilon monofilament thread for skin suturing were used. No drainage was used to avoid loss of any of the bone substitute material when removing the drain. The healing process of the fracture site after implantation was radiologically monitored using Siemens Multix Pro X-ray device produced in 1999 (as well as by physical examination immediately after the surgery and then in time intervals selected individually for each patient). At the same time intervals, the analysis of CRP level in patients' blood was performed (using Roche Diagnostic Cobas 6000 device).

In vitro soaking in human serum, weight and volume measurements, ions concentration evaluation

Five samples from each group were placed individually in wells of sterile culture plate and soaked in human serum of neutral pH (7.4; 4 mL per well with ø 5 mm sample and 10 mL per well with ø 13 mm sample) collected from healthy persons. Incubation was carried out for 5 days at 37°C to simulate the temperature of human body. Another five samples from each group were preincubated in Ringer solution for 30 min, and then incubated in human serum in the similar manner. Serum was replaced every 24 h with a fresh portion of the liquid during 5 days of experiment. In each experimental step, human serum was supplemented with 1% antibiotic/antimycotic solution (Sigma-Aldrich®) to prevent bacterial or fungal growth.

All samples were weigh on an analytical balance with accuracy 0.0000 g (XS205, Mettler-Toledo, Switzerland) before and during incubation in serum at defined time points (after 1, 3, 10 min and 1, 2, 24, 48, 72, 96, 120 h). Excess of the serum was removed before weight measurement using Whatman article. To measure the volume of the samples at the same time points, Archimedes method was applied. However, one should note that this method did not allow to define the volume of samples before the incubation (in time point "0"). Prior to the statistical analysis, values of sample weight and volume were normalized (100% = maximum weight or volume of each sample during five days of experiment). Obtained results were expressed at the graphs as mean values ± standard deviation (SD).

After the experiment, all samples were washed several times in deionized water and dried at 37°C until constant weight, to calculate the change of dry weight before and after the soaking experiment. The same samples were then subjected to evaluation of physicochemical parameters using different techniques (microcomputed tomography [microCT], X-ray diffraction [XRD], Fourier-transform infrared spectroscopy [FTIR], macroscopic observations, scanning electron microscopy [SEM], mercury porosimetry, and mechanical testing).

In parallel, ion reactivity of the composites was determined by analysis of the Ca^{2+}, Mg^{2+}, and PO_4^{3-} concentrations in incubation serum every 24 h for five days. Ions concentration in the serum was measured spectrophotometrically using commercial diagnostic reagents: Calcium CPC, Magnesium and Phosphorus (Biomaxima, Inc., Poland), respectively. The pH of the incubation solution was controlled every 24 h with calibrated pH meter (CPO-551, Elmetron Ltd., Poland).

MicroCT evaluation

The microstructure evaluation of tested wet composite samples was performed by X-ray computed tomography (using Skyscan 1172, Bruker microCT, Belgium). After scanning the reconstruction process was performed, whereby a set of cross sections was obtained with the resolution of 6 μm (for samples ø 5 mm) and 12 μm (for samples ø 13 mm) in each axis. The region of interest used in analysis was set on entire volume of the specimens. The purpose of the microCT analysis was to assess the microstructure of tested materials and the recognition of changes occurred during incubation.

XRD and FTIR analysis

Samples for XRD and FTIR analysis were ground and dried at 115°C for 20 h to remove traces of adsorbed water prior to the analysis.

X-ray diffraction data were acquired using the diffractometer type HZG-4 (Carl Zeiss Jena, Germany) equipped with Cu-anode conventional X-ray tube (operated at 40 kV and 20 mA) with Ni filter and scintillation detector in a Bragg-Brentano geometry. Diffraction data were collected by step counting in the range $2\Theta = 20 - 50°$ at 0.01° intervals for 2 s per data point. Identification of phases contained in tested materials before, and created after incubation, based on diffraction patterns were done using the PDF-4 database International Centre for Diffraction Data (ICDD).

The FTIR-attenuated total reflectance (ATR) spectra were taken in Vertex 70 spectrometer equipped with diamond crystal (Bruker), 64 scans, resolution 4 cm^{-1} and analyzed by OPUS 7.0 software (Bruker).

Macroscopic and SEM evaluation

Macroscopic observations were performed using SMZ1500 Nikon stereoscopic microscope equipped with digital camera (Nikon Instruments Inc.). SEM images were obtained using

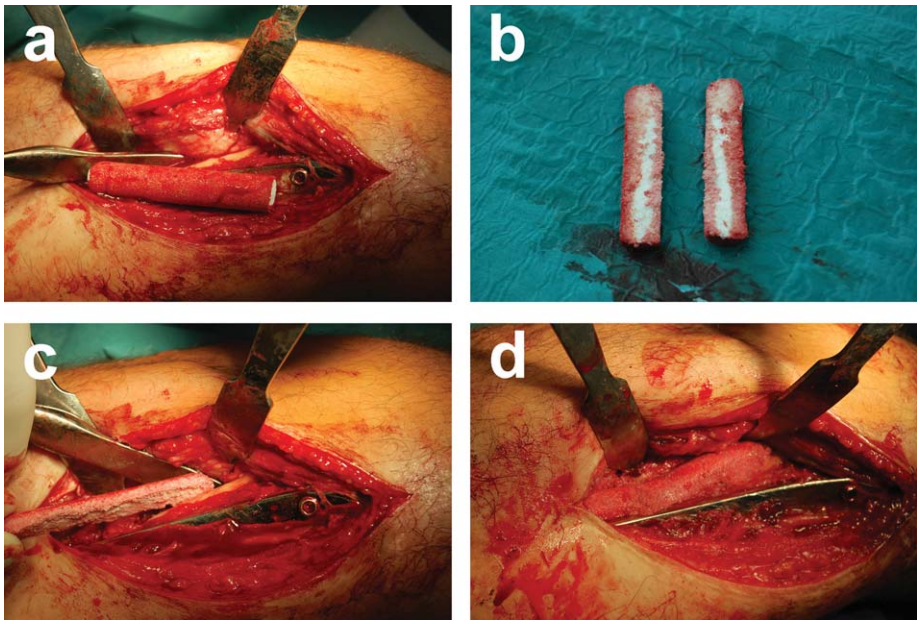

FIGURE 1. Procedure of HAp/glucan composite implantation. (a) Intraoperative photograph showing the defect area in the femur and the ø 13 mm composite partially soaked in blood. (b) Longitudinal section of the composite moistened with patient's blood. (c) Insertion of the implant into bone defect. (d) Composite material in final position before skin suturing.

Nova NanoSEM 450 (FEI) in low and high vacuum conditions.

Pore size distribution determination

Pore size distribution (PSD) in the composites was evaluated according to the ISO 15901–1:2005 standard[25] using Autopore IV 9500 (Micrometrics Inc.) mercury porosimeter. Prior to the experiment, samples were dried (105°C) and degassed in vacuum (6.67 Pa, 20°C). Mercury intrusion was performed applying a stepwise pressure increments in the range between 0.0036 and 413 MPa. PSD, as a function of pore radius, was determined based on the Washburn equation:

$$P = \frac{2\gamma_{Hg}\cos\theta}{r}$$

where P is the external pressure (Pa) applied in the vacuum chamber; γ_{Hg} is the surface tension of mercury (0.485 J/m^2); Θ is the contact angle of mercury (140°); and r is the pore radius of pore aperture (m) for a cylindrical pore.

This approach allows the determination of pore radii ranging from 0.0018 to 206 μm. Average pore radius ($2V/A$) was calculated by assuming that all pores are cylindrical. Therefore, when the volume ($V = \pi r^2 L$) is divided by the pore area ($A = 2\pi rL$), the average pore radius (r) equals $2V/A$.

Mechanical behavior

The behavior of the composites during compression was assessed on wet samples (ø 13, 15 mm length). Zwick Roell Z2.5 testing machine was used to conduct compression testing with preload value of 1 N with crosshead moving speed 10 mm/min, followed by basic load rate of 0.5 mm/min. Load rate was lower than standardized for most of materials because of viscoelasticity nature of tested composites. The mechanical compression was carried out until 40% of strain was reached and the obtained data allowed the stress–strain characteristics.

Statistical analysis

Normality of data was tested using the Shapiro–Wilk test ($\alpha = 0.05$). Obtained values of sample weight and volume were compared using Two-way repeated-measures (RM) analysis of variance (ANOVA) ($\alpha = 0.05$) with Sidak's multiple comparisons *post hoc* test using GraphPad Prism (GraphPad Software Inc., version 6.01 for Windows, San Diego) to reveal significant differences between samples treated and nontreated with Ringer solution or between samples of different size. The first null hypothesis was no influence of Ringer treatment on composite mass or composite volume or glucan mass or glucan volume (eight independent analysis). The null hypothesis in second analysis was no influence of sample size on composite/glucan mass/volume (next eight independent analysis). Statistical significance was indicated as follows: * indicates $p < 0.05$; ** indicates $p < 0.01$, *** indicates $p < 0.001$, **** indicates $p < 0.0001$.

The compressive strength of samples was analyzed statistically to reveal significant differences between samples. Two variables (Ringer treatment and sample size) were tested using Two-way RM ANOVA ($\alpha = 0.05$) with Tukey's multiple comparisons *post hoc* test using GraphPad Prism. The null hypothesis was no influence of sample size or Ringer treatment on the compressive strength.

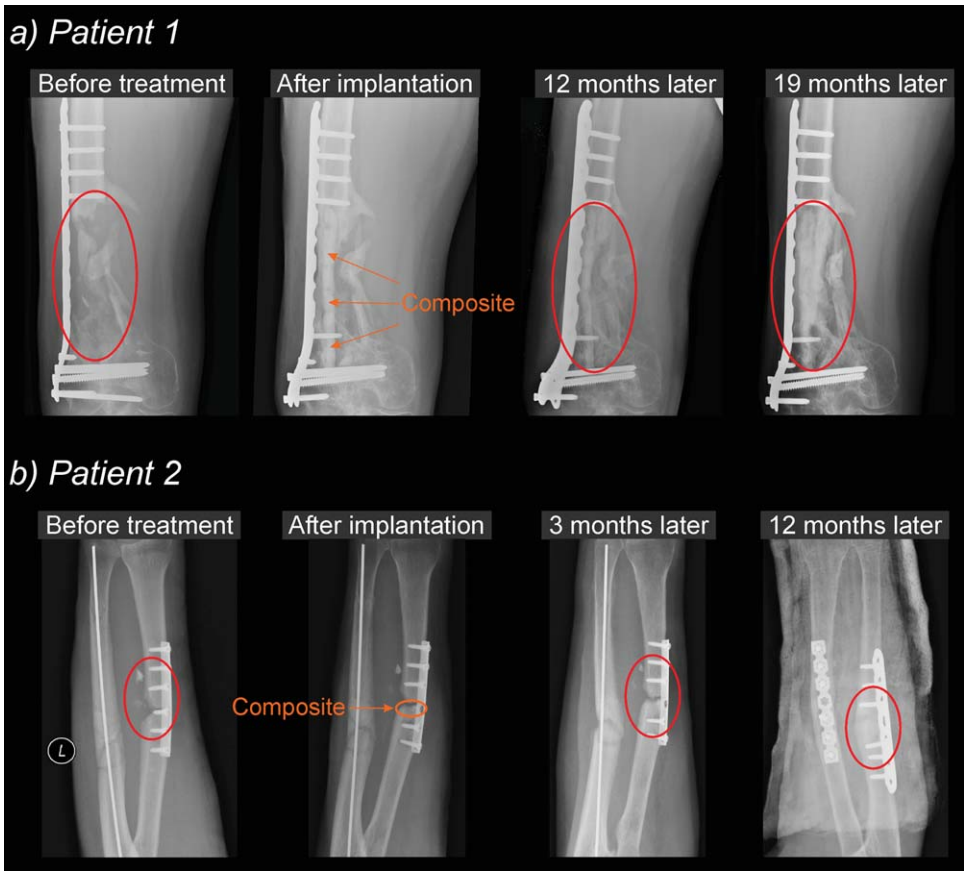

FIGURE 2. X-ray images of bone defects treated with HAp/glucan composite. (a) Patient 1, femur implanted with ø 13 mm composite. The AP projections taken before implantation, immediately after implantation, 12 and 19 months after implantation. (b) Patient 2, radius implanted with ø 5 mm composite. The AP projections taken before implantation, immediately after implantation, 3 and 12 months after implantation. Arrows indicate the position of composite insertion.

RESULTS

Exemplary procedure of HAp/glucan composite implantation, including partial soaking of sterile composite cylinder in patient's blood (directly within the wound created for the purpose of implantation), was presented in Figure 1. Clinical observation during perioperative period as well as control visits showed no signs of compartment syndrome (swelling of the limbs, pain, increased tension of soft tissues in the operated field). Moreover, patients themselves did not report any symptoms of this syndrome. CRP level in the patients' blood, collected during the control visits, remained within the range 1.5–10.3 mg/L within the postoperative period (normal range: 0.08–5 mg/L). Figure 2 presents the results of X-ray examination of two selected patients subjected to the implantation procedure. Thick and long composite cylinder (ø 13, 70 mm length) was implanted in patient's femur (patient 1). Twelve months after the surgery, significant mineralization of the implant, penetration of newly formed osseous tissue into the three-dimensional structure of composite and bone tissue rearrangement appeared. Nineteen months after the surgery, high mineralization, hierarchical organization, and complete regeneration of bone tissue in the implant site was observed [Figure 2(a)]. In patient 2, smaller composite piece (ø 5, 15 mm length) was implanted into the tibial bone defect. Three months after the surgery, slight mineralization of implanted composite and radiological bony bonding features was observed. Twelve months after the surgery, high radiopacity and complete synostosis of bone tissue in the implant site was noticed [Figure 2(b)]. Thus, composite-assisted bone regeneration monitored in presented clinical cases preceded without significant complications and was not correlated with the appearance of inflammation (as suggested by CRP levels).

In clinical cases described above, no signs of inflammation were observed. We hypothesized that the healing of bone long defects without side effects proceeded due, among others, to the lack of local acidification of tissue liquids which usually appears in inflamed tissue. To verify this hypothesis, we examined the behavior of HAp/glucan composite in blood serum collected from healthy people (of neutral pH) *in vitro*. We took into consideration that, prior to the operation, the surgeons soaked the composite cylinders in either blood collected from the operating field or in saline solution. Therefore, the *in vitro* experiments were also performed in two versions: incubation exclusively in human serum or preincubation in Ringer solution prior to human serum (as shown in Table I).

TABLE I. Samples Description

ø of Samples (mm)	Incubation Liquid		Sample Name
	Ringer Solution (30 min)	Human Serum (5 days)	
5	–	+	5 mm S
	+	+	5 mm S+R
13	-	+	13 mm S
	+	+	13 mm S+R

Relative weight and volume of composites presoaked in Ringer solution did not change significantly between the beginning and the end of soaking process. The changes varied slightly: 2.5–6% (relative weight) and 2–5% (relative volume) for small and big samples (ø 5 and 13 mm, respectively) [Figure 3(a,b)]. This suggests that presoaked composites, after implantation, are not likely to cause any increase of intraosseous pressure within the defect. In contrary,

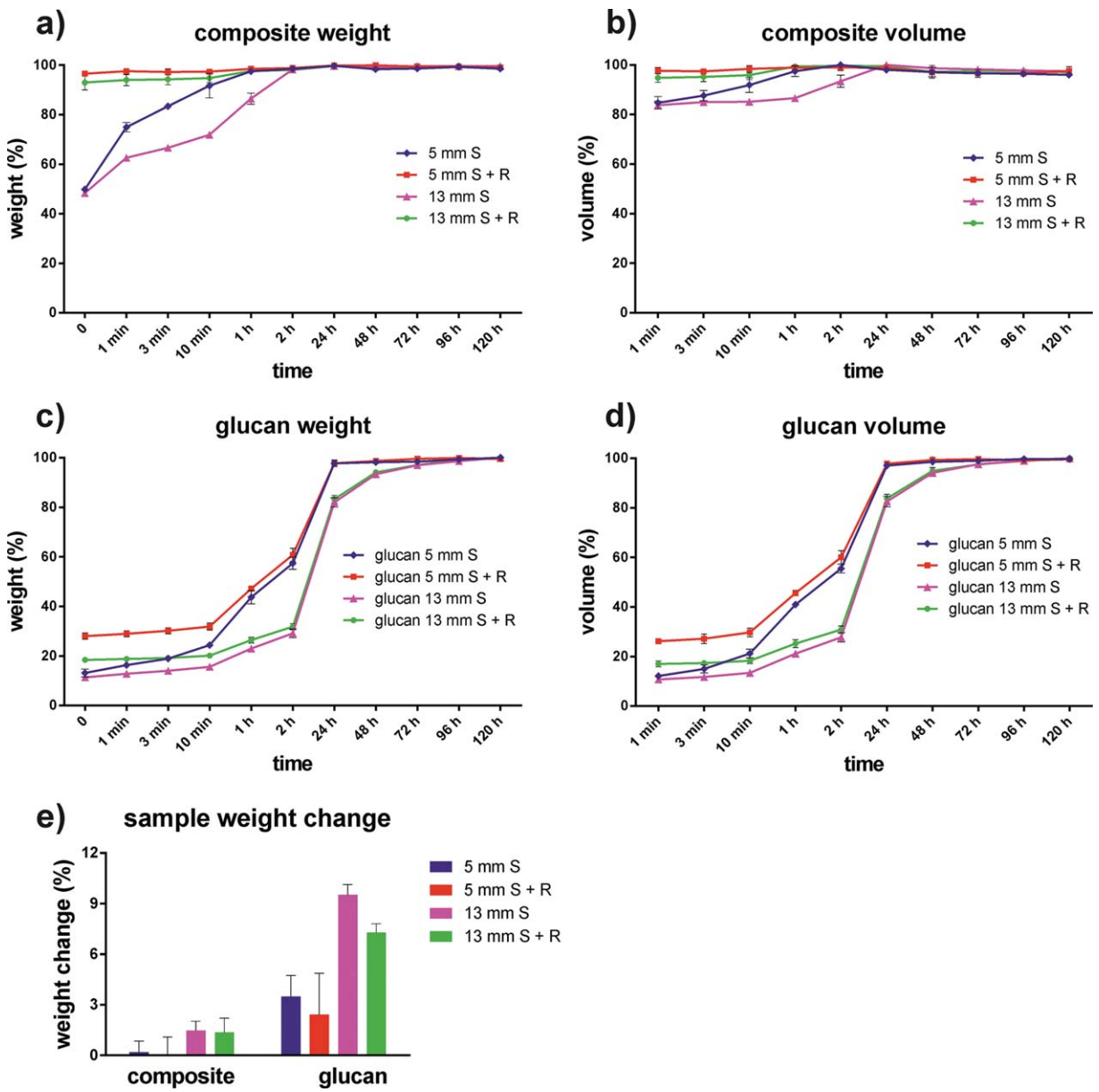

FIGURE 3. Relative weight (a,c) and relative volume (b,d) of HAp/glucan composite (a,b) and glucan (c,d) samples soaked either in serum (S) or in Ringer solution followed by serum (S + R) during 5 days long experiment; (e) percent change of dry composite and glucan weight before and after the experiment; (mean ± SD, $n = 5$).

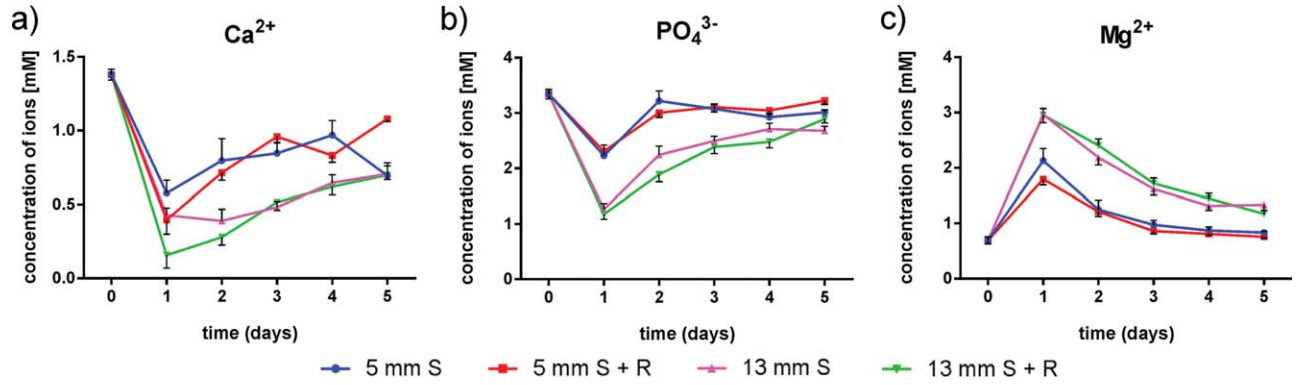

FIGURE 4. Profiles of Ca^{2+} (a), PO_4^{3-} (b), and Mg^{2+} (c) concentration in serum incubated with samples of composite with and without presoaking in Ringer solution ((mean ± SD, $n = 27$). Every 24 h, serum was collected for ions measurement and replaced by a fresh serum portion.

more significant changes in relative weight and volume of samples were observed for samples soaked exclusively in serum, without presoaking in Ringer solution. The changes varied within the following ranges: 50–52% (relative weight) and 15–16% (relative volume) for small and big samples (5 and 13 mm, respectively) [Figure 3(a,b)]. It was the most important that complete soaking of tested composite (to its maximum volume) was observed earlier than 24 h after the implantation (for ø 5 mm cylinders—even after 1 h) [Figure 3(b)]. Afterward, the volume of soaked material did not change. The soaking of composite samples, found in this experiment, was not straightly related to soaking of curdlan polymer. Curdlan samples reached their final wet weight and volume after 24 h (for ø 5 mm cylinders) or 96 h (for ø 13 mm cylinders) of soaking, increasing their volume 6–9 times [Figure 3(c,d)]. Calculation of the difference between dry polymer weight before and after soaking showed that glucan was able to bind significant amount (up to 10% content) of water [Figure 3(e)]. The same observation was made for dry composite samples but the content of bound water was much less (approximately 1.5%; for ø 13 mm cylinders) [Figure 3(e)]. Thus, the interaction of ceramic granules with polymer fibers probably played a role in soaking rate and capacity of HAp/curdlan composite.

According to the two-way RM ANOVA analysis, the Ringer treatment significantly influenced weight and volume in all groups (Supporting Information, Table S1). The Sidak's test revealed that the mass and volume of 5 mm composite was significantly affected by the Ringer solution treatment up to 10 min. In the case of 13 mm composite the mass was statistically different up to 1 h, while the volume up to 2 h. The mass and volume of glucan samples (both 5 and 13 mm) preincubated in Ringer solution were significantly higher up to 2 h. The second set of analyses regarded the influence of the sample size (diameter 5 mm vs. 13 mm) on physical parameters of samples (Supporting Information, Table S2). According to the two-way RM ANOVA, the mass and volume of composite and glucan were significantly different in all groups, except the volume of composite group treated with Ringer solutions followed by serum. The *post hoc* test revealed that the initial mass and volume of samples treated with serum only was similar, while in groups preincubated in Ringer solution they varied significantly. Volume of composite treated with serum only varied significantly between 10+ min and 2 h, while when treated with Ringer solutions followed by serum there was no significant difference after 3 min and later.

Samples of serum incubated with tested composites were evaluated for changes in Ca^{2+}, $PO_4{}^3$–, and Mg^{2+}. During the first exchange of serum, significant uptake of Ca^{2+} and $PO_4{}^3$– ions, assisted by huge release of Mg^{2+} ions, was observed [Figure 4 (a–c)]. In serum samples collected during further medium exchanges, the tendencies for ions uptake/release remained the same but with decreasing intensity. Presoaking in Ringer solution does not seem to exert any significant effect on this phenomenon both for PO_4^{3-} and Mg^{2+} ions—it is more evidently related to sample dimension. In turn, presoaking in Ringer solution increased the adsorption of Ca^{2+} ions but only during two initial serum exchanges [Figure 4(a)].

MicroCT images of composite cylinders after five days of incubation in experimental liquids revealed that preincubation in Ringer solution prior to the soaking in human serum did not cause any dimensional and structural changes in composite samples [Figure 5(a–d)]. Chemical structure and phase composition, as proved by FTIR and XRD techniques, neither changed due to the incubation in human serum (or Ringer solution followed by human serum) [Figure 6(a,b)]. FTIR spectra did not show the presence of bands characteristic for CO_3^{2-} groups, which should be expected around 1456, 1411, and 880 cm^{-1}. Moreover, FTIR spectra did not reveal the presence of serum proteins adsorbed to the composite, as showed by the absence of amide I and II bands [1650 and 1550 cm^{-1} Figure 6(a)]. Absorbance of serum proteins was expected, due to a high content of highly porous HAp in the composite, because HAp of high surface area adsorbs serum albumin easily.[26] However, in our study, not surface area of studied samples were evaluated for amide bands presence but entire ground composite sample; therefore, the quantity of adsorbed protein was likely to be below the detection limit. Similarly, no changes of composites surface structure and lack of apatite layer deposition

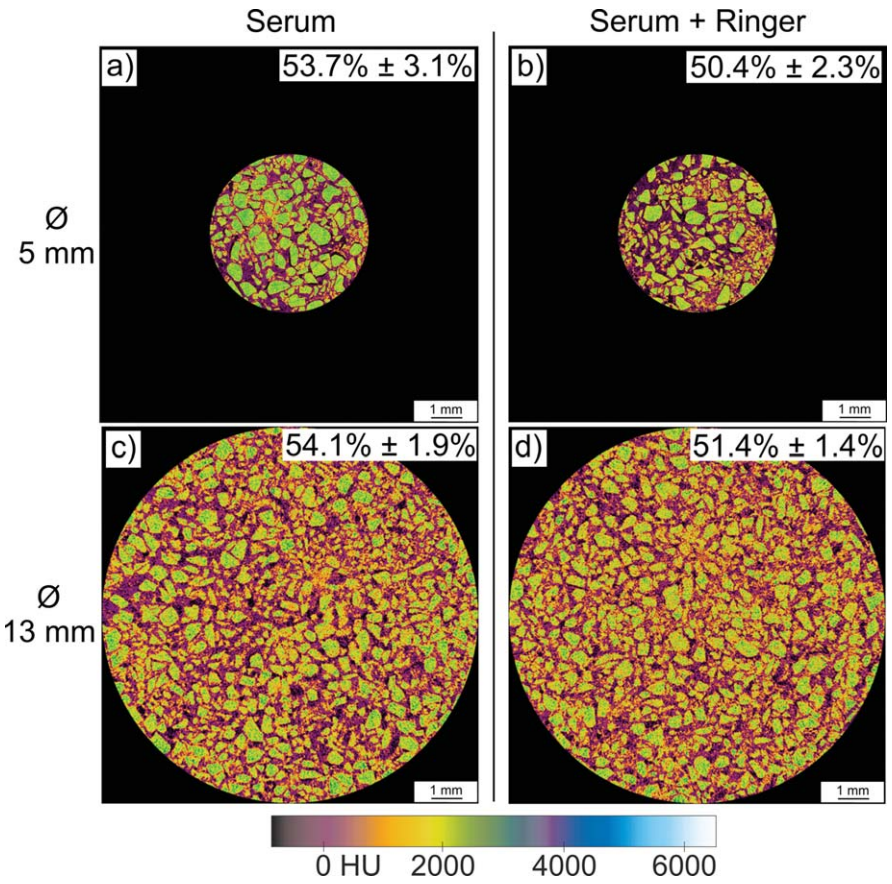

FIGURE 5. Influence of presoaking in Ringer solution on microstructure (microCT; a–d; color bar of Hounsfield units (HU) below the images) of samples incubated in serum or in Ringer solution followed by serum. Percentage of surface covered by ceramic granules was shown in insets in upper right corners. MicroCT images of samples of ø 5 mm were rescaled to keep the real size proportions to samples of ø 13 mm.

were detected by SEM technique (Figure 7). Evaluation of samples porosity revealed that presoaking in Ringer solution did not induce any noticeable changes in composite porous structure—only the amount of large pores (approximately 1 μm) and very small pores (approximately 0.03 μm) was slightly higher in ø 13 mm samples (Figure 8). This is in agreement with slightly smaller total pore area, density and porosity of ø 13 mm samples when compared with ø 5 mm ones (Table II). Profiles of compressive stress and mechanical stress values of all tested samples are also similar, in particular for ø 13 mm samples (Figure 9). Only for ø 5 mm samples, profiles of compressive strength are slightly different but differences are not particularly meaningful.

DISCUSSION

Hydrogel-based materials for medical applications may exhibit both beneficial and unfavorable properties. For example, due to their soft consistency, their implantation into bone defects is associated with lower risk of frictional irritation. On the other hand, hydrogel swelling in tissue liquids (of neutral or acidic pH) may increase the local strain within soft tissues, leading to a partial evacuation of biomaterial because of its soft consistency. Therefore, different phenomena including the effect of swelling of hydrogels and hydrogel-based composites must be taken into account in design of biomaterials for bone repair.

In this work, results of HAp/glucan composite implantation into defects in long bones were evaluated in clinical case report concerning five patients, during up to 19-month-long postoperative period. In this clinical case report, the repair of bone defects seemed to proceed without complications, even for big defects (7 cm in length). During the surgeries, little volume increase of implanted material was observed after soaking with blood but this phenomenon was obviously not dangerous for surrounding tissues and resulted in better fixation of implanted material. Clinical observations and CRP levels monitored after the operation suggested that the lack of significant inflammation during the postoperative period. These results were completely different than those reported earlier for the implantation of the same biomaterial to alveolus extraction socket.[22] Then, the failure of therapy was found to be correlated with significant biomaterial swelling and remodeling in acidic medium (which usually appears in inflamed tissue).[22] Therefore, in this work, the impact of neutral human serum (protein-rich medium) on behavior of HAp/glucan composite was studied *in vitro*. This experiment, moreover, considered the surgeons suggestions that biomaterial presoaking prior to the implantation (e.g., in patient's blood or in saline

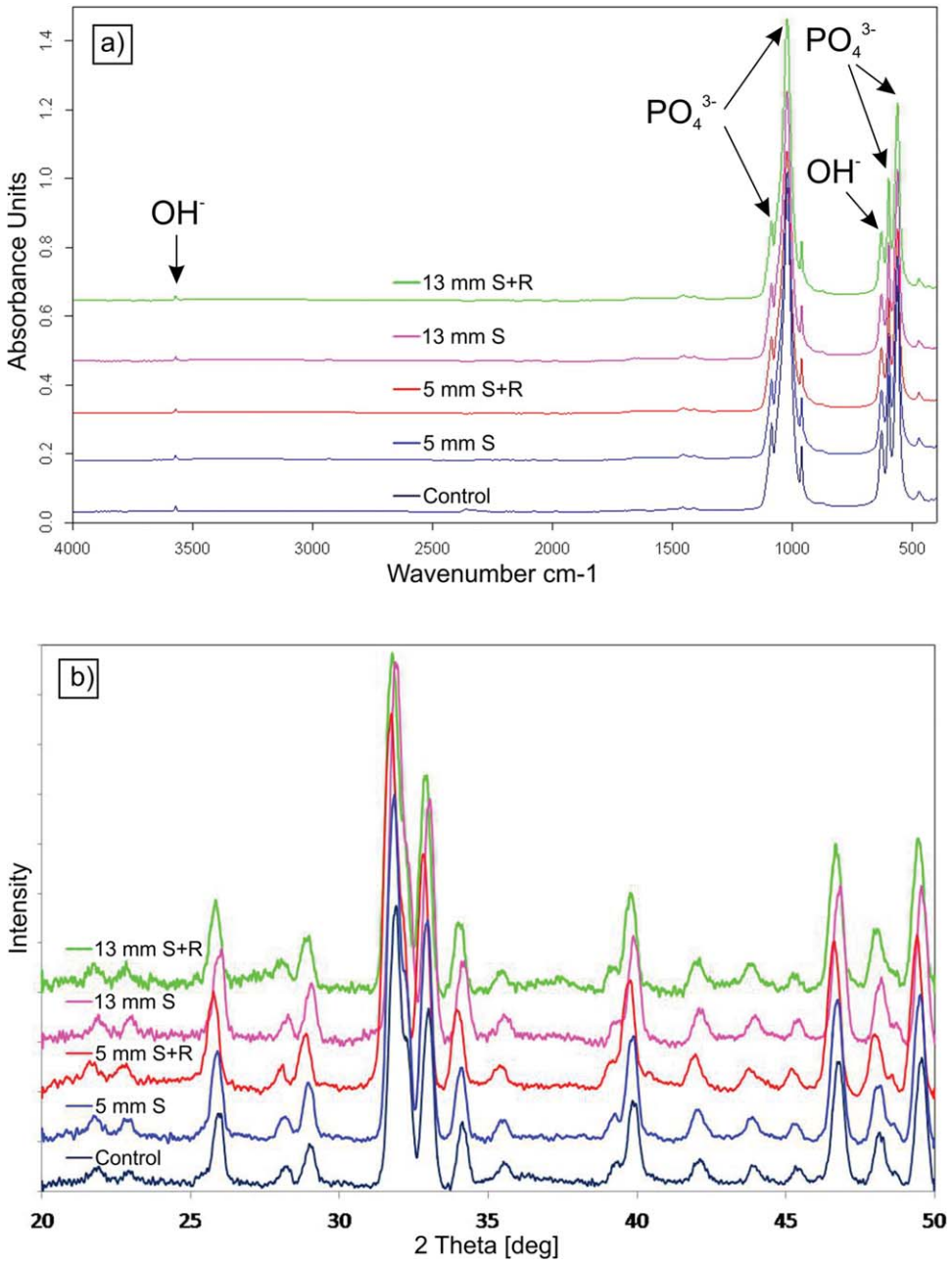

FIGURE 6. Chemical structure (FTIR; a) and phase composition (XRD; b) of samples incubated in serum or in Ringer solution followed by serum.

solution) is advised in some cases to enable its cutting and better fitting to the size of defect. Therefore, either dry or presoaked (in Ringer solution) composite samples were subjected to incubation in neutral human serum.

According to the statistical analysis, the first null hypothesis (no influence of Ringer treatment on the weight and the volume of tested samples) was rejected ($p < 0.05$). Moreover, the second null hypothesis (no influence of sample size on composite/glucan mass/volume) was rejected in most cases, except the volume of composite group treated with Ringer solutions followed by serum ($p > 0.05$).

Some conclusions may be withdrawn on a base of obtained results, confirmed by the statistical analysis. First, all significant changes in weight and volume of composite appeared during the first hours of incubation in tested liquids of neutral pH (up to 24 h); then the parameters remained unchanged. These observations were in agreement with our previous results of the composite soaking in protein-free buffer of neutral pH (7.4).[22] They differed, however, from the observations of composite incubated in acidic buffer: the composite swelled during first 24 h due to the water adsorption and then again between 3 and 5 day due to HAp dissolution/precipitation and composite remodeling in acidic medium.[22] Therefore, *in vitro* behavior of tested composite in media of neutral pH may suggest that *in vivo* in noninflamed tissue the material may probably react

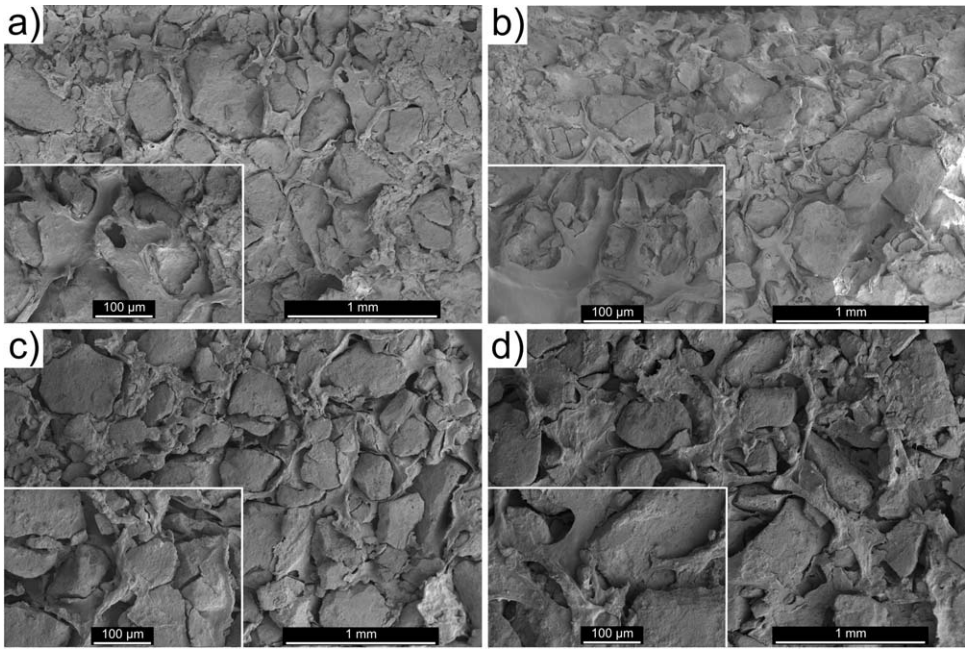

FIGURE 7. SEM images of composite samples incubated in human serum without (a,c) and with presoaking in Ringer solution (b,d). Samples of ø 5 mm (a,b) and ø 13 mm (c,d). In insets: magnification of sample surfaces.

similarly and may not induce any side effects related to significant swelling and volume increase. Moreover, water adsorption by the composite, observed also in media of neutral pH, may be beneficial for bone tissue healing process because water reservoir is thought to be required for osteochondral and cartilage defects regeneration. Water adsorption by HAp/curdlan composite is most probably related to the presence of -OH groups (both in curdlan and HAp) because some water remains in the composite even after drying [Figure 3(e)].

Second, during the presoaking step (in Ringer solution), almost complete increase of volume of tested composites (both for small and large pieces) was observed during 20–30 min. Most probably low viscosity of saline solution was a factor which allowed this liquid to penetrate the pores of composite easily and enabled its quick swelling. Therefore, surgeons should not expect any volume changes and related side effects (as compartment syndrome) while using presoaked samples of HAp/glucan composite for the implantation, as confirmed by statistical analysis (according to the data in Supporting Information, Tables S1 and S2).

Third, soaking of dry composite in human serum takes more time to reach the maximum volume than soaking in Ringer solution followed by serum. Such a difference between the behavior of biomaterial incubated in various liquids (e.g., of high and low viscosity) was expected. It was shown that hydrogels swell in different manner in water and saline solution.[27] Similar observations were made for Haemaccel, when blood serum and blood plasma were tested as media for hydrogels behavior.[28] Moreover, it should be noted that composite size affects the soaking profile. Complete soaking in serum for small (ø 5 mm) samples of HAp/glucan composite took 1 h. However, volume of big samples (ø 13 mm) did not change particularly during that time—it reached its maximum (by approximately 15%) between 2nd and 24th hour of incubation in serum [Figure

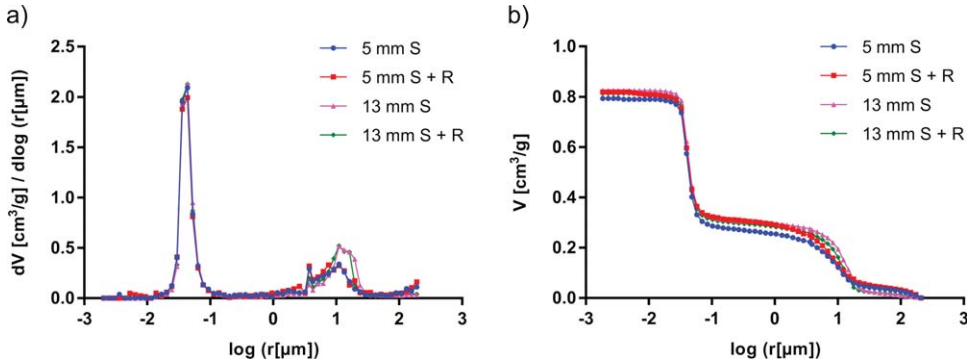

FIGURE 8. Porosity of the composites incubated in human serum without and with presoaking in Ringer solution. (a) Differential and (b) cumulative curves.

TABLE II. Selected Porosity Parameters of Composite Samples (ø 5 or 13 mm) Incubated in Ringer Solution Followed by Human Serum or Exclusively in Human Serum

Sample	Volume of Pores (cm^3/g)	Total Pore Area (m^2/g)	Median Pore Radius (Volume) (μm)	Median Pore Radius (Area) (μm)	Average Pore Radius ($2V/A$) (μm)	Bulk Density at 0.0036 MPa (g/cm^3)	Apparent (Skeletal) Density (g/cm^3)	Porosity (%)
5 mm S	0.79	27.19	0.049	0.032	0.058	0.91	3.29	72.3
5 mm S+R	0.82	27.91	0.051	0.032	0.059	0.90	3.37	73.4
13 mm S	0.82	24.76	0.052	0.034	0.067	0.85	2.79	67.7
13 mm S+R	0.81	25.16	0.051	0.033	0.065	0.86	2.82	69.7

3(b)]. Such a partial composite soaking in patient's blood was also observed during the implantation procedure presented in this work, as shown in Figure 1(b). Thus, the most significant volume increase (by approximately 15%) for big composite samples should be expected by surgeons after wound closure. The question remains whether such increase of implant volume is likely to cause any negative effect on results of bone defect repair. First, the 15% increase of biomaterial volume *in situ* is not necessarily encumbered with a risk of appearance of compartment syndrome for soft hydrogel-based material. Second, in surgical procedures described in this work, the implanted composite is surrounded by soft tissue. Compartment syndrome appears in closed, nonelastic muscle compartment (as osseofascial compartments) and is very rarely presented after elective orthopedic surgery and especially joint arthroplasty.[29] Therefore, for HAp/glucan composite soaked *in vivo* in neutral human serum, the appearance of side effects related to volume increase is probably unlikely to appear.

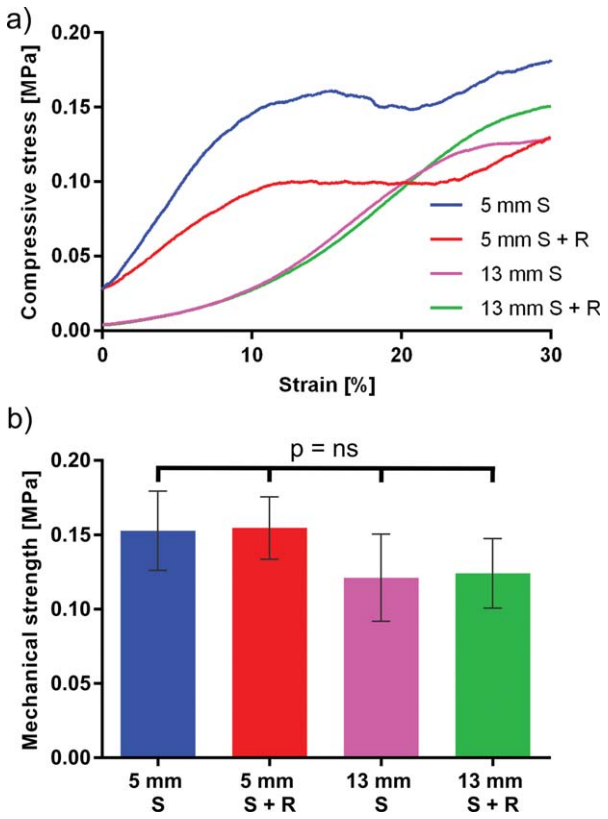

FIGURE 9. Mechanical parameters of composites incubated in human serum with and without presoaking in Ringer solution. (a) The representative stress–strain curves for compression and (b) the compressive strength of samples (mean ± SD, $n = 3$).

Intense apatite precipitation (which may also cause *in vivo* remodeling of composite) did not appear in tested neutral human serum. Although uptake of Ca^{2+} and PO_4^{3-} ions, suggesting the apatite precipitation, was observed (Figure 4) its intensity decreased with time. It was neither confirmed by FTIR technique (lack of carbonate bands suggesting the precipitation of biological apatite) nor by SEM observations. Perhaps traces of apatite were formed on the composite surface but they were deposited within micropores of HAp granules (approximately 100 nm in size, as reported elsewhere[14]) and this could explain the decreasing rate of Ca^{2+} and PO_4^{3-} ions uptake. Following this hypothesis, such a deposition and pores clogging could probably reduce the surface area of HAp granules and slow down the rate of further uptake of Ca^{2+} and PO_4^{3-} ions. Release of Mg^{2+} ions, parallel with the uptake of Ca^{2+} and PO_4^{3-} ions, is in agreement with our earlier results where such a specific Mg^{2+}/Ca^{2+} uptake/release equilibrium was observed for HAp/gypsum composite.[30] One should remember, however, that the phenomenon of ions absorption-desorption from the composite was observed for five days only.

Overall, the obtained results suggest that HAp/glucan composite does not significantly change its volume and structure when incubated in protein-free and protein-rich media of neutral pH. Volume increase observed *in vitro* is size-dependent but limited to the first 24 h of incubation. Moreover, the increase does not exceed 15% of relative volume. According to the observations, the composite presoaking before the implantation in saline solution or even in patient's blood is likely to prevent or limit the implant volume increase *in vivo*. This presoaking strategy may be additionally beneficial: the saline solution may contain drugs (e.g., antibiotics) preventing the postoperative bacterial infection of the implant. HAp/glucan composite was previously reported to absorb antibiotics in biphasic mode.[31] Thus, this property can be adapted to increase the safety of HAp/glucan composite for repair of defects in long bones. However, it should be noted that hypothetic safety of HAp/glucan composite in clinical practice is predicted in this article exclusively on the basis of five clinical cases

(implantation into defects in long bones) and extended *in vitro* experiment concerning the behavior of biomaterial in media of neutral pH. To verify these hypothetic conclusions and confirm the clinical safety of HAp/glucan composite, an extended *in vivo* study (randomized, double-blind, parallel-group controlled trial) is necessary.

CONCLUSIONS

Presented results of HAp/glucan composite behavior in media of neutral pH suggest that the composite swelling is size-dependent, time-limited (appear during up to 24 h of incubation) and that human serum penetrates the composite structure relatively slowly, in comparison with low-viscous media. Neutral pH of incubation medium allows to prevent the excessive increase of composite volume (in contrast to our previous observations concerning the acidic medium. Additionally, pre-incubation in Ringer solution (protein-free medium) protected against undesirable composite swelling. Therefore, presoaking of the composite prior to the implantation (e.g., in saline or drug solution) is highly recommended to reduce the risk of postoperative side effects.

ACKNOWLEDGMENT

The authors want to express their gratitude to Medical Inventi Ltd. (owner of intellectual property for HAp/glucan composite) for its permission to study the composite behavior.

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