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Biomimetic mineralisation on cellulose/cuttlebone scaffolds

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The impact of cuttlebone microparticles on the *in vitro* bioactivity and physical-chemical characteristics of cellulose-based scaffolds during biomimetic mineralisation in a simulated body fluid (SBF) was studied. *In vitro* bioactivity of cellulose/cuttlebone scaffolds was shown, because hydroxyapatite precursors were formed on the surface of scaffolds during soaking in SBF. It was also shown that the incorporation of cuttlebone microparticles into the cellulose-based matrix positively affected the physical-chemical properties of scaffolds, such as water retention, swelling degree and degradation profile (by means of weight loss). Additionally, neutral pH values of SBF during the prolonged soaking (up to 24 weeks) of scaffolds clearly argued in favour of developed scaffolds for bone tissue engineering applications.

Keywords: cuttlebone, scaffolds, simulated body fluid

INTRODUCTION

Filling bone defects remains a routine activity in clinical practice. A number of physical, chemical, biological, immunological and other biomedical characteristics of bone graft substitutes (i.e. scaffolds) are established to fulfil patient needs. Series of tests are performed *in vitro* at the first step of the new-type scaffold development. For this reason, studies in SBF remain an initial and irreversible method for the evaluation of physical-chemical characteristics of scaffolds. Biomimetic mineralisation is used for the evaluation of *in vitro* bioactivity of scaffolds. Moreover, physical parameters of scaffolds, such as water uptake and retention, swelling ratio and biodegradability, play an important role in the prediction of *in vivo* performance of bone graft substitutes.

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Despite substantial achievements in scaffold fabrication techniques and bioactivity enhancement, some challenges in bone tissue engineering still remain. One of the critical issues is biocompatibility of synthetic polymeric scaffolds. According to the literature, acidic degradation products of poly(lactic) acid, poly(glycolic) acid and their co-polymers have been implicated in adverse tissue reactions, as local acidity leads to inflammation and other foreign body reactions in the organism [1, 2]. Therefore, even commercial products could attain a rejection in clinical practice. For these, and some other reasons, natural materials gain an increased interest for scientific research and further practical implementation.

Composite scaffolds have potential applications in bone tissue engineering, because polymeric matrices reinforced with mineral fillers more likely mimic the structure and physical-chemical properties of the natural bone. Cellulose is a widespread polysaccharide commonly used in

biomedicine, because it is characterised as a biocompatible and non-immunogenic natural material. Synthetic hydroxyapatite (HAp, $Ca_{10}(PO_4)_6(OH)_2$) is most often used in filling bone defects. However, magnesium, manganese, iron, zinc, cuprum and other biologically active ions could significantly enhance bioactivity of synthetic stoichiometric HAp. Therefore, HAp precursors of natural origin with nature-adjusted amounts of bioactive trace elements are highly desirable for bone tissue engineering. Natural calcium sources, like cuttlebone, are ideal starting materials for scaffold design. Cuttlebone is known as a biocompatible, osteoconductive and bioactive marine-derived material [3].

In our study 3D macroporous cellulose-based scaffolds reinforced with cuttlebone microparticles were developed; *in vitro* bioactivity and physical-chemical properties of the scaffolds during the prolonged soaking in SBF were discussed.

EXPERIMENTAL

Materials

Cuttlebones were supplied by Vital Pet Products Ltd, UK. The cuttlebones were crushed, then milled (vibrating cup mill Pulverisette 9, Fritsch) and sieved (sieve shaker Haver EML, Haver & Boecker); the fraction with the particle size <80 μm was collected. Acetylcellulose (Sigma-Aldrich) and all reagents for SBF preparation (Eurochemicals), such as NaCl, $NaHCO_3$, KCl, KH_2PO_4, $MgCl_2 \cdot 6H_2O$, $CaCl_2$, were of reagent grade.

Preparation and characterisation of cellulose-based scaffolds with cuttlebone microparticles

Cellulose-based scaffolds with a powdered cuttlebone filler (Cel-CB scaffold) were prepared by mechanical immobilisation of cuttlebone microparticles within cellulose gel as described previously [4]. The porous morphology of scaffolds was achieved by lyophilisation (Christ Alpha 2–4 LSC, Martin Christ GmbH). In this study, scaffolds without any mineral filler (Cel scaffold) were used for comparison. Scanning electron microscopy (SEM) equipped with an energy dispersive spectrometer (EDS) Quanta 200 with a detector XFlash 4030 (Bruker AXS) was used for structural and elemental analyses.

Soaking of scaffolds in SBF

Generally, SBF is used in those *in vitro* tests, where mimicking of blood mineral composition is required. The ion concentrations of SBF is therefore similar to the ion concentrations of blood plasma (Table). SBF was prepared according to the recommendations of Kokubo and co-authors [5]. The calculated amounts of reagents were added into distilled water in a certain order and buffered with Tris/HCl and HCl. Cylindrical shape samples were soaked in SBF at 37 °C and 120 rpm speed (incubator shaker KS 4000 i control) up to 24 weeks. SBF was renewed every 2 weeks.

Analysis of scaffold samples after soaking in SBF

The physical characteristics of the scaffolds, such as water retention (*WR*), swelling ratio (α) and degradation ratio by means of weight changes (*WCh*) of samples, were evaluated.

The *WR* characteristic was calculated according to Eq. 1. To ensure the right filling of all scaffold pores with a solution, the immersed samples were placed into an ultrasonic bath for 5 min, and the residual solution was removed by filter paper before weighing:

$$WR(\%) = (m_1 - m_0) / m_0 \times 100. \quad (1)$$

Here m_0 is the weight of a dry sample, g; m_1 is the weight of a wet sample removed from distilled water after 2 h of soaking, g.

The swelling ratio (α) as a measure of scaffold expansion in physiological conditions was calculated using Eq. 2:

$$\alpha = (V_1 - V_0) / V_0. \quad (2)$$

Here V_0 is the volume of a dry sample and V_1 is the volume of a sample after soaking in distilled water for 2 h. The volume of the samples was calculated from the measured parameters of a scaffold, such as the height (h) and diameter (d), using Eq. 3:

$$V\ (cm^3) = \pi \times (d^2/4) \times h. \quad (3)$$

The *in vitro* degradation ratio of scaffolds was evaluated by calculation of *WCh* of the samples using Eq. 4:

$$WCh\ (\%) = (m_1 - m_0) / m_0 \times 100. \quad (4)$$

Here m_0 is the weight of a dry sample, g; m_1 is the weight of a wet sample taken out from SBF, g.

Analysis of SBF during soaking of scaffolds

The SBF solution was analysed for calcium concentration and pH at the end of the time points. Flame atomic absorption spectrometry (Analyst 400, Perkin Elmer) analysis was

Table. **Ion concentrations and pH values of SBF and human blood plasma**

	Ion concentration, mmol/L								
	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	HCO_3^-	HPO_4^{2-}	SO_4^{2-}	pH
SBF	142.0	5.0	1.5	2.5	147.8	4.2	1.0	0.5	7.4
Blood plasma	142.0	5.0	1.5	2.5	103.0	27.0	1.0	0.5	7.4

performed for the determination of Ca^{2+} ions concentration in SBF. The pH values of the medium were monitored by a benchtop meter (Orion™ Star A111, Thermo Fisher Scientific).

Statistical analysis

All measurements were performed in triplicate. Data are expressed as the mean value ± standard error of deviation (SED). $P \leq 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Characterisation of scaffolds with cuttlebone microparticles

Two groups of samples were prepared: (a) Cel-CB scaffolds and (b) Cel scaffolds, used for comparison (Fig. 1a, c, respectively). 3D structure of all these scaffolds was achieved by lyophilisation (Fig. 1b, d). Additionally, complex scaffold shapes, preferable for bone tissue engineering applications, were developed (Fig. 1e).

As could be observed, different shapes of porous cellulose-based scaffolds are possible to be prepared. This includes but is not limited to a cylindrical, tubular, block, rod, special U shape or a special L shape. The possibility to modify the scaffold shape is an important characteristic of scaffolds, because bone defects are usually unique and require individual fitting.

Characterisation of scaffold samples after soaking in SBF

The ability of any bone graft substitute to generate apatite layer formation on its surface obviously argues for bioactivity of a material. This phenomenon has a positive impact on tight bone–scaffold interaction and further successful bone tissue remodelling *in vivo*. For this reason biomimetic mineralisation is often used to evaluate the *in vitro* bioactivity of scaffolds.

In our study the surface of the Cel-CB scaffold was covered by microsphere-shape particles almost after one week of soaking in SBF (Fig. 2a). This type of particles is typical of HAp, i.e. the main mineral component of a bone. EDS mapping also revealed the appearance of phosphorus on the Cel-CB scaffold surface (Fig. 2b, c). However, after one week of soaking in SBF, the Ca:P molar ratio was 3:1, indicating that further phosphorus deposition on the scaffold surface is available.

The surface of the Cel scaffold was not covered by microsphere-shape particles, or any other HAp characteristic shape particle (Fig. 2d). EDS mapping revealed that there was no phosphorus on the Cel scaffold surface, while the calcium concentration was negligible (Fig. 2e, f). The results confirmed that the surface of the Cel scaffold remained bioinert during soaking in SBF, because any bioactive filler or functional groups that enhance bioactivity, such as silanol, phosphate, carboxyl, etc., were not incorporated during the preparation of this type of scaffold. In contrast, the immobilisation of a bioactive cuttlebone material into the cellulose-based scaffold induced HAp precursor formation on its surface during soaking in SBF. This fact argues for the *in vitro*-evaluated bioactivity of the developed Cel-CB composite scaffold.

Figure 3a depicts the water retention and swelling behaviour of the scaffolds. All scaffolds showed a high water retention. The Cel-CB scaffolds showed the water retention up to 240%, that was two-fold greater than the weight of the scaffold itself. The water retention of the Cel scaffolds was up to 450% (greater compared to that of Cel-CB scaffolds). This phenomenon could be explained by the fact that the cellulose

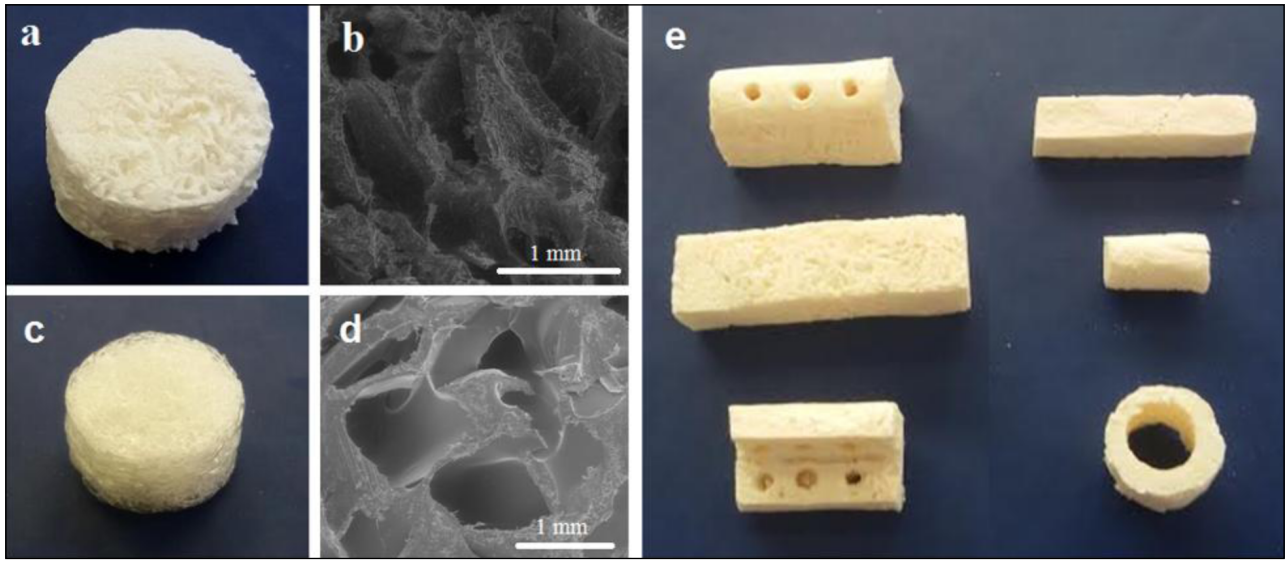

Fig. 1. Photographs of (a) Cel-CB scaffold and (c) Cel scaffold in natural size; (b, d) corresponding microphotographs; and (e) Cel-CB scaffolds of different shapes. Coloured online

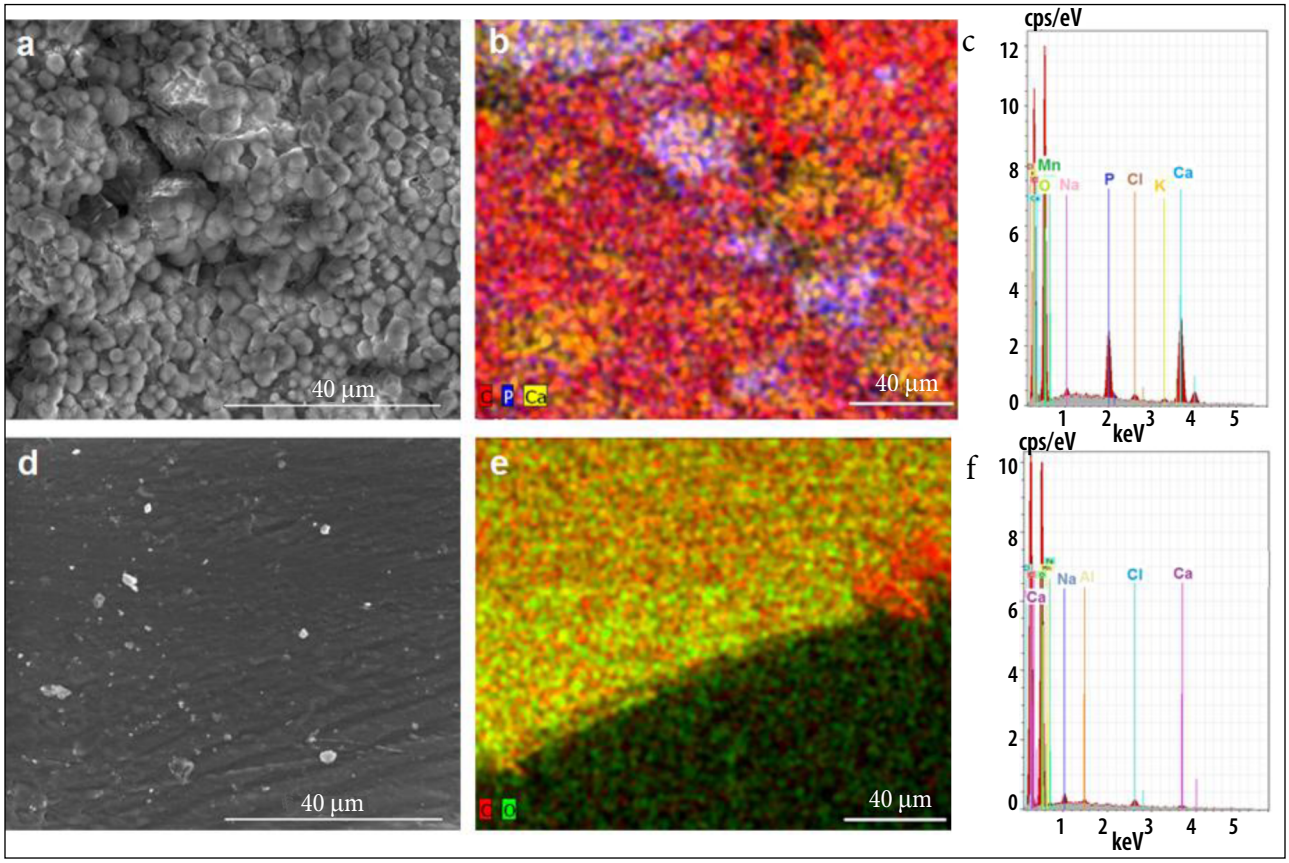

Fig. 2. Morphological and chemical characterization of the scaffolds surface after 1 week of soaking in SBF: (a, d) microphotograph of Cel-CB and Cel scaffold samples, respectively; (b, c) EDS mapping of Cel-CB scaffold; (e, f) EDS mapping of Cel scaffold. Coloured online

matrix is more hydrophilic than the cellulose-based composite scaffold with a mineral filler. Hydroxyl groups of cellulose readily form hydrogen bonds with a water molecules and therefore they are characterised as more hydrophilic. Moreover, pores of the Cel scaffolds were larger in diameter that allowed better water penetration into this type of scaffolds.

Water retention is also closely related to the swelling properties of a material. However, the swelling ratio was found to be similar for both types of the cellulose-based scaffolds. In the literature, osteoblastic cell proliferation and differentiation are highly dependent on water uptake and retention, whereas the swelling ratio is critical for structural stability and mechanical properties of a scaffold [6, 7]. Water retention and swelling behaviour profiles as shown for Cel-CB scaffolds are preferable for bone tissue engineering applications.

To date, the biodegradability profiles of various scaffolds have been investigated thoroughly because this physical-chemical parameter plays a crucial role in opportune bone biorestoration. The degradation behaviour of a scaffold by means of the degradation rate and non-cytotoxicity of its metabolic products are also primarily evaluated *in vitro* [8].

From the data obtained in our study (Fig. 3b), it can be seen that the Cel-CB scaffolds gained ~5% weight during the first 3 weeks of soaking in SBF, which indicated the deposition of newly formed apatite particles on the surface of the scaffold as was described earlier (Fig. 2a). The Cel-CB scaffolds lost 15% of their weight at the end of 12 weeks which was similar for the chemically modified matrices of regenerated cellulose in which the degradation of the polymer network was first observed after 9 weeks of incubation in SBF [9]. The *WCh* of the Cel-CB scaffolds was followed by a slight increase up to 10% of weight loss at the end of 24 weeks. This phenomenon is presumably related to a continued apatite layer formation during the prolonged soaking in SBF as described similarly in the literature for the HAp/chitosan/carboxymethyl cellulose composite scaffold [10].

In contrast, *WCh* of the Cel scaffolds were considerably stable and do not exceed 2% even at the end of the experiment. Non-enzymatic hydrolysis of cellulose through the cleavage of β(1→4)-bonds between glucose moieties leads to non-cytotoxic soluble sugars formation under biological conditions [11]. The high degree of cellulose crystallinity and the absence of cellulase in animal and human tissue lower biodegradation rates significantly and define cellulose-based implants as biodurable, leading to unwanted biological responses. However, the crystallinity and swelling behaviour of regenerated cellulose is known to be different from that of bacterial or plant cellulose due to its higher degradation rate and quick resorption [12, 13]. According to the results of our study, the Cel-CB scaffolds and Cel scaffolds have demonstrated different *in vitro* degradation

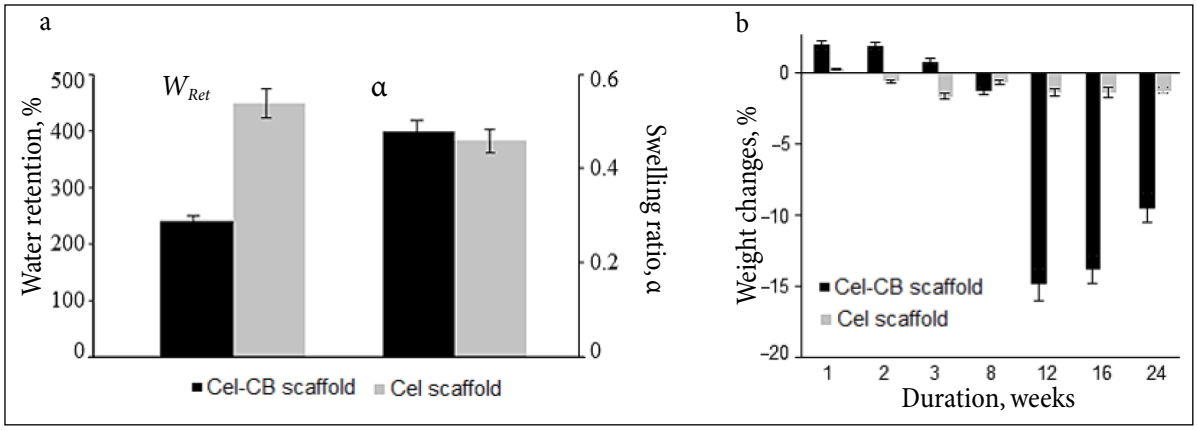

Fig. 3. Physical characteristics of the scaffolds: (a) water retention and swelling ratio after immersion in distilled water for 2 h; (b) weight changes during 24-week soaking in SBF

profiles by means of sample *WCh*, which showed that the incorporation of cuttlebone microparticles into cellulose gel during the scaffold preparation accelerated the degradation process by more than 15% during 12 weeks of soaking in SBF. The reason for this phenomenon may be that cuttlebone microparticles disturb the ordered arrangement of hydrogen bonds in cellulose macromolecules during the scaffold preparation stage and therefore determine the reduction of cellulose crystallinity [13].

Importantly, biodegradation/bioresorption is a required characteristic of bone graft substitutes. Considering that replacement of a scaffold by a newly-formed bone is dependent on the acting site and defect size as well as on human age, adjacent diseases, metabolic disorders, lifestyle, and other physiological and biological parameters, the cellulose:cuttlebone ratio in scaffolds could therefore be varied to match the degradation rate for particular bone defects.

Characterisation of SBF during soaking of scaffolds

Calcium ion release from the cellulose-based scaffolds into SBF and proton concentrations during soaking of the scaffolds are plotted in Fig. 4.

As shown in Fig. 4a, the solution pH, where the samples of Cel-CB scaffolds were soaked, was neutral to slightly alkaline during all measurement (7.3 < pH > 7.9). The calcium concentration in SBF after 4 weeks was 415 ppm (pH of 7.5), while after 12 weeks it was 462 ppm (pH of 7.3), and finally after 24 weeks it was 620 ppm (pH of 7.5). As shown in Fig. 4b, the solution pH, where the samples of Cel scaffolds were soaked, was also neutral during all measurement (7.3 < pH > 7.5). However, the calcium concentration was almost three-fold lower compared to the SBF of Cel-CB scaffolds. The calcium concentration of Cel scaffolds in SBF after 4 weeks was 154 ppm (pH = 7.3), after 12 weeks it was 293 ppm (pH = 7.4), and finally after 24 weeks it was 182 ppm (pH = 7.4).

As can be observed, for the group as a whole the pH variations during the prolonged soaking in SBF were minimal (pH range 7.3–7.9). Small pH variations during the scaffolds soaking correlate well with the normal blood plasma pH, indicating non-acidic environment, additionally sustained by calcium ion release from the samples (Fig. 4a, b). The observed phenomenon might have a favourable effect on a collagen type 1 expression in bone remodelling and

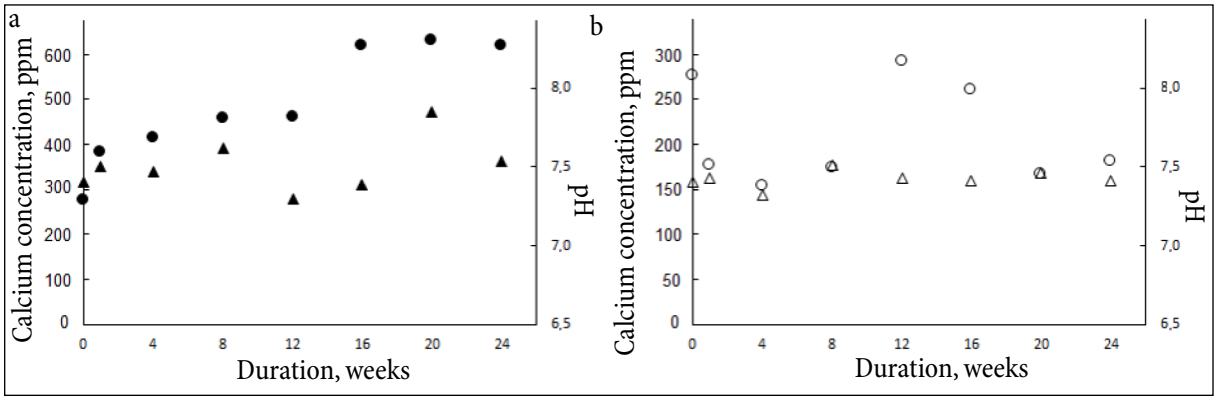

Fig. 4. Calcium concentrations and pH values of SBF during soaking of scaffolds up to 24 weeks for: (a) Cel-CB scaffolds and (b) Cel scaffolds. A round-shape symbol indicates calcium concentration; a triangle-shape symbol indicates pH values

modulate a local inflammatory response to a minimum expression [14, 15]. The Cel-CB scaffold samples showed an increase of calcium concentration in SBF during soaking (Fig. 4a). This indicates that calcium ions, dissolved from the mineralised scaffold, appear to form an apatite layer on the scaffold surface [16]. In contrast to the mineralised scaffolds, the Cel scaffolds showed a decrease of calcium concentration in SBF within the soaking period (Fig. 4b), which could be explained by a steady calcium precipitation from SBF.

CONCLUSIONS

It can be concluded that cellulose-based scaffolds reinforced with cuttlebone microparticles demonstrate *in vitro* bioactivity by means of hydroxyapatite precursor formation on a scaffold surface during biomimetic mineralisation. Proper physical-chemical characteristics of these composite scaffolds evaluated *in vitro* arguably define them as a promising material for bone tissue engineering applications.

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BIOIMITACINĖ MINERALIZACIJA CELIULIOZĖS / SEPIJOS KAULO KARKASUOSE

S a n t r a u k a

Įvertinta sepijos kaulo mikrodalelių įtaka celiuliozės / sepijos kaulo karkasų *in vitro* bioaktyvumui ir jų fizikiniams-cheminiams parametrams vykstant bioimitacinei mineralizacijai modeliniame kūno skystyje. Nustatyta, kad tyrimo metu modeliniame kūno skystyje celiuliozės / sepijos kaulo karkasai pasižymėjo bioaktyvumu dėl hidroksiapatito pirmtakų formavimosi ant jų paviršiaus. Pažymėta teigiama sepijos kaulo mikrodalelių įtaka karkasų vandens sulaikymo, brinkimo laipsnio ir masės kitimo rodikliams, kurie yra tinkami kaulo audinio inžinerijos tikslams. Nustatyta, kad modelinio kūno skysčio pH liko neutralus viso tyrimo metu (24 sav.) ir tai leidžia daryti prielaidą, kad karkasų irimo metu formuojasi tik neutralūs reakcijos tarpiniai produktai. Šis faktas yra palankus fizikinis veiksnys kaulo audinio inžinerijoje, kadangi išvengiama uždegiminių reakcijų *in vivo.*