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Preparation of cellulose-nanohydroxyapatite composite scaffolds from ionic liquid solutions

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Abstract

Cellulose and cellulose-nanohydroxyapatite composite (CNH) tissue engineering scaffolds were fabricated by a particulate leaching technique, with poly(methyl methacrylate) (PMMA) particles as the porogen. Two different solvents were used for the dissolution of cellulose. The first solvent was a mixture of *N*,*N* dimethylacetamide (DMA) + lithium chloride (LiCl) and the second the ionic liquid 1-*n*-butyl-3-methylimidazolium chloride (BmimCl). By taking advantage of the properties of BmimCl, a better dispersion of hydroxyapatite in cellulose was accomplished compared with the dispersion when using DMA + LiCl as the solvent. Although the solvent influences the behavior of the compact materials, such as water sorption and thermal degradation, it does not play any role in the production of porous materials since highly interconnected pore structures similar to each other were obtained regardless of the solvent used. After the regeneration process of cellulose, BmimCl was easily recycled by vacuum distillation. The feasibility of encapsulation of an antibiotic drug into the cellulose-hydroxyapatite matrix was examined.

Keywords: Cellulose; Hydroxyapatite; Scaffold; Ionic liquid; Particulate leaching

1. Introduction

The research interest in the field of tissue engineering has increased rapidly over the past two decades. The goal of tissue engineering is the regeneration, modification, growth, and maintenance of living tissues by combining the principles of engineering and medical sciences. One of the major challenges stemming from this goal, is the construction of an appropriate temporary artificial extracellular matrix, called scaffold, which will support three-dimensional tissue formation (Pancrazio, Wang, & Kelley 2007; Quirk, France, Shakesheff, & Howdle, 2004).

A scaffolding material must have specific properties such as biocompatibility, biodegradation, high open (interconnected) porosity, and mechanical strength. It is also desirable that the scaffold act as a controlled release device for growth factors and/or pharmaceutical substances (anti-inflammatory agents, for example) (Karageorgiou & Kaplan, 2005; Ma, 2004).

Natural and synthetic biodegradable polymers have been used extensively as scaffolding materials (Ma, 2004; Pancrazio et al. 2007). For the mechanical reinforcement of polymers or for certain applications such as bone regeneration, composites materials of biopolymers and bioceramics have been produced. The most frequently used bioceramic material is hydroxyapatite (Hap). Besides Hap, bioglasses have been used as scaffolding materials alone or as additives to polymers (Blacher, Maquet, Jerome, Pirard, & Boccaccini, 2005; Chen, Thomson, & Boccaccini, 2006; Karageorgiou & Kaplan, 2005; Ma, 2004; Thomson, Yaszemski, Powers, & Mikos, 1998; Wei & Ma, 2004).

One important category of natural biopolymers is polysaccharides. Cellulose is the best example since it is the most abundant renewable polysaccharide found on earth. The crystalline structure of cellulose, due to the hydrogen

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bonds between and within its chains, in combination with the high molecular weight, give it unique properties like chemical stability and mechanical strength and, of course, biocompatibility and biodegradation. These advantages though, make cellulose's treatment very difficult. For example, cellulose exhibits zero solubility in water under mild conditions (it is soluble in supercritical water, however) and in common organic solvents. As a consequence, more attention has been paid to cellulose derivatives (cellulose acetate is a representative example) or bacterial cellulose (BC). A nano-fibrous scaffold has been produced recently via electrospinning of cellulose acetate (Han & Gouma, 2006). BC has a fibrous structure similar to structures obtained by electrospinning of synthetic polymers and exhibits high crystallinity and mechanical strength.

Composites of BC and hydroxyapatite have been produced by immersing BC in an appropriate aqueous solution, such as simulated body fluid (SBF), and by depositing hydroxyapatite particles on the fibers (Czaja, Young, Kawecki, & Brown, 2007; Hutchens, Benson, Evans, O'Neill, & Rawn, 2006; Muller et al., 2006; Wan et al., 2007). Disadvantages of this procedure with BC are the inability to control the structure morphology (fiber diameter, pore size) and the inability to encapsulate desirable substances inside the BC matrix. In addition, the composites produced by this way, although suitable for bone regeneration, they do not have significantly improved thermal and mechanical properties.

Recently, ionic liquids (ILs) were found to dissolve native cellulose (Remsing, Swatloski, Rogers, & Moyna, 2006; Swatloski, Spear, Holbrey, & Rogers, 2002; Zhang, Wu, Zhang, & He, 2005). ILs are organic salts with polar character and low melting point (<100 °C). They are thermally and chemically stable and have a practically non-detectable vapor pressure. Their non-volatile nature allows for easy recycling after usage and, thus, places them high in the realm of green chemistry (Kubisa, 2005; Zhu et al., 2006). Quite few ionic liquids are known (and have been used) to dissolve cellulose. The most frequently used are the hydrophilic ILs 1-*n*-butyl-3-methylimidazolium chloride (BmimCl) and 1-allyl-3-methylimidazolium chloride (AmimCl). Cellulose films and composites with encapsulated enzymes have been produced using a dissolution in ionic liquid regeneration process (Turner, Spear, Holbrey, Daly, & Rogers, 2005; Turner, Spear, Holbrey, & Rogers, 2004). Recently, a micro- and nano-sized fibrous scaffold has been prepared via electrospinning by using an ionic liquid as the solvent (Viswanathan et al., 2006).

In the present study, cellulose and cellulose-hydroxyapatite composite scaffolds were successfully prepared from DMA + LiCl and from ionic liquid solutions. An attempt to encapsulate amoxicillin, an antibiotic drug, into the produced scaffolds was made. In what follows we report on the experimental methods of preparation and characterization of the above mentioned scaffolds and on their use for drug encapsulation.

2. Experimental

2.1. Materials and methods

Cellulose (1486 soft pulp, DP = 1283) was purchased from Lenzing Aktiengesellschaft (Austria). *N*,*N* dimethylacetamide (purity >98%) was purchased from Fluka. BmimCl was purchased from Sigma–Aldrich (undefined purity). Hydroxyapatite (>97%) nano-powder (~100 nm) was purchased from Sigma–Aldrich. Amoxicillin (trihydrate) was obtained from Ranbaxy Laboratories (India, 99% purity). All other chemicals were purchased from Sigma–Aldrich. They were of analytical grade and were used as received.

The morphology of porous samples was examined using a Jeol JSM-840A scanning electron microscope. For particle dispersion an ultra-sonicator (HEAT SYSTEMS-ULTRASONICS, INC, W-375) was used. For further characterization of the samples a Rigaku Miniflex X-ray diffractometer (Cu, $\lambda = 1.5405$ Å), a thermogravimetric analyzer (Shimadzu, model TGA-50), and a reflectance measurement spectrophotometer (HunterLab, model mini Scan XE plus) were used.

The average pore diameter of the samples was calculated from the SEM pictures with appropriate software (ImageJ 1.32j). The PMMA particle size distribution was calculated by screening the particles with appropriate sieves. The porosity of the samples was estimated by the following equation:

$$P = (1 - d_s/d_b) \times 100, \quad (1)$$

where d_s is the density of the scaffold and d_b the density of the bulk material (Karageorgiou & Kaplan, 2005). The density of porous samples was calculated by dividing their mass with their volume. The volume, in turn, was calculated by measuring their dimensions with a digital micrometer. The density of the bulk samples was measured using the buoyancy method (ASTM D-792) with ethylene glycol as the liquid of known density. When calculating densities, errors have been taken into account (for example the scale's accuracy) and the reported results are statistical averages obtained after appropriate statistical analysis.

2.2. Preparation of cellulose solutions in DMA + LiCl mixture and in BmimCl

For the preparation of DMA + LiCl solution, cellulose was first treated with a 5% w/v LiCl aqueous solution for 24 h, filtered, and dried under vacuum for another 24 h. Subsequently, it was dissolved in DMA containing 6% LiCl by magnetic stirring for 24 h at 60 °C and for another 24 h at room temperature (~22 °C). Since there were cellulose losses during filtering, the actual concentration was calculated by removing with water the solvent system of a solution of known mass and then drying and weighing the polymer. The true concentration was found to be 1.72% w/w.

In precisely known mass of solution an appropriate mass of nanohydroxyapatite (NH) was added in order to obtain composites with NH load of 0.16 (16% NH and 84% cellulose, w/w) and 0.30. The NH particles were dispersed in the solutions by ultra-sonication for 40 min.

The solution of cellulose in BmimCl (1.72% w/w) was obtained by continuous stirring at ~95 °C for 5 h. After complete dissolution of cellulose, NH particles were added to the solution in order to obtain composites of the same final NH loads of 0.16 and 0.30, and stirred for 15 min. Then sonication was applied four times for 5 min each time, a total of 20 min. This was chosen because the BmimCl solutions were at high temperature (above 70 °C), and sonication was applied for 5 min, followed by 5 min cooling and stirring in order to avoid thermal degradation of cellulose and decomposition of the ionic liquid due to local overheating. The procedure was repeated 4 times. In the case of DMA + LiCl the viscosity of the solutions was much lower even at room temperature and local overheating due to ultra-sonication was not as high. Thus, in this case, ultra-sonication was interrupted only once (at 20 min), followed by 5 min cooling.

2.3. Preparation of compact and porous cellulose and cellulose-NH composite samples

Porous materials from DMA solutions were prepared by the following procedure: Solutions of cellulose with and without dispersed NH particles were mixed with PMMA particles ($250 < d_p < 500$ μm) in a mass proportion of solution to PMMA equal to 1:2. The suspensions were allowed to stand at room temperature for 24 h (enough time for gelation), immersed in water and methanol (extensive washing), and dried at 40 °C. PMMA particles were then leached with dichloromethane.

Compact (regenerated) cellulose and cellulose – NH composite materials were prepared by a procedure similar to the one used for porous samples but without PMMA particles. After water extraction with methanol, the samples were placed on plates to ensure flatness and were dried at 40 °C.

Porous and compact samples from BmimCl solutions were prepared by the same procedure as from DMA + LiCl solutions with the only difference that, after their preparation, the solutions were allowed to stand for 30 min at 4 °C and immediately after this they were immersed in water and kept at 4 °C. In this case the PMMA particles for the porous samples had a size distribution between 120 and 250 μm.

Compact samples were examined by X-ray diffraction and thermogravimetric analysis (air, 10 °C/min until 80 °C, hold for 5 min, and then with a heating rate of 10 °C/min until 600 °C. The mass at 150 °C was set as the 100% mass). The water uptake capacity was also measured at room temperature (~27 °C). To ensure saturation, the samples were placed in excess of water and the uptake was taken from the final weight when remained unchanged for at least 24 h. Porous samples were immersed in liquid N_2 and were cut with a razor blade in order to prevent deformation of their structure, coated with carbon, and examined with Scanning Electron Microscope. The density of, both, porous and compact materials was also measured.

2.4. Encapsulation of amoxicillin into cellulose-0.16 NH composite from BmimCl solution

For the preparation of cellulose-hydroxyapatite composite of 0.16 NH load with encapsulated amoxicillin, the same procedure was followed as for the plain cellulose-0.16 NH composite from BmimCl solution, with the only difference that amoxicillin was added in the solution in a proportion of 0.08% of cellulose mass. Both, compact and porous materials were produced. The compact regenerated material was washed with ethanol in order to remove the non-encapsulated drug from the surface. For a qualitative examination of encapsulation, the yellowness index of the 0.16 NH composites with and without amoxicillin was measured with the reflectance spectrophotometer. Also, the thermal stability of native amoxicillin was examined thermogravimetrically at three different temperatures (100, 150, and 200 °C) for 120 min and in air atmosphere. This was deemed necessary since the encapsulation procedure required relatively high temperatures (70–100 °C) and possible thermal decomposition of amoxicillin had to be avoided.

3. Results and discussion

3.1. Comparison of compact regenerated materials from DMA + LiCl and BmimCl solutions

Six compact samples were prepared in total. Transparent cellulose films were obtained after regeneration from, both, DMA + LiCl and BmimCl solutions. In the case of composite materials, the ones from BmimCl had identical top and bottom surface while the composites regenerated from DMA + LiCl solution had a bottom surface of higher roughness. This difference was due to the fact that, until gelation occurred, NH particles agglomerated and deposited (macroscopic phase separation) while some part of it remained dispersed in cellulose. The phase separation was visible by naked eye for the composite samples from DMA + LiCl and it was more intense in the composite with the higher (0.30) load of hydroxyapatite. This is indicative of the absence of strong interactions between hydroxyapatite and cellulose molecules in DMA + LiCl solutions.

On the other hand, cellulose solutions in BmimCl, exhibit the typical behavior of a gel (syneresis, uniform shrinking) with spontaneous gelation, in contrast to DMA + LiCl solutions which require time for gelation. The low temperature was selected in order to quickly solidify the solution and prevent hydroxyapatite sedimentation. In BmimCl solution, hydroxyapatite and cellulose molecules do not

interact strongly, but the spontaneous gelation and the concomitant increase of viscosity suffice to keep the particles of hydroxyapatite dispersed in the cellulose matrix. A better dispersion was then expected in the composites from BmimCl and, indeed, no phase separation could be observed. As we will see later, due to homogeneity, these materials exhibit better properties than the respective composite materials from DMA + LiCl solution.

In Fig. 1a are shown the XRD patterns of native and regenerated celluloses from DMA + LiCl and BmimCl solutions. Both regenerated celluloses exhibit a different degree of crystallinity from native cellulose. The diffraction peaks of native cellulose are the diffraction peaks of cellulose I, while regenerated cellulose gives diffraction peaks of cellulose II (Zhang et al., 2005). Similar results have been reported in the literature for cellulose regenerated from AmimCl (Zhang et al., 2005). The degree of crystallinity can be approximately determined from the peak areas and by taking into account the thickness of the samples. The cellulose film regenerated from BmimCl, although thicker than the film from DMA + LiCl, exhibited a smaller peak indicating less crystallinity. Of course, the large difference in the peak area between native and regenerated celluloses arises from the higher crystallinity as well as the thickness of the native cellulose film.

In order to accomplish cellulose dissolution, the inter- and intra-molecular hydrogen bonds must be disrupted. The chloride ions (in both solvents) are believed to form (new) hydrogen bonds with hydroxyl groups of the molecules of cellulose during the dissolution process (Swatloski et al., 2002; Zhang et al., 2005). Since BmimCl is a more powerful solvent for cellulose as it can dissolve cellulose up to 25% w/w without pretreatment (Swatloski et al., 2002), it should have a more drastic influence on the crystalline structure of native cellulose. In addition, the regeneration process (solidification) in the case of DMA + LiCl solution is slower because the gelation (self cross-linking) process takes time. These might be two possible explanations for the higher crystallinity of cellulose regenerated from DMA + LiCl solution.

The XRD patterns of the composite materials are shown in Fig. 1b and c. It is worth mentioning that in the 0.30 Hap composites, although the cellulose peaks in both samples are similar, the peaks of hydroxyapatite are higher in the composite from DMA + LiCl solution. This is due to the phase separation mentioned above (the almost pure hydroxyapatite layer at the bottom surface of the sample results in a more intense diffraction).

Concerning thermogravimetric analysis, although native cellulose is thermally the most stable from all cellulose samples, it had the highest decomposition rate and the highest mass loss. Similar results have been reported in the literature for another type of cellulose regenerated from BmimCl (Swatloski et al., 2002). Comparing the two regenerated cellulose samples, the one from BmimCl starts to

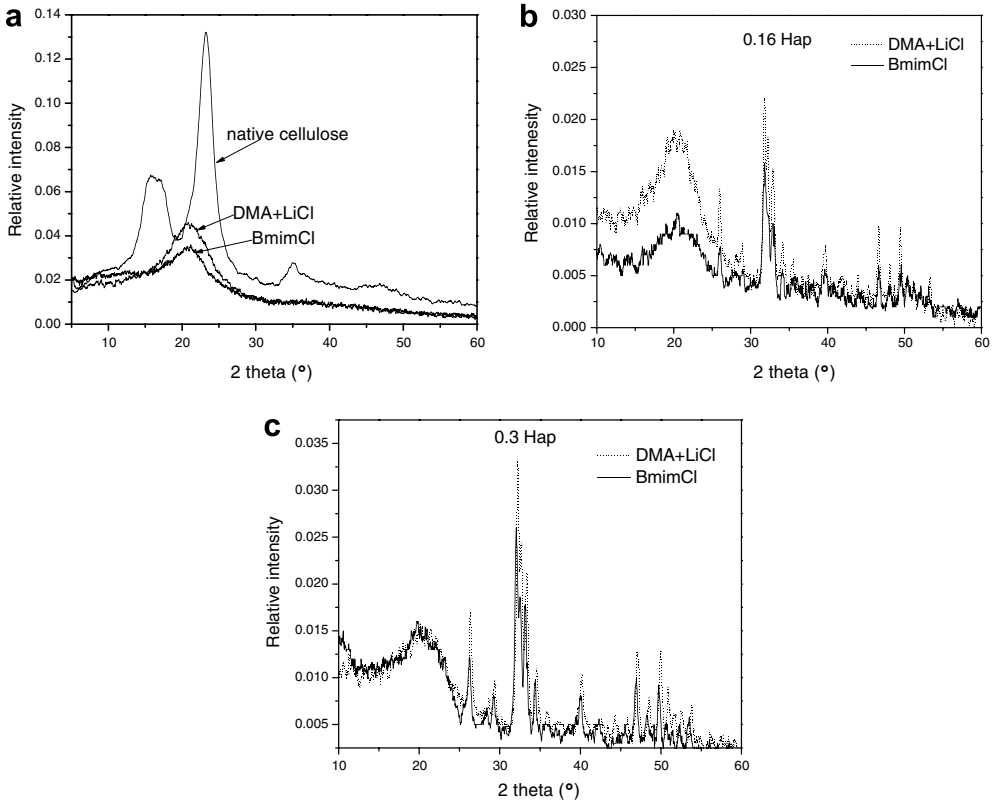

Fig. 1. XRD patterns of native cellulose and regenerated materials (a) celluloses (b) 0.16 NH composites (c) 0.30 NH composites.

decompose at a higher temperature and it has lower mass loss and lower decomposition rate.

The thermal stability of the composite materials is increased by increasing the hydroxyapatite content. The composites from BmimCl are more stable compared to the respective composites from DMA + LiCl. The 0.16 Hap composite from BmimCl solution exhibits a thermal stability even higher than that of the 0.30 Hap composite from DMA + LiCl solution up to 260 °C. Two factors contribute to this behavior: The inherent stability of cellulose and the better dispersion of Hap in the BmimCl composites. The 0.30 Hap composite from BmimCl solution exhibits a thermal stability similar to that of native cellulose with a lower decomposition rate. The decomposition rates for regenerated cellulose samples and 0.16 Hap composites are the same but are different for the 0.30 Hap composites. Representative data from the thermogravimetric analysis are summarized in Table 1.

The water absorbance of the samples is shown in Fig. 2. Samples from BmimCl appear to absorb less water. By increasing hydroxyapatite content less water is absorbed per gram of dry material. All regenerated materials have smaller water absorbance capacity compared to native cellulose.

3.2. Characterization of porous composites from DMA + LiCl and BmimCl solutions

The produced scaffolds had a diameter of around 0.7 cm, a thickness of 0.4 cm, and weighted about 0.0025 g. Representative SEM micrographs of these scaffolds are shown in Fig. 3a–d. In all samples a surface porosity is present and the pores are highly interconnected and fairly uniform. Pore size distributions are close to the distribution of PMMA particles used, the only exception being the cellulose sample from BmimCl solution, which exhibits a larger pore size distribution. This unexpected result could be explained by taking into account the higher surface that a sample gets as it is ground finely. Since the PMMA particles in BmimCl solution were more finely ground (120–250 μm compared to 250–500 μm in the DMA + LiCl solution), the contact surface between scaffolding material and porogen was larger and thus resulted in some degree of pore coalescence by the breaking of the cellulose wall between two pores. In the composite scaffolds from BmimCl this was not observed due to the lower proportion of scaffolding material to porogen and to the mechanical reinforcement of cellulose by the evenly dispersed hydroxyapatite.

Table 1
Results from thermogravimetric analysis

Material	Temperature of 98% remaining mass (°C)	Temperature of maximum decomposition rate (°C)
Native cellulose	275	345
Cellulose DMA + LiCl	225	310
Cellulose-0.16 Hap DMA + LiCl	245	310
Cellulose-0.3 Hap DMA + LiCl	255	315
Cellulose BmimCl	235	315
Cellulose-0.16 Hap BmimCl	260	315
Cellulose-0.3 Hap BmimCl	275	335

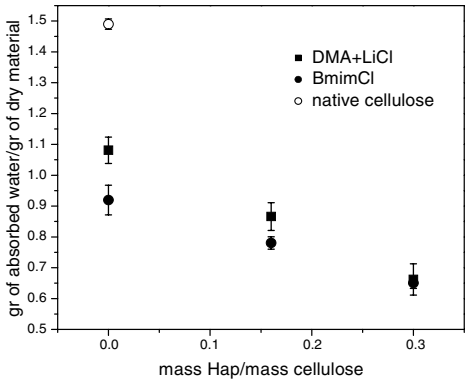

Fig. 2. Water absorption of non-porous samples.

All samples exhibited high porosity (>96%). It is worth mentioning that, by immersing a scaffold in dichloromethane, the scaffold absorbed dichloromethane about 70 times its weight within 5 s. This indicates both high and open (interconnected) porosity. The characteristics of the porous samples are summarized in Table 2.

3.3. Encapsulation of amoxicillin

Since BmimCl is an ionic liquid with relatively high melting point (70 °C), the procedures of dispersion or dissolution require high temperatures. Thus, the thermal stability of amoxicillin was first examined. Its mass loss at three different temperatures is shown in Fig. 4. As shown, a rapid decomposition occurs at 200 °C while at 150 °C the decomposition is much slower. At 100 °C, after water evaporation (of the trihydrate form of amoxicillin), the dry mass remained stable for at least 35 min (time greater than the time needed for dispersion of amoxicillin). Thus, by avoiding temperatures in excess of 100 °C, amoxicillin can be dispersed in BmimCl – cellulose solution without thermal decomposition.

In Table 3 are reported the color measurements of the encapsulated sample in the color space CIE 1976 $L^*a^*b^*$ from the reflectance spectrophotometer. From the increases in index b^* and yellowness index, yi, it can be concluded that this sample is more yellow compared to the same material without amoxicillin and this is due to the light yellow color of amoxicillin. By this way, a qualitative determination of the extent of encapsulation is made, which suffices for the purposes of the present work. For quantitative determination, the polymer is usually

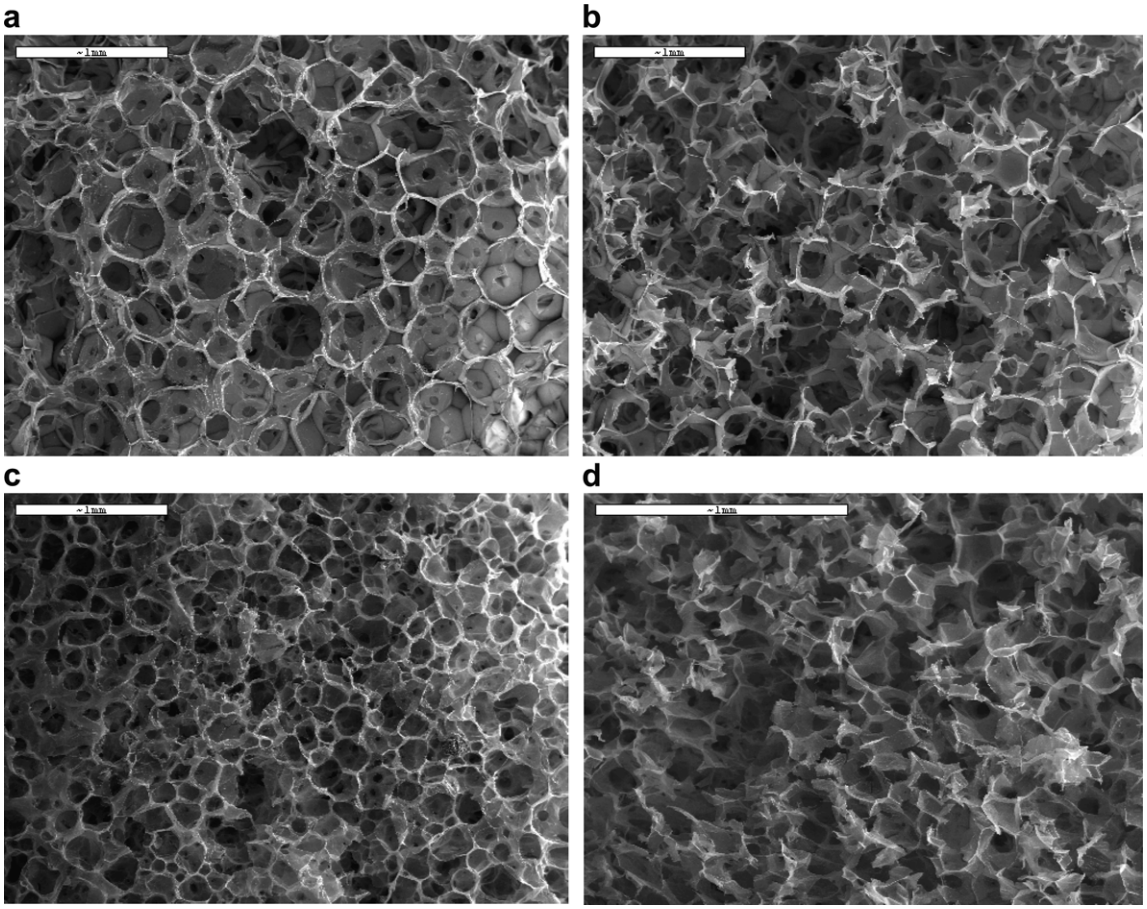

Fig. 3. Representative SEM pictures (magnification 30×): (a) External surface of cellulose scaffold prepared from DMA + LiCl; (b) Cross-section of cellulose-0.16 Hap composite scaffold prepared from DMA + LiCl; (c) External surface of cellulose-0.16 Hap composite scaffold from BmimCl; (d) Cross-section of cellulose-0.3 Hap composite scaffold from BmimCl (magnification 50×).

Table 2
Total porosity and average pore size of the produced scaffolds

	Total porosity (%)	Average pore size (μm)	PMMA particles average size (μm)
Cellulose (DMA + LiCl)	98–99	311 ± 78	250–500
Cellulose-0.16 Hap (DMA + LiCl)	97–99	338 ± 77	250–500
Cellulose-0.30 Hap (DMA + LiCl)	97–99	322 ± 82	250–500
Cellulose (BmimCl)	98–99	264 ± 60	120–250
Cellulose-0.16 Hap (BmimCl)	97–99	173 ± 51	120–250
Cellulose-0.30 Hap (BmimCl)	97–99	172 ± 44	120–250

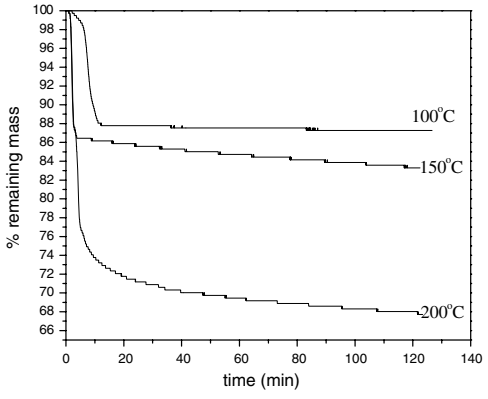

Fig. 4. Mass loss of amoxicillin at three different temperatures.

redissolved and the encapsulated drug is selectively extracted. This procedure with regenerated cellulose cannot be applied here, since re-dissolution is difficult or even impossible (due to the structure of regenerated cellulose). Even if the composite were redissolved, still there would be the problem of finding a solvent for amoxicillin not miscible with BmimCl. One possible solution (though time consuming) would be the release of amoxicillin by the

Table 3
Spectrophotometric results for cellulose-0.16 Hap compact composite with and without encapsulated amoxicillin

	Without amoxicillin	With encapsulated amoxicillin
L^*	73.39	68.11
a^*	−1.12	−1.62
b^*	2.19	3.76
yi	3.89	7.28

enzymatic degradation of the composite in aqueous environment. Work is underway in our Laboratory for the quantitative determination of encapsulation.

4. Conclusions

Cellulose-nanohydroxyapatite composite scaffolds with high and open porosity were successfully prepared by PMMA particulate leaching. Besides DMA + LiCl, which is a well known solvent for cellulose, an ionic liquid, BmimCl, was used to dissolve cellulose. By taking advantage of BmimCl's properties (powerful solvent, highly viscous, leads to spontaneous gelation behavior), a better dispersion of hydroxyapatite in cellulose was achieved. In addition, the materials regenerated from BmimCl solutions had different properties (such as thermal stability and water absorption capacity) from the materials regenerated from DMA + LiCl solutions. Thus, ionic liquids open new possibilities for the otherwise difficult treatment of biomacromolecules. The properties of these new materials make them promising especially for bone regeneration applications. Besides the different properties of the produced materials, the scaffold fabrication process by using ionic liquids is faster, cheaper, and environmentally friendly. In addition, it was shown that encapsulation of desirable active substances in them is feasible. Of course, the high melting point of the specific ionic liquid (BmimCl) becomes a restricting factor for encapsulation of thermally sensitive substances such as proteins. For these substances a room temperature ionic liquid could be more appropriate.

In this study two lines of green chemistry were combined for the fabrication of tissue engineering scaffolds: the biodegradable and renewable sources (cellulose) and the ionic liquids. The use of dichloromethane for the PMMA leaching could be avoided by using a polymer of very low molecular weight and by leaching it with supercritical carbon dioxide, thus, adding a third line of green chemistry to scaffold preparation.

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