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Salt leaching using powder (SLUP) process for glass/chitosan scaffold elaboration for biomaterial applications

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Abstract

Tissue engineering has emerged as an alternative approach to create bone tissue by growing cells on 3D scaffolding. The aim of this study was to synthesize a composite glass/chitosan (BG-CH) by using new salt leaching using powder (SLUP) process in order to control the porosity rate and then the chemical reactivity of the final product. SLUP process consists on the cavity creation with desired pore sizes. It does not require heat treatment. This process is based on washing out the NaCl particles used for that. It is due to its high solubility in aqueous media. This work focuses on the elaboration, physicochemical, and chemical reactivity studies of pure bioactive glass and bioactive glass associated with chitosan. A range of composite scaffolds with different bioactive glass/chitosan contents has been synthesized. NaCl with a distinct range size was used with the aim of optimizing the pore network. Obtained results show that the specific surface area and pore volume increase with increasing of chitosan and porogen content. The same observations for pore volume were registered. The obtained scaffolds had high porosity (90%) with good pore connectivity. SEM images revealed strong dependence of sizes and shapes of pores on the salt/composite ratios.

Keywords Bioactive glass · Chitosan · Biomaterial · Salt leaching using powder · Porosity

Introduction

Large, nonhealing bone defects caused by trauma, tumor resection, or disease pose major clinical and socioeconomic problems. In situations with significant bone loss, bone grafts are used to fill the defect bone and promote bone tissue formation [1, 2]. More than 500,000 bone-grafting procedures are performed every year [3]. This number is projected to increase steadily due to population aging and correspondingly rising incidence of degenerative musculoskeletal diseases. At present, there is no ideal treatment for large bone defects. Bone allografts could be interesting; however, their clinical utility is limited by the risk of disease transmission and high cost [4–6]. To overcome the shortcomings of bone grafting, much research effort is focused on the development of

synthetic bone scaffolds as bone graft substitutes. They are widely regarded to be one of the key items along with cells and a dynamic environment for regeneration of damaged tissue [7–9]. Scaffolds are three-dimensional porous structures that act as templates for in situ bone regeneration. The successful design of a bone scaffold needs to incorporate both biological physicochemical and mechanical considerations [10]. The basic biological requirement of any bone scaffold is osteoconductivity, i.e. the ability to support the attachment of bone cells and to provide an interconnected pore structure through which cell migration and vessel formation can take place. To satisfy these conditions, scaffolds need well-interconnected pores and suitable porosity [11, 12] and large specific surface and uniform pore distribution with a degradation or resorption rate that the tissue replacement needs [13]; also, it needs to be biocompatible and biodegradable. Scaffolds are desired to minimize the amount of implanted material and to increase the specific surface area for cell attachment, a vibrant environment for regeneration of damaged tissue and tissue in growth [14, 15]. Cells are developed on a biodegradable porous scaffold in which the tissue is forming and growing; the scaffold gradually degrades.

For this reason, several methods have been developed to prepare porous scaffolds with well pore interconnectedness

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Fig. 1 SLUP process

According to the technique and chitosan rate, they were simply called SLUPS29, SLUPS50, SLUPS71, and SLUPS90.

Preparation of lyophilized scaffolds LS

The same range was synthesized by using lyophilization process: The first step has consisted to dissolve chitosan powder, with a medium molecular weight, in acetic acid solution 1% v/v. The solution was stirred under magnetic stirring at room temperature during 24 h in order to dissolve all the chitosan powder. The final step is the preparation of four scaffolds having the same CH and BG ratio as those prepared by SLUP method: LS:29CH, LS:50CH, LS:71CH, and LS:90CH presenting different ratios chitosan/glass (CH/BG). The glass was added in the chitosan solution. The mixtures were homogenized for 24 h in an ultrasonic bath. The solutions were cast into well plates. They were frozen at -20°C all-night to freeze the core of the material and placed in a freeze-dryer at 0.65 mbar at -60°C during 24 h to remove all solvents in samples. Obtained scaffolds were washed in a NaOH solution 0.4% w/v and distilled water to eliminate traces of acetic acid.

Physicochemical characterization of scaffolds

To verify that the synthesized material is an amorphous glass, its structure has been studied by using X-ray diffraction (XRD). XRD measurements were performed with a PAN analytical model operate at 40 kV and 30 mA using Cu K α radiation. The detector was scanned over a range of 2θ angles from 5° to 90° at a step size of 0.026° and well time of 40 s per step. The Fourier transform infrared spectroscopic analysis (FT-IR) was used to determine the functional groups composed the obtained scaffolds. A Bruker Equinox 55 spectrometer was employed for the FT-IR spectroscopy analysis. The samples of scaffolds powders were mixed with KBr powder and pressed into a disk suitable for FT-IR measurement. The FT-IR spectra were recorded from 4000 to 400 cm^{-1} . The morphology of the scaffolds was observed using scanning electron microscopy (SEM) (Jeol JSM 6301). The flow of electrical charges at the surface is necessary for the SEM analysis. For that, samples have been metalized by gold-palladium layer (a few μm of thickness).

Specific surface area and pore size distribution of the samples have been calculated by using Brunauer–Emmett–Teller

Table 1 Chemical composition of scaffolds

Sample	Solvent volume 1% (ml)	Mass of chitosan (g)	Concentration of the chitosan solution (%)	Mass of used glass (g)	% by weight of chitosan in composites	Composite notation	NaCl/Composite
SLUP29	80	0.80	1	2.00	29	46S6:29CH	1
SLUP50	140	1.40	1	1.40	50	46S6:50CH	1
SLUP71	200	2.00	1	0.80	71	46S6:71CH	1
SLUP90	252	2.52	1	0.28	90	46S6:90CH	1

(BET) N₂ adsorption-desorption method (Flowsorb II 2300, Coultronics France SA).

The Helium Pycnometer device measured porosity of the scaffolds. The experiments were performed five times, and the average porosity value was obtained. The porosity (P) of the scaffolds was obtained by the following equation.

$$\text{Porosity} = \frac{V_0 - \frac{m}{\rho}}{V_0} \times 100(\%)$$

where

V₀: The apparent volume of scaffold, which is calculated using the outer dimension of scaffold;

m: Mass of scaffold from which NaCl is leached out;

ρ: Density of sample. The following curve shows the evolution of porosity rate according to the composition of SLUP scaffolds from 73 to 90%.

In vitro chemical reactivity study

In vitro assays, as described in a previous work [31], were realized in a SBF whose the chemical composition is similar to the blood plasma (Table 2) [31, 32]. A total of 30 mg of powder samples were immersed in triplicate in 60 ml of SBF at a temperature of 37 °C and maintained under controlled stirring at 50 rpm. At the end of soaking time, the powder samples were removed and rinsed with deionized water to stop the exchange reactions. The powder samples were dried and stored for further investigation of the formation of HA layer on the surface of powder samples. This hypothesis was verified by X-ray diffraction. The SBF solutions were stored in fridge to evaluate the ionic exchanges between materials and SBF by ICP-OES (Spectro Ciros Vision Ametek).

Results and discussion

Morphological study of scaffolds

Binocular microscopy

The obtained images of SLUP scaffolds before and after the salt leaching using binocular microscopy are shown in Fig. 2.

Table 2 Ion concentrations (mM) in SBF and in human blood plasma [32]

		Na ⁺	K ⁺	Ca ²⁺	Mg ²⁺	Cl ⁻	HCO ₃ ⁻	HPO ₄ ²⁻
t2.3	SBF	142	5	2.5	1.5	148.8	4.2	1
t2.4	Blood plasma	142	5	2.5	1.5	103	27	1

The porogen was used with the aim of optimizing the pores network; the solid porogen agent spreads in the structure in a controlled manner. It makes it possible to have better control of the pore size, particularly access to large sizes and better control of the pore's organization. The porogenic agent chosen (in particulate form, preferably (quasi-spherical) may be preferentially removed to obtain the porous layer whose pores may have substantially the shape and size of this pore-forming agent.

As depicted in Fig. 2, porosity appears in Fig. 2a just after salt-leaching out. The obtained images show that the composite before leaching (Fig. 2a) is characterized by a compact surface covered by a layer of porogen. After leaching (Fig. 2b), we clearly observe a radical change in the surface of the composite with the appearance of an organized porosity that occupies the entire surface of the composite. The efficiency of the process is directly related to the porogen.

SEM

Morphology of the surface and of the interconnectivity of the fabricated scaffolds is presented in Fig. 3. Porous structure was observed on both; lyophilized scaffolds (Fig. 3a) and SLUP scaffold (Fig. 3b) at BG:chitosan contents of 50:50. A regular porous structure was observed in SLUPS; however, that of LS was irregular.

From a morphological point of view, the porosity of SLUP scaffold is much greater than that of LS scaffolds. SEM images showed the influence of the salt particles on the pore size. It highlights the form and pore size uniformity. It shows the surface image and macrostructure of original salt-leaching scaffold with NaCl treatment. It demonstrates that pores create an interconnected network and are ideal for the vascularization. Therefore, the microstructure of the SLUP scaffolds is extremely promising since it is highly porous, with a well-developed network of interconnected pores.

This analysis aims also to observe the distribution and architecture of the pores and to understand the association between bioactive glass and chitosan matrix in a regular porosity. We have noticed from the SEM images that this phenomenon is very important in the SLUP scaffolds. Good interconnectivity of the global pores was observed, and the coexistence of orders in obtained pores was verified.

The pores and caves structure in each sample can be clearly observed; with the pore size of around 30 μm sieving process in the SLUP scaffold (Fig. 3a) and heterogeneous pore size in the lyophilized scaffold as depicted in Fig. 3b.

Image of lyophilized scaffold (Fig. 3b) showed irregular pore structure and hole presence. There are no distinct and deep pores. The size and the shape of the pores were nonuniform, and the pores tended to be piled up pell-mell with each other. For tissue engineering applications, irregular structure is unfavorable for the cell migration and growth because of the

Fig. 2 Images of SLUP scaffolds before (a) and after (b) the salt leaching using binocular microscopy

pore architecture wish going from the surface to interior of matter. However, the pore structure became more and more clear and integral with the increase of NaCl content. Especially, obvious honeycomb-like structure could be observed especially in the samples with higher NaCl (NaCl: composite = 1) as shown in Fig. 3a. Upon salt particles, the obtained results show a soft structure with regular pores. Before leaching, there is a lot of obvious configuration like oval-shaped salt powder, which is leached out. The picnometric measurements revealed that 90% of pore size corresponds to the particle size of NaCl used as porogen.

The salt particles spread to the bottom of the scaffold matrix creating a high porosity as shown in Fig. 4. Therefore, the porosity of SLUP scaffold is higher than that of traditional scaffold. The porosity of the material is one of the key parameters successes in tissue engineering applications. Its pore volume (void volume inside), pore size, and specially the interconnection between pores have a major influence on the ability of the bone-implant vascularization and gradually biomineralization. They subsequently influence adhesion, cell migration, and tissue penetration within structures. It is therefore an important parameter for tissue engineering. Other studies have

Fig. 3 Morphology of the surface and of the interconnectivity of the fabricated scaffolds. a Lyophilized scaffolds; b SLUP scaffold

Fig. 4 Salt particles spread to the bottom of the scaffold matrix creating a high porosity

reported that the induced porogen on the composite matrix generates a regular porosity [27, 33].

XRD and BET

The obtained diffractograms are presented in Fig. 5. The 46S6-CH composite before the addition of progen presents a diffraction halo between 16 and 24 ($2\theta^\circ$) that we also observe on the diffractogram of chitosan alone. We also note the appearance of a halo at 30 ($2\theta^\circ$) which highlights interactions between bioactive and chitosan. After the addition of porogen, we notice the appearance of the peaks corresponding to NaCl (31.8 and 45.4 ($2\theta^\circ$)), which disappear just after salt-leaching out. They assert that the crystalline structure of the scaffolds

after salt leaching retains its amorphous starting structure, which proves that the totality of porogen was leached out even that incorporated on the scaffolds structure leaving voids interconnected between them. After water leaching, the NaCl was dissolved in water and the peaks of this phase in the XRD pattern disappeared accordingly after salt-leaching out as shown in Fig. 1, and no streak attributed to NaCl was detected on the scaffold diffractogram. This result was also confirmed by EDS analysis as shown in Fig. 6, which shows the disappearance of the porogen within the matrix of the composite.

Porosity calculation

SLUP scaffolds possessed highest porosity (90%) as presented in Fig. 7. The high porosity of a scaffold can provide benefits for cell growth and migration. We observe more that the BG/CH ratio decreases towards a more porous structure. Table 3 presents the evolution of porosity rate according to the composition of our SLUP scaffolds and LS. Open porosity varies from 73 to 90% of SLUP when the open porosity rate of LS was too low and varies from 12 to 31%.

Comparing SLUPS with LS, BET measures show an important evolution of pore volume in the case of SLUPS instead of LS as depicted in Fig. 8. In the case of LS, the pore volume varies between 3.8 and 6.5 $\text{mm}^3 \text{g}^{-1}$ depending on the BG:CH ratio. However, it varies between 5 and 6 in the case of SLUP scaffolds.

This characteristic justifies more and more the advantages of the technique “SLUP” and allows us to understand the physical behavior at the interior of the scaffolds matrix. In fact, SLUP is pressure free; there was a definite space between the powder particles, and this space does not shrink while melting composite, which leads to create a large pore volume.

Fig. 5 Diffractograms

Fig. 6 EDS analysis

Specific surface area

The specific surface area represents the total area per 1 g of sample. It is therefore necessary to consider the entire surface of each particle, open porosity included. The physical

Fig. 7 SLUPS composition

principle, recognized for the determination of the specific surface, is based on the adsorption of gas at low temperature. It allows a measurement without modification of the geometrical texture of the sample and determining the area of the entire surface of the powder particles, including the surface of the open pores accessible to the external gas molecules.

Figure 9 shows a progression of the specific surface area of SLUP scaffolds by increasing the chitosan rate in there, which varies between 20 and 37 m² g⁻¹. This increase is much more remarkable when applying the salt-leaching technique compared with the loyalty method, which showed a slight increase in a low specific surface area.

Table 3 Porosity rate according to the composition of SLUP scaffolds

Sample: SLUP	Porosity %	Sample LS	Porosity %
SLUP29	73	LS29	12
SLUP50	75	LS50	16
SLUP71	95	LS71	22
SLUP90	90	LS90	31

Fig. 8 BET measures comparing SLUPS with LS

This increase is much more important when applying the SLUP technique compared with the conventional chemical method, which showed a slight increase in a low specific surface area.

BET analysis nitrogen

Porosity was evaluated by Brunauer–Emmet–Teller (BET) method using Micromeritics ASAP analyzer Isotherms of SLUP scaffolds as shown in Fig. 10.

All samples exhibited isotherms of type IV based on the IUPAC classification. According to the International Union of Pure and Applied Chemistry (IUPAC) classification, the obtained isotherms (Fig. 10) belong to type H3. The curves of adsorption and desorption form a hysteresis loop. The hysteresis cycle is due to the capillary condensation phenomenon characteristic of mesoporous material (pore diameter between 2 nm and less than 50 nm). On each hysteresis cycle, shoulders, characteristic of several families of mesoporous materials, are observed at $P/P_0 = 0.5$.

Pores size control

In this process, we used porogen as a porosity control tool. This analysis was done to verify the hypothesis of the dependence of the pore size as well as the homogeneity of porosity on the particle size of the porogen. For this purpose, we have worked with a range of porogen varying each time the particle size. The pores have the same size as the porogen particles. The SEM photos of these composites clearly show the change in pore size from one composite to another relative to the change in pore size. We can deduce that pores take the same size of porogen particles as confirmed by SEM graphs (Fig. 11).

Influence of porogen rates on the specific surface and porosity volume of scaffold SLUPS

Using the proposed SLUP technique, the specific surface and porosity volume could be easily controlled via a simple procedure, as depicted in Fig. 12. It has been noticed

Fig. 9 Progression of the specific surface area of SLUP scaffolds by increasing the chitosan rate

Fig. 10 Porosity evaluated by Brunauer–Emmet–Teller (BET) method using Micromeritics ASAP analyzer Isotherms of SLUP scaffolds

that the pore volume increases by increasing the rate of porogen in the composition of the scaffold (Fig. 12), which allows to control pore's volume and to have the pore volume of the requested pore.

Kinetic of the chemical reactivity of SLUPS

The XRD patterns of the glass after soaking in SBF for different periods confirm the formation of carbonated

Fig. 11 SEM graphs of parogen particles

Fig. 12 Pore volume increases by increasing the rate of porogen in the composition of the scaffold

hydroxyapatite (HCA) on the surface of SLUPS. Figure 13 shows the XRD patterns for SLUPS 50 before and after soaking in SBF at different time intervals (24, 48, and 72 h). After 24 h of soaking in SBF solution, two characteristic peaks of hydroxyapatite at 25.80° and 31.79° were observed. The intensity of the peaks increases with increasing time of soaking to 3 days. These results highlight the high bioactivity of SLUP scaffolds.

Porogen effects on the kinetic of the chemical reactivity

Evolutions of silicon, calcium, and phosphorus concentrations in SBF, measured by ICP-OES, versus soaking time, are presented in Fig. 14. The evolution of the concentrations of all these elements followed a similar trend: strong evolution within the first 3 days. The concentration of silicon increased during the first 24 h. At the same time, the calcium and phosphorus concentration decreased. The evolution of silicon concentration in SBF clearly indicated that a dissolution of the glass occurred during the first hour after soaking. This evolution is due to the high porosity of SLUP scaffold. The decrease of the calcium and phosphorous concentration

indicated that there was a calcium and phosphorus uptake by the glass surface.

The effect of the presence of porogen on the reactivity of the SLUPS surface has been studied. A total of 50 SLUPS and 50 LS were selected for testing in vitro. Evolution of silicon, calcium, and phosphorus concentrations in SBF, measured by ICP-OES versus soaking time, are presented on Fig. 14a-c, respectively.

For SLUPS, the Si concentration increases from 0 to 52 ppm in the first 24 h, while for the LS, the Si concentration increases to only 25 ppm. Therefore, the release of the silica and, consequently, the dissolution of SLUPS occur during 24 h. The same applies to the calcium and phosphorus, their concentrations in SBF decrease after 24 h in the case of SLUPS than LS. For more information on evolution of calcium and phosphorus concentration in SBF, we have analyzed these concentrations for a short soaking time. After 24 h, the concentration of calcium increases from 180 to 370 ppm and of phosphorus from 75 to 65 ppm during the first 24 h. In conclusion, the presence of porogen affects fragility and porosity, which increases the ion exchanges and consequently the bioactivity improvement.

Fig. 13 XRD patterns for SLUPS 50 before and after soaking in SBF at different time intervals (24, 48, and 72 h)

Fig. 14 Evolutions of silicon (a), calcium (b), and phosphorus (c) concentrations in SBF, measured by ICP-OES, versus soaking time

Conclusion

In this work, we proposed a novel method known as SLUP, which complements the disadvantages of the conventional salt-leaching method. Thus, SLUP does not require solvent or high pressure. Furthermore, SLUP provides scaffolds with higher porosity than that of the conventional salt-leaching scaffolds. The present study demonstrates a new method to modify the surface of porous scaffolds based on the solution casting and salt-leaching technique of chitosan-based hybrid polymer scaffolds. The porosity of the scaffolds showed high porosity. The SEM observations showed the increases of pore size interconnectivity. It can be concluded that salt leaching is a potential technique for chitosan-based scaffolds and can contribute to improve significantly their physicochemical properties. This simple salt-leaching process can be potentially applied to various biodegradable scaffolds for tissue engineering. This phenomenon can be understood as a result of the well-interconnected structure shown in the SEM image of the SLUP scaffold. Moreover, to reveal the simplicity and expandability of the proposed scheme, that is, SLUP, a three-

layer scaffold, with each layer having a different porosity and pore size, was fabricated. Thus, the SLUP (salt leaching using powder) technique may be a promising method for fabricating a scaffold in lab-scale experiments because when using SLUP, it is easy and facile to fabricate a well-interconnected scaffold with well-defined geometry.

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