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Ceramics International 37 (2011) 2445–2451
CERAMICS INTERNATIONAL
www.elsevier.com/locate/ceramint

Gentamicin sulphate release from lost foam wollastonite scaffolds using poly(DL-lactide-co-glycolide) acid

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Received 21 January 2011; received in revised form 24 March 2011; accepted 25 March 2011
Available online 6 April 2011

Abstract

A study on the gentamicin sulphate release from lost foam wollastonite scaffolds using poly(DL-lactide-co-glycolide) acid (PLGA) was performed. Scaffolds were made through the lost foam technique using two different evaporative-patterns: poly(methyl-methacrylate) (PMMA) spheres and polyurethane (PUR) sponge. With the aim to control the rate of the gentamicin sulphate delivery, poly(DL-lactide-co-glycolide) acid was used. The porous scaffolds were gentamicin sulphate loaded by different ways. All methods showed the same gentamicin sulphate release pattern: a high rate of release in the first 24 h followed by a slow rate for a period longer than 300 h. The compression strength of the PLGA filled pores scaffolds after a degradation test was measured and compared with that of empty pores scaffolds, having as a result a notable increase in the compression strength. A hemolysis test with human blood was made to each delivery system. With only one exception, every system showed a hemolysis lower than 5%, proving to be hemocompatible. The scaffolds made by using PMMA spheres showed a better behavior in the drug release process, as well as higher mechanical properties.

Keywords: Drug delivery; Gentamicin; Wollastonite scaffolds; Poly(DL-lactide-co-glycolide) acid

1. Introduction

The donor tissue is preferred to perform bone substitutions. Even if these tissues have good biocompatibility, they are expensive, hard to get, and present risk of disease transmission. The ceramic materials used in reconstructive surgery can be classified in two main groups: bioinerts and bioactives. The bioinert materials have none or a very little influence in the surrounding tissue. The bioactive materials bond to living tissue. The surgeons apply these ceramics to fill and/or substitution of bones, and depending of the situation, sometimes they are required in form of pellets, scaffolds or dense forms. In cases where mechanical properties are only achieved by metals a ceramic coat is applied to the metallic prostheses [1]. The use of porous materials presents some benefits the most important of them is the ability of the surrounding bone tissue to grow into the pores of the implant, resulting in a mechanical bond. The bone growth into the material stabilizes it and gives strength to the implant [2]. Several studies mention an optimal pore size, but not all the authors obtained the same results: Chang et al. [3] mention pores of 50 μm or bigger, Klawitter [4] recommends a minimum size of 200 μm, Kuboki et al. [5] find the optimal pore size between 300 and 400 μm and Gauthier et al. [6] report that 565 μm pores are better than the 300 μm ones. An optimal pore size does not assure the bone growth and proliferation. The pore interconnectivity also plays an important role [7]. In addition, the interconnectivity and the architectural characteristics play a very important role in the revascularization of the scaffolds. Bai et al. [8] studied the effect of the porous structure parameters on vascularization of biomaterials. Using assembled organic microspheres as templates combined with casting technique they produced a series of disk-type β-tricalcium phosphate (β-TCP) with variable pore sizes and variable interconnections. The increase in pore size only resulted in an increase in size of the blood vessels growing into the macroporous of the bioceramic scaffolds. However, with the increase in size of interconnection,

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0272-8842/$36.00
doi:10.1016/j.ceramint.2011.03.035

both the size and the number of the blood vessels formed in the macroporous increased, concluding that the size of the interconnections is more important for vascularization in the scaffold compared with the pore size. Statistics reveals that infections in hip prosthetics are presented between 2 and 4% of the cases, while bone infections rise up to 45% in nails used in external fixation. One of the principal aims is to reach the infected zone of the bone to deploy the antibiotic. Another advantage of the implant fabrication would be to include drugs like antibiotics [9,10]. The aim of the present work is the development of an inorganic–organic composite for a local delivery of antibiotic to bone that retards the antibiotic release in a proper way. The selected material to make the scaffold was wollastonite. Even thought calcium phosphates are the preferred materials for these applications due to the similarity to biological apatites, the materials that bond at a faster rate to the bone tissue are those that contain silicon, such as bioactive silicate glasses and wollastonite. Xu et al. [11] compared the *in vivo* bone-regenerative capacity and resorption of the porous β-calcium silicate (β-$CaSiO_3$, β-CS) bioactive ceramics with porous β-tricalcium phosphate (β-$Ca_3(PO_4)_2$, β-TCP) bioceramics. The resorption of β-CS was much higher than that of β-TCP and more bone formation was observed with β-CS as compared with β-TCP. Furthermore, few works have been reported about the use of wollastonite for scaffolds manufacture. An additional possible advantage of this material is the fact that it can be heat treated in order to achieve higher mechanical properties. In order to retard the delivery of gentamicin in the system, poly(DL-lactide-co-glycolide) acid (PLGA) was chosen to capture the antibiotic due to its biocompatibility and degradation rate, which will release gentamicin and promote new bone formation [12].

2. Materials and methods

2.1. Fabrication of scaffolds

Wollastonite (Gosa, S.A., Mexico), with an average particle size of 18.36 μm, was used as a raw material to make the scaffolds and its chemical composition is shown in Table 1. Two methods were employed to elaborate the wollastonite scaffolds used in the experiments: *Lost sponge method:* A polyurethane sponge (average pore size 517.08 μm, pore size range 200–1000 μm) was soaked in a slurry made by adding 3.14 g of wollastonite and 0.99 g of sodium silicate, which acts as a bonding agent, to 3 ml of deionized water. In this case, the slurry had to be fluid enough to penetrate the whole sponge. After the impregnation, the loaded sponge was dried at 300 μC for 2 h (heating rate of 1 °C/min) and finally sintered at 1250 °C for 10 h (heating rate of 10 °C/min). During this last step, the polyurethane was burned leaving behind interconnected porosity. To obtain a cylinder form required for the compression strength test of the scaffolds, a hot ring of iron wire (6 mm in diameter) was pressed against the sponge. Lost spheres method: In this method 3.6 g of poly(methyl-methacrylate) spheres (ranging from 50 to 100 μm in diameter) were added directly to a slurry composed by 3.14 g of wollastonite, 0.99 g sodium silicate and 3 ml of deionized water. In order to obtain interconnected porosity, it was necessary to ensure the contact among spheres, thus the mixture was pressed into a steel piston (6 mm of internal diameter) applying a load of 500 kg using a hydraulic press. The hardened mixture was dried and sintered at the conditions mentioned above.

Table 1
Chemical composition of wollastonite used in the experiments (mol%).

CaO	SiO_2	Fe_2O_3	Al_2O_3	Na_2O	MgO	K_2O
45.91	52.85	0.179	0.298	0.041	0.663	0.003

2.2. Incorporation of gentamicin sulphate to the scaffolds

In order to fill and coat the porous structures with gentamicin sulphate, three methods were used.

S+R method: As a first step the sintered scaffolds were immersed in an aqueous solution of gentamicin sulphate (0.27 g of gentamicin sulphate in 1 ml of deionized water) and then they were air-dried (*S* stands for the layer of crystallized gentamicin sulphate). As a second step the treated scaffolds were immersed in a solution made of PLGA in dichloromethane applying a slow suction under vacuum to force the solution to fill the structure and then they were air-dried (*R* stands for the PLGA layer). This procedure was repeated for 3 times. *Sus method:* The filling and coating material was a composite. The sintered scaffolds were dipped in a suspension of gentamicin sulphate (0.27 g) in a solution made of PLGA in dichloromethane (0.3 g of PLGA in 1 ml of dichloromethane) applying a slow suction under vacuum to force the suspension to fill the structure and then they were air-dried (3 times, *Sus* stands for the layer of a composite PLGA–gentamicin sulphate). *S+Sus method:* The sintered scaffolds were treated as mentioned above to obtain, firstly, a layer of crystallized gentamicin sulphate and then a layer of a composite PLGA–gentamicin sulphate. This procedure was repeated for 3 times.

2.3. Drug release

Each antibiotic loaded scaffold was immersed in 50 ml of SBF solution [13] using individual polyethylene flasks up to 30 days at 37 °C. The sampling was made taking 4 ml of solution and then replacing them with 4 ml of fresh SFB. The concentration was measured by UV/visible light spectrophotometry using a wavelength of 210 nm. The experimental design for the drug delivery tests, as well as the loading amount of gentamicin, are shown in Table 2.

2.4. Mechanical testing

To determine the effect of the filling-pore material on the compressive properties of the scaffolds, two sets of scaffolds were tested. The first set consisted of empty-pore scaffolds and the second set consisted of filled-pore scaffolds. The scaffold pores were filled with PLGA according to the procedure mentioned in Section 2.2. These treated scaffolds were then

Table 2
Characteristics of the systems used for gentamicin sulphate release.

System ID	Scaffold fabrication method	Layers of crystallized gentamicin sulphate	Layers of PLGA	Layers of composite PLGA–gentamicin sulphate	Loading amount of gentamicin sulphate (mg)
S+R(P)	Lost Spheres	X	X		126.5
S+R(E)	Lost Sponge	X	X		169.9
S+Sus(P)	Lost Spheres	X		X	214.3
S+Sus(E)	Lost Sponge	X		X	139.7
Sus(P)	Lost Spheres			X	112.7
Sus(E)	Lost Sponge			X	79.4

immersed in SBF for a period of four weeks at 37 °C in order to emulate the degradation that the polymer would exhibit during the drug delivery period. Five samples (6 mm in diameter, 12 mm in height) of each designed scaffold were tested using a Controls uniaxial testing system (model 50-C7024) with a 15 kN load cell. The crosshead speed was set at 0.5 mm/min and the load was applied until the scaffold was cracked. The mechanical strength evaluation was performed according to the ASTM F 451-95 standard.

2.5. Physical properties

The pore size and porosity of the scaffolds were measured using the specific techniques of the Image Pro Plus® software. Fifty fields of each sample were used to measure both porosity and the pore size. A liquid displacement method was used to evaluate the scaffolds density. A scaffold of weight W was immersed in a graded cylinder containing a known volume (V_1) of water. The cylinder was placed under vacuum to force the water into the pores of the scaffold until no air bubble emerged. The total volume of the water and scaffold was then recorded as V_2. The volume difference ($V_2 - V_1$) was the volume of the skeleton of the scaffold. The total volume of the scaffold, V, was then: $V = V_2 - V_1$. The apparent density of the scaffold, ρ, was evaluated using $\rho = W/(V_2 - V_1)$. The scaffold morphology (surfaces and fracture surfaces) was studied using scanning electron microscopy (Philips XL30 ESEM).

2.6. Hemolysis test

The basis of the hemolysis test is the following: when the external membrane of the erythrocytes is destroyed, hemoglobin is released. It is possible to estimate the amount of destroyed erythrocytes in a given test by measuring the quantity of hemoglobin in a sample. This works as a reference to know the toxicity grade of a dispositive that will be in contact with human blood. In order to make this experiment, negative and positive controls are needed in each run [14]. The negative control (0% hemolysis) should yield the less amount of hemoglobin, and it is done by adding erythrocytes to an isotonic solution. The solution used was the Alsever, made by adding 3.97035 g of sodium citrate and 2.07462 g of sodium chloride to 500 ml of deionized water. In the positive control (100% hemolysis) all the erythrocytes are destroyed when they are added to deionized water. To obtain the erythrocytes a donor's blood was centrifuged at 3000 rpm for 4 min. This step was done at 4 °C. Afterwards the plasma was decanted, the erythrocytes were washed with Alsever solution to be centrifuged one more time. This step was repeated three times. The solution used in the experiment was made by adding 100 μl of erythrocytes to 10 ml of cold Alsever solution in a Falcon tube gently stirred. A quantity of 150 μl of this solution was used for each individual tube in the experiment. The positive control tube had 1850 μl of deionized water, while the negative control tube and the tubes with scaffolds had 1850 μl of Alsever solution. The tubes were placed in a water bath shaker at 37 °C for 1 h. Finally, the tubes were centrifuged one last time to measure the hemoglobin by UV-visible light spectrophotometry using a wavelength of 415 nm (Spectronic, model Genesis 5). The test for each delivery system was done three times.

3. Results and discussion

3.1. Physical and mechanical properties

Fig. 1 presents SEM images of the fracture surfaces of scaffolds made by the lost sponge and the lost spheres methods, as observed, the pores in the scaffolds are interconnected presenting irregular and spherical morphologies, respectively. Table 3 shows the physical properties of the scaffolds made by the two described methods. The size pore range for the scaffolds made by the lost sponge method varies from 170 to 891 μm, while for the scaffolds made by the lost spheres method varies from 23 to 143 μm. Therefore, the two different pore sizes are compatible with osteoconduction. The values of the interconnected porosity for both scaffolds confirm that a high percentage of the pores are interconnected. The measured apparent densities of the scaffolds are in the range of the apparent density of the trabecular bone (0.14–1.1 g/cm^3) [15]. Fig. 2 shows SEM images of the fracture surfaces of scaffolds made by the lost sponge and the lost spheres methods with the pores filled of PLGA. The pores were filled in order to determine the effect of the filling-pore material on the compressive properties of the scaffolds. As it can be seen, the pores were almost completely filled of PLGA.

Fig. 3 and Table 4 show the results of the compression tests. Both types of as-fabricated scaffolds showed a brittle fracture. The strength of both types of as-fabricated scaffolds was in the range of human trabecular bone and lower than that of human cortical bone. The strength of the scaffolds made by the lost spheres method (4.486 MPa) was significantly greater than the obtained for the scaffolds made by the lost sponge method

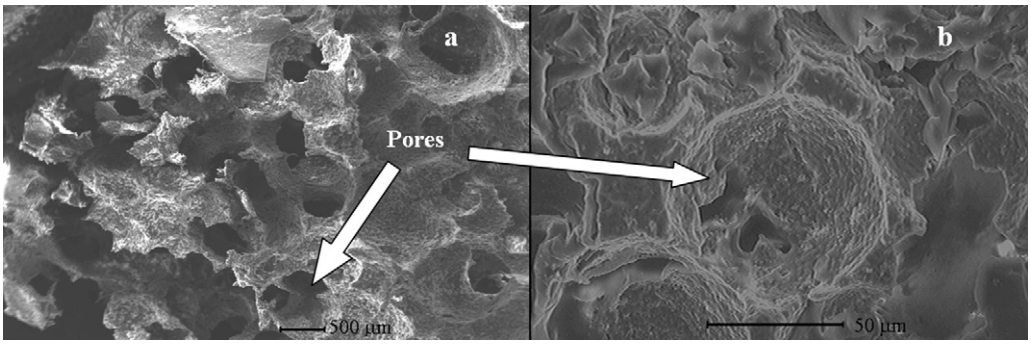

Fig. 1. Fracture surfaces of scaffolds made by (a) the lost sponge and (b) the lost spheres methods.

Table 3
Physical properties for the scaffolds used in the experiments.

Fabrication method	Density (g/cm^3)	Interconnected porosity (%)	Pores	
			Average size (μm)	Size range (μm)
Lost sponge	0.73847956	60.769108	442.126098	170–891
Lost spheres	0.57241846	65.383003	66.8173031	23–143

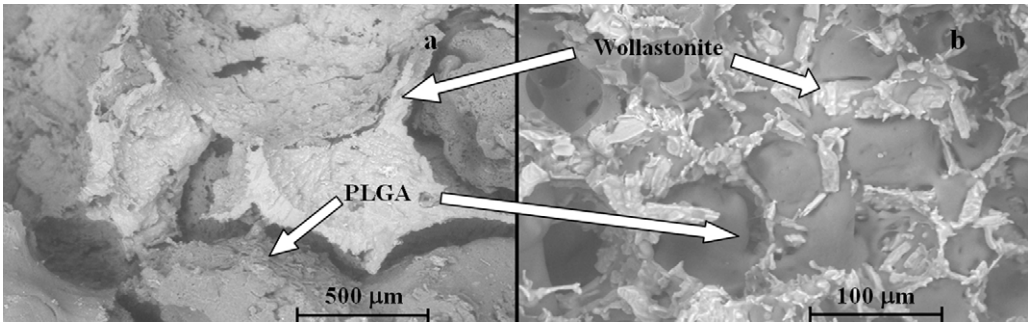

Fig. 2. Fracture surfaces of scaffolds with the pores filled with PLGA made by (a) the lost sponge and (b) the lost spheres methods.

(3.104 MPa) (Two-way ANOVA, $p < 0.002$). The higher strength value was obtained for the scaffolds made by the lost spheres method, despite its lower density, this can be attributed to its more regular form and pore distribution. The strength of both types of scaffolds with the pores filled of PLGA was significantly higher than those obtained for the as-fabricated scaffolds, increasing the value of this property in 44% and 79% for the scaffolds made by the lost sponge and the lost spheres methods, respectively. These strength values are higher than those reported for porous calcium silicate systems, made by the lost sponge method as-fabricated and with the pores filled of PLA (strength of 0.3 MPa and 1.45 MPa, respectively) [16]. Porous systems of wollastonite–chitosan composites also presented lower compressive strength (0.12 MPa) [17]. The strength obtained by the scaffold made using the lost spheres method with PLGA filled pores presented values comparable to those obtained by dense wollastonite–titania composites (6.8–9.97 MPa) [18]. The strength of the scaffolds obtained in this work is higher than those obtained by porous TCP scaffolds obtained by selective laser sintering (2.26 MPa) [19], however they are lower than those presented by porous HA–gelatin composites (9.7–32.1 MPa) [20]. Lin et al. [21] worked with macroporous calcium silicate ceramics (MCSC) with different pore sizes and porosities, using polyethylene glycol (PEG) as a porogen instead of PMMA. The compressive strength and porosity of MCSC was controlled between 4.92 and 65.21 MPa and 41.28 and

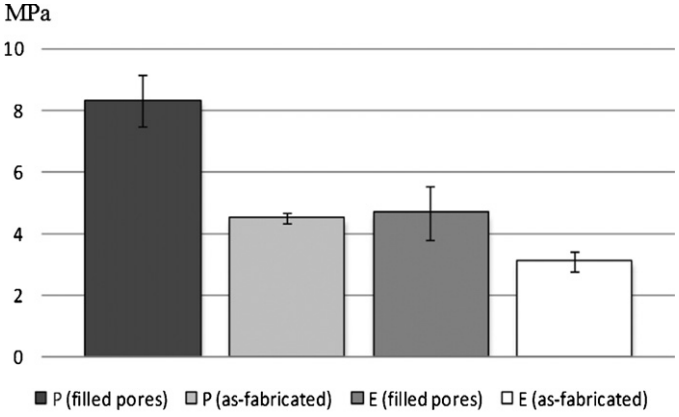

Fig. 3. Compressive strength of the scaffolds made by the lost spheres and the lost sponge methods both as-fabricated and with filled pores.

Table 4
Compressive properties of the scaffolds made by both the lost spheres and the lost sponge methods as-fabricated and with filled pores (data for human bone are presented for comparison).

Sample	Fabrication method	PLGA filled pores	Compressive strength (MPa)
P polymer	Lost Spheres	Yes	8.32
P normal	Lost Spheres	No	4.486
E polymer	Lost Sponge	Yes	4.65
E normal	Lost Sponge	No	3.104
Human trabecular bone			0.7–15.0 [12]
Human cortical bone			131.1–206 [12]

73.60%, respectively, through regulation of the weight fraction and the particle size of the $CaSiO_3$ and PEG.

3.2. Gentamicin sulphate release

Fig. 4 shows the behavior of each of the six drug delivery systems immersed in SBF. As it can be observed there is a burst of the gentamicin sulphate concentration in the SBF during approximately the first 12 h followed by a slow gentamicin sulphate release for a long time period for all the systems. When examined by SEM, a thick layer of PLGA was observed on the surface of the scaffolds made by the lost spheres method (Fig. 5). Due to the larger pore size of the scaffolds made by the sponge lost method, the polymer penetrated inside the piece

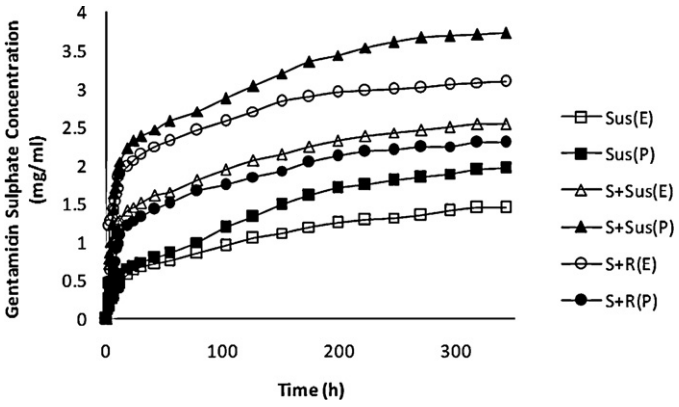

Fig. 4. Gentamicin sulphate release from the scaffolds in SBF. System ID is shown in Table 2.

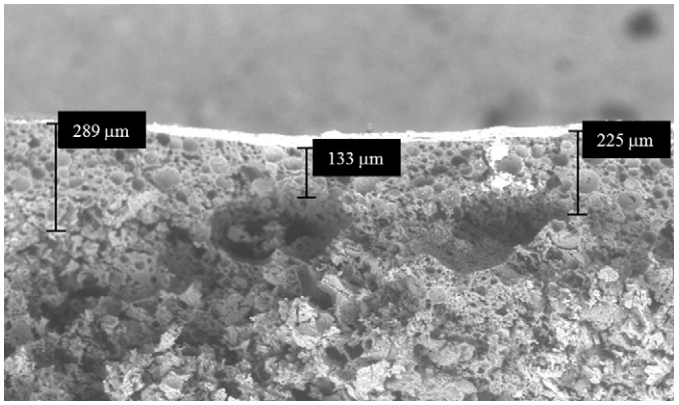

Fig. 5. PLGA layer on the surface of a scaffold made by the lost spheres method.

Table 5
Average absorbances and hemolysis percentages.

Sample group	Sample	Average absorbance	Hemolysis %
Scaffolds made by the lost spheres method	Control +	1.3133	100
	Control −	0.0266	0
	Sus(*P*)	0.0470	1.5803
	S+*S*(*P*)	0.0575	2.3963
	S+*R*(*P*)	0.0440	1.3471
Scaffolds made by the lost sponge method	Control +	2.2703	100
	Control −	0.0210	0
	Sus (E)	0.0805	2.6452
	S+S (E)	0.1780	6.9798
	S+R (E)	0.0636	1.8968
Scaffolds without antibiotic fixation	Control +	2.0256	100
	Control −	0.0380	0
	P	0.0505	0.6288
	E	0.0965	2.9431

easier, avoiding the formation of a thick PLGA layer on their surface. For the case of the *S*+*Sus*(*P*) system the layer of composite PLGA–gentamicin sulphate is a good source of antibiotic, but in the case of the *S*+*R*(*P*) system the layer of PLGA retards the drug release. Because of its two gentamicin sulphate sources (the layer of crystallized gentamicin sulphate and the layer of PLGA–gentamicin sulphate composite), the *S*+*Sus*(*P*) system was the one that managed to capture and release the highest amount of gentamicin sulphate. Taking into account the behavior of the *Sus*(*E*) and *Sus*(*P*) systems, it looks like the scaffolds capture a higher amount of antibiotic when they are immersed in the gentamicin sulphate solution

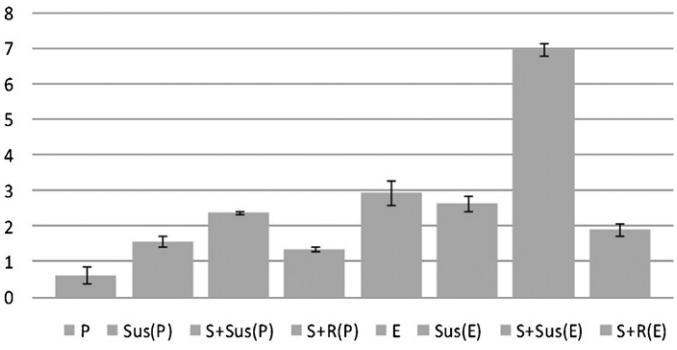

Fig. 6. Hemolysis percentage of each drug delivery system.

Table 6
Gentamicin sulphate delivered to SBF during the first 12 h and order of kinetic constant values for the delivery process.

System	12 h		12–342 h	
	Gentamicin concentration (mg/ml)	Gentamicin delivered (%)	Order (n)	Constant (k, h^{-1})
Sus(*E*)	0.45	31.7	0	0.0027
Sus(*P*)	0.57	29.3	0	0.0043
S+*Sus*(*E*)	1.29	51.0	0	0.0039
S+*Sus*(*P*)	2.05	54.9	0	0.0064
S+*R*(*E*)	1.87	60.6	0	0.0047
S+*R*(*P*)	1.08	47.0	0	0.0040

comparing with the amount that they capture when immersed into the suspension of gentamicin sulphate in PLGA–dichloromethane. Table 5 shows the gentamicin delivered to SBF during the first 12 h. As expected, the amount of gentamicin delivery is lower for the *Sus* systems (*E* and *P*). This indicates that the external composite layer retards the delivery process. On the other hand, in the systems where a layer of crystallized gentamicin is present (*S* systems) a higher burst of gentamicin release is observed. Other studies about drug delivery systems using wollastonite [18] report the highest gentamicin sulphate concentrations measured in SBF as 0.8 mg/ml, which were reached in 4 h. The systems tested in this study showed higher antibiotic charges reaching until 3.5 mg/ml of gentamicin sulphate in the SBF and release periods longer than 300 h. As observed in Fig. 5, the delivery occurred in two stages: the first stage when a burst of gentamicin sulphate happens and a second stage when a slow delivery of gentamicin sulphate occurs. Due to this, the kinetic study was only performed for analyzing the second stage. Table 5 shows the order and kinetic constant values for the delivery process in SBF from 12 to 342 h for the studied systems. In this period of time all the systems demonstrated a delivery of order 0, indicating that the rate of delivery is independent on the gentamicin concentration. The highest rate constant value corresponds to the *S*+*Sus*(*P*) system, because of its two gentamicin sources. On the other hand, the lowest rate constant value corresponds to the *Sus*(*E*) due to the fact that the gentamicin is only one of the phases of the composite. The rest of the systems have a similar rate constant value.

3.3. Hemolysis test results

In order to obtain the hemolysis percentage (erythrocites destruction) for each drug delivery system, the following equation was used: $H_i = 100 * (A_i - C^-)/(C^+ - C^-)$, where: H_i = hemolysis percentage of the sample i, A_i = absorbance measured of the sample i, C^- = absorbance of the negative control (0% hemolysis), and C^+ = absorbance of the positive control (100% hemolysis). Table 5 shows the average measured absorbances as well as their respective hemolysis percentage. The analysis was performed using 2 groups of 15 experimental units, where the drug delivery systems made by the lost sponge and the lost spheres methods were compared. In addition, 1 group of 12 experimental units of scaffolds without antibiotic fixation was used as blank. Fig. 6 shows the hemolysis results for each system. Considering the results presented in Fig. 6 and Table 6 the hemolysis is notably higher for the scaffolds made by the lost sponge method, being the *S*+*S*(*E*) system the most hemolytic one. In the same way, among the systems of the scaffolds made by the lost spheres method, the *S*+*Sus*(*P*) system showed the highest hemolysis. This event can be explained taking into account that the antibioticfixation method *S*+*Sus* is the one that captures the highest amount of gentamicin sulphate, thus resulting in being the most toxic as well. With the exception of the *S*+*Sus*(*E*) system, the systems showed to be hemocompatibles (under 5% of hemolysis) [22]. Another detail is that the blank scaffolds made by the lost sponge method (E) showed a higher hemolysis than that observed in the *Sus*(*E*) and *S*+*R*(*E*) systems, while the blank scaffolds made by the lost sphere method (*P*) show the lowest hemolysis of all the samples. This is a morphologic effect rather than a chemical one, due to the structure of the sample E which has edgy borders that manage to destroy the erythrocites. These edges are coated with PLGA during the antibiotic fixation diminishing the morphologic effect, but never managing a surface as smooth as the systems obtained by the spheres lost method. The pores of the sample *P* are very small and do not present this problem.

4. Conclusions

The feasibility of obtaining wollastonite scaffolds by the lost sponge and the lost spheres methods has been demonstrated. The difference in the average pore size of the scaffolds obtained by these two methods is notably different. However, both types of scaffolds can be used for antibiotic delivery in bone applications. Appropriated compressive strength was observed in all the cases (from 3 to 8 MPa). The gentamicin sulphate release to SBF takes place in two stages; in the first stage a burst occurs (from 0 to 12 h) followed by the second stage, when a slow delivery of gentamicin is presented (up to 342 h). Practically all the systems tested were hemocompatible. The results of this work indicate that these scaffolds are highly potential materials for trabecular bone reconstruction as drug delivery systems.

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