PREPARATION OF INTELLIGENT CHITOSAN-COLLAGEN BASED SCAFFOLDS FOR DRUG DELIVERY

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Chitosan-collagen scaffolds were produced by freeze-drying method and coated with a thermoresponsive polymer poly(N, N-diethylacrylamide) (PDEAAm) to confer them temperature sensitivity using a non residue technology, the supercritical carbon dioxide ($scCO_2$). All native and coated scaffolds were characterized by SEM, intrusion mercury porosimetry, swelling, biodegradability and mechanical analysis.
Microarchitectural analysis by scanning electron microscopy and mercury intrusion porosimetry demonstrated that the coating of the pores inner structure could be efficiently achieved without a considerable loss of porosity. Collagen scaffolds lead to larger porous while chitosan scaffolds originated smaller ones. Collagen scaffolds also showed to be more biodegradable with higher swell capacity than chitosan, however chitosan scaffolds are more stiffness than collagen scaffolds. Scaffolds from mixtures of chitosan and collagen showed intermediate properties between chitosan and collagen appearing as a promising option as a biomedical device.
Different release profiles of BSA and ibuprofen were achieved from different native and coated scaffolds. The maximum amount of BSA and ibuprofen were 250 and 180 mg/g $_{scaffold}$, respectively, from collagen scaffolds.

INTRODUCTION

The first decade of the 21st century saw an increasing interest in the development of devices for delivery of bioactive substances that could react to external stimuli [1]. Herein we report a new strategy for the production of smart partially-biodegradable scaffolds that exhibit a temperature-responsive behavior and their effects on the release of a model protein and a drug of low molecular weight.
Collagen and chitosan are amongst the most abundant polymers in nature [1, 2]. Collagen is a natural, biocompatible and biodegradable polymer that contains both acid and basic amino acid residues and may bear either a positive or negative charge depending on the environments. Scaffolds made of collagen have been observed to promote cell and tissue attachment and growth [3]. Chitosan is a linear polysaccharide containing two β-1,4-linked sugar residues, *N*-acetyl-d-glucosamine and d-glucosamine, distributed randomly along the polymer chain. It is obtained commercially by partial de-*N*-acetylation of chitin. The chitosan used in this study contained approximately 15 mol % *N*-acetyl-d-glucosamine residues. [4]. Since chitosan exhibits favorable biological properties such as nontoxicity, biocompatibility, and biodegradability, it has attracted great attention in the pharmaceutical and fields [5], such as wound dressings and drug delivery systems [3], [4]. Chitosan has both reactive amino and hydroxyl groups that can be chemically and physically modified leading to different pore structures, being a high potential in tissue engineering applications [6]. Collagen and chitosan do not exist together as blends in nature, but the specific properties of each may be used to

produce man-made blends that confer unique structural and mechanical properties. It has been shown that chitosan can modify the properties of collagen when the biological or mechanical properties are considered [1]. Blends of collagen and chitosan have been used to prepare polymeric scaffolds for the in vitro culture of human epidermoid carcinoma cells [2], as membrane for controlled release [7], and as implant fibers [8].
In order to turn this combination between chitosan and collagen much more attractive, in this work, the scaffolds were coated with a thermo responsive polymer, poly(N, N-diethylacrylamide) (PDEAAm), (*in situ* polymerization) using supercritical carbon dioxide [9], turning them "intelligent" on-off devices to drug delivery. The same supercritical carbon dioxide ($scCO_2$) methodology was used to homogeneously distribute the monomer, initiator and cross-linker within the micropores of a polymeric substrate and to perform the polymerization reaction reaction using the pores of the scaffolds as microreactors [10]. BSA and ibuprofen were the selected model molecules with different sizes to investigate the release profiles exhibited by the fabricated devices as a function of different environment temperature conditions.

MATERIALS AND METHODS

Materials

Acetic acid (AA purity≥99%), 2,2'-azobis(isobutyronitrile) (AIBN, purity ≥ 98%), bovine serum albumin (BSA, purity≥98%), chitosan (CHT), collagen from bovine Achilles tendon type I (CLG), glutaraldialdehyde 50 wt% solution in water and ibuprofen were purchased from Sigma-Aldrich. Methylenebisacrylamide (MBA, purity≥98%) was purchased from Fluka. N-N-diethylacrylamide was purchased from Polysciences Inc. Carbon dioxide (CO_2) was supplied by Air Liquide with 99.998% purity. All reagents were used without any further purification.

Methods

Scaffolds preparation

Scaffolds were prepared by freeze-drying method, as described by Madihally et al [11]. The casting solutions with concentration 3% (w/v) are prepared dissolving chitosan and collagen in acetic acid (1% v/v) at 90 ºC with stirring. Similar casting solutions were also prepared in the same conditions but adding the serum bovine albumin (BSA) or ibuprofen (0.8 % (w/v)). The homogeneous solutions at room temperature are placed in sample tubes with an inner diameter of 1.2 cm and 3 cm height. Different sample tubes are immersed in freezing bath containing ethanol at -20ºC, during ten minutes and lyophilized until dry in a *Telstar cryodos-50*. After, scaffolds were treated with an aqueous solution of glutaraldehyde (1% v/v) on a high pressure apparatus similar to the one used in our group for membranes preparation (first high pressure cell), but in this case using an additional high pressure cell where the crosslinker solution was placed [12]. Initially, a CO_2 flow passes through the high pressure cell, where the crosslinker agent was placed, to saturate the CO_2 stream in glutaraldehyde. The first high pressure cell is placed in contact with the second high pressure cell, where the scaffold samples were previously sited. During twenty minutes [12], the CO_2 saturated in glutaraldehyde at a constant flow rate of 9.8 g/min passes through the second high pressure cell and contacts with the scaffold samples. Pressure (20 MPa) and temperature (40 ºC) are controlled as described above. After crosslinking treatment, the first high pressure cell is disconnected of the system and a pure CO_2 flow passes through the second high pressure cell during one hour to clean the remaining residues of glutaraldehyde. At the end of operation, the system is slowly depressurized during 10 minutes.

Scaffolds coating/impregnation with PDEAAm

In a typical procedure, the *in situ* polymerization of PDEAAm within the scaffold pores is performed by loading the scaffolds and reactants, monomer, cross-linking agent (1 wt %) and initiator (2 wt %), into the high-pressure cell [10].

The polymerization reaction was performed in a 33 mL stainless steel high-pressure cell equipped with two aligned sapphire windows in both tops stamped with Teflon o-rings. The cell is immersed in a thermostatted water bath with ± 0.01ºC of stability. Temperature control was done with a RTD with a precision of ±0.001 K in a probe contacting the cell, connected to a Hart Scientific PID controller. The internal agitation is assured by magnetic stirring. The pressure is controlled with a manometer (SETRA, model Datum 2000) with a precision of ±0.01Mpa [13].

The cell was charged with 1 mL of DEAAm, initiator (2% w/w of AIBN with respect to total mass of monomer) and crosslinker (1% w/w of *N,N'*-methylenebisacrylamide (MBAAm) with respect to total mass of monomer). All experiments were performed at 65ºC and 28 MPa during 24 hours under stirring. At the end of the reactions the resulting polymeric materials were slowly washed with fresh high-pressure CO_2 for one hour in order to remove unreacted monomer.

Scaffolds characterization

Scaffolds morphology was investigated using scanning electron microscopy (SEM) in a Hitachi S-2400 equipment, with an accelerating voltage set to 15 kV. Scaffolds samples were frozen and fractured in liquid nitrogen for cross-section analysis. All samples were gold coated before analysis.

Scaffold porosity and pore size distribution were determined by mercury porosimetry (micromeritics, autopore IV).

The swelling tests were performed in order to determine the scaffold's water uptake ability. Dry scaffolds samples were weighted and immersed in beakers containing approximately 20 mL of PBS solution at pH 7.4 and temperatures 20 and 37 ºC. Periodically, the samples were removed from the swelling medium (20 ºC), wiped to remove excessive water of the surface and weighted. After 24 hours, when the mass of polymers reached a plateau value, the samples were transferred to a swelling medium at 37 ºC maintaining the pH 7.4. After another 24 hours, a new plateau was reached, and the samples were transferred again to the bath at 37 ºC. Thus dynamic swelling and shrinking was studied during one week. Equilibrium hydration or swelling degree W (%) of the synthesized samples was determined as defined by Equation 1:

$$Swelling\ degree\ \left(W\ (\%)\right) = \frac{W_w - W_d}{W_d} \times 100 \quad (1)$$

The scaffolds degradation *in vitro* was studied by degrading chitosan and collagen in PBS solution at 37ºC containing 2 µg/mL of lysozyme. Percentage remaining weight was calculated using the following equation:

$$\text{Remaining weight, } W(\%) = 100 - \left(\frac{W_i - W_f}{W_i} \times 100\right) \quad (2)$$

where W_i is the initial dry weight of sample and W_f is the dry weight of sample after degradation.

The compression modulus of the scaffolds was determined with tensile testing equipment (*MINIMAT firm-ware* v.3.1). The samples with length of 1.5 cm^{-1} and width of $1cm^{-1}$ were compressed until their deformation. The used speed of testing was 1 mm min^{-1}. A full scale load of 20 N was used. The compression modulus was calculated from the slope of the linear portion of the stress-strain curve. All samples were tested in dry state at room temperature.
Load graphs were obtained during testing and converted to stress strain curves applying equations (3) and (4):

$$Stress = \sigma = \frac{F}{A} \tag{3}$$

$$Strain = \varepsilon = \frac{\Delta l}{L} \tag{4}$$

where F is the applied force, A the cross sectional area, Δl is the change in length and L is the length between clamps.

Controlled release studies

Small portions of the scaffold (around 20 mg) were placed inside a 50 mL of buffer solutions at pH 7.4. Solution of native scaffolds were immersed in water bath at 37 ºC while coated scaffolds solutions were immersed at 20 ºC and 37 ºC in each 24 h in order to evaluate their ON-OFF behavior. 1 mL aliquots were withdraw periodically from the solutions and collected in eppendorfs. The release medium was refreshed with buffer solution (1 mL) after sampling.
The amounts of BSA and ibuprofen released were analyzed in a Helius Alpha Double-Beam UV/VIS spectrophotometer at 264 and 280 nm, respectively.

RESULTS

In order to study the morphological and mechanical properties of scaffolds produced from chitosan and collagen, different scaffolds were prepared with compositions of: chitosan 100% (CHT), 50% (5050) and 0% (CLG).
SEM micrographs, Figure 1 A, show the different structures obtained for all native scaffolds. Chitosan scaffolds exhibit small, spherical and homogeneous pores while collagen scaffolds present pores more elongated as sheets. Scaffolds resulted from blend of chitosan and collagen have both types of pores, some spherical and others longer and larger.
Figure 1 B shows the scaffolds already coated with PDEAAm, the polymer (PDEAAm) particles across and into the scaffold pores assure the efficiency of the *in situ* DEAAm polymerization using $scCO_2$.

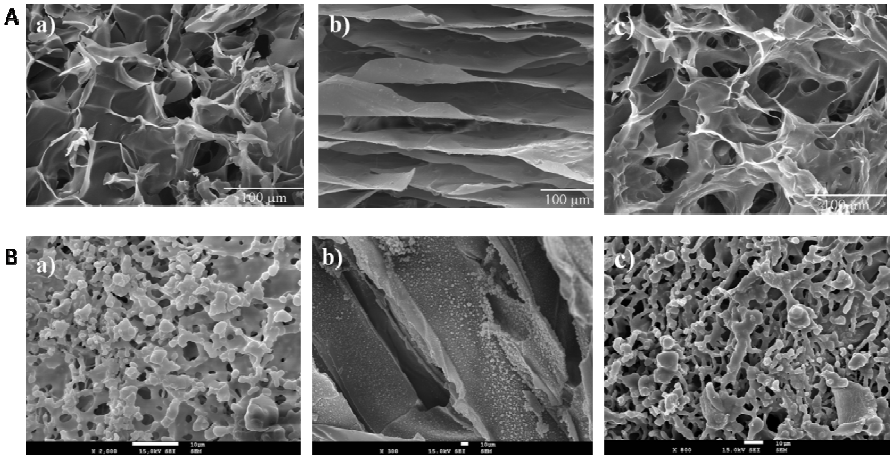

Figure 1: SEM images: A) native scaffolds and B) coated scaffolds. a) chitosan, b) 5050 and c) collagen.

In order to analyze the differences between pore size of native and coated scaffolds, mercury porosimetry analysis were performed. Table 1 shows the different values obtained for the average pore size diameters and porosities. The values are in agreement with the SEM results. The native collagen and 5050 scaffolds present larger average pore size diameters, around 35 µm, while chitosan scaffold presented an average pore size diameter value of 16 µm. The same trend was followed by the coated scaffolds which suffered a general decrease on the mean pore size as it was expected.

Figure 2 shows the swelling profiles at pH 7.4 and at different temperatures (20 and 37 ºC). All scaffolds coated with PDEAAm showed the expected swelling behavior. At 20 ºC, the PDEAAm chains are expanded interacting predominantly with water and a large water uptake is observed while above the LCST, the chains of PDEAAm collapse blocking the pores and consequently the water uptake decrease. The swelling capacity decreased with the collagen percentage ratio.

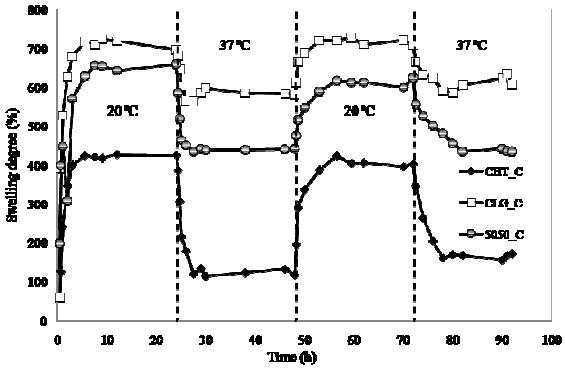

Figure 2: Swelling profiles of scaffolfs at pH 7.4 and at different temperatures: 20 and 37ºC

In order to analyze the scaffolds biodegradability, scaffolds (native and coated) were immersed in a lysozyme solution, the main enzyme responsible to degrade chitosan and collagen [11]. Different biodegradation profiles can be observed in Figure 3 along 30 days. As it is possible to observe, collagen scaffolds always present the faster biodegradation profile. Chitosan native scaffold lost just 10 % of initial weight at the end of experience time while 5050 and collagen native scaffolds lost approximately 60%. By comparison of biodegradable profiles between native and coated scaffolds it is clear that the coating procedure increased the biodegrability of the materials. The scaffolds that were not cross linked dissolved in only few hours of immersion in the lysozyme solution.

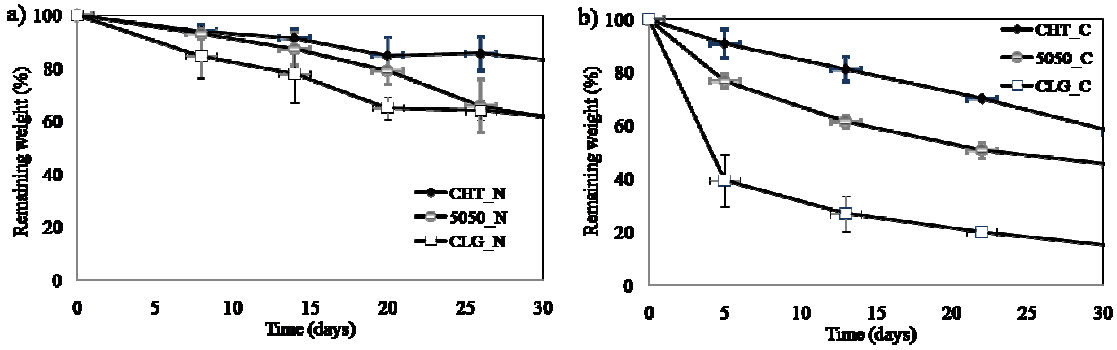

Figure 3: Biodegradability profiles of native and coated scaffolds

DMA was used to characterize the mechanical properties of the scaffolds. The uniaxial compression tests provide an indication of the compressibility of the scaffolds which is an important factor to consider in potential applications. The measured compressive modulus of each scaffold are included in Table 1. Native and coated chitosan scaffolds showed to be stiffer than collagen scaffolds in the same conditions. The blended scaffolds, show an intermediate mechanical behaviour. The compression modulus values obtained to chitosan and collagen scaffolds are very close to the values described in the literature [14]. The coated scaffolds presented values of compression modulus higher than the values of native scaffolds, independently of the scaffold material.

Table 1: Effect of scaffold material in mean pore size diameter, porosity and compression modulus

Scaffold	Mean Pore Size Diameter (μm)	Porosity (%)	Compression Modulus (MPa)
CHT_N	15.83	58	4.8
5050_N	36.53	78	2.0
CLG_N	34.64	73	1.2
CHT_C	10.76	57	13.6
5050_C	28.89	71	9.9
CLG_C	28.29	58	3.4

In order to use these scaffolds as "smart" drug delivery devices, it was necessary to study the drug release profiles under different physiological conditions and to analyze their temperature responsiveness.

BSA and ibuprofen release profiles from native and coated scaffolds at different conditions are plotted in Figure 4 and 5.

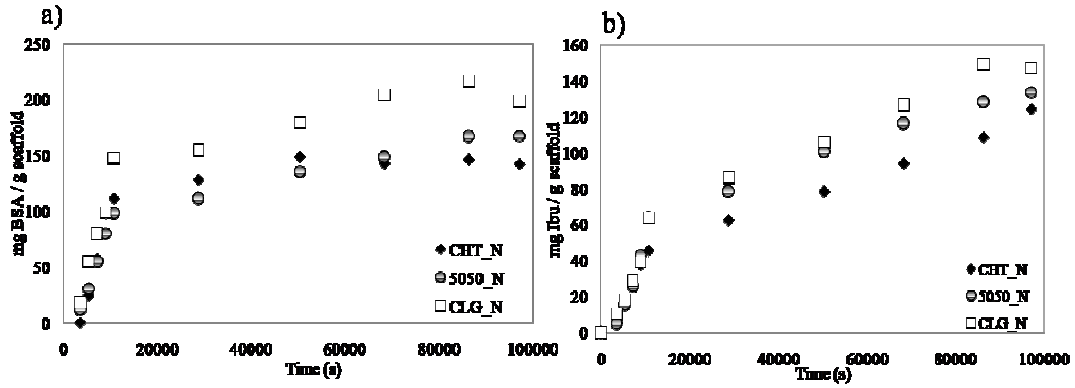

Figure 4: Release profiles of native scaffolds at pH 7.4 and 37 ºC: a) BSA release and b) ibuprofen release

The amino group of chitosan has a pK_a value of 6.5, the pK_a of collagen is 4.4 and the isoelectric point of BSA is 4.8. At pH 7.4, chitosan, collagen and BSA are all deprotonated and so negatively charged leading to high BSA release. The higher amount of BSA released at pH 7.4 by collagen scaffolds than chitosan is explained by the opened morphology of collagen scaffolds and their higher swelling capacity. The 5050 scaffold shows values of protein released between the values from chitosan and collagen scaffolds as it can be seen in Table 2. Ibuprofen is a low molecular weight compound and is also deprotonated at pH 7.4 so easy release occurs. The 5050 scaffolds show again intermediate ibuprofen release profiles between chitosan and collagen scaffolds profiles but closer to the values of collagen scaffolds. Comparing the values of BSA and ibuprofen released it is possible to observe that the total value of BSA released by gram of scaffold is always higher than the amount of ibuprofen

released. This makes sense because larger molecules cannot penetrate in the smaller scaffold pores. Due to its high volume BSA diffuses quickly through the scaffold but ibuprofen which has a small size can pass across the smaller pores of the scaffolds and, consequently, is more retained and a lower released amount of ibuprofen is achieved.
In order to analyze the on-off release control of coated scaffolds, they were immersed in a buffer solution at pH 7.4 and 20 ºC. After 24 h a thermo pulse (37 ºC) was introduced and this procedure was repeated during four days in order to evaluate the drug released at 20 and 37 ºC (ON-OFF profile). Figure 5 shows the different BSA (a)) and ibuprofen (b)) ON-OFF profiles from the different coated scaffolds.

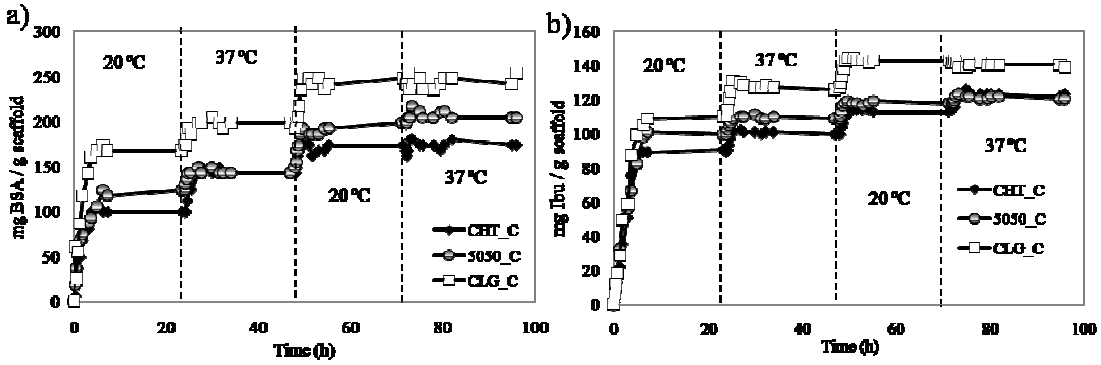

Figure 5: ON-OFF profiles of coated scaffolds with PDEAAm: a) BSA release and b) ibuprofen release

At 20 ºC, the scaffolds present the highest swelling values and the PDEAAm chains are in the hydrophilic state interacting predominantly with water. In this situation, the surface of unit cells become less crowded and the PDEAAm chains extend outside the unit cell allowing enough space for the protein to cross the thermoresponsive layer and be released to the surrounding environment. In the first five hours, at 20 ºC, a burst release occurs, possible due the BSA and ibuprofen located near the surface [15]. However with the assays continuation, introducing the thermo pulse, it is visible that at 37 ºC this burst is not intense as well as all the amount of BSA and Ibu released. When the pulse is introduced at 20 ºC the BSA amount released is always higher than at 37 ºC (Figure 5 a)). In case of ibuprofen (Figure 5 b)), the amount released is very similar at both temperatures; in the case of a low molecular weight drug the release is mainly controlled by diffusion [16]. The hydrogel temperature responsive character always influences the drug release, however might not be enough to prevail to the effect of drug size to diffusion coefficient. This is a possible explanation to the observed small differences of ibuprofen amounts released at 20 and 37 ºC.

Table 2: Resume of amount of BSA and ibuprofen released from native and coated scaffolds

	Native Scaffolds			Coated Scaffolds		
	CHT	5050	CLG	CHT	5050	CLG
BSA ($mg/g_{scaffold}$)	130	142	180	173	204	253
Ibu ($mg/g_{scaffold}$)	116	128	149	120	123	140

CONCLUSIONS

Herein we proposed a completely clean strategy to develop potential thermo and pH on-off devices for drug delivery, combining the preparation of chitosan-collagen based scaffolds by freeze drying and the *in situ* synthesis of a thermoresponsive polymer.

The preparation of chitosan-collagen based scaffolds by freeze drying method with and without BSA and ibuprofen was also investigated. By varying the scaffold material composition it was possible to control the scaffold's properties in terms of mean pore size, biodegradability, swelling and mechanical behavior.
The scaffolds coated with PDEAAm in $scCO_2$, were also fully characterized. Different release profiles of BSA and ibuprofen were achieved at different temperature external conditions which assure the huge capacity of the prepared scaffolds as promising intelligent devices for drug delivery.

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