SYNTHESIS AND CHARACTERIZATION OF 2-OXAZOLINE-BASED POLYMERS FOR ANTI-FOULING AND ANTIMICROBIAL SURFACE ACTIVITY

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Living 2-oxazoline-based polymers were synthesized in supercritical carbon dioxide ($scCO_2$) and further end-capped with a linear amine in order to gain antimicrobial activity. The polymers were characterized by FT-IR, NMR, MALDI-TOF and GPC and microbiological assays were also performed in order to evaluate their bactericidal activity. Poly(2-methyl-2-oxazoline) and poly(bisoxazoline) end-capped with *N*,*N*-dimethyldodecylamine (DDA) and linear poly(ethyleneimine) hydrochloride (LPEI.HCL), obtained by the hydrolysis of polyoxazolines, resulted in efficient polymeric materials with high levels of antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli*, two well known and important pathogens.
Chitosan scaffolds were modified with functionalized polyoxazolines and with LPEI and characterized by SEM and DMA in order to evaluate their morphology and mechanical performance. Cytotoxicity and anti-fouling assays were also performed to investigate the cytotoxicity of the scaffolds against mammalian cells and their fouling resistance. The preliminary results suggest that CHT-PBisOxDDA scaffold is the best candidate for further biomedical or biological applications.

INTRODUCTION

The emergent demand in medicine and in the biomaterial field for polymers with a more efficient antimicrobial and anti-fouling activity has driven our research to develop new synthetic methods for the production of functionalized polymers. Polyoxazolines represent a unique approach to new antibiotics and are strong candidates for the development of new polymeric therapeutics [1].
It was previously demonstrated in our group, that the polymerisation of 2-substituted-2-oxazoline can be successfully performed in supercritical CO_2, in alternative to conventional organic solvents. Low molecular weight polymers were obtained in high yield and low polydispersity from three different monomers using boron trifluoride etherate ($BF_3.OEt_2$) as initiator [2]. The polymerization of 2-substituted-2-oxazoline *via* a *living* cationic ring-opening mechanism provides an extraordinary control of the morphology of the polymers as well as their later functionalization with tertiary amines in order to obtain an ammonium-type end-capping [1, 3]. Several studies related with antimicrobial activity are based in this type of compounds and materials possessing quaternary ammonium salts in their structure [4, 5].
Linear poly(ethyleneimine (LPEI) was also synthesized by acid hydrolysis of poly(2-metil-2-oxazoline). LPEI contains secondary amine groups in the backbone offering many possibilities for further chemical modifications [6]. Moreover, it possesses a high number of

advantages as it can act as chelating agent, it has high water solubility and good physical and chemical stability [7] under normal physiological conditions (pH from 6.8 to 7.4).
Chitosan is a linear polysaccharide containing two β-1,4-linked sugar residus, *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. It is only soluble in aqueous solutions of low pH, because its pK_a value is ~6.3. [8]. Since chitosan exhibits favorable biological properties such as nontoxicity, biocompatibility, and biodegradability, it has attracted great attention in the biomedical and pharmaceutical fields [9], such as wound dressings and drug delivery systems [10, 11, 12]. Chitosan has also been proposed as a scaffold to sustain cell adhesion and proliferation for application in tissue engineering [13, 14].
In this work, we developed a simple approach to render chitosan scaffolds surface with antimicrobial properties by blending it with poly(oxazoline)s end capped with DDA group previously synthesized in $scCO_2$. Scaffolds were evaluated in terms of cytotoxicity following International Standard guidelines, as well as in terms of fouling resistance envisaging the future use of these devices in biological and medical applications.

MATERIALS AND METHODS

MATERIALS

The monomer 2-methyl-2-oxazoline (MetOx), the initiator boron trifluoride diethyl etherate ($BF_3.OEt_2$), chitosan (75-85% deacetylated, *M*w = (190 – 310) kg mol^{-1}), glacial acetic acid (purity ≥ 99%), glutaraldialdehyde 50 wt% solution in water, phenol (purity≥95%), phosphate buffered saline (PBS), accutase™ and cell growth determination Kit MTT-based were purchased from Sigma-Aldrich. The monomer bisoxazoline (BisOx) was synthesized as described in the literature.[15] *N*,*N*-dimethyldodecylamine was purchased from Fluka. Mueller-Hinton broth medium was purchased from Oxoid. Acetone (p.a.) was purchased from Pronalab. RPMI-1640 (a Roswell Park Memorial Institute medium), trypan blue and fetal bovine serum (FBS) used in cell culture were purchased from Invitrogen. L929 cells were obtained from DSMZ, Germany and from Sigma. Carbon dioxide was supplied by Air Liquide with a purity of 99,998%. All materials and solvents were used as received without any further purification.

SYNTHESIS OF 2-OXAZOLINE-BASED POLYMERS

Polymerization reactions were carried out in a 11 mL stainless-steel reactor equipped with two aligned sapphire windows in both tops stamped with Teflon o-rings. Two 2-substituted oxazoline monomers were studied (MetOX, and BisOx) and boron trifluoride etherate ($BF_3.Et_2O$) was used as the initiator. The monomer/initiator ratio used to each polymerization was, respectively: [M]/[I]=15 (MetOx) and [M]/[I]=7,5 (BisOx), according to the results previously reported in the literature.[2]
The reactor cell was charged with the 2-substituted oxazoline, the initiator, a magnetic stirring bar, and then immersed in a thermostatized bath. For polymerizations performed at 60 ºC a water bath was used, for higher temperatures, 70 ºC, an oil bath was chosen. CO_2 was introduced in the reactor in order to achieve the desired reaction pressure (from 16 to 20 MPa). After 20 hours of reaction, the pressure was slowly released and the reactor reached the

room temperature. Inside the reactor, and according to the adopted conditions of temperature and pressure, a viscous foam or a solid, was obtained – the so called *living polymer*. The *living polymer* allows the direct functionalization by end-capping with different molecules. (please refer to Figure 1).

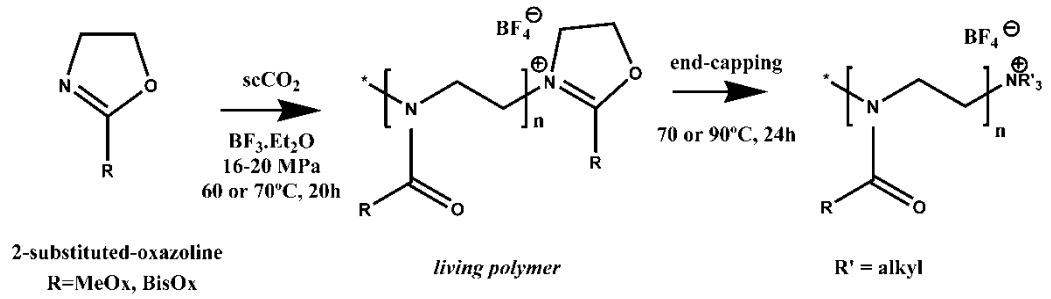


Figure 1: Green synthesis of 2-oxazoline-based polymers and its functionalization by amine end-capping.

Functionalization of the *living polymer* can be performed with the addition of a tenfold excess of amine to relation of the amount of initiator. The mixture was kept at 70 ºC under stirring during 24 hours (Figure 2). When the final polymer was a solid, it was washed with diethyl ether and dried in vacuum. Oily polymers were purified by extraction with diethyl ether and water, once the polymer is miscible in water. Finally, the aqueous phase is evaporated to dryness.

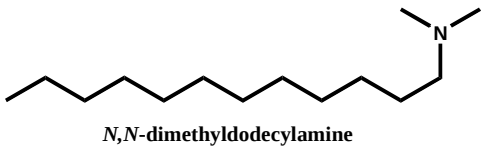


Figure 2: Chemical structure of *N,N*-dimethyldodecylamine used in *living polymer* end-capping.

LPEI was prepared by hydrolysis of poly(2-methyl-2-oxazoline) end-capped with *N,N*-dimethyldodecylamine (M_w= 1248 g/mol, PD=1.13) in a 5 M HCl (hydrochloric acid) solution under reflux (100 ºC) for 9 hours (figure 3). After this period the polymer precipitates as the hydrochloride salt. The mixture is then filtered off, washed with acetone and dried in vacuum.[16] LPEI is obtained as a white solid.

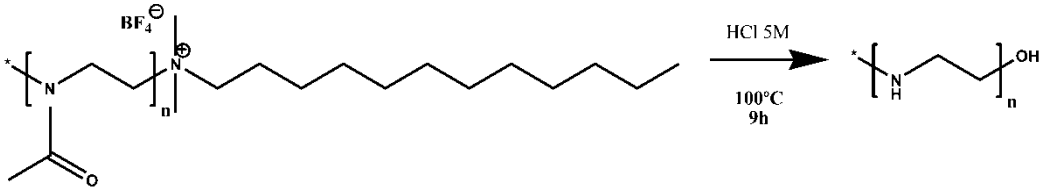


Figure 3: Preparation of LPEI by acid hydrolysis of PMetOx-DDA.

MORPHOLOGYCAL AND PHYSICO-CHEMICAL CHARACTERIZATION OF 2-OXAZOLINE-BASED POLYMERS

The infrared spectra were obtained using a FT-IR ‘‘Nicolet Nexus’’ equipment. The NMR spectra were acquired in a Bruker ARX 400 spectrometer. The MALDI-TOF mass spectrometry was performed on a AUTOFLEX Bruker apparatus using dithranol as the matrix. The samples were prepared by mixing aqueous solutions of the polymer (10^{-4} M) and matrix in a typical ratio 1:1 (v/v). Average molecular weight of the polymer samples were determined by Gel Permeation Chromatography (GPC) using a KNAUER system with an evaporative light scatter detector from Polymer laboratories using dimethylformamide (DMF) as the eluent (temperature of the evaporator: 175 ºC, temperature in nebulisation camera: 85 ºC, eluent rate flow: 1.8 mL min^{-1}). Two Polypore columns were used in series. MALDI-TOF mass spectrometry was carried out at Riaidt in Spain. All the other polymer analyses were carried out at REQUIMTE, Associated Laboratory.

CHARACTERIZATION OF THE ANTIMICROBIAL EFFECT OF FUNCTIONALIZED 2-OXAZOLINE-BASED POLYMERS

To evaluate their antimicrobial profile *Staphylococcus aureus NCTC8325-4* and *Escherichia coli AB1157* strains were used in all experiments and were grown in Mueller-Hinton broth medium. Cultures were grown overnight in broth at 37ºC, diluted 1/200 and further incubated at 37 ºC, with aeration and vigorous shaking to an optical density at 600nm of 0.5-0.6. In order to determine MIC values, 2-oxazoline-based polymers were dissolved in the Mueller Hinton Broth medium. The polymers solutions were placed in a sterile 96-well microtiter plate and serially diluted. Then, 5 μL of culture (10^5 cells) were added to each well containing a volume of 100 μL of medium with a specific concentration of polymer (sequential 2 fold dilutions). Plates were incubated with shaking (100 rpm) at 37 ºC overnight. Sterilization controls of the medium and polymer were also carried out. MIC results were determined after 24 hours of incubation. The tests were performed in duplicate and with both *S.aureus* and *E.coli* strains. To determine their killing times, a solution of polymer in growth medium with a concentration 5 times the obtained MIC value was added to *S.aureus* and *E.coli* cultures in exponential growth phase (OD_{600nm}= 0,5-0,6). Samples of the culture were taken before and after the addition of polymers at specific time points. Each sample was serially diluted to 10^0, 10^{-2} and 10^{-4} in growth medium. 100 μL of each dilution were plated on Mueller Hinton agar and incubated at 37 ºC. After 24 hours, the number of viable colony was determined. Optical density was also measured during time assay. Killing curves were constructed by plotting colony-forming units (CFU) against time, in a log scale.

SCAFFOLDS PREPARATION

Scaffolds were prepared by freeze-drying method, as described by Madihally et al.[17] The casting solutions with concentration 3 % (w/w) are prepared dissolving chitosan and different 2-oxazoline-based polymers in acetic acid (1% v/v) at 90 ºC with stirring. The casting solutions were prepared using 90 % (w/wt) of chitosan and 10 % (w/wt) of each 2-oxazoline-based polymers. 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 20 minutes and lyophilized until dry in a *Telstar cryodos-50*. After, scaffolds were treated with a acetone solution of glutaraldehyde

(0.5% 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.[18]
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 ten minutes, 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. After crosslinking treatment, the first high pressure cell is disconnected of the system and a mixture of CO_2 and acetone flow passes through the second high pressure cell during one hour and finally just a pure CO_2 flow during another hour to clean efficiently the remaining residues of glutaraldehyde. At the end of operation, the system is slowly depressurized during 10 minutes.

MORPHOLOGICAL AND MECHANICAL CHARACTERIZATION OF PREPARED SCAFFOLDS

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.
The compression modulus of the scaffolds was determined with tensile testing equipment (*MINIMAT firm-ware* v.3.1) using samples with length of 1.5 cm and 1 cm width. 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 compression graphs were obtained during testing and converted to stress strain curves applying equations:

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

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

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

SCAFFOLDS CYTOXICITY EVALUATION

The scaffolds cytotoxicity evaluation was performed following the ISO 10993-5 guidelines. Briefly, triplicates of scaffolds with a width of 1 cm were placed in polystyrene tubes containing 4 mL of RPMI – 1640 with 10% (v/v) of FSB and kept in an incubator (37ºC, 5% CO_2, fully humidified) for 1 day. The liquid extracts were used to culture L929 mouse fibroblasts (initial density $10x10^4$ cells/mL) in 24-well plates for 24 hours. The cell metabolic activity was determined by analyzing the conversion of MTT (yellowish color) to its formazan derivative (purple – absorbance at 570 nm after a 3 hour incubation at 37 ºC) using a MTT-based cell growth determination kit. The results were normalized to the negative control for cytotoxicity (fresh RPMI medium) and compared to the positive control (0.01M Phenol).

STUDY OF SCAFFOLDS FOULING RESISTANCE

To evaluate the anti-fouling performance of the prepared scaffolds, two different procedures, one static and one dynamic, were followed. BSA was used as a model protein. In the static procedure, scaffold samples with 50 mg were immersed under stirring in 3 mL of BSA solution (3 mg mL^{-1}) in PBS (pH 7.4). After 24 h, scaffold samples were immersed in 2 mL of PBS in order to wash the scaffolds, and absorbance of washes was determined. The washing procedure was repeated until the absorbance at 280 nm was zero. The amount of BSA adsorbed in the scaffolds was determined by measuring the difference between the initial and final concentration of BSA in the solution and the amount of BSA quantified in washes. In the dynamic procedure a stirred cell composed by two chambers [19] was used. The scaffold sample with 25 mg was clamped between the chambers at room temperature. In a typical procedure, the feed chamber is filled with 3 mL of BSA in phosphate buffer solution (PBS pH 7.4) (3 mg mL^{-1}) and the receptor chamber is filled with 3mL of PBS pH 7.4. 1 mL aliquots were withdrawn periodically from the solutions and collected in *eppendorfs* in order to evaluate the protein capacity to cross over the scaffold sample avoiding BSA adhesion on the scaffold surface. The volume was kept constant in each compartment by adding net BSA solution to the receptor. A tune stirring of each cell compartment was carried out to eliminate the BSA adhesion in the chambers. At the end of assays (72 hours) the scaffolds were removed and washed with PBS in order to remove the BSA absorbed into the scaffold. The washes were repeated until absorbance at 280 nm was zero. BSA was quantified by UV spectroscopy (Helios Alpha Double- Beam UV/VIS Spectrophotometer) at a wavelength of 280 nm. To correlate the absorbance with the BSA concentration, calibration measurements were performed.

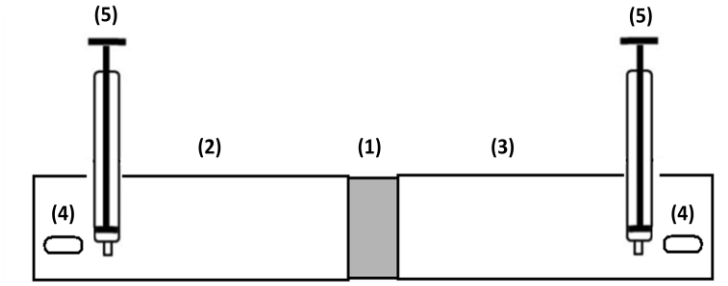
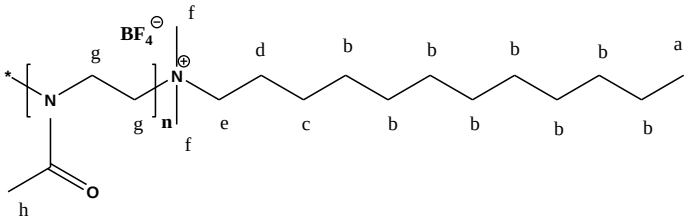


Figure 4: Schematic representation of the dynamic assay apparatus. (1) – Scaffold sample; (2) – feed chamber; (3) –permeated chamber; (4) magnetic stirrer; (5) – syringe for sampling.

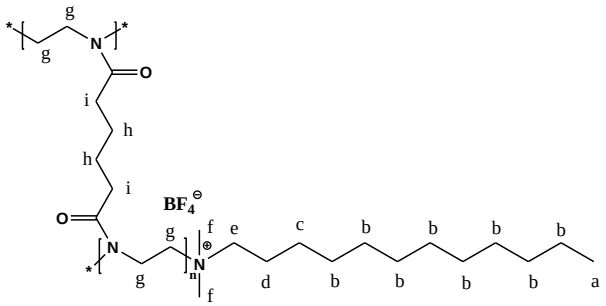
RESULTS

2-oxazoline-based polymers characterization. Polymers were characterized using FT-IR, ^{1}H-NMR, ^{13}C-NMR, GPC and MALDI-TOF techniques, as long as they were soluble in the required solvents.

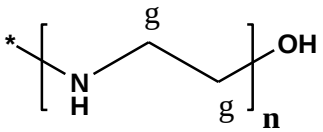
Poly(2-methyl-2-oxazoline) end-capped with *N,N*-dimethyldodecylamine (PMetOx-DDA). Yellow solid. Water soluble. Hygroscopic. Yield: 68 %. FT-IR: $\nu_{máx}/cm^{-1}$ 1738 (w, HO(C=O)N-), 1634 (s, Me(C=O)N-). ^{1}H-NMR (400MHz, $CDCl_3$) δ/ppm= 0.87 (3H, t, J=6.3 Hz, Ha), 1.24 (16H, bs, Hb), 1.71 (2H, bs, Hc), 2.13 (3H, bs, Hh), 3.01 (2H, t, J=8.0 Hz, Hd), 3.15 (2H, bs, He), 3.44 (10H, bs, Hg+Hf, overlapped signals). ^{13}C-NMR (400MHz, $CDCl_3$) δ/ppm= 14.10, 21.15, 22.65, 24.48, 26.44, 29.80, 29.35, 29.56, 31.86, 43.49, 44.95, 47.05, 47.84, 58.63, 170.88 (C=O), 171.62 (C=O). GPC: M_n= 17 235 g/mol M_w/M_n= 1.13. MALDI-TOF (after hydrolysis): M_n= 1248 g/mol *n*= 12.



Poly(bisoxazoline) end-capped with *N,N*-dimethyldodecylamine (PBisOx-DDA). Brown solid. Water soluble. Yield: 67 %. FT-IR: $\nu_{máx}/cm^{-1}$ 1744 (w, HO(C=O)N-), 1652 (w, N-(C=O)). ^{1}H-NMR (400MHz, CD_3OD) δ/ppm= 0.89 (3H, t, J= 6.2 Hz, Ha), 1.29-1.37 (16H, bs, Hb), 1.62 (4H, bs, Hh), 1.70 (2H, bs, Hc), 2.22 (4H, bs, Hi), 3.02 (2H, t, J=5.3 Hz, Hd), 3.07-3.11 (2H, bs, He), 3.26-3.30 (14H, bs, Hg+Hf, signals overlapped with the signal of the solvent). ^{13}C-NMR (400MHz, CD_3OD) δ/ppm= 14.41, 17.08, 23.71, 26.63, 27.42, 30.18, 30.45, 30.47, 30.61, 30.71, 33.04, 36.66, 42.80, 42.93, 43.43, 61.60, 176.18 (C=O) M_n= 786 g/mol *n*= 3. GPC: M_n= 4 897 g/mol M_w/M_n= 1.05. MALDI-TOF: n.a.



Linear Poly(ethyleneimine) hydrochloride (LPEI). White solid. Water soluble. Polymer insoluble in dimethylformamide. Yield: 74 %. FT-IR: $\nu_{máx}/cm^{-1}$ 3420 (s, NH), 2659 (s, CH). ^{1}H-NMR (400MHz, D_2O) δ/ppm= 3.48 (4H, s, Hg). ^{13}C-NMR (400MHz, D_2O) δ/ppm= 43.83. GPC: n.a. MALDI-TOF: M_n= 548 g/mol *n*= 12.



FT-IR spectroscopy allowed the identification of the main functional groups of all polymers. To determine the molecular weight of each polymer, NMR spectroscopy, GPC and MALDI-TOF techniques were used. The analysis of the NMR spectra also allowed concluding that the end-capping was successfully performed in both *living polymers*. It was also possible to conclude from the NMR spectra of LPEI that the complete hydrolysis of both oxazoline's acetyl groups and the amine end-capping has been achieved.
Characterization of 2-oxazoline-based polymers antimicrobial activity was performed by determining MIC values and killing times for two bacteria.

Table 1: MIC determination of water soluble 2-oxazoline-based polymers.

Polymer	MIC value (mg/mL)		Killing time (min)	
	S.aureus NCTC 8325-4	*E.coli AB1157*	*S.aureus NCTC 8325-4*	*E.coli AB1157*
PMetOX-DDA	1,5	0,75	< 5	< 5
	0,75	0,75		
PBisOx-DDA	0,37	0,37	< 5	< 5
	0,19	0,37		
LPEI	0,09	0,09	240	45
	0,05	0,19		

Minimum inhibitory concentrations (MIC)s and killing rates of the synthesized polymers against *S. aureus* and *E. coli* were determined. The end-capping with *N,N*-dimethyldodecylamine of poly(2-methyl-2-oxazoline) and poly(bisoxazoline) led to materials with high MIC values and fast killing rates while LPEI.HCL, the polymer with lower MIC value, takes more time to achieve 100 % killing for both bacteria.

CHT and CHT blended with antimicrobial oxazoline-based polymer scaffolds.

Scaffolds are crucial to tissue engineering strategies for a number of reasons; as a threedimensional structure they provide volume fill, mechanical integrity and a surface that can provide chemical and architectural guidance for regenerating tissues. Figure 5 shows the different structures obtained for all scaffolds produced. The comparison of SEM images show clearly that CHT-PBisOx-DDA scaffold presents a morphological structure completely different from CHT-MetOx-DDA and CHT-LPEI morphological structure. CHT-PBisOx-DDA scaffold presents, in general, small and spherical pores while CHT-MetOx-DDA and CHT-LPEI scaffolds have large elongated pores with deep channels. These results demonstrate that the morphology of the porous device can be designed by changing the 2-oxazoline-based polymer that was blended with chitosan.

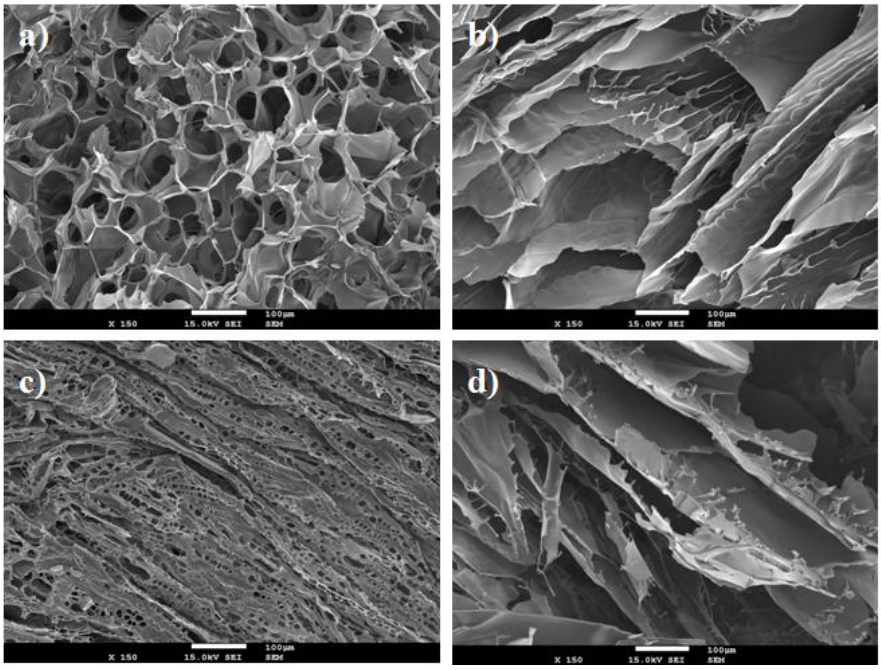


Figure 5: SEM images: a) CHT, b) CHT-PMetOx-DDA, c) CHT-PBisOx-DDA, and d) CHT-LPEI

The addition of end capped oxazoline-based polymers to CHT also leads to dramatic changes on the mechanical properties of CHT-based scaffolds. DMA was used to determine the compressive modulus of each scaffold. The results are summarized in Table 2.

Table 2 : Compression modulus of prepared scaffolds

Scaffold	Compression Modulus (KPa)
CHT	0.25
CHT-PMetOx-DDA	0.2
CHT-PBisOx-DDA	0.05
CHT-LPEI	0.23

CHT scaffold showed the highest compression modulus (0.25 KPa). The addition of oxazoline based polymers leads to lower compression modulus. CHT-PBisox-DDA scaffold presented the lowest value (0.05 KPa).

Scaffolds biocompatibility and fouling resistance evaluation

The use of biomaterials in the settings of tissue engineering and regenerative medicine requires a cytotoxicity evaluation to test cell responses when interacting with the material. All prepared scaffolds started to be evaluated in terms of cytotoxicity following International Standard guidelines. The preliminary results showed relatively low values of viable cells for all scaffolds. Approximately, 25% viable cells after 24 hours of incubation were obtained with CHT scaffolds and 35% viable cells were observed with CHT-PBisOx-DDA scaffold. The low biocompatibility exhibited by the tested scaffolds indicate that further improvement of experimental procedures are needed, namely the reticulation and CO_2 cleaning procedures.

To further investigate the scaffolds fouling resistance, static and dynamic assays were performed. Table 3 shows the BSA percentages remaining in feed solution, the amount of BSA recovered from washes and BSA retained in the scaffolds.

Table 3 - BSA amount at different stages in the static assays

	BSA $^{(a)}_{\text{after 24h}}$ **(%)**	**BSA** $^{(b)}_{\text{recovered}}$ **(%)**	**[BSA]** $_{\text{retained}}$ **(%)**
CHT	21,1	25,0	53,9
CHT-PMetOx-DDA	2,7	66,3	31,0
CHT-PBisOx-DDA	4,5	60,7	34,8
CHT-LPEI	50,4	23,7	25,9

(a) obtained from remaining solution
(b) obtained from washes

Data from Table 3 suggest that the addition of 2-oxazoline-based polymers to CHT solution improved the scaffolds resistance to protein irreversible fouling. After 24 hours of scaffold

immersion in a concentrated BSA solution, CHT-LPEI scaffold retained only half of the BSA retained by the CHT scaffold. CHT-PMetOx-DDA and CHT-PBisOx-DDA scaffolds revealed similar irreversible antifouling behaviour. The higher efficiency of CHT-LPEI scaffold could be related with the fact that LPEI is segregated to pores surface during the lyophilisation process as it is visible in SEM image (Figure 5d).
Figure 6 shows the permeation BSA profiles through the different scaffolds in the dynamic assays.

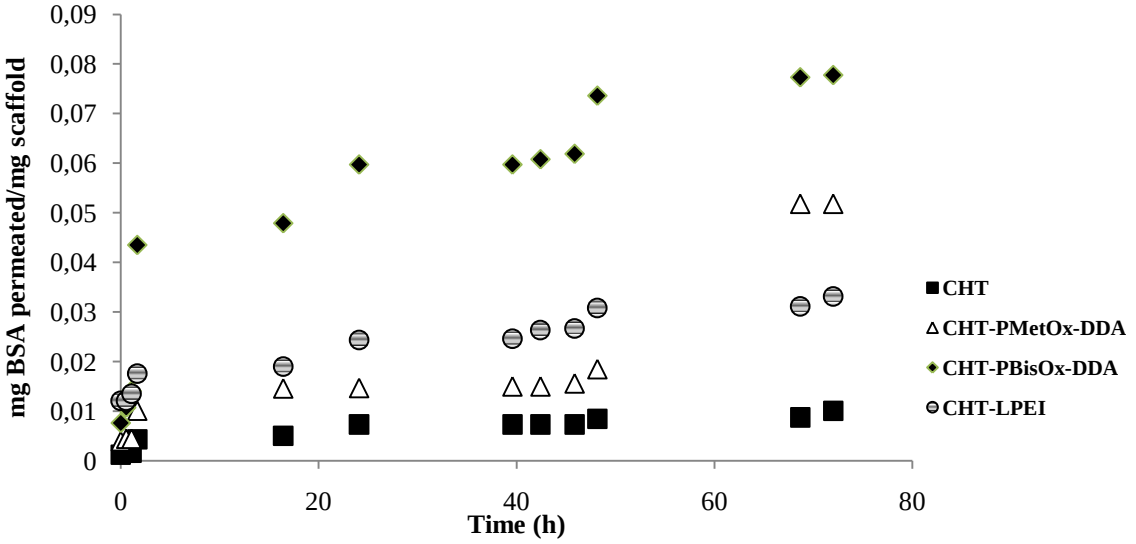


Figure 6: Mass of BSA permeated per mass of scaffold during time assay.

From data presented in Figure 6, it can be concluded that the blending of CHT with LPEI or end capped polyoxazolines leads to an improvement of BSA permeation. Using equivalent mass of scaffolds, CHT only permeated 0.01 mg of BSA after 72 hours, but using CHT- LPEI scaffold 0.03 mg of BSA was permeated and with CHT-PMetOx-DDA and CHT-PBisOx-DDA scaffolds 0.05 mg and 0.08 mg of BSA were permeated, respectively.
However, it was important to investigate if the retained BSA was still in the feed chamber or if it was adsorbed on the scaffold. Thus, after 72 hours of permeation experiments, the scaffolds were washed with PBS and the absorbances of washes as well as the absorbance of the feed chamber were determined, in order to quantify the BSA withheld on the scaffold and the BSA that remained in the feed chamber at the end of the assays. The results are summarized in Table 4.

Table 4: BSA amount at different stages in the dynamical assays

	Feed chamber (%)	Rejection (%) [a]	Recovery (%) [b]	Retained (%)
CHT	76.1	3.0	6.1	14.8
CHT-LPEI	77.1	11.8	11.0	0.1
CHT-PMetOx-DDA	77.0	16.9	2.6	3.5
CHT-PBisOx-DDA	67.1	27.8	4.9	0.2

[a] obtained during the permeation stage
[b] obtained during the washing stage

The values presented in Table 4 allow us to infer that: large BSA amounts were retained in the feed chamber; CHT scaffold is the one that rejected less amount of BSA and the addition of 2-oxazoline-based polymers to the chitosan improved the fouling resistance. CHT-LPEI and CHT-PBisOx-DDA scaffolds showed to be the scaffolds which adsorbed less amount of BSA. Comparing both static and dynamic studies, similar and consistent results were obtained, but further assays are required.

CONCLUSION

This work represents a new alternative to develop new antimicrobial and antifouling devices with application in biological and biomedical fields using green technologies and without any traces of organic solvents.
All end capped 2-oxazoline-based polymers were synthesized with success in $scCO_2$ and were incorporated into CHT-based scaffolds. Our preliminary results from cytotoxicity, antimicrobial and antifouling assays together with the morphological and mechanical analysis suggest that CHT-polyoxazoline scaffolds are promising for further applications in separation processes and in biomedical field.

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