Chemical Functionalisation of the Surface of PCL Nanofibers for Collagen Immobilization

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RESUMO

Engenharia tecidual é uma proeminente área em diversas especialidades médicas e odontológicas. Polímeros sintéticos tem se mostrado uma excelente alternativa de biomaterial para ser usado como matriz de suporte celular para a regeneração tecidual. Apesar da eletrofiação ter se mostrado como uma eficiente técnica de processamento para o desenvolvimento de biomateriais, o controle das propriedades de superfície das nanofibras geradas ainda é um desafio. Nesse estudo, foi apresentado como a superfície do poli- ε-caprolactona (PCL) pode ser modificada quimicamente para ter o colágeno imobilizado a ela. As reações de aminólise e hidrolise foram otimizadas para criar grupamentos de ancoragem na superfície do PCL e glutaraldeído e carbodiimida foram utilizados para ligar o colágeno a estes sítios. Alterações de cristalinidade foram mensuradas por meio de calorimetria exploratória diferencial (DSC) e difração de raio-X. A adesão e proliferação de osteoblastos foi determinada por meio de ensaio MTT. Os dados foram submetidos a ANOVA de um fator para cada ensaio. Os resultados mostram que a cristalinidade do PCL não é alterada pelo processo de eletrofiação, o que aumenta a resistência às reações de aminólise e hidrólise. A quantidade de colágeno imobilizado está relacionada com a quantidade de grupamentos funcionais inseridos na matriz de suporte celular. O mínimo de 1,5% de colágeno, em peso, é necessário ser inserido à matriz para aumentar significantemente a adesão e proliferação celular. As técnicas de aminólise e hidrolise foram otimizadas e eficazes para imobilização de colágeno ao PCL e consequentemente à melhora na adesão celular.

Descritores: poliésteres; colágeno; membranas artificiais; materiais biocompatíveis.

ABSTRACT

Tissue Engineering is a prominent areal in several medical and dental specialties. The synthetics polymer has been shown as an excellent alternative of biomaterial to be used as scaffold in tissue regeneration. While electrospinning has proven to be a useful processing technique for membranes development, the control of the surface properties of the generated nanofibers is still a challenge. In this study, it was showed how the surface of poly-ε-caprolactone (PCL) can be modified chemically, followed by collagen immobilisation. While aminolysis and hydrolysis reactions were optimized to create surface anchors in PCL, glutaraldehyde and carbodiimide were used to bind collagen. Crystallinity changes were assessed by differential scanning calorimetry and X-ray diffraction. Osteoblast adhesion and proliferation were measured using an MTT assay. The data were submitted to a one-way ANOVA for each test. The results indicate that the PCL crystallinity remained constant after electrospinning, giving a higher resistance towards aminolysis and hydrolysis reactions. The amount of immobilised collagen is in agreement with the number of functional groups inserted into the scaffolds, whereas the rate of cell proliferation follows the total quantity of collagen attached. A minimum of 1.5% collagen attached to the scaffolds was necessary to significantly increase cell attachment and proliferation. So, the aminolysis and hydrolysis reactions were optimized and efficient in order to immobilise the collagen to PCL and consequently to increase the cell adhesion.

Keywords: polyesters; collagen; artificial membranes; biocompatible materials;

INTRODUCTION

Tissue engineering has been intensively studied within the medical and dentistry community as a potential treatment in several areas and specialties. These studies range from the regeneration of small defects up to complete organ replacement [1, 2, 3]. The use of synthetic polymers as scaffolds has proven an excellent alternative for regenerative techniques because they do not develop immunological reactions, are easily handled and can be sterilised. Furthermore, these synthetic polymers can also mimic the extracellular matrix [4].

Poly-ε-caprolactone (PCL), a semi-crystalline polymer, whose melting temperature is approximately 60°C, is an extensively studied aliphatic polyester and has also been used for tissue engineering. Although PCL's mechanical properties are poorer than other synthetic polymers, such as of PLLA [5, 6], its degradation is slower, making it suitable for drug delivery [4].

However, PCL is hydrophobic (contact angles of 93) [7] and It exhibit little interaction with cells and hinder the flow of nutrients in porous devices. Furthermore, they do not have specific binding sites for cell adhesion proteins [8, 9]. The immobilisation of natural polymers such as collagen, gelatin or chitosan to the polyesters surface is an efficient alternative for increasing its hydrophilicity and, thus, its ability to promote cell adhesion and proliferation [8, 10, 11] while maintaining the cell's functionality [12, 13]. This immobilisation can be obtained by attaching amine or carboxyl groups onto the polymer surface through, for example, aminolysis or hydrolysis reactions [14]. Although the use of these methodologies has been successfully employed and intensively studied in films or sintered 3D matrix [10, 15], to our knowledge their use in electrospun scaffold has not been described. It has been shown the decrease or complete loss of the polymer crystallinity during the electrospinning process [16, 17], which could directly affect the properties and the reactivity of the material, thus affecting aminolysis and hydrolysis reaction rates. Therefore, the development of systematic studies for surface modification and control on polymeric electrospun scaffolds is of paramount importance.

A maximum amine group insertion was observed in PCL when films were submitted to a solution of 10% 1,6-hexanediamine (HDA) at 37°C for 45 min [18]. The final concentration of the amine groups was directly

related to the reaction time and HDA concentration [11, 18].

In contrast, the hydrolysis treatment of polyesters has not been systematised. Cui et al. [19] demonstrated that gelatin immobilisation on PLLA films hydrolysed in a 1 M NaOH solution at 50°C for 1 h improved the adhesion and proliferation of chondrocytes. Mats formed from electrospun PLLA hydrolysed in 0.5 M NaOH at room temperature for 5 min demonstrated a good relationship between functionalisation and mechanical properties [20].

After surface treatment, promising results have been obtained by immobilising macromolecules on the polymer surface using GTA or carbodiimide [21, 22]. However, no studies have compared the materials obtained with different immobilisation techniques or functionalisation types.

The aim of this study was to optimise the aminolysis and hydrolysis conditions for PCL electrospun scaffolds and to compare the two functionalities against collagen immobilisation using glutaraldehyde (GTA) or carbodiimide. In addition, the materials were tested for osteoblast attachment and proliferation.

MATERIALS AND METHODS

Scaffold Preparation and Functionalisation

PCL scaffolds were obtained through electrospinning using the following parameters: a flow rate of 4 mL/h, a distance between the needle and the collector of 18 cm and 25 kV. For the aminolysis reaction, the PCL scaffolds were immersed, in different time intervals in an ethanol solution containing 20% ethylenediamine (EDA) at 37°C. For hydrolyses, the PCL scaffolds were immersed, during several times interval in a solution of 1 M NaOH, at 37°C.

Aminolysis Quantification

The quantity of amine inserted onto the scaffold was measured using a ninhydrin test. The scaffolds were immersed in an ethanol solution containing 0.01 M ninhydrin for 45s, transferred to a clean glass tube and heated to 70°C for 10 min. The scaffolds were solubilised in equal parts of dichloromethane and isopropanol. The absorbance at 540 nm was measured and compared to a calibration curve to determine the amine concentration in the scaffolds.

Hydrolysis Quantification

The number of carboxyl groups inserted into the scaffolds was measured using the rhodamine method. A 4-mg sample of rhodamine 6G was diluted in a phosphate buffer (pH 12), 100 mL toluene was added to this solution under vigorous shaking, and the rhodamine was extracted. The PLLA and PCL scaffolds were solubilised in dichloromethane to which the rhodamine solution was added. After 1 h, the absorbance at 535 nm was measured and compared to a calibration curve to determine the carboxyl concentration in the scaffolds.

Immobilisation and Quantification of Collagen

A 2-mg/mL collagen solution was prepared in aqueous acetic acid (3 vol%). The aminolysis of PLLA and PCL was performed over 5 min and 24 h, respectively, according to the parameters described above. The hydrolysis was performed over 3 min and 4 h for PLLA and PCL, respectively, as above described.

The collagen was immobilised on the scaffolds using three methodologies. In the first, the scaffolds with inserted amine were immersed in a collagen solution for 2 h, frozen, lyophilised and then immersed in a 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide chloride (EDC) solution with 48 mM EDC, 6 mM N-hydrosuccinimide and 50 mM MES (2-(*N*-morpholino)ethanesulfonic acid) buffer (pH 5.0) for 24 h at 37°C [21]. The scaffolds with the inserted amine were also used for the second immobilisation method. The scaffolds were immersed for 3 h at room temperature in a solution containing 1 wt% GTA in PBS (pH 7.4), rinsed with deionised water for 2 h and immersed in a collagen solution at 4°C for 24 h [10]. Finally, in the third method, scaffolds with inserted carboxyl groups were immersed in an EDC solution (similar to that in method 1) for 24 h at 4°C, rinsed with deionised water for 2 h and immersed in a collagen solution for 24 h at 4°C [13].

The quantity of inserted collagen was measured using the ninhydrin test. For degradation, the treated scaffolds were immersed in a 6 M HCl solution for 24 h, at 120°C under a nitrogen atmosphere. The HCl solution was evaporated, and a combination of a ninhydrin solution (0.04 M) and a 0.1 M citric acid buffer solution (pH 5.5) was added. The final solution was heated to 70°C for 10 min and cooled to 4°C for 5 min. The absorbance at 560 nm was measured and compared

to a calibration curve to determine the collagen concentration in the scaffolds.

Differential Scanning Calorimetry (DSC)

After electrospinning, the polymer pellet and scaffold, without heat treatment or with submission to heat treatments similar to the steps that occur during the aminolysis and hydrolysis reactions, were analysed for PCL. The specimens (10 mg) were analysed from −50°C to 250°C at a rate of 10°C/min under a 50 mL/min nitrogen flow. The crystallinity (X_c) was measured according to the following formula:

$$X_c = \Delta H_m / \Delta H_f^o$$

in which ΔH_m is the enthalpy of fusion and ΔH^0_f is the specific heat of fusion (J/g), which were 93 and 140 J/g for PLLA and PCL, respectively.

Wide Angle X-ray Diffraction (WAXD)

X-ray diffraction was performed on the PCL films (prior to electrospinning) and the scaffolds both without heat treatment and with the same heat treatment used in the aminolysis and hydrolysis reactions. The analysis was performed using a monochromatic beam with wavelength of 0.154 nm (CuKα), at 30 kV and 15 mA. The intensities were obtained from 2θ values ranging from 3 to 60°, at steps of 0.05°.

Cell Adhesion and Proliferation

A cell line of mouse osteoblasts (OFCOLL II) obtained from the cell bank of Rio de Janeiro was used. A cell suspension of 1×10^4 cell/mL was added to the culture dish and cultivated in Dulbecco's modified Eagle's medium (DMEM) with 15% foetal bovine serum and 1% penicillin/streptomycin (10,000 U mL^{-1}/10,000 μg mL^{-1}). After a specified time interval, the scaffolds containing the cultivated cells (n=3) were immersed in DMEM with a 20% aqueous solution (5 mg/mL) of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). After 4 h in an oven, the crystals were dissolved in dimethyl sulfoxide. Their absorbance was measured at 570 nm.

Statistical Analysis

For each test, the data were submitted to a one-way ANOVA and Tukey's test (α=0.05), according to the homoscedasticity and normality requirements.

RESULTS AND DISCUSSION

To optimise the parameters and to improve the functionality of the polymer scaffolds, PLC was submitted to aminolysis and hydrolysis reactions at several time intervals. The aminolysis reaction broke the polymer ester linkages and inserted an amine while releasing water. The yield of amine insertion depends on the temperature, reaction time, type of amine and concentration [11]. However, because the electrospun fibres are produced in nanometre sizes and because the process can decrease the crystallinity in some cases, the conditions necessary for producing similar quantities of amino (via aminolysis) and carboxyl groups (via hydrolysis) are milder than for films and other thicker matrixes [19, 23].

In this study, the aminolysis of PCL nanofibrous scaffolds at 37°C in a 20 vol% EDA solution demonstrated a direct relationship between the reaction time and the total amine insertion, as indicated in Table 1. The maximum amine insertion was obtained after 24 h of reacting. After 24 h, the scaffold significantly degraded and lost its integrity. In contrast, the PCL nanofibrous scaffold was hydrolysed in a 1 M NaOH solution at 37°C, and an increase in the carboxyl groups was noted at 30 min, which became stable over time, according to Table 2. The hydrolysis reaction could continue for a maximum of 4 h before any loss of integrity became apparent. To our knowledge, only one previous study has evaluated the use of hydrolysis to melt a PCL membrane covered with a nanofibrous scaffold in which a 5 M NaOH solution was used over 3 h [20]. These parameters are reasonable when compared with the milder conditions in the present study, which treats nanofibrous structures instead of melted polymers.

Material	Amine type	Aminolysis time (h)	Amine group concentration (mol/g)
PCL	EDA	1	$2.8\pm0.8\times10^{-3}$ (B)
		6	$3.6\pm0.7\times10^{-3}$ (B)
		12	$3.6\pm0.5\times10^{-3}$ (B)
		24	$10.6\pm0.2\times10^{-3}$ (A)

Table 1: Total amine group concentration (mol/g) inserted into PCL scaffolds based on the reaction time (h) using EDA. Similar letters indicate an absence of a significant difference (α=0.05).

Material	Hydrolysis Time (h)	Carboxyl group concentration (mol/g)
PCL	0	$1.1\pm0.2\times10^{-5}$ (B)
	0,5	$1.9\pm0.1\times10^{-5}$ (A)
	1	$2.0\pm0.3\times10^{-5}$ (A)
	2	$2.0\pm0.2\times10^{-5}$ (A)
	4	$2.1\pm0.2\times10^{-5}$ (A)

Table 2: Total carboxyl group concentration (mol/g) inserted in PCL scaffolds, based on reaction time (h), using 1M NaOH. Similar letters show absence of statistical difference (α=0.05).

Several techniques can be used to measure crystallinity. X-ray diffraction is known for its higher rigidity and allows for a 3D analysis of the specimen. In contrast, DSC is simpler and more widely used [24]. Because both techniques have advantages and limitations, both were used in a complementary way. Table 3 shows the PCL crystallinity values before and after electrospinning and in electrospun scaffolds both with and without heat treatment (annealing). These conditions simulated both aminolysis and hydrolysis reactions. The PCL shows no change in its crystallinity after electrospinning or heat treatment. These data agree with other studies involving PCL electrospun fibres or sponges [17, 25]. The crystallisation of PCL may be facilitated by the fibres' alignment and orientation after electrospinning or heat treatment due to its low T_g [17, 25]. With a more crystalline structure than PLLA for example, the PCL scaffold is more resistant to degradation, which occurs preferentially in the amorphous portion of the material and thus allows an increased insertion of amine groups per gram of material.

Material	Crystallinity (%)	
	WAXD	DSC
PCL	47±3% (A)	53.2±0.2 (A)
Electrospun PCL without heat treatment	45±10% (A)	55±3 (A)
Electrospun PCL + 4 h at 37°C	46±7% (A)	55±3 (A)
Electrospun PCL + 24 h at 37°C	48±9% (A)	55±3 (A)

Table 3: PCL crystallinity before and after electrospinning obtained via WAXD and DSC and in electrospun scaffolds with or without heat treatment. Similar letters indicate an absence of a significant difference for each material in each test (α=0.05). Upper case letters shows comparison in PCL materials for each column.

Scaffolds treated under the conditions for the highest degree of aminolysis and hydrolysis for each material and obtained over a longer time were used to immobilise the collagen. The collagen was immobilised onto the aminolysis-treated scaffolds via two methodologies: (i) reaction with carbodiimide and (ii) reaction with GTA. The collagen was only inserted into the hydrolysed scaffolds using a carbodiimide reaction. Table 4 provides the quantification of the collagen (%) immobilised via the three given methods. The quantity of immobilised collagen mirrored the functionality obtained by hydrolysis or aminolysis. Accordingly, a higher proportion of free amine groups could be created in the electrospun PCL scaffolds than in the carboxyl groups. Therefore, the maximum amount of collagen was immobilised on scaffolds containing more amine groups. In fact, studies that evaluated films with increased amine or carboxyl group concentrations have demonstrated a direct relationship between these concentrations and the amount of collagen incorporated [10, 26]. The collagen quantity inserted using GTA was similar to that using EDC. Thus, one can conclude that the number of previously inserted functional groups is more important than the method used to immobilise the collagen.

Material	Functionalisation	Link	Immobilised collagen (%)
PCL	Aminolysis	EDC	1.6±0.5 (a)
	Aminolysis	GTA	1.5±0.1 (a)
	Hydrolysis	EDC	0.30±0.02 (b)

Table 4: Total quantity of immobilised collagen in PCL, using different methodologies. Similar letters indicate an absence of a significant difference for each material.

Finally, the PCL scaffolds containing immobilised collagen were submitted to cell culture to examine cell adhesion, cell proliferation and material cytotoxicity. Figure 1 shows cell adhesion and proliferation from day 0 to day 8 of culture for PCL. The cell adhesion observed on day 0 of culture showed no differences among the groups; apparently, on day 0, all of the materials were satisfactory for cell adhesion at the initial number of cells. However, with an increase in the number of cells, collagen insertion increased the cell adhesion and cell proliferation [10, 27]. Differences in cell proliferation were observed only after the 5th day for PCL, defining the optimal conditions being the aminolysis followed by collagen immobilisation with EDC. These data are consistent with the degree of functionalisation in the scaffolds and indicate a possible association between the degree of functionalisation and the amount of incorporated collagen.

When we compared the use of EDC or GTA as the crosslinking agent in scaffolds treated with aminolysis, no significant differences were observed between the groups with regard to collagen immobilisation or cell proliferation. This result indicated that none of the crosslinking agents had a cytotoxic effect and that the total quantity of collagen inserted is the most important parameter in controlling cell proliferation. In the present study, an attachment of at least 1.5% of the collagen to the scaffolds was required to guarantee sufficient cell proliferation and to develop significant improvements in the materials after 8 days of culture.

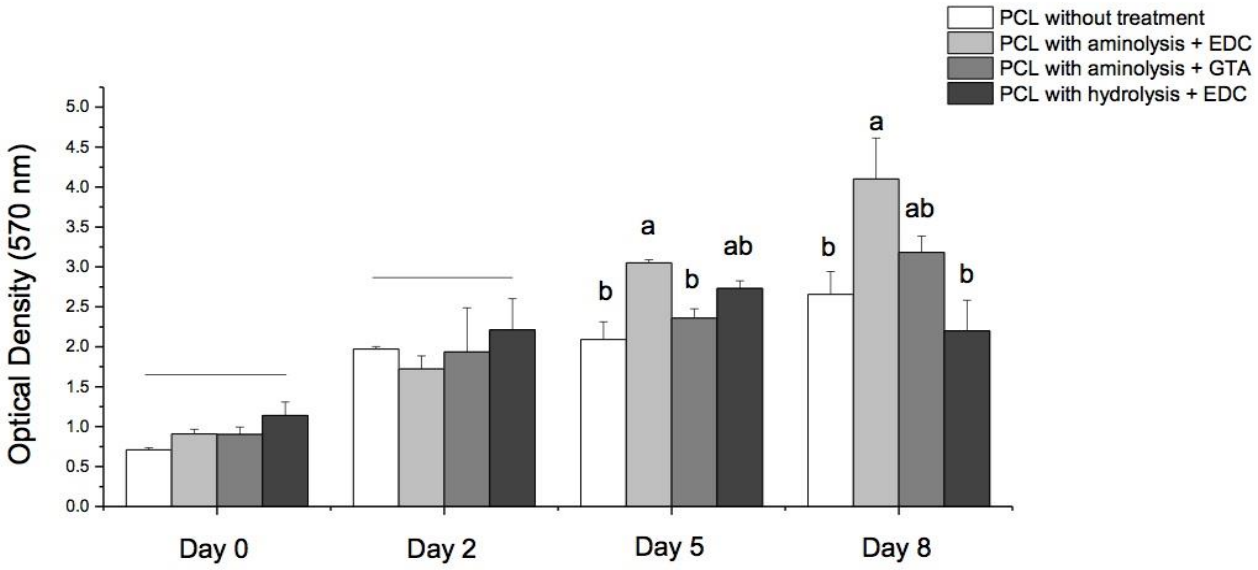

Figure 1: Mean and standard deviation of optical density related do osteoblast proliferarion (OFCOLL-II), obtained at 0, 2, 5 and 8 days in PCL scaffolds. Similar letters show absence of statistical differencefor each material (α=0,05).

CONCLUSION

Considering the presented results, we conclude that PCL electrospun mats as scaffolds in tissue engineering were successfully functionalised by aminolysis or hydrolysis. The parameters described and discussed here are presented as a general guide for optimising this process. Also, the functionalisations followed by collagen immobilisation were able to improve the cell attachment and proliferation when 1.5% de collagen was inserted. The choice of methodology for collagen immobilisation is related to the total number of inserted functional groups.

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