Novel hybrid membrane of chitosan/poly(ε-caprolactone) for tissue engineering

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We investigated the potential use of 3D hybrid membrane: poly(ε-caprolactone) (PCL) mesh using rotary jet spinning with subsequent chitosan (CH) coating. The morphological examinations by scanning electron microscopy (SEM) were proved the efficiency of this technique on obtaining relative homogeneous PCL fiber mats (15.49 ± 4.1µm), with high surface porosity (1.06 ± 0.41µm) and effective CH coating. The feasibility of rotary jet spinning allowed the solvent evaporation during the process; this fact was verified by differential scanning calorimetry (DSC), indeed also had verified changes in thermal properties on the hybrid membrane, since the present of CH. It was investigated the mechanical properties of the hybrid membrane and CH film, the data were that the samples presents good tensile modulus but low strain at the break. In addition, it was verified the biocompatibility properties in vitro using Vero cells. PCL mesh demonstrated cells more spread vastly in the pore surface, with attachments in between fibers indicating the potential for cell adhesion. The films samples (CH and hybrid membrane) resulted in a cells layer on the surfaces with an intense staining (metachromasy), which is the result of cells more active. The cell counting -5 days of culture- and the MTT assay -21 days of culture- demonstrated that the materials tested proved to be different from the positive control and equal to each other and this fact, in our view, this indicates a satisfactory proliferation. Thus, based on the results here, this novel hybrid membrane provides an attractive material for tissue engineering applications.

Introduction

Chitosan (CH)-based membranes seem to be excellent materials that could be very used in many biomedical applications. This biopolymer ensures not only biodegradation, but also its natural characteristic of being an antimicrobial agent and guaranty biocompatibility with cell culture. However, less flexibility in regulating the mechanical properties limits its usage.1

Indeed the polymer poly (ε-caprolactone) (PCL), well-known semicrystalline aliphatic polyester used extensively and FDA-approved for biomedical applications, presents highly crystalline structure that is beneficial to its mechanical properties. But it has low surface energy and hydrophobicity limits its biocompatibility and has a slow degradation rate (up to 6 to 36 mo).2

The search for ideal biomaterials is still on-going for tissue regeneration where the properties of scaffolds are dictated concurrently by many factors, e.g., cell-material interaction, mechanical solicitation, degradation rate and metabolic route. Blending polymers is an approach to develop new biomaterials exhibiting combinations of properties that cannot be reaching by individual polymer. Indeed, the research of CH and PCL blend brings to a new direction for tissue engineering.3-5

In tissue engineering scaffolds can be produce by many technologies. With the focus to reach nanofibers the technique widely used is electrospinning, which can be classified by the method of polymeric preparation into solution and melt electrospinning. Since 1934, many researches attempt to improve the melt and solution electrospinning drawbacks. The solution electrospinning process suffers from low productivity (up to 300mg/hr); requirement of additional solvent extraction process; and environmental concerns (toxic solvents are used). In other hand, the melt electrospinning is free from those drawbacks, but present difficulties inherent in finer fiber formation, higher viscosity of molten polymer and the electrical discharge problem associated with the application of high voltage to polymeric melt.

The search to overcome the use of electrostatic force lead to the creation of different methods such as meltblowing, drawing, biocomponent spinning, forcespinning, phase-separation, and flash-spinning. Indeed all the methods present advantages and disadvantages comparing with electrospinning. Simple processes, free solvent use, high production rates and environmental advantages are examples of the mains vantages; however the nonuniform fiber size, higher variation of fiber diameter, complex process and machine, and polymeric heating are the principals’ drawbacks.

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The rotary jet spinning (RJS) was created in 2010 by Badrossamay. This system fabricates three-dimensional aligned nanofibers by exploiting a high-speed rotating nozzle to form a polymer jet which undergoes stretching before solidification. Some aspects can be controlled by varying rotational speed nozzle geometry and solution properties e.g., fiber diameter, morphology and web porosity. Comparing with the most common method to obtain fibers the electrospinning, the RJS has several advantages, e.g., no requirement of high voltage, fiber fabrication is independent of solution conductivity, can be applicable to polymeric emulsions and suspensions and high productivity.6

This study explored the application of the PCL mesh produce by rotary jet spinning technique (RJS). The aims of this research were to investigate the potential use in tissue engineering of the 3D hybrid structure: Chitosan coating/Poly (ε-caprolactone) mesh (hybrid membrane).

Results

Characterization of the samples

SEM images

We produced using the technique rotary jet spinning PCL fibers in a morphology of non woven membrane showing high porosity surface the average was 1.06 ± 0.41µm (Fig. 1A and B).

Also, analyzing the SEM images was possible to note the full coating of the biomaterial with chitosan (Fig. 1C and D). The size of the PCL fiber were determinate by the use of the software Image Tool, a total of 54 fibers were measured at 3 randomly selected places, the average was 15.49 ± 4.1 µm, as demonstrated in the histogram (Fig. 2).

Thermal properties

PCL is a crystalline polymer: pure PCL melts at 60 °C and its glass transition temperature around –60 °C. On the other
hand, the biopolymer chitosan is an amorphous biopolymer, the transition temperatures may vary significantly based on deacetylation degree reach for the used CH. Figure 3 showed the second heating thermograms for a control PCL mesh and the hybrid membrane PCL/CH. Into figure is shown the melting peak for each sample and the enthalpy involved during the melt process. On the upper corner on the graphic is shown the crystallization process for each sample were is clear the difference between the peak crystallization temperature reach after erase the thermal history.

Table 1. Tensile modulus, peak load and strain at break of the CH and hybrid membrane samples

| Samples               | Tensile Modulus (MPa) | Peak load (N) | Strain at break (%) |
|-----------------------|-----------------------|---------------|---------------------|
| CH film               | 2836,92 (± 276)       | 24,06 (± 1,5) | 1,88 (± 0,31)       |
| Hybrid membrane (CH/PCL) | 1342,25 (± 187)   | 16,58 (± 3,5) | 2,82 (± 0,82)       |

The samples were tested in a dry state. Table 1 shows the results the samples were very brittle, exhibiting a break strain as low as 1.8–2.8% and an elastic modulus of 2836–1342 MPa. All the samples had a uniform thickness of 14 ± 0.08 μm and 12 ± 0.03 μm hybrid membrane and CH film, respectively.

Mechanical tensile test

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In vitro tests

Figure 4 we observed cells growing on the materials with 1 and 2 d of culture with light microscopy. Figure 5 shows we could see cells culture by 3 d and stained with TB and CB. We see that the spacing between the material fibers interfere with the cell adhesion and growth on biomaterials. The cell growth analysis showed that after one day of culture, the positive control was different from the other samples. After two days of incubation, the sample PCL mesh was similar to negative control and both were different from others samples studied. With three days of culture, the biomaterials tested were shown to be equal to each other and different from controls.

During the culture period cells were not able to reach confluence on the substrates (Fig. 6). In all samples, at different time, we detected cells strongly stained by TB, which are basophilic dye. On PCL mesh, we found ortochromatic cells. On the other hand, on hybrid membrane and CH film we found metachromatic cells. The cells were also stained with CV, a general dye used for morphological studies. With this dye we could see cells with irregular morphology on biomaterials.

Figure 7 showed the absorbance obtained from an MTT assay of Vero cells with were cultured on chitosan films, PCL films and hybrid membrane for 21 d. The results indicate that Vero cells had viability pattern similar to positive control used. Thus, after 21 d of cultured, we found on polymers studied a number of living cells similar to culture plate.

Discussion

The development of new technologies to manufacture nanofibers and microfibers (e.g., drawing out, molecular self
assembly, thermally induced phase separation, electrospinning) are increasing nowadays. Modifications of electrospinning techniques are co-axial electrospinning and electrohydrodynamic printing, indeed those processes use needle for the polymer ejection. Needleless technology is a promising process, since it is very flexible and enables the creation of nanofibers with high production capacity on an industrial scale, the process use liquid surfaces on a rotary spinning roller or wire. However the utilization is limited by the major disadvantages like low production rate and low safety features.

The need of improvement techniques attempted to new method without application of high voltage, in this case, centrifugal spinning system (rotary jet spinning) offers several appealing features such as the obtained fibrous web, with interconnected pores, facile and low cost effective process. In this study we investigate the use of a Poly (caprolactone) (using

Figure 4. Images with 24 (A1-C1) and 48 (A2-C2) hours of culture, images of the optical microscopy without staining, magnification of 100x (A-PCL mesh; B-hybrid membrane; C-CH film).
chloroform as a high volatile solvent) mesh by rotary jet spinning process with Chitosan (dissolved by acetic acid) coating for tissue engineering applications.

The solvent choice brings others possibilities at the methodology, e.g., the evaporation and viscosity control. The volatility of the solvent act at the solidification and contraction of the jet, therefore when use highly volatile solvents the jet form thicker fibers. Also, the fiber diameter is correlated with the dynamic viscosity, that increase proportional. The PCL fibers had the diameters ranging from 11.39 to 19.59 μm (Fig. 2). The literature demonstrated results lower than we obtained; with an orifice diameter of 340 μm, 12000 rpm rotation speed and 10

**Figure 5.** In vitro test Vero cells: Images with staining TB (A1-C1) and CV (A2-C2) with 72 h of culture, magnification of 200x (A- PCL mesh; B- hybrid membrane; C-CH film).
melt temperature is around 3 °C, been bigger for the PCL in the first heating shown and the difference between PCL and Chitosan. After the crystallization process, in the main peaks of PCL shown in Figure 3 in many biomedical applications. Improves water absorption capability, which is desirable property for implantation. The hybrid membrane composed of PCL and CH had higher strain at break which is important to ensure dimensional stability properties to this system against external forces. But the high stretching caused by the rotary jet spinning technique over the non-woven PCL fibers, coupled with the presence of layers of chitosan (a very rigid but fragile biopolymer), is what gives it these low properties to this hybrid membranes.

Cytotoxicity tests were conducted to investigate the effects of the biomaterials on animal cells, which is one of the prerequisites for implantation. The Figure 4 showed the samples PCL mesh, hybrid membrane and CH film during 1-d and 2-d of culture, also it was possible to observe the morphology of the Vero cells, all the samples presents a compatibly surfaces, without any toxicity. In order to see the growth and morphology of the cells on the surface were staining with toluidine blue (TB) and crystal violet (CV) at 3-d of culture (Fig. 5). With CV it was seen that the cells had different interactions with different material structures. The sample with fibers demonstrated cells more spread vastly in the pore surface, with attachments in between fibers indicating the potential for cell adhesion. The films samples (CH and hybrid membrane) resulted in a cells layer on the surfaces. Cytochemistry revealed changes in cell behavior induced by materials. TB-stained basophilic cells were observed in all samples. At pH 4.0, TB stains nucleic acids and glycosaminoglycans. Although Vero cells synthesize glycosaminoglycans, they produce them in soluble form in the culture medium. Thus, the presence of variations on basophilic cytoplasm suggests variation on cell active. We found a more intense staining (metachromasy) at cells on hybrid membrane and CH film than PCL mesh. These results suggest that cells were more active on hybrid membrane and CH film.

Once attached, Vero cells proliferated on all types of samples and were quantified by counting cell number. The 1-d results were without difference of the proliferation rate, except by...
negative control. With two days incubation, the sample PCL mesh was similar to negative control and both were different from other samples studied. With three days of culture, the biomaterials tested were shown to be equal to each other and different from controls (Fig. 6).

The culture plates, used as negative control, are prepared to stimulate cell growth. Accordingly, it was expected that these samples had higher cell proliferation. The materials tested proved to be different from the positive control and equal to each other. In our view, this indicates a satisfactory proliferation standard. Similar results were obtained with Vero cells with different blends of PLLA/PHBV and different porous PLLA scaffolds.14,15

With the goal to investigate the cell viability of the samples with Vero Cells, we produced the cell culture during 21 d, we made a MTT assay with this time of incubation. The results obtained with samples chitosan, chitosan / PCL and PCL shown that these did not alter the proliferative index, because the cells grew and multiplied on them in a pattern similar to positive control (Fig. 7). These data support the interpretation that the cells grow in a satisfactory manner on these polymers. The biodegradable materials have a good ability to support cell growth. In this sense, our data are consistent with other reports.15

Materials and Methods

Materials

The polymer (poly(D-glucosamine) name chitosan (CH) with viscometer molecular weight 130,000 g/mol and 80 KDa polycaprolactone (PCL) were purchased from Sigma Aldrich, the solvent chloroform from Merck and acetic acid form Synth.

Formation of the mesh

PCL pellets were dissolved in chloroform at a concentration of 10% (w/v) under stirring. After the homogenization, 6 h, the solution was prepared to use at the rotary jet spinning system (Fig. 8).

The equipment consisted of a reservoir with four side wall orifices, with the diameter of 0.5 mm that is attached to the shaft of a motor with one rotation speed (3500 rpm). To facilitate the fiber collection an aluminum foil is placed on the cylindrical collector held against the collector wall at a distance of 300 mm. The process allows obtain fibers through a drop by stretching a component of centripetal force (tangential) when from a container in rotation, is ejected a viscoelastic solution.16 This is how on the inner walls of the drum, a film type non woven mesh is created.

Fabrication of hybrid membrane

Layer by layer CH/PCL/CH was prepared using the PCL mesh, obtained by rotary jet spinning, and solution of CH/acetic acid. The 50% of the solution was placed into a Teflon mold and cover with the PCL mesh, after 24 h was added the 50% of the CH/acetic acid solution to form a uniform coating (Fig. 9).

Fabrication of the films

Like a control a film of PCL was casted into a glass petri dish by dissolving in chloroform (10% w/v) at room temperature. The dried film was collected and vacuum dried for 48 h. The same preparation method was used for CH film, but the solvent used was 0.3 M acetic acid solution with the CH/solvent proportion of 0.17:50% w/v.

Characterization

Thickness and fiber dimensions

The thicknesses of all samples were measured using a micrometer and the average value was computed from 6 measurements of different scaffolds for the same sample batch. The PCL fiber dimensions were characterized using Image
Stained with Toluidine Blue at pH 4.0 (TB) and Crystal Violet (in PBS 0.1 M, pH 7.2), washed twice in distilled water, and at 5 d of culture, the samples were also fixed in 10% formalin. For the second scan, the CH samples were heated up to 180 °C and maintained at the temperature for about 5 min in order to erase the thermal history. The melting temperature of PCL was taken as the temperature at which the endothermic peak occurred in the second scan. For the CH samples, it was heated to 180 °C and maintained at the temperature for about 5 min, then were cooled to 25 °C from where they were heated back to 300 °C.

Tensile mechanical tests

Tensile tests of samples were performed at MTS tensile tester (FEM-Unicamp). Rectangular strips (~7 mm × ~0.5 mm × ~0.15 mm) were subjected to a constant strain rate (20 mm/min) in tension until broken. From the stress-strain curves, tensile modulus (MPa), peak load (N), and strain at break (%) were determined. The reported values are an average of five measurements.

In vitro tests

Vero cells, a fibroblastic cell line established from the kidney of the African green monkey (Cercopithecus aethiops), were obtained from the Adolfo Lutz Institute, São Paulo, Brazil. Vero cells are recommended for studies of cytotoxicity and cell-substratum interactions in biomaterial research. The cells were cultured in 5% CO2 at 37 °C in 199 medium (Lonza Group Ltd) supplemented with 10% fetal calf serum (FCS, from Nutricell). The significant differences were analyzed by one-way analysis of variance (ANOVA). Differences were regarded as statistically significant at \( P < 0.05 \).

Conclusions

In this paper, we present data which show the extent of the potential of the PCL fibers, produced by rotary jet spinning, coated with chitosan forming a 3D hybrid membrane with high potential to be use in tissue engineering. It was investigated the surface morphology, thermal and mechanical properties, and biocompatibility of the novel hybrid biomaterial. The rotary jet spinning was investigated and successfully used to produce PCL 3D porous mesh. Also, the technique of casting promoted the combine of PCL mesh with CH membrane. Finally, the successful addition of cells is creating and even more versatile and biocompatible 3D hybrid structure.
This preliminary investigations indicate that these strategies have great potential to improve current biomaterials and in the development of new scaffolds for tissue engineering.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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