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Cytocompatibility evaluation in cell-culture systems of chemically crosslinked chitosan/PVA hydrogels

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ABSTRACT

In the present study we report the preparation, characterization and cytocompatibility of novel polymeric systems based on blends of chitosan and poly(vinyl alcohol) (PVA) and chemically crosslinked by glutaraldehyde for biomedical applications. The structure of the hydrogels was characterized through Fourier Transform Infrared spectroscopy (FTIR) and their swelling behavior was investigated as preliminary *in vitro* test. Bioactivity, cytotoxicity and cell viability were assessed via MTT assay with 2 cell cultures and cell spreading-adhesion analysis. Moreover, the cell viability and potential biocompatibility were assessed by the secretion of nitric oxide by activated macrophages with gamma interferon (IFN-γ) cytokine and lipopolysaccharide (LPS). It was found that by increasing the chitosan to PVA ratio the swelling behavior was significantly altered. In addition, all tested hydrogels have clearly presented adequate cell viability, non-toxicity and suitable properties which can be tailored for prospective use in tissue engineering.

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1. Introduction

Chitosan is a copolymer of glucosamine and *N*-acetylglucosamine derived from the natural polymer chitin, which is commercially available. Chitosan can be considered as one of the most investigated materials in recent years. The non-toxicity, high biocompatibility, and antigenicity of chitosan have driven the interest of the scientific community due to its potential applications in biomedical field. It was reported that chitosan could regulate cell biology such as differentiation, proliferation, and cytokine production [1–4]. The biocompatibility of chitosan brings its utility as biomaterial, in tissue engineering, membranes and drug delivery systems [1–4]. But chitosan has some drawbacks, it is only soluble in aqueous medium in the presence of a small amount of acid such as acetic acid and its mechanical properties are not readily suitable for some biomedical applications. Also, it has been reported the toxicity of chitosan to some organisms associated with its bacteriostatic effect [4–7]. That has raised some concern about using chitosan as a fully biocompatible polymer. So, it is important to move beyond standard regular bioactivity methods in order to properly evaluate chitosan-derived hydrogels. As a result many researchers have tried to modify its properties without compromising the viability of future use in living organisms. One path to be pursuit is the utilization of natural or synthetic polymers, separately or blended, with grafted or crosslinked networks, in order to match the required properties. In other words, the polymer blended crosslinked system may present differential degradation behavior under physiological fluid conditions, where part of polymeric network may undergo to fast solvation and another portion may experience slow degradation by de-polymerization. Hence, chitosan joined to other polymers opened a window of research for altering or tailoring the property of interest [4–7]. Among organic polymers, poly(vinyl alcohol) (PVA) is one of the very few vinyl polymers soluble in water that has been studied intensively because of its attractive features for medical applications such as high hydrophilicity, good film forming and processability [7,8]. In addition, the published literature has indicated that no significant difference was found in quantitative or in qualitative cytotoxicity evaluation of PVA. It was also shown that the amount of PVA accumulated in organs was too small to affect the biological fate, which suggested that PVA is excreted to the same extent as for example PEG, supporting the cytocompatibility of PVA [9]. Focusing on modifying the polymeric network, glutaraldehyde has been broadly used as an active chemical crosslinker due to the formation of intra–interchain covalent bondings. Nevertheless, this synthetic crosslinking reagent has been reported as highly cytotoxic that may impair the biocompatibility of the crosslinked biomaterials [10]. That disadvantage can be overcome by assuring that all aldehyde functional groups are actually crosslinking the polymeric network or they are effectively blocked with molecules such as aminoacids and proteins which are widely present in living organism serum. MTT and cell adhesion have been used for preliminary evaluation of biocompatibility of biomaterials for quite some time. More recently, nitric oxide (NO) which is a potent

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signaling molecule secreted by healthy endothelial cells has been utilized to assess the activity of cells. NO is a highly reactive free radical involved in a number of physiological and pathological processes [11]. As a result, nitric oxide production by macrophages can be examined as indicator of cell activation. A prospective biomaterial has to undergo extensive *in vitro* and *in vivo* tests and should ensure non-toxicity to the biological hosting site where it will be utilized. Cell-based compatibility assays must be properly conducted as a reliable tool of predicting the biomaterial behavior *in vivo* and future clinical performance.

Thus, in the present research we have developed a novel system by blending chitosan with PVA (low degree of hydrolysis), at different ratios followed by chemical crosslinking with glutaraldehyde, aiming for altering the polymeric network. These synthesized chitosan/PVA films were spectroscopically and structurally characterized via FTIR. Moreover, cell cultures were used for assessing cytocompatibility and cell viability cell behavior using MTT method, cell spreading-adhesion via SEM analysis and nitric oxide production by activated macrophages with gamma interferon (IFN-γ) cytokine and lipopolysaccharide (LPS).

2. Materials and methods

2.1. Materials

All salts and reagents used were of analytical degree and Milli-Q water was used in all solutions (18.0 MΩ). Poly (vinyl alcohol-co-vinyl acetate) (PVA) supplied from Sigma-Aldrich with 80% degree of hydrolysis and molar weight M_W = 9000–10,000 g/mol. Chitosan (Aldrich Chemical) powder, medium molecular weight, degree of deacetylation (DD) = 80%, was used without further purification. Glutaraldehyde (GA) or 1,5-pentane-dial (Aldrich Chemical) used as covalent chemical crosslinking reagent was purchased as a 25% (wt.%) aqueous solution.

2.2. Methods

2.2.1. Chitosan and PVA solution preparation

Briefly, PVA hydrogels were prepared by fully dissolving 5.0 g of polymer powder without further purification in 100 mL of Milli-Q water, under magnetic stirring, at temperature of 75 °C ± 2 °C, as previously reported by our group [7,8,12]. PVA 5% solution was cooled down to room temperature and the pH was corrected to (2.00 ± 0.05) with 1.0 M HCl (Sigma). Chitosan hydrogels (Chi) were produced in a similar procedure by fully dissolving 2.5 g in 250.0 mL of Milli-Q water with 2% of CH_3COOH (Sigma), under constant magnetic stirring for 48 h.

2.2.2. Chitosan, PVA and blends films preparation

Different quantities of PVA were added into the 1.0% chitosan solution in order to obtain chitosan/PVA mass ratios of (0:1), (1:3), (1:1), (3:1) and (1:0) and pH was corrected to (4.00 ± 0.05) with 1.0 M NaOH solution. The mixture was kept under stirring for 5 min until the PVA and chitosan completely formed a clear solution. Then, the crosslinker reagent (glutaraldehyde) was slowly added under constant stirring. The final concentration of glutaraldehyde in the gel solution precursors was 1% and 5% (wt.%). Further in the sequence, the solution was poured into plastic moulds (polyethylene, round-plate shape, diameter = 85 mm, height = 10 mm) and let drying for 72–120 h at room temperature, and finally dried at 40 °C for 24 h (constant weight). Chitosan/PVA samples chemically crosslinked were identified by (*X*:*Y*:*Z*) that is, *X* as chitosan content, *Y* as PVA content and *Z* as glutaraldehyde (wt.%). For instance, sample labeled as Chi/PVA/GA (1:3:1) represents the following proportion of reagents: 25% chitosan, 75% PVA and crosslinked with 1.0% GA (wt.%). The dried gel was stored in a desiccator before all subsequent characterization procedures. The average film thickness produced was assessed with a Mitotoyo (± 10 μm) micrometer (4 measurements each sample).

2.3. Characterization

2.3.1. Fourier transform infrared spectroscopy (FTIR)

FTIR spectroscopy analysis was performed to characterize the presence of specific chemical groups in the hydrogels and also the effectiveness on the crosslinking process. Chitosan, PVA and their blends before and after crosslinking with GA (Chi/PVA/GA) were prepared as 100 to 200 μm thick films and analyzed by FTIR using ATR (attenuated total reflection) modes. FTIR spectra were collected with wavenumber ranging from 4000 to 650 cm^{-1} during 64 scans, with 2 cm^{-1} resolution (Paragon 1000, Perkin-Elmer, USA). The FTIR spectra were normalized and major vibration bands were identified and associated with the main chemical groups.

2.3.2. Simulated body fluid assay (SBF)

Preliminary cytocompatibility was investigated in the present work, by using a protein-free acellular simulated body fluid medium (SBF or Kokubo solution) with pH (7.40) and ionic composition (Na^+ 142.0, K^+ 5.0, Ca^{2+} 2.5, Mg^{2+} 1.5, Cl^- 147.8, HCO_3^- 4.2, HPO_4^{2-} 1.0, SO_4^{2-} 0.5 mM) equal to those in blood plasma [12]. Hence, chitosan/PVA hydrogel samples were cut into 5.0 mm × 5.0 mm square pieces and soaked in SBF at pH 7.4 for swelling assay. Samples were evaluated at 30 min, 2 h, 4 h, 24 h, 96 h and 192 h. At the end of each soaking period, the remaining solution excess on the gels was wiped with a lint-free tissue paper, and dried at 40 °C in an oven for 24 h.

2.3.2.1. Swelling assay. Fluid absorption studies are of paramount importance for preliminary analysis of biodegradable materials. For fluid-uptake measurements, all the specimens of the chitosan/PVA hydrogels with molar ratios of 0:1, 1:3, 1:1, 3:1 and 1:0 were prepared as described in the previous section, were weighed (W_0) before being immersed in SBF at 37 °C. After immersion for different time periods, the samples were carefully removed from the medium and, after wiping off water excess on the surface with filter paper, they were weighed for the determination of the wet weight (W_f) as a function of the immersion time [12]. Swelling index (S) is given by the Eq. (1):

$$S = \frac{W_f - W_0}{W_0} \times 100\% \quad (1)$$

Each SBF absorption experiment was repeated three times and the average value was taken to validate the results.

2.3.3. Cytocompatibility, cell viability and bioactivity assays on polymeric blends

2.3.3.1. Neutralization procedures. Phosphate buffered saline (PBS) was used in the procedure to neutralize remaining cytotoxic groups of non-reacted glutaraldehyde crosslinker. The chitosan/PVA films were immersed in polyethylene flasks with 75 mL PBS solution without cells and with an area/volume ratio ranging from 0.5 to 1.0 cm^{-1}. The flasks were placed in an incubator with controlled temperature of 37 °C for 2.5 h. Later the samples were washed in de-ionized water, and dried at 40 °C for 48 h. All the samples submitted to the cytotoxicity experiment have been previously sterilized by exposure to saturated steam of ethylene oxide. It is rather important to emphasize that despite of being reported cytotoxic, glutaraldehyde reacts under acid condition with both amine and hydroxyls groups from chitosan and PVA. Yet, any remaining aldehyde unreacted group is almost immediately blocked by aminoacids and proteins present in living organisms sera.

2.3.3.2. Bioviability of chitosan/PVA blends in VERO cell culture. As previously reported by our group [12,13], African green monkey kidney VERO cells, a fibroblastic cell line, were used for the experiments of cell biocompatibility MTT (3-[4,5-dimethyltriazol-2-y1]-2,5-diphenyl tretrazolium) and adhesion assays. It is worth to point out that a fully

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