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Comparison of the properties of membranes produced with alginate and chitosan from mushroom and from shrimp

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ABSTRACT

Dense and porous chitosan-alginate membranes (1:1 in mass) useful as coverages of skin wounds treated through cell therapy were produced using chitosan of different chain sizes from fungal (white mushrooms) and animal (shrimp shells) sources. Porous materials were obtained by adding the surfactant Poloxamer 188 to the formulations. The influence of chitosan type on membranes physicochemical properties and toxicity to fibroblasts was evaluated. Porosity was noticed to be more pronounced in membranes obtained with fungal chitosan and increased with its molecular mass. These formulations showed the highest values of thickness, roughness, opacity, liquid uptake and water vapor permeability. The membranes were not toxic to fibroblasts, but the lowest cytotoxicity values (0.16–0.21%) were observed for membranes prepared with fungal chitosan in the presence of surfactant. In conclusion, it is possible to replace chitosan from animal sources by chitosan of fungal origin to produce membranes with negligible cytotoxicity while maintaining appropriate physicochemical properties.

1. Introduction

The morbidity and mortality associated with chronic skin lesions, such as pressure and diabetic ulcers, are a significant healthcare problem worldwide and different therapies have been proposed for them lately. Cell therapy is a recent approach, consisting in the application of cultured healthy cells (frequently autologous) on the lesion to promote proliferation and growth of dermal and/or epidermal cells, creating an appropriate environment for healing [1]. Coating of the wound with appropriate dressings, such as those produced from carbohydrates, mostly polysaccharides of animal, plant or microbial sources, is often required after cell application to provide mechanical protection, prevent dehydration and protect against external agents.

The differences in chemical structure, molecular mass and chain shapes of the polysaccharides determine their solubility, surface, gelation and film-forming properties. Because of their functional diversity, non-toxicity, frequently good hemocompatibility and suitable interactions with living cells, polysaccharides such as chitosan and alginate are widely exploited for the production of wound dressings [2].

Chitosan is one of the most promising and versatile carbohydrates for medical and biological applications. Its properties include stability over long time periods under various conditions, gradual biodegradability, high biocompatibility, and bacteriostatic activity. In acidic media, chitosan becomes a cationic polymer, which allows immobilization of ligands, such as glycoproteins, to promote cell adhesion and proliferation [3]. The association of cationic chitosan with anionic polymers produces polyelectrolyte complexes (PECs) with unique physical and chemical properties [4], such as those obtained from chitosan-alginate PECs [5]. The advantages of PECs comprise greater mechanical stability, resistance to changes in pH and enhanced capacity to carry active agents [6].

Commercial chitosan can be obtained at relatively low cost from alkaline deacetylation of chitin, which is abundant in the exoskeleton of crustaceans. For this reason, most of the works focusing on the development of chitosan-alginate PECs useful as wound dressings are carried out with animal-derived chitosan, frequently with molecules of high molecular mass [7–11]. However, the diversity of animal sources, changes in seasonal supply, and difficulties in raw materials standardization result in high batch-to-batch variability in terms of deacetylation degree and molecular mass. These problems contribute to reduce experiment reproducibility and to yield conflicting results regarding the evaluation of

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important biological properties of chitosan for cell therapy applications. Moreover, chitosan of crustacean origin may also induce allergic reactions [12,13]. In the traditional process of chitin extraction from crustaceans, calcium and proteins are removed by HCl and NaOH, respectively. The remaining material is usually bleached with $KMnO_4$ or H_2O_2 and deacetylation is performed with hot concentrated alkaline or acidic solution. These harsh treatments can result in considerable amounts of waste [14] and deleterious trace contaminants.

Chitosan obtained from non-animal sources, such as from *Zygomycetes* fungi cultivated under controlled conditions, is an alternative potentially more suitable for the production of medical and pharmaceutical biomaterials than animal-derived chitosan, avoiding many of these inconveniences. Fungi-derived chitosan is a high-purity product with defined physicochemical properties and significantly lower health risks than the animal-derived product [15]. Whereas the exoskeleton of crustaceans is composed exclusively of chitin, the cell walls of fungi contain both chitin and chitosan [16]. Thus, it is possible to obtain chitosan directly from fungal wall using a mild weak acid treatment that has little or no effect on the polysaccharide structure and, in addition, generates less waste. Different species of mushrooms (*Agaricus bisporus*, *Auricularia auriculajudae*, *Lentinula edodes*, etc.) can produce chitin and chitosan. The extraction process of chitin is similar to that employed for crustacean shells, but due to the low levels of inorganic materials in cell wall, less concentrated solutions of acids may be used during the process [17].

Chitosan from fungi walls has low molecular mass and high polydispersity [16]. Its quantity and quality depend highly on the fermentation conditions (pH, culture medium composition, temperature) and the fungal species involved [18]. Several of the studies performed focusing on these factors aim at tailoring chitosan properties and increasing its yield. Moreover, efforts are also being made to reduce the production costs of fungal chitosan by using fermentation biowastes [19,20].

In spite of all these efforts, studies on the use of fungal chitosan for the production of biomaterials for tissue engineering are still scarce. One of the few studies comparing scaffolds based on fungal (*Gongronella butleri*) and shrimp chitosan reports that fungal chitosan scaffolds obtained by freeze-drying showed excellent mechanical, water absorption and lysozyme degradation properties [16]. The influence of chitosan source on the properties of more elaborate biomaterials composed of mixtures of polymers, such as chitosan-alginate PECs obtained in the presence or absence of surfactants as porogenic agents is not yet reported.

Hence, in the present study, dense and porous chitosan-alginate membranes were prepared using high molecular mass chitosan from animal and fungal sources with three different molecular masses to better account for the potential of replacement of animal chitosan in the production of wound dressings. The effects of chitosan source on membranes physicochemical properties and on their indirect cytotoxicity to fibroblasts cells were evaluated.

2. Materials and methods

2.1. Materials

Chitosan-alginates membranes were produced with high purity chitosan from white mushroom with three different molecular masses (40–60 kDa, 110–150 kDa and 140–220 kDa, deacetylation degree around 80%), chitosan from shrimp shells (C-3646, deacetylation degree of 88%, average molecular mass of 1260 kDa, intrinsic viscosity of 848 mL/g at 25 °C) and alginic acid sodium salt (A-2033, average molecular mass of 90 kDa, intrinsic viscosity of 690 mL/g at 25 °C, composed of 61% mannuronic acid and 39% guluronic acid), all from Sigma-Aldrich (St. Louis, USA).

The surfactant Poloxamer 188 (Sigma-Aldrich, USA), formerly commercialized as Pluronic F68, was used as porogenic agent. Other reagents include calcium chloride dihydrate and sodium hydroxide (Merck, Germany), acetic acid (Synth, Brazil), fetal bovine serum (FBS), α-MEM and RPMI-1640 cell culture medium (Nutricell, Brazil), thiazolyl blue tetrazolium bromide (MTT, Sigma-Aldrich, USA), sodium dodecyl sulfate (Merck, Germany) and dimethyl sulfoxide (Sigma-Aldrich, USA).

2.2. Membrane preparation

The alginate-chitosan membranes (1:1 in mass) were prepared based on the procedure described by Bueno et al. [9], with some modifications. For formulation description purposes, chitosan (C) and alginate (A) dense and porous membranes were designated C_i-A and C_i-A-P, respectively, where the subscript i in C denotes chitosan from fungal (C_F) or animal (C_A) origin. Fungal chitosan of different molecular masses were designated as C_{FV} (very low molecular mass, 40–60 kDA), C_{FL} (low molecular mass, 110–150 kDa), C_{FM} (medium molecular mass, 140–220 kDa) and C_{AH} (high molecular mass, 1260 kDa).

Briefly, aqueous solutions of chitosan at 1% (w/v) were prepared in 1% acetic acid, while alginate was dissolved in deionized water at concentration of 1% w/v. The chitosan solution (200 mL) was added to the alginate solution (200 mL) with a peristaltic pump (Model Minipuls 3, Gilson, USA) at a flow rate of 200 mL/h, at 25 °C and under stirring at 500 rpm in a stainless steel tank with internal diameter of 10 cm and height of 20 cm. Following, the pH of the suspension was elevated to 7.0 by adding 1 M NaOH, maintaining the stirring at 1000 rpm for 10 min. Afterwards, the carboxyl groups of alginate which were not bound to chitosan amino groups were crosslinked by the addition of 10 mL of 1% (w/v) $CaCl_2$ aqueous solution.

The mixture was then degassed with a vacuum pump (model Q-355B, Quimis) for 2 h, transferred in aliquots of 85 g to polystyrene Petri dishes (15 cm in diameter) and dried in an oven with air circulation (model 410, Nova Etica) at 37 °C for 24 h. The crosslinking of the dried membranes was complemented by total immersion of the films in 150 mL of an aqueous calcium chloride solution (2% w/v) for 30 min, followed by two washing steps in 200 mL of deionized water for 30 min each. A final drying step was carried out at room temperature for 24 h.

Porous membranes were prepared by adding Poloxamer 188 to the alginate solution at 0.2% (w/v). Except for the deaeration under vacuum, which was not performed for these formulations, the remaining steps were the same as those described previously.

2.3. Membrane characterization regarding physical and chemical properties

Membrane thickness, water vapor permeability (WVP), mechanical properties, opacity and morphological analysis were performed following the procedures reported by Bierhalz et al. [21]. Basically, the thickness of the membranes was measured with a digital micrometer (MDC-25S; Mitutoyo, Japan) at 10 random positions. The opacity of the membranes was measured with a colorimeter (Coloquest II; Hunterlab, USA), operating in the transmittance mode. The morphology was evaluated with a scanning electron microscope (SEM; model LEO440i; LEO Electron Microscopy, UK) operating at 15 kV and 100 pA after coating the samples with gold.

For water vapor permeability (WVP) determination, the sample under analysis was sealed over a circular opening of a Plexiglas® permeation cell filled with anhydrous calcium chloride (condition