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Characterization of a Gelatin/Chitosan/Hyaluronan scaffold-polymer

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Abstract Gelatin, chitosan and hyaluronic acid are natural components used to prepare polymeric scaffold in tissue engineering. The physical properties of these materials confer an appropriate microenvironment for cells, which can be used as a regeneration system for skin and cartilage. In this work, we prepared and characterized a Gelatin/Chitosan/Hyaluronan lyophilized-polymer. Physical properties of lyophilized-polymer changed slightly with moisture, but when polymer was totally hydrated the elasticity changed significantly. Thermophysical characterisation indicated that temperatures higher than 30ºC could modify irreversibly the polymeric matrix probably due to protein denaturation. Besides, we used the polymer as scaffold to prepare a biosynthetic-skin, reporting biological behaviour and its mechanical properties.

Keywords: chitosan, fibrin, gelatin, hyaluronic acid, scaffold

INTRODUCTION

In recent years, biologically active scaffolds used in tissue engineering and regenerative medicine have been generating promising results in skin replacement and cartilage regeneration. Many kinds of synthetic and natural polymer materials have been used as scaffolds. Their function is to provide mechanical support, allow

the adhesion and proliferation of the cells embedded on the scaffold or for cells migrating from surrounding tissue.

A scaffold should be biocompatible, non-antigenic, biodegradable and have a three-dimensional structure with adequate porosity and pore size (Dainiak et al. 2010). Synthetic polymers such us poly(lactic acid) (PLA) and poly(glycolic acid) (PGA) have good mechanical properties and stability, but have poor cell-matrix interaction due to lack of appropriate informational structure for cell attachment, also, they can be toxic for the implanted cells as they undergo hydrolytic degradation producing lactic and glycolic acid (Chen et al. 2002; Tan et al. 2009).

By contrast, natural biomaterials such as gelatin, chitosan and hyaluronic acid, among others, exhibit multiple bioactivities that are beneficial for the embedded cells, because they are either, components of extracellular matrix (ECM) or originated from organisms, thus containing cell-specific domains such as the RGD (Arg-Gly-Asp) cell adhesion sequence (Hunt and Grover, 2010). They are also bioabsorbable allowing the generation of a new ECM by the implanted cells. It is therefore recognized the use of these biomaterials to develop scaffold for skin and cartilage regeneration (Liu et al. 2004; Tan et al. 2009). The favorable properties of these biopolymers are related to particular characteristics of its main components that in combination make the polymer a very versatile and functional scaffold for tissue engineering.

Gelatin is an irreversible hydrolyzed form of collagen, formed by breaking apart its natural triple-helix structure into single-strand molecules. It is non-immunogenic compared to collagen; it retains informational signaling capacity such as the RGD sequence and is completely reabsorbable *in vivo* (Xia et al. 2004; Dainiak et al. 2010). Due to its inferior mechanical properties, generally, gelatin is used in combination with other scaffolds forming materials.

Chitosan is a partially deacetylated derivative of chitin, an abundant natural polymer and mayor constituent of the shells of some crustacean. It is a linear polysaccharide consisting of glucosamine and N-acetylglucosamine, and is structurally similar to glycosaminoglycans (GAG), an important structural element of the extracellular matrix of many tissues. This is a promising biomaterial because of its biocompatibility, biodegradability, low immunogenicity, gel-forming ability, high adsorption capacity, being also bacterio-fungistatic and nontoxic *in vivo* (Xia et al. 2004; Shi et al. 2006). The versatility of chitosan in terms of its modification and combination with other polymers has allowed its use for the development of a wide range of tissues such as bone, liver, neural tissue, vascular grafts, cartilage and skin (Shi et al. 2006). Gelatin and chitosan have been combined to synthesize scaffolds, characterized with an excellent ability to be processed into both, monolayer and bilayer porous scaffolds for use in human skin fibroblast and keratinocyte transplantation and skin regeneration (Mao et al. 2003).

Hyaluronic acid or hyaluronan (HA) is a naturally occurring, water soluble, polysaccharide that is widely distributed throughout the ECM of all connective tissues in human and other animals. It is the only nonsulfated GAG consisting of multiple disaccharide units of glucuronic acid and N-acetylglucosamine. The coiled structure of HA can trap 1000 times its weight in water. These characteristics give it unique physicochemical properties as well as distinctive biological functions. As a consequence, HA and its derivatives have been widely investigated as materials for tissue engineering, for instance, the HA properties include many special interactions

with growth factors, receptors and adhesion proteins that suggest that its incorporation into a chitosan-gelatin network it may modify the material bioactivity generating a hydrogel in which other matrix molecules can assemble (Mao et al. 2003; Tan et al. 2009).

Polyeletrolytic complexing has been suggested as joining mechanism of collagen-chitosan-hyaluronan (Collombel et al. 1992). Indeed is has been described that the acylation degree of chitosan would have a significant effect on the chemical interactions between the polymer, collagen and GAG. Miyata et al. (1993) indicated that an acylation degree between 70 and 100% could generate 45% of free NH_3^+ amine functions, limiting the bonding interactions of these groups with COO- of collagen or SO_4^{-2} and COO^{-2} groups of chondroitins sulfate, a common GAG used in scaffold preparation. Thus generating lower number of ionic bonds between constituents and therefore having a significant effect on the system integrity (*e.g.* salting out of GAG on solution). In order to generate a stable structure, a chemical crosslinking is generally recommended (*e.g.* hexamethlyne diidocyanate or glutaraldehyde) (Collombel et al. 1992).

Gelatin/Chitosan/Hyaluronan based scaffold-polymer has been combined to obtain a variety of biocompatible products for tissue engineering (Liu et al. 2004; Tan et al. 2009), but little information related to their physical properties is available. The biomaterial characterization is necessary in scaling-up from bench lab, pilot and industrial scale production. It is also a requirement for packaging design and optimal storage conditions (Marreco et al. 2004). Additionally, the production of Gelatin/Chitosan/Hyaluronan scaffold is usually aimed to obtain a dry polymer, with specific physical properties that can significantly vary at the moist environment necessary for cells loading used in tissue regeneration. Therefore a full characterization, including all steps of scaffold-polymer manipulation, is strongly recommended to improve the understanding of the structure of these systems in order to optimize the product performance and stability.

Physical and thermal properties of a biomaterial can provide important information related to its structure. Furthermore they provide general information on stability, shrinkage, expansion and others properties (Nazhat, 2008). The aim of this study was to characterize the Gelatin/Chitosan/Hyaluronan polymer used as scaffold for skin tissue-engineering by mechanical, thermophysical and biological assays.

MATERIALS AND METHODS

Preparation of Gelatin/Chitosan/Hyaluronan polymer

Gelatin (from bovine, USP grade) was purchased from MERCK (Germany). Chitosan (from crab shells, minimum 85% deacetylated) and hyaluronic acid (from human umbilical cord) were purchased from Sigma-Aldrich (USA). The preparation of a 3 mm thickness Gelatin/Chitosan/Hyaluronan polymer was followed as described elsewhere (Liu et al. 2004).

Gelatin (1% w/v) was mixed with chitosan (2% w/v in 1% v/v acetic acid) and hyaluronic acid (0.01% w/v) in 7:2:1 proportion at 50ºC. Then, the solution was poured on a Petri dish, cooled, frozen and lyophilized. The matrix was cross-linked by

the use of a MES/EDC/NHS solution (ethanol 90% v/v as solvent): MES 50 mM (2-morpholine-ethane sulfonic acid), EDC 20 mM (1-ethyl-(3,3-dimethyl-aminopropyl)-carbodiimide) and NHS 8 mM (N-hydroxysuccinimide); after that, it was washed with ethanol. The cross-linked matrix was frozen and lyophilized, obtaining a stable dry polymer (Liu et al. 2004).

The lyophilized polymers were equilibrated to different moisture contents by storage at 20ºC under saturated salt solutions (LiCl, $NaNO_3$ and $MgCl_2$). The equilibrated samples were assessed mechanically by texture analysis and thermically by differential scanning calorimetry.

Preparation of biosynthetic-skin

The Gelatin/Chitosan/Hyaluronan polymer was disinfected into ethanol 70% v/v for 24 hrs (Marreco et al. 2004; Weinstein-Oppenheimer et al. 2010). Then, the polymer was washed with sterile PBS (filtered 0.22 µm) and hydrated using culture media (DMEM). Human skin-fibroblasts were loaded into the scaffold (4 x 10^4 cell/cm^2 or 1 x 10^5 cell/cm^3) (Weinstein-Oppenheimer et al. 2010), using *in-situ* gelification of fibrin (200 µL/cm^2) (Young et al. 2007). The biosynthetic-skin was incubated in DMEM (10% FBS) (Gibco-Invitrogen, USA) with 5% CO_2 at 37ºC.

The fibrin was prepared mixing equal parts of fibrinogen solution (15 mg/ml; Sigma-Aldrich, USA) and thrombin solutions (130 NIH/ml; ICN-Biomedicals, USA) at 37ºC. The thrombin (carrying the cells) was prepared in sterile mQ-water with $CaCl_2$ (30 mM) and NaCl to adjust the osmolarity to 300 mosM (Acevedo et al. 2009; Acevedo et al. 2010).

Fibroblasts were isolated from foreskin of a healthy donor using the methods described by Acevedo et al. (2009), under informed consent and approval of the surgeon in charge.

Physical properties

Mechanical properties of the polymer and biosynthetic-skin were evaluated in a tensile testing machine ZWICK/ROELL (Germany) model DO-FB0.5TS with a load cell of 500N. The testing speed was 50 mm/min. The parameters measured were force, elongation % at brake and maximum force. The Young's modulus of elasticity was defined as the slope of the linear zone on the stress-strain curved. The measurements were performed in six replicates.

The moisture content of each sample was measured gravimetrically in triplicates at 105ºC for 24 hrs using a convective oven.

The porosity was measure with a gas pycnometer (MVP 1305, Micrometrics, USA), using nitrogen as displacement gas. The set pressure was 20 psi (137.9 MPa) and the insert used was for 5-35 ml. Three replicates were measured for each sample.

Diffusivity

Glucose diffusivity in hydrated polymer was estimated as described by Villalobos et al. (2010), with minimal modifications. Pieces of hydrated polymer (2 x 2 cm) were immersed in glucose solution (5% w/v). Every minute, a sample was taken to measure the total glucose adsorbed. Glucose concentration was measured by using a commercial GOD/PAP kit (Glucose Liquicolor) (Human GmbH, Germany). The experimental data set was fitted to an integrated form of the Fick's second law to obtain the diffusion coefficient.

Differential Scanning Calorimetry (DSC)

Samples of 1 mg were weighted and loaded into standard 30 µl aluminium pans (PE No. 0219-0041). A Differential Scanning Calorimeter (Diamond Hyper DSC, Perkin Elmer, U.S.A.) was used, previously calibrated using indium as a reference material, with a melting enthalpy (ΔH) of 28.45 J/g and melting temperature (Tm) of 156.6ºC. The analytic method performed was a first scan from -10ºC a 180ºC at 10ºC/min, a cooling stage from 180ºC to -10ºC at 40ºC/min and a second run -10ºC a 180ºC at 10ºC/min.

MTT-assay

MTT-assay measures the viable biomass, and it was used to evaluate the cytotoxicity of polymer and cell growth into the biosynthetic-skin (Acevedo et al. 2009; Acevedo et al. 2010). The MTT solution (0.5%; Sigma, USA) was prepared in PBS. The polymer and biosynthetic-skin were incubated with medium and MTT solution (4:1) for 4 hrs at 37ºC. Subsequently, a lysis buffer (3% w/v SDS and 40 mM HCl, in isopropanol) and ultrasound were applied to make the formazan soluble. The parameter used to estimate viable biomass was the total formazan production after reaching a plateau level (assay end-point).

Scanning electron microscopy (SEM)

To observe the morphology of the polymer, pieces of 1 mm^2 were cut and dehydrated in liquid CO_2 (critical point), mounted onto aluminium stubs with double-sided carbon tape, and sputter coated with gold-palladium (80/20). Samples were examined under a Scanning electron microscopy (SEM) Jeol JSM-25-SII. Scaffold pore size was directly estimated from a scale rule given by image analysis software built into the microscope.

Histochemistry

The biosynthetic-skin was fixed in Bouin's solution for 24 hrs, dehydrated and embedded in Paraplast-Plus (Sigma, USA). Five µm-thick serial sections were obtained and mounted on xylane-coated microscope slides, deparaffinized and rehydrated. The sections were stained with a modified Arteta trichrome stain (Hematoxylin / Erythrosine B - Orange G/Methyl blue) (Acevedo et al. 2009).

RESULTS AND DISCUSSION

Physical characterization of polymer

Morphology of the polymer is regular with an average porous diameter close to 100 µm (Figure 1a) and high porosity (void-volume fraction) 99.0% (± 0.03%). Since that theoretical porosity of our matrix is 99.2% (based on its solid content), the lyophilizing and cross-linking contractions did not change significantly the matrix void-volume. These values are in the range used for scaffolds in tissue engineering. Porous sizes below 20 µm would not allow free access to the skin cells, and pores with mean sizes greater than 125 µm would not allow proper cell attachment (Chin et al. 2008). Literature indicated that porosities greater than 95% are appropriate for tissue engineering (Chen et al. 2002).

Mechanical properties of lyophilized polymer did not change significantly when the samples were equilibrated to different moisture contents. In particular, the Young's elastic modulus was close to 1.3 MPa, and there was not significant differences in its

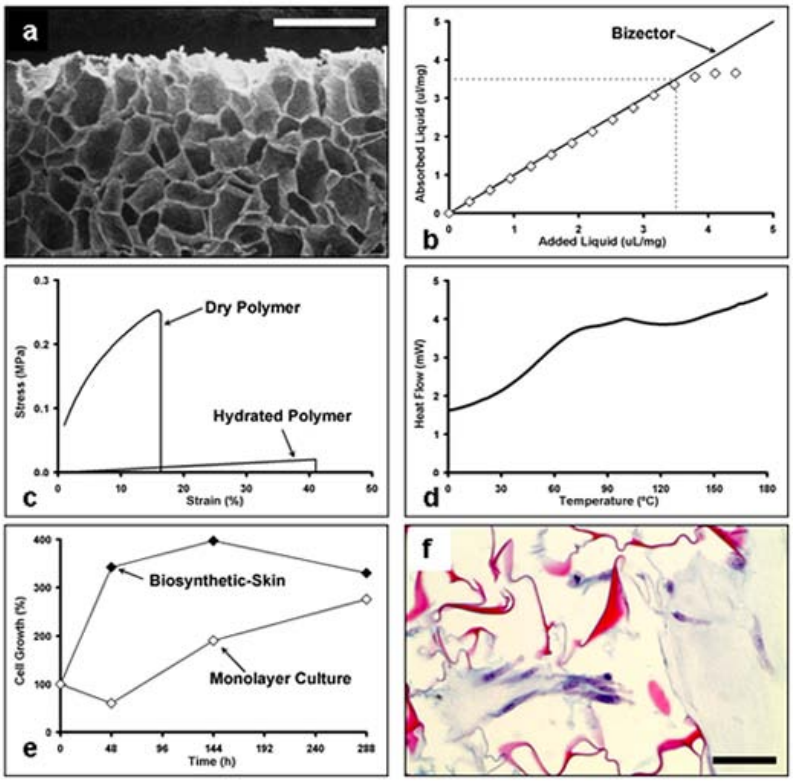

Fig. 1 Characterization of polymer and biosynthetic-skin. (a) SEM-Microestructure of dry polymer (bar 500 µm). (b) Retention water capacity of polymer. (c) Stress-Strain curve of dry and hydrated polymer. (d) DSC-Thermogram of dry polymer. (e) Cell growth on biosynthetic-skin and monolayer culture as control. (f) Histology of biosynthetic-skin (bar 50 µm). Fibrin is blue staining and polymer is red staining.

values ($p < 0.05$) between different moisture levels (~10-14%). However, obtained values for the maximum stress and strength decreased slightly with moisture (Table 1).

The polymer has a high water swelling capacity, allowing rapid hydration when fluid or culture medium was added (Figure 1b). The dry polymer can contain 3.5 µL of fluid per milligram (reaching 80% humidity). However, when hydrated with culture medium it presented significant variations ($p < 0.05$) in its mechanical properties (Table 1), decreasing its rigidity (stress) and increasing its deformability (strain) (Figure 1c). The hydration of the polymer is a necessary step for incorporation and viability of cells, because they can not live in dry environments. The hydrated polymer became more plastic and fragile material that can be deformed and broken with the application of small forces (Figure 1c).

Glucose diffusivity was experimentally estimated soaking the hydrated polymer (see Materials and Methods). We observed that glucose concentration increased quickly in the first seconds (< 60 sec), likely by a high capacity of surface sorption. After that, the concentration increased slowly, showing a diffusional behaviour. Diffusivity was estimated fitting the data to Fick's law in 6.3×10^{-10} (m^2/s). This value is comparatively similar to the diffusion value of glucose in pure water (6.9×10^{-10} m^2/s) and others hydrated biomaterials such calcium-alginate (6.4×10^{-10} m^2/s) (Villalobos et al. 2010).

Thermophysical characterization of polymer

In recent years, Differential Scanning Calorimetry (DSC) has been used to assess decomposition and protein denaturation of biomaterials used in tissue engineering such as in natural silk fibroin based (Kasoju et al. 2009) and mineralized type I collagen scaffolds (Gelinsky et al. 2008) respectively. DSC performed on the different set of dry polymers showed an irreversible endothermic transition, with maximum

Table 1. Properties of polymer and biosynthetic-skin.

Sample	Stress at Break (MPa)	Maximum Stress (MPa)	Strain at Brake (%)	Transition Onset Temperature (ºC)	Transition Enthalpy (J/g)
Dry Polymer (10.6% Moisture)	0.44	0.45	25.6	30.0	199.8
Dry Polymer (11.2% Moisture)	0.34	0.35	17.6	32.6	221.3
Dry Polymer (13.6% Moisture)	0.24	0.25	15.8	35.7	243.6
Hydrated Polymer (~80% Moisture)	0.01	0.01	41.0	-	-
Biosynthetic-Skin (~90% Moisture)	0.02	0.02	10.8	-	-

peak close to 70ºC and an onset temperature close to 30ºC (Table 1). Two merged shoulders were detected on all the DSC traces, suggesting the presence of an integrated two phase system (Figure 1d). The enthalpy associated to this transition is close to 200 J/g, increasing slightly with moisture content.

Experimental work on collagen based systems has showed similar thermal profiles associated to protein denaturation. Gelinsky et al. (2008) showed the effect of the crosslinking of EDC on water swollen collagen. The cross-linked protein showed higher denaturation temperature than non-crosslinked sample. Literature also indicates that the glass transition temperature (Tg) of each components forming the scaffold is relatively higher that the detected transition temperature. Indeed, Lazaridou and Biliaderis (2002) detected a Tg ~70^{o}C for chitosan casted films and Yakimets et al. (2005) measured a Tg ~70^{o}C for a gelatin film containing similar moisture contents. Therefore the occurrence of the endothermic transition could be explained also by some relaxation process of the matrix stored in the glassy state (dry polymer).

Regarding the structure integrity, it is unlikely to observe by DSC on the scan temperature trace (-10ºC a 180ºC) a disruption of the structure of the crosslinked scalfold into its main constituents collagen, chitosan and hyoluronic acid. Berger et al. (2004) suggest chemical crosslinked chitosan hydrogels involving covalent bondings as irreversible. Chemical alterations of the structure would require higher energy levels indicated by endoderm at greater temperatures.

In terms of the scaffold preparation protocol, these results indicate that the maximum storage temperature of the polymer cannot exceed 30ºC, because structural changes could appear for long times of storage. In addition, the sterilization process by heating can not be used, because the maximum transition changes occur at 70ºC, altering irreversibly the polymer structure.

Polymer as scaffold to biosynthetic-skin

The capacity of the polymer as a carrier to load cell suspensions was evaluated. Prior to any manipulation, the polymer was sterilized, washed with PBS and rehydrated with medium (DMEM).

The MTT assay showed that the hydrated polymer loaded with cells (4 x 10^4 cell/cm^2 or 1 x 10^5 cell/cm^3) has not cytotoxic properties after 24 and 48 hrs ($p > 0.05$), validating that the polymer is an excellent scaffold matrix to construction of dermal implants.

The polymer mixture was used as scaffold to prepare a biosynthetic-skin, loading 100 µL of thrombin per squared centimeter of polymer (100 µL/cm^2), and then gelling *in situ* with an equal part of fibrinogen solution (100 µL/cm^2). Since the optimum absorption was 3.5 µL per 1 mg of polymer, the same concentration of fibrin was added (equivalent at 200 µL/cm^2). Thrombin was used as carrier of human fibroblasts, loading the cells at 4 x 10^4 cell/cm^2 (or 1 x 10^5 cell/cm^3) (Young et al. 2007).

The cell growth on biosynthetic-skin, measured with MTT assay, was faster compared to monolayer culture ($p < 0.05$) and did not show a lag phase ($p < 0.05$) (Figure 1e). The population doubling time (calculated from Figure 1e), was close to 27 hrs.

Histological analysis of a section of the biosynthetic-skin showed an excellent cell distribution within the scaffold, where cells are distributed between both phases and there is an adequate interaction between the two phases of the system. It is also observed that fibrin is bonding with the polymer, showing a correct integration of the system (Figure 1f).

Mechanical properties of the biosynthetic-skin are stronger than simple hydrated polymer (Table 1). The strength decreased significantly ($p < 0.05$) when the hydrated polymer is coated with fibrin. Elastic module of the fibrin without ligation (without blood transglutaminase - Factor XIIIa) has been reported as 1.4 MPa (Collet et al. 2005). Our data show that the elastic modulus for the biosintetic-skin was close to 0.2 MPa, indicating that this material is mechanically softer than the wound-healing matrix.

CONCLUDING REMARKS

Morphology of the obtained polymer is regular. Porosity and porous diameter of polymer is in the range used in tissue engineering making viable for tissue engineering applications.

Mechanicals properties of dry polymer did not change significantly for the studied moisture range (~10-14%). This would indicate that at the moisture content range studied the polymer was in the glassy state.

Young's elastic module was close to 1.3 MPa. Thermophysical properties indicate that temperatures higher than 30ºC could modify irreversibly the dry polymer and relaxation process may occur on storage. As expected, the hydration process changes significantly the elasticity of polymer generating a more plastic material.

Hydrated polymer did not show cytotoxical properties. It can be loaded with other liquids used as cell-carrier, and used as scaffold to prepare a biosynthetic-skin.

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