Materials Letters 106 (2013) 369–372

Contents lists available at SciVerse ScienceDirect

Materials Letters

journal homepage: www.elsevier.com/locate/matlet

A biological scaffold filled with silica and simultaneously crosslinked with polyurethane

Birzabith Mendoza-Novelo [a,*], Gerardo González-García [b], José L. Mata-Mata [b], ĆLaura E. Castellano [c], Patricia Cuéllar-Mata [d], Eva E. Ávila [d]

[a] Depto. Ingenierías Química, Electrónica y Biomédica, DCI, Universidad de Guanajuato Campus León, Loma del Bosque 103, 37150 León, Gto, Mexico
[b] Depto. Química, DCNE, Universidad de Guanajuato, Campus Guanajuato, Noria Alta s/n, 36050 Guanajuato, Gto, Mexico
[c] Depto. Ciencias Aplicadas a la Salud, DCS, Universidad de Guanajuato, Campus León, Garza Sada 572, 37150 León, Gto, Mexico
[d] Depto. Biología, DCNE, Universidad de Guanajuato, Campus Guanajuato, Noria Alta s/n, 36050 Guanajuato, Gto, Mexico

ARTICLE INFO

Article history:
Received 21 February 2013
Accepted 18 May 2013
Available online 28 May 2013

Keywords:
ECM scaffold
Polyurethane prepolymer
TEOS
Filling
Crosslinking

ABSTRACT

The present study investigates the use of SiO_2 networks combined with polyurethane prepolymer (PUP) as filler and crosslinker for biological scaffolds. We also studied its effect on the *in vitro* biostability, the macrophage adhesion/proliferation and the tensile viscoelastic response. The biocomposite was prepared by the simultaneous crosslinking/filling of pericardial extracellular matrix (ECM) with PUP and hydrolysis-condensation of tetraethylorthosilicate (TEOS). This modification was used to regulate some biological and structural features of the tissue engineering scaffold. The PUP—SiO_2—ECM material shows equivalent viscoelastic response to that densely-crosslinked matrix (either glutaraldehyde or PUP), and equal macrophage adhesion and *in vitro* biostability that non-crosslinked ECM scaffold.

1. Introduction

Hybrid biomaterials have the advantage of combining properties to provide biological functions and control over their compositional, structural and mechanical features [1,2]. The crosslinking of tissue-derived scaffolds impacts the structural features and consequently the mechanical and biological responses of the extracellular matrix (ECM) material [3,4]. Recently, an effective method was reported that stabilizes the collagen network after its treatment with water-soluble blocked polyurethane prepolymers (PUPs) with carbamoyl sulfonate-protected isocyanate groups [5]. The use of silicates as coating or filler into degradable matrices have shown promising results in the bioactive scaffolds fabrication for bone tissue engineering [6,7], the cell immobilization [8] or the controlled drug delivery [9,10].

Herein we reported the preparation of a novel biocomposite comprising pericardial ECM scaffold, polyurethane and Si–O–Si hydrogel. The biostability, macrophage adhesion and viscoelastic stress relaxation for the biocomposite and the acellular and crosslinked biological scaffold were studied.

2. Materials and methods

PUP-crosslinking and silica-filling of the ECM material: Water-soluble blocked polyurethane prepolymer (PUP) was synthesized as previously described elsewhere [11]. (A) Prepolymer—A poly (ethylene glycol) (1000 Da, SigmaAldrich) was reacted with hexamethylene diisocyanate (HDI Aliphatic isocyanates Luxanate HM, Lyondell) in a molar NCO/OH ratio of 4.0:1.0 for 2 h at 100 °C. The temperature was reduced to 60 °C. (B) Blocked prepolymer—Sodium bisulfate was dissolved in water (40 w%) and this solution was added slowly above prepolymer and the reaction was continued for 2 h at 40 °C. The solution was diluted with water to give a product containing about 30% solids by weight. Water-soluble blocked PUP containing tetraethylorthosilicate—the prepolymer was converted in a similar manner. The tetraethylorthosilicate (TEOS, SigmaAldrich) in ethanol was added (5 w%) before the sodium bisulfate solution. Glutaraldehyde (GA), magnesium oxide (MgO), disodium piperazine-1,4-diethanesulphonate hydrate (PIPES), 2,4,6-Trinitrobenzenesulfonic acid (TNBS), 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and collagenase type I were purchased from SigmaAldrich. The ECM material was isolated from bovine pericardium and processed as previously described [12]. Briefly, pericardial tissue was decellularized by the reversible alkaline swelling method, as the ultrastructure and tensile properties were preserved after processing of the native tissue [12]. The strips ($5 \times 30\ mm^2$) were

* Corresponding autor. Tel.: +52 477 7885100x8464; fax: +52 477 7885107.
E-mail address: bmnovelo@dci.ugto.mx (B. Mendoza-Novelo).

0167-577X/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.matlet.2013.05.088

obtained in two orthogonal directions, using the root-to-apex direction on pericardial ventral zone (labeled as 0°) as reference. One group was assessed after the decellularization (Group I) and three groups were assessed after the modifications described below.

Group II—Combined PUP-crosslinking and SiO_2-filling of the ECM scaffold. Samples were conditioned in PIPES buffered solution (PSS, 0.03 M, pH 7.4) containing PUP/TEOS (15 w%, RT, 3 h) under agitation at 30 rpm. Then, pH was increased to 9 by the addition of MgO and the reaction was continued for 24 h.

Group III—PUP-crosslinking of the ECM scaffold. This was similar to the procedure used for Group II except that PUP without TEOS was used.

Group IV—GA-crosslinking of the ECM scaffold. Samples were exposed to PSS containing GA (0.625%, RT, 24 h).

Characterization of the biocomposite: (*Assessment of the in vitro biostability*): Material-amine content was determined by the TNBS assay. Briefly, samples were exposed to $NaHCO_3$ (4 w%, RT, 30 min), reacted with TNBS (0.5 w%, 2 mL, 40 °C, 2 h), rinsed (Supplementary data), lyophilized, weighed and hydrolyzed with HCl (25 w%, 2 mL). The hydrolyzates were diluted and their absorbances were measured at 344 nm. Residual amine content was calculated using a molar absorption coefficient of 14,600 ml/mol cm, and expressed as mmol/g of dry sample.

Denaturation temperature (T_d) was determined by differential scanning calorimetry (DSC). Samples were placed in volatile-sample aluminum pans. The experiments were performed from 55 to 100 °C at a heating rate of 10 °C/min. T_d is reported as the temperature at the peak of the endothermic transition during the first trace.

Collagenase-resistance was determined by the lost weight and the released amine amount when samples were exposed to enzyme. Samples were incubated with collagenase type I (72 U/ml, 50 mM Tris · HCl, 5 mM $CaCl_2 \cdot 2H_2O$, 0.03 w% NaN_3, pH 7.4, 37 °C, 20 h), washed (5 mM EDTA), lyophilized and weighed. The weight loss was determined using wet/dry ratios of degraded and non-degraded samples, whereas the released amines from samples were quantified in the supernatant using the ninhydrin assay (1%, pH 5, 95 °C, 20 min).

(*Assessment of the tensile viscoelastic response*): The mean thickness of the strips was determined averaging four measurements using a Mitutoyo digital micrometer. The strips were placed in an electromechanical tensile testing machine (Instron 4411, adapted with a transparent chamber) with an effective gauge length of 20 mm and submerged in saline solution heated at 37 °C [13]. The stress relaxation tests were performed by stretching the strip to a given stress of 1000 kPa (cross-head speed of 50 mm min^{-1}), stopping the movable cross-head and recording load over 100 s. The axial force was measured by means of a 100 N load cell and the elongation by the internal LVDT sensor. The ratio of stress at a time t, $\sigma(t)$, to the initial stress (σ_o) was plotted vs. time on a logarithmic scale. The stress relaxation ratio at 100 s was calculated as the ratio of σ_o to $\sigma_{(100\ s)}$.

(*Assessment of the macrophage adhesion/proliferation*): Mouse monocyte macrophage cell line RAW264.7 was routinely grown in RPMI-1640 medium supplemented with 10% fetal bovine serum. Cells were passaged and seeded onto lyophilized material (5×5 mm^2) in 96-well plate and cultured at 37 °C, 5% CO_2 and 100% humidity. After 24 h of incubation, the materials were transferred to empty wells to assess the cell viability on both wells and materials. The cell viability was assessed by MTT assay. The viability of macrophages cultured in the absence of material represented 100% viability.

Statistical analysis: The statistical analysis was performed with ANOVA. The Sidak–Holm Test was used for the comparison between data groups. The results were considered significantly at p-values ≥0.05.

3. Results and discussion

Porosity, degradation and surface chemistry are features required for regenerative activity of biological scaffolds [3]. The combination of pericardial ECM scaffold with polyurethanes offers advantages for the control of the crosslinking density and consequently for the degradation rates [5,14]. On the other hand, Si is able to induce the hydroxyapatite precipitation from aqueous solution and also enhance osteoblast proliferation, differentiation and collagen production under *in vitro* conditions [15]. These favorable effects are motivating the incorporation of Si into scaffolds intended to bone tissue replacement [2,6]. With this in mind, in this study we reported a hybrid scaffold comprising ECM, Si and polyurethane intended to combine the bioactivity, biodegradability and biocompatibility of the components.

Thermal stability of the crosslinked and filled matrix: The shrinkage of collagen materials upon heating is attributed to the transition from collagen triple helix to a random coil conformation and/or the melting of the crystalline structures present in collagen. The crosslinking density of scaffolds was assessed by means of the increase in the temperature of the collagen denaturation. DSC thermograms confirmed the stabilization of the ECM scaffold that was modified with all three protocols. T_d for non-crosslinked ECM was increased by 10 °C after that matrix was treated with PUP and with or without TEOS. An increase of 14 °C was observed after the GA treatment (Fig. 1). These results indicate the stabilization of the ECM material after treatment with PUP containing Si.

Residual amine and water content of the crosslinked and filled matrix: A second indicator of the biostability of the scaffolds was the residual amine content. The scaffold treated by any of the three stabilization protocols showed lower residual amine content compared to non-crosslinked matrix (see bar graphic in Fig. 2a). About 83 and 78% of the material-amines were blocked after that matrix was crosslinked with GA and PUP, respectively. On the other hand, it was 27% for matrix treated with PUP and SiO_2. This result indicates that simultaneous use of PUP with silica protected over 70% of the amines on the ECM, allowing amines remain free. A competitive diffusion between the orthosilicate hydrolysis products and PUP molecules, and the spatial restriction after the formation of the hydrated-silica network can limit the nucleophilic attack by amines on isocyanate groups. In crosslinking protocols using GA or PUP, the water content decreased, while for the PUP–SiO_2–ECM biocomposite the water content was not significantly altered (Fig. 2a). This indicates that PUP–SiO_2–ECM material maintained hydrophilicity equal to the non-crosslinked scaffold. The loss of water from scaffold can be limited by the SiO_2 network, which can act as a barrier for water diffusion [9]. In fact, increasing the hydrophilicity of bone implants has been associated with an increase of the osseointegration process [16].

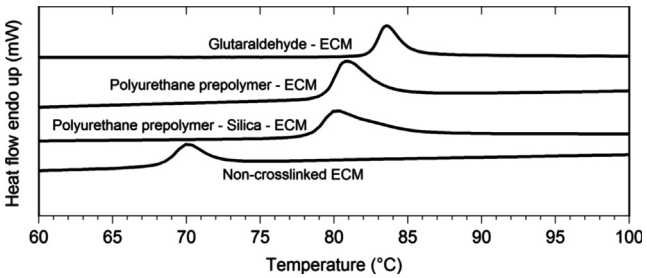

Fig. 1. Typical DSC thermograms for the decellularized, crosslinked and filled pericardial matrix.