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ScienceDirect
Biomaterials 29 (2008) 1630–1637
Biomaterials
www.elsevier.com/locate/biomaterials

Preparation and rheological characterization of a gel form of the porcine urinary bladder matrix

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Received 24 July 2007; accepted 15 December 2007
Available online 16 January 2008

Abstract

Biologic scaffolds composed of extracellular matrix (ECM) have been used to facilitate the repair and reconstruction of a variety of tissues in clinical and pre-clinical studies. The clinical utility of such scaffolds can be limited by the geometric and mechanical properties of the tissue or organ from which the ECM is harvested. An injectable gel form of ECM could potentially conform to any three-dimensional shape and could be delivered to sites of interest by minimally invasive techniques. The objectives of the present study were to prepare a gel form of ECM harvested from the urinary bladder (urinary bladder matrix or UBM), to characterize the rheological properties of the gel, and finally to evaluate the ability of the gel to support *in vitro* growth of smooth muscle cells. Following enzymatic solubilization with pepsin, UBM was induced to self-assemble into a gel when brought to physiological conditions. The UBM gel supported the adhesion and growth of rat aortic smooth muscle cells when cultured under static *in vitro* conditions. The present study showed that an intact form of UBM can be successfully solubilized without purification steps and induced to repolymerize into a gel form of the UBM biologic scaffold material.

Keywords: Extracellular matrix; Viscoelasticity; Rheology; Scaffold; Gel

1. Introduction

Biologic scaffolds composed of extracellular matrix (ECM) have been used for the repair of a variety of tissues including the lower urinary tract [1,2], esophagus [3,4], myocardium [5–7], and musculotendinous [8–10] tissues, often leading to tissue-specific constructive remodeling with minimal or no scar tissue formation. These biologic scaffolds are typically prepared by decellularization of intact tissues or organs. The resulting ECM scaffold materials are composed of the structural and functional molecules that characterize the native tissue ECM such as collagen, laminin, fibronectin, growth factors, glycosaminoglycans, glycoproteins, and proteoglycans [11–13]. However, the resulting decellularized ECM is usually characterized by a two-dimensional sheet with limited ability to conform to irregular three-dimensional shapes and sizes. Therefore, the clinical utility of an ECM biologic scaffold for many clinical applications is typically restricted to topical administration or to invasive surgical procedures that can accommodate variations of the two-dimensional sheet forms.

An ECM scaffold derived from the porcine urinary bladder, referred to as urinary bladder matrix (UBM), has been previously investigated in pre-clinical studies as a biologic scaffold for the reconstruction of damaged laryngeal tissue [14], for the

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doi:10.1016/j.biomaterials.2007.12.014

reconstruction of esophageal tissue [4,15], as a treatment for urinary incontinence [2], and as a myocardial patch [6,7,16] with promising results. Suspensions made from a particulate (powdered) form of lyophilized UBM have been successfully used as an injectable scaffold for the treatment of urinary incontinence in pre-clinical studies [2] but the needle size required to accommodate the particle size was prohibitive for most clinical applications, and substances such as glycerin were required to increase the viscosity of the solution.

A soluble form that can be induced to polymerize into a gel form could expand the clinical utility of ECM biologic scaffolds. ECM gels with appropriate viscosities could be delivered via minimally invasive surgical techniques with the ability to conform to three-dimensional spaces after injection. Ideally this soluble form would retain the bioactivity of the ECM scaffold and its preparation would minimize or avoid purification steps that could remove growth factors and low molecular weight peptides present in the native ECM. Previous studies have described a gel form of an ECM derived from the small intestine but the preparation required an aggressive purification process that may have resulted in the loss of bioactive molecules, and the rheological properties of the gel were not determined [17]. Gels have been formed from individual components of the ECM such as collagen and from the secreted products of cell lines (Matrigel™). Matrigel™ is not used therapeutically due to its source (tumor cell line), and individual ECM components lack the bioactivity found in minimally processed ECM scaffolds in which structural and functional molecules are present in physiologically relevant amounts.

The long-term objective of this work is to develop a gel form of the UBM scaffold that retains the bioactivity and ability to promote constructive tissue remodeling; properties that are characteristic of the 2-D sheet form of UBM. The present study is the first step towards this goal and describes a method to solubilize UBM without purification steps and a method to repolymerize the soluble UBM–ECM under physiological conditions. The present study characterizes the gelation kinetics, the rheological properties, and the static *in vitro* cytocompatibility of the UBM gel.

2. Materials and method

2.1. ECM preparation

The preparation of UBM has been previously described [20,21]. In brief, porcine urinary bladders were harvested from 6-month-old 108–118 kg pigs (Thoma Meat Market) immediately following euthanasia. The excess connective tissue and residual urine were removed. The tunica serosa, tunica muscularis externa, the tunica submucosa, and majority of the tunica muscularis mucosa were mechanically removed. The urothelial cells of the tunica mucosa were dissociated from the luminal surface by soaking the tissue in 1.0 N saline solution. The resulting biomaterial, which was composed of the basement membrane of the urothelial cells plus the subjacent lamina propria, was referred to as urinary bladder matrix (UBM). UBM sheets were placed in a solution containing 0.1% (v/v) peracetic acid (Sigma), 4% (v/v) ethanol (Sigma), and 95.9% (v/v) sterile water for 2 h. Peracetic acid residue was then removed with two 15-min phosphate-buffered saline (pH = 7.4) washes, followed by two washes with sterile water for 15 min each. The decellularized UBM sheets were then lyophilized using an FTS Systems Bulk Freeze Dryer Model 8-54 and comminuted to a particulate form using a Wiley Mini Mill (Fig. 1A).

2.2. ECM digestion and solubilization

One gram of lyophilized UBM powder (Fig. 1A) and 100 mg of pepsin (Sigma, ~2000–2300 U/mg) were mixed in 100 ml of 0.01 M HCl and kept at a constant stir for ~48 h at room temperature (25 °C). The resultant viscous solution of digested UBM or pre-gel solution had a pH of approximately 3.0–4.0 (Fig. 1B). The activity of pepsin was irreversibly inactivated when the pH was raised to 7.4 (see Section 2.4).

2.3. Gel characterization

UBM and rat-tail collagen type I (BD, Biosciences) solutions were electrophoresed on 7.5% polyacrylamide gels under reducing conditions (5% 2-mercaptoethanol). The proteins were visualized with Gel-Code Blue (Bio-Rad), and images recorded by a Kodak imaging station.

Collagen and sulfated glycosaminoglycan (S-GAG) content of the UBM gel were determined using the hydroxyproline assay [18] and the Blyscan™ assay kit (Biocolor), respectively. The Blyscan™ assay was performed according to the manufacturer's instructions. The hydroxyproline content was determined by hydrolyzing the samples with 2 M NaOH (100 μl total volume) in an autoclave at 120 °C for 20 min. The samples were neutralized with 50 μl of 4 M HCl and reacted with 300 μl of 0.056 M chloramine-T (Spectrum), mixed gently, and allowed to oxidize for 25 min at room temperature. The samples were then mixed with 300 μl of 1 M Ehrlich's aldehyde (Spectrum) and incubated at 65 °C for 20 min. A standard curve was generated using rat-tail collagen type I (BD, Biosciences) and used to calculate the total amount of collagen present in the digested UBM solutions. The colorimetric change was determined by the absorbance at 550 nm using a SpectraMax spectrophotometer (Molecular Devices). Three different batches of UBM were tested ($n = 3$).

2.4. Gelation

UBM and rat-tail collagen type I gels were formed by mixing 0.1 N NaOH (1/10 of the volume of pre-gel solution) and 10× PBS pH 7.4 (1/9 of the volume of pre-gel solution) at 4 °C. The solution was brought to the desired volume/concentration using cold (4 °C) 1× PBS pH 7.4 and placed at 37 °C for gelation to occur. Collagen and UBM gels are shown in Fig. 1C.

Turbidimetric gelation kinetics was determined spectrophotometrically as previously described [19]. Final gel solutions were kept at 4 °C and transferred to a cold 96-well plate by placing 100 μl/well in triplicate. The SpectraMax spectrophotometer (Molecular Devices) was pre-heated to 37 °C, the plate placed in the spectrophotometer, and the turbidity of each well was measured at 405 nm every 2 min for 1.5 h. The absorbance values for each well were recorded and averaged. Three individual tests were performed on the same batch of collagen type I ($n = 3$) and five ($n = 5$) individual tests were performed on different batches of the UBM gel. Each individual test was conducted in triplicates and averaged. The time needed to reach 50% of the maximum turbidity measurement (e.g. maximum absorbance value) was defined as $t_{1/2}$ and the lag phase (t_{lag}) was calculated by extrapolating the linear portion of the curve (see Fig. 5B). The speed (S) of the gelation based on turbidimetric measurements was determined by calculating the maximum slope of the growth portion of the curve as shown in Fig. 5B.

2.5. Rheological measurements

The sample was subjected to an oscillatory strain of

$$\gamma(t) = \gamma_0 \cos(2\pi f t) \quad (1)$$

where γ_0 was the amplitude of the sinusoidal strain, t was the time, and f was the frequency. The sample developed a sinusoidal stress described as follows:

$$\sigma(t) = |G^*|\gamma(t) \quad (2)$$

where G^* was the frequency dependent complex modulus of the sample. The real part of G^*, denoted G', was in phase with the applied strain and was called the storage modulus since it corresponded to storage of mechanical energy in