Materials Science and Engineering C 42 (2014) 146–154

Contents lists available at ScienceDirect

Materials Science and Engineering C

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

Porous hydrogel of wool keratin prepared by a novel method: An extraction with guanidine/2-mercaptoethanol solution followed by a dialysis

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ARTICLE INFO

Article history:
Received 11 February 2014
Received in revised form 28 April 2014
Accepted 13 May 2014
Available online 20 May 2014

Keywords:
Keratin
Wool
Guanidine
Hydrogel
Sponge

ABSTRACT

In this study, we show a novel simple method to prepare a sponge-like porous keratin hydrogel through the extraction of wool keratin in a solution containing guanidine hydrochloride and 2-mercaptoethanol followed by dialysis for both aggregation of keratin and recrosslink. The gel had a highly porous structure and a fast-swelling property in rehydration after freeze-drying. It had also high mechanical strength both in the tensile test and the measurement of dynamic viscoelasticity. Three types of animal cells, PC12 cells, HOS cells and murine embryonic fibroblasts, well attached and grew on the surface of the porous hydrogel.

1. Introduction

Keratin is contained rich in animal epidermal tissues and also common proteins in our daily life. People have been discarding most of them (ex. hair, and nail) produced by organisms with hair cut or clipping nails and so on. Furthermore, a large quantity of animal hair has been scrapped as nonedible parts and industrial waste in processing meat and leather after slaughtering livestock [1].

Keratin, especially hard-α-keratin, can be extracted from the cortex, the inner part of hair covered with cuticle [2,3]. It's noted that keratin belongs to the intermediate filament cytoskeleton protein family, and that the α-helical rod domain consisting of two major components, type I and type II subunits, dimerizes to form a coiled-coil structure [4]. As presented above, keratin has multiple components [5]. Crude extract of hair keratin includes 27 keratin-associated proteins [6] (called as "γ-fraction" by Mark Van Dyke et al. [7]) in addition to those two subunits. It's considered that these proteins act like "glue" which crosslink between the chains of the keratin subunit [7].

Keratin is known as a relatively cysteine-rich protein. The most important attribute of keratin is their stability against proteolytic degradation resulted from a number of disulfide bonds. Because mammals typically don't have keratinase, keratin-degrading enzyme, keratin maintains the stability both in vitro and in vivo [7].

Biocompatibility and absorptivity are essentially important in designing the scaffold for cell culture in tissue engineering. Those properties highly depend on the proteins and other biopolymers consisting of the scaffold. Extracellular matrix (ECM) proteins, those that are widely used as scaffolds (ex. collagen, fibronectin, and laminin), satisfied those requirements. However they rarely have enough mechanical strength without the chemical crosslinking or modification. Easily absorptive ECM proteins are not suitable as scaffolds for long-term cell culture in some cases of medical application.

As a scaffold with stability for the long-term cell culture in vitro, or also for the stable implantable scaffold in vivo, keratin had been studied for utilization as biomaterial [8,9]. In particular, we consider that sponge and hydrogel consisting of keratin protein are worth to be studied for medical application because they can provide large amounts of materials of higher value. Tachibana et al. first reported about keratin biomaterial derived from wool in 2001 [10]. Since then, several groups have prepared various porous keratin hydrogels in different methods [11–14]. However, those porous hydrogels need cumbersome protocol such as use of reagent containing metal ion [11], and treatment of lyophilization [12,13].

In this study, we show a novel simple method of preparing a sponge-like porous keratin hydrogel through the extraction in a guanidine/2-mercaptoethanol solution followed by dialysis to cause self-assembly of the protein. Characterization on the mechanical strength of the gel and their properties for cell culture is evaluated in vitro. Keratin hydrogel with novel architecture has a possibility to prevail as a convenient material alternative to traditional scaffold derived from ECM or other proteins.

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http://dx.doi.org/10.1016/j.msec.2014.05.018
0928-4931/

2. Materials and methods

2.1. Materials

Wool (*Ovis aries*) was purchased from a local farm (Kobe Municipal Rokkosan Pasture, Kobe, Japan). Cellulose tubes (3500 MWCO, D304-50) for dialysis were purchased from BioDesign (Mt. Carmel, NY). Guanidine hydrochloride, 2-mercaptoethanol and Cell Count Reagent SF (07553-44) were from Nacalai tesque (Kyoto, Japan). Fetal bovine serum (FBS) (171012), horse serum (HS) (16050-122), DMEM-low glucose (12800-017), DMEM-high glucose (12800-017) and MEM (12800-017) were from Gibco®, Life Technologies (Carlsbad, CA). Twenty-four-well cell culture plates (167008) and 96-well cell culture plates (167008) were from Nunc (Roskilde, Denmark). DAPI solution (340-07971) was from Dojindo (Kumamoto, Japan). Collagen BM (340-07971) was from Nitta Gelatin (Osaka, Japan). Water-soluble carbodiimide, hydrochloride (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; EDC) (340-07971) was from Peptide Institute, Inc. (Osaka, Japan). All other reagents were of analytical grade.

2.2. Preparation of keratin hydrogel

Wool (2.0 g) was incubated in a 20-mL solution containing 8 M guanidine hydrochloride, and 1.66 M 2-mercaptoethanol (2-ME) for 18 h at 60–70 °C in an Erlenmeyer flask. The extract was filtered through a stainless steel mesh. A hand-made apparatus for dialysis was used as described below. A hole ($\phi = 2.0$ cm) was made in the cap of the 50-mL disposable test tube (BD Falcon REF 352096, Becton Dickinson, Franklin Lakes, NJ). The keratin extract (2.5 mL) was poured into the tube. Thus the hole was covered with a cellulose membrane (3500 MWCO) and then tightly sealed with the cap. The tube containing the extract was placed upside down for dialysis against distilled water (1 L) as shown in Fig. 1. After dialysis for 6 h, a disk of the microporous white keratin hydrogel was obtained. The hydrogel was sterilized by steam autoclaving in distilled water (121 °C, 15 min). Aliquot of the hydrogels was dissolved again in the solution containing 8 M urea, 0.26 M sodium dodecylsulfate (SDS), and 1.66 M 2-ME for 16 h at 37 °C to analyze the subunit composition of keratin by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.3. FTIR spectroscopy

The samples were dried in a vacuum, were crushed into a powder form, and were put on the sample folder for the measurement of the Fourier-transformed infrared (FTIR) spectra of the keratin hydrogel in an ATR mode using a FTIR spectrometer (IRAffinity-1, Shimadzu, Kyoto). The scan was repeated 40 times to average the data from 700 to 4000 cm^{-1} with a resolution of 2.0 cm^{-1} (data interval 1.93 cm^{-1}).

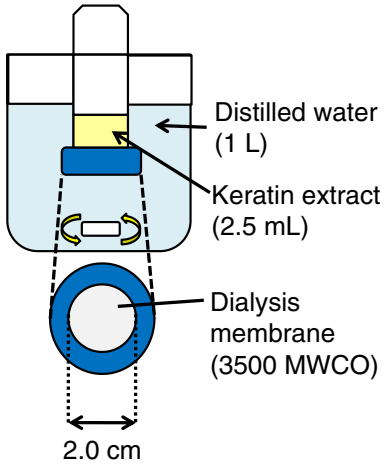

Fig. 1. Schematic representation of an apparatus to prepare keratin hydrogel by dialysis.

2.4. Mechanical property of keratin hydrogel

In this study, chemically-crosslinked collagen gel was prepared according to the method previously reported [15] and then compared with keratin hydrogel. Type I collagen is an ECM protein widely used as a scaffold for cell culture. Collagen BM (type I collagen derived from porcine skin, acid-soluble, 0.6% (w/v), pH 3.0) was used to prepare the collagen gel. The gel was crosslinked with EDC. EDC enhances the mechanical strength of the gel by forming intra- and inter- molecular crosslinks with dehydration condensation between the amino group and the carboxyl group. Briefly, 1.6 mL of collagen BM, 0.2 mL of ×10 concentrated phosphate buffered saline (−) (PBS (−)) and 0.2 mL of buffer solution containing 50 mM NaOH, 260 mM $NaHCO_3$, and 200 mM HEPES, were mixed and incubated for 24 h at 37 °C in a 12-well plate to prepare the collagen gel. The collagen gels taken out from the well were incubated overnight at 37 °C in 10 mL of aqueous solution containing 125 mM EDC. Those collagen gels and keratin hydrogels prepared as described above were compared with each other in the rheological test, tensile test, and swelling test according to the methods previously reported [15].

2.4.1. Measurements of dynamic viscoelasticity

Collagen gel disks (diameter, $\phi = 20$ mm, thickness = 2.5 mm) and keratin hydrogel disks (diameter, $\phi = 20$ mm, thickness = 1.2 mm) were prepared and used for measurement of dynamic viscoelasticity using a rheometer (Physica MCR301, Anton Paar, Austria) with an oscillatory rotating disk to obtain values for the storage modulus (G′) and the loss modulus (G″) [15]. Measurements were carried out with a deflection angle of 0.167–0.501 m rad and a range of angular frequency ω from 0.1 to 100 rad/s at 25 °C. This test was performed in the linear viscoelastic region (VLR) at least from 0.1 to 10 rad/s in this study. Four samples (n = 4) were measured for each condition.

2.4.2. Tensile test

Rectangle-shaped crosslinked collagen gels (18 mm × 10 mm, thickness = 2.5 mm) and keratin hydrogels (18 mm × 10 mm, thickness = 1.2 mm) were prepared. They were used for the tensile test at room temperature (approximately 25 °C) using a tensile tester with an extension speed of 2 mm/min [15]. To prevent slippage of the gels, the arms of

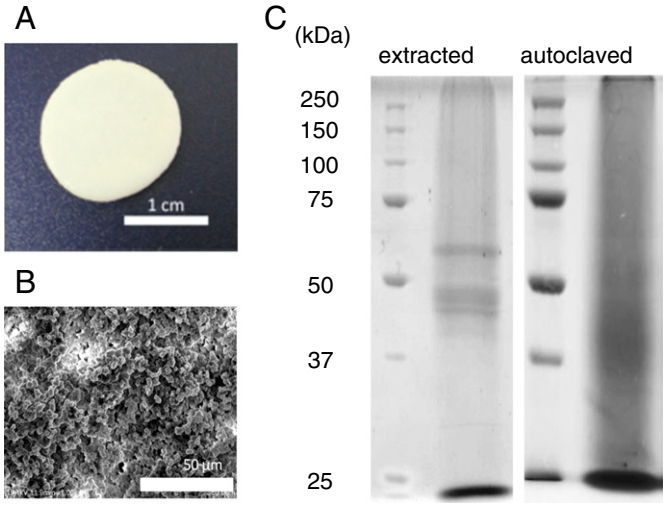

Fig. 2. A photograph of a keratin hydrogel (A), an electron micrograph (SEM image) of the surface of the keratin hydrogel (B) and results of the SDS-PAGE (C) of the keratin hydrogel before (left side of picture) and after (right side of picture) autoclave with molecular weight markers (left lane of each picture).