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Wool fibril sponges with perspective biomedical applications

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ABSTRACT

Sheep's wool was used as a natural source to prepare keratin microfibril sponges for scaffolding, by disruption of the histological structure of the fibres through mild alkali treatment, followed by ultrasonication, casting and salt-leaching.

The wool sponges showed highly interconnected porosity (93%) and contain intrinsic sites of cellular recognition that mimic the extracellular matrix (ECM). They displayed good thermal and water stability due to the conversion of disulphide cystine bonds into shorter monosulphide lanthionine intermolecular bonds, but significantly swelled in water, because of the high hydrophilicity and porosity, with a volume increasing up to 38%. Nevertheless, sponges were stable in water without structural changes, with a neutral pH in aqueous media, and showed excellent resilience to repeated compression stresses.

According to in vitro biocompatibility assays, wool fibril sponges showed a good cell adhesion and proliferation as proved by MTT, FDA assays and SEM observations. The unique structure of the cortical cell network made by wool keratin proteins with controlled-size macro-porosity suitable for cell guesting, and nutrient feeding, provides an excellent scaffold for future tissue engineering applications.

1. Introduction

Many natural polymers have been studied for biomedical applications seeking for biocompatibility, biodegradability and, in some cases, enhanced cellular adhesion; all these properties are highly requested in scaffolding systems. Protein-based biomaterials from collagen, albumin, gelatine, fibroin and keratin, have been proposed for many biomedical and biotechnological applications due to their ability to work as an extracellular matrix that facilitates cell–cell and cell–matrix interactions. Among them, keratin-based materials have emerged as promising candidates because of their biocompatibility, biodegradability and mechanical strength.

Keratin is one of the most abundant non-food proteins found in hairs, feathers, wool, horns and nails of mammals, reptiles, and birds. It is highly hydrophilic and contains a high amount of the amino acid cystine, a sulphur-containing amino acid that gives rise to intra- and inter-molecular disulphide cystine bonds which largely influence its mechanical and chemical properties [1].

Keratin can be extracted from the natural sources using reducing or oxidising agents, and successively regenerated in the forms of films [2–10], nanofibres [11–17], sponges [12–21] and hydrogels [22,23], which have been extensively studied and proposed for biomedical applications.

Moreover, literature reports that keratin improves the production of anti-inflammatory cytokines and decreases the quantity of pro-inflammatory cytokines; its use in regenerative medicine may therefore provide additional benefits by regulating a positive remodelling response [24].

Tachibana et al. demonstrated that reduced keratin extracted from wool fibres, is suitable for fibroblast [25] and osteoblast [26] scaffolding, owing cell adhesion sequences (arginine–glycine–aspartic acid: RGD and leucine–aspartic acid–valine: LDV), while the high cysteine content (about 20 mol%) is suitable for modification targets.

Novel biomaterials were recently prepared via ultrasonic fibrillation of wool without any chemical extraction, exploiting the intrinsic histological structure of the wool fibre [27,28] which is made of three main

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morphological components, namely the cuticle, the cell membrane complex and the cortex. The cuticle of the wool fibre consists of a thin envelope of flat overlapping 'cuticle cells' arranged like roof tiles surrounding the cortex, which is made up of spindle shaped 'cortical cells' oriented parallel to the fibre axis. The cell membrane complex, sometimes referred to as intercellular cement, performs the function of cementing cortical and cuticle cells together [29].

Fibrillation of the hierarchic cell assembly produces a suspension of cortical cells (fibrils) into the aqueous protein medium resulting from degradation of the other histological components that were cast into porous films made of randomly oriented cortical cells stuck to each other by the hydrolysed keratin matrix.

In this work, the above methodology has been combined with salt-leaching to prepare micro- and macro-porous keratin sponges with the aim of mimicking native tissues for cell proliferation and cell-guided tissue formation.

2. Experimental

2.1. Materials

Botany wool, 20.3 μm mean fibre diameter, in the form of top (the fibre sliver obtained from raw wool by scouring, carding, and combing processes) was supplied by The Woolmark Co., Italy. All analytical grade chemicals were purchased from Sigma-Aldrich, except otherwise specified.

2.2. Methods

2.2.1. Preparation of the bio-composite sponges

8 g wool fibres weighted in standard atmosphere (20 °C and 65% RH), were cut into snippets of some millimetres and bathed in 400 ml 0.1 N NaOH (material to liquor ratio 1:50) for 24 h at 60 °C without stirring. The snippets were rinsed with tap water until neutral pH on a stainless steel sieve (120 mesh), then soaked in deionised water (total volume 150 ml) and submitted to ultrasonic irradiation for 30 min with a Sonics Vibracell 750 (Cole Parmer) sonicator equipped with a stainless steel 1/2 inch "solid" probe. Power was tuned to 600 W at the frequency of 20 kHz, with control temperature set at 50 °C. This treatment produces a suspension of cortical cells and fibre fragments in the aqueous protein solution. Coarse fibre fragments were removed by filtration with a stainless steel, 120 mesh sieve. The permeate cell suspension was centrifuged at 12,000 rpm for 15 min, and the supernatant was removed.

The solid precipitate was added with 16 ml deionised water and kept on stirring until the complete suspension of the cortical cells that were arranged to 0.05 g/ml. This suspension was added with 1.17 g/ml controlled size NaCl (400–500 μm) then cast at 50 °C until dry.

The resulting material was washed 5 times with deionised water in order to completely remove the salt, giving rise to a wool fibril sponge that was dried again at 50 °C.

An additional thermal treatment at 180 °C for 2 h was carried out to improve the water stability of the sponge, increasing isopeptide bonding and other crosslinks through dehydration, as reported in literature [30,31].

2.2.2. Morphological characterisation

SEM investigation was performed with a LEO (Leica Electron Optics) 135 VP SEM, at 15 kV acceleration voltage and 30 mm working distance. The samples were mounted on aluminium specimen stubs with double-sided adhesive tape and sputter-coated with 20 nm thick gold layer in rarefied argon, using an Emitech K 550 Sputter Coater with 20 mA current for 180 s. Cross sections of the films were obtained by fragile fracture in liquid nitrogen.

2.2.3. Porosity

Porosity was measured by an inert gas stereopicnometer Quantachrome, model SPY-3. Sponge samples were placed 24 h in standard atmosphere (20 °C, 65% RH) before being placed into the pycnometer cell, and fluxed with argon for 10 min to remove moisture and air. After that, the measurement of the volume has been performed. Once the real volume has been measured, the samples were weighed in order to calculate the true density.

2.2.4. Degree of crosslinking

The crosslinking degree of the sponges before and after the thermal treatment was determined by the ninhydrin assay [32–33]. Sponges (10 mg) were heated in a ninhydrin solution (2% (*w*/*v*)) at 100 °C for 20 min. The optical absorbance of the resulting solution was read at a wavelength of 570 nm using a Perkin Elmer Lambda 35 spectrometer. The concentration of free NH_2 groups in the sample was determined using standard curve of glycine concentration vs. absorbance. Non-crosslinked sponges were used as control materials. Triplicate samples were evaluated.

The degree of crosslinking was determined by the following equation:

$$\text{Degree of crosslinking } (\%) = \left[(NH_2)_{nc} - (NH_2)_c * 100\right] / (NH_2)_{nc}$$

where $(NH_2)_{nc}$ and $(NH_2)_c$ are, respectively, the mole fraction of free NH_2 in non-crosslinked and crosslinked samples.

2.2.5. Amino acid composition

The wool fibril sponges, before and after the thermal treatment at 180 °C, were submitted to amino acid analysis compared with the original wool. All samples (40 mg) were hydrolyzed with 15 ml HCl (6 M) at 110 °C for 24 h in sealed tubes. Free amino acid residues were derivatized with hydroxysuccinimidyl carbamate (AQC by Waters) and eluted on a 15 cm × 0.39 cm reversed-phase column (Waters). An Alliance high-performance liquid chromatograph (HPLC; Waters) was used, and the eluate was detected at 254 nm. The quantitative amino acid composition was determined by calibration with the Amino Acid Standard H (Pierce), cysteic acid and lanthionine (TCI Europe) as external standards, and α-aminobutyric acid as internal standard.

2.2.6. SDS-PAGE

The samples (30 mg) were extracted in 1 ml solution containing Tris/HCl (550 mM; pH 8.6), DTT (140 mM), ethylenediaminetetraacetic acid (5 mM) and urea (8 M) for 4 h under nitrogen atmosphere, according to the Marshall method [34]. The protein concentration of the extract was determined with the Bradford protein assay method (Bio-Rad) using bovine serum albumin standard. Samples were dissolved into a buffer containing the NuPAGE LDS sample buffer and the NuPAGE sample reducing agent as recommended by the Invitrogen protocol, to deliver 30 mg of sample to the gel (NuPAGE reducing agent contains 500 mM DTT at a ready-to-use 10 concentration in a stabilised liquid form; NuPAGE buffer contains lithium dodecyl sulphate at a pH of 8.4), which allows for maximal activity of the reducing agent. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using an Xcell SureLock Mini-Cell (Invitrogen) on Bis-tris 4–12% polyacrylamide pre-cast gel (Invitrogen) using the NuPAGE MOPS SDS running buffer. This is suitable for proteins with molecular weights from 191 to 14 kDa, referring to myosin, phosphorylase, bovine serum albumin, glutamic dehydrogenase, alcohol dehydrogenase, carbonic anhydrase, myoglobin red, and lysozyme as the molecular weight markers (see blue pre-stained standard, from Invitrogen).

2.2.7. pH of the water extract

In accordance with the International Wool Textile Organization standard IWTO-2-96, the wool fibril sponges were bathed in distilled water at 20 °C and stirred for an hour before the pH measurement.