Materials Science and Engineering C 68 (2016) 163–171

Contents lists available at ScienceDirect

Materials Science and Engineering C

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

Ovine tendon collagen: Extraction, characterisation and fabrication of thin films for tissue engineering applications

M.B. Fauzi [a], Y. Lokanathan [a], B.S. Aminuddin [a,b], B.H.I. Ruszymah [a,c], S.R. Chowdhury [a,*]

[a] Tissue Engineering Centre, UKM Medical Centre, Jalan Yaacob Latiff, Bandar Tun Razak, 56000 Cheras, Kuala Lumpur, Malaysia
[b] Ear, Nose & Throat Consultant Clinic, Ampang Puteri Specialist Hospital, Taman Dato Ahmad Razali, 68000 Ampang, Selangor, Malaysia
[c] Department of Physiology, UKM Medical Centre, Jalan Yaacob Latiff, Bandar Tun Razak, 56000 Cheras, Kuala Lumpur, Malaysia

ARTICLE INFO

Article history:
Received 13 February 2016
Received in revised form 14 April 2016
Accepted 23 May 2016
Available online 24 May 2016

Keywords:
Ovine collagen
Collagen film
Collagen type I
Tissue engineering
Biocompatibility
Cell alignment
Unidirectional alignment

ABSTRACT

Collagen is the most abundant extracellular matrix (ECM) protein in the human body, thus widely used in tissue engineering and subsequent clinical applications. This study aimed to extract collagen from ovine (*Ovis aries*) Achilles tendon (OTC), and to evaluate its physicochemical properties and its potential to fabricate thin film with collagen fibrils in a random or aligned orientation. Acid-solubilized protein was extracted from ovine Achilles tendon using 0.35 M acetic acid, and 80% of extracted protein was measured as collagen. SDS-PAGE and mass spectrometry analysis revealed the presence of alpha 1 and alpha 2 chain of collagen type I (col I). Further analysis with Fourier transform infrared spectrometry (FTIR), X-ray diffraction (XRD) and energy dispersive X-ray spectroscopy (EDS) confirms the presence of triple helix structure of col I, similar to commercially available rat tail col I. Drying the OTC solution at 37°C resulted in formation of a thin film with randomly orientated collagen fibrils (random collagen film; RCF). Introduction of unidirectional mechanical intervention using a platform rocker prior to drying facilitated the fabrication of a film with aligned orientation of collagen fibril (aligned collagen film; ACF). It was shown that both RCF and ACF significantly enhanced human dermal fibroblast (HDF) attachment and proliferation than that on plastic surface. Moreover, cells were distributed randomly on RCF, but aligned with the direction of mechanical intervention on ACF. In conclusion, ovine tendon could be an alternative source of col I to fabricate scaffold for tissue engineering applications.

1. Introduction

Scaffolds that mimic the microenvironment of native tissues are an essential component in tissue engineering applications [1,2]. The properties of scaffolds, such as biomaterial composition, architecture and mechanical strength determine the efficiency of cellular interaction, successful integration with the host tissue and functional effectiveness in regeneration [3]. Natural, synthetic and composite biomaterials are extensively used in fabricating scaffolds for tissue engineering applications [1]. However, natural biomaterials containing extracellular matrix (ECM) components are preferred over synthetic materials as they provide advantages regarding their biological properties and other related bio-mechanistic factors [4].

Among the natural biomaterials, collagen is widely used due to its excellent biocompatibility and biodegradability, low immunogenicity and superior versatility for fabricating scaffolds in different forms (sheets, sponges, gels and nanofibres) [5]. Collagen is the major extracellular matrix protein in humans, and is thus in high demand in the tissue engineering, regenerative medicine and cosmetic industries [6]. Collagen from animal sources has been extensively studied and is commonly used in various applications [7,8]. Due to its abundance, collagen has been isolated from various mammalian (bovine, porcine, goat and rat) and non-mammalian (fish, amphibian and sea plant) sources [7–11]. Collagen isolated from different sources differs in terms of molecular organisation, stability and immunogenicity, which affect the functionality of the engineered tissue [9]. Recombinant collagen has emerged as an alternative source as it is biocompatible and mimics the natural micro-environment [12]. However, the absence of post-translational modifications on recombinant collagen affects normal biological functions upon implantation [13]. Thus, careful selection of the collagen source is important for the fabrication of scaffolds and the subsequent clinical outcome.

There are 29 different types of collagen that have been described in the literatures; nine of them are commonly available, including collagen types I, II, III, IV, V, VII, IX, XI and XII [14,15]. Among them, collagen type I (col I) comprises about 90% of the total collagen of the human body, and is found abundantly in skin, tendon, bone, ligament and cornea [16,17]. Col I is most often used in the fabrication of scaffolds for tissue

* Corresponding author at: Tissue Engineering Centre, Universiti Kebangsaan Malaysia Medical Centre, Jalan Yaacob Latif, Bandar Tun Razak, 56000 Cheras, Kuala Lumpur, Malaysia.
E-mail addresses: shiplu@ppukm.ukm.edu.my, shiplu56@gmail.com (S.R. Chowdhury).

http://dx.doi.org/10.1016/j.msec.2016.05.109
0928-4931/

engineering applications, especially for the aforementioned tissues [6]. Similarly, in other mammalians such as bovine, porcine, ovine etc., col I is abundant in skin and tendon, and commonly isolated from these tissues. Skin mostly contains col I and col III, whereas tendon contains mostly col I, a small portion of elastin and proteoglycan and some inorganic components [18]. Col I from these tissues isolated via acid extraction using acetic or citric acid [19], enzymatic digestion using pepsin [20], neutral salt extraction using disodium phosphate [21], organic extraction using urea [22] and combinations of these methods.

In the native state, the orientation and distribution of col I fibrils varies between tissues, and have an effect on cellular organisation. Alterations in the structure may cause functional defects [23,24]. For example, collagen fibrils are randomly distributed in the dermal layer of normal skin. A uniaxial alignment of collagen fibrils in the dermal layer results in scar formation and, in severe cases, keloid formation [25,26]. In contrast, collagen fibrils in the corneal stroma need to be aligned, and alterations from this cause blindness [27]. Thus, consideration should be given to the organisation of collagen fibrils during the fabrication of scaffolds for tissue engineering applications. The fabrication of collagen scaffolds normally produces a random distribution of collagen fibrils. However, the application of an external intervention such as mechanical, electrical or magnetic stimulation can align collagen fibrils in the scaffold [28,29]. These methods include the application of a static magnetic field to dilute collagen gels [30], running an electrical current through a collagen solution [31], shear flow deposition using a micro-fluidic device [32], flow processing [33], electrospinning of a collagen solution with conductive organic and non-organic solvents [34,35], spin coating [36], micro-patterning of surfaces [37], stretching of the collagen film on a PDMS substrate [38] or even combined techniques such as electrospinning associated with a magnetic field [29]. Although these methods have excellent potential for aligning collagen fibrils, specified preparations and tedious techniques limit their extensive application in tissue engineering and cell biology. Moreover, the application of extreme conditions such strong magnetic [39] and electric fields as well as the use of organic solvents will likely to lead to collagen denaturation [40].

In our previous studies, ovine (*Ovis aries*) tendon collagen (OTC) was extracted, and its biocompatibility towards human dermal fibroblasts (HDF) was shown [41,42]. However, the physico-chemical properties of the collagen were not tested. The aims of the current study were to characterise and identify the major constituents in the isolated OTC. Furthermore, an attempt was made to fabricate collagen films with aligned collagen fibrils using simple mechanical intervention, and to elucidate its effect on HDF attachment, proliferation and alignment.

2. Materials & methods

2.1. Collagen extraction and purification from ovine tendon

Crude tendon was cleaned of fascia and muscle tissues and freeze-dried for 48 h. The dried tendon was cut into small pieces and dissolved in 0.35 M acetic acid (Analar, USA) at 4 °C for 24–48 h to extract collagen. Sodium chloride (0.05 g/ml; Sigma, USA) was added to the collagen solution and incubated at 4 °C for 24–48 h, followed by centrifugation at 10,000 rpm for 45 min. The collagen pellet was then dialysed for 72 h using dialysis tube (molecular weight cut off = 14 kDa) (Sigma) with alternate change of disodium hydrogen phosphate solution (Na_2HPO_4; 0.2 M) and phosphate buffer saline (PBS; 1x) as dialysis buffer every 12 h. Dialyzed collagen was freeze-dried for 24–48 h and re-dissolved in 0.35 M acetic acid.

2.2. Total protein and total collagen quantification

Quantification of total protein in the OTC solution was performed by the bicinchoninic acid (BCA) assay kit (Sigma) according to the manufacturer's protocol. In brief, BCA working solution was prepared by mixing of reagent A and reagent B. Then, 12.5 μl of collagen solution was mixed with 100 μl of BCA working solution, followed by incubation at 37 °C for 30 min. The absorbance was recorded at 562 nm using a spectrophotometer. Bovine serum albumin (Sigma) was used as the standard.

The concentration of total collagen in OTC solution was determined with the collagen Sircol assay (Biocolor, UK), performed according to the manufacturer's instructions. Bovine collagen (Biocolor, UK) was used as the standard. Briefly, 1 ml of Sircol dye was added to an equal volume of standard and test samples. The solution was shaken gently for 30 min and centrifuged at 12,000 rpm for 10 min. The pellet was re-suspended in 0.75 ml of ice-cold acid salt wash reagent (contains acetic acid, sodium chloride and surfactants) followed by centrifugation at 12,000 rpm for 10 min. The pellet was then mixed with 0.2 ml of alkali reagent (contain of 0.5 M sodium hydroxide) and absorbance was measured at 555 nm using a spectrophotometer.

2.3. Identification of protein in the collagen solution

Protein identification in the OTC solution was executed via 1D SDS-PAGE and MALDI-TOF/TOF mass spectrometry. 1D SDS-PAGE was performed using 5% acrylamide gel at a constant current 400 mA, 150 V on miniVE apparatus (GE, USA) for 2 h. Later, the gel was stained with Coomassie blue and an image of the gel was captured using ImageScanner (GE, USA). The protein bands from the SDS gel were cut separately and analysed on a MALDI-TOF/TOF mass spectrometer using mascot sequence matching software (Matrix Science) with the Ludwig NR Database.

2.4. Chemical characterisation of OTC

Fourier transform infrared spectrometry (FTIR), X-ray diffraction (XRD) and energy dispersive x-ray spectroscopy (EDS) were used for the chemical characterisation of OTC. FTIR, XRD and EDS analyses were conducted using thin films of air-dried OTC. To analyse the chemical compound, FTIR spectra were recorded from 4000 to 650 cm^{-1} using a Perkin Elmer Spectrum 400 FT-Infrared (IR) Spectrometer with Spotlight 400 Imaging System (PerkinElmer, USA). The graph containing different peaks was analysed with references. The OTC film was also tested using X-ray diffraction (XRD; Bruker D8 Advance, USA) with a setting of 0.02° step size and a scan rate of 1°/min with CuKα radiation ($\lambda = 1.54$ nm). The OTC film was scanned using a Phenom ProX microscope (Phenom, Netherland) with EDS to obtain the percentage of elements available in OTC. Data were analysed using integrated software (ProSuite-Element Identification). For both analyses, rat tail col I (Sigma) was used as the control.

2.5. Fabrication of OTC films

Simple mechanical intervention by means of directional movement was applied using a platform rocker to fabricate the aligned collagen film (ACF). In brief, the collagen solution was poured into 12-well culture plates (1.5 ml/well) and placed on a unidirectional rocker for 6 h followed by drying at 37 °C in an incubator. The random collagen film (RCF) was fabricated without applying any mechanical intervention. Collagen films, both ACF and RCF, were sterilised via subsequent washing with sterile phosphate buffer saline (PBS; Sigma) and 70% ethanol (Analar). Prior to use for cell culture, OTC films were washed with a large amount of PBS and air-dried in a biosafety cabinet.

2.6. Assessment of the collagen orientation in OTC films

Dried films were observed under differential interference contrast (DIC) to visualise collagen fibril orientation using A1R confocal laser scanning microscope (CLSM; Nikon, Japan). The orientation of collagen fibrils in OTC films was also visualised via scanning electron microscopy