Biomaterials 29 (2008) 3278–3288

Contents lists available at ScienceDirect

Biomaterials

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

An electrochemical fabrication process for the assembly of anisotropically oriented collagen bundles

Xingguo Cheng [a], Umut A. Gurkan [a], Christopher J. Dehen [b], Michael P. Tate [c], Hugh W. Hillhouse [c], Garth J. Simpson [b], Ozan Akkus [a,*]

[a] Weldon School of Biomedical Engineering, Purdue University, West Lafayette, IN 47907, USA
[b] Department of Chemistry, Purdue University, West Lafayette, IN 47907, USA
[c] School of Chemical Engineering, Purdue University, West Lafayette, IN 47907, USA

ARTICLE INFO

Article history:
Received 23 January 2008
Accepted 14 April 2008
Available online 9 May 2008

Keywords:
Assembly
Collagen
Electrochemistry
Biomimetic materials
Connective tissues

ABSTRACT

Controlled assembly of collagen molecules *in vitro* remains a major challenge for fabricating the next generation of engineered tissues. Here we present a novel electrochemical alignment technique to control the assembly of type-I collagen molecules into highly oriented and densely packed elongated bundles at the macroscale. The process involves application of electric currents to collagen solutions which in turn generate a pH gradient. Through an isoelectric focusing process, the molecules migrate and congregate within a plane. It was possible to fabricate collagen bundles with 50–400 μm diameter and several inches length via this process. The current study assessed the orientational order, and the presence of fibrillar assembly in such electrochemically oriented constructs by polarized optical microscopy, small angle X-ray scattering, second harmonic generation, and electron microscopy. The mechanical strength of the aligned crosslinked collagen bundles was 30-fold greater than its randomly oriented-crosslinked counterpart. Aligned crosslinked collagen bundles had about half the strength of the native tendon. Tendon-derived fibroblast cells were able to migrate and populate multiple macroscopic bundles at a rate of 0.5 mm/day. The anisotropic order within biocompatible collagenous constructs was conferred upon the nuclear morphology of cells as well. These results indicate that the electrochemically oriented collagen scaffolds carry baseline characteristics to be considered for tendon/ligament repair.

1. Introduction

As a natural protein, collagen is mainly responsible for the mechanical and structural integrity of load-bearing extracellular matrices in all vertebrates and several invertebrates. Rod-like collagen molecules self-assemble in a crystal-like fashion with different packing patterns and the resulting structures are cross-linked (CX) to form mechanically robust tissues *in vivo* [1]. More importantly, as in tendon and ligaments, type-I collagen molecules are anisotropically oriented in a preferred direction along the dominant physiological loading pattern [2]. As a biomaterial, collagen has been a key ingredient in tissue engineering scaffolds, wound healing biomaterials, drug/gene delivery agents, and nerve guide substrates [3,4]. The application range of collagen would be greatly broadened if the assembly process could be better controlled to facilitate the synthesis of dense and oriented tissue-like constructs [5].

There have been several efforts to render synthetic collagen structures with some degree of orientational anisotropy. The alignment of molecules by flow, mechanical extrusion, microfluidic channels, or anisotropic chemical nanopatterns has limitations in attaining high packing density, elastic deformability, and the final size of the construct [6–11]. Also, orienting collagen molecules by magnetic fields require tesla-order superconducting magnets due to low diamagnetic constant of collagen [12]. Collagen structures with liquid crystalline order can be synthesized by slowly increasing the collagen solution concentration during a lengthy (weeks to months) dehydration process [13]. Oriented collagen nanofibers can also be achieved by the electrospinning process [14]. However, this process has some limitations in terms of its cost-effectiveness and it involves the use of toxic and corrosive solvents. Therefore, there is currently a need to find alternative means of forming highly oriented and densely packed macroscale collagenous constructs with preparation timescales in the order of minutes to hours.

One alternative approach for providing high orientational order and packing density involves manipulation of the electrochemical environment surrounding collagen molecules. Previous studies

* Corresponding author. Tel.: +1 765 496 7517; fax: +1 765 496 1992.
E-mail address: ozan@purdue.edu (O. Akkus).

0142-9612/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2008.04.028

investigated whether small electrical currents applied directly to collagen solutions played a role in the fibrillogenesis of collagen *in vitro* from a physiological bioelectricity point of view [15–17]. These studies demonstrated the emergence of a birefringent band between two electrodes which are dipped in collagen solutions. Early literature made a limited attempt to characterize electrochemically synthesized collagen structures and it was reported that there was a general lack of orientational anisotropy and the D-banding pattern was absent [16,17]. Therefore, the degree of fibrillar orientation, mechanical properties and other physicochemical characteristics of collagen constructs produced as such are currently ambiguous. Accordingly, the electrochemical process has been abandoned since the last 40 years.

Earlier attempts assumed the voltage and the current as the key variables in the electrochemical process, whereas the potential effects of peripheral factors such as the dimension and configuration of electrochemical cell were not taken into account. The first aim of this study was to improve the electrochemical synthesis process to form highly oriented and mechanically robust collagenous bundles (ropes) by using parallel wire electrodes. The level of improvement in the structural organization and mechanical properties were characterized by scanning electron microscopy (SEM), transmission electron microscopy (TEM), two-dimensional (2D) small angle X-ray spectroscopy (SAXS), second harmonic generation (SHG) methods, and tensile tests. Our second aim was to assess the baseline feasibility of the electrochemically synthesized collagen bundles for tissue engineering of connective tissues. The second aim was achieved by assessing the migration and proliferation, morphology, and orientation of cells seeded on the material. Three-dimensional (3D) constructs were constructed by grouping multiple oriented bundles together and the ability of the cells to populate the material was monitored.

2. Materials and methods

2.1. Preparation of aligned collagen bundles

The overall process included the following basic stages: (1) subjecting dialyzed collagen solution to electrochemical process which results in the formation of aligned collagen bundle, (2) treatment of the bundle in phosphate buffered saline (PBS) at 37 °C to promote fibrillogenesis, and (3) crosslinking in genipin to provide the final strength.

Fully dialyzed collagen was used as the electrolyte for the electrochemical alignment process. Ten milliliters of type-I collagen (6 mg/mL 97% from bovine hide; INAMED Corporation, Santa Barbara, CA) was dialyzed ($MW_{cut\ off} = 3.5$ kDa) against ultrapure water at 5 °C for 72 h to remove salts. The dialyzed collagen had the characteristics of normal acidic soluble monomeric collagen and did not undergo fibrillogenesis before the onset of the electrochemical process which took place at room temperature (Fig. 1a). The dialyzed collagen underwent fibril formation only after the addition of 10× PBS and adjustment of the temperature to 37 °C at pH 7.4 (Fig. 1a). This indicates that the dialyzed collagen mainly existed in molecular form instead of fibrillar form at the onset of electric current application.

The electrochemical process was carried out in a custom-made two-electrode electrochemical cell (Fig. 1b). Two stainless steel electrode wires (0.254 mm diameter, 25 mm length; Sigmund Cohen Corporation, New York) were stripped of their insulating sheet along an inch-long segment and positioned parallel to each other with about 1 mm spacing on a glass slide. The gap between the electrodes was filled with the dialyzed collagen solution which was a transparent viscous fluid. The electrochemical cell was placed in a closed glass Petri dish lined with moist bench paper to maintain humidity at room temperature. Electrodes were connected to a DC voltage source and a 1 MΩ resistor in series (Fig. 1b). At a supply voltage of 6 V, the current through the collagen solution was measured to be 3.5 μA and 2.5 V DC. The orientation of the forming collagen band was monitored by a polarized optical microscope (Olympus BX51, Melville, NY, USA) with the application of first order wavelength gypsum plate. For a positive birefringent material such as collagen, molecules which are aligned along the slow axis of this plate appear blue while those molecules which are aligned perpendicular to the slow axis appear yellow [18]. After the confirmation of the congregation and alignment of molecules along a band by polarized imaging, the current was interrupted, PBS was added, and the sample was collected with a pair of tweezers. This freshly aligned collagen bundles were incubated in 10× PBS solution (pH 7.4, 37 °C) for 12 h prior to crosslinking. The average dimension of bundles varied in the range of 50–400 μm diameter and 3–7 cm length depending on the length of electrodes. These aligned collagen bundles were crosslinked in 15 ml of 0.625% genipin (Wako Pure Chemical, Osaka, Japan) in a sterile 1× PBS solution at 37 °C for 3 days [19].

Randomly oriented collagen networks were used as controls to compare with aligned collagen made by the above electrochemical method. Randomly oriented collagen was prepared by mixing the dialyzed monomeric collagen solution with 10× PBS, casting on a glass surface as a layer, and by gelling at 37 °C. Strips were cut from this layer after crosslinking with genipin using fresh surgical blades. Native tendon bundles were obtained at the origin of the biceps muscle of a canine that was euthanized under the approval of the Purdue Animal Care and Use Committee. The tendon fibers were bleached in 1% NaOCl solution for 90 s to expose the collagen phase by removing the non-collagenous matter [20].

2.2. Small angle X-ray scattering (SAXS)

SAXS patterns were collected using a three-pinhole SAXS camera (Molecular Metrology, Texas, USA) with a microfocus X-ray source, an Osmic MaxFlux confocal X-ray optic, and a 2D Fujifilm image plate detector at a camera length of 1647 mm. The detector was calibrated using a silver behenate powder standard ($q = 0.107623$ Å^{-1}). The main beam intensity was attenuated with a beam stop blocking all scattering below $q = 0.11$ nm^{-1}. Plots of the intensity versus the magnitude of the scattering vector (q) were produced from a radial line plot of the 2D SAXS data along the direction of the fiber axis. The 2D SAXS scattering data was processed to calculate the order parameter as described earlier [21]. The intensity $I(\phi)$ of the strongest arc versus the azimuthal angle ϕ was obtained for tendon and the aligned crosslinked collagen. The integrals in Eq. (1) were carried out numerically and the order parameter S was obtained using Eq. (2). For an isotropic material, $S = 0$, and for an ideal uniaxially oriented material, $S = 1$.

$$\langle \cos^2 \phi \rangle = \frac{\int_0^{\pi/2} I(\phi) \sin(\phi) \cos^2 \phi \, d\phi}{\int_0^{\pi/2} I(\phi) \sin(\phi) d\phi} \quad (1)$$

$$S = \frac{1}{2}\left(3\langle \cos^2 \phi \rangle - 1\right) \quad (2)$$

2.3. Second harmonic generation (SHG) analysis

SHG analysis was performed to quantify the degree of orientation by using the discrete retardance nonlinear optical ellipsometer described previously [22] on samples of random-CX collagen gel, aligned-CX collagen bundles, and native tendon bundles. The SHG intensities were measured using different combinations of vertically (V) and horizontally (H) linearly polarized light for the incident and detected beams, with the vertical axis corresponding to the longer axis of the bundles. Samples were air dried, placed between two glass slides, and measured in the transmission configuration (0° tilt) as described earlier [22]. The SHG intensity was measured 9 times for each of the three samples, with 64 laser pulses per data point, and normalized to HHH. Reported standard deviations are dominated by sample-to-sample variance.

2.4. Scanning electron microscopy (SEM) and transmission emission microscopy (TEM)

Collagen samples were prepared using a procedure similar to that developed by Raub et al. for SEM imaging of collagen gels [23]. Dried samples were mounted on holders and coated with Pt for 40 s prior to imaging (FEI NOVA nanoSEM, FEI Company, Hillsboro, OR) using through-the-lens and Everhart–Thornley detectors at a 5 kV accelerating voltage. For TEM analysis, collagen samples were macerated in a depression slide using a scalpel and forceps in ultrapure water. About 10 μl of the supernatant was put on a TEM grid and allowed to settle for 1 min. Samples were stained with 1% phosphotungstic acid and dried prior to imaging by TEM (FEI/Philips CM-100, FEI Company, Hillsboro, OR).

2.5. Mechanical testing

Random-CX collagen bundles, aligned-CX collagen bundles prepared by the current electrochemical process, bleached tendon-CX, and native tendon fibers ($N = 10$/group) were tested for their tensile mechanical properties under similar conditions. Prior to tensile tests, samples were washed with deionized water, serially dehydrated in EtOH/H_2O solutions, and air dried. Both ends of the dried bundles were fixed on plastic tabs using epoxy and rehydrated with a 1× PBS. The thicknesses of rehydrated samples were measured using a confocal microscope (Olympus FV1000) and the width was measured using a calibrated video-microscope at five different locations along the gage length of bundles. The area that is measured the closest to the failure location was utilized for stress calculations. Samples were mounted on fixtures of an electromagnetically controlled materials testing machine (Testresources 800L, Shakopee, MN) and loaded in tension to failure monotonically in displacement control (10 mm/min). Load was measured by a load cell (10 lb, Omega Inc., Stamford, CT) and load values were divided by the area of the sample to calculate stress. The extension was normalized with the original gage length to calculate the strain. Elastic modulus was obtained by a linear regression fit. The