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Biomimetic wet-stable fibres via wet spinning and diacid-based crosslinking of collagen triple helices

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ABSTRACT

One of the limitations of electrospun collagen as bone-like fibrous structure is the potential collagen triple helix denaturation in the fibre state and the corresponding inadequate wet stability even after crosslinking. Here, we have demonstrated the feasibility of accomplishing wet-stable fibres by wet spinning and diacid-based crosslinking of collagen triple helices, whereby fibre ability to act as bone-mimicking mineralisation system has also been explored. Circular dichroism (CD) demonstrated nearly complete triple helix retention in resulting wet-spun fibres, and the corresponding chemically crosslinked fibres successfully preserved their fibrous morphology following 1-week incubation in phosphate buffer solution (PBS). The presented novel diacid-based crosslinking route imparted superior tensile modulus and strength to the resulting fibres indicating that covalent functionalization of distant collagen molecules is unlikely to be accomplished by current state-of-the-art carbodiimide-based crosslinking. To mimic the constituents of natural bone extra cellular matrix (ECM), the crosslinked fibres were coated with carbonated hydroxyapatite (CHA) through biomimetic precipitation, resulting in an attractive biomaterial for guided bone regeneration (GBR), e.g. in bony defects of the maxillofacial region.

1. Introduction

Bone tissue engineering (TE) is a fascinating field of research with substantial focus on delivering materials that mimic natural constituents of bone. Especially in the maxillofacial context, natural polymers, such as collagen, have been widely employed for the design of biomimetic membranes for guided tissue regeneration (GBR) [1], aiming to accomplish selective, endogenous bone tissue growth into a defined space maintained by tissue barriers [2]. The fabrication of tissue-mimicking biomaterials is a key to successful GBR. In this regards, the extra cellular matrix (ECM), which rules the structure, properties and functions of bone and comprises both non-mineralized organic and mineralized inorganic components should be greatly considered [3]. The main organic component of bone is type I collagen, which forms more than 90% of its organic mass [4,5]. Although the architectures and roles of these collagens vary widely, they all comprise triple helix bundles at the molecular scale, where the collagen molecule consists of a right-handed triple helix composed of about 1000 amino acids with two identical α_1(I) and α_1(II) chains, and one α_2(I) chain. These three chains, staggered by one residue relative to each other, are supercoiled around a central axis in a right-handed manner to form the triple helix which is around 300 nm in length and 1.5 nm in diameter [4,6–9]. The strands are held together mostly by hydrogen bonds between adjacent –CO and –NH groups [10]. On the other hand what defines bone as a mineralized tissue is the deposition of inorganic carbonated apatite, and this mineral deposition is mainly accomplished by the precipitation of the apatite phase. Initially the precipitation occurs via matrix vesicle nucleation alone but, ultimately, requires collagen structures [5]. Thus, carbonated apatite coated collagen fibres, which are obtained from the assembly of collagen triple helices and are mineralized with apatite, are considered as the building blocks of ECM. In order to mimic this unique material constituents present *in vivo*, the formation of triple-helical collagen fibres and respective biomimetic mineralization with carbonated apatite has been addressed in this paper.

Owing to the excellent biological features and physiochemical properties of collagen, it has been among the most widely used biomaterials for biomedical applications [6,10–12] particularly when delivered in the form of gels, films, injectables and coatings

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[13–17]. To further extend the utility of collagen for use in medical devices and to address issues such as fixation in defect sites and stability for TE and GBR, the ability to manufacture mechanically robust fibres and fabrics is important. However, avoiding denaturation of the native triple helical structure and the instability of regenerated collagen materials in the hydrated state still remains highly challenging [18]. Electrospinning, which has been the main collagen fibre manufacturing process for use in TE, has limitations. The organic reagents required to prepare collagen electrospinning solutions such as those based on fluoroalcohols [19,20] are known to be highly toxic and partially denature the native structure of collagen [18,20]. To address this issue non-toxic solvents such as PBS/ethanol or acetic acid have been successfully introduced [21,22]. Among the different fibre manufacturing processes available, wet spinning has the potential to convert biomolecules into fibres without need for high voltage during manufacture and is less likely to be associated with denaturation [23,24]. Wet spinning was developed by the textile industry in the early 1900s as a means of producing man-made fibres such as viscose rayon. This fibre spinning technology is based on non-solvent induced phase separation, whereby polymer dope solutions are extruded through a spinneret into a non-solvent coagulation bath in which liquid polymer streams turn into solid filaments. In this context, using wet spinning to manufacture fibres from collagen triple helices suspension utilising non-toxic solvents such as acetic acid, whilst avoiding the addition of any synthetic phase, could be promising.

Many studies have been conducted to stabilize collagen fibres via covalent crosslinking [25–27]. The most popular crosslinking method is via carbodiimide, especially 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC), often used in the presence of N-hydroxysuccinimide (NHS). This method leads to activation of carboxylic functions and subsequent formation of amide net-points between amino and carboxylic functions of collagen [25–27]. One of the main reason of the popularity of EDC/NHS treatment is its compatibility compared to the commonly used bifunctional crosslinker, glutaraldehyde (GTA) [27]. However, EDC/NHS mostly links carboxylic acid and amino groups that are located within 1.0 nm of each other, which eventually means functional groups that are located on adjacent collagen molecules are too far apart to be bridged by carbodiimide [28,29]. A high molecular weight diacid could be a potential candidate to crosslink collagen fibres in presence of EDC/NHS. To address this hypothesis, we have evaluated the utility of 1, 3-phenylenediacetic acid (Ph) and poly(ethylene glycol) bis(carboxymethyl) ether (PEG) as bifunctional crosslinkers of varied segment length. Ph has been used to form biocompatible stable collagen hydrogels [30]; and PEG, which is an FDA approved chemical for several medical and food industries [31], has also been used to stabilize collagen [32–34] and polysaccharides [35].

Besides the nonmineralized collagen component, another important constituent of bone ECM is a mineralized inorganic component. Therefore, in order to develop attractive biomaterials for GBR, a composite of collagen and mineralized component should be considered. Initially the apatite was assumed to be hydroxyapatite, however, due to the presence of significant amount of carbonate, it is better defined as carbonate hydroxyapatite (CHA) [36]. Composites of biopolymer and hydroxyapatite (HA) were investigated in this regard, however, in a composite the functionality of HA is reduced due to the masking of apatite particles by biopolymers [37,38]. Therefore, a biomimetic coating of apatite on a collagen template can be considered an efficient approach [39,40]. By mimicking the natural biomineralization process Kokubo et al. first reported the use of simulated body fluid (SBF) for biomimetic growth of apatite coatings on bioactive $CaO–SiO_2$ glasses [41]. SBF has also been used to form apatite coating on collagen formulations [39,42,43]. However, SBF possesses limitations, particularly the time consuming nature of the process, necessity of a constant pH and constant replenishment to maintain super-saturation for apatite crystal growth [37,39,44,45]. Therefore, an alternative, simple and efficient approach for biomimetic coating on collagen fibre is needed.

The aim of the current study was to achieve wet-stable fibres via wet spinning and covalent crosslinking of collagen triple helices, and also to mimic the constituents of natural bone ECM through the precipitation of CHA on the as-formed wet-stable fibres. Ph and PEG were used as diacids of varied molecular weight to increase the likelihood of crosslinking distant collagen molecules was compared to the state-of-the-art zero length crosslinker EDC. Finally, CHA was coated through a biomimetic precipitation process on the crosslinked wet-spun fibres.

2. Materials and methods

2.1. Materials

1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) and 1, 3 phenylenediacetic acid (Ph) were purchased from Alfa Aesar. 2,4,6-trinitrobenzenesulfonic acid (TNBS), acetic acid (CH_3COOH), calcium chloride ($CaCl_2$), phosphoric acid (H_3PO_4), sodium carbonate (Na_2CO_3), Potassium phosphate dibasic trihydrate ($K_2HPO_4.3H_2O$), poly(ethylene glycol) bis(carboxymethyl) ether (PEG) and Dulbecco's Phosphate Buffered Solution (PBS) were purchased from Sigma Aldrich. Tissue culture media, Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FBS) and penicillin–streptomycin (PS) were purchased from Gibco. CellTiter® 96 AQueous one solution cell proliferation assay were purchased from Promega UK Ltd.

2.2. Isolation of type I collagen from rat tail tendons

Type I collagen was isolated through acidic treatment of rat tail tendons as described in previous papers [30,46]. In brief, frozen rat tails were thawed in 70% ethanol for about 15 min. Individual tendons were pulled out of the tendon sheath and placed in 50 ml of 17.4 mM acetic acid solution for each rat tail at 4 °C in order to extract collagen. After three days the supernatant was centrifuged at 10,000 r min^{-1} for half an hour. The mixture was then freeze-dried in order to obtain type I collagen. The resulting product showed only the main electrophoretic bands of type I collagen during sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-page) analysis [46].

Collagen dissolved in 17.4 mM acetic acid was studied by reading the optical density at 310 nm using a photo spectrometer. 0.25% (w/v) of collagen was mixed in 17.4 mM acetic acid to carry out the experiment. The optical density of the hydrolysed fish collagen solution was also studied as a control, given the absence of triple helix in respective CD spectrum in the same conditions [47].

2.3. Formation of wet-spun fibres (Fs)

To accomplish Fs from collagen triple helices, collagen was dissolved in 17.4 mM acetic acid in different concentrations (0.8–1.6% wt/vol) at 4 °C overnight. Resulting collagen suspensions were transferred into a 10 ml syringe having 14.5 mm internal diameter. The collagen suspensions were then ejected from the syringe through a syringe pump at a dispensing rate of 12 ml h^{-1} with the syringe tip submerged in a coagulation bath containing 1 L of pure ethanol at room temperature. The as-formed fibres were then removed from the ethanol and dried separately at room temperature.