Carbohydrate Polymers 82 (2010) 1264–1270

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Assembly of collagen fibrillar networks in the presence of alginate

Lin Sang, Xiaoliang Wang, Zhenhua Chen, Jiao Lu, Zhongwei Gu, Xudong Li*

National Engineering Research Center for Biomaterials, Sichuan University, Chengdu 610064, PR China

ARTICLE INFO

Article history:
Received 23 October 2009
Received in revised form 6 May 2010
Accepted 2 July 2010
Available online 13 July 2010

Keywords:
Collagen
Self-assembly
Alginate
Biomimetic
Networks

ABSTRACT

This study reports in vitro reconstitution of collagen–alginate blends with a wide range of alginate/collagen ratios, aiming at revealing the nanostructured fibrillar networks assembled at high collagen concentrations. The aggregation states of collagen monomeric solutions at varying concentrations from 1 to 7 mg/ml, in the absence or presence of alginate, were examined by using scanning electron microscopy and atomic force microscopy. Compared with collagen, the blends with increasing alginate/collagen ratios assembled into looser three-dimensional fibrillar networks interlaced by larger twisted fibrils which were irregular and dense aggregates of smaller fibrils with the normal banding periodicity. Typical turbidimetric assays revealed the different kinetics in presence of alginate and alginate-eluted quantitation further confirming the capturing of alginate in the collagen–alginate fibrillar networks. The electrostatic complexation between collagen and alginate was postulated to play a crucial role in this abnormal aggregation of collagen–alginate blends.

1. Introduction

Collagen is the primary constituent of extracellular matrices (ECM). As the most widely used natural macromolecule, collagen has been extensively applied to fabricate various three-dimensional scaffolds for tissue engineering purposes. In order to overcome the mechanically weak and easily degradable nature of collagen biomaterial, natural and synthetic biopolymers are usually introduced to achieve composite scaffolds with improved properties during which chemical crosslinking of active groups between collagen and biopolymers is generally involved (Choi, Hong, Lee, & Song, 1999; Lin, Tan, Marra, Jan, & Liu, 2009; Ma et al., 2003; Nam, Kimura, & Kishida, 2008).

However, the nativelike fibril nature of collagen was absent in those composite scaffolds obtained by simply mixing monomeric collagen with synthetic polymers (Chen, Ushida, & Tateishi, 2002) and with polysaccharides such as chitosan (Ma et al., 2003), alginate (Choi et al., 1999; Lee, Lo, Cheung, Wong, & Chan, 2009) and hyaluronan (Lin et al., 2009). In the tissues of vertebrates and many other multicellular organisms, the formation, structure and organization of collagen fibrils with 67 nm cross-striated patterns determine specific tissue architecture, stability and mechanical attributes (Canty & Kadler, 2005; Parry, Barners, & Craig, 1978). Lately reported studies confirmed that the fibrillar collagen carrier with unique D-patterns exhibited better cellular responses due to its analogous nature to ECM, indicating that the presence of nanostructured fibrillar assemblies is an important attribute for biomimetic ECM (Tsai, Chen, Chen, & Hsu, 2009).

The self-assembly formation of collagen fibrils, i.e. in vitro reconstitution or fibrillogenesis, has long received extensive investigations. Varied influencing factors upon in vitro reconstitution of monomeric collagen type I have been evaluated such as temperature, pH, ionic strength, type of salts and solutes (Cisneros, Hung, Franz, & Muller, 2006; Gobeaux et al., 2008; Jiang, Hörber, Howard, & Müller, 2004; Kuznetsova, Chi, & Leikin, 1998; Wood & Keech, 1960; Wood, 1960a; X.L. Wang et al., 2009), and presence of proteoglycans/glycosaminoglycans and polysaccharides (Brightman et al., 2000; Keech, 1961; Raspanti, Viola, Sonaggere, Tira, & Tenni, 2007; Raspanti et al., 2008; Salchert et al., 2004; Stamov, Grimmer, Salchert, Pompe, & Werner, 2008; Tsai, Liu, Hsu, & Chen,2006; Wood, 1960b). These studies mainly focused on the kinetic behaviors of the collagen molecule-fibril transition and the aggregated fibril morphologies, having a major impact on our contemporary knowledge of collagen self-assembly. However, dilute monomeric collagen solutions generally at less than 1 mg/ml were mostly applied in these studies, which only yielded mechanically weak assemblies.

To achieve useful biomimetic ECM analogues with nativelike fibrils, in vitro reconstitution of collagen monomeric solutions at high concentrations for elastic gels is prerequisite (Gobeaux et al., 2008). Owing to the facile crosslinking feature between collagen and polysaccharides, assembly of collagen–polysaccharide fibrillar networks followed by chemical crosslinking treatments is believed to be an efficient strategy to produce the mechanically robust

* Corresponding author at: National Engineering Research Center for Biomaterials, Sichuan University, Wangjianglu #29, Chengdu 610064, Sichuan, PR China. Tel.: +86 28 8541 2102; fax: +86 28 8541 2102.
E-mail address: xli20004@yahoo.com (X. Li).

0144-8617/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2010.07.005

biomimetic scaffolds. Obviously, investigations into the assembled collagen–polysaccharide fibrillar networks at high concentrations are important to realize this purpose. According to the best of our knowledge, despite the previous reports on collagen aggregation patterns and kinetics in the presence of polysaccharides, in vitro reconstitution of collagen–polysaacharide blends at collagen concentrations high enough to form strong gels for tissue engineering applications is yet to be explored.

Alginate is a linear anionic polysaccharide consisting of (1→4) linked β-D-mannuronate (M units) and its C-5 epimer α-L-guluronate (G units) in varying composition and sequence, abundant in brown algae. Owing to its good elasticity, biodegradability and excellent biocompatibility (Augst, Kong, & Mooney, 2006), alginate has been used for encapsulating cells (Gregory et al., 1999), drug carrier systems (Canty & Kadler, 2005) and scaffoldings for different tissue regenerations such as skin (Lee et al., 2009), bone (Li, Ramay, Hauch, Xiao, & Zhang, 2005), cartilage (Beekman, Verzijl, Ban, Von der Mark, & TeKoppele, 1997) and liver (Dvir-Ginzberg, Gamlieli-Bonshtein, Agbaria, & Cohen, 2003). The great interest in alginate was originated from its ability to form hard gels in the presence of divalent ions or to strong composite biomaterials including with collagen by chemical crosslinking (Choi et al., 1999; Lee et al., 2009).

In addition, the choice of alginate as a typical polysaccharide in this study is further based on the reported disagreeing experimental evidences for the role of alginate on collagen formation in alginate cultures of chondrocytes (Beekman et al., 1997; Gregory et al., 1999). Accordingly, the object of this study is to investigate the nanostructured fibrillar networks of collagen and collagen–alginate with varying alginate/collagen ratios and to focus especially on revealing their difference assembled at the initial collagen concentration 7 mg/ml which is sufficient to produce fibrillar gels directly for grafting biomaterials (X.L. Wang et al., 2009; X.M. Wang et al., 2009). We expect that this study would provide useful results for understanding the influence of alginate on collagen assembly and meanwhile lay a foundation for developing mechanically strong biomimetic collagen–alginate scaffolds.

2. Experimental

Type I collagen was extracted from calf skin by pepsin digestion. Briefly, the clean calf skin was cut into pieces. After defatted, the skin pieces were swollen in 0.5 M acetic acidic solution at 4 °C and then digested with 0.3% (w/w) pepsin (EC 3.4.23.1, 800–2500 U/mg protein, Sigma–Aldrich, St. Louis). The pepsin digestion was lasted for 6 days and then inactivated by adding 2 M sodium hydroxide. The digested supernatant was precipitated with sodium chloride, collected by centrifugation and redissolved in hydrochloric acid (pH 2). After a second NaCl precipitation and thorough dialysis, the extracted collagen was stored in 10 mM HCl at 4 °C for the following experiments. The purity of extracted collagen was demonstrated by collagenase digestion (EC 3.4.24.3, 265 U/mg, Sigma–Aldrich, St. Louis) and examination by SDS polyacrylamide gel electrophoresis. The amino acid composition of the collagen showed the relatively high contents of glycine, alanine, proline and hydroxyproline, 327, 135, 125 and 91 residues per thousand residues, respectively. The detailed results and biological evaluation of the extracted collagen were given elsewhere (Lu et al., 2005). Sodium alginate (2400 cps for a 2 wt.% aqueous solution at 25 °C) and other chemicals (Kelong Chem Co., Chengdu) were used as received. The deionized water (18 MΩ/cm) was used in the present experiments.

2.1. Formation of collagen–alginate blends

At the water bath of 4 °C, the collagen acidic solution (7 mg/ml) was neutralized to pH 7.2 with 2 M NaOH, and 0.3 M Na_2HPO_4 solution was introduced to achieve a final phosphate concentration of 20 mM. To form a collagen–alginate blend, aliquots of the 2 wt.% alginate aqueous solutions was dropwise added with stirring to the neutral collagen solution. The obtained homogeneous collagen–alginate blend was finally degassed by centrifugation at 4 °C. A wide range of alginate/collagen ratios, 20/80, 50/50 and 70/30 (by weight), were considered in this study to form collagen–alginate blends, and their assembled products were thereafter designated respectively as 20/80, 50/50 and 70/30 hydrogels or assemblies in the text when referred to. Along with the collagen–alginate blends, pure collagen solutions at typical concentrations were also prepared in order to reveal the effect of alginate on collagen fibrillogenesis.

2.2. Assemblies by in vitro reconstitution

In vitro reconstitution was initiated by the temperature elevation to 25 °C. Three types of reconstituted products, fibrillar gels, critical-point-dried and air-dried thin assemblies on clean glass slides, were prepared in this study. The gels obtained with the blends were thoroughly washed by soaking in deionized water, monitored by conductimetric measurements. The critical-point-dried samples were directly obtained with the blends whereas the diluted blends were used for the air-dried assemblies.

A drop of 100 μl collagen–alginate blend or collagen solution was placed on the top of clean glass slides (2 cm × 2 cm). After fibrillogenesis initiated at 25 °C, the resultant assemblies on glass slides were rinsed six times with deionized water, and then fixed in 2.5% glutaraldehyde/0.01 M PBS, pH 7.4 for 12 h at room temperature, and finally dehydrated using graded ethanols (30, 50, 70, 90, and 100% ethanol for 15 min each) followed by critical-point drying.

The aforementioned blends were also diluted with 10 mM PBS in order to investigate the difference in the aggregation states of the collagen–alginate blends at different collagen concentrations. For this purpose, the final collagen concentration of the diluted 50/50 blend and pure collagen was set to 1 mg/ml. 100 μl diluted blend or collagen was deposited on the surface of clean glass slides (2 cm × 2 cm) and allowed to form a gel layer at 25 °C. The resultant layers were carefully washed with deionized water and then dried under a laminar flow of air.

2.3. Scanning electron microscopy (SEM)

The critical-point-dried collagen and collagen–alginate assemblies on glass slides were examined on a scanning electron microscope (SEM, JSM-5900LV, JEOL), operated at 10 kV. For SEM observations, a thin layer of gold was coated on the surface of the critical-point-dried samples.

2.4. Atomic force microscopy (AFM)

The surface topography of air-dried collagen and 50/50 assemblies was observed in the tapping mode on an atomic force microscope (AFM, MFP-3D-BIO, Asylum Research) equipped with square pyramidal silicon nitride tips (Olympus AC 160TS) with spring constant ~1 N/m. Both height and amplitude images were recorded at a scan rate of 1.0 Hz in air. Fibril diameters, lengths and banding periodicities were measured by using the corresponding topography section analysis software provided with the instrument.

2.5. Turbidimetric measurement

For turbidity measurements the diluted 50/50 blend and collagen solution were poured into a photometer cuvette immediately after the final collagen concentration 1 mg/ml was achieved with

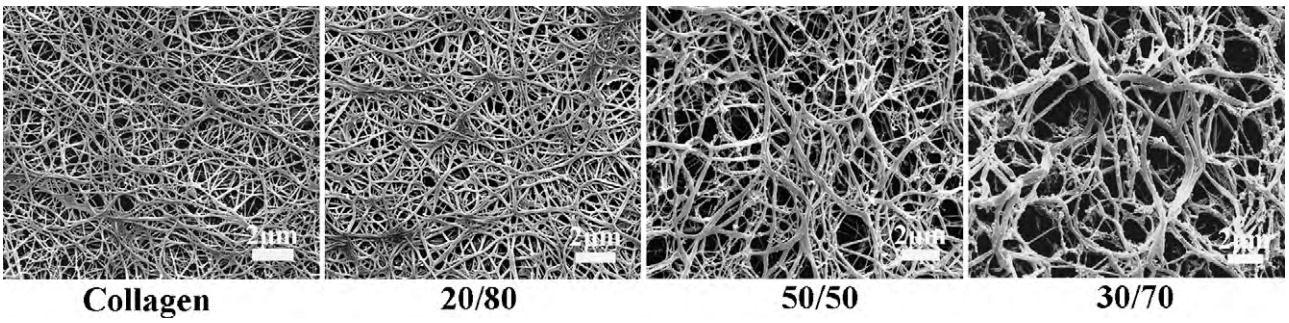

Fig. 1. SEM images of the critical-point-dried (a) collagen, (b) 20/80, (c) 50/50 and (d) 70/30 assemblies obtained at the initial collagen concentration of 7 mg/ml.

PBS. Fibril formation and aggregation kinetics at 25 °C were monitored on a Perkin Elmer Lambda 650 s UV/VIS spectrometer. The time course of the optical density was recorded at 330 nm.

2.6. Alginate quantitation

After gelling via collagen fibrillogenesis at 25 °C, the gels assembled with the 20/80 and 50/50 blends were directly soaked in deionized water, and the eluted alginate after soaking for 2 days and 7 days was measured to reveal the amount of alginate present in the collagen–alginate gels. For this quantitation, 2 ml of each blend was used to form an elastic gel by elevating the temperature from 4 °C to 25 °C, and the resultant gels were then soaked in 20 ml deionized water. The carbazole method was applied to measure the concentration of alginate eluted from the gels with some modifications (Bitter & Muir, 1962). Briefly, 0.5 ml of the soaking solution was extracted at the designated soaking intervals, and then the extracted solution was added, in ice bath, to the tubes containing sodium tetraborahydrate–sulfuric acid solution. The tube was sealed, vortex shaken for 1 min, and then heated in a vigorously boiling water bath for 10 min and finally cooled to room temperature. Carbazole was subsequently added, shaken again, and the tube was kept in the boiling water for another 15 min. The absorption of the analytical solutions was measured at 530 nm on a Perkin Elmer Lambda 650 s UV/VIS spectrometer. The eluted alginate amounts were calculated using a standard curve.

3. Results and discussion

Alginate, a water-soluble polymer, was introduced in this study to collagen monomeric solutions to form homogeneous blends with a wide range of the alginate/collagen ratios (20/80, 50/50 and 70/30 by weight). The self-assembly of collagen in the presence of a high amount of alginate was first investigated by the microscopic observations of critical-point-dried and air-dried assemblies on the glass slides. Comparative SEM images of critical-point-dried collagen and collagen–alginate assemblies are shown in Fig. 1. It is evident that, by using the initial concentration 7 mg/ml of collagen monomeric solutions, fibrillogenesis at 25 °C gave rise to a dense three-dimensional fibril network in all the samples.

In the case of collagen (Fig. 1a), fibrils were small, variable in diameter. The obtained network was interwoven mainly by straight and long small fibrils due to the accretion of collagen molecules. The lateral aggregation and fusion of fibrils became conspicuous in the 20/80 assemblies (Fig. 1b). With further increasing the alginate/collagen ratio, the collagen–alginate blend yielded a notably loose three-dimensional fibril network and the presence of fewer and thicker twisted fibrils was much prominent. In the 50/50 assemblies (Fig. 1c), very thick and twisted fibrils together with some small fibrils formed a three-dimensional network with a much higher open porosity compared with collagen assemblies (Fig. 1a). Fig. 1d represents a loose fibrillar network exhibiting the highest mesh size, interlaced by extremely thick and twisted fibrils, which was assembled with the 70/30 blend containing the highest amount of alginate.

Note that the concentration of collagen solutions has a major impact on their aggregation states. At physiological conditions, a low concentration corresponds to fewer nucleation sites, and thus gives rise to fewer but thicker fibrils by the consecutive aggregation of the remaining collagen molecules (Gobeaux et al., 2008; Raspanti et al., 2007; Wood, 1960a; Wood & Keech, 1960). As the initial concentration 7 mg/ml of a collagen solution was used to prepare the collagen–alginate blend with a varying ratio, the final concentration of collagen containing in the blend actually reduced

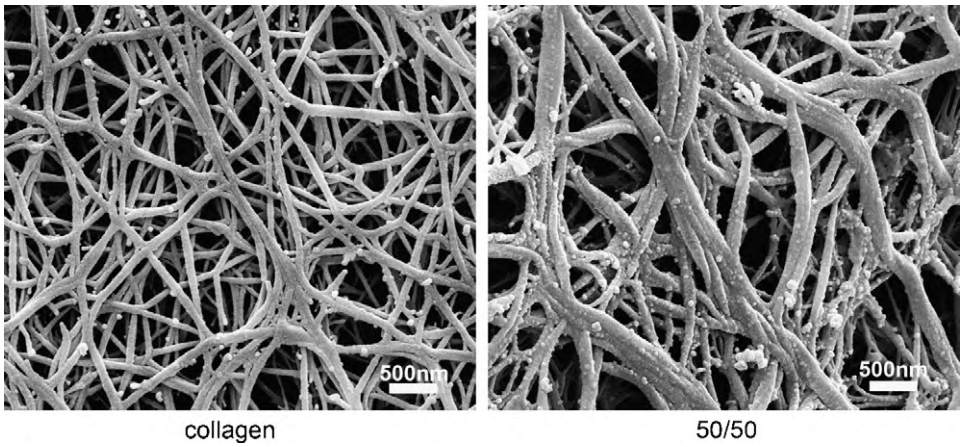

Fig. 2. Comparative SEM images of the critical-point-dried collagen and 50/50 assemblies obtained at the same final collagen concentration (4.3 mg/ml), confirming the predominant effect of alginate on the formation of thick fibrils and the loose three-dimensional network.

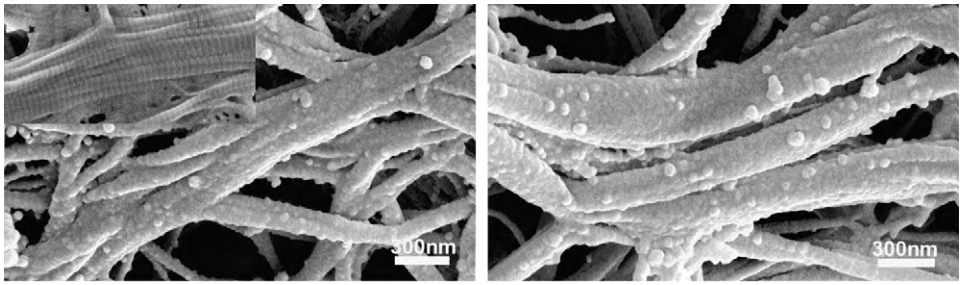

Fig. 3. Higher magnification SEM images of the critical-point-dried (a) 50/50 and (b) 70/30 assemblies obtained at the initial collagen concentration of 7 mg/ml, revealing the microstructural details of fibrils.

with a higher alginate/collagen ratio. To clarify which factor, either the reduced concentration of collagen or the presence of a higher amount of alginate, contributed to the special aggregation states of collagen–alginate assemblies (Fig. 1), a control experiment was conducted with the collagen solution at a concentration equivalent to the final collagen concentration (4.3 mg/ml) of the 50/50 blend. The comparative SEM images of their critical-point-dried assemblies after fibrillogenesis at 25 °C are shown in Fig. 2. It is evident that the abnormally aggregated morphology of 50/50 assemblies is absent in the control collagen assemblies, verifying the predominant effect of alginate on the formation of a loosely fibrillar network interlaced by thick and twisted fibrils.

Higher magnification SEM image of the 50/50 assemblies (Fig. 3a) revealed that the large but twisted fibrils were formed by the densely lateral aggregation of small fibrils and their merging. The inserted image clearly showed the presence of periodic cross-striated patterns along the longitudinal direction of small fibrils, and the densely lateral coalescence of these small striated fibrils into a thick aggregate, i.e. a bundle of small striated fibrils, was evident. The existence of extremely thick fibrils was also observed in the critical-point-dried assemblies of the 70/30 blend (Fig. 3b), which actually yielded a loose fibrillar network with the highest open porosity among all the samples according to Fig. 1. The fibril width of collagen and collagen–alginate assemblies is given in Fig. 4. A steady increment from 180 nm for collagen (0/100) to 610 nm for 70/30 assemblies was found with increasing alginate/collagen ratios. It should be pointed out that very small fibrils about 50 nm thick were discernible in all the critical-point-dried assemblies. To better reveal the alginate-dependent abnormal fibril coalescence among the assemblies, these very small fibrils were deliberately excluded.

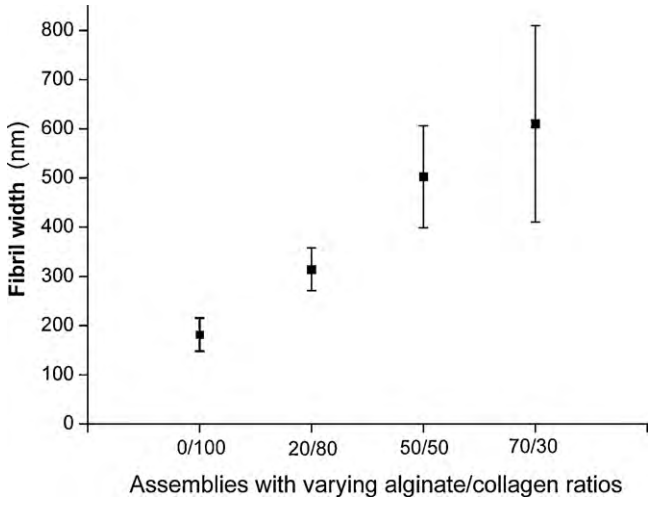

Fig. 4. Fibril width of the assemblies with varying alginate/collagen ratios.

The comparative aggregation states of collagen and a typical collagen–alginate blend were further investigated by AFM. Fig. 5 is the representative AFM images of the air-dried collagen and 50/50 assemblies reconstituted at 25 °C at the same final collagen concentration (1 mg/ml). Pure collagen yielded long and straight fibrils with distinct and regular cross-striated patterns, and partially lateral aggregation and fusion of fibrils were discernible but these cases were not dominant. This assembly feature was in agreement with the reported AFM results at the collagen concentration of 1 mg/ml (Raspanti et al., 2008). In contrast, fibril aggregations were prevailing in the 50/50 assemblies, and very huge and twisted fibrils formed as compared to the size of collagen fibrils. Topography cross-sectional analyses indicated that the nominal diameters ranged within 120–400 nm and from 300 nm to 1.2 μm, respectively for collagen assemblies and 50/50 assemblies. It is noteworthy that AFM measured values were incomparable with those of SEM, due to the difference in the air-drying and critical-point drying techniques.

AFM observations further revealed that the large fibrils of 50/50 assemblies were dense aggregates of smaller fibrils appeared in an irregularly bundled way. The irregular coalescence of small fibrils into large and twisted fibrils could also be judged by their respective cross-striated patterns. These small fibrils had the normal banding periodicity of collagen fibrils according to the topography longitudinal section analyses, confirming that the self-assembly nature of collagen in the blends was not altered, in agreement with the reported results (Keech, 1961; Kuo, Wang, Weng, Lu, & Chang, 2005; Tsai et al., 2006; Wood, 1960b). These comparative AFM results achieved at the equal collagen concentration 1 mg/ml were consistent with SEM observations in Figs. 1 and 2, validating the formation of dense aggregates in the presence of a higher amount of alginate. This abnormal coalescence of small fibrils to large dense aggregates suggests the alteration of the lateral rearrangement of the collagen molecules during the normal assembly (Cisneros et al., 2006). It is worthy to note that the lateral aggregation of the fibrils was considered as a possible result of the quite large forces developed in a stream of air at room temperature (Wood & Keech, 1960). In the present study, irregular bundles of small striated fibrils were observed in both critical-point-dried SEM and air-dried AFM samples although we could not definitely confirm whether or not they were present in the gel state assembled at varying alginate/collagen blends containing 1–7 mg/ml collagen. But, this abnormal coalescence of small fibrils was completely absent in the reported alginate-dependent fibril formation (Kuo et al., 2005; Tsai et al., 2006).

The corresponding kinetic assay of the reconstitution of both pure collagen solution and 50/50 blend recorded a notable difference (Fig. 6). Compared with the turbidity curve of pure collagen, the 50/50 blend yielded a remarkably prolonged lag phase (period)

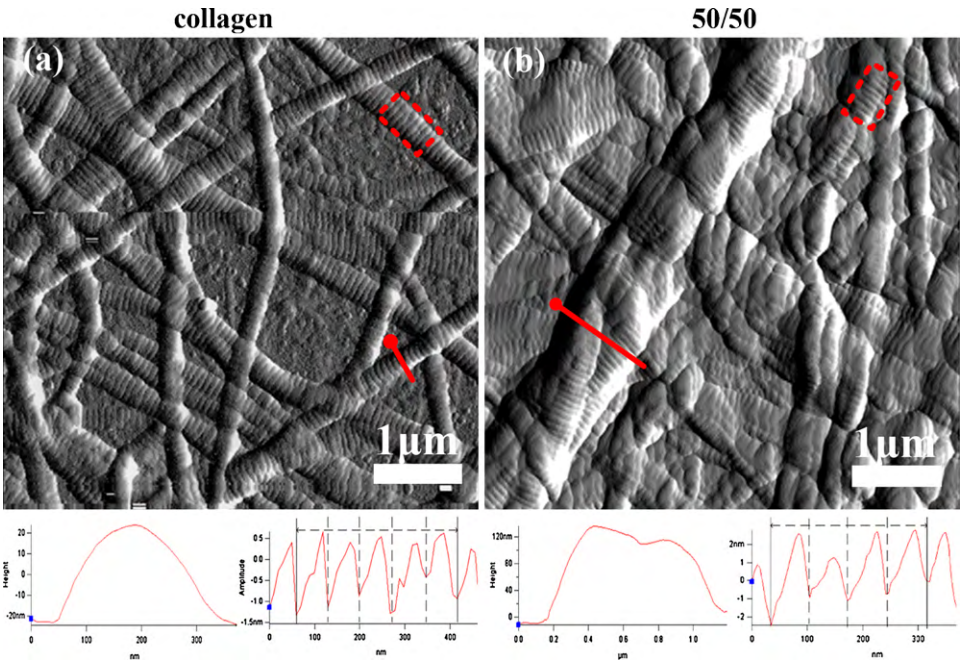

Fig. 5. AFM images and corresponding selective site longitudinal and cross-sections of air-dried collagen and 50/50 assemblies on glass slides. The 50/50 blend for the assemblies was diluted with PBS and the final concentration of collagen in both diluted blend and collagen control was 1 mg/ml.

with a comparable polymerization rate. The equilibrium optical density value of pure collagen was 1.42 whereas this value rose up to 1.72 for the 50/50 blend. Higher optical density values generally correspond with larger size and/or amount of fibrils (Brightman et al., 2000; Salchert et al., 2004; Stamov et al., 2008; Wood & Keech, 1960). As the final collagen concentration of pure collagen solution and 50/50 blends was constant, i.e. 1 mg/ml, these differences in the turbidimetric measurements further confirmed the influence of alginate on the collagen fibril formation and also suggested the probable capturing of alginate in the collagen fibrillar network.

The carbazole method was thus used to measure the possible capturing of alginate in collagen assemblies. After collagen–alginate gels were obtained, the gels were soaked in deionized water, and the amounts of alginate eluted from the 20/80 and 50/50 gels after soaking for 1 day or 7 days are given in Fig. 7. In general, a higher eluted amount was recorded in the gel prepared with a higher alginate/collagen ratio. The value after 1 day of soaking was 2.5% for the 20/80 gel and 4.8% for the 50/50 gel. However, after 7 days of soaking, the 20/80 gel only yielded a slight higher elution of alginate compared to the 50/50 gel. The net incremental amount compared with the eluted value after 1 day of soaking was very close for two gels, 2% for the 20/80 gel and 2.8% for the 50/50 gel. Considering that alginate is a water-soluble polymer, a higher amount of eluted alginate for 1 day was probably due to the elution of the possible residual and poorly bound alginate. Therefore, these quantitative data indicated that a large amount of alginate was contained in collagen fibrillar gels.

The aggregated morphologies of fibrils in the presence of glycosaminoglycans or polysaccharides was found to be case-dependent and dose-dependent, but extensive investigations associated with heparin, decorin, chondroitin sulphate, hyaluronan and alginate confirmed unanimously that the typical banding

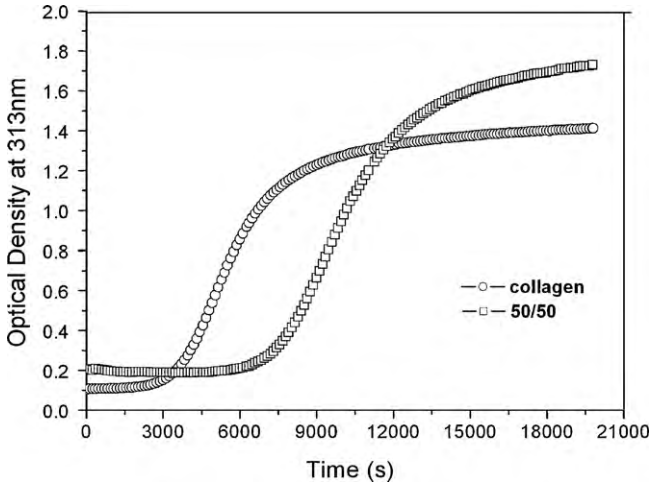

Fig. 6. Turbidity curves at 25 °C of collagen and 50/50 blends. The blends were diluted with PBS and the final concentration of collagen in 50/50 blend and collagen control was 1 mg/ml.

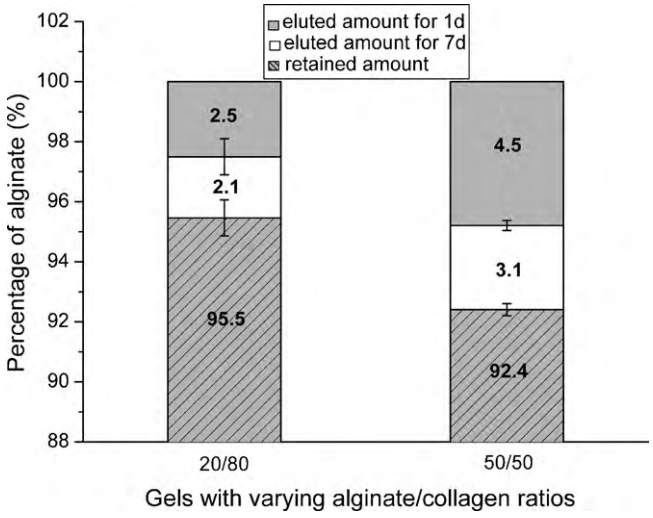

Fig. 7. Alginate-eluted amounts of 20/80 and 50/50 gels after soaked in PBS for 1 day and 7 days.

patterns of normal collagen fibrils (about 67 nm) was preserved (Brightman et al., 2000; Keech, 1961; Raspanti et al., 2007, 2008; Salchert et al., 2004; Tsai et al., 2006; Wood, 1960b). In terms of collagen assembly in the presence of alginate, the previously reported TEM results indicated that at the concentration ≤0.5 mg/ml of collagen type I and II, the size of assembled fibrils increased with increasing alginate concentration (Kuo et al., 2005; Tsai et al., 2006). Our coherent SEM and AFM results revealed the abnormal dense coalescence of smaller fibrils with the normal banding periodicity, based on the microscopic examinations of the three-dimensional fibrillar networks obtained either at the final concentration of 1 mg/ml or at the initial concentration of 7 mg/ml.

Collagen fibril formation is a spontaneous process primarily driven by the formation of hydrogen-bonded water clusters bridging recognition sites on opposing helices and also by hydrophobic interactions between opposing nonpolar amino acid side chains (Cisneros et al., 2006; Kuznetsova et al., 1998; Salchert et al., 2004; Wood & Keech, 1960). In a specific experiment, collagen fibril organization is governed by the assembly conditions, concentrations of triple helices, other non-collagenous components available in the initial solution, their interactions and thereby imposed limitations on the mobility of collagen molecules in the forming gel (Brightman et al., 2000; Cisneros et al., 2006; Gobeaux et al., 2008; Kuznetsova et al., 1998; Raspanti et al., 2007; Wood, 1960a,b; Wood & Keech, 1960). Collagen precipitation is known to follow a two-step mechanism of fibril formation whereas the precipitation curves show a lag period followed by a sigmoid growth curve. The first step is mainly related, occurring during the lag period, to the formation of asymmetric nuclei while the second consists of the growth of these nuclei into fibrils. It is found that the width of collagen fibrils is determined by the number and the shape of the nuclei and is thus decided during the lag period (Wood, 1960a), somewhat like crystal formation. As shown in Fig. 6, the prolonged lag period of the alginate/collagen blend, compared with that of pure collagen, right corresponded to the formation of very thick aggregates (Fig. 5), indicating that alginate suppressed the nuclei formation or exerted some influence over the initial consecutive accretion of collagen molecules. As alginate is a linear anionic polysaccharide containing plenty of carboxylic and hydroxyl groups, we postulate that the electrostatic complexation of alginate with collagen had a major impact on the formation of irregular and dense aggregates of small fibrils with normal banding periodicity.

Firstly, the alginate intervention directly influenced the spontaneous process of collagen normal assembly because the hydrophobic characteristics as well as the net charge of collagen molecules would be affected by this electrostatic complexation (Salchert et al., 2004). Secondly, collagen assembly in the presence of plentiful alginate could be considered as a kind of template polymerization involving a pick-up mechanism (Cristallini et al., 2001). Because of the electrostatic complexation between collagen and alginate, there was no preferential adsorption and collagen polymerization initiated in the bulk. When the consecutive accretion of collagen monomers or multimers had reached a critical length, the co-operative effects allowed for the adsorption of the growing microfibrils and alginate. Accordingly, in contrast to the normal linear and lateral addition of collagen monomers into microfibrils for long and large collagen fibrils, the adsorbed alginate suppressed the lateral fusion/addition of collagen molecules from the blend but facilitated the lateral aggregation of growing fibrils, finally leading to the dense and twisted large aggregates organized irregularly by small fibrils. The alginate-eluted experiments (Fig. 7) further revealed the capturing of alginate within the fibrillar networks, supporting that there existed electrostatic interactions between collagen and alginate. While this does not preclude the lateral packing effect of fibrillar collagen imposed by anionic alginate through osmotically applied pressure. Extrafibrillar proteoglycans were reported to osmotically regulate the molecular packing of collagen in cartilage, hence influencing the mechanical properties of the fibrils (Katz, Wachtel, & Maroudas, 1986). Obviously, the in-depth elucidation of the underlying mechanism for the present results is expected to further investigate, probably involving systematic turbidimetric, conductimetric and rheological analyses in combination with considering the different ionic strengths and charge effects.

This study is believed also to provide valuable results for developing ECM analogues. The variation in the width of biological fibrils and their orderly aggregated states determine collagen functional purposes by influencing stiffness and tensile strength of connective tissues (Canty & Kadler, 2005; Parry et al., 1978). To fabricate biomimetic collagen materials, cyclic mechanical loading of collagen gels was reported to promote fibril fusion for enhanced mechanical properties with larger fibrils (Cheema, Chuo, Sarathchandra, Nazhat, & Brown, 2007). The present study indicated that large bundles of small striated fibrils could be obtained simply by the introduction of alginate, and the similar organization of fibrils is found to exist in tissue for achieving elasticity through the enhancing interfibrillar non-covalent crosslinks between the collagen fibrils and the components of the matrix (Parry et al., 1978). Furthermore, the captured alginate in the fibrillar gels permits the subsequent facile use of chemical crosslinking with collagen to fabricate mechanically strong biomimetic fibrillar scaffolds for tissue engineering purposes.

4. Conclusion

Collagen reconstitution in the absence or presence of alginate was evaluated with an emphasis on at the high initial collagen concentration up to 7 mg/ml, which yield elastic gels strong enough to fabricate three-dimensional scaffolds for tissue engineering purposes. With increasing alginate/collagen ratios (20/80, 50/50 and 70/30 by weight), the collagen–alginate blends assembled into three-dimensional fibrillar networks with a higher open porosity, constituted predominantly by larger twisted fibrils which were dense and irregular aggregates of small fibrils with normal banding periodicity. The capturing of alginate in the fibrillar networks suggests the feasibility to develop mechanically strong biomimetic scaffolds with nativelike fibrils for tissue engineering purposes with the aid of a chemical crosslinking treatment.

Acknowledgments

This work is supported by the National Natural Science Foundation of China (No. 30670561 and 50830105), and the National Basic Research Program of China (No. 2005CB623903 and 2007CB936102).

References

Augst, A. D., Kong, H. J., & Mooney, D. J. (2006). Alginate hydrogels as biomaterials. *Macromolecular Bioscience*, *6*, 623–633.

Beekman, B., Verzijl, N., Ban, R. A., Von der Mark, K., & TeKoppele, J. M. (1997). Synthesis of collagen by bovine chondrocytes cultured in alginate: Posttranslational modifications and cell-matrix interaction. *Experimental Cell Research*, *237*, 135–141.

Bitter, T., & Muir, H. M. (1962). A modified uronic acid carbazole reaction. *Analytical Biochemistry*, *4*, 330–334.

Brightman, A. O., Rajwa, B. P., Sturgis, J. E., McCallister, M. E., Robinson, J. P., & Voytik-Harbin, S. L. (2000). Time-lapse confocal reflection microscopy of collagen fibrillogenesis and extracellular matrix assembly in vitro. *Biopolymers*, *54*, 222–234.

Canty, E. G., & Kadler, K. E. (2005). Procollagen trafficking, processing and fibrillogenesis. *Journal of Cell Science*, *118*, 1341–1353.

Cheema, U., Chuo, C. B., Sarathchandra, P., Nazhat, S. N., & Brown, R. A. (2007). Engineering functional collagen scaffolds: Cyclical loading increase materials strength and fibril aggregation. *Advanced Functional Materials*, *17*, 2426–2431.

Chen, G., Ushida, T., & Tateishi, T. (2002). Scaffold design for tissue engineering. *Macromolecular Bioscience*, *2*, 67–77.

Choi, Y. S., Hong, S. R., Lee, Y. M., & Song, K. W. (1999). Study on gelatin-containing artificial skin: I. Preparation and characteristics of novel gelatin-alginate sponge. *Biomaterials*, *20*, 409–417.

Cisneros, D. A., Hung, C., Franz, C. M., & Muller, D. J. (2006). Observing growth steps of collagen self-assembly by time-lapse high-resolution atomic force microscopy. *Journal of Structural Biology*, *154*, 232–245.

Cristallini, C., Barbani, N., Glusti, P., Lazzeri, L., Cascone, M. G., & Ciardelli, G. (2001). Polymerisation onto biological templates, a new way to obtain bioartificial polymeric materials. *Macromolecular Chemistry and Physics*, *202*, 2104–2113.

Dvir-Ginzberg, M., Gamlieli-Bonshtein, I., Agbaria, R., & Cohen, S. (2003). Liver tissue engineering within alginate scaffolds: Effects of cell-seeding density on hepatocyte viability, morphology, and function. *Tissue Engineering*, *9*, 757–766.

Gobeaux, F., Mosser, A., Anglo, A., Panine, P., Davidson, P., Giraud-Guille, M. M., et al. (2008). Fibrillogenesis in dense collagen solutions: A physicochemical study. *Journal of Molecular Biology*, *376*, 1509–1522.

Gregory, K. E., Marsden, M. E., Anderson-Mackenzie, J., Bard, J. B. L., Bruckner, P., Farjanel, J., et al. (1999). Abnormal collagen assembly, though normal phenotype, in alginate bead cultures of chick embryo chondrocytes. *Experimental Cell Research*, *246*, 98–107.

Jiang, F. Z., Hörber, H., Howard, J., & Müller, D. J. (2004). Assembly of collagen into microribbons: Effects of pH and electrolytes. *Journal of Structural Biology*, *148*, 268–278.

Katz, E. P., Wachtel, E. J., & Maroudas, A. (1986). Extrafibrillar proteoglycans osmotically regulate the molecular packing of collagen in cartilage. *Biochimica Biophysica Acta*, *882*, 136–139.

Keech, M. K. (1961). The formation of fibrils from collagen solutions. IV. Effect of mucopolysaccharides and nucleic acids: An electron microscope study. *Journal of Biophysical and Biochemical Cytology*, *9*, 193–209.

Kuo, S. M., Wang, Y. J., Weng, C. L., Lu, H. E., & Chang, S. J. (2005). Influence of alginate on type II collagen fibrillogenesis. *Journal of Materials Science: Materials in Medicine*, *16*, 525–531.

Kuznetsova, N., Chi, S. L., & Leikin, S. (1998). Sugars and polyols inhibit fibrillogenesis of type I collagen by disrupting hydrogen-bonded water bridges between the helices. *Biochemistry*, *37*, 11888–11895.

Lee, M., Lo, A. C., Cheung, P. T., Wong, D., & Chan, B. P. (2009). Drug carrier systems based on collagen–alginate composites structures for improving the performance of GDNF-secreting HEK293cells. *Biomaterials*, *30*, 1214–1221.

Li, Z. S., Ramay, H. R., Hauch, K. D., Xiao, D. M., & Zhang, M. Q. (2005). Chitosan-alginate hybrid scaffolds for bone tissue engineering. *Biomaterials*, *26*, 3919–3928.

Lin, Y. C., Tan, F. J., Marra, K. G., Jan, S. S., & Liu, D. C. (2009). Synthesis and characterization of collagen/hyaluronan/chitosan composite sponges for potential biomedical applications. *Acta Biomaterialia*, *5*, 2591–2600.

Lu, J., Lin, X. Y., Jiang, B., Li, X. D., Chen, J. Y., & Zhang, X. D. (2005). Preparation and characterization of collagen by hydrogel formation method. *Key Engineering Materials*, *288–289*, 377–380.

Ma, L., Gao, C. Y., Mao, Z. W., Zhou, J., Shen, J. C., & Hu, X. Q. (2003). Collagen/chitosan porous scaffolds with improved biostability for skin tissue engineering. *Biomaterials*, *24*, 4833–4841.

Nam, K., Kimura, T., & Kishida, A. (2008). Controlling coupling reaction of EDC and NHS for preparation of collagen gels using ethanol/water co-solvents. *Macromolecular Bioscience*, *8*, 32–37.

Parry, D. A. D., Barners, G. R. G., & Craig, A. S. (1978). A comparison of the size distribution of collagen fibrils in connective tissues as a function of age and a possible relation between fibril size distribution and mechanical properties. *Proceedings of the Royal Society of London, Series B: Biological Sciences*, *203*, 305–321.

Raspanti, M., Viola, M., Forlino, A., Tenni, R., Gruppi, C., & Tira, M. E. (2008). Glycosaminoglycans show a specific periodic interaction with type I collagen fibrils. *Journal of Structural Biology*, *164*, 134–139.

Raspanti, M., Viola, M., Sonaggere, M., Tira, M. E., & Tenni, R. (2007). Collagen fibril structure is affected by collagen concentration and decorin. *Biomacromolecules*, *8*, 2087–2091.

Salchert, K., Streller, U., Pompe, T., Herold, N., Grimmer, M., & Werner, C. (2004). In vitro reconstitution of fibrillar collagen type I assemblies at reactive polymer surfaces. *Biomacromolecules*, *5*, 1340–1350.

Stamov, D., Grimmer, M., Salchert, K., Pompe, T., & Werner, C. (2008). Heparin intercalation into reconstituted collagen I fibrils: Impact on growth kinetics and morphology. *Biomaterials*, *29*, 1–14.

Tsai, S. W., Chen, C. C., Chen, P. L., & Hsu, F. Y. (2009). Influence of topography of nanofibrils of three-dimensional collagen gel beads on the phenotype, proliferation, and maturation of osteoblasts. *Journal of Biomedical Material Research Part A*, *91*, 985–993.

Tsai, S. W., Liu, R. L., Hsu, F. Y., & Chen, C. C. (2006). A study of the influence of polysaccharides on collagen self-assembly: Nanostructure and kinetics. *Biopolymers*, *83*, 381–388.

Wang, X. L., Wang, X. M., Tan, Y. F., Zhang, B., Gu, Z. W., & Li, X. D. (2009). Synthesis and evaluation of collagen–chitosan–hydroxyapatite nanocomposites for bone grafting. *Journal of Biomedical Material Research Part A*, *89*, 1079–1087.

Wang, X. M., Wang, X. L., Ma, J. F., Jiang, J. M., Zheng, G. Q., Chen, Z. H., et al. (2009). Versatile nanostructured processing strategy for bone grafting nanocomposites based on collagen fibrillogenesis. *Advances in Applied Ceramics*, *108*, 384–388.

Wood, G. C. (1960a). The formation of fibrils from collagen solutions. 2. A mechanism of collagen fibril formation. *Biochemical Journal*, *75*, 598–605.

Wood, G. C. (1960b). The formation of fibrils from collagen solutions. 3. Effect of chondroitin sulphate and some other naturally occurring polyanions on the rate of formation. *Biochemical Journal*, *75*, 605–612.

Wood, G. C., & Keech, M. K. (1960). The formation of fibrils from collagen solutions. 1. The effect of experimental conditions: Kinetic and electron microscope studies. *Biochemical Journal*, *75*, 588–598.