JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE **16** (2005) 525–531

Influence of alginate on type II collagen fibrillogenesis

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Collagen II is the majority of extracellular matrix components in articular cartilage, which with the major functions of preventing expansion of the tissue and distributing the load of body weight. To obtain man-made ECM, the reconstitution of collagen could be conducted in the presence of negatively charged polysaccharide, such as alginate. Alginate is an anionic polysaccharide capable of eversible gelated in calcium ion solution to prepare different shapes of biomaterials. Its well-known biocompatibility makes it an ideal material in biomedical applications. Thus, the aim of this study was to evaluate the effects of alginate on the fibrillogenesis of type II collagen. The preliminary results revealed that inclusion of alginate into soluble type II collagen solution could inhibit the development of turbidity of collagen solution, and the apparent rate constants in lag and growth phases decreased during collagen formation period, both rate constants decreased to about one-third of the original constants, respectively. From TEM observations, the collagen fibrils were significantly thicker in 0.05% and 0.1% alginate as compared with pure collagen solution. Furthermore, the D-periods of collagen fibers kept unchanged significantly under all reconstituted conditions, which meant the packing of collagen monomer was probably not affected by adding these amounts of alginate.
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1. Introduction

The development of new biomaterial scaffolds for tissue engineering requires a better understanding of how cells interact with its surrounding extracellular matrix (ECM). Since bindings of ECM proteins and their respective membrane receptors occur in the scale of nanometer, thus, an ideal artificial bio-matrix should be designed and fabricated by a bottom-up process to meet these requirements. Besides, many researches studied the interactions of cells with ECM for years and concluded that these ideas were the foundation of development of scaffolds for tissue engineering applications.

Collagen is the major component of ECM, where provides a skeleton structure for cell adhesion, and is known that collagen could affect the growth and differentiation of a variety of cells [1–3]. Among the collagen family, type I collagen contains amino acid motifs RGD (Arg-Gly-Asp) which could mediate cell adhesion, and thus leading to changes in the gene expression profile of the interacting cells. Type II collagen is the majority of extracellular matrix in articular cartilage, the mainly function of type II collagen is preventing expansion of the tissue and distributing load on the articular cartilage. Generally speaking, collagen is composed of three polypeptides forming triple helical structure (monomer). Under normal physiological conditions, collagen could undergo spontaneous self-assembly reaction and then form fibrillar and fibrous structures. This self-assembly process also takes place *in vitro* where collagen monomers could be reconstituted into fibrils. Members of the collagen family, including type I, II, III and V, are able to from fibrils that contain characteristic D-periodic cross-striated pattern. The collagen reconstitution process involves packing of triple-helix monomer staggered to the preceding one, leading to a continuous fibrillar structure with alternating gap and overlapping regions [4]. Because of this unique self-assembly, collagen could exist and express in different morphologies and spatial arrangements. The effect of differentially organized collagen structures on cellular fates and behaviors is an exciting and interesting area that still remains to be explored.

Type II collagen is the major component of cartilage, and combines hyaluronan and proteoglycans to form the extracellular matrix of cartilage. Type II collagen is synthesized by chondrocytes and has the triple helix structure, which formed by three $\alpha 1$(II) chains. Type II collagen could undergo self-assembly reaction

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to form fibers and thereafter form a dense network with a specific ultra-structural organization, which possessing superior tensile properties in the cartilage. Collagen is the most commonly used biomaterial in scaffold construction for tissue engineering applications today. To obtain man-made ECM, the reconstitution process of collagen could be conducted in the presence of negatively charged polysaccharide, such as alginate. Most of the researchers had focused on the *in vitro* reconstitution experiments and morphological studies of type I collagen [5–10]. However, little is known on the kinetics and the fibril structure of type II collagen fibrillogenesis under the presence of polysaccharides.

Alginate is an anionic polysaccharide that capable of reversible gelated in the presence of calcium ion to prepare different shape of biomaterials. Thus, alginate has been widely used in the applications for microencapsulation of tissues and cells [11–15]. It is also utilized as a carrier material for repairing damaged articular cartilage. Research findings indicate that this alginate-calcium gelating system is a promising methodology for chondrocytes culture applications [16–18]. Whereas, the nano-structure of the type II collagen matrix produced in the presence of alginate has not been completely studied and described. Thus, it is of great important and interest to investigate the effects of alginate on the morphology of the reconstituted collagen.

The aim of this study is to evaluate and analyze the type II collagen fibrillogenesis by adding variant alginate concentrations. Here we evaluate the extents of type II collagen fibrillogenesis in the presence of alginate by examining the turbidity of mixture solution. Transmission electron microscopy (TEM) is also utilized to examine the morphological structures of reconstituted type II collagen fibers. Furthermore, the type II collagen/alginate matrix fabrication and the relating chondrocytes cell cultures are currently underway in our lab.

2. Materials and methods

2.1. Materials

Alginate was purchased from Sigma (St. Louis, MO), with the molecular weight of 150,000. Guanidinium chloride and pepsin were purchased form Sigma. Acetic acid was purchased from Merck-Schuchardt (Germany). All chemicals used in this study were of reagent grade. UV-VIS spectrophotometer (8453, Germany) was supplied by Agilent.

2.2. Purification of type II collagen

Collagen II was purified from fetal bovine articular cartilage by using a pepsin digestion and salt precipitation. Briefly, cartilages were cut into small pieces about 1 mm^3 and thereafter rinsed with distilled water. In order to remove the noncollagenous components of the cartilages, these small pieces were extracted with 15 vol. of 4.0 M guanidinium chloride (0.05 M Tris, pH 7.2) for 48 h. The pieces were then collected by centrifugation and homogenized in 0.5 N acetic acid solution that containing 0.5 mg/ml pepsin for 48 h (at 4 °C). The insoluble parts were removed by centrifugation at 16,700 g for 1 h. The supernatant was collected and then NaCl was slowly added to a final concentration of 0.86 M to precipitate type II collagen. The resulting type II collagen was dissolved in 0.05 M Tris-HCl (pH 7.2). Finally, this type II collagen solution was dialyzed against 0.05 M Tris-HCl 0.5 M NaCl, 0.05 M Tris-HCl 0.2 M NaCl and 0.02 M PBS, respectively and characterized by SDS-polyacrylamide gel electrophorese in 4–12% gradient gel (Gibco, NuPAGE^{TM} 4–12% Bis-Tris Gel).

2.3. Fabrication of collagen fibrils by reconstitution

Owning to the prepared collagen solution perhaps contained small amounts of aggregates; we centrifuged this collagen solution before the reconstitution experiments. A final 0.2–0.5 mg/ml collagen solution was prepared by adding a proper aliquot of iced PBS solution. Alginate concentration was set between 0.05 and 0.2% to assay the collagen fibrillogenesis changes. All the formations of collagen fibers were measured by the turbidity-time assay and observed by TEM. The relating parameters were derived by these obtained results.

2.4. Turbidity-time assay

Collagen fibrillogenesis analysis was carried out by the turbidity-time assay described by Silver *et al.* [19, 20]. The samples were transferred to a cuvette and immediately placed in a UV-VIS spectrophotometer that was pre-warmed to 37 °C. The event of collagen fibril formation was real time monitored under the absorbance wavelength set at 310 nm. The turbidity was defined as 2.303 times the absorbance values obtained at 310 nm. All experimental samples were measured in triplicate.

Under typical assembly conditions, the absorbance curve was a function of time and with a sigmoidal profile. In general, there were three characteristic phases; with initial lag phase without turbidity change, a growth phase which there was an increase in turbidity and, finally, a final plateau that no turbidity change, an example of that was shown in Fig. 1. The apparent rate constants were attained from the lag and growth phases of collagen fibrillogenesis by plotting c/t_{lag} and c/t_g

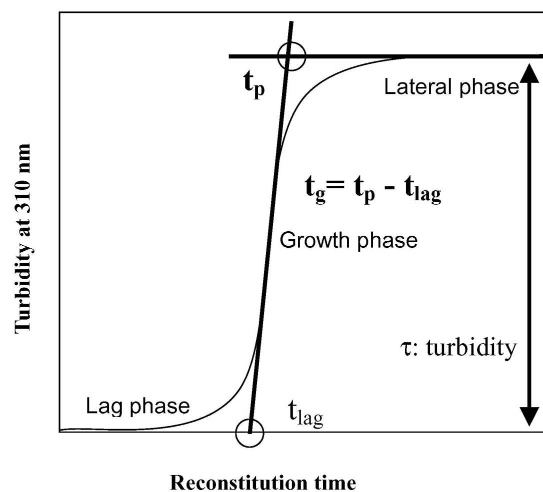


Figure 1 The characterization of the turbidity-time curve.

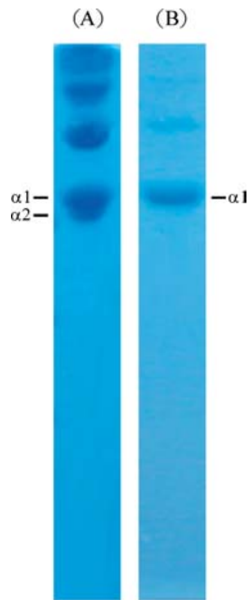


Figure 2 SDS-polyacrylamide gel electrophoresis of type I collagen from Vitrogen (A) and purified type II collagen (B).

versus collagen concentration. The slopes of these plots were proportional to the rate constant, and were defined as the apparent rate constant for the lag (k_{lag}) and growth (k_g) phases.

2.5. Analysis of collagen structure

The reconstituted type II collagen fibrils were observed by TEM and then analyzed to obtain the fibril diameter and D-periods of each fibril. In Brief, twenty fibrils were haphazardly picked to gauge the diameter for each experimental condition, and five D-periods of each fibril were calibrated to obtain the average D-pattern distance. The collagen fibril samples were took at every period of fibrillogenesis, and then placed on formvar coated copper grids. These grids were then negatively stained with 1% phosphotungstic acid. After that, these dry grids were examined in transmission electron microscope (Hitachi, Model HF-2000 Field Emission, Japan).

3. Results and discussion

3.1. Type II collagen isolation and identification

Type II collagen was isolated and purified from bovine articular cartilage. SDS-PAGE showed a typical profile for type II collagen, as can be seen, there was one characteristic band of type II collagen and thus revealed that this purification process was bale to isolate the collagen component and free from other contaminating proteins (Fig. 2).

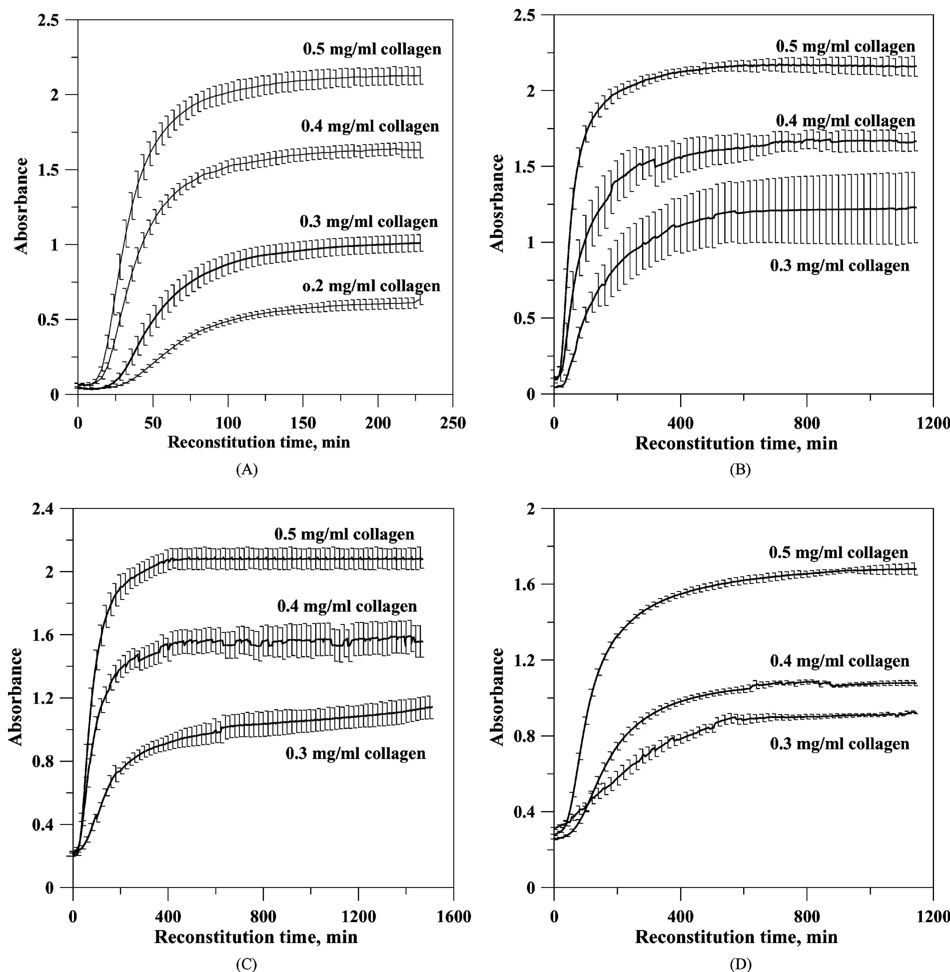
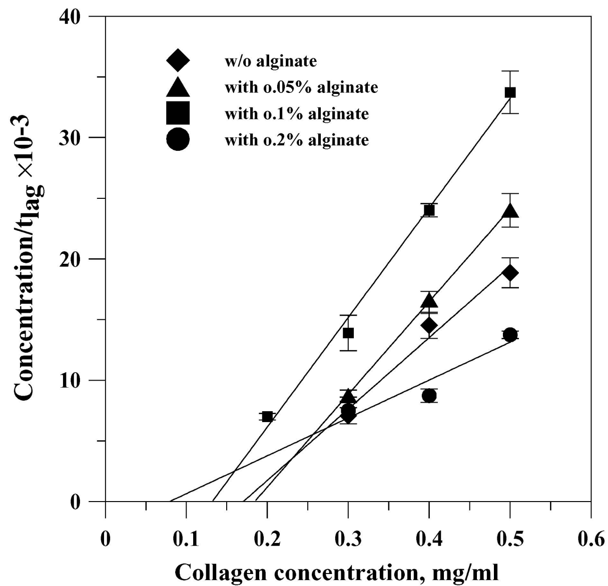
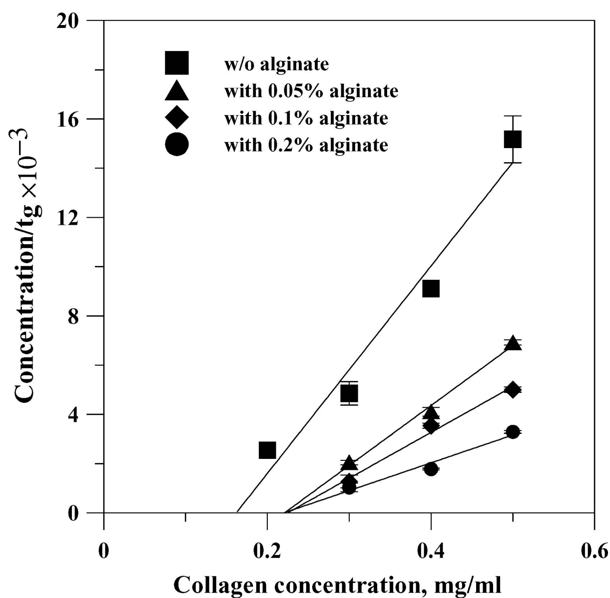


Figure 3 The turbidity-time curves for type II collagen assembly: (A) pure collagen solution, (B) collagen with 0.05% alginate, (C) collagen with 0.1% alginate and (D) collagen with 0.2% alginate. Value are means ± SD.



(A)



(B)

Figure 4 Determination of apparent rate constants: (A) K_{lag} for the lag phase and (B) K_g for growth phase.

3.2. The effect of alginate on the type II collagen fibrillogenesis

Fig. 3a illustrated four turbidity-time curves at various collagen concentrations, as shown, an obviously greater slope (the growth phase) and larger turbidity (plateau phase) was appeared at higher collagen concentration. These turbidity-time curves were similar to those reported in other investigations. Fig. 3b–d showed the similar profile of curves after addition of various concentrations of alginate, whereas, these curves had higher variation at the lower concentration of alginate that indicated the random reconstitution condition of collagen. In addition, the final turbidity of the plateau phase was decreased on the increasing concentration of alginate, and decreased significantly at the concentration of 0.2% as compared. Apparent rate constants were established and calculated from the lag and growth phase of turbidity-time curve (Fig. 4) and the relating

TABLE I The apparent rate constants of type II collagen fibrillogenesis[a]

	Lag phase[b]	Growth phase[b]
Alginate (%) concentration	K_{lag}	K_g
0	90.28	42.14
0.05	76.4	24.37
0.1	58.85	18.63
0.2	26.48	11.31

[a]The apparent rates constants of the lag phase and growth phase were obtained from Fig. 4.
[b]Values were min-1$\times 10^{-3}$.

rate constants were shown in Table I. As shown, the K_{lag} rate constant decreased from 90.28 to 26.48 $\times$ 10^{-3} at the increasing concentration of alginate, meanwhile, the k_g rate constant also decreased from 42.14 to 11.31 $\times$ 10^{-3}. The resulting decreasing of rate constants indicated that the retarding effect of alginate on collagen fibrillogenesis. In other words, the collagen fibrillogenesis was inhibited in the presence of alginate.

The nano-structure and the relating information of collagen fibers was examined and characterized by TEM (Fig. 5). From TEM observations, all collagen fibers showed the D-periods pattern in the absence and presence of various concentrations of alginate. The effect of alginate on the diameter of reconstituted collagen fibers was showed in Fig. 6. We found that the collagen fibers were thicker at the lower alginate concentration. And the diameter of collagen was increased at the increasing incubation time and kept constantly after 6 h. Furthermore, the D-periods of collagen fibers unchanged significantly under all experimental conditions, which meant the packing of collagen monomer could was not affected by alginate during the reconstitution process (Fig. 7). From the TEM results, we could conclude that the diameter of reconstituted collagen fibers was dependent and influenced on the presence and concentrations of alginate.

Collagen and alginate do not exist together as mixtures in nature, but the specific properties of each may be used to make the new bio-matrix that provide novel structural and biological properties. Wang *et al.* had reported that type I collagen–alginate microspheres were prepared by alginate-calcium gelating system [21]. These microspheres have been used as scaffolds for *in vitro* culture of osteoblast cells. The results demonstrated that type I collagen was capable of reconstitution in the presence of alginate and the microspheres provided a suitable growth environment for osteoblast cells. On the contrary, type II collagen occasionally failed to reconstitute to form gel under normal thermal condition and this restrict their applications in biomedical fields. Many studies had utilized the fiber-suspension solution or the freeze-dried matrix of type II collagen to investigate the applicability of the culture of the chondrocytes [22–25]. Alternatively, we used the alginate-calcium gelating system to prepare the type II collagenous microspheres that could encapsulate the chondrocytes in our lab. Therefore, it was very important to point out whether the type II collagen could be

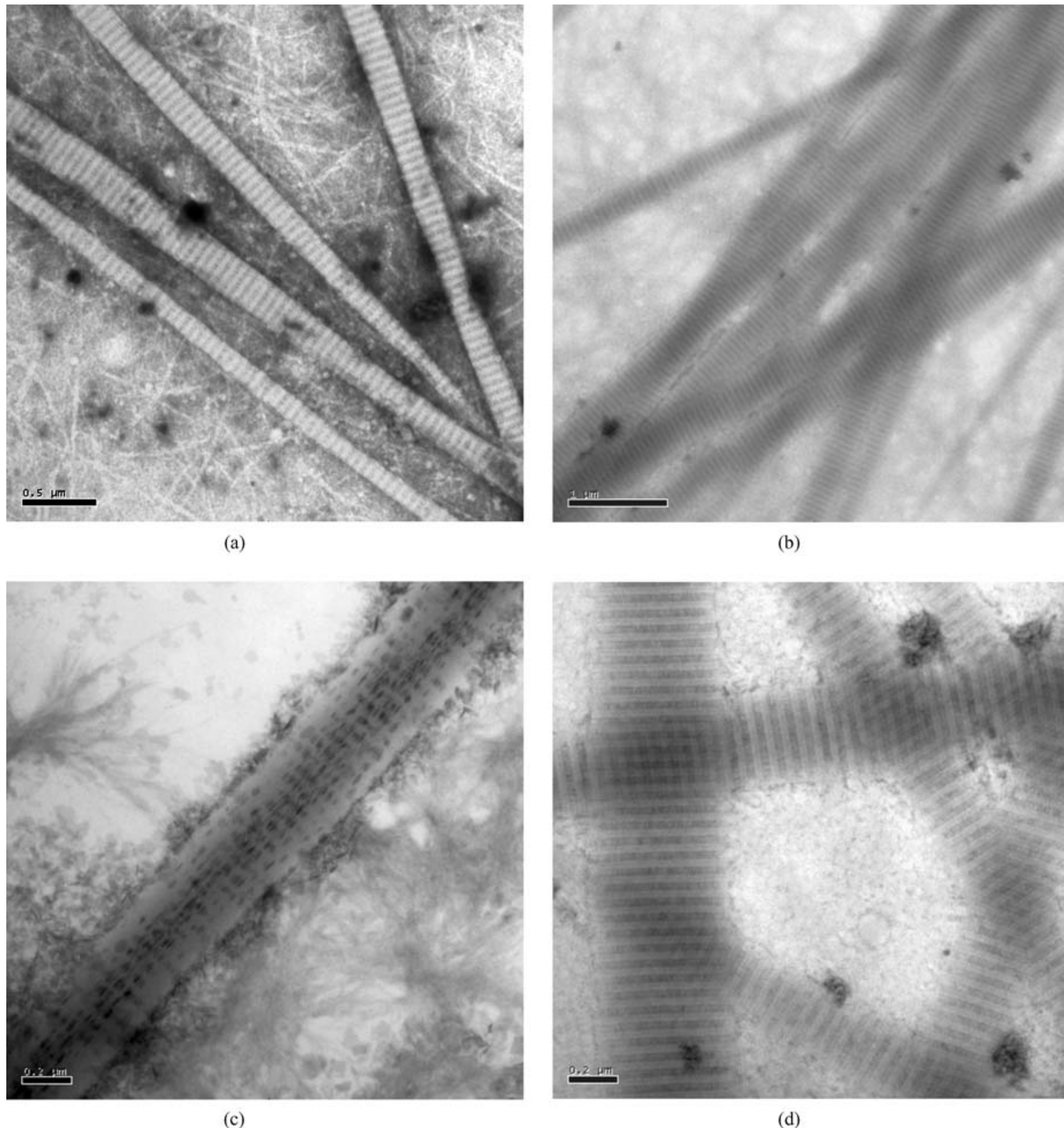


Figure 5 Electron micrographs of type II collagen fibers: (a), pure collagen fibers formed at 3 hour of incubation; (b), (c), and (d) fibers formed in the 0.1% alginate at 3, 6, 12 h, respectively.

capable of reconstitution in the presence of alginate or not. We believed that a detailed understanding of type II collagen fiber formation in the presence of alginate would provide important guidelines and information for designing a suitable scaffold for chondrocytes culturing.

Generally, the process of collagen reconstitution could be divided into two major steps: nucleation and propagation. The formation of nucleus (aggregates of 2 to 15 monomers) occurred at an early stage, which was often appeared at the lag phase. This nucleation was generally the rate-limiting step of collagen reconstitution. The results obtained in this study showed that the lag time of type II collagen fiber formation was elongated after adding alginate. It seemed that the nucleation of collagen fibrillogenesis was obstructed by alginate. We presumed that the elongated lag time could be contributed by the interference of alginate in the nucleation of the collagen reconstitution process, and then the formation of the nucleus slow down. Therefore, apparent rate constants at lag phase had dropped drastically (Table I). In the propagation process of fibrillogenesis, the growth rate of collagen fiber formation also decreased on the existence of alginate. The growth time was elongated and the apparent rate constants plunged in the presence of alginate. Due to alginate was an anionic polymer and perhaps could cohere on the collagen surface. This coherence of alginate reduced the hydrophobic area of collagen to interact with other molecules and yielded inefficiently collagen assembly reaction. From the TEM observations, we discovered the collagen monomers did not bind onto the fibrils easily in the presence of alginate (before 3 h incubation), and then the resulting collagen fibers were thin as compared with that without adding alginate. However, the diameter of collagen fibers was wider in the lower concentrations of alginate (0.05 and 0.1%) after 3-h reconstitution time. On the contrary, collagen fibers

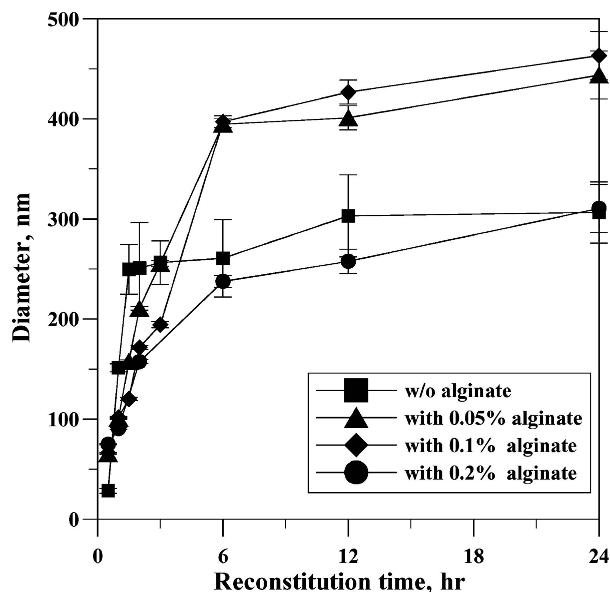


Figure 6 Diameters of type II collagen II as a function of reconstitution time in the presence of alginate. (value are means ± SD).

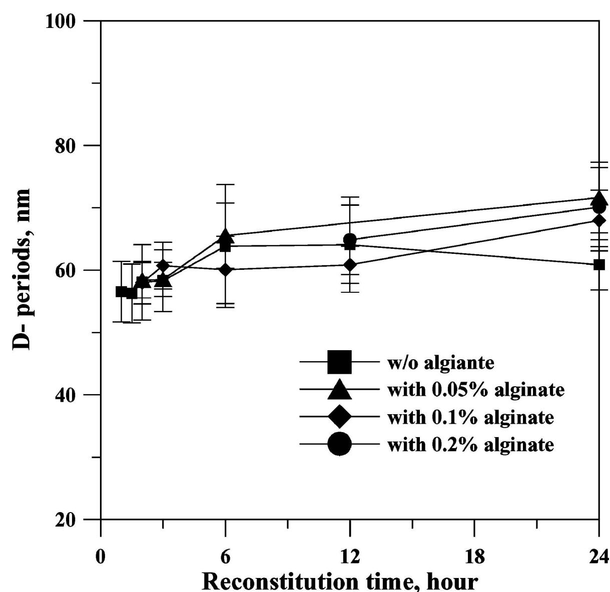


Figure 7 D-periods of type II collagen II as a function of reconstitution time in the presence of alginate. (value are means ± SD).

were smaller under the higher concentration of alginate (0.2%). The viscosity of mixture solution increased after adding high amount of alginate, this could restrict the movability of collagen monomers in the solution. Therefore, collagen fiber formation would be retarded in high concentration of alginate.

4. Conclusion

The present data showed that type II collagen monomers could reconstitute into fibrils in the presence of alginate. Incidentally, the fibrillogenesis rate and the diameters of type II collagen could be easily varied by adjusting the concentration of alginate. The results of this study indicated that the alginate could affect type II collagen fibrillogenesis and fibers sizes in vitro environment. These obtained results and findings would provide us an elementary criterion for fabricating various type II collagen matrixes as a carrier for chondrocytes or in other biomedical applications.

Acknowledgment

This work was supported by a grant from National Science Council, ROC. (NSC-92-2120-M-010-001).

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Received 15 August
and accepted 15 October 2004