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Influences of hyaluronan on type II collagen fibrillogenesis in vitro

Shyh Ming Kuo · Yng Jiin Wang · Gregory Cheng-Chie Niu · Huai En Lu · Shwu Jen Chang

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Abstract The effect to the kinetics of type II collagen fibrillogenesis with the addition of hyaluronan (HA), (M_w of 1.8×10^6 Da), at various concentrations of HA (0.01, 0.05 and 0.1 wt.%) for a series of fibril formation systems was examined in this study. Evidences deduced from the turbidity–time curves revealed that the inclusion of HA had minor or no impact to the fibrillogenesis of type II collagen (collagen conc. at 0.2 mg/mL). The apparent rate constants, k_{lag} (lag phase) increased slightly but k_g (growth phase) decreased not very significantly with addition of HA, as compared to the case of pure collagen. This leads us to believe tentatively that, with the addition of HA to collagen solutions, the nucleation process of the fibril formation might have been sped up slightly whereas the growth process slowed up slightly. However, data from TEM observations on the resulting fibrils indicated that the presence of HA did not significantly affect the diameters and the characteristic D-banding periods of the collagen fiber formed. And, from the statistical analyses, we found only insignificant difference ($P > 0.05$) between the specimens from the various experimental groups. It seems to indicate that the ultimate packing of collagen monomers was probably not interfered or affected significantly by the presence of HA in vitro.

S. M. Kuo · G. C.-C. Niu · H. E. Lu · S. J. Chang (✉)
Department of Biomedical Engineering, I-SHOU University, Kaohsiung County, Taiwan
e-mail: sjchang@siu.edu.tw

Y. J. Wang
Institute of Biomedical Engineering, Yang Ming University, Taipei, Taiwan

Introduction

Extracellular matrix (ECM), the actual cellular glue synthesized by cells, consists of macromolecules such as collagenous molecules, glycoproteins, proteoglycans and glycoaminoglycans, etc. Conversely, ECM holds the cells together by providing physical support for cellular anchorage, where cells can adhere and signal to each other to determine the cell orientation and scaffolding of tissue renewal. It has been known that matrix components markedly influence the migration, proliferation and differentiation of cells through receptors from ECM molecules on cell surfaces. As such, the development of new scaffolding matrix requires a better understanding of how the component interacts with each other and their interactions with its surrounding cells.

Both collagen and hyaluronan are major components of the native ECM and tissues. Collagen as the major component of ECM, is responsible for providing the associated tissues with tensile strength also serves as a structural scaffold for cell adhesion and growth [1–3]. Under normal physiological conditions, collagen can undergo spontaneous self-assembly reaction and then form fibrous structures. This self-assembly process, as termed fibrillogenesis is an entropy driven process and is influenced by the hydrogen bonding and effective charges on the collagen molecules. It may take place in vivo and in vitro where collagen monomers could be self-organized into fibrils. Following a still unclear mechanism of nucleation process, the triple-helix monomers tend to pack and grow further by staggered and aggregated on to growing fibril chains assemblies. The resulting fibrils have a distinct periodicity known as D-banding [4]. Recent studies have showed that collagen fibril morphology is critically important in determining the tensile strength of the associated tissues and, thus, provides

a scaffold for the cell adhesion [5, 6]. Some experimental data showed that the collagen morphological structures could affect the adhering, growth and differentiation of a variety of cells in cell culture applications [7, 8].

Type II collagen, as the major structural component of the ECM of cartilage and vitreous humor, has provided critical domains as sites for the molecular interactions involving in fibril formation, entanglement and binding with other components such as hyaluronan and some proteoglycans to form articular cartilage. Type II collagen consists of a triple helix structure, which formed by three α1(II) chains. These macromolecules undergo self-assembly reaction first to form fibrils. The fibrils aggregated to form dense fibers and further stagger into networks with specific ultra-structural organization. The resulting collagen structures possess superior tensile properties to prevent expansion of the tissue and distributing load on the articular cartilage.

Hyaluronan is a linear polysaccharide made of many repeats of disaccharide units of D-glucuronic acid and *N*-acetyl-D-glucosamine. It binds to a large amount of water to form a viscous hydrated gel that gives connective tissue an ability to spread the pressure around and to resist compression loads. Furthermore, it contributes to the maintaining of the architecture and visco-elastic properties of tissue and the modulating of cell functions such as adhesion, migration and proliferation via interaction with specific cell surface receptors (such as CD44). Besides, purified high molecular weight hyaluronan is mostly used as a biomedical material. Many applications that take advantage of the exceptionally high viscosity characteristics to facilitate a thickened gel-like barrier were first found almost indispensable for surgery procedures in ophthalmology, such as intra-ocular lenses implantation. Other uses of hyaluronan were reported for the treatment of degenerative joint dysfunction in horses and gradually expanded to the experimental treatment to humans [9–12].

Collagen is one of the most commonly used biomaterials in scaffold construction for tissue engineering applications today. The effect of differentially organized collagen structures on cellular fates and behaviors is an exciting and interesting area that still remains to be explored [13–15]. Many of the researchers have focused on the in vitro reconstitution experiments and morphological studies on type I collagen [16–18]. However, to obtain man-made ECM, the materials used must simultaneously promote a series of desirable cellular functions for a specific application i.e. adhesion, proliferation, differentiation and thereafter the tissue development. And for that sake, we evaluate the kinetics and survey, if any, of type II collagen fibrillogenesis in the presence of hyaluronan by examining the turbidity of mixture solutions. Also, transmission electron microscopy (TEM) is utilized to examine the morphological structures of reconstituted type II collagen fibers. Along the way, the type II collagen–hyaluronan matrix formation that associated with chondrocytes cell cultures is currently being studied in our lab to expand the scope of applications of collagen type II in the use of ECM.

Materials and methods

Materials

HA with the molecular weight of 1.8×10^6 Da (from *Streptococcus zooepidemicus*) was purchased from Fluka (Switzerland). Guanidinium chloride and pepsin was purchased from Sigma (St. Louis, MO). Acetic acid was purchased from Merck-Schuchardt (Germany). All chemicals used in this study were of reagent grade. UV-VIS spectrophotometer model was supplied by Agilent 8453 (Germany).

Extraction and purification of type II collagen

Collagen II was extracted and purified from bovine articular cartilage by using pepsin digestion and differential salt precipitation as described previously [19]. The purity of obtained collagen was characterized by SDS-polyacrylamide gel electrophorese in 4–12% gradient gel (Gibco, NuPAGE^{TM} 4–12% Bis-Tris Gel). Lyophilized products were stored at –20 °C.

Fibril formation

Before the reconstitution experiments, the collagen solution was centrifuged at 32,000 rpm (himac CP75, Hitachi) for 3 h to remove a small amount of aggregates of collagen. A final concentration of collagen solution from 0.2 to 0.5 mg/mL was adjusted by adding a proper aliquot of iced PBS solution. The final concentration of hyaluronic acid was each set at 0.01%, 0.05% or 0.1% in the ice-cooled type II collagen solution. For reconstitution of collagen monomers to the fibrils, the collagen mixture was at 37 °C over a time period of 48 h.

Turbidity–time assay

The formation of collagen fibers and the relating kinetic behavior of collagen fibrillogenesis were conducted by the turbidity–time assay [20, 21]. Briefly, the prepared mixtures were transferred to quartz cuvettes and covered. Fibrillogenesis was initiated by placing cuvettes into the temperature-controlled cell holder of the Agilent spectrophotometer that was pre-warmed at 37 °C. Fibrillogenesis, indicated by the development of turbidity in the mixtures

solution was real-time monitored under the absorbance wavelength set at 310 nm. The turbidity was calculated by 2.303 times of the absorbance values that obtained at 310 nm. All experiments were measured in triplicate.

Electron microscopy to confirm fibrillogenesis

In order to obtain electron micrographs of the formed collagen fibrils in the presence of hyaluronic acid for assaying, samples of the reconstituted type II collagen fibrils were taken at pre-determined periods of time and 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 (Philips, Model CM 200, Holland). Twenty collagen 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.

Statistical analysis

Collagen fibrils diameters were presented by means ± standard deviation. Each result was statistically analyzed by the student's *t*-test. The values of $P < 0.05$ were considered to be statistically significant.

Results and discussion

Both type II collagen and HA are major components of the ECM of articular cartilage. To obtain a man-made ECM, it is very important to understand the interactions between these ingredients and their influences on the fibrillogenesis of type II collagen. To simulate the fibrillogenesis of type II collagen in the cartilage tissue, we utilized the HA with MW of 1.8×10^6 Da being similar to HA existed in cartilage tissue to study the in vitro fibrillogenesis of type II collagen [22].

Under a given assembly condition, a turbidity–time curve showed three characteristic phases: an initial lag phase where there was no detectable change in turbidity, a growth phase during which turbidity changed rapidly and a plateau region where turbidity again remained constant. Figure 1 showed the turbidity–time curves at four experimental concentrations of type II collagen. All curves had the similar profile, and a greater slope (relating to the growth phase) and larger turbidity (relating to plateau phase) were appeared at higher concentration of collagen, which meant an increasing in the rate of fibril formation at a higher initial collagen concentration. Figure 2 showed the turbidity–time curves after addition of various amounts of HA (type II collagen concentration was kept constant at 0.2 mg/mL). It appeared that all curves exhibited a similar

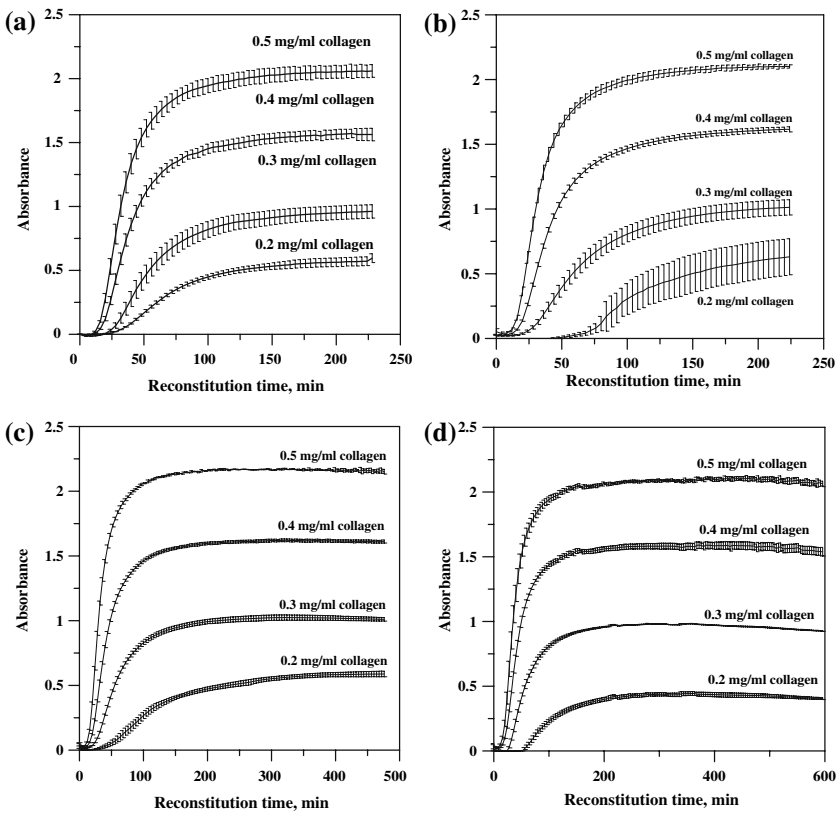

Fig. 1 Turbidity–time curve for collagen II (**a**) with (**b**) 0.01%, (**c**) 0.05%, (**d**) 0.1% hyaluronan at 37 °C. Values are means ± SD

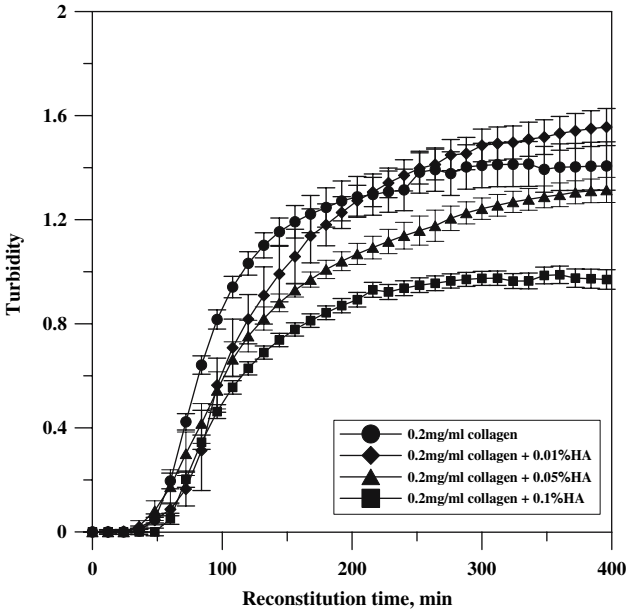

Fig. 2 The turbidity–time curves for type II collagen assembly for different amounts of HA. Values are means ± SD

profile, and after addition of HA, the growth phase decreased a little bit as compared with the pure collagen. Besides, the final turbidity of the plateau phase was only slightly changed with the amounts of HA added.

Table 1 listed the lag time, the growth time and final turbidity for these turbidity–time curves. The corresponding apparent rate constants of collagen fibrillogenesis was obtained from the lag and growth phases by plotting c/t_{lag} and c/t_g against to collagen concentration. The slopes of these plots were defined as the apparent rate constant, k_{lag} (lag phase) and k_g (growth phase). In summary, the apparent rate constants in this study were shown in Fig. 3 and Table 2. As shown, the k_{lag} and k_g did not change significantly after addition of various amounts of HA. The rate constant, k_{lag}, increased from 90.29×10^{-3} min^{-1}, for pure type II collagen, to about 104.20×10^{-3} min^{-1} with 0.01% HA added. The increasing of lag phase rate constant seemed to indicate that the initial linear and lateral aggregation of collagen monomers into oligomers and then into sub-fibrillar components during collagen fibrillogenesis were being accelerated by the addition of HA. However, the growth phase rate constants, k_g, decreased from 42.14×10^{-3} min^{-1} for collagen, to about 33.53×10^{-3} min^{-1} with 0.01% HA added. In other words, k_g changed in opposite direction to k_{lag}. It appeared that the collagen II fibrils formation might have been interfered in the periods of lateral growth of fibrils, and later on, of fibril bundles due to much greater domain size expansion in the presence of HA.

The nano-structures of reconstituted type II collagen fibers in the existence of HA were examined and characterized by TEM (Fig. 4). From TEM observations, collagen monomers could reconstitute into collagen fibers after addition of various amounts of HA and showed the characteristic D-periodic bandings of collagen (about 65 nm). The effects of HA on the diameters of reconstituted collagen fibers were showed in Fig. 5. As can be seen, the diameter of collagen increased significantly at the initial incubating time and reached a constant diameter after 6 h of incubation. The collagen fibers were thinner in diameter upon addition of HA before 24 h incubation period (as

Table 1 The lag time, growth time and turbidity from the turbidity–time curves

	Type II collagen			
	COL: 0.2 mg/mL	COL: 0.3 mg/mL	COL: 0.4 mg/mL	COL: 0.5 mg/mL
t_{lag} (min)	28.65 ± 5.29	22.07 ± 3.97	16.68 ± 0.65	14.91 ± 1.37
t_g (min)	78.58 ± 1.66	63.08 ± 10.96	44.01 ± 2.42	33.23 ± 0.73
Turbidity (H)	1.42 ± 0.09	2.28 ± 0.16	3.62 ± 0.11	4.71 ± 0.21
Type II collagen with 0.1% HA				
t_{lag} (min)	38.47 ± 1.27	23.47 ± 1.32	18.83 ± 0.84	16.11 ± 1.04
t_g (min)	93.15 ± 2.57	55.31 ± 6.51	46.53 ± 3.03	40.86 ± 1.41
Turbidity (H)	1.23 ± 0.03	24.18 ± 0.02	3.61 ± 0.05	4.78 ± 0.04
Type II collagen with 0.05% HA				
t_{lag} (min)	41.56 ± 6.32	25.73 ± 2.30	17.54 ± 0.07	14.43 ± 0.13
t_g (min)	124.01 ± 2.11	55.21 ± 5.30	47.21 ± 0.03	39.30 ± 0.13
Turbidity (H)	1.36 ± 0.07	2.34 ± 0.08	3.69 ± 0.07	4.94 ± 0.02
Type II collagen with 0.01% HA				
t_{lag} (min)	57.21 ± 12.06	20.76 ± 1.62	15.47 ± 1.71	13.68 ± 1.51
t_g (min)	142.33 ± 3.06	80.95 ± 9.50	53.72 ± 4.76	39.43 ± 5.65
Turbidity (H)	1.53 ± 0.11	2.4 ± 0.17	3.73 ± 0.02	4.75 ± 0.05

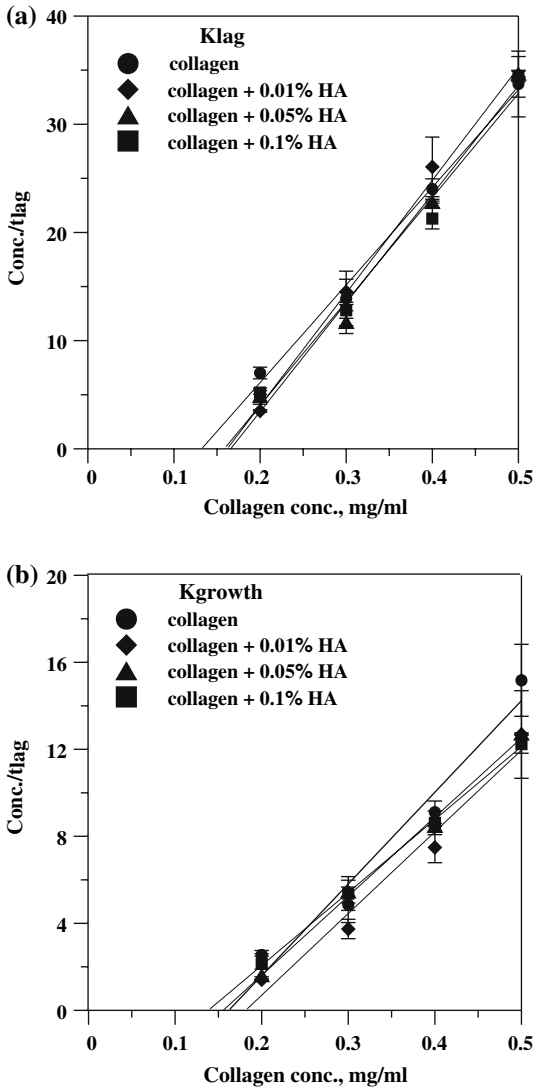

Fig. 3 Determination of apparent rate constants of type II collagen fibrillogenesis: (**a**) k_{lag} for the lag phase and (**b**) k_g for growth phase

Table 2 The apparent rate constants of type II collagen fibrillogenesis

HA concentration (%)	Lag phase		Growth phase	
	k^a_{lag}	R^2	k^a_g	R^2
0	90.28	0.99355	42.14	0.96179
0.01	104.20	0.99539	37.56	0.97181
0.05	100.49	0.98651	36.35	0.99620
0.1	96.16	0.98207	33.53	0.99920

[a] Values were $min^{-1} \times 10^{-3}$

compared with the pure collagen) and then increased to the same range of diameter after 48 h incubation. On the contrary, the characteristic D-periodic bands were not changed significantly under all experimental conditions, the D-periods kept at the same range at about 60 nm throughout the entire process (Fig. 6). These findings revealed that the spontaneously packing of collagen monomers was not affected by HA after a longer incubation period (48 h), although the initial lag phase and growth phase of collagen fibrillogenesis would be slightly influenced with the addition of HA. The statistical analyses revealed that after 48 h of incubation there were insignificant differences on the sizes of fibril diameter as well as D-bands between the ones from pure collagen and those with addition of HA.

Perhaps, it is worthy of mentioning that the majority of the kinetics studies about assembly of collagen molecules have concentrated on pure collagen along [23–27]. Actually, in an assembly process either in vivo or in vitro, there are many other molecules, such as hyaluronic acid and chondroitin sulfate in co-existence of that might all contribute to the ultimate kinetics in different ways. In fact, it is not surprising to see that kinetics studies of type I collagen assembly in co-existing with those molecules have become subjects of interest lately and some initial results seem to be quite encouraging [28–30]. We hope that by studying the roles played by those molecules in the kinetics of type II collagen assembly in vitro would be eventually helpful in the formulation of some proper ECM material.

Generally speaking, the ability of collagen molecules to spontaneously self-assemble into fibrils permits the investigation of collagen fibrillogenesis in an in vitro system. Turbidity–time curves typically reflect the behaviors of collagen fibrillogenesis in vitro. From these curves, based on various mixing ratios, a phase lags stage that correlated to the linear and lateral aggregation of collagen monomers into sub-fibrillar components (nucleation stage) and, subsequently, a growth stage that correlated to the lateral growth of fibrils and fibril bundles (propagation stage) could be both obtained. The results showed that the lag time of type II collagen fiber formation was not markedly influenced with the addition of various amounts of HA. The apparent rate constants at lag phase increased slightly about 10% (cf. Table 2). In the propagation stage of fibrillogenesis, the growth rate of collagen fiber formation decreased in the presence of HA (with exception of addition of 0.05%, w/w, HA, where a small increment of growth rate was observed). The growth time was prolonged and the apparent rate constants plunged by 10% in the presence of HA.

Since HA is an anionic polymer, it is quite probable that the polymer chains could aggregate or adhere onto the positively charged regions of a collagen surface and thus interfered the chance of collagen monomers from interacting to each other and thus retarded the collagen fibrillogenesis reaction. From the consideration of domain sizes, the HA (M_w of 1.8×10^6 Da) used in this study being greater than that of collagen monomer (M_w of

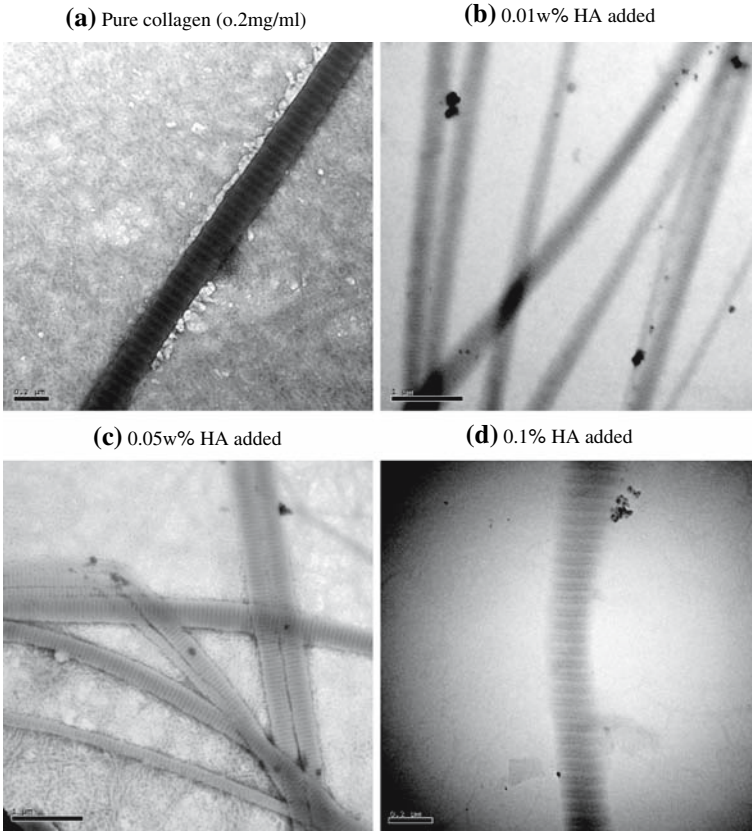

Fig. 4 Transmission electron micrographs of type II collagen fibers: (**a**) pure collagen, (**b**) 0.01 wt.% HA added, (**c**) 0.05 wt.% HA added and (**d**) 0.1 wt.% HA added. Type II collagen concentration was 0.2 mg/mL, incubation time: 48 h

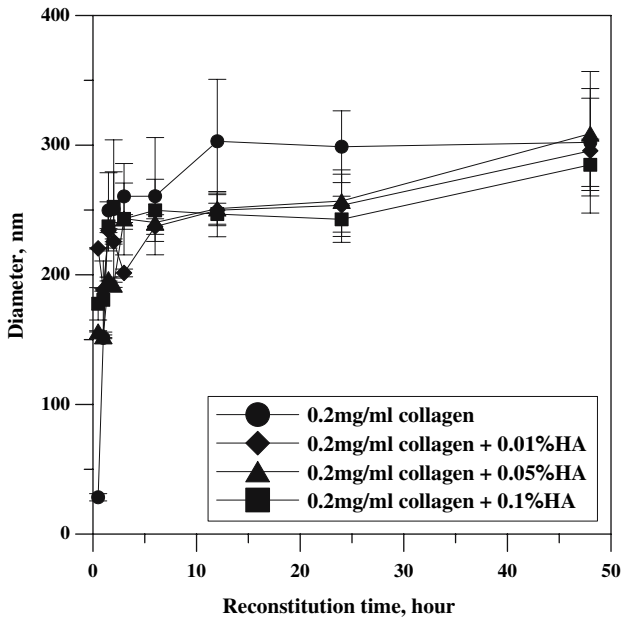

Fig. 5 Diameters of type II collagen as a function of reconstitution time in the presence of HA. Values are means ± SD

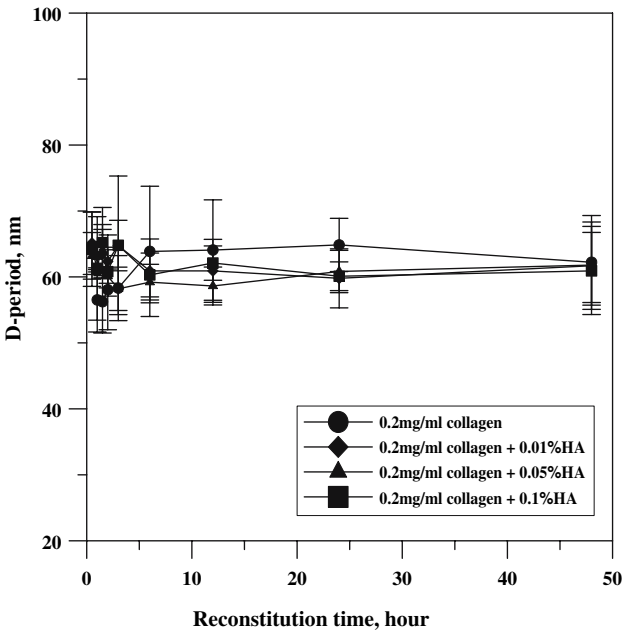

Fig. 6 D-periods of type II collagen as a function of reconstitution time in the presence of HA. Values are means ± SD

3.0×10^5 Da), could promote the aggregation of collagen monomers but might obstruct the processes of fibrillogenesis that follows. From the TEM observations, the collagen fibrils were found smaller in diameter with the present of HA in initial incubation periods. In addition, the distribution of collagen fibril diameters was wider than the ones

with the addition of HA during 3 h incubation. Ultimately, the diameters of collagen were found no significant difference between the various experimental groups from the statistical analyses for samples that undergoing 48 h of thermal incubation. It seemed that HA could affect the reconstitution of collagen in the early period of fibrillogenesis, but the final diameters of collagen fibers would not be influenced significantly with the presence of HA if the thermal incubation period was carried out long enough.

In a pervious study on the influence of alginate to collagen II fibrillogenesis [19], the results showed that both apparent rate constants, k_{lag} and k_g, in the presence of alginate, decreased to about one-third of the original value. A indicated that alginate would retard the formation of collagen II fibers. By contrast, collagen II fibrillogenesis seemed not to be influenced by the presence of HA from the results of this study. Although alginate and HA both are the natural occurring anionic polysaccharides with similar structures, the influences on the type II collagen fibrillogenesis are distinctively different. All the apparent rate constants changed slightly with addition of HA to the solutions. More detailed studies, such as using HA with wider range of molecular weights, and the addition of one of the minute yet critical components, such as, glycosaminoglycan to the collagen fibrillogenesis process, are currently in progress in our lab. Preliminary data indicated that the change of both apparent rate constants in the presence of HA was smaller than that in the presence of alginate.

Conclusion

The present data showed that the ultimate packing of collagen was probably not interfered or affected significantly by the presence of HA in vitro environment. The inclusion of HA had minor or no impact to the fibrillogenesis and fibers sizes of type II collagen in vitro. These preliminary results and findings would provide us with an improved elementary criterion, such as formulation of systems with a wide range of viscosity characteristics, for fabricating various type II collagen matrixes as a carrier for chondrocytes or in the field biomedical applications.

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