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Insight into the collagen assembly in the presence of lysine and glutamic acid: An in vitro study

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ABSTRACT

The aim of this study is to evaluate the effects of two different charged amino acids in collagen chains, lysine and glutamic acid, on the fibrillogenesis process of collagen molecules. The turbidity, zeta potential, and fiber diameter analysis suggest that introducing the positively charged lysine into collagen might improve the sizes or amounts of the self-assembled collagen fibrils significantly. Conversely, the negatively charged glutamic acid might restrict the self-assembly of collagen building blocks into a higher order structure. Meanwhile, the optimal fibrillogenesis condition is achieved when the concentration of lysine reaches to 1 mM. Both scanning electron microscopy (SEM) and atomic force microscope (AFM) analysis indicates that compared to pure collagen fibrils, the reconstructed collagen-lysine co-fibrils exhibit a higher degree of inter-fiber entanglements with more straight and longer fibrils. Noted that the specific D-period patterns of the reconstructed collagen fibrils could be clearly discernible and the width of D-banding increases steadily after introducing lysine. Besides, the kinetic and thermodynamic collagen self-assembly analysis confirms that the rate constants of both the first and second assembly phase decrease after introducing lysine, and lysine could promote the process of collagen fibrillogenesis obeying the laws of thermodynamics.

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1. Introduction

Collagens constitute one of the main components in extracellular matrices of the connective tissues in all multicellular organisms [1]. Due to its analogous nature to ECM, collagens have achieved much attention and been extensively utilized in the yield of biomedical materials, including hemostatic [2], drug delivery systems [3], wound dressings [4], and tissue engineering scaffolds [5–7]. Nevertheless, collagen-rich tissues are typically based on the insoluble collagen fibrils with around 67 nm cross-striated patterns as an elementary building block according to the collagen's biosynthesis in vivo [8]. More to this point, in the extracellular matrix, collagen molecules are ready to self-assemble at the nano-, micro-, and macro-scales into more advanced collagen aggregates in a complex hierarchical way [9]. Evidence to date indicates that these suprafibrillar architectures play a pivotal role in maintaining the fundamental shapes, physical characteristics, functional features and structural integrity of the connective tissues [10,11]. Moreover, collagen aggregates are serving as a docking site for several growth factors, such as proteoglycan and cytokines, which are tightly associated with the differentiation, survival, polarity and motility of cells [12]. Meanwhile, collagen aggregates could profoundly influence many physiological processes of biological adaptations and tissue regeneration [13–15]. Besides, the aggregated architectures could not only reinforce the mechanical attributes of tissues, but also promote their thermo- and structural stability [16]. Recently, the literatures have reported that collagen aggregates might exhibit better cellular responses compared to monomeric collagen molecules, indicating that the suprafibrillar assemblies could more preferably bio-mimic the biological characteristics of the native extracellular matrix [17]. Many interests have been focused on the formation of collagen fibrils in vitro for over 60 years. The self-assembly approach to reconstitute the native-like collagen fibrils in vitro has always attracted widespread concerns owing to the fibrillogenesis feature of monomeric collagen molecules. Collagen molecules could self- or induced- aggregate in several different patterns depending on the external micro-circumstances frequently. Various influencing factors upon the fibrillogenesis of the regenerated native-type fibrils have been investigated extensively [18], including temperature, pH, ionic strength, type of salts and buffer, intactness of the N- and C-telopeptides, presence of proteoglycans/glycosaminoglycans, decorin and polysaccharides [13,19–23]. The microstructure of the reconstituted fibrils could be fine-tuned by these parameters, which play a vital role in collagen biochemistry and biology.

Generally, the charged amino acid residues constitute approximately 15–20% of total amino acid residues throughout the collagen triple helical domains [24]. Lysine and glutamic acid are two disparate types of charged amino acids in collagen chains therein [25]. As is well

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known, the hallmark of collagens is a highly repetitive sequence based on a triplet of amino acids $(Gly–X_{AA}–Y_{AA})_n$. The basic lysine residues routinely occupy the Y_{AA} position, while the acidic glutamic acid could be commonly observed in the X_{AA} position [25]. The prevalence of intermolecular and intramolecular interactions among the ionic amino acid residues are essential for the stability of collagen's triple helical conformation [26]. Moreover, the presence of ionic residues in recognition sites along collagen molecules could regulate the fibril formation and induce intermolecular interactions with other connective tissue macromolecules or cell receptors [25]. More to the point, it is reported that the amine groups of lysine of collagen could cause chemical reactions with the carboxyl groups of glutamic acid residues to generate inter and intra- molecular crosslinking [27]. Meanwhile, the groups of —OH, —COOH and $—NH_2$ in the lysine and glutamic acid have been considered as potential interacting sites for the formation of H-bonds or hydrophobic interactions with collagen [25], which may play an influential part in the stabilisation of collagen and bring a substantial contribute to the quartered-stagger of molecules and periodicity in the regenerated collagens fibrils [28]. Further, molecular modelling studies indicate that the binding energy of the interactions between lysine and collagen presents the lowest level indicating the strongest interactions compared to glutamic acid [27]. Therefore, after introducing lysine, collagen could exhibit distinctly improved mechanical properties and biological stability. Besides, lysine and glutamic acid have been subjected to extensive investigations that have wide applications in the fields of pharmaceutical industry. Previous literatures have reported that lysine could enhance the immune function and improve the functions of the central nervous tissue [29]. And glutamic acid could improve the function of nervous centralize and cortical brain for neurasthenia patients [30]. Hence, introducing lysine and glutamic acid is benefit to make collagen more functional.

From the foregoing, the reconstituted collagen fibrils in the presence of lysine and glutamic acid have the potential to serve as a kind of functional biomimetic ECM analogues. The reconstituted collagen co-fibrils in vitro might serve as a better alternative source of collagens for further biological applications due to their natural biocompatibility in vivo. Nevertheless, the effects of lysine and glutamic acid on the fibrillogenesis of the reconstituted native-type collagen fibrils in vitro are still remained unknown. Accordingly, the objective of this research is to elaborate the optimum assembly conditions, and focus especially on revealing the mechanisms underlying the collagen self-assembly process in the presence of lysine and glutamic acid via turbidity measurements, zeta potential and fiber diameter measurements, pyrene fluorescence measurements, scanning electron microscopy (SEM) analysis and atomic force microscope (AFM) analysis. In addition, the self-assembly kinetic and thermodynamics parameters of collagen after introducing the charged amino acid are further investigated. We expect that this study would provide useful results for understanding the influence of the charged amino acids on collagen assembly and lay a foundation for developing various biomimetic forms for biomedical applications.

2. Experiments

2.1. Materials

The type I pepsin-solubilized collagen with molecular weight about 300 kDa was self-prepared according to our previous report [31]. Unless noted otherwise, all chemicals and reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Preparation of collagen-lysine and collagen- glutamic acid blended solutions

The acid soluble collagen was dissolved in 10 mM sodium phosphate (pH 7.2) containing 110 mM NaCl to an initial concentration of 1 mg/mL at 4 °C. Subsequently, both lysine and glutamic acid were introduced to the collagen solutions to the concentrations of 1 mM, 5 mM, 8 mM and 10 mM, respectively. Then prior to fibrillogenesis, the aforementioned blended solutions were sufficiently stirred at 4 °C for 30 min. Finally, the resulting mixtures were homogenized and incubated in a biochemical incubator at 37 °C for 12 h to obtain the reconstituted assemblies. Scheme 1 shows the effects of lysine and glutamic acid on the collagen fibrillogenesis and the D-periodicity of the resulting collagen fibrils.

2.3. Turbidity measurement of collagen-lysine and collagen- glutamic acid assemblies

The fibril formation and aggregation kinetics of collagen-lysine and collagen-glutamic acid blended specimens were monitored by turbidimetric measurements on a Perkin Elmer Lambda 25 UV/VIS spectrometer equipped with a thermostat holder. Then the specimens were maintained at the desired temperature by water circulation and the temperature fluctuation was controlled within ± 0.1 °C. Finally, the blended solutions were poured into a quartzose cuvette (1 cm) and immediately transferred to the spectrophotometer. The time course of optical density (turbidity) was measured at 313 nm every 30 s [32]. Besides, the self-assembly kinetic parameters were characterized quantitatively according to the method of Yan et al. [33].

2.4. Zeta potential and fiber diameter measurements of collagen-lysine and collagen- glutamic acid blended solutions

The zeta (ζ) potential was tested by using a zeta potential analyzer (Powepac Basic, Malvern, UK). In order to avoid precipitation, the measurements were carried out immediately when the aforementioned blended solutions were obtained. Zeta potential was calculated using the Smoluchowski equation. Besides, the fiber diameters of the reconstituted assemblies were measured by mastersizer 2000 (Nano-ZS, England). All the assays were conducted at least in triplicate on separate occasions.

2.5. Pyrene fluorescence measurements

Fluorescence measurements of pyrene in the collagen and collagen-lysine blended solutions were conducted according to the method of Nakashima et al. [34] with slight modification. Pyrene, used as a hydrophobic probe, was dissolved in methanol to give a stock concentration of 400 μM. 50 μL of pyrene solution was transferred to 10-ml brown glass volumetric flask followed by gentle evaporation of the solvent under nitrogen-gas stream, and then 10 mL of collagen and collagen-lysine blended solutions were added into the volumetric flasks. After ultrasonic processing for 3 min, the samples were stored in the dark thermostatically at 37 °C for 1 day before the spectroscopic measurements. Emission fluorescence measurements were conducted on a Hitachi F- 4010 spectrofluorometer. All the specimens were excited at 343 nm and the emission spectra of 360–460 nm were collected with a scanning rate of 120 nm/min. The excitation and emission slits were set at 5 nm and 2.5 nm, respectively [35]. Further, the critical aggregation mass concentrations of collagen in the presence of lysine at 25 °C, 29 °C, 32 °C, 35 °C and 39 °C were determined due to the so-called pyrene 1:3 ratio method [36].

2.6. Scanning electron microscopy (SEM)

After the aforementioned blended solutions were incubated at 37 °C for 12 h, 200 μL of all the resultant reconstituted assemblies were dropped on the top of coverslips. Subsequently, the reconstituted assemblies were rinsed for 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. Finally the specimens were dehydrated using graded ethanols (30, 50, 70, 90, and 100% ethanol for 15 min each) followed by the

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