In vitro formation and thermal transition of novel hybrid fibrils from type I fish scale collagen and type I porcine collagen

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Abstract

Novel type I collagen hybrid fibrils were fabricated by neutralizing a mixture of type I fish scale collagen solution and type I porcine collagen solution with a phosphate buffer saline at 28°C. Their structure was discussed in terms of the volume ratio of fish/porcine collagen solution. Scanning electron and atomic force micrographs showed that the diameter of collagen fibrils derived from the collagen mixture was larger than those derived from each collagen, and all resultant fibrils exhibited a typical D-periodic unit of ∼67 nm, irrespective of volume ratio of both collagens. Differential scanning calorimetry revealed only one endothermic peak for the fibrils derived from collagen mixture or from each collagen solution, indicating that the resultant collagen fibrils were hybrids of type I fish scale collagen and type I porcine collagen.

Keywords: type I collagen, hybrids, DSC measurement

1. Introduction

Hybrid collagen fibrils are widely present in biological tissue and are of great biological significance. For example, collagen type II and III hybrid fibrils have been found in human articular cartilage [1] and collagen type I and III hybrid fibrils were observed in the normal rat liver [2]. Until now, although more than 28 types of collagens have been found in human tissues, research interest has been mainly focused on type I collagen-based fibrils, because they are the main structural protein of the skeletal system and have a cross-striated structure with a typical D-periodic band of 60–70 nm. These fibrils are crucial to cell attachment and growth as well as nutrition diffusion of transparent corneal tissue [3] and are responsible for the strength of tendon and bone [4]. Through neutralization of acid-solubilizing type I collagen with phosphate buffer saline (PBS), type I collagen fibrils are widely re-assembled in vitro and exhibit similar biological and mechanical properties to those in vivo [5].

In vivo, type I collagen fibrils preferentially form hybrid fibrils with other types of collagen. Birk et al for the first time definitively demonstrated, with immunoelectron microscopy, that type I and type V collagen were co-distributed within the same fibrils in avian corneal stroma [6]. They also found that chick corneal stroma were heterotypic and composed of both collagen types I and V, and that the interaction of type V with type I collagen was one mechanism that modulates fibril diameter [7]. Fleischmajer et al confirmed that dermal collagen fibrils were hybrids of type I and type III collagens [8]. Adachi et al obtained hybrid fibrils of type I and...
type V collagen and found that the latter had a limited effect on the growth of the former [9]. These studies indicate great biological importance of type I collagen-based hybrid fibrils. However, little attention has been paid to the hybrid fibrils of various type I collagens extracted from different species.

Recently, a type I collagen derived from fish scales attracted much interest as one of the alternative collagen sources for artificial substitutes. This is because it has been found that fish scales are a composite of hydroxyapatite and type I collagen fibrils and have plywood structure of aligned fibril sheets [10, 11]. The structural features are similar to those of bones, tendons and corneas in the human body. The tilapia fish scale collagen has a higher denaturation temperature (about 36°C) than that of the collagen extracted from other fish species. Moreover, re-assembled fish scale collagen fibril sponges exhibited a biocompatible property similar to that of the porcine collagen after being implanted into the back muscle of a rabbit [12]. These advantages suggest that tilapia fish collagen fibrils can be used as artificial substitutes for bone, tendon and cornea tissue. Thus, research on the in vitro re-constitution of fish scale collagen fibrils is particularly important. However, no published work has yet described the preparation and characterization of type I fish scale collagen-based hybrid fibrils.

The type I collagen extracted from porcine dermis is one of the conventional collagen sources for biomedical applications because of its excellent biocompatibility, biodegradability and high (about 42°C) denaturation temperature [13, 14]. This study is the first attempt to prepare novel hybrid collagen fibrils of type I fish scale collagen and the conventional type I porcine collagen fibrils.

2. Experimental details

2.1. Fabrication of hybrid fibrils

Tilapia fish scale type I collagen (Taki chemical Co., Ltd) and porcine dermis type I collagen (Nitta Gelatin Inc.), which were digested with pepsin, were dialyzed four times in distilled water at 4°C for 12 h and then lyophilized at −10°C for 48 h. Both freeze-dried collagens were dissolved in a hydrochloric acid (HCl; 1 mM, pH 3.0) solution at 4°C to obtain two 1% (w/v) collagen solutions. The solutions were mixed together at different ratios and stirred overnight at 4°C to obtain three separate collagen mixtures, the fish collagen/porcine collagen volume ratios of which were fixed at 3, 1 and 0.3. The three collagen mixtures were then well mixed with 10 × PBS (pH 7.4, 1370 mM NaCl, 27 mM KCl, 80 mM Na2HPO4 and 15 mM KH2PO4) for about 30 s at 4°C, with a collagen mixture/10 × PBS volume ratio of 9, and then quickly poured into a silicone rubber mold. The mold was then kept at 28°C for 3 h to produce opaque collagen fibril hydrogels. The collagen fibrils derived from the three collagen mixtures were abbreviated as FP3, FP1 and FP03 (from their fish collagen/porcine collagen volume ratios of 3, 1 and 0.3) and those derived from a single fish collagen solution and a single porcine collagen solution were named Fish and Porcine, respectively.

2.2. Characterization

The structure and denaturation behavior of both collagens and the collagen mixtures were characterized with a circular dichroism (CD) spectrometer (JASCO J-725, Jasco Corp, Tokyo, Japan). Appropriate amount of 0.04% (w/v) each collagen and collagen mixture were separately filled in a quartz cell with a path length of 1 mm and the temperature was controlled with a Peltier cooling unit. For structural analysis, the wavelength was scanned from 190 to 250 nm at a fixed temperature of 28°C. For studying denaturation behavior, the wavelength was fixed at 221 nm and the temperature was increased from 20 to 50°C at a rate of 1°C min⁻¹.

The microstructure of the collagen fibrils was observed with a scanning electron microscope (SEM; JEOL 5600LV, Tokyo, Japan) at an accelerating voltage of 20 kV. Before observation, all collagen fibril hydrogels were sequentially soaked in 50, 70, 90 and 100% ethanol solutions for 30 min each, then in a 1-butyl alcohol solution for three 30 min periods, and finally freeze-dried in the 1-butyl alcohol solution at −10°C.

The nanostructure of the re-assembled collagen fibrils was observed with an atomic force microscope (AFM; SPM-9600, Shimadzu, Kyoto, Japan) as phase contrast images. The as-prepared collagen fibril hydrogels were mechanically dispersed in ethanol and deposited on a slide glass before observation.

 Thermal transitions of the re-assembled fibrils were analyzed with a differential scanning calorimeter (DSC; TAS-100, Rigaku Co., Tokyo, Japan). Between 2 and 3 mg of the freeze-dried samples (see SEM sample preparation) was swollen in 20 µl of PBS overnight in capped Al pans. PBS was used as the reference substance. The measurements were performed between 25 and 100°C at a heating rate of 5°C min⁻¹ in a nitrogen atmosphere.

3. Results and discussion

Figure 1(left) presents the CD spectrum of 0.04% (w/v) for the fish collagen (a), the collagen mixtures with fish collagen/porcine collagen volume ratios of 3 (b), 1 (c) and 0.3 (d), and the porcine collagen (e) at 28°C. Despite the changes in the volume ratio of the collagens, the CD
spectra of the five solutions are very similar and contain two distinct peaks at 196 and 221 nm, which were previously attributed to the characteristic triple-helical structure of normal collagens [10]. Figure 1(right) shows the denaturation behavior of the five solutions. The denaturation curves of the solutions between 20 and 50 °C are similar and consist of three sequential steps: a plateau region with no change in CD value at the early stage, a degradation region with a rapid decrease in CD value at the middle stage and a stabilized region with no change in CD value at the later stage. The denaturation temperature range differs significantly with the type of collagen solution. The fish collagen solution degrades between 31 and 40 °C, while the porcine collagen solution degrades between 34 and 45 °C. In contrast, despite the change in volume ratio for the three mixtures, their denaturation temperature range is almost the same (i.e. between 31 and 45 °C). Interestingly, the collagen mixtures exhibit two steps of denaturation ascribed to both fish and porcine collagens, and the ratio of CD values between 31–40 °C and 40–50 °C almost matches the mixing volume ratio. This reveals that the co-existence of both collagens has no strong effect on their triple-helical structure and each collagen maintains its characteristics. Besides, upon just mixing, monomeric collagens they never form hybrid fibrils.

Figure 2 shows SEM images of the resultant collagen fibrils Fish (a), FP1 (b) and Porcine (c) after fibrogenesis treatment in PBS at 28 °C. The fibrils’ appearance indicates that fibrogenesis occurred in all solutions and that it was not affected by the co-existence of both collagens. All collagen fibrils showed a highly entangled structure. The average diameters of 100 collagen fibrils were calculated using SmileViewer software and found to be 79 ± 2 nm (Fish), 100 ± 3 nm (FP1) and 91 ± 2 nm (Porcine). The co-existence of both collagens resulted in larger hybrid fibrils. As mentioned at the beginning of the paper, the presence of other types of collagens has been found to play an important role in modulating the diameter of type I collagen fibrils. Here, we confirmed that the type I collagen species also strongly affects the resultant fibril diameters.

Figure 3 presents typical phase contrast images of Fish (a), FP1 (b) and Porcine (c). It shows the nanostructures of the re-assembled fibrils and further reveals the interaction between fish collagen and porcine collagen. All re-assembled fibrils were characterized by a transverse D-banding periodic pattern consisting of subsequent grooves and ridges, and the average distance between the neighboring grooves was 67 nm. All collagen fibrils showed a characteristic D-period value of 67 nm in the normal type I collagen fibrils [15]. The co-existence of both collagens did not change the nanostructure of the collagen fibrils.

The co-existence of both collagens might produce either fibril mixtures or fibril hybrids. To clarify this and to characterize the resultant fibrils, we used a thermal analysis technique that is simpler than the conventional and complex immunochemical assay. Figure 4 shows the DSC curves of the resultant collagen fibrils. The denaturation temperature of fish collagen fibrils was the lowest and that of porcine collagen was the highest. The denaturation temperature of the resultant fibrils from the collagen mixtures differed depending on the fish collagen/porcine collagen volume ratio; i.e. it increased when the amount of porcine collagen in the starting mixture was increased. However, it was surprising to find
that the resultant fibrils from both collagens showed only one endothermic peak, in the same manner as for the single fish and porcine collagen fibrils. This indicates that these fibrils were hybrid fibrils from both collagens. Because this premise is somewhat speculative, we attempted to confirm it by preparing a fibril mixture by mechanically mixing both purified fish and porcine collagen fibrils at the same weight. The DSC curve of such fibril mixture showed two distinct endothermic peaks, one corresponding to the single fish collagen fibrils and another to porcine collagen fibrils. This indicates that there was no strong interaction between both fibrils in the fibril mixture. However, only one endothermic peak was obtained for the fibrils derived from both collagen mixtures. We thus confirmed that the co-existence of both type I collagens resulted in hybrids of both collagen fibrils, not the mixtures of both fibrils during fibrogenesis. Moreover, the hybridization was not affected by the volume ratio of both collagens. To the best of our knowledge, this is the first report of the hybridization of type I fish collagen fibrils and type I porcine collagen fibrils. Type I collagen molecules extracted from mammals are composed of three polypeptide chains, i.e. two $\alpha 1$ chains and one $\alpha 2$ chain. In contrast, fish collagen generally contains three chains, i.e. $\alpha 1$, $\alpha 2$ and $\alpha 3$ chains [10]. Although the amino sequences and peptide chains of collagen fibrils are different, fish scale collagen molecules also form cross-striated fibrils with the typical D-periodic band of $\sim 67$ nm, in the same way as conventional porcine collagen fibrils. The difference in amino residues and polypeptide did not significantly affect the fibrogenesis of fish collagen. As found in vivo, the hybridization of various types of collagen was related to the similar triple-helical structure of collagen molecules. It is thus reasonable to assume that the reported here hybridization of fish and porcine collagens took place due to the similar triple-helical structure, as confirmed in figure 1. As stated by Silver et al [16] and Ciarletta and Ben Amar [17], the diameter of collagen fibrils has a strong effect on the mechanical properties of the tendon and ligaments. Therefore, the present hybridization process can be designed to provide specific mechanical properties to the engineered hybrids for biomedical applications, such as tissue engineering, and it can become a useful fabrication method for healing and replacement techniques.

4. Conclusions

Novel type I collagen fibrils were prepared by neutralizing a mixture of type I fish scale collagen solution and type I porcine collagen solution with a phosphate buffer saline at 28 $^\circ$C. AFM phase images showed that those fibrils had a typical cross-striated periodic unit of 67 nm and DSC curves confirmed that they were hybrids of type I fish scale collagen and type I porcine collagen. Such hybrid fibrils are advantageous for extending applications of biomaterials in drug delivery systems and tissue engineering.

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Figure 4. DSC curves of collagen fibrils (a) fish, (b) FP3, (c) FP1, (d) FP03, (e) porcine and (f) a mixture of freeze-dried fish collagen and porcine collagen fibrils of equal weight.