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The Preparation of Nano-SiO₂/Dialdehyde Cellulose Hybrid Materials as a Novel Cross-Linking Agent for Collagen Solutions

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Abstract: Nano-SiO₂ was immobilized onto dialdehyde cellulose (DAC) to prepare SiO₂/DAC hybrid materials. Fourier transform infrared spectra (FTIR), thermogravimetric analysis and field emission scanning electron microscopy of SiO₂/DAC indicated that nano-SiO₂ had been successfully hybridized with DAC. X-ray diffraction suggested that the structure of DAC was influenced by the nano-SiO₂. SiO₂/DAC was then used as the cross-linker of collagen solutions. Gel electrophoresis patterns and FTIR reflected that cross-linking occurred between DAC and collagen, but that collagen retained the native triple-helix, respectively. Differential scanning calorimetry indicated that the thermal stability of collagen could be effectively improved by SiO₂/DAC. Dynamic rheology tests revealed that the flowability of collagens cross-linked by SiO₂/DAC was superior to that of those cross-linked by DAC; meanwhile, collagens cross-linked by SiO₂/DAC possessed a more homogeneous morphology compared to those cross-linked by DAC. The hybridization of SiO₂/DAC as a cross-linker for collagen could effectively prevent the gelation caused by excessive cross-linking, and significantly improve the thermostability of collagen, which could be helpful for collagen being applied in fields including biomaterials, cosmetics, etc.

Keywords: dialdehyde cellulose; nano-silicon dioxide (nano-SiO₂); collagen; hybridization; flowability; thermal stability

1. Introduction

Collagen, the major component of extracellular matrices, has become a popular biomaterial and has been widely used in pharmacy, food, cosmetics and tissue engineering due to its bioactivity, biocompatibility and biodegradability [1–3]. Usually, aqueous preparations of collagen can be used for medial injection [4] or used as drug carriers and so on [5,6]; additionally, collagen solutions may also be processed into a variety of formats, including sheets, fibers, sponges and packaging films [7–10]. During these applications and processes, the thermal stability of collagen in solutions plays an essential role in keeping collagen against denaturation, as the excellent characteristics of the native collagen would be lost if the collapse of the triple helix to a random coil occurred.

The thermal stability of collagen in solutions has been studied widely, and all of these studies suggest that collagen has a relatively weak thermal stability, which limits its industrial and biological applications. Chemical cross-linking procedures are a useful method for reinforcing the collagen structure, and several cross-linkers, such as glutaraldehyde (GA) [11], formaldehyde [12] and diisocyanates [13], have been used. However, these cross-linkers usually show toxicity. Some researchers have developed cross-linking agents with low toxicity, such as carbodiimide [14],

but nevertheless, incomplete cross-linking under a sufficiently high carbodiimide concentration could be caused by side reaction of the carbodiimide [15]. Therefore, it was necessary to develop an alternative cross-linking agent with good biocompatibility and low cytotoxicity.

Cellulose is the most abundant natural polymer and has some fascinating properties, such as biocompatibility, biodegradability and desirable mechanical properties, and thus a variety of cellulose derivatives that can be widely used in many fields have been produced by chemical modifications such as esterification, halogenation, oxidation and etherification [16], or by chemical grafting [17]. Amongst these derivatives, periodate oxidized cellulose is prepared by specific cleavage of the C2–C3 bond of the glucopyranoside ring, producing two aldehyde groups per unit [18]. Thus, it is named as dialdehyde cellulose (DAC), which is an open chain polymer containing aldehyde groups. Considering the functional aldehyde groups could produce cross-links with free amino groups, DAC can be used as a novel cross-linking reagent for collagen. Kanth et al. investigated the effect of DAC on cross-linking efficiency, hydrothermal and enzymatic stability of collagen fibers using DAC as a cross-linker. The results suggested that the hydrothermal stability of collagen fibers could be improved significantly [19]. However, as for collagen in solutions, a solid-like network structure would be gradually developed, and thus gelation is prone to occur when cross-linking reagent is added [20]. In particular, compared to cross-linkers with low molecular weight, the gelation of collagen solutions was more difficult to avoid, due to the presence of a large number of aldehyde groups in the long chain of DAC. Hence, DAC needs to be modified in order to maintain the flowability of the cross-linked collagen in solutions.

SiO₂, which was formed by the hydrolysis of tetraethoxy silane, has been introduced into leather to improve the hydrothermal stability [21]. With collagen as a model of leather, it was revealed that hydrogen bonding between the silanol of the silica and the carboxyl hydroxyl, as well as amino groups of collagen, occurred between SiO₂ and collagen [22]. Obviously, this effect was weaker than covalent cross-linking induced by DAC, so the gelation of collagen solutions could be expected to be inhibited if part of the aldehyde group of DAC were replaced by SiO₂; meanwhile, the thermal stability of collagen solutions could still be improved by the effects from both DAC and SiO₂. Therefore, in the present work, we attempted to prepare SiO₂/DAC hybrid materials via organic/inorganic hybridization of nano-SiO₂ and DAC to be a novel cross-linking agent for collagen.

In the present study, the structure and morphology of the SiO₂/DAC hybrid materials were investigated by the methods of Fourier transform infrared (FTIR), X-ray diffraction (XRD) and thermogravimetric analysis (TGA). Then the hybrid materials were used as a novel cross-linking agent for collagen solutions. Subsequently, the modified collagens were characterized, and the effect of modification was evaluated via sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), differential scanning calorimetry (DSC), oscillatory rheological measurements and Field emission scanning electron microscopy (FESEM). New insights into and understanding of the mechanism of stabilization of collagen by SiO₂/DAC hybrid materials via organic/inorganic hybridization of nano-SiO₂ and DAC were gained.

2. Materials and Methods

2.1. Materials

Collagen was self-prepared from calf skins according to our previous work [23]. Briefly, the supernatants extracted from the delimed and neutralized bovine split pieces by 0.5 mol/L acetic acid containing 3% pepsin (1:3000) were collected by refrigerated centrifugation at 9000× *g*. After this step, the supernatants were salted out by the addition of NaCl to a final concentration of 0.7 mol/L, and then the precipitate was again dissolved in 0.5 mol/L acetic acid and dialyzed against 0.1 mol/L acetic acid for 3 days. Finally, pepsin-soluble collagen solution (about 3 mg/mL) was lyophilized by a freeze dryer (Labconco Freeze Dryer FreeZone 6 Liter, LABCONCO Co., Kansas City, KS, USA) at −50 °C for 2 days and stored at 4 °C until used.

Microcrystalline cellulose (degree of polymerization = 200) was purchased from Aladdin Co., Ltd. (Shanghai, China). Sodium periodate (NaIO_4), N-(β -aminoethyl)- γ -aminopropyl trimethoxy-silane (AEAPTS) and tetraethyl siloxane (TEOS) with analytical pure grade were purchased from National Pharmaceutical Group (Shanghai, China).

2.2. Preparation of SiO_2/DAC Hybrid Materials

Dialdehyde cellulose (DAC) was prepared according to the methods reported earlier, with modifications as described [24]. Microcrystalline cellulose (25 g) was hydrolyzed in 5 mol/L hydrochloric acid for 12 h. The hydrolyzed cellulose was suspended in deionized water, and then 25 g of sodium periodate was added, stirring for 4 h with a magnetic stirrer. The pH of the solution was maintained at 3.5 during the reaction and the reaction was performed in the dark at 40 °C. Ethylene glycol (5 mL) was added to terminate the reaction, and then the product was extracted with centrifugation in *t*-butyl alcohol for three times. Finally, the resultant was lyophilized by the freeze dryer at -50 °C for 2 days. The obtained DAC were converted to nitrogen-containing compounds using the Schiff base reaction with hydroxylamine hydrochloride, according to the method of reported previously [25]. Elemental composition of nitrogen-containing derivatives was determined for C, H, and N by an elemental analyzer (Elementar Vario EL, Elementar Co., Hanau, Germany), and then the dialdehyde content was calculated to be 21.2%.

TEOS (50 mL) was added to a 50% (*v/v*) ethanol solution (250 mL); after stirring at room temperature for 1 h, glacial acetic acid (2.5 mL) was added and then stirred at 75 °C for 15 min. After this step, AEAPTS was dropwise added into the solution until a gel was formed. The gel was washed with 50% (*v/v*) ethanol solution for three times, and then $\text{SiO}_2\text{-NH}_2$ was lyophilized by the freeze dryer at -50 °C for 2 days.

The SiO_2/DAC hybrid materials were then prepared as follows: a mixture was prepared by dispersed $\text{SiO}_2\text{-NH}_2$ and DAC together in 50% (*v/v*) ethanol solution with the mass ratios (SiO_2/DAC) of 1/20, 1/10, 1/5 and 1/2, respectively. After stirring at 65 °C for 18 h, the resultant was washed by deionized water three times, and then hybrid materials were obtained by lyophilization with the freeze dryer at -50 °C for 2 days and were stored at 4 °C until use. The hybrid materials were denoted as SiO_2/DAC (1/20), SiO_2/DAC (1/10), SiO_2/DAC (1/5) and SiO_2/DAC (1/2), respectively, according to the initial mass ratios of SiO_2/DAC . The nano- SiO_2 in the following description was called SiO_2 for short.

2.3. Characterization of SiO_2/DAC Hybrid Materials

2.3.1. Fourier Transform Infrared (FTIR) Spectra of SiO_2/DAC

FTIR spectra of the SiO_2/DAC hybrid materials were recorded with a FTIR spectrophotometer (Thermo Scientific Nicolet IS10, Thermo Fisher Scientific Co., Waltham, MA, USA) using potassium bromide (KBr) pellets. The measurements were performed at a data acquisition rate of 2 cm^{-1} per point and in the range from 400 to 4000 cm^{-1} . To obtain a nice signal-to-noise ratio, 48 scans were performed for each sample.

2.3.2. X-ray Diffraction (XRD) of SiO_2/DAC

The X-ray diffraction patterns of the SiO_2/DAC hybrid materials were recorded using $\text{Cu K}\alpha$ radiation ($\lambda = 0.154056\text{ nm}$) on a diffractometer (Panalytical X'pert Pro MPD, PANalytical B.V. Co., Almelo, The Netherlands) at a scanning rate of $1^\circ/\text{min}$ in the 2θ range from 5 to 70° .

2.3.3. Thermogravimetric Analysis (TGA) of SiO_2/DAC

Thermogravimetric analysis of the SiO_2/DAC hybrid materials was performed with a thermal analyzer (Netzsch TG 209, NETZSCH Co., Selb, Germany). Samples ($\sim 2.0\text{ mg}$) were heated from 30 to $800\text{ }^\circ\text{C}$ (ramp of $10\text{ }^\circ\text{C}/\text{min}$) in a high-purity nitrogen atmosphere flowing at $80\text{ cm}^3/\text{min}$.

2.4. Cross-Linking of Collagen with SiO₂/DAC

The freeze-dried collagen was dissolved in water acidified to pH 4.0 using dilute HCl to obtain a concentration of 6 mg/mL, and then the pH of the solution was adjusted to 11.0 by dropwise addition of NaOH (1.0 mol/L). DAC, SiO₂/DAC hybrid materials and SiO₂-NH₂ in powder format were slowly added to the collagen solutions so that the final mass ratio of cross-linker/collagen reached 1/50 for all the samples. These solutions were gently stirred for 12 h at 20 °C, during which time all of the cross-linkers were dissolved, due to the effect of NaOH, and the resultant solutions were clear. The mixtures were further kept in the refrigerator for 24 h at 10 °C. The cross-linking reaction between cross-linkers and collagen were then terminated by adding excessive glycine, since the amino group in glycine is able to react with the active aldehyde groups in DAC or SiO₂/DAC. After this step, these solutions were dialyzed against 0.05 mol/L acetic acid solution, and thus the residual cross-linkers were removed. Finally, these resultants were lyophilized in the freeze dryer.

2.5. Characterization of Cross-Linked Collagen

2.5.1. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Collagens

Electrophoretic patterns were measured according to the method of Laemmli [26] with a slight modification, using 10% resolving gel and 4% stacking gel. Collagens were mixed with a sample buffer containing 10% β-Mercaptoethanol, to reach a final collagen concentration of 1 mg/mL, and the mixed solution was boiled for 5 min. After electrophoresis, the gel was stained for 45 min using 0.25% Coomassie brilliant blue R250 solution and destained using 7.5% acetic acid and 5% methanol.

2.5.2. FTIR of Collagens

FTIR spectra of the collagen samples were recorded with a FTIR spectrophotometer (Thermo Scientific Nicolet IS10, Thermo Fisher Scientific Co., Waltham, MA, USA) using potassium bromide (KBr) pellets. The measurements were performed at a data acquisition rate of 2 cm⁻¹ per point and in the range from 450 to 4000 cm⁻¹. 48 scans were performed for each sample.

2.5.3. Differential Scanning Calorimetry (DSC) of Collagens

The cross-linking effect on collagen imposed by the SiO₂/DAC hybrid materials was evaluated by DSC (Netzsch DSC 200PC, NETZSCH Co., Selb, Germany). The freeze-dried collagens (~2 mg) were weighted accurately into aluminum pans and sealed; they were scanned over the range from 30 to 60 °C at a heating rate of 3 °C/min in a nitrogen atmosphere. Liquid nitrogen was used as a cooling medium, and empty pans were used as the reference.

2.5.4. Oscillatory Rheological Measurements of Collagens

The rheological measurements of solutions were performed on a Rheometer System (MARS III, HAAKE Co., Karlsruhe, Germany) equipped with a cone-and-plate geometry (diameter 30 mm; angle 2°). For the precise control of sample temperature, temperature was controlled to within an accuracy of ±0.10 °C. The samples for oscillatory rheological tests were measured after each equilibration time for 3 min. Rheological properties were measured by dynamic frequency sweeps at a constant strain of 5% within the linear range. Dynamic frequency sweep tests for all the samples were performed from 0.1 to 10 Hz at constant temperature of 20 °C. The storage modulus (*G'*), loss modulus (*G''*) and tan δ were recorded.

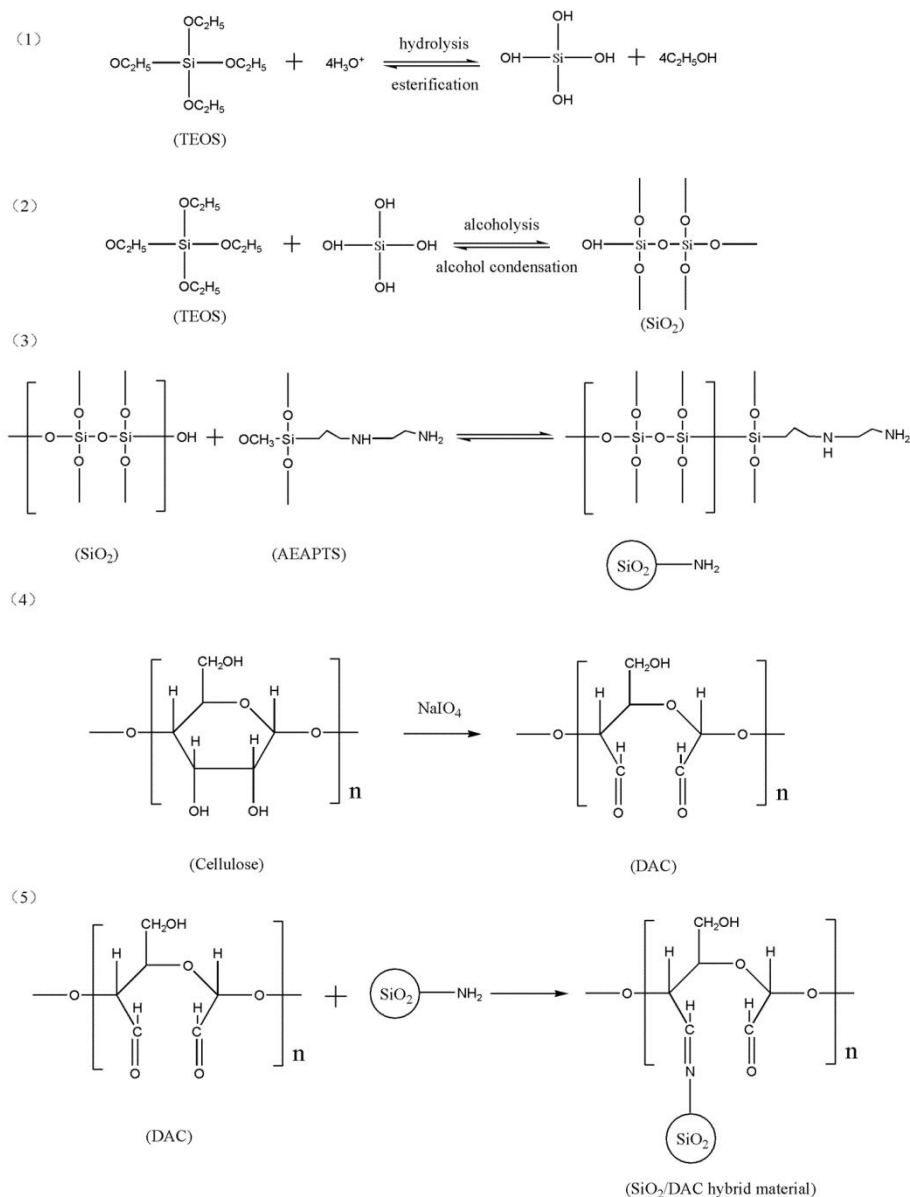
2.5.5. Field Emission Scanning Electron Microscopy (FESEM) of Collagens

The morphologies of lyophilized DAC, SiO₂-NH₂ and the modified collagen were examined with a field-emission scanning electron microscope (Nova FEI NanoSEM 230, FEI Co., Hillsboro, OR, USA), operated at an accelerating voltage of 10 or 5 kV.

3. Results and Discussion

3.1. Preparation and Characterization of SiO₂/DAC Hybrid Materials

Scheme 1 displays the synthetic routes of SiO₂, DAC and the SiO₂/DAC hybrid materials. After the Schiff base reaction between the aldehyde groups of DAC and the amino group of SiO₂-NH₂, SiO₂ was immobilized onto the chains of DAC. Therefore, the prepared hybrid materials can be described as SiO₂ immobilized DAC, possessing both of the aldehyde groups and SiO₂ as the functional constituents reacting with collagen.



Scheme 1. Schematic illustration of the preparation of SiO₂/DAC hybrid materials.

As shown in Figure 1, the FTIR spectra of DAC showed two characteristic peaks at 1726 cm⁻¹ and 880 cm⁻¹, which is in line with results reported previously [27,28]. A diffuse band at 880 cm⁻¹ can be assigned to the hemiacetal and hydrated form [29]. The sharp peak at 1726 cm⁻¹ is a characteristic band of carbonyl groups [28]. The absorption band at 3452 cm⁻¹ of the SiO₂ indicated that there were plenty of hydroxyl groups on its surface. While the absorptions at 1081 cm⁻¹, 798 cm⁻¹ and

469 cm^{-1} were attributed to the antisymmetric stretching vibration, symmetric stretching vibration and flexural vibration of Si–O–Si, respectively [30]. After reaction with SiO_2 , the hybrid materials SiO_2/DAC exhibited new characteristic peaks located at 1067, 795 and 468 cm^{-1} , as well as 1060, 801 and 469 cm^{-1} , for samples with SiO_2/DAC of 1/10 and 1/2, respectively, which were derived from the absorption of Si–O–Si. It is therefore suggested that SiO_2 was probably immobilized onto the DAC molecules.

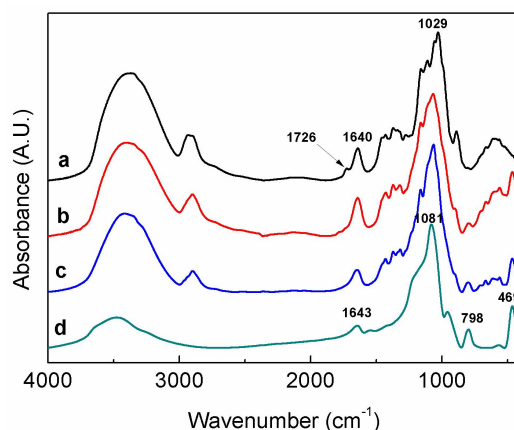


Figure 1. FTIR spectra of DAC (a), SiO_2 (d) and the SiO_2/DAC hybrid materials with ratios of 1/10 (b) and 1/2 (c).

The TG thermographs and the DTG curves (the inserts) for samples are shown in Figure 2. As indicated by the high residual masses after the decomposition step of the TG curves, it was found that DAC had a low char yield on pyrolysis, while the char yields were increased with the increase of SiO_2/DAC ratios. This could be attributed to the hybridization of DAC with $\text{SiO}_2\text{-NH}_2$, as the latter is an inorganic matter and exhibited a high char yield, as shown in Figure 2.

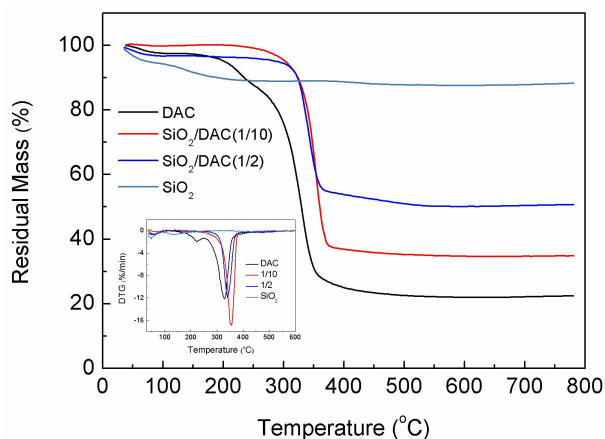


Figure 2. Thermogravimetric curves of the SiO_2/DAC hybrid materials.

From the DTG curves, the corresponding temperature of the maximum decomposition rate (T_{max}) could be obtained. We have previously observed that the native cellulose had a T_{max} of 359.6 $^{\circ}\text{C}$ [31]. After treatment with NaIO_4 , the T_{max} was reduced to 328.2 $^{\circ}\text{C}$ for the resultant DAC. The reduction in decomposition temperature for DAC compared to that of cellulose has been reported by Kim et al. [25]. However, the SiO_2/DAC hybrid materials had T_{max} of 355.4 and 341.8 $^{\circ}\text{C}$ for SiO_2/DAC ratios of 1/10 and 1/2, respectively, indicating that the thermal stability of DAC could be improved by the

introduction of inorganic matter. It should be noted that there was no distinct weight loss peak for the pure SiO_2 , suggesting that the sample was inorganic.

Figure 3 shows the XRD patterns of all the samples. For DAC, the diffraction peaks observed at 14.8° , 16.3° and 22.4° were attributed to the cellulose I pattern [32], but it was also reported that the crystallinity would be lower than that of cellulose [33]. SiO_2 exhibited a broad peak at $\sim 23.5^\circ$, which was in accordance with the reported result and suggested an amorphous form for SiO_2 [34]. In the case of hybrid material with SiO_2/DAC mass ratios of 1/10 and 1/2, the diffraction peaks disappeared significantly and disproportionately in terms of the SiO_2/DAC mass ratios, indicating the obvious decline in the degree of crystallinity of DAC. This observation suggests that there might be an interaction between DAC and SiO_2 particles, restricting the capacity of DAC to form a well-defined order.

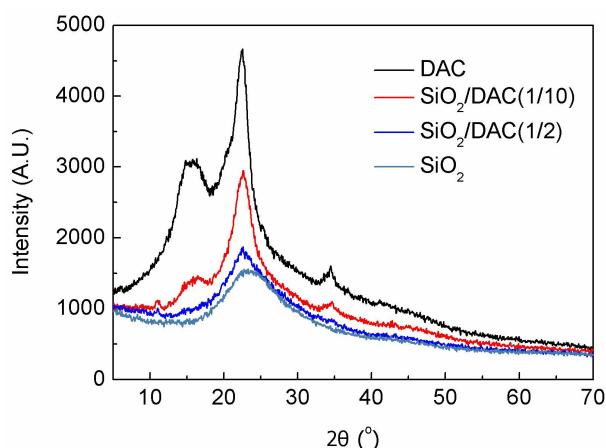


Figure 3. XRD patterns of the SiO_2/DAC hybrid materials.

3.2. FESEM of $\text{SiO}_2\text{-DAC}$

Figure 4 shows the FESEM images for DAC and SiO_2/DAC (1/2). It can be seen that DAC exhibited long and short rods (Figure 4a). For SiO_2/DAC , there were many particles of $\text{SiO}_2\text{-NH}_2$ bonded to surface of DAC, which further demonstrates that interaction occurred between the molecules of $\text{SiO}_2\text{-NH}_2$ and DAC. In addition, it was obviously found that the size of $\text{SiO}_2\text{-NH}_2$ was tens to hundreds of nanometers. For simplicity, in the following, SiO_2 refers to $\text{SiO}_2\text{-NH}_2$.

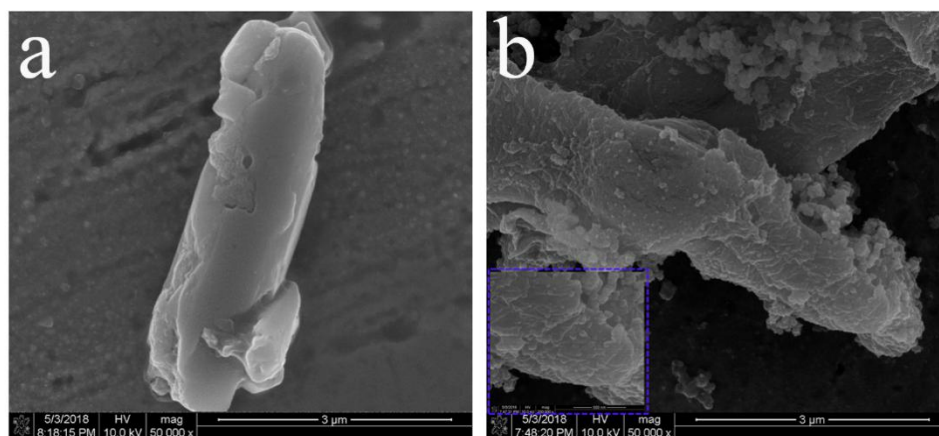
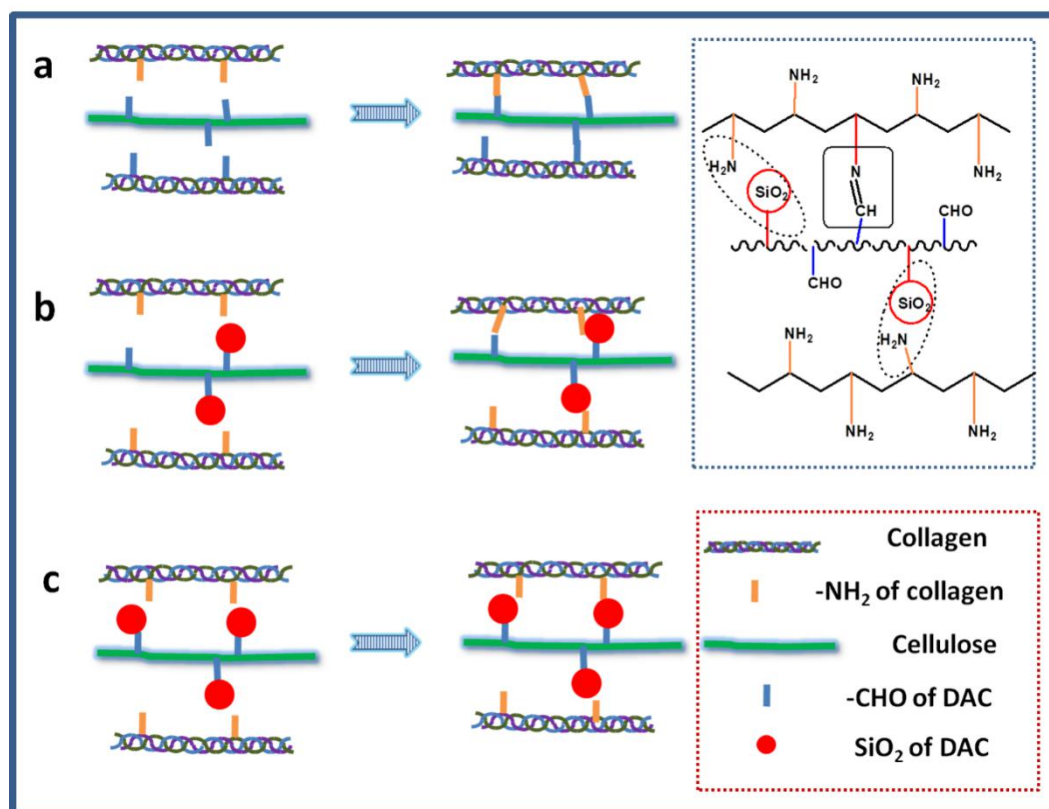


Figure 4. FESEM images (scale bar = $3\ \mu\text{m}$) of DAC (a), and SiO_2/DAC (1/2) (b) (Small figure at bottom left has a magnification of 200,000 (the blue dashed outline). Bars, 500 nm.).

3.3. The Cross-Linking of Collagen Using SiO₂/DAC Hybrid Materials as Modifiers

The prepared SiO₂/DAC hybrid materials, as well as the pure DAC and SiO₂, were applied in the modification of collagen solution. As the modifiers were added into collagen solution, the powders were dispersed quickly with stirring and then dissolved gradually due to the effect of basic pH. It should be pointed out that although the pH was just 11.0, DAC could be dissolved because its structure has been destroyed during the previous processes, which included hydrolysis and oxidation. After reaction, clear mixtures could be obtained. Scheme 2 presents the cross-linking mechanism of collagen with SiO₂/DAC, which could possibly be ascribed to two types of reaction in the mixtures. Firstly, there exists the Schiff base reaction between the amino groups of collagen and the paired aldehyde groups of the cross-linking reagents [19]; secondly, the hydroxyl groups of SiO₂ in the hybrid materials can form hydrogen bonds with the hydroxyl groups or amino groups of collagen [21]. In fact, the cross-linking mechanism might be more complex. The cross-linking products were then characterized to evaluate the influences of SiO₂/DAC hybrid materials on the structural properties of collagen solutions.



Scheme 2. Schematic illustration of the cross-linking mechanism of collagen with SiO₂/DAC. (a) Interaction between the amino groups of collagen and the paired aldehyde groups of DAC; (b) hydrogen bonds formed between the hydroxyl groups of SiO₂ in SiO₂/DAC and the hydroxyl groups or amino groups of collagen; (c) more SiO₂ in SiO₂/DAC replaced aldehyde groups and bonded to the molecules of collagen).

SDS-PAGE was performed to identify the molecular weight distribution of collagens. As can be seen from Figure 5, the pattern for all the collagens consists of two α -chains ($\alpha 1$ and $\alpha 2$) as the major constituents. Components with higher molecular weight (MW), including β - and γ -components, were also observed. This suggests that the native triple-helix of collagen was not destroyed by the introduction of cross-links, which was confirmed by the quite similar characteristics observed between the FTIR spectra of all of the collagens (as shown in Figure 6). In the case of collagen modified by

DAC, the band intensity for α -chains and β -components was significantly reduced, which could be due to the formation of cross-linked aggregates with higher MW that could not get through the gel [35]. However, as displayed in the gel patterns, the chemical cross-linking effect on collagen became weaker when SiO_2/DAC or pure SiO_2 were employed.

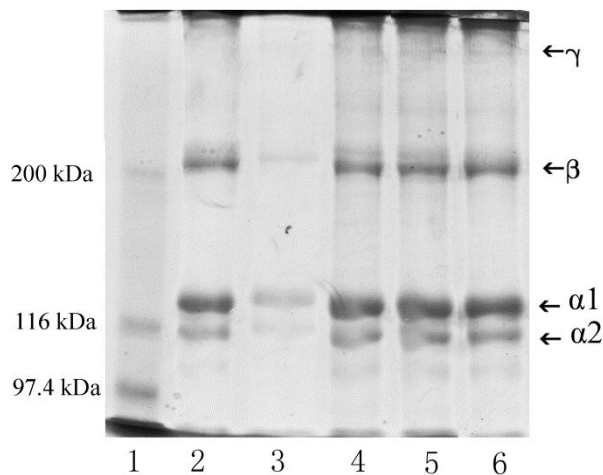


Figure 5. SDS-PAGE patterns of native collagen and collagens modified by the SiO_2/DAC hybrid materials: (lane 1) protein markers; (lane 2) native collagen; (lane 3) collagen modified by DAC; (lane 4) collagen modified by SiO_2/DAC (1/10); (lane 5) collagen modified by SiO_2/DAC (1/2); (lane 6) collagen modified by SiO_2 .

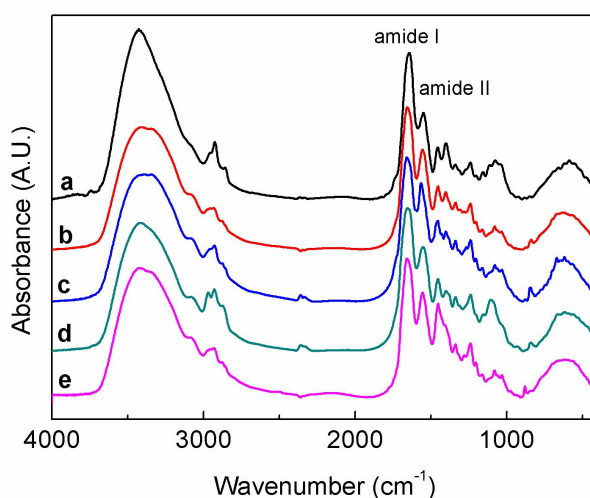


Figure 6. FTIR spectra of native collagen (a), and collagen cross-linked by the SiO_2/DAC hybrid materials: collagen cross-linked by DAC (b); collagen cross-linked by SiO_2/DAC (1/10) (c); collagen cross-linked by SiO_2/DAC (1/2) (d); and collagen cross-linked by SiO_2 (e).

3.4. DSC Thermograms of Cross-Linked Collagens

DSC thermograms of native collagen and collagen modified by the SiO_2/DAC hybrid materials are shown in Figure 7. Endothermic peaks associated with the helix-coil transition due to the thermal disruption of hydrogen bonds, which was thought to stabilize the triple helix of collagen, were detectable from the heat flow curves as a function of temperature. The un-modified collagen had a denaturation temperature (T_d) of 38.9 °C, which was in line with previous reports [36,37]. As for collagen cross-linked by DAC, the T_d was significantly increased by 14.4 °C. However, the T_d of cross-linked collagen by SiO_2/DAC decreased with increasing SiO_2/DAC ratios, but was still

obviously higher than that of the un-modified collagen. Note that the cross-linking of pure SiO₂ had a slight effect on the thermal stability of collagen.

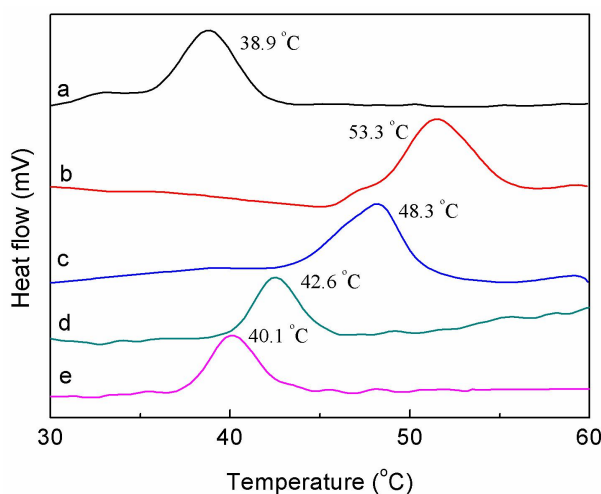


Figure 7. DSC thermograms of native collagen (a), and collagen cross-linked by the SiO₂/DAC hybrid materials: collagen cross-linked by DAC (b); collagen cross-linked by SiO₂/DAC (1/10) (c); collagen cross-linked by SiO₂/DAC (1/2) (d); and collagen cross-linked by SiO₂ (e).

3.5. Dynamic Rheology Tests of Cross-Linked Collagens

It was reported that cross-linked collagen was more resistant to deformation and to flow than native collagen [38], which was reflected in its viscoelastic properties. The frequency dependence of G' , $\tan \delta$ and η^* for all the samples over the frequency range of 0.01–10 Hz at 25 °C is depicted in Figure 8. As can be seen from Figure 8, the values of G' were increased exponentially with the addition of DAC. However, the values of G' decreased remarkably for the collagen modified by SiO₂/DAC (1/10), although they were still greater than those of the un-modified collagen. With increasing SiO₂/DAC ratios, the values of G' decreased continuously. It was interesting to note that collagen modified by SiO₂ displayed a higher G' than that of native collagen when the shearing frequency was no more than 0.2 Hz, while the values were close to those of native collagen when the shearing frequency further increased to 10 Hz. It seemed that the entanglement network of collagen was not stable enough because of the weak interactions between collagen and SiO₂, and thus the network was easily destroyed when the samples underwent a strong mechanical treatment.

The technique of dynamic oscillation is useful in resolving the structural properties of materials into a solid-like and a liquid-like response (G' and G'' , respectively) [39]. Generally, the ratio G''/G' , which is defined as the loss tangent ($\tan \delta$), was used to describe the flowing behavior of molecules, and it crossed the threshold ($\tan \delta = 1$) from solid-like to liquid-like behavior [40]. That is, the smaller the value of $\tan \delta$, the more rubbery or elastomeric the behavior of the material was [41]. The $\tan \delta$ value of all samples was smaller than 1 under our experimental conditions, indicating a solid-like network of collagen solution. However, as DAC was added, the modified collagen exhibited $\tan \delta$ values below 0.17, indicating the absolute solid-like behavior of this sample. When the SiO₂/DAC hybrid materials were used, the $\tan \delta$ of collagen increased to different extents, suggesting that the solid-like network tended to become a liquid-like network, due to the moderate cross-linking effect of the hybrid modifier. With respect to the sample modified by SiO₂, the $\tan \delta$ curves displayed a trend similar to that of G' when the shearing frequency was increased, which again confirmed the weak interaction between collagen and SiO₂.

From the changes in the complex viscosity (η^*) (Figure 8c), it could be seen that all samples showed a shear thinning behavior. Note that the η^* values of collagen cross-linked by DAC were far larger than those for the other samples, indicating that a rapid cross-linking reaction had occurred between

collagen and DAC, while the η^* values of collagen cross-linked by SiO₂/DAC or SiO₂ decreased as the ratio of SiO₂ in SiO₂/DAC increased. The decrease in viscosity due to the introduction of SiO₂ was in favor of processing for collagen solution.

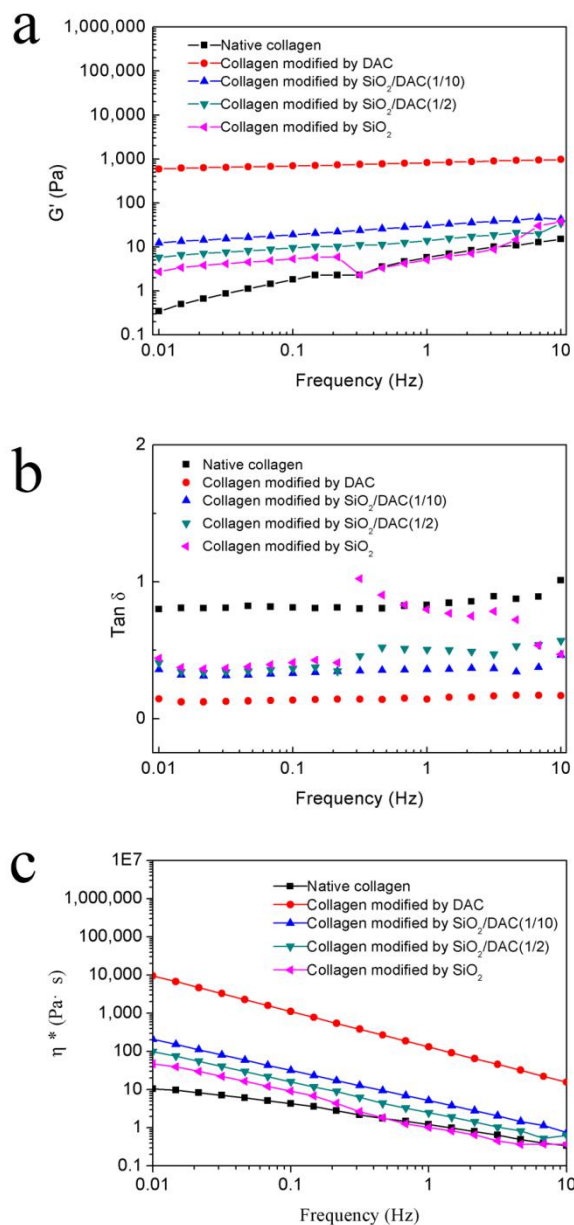


Figure 8. G' (a), $\tan \delta$ (b) and η^* (c) of native collagen and collagen cross-linked by the SiO₂/DAC hybrid materials.

3.6. FESEM of Cross-Linked Collagens

The FESEM images, as shown in Figure 9, displayed a well-ordered porous structure for native collagen (Figure 9a). As for collagen modified by DAC (Figure 9b), the sponge had a heterogeneous structure, which was a result of the excessively fast cross-linking effect. It could be observed from Figure 8b that the permeability of pores was decreased, and the aggregation of collagen was enhanced due to the cross-linking effect of DAC. In contrast, with the increased ratio of SiO₂/DAC, the morphological characteristics of porosity and fibrillar network of modified collagen became evident, particularly for the sample modified by SiO₂/DAC(1/2) (Figure 9d), suggesting that a moderate cross-linking effect on collagen could be imposed by the hybrid modifier. It should be noted

that collagen modified by the pure SiO₂ had a distinct morphology compared to the other samples; that is, a large number of fibers were visible. This morphology was quite similar to that reported previously [34], indicating that the aggregation behavior of collagen was influenced by the addition of nano-SiO₂.

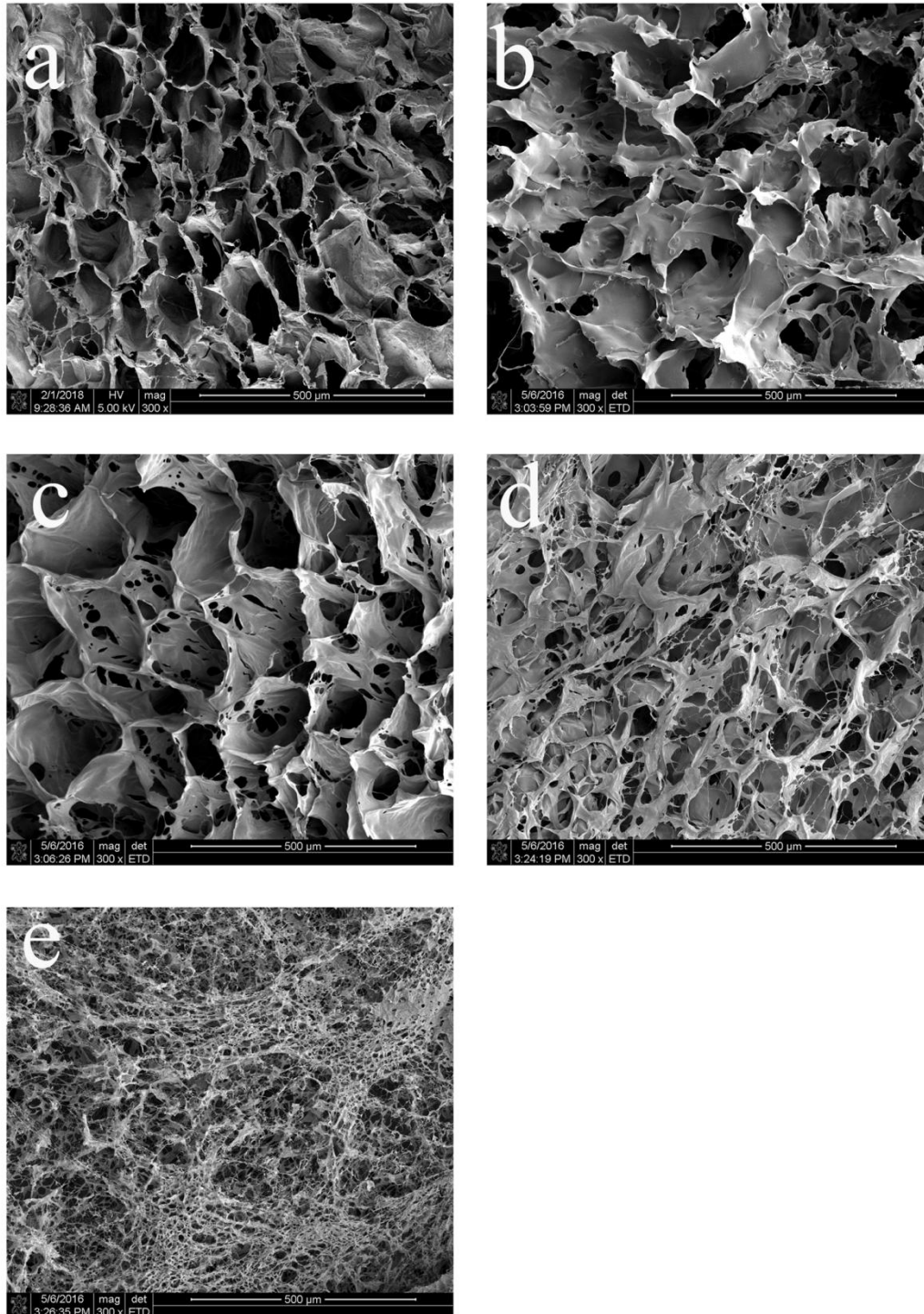


Figure 9. FESEM images (scale bar = 500 μm) of native collagen (a), and collagen cross-linked by the SiO₂/DAC hybrid materials: collagen cross-linked by DAC (b); collagen cross-linked by SiO₂/DAC(1/10) (c); collagen cross-linked by SiO₂/DAC(1/2) (d); and collagen cross-linked by SiO₂ (e).

4. Conclusions

A novel cross-linking agent of collagen was prepared via the hybridization of DAC and nano-SiO₂. The structure of the SiO₂/DAC hybrid materials was studied. Then, SiO₂/DAC hybrid materials were added into collagen solutions. Multiple sources of evidence were provided that the triple-helix of collagen was not destroyed; rather, the thermal stability could be significantly improved due to the interactions between SiO₂/DAC and collagen. Compared to collagen modified by pure DAC, collagen modified by the SiO₂/DAC hybrid materials exhibited a better flowability, protecting collagen solutions against excessive gelation. This will be of significance for the development of new cross-linkers and for the process and design of materials based on collagen in the format of solution.

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References

1. Patino, M.G.; Neiders, M.E.; Andreana, S.; Noble, B.; Cohen, R.E. Collagen: An overview. *Implant Dent.* **2002**, *11*, 280–285. [[CrossRef](#)] [[PubMed](#)]
2. Müller, W.E.G.; Relkovic, D.; Ackermann, M.; Wang, S.; Neufurth, M.; Radicevic, A.P.; Ushijima, H.; Schröder, H.C.; Wang, X. Enhancement of Wound Healing in Normal and Diabetic Mice by Topical Application of Amorphous Polyphosphate. Superior Effect of a Host–Guest Composite Material Composed of Collagen (Host) and Polyphosphate (Guest). *Polymers* **2017**, *9*, 300. [[CrossRef](#)]
3. Aravamudhan, A.; Ramos, D.M.; Nip, J.; Kalajzic, I.; Kumbar, S.G. Micro-Nanostructures of Cellulose-Collagen for Critical Sized Bone Defect Healing. *Macromol. Biosci.* **2017**, *18*, 1700263. [[CrossRef](#)] [[PubMed](#)]
4. Ford, C.N.; Bless, D.M.; Loftus, J.M. Role of injectable collagen in the treatment of glottic insufficiency: A study of 119 patients. *Ann. Otol. Rhinol. Laryngol.* **1992**, *101*, 237–247. [[CrossRef](#)] [[PubMed](#)]
5. Toung, J.S.; Ogle, R.C.; Morgan, R.F.; Lindsey, W.H. Insulinlike Growth Factor 1– and 2–Augmented Collagen Gel Repair of Facial Osseous Defects. *Arch. Otolaryngol. Head Neck Surg.* **1999**, *125*, 451–455. [[CrossRef](#)] [[PubMed](#)]
6. Ehrlich, H. Chitin and collagen as universal and alternative templates in biomineralization. *Int. Geol. Rev.* **2010**, *52*, 661–699. [[CrossRef](#)]
7. Cuq, B.; Gontard, N.; Guilbert, S. Proteins as agricultural polymers for packaging production. *Cereal Chem.* **1998**, *75*, 1–9. [[CrossRef](#)]
8. Friess, W. Collagen–biomaterial for drug delivery¹. *Eur. J. Pharm. Biopharm.* **1998**, *45*, 113–136. [[CrossRef](#)]
9. Heinemann, S.; Heinemann, C.; Ehrlich, H.; Meyer, M.; Baltzer, H.; Worch, H.; Hanke, T. A novel biomimetic hybrid material made of silicified collagen: Perspectives for bone replacement. *Adv. Eng. Mater.* **2007**, *9*, 1061–1068. [[CrossRef](#)]
10. Ehrlich, H.; Wysokowski, M.; Żółtowskaaksamitowska, S.; Petrenko, I.; Jesionowski, T. Collagens of Poriferan Origin. *Mar. Drugs* **2018**, *16*, 79. [[CrossRef](#)] [[PubMed](#)]
11. Gough, J.E.; Scotchford, C.A.; Downes, S. Cytotoxicity of glutaraldehyde crosslinked collagen/poly (vinyl alcohol) films is by the mechanism of apoptosis. *J. Biomed. Mater. Res.* **2002**, *61*, 121–130. [[CrossRef](#)] [[PubMed](#)]
12. Usha, R.; Ramasami, T. Effect of crosslinking agents (basic chromium sulfate and formaldehyde) on the thermal and thermomechanical stability of rat tail tendon collagen fibre. *Thermochim. Acta* **2000**, *356*, 59–66. [[CrossRef](#)]
13. Rault, I.; Frei, V.; Herbage, D.; Abdul-Malak, N.; Huc, A. Evaluation of different chemical methods for cross-linking collagen gel, films and sponges. *J. Mater. Sci. Mater. Med.* **1996**, *7*, 215–221. [[CrossRef](#)]

14. Scheffel, D.L.S.; Hebling, J.; Scheffel, R.H.; Agee, K.A.; Cadenaro, M.; Turco, G.; Breschi, L.; Mazzoni, A.; de Souza Costa, C.A.; Pashley, D.H. Stabilization of dentin matrix after cross-linking treatments, in vitro. *Dent. Mater.* **2014**, *30*, 227–233. [[CrossRef](#)] [[PubMed](#)]
15. Saito, H.; Murabayashi, S.; Mitamura, Y.; Taguchi, T. Characterization of alkali-treated collagen gels prepared by different crosslinkers. *J. Mater. Sci. Mater. Med.* **2008**, *19*, 1297–1305. [[CrossRef](#)] [[PubMed](#)]
16. O'Connell, D.W.; Birkinshaw, C.; O'Dwyer, T.F. Heavy metal adsorbents prepared from the modification of cellulose: A review. *Bioresour. Technol.* **2008**, *99*, 6709–6724. [[CrossRef](#)] [[PubMed](#)]
17. Kang, H.; Liu, R.; Huang, Y. Graft modification of cellulose: Methods, properties and applications. *Polymer* **2015**, *70*, A1–A16. [[CrossRef](#)]
18. Li, J.; Wan, Y.; Li, L.; Liang, H.; Wang, J. Preparation and characterization of 2,3-dialdehyde bacterial cellulose for potential biodegradable tissue engineering scaffolds. *Mater. Sci. Eng. C* **2009**, *29*, 1635–1642. [[CrossRef](#)]
19. Kanth, S.V.; Ramaraj, A.; Rao, J.R.; Nair, B.U. Stabilization of type I collagen using dialdehyde cellulose. *Process Biochem.* **2009**, *44*, 869–874. [[CrossRef](#)]
20. Zhang, M.; Li, J.; Ding, C.; Liu, W.; Li, G. The rheological and structural properties of fish collagen cross-linked by N-hydroxysuccinimide activated adipic acid. *Food Hydrocoll.* **2013**, *30*, 504–511. [[CrossRef](#)]
21. Fan, H.; Li, L.; Shi, B.; He, Q.; Peng, B. Characteristics of leather tanned with nano-SiO₂. *J. Am. Leather Chem. Assoc.* **2005**, *100*, 22–28.
22. Niu, L.; Jiao, K.; Qi, Y.; Yiu, C.K.Y.; Ryou, H.; Arola, D.D.; Chen, J.; Breschi, L.; Pashley, D.H.; Tay, F.R. Infiltration of Silica Inside Fibrillar Collagen. *Angew. Chem. Int. Ed.* **2011**, *123*, 11892–11895. [[CrossRef](#)]
23. Zhang, M.; Yang, J.; Ding, C.; Huang, L.; Chen, L. A novel strategy to fabricate water-soluble collagen using poly(γ -glutamic acid)-derivatives as dual-functional modifier. *React. Funct. Polym.* **2018**, *122*, 131–139. [[CrossRef](#)]
24. Varma, A.; Kulkarni, M. Oxidation of cellulose under controlled conditions. *Polym. Degrad. Stab.* **2002**, *77*, 25–27. [[CrossRef](#)]
25. Kim, U.-J.; Kuga, S. Thermal decomposition of dialdehyde cellulose and its nitrogen-containing derivatives. *Thermochim. Acta* **2001**, *369*, 79–85. [[CrossRef](#)]
26. Laemmli, U.K. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature* **1970**, *227*, 680–685. [[CrossRef](#)] [[PubMed](#)]
27. Jiang, X.; Yang, Z.; Peng, Y.; Han, B.; Li, Z.; Li, X.; Liu, W. Preparation, characterization and feasibility study of dialdehyde carboxymethyl cellulose as a novel crosslinking reagent. *Carbohydr. Polym.* **2016**, *137*, 632–641. [[CrossRef](#)] [[PubMed](#)]
28. Kim, U.-J.; Kuga, S.; Wada, M.; Okano, T.; Kondo, T. Periodate oxidation of crystalline cellulose. *Biomacromolecules* **2000**, *1*, 488–492. [[CrossRef](#)] [[PubMed](#)]
29. Rowen, J.W.; Forziati, F.H.; Reeves, R.E. Spectrophotometric Evidence for the Absence of Free Aldehyde Groups in Periodate-oxidized Cellulose¹. *J. Am. Chem. Soc.* **1951**, *73*, 4484–4487. [[CrossRef](#)]
30. Tang, S.; Zou, P.; Xiong, H.; Tang, H. Effect of nano-SiO₂ on the performance of starch/polyvinyl alcohol blend films. *Carbohydr. Polym.* **2008**, *72*, 521–526. [[CrossRef](#)]
31. Zhang, M.; Ding, C.; Chen, L.; Huang, L. The preparation of cellulose/collagen composite films using 1-ethyl-3-methylimidazolium acetate as a solvent. *BioResources* **2014**, *9*, 756–771. [[CrossRef](#)]
32. Keshk, S.; Sameshima, K. Influence of lignosulfonate on crystal structure and productivity of bacterial cellulose in a static culture. *Enzym. Microb. Technol.* **2006**, *40*, 4–8. [[CrossRef](#)]
33. Varma, A.J.; Chavan, V.B. A study of crystallinity changes in oxidised celluloses. *Polym. Degrad. Stab.* **1995**, *49*, 245–250. [[CrossRef](#)]
34. Eglin, D.; Maalheem, S.; Livage, J.; Coradin, T. In vitro apatite forming ability of type I collagen hydrogels containing bioactive glass and silica sol-gel particles. *J. Mater. Sci. Mater. Med.* **2006**, *17*, 161–167. [[CrossRef](#)] [[PubMed](#)]
35. Zhang, M.; Wu, K.; Li, G. Interactions of collagen molecules in the presence of N-hydroxysuccinimide activated adipic acid (NHS-AA) as a crosslinking agent. *Int. J. Biol. Macromol.* **2011**, *49*, 847–854. [[CrossRef](#)] [[PubMed](#)]
36. Ding, C.; Zhang, M.; Li, G. Rheological properties of collagen/hydroxypropyl methylcellulose (COL/HPMC) blended solutions. *J. Appl. Polym. Sci.* **2014**, *131*. [[CrossRef](#)]

37. Liu, W.; Li, G. Non-isothermal kinetic analysis of the thermal denaturation of type I collagen in solution using isoconversional and multivariate non-linear regression methods. *Polym. Degrad. Stab.* **2010**, *95*, 2233–2240. [[CrossRef](#)]
38. Wallace, D.G.; Rhee, W.; Reihanian, H.; Ksander, G.; Lee, R.; Braun, W.B.; Weiss, B.A.; Pharriss, B.B. Injectable cross-linked collagen with improved flow properties. *J. Biomed. Mater. Res.* **1989**, *23*, 931–945. [[CrossRef](#)] [[PubMed](#)]
39. Yang, Y.-L.; Kaufman, L.J. Rheology and confocal reflectance microscopy as probes of mechanical properties and structure during collagen and collagen/hyaluronan self-assembly. *Biophys. J.* **2009**, *96*, 1566–1585. [[CrossRef](#)] [[PubMed](#)]
40. Al-Ruqaie, I.M.; Kasapis, S.; Abeysekera, R. Structural properties of pectin-gelatin gels. Part II: Effect of sucrose/glucose syrup. *Carbohydr. Polym.* **1997**, *34*, 309–321. [[CrossRef](#)]
41. Korhonen, M.; Hellen, L.; Hirvonen, J.; Yliruusi, J. Rheological properties of creams with four different surfactant combinations-effect of storage time and conditions. *Int. J. Pharm.* **2001**, *221*, 187–196. [[CrossRef](#)]



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