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Potent and regulable crosslinking of ultrafine fibrous protein scaffolds for tissue engineering using cytocompatible disaccharide derivative

Helan Xu,[a] Peng Liu,[b] Xiang Mi,[b] Lan Xu,[c] Yiqi Yang,[a,d,e,*]

[a] Department of Textiles, Merchandising and Fashion Design, 234, HECO Building, University of Nebraska-Lincoln, Lincoln, NE 68583-0802, United States

[b] Key Laboratory of Science and Technology of Eco-Textiles, Ministry of Education, Donghua University, Shanghai 201620, China

[c] Department of Agronomy and Horticulture, University of Nebraska-Lincoln, 279 Plant Science Hall, Lincoln, NE 68583-0915, United States

[d] Department of Biological Systems Engineering, 234, HECO Building, University of Nebraska-Lincoln, Lincoln, NE 68583-0802, United States

[e] Nebraska Center for Materials and Nanoscience, 234, HECO Building, University of Nebraska-Lincoln, Lincoln, NE 68583-0802, United States

* Corresponding Author. Tel: +001 402 472 5197; Fax: +001 402 472 0640; E-mail: yyang2@unl.edu

Abstract

Sucrose, a naturally-occurring disaccharide, could be oxidized into polar polyaldehydes to improve the performance properties of tissue engineering scaffolds composed of three-dimensionally arranged ultrafine protein fibers in a controllable manner. With significantly better water stability, *in vitro* study demonstrated that biocompatibility of the oxidized sucrose crosslinked scaffolds was similar to the citric acid crosslinked ones. Due to the structural similarity to the major component in native extracellular matrices (ECMs), proteins had advantages over other macromolecules for development of tissue engineering scaffolds. We have successfully developed three-dimensional (3D) ultrafine fibrous structures from proteins that could mimic the authentic 3D architectures of native ECMs. However, the enlarged contacting area exposed to water worsened the poor water stability of proteins, and thus necessitated potent and non-toxic crosslinking. Citric acid, a biobased crosslinker, was widely recognized as safe and showed good crosslinking efficiency among non-toxic crosslinkers for proteins, though still less potent than aldehydes. In this research, sucrose was oxidized into non-volatile polyaldehydes with high polarity and lower toxicity. Comparing to those crosslinked with citric acid, the 3D ultrafine fibrous zein scaffolds crosslinked with oxidized sucrose showed significantly better water stability and similar cytocompatibility via i*n vitro* study with preosteoblasts. In summary, oxidized sucrose could be a safe and potent crosslinker to improve water stability of macromolecule-based materials for medical and industrial applications.

Keywords: 3D scaffold; Electrospinning; Protein; Oxidized sucrose; Non-toxic crosslinking; Water-stable.

Introduction

With specific advantages over other natural macromolecules and synthetic polymers, proteins, such as gelatin, silk fibroin and zein, could be preferred materials in biomedical applications. Besides their biocompatibility and biodegradability, proteins have similar molecular structures to collagens, the major components in native tissues and organs.[1] Proteins are also versatile in delivery of diverse therapeutic agents, as the surface charge of proteins could be tuned by adjusting environmental pH, and the hydrophobic domains in polypeptides might facilitate loading of hydrophobic payloads.[2, 3] Proteins in three-dimensional (3D) ultrafine fibrous architectures could mimic native ECMs in both aspects of materials and structures, and thus could better fulfill the basic goal of restoration of the functions of native ECMs in tissue engineering.

Electrospinning has been widely used to develop 3D structures, due to its versatility and high efficiency comparing to other methods.[4] The electrospinning methods that could produce 3D and two-dimensional (2D) fibrous structures were defined as 3D electrospinning and 2D electrospinning, respectively. Till now, most 3D electrospinning was achieved via incorporation of coarse fibers,[5] porogens (ice crystals, salts, etc.)[6] and coagulation bath[7] in conventional 2D electrospinning. These approaches only increased distances among the fibers, but could not change planar deposition of fibers. Resultantly, the final structures did not have stereoscopically random fiber orientation to sufficiently resemble the native ECMs.

Development of 3D ultrafine fibrous scaffolds from well-recognized proteins in biomedical areas remained challenging due to their further decreased water stability. Most proteins have water stability inferior to other macromolecules. Comparing to forms of films, 2D fibrous mats, 3D non-fibrous sponges, 3D fibrous proteins had the worst wet dimensional stability and mechanical properties due to their much larger surface area exposed to water. We developed 3D randomly oriented ultrafine fibers via conductivity regulation of the spinning dopes for electrospinning.[8] However, all the scaffolds with better water stability were produced from less recognized highly crosslinked proteins, such as feather keratin,[9] soy protein[10] and wheat

protein.[11] Regarding other known proteins for tissue engineering, such as gelatin and zein, non-toxic and potent chemical crosslinking method was in need to overcome their poor water stability before they could be fabricated into 3D ultrafine fibrous forms.[12, 13]

Citric acid, a widely studied non-toxic crosslinker for protein, was a polycarboxylic acid fermented from starch. Multiple proteins, including collagen,[14] gelatin,[15] peanut protein[16] and wheat gliadin,[17] in the forms of nanofibers, microfibers and films all have been crosslinked using citric acid. In vitro study in the above work proved the biocompatibility of citric acid crosslinking. Crosslinking effects of CA were better than most existing non-cytotoxic crosslinkers, however, still inferior to small molecule aldehydes.

To the best of our knowledge, the most potent crosslinker might be small molecule aldehydes, such as glutaraldehyde and formaldehyde.[18] Due to their high reactivity and volatility, small molecule aldehydes could readily react with proteins under room temperature in the form of vapor.[14] For the same reason, small aldehydes are usually toxic and carcinogenic as they could readily react with proteins and DNAs in body under physiological conditions.[19, 20] Besides, the highly volatile small molecule aldehydes were harmful to the environment and operators.[21]

Toxicity could be remarkably decreased via reducing volatility and enhancing polarity of aldehydes.[22] Boiling points of aldehydes could be raised by increasing number of carbons and polarity of the backbones. The resultant steric hindrance and electrical repulsion could also alleviate the reactivity of aldehydes. To reduce the volatility and increase polarity of aldehydes, multiple polysaccharides have been oxidized into polar polyaldehydes and used to improve water stability and mechanical properties of macromolecules for multiple applications.[23, 24, 25] However, till now, no work has been conducted to evaluate safety and effectiveness of using oxidized polysaccharides to crosslink the 3D ultrafine fibrous scaffolds.

Experimental

Materials

Zein (F-4000), the corn protein, was purchased from Freeman Industries LLC, Tuckahoe, NY, USA. Sodium dodecyl sulfate (SDS), citric acid (CA), sodium hypophosphite (SHP), sucrose, glycerol, barium dichloride, sodium periodate, potassium chloride, methylene blue, hydrochloric acid, glycine, acetone, Dimethylacetamide (DMAc), acrylamide, 1,4-dithiothreitol, trimethyloaminomethane, N,N,N,N-tetramethyethylenediamine, N,N-methylene diacrylamide and coomassie brilliant blue G250 were purchased from Sinopharm Chemical Reagent Co., Ltd, Shanghai, China. Standard protein markers with molecular weight varying from 10-250 kDa were supplied by Bio-Rad Chemical Co., Ltd, Hercules, CA, USA. Bovine serum albumin (BSA) was purchased from Amresco, Solon, OH, USA. All reagents were AR grade and used as received.

Preparation of oxidized sucrose (OS)

Oxidized sucrose was prepared according to our previous study.[26] In brief, 10.26 g of sucrose and 19.50 g of sodium periodate were dissolved in 300 g of water, stirred in nitrogen atmosphere at 25 °C for 26 h. To terminate the reaction, 11.20 g of barium dichloride was added into the solution, stirred at 5 °C for 1 h. The filtrate containing 6 wt% of OS, actually polyaldehyde derivatives, was obtained and stored at 5 °C.

As the OS was a mixture of polyaldehydes with different numbers of aldehyde groups in each molecule, the molecular weight (MW) was difficult to estimate. As the highest MW reduction would be due to loss of 1 carbon, 1 oxygen and 8 hydrogen atoms, the possible lowest MW might be 342.30 of sucrose – 12.01 of carbon – 15.999 of oxygen – 1.008 of hydrogen × 8 = 308.243. The average MW of 325.272 of OS mixture was estimated by averaging the MWs of unreacted sucrose and the polyaldehyde with the lowest possible MW. The MW was used in the following estimation of OS concentrations.

To verify generation of aldehyde groups, the OS solution was characterized using ^{1}H nuclear magnetic resonance (^{1}H NMR). The filtrate was dried under reduced pressure and re-

dissolved in D_2O, and then analyzed on a Bruker AV-400 FT-NMR spectrometer (Bruker, Billerica, MA, USA).

Electrospinning and crosslinking of 3D ultrafine zein fibrous scaffolds

The 3D electrospinning of ultrafine zein fibrous scaffolds was adapted from previous work.[8, 9] To prepare original zein spinning dope, 25 wt% of zein and 25 wt% of SDS was dissolved in water and stirred at 70 °C for 1 hr.

To study the crosslinking effects, about 0.307, 0.922, 1.537, 2.459 and 3.074 mmol kg^{-1} (based on the weight of zein) of OS were added to the original zein spinning dope. The concentrations were equivalent to 1, 3, 5, 8 and 10 wt% of OS based on the weight of zein. For comparison, 1.39, 4.17, 6.95, 11.12, 13.90 mmol kg^{-1} of CA and SHP (based on the weight of zein) were added into the original zein spinning dope.[27] The concentration was also equivalent to 1, 3, 5, 8 and 10 wt% of CA based on the weight of zein. All the electrospinning parameters, including extrusion speed of 2 mL h^{-1}, voltage of 42 kV, and distance of 25 cm between needle and collecting board, were used for all the samples. The needle was negatively charged, and the collecting board was positively charged.

The obtained 3D zein fibrous scaffolds were heated at 150 °C for 2 hr to complete the crosslinking reaction, and then coagulated in solution containing 70% methanol/10% acetic acid for 30 min under 25 °C. To completely remove SDS, the fibrous scaffolds were subject to multiple times of aqueous 60% acetone solution with saturated KCl until SDS could not be detected using methylene blue assay kit. Subsequently, the scaffolds were rinsed in distilled water for at least three times and then freeze dried.

Morphologies of 3D zein scaffolds before and after crosslinking

Morphologies of the 3D scaffolds before and after crosslinking with OS and CA under dry and wet states were observed using a scanning electron microscope (JSM-5600LV, Akishima-Shi, Tky, Japan) and a Nikon A1 confocal laser scanning microscope (Nikon Inc.,

Melville, NY, USA), respectively. The average diameters of ultrafine zein fibers were measured on a software, Image J with 100 times of measurements for each data point.

Verification of reaction between OS and zein

The reaction between 8 wt% of OS and zein was confirmed using ^{1}H nuclear magnetic resonance (^{1}H NMR). The proteins with and without crosslinking were dissolved in deuterated DMSO and then analyzed on A Bruker AV-700 FT-NMR spectrometer (Bruker, Billerica, MA, USA).

Molecular weight distribution of zein before and after crosslinking

Molecular weight distribution of zein before and after crosslinking with OS and CA were evaluated using sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE). About 5 mg mL^{-1} Protein samples were dissolved in 1x SDS-PAGE sample buffer (Invitrogen, Grand Island, NY, USA), heated at 70 °C for 10 min, and then loaded into each lane (10 μL lane^{-1}) of a NuPAGE 4-12 % Bis-Tris gel (Invitrogen, Grand Island, NY, USA). After electrophoresis, the gel was fixed in 10% acetic acid in 25% of isopropanol, stained in the 0.06 wt% Coomassie Brilliant Blue G 250 solution in 10% acetic acid, followed by destaining in 10% acetic acid for 9 h with gentle shaking. Images of the gel were taken for comparison.

Water stability of 3D ultrafine fibrous scaffolds

Effects of OS on the stability of 3D ultrafine fibrous zein scaffolds was studied in a physiological condition and compared with CA. The percentages for crosslinking were 8 wt% for OS and 10 wt% for CA. Crosslinked zein samples (100 mg each) were incubated in 4g of phosphate buffered saline (PBS, pH 7.4) at 37 °C for 30 days, removed and washed in distilled water for three times, freeze dried and conditioned. The weights of scaffolds before and after incubation were measured on a 5 digital balance (New Classic MS 105, Mettler Toledo, Columbus, OH). The % weight retention was calculated according the following equation (1).

$$\%\ \text{weight retention} = \frac{Weight\ of\ scaffolds\ after\ incubation\ (mg)}{Initial\ weight\ of\ scaffolds\ (mg)} \times 100\% \quad (1)$$

Mechanical analysis

The wet and dry compressive properties of 3D electrospun zein scaffolds crosslinked with CA and OS, were tested on a TA.XT.Plus texture analyzer (Texture Technologies Corp., Scarsdale, NY, USA). The electrospun 3D zein scaffolds under dry state, immersed in PBS for 12 hr at 37 °C were tested. All of the samples had a thickness between 20 and 30 mm. The unconfined compression property was measured using a 0.5-in.-diameter flat plastic plunger at a speed of 1 mm/s until 40% strain was reached.

Cytocompatibility evaluation

In vitro study with preosteoblast cells were used to evaluate the cytocompatibility of crosslinked 3D ultrafine fibrous zein scaffolds. Each specimen at about 10 mg was prepared and sterilized under 120 °C for 1 hr, and then placed in 48-well culture plates.

It has been proved that CA crosslinked zein scaffolds were favorable substrates that could effectively promote cell attachment and proliferation.[8,28,29] To specifically elucidate the influence of OS crosslinking on the cytocompatibility of scaffolds in this study, CA crosslinked 3D ultrafine fibrous zein scaffolds with morphological structures similar to the OS crosslinked ones were selected as controls. In this way, different cell accessibility in the scaffolds caused by different scaffold structures could be avoided.

MC 3T3-E1 preosteoblast cells were cultured in alpha-Modified Eagle's Medium (α-MEM, Gibco, Invitrogen, Grand Island, NY, USA) containing 10% Fetal Bovine Serum (FBS, Gibco, Invitrogen, Grand Island, NY, USA) with no ascorbic acid. The cells were seeded onto the scaffolds (1×10^5 cells mL^{-1}, 500 μL $well^{-1}$) and then cultured at 37 °C in a humidified 5% CO_2 atmosphere for different time intervals. At each time point, the samples were washed with PBS, placed in new 48-well plates containing 450 μL $well^{-1}$ 20% methanethiosulfonate (MTS) reagent (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA) in α-MEM and incubated at 37 °C in a humidified 5% CO_2 atmosphere for 3 h. After incubation,

150 μL of the solution from each well was pipetted into a 96-well plate and the optical densities were measured at 490 nm using a UV/vis multiplate spectrophotometer (Multiskan Spectrum, Thermo Scientific Waltham, MA, USA). The MTS solution in α-MEM without cells served as the blank.

Immnofluorescent observation was done in order to compare spreading and penetration of cells on OS and CA crosslinked scaffolds, a Nikon A1 confocal laser scanning microscope (Nikon Inc., Melville, NY, USA). After 3 days of culture, samples were washed with PBS, fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) and permeabilized with 0.1% Triton X-100 solution (Invitrogen, Grand Island, NY, USA) for 5 min. After another wash with PBS, cells were stained by Hoechst 33342 solution (Invitrogen, Grand Island, NY, USA) and observed under 488 nm for illustration of the nuclei.

Protein sorption

Protein sorption was performed by incubating 2.5 mg of scaffolds in 1 mL of phosphate buffered saline (PBS, 0.1 m, pH 7.4) containing bovine serum albumin (BSA) at concentrations of 417.4 μg mL^{-1} and 1134.0 μg mL^{-1}. After incubated under 37 °C for 12 hr, the mixture was centrifuged under 8000 rcf for 20 min, and the supernatant was collected. The concentration of the protein in the supernatant was then quantified with using a Bradford protein assay kit (DC protein assay kit, BioRad, Hercules, CA). A standard curve obtained with BSA in the range of 0.2-1 mg mL^{-1}. The amount of protein released from zein scaffolds during incubation was subtracted as blank in calculation. For comparison, the amounts of protein sorption were converted into unit of weight ratio using parameters existed in other publications.

Statistical analysis

A one-way analysis of variance with Tukey's pairwise multiple comparison was employed to analyze the data. The confidence interval was set at 95% and a P value less than 0.05

was considered to be a statistically significant difference. Data points labeled with the same symbols or characters were not significantly different from each other. The error bars shown in the figures indicate the standard deviations of the data.

Results and Discussion

To meet the requirements of water stability for biomedical applications, 3D ultrafine fibrous scaffolds needed stronger crosslinking than 2D fibrous scaffolds and 3D non-fibrous sponges, due to their much larger surface area.

Crosslinking of 3D ultrafine fibrous scaffolds with oxidized sucrose

Attributed to the presence of multiple aldehyde groups and highly polar backbones, polyaldehydes in oxidized sucrose might simultaneously have high reactivity and safety. Aldehyde groups could be produced via oxidization of vicinal diols in sucrose molecules by sodium periodate.[30] Oxidized sucrose was a mixture of highly polar tetraaldehydes, other polyaldehydes and their hemiacetal forms. A typical product among them was a tetraaldehyde with highly polar backbones with a number of hydroxyl groups as shown in Fig. 1.[31] The non-volatile oxidized sucrose showed lower reactivity but lower risk than glutaraldehyde, due to the bulky polar backbones. Oxidized sucrose could not only react with primary amines ($–NH_2$), but also with hydroxyl groups (-OH) to form hemiacetal, as shown in Fig. 1.

In Fig. 1, the ^{1}H-NMR spectra showed one proposed structure of hemiacetal derivatives of oxidized sucrose, in accordance with the results reported by de Wit.[31] The peaks representing one free aldehyde group with chemical shift at 8-8.5 ppm and four hemiacetal groups between 4.5 and 5 ppm, successfully proved that oxidation of sucrose generated polyaldehyde derivatives.

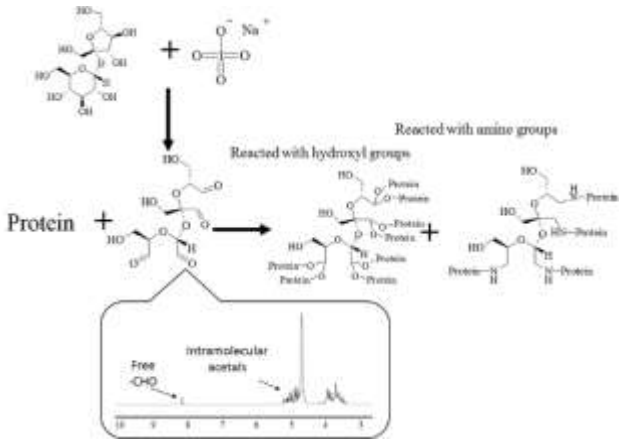

Fig. 1. Scheme of oxidation of sucrose by sodium periodate; reaction between OS and amine and hydroxyl groups on protein; and ^{1}H-NMR spectra of oxidized sucrose.

Morphology of 3D zein scaffolds under dry and wet conditions

In Fig. 2, all the 3D electrospun ultrafine zein fibrous scaffolds with or without crosslinking were composed of uniform continuous fibers, proving the good solubility and spinnability of zein with addition of the crosslinkers. The fibers without and with crosslinking did not show preferred directions of alignment in both front and side views, suggesting the stereoscopic random orientations of fibers. From the CLSM images in the right column, side views of the stereoscopic distribution of fibers in the scaffolds could be seen in all the three

scaffolds.

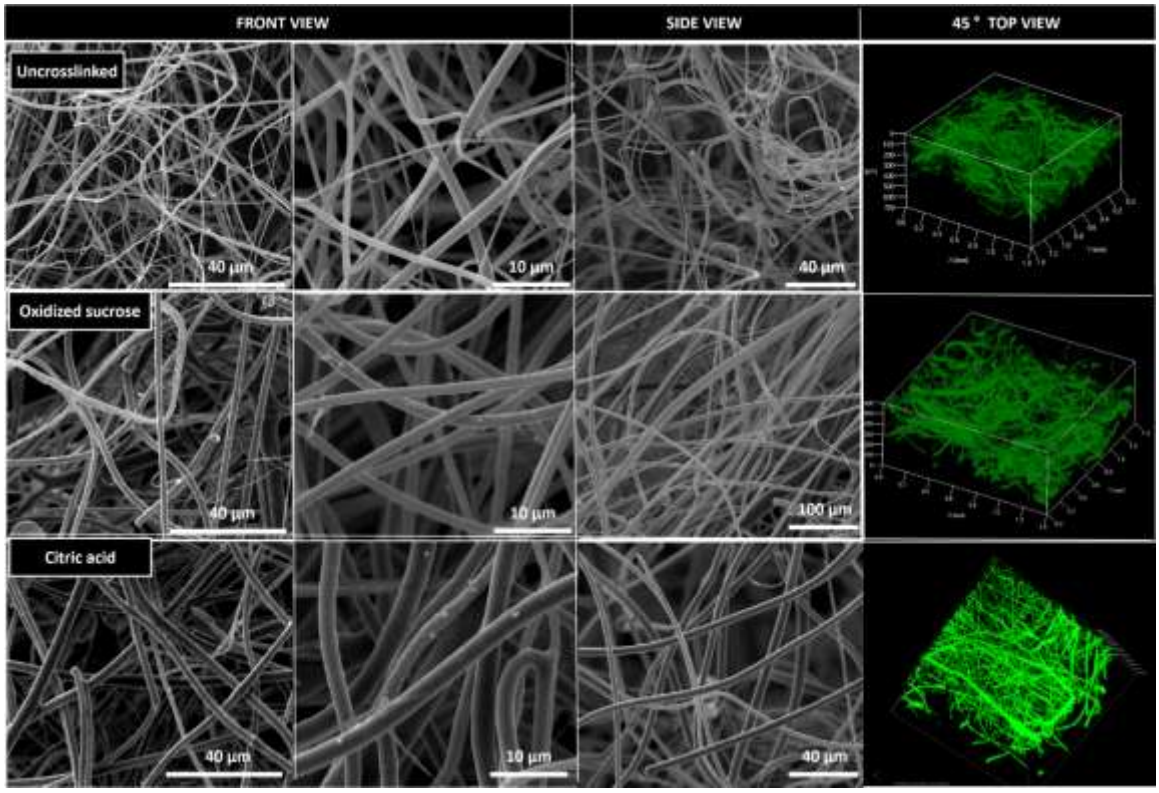

Fig. 2. SEM images of the front views (first and second columns) and side views (third column), and CLSM images of the top 45° view (fourth column) of the electrospun 3D ultrafine fibrous zein scaffolds without crosslinking, 5 wt% of OS and 10 wt% of CA. The spinning dopes were prepared by dissolving 25 wt% of zein, 25 wt% of SDS and crosslinkers in distilled water.

The diameters of individual fibers in the 3D ultrafine fibrous scaffolds increased from 1.12 ± 0.50 μm to 1.89 ± 0.83 μm after OS crosslinking and to 2.23 ± 0.98 μm after CA crosslinking. The pH change caused by incorporation of crosslinkers could lead to variation of fiber diameters. The isoelectrical point of zein was around pH 6,[32] and thus there was weaker electrical intermolecular repulsion when the pH of zein solution was closer to the pI. Here, The pH was 5.524 for the uncrosslinked zein solution, 4.276 for the OS added one and 3.152 for the CA added one. At higher pH, the electricl repulsion became weaker, and thus the fibers could be finer. The decreasing trend of pH was in accordance to the increasing trend of the fiber diameter.

Molecular weight of zein after OS and CA crosslinking

In Fig. 3i and 3ii, changes in molecular weight of zein crosslinked with different wt% of CA and OS were demonstrated. As the weight ratio of CA increased from 1% to 10%, the band at 20 kDa, the bands between 37 and 250 kDa became lighter and lighter though still existed at 10%. The band above 250 kDa became increasingly dark. As the weight ratio of OS increased from 1% to 10%, the band at 20 kDa, the bands between 37 and 250 kDa gradually faded due to the significantly decreased solubility.

The solubility of 3D crosslinked scaffolds in SDS-PAGE sample buffer was illustrated in Fig. 3iii. The uncrosslinked zein not listed here dissolved in SDS-PAGE completely after heated for 30 min at 70 °C. The solubility decreased as the concentration of crosslinker increased for both OS and CA. However, the overall solubility of OS crosslinked zein ultrafine fibers was much higher than the CA crosslinked ones. It could be inferred that more complicated protein networks could be built up via crosslinking with OS.

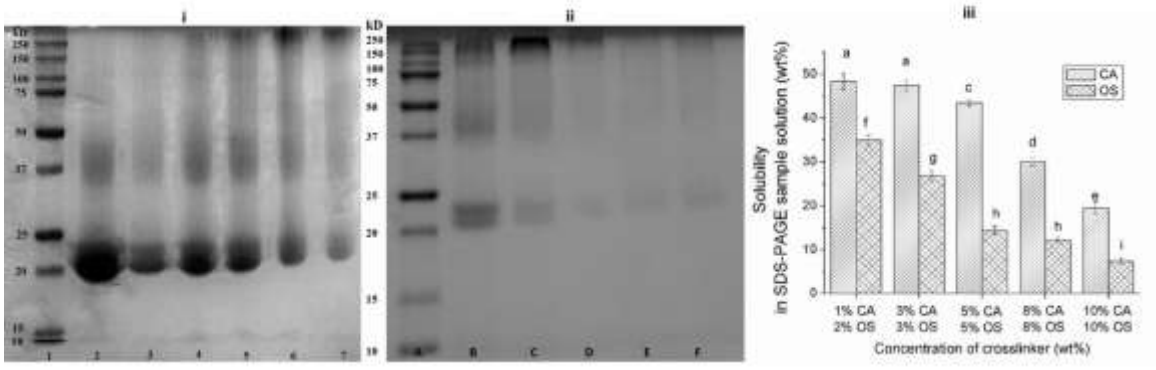

Fig. 3. i) SDS-PAGE of, Lane 1, standard protein marker; Lane 2, uncrosslinked zein; zein crosslinked with CA at concentrations of 1 wt% (Lane 3); 3 wt% (Lane 4); 5 wt% (Lane 5); 8 wt% (Lane 6); 10 wt% (Lane 7). **ii)** SDS-PAGE of, Lane A, standard protein marker; zein crosslinked with OS at concentrations of 2 wt% (Lane B); 3 wt% (Lane C); 5 wt% (Lane D); 8 wt% (Lane E); 10 wt% (Lane F). **iii)** Solubility of the samples in i) and ii) in the SDS-PAGE sample buffer. Different letters in the figure indicated significant difference. All the crosslinking reactions were carried out under 150 °C for 2 hr.

Verification of reaction between oxidized sucrose and zein

The ^{1}H-NMR spectra of uncrosslinked zein and 8 wt% OS crosslinked zein were illustrated in Fig. 4, verifying occurrence of reaction between OS and zein. Zein presented a typical NMR spectra of proteins with multiple hydrogen in various functional groups, such as –OH, –COOH, –CONH–, etc. However, in the spectra of OS crosslinked zein, two extra peaks at 8.4 ppm and 10.2 ppm could be observed, representing two types of aldehyde groups, respectively. The peak at 8.4 ppm, representing free –CHO, was also found in the spectra of OS as shown in Fig. 1. This might be due to the reduced reactivity of OS attributed to steric hindrance caused by various adjacent polar groups and the peak. In addition, the peak at 10.2 ppm might indicate aldehyde groups with adjacent electrophilic groups, such as –COOH and –CONH–, which could reduce the shielding effects by decreasing electron density of H in aldehyde groups. These aldehyde groups might be on the OS reacted on proteins, which contained abundant electrophilic groups, such as –COOH and –CONH–. The peak at 9.2 ppm represented the hydrogen in amide groups which ever under the influence of electrophilic carbonyl groups in carboxylic acids.

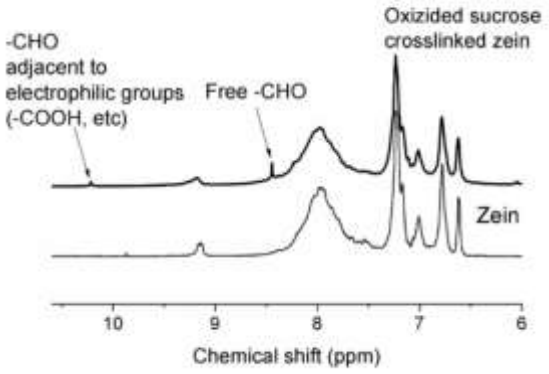

Fig. 4. ^{1}H-NMR spectra of uncrosslinked zein and OS crosslinked zein (crosslinking condition: 5% oxidized sucrose, heated at 150 °C for 2 hr)

Stability of 3D ultrafine fibrous zein scaffolds with CA and OS crosslinking in physiological conditions

The poor long-term stability in physiological conditions restricted applications of proteins as tissue engineering scaffolds. Fig. 5 demonstrated weight retention of 3D ultrafine fibrous zein scaffolds crosslinked with OS and CA in PBS after 30 days. The initial rate of weight reduction of the OS crosslinked scaffolds was much lower than the CA crosslinked ones. The OS crosslinked scaffolds preserved at least 88.0% of their weight, significantly higher than the 85.7% of CA crosslinked ones statistically. It could be inferred that oxidized sucrose could form more stable crosslinking networks in protein than CA.

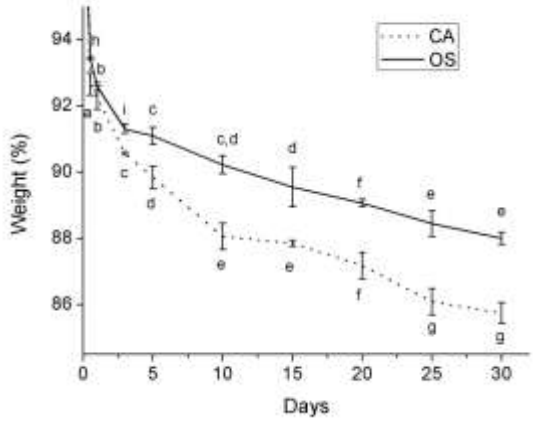

Fig. 5. Weight loss of electrospun 3D ultrafine fibrous zein scaffolds crosslinked with CA and OS over 30 days. About 100 mg of samples was incubated in 4 g of 1x PBS at 37 °C. Significant differences between samples are indicated by different letters.

Compressive properties of 3D zein fibrous scaffolds crosslinked with CA and OS

Fig. 6 demonstrates the unconfined compression property of electrospun ultrafine fibrous 3D scaffolds from zein after CA and OS crosslinking under dry and wet conditions. The compressive modulus of 3D zein scaffolds was around 1.8 kPa under dry state and decreased to around 0.4 kPa after being soaked in PBS for 24 h. The properties were similar to that of other 3D porous scaffolds from other proteins, such as silk fibroin[33] and gelatin.[34] However, these scaffolds

from fibroin and gelatin had smaller pores (less than 100 μm) encompassed in slices and thus were much denser than the 3D fibrous structures developed in this study. The mechanical strength of the keratin scaffolds still needed to be improved in a future study.

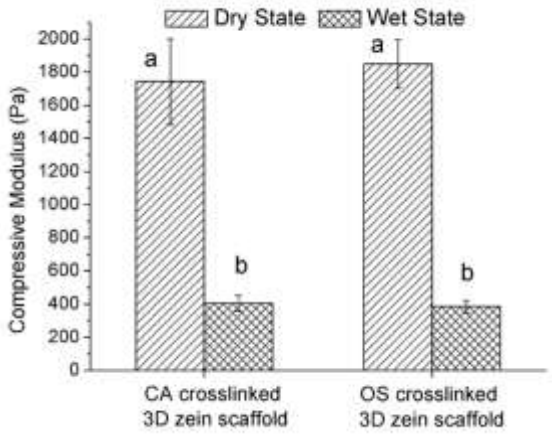

Fig. 6. Compressive modulus of electrospun 3D zein fibrous scaffolds crosslinked with CA or OS under dry and wet states. Data labeled with the same symbols were not significantly different from each other.

Cytocompatibility of 3D zein fibrous scaffolds crosslinked with CA and OS

Fig. 7 demonstrates the effect of crosslinking on growth of MC 3T3-E1 preosteoblast cells cultured on 3D ultrafine fibrous zein scaffolds evaluated via MTS assay. After culture for 4 h, similar amount of cells attached onto the 3D scaffold crosslinked with CA and OS. The number of cells kept increasing for 5 days on both of the 3D scaffolds crosslinked with OS and CA. The 3D ultrafine fibrous zein scaffolds showed high accessibility to cells due to the high porosity of the structures. The remarkably enhances water stability of the crosslinked scaffolds rendered preservation of the 3D structures during the time period of cell culture. It also could be inferred that, during the cell culture time period, there were no significant difference between the degradation products of both CA and OS crosslinked scaffolds on cell growth.

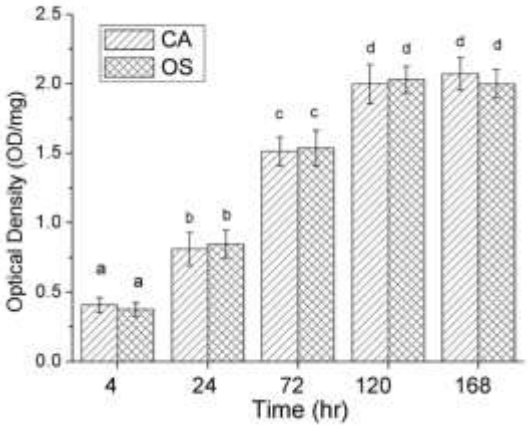

Fig. 7. Attachment and proliferation of preosteoblast cells on OS crosslinked and CA crosslinked 3D ultrafine fibrous zein scaffolds evaluated by MTS assay. Significant differences between samples are indicated by different letters.

Fig. 8 demonstrated that, after cultured for 7 days, proliferation of preosteoblast cells on a plane, which was 45 μm below the scaffold surface of OS crosslinked 3D ultrafine fibrous zein scaffolds was similar to that at the same layer of CA crosslinked scaffolds. The amounts and shapes of cells in the two views were similar. The results were in consistence with the results as shown in Fig. 7.

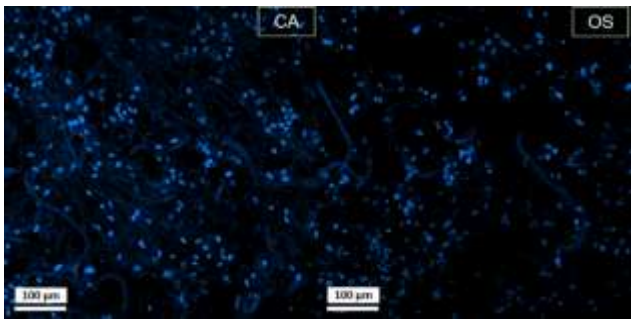

Fig. 8. Spreading of MC 3T3 cells on the plane at the depth of 45 μm in the electrospun 3D ultrafine fibrous zein scaffolds crosslinked with oxidized sucrose and glutaraldehyde after cultured for 7 days.

Combining the stability result in Fig. 5, the higher weight reduction of CA crosslinked scaffolds still might render poorer stability of CA crosslinked scaffolds than the OS crosslinked

ones. Long-term cell culture study should be carried out to compare the two crosslinking methods in the future.

Protein sorption on 3D zein fibrous scaffolds crosslinked with CA and OS

Table 1. Adsorption parameters of bovine serum albumin (BSA) onto the 3D zein ultrafine scaffolds crosslinked with CA and OS, respectively. (Conditions: 2.5 mg of scaffolds in 1 mL of PBS (0.1 m, pH 7.4), incubated at 37 °C for 12 hr)

	Concentration of BSA in PBS ($\mu g\ mL^{-1}$)	Solid to liquor ratio (mg:mg)	Amount of sorbed BSA/initially added BSA (%)	Amount of sorbed BSA at equilibrium ($mg\ g^{-1}$)	Ref
CA crosslinked 3D ultrafine zein scaffold	417.4	1:400	34.97 ± 2.63	58.39 ± 4.01	This work
CA crosslinked 3D ultrafine zein scaffold	1134.0	1:400	27.21 ± 4.58	123.41 ± 8.94	This work
OS crosslinked 3D ultrafine zein scaffold	417.4	1:400	38.02 ± 4.24	63.49 ± 3.85	This work
OS crosslinked 3D ultrafine zein scaffold	1134.0	1:400	29.82 ± 1.92	135.26 ± 5.33	This work
Nano-hydroxyapatite and poly(l-lactic acid) (PLLA)*	3500-5000	Infinite bath	-	8	35
Poly(d,l-lactide) (PDLA) nanofibrous scaffolds*	3500-5000	Infinite bath	-	22	36
Hydroxyapatite and poly(l-lactic acid) (PLLA) scaffolds*	3500-5000	Infinite bath	-	30	37

*The protein concentrations in the bath was calculated considering the protein contents in FBS ranged from 3.5 wt% to 5 wt%. The protein concentrations in PBS with 10% and 5% FBS were about 3500 to 5000 $\mu g\ mL^{-1}$ and about 1750-2500 $\mu g\ mL^{-1}$, respectively. The adsorption amounts were calculated using information provided in the paper, such as protein adsorbed on each scaffold, densities and sizes of scaffolds.

Table 1 demonstrates weight ratios of sorbed BSA/initially added BSA and amounts of BSA sorption on CA and OS crosslinked 3D ultrafine zein scaffolds. The weight ratios of sorbed BSA over the initially added BSA decreased as the concentrations of BSA solution increased. Under different conditions, the OS crosslinked zein scaffolds did not have significantly different BSA sorption amounts, comparing to the CA crosslinked ones. It were reported that, poly(d,l-lactide) (PDLA) nanofibrous scaffolds[35], nano-hydroxyapatite and poly(L-lactic acid) (PLLA) composite scaffolds[36] and hydroxyapatite and poly(l-lactic acid) (PLLA) scaffolds[37] adsorbed approximate 22, 8 and 30 mg g^{-1} of proteins under high protein concentrations at infinite sorption bath.

It could be inferred that, even at much lower initial protein concentrations under much lower liquor ratio, the crosslinked zein scaffolds could sorb much higher amounts of proteins than nanoscale PLA scaffolds. Due to more similar molecular structures, zein had much higher affinity, and thus could sorb higher amount of BSA than PLA. The higher accessibility of 3D ultrafine fibrous structures of zein scaffolds might also contribute to the higher loading of BSA.

The high protein sorption amounts could render crosslinked 3D ultrafine fibrous zein scaffolds favorable micro-environments for preosteoblast cells, comparing to the synthetic polymers. However, there were not significantly differences between the CA and OS crosslinkers. The protein sorption on the two scaffolds matched with the cell culture results.

Conclusions

Three-dimensional ultrafine fibrous zein scaffolds were crosslinked with potent non-toxic polyaldehydes derived from sucrose for tissue engineering applications and compared with those crosslinked with CA. The OS crosslinked 3D ultrafine fibrous zein scaffolds showed remarkable

increase in molecular weight and water stability. NMR analysis confirmed successful reaction between OS and zein. The weight loss of 3D zein ultrafine fibrous scaffolds crosslinked with OS in physiological environments was significantly lower than those crosslinked with CA. However, the protein sorption and mechanical properties of CA and OS crosslinked 3D zein scaffolds were not significantly different. Via *in vitro* study using preosteoblast cells, OS crosslinked scaffolds showed similarly good cytocompatibility comparing to CA. In summary, oxidized sucrose with multiple polar multi-carbon aldehydes could be non-toxic and effective crosslinker for proteins.

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Sucrose derived crosslinker enhanced water stability of ultrafine fibrous protein scaffolds efficiently and showed biocompatibility similar to citric acid.
80x40mm (300 x 300 DPI)