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# Non-toxic and clean crosslinking system for protein materials: Effect of extenders on crosslinking performance

Kaili Song [a, b], Helan Xu [b], Bingnan Mu [b], Kongliang Xie [a], Yiqi Yang [b, c, d, *]

[a] Key Laboratory of Science and Technology of Eco-Textiles, Ministry of Education, College of Chemistry, Chemical Engineering and Biotechnology, Donghua University, Shanghai, 201620, China
[b] Department of Textiles, Merchandising and Fashion Design, 234, HECO Building, University of Nebraska-Lincoln, Lincoln, NE, 68583-0802, United States
[c] Department of Biological Systems Engineering, 234, HECO Building, University of Nebraska-Lincoln, Lincoln, NE, 68583-0802, United States
[d] Nebraska Center for Materials and Nanoscience, 234, HECO Building, University of Nebraska-Lincoln, Lincoln, NE, 68583-0802, United States

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## ABSTRACT

One of the biggest disadvantages of protein cross-linking method is the toxicity of cross-linking agents. In this paper, a clean and non-toxic cross-linking system for protein materials using citric acid (CA) as crosslinker and polyols as cross-linking extender was established. Incorporation of polyols could enhance cross-linking performance of CA by forming branched network among protein molecules. Effect of polyol structure on the cross-linking performance was studied systematically, and keratin fiber was used as an example of protein materials. Extenders containing both primary and secondary hydroxyl groups were more effective in enhancing cross-linking efficiency. Changes in the amounts of carboxyl group, amine group and sulfhydryl group of keratin fiber after cross-linking were measured via potentiometric and conductometric titrations to quantify the cross-linking efficiency. Cross-linking mechanism was also illustrated. With proper numbers of primary hydroxyl groups, secondary hydroxyl groups and backbone length, polyols could be efficient in enhancing performance of this cross-linking system. This novel clean cross-linking system could be used for not only protein engineering, but also other areas where utilization of cross-linking agents are contemplated.

## 1. Introduction

Bio-based polymeric materials exhibit advantages over synthetic polymers due to their inherent biocompatibility, biodegradability, easy availability from reproducible resources and low cost (Kapoor and Kundu, 2016). Recently, there is an increasing interest in using biopolymers to replace and reduce the consumption of conventional polymers derived from petroleum (Xia and Larock, 2010). Protein, one of the naturally derived biopolymers, displays unique physico-chemical characteristics that confer them significant potential application in various fields such as food industry (Hosseini et al., 2016), medical field (Elzoghby et al., 2016), tissue engineering (Xu et al., 2014), environmental treatment (Song et al., 2017), and so on. However, protein materials show poor water stability due to a variety of hydrophilic groups such as carboxyl, hydroxyl and amide groups on its side chains. It is commonly known that the mechanical properties of protein materials are not satisfactory for practical application.

Cross-linking is a viable method to improve properties of protein materials. Protein molecules can achieve stronger inter or intro-molecular covalent bonds, closer chain packing as well as reduced molecule mobility by means of cross-linking (Sionkowska et al., 2010). A variety of cross-linking methods have been developed to improve the properties of protein materials including thermal cross-linking, enzymatic cross-linking (Qin et al., 2016), radiation cross-linking and chemical cross-linking (Xu et al., 2016). Upon heating under high temperature, the hydrogen bonds and non-polar hydrophobic interactions in protein molecules could be disrupted and cause the denaturation and thermal cross-linking of protein. However, it is not applicable in some areas, such as medical field, where the properties of protein material are highly important for its further application. Enzymatic cross-linking is usually done with the help of transglutaminase, a specific enzyme that can catalyze the formation of $\varepsilon$ -(glutamyl)-lysine crosslink in proteins

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* Corresponding author. Department of Textiles, Merchandising and Fashion Design, 234, HECO Building, University of Nebraska-Lincoln, Lincoln, NE 68583-0802, United States.

*E-mail address:* yyang2@unl.edu (Y. Yang).

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via acyl transfer reaction (Gaspar and De Góes-Favoni, 2015). However, due to the natural of enzyme, it is only suitable for several specific protein materials. Cross-linking by radiation is to expose proteins under ionizing radiation, which will cause oxidation of amino acids, rupture of covalent bonds, formation of protein free radicals, and induce polymerization within protein molecules (Benbettaieb et al., 2016). However, the energy of radiation can destroy the native structure of protein and cause degradation of protein materials. Chemical cross-linking is a relatively convenience and effective method. In most chemical cross-linking, bifunctional agents such as glutaraldehyde, diisocyanates and carbodiimides have been used. Glutaraldehyde is a high effective and widely used cross-linker for proteins. However, it possesses high toxicity not only to cell and biological system but also to the environment (Zimmer and McKinley, 2008). Therefore, it is imperative to develop non-toxic and cleaner cross-linking agents to replace the current counterparts.

Citric acid (CA) (Ghorpade et al., 2016), which is a common tricarboxylic acid with advantages such as low cost, renewability, and non-toxicity that can be used as cross-linking agents to crosslink cellulosic materials. CA was widely applied to improve the water stability and mechanical strength of different protein materials, such as, peanut film (Reddy et al., 2012), zein scaffold (Xu et al., 2015a), wool fabric (Mohsin et al., 2014), wheat gluten film (Xu et al., 2015b). However, the cross-linking efficiency is still not satisfying compared with that of glutaraldehyde. The extender could play significant role in CA cross-linking system (Jiang et al., 2013). We found that incorporation of polyols as cross-linking extenders could enhance cross-linking effects. By using polyols, it can form branched cross-linking network which could improve the cross-linking efficiency. In addition, polyols are most naturally derived and nontoxic as well as low in cost (Yao et at., 2013) and it should be more environmentally and economically feasible to use the combination of polycarboxylic acids and polyols for cross-linking (Liu et al., 2016). However, till now, there has been no reports published on the effects of chemical structures of polyols on the cross-linking results and relevant research should be carried out.

In this research, we chose hair keratin fiber as an example of protein materials for cross-linking study since the cross-linking agents currently in use for cross-linking hair fiber is high toxic and carcinogenic (Couto et al., 2013). Also the consumption of hair care products increases dramatically with the civilization of humankind and female liberation (Popescu and Höcker, 2007). Due to the increasingly strict rules and more awareness among the customers, it is highly demanded and necessary to develop cleaner nontoxic and environmentally-friendly productions for hair keratin fiber cross-linking. By adopting hair keratin fiber as protein material not only can study the cross-linking mechanism but also can replace the toxic hair cross-linking products. Six polyols with different backbone length and hydroxyl groups were systematic studied to investigate the effect of the structure of polyol on its performance. Reaction mechanism between CA, extender and hair keratin fiber was proposed. Changes in the amounts of carboxyl groups, amine groups and sulfhydryl groups after different cross-linking were measured via potentiometric and conductometric titrations to quantify the cross-linking efficiency. Combined methods of Raman spectrometry, wide-angle X-ray diffraction (WXRD) were used to investigate the secondary structures of keratin fibers after cross-linking reaction.

## 2. Experimental

### 2.1. Materials

The straight hair fiber was obtained from local hair salon in Shanghai, China. Cysteine (98.0%), sodium dodecyl sulfate (SDS, 99.0%), sodium hypophosphite (SHP, 99.0%), citric acid (CA, 99.0%), sodium carbonate (98.0%), 1,3-propanediol (99.0%), 1,4-butanediol (99.0%), 1,5-pentanediol (99.0%), 1,6-hexanediol (99.0%), 1,2,4-butatriol (99.0%), 1,2,3,4-butanetetrol (99.0%) were purchased from EMD Chemicals Inc. Gibbstown, NJ. 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, 99.0%) was purchased from G-Biosciences, St. Louis, MO. Urea (99.0%) was purchased from Oak Chemical, Inc. West Columbia, SC. The purity of chemicals was considered in all the calculations of concentrations.

### 2.2. Hair fiber cross-linking

Before cross-linking, all hair samples were rinsed and dried thoroughly. Hair cross-linking was following this two steps, reduction and cross-linking. In the reduction step, hair bundle was wounded up onto a glass rod tightly to create curls. Then soaked the glass rod into the reduction solution containing 2 M urea and 5 wt% cysteine, adjusted pH to 9.5 using sodium carbonate at a ratio of 1:50 under 50 °C for 20 min. After that, rinse the hair bundle thoroughly using tape water. Then immersed the hair bundle into cross-linking solution which containing 15 wt% citric acid, 50% of SHP based on the weight of citric acid and certain amount of polyol under ultrasonic for 5 min and then dried under 80 °C for 10 min. The hair bundle with cross-linking agent was heated at 180 °C for 4 min to complete cross-linking reaction after it was dried completely and washed thoroughly. After cross-linking treatment hair fiber will be set into a new shape (curly in this study), the cross-linking performance which is calculated by Equation (1) and the retention of mechanical properties of hair fiber were adopt to evaluate the cross-linking performance:

$$\text{Crosslinking performance} = \frac{\text{Number of loops after crosslinking}/\text{Fiber length after crosslinking}}{\text{Number of loops before crosslinking}/\text{Fiber length before crosslinking}} \times 100 \tag{1}$$

We also treated hair fiber using commercial products according to the recommendation procedures of each product as comparison. Ten commercial products with different brands and prices were randomly chosen from the local market. A commercial product with the highest perming performance among those was selected as the comparison.

### 2.3. Tensile properties

All hair samples were conditioned in conditioning room (21 °C, 65% relative humidity) for two days before testing. For wet tensile properties testing, hair samples were immersed in distilled water for 40 min and dried with lab paper before testing. Tensile properties of hair fiber were tested using an Instron tensile testing machine (Model 4400, Norwood, MA) according to ASTM standard