1. A non-toxic, cleaner and effective protein crosslinking system was developed.
2. Effect of polyols as extenders in the crosslinking reactions was investigated and the mechanism was illustrated.
3. Polyols with three or four carbons and three to four hydroxyl groups showed the best effect.
4. Development of performance-based predictive models for different structure of polyols using Response Surface Methodology.

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

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ABSTRACT

One of the biggest disadvantages of protein cross-linking method is 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.

KEYWORDS: Non-toxic cross-linking; Polyol; Keratin fiber; Citric acid.

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 have 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 applications.

Cross-linking is a viable method to improve properties of protein materials. Protein molecules can achieve stronger inter or intra-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 ϵ -(glutamyl)-lysine

crosslinks in proteins via acyl transfer reaction (Gaspar and De Góes-Favoni, 2015). However, due to the nature 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 convenient and effective method. In most chemical cross-linking, bifunctional agents such as glutaraldehyde, diisocyanates and carbodiimides have been used. Glutaraldehyde is a highly 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 a significant role in CA cross-linking system. We found that incorporation of polyols as cross-linking extenders could enhance cross-linking effects. By using polyols, it can form a 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 (Wang et al., 2001) and it should be more environmentally and economically feasible to use the

combination of polycarboxylic acids and polyols for cross-linking (Yazdani and Gonzalez, 2007). 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 highly 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 et al., 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 systematically 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 (WAXRD) 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 wound up onto a glass rod tightly to create curls. Then soaked the glass rod into the reduction solution containing 2M 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 tap 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:

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

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 D-3822. During testing, a gauge length of 1 inch and crosshead speed of 18 mm min⁻¹ was used. About 40 specimens were tested for each treatment condition.

2.4. Intrinsic viscosity of the hydrolyzed hair fiber solution

Molecular weight of hair fiber could increase with the increase of cross-linking degree. To verify the change in molecular weight, we determined the intrinsic viscosity of solution of hydrolyzed hair fibers cross-linked to different extents. Since hair fiber could not be dissolved without degradation of the keratin backbone, we first hydrolyzed hair fiber to make it soluble then crosslink the soluble hydrolyzed hair fiber according to the cross-linking process to verify the cross-linking reactions. Intrinsic viscosities of the hair fiber solutions were determined according to ASTM standard D 2857. An Ubbelohde capillary viscometer (Shanghai Liangjing Glassware Company, China) with capillary diameter of 0.5-0.6 mm was used. Formic acid was used as solvent. About 1 g of de-lipid hair fiber was first dissolved in 20 mL solution containing 1 M NaOH and 8M urea at 40 °C for 3 h, and then centrifuged. The supernatant was dialyzed against distill water for 3 days, and then precipitated using 0.1 N HCl at pH 4.6 to obtain the hydrolyzed hair keratin. The hydrolyzed hair protein was cross-linked under the same condition used in the second step of cross-linking.

2.5. Titration of sulfhydryl groups, carboxyl groups and amine groups

Solid-phase assay for total thiol group content was carried out according to the reported colorimetric reaction method (Chan and Wasserman, 1993). About 60 mg of chopped hair fiber was suspended in 2.0 mL of reaction buffer consisting of 8M urea, 10 mM DTNB, 3 mM ethylene-diaminetetraacetic acid (EDTA), and 0.2 M Tris-HCl, pH 8.0. Then it was incubated for 15min under N₂ followed by centrifuged at 10000 ×g for 20 min to get supernatant. The absorbance of the supernatant was read at 412 nm using UV/Vis spectrophotometer (Model DU[®] 720, Beckman Coulter Inc. Brea, CA). Three replica experiments were conducted for each data point. The total thiol group content was calculated by the following Equation (2):

$$\text{Thiol group content } \left(\frac{\text{mmol}}{\text{g}} \right) = \frac{(A/13600) \times V}{M} \quad (2)$$

Where A is the value of absorbance, V is the volume of reaction buffer; M is the weight of hair fiber.

Contents of amino group and carboxyl group in hair fiber were determined by titration using a Mettler Toledo SevenMulti[™] S47 pH/conductivity meter equipped with an Inlab[®] Expert Pro electrode and an Inlab[®] 730 probe for pH and conductivity measurements according to the previously reported method (Song et al., 2016). Around 2 g of delipidized and chopped hair fiber was weighed precisely and dispersed in 40 mL of standard 0.05 N HCl to protonated the carboxylic and amino groups in hair fiber. Standardized 0.05 N NaOH solution was used in titration. Conductivity and pH values were recorded after addition of about 0.1 mL NaOH solution each time. Three replica experiments were conducted for each data point.

2.6. Raman spectroscopy

Raman spectroscopy (InVia H 18415, Renishaw, Illinois, IL) was used to determine the secondary structure of keratin fiber before and after cross-linking. The hair samples were

171 embedded in epoxy resin, cured, and then microtomed to thickness of 2.0 μm , and mounted on
172 slide glasses. All Raman spectra were recorded 25 μm under the surface of hair fiber. The laser
173 excitation was provided by an argon ion laser at a cross slit of 100-200 μm with power of 30-50
174 mW at a wavelength of 632 nm.

175 2.7. Wide-angle X-ray diffraction (WAXD) measurement

176 X-ray diffraction studies were conducted using the Rigaku SmartLab Diffractometer (Rigaku
177 Co., Tokyo). Nickel-filtered $\text{CuK } \alpha$ radiation ($\lambda=0.15406$ nm) generated at 40 kV and 44 mA
178 was used. The incident beam was collimated through a collimator 100 μm and the diffraction
179 beam was received through a 1.8 mm wide and 5.8 mm long slit. For equatorial WAXD pattern,
180 hair fibers were placed in a plane perpendicular to the hair axis and the scanning speed was 0.01°
181 (2θ) per second. For meridional WAXD pattern, hair fibers were placed in a plane that was
182 parallel to the hair axis and the scanning speed was 0.02° (2θ) per second.

183 2.8. Response surface methodology (RSM) used for data analysis

184 R 3.3.0 software (R Development Core Team, 2016) was used for RSM design. A five-level-
185 three factor central composite design (CCD), which was generated by a five-level-three factor,
186 was applied to analyze data. The backbone length of extender (A), number of carboxyl groups of
187 extender (B) and the cross-linking performance (PE) were chosen as the three independent

experimental factor. Retention of tensile strength (RTS) and retention of elongation (RE) was the two response, as shown in Table 1. For the three-dimensional response surface plots in Fig. 8, the PE value was kept at 69%, which was the obtained optimum value.

Table 1

CCD Matrix for design Arrangement and Responses.

Run order	Response 1:RTS	Response 2:RE	Factor		
			PE: Cross-linking performance (%)	A: Backbone length of extender	B: Number of hydroxyl groups of extender
1	53	84	41	3	2
2	55	109	46	4	2
3	60	95	42	5	2
4	54	102	34	6	2
5	43	86	41	4	2
6	50	70	50	4	3
7	60	90	47	4	3
8	57	100	44	4	4
9	55	95	37	3	2
10	51	98	44	6	3
11	54	90	56	6	2
12	63	95	54	5	2
13	69	118	43	4	2
14	55	120	46	4	4
15	52	90	70	4	3
16	60	97	67	4	4
17	80	105	61	3	2
18	76	122	52	4	2
19	62	108	45	5	2

2.9. Statistical Analysis

All the data points were compared using the one-way analysis of variance with the Scheffé test with a confidence interval of 95%. A p value smaller than 0.05 indicated a statistically significant difference. Standard deviations are shown by the error bars in the figures, and the data in the figures labeled with different numbers or characters indicate significant differences among them.

3. Results and discussion

3.1. 1,2,3,4-butanetetrol as the cross-linking extender

As shown in Table 2, after incorporation of 1,2,3,4-butanetetrol as extender, the cross-linking performance of CA cross-linked hair could be significantly increased from 45% to 75%, the dry and wet tensile strength of permed hair was also remarkably enhanced for 20% and 19%. Extender with multiple -OH groups could react with -COOH groups in CA. The single cross-linking unit of individual CA molecules were enlarged to a bulkier one composed of a number of CA molecules connected by extenders. And resultantly, the area that the networked cross-linker could reach was enlarged, leading to better cross-linking result that enabled the cross-linking with higher efficiency, as well as higher retention of mechanical properties of hair fibers.

Table 2

Comparison of cross-linking performance and mechanical properties of hair using CA cross-linking with and without extender.

Cross-linking agent	Cross-linking performance (%)	Retention of tensile strength (%)		Retention of elongation (%)	
		Dry	Wet	Dry	Wet
CA	45	60	51	105	112
CA+1,2,3,4-butanetetrol ^a	75	80	70	121	130

^a CA:1,2,3,4-butanetetrol (molar ratio) =1:0.7

To clarify effects of molecular structures of extenders on cross-linking performance and mechanical properties, polyols with different length of carbon backbones, position of hydroxyl groups and number of hydroxyl groups in the backbones were used in this study.

3.2. Effects of polyols structure on its performance

The shape and mechanical properties of hair were maintained by the intermolecular hydrogen bonds, ionic linkages and disulfide bonds. Thioglycolate, the main chemical used in cross-linking, is toxic and harmful to hair dressers and customers via inhalation and dermal adsorption.

220 A green and non-toxic approach was developed using cysteine in the first step and citric acid in
221 the second step for hair cross-linking. The cysteine functioned as an equivalent of thioglycolate
222 to dissociate the intermolecular forces for re-shaping, while citric acid was used as cross-linker
223 to fix the keratin molecules at new relevant positions. The efficiency of cross-linking was critical
224 to the cross-linking results. Adding bio-based polyols as extenders could further enhance the
225 cross-linking results of citric acid. Hydroxyl groups in polyols could react with carboxyl groups
226 in citric acid to form larger cross-linking units, and enlarged the distance and increased the
227 numbers of carboxyl groups available for cross-linking per unit. Consequently, the cross-linking
228 density of citric acid/polyol system could be increased.

229 As has been indicated in our previous study (Song et al., 2016), utilization of polycarboxylic
230 acids with longer backbones and more carboxyl groups could lead to improved cross-linking
231 efficiencies. However, the best polycarboxylic acid, BTCA, is high in cost, and could only be
232 obtained via synthesis, and is not green and renewable. Polyols, such as glycerol, are usually
233 naturally originated, and sold at low prices. Using citric acid and polyols could be a more
234 feasible approach to achieve non-toxic high efficiency cross-linking. Extenders could effectively
235 inhibit decarboxylation of CA and react with CA to form large cross-linking unit and facilitate
236 cross-linking of keratin molecules with relatively long distances.

237

239

240 **Fig. 1.** Cross-linking performance (A) and mechanical properties (B) (C) of hair treated with diol
 241 extenders with different length of backbones. CA: extender (molar ratio) = 1:0.7.

242 As shown in Fig. 1A, addition of diols during CA cross-linking could significantly affect the
 243 cross-linking performance. Increasing the backbone length of diols from 3 to 4 first increased
 244 cross-linking performance from 42% to the highest 47%, and then decreased to 37% by
 245 continuing increasing the number of carbon to 6. There were similar trends for the tensile
 246 strength and elongation. Polyols functioned as extenders for CA by linking individual CA
 247 molecules together to form a larger cross-linking unit. The enlargement of the cross-linking unit
 248 extended by 1,3-propanediol was limited due to its short backbone. The cross-linking unit that
 249 was extended by 1,6-hexanediol was also limited probably due to the overlong distance of
 250 among the cross-linked keratin molecules. The long connections built among keratin molecules
 251 could not preventing them from slippage, leading to reduced cross-linking performance. There

was an optimal length of diol backbones as extenders. Among those diols, both 1,4-butanediol, 1,5-pentanediol showed similar or higher cross-linking performance compare to commercial product as a result of increased cross-linking performance within keratin chain.

The effect of length of diol backbone on the tensile properties of hair keratin were shown in Fig. 1B and C. Both diols with backbone length of 3 to 4 resulted in higher retention of tensile strength and elongation than commercial product. Retention of tensile strength and elongation increased and then decreased as the backbone length of extender increased from 3 to 4 and then from 4 to 6. The highest tensile retention was achieved using 1,4-butanediol, which reached a balance between extending the cross-linking network to relatively distant molecules, and rendering certain numbers of cross-linked molecules to withstand stress simultaneously.

Primary hydroxyl groups in polyols are more reactive to undergo esterification with CA, decreasing feasible dehydration of CA which is unfavorably for cross-linking. Polyols with different numbers of -OH groups with the same backbones and with -OH groups located at different positions were selected to further investigate the effect of -OH groups in extender on the cross-linking performance.

269

270 **Fig. 2.** Cross-linking performance of hair treated with CA and polyol extenders at various molar
271 ratios of -COOH/-OH.

272 Fig. 2 demonstrates that, with four carbons in the backbones, polyols with 4, 3 and 2 -OH
273 groups showed high, medium and low cross-linking performance on hair. The -OH groups in
274 polyols could react with -COOH groups in CA molecules, the cross-linking complex could have
275 higher cross-linking capability due to their increasing number of -COOH groups per molecule.
276 With the same amounts of -COOH groups available from CA as the crosslinker, more -OH
277 groups available per extender meant more CA molecules could be connected to form cross-
278 linking complex. Each of the cross-linking units with multiple -COOH groups could crosslink
279 multiple keratin molecules to maintain the newly set hair styles.

280

Fig. 3. Effects of CA and extenders at various molar ratio of -COOH/-OH on the dry (A) and wet (B) mechanical properties of hair fiber after cross-linking treatments.

Fig. 3 shows the effects of extenders with different numbers of primary and secondary hydroxyl groups on the retention of mechanical properties of hair. All those polyols used in this study resulted in higher retention of tensile strength than commercial products. Under the same molar ratio of -COOH/-OH groups, 1,2,3,4-butanetetrol and 1,2,4-butanediol as extenders could retain both dry and wet tensile strength and strain to the highest and lowest degree. Retention of tensile properties were also highly correlated to the rebuilding of cross-linking network after breakage of disulfide bonds in the first reduction step. When the network was more completely re-constructed, the hair fibers could better regain the strength and elongation. Because the total quantity of hydroxyl group added was kept constant, polyols featuring a higher secondary hydroxyl ratio could form more branched network through the reaction of secondary hydroxyl and -COOH group on CA. Although secondary hydroxyls are less as reactive as primary one, their presence may enhance the hydrogen bonding between keratin molecules and mechanical properties. With more -OH groups per extender molecule, more CA could be connected into larger cross-linking units with more -COOH groups per unit, leading to a more stable cross-linking network. However, for the same extender, retention of tensile strength and elongation first increased and then decreased as the molar ratio of -COOH/-OH group increased from 1:0.3 to 1:3. The highest retention of tensile strength and elongation was achieved at molar ratio of -COOH/-OH groups of 1:1 and 1:0.7. Increasing the amount of extender to increase the amount of -OH groups could be advantageous to enlarge the cross-linking units for better cross-linking performance. However, when the amount of -OH group was too high, extenders excessively consumed -COOH groups in CA, leaving less of them available for reaction with hair keratin. In

summary, with more -OH groups per carbon of the extender and appropriate molar ratio of -COOH/-OH groups, the retention of tensile properties of hair could be improved comparing to the cross-linking without extender.

3.3. Reaction mechanism

Polyols with different backbone length and hydroxyl groups played a critical role in branching the cross-linking reaction between keratin molecules to enhance the cross-linking network, and improve the cross-linking performance and mechanical properties of hair fiber. The intrinsic viscosity was summarized in Table 3. The hydrolyzed hair fiber show the lowest intrinsic viscosity compare to those of cross-linked which could be attribute to the increased molecular weight as a result of cross-linking reaction. From 1,4-butanediol, 1,2,4-butanetriol to 1,2,3,4-butanetetrol, with the number of hydroxyl group and backbone length increased the branched cross-linking network within hair fiber formed and the intrinsic viscosity was increased.

Table 3

Intrinsic viscosities of the cross-linked hair fiber.

Treatment	Intrinsic viscosity, dL/g
Hydrolyzed hair fiber	0.183
CA	0.907
1,4-butanediol	1.097
1,2,4-butanetriol	1.483
1,2,3,4-butanetetrol	1.978

Scheme 1. Step 1-Reduction: Cleave disulfide bones in keratin molecules in untreated hair into sulfhydryl groups using cysteine. Step 2-Cross-linking: Cross-link hydroxyl groups, amine groups, and sulfhydryl groups in the reduced hair keratin using CA and extenders.

Scheme 1 illustrates the reduction and cross-linking reactions occurred on hair step by step. The premise for re-shaping hair was to break the disulfide bonds that maintain the morphology

of the original hair. As shown in Step 1, cysteine was used to reduce the disulfide bonds into sulfhydryl groups. After that, the reduced hair was curled to designated shape, citric acid was used to create new covalent bonds between its carboxylic groups and amine groups, sulfhydryl groups and hydroxyl groups in hair keratin molecules. The keratin molecules were fixed at their new relative positions which resulted in formation of new hair styles.

In Step 2, upon heating to 180 °C, one anhydride was formed between two adjacent carboxylic groups in one citric acid molecule after removal of one molecule of water. The anhydride reacted with SHP via acetylation to form the intermediate (1), in which, the carbon carrying positive charge attacked the negatively charged oxygen, sulfur and nitrogen in the nucleophilic hydroxyl groups, sulfhydryl groups and amine groups to form product (2). One molecule of SHP and one carboxylic group in the citric acid was released after the S_N2 nucleophilic substitution. If there were other available carboxylic group in the same molecule of citric acid, another anhydride could be formed in the same molecule of citric acid to continue the previous S_N2 nucleophilic substitution, resulting in cross-linking of keratin molecules in hair. In general, with more carboxylic groups and shorter distances among them in one citric acid molecule, the possibility of anhydride formation and subsequent cross-linking became much higher.

3.4. Quantification of functional groups via titration

Fig. 4. Titration curves of keratin fiber treated with CA and extenders with different chain length.

To quantify the cross-linking reaction, amounts of -SH groups, -COOH groups and -NH₂ groups in hair were titrated. After step 1 of reduction, the amount of -SH groups increased as the disulfide bonds were broken by cysteine. In the following reaction, the -SH groups reacted with -COOH groups in CA to form thioester linkage and reacted with -SH groups to rebuild disulfide bonds. As the -COOH groups in CA could also react with -NH₂ and -OH in hair keratin to form amino groups and ester groups, the amounts of the latter two groups should decrease. As it is of low possibility for all the -COOH groups in CA to participate in the reaction, the overall -COOH amount in hair increased after CA cross-linking.

As shown in Fig. 4, the amount of -SH groups in hair increased to 39 μmol/g after cross-linked with CA comparing with that of 18 μmol/g in original hair, and kept in the range of 27-32 μmol/g after extenders were added into the cross-linking systems. The amount of -NH₂ groups in hair decreased for 47% after cross-linked with CA, and further for 65% after extenders were added into the cross-linking systems. The changes of -SH and -NH₂ groups after adding extenders could be attributed to enhanced cross-linking of CA induced by extenders. On the contrary, the amount of -COOH groups in hair increased for 11 times after cross-linked with CA,

and then decreased slightly for 9.4 times after extenders were added into the cross-linking systems. The result indicated that longer polyols were less effective in keeping keratin molecular chain from slipping. It could be assumed that reaction between CA and extenders as well as enhanced cross-linking between CA and hair keratin consumed substantial amounts of -COOH groups. Among extenders with -OH groups at different position, the change in amounts of -SH, -NH₂ and -COOH groups were not significantly different from each other.

Fig. 5. Titration curves of keratin fiber treated with CA and extenders containing different amounts of hydroxyl groups (COOH/OH molar ratio).

From Fig. 5, it could be observed that all the hair cross-linked with CA had less amount of -SH and -NH₂ groups and higher amount of -COOH groups than the reduced hair. When the polyol extenders were added into the cross-linking system, the amount of -SH, -NH₂ and -COOH groups first decreased as the ratio of -COOH/-OH increased from 1:0.3 to 1:0.7 and then increased with increasing of -COOH/-OH molar ratio. With proper amount of polyol to participate in the cross-linking reaction, CA could react with polyol to form a larger cross-

linking unit and react more effectively with keratin molecules. However, when there were too many polyol molecules in the system, the reaction between polyol and CA might dominate and consume too many CA molecules, leading to slight increase in the amounts of -SH and -NH₂ groups.

3.5. Analysis of structural change of keratin fiber by Raman spectroscopy

Fig. 6. Raman spectroscopy of hair fiber (A) Curving fitting of the amide I band region of original hair Raman spectrum. 1: α -helix form; 2: β -sheet and/or random coil form peak fitting (B) and Amide-I of hair fibers (C) treated with citric acid and different extender.

The representative cortex Raman spectra of hair samples are shown in Fig. 6A. Comparing with original hair, Raman spectra of the cross-linked hair had new band at 645 cm⁻¹, representing thioester groups formed via esterification of thiol groups in keratin molecules and carboxylic groups in citric acid as shown in scheme 1. The S-O band at 1040 cm⁻¹, assigned to sulfonic acid group (Schlücker et al., 2006), slightly increased after cross-linking. This suggests that, after cross-linking, most of the -SH groups generated in reduction were converted into three

forms, disulfide bonds due to oxidation, thioester groups due to esterification, and sulfonic acid groups also due to oxidation. It has been reported that β -sheet and/or random coil content (β /R) and the α -helix content in human hair fibers could be estimated by amide I band analysis (Kuzuhara, 2013). In this study, proportions of band components of the hair samples were evaluated by spectrum simulation of the amide I band region (Fig. 6C) with Gaussian line shapes and appropriate line width, as shown in Fig. 6B. The bands at 1671 cm^{-1} and 1652 cm^{-1} were assigned to β -sheet and/or random coil forms and α -helix form.

After deconvolution, the β -sheet and/or random coil content (β /R) and the α -helix content of original and cross-linked hair samples are summarized in Table 4. It could be observed that, the α -helix contents decreased while the β -sheet and/or random coil content increased after cross-linking. After reduction, the α -helix conformation was converted to random coil due to cleavage of disulfide bonds that stabilized the secondary structures of keratin in original hair. Cross-linking in second step could stabilize the new conformation of β -sheet and/or random coil, and led to increase in their amounts. To distinguish β -sheet from random coil, XRD analysis was carried out.

Table 4

The α -helix, random-coil and β -sheet contents in hair samples.

Treatment	α -helix (%)	Random coil and β -sheet (%)
Original hair	19.04	80.99
CA	14.82	85.18
1,4-butanediol	14.72	85.28
1,2,4-butanetriol	15.72	84.18
1,2,3,4-butanetetrol	14.68	85.32

3.6. Analysis structural change of keratin fiber by WXR

The changes in the equatorial and meridional WAXD patterns of hair fibers treated with citric acid and different extenders are shown in Fig. 7 (A) and (B). In the equatorial WAXD pattern of hair fiber (Fig. 7A), the peak at $2\theta=9^\circ$ (0.98 nm) was commonly associated with both α -helix and β -sheet structures, and the reflection at $2\theta=20^\circ$ (0.465 nm) was usually assigned to β -sheet structure. In meridional WAXD patterns in Fig. 7B, the peak at $2\theta=18^\circ$ (0.51 nm) was associated with the α -helix structure of hair keratin fiber (Nishikawa et al., 1998). It could be observed that, original hair showed higher intensity at 9° and 20° comparing to processed hair, indicating the decrease of total amounts of α -helix and β -sheet conformations after treatment. From Fig. 7A, it could be seen that, addition of extenders could increase the amount of β -sheet in the treated hair fiber, inferring the decrease in the amount of α -helix structure of the treated hair fiber. The cross-linking performance enhanced by extenders resulted in preservation of β -sheet induced during stretching during hair cross-linking. Among all those treated hair fibers, incorporation of 1,2,3,4-butanetetrol as a cross-linking extender led to increase in the diffraction peak at 9° and 20° (Fig. 7A), indicating the highest amounts of α -helix and β -sheet conformations and the best preservation of the original hair structure due to its highest cross-linking efficiency.

Fig. 7. Equatorial (A) and meridional (B) WAXD patterns of hair fibers treated with citric acid and different extender.

3.7. Relationship between extender structure and mechanical property of hair fiber

RSM is a useful technique not only for optimizing experimental design, but also for predicting the performance under a given experimental condition. The most important indicator in evaluating the result of hair cross-linking is the mechanical property of hair fiber. Retention of tensile strength (RTS) and elongation (RE) of hair fiber after cross-linking is modeled as the response to three variable cross-linking parameters (PE, A, B). The result obtained from the run of 19 experimental sets, suggested by CCD models of RSM were analyzed by R software. The obtained equation and the ANOVA results are summarized in Table 5.

Table 5

ANOVA results for the two responses obtained by RSM.

Response	Obtained equation	<i>P</i> value	F value	SD	R_{adj}^2
RTS	RTS= -0.583304+0.018021PE+0.191652A +1.97712B-0.004789PE*A- 0.049273PE*B- 0.495959A*B+0.012366PE*A*B	<0.00001	20.5	0.00344	0.8778
RE	RE=-71.660+18.231PE+21.853A- 304.432B-2.454PE*A- 2.355PE*B+100.244A*B	0.0000413	10.14	33.98	0.7529

Fig. 8. Effects of backbone length of extender and number of hydroxyl groups of extender on retention of tensile strength and retention of elongation based on RSM.

ANOVA results in Table 5 indicate that the model was significant at 95% confidence level for two responses since their p values were less than 0.0001 and the adjusted regression coefficients (R_{adj}^2) were high enough to indicate good overall performance of the model. Based on the coefficients shown in Table 5, the factor B (number of hydroxyl groups of extender) showed higher impact on the two responses comparing to factor A (backbone length of extender). The hydroxyl groups in the extenders showed greater impact on the final mechanical properties of permed hair fibers, by forming branched network between citric acid and keratin molecules. In order to better understand the effect of extender structure on tensile strength and elongation of the treated hair fibers, three-dimensional response surface plots of the retention of tensile strength and retention of elongation versus backbone length and number of hydroxyl groups of extender were drawn and the results are shown in Fig. 8.

From Fig. 8A, the retention of tensile strength increased with the increase of backbone length of extender first, then decreased when the backbone was longer than four carbons. However, retention of tensile strength exhibited a gradually rising trend as the number of hydroxyl groups of extender increased. This was mainly attributed to the fact that more hydroxyl groups on the extender could promote the formation of dense and branched cross-linking network within keratin molecules. The number of hydroxyl groups of extender has greater impact on the mechanical property of treated hair fiber compared with backbone length of extender. Moreover, the contour plot is elliptical, implying that the interactive effect of backbone length of extender and hydroxyl groups of extender on retention of tensile strength is significant.

Fig. 8B shows the interaction of backbone of extender and hydroxyl groups of extender on the retention of elongation of hair fiber. It seems that the backbone length of extender did not play a significant role in preserving of elongation of hair fiber as the surface showed a slight change

with increasing backbone length of extender. However, retention of elongation increased rapidly at first and then became constant with the rising of hydroxyl groups of extender. The backbone of extenders formed bridges between citric acid and keratin molecules and the hydroxyl groups on the extender facilitated formation of branched cross-linking network. Overall, the hydroxyl groups of extender showed a greater impact on both tensile strength and elongation of hair fibers.

The optimum conditions suggested by the RSM for the structure of extender are four backbone length and with four hydroxyl groups on its backbone. Verification for the optimum operation condition was conducted, and the results are summarized and compared to the RSM predicted values in Table 6. The obtained values for retention of tensile strength and elongation were 73% and 118%, which were not significantly different from the predicted value of 69% and 115% at 95% confidence interval. The insignificant differences indicated the validity of the model.

Table 6

Comparison of experimental results and predicted results for cross-linking of hair fiber at optimized conditions.

	RSM predicted	Experimental	Percentage error
Retention of tensile strength (%)	69	73	5%
Retention of elongation (%)	115	118	2%

4. Conclusions

In this work, polyols were added into non-toxic citric acid cross-linking system aiming establishment of a high effective cross-linking system for protein material to replace highly-toxic or ineffective protein cross-linking formula currently in use. Regarding the structures of polyols, increasing number of secondary hydroxyl groups in polyol with optimal backbone length was

485 favorable for branched network formation. From Raman spectroscopy and XRD analysis, it was
486 found that the α -helix in the secondary structure of hair keratin was partially disrupted, while the
487 amounts of β -sheet and random coil increased after cross-linked by CA and polyols. In summary,
488 the non-toxic and green cross-linking system using polyol as extender and CA as cross-linker
489 could obtain satisfactory performance with enhanced cross-linking efficiency, and could be a
490 promising replacement for the toxic cross-linking formula currently in use.

491

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- 564

Figure captions

Fig. 1. Cross-linking performance (A) and mechanical properties (B) (C) of hair treated with diol extenders with different length of backbones. CA: extender (molar ratio) = 1:0.7.

Fig. 2. Cross-linking performance of hair treated with CA and polyol extenders at various molar ratios of -COOH/-OH.

Fig. 3. Effects of CA and extenders at various molar ratio of -COOH/-OH on the dry (A) and wet (B) mechanical properties of hair fiber after cross-linking treatments.

Scheme 1. Step 1-Reduction: Cleave disulfide bonds in keratin molecules in untreated hair into sulfhydryl groups using cysteine. Step 2-Cross-linking: Cross-link hydroxyl groups, amine groups, and sulfhydryl groups in the reduced hair keratin using CA and extenders.

Fig. 4. Titration curves of keratin fiber treated with CA and extenders with different chain length.

Fig. 5. Titration curves of keratin fiber treated with CA and extenders containing different amounts of hydroxyl groups (COOH/OH molar ratio).

Fig. 6. Raman spectroscopy of hair fiber (A) Curving fitting of the amide I band region of original hair Raman spectrum. 1: α -helix form; 2: β -sheet and/or random coil form peak fitting (B) and Amide-I of hair fibers (C) treated with citric acid and different extender.

Fig. 7. Equatorial (A) and meridional (B) WAXD patterns of hair fibers treated with citric acid and different extender.

Fig. 8. Effects of backbone length of extender and number of hydroxyl groups of extender on retention of tensile strength and retention of elongation based on RSM.

586 **Table 1**

587 CCD Matrix for design Arrangement and Responses.

Run order	Response 1:RTS	Response 2:RE	Factor		
			PE: Cross- linking performance (%)	A: Backbone length of extender	B: Number of hydroxyl groups of extender
1	53	84	41	3	2
2	55	109	46	4	2
3	60	95	42	5	2
4	54	102	34	6	2
5	43	86	41	4	2
6	50	70	50	4	3
7	60	90	47	4	3
8	57	100	44	4	4
9	55	95	37	3	2
10	51	98	44	6	3
11	54	90	56	6	2
12	63	95	54	5	2
13	69	118	43	4	2
14	55	120	46	4	4
15	52	90	70	4	3
16	60	97	67	4	4
17	80	105	61	3	2
18	76	122	52	4	2
19	62	108	45	5	2

588

Table 2

Comparison of cross-linking performance and mechanical properties of hair using CA cross-linking with and without extender.

Cross-linking agent	Cross-linking performance (%)	Retention of tensile strength (%)		Retention of elongation (%)	
		Dry	Wet	Dry	Wet
CA	45	60	51	105	112
CA+1,2,3,4-butanetetrol ^a	75	80	70	121	130

^a CA:1,2,3,4-butanetetrol (molar ratio) =1:0.7

594 **Table 3**

595 Intrinsic viscosities of the cross-linked hair fiber.

Treatment	Intrinsic viscosity, dL/g
Hydrolyzed hair fiber	0.183
CA	0.907
1,4-butanediol	1.097
1,2,4-butanetriol	1.483
1,2,3,4-butanetetrol	1.978

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598 **Table 4**

599 The α -helix, random-coil and β -sheet contents in hair samples.

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Table 6

Comparison of experimental results and predicted results for cross-linking of hair fiber at optimized conditions.

	RSM predicted	Experimental	Percentage error
Retention of tensile strength (%)	69	73	5%
Retention of elongation (%)	115	118	2%

611 **Graphical abstract**

612

613 **A non-toxic and effective cross-linking system based on citric acid, featuring polyols as**

614 **cross-linking extenders is developed.** The structure of extender had a remarkable impact on

615 cross-linking performance. Extenders with 3 or 4 carbons and 3 to 4 hydroxyl groups showed the

616 best effect.