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(54) Title: METHODS FOR PRODUCING, FILMS COMPRISING, AND METHODS FOR USING HETEROGENEOUS CROSSLINKED PROTEIN NETWORKS

(57) Abstract: Methods for producing biocompatible heterogeneous proteinaceous networks crosslinked with a heterogeneous crosslinking agent, and the novel crosslinked networks.

**TITLE: METHODS FOR PRODUCING, FILMS COMPRISING, AND
METHODS FOR USING HETEROGENEOUS CROSSLINKED
PROTEIN NETWORKS**

5 **Cross Reference to Related Applications**

The present application claims the filing date of U. S. Patent Application Serial No. 10/119,477 filed April 10, 2002. The present application also claims the benefit of the filing date of the following application: U.S. Patent Application Serial No. 10/133,885, filed April 26, 2002. Related applications, to which priority is claimed as
10 may be required, are U.S. Patent Application Serial No. 10/127,523, filed April 22, 2002, and U.S. Provisional Application No. 60/393,958, filed July 5, 2002. A related application is Application Serial No. 10/254,364, filed September 25, 2002, claiming the benefit of U.S. Provisional Application No. 60/324,709.

Field of the Invention

15 The present invention is directed to methods for producing biocompatible heterogeneous proteinaceous networks crosslinked with a heterogeneous crosslinking agent. Preferred proteins for use in forming the networks are α -keratins, or high molecular weight keratins (HMWK's). The crosslinking agent preferably reacts with reactive pendant groups existing on the keratin molecules and either produces no
20 byproducts, produces biocompatible byproducts, such as hydrogen, water, and carbon dioxide, or produces byproducts that can be removed from the network.

Background of the Invention

Proteins, such as keratin proteins, are beneficial in healing damaged epithelial tissues. Unfortunately, the chemical and engineering properties of keratin proteins have
25 been relatively limited to those achieved using oxidative and reductive chemistries, and

side chain protein crosslinks. A need exists for proteins, and methods for crosslinking proteins, preferably α -keratins, to form films having a broad scope of chemical and engineering properties so that the potential applications of protein-based materials can be expanded.

5 Summary of the Invention

A method is provided for producing functionalized proteins comprising reactive pendant groups effective to react with heterogeneous crosslinking agents and to form heterogeneous proteinaceous networks. The proteins are treated with a reactive
10 nucleophile under conditions effective to convert thiol groups to thiolate anions. The thiolate anions are exposed to a functionalization agent comprising a substitution end and at least one reactive end under conditions effective to induce said thiolate anions to react with said substitution end, thereby producing a plurality of functionalized proteins comprising reactive pendant groups comprising said reactive end. In a preferred
15 embodiment, the reactive end is an epoxide group, and the functionalized proteins are exposed to conditions effective to induce the epoxide groups to react with reactive amines pendant on the proteins, producing a heterogeneous cross-linked protein network. The protein preferably is selected from the group consisting of α -keratin, collagen, and elastin. The functionalization agent preferably is epichlorohydrin.

20 In another aspect, a method is provided comprising exposing said plurality of functionalized proteins, preferably functionalized α -keratins, to a crosslinking agent comprising at least a first terminus reactive toward said reactive end and a second terminus reactive toward an entity selected from the group consisting of said reactive end and other reactive pendant groups, said exposing occurring under conditions effective to
25 induce said first terminus on a plurality of molecules of said crosslinking agent to react

with said reactive end on a plurality of first functionalized α -keratin molecules, and to induce said second terminus on a plurality molecules of said crosslinking agent to react with said entity on a plurality of second functionalized α -keratin molecules, producing a cross-linked protein network. The reactive end preferably is selected from the group consisting of an anhydride group, a carboxylic acid group, an epoxide group, and an isocyanate group. The proteins are selected from the group consisting of keratin, collagen, and elastin, most preferably α -keratin.

A method is provided for making a keratin network comprising a heterogeneous crosslinking agent, said method comprising exposing α -keratins comprising reactive pendant groups to a heterogeneous crosslinking agent comprising a first functional group and a second functional group adapted to react with said reactive pendant groups under conditions effective to induce a first reaction between said first functional groups on a plurality of molecules of said crosslinking agent and first reactive pendant groups on a plurality of first α -keratin molecules and to induce a second reaction between said second functional groups on a plurality of molecules of said crosslinking agent and second reactive pendant groups on a plurality of second α -keratin molecules, thereby producing a heterogeneous cross-linked keratin network

In one aspect, the method comprises exposing α -keratins comprising reactive pendant groups to a nucleophilic substitution agent comprising a substitution end and at least one terminal epoxide under conditions effective to induce said reactive pendant groups to react with said substitution end, thereby producing a plurality of epoxidized α -keratins comprising epoxidized pendant groups. The reactive pendant groups preferably are thiolate anions, and the functionalization agent preferably is epichlorohydrin. In another aspect, the method further comprises treating said plurality of epoxidized α -

keratins with a multi-functional crosslinking agent comprising at least a first nucleophilic group and a second nucleophilic group under conditions effective to induce first epoxidized pendant groups on a plurality of first epoxidized α -keratin molecules to react with said first nucleophilic group on a plurality of molecules of said crosslinking agent, and to induce said second epoxidized pendant groups on a plurality of second epoxidized α -keratin molecules to react with said second nucleophilic group on a plurality of molecules of said crosslinking agent, producing said cross-linked α -keratin network.

In another aspect, the method comprises exposing α -keratins comprising reactive pendant groups to a crosslinking agent comprising at least a first terminal epoxide and a second terminal epoxide under conditions effective to induce a first reaction between said first terminal epoxides on a plurality of molecules of said crosslinking agent and first reactive pendant groups on a plurality of first α -keratin molecules, and to induce a second reaction between said second terminal epoxides on a plurality of molecules of said crosslinking agent and second reactive pendant groups on a plurality of second α -keratin molecules, thereby producing a heterogenous cross-linked keratin network. In a preferred embodiment, said reactive pendant groups are selected from the group consisting of thiol groups and reactive amines.

In another aspect, the method comprises exposing α -keratins comprising reactive pendant groups to a crosslinking agent comprising at least a first functionality and a second functionality independently selected from the group consisting of an ester group and a carboxylic acid group under conditions effective to induce first reactions between said first functionality on a plurality of molecules of said crosslinking agent and first reactive pendant groups on a plurality of first α -keratin molecules, and to induce second reactions between said second functionality on a plurality of molecules of said

crosslinking agent and second reactive pendant groups on a plurality of second α -keratin molecules, thereby producing a heterogeneous cross-linked keratin network. Preferred crosslinking agents include, but are not necessarily limited to anhydrides and dicarboxylic acids. More preferred crosslinking agents are phthalic anhydride and terephthalic acid. Preferred reactive pendant groups are selected from the group consisting of thiols, hydroxyls, and reactive amines, preferably thiols.

In yet another aspect, the method comprises exposing α -keratins comprising reactive pendant groups to a crosslinking agent comprising at least a first isocyanate group and a second isocyanate group under conditions effective to induce a first reaction between said first isocyanate groups on a plurality molecules of said crosslinking agent and first reactive pendant groups on a plurality of first α -keratin molecules, and to induce a second reaction between said second isocyanate groups on a plurality of molecules of said crosslinking agent and said second reactive pendant groups on a plurality of second α -keratin molecules, thereby producing a heterogenous cross-linked keratin network.

The crosslinking agent preferably is a diisocyanate selected from the group consisting of aryl diisocyanates, including benzyl diisocyanate, alkyl diisocyanates, and allyl diisocyanates having from about 1 to about 8 carbon atoms. A preferred diisocyanate is hexanediisocyanate. Reactive pendant groups preferably are selected from the group consisting of hydroxyl groups, thiol groups, and reactive amine groups.

In another aspect, the method comprises exposing proteins comprising reactive pendant groups to a heterogeneous crosslinking agent comprising a first functional group and a second functional group adapted to react with said reactive pendant groups under conditions effective to induce a first reaction between said first functional groups on a plurality of molecules of said crosslinking agent and first reactive pendant groups on a

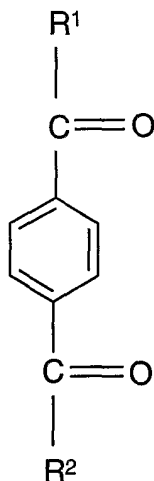
plurality of first protein molecules, and to induce a second reaction between said second functional groups on a plurality of molecules of said crosslinking agent and second reactive pendant groups on a plurality of second soluble protein molecules, thereby producing a heterogenous cross-linked proteinaceous network. The proteins are selected
5 from the group consisting of keratin, collagen, and elastin, most preferably α -keratin.

In another embodiment, a heterogenous crosslinked protein network is provided comprising a plurality of protein molecules interlinked by at least one crosslinking agent, said network comprising first bonds between first functional groups on a plurality of molecules of said crosslinking agent and first reactive pendant groups on a plurality of
10 first protein molecules and second bonds between second functional groups on a plurality of molecules of said crosslinking agent and second reactive pendant groups on a plurality of second protein molecules. The protein molecules suitably are selected from the group consisting of keratin, collagen, and elastin, most preferably α -keratin. In a preferred embodiment, the crosslinking agent is heterogeneous and comprises a plurality
15 of said functional groups. Suitable functional groups are selected from the group consisting of alkoxide groups, vinyl groups, hydroxyl groups, amine groups, aldehyde groups, isocyanate groups, ester groups, and anhydride groups. Preferred alkoxides are epoxides.

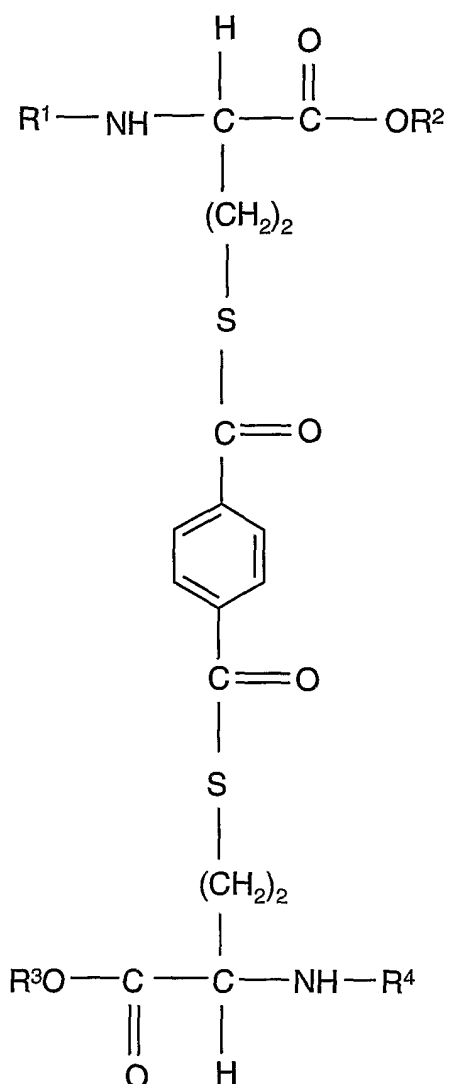
In a preferred embodiment, the heterogeneous crosslinked keratin network
20 comprises a plurality of α -keratin molecules interlinked by a crosslinking agent, said network comprising first bonds between first functional groups on a plurality of molecules of said crosslinking agent and first pendant groups on a plurality of first α -keratin molecules and second bonds between second functional groups on a plurality of molecules of said crosslinking agent and second reactive pendant groups on a plurality of

second α -keratin molecules. In a most preferred embodiment, the first bonds and the second bonds are covalent bonds. Suitable functional groups are selected from the group consisting of alkoxide groups, vinyl groups, hydroxyl groups, amine groups, aldehyde groups, isocyanate groups, ester groups, and anhydride groups. Preferred reactive pendant groups are selected from the group consisting of hydroxyl groups, thiol groups, reactive amine groups, and alkoxides. Preferred alkoxides are epoxides.

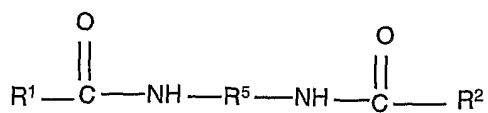
In a preferred embodiment, the heterogeneous crosslinked network comprises the following crosslinks:



10 wherein R¹ and R² independently are amino acid residues from separate protein molecules, the residues being selected from the group consisting of cysteine, arginine, serine, lysine, asparagine, glutamine, tyrosine, tryptophan, and histidine. Preferably, R¹ and R² independently are selected from the group consisting of cysteine and arginine. In a most preferred embodiment, R¹ and R² are cysteine, as shown below:

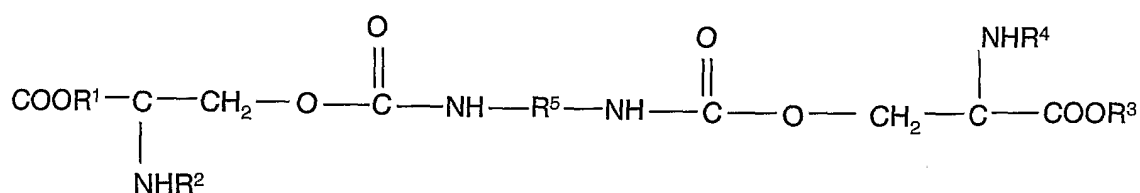


In another preferred embodiment, the heterogeneous crosslinked network comprises the following crosslinks:

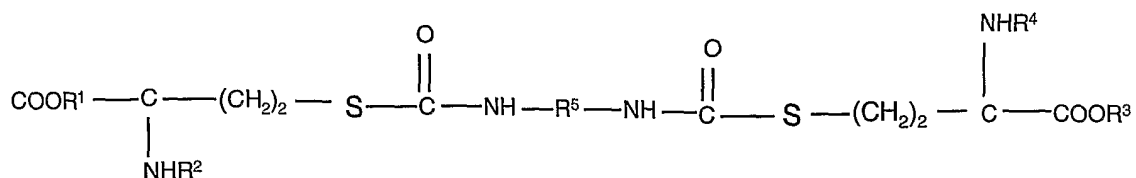


wherein R¹ and R² independently are amino acid residues from separate protein molecules, the residues being selected from the group consisting of cysteine, arginine, serine, lysine, asparagine, glutamine, tyrosine, tryptophan, and histidine; and, R⁵ is selected from the group consisting of alkoxy groups, alkylene groups, and alkenyl groups having from about 1 to about 50 carbon atoms, alone, or in combination with cyclic alkyl groups or aromatic groups. Preferably, R¹ and R² independently are selected from the group consisting of glutamic acid and aspartic acid.

In another preferred embodiment, the heterogeneous crosslinked network comprises the following crosslinks, wherein R¹ and R² are the remainder of a first protein molecule and R³ and R⁴ are the remainder of a second protein molecule:

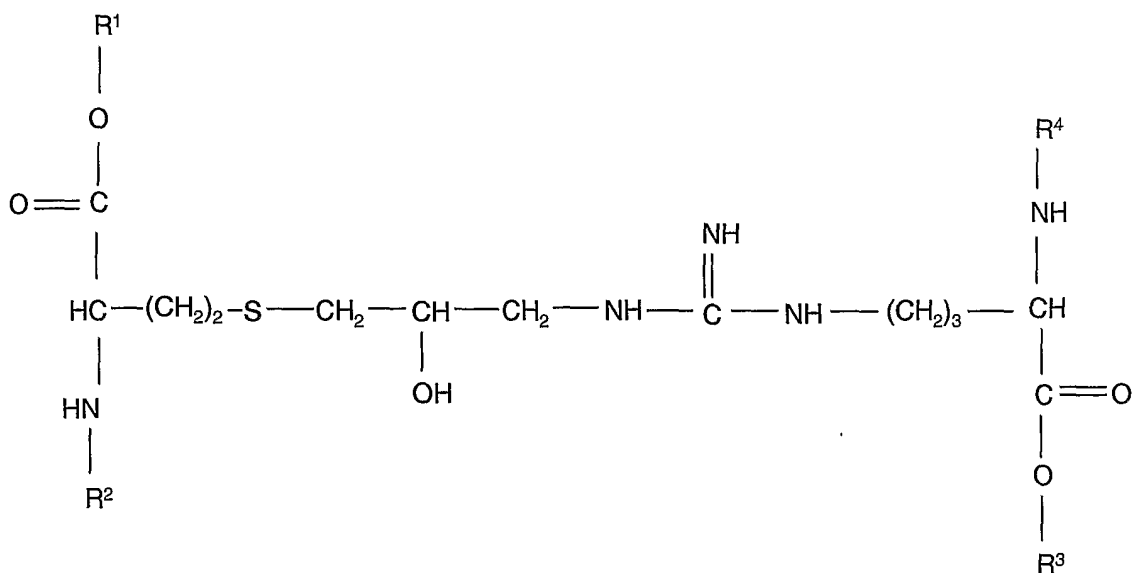


In another preferred embodiment, the heterogeneous crosslinked network comprises the following crosslinks, wherein R¹ and R² are the remainder of a first protein molecule and R³ and R⁴ are the remainder of a second protein molecule:



In another preferred embodiment, the heterogeneous crosslinked network comprises the following crosslinks, wherein R¹ and R² are the remainder of a first protein molecule and R³ and R⁴ are the remainder of a second protein molecule:

In another preferred embodiment, the heterogeneous crosslinked network comprises the following crosslinks, wherein R¹ and R² are the remainder of a first protein molecule and R³ and R⁴ are the remainder of a second protein molecule:



- 5 Another preferred aspect is a film made using any of the foregoing processes, or comprising any of the foregoing crosslinks.

Detailed Description of the Invention

The present invention is directed toward methods for crosslinking proteins, preferably using heterogeneous crosslinking agents to form heterogeneous proteinaceous networks or films. As used herein, the term "heterogeneous" refers to a proteinaceous network or film, preferably comprising protein molecules having a relatively high molecular weight of from about 50 to about 85 kDa, or derivatives therefrom. The protein molecules are interlinked by a non-proteinaceous crosslinking material.

The methods described herein may be used to treat a wide variety of proteins to form network structures, preferably elastomeric films. Examples of suitable naturally occurring proteins include, but are not necessarily limited to keratin, collagen, and elastin. The proteins may be natural, synthetic, or recombinant. Preferred proteins are

relatively high in cysteine content. Most preferred proteins are keratin proteins, even more preferably α -keratin proteins, also sometimes called high molecular weight keratins (HMWK's).

A preferred source of keratin proteins is hair or fur. The hair may be animal, or
5 human. Keratins are loosely defined as the hardened and insolubilized proteins found in the epidermal cells of vertebrates. Human hair is composed almost entirely of keratins.

Human hair has a cuticle, which is a tough tubular outer layer made up of flattened cells arranged in a scaly, overlapping profile. The inner bulk of the hair is called the cortex and is constructed from elongated cells that are densely packed with
10 fibrous keratins. The fibrous keratins are arranged in bundles referred to as microfibrils and possess an α -helical tertiary structure. The microfibrils are bound together with an amorphous keratin matrix.

The amorphous keratin matrix and the microfibrils vary in function and composition. The matrix is the "glue" that holds the microfibrils together. This matrix
15 "glue" is high in sulfur content, and is comprised of low molecular weight keratins (LMWK) which typically have an average molecular weight of from about 10 to about 15 kDa. The microfibrils are comprised of high molecular weight keratins (HMWK) having a relatively lower sulfur content, but having a higher average molecular weight of typically from about 50 to about 85 kDa. HMWK's and LMWK's vary in chemical
20 properties, such as reactivity and solubility.

Keratins are afforded their structural integrity, in large part, by the presence of disulfide crosslinks which form a three dimensional network of polypeptide chains. This network structure renders keratins insoluble. Keratins can, however, be made water soluble by destroying this three dimensional structure via disulfide bond scission.

Disulfide bond scission can be performed either oxidatively, reductively, or using some combination of both types of bond scission. Oxidative bond scission with hydrogen peroxide, for example, results in the formation of sulfonic acid residues produced from cystine. The material produced using hydrogen peroxide for disulfide bond scission is highly ionic and has excellent water solubility. Reductive bond scission with mercaptoethanol, for example, results in the formation of cysteine residues produced from cystine. The material produced using this reductive technique is highly reactive and will readily re-crosslink.

Disulfide bond scission and keratin extraction

The proteins, preferably α -keratins, may be processed and/or isolated in any manner that renders them sufficiently soluble in the reaction media for crosslinking reaction(s) to occur. A number of the reactions described below call for an anhydrous solvent. Persons of ordinary skill in the art will recognize that anhydrous solvents include a large number of solvents, including, but not necessarily limited to 1,2-dimethoxyethane, dimethylformamide, dimethylsulfoxide (DMSO), N-methylpyrrolidone, and others. Generally, the reactions require the presence of at least some water.

Oxidation/Reduction of Cystine Residues

In a preferred embodiment, which uses keratins as a source material (e.g. human hair), the hair is oxidized by a suitable oxidizing agent. Suitable oxidizing agents include, but are not necessarily limited to hydrogen peroxide, peracetic acid, percarbonates, persulfates, chlorine dioxide, sodium and calcium peroxides, perborates, and hypochlorite. The oxidants are used at a concentration of up to about 35%,

preferably at from about 0.1% to about 10%. The oxidation preferably occurs at reflux temperatures.

In a preferred embodiment, the hair is treated with hydrogen peroxide (H_2O_2), at from about 0.1% to about 10%, most preferably 1%, in order to disrupt the cuticle and
5 swell the keratin source material. This process also converts some fraction of the cystine residues into sulfonic acid groups. The amount of oxidation may be controlled by varying the time of oxidation, preferably from about 0 hours to about 4 hours, while retaining the other conditions of the oxidation reaction constant. These conditions include concentration and type of oxidant, temperature, and ratio of extracting media to
10 keratin source material. After the reaction is complete, the oxidized hair is filtered and rinsed, preferably with deionized water. The filtrate is discarded and the hair allowed to dry.

Where other conditions of oxidation are maintained constant, the conversion rate of cystine to sulfonic acid residues is roughly proportional to the amount of time used for
15 the oxidation. Residual cystines in the resulting oxidized keratin solids are converted to other sulfur-containing moieties using reductive techniques. Preferably, the disulfide-bridged cystine group is converted to a thiol group, which has utility of its own, or can be modified using a variety of chemical techniques.

Reaction with a Reducing Agent

20 If oxidized, the oxidized hair preferably is treated with a reducing agent. Treatment of oxidized keratin proteins with reducing agents facilitates the formation of cysteine from cystine, but tends to leave the previously oxidized groups unaltered. Suitable reducing agents include, but are not necessarily limited to thioglycolic acid and salts thereof, mercaptoethanol, dithiothreitol, thioglycerol, thiolactic acid, glutathione,

cysteine, sodium sulfide, and sodium hydrosulfide. Preferred reducing agents are thioglycolic acid and mercaptoethanol, most preferably thioglycolic acid.

In order to treat the oxidized hair with the reducing agent, the previously oxidized hair is suspended in the reducing agent typically at a concentration of up to about 10N, preferably from about 0.1N and 1N; at a pH greater than about 7, preferably equal to or greater than 9, most preferably 9; a temperature of from about 25 to about 80 °C, preferably about 60 °C, preferably for a time period of from about 1 to about 72, most preferably about 24 hours. The reaction occurs under an inert atmosphere, preferably nitrogen. The liquid fraction is separated from any remaining solids using known means, including but not necessarily limited to filtration, or cannulation and/or centrifugation, preferably under inert atmosphere. A preferred method of separation is filtration. Once the solids are removed, the soluble keratin proteins are isolated from the solution by addition of a water-miscible non-solvent, or by spray drying. Water-miscible non-solvents include, but are not necessarily limited to ethanol, methanol, isopropyl alcohol, tetrahydrofuran, acetone, dioxane, and the like, again under inert atmosphere. A preferred non-solvent is ethanol. The precipitate is separated from the non-solvent using known means, preferably by filtration and rinsing using additional aliquots of the non-solvent. The resulting keratin proteins are dried using known techniques, preferably overnight under vacuum at room temperature. This process results in the keratins having both sulfonic acid groups and thiol groups.

Thiols possess reactivities similar to alcohols, and can be used to perform a multitude of known organic chemical reactions, such as those described in McMurry, J., *Organic Chemistry*, Brooks/Cole Publishing Co., Monterey, CA (1984); Scudder, P. H., *Electron Flow in Organic Chemistry*, John Wiley & Sons, New York, NY (1992);

Stowell, J. C., *Intermediate Organic Chemistry*, John Wiley & Sons, New York, NY (1994), incorporated herein by reference. The ratio of sulfonic acid to thiol is primarily controlled by the quantity of primary reactive sites remaining after oxidation. Of course, the rate of reduction will also be affected by reagent concentration(s), reaction
5 temperature(s), and exposure time(s).

Reductive/reductive extraction

Reductive chemistries also are known for disulfide bond scission in keratins: See Wardell, J. L., "Preparation of Thiols" in *The Chemistry of the Thiol Group*, Patai, S. (Editor), pp. 163-353, John Wiley & Sons, New York, NY (1974), incorporated herein
10 by reference. HMWK's may be extracted from hair using at least two reductive extractions, as described in Crewther, W. G., Fraser, R. D. B., Lennox, F. G., and Lindley, H., "The Chemistry of Keratins" in *Advances in Protein Chemistry*, Anfinsen, C. B., Jr., Anson, M. L., Edsall, J. T., and Richards, F. M. (Editors), Academic Press, New York, pp. 191-346 (1965), incorporated herein by reference.

15 Briefly, in a first reductive extraction, the hair is treated with a first reducing agent under first conditions effective to selectively extract matrix keratins, producing a first solution comprising soluble reduced matrix keratins and remaining hair solids. The remaining hair solids and the first solution are separated, and the remaining hair solids are exposed to a second extraction solution under second conditions effective to
20 solubilize α -keratins, producing a second solution comprising soluble reduced α -keratins and solid cuticle. Once the second extraction is complete, the remaining solids essentially are the empty, intact cuticle.

The liquid fraction is separated from the solid cuticle using known means, including but not necessarily limited to filtration, or cannulation and/or centrifugation,

preferably under inert atmosphere. A preferred method of separation is filtration. Once the solids are removed, the soluble keratin proteins are isolated from the solution by addition of a water-miscible non-solvent, or by spray drying. Water-miscible non-solvents include, but are not necessarily limited to ethanol, methanol, isopropyl alcohol, tetrahydrofuran, acetone, dioxane, and the like, again under inert atmosphere. A preferred non-solvent is ethanol. The precipitate is separated from the non-solvent using known means, preferably by filtration and rinsing using additional aliquots of the non-solvent. The resulting keratin proteins are dried using known techniques, preferably overnight under vacuum at room temperature. The dried keratin proteins are ground into a powder, sometimes referred to as "HMWK powder."

Network formation

Thiols and other chemical moieties contained in amino acid residues have utility as labile sites for crosslinking reactions to form protein networks, preferably networks having the properties of an elastomeric film. Preferred reactions and crosslinking agents are those which produce biocompatible byproducts, preferably hydrogen, water, carbon dioxide, or any other biocompatible byproduct that is readily metabolized or excreted, removed from the network, or at least is not toxic to the human body. In order to prepare the networks, the desired crosslinking agent(s) are determined. Crosslinking agents other than glutaraldehyde having either two or more of the same functional groups, or two or more different functional groups are suitable. Preferable crosslinking agents have two or more of the same functional group, as described below.

In a preferred embodiment, which uses HMWK proteins, the HMWK proteins are dissolved in an aqueous solvent. Preferably, about 2 g of HMWK powder is mixed in water containing a suitable base, and the mixture is stirred and heated to a temperature

effective to dissolve the keratin, typically not more than 60°C. The pH of the solution is maintained at about 9 to 11 using a suitable base. Suitable bases include, but are not necessarily limited to ammonium hydroxide, sodium hydroxide, and potassium hydroxide, preferably ammonium hydroxide. At least about 5 wt.%, preferably about 10
5 wt.%, relative to the keratin, of a multifunctional crosslinking agent is added to the mixture, forming a network precursor solution. Depending upon the crosslinking agent, a catalyst or promotor may be added. The network precursor solution is distributed over an appropriate surface or mold, preferably to a thickness of from about 1 to about 10 mm, and cured by exposure to suitable energy, such as a heat lamp, an autoclave, a
10 microwave, or a UV lamp. In a preferred embodiment, the solutions are placed under a heat lamp effective to produce a temperature of at least about 60°C for from about 30 to 300 minutes.

Crosslinking reactions

Crosslinking of the proteins and network formation occurs, generally, when a
15 non-protein reactant which is at least difunctional, or has at least two reactive groups, is used to crosslink between reactive pendant groups on two different keratin molecules. The non-protein reactant creates a bridge between keratin molecules, and thus produces a three-dimensional network.

Proteins comprise amino acids, which generally have the formula:

20

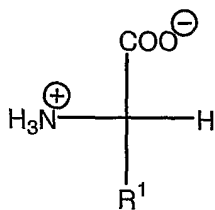
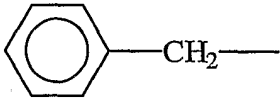
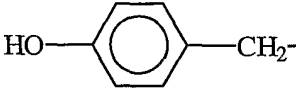
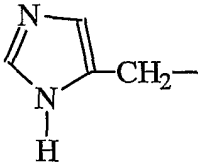
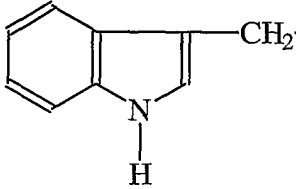


Table 1 summarizes the amino acid residues found in human hair, for example, and shows the "R¹" groups associated with each residue.

Amino Acid	R ¹ Group	Nature	pKa	Iso-electric Point (pH)	Percent Composition in Hair
Cysteine	H—S—CH ₂ —	Nonpolar	8.4	5.02	17.3
Glutamic Acid	$\begin{array}{c} \text{O} \\ \parallel \\ \text{HO}-\text{C}-\text{CH}_2-\text{CH}_2- \end{array}$	Polar	4.5	3.22	13.9
Arginine	$\begin{array}{c} \text{NH} \\ \parallel \\ \text{NH}_2-\text{C}-\text{N}-(\text{CH}_2)_3 \\ \\ \text{H} \end{array}$	Polar	12.5	11.15	9.85
Serine	HO—CH ₂ —	Polar	None	5.68	9
Threonine	$\begin{array}{c} \text{OH} \\ \\ \text{CH}_3-\text{CH}- \end{array}$	Polar	None	5.64	7.75
Leucine	$\begin{array}{c} \text{CH}_3 \\ \diagdown \\ \text{CH}-\text{CH}_2- \\ \diagup \\ \text{CH}_3 \end{array}$	Hydrophobic	None	5.98	7.35
Proline	$\begin{array}{c} \text{CH}_2- \\ \diagup \quad \diagdown \\ \text{CH}_2 \quad \text{CH}_2 \end{array}$	Hydrophobic	None	6.3	6.95
Aspartic Acid	$\begin{array}{c} \text{O} \\ \parallel \\ \text{HO}-\text{C}-\text{CH}_2- \end{array}$	Polar	4.5	2.77	5.8
Valine	$\begin{array}{c} \text{CH}_3 \\ \diagdown \\ \text{CH}- \\ \diagup \\ \text{CH}_3 \end{array}$	Hydrophobic	None	5.96	5.7
Isoleucine	$\begin{array}{c} \text{CH}_3 \\ \\ \text{CH}_3-\text{CH}_2-\text{CH}- \end{array}$	Hydrophobic	None	5.94	4.75

Glycine	H—	Nonpolar	None	5.65	4.15
Phenylalanine		Hydrophobic	None	5.48	3
Alanine	CH ₃ —	Hydrophobic	None	6	2.8
Tyrosine		Hydrophobic	None	5.66	2.6
Lysine	NH ₂ —(CH ₂) ₄ —	Polar	10.4	9.59	2.5
Histidine		Aromatic	6.2	7.47	0.9
Methionine	CH ₃ —S—CH ₂ —CH ₂ —	Hydrophobic	None	5.74	0.85
Tryptophan		Hydrophobic	None	5.89	0.85

The most abundant amino acid in human hair is cysteine, which is found in the form of disulfide-bridged cystine groups. As discussed above, this group can be converted to other sulfur containing moieties, most notably thiol. Thiols theoretically

5 can be reacted with reactive ends of a crosslinking agent using a number of chemical techniques, such as those described in S. Patai (Ed.), *the Chemistry of the Thiol Group*, Parts 1 and 2, John Wiley & Sons, New York, NY (1974), incorporated herein by reference. Other reaction scenarios, such as those directed toward polymer synthesis, also are useful to convert thiols to an assortment of desirable functional residues,

10 including those described in Rempp, P. and Merrill, E. W., *Polymer Synthesis*, Huethig & Wepf Verlag Basel, Heidelberg, Germany (1986); Young, R. J. and Lovell, P. A., *Introduction to Polymers*, Chapman & Hall, London (1991); Odian, G., *Principles of*

Polymerization, John Wiley & Sons, New York, NY (1991), incorporated herein by reference.

In addition to cysteine, the following amino acids have pendant groups comprising nitrogen or oxygen which may be useful as reactive pendant groups; 5 arginine, serine, glutamic acid, threonine, aspartic acid, lysine, asparagine, glutamine, tyrosine, tryptophan, and histidine. Where the protein is α -keratin, preferred amino acid residues comprising reactive pendant groups for crosslinking are cysteine, arginine, serine, and glutamic acid, most preferably cysteine and arginine.

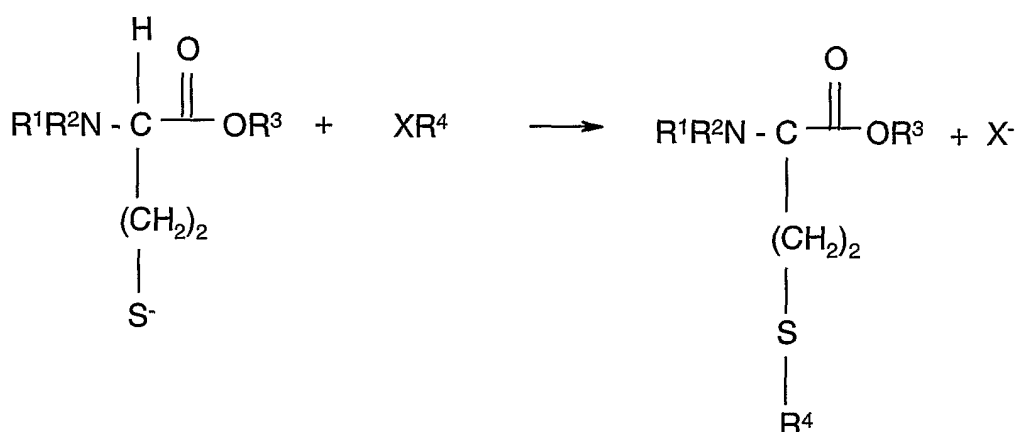
Crosslinking agents comprise at least one reactive group, preferably at least two 10 reactive groups. Preferred reactive groups are selected from the group consisting of epoxide groups, isocyanate groups, and carboxyl groups. Most preferred crosslinking agents are diepoxides, diisocyanates, and dicarboxylates, including anhydrides and hydrolyzed diacids thereof. For convenience, the crosslinking agents described herein sometimes are referred to as "di-" functional. However, unless a crosslinking agent is 15 expressly claimed or expressly stated to be di-functional only, it is to be understood that the crosslinking agents described herein may also be multi-functional, e.g., di-, tri, tetra-, etc. The non-functional portion of the molecule (R^5 , below) generally forms the remainder of the "bridge" crosslinking the protein molecules. R^5 is biocompatible and typically is an organic moiety. Suitable organic moieties include, but are not necessarily 20 limited to alkoxy groups, alkylene groups, and alkenyl groups having from about 1 to about 50 carbon atoms. The alkoxy groups, alkylene groups, or alkenyl groups may be present alone, or in combination with cyclic alkyl groups or aromatic groups.

Without limiting the invention to a particular theory or mechanism of action, unless expressly claimed, the following are crosslinking chemistries involved in producing the heterogeneous crosslinked protein networks:

5 Production of thioether

A preferred reductive modification is the formation of a thiolate anion, followed by nucleophilic substitution employing an appropriate leaving group, yielding a thioether, preferably an alkoxy functional thioether (or a thioester). A preferred alkoxy functional thioether is an epoxy-functional thioether. The general reaction is shown

10 below:

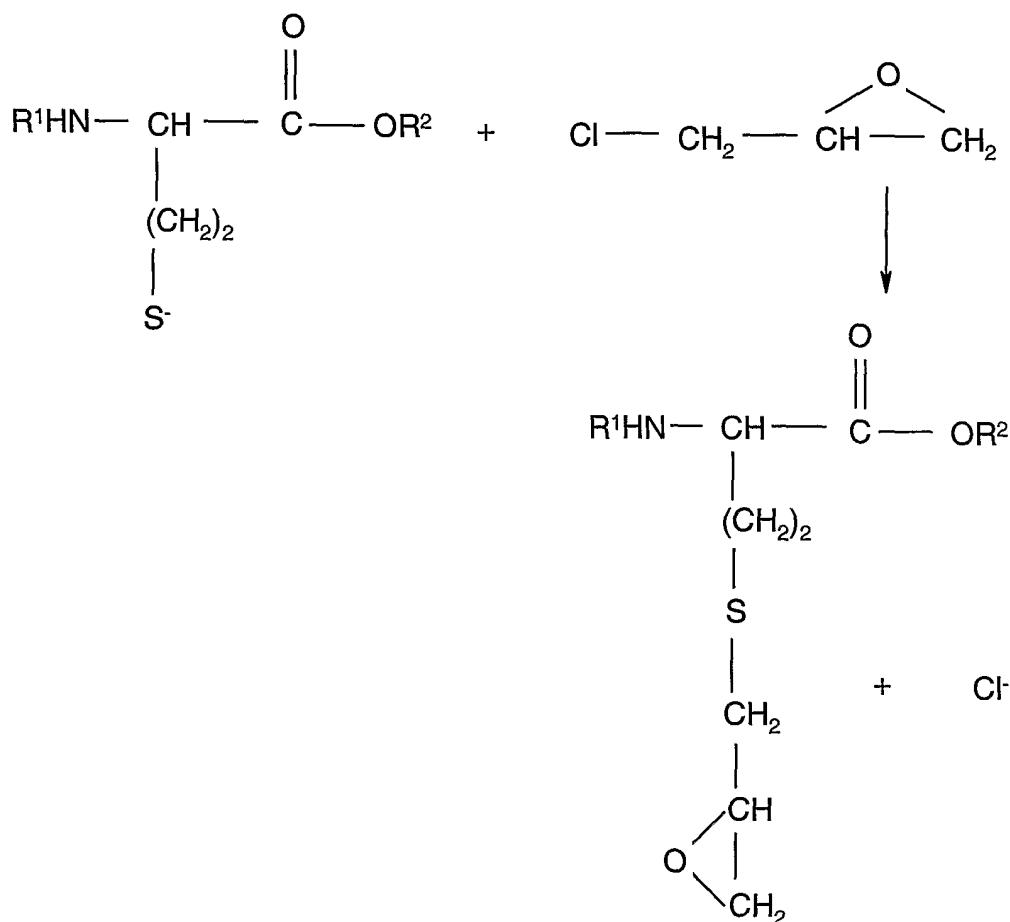


wherein R¹ and R² comprise entities selected from the group consisting of hydrogen and the remainder of the N-terminal portion of the protein molecule; R³ comprises the remainder of the carboxy-terminal portion of the protein molecule; and, R⁴ is a group adapted to form a thioether, preferably an alkoxy functional thioether. Suitable R⁴ groups comprise a "substitution end," which bonds with the sulfur and a "reactive end" which reacts with the crosslinking agent. Suitable substitution ends include, but are not necessarily limited to unsubstituted and halo-substituted alkyl groups and alkylene groups having from about 1 to about 8 carbon atoms, including resonance hybrids, such

as allyl groups, and unsubstituted and halo-substituted aryl groups. Suitable reactive ends include, but are not necessarily limited to acyl groups, and polyalkylethers containing from about 1 to 50 repeat groups, isocyanate groups, silane groups, and silicone groups. Preferred reactive ends include, but are not necessarily limited to 5 carboxyl groups, isocyanate groups, and alkoxide groups. A most preferred reactive end is an epoxide group. In the foregoing formula, X may be any appropriate leaving group. Suitable leaving groups include, but are not necessarily limited to halide groups, tosylate groups, acetate groups, hydroxyl groups, alkoxy groups, and amine groups. Preferred X groups are halides, most preferably chlorine. In a most preferred embodiment, XR⁴ is 10 epichlorohydrin.

The thiolate anion can be generated from thiol, or more directly from the water soluble peptide feedstock, preferably a keratin feedstock, by reaction with a reactive nucleophile. Suitable nucleophiles include alkyl and aryl functional sulfide salts, sulfonates, isocyanates, thiocyanates, halides, hydrosulfide, hydroxide, alkoxides, azides, 15 and acetates preferably alkyl and aryl sulfide salts, hydrosulfide, hydroxide, alkoxides, azides, and acetates.

The reaction where RX is epichlorohydrin is shown below:

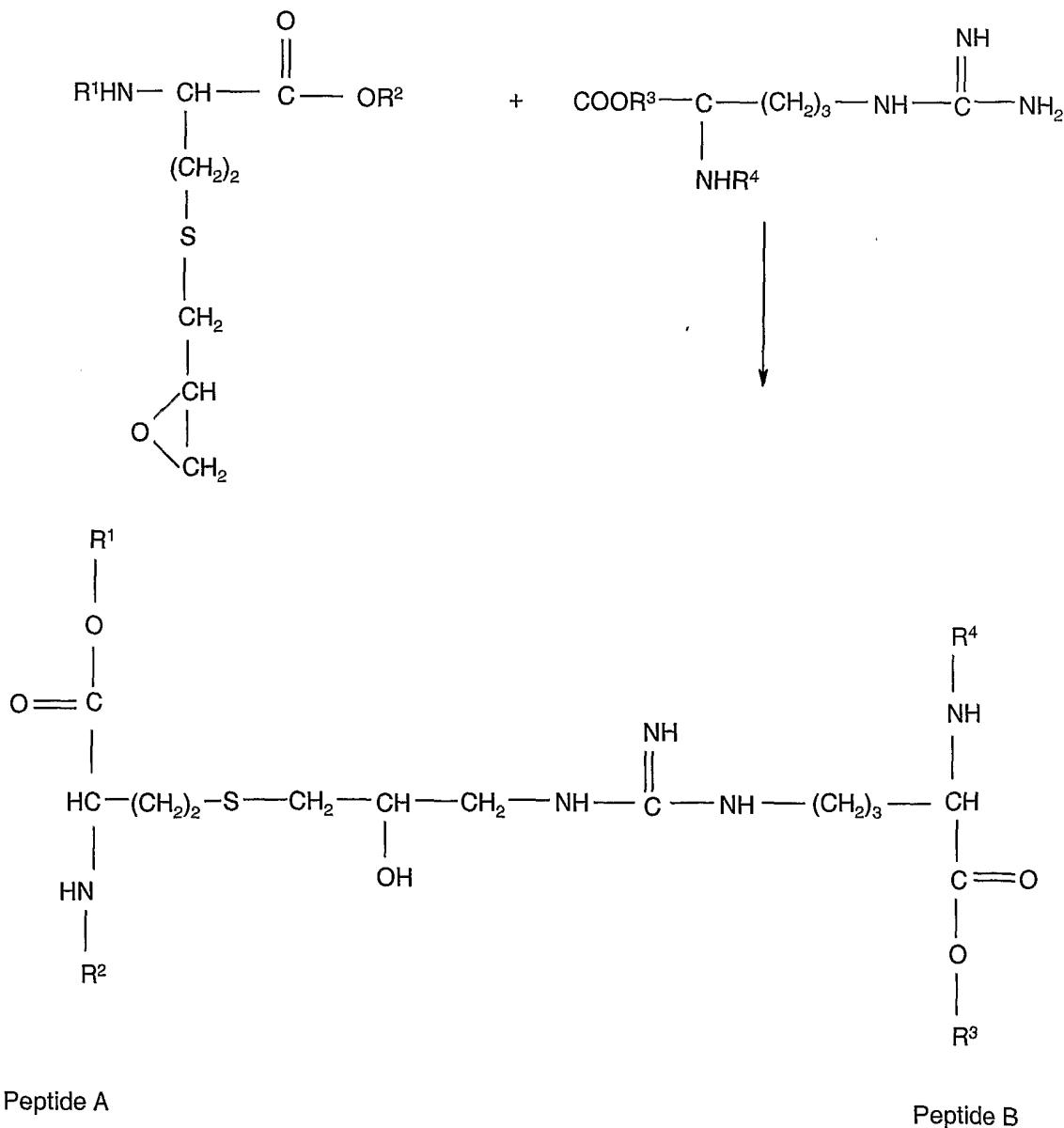


wherein R¹ and R² are the remainder of the water soluble peptide molecule of which cysteine is a part.

- 5 In order to form the foregoing epoxide functionalized water soluble peptides, preferably water soluble keratins, a water soluble keratin source material is first produced, preferably as described above. The water soluble keratins are then exposed to a solution of "RX", preferably epichlorohydrin, in aqueous solution at a pH of from about 9 to about 11. The RX is typically at a concentration of up to about 20 mole
- 10 percent relative to keratin, preferably from about 5 to 10 mole percent relative to keratin, most preferably about 10 mole.%. The pH is greater than about 7, preferably greater than 9. The temperature is from about 20 to about 100 °C, preferably about 60°C. The

reaction continues for a time period of from about 1 to about 72 hours, most preferably about 24 hours. The result is epoxidized thiol groups.

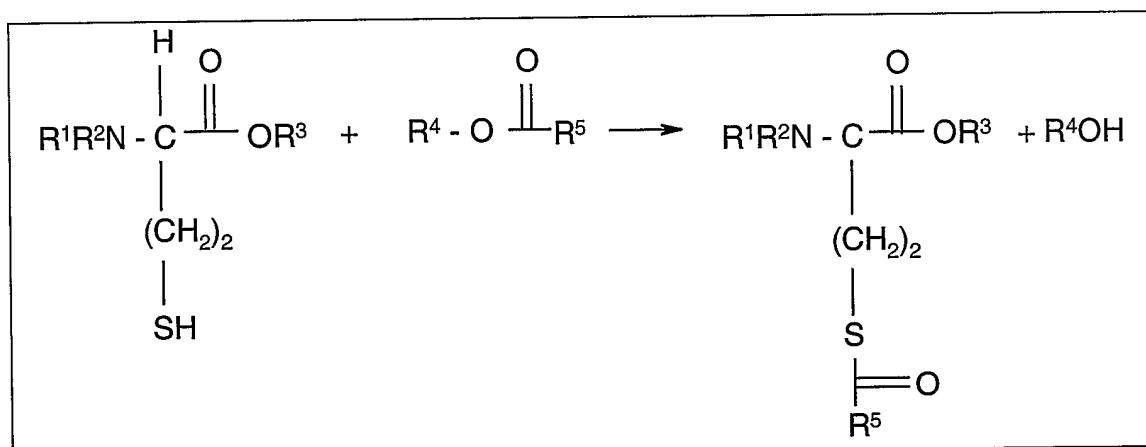
The resulting epoxy functional thioether is reacted with a reactive pendant group on a second water soluble keratin peptide, including but not necessarily limited to a thiol group and a reactive nitrogen-containing group, such as an amine group. The following is an illustration of a crosslinking reaction between an epoxy functional thioether on one water soluble peptide and an arginine residue on another water soluble peptide:



R¹ and R² are the remainder of the water soluble peptide "A" bearing the epoxy functionalized cysteine, and R³ and R⁴ are the remainder of the water soluble peptide molecule B, containing the arginine residue. Although it is theoretically possible for water soluble peptide molecule A and B to be the same molecule, it is preferred for peptides A and B to be different molecules, preferably different water soluble α -keratin molecules.

Conversion of thiol by condensation

Condensation reactions such as transesterification, for example, can be used to generate thioesters. An example of a transesterification reaction is shown in Scheme 3.

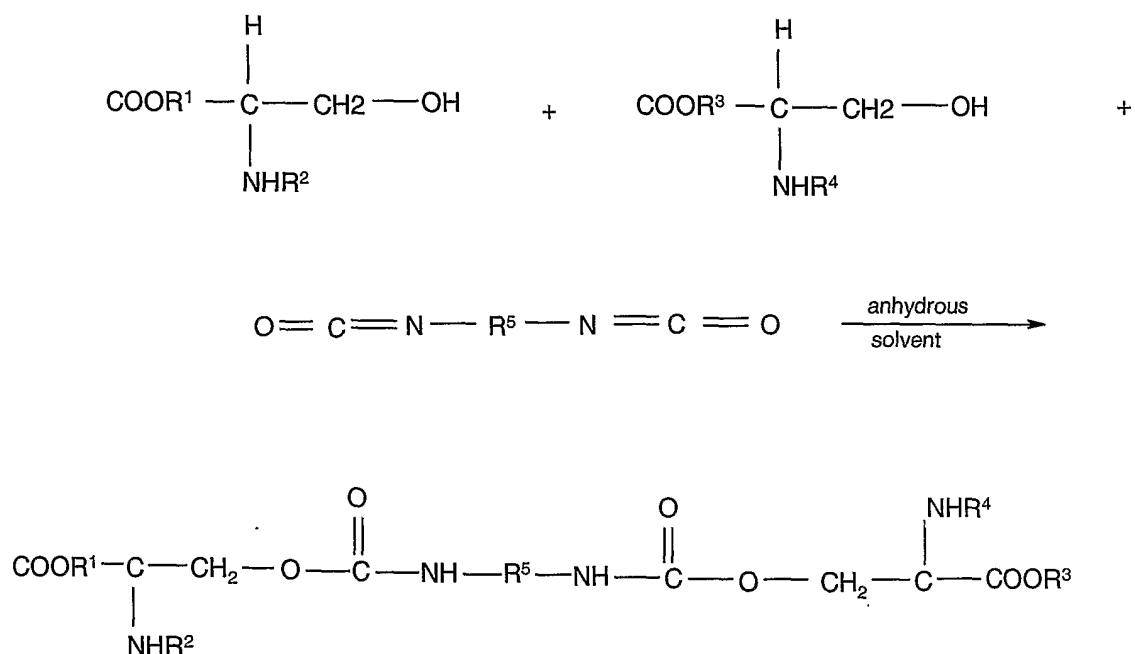


wherein R¹ and R² comprise entities selected from the group consisting of hydrogen and the remainder of the N-terminal portion of the protein molecule; R³ comprises the remainder of the carboxy-terminal portion of the protein molecule; R⁴ is an appropriate leaving group; and, R⁵ is a functional hydrocarbon. Suitable R⁴ groups include, but are not necessarily limited to hydrogen, alkyl groups having from about 1 to 6 carbon atoms, and aryl groups, including benzyl groups. Suitable R⁵ groups include, but are not necessarily limited to aryl groups, including benzyl groups, and alkyl and allyl groups

having from about 1 to about 20 carbon atoms in combination with any number of heteroatoms, such as oxygen and nitrogen, and polyalkylethers comprising from about 1 to 50 repeat groups.

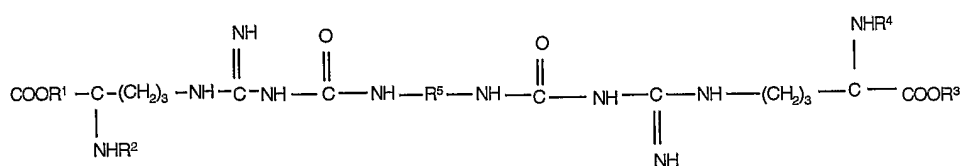
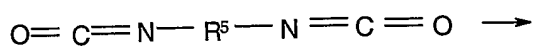
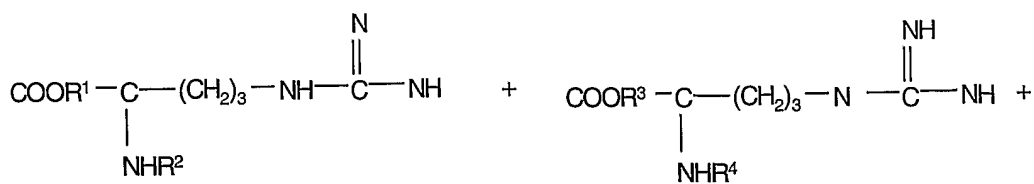
5 Addition of isocyanate to hydroxyl groups

In another preferred embodiment, a diisocyanate is reacted with hydroxyl groups in the keratin, such as those contained in serine. The reaction is shown below:

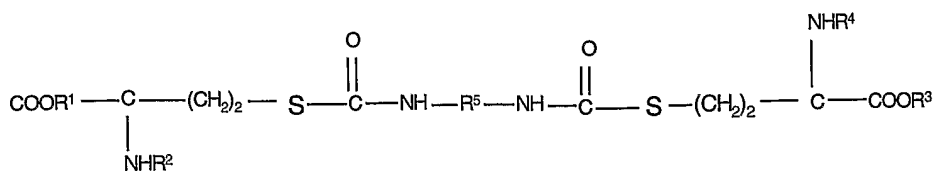
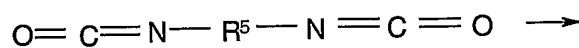
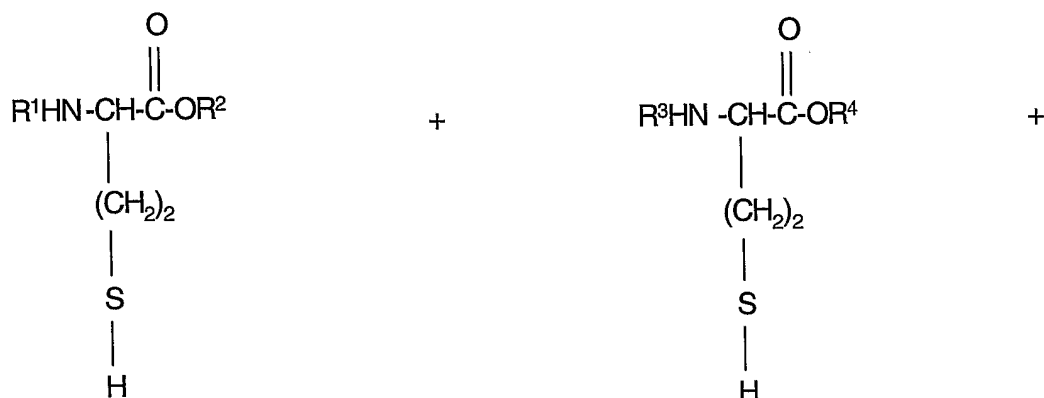


wherein R^1 and R^2 represent the remainder of one protein molecule, and R^3 and R^4 represent the remainder of a second protein molecule preferably a different α -keratin molecule. R^5 may be a variety of organic moieties effective to produce films having the desired properties. In a preferred embodiment, R^5 is selected from the group consisting of aryl groups, including benzyl groups, and alkyl and allyl groups having from about 1 to about 8 carbon atoms. In a preferred embodiment, R^5 is an alkyl group having 6 carbon atoms.

A similar reaction occurs with arginine:



A similar reaction occurs with cysteine, as follows:

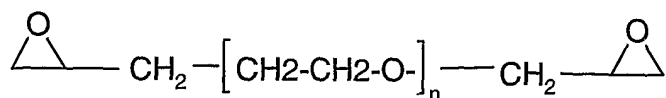


In order to perform these reactions, α -keratin proteins are dissolved in a suitable anhydrous solvent. Preferably, about 2 g of HMWK powder is mixed in an aprotic solvent containing a suitable base, preferably KOH, and the mixture is stirred and heated to a temperature effective to dissolve the keratin, typically about 75 °C. Suitable aprotic solvents include, but are not necessarily limited to isopropyl alcohol, methyl sulfoxide, or DMF.

The resulting solutions are exposed to the isocyanate-containing reagent, including but not limited to aryl diisocyanates, including benzyl diisocyanate, and alkyl and allyl diisocyanates having from about 1 to 8 carbon atoms. A preferred diisocyanate is hexanediisocyanate. The solutions comprise an aprotic solvent and in the presence of an appropriate metal catalyst, preferably an organotin compound, most preferably dibutyltin dilaurate. The concentration of diisocyanate is about 20 mole percent relative to keratin dissolved in the same aprotic solvent, preferably between 5 and 10 mole percent relative to keratin, most preferably about 10 %; at a temperature of from about 0 to about 100°C, preferably about 60-75°C, preferably for a time period of from about 0 to about 72 hours, most preferably about 24 hours or less, most preferably about 6 hours or less. The result is an elastomer which does not hydrolyze upon exposure to an aqueous buffer having a pH of about 7 for at least about 2 days, preferably at least about 7 days.

Addition of Amine Groups

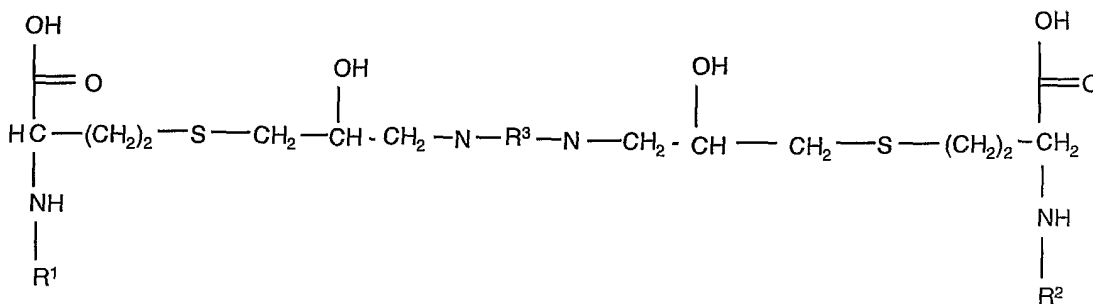
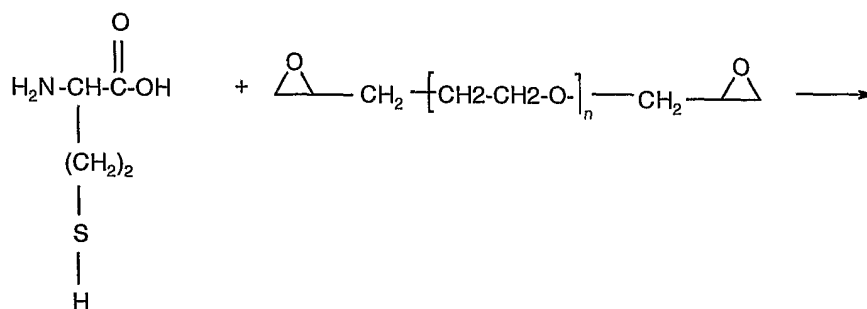
Addition reactions between reactive amine groups and oxirane compounds occur readily without the aid of a catalyst. The crosslinking reaction with arginine is as follows:



wherein n is from about 1 to about 50. Preferred epoxides include DERTM 332 and DERTM 736, available from the Dow Chemical Company.

A similar reaction occurs when a diepoxide reacts with cysteine residues:

5



wherein n is from about 1 to about 50, R¹ and R² are the remainder of a first protein molecule, and R³ and R⁴ are the remainder of a second protein molecule.

10

Persons of ordinary skill in the art will recognize that many of the crosslinking agents described herein will react with a variety of amino acid residues having pendant groups comprising a reactive nitrogen atom, sulfur atom, or oxygen atom. Hence, one end of a diepoxide may react with a cysteine residue while the other end of the diepoxide reacts with an arginine residue, as follows:

hydrophobic. The “dissolution rate” of the resulting network or film is measured by determining how long the film resists hydrolysis upon exposure to an aqueous buffer having a pH of about 7. A desirable “dissolution rate” will depend upon the application in which the film is to be used.

5 The invention will be better understood with reference to the following Examples, which are illustrative only:

Example 1

Reduced, HMWK was prepared by placing 40 g of clean, dry human hair into a 1000 mL wide mouth glass reactor. 800 mL of a 0.8M solution of thioglycolic acid at
10 pH 10.2 (adjusted with potassium hydroxide) was added and the mixture was stirred at room temperature under a nitrogen atmosphere for 18 hours. The solution was filtered and the liquid discarded. The hair was rinsed with copious amounts of deionized (DI) water, then placed back into the reactor. 400 mL of a 7M urea solution was added and the mixture was stirred at room temperature under a nitrogen atmosphere for 24 hours.
15 After urea extraction, the solids were separated from the liquid by centrifugation. The liquid was added dropwise to a 10-fold volume excess of ethanol, thereby forming a keratin precipitate. The precipitated keratins were isolated by filtration and dried under vacuum. The resulting HMWK powder was ground by hand using a mortar and pestle.

Example 2

500 g of clean, dry human hair was placed in a 12000 mL round bottom flask. 8350 mL of 1 weight/volume percent of hydrogen peroxide was added and the reaction heated to reflux for 180 minutes. The hair was separated from the liquid by filtration and the liquid discarded. The hair was rinsed with copious amounts of water and allowed to air dry. 100 g of the dried, oxidized hair was placed in a 2000 mL round bottom flask. 1000 mL of 1M thioglycolic acid at pH 9 (adjusted with ammonium hydroxide) was added and the mixture was heated to 60°C under a nitrogen atmosphere for 24 hours. After reductive extraction, the solids were separated from the liquid by centrifugation. The liquid was added dropwise to a 8-fold volume excess of ethanol, thereby forming a keratin precipitate. The precipitated HMWK keratins were isolated by filtration and dried under vacuum.

A solution was prepared by mixing 2 g of HMWK with 1 mL of 30% ammonium hydroxide and 10 mL of dimethyl sulfoxide. The solution was stirred and heated to ca. 75°C to effect dissolution of the keratins. The solution was split into 4 volumes and placed into separate vials. To each of these 4 solutions was added 5, 10, 15, and 20 weight percent (relative to HMWK) of hexanediisocyanate, respectively, and 0.05, 0.1, 0.15, and 0.2 weight percent (relative to HMWK) of butyldilauryltin catalyst, respectively. The solutions were mixed using a vortex mixer, poured into separate petri dishes, and placed under a heat lamp. After 120 minutes of exposure, the samples were removed from the heat and peeled from the petri dishes. After curing, a small piece of each elastomer was immersed in a pH 7 aqueous buffer solution. After exposure to aqueous buffer for 48 hours, elastomers made with 5, 15, and 20 weight percent

hexanediisocyanate were unchanged. The sample made with 10 weight percent hexanediisocyanate began to slowly hydrolyze after only 24 hours.

Example 3

4.5 g of the HMWK sample from Example 2 was dissolved in 2.25 mL of 30% ammonium hydroxide and 22.5 mL of dimethyl sulfoxide by heating to ca. 75°C and stirred. The solution was split into 9 separate vials and used to prepare the solutions described in the following Table.

Sample	Terephthalic Acid (grams)	Phthalic Anhydride (grams)	DER™ 332 Resin (grams)	Sodium Acetate Catalyst (grams)
1a (control)	-	-	-	-
2a	0.025	-	-	0.005
2b	0.050	-	-	0.005
3a	-	0.025	-	0.005
3b	-	0.050	-	0.005
4a	-	-	0.025	-
4b	-	-	0.050	-

The solutions were poured into separate petri dishes and placed under a heat lamp for ca. 240 minutes. After curing, a small piece of each elastomer was immersed in a pH 7 aqueous buffer solution. After exposure to aqueous buffer for 48 hours, elastomers 1a, 2a, 2b, 3a, and 3b had disintegrated and partially dissolved. After 6 days, elastomers 4a and 4b remained intact.

Persons of ordinary skill in the art will recognize that many modifications may be made to the present invention without departing from the spirit and scope of the present invention. The embodiment described herein is meant to be illustrative only and should not be taken as limiting the invention, which is defined in the claims.

I Claim:

1 1. A method for making a keratin network comprising a heterogeneous
2 crosslinking agent, said method comprising exposing α -keratins comprising reactive
3 pendant groups to a heterogeneous crosslinking agent other than gluteraldehyde
4 comprising a first functional group and a second functional group adapted to react with
5 said reactive pendant groups under conditions effective to induce a first reaction between
6 said first functional groups on a plurality of molecules of said crosslinking agent and first
7 reactive pendant groups on a plurality of first α -keratin molecules and to induce a second
8 reaction between said second functional groups on a plurality of molecules of said
9 crosslinking agent and second reactive pendant groups on a plurality of second α -keratin
10 molecules, thereby producing a heterogenous cross-linked keratin network.

1 2. The method of claim 1 wherein said first reaction is effective to produce
2 covalent bonds between said first functional group and said first reactive pendant group
3 and said second reaction is effective to produce covalent bonds between said second
4 functionality and said second reactive pendant group.

1 3. The method of any of claims 1 or 2 wherein said first functional group
2 and said second functional group are independently selected from the group consisting of
3 alkoxide groups, vinyl groups, hydroxyl groups, amine groups, aldehyde groups,
4 isocyanate groups, ester groups, and anhydride groups.

1 4. The method of any of claims 1-3 wherein said crosslinking agent is
2 selected from the group consisting of a a multi-functional alkoxide, a multifunctional
3 vinyl, a multi-functional hydroxyl, a multifunctional amine, a multi-functional aldehyde,
4 a multi-functional isocyanate, a multifunctional ester, and an anhydride.

1 5. A method for making a keratin network comprising a heterogeneous
2 crosslinking agent, said method comprising:
3 treating soluble α -keratin proteins comprising disulfide bonds under first
4 conditions effective to break said disulfide bonds and to convert cystine
5 residues to thiolate anions;
6 exposing said thiolate anions to a crosslinking agent other than glutaraldehyde
7 comprising at least a first functional group and a second functional group
8 reactive with thiolate anions under conditions effective to induce a first
9 reaction between said first functional group on a plurality of molecules of
10 said crosslinking agent and first thiolate anions on a plurality of first α -
11 keratin protein molecules, and to induce a second reaction between said
12 second functional group on a plurality of molecules of said crosslinking
13 agent and second thiolate anions on a plurality of second α -keratin
14 proteins, thereby producing a heterogenous cross-linked keratin network.

1 6. The method of claim 5 wherein said first reaction and said second
2 reaction produce thiolate ethers.

1 7. The method of any of claims 5-6 wherein said crosslinking agent has the
2 following general structure:



4 wherein R is an organic group adapted to covalently bond with sulfur;

5 X is adapted to be displaced from R by a sulfur anion.

1 8. The method of any of claims 5-7 wherein
2 R comprises a substitution end which bonds with the sulfur and a reactive end
3 which reacts with the crosslinking agent, said substitution end being

4 selected from the group consisting of unsubstituted and halo-substituted
5 alkyl groups and mono- or multialkylene groups having from about 1 to
6 about 8 carbon atoms, and unsubstituted and halo-substituted aryl groups.
7 said reactive ends being selected from the group consisting of acyl
8 groups, and polyalkylethers containing from about 1 to 50 repeat groups,
9 isocyanate groups, organosilane groups, and silicone groups; and

10 X is selected from the group consisting of sulfide groups, sulfonate groups,
11 cyanate groups, thiocyanate groups, halide groups, hydrosulfide groups,
12 hydroxide groups, alkoxide groups, azide groups, tosylate groups, and
13 acetate groups.

1 9. The method of any of claims 7-8 wherein X is a halide group.

1 10. A method for functionalizing α -keratin proteins comprising exposing said
2 α -keratin proteins comprising reactive pendant groups to a nucleophilic substitution
3 agent comprising at least one terminal epoxide under conditions effective to induce a
4 plurality of said reactive pendant groups to react with said nucleophilic substitution
5 agent, thereby producing a plurality of epoxidized α -keratin molecules.

1 11. The method of claim 10 wherein said reactive pendant groups are thiolate
2 anions.

1 12. The method of any of claims 10-11 wherein said nucleophilic substitution
2 agent is epichlorohydrin.

1 13. The method of any of claims 10-12 further comprising treating said
2 plurality of epoxidized α -keratin molecules under conditions effective to induce first
3 epoxidized pendant groups on a plurality of first epoxidized α -keratin molecules to react

4 with nucleophilic pendant groups on a plurality of second α -keratin molecules, producing
5 said cross-linked α -keratin network.

1 14. The method of any of claims 10-13 wherein said nucleophilic pendant
2 group is a reactive amine group.

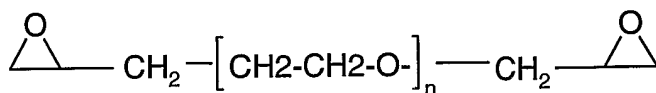
1 15. A method for making a keratin network comprising a heterogeneous
2 crosslinking agent, said method comprising exposing α -keratins comprising reactive
3 pendant groups to a crosslinking agent comprising at least a first terminal epoxide and a
4 second terminal epoxide under conditions effective to induce a first reaction between
5 said first terminal epoxide on a plurality of molecules of said crosslinking agent and first
6 reactive pendant groups on a plurality of first α -keratin proteins molecules, and to induce
7 a second reaction between said second terminal epoxides on a plurality of molecules of
8 said crosslinking agent and second reactive pendant groups on a plurality of second α -
9 keratin proteins molecules, thereby producing a heterogenous cross-linked keratin
10 network.

1 16. The method of claim 15 wherein said reactive pendant groups comprise
2 an element selected from the group consisting of nitrogen, sulfur, oxygen, and a
3 combination thereof.

1 17. The method of any of claims 15-16 wherein an amino acid comprises said
2 reactive pendant group, said amino acid being selected from the group consisting of
3 cysteine, arginine, serine, lysine, asparagine, glutamine, tyrosine, tryptophan, and
4 histidine.

1 18. The method of any of claims 15-16 wherein an amino acid comprises said
2 reactive pendant group, said amino acid being selected from the group consisting of
3 cysteine, arginine, and serine.

1 19. The method of any of claims 15-18 wherein said crosslinking agent is a
2 diepoxide resin having the following general structure:



5

6 wherein n is from about 1 to about 50.

1 20. The method of any of claims 15-19 wherein said nucleophilic substitution
2 agent is a diepoxide resin selected from the group consisting of a diglycidyl ether of
3 bisphenol A and a diglycidyl ether of polyethylene glycol.

1 21. A method for making a keratin network comprising a heterogeneous
2 crosslinking agent, said method comprising exposing soluble α -keratin proteins
3 comprising reactive pendant groups to a crosslinking agent comprising a plurality of
4 carboxylic acid groups under conditions effective to induce first reactions between first
5 carboxylic acid groups on a plurality of molecules of said crosslinking agent and first
6 reactive pendant groups on a plurality of first α -keratin protein molecules, and to induce
7 second reactions between second carboxylic acid groups on a plurality of molecules of
8 said crosslinking agent and second reactive pendant groups on a plurality of second α -
9 keratin protein molecules, thereby producing a heterogenous cross-linked keratin
10 network.

1 22. The method of claim 21 wherein said reactive pendant groups are thiol
2 groups and said first and second reactions produce thiolate esters.

1 23. The method of any of claims 21-22 wherein said crosslinking agent has
2 the following general structure:

3 $R(\text{COOH})_n$

4 wherein

5 R is selected from the group consisting of alkylene groups having from about 1 to
6 about 12 carbon atoms, alkenylene groups having from about 2 to about
7 12 carbon atoms, aryl groups, silyl groups, and silicone groups;
8 n is from about 2 to about 6.

1 24. A method for making a keratin network comprising a heterogeneous
2 crosslinking agent, said method comprising exposing soluble α -keratin proteins
3 comprising reactive pendant groups to phthallic anhydride under conditions effective to
4 induce a first reaction between first reactive pendant groups on a plurality of first α -
5 keratin protein molecules and first carbox-oyl groups of a plurality of molecules of said
6 phthallic anhydride, and to induce a second reaction between second reactive pendant
7 groups on a plurality of second α -keratin protein molecules and second carbox-oyl
8 groups of a plurality of molecules of said phthallic anhydride, thereby producing a
9 heterogenous cross-linked keratin network.

1 25. The method of claim 24 wherein said pendant groups are thiol groups.

1 26. A method for making a keratin network comprising a heterogeneous
2 crosslinking agent, said method comprising exposing soluble α -keratin proteins
3 comprising reactive pendant groups to a crosslinking agent comprising a plurality of
4 cyanate groups under conditions effective to induce a first reaction between first cyanate
5 groups on a plurality molecules of said crosslinking agent and first reactive pendant
6 groups on a plurality of first α -keratin proteins molecules, and to induce a second
7 reaction between second cyanate groups on a plurality of molecules of said crosslinking

8 agent and second reactive pendant groups on a plurality of second α -keratin protein
9 molecules, thereby producing a heterogenous cross-linked keratin network.

1 27. The method of claim 26 wherein said reactive pendant groups are
2 hydroxyl groups.

1 28. The method of any of claims 26-27 wherein said crosslinking agent has
2 the following general structure:



4 wherein R is selected from the group consisting of alkylene groups having from about 1
5 to about 12 carbon atoms, alkenylene groups having from about 2 to about 12 carbon
6 atoms, aryl groups, silyl groups, and silicone groups.

1 29. A method for making a keratin network comprising a heterogeneous
2 crosslinking agent, said method comprising exposing soluble α -keratin proteins
3 comprising pendant thiol groups to phthallic anhydride under conditions effective to
4 induce a first reaction between a first thiol group on a plurality of first α -keratin proteins
5 molecules and first carbox-oyl groups of a plurality of said phthallic anhydride
6 molecules, and to induce a second reaction between second thiol groups on a plurality of
7 second α -keratin protein molecules and second carbox-oyl groups of a plurality of said
8 phthallic anhydride molecules, thereby producing a heterogenous cross-linked keratin
9 network.

1 30. A method for making a keratin network comprising a heterogeneous
2 crosslinking agent other than glutaraldehyde, said method comprising exposing α -keratin
3 proteins comprising carboxylic acid pendant groups to a crosslinking agent comprising
4 at least a first nucleophilic group and a second nucleophilic group under conditions
5 effective to induce a first reaction between said first nucleophilic group on a plurality of

6 molecules of said crosslinking agent and first carboxyl group on a plurality of first α -
7 keratin protein molecules, and to induce a second reaction between said second
8 nucleophilic group on a plurality of molecules of said crosslinking agent and second
9 carboxylic acids on a plurality of second α -keratin protein molecules, thereby producing
10 a heterogenous cross-linked keratin network.

1 31. The method of claim 30 wherein said nucleophilic group is selected from
2 the group consisting of esters, amines, alcohols, and halogenated reagents.

1 32. A method for making a protein network comprising a heterogeneous
2 crosslinking agent other than glutaraldehyde, said method comprising exposing soluble
3 proteins comprising reactive pendant groups to a heterogeneous crosslinking agent
4 comprising a first functional group and a second functional group adapted to react with
5 said reactive pendant groups under conditions effective to induce a first reaction between
6 said first functional groups on a plurality of molecules of said crosslinking agent and first
7 reactive pendant groups on a plurality of first soluble protein molecules, and to induce a
8 second reaction between said second functional groups on a plurality of molecules of
9 said crosslinking agent and second reactive pendant groups on a plurality of second
10 soluble protein molecules, thereby producing a heterogenous cross-linked protein
11 network.

1 33. The method of claim 32 wherein said first reaction is effective to produce
2 covalent bonds between said first functional group and said first reactive pendant group
3 and said second reaction is effective to produce covalent bonds between said second
4 functionality and said second reactive pendant group.

1 34. A heterogenous crosslinked protein network comprising a plurality of
2 protein molecules interlinked by a crosslinking agent other than glutaraldehyde, said

3 network comprising first bonds between first functional groups on a plurality of
4 molecules of said crosslinking agent and first pendant groups on a plurality of first
5 protein molecules and second bonds between second functional groups on a plurality of
6 molecules of said crosslinking agent and second reactive pendant groups on a plurality of
7 second protein molecules.

1 35. The network of any of claims 32-34 wherein said proteins are selected
2 from the group consisting of keratins, collagens, and elastins.

1 36. A heterogenous crosslinked keratin network comprising a plurality of α -
2 keratin molecules interlinked by a crosslinking agent other than glutaraldehyde, said
3 network comprising first bonds between first functional groups on a plurality of
4 molecules of said crosslinking agent and first pendant groups on a plurality of first
5 protein molecules and second bonds between second functional groups on a plurality of
6 molecules of said crosslinking agent and second reactive pendant groups on a plurality of
7 second protein molecules.

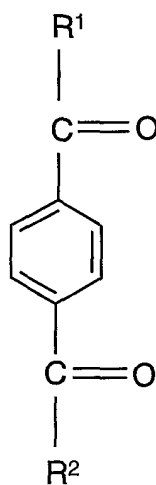
8 37. The method of any of claims 32-35 wherein said first functional group(s)
9 and said second functional group(s) independently are selected from the group consisting
10 of alkoxide groups, vinyl groups, hydroxyl groups, amine groups, aldehyde groups,
11 isocyanate groups, ester groups, and anhydride groups.

1 38. The method of any of claims 32-37 wherein said crosslinking agent is
2 selected from the group consisting of a multifunctional alkoxide, a multifunctional vinyl,
3 a multifunctional hydroxyl, a multifunctional amine, a multifunctional aldehyde, a
4 multifunctional isocyanate, an anhydride, and a multifunctional carboxylic acid.

1 39. The network of claim 34-38 wherein said first bonds and said second
2 bonds are covalent bonds.

1 40. The network of any of claims 32-39 wherein said reactive pendant groups
2 are selected from the group consisting of hydroxyl groups, thiol groups, reactive amine
3 groups, and epoxides.

1 41. A heterogeneous crosslinked proteinaceous network comprising the
2 following crosslinks:



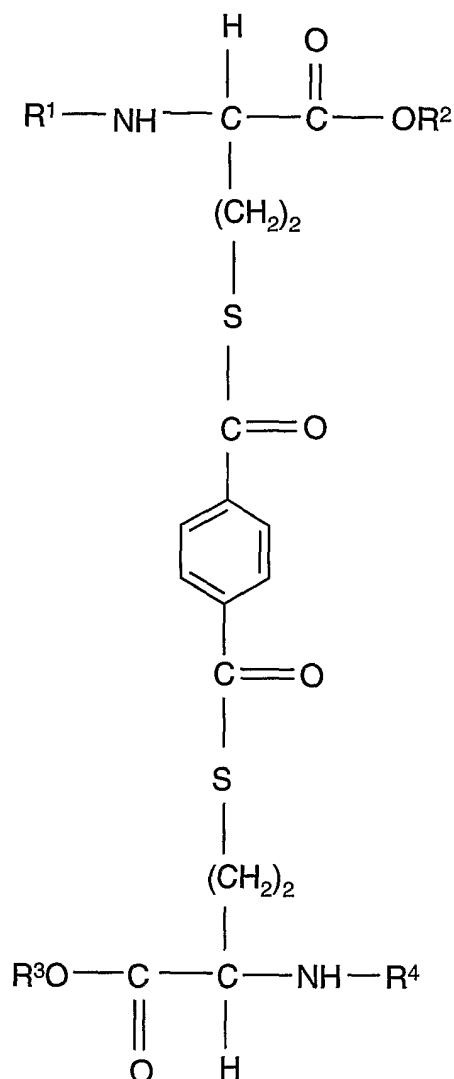
3
4 wherein R¹ and R² independently are amino acid residues from separate protein
5 molecules, the residues being selected from the group consisting of cysteine, arginine,
6 serine, lysine, asparagine, glutamine, tyrosine, tryptophan, and histidine.

1 42. The network of claim 41 wherein said protein molecules are keratin
2 molecules.

1 43. The method of any of claims 41-42 wherein R¹ and R² independently are
2 selected from the group consisting of cysteine and arginine.

1 44. A heterogeneous cross-linked proteinaceous network comprising the
2 following crosslinks:

3



4

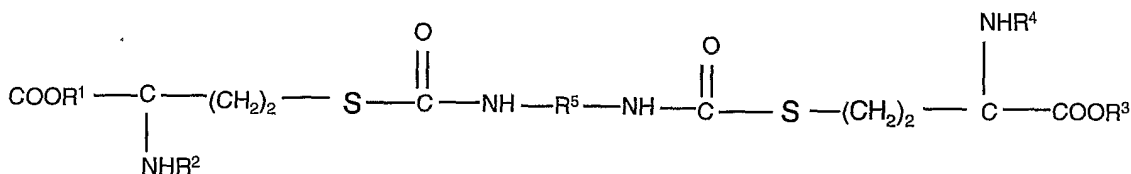
5 wherein R¹ and R² independently are amino acid residues from separate protein
 6 molecules, the residues being selected from the group consisting of
 7 cysteine, arginine, serine, lysine, asparagine, glutamine, tyrosine,
 8 tryptophan, and histidine.

1 45. The network of claim 44 wherein said protein molecules are keratin
 2 molecules.

1 46. A heterogeneous crosslinked proteinaceous network comprising the
 2 following crosslinks:

1 50. A heterogeneous crosslinked proteinaceous network comprising the
2 following crosslinks:

3

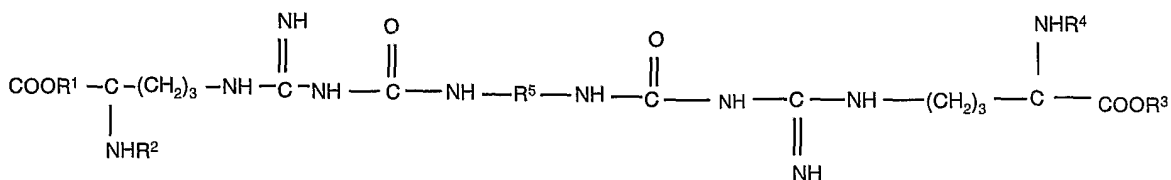


4
5

6 wherein R¹ and R² are the remainder of a first protein molecule; and,
7 R³ and R⁴ are the remainder of a second protein molecule.

1 51. The network of claim 50 wherein said first and second protein molecules
2 are keratin molecules.

1 52. A heterogeneous crosslinked proteinaceous network comprising the
2 following crosslinks:



3
4

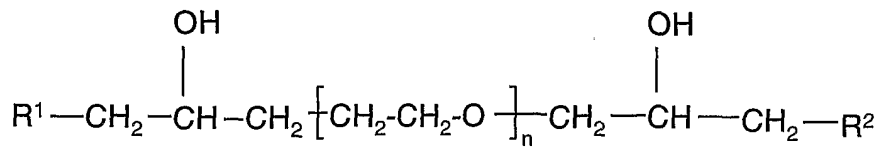
5 wherein R¹ and R² are the remainder of a first protein molecule; and,
6 R³ and R⁴ are the remainder of a second protein molecule.

1 53. The network of claim 52 wherein said first and second protein molecules
2 are keratin molecules.

1 54. A heterogeneous crosslinked network comprises the following crosslinks:

2

3



4

5

6 wherein

7 n is from about 1 to about 50; and,

8 R¹ and R² independently are amino acid residues from separate protein

9 molecules, the residues being selected from the group consisting of

10 cysteine, arginine, serine, lysine, asparagine, glutamine, tyrosine,

11 tryptophan, and histidine.

1 55. The network of claim 54 wherein R¹ and R² independently are selected

2 from the group consisting of cysteine and arginine.

1 56. The network of any of claims 54-55 wherein said protein molecules are

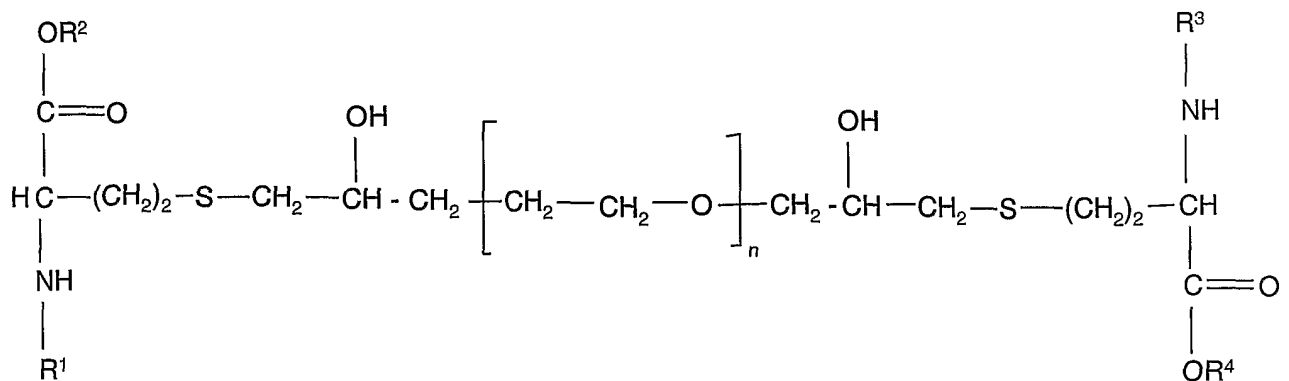
2 keratin molecules.

1 57. A heterogeneous crosslinked proteinaceous network comprising

2 crosslinks having the following structure:

3

4



5
6

7 wherein

8 n is from 1 to 50;

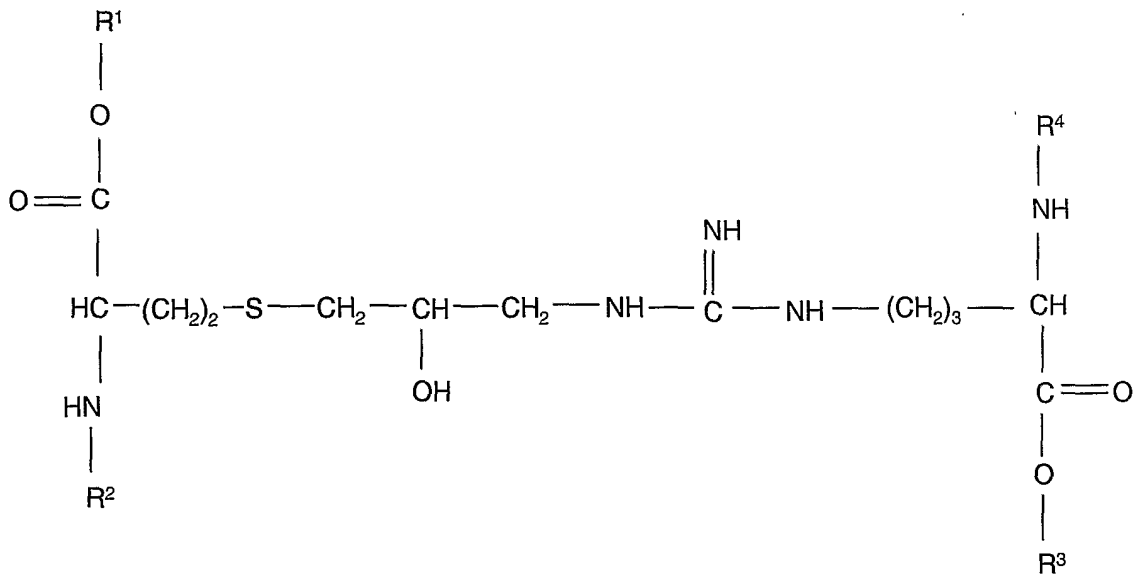
9 R¹ and R² are the remainder of a first protein molecule; and,

10 R³ and R⁴ are the remainder of a second protein molecule.

1 58. The network of claim 57 wherein said first and second protein molecules
2 are keratin molecules.

1 59. A heterogeneous crosslinked proteinaceous network comprising the
2 following crosslinks:

3



4
5

6

7 wherein

8 R¹ and R² are the remainder of a first protein molecule; and

9 R³ and R⁴ are the remainder of a second protein molecule.

10 60. A method for increasing or decreasing the dissolution rate of a
11 heterogeneous crosslinked proteinaceous network comprising controlling a quantity of

12 crosslink bonds having a characteristic selected from the group consisting
13 hydrophobicity, hydrolytic stability, and steric hindrance.

1 61. The method of any of claims 1-60 wherein said α -keratins are derived
2 from human hair.

1 62. The method of any of claims 1-61 wherein said cross-linked keratin
2 network resists hydrolysis upon exposure to an aqueous buffer having a pH of about 7
3 for at least 7 days.

1 63. A network or method as described in the present application.

1 64. A heterogeneous, cohesive keratin network and method for making same.