(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
27 February 2020 (27.02.2020)

WIPO | PCT

(10) International Publication Number
WO 2020/041557 A1

(51) International Patent Classification:
C07K 1/107 (2006.01) *C09J 189/00* (2006.01)
C07K 14/00 (2006.01)

(21) International Application Number:
PCT/US2019/047639

(22) International Filing Date:
22 August 2019 (22.08.2019)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/721,112 22 August 2018 (22.08.2018) US

(71) Applicant: **PURDUE RESEARCH FOUNDATION** [US/US]; 1801 Newman Road, West Lafayette, Indiana 47906 (US).

(72) Inventors: **LIU, Julie C.**; 3606 Swansea Drive, West Lafayette, Indiana 47906 (US). **HOLLINGSHEAD, Sydney E.**; 1606 Mayview Avenue, Cleveland, Ohio 44109 (US). **LIN, Charng-yu**; 2550 Yeager Road, Apt. 7-10, West Lafayette, Indiana 47906 (US). **WILKER, Jonathan J.**; 410 South 7th Street, Lafayette, Indiana 47901 (US).

(74) Agent: **YAN, LIANG ZENG**; 1801 Newman Road, West Lafayette, Indiana 47906 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ,

(54) Title: PROTEIN-BASED ADHESIVE AND ITS MODIFICATION

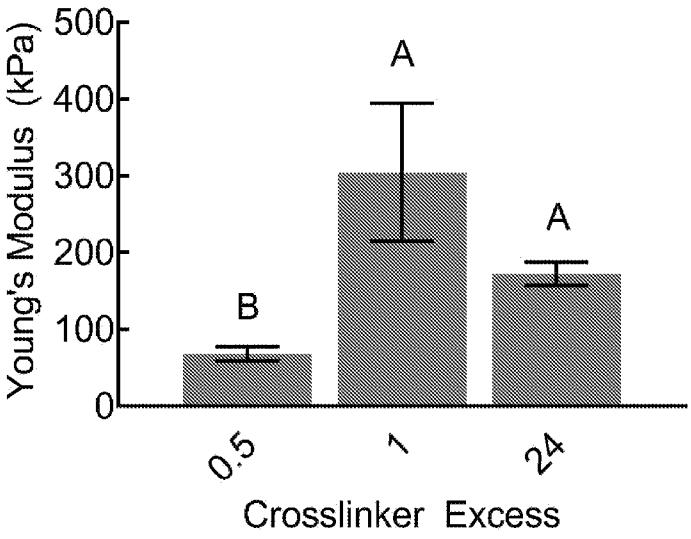

FIG. 2

(57) Abstract: This invention relates to protein-based adhesives that are capable to adhere to a substrate in a dry, wet, moist, or an aqueous environment. Particularly, said adhesives comprise a crosslinking agent and an elastin-like polypeptide (ELP) that contain tyrosine, lysine, cysteine, dihydroxyphenylalanine (DOPA) or trihydroxyphenylalanine (TOPA) amino acid residue, or a combination thereof, on the polypeptide chain of said ELP. Among others, the adhesives disclosed herein may find broad applications in medical treatments and surgical operations. Compositions and methods of use are within the scope of this application.

WO 2020/041557 A1

[Continued on next page]

UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

PROTEIN-BASED ADHESIVE AND ITS MODIFICATION

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefits of United States Provisional Application Serial No. 62/721,112, filed August 22, 2018, the contents of which are incorporated herein in their entirety.

GOVERNMENT RIGHTS

[0002] This invention was made with Government support under DMR1309787, awarded by the National Science Foundation. The Government has certain rights in the invention.

STATEMENT OF SEQUENCE LISTING

[0002] A computer-readable form (CRF) of the Sequence Listing is submitted with this application. The file, generated on August 21, 2019, is entitled 68335-02_ST25_txt, the contents of which are incorporated herein in their entirety. Applicant states that the content of the computer-readable form is the same and the information recorded in computer readable form is identical to the written sequence listing.

TECHNICAL FIELD

[0003] This invention relates to protein-based adhesives that are capable to adhere to a substrate in a dry, wet, moist, or an aqueous environment, particularly, to adhesives comprising a crosslinking agent and an elastin-like polypeptide (ELP) that contain tyrosine, lysine, cysteine, dihydroxyphenylalanine (DOPA) or trihydroxyphenylalanine (TOPA) amino acid residue, or a combination thereof, on the polypeptide chain. Among others, the adhesives disclosed herein may find various applications in medical treatments or surgical operations.

BACKGROUND

[0004] This section introduces aspects that may help facilitate a better understanding of the disclosure. Accordingly, these statements are to be read in this light and are not to be understood as admissions about what is or is not prior art.

[0005] There has been a wealth of recent interest in the development of adhesive materials that function in wet or underwater environments. In particular, much of this focus has been placed on adhesive development for biomedical applications, as a suitable biomedical adhesive could have an immense impact on health and the economy. Each year, over 230 million major surgeries are performed worldwide, and over 12 million traumatic wounds are treated in the U.S. alone. Approximately 60% of these wounds are closed using mechanical methods such as sutures and staples. Sutures and staples have several disadvantages relative to adhesives, including patient discomfort, higher risk of infection, and the inherent damage to surrounding healthy tissue.

[0006] Current FDA-approved adhesives and sealants face several challenges. First, numerous adhesives exhibit toxic characteristics. For example, cyanoacrylate-based adhesives like Dermabond® and SurgiSeal® can only be applied topically due to carcinogenic degradation products. Fibrin sealants like Tisseel and Artiss are derived from blood sources and therefore carry the potential for blood-borne pathogen transmission. Poly(ethylene glycol) (PEG) adhesives are approved as a suture sealants but, due to intense swelling when wet, have the potential to cause moderate inflammatory responses. TissuGlu®, a following subcutaneous implantation, and, in clinical trials, seroma formation occurred in 22% of patients. More important, however, is that most of these adhesives do not possess strong adhesion in an excessively wet environment and are not approved for application in wound closure. In fact, many of these materials specifically advise to dry the application area as much as possible.

[0007] In approaching the challenge of developing a strong adhesive for wet applications, many researchers have been inspired by natural glues. Specifically, underwater application and bonding has been demonstrated with materials based on organisms such as sandcastle worms and mussels. Both of these organisms produce proteins containing the non-canonical amino acid 3,4- dihydroxyphenylalanine (DOPA), which has been shown to provide adhesion strength, even in wet environments. In the case of a mussel-mimetic polymer, underwater application was achieved by dissolving the polymer in a chloroform/methanol solution to maintain phase separation from the aqueous environment. The use of toxic organic solvents, however, is not appropriate for biomedical applications.

[0008] An alternative method for underwater application uses the phenomenon of coacervation, a form of aqueous liquid-liquid phase separation that is implicated in the adhesion mechanism of sandcastle worms, caddisfly larvae, and mussels. Adhesive coacervate materials mimicking both mussels and sandcastle worms have been developed. To form these coacervates, multiple components needed to be mixed in specific conditions and thus limited their overall applicability.

[0009] As it can be seen, there is a need for a strong adhesive that functions in a wet environment. It would also be desirable if this adhesive could be manipulated in forming a strong seal in the desired environment. It would be further desirable if the adhesive was also non-toxic and may be used in biomedical applications.

BRIEF SUMMARY

[0010] The present disclosure provides protein-based adhesives that are capable to adhere to a substrate in a dry, wet, moist or even an aqueous environment. Particularly, said adhesives comprise a crosslinking agent and an elastin-like polypeptide (ELP) that contain tyrosine, lysine, cysteine, dihydroxyphenylalanine (DOPA) or trihydroxyphenylalanine (TOPA) amino acid residue, or a combination thereof, on the polypeptide chain of ELP. The ELP-based adhesives of the present disclosure show high cytocompatability and are appropriate for use in biomedical applications, including broad applications in medical operations or surgeries.

[0011] In some embodiments, the present disclosure provides protein-based adhesives that are capable to adhere to a substrate in a dry, wet, moist or an aqueous environment. Particularly, the physical and chemical properties, including the cure time and strength, of said adhesives comprising a crosslinking agent and an elastin-like polypeptide (ELP) that contain tyrosine, lysine, cysteine, dihydroxyphenylalanine (DOPA) or trihydroxyphenylalanine (TOPA) amino acid residue, or a combination thereof, on the polypeptide chain of ELP, may be modified and controlled by adjusting the type and amount of said crosslinking agent.

[0012] In some illustrative embodiments, the present invention relates to an adhesive comprising a crosslinking agent and an elastin-like polypeptide (ELP), wherein polypeptide chain of said ELP contains tyrosine, lysine, cysteine, dihydroxyphenylalanine (DOPA) or trihydroxyphenylalanine (TOPA) amino acid residue, or a combination thereof.

[0013] In some other embodiments, the present invention relates to an adhesive comprising a crosslinking agent, an elastin-like polypeptide (ELP), wherein polypeptide chain of said ELP contains tyrosine, lysine, cysteine, dihydroxyphenylalanine (DOPA) or trihydroxyphenylalanine (TOPA) amino acid residue, or a combination thereof, and an oxidizing agent.

[0014] In some illustrative embodiments, the present invention relates to an adhesive disclosed herein, wherein said oxidizing agent comprises a ferric salt, hydrogen peroxide, sodium periodate, or an enzyme that transforms the phenolic side chain of tyrosine residues of said ELP.

[0015] In some illustrative embodiments, the present invention relates to an adhesive disclosed herein, wherein said DOPA of polypeptide chain of said ELP is generated by using a tyrosinase.

[0016] In some illustrative embodiments, the present invention relates to an adhesive disclosed herein, wherein said adhesive is capable of adhering to a substrate when applied to said substrate in a dry, wet, moist, or an aqueous environment.

[0017] In some illustrative embodiments, the present invention relates to an adhesive disclosed herein, wherein said adhesive is compatible for biomedical applications.

[0018] In some illustrative embodiments, the present invention relates to an adhesive disclosed herein, wherein said adhesive is useful in medical treatments or surgical operations.

[0019] In some illustrative embodiments, the present invention relates to an adhesive disclosed herein, wherein said crosslinking agent is an oxidizing agent.

[0020] In some illustrative embodiments, the present invention relates to an adhesive disclosed herein, wherein said oxidizing agent comprises a ferric salt, hydrogen peroxide, sodium periodate, or an enzyme that transforms the phenolic side chain of tyrosine residues of said ELP.

[0021] In some illustrative embodiments, the present invention relates to an adhesive disclosed herein, wherein said crosslinking agent comprises trishydroxymethylphosphine (THP), tetrakis(hydroxymethyl)phosphonium chloride (THPC), an N-hydroxysuccinyl ester (NHS), genipin, pyridyl disulfide, a maleimide, a vinylsulfone, a haloacetyl, or a combination thereof.

[0022] In some illustrative embodiments, the present invention relates to a method of manufacturing an adhesive comprising the steps of:

providing a crosslinking agent; and

providing an elastin-like polypeptide (ELP), wherein polypeptide chain of said ELP contains tyrosine, lysine, cysteine, dihydroxyphenylalanine (DOPA) or trihydroxyphenylalanine (TOPA) amino acid residue, or a combination thereof.

[0023] In some illustrative embodiments, the present invention relates to a method of manufacturing an adhesive disclosed herein, wherein the method further comprises a step of adding an oxidizing agent.

BRIEF DESCRIPTION OF DRAWINGS

[0024] The disclosed embodiments and other features, advantages, and disclosures contained herein, and the matter of attaining them, will become apparent and the present disclosure will be better understood by reference to the following description of various exemplary embodiments of the present disclosure taken in conjunction with the accompanying drawings, wherein:

[0025] Fig. 1, changes occur in the mass of YKV hydrogels after swelling across a range of pH (square marker) and absolute zeta potential of YKV solution across a range of pH (circle marker). YKV was crosslinked into hydrogels using THP. Zeta potential is a measure of overall surface charge of a protein. Actually, a change in mass less than 100% indicates a shrinking. The hydrogels tended to shrink at pH where the overall charge (zeta potential) was low. Shrinking near physiological pH is useful for surgical adhesives, as it reduces the risk of pressure on nearby tissues, blood vessels, and nerves.

[0026] Fig. 2 shows Young's modulus (stiffness) of YKV hydrogels at varying THP crosslinker amounts swelled at pH 7. Crosslinker excess indicates the molar amount of hydroxyl groups in THP compared to the molar amount of amines in YKV. The amount of THP incorporated into a YKV hydrogel will affect the stiffness of the gel. A very high (24) or low (0.5) excess of THP will create a less connected hydrogel network, resulting in a softer gel. An equimolar amount of THP hydroxyl groups and YKV amine groups (1) creates a more highly connected network, with higher stiffness.

[0027] Fig. 3A shows Young's Modulus of YKV hydrogels at varying THP crosslinking ratios swelled across a range of pH. At all crosslinking ratios, YKV hydrogels tended to become stiffest near physiological pH. Changing the THP crosslinking ratio affected the amount of pH response in the hydrogels.

[0028] Fig. 3B shows swelling and opacity of hydrogels was affected by crosslinking ratio and acetylation. ELP[YKV-48] hydrogels with 24:1, 1:1, and 1:2 crosslinking ratio, and HDØ-ELP[YKV-48] and acELP[YKV-48] hydrogels with 1:2 crosslinking ratio, swelled at pH 3 to 11 for six days. Scale bar is 5 mm.

[0029] Fig. 4A describes the mass fraction of supernatant and coacervate after phase separation across a range of initial solution concentrations. As the initial solution concentration increases, the mass fraction of coacervate after phase separation increases.

At ~400 mg/mL or higher, the coacervate phase has a mass fraction close to 1 (data not shown).

[0030] Fig. 4B depicts SDS PAGE gel of purified ELP[YKV-48] (Lane #1), HDØ-ELP[YKV-48] (Lane #2), and acELP[YKV-48] (Lane #3).

[0031] Fig. 5 shows the concentrations of supernatant and coacervate after phase separation across a range of initial solution concentrations. As initial solution concentration increases, the concentration of the supernatant plateaus. The coacervate concentration has more variability due to measuring error, but also seems to plateau.

[0032] Fig. 6 depicts adhesive strength of YKV dissolved in water (black) and PBS (light grey), applied to aluminum and cured at 37 °C for 24 hours under dry conditions. Increasing protein concentration increases the adhesive strength. Using water as the solvent increases the adhesive strength compared to PBS. Isolated coacervate has similar adhesive strength to high concentration protein solutions. n=3 for water and 4 for PBS. No statistical analysis.

[0033] Fig. 7 shows adhesive strength of YKV dissolved in water (black) and PBS (light grey) and mYKV dissolved in PBS (dark grey), applied to aluminum and cured at 37 °C for 24 hours under humid conditions. The error bars of "YKV in water" are too small to be shown. Increasing the concentration improves adhesive strength. Using PBS increases adhesive strength compared to water. At lower concentrations, mYKV has a higher adhesive strength than YKV. At higher concentrations, YKV and mYKV have similar adhesive strength. Isolate coacervate has similar adhesive strength to high concentration protein solutions. No statistical analysis.

[0034] Fig. 8 demonstrates the adhesion strength of BSA (grey striped), YKV (light grey), and mYKV (black), in water at pH 7.4 on pig skin in physiological conditions (humid, 37 °C) across a range of concentrations. The addition of DOPA to YKV improves adhesion.

[0035] Fig. 9 shows the adhesive strength of YKV (light grey) and mYKV (black) mixed with THP at various ratios and applied to pig skin under physiological conditions (humid, 37 °C) and cured for 24 hours. 1:0 indicates protein mixed with no THP. Increasing the concentration of THP increased the adhesive strength of the protein, up to 1:50. At 1:10 and 1:50, YKV and mYKV have similar adhesive strength.

[0036] Fig. 10 describes the adhesive strength of commercial glue Tisseel (dark grey), and THP mixed at 1:50 with water (white), BSA (grey stripe), YKV (light grey), and mYKV (black). Various proteins mixed with THP have higher adhesion strength than Tisseel.

[0037] Fig. 11 shows the adhesive strength of YKV (light grey) and mYKV (black) mixed with iron nitrate nonahydrate at various ratios and applied to pig skin under physiological conditions (humid, 37 °C) and cured for 24 hours. 3:0 indicates protein mixed with no iron. Increasing the concentration of iron to a ratio of 3:10 increased the adhesive strength of the protein. At 3:1 and 3:10, mYKV had higher adhesion strength than YKV.

[0038] Fig. 12 describes the adhesive strength of commercial glue Tisseel (dark grey), and iron mixed at 3:10 with water (white), BSA (grey stripe), YKV (light grey), and mYKV (black). The addition of DOPA improves adhesion in the presence of iron. mYKV and BSA mixed with iron have higher adhesive strength than Tisseel.

[0039] Fig. 13 shows the adhesive strength of mYKV mixed with sodium periodate at various ratios (catechol: periodate) and applied to pig skin under physiological conditions (humid, 37C) and cured for 24 hours. 1:0 indicates protein mixed with no iron. Increasing the concentration of periodate up to 1:0.1 increased the adhesive strength of the protein.

[0040] Fig. 14 shows the adhesive strength of Tisseel (dark grey), and periodate mixed at 1:0.1 with water (white), BSA (grey stripe), and mYKV (black). Periodate mixed with water or BSA has negligible adhesive strength. Periodate mixed with mYKV has a higher adhesive strength than Tisseel.

[0041] Fig. 15A shows adhesive strength of water mixed with crosslinker combinations of THP (T) at 1:50, Iron (I) at 3:10, and Periodate (P) and 1:0.1. Water mixed with TP had higher adhesion strength than tisseel.

[0042] Fig. 15B shows adhesive toughness of water mixed with crosslinker combinations of THP (T) at 1:50, Iron (I) at 3:10, and Periodate (P) and 1:0.1. Water mixed with TP had higher adhesion strength than tisseel.

[0043] Fig. 16A shows adhesive strength of YKV mixed with crosslinker combinations of THP (T) at 1:50, Iron (I) at 3:10, and Periodate (P) and 1:0.1. YKV mixed with T, TP,

and TIP had the highest adhesive strength and toughness. YKV with T, TP, and TIP had 17X higher adhesion strength than Tisseel.

[0044] Fig. 16B depicts toughness of YKV mixed with crosslinker combinations of THP (T) at 1:50, Iron (I) at 3:10, and Periodate (P) and 1:0.1. YKV mixed with T, TP, and TIP had the highest adhesive strength and toughness. YKV with T, TP, and TIP had 17X higher adhesion strength than Tisseel.

[0045] Fig. 17A shows adhesive strength of mYKV mixed with crosslinker combinations of THP (T) at 1:50, Iron (I) at 3:10, and Periodate (P) and 1:0.1. Adhesion strength was higher than Tissel with T, I, TP, and TIP. Toughness was higher than Tisseel with I, TI, and TP.

[0046] Fig. 17B shows toughness of mYKV mixed with crosslinker combinations of THP (T) at 1:50, Iron (I) at 3:10, and Periodate (P) and 1:0.1. Adhesion strength was higher than Tissel with T, I, TP, and TIP. Toughness was higher than Tisseel with I, TI, and TP.

[0047] Fig. 18A depicts adhesive strength of water, YKV and mYKV mixed with crosslinker combinations of THP (T) at 1:50, Iron (I) at 3:10, and Periodate (P) and 1:0.1. Data are the same as Figs. 1.8 to 1.10. YKV and mYKV had higher adhesion strength than water when mixed with T. YKV had higher adhesion strength and toughness than YKV when mixed with I. YKV had higher adhesion strength and toughness than mYKV when mixed with TIP.

[0048] Fig. 18B shows toughness of water, YKV and mYKV mixed with crosslinker combinations of THP (T) at 1:50, Iron (I) at 3:10, and Periodate (P) and 1:0.1. Data are the same as Figs. 1.8 to 1.10. YKV and mYKV had higher adhesion strength than water when mixed with T. YKV had higher adhesion strength and toughness than YKV when mixed with I. YKV had higher adhesion strength and toughness than mYKV when mixed with TIP.

[0049] It should be appreciated that not all of the features of the components of the figures are necessarily described. Some of these non-discussed features, such as various couplers, etc., as well as discussed features are inherent from the figures themselves. Other non-discussed features may be inherent in component geometry and/or configuration.

DETAILED DESCRIPTION

[0001] For the purposes of promoting an understanding of the principles of the present disclosure, reference will now be made to the embodiments illustrated in the drawings, and specific language will be used to describe the same. The following detailed description is of the best currently contemplated modes of carrying out the disclosure. The description is not to be taken in a limiting sense, but is made merely for the purpose of illustrating the general principles of the disclosure, since the scope of the disclosure is best defined by the appended claims.

[0002] As used herein, the following terms and phrases shall have the meanings set forth below. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art.

[0003] In the present disclosure the term "about" can allow for a degree of variability in a value or range, for example, within 20%, within 10%, within 5%, or within 1% of a stated value or of a stated limit of a range.

[0004] In the present disclosure the term "substantially" can allow for a degree of variability in a value or range, for example, within 80%, within 90%, within 95%, or within 99% of a stated value or of a stated limit of a range.

[0005] In this document, the terms "a," "an," or "the" are used to include one or more than one unless the context clearly dictates otherwise. The term "or" is used to refer to a nonexclusive "or" unless otherwise indicated. In addition, it is to be understood that the phraseology or terminology employed herein, and not otherwise defined, is for the purpose of description only and not of limitation. Any use of section headings is intended to aid reading of the document and is not to be interpreted as limiting. Further, information that is relevant to a section heading may occur within or outside of that particular section. Furthermore, all publications, patents, and patent documents referred to in this document are incorporated by reference herein in their entirety, as though individually incorporated by reference. In the event of inconsistent usages between this document and those documents so incorporated by reference, the usage in the incorporated reference should be considered supplementary to that of this document; for irreconcilable inconsistencies, the usage in this document controls.

[0006] The term "patient" includes human and non-human animals such as companion animals (dogs and cats and the like) and livestock animals. Livestock animals are animals raised for food production. The patient to be treated is preferably a mammal, in particular a human being.

[0007] For reference, the following amino acid abbreviations and names may be identified herein as applying to one or more polypeptides or proteins of the present disclosure:

Three-letter Abbreviation	Single-letter Abbreviation	Amino Acid Name
Ala	A	Alanine
Arg	R	Arginine
Asn	N	Asparagine
Asp	D	Aspartic acid (Aspartate)
Cys	C	Cysteine
Gln	Q	Glutamine
Glu	E	Glutamic acid (Glutamate)
Gly	G	Glycine
His	H	Histidine
Ile	I	Isoleucine
Leu	L	Leucine
Lys	K	Lysine
Met	M	Methionine
Phe	F	Phenylalanine
Pro	P	Proline
Ser	S	Serine
Thr	T	Threonine
Trp	W	Tryptophan
Tyr	Y	Tyrosine
Val	V	Valine
Asx	B	Aspartic acid or Asparagine
Glx	Z	Glutamine or Glutamic acid
Xaa	X	(any amino acid, or a group of amino acids, as may be referenced herein)

[0008] Broadly speaking, the present disclosure provides protein-based adhesives that are capable to adhere to a substrate in a dry, wet, moist or even an aqueous environment. Particularly, said adhesives comprise a crosslinking agent and an elastin-like polypeptide (ELP) that contain tyrosine, lysine, cysteine, dihydroxyphenylalanine (DOPA) or trihydroxyphenylalanine (TOPA) amino acid residue, or a combination thereof, on the polypeptide chain of ELP. The ELP-based adhesives of the present disclosure show high

cytocompatability and are appropriate for use in biomedical applications, including broad applications in medical operations or surgeries. Compositions and methods of use are within the scope of this application.

[0009] In some embodiments, the present disclosure provides protein-based adhesives that are capable to adhere to a substrate in a dry, wet, moist or an aqueous environment. Particularly, the physical and chemical properties, including the cure time and strength, of said adhesives comprising a crosslinking agent and an elastin-like polypeptide (ELP) that contain tyrosine, lysine, cysteine, dihydroxyphenylalanine (DOPA) or trihydroxyphenylalanine (TOPA) amino acid residue, or a combination thereof, on the polypeptide chain of ELP, may be modified and controlled by adjusting the type and amount of said crosslinking agent.

[0010] In some embodiments, an oxidizing agent is a crosslinking agent. In some other embodiments, a crosslinking agent is an oxidizing agent. In some embodiment, a crosslinking agent is usually incorporated in the final network of the adhesive, while an oxidizing agent may or may not be incorporated in the final network of the adhesive. For example, THP act as a crosslinker because they are incorporated into the final network of said adhesive. On the other hand, periodate, acts as an oxidizer to the protein to the crosslinking process, but they are not incorporated into the final network of the adhesive. Iron acts as both an oxidizer and a crosslinker because it does oxidize the protein, and it is also incorporated into the final network.

[0011] In some illustrative embodiments, the present invention relates to an adhesive comprising a crosslinking agent and an elastin-like polypeptide (ELP), wherein polypeptide chain of said ELP contains tyrosine, lysine, cysteine, dihydroxyphenylalanine (DOPA) or trihydroxyphenylalanine (TOPA) amino acid residue, or a combination thereof.

[0012] In some other embodiments, the present invention relates to an adhesive comprising a crosslinking agent, an elastin-like polypeptide (ELP), wherein polypeptide chain of said ELP contains tyrosine, lysine, cysteine, dihydroxyphenylalanine (DOPA) or trihydroxyphenylalanine (TOPA) amino acid residue, or a combination thereof, and an oxidizing agent.

[0013] In some illustrative embodiments, the present invention relates to an adhesive disclosed herein, wherein said oxidizing agent comprises a ferric salt, hydrogen peroxide, sodium periodate, or an enzyme that transforms the phenolic side chain of tyrosine residues of said ELP.

[0014] In some illustrative embodiments, the present invention relates to an adhesive disclosed herein, wherein said DOPA of polypeptide chain of said ELP is generated by using a tyrosinase.

[0015] In some illustrative embodiments, the present invention relates to an adhesive disclosed herein, wherein said adhesive is capable of adhering to a substrate when applied to said substrate in a dry, wet, moist, or an aqueous environment.

[0016] In some illustrative embodiments, the present invention relates to an adhesive disclosed herein, wherein said adhesive is compatible for biomedical applications.

[0017] In some illustrative embodiments, the present invention relates to an adhesive disclosed herein, wherein said adhesive is useful in medical treatments or surgical operations.

[0018] In some illustrative embodiments, the present invention relates to an adhesive disclosed herein, wherein said crosslinking agent is an oxidizing agent.

[0019] In some illustrative embodiments, the present invention relates to an adhesive disclosed herein, wherein said oxidizing agent comprises a ferric salt, hydrogen peroxide, sodium periodate, or an enzyme that transforms the phenolic side chain of tyrosine residues of said ELP.

[0020] In some illustrative embodiments, the present invention relates to an adhesive disclosed herein, wherein said crosslinking agent comprises trishydroxymethylphosphine (THP), tetrakis(hydroxymethyl)phosphonium chloride (THPC), an N-hydroxysuccinyl ester (NHS), genipin, pyridyl disulfide, a maleimide, a vinylsulfone, a haloacetyl, or a combination thereof.

[0021] In some illustrative embodiments, the present invention relates to a method of manufacturing an adhesive comprising the steps of:

providing a crosslinking agent; and

providing an elastin-like polypeptide (ELP), wherein polypeptide chain of said ELP contains tyrosine, lysine, cysteine, dihydroxyphenylalanine (DOPA) or trihydroxyphenylalanine (TOPA) amino acid residue, or a combination thereof.

[0022] In some illustrative embodiments, the present invention relates to a method of manufacturing an adhesive disclosed herein, wherein the method further comprises a step of adding an oxidizing agent.

[0023] In some illustrative embodiments, the present invention relates to a method of manufacturing an adhesive disclosed herein, wherein said oxidizing agent comprises a ferric salt, hydrogen peroxide, sodium periodate, or an enzyme that transforms the phenolic side chain of tyrosine residues of said ELP.

[0024] In some illustrative embodiments, the present invention relates to a method of manufacturing an adhesive disclosed herein, wherein said crosslinking agent is trishydroxymethylphosphine (THP), tetrakis(hydroxymethyl)phosphonium chloride (THPC), an N-hydroxysuccinyl ester (NHS), genipin, pyridyl disulfide, a maleimide, a vinylsulfone, a haloacetyl, or a combination thereof.

[0025] In some illustrative embodiments, the present invention relates to a method of manufacturing an adhesive disclosed herein, wherein said adhesive is capable of adhering to a substrate when applied to said substrate in a dry, wet, moist, or an aqueous environment.

[0026] In some illustrative embodiments, the present invention relates to a method of manufacturing an adhesive disclosed herein, wherein said adhesive is useful in medical treatments or surgical operations.

[0027] In some illustrative embodiments, the present invention relates to a method of manufacturing an adhesive disclosed herein, wherein said DOPA of polypeptide chain of said ELP is generated by using a tyrosinase.

[0028] In some illustrative embodiments, the present invention relates to a method of manufacturing an adhesive disclosed herein, wherein said adhesive is capable of adhering to a substrate when applied to said substrate in a dry, wet, moist, or aqueous environment.

[0029] In another embodiment of the present disclosure, the ELPs comprise tyrosine, lysine or cysteine residues. In other embodiments, some or all of the tyrosine residues are replaced by DOPA and/or TOPA, in any combination thereof. The DOPA and TOPA

may be formed enzymatically through treatment with an enzyme such as a tyrosinase of ELP polypeptides containing tyrosine residues. Alternative, they may be formed chemically using methods well known in the art. Another method would be to produce the ELPs synthetically, adding DOPA and TOPA to the peptides. Yet in some other embodiments, the ELPs of the present disclosure may be produced recombinantly using methods known in the art, substituting DOPA and/or TOPA for the tyrosine.

[0030] Certain embodiments of the present disclosure may be better understood through the following non-limiting examples.

[0031] Many "smart" materials have been designed to respond to environmental triggers such as temperature, light, and pH. [1] Smart materials are especially useful in applications like controlled drug release [2,3], tissue engineering [4], and microfluidics [5–7], where changes in pH or temperature can be harnessed to regulate or affect surrounding systems. Hydrogels are commonly used as a material for these systems due to their biocompatibility, softness, and controlled permeability to small molecules. [7] The softness of hydrogels can be tuned by changing the crosslinked network's density. If the hydrogel was made with a chemical crosslinker, the density can be tuned by changing the concentration of crosslinker. pH-sensitivity can be induced in hydrogels by including ionizable functional groups. [8] When a gel has a higher charge, it is more hydrophilic and will swell and soften due to attracted water. [9] When the gel has a charge close to neutral, it will become more hydrophobic and expel water while shrinking and stiffening. [9] These pH-sensitive hydrogels can be made from synthetic or natural polymers. Engineered natural polymers like recombinant proteins allow for precise sequence control and lower dispersity than synthetic polymer systems.

[0032] Non-uniform populations in pH-sensitive charged systems could cause shifts in charge to be less distinct since the more diverse polymer population does not have a homogeneous response. Uniform populations like recombinant proteins are more likely to have a homogeneous response to environmental changes. Additionally, recombinant protein systems allow for tighter control over small changes in charge since very similar proteins can be made that only differ by a few amino acids. Recombinant proteins can

also be designed to contain modular protein "tags" that add functionality to the protein, including purification, identification, [10] and environmental sensitivity. [11] Elastin-like polypeptides (ELPs) are recombinant mimics of native elastin with similar elastic properties [12], and improved solubility. [13] ELPs are composed of the repeating pentapeptide sequence VPGXG (**SEQ ID NO: 11**), where X is any amino acid except proline. [14] ELPs are well-studied elastomeric proteins that are hydrophilic, [15] biocompatible, [16, 17] and easy to produce in large quantities. [18] ELPs can also hase separate upon heating above the lower critical solution temperature (LCST). [19] ELP-based hydrogels are elastic, highly resilient, and can be tuned to the stiffness range of soft tissues. [12] Ionizable amino acids have been used to induce pH sensitivity in uncrosslinked ELPs. [15, 20, 21] LCST behavior of ELPs is driven by interactions with water, and ELPs that are more hydrophilic have higher LCSTs. [15] When ionizable amino acids were incorporated into ELP proteins, the LCST of the protein became pH sensitive. If the ELP was in solution at a pH where the ionizable amino acid was charged, the ELP became more hydrophilic. This caused the LCST of the ELP to increase. [15, 20, 21] Recombinant proteins and synthetic polymers that are pH sensitive can remain pH sensitive after crosslinking. Polymer-based hydrogels containing polar molecules show pH-dependent swelling and mechanical properties. [22–24] If the polar groups in these hydrogels are blocked through chemical modification, the gel will become more hydrophobic and thus experience lower swelling ratios and higher stiffness. [25] In particular, acetylation can be used to block lysine [25,26] and tyrosine [27,28] groups.

[0033] Materials and Methods

[0034] This present disclosure is a further development of the subject matter technology of our previous U.S. patent application No. 15/230,762, filed August 8, 2016, the content which is incorporated hereby into the instant application in its entirety.

[0035] *Reagents:* All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Avantor Performance Materials (Center Valley, PA) unless stated otherwise. Water was ultra-purified with a Milli-Q ultra-purification system (Millipore, Billerica, MA). NIH/3T3 fibroblasts were a generous gift from Dr. Alyssa Panitch (Purdue University). Tisseel was generously donated by Baxter BioSurgery (Deerfield, IL).

[0036] The method of preparing various ELP proteins and their characterization was reported in the U.S. patent application No. 15/230,762, filed August 8, 2016, and is further elaborated below. A new variation has been developed, which contains thiol groups from cysteine guest residues. In this document, "YKV" refers to an ELP protein containing equal amounts of Y, K, and V in its guest residue positions, and "mYKV" refers to the same protein that has been modified to include DOPA.

[0037] The proteins are dissolved into water or phosphate-buffered saline at varying concentrations. Coacervate is isolated by dissolving protein into a solvent, heating the solution, and centrifuging the solution. The supernatant (protein-poor layer) is removed and the remaining coacervate (protein-rich layer) is used for testing. The protein solutions were mixed with the following chemicals for the following effects: Tris-hydroxymethyl phosphine (THP), (or tris-hydroxymethyl phosphonium chloride THPC) which chemically binds to amine groups in the protein (and potentially to amines in the surface of biological tissues). The addition of THP can create a crosslinked hydrogel in the presence or absence of DOPA. The amount of THP added affects the stiffness, swelling, and water content of the resulting hydrogel. If THP is added to a protein containing DOPA, it can also improve adhesive strength. This may occur through the binding of the protein to itself and to the surface of the tissue.

[0038] Iron (iron nitrate nonahydrate) (or various other metal ions), which chelates with DOPA groups. When iron is mixed with DOPA-containing proteins, three DOPA groups chelate with one iron molecule. The chelated DOPA can then covalently bind to other chelated DOPA to form bonds within the proteins, or the chelated DOPA can covalently bind to surfaces. Sodium periodate (or other oxidizers), which oxidizes DOPA and causes it to become more reactive. Oxidized DOPA is more likely to form cohesive bonds. Hydrogen perioxide (or other oxidizers), which oxidizes thiol groups and cause them to bind to other thiol groups.

[0039] Tisseel (by Baxter International Inc.) was used as a comparison in adhesive testing. Tisseel is a protein based sealant used to prevent the leaking of fluid from sutured wounds inside of the body. It is *not* used for wound closure without the use of mechanical support, but is a commonly used commercial product for comparison in adhesive testing.

[0040] To measure the swelling of hydrogels, protein solution was mixed with THP and put into a custom mold. After crosslinking, the hydrogel was submerged in a pH-controlled liquid, and the mass of the hydrogel was monitored over time. After seven days, the hydrogel was removed and dried and its dry mass was measured. Swelling ratio and water content were calculated as:

$$\text{Swelling Ratio} = \frac{m_w}{m_d}$$

$$\text{Water Content } (\%) = 100 \times \frac{m_w - m_d}{m_w}$$

m_d = mass of dried hydrogel

m_w = mass of swollen hydrogel

[0041] The stiffness of hydrogels was measured by compressing hydrogels at a fixed rate and calculating the slope of the stress-strain curve between 5 and 20% strain.

[0042] Adhesive strength was measured with lap-shear adhesion. Aluminum or pig skin substrates were used, and the samples were applied to adherends and cured in a 37 °C humid environment until testing. To test, a constant force was applied and the adhesive strength was calculated as the maximum force before failure divided by the overlap area of the adherends. Samples that failed when handled before testing were not measured, and the failure rate on handling is shown below each figure in a table inset. Unless noted otherwise, six replicates were created for each group. If a group had two or fewer samples that were tested (i.e. did not fail upon handling), the group is shown without an error bar and is excluded from statistical analysis.

[0043] Pig skin adhesion was based on ASTM standard F2255-05. Testing adherends were prepared by cutting pig skin (supplier) into squares and storing at -80°C until use. Custom aluminum adherends were made with a XxX cut out. Pig skin was thawed in PBS and applied to aluminum adherends using cyanoacrylate glue. To prepare adhesive, protein was dissolved in water at the desired concentration and the pH was adjusted to 7.4±0.1 using sodium hydroxide and hydrochloric acid. Crosslinker solutions were prepared in water at concentrations necessary to reach the desired ratio to the reactive group in YKV. All solutions were kept on ice during use. 10 µL of protein solution was applied to one pig skin adherend face. If used, 5 µL of crosslinker was applied on top of

the protein solution. The other pig skin adherend was immediately applied on top of the protein solution. The sample was covered in a water moistened paper towel and weighted with a mass of X grams. The samples were placed into a humid chamber and incubated at 37°C for the necessary time. Humid chambers were removed from the incubator 30 minutes before testing to allow the samples to come to room temperature without drying out. Samples were loaded into a BOSE X, and pulled at a constant rate of X mm/min until failure. Overlap area was measured with calipers. Adhesive strength was calculated as the maximum force divided by overlap area, and toughness was calculated as the area under the stress-strain curve divided by overlap area. Six replicates of each group were prepared unless noted otherwise.

[0044] If two groups were compared, a two-sample t-test was used and statistical significance is noted by * ($p<0.05$) or ** ($p<0.01$). If more than two groups were compared, ANOVA was used. If the variance was equal, Tukey groupings were used. If the variance was unequal, Games-Howell groupings were used. Groupings are noted by the use of letters, where groups that do not share a letter are statistically different ($p<0.05$).

[0045] Protein [YKV-48], also known as EL(YKV)16 or ELY_{16}. SEQ ID NO: 1 (DNA) and SEQ ID NO: 2 (protein) in the CRF file of 68335-02_ST25.txt

- Amino acid sequence:

MMASMTGGQQMGHHHHHHHDDDDKLDGTLPGYGVPGKGVPGVGVPGYGVP
GKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGVPGV
GVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPGYGV
PGKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGVPG
VGVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPGYG
VPGKGVPGVGVPVADRGMRLE (**SEQ ID NO: 2**)

[0046] Protein [YKV-72], SEQ ID NO: 3 (DNA) and SEQ ID NO: 4 (protein) in the CRF file of 68335-02_ST25.txt

- Amino acid sequence:

MMASMTGGQQMGHHHHHHHDDDDKLDGTLPGYGVPGKGVPGVGVPGYGVP
GKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGVPGV
GVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPGYGV

PGKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGVPG
VGVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPGYG
VPGKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGVP
GVGVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPGY
GVPGKGVPGVGVPGYGVPGKGVPGVGVPV ADRGMRLE (**SEQ ID NO: 4**)

[0047] Protein [YKV-96], SEQ ID NO: 5 (DNA) and SEQ ID NO: 6 (protein) in the CRF file of 68335-02_ST25.txt

- Amino acid sequence:

MMASMTGGQQMGHHHHHHHHDDDDKLDGTLPGYGVPGKGVPGVGVPGYGVP
GKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGVPGV
GVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPGYGV
PGKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGVPG
VGVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPGYG
VPGKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGVP
GVGVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPGY
GVPGKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGV
PGVGVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPG
YGVPGKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPV ADRGMR
LE (**SEQ ID NO: 6**)

[0048] Protein [YKV-48] without tags (also known as HDØ-EL(YKV)16), SEQ ID NO: 7 (DNA) and SEQ ID NO: 8 (protein) in the CRF file of 68335-02_ST25.txt

- Amino acid sequence:

MSKGPGVDGTLPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGVP
GVGVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPGY
GVPGKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGV
PGVGVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPG
YGVPGKGVPGVGVPGYGVPGKGVPGVGVPGYGVPGKGVPGVGVPV ADRGMR
LDKEFLE (**SEQ ID NO: 8**)

[0049] Protein [CKVYKV-72], SEQ ID NO: 9 (DNA) and SEQ ID NO: 10 (protein) in the CRF file of 68335-02_ST25.txt

- Amino acid sequence:

MSKGPGVDGTLPGYGVPGKGVPGVGVPGCGVPGKGVPGVGVPGYGVPGKGVP
GVGVPGCGVPGKGVPGVGVPGYGVPGKGVPGVGVPGCGVPGKGVPGVGVPGY
GVPGKGVPGVGVPGCGVPGKGVPGVGVPGYGVPGKGVPGVGVPGCGVPGKGV
PGVGVPGYGVPGKGVPGVGVPGCGVPGKGVPGVGVPGYGVPGKGVPGVGVPG
CGVPGKGVPGVGVPGYGVPGKGVPGVGVPGCGVPGKGVPGVGVPGYGVPGKG
VPGVGVPGCGVPGKGVPGVGVPGYGVPGKGVPGVGVPGCGVPGKGVPGVGVP
GYGVPGKGVPGVGVPGCGVPGKGVPGVGVPGYGVPGKGVPGVGVPGCGVPGK
GVPGVGVPVADRGMRLEHHHHHH (**SEQ ID NO: 10**)

[0050] Proteins were constructed with a cloning scheme adapted from one previously developed by our lab. [29] ELP[YKV-48] was constructed as previously described. [30] The modified scheme used AgeI and AvaI restriction enzymes (New England Biolabs, Ipswich MA) to create uninterrupted repeats of the ELP pentapeptide sequence. To construct HDØ-ELP[YKV-48], pET21b plasmid was first modified by removing the T7-tag and His-tag followed by inserting a DNA oligo encoding amino acid sequence Ser-Lys-Gly-Pro-Gly (**SEQ ID NO: 12**). The previously cloned ELP[YKV-48] encoding DNA fragment was then inserted into the modified pET21b after the leading sequence. Standard molecular cloning techniques were used. [31] Proteins were expressed using *E. coli* Rosetta 2(DE3) pLysS (Novagen, Burlington MA) bacterial expression strains. ELP[YKV-48] was expressed as previously described. [30] Cells were initially cultured for 14 h overnight at 37 °C in 2xYT medium using the appropriate antibiotics. For ELP[YKV-48], overnight cell cultures were then diluted 1:25 into a fermentor (BioFlo 100, 14 L capacity, New Brunswick Scientific, Enfield CT) with 10 L of Terrific Broth using the appropriate antibiotics. At an optical density (OD) of 4-5, protein expression was induced using 2.5 mM isopropyl β-D-1- thiogalactopyranoside (IPTG, VWR, Radnor PA). Fermentation continued until the OD was ~10. Cells were then harvested by centrifugation at 4 °C. For HDØ-ELP[YKV-48], overnight cell cultures were diluted 1:100 into 4 L flasks containing 1 L of 2xYT medium and the appropriate antibiotics. At an OD of ~1, protein expression was induced using 1 mM IPTG. After 3.5 h of additional culture, cells were harvested by centrifugation at 4 °C. Both proteins were purified as described previously. [30] Expression and purification were confirmed with SDS-PAGE imaging (Fig. 4B).

[0051] Standard molecular cloning techniques were used. [31] YKV proteins were expressed using E. coli Rosetta 2(DE3) pLysS (Novagen, Burlington MA) bacterial expression strains as previously described. [30] Cells were initially cultured for 14h overnight at 37 °C in 2xYT medium using the appropriate antibiotics. Overnight cell cultures were then diluted 1:25 into a fermentor (BioFlo 100, 14 L capacity, New Brunswick Scientific, Enfield CT) with 10 L of Terrific Broth using the appropriate antibiotics. At an optical density (OD) of 4-5, protein expression was induced using 2.5 mM isopropyl β-D-1- thiogalactopyranoside (IPTG, VWR, Radnor PA). Fermentation continued until the OD was ~10. Cells were then harvested by centrifugation at 4 °C. YKV was purified as described previously. [30] Expression and purification were confirmed with SDS-PAGE and Western Blot imaging. Modified EL YKV was produced by mushroom tyrosinase reaction, purification.

[0052] Hydrogel Formation

[0053] Protein was dissolved into 200 mM buffer composed of 80 mM citric acid (MP Biomedicals, Santa Ana CA), 40 mM sodium phosphate dibasic (Sigma Aldrich, St. Louis MO), 40 mM 1,3-bis(tris(hydroxymethyl)methylamino)propane (Alfa Aesar, Haverhill MA), and 40 mM N-cyclohexyl-3-aminopropanesulfonic acid (Sigma Aldrich) in Milli-Q filtered water, pH 7. Hereafter this buffer mixture is referred to as CPPC buffer. Tris(hydroxymethylphosphine) (THP, Acros Organics, Geel, Belgium) was dissolved into 200 mM CPPC buffer (pH 7). Protein solution (13 wt% final oncentration) and crosslinker solution were added at a 9:1 volume ratio and crosslinked in a custom silicone mold at 15 °C for 18 h. Gels for swelling tests were 0.8 mm thick and 4 mm in diameter, and gels for mechanical testing were 1.4 mm thick and 4 mm in diameter. After crosslinking, gels were removed from molds and briefly rinsed in water.

[0054] Chemical Modification

[0055] To acetylate protein in solution, lyophilized protein was dissolved at 14 wt% into 0.05 M sodium borate (MP Biomedical) at pH 9.8. Acetic anhydride (Alfa Aesar) was added at 1000x excess of acetic anhydride to amine groups. After reacting for 18 h at 4 °C, the solution was diluted to 10 mg/mL using water and dialyzed against water. The solution was then lyophilized. To acetylate crosslinked hydrogels, gels were swelled in 0.05 M sodium borate buffer at pH 9.8 for at least 5 h. Gels were then immersed in acetic

acid at 1000x excess of acetic anhydride to amine groups. After reacting for 18 h at 4 °C, gels were rinsed for 5 min in 1 mL of water, rinsed for 5 min in 1 mL of the required buffer, and then immersed in 4 mL of the required buffer for further testing.

[0056] Protein Sequence and Charge Modification Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) was performed on lyophilized, uncrosslinked protein (Purdue University Center for Cancer Research). Amino acid analysis was completed on lyophilized, uncrosslinked protein and on lyophilized, crosslinked hydrogels (UC Davis Molecular Structure Facility). Electrospray Ionization Mass Spectrometry was completed by Charng-Yu Lin. Amine content was determined using an o-phthalaldehyde (OPA) assay. The assay reagent was composed of 5.96 mM ortho-phthalaldehyde (Alfa Aesar), 342.6 mM ethanol (Decon Labs, King of Prussia PA), 28.4 mM β-mercaptoethanol (Sigma Aldrich), 43.9 mM sodium borate (MP Biomedical), and 34.6 mM sodium dodecyl sulfate (Sigma Aldrich) in Milli-Q water. 10 uL of a protein and Milli-Q water solution was mixed with 300 uL of assay reagent. After 10 min of incubation, samples were measured with an excitation at 350 nm and an emission at 455 nm. ELP[YKV-48] was used to construct a standard curve. Tyrosine content was determined by measuring the autofluorescence of tyrosine residues. Protein solutions dissolved in Milli-Q water were measured with an excitation at 276 nm and an emission at 305 nm. ELP[YKV-48] was used to construct a standard curve. Zeta potential was measured using a Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern, United Kingdom) with a minimum of 10 runs per sample. Protein was dissolved at 0.1957 mM in 10 mM CPPC buffer (200 mM CPPC buffer as previously described, diluted with Milli-Q water) pre-adjusted to the required pH using HCl and NaOH. The solution was filtered and then tested for zeta potential.

[0057] Protein Design and Cloning: The elastin-like polypeptide (ELP) labeled as ELY_{16} was designed with Geneious software (Biomatters Inc., San Francisco, CA) using the repeated amino acid sequence Val-Pro-Gly-Xaa-Gly (**SEQ ID NO: 13**); the guest residues Xaa were evenly divided among Tyr, Lys, and Val. The complete amino acid sequence for full-length ELY_{16} may be found in the Sequence Listing concomitantly filed. Cloning was performed using standard techniques (Ausubel FM *et al.*, editors.

Current Protocols in Molecular Biology. New York: John Wiley & Sons; 2003) and a scheme modified from one previously developed (Renner, JN *et al.*, Protein Expr Purif 2012;82:90-6). The new scheme utilized AgeI and AvaI restriction enzymes (New England Biolabs, Ipswich, MA) to achieve seamless repeats of the elastin-like sequence.

[0058] Protein Expression and Purification: ELY_{16} was transformed into the Rosetta2(DE3)pLysS *E. coli* expression host (EMD Chemicals, Gibbstown, NJ). Bacterial colonies were inoculated into 2xYT medium containing 50 µg/mL kanamycin and 34 µg/mL chloramphenicol and grown 16-18 h at 37°C and 300 rpm. The overnight culture was diluted 1:250 for expression in a 14 L-capacity fermenter (BioFlo 100, New Brunswick Scientific, Enfield, CT) with 10 L of Terrific Broth (TB). When the optical density (OD) at 600 nm reached 5-6, protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG, EMD Chemicals) at a final concentration of 2.5 mM. Upon reaching stationary phase, cells were harvested by centrifugation and immediately resuspended in Buffer B (8 M urea, 100 mM NaH_2PO_4, 100 mM Tris-Cl, pH 8.0) before being frozen at -80°C.

[0059] Purification was performed by a salting and heating method that was modified from a previously described protocol (Renner, JN *et al.*, Biomacromolecules 2012;13:3678-85; Kim, Y *et al.*, Biomater Sci 2014;2:1110-9). Cells were lysed by multiple freeze-thaw cycles in combination with sonication (Misonix XL-2000, Qsonica, Newtown, CT) for 1 min followed by a 1 min incubation on ice. Total sonication time was at least 2 h. The cell lysate was then centrifuged at 10000*g* for 45 min and 4°C to remove the cell debris. To salt out undesired proteins, 10% (w/v) ammonium sulfate was added to the cleared supernatant. The mixture was incubated on ice for 310 min followed by centrifugation for 45 min at 10000*g* and 4°C. The supernatant was decanted from the pellet, and an additional 10% (w/v) ammonium sulfate was added to precipitate ELY_{16}. The solution was incubated on ice and centrifuged as before. The pellet was then resuspended in water at 500 mg/mL based on wet weight, heated to 80°C, vortexed, and heated again to 80°C. The heated solution was centrifuged for 45 min at 10000*g* and 25°C, and the supernatant was dialyzed extensively against reverse osmosis water at 10°C before lyophilization.

[0060] Expression and purification of ELY_{16} were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot using standard techniques (Bonifacino JS *et al.*, editors. Current Protocols in Cell Biology. New York: John Wiley & Sons; 2002). SDS-PAGE gels were stained with Coomassie Brilliant Blue R-250. The protein was detected in the Western blot using an anti-T7 tag antibody conjugated to horseradish peroxidase (EMD Chemicals, Gibbstown, NJ) in combination with a 1-component 3,3',5,5'- tetramethylbenzidine (TMB) colorimetric substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Purity was assessed using densitometry analysis with lmageJ software (NIH, Bethesda, MD) (Abramoff, MD *et al.*, Biophotonics Int 2004;11:36-41).

[0061] The molecular weight was confirmed using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) (Dr. Connie Bonham, Campus- Wide Mass Spectrometry Center, Purdue University) with sinapinic acid as the matrix. Briefly, the MALDI mass spectra were obtained on a Voyager DE-Pro TOF mass spectrometer (Applied Biosystems, Framingham, MA) in the linear mode with delayed extraction. Positive-ion spectra were obtained with an acceleration voltage of 25000 V.

[0062] The amino acid composition was verified with amino acid analysis (John Schulze, Molecular Structure Facility, University of California, Davis). Briefly, the sample underwent liquid phase hydrolysis in 2 N HCl/1% phenol at 110°C for 24 h before being dried. The sample was then dissolved in norleucine dilution buffer to a final volume of 1 mL, vortexed, and spun down. Injection volume was 50 µL at a 2.0 nmol scale.

[0063] Tyrosinase Modification: To convert tyrosine residues to DOPA, ELY_{16} was dissolved at 2 mg/mL in 0.1 M sodium acetate buffer with 0.1 M ascorbic acid, pH 5.5. Mushroom tyrosinase was added to a final concentration of 150 U/mL, and the mixture was incubated at 37°C and 200 rpm for 8 h. Enzyme activity was halted with 0.2 mL of 6 N HCl per mL of reaction as described previously (Marumo K & Waite JH, Biochem Biophys Acta 1986;872:98-103.). The tyrosinase-modified ELY_{16} ($mELY_{16}$) solution was dialyzed extensively in 5% acetic acid at 4°C and lyophilized.

[0064] The extent of conversion was measured with difference spectrophotometry (Waite JH, Anal Chem 1984; 56:1935-9) and comparison to standard solutions of L-DOPA. The

increase in molecular weight due to conversion was confirmed by MALDI-TOF and SDS-PAGE. DOPA content was also assessed with amino acid analysis using a procedure similar to that described above with the modifications of using a 5.0 nmol scale and S-2-aminoethyl-L-cysteine as a diluent. The DOPA elution peak was compared with that of an L-DOPA control solution.

[0065] Protein Adsorption to Coverslips: Acid-washed coverslips (12 mm diameter, VWR, Radnor, PA) were incubated overnight at 4°C with ELY_{16}, $mELY_{16}$, or bovine serum albumin (BSA, Fraction V, EMD Chemicals, Gibbstown, NJ) dissolved at 1 mg/mL in water. Protein surface density was measured by washing coverslips three times with MilliQ water and performing a bicinchoninic acid (BCA) colorimetric assay. Separate standard solutions for ELY_{16} and BSA were used to determine adsorbed protein concentration. Four replicates were tested for each sample.

[0066] Cell Culture: NIH/3T3 fibroblasts were generously donated by Dr. Alyssa Panitch (Purdue University). Fibroblasts were cultured at 37°C and 5% CO2 in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 100 U/mL penicillin-streptomycin (Gibco, Carlsbad, CA) and 10% bovine calf serum. Cells were subcultured at 60-80% confluency.

[0067] Cytocompatibility Testing: Coverslips coated in adsorbed protein sterilized by incubation in 70% ethanol for 5 min, blocked in sterile-filtered BSA (1 mg/mL in water) for 30 min, and rinsed with phosphate-buffered saline (PBS, 4.2 mM $NaHPO_4$, 0.8 mM KH_2PO_4, 50 mM NaCl, pH 7.4). Fibroblasts were seeded onto coverslips at 2500 cells per cm^2 in a 24-well plate (BD Falcon, Durham, NC). For a positive control, acid-washed coverslips were incubated for 5 min in 0.01% poly-L-lysine (PLL, Trevigen, Gaithersburg, MD) then rinsed three times in PBS. Images were taken with a Nikon Ti-E C-1 Plus microscope. All groups were tested in triplicate.

[0068] To assess cell viability, cells were cultured for 2 days and tested with a LIVE/DEAD viability/cytotoxicity kit (Molecular Probes, Carlsbad, CA). Cells were incubated in staining solution (1.5 μM ethidium homodimer-1 and 0.5 μM calcein acetoxymethyl ester (calcein AM) in PBS), rinsed three times with PBS, and imaged with

a 10x objective. All PBS was supplemented with 0.01% $CaCl_2$ and 0.01% $MgCl_2$ to prevent cell detachment. As a negative control, cells on PLL were incubated in 70% ethanol for 30 min at 37°C prior to staining. Cells were counted using NIS-Elements software (Nikon, Tokyo, Japan), and at least 90 cells were counted per replicate. Viability was calculated as the number of living cells divided by the total number of cells in each replicate.

[0069] Cell morphology was assessed via actin staining. After culturing for 2 days, cells were fixed in ice-cold acetone for 1 min and then washed three times with filtered PBS. Coverslips were then incubated for 20 min with Alexa Fluor 488 phalloidin (Molecular Probes, Carlsbad, CA) at a 1:40 dilution in PBS. Following three 10 min washes with PBS, cells were then counterstained for 30 min with DRAQ5 (Biostatus Limited, Leicestershire, UK) diluted 1:500 in PBS. Finally, coverslips were rinsed twice in PBS, mounted with Vectashield (Vector Laboratories), and sealed with nail polish. Confocal imaging was performed with EZ-C1 software using a 40x objective.

[0070] Turbidity Testing: Lower critical solution temperatures (LCSTs) of ELY_{16} and ELY_{16} were assessed using turbidity readings from a Crystal16 (Technobis Group, Alkmaar, the Netherlands). Protein samples were held at 10°C for 15 min, ramped at 1°C/min to 50°C, then held at 50°C for 2 min. Light transmission data was recorded and normalized to the maximum transmission for each sample. The LCST was calculated as the inflection point of the transmission vs. temperature curve.

[0071] Lap Shear Adhesion: Aluminum adherends were prepared and cleaned using ASTM standard D2651-01 (Standard D2651: Preparation of metal surfaces for adhesive bonding. West Conshohocken, PA: ASTM International; 2008). Bulk lap shear adhesion bonding was tested with a modified version of the ASTM D1002 standard, as previously described (Jenkins, CL *et al.,* ACS Appl Mater Interfaces 2013;5:5091-6; Standard D1002: Apparent shear strength of single-lap-joint adhesively bonded metal specimens by tension loading (metal-to-metal). West Conshohocken, PA: ASTM International; 2010). Briefly, protein was resuspended at 150 mg/mL in water, and 5 µL of this solution was spread onto each aluminum adherend. Tisseel was prepared according to the manufacturer's instructions and tested by applying an equivalent total mass of protein (1.5

mg per test) based on the stated protein content of Tisseel. Adherends were overlapped with an area of 1.2 cm x 1.2 cm and were cured for 24 h at 37°C. Bond strengths were quantified using an Instron 5544 Materials Testing System (Norwood, MA) with a 2000 N load cell and a loading rate of 2 mm/min. Maximum force was divided by overlap area to determine the adhesion strength. Each condition was tested with at least 5 samples.

[0072] For humid curing, adherends were covered with a layer of damp paper towels followed by a layer of plastic wrap to prevent them from drying. For underwater curing, protein solution (either ELY_{16} or $mELY_{16}$) was adjusted to pH 7.5. Aluminum adherends were placed in a PBS bath at 37°C. Protein solution (10 μL) was applied to one adherend, and the other adherend was overlapped as before. For underwater testing, at least 7 samples were tested for each group.

[0073] Statistical Analysis: Data are represented as the mean ± the standard deviation. All data were first examined for outliers using Grubbs' test; any outliers were discarded from further analysis. Next, Levene's test was used to assess equality of variances, and data were analyzed with one-way analysis of variance (ANOVA) followed by Tukey's Honestly Significant Difference (HSD) or the Games-Howell (for unequal variances) *post hoc* test. Finally, the normality of the ANOVA residuals was assessed with the Kolmogorov-Smirnov test. If the residuals were not normally distributed, the original data were transformed with the Box-Cox method, and the analysis was repeated on the transformed data. If only two groups were being compared, an unpaired *t*-test was used instead of ANOVA to assess statistical difference. All statistical analyses were performed with GraphPad online software (La Jolla, CA) or Minitab 17 (State College, PA). A *p*-value ≤ 0.05 was considered significant.

[0074] As shown in Fig. 1, changes occur in the mass of YKV hydrogels after swelling across a range of pH (square marker) and absolute zeta potential of YKV solution across a range of pH (circle marker). YKV was crosslinked into hydrogels using THP. Zeta potential is a measure of overall surface charge of a protein. Actually, a change in mass less than 100% indicates a shrinking. The hydrogels tended to shrink at pH where the overall charge (zeta potential) was low. Shrinking near physiological pH is useful for surgical adhesives, as it reduces the risk of pressure on nearby tissues, blood vessels, and nerves.

[0075] Fig. 2 shows Young's modulus (stiffness) of YKV hydrogels at varying THP crosslinker amounts swelled at pH 7. Crosslinker excess indicates the molar amount of hydroxyl groups in THP compared to the molar amount of amines in YKV. The amount of THP incorporated into a YKV hydrogel will affect the stiffness of the gel. A very high (24) or low (0.5) excess of THP will create a less connected hydrogel network, resulting in a softer gel. An equimolar amount of THP hydroxyl groups and YKV amine groups (1) creates a more highly connected network, with higher stiffness.

[0076] Fig. 3A shows Young's Modulus of YKV hydrogels at varying THP crosslinking ratios swelled across a range of pH. At all crosslinking ratios, YKV hydrogels tended to become stiffest near physiological pH. Changing the THP crosslinking ratio affected the amount of pH response in the hydrogels.

[0050] Fig. 3B shows swelling and opacity of hydrogels was affected by crosslinking ratio and acetylation. ELP[YKV-48] (**SEQ ID NO: 2**) hydrogels with 24:1, 1:1, and 1:2 crosslinking ratio, and HDØ-ELP[YKV-48] (**SEQ ID NO: 8**) and acELP[YKV-48] (**SEQ ID NO: 14**) hydrogels with 1:2 crosslinking ratio, swelled at pH 3 to 11 for six days. Scale bar is 5 mm.

[0051] Fig. 4A describes the mass fraction of supernatant and coacervate after phase separation across a range of initial solution concentrations. As the initial solution concentration increases, the mass fraction of coacervate after phase separation increases. At ~400 mg/mL or higher, the coacervate phase has a mass fraction close to 1.

[0052] Fig. 4B depicts SDS PAGE gel of purified ELP[YKV-48] (**SEQ ID NO: 2**) (Lane #1), HDØ-ELP[YKV-48] (**SEQ ID NO: 8**) (Lane #2), and acELP[YKV-48] (**SEQ ID NO: 14**) (Lane #3).

[0077] Fig. 5 shows the concentrations of supernatant and coacervate after phase separation across a range of initial solution concentrations. As initial solution concentration increases, the concentration of the supernatant plateaus. The coacervate concentration has more variability due to measuring error, but also seems to plateau.

[0078] Fig. 6 depicts adhesive strength of YKV dissolved in water (black) and PBS (light grey), applied to aluminum and cured at 37 °C for 24 hours under dry conditions. Increasing protein concentration increases the adhesive strength. Using water as the solvent increases the adhesive strength compared to PBS. Isolated coacervate has similar

adhesive strength to high concentration protein solutions. n=3 for water and 4 for PBS. No statistical analysis.

[0079] Fig. 7 shows adhesive strength of YKV dissolved in water (black) and PBS (light grey) and mYKV dissolved in PBS (dark grey), applied to aluminum and cured at 37 °C for 24 hours under humid conditions. The error bars of "YKV in water" are too small to be shown. Increasing the concentration improves adhesive strength. Using PBS increases adhesive strength compared to water. At lower concentrations, mYKV has a higher adhesive strength than YKV. At higher concentrations, YKV and mYKV have similar adhesive strength. Isolate coacervate has similar adhesive strength to high concentration protein solutions. No statistical analysis.

[0080] Fig. 8 demonstrates the adhesion strength of BSA (grey striped), YKV (light grey), and mYKV (black), in water at pH 7.4 on pig skin in physiological conditions (humid, 37 °C) across a range of concentrations. The addition of DOPA to YKV improves adhesion.

[0081] Fig. 9 shows the adhesive strength of YKV (light grey) and mYKV (black) mixed with THP at various ratios and applied to pig skin under physiological conditions (humid, 37 °C) and cured for 24 hours. 1:0 indicates protein mixed with no THP. Increasing the concentration of THP increased the adhesive strength of the protein, up to 1:50. At 1:10 and 1:50, YKV and mYKV have similar adhesive strength.

[0082] Fig. 10 describes the adhesive strength of commercial glue Tisseel (dark grey), and THP mixed at 1:50 with water (white), BSA (grey stripe), YKV (light grey), and mYKV (black). Various proteins mixed with THP have higher adhesion strength than Tisseel.

[0083] Fig. 11 shows the adhesive strength of YKV (light grey) and mYKV (black) mixed with iron nitrate nonahydrate at various ratios and applied to pig skin under physiological conditions (humid, 37 °C) and cured for 24 hours. 3:0 indicates protein mixed with no iron. Increasing the concentration of iron to a ratio of 3:10 increased the adhesive strength of the protein. At 3:1 and 3:10, mYKV had higher adhesion strength than YKV.

[0084] Fig. 12 describes the adhesive strength of commercial glue Tisseel (dark grey), and iron mixed at 3:10 with water (white), BSA (grey stripe), YKV (light grey), and

mYKV (black). The addition of DOPA improves adhesion in the presence of iron. mYKV and BSA mixed with iron have higher adhesive strength than Tisseel.

[0085] Fig. 13 shows the adhesive strength of mYKV mixed with sodium periodate at various ratios (catechol: periodate) and applied to pig skin under physiological conditions (humid, 37C) and cured for 24 hours. 1:0 indicates protein mixed with no iron. Increasing the concentration of periodate up to 1:0.1 increased the adhesive strength of the protein.

[0086] Fig. 14 shows the adhesive strength of Tisseel (dark grey), and periodate mixed at 1:0.1 with water (white), BSA (grey stripe), and mYKV (black). Periodate mixed with water or BSA has negligible adhesive strength. Periodate mixed with mYKV has a higher adhesive strength than Tisseel.

[0087] Fig. 15A shows adhesive strength of water mixed with crosslinker combinations of THP (T) at 1:50, Iron (I) at 3:10, and Periodate (P) and 1:0.1. Water mixed with TP had higher adhesion strength than tisseel.

[0088] Fig. 15B shows adhesive toughness of water mixed with crosslinker combinations of THP (T) at 1:50, Iron (I) at 3:10, and Periodate (P) and 1:0.1. Water mixed with TP had higher adhesion strength than tisseel.

[0089] Fig. 16A shows adhesive strength of YKV mixed with crosslinker combinations of THP (T) at 1:50, Iron (I) at 3:10, and Periodate (P) and 1:0.1. YKV mixed with T, TP, and TIP had the highest adhesive strength and toughness. YKV with T, TP, and TIP had 17X higher adhesion strength than Tisseel.

[0090] Fig. 16B depicts toughness of YKV mixed with crosslinker combinations of THP (T) at 1:50, Iron (I) at 3:10, and Periodate (P) and 1:0.1. YKV mixed with T, TP, and TIP had the highest adhesive strength and toughness. YKV with T, TP, and TIP had 17X higher adhesion strength than Tisseel.

[0091] Fig. 17A shows adhesive strength of mYKV mixed with crosslinker combinations of THP (T) at 1:50, Iron (I) at 3:10, and Periodate (P) and 1:0.1. Adhesion strength was higher than Tissel with T, I, TP, and TIP. Toughness was higher than Tisseel with I, TI, and TP.

[0092] Fig. 17B shows toughness of mYKV mixed with crosslinker combinations of THP (T) at 1:50, Iron (I) at 3:10, and Periodate (P) and 1:0.1. Adhesion strength was

higher than Tissel with T, I, TP, and TIP. Toughness was higher than Tisseel with I, TI, and TP.

[0093] Fig. 18A depicts adhesive strength of water, YKV and mYKV mixed with crosslinker combinations of THP (T) at 1:50, Iron (I) at 3:10, and Periodate (P) and 1:0.1. Data are the same as Figs. 1.8 to 1.10. YKV and mYKV had higher adhesion strength than water when mixed with T. YKV had higher adhesion strength and toughness than YKV when mixed with I. YKV had higher adhesion strength and toughness than mYKV when mixed with TIP.

[0094] Fig. 18B shows toughness of water, YKV and mYKV mixed with crosslinker combinations of THP (T) at 1:50, Iron (I) at 3:10, and Periodate (P) and 1:0.1. Data are the same as Figs. 1.8 to 1.10. YKV and mYKV had higher adhesion strength than water when mixed with T. YKV had higher adhesion strength and toughness than YKV when mixed with I. YKV had higher adhesion strength and toughness than mYKV when mixed with TIP.

[0095] While various embodiments of protein-based adhesives and methods of producing the same have been described in considerable detail herein, the embodiments are merely offered as non-limiting examples of the disclosure described herein. It will therefore be understood that various changes and modifications may be made, and equivalents may be substituted for elements thereof, without departing from the scope of the present disclosure. The present disclosure is not intended to be exhaustive or limiting with respect to the content thereof.

[0096] Further, in describing representative embodiments, the present disclosure may have presented a method and/or a process as a particular sequence of steps. However, to the extent that the method or process does not rely on the particular order of steps set forth therein, the method or process should not be limited to the particular sequence of steps described, as other sequences of steps may be possible. Therefore, the particular order of the steps disclosed herein should not be construed as limitations of the present disclosure. In addition, disclosure directed to a method and/or process should not be limited to the performance of their steps in the order written. Such sequences may be varied and still remain within the scope of the present disclosure.

[0097] Those skilled in the art will recognize that numerous modifications can be made to the specific implementations described above. The implementations should not be limited to the particular limitations described. Other implementations may be possible.

[0098] While the inventions have been illustrated and described in detail in the drawings and foregoing description, the same is to be considered as illustrative and not restrictive in character, it being understood that only certain embodiments have been shown and described and that all changes and modifications that come within the spirit of the invention are desired to be protected. It is intended that the scope of the present methods and apparatuses be defined by the following claims. However, it must be understood that this disclosure may be practiced otherwise than is specifically explained and illustrated without departing from its spirit or scope. It should be understood by those skilled in the art that various alternatives to the embodiments described herein may be employed in practicing the claims without departing from the spirit and scope as defined in the following claims.

CLAIMS:

1. An adhesive comprising a crosslinking agent and an elastin-like polypeptide (ELP), wherein polypeptide chain of said ELP comprises tyrosine, lysine and cysteine residues, or a derivative thereof.
2. The adhesive of claim 1 further comprising an oxidizing agent.
3. The adhesive of claim 2, wherein said oxidizing agent comprises a ferric salt, hydrogen peroxide, sodium periodate, or an enzyme that transforms the phenolic side chain of tyrosine residues of said ELP.
4. The adhesive of claim 1, wherein said crosslinking agent is an oxidizing agent.
5. The adhesive of claim 4, wherein said oxidizing agent comprises a ferric salt, hydrogen peroxide, sodium periodate, or an enzyme that transforms the phenolic side chain of tyrosine residues of said ELP.
6. The adhesive of claim 1, wherein said crosslinking agent comprises trishydroxymethylphosphine (THP), tetrakis(hydroxymethyl)phosphonium chloride (THPC), an N-hydroxysuccinyl ester (NHS), genipin, pyridyl disulfide, a maleimide, a vinylsulfone, a haloacetyl, or a combination thereof.
7. The adhesive of claim 1, wherein said derivative of tyrosine residue of ELP polypeptide chain is dihydroxyphenylalanine (DOPA) or trihydroxyphenylalanine (TOPA), transformed chemically or enzymatically from said tyrosine residue.
8. The adhesive of claim 1, wherein said tyrosine residue of ELP polypeptide chain is transformed to dihydroxyphenylalanine (DOPA) or trihydroxyphenylalanine (TOPA), or a combination thereof, chemically or enzymatically from said tyrosine residue.
9. The adhesive of claim 8, wherein said transformation of tyrosine residue of ELP polypeptide chain to dihydroxyphenylalanine (DOPA) or trihydroxyphenylalanine (TOPA), or a combination thereof, is carried out enzymatically by using a tyrosinase.
10. The adhesive of claim 8, wherein said transformation of tyrosine residue of ELP polypeptide chain to dihydroxyphenylalanine (DOPA) or trihydroxyphenylalanine (TOPA), or a combination thereof, is carried out chemically by using an oxidizing agent.

11. The adhesive according to claims 1-10, wherein said adhesive is capable of adhering to a substrate when applied to said substrate in a dry, wet, moist, or an aqueous environment.
12. The adhesive of claims 1-11, wherein said adhesive is compatible for biomedical research and clinical applications.
13. The adhesive of claims 1-12, wherein said adhesive is useful in a medical surgery.
14. A method of generating an adhesive comprising the steps of:
    preparing a crosslinking agent; and
    adding an elastin-like polypeptide (ELP), wherein polypeptide chain of said ELP comprises tyrosine, lysine and cysteine residues, or a derivative thereof.
15. The method of claim 14, further comprising the step of adding an oxidizing agent.
16. The method of claim 15, wherein said oxidizing agent comprises a ferric salt, hydrogen peroxide, sodium periodate, or an enzyme that transforms the phenolic side chain of tyrosine residues of said ELP.
17. The method of claim 14, wherein said crosslinking agent is trishydroxymethylphosphine (THP), tetrakis(hydroxymethyl)phosphonium chloride (THPC), an N-hydroxysuccinyl ester (NHS), genipin, pyridyl disulfide, a maleimide, a vinylsulfone, a haloacetyl, or a combination thereof.
18. The method according to claim 14, wherein said derivative of tyrosine residue of ELP polypeptide chain is dihydroxyphenylalanine (DOPA), trihydroxyphenylalanine (TOPA), or a combination thereof, transformed chemically or enzymatically from said tyrosine residue.
19. The method according to claim 14, wherein said tyrosine residue of ELP polypeptide chain is transformed to dihydroxyphenylalanine (DOPA) or trihydroxyphenylalanine (TOPA), or a combination thereof, chemically or enzymatically from said tyrosine residue.
20. The method according to claims 18-19, wherein said transformation of tyrosine residue of ELP polypeptide chain to dihydroxyphenylalanine (DOPA) or trihydroxyphenylalanine (TOPA), or a combination thereof, is carried out enzymatically by using a tyrosinase.

21. The method according to claims 18-19, wherein said transformation of tyrosine residue of ELP polypeptide chain to dihydroxyphenylalanine (DOPA) or trihydroxyphenylalanine (TOPA), or a combination thereof, is carried out chemically by using an oxidizing agent.
22. The method of claims 14-21, wherein said adhesive is capable of adhering to a substrate when applied to said substrate in a dry, wet, moist, or an aqueous environment.
23. The method of claims 14-22, wherein said adhesive is compatible for biomedical applications.
24. The method of claims 14-23, wherein said adhesive is useful in a medical surgery.
25. An adhesive prepared according to the method of claims 14-24.
26. The adhesive of claim 25, wherein said adhesive is capable of adhering to a substrate when applied to said substrate in a dry, wet, moist, or aqueous environment.
27. The adhesive of claim 25, wherein said adhesive is compatible for biomedical research and clinical applications.
28. The adhesive of claim 25, wherein said adhesive is useful in a medical surgery.

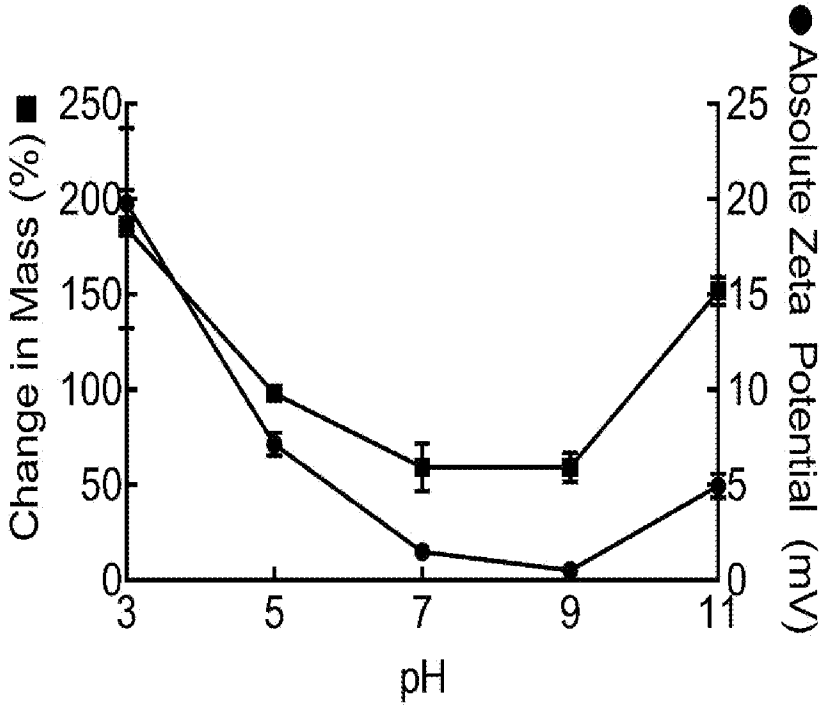
Change in Mass (%)
Absolute Zeta Potential (mV)
250
200
150
100
50
0
25
20
15
10
5
0
3
5
7
9
11
pH

FIG. 1

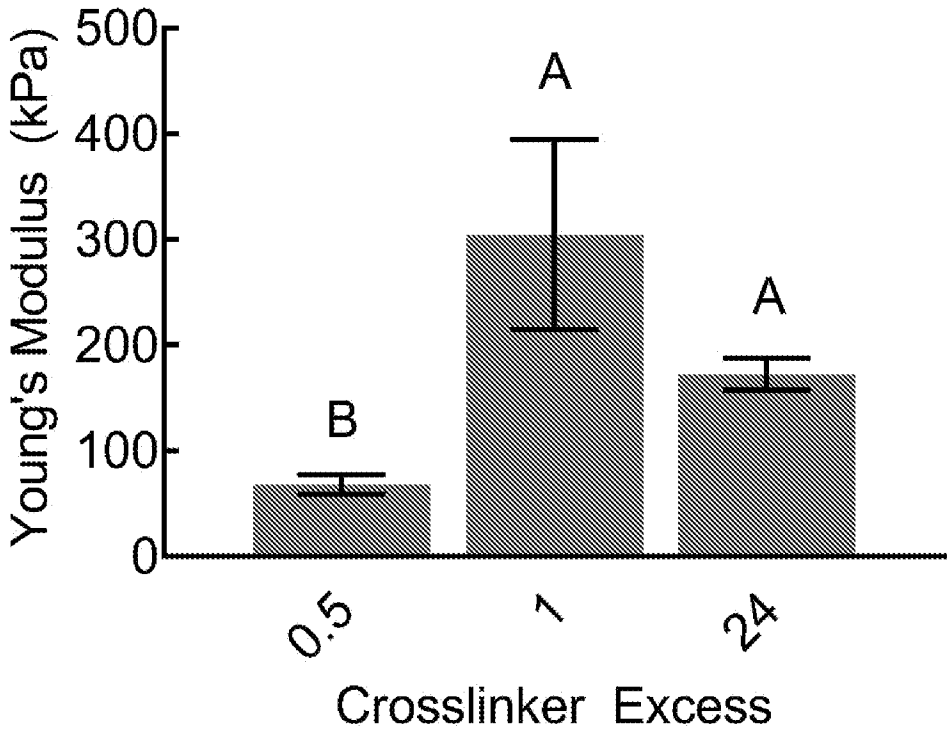
Young's Modulus (kPa)
500
400
300
200
100
0
A
A
B
0.5
1
24
Crosslinker Excess

FIG. 2

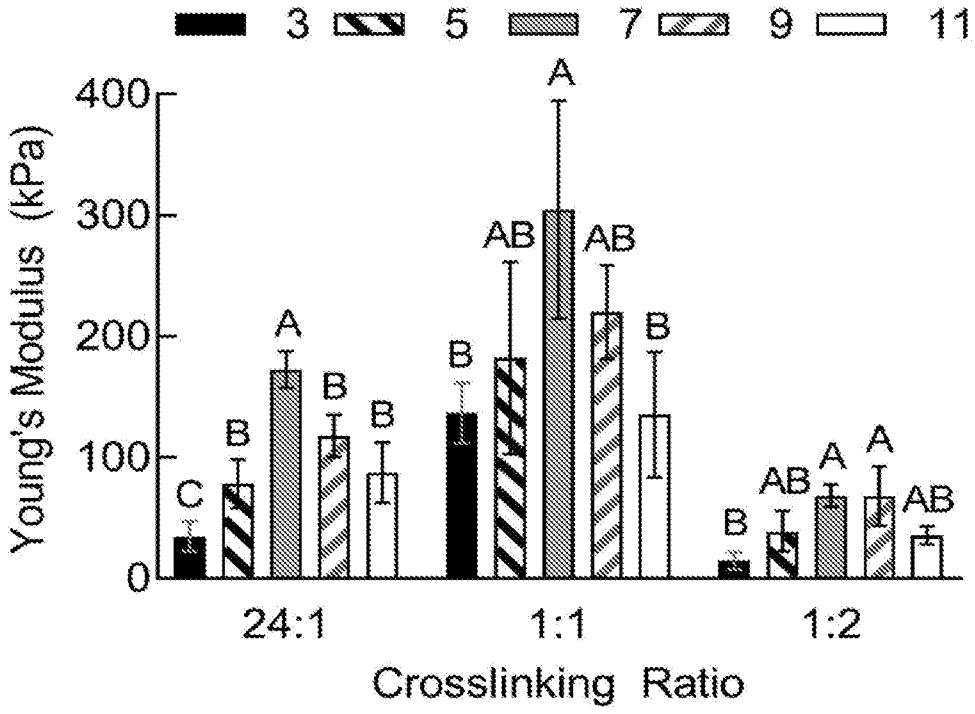
3
5
7
9
11
Young's Modulus (kPa)
400
300
200
100
0
C
B
A
B
B
B
AB
A
AB
B
B
AB
A
A
AB
24:1
1:1
1:2
Crosslinking Ratio

FIG. 3A

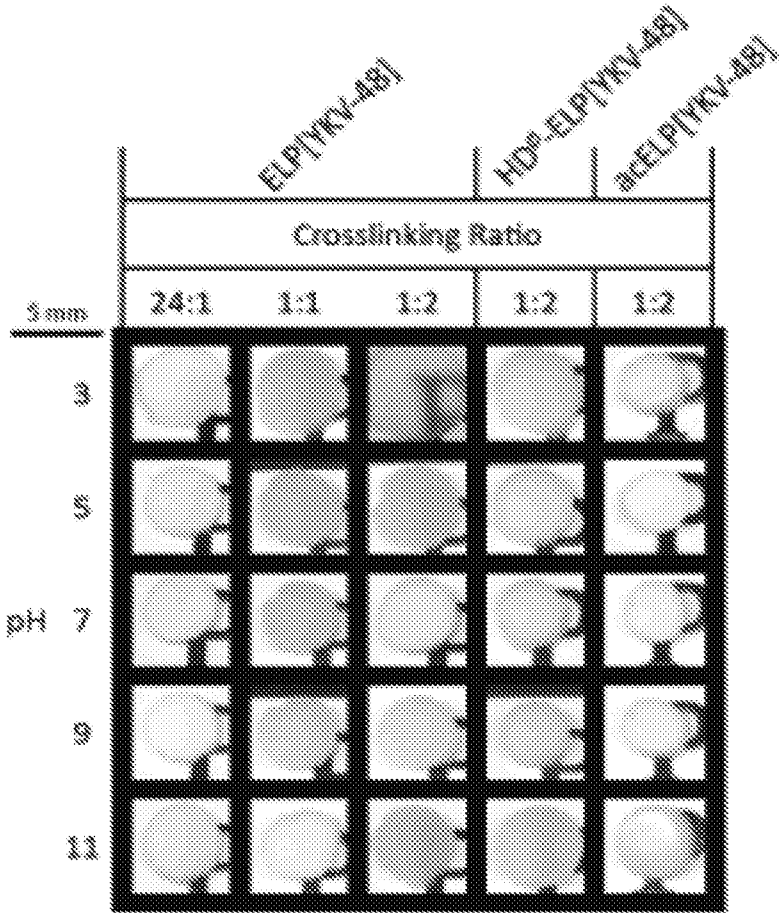
ELP[YKV-48]
HD^P-ELP[YKV-48]
ac-ELP[YKV-48]
Crosslinking Ratio
24:1
1:1
1:2
1:2
1:2
5 mm
3
5
pH 7
9
11

FIG. 3B

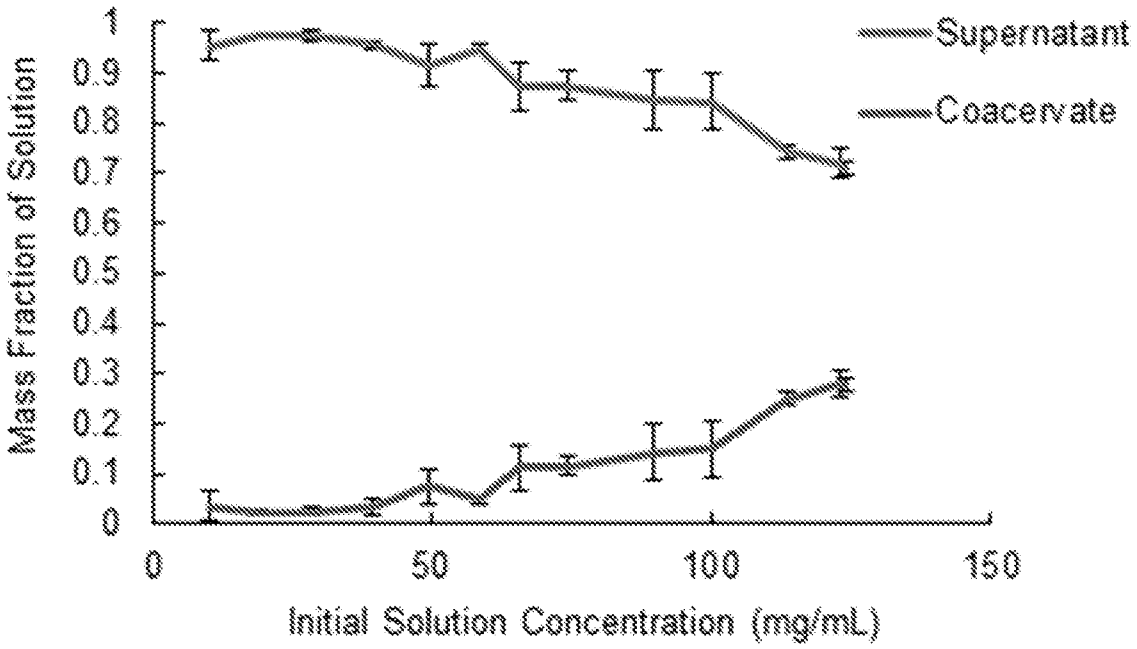
Supernatant
Coacervate
Mass Fraction of Solution
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
50
100
150
Initial Solution Concentration (mg/mL)

FIG. 4A

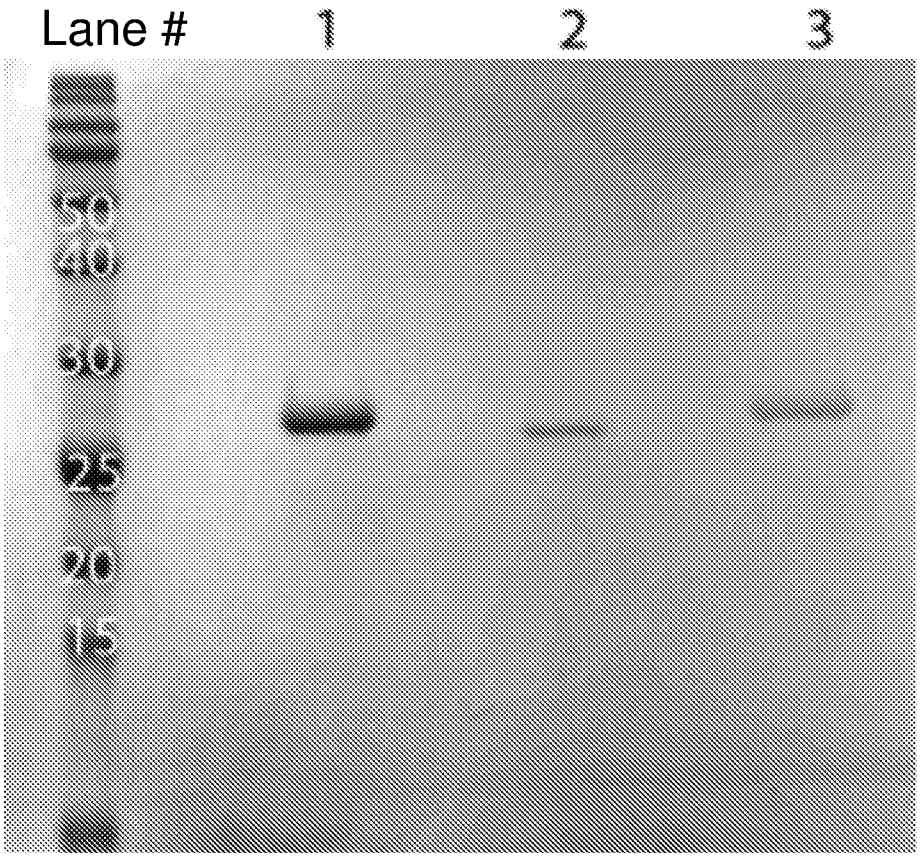
Lane #
1
2
3

FIG. 4B

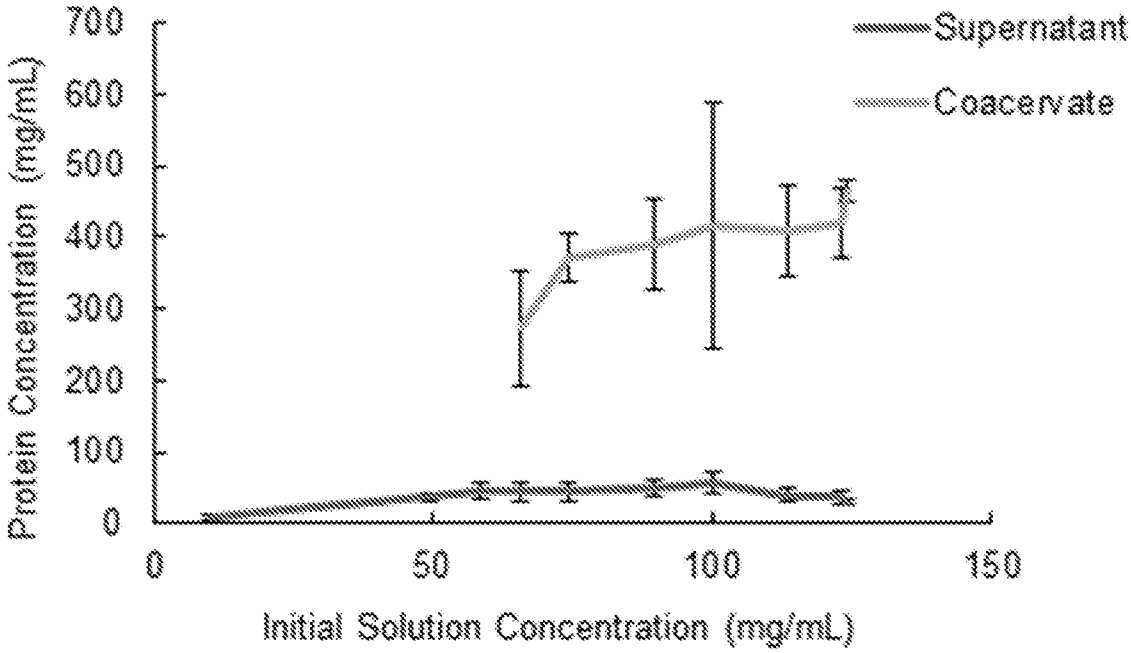
Protein Concentration (mg/mL)
700
600
500
400
300
200
100
0
0
50
100
150
Initial Solution Concentration (mg/mL)
Supernatant
Coacervate

FIG. 5

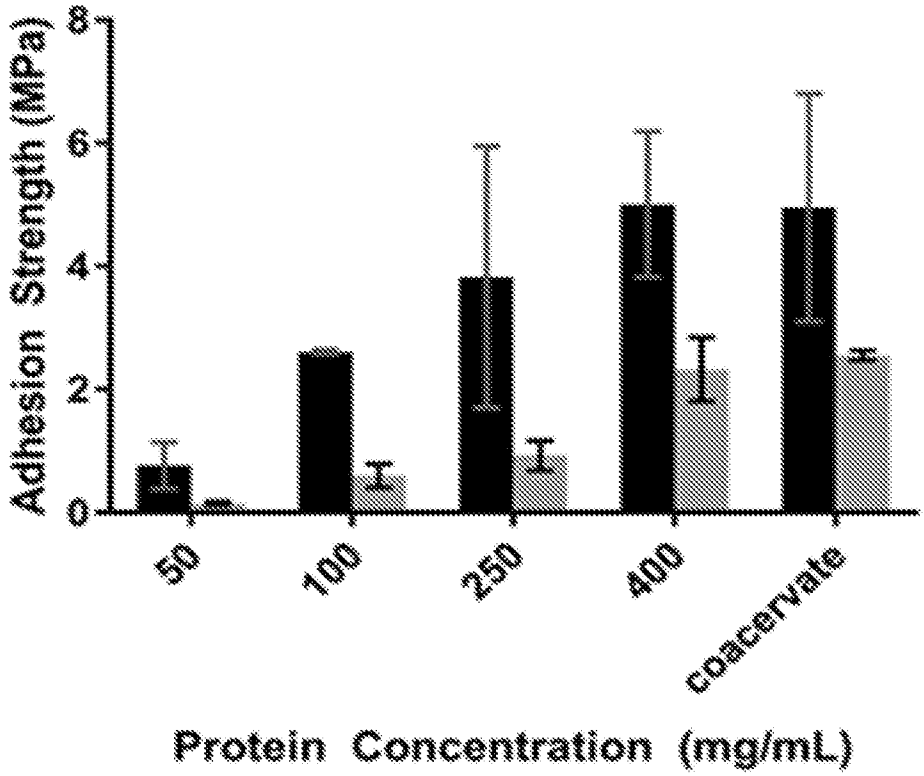
Adhesion Strength (MPa)
8
6
4
2
0
50
100
250
400
coacervate
Protein Concentration (mg/mL)

FIG. 6

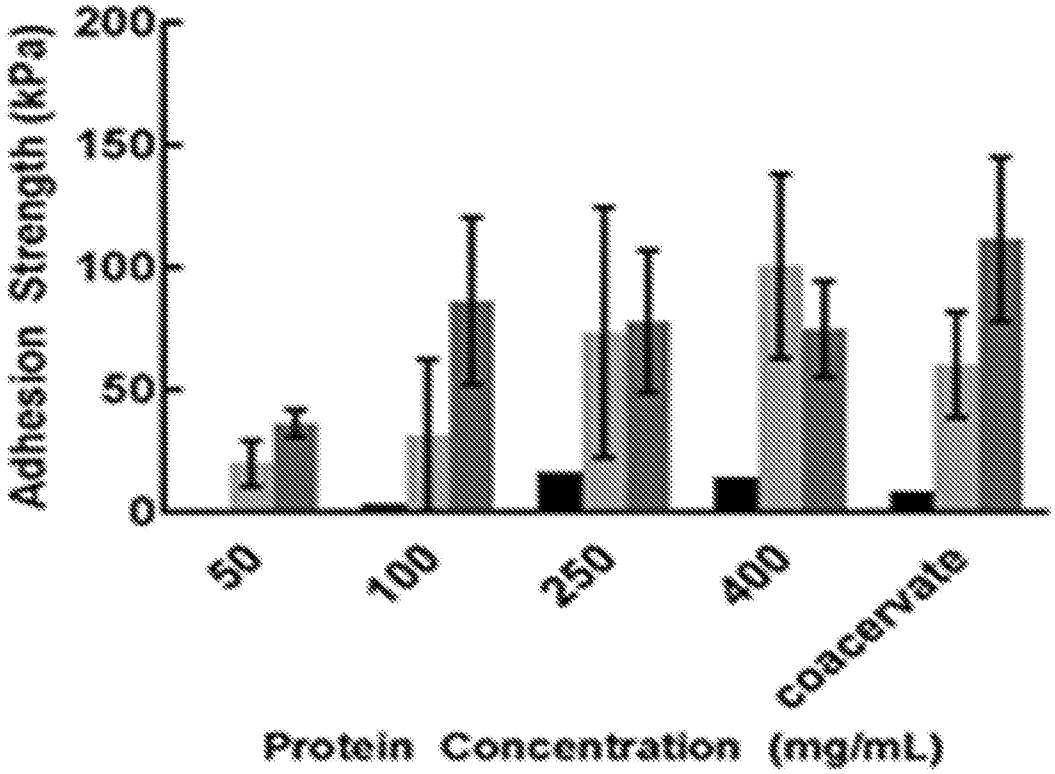
Adhesion Strength (kPa)
200
150
100
50
0
50
100
250
400
coacervate
Protein Concentration (mg/mL)

FIG. 7

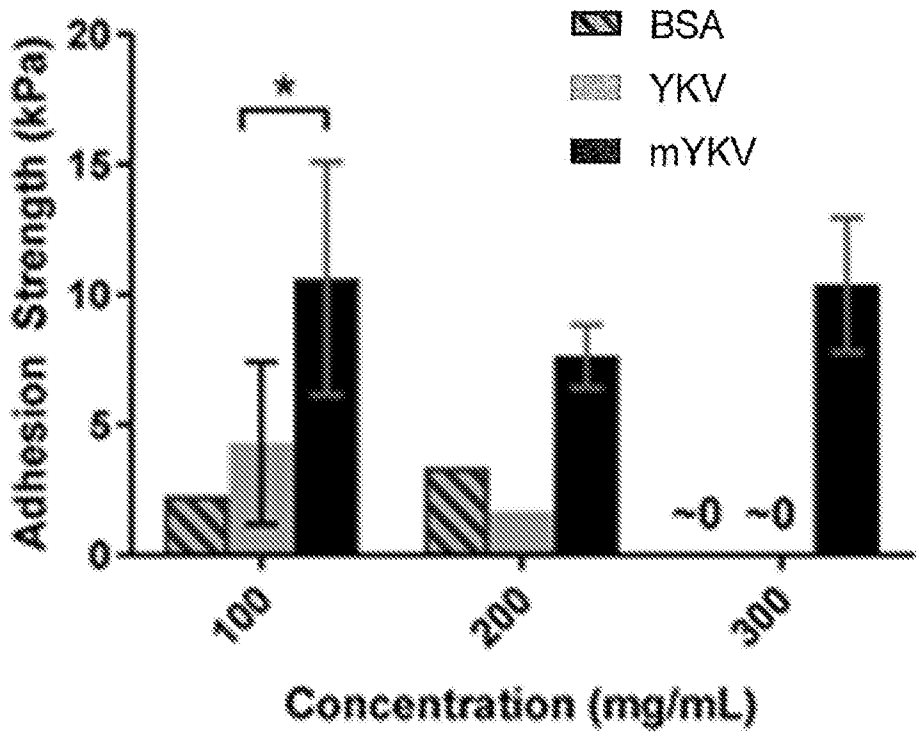
BSA
YKV
mYKV
Adhesion Strength (kPa)
20
15
10
5
0
*
~0
~0
100
200
300
Concentration (mg/mL)

FIG. 8

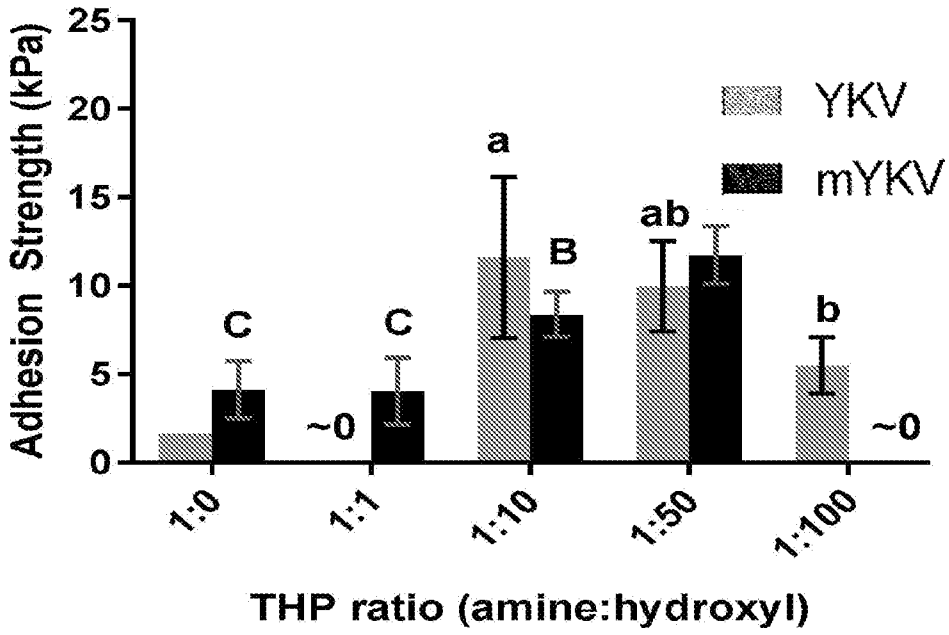
Adhesion Strength (kPa)
25
20
15
10
5
0
YKV
mYKV
C
C
a
B
ab
b
~0
~0
1:0
1:1
1:10
1:50
1:100
THP ratio (amine:hydroxyl)

FIG. 9

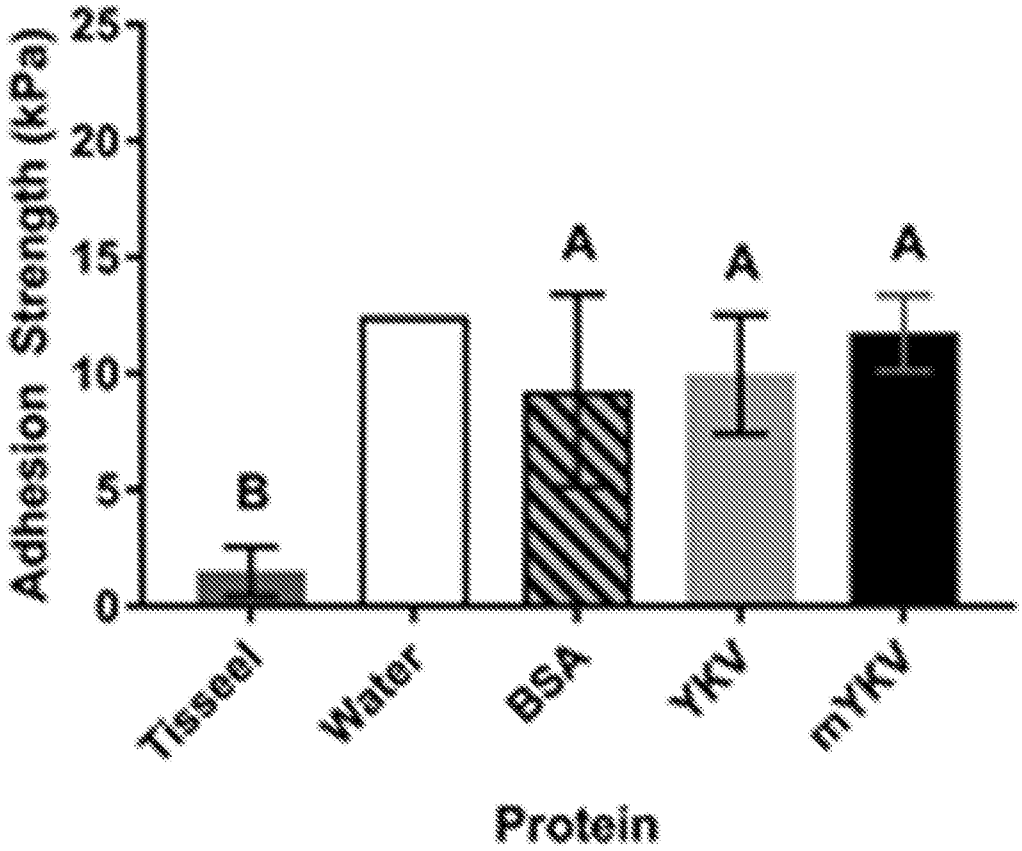
Adhesion Strength (kPa)
25
20
15
10
5
0
B
A
A
A
Tissseel
Water
BSA
YKV
mYKV
Protein

FIG. 10

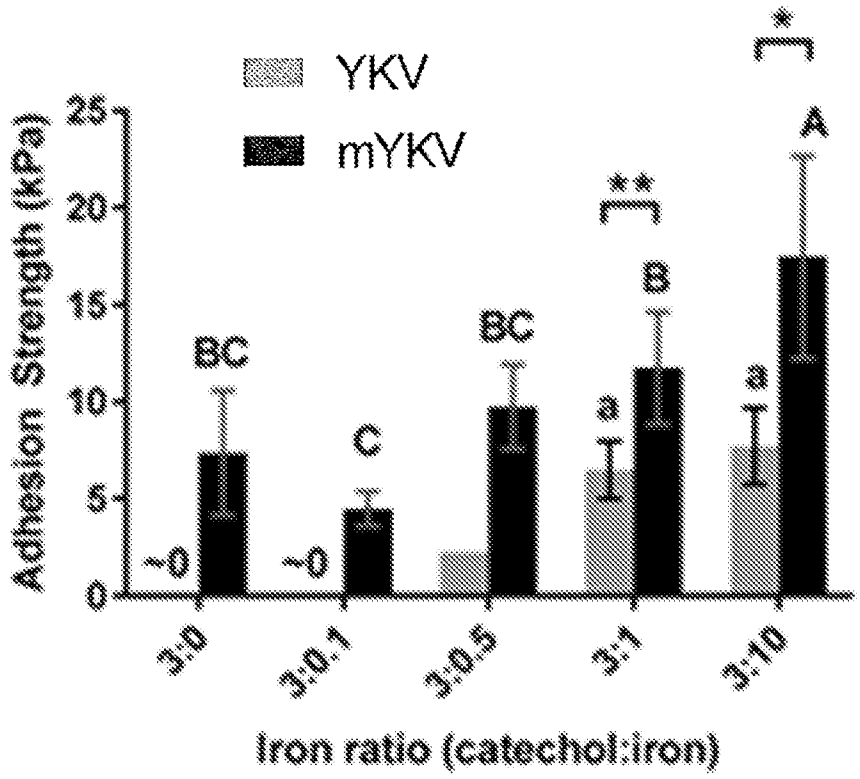
YKV
mYKV
Adhesion Strength (kPa)
25
20
15
10
5
0
~0
~0
BC
C
BC
a
B
a
A
**
*
3:0
3:0.1
3:0.5
3:1
3:10
Iron ratio (catechol:iron)

FIG. 11

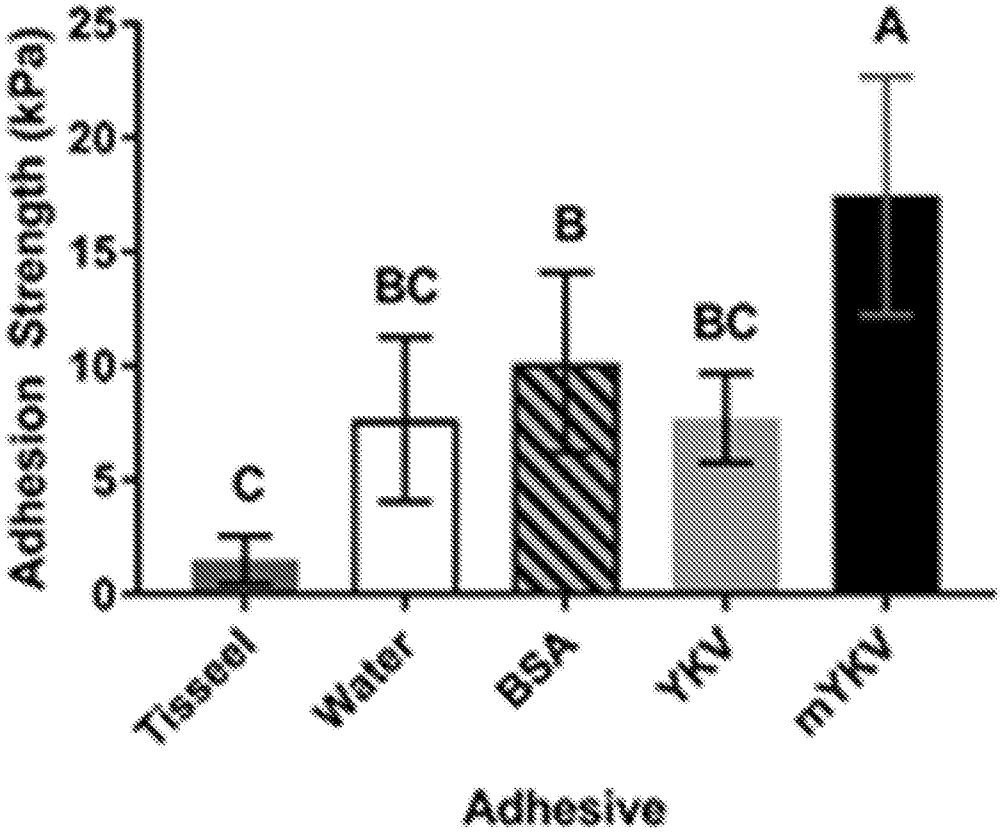
Adhesion Strength (kPa)
25
20
15
10
5
0
C
BC
B
BC
A
Tissseal
Water
BSA
YKV
mYKV
Adhesive

FIG. 12

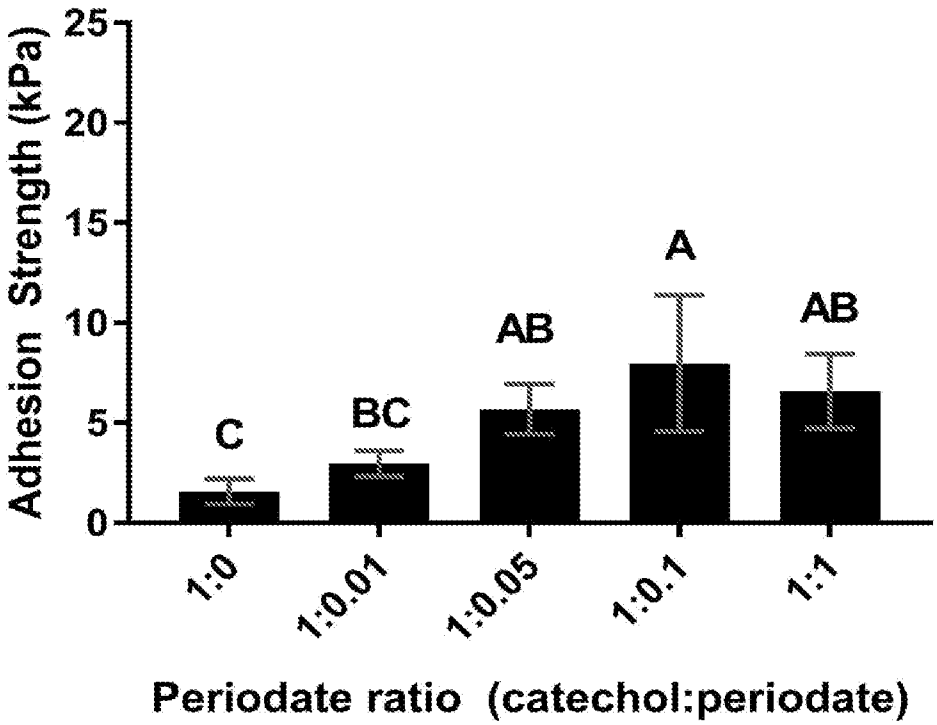
Adhesion Strength (kPa)
25
20
15
10
5
0
C
BC
AB
A
AB
1:0
1:0.01
1:0.05
1:0.1
1:1
Periodate ratio (catechol:periodate)

FIG. 13

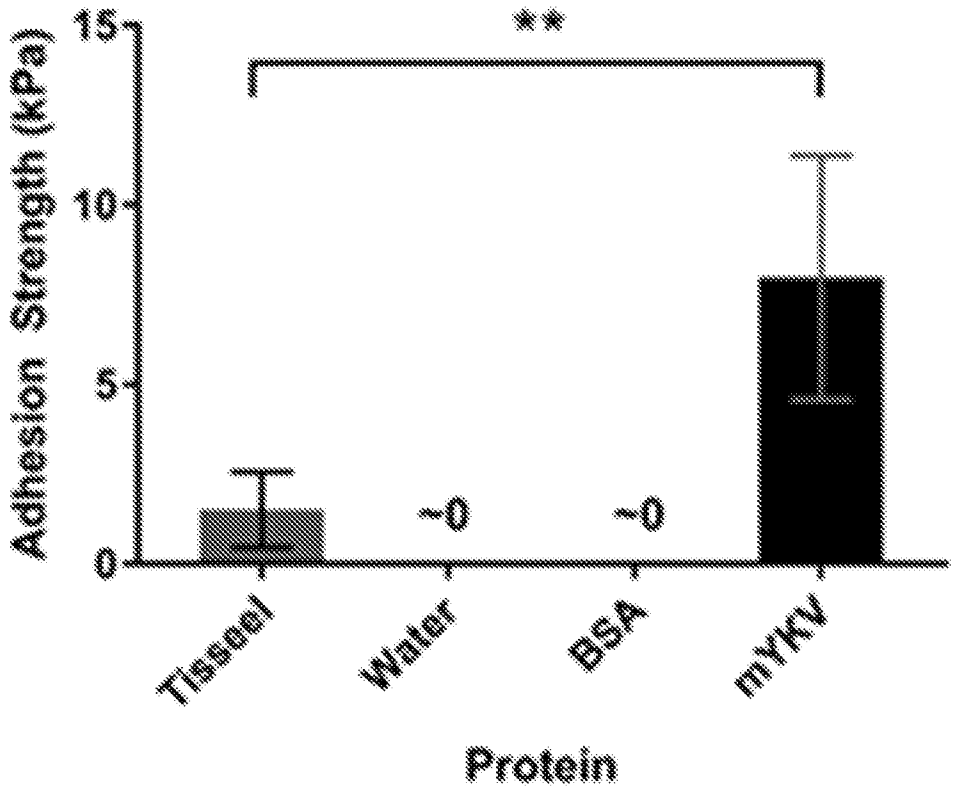
Adhesion Strength (kPa)
15
10
5
0
**
~0
~0
Tissseel
Water
BSA
mYKV
Protein

FIG. 14

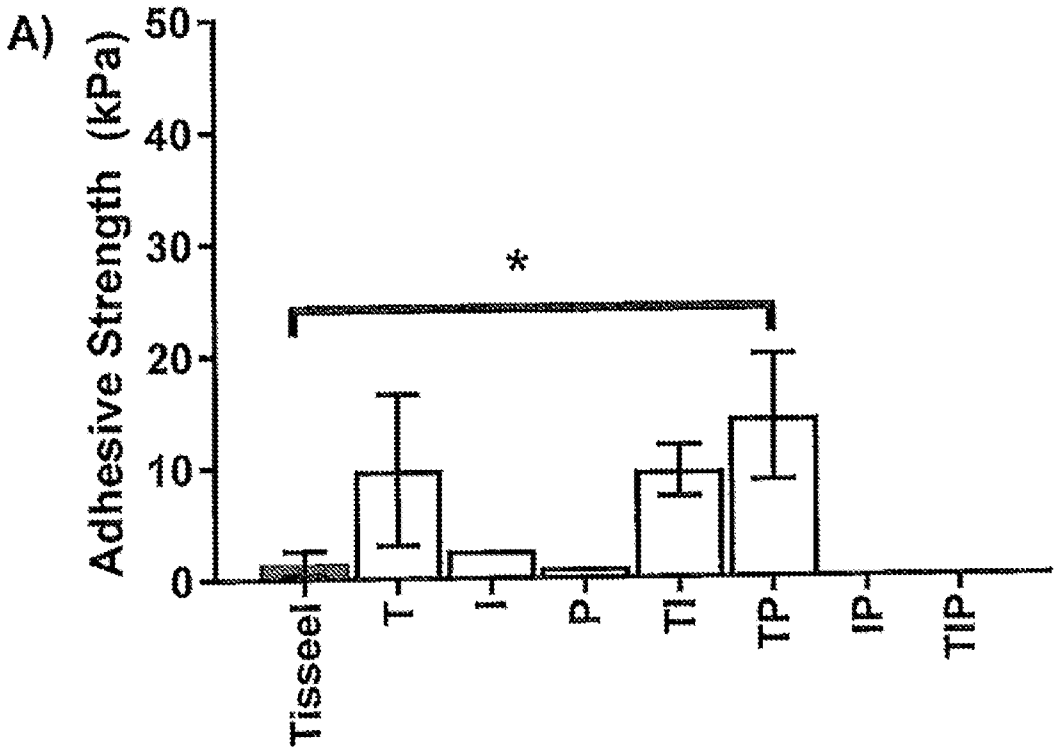

FIG. 15A

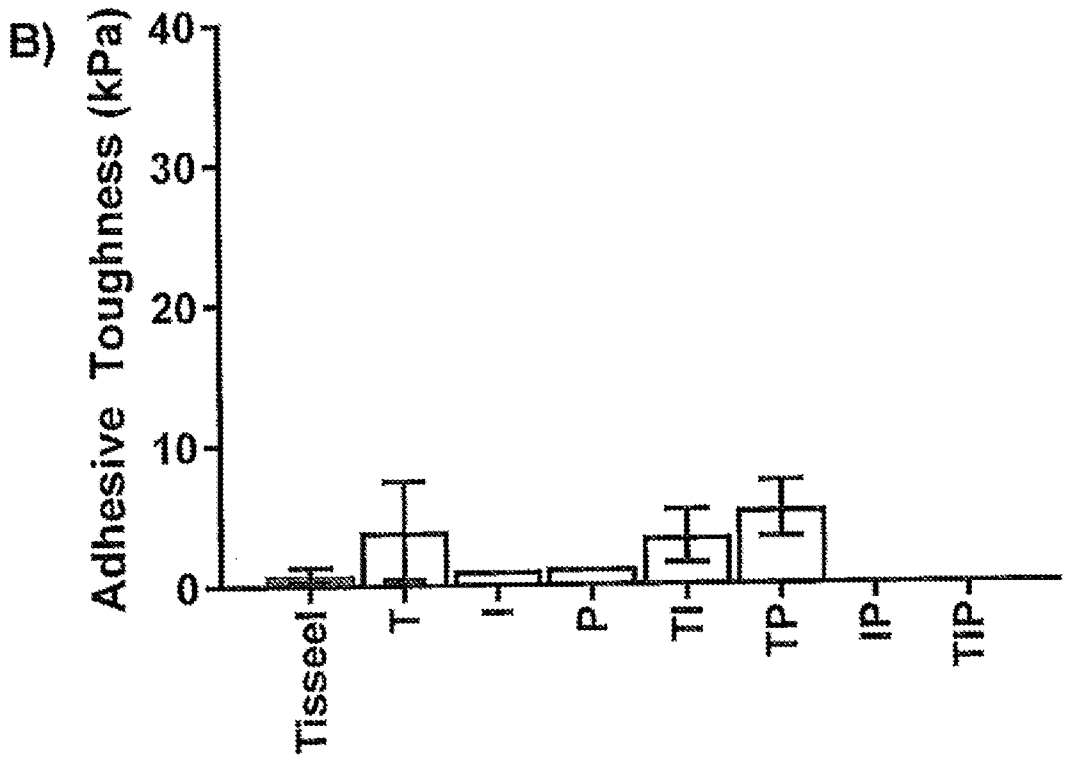

FIG. 15B

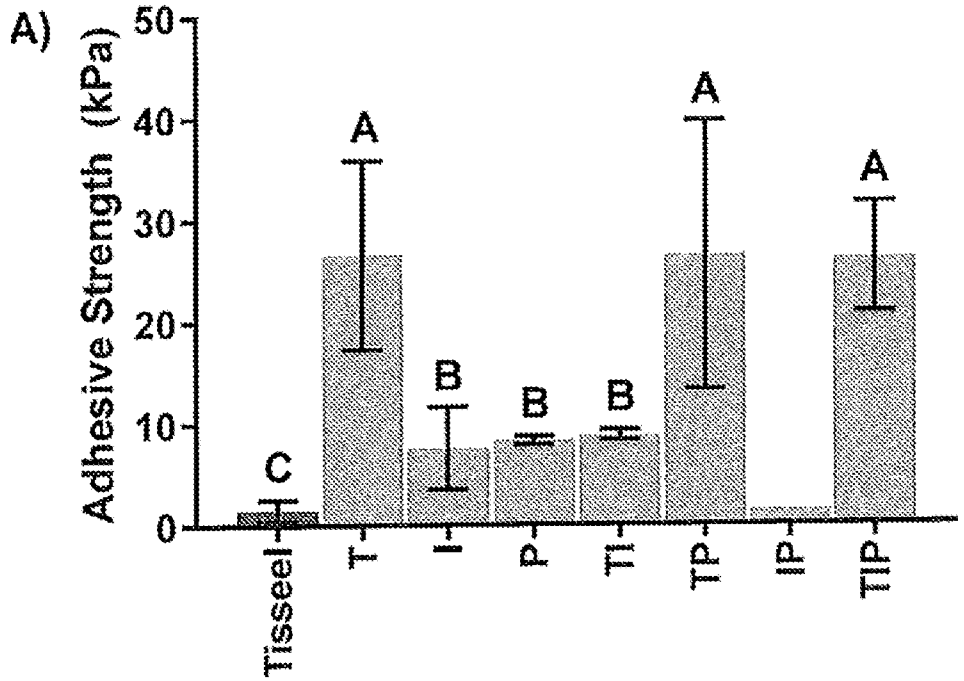

FIG. 16A

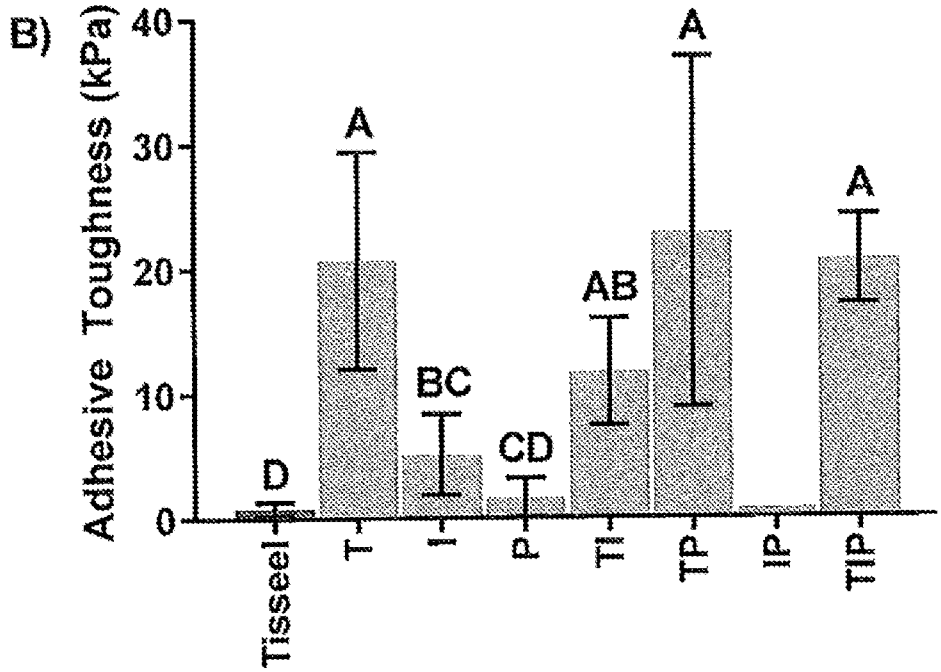

FIG. 16B

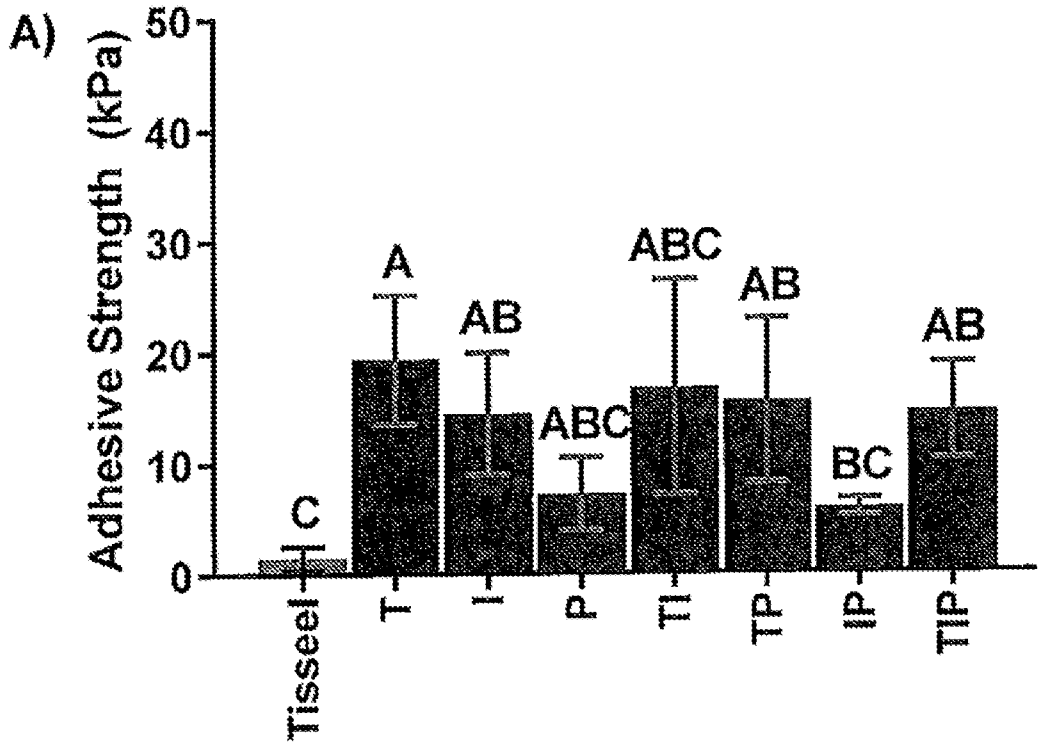

FIG. 17A

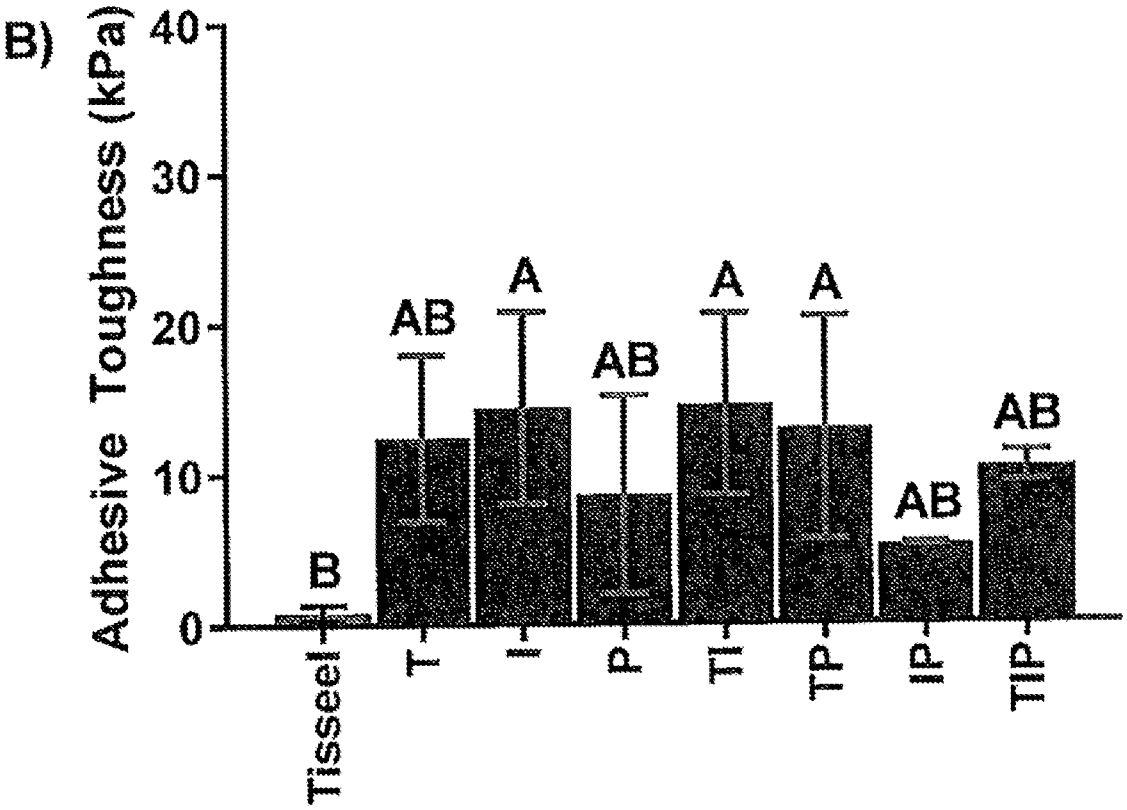

FIG. 17B

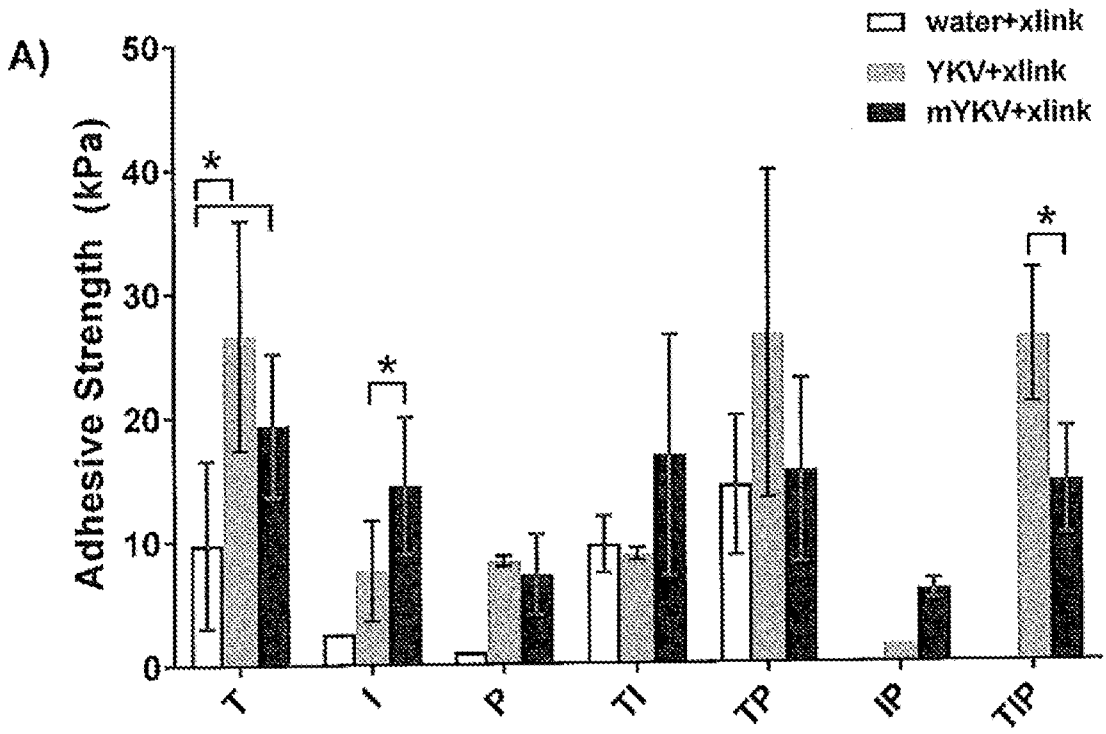

FIG. 18A

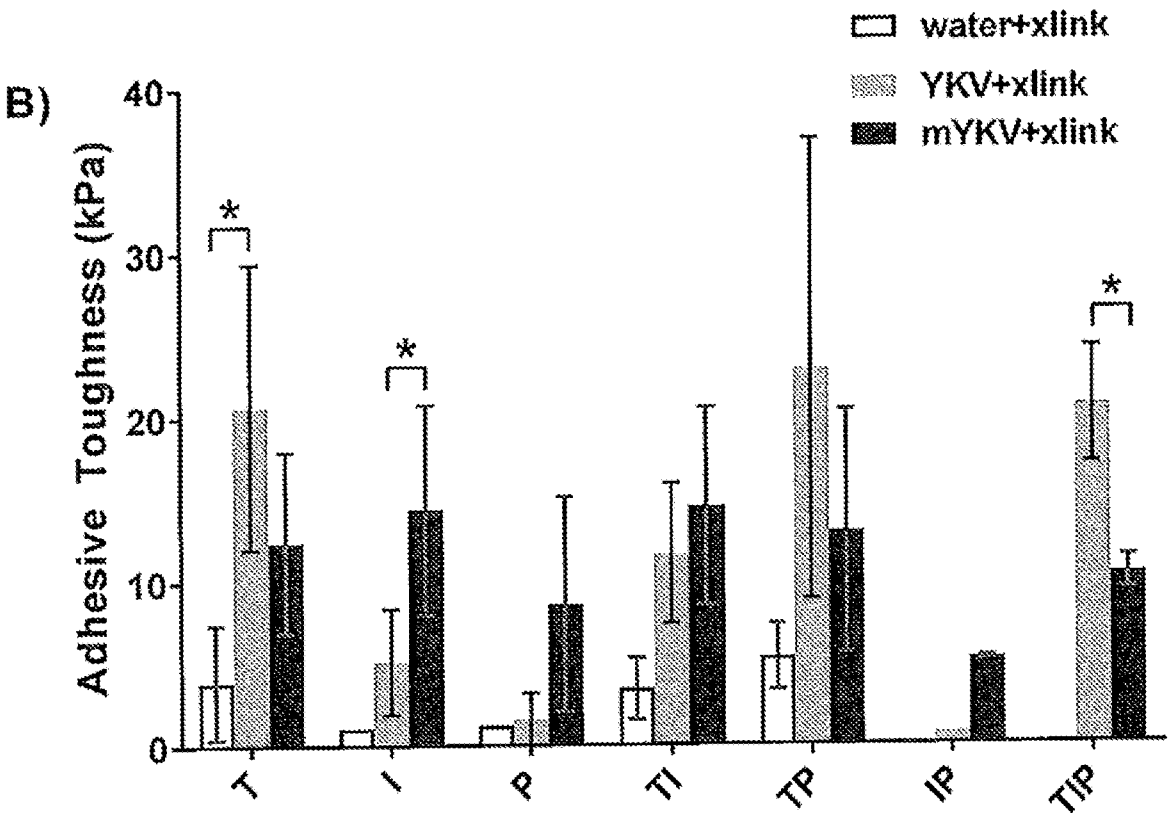

FIG. 18B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 19/47639

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C07K 1/107; C07K 14/00; C09J 189/00 (2019.01)
CPC - C07K 14/78; C07K 2319/00; C07K 7/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 2017/0015885 A1 (Liu et al.) 19 January 2017 (19.1.2017) abstract, para [0041], [0079], [0137]	1, 6-9, 14, 17-19 ---------- 2-5, 10, 15-16
Y	US 2016/0346424 A1 (KENSEY NASH CORPORATION) 1 December 106 (1.12.2016) para [0129]-[0130],	2-5, 15-16
Y	Van et al. FORMATION OF DIHYDROXYPHENYLALANINE FROM TYROSINE BY COUPLED OXIDATION WITH ASCORBIC ACID. THE JOURNAL OF INVESTIGATIVE DERMATOLOGY. 1949 Jan;12(1):11-7. title	10
Y	US 2010/0003329 A1 (ELISSEEFF) 7 January 2010 (7.1.2010) abstract	1
Y	US 5197973 A (Pang et al.) 30 March 1993 (30.3.1993) abstract	1
Y	US 4585585 A (Waite) 29 April 1986 (29.4.1986) abstract	1
Y	Numata et al. Synthesis of Adhesive Peptides Similar to Those Found in Blue Mussel (Mytilus edulis) Using Papain and Tyrosinase. Biomacromolecules 2014, 15, 3206-3212, abstract	1
Y	Brennan et al. A bioinspired elastin-based protein for a cytocompatible underwater adhesive. Biomaterials 124 (2017) 116-125, abstract	1

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 October 2019	18 NOV 2019
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 19/47639

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 11-13, 20-28
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.