

DESCRIPTION

TITLE OF INVENTION: [SALAMANDER SKIN SECRETION HYDROLYSATE, AND PREPARATION METHOD AND USE THEREOF]

[0001] The present application claims priority from Chinese invention patent application CN2021113742137 “A PREPARATION METHOD OF A SALAMANDER SKIN SECRETION HYDROLYSATE” filed on November 19, 2021, which is incorporated by reference in its entirety.

TECHNICAL FIELD

[0002] The present invention relates to the field of biological materials, and in particular to a salamander skin secretion hydrolysate, and preparation method and use thereof.

BACKGROUND

[0003] Wound closure is critical in surgical procedures. Clinically, 60% of wounds are held by sutures or staples after surgery. Due to the fragility of soft tissue and local stress, mechanical closure may cause secondary injury and require removal of non-degradable sutures or staples. When suturing wounds, hypertrophic scars may occur due to poor alignment, excessive skin treatment, or deep skin abrasions.

[0004] To date, most of the adhesives available in the prior art for wound closure have limitations. For example, the most widely used commercial synthetic adhesive—cyanoacrylate (CA)—although it has excellent adhesive strength, its cytotoxicity and non-biodegradability cannot be ignored. In addition, cyanoacrylate has poor elasticity and cannot adapt to the adhesion of soft tissues with large mobility such as joints and skin. Compared with cyanoacrylate, bio-based adhesives (such as fibrin glue, gelatin, hyaluronic acid, etc.) have good biocompatibility, but their adhesive strength is low. They can only be used as an auxiliary to sutures and cannot be used alone, thereby limiting their application.

SUMMARY

[0005] In a first aspect, the present invention provides a method of a salamander skin secretion hydrolysate, comprising the following steps:

S1 mixing a disulfide bond reducing agent and a first solvent to obtain a second solvent;

S2 mixing a lyophilized powder of salamander skin secretion and the second solvent to obtain a first mixed solution;

S3 fully hydrolyzing the first mixed solution to obtain a second mixed solution;

S4 centrifuging the second mixed solution and collecting a supernatant;

S5 transferring the supernatant into a dialysis bag with a molecular weight cut-off ≤ 1000 and placing the dialysis bag in the first solvent for full dialysis to obtain the salamander skin secretion hydrolysate;

wherein the disulfide bond reducing agent comprises a specific disulfide bond reducing agent, the specific disulfide bond reducing agent is Tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl), and the first solvent comprises one or more of ultrapure water, deionized water, water for injection, and pure water.

[0006] In some embodiments, a mass ratio of the first solvent to the lyophilized powder of salamander skin secretion is at least 4:1.

[0007] In some embodiments, a mass ratio of the lyophilized powder of salamander skin secretion to the disulfide bond reducing agent is at least 3:1.

[0008] In some embodiments, hydrolysis time for the hydrolyzing comprises 2 - 168 hours.

[0009] In some embodiments, hydrolysis time for the hydrolyzing is 12 - 48 hours.

[0010] In some embodiments, temperature condition for the S3, the S4 and the S5 comprises 0 - 8°C.

[0011] In some embodiments, temperature condition for the S3, the S4 and the S5 is 0 - 4°C.

[0012] In some embodiments, the S3 is carried out in a vacuum reactor, and a vacuum degree of the vacuum reactor is < 5 kPa.

[0013] In some embodiments, the disulfide bond reducing agent further comprises vitamin C.

[0014] In some embodiments, dialysis time for the dialysis comprises 48 hours.

[0015] In some embodiments, a concentration of the salamander skin secretion hydrolysate obtained comprises 3% - 7%.

[0016] In some embodiments, the method further comprises S6 concentrating the salamander skin secretion hydrolysate to obtain a concentrated salamander skin secretion hydrolysate.

[0017] In some embodiments, a method for the concentrating comprises centrifuging the salamander skin secretion hydrolysate loaded into a concentrated centrifuge tube with a molecular weight cut-off $\leq 3K$ or evaporating and concentrating the salamander skin secretion hydrolysate.

[0018] In the second aspect, the present invention also provides a salamander skin secretion hydrolysate, wherein the salamander skin secretion hydrolysate is prepared by the above method.

[0019] In the third aspect, the present invention also provides a use of a salamander skin secretion hydrolysate in the preparation of a bioadhesive, wherein the salamander skin secretion hydrolysate is prepared by the above method.

[0020] In some embodiments, a concentration of the salamander skin secretion hydrolysate comprises 5% - 7%.

[0021] In some embodiments, after the bioadhesive is applied to a wound, the bioadhesive penetrates into interior of tissue of the wound by itself or through external pressure to form a microgel structure.

[0022] In some embodiments, the wound comprises a skin wound.

[0023] In some embodiments, the bioadhesive is used for promoting healing of the wound.

[0024] In the fourth aspect, the present invention also provides a use of a salamander skin secretion hydrolysate in the preparation of a wound dressing, wherein the salamander skin secretion hydrolysate is prepared by the above method.

[0025] In the fifth aspect, the present invention also provides a use of a salamander skin secretion hydrolysate in the preparation of a photosensitive hydrogel, wherein the photosensitive hydrogel comprises a concentrated salamander skin secretion hydrolysate and a photoinitiator; under exposure to visible light, the concentrated salamander skin secretion hydrolysate is cured to form the photosensitive hydrogel; the concentrated salamander skin secretion hydrolysate is prepared by the above method, and the photoinitiator comprises Ru/SPS system.

[0026] In some embodiments, an intensity of the visible light comprises 7000W.

[0027] In some embodiments, the photosensitive hydrogel is used as a hemostatic agent.

[0028] In some embodiments, the photosensitive hydrogel is used as a bioadhesive.

[0029] In some embodiments, the photosensitive hydrogel is used as a wound dressing.

[0030] As used in the present invention, the term "bioadhesive" refers to a biocompatible substance that can adhere to and remain in biological tissue. In some embodiments, the bioadhesive is bioglue. In some embodiments, the bioadhesive can replace sutures/staples, serving to close wounds and stop bleeding. In some embodiments, the bioadhesive also has the functions of anti-infection, promoting healing, and inhibiting scar hyperplasia. The salamander skin secretion hydrolysate prepared by the preparation method provided by the present invention can be used as a bioadhesive to achieve effects such as adhesion, wound closure (even hemostasis, inhibition of bacteria), and promotion of wound healing, which can reduce the time required for suturing and technical sensitivity, decrease the difficulty of postoperative care, avoid secondary injury to patients caused by the removal of sutures/staples, and reduce the number and duration of patient visits.

[0031] As used in the present invention, the term "hydrogel" refers to a natural or synthetic polymer network that is highly absorbent (e.g., can absorb and/or retain large volumes of water).

[0032] As used in the present invention, the term "microgel" refers to a hydrogel particle with an intramolecular cross-linked structure and a micron-scale particle size.

[0033] As used in the present invention, the term "wound dressing" refers to a material used to cover the surface of a wound and to treat or protect the wound. In some embodiments, the wound dressings are divided into: (1) passive dressings (i.e. traditional dressings), which passively cover the wound surface and absorb exudate, and provide limited protection for the wound surface; (2) interactive dressings. There are many forms of interaction between the dressing and the wound surface, such as absorbing exudate and toxic substances, allowing gas exchange, thereby creating an ideal environment for healing; providing a barrier outer structure to prevent the intrusion of microorganisms in the environment and cross-infection in the wound

surface, etc. The salamander skin secretion hydrolysate and the formed photosensitive hydrogel involved in the present invention belong to the interactive dressing.

[0034] As used in the present invention, the term "biological tissue" or "tissue" refers to any tissue of a living or dead organism or any tissue derived from a living or dead organism. A biological tissue may comprise any single tissue (e.g., a collection of interconnected cells) or a group of tissues that make up an organ or part or region of an organism. The biological tissue comprises one or more of animal tissues including mucosal tissue, epidermal tissue, dermal tissue, and subcutaneous tissue. Different biological tissues can form organs with specific functions. The organism (i.e., subject or individual) related to or derived from a biological tissue can be any animal, including mammals and non-mammals (e.g., invertebrates). Biological tissue may be intact, or may have one or more incisions, cracks, defects, or other types of wounds. In some embodiments, the biological tissue is mammalian tissue.

[0035] As used herein, "wound" refers to a physical disruption of the continuity or integrity of a biological tissue structure. Wounds may result from cuts, abrasions, avulsions, lacerations, punctures, cancer, diabetic ulcers or lesions, burns, surgery, or other injuries. In some embodiments, the wound may result in bleeding.

[0036] As used herein, "wound healing" refers to partial or complete restoration of tissue integrity. In some embodiments, the tissue is skin, i.e. the wound is a skin wound, such as a dermal or epidermal wound. In some embodiments, wound healing comprises temporal and spatial healing programs that comprise wound closure and processes involving wound closure. "Promoting wound healing" is understood as restoring tissue from a break in continuity or integrity. In some embodiments, promoting wound healing comprises achieving the same level of healing with less healing time required. In some embodiments, promoting wound healing comprises achieving a greater level of healing within the same healing time. Specifically, one or more of: neovascularization; migration of fibroblasts, endothelial cells, and epithelial cells; extracellular matrix deposition; epidermal replantation; and remodeling may be involved.

[0037] As used herein, the term "wound closure" refers to the closure of a wound, including closing the wound without exposing the internal tissues. In some embodiments, the wound closure comprises rejoining the edges of the wound to

form a continuous barrier (e.g., intact skin). In some embodiments, the wound closure further comprises hemostasis.

[0038] As used herein, "full-thickness wound" refers to a full-thickness disruption of the dermis (and epidermis).

[0039] As used herein, "scar" refers to the appearance, morphology and histopathological changes of tissues caused by trauma, which is an inevitable product of the body's wound repair process. When the scar grows beyond a certain limit, various complications may occur (such as the destruction of appearance and functional disturbance, etc.), which will bring huge physical pain and mental pain to the patient (especially scars left after burns, scalds, and severe trauma). "Scarless healing" means less, significantly less or no scarring that would result from natural wound healing.

[0040] As used in the present invention, the term "biocompatible" refers to a material that is substantially non-toxic in its intended in vivo environment and is not substantially rejected by an individual's physiological system. The evaluation of biocompatibility of biomaterials should follow the two principles of biosafety and biofunctionality, which require that biomaterials have very low toxicity, and at the same time require that biomaterials can properly stimulate the corresponding functions of the body in specific applications (such as the material interacts with the body environment). In some embodiments, the biocompatibility meets FDA requirements. The immune response and tissue repair process in the body are very complex, and it is often not sufficient to determine the biocompatibility of a certain material through one kind of cell or tissue. The present invention confirms that the salamander skin secretion hydrolysate has a higher biocompatibility on organisms (especially in the wound environment) by a series of in vitro and in vivo experiments with reference to the requirements of the International Standards Organization (ISO) 10993 and the national standard GB/T16886.

[0041] As used herein, "regeneration" is a repair process in which a tissue or organ suffers partial loss due to an external factor, and a structure identical in shape and/or function to the missing part regrows on the basis of the remaining part.

[0042] As used herein, "biosourced" means derived from or taken from naturally occurring organisms and parts of organisms. In other words, the lyophilized powder

of salamander skin secretion and the salamander skin secretion hydrolysate involved in the present invention are not obtained through gene recombination technology.

[0043] As used herein, the term "substantially free" or "substantially free of" means that the referred substance is absent or present in a non-detectable amount in a certain substance.

DESCRIPTION OF DRAWINGS

[0044] To make the embodiments of the present invention or the technical solutions in the prior art clearer, the drawings required to be used in the description of the embodiments or the prior art will be briefly introduced below. It is obvious that the drawings described below are some embodiments of the present invention, and that other drawings can be obtained from these drawings for those of ordinary skill in the art without making inventive effort.

[0045] **[Fig. 1]:** illustrates representative real views of the lyophilized powder of salamander skin secretion and the hydrogel formed by the lyophilized powder of salamander skin secretion and water;

[0046] **[Fig. 2a]:** illustrates representative images of salamander skin secretion hydrolysate before hydrolysis, after hydrolysis, and the obtained adhesive supernatant;

[0047] **[Fig. 2b]:** illustrates representative images of the supernatant in Fig. 2a before, during, and after dialysis;

[0048] **[Fig. 2c]:** illustrates representative real views of adhesive salamander skin secretion hydrolysate provided by the present invention;

[0049] **[Fig. 3a]** and **[Fig. 3c]:** illustrates the experimental results of the detection of the cytotoxicity of salamander skin secretion hydrolysate;

[0050] **[Fig. 3b]:** illustrates the high cytocompatibility of the hydrolysate of the TCEP group in the present invention;

[0051] **[Fig. 3d]** and **[Fig. 3e]:** illustrates the experimental results of the promotion of the expression of angiogenesis-related genes in the cells by the salamander skin secretion hydrolysate on the 7th day and the 14th day of culture respectively;

[0052] [Fig. 3f]: illustrates the scratch assay results of vitamin C group and TCEP group;

[0053] [Fig. 3g]: illustrates the Transwell assay results of vitamin C group and TCEP group;

[0054] [Fig. 3h]: illustrates the permeability of salamander skin secretion hydrolysate;

[0055] [Fig. 3i]: illustrates representative SEM images of the adhesion interface of salamander skin secretion hydrolysate to porcine skin;

[0056] [Fig. 4a]: illustrates the macroscopic experimental results of the skin incision healing experiment of each experimental group (blank control group, hydrolysate group, fibrin adhesive group, suture group, and cyanoacrylate group);

[0057] [Fig. 4b]: illustrates the experimental results of HE staining of the skin incision healing experiment of each experimental group (blank control group, hydrolysate group, fibrin adhesive group, and cyanoacrylate group);

[0058] [Fig. 4c]: illustrates the macroscopic experimental results of the skin incision healing experiment of each experimental group (blank control group, vitamin C group, TCEP group, fibrin adhesive (FB) group, cyanoacrylate (CA) group, and suture group);

[0059] [Fig. 4d]: illustrates the experimental results of HE staining of the skin incision healing experiment of each experimental group (blank control group, vitamin C group, TCEP group, fibrin adhesive (FB) group, cyanoacrylate (CA) group, and suture group);

[0060] [Fig. 5a]: illustrates the experimental results of the co-culture of salamander skin secretion hydrolysate + photosensitizer Ru/SPS and cells;

[0061] [Fig. 5b]: illustrates the hemostatic effect of the photosensitive hydrogel formed by curing salamander skin secretion hydrolysate + photosensitizer Ru/SPS (abbreviated as photosensitive hydrogel) as a photosensitive hemostatic agent;

[0062] [Fig. 5c]: illustrates the effect of photosensitive hydrogel as a wound dressing;

[0063] [Fig. 6a]: illustrates the macroscopic effect of salamander skin secretion hydrolysate used as a liquid wound dressing to promote wound healing of a full-thickness skin defect;

[0064] [Fig. 6b]: illustrates the experimental results of HE staining of salamander skin secretion hydrolysate used as a liquid wound dressing to promote wound healing of a full-thickness skin defect;

[0065] [Fig. 6c]: illustrates the macroscopic experimental results of the skin incision healing experiment of each experimental group (blank control group, hydrolysate group, fibrin adhesive (FG) group, cyanoacrylate (CA) group and suture group);

[0066] [Fig. 7]: illustrates the summary diagram of the performances and the specific uses of salamander skin secretion hydrolysate provided by the present invention;

[0067] [Fig. 8a]: illustrates representative SEM images and adhesion of unconcentrated and concentrated salamander skin secretion hydrolysates cured by visible light;

[0068] [Fig. 8b]: illustrates the ultimate adhesion strength of CA, the concentrated hydrolysate, the unconcentrated hydrolysate and the photosensitive hydrogel, and the schematic diagram of the detection device;

[0069] [Fig. 8c]: illustrates the in vivo biodegradable properties of the photosensitive hydrogel;

[0070] [Fig. 8d]: illustrates the operation process of the liver hemorrhage model constructed by the present invention;

[0071] [Fig. 8e]: illustrates the SEM images of the photosensitive hydrogel tightly bound to the liver after hemostasis on the bleeding liver;

[0072] [Fig. 8f]: illustrates the hemostatic effect of each experimental group (blank group, hydrolysate group, fibrin glue (FB) group and photosensitive hydrogel group) on the liver;

[0073] [Fig. 9a]: illustrates the Raman spectra of different samples (lyophilized powder of salamander skin secretion, hydrogel of salamander skin secretion, TCEP group, and vitamin C group);

[0074] [Fig. 9b]: illustrates the infrared spectrum analysis of different samples (lyophilized powder of salamander skin secretion, hydrogel of salamander skin secretion, TCEP group, and vitamin C group).

DETAILED DESCRIPTION

[0075] To make the objective, the technical solutions and advantages of the embodiments of the present invention clearer, the technical solutions of the embodiments of the present invention will be clearly and completely described below in combination with drawings. It is obvious that the described embodiments are some of the embodiments of the present invention, not all of the embodiments. Based on the embodiments of the present invention, all other embodiments obtained by those of ordinary skill in the art without making inventive effort shall belong to the protection scope of the present invention.

[0076] It should be noted that the term “include”, “comprise” or any variant thereof is intended to encompass nonexclusive inclusion so that a process, method, article or device including a series of elements includes not only those elements but also other elements which have been not listed definitely or an element(s) inherent to the process, method, article or device. Moreover, the expression “comprising a(n) . . .” in which an element is defined will not preclude presence of an additional identical element(s) in a process, method, article or device comprising the defined element(s) unless further defined.

[0077] As used herein, the term “about”, typically means $\pm 5\%$ of the stated value, more typically $\pm 4\%$ of the stated value, more typically $\pm 3\%$ of the stated value, more typically, $\pm 2\%$ of the stated value, even more typically $\pm 1\%$ of the stated value, and even more typically $\pm 0.5\%$ of the stated value.

[0078] Throughout this application, embodiments of this invention may be presented with reference to a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as “from 1 to 6” should be considered to have specifically disclosed subranges such as “from 1 to 3”, “from 1 to 4”, “from 1 to 5”, “from 2 to 4”, “from 2 to 6”, “from 3 to 6”, etc.; as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

[0079] A preparation method of a salamander skin secretion hydrolysate provided by the present invention comprises the following steps:

[0080] S1 mixing a disulfide bond reducing agent and a first solvent to obtain a second solvent.

[0081] A suitable first solvent should be substantially free of ions, such as ultrapure water, deionized water, water for injection, pure water and the like.

[0082] S2 mixing a lyophilized powder of salamander skin secretion and the second solvent to obtain a first mixed solution.

[0083] In fact, there is no specific limitation on the mixing order of the disulfide bond reducing agent, the first solvent, and the lyophilized powder of salamander skin secretion (i.e., the step of obtaining the first mixed solution). For example, the first mixed solution can be obtained by adding and mixing the disulfide bond reducing agent, the first solvent, and the lyophilized powder of salamander skin secretion together. It can also be obtained by mixing the disulfide bond reducing agent with the first solvent followed by adding and mixing the lyophilized powder of salamander skin secretion. Alternatively, it can be obtained by mixing the lyophilized powder of salamander skin secretion with the first solvent followed by adding and mixing the disulfide reducing agent. It can also be obtained by mixing the disulfide reducing agent with the lyophilized powder of salamander skin secretion followed by adding and mixing the first solvent. The ratios of the disulfide reducing agent, the first solvent, and the lyophilized powder of salamander skin secretion can be adjusted according to actual needs, and the present invention does not limit this. For example, a mass ratio of the lyophilized powder of salamander skin secretion to the disulfide reducing agent may be 10:1, 9:1, 8:1, 7:1, 6:1, 5:1, 4:1, or 3:1. A mass ratio of the first solvent to the lyophilized powder of salamander skin secretion may be 12:1, 11:1, 10:1, 9:1, 8:1, 7:1, 6:1, 5:1, or 4:1. A concentration of the second solvent maybe about 1% - 5% (specific values can be selected as about 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5%, or 5%, w/w%). The lower limit (minimum value) of the mass ratio of the lyophilized powder of salamander skin secretion to the disulfide reducing agent may be 3:1. The lower limit (minimum value) of the mass ratio of the first solvent to the lyophilized powder of salamander skin secretion may be 4:1.

[0084] An exemplary preparation method of the lyophilized powder of salamander skin secretion is as follows (taking Chinese giant salamander (*Andrias dividi*anus) as

an example): adult healthy male Chinese giant salamander (3-6 years old, body length 65-110cm, body weight $2.8 \pm 0.7\text{kg}$) is washed with clean water. Under mild mechanical stimulation, mucus secretion is induced from the dorsal skin of the Chinese giant salamander and collected in a clean tube. After collection, the skin secretion mucus of Chinese giant salamander is washed with sterilized PBS or other suitable solvents, shaken, and centrifuged, and the supernatant is collected. The supernatant is lyophilized (freeze-dried) for 24 hours, and grinded into a powder (i.e. lyophilized powder of skin secretion of *Andrias dividiianus*, SSAD for short) by a freeze ball mill, as shown in the left figure of Fig. 1. The lyophilized powder of salamander skin secretion is stored at -20°C until further use. The SSAD is preferably freshly prepared. In the present invention, lyophilized powder of skin secretion of *Andrias dividiianus*, SSAD, SSAD lyophilized powder and SSAD powder have the same meaning unless otherwise specified. The skin is an important respiratory organ of salamanders, with mucus glands and granular glands distributed. The reason for choosing the skin secretion of *Andrias dividiianus* in the present invention is that it can represent the mucus secreted by amphibians like salamanders when stimulated. Electrical stimulation and skin scratching can promote mucus secretion.

[0085] S3 fully hydrolyzing the first mixed solution to obtain a second mixed solution.

[0086] The temperature condition for the hydrolysis comprises $0-8^{\circ}\text{C}$ (preferably $0-4^{\circ}\text{C}$). The hydrolysis time is related to factors such as the specific hydrolysis temperature, the concentration of the second solvent, the concentration of the lyophilized powder of salamander skin secretion, etc. Within the temperature range of $0-8^{\circ}\text{C}$, the higher the temperature, the shorter the hydrolysis time; the higher the concentration of the second solvent, the shorter the hydrolysis time; the higher the concentration of the lyophilized powder of salamander skin secretion, the longer the hydrolysis time. The hydrolysis time may also be related to the specific disulfide bond reducing agent used. Therefore, the hydrolysis time can be adjusted according to actual needs, and the present invention does not limit this. Exemplary hydrolysis time comprises 2 - 168h, preferably 12 - 48h. Mixing can be carried out by a commonly used mixing method in the art, such as shaking and vortex mixing.

Preferably, S3 can be carried out in a low-temperature vacuum reactor, and the vacuum degree of the vacuum reactor is < 5kPa.

[0087] S4 centrifuging the second mixed solution and collecting a supernatant.

[0088] The specific condition for centrifugation can be adjusted according to actual needs, and the present invention does not limit this. Exemplary centrifugation condition comprises centrifugation at 4500 rpm for 10 min at 4°C.

[0089] S5 transferring the supernatant into a dialysis bag with a molecular weight cut-off ≤ 1000 and placing the dialysis bag in the first solvent for full dialysis to obtain the salamander skin secretion hydrolysate.

[0090] The temperature condition for the dialysis comprises 0-8°C (preferably 0 - 4°C). A dialysis bag with a molecular weight cut off of 500 or 1000 is preferred. The dialysis time is actually related to the treatment method during the dialysis. For example, the dialysis time can be shortened by increasing the frequency of changing the first solvent in which the dialysis bag is placed. Therefore, the dialysis time can be adjusted according to actual needs, and the present invention does not limit this.

[0091] Wherein, the disulfide bond reducing agent comprises a specific disulfide bond reducing agent. In the present invention, "specific disulfide bond reducing agent" refers to a disulfide bond reducing agent capable of reducing an intermolecular disulfide bond in a protein or polypeptide to be reduced while keeping an intramolecular disulfide bond from being reduced as much as possible. The specific disulfide bond reducing agent comprises Tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl). Of course, in the method of preparing the salamander skin secretion hydrolysate provided by the present invention, it can also be used together with common protein denaturants/organic solvents known in the art, such as vitamin C, acetic acid, hydrochloric acid, β -mercaptoethanol, dithiothreitol, EDTA, urea, ethanol, acetone, guanidine hydrochloride, sodium dodecyl sulfate, sodium hydroxide, and proteases such as pepsin, trypsin, papain, and subtilisin.

[0092] Further, the method of preparing the salamander skin secretion hydrolysate provided by the present invention may also comprise S6 concentrating the salamander skin secretion hydrolysate to obtain a concentrated salamander skin secretion hydrolysate.

[0093] An exemplary concentration method comprises centrifuging the salamander skin secretion hydrolysate loaded into a concentrated centrifuge tube

with a molecular weight cut-off $\leq 3K$ or evaporating and concentrating the salamander skin secretion hydrolysate.

[0094] Preferably, any of the above steps are carried out under vacuum or inert atmosphere.

[0095] Preparation Example 1: Preparation of SSAD hydrolysate dialysis solution using TCEP-HCl (referred to as "TCEP").

[0096] Weigh 100 mg of TCEP and dissolve it in 5 mL of deionized water, and weigh 500 mg of SSAD powder into it, shake and mix to obtain a mixed solution. The mixed solution is hydrolyzed at 4°C for 48 hours, and the mixed solution after 48-hour hydrolysis is centrifuged at 4000 rpm for 20 minutes at 4°C to obtain about 3.5 mL of supernatant. Dialyze the supernatant in deionized water at low temperature (4°C) using a dialysis bag with a molecular weight cutoff of 1000 for 48 hours to obtain SSAD hydrolysis dialysis solution.

[0097] The SSAD powder is hydrolyzed at mixing ratios (mass ratio) of SSAD powder to TCEP of 10:1, 5:1, and 3:1 respectively. It is found that after 1 day of hydrolysis, the colloid is dispersed in a flocculent form, and the obtained supernatant maintains the adhesive property of SSAD itself, while maintaining the activity of the protein and polypeptide contained in SSAD. The obtained lower precipitate is non-adhesive. Preferably, the mass ratio of SSAD powder to TCEP may be 5:1, and the mass ratio of SSAD powder to deionized water may be 10:1.

[0098] The representative images of SSAD hydrolysate before hydrolysis, after hydrolysis, and the obtained adhesive supernatant (taking the mass ratio of SSAD powder to TCEP as 5:1 as an example) are shown in Fig. 2a. The mixed solution obtained after hydrolysis is centrifuged, and the obtained supernatant has obvious adhesion. Representative images of the supernatant before, during, and after dialysis are shown in Fig. 2b. The experiment reveals that the clear and transparent liquid before dialysis turns pale milky white after dialysis. The pale milky appearance may be caused by oxidation during dialysis. Therefore, if conditions permit, any step in the preparation method provided by the present invention is preferably carried out under vacuum or inert atmosphere. Fig. 2c illustrates representative images of the obtained SSAD hydrolysate. In the image, the left figure illustrates the prepared SSAD hydrolysate, presenting a uniform semi-transparent appearance. The upper middle figure illustrates the SSAD hydrolysate loaded in a syringe. The lower middle

figure illustrates a drop of SSAD hydrolysate on a fingertip, indicating its good flowability and injectability. The right figure illustrates that after the SSAD hydrolysate is dropped to the fingertip and pinched by two fingers within less than 1 minute, the SSAD hydrolysate can stick the upper and lower fingers, highlighting its noticeable adhesion. In this invention, SSAD hydrolysis dialysis solution, SSAD hydrolysate, and hydrolysate have the same meaning unless otherwise specified.

[0099] Preparation Example 2: Preparation of SSAD hydrolysis dialysis solution Using Vitamin C

[0100] Weigh 66mg of vitamin C and dissolve it in 5mL of deionized water, and weigh 500mg of SSAD powder into it, shake and mix to obtain a mixed solution. The mixed solution is hydrolyzed at 4°C for 48 hours, and the mixed solution after 48-hour hydrolysis is centrifuged at 4000 rpm for 20 minutes at 4°C to obtain about 3.5 mL of supernatant. Dialyze the supernatant in deionized water at low temperature (4°C) using a dialysis bag with a molecular weight cutoff of 1000 for 48 hours to obtain SSAD hydrolysis dialysis solution.

[0101] The concentration of the salamander skin secretion hydrolysate prepared by the preparation method provided by the invention is usually between 3% and 7%. The concentration of salamander skin secretion hydrolysate (such as SSAD hydrolysate) is calculated by weighing after freeze-drying, unless otherwise specified. Unless otherwise specified (for example, vitamin C group), "salamander skin secretion hydrolysate" and "hydrolysate" in the present invention both refer to the salamander skin secretion hydrolysate prepared using TCEP.

[0102] In fact, the present invention also attempts to use other common protein denaturants/organic solvents in the art to hydrolyze the lyophilized powder of salamander skin secretion, including acetic acid solutions with concentrations of 25%, 50%, 75%, and 99.5%, hydrochloric acid solutions with concentrations of 21.6% and 36%, urea aqueous solutions with concentrations of 25%, 50%, and 57% and β -mercaptoethanol, dithiothreitol (DTT), EDTA, sodium hydroxide, ethanol, acetone, guanidine hydrochloride, sodium dodecyl sulfate (SDS) and proteases (pepsin, trypsin, papain, subtilisin, etc.) etc. The experimental results show that even if a strong base is added to adjust the pH, the supernatant obtained by hydrolysis with acetic acid or hydrochloric acid is still too acidic and has extremely low adhesion; while the colloid swells after hydrolysis with urea aqueous solution for 5 hours, the

adhesion of the supernatant obtained is medium to low. The resulting lower precipitate is instead moderately adhesive.

[0103] Embodiment one: the characteristic of salamander skin secretion hydrolysate

[0104] 1.1 Adhesion of salamander skin secretion hydrolysate

[0105] The lyophilized powder of salamander skin secretion (such as powdered SSAD) will immediately form a highly adhesive hydrogel when it meets water. But it is difficult to disperse (that is, insoluble) in aqueous solution, as shown in the right figure of Fig. 1.

[0106] Compared with the previously prepared lyophilized powder of salamander skin secretion, the salamander skin secretion hydrolysate (taking SSAD hydrolysate as an example) provided by the present invention has better biocompatibility and similar adhesion (Table 1) and the adhesion is adjustable. Different from the adhesion result (that is, the adhesion of the salamander skin secretion hydrolysate increases with the increase of its concentration) according to the pre-experiment speculation, the present invention unexpectedly finds that the lower concentration of the salamander skin secretion hydrolysate (for example, the hydrolysate with concentration of 5%, 7% below) has higher adhesion strength than that of the higher concentration of the salamander skin secretion hydrolysate (for example, the hydrolysate with concentration of 10%, 15%, 20% below). In other words, the correlation between adhesion and concentration of the salamander skin secretion hydrolysate presents a downward-opening parabola. When the concentration is 5-7%, the adhesion of the salamander skin secretion hydrolysate is the highest; the adhesion of skin secretion hydrolysate increases with the increase of concentration. When the concentration is >7%, the adhesion of the salamander skin secretion hydrolysate decreases with the increase of concentration.

[0107] Table 1: Adhesion of SSAD hydrolysate

Concentration of SSAD hydrolysate	Adhesion to skin
3%	10.8 kPa±2.3 kPa
5%	26.5kPa±3.9 kPa
7%	27.6 kPa±3.2 kPa
10%	21.8 kPa±2.7 kPa
15%	17.7 kPa±3.6 kPa
20%	16.3 kPa±2.1 kPa

[0108] 1.2 Biocompatibility of salamander skin secretion hydrolysate

[0109] Biocompatibility of salamander skin secretion hydrolysate is assessed using the Cell Viability Assay Kit (CCK-8) following the manufacturer's instructions. As shown in Fig. 3a and Fig. 3c (Fig. 3a and Fig. 3c are different representations of the same data), the cytotoxicity of different concentrations (1/100, 1/1000, 1/10000 w/w%) of powder leach liquor, dialyzed supernatant, and non-dialyzed supernatant on skin fibroblast cells is detected. The results prove that the dialyzed supernatant (i.e. SSAD hydrolysate) has good biocompatibility. It not only has no obvious cytotoxicity, but also has the effect of promoting cell proliferation.

[0110] Using polymerase chain reaction (PCR) technology, the expression of angiogenesis-related genes in human umbilical vein endothelial cells (HUVEC) co-cultured with different concentrations of SSAD hydrolysate is detected. Fig. 3d and Fig. 3e show the changes in the expression levels of angiogenesis-related genes in HUVECs. As shown in Fig. 3d (co-culture for 7 days) and Fig. 3e (co-culture for 14 days), in the 1/50 group (the concentration of SSAD hydrolysate is about 5%, mixed with the cell culture medium at a volume ratio of 1:49) and 1 /100 group (the concentration of SSAD hydrolysate is about 5%, mixed with the cell culture medium at a volume ratio of 1:99), CD31, VEGF, EphrinB2 and other angiogenesis-related genes are significantly highly expressed in a dose-dependent manner. In other words, as the concentration of SSAD hydrolysate increases, the expression of angiogenesis-related genes in cells increases in a concentration-dependent effect, indicating that SSAD hydrolysate could promote angiogenesis.

[0111] Vitamin C, also known as ascorbic acid, is a strong reducing agent. Meanwhile, vitamin C is an essential nutrient for living organisms, especially humans. Thus it is considered to be high in biosafety. Using the Cell Viability Assay Kit (CCK-8), the present invention further compares the biocompatibility of the hydrolysate prepared in Preparation Example 1 (abbreviated as TCEP group, the same below) and the hydrolysate prepared in Preparation Example 2 (abbreviated as vitamin C group, the same below). Surprisingly, however, as shown in Fig. 3b, the biocompatibility of the TCEP group is higher than that of the vitamin C group on day 1, day 3, and day 5. Scratch assay (Fig. 3f) and Transwell assay (Fig. 3g) also confirm that the biocompatibility of TCEP group is higher than that of vitamin C group.

[0112] An exemplary experimental method of the scratch assay comprises: about 5×10^5 cells are seeded in each well of a 6-well plate and cultured for 24 hours under suitable conditions. After 24 hours, use a pipette tip to vertically scratch the bottom of the well with five intersecting lines each. The cells are washed 3 times with PBS to remove the scratched cells, and then serum-free medium is added. The 6-well plate is placed in an incubator at 37°C, 5% CO₂ for culture, and sampled and photographed at 0, 12, 24, 48 h. An exemplary experimental method of the Transwell assay comprises: take cells in the logarithmic growth phase, digest the cells routinely, resuspend the cells in a medium containing 1% FBS, and adjust the cell density to $5 \times 10^5/\text{mL}$. 100 μL of cell suspension is seeded in the upper chamber of the Transwell chamber (24-well plate), and 600 μL of medium containing 10% FBS is added to the lower chamber, and 1% SSAD hydrolysate is added to the control group. After culturing the cells for 24h or 48h, the chamber is taken out and rinsed in a beaker. Then methanol is added to the chamber at 800 $\mu\text{L}/\text{well}$. The liquid in the upper chamber is removed completely, and fixation with 4% paraformaldehyde is performed at room temperature for 10-30 min. After removing the fixative solution in the upper chamber, add 0.1% crystal violet staining solution and stain at room temperature for 20 min. The chamber is gently rinsed with running water and soaked 3 times for destaining. Remove the liquid in the upper chamber completely and wipe off the unigrated cells on the membrane surface at the bottom of the upper chamber with a cotton swab gently. Cells in 5 fields of view are randomly observed under a microscope and photographed and recorded (images are processed using ImageJ software).

[0113] In this embodiment, Raman spectroscopy and infrared spectroscopy are used to further analyze the hydrolysate of TCEP group and vitamin C group. Raman spectroscopy and infrared spectroscopy are based on different physical principles to detect the vibrational properties of molecules, and have the advantages of simplicity and no damage to samples. Infrared spectroscopy is based on the absorption properties of the sample, where the signal intensity follows Beer's law. Raman spectroscopy relies on the photons scattered inelastically by the sample being detected, and the intensity of the Raman shift is proportional to the molecular concentration.

[0114] Fig. 9a shows that, compared with the lyophilized powder of salamander skin secretion and the hydrogel of salamander skin secretion, the TCEP group and the vitamin C group have no peak at the wavelength corresponding to the disulfide bond. Specifically, as shown in Fig. 9a, the lyophilized powder of salamander skin secretion (referred to as "powder") has a corresponding S-S vibration mode at 499 cm^{-1} , and the hydrogel of salamander skin secretion (referred to as "hydrogel") has a corresponding S-S vibration mode at 492 cm^{-1} . But the corresponding S-S vibration mode does not appear in the TCEP group and the vitamin C (referred to as VC) group, indicating that the disulfide bonds in the hydrolysate of the TCEP group and the vitamin C group may be mostly reduced. Based on this, this embodiment further analyzes the disulfide bond (S-S) sub-conformation of different samples.

[0115] The disulfide bond (S-S) will undergo stretching vibration, and the Raman spectrum around $500\text{-}550\text{ cm}^{-1}$ belongs to the characteristic band of the disulfide bond. The Raman peak here can be mainly divided into three sub-conformations of S-S stretching vibration. Among them, the Raman peak around 508 cm^{-1} corresponds to the S-S stretching vibration of the gauche-gauche-gauche (ggg) conformation, the Raman peak around 525 cm^{-1} corresponds to the S-S stretching vibration of the gauche-gauche-trans (ggt) conformation, and the Raman peak around 545 cm^{-1} corresponds to the S-S stretching vibration of the trans-gauche-trans (tgt) conformation. The tgt and ggg conformations belong to the intermolecular and intramolecular disulfide bond configurations, respectively. The results are shown in Table 3. Compared with the powder alone, the three S-S sub-conformations of the hydrogel are significantly changed. Compared with the hydrogel, the vitamin C group has a small change, only a slight change in the tgt sub-conformation. The TCEP group has a large change, and there are large changes in the ggt and tgt. It can be seen that compared with vitamin C treatment, TCEP treatment may have a greater impact on the change of protein spatial structure. Further analysis shows that compared with powder, the content of ggg and tgt conformation in the vitamin C group decreases. The content of ggg conformation decreases and the content of tgt conformation increases in the TCEP group, indicating that vitamin C treatment damages some of the intermolecular disulfide bonds, thereby leading to a decrease in the intermolecular force, and ultimately leading to a decrease in the stability of the protein structure after vitamin C treatment. This also shows that TCEP treatment

increases the content of intermolecular disulfide bonds, which may lead to an increase in the intermolecular force, and ultimately lead to an increase in the stability of the protein structure after TCEP treatment.

[0116] Table 3 S-S sub-conformations (Raman spectrum) of different samples

	ggg	ggt	tgt
Hydrogel	45.47%	48.62%	5.91%
Powder	55.84%	31.04%	13.12%
Vitamin C	44.20%	47.13%	8.67%
TCEP	47.29%	34.00%	18.71%

[0117] The Raman spectrum also shows that the TCEP group has an amide I band vibration at 1667 cm^{-1} , and the C-C stretching vibration at 1453 cm^{-1} . In the vitamin C group, the absorption peak of the amide I band appears at 1659 cm^{-1} , and the C-C stretching vibration appears at 1445 cm^{-1} . The powder has an absorption peak of the amide I band at 1670 cm^{-1} , C-C stretching vibration at 1448 cm^{-1} , C-C stretching vibration at 1003 cm^{-1} , and S-S vibration mode at 499 cm^{-1} . The hydrogel has an amide I band vibration at 1674 cm^{-1} , amide II band vibration at 1585 cm^{-1} , C-C stretching vibration at 1454 cm^{-1} , amide III band vibration at 1307 cm^{-1} , C-C stretching vibration at 1007 cm^{-1} , and S-S stretching vibration at 492 cm^{-1} (as shown in Fig. 9a and Table 4).

[0118] Table 4 Amide I band, amide II band, amide III band and C-C conformation (Raman spectrum) of different samples

	Amide I band	Amide II band	Amide III band	C-C
Hydrogel	1674 cm^{-1}	1585 cm^{-1}	1307 cm^{-1}	$1007, 1454\text{ cm}^{-1}$
Powder	1670 cm^{-1}	-	-	$1003, 1448\text{ cm}^{-1}$
Vitamin C	1659 cm^{-1}	-	-	1445 cm^{-1}
TCEP	1667 cm^{-1}	-	-	1453 cm^{-1}

[0119] Fig. 9b and Table 5 show the infrared spectroscopy analysis of different samples. Disulfide bonds play an important role in stabilizing the tertiary structure of proteins. During the preparation of the hydrolysate, the disulfide bonds are destroyed and the protein needs to form a new tertiary structure, so that all the atoms in the peptide chain can be rearranged in space. The appearance of the aromatic C-H structure in the infrared spectrum shows that the protein molecules in the

hydrolysate have aromatic amino acids such as tyrosine and tryptophan inside, which further indicates that the preparation process of the hydrolysate will change the conformation of the protein, thereby making the exposure of internal structure occurs. Compared with the powder, the amide II band of the TCEP group gradually weakens, and the amide II band even disappears in the hydrogel, indicating that the sample generation process of the TCEP group and the hydrogel has a certain influence on the protein content and structure. However, C-H vibration peaks appear at 931 and 3080 cm^{-1} in the vitamin C group, indicating that the protein structure and composition changes significantly, and a large number of aromatic proteins (tyrosine, tryptophan, etc.) appear. The increase in the relative content of aromatic proteins may change the hydrophobicity and structural stability of the hydrolysate treated with vitamin C, resulting in an increase in hydrophobicity and a decrease in structural stability of the hydrolysate treated with vitamin C. This indicates that vitamin C treatment will not only reduce the intermolecular and intramolecular disulfide bonds, but also reduce oxidized groups such as aldehyde groups. The obvious increase of methyl and methylene intensity indicates that the content of aliphatic hydrocarbons in the TCEP group and hydrogel is higher, which may be mainly derived from the structure of peptidoglycan.

[0120] Table 5 Infrared spectroscopy analysis of different samples

	Aromatic C-H	Amide II band	Amide III band	Methyl and methylene
Hydrogel	877 cm^{-1}	-	-	2924 cm^{-1}
Powder	-	1544 cm^{-1}	-	2927 cm^{-1}
Vitamin C	931, 3080 cm^{-1}	1533 cm^{-1}	1235 cm^{-1}	2967, 2927, 2870 cm^{-1}
TCEP	868 cm^{-1}	1544 cm^{-1}	-	2924 cm^{-1}

[0121] In addition, as shown by semi-quantitative analysis of infrared spectroscopy (Table 6), compared with samples from other groups, the secondary structure of the vitamin C group changes significantly, the relative content of β -turn and Random decreases, and β -turn and Random transform into a β -sheet structure.

[0122] Table 6 Semi-quantitative analysis of infrared spectroscopy of different samples

	α -helix	β -sheet	β -turn	Random
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Powder	14.78%	27.92%	24.36%	32.94%
TCEP	15.23%	26.51%	25.32%	32.94%
Hydrogel	16.81%	26.09%	23.23%	33.87%
Vitamin C	14.24%	32.02%	22.57%	31.17%

[0123] It is reported that the tertiary structure models of regular proteins can be divided into four categories: (1) all- α type: dominated by α -helix structure, the content of α -helix is greater than 40%, while the content of β -sheet is less than 5%; (2) all- β type: dominated by β -sheet structure, the content of β -sheet is greater than 40%, while the content of α -helix is less than 5%; (3) α + β type: the content of both α -helix and β -sheet is greater than 15%. These two structures are separated in space, and more than 60% of the folded chains are antiparallel; (4) α / β type: the content of both α -helix and β -sheet is greater than 15%. These two structures are alternate in space, and more than 60% of the folded chains are aligned in parallel. As shown by the circular dichroism spectrum analysis (Table 7), the protein sample in the powder leach liquor group is an α + β type protein with a relatively stable structure. The protein conformation of the vitamin C group undergoes significant changes, with its secondary structural features basically disappear, mainly presenting a Random (random coil) conformation with a relative content of more than 90%. This further proves that that vitamin C causes significant denaturation of the protein. The secondary structure of proteins in the powder (acetic acid) group (SSAD lyophilized powder treated with 0.75 mol/L acetic acid + 500 mg SSAD lyophilized powder + 5 mL ddH₂O) and TCEP group transforms mainly to β -sheet and Random. This may be due to the combination of acetic acid or TCEP with the amino and side chain groups of amino acids in the α -helix structure of the proteins, thereby causing the unfolding of protein molecules to change their spatial conformation.

[0124] Table 7 Circular dichroism analysis of different samples

	α -helix	β -sheet	β -turn	Random
Powder (acetic acid)	20.7%	24.1%	6.9%	48.3%
TCEP	20.7%	24.1%	19.0%	36.2%
Powder leach liquor	31.7%	21.6%	26.4%	20.3%
vitamin C	4%	4%	2%	90%

[0125] In addition, compared with SSAD lyophilized powder, SSAD hydrolysate has a lighter odor. This may be due to the fact that TCEP weakens or decomposes dimethyl disulfide (which may be the source of odor and low toxicity of SSAD powder) during the hydrolysis of SSAD powder, which further reduces the odor and toxicity of SSAD hydrolysate.

[0126] In summary, the present invention uses a specific disulfide bond reducing agent (such as TCEP-HCl), which can reduce the intermolecular disulfide bonds in the lyophilized powder of salamander skin secretion, while keeping intramolecular disulfide bonds and oxidized groups from being reduced (even a slight excess of this specific disulfide reducing agent will not significantly affect intramolecular disulfide bonds). Furthermore, during the hydrolysis process of this specific disulfide bond reducing agent, the protein structure of the original lyophilized powder of salamander skin secretion is rearranged, so that the content of the intramolecular disulfide bonds of the obtained salamander skin secretion hydrolysate increases, leading to the improvement of the stability of the salamander skin secretion hydrolysate. In addition, this specific disulfide bond reducing agent can also reduce the toxic and odorous substances (for example, dimethyl disulfide) in the lyophilized powder of the salamander skin secretion to reduce the odor and toxicity of the salamander skin secretion hydrolysate. Therefore, the present invention uses a specific disulfide bond reducing agent to hydrolyze the lyophilized powder of salamander skin secretion to prepare a water-soluble, injectable hydrolysate that maintains adhesion and improves biological safety (compared with the hydrogel formed by the lyophilized powder of salamander skin secretion meeting water).

[0127] Using the three-dimensional gel formed by rat tail collagen capable of simulating extracellular matrix as an in vitro tissue model, the present invention evaluates the tissue permeability of SSAD hydrolysate. Add 100 μ L of rat tail collagen solution dropwise to a clean glass slide and cover the slide with a cover glass, then place it on a shaker at 37° C, and wait for it to solidify (about 3 min). Fluorescein-labeled hydrolysate is added dropwise around the glass slide to allow it to permeate naturally (the whole process is observed under an immunofluorescence microscope (magnification 4 $\times$) and photographed and recorded). Fig. 3h shows the penetration of SSAD hydrolysate into rat tail collagen at 0 min, 3 min, 5 min and 7 min, in which the upper layer is rat tail collagen and the lower layer is fluorescein-

labeled hydrolysate. The results show that the gradual penetration of SSAD hydrolysate into the rat tail collagen is observed, indicating that the appropriate concentration of SSAD hydrolysate has good permeability as a bioadhesive and can enter the extracellular matrix-like environment over time.

[0128] An incision with a length of 1.5 cm is made on the dorsal skin of SD rats. As shown in the macroscopic image of Fig. 6c, the adhesive performance of the photosensitive hydrogel group (see below for the preparation method of the photosensitive hydrogel) is better than that of the blank group, cyanoacrylate (CA) group, and fibrin glue (FB) group, exhibiting its potential as an adhesive. Among these 6 groups, SSAD hydrolysate has better healing speed and healing effect, and could even promote scarless wound healing. The incision bonded with SSAD hydrolysate is sliced, and placed under a scanning electron microscope (SEM) to observe the adhesion interface of the incision. As shown in the scanning electron microscope image of Fig. 3i, after the SSAD hydrolysate is bonded to the skin tissue, the gap in the incision is closed well, and the incision edge is hardly visible.

[0129] As summarized in Fig. 7, when the salamander skin secretion hydrolysate in solution form is applied to the wound at a low concentration (e.g., 5%-7%), it can cover the wound surface in an injectable, thinner, and uniform form. It adheres more closely to the wound, requiring only slight pressure (e.g., using finger pressure) to achieve stable adhesion to the wound. The entire process is simpler and more controllable.

[0130] Surprisingly, the present invention finds that the actual adhesive effect of the salamander skin secretion hydrolysate is not the higher the concentration, the higher the adhesion, and the better the adhesive effect as generally believed. When it is at a relatively low concentration (for example 5%-7%), the adhesive effect of the salamander skin secretion hydrolysate is better. Before application to the wound, a relatively low concentration (e.g., 5% - 7%) of the salamander skin secretion hydrolysate is in a "saturated" state (i.e., a "solution" state). Before reaching the "saturated" state, the adhesion of salamander skin secretion hydrolysate will increase with the increase of concentration. After reaching the "saturated" state, the adhesion of salamander skin secretion hydrolysate will decrease with the increase of concentration. When it is applied to a wound, under slight pressure, it will adhere to the wound and can penetrate deeper into the interior of the biological tissue along

with the solvent (such as water in the hydrolysate). As the solvent enters the tissue, the local concentration of salamander skin secretion hydrolysate increases (for example, to a concentration $> 7\%$) and reaches a "supersaturated" state (i.e., a "microgel" state). It is able to interact with the proteins in the biological tissue to form a cross-linked network between tissues. Eventually, the microgel can form a gel inside the tissue. In other words, after being applied to the wound and subjected to a certain pressure, the solvent (for example, water) in the salamander skin secretion hydrolysate provided by the present invention can penetrate deeper into the tissue, so that the local concentration of salamander skin secretion hydrolysate increases and forms a microgel structure with a cross-linked network between tissues. Microgel particles with a smaller particle size can self-assemble at the wound tissue, so the formed cross-linked network (i.e. microgel structure) is denser and more adaptable to the wound environment. Therefore, the microgel structure can adhere to the tissues at the wound more tightly, strengthen the connection between the tissues at the wound, improve the continuity and integrity of the tissue at the wound, and finally promote the healing of the wound. The salamander skin secretion hydrolysate provided by the invention can change in concentration and state at the wound, which provides a suitable healing environment for the wound. Furthermore, compared to the amount required when using the lyophilized powder of salamander skin secretion, less amount is required when using the salamander skin secretion hydrolysate provided by the present invention to cover the same area of the wound. It also results in lower inflammation and shorter wound recovery time, demonstrating superior wound healing properties.

[0131] 1.3 Wound-healing properties of SSAD hydrolysate

[0132] Previous studies have shown that SSAD powder can promote scarless wound healing, and the healing effect is the best among the groups tested (fibrin glue group, hemostatic control group, cyanoacrylate group, and suture group). No signs of infection or inflammation are seen, hair regrowth is observed without scarring, and SSAD promotes overall wound healing. Fig. 4a shows the comparison of in vivo effect of SSAD hydrolysate (which can be understood as a bioadhesive) with other four wound closure methods (negative control group (blank), suture, cyanoacrylate adhesive (commercial glue), fibrin glue (biological glue)). Images of 0, 1, 3, and 5 days after incision (incision length is 2cm) in the rat back show that SSAD

hydrolysate maintains the ability to promote cell proliferation, migration, wound regeneration, and angiogenesis, which is similar to the ability to promote wound healing of the above-mentioned SSAD powder. HE staining images of damaged tissue (epidermal (E), dermis (D), scab (SC), incision site (★)) in Fig. 4b show that the remaining crack width of the SSAD hydrolysate group is significantly narrower than that of the blank group, fibrin glue group and cyanoacrylate group. The number of proliferating epithelial cells in the SSAD hydrolysate group is significantly higher than the other groups, and the granulation tissue of the SSAD hydrolysate group is less.

[0133] Fig. 4c is the macroscopic image of incision in rat back skin (incision length is 2cm) model showing that on the 5th day after operation, in the vitamin C group and TCEP group, the wounds have healed and scab, and the healing speed and effect are better than cyanoacrylate glue (CA) group, fibrin glue (FB) group and suture group and even better than blank control group. The histological sections further confirm that on the 5th day after operation, the healing effects of the TCEP group and the vitamin C group are better, and the inflammation of the incision edge in the TCEP group is the lightest (Fig. 4d, E represents the epidermis, D represents the dermis, "CA" in the lower figure in CA group represents that CA cannot be degraded).

[0134] This embodiment further compares the characteristics of SSAD hydrolysate and SSAD lyophilized powder, as shown in Table 2.

[0135] Table 2 Comparison of SSAD hydrolysate and SSAD lyophilized powder

	SSAD lyophilized powder	SSAD hydrolysate
Physical state	Powder	Solution
Changes upon contact with water	Become a hydrogel	Diluted
Preparation process	After giant salamander skin mucus is cleaned, freeze-dried, and ground.	After SSAD powder is hydrolyzed and dialyzed.
Odor	Obvious pungent smell	Faint weak protein powder smell
Adhesion (porcine skin)	32 ± 5 kPa	29 ± 3 kPa
Injectability	No	Yes
Biocompatibility	High-concentration leach liquor (1%) has weak cytotoxicity.	No significant cytotoxicity (At 5% concentration, cells can still grow well).

Promotion of cell migration, proliferation, regeneration, wound healing	Low-concentration leach liquor (0.01-0.05%) promotes cell proliferation, migration, angiogenesis.	Maintains promotion of cell proliferation, migration, wound regeneration, angiogenesis, and exhibits dose-dependent effects within the range of 0.01%-5% (or 0.01%-7%), with higher concentrations yielding better effects on cell proliferation, migration, and angiogenesis.
Degradability	Completely degradable in vivo.	Completely degradable in vivo.
Wet adhesion	Yes (It forms a gel upon contact with water. It can be used for exudative or bleeding wounds. It can be used for dry wounds in combination with solution.)	No (It has already been fully hydrated. It can be applied to dry wounds or wounds treated with basic methods such as gauze.)

[0136] In conclusion, the salamander skin secretion hydrolysate not only has excellent adhesion properties, good biocompatibility, biodegradability, and promotes tissue regeneration, but also has injectable properties, low inflammatory response, and deeper penetration depth in tissues, etc. which can be applied to the wound site in an easy way.

[0137] Embodiment two: salamander skin secretion hydrolysate as bioadhesive and liquid dressing

[0138] The above experiments show that the salamander skin secretion hydrolysate can be used as a bioadhesive (Fig. 6b). After the salamander skin secretion hydrolysate is used to adhere to the skin tissue, the gap in the incision is closed better, the incision edge is almost invisible, the inflammatory response is lower, and the recovery time of the wound is shorter. In addition, the rat skin full-thickness defect model (a circle with a diameter of 1.5 cm) shown in Fig. 6a shows that the SSAD hydrolysate can also be used as a liquid dressing to promote wound healing and regeneration.

[0139] Embodiment three: salamander skin secretion hydrolysate as photosensitive hydrogel

[0140] Concentration method can be used to further concentrate the salamander skin secretion hydrolysate. SEM images and adhesion of unconcentrated and concentrated hydrolysates after visible light curing are shown in Fig. 8a. The concentrated hydrolysate (concentration 1 time means that the volume of the

concentrated hydrolysate is reduced to half of that before concentration) has a denser pore size and higher adhesion than the unconcentrated hydrolysate. A suitable concentrating method comprises centrifuging the salamander skin secretion hydrolysate loaded into a centrifuge tube with a molecular weight cut-off $\leq 3K$ or evaporating and concentrating the salamander skin secretion hydrolysate.

[0141] An exemplary preparation method of the photosensitive hydrogel comprises: 500 μL of the concentrated SSAD hydrolysate (concentration is about 15%) and photoinitiator Ru/SPS (ruthenium (II) compound and sodium persulfate) are mixed in the ratio of 50:1 (that is, adding 10 μL RU+10 μL SPS, the concentration of Ru/SPS is 1/25 mM) in a clean EP tube (the entire process is carried out using aluminum foil to avoid light.). Then, the mixture is added to a 96-well plate (50 μL /well), and repeated for 8 wells. Subsequently, it is cured under 7000w visible light for 30 seconds (the stronger the intensity of the visible light, the shorter the curing time; the intensity of visible light and curing time can be adjusted according to the actual situation). The macroscopic images of the photosensitive hydrogel before and after light exposure (irradiation) are shown in the lower part of Fig. 7. The photosensitive hydrogel before light exposure is in a liquid state, while the photosensitive hydrogel after light exposure is in a solid state.

[0142] Co-culturing cells and the photosensitive hydrogel. Preparation of medium: add 5mL FBS and 500 μL Penicillin-Streptomycin solution to a 50mL centrifuge tube. Then, bring the volume up to 50 mL with basic DMEM medium to obtain 2 tubes of 50 mL of complete medium (1% Penicillin-Streptomycin + 10% FBS + basic DMEM medium). Add 2.5 mL of FBS and 1.25 mL of Penicillin-Streptomycin solution to a 50 mL centrifuge tube, and bring the volume up to 25 mL with basic DMEM medium to obtain 25 mL of complete medium (5% Penicillin-Streptomycin + 10% FBS + basic DMEM medium) in 1 tube. Digest adherent cells. Take the 10 cm culture dish out of the incubator when the cells reach 70%-80% confluency. Transfer it to a biosafety cabinet, discard the original medium, rinse once with PBS, aspirate, and add 1 ml of trypsin for digestion for 2-3 minutes. When the cells round up, add 3 ml of complete medium to stop the digestion. Pipette up and down to disperse the cells, collect the cell suspension into a 15 ml centrifuge tube, and centrifuge at 1000 rpm for 3 minutes. Discard the supernatant and resuspend the cells in 3 ml of complete medium (5% Penicillin-Streptomycin + 10% FBS + basic DMEM medium) for re-

suspension. Cell counting: transfer 200 μL of the cell suspension to an EP tube, add 600 μL of culture medium with 5% Penicillin-Streptomycin to dilute the original medium 4 times. Take 10 μL of the cell suspension and count the cells using a hemocytometer. Add the cell suspension (6000 cells/well) at 3 μL /well into the 96-well plate containing the photosensitive hydrogel, then add 100 μL of culture medium with 5% Penicillin-Streptomycin. Transfer the 96-well plate to the incubator and culture at 37°C with 5% CO_2 .

[0143] Next, observe whether there is cell growth inside the photosensitive hydrogel after 24 and 48 hours of co-culture using the live/dead staining method. Observation under a fluorescence microscope (Fig. 5a) shows L929 cells adhering to the hydrogel, indicating the good cytocompatibility in vitro of the photosensitive hydrogel. An exemplary experimental method includes: first, prepare the live/dead staining working solution. Thaw the live/dead staining storage solution in the dark in advance. Take a clean EP tube, cover it with aluminum foil to avoid light, add 800 μL of PBS, 1.6 μL of Ca, and 2.4 μL of PI, and mix well for further use. Then, the 96-well plate is taken out of the incubator and centrifuged. The supernatant is discarded. The cell pellet is rinsed with PBS once. After removing the PBS completely, add 100 μL of the staining working solution per well, and incubate in the dark for 15 minutes. Finally, observe and photograph under a fluorescence microscope.

[0144] As shown in the setup in Fig. 8b, after the edge-to-edge adhesion of the porcine skin, a shear force is applied at a rate of 0.1 N/s using a universal mechanical tester to measure the ultimate adhesion strength (ultimate shear stress) of CA (cyanoacrylate), concentrated hydrolysate, unconcentrated hydrolysate and photosensitive hydrogel. The results show that the ultimate adhesion strength of the concentrated hydrolysate and the photosensitive hydrogel is not as good as that of the unconcentrated hydrolysate.

[0145] Fig. 8c shows the staining results of the sample sections after 7 days and 14 days after the subcutaneous implantation of 100 mg of photosensitive hydrogel at the back incision of SD rats. Both HE staining and Masson staining indicate that the photosensitive hydrogel can be degraded in vivo (it can be completely degraded at 21 days after implantation, experimental data not shown) and has good biocompatibility.

[0146] Fig. 8d shows the surgical procedure of the rat liver hemorrhage model. First, an 8-mm defect is created on the liver, and then the liver is excised. Then, gauze is used to stop bleeding from the liver. Finally, the photosensitive hydrogel is injected and exposed to visible light. As shown in Fig. 5b, the prepared SSAD photosensitive hydrogel can be used as a photosensitive hemostatic agent. Compared with the control group and the fibrin glue (FB) group, the photosensitive hydrogel group significantly reduces the blood loss and bleeding time. The SEM images of Fig. 8e (350× magnification on the left and 1000× on the right) show that the photosensitive hydrogel can bind very tightly to tissues that require hemostasis (e.g., the liver). Fig. 8f further shows the hemostatic effect of each experimental group (blank group, hydrolysate group, fibrin glue (FB) group, and photosensitive hydrogel group) on the liver at 0 min, 2 min, 4 min, and 6 min. The results show that the photosensitive hydrogel can stop the massive hemorrhage of the liver within 1 minute, while the other groups can't control the bleeding within 6 minutes.

[0147] The rat skin full-thickness defect model (a circle with a diameter of 1.5 cm) shown in Fig. 5c shows that the SSAD photosensitive hydrogel is also effective in promoting wound healing (as a liquid dressing), and can promote wound healing and regeneration.

[0148] In addition, the SSAD hydrolysate added with a photoinitiator (such as Ru/SPS) can also be used alone or in combination with other methacrylated bioink materials (such as GelMA, HAMA, FibMA, CSMA, Col1MA, HepMA, AlgMA, DexMA, ChsMA, CHIMA, PCLMA, ElaMA, etc.) for 3D printing bioink. This also shows that the salamander skin secretion hydrolysate is an excellent novel biomaterial with stronger and adjustable adhesion, higher biocompatibility and excellent ability to promote regeneration and proliferation, which can be applied to many fields.

[0149] The embodiments of the present invention are described above with reference to the accompanying drawings, but the present invention is not limited to the aforementioned specific embodiments. The aforementioned embodiments are merely illustrative and not limiting. For those of ordinary skill in the art, many forms can be made under the teaching of present invention without departing from the spirit of the present invention and the scope of the claims, all of which shall fall within the protection scope of the present invention.

CLAIMS

Claim 1. A preparation method of a salamander skin secretion hydrolysate, comprising the following steps:

S1 mixing a disulfide bond reducing agent and a first solvent to obtain a second solvent;

S2 mixing a lyophilized powder of salamander skin secretion and the second solvent to obtain a first mixed solution;

S3 fully hydrolyzing the first mixed solution to obtain a second mixed solution;

S4 centrifuging the second mixed solution and collecting a supernatant;

S5 transferring the supernatant into a dialysis bag with a molecular weight cut-off ≤ 1000 and placing the dialysis bag in the first solvent for full dialysis to obtain the salamander skin secretion hydrolysate;

wherein the disulfide bond reducing agent comprises a specific disulfide bond reducing agent, the specific disulfide bond reducing agent is Tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl), and the first solvent comprises one or more of ultrapure water, deionized water, water for injection, and pure water.

Claim 2. The method according to claim 1, wherein a mass ratio of the first solvent to the lyophilized powder of salamander skin secretion is at least 4:1.

Claim 3. The method according to claim 1, wherein a mass ratio of the lyophilized powder of salamander skin secretion to the disulfide bond reducing agent is at least 3:1.

Claim 4. The method according to claim 1, wherein hydrolysis time for the hydrolyzing comprises 2 - 168 hours, optionally 12 - 48 hours.

Claim 5. The method according to claim 1, wherein temperature condition for the S3, the S4 and the S5 comprises 0 - 8°C.

Claim 6. The method according to claim 1, wherein the S3 is carried out in a vacuum reactor, and a vacuum degree of the vacuum reactor is < 5 kPa.

Claim 7. The method according to claim 1, wherein the disulfide bond reducing agent further comprises vitamin C.

Claim 8. The method according to claim 1, wherein dialysis time for the dialysis comprises 48 hours.

Claim 9. The method according to claim 1, wherein a concentration of the salamander skin secretion hydrolysate obtained comprises 3% - 7%.

Claim 10. The method according to any one of claims 1-9, wherein the method further comprises S6 concentrating the salamander skin secretion hydrolysate to obtain a concentrated salamander skin secretion hydrolysate.

Claim 11. The method according to claim 10, wherein a method for the concentrating comprises centrifuging the salamander skin secretion hydrolysate loaded into a concentrated centrifuge tube with a molecular weight cut-off $\leq 3K$ or evaporating and concentrating the salamander skin secretion hydrolysate.

Claim 12. A salamander skin secretion hydrolysate, wherein the salamander skin secretion hydrolysate is prepared by the method according to any one of claims 1-11.

Claim 13. Use of a salamander skin secretion hydrolysate in the preparation of a bioadhesive, wherein the salamander skin secretion hydrolysate is prepared by the method according to any one of claims 1-9.

Claim 14. The use according to claim 13, wherein a concentration of the salamander skin secretion hydrolysate comprises 5% - 7%.

Claim 15. The use according to claim 13, wherein after the bioadhesive is applied to a wound, the bioadhesive penetrates into interior of tissue of the wound by itself or through external pressure to form a microgel structure.

Claim 16. The use according to claim 15, wherein the wound comprises a skin wound.

Claim 17. The use according to claim 15, wherein the bioadhesive is used for promoting healing of the wound.

Claim 18. Use of a salamander skin secretion hydrolysate in the preparation of a wound dressing, wherein the salamander skin secretion hydrolysate is prepared by the method according to any one of claims 1-11.

Claim 19. Use of a salamander skin secretion hydrolysate in the preparation of a photosensitive hydrogel, wherein the photosensitive hydrogel comprises a concentrated salamander skin secretion hydrolysate and a photoinitiator; under exposure to visible light, the concentrated salamander skin secretion hydrolysate is cured to form the photosensitive hydrogel; the concentrated salamander skin secretion hydrolysate is prepared by the method according to claim 10, and the photoinitiator comprises Ru/SPS system.

Claim 20. The use according to claim 19, wherein an intensity of the visible light comprises 7000W.

Claim 21. The use according to claim 19, wherein the photosensitive hydrogel is used as a hemostatic agent.

Claim 22. The use according to claim 19, wherein the photosensitive hydrogel is used as a bioadhesive.

Claim 23. The use according to claim 19, wherein the photosensitive hydrogel is used as a wound dressing.