

BIO-INK AND PREPARATION METHOD AND APPLICATION THEREOF

TECHNICAL FIELD

The present invention relates to a bio-ink, and more particularly to a bio-ink for 3D printing and a preparation method and application thereof.

BACKGROUND

At present, the rapid development and application of 3D printing technology (also known as rapid prototyping and additive manufacturing technology) is bringing about a technological innovation in many traditional fields, such as construction engineering, mold processing, industrial design, education and medical care. 3D printing technology mainly generates a 3D model of a printing subject based on computer-aided design (CAD), breaks it into different two-dimensional planes, and then rapidly forms a three-dimensional entity according to the principle of "layer-by-layer printing, layer-by-layer overlay". The printing process is not limited by geometry, while the printing speed is adjustable and different molding methods can be selected according to different printing materials. This customizable feature has led to widespread attention in biomedical applications such as surgical models, drug screening, tissue engineering and regenerative medicine, allowing the emergence of 3D bioprinting technology. However, due to the complexity and sensitivity of biological tissue structures, the use of 3D bioprinting technology to create implantable organs and tissues still faces enormous challenges. The development of biomaterials suitable for 3D printing is the key to this technology.

First of all, 3D bio-printing materials need to meet the requirements of printing technology for physicochemical properties of materials by having good biocompatibility and degradability, suitable mechanical properties, and a degradation product with no obvious toxic and side effects that can be metabolized and discharged. At present, bioprinting materials can be mainly divided into two categories: artificially synthesized biopolymer materials such as degradable polylactic acid (PLA), polycaprolactone (PCL), polyglycolic acid (PGA), polyethylene glycol (PEG), the copolymers thereof, and the like. The such

synthetic polymers have a controllable molecular weight, good processing property, adjustable mechanical properties, and bio-degradable. However, due to their structural difference from the living organisms, the biological affinity thereof is yet to be improved, and the degradation products have some degree of toxicity and side effects. Another type of bioprinting material is mainly natural polymer materials such as collagen, fibrin, silk protein, alginate, chitosan, hyaluronic acid, and the like. Such protein and polysaccharide materials tend to have good bio-affinity, degradability and cell adhesion, with degradation products absorbable and utilizable by organisms. However, due to their relatively fixed composition and structure, the physical and chemical properties are difficult to control and the processability is poor. Moreover, the extraction and separation thereof are cumbersome, which increases the cost of use and limits its large-scale application.

Secondly, current 3D bioprinting technologies mainly use direct-writing 3D printing of fused deposition modelling (FDM) and extrusion curing, as well as inkjet printing based on solution inkjet curing. The fused deposition modelling technique heats the thermoplastic resin to a fluid state and then models the same by extrusion and solidification deposition. It is not suitable for materials such as heat sensitive proteins, while its resolution is too low to meet the accuracy requirements of scaffold for tissue engineering. Direct injection printing adopts a method of solution extrusion curing, which requires a low-viscosity polymer solution, resulting in poor mechanical strength of the molded product which cannot fulfill the strength requirements of the tissue engineering support. Inkjet printing enables high-precision printing in two-dimension directions, but it does not allow for fabrication of a suspending, porous structures in a three-dimensional direction. Therefore, the development of new 3D printing technology and printing equipment is another direction for the development of 3D bio-printing. Near-field electro-hydro dynamic jet (EHD-jet) molding is an emerging 3D printing technology that can be used to make scaffold for tissue engineering. It combines electrospinning technology and 3D printing mechanism, using a high-viscosity polymer solution as the "ink", which is solidified to form fibers under the action of electric field force, printing layer by layer and overlaying layer by layer to form a three-dimensional scaffold for tissue engineering with high precision and high strength. However, bio-inks currently available for this technology are scarce, causing a bottleneck for its development and application.

SUMMARY OF THE INVENTION

In view of the above deficiencies of the prior art and lack of bio-inks, one object of the present invention is to provide a novel bio-ink which forms a composite bio-ink by blending, and has combined advantages of a synthetic polymer and a natural polymer.

In view of this, in a first aspect of the invention, there is provided a bio-ink including, by mass percentage, the following components:

1% to 70% of a synthetic degradable polymer;

1% to 70% of a natural polymer; and

28% to 98% of a solvent;

wherein the solvent is water, an organic solvent or any combination thereof.

In the present invention, any synthetic degradable polymer can be used for this purpose as long as it is biodegradable, including, for example, but not limited to, various types of polyesters and polyamides. In one embodiment of the bio-ink, the synthetic degradable polymer is preferably a polyester polymer, with the advantage of good solubility in organic solvents, high degree of mixing with natural polymers, controllable molecular weight, and the technical effect of diversified shapes of the printed scaffold, therefore being suitable for the use. Polyester polymers suitable for this purpose include, but are not limited to, any one or any combination selected from the group consisting of poly lactic acid (PLA), poly- ϵ -caprolactone (PCL), polyanhydride, polyorthoester, poly-D, L-lactide (PDLLA), polyglycolic acid (PGA), and poly(p-dioxanone). The preferred polyester polymer is polycaprolactone (e.g., an average molecular weight ranging from 10 kDa to 20 kDa, and a melting point of 30 to 100 °C), but not limited thereto. Polycaprolactone is particularly preferred because of a high solubility in an organic solvent and ideal viscosity and electrical conductivity to achieve a desired 3D printing effect.

In some embodiments, the mass percentage of the synthetic degradable polymer is preferably ranged from 10 to 60%, more preferably from 20 to 50%, and still more preferably from 30 to 40%.

In one embodiment of the bio-ink, the natural polymer is a plant-derived protein, i.e., a plant protein. Any plant-derived protein can be used for this purpose as long as it has a certain degree of solubility in an organic solvent, including but not limited to any one or any

combination selected from the group: vegetable protein, cereal protein, legume protein, nut protein, walnut protein, almond protein, lotus seed protein, white kidney bean protein, corn protein, barley protein, wheat protein, soy protein, rice protein, and peanut protein. In a preferred embodiment, the plant protein is corn protein.

Preferably, the plant-derived protein is a plant prolamin, with the advantages for the purpose of use herein being a high solubility in an organic solvent, controllable solution viscosity and conductivity, and strong mechanical properties. The 3D-printed materials obtained from bio-ink using plant prolamin have good mechanical properties, good bio-affinity, good biodegradability, and adjustable technical effects. Plant prolamin suitable for this purpose includes, but is not limited to, any one or any combination selected from the group consisting of legume prolamin, cereal prolamin, vegetable prolamin, *Leymus* prolamin, coconut prolamin, corn protein, barley prolamin, wheat prolamin, soy prolamin, peanut prolamin, pea prolamin, potato prolamin, rice prolamin, and oat prolamin. Preferred plant prolamins are wheat prolamin, corn protein, and the like, which are particularly preferred because of their ideal molecular weight, electrical conductivity, viscosity, bio-affinity, and biodegradability, which allows the formulated ink to be suitable for printing ideal 3D biological scaffolds. Particularly preferred is corn protein (for example, α -zein having a molecular weight of 21 to 26 kDa) and wheat prolamin, but not limited thereto.

In some of the embodiments, the mass percentage of the natural polymer is preferably 2 to 70%, more preferably 5 to 60%, still more preferably 5 to 50%, even more preferably 5 to 40%, still more preferably 5 to 30%, and more preferably 10 to 20%."

Any solvent can be used for the purpose of the present invention as long as it satisfies the conditions of dissolving solutes of the protein and the synthetic polymers. Solvents suitable for this purpose include, but are not limited to, water, methanol, alcohol, isopropanol, n-propanol, n-butanol, isobutanol, ethylene glycol, ethylene glycol monomethyl ether, ethylene glycol dimethyl ether, diethylene glycol, diethylene glycol monomethyl ether, diethylene glycol dimethyl ether, 1,2-propanediol, 1,3-propanediol, glycerol, acetone, butanone, pentanone, methyl formate, ethyl formate, propyl formate, N,N-dimethylformamide, methyl acetate, ethyl acetate, propyl acetate, butyl acetate, N,N-dimethylacetamide, methyl propionate, ethyl propionate, methyl butyrate, ethyl butyrate, diethyl ether, butyl ether, tetrahydrofuran, dioxane, formic acid, acetic acid, propionic acid, butyric acid, dichloromethane, dichloroethane, trichloromethane, tetrachloroethane, benzene,

toluene, xylene, acetonitrile, o-dichlorobenzene, fluorobenzene, and any combination thereof. Acetic acid is preferred, especially acetic acid having a purity of > 99.5%, but is not limited thereto. Acetic acid is particularly preferred because it strongly dissolves proteins and synthetic polymers, allowing the bio-ink formed therefrom to obtain a desired viscosity for printing of a desired 3D biological scaffold.

In some embodiments, the mass percentage of the solvent is preferably from 30 to 80%, more preferably from 40 to 70%, and still more preferably from 50 to 60%.

In a particular embodiment of the bio-ink, the bio-ink further includes from 0.0001% to 5% by mass percentage of a fluorescently labeled molecule.

In some embodiments, the fluorescently labeled molecule is a fluorescently labeled natural polymer. In some embodiments, the fluorescently labeled molecule may be derived from fluorescent modification of part of the natural polymer component of the bio-ink of the present invention, thereby printing a product having fluorescent properties.

In some embodiments, the fluorescently labeled molecule is a fluorescently labeled protein. In some embodiments, the fluorescently labeled protein is a plant-derived protein. A fluorescent molecule can be used for labeling as long as it has a certain degree of fluorescent intensity, wherein the fluorescent luminophore suitable for this purpose includes, but is not limited to, any one or any combination selected from the group consisting of coumarin (blue fluorescence), fluorescein, rhodamine, pyrene, Nile red (red fluorescence), fluoroboron (green fluorescence), IR-780 (near-infrared fluorescence), and derivatives thereof. Particularly preferred is coumarin (maximum emission wavelength of 435 nm), such as 6,7-dimethoxycoumarin, which is particularly preferred because of its strong fluorescence intensity.

In some embodiments, the mass percentage of the fluorescently labeled molecule is preferably from 0.001% to 5%, more preferably from 0.01% to 5%, and still more preferably from 0.1% to 3%, and most preferably from 0.5% to 2%.

The present invention also provides a method for preparing any one of the above bio-inks, including the steps of:

first of all, weighing the natural polymer, or weighing the natural polymer and the fluorescently labeled molecule, according to the mass percentages defined above, and

completely dissolving the same in the solvent to obtain a clear solution, wherein, according to the material chosen, this step may be carried out, for example, at room temperature, and wherein ultrasound or the like may be used to facilitate the dissolution, though the dissolution time should not be too long to prevent the protein from gelation;

next, weighing the synthetic degradable polymer according to the mass percentage defined above, dissolving the same in the clear solution and mixing uniformly to obtain a solution of uniform color, i.e., the bio-ink; wherein, according to the material chosen, a 40 to 60 °C water bath, ultrasound or the like may be used to facilitate the dissolution, with appropriate stirring, for example, stirring once every 10 to 20 minutes to mix well.

In one embodiment, the resulting uniform-color solution (bio-ink) may be further stood at a constant temperature of 20 to 40 °C (for example, in a 30 °C incubator) for a period of time, such as 30 minutes or more, as needed, so as to remove bubbles and further optimize ink quality.

The above preparation method utilizes the unique dissolution characteristics of plant prolamin and the synthetic polyester polymer, and uses the methods of heating, ultrasonication, and stirring to complete blending and modification of the components in the solution to form a uniform and stable 3D printing bio-ink.

The present invention also provides a scaffold, such as a scaffold for tissue engineering, made using any of the above-described bio-inks.

In some embodiments, the scaffold is printed using any of the above-described bio-inks via 3D printing techniques.

The invention also provides the use of the above bioscaffold for *in vitro* cell growth.

The invention also provides the use of any of the above bio-inks for 3D printing.

In some embodiments, the 3D printing with any of the above-described bio-inks is performed using a 3D printing device based on EHD-jet.

In some embodiments using an EHD-jet 3D printing device, a three-dimensional scaffold for *in vitro* cell culturing with variable pore size, diverse pattern forms, good mechanical strength, and cell affinity can be formed by adjusting the printing parameters during printing.

The present invention also provides a method of performing 3D printing using an EHD-jet 3D printing apparatus with any of the above-described bio-inks. The 3D printing device/system based on the electro-hydro dynamic injection (EHD-jet) technology mainly includes a syringe pump, a connecting hose, a nozzle, a high voltage DC power source, a printing plate, a three-dimensional motion platform, software and a control system. When the bio-ink of the present invention is used, it is possible to control the diameter of the fiber after curing and the rate and stability of the printing process by adjusting the printing parameters. The aperture size and void ratio of the molded scaffold can be controlled by a preset print path and a stacked manner. For example, the print parameters can be set to: ink supply rate: 0.1 to 100 $\mu\text{l}/\text{min}$; DC voltage: 1 to 3 kV; distance from nozzle to base plate: 1 to 10 mm; platform moving rate: 1 to 300 mm/s; temperature: 20 to 90 $^{\circ}\text{C}$. The ink supply rate is preferably from 0.1 to 50 $\mu\text{l}/\text{min}$, or more preferably from 0.2 to 10 $\mu\text{l}/\text{min}$, or most preferably from 0.5 to 5 $\mu\text{l}/\text{min}$. The platform movement rate is preferably 5 to 300 mm/s, and more preferably 10 to 300 mm/s. The temperature is preferably from 10 to 60 $^{\circ}\text{C}$, more preferably from 15 to 50 $^{\circ}\text{C}$, and most preferably from 20 to 40 $^{\circ}\text{C}$.

The first aspect of the present invention has the beneficial effects of providing a composite bio-ink formed by blending with a non-toxic solvent as a blending carrier and by allowing a synthetic polymer and a natural polymer to blend in a solution at a molecular level. The resultant bio-ink combines the advantages of both the synthetic polymer and the natural polymer. The bio-ink formed by blending is uniform and stable, which can form a 3D scaffold for tissue engineering with controllable pore size by EHD-jet 3D printing technology. The scaffold is degradable with a degradation rate alterable by adjusting the proportion of each component in the bio-ink. Therefore, the present invention overcomes the deficiencies of the prior 3D printing ink solution, such as simple composition, uncontrollable biodegradability, and difficulties in taking into account cell compatibility, degradability and mechanical properties. The bio-ink is provided with good bio-affinity and capable of rapid curing, whereby the 3D-printed scaffold with such ink has sufficient mechanical strength, controllable cell affinity and degradability.

In some embodiments, the bio-ink of the present invention may further include a fluorescently labeled molecule, so that the three-dimensional scaffold formed by 3D printing using the bio-ink has a fluorescent property. The variation in fluorescence intensity during degradation can be used to quantitatively describe the degree of degradation. This can thus

be used to monitor the degree of degradation of the scaffold while performing cell culture, providing a novel method for accurate assessment of material degradability.

In a second aspect of the invention, there is provided a method for preparation of a fluorescently labeled protein, including the steps of:

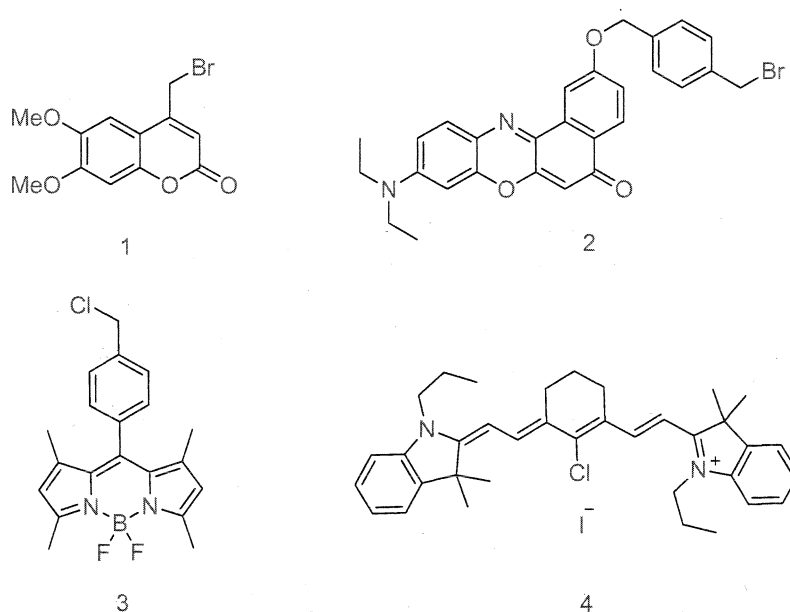
dissolving a derivative of a fluorescent molecule and a protein simultaneously dissolved in an organic solvent;

adding a base thereto and stirring to allow reaction at a temperature of 20 °C to 150 °C for 1 to 24 hours; wherein the temperature is preferably 20 °C to 100 °C, more preferably 20 °C to 90 °C, and most preferably 20 to 80 °C;

pouring the reaction solution, after cooling, into a saturated salt solution (for example, at least 20 times by volume) to precipitate the protein, filtrating, and washing to obtain the fluorescently labeled protein and the fluorescently labeled protein is obtained by filtration and washing; wherein, in some of the embodiments, the saturated salt solution is a saturated aqueous solution of ammonium sulfate.

In some of the embodiments, the above method of the present invention further includes a purifying step and a drying step: re-dissolving the obtained protein in an aqueous ethanol solution, filtering, removing the solvent by rotary evaporation, and vacuum-drying. In one embodiment, the aqueous ethanol solution is a 70% aqueous ethanol solution.

In some embodiments, the derivative of a fluorescent molecule used in the above method is a halogenated derivative of a fluorescent molecule, such as a brominated, chlorinated or iodinated derivative. The fluorescent molecule can be used for labeling as long as it has a certain degree of fluorescent intensity. Fluorescent luminophores suitable for this purpose include, but not limited to, a brominated or chlorinated derivative of the fluorescent molecule that is any one or any combination selected from the group consisting of 4-bromomethyl-6, 7-dimethoxycoumarin (1), benzyl bromide-substituted Nile Red (2), benzyl chloride-substituted fluoroboron (3), and IR-780 iodide (4) (the structural formula is as follows). Preferred is 4-bromomethyl-6,7-dimethoxycoumarin, which is particularly preferred because it readily reacts with a protein to obtain a protein derivative carrying a fluorescent chromophore.



In some embodiments, the derivative of a fluorescent molecule is added in a molar amount of 0.0001 to 10 times the average molar amount of the protein.

In some embodiments, the protein used in the above method is a plant-derived protein, i.e., a vegetable protein. Any plant-derived protein can be used for this purpose as long as it has a certain degree of solubility in an organic solvent, including but not limited to any one or any combination selected from the group: vegetable protein, cereal protein, legume protein, nut protein, walnut protein, almond protein, lotus seed protein, white kidney bean protein, corn protein, barley protein, wheat protein, soy protein, rice protein, and peanut protein. In a preferred embodiment, the plant protein is corn protein. Plant prolamin suitable for this purpose includes, but is not limited to, any one or any combination selected from the group consisting of legume prolamin, cereal prolamin, vegetable prolamin, Leymus prolamin, coconut prolamin, corn protein, barley prolamin, wheat prolamin, soy prolamin, peanut prolamin, pea prolamin, potato prolamin, rice prolamin, and oat prolamin. Preferred plant prolamins are wheat prolamin, corn protein, and the like.

In some embodiments, the fluorescently labeled protein prepared by the above method is used in a bio-ink for 3D printing. The advantage of plant protein for this purpose includes high solubility, controllable solution viscosity and conductivity in organic solvents, and strong mechanical properties. Preferably, the vegetable protein is a plant prolamin. The 3D-printed materials obtained from bio-ink using plant prolamin have good mechanical properties, good bio-affinity, good biodegradability, and adjustable technical effects. The

plant prolamin suitable for this purpose is particularly preferred because of their ideal molecular weight, electrical conductivity, viscosity, bio-affinity, and biodegradability, which allows the formulated ink to be suitable for printing ideal 3D biological scaffolds. Particularly preferred is corn protein (for example, α -zein having a molecular weight of 21 to 26 kDa) and wheat prolamin, but not limited thereto.

Any solvent can be used for the purpose of this aspect of the present invention as long as it satisfies the conditions of dissolving the above solutes of the protein and the synthetic polymers. Solvents suitable for this purpose include, but are not limited to, methanol, alcohol, isopropanol, n-propanol, n-butanol, isobutanol, ethylene glycol, ethylene glycol monomethyl ether, ethylene glycol dimethyl ether, diethylene glycol, diethylene glycol monomethyl ether, diethylene glycol dimethyl ether, 1,2-propanediol, 1,3-propanediol, glycerol, acetone, butanone, pentanone, methyl formate, ethyl formate, propyl formate, N,N-dimethylformamide, methyl acetate, ethyl acetate, propyl acetate, butyl acetate, N,N-dimethylacetamide, methyl propionate, ethyl propionate, methyl butyrate, ethyl butyrate, diethyl ether, butyl ether, tetrahydrofuran, dioxane, formic acid, acetic acid, propionic acid, butyric acid, dichloromethane, dichloroethane, trichloromethane, tetrachloroethane, benzene, toluene, xylene, acetonitrile, o-dichlorobenzene, fluorobenzene, dimethyl sulfoxide (DMSO), and any combination thereof. Acetic acid is preferred, especially acetic acid having a purity of > 99.5%, but is not limited thereto. Acetic acid is particularly preferred because it strongly dissolves polymer.

In some of the embodiments, a base may be used in the above method as long as it can neutralize the acid generated from the reaction. Bases suitable for this purpose include, but are not limited to, any one or any combination selected from the group consisting of sodium carbonate, potassium carbonate, triethylamine, and diisopropylmethylamine, but are not limited thereto. The base is preferably triethylamine, because of its moderate boiling point and easy removal.

In some embodiments, the base is added in a molar amount of 0.1 to 50 times, preferably 1 to 10 times, more preferably 3-5 times the molar amount of the derivative of a fluorescent molecule.

The present invention also provides a fluorescently labeled protein prepared according to the above method.

The present invention also provides the use of a fluorescently labeled protein prepared according to the above method for producing a bio-ink for 3D printing.

The present invention also provides a bio-ink including a fluorescently labeled protein prepared according to the above method, the bio-ink including the following components in mass percent:

1% to 70% of a synthetic degradable polymer;

1% to 70% of a natural polymer;

0.0001% to 5% of a fluorescently labeled molecule; and

28% to 98% of a solvent;

wherein the solvent is water, an organic solvent or any combination thereof, and wherein the fluorescently labeled molecule is a fluorescently labeled protein prepared according to the above method.

In some embodiments, the mass percentage of the synthetic degradable polymer is preferably from 10 to 60%, more preferably from 20 to 50%, and still more preferably from 30 to 40%.

In some of the embodiments, the mass percentage of the natural polymer is preferably from 2 to 70%, more preferably from 5 to 60%, still more preferably from 5 to 50%, still more preferably from 5 to 40%, still more preferably 5 to 30%, more preferably 10-20%.

In some embodiments, the mass percentage of the solvent is preferably from 30 to 80%, more preferably from 40 to 70%, and still more preferably from 50 to 60%.

In some embodiments, the mass percentage of the fluorescently labeled molecule is preferably from 0.001% to 5%, more preferably from 0.01% to 5%, more preferably from 0.1% to 3%, and most preferably from 0.5% to 2%.

In this aspect of the invention, any synthetic degradable polymer can be used for this purpose as long as it is biodegradable, including, for example, but not limited to, various types of polyesters and polyamides. In one embodiment of the bio-ink, the synthetic degradable polymer is preferably a polyester polymer, with the advantage of good solubility in organic solvents, high degree of mixing with natural polymers, controllable molecular weight, and the technical effect of diversified shapes of the printed scaffold, therefore being

suitable for the use. Polyester polymers suitable for this purpose include, but are not limited to, any one or any combination selected from the group consisting of poly lactic acid (PLA), poly- ϵ -caprolactone (PCL), polyanhydride, polyorthoester, poly-D, L-lactide (PDLLA), polyglycolic acid (PGA), and poly(p-dioxanone). The preferred polyester polymer is polycaprolactone (e.g., an average molecular weight ranging from 10 kDa to 20 kDa, and a melting point of 30 to 100 °C), but not limited thereto. Polycaprolactone is particularly preferred because of a high solubility in an organic solvent and ideal viscosity and electrical conductivity to achieve a desired 3D printing effect.

In one embodiment of the bio-ink, the natural polymer described in the above bio-ink is a plant-derived protein, i.e., a vegetable protein. Any plant-derived protein can be used for this purpose as long as it has sufficient solubility in organic solvents, including but not limited to: vegetable protein, cereal protein, legume protein, nut protein, walnut protein, almond protein, lotus seed protein, white kidney bean protein, corn protein, barley protein, wheat protein, soy protein, rice protein, and peanut protein. In a preferred embodiment, the plant protein is corn protein.

Preferably, the plant-derived protein is a plant prolamin, with the advantages for the purpose of use herein being a high solubility in an organic solvent, controllable solution viscosity and conductivity, and strong mechanical properties. The 3D-printed materials obtained from bio-ink using plant prolamin have good mechanical properties, good bio-affinity, good biodegradability, and adjustable technical effects. Plant prolamin suitable for this purpose includes, but is not limited to, any one or any combination selected from the group consisting of legume prolamin, cereal prolamin, vegetable prolamin, *Leymus* prolamin, coconut prolamin, zein, barley prolamin, wheat prolamin, soy prolamin, peanut prolamin, pea prolamin, potato prolamin, rice prolamin, and oat prolamin. Preferred plant prolamins are wheat prolamin, zein, and the like, which are particularly preferred because of their ideal molecular weight, electrical conductivity, viscosity, bio-affinity, and biodegradability, which allows the formulated ink to be suitable for printing ideal 3D biological scaffolds. Particularly preferred is zein (for example, α -zein having a molecular weight of 21 to 26 kDa) and wheat prolamin, but not limited thereto.

Any solvent can be used for the purpose of the present invention as long as it satisfies the conditions of dissolving solutes of the protein and the synthetic polymers. Solvents suitable for this purpose include, but are not limited to, water, methanol, alcohol, isopropanol,

n-propanol, n-butanol, isobutanol, ethylene glycol, ethylene glycol monomethyl ether, ethylene glycol dimethyl ether, diethylene glycol, diethylene glycol monomethyl ether, diethylene glycol dimethyl ether, 1,2-propanediol, 1,3-propanediol, glycerol, acetone, butanone, pentanone, methyl formate, ethyl formate, propyl formate, N,N-dimethylformamide, methyl acetate, ethyl acetate, propyl acetate, butyl acetate, N,N-dimethylacetamide, methyl propionate, ethyl propionate, methyl butyrate, ethyl butyrate, diethyl ether, butyl ether, tetrahydrofuran, dioxane, formic acid, acetic acid, propionic acid, butyric acid, dichloromethane, dichloroethane, trichloromethane, tetrachloroethane, benzene, toluene, xylene, acetonitrile, o-dichlorobenzene, fluorobenzene, and any combination thereof. Acetic acid is preferred, especially acetic acid having a purity of > 99.5%, but is not limited thereto. Acetic acid is particularly preferred because it strongly dissolves proteins and synthetic polymers, allowing the bio-ink formed therefrom to obtain a desired viscosity for printing of a desired 3D biological scaffold.

In the second aspect of the present invention, a method for preparing a fluorescently labeled protein is provided with the following beneficial effect. In the prior art, the labeling site of most fluorescently labeled dyes is an amino group on the lysine side chain or a sulfhydryl group on the cysteine side chain of the protein, making this method difficult for use in labeling proteins almost free of lysine, cysteine or with a very low content thereof, such as plant-derived prolamin. The technique of the second aspect of the invention provides a simple and efficient fluorescently labeled protein, especially a plant-derived prolamin. The labeling site is mainly an amino acid including a free hydroxyl or carboxyl group in the protein, with high labeling efficiency and stable product yield. Therefore, the deficiencies of existing marking methods are overcome or at least partly alleviated.

Further, using the fluorescently labeled protein prepared according to the method of the second aspect of the present invention, a bio-ink having a specific fluorescence can be produced, which can be used to produce (for example, by 3D printing) a scaffold for tissue engineering with fluorescent properties. Variation in fluorescence intensity thereof can be used to assess the extent of scaffold degradation in real time. At present, the evaluation of the degradation properties of degradable materials mainly employs a gravimetric method and a SEM observation method, wherein acid, alkali and enzyme are used to catalyze the degradation reaction to accelerate the degradation rate, after which the material degradability is assessed by measuring the weight change of the material or observe the variation in

surface morphology thereof. These methods cannot truly reflect the actual degradation of the material *in vivo*, while the operation steps are cumbersome and the accuracy is poor. The bio-ink having the fluorescent property of the invention and the scaffold for tissue engineering made thereof utilize the sensitivity of fluorescence to characterize the material degradation by detecting the change in fluorescence intensity of the material during the degradation process. It is possible to monitor the degradation simultaneously as the three-dimensionality cell cultured on the molded scaffold, which provides a new solution and method for evaluation of the degradation properties of degradable materials.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig.1 shows a three-dimensional schematic view of a nozzle moving path (single layer) and a post-cure scaffold in a bio-ink 3D printing process according to Examples 1-3 and 5-6 of the present invention.

Fig.2 shows an optical micrograph of a scaffold formed by 3D printing of a bio-ink prepared in an embodiment of the present invention, wherein Fig. 2a, 2b, and 2c respectively show the scaffolds printed in Examples 1, 2, and 3 (a PCL scaffold, a PCL-Zein (6-1) scaffold, and a PCL-Zein (5-2) scaffold, respectively).

Fig.3 shows a scanning electron microscope (SEM) photograph of a front side, a back side, and a cross section of a scaffold formed by 3D printing of the bio-ink according to Example 2 of the present invention.

Fig.4 shows 4a) an optical microscope image of a scaffold formed by 3D printing of the bio-ink in Example 4 of the present invention and 4b) a photograph taken after the scaffold was peeled off from the printing place after being placed in the air for 3 hours.

Fig.5 shows a photograph under normal light (left) of a bio-ink prepared in Example 6 of the present invention and a fluorescent photograph thereof (right; ultraviolet excitation, wavelength 365 nm).

Fig.6 shows a laser confocal micrograph of a scaffold formed by 3D printing of a bio-ink prepared in Example 6 of the present invention (left: top view; right: three-dimensional side view).

Fig.7 shows a confocal micrograph of the scaffolds made by the 3D printing of the

bio-ink from Examples 1, 2, and 3 of the present invention on the first and fourth days of the cell culture experiment of Example 8, the PCL, PCL-Zein (6-1) and PCL-Zein (5-2) show the scaffolds obtained by printing with the bio-inks from Examples 1, 2, and 3, respectively.

Fig.8 shows a statistical diagram illustrating the number of cell adhesions on the scaffolds made by the 3D printing of the bio-ink from Examples 1, 2, and 3 of the present invention on the first and fourth days in the cell culture experiment of Example 8, the PCL, PCL-Zein (6-1) and PCL-Zein (5-2) as shown show the scaffolds obtained by printing with the bio-inks from Examples 1, 2, and 3, respectively.

Fig.9 shows a graph showing the weight loss against time in the *in vitro* enzyme-accelerated degradation experiment of the scaffolds made by the 3D printing of the bio-ink from Examples 1, 2, and 3 of the present invention, the PCL, PCL- Zein (6-1) and PCL-Zein (5-2) as shown show the scaffolds obtained by printing with the bio-inks from Examples 1, 2, and 3, respectively.

Fig.10 shows a scanning electron microscope (SEM) photograph of the fiber surface before degradation and after protease degradation for 12 day of the scaffolds in the scaffold degradation experiment of Example 9 performed on the scaffolds made by the 3D printing of the bio-ink from Examples 1, 2, and 3 of the present invention, the PCL, PCL- Zein (6-1) and PCL-Zein (5-2) as shown show the scaffolds obtained by printing with the bio-inks from Examples 1, 2, and 3, respectively.

DETAILED DESCRIPTION OF EMBODIMENTS

Specific embodiments of the present invention will be described in detail below with reference to the accompanying drawings. The embodiments are implemented based on the concept of the technical solution of the present invention, while the detailed embodiments and the operation procedures are given. However, the scope of the present invention is not limited to the following embodiments, and the specific scope of protection is defined only by the claims.

Example 1

This embodiment is an example for preparation of a bio-ink as a control group (hereinafter referred to as 70% PCL control material).

The bio-ink of this embodiment was prepared from the following raw materials in terms of mass concentration percentage:

Polycaprolactone particles (average molecular weight 80 kDa): 70%; and

Glacial acetic acid (purity > 99.5 %): 30%.

The preparation method of the 70% PCL reference material of the present embodiment was as follows: at room temperature, polycaprolactone particles (3.500 g) were added to 5 ml of glacial acetic acid, sonicated in a 50 °C water bath, and stirred once every 15 minutes, completely dissolved and uniformly mixed to obtain a uniform color solution, that is the bio-ink. The bio-ink solution was allowed to stand still in a 30°C incubator for half an hour, had the bubbles removed, and stored for later use.

The printing process and parameter setting of the 70% PCL control material of this embodiment were as follows: the above bio-ink is transferred to a 5 ml syringe, placed in a micro-injection pump, and connected to a hose and a nozzle, and the nozzle was then fixed at a position perpendicular to the printing plate.

The injection pump was adjusted to an injection rate of 5 $\mu\text{L}/\text{min}$, a vertical distance between the nozzle and the print substrate of 6 mm, a platform moving rate of 100 mm/s, a stable voltage of 1 kV, and a printing temperature of room temperature. The print program was set to have a print path is as shown in Fig.1, with a hole size of 400 μm and the number of print layer being 24. After the ink extrusion rate is stabilized, the printing substrate was connected to the high voltage power supply to complete the printing according to the preset path.

Optical microscope images of a scaffold obtained by printing with 70% PCL control material (hereinafter referred to as a 70% PCL scaffold) are shown in Fig. 2a. The scaffold was estimated to have an average diameter of 24.8 μm and an average pore size of 374 μm .

Example 2

The present embodiment describes an example of preparing the bio-ink for 3D printing of the present invention and the use thereof in printing a scaffold, wherein polycaprolactone, zein and glacial acetic acid, for purpose of example only, were used as the synthetic degradable polymer, the plant-derived prolamin, and the solvent, respectively, but not limited

thereto.

The bio-ink of this embodiment was prepared from the following raw materials in terms of mass concentration percentage:

Polycaprolactone particles (average molecular weight 80 kDa): 60%;

Zein powder (purity > 98%): 10%; and

Glacial acetic acid (purity > 99.5 %): 30%.

The preparation method of the bio-ink of the present embodiment is as follows: firstly, at room temperature, zein (0.500 g) was weighed, added to 5 ml of glacial acetic acid, and completely dissolved by water bath-ultrasonic treatment to obtain a yellow clear solution; secondly, polycaprolactone particles (3.000 g) were added to the solution, sonicated in a 50 °C water bath, stirred once every 15 minutes, completely dissolved and uniformly mixed to obtain a uniform color solution, that is the bio-ink. Finally, the solution was allowed to stand still in a 30 °C incubator for half an hour, had the bubbles removed, and stored for later use.

The bio-ink printing procedure of this embodiment was the same as Example 1, with the following parameter settings: an injection speed of the injection pump of 1 $\mu\text{L}/\text{min}$, a vertical distance between the nozzle and the printing substrate of 3 mm, and a controlled temperature of 26 °C. In practice, the settings of the above print procedure and parameters can be adjusted as needed.

Optical microscope images of a scaffold obtained by printing the bio-ink are shown in Fig.2b. It has been measured to have an average diameter of 25.0 μm and an average pore size of 384 μm . The stereostructure of the scaffold is shown in the scanning electron microscope (SEM) photograph of Fig.3.

Example 3

The present embodiment describes an example of preparing the bio-ink for 3D printing of the present invention and the use thereof in printing a scaffold, wherein polycaprolactone, zein and glacial acetic acid, for purpose of example only, were used as the synthetic degradable polymer, the plant-derived prolamin, and the solvent, respectively, but not limited

thereto.

The bio-ink of this embodiment was prepared from the following raw materials in terms of mass concentration percentage:

Polycaprolactone particles (average molecular weight 80 kDa): 50%;

Zein powder (purity > 98%): 20%; and

Glacial acetic acid (purity > 99.5 %): 30%.

The preparation method was the same as in Example 2, except that the amount of zein used was 1.000 g and the amount of polycaprolactone particles was 2.500 g.

The print procedure setting for this bio-ink is the same as in Example 1, and the parameter settings are as follows: the syringe pump injection rate is adjusted to 1 $\mu\text{L}/\text{min}$, the vertical distance between the nozzle and the print substrate is 3 mm, the platform moving rate is 150 mm/s, the stable voltage is 2.8 kV, and the printing temperature is 27 $^{\circ}\text{C}$.

Optical microscope images of a scaffold by printing the bio-ink are shown in Fig. 2c. The scaffold is estimated to have an average diameter of 28.5 μm and an average pore size of 380 μm . The scaffold stereostructure was similar to that shown in the scanning electron micrograph of Example 1.

Example 4

The present embodiment describes an example of preparing the bio-ink for 3D printing of the present invention and the use thereof in printing a scaffold, wherein polycaprolactone, wheat protein and glacial acetic acid, for purpose of example only, were used as the synthetic degradable polymer, the plant-derived protein, and the solvent, respectively, but not limited thereto.

The bio-ink of this embodiment was prepared from the following raw materials in terms of mass concentration percentage:

Polycaprolactone particles (average molecular weight 80 kDa): 60%;

Freeze-dried wheat protein powder: 10%; and

Glacial acetic acid: 30%.

The preparation method of the bio-ink of this embodiment was as follows: firstly, wheat protein (0.500 g) was weighed, added to 5 ml of acetic acid, and completely dissolved by water bath-ultrasonic treatment to obtain a solution; next, polycaprolactone (3.000 g) was added to the solution, sonicated in a water bath, stirred once every 30 minutes, completely dissolved and uniformly mixed to obtain a uniform color solution, i.e., the bio-ink; finally, the solution was allowed to stand still in a 30°C incubator for half an hour, had the bubbles removed, and stored for later use.

The bio-ink printing procedure of this embodiment was the same as Example 1, with the following parameter settings: an injection speed of the injection pump of 0.1 to 40 $\mu\text{L}/\text{min}$, a vertical distance between the nozzle and the printing substrate of 1 to 10 mm, a platform moving rate of 10 to 2000 mm/s, and a stable voltage of 0.5-5.0 kV. In practice, the settings of the above print procedure and parameters can be adjusted as needed.

An optical microscope image of a scaffold by printing the bio-ink is shown in Fig.4a. It has been measured to have an average diameter of 73.0 μm and an average pore size of 330 μm . The bio-ink of this embodiment, after printing, could be easily peeled off from the printing substrate after being placed in the air for 3 hours, as shown in Fig. 4b.

Example 5

The present embodiment describes an example of the method for producing a fluorescently labeled protein of the present invention, in which a coumarin dye and zein are used as examples of a fluorescent molecule and a prolamin, respectively, but not limited thereto.

The preparation method was as follows:

4-bromomethyl-6,7-dimethoxycoumarin (30 mg) and zein (1 g) were weighed and dissolved simultaneously into N, N-dimethylformamide (15 ml) in a reaction flask.

Potassium carbonate powder (120 mg) was added to the reaction flask, and the reaction was stirred at 65 °C for 12 hours.

After the reaction solution was cooled, it was poured into 400 ml of a saturated ammonium sulfate solution, and the precipitated protein was filtered under reduced pressure. The obtained solid was washed with deionized water (3×100 ml), followed by ethyl acetate

(3 × 50 ml), dichloromethane (3 × 50 ml), acetone (3 × 50 ml), and finally rinsed with deionized water (3 × 100 ml).

The filtered and washed solid was redissolved into a 70% aqueous solution of ethanol, had the insoluble impurities and the solvent removed by filtration and by rotary evaporation, respectively, and dried under vacuum to obtain coumarin-labeled zein.

Example 6

This embodiment describes a further example of preparing the bio-ink for 3D printing of the present invention and using it for printing a scaffold, wherein polycaprolactone, zein, 6, 7-dimethoxy coumarin, and glacial acetic acid were used as, for purpose of example only, the synthetic degradable polymer, plant-derived protein, fluorescently labeled molecule, and solvent, but not limited thereto.

The bio-ink for 3D printing described in this embodiment is prepared from the following raw materials in terms of mass concentration percentage:

Polycaprolactone particles (average molecular weight of 80 kDa): 60%;

Zein powder (purity > 98%): 9 %;

6, 7-dimethoxycoumarin labeled zein powder: 1%; and

Glacial acetic acid (purity > 99.5 %): 30%.

The preparation method was the same as in Example 2 except that the zein used was 0.450 g, the polycaprolactone particles were 3.000 g, and the 6,7-dimethoxycoumarin-labeled zein powder was 0.050 g.

Before preparation of the above bio-ink, the coumarin dye-labeled zein could be prepared according to the method described in Example 4.

The bio-ink for 3D printing prepared in this example showed significant blue fluorescence under 365 nm light excitation, as shown in Fig.5. A scaffold printed with this bio-ink and molded in accordance with the printing parameters of Example 2 was subjected to three-dimensional fluorescence imaging (laser excitation wavelength of 405 nm) by a laser confocal microscope, as shown in Fig.6.

Example 7

This embodiment describes still another example of preparation of the bio-ink for 3D printing of the present invention, in which polycaprolactone, zein, 6,7-dimethoxycoumarin and glacial acetic acid are respectively used as examples of the synthetic degradable polymer, plant-derived protein, fluorescently labeled molecule, and solvent, but not limited thereto.

The bio-ink for 3D printing described in this embodiment was prepared from the following raw materials in terms of mass concentration percentage:

Polycaprolactone particles (average molecular weight of 80 kDa): 50%;

Zein powder (purity > 98%): 18%;

6, 7-dimethoxycoumarin labeled zein powder: 2%; and

Glacial acetic acid (purity > 99.5 %): 30%.

The method as described in Example 4 could be used to prepare the coumarin-labeled zein as used above.

The bio-ink of the present embodiment was prepared in the same manner as in Example 2, except that the zein used was 0.900 g, the polycaprolactone particles 2.500 g, and the 6,7-dimethoxycoumarin-labeled zein powder 0.100 g.

The bio-ink for 3D printing prepared in this example has a fluorescent property and a print-formed scaffold, which is similar to that of the embodiment 5.

Example 8

This embodiment describes an example of a comparative experiment of cell culture, in which composite scaffolds obtained by 3D printing of bio-inks in Examples 1, 2, and 3 were used.

Experimental method:

1) Cell culture comparative experiment steps were as follows:

The scaffolds of Examples 1, 2, and 3 were cut into square samples of size 1 cm * 1 cm.

The scaffold sample was sterilized before being subjected to cell culture experiments. H1299 human lung cancer cells (non-small cell lung cancer) were inoculated with an inoculation density of $4 \times 10^6/\text{ml}$ in the cell culture medium of RPMI-1640 (including 10% newborn fetal calf serum and 1% antibiotics) in a 24-well ultra-low adsorption cell culture dish (Corning) as the culture container. The initial inoculation density and the inoculation volume of the cells were $4 \times 10^6/\text{ml}$ and 15 μl , respectively. After inoculation, the cells were allowed to stand still in the cell culture incubator for 1.5 hours, and then 985 μl of RPMI-1640 medium was added for culturing (37 °C, 5.1% CO₂). The cell culture medium was replaced every two days.

2) Staining was conducted as follows in order to observe the adhesion and proliferation of cells:

The pre-treated scaffolds were transferred to new cell culture dishes, washed three times with phosphate buffered saline (PBS), and stained with 1 ml of Hoechst 33342 (10 $\mu\text{g}/\text{ml}$ in RPMI-1640) for 15 minutes. After staining, the scaffolds were washed 3 times with PBS and imaged with a laser confocal microscope.

3) The cells were desorbed and counted using a flow cytometer as follows in order to quantify the amount of cell adhesion:

The scaffolds after cell culture were transferred to new culture dishes, treated with 1 ml of trypsin (0.25% in PBS) for 5-10 minutes, and observed under a microscope so that the cells were in a predissociated state. The protease solution was gently removed, and 1 ml of filtered PBS solution (0.22 μm filter) was added. The cells adhered to the scaffold were blown with a pipette tip to disperse separately in solution. Such suspension was quantitatively transferred to a centrifuge tube and added with 1 ml of filtered PBS to washing the remaining cells. The suspension was then combined for cell counting.

Experimental results:

The results of laser confocal microscopy imaging of adhesion and proliferation of cells are shown in Fig. 7, while the results of cell counting using a flow cytometer after cell desorption are shown in Fig. 8.

The adhesion and proliferation of cells of the scaffolds of Examples 1, 2, and 3 on the first and fourth days are shown in the laser confocal micrograph of Fig.7, respectively, in

which the cells were subjected to staining by DiI cell membrane dye and Hoechst 33342 nuclear dye, respectively. As can be seen from Fig. 7, the PCL-Zein scaffolds of Examples 2 and 3 had significantly more cell adhesion than the pure PCL scaffolds on the first day of culture, as compared to the PCL scaffolds prepared in Example 1. After the fifth day of culture, the number of proliferated cells on the PCL-Zein scaffold in Examples 2 and 3 was still significantly greater than that of the PCL scaffold. It can thus be seen that the scaffold printed by the zein-modified bio-ink was more conducive to cell adhesion and proliferation, with the cell affinity significantly improved.

The cell counts of the scaffolds of Examples 1, 2, and 3 on the first and fourth days of cell culture are shown in Fig. 8. The quantitative results of cell adhesion and proliferation were in accordance with the qualitative results by the laser scanning confocal microscopy, wherein corn protein-modified scaffolds exhibited better cell affinity.

Example 9

This embodiment describes an example of a degradability comparison experiment of composite scaffolds obtained by 3D printing of bio-inks in Examples 1, 2, and 3, respectively.

Experimental method:

1) *In-vitro* enzyme-accelerated degradation experiment for evaluation of scaffold degradability: The scaffolds printed in Examples 1, 2, and 3 were first dried in a vacuum oven (40 °C) to a constant weight and weighed for the initial scaffold mass (W_i). 5 mL of phosphate buffer (pH=7.4, including 100 UI/mL penicillin and 100 µg/mL streptomycin) and 25 µl of proteinase k (Proteinase k, 20 mg/ml, PCR grade) were added to the dish, so that the final concentration of protease in the solution was 0.1 mg/ml. The scaffold was sterilized, added to the culture dish, and placed in a biological incubator (37 °C, 5.1% CO₂). The scaffold was taken out every two days, washed 3 times with deionized water, and placed in a vacuum oven (40 °C) for drying to a constant weight. The weight of the scaffold after j days was recorded as W_j . Then, after replacing with new phosphate buffer and protease, the degradation experiment continued.

2) Degradation status of the scaffold: expressed by the weight loss (Weight Loss %) and

degradation time (Day) curve, wherein the weight loss of the scaffold was calculated by the following formula:

$$\text{Weight loss (\%)} = (W_i - W_f) / W_i * 100 \text{ \%}.$$

3) Fiber surface structure before and after degradation of the scaffold: could be shown by scanning electron microscopy (SEM), wherein all the samples tested were surface-sputtered with platinum (Pt).

Experimental results:

The experimental results of degradation of the scaffolds in Examples 1, 2, and 3 are shown in Fig. 9, respectively, while the fiber surface structures before and after the degradation of the scaffold are shown in Fig. 10.

As can be seen from the comparison in this example, the zein-modified scaffold exhibits better degradation performance in an *in vitro* protease-accelerated assay, while the degradation rates of the scaffold could be regulated by adjusting the ratio of protein to polylactone in the material. It can be seen from the SEM image that the protein- including scaffold degraded to form a nano-scale porous scaffold, which greatly increased the specific surface area of the scaffold.

The above-mentioned embodiments are merely illustrative of several embodiments of the present invention. The description thereof is specific and detailed, but is not to be construed as limiting the scope of the invention. It should be noted that numerous variations and modifications may be made by those skilled in the art without departing from the spirit and scope of the invention. Therefore, the scope of the invention should be defined only by the appended claims.

CLAIMS

1. A bio-ink comprising the following components by mass percentage:
1% to 70% of a synthetic degradable polymer;
1% to 70% of a natural polymer; and
28% to 98% of a solvent;
wherein the solvent is water, an organic solvent or any combination thereof.
2. The bio-ink according to claim 1, wherein the synthetic degradable polymer is a polyester macromolecule and the natural polymer is a plant-derived protein.
3. The bio-ink according to claim 1 or 2, wherein the bio-ink further comprises 0.0001% to 5% by mass of a fluorescently labeled molecule.
4. A method for preparing a bio-ink according to any one of claims 1-3, comprising the steps of:

 weighing the natural polymer, or weighing the natural polymer and the fluorescently labeled molecule, according to the mass percentages defined, and dissolving the same in the solvent to obtain a clear solution; and

 weighing the synthetic degradable polymer according to the mass percentage defined, dissolving the same in the clear solution and mixing uniformly to obtain a solution of uniform color, that is, the bio-ink.
5. A scaffold for tissue engineering obtained by 3D printing with the bio-ink according to any one of claims 1 to 3 or a bio-ink prepared by the method according to claim 4.
6. A method for preparing a fluorescently labeled protein, comprising the steps of:

 dissolving a derivative of a fluorescent molecule and a protein in an organic solvent simultaneously;

 adding a base thereto and stirring to allow reaction at a temperature of 20 °C to 150 °C for 1 to 24 hours; and

 pouring the reaction solution, after cooling, into a saturated salt solution to precipitate the protein, filtering, and washing to obtain the fluorescently labeled protein.

7. The method according to claim 6, further comprising a purifying and drying step, that is, re-dissolving the obtained protein in an aqueous ethanol solution, filtering, removing the solvent by rotary evaporation, and vacuum-drying.

8. The method according to claim 6 or 7, wherein the derivative of a fluorescent molecule is a halogenated derivative of a fluorescent molecule, the protein is a plant-derived prolamin, and the derivative of a fluorescent molecule is added in a molar amount that is 0.0001 - 10 times of the average molar amount of the protein.

9. A fluorescently labeled protein produced by the method of any one of claims 6-8.

10. A bio-ink comprising the following components by mass percentage:

1% to 70% of a synthetic degradable polymer;

1% to 70% of a natural polymer;

0.0001% to 5% of a fluorescently labeled molecule; and

28% to 98% of a solvent;

wherein the solvent is water, an organic solvent or any combination thereof, and wherein the fluorescently labeled molecule is the fluorescently labeled protein according to claim 9.