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Title of invention:

Recombinant collagen and recombinant collagen sponge material

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RECOMBINANT COLLAGEN AND RECOMBINANT COLLAGEN SPONGE MATERIAL

Technical field

The present disclosure relates to the technical field of medical technology and surgery, particularly relates to a recombinant collagen, a recombinant collagen sponge material, a method for preparation thereof, and use thereof, and more particularly to a recombinant collagen sponge material capable of hemostasis and wound surface repair, a method for preparation thereof, and use thereof.

Background

In our daily lives there are many scenarios where uncontrolled bleeding is the main cause of sudden accidents and massive bleeding in medical operations, such as first-aid treatment for emergencies and hemostasis in trauma during surgery, and thus effective and rapid local hemostasis for patients is of particular importance. Therefore, shortening the hemostasis time of hemostatic materials and improving the quality of hemostasis has become an optimal strategy to reduce mortality among patients. Clinically commonly used hemostatic materials, such as cellulose-based hemostatic gauze, hemostatic fibers, and hemostatic bandages, have limitations in use, for example long hemostasis time, inability to treat wound infection and suppuration, and inability to induce regeneration in wounds. Therefore, a product having a superior hemostatic effect and an excellent tissue repairing effect is one of the clinical products urgently needed in the field of surgery.

CN101890179A provides a water-soluble hemostatic material which is mainly composed of oxidized regenerated cellulose ether, has a significant hemostatic effect but insufficient strength, is difficult to degrade in the body, and may be deposited in other organs of the human body after absorption.

CN104558675A provides a hemostatic microfibrous collagen sponge having a good hemostatic effect. However, it is difficult to ensure a controllable structure and quality of the product because only physical thermal cross-linking is applied, and there are also risks posed by immunogenicity and animal viruses.

CN103432620A provides an anti-adhesion hemostatic film mainly composed of dextran and prepared by electrospinning after cross-linking, the production process thereof is relatively complex, the cost is high, and the biocompatibility, which is a strict requirement set upon clinical products, has not been evaluated.

Summary

Due to the defects in the prior art, the first objective of the present disclosure is to provide a recombinant collagen; the second objective of the present disclosure is to provide a recombinant collagen sponge material having a hemostatic and wound surface repairing effect; the third objective of the present disclosure is to provide a method for preparing the recombinant collagen sponge material; and the fourth objective of the present disclosure is to provide use of the recombinant collagen sponge material as a hemostatic product in hemostasis and wound surface repair.

The objectives of the present disclosure are achieved through the following technical solutions.

In one aspect, the present disclosure provides a recombinant collagen, comprising:
(a) a protein composed of the amino acid sequence represented by SEQ ID NO: 2;

SEQ ID NO: 2

GPPGEPGNPGKPGSPGPAGSNGEPGPAGSPGEKGSQGSNGNPGPAGNQGQPG

NKGSPGNPGKPGEPGSNGPQGEPGSQGNPGKNGQPGSPGSQGSPGNQGQPGK
PGQPGEQGSPGNQGPAGNEGPKGQPGQNGKPGSPGPPGEPGNPGKPGSPGPA
GSNGEPGPAGSPGEKGSQGSNGNPGPAGNQGQPGNKGSPGNPGKPGEPGSNG
PQGEPGSQGNPGKNGQPGSPGSQGSPGNQGQPGKPGQPGEQGSPGNQGPAGN
EGPKGQPGQNGKPGTPGPPGEPGNPGKPGSPGPAGSNGEPGPAGSPGEKGSQG
SNGNPGPAGNQGQPGNKGSPGNPGKPGEPGSNGPQGEPGSQGNPGKNGQPGS
PGSQGSPGNQGQPGKPGQPGEQGSPGNQGPAGNEGPKGQPGQNGKP

and/or

(b) a protein which has the same function as (a) and is derived from (a) by substitution, deletion and/or addition of one or more amino acids in SEQ ID NO:2.

Based on the Gly-X-Y repeats of human type I collagen as the smallest repeating unit, the inventors creatively used the hydrophilic Gly-X-Y for permutation and combination, and designed a collagen with a length of 411 amino acids (represented by SEQ ID NO: 2). A corresponding nucleotide sequence (represented by SEQ ID NO:1) was also designed according to the codon preference in *Pichia*, synthesized, and inserted into the expression vector pPIC9K of *Pichia* to construct a pPIC9K-COL expression vector. The vector was transformed into a *Pichia* host strain GS115 by electrotransformation, high-copy number strains were picked through screening with the antibiotic G418, and finally a high-expression engineered strain of *Pichia* was obtained through shaking flask screening. This engineered strain was subjected to large-scale biological fermentation to obtain the raw material of the recombinant collagen. The recombinant collagen according to the present disclosure shows excellent cell attachment and hydrophilicity, and is an optimal raw material for preparing a recombinant collagen sponge material.

In another aspect, the present disclosure further provides a polynucleotide encoding the amino acid sequence of the recombinant collagen above, and the DNA sequence of the polynucleotide comprises the DNA sequence represented by SEQ ID NO:1.

SEQ ID NO: 1

GGTCCTCCCGGCGAACCAGGTAATCCTGGTAAACCTGGTTCTCCCGGCCCA
GCTGGTTCCAACGGGGAGCCGGGTCCTGCCGGCTCACCCGGAGAAAAGGG
GTCGCAAGGTAGTAATGGCAACCCAGGACCGGCAGGGAATCAGGGTCAAC
CTGGCAACAAAGGAAGCCCCGGGAATCCAGGTAAGCCGGGCGAGCCTGGA
TCTAACGGGCCCCAGGGTGAACCAGGCTCCCAAGGAAATCCGGGGAAAAA
CGGTCAGCCTGGCTCACCCGGATCGCAAGGGAGTCCAGGTAATCAGGGCC
AACCGGGAAAGCCTGGGCAGCCCGGTGAGCAAGGCAGCCCAGGAAACCA
GGGGCCGGCGGGTAATGAAGGCCCTAAAGGACAACCCGGGCAGAACGGT
AAGCCAGGATCCCCGGGTCCTCCCGGCGAACCAGGTAATCCTGGTAAACCT
GGTTCTCCCGGCCCAGCTGGTTCCAACGGGGAGCCGGGTCCTGCCGGCTC
ACCCGGAGAAAAGGGGTCGCAAGGTAGTAATGGCAACCCAGGACCGGCA
GGGAATCAGGGTCAACCTGGCAACAAAGGAAGCCCCGGGAATCCAGGTAA
GCCGGGCGAGCCTGGATCTAACGGGCCCCAGGGTGAACCAGGCTCCCAAG
GAAATCCGGGGAAAAACGGTCAGCCTGGCTCACCCGGATCGCAAGGGAGT
CCAGGTAATCAGGGCCAACCGGGAAAGCCTGGGCAGCCCGGTGAGCAAG
GCAGCCCAGGAAACCAGGGGCCGGCGGGTAATGAAGGCCCTAAAGGACA
ACCCGGGCAGAACGGTAAGCCAGGTACCCCAGGTCCTCCCGGCGAACCAG
GTAATCCTGGTAAACCTGGTTCTCCCGGCCCAGCTGGTTCCAACGGGGAGC
CGGGTCCTGCCGGCTCACCCGGAGAAAAGGGGTCGCAAGGTAGTAATGGC
AACCCAGGACCGGCAGGGAATCAGGGTCAACCTGGCAACAAAGGAAGCC
CCGGGAATCCAGGTAAGCCGGGCGAGCCTGGATCTAACGGGCCCCAGGGT
GAACCAGGCTCCCAAGGAAATCCGGGGAAAAACGGTCAGCCTGGCTCACC
CGGATCGCAAGGGAGTCCAGGTAATCAGGGCCAACCGGGAAAGCCTGGGC
AGCCCGGTGAGCAAGGCAGCCCAGGAAACCAGGGGCCGGCGGGTAATGA
AGGCCCTAAAGGACAACCCGGGCAGAACGGTAAGCCA

In yet another aspect, the present disclosure further provides an expression vector containing the above-mentioned polynucleotide.

In yet another aspect, the present disclosure further provides a host strain containing the above polynucleotide, the host strain being an engineered *Pichia* strain that has been deposited (Date of deposit: Jan 8, 2020; Depository authority: China General Microbiological Culture Collection Center (CGMCC); Address of depository authority: Institute of Microbiology Chinese Academy of Sciences, Building 3, No.1 West Beichen Road, Chaoyang District, Beijing; Accession number: CGMCC No. 19314; Taxonomic name: *Pichia sp.*). The recombinant collagen according to the present disclosure is obtained by fermentation of the engineered *Pichia* strain.

In a further aspect, the present disclosure also provides a recombinant collagen sponge material, obtained by sequential physical cross-linking and chemical cross-linking of the aforementioned recombinant collagen; wherein the recombinant collagen sponge material has a moisture absorption capacity of 40-50, and porosity of 90% or higher.

In the present disclosure, the moisture absorption capacity refers to the ratio of the weight of the recombinant collagen sponge material after water absorption to the weight of the recombinant collagen sponge material before water absorption. Porosity refers to the percentage of the pore volume in the recombinant collagen sponge material to the total volume of the recombinant collagen sponge material in its natural state.

The present disclosure achieves high porosity by low-degree cross-linking and freeze-drying techniques, as low-degree cross-linking (10%-20%) can ensure a good swelling property of the product, and freeze-drying can ensure uniform and stable pore distribution. The high water absorption according to the present disclosure is mainly determined by the designed amino acid sequence of the recombinant protein and the interconnected high porosity. Specifically, the designed amino acids increased the number of hydrophilic amino acids to improve water absorption, and the interconnected high porosity can provide water absorption and water retention.

In yet another aspect, the present disclosure provides a method for preparing the above-mentioned recombinant collagen sponge material, comprising the following steps:

- dissolving the aforementioned recombinant collagen in water to obtain a recombinant collagen solution;
- lyophilizing the recombinant collagen solution by a freeze-drying method; and
- subjecting the lyophilized and formed recombinant collagen to physical cross-linking and chemical cross-linking in sequence to obtain a recombinant collagen sponge material.

The recombinant collagen raw material according to the present disclosure is water-soluble, has excellent cell attachment property and hydrophilicity, has a low crosslinking degree after the physical crosslinking and chemical crosslinking to ensure the mechanical strength of the sponge while avoiding a risk of inflammation caused by prolonged retention of residual material in the body. After the low-degree cross-linking and freeze-drying process, a collagen sponge with good water absorption is obtained. The special amino acid sequence design improves the attachment of platelets, while the high water absorption can concentrate the platelets in the blood to rapidly stop bleeding.

Generally, by physical crosslinking alone it is difficult to obtain a uniform degree of crosslinking and the quality is uncontrollable, while chemical crosslinking alone often requires involvement of a high level of chemical crosslinking agents, which affects the biocompatibility of the final product. The present disclosure adopts the "physical plus chemical" cross-linking and can obtain a recombinant collagen sponge material having a low cross-linking degree and excellent biocompatibility.

In the above method, preferably, the recombinant collagen sponge material obtained after the cross-linkings is further subjected to washing, drying, and sterilization.

In the above method, preferably, the device used for the washing includes a rotating rod and a porous clamp box fixed on the rotating rod; wherein the porous clamp box is used to hold the recombined collagen sponge, and the rotating rod is used to rotate and drive the porous clamp box to flip in a cleaning medium, so as to wash off the residual reagent in the recombinant collagen sponge material.

The washing device according to the present disclosure is an independently designed device, wherein the designed porous clamp box has an effect of fixing and shaping the recombined sponge, and the rotation of the rotating rod drives the clamp box to flip in a cleaning medium, which can improve the efficiency of washing off residual reagents in the recombined sponge. Due to the high efficiency of the washing device and the use of low-degree crosslinking agents, the biocompatibility of the product is ensured.

In the above method, preferably, the drying includes one or a combination of more of oven drying, freeze drying and vacuum drying.

In the above method, preferably, the sterilization is performed by 15 to 25 kGy Co^{60} irradiation.

In the above method, preferably, the physical crosslinking includes one or a combination of more of thermal crosslinking, radiation crosslinking, and repeated freezing-reconstitution.

In the above method, preferably, the temperature for thermal crosslinking is 110°C, and the crosslinking duration is 2h.

In the above method, preferably, the radiation source for the radiation cross-linking includes ultraviolet rays and/or gamma rays.

In the above method, preferably, the chemical crosslinking is performed by addition of a chemical crosslinking agent including one or a combination of more of glutaraldehyde, carbodiimide, and genipin.

In the above method, preferably, the concentration of the chemical crosslinking agent is 0.005 to 0.015 mol/L.

In the above method, preferably, the mass ratio of the chemical crosslinking agent to the recombinant collagen is 1:1 to 5.

After cross-linking, the recombinant collagen sponge material according to the present disclosure has a low degree of cross-linking of 10%-20%, as determined by percentage analysis of free amino groups on the side chains of amino acids.

In the above method, preferably, the chemical crosslinking duration is 1 to 5 hours.

In the above method, preferably, the concentration of the recombinant collagen solution is 1% to 5%.

In the above method, preferably, before freeze-drying the recombinant collagen solution, it further includes injecting the recombinant collagen solution into a mold for forming.

In the above method, preferably, the recombinant collagen solution is freeze-dried at a gradient from -50°C to 30°C.

In another aspect, the present disclosure further provides use of the above-mentioned recombinant collagen sponge material as a hemostatic product in hemostasis and wound surface repair. It is mainly applicable in the field of medical surgery, for hemostasis of acute and chronic wounds during surgeries and tissue repair at wound surfaces.

The recombinant collagen raw material according to the present disclosure is water-soluble, shows excellent cell attachment property and hydrophilicity, and provides a collagen sponge having good water absorption after being subjected to low-degree cross-linking and freeze-drying processes. The high efficiency of the washing device and use of a low-degree crosslinking agent ensure the biocompatibility of the product. The special amino acid sequence design improves the platelet attachment ability, and the high water absorption can concentrate the platelets in the blood so as to quickly stop bleeding. In summary, the recombinant collagen sponge material according to the present disclosure is capable of hemostasis, wound surface repair, moisture absorption and platelet aggregation, and has high moisture absorption, a significant hemostatic effect and good biocompatibility, assuming great clinical significance in the field of surgery.

Description of drawings

Figure 1 shows the results of an electrophoresis of protein expression levels in different strains according to the present disclosure.

Figure 2 is a schematic representation of the structure of the washing device used in an example of the present disclosure.

Figure 3 shows a comparative experiment of cell proliferation in the recombinant collagen sponge material in an example of the present disclosure.

Figure 4 shows a comparative experiment of hemostatic performance of the recombinant collagen sponge material on a New Zealand rabbit liver over 20 seconds in an example of the present disclosure.

Figure 5 is a schematic representation of a wound surface model on the back of a rat in an example of the present disclosure.

Figure 6 shows the repairing of wound surfaces with the materials in various groups at different time points in an example of the present disclosure.

Figure 7 shows a liquid chromatography profile for a compositional analysis of the amino acid components of the recombinant collagen in an example of the present disclosure.

Figure 8 is a mass spectrometry profile of the molecular weight of the recombinant collagen in an example of the present disclosure.

Deposition of Microorganisms for the Purpose of Patent Procedures

The engineered *Pichia* strain according to the present disclosure:

Date of deposit: Jan 8, 2020;

Depository authority: China General Microbiological Culture Collection Center

(CGMCC);

Address of depository authority: Building 3, No.1 West Beichen Road, Chaoyang District, Beijing;

Accession number: CGMCC No. 19314;

Taxonomic name: *Pichia sp.*

Detailed Description of Invention

In order to provide a clearer understanding of the technical features, objectives and beneficial effects of the present disclosure, the technical solutions of the present disclosure will be described in detail below, but are not to be construed as limiting the implementable scope of the present disclosure.

The recombinant collagen raw material used in the following examples was obtained by constructing a genetically engineered strain, producing a recombinant collagen having a molecular weight of 38 kDa by microbial fermentation, purifying and then freeze-drying the recombinant collagen.

The specific process was as follows.

Based on the Gly-X-Y repeats of human type I collagen as the smallest repeating unit, the inventors creatively used the hydrophilic Gly-X-Y for permutation and combination, and designed a collagen with a length of 411 amino acids (represented by SEQ ID NO: 2). A corresponding nucleotide sequence according to the codon preference in *Pichia* (represented by SEQ ID NO:1) was also designed, synthesized, and inserted into the expression vector pPIC9K of *Pichia* to construct a pPIC9K-COL expression vector. The vector was transformed into a *Pichia* host strain GS115 by electrotransformation, high-copy number strains were picked through screening with increasing concentrations of Geneticin G418 in the medium, transformants with a high copy number were picked for an expression test in shaking flasks, and strains showing a high level of expression were selected as the genetically engineered strain for production. Under the same electrophoresis conditions, the strain B31 showed a relatively high expression level (as shown in Figure 1), and was deposited as an engineered strain (accession number: CGMCC No. 19314) for scale-up production. The engineered strain was used to carry out large-scale biological fermentation to obtain the raw material of the recombinant collagen.

The process of obtaining the recombinant collagen by fermenting the engineered strain according to the present disclosure was as follows.

(1) Primary seed cultivation

The engineered strain of the present disclosure (Accession number: CGMCC No. 19314) was inoculated into an Erlenmeyer flask containing a BMGY medium, and incubated in a thermostatic culturing shaker at 29°C, 225 rpm for 60 to 70 hours to obtain a primary seed liquid.

(2) Secondary seed cultivation

The primary seed liquid was fed into a seed tank, and then cultured in the tank at a temperature controlled at 29.0±1.0°C, a tank pressure of 0.050±0.010 MPa, and pH 5.0. During the culturing, the aeration and stirring speed were adjusted to maintain the dissolved oxygen at about 30%. The secondary seed cultivation was performed for about 16 hours to obtain a secondary seed liquid.

(3) Fermentation in fermenter

<u>Base material cultivation stage</u>

After the secondary seed cultivation was complete, the secondary seed liquid was transferred to a fermenter, the culturing temperature was controlled at 29.0±1.0°C, the tank pressure was controlled at 0.050±0.010 MPa, and the DO was controlled at about 30% by manual adjustment of the aeration, oxygen level and rotation speed. After 12 to 18 hours of cultivation, the feeding cultivation stage started.

<u>Glycerin feeding stage</u>

When the feeding stage started, the oxygen supply was immediately turned off, and the DO was lowered to about 40% by reducing the stirring speed. An automatic feeding system was started with an initial flow rate of a glycerin solution of 0.8 mL/min (1s/60s). After 12 hours of feeding, a sample was taken to measure the wet strain weight of the fermentation broth. When the wet strain weight of the fermentation broth reached 200 g/L, the glycerin feeding was stopped and starvation was started.

<u>Starvation stage</u>

The DO was controlled at 30-40% by adjusting the aeration volume and reducing the stirring speed, and the starvation state was maintained for 1.0 h.

<u>Methanol induction stage</u>

The methanol flow rate was increased according to the actual DO. The methanol flow rate was generally controlled within 8.0 mL/min (10s/60s), and the methanol induction duration was generally controlled at 40 to 48 h. The DO during methanol induction should be controlled at 20 to 35%, and it should be confirmed that no excessive methanol was accumulated at this stage.

<u>Discharge from fermenter</u>

After induction for 44 to 48 hours, the recombinant collagen was discharged from the fermenter, harvested, and sampled for testing.

The amino acid sequence of the recombinant collagen is represented by SEQ ID NO: 2:

SEQ ID NO: 2

GPPGEPGNPGKPGSPGPAGSNGEPGPAGSPGEKGSQGSNGNPGPAGNQGQPG
NKGSPGNPGKPGEPGSNGPQGEPGSQGNPGKNGQPGSPGSQGSPGNQGQPGK
PGQPGEQGSPGNQGPAGNEGPKGQPGQNGKPGSPGPPGEPGNPGKPGSPGPA
GSNGEPGPAGSPGEKGSQGSNGNPGPAGNQGQPGNKGSPGNPGKPGEPGSNG
PQGEPGSQGNPGKNGQPGSPGSQGSPGNQGQPGKPGQPGEQGSPGNQGPAGN

EGPKGQPGQNGKPGTPGPPGEPGNPGKPGSPGPAGSNGEPGPAGSPGEKGSQG
SNGNPGPAGNQGQPGNKGSPGNPGKPGEPGSNGPQGEPGSQGNPGKNGQPGS
PGSQGSPGNQGQPGKPGQPGEQGSPGNQGPAGNEGPKGQPGQNGK

The recombinant collagen according to the present disclosure shows excellent cell attachment property and hydrophilicity, and is an optimal raw material for preparing a recombinant collagen sponge material.

The washing device used in the following Examples was shown in Figure 2. The washing device includes a rotating rod and a porous clamp box fixed on the rotating rod; wherein the porous clamp box is used to hold the recombined collagen sponge, and the rotating rod is used to rotate and drive the porous clamp box to flip in a cleaning medium, so as to wash off residual reagents in the recombinant collagen sponge material.

Example 1

This Example provides a recombinant collagen sponge material and a method for preparation thereof. The method comprised:

(1) preparing a 3% solution of a recombinant collagen (molecular weight: 38 kDa) and stirring it thoroughly; pouring the recombinant collagen solution into a mold for forming, and lyophilizing it by a freeze-drying method with the following lyophilization parameters: -50°C for 5h, -30°C for 3h, -20°C for 3h, -10°C for 2h, 0°C for 1h, 10°C for 5h, 20°C for 20h, 30°C for 60h;

(2) performing physical crosslinking with ultraviolet radiation at a radiation distance of 20 cm for 6h, and then performing chemical crosslinking with 0.01 mol/L carbodiimide (the mass ratio of carbodiimide to recombinant collagen was 1:1) at a reaction temperature of 4°C for 5h;

(3) washing in the washing device according to the present disclosure 5 times, with 20

minutes each time;

(4) drying the washed recombinant collagen sponge at a temperature of 60°C for 3 hours; encapsulating a sample thereof and sterilizing it with 15 kGy Co^{60} irradiation to obtain a recombinant collagen sponge material.

Example 2

This Example provides a recombinant collagen sponge material and a method for preparation thereof. The method comprised:

(1) preparing a 5% solution of a recombinant collagen (molecular weight: 38 kDa) and stirring it thoroughly; pouring the recombinant collagen solution into a mold for forming, and lyophilizing it by a freeze-drying method with the following lyophilization parameters: -50°C for 6h, -30°C for 2h, -20°C for 2h, -10°C for 2h, 0°C for 2h, 10°C for 10h, 20°C for 20h, 30°C for 50h;

(2) performing physical crosslinking at a high temperature of 110°C for 2h, and then performing chemical crosslinking with a 0.005 mol/L solution of genipin (the mass ratio of genipin to recombinant collagen was 1:4) at a reaction temperature of 25°C for 2h;

(3) washing in the washing device according to the present disclosure 6 times, with 15 minutes each time;

(4) freeze-drying the washed recombinant collagen sponge with the same lyophilization parameters as in the first washing; encapsulating a sample thereof and sterilizing it with 25 kGy Co^{60} irradiation to obtain a recombinant collagen sponge material.

Example 3

This Example provides a recombinant collagen sponge material and a method for preparation thereof. The method comprised:

(1) preparing a 1% solution of a recombinant collagen (molecular weight: 38 kDa)

and stirring it thoroughly; pouring the recombinant collagen solution into a mold for forming, and lyophilizing it by a freeze-drying method with the following lyophilization parameters: -50°C for 6h, -30°C for 2h, -10°C for 4h, 0°C for 2h, 10°C for 5h, 20°C for 20h, 30°C for 50h;

(2) performing physical crosslinking with γ rays at a radiation dose of 35 kGy, and then performing chemical crosslinking with a 0.015 mol/L solution of glutaraldehyde (the mass ratio of glutaraldehyde to recombinant collagen was 1:4) at a reaction temperature of 25°C for 1h;

(3) washing in the washing device according to the present disclosure 9 times, with 10 minutes each time;

(4) freeze-drying the washed recombinant collagen sponge with the same lyophilization parameters as in the washing; encapsulating a sample thereof and sterilizing it with 25 kGy Co^{60} irradiation to obtain a recombinant collagen sponge material.

Test experiments

1. Cell proliferation test on recombinant collagen sponge material

A sample material with an appropriate size (Example 1) was put in a well plate, and L929 cells growing in the exponential phase was inoculated onto the material placed in the well plate at a density of 2×10^5 cells. One hour later, 1 ml medium was supplemented to each well and the culturing was continued. After 20 hours, the material was gently rinsed with PBS 3 times and transferred into a new well, and the cell quantity was measured by a CCK-8 method. 1 ml of a medium containing 10% (volume fraction) CCK-8 reagent was added to each well. After incubation for 2h in an incubator, the absorbance at 450 nm was measured with a microplate reader. In the proliferation test, after the cells were inoculated and cultured for 1d, 3d, 5d, and 7d, the number of cells was measured by the CCK-8 method, and the growth of the cells in the material was observed. The experimental results are shown in Figure 3.

It can be seen from Figure 3 that the cell quantity in the blank control group began to decrease after 3 days, and the cell quantity in the bovine collagen sponge control group began to decrease after 5 days. The test group showed an increasing trend from 1 to 7 days, indicating that the recombinant collagen sponge material can provide a more effective space and growth environment for cell proliferation, and has a good effect of guiding tissue repair.

2. Water absorption performance test on recombinant collagen sponge material

The weight of the sample (Example 2) was measured as m1, and the weight after sufficient water absorption in physiological saline (10s) was recorded as m2. According to the equation: Water absorption rate = (m2-m1)/m1, the water absorption rate of each sample was calculated. The results are shown in Table 1 below which shows a comparison of the water absorption rate between samples in two groups.

Table 1

Group	**Water absorption rate**
Control (Bovine collagen sponge)	22.6±2.1
Test (The recombinant collagen sponge of Example 2)	44.3±5.8*

It can be seen from Table 1 that the moisture absorption rate of the test group was significantly higher than that of the control group, and the difference was statistically significant ($P<0.05$).

3. Hemostatic performance test on recombinant collagen sponge material

The evaluation was carried out by the liver hemostasis test in New Zealand rabbits. Specifically, a New Zealand rabbit was laparotomized layer by layer and the liver was exposed. A 0.5cm*1.0cm bleeding wound was made on the liver lobe of the rabbit with a razor blade. The bleeding site was immediately subjected to hemostatic treatment with the recombinant collagen sponge or a natural collagen sponge, and the hemostatic effect and duration for local hemostasis were observed. After observation for a certain period of time, the hemostatic material was removed to observe whether the bleeding continued. The experimental results are shown in Figure 4.

It can be seen from Figure 4 that, as observed after 20s hemostatic treatment with the control group (bovine collagen sponge) and the test group (recombinant collagen sponge) and removal of the material, the test group completely stopped bleeding, while the control group still showed bleeding and did not complete the hemostasis.

It can be known from the above measurement and evaluation results that the recombinant collagen sponge material prepared according to the present disclosure has good clinical effectiveness and can be applied to hemostasis and wound repair in the field of medical surgeries.

4. Wound surface repair test on recombinant collagen sponge material

(1) Rats were allowed to adapt to the environment for one week after purchased to the laboratory.

(2) The rats were anesthetized and subjected to skin preparation, where intraperitoneal anesthetization was performed with 3% sodium pentobarbital at an anesthetic dose of 30 mg/kg, and after successful anesthesia, the back was shaved with an electric clipper.

(3) Three round full-thickness skin excision wounds having a diameter of 15 mm and an area of 1.766 cm^2 were created on the back of the animals with a modelling skin sampler (diameter 15 mm), and marked with Indian ink. The wounds were A, B, C, respectively corresponding to the group of the recombinant collagen sponge material

of Example 1 (i.e., Material group A), the bovine collagen group (i.e., Control group B), and the blank control group (i.e., Blank group C). After the model was established, the wound was covered with the corresponding sponge and fixed with transparent waterproof medical tape (as shown in Figure 5).

The wound surface repairing was observed for 3 to 18 days after the surgery, and the results are shown in Figure 6 (the ordinate represents the relative value of the area of damaged skin, and the abscissa represents the postoperative recovery time). It can be seen from Figure 6 that the wound surfaces in the three groups were all repaired and shrunk over time, wherein the recombinant collagen sponge material group was significantly better than the control group and the blank group, with statistical significance ($P<0.05$).

5. Validation experiment on recombinant collagen raw material

The engineered *Pichia* strain according to the present disclosure was used to carry out large-scale biological fermentation to obtain the recombinant collagen raw material, and the sequence and molecular weight of the raw material protein were determined by N-terminal sequencing, amino acid analysis and mass spectrometry. The results are as follows.

1) N-terminal sequencing

The N-terminal sequence of the sample was determined by Edman degradation as: NH_2-Gly-Pro-Pro-Gly-Glu-Pro-Gly-Asn-Pro-Gly-Lys-Pro-Gly-Ser-Pro (shown in SEQ ID NO: 3), which is consistent with the designed sequence.

2) Amino acid analysis

It can be seen from Figure 7 that the amino acid composition of the prepared sample was G, E, S, T, A, P, K, N, which are consistent with the designed amino acid composition.

3) Mass spectroscopy

It can be seen from the mass spectrometry profile in Figure 8 that the molecular weight of the sample was about 38 kDa, which is consistent with the designed value.

Conclusion: The prepared recombinant collagen is consistent with the designed requirements and is a 38 kDa recombinant collagen.

CLAIMS

1. A recombinant collagen, comprising:

(a) a protein composed of the amino acid sequence represented by SEQ ID NO: 2; and/or

(b) a protein which has the same function as (a) and is derived from (a) by substitution, deletion and/or addition of one or more amino acids in SEQ ID NO:2.

2. The recombinant collagen according to claim 1, wherein the DNA sequence of the polynucleotide encoding the amino acid sequence represented by SEQ ID NO: 2 comprises the DNA sequence represented by SEQ ID NO:1.

3. An engineered *Pichia* strain deposited with the accession number of CGMCC No. 19314, for use in fermentation to obtain the recombinant collagen according to claim 1 or 2.

4. A recombinant collagen sponge material, obtained by sequential physical cross-linking and chemical cross-linking of the recombinant collagen according to claim 1 or 2; wherein the recombinant collagen sponge material has a moisture absorption capacity of 40-50, and porosity of 90% or higher.

5. A method for preparing the recombinant collagen sponge material according to claim 4, comprising the following steps:

- dissolving the recombinant collagen according to claim 1 or 2 in water to obtain a recombinant collagen solution;
- lyophilizing the recombinant collagen solution by a freeze-drying method; and
- subjecting the lyophilized and formed recombinant collagen to physical cross-linking and chemical cross-linking in sequence, to obtain the recombinant collagen sponge material.

6. The method according to claim 5, wherein the recombinant collagen sponge material obtained after the cross-linkings is further subjected to washing, drying, and sterilization.

7. The method according to claim 6, wherein the device used for the washing includes a rotating rod and a porous clamp box fixed on the rotating rod; wherein the porous clamp box is used to hold the recombined collagen sponge, and the rotating rod is used to rotate and drive the porous clamp box to flip in a cleaning medium, so as to wash off residual reagents in the recombinant collagen sponge material.

8. The method according to claim 6, wherein the drying includes one or more of oven drying, freeze drying, and vacuum drying.

9. The method according to claim 6, wherein the sterilization is performed by 15 to 25 kGy Co^{60} irradiation.

10. The method according to claim 5, wherein the physical crosslinking includes one or more of thermal crosslinking, radiation crosslinking, and repeated freezing-reconstitution.

11. The method according to claim 5, wherein the temperature for thermal crosslinking is 110°C, and the crosslinking duration is 2h;
preferably, the radiation source for the radiation cross-linking includes ultraviolet rays and/or gamma rays.

12. The method according to claim 5, wherein the chemical crosslinking is performed by addition of a chemical crosslinking agent including one or more of glutaraldehyde, carbodiimide, and genipin.

13. The method according to claim 5, wherein the concentration of a chemical

crosslinking agent is 0.005 to 0.015 mol/L;

preferably, the mass ratio of the chemical crosslinking agent to the recombinant collagen is 1:1 to 5;

preferably, the chemical crosslinking duration is 1 to 5 hours.

14. The method according to claim 5, wherein the concentration of the recombinant collagen solution is 1% to 5%.

15. The method according to claim 5, further comprising injecting the recombinant collagen solution into a mold for forming before the recombinant collagen solution is subjected to freeze-drying;

preferably, the recombinant collagen solution is freeze-dried at a gradient from -50°C to 30°C.

ABSTRACT

The present disclosure provides a recombinant collagen and a recombinant collagen sponge material. The recombinant collagen comprises: (a) a protein composed of the amino acid sequence represented by SEQ ID NO: 2; and/or (b) a protein which has the same function as (a) and is derived from (a) by substitution, deletion and/or addition of one or more amino acids in SEQ ID NO:2. The recombinant collagen sponge material is obtained by sequential physical cross-linking and chemical cross-linking of the recombinant collagen. The recombinant collagen sponge material according to the present disclosure is capable of hemostasis, wound surface repair, moisture absorption and platelet aggregation, and has high moisture absorption, a significant hemostatic effect and good biocompatibility, assuming great clinical significance in the field of surgery.

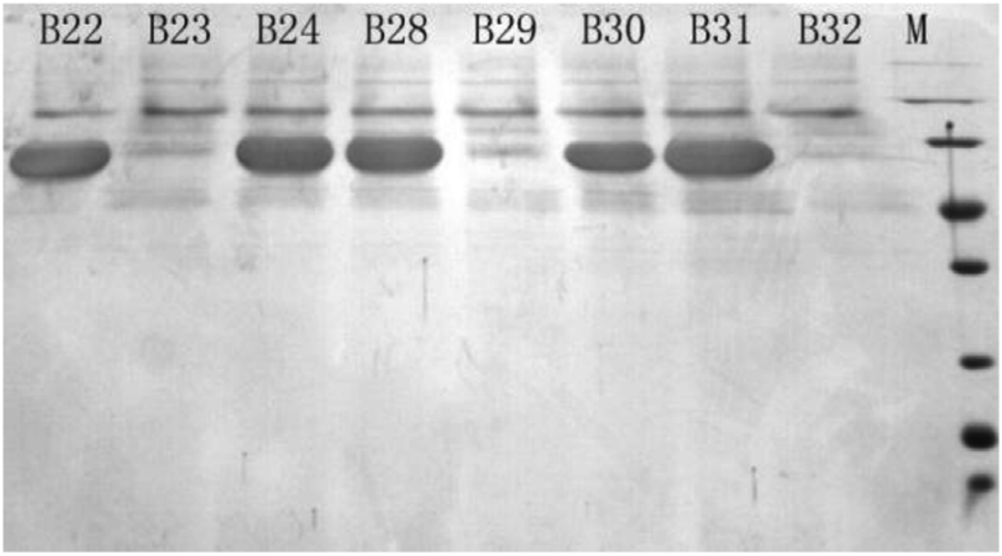


FIG. 1

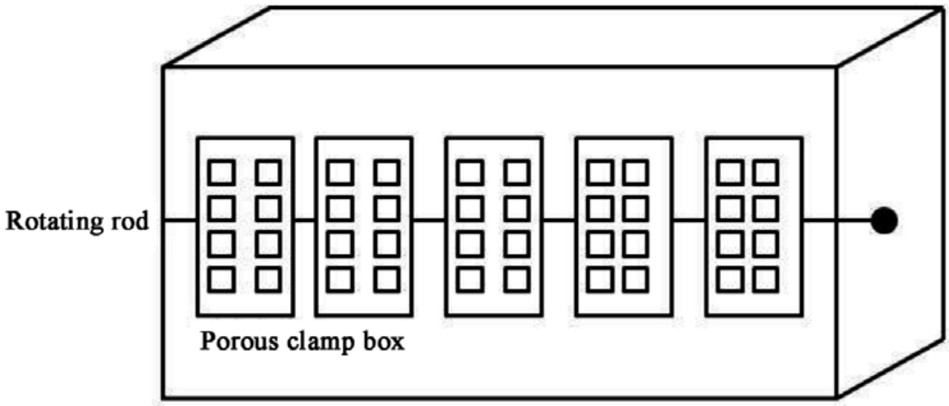


FIG. 2

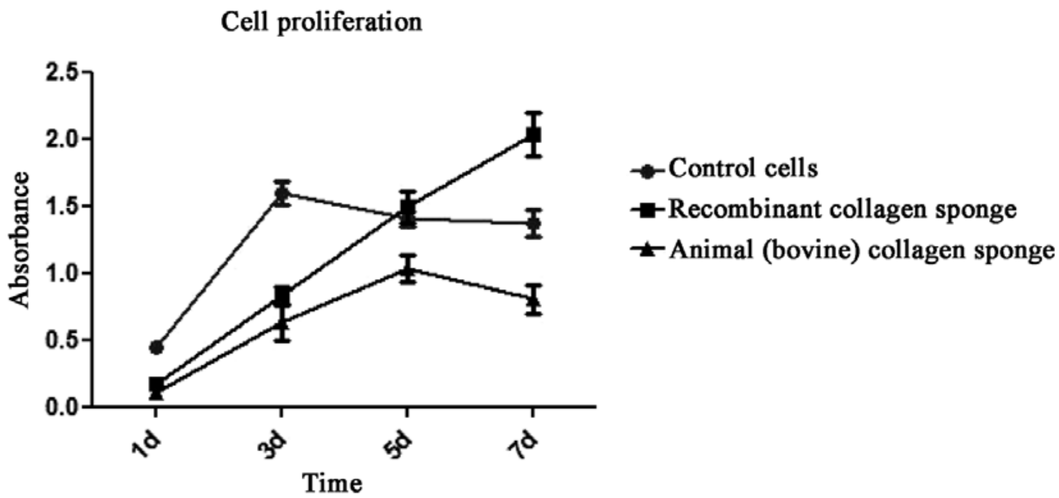

Cell proliferation
2.5
2.0
1.5
1.0
0.5
0.0
Absorbance
1d
3d
5d
7d
Time
Control cells
Recombinant collagen sponge
Animal (bovine) collagen sponge

FIG. 3

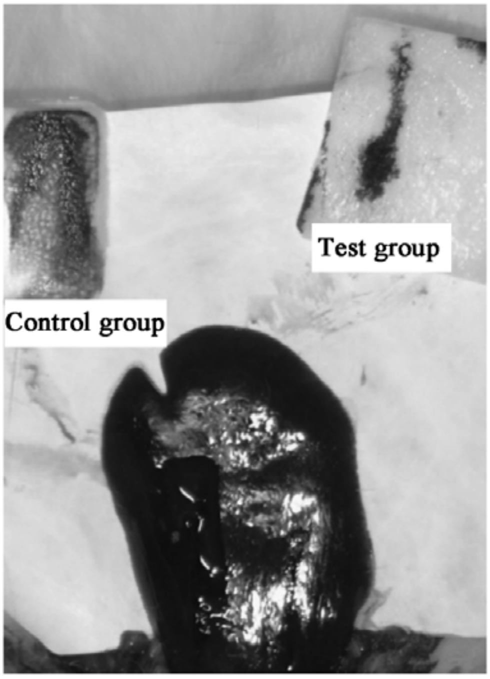


FIG. 4

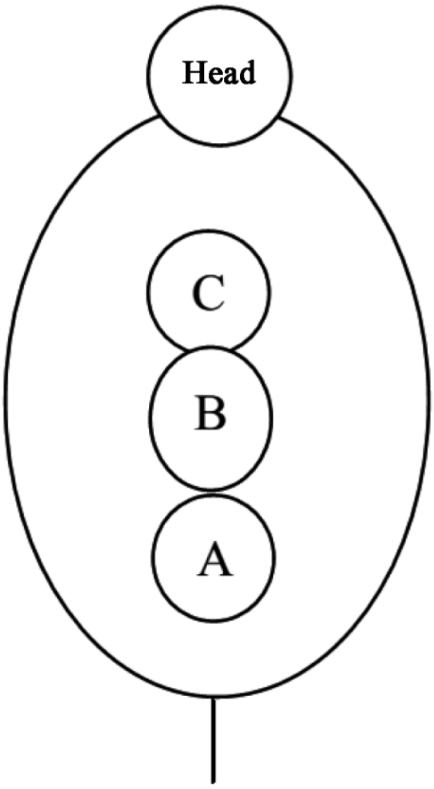


FIG. 5

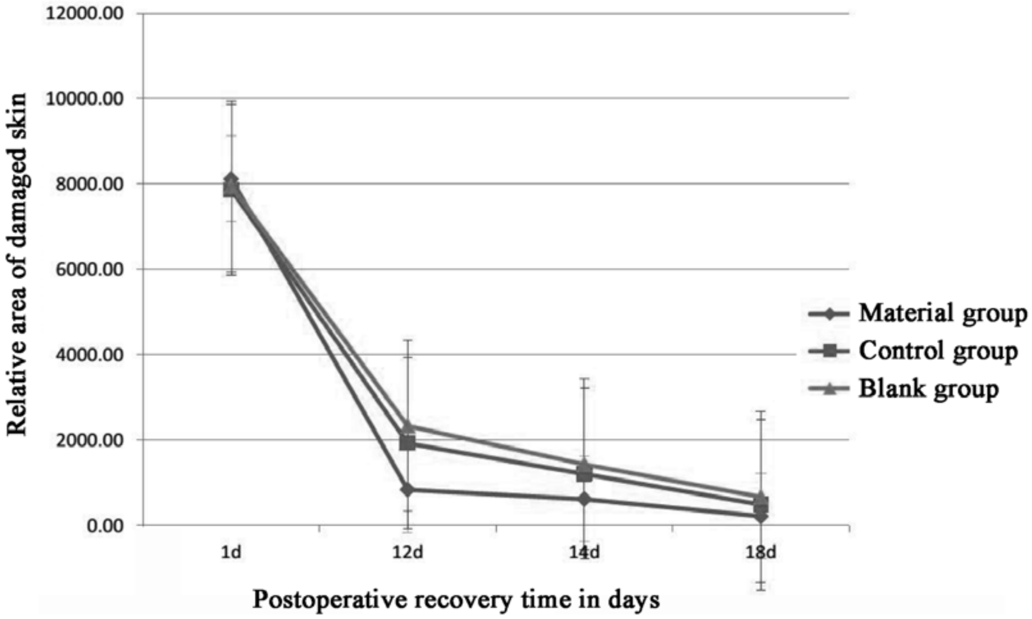

12000.00
10000.00
8000.00
6000.00
4000.00
2000.00
0.00
Relative area of damaged skin
1d
12d
14d
18d
Postoperative recovery time in days
Material group
Control group
Blank group

FIG. 6

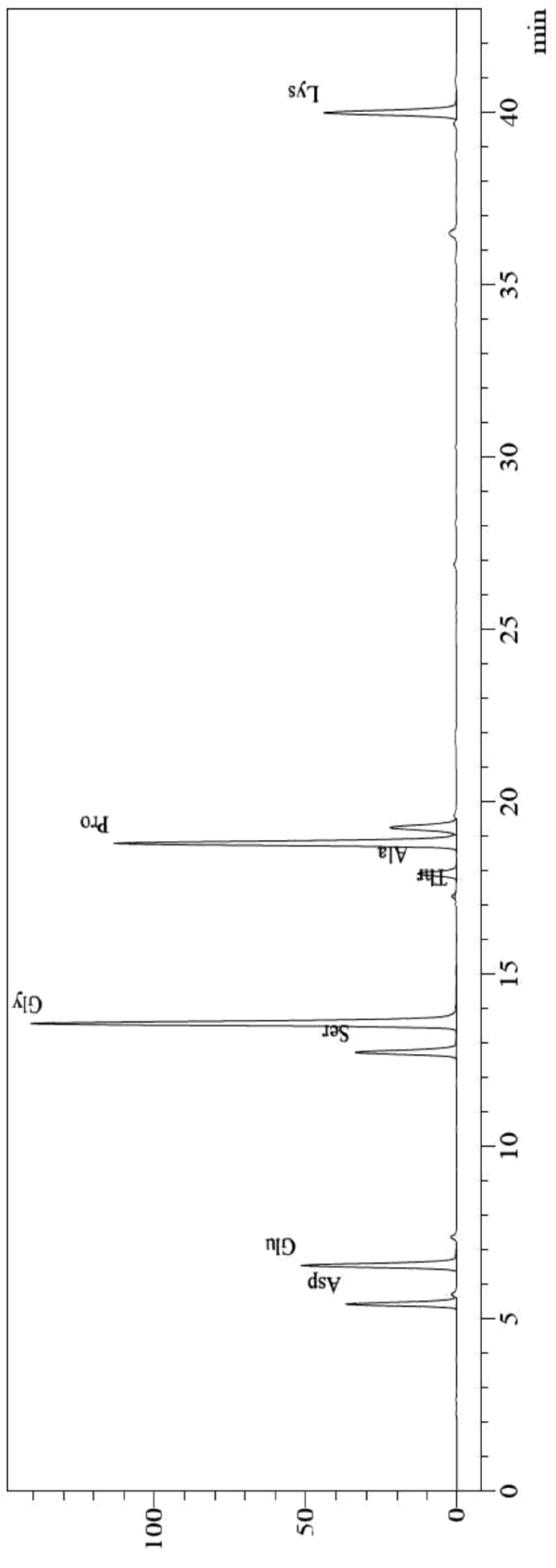
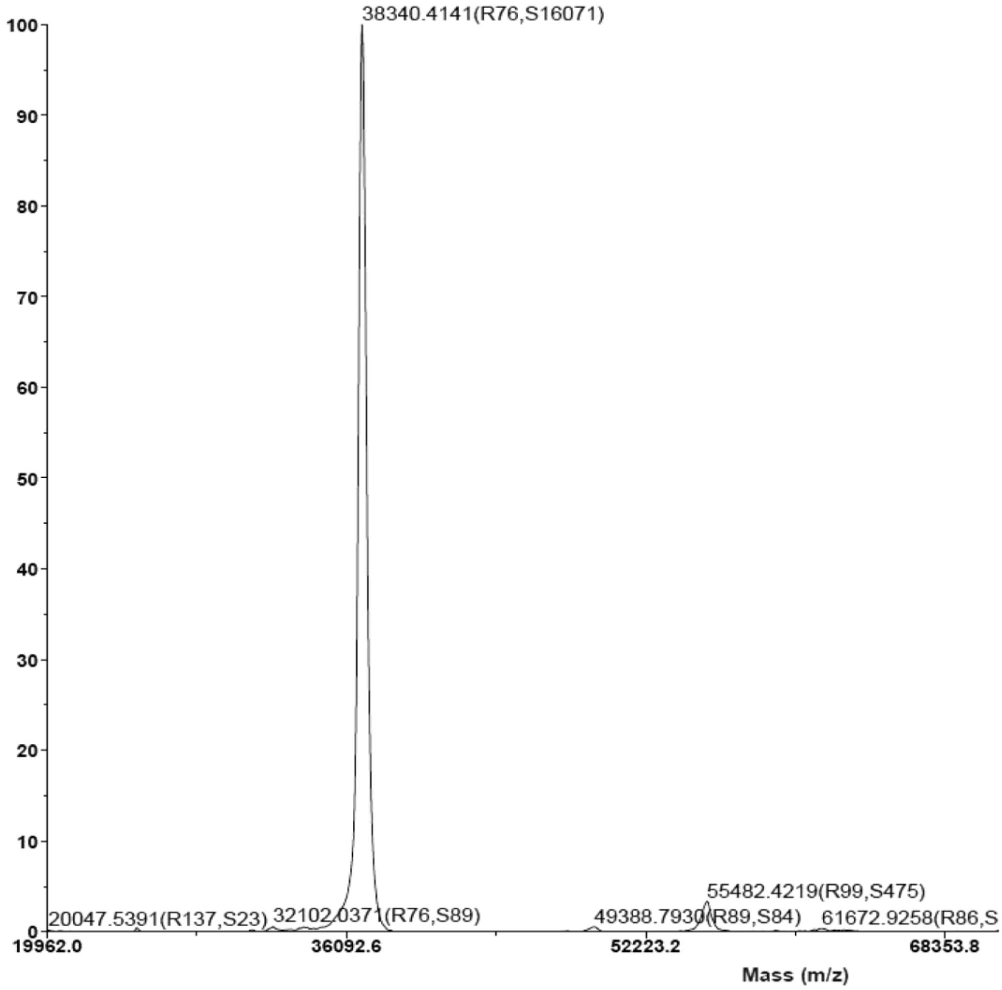


FIG. 7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80
70
60
50
40
30
20
10
0
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32102.0371(R76,S89)
49388.7930(R89,S84)
55482.4219(R99,S475)
61672.9258(R86,S
19962.0
36092.6
52223.2
68353.8
Mass (m/z)

FIG. 8