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(54) Title: SCHIFF-BASED ALDEHYDIC HYALURONIC ACID-CHITOSAN HYDROGEL COMPOSITIONS AND USES THEREOF

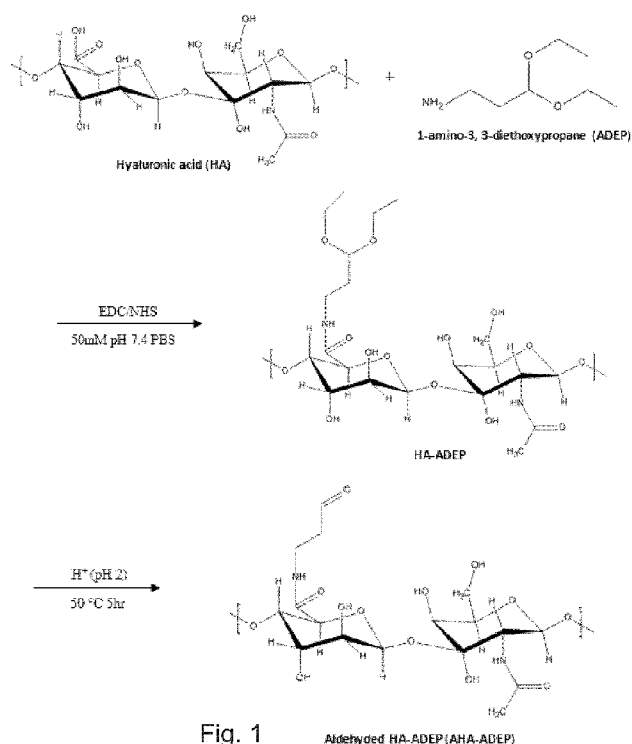


Fig. 1

Aldehyde HA-ADEP (AHA-ADEP)

(57) Abstract: A wound dressing composition, comprising a flexible pharmaceutically acceptable carrier material, and a hydrogel composition coated onto the flexible pharmaceutically acceptable material. The hydrogel composition comprises an aldehydic hyaluronic acid prepared by modifying a hyaluronic acid with a 1-amino-3, 3-diethoxy-propane, and a chitosan conjugated to the aldehydic hyaluronic acid by a Schiff base linkage. The carrier material may be a woven material comprising natural fibers and/or synthetic fibers. Alternatively, the carrier material may be a polymeric sheet material. The wound dressing composition may additionally comprise one or more of a buffer component, a softening agent, and an antimicrobial composition.

TITLE: SCHIFF-BASED ALDEHYDIC HYALURONIC ACID-CHITOSAN
HYDROGEL COMPOSITIONS AND USES THEREOF

TECHNICAL FIELD

The present disclosure pertains to hydrogel compositions. More particularly, the
5 present disclosure pertains to aldehydic hyaluronic acid-chitosan hydrogel compositions for
use in wound dressings, and to methods for preparing the hydrogel compositions.

BACKGROUND

Burn injuries and chronic skin ulcers resulting from, for example, diabetic disease,
long-term bedcare, are major public health issues, as both of these conditions can lead to
10 severe physiological stress and can be life threatening.

Damage to the skin, whether by way of a burn, a skin ulcer, or otherwise, induces a
series of events, including fluid loss, hypothermia, infection, and scar tissue. Consequently,
the process to heal wounds is also a complex series of events, involving responses from many
different cell types and growth factors. These wound healing responses include coagulation,
15 inflammation, matrix degradation, synthesis and deposition, angiogenesis, fibroplasia, re-
epithelialization, contraction, and remodeling.

For the last several decades, tissue-engineered skin has been in clinical use for
treating chronic skin wounds and wounds due to burns. Skin tissue engineering generally
requires a biomimetic extracellular matrix (ECM) that can be assimilated into the body when
20 the new skin tissue is regenerated. Examples of biomaterials that can be used for such a
matrix include, naturally occurring substances, such as collagen, and biodegradable synthetic
polymers and hydrogels (8). In fact, synthetic polymers, such as polymeric fibers and
hydrogels, have recently been used to deliver bioactive molecules and cells to help in
accelerating angiogenesis, epithelial migration and integration during wound healing.

25 Despite progress in the technology of tissue-engineered skin, no synthetic skin has yet
been able to completely replicate normal, healthy skin. Many existing tissue-engineered skin
grafts, for example, have limited, if any, functionalities for cellular proliferation,
angiogenesis and re-epithelialization, which are critical determinants for skin regeneration
and complete wound healing. Skin regeneration after severe burns, such as third-degree

burns, and with chronic wound ulcers of the skin, therefore, still presents a clinical challenge.

Hyaluronic acid (HA) is a naturally occurring substance that is part of the ECM. It has been widely used in tissue engineering as a biocompatible, non-sulfated, glycosaminogenic and non-inflammatory biomaterial. It consists of disaccharide units of [β (1, 4)-D-glucuronic acid- β (1, 3)-N-acetyl-D-glucosamine] linkages. HA can offer a temporary structure in the early stages of wound healing. It directly influences tissue organization by interacting with cell-surface receptors, enhancing the migration of cells, and facilitating ECM remodeling. HA may be modified for its application as an injectable hydrogel using several different methods. One approach is to oxidize HA to obtain aldehyde groups that can react with amino groups from other polymers to form a hydrogel. This oxidization process, however, involves the uses of HIO_4 , NaIO_4 or H_2O_2 to break the HA, causing a loss of the integral structure of HA and further degradation problems. Another approach is to endow HA with alkene bonds that can be used in polymerization to obtain various injectable hydrogels, including those with thermal sensitivity. Initiators and chemical cross-linkers introduced in this approach, however, cause an unavoidable toxicity to host tissues, and therefore, a reduction in biocompatibility.

SUMMARY

The exemplary embodiments of the present disclosure pertains to an aldehydic hyaluronic acid-chitosan hydrogel composition prepared via Schiff base linkage, methods for preparing the hydrogel composition and uses thereof.

The hydrogel composition of the present disclosure comprises hyaluronic acid (HA) aldehyded with 1-amino-3, 3-diethoxy-propane (ADEP) to produce an ADEP-HA combination that is then conjugated to chitosan (CS) using Schiff base linkage to produce an AHA-CS composition. The HA compound in the hydrogel composition retains its natural polysaccharide structure as no crosslinking agent is used that breaks the backbone of the HA polysaccharide structure. The hydrogel composition does not comprise any growth factors, cytokines or additional cells. The hydrogel composition is injectable, bioactive and biocompatible, and thereby can be administered to a subject in need thereof to improve the wound healing process.

An exemplary method for preparing a hydrogel composition of the present disclosure

is provided herein, in which the HA polysaccharide of the hydrogel composition retains its natural polysaccharide structure, and the method generally comprises the steps of providing an aqueous solution of HA, mixing the aqueous solution of HA with ADEP for a sufficient amount of time to allow HA to be aldehyded by ADEP, and thereafter, purifying the HA-ADEP product. The purified HA-ADEP product is then combined with an aqueous solution of CS in the presence of a Schiff base to produce the AHA-CS hydrogel composition of the present disclosure.

An exemplary method for treating a wound in a subject in need thereof comprising the administration or application of the hydrogel composition of the present disclosure to the wound is provided. The hydrogel composition of the present disclosure may be used to treat any type of wound described herein, including, without limitation: chronic skin ulcers, such as, without limitation, diabetic foot ulcers, venous stasis ulcers, pressure ulcers, and ulcers and lacerations of any type that require advanced wound management; and burns, such as without limitation, second, third or fourth degree burns, or any type of burn that requires wound management and treatment. The hydrogel composition of the present disclosure may generally be used to regenerate tissue that has been injured, such as skin tissue.

DESCRIPTION OF THE DRAWINGS

The present disclosure will be described in conjunction with reference to the following drawings in which:

Fig. 1 is a schematic flow chart showing an exemplary process for synthesizing aldehyded 1-amino-3, 3-diethoxy-propane (ADEP)-hyaluronic acid (HA) (referred to as "AHA"), followed by hydrolysis;

Fig. 2 is a schematic flow chart showing an exemplary AHA-CS hydrogel composition of the present disclosure synthesized by combining AHA with chitosan (CS) via Schiff base linkage;

Figs. 3(A) and 3(B) are Fourier-transformed infrared (FTIR) spectroscopy images of the AHA-CS hydrogel compositions and related polysaccharide derivatives disclosed herein; ADEP to form an aldehydic hyaluronic acid;

Figs. 4(A) and 4(B) are charts showing the rheological properties of an exemplary

hydrogel composition according to one embodiment of the present disclosure. Figure 4(A) is a graph illustrating the G' (storage modulus) and G'' (loss modulus) of the hydrogel composition versus time during the gelation process of the AHA and CS mixtures, and Figure 4(B) is a graph illustrating the G' and G'' of an exemplary hydrogel composition versus frequency in Hertz (Hz);

Figs. 5(A) and 5(B) are confocal microscopy images of mesenchymal stem cells (MSCs) that were cultured on an exemplary hydrogel composition of the present disclosure, taken on day 1 (Fig. 5(A)) and on day 14 (Fig. 5(B)) (scale bar = 100 μ m);

Fig. 6 is a chart comparing the viability of mesenchymal stem cells cultured on an exemplary hydrogel ("hydrogel") the viability of control mesenchymal stem cells cultured on Tissue culture polystyrene (TCPS) for 48 h;

Fig. 7 shows representative images of the wound contours of a control group of 24 C57BL/6J mice and a group of 24 C57BL/6J mice treated with an exemplary AHA-CS hydrogel composition of the present disclosure, on days 0, 3, 7, 10, 14, and 21;

Fig. 8 is a chart showing the time-course changes on the raw surface % of the skin wounds of the treated mice (AHA-CS) as compared to control mice (control) on days 0, 3, 7, 10, 14, and 21 (** $P \leq 0.01$);

Fig. 9 is a showing showing wound contraction % of the wounds in treated mice (AHA-CS) compared to control mice (control) on days 0, 3, 7, 10, 14, and 21 (* $P \leq 0.05$ and ** $P \leq 0.01$);

Figs. 10(A), 10(B), 10(C), 10(D) are micrographs showing representative images of H&E stained histological sections of skin wounds and surrounding tissue excised from control mice (control group) and treated mice (AHA-CS group) after 7 days and 14 days of treatment (scale bar = 100 μ m);

Figs. 11(A), 11(B) and 11(C) are charts showing increases in vascularity in the treated mice (AHA-CS) compared to control mice (control) wherein Figure 11(A) is a graph showing the quantification of capillary density in wound beds treated with the hydrogel composition as compared with the control group, and Figures 11(B) and (C) are graphs showing the relative mRNA expression of VEGF-A (Figure 11(B)) and SDF-1 (Figure 11(C)) in control

mice and in treated mice on days 7 and 14 ($P \leq 0.05$);

Figs. 12(A) are micrographs of representative confocal laser scanning microscopy images of wound healing tissues in control mice (control group) while Figs. 12(B) micrographs of representative confocal laser scanning microscopy images of wound healing tissues in treated mice (scale bar = 100 μ m);

Fig. 13 is a graph showing the quantification of Ki67-positive cells in the granulation tissues of control mice (control) and treated mice (AHA-CS) on days 7 and 14, respectively ($**P \leq 0.01$);

Fig. 14 is a chart showing granulation tissue formation and volume quantification of the wound tissue of control mice (control) compared to treated mice (AHA-CS) on day 7 ($P \leq 0.05$);

Fig. 15 shows representative images of H&E stained histological sections of skin wounds and surrounding tissue excised from control mice (control group) and treated mice (AHA-CS group) 7 days and 14 days after treatment (Scale bar = 1mm);

Figs. 16(A) are micrographs showing representative confocal laser scanning microscopy images of p63 immunohistochemical staining of wound healing tissues in control mice (control group), and Figs. 16(B) are micrographs showing representative confocal laser scanning microscopy images of p63 immunohistochemical staining of wound healing tissues in treated mice (AHA-CS group) (scale bar = 100 μ m);

Fig. 17 is a chart showing the percentage of p63-positive cells in wound tissues of treated mice (AHA-CS) compared to control mice (control) on days 7 and 14 ($P \leq 0.05$); and

Figs. 18(A) is a chart showing the relative mRNA expression of MMP9 while Fig. 18(B) is a chart showing relative mRNA expression of MMP3 on days 7 and 14 in control mice (control) and treated mice (AHA-CS) ($P \leq 0.05$).

DETAILED DESCRIPTION

The exemplary embodiments of the present disclosure pertain to aldehydic hyaluronic acid-chitosan hydrogel compositions prepared via Schiff base linkages, that are useful for the treatment of wounds. Some exemplary embodiments pertain to methods for preparing the

hydrogel compositions disclosed herein. Some exemplary methods pertain to uses of the hydrogel compositions disclosed herein.

Healing of wounds is a complex process, including a variety of different cell types and growth factors associated with coagulation, inflammation, matrix degradation, synthesis and deposition, angiogenesis, fibroplasias, re-epithelialization, contraction and remodeling.

Tissue-engineered skin has been used for several decades for treatment of chronic skin wounds, and wounds resulting from burns or other injuries. However, a tissue-engineered skin that is able to completely replicate normal healthy skin is still not available treatment such wounds in patients. For example, many existing tissue-engineered skin grants have limited, if any, functionalities for initiating and/or sustaining thereon and therein cellular proliferation, angiogenesis and re-epithelialization, all of which are critical determinants for successful skin regeneration and complete wound healing. Many tissue-cultured skins incorporate hyaluronic acid (HA) because it is a biocompatible, non-sulfated, glycosaminogenic and non-inflammatory biomaterial. However, the current use of HA hydrogels for skin regeneration strategies is limited due to the harsh chemical modifications that occur to the HA molecules during their production. The chemical modifications result in losses in the structural integrity of the HA molecules and/or in reduced bio-compatibility with mammalian tissues.

The term “a cell” includes a single cell as well as a plurality or population of cells. Administering an agent to a cell includes both *in vitro* administrations and *in vivo* administrations.

The term “about” or “approximately” means within 20%, preferably within 10%, and more preferably within 5% of a given value or range.

The word “comprise” or variations such as “comprises” or “comprising” will be understood to imply the inclusion of a stated integer or groups of integers but not the exclusion of any other integer or group of integers.

The word “complexed” as used herein means attached together by one or more linkages.

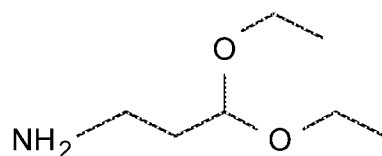
As used herein, “hydrogel” means the polymeric materials of the present disclosure

that are extensively swollen in aqueous medium and retain a significant fraction of the aqueous medium within a three dimensional network (cross-linked structure) without dissolving in the aqueous medium.

As used herein, “hyaluronic acid” and “HA” means the compound constituted of series of repeating dimeric units of D-glucuronic acid and N-acetylglucosamine. The term “hyaluronic acid” is also intended to include not only elemental hyaluronic acid, but hyaluronic acid with traces of other elements or in various compositions with other elements, as long as the chemical and physical properties of hyaluronic acid remain unchanged. In addition, the term “hyaluronic acid” as used in the present disclosure includes natural formulas, synthetic formulas or a combination of these natural and synthetic formulas, and derivatives thereof.

The HA used in the present disclosure may be extracted from animal tissue exemplified by rooster combs and umbilical cords, or alternatively from bacterial cultures exemplified as those of hemolytic group A or C Streptococci. Those skilled in the art will appreciate that the HA of the present disclosure may be obtained from any other source so long as it is pure enough to avoid provoking an adverse or toxic reaction in the human in which it is introduced.

As used herein, “1-amino-3, 3-diethoxy-propane” and “ADEP” means the compound having the chemical structure of:



Eq.

As used herein, “chitosan” or “CS” means the polysaccharide derived from chitin with a linear structure consisting of β -(1-4)-linked 2-amino-2-deoxy-*D*-glucose and 2-acetamido-2-deoxy-*D*-glucose units. Chitosan is a naturally occurring material found in crustacean shells and some fungi, and is a biocompatible, hydrophilic polymer with hemostatic and antimicrobial characteristics. Chitosan is useful in the present composition for its cell adhesion properties, due to its GAG-like structure, and for its anti-infection functions and low cytotoxicity. The chitosan of the present disclosure may be unmodified or may be

modified with other chemical groups, such as primary amine groups or succinyl groups, and may be obtained from a naturally occurring source or may be synthetically derived. A person skilled in the art would appreciate that any suitable form of chitosan may be used in the present exemplary hydrogel compositions.

5 As used herein, “AHA-CS” means the exemplary aldehydic hyaluronic acid-chitosan hydrogel compositions of the present disclosure.

 As used herein, “Schiff base” means a compound with a functional group that contains a carbon-nitrogen double bond with the nitrogen atom connected to an aryl or alkyl group, not hydrogen. The “Schiff base” in the present disclosure may have the general
10 formula $R^1R^2C=NR^3$, where R is an organic side chain. Any Schiff base satisfying these criteria may be used in the present disclosure for the AHA-CS hydrogel composition. As used herein, “Schiff base linkage” means the use of a Schiff base of the present disclosure in linking two polysaccharide derivatives together, in particular, the aldehyded HA-ADEP compound and chitosan.

15 The term “MSCs” as used herein means bone-marrow-derived mesenchymal stem cells.

 The term “antimicrobial” as used herein means antibiotic, antiseptic, disinfectant. The term “subject” as used herein includes all members of the animal kingdom, and specifically includes humans.

20 The term “pharmaceutically acceptable carrier” as used herein means a flexible material that is capable of: (i) supporting and retaining an exemplary AHA-CS hydrogel composition of the present disclosure applied onto and/or within a surface of the flexible material, (ii) conforming to an external surface of a mammalian subject with which it comes into contact, (iii) maintaining the contact with the subject’s external surface for an
25 extended period of time, so as to facilitate topical application of the composition to the surface without any adverse physiological response, and without being appreciably decomposed by aqueous contact during contact with the subject’s external surface.

 The present disclosure pertains to hydrogel compositions comprising HA aldehyded with 1-amino-3, 3-diethoxy-propane (ADEP) to produce an ADEP-HA combination which is
30 then conjugated to chitosan (CS) using Schiff base linkage. The HA molecules in the present

hydrogel compositions retain their natural polysaccharide structure. The present hydrogel compositions are injectable, bioactive, biocompatible, and therefore can be administered to a subject in need thereof to improve wound healing. The exemplary hydrogel compositions do not comprise any additional growth factors, cytokines or cells, or any cross-linking agents
5 that break the backbone of the HA polysaccharide structure.

The amount of HA present in the present exemplary hydrogel compositions may vary from approximately 10% to approximately 85% of the hydrogel composition, or any amount therebetween. By way of example, the amount of HA may vary from approximately 10% to approximately 80%, or any amount therebetween, from approximately, 10% to approximately
10 65%, or any amount therebetween, from approximately 10% to approximately 45%, or any amount therebetween, from approximately 10% to approximately 30%, or any amount therebetween, from approximately 10% to approximately 25%, or any amount therebetween, from approximately 30% to approximately 65%, or any amount therebetween, from approximately 30% to approximately 45%, or any amount therebetween, from approximately
15 45% to approximately 65%, or any amount therebetween, of the hydrogel composition. For example, the amount of HA present in the hydrogel composition may be 10%, 12%, 15%, 18%, 20%, 22%, 25%, 28%, 30%, 32%, 35%, 38%, 40%, 42%, 45%, 48%, 50%, 52%, 55%, 58%, 60%, 62%, 65%, 68%, 70%, 72%, 75%, 78%, 80%, 81%, 82%, 83%, 84% or any amount therebetween, of the hydrogel composition.

The amount of ADEP present in the present exemplary hydrogel compositions may vary from approximately 0.1% to approximately 35% of the hydrogel composition, or any amount therebetween. By way of example, the amount of ADEP may vary from approximately 0.1% to approximately 30%, or any amount therebetween, from approximately 0.1% to approximately 20%, or any amount therebetween, from approximately 0.1% to approximately
20 approximately 19%, or any amount therebetween, from approximately 0.1% to approximately 8%, or any amount therebetween, from approximately 9% to approximately 18%, or any amount therebetween, from approximately 9% to approximately 20%, or any amount therebetween, from approximately 19% to approximately 20%, or any amount therebetween, of the hydrogel composition. For example, the amount of ADEP present in the hydrogel
25 composition may be approximately 0.2%, 0.5%, 0.8%, 1%, 2%, 5%, 8%, 10%, 12%, 15%, 16%, 17%, 18%, 19%, 20%, 22%, 25%, 28%, 30%, 31%, 32%, 33%, 34% or 35%, or any amount therebetween, of the hydrogel composition.

The amount of CS present in the present exemplary hydrogel compositions may vary from approximately 10% to approximately 80% of the hydrogel composition, or any amount therebetween. By way of example, the amount of CS may vary from approximately 10% to approximately 70%, or any amount therebetween, from approximately 10% to approximately 51%, or any amount therebetween, from approximately 10% to approximately 50%, or any amount therebetween, from approximately 10% to approximately 29%, or any amount therebetween, from approximately 10% to approximately 29%, or any amount therebetween, from approximately 20% to approximately 80%, or any amount therebetween, from approximately 30% to approximately 70%, or any amount therebetween, from approximately 30% to approximately 50%, or any amount therebetween, or from approximately 51% to approximately 70%, or any amount therebetween, of the hydrogel composition. For example, the amount of CS present in the hydrogel composition may be approximately 11%, 12%, 15%, 18%, 20%, 22%, 25%, 28%, 29%, 30%, 32%, 35%, 38%, 40%, 42%, 45%, 48%, 50%, 52%, 55%, 58%, 60%, 65%, 68%, 70%, 72%, 75%, 78%, 79% or 80%, or any amount therebetween, of the hydrogel composition.

The present exemplary hydrogel compositions can optionally comprise salts and/or buffers. Suitable salts are exemplified by sodium chloride, potassium chloride and the like. Suitable buffers are exemplified by ammonium, phosphate, borate, bicarbonate, carbonate, cacodylate, citrate, and other organic buffers such as tris(hydroxymethyl) aminomethane (TRIS), morpholine propanesulphonic acid (MOPS), and N-(2-hydroxyethyl) piperazine-N'(2-ethanesulfonic acid) (HEPES). Suitable buffers can be chosen based on a desired pH range for an exemplary hydrogel composition.

Additional additives may be present in the formulation for modification of the mechanical properties of the composition. Suitable additives are exemplified by fillers, softening agents and stabilizers. Suitable fillers are exemplified by carbon black, metal oxides, silicates, acrylic resin powder, and various ceramic powders. Suitable softening agents are exemplified by dibutyl phosphate, dioctylphosphate, tricresylphosphate, tributoxyethyl phosphates and other esters. Suitable stabilizers are exemplified by trimethyldihydroquinone, phenyl-beta-naphthyl amine, p-isopropoxydiphenylamine, diphenyl-p-phenylene diamine and the like.

The present exemplary hydrogel compositions can optionally comprise one or more antimicrobial compositions. Classes of suitable antibiotic compositions for incorporation into

the present exemplary hydrogel compositions include aminoglycosides exemplified by tobramycin, gentamicin, neomycin, streptomycin, and the like; azoles exemplified by fluconazole, itraconazole, and the like; β -lactam antibiotics exemplified by penams, cepheids, carbapenems, monobactams, β -lactamase inhibitors, and the like; cephalosporins exemplified by cefacetrile, cefadroxyl, cephalexin, cephalozin, cefproxil, cefbuperazone, and the like; chloramphenicol; clindamycin; fusidic acid; glycopeptides exemplified by vancomycin, teicoplanin, ramoplanin, and the like; macrolides exemplified by azithromycin, clarithromycin, dirithromycin, erythromycin, spiramycin, tylosin, and the like; metronidazole; mupirocin; penicillins exemplified by benzylpenicillin, procaine benzylpenicillin, benzathine benzylpenicillin, phenoxymethylpenicillin, and the like; polyenes exemplified by amphotericin B, nystatin, natamycin, and the like; quinolones exemplified by ciprofloxacin, ofloxacin, danofloxacin, and the like; rifamycins exemplified by rifampicin, rifabutin, rifapentine, rifaximin, and the like; sulfonamides exemplified by sulfacetamine, sulfadoxine, and the like; tetracyclines exemplified by doxycycline, minocycline, tigecycline, and the like; and trimethoprim, among others.

Some exemplary embodiments of the present disclosure pertain to methods of preparing the present exemplary hydrogel compositions wherein the HA retains its natural polysaccharide structure.

The exemplary methods generally comprise the steps of providing mixing together an aqueous solution of HA with an aqueous solution of ADEP for a sufficient amount of time to allow HA to be aldehyded by ADEP, after which, the HA-ADEP product is purified (Fig. 1). The purified HA-ADEP product is then combined with an aqueous solution of CS in the presence of a Schiff base to produce the AHA-CS hydrogel composition of the present disclosure (Fig. 2).

Different ratios of the aqueous component solutions may be used provided the properties of the solutions and the ratios are such that they crosslink to form the hydrogel composition of the present disclosure when combined. A person skilled in the art can achieve different gelation rates of the hydrogel compositions by manipulating the mass ratios of AHA:CS. Suitable mass ratios of AHA:CS used in the present disclosure may vary from about 0.1:0.5 to about 0.1:3.0, or any amount therebetween. For example, about 0.1:1.0, about 0.1:1.5, about 0.1:2.0, about 0.1:2.5, about 0.1:3.0. Suitable mass ratios of AHA:CS may also vary from about 0.6:1.0 to about 0.6:3.0, or any amount therebetween. For example,

about 0.6:1.5, about 0.6:2.0, about 0.6:2.5, about 0.6:3.0. Suitable mass ratios of AHA:CS may also vary from about 1.0:1.5 to about 1.0:3.0, or any amount therebetween. For example, about 1.0:2.0, about 1.0:2.5.

In one embodiment, an aqueous solution of HA comprises dissolving HA in a suitable
5 neutral or basic buffer, and then mixing the HA with N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-hydroxy-succinimide (NHS) either prior to adding ADEP or alternatively, simultaneously with the addition of ADEP.

In another embodiment, the HA-ADEP product that is purified after the mixing of HA with ADEP may be dissolved in a solution such that the pH of the resulting HA-ADEP
10 solution is acidic for hydrolyzing the aldehyde group for the aldehyde process. After a suitable period of time, such as, but not limited to 24 hours, the pH of the HA-ADEP solution may be substantially neutralized.

In another embodiment, the hydrogel composition is formed prior to application to a target area on a subject. The component solutions may be mixed in an appropriate vessel and
15 allowed to cure to a suitable state of gelation for use.

In another embodiment, the hydrogel is formed *in situ*. Aqueous solutions of the components of the hydrogel composition can be simultaneously applied or deposited to a target area by spraying, streaming, injecting, painting or pouring of the solutions. The components mix together upon application to the target area and then crosslink to form a
20 polymer network. The formation of the polymer network in an aqueous media creates the hydrogel. Alternatively, the components may mix during delivery to the target area. This may happen in the air in the case of the aerosolized or spray applications, or alternatively, in the lumen of a delivery device in the case of a streaming or injection delivery. The mixing of the component solutions may be aided by the use of static or active mixing elements in either
25 delivery, such as inclusion of non-reactive elements exemplified by beads.

Another method of application would be to mix the aqueous component solutions prior to delivery provided the cure time of the hydrogel was appropriately chosen.

The component aqueous solutions may be applied or deposited simultaneously to the target area, or in an iterative fashion (application of an initial component solution followed by
30 application of a second component solution, etc.). The method of application or deposition

may be any of those described above. Furthermore, various methods and devices as are known in the art may be used for performing *in situ* curing of a multi-component system.

As used herein, the term “gelation” means the formation of a three-dimensional network and thus the transition from a liquid composition to a viscous composition. An *in situ* formation of the hydrogel is due to the transition from a liquid state to a solid state at the site of application in the body. The mechanism of gelation of the present disclosure is based on the Schiff base reaction between amino and aldehyde groups on the polysaccharide derivatives of the hydrogel composition (Fig. 2).

As is evident from the description above and the exemplary embodiments illustrated in Figs. 1 and 2, a cross-linking agent that breaks the backbone of the HA polysaccharide structure is not introduced during the preparation and gelation of the hydrogel composition. Those skilled in the art will appreciate that such exemplary methods will, therefore, allow the HA in the hydrogel composition to retain its natural polysaccharide structure since the structure of the polysaccharide backbone of HA is not compromised, as reported in prior art.

Other exemplary embodiments of the present disclosure pertain to methods for treating wound comprising application of the exemplary hydrogel compositions to target surfaces on mammalian subjects. As used herein, the term “wound” means any injury to external skin surfaces of a subject’s body, caused by a cut, blow, fire, heat, irritation, inflammation or any biochemical or biological condition of a subject, that involves the laceration or breaking of the surface layers of the skin.

For example, the present hydrogel compositions may be used to act as an agent for the treatment of diabetic foot ulcers, venous stasis ulcers, pressure ulcers, or ulcers and lacerations of any type that require advanced wound management.

The present hydrogel compositions may be used to act as a surface agent for the treatment of severe burns exemplified by second, third or fourth degree burns, or any other type of burn that requires wound management and treatment.

The hydrogel compositions of the present disclosure may generally be used to facilitate regeneration of tissues. As used herein, “regenerate” means to grow back a portion or all of a tissue. For example, methods of regenerating skin to aid in the healing of diabetic foot ulcers, pressure sores, and venous insufficiency are described herein. Tissues which may

be regenerated include, but are not limited to, skin, bone, nerve, blood vessel, and cartilage tissue.

The present hydrogel compositions may be applied as spray coatings. The multiple components of the compositions may be applied sequentially or concurrently to enable curing
5 via partial or full crosslinking of the polysaccharide components at the target site. Spray coatings may be applied to wounds or lesions to aid in or accelerate wound healing.

The present hydrogel compositions may be applied as cured materials i.e., substantially fully crosslinked material that may or may not be hydrated. This may be used, for example, without limitation, in wound healing as a preformed covering with or without an
10 adhesive backing (as commonly used in bandage form).

The present hydrogel compositions may be formulated as a powder for rehydration. The powder may consist of the cured (partially or fully crosslinked) hydrogel composition of the present disclosure that has been ground, milled, chopped, cryomilled, fragmented through syringe to syringe mixing, or any other process that may be used to reduce the size of a
15 material to a desired particle size and subsequently rehydrated. This embodiment may have application in the following exemplary areas: the treatment of diabetic ulcers, the treatment of sinus and mucosal lesions, the treatment of wounds from burns, and as a protective coating for wounds and other types of lesions.

The present exemplary hydrogel compositions may be used for coating onto and/or
20 into flexible pharmaceutically acceptable carrier materials to produce wound dressings suitable for topical application onto a wound site on a subject's body surface. Suitable flexible pharmaceutically acceptable carrier materials are exemplified by woven materials comprising natural fibers and/or synthetic fibers. Suitable natural fibers are exemplified by cotton fibers, linen fibres, hemp fibres and the like. Suitable synthetic fibers comprise
25 filaments formed from polymers exemplified by polyamides, polyesters, polystyrenes, polyacrylates, vinyl polymers (e.g., polyethylene, polytetrafluoro-ethylene, polypropylene and polyvinyl chloride), polycarbonates, polyurethanes, poly dimethyl siloxanes, cellulose acetates, polymethyl methacrylates, ethylene vinyl acetates, polysulfones, nitrocelluloses and similar copolymers. Suitable polymers also include biological polymers which can be
30 naturally occurring or produced *in vitro* by fermentation and the like. Suitable biological polymers are exemplified by collagen, elastin, silk, keratin, and copolymers thereof. The

woven materials may be gauze-like in that they may be very thin and loosely woven. Alternatively, the woven materials may more tightly woven from thicker fibers.

Alternatively, suitable flexible pharmaceutically acceptable carrier materials are exemplified by synthetic polymeric sheets comprising materials exemplified by polyethylene
5 films, polypropylene films, polyamide films and the like. Particularly suitable are liquid-impermeable, vapor-permeable membranes allow vapors to egress from a wound site through the material while preventing liquids from ingressing or egressing through the material. Such membranes are commonly referred to as high moisture vapor transmission rate (MVTR) films formed from hydrophilic polymers exemplified by polyvinyl alcohol, polyvinyl acetate,
10 cellulose-based materials (e.g., ethers, esters, nitrates, and the like) polyvinyl pyrrolidone, polyurethanes, polyamides, polyesters, polyacrylates, polymethacrylates, and polyacrylamides. Such MVTR films may be cross-linked, or blended, or grafted, or copolymerized with each other. The hydrophilicity of MVTR films may be enhanced by surface treatments exemplified by chemical, plasma, light (UV), corona, or other ionizing
15 radiation.

It is optional for a wound dressing comprising a flexible pharmaceutically acceptable carrier material onto and/or into which has been provided an exemplary hydrogel composition of the present disclosure, to have an adhesive material around the periphery of wound dressing to facilitate adherence of the wound dressing in contact with a target wound
20 site after the wound dressing is applied in place.

The present hydrogel compositions may be applied or administered to a target site for between 1 day to 30 days, or any number of days therebetween. For example, without limitation, the hydrogel composition may be applied or administered to a target site for a period of about 2, 4, 6, 8, 11, 12, 13, 15, 16, 17, 19, 20, 22, 23, 24, 26, 27, 28 or 29 days.

It will be appreciated that the hydrogel composition of the present disclosure is useful
25 for treatment of skin defects and tissue injuries. For example, the present hydrogel compositions facilitate acceleration of wound closure (Figs. 7-9), enhance angiogenesis and promote numbers of capillaries at the target application sites (Figs. 10, 11(A)-11(C)), promote cell migration to injury sites (Figs. 12, 13), increase granulation tissue volume to
30 assist in the early wound healing processes (Figs. 14, 15), accelerate re-epithelialization and the building of new tissues after tissue injury (Figs. 16, 17), and facilitate extracellular matrix

(ECM) remodelling during the wound healing processes (Fig. 18).

Some exemplary embodiments pertain to kits for use in packaging and delivering the components of the exemplary hydrogel compositions of the present disclosure. The components may be present in separate containers in the kit. The requisite buffer solutions for the hydrogel composition may be additionally provided in separate containers. Containers are understood to refer to any structure that may hold or surround the components of the hydrogel composition of the present disclosure; exemplary containers include syringes, vials, pouches, capsules, carpules, ampules, cartridges, and the like. The containers may be shielded from visible, ultraviolet, or infrared radiation through the use of additional components (e.g. a foil pouch surrounding a syringe) or through selection of the material properties of the container itself (e.g. an amber glass vial or opaque syringe).

The exemplary kits may also include one or more mixing devices. The kits may additionally include a delivery device (which may or may not comprise a mixing element) exemplified by bandages, dressings, splints, gauze, and the like, for applying the hydrogel composition to an injured tissue or a wound.

The exemplary kits may additionally comprise other components exemplified by desiccants or other means of maintaining control over moisture content in the kit, oxygen scrubbers or other means of maintaining control over oxygen content within the kit, an inert gas atmosphere (for example, without limitation, nitrogen or argon), indicators to convey the maximum temperature experienced by the kit, indicators to convey exposure to sterilizing radiation, ethylene oxide, autoclave conditions, and the like, retaining or positioning structures to prevent damage to the components (for example, without limitation, trays or packaging card), which are required to maintain the product in good condition during transport and storage.

The exemplary kits may additionally include instructions and directions for use.

EXAMPLES

Materials and Methods

Materials

Medium molecular weight chitosan (CS), hyaluronic acid (HA), 1-amino-3, 3-diethoxy-propane (ADEP), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-hydroxy-succinimide (NHS) were all purchased from SIGMA-ALDRICH[®] (SIGMA-ALDRICH is a registered trademark of Sigma-Aldrich Co. LLC, Saint Louis, MO, USA). Bone marrow-derived mesenchymal stem cells (MSCs) were ordered from the American Type Culture Collection (Manassas, VA, USA). LIVE/DEAD[®] Viability/Cytotoxicity Kit (LIVE/DEAD is a registered trademark of Molecular Probes Inc., Eugene, OR, USA) was purchased from Biotium Inc. (Hayward, CA, USA) and a VYBRANT[®] MTT Cell proliferation Assay Kit (VYBRANT is a registered trademark of Molecular Probes Inc., Eugene, OR, USA) was purchased from INVITROGEN[®] (INVITROGEN is a registered trademark of Life Technologies Corp., Carlsbad, CA, USA).

Method of Preparing an Embodiment of the Hydrogel Composition

Hyaluronic acid (HA, 250mg) was dissolved in 10 ml of 0.1M PBS (pH=7.4). EDC (125mg) and NHS (100mg) were added into the solution with magnetic stirring for approximately 30 minutes. ADEP (389mg, 0.427ml) was then added to the HA solution. After overnight, the solution was purified via acetone precipitation and dialysis before freeze-drying. The purified product was re-dissolved in H₂O and the pH of the solution was adjusted to 2.0 using 1M HCl for the aldehyde process. After 24hr, the pH was changed to 6-7 before dialysis and freeze-drying. 2.5% (w/v) chitosan (CS) solution was prepared by dissolving 25 mg of CS in 1% acetic acid solution. The hydrogel can be formed by mixing both solutions of AHA-ADEP:CS with 1:2 mass ratio in a same volume. This exemplary method is illustrated in the schematic flowcharts shown in Figs. 1 and 2.

Analytical instrumentation

The chemical structures of the hydrogel composition and its components were characterized using fourier-transformed infrared (FTIR) spectroscopy. For FTIR analysis, the

materials were powdered and mixed with dry potassium bromide (KBr) at a volume ratio of 1:200. Samples were recorded with a Nicolet Avatar 360 FTIR spectrometer (Thermo Scientific, Waltham, MA, USA) against a blank KBr pellet background.

Characterization of gelation property

5 To evaluate the working ability of the hydrogel composition, measurements of its storage modulus (G') and loss modulus (G'') were conducted. The gelation dynamics of the hydrogel composition were characterized via a viscoelasticity test on an AR2000 Rheometer (TA Instruments, New Castle, DE, USA). The viscosity, storage modulus (G') and loss modulus (G'') were measured at various frequencies at room temperature.

10 *Characterization of the hydrogel composition*

Morphology of the hydrogel was examined using scanning electron microscopy (SEM; Cambridge Stereoscan 120) with an accelerating voltage of 20 kV. The swollen hydrogel samples at their maximum swelling ratio in distilled water were quickly frozen in liquid nitrogen and then freeze-dried for 3 days. The freeze-dried samples were vacuum-
15 coated with a gold layer prior to SEM examination.

Preparation of MSCs for Cell Culture Studies

Bone marrow-derived mesenchymal stem cells (MSCs) were cultured with GIBCO[®] Dulbecco's Modified Eagle's Medium (DMEM; GIBCO is a registered trademark of Life Technologies Corp., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS),
20 1.0×10^5 U/l penicillin, and 100 mg/l streptomycin, at 37°C in 5% CO₂. The two pre-gel solutions (100µl each) were dropped onto two separate coverslips. Gelatinization was allowed to take place after placing one coverslip over the other, for 30 min at 37°C. The resulting hydrogel was then washed three times in fresh DMEM medium. 5×10^4 cells were seeded onto the hydrogel and then incubated.

25 *Cell Staining*

The MSC morphologies on the hydrogel compositions were investigated using confocal laser-scanning microscopy. The cells-laden hydrogels were fixed with 4% paraformaldehyde solution at room temperature for 20 min. After being washed with PBS, samples were permeabilized using 0.5% Triton X-100 in PBS solution at room temperature

for 5 min. The samples were then blocked in 1% bovine serum albumin PBS solution at room temperature for 10 min. The samples were incubated in BODIPY[®] FL phalloidin solution (BODIPY is a registered trademark of Molecular Probes Inc.) for 20 minutes at room temperature. The cell nuclei were stained with TO-PRO[®]-3 (TO-PRO is a registered trademark of Molecular Probes Inc.). Images were taken using a confocal laser scanning microscopy (Olympus FV1000,; Olympus America, Center Valley, PA, USA).

Cell Viability

The gelation of the hydrogel composition was conducted in 96-well culture plates for 5 min at 37°C. MSCs were used to test cell viability. Cells were first seeded onto the hydrogels at a density of 5×10^4 cells/well in a 96-well culture plate. The culture media was changed every 2-3 days. At designated times, each well was washed with PBS solution for 3 times, then 10µl MTT reagent was added, and the culture plate was further incubated for 4 hrs. After that, the solution was removed from the wells and the formazan crystals were dissolved in 200 µl of DMSO and the absorbance was measured at 570 nm (n=3).

Skin Defect Mouse Model

24 C57BL/6J mice (6-8 weeks, weighing between 20 and 25 g at the time of the experiments) were used under an approved animal protocol in Southern Medical University (Tonghe GangZhou, China). Twenty-four hours prior to surgery, the dorsal skin was depilated with a hair remover. On the day of the surgery, animals were anesthetized with 60 mg/kg Nembutal (Sigma-Aldrich, CHN). A square template with the size of 1cm×1cm was marked on the dorsal skin of the mice. For a complete thickness skin defect model, the skin, including its panniculus carnosus, was excised. The wound was first moistened with a drop of sterile saline solution, then either evenly topped with 0.5ml of the hydrogel composition (the treated group) or left untreated (the control group). Next, the wounds were covered with the COMBIDERM[®] dressings (ConvaTec Inc., Skillman, NJ, USA; COMBIDERM is a registered trademark of Rhythm and Blue Inc., West Sussex, UK), which were changed every 3 days. The wound contours were photographed on days 0, 3, 7, 10, 14, and 21. On day 7, 6 animals of each group and on day 14, 6 animals of each group were euthanized and the entire wound, including surrounding tissue, within 5mm were harvested. Each harvested wound was cut through the middle into two halves. One half of the wound sample was fixed in 10% neutral-buffered formalin solution for 48 hours and then stored in 70% alcohol at 4°C for

further H&E staining. The remaining half of the wound sample was frozen in liquid nitrogen for further RT-PCR analysis.

Histological analysis

The wound from control and treated mice, including surrounding tissue, were fixed in formalin and embedded in paraffin for routine histological processing. A 3µm section obtained from each paraffin block was stained using routine H&E protocol, and digital images of each H&E stained section were taken and analyzed using ADOBE® PHOTOSHOP® CS Software (ADOBE and PHOTOSHOP are registered trademarks of Adobe Systems Inc., San Jose, CA, USA). Area and thickness of granulation tissues were measured using Image J software (National Institutes of Health, Bethesda, MD, USA) by 2 independent observers, blinded to the treatment, quantifying the area of granulation tissue at 4× magnification.

RT-PCR

RNA was extracted from frozen tissue samples using RNEASY® Mini Kit (RENEASY is a registered trademark of Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. After DNase digestion, cDNA was synthesized using a SUPERScript® III First Strand Synthesis System for RT-PCR (SUPERScript is a registered trademark of Life Technologies Corp.). Real-time reverse-transcriptase polymerase chain reaction was conducted with primers designed for this study in an ABI PRISM® 7300 system (ABI PRISM is a registered trademark of Applied Biosystems, Foster City, CA, USA) using DYNAMO® SYBR® Green qPCR Kit (DYNAMO and SYBR are registered trademarks of Molecular Probes Inc.). The amount of each RNA sample was normalized using mouse glyceraldehyde-3-phosphate de-hydrogenase (GAPDH) as an internal control. Primers used are set out in Table 1:

TABLE 1: Primers

Primer Name*	Sequence	SEQ ID NO
MMP3-F	5' GACGATGATGAACGATGGA	1
MMP3-R	5' CCATAGAGGGACTGAATACCA	2
MMP9-F	5'TCCAGTACCAAGACAAAGC	3
MMP9-R	5'GAGCCCTAGTTCAAGGGCAC	4
SDF1-F	5' GCCAGTCCCTCTGTTACAA	5
SDF1-R	5'CTGCACTTCCTTGCTAAAGTC	6
VEGF-F	5'TGCCGGTTCCAACCAGAA	7
VEGF-R	5'GTGGAGGAGCGAGCTGAA	8
GAPDH-F	5' GGCCTCCAAGGAGTAAGAAA	9
GAPDH-R	5' GCCCCTCCTGTTATTATGG	10

* MMP3 = matrix metalloproteinase-3; MMP9 = matrix metalloproteinase-9; SDF-1 = stromal cell-derived factor-1; VEGF = vascular endothelial growth factor-A; and GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

5 Ct values (cycle threshold) were used to calculate the amount of amplified PCR product in comparison to the housekeeping gene GAPDH. The relative amount of mRNA was calculated as $2^{-\Delta C_t}$.

Immunohistochemical study for Ki67 and p63

Each wound specimen from each of the euthanized animals was cut into two sections sequentially and placed on the glass slide. Two neighboring slides were stained with mouse anti-Ki67 and p63 (Santa Cruz Biotechnology Inc., Dallas, TX, USA) primary antibodies, respectively, and followed by FITC-labeled secondary antibodies (Life Technologies Corp.). Sections were counterstained with DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) (Life Technologies Corp.) before examination. All evaluations were conducted in a blinded way.

Statistical analysis

Statistical significance was tested by using a one way analysis of variance (ANOVA) with 95% confidence interval. When $P \leq 0.05$, differences were considered to be statistically significant.

5 Example 1: Analysis and Characterization of the Hydrogel Composition*FTIR Spectroscopy Results*

The chemical structures of the hydrogel composition prepared according to the method described above and its components were characterized using Fourier-transformed infrared (FTIR) spectroscopy

10 Fig. 3(A) shows the FTIR spectral image of the polysaccharide derivatives (CS, AHA-ADEP and HA) and the hydrogel composition (AHA-CS hydrogel) of the present disclosure. Fig. 3(B) shows the FTIR spectral image of AHA-ADEP and the hydrogel composition (AHA-CS hydrogel) of the present disclosure between 1500 and 1900 cm^{-1} . The newly appeared characteristic peaks at 1730 cm^{-1} and 2780 cm^{-1} associate with the $-\text{CH}=\text{O}$ stretch vibration and C-H stretching of the aldehyde group on AHA-ADEP. These two peaks
15 became invisible, attributing to the consumption of aldehyde to form the imine bond between AHA-ADEP and CS. FTIR spectrum of the AHA-CS hydrogel composition shows a slight increase of absorption at 1680 cm^{-1} corresponding to the C=N bond. It is clearly showed that there is a minor symmetric vibrational band for the aldehyde group of the modified HA at
20 1730 cm^{-1} , which disappears after covalent linking with amino group on chitosan to form a Schiff base, which appears as a shoulder at 1680 cm^{-1} in Fig. 3(B).

Characterization of gelation properties

To evaluate the working ability of the hydrogel composition, measurements of its storage modulus (G') and loss modulus (G'') were conducted using a viscoelasticity test on
25 an AR2000 Rheometer, as described above.

The gelation time was defined as the time interval between the mixture of the two liquids and the formation of the gel, and the cross point (gel point) of G' and G'' curves was considered as the initiation of sol-gel transition (solid to gelation transition), as shown in Fig.

4(A). The measurements were taken at a frequency of 0.1 Hz. The result suggests that AHA-ADEP and CS form a hydrogel. The viscoelastic properties of the AHA-CS hydrogel composition were then evaluated using a dynamic oscillatory rheometry in which G' and G'' moduli were monitored as a function of sweep frequency at a low strain amplitude. As shown
5 in Fig. 4(B), the loss modulus G'' remains smaller than the storage modulus G' , indicating the AHA-CS hydrogel composition displays an elastic-like behavior.

Example 2: Cell Culture Studies of MSCs Seeding on Hydrogel Compositions

Cell staining

The morphologies of MSCs seeded on hydrogels were investigated using confocal
10 laser-scanning microscopy. 5×10^4 MSCs were seeded on hydrogels on day 0. Samples on day 1 and day 14 were investigated using confocal laser-scanning microscopy.

As shown in Fig. 5(A), on day 1, MSCs were observed to display a relatively homogenous population that exhibited a spindle-shaped phenotype. After being cultivated for 2 weeks, the MSCs exhibited an elongated and tubular shape on the hydrogel on day 14
15 (Fig. 5(B)).

Cell viability

Cell viability of the MSCs seeded on the hydrogel compositions was assessed using an MTT assay after 48 hr cultivation.

Cell viability value of the control (TCPS) in the MTT assay was set as the control.
20 The viability of MSCs seeded on the AHA-CS hydrogel composition was found to be around 55% (Fig. 6).

Example 3: Acceleration of Wound Closure by the Hydrogel Composition

Wound Closure Analysis

The skin defect mouse model described above was used to examine the ability of the
25 hydrogel composition to accelerate wound closure.

After the mice were anesthetized, each entire wound was photographed immediately

by a digital camera (Canon 550D, Tokyo, Japan). Closure measurement was conducted on days 0, 3, 7, 10, 14 and 21 on all mice. Wound sizes at different times (see Fig. 8), and wound contraction (see Fig. 9) were measured using Image J software. The percentage of wound closure was calculated as follows:

$$\text{Wound closure} = \frac{A_0 - A_t}{A_0} \times 100\% \quad \text{Eq. 2}$$

where A_0 and A_t denote the original area of a wound and actual area of the wound on day t , respectively. The original area of the wound is the dimension of the full-thickness skin wound area (1cm×1cm) on day 0. The investigators measuring samples were blinded to the control and treatment groups.

10 Results

The AHA-CS hydrogel composition promoted faster wound closure over time as compared with the control group in the mouse model (Figures 7 and 8). Wound area shrank by 86% in the hydrogel composition-treated group on day 10, as compared with 49% in the control group (Fig. 8, $P \leq 0.001$). Contraction in the AHA-CS hydrogel composition-treated wounds increased to 62% as compared with 31% in the control wounds on day 14 (Fig. 9, $*P \leq 0.05$, $**P \leq 0.01$).

Example 4: Enhancement of Angiogenesis by the Hydrogel Composition

A study was done using the skin defect mouse model described above to assess whether the hydrogel composition of the present disclosure promotes blood vessel formation as blood vessel supply is crucial for wound healing and final integration of implanted avascular tissue scaffolds.

Blood Vessel Density Quantitation

To test whether the hydrogel composition of the present disclosure promotes blood vessel formation, the numbers of capillary in the H&E stained histological sections was quantitated on days 7 and 14.

Capillary density was evaluated using H&E stained histological sections at 20× magnification. The images were analyzed using Adobe® Photoshop® CS Software and blood

vessels were marked and counted (see Fig. 10).

RT-PCR

RT-PCR was performed on RNA extracted from frozen tissue samples from hydrogel composition-treated mice and control mice using the SDF-1 (SEQ ID NO:5, SEQ ID NO:6) and VEGF-A primers (SEQ ID NO:7, SEQ ID NO:8) listed in Table 1. The GAPDH primers (SEQ ID NO:9, SEQ ID NO:10) were used as an internal control. The angiogenic chemokine, CXC chemokine ligand 12 (CXCL12; previously referred to in the prior art as stromal cell-derived factor-1 or SDF-1), has important effects on angiogenesis by recruiting endothelial progenitor cells. During endogenous angiogenesis, vascular endothelial growth factor-A (VEGF-A) has been shown to enhance angiogenesis by promoting endothelial cell mitogenesis and cell migration.

Results

The hydrogel composition of the present disclosure resulted in about 2.5 and 2-fold increase in blood vessel density in wound beds as compared with the control group on days 7 and 14, respectively (see Figs. 10 and 11(A), ** $P \leq 0.001$). The mRNA expression of VEGF and SDF-1 by RT-PCR showed a similar tendency with increased numbers of blood vessel density in hydrogel composition-treated groups as compared with control groups. VEGF expression had approximately a 1.3-fold increase as compared with control groups on day 7 (see Fig. 11(B), $P \leq 0.05$) and SDF-1 expression had a 2.1-fold and 1.6-fold increase as compared with control groups on day 7 and 14, respectively (see Fig. 11(C), $P \leq 0.05$).

Example 5: Promotion of Cell Proliferation by the Hydrogel Composition

A study was conducted to assess whether the hydrogel composition of the present disclosure promotes cell proliferation, because insufficient local cellular proliferation is a profound phenomenon occurring in complex tissue defects resulting from third-degree burns and chronic ulcers.

Immunohistochemical study for Ki67 and p63

To investigate whether the hydrogel composition of the present disclosure enhances cell proliferation in wound sites, immunofluorescence of Ki67 in the wound beds was

quantified using the immunohistochemical method described above. Ki67 is a cellular marker for proliferation.

Results

Fig. 12 shows the immunofluorescence of Ki67 in green and nuclear counterstain with DAPI in blue. The numbers of Ki67 fluorescence-positive cells in the granulation tissues of the treated wounds was quantitated and found to be 2.1 and 1.9 times as much as in the control wounds on days 7 and 14, respectively (see Fig. 13, $P \leq 0.001$).

Example 6: Increase of Granulation Tissue Volume by the Hydrogel Composition

Granulation tissue can serve as a temporary ECM for the body to start rebuilding in early wound healing after injury.

Granulation Tissue Volume

To test whether the hydrogel composition of the present disclosure can assist in increasing granulation tissue volume and consequently assist in wound healing, granulation tissue volume in hydrogel composition-treated groups was compared with control groups.

Histological analysis

After the treated and control mice were euthanized, the wounds of each mice, including its surrounding tissues, were fixed in formalin and embedded in paraffin for routine histological processing, as described above in the Materials and Methods section. The area and thickness of the granulation tissue was measured by 2 independent observers using Image J software, blinded to the treatment, quantifying the area of granulation tissue at 4× magnification.

Results

As shown in Fig. 14, the newly formed granulation tissue in the treated wounds were 2.1 times greater than in the control wounds (see Fig. 14, $P \leq 0.05$). The histological analysis showed the structure of wound beds in the control group as being characterized by a thin and loosely disordered granulation tissue, whereas the hydrogel-treated wounds showed a thick granulation layer in the wound defect. Interestingly, on days 7 and 14, small amounts of

adipocytes and neoformed hair follicles were observed in the granulation tissue and epidermis layer, respectively (see Fig. 15).

Example 7: Acceleration of Re-epithelialization by the Hydrogel Composition

It is known that re-epithelialization is a crucial marker of new tissues being built after skin injury. Keratinocyte migration in the early healing stage is a critical step to accelerating re-epithelialization.

Immunohistochemical Staining of p63

To assess whether the hydrogel composition of the present disclosure is able to accelerate re-epithelialization of new tissues, migration of keratinocytes was examined using p63 immunohistochemical staining.

Results

As shown in Fig. 16, migration of keratinocytes was detected by p63 immunohistochemical staining. p63-positive cells were found on the wound surface including wound edge, as well as in the deep granulation tissue in the treated wound. The numbers of p63-positive cells in the wound tissues of the treated wounds were 1.7 times and 2.7 times as much as that of the control on days 7 and 14, respectively (Fig. 17, $P \leq 0.05$).

Without wishing to be bound by theory, it is thought that this kind of keratinocyte migration may be stimulated by the interactions between the epidermal stem cells and the bioactive hydrogel composition of the present disclosure.

Example 8: Up-Regulating MMPs by the AHA-CS Hydrogel Composition

Matrix metalloproteinases (MMPs) are related to extracellular matrix depositing and remodeling, which is a crucial target for wound healing.

RT-PCR

To test whether treatment with the hydrogel composition of the present disclosure upregulates MMP expression, RT-PCR was conducted using the MMP-3 primers (SEQ ID NO:1, SEQ ID NO:2) and the MMP-9 primers (SEQ ID NO:3, SEQ ID NO:4) listed in Table 1.

Results

MMP-3 expression in the hydrogel-treated wound was 1.6 times as much as that in the control (day 14, $P \leq 0.05$), and MMP-9 expression levels in the treated wounds were 2.3 times (day 7, $P \leq 0.05$) and 9.1 times (day 14, $P \leq 0.01$) as much as that in the control (Figs. 18(A) and (B)). These findings suggest that the hydrogel composition can promote matrix remodeling.

The Examples described herein demonstrate that the hydrogel composition of the present disclosure can stimulate the wound healing mechanism by significantly accelerating wound closure, enhancing cell proliferation and increasing keratinocyte migration. The hydrogel composition also significantly promotes formation of granulation tissue and capillary number in the wounds. Up-regulation of the expression of markers of angiogenesis (VEGF), angiogenic chemotactic factors (SDF-1) and ECM remodeling MMPs (MMP-3, MMP-9) was seen in the hydrogel-treated wounds.

The present disclosure has been described with regard to one or more embodiments. However, it will be apparent to persons skilled in the art that a number of variations and modifications can be made without departing from the scope of the invention as defined in the claims. Examples of such modifications include the substitution of known equivalents for any aspect of the invention in order to achieve the same result in substantially the same way.

CLAIMS

What is claimed is:

1. A wound dressing composition, comprising:
a flexible pharmaceutically acceptable carrier material, and
a hydrogel composition coated onto the flexible pharmaceutically acceptable material, said hydrogel composition comprising an aldehydic hyaluronic acid prepared by modifying a hyaluronic acid with a 1-amino-3, 3-diethoxy-propane, and a chitosan conjugated to the aldehydic hyaluronic acid by a Schiff base linkage.
2. The wound dressing composition of claim 1, wherein the flexible pharmaceutically acceptable carrier material is a woven material comprising at least one of natural fibers, synthetic fibers, and biological polymers.
3. The wound dressing composition of claim 2, wherein the natural fibers contain at least one of cotton fibers or linen fibers or hemp fibers.
4. The wound dressing composition of claim 2, wherein the synthetic fibers contain at least one of polyamides, polyesters, polystyrenes, polyacrylates, vinyl polymers (e.g., polyethylene, polytetrafluoro-ethylene, polypropylene and polyvinyl chloride), polycarbonates, polyurethanes, poly dimethyl siloxanes, cellulose acetates, polymethyl methacrylates, ethylene vinyl acetates, polysulfones, and nitrocelluloses.
5. The wound dressing composition of claim 2, wherein the biological fibers contain at least one of collagen, elastin, silk, and keratin.
6. The wound dressing composition of claim 1, wherein the flexible pharmaceutically acceptable carrier material is a polymeric sheet material.
7. The wound dressing composition of claim 6, wherein the polymeric sheet material is one of a polyethylene film, a polypropylene film, and a polyamide film.

8. The wound dressing composition of claim 6, wherein the polymeric sheet material is a high moisture vapor transmission rate film formed from a hydrophilic polymer.
9. The wound dressing composition of claim 7, wherein the high moisture vapor transmission rate film is formed from one of a polyvinyl alcohol, a polyvinyl acetate, a cellulose-based material, a polyvinyl pyrrolidone, a polyurethane, a polyamide, a polyester, a polyacrylate, a polymethacrylate, and a polyacrylamide.
10. The wound dressing composition of claim 7, wherein the hydrophylicity of the high moisture vapor transmission rate film is enhanced by one of a chemical treatment, a plasma treatment, a UV light treatment, or an ionizing radiation treatment.
11. The wound dressing composition of claim 1, additionally comprising a buffer component.
12. The wound dressing composition of claim 11, wherein the buffer component is one of a sodium chloride buffer, a potassium chloride buffer, an ammonium buffer, a phosphate buffer, a borate buffer, a bicarbonate buffer, a carbonate buffer, a cacodylate buffer, a citrate buffer, a tris(hydroxymethyl) aminomethane buffer, a morpholine propanesulphonic acid buffer, and a N-(2-hydroxyethyl) piperazine-N'(2-ethanesulfonic acid) buffer.
13. The wound dressing composition of claim 1, additionally comprising a softening agent.
14. The wound dressing composition of claim 13, wherein the softening agent is one of dibutyl phosphate, dioctylphosphate, tricresylphosphate, and tributoxylethyl phosphate.
15. The wound dressing composition of claim 1, additionally comprising an antimicrobial composition.
16. The wound dressing composition of claim 15, the antimicrobial composition is one of tobramycin, gentamicin, neomycin, streptomycin, fluconazole, itraconazole, penams,

cephems, carbapenems, monobactams, β -lactamase inhibitors, cefacetrile, cefadroxyl, cephalexin, cephalixin, cephazolin, cefprozil, cefbuperazone, chloramphenicol, clindamycin, fusidic acid, vancomycin, teicoplanin, ramoplanin, azithromycin, clarithromycin, dirithromycin, erythromycin, spiramycin, tylosin, metronidazole, mupirocin, benzylpenicillin, procaine benzylpenicillin, benzathine benzylpenicillin, phenoxymethylpenicillin, amphotericin B, nystatin, natamycin, ciprofloxacin, ofloxacin, danofloxacin, rifampicin, rifabutin, rifapentine, rifaximin, sulfacetamide, sulfadoxine, doxycycline, minocycline, tigecycline, and trimethoprim.

17. A hydrogel composition for dressing a wound, the composition comprising:
 - an aldehydic hyaluronic acid prepared by modifying a hyaluronic acid with a 1-amino-3, 3-diethoxy-propane; and
 - a chitosan conjugated to the aldehydic hyaluronic acid by a Schiff base linkage.
18. The hydrogel composition of claim 17, additionally comprising a buffer component.
19. The hydrogel composition of claim 18, wherein the buffer component is one of a sodium chloride buffer, a potassium chloride buffer, an ammonium buffer, a phosphate buffer, a borate buffer, a bicarbonate buffer, a carbonate buffer, a cacodylate buffer, a citrate buffer, a tris(hydroxymethyl) aminomethane buffer, a morpholine propanesulphonic acid buffer, and a N-(2-hydroxyethyl) piperazine-N'(2-ethanesulfonic acid) buffer.
20. The hydrogel composition of claim 17, additionally comprising a softening agent.
21. The hydrogel composition of claim 20, wherein the softening agent is one of dibutyl phosphate, dioctylphosphate, tricresylphosphate, and tributoxethyl phosphate.
22. The hydrogel composition of claim 17, additionally comprising an antimicrobial composition.
23. The hydrogel composition of claim 22, wherein the antimicrobial composition is one of tobramycin, gentamicin, neomycin, streptomycin, fluconazole, itraconazole, penams,

cephems, carbapenems, monobactams, β -lactamase inhibitors, cefacetrile, cefadroxyl, cephalexin, cephalosin, cefprozil, cefbuparazone, chloramphenicol, clindamycin, fusidic acid, vancomycin, teicoplanin, ramoplanin, azithromycin, clarithromycin, dirithromycin, erythromycin, spiramycin, tylosin, metronidazole, mupirocin, benzylpenicillin, procaine benzylpenicillin, benzathine benzylpenicillin, phenoxymethylpenicillin, amphotericin B, nystatin, natamycin, ciprofloxacin, ofloxacin, danofloxacin, rifampicin, rifabutin, rifapentine, rifaximin, sulfacetamide, sulfadoxine, doxycycline, minocycline, tigecycline, and trimethoprim.

24. A method for preparing a hydrogel composition for dressing a wound, the method comprising the steps of:

mixing together an aqueous solution of hyaluronic acid with an aqueous solution of hyaluronic acid to produce an aldehydic hyaluronic acid product;

purifying the aldehydic hyaluronic acid product;

conjugating with a Schiff base linkage, a chitosan to the aldehydic hyaluronic acid product to produce an aldehydic hyaluronic acid-chitosan complex

25. The method of claim 24, additionally comprising mixing a buffer with the aldehydic hyaluronic acid-chitosan complex.

26. The hydrogel composition of claim 25, wherein the buffer component is one of a sodium chloride buffer, a potassium chloride buffer, an ammonium buffer, a phosphate buffer, a borate buffer, a bicarbonate buffer, a carbonate buffer, a cacodylate buffer, a citrate buffer, a tris(hydroxymethyl) aminomethane buffer, a morpholine propanesulphonic acid buffer, and a N-(2-hydroxyethyl) piperazine-N'(2-ethanesulfonic acid) buffer.

27. The method of claim 24, additionally comprising mixing a softening agent with the aldehydic hyaluronic acid-chitosan complex.

28. The method of claim 27, wherein the softening agent is one of dibutyl phosphate, dioctylphosphate, tricresylphosphate, and tributoxyethyl phosphate.

29. The method of claim 24, additionally comprising mixing an antimicrobial composition with the aldehydic hyaluronic acid-chitosan complex.

30. The method of claim 29, wherein the antimicrobial composition is one of tobramycin, gentamicin, neomycin, streptomycin, fluconazole, itraconazole, penams, cepheems, carbapenems, monobactams, β -lactamase inhibitors, cefacetrile, cefadroxyl, cephalexin, cephalosin, cefprozil, cefbuparazone, chloramphenicol, clindamycin, fusidic acid, vancomycin, teicoplanin, ramoplanin, azithromycin, clarithromycin, dirithromycin, erythromycin, spiramycin, tylosin, metronidazole, mupirocin, benzylpenicillin, procaine benzylpenicillin, benzathine benzylpenicillin, phenoxymethylpenicillin, amphotericin B, nystatin, natamycin, ciprofloxacin, ofloxacin, danofloxacin, rifampicin, rifabutin, rifapentine, rifaximin, sulfacetamide, sulfadoxine, doxycycline, minocycline, tigecycline, and trimethoprim.

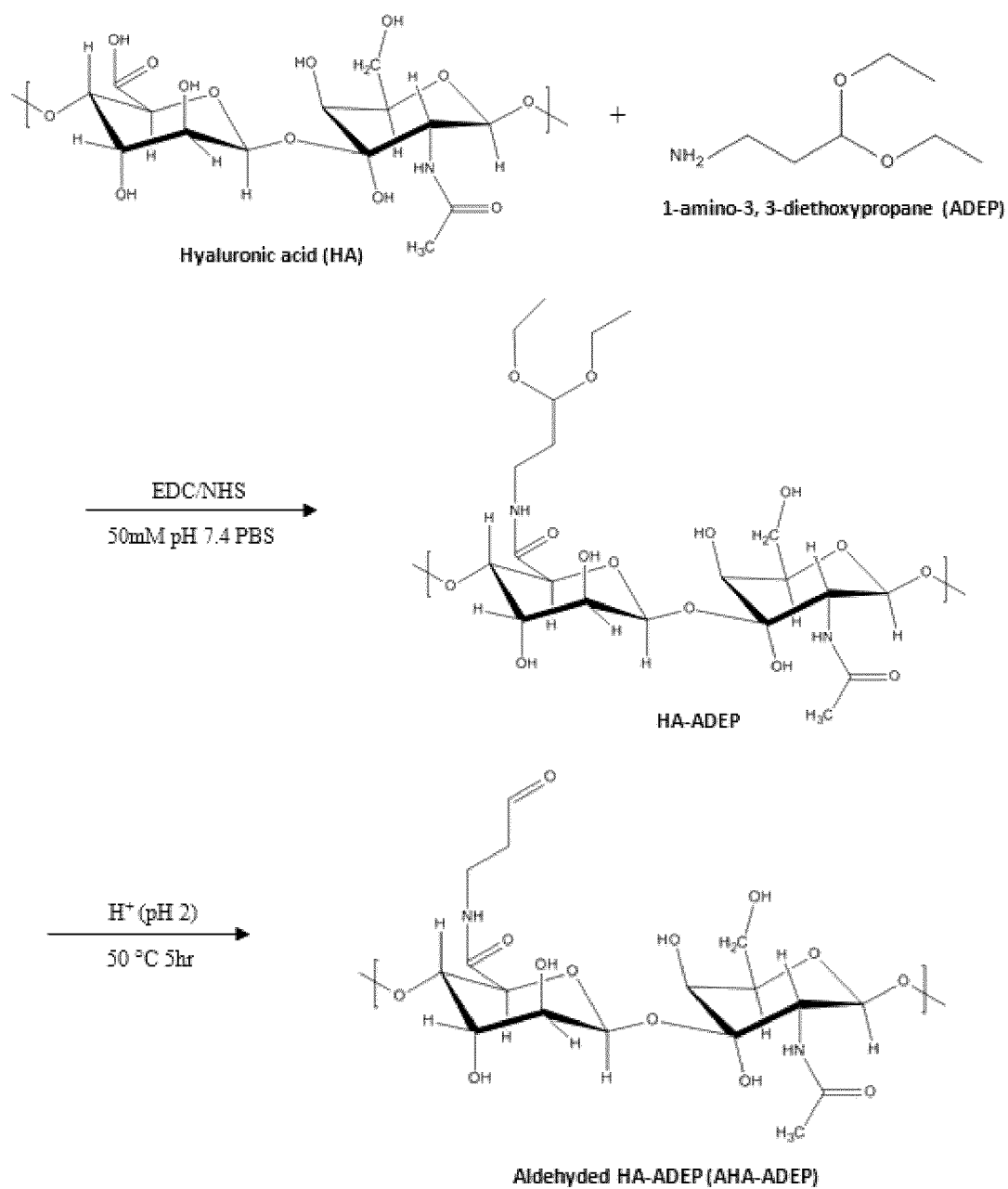


Fig. 1

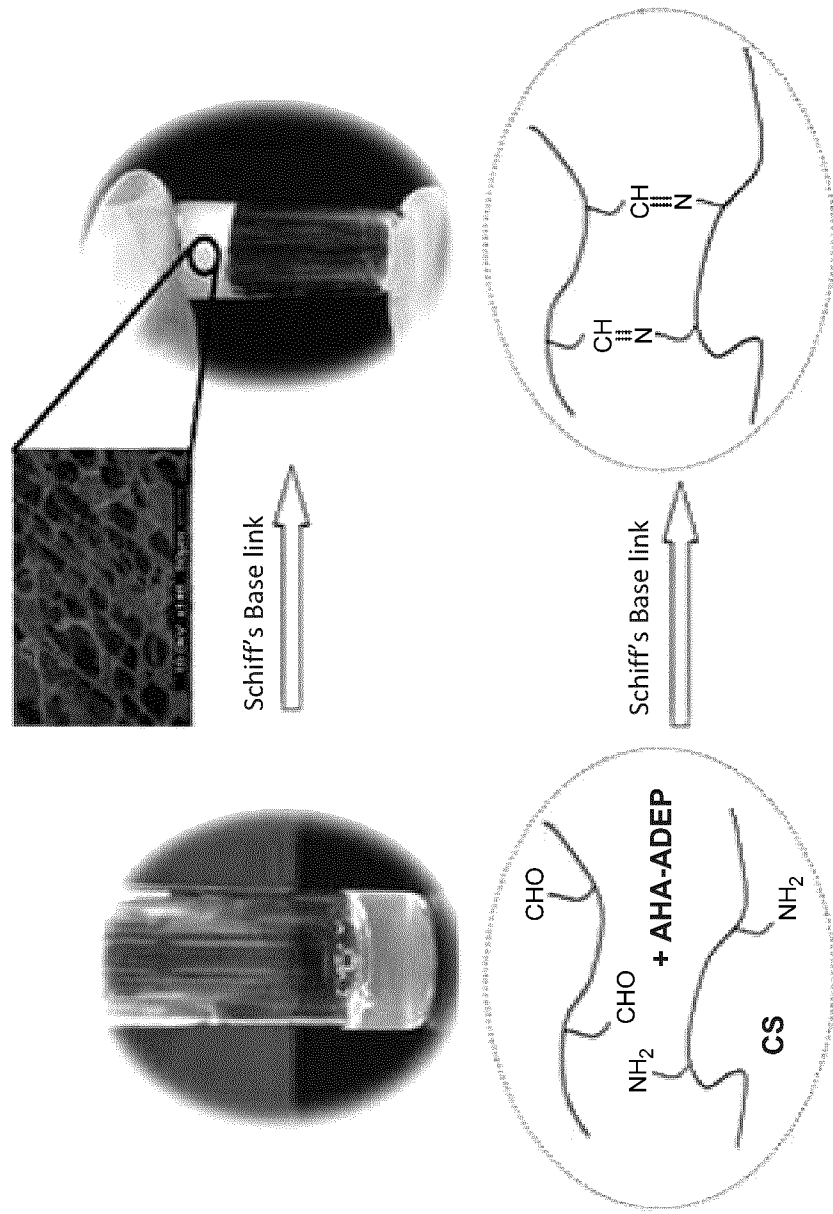


Fig. 2

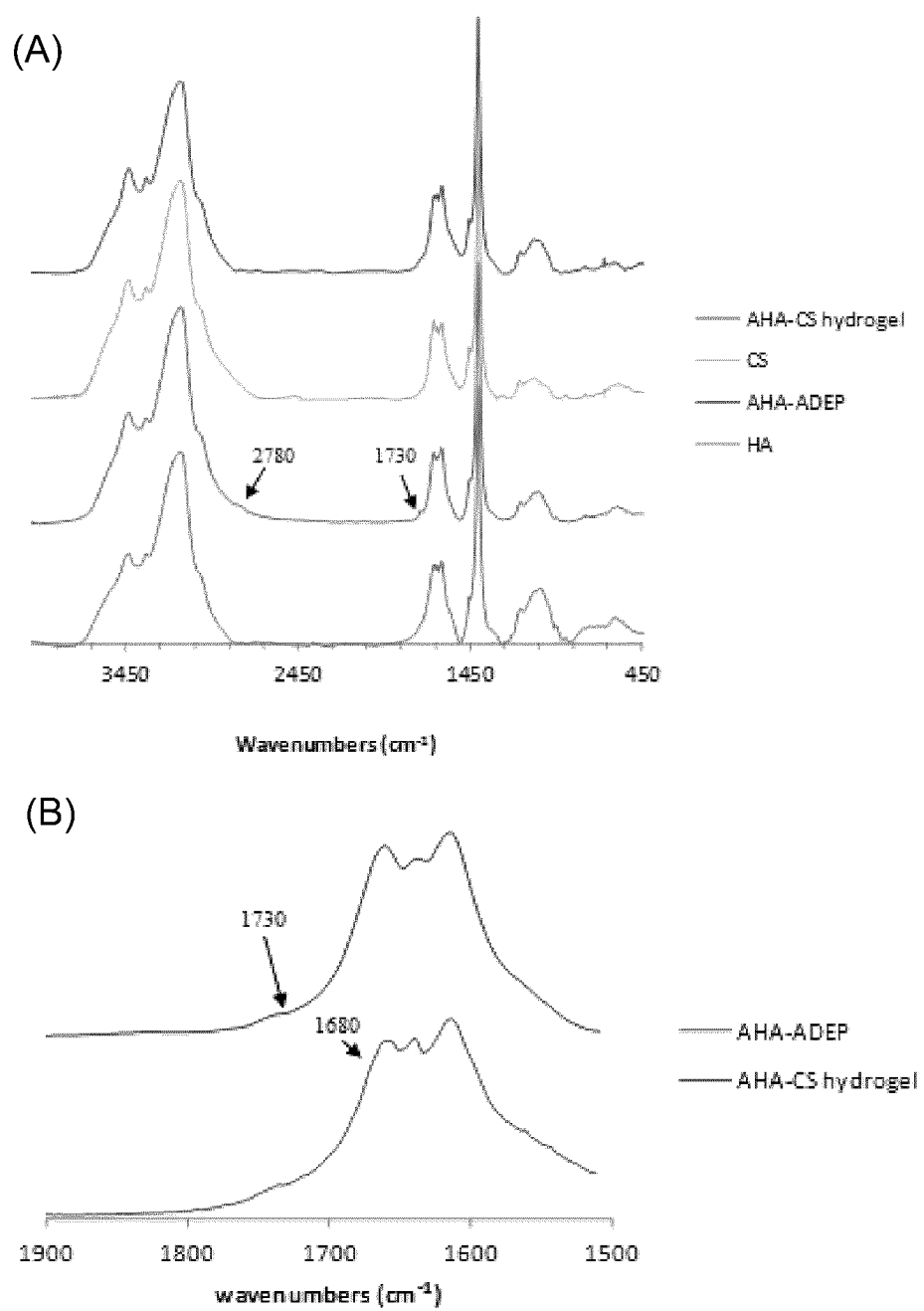


Fig. 3

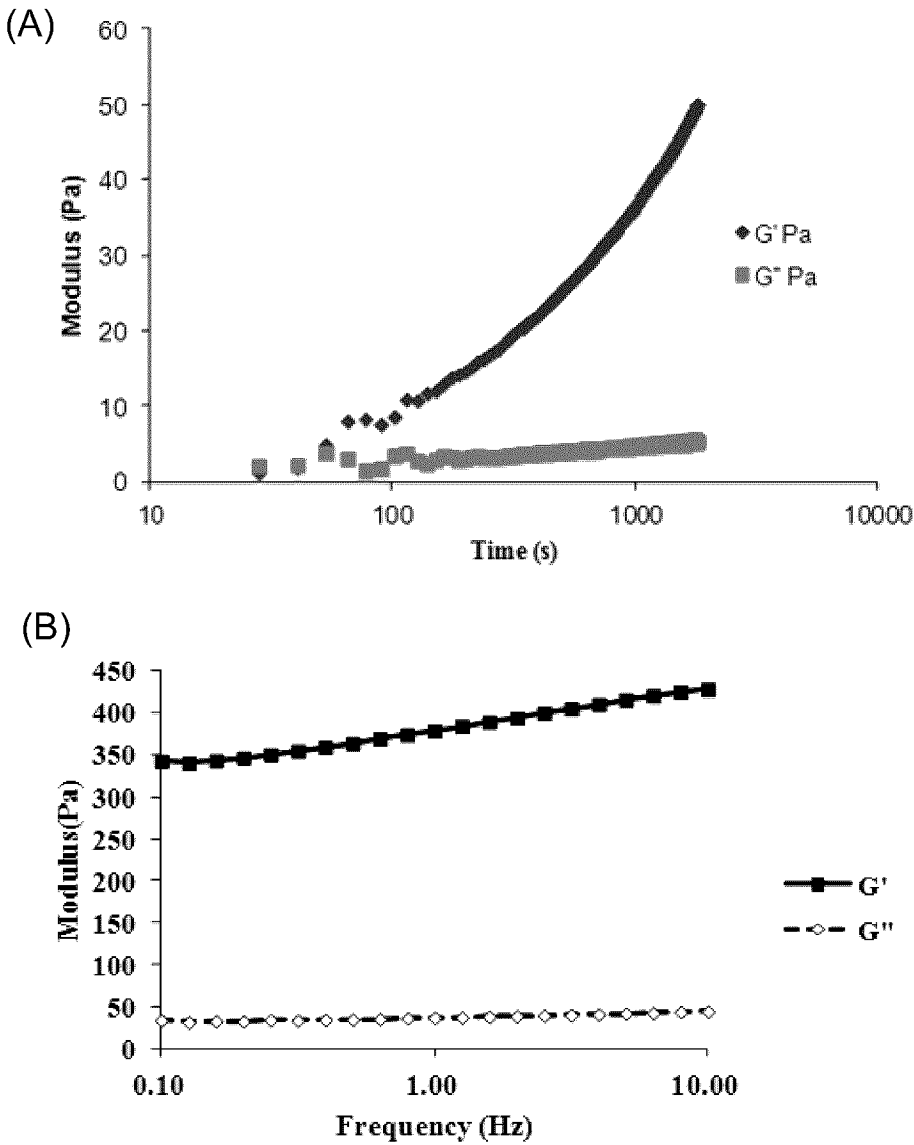


Fig. 4

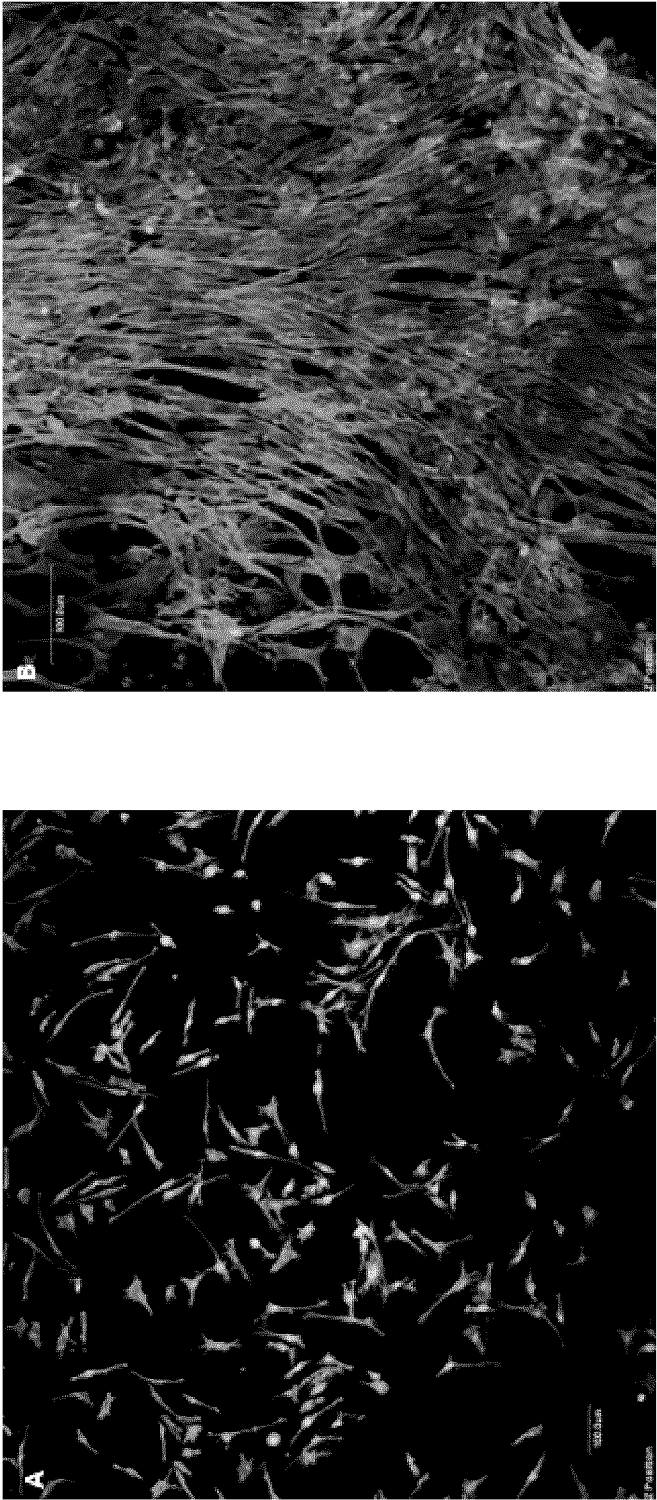


Fig. 5

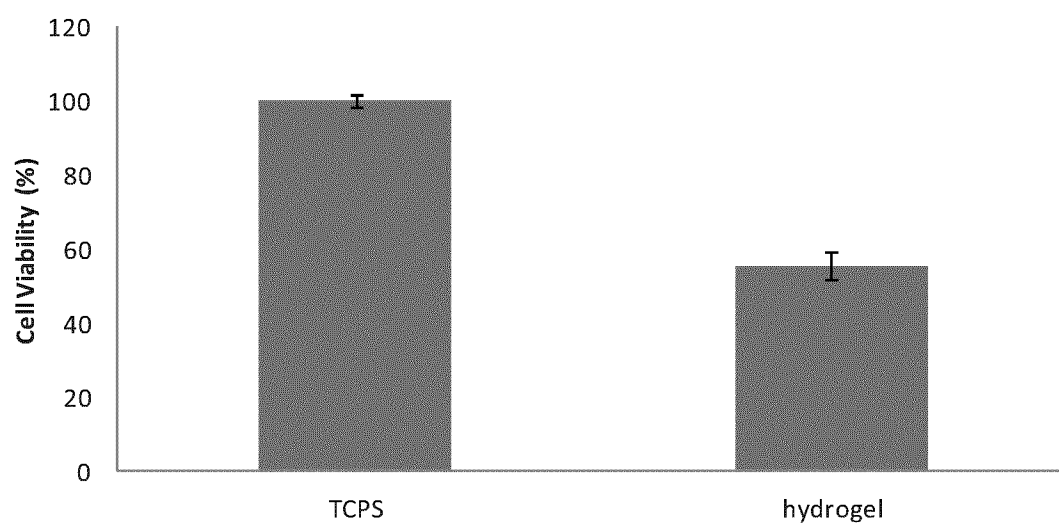


Fig. 6

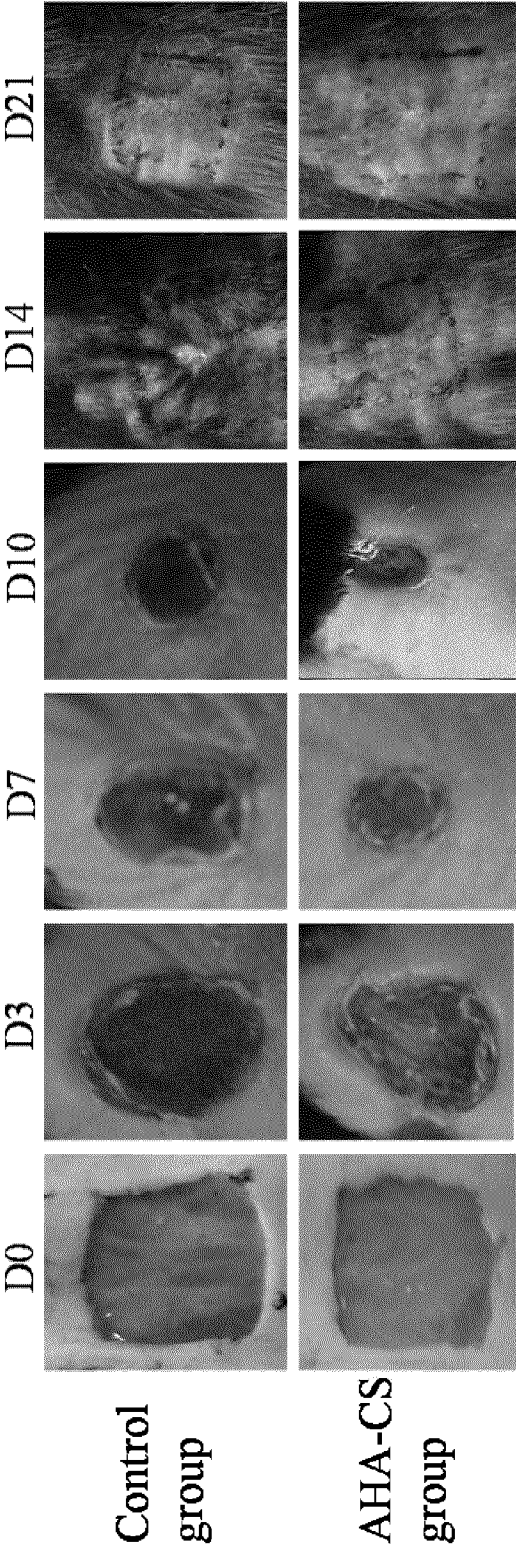


Fig. 7

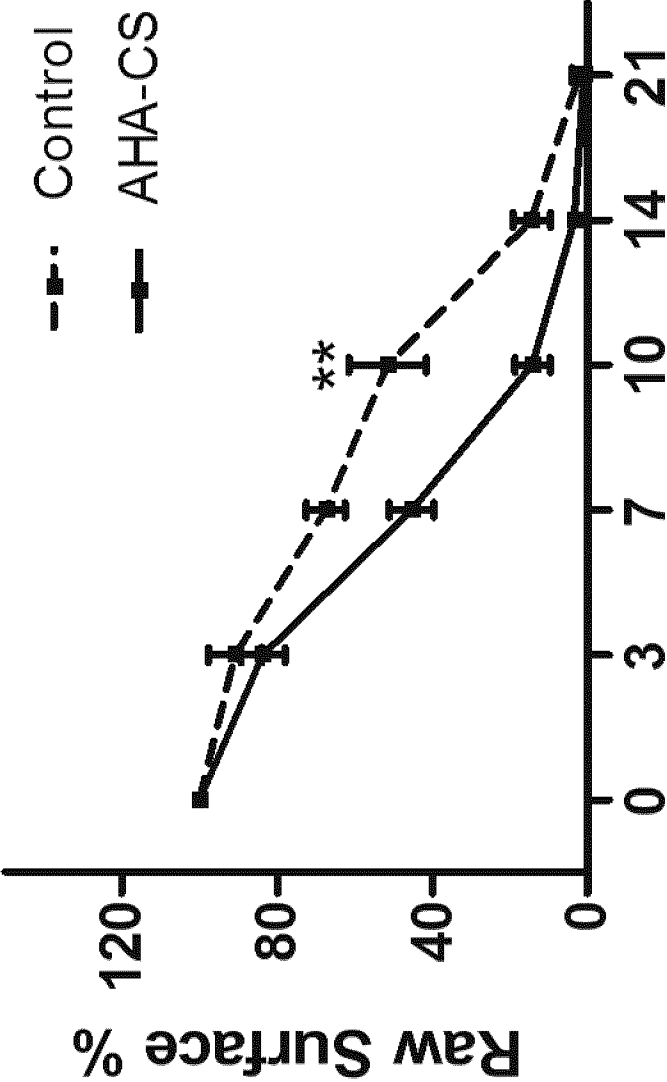


Fig. 8

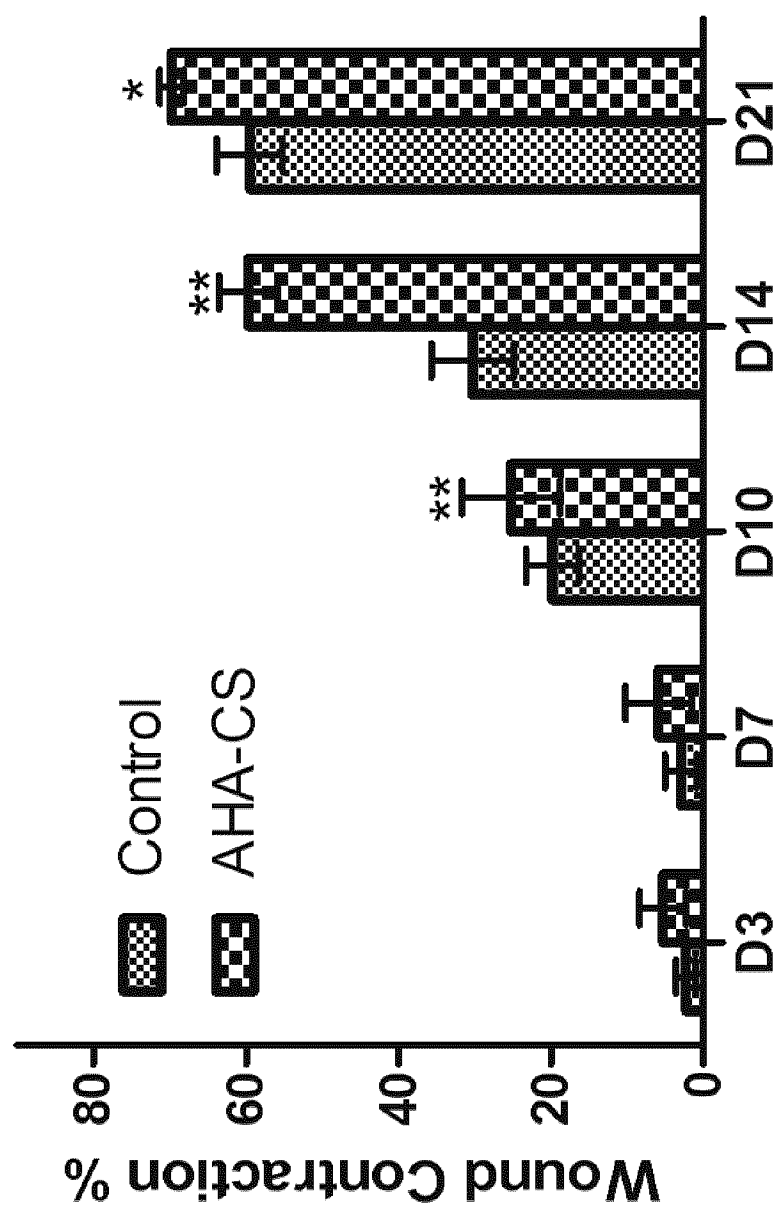


Fig. 9

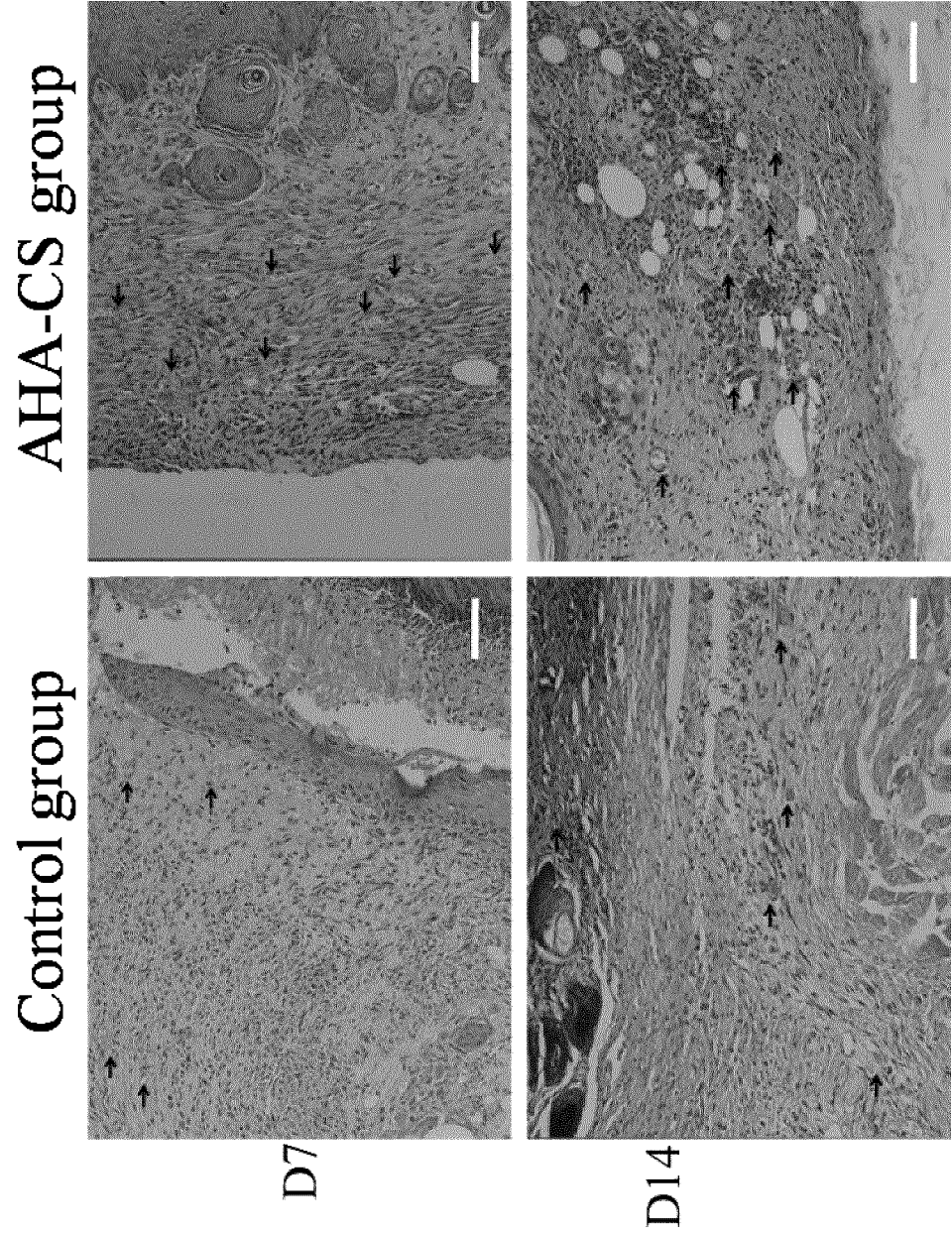


Fig. 10

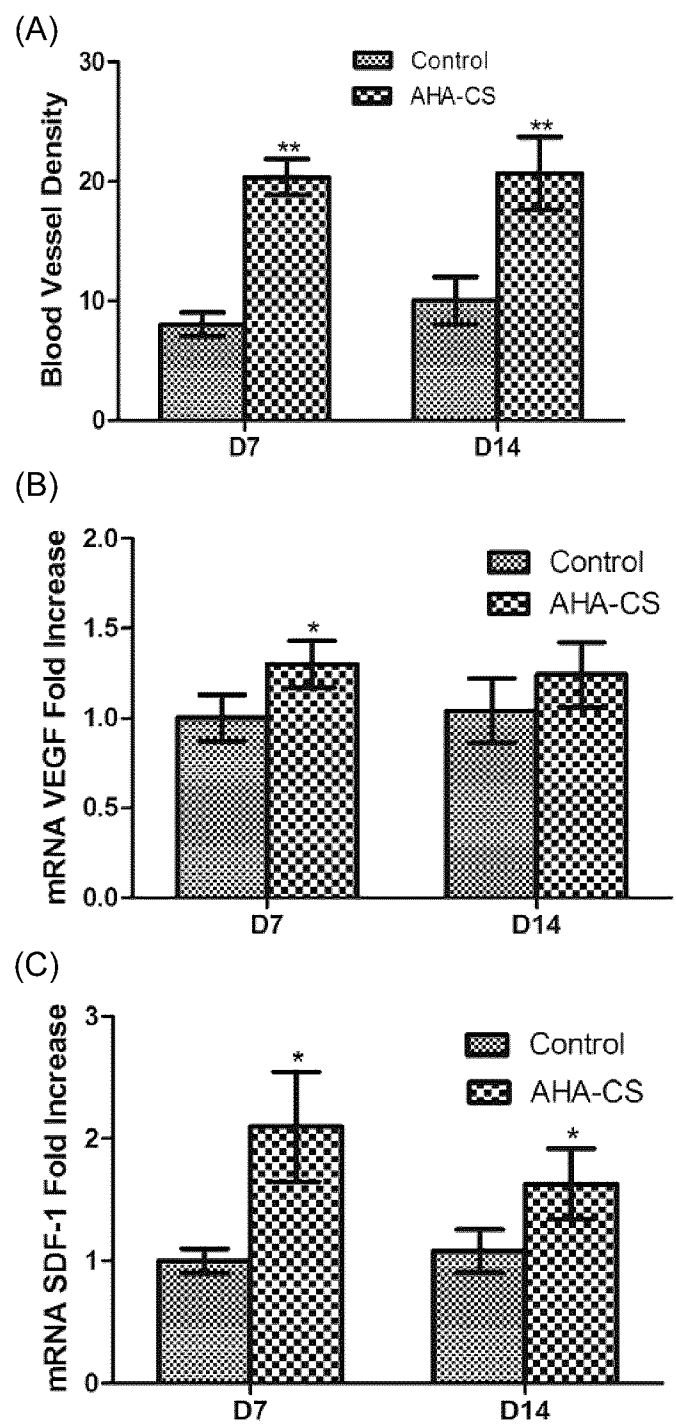


Fig. 11

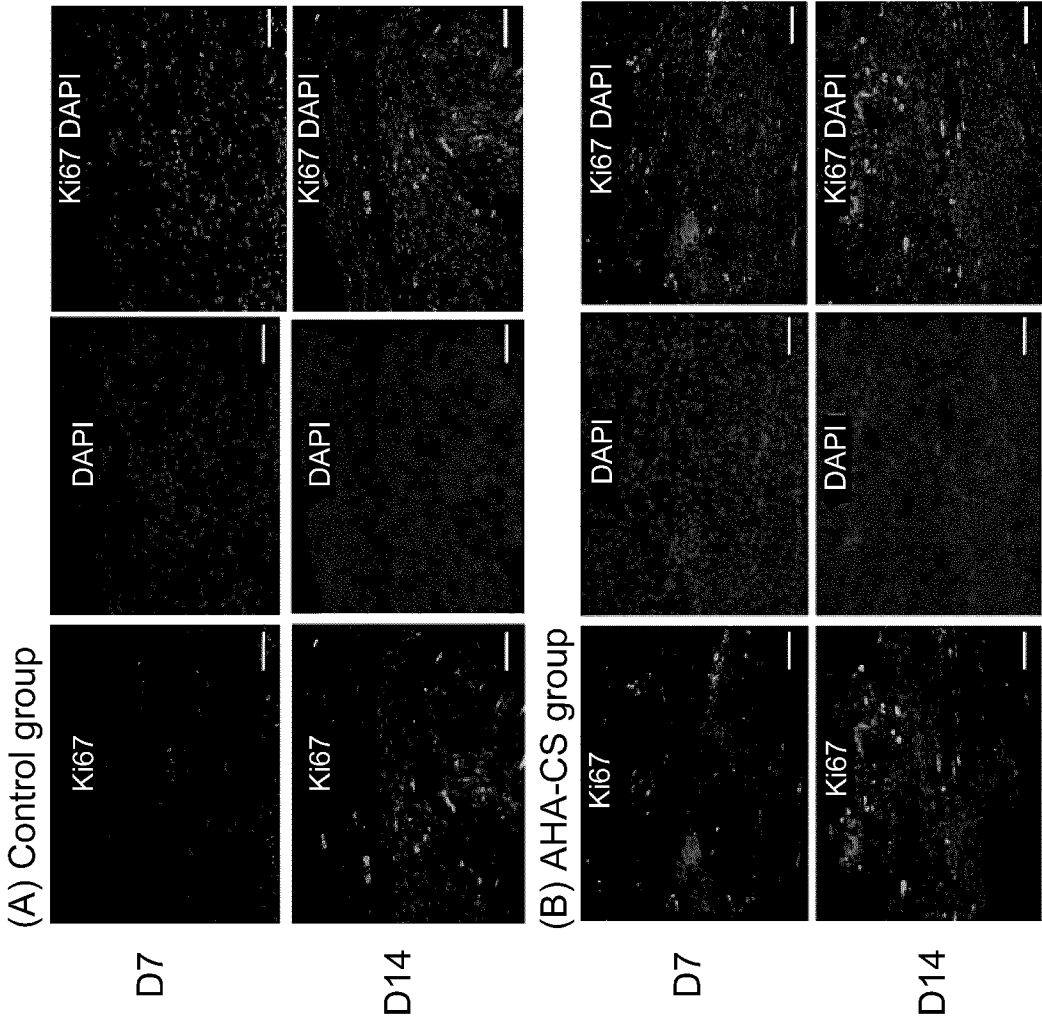


Fig. 12

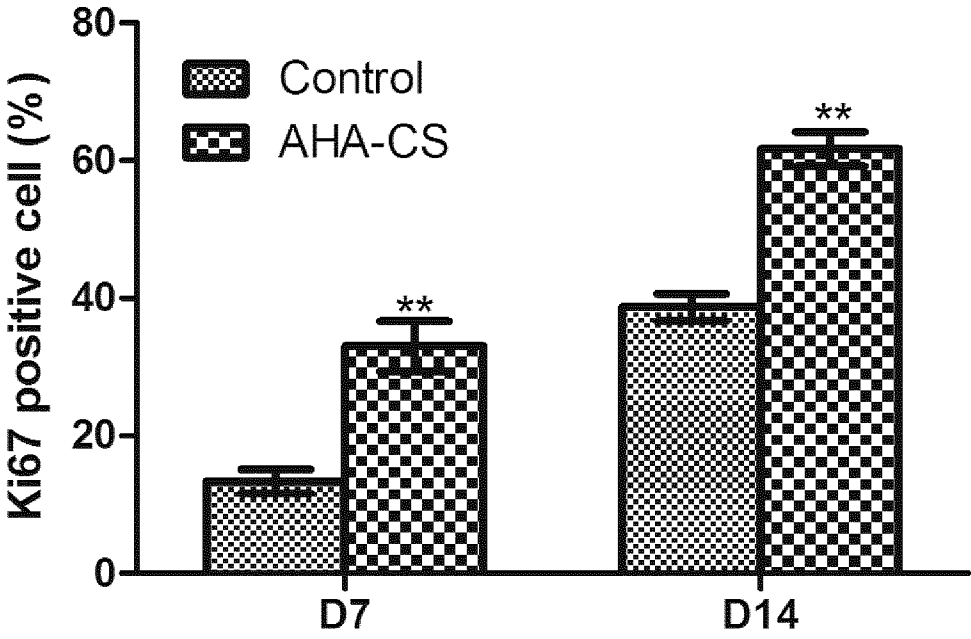


Fig. 13

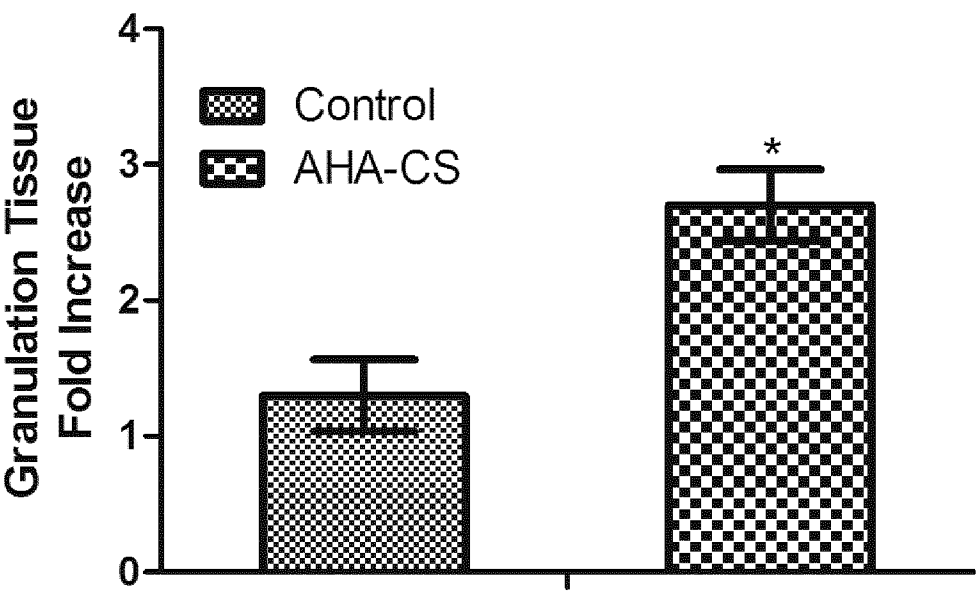


Fig. 14

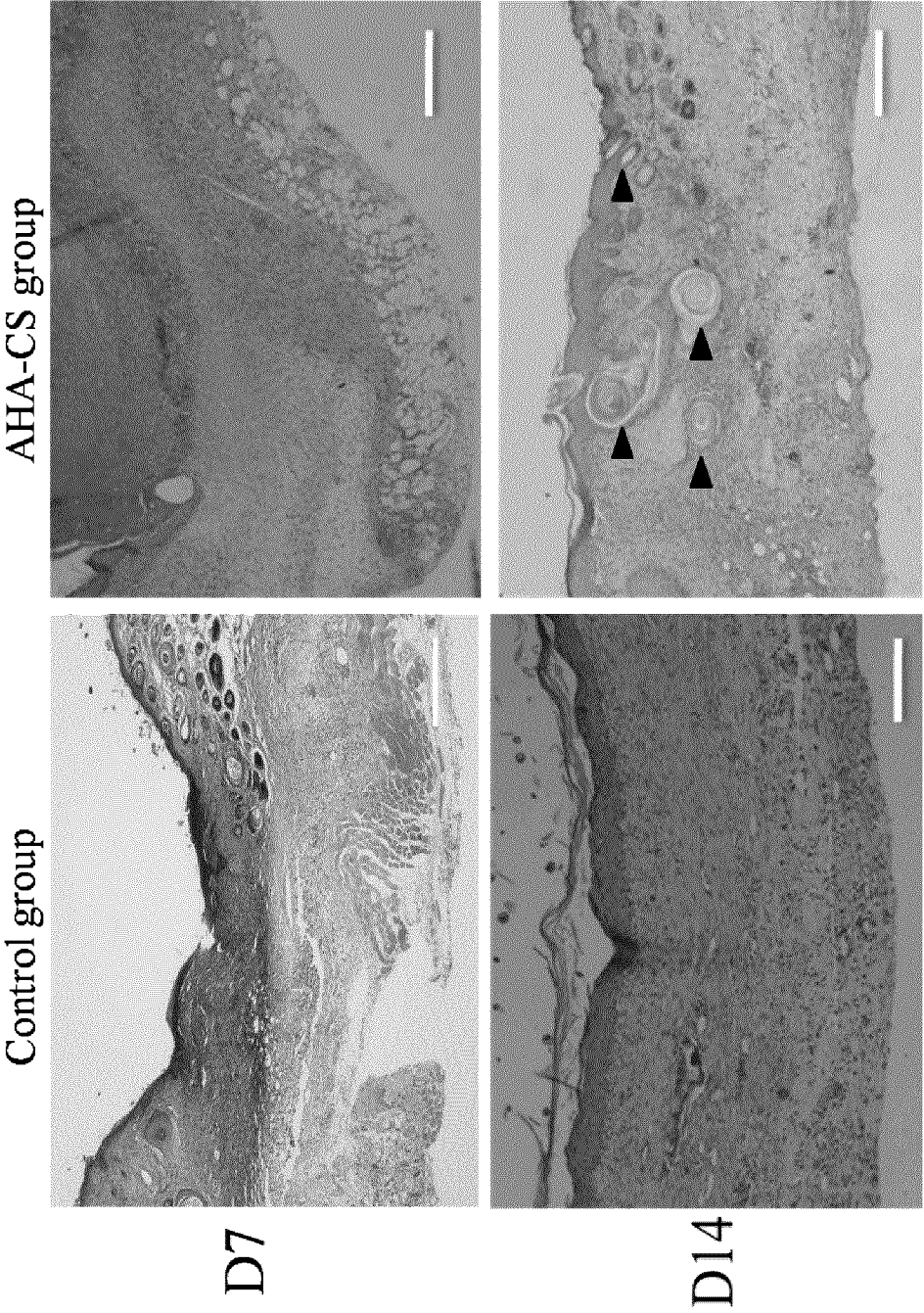


Fig. 15

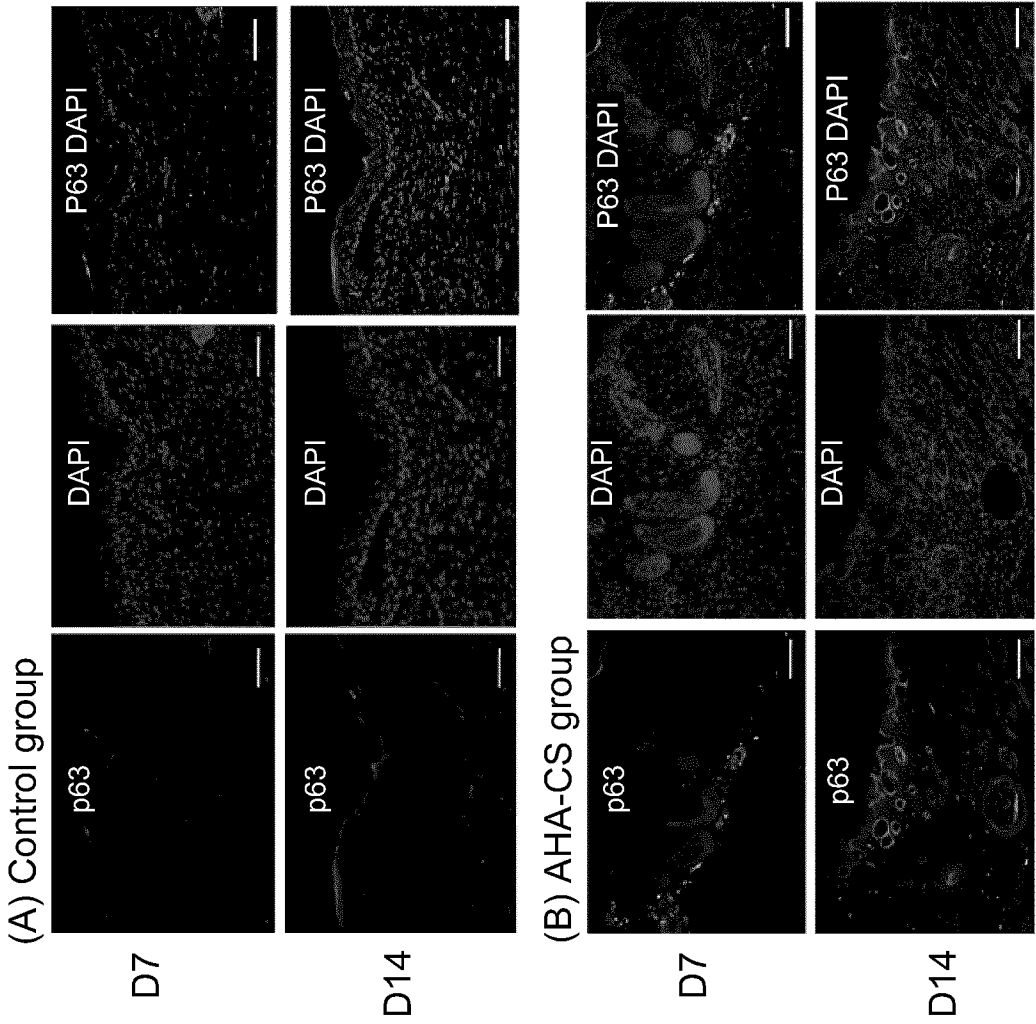


Fig. 16

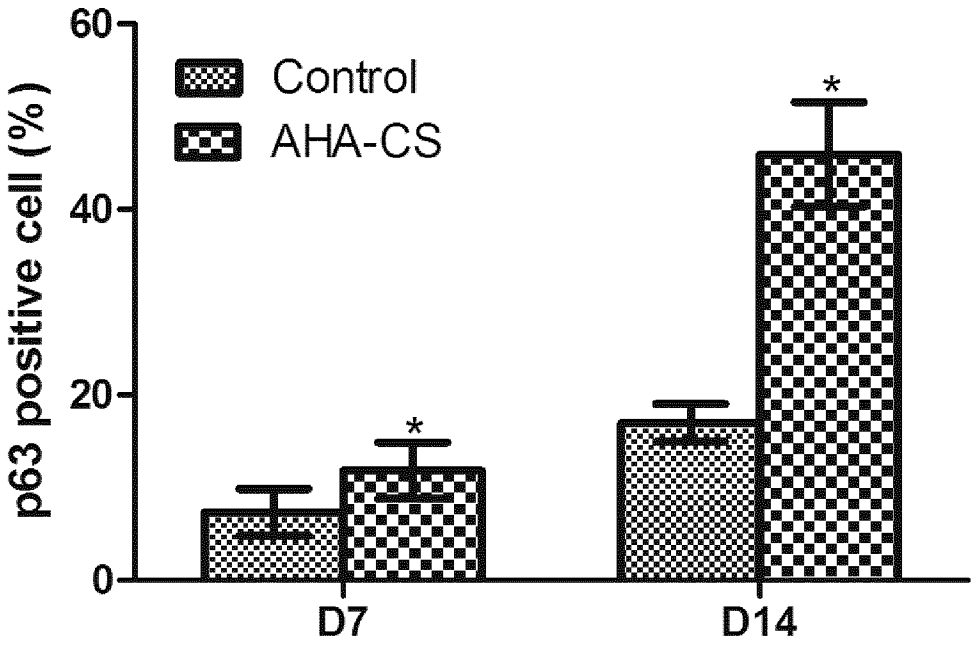


Fig. 17

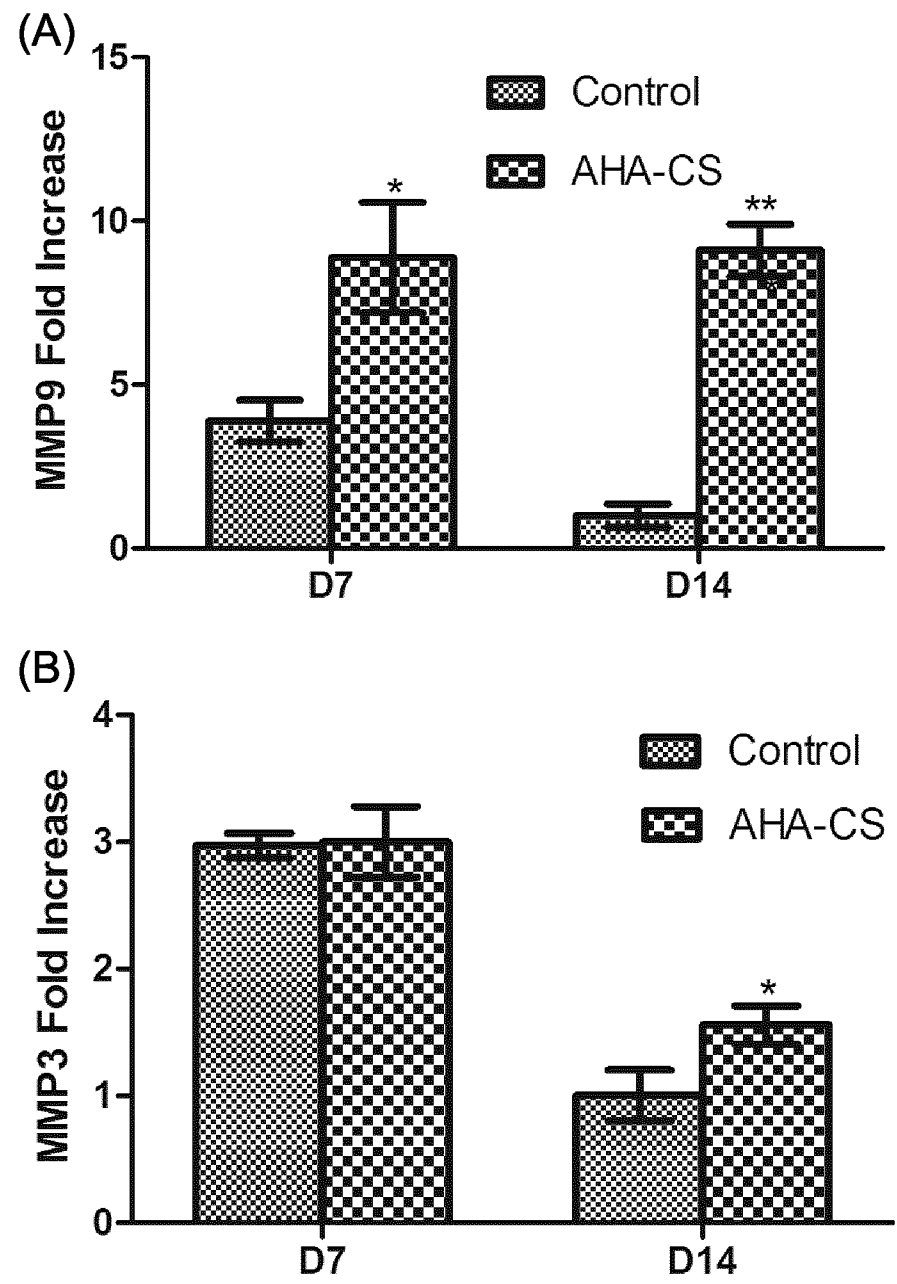


Fig. 18

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA2014/050311

A. CLASSIFICATION OF SUBJECT MATTER

IPC: **A61K 47/36** (2006.01), **A61K 9/10** (2006.01), **A61L 15/28** (2006.01), **A61P 17/02** (2006.01)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: **A61K 47/36** (2006.01), **A61K 9/10** (2006.01), **A61L 15/28** (2006.01), **A61P 17/02** (2006.01)

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

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)

Epoque, Canadian patent database, Scopus (chitosan, hyaluronic acid, hyaluronate, hyaluronan, diethoxy propane, hydrogel, and wound)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2005/113608 [WANG, W.] 1 December 2005 (01-12-2005)	1-30
A	US 2007/0031467 [ABRAHAMS, J. M. et al.] 8 February 2007 (08-02-2007)	1-30
A	CA 2 808 235 [CHEN, W. et al] 1 November 2007 (01-11-2007)	1-30

Further documents are listed in the continuation of Box C.

See patent family annex.

* "A" "E" "L" "O" "P"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance earlier application or patent but published on or after the international filing date document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) document referring to an oral disclosure, use, exhibition or other means document published prior to the international filing date but later than the priority date claimed	"T" "X" "Y" "&"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art document member of the same patent family
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Date of the actual completion of the international search
30 May 2014 (30-05-2014)

Date of mailing of the international search report

26 June 2014 (26-06-2014)

Name and mailing address of the ISA/CA
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Authorized officer

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INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CA2014/050311

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
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		US2005271729A1	08 December 2005 (08-12-2005)
		US7651702B2	26 January 2010 (26-01-2010)
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		US2007031468A1	08 February 2007 (08-02-2007)
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		EP2013237A2	14 January 2009 (14-01-2009)
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		US8513217B2	20 August 2013 (20-08-2013)
		WO2007124198A2	01 November 2007 (01-11-2007)
		WO2009017753A2	05 February 2009 (05-02-2009)
		US2014107065A1	17 April 2014 (17-04-2014)