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(54) Title: HYDROGELS WITH TUNABLE ELECTROSTATIC PROPERTIES

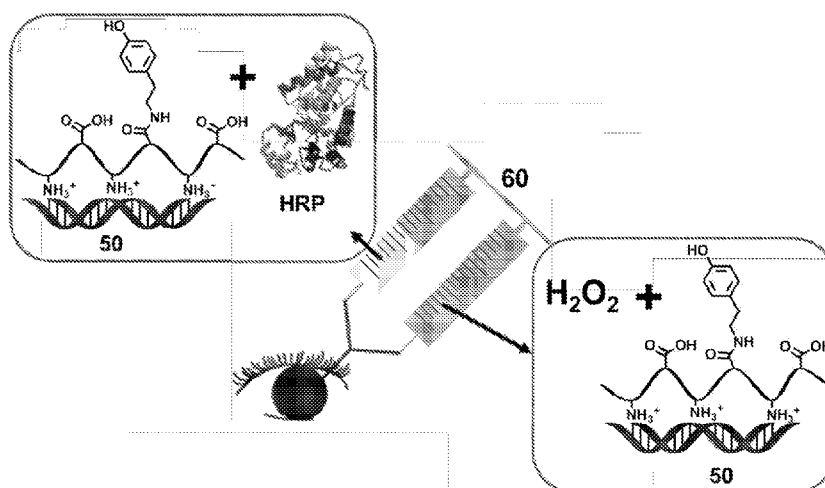


FIG. 2

(57) Abstract: Disclosed herein is a sustained release composition comprising an active agent having a positive or a negative charge when placed in an aqueous environment, and a crosslinked hydrogel tuned to have an overall charge that facilitates electrostatic interaction between the crosslinked hydrogel and the active agent, wherein the crosslinked hydrogel is formed from a heterobifunctional polymer (e.g. gelatin) and a heterobifunctional crosslinking agent (e.g. tyramine or hydroxyphenyl propionic acid).



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HYDROGELS WITH TUNABLE ELECTROSTATIC PROPERTIES

Field of Invention

- 5 This invention relates to a sustained release composition comprising a crosslinked hydrogel and an active agent, and the use of said composition to treat a disease or a medical condition.

Background

- 10 The listing or discussion of a prior-published document in this specification should not necessarily be taken as an acknowledgement that the document is part of the state of the art or is common general knowledge.

Glaucoma is an eye disease caused by elevated eye pressure that can result progressive
15 damage to the optic nerve, leading to loss of vision and blindness. Normal eye pressure, where the eye is soft, is usually from 8 to 22 mmHg. However, patients with glaucoma have an elevated eye pressure of more than 22 mmHg, which can lead to hardening of the eyes. This in turn damages the delicate optic nerve fibre and decreases blood flow to the nerve. The elevated pressure is mostly due to the clogging or blocking of drainage channel or trabecular
20 meshwork of the eye, which leads to build-up of aqueous humour in the eye (<https://www.medicinenet.com/glaucoma/article.htm>). Currently, glaucoma affects almost 70 million cases worldwide and is also the leading cause of irreversible blindness worldwide. With an ageing population, it is estimated that by 2020, the number of glaucoma cases will rise to 80 million, in which approximately 11 million people will be blind due to glaucoma. In Singapore,
25 approximately 3% and 10% of the people over 50 years old and 70 years old, respectively, are diagnosed with glaucoma, with many more people who are not diagnosed due to lack of symptoms in the early stages of the disease. Therefore, there is an increasing demand for new and effective treatments for glaucoma.

- 30 The current first-line treatment for glaucoma is the use of an anti-hypertensive eye drop. There are mainly five types of anti-hypertensive drugs, which include prostaglandin analogues, beta-blockers, carbonic anhydrase inhibitors, alpha-2 agonists and cholinergic agents. These drugs function mainly by decreasing the production of aqueous humour in the eye or increasing outflow of aqueous humour, which lowers the intraocular pressure (IOP) to prevent the
35 progression of glaucoma. However, these drugs have major limitations, such as serious side effects, poor drop instillation technique, and high costs, which therefore lead to poor adherence to the treatment regime. Further, statistics have shown that the failure rate for first-

line mono-treatment after two years is approximately 40-75%. Therefore, glaucoma treatment still relies on second- or third-line treatments, such as using a second drug, laser trabeculoplasty and surgery, to improve the overall glaucoma treatment. However, some patients may not respond to certain drugs, which therefore leads to lower success when using a second drug. Further, combined therapies using different drugs may also lead to more adverse effects and complications, which is undesirable. As such, laser trabeculoplasty and surgery are important treatment options for treating glaucoma.

Glaucoma filtration surgery is a common treatment option that reduces the IOP by creating an additional channel in the sclera (white part of the eye) to allow the outflow of aqueous humour. This is an invasive method that might cause trauma, scarring, and fibrosis to the patient's eye. Subconjunctival scarring after the glaucoma filtration surgery will cause poorly filtering blebs, which can subsequently lead to a rise in IOP and failure of the treatment. Further, studies on mainly Chinese and Malay patients from Singapore and Malaysia showed low success rate (~45-62%) of the glaucoma filtration surgery two years after the surgery. As such, post-surgery scarring management plays an important role in ensuring the success of glaucoma surgical treatment. Currently, anti-fibrotic agents such as 5-fluorouracil (5FU) and mitomycin-C (MMC) are being widely used to prevent scarring after glaucoma filtration surgery. However, their effects are non-specific and cause major side effects such as hypotonous maculopathy and bleb-related endophthalmitis. Safer and more effective methods are highly desirable for managing post-glaucoma surgery to improve the anti-scarring effect, so as to lead to the overall success of the glaucoma treatment.

Given the above, there remains a need to develop new compositions or drug delivery systems to improve the treatment of glaucoma. More importantly, such compositions or systems have to be safe, easy to handle and effective in delivering the active agents to the areas to be treated. In addition, such compositions or systems have to be versatile so that they can be used with other types of active agents to improve the treatment of other diseases or conditions.

Gelatin is commonly used in food and pharmaceutical industries, and is recognised by the U.S. Food & Drug Administration (FDA) as "Generally Recognized as Safe" (GRAS). Gelatin is well-known to be biocompatible, biodegradable and can be processed easily.

Summary of Invention

In a first aspect of the invention, there is provided a sustained release composition comprising:

an active agent having a positive or negative charge when placed in an aqueous environment;

a crosslinked hydrogel tuned to have an overall charge that facilitates electrostatic interaction between the crosslinked hydrogel and the active agent, wherein:

the crosslinked hydrogel is formed from:

a heterobifunctional polymer comprising a first set of functional groups that are positively charged and a second set of functional groups that are negatively charged in an aqueous environment; and

a heterobifunctional crosslinking agent, where the heterobifunctional crosslinking agent comprises:

at least one functional group capable of forming a bond to a further molecule of said heterobifunctional crosslinking agent; and

at least one functional group suitable to form a bond with the first or second set of functional groups of the heterobifunctional polymer, so as to reduce the number of the first or second functional groups in the first or second sets of functional groups, thereby modifying the overall charge of the crosslinked hydrogel and facilitating the electrostatic interaction between the crosslinked hydrogel and the active agent.

In embodiments of the first aspect of the invention:

(a) the active agent may be a biomolecule and/or a small molecule active agent (e.g. cisplatin or paclitaxel). For example, the biomolecule may be a charged biomolecule selected from one or more of clustered regularly interspaced short palindromic repeats (CRiSPR) components, a nucleic acid, a microRNA and a siRNA. When the biomolecule is microRNA it may be selected from one or more of 5'-UUGUGCUUGAUCUAACCAUGU-3' (miR-218), 5'-UAGCACCAUUUGAAAUCAGUGUU-3' (miR-29b duplex), 5'-AUCACAUUGCCAGGGAUUACC-3' (miR-23b), and 5'-UGAAAUGUUUAGGACCACUAG-3' (miR-203). When the biomolecule is siRNA it may be selected from one or more of 5'-AACAAGACCUUCGACUCUCC-3' (SPARC), 5'-AACCTGAAGATCTTCAACAACCCTGTCTC-3' (Smad3), 5'-AACCUGCUGAAGGAUGGUGAC-3' (p53), 5'-CCAAGAACCGGAACCGUCUTT-3' (MC1R), 5'-GCAGUACCUUUCUACCACUTT-3' (MITF), and 5'-UCACUUACAGGAUCUAUAAUU-3' (Elastase);

(b) the heterobifunctional polymer may be selected from one or more of gelatin, collagen, silk fibroin, elastin and $\text{H}_2\text{N-PEG-CO}_2\text{H}$. For example, the heterobifunctional polymer may be gelatin;

(c) the heterobifunctional crosslinking agent comprises at least one functional group capable of forming a bond to a further molecule of said heterobifunctional crosslinking agent, wherein the at least one functional group capable of forming a bond to a further molecule of said heterobifunctional crosslinking agent is a functional group that is not charged, or is zwitterionic, in an aqueous environment. Examples of suitable functional groups capable of forming a bond to a further molecule of said heterobifunctional crosslinking agent may be selected from one or more of norbornene, tetrazine, methacrylate, OH, SH, azide, C_2 to C_{10} alkene, C_2 to C_{10} alkyne, particularly the at least one functional group capable of forming a bond to a further molecule of said heterobifunctional crosslinking agent may be SH or, more particularly, OH;

(d) when the at least one functional group suitable to form a bond with the first or second set of functional groups is suitable to form a bond with the first set of functional groups, then it may be selected from one or more of a carbonyl group, an ester, a peroxy acid and CO_2H ; and when the at least one functional group suitable to form a bond with the first or second set of functional groups is suitable to form a bond with the second set of functional groups, then it may be selected from one or more of amide and amino;

(e) the heterobifunctional crosslinking agent may be selected from molecules suitable for undergoing Click reactions together, molecules suitable for photocrosslinking to one another, thiol-containing molecules, phenolic molecules with carboxylic acid groups, and phenolic molecules with amine groups. Molecules suitable for undergoing Click reactions together may be selected from two or more of 5-norbornene-2-carboxylic acid, tetrazine acid, 3-azido-1-propanamine, 3-azidopropanoic acid, N-hydroxysuccinimide (NHS) esters, dibenzocyclooctyne-amine, 2-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)ethan-1-amine and 3-(4-(prop-2-yn-1-yloxy)benzoyl)benzoic acid. Molecules suitable for photocrosslinking to one another may be selected from one or more of methacrylic acid, and thiol-containing molecules suitable to crosslink with ene-containing molecules. Thiol-containing molecules may be selected from one or more of 5-(4-aminophenyl)-1,3,4-oxadiazole-2-thiol, 3-amino-1,2,4-triazole-5-thiol, and cysteamine. Phenolic molecules with carboxylic acid groups may be selected from one or more of 3-(4-hydroxyphenyl)propionic acid and 3,4-dihydroxyphenylacetic acid. Phenolic molecules with amine groups may be selected from one or more of 4-hydroxybenzylamine, dopamine, and tyramine;

(f) the overall charge of the crosslinked hydrogel also facilitates the sustained release effect of the composition, optionally wherein the sustained release effect may be from 1 to 30 days, such as from 2 to 25 days, such as 5 to 20 days;

(g) when the active agent is a siRNA, then the crosslinked hydrogel may be formed from gelatin and tyramine, where the zeta potential of the crosslinked hydrogel is from -1 mV to +10 mV, such as from 0 mV to +9 mV, such as from +0.4 mV to +8.5 mV, such as from +0.6 mV to +1 mV;

- 5 (h) when the active agent is cisplatin, then the crosslinked hydrogel may be formed from gelatin and 3-(4-hydroxyphenyl)propionic acid, where the zeta potential of the crosslinked hydrogel is from -4 mV to -10 mV.

10 In a second aspect of the invention, there is provided a use of a sustained release composition as described in the first aspect of the invention or any technically sensible combination of its embodiments in medicine.

In a third aspect of the invention, there is provided:

- 15 (Ci) a use of a sustained release composition as described in the first aspect of the invention or any technically sensible combination of its embodiments in the preparation of a medicament for the treatment and/or prevention of scarring, wherein the active agent of the sustained release composition is an anti-scarring agent (e.g. the anti-scarring agent is a siRNA, such as 5'-AACAAGACCUUCGACUCUCC-3'), optionally wherein the use relates to the treatment and/or prevention of scarring in a subject who
- 20 has undergone surgery (e.g. eye surgery for glaucoma);
- (Cii) a sustained release composition as described in the first aspect of the invention or any technically sensible combination of its embodiments for the treatment and/or prevention of scarring, wherein the active agent of the sustained release composition is an anti-scarring agent (e.g. the anti-scarring agent is an siRNA, such as 5'-
- 25 AACAAGACCUUCGACUCUCC-3'), optionally wherein the treatment and/or prevention of scarring relates to a subject who has undergone surgery (e.g. eye surgery for glaucoma);
- (Ciii) a method of treating and/or preventing scarring, wherein the method comprises providing to a subject in need thereof a pharmaceutically effective amount of a
- 30 sustained release composition as described in the first aspect of the invention or any technically sensible combination of its embodiments, wherein the active agent of the sustained release composition is an anti-scarring agent (e.g. the anti-scarring agent is an siRNA, such as 5'-AACAAGACCUUCGACUCUCC-3'), optionally wherein the treatment and/or prevention of scarring relates to a subject who has undergone surgery
- 35 (e.g. eye surgery for glaucoma).

In a fourth aspect of the invention, there is provided:

- (Di) a use of a sustained release composition as described in the first aspect of the invention or any technically sensible combination of its embodiments in the preparation of a medicament for the treatment and/or prevention of cancer, wherein the active agent of the sustained release composition is an anti-cancer agent (e.g. the anti-cancer agent is cisplatin or paclitaxel);
- (Dii) a sustained release composition as described in the first aspect of the invention or any technically sensible combination of its embodiments for the treatment and/or prevention of cancer, wherein the active agent of the sustained release composition is an anti-cancer agent (e.g. the anti-cancer agent is cisplatin or paclitaxel);
- (Diii) a method of treating and/or preventing cancer, wherein the method comprises providing to a subject in need thereof a pharmaceutically effective amount of a sustained release composition as described in the first aspect of the invention or any technically sensible combination of its embodiments, wherein the active agent of the sustained release composition is an anti-cancer agent (e.g. the anti-cancer agent is cisplatin or paclitaxel).

In a fifth aspect of the invention, there is provided a pharmaceutical composition comprising a sustained release composition as described in the first aspect of the invention or any technically sensible combination of its embodiments in combination with one or more of a pharmaceutically acceptable excipient, diluent or carrier.

In a sixth aspect of the invention, there is provided a kit of parts, comprising:

(Aa) a first composition comprising:

a complex of an active agent having a positive or negative charge when placed in an aqueous environment and an un-crosslinked hydrogel tuned to have an overall charge that facilitates electrostatic interaction between the un-crosslinked hydrogel and the active agent; and

an enzyme suitable to crosslink the un-crosslinked hydrogel; and

(Bb) a second composition comprising an oxidation source (e.g. H_2O_2), wherein:

the un-crosslinked hydrogel comprises a heterobifunctional polymer that has a first set of functional groups that are positively charged and a second set of functional groups that are negatively charged in an aqueous environment, where a portion of the first or second set of functional groups are capped by a heterobifunctional crosslinking agent, such that the overall charge of the un-crosslinked hydrogel is modified to facilitate the formation of the complex between the un-crosslinked hydrogel and the active agent, where:

the heterobifunctional crosslinking agent comprises at least one functional group capable of forming a bond to a further molecule of said heterobifunctional crosslinking agent

when the first and second compositions are combined, and at least one functional group that has formed a bond to a portion of the first or second set of functional groups of the heterobifunctional polymer, so as to reduce the number of the first or second functional groups, thereby modifying the overall charge of the un-crosslinked hydrogel and facilitating the electrostatic interaction between the un-crosslinked hydrogel and the active agent.

Brief Description of Drawings

Fig. 1 Depicts: (a) a schematic representation of the preparation of the crosslinked hydrogel of siRNA-Gtn-Tyr (**55**) of the current invention; and (b) a schematic representation illustrating the crosslinking of adjacent polyplexes (**50**) or Gtn-Tyr precursors (**30**) in the presence of horseradish peroxidase (HRP) and H_2O_2 .

Fig. 2 Depicts the dual-syringe delivery of siRNA-Gtn-Tyr polyplexes (**50**) into the conjunctiva to allow the formation of the crosslinked hydrogel (**55**) *in situ*, after glaucoma filtration surgery for anti-fibrosis therapy. The mixture of HRP and siRNA-Gtn-Tyr polyplexes (**50**), and the mixture of H_2O_2 and siRNA-Gtn-Tyr polyplexes (**50**) were stored in two separate syringes to prevent the crosslinking from occurring before administration.

Fig. 3 Depicts the: (a) initial; and (b) subsequent studies relating to the correlation of the zeta potentials of various Gtn-Tyr precursors (**30**) to the amount of phenol (which corresponds to the amount of tyramine conjugated to the gelatin backbone).

Fig. 4 Depicts the physical appearance (photographs) of crosslinked Gtn-Tyr hydrogel with: (a) increasing amount of Tyr, showing increased opacity of the hydrogel; and (b) increasing concentration of H_2O_2 , showing increased stiffness of the hydrogel; and (c) photographs of a 32 gauge Hamilton syringe showing a small volume of the Gtn-Tyr precursor (**30**), with HRP and H_2O_2 , passing through the syringe and the formation of a 10 μ L hydrogel (in circle).

Fig. 5 Depicts: (a) the release profile of FAM-SPARC from the as-prepared crosslinked siRNA-Gtn-Tyr hydrogel (**55**) prepared from Gtn-Tyr precursors (**30**) with small and large positive surface charges, respectively; and (b) degradation profile of crosslinked Gtn-Tyr hydrogel prepared using Gtn-Tyr precursors F1 and F2 (from Table 1b).

Fig. 6 Depicts: (a) the proliferation profile of C57Bl6/J MTF cultured on the crosslinked Gtn-Tyr hydrogel synthesised using Gtn-Tyr precursor (**30**) with small and large surface positive charge for 1-7 days; and (b) fluorescence images of live C57Bl6/J MTFs cells (green

fluorescence) cultured on the crosslinked Gtn-Tyr hydrogel synthesised using Gtn-Tyr precursor **30** with small and large surface positive charge for 1-7 days. Red fluorescence was not observed which indicated the absence of dead cells. Scale bar=200 μ m.

Fig. 7 Depicts the SPARC expression of C57Bl6/J MTFs at: (a) day 2; and (b) day 7 after treatment with crosslinked siSPARC-Gtn-Tyr hydrogels made from the respective Gtn-Tyr precursors (R3-R7, Table 1a) with increasing surface charge and siSPARC as the siRNA. Negative controls were carried out using a non-silencing scrambled siRNA (SCRAMBLED). The SPARC expression was normalised to housekeeping gene 18s.

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Fig. 8 Depicts a schematic representation of the delivery mechanism of the as-prepared crosslinked siSPARC-Gtn-Tyr hydrogels (**55**) to the cells (**58**), which involves electrostatic protection and delivery of the siSPARC into the cellular environment. The crosslinked hydrogels (**55**) can undergo degradation (**56**) to form siSPARC-Gtn-Tyr polyplexes (**57**) which are internalised into the cells (**58**).

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Fig. 9 Depicts the *in vivo* imaging of operated tissues in a rabbit model of glaucoma filtration surgery with insertion of a 24-gauge cannula: (a-c) slit-lamp microscopy images of the week 4 postoperative tissues treated with MMC, crosslinked siSCRAMBLED- and siSPARC-Gtn-Tyr hydrogels, respectively. While blebs were no longer visible in all of the tissues treated with MMC and crosslinked siSCRAMBLED-Gtn-Tyr hydrogel, majority of the tissues treated with crosslinked siSPARC-Gtn-Tyr hydrogel showed visible blebs at week 4; and (d-l) *in vivo* confocal images of the week 4 operated area. * indicates microcysts, arrowheads indicate large vasculature, while arrows indicate fine vasculature.

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Fig. 10 Depicts the histochemical analyses of operated tissues in a rabbit model of glaucoma filtration surgery with insertion of a 24-gauge cannula: (a, d, g) Hematoxylin and eosin (H&E) staining of week 4 postoperative tissues treated with MMC, crosslinked siSCRAMBLED- and siSPARC-Gtn-Tyr hydrogels, respectively; (b, e, h) Masson's trichrome staining of consecutive sections of the same eyes; and (c, f, i) Picrosirius red staining of consecutive sections of the same eyes viewed by polarised microscopy. Vertical double-ended arrow indicate extent of the subconjunctiva matrix, while "S" indicates sclera. Scale bar=100 μ m.

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Fig. 11 Depicts a schematic representation of the preparation of the crosslinked Gtn-HPA hydrogel (**36**). A photograph of the transparent hydrogel is as shown in the insert.

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Fig. 12 Depicts a schematic representation of the preparation of the crosslinked cisplatin-Gtn-HPA hydrogel (**47**) of the current invention.

Fig. 13 Depicts the correlation of the zeta potentials of various Gtn-HPA precursor (**35**) to the amount of HPA (which corresponds to the amount of HPA conjugated to the gelatin backbone).

Fig. 14 Depicts the release profile of cisplatin from the as-prepared crosslinked cisplatin-Gtn-HPA hydrogel (**47**) prepared from Gtn-HPA precursors (**35**) with negative and positive charges (S1 and S2), respectively.

Fig. 15 Depicts: (a) the proliferation profile; (b) cell doubling rate of MDA-MB-231 human breast cancer cells cultured on the crosslinked cisplatin-Gtn-HPA hydrogel (**47**), prepared from precursors S1 and S2 respectively, for 1-7 days; and (c) fluorescence images of live MDA-MB-231 cells (green fluorescence) after cultured on the crosslinked cisplatin-Gtn-HPA hydrogels for 7 days.

Description

It has been surprisingly found that the use of a crosslinked gelatin-based hydrogel that contains an active agent allows a sustained release of the active agent to the targeted sites for enhanced therapeutic effects. Advantageously, the crosslinked hydrogel can be tuned accordingly to have an overall charge that controls the release of the active agent(s) in a suitable manner. The crosslinked hydrogel also provides encapsulation to the active agent, which helps to reduce degradation of the active agent. Therefore, said composition allows a more effective and sustained delivery of active agent for treating diseases or other medical conditions.

Thus, in a first aspect of the invention, there is provided a sustained release composition comprising:

an active agent having a positive or negative charge when placed in an aqueous environment;

a crosslinked hydrogel tuned to have an overall charge that facilitates electrostatic interaction between the crosslinked hydrogel and the active agent, wherein:

the crosslinked hydrogel is formed from:

a heterobifunctional polymer comprising a first set of functional groups that are positively charged and a second set of functional groups that are negatively charged in an aqueous environment; and

a heterobifunctional crosslinking agent, where the heterobifunctional crosslinking agent comprises:

at least one functional group capable of forming a bond to a further molecule of said heterobifunctional crosslinking agent; and

at least one functional group suitable to form a bond with the first or second set of functional groups of the heterobifunctional polymer, so as to reduce the number of the first or second functional groups in the first or second sets of functional groups, thereby modifying the overall charge of the crosslinked hydrogel and facilitating the electrostatic interaction between the crosslinked hydrogel and the active agent.

In embodiments herein, the word “comprising” may be interpreted as requiring the features mentioned, but not limiting the presence of other features. Alternatively, the word “comprising” may also relate to the situation where only the components/features listed are intended to be present (e.g. the word “comprising” may be replaced by the phrases “consists of” or “consists essentially of”). It is explicitly contemplated that both the broader and narrower interpretations can be applied to all aspects and embodiments of the present invention. In other words, the word “comprising” and synonyms thereof may be replaced by the phrase “consisting of” or the phrase “consists essentially of” or synonyms thereof and *vice versa*.

When used herein, the term “active agent having a positive or negative charge when placed in an aqueous environment”, refers to an active agent that has a net positive or net negative charge in an aqueous environment. The aqueous environment may be any suitable aqueous environment, but may be particularly ones having a pH value of from 5 to 8, such as from 6.4 to 7.5, such as from 7.35-7.45. For the avoidance of doubt, when a number of different numerical ranges are provided, all possible combinations of the numerical point values provided to create further ranges are explicitly contemplated within the invention. For example, the ranges above provide the following pH ranges:

from 5 to 6.4, from 5 to 7.35, from 5 to 7.45, from 5 to 7.5, from 5 to 8;

from 6.4 to 7.35, from 6.4 to 7.45, from 6.4 to 7.5, from 6.4 to 8;

from 7.35 to 7.45, from 7.35 to 7.5, from 7.35 to 8;

from 7.45 to 7.5, from 7.45 to 8; and

from 7.5 to 8.

Crosslinked hydrogels are hydrophilic, three dimensional cross-linked polymer systems capable of imbibing large amounts of water or biological fluids between their polymeric chains to form aqueous semi-solid/solid gel networks. The crosslinked hydrogels used in the current

invention are formed from a heterobifunctional polymer and a heterobifunctional crosslinking agent.

The heterobifunctional polymers used herein are materials that contain two (i.e. a first and a second) oppositely charged sets of functional group types when placed in an aqueous environment (e.g. an aqueous environment having the pH ranges discussed above). When discussed herein, the first set of functional groups are positively charged and the second set of functional groups are negatively charged. In certain embodiments, the heterobifunctional polymer may naturally have an overall neutral charge (i.e. it is essentially zwitterionic), it may have an overall negative charge or it may have an overall positive charge. When used herein, the term "overall charge" is used to refer to the net charge of the material being discussed. Suitable heterobifunctional polymers include, but are not limited to gelatin, collagen, silk fibroin, elastin, H₂N-PEG-CO₂H and combinations thereof. In particular embodiments that may be mentioned herein, the heterobifunctional polymer may be gelatin.

As will be appreciated, the term "PEG" used herein is an abbreviation of polyethylene glycol. Moreover, it is used herein as a banner term to cover polyethylene glycol and polyethylene oxide, which are both terms for a polymer/oligomer of the formula H-(O-CH₂-CH₂)_n-OH, but which differ on molecular weight. The term PEG when used herein may refer to a polymeric material having the formula H-(O-CH₂-CH₂)_n-OH with a molecular mass below 20,000 g/mol, based on the number average molecular weight. The term PEO when used herein may refer to a polymeric material having the formula H-(O-CH₂-CH₂)_n-OH with a molecular mass above 20,000 g/mol, based on the number average molecular weight.

As noted above, the heterobifunctional crosslinking agent comprises:

at least one functional group capable of forming a bond to a further molecule of said heterobifunctional crosslinking agent; and

at least one functional group suitable to form a bond with the first or second set of functional groups of the heterobifunctional polymer.

The at least one functional group (of the heterobifunctional crosslinking agent) suitable to form a bond with the first or second set of functional groups of the heterobifunctional polymer react with at least a portion of the first or second set of functional groups on the heterobifunctional polymer, such that the overall charge of the resulting material is modified. The "at least a portion of the first or second set of functional groups" may be from 0.01% to 100% of the first or second set of functional groups on the heterobifunctional polymer. For example, while the heterobifunctional polymer might have an overall negative charge, the reaction with the

heterobifunctional crosslinking agent may cause the material to end up with an overall neutral or overall positive charge. Thus, in this example, the heterobifunctional crosslinking agent is selected to have a functional group that can react with the second set of functional groups on the heterobifunctional polymer, thereby reducing the negative charges present in the polymeric backbone and thereby changing the overall charge of the resulting material. Whether the resulting polymer ends up with an overall negative, neutral charge or positive charge depends on the amount of heterobifunctional crosslinking agent used relative to the amount of the second set of functional groups on the heterobifunctional polymer.

When the at least one functional group suitable to form a bond with the first or second set of functional groups is suitable to form a bond with the first set of functional groups, then it may be selected from the group comprising, but not limited to, a carbonyl group, an ester, a peroxy acid and CO_2H . When the at least one functional group suitable to form a bond with the first or second set of functional groups is suitable to form a bond with the second set of functional groups, then it may be selected from one or more of amide and amino.

As will be appreciated, the at least one functional group suitable to form a bond with the first or second set of functional groups (of the heterobifunctional polymer) may itself be a functional group that has the opposite polarity to the functional group set to which it forms a bond to. For example, when the at least one functional group suitable to form a bond with the first or second set of functional groups of the heterobifunctional polymer is suitable to form a bond with the first set of said functional groups, then it may be negatively charged in an aqueous environment (e.g. at the pH ranges mentioned above) and may be selected from a peroxy acid or, more particularly, CO_2H . Correspondingly, when the at least one functional group suitable to form a bond with the first or second set of functional groups of the heterobifunctional polymer is suitable to form a bond with the second set of said functional groups, then it may be positively charged in an aqueous environment (e.g. at the pH ranges mentioned above) and may be selected from an amino group. For the avoidance of doubt, reference to an amino group in the context of forming a bond with the second set of functional groups refers to an amino functional group capable of forming a covalent bond and so it excludes quaternary and tertiary amino groups.

The heterobifunctional crosslinking agent also has at least one functional group capable of forming a bond to a further molecule of said heterobifunctional crosslinking agent. This at least one functional group capable of forming a bond to a further molecule of said heterobifunctional crosslinking agent may be one that is not charged, or is zwitterionic, in an aqueous environment (e.g. in the pH ranges described above). Suitable functional groups include, but

are not limited to norbornene, tetrazine, methacrylate, OH, SH, azide, C₂ to C₁₀ alkene, and C₂ to C₁₀ alkyne and combinations thereof (e.g. norbornene and tetrazine combinations). In particular embodiments that may be mentioned herein, the at least one functional group capable of forming a bond to a further molecule of said heterobifunctional crosslinking agent may be SH or, more particularly, OH.

For the avoidance of doubt, the at least one functional group capable of forming a bond to a further molecule of said heterobifunctional crosslinking agent may be capable of forming the bond directly or indirectly with another molecule of itself. For example, when the heterobifunctional crosslinking agent has a phenolic OH group (e.g. tyramine), the OH group of one of the molecules of tyramine may react directly with an aromatic carbon atom on the other molecule of tyramine to form an O-C bond. Alternatively, the OH group on tyramine may cause the formation of a carbon radical on the benzene ring, which can then form a C-C bond with a correspondingly activated molecule of tyramine (said C-C bond caused by the presence of the OH groups on the tyramine molecules). As will be appreciated, the reactions described above are discussed in relation to individual molecules of tyramine for clarity. In the current invention, the molecules of tyramine are bonded (by the amino group) to the polymeric backbone of the heterobifunctional polymer and when two tyramine molecules so bound react together, they form a crosslink between the respective polymeric backbones.

In other embodiments, the at least one functional group capable of forming a bond to a further molecule of said heterobifunctional crosslinking agent may refer to two different, but complementary, functional groups. As will be understood, this means that the heterobifunctional crosslinking agent may comprise two separate kinds of molecules with complementary functionalities that react together to form at least one covalent bond. An example of such functional groups are norbornene and tetrazine, which may react together in a Diels-Alder reaction to generate the desired crosslink.

Given the above, the heterobifunctional crosslinking agent may be selected from one or more of molecules suitable for undergoing Click reactions together, molecules suitable for photocrosslinking to one another, thiol-containing molecules, phenolic molecules with carboxylic acid groups, and phenolic molecules with amine groups. Examples of molecules suitable for undergoing Click reactions together include, but are not limited to, two or more of 5-norbornene-2-carboxylic acid, tetrazine acid, 3-azido-1-propanamine, 3-azidopropanoic acid, N-hydroxysuccinimide (NHS) esters, dibenzocyclooctyne-amine, 2-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)ethan-1-amine and 3-(4-(prop-2-yn-1-yloxy)benzoyl)benzoic acid (it will be appreciated that the molecules selected need to have compatible functional groups that

undergo a Click chemical reaction together). Examples of molecules suitable for photocrosslinking to one another includes, but is not limited to, methacrylic acid, and thiol-containing molecules suitable to crosslink with ene-containing molecules. Examples of thiol-containing molecules include, but are not limited to, 5-(4-aminophenyl)-1,3,4-oxadiazole-2-thiol, 3-amino-1,2,4-triazole-5-thiol, cysteamine and combinations thereof. Examples of phenolic molecules with carboxylic acid groups include, but are not limited to, 3-(4-hydroxyphenyl)propionic acid, 3,4-dihydroxyphenylacetic acid and combinations thereof. Examples of phenolic molecules with amine groups include, but are not limited to, 4-hydroxybenzylamine, dopamine, tyramine and combinations thereof. Ene-containing molecules (that may be used in photo-crosslinking reactions with the thiol-containing molecules above) include, but are not limited to, 2-propen-1-amine, allylamine, 3-butenylamine, and norbornene containing molecules (e.g. 5-norbornene-2-carboxylic acid, 5-norbornene-endo-2,3-dicarboxylic acid and 5-norbornene-2-endo-acetic acid) and combinations thereof. In particular embodiments of the invention that may be disclosed herein, the heterobifunctional crosslinking agent may be selected from 3-(4-hydroxyphenyl)propionic acid, 3,4-dihydroxyphenylacetic acid or, more particularly, tyramine.

As will be appreciated any active agent that carries a positive or negative charge in an aqueous environment (e.g. an aqueous environment having the pH ranges discussed above) may be used herein. As will be apparent, "active agent" may refer to a material that has a pharmaceutical or cosmetic effect on a subject – particularly a pharmaceutical effect.

The active agent may be a biomolecule or a small molecule active agent. Any suitable small molecule active agent may be used, such as a cosmetically active agent or, more particularly, a pharmaceutically active agent. Examples of suitable small molecule active agents include, but are not limited to, cisplatin and paclitaxel. It will be noted that cisplatin is itself a neutral molecule, but it is hydrolysed to $[\text{PtCl}(\text{NH}_3)_2(\text{H}_2\text{O})]^+$ when added to water, and it is this hydrolysed version that electrostatically interacts with the crosslinked hydrogel described herein. Examples of suitable biomolecules include, but are not limited to clustered regularly interspaced short palindromic repeats (CRISPR) components, a nucleic acid, a microRNA and a siRNA. As will be appreciated, these biomolecules are charged.

Suitable microRNAs may include, but are not limited to, 5'-UUGUGCUUGAUCUAACCAUGU-3' (miR-218), 5'-UAGCACCAUUUGAAAUCAGUGUU-3' (miR-29b duplex), 5'-AUCACAUUGCCAGGGAUUACC-3' (miR-23b), and 5'-UGAAAUGUUUAGGACCACUAG-3' (miR-203). Suitable siRNAs may include, but are not limited to, 5'-AACAAAGACCUUCGACUCUCC-3' (SPARC), 5'-

AACCTGAAGATCTTCAACAACCCTGTCTC-3' (Smad3), 5'-AACCUGCUGAAGGAUGGUGAC-3' (p53), 5'-CCAAGAACCGGAACCUGCUTT-3' (MC1R), 5'-GCAGUACCUUUCUACCACUTT-3' (MITF), and 5'-UCACUUACAGGAUCUAUAAUU-3' (Elastase).

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Indications associated with the above-mentioned biomolecules include, but are not limited to:

- wound healing and/or anti-scarring effects associated with the siRNAs 5'-AACCAAGACCUUCGACUCUUC-3' (SPARC), 5'-AACCTGAAGATCTTCAACAACCCTGTCTC-3' (Smad3) and 5'-AACCUGCUGAAGGAUGGUGAC-3' (p53);
- treatment and/or prevention of skin pigmentation associated with the siRNAs 5'-CCAAGAACCGGAACCUGCUTT-3' (MC1R) and 5'-GCAGUACCUUUCUACCACUTT-3' (MITF) and the microRNA 5'-UUGUGCUUGAUCUAACCAUGU-3' (miR-218);
- anti-wrinkle effects associated with the siRNA 5'-UCACUUACAGGAUCUAUAAUU-3' (Elastase) and the microRNA 5'-UAGCACCAUUUGAAAUCAGUGUU-3' (miR-29b duplex);
- anti-cancer effects associated with the microRNA 5'-AUCACAUUGCCAGGGAUUACC-3' (miR-23b); and
- psoriasis treatment associated with the microRNA 5'-UGAAAUGUUUAGGACCACUAG-3' (miR-203).

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It will be appreciated that more than one of the active agents mentioned herein may be present in any given formulation, thereby allowing combination treatments. Examples of such combinations formulations may involve cisplatin and an anti-cancer siRNA/microRNA or paclitaxel and an anti-cancer siRNA/microRNA. As will be appreciated, while it is preferred that the overall charge of the crosslinked hydrogel is opposite to the charge of the active agent(s) to which it interacts, this does not always need to be the case. This is demonstrated in the examples below.

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In an embodiment of the invention discussed in more detail below, the active agent is a siRNA and the crosslinked hydrogel is formed from gelatin and tyramine, where the zeta potential of the crosslinked hydrogel is from -1 mV to +10 mV, such as from 0 mV to +9 mV, such as from +0.4 mV to +8.5 mV, such as from +0.6 mV to +1 mV. Particular values for the zeta potential of the crosslinked hydrogel when the active agent is a siRNA and the crosslinked hydrogel is

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formed from gelatin and tyramine may be around 0 mV, such as ± 0.14 mV, such as ± 0.56 mV. For example, the siRNA might be 5'-AACAAGACCUUCGACUCUCC-3'.

In a further embodiment of the invention discussed in more detail below, the active agent is cisplatin and the crosslinked hydrogel is formed from gelatin and 3-(4-hydroxyphenyl)propionic acid, where the zeta potential of the crosslinked hydrogel is from -4 mV to -10 mV. In further embodiments, the sustained release composition may contain both cisplatin and 5'-AACAAGACCUUCGACUCUCC-3' SPARC.

In a yet further embodiment, the active agent may be paclitaxel and an anti-cancer siRNA and/or microRNA and the crosslinked hydrogel is formed from gelatin and tyramine, where the zeta potential of the crosslinked hydrogel is from -1 mV to +10 mV, such as from 0 mV to +9 mV, such as from +0.4 mV to +8.5 mV, such as from +0.6 mV to +1 mV.

It is believed that the crosslinked hydrogel facilitates the sustained release effect of the composition. Without wishing to be bound by theory, this effect may be particularly apparent when the overall charge of the crosslinked hydrogel is opposite to that of the active agent bound to it.

For example, a more positively charged crosslinked polymer was shown to provide a more sustained release of a negatively charged active agent (siRNA) over a period of time (see Example 3 and Fig. 5a). Similarly, a more negatively charged crosslinked polymer was shown to provide a more sustained release of a positively charged active agent (see Example 8 and Fig. 14). However, this does not always need to be the case as a crosslinked hydrogel and active agent with similar charges can also provide a sustained release to some extent.

In other words, when the charges of the active agent and the crosslinked hydrogel match, there may still be a sustained release effect, but the period of this sustained release effect is generally less than that obtained when using an active agent and a crosslinked hydrogel that are oppositely charged. Thus, the compositions disclosed herein can be selected to provide a tuned release rate to suit the condition to be treated. For example, in embodiments that relate to the prevention of scarring following a surgical procedure to treat glaucoma, it would be desirable to minimise discomfort to the subject by only having to administer a suitable anti-scarring composition once – and for the composition to provide a release rate that maintains a sufficient concentration of the active agent(s) for a period of time that prevents the need for any further surgical procedures. In such embodiments, the skilled person may select a composition comprising one or more negatively charged active agents and a crosslinked

hydrogel that is tuned to have an overall positive charge – the dose provided to the subject would be selected to provide the sustained release effect for a period of time expected to be sufficient to avoid the need for further surgical interventions in the future.

- 5 In all cases disclosed herein, the compositions disclosed herein may provide a sustained release effect of from 1 to 30 days, such as from 2 to 25 days, such as 5 to 20 days.

As will be appreciated, the overall charge of the crosslinked hydrogel may be tuned to match the desired release profile of the active agent(s) to be released. As such, the overall charge
10 of the hydrogel may be from -10 mV to +10mV.

The composition described herein may be provided as a kit of parts that allows for the assembly of the sustained release composition described above just before use. In certain embodiments, where the crosslinked hydrogel may be formed by an oxidising enzymatic
15 reaction, the kit of parts may comprise:

(Aa) a first composition comprising:

a complex of an active agent having a positive or negative charge when placed in an aqueous environment and an un-crosslinked hydrogel tuned to have an overall charge that facilitates electrostatic interaction between the un-crosslinked hydrogel and the active agent; and
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an enzyme suitable to crosslink the un-crosslinked hydrogel; and

(Bb) a second composition comprising an oxidation source (e.g. H_2O_2), wherein:

the un-crosslinked hydrogel comprises a heterobifunctional polymer that has a first set of functional groups that are positively charged and a second set of functional groups
25 that are negatively charged in an aqueous environment, where a portion of the first or second set of functional groups are capped by a heterobifunctional crosslinking agent, such that the overall charge of the un-crosslinked hydrogel is modified to facilitate the formation of the complex between the un-crosslinked hydrogel and the active agent, where:

the heterobifunctional crosslinking agent comprises at least one functional group
30 capable of forming a bond to a further molecule of said heterobifunctional crosslinking agent when the first and second compositions are combined, and at least one functional group that has formed a bond to a portion of the first or second set of functional groups of the heterobifunctional polymer, so as to reduce the number of the first or second functional groups, thereby modifying the overall charge of the un-crosslinked hydrogel and facilitating the
35 electrostatic interaction between the un-crosslinked hydrogel and the active agent.

As will be appreciated, when the two components (Aa) and (Bb) are mixed together, the crosslinked hydrogel is formed by way of an enzymatic oxidative crosslinking reaction. The formation of the crosslinked hydrogel just prior to use ensures that crosslinked hydrogel is at its optimum form (with little or no degradation) when administered to the subjects. This ensure
5 that the integrity of the crosslinked hydrogel and the active ingredient is not compromised, such that an effective delivery of the active agent can be achieved. Importantly, the enzymatic oxidative crosslinking reaction has a fast gelation rate (in minutes), which allows the crosslinked hydrogel to be formed prior to use

10 Examples of the heterobifunctional polymer and heterobifunctional crosslinking agent suitable for use in such an enzymatic oxidation crosslinking reaction include, but are not limited to gelatin and tyramine, respectively.

It will be appreciated that other kits of parts may be formed depending on the components
15 used and how they react together to form the crosslinked hydrogel. These are explicitly contemplated herein and are readily derivable based on the knowledge of a person skilled in this field.

The sustained release compositions disclosed above may be used in medicine. As will be
20 appreciated, the sustained release compositions disclosed above may be suitable for use in medicine when they contain a pharmaceutically active agent.

In embodiments of the invention that may be mentioned herein there is disclosed:

- (Ci) a use of a sustained release composition as described above in the preparation of a medicament for the treatment and/or prevention of scarring, wherein the active agent
25 of the sustained release composition is an anti-scarring agent;
- (Cii) a sustained release composition as described above for the treatment and/or prevention of scarring, wherein the active agent of the sustained release composition is an anti-scarring agent; and
- (Ciii) method of treating and/or preventing scarring, wherein the method comprises
30 providing to a subject in need thereof a pharmaceutically effective amount of a sustained release composition as described above, wherein the active agent of the sustained release composition is an anti-scarring agent.

As will be appreciated, any active agent (or combination of active agents) that can provide the
35 effect mentioned above may be used in these sustained release compositions. For example, the anti-scarring agent may be an siRNA, such as 5'-AACAAAGACCUUCGACUCUCC-3'. Other siRNAs that have this effect are described above and may also be used in place of or

in combination with 5'-AACAAAGACCUUCGACUCUCC-3'. While the anti-scarring effect may apply to any potential use (e.g. in the prevention or treatment of a pre-existing scar), it may be particularly useful in the treatment and/or prevention of scarring in a subject who has undergone surgery (e.g. eye surgery for glaucoma).

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When used herein, the term "treatment and/or prevention of scarring" is also intended to cover the treatment of wounds too.

In embodiments of the invention that may be mentioned herein there is disclosed:

- 10 (Di) a use of a sustained release composition as described above in the preparation of a medicament for the treatment and/or prevention of cancer, wherein the active agent of the sustained release composition is an anti-cancer agent (e.g. the anti-cancer agent is cisplatin or paclitaxel);
- (Dii) a sustained release composition as described above for the treatment and/or
15 prevention of cancer, wherein the active agent of the sustained release composition is an anti-cancer agent (e.g. the anti-cancer agent is cisplatin or paclitaxel);
- (Diii) a method of treating and/or preventing cancer, wherein the method comprises providing to a subject in need thereof a pharmaceutically effective amount of a sustained release composition as described above, wherein the active agent of the
20 sustained release composition is an anti-cancer agent (e.g. the anti-cancer agent is cisplatin or paclitaxel).

As will be appreciated, the treatment of which cancer(s) will be determined by the active agent(s) present in the selected sustained release composition. Examples of a suitable anti-cancer agent include, but are not limited to 5'-AUCACAUUGCCAGGGAUUACC-3' (miR-23b) and 5'-AACAAAGACCUUCGACUCUCC-3'.

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In embodiments of the invention that may be mentioned herein there is disclosed:

- (Ei) a use of a sustained release composition as described above in the preparation of a
30 medicament for the treatment and/or prevention of psoriasis, wherein the active agent of the sustained release composition is an anti-psoriasis agent (e.g. the anti-psoriasis agent is 5'-UGAAAUGUUUAGGACCACUAG-3' (miR-203));
- (Eii) a sustained release composition as described above for the treatment and/or prevention of psoriasis, wherein the active agent of the sustained release composition
35 is an anti-psoriasis agent (e.g. the anti-psoriasis agent 5'-UGAAAUGUUUAGGACCACUAG-3' (miR-203));

- (Eiii) a method of treating and/or preventing psoriasis, wherein the method comprises providing to a subject in need thereof a pharmaceutically effective amount of a sustained release composition as described above, wherein the active agent of the sustained release composition is an anti-psoriasis agent (e.g. the anti-psoriasis agent is 5'-UGAAAUGUUUAGGACCACUAG-3' (miR-203)).

In embodiments of the invention that may be mentioned herein there is disclosed:

- (Fi) a use of a sustained release composition as described above in the preparation of a medicament for the treatment and/or prevention of skin pigmentation, wherein the active agent of the sustained release composition is an anti-skin pigmentation agent (e.g. the anti-skin pigmentation agent is selected from the siRNA 5'-CCAAGAACCGGAACCUGCUTT-3' (MC1R), the siRNA 5'-GCAGUACCUUUCUACCACUTT-3' (MITF) and/or the microRNA 5'-UUGUGCUUGAUCUAACCAUGU-3' (miR-218));

- (Fii) a sustained release composition as described above for the treatment and/or prevention of skin pigmentation, wherein the active agent of the sustained release composition is an anti-skin pigmentation agent ((e.g. the anti-skin pigmentation agent is selected from the siRNA 5'-CCAAGAACCGGAACCUGCUTT-3' (MC1R), the siRNA 5'-GCAGUACCUUUCUACCACUTT-3' (MITF) and/or the microRNA 5'-UUGUGCUUGAUCUAACCAUGU-3' (miR-218));

- (Fiii) a method of treating and/or preventing skin pigmentation, wherein the method comprises providing to a subject in need thereof a pharmaceutically effective amount of a sustained release composition as described above, wherein the active agent of the sustained release composition is an anti-skin pigmentation agent ((e.g. the anti-skin pigmentation agent is selected from the siRNA 5'-CCAAGAACCGGAACCUGCUTT-3' (MC1R), the siRNA 5'-GCAGUACCUUUCUACCACUTT-3' (MITF) and/or the microRNA 5'-UUGUGCUUGAUCUAACCAUGU-3' (miR-218)).

In embodiments of the invention that may be mentioned herein there is disclosed:

- (Gi) a use of a sustained release composition as described above in the preparation of a medicament for the treatment and/or prevention of wrinkles, wherein the active agent of the sustained release composition is an anti-wrinkle agent (e.g. the anti-wrinkle agent is selected from the siRNA 5'-UCACUUACAGGAUCUAUAAUU-3' (Elastase) and/or the microRNA 5'-UAGCACCAUUUGAAAUCAGUGUU-3' (miR-29b duplex));

- (Gii) a sustained release composition as described above for the treatment and/or prevention of wrinkles, wherein the active agent of the sustained release composition

is an anti-wrinkle agent ((e.g. the anti-wrinkle agent is selected from the siRNA 5'-UCACUUACAGGAUCUAUAAUU-3' (Elastase) and/or the microRNA 5'-UAGCACCAUUUGAAAUCAGUGUU-3' (miR-29b duplex));

(Giii) a method of treating and/or preventing wrinkles, wherein the method comprises providing to a subject in need thereof a pharmaceutically effective amount of a sustained release composition as described above, wherein the active agent of the sustained release composition is an anti-wrinkle agent ((e.g. the anti-wrinkle agent is selected from the siRNA 5'-UCACUUACAGGAUCUAUAAUU-3' (Elastase) and/or the microRNA 5'-UAGCACCAUUUGAAAUCAGUGUU-3' (miR-29b duplex)).

In embodiments of the invention that may be mentioned herein there is provided a pharmaceutical composition comprising a sustained release composition as described in the first aspect of the invention or any technically sensible combination of its embodiments in combination with one or more of a pharmaceutically acceptable excipient, diluent or carrier.

Further aspects and embodiments of the invention will now be described by reference to the following non-limiting examples.

Examples

Materials

Gelatin and Horseradish peroxidase were purchased from Wako Pure Chemical Industries, Ltd., Japan. Tyramine chloride, 3-(4-hydroxyphenyl)propionic acid, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride, N-hydroxysuccinimide, cisplatin, N,N-dimethylformamide (anhydrous, 99.8%), o-phenylenediamine, sodium bicarbonate and Dulbecco's Modified Eagle's Medium (DMEM) high glucose were purchased from Sigma-Aldrich, US. Hydrogen peroxide (30 wt%) was obtained from Acros Organics, Belgium. A 21-base double-stranded small interfering RNA for SPARC (siSPARC: 5'-AACAAGACCUUCGACUCUCC-3'), a non-silencing scrambled control (siSCRAMBLED: 5'-GCUCACAGCUCAAUCCUAAUC-3') and fluorescein-tagged SPARC (FAM-SPARC) were synthesised by Simply Science, Singapore. The siRNAs were also synthesised and purified by Bioneer (Daedeok-gu, Korea). All tissue culture reagents were obtained from Invitrogen Corp. (CA, USA) unless otherwise stated. Fetal bovine serum and antibiotic-antimycotic (ABAM) (100x) were obtained from Thermo Fisher Scientific, USA. All chemicals and reagents were used as purchased without further purification.

General Synthesis 1 – Synthesis of gelatin-tyramine (Gtn-Tyr) precursor (30)

Gelatin was conjugated with tyramine using the carbodiimide crosslinking reaction as previously established in the procedures in Wang, L.-S., *et al.*, *Biomaterials*, **2010**, *31*, 8608-8616. Briefly, 2 w/v% of gelatin (Wako Pure Chemical Industries, Ltd., Japan) was dissolved in deionised (DI) water by heating the solution at 60 °C. 1 g (5.76 mmol) of Tyramine chloride (Tyr.Cl; Sigma-Aldrich, US; excess) was then added to this solution. To initiate the conjugation reaction, 0.32 to 4.25 mmol N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC.HCl) and 0.16 to 2.13 mmol N-hydroxysuccinimide (NHS) were added into the mixture. The ratio of EDC: NHS was kept at 2.0. The pH of the mixture was adjusted to 4.7 and the reaction was allowed to proceed for 24 h. After 24 h, the pH of the mixture was adjusted to 7.0 to stop the reaction. The mixture was then dialysed against DI water for 2 days using a dialysis tube with MWCO 3500 (Thermo Fisher Scientific, US). Finally, the purified mixture was freeze-dried to give the hydrogel precursor (**30**).

The precursor was dissolved in deionised water and the amount of tyramine conjugated onto the gelatin backbone was quantified using a UV-vis spectrometer via the absorbance at 275 nm. The absorbance of the precursor was compared with the absorbance of a known amount of Tyr. Each sample was tested three times and the average of three different samples synthesised using the same condition was calculated.

General Synthesis 2 – Synthesis of gelatin-3-(4-hydroxyphenyl)propionic acid (Gtn-HPA) precursor (35)

Gelatin-3-(4-hydroxyphenyl)propionic acid (Gtn-HPA) conjugates (**35**) were synthesised according to the carbodiimide crosslinking reaction in General Synthesis 1. Briefly, 1 g of 3-(4-hydroxyphenyl) propionic acid (HPA) and 0.016 to 0.158 g N-hydroxysuccinimide (NHS) were first dissolved in 20 mL of DI water mixed with 30 mL of N,N-dimethylformamide (DMF). 0.053 to 0.525 g of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC.HCl) was added to this solution to initialise the reaction. The pH of the mixture was adjusted and maintained at 4.7 for 2 h. Thereafter, 4 w/v % Gtn (2 g in 50 mL of DI water) solution was added to this mixture and the pH was again adjusted and maintained at 4.7. The reaction was then allowed to proceed overnight at room temperature. After the reaction was completed, the pH of the mixture was adjusted to 7.0. The mixture was then dialysed against 0.1 M sodium chloride (NaCl) solution for 2 days, followed by against 25% ethanol, and deionised (DI) water in sequence for 1 day each. The dialysed product was freeze-dried to give the hydrogel precursor (**35**).

Similar to General Synthesis 1, the precursor was dissolved in deionised water and the amount of HPA conjugated onto the gelatin backbone was quantified using a UV-vis spectrometer *via* the absorbance at 276 nm.

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General Methods

Zeta potential measurements of Gtn-Tyr and Gtn-HPA precursors (30 and 35, respectively)

10 The zeta potentials of Gtn-Tyr and Gtn-HPA precursors with different conjugation degrees were measured using the Malvern Zetasizer. The freeze-dried precursors were dissolved in deionised (DI) water at a concentration of 5 w/v%. The zeta potential was measured in DTS1070 disposable folded capillary cells at the temperature of 25 °C. The instrument was calibrated using a latex with known zeta potential. Each sample was tested three times and
15 the average of three different samples synthesised using the same condition was calculated. The zeta potential of precursors is abbreviated as “ZP” in some of the examples as described below.

Statistical analysis

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All data were expressed in mean $\pm$ standard deviation with a replicate of $n=3$ unless otherwise specified. The differences between the values were assessed using one-way ANOVA, where $p < 0.05$ was considered statistically significant.

25 **Example 1.** Preparation of polyplexes of siRNA-Gtn-Tyr (50) and crosslinked hydrogel of siRNA-Gtn-Tyr (55)

The crosslinked hydrogel of siRNA-Gtn-Tyr (55) of the current invention was prepared using the Gtn-Tyr precursor (30) synthesised in accordance to General Synthesis 1. A schematic
30 representation of the preparation of the crosslinked hydrogel of siRNA-Gtn-Tyr 55 of the current invention is as shown in Fig. 1a. As will be appreciated, the siRNA can be any form of siRNA (i.e. siSPARC) or any other active agent.

Experimental procedure

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Typically, the Gtn-Tyr precursor (**30**) can be crosslinked (with or without the siRNA) by first dissolving the freeze-dried precursor (**30**), from General Synthesis 1, in 1x PBS to form a 5 w/v% precursor solution. This was then followed by the addition of horseradish peroxidase (HRP) and H₂O₂ to form the crosslinked Gtn-Tyr hydrogel with different properties. The final concentrations of HRP and H₂O₂ in the mixture were 0.05-0.15 units/mL and 1-9 mM, respectively.

To prepare the crosslinked hydrogel of siRNA-Gtn-Tyr (**55**), a 5 w/v% Gtn-Tyr solution was first prepared by dissolving freeze-dried precursor (**30**) in 1x PBS. The siSPARC (**40**) (50 to 2000 pmol) was then added into the 0.5 ml Gtn-Tyr solution and allowed to incubate for 15 min. Thereafter, HRP (3 μ L) and H₂O₂ (5.1 μ L) were added to the mixture and the sample was allowed to set for 0.5 h to give the crosslinked hydrogel (**55**). The final concentrations of HRP and H₂O₂ in the mixture were 0.15 units/mL and 3 mM, respectively.

Results and discussion

The schematic diagram in Fig. 1a shows the conjugation of tyramine (**20**) onto the gelatin (**10**) backbone through carbodiimide crosslinking reaction in aqueous condition to form the Gtn-Tyr precursor (**30**). The positively charged amine groups (in the form of ammonium) of gelatin allowed electrostatic interaction with the negatively charged siRNA (**40**) to form the polyplexes (**50**). The crosslinking reaction was then performed on the polyplexes (**50**) via the peroxidase-mediated reaction with the use of horseradish peroxidase (HRP) and H₂O₂ to form the crosslinked siRNA-Gtn-Tyr hydrogel (**55**) of the current invention. Typically, the crosslinks between the adjacent polyplexes were formed through the covalent bonds formed between the phenol groups of the tyramine molecules (Fig. 1b). This enzymatic crosslinking method is suitable for this application as it is biocompatible, has a fast gelation rate (in minutes) and can be performed under mild reaction conditions (Teixeira, L.S.M., *et al.*, *Biomaterials*, **2012**, 33, 1281-1290; Sakai, S., *et al.*, *Biomaterials*, **2009**, 30, 3371-3377; Lee, F., *et al.*, *Soft Matter*, **2008**, 4, 880-887; Wang, L.-S., *et al.*, *Biomaterials*, **2014**, 35, 2207-2217).

To provide post-glaucoma surgery anti-scarring management, the siSPARC-Gtn-Tyr polyplexes (**50**) can be administered into the patient's conjunctiva using a dual syringe (**60**) after the glaucoma filtration surgery, to allow the formation of the crosslinked hydrogel (**55**) *in situ* (Fig 2). The mixture of HRP and siRNA-Gtn-Tyr polyplexes (**50**), and the mixture of H₂O₂ and siRNA-Gtn-Tyr polyplexes (**50**) were stored in two separate syringes to prevent the crosslinking from occurring before administration. The injection of the dual syringe (**60**) allows the mixing of the two components, therefore, allowing the crosslinking to take place. This

allows the crosslinked siRNA-Gtn-Tyr hydrogel (**55**) to be formed *in situ* to give a more effective delivery of the siSPARC.

Example 2. Effects of various degrees of conjugation of tyramine to gelatin on the properties of crosslinked hydrogel of siRNA-Gtn-Tyr (55**) of the current invention**

To understand the effect of conjugating tyramine to gelatin on the crosslinked siRNA-Gtn-Tyr (**55**) of the current invention, different Gtn-Tyr precursors (**30**) were synthesised using various amount of EDC and NHS (in accordance to General Synthesis 1). The properties of the as-synthesised precursors (**30**) and crosslinked hydrogel (**55**) were then studied.

Different Gtn-Tyr precursors (**30**) were synthesised using various amount of EDC and NHS (with reaction time of 24 h) as shown in Tables 1a and 1b below. The gelatin without conjugation process (R1) was shown to comprise 52.37 ± 0.47 $\mu\text{mol/g}$ phenylalanine and tyrosine residues. The increase in the amount of EDC and NHS led to the increased rate of conjugation, therefore resulting in a higher amount of tyramine conjugated onto the gelatin backbone. The conjugation of tyramine also reduced the amount of “free” unreacted carboxylic acid groups on the gelatin, resulting in the increase in positive surface charge due to the unchanged amount of amine groups (can be protonated to form positively charged ammonium groups). Fig. 3a and b depict the initial and subsequent studies relating to the correlation of the zeta potentials of various Gtn-Tyr precursors (**30**) to the amount of phenol (which corresponds to the amount of tyramine conjugated to the gelatin backbone). Both Fig. 3a and b show that the zeta potential of Gtn-Tyr precursor (**30**) increased linearly with increasing amount of Tyr. The increase in zeta potential was proposed to increase the interaction of the gelatin with the negatively charged siRNA, thus improving the encapsulation capacity of the hydrogel for the siRNA.

Table 1a. Initial synthesis of Gtn-Tyr precursor (**30**) using various amounts of EDC and NHS.

Sample name	Tyramine (mmol)	EDC (mmol)	NHS (mmol)	Amount of phenol ($\mu\text{mol/g}$)		Zeta Potential (mV) (for subsequent studies)
				Initial studies (Fig. 3a)	Subsequent studies (Fig. 3b)	
R1	-	-	-	52.37 ± 0.47	62.70 ± 2.70	-3.25 ± 0.45
R2	5.76	0.53 (0.32)*	0.27 (0.16)*	118.87 ± 2.25	-	-
R3	5.76	0.64 (0.53)*	0.32 (0.27)*	137.65 ± 1.48	185.74 ± 5.08	-1.24 ± 0.44

Sample name	Tyramine (mmol)	EDC (mmol)	NHS (mmol)	Amount of phenol ($\mu\text{mol/g}$)		Zeta Potential (mV) (for subsequent studies)
				Initial studies (Fig. 3a)	Subsequent studies (Fig. 3b)	
R4	5.76	0.74	0.37	177.76 ± 1.68	224.45 ± 9.32	-0.56 ± 0.35
R5	5.76	1.06	0.53	196.13 ± 3.70	264.47 ± 10.17	$+0.45 \pm 0.13$
R6	5.76	2.12	1.06	298.69 ± 1.35	393.98 ± 15.23	$+3.21 \pm 0.24$
R7	5.76	3.18	1.59	355.82 ± 7.63	488.99 ± 12.78	$+6.29 \pm 0.43$
R8	5.76	4.25	2.13	408.61 ± 5.41	-	-

* The values in bracket refer to the amounts used in subsequent studies.

Table 1b. Subsequent synthesis of Gtn-Tyr precursor (**30**) using various amounts of EDC and NHS.

Sample name	Tyramine (mmol)	EDC (mmol)	NHS (mmol)	Amount of phenol ($\mu\text{mol/g}$)	Zeta potential (mV)
Gelatin	-	-	-	62.8 ± 2.7	-3.29 ± 0.35
F1	5.76	0.74	0.37	220.5 ± 11.9	-0.30 ± 0.26
F2	5.76	3.07	1.54	385.0 ± 27.7	$+6.34 \pm 0.45$

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To understand the properties of the crosslinked hydrogels formed from the various precursors (**30**), peroxidase-mediated coupling reaction on the precursors was performed (without the siRNA). In the formation of the crosslinked hydrogel, the phenol crosslinking sites of Gtn-Tyr were oxidised through peroxidase-mediated coupling reaction to form the hydrogel network. As shown in Fig. 4a, after the addition of HRP and H_2O_2 , the Gtn-Tyr precursor (**30**) with a low amount of tyramine (R3-R5 of Table 1a) formed a transparent hydrogel, while the precursor (**30**) with a higher amount of tyramine (R6-R7 of Table 1a) gave a crosslinked hydrogel with a higher opacity (when fabricated with the same amount of crosslinkers, but different amount of NHS and EDC).

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In addition, increasing the H_2O_2 concentration (from 1 mM to 6 mM) increased the stiffness of the crosslinked Gtn-Tyr hydrogel (Fig. 4b). Further, to demonstrate the ease of injectability of the precursor and crosslinked hydrogel, Gtn-Tyr precursor (**30**) together with HRP and H_2O_2 were passed through a 32 gauge Hamilton syringe (with 0.24 mm outer diameter and 0.11 mm inner diameter). As shown in Fig. 4c, the Gtn-Tyr precursor has no issue passing through the 32 gauge needle and to form 10 μL of the crosslinked Gtn-Tyr hydrogel.

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Overall, the results showed that the properties of the crosslinked Gtn-Tyr hydrogel (i.e. electrostatic properties, opacity and stiffness) can be easily adjusted to cater for different

applications. The Gtn-Tyr precursor and hydrogel also showed their superior injectability through needle with very small gauge, therefore can be used as a minimally invasive treatment for patients.

5 **Example 3. Effects of electrostatic properties of the Gtn-Tyr precursors (30) on the release profile of siRNA from the siRNA-Gtn-Tyr crosslinked hydrogel (55), and the degradation profile of the crosslinked Gtn-Tyr hydrogels**

10 The release profile of the crosslinked siRNA-Gtn-Tyr hydrogel (55) of the current invention was investigated to understand the release of siRNA from the crosslinked hydrogel. To allow the determination of the release rate, a fluorescein-tagged siSPARC (FAM-SPARC) was used in place of the actual siRNA (siSPARC). In addition, the degradation profile of crosslinked Gtn-Tyr hydrogels in the presence of collagenase were also investigated.

15 *Experimental procedure*

In this study, Gtn-Tyr precursor (30) with small surface positive charge ($ZP = + 0.61 \pm 0.01$ mV) and another one with large surface positive charge ($ZP = + 7.83 \pm 0.45$ mV) were used. A 5 w/v% Gtn-Tyr precursor solution was prepared by dissolving the freeze-dried precursor (30) in 1x PBS. Different amount of FAM-SPARC (50 to 2000 pmol) was then loaded into the 0.5 mL Gtn-Tyr solution and allowed to incubate for 15 min. Thereafter, HRP (0.1 mL) and H_2O_2 (0.1 mL) were added to the mixture (to give final concentrations of 0.15 units/mL and 3 mM, respectively) and then cast into a well in a 96-well plate. The sample was then allowed to set for 0.5 h before it was topped up with 0.1 mL of 1x PBS as the release buffer solution. At certain time points, the release buffer solution was collected and replenished with fresh solution. The release buffer solution was then characterised by a fluorescence spectrometer at an excitation wavelength of 488 nm and an emission wavelength of 518nm (Tecan Infinite M2000, Tecan Group Ltd, Switzerland). The fluorescence intensity was compared to the fluorescence intensity of known amount of FAM-SPARC to determine the amount of FAM-SPARC released.

Degradation profile of crosslinked Gtn-Tyr hydrogels

For the degradation study, Gtn-Tyr precursors F1 and F2 were used (Table 1b). Typically, 5 w/v% Gtn-Tyr solution was first prepared by dissolving the freeze-dried precursor in 1x PBS. Thereafter, HRP and H_2O_2 were added to 0.2 mL precursor solution in a 2 mL microtube. The final concentrations of HRP and H_2O_2 in the mixture were 0.05 units/mL and

3 mM, respectively. The sample was allowed to set for 0.5 h before it was topped up with 0.2 mL collagenase type I with a concentration of 0.5 units/mL. At a designated time point, the collagenase solution was removed and the sample was weighed before further fresh collagenase solution was added. The percentage weight loss at each time point was calculated using formula below:

$$\text{Percentage weight loss (\%)} = \frac{W_i - W_t}{W_i} \times 100\%$$

where W_i and W_t are the sample's initial weight and weight at the designated time points respectively.

Results and discussion

Fluorescence-tagged SPARC (FAM-SPARC) was used to study the release profile of as-prepared crosslinked siRNA-Gtn-Tyr hydrogel synthesised using Gtn-Tyr precursor (**30**) with small surface positive charge ($ZP = +0.61 \pm 0.01$ mV) and large surface positive charge ($ZP = +7.83 \pm 0.45$ mV).

As shown in Fig. 5a, the sample with small positively charged surface showed a very large initial burst release (~34 %) of the FAM-SPARC after day 1, followed by a first order release (with a relatively constant amount of FAM-SPARC release over time). On the other hand, the sample with large surface positive charge gave very small initial burst release (~9 %) after day 1, followed by linear release for 7 days. It was observed that the release profile was correlated with the electrostatic properties of Gtn-Tyr precursor (**30**), which was indirectly related to the amount of tyramine conjugated to gelatin.

The Gtn-Tyr with smaller surface positive charge showed weaker interaction with the negatively charged siRNA, therefore resulting in the large initial burst release. In contrast, the Gtn-Tyr with larger surface positive charge demonstrated stronger interaction with the siRNA, which enabled a tighter binding of the siRNA, resulting in smaller initial burst release and linear release. Overall, the amount of tyramine conjugated onto the gelatin affects the electrostatic properties of gelatin, which ultimately controls the release kinetics of the siRNA from the crosslinked hydrogel formed.

For the degradation study, it was observed that crosslinked Gtn-Tyr hydrogels prepared from precursors with higher amount of tyramine (F2) showed a slower degradation rate than that

prepared from precursor with less amount of tyramine (F1) (Fig. 5b). This was probably due to a higher crosslinking density within the hydrogel with more conjugated tyramines. As will be appreciated, this shows that the degradation rate of the crosslinked hydrogel can be easily controlled to cater to different needs of the application.

Example 4. *In vitro* cytocompatibility of crosslinked Gtn-Tyr hydrogel with different surface charges on C57Bl6/J MTF cells

To demonstrate the *in vitro* cytocompatibility of the crosslinked Gtn-Tyr hydrogel of the current invention, the crosslinked hydrogel was tested on C57Bl6/J mice tenon fibroblasts (MTFs)

Experimental procedure

Cell culture of C57Bl6/J mice tenon fibroblasts (MTFs)

Small biopsy samples containing subconjunctival mice tenon fibroblasts (MTFs) were obtained from C57Bl6/J mice during standard intraocular surgery with approval by the institutional ethics committee. The mice tenon explants were placed on a culture dish with a drop of fetal bovine serum (FBS) for 15 mins before adding in DMEM supplemented with 10 % FBS and Penicillin-Streptomycin (100 U/ml and 100 µg/ml respectively). The explants were incubated at 37°C in a humidified incubator with 5% CO₂. Primary MTFs that migrated out from the tissue were propagated in the same medium.

Cytocompatibility of the crosslinked Gtn-Tyr hydrogel (without siRNA)

A 5 w/v% Gtn-Tyr solution is prepared by dissolving freeze-dried Gtn-Tyr precursor (**30**), from General Synthesis 1, in Dulbecco's Modified Eagle Media (DMEM) high glucose supplied with 10% fetal bovine serum (FBS) and 1% penicillin. This was followed by the addition of HRP and H₂O₂ to the 0.5 mL Gtn-Tyr precursor solution to crosslink the sample, which was then cast into a well in a 24-well plate. The final concentrations of HRP and H₂O₂ in the mixture were 0.15 units/mL and 3 mM, respectively. C57Bl6/J MTFs of less than passage 8 were used in this study. C57Bl6/J MTFs were then seeded on top of the hydrogel at a density of 2 x 10⁴ cells per well. The mixture of hydrogel and cells in each well was then topped up with 0.5 mL culture medium and incubated at 37 °C and 5% CO₂.

At day 1, 3, 5 and 7, the proliferation of C57Bl6/J MTFs was characterised using PrestoBlue™ cell viability reagent (Thermo Fisher Scientific, US) according to the manufacturer's protocol.

The cells were also visualised under Axio Observer Z1 inverted microscope (Carl Zeiss Pte. Ltd., Germany) using LIVE/DEAD™ cell viability assay at day 1, 3, 6 and 7, with excitation/emission wavelengths at 494/517 nm and 517/617 nm for the green (for staining live cells) and red dyes (for staining dead cells), respectively.

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Results and discussion

The cytocompatibility of the crosslinked Gtn-Tyr hydrogel synthesised using Gtn-Tyr precursor (30) with small surface charge ($ZP = +0.61 \pm 0.01$ mV) and large surface charge ($ZP = +7.83 \pm 0.45$ mV) was investigated to compare the effect of tyramine or surface charge on the viability and proliferation of the cells.

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The proliferation profile as shown in Fig. 6a indicated that the increase in the amount of tyramine or surface charge did not affect the cytocompatibility of the crosslinked hydrogel on the cells. In addition, C57Bl6/J MTFs were shown to be able to attach to the surface of the plate/hydrogel and showed positive proliferation rate for 7 days on both samples with small and large surface positive charges. The fluorescence images of the cells in Fig. 6b visually confirmed that the C57Bl6/J MTFs were attached and alive at day 1, 3, 6 and 7 in samples with small and large positive charges.

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Overall, it was observed that the crosslinked Gtn-Tyr hydrogel demonstrated efficacy support towards supporting attachment and proliferation of eye fibroblast cells, without causing any toxic effects. The crosslinked Gtn-Tyr hydrogel demonstrated its excellent cytocompatibility, regardless of the amount of conjugated tyramine on the gelatin or the surface charges. This shows that the crosslinked Gtn-Tyr hydrogel is a potential delivery system with tunable electrostatic properties for ocular therapies.

Example 5. *In vitro* siSPARC silencing of C57Bl6/J MTF cells using the crosslinked siSPARC-Gtn-Tyr hydrogel (55) with different surface charges

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The *in vitro* efficacy of siSPARC-Gtn-Tyr hydrogel (55) of the current invention in silencing the SPARC expression in live cells were carried out to understand the potential of the current invention for anti-scarring therapy.

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Experimental procedure

Freeze-dried Gtn-Tyr precursor (30) (R3-R7 of Table 1a) was dissolved in DMEM high glucose without FBS and loaded with siSPARC (20 μ L, 4 nmol/mL). The mixture was incubated for 15

min. The mixture (0.5 mL) was then crosslinked with HRP (3 μ L) and H₂O₂ (5.1 μ L) and cast into a well of a 12-well plate. The final concentrations of HRP and H₂O₂ in the mixture were 0.15 units/mL and 3 mM, respectively. C57Bl6/J MTFs were then seeded on top of the hydrogel with a density of 3×10^4 cells per well. The sample was topped up with 0.5 mL culture medium and incubated at 37 °C and 5% CO₂.

At days 2 and 7, the total RNA was recovered from the samples with Trizol Reagent (Invitrogen Corp.) according to the manufacturer's recommendations. First-strand cDNA was synthesised using 500 ng of total RNA extract and 1 μ L of 50 ng/ μ L random hexamer primer (Invitrogen Corp.) with Superscript III reverse transcriptase (Invitrogen Corp.) according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed in a total volume of 10 μ L in 384-well microtiter plates. Each reaction consisted of 1 μ L of the first-strand reaction product, 0.5 μ L each of upstream and downstream primers (10 μ M each), 4 μ L of Power SYBR Green PCR Master Mix (Applied BioSystems, CA, USA), and 4 μ L of DNase-RNase-free distilled water (Sigma-Aldrich Corp., MO, USA). Amplification and analysis of the cDNA fragments were carried out using the Roche LightCycler 480 System (Roche Diagnostics Corp, Indianapolis, USA). All PCR reactions were performed in triplicate. All mRNA levels were measured at cycle threshold (CT) levels and were normalised with the corresponding β -actin CT values. Values were expressed as fold increase over the corresponding values for untreated WT control by the $2^{-\Delta\Delta CT}$ method.

Negative controls were carried out using a crosslinked siRNA-Gtn-Tyr hydrogel prepared using a non-silencing scrambled siRNA (siSCRAMBLED), instead of siSPARC.

Results and discussion

The SPARC silencing effect was studied at day 2 and 7 using crosslinked siSPARC-Gtn-Tyr hydrogels made from Gtn-Tyr precursors R3 to R7 (in Table 1a) with increasing zeta potential.

As shown in Fig. 7a, all the sample groups except R3 showed SPARC silencing effect at day 2. However, only the crosslinked siSPARC-Gtn-Tyr hydrogels of R4 (* $p=2.9 \times 10^{-5}$), R6 (* $p=3.9 \times 10^{-4}$) and R7 (* $p=4.0 \times 10^{-3}$) showed significant down-regulation of SPARC expression as compared to the "SCRAMBLED" group. At day 7, the crosslinked hydrogels of R3 (* $p=3.3 \times 10^{-3}$), S4 (* $p=1.6 \times 10^{-6}$) and R6 (* $p=8.8 \times 10^{-3}$) showed significant SPARC silencing as compared to "SCRAMBLED" group (Fig. 7b).

Overall, only the crosslinked siSPARC-Gtn-Tyr hydrogels of R4 and R6 showed sustained SPARC silencing over 7 days. In addition, the crosslinked hydrogels of R4 showed the most statistically significant SPARC downregulation as compared to other sample groups at day 2 and 7. Given this, under the same fabrication conditions, it was observed that the crosslinked siSPARC-Gtn-Tyr of R4 with zeta potential of $+0.14 \pm 0.03$ mV (based on initial studies) appears to have the optimum parameters for sustained SPARC silencing.

The amount of tyramine conjugated to gelatin was shown to significantly affect the properties of the Gtn-Tyr hydrogel formed (such as the stiffness and electrostatic properties) which ultimately affect the effectiveness of siSPARC delivery and silencing effect. The results suggested that Gtn-Tyr precursor (**30**) with sufficient crosslinking sites (~ 180 $\mu\text{mol/g}$ for R4, based on initial studies) and surface charge close to $+0$ mV has suitable physical or material properties for a delivery system with good siSPARC release for anti-scarring effect. It was observed that the crosslinked hydrogels made from precursors with too few crosslinking sites were easily degraded and they produced inconsistent results (large standard deviation). On the other hand, crosslinked hydrogels made from precursors with high surface charge showed SPARC silencing in the initial stage (day 2), but the effect was observed to be diminished slowly at day 7. This may be due to the strong surface charge that restricts the effective amount of siSPARC to be released out, resulting in decreased downregulation effect.

Fig. 8 shows the schematic representation of the delivery mechanism of the as-prepared crosslinked siSPARC-Gtn-Tyr hydrogel (**55**) into the cells (**58**), which involves electrostatic protection and delivery of the siSPARC (**40**) into the cellular environment. Upon crosslinking (using HRP and H_2O_2), the crosslinked Gtn-Tyr hydrogel possesses a net positive charge, which stabilises and encapsulates the negatively charged siRNA (i.e. siSPARC). After administering the hydrogel (**55**) to a target subject, the hydrogel (**55**) can undergo degradation (**56**) over time (under ambient conditions, by enzymes etc) to form siSPARC-Gtn-Tyr polyplexes (**57**), which are then internalised into the cells (**58**). Such polyplexes (**57**), which typically comprise the net positively charged gelatin backbone and the net negatively charged siSPARC, are able to prevent the siSPARC from being degraded by the extracellular matrix. At the same time, the polyplexes (**57**) also facilitate cellular internalisation through the cell membrane which is usually negatively charged *via* a clathrin-mediated endocytotic pathway

As such, this delivery mechanism of the current invention is able to provide an efficient delivery of the active agent (i.e. siSPARC) into the targeted cells, which can improve the therapeutic efficacy of the active agent. Importantly, such crosslinked hydrogels also provide tunability of

the properties (i.e. electrostatic properties, octanol-water partitioning), which are necessary for dermal drug delivery, skin transport and cell internalisation.

Example 6. *In vivo* efficacy of crosslinked siSPARC-Gtn-Tyr hydrogel (55) on wound healing and scar prevention after surgery

To demonstrate the *in vivo* efficacy of the crosslinked siSPARC-Gtn-Tyr hydrogel (55) of the current invention, a modified version of an established rabbit model of glaucoma filtration surgery was used to investigate the effects of the hydrogel in healing wound and preventing scar formation after surgery.

Glaucoma surgery (trabeculectomy) typically involves making a small incision in the eye wall (sclera) and to allow the aqueous humour in the eye to be drained through a small reservoir or bleb. In this way, fluids in the eye are drained out into the bleb, therefore reducing the intraocular pressure (IOP). However, a common cause of glaucoma surgery failure is subconjunctival fibrosis in the bleb, which can lead to lack of filtration and a flat bleb with subsequent increases in IOP. Given this, it is important that the current invention is able to prevent or reduce fibrotic response in the blebs to prevent the occurrence of failing blebs.

Experimental procedure

Surgical Procedure

All animal procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Fifteen New Zealand White rabbits (2–2.4 kg, 12–14 weeks old; SEMC, Duke-NUS, NCCS Animal Facility, Sembawang, Singapore) were acclimatised for 7 days before the experiment was commenced. The animals were anaesthetised with a combination of ketamine (Ketaset; Fort Dodge Animal Health, Southampton, UK) and medetomidine HCl (Domitor; Pfizer Animal Health, Sandwich, UK). A fornix based conjunctival flap was raised and blunt dissection of the subconjunctival space was performed at approximately 3 mm along the limbus and 5 mm posteriorly. A 24-gauge, 25-mm intravenous cannula (Venflon 2; Becton Dickinson, Oxford, UK) was used to create a sclerostomy, starting 2.5mm behind the limbus, passing into clear cornea before entry into the anterior chamber. Once the cannula and needle were seen in the anterior chamber, the needle was withdrawn and removed as the cannula was advanced into the mid-pupillary area. The cannula was then trimmed and bevelled at its scleral end to protrude 1mm from the insertion point. A 10-0 nylon suture (B/V 100-4; Ethicon) fixed the tube to the scleral surface.

Water tight closure of the conjunctival incision was performed using 10-0 nylon *via* purse-string sutures and where necessary a mattress suture. One drop each of guttae chloramphenicol and Betnesol-N (Glaxo Wellcome, Uxbridge, UK) ointment was instilled at the end of surgery. The surgery was executed by the same masked individual. Only the left eye was operated on, and the surgical procedure was performed at the same site superiorly in each animal.

Treatment Regimen

Five rabbits each were randomly allocated to one of three treatment groups: (1) subconjunctival injection of 0.1 mL of crosslinked siSPARC-Gtn-Tyr hydrogel (R4 of Table 1a); (2) subconjunctival injection of 0.1 mL crosslinked siSCRAMBLED-Gtn-Tyr hydrogel; and application of Mitomycin C (MMC) application for 1 min to the subconjunctival space during surgery.

Groups receiving the subconjunctival injection had it administered immediately after surgery and on Days 3, 7 and 9 post operatively. The topical antibiotic and steroid drops were administered daily for 2 weeks post-operatively. The animals were sacrificed on day 30. Only the left eye was enucleated and histologically analysed.

Examination and Clinical Evaluation

Baseline recordings included measurement of intraocular pressure (IOP) and the appearance of the superior bulbar conjunctiva. IOPs were recorded in both eyes with a handheld tonometer (Tono-pen; Mentor, Norwell, MA), after topical instillation of 0.5% Lignocaine HCL.

Postoperative observations were performed at weekly intervals until sacrifice. A single, masked independent investigator objectively graded each bleb for survival and vascularity based on slit lamp examination and photography. The primary outcome metric was bleb histology and bleb survival, which was defined as the presence of an elevated subconjunctival fluid pocket at the surgical site. Slit-lamp microscopy was performed using Righton LED slit lamp MW50D (Right Mfg Co Ltd, Japan). *In vivo* confocal microscopic examinations of the operated and treated conjunctiva were performed using Hrt3 microscope (Heidelberg Engineering, Heidelberg, Germany). Optical coherence tomography angiography of the bleb vasculature was captured Optovue AngioVue™ (Optovue, Inc., Freemont, CA).

Histologic Evaluation

Both eyes were enucleated. The upper lid was removed together with the whole eye to preserve the bleb and superior conjunctiva. Histochemical evaluation of operated conjunctival cryosections by H&E, picro-sirius red and Masson's Trichrome staining was performed.

Qualitative clinical and histological evaluation was important for evaluating the efficacy of the crosslinked hydrogels in the *in vivo* study.

Results

To ascertain the effectiveness and translational potential of the crosslinked siSPARC-Gtn-Tyr hydrogel in enhancing anti-scarring effect, *in vivo* studies on a rabbit model that have undergone glaucoma filtration surgery with insertion of a 24-gauge cannula were carried out. Rabbits administered with crosslinked siSCRAMBLED-Gtn-Tyr hydrogel and MMC were included as negative and positive controls, respectively. The operated conjunctiva treated with the crosslinked siSPARC-Gtn-Tyr hydrogel, crosslinked siSCRAMBLED-Gtn-Tyr hydrogel and MMC were imaged and analysed *via* various techniques as discussed.

Slit lamp microscopy

Characterisation of the eyes by slit-lamp microscopy showed that the one-time application of MMC at 0.2 mg/mL was unable to prevent bleb loss which occurred by 2 weeks post-surgery (data not shown), and the blebs were clearly not visible at the 4th week (Fig. 9a). Similarly, crosslinked siSCRAMBLED-Gtn-Tyr hydrogel treatment also failed to maintain bleb survival at the 4th week (Fig. 9b). In contrast, a shallow but visible bleb can still be observed upon treatment with crosslinked siSPARC-Gtn-Tyr hydrogel for at least 4 weeks (Fig. 9c).

In vivo confocal microscopy

When the week 4 postoperative tissues were examined by *in vivo* confocal microscopy, it was observed that MMC treatment had grossly loosened the subconjunctival matrix with numerous microcysts retained in the failed bleb (Fig. 9d). This morphology was different from the subconjunctiva treated with crosslinked siSCRAMBLED-Gtn-Tyr hydrogel which showed densely organised matrix fibers and the absence of microcysts (Fig. 9e). On the other hand, treatment with crosslinked siSPARC-Gtn-Tyr hydrogel resulted in an ostensibly perturbed matrix fiber organisation and the presence of numerous microcysts (Fig. 9f). Moreover,

treatment with the crosslinked siSPARC-Gtn-Tyr hydrogel gave mainly straight vasculature which included both large and fine vessels in the subconjunctiva (Fig. 9i). This vasculature architecture is a distinguishing feature against the mainly tortuous vessels found in both MMC and crosslinked siSCRAMBLED-GTn-Tyr hydrogel treated tissues (Fig. 9g and h). Finally, while hyper-reflective dots characteristic of MMC treatment were easily visible (Fig. 9j), the hydrogel remnants were visualised within the treated subconjunctivas as a cobbled stone-like network (Fig. 9k and l).

Histology

Histological examination of the treated conjunctivas provided corroborating evidence for the effectiveness of the crosslinked siSPARC-Gtn-Tyr hydrogels treatments in facilitating bleb survival. Hematoxylin and eosin (H&E), Masson Trichrome and picrosirius staining revealed that although a cleared area almost devoid of matrix was present in the MMC-treated conjunctiva after 4 weeks (*, Fig. 10a-c), a surprising relatively large portion of the tissue was occupied by dense collagen fibres (ψ , Fig. 10a-c). This unusual deposition of collagen scar protein suggests that MMC drug activity was very localised and affected scarring only in areas reached by the administered drug.

In contrast, the tissue treated with crosslinked siSCRAMBLED-Gtn-Tyr hydrogel showed less focal deposition of dense collagen fibres, with some areas comprising of disorganised and sparse collagen deposition (Fig. 10d-f). This observation suggests that hydrogel *per se* may provide a physical barrier to normal scar deposition in the treated area. In both MMC- and crosslinked siSCRAMBLED-Gtn-Tyr hydrogel-treated tissues, the collagen fibres present in the operated area were mainly yellowish-orange when visualised using picrosirius red staining (Fig. 10c and f), suggesting that the mature form of collagen had settled in the operated areas by 4 weeks post-surgery. In contrast, treatment with crosslinked siSPARC-Gtn-Tyr hydrogel resulted in a collagen matrix that was vastly distinct from the former two treatments. The collagen matrix in the area treated with crosslinked siSPARC-Gtn-Tyr hydrogel was sparse, diffused and appeared to consist of seemingly short and disrupted fibres (Fig. 10g and h). Visualisation of the sections stained by picrosirius red further revealed that the collagen fibres assembled in response to the crosslinked siSPARC-Gtn-Tyr hydrogel treatment were predominantly greenish-yellow in birefringence (Fig. 10i). This suggests that there may be suppression of maturation of newly-deposited collagen fibres with such treatment.

Discussion

SPARC is a protein that mediates fibrosis by regulating collagen production and assembly in the extracellular matrix. This is done *via* modulating the interaction of the cell surface and collagen and also *via* regulating the incorporation of collagen to fibrils. As such, attempts at anti-fibrosis and anti-scarring therapy would involve disrupting collagen, other extracellular and matricellular proteins, and would lead to the maintenance of a bleb.

The result as shown from the rabbit model suggests that SPARC silencing, facilitated by hydrogel as a delivery vehicle, can effectively modulate several biological responses associated with bleb survival. First, treatment with crosslinked siSPARC-Gtn-Tyr hydrogel resulted in straight vasculature and the generation of multiple microcysts, which have been reported to correspond to a functioning bleb in human. Second, the deposition of diffused, disrupted and immature collagen fibres resulting from the crosslinked siSPARC-Gtn-Tyr hydrogel treatment implies the reduction or delay of mature scar formation, which is a major cause of bleb failure. Notably, it appears that the hydrogel component also likely contributed to the mechanical disruption of collagen deposition, further supporting the diffused morphology that was observed in the confocal and histology images. Overall, the presence of Gtn-Tyr hydrogel allows the controlled, slow release of siSPARC at the target site as the hydrogel degraded over a specific time frame. In addition, the gelatin can help to facilitate the internalisation of the siSPARC into the cells to initialise treatment. Further, another advantage of using the hydrogel as the delivery vehicle is that the volume of the hydrogel allows the mechanical preservation of a large space within the subconjunctival space, which possibly provided a further obstacle to fibrosis.

The crosslinked siSPARC-Gtn-Tyr system was also compared with a commonly used anti-scarring agent MMC. Rabbit models typically demonstrate more intense fibrosis and scar formation and the use of very high dose MMC is usually required to maintain bleb survival. However, this can lead to significant tissue destruction and toxicity to the subjects. In this study, the MMC dose amount which would be used on humans was administered and scar formation was noted within the first week of surgery. This highlights the importance of seeking an alternate anti-fibrotic agents, and in this case, it was observed that targeting the SPARC proteins using siSPARC gave positive results. The eyes treated with crosslinked siSPARC-Gtn-Tyr hydrogel showed significantly less subconjunctival scarring, which led to prolonged bleb survival and the observed vascularity at week 4 after the surgery.

Example 7. Preparation of crosslinked Gtn-HPA hydrogel (36) and crosslinked cisplatin-Gtn-Tyr hydrogel (47)

The crosslinked Gtn-HPA hydrogel (36) and cisplatin-Gtn-Tyr hydrogel (47) of the current invention were prepared using the Gtn-HPA precursor (35) synthesised in accordance to General Synthesis 2. As will be appreciated, the cisplatin can be replaced with any active agent, preferably with cation charges for interaction with the crosslinked Gtn-HPA hydrogel.

Experimental procedure

Typically, the Gtn-HPA precursor (35) can be crosslinked (with or without the active agent) by first dissolving the freeze-dried precursor (35), from General Synthesis 2, in 1x PBS to form a 5-10 w/v% precursor solution. This was followed by the addition of horseradish peroxidase (HRP) and H₂O₂ to form the crosslinked Gtn-HPA hydrogel (36) (Fig. 11), with a setting time of 0.5 h. The final concentrations of HRP and H₂O₂ in the mixture were 0.10-0.15 units/mL and 1-9 mM, respectively.

To prepare the crosslinked hydrogel of cisplatin-Gtn-HPA (47), a 5 w/v% Gtn-Tyr solution was first prepared by dissolving freeze-dried precursor (35) in 1x PBS and was loaded with 50 µg of cisplatin (37). The mixture precursor (0.5 mL) was then added with HRP (3 µL) and H₂O₂ (5.1 µL) and the sample was allowed to set for 0.5 h to give the crosslinked hydrogel (47) (Fig. 12). The final concentrations of HRP and H₂O₂ in the mixture were 0.15 units/mL and 3 mM, respectively.

Results and discussion

HPA (25) were conjugated to gelatin (10) using the carbodiimide crosslinking reaction, to form the Gtn-HPA precursor (35) as shown in Fig. 12. HPA, which contains a carboxylic group, can react with the amine group found on gelatin. As a result, a portion of the amine groups will react with the HPA molecules, resulting in less positively charged amine groups available (in ammonium form) and "excess" negatively charged carboxylic groups (in carboxylate form) exposed to the environment. Therefore, a negatively charged gelatin precursor will be produced and can be used to form a negatively charged hydrogel that may be used for encapsulation and delivery of cationic drugs such as Brinzolamide, Acetazolamide (glaucoma drugs), doxorubicin and cisplatin (cancer drugs). Similar to the Gtn-Tyr precursor (30) in Example 1, the presence of HPA molecules, which contain phenol, allow the crosslinking of Gtn-HPA precursor through peroxidase-mediated crosslinking reaction (Fig. 1b). The

crosslinking of Gtn-HPA precursor using horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2) results in the formation of a transparent crosslinked hydrogel (**36**) (Fig. 11).

It was observed that controlling the amount of EDC and NHS is able to control the amount of HPA molecules conjugated onto the gelatin, and varying the amount of conjugated HPA will result in hydrogels with various zeta potentials (Table 2). This is because as more HPA molecules react with the amine groups, a more negatively charged precursor will be produced (Fig. 13). Other possible synthesis parameters that can potentially control the amount of HPA conjugated onto gelatin include pH, reaction time and the ratio between EDC and NHS.

Table 2. Synthesis of Gtn-HPA precursor (**35**) using various amounts of EDC and NHS.

Sample name	Tyramine (mmol)	EDC (mmol)	NHS (mmol)	Amount of phenol ($\mu\text{mol/g}$)	Zeta Potential (mV)
Gelatin	-	-	-	62.70 ± 2.70	-3.25 ± 0.45
T1	6.02	0.46	0.23	165.44 ± 1.98	-4.78 ± 0.04
T2	6.02	1.37	0.68	248.81 ± 11.04	-5.46 ± 0.20
T3	6.02	1.83	0.91	367.97 ± 6.92	-7.88 ± 0.27
T4	6.02	3.65	1.83	486.85 ± 10.77	-9.68 ± 0.44

Example 8. Effects of electrostatic properties of the Gtn-HPA precursors (35**) on the release profile of cisplatin from the crosslinked cisplatin-Gtn-HPA hydrogel (**47**) of the current invention**

The release profile of the crosslinked cisplatin-Gtn-HPA hydrogel (**47**) of the current invention was investigated to understand the release of cisplatin from the crosslinked hydrogel.

Experimental procedure

5 w/v % Gtn-HPA precursor solution was prepared by dissolving freeze-dried precursor (**35**) in 1x PBS. Gtn-HPA precursors S1 (ZP = -7.58 ± 0.37 mV) and S2 (ZP = $+6.20 \pm 0.12$ mV) were used in this study. 50 μg of cisplatin was then loaded into a 0.5 mL Gtn-HPA precursor solution and was allowed to incubate for 15 min. Thereafter, HRP (3 μL) and H_2O_2 (5.1 μL) were added to the mixture and then cast into a well of a 24-well plate. The final concentrations of HRP and H_2O_2 in the mixture were 0.15 units/mL and 3 mM, respectively. The sample was allowed to set for 0.5 h before it was topped up with 1 mL of 1x PBS as the release buffer solution. At a specific time point, the release buffer solution was collected and replenished with fresh solution. The crosslinked cisplatin-Gtn-HPA hydrogels prepared from precursors S1

and S2 are denoted as “crosslinked cisplatin-Gtn-HPA hydrogel (S1)” and “crosslinked cisplatin-Gtn-HPA hydrogel (S2)”, respectively.

The amount of cisplatin released into the buffer solution was characterized according to the method developed by Basotra *et al.* (Basotra, M. *et al.*, ISRN Analytical Chemistry, vol. 2013, Article ID 936254, 8 pages, 2013). Briefly, 1.4 mg/mL *o*-phenylenediamine (OPDA) was added to the release buffer with a volume ratio of release buffer: OPDA = 1:2. The mixture was heated at 100 °C for 10 min. Next, the mixture was allowed to cool to room temperature and topped up with DMF with a volume ratio of release buffer: OPDA: DMF = 1:2:7. Finally, the mixture was scanned using an absorbance wavelength of 706 nm (SpectraMax™ M2 microplate readers, Molecular Devices, USA). The absorbance value was then compared with the absorbance values of known amount of cisplatin to determine the amount of cisplatin released.

Results and discussion

Cisplatin, an anti-cancer drug, was used to study the encapsulation and delivery efficacy of the crosslinked Gtn-HPA hydrogel. Cisplatin ($\text{PtCl}_2(\text{NH}_3)_2$) is a platinum-based drug which forms a cationic species $[\text{PtCl}(\text{NH}_3)_2(\text{H}_2\text{O})]^+$ when dissolved in water. Therefore, it is likely that the cationic species of cisplatin is the active compound that interacts with the negatively charged carboxylate of the gelatin backbone.

In this study, the cisplatin release profiles of crosslinked cisplatin-Gtn-HPA hydrogels (S1) and (S2) were compared to each other. As shown in Fig. 14, the negatively charged precursor S1 gave a crosslinked cisplatin-Gtn-HPA hydrogel with a slower release rate as compared to that of the positively charged precursor S2. The slower release rate from the Gtn-HPA hydrogel may be due to the stronger electrostatic interaction between the positively charged cationic species of cisplatin and the negatively charged carboxylate groups on the gelatin. Overall, this release study showed that the negatively charged crosslinked cisplatin-Gtn-HPA hydrogel S1 has a stronger ionic interaction with cationic drugs, therefore causing the slower release to the environment.

At day 9, the crosslinked cisplatin-Gtn-Tyr hydrogel (S2) showed a significantly ($*p < 0.05$) higher cumulative release percentage as compared to the crosslinked cisplatin-Gtn-HPA hydrogel (S1) (Fig. 14). As such, this shows that the negative charges on the hydrogel can be controlled to give different degrees of electrostatic interaction with the cationic drugs, such that the release rate of the drugs from the crosslinked hydrogel can be tuned accordingly.

Example 9. *In vitro* anti-cancer activity of crosslinked cisplatin-Gtn-HPA hydrogel (47) on MDA-MB-231 human breast cancer cells

To demonstrate the *in vitro* anti-cancer activity of the crosslinked cisplatin-Gtn-HPA hydrogel (47) of the current invention, the crosslinked hydrogel was tested on MDA-MB-231 breast cancer cells.

Experimental procedure

Freeze-dried Gtn-HPA precursor (35), with zeta potential of -7.58 ± 0.37 mV (S1), was dissolved in 1x PBS to form a precursor solution. 50 μ g of cisplatin was then added into a 0.5 mL precursor solution and was allowed to incubate for 15 min. Thereafter, HRP (3 μ L) and H₂O₂ (5.1 μ L) were added to the mixture and then cast into a well of a 24-well plate. The sample was allowed to set for 0.5 h to give the crosslinked hydrogel. The final concentrations of HRP and H₂O₂ in the mixture were 0.15 units/mL and 3 mM, respectively. MDA-MB-231 human breast cancer cells were then seeded on top of the hydrogel with a density of 5×10^4 cells per well. The sample was topped up with 0.5 mL of DMEM supplemented with 10 % FBS and 1 % ABAM, and incubated at 37 °C and 5 % CO₂. The metabolic activity or viability of MDA-MB-231 breast cancer cells at day 1, 3, 5 and 7 was quantified using PrestoBlue™ cell viability reagent according to manufacturer's protocol. At day 7, the cells were treated with LIVE/DEAD™ cell viability assay and the fluorescence images of the cells were captured using a Zeiss Axio Observer.Z1-inverted microscope (Carl Zeiss, Germany). The treatment result was compared to crosslinked cisplatin-Gtn-HPA hydrogel prepared from Gtn-HPA precursor with zeta potential of $+6.20 \pm 0.12$ mV (S2). The crosslinked cisplatin-Gtn-HPA hydrogels prepared from precursors S1 and S2 are denoted as "crosslinked cisplatin-Gtn-HPA hydrogel (S1)" and "crosslinked cisplatin-Gtn-HPA hydrogel (S2)", respectively.

Results and discussion

In vitro anti-cancer activity of the as-prepared crosslinked cisplatin-Gtn-HPA hydrogels (S1 and S2) was carried out by culturing the MDA-MB-231 breast cancer cells on the surface of the crosslinked cisplatin-Gtn-HPA hydrogels. As shown by the proliferation study in Fig. 15a, the crosslinked cisplatin-Gtn-HPA hydrogel (S1) showed inhibition of the proliferation of MDA-MB-231 breast cancer cells from day 1 to 3, with significant decrease ($*p < 0.05$) in the cell number from day 3 to 5 and day 5 to 7. The results showed that the crosslinked cisplatin-Gtn-HPA hydrogel, prepared from the negatively charged S1 precursor, effectively inhibited the growth of the MDA-MB-231 breast cancer cells and resulted in cell death from day 3 to 7.

On the other hand, the crosslinked cisplatin-Gtn-HPA hydrogel (S2), prepared from the positively charged Gtn-HPA precursor S2, showed significant increase ($*p < 0.05$) in cell number from day 1 to 3. Therefore, the crosslinked cisplatin-Gtn-HPA hydrogel (S2) did not inhibit the growth of MDA-MB-231 breast cancer cells in the initial phase. However, the cell number was observed to decrease significantly ($*p < 0.05$) from day 3 to 5 and day 5 to 7. This may be due to the high amount of cisplatin which were released at the later stage (~day 4-7) of the study.

The cumulative cell doubling rate at day 7 showed that the MDA-MB-231 cancer cells had a more significant negative growth rate when treated with the crosslinked cisplatin-Gtn-HPA S1 (negatively charged precursor) than that of S2 (positively charged precursor) (Fig. 15b). As demonstrated by the LIVE/DEAD™ fluorescence images of the cells (Fig. 15c), both types of hydrogels also showed only a few live cells remaining on the surface at day 7. These images further confirmed the killing of the MDA-MB-231 cancer cells by the as-prepared crosslinked cisplatin-Gtn-HPA hydrogels (Fig. 15c).

In this study, it was observed that the crosslinked cisplatin-Gtn-HPA hydrogel (S1) inhibited the growth of the cancer cells at day 3 and effectively killed the cells on day 5 and 7. Therefore, such Gtn-HPA hydrogel can be an effective delivery system for cancer treatment. Even though the release rate of cisplatin-Gtn-HPA (S1) was slower than that of S2, it was demonstrated to be more effective for *in vitro* cancer treatment. This observation may be due to the negative surface charges on the hydrogel which is not favorable towards the cancer cells. The elevated glycolysis in the cancer cells can lead to a higher-level secretion of lactate, which creates high negative surface charges. Therefore, cancer cells with high negative surface charge may not be able to attach well onto the negatively charged hydrogels and may be “repelled” by the negative charges on the crosslinked cisplatin-Gtn-HPA hydrogel (S1). As such, this can reduce the amount of cisplatin needed to kill the cancer cells, which potentially reduces the side effects caused by cisplatin. Overall, the combined effect of both cisplatin and negative surface charge makes the crosslinked cisplatin-Gtn-HPA hydrogel of the current invention a promising delivery system for cancer treatment.

Claims

1. A sustained release composition comprising:
 - an active agent having a positive or negative charge when placed in an aqueous environment;
 - a crosslinked hydrogel tuned to have an overall charge that facilitates electrostatic interaction between the crosslinked hydrogel and the active agent, wherein:
 - the crosslinked hydrogel is formed from:
 - a heterobifunctional polymer comprising a first set of functional groups that are positively charged and a second set of functional groups that are negatively charged in an aqueous environment; and
 - a heterobifunctional crosslinking agent, where the heterobifunctional crosslinking agent comprises:
 - at least one functional group capable of forming a bond to a further molecule of said heterobifunctional crosslinking agent; and
 - at least one functional group suitable to form a bond with the first or second set of functional groups of the heterobifunctional polymer, so as to reduce the number of the first or second functional groups in the first or second sets of functional groups, thereby modifying the overall charge of the crosslinked hydrogel and facilitating the electrostatic interaction between the crosslinked hydrogel and the active agent.
2. The sustained release composition according to Claim 1, wherein the active agent is a biomolecule and/or a small molecule active agent (e.g. cisplatin or paclitaxel).
3. The sustained release composition according to Claim 2, wherein the biomolecule is a charged biomolecule selected from one or more of CRISPR components, a nucleic acid, a microRNA and a siRNA, optionally wherein:
 - the microRNA is selected from one or more of 5'-UUGUGCUUGAUCUAACCAUGU-3' (miR-218), 5'-UAGCACCAUUUGAAAUCAGUGUU-3' (miR-29b duplex), 5'-AUCACAUUGCCAGGGAUUACC-3' (miR-23b), and 5'-UGAAAUGUUUAGGACCACUAG-3' (miR-203); and
 - the siRNA is selected from one or more of 5'-AACAAGACCUUCGACUCUUCC-3' (SPARC), 5'-AACCTGAAGATCTTCAACAACCCTGTCTC-3' (Smad3), 5'-AACCUGCUGAAGGAUGGUGAC-3' (p53), 5'-CCAAGAACCGGAACCUGCUTT-3' (MC1R), 5'-GCAGUACCUUUCUACCACUTT-3' (MITF), and 5'-UCACUUACAGGAUCUAUAAUU-3' (Elastase).

4. The sustained release composition according to any one of the preceding claims, wherein the heterobifunctional polymer is selected from one or more of gelatin, collagen, silk fibroin, elastin and $\text{H}_2\text{N-PEG-CO}_2\text{H}$.

5. The sustained release composition according to Claim 4, wherein the heterobifunctional polymer is gelatin.

6. The sustained release composition according to any one of the preceding claims, wherein the heterobifunctional crosslinking agent comprises at least one functional group capable of forming a bond to a further molecule of said heterobifunctional crosslinking agent, wherein the at least one functional group capable of forming a bond to a further molecule of said heterobifunctional crosslinking agent is a functional group that is not charged, or is zwitterionic, in an aqueous environment.

7. The sustained release composition according to Claim 6, wherein the at least one functional group capable of forming a bond to a further molecule of said heterobifunctional crosslinking agent is selected from one or more of norbornene, tetrazine, methacrylate, OH, SH, azide, C_2 to C_{10} alkene, and C_2 to C_{10} alkyne, optionally wherein the at least one functional group capable of forming a bond to a further molecule of said heterobifunctional crosslinking agent is SH or, more particularly, OH.

8. The sustained release composition according to any one of the preceding claims, wherein:

(A) the at least one functional group suitable to form a bond with the first set of functional groups is selected from one or more of a carbonyl group, an ester, a peroxy acid and CO_2H ; or

(B) the at least one functional group suitable to form a bond with the second set of functional groups is selected from one or more of amide and amino.

9. The sustained release composition according to any one of the preceding claims, wherein the heterobifunctional crosslinking agent is selected from molecules suitable for undergoing Click reactions together, molecules suitable for photocrosslinking to one another, thiol-containing molecules, phenolic molecules with carboxylic acid groups, and phenolic molecules with amine groups, optionally wherein:

molecules suitable for undergoing Click reactions together are selected from two or more of 5-norbornene-2-carboxylic acid, tetrazine acid, 3-azido-1-propanamine, 3-

azidopropanoic acid, N-hydroxysuccinimide (NHS) esters, dibenzocyclooctyne-amine, 2-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)ethan-1-amine and 3-(4-(prop-2-yn-1-yloxy)benzoyl)benzoic acid;

molecules suitable for photocrosslinking to one another are selected from one or more of methacrylic acid, and thiol-containing molecules suitable to crosslink with ene-containing molecules;

thiol-containing molecules are selected from one or more of 5-(4-aminophenyl)-1,3,4-oxadiazole-2-thiol, 3-amino-1,2,4-triazole-5-thiol, and cysteamine;

phenolic molecules with carboxylic acid groups are selected from one or more of 3-(4-hydroxyphenyl)propionic acid and 3,4-dihydroxyphenylacetic acid; and

phenolic molecules with amine groups are selected from one or more of 4-hydroxybenzylamine, dopamine, and tyramine.

10. The sustained release composition according to any one of the preceding claims, wherein the overall charge of the crosslinked hydrogel also facilitates the sustained release effect of the composition, optionally wherein the sustained release effect is from 1 to 30 days, such as from 2 to 25 days, such as 5 to 20 days.

11. The sustained release composition according to any one of the preceding claims, wherein the active agent is a siRNA and the crosslinked hydrogel is formed from gelatin and tyramine, where the zeta potential of the crosslinked hydrogel is from -1 mV to +10 mV, such as from 0 mV to +9 mV, such as from +0.4 mV to +8.5 mV, such as from +0.6 mV to +1 mV.

12. The sustained release composition according to any one of Claims 1 to 10, wherein the active agent is cisplatin and the crosslinked hydrogel is formed from gelatin and 3-(4-hydroxyphenyl)propionic acid, where the zeta potential of the crosslinked hydrogel is from -4 mV to -10 mV.

13. Use of a sustained release composition as described in any one of Claims 1 to 12 in medicine.

14. Use of a sustained release composition as described in any one of Claims 1 to 11 in the preparation of a medicament for the treatment and/or prevention of scarring, wherein the active agent of the sustained release composition is an anti-scarring agent (e.g. the anti-scarring agent is a siRNA, such as 5'-AACAAGACCUUCGACUCUCC-3'), optionally wherein the use relates to the treatment and/or prevention of scarring in a subject who has undergone surgery (e.g. eye surgery for glaucoma).

15. Use of a sustained release composition as described in any one of Claims 1 to 10 and 12 in the preparation of a medicament for the treatment and/or prevention of cancer, wherein the active agent of the sustained release composition is an anti-cancer agent (e.g. the anti-cancer agent is cisplatin).

16. A pharmaceutical composition comprising the sustained release composition as described in any one of Claims 1 to 12 in combination with one or more of a pharmaceutically acceptable excipient, diluent or carrier.

17. A kit of parts, comprising:

(Aa) a first composition comprising:

a complex of an active agent having a positive or negative charge when placed in an aqueous environment and an un-crosslinked hydrogel tuned to have an overall charge that facilitates electrostatic interaction between the un-crosslinked hydrogel and the active agent; and

an enzyme suitable to crosslink the un-crosslinked hydrogel; and

(Bb) a second composition comprising an oxidation source (e.g. H_2O_2), wherein:

the un-crosslinked hydrogel comprises a heterobifunctional polymer that has a first set of functional groups that are positively charged and a second set of functional groups that are negatively charged in an aqueous environment, where a portion of the first or second set of functional groups are capped by a heterobifunctional crosslinking agent, such that the overall charge of the un-crosslinked hydrogel is modified to facilitate the formation of the complex between the un-crosslinked hydrogel and the active agent, where:

the heterobifunctional crosslinking agent comprises at least one functional group capable of forming a bond to a further molecule of said heterobifunctional crosslinking agent when the first and second compositions are combined, and at least one functional group that has formed a bond to a portion of the first or second set of functional groups of the heterobifunctional polymer, so as to reduce the number of the first or second functional groups, thereby modifying the overall charge of the un-crosslinked hydrogel and facilitating the electrostatic interaction between the un-crosslinked hydrogel and the active agent.

Figures

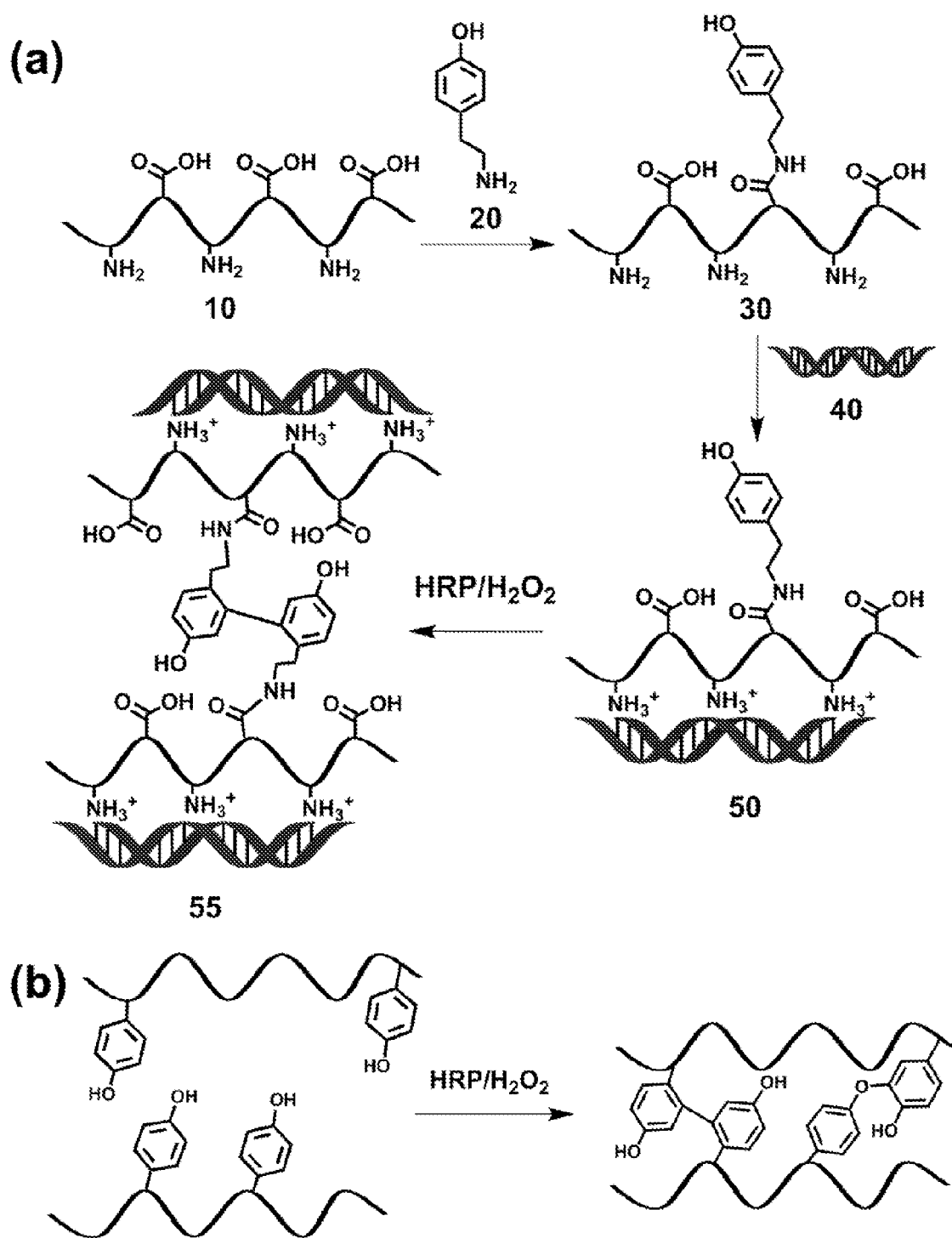


FIG. 1

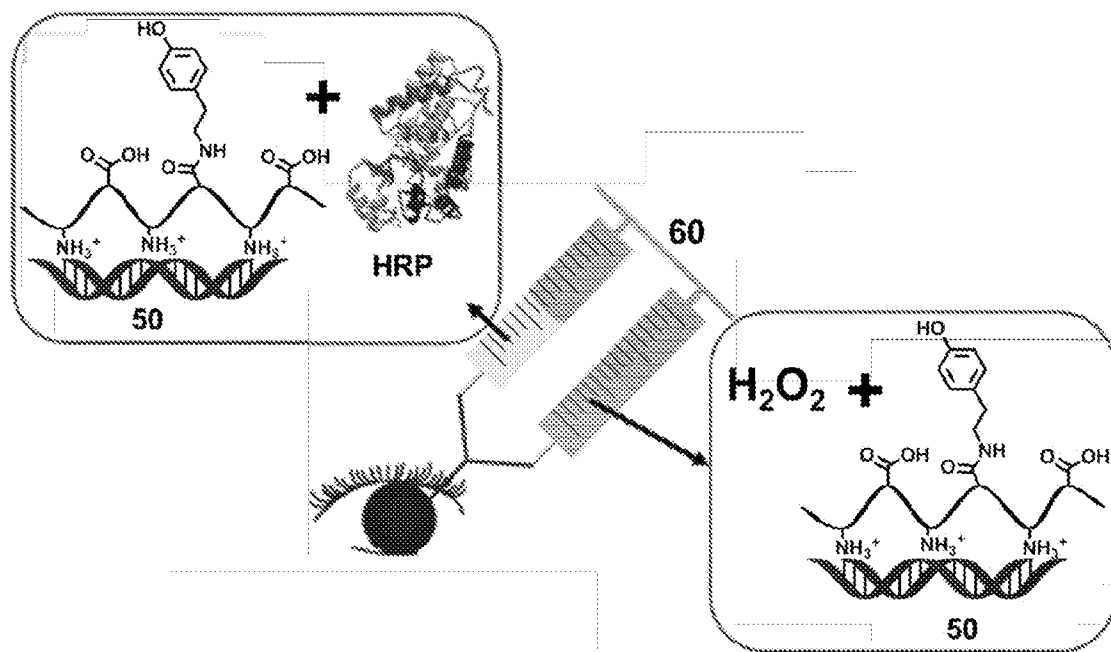


FIG. 2

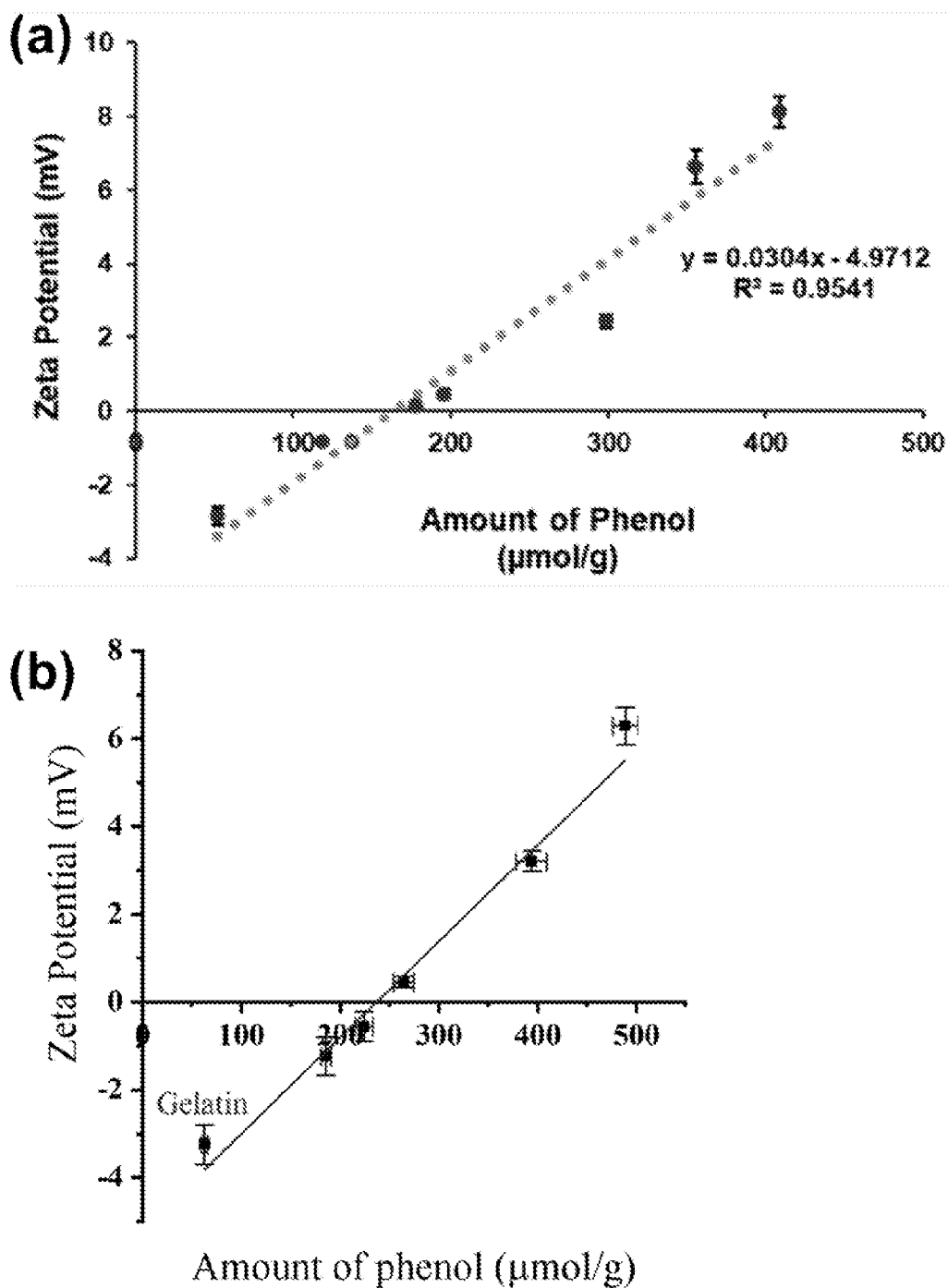


FIG. 3

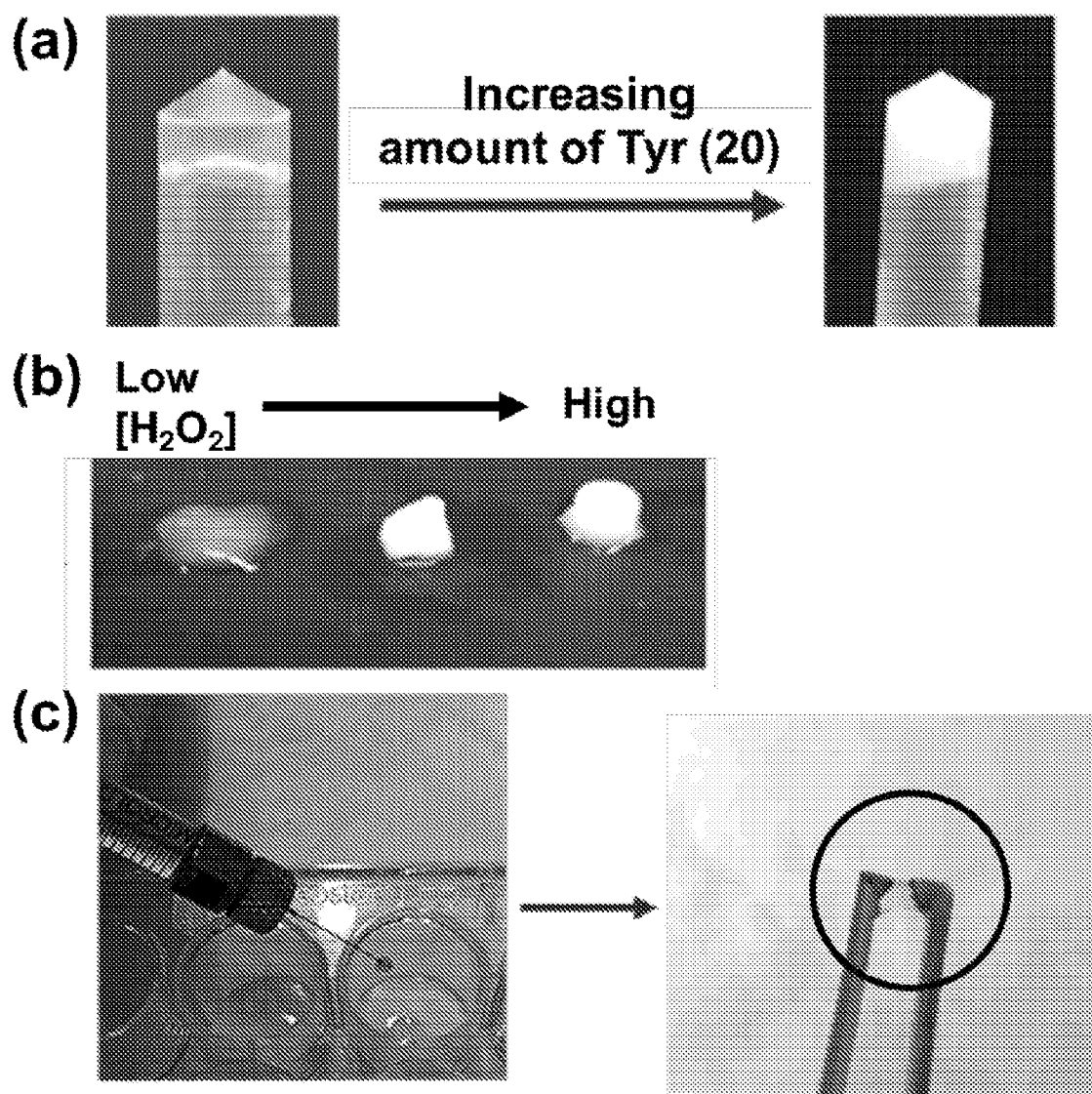


FIG. 4

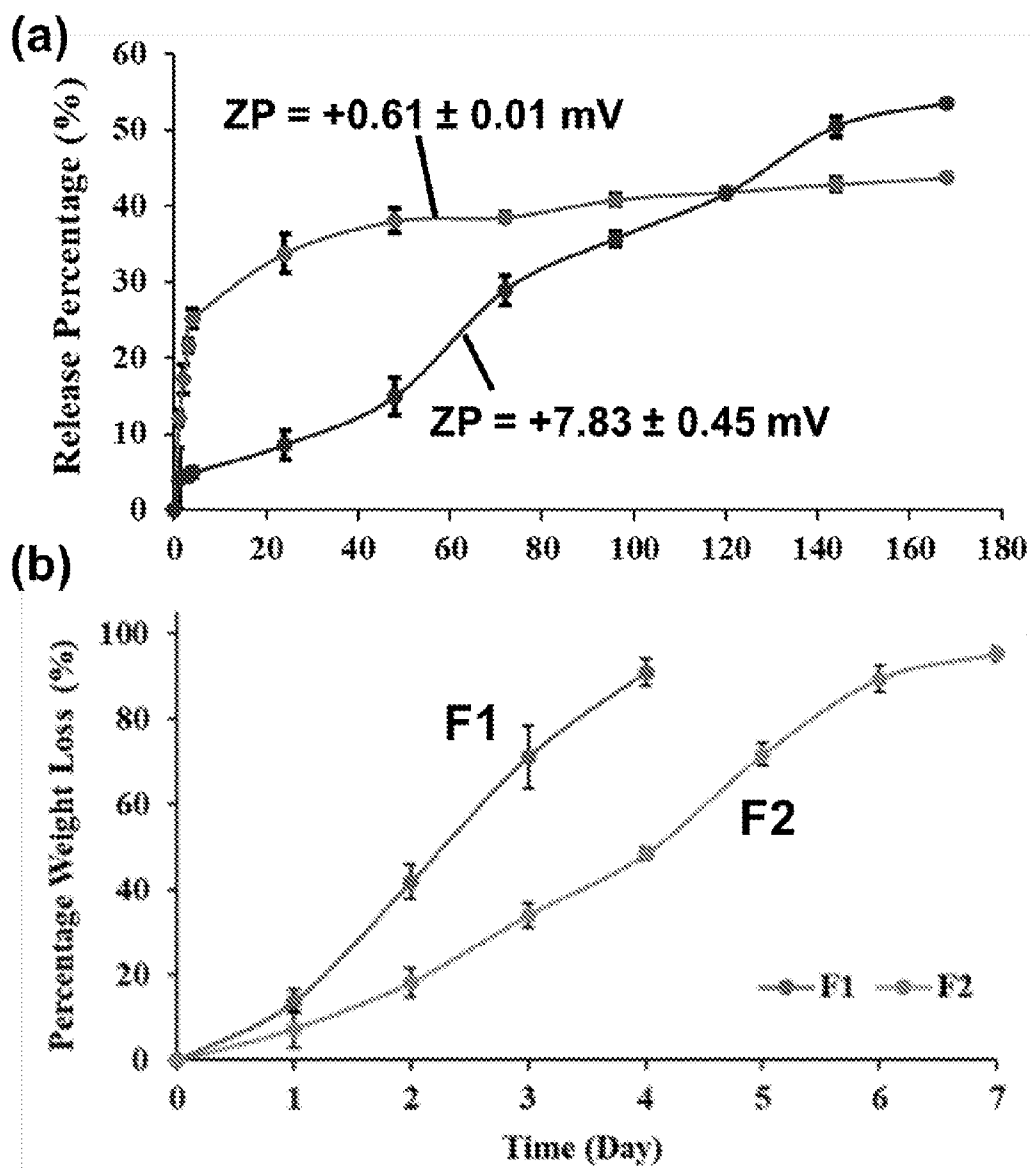


FIG. 5

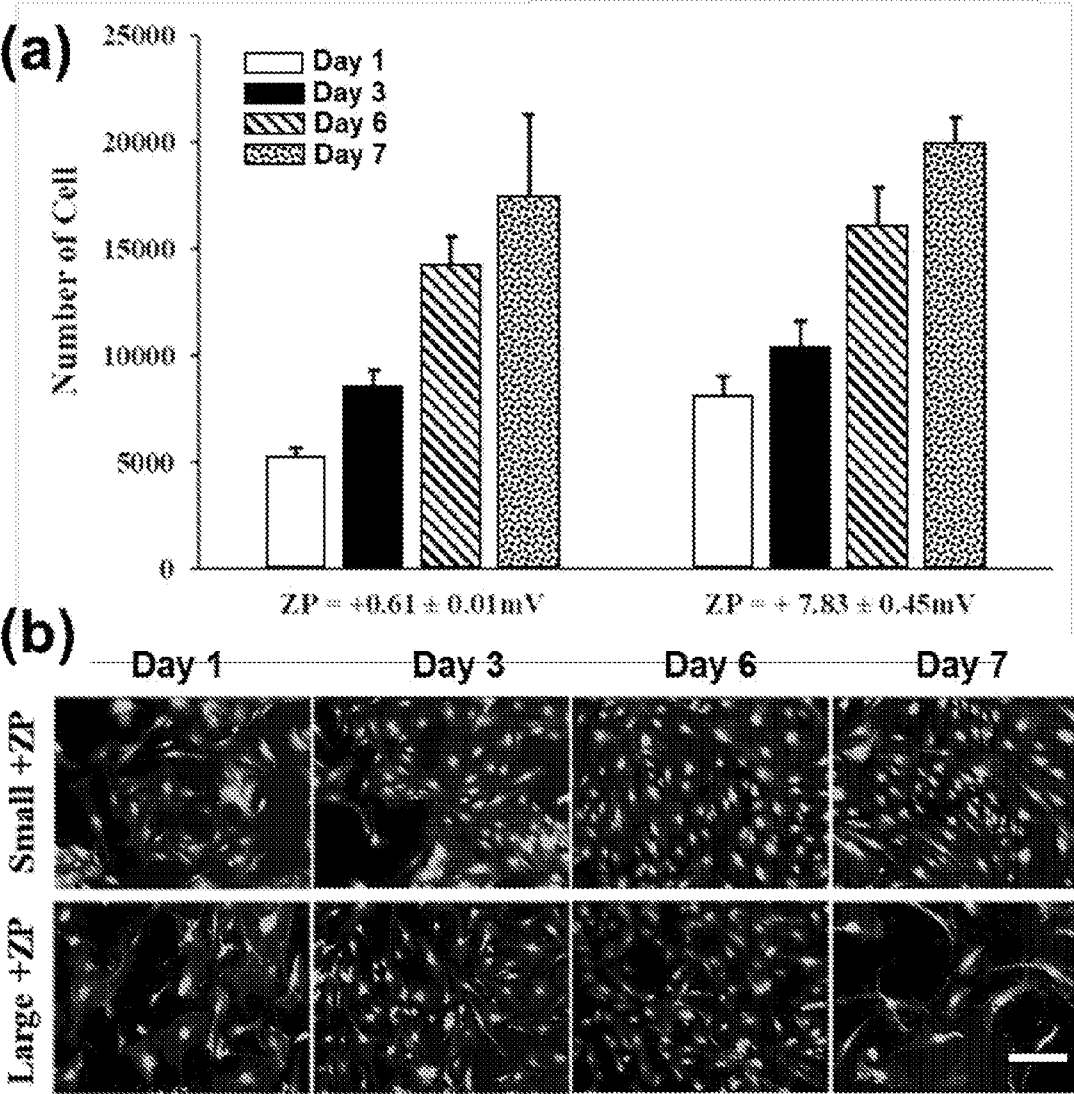


FIG. 6

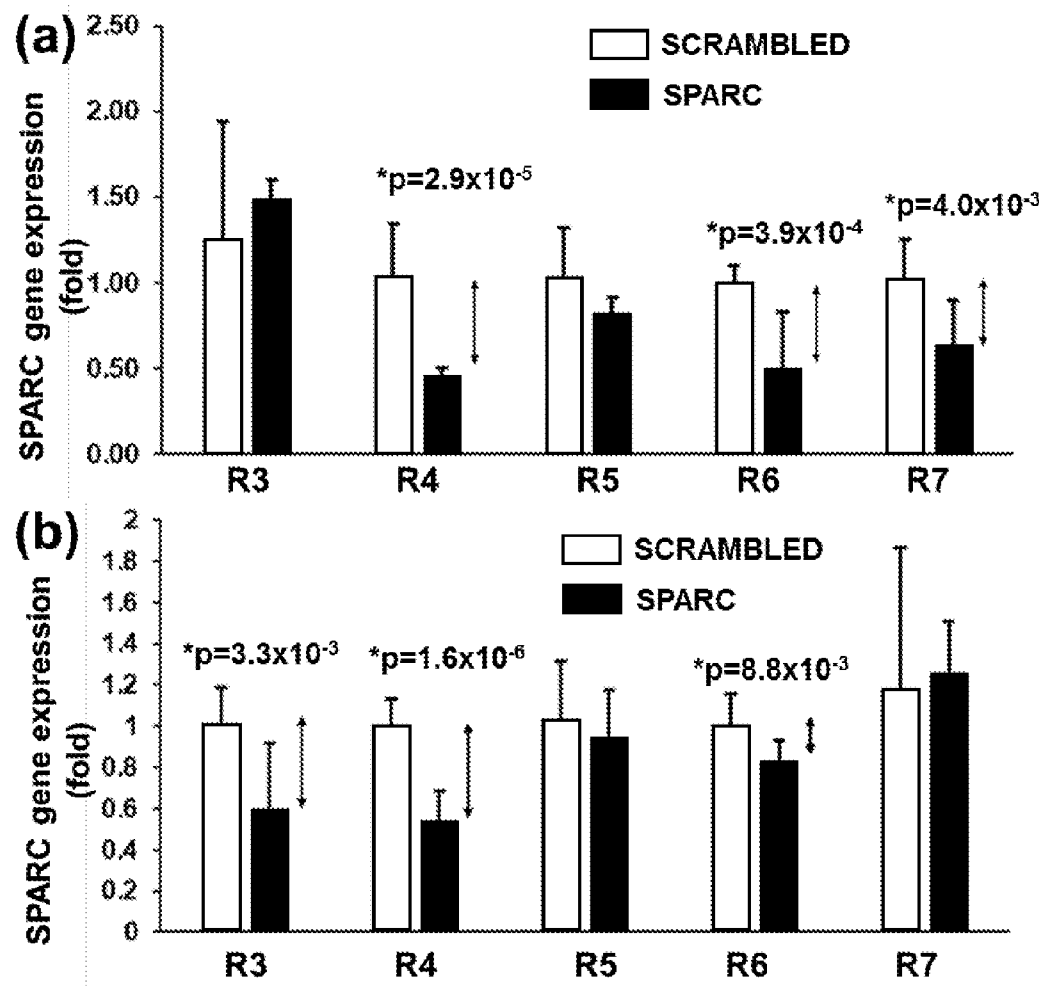


FIG. 7

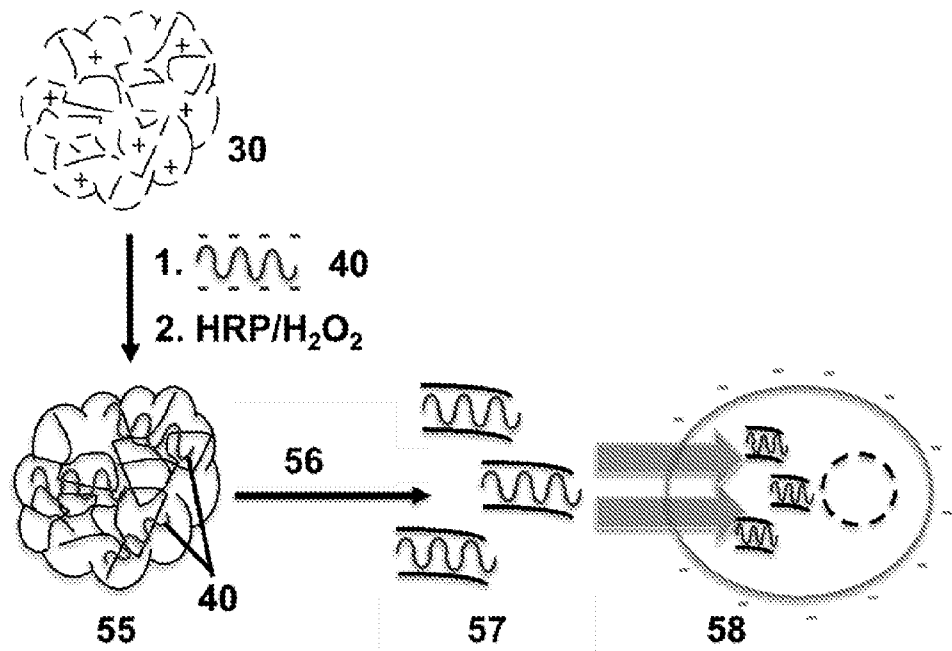


FIG. 8

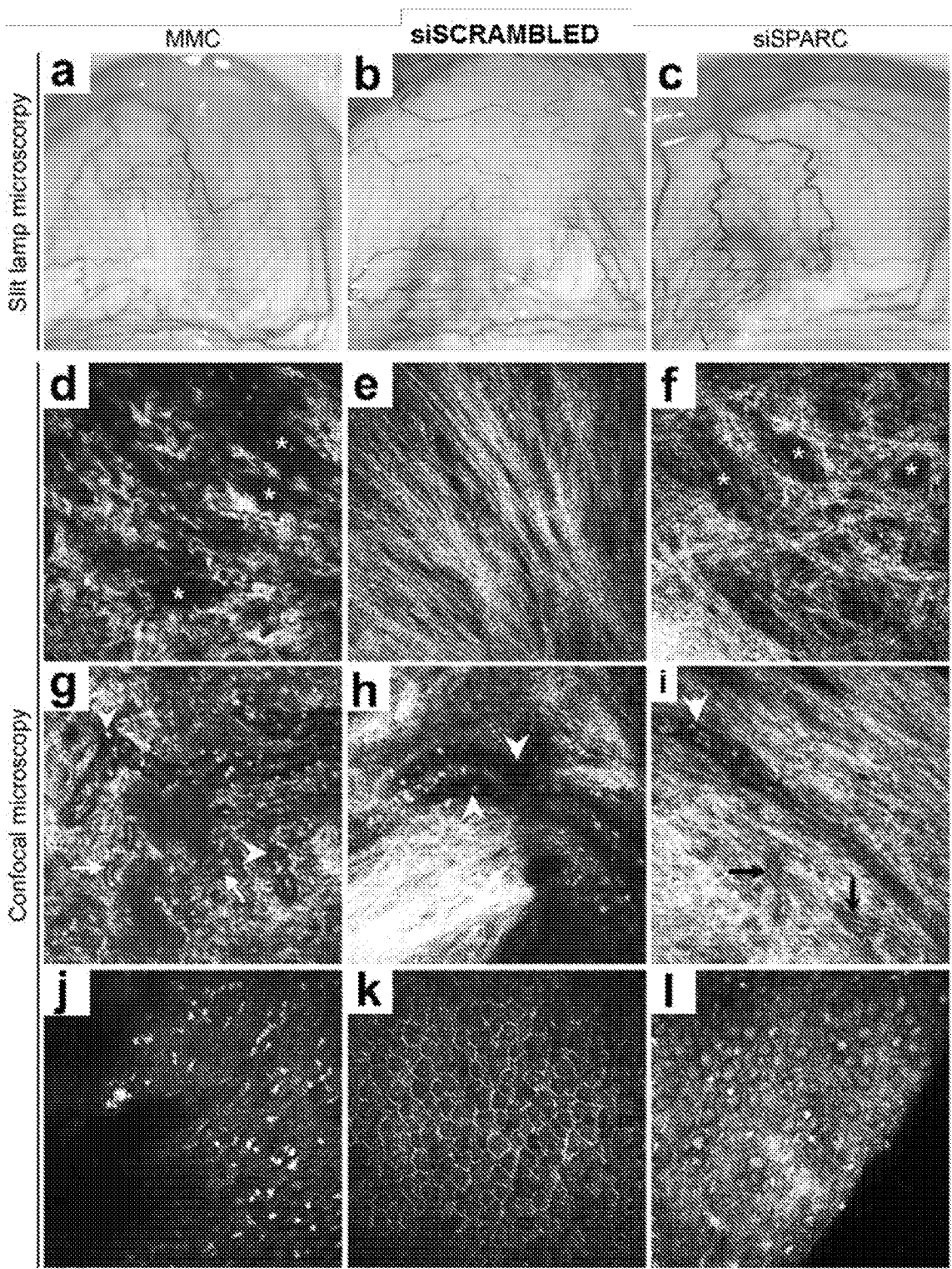


FIG. 9

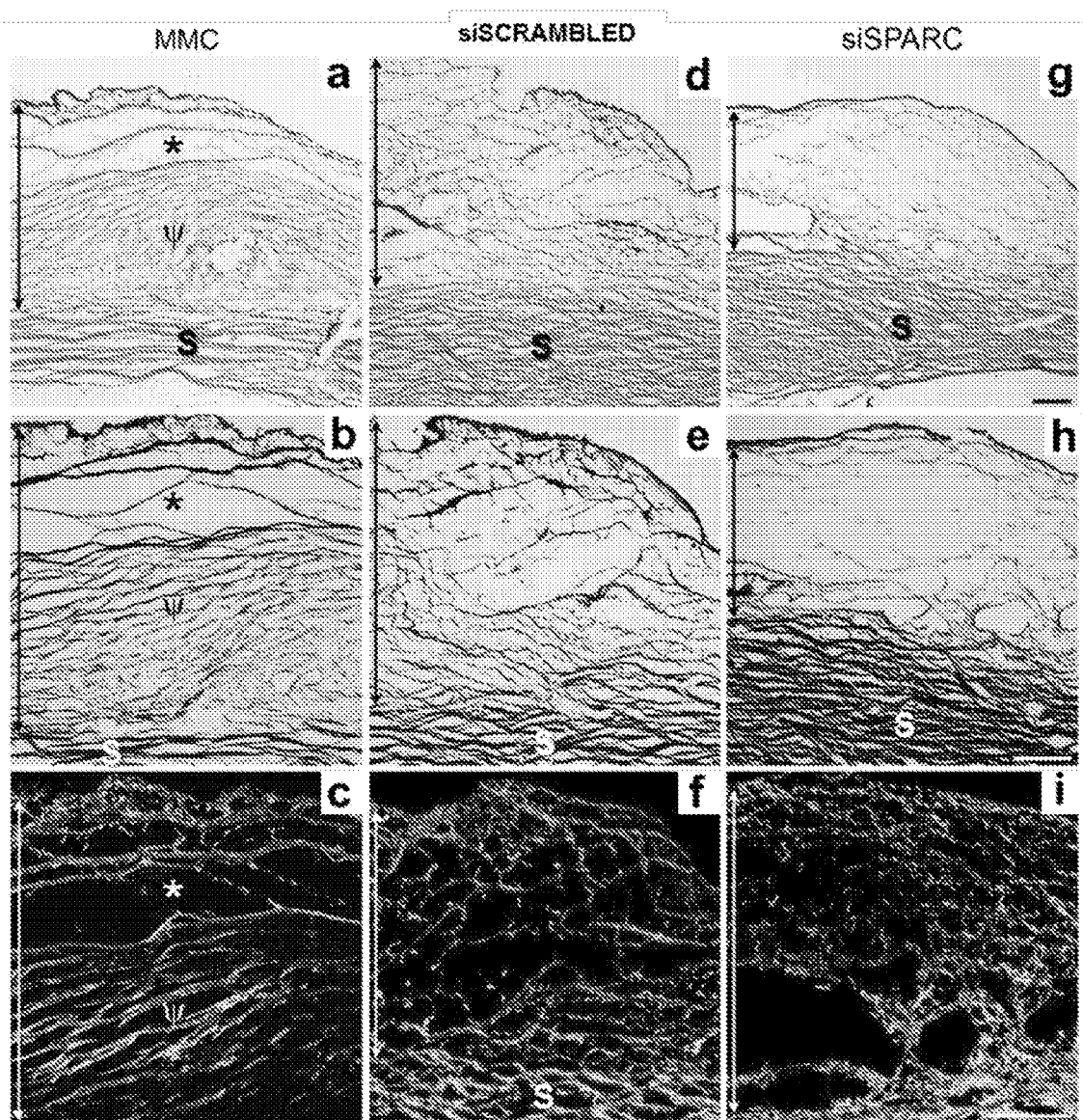


FIG. 10

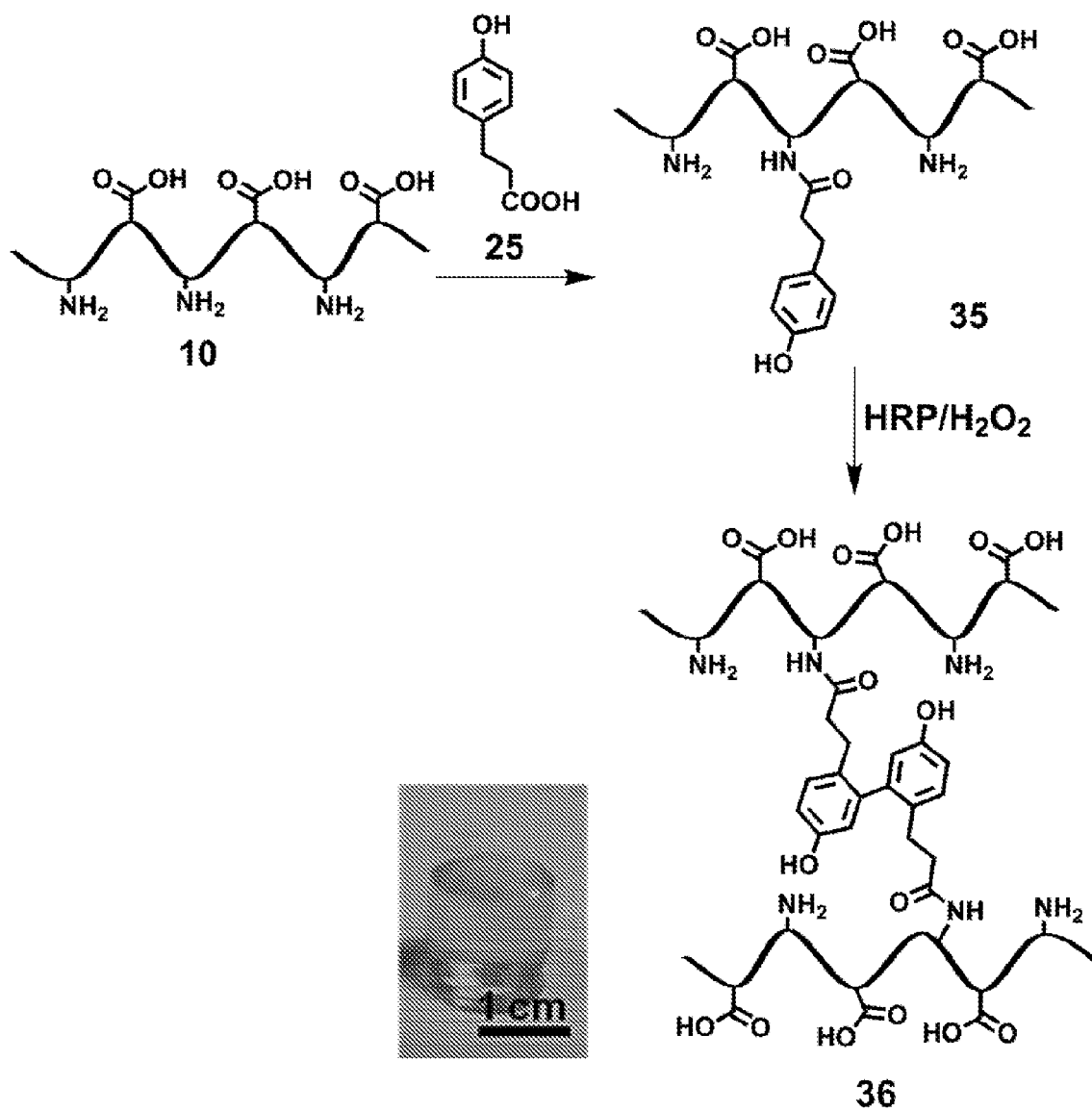


FIG. 11

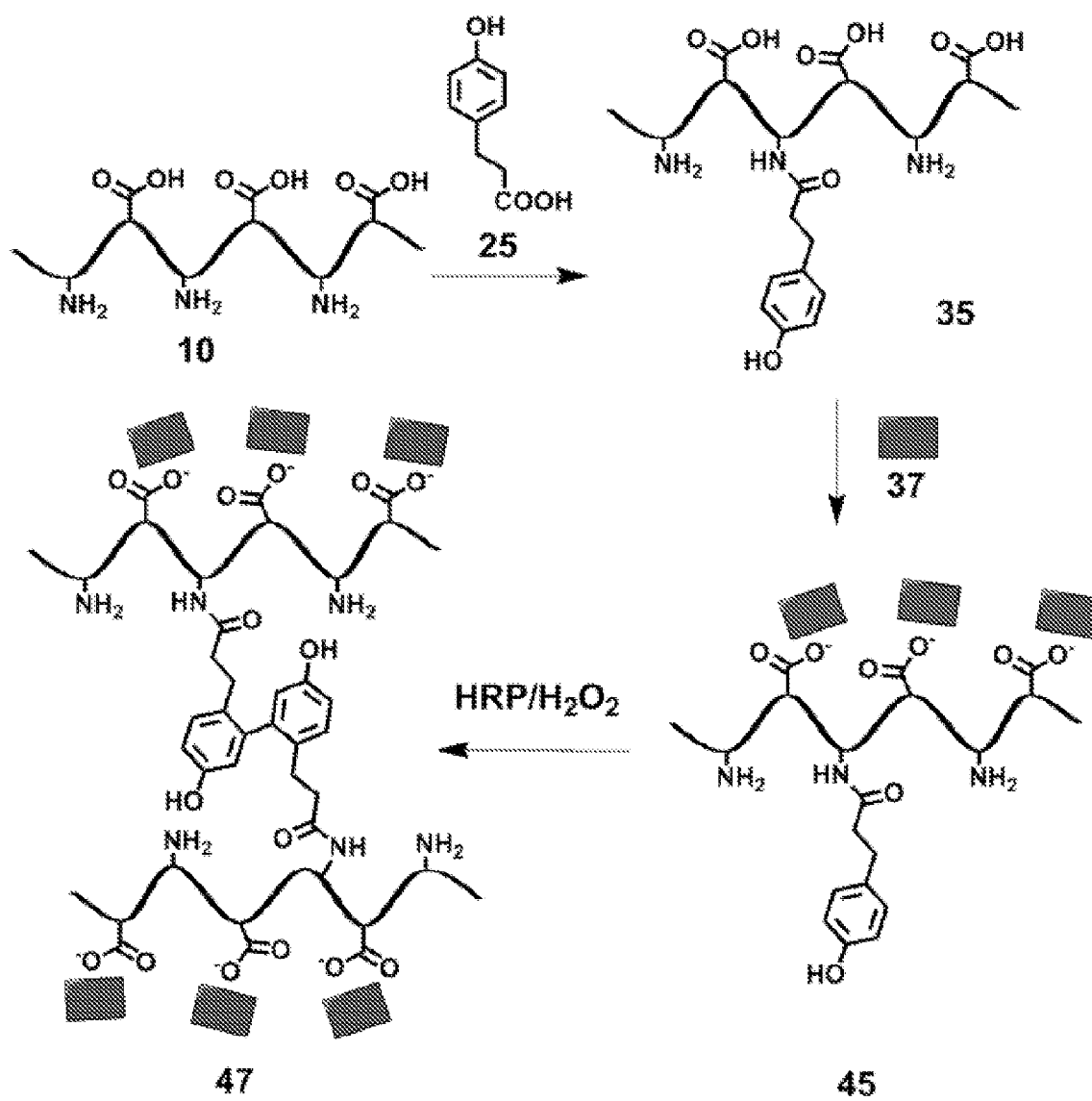


FIG. 12

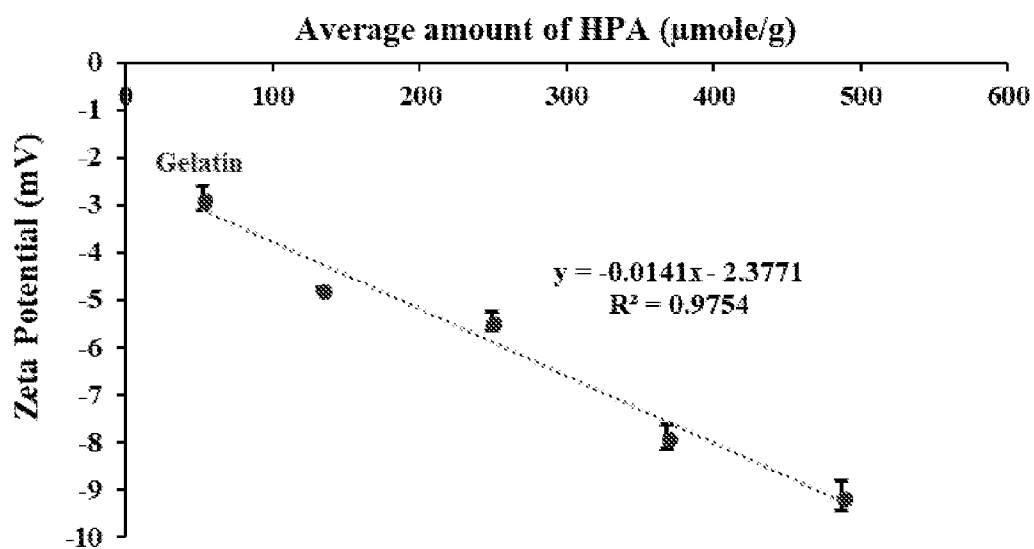


FIG. 13

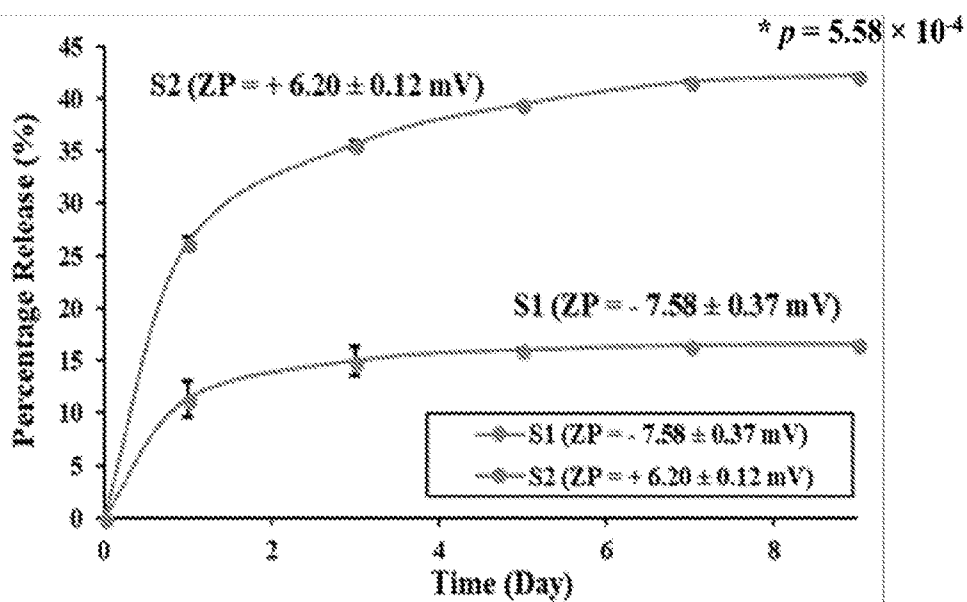


FIG. 14

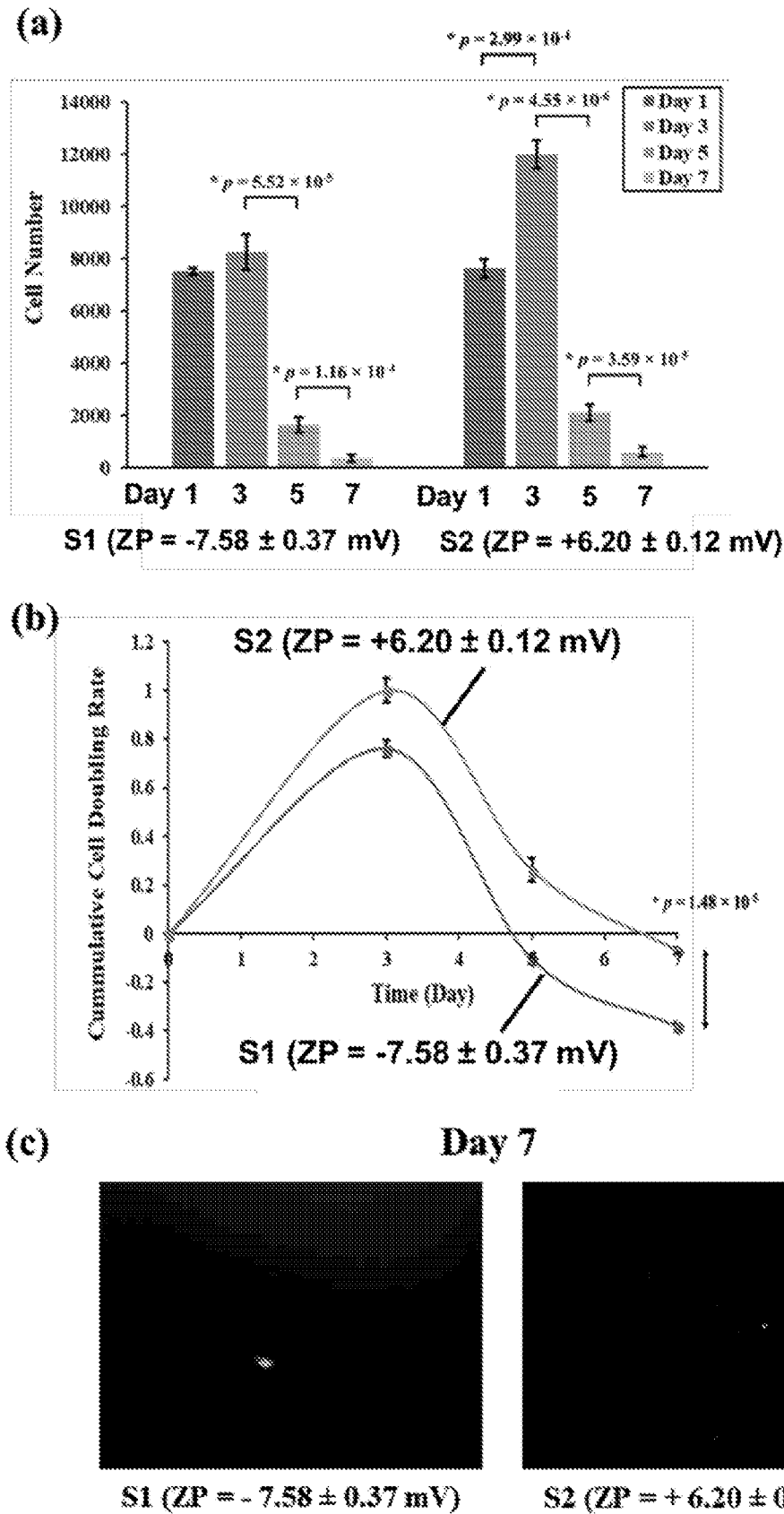


FIG. 15

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2019/050448

A. CLASSIFICATION OF SUBJECT MATTER

See Supplemental Box

According to International Patent Classification (IPC)

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K, A61P

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

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

FAMPAT, CAPLUS, EMBASE, MEDLINE, BIOSIS: sustained release, hydrogel, crosslink, heterobifunctional polymer, gelatin, heterobifunctional crosslinker, tyramine, hydroxyphenylpropionic acid and related terms.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2015/0250890 A1 (PARK, K. D. ET AL.) 10 September 2015 Paragraphs [0050], [0052], [0053], [0058]-[0062], [0064], [0093]-[0096], [0101]-[0108], [0129], [0130], [0136]-[0138], figures 5, 7, 12, 15	1-17
X	US 2012/0100103 A1 (PARK, K. D. ET AL.) 26 April 2012 paragraphs [0037], [0038], [0042]-[0046], [0075]-[0088], [0100]-[0102], figures 1-4, 10	1-17
X	THI, T. T. H. ET AL., Oxidized cyclodextrin-functionalized injectable gelatin hydrogels as a new platform for tissue-adhesive hydrophobic drug delivery. <i>Royal Society of Chemistry</i> , 6 July 2017, Vol. 7, pages 34053-34062 [Retrieved on 2019-10-25] <DOI: 10.1039/C7RA04137C> Page 34054 right column second – third paragraph, page 34059 right column last paragraph – page 34060 left column first paragraph, page 34061 left column first paragraph, figures 1(A), 1(B), 5(B)	1-17

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

*Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

25/10/2019

(day/month/year)

Date of mailing of the international search report

30/10/2019

(day/month/year)

Name and mailing address of the ISA/SG



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INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2019/050448

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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