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(54) Title: DUAL-CROSSLINKED HYDROGEL AND PREPARATION METHOD THEREOF

(57) Abstract: A process to prepare a dual-crosslinked hydrogel, comprising the following steps: 1) functionalizing a glycosaminoglycan such as hyaluronic acid with a methacrylate on hydroxyl group and with a dihydrazide on carboxyl group to obtain a methacrylate and hydrazide group functionalized glycosaminoglycan, wherein the degree of methacrylation is 5-200%, and the degree of hydrazide group modification is 8-70%; oxidizing a second-polysaccharide such as dextran to obtain a second-polysaccharide with aldehyde groups, the degree of aldehyde modification in the second-polysaccharide is 10-95%; 2) crosslinking the methacrylate and hydrazide group functionalized glycosaminoglycan and the second-polysaccharide-aldehyde in an aqueous solvent, to form a dynamic acylhydrazone bond cross-linked hydrogel; and 3) photopolymerizing the dynamic acylhydrazone bond cross-linked hydrogel by irradiation under the presence of a photoinitiator. The dual-crosslinked hydrogel prepared according to the process, a hydrogel precursor to prepare a dual-crosslinked hydrogel, and use thereof are also provided.

Dual-crosslinked hydrogel and preparation method thereof

Technical Field

The invention relates to an injectable hydrogel with secondary autonomous covalent crosslinking and a preparation method thereof.

Background art

Glycosaminoglycans (GAGs) are long linear (unbranched) polysaccharides consisting of repeating disaccharide (double sugar) units. Glycosaminoglycans have good biocompatibility. Hyaluronic acid (HA; conjugate base hyaluronate), also called hyaluronan, is an anionic, nonsulfated glycosaminoglycan. It is a linear polysaccharide that consists of alternating units of a repeating disaccharide, β-1,4-D-glucuronic acid-β-1,3-N -acetyl-D-glucosamine. It is abundant in cartilage and skin and plays a key structural role in the organization of the extracellular matrix as an organizing structure for the assembly of a proteoglycan. Viscous solutions of high molecular-weight HA and its derivatives are being used in therapy for promoting wound healing in various tissues, such as a surgical aid in eye and skin. However, these solution systems are limited in application, due to undesirable loss of material from the injection site and minimal control over important material properties (e.g., mechanics and degradation).

Injectable hydrogels may be surgically implanted to fit variable target sites in patients using minimally invasive methods, making them particularly attractive for clinical use. For these purposes, a variety of injectable HA-based hydrogels have been explored by numerous chemical crosslinking mechanisms including photo-crosslinking, dynamic-chemistry, physical-assembly and non-covalent interactions.

For example, in CN104892962A, HA was functionalized with thiol groups and subjected to a one-step crosslinking protocol for an injectable HA hydrogel basing on oxidation-reduction reactions between thiols and disulfide bond. Unfortunately, these materials are inherently limited in that they typically exhibit low mechanical strength and may exhibit rapid degradation.

Ying Luo et.al. (Journal of Biomedical Materials Research B: Applied Biomaterials | May 2010 Vol 93b, Issue 2 387) disclosed an injectable hyaluronic acid-dextran hydrogel, wherein hyaluronic acid (HA) and dextran were chemically modified and subsequently crosslinked via formation of hydrazone (acylhydrazone) bonds in phosphate buffer. The compressive moduli of the hydrogels were around or above 10 kPa.

Hydrogels formed through covalent means display great versatility with the allowed inclusion of controlled network degradation and mechanical strength. In CN106188584A, a UV-crosslinked, stiff HA hydrogel was prepared by free-radical photopolymerization of methacrylates, where HA were functionalized with both 2-aminoethyl methacrylate hydrochloride and L-lysine. However, as a result of the

single covalent bonding structure, hydrogels are unable of self-healing, which limits them in their applications as an injectable material.

Hoang D. Lu et.al. (Adv Healthc Mater. 2013 Jul; 2(7):1028-36) disclosed a secondary photo-crosslinking of injectable shear-thinning dock-and-lock hydrogel, wherein the hydrogel was stabilized through light-initiated radical polymerization of methacrylate functional groups to tune gel mechanics and erosion kinetics. By tuning material compositions, hydrogels with storage modulus (G′) as high as ~ 1 kPa could be formed.

For more physically demanding applications, non-covalently and covalently crosslinked systems may be more appropriate to modulate the injectability, self-healing properties and the degradation and mechanical strength. For example, in CN104910396A, an injectable dual-crosslinked hydrogel system was prepared from amino-/vinyl- HA and oxidized HA. The hydrogels first undergo dynamic Schiff-based crosslinking through amino- and aldehyde- groups, then a secondary covalent crosslinking occurs in situ via vinyl- to stabilize the network. The compression moduli were around 7.4kPa～18.9kPa.

Sudhir Khetan et.al. (Nat Mater. 2013 May;12(5):458-65) disclosed a MeHA hydrogel with compression moduli around 4~91 kPa. The final storage modulus would be even lower according to the relationship between compression modulus and storage modulus: $E = 2 \times (1+\mu) G$. Here, E is Young's modulus. μ is Poisson's ratio, which is from 0 to 0.5. G is shear modulus of elasticity.

In particular, a disadvantage with the Schiff-based reaction is exceedingly fast gelation, which may result in premature crosslinking and delivery failure. For ease of clinical application, the hydrogel must therefore undergo crosslinking with gentle reaction kinetics to prevent premature crosslinking and delivery failure.

There was thus the problem of providing a novel, mild HA-dextran hydrogel system which can self-heal immediately after shear induced flow, is cytocompatible, and can be stabilized through light-initiated radical polymerization of methacrylate functional groups to tune gel mechanics and degradation kinetics. Further, there was the problem of providing a process for the production of such hydrogels.

Stiffness has been regarded as a key metric for how the matrix resists cellular traction forces to regulate stem cell fate. For example, stiff substrates with moduli in the range of 30−35 kPa promote stem cell differentiation into osteoblasts. On the other hand, the phenotype of stem cell can be better maintained when they are cultured on soft substrates with a modulus of 0.5 kPa, as compared to those with a modulus of 30 kPa. From soft fat to stiff articular cartilage and bone, the moduli of these tissue are from 0.02 kPa to 950 kPa (see, Akon Higuchi et al. Chem. Rev. 2013, 113, 3297−3328; Akon Higuchi et al. Chem. Rev. 2012, 112, 4507−4540; and Biji Balakrishnan et al. Chem. Rev. 2011, 111, 4453–4474). Therefore, a key goal is

to achieve high moduli for hydrogel produced from natural materials thus with good biocompatibility and good tissue regeneration.

Summary of the invention

To address these inherent limitations of current injectable hydrogel systems, a novel, mild hydrogel system was developed by using mild dual-crosslinking conditions. To accomplish this, according to the present invention, a glycosaminoglycan such as HA is first functionalized with methacrylate and hydrazide groups, and a second-polysaccharide such as dextran is oxidized to obtain aldehyde groups. The mixture of functionalized glycosaminoglycan and second-polysaccharide-aldehyde first undergo gentle dynamic covalent acylhydrazone bond crosslinking through hydrazide and aldehyde groups to obtain a dynamic acylhydrazone bond cross-linked hydrogel. Acylhydrazone bond formation is an efficient, biocompatible chemistry, often used for bioconjugation, but also can be tuned to be dynamically covalent, depending on the chemical structures. See C. Sun et al. Polymer 160 (2019) 246–253 for more description on acylhydrazone bond. This mechanism permits them to possess desirable properties like shear-thinning and rapidly self-healing. After injection of the dynamic acylhydrazone bond cross-linked hydrogel into a target site, a secondary covalent crosslinking occurs in situ via photopolymerization of methacrylates to stabilize the network and modulate mechanics to obtain the dual-crosslinked hydrogel.

The properties of the dual-crosslinked hydrogels permit them to be used as injectable and photo-stabilizing cell carriers. Cells such as stem cells can be homogenously encapsulated within the hydrogels under constant conditions by, for example, simply resuspending cells with for example one glycosaminoglycan component dissolved in growth media and subsequently mixing with the second polysaccharide component such as dextran-aldehyde. The dual-crosslinked hydrogels have good cytocompatibility and viable cells may be remained above 90 % for 14 days.

Without wishing to be bound by theory, it is believed that the dual-crosslinking, i.e., acylhydrazone crosslinking and methacrylate covalent crosslinking makes the dual-crosslinked hydrogel of the invention possesses advantages such as improved mechanical properties. The dual-crosslinked hydrogel of the invention may have a final storage modulus up to 1000 kPa or more. Apparently, there is a synergetic effect between acylhydrazone crosslinking and methacrylate covalent crosslinking as none of the acylhydrazone crosslinking and methacrylate covalent crosslinking alone may achieve such high modulus. Such high modulus is particularly important and valuable for the applications such as scaffold for cartilage in tissue engineering. Thus, the dual-crosslinked hydrogel of the invention may be applied in applications which requires a high final storage modulus.

Compared with conventional hydrogels with dynamic acylhydrazone-crosslinking only, the dual-crosslinked hydrogel systems exhibit a slow reduction in mass loss at all observed time points. Moreover, the final storage modulus from photopolymerization of the dual-crosslinked hydrogel significantly increases from

0.1 kPa to 1000 kPa or more, which is very surprising for and beyond expectation of a person skilled in the art.

The invention provides a process to prepare a dual-crosslinked hydrogel, comprising the following steps,

1) functionalizing a glycosaminoglycan such as hyaluronic acid with a methacrylate on hydroxyl group and with an dihydrazide on carboxyl group to obtain a methacrylate and hydrazide group functionalized glycosaminoglycan, which can be either a glycosaminoglycan with both methacrylate and hydrazide group modification, or a glycosaminoglycan with methacrylate modification only and a glycosaminoglycan with hydrazide group modification only, wherein the degree of methacrylation is 5-200%, preferably 85-165%, and the degree of hydrazide group modification is 8-70%, preferably 10-65%, more preferably 10-40%; oxidizing a second-polysaccharide such as dextran to obtain a second-polysaccharide with aldehyde groups, the degree of aldehyde modification in the second-polysaccharide is 10-95%, preferably 85~95%;

2) crosslinking the methacrylate and hydrazide group functionalized glycosaminoglycan and the second-polysaccharide-aldehyde in an aqueous solvent, such as phosphate buffer saline (PBS) or a cell culture medium, to form a dynamic acylhydrazone bond cross-linked hydrogel; and

3) photopolymerizing the dynamic acylhydrazone bond cross-linked hydrogel by irradiation under the presence of a photoinitiator.

The dual-crosslinked hydrogel of the invention comprises a first dynamic acylhydrazone bond crosslinking between hydrazide groups of hydrazide groups modified glycosaminoglycan and aldehyde groups of a second-polysaccharide-aldehyde, and a secondary covalent crosslinking formed in situ via photopolymerization of the methacrylate groups modified on the glycosaminoglycan.

The glycosaminoglycan of the invention is water soluble. The glycosaminoglycan is selected from glycosaminoglycans that may be modified by with a methacrylate on a hydroxyl group and with a dihydrazide on a carboxyl group. The glycosaminoglycan is preferably selected from the group consisting of hyaluronic acid, chondroitin sulfate (e.g. chondroitin-6-sulfate, and chondroitin-4-sulfate), dermatan sulfate, heparan sulfate and heparin, especially hyaluronic acid. Unless otherwise specified, the term “hyaluronic acid” in the invention refers to hyaluronic acid or a salt thereof, such as sodium hyaluronate. The salt of hyaluronic acid refers to water-soluble salt of hyaluronic acid. Non-limiting examples may be selected from sodium hyaluronate, hyaluronic acid potassium salt, tetrabutylammonium hyaluronate (HA-TBA), etc.

The second-polysaccharide of the invention is a water-soluble polysaccharide. The second-polysaccharide comprises a 1,4-linkage and/or a 1,6-linkage. In addition,

the second-polysaccharide comprises an ortho-hydroxyl on C2, C3 or C4 of the sugar ring of the polysaccharide. The second-polysaccharide does not include those with 1,3-linkage only. The second-polysaccharide may be glucans such as β-1,3/1,6-glucan, α-1,6-glucan with α-1,3-branches, α-1,6- glucan, α -1,4/1,6-glucan, alginate and pectin, etc. Non-limiting examples of the glucan include dextran, laminarin (β-1,3- and β-1,6-glucan), water soluble cellulose derivatives, especially dextran.

The term "methacrylate" refers to hydrolyzable methacrylates. Non-limiting examples of methacrylate include methacrylic anhydride, 2-aminoethyl methacrylate hydrochloride, glycidyl methacrylate.

The dihydrazide in the invention refers to a dihydrazide that may, after hydrazide group modification of the glycosaminoglycan, further react with an aldehyde group to form an acylhydrazone bond. The dihydrazide may be selected from water-soluble dihydrazides. Non-limiting examples may be selected from adipic dihydrazide, ethanedihydrazide, oxalyldihydrazide, dodecanedioic dihydrazide.

In some embodiments, the dual-crosslinked hydrogel is a dual-crosslinked hyaluronic acid-dextran hydrogel.

Methacrylate may react with the free hydroxyl groups at different positions such as C6 of the hyaluronic acid repeating unit, although hydroxyl group of C6 is typically the most reactive one.

In the invention, the term "second-polysaccharide with aldehyde groups" is used interchangeably with "second-polysaccharide-aldehyde". Both terms mean second-polysaccharide with aldehyde groups oxidized from hydroxyl groups on the original second-polysaccharide molecule. Similarly, the term "dextran with aldehyde groups" is used interchangeably with "dextran-aldehyde". Both terms mean dextran with aldehyde groups oxidized from hydroxyl groups on the original dextran molecule.

The term "degree of methacrylation" refers to:
(the mole of methacrylated hydroxyl groups / the total mole number of repeating units contained in the polymer) * 100%

See S.K. Seidlits et al. Biomaterials 31 (2010) 3930–3940 for more description on methcarylation degree and the determination method thereof.

The degree of methacrylation may be 5-200%, preferably 85-165%, for example 100-165%, 110-165%,120-165%,130-165%, 140-165%, or 150-165%.

The term "degree of hydrazide group modification" refers to:
(the mole of hydrazide group modified repeating units/ the mole of repeating units in the polymer) * 100%.

See Paul Bulpitt et al. Journal of biomedical materials research, 47(2), 152-169 for the determination method thereof.

The degree of hydrazide group modification may be 8-70%, preferably 10-65%, for example 10-40%, 20-65%, or 20-40%.

When adipic dihydrazide (ADH) and dextran is used, acylhydrazone bonds are formed between the aldehyde groups of dextran-aldehyde (oxidized dextran or ODEX) and the acylhydrazide bonds of ADH in step 2).

A person skilled in the art may control the oxidation degree of the second-polysaccharide-aldehyde, e.g. through reaction time and the amount of the oxidant. The degree of aldehyde modification in the second-polysaccharide such as dextran product may be determined by e.g. measuring the number of aldehyde groups in the polymer using t-butyl carbazate and trinitrobenzenesulfonic acid, see for example, Jia X, Colombo G, Padera R, Langer R, Kohane DS. Prolongation of sciatic nerve blockade by in situ cross-linked hyaluronic acid. Biomaterials 2004; 25:4797-4804; or Bouhadir KH, Housman DS, Mooney DJP. Synthesis of crosslinked poly(aldehyde guluronate) hydrogels. Polymer 1999; 40: 3575-3584.

The degree of aldehyde modification in the second-polysaccharide maybe 10-95%, for example 50-95%, 60-95%, 70-95%, or 80-95%, preferably 85~95%.

In order to obtain optimized gelation, a person skilled in the art may adjust the concentration of the modified glycosaminoglycan and modified second-polysaccharide; preferably the concentration of modified glycosaminoglycan such as modified hyaluronic acid is 0.5-3wt.%, preferably 1~2wt.%, the concentration of second-polysaccharide-aldehyde such as dextran-aldehyde is 4-8wt.%, preferably 4~6wt.% based on the total weight of the reaction mixture in step 2).

In some embodiments, the glycosaminoglycan, such as hyaluronic acid may have a molecular weight of 50~2000 kDa, preferably 50~1000 kDa.

In some embodiments, the second-polysaccharide, such as dextran may have a molecular weight of 10-1000 kDa, such as 10-100 kDa,10~70 kDa, preferably 10~20 kDa.

In step 1), glycosaminoglycan such as hyaluronic acid is modified with methacrylate to obtain a methacrylated glycosaminoglycan, then further modified with hydrazide groups. Alternatively, glycosaminoglycan may be modified with methacrylate and hydrazide groups separately (i.e., not in the same molecule), then in step 2) crosslinking both the methacrylated glycosaminoglycan and hydrazide modified glycosaminoglycan with the second-polysaccharide-aldehyde to form a dynamic acylhydrazone bond cross-linked hydrogel. In addition, in step 1), the second-polysaccharide is oxidized to prepare a second-polysaccharide-aldehyde.

In step 2), the modified glycosaminoglycan and second-polysaccharide -aldehyde is crosslinked via formation of acylhydrozone bonds, preferably in a phosphate buffer saline (PBS) or a cell culture medium.

In step 3), the methacrylate functional groups of the dynamic acylhydrazone bond cross-linked hydrogel are polymerized. In some examples, the polymerization is a light-initiated radical polymerization.

The irradiation may be 320-500 nm irradiation, sunlight irradiation, etc., preferably 405 nm blue light. In some embodiments, the dynamic acylhydrazone bond cross-linked hydrogels were crosslinked by using 405 nm blue-light, at a power of 2-4 W, a light intensity of 6~10 mW/cm^2 for 1~3 min.

The photoinitiator may be those conventional in the art, for example lithium phenyl-2, 4, 6-trimethylbenzoylphosphinate (LAP). In some embodiments, the photoinitiator may be dissolved in the aqueous solvent of step 2).

The invention further provides a dual-crosslinked hydrogel prepared according to the above process of the invention.

The invention further provides a hydrogel precursor to prepare a dual-crosslinked hydrogel, wherein the hydrogel precursor is prepared by a method comprising the following steps:

1) functionalizing a glycosaminoglycan such as hyaluronic acid with methacrylate on hydroxyl group and with a hydrazide on carboxyl group to obtain a methacrylate and hydrazide group functionalized glycosaminoglycan, which can be either a glycosaminoglycan with both methacrylate and hydrazide group modification, or a glycosaminoglycan with methacrylate modification only and a glycosaminoglycan with hydrazide group modification only, wherein the degree of methacrylation is 5-200%, preferably 85-165%, and the degree of hydrazide group modification is 8-70%, preferably 10-65%, more preferably 10-40%; oxidizing a second-polysaccharide such as dextran to obtain a second-polysaccharide with aldehyde groups, the degree of aldehyde modification in the second-polysaccharide is 10-95%, preferably 85~95%; and

2) crosslinking the methacrylate and hydrazide group functionalized glycosaminoglycan and the second-polysaccharide with aldehyde groups in an aqueous solvent, such as phosphate buffer saline (PBS) or a cell culture medium, to form a dynamic acylhydrazone bond cross-linked hydrogel.

The hydrogel precursor is the dynamic acylhydrazone bond cross-linked hydrogel prepared in step 2). It is injectable and may be used to prepare the dual-crosslinked hydrogel of the invention.

In use, the hydrogel precursor may optionally carry active ingredients like cells and be injected into the desired part of a subject. Then the hydrogel precursor may be

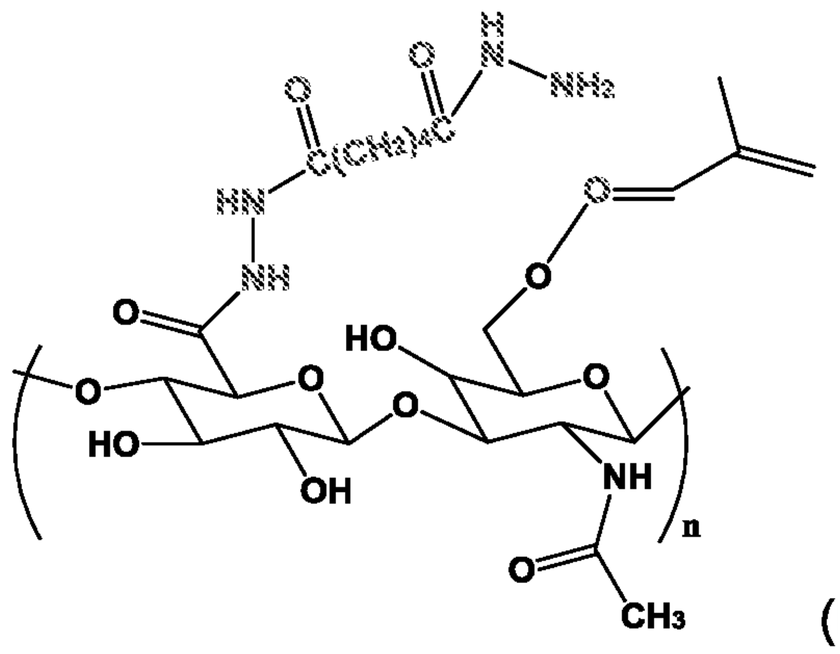
photopolymerized by exposure to irradiation (e.g. 405 nm blue light) in situ, thereby a dual-crosslinked hydrogel may be prepared.

The invention further provides use of the dual-crosslinked hydrogel of the invention or prepared according to the process of the invention or the hydrogel precursor of the invention in tissue engineering, bioapplications such as drug delivery system, biosensors and soft tissue filling agents, etc., or as a carrier of at least one active ingredient such as cell. The cell includes stem cells. The cell carrier may have a high cell viability. Notably, stem cells can be homogenously encapsulated within the dual-crosslinked hydrogel and the viable cells may be remained above 90 % for 14 days. In use, the cells may be incorporated into the dual-crosslinked hydrogel by various ways. For example, the cells may be incorporated into the dual-crosslinked hydrogel by impregnating the dynamic acylhydrazone bond cross-linked hydrogel in a cell suspension; or preferably cells may be added into the reaction mixture before forming the dynamic acylhydrazone bond cross-linked hydrogel, for example cells may be mixed with the functionalized glycosaminoglycan and/or second-polysaccharide with aldehyde groups.

The dual-crosslinked hydrogel with cell may be used as tissue induced scaffold. The dual-crosslinked hydrogel without cell may be used as tissue engineering scaffold.

Chemical crosslinking can be tuned by the dynamic acylhydrazone crosslinking and secondary photo-irradiation, and the extent of chemical crosslinking can be tuned by adjusting irradiation exposure time. The gelation time and equivalent time can be controlled within 20 s-60 s and 10-30 mins, respectively for the dynamic acylhydrazone bond cross-linked hydrogel. With secondary crosslinking, the moduli of the hydrogels can be controlled within 0.1 kPa~2000 kPa, or be controlled from 100 kPa, 200 kPa, 300 kPa, 400 kPa, 500 kPa, 600 kPa, 700 kPa, or 800 kPa, to 1000 kPa, even up to 1100 kPa, 1200 kPa, 1500 kPa, 1600 kPa, 1700 kPa, 1800 kPa, 1900 kPa, 1950 kPa, or 2000 kPa, by e.g. controlling the degree of degree of methacrylation, the degree of hydrazide group modification, the degree of aldehyde modification, the molecular weight of glycosaminoglycan and second-polysaccharide, the concentration of modified glycosaminoglycan such as hyaluronic acid, the concentration of second-polysaccharide-aldehyde, and the irradiation exposure time between for example 10 s, 20 s, 30 s, 40 s, 50 s, or 60 s to 2 mins, 3 mins, 5 mins, 10 mins, or 20 mins, such as 20 s to 20 mins, 30 s to 20 mins, 40 s to 20 mins, 50 s to 20 mins, 60 s to 20 mins.

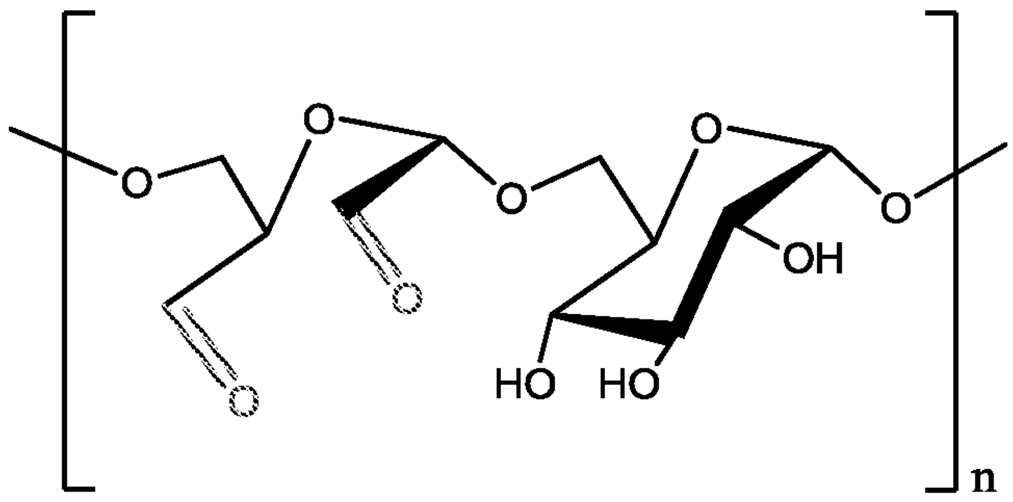
In some embodiments, the chemical structure of dual-functionalized HA with dihydrazide- and methacrylate- modification may be schematically shown as formula I:



(I)

In some embodiments, the molecular weight of sodium hyaluronate is 50~2000 kDa, for example, 50~1500 kDa, preferably 200~1000 KDa. In one embodiment, the molecular weight of sodium hyaluronate is 200~500 KDa.

In some embodiments, the structure of dextran-aldehyde (oxidized dextran or ODEX) may be schematically shown as formula II:



(II)

In some embodiments, the dual-crosslinked hydrogel according to the invention is prepared according to the method as follows,

Preparation of Methacrylated HA (MeHA)
Dissolving sodium hyaluronate (e.g.200-500 kDa) at 0.5-1.5 wt.% in deionized water (DI H_2O);
Adding methacrylic anhydride dropwise (~1.5-3 mL per g HA) with stirring at 2~5 °C;
Maintaining the stirring mixture at pH 7.5-8.5 by continuously adding 5-10 mM NaOH for 8-12 h;
Reacting overnight at 2~5 °C;
Adding MA (1-2 mL MA per g HA) with pH maintenance for 4-6 h; and
Purifying the methacrylated HA.

In some embodiments, the purification is done bydialyzing the macromer solution against DI H_2O (M_W cutoff 8,000-14,000) for 3-5 days.

The purified methacrylated HA may be frozen at -60 to -80 °C, lyophilized, and stored at -80 °C in powder form.

Preparation of Dihydrazide-HA (DHA-HA) or Dihydrazide-MeHA (DHA-MeHA)
Dissolving sodium hyaluronate or MeHA in H_2O at a concentration of 2-3 mg/mL;
Adding a 30-fold molar excess of adipic dihydrazide to this solution;
Adjusting the pH of the reaction mixture to 6.5-7.0, e.g. with 0.1-0.5 M NaOH/0.1-0.5M HCl;
Dissolving 1-Ethyl-3-[3 (dimethylamino)propyl]-carbodiimide (EDC) (1-2 mmol) and 1-hydroxybenzotriazole (HOBt) (1-2 mmol) in dimethylsulfoxide (DMSO)/H_2O (1:1, 1 mL);
After mixing, maintaining the pH of the reaction at 6.5-7.0 e.g. by the addition of 0.1-0.5 M NaOH and allowing the reaction to proceed 12-15 h;
Adjusting the pH to 7.0-7.4, e.g. with 0.1-0.5M NaOH; and
Purifying the obtained derivatized HA.

In some embodiments, the purification is done by exhaustively dialyzing (MW cutoff 8,000–14,000) against distilled H_2O for 3-5 days.

The yield of product may typically be around 80%.

Preparation of dextran-aldehyde
Dissolving dextran in water at a concentration of 200-250 mg/mL;
Adding dropwise an aqueous solution of sodium periodate (0.1–0.5 g dissolved in 5 mL water);
Stirring the obtained mixture for 2 h at room temperature in the dark;
Adding ethylene glycol to inactivate any unreacted periodate; and
Purifying the obtained dextran-aldehyde.

In some embodiments, the purification is done by exhaustive dialysis against water for 3-5 days. Dry product of the dextran-aldehyde may be obtained by freeze drying.

Dual-crosslinked MeHA/DHA-HA/ODEX or DHA-MeHA/ODEX hydrogel formation
Preparing MeHA/DHA-HA or DHA-MeHA solutions (ranging from 1.0–2 wt.%) in PBS (pH 7.4) containing 0.1-0.2 wt.% lithium phenyl-2, 4, 6-trimethylbenzoylphosphinate (LAP) photoinitiator;
mixing a solution of ODEX (e.g. 50–60% w/v in PBS) with a solution of DHA-HA or DHA-MeHA (1-2% w/v) to obtain a dynamic acylacylhydrazone bond cross-linked hydrogel; and
Photopolymerizing the dynamic acylhydrazone bond cross-linked hydrogel by irradiation, to obtain the dual-crosslinked MeHA/DHA-HA/ODEX or DHA-MeHA/ODEX hydrogel.

In some embodiments, 0.1 ml solutions of ODEX (50–60% w/v in PBS (Invitrogen)) were pipetted into 0.9 mL DHA-HA or DHA-MeHA (1-2% w/v) contained in a cylindrical mold made from a truncated 1-mL syringe. The mixture was vigorously stirred using the pipet tip and then put on a shaker for gelation which typically took

about 0.5-2 min. The resulting round hydrogel disks were exposed to 10 mW cm^{-2} 405 nm blue light for 1-3 min on each side. Disk shaped hydrogels (~4 mm width, ~1 mm height) were cored out of the gel slab and immersed and equilibrated with PBS for 12-16 h.

Therefore, the present invention provides a novel, mild dual-crosslinked hydrogel which has advantageous properties like rapid self-healing (although worse than the hydrogel precursor of the invention), and good cytocompatibility. In addition, the hydrogel precursor, i.e., dynamic acylhydrazone bond cross-linked hydrogel is injectable and has the property of shear-thinning before irradiation; the hydrogel can self-heal immediately after shear induced flow and can be stabilized through light-initiated radical polymerization of methacrylate functional groups to tune gel mechanics and degradation kinetics.

Other advantages of the present invention would be apparent for a person skilled in the art upon reading the specification.

Brief Description of Drawings

Figure 1 shows ^{1}H NMR spectra of functionalized HA and dextran-aldehyde, including (a) MeHA, (b) DHA-HA, (c) DHA-MeHA and (d) ODEX. MeHA, DHA-MeHA and ODEX samples were prepared during Example 1 and DHA-HA sample was prepared during Example 4.

Figure 2 shows the shear-thinning (a) upon the continuous step shear rates between 1 s^{-1} to 10 s^{-1} and self-healing (b) upon alternative shear rate between 0.1 s^{-1} and 1 s^{-1} at 37 °C of the dynamic acylhydrazone bond cross-linked hydrogel prepared during Example 1.

Figure 3 shows the representative curves of storage (G') and loss (G'') moduli during irradiation of:
the dynamic acylhydrazone bond cross-linked hydrogel with dynamic-crosslinking only prepared during Example 1(Figure 3a);
the photo-crosslinked HA-based hydrogel without ODEX treatment prepared in Comparative Example 1 with light irradiation (10 mW/cm^2) (Figure 3b); and
the dynamic acylhydrazone bond cross-linked hydrogel with ODEX treatment prepared in Example 1 (Figure 3c). The in-situ crosslinking process involved both dynamic-crosslinking and light exposure. After light irradiation, double-bond crosslinking formed in the dynamic acylhydrazone bond cross-linked hydrogel and the hydrogel became a dual-crosslinked HA-dextran hydrogel finally prepared in Example 1.

Figure 4 shows degradation kinetics of non-UV-treated hydrogel (dynamic acylhydrazone bond cross-linked hydrogel prepared during Example 1 before irradiation) and UV-treated hydrogel finally prepared in Example 1. For all time points, the percentage of HA release was greater from non-UV-treated hydrogel relative to UV-treated hydrogel ($p<0.01$). Error bars represent standard error of the mean.

Figure 5 shows quantification of cell viability from LIVE/DEAD assay (n=3).

Figure 6 shows gross appearance of defect site at 12 weeks of surgery in control and dual-crosslinked HA-dextran hydrogel prepared in Example 1, where dual-crosslinked HA-dextran hydrogel was in-situ implanted to the right knee joint while the empty defects of left knee joint were acted as control group.

Detailed description of the invention

The invention is now described in detail by the following examples. The scope of the invention should not be limited to the embodiments of the examples.

Example 1

Methacrylated HA (MeHA)

Sodium hyaluronate (Evonik Industries AG, 200-500 kDa) was dissolved at in deionized water (DI H_2O) to prepare a 1 wt% solution. Methacrylic anhydride (MA; Sigma) was added dropwise (~2.5 mL per g HA) with stirring at 4 °C. The stirring mixture was maintained at pH ~8 by continuously adding 10 mM NaOH for 8 h, followed by reaction overnight at 4 °C and further addition of MA (~1 mL MA per g HA) with pH maintenance for ~4 h. The obtained macromer solution was dialyzed against DI H_2O (M_W cutoff 8,000-14,000) for 3 d, frozen at -80 °C, lyophilized, and stored at -80 °C in powder form. The modification degree of MeHA was 160% according to ^{1}H-NMR analysis.

Dihydrazide-MeHA (DHA-MeHA)

MeHA prepared above was dissolved in H_2O at a concentration of 3 mg/mL. To this solution a 30-fold molar excess of adipic dihydrazide was added. The pH of the reaction mixture was adjusted to 6.8 with 0.1 M NaOH/0.1M HCl. 1-Ethyl-3-[3 (dimethylamino)propyl]-carbodiimide (EDC) (192 mg, 1 mmol; Aldrich Chemical Co.) and 1-hydroxybenzotriazole (HOBt) (135 mg, 1 mmol; Aldrich Chemical Co.) was dissolved in dimethylsulfoxide (DMSO)/H_2O (1:1, 1 mL). After mixing, the pH of the reaction was maintained at 6.8 by the addition of 0.1M NaOH and the reaction was allowed to proceed overnight. The pH was subsequently adjusted to 7.0 with 0.1M NaOH and the derivatized HA exhaustively was dialyzed against DI H_2O (M_W cutoff 8,000–14,000) for 3 d, frozen at -80 °C, lyophilized, and stored at -80 °C in powder form. The dihydrazide modification degree of DHA-MeHA was 36% according to ^{1}H-NMR analysis.

Dextran-aldehyde (ODEX)

To functionalize dextran (molecular weight: 20 kDa) with aldehyde groups, 5 g dextran was dissolved in water at a concentration of 250 mg/mL. An aqueous solution of sodium periodate (0.3 g dissolved in 5 mL water) was added dropwise, and the reaction mixture was stirred for 2 h at room temperature in the dark. Ethylene glycol was then added to inactivate any unreacted periodate. The solution was purified by exhaustive dialysis against water for 3 days, and the dry product of the dextran-aldehyde was obtained by freeze drying. The modification degree of dextran-aldehyde was 91% according to trinitrobenzene sulfonate assay.

Dual-crosslinked DHA-MeHA/ODEX hydrogel formation
A 2.0 wt.% DHA-MeHA solution was prepared by dissolving DHA-MeHA in PBS (pH 7.4) containing 0.1 wt.% lithium phenyl-2, 4, 6-trimethylbenzoylphosphinate (LAP) photoinitiator (Tokyo Chemical Industry Co., Ltd.). 0.1 ml ODEX (60% w/v prepared by dissolving ODEX in PBS (Invitrogen)) solution was pipetted into 0.9 mL DHA-MeHA (2% w/v in PBS) solution contained in a cylindrical mold made from a truncated 1-mL syringe. The mixture was vigorously stirred using the pipet tip and then put on a shaker for gelation which typically took about 60 s. The resulting round hydrogel disk, which was a dynamic acylhydrazone bond cross-linked hydrogel, was exposed to 10 mW/cm^2 405 nm blue light for 60 s on each side. The disk shaped dual-crosslinked hydrogel (~4 mm width, ~1 mm height) was cored out of the gel slab and immersed and equilibrated with PBS for 12 h. Multiple dual-crosslinked hydrogel samples were prepared in Example 1. The storage modulus of the obtained hydrogel was about 1910 kPa.

<u>Comparative Example 1: DHA-MeHA hydrogel</u>
A 2.0 wt.% solution of DHA-MeHA prepared during Example 1 was prepared in PBS (pH 7.4) containing 0.1 wt.% lithium phenyl-2, 4, 6-trimethylbenzoylphosphinate (LAP) photoinitiator. LAP was chosen due to its aqueous solubility and good cytocompatibility. The mixtures were pipetted between glass slides and exposed to 10 mW/cm^2 405 nm blue light for 1 min on each side. Disk shaped hydrogels (~4 mm width, ~1 mm height) were cored out of the gel slab.

The modulus of the photo-crosslinked DHA-MeHA hydrogel was 3.08 kPa. The determination method was the same as the method described below used for Example 1.

<u>Example 2</u>
MeHA and dihydrazide-MeHA (DHA-MeHA) was prepared according to the same method as Example 1.

Dextran-aldehyde was prepared according to the same method as Example 1.

Dual-crosslinked DHA-MeHA/ODEX hydrogel formation
Dual-crosslinked DHA-MeHA/ODEX hydrogel was prepared according to the same method as Example 1 except that the resulting dynamic acylhydrazone-crosslinked hydrogel was exposed to 10 mW/cm^2 320-500 nm irradiation (comprising 405 nm blue light) instead of 405 nm blue light. The storage modulus of the obtained hydrogel was about 780 kPa. As the irradiation was not done under the optimum irradiation wavelength 405 nm, the storage modulus was lower than that in Example 1.

<u>Example 3</u>
MeHA and dihydrazide-MeHA (DHA-MeHA) was prepared according to the same method as Example 1.

Dextran-aldehyde was prepared according to the same method as Example 1 except that the aqueous solution of sodium periodate used 0.25 g instead of 0.3 g sodium periodate dissolved in 5 mL water. The modification degree of dextran-aldehyde was 86% according to trinitrobenzene sulfonate assay.

Dual-crosslinked DHA-MeHA/ODEX hydrogel formation
A 1 wt.% DHA-MeHA solution was prepared in PBS (pH 7.4) containing 0.2 wt.% lithium phenyl-2, 4, 6-trimethylbenzoylphosphinate (LAP) photoinitiator. 0.1 ml ODEX (60% w/v in PBS) solution was pipetted into 0.9 mL DHA-MeHA (2% w/v in PBS) solution contained in a cylindrical mold made from a truncated 1-mL syringe. The mixture was vigorously stirred using the pipet tip and then put on a shaker for gelation which typically took about 60 s. The resulting round hydrogel disk was exposed to 10 mW/cm^2 320-500 nm irradiation (comprising 405 nm blue light) for 20 s on each side. The disk-shaped hydrogel (~4 mm width, ~1 mm height) was cored out of the gel slab and immersed and equilibrated with PBS for 12 h. The storage modulus of the obtained hydrogel was about 295 kPa. As the irradiation was not done under the optimum irradiation wavelength 405 nm, the storage modulus was lower than that in Example 1.

Example 4: separate modification to HA by MA and DHA
Methacrylated HA (MeHA) was prepared according to the same method as Example 1.

Dihydrazide-HA (DHA-HA)
Sodium hyaluronate (Evonik Industries AG, 200-500 kDa) was dissolved in H_2O at a concentration of 3 mg/mL. To this solution a 30-fold molar excess of adipic dihydrazide was added. The pH of the reaction mixture was adjusted to 6.8 with 0.1 M NaOH/0.1M HCl. 1-Ethyl-3-[3 (dimethylamino)propyl]-carbodiimide (EDC) (192 mg, 1 mmol) and 1-hydroxybenzotriazole (HOBt) (135 mg, 1 mmol) was dissolved in dimethylsulfoxide (DMSO)/H_2O (1:1, 1 mL). After mixing, the pH of the reaction was maintained at 6.8 by the addition of 0.1M NaOH and the reaction was allowed to proceed overnight. The pH was subsequently adjusted to 7.0 with 0.1M NaOH and the derivatized HA exhaustively was dialyzed against DI H_2O (M_W cutoff 8,000–14,000) for 3 d, frozen at -80 °C, lyophilized, and stored at -80 °C in powder form.

Dextran-aldehyde was prepared according to the same method as Example 1 except that the aqueous solution of sodium periodate used 0.5 g instead of 0.3 g sodium periodate dissolved in 5 mL water. The modification degree of dextran-aldehyde was 95% according to trinitrobenzene sulfonate assay.

Dual-crosslinked DHA-HA/MeHA/ODEX hydrogel formation
Dynamic acylhydrazone bond cross-linked hydrogel was prepared by mixing reactive DHA-HA, MeHA and dextran-aldehyde polymer solutions containing 0.2 wt.% lithium phenyl-2, 4, 6-trimethylbenzoylphosphinate (LAP) photoinitiator. Specifically, 0.1 ml ODEX (50% w/v in PBS) solution was pipetted into 0.9 mL DHA-HA/MeHA (1:1, 2% w/v in PBS, which was prepared by mixing 2% w/v

DHA-HA in PBS with 2% w/v MeHA in PBS) solution containing 0.2 wt.% LAP photoinitiator contained in a cylindrical mold made from a truncated 1-mL syringe. The mixture was vigorously stirred using the pipet tip and then put on a shaker for gelation which typically took about 60 s. The resulting round hydrogel disk was exposed to 10 mW/cm^2 320-500 nm irradiation (comprising 405 nm blue light) for 20 s on each side. The resulting round hydrogel disk was ejected from the cylindrical mold, and immersed and equilibrated with PBS for 12 h. The storage modulus of the obtained hydrogel was about 202 kPa. As the irradiation was not done under the optimum irradiation wavelength 405 nm, the storage modulus was lower than that in Example 1.

Example 5: separate modification to HA by MA and DHA
Methacrylated HA (MeHA) was prepared according to the same method as Example 1.

Dihydrazide-HA (DHA-HA) was prepared according to the same method as Example 4.

Dextran-aldehyde was prepared according to the same method as Example 1.

Dual-crosslinked DHA-HA/MeHA/ODEX hydrogel formation
Dynamic acylhydrazone bond cross-linked hydrogel was prepared by mixing reactive DHA-HA, MeHA and dextran-aldehyde polymer solutions containing 0.1 wt.% lithium phenyl-2, 4, 6-trimethylbenzoylphosphinate (LAP) photoinitiator. Specifically, 0.1 ml ODEX (55% w/v in PBS) solution was pipetted into 0.9 mL DHA-HA/MeHA (1:1, 1% w/v in PBS, which was prepared by mixing 1% w/v DHA-HA in PBS with 1% w/v MeHA in PBS) solution containing 0.2 wt.% LAP photoinitiator contained in a cylindrical mold made from a truncated 1-mL syringe. The mixture was vigorously stirred using the pipet tip and then put on a shaker for gelation which typically took about 60 s. The resulting round hydrogel disk was exposed to 10 mW/cm^2 320-500 nm irradiation (comprising 405 nm blue light) for 30 s on each side. The disk shaped dual-crosslinked hydrogel (~4 mm width, ~1 mm height) was cored out of the gel slab and immersed and equilibrated with PBS for 12 h. The storage modulus of the obtained hydrogel was about 185 kPa. As the irradiation was not done under the optimum irradiation wavelength 405 nm, the storage modulus was lower than that in Example 1.

Characterization of the dual-crosslinked hydrogel
The MeHA, DHA-MeHA, and ODEX samples prepared during Example 1 and DHA-HA sample prepared during Example 4 were analyzed by using NMR spectrometer. The results were shown in Figure 1.

The chemical structure of functional HA derivatives including MeHA, DHA-HA, DHA-MeHA and dextran-aldehyde was determined on a Bruker Advance III spectrometer using D_2O as the solvent. Proton-NMR of the modified HA revealed distinct methylene (HA 6.1 and 5.6 ppm). The degree of methacrylate modification

was determined from the relative integrations of the methacrylate (1H each) to the HA backbone protons (10H).

As shown in Figure 1a, for the MeHA, the signals of vinyl protons clearly appeared at 5.6 and 6.1 ppm. According to the peak areas of 5.6 ppm and 6.1 ppm relative to the sugar ring of HA (3.1-3.8 ppm, 10H) in the MeHA spectrum, the degree of methacrylation was about 160%.

The chemical structure of DHA-HA was shown in Figure 1b, the apparent degree of modification calculated by the ratio of the protons of adipic (4H, CH_2CH_2, 1.35-1.55 ppm) to the sugar ring of HA (3.1-3.8 ppm, 10H) was about 36%.

In the spectrum of the DHA-MeHA, the vinyl protons (5.6 ppm, 6.1 ppm) and adipic protons (4H, CH_2CH_2, 1.35-1.55 ppm) were shown in Figure 1c, where the degree of methacrylation was 160%, and the degree of hydrazide groups modification was 32%.

In the spectra of dextran-aldehyde, ODEX presents several additional peaks in the range of 5.0−5.8 ppm, which were assigned to the protons from the hemiacetal structures (Figure 1d).

Furthermore, the rheology tests (moduli, shear-thinning and self-healing) as shown below and in Figure 3 confirmed that the desired dual-crosslinking structure were formed in the dynamic acylhydrazone bond cross-linked hydrogel prepared during Example 1.

Figure 3 (a) indicated that dynamic acylhydrazone bond were formed in the dynamic acylhydrazone bond cross-linked hydrogel prepared during Example 1.

Figure 3 (b) indicated that when irradiated, the dihydrazide-MeHA (DHA-MeHA) prepared during Comparative Example 1 may form double-bond crosslinking due to the existence of double-bonds in the methacrylation in the modified HA.

Figure 3 (c) indicated that when irradiated, dual-crosslinking were formed in the dual-crosslinked HA-dextran hydrogel prepared in Example 1.

<u>Performance test of hydrogel</u>

The hydrogels prepared during Example 1 were analyzed according to the methods as follow. The other hydrogels prepared in the description were analyzed according to the same methods.

Moduli, shear-thinning and self-healing:

Moduli, shear-thinning and self-healing of HA-dextran hydrogels prepared during Example 1 including the dynamic acylhydrazone bond cross-linked hydrogel and the dual-crosslinked DHA-MeHA/ODEX hydrogel were assessed with shear rate sweeps and time sweeps using a DHR-2 rheometer (TA Instruments, Delaware, United States) with a quartz plate connected to a blue light source. A plate

geometry with a solvent trap, 20 mm diameter, and 500 μm gap distance was used. Dynamic acylhydrazone bond cross-linked HA-dextran hydrogels prepared during Example 1 were formed by homogenously mixing together HA/dextran components, and loaded onto the rheometer. Shear rate swept between 0.1 to 10 s^{-1} was tested at 37 °C. Time sweeps were performed for shear recovery experiments. Hydrogels were deformed using 1 s^{-1} and allowed to recover at 0.1 s^{-1} shear rate at 37 °C. To measure the response of rheological properties to photopolymerization, in situ polymerization was performed with in-situ dynamic crosslinking with 405 nm wavelength irradiation at 10 mW/cm^2 light intensity using a dental lamp attached to a light guide for different formulations for 5-10 min via a light-curing stage during oscillatory time sweeps at a frequency of 1 Hz and a strain of 0.5%. Experiments were repeated for a minimum of three times, and representative data was presented.

As shown in Figure 2a, the viscosity of the dynamic acylhydrazone bond cross-linked hydrogel continuously decreased with shear rate increased from 1 s^{-1} to 10 s^{-1}, which suggests that such hydrogels exhibit shear-thinning property suitable for easy injection. Moreover, these hydrogels were able to self-heal after shear injection. As shown in Figure 2a, at 1 s^{-1} shear rate, the viscosity of the dynamic acylhydrazone bond cross-linked HA-dextran hydrogel abruptly decreased from 418 Pa·s to 82 Pa·s. In contrast, as the shear rate was jumped to 0.1 s^{-1}, the viscosity rapidly recovered to its original value. These values demonstrate that the dynamic acylhydrazone bond cross-linked HA-dextran hydrogels self-healed rapidly into hydrogels and the methacrylate functionality did not interfere with the shear-thinning and self-healing properties of the dynamic hydrogels.

The representative curves of storage (G') and loss (G") moduli of the hydrogels prepared during Example 1 and Comparative Example 1 were shown in Figure 3. The storage modulus (G') and the loss modulus (G") of the hydrozide-crosslinking DHA-MeHA/ODEX were firstly monitored with an oscillatory time sweep.

Figure 3a shows the dynamic acylhydrazone bond crosslinking step during Example 1, the injectable liquid-like property of the precursor (before dynamic acylhydrazone bond crosslinking) was shown from the larger G" than G'. After crosslinking of 25 s, the injectable precursor was converted into a gel (dynamic acylhydrazone bond cross-linked HA-dextran hydrogel), where the G' was larger than G". Allowing further crosslinking, the G' of dynamic DHA-MeHA/ODEX hydrogels reached ~40 Pa at about 1200 s of crosslinking. These values suggested that the dynamic acylhydrazone bond cross-linked DHA-MeHA/ODEX hydrogels were soft and weak, which were beneficial for injection.

Figure 3b shows the formation of DHA-MeHA hydrogel prepared during Comparative Example 1. Upon exposure to free-radical generating irradiation using a 405 nm blue-light, the precursor (DHA-MeHA without ODEX component) exhibit increased in both G' and G", and was rapidly converted into a hydrogel (< 30 s), as reflected by the increase in G' over the G". Allowing crosslinking to proceed to

completion, the G' of DHA-MeHA hydrogels reached as high as ~3.08 kPa at about 180 s of irradiation.

Figure 3c shows the formation of dual-crosslinked DHA-MeHA hydrogel prepared during Example 1. As shown in Figure 3(c), the DHA-MeHA/ODEX hydrogels were firstly crosslinked by dynamic hydrazone and subsequently strengthened via chemical crosslinking. The dual-crosslinked DHA-MeHA/ODEX hydrogels resulted in a more rigid viscoelastic solid. The G' of the dual-crosslinked DHA-MeHA/ODEX hydrogels reached as high as ~1910 kPa at about 600 s of irradiation, which was much higher than both of the DHA-MeHA/ODEX hydrogels without irradiation (dynamic acylhydrazone bond cross-linked HA-dextran hydrogel) and the DHA-MeHA without ODEX components, due to dynamic acylhydrazone crosslinking and chemical crosslinking between methacrylate groups.

Chemical crosslinking can be terminated by simply removing irradiation, and the extent of chemical crosslinking can be tuned by adjusting irradiation exposure time. The G' of such dual-crosslinking hydrogel could be adjust from 0.1~1000 kPa, which was beyond expectation and very surprising for a person skilled in the art.

Degradation kinetics:
To quantitatively assess degradation kinetics, HA-dextran hydrogels prepared during Example 1, including the dynamic acylhydrazone bond cross-linked hydrogel and the dual-crosslinked DHA-MeHA/ODEX hydrogel (8 mm diameter, 3 mm thickness) were incubated in separate wells of a 24-well plate containing 5 U/mL exogenous hyaluronidase in 1.0 mL PBS on an orbital shaker at 37 ºC. The solutions were refreshed every 48 hours until day 14. Hydrogel samples were removed from the cultures after 1-2 d, washed with distilled water, and lyophilized in a freeze dryer at -65 °C for 3 days and weighed. The extent of degradation was estimated from the weight loss of the polymer based on the following equation:

$$W=(W_o-W_d)/W_o*100\%$$

Where W_o is the original weight of the hydrogel samples, and W_d is the weight of dry sample after being degraded by hydrolysis in PBS.

Degradation kinetics of the dynamic acylhydrazone bond cross-linked hydrogels and dual-crosslinked hydrogels after irradiation prepared during Example 1 in the presence of 5 U/ml exogenous hyaluronidase for 14 d were shown in Figure 4. Dynamic acylhydrazone bond cross-linked hydrogels exhibited rapid HA release, whereas irradiated hydrogels exhibited little HA release, which suggested that secondary polymerization inhibited the degradation of the hydrogels.

<u>Cell viability test:</u>
Bone marrow stromal cells (BMSCs) were isolated from neonatal SD rats. Cells were cultured in low-glucose Dubecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco). These cells were located in humidified incubator at 37 °C with a 5% CO_2 atmosphere. After 3 days,

non-adherent cells were removed and fresh media was added. The cells were passaged upon almost confluence. Only three-passage BMSCs were used for hydrogel studies.

The same preparation method as Example 1 were performed except that cells were incorporated into the hydrogel in the stage of "Dual-crosslinked DHA-MeHA/ODEX hydrogel formation". Single cell suspensions of BMSCs were mixed with DHA-MeHA and ODEX solutions prepared in Example 1. The final concentration of encapsulated BMSCs in the Dual-crosslinked DHA-MeHA/ODEX hydrogel prepared in Example 1 was 1×10^6 cells mL^{-1}. The hydrogels (50 µL each) encapsulated with BMSCs were incubated a 24-well culture plate at 37 °C in 5% CO_2 with medium change every two days. Cell viability and proliferation of the encapsulated hASCs were determined by LIVE/DEAD assays according to the manufacturer's protocol (Biovision). For quantitative analysis of LIVE/DEAD result, images were taken randomly from three hydrogel samples at 1, 7 and 14 d after encapsulation using a Leica inverted microscope. The cell viability was defined as the ratio between the number of live cells and the total cell number in the field.

The cell viability was demonstrated by using LIVE/DEAD assay (n=3). As shown in Figure 5, the viable stem cells remained above 90 % after 14 days, which indicated the dual-crosslinked hydrogel are cytocompatible and the photo-irradiation does not harm the cells.

<u>Cartilage tissue regeneration</u>

Twelve healthy mature male New Zealand white rabbits weighing around 2.5~3 kg were used for cartilage repair experiment. The full thickness cartilage defects with ~4 mm diameter were created on each knee joint. The empty defects of left cartilage were acted as control group. All surgical procedures were performed under aseptic conditions. The rabbits were anaesthetized by using chloral hydrate (0.15 g/kg body weight). The hind limb was shaved, prepped with betadine and 70% alcohol. A parapatellar, longitudinal incision was made and the patella was luxated medially exposing the femoropatellar groove. Using a dental drill of 3.5 mm diameter, a circular defect (~4 mm in diameter and ~4 mm in depth) was created at the femoro patellar groove by increasing the defect size sequentially to full osteochondral thickness. The drilling was done under continuous irrigation with saline solution for cooling and removing residual tissue while creating the defect. A sterile gauss was used to blot blood at the defect and to obtain homeostasis. Aseptically prepared dynamic acylhydrazone bond cross-linked hydrogel hydrogels prepared during Example 1 (without cells) of each rabbit group were loaded into a syringe. The defect was filled with injectable hydrogels (150 µL) using 35G needle and then was exposed to 10 mW cm^{-2} 405 nm blue light for 180 s. The patella was repositioned, followed by suturing the muscle and skin separately. The osteochondral defect was also created in contralateral limb by repeating the same procedure. Bilateral surgery was performed in the articulating knee joints and the approach was identical for all rabbits. Animals were moved to their cages after they recovered from anesthesia. Post and pre-surgical care was taken by intra-muscular administration of penicillin (400,000 units). At 12 weeks, animals were euthanized

by carbon dioxide inhalation and the femoral end of knee joints was dissected for further analysis.

As shown in Figure 6, after 12 weeks, the hydrogel injected groups exhibited closure of defect with no adverse degeneration of tissue when compared control groups. Moreover, more deposition of newly formed tissue was observed in animals treated with hydrogel group.

As used herein, terms such as “comprise(s)” and the like as used herein are open terms meaning 'including at least' unless otherwise specifically noted.

All references, tests, standards, documents, publications, etc. mentioned herein are incorporated herein by reference. Where a numerical limit or range is stated, the endpoints are included. Also, all values and subranges within a numerical limit or range are specifically included as if explicitly written out.

The above description is presented to enable a person skilled in the art to make and use the invention, and is provided in the context of a particular application and its requirements. Various modifications to the preferred embodiments will be readily apparent to those skilled in the art, and the generic principles defined herein may be applied to other embodiments and applications without departing from the spirit and scope of the invention. Thus, this invention is not intended to be limited to the embodiments shown, but is to be accorded the widest scope consistent with the principles and features disclosed herein. In this regard, certain embodiments within the invention may not show every benefit of the invention, considered broadly.

Claims

1. A process to prepare a dual-crosslinked hydrogel, comprising the following steps:
1) functionalizing a glycosaminoglycan such as hyaluronic acid with a methacrylate on hydroxyl group and with a dihydrazide on carboxyl group to obtain a methacrylate and hydrazide group functionalized glycosaminoglycan, which can be either a glycosaminoglycan with both methacrylate and hydrazide group modification, or a glycosaminoglycan with methacrylate modification only and a glycosaminoglycan with hydrazide group modification only, wherein the degree of methacrylation is 5-200%, preferably 85-165%, and the degree of hydrazide group modification is 8-70%, preferably 10-65%, more preferably 10-40%; oxidizing a second-polysaccharide such as dextran to obtain a second-polysaccharide with aldehyde groups, the degree of aldehyde modification in the second-polysaccharide is 10-95%, preferably 85~95%;

2) crosslinking the methacrylate and hydrazide group functionalized glycosaminoglycan and the second-polysaccharide-aldehyde in an aqueous solvent, such as phosphate buffer saline or a cell culture medium, to form a dynamic acylhydrazone bond cross-linked hydrogel; and

3) photopolymerizing the dynamic acylhydrazone bond cross-linked hydrogel by irradiation under the presence of a photoinitiator.

2. The process of claim 1, wherein the glycosaminoglycan is water soluble and is selected from glycosaminoglycans that may be modified with a methacrylate on a hydroxyl group and with a dihydrazide on a carboxyl group; preferably selected from the group consisting of hyaluronic acid, chondroitin sulfate, dermatan sulfate, heparan sulfate and heparin, more preferably is hyaluronic acid, especially hyaluronic acid with a molecular weight of 50~2000 kDa, preferably 200~1000 kDa.

3. The process of claim 1, wherein the second-polysaccharide is a water soluble polysaccharide, and comprises a 1,4-linkage or a 1,6-linkage, and comprises an ortho-hydroxyl on C2, C3 or C4 of the sugar ring; preferably is selected from the group consisting of glucans such as β-1,3/1,6-glucan, α-1,6-glucan with α-1,3-branches, α-1,6- glucan, α -1,4/1,6-glucan, alginate and pectin, especially dextran, laminarin (β-1,3- and β-1,6-glucan), or water soluble cellulose derivatives; more preferably is dextran, especially dextran with a molecular weight of 10-1000kDa, such as 10-100kDa, 10~70 kDa, preferably 10~20 kDa.

4. The process of claim 1, wherein the methacrylate is selected from hydrolyzable methacrylates; preferably is selected from the group consisting of methacrylic anhydride, 2-aminoethyl methacrylate hydrochloride, and glycidyl methacrylate; more preferably is methacrylic anhydride or glycidyl methacrylate.

5. The process of claim 1, wherein the dihydrazide is water-soluble and is a dihydrazide that may, after hydrazide group modification of the glycosaminoglycan,

further react with an aldehyde group to form an acylhydrazone bond; preferably is selected from the group consisting of adipic dihydrazide, ethanedihydrazide, oxalyldihydrazide, and dodecanedioic dihydrazide.

6. The process of claim 1, wherein the concentration of modified glycosaminoglycan such as modified hyaluronic acid is 0.5-3wt.%, preferably 1~2wt.%, the concentration of second-polysaccharide-aldehyde such as dextran-aldehyde is 4-8wt.%, preferably 4~6wt.% based on the total weight of the reaction mixture in step 2).

7. The process of claim 1, wherein the irradiation is 405 nm blue light, for example at a light intensity of 6~10 mW/cm^2 for 1~3 min.

8. The process of claim 1, wherein cells are incorporated into the dual-crosslinked hydrogel by impregnating the dynamic acylhydrazone bond cross-linked hydrogel in a cell suspension; or by adding cells into the reaction mixture before forming the dynamic acylhydrazone bond cross-linked hydrogel, preferably by mixing cells with the functionalized glycosaminoglycan and/or the second-polysaccharide-aldehyde.

9. A dual-crosslinked hydrogel prepared according to the process of any one of claims 1-8.

10. A hydrogel precursor to prepare a dual-crosslinked hydrogel, which is prepared by the following steps:

1) functionalizing a glycosaminoglycan such as hyaluronic acid with methacrylate on hydroxyl group and with a dihydrazide on carboxyl group to obtain a methacrylate and hydrazide group functionalized glycosaminoglycan, which can be either a glycosaminoglycan with both methacrylate and hydrazide group modification, or a glycosaminoglycan with methacrylate modification only and a glycosaminoglycan with hydrazide group modification only, wherein the degree of methacrylation is 5-200%, preferably 85-165%, and the degree of hydrazide group modification is 8-70%, preferably 10-65%, more preferably 10-40%; oxidizing a second-polysaccharide such as dextran to obtain a second-polysaccharide with aldehyde groups, the degree of aldehyde modification in the a second-polysaccharide is 10-95%, preferably 85~95%; and

2) crosslinking the methacrylate and hydrazide group functionalized glycosaminoglycan and the second-polysaccharide-aldehyde in an aqueous solvent, such as phosphate buffer saline (PBS) or a cell culture medium, to form a dynamic acylhydrazone bond cross-linked hydrogel.

11. Use of the dual-crosslinked hydrogel of claim 9 or the hydrogel precursor of claim 10 in tissue engineering, bioapplications such as drug delivery system, biosensors and soft tissue filling agents, etc., or as a carrier of at least one active ingredient such as cell.

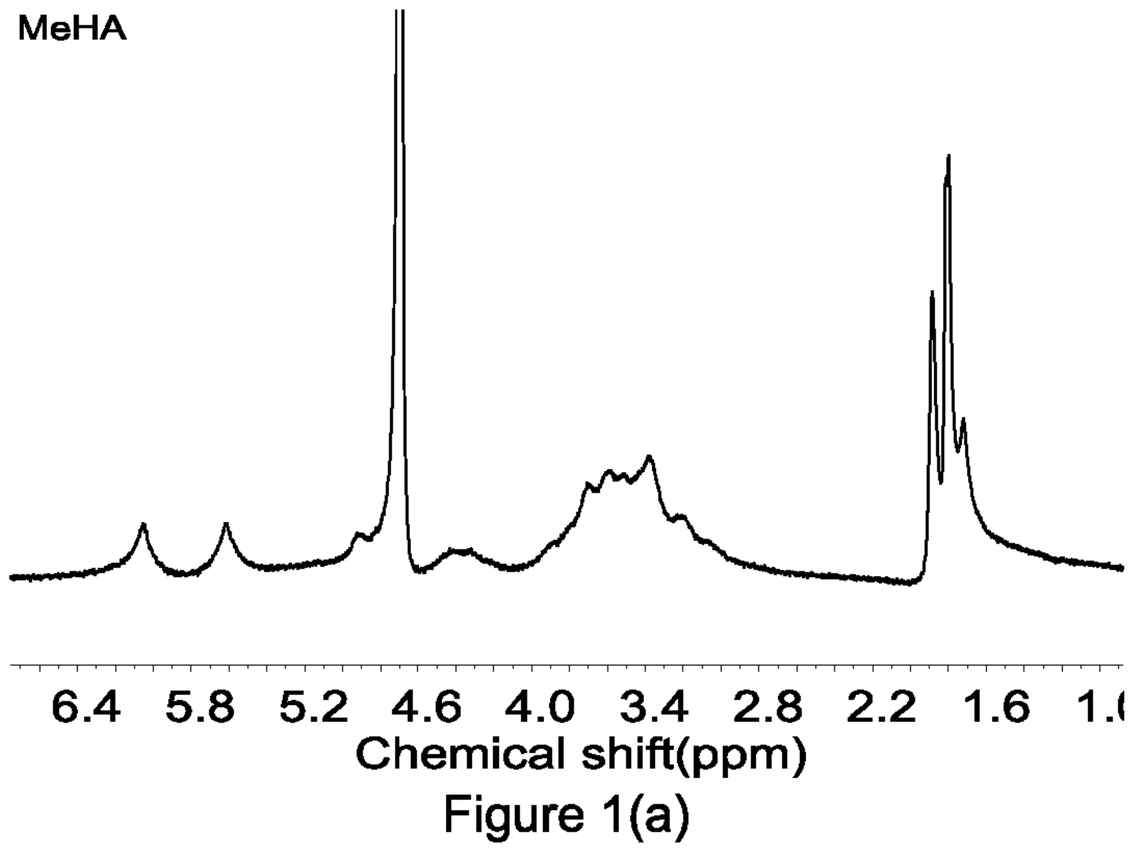


Figure 1(a)

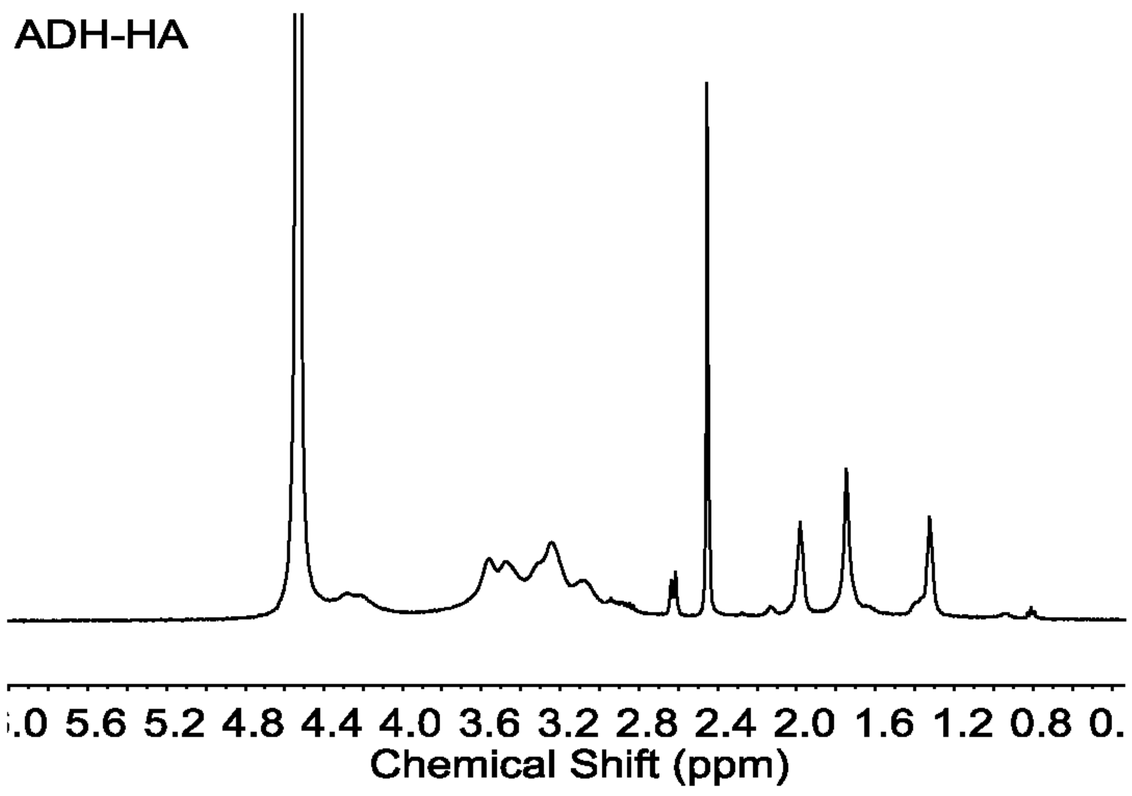


Figure 1(b)

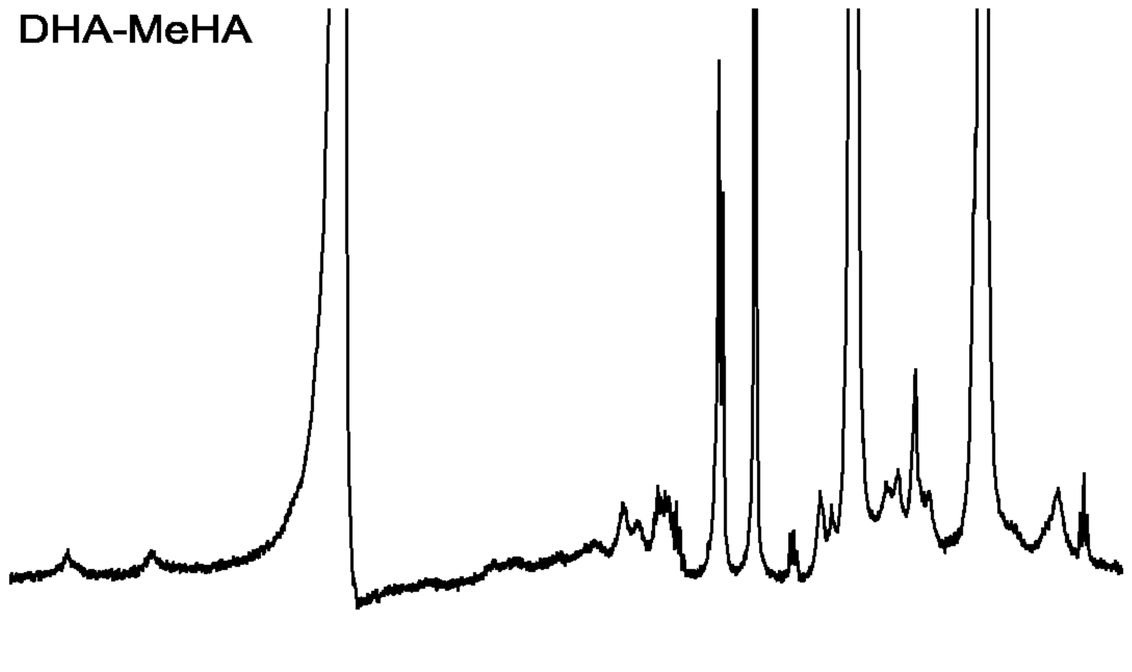


Figure 1(c)

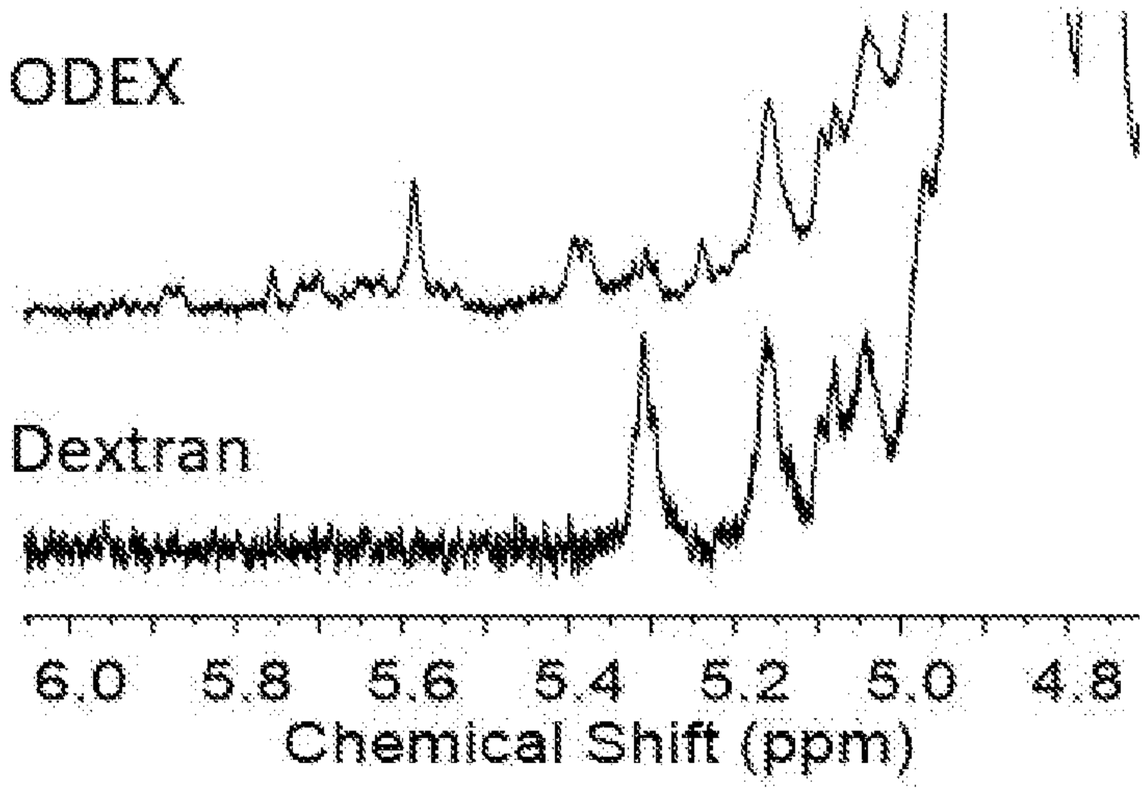


Figure 1(d)

Figure 1

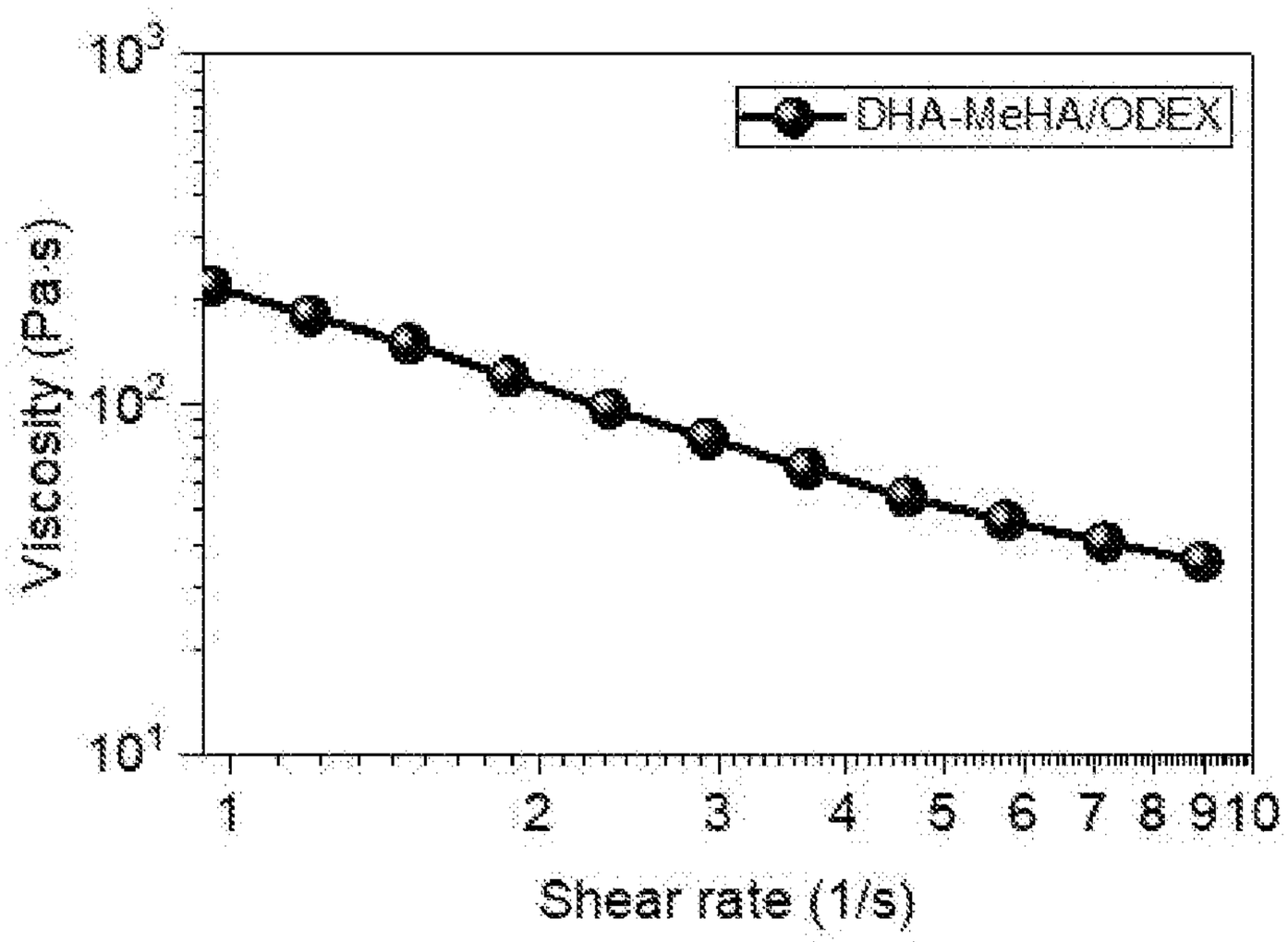


Figure 2 (a)

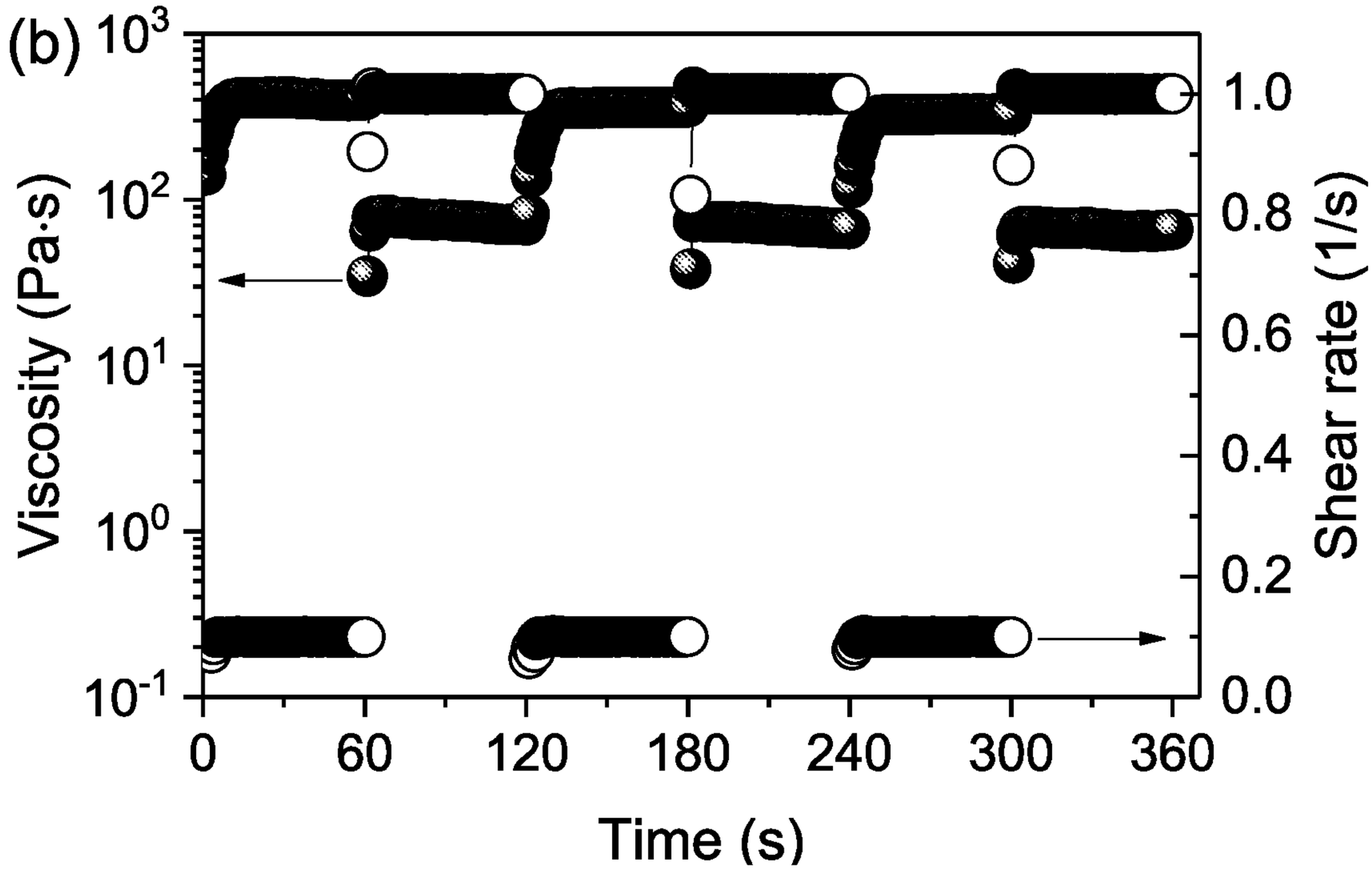


Figure 2 (b)

Figure 2

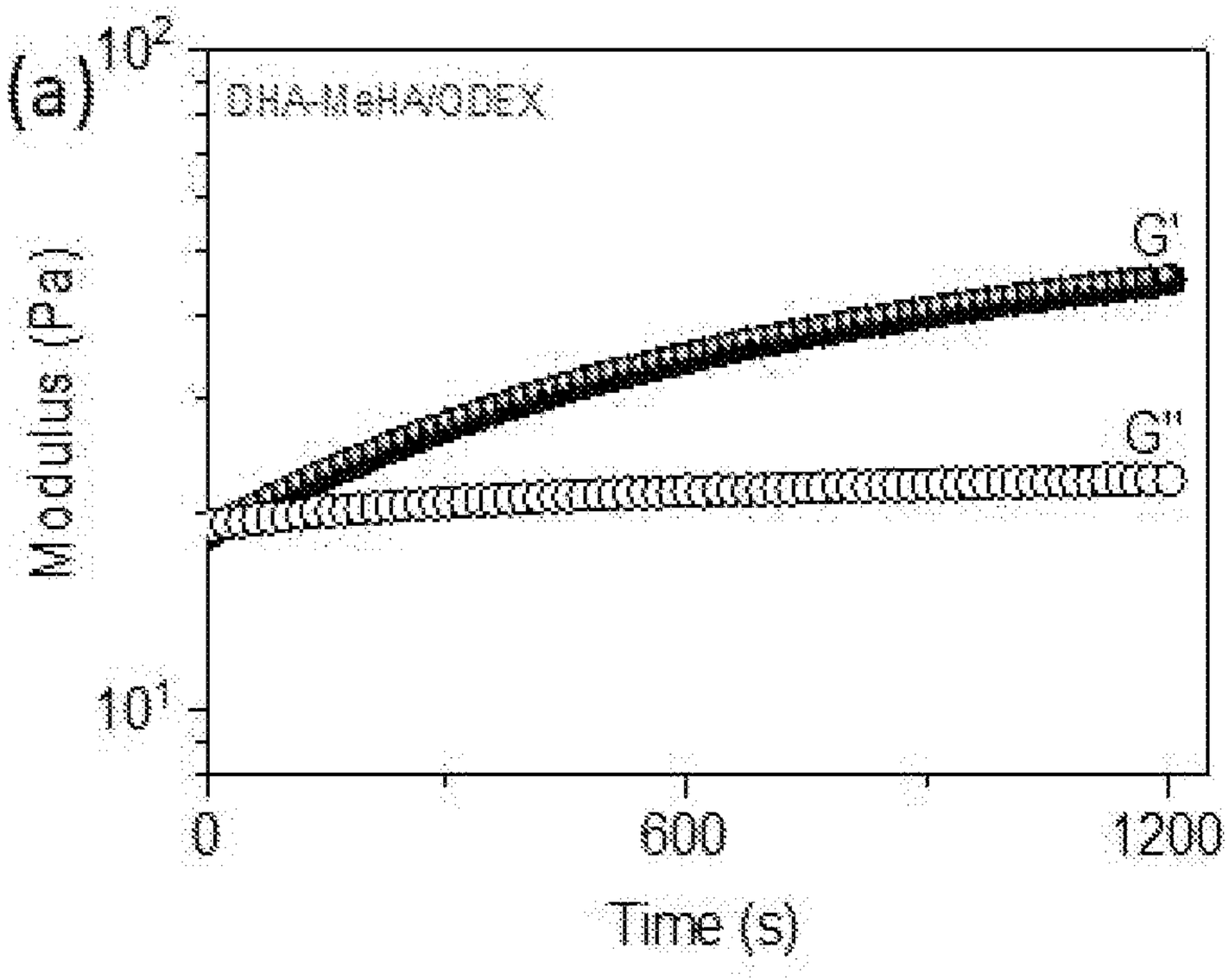


Figure 3 (a)

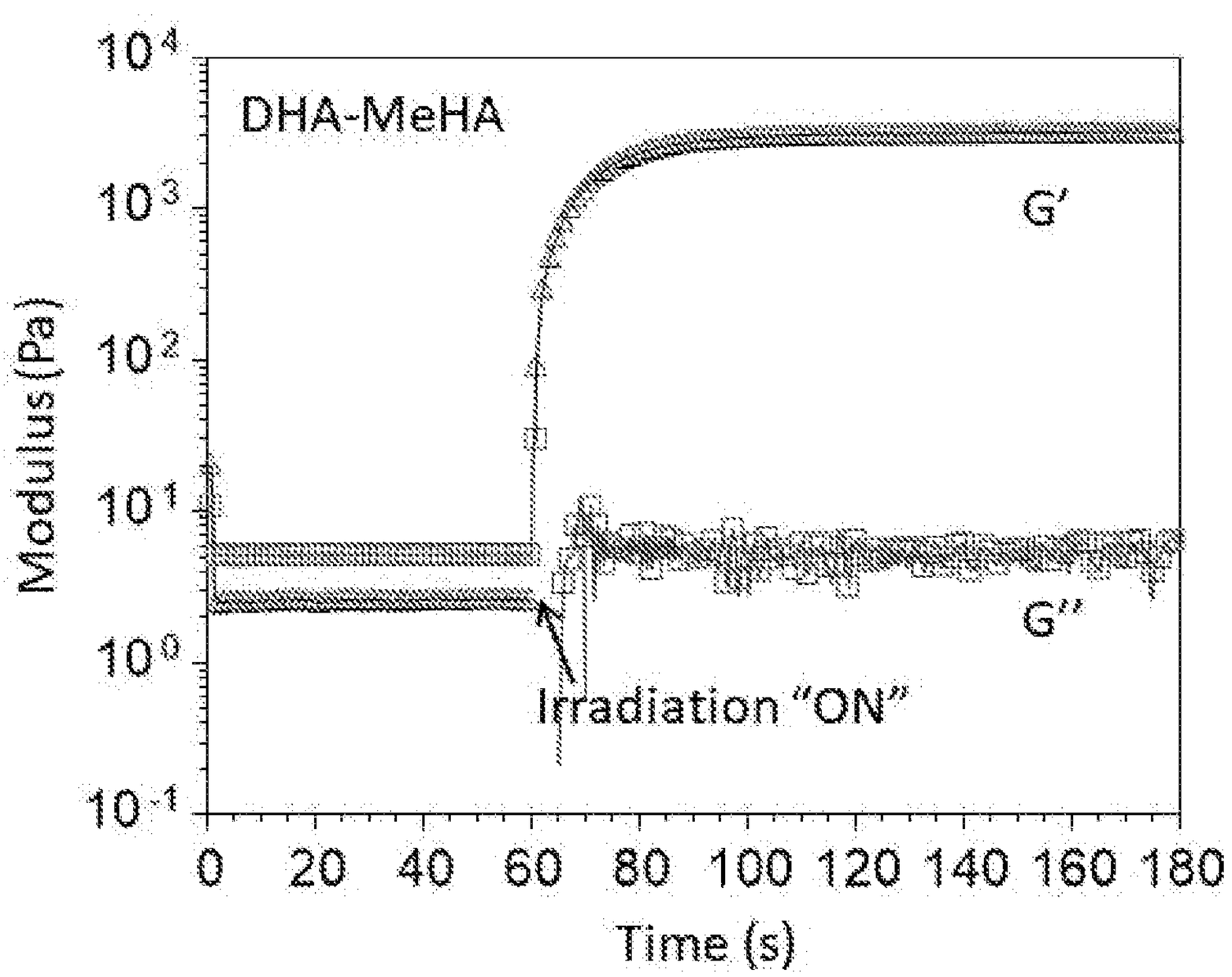


Figure 3 (b)

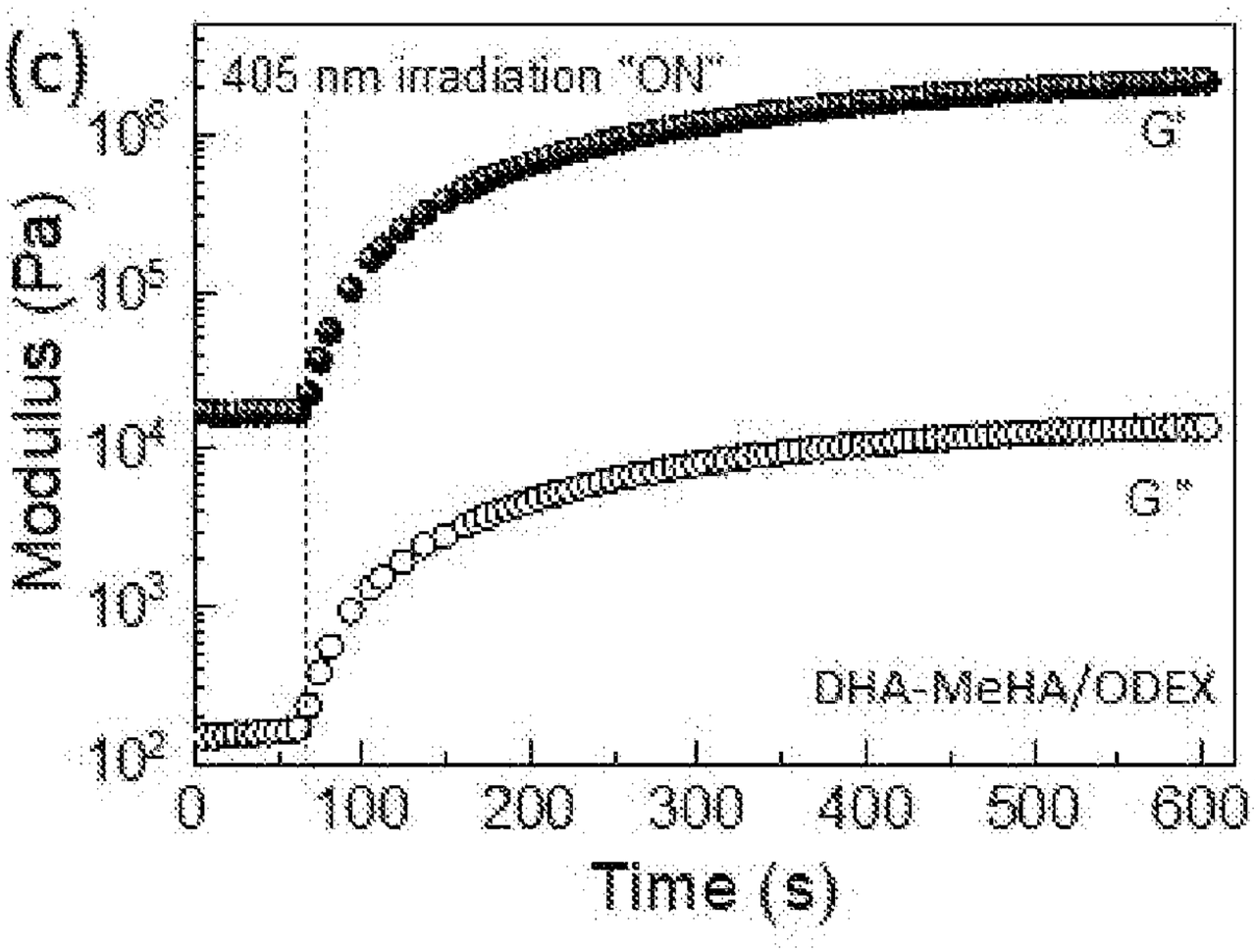


Figure 3 (c)

Figure 3

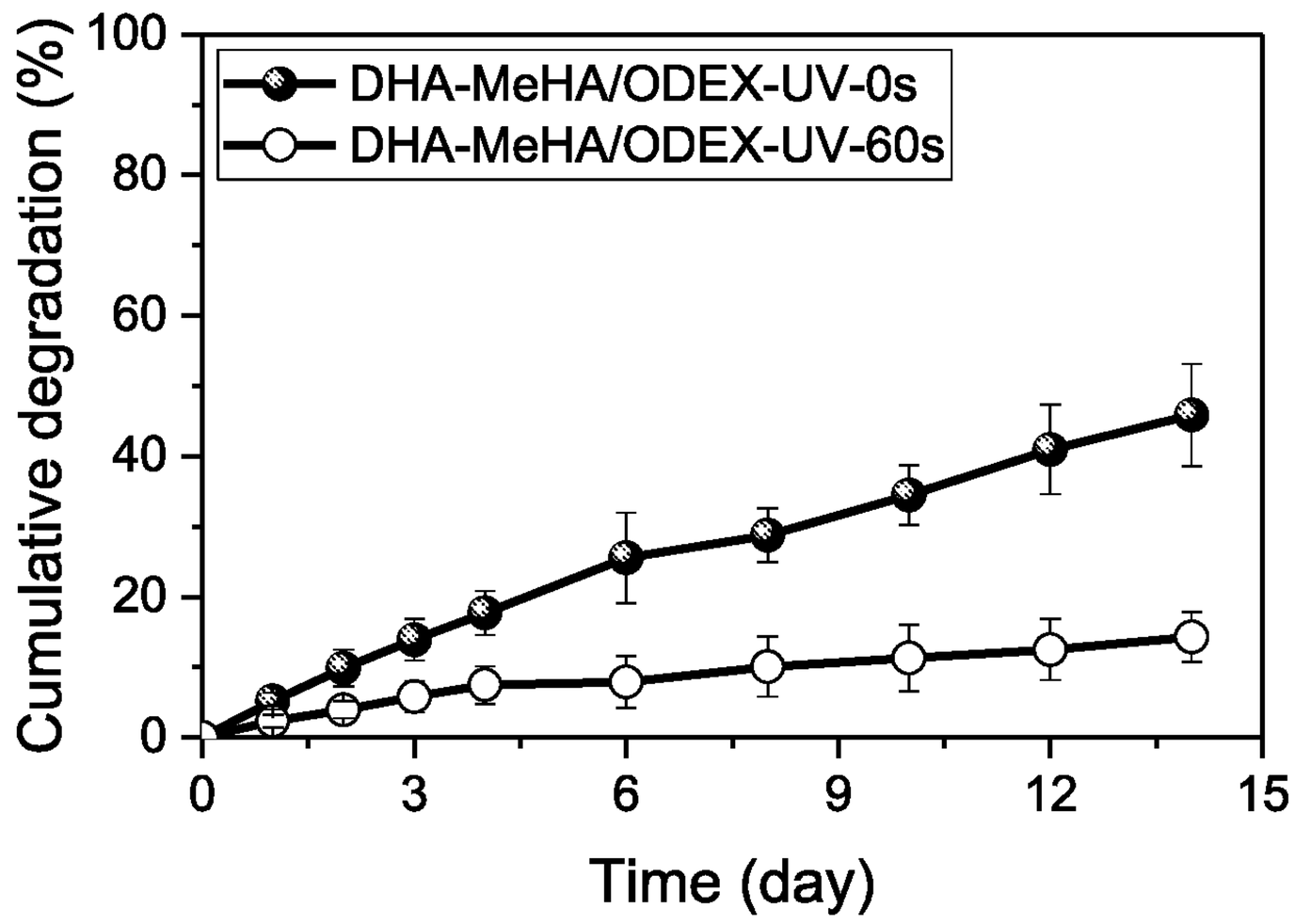


Figure 4

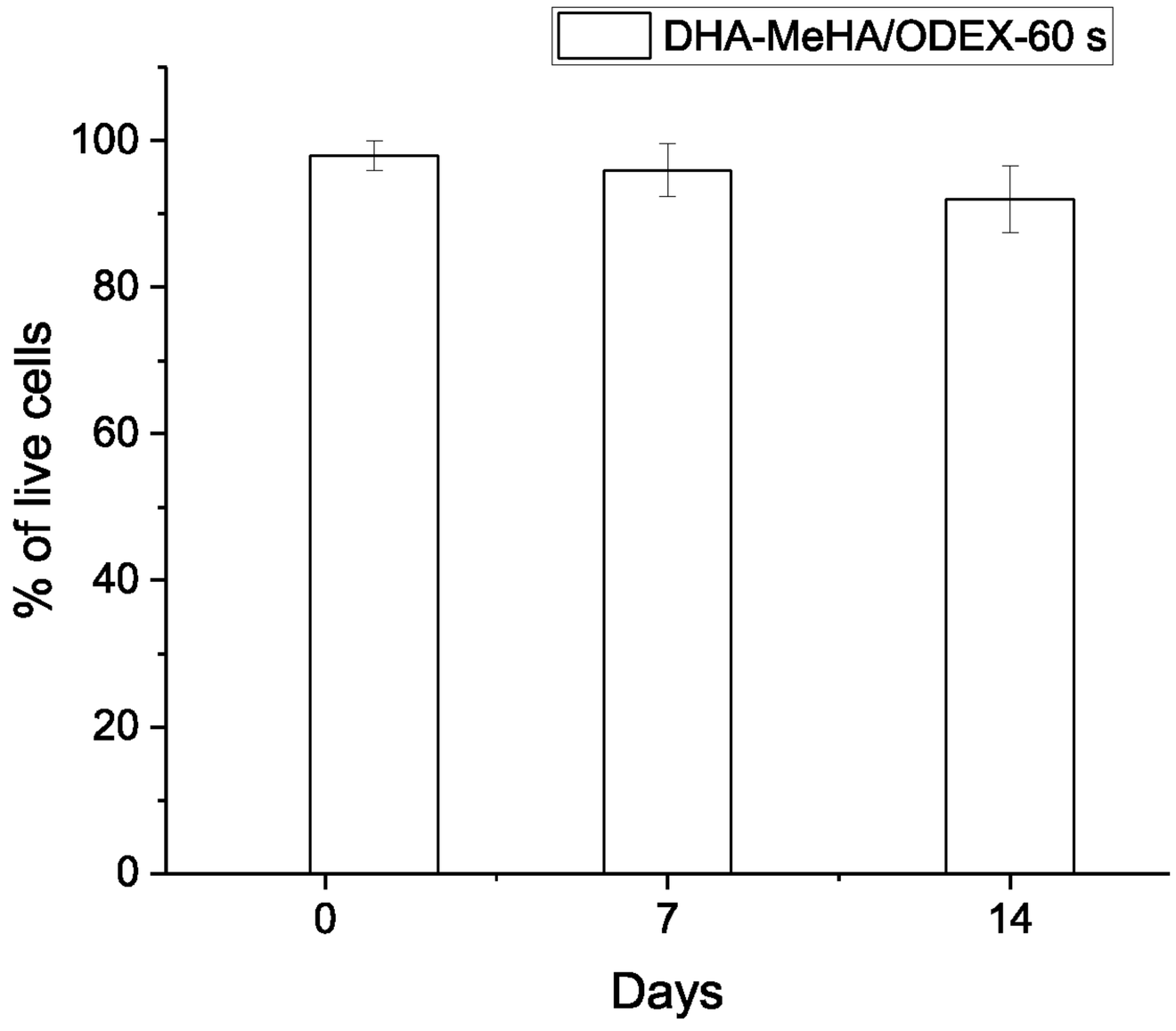


Figure 5

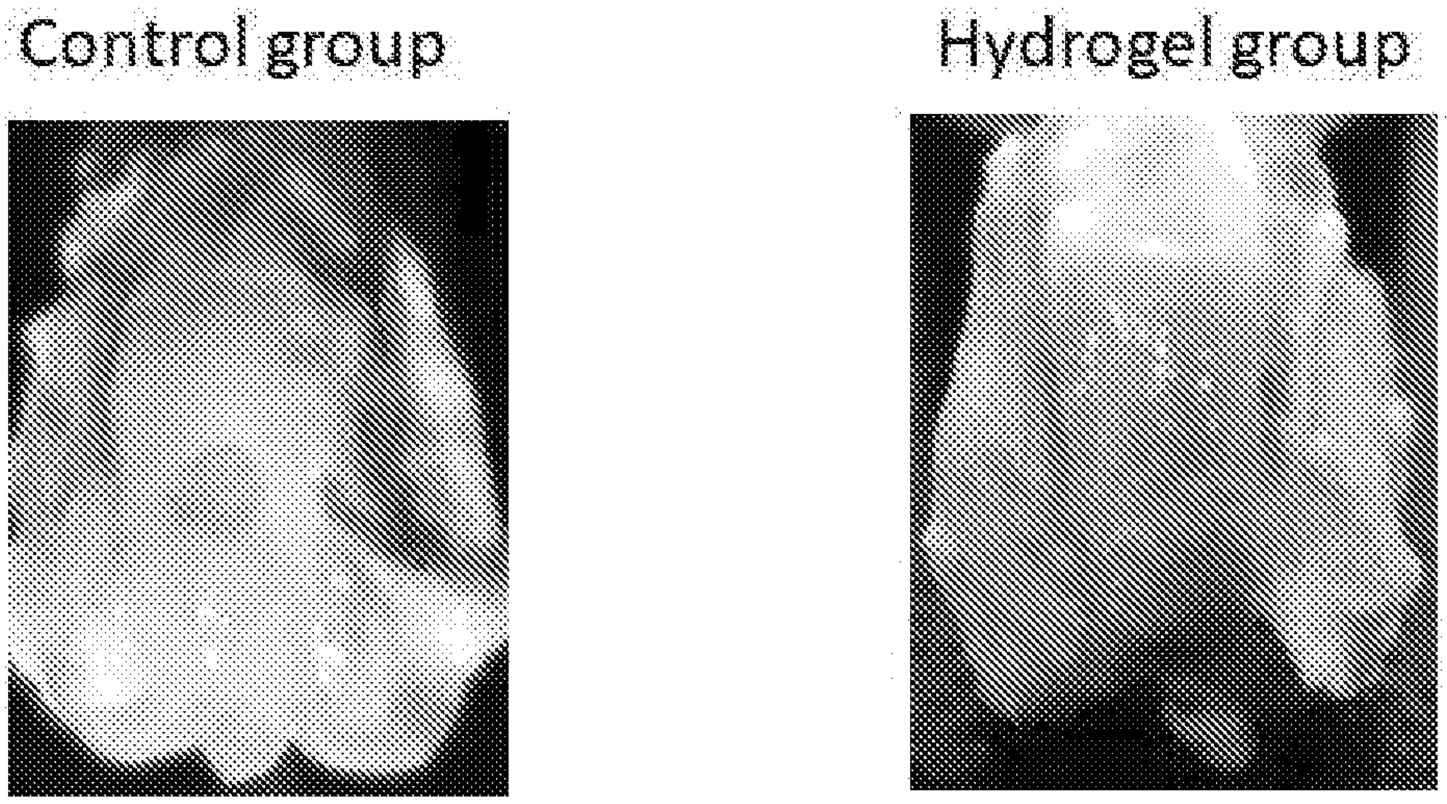


Figure 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2019/127320

A. CLASSIFICATION OF SUBJECT MATTER

C08J 3/075(2006.01)i; C08L 5/08(2006.01)i; A61L 27/20(2006.01)i

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C08J; C08L; A61L

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)

CNKI,CNABS,DWPI,EPODOC,CA: dual, crosslink+, hydrogel, glycosaminoglycan, hyaluronic acid,chondroitin sulfate, dermatan sulfate, heparan sulfate, heparin, +dihydrazide, dextran

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CN 104910396 A (UNIV. XI AN JIAOTONG) 16 September 2015 (2015-09-16) abstract, examples 1-7	1-11
A	CN 104910392 A (UNIV. XI AN JIAOTONG) 16 September 2015 (2015-09-16) abstract, examples 1-7	1-11
A	WO 2019210496 A1 (SHANGHAI QISHENG BIOLOGICAL PREPARATION CO. LTD.) 07 November 2019 (2019-11-07) claims 1-37	1-11
A	US 2017065746 A1 (UNIV. ARIZONA STATE) 09 March 2017 (2017-03-09) paragraphs 【0068】 - 【0071】	1-11
A	CN 101296709 A (EVONIK DEGUSSA GMBH) 29 October 2008 (2008-10-29) claims 1-16	1-11
A	CN 110240712 A (UNIV. DALIAN TECHNOLOGY) 17 September 2019 (2019-09-17) claims 1-11	1-11

☐ 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
"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	Date of mailing of the international search report
11 September 2020	**24 September 2020**
Name and mailing address of the ISA/CN **National Intellectual Property Administration, PRC** **6, Xitucheng Rd., Jimen Bridge, Haidian District, Beijing 100088** **China** Facsimile No. **(86-10)62019451**	Authorized officer **LIU,Yuxiong** Telephone No. **86-(10)-53962216**

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN2019/127320

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
CN	104910396	A	16 September 2015	CN	104910396	B	20 October 2017
CN	104910392	A	16 September 2015	CN	104910392	B	15 December 2017
WO	2019210496	A1	07 November 2019	None			
US	2017065746	A1	09 March 2017	US	10265439	B2	23 April 2019
CN	101296709	A	29 October 2008	AU	2006308083	B2	19 July 2012
				US	9480654	B2	01 November 2016
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				US	8313778	B2	20 November 2012
				IL	190697	D0	03 November 2008
				AT	526040	T	15 October 2011
				SI	EP1951314	T1	29 February 2012
				BR	PI0617775	B1	22 October 2019
				PL	1951314	T3	29 February 2012
				AU	2006308083	A1	03 May 2007
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				IL	190697	A	30 April 2015
				US	2009011038	A1	08 January 2009
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				HK	1198429	A1	24 April 2015
				JP	2009512722	A	26 March 2009
				CA	2625856	C	28 January 2014
CN	110240712	A	17 September 2019	None			