As used herein, the term “pH-adjusting agent” refers to a chemical compound that is used to shift the pH of the droplets within an emulsion, such as within the emulsion of step (a) and to initiate and/or accelerate the crosslinking reaction between the first and second functionalized HA.

As used herein, the term “blocking reagent” refers to a chemical compound that is used to cap unreacted functional groups, such as -FG_1 or -FG_2.

As used herein, the term “flow system” or “continuous flow system” refers to a system where a process, such as precipitation of a polymer, is run in a continuously flowing stream rather than in a batch production.

As used herein, the term “a setup for precipitating and isolating a polymer” refers to an arrangement or equipment comprising a flow system that is connected to a collecting assembly.

As used herein, the term “anti-solvent” refers to a solvent in which a polymer, such as a functionalized HA, is insoluble. The term “insoluble” with reference to a polymer means that less than one gram of said polymer can be dissolved in one liter of said solvent at room temperature (room temperature may range from 17 °C to 30 °C, such as from 17 °C to 25 °C) to form a homogenous solution.

As used herein “a screen scroll centrifuge” refers to a filtering centrifuge which separates solids and liquid from a solid-liquid mixture. In a typical screen scroll centrifuge, the basic principle is that the entering feed is separated into liquid and solids as two products.

In general, the term “comprise(s)” or “comprising” also encompasses “consist(s) of” or “consisting of”.

The present invention relates to a method for preparing hydrogel microspheres or pharmaceutically acceptable salts thereof comprising a crosslinked hyaluronic acid (HA), wherein the method comprises the steps of:

(a) mixing a solution A with a solution B to form an emulsion, wherein solution A comprises a first functionalized HA that is modified with one or more -FG_1 and optionally further functional groups and a second functionalized HA that is modified with one or more -FG_2 and optionally further functional groups, wherein -FG_1 and -FG_2 are functional group moieties that are different from each other and wherein -FG_1 on the first functionalized HA reacts with -FG_2 on the second functionalized HA to form a plurality of crosslinks which results in the formation of hydrogel HA microspheres;
(b) optionally, adding a pH-adjusting agent to the emulsion of step (a); and
(c) collecting the obtained hydrogel HA microspheres of step (a) or (b).

It will be recognized by the person skilled in the art that the method of the present invention for preparing hydrogel microspheres comprising a crosslinked HA or pharmaceutically acceptable salt thereof may be applied for the preparation of any hydrogel microspheres comprising any crosslinked glycosaminoglycan. In other words, the method of the present invention may equally be applied for preparing hydrogel microspheres comprising a crosslinked heparin, heparan sulfate, heparosan, chondroitin sulfate, dermatan sulfate or keratan sulfate. In particular, said method may be applied for preparing hydrogel microspheres comprising a crosslinked heparosan.

The polymerization within the method of the present invention occurs via suspension polymerization. In step (a) solutions A and B form an emulsion and after a sufficient mixing time, solution A becomes a dispersed phase, while solution B becomes a continuous phase. The dispersed phase should not be miscible with the continuous phase. Also, in step (a), both the functionalized HA are predominantly or exclusively linear HA strands.

In step (a) the mixing of solutions A and B which form the emulsion may require vigorous agitation, pressure or other forces that may be achieved by stirring, such as by stirring with a pitched blade stirrer in combination with a baffle; shaking, such as by shaking in a vessel, such as in a falcon tube (closed tube); by using a rotor or stator; by using an ultrasound device; by using a static mixer, such as by using a packed bead column or flow plate; by using a membrane with defined pores, such as by using a cross-flow membrane emulsification technique in which the liquid form of the material to be formed into microparticles is pushed through a membrane

comprising micro-scale pores into a flowing solution of the dispersed phase or stirred cell membrane emulsification; by using a tubular membrane having a surface made from a hydrophobic plastic, such as the one disclosed in WO 2022/198052 A2 which is herewith incorporated by reference in its entirety; by using a microfluidic droplet generator; or by spray polymerization in the air, such as by using an ultrasonic atomizer. Suitably, the mixing in step (a) is achieved by stirring, such as by stirring with a pitched blade stirrer in combination with a baffle or by using a microfluidic droplet generator.

In certain embodiments, the method of the present invention, comprises the steps of:

(a) mixing a solution A with a solution B to form an emulsion, wherein solution A comprises a first functionalized HA that is modified with one or more -FG_1 and optionally further functional groups and a second functionalized HA that is modified with one or more -FG_2 and optionally further functional groups, wherein -FG_1 and -FG_2 are functional group moieties that are different from each other and wherein -FG_1 on the first functionalized HA reacts with -FG_2 on the second functionalized HA to form a plurality of crosslinks which results in the formation of hydrogel HA microspheres;
(b) adding a pH-adjusting agent to the emulsion of step (a); and
(c) collecting the obtained hydrogel HA microspheres of step (a) or (b).

In certain embodiments, all -FG_1 are the same and all -FG_2 are the same.

Suitably, the first and second functionalized HA of the method of the present invention are not optionally modified with further functional groups.

The method of the present invention optionally further comprises the step of size fractionating the obtained hydrogel microspheres of step (c) to obtain microspheres with a particular particle size distribution.

Also, it will be recognized by the person skilled in the art that throughout the specification there could be optional washing or purification steps in between any of the steps of the method of the present invention.

In certain embodiments, the method of the present invention comprises the steps of:

(a) mixing a solution A with a solution B to form an emulsion, wherein solution A comprises a first functionalized HA that is modified with one or more -FG_1 and optionally further functional groups and a second functionalized HA that is modified with one or more -FG_2 and optionally further functional groups, wherein -FG_1 and -FG_2 are functional group moieties that are different from each other and wherein -FG_1 on the first functionalized HA reacts with -FG_2 on the second functionalized HA to form a plurality of crosslinks which results in the formation of hydrogel HA microspheres;

(b) optionally, adding a pH-adjusting agent to the emulsion of step (a);

(c) collecting the obtained hydrogel HA microspheres of step (a) or (b);

(d) optionally, size fractionating the obtained hydrogel HA microspheres of step (a), (b) or (c) to obtain microspheres with a particular particle size distribution;

(e) optionally, washing the microspheres obtained in step (a), (b), (c) or (d);

(f) optionally, incubating the hydrogel HA microspheres of step (a), (b), (c), (d) or (e) in a buffering agent of a pH ranging from about 7 to about 14, such as from about 8 to about 12 or such as from about 8.5 to about 10;

(g) optionally, incubating the hydrogel HA microspheres of step (a), (b), (c), (d), (e) or (f) with a reducing agent;

(h) optionally, washing the microspheres obtained in step (f) or (g); and

(i) optionally, collecting the hydrogel HA microspheres of step (d), (e), (f), (g) or (h).

It is understood that step (d) may also be performed after steps (e), (f), (g) or (h).

In certain embodiments, the molecular weight of the first and second functionalized HA independently ranges from about 80 kDa to about 250 kDa, such as from about 90 kDa to about 200 kDa or such as from about 100 kDa to about 150 kDa. Suitably, the molecular weight of the first and second functionalized HA ranges from 100 kDa to 150 kDa. It is understood that said HA may be polydisperse and comprise polymer chains of unequal length and so the molecular weight is not a single value, i.e. said HA exists as a distribution of chain lengths and molecular weights. For example, if the molecular weight of the first or second functionalized HA is 125 kDa, said HA will comprise polymeric HA strands ranging from about 30 kDa to about 400 kDa.

In certain embodiments, in step (d) the obtained microspheres have a diameter ranging from about 1 μm to about 1000 μm, such as from about 50 μm to about 900 μm, such as from about 100 μm to about 700 μm, such as from about 200 μm to about 500 μm or such as from about 50 μm to about 500 μm, as determined by flow microscopy or other similar methods known in the art. In certain embodiments, in step (d) the obtained microspheres have a diameter ranging from 1 μm to 1000 μm, such as from 50 μm to 900 μm, such as from 100 μm to 700 μm, such as from 200 μm to 500 μm or such as from 50 μm to 500 μm, as determined by flow microscopy. In certain embodiments, in step (d) the obtained microspheres have a diameter ranging from 50 μm to 500 μm, as determined by flow microscopy. In certain embodiments, in step (d) the obtained microspheres have a diameter ranging from 100 μm to 200 μm, as determined by flow microscopy.

Suitably, in step (d) the obtained microspheres have a d_{10} value of ≥50 μm and a d_{90} value of ≤900 μm, such as a d_{10} value of ≥100 μm and a d_{90} value of ≤700 μm or a d_{10} value of ≥200 μm and a d_{90} value of ≤500 μm, as determined by flow microscopy. In certain embodiments, in step (d) the obtained microspheres have a d_{10} value of ≥100 μm and a d_{90} value of ≤200 μm. Preferably, for these measurements said microspheres are in succinate buffer.

As used herein, the parameter d_{10} value signifies the point in the size distribution, below which 10% of the total volume of material in the sample is contained. Similarly, the d_{90} value is the size below which 90% of the volume of the material is contained. It is understood that the swelling of the HA microspheres may be influenced by the buffering agent in which the microspheres are stored during the measurement and/or pH, osmolality and ionic strength, and accordingly this may have an impact on the d_{10} and values d_{90}.

In certain embodiments, in step (f) the buffering agent has a pH ranging from 8 to 12. In certain embodiments, in step (f) the buffering agent has a pH ranging from 8.5 to 10. In certain embodiments, in step (f) the buffering agent has a pH of about 9. In certain embodiments, in step (f) the buffering agent has a pH of 9.

In certain embodiments, the method of the present invention comprises the steps of:

(a) mixing a solution A with a solution B to form an emulsion, wherein solution A comprises a first functionalized HA that is modified with one or more -FG_1 and optionally further functional groups and a second functionalized HA that is modified with one or more -FG_2 and optionally further functional groups, wherein -FG_1 and -FG_2 are functional group moieties that are different from each other and wherein -FG_1 on the first functionalized HA reacts with -FG_2 on the second functionalized HA to form a plurality of crosslinks which results in the formation of hydrogel HA microspheres;
(b) adding a pH-adjusting agent to the emulsion of step (a);
(c) collecting the obtained hydrogel HA microspheres of step (b);
(d) size fractionating the obtained hydrogel HA microspheres of step (c) to obtain microspheres with a particular particle size distribution;
(e) optionally, washing the microspheres obtained in step (c) or (d);
(f) incubating the hydrogel HA microspheres of step (c), (d) or (e) in a buffering agent of a pH ranging from about 7 to about 14, such as about 8 to about 12, or such as about 8.5 to 10;
(g) incubating the hydrogel HA microspheres of step (c), (d), (e) or (f) with a reducing agent;
(h) washing the microspheres obtained in step (f) or (g); and
(i) collecting the hydrogel HA microspheres of step (d), (e), (f), (g) or (h).

The present invention also relates to hydrogel HA microspheres or pharmaceutically acceptable salts thereof obtainable or obtained by any of the methods of the present invention.

It was surprisingly found that the method of the present invention allows for the use of linear functionalized HA strands with low degree of substitution in the synthesis of hydrogel HA microspheres that are effectively crosslinked. It was also observed that owing to the size of the microspheres, the injectability of the HA microspheres and drug conjugates comprising hydrogel HA microspheres was significantly improved over the injectability of a coherent gel, such as of the coherent gel disclosed in WO 2018/175788 A1. Another advantage of using HA hydrogel microspheres as carriers for drug conjugates is that prior to the administration, the microspheres can be washed, and thus soluble by-products can be easily removed. On the other hand, the coherent gel disclosed in WO 2018/175788 A1 cannot undergo washing steps, because said gel is obtained via polymerization in a syringe and thereafter it is directly injected into the eye.

Furthermore, the coherent gel disclosed in WO 2018/175788 A1 is prone to hardening while being filled in the injection syringe making the process of filling the syringe more challenging. In contrast, the HA hydrogel microspheres of the present invention can be stored after synthesis and then easily filled or further conjugated to drug moieties in the final container at a later time point.

Suitably, solution A of step (a) comprises the first and second functionalized HA and a solvent in which said functionalized HA can be dissolved.

In certain embodiments, solution A of step (a) comprises the first and second functionalized HA and dimethyl sulfoxide, DMF, DMA or a mixture thereof. In certain embodiments, solution A of step (a) comprises the first and second functionalized HA and dimethyl sulfoxide.

In certain embodiments, solution A of step (a) comprises the first and second functionalized HA, dimethyl sulfoxide and water. In certain embodiments, solution A of step (a) comprises the first and second functionalized HA, dimethyl sulfoxide and a buffering agent.

In certain embodiments, solution A of step (a) is an aqueous solution and comprises the first and second functionalized HA and a buffering agent.

It is clear to the person skilled in the art that in general the phrase “a buffering agent” may refer to one buffering agent or to a mixture of two or more buffering agents.

Exemplary buffering agents may be selected from the group consisting of N-(2-acetamido)-2-aminoethanesulfonic acid (ACES), acetate, 2,2′,2″-nitrilotriacetic acid (ADA), adipate, alanine, ammonium, 2-amino-2-methyl-1-propanol (AMP), 2-amino-2-methyl-1,3-propanediol (AMPD), N-(1,1-dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid (AMPSO), arginine, ascorbate, aspartate, benzoate, N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), bicarbonate, N,N-bis(2-hydroxyethyl)glycine, bis-(2-hydroxy-ethyl)-amino-tris(hydroxymethyl)-methane, 1,3-bis(tris(hydroxymethyl)methylamino)propane, borate, 4-(cyclohexylamino)butane-1-sulfonic acid (CABS), N-cyclohexyl-3-aminopropanesulfonic acid (CAPS), 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (CAPSO), carbonate, N-cyclohexyl-2-

aminoethanesulfonic acid (CHES), citrate, diethanolamine, 3-(N,N-bis[2-hydroxyethyl]amino)-2-hydroxypropanesulfonic acid (DIPSO), edetate, ethanolamine, ethylenediamine, formate, fumarate, gluconate, glutamate, glycine, glycylglycine, guanidine, N-(2-hydroxyethyl)piperazine-N'-(4-butanesulfonic acid) (HEPBS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 3-[4-(2-hydroxyethyl)piperazin-1-yl]propane-1-sulfonic acid (HEPPS), N-(2-hydroxyethyl)piperazine-N'-(propanesulphonic acid) (HEPPSO), histidine, hydrazine, imidazole, lactate, lysine, malate, maleate, 2-(N-morpholino)ethanesulfonic acid (MES), metaphosphate, methylamine, 4-(4-morpholinyl)butanesulfonic acid (MOBS), 3-(N-morpholino)propanesulfonic acid (MOPS), 2-hydroxy-3-morpholinopropanesulfonic acid (MOPSO), pentetate, phosphate, piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES), piperazine, piperidine, piperazine-N,N′-bis(2-hydroxypropanesulfonic acid) (POPSO), propionate, pyridine, pyrophosphate, pyruvate, sorbate, succinate, N-tris(hydroxymethyl)methyl-4-aminobutanesulfonic acid (TABS), ([tris(hydroxymethyl)methylamino]propanesulfonic acid (TAPS), 2-hydroxy-3-[tris(hydroxymethyl)methylamino]-1-propanesulfonic acid (TAPSO), tartrate, taurine, 2-{[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino}ethane-1-sulfonic acid (TES), tricine, triethanolamine, tromethamine and α-ketoglutarate.

It is clear to the person skilled in the art that the corresponding conjugate acids, bases or salts of the buffering agents and mixtures thereof are also included.

In certain embodiments, the aqueous solution comprises the first and second functionalized HA and a buffering agent, such as a buffering agent selected from the group consisting of citrate and histidine or a mixture thereof. In certain embodiments, the buffering agent comprises a mixture of citrate and histidine. In certain embodiments, the buffering agent consists of a mixture of citrate and histidine.

As defined herein, the term "histidine" is intended to encompass both D-histidine and L-histidine and mixtures thereof. In certain embodiments, the term "histidine" refers to L-histidine. In certain embodiments, the term "histidine" refers to D-histidine. In certain embodiments, the term "histidine" refers to a mixture of L-histidine and D-histidine.
In certain embodiments, the buffering agent is L-histidine.

In certain embodiments, the buffering agent consists of a mixture of citrate, L-histidine and sodium chloride.

In certain embodiments, the aqueous solution comprises the first and second functionalized HA, citrate and sodium chloride.

In certain embodiments, the aqueous solution consists of the first and second functionalized HA, citrate and sodium chloride. In certain embodiments, the aqueous solution comprises the first and second functionalized HA, histidine and sodium chloride.

In certain embodiments, the aqueous solution comprises the first and second functionalized HA and an emulsifying agent, while solution B comprises a solvent.

The buffering agent may be added in general in an amount of about 0.01 mM to about 500 mM. In certain embodiments, the buffering agent has a concentration ranging from about 0.5 mM to about 350 mM. In certain embodiments, the buffering agent has a concentration ranging from about 1 mM to about 250 mM. In certain embodiments, the buffering agent has a concentration ranging from about 5 mM to 100 mM. In certain embodiments, the buffering agent has a concentration of about 100 mM. In certain embodiments, the buffering agent has a concentration of 100 mM. In certain embodiments, the buffering agent has a concentration of about 5 mM. In certain embodiments, the buffering agent has a concentration of 5 mM.

Suitably, solution B of step (a) comprises an emulsifying agent and a solvent.

It is clear to the person skilled in the art that in general the phrase “a solvent” may refer to one solvent or to a mixture of two or more solvents and that the phrase “an emulsifying agent” may refer to one emulsifying agent or to a mixture of two or more emulsifying agents.

Exemplary emulsifying agents may be selected from the group consisting of sorbitan esters such as sorbitan monolaurate (Span® 20), sorbitan monooleate (Span® 80), sorbitan monopalmitate (Span® 40), sorbitan monostearate (Span® 60), sorbitan sequioleate (Span® 83), sorbitan trioleate