



(51) International Patent Classification:

A61L 27/16 (2006.01) A61L 27/24 (2006.01)

A61L 27/18 (2006.01) B33Y 70/00 (2020.01)

A61L 27/22 (2006.01) B33Y 80/00 (2015.01)

(21) International Application Number:

PCT/US2022/072084

(22) International Filing Date:

03 May 2022 (03.05.2022)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

63/183,423 03 May 2021 (03.05.2021) US

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

(54) Title: FACILE CURABLE BIOINK FORMULATIONS

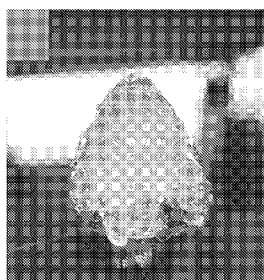


FIG. 1A

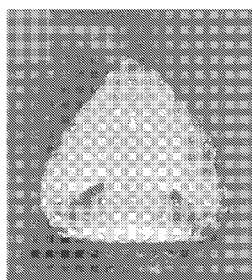


FIG. 1B

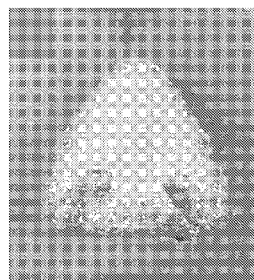


FIG. 1C

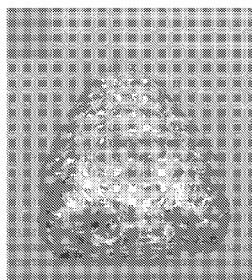


FIG. 1D

FIG. 1

(57) **Abstract:** Bioink formulations including an ionically crosslinkable polymer; the ionically crosslinkable polymer includes a plurality of ionically crosslinkable groups, preferably, anionic functional groups. Also provided are bioink formulation kits including the ionically crosslinkable polymers and a cationic crosslinker, wherein the crosslinker, when contacted with the bioink formulation, reacts with the ionically crosslinkable functional group of the ionically crosslinkable polymer to form a polymer network structure.



Published:

— *with international search report (Art. 21(3))*

FACILE CURABLE BIOINK FORMULATIONS

Cross-Reference to Related Applications

[0001] The present application claims the benefit of priority of U.S. provisional patent application no. 63/183,423, filed on May 3, 2021, the entire content of which is incorporated herein in its entirety.

Background

[0002] A major goal in the tissue engineering is the design of scaffold for the formation of new viable tissues. Scaffolds are generally fabricated using materials of natural or synthetic origin and capable of recreating the in vivo microenvironment provided mainly by the extracellular matrix (ECM) in living organs. Thereby, scaffold structures should incorporate the appropriate biophysical, biomechanical and biochemical cues that guide cell proliferation, differentiation, maintenance and function.

[0003] Bioprinting is a scaffold fabrication technique where material composition, known as bioink, is deposited along with cells in layer-by-layer fashion to create spatially controlled three-dimensional tissue-like structure. Bioinks are bioprintable hydrogel materials normally derived from substances that provide an ECM-like environment to the living cells.

[0004] One important step in creating the functional scaffold is the crosslinking of bioprinted construct to preserve its 3D structure in the cell culture conditions. This is currently accomplished by using bioink compositions containing chemically or photochemically crosslinkable functional groups and employing a suitable crosslinking process during or after bioprinting. For photochemically crosslinking, i.e., crosslinking with light, the bioink composition requires inclusion of photoinitiators—additive molecules that are known to be cytotoxic and detrimental to cell health. Effective crosslinking by photoinitiation often requires exposure to high intensity light at harmful wavelengths, such as in the ultraviolet or infrared regions of the spectrum, which is also detrimental to living cells. Other methods of crosslinking include use of chemicals

which are cytotoxic such as formaldehyde, glutaraldehyde, and similar. Therefore, there is a need for bioink compositions which can be crosslinked using agents that are not toxic or otherwise detrimental to cells, as well as new processes that are conducive to high cell viability.

Summary

[0005] Provided are bioink formulations including an ionically crosslinkable polymer, wherein the ionically crosslinkable polymer includes a plurality of ionically crosslinkable groups, preferably, the ionically crosslinkable groups are anionic functional group. In some embodiments, the ionically crosslinkable polymer is a natural polymer, such as gelatin, collagen, hyaluronic acid, chitosan, alginate, cellulose, pectin, agarose, ulvan, silk, or fibrin, wherein the natural polymer has been modified to have anionic functional groups available for ionic crosslinking. In other embodiments, the ionically crosslinkable polymer is a synthetic polymer, such as poly(ethylene glycol) (PEG), polyvinyl alcohol (PVA), poly(acrylic acid) (PAA), poly(2-hydroxyethyl methacrylate) (PHEMA), poly(N-isopropylacrylamide) (PNIPAM), copolymers thereof, wherein the synthetic polymers include monomers having anionic functional groups, or which have otherwise been modified to have anionic functional groups available for ionic crosslinking.

[0006] In various embodiments, the ionically crosslinkable polymer has molecular weight in the range from 1000 Da to 100M Da. In a preferred embodiment, the ionically crosslinkable has a molecular weight in the range from 10,000 Da (10 kDa) to 10 MDa. In a more preferred embodiment, the ionically crosslinkable polymer has a molecular weight in the range from 20,000 Da (20 kDa) to 1.3 MDa.

[0007] In various embodiments, the bioink formulation includes the ionically crosslinkable polymer is in solid, hydrogel or solution form. In hydrogel or solution form, the bioink formulation includes from 1% (w/v) to 99% (w/v) of ionically crosslinkable polymer in water. In some embodiments, the bioink formulation includes from 2% (w/v) to 30% (w/v) of ionically crosslinkable polymer in water. In still other embodiments, the bioink formulation includes from 2% (w/v) to 20% (w/v) of ionically crosslinkable polymer in water.

[0008] Also provided are bioink formulation kits including a bioink formulation as described above and a crosslinker, wherein the crosslinker, when contacted with the bioink formulation, reacts with the ionically crosslinkable functional group of the ionically crosslinkable polymer to form a polymer network structure.

[0009] Preferably the crosslinker is selected from of Be^{2+} , Mg^{2+} , Ca^{2+} , Sr^{2+} , Ba^{2+} , Ra^{2+} , B^{3+} , Al^{3+} , Ga^{3+} and combinations thereof. In a preferred embodiment, the crosslinker is Ca^{2+} .

[0010] In some embodiments, the bioink formulation kit includes the crosslinker provided as a solid salt. In other embodiments, the bioink formulation kit includes the crosslinker as a solution, preferably an aqueous solution.

Brief Description of the Drawings

[0011] FIG. 1A shows a nose structure (~1.6 cm height) printed and crosslinked with Formulation 1, described below; FIG. 1B shows a nose structure printed and crosslinked with Formulation 2; FIG. 1C shows a nose structure printed and crosslinked with Formulation 3; and FIG. 1D shows a nose structure printed and crosslinked with Formulation 4.

[0012] FIG. 2A shows a structure printed using Formulation 3; FIG. 2B shows the same structure printed using Formulation 5; dye was added for visualization.

[0013] FIG. 3 shows the compressive moduli before and after treating with calcium chloride solution for Formulations 1 and 2.

[0014] FIG. 4 shows the shear moduli before and after treating with calcium chloride solution for Formulations 3, 4, and 5.

[0015] FIG. 5A is a graph showing the normalized fluorescence intensity of encapsulated hMSCs in Formulations 3 and 5 after 1, 7, and 14 days of culture. FIG. 5B is a graph showing the percent cell viability of encapsulated hMSCs in Formulations 3 and 5 after 1, 7, and 14 days of culture. FIG. 5C is a fluorescent image of printed constructs of encapsulated hMSCs in Formulations 3 and 5 after 1, 7, and 14 days of culture.

Detailed Description

[0016] As already noted, many currently available bioink formulations contain cytotoxic additives, such as thermal or photo-initiator molecules and crosslinkers and require crosslinking by exposure to harmful/high intensity light or by using cytotoxic chemicals. Provided herein are new bioink compositions and a facile crosslinking process for bioink compositions favorable for high cell viability. The bioink composition described herein contains chemically functionalized components derived from extracellular matrix (ECM) of natural tissues. The said chemical functional groups promote crosslinking of bioink constituents when mixed with non-cytotoxic ions such as calcium ions.

[0017] The bioink formulations provided herein are cell friendly, utilizing mild conditions conducive to high cell viability, and user friendly, as users are not required to handle any harmful light energy or harmful chemical agents such as aldehydes, such as formaldehyde or glutaraldehyde, for crosslinking.

[0018] The bioink formulations provided herein include one or more ionically crosslinkable polymers, that is, a polymer having one or more ionically crosslinkable groups, and more specifically, one or more anionic functional groups as the ionically crosslinkable group. Suitable anionic functional groups include monoanionic functional groups, dianionic functional groups and polyanionic functional groups. In some embodiments, the ionically crosslinkable polymer includes a plurality of identical anionic functional groups. In other embodiments, the ionically crosslinkable polymer includes a combination of two or more different anionic functional groups.

[0019] The term anionic functional group is understood to encompass functional groups that are anionic in aqueous solution, and thus encompass, for example, acids and their conjugate bases and salts of organic acids. Particularly suitable classes of anionic functional groups include carboxylic acids and their conjugate bases, salts of carboxylic acids, sulfonic acids and their conjugate bases and salts of sulfonic acids.

[0020] When the anionic functional group is a carboxylic acid, it may be an aliphatic carboxylic acid or salt thereof or an aromatic carboxylic acid or salt thereof. Suitable carboxylic acid groups include, but are not limited to acrylic acid, methacrylic acid, maleic acid, succinic acid, benzoic acid, naphthalic acid, polyacrylic acid,

polymethacrylic acid, polymaleic acid, alginic acid, guluronic acid and conjugate acids and salts of any of these.

[0021] When the anionic functional group is a sulfonic acid, it may be an alkyl sulfonic acid or salt thereof or an aromatic sulfonic acid or salt thereof. In some embodiments, the alkyl sulfonic acid is a C₁-C₅ alkyl sulfonic acid. Suitable sulfonic acid groups include but are not limited to methyl sulfonic acid, ethyl sulfonic acid and benzene sulfonic acid.

[0022] Particularly preferred anionic salt ionically crosslinkable functional groups include carboxylate salts, sulfonate salts, maleate salts, succinate salts, polyacrylate salts, polymethacrylate salts, polymaleate salts, alginate salts, guluronate salts and combinations thereof.

[0023] When the anionic functional group is the salt of a carboxylic acid or the salt of a sulfonic acid, it further includes a counterion. Suitable counterions include, but are not limited to Na⁺, Li⁺, K⁺, Cs⁺ and alkylammonium ions N⁺R₁R₂R₃R₄, wherein R₁ is selected from C₁-C₁₀ alkyl, C₁-C₆ aryl, and C₁-C₁₀ alkylaryl, and R₂, R₃, and R₄ are individually selected from H, C₁-C₁₀ alkyl, C₁-C₆ aryl, and C₁-C₁₀ alkylaryl. Particularly preferred alkylammonium ions include trialkylammonium salts, such as trimethylammonium salt, triethylammonium salt, and tributylammonium salt, or quaternary ammonium salts, such as tetramethyl ammonium salt, tetraethylammonium salt and tetrabutylammonium salt.

[0024] Some exemplary difunctional anionic groups include maleic acid, succinic acid, salts thereof and combinations thereof. Some exemplary polyionic polymers include but not limited to polyacrylic acid, polymethacrylic acid, salt of polymethacrylic acid, poly maleic acid, salts thereof, and combinations thereof.

[0025] In some embodiments, the ionically crosslinkable polymer is obtained by the polymerization of monomers wherein at least one monomer includes an ionically crosslinkable functional group that will be available for crosslinking. In other embodiments, the ionically crosslinkable polymer is obtained by chemically modifying the (already formed) polymer through the addition of an ionically crosslinkable functional group.

[0026] In some embodiments described herein, the bioink formulation includes an ionically crosslinkable natural polymer. Some particularly suitable natural polymers include gelatin, collagen, hyaluronic acid, chitosan, alginate, cellulose, pectin, agarose, ulvan, silk and fibrin, each having one or more ionically crosslinkable groups.

[0027] In other embodiments described herein, the bioink formulation includes an ionically crosslinkable synthetic polymer. Some exemplary synthetic polymers useful in the bioink formulations described herein include poly(ethylene glycol) (PEG), polyvinyl alcohol (PVA), poly(acrylic acid) (PAA), poly(2-hydroxyethyl methacrylate) (PHEMA), poly(N-isopropylacrylamide) (PNIPAM) and copolymers thereof, each having one or more ionically crosslinkable groups.

[0028] Some preferred ionically crosslinkable polymers for use in the bioink formulations and methods provided herein include carboxylic acid functionalized gelatin, sodium alginate, polyacrylic acid grafted gelatin (GelPAA), guluronic acid grafted gelatin, and combinations thereof.

[0029] In various embodiments of the bioink formulations described herein, the ionically crosslinkable polymer has a molecular weight in range from 1000 Da (1 kDa) to 100 MDa. In some preferred embodiments, the ionically crosslinkable polymer in the bioink formulation has a molecular weight in the range from 10,000 Da (10 kDa) to 10 MDa. In some particularly preferred embodiments, the ionically crosslinkable polymer in the bioink formulation has a molecular weight in the range from 20,000 Da (20 kDa) to 1.3 MDa.

[0030] In various embodiments, the bioink formulations described herein include an ionically crosslinkable polymer in solid form. In other embodiments, the ionically crosslinkable polymer is in hydrogel form. In still other embodiments, the ionically crosslinkable polymer is in solution form.

[0031] In embodiments in which the ionically crosslinkable polymer is in hydrogel form or solution form, the bioink formulation may contain from 0.1% (w/v) to 99% (w/v) of ionically crosslinkable polymer or combinations of polymers in water. In some embodiments, the ionically crosslinkable polymer is included in the bioink formulation in the range from 0.1% (w/v) to 60% (w/v). In other embodiments, the ionically crosslinkable polymer is included in the bioink formulation in the range from 1% (w/v)

to 60% (w/v). In other embodiments, the ionically crosslinkable polymer is included in the bioink formulation in the range from 1% (w/v) to 50% (w/v). In some preferred embodiments, the ionically crosslinkable polymer is included in the bioink formulation in the range from 1% (w/v) to 30% (w/v). In still preferred embodiments, the ionically crosslinkable polymer is included in the bioink formulation in the range from 2% (w/v) to 20% (w/v). Each of these ranges is meant to include and encompass smaller ranges within, for example, 0.1% (w/v) to 60% (w/v) also includes 0.2%, 0.3%, 0.4%, 0.5%, and so forth, as well as 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, etc., all (w/v).

[0032] In embodiments in which the bioink formulation includes more than one ionically crosslinkable polymer, the ionically crosslinkable polymers may be included in a 1:1 ratio or in another ratio, e.g., 10:1 to 1:300. In some embodiments, the range of first ionically crosslinkable polymer to second ionically crosslinkable polymer in the bioink formulation is in the range from 6:1 to 1:300. In some embodiments the range of first ionically crosslinkable polymer to the second ionically crosslinkable polymer may be, for example 60:1, 30:1, 25:1, 15:1, 10:1, 3:1, 1:1, 1:10, 1:15, 1:20, 1:100, 1:200, 1:300 1:500, 1:1000, 1:2000 and so forth, including values in-between.

[0033] In some bioink formulations including two or more ionically crosslinkable polymers, the first ionically crosslinkable polymer is present in the range from 2% (w/v) to 20% (w/v) and the second ionically crosslinkable polymer is present in the range from 0.001% (w/v) to 50% (w/v). In some preferred embodiments, the second ionically crosslinkable polymer is present in the bioink formation at a concentration in the range from 0.01% (w/v) to 30% (w/v); in other preferred embodiments, the second ionically crosslinkable polymer is present at a concentration in the range from 0.1% (w/v) to 10% (w/v).

[0034] In some embodiments, the bioink formulations provided herein include one ionically crosslinkable polymer. In other embodiments, the bioink formulations include a combination of two or more ionically crosslinkable polymers.

[0035] Crosslinking of the ionically crosslinkable polymer can be done by bivalent, trivalent or tetravalent cations—referred to as a “crosslinking additive” or more simply, a “crosslinker.” The crosslinking additive or crosslinker reacts with two or more of the

anionic functional groups on the ionically crosslinkable polymer to form polymer network structure. Exemplary crosslinkers used with the ionically crosslinkable polymers provided herein include, but are not limited to, Ca^{2+} , Mg^{2+} , Ba^{2+} , Be^{2+} , Sr^{2+} , Ra^{2+} , B^{3+} , Al^{3+} , Ga^{3+} cations. Salts of these cations may be dissolved in aqueous solution to provide the cation crosslinkers. The salts may be any suitable salts, i.e., any salt in which the counterion is not cytotoxic and does not negatively affect the bioink formulation. Some preferred salts of the crosslinking cation include, but are not limited to halide salts, such as chloride salts, carbonate salts and sulfate salts. In a preferred embodiment, Ca^{2+} is the crosslinker. In some embodiments CaCl_2 salt is dissolved in aqueous solution to provide the crosslinking cations. The crosslinker cations are provided at a concentration sufficient to crosslink the ionically crosslinkable polymer. In various embodiments, the concentration of cations is in the range from 1 mM to 10 M. In some preferred embodiments, the concentration of cations is in the range from 10 mM to 1M. In some particularly preferred embodiments, the concentration of cations is in the range from 100 mM to 200 mM.

[0036] Also provided are bioink formulation kits including at least one ionically crosslinkable polymer and at least one crosslinker. The kits may also include, for example, directions for use, information about particular applications, additional components, and so forth. In some embodiments, the kits may include two or more ionically crosslinkable polymers and/or two or more crosslinkers. In some embodiments, the kits may include several different ionically crosslinkable polymers and one or more crosslinkers, allowing the user to test several different formulations for a particular application.

[0037] In some embodiments, the ionically crosslinkable polymers in the kits may be provided as a solid. In other embodiments, the ionically crosslinkable polymers may be provided as hydrogel. In other embodiments, the ionically crosslinkable polymers may be provided as a solution. When the ionically crosslinkable polymer is provided as a hydrogel or as a solution, it may be provided in as a concentrated hydrogel or solution to be diluted to the desired percent (w/v) of polymer, by the user, for their particular application. In other embodiments, the kit may include the ionically crosslinkable polymer as a hydrogel or solution at concentrations ready-to-use without further dilution. In some embodiments, the kit may include one or more ionically crosslinkable

polymers provided at several different percentages (w/v) in water, allowing a user to try bioink formulations with varying percent (w/v) levels of ionically crosslinkable polymer for their particular application without having to prepare several dilutions.

[0038] The crosslinkers may be provided in the kits as a salt in solid form, or in solution. When provided as a solid, the user of the kit may dissolve to form an aqueous solution of the desired concentration. When provided as a solution, the solution may be provided as a concentrate that can be diluted to the desired concentration by the end user, or the solution may be provided at a ready-to-use concentration. Exemplary crosslinkers include Be^{2+} , Mg^{2+} , Ca^{2+} , Sr^{2+} , Ba^{2+} , Ra^{2+} , B^{3+} , Al^{3+} , Ga^{3+} . In some embodiments, the kit contains a crosslinking additive in solid or solution form. In a preferred embodiment, when the crosslinking additive is in solution form, the solution is an aqueous solution.

[0039] One specific example of chemically functionalized components derived from ECM that is useful in the ionically crosslinkable bioink formulations described herein is the carboxylic acid functionalized gelatin, abbreviated as GelCOOH. The GelCOOH is derived from the reaction of type A gelatin (available from MilliporeSigma www.sigmaaldrich.com, product number G2500) reacted with succinic anhydride.

[0040] In some embodiments, the ionically crosslinkable bioink described herein is formulated with an aqueous solution of GelCOOH. In some preferred embodiments, the GelCOOH is present in the bioink formulation at a concentration in the range from 0.1% (w/v) to 60% (w/v) in water, preferably from 1% (w/v) to 30% (w/v), and more preferably, from 2% (w/v) to 20% (w/v). The bioink is crosslinked with aqueous solution crosslinker cation. In a preferred embodiment, the crosslinker cation is calcium chloride, with the concentration of the calcium chloride in the range from 1 mM to 10 M, preferably from 10 mM to 1M, and more preferably, from 100 mM to 200 mM.

[0041] In another embodiment, a group of bioink compositions includes an aqueous solution of carboxylic acid functionalized gelatin with concentration varied between 2% (w/v) to 20% (w/v), and sodium alginate (available from MilliporeSigma www.sigmaaldrich.com, product number A2033), with concentration varied between 0.001% (w/v) to 50% (w/v) in water, preferably from 0.01% (w/v) to 30% (w/v), and more preferably, from 0.1% (w/v) to 10% (w/v). The bioink is crosslinked with aqueous

solution crosslinker cation. In a preferred embodiment, the crosslinker cation is calcium chloride, with the concentration of the calcium chloride in the range from 1 mM to 10 M, preferably from 10 mM to 1M, and more preferably, from 100 mM to 200 mM.

[0042] Yet another group of bioink compositions described herein include an aqueous solution of polyacrylic acid grafted gelatin (GelPAA) and sodium alginate, with concentration varied between 0.1% (w/v) to 60% (w/v), preferably from 1% (w/v) to 30% (w/v), and more preferably, from 2% (w/v) to 20% (w/v). The bioink is crosslinked with aqueous solution crosslinker cation. In a preferred embodiment, the crosslinker cation is calcium chloride, with the concentration of the calcium chloride in the range from 1 mM to 10 M, preferably from 10 mM to 1M, and more preferably, from 100 mM to 200 mM.

[0043] Further yet, another group of bioink formulations as described herein include an aqueous solution of guluronic acid grafted gelatin, with concentration varied between 0.1% (w/v) to 60% (w/v), crosslinked with an aqueous solution of calcium chloride, preferably from 1% (w/v) to 30% (w/v), and more preferably, from 2% (w/v) to 20% (w/v). The bioink is crosslinked with aqueous solution crosslinker cation. In a preferred embodiment, the crosslinker cation is calcium chloride, with the concentration of the calcium chloride in the range from 1 mM to 10 M, preferably from 10 mM to 1M, and more preferably, from 100 mM to 200 mM.

[0044] And another bioink formulation as described herein includes an aqueous solution of alginic acid functionalized gelatin, with concentration varied between 0.1% (w/v) to 60% (w/v), preferably from 1% (w/v) to 30% (w/v), and more preferably, from 2% (w/v) to 20% (w/v). The bioink is crosslinked with aqueous solution crosslinker cation. In a preferred embodiment, the crosslinker cation is calcium chloride, with the concentration of the calcium chloride in the range from 1 mM to 10 M, preferably from 10 mM to 1M, and more preferably, from 100 mM to 200 mM.

[0045] The present disclosure further provides the following embodiments set forth in the clauses below:

[0046] {1} A bioink formulation comprising an ionically crosslinkable polymer, wherein the ionically crosslinkable polymer comprises an ionically crosslinkable group.

[0047] {2a} The bioink formulation of clause {1}, wherein the ionically crosslinkable polymer comprises an anionic functional group.

[0048] {2b} The bioink formulation of clause {1}, wherein the ionically crosslinkable polymer includes an anionic functional group.

[0049] {3a} The bioink formulation of any of clauses {1}, {2a}, or {2b} wherein the ionically crosslinkable polymer comprises a natural polymer, a synthetic polymer, or a combination of a natural polymer and a synthetic polymer.

[0050] {3b} The bioink formulation of any of clauses {1}, {2a}, or {2b} wherein the ionically crosslinkable polymer comprises a natural polymer.

[0051] {3c} The bioink formulation of any of clauses {1}, {2a}, or {2b} wherein the ionically crosslinkable polymer consists essentially of a natural polymer.

[0052] {3d} The bioink formulation of any of clauses {1}, {2a}, or {2b} wherein the ionically crosslinkable polymer consists of a natural polymer.

[0053] {3e} The bioink formulation of any of clauses {1}, {2a}, or {2b} wherein the ionically crosslinkable polymer is a natural polymer.

[0054] {3f} The bioink formulation of any of clauses {1}, {2a}, or {2b} wherein the ionically crosslinkable polymer comprises a synthetic polymer.

[0055] {3g} The bioink formulation of any of clauses {1}, {2a}, or {2b} wherein the ionically crosslinkable polymer consists essentially of a synthetic polymer.

[0056] {3h} The bioink formulation of any of clauses {1}, {2a}, or {2b} wherein the ionically crosslinkable polymer consists of a synthetic polymer.

[0057] {3i} The bioink formulation of any of clauses {1}, {2a}, or {2b} wherein the ionically crosslinkable polymer is a synthetic polymer.

[0058] {3j} The bioink formulation of any of clauses {1}, {2a}, or {2b} wherein the ionically crosslinkable polymer comprises a combination of a natural polymer and a synthetic polymer.

[0059] {3k} The bioink formulation of any of clauses {1}, {2a}, or {2b} wherein the ionically crosslinkable polymer is a combination of a natural polymer and a synthetic polymer.

[0060] {4a} The bioink formulation of any of clauses {3a}, {3b}, {3c}, {3d}, {3e}, {3j}, and {3k} wherein the natural polymer comprises gelatin, collagen, hyaluronic acid, chitosan, alginate, cellulose, pectin, agarose, ulvan, silk, fibrin or a combination thereof.

[0061] {4b} The bioink formulation of any of clauses {3a}, {3b}, {3c}, {3d}, {3e}, {3j}, and {3k} wherein the natural polymer is selected from the group consisting of gelatin, collagen, hyaluronic acid, chitosan, alginate, cellulose, pectin, agarose, ulvan, silk, fibrin and combinations thereof.

[0062] {5a} The bioink formulation of any of clauses {3a}, {3f}, {3g}, {3h}, {3i}, {3j}, and {3k} wherein the synthetic polymer comprises poly(ethylene glycol) (PEG), polyvinyl alcohol (PVA), poly(acrylic acid) (PAA), poly(2-hydroxyethyl methacrylate) (PHEMA), poly(N-isopropylacrylamide) (PNIPAM), copolymers thereof, or combinations thereof.

[0063] {5b} The bioink formulation of any of clauses {3a}, {3f}, {3g}, {3h}, {3i}, {3j}, and {3k} wherein the synthetic polymer is selected from the group consisting of poly(ethylene glycol) (PEG), polyvinyl alcohol (PVA), poly(acrylic acid) (PAA), poly(2-hydroxyethyl methacrylate) (PHEMA), poly(N-isopropylacrylamide) (PNIPAM), copolymers thereof, and combinations thereof.

[0064] {6a} The bioink formulation of any of clauses {1}, {2a}, {2b}, {3a} through {3k}, {4a}, {4b}, {5a} or {5b}, wherein the ionically crosslinkable group comprises an anionic functional group selected from a monoanionic functional group, a dianionic functional group, a polyanionic functional group, and combinations thereof.

[0065] {6b} The bioink formulation of any of clauses {1}, {2a}, {2b}, {3a} through {3k}, {4a}, {4b}, {5a} or {5b}, wherein the ionically crosslinkable group is an anionic functional group selected from a monoanionic functional group, a dianionic functional group, a polyanionic functional group, and combinations thereof.

[0066] {7a} The bioink formulation of any of clauses {1}, {2a}, {2b}, {3a} through {3k}, {4a}, {4b}, {5a}, {5b}, {6a}, or {6b} wherein the ionically crosslinkable group comprises a carboxylic acid, a salt of a carboxylic acid, a sulfonic acid, a salt of a sulfonic acid, or combinations thereof.

[0067] {7b} The bioink formulation of any of clauses {1}, {2a}, {2b}, {3a} through {3k}, {4a}, {4b}, {5a}, {5b}, {6a}, or {6b} wherein the ionically crosslinkable group is selected from the group consisting of carboxylic acids, salts of carboxylic acids, sulfonic acids, salts of sulfonic acids, and combinations thereof.

[0068] {8a} The bioink formulation of either of clauses {7a} or {7b} wherein the ionically crosslinkable group is a carboxylic acid or salt of a carboxylic acid comprising acrylic acid, methacrylic acid, maleic acid, succinic acid, benzoic sulfonic, naphthalic acid, polyacrylic acid, poly(methacrylic acid), polymaleic acid, alginic acid, guluronic acid, carboxylate, maleate, succinate, polyacrylate, polymethacrylate, polymaleate, alginic acid, guluronic acid or a combinations thereof.

[0069] {8b} The bioink formulation of either of clauses {7a} or {7b} wherein the ionically crosslinkable group is a carboxylic acid or salt of a carboxylic acid selected from the group consisting of acrylic acid, methacrylic acid, maleic acid, succinic acid, benzoic sulfonic, naphthalic acid, polyacrylic acid, poly(methacrylic acid), polymaleic acid, alginic acid, guluronic acid, carboxylate, maleate, succinate, polyacrylate, polymethacrylate, polymaleate, alginic acid, guluronic acid and combinations thereof.

[0070] {9a} The bioink formulation of either of clauses {7a} or {7b} wherein the ionically crosslinkable group is a sulfonic acid or salt of a sulfonic acid comprising alkyl sulfonic acids, aromatic sulfonic acids, alkyl sulfonates, aromatic sulfonates, or combinations thereof.

[0071] {9b} The bioink formulation of either of clauses {7a} or {7b} wherein the ionically crosslinkable group is a sulfonic acid or salt of a sulfonic acid selected from the group consisting of alkyl sulfonic acids, aromatic sulfonic acids, alkyl sulfonates, aromatic sulfonates, and combinations thereof.

[0072] {10} The bioink formulation of any of clauses {1}, {2a}, {2b}, {3a} through {3k}, {4a}, {4b}, {5a}, {5b}, {6a}, {6b}, {7a}, {7b}, {8a}, {8b}, {9a}, or {9b}

wherein the ionically crosslinkable polymer has molecular weight in the range from 1000 Da to 100M Da, preferably in the range from 10,000 Da (10 kDa) to 10 MDa, and more preferably in the range from 20,000 Da (20 kDa) to 1.3 MDa.

[0073] {11} The bioink formulation of any of clauses {1}, {2a}, {2b}, {3a} through {3k}, {4a}, {4b}, {5a}, {5b}, {6a}, {6b}, {7a}, {7b}, {8a}, {8b}, {9a}, {9b}, or {10} wherein the ionically crosslinkable polymer is in solid, hydrogel or solution form.

[0074] {12} The bioink formation of clause {11} comprising from 0.1% (w/v) to 99% (w/v), preferably from 0.1% (w/v) to 60% (w/v), more preferably from 1% (w/v) to 30% (w/v), and even more preferably from 2% (w/v) to 20% (w/v) of ionically crosslinkable polymer in aqueous solution.

[0075] {13} The bioink formulation of clause {12}, wherein the bioink comprises two ionically crosslinkable polymers, wherein the first ionically crosslinkable polymer has a concentration in the range from 2% (w/v) to 20% (w/v), and the second ionically crosslinkable polymer has a concentration in the range from 0.001% (w/v) to 20% (w/v), preferably from 0.01% (w/v) to 30% (w/v), and more preferably from 0.1% (w/v) to 10% (w/v), in aqueous solution.

[0076] {14} A bioink formulation kit comprising a bioink formulation of any of clauses {1}, {2a}, {2b}, {3a} through {3k}, {4a}, {4b}, {5a}, {5b}, {6a}, {6b}, {7a}, {7b}, {8a}, {8b}, {9a}, {9b}, {10}, {11}, {12}, or {13} and a crosslinker, wherein the crosslinker, when contacted with the bioink formulation, reacts with the ionically crosslinkable functional group of the ionically crosslinkable polymer to form a polymer network structure.

[0077] {15} The bioink formulation kit of clause {14} wherein the crosslinker is selected from the group consisting of Be^{2+} , Mg^{2+} , Ca^{2+} , Sr^{2+} , Ba^{2+} , Ra^{2+} , B^{3+} , Al^{3+} , Ga^{3+} and combinations thereof, and is preferably Ca^{2+} .

[0078] {16} The bioink formulation kit of either of clauses {14} or {15} wherein the crosslinker is provided as a solid salt, preferably a salt selected from the group consisting of chloride salts, carbonate salts, and sulfate salts.

[0079] {17} The bioink formulation kit of any of clauses {14}, {15}, or {16} wherein the crosslinker is a solution, preferably an aqueous solution.

[0080] {18} A composition including a bioink formulation of any of clauses {1}, {2a}, {2b}, {3a} through {3k}, {4a}, {4b}, {5a}, {5b}, {6a}, {6b}, {7a}, {7b}, {8a}, {8b}, {9a}, {9b}, {10}, {11}, {12}, or {13} and a crosslinker, wherein the crosslinker, when contacted with the bioink formulation, reacts with the ionically crosslinkable functional group of the ionically crosslinkable polymer to form a polymer network structure.

[0081] {19} A composition of clause {18} wherein the crosslinker is a cation selected from the group consisting of Be^{2+} , Mg^{2+} , Ca^{2+} , Sr^{2+} , Ba^{2+} , Ra^{2+} , B^{3+} , Al^{3+} , Ga^{3+} and combinations thereof.

[0082] {20} The composition of either of clauses {18} or {19} wherein the crosslinker is Ca^{2+} .

[0083] {21} A composition of any of clauses {18}, {19}, or {20} wherein the crosslinker is derived from a salt, preferably a salt selected from the group consisting of chloride salts, carbonate salts, and sulfate salts.

[0084] {22} A composition of any of clauses {18}, {19}, {20}, or {21} wherein the crosslinker is in a solution.

[0085] {23} The composition of any of clauses {18}, {19}, {20}, {21}, or {22} wherein the crosslinker is in an aqueous solution.

[0086] Examples: Following are the specific examples of bioink formulations of the present invention.

[0087] Example 1. General procedure for preparation of Gel-COOH.

[0088] Succinylation of gelatin (Gel-COOH) was carried out as follows. 5% (w/v) aqueous gelatin solution was prepared by heating it in a water bath at 40°C. The gelatin solution was adjusted to pH 9 using 1N NaOH solution. Varying amounts of succinic anhydride, ranging from 0.25 to 1.25 g were dissolved in 5 mL of acetone. To 5% gelatin solution 5 mL of acetone containing various amounts of succinic anhydride were

added giving a ratio range of 0.05–0.25 mg succinic anhydride per mg of gelatin. After adding succinic anhydride, the solution was stirred for 3 h maintaining the pH at 9 during the reaction. The reacted gelatin solution was dialyzed against distilled water for 48 h. Gel-COOH was obtained as powder upon lyophilization.

[0089] Example 2. Bioink Formulation 1: aqueous bioink formulation with 10% (w/v) Gel-COOH.

[0090] Gel-COOH (1g) was dissolved in PBS (10 mL) at 60°C. The pH was adjusted to neutral and the solution was filtered through a 0.22 µm PES membrane. The bioink was crosslinked with 0.1M aqueous calcium chloride solution. Bioprinting of formulation 1 was done using a Cellink Inkredible+ bioprinter at room temperature, 10 mm/s, 60-90 kPa with a 22G tapered plastic needle onto a 90 mm petri dish.

[0091] Example 3. Bioink Formulation 2: aqueous bioink formulation with a combination of 10% (w/v) Gel-COOH and 1% (w/v) sodium alginate.

[0092] Sodium alginate (100 mg) and Gel-COOH (1g) was dissolved in PBS (10mL) at 60°C. The pH was adjusted to neutral and the solution was filtered through a 0.22 µm PES membrane. The bioink was crosslinked with 0.1M aqueous calcium chloride solution.

[0093] Bioprinting of Formulation 2 was done using a Cellink Inkredible+ bioprinter at room temperature, 10 mm/s, 60-90 kPa with a 22G tapered plastic needle onto a 90mm petri dish.

[0094] Example 4. General procedure for preparation of gelatin-guluronate (Gel-Gul).

[0095] To prepare the precursor gelatin-guluronate (Gel-Gul), guluronate (2.00 g; 10.30 mmol; 1.00 eq.) was dissolved in 200 mL DI water (1% w/v) in a beaker equipped with a magnetic stir bar and pH probe. The solution was heated to 40 °C and the pH adjusted to 4.5 - 5.0. The gelatin was prepared by adding 300 bloom Gelatin (2.00 g; 2.31 mmol; 0.22 eq.) to 50 mL DI water (1% w/v) in a 250 mL round-bottom flask and placing the flask in a hot water bath (45 °C) while stirring. N-hydroxysuccinimide (355.68 mg; 3.09 mmol; 0.30 eq.) was added to the guluronate solution. Then EDC

(197.49 mg; 1.03 mmol; 0.10 eq.) was added to the guluronate solution. The solution was stirred for 15 min. The gelatin solution was added to the guluronate solution, the pH adjusted to 7.5, and stirred for one hour. The reacted gelatin solutions was dialyzed for 3 days against pure DI water to purify and lyophilized to yield a white solid.

[0096] Example 5. Bioink Formulation 3: aqueous bioink formulation with guluronic acid functionalized gelatin 10% (w/v), crosslinked with 0.2 M aqueous calcium chloride solution.

[0097] Gel-Gul (1g) was dissolved in DPBS (10mL) at 60°C. The pH of the bioink was neutralized using 1M NaOH. The bioink solution was sterile filtered using a 0.22 µm PES membrane.

[0098] The bioink was printed using a Cellink BioX and Allevi 3 bioprinters at 22°C, 10 mm/s, 100 kPa with a 22G tapered plastic needle onto a 90mm petri dish. The bioink was ionically crosslink by the addition of a calcium chloride solution (0.2 M) for 1 min.

[0099] Example 6. General procedure for preparation of gelatin-alginate (Gel-Alg).

[0100] To prepare the precursor gelatin-alginate (Gel-Alg), low viscosity Alginate (2.00 g; 10.30 mmol; 1.00 eq.) was dissolved in 200 mL DI water (1% w/v) in a beaker equipped with a magnetic stir bar and pH probe. The solution was heated to 40 °C and pH adjusted to 4.5 - 5.0. The gelatin solution was prepared by adding 300 bloom Gelatin (2.00 g; 2.31 mmol; 0.22 eq.) to 50 mL DI water (1% w/v) in a 250 mL round-bottom flask and placing the flask in a hot water bath (45°C) while stirring. N-hydroxysuccinimide (355.68 mg; 3.09 mmol; 0.30 eq.) was added to the alginate solution. EDC (197.49 mg; 1.03 mmol; 0.10 eq.) was then added to the alginate solution. The resulting solution was stirred for 15 min. The gelatin solution was then added to the alginate solution. The pH was adjusted to 7.5 and stirred for one hour. The reacted gelatin was dialyzed for 3 days against pure DI water to purify and lyophilized to yield a white solid.

[0101] Example 7. Formulation 4: Aqueous bioink formulation of 8% (w/v) Gel-Alg, crosslinked with 0.2 M aqueous calcium chloride solution.

[0102] Gel-Alg (800 mg) was dissolved in HBSS (10mL) at 60°C. The pH of the bioink was neutralized using 1M NaOH. The bioink solution was sterile filtered using a 0.22 µm PES membrane.

[0103] The bioink was printed using a Cellink BioX bioprinter at 20°C, 10 mm/s, 100 kPa with a 22G tapered plastic needle onto a 90mm petri dish. The bioink was ionically crosslink by the addition of a calcium chloride solution (0.2M) for 1 min.

[0104] Images of structures printed using Cellink Inkredible+ or BioX bioprinters and crosslinked with the formulations described in example Formulations 1-4 are shown in FIG. 1. Images of structures printed using Allevi 3 bioprinter with formulations described in example Formulated 3 and 5 are shown in FIG. 2. Purple dye was added for visualization purposes.

[0105] Example 8. To demonstrate the ionic crosslinking of the bioink formulations of the present invention, mechanical properties of the polymer hydrogel were analyzed before and after treating with calcium chloride solution as described below.

[0106] Bioink formulations from Formulation 1 & Formulation 2 were printed into disc shaped samples with a diameter of 40 mm and thickness of 1.5-2 mm. For crosslinking studies, samples were submerged in 0.1M calcium chloride solution for 1 hour to crosslink. Compressive moduli of the samples were measured using DHR2 dynamic hybrid rheometer from TA instruments before and after crosslinking with calcium chloride and data were plotted in FIG. 3. Increase in moduli after CaCl₂ treatment indicated successful crosslinking with exposure to Ca²⁺ ions.

[0107] Separately, bioink formulations from Formulation 3, Formulation 4, and Formulation 5 were studied under oscillation for increased stiffness with respect to ionic crosslinking. For crosslinking studies, samples were submerged in 0.2M calcium chloride solution for 1 hour to crosslink. Storage moduli of the samples were measured using DHR2 dynamic hybrid rheometer from TA instruments before and after crosslinking with calcium chloride, shown below in FIG. 4. An increase in moduli after CaCl₂ treatment indicated the successful ionic crosslinking of the formulation with the Ca²⁺ ions.

[0108] Example 9. General procedure for preparation of hyaluronic acid-gulonate (HA-Gul).

[0109] To prepare hyaluronic acid adipic dihydrazide (HA-ADH), 5.0 g hyaluronic acid was dissolved in 625 mL DI water followed by addition of 90.0g(0.51 moles) adipic dihydrazide. The pH of the solution was adjusted to 4.75 using 0.1M aqueous hydrochloric acid. Once the pH was approximately 4.75, 10g (0.04 moles) of N-ethyl-N'-(3-(dimethylamino)propyl)carbodiimide (EDC) was added to the solution and the pH was readjusted to 4.75. The solution was then stirred overnight in ambient conditions. The reacted solution was then dialyzed for 3 days against pure DI water to purify and lyophilized to yield a white solid.

[0110] To prepare the precursor hyaluronic acid-gulonate (HA-Gul), a HA-ADH solution was prepared by adding HA-ADH (2.00 g) to 50 mL DI water (1% w/v) in a 250 mL round-bottom flask. The solution was kept stirring on a magnetic stirrer. Separately, gulonate (2.00 g; 10.30 mmol; 1.00 eq.) was dissolved in 200 mL DI water (1% w/v) in a beaker using magnetic stirrer. The pH of the solution was adjusted to 4.5 - 5.0 using 0.1N aqueous hydrochloric acid. N-hydroxysuccinimide (355.68 mg; 3.09 mmol; 0.30 eq.) was added to the gulonate solution. After 10 minutes, EDC (197.49 mg; 1.03 mmol; 0.10 eq.) was added to the gulonate solution and continued stirring for 15 min. Previously prepared HA-ADH solution was then added to the gulonate solution, and the pH adjusted to 7.5, and stirred for one hour. The reacted solution was dialyzed for 3 days against pure DI water to purify and lyophilized to yield a white solid.

[0111] Example 10. Formulation 5: aqueous bioink formulation with guluronic acid functionalized gelatin 10% (w/v) and guluronic acid functionalized Hyaluronic Acid 2% (w/v), crosslinked with 0.2 M aqueous calcium chloride solution.

[0112] Gel-Gul (1g) and HA-Gul (0.2g) was dissolved in 50 mM HEPES (8.8 mL) at 60°C. The pH of the bioink was neutralized using 1M NaOH. The bioink solution was sterile filtered using a 0.22 µm PES membrane.

[0113] The bioink was printed using a Allevi 3 bioprinter at 19°C, 6 mm/s, 35 psi with a 22G blunt needle onto a 90mm petri dish. The bioink was ionically crosslinked by the addition of a calcium chloride solution (0.2 M) for 1 min.

[0114] Example 11. To demonstrate the cytocompatibility of the bioink formulations described herein, viability and metabolic activity of encapsulated human mesenchymal stem cells (hMSCs) was assayed as follows:

[0115] Cultured hMSCs were trypsinized and dispersed in formulations 3 and 5 at a concentration of 2×10^6 cells per mL of bioink. The formulations containing dispersed cells were then printed into 12 well and 96 well plates to assay viability and metabolic activity, respectively. To assay viability, printed constructs were stained with Calcein AM (1:1000 dilution) and propidium iodide (1:1000 dilution) for 15 min before imaging with a fluorescent microscope after 1, 7 and 14 days of culture. To assay metabolic activity, printed constructs were incubated with PrestoBlue (1:10 dilution) for 4 hours before fluorescent reading with a plate reader after 1, 7 and 14 days of culture. These results are shown in FIG. 5.

[0116] The examples herein are for illustrative purposes only and are not meant to limit the scope of the invention as set forth in the claims.

The invention claimed is:

1. A bioink formulation comprising an ionically crosslinkable polymer, wherein the ionically crosslinkable polymer comprises an ionically crosslinkable group.
2. The bioink formulation of claim 1 wherein the ionically crosslinkable polymer comprises an anionic functional group.
3. The bioink formulation of claim 2 wherein the ionically crosslinkable polymer comprises a natural polymer, a synthetic polymer, or a combination of a natural polymer and a synthetic polymer.
4. The bioink formulation of any of claims 1-3 wherein the ionically crosslinkable polymer comprises a natural polymer having an anionic functional group, wherein the natural polymer is selected from the group consisting of gelatin, collagen, hyaluronic acid, chitosan, alginate, cellulose, pectin, agarose, ulvan, silk, fibrin, and combinations thereof.
5. The bioink formulation of any of claims 1-3 wherein the ionically crosslinkable polymer comprises a synthetic polymer having an anionic functional group, wherein the synthetic polymer is selected from the group consisting of poly(ethylene glycol) (PEG), polyvinyl alcohol (PVA), poly(acrylic acid) (PAA), poly(2-hydroxyethyl methacrylate) (PHEMA), poly(N-isopropylacrylamide) (PNIPAM), copolymers thereof, and combinations thereof.
6. The bioink formulation of any of claims 1-5 wherein the ionically crosslinkable group comprises an anionic functional group selected from the group consisting of a monoanionic functional group, a dianionic functional group, a polyanionic functional group, and combinations thereof.
7. The bioink formulation of any of claims 1-5 wherein the ionically crosslinkable group is selected from the group consisting of carboxylic acids, salts of carboxylic acids, sulfonic acids, salts of sulfonic acids, and combinations thereof.
8. The bioink formulation of any of claims 1-7 wherein the ionically crosslinkable group is a carboxylic acid or salt of a carboxylic acid selected from the group consisting of acrylic acid, methacrylic acid, maleic acid, succinic acid, benzoic sulfonic,

naphthalic acid, polyacrylic acid, poly(methacrylic acid), polymaleic acid, alginic acid, guluronic acid, carboxylate, maleate, succinate, polyacrylate, polymethacrylate, polymaleate, alginic acid, guluronic acid and combinations thereof.

9. The bioink formulation of any of claims 1-7 wherein the ionically crosslinkable group is a sulfonic acid or salt of a sulfonic acid selected from the group consisting of alkyl sulfonic acids, aromatic sulfonic acids, alkyl sulfonates, aromatic sulfonates, and combinations thereof.

10. The bioink formulation of any of claims 1 to 9 wherein the ionically crosslinkable polymer has molecular weight in the range from 1000 Da to 100M Da, preferably in the range from 10,000 Da (10 kDa) to 10 MDa, and more preferably in the range from 20,000 Da (20 kDa) to 1.3 MDa.

11. The bioink formulation of any of claims 1 to 10 wherein the ionically crosslinkable polymer is in solid, hydrogel or solution form.

12. The bioink formation of claim 11 comprising from 0.1% (w/v) to 99% (w/v), preferably from 0.1% (w/v) to 60% (w/v), more preferably from 1% (w/v) to 30% (w/v), and even more preferably from 2% (w/v) to 20% (w/v) of ionically crosslinkable polymer in aqueous solution.

13. The bioink formulation of claim 12, wherein the bioink comprises two ionically crosslinkable polymers, wherein the first ionically crosslinkable polymer has a concentration in the range from 2% (w/v) to 20% (w/v), and the second ionically crosslinkable polymer has a concentration in the range from 0.001% (w/v) to 20% (w/v), preferably from 0.01% (w/v) to 30% (w/v), and more preferably from 0.1% (w/v) to 10% (w/v), in aqueous solution.

14. A bioink formulation kit comprising a bioink formulation of any of claims 1 to 13 and a crosslinker, wherein the crosslinker, when contacted with the bioink formulation, reacts with the ionically crosslinkable functional group of the ionically crosslinkable polymer to form a polymer network structure.

15. The bioink formulation kit of claim 14 wherein the crosslinker is selected from the group consisting of Be^{2+} , Mg^{2+} , Ca^{2+} , Sr^{2+} , Ba^{2+} , Ra^{2+} , B^{3+} , Al^{3+} , Ga^{3+} and combinations thereof, and is preferably Ca^{2+} .
16. The bioink formulation kit of either of claims 14 or 15 wherein the crosslinker is provided as a solid salt, preferably a salt selected from the group consisting of chloride salts, carbonate salts, and sulfate salts.
17. The bioink formulation kit of either of claims 14 or 15 wherein the crosslinker is in a solution, preferably an aqueous solution.
18. A composition including a bioink formulation of any of claims 1-13 and a crosslinker, wherein the crosslinker, when contacted with the bioink formulation, reacts with the ionically crosslinkable functional group of the ionically crosslinkable polymer to form a polymer network structure.
19. The composition of claim 18 wherein the crosslinker is selected from the group consisting of Be^{2+} , Mg^{2+} , Ca^{2+} , Sr^{2+} , Ba^{2+} , Ra^{2+} , B^{3+} , Al^{3+} , Ga^{3+} and combinations thereof, and is preferably Ca^{2+} .
20. The composition of either of claims 18 or 19 wherein the crosslinker is derived from a salt, preferably selected from the group consisting of chloride salts, carbonate salts and sulfate salts.

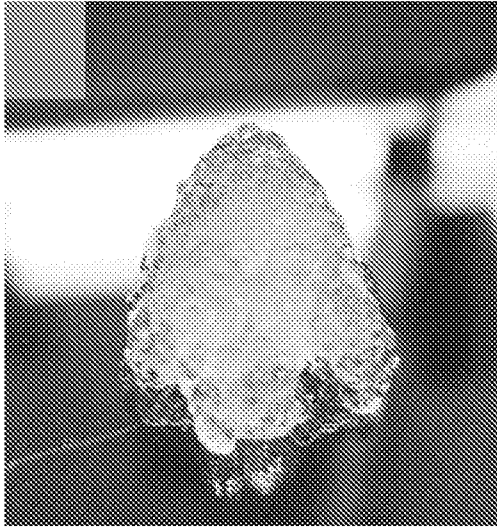


FIG. 1A

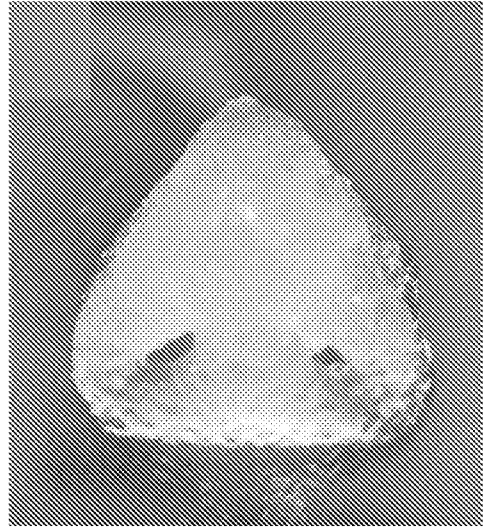


FIG. 1B

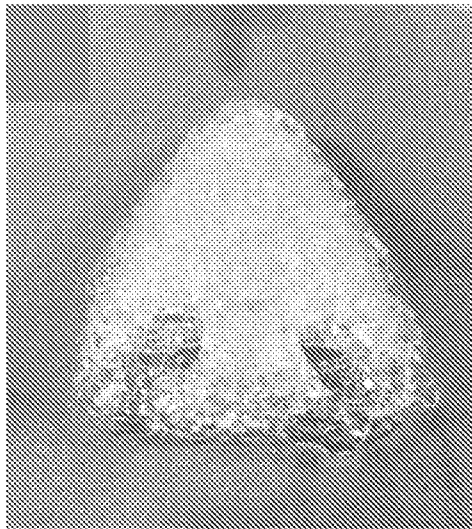


FIG. 1C

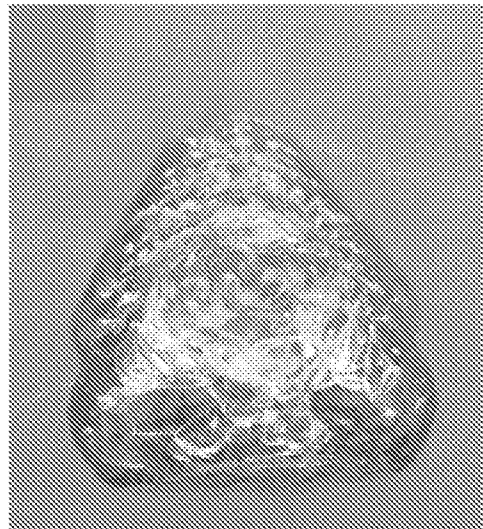


FIG. 1D

FIG. 1

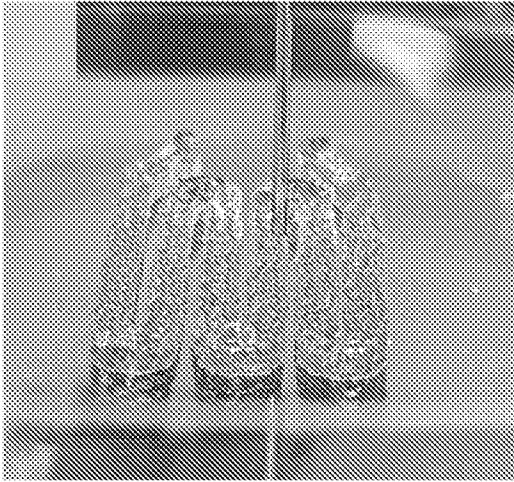


FIG. 2A

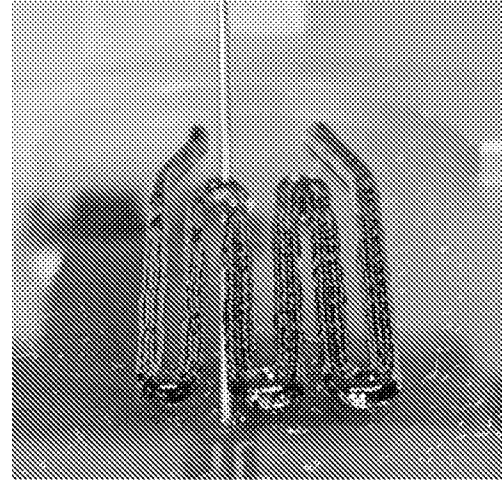


FIG. 2B

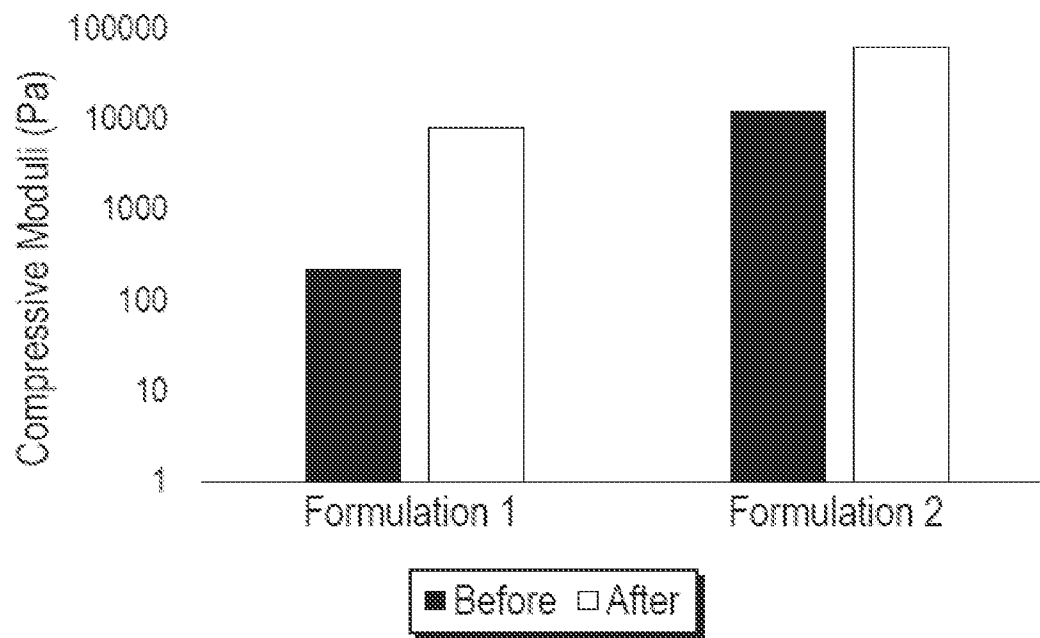


FIG. 3

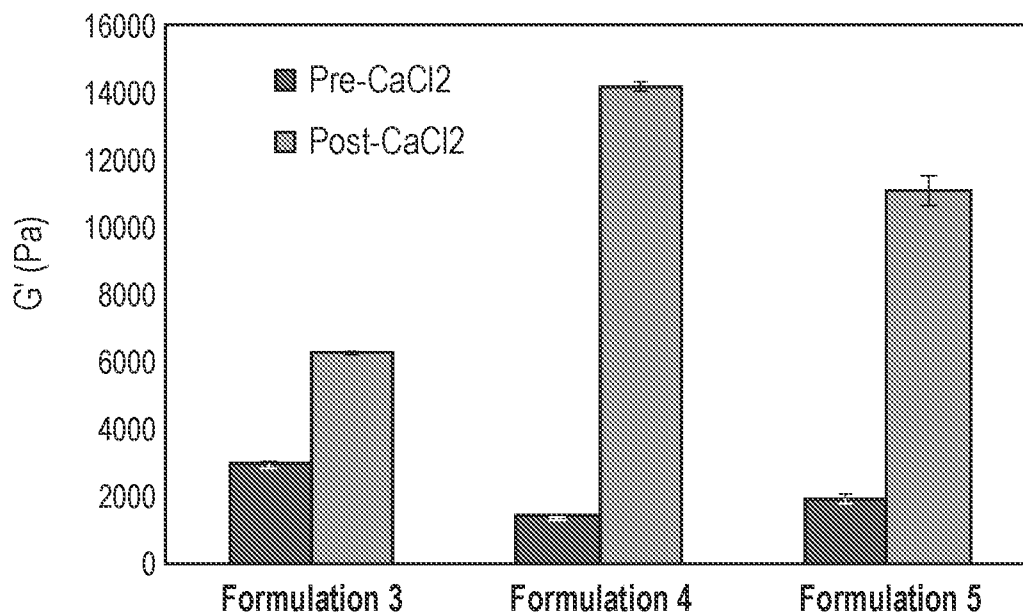


FIG. 4

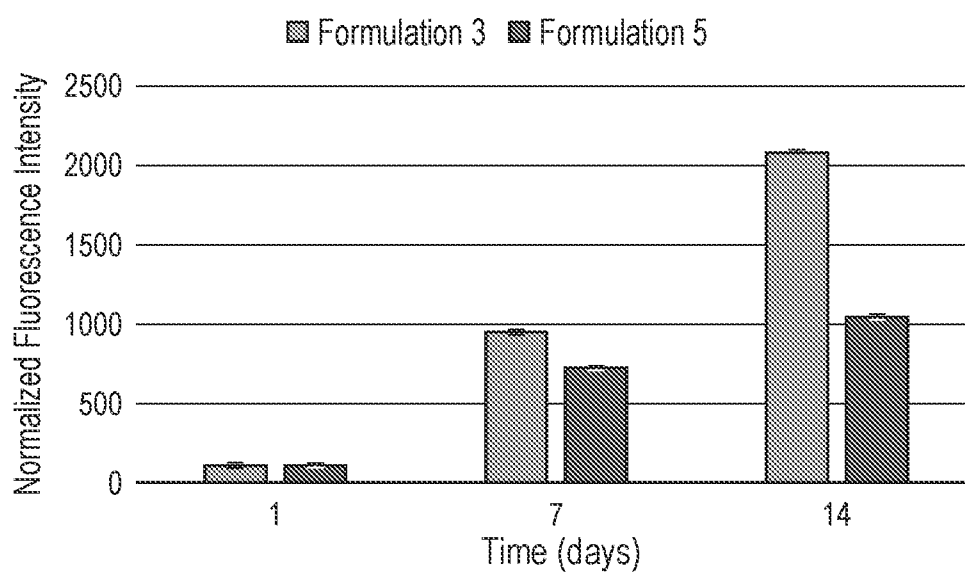


FIG. 5A

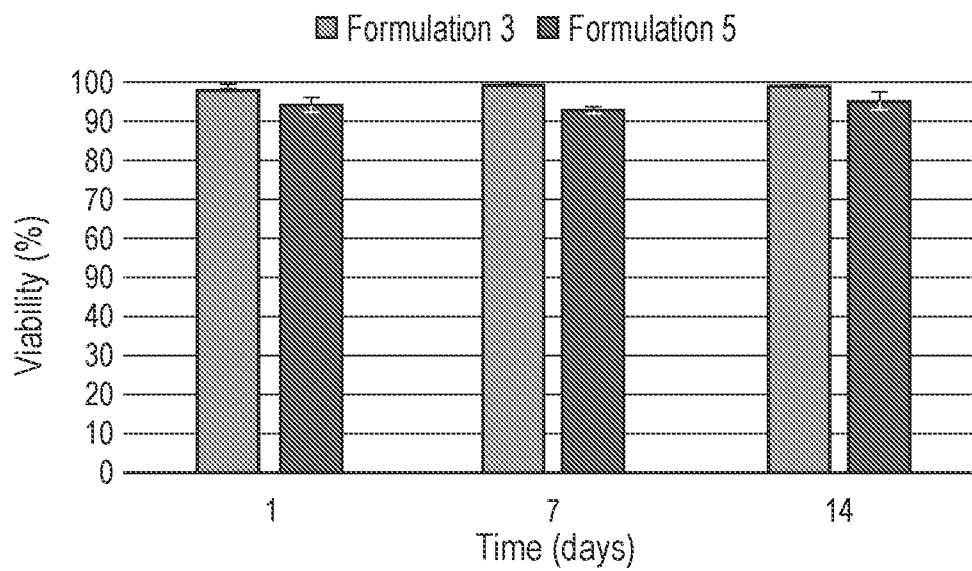


FIG. 5B

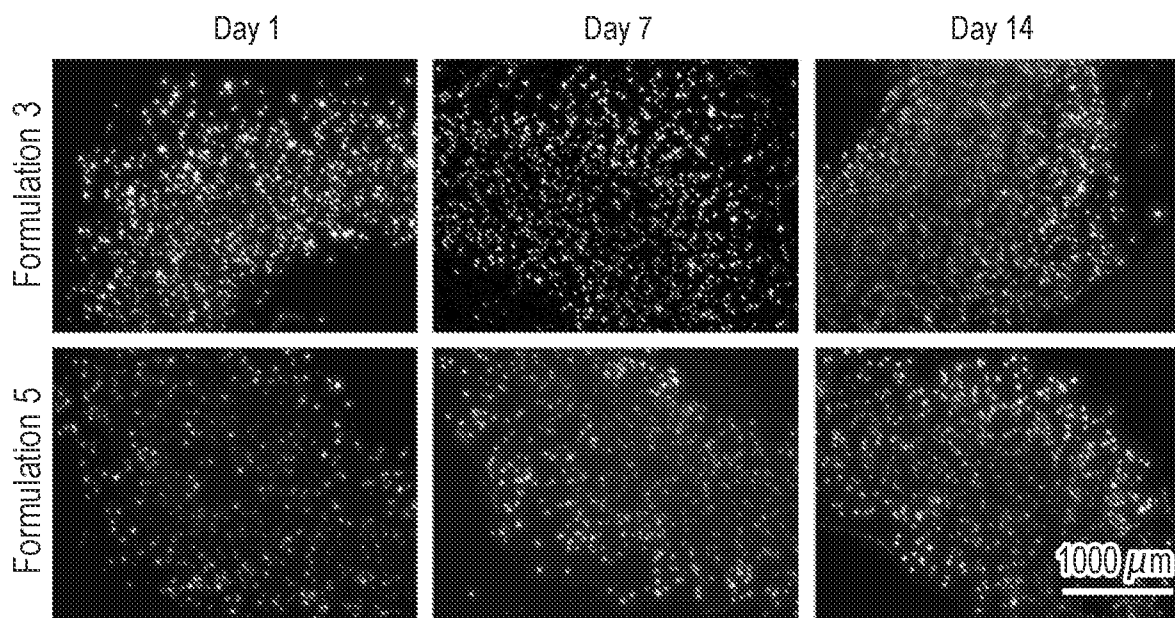


FIG. 5C

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2022/072084

A. CLASSIFICATION OF SUBJECT MATTER INV. A61L27/16 A61L27/18 A61L27/22 A61L27/24 B33Y70/00 B33Y80/00 ADD. 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) A61L B33Y 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) EPO-Internal, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2021/069378 A1 (NELSON KIMBERLY [US] ET AL) 11 March 2021 (2021-03-11) page 2, paragraph 0020 - paragraph 0023 page 3, paragraphs 0036, 0037, 0047, 0049 -----	1-4, 6-8, 10-12, 14-20
X	CN 107 998 449 A (HANGZHOU JIENUOFEI BIOLOGICAL TECH CO LTD) 8 May 2018 (2018-05-08) paragraphs [0001], [0027], [0029] -----	1-4, 6-8, 11-20
X	US 6 497 902 B1 (MA PETER X [US]) 24 December 2002 (2002-12-24) column 2, line 21 - line 47 column 4, line 43 - line 46 column 5, line 55 - line 61 column 6, line 8 - column 7, line 10 column 7, line 17 - line 26 -----	1-9, 11, 14-20
☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
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Date of the actual completion of the international search 14 July 2022		Date of mailing of the international search report 25/07/2022
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Dudás, Eszter

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2022/072084

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