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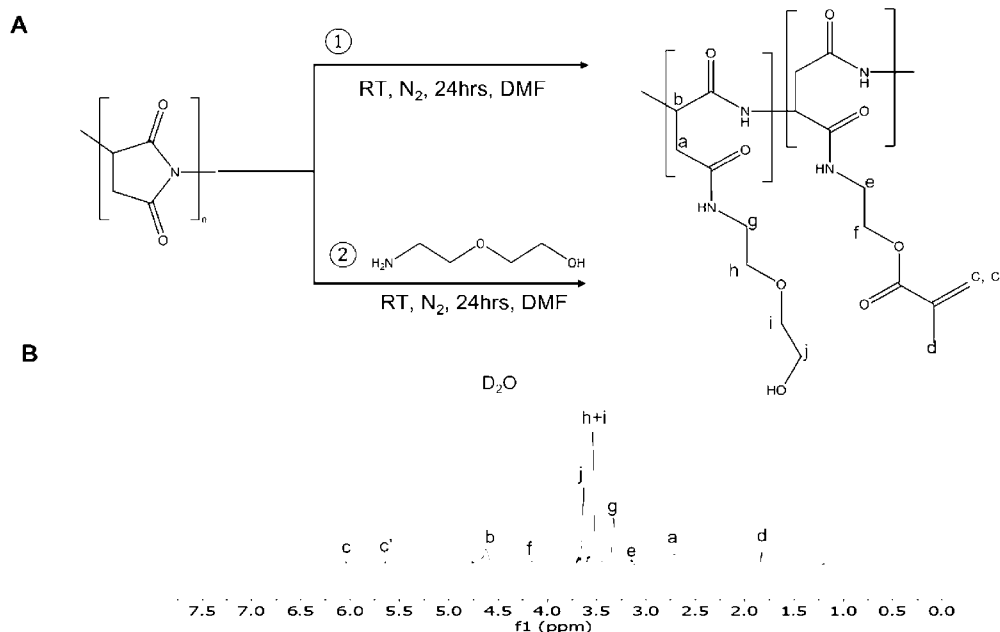
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(54) Title: SYNTHETIC BIOINK FORMULATIONS

Fig. 1



(57) Abstract: Provided is a xeno-free, synthetic bioink formulation comprising polymers derived from poly(aspartamide) (PAspAm) for 3D bioprinting applications, a method for producing a three-dimensional object using said synthetic bioink formulation and a three-dimensional object obtainable by said method.

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SYNTHETIC BIOINK FORMULATIONS

Sequence Listing

[0001] The present application claims the benefit of priority of U.S. provisional patent application no. 63/582,304, filed on September 13, 2023, the entire contents of which are hereby incorporated by reference in their entirety.

[0002] The instant application contains a Sequence Listing which has been submitted electronically in XML file format and is hereby incorporated by reference in its entirety. Said XML copy, created on July 25, 2024, is named P23-105-WO-PCT_SL.xml and is 11,772 bytes in size.

Field of Invention

[0003] The present invention provides xeno-free, synthetic bioink formulations comprising polymers based on a poly(aspartamide) (PAspAm) scaffold for 3D bioprinting applications. The bioink formulations of the present invention are biocompatible, biodegradable, and promote cellular functions. In addition, they show better printability and crosslinkability to ensure high dimensional fidelity and structural integrity of printed structures created therefrom. Furthermore, the bioink formulations of the present invention can be tuned to exhibit beneficial shear thinning properties.

[0004] There is further provided a method for producing a three-dimensional object, wherein said bioink formulation is applied to a 3D bioprinting technique to produce said three-dimensional object. Moreover, there is provided a three-dimensional object obtainable by said method. Said three-dimensional object can be used for tissue engineering, regenerative medicine, cell delivery, drug delivery, drug discovery, wound dressing, biosensors, cosmetics, hygiene products, medical devices, implantable electronics and/or other biomedical applications.

Background

[0005] Biofabrication is an interdisciplinary field that combines principles of engineering, biology, and material science and holds the potential to generate constructs that closely mimic the composition and hierarchical architecture of native

tissues. Such constructs can be applied for various biomedical uses including tissue engineering, regenerative medicine, and drug discovery.

[0006] Among several fabrication methods, 3D bioprinting offers tremendous potential in the fabrication of functional tissue as it provides better control over the spatial arrangement of the matrix, architectural fidelity, and compositional reproducibility of the constructs. 3D bioprinting techniques allow precise placement of cells in spatially predefined locations within confined 3D structures.

[0007] The most common 3D bioprinting techniques include DLP-based bioprinting, droplet-based bioprinting, extrusion-based bioprinting, forward transfer bioprinting, inkjet bioprinting, integrated bioprinting, laser-induced bioprinting, stereolithography-based bioprinting, magnetic bioprinting, and volumetric bioprinting. These 3D bioprinting techniques allow precise placement/positioning in spatially predefined locations within confined 3D structures.

[0008] One of the critical components of 3D bioprinting is the bioink used for creating tissue constructs. The bioink is an aqueous solution of one or more biomaterials in the form of a hydrogel, which optionally contains one or more desired cell types. Hence, however, bioink should be able to transport cells during printing and support cell growth after printing. To this end, a bioink should satisfy certain material and biological requirements.

[0009] Typical material properties include printability, crosslinkability, matrix stiffness, protection against shear stresses during the printing process, and dimensional fidelity of printed structures during subsequent cell cultivation.

[0010] Biological requirements mainly include degradability, cytocompatibility, and promotion of cell bioactivity during subsequent cell cultivation.

[0011] Among the different biomaterials, hydrophilic polymers are the most prominent materials which are used in bioink formulations. Hitherto, natural polymers, such as collagen, gelatin, hyaluronic acid, alginate, chitosan, have claimed central roles in bioink formulations due to their availability and ability to provide adapted scaffolding systems for the structural and functional organization of

cells.

[0012] Natural polymers possess unique properties such as biocompatibility and biodegradability, thus making them well-adapted for many biomedical applications. However, natural polymers have several inherent disadvantages, including the high degree of variability in the structure of polymers derived from different animals and poor batch-to-batch consistency in the manufacturing process. Natural polymers are also structurally more complex and are difficult to chemically modify to achieve desired rheological and mechanical properties. Besides, natural polymers also pose a high risk of adventitious agent contamination. Additionally, there are both regulatory and ethical problems with using polymers sourced from animals. Therefore, there is a need for bioink formulations that are derived from xeno-free materials such as, for example, synthetic polymers.

[0013] Bioinks derived from synthetic polymers can easily overcome some of the inherent challenges of natural polymer-based bioinks. For example, synthetic polymers have well-defined, less complex structures, and these structures can be easily modified using several functionalization strategies to provide cell-mediated degradation and greater control over the cell functions, including adhesion, proliferation, and morphogenesis. Besides, synthetic polymers can be manufactured on a large scale with a high degree of batch consistency necessary for reproducible results.

[0014] KR 20180025117 A relates to a bioink containing a polyester-based copolymer with a controlled melting point, a biocompatible structure containing the same, and a manufacturing method thereof. The polyester-based copolymer is synthesized using at least one alcohol-based initiator selected from the group consisting of diethylene glycol ethyl ether, polypropylene glycol, and methoxy(ethylene glycol).

[0015] KR 20200017606 A relates to a scaffold for tissue regeneration, method for its manufacture, and a bioink material for 3D printing using the same. The bioink material for 3D printing is based on a physically crosslinked casein-polyvinyl alcohol hydrogel.

[0016] WO 2019/236891 A1 relates to pharmaceutical bioinks, pharmaceutical

formulations, and methods of printing pharmaceutical bioinks. The pharmaceutical bioink comprises a hydrophobic photocurable resin capable of being cured by visible light, where, in particular, poly(ethylene glycol)diacrylate (PEGDA) is used as such hydrophobic photocurable resin.

[0017] WO 2021/234141 A1 relates to a hydrogel formed with poly(ethylene glycol) (PEG) combined with heparin and a positively charged immune molecule.

[0018] Many of these bioink formulations made with synthetic polymers contain non-degradable backbone structures and can hinder cellular functions. Besides, they do not contain the structural units that promote cellular functions such as adhesion, proliferation, and morphogenesis.

[0019] Hence, there is still a continuous need to develop improved bioink formulations suitable for 3D bioprinting applications, which overcome the disadvantages known from the prior art. In particular, there is a need for improved bioink formulations suitable for 3D bioprinting, which are xeno-free, biocompatible, biodegradable and promote cellular functions, show better printability and crosslinkability to ensure high dimensional fidelity and structural integrity of printed structures created therefrom, and can be tuned to exhibit beneficial shear thinning properties.

Objective

[0020] The present invention aims to overcome the disadvantages of the bioinks known from the prior art, which include bioinks derived from natural polymers and synthetic polymers. These disadvantages include inter alia batch-to-batch inconsistency, difficulty in fine-tuning the polymer's properties, scale-up manufacturing, risk of adventitious agent contamination, non-degradability of synthetic polymers and lack of structures promoting cellular functions.

[0021] Therefore, the main objective of the present invention is to provide bioink formulations suitable for use in 3D bioprinting.

[0022] Another objective of the present invention is to provide bioink formulations

that are xeno-free (i.e. free of one or more animal derived components).

[0023] Another objective of the present invention is to provide bioink formulations that are biocompatible, biodegradable, and show no cell toxicity.

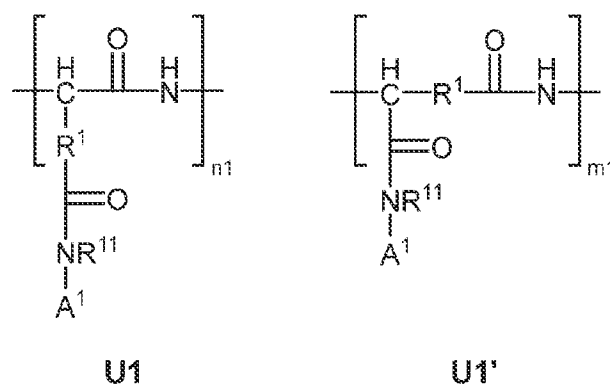
[0024] Yet another objective of the present invention is to provide bioink formulations that maintain high cell viability and promote cellular functions including, but not limited to, attachment, proliferation, metabolism, differentiation, and extracellular matrix deposition.

[0025] Yet another objective of the present invention is to provide bioink formulations that show better printability and crosslinkability to ensure high dimensional fidelity and structural integrity of printed structures created therefrom so to maintain 3D bioprinted structures.

[0026] Yet another objective of the present invention is to provide bioink formulations that exhibit beneficial shear thinning properties.

Summary

[0027] The embodiments of the present invention described hereinafter provide a technical solution to the aforementioned objectives. In a first embodiment, the present invention provides a bioink formulation comprising a polymer, wherein the polymer comprises a repeating unit U1 and a repeating unit U1':



wherein:

R^1 is selected from alkylene, preferably from C_1 - C_{20} alkylene, more preferably from C_1 - C_{10} alkylene, most preferably from methylene, ethylene and propylene;

R^{11} is selected from hydrogen or alkyl, preferably from hydrogen or C_1 - C_{20} alkyl, more preferably from hydrogen or C_1 - C_{10} alkyl, most preferably from hydrogen, methyl, ethyl and propyl;

A^1 is a moiety imparting hydrophilicity to the polymer;

$n1$ is an integer from 1 to 5,000, preferably from 10 to 5,000, more preferably from 100 to 5,000; and

$m1$ is an integer from 1 to 5,000, preferably from 10 to 5,000, more preferably from 100 to 5,000.

[0028] In a second embodiment, the present invention provides a method for producing a three-dimensional object, wherein the bioink formulation according to the first embodiment of the present invention is applied to a 3D bioprinting technique to produce said three-dimensional object.

[0029] In a third embodiment, the present invention provides a three-dimensional object, which is obtainable or obtained by the method for producing a three-dimensional object according to the second embodiment of the present invention.

[0030] The present invention is further described in the embodiments following hereinafter in the detailed description.

Brief Description of the Figures

[0031] Fig. 1: A) Schematic synthesis of PAspAm-MA (**1**). B) 1H NMR spectrum of PAspAm-MA (**1**).

[0032] Fig. 2: Rheological analysis of time-dependent shear storage modulus (G') and shear loss modulus (G'') on visible light (405 nm) irradiated PAspAm-MA (**1**) at 5% and 10% wt/v concentrations under room temperature.

[0033] Fig. 3: Cell viability in PAspAm-MA (**1**) hydrogel. A) Live/Dead stained 3T3 fibroblasts encapsulated in PAspAm-MA (**1**) hydrogel after 1 and 3 days of

culture. Scale bar = 300 μm . B) Cell viability calculated as percentage of live cells from the Live/Dead staining images. C) Cell metabolism measured by PrestoBlue® fluorescence assay. * $p < 0.05$.

[0034] Fig. 4: Printability of PAspAm-MA (1) bioink by digital light processing (DLP). A M shape was printed with dimensions at 25mmx13mmx0.6mm.

[0035] Fig. 5: A) Schematic synthesis of PAspAm-Tyr (2). B) ^1H NMR spectrum of PAspAm-Tyr (2).

[0036] Fig. 6: Rheological analysis of time-dependent shear storage modulus (G') on visible light (405 nm) irradiated PAspAm-Tyr (2).

[0037] Fig. 7: Printability of PAspAm-Tyr (2) bioink by digital light processing (DLP). A) A grid shape with dimension at 9 mm x 9 mm x 0.5 mm. B) A word, "BIOINK", was printed with dimension at 34 mm x 7.2 mm x 0.5mm.

[0038] Fig. 8: Cell growth (hMSCs) in DLP printed PAspAm-Tyr (2) bioink hydrogels. A) Live/Dead stained hMSCs encapsulated in bioprinted PAspAm-Tyr (2) hydrogel after 1, 7 and 14 days of culture. Scale bar = 750 μm . B) Cell metabolism measured by PrestoBlue® fluorescence assay. * $p < 0.05$. C) Cell viability calculated as percentage of live cells (gray) from the Live/Dead staining images.

[0039] Fig. 9: Cell growth (3T3 fibroblasts) in DLP printed PAspAm-Tyr-RGD+ (3) and PAspAm-Tyr (2) bioink hydrogels. PEGDA(6k) is used as the control group. Live/Dead stained 3T3 fibroblasts encapsulated in bioprinted PAspAm-Tyr-RGD+ (3), PAspAm-Tyr (2) and PEGDA(6k) hydrogels after 1, 7, 14 and 21 days of culture. Scale bar = 750 μm .

[0040] Fig. 10: Cell growth (3T3 fibroblasts) in DLP printed PAspAm-Tyr-RGD+ (3) and PAspAm-Tyr (2) bioink hydrogels. PEGDA(6k) is used as the control group. A) Cell metabolism measured by PrestoBlue® fluorescence assay. * $p < 0.05$. B) Cell viability calculated as percentage of live cells (gray) from the Live/Dead staining images.

[0041] Fig. 11: A) Schematic synthesis of PAspAm-Nor (4). B) ^1H NMR spectrum

of PAspAm-Nor (**4**).

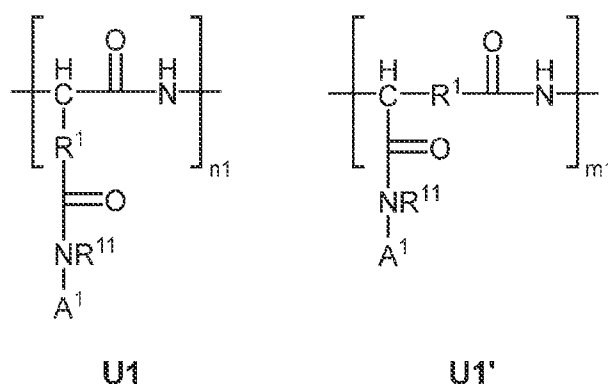
[0042] Fig. 12: Crosslinking mechanism of the PAspAm-Nor-RGD++ (**4a**) bioink based on thiol-ene click chemistry.

[0043] Fig. 13: Rheological analysis of time-dependent shear storage modulus (G') on visible light (405 nm) irradiated PAspAm-Nor (**4**) and PAsp-Am-Nor-RGD++ (**4a**) bioinks.

[0044] Fig. 14: Cell growth (3T3 fibroblasts) in DLP printed PAspAm-Nor (**4**) and PAspAm-Nor-RGD++ (**4a**) bioink hydrogels. A) Live/Dead stained 3T3 fibroblasts encapsulated in bioprinted PAspAm-Nor (**4**) and PAspAm-Nor-RGD++ (**4a**) hydrogels after 1, 7, 14, 21 and 28 days of culture. Scale bar = 1500 μm . B) Cell metabolism measured by PrestoBlue® fluorescence assay.

Detailed Description

[0045] In a first embodiment of the present invention, a bioink formulation is provided comprising a polymer, wherein the polymer comprises a repeating unit U1 and a repeating unit U1':



wherein:

R^1 is selected from alkylene, preferably from C_1 - C_{20} alkylene, more preferably from C_1 - C_{10} alkylene, most preferably from methylene, ethylene and propylene;

R^{11} is selected from hydrogen or alkyl, preferably from hydrogen or C_1 - C_{20} alkyl, more preferably from hydrogen or C_1 - C_{10} alkyl, most preferably from hydrogen, methyl, ethyl and propyl;

A^1 is a moiety imparting hydrophilicity to the polymer;

$n1$ is an integer from 1 to 5,000, preferably from 10 to 5,000, more preferably from 100 to 5,000; and

$m1$ is an integer from 1 to 5000, preferably from 10 to 5,000, more preferably from 100 to 5,000.

[0046] A^1 in the repeating units $U1$ and $U1'$ of the polymer is a moiety imparting hydrophilicity to the polymer. A moiety imparting hydrophilicity to the polymer means a moiety that comprises at least one polar group and/or ionic group capable of interacting with water by forming secondary bonds such as, for example, hydrogen bonds, dipolar bonds, electrostatic interaction, and/or van-der-Waals interaction with water molecules.

[0047] Preferably, A^1 in the repeating units $U1$ and $U1'$ of the polymer comprises one or more groups selected from -OH, -SH, -NH₂, -CO-NH₂ and -CO₂H.

[0048] Preferably, A^1 in the repeating units $U1$ and $U1'$ of the polymer comprises a water-soluble moiety. Such water-soluble moiety includes water-soluble polymer moieties such as, but not limited to, polyethylene oxide, polyvinyl alcohol, and polyacrylamide moieties.

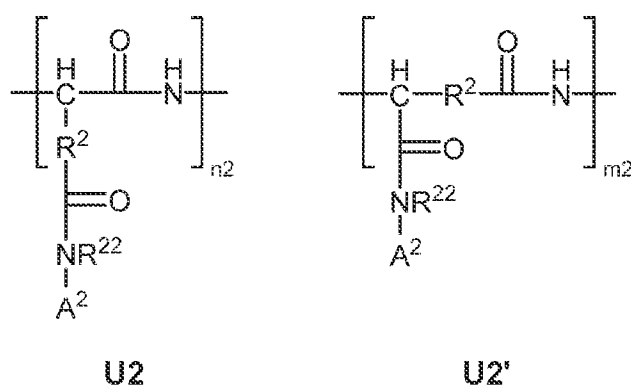
[0049] Preferably, A^1 in the repeating units $U1$ and $U1'$ of the polymer comprises an alkylene unit $-(CH_2)_x-$ and/or an ethylene oxide unit $-(CH_2-CH_2-O)_y-$, wherein x and y are independently from each other integers from 1 to 2,000, preferably from 1 to 100, more preferably from 1 to 10, most preferably 1.

[0050] More preferably, A^1 in the repeating units $U1$ and $U1'$ of the polymer comprises one or more groups selected from -OH, -SH, -NH₂, -CO-NH₂ and -CO₂H; and an alkylene unit $-(CH_2)_x-$ and/or an ethylene oxide unit $-(CH_2-CH_2-O)_y-$, wherein x and y are independently from each other integers from 1 to 2,000, preferably from 1

to 100, more preferably from 1 to 10, most preferably 1.

[0051] Most preferably, A^1 in the repeating units $U1$ and $U1'$ of the polymer is selected from the list consisting of $-\text{CH}_2-\text{CH}_2-\text{OH}$, $-\text{CH}_2-\text{CH}_2-\text{O}-\text{CH}_2-\text{CH}_2-\text{OH}$, $-\text{CH}_2-\text{CH}_2-\text{O}-\text{CH}_2-\text{CH}_2-\text{O}-\text{CH}_2-\text{CH}_2-\text{OH}$, $-\text{CH}_2-\text{CH}_2-\text{O}-\text{CH}_2-\text{CH}_2-\text{OCH}_3$, $-\text{CH}_2-\text{CH}_2-\text{O}-\text{CH}_2-\text{CH}_2-\text{O}-\text{CH}_2-\text{CH}_2-\text{OCH}_3$, $-\text{CH}_2-\text{CH}_2-\text{SH}$, $-\text{CH}_2-\text{CH}_2-\text{O}-\text{CH}_2-\text{CH}_2-\text{SH}$, $-\text{CH}_2-\text{CH}_2-\text{NH}_2$, $-\text{CH}_2-\text{CH}_2-\text{O}-\text{CH}_2-\text{CH}_2-\text{NH}_2$, $-\text{CH}_2-\text{CH}_2-\text{CO}_2\text{H}$, $-\text{CH}_2-\text{CH}_2-\text{O}-\text{CH}_2-\text{CH}_2-\text{CO}_2\text{H}$, $-\text{CH}_2-\text{CH}_2-\text{CO}_2\text{H}$, and $-\text{CH}_2-\text{CH}_2-\text{O}-\text{CH}_2-\text{CH}_2-\text{CO}_2\text{H}$.

[0052] In a preferred embodiment of the present invention, the polymer further comprises a repeating unit $U2$ and a repeating unit $U2'$:



wherein:

R^2 is independently from each other selected from alkylene, preferably from $\text{C}_1\text{-C}_{20}$ alkylene, more preferably from $\text{C}_1\text{-C}_{10}$ alkylene, most preferably from methylene, ethylene and propylene;

R^{22} is independently from each other selected from hydrogen or alkyl, preferably from hydrogen or $\text{C}_1\text{-C}_{20}$ alkyl, more preferably from hydrogen or $\text{C}_1\text{-C}_{10}$ alkyl, most preferably from hydrogen, methyl, ethyl and propyl;

A^2 is a moiety containing at least one reactive group which can be further reacted to crosslink two or more polymer chains;

$n2$ is an integer from 1 to 5,000, preferably from 10 to 5,000, more preferably from 100 to 5,000; and

m2 is an integer from 1 to 5,000, preferably from 10 to 5,000, more preferably from 100 to 5,000.

[0053] Preferably, the reactive group contained in A² in the repeating units U2 and U2' is selected from the list consisting of groups capable to undergo polymerization crosslinking reactions, groups capable to undergo Michael-type or click chemistry-type crosslinking reactions, groups capable to undergo oxidative crosslinking reactions, and groups capable to undergo redox crosslinking reactions.

[0054] More preferably, the reactive group contained in A² in the repeating units U2 and U2' of the polymer is selected from groups capable to undergo polymerization crosslinking reactions, wherein preferably said groups contain one or more vinyl or allyl groups.

[0055] More preferably, the reactive group contained in A² in the repeating units U2 and U2' of the polymer is selected from groups capable to undergo Michael-type or click chemistry-type crosslinking reactions, wherein preferably said groups contain one or more acrylate, methacrylate, n-alkyne, cyclo-alkyne, amine, azide, carboxyl, hydrazide, hydroxyl, maleimide, norbornene, tetrazine and/or thiol groups. Most preferably, A² in the repeating units U2 and U2' of the polymer is selected from tyramine moieties and tyrosine moieties.

[0056] More preferably, the reactive group contained in A² in the repeating units U2 and U2' of the polymer is selected from groups capable to undergo oxidative crosslinking reactions, wherein preferably said groups contain one or more aromatic moieties with one or more aromatic hydroxyl groups, wherein more preferably said groups contain one or more mono-phenol, di-phenol, tri-phenol, oligo-phenol and/or poly-phenol moieties, wherein particularly preferably said groups contain a mono-phenol moiety. Most preferably, A² in the repeating units U2 and U2' of the polymer is selected from -CH₂-CH₂-C₆H₄-OH and -CH(CO₂H)-CH₂-C₆H₄-OH.

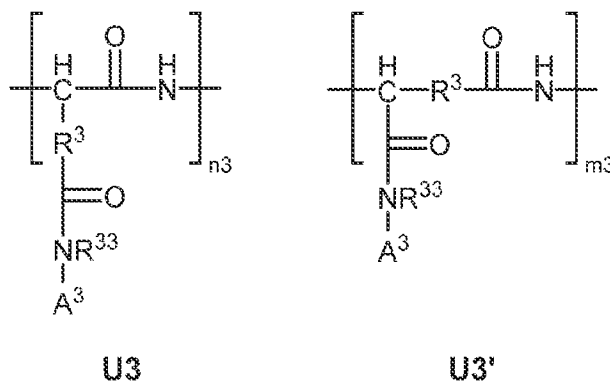
[0057] More preferably, the reactive group contained in A² in the repeating units U2 and U2' of the polymer is selected from groups capable to undergo redox crosslinking reactions, wherein preferably said groups contain one or more thiol and/or amine groups. Most preferably, A² in the repeating units U2 and U2' of the

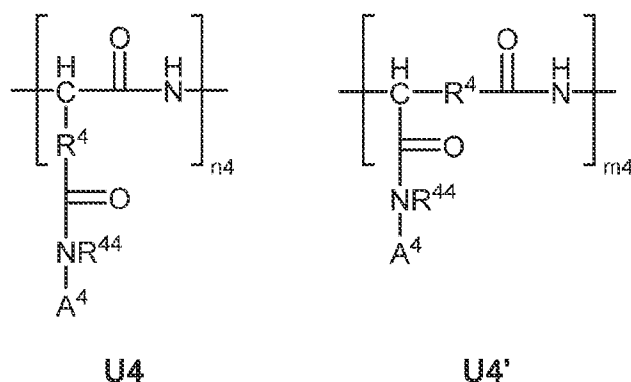
polymer is selected from glutathione moieties and histidine moieties.

[0058] In a preferred embodiment of the present invention, the bioink formulation comprises a reactive peptide compound capable to react with A². Preferably, the reactive peptide compound comprises one or more functional groups capable to react with the reactive group contained in A². Preferred functional groups capable to react with the reactive group contained in A² are selected from the list consisting of allyl, styryl, vinyl, acrylate, methacrylate, maleimide and thiol.

[0059] Preferred reactive peptide compounds are RGD containing peptide compounds having one or more functional groups selected from the list consisting of allyl, styryl, vinyl, acrylate, methacrylate, maleimide and thiol. A particularly preferred reactive peptide compound is H-Gly-Arg-Gly-Asp-Ser-Pro-Cys-OH (SEQ ID NO: 11) having a thiol group.

[0060] In a more preferred embodiment of the present invention, the polymer further comprises (i) a repeating unit U3 and a repeating unit U3'; or (ii) a repeating unit U4 and a repeating unit U4'; or (iii) a repeating unit U3, a repeating unit U3', a repeating unit U4 and a repeating unit U4':





wherein:

R^3 and R^4 are independently from each other selected from alkylene, preferably from C_1 - C_{20} alkylene, more preferably from C_1 - C_{10} alkylene, most preferably from methylene, ethylene and propylene;

R^{33} and R^{44} are independently from each other selected from hydrogen or alkyl, preferably from hydrogen or C_1 - C_{20} alkyl, more preferably from hydrogen or C_1 - C_{10} alkyl, most preferably from hydrogen, methyl, ethyl and propyl;

A^3 is a moiety imparting cellular function to the polymer;

A^4 is a moiety imparting shear thinning properties to the polymer;

wherein in case (i):

n_3 is an integer from 1 to 5,000, preferably from 10 to 5,000, more preferably from 100 to 5,000;

m_3 is an integer from 1 to 5,000, preferably from 10 to 5,000, more preferably from 100 to 5,000; and

the sum of n_1 , n_2 , n_3 , m_1 , m_2 and m_3 is greater than 5, preferably greater than 50, more preferably greater than 500; or

wherein in case (ii):

n_4 is an integer from 1 to 5,000, preferably from 10 to 5,000, more preferably from 100 to 5,000;

m4 is an integer from 1 to 5,000, preferably from 10 to 5,000, more preferably from 100 to 5,000; and

the sum of n1, n2, n4, m1, m2, and m4 is greater than 5, preferably greater than 50, more preferably greater than 500, or

wherein in case (iii):

n3 is an integer from 1 to 5,000, preferably from 10 to 5,000, more preferably from 100 to 5,000;

m3 is an integer from 1 to 5,000, preferably from 10 to 5,000, more preferably from 100 to 5,000;

n4 is an integer from 1 to 5,000, preferably from 10 to 5,000, more preferably from 100 to 5,000;

m4 is an integer from 1 to 5,000, preferably from 10 to 5,000, more preferably from 100 to 5,000; and

where in case (iii) the sum of n1, n2, n3, n4, m1, m2, m3 and m4 is greater than 7, preferably greater than 50, more preferably greater than 500.

[0061] A³ in the repeating units U3 and U3' of the polymer is a moiety imparting cellular function to the polymer. Preferred cellular function includes, but is not limited to, attachment, proliferation, metabolism, differentiation, and extracellular matrix deposition.

[0062] Preferably, A³ in the repeating units U3 and U3' of the polymer contains one or more synthetic functional peptide sequences found in native proteins, wherein preferably said native proteins are fibronectin, laminin, collagen, elastin, albumin, immunoglobulin or silk.

[0063] Preferred synthetic functional peptide sequences are selected from the list consisting of RGD, YIGSR (SEQ ID NO: 1), IKVAV (SEQ ID NO: 2), DGEA (SEQ ID NO: 3), PHSRN (SEQ ID NO: 4), PRARI (SEQ ID NO: 5), (GAGAGS)_p (SEQ ID NO: 6), (VPGXG)_n (SEQ ID NO: 7), (GPO)_m, and Gly-Lys-Gly-Tyr-Gly-Arg-Gly-Asp-Ser-Pro-Gly (SEQ ID NO: 8), wherein p, n and m are independently from each

other integers of ≥ 1 .

[0064] A^4 in the repeating units U4 and U4' of the polymer is a moiety imparting shear thinning properties to the polymer.

[0065] Preferably, A^4 in the repeating units U4 and U4' of the polymer is a moiety imparting shear thinning properties due to supramolecular interactions.

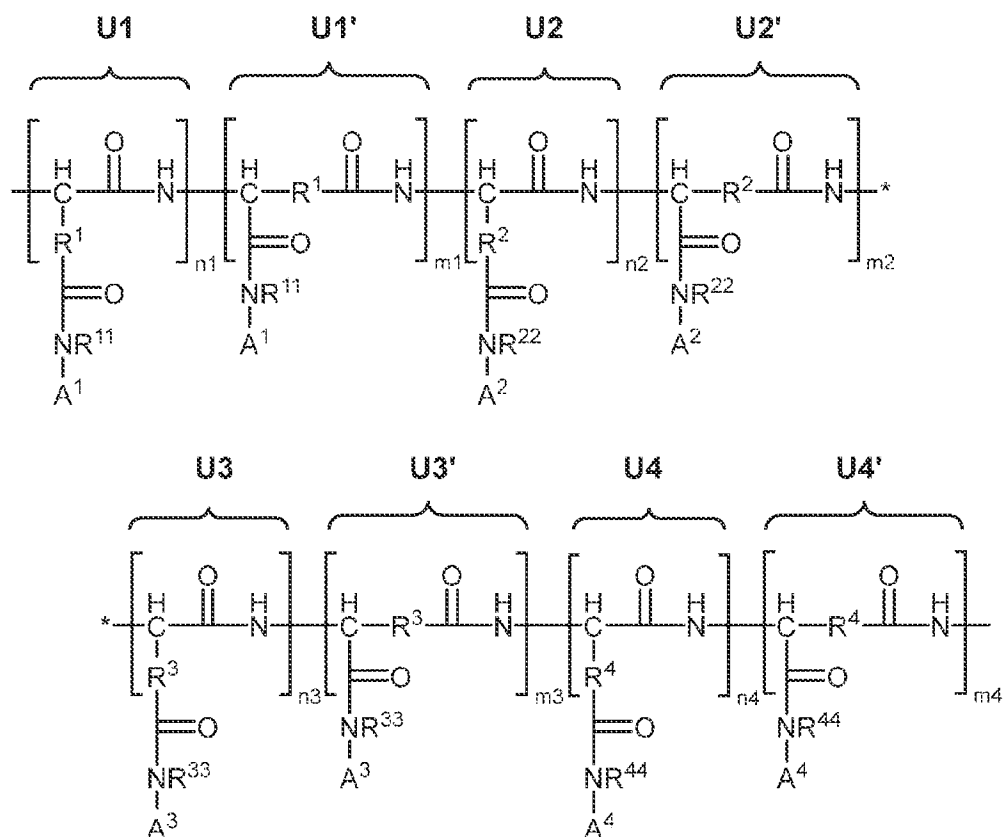
[0066] Preferred supramolecular interactions are host-guest or self-assembly interactions, wherein such interactions preferably include interactions between cyclodextrin, adamantane, cucurbit[8]uril, cholesterol, polyethylene glycol and ureidopyrimidinone.

[0067] In a preferred embodiment of the present invention, A^4 in the repeating units U4 and U4' of the polymer contains one or more cyclodextrin, adamantane, cucurbit[8]uril, cholesterol, polyethylene glycol or ureidopyrimidinone moieties.

[0068] In a more preferred embodiment of the present invention, A^4 in the repeating units U4 and U4' of the polymer is a cyclodextrin, adamantane, cucurbit[8]uril, cholesterol, polyethylene glycol or ureidopyrimidinone moiety.

[0069] In a particularly preferred embodiment of the present invention, the polymer

is represented by Formula I:



Formula I

wherein:

R^1 , R^2 , R^3 and R^4 are independently from each other selected from alkylene, preferably from C_1 - C_{20} alkylene, more preferably from C_1 - C_{10} alkylene, most preferably from methylene, ethylene and propylene;

R^{11} , R^{22} , R^{33} and R^{44} are independently from each other selected from hydrogen or alkyl, preferably from hydrogen or C_1 - C_{20} alkyl, more preferably from hydrogen or C_1 - C_{10} alkyl, most preferably from hydrogen, methyl, ethyl and propyl;

A^1 is a moiety imparting hydrophilicity to the polymer;

A^2 is a moiety containing at least one reactive group which can be further reacted to crosslink two or more polymer chains;

A³ is a moiety imparting cellular function to the polymer;

A⁴ is a moiety imparting shear thinning properties to the polymer;

n1 is an integer from 1 to 5,000, preferably from 10 to 5,000, more preferably from 100 to 5,000;

m1 is an integer from 1 to 5,000, preferably from 10 to 5,000, more preferably from 100 to 5,000;

n2 is an integer from 1 to 5,000, preferably from 10 to 5,000, more preferably from 100 to 5,000;

m2 is an integer from 1 to 5,000, preferably from 10 to 5,000, more preferably from 100 to 5,000;

n3 is an integer from 0 to 5,000, preferably from 1 to 5,000, more preferably from 10 to 5,000, most preferably from 100 to 5,000;

m3 is an integer from 0 to 5,000, preferably from 1 to 5,000, more preferably from 10 to 5,000, most preferably from 100 to 5,000;

n4 is an integer from 0 to 5,000, preferably from 1 to 5,000, more preferably from 10 to 5,000, most preferably from 100 to 5,000; and

m4 is an integer from 0 to 5000, preferably from 1 to 5,000, more preferably from 10 to 5,000, most preferably from 100 to 5,000;

wherein the sum of n1, n2, n3, n4, m1, m2, m3 and m4 is greater than 5, preferably greater than 7, more preferably greater than 50, most preferably greater than 500.

[0070] The repeating units U1, U1', U2, U2', U3, U3', U4 and U4' can be distributed in the polymer represented by Formula I in any order. The polymer can thus be present, for example, as a random copolymer, as an alternating copolymer, and/or as a block copolymer. The asterisk * denotes a linkage of the two parts of the polymer shown in Formula I.

[0071] Preferred embodiments for the moieties A¹ to A⁴ in the polymer represented

by Formula I are as indicated above.

[0072] In a preferred embodiment of the present invention, the polymer in the bioink formulation has a weight average molecular weight (M_w) in the range from 1,000 Da (1 kDa) to 100,000,000 Da (100 MDa), preferably from 10,000 Da (10 kDa) to 10,000,000 Da (10 MDa), more preferably from 20,000 Da (20 kDa) to 5,000,000 Da (5 MDa), most preferably from 30,000 Da (30 kDa) to 1,000,000 Da (1 MDa). The weight average molecular weight (M_w) can be determined by any standard method known to those skilled in the art, such as GPC.

[0073] In a preferred embodiment of the present invention, the mass concentration of the polymer in the bioink formulation is from 0.1% (wt/v) to 99% (wt/v), preferably from 1% (wt/v) to 70% (wt/v), more preferably from 2% (wt/v) to 40% (wt/v), particularly preferably from 5% (wt/v) to 30% (wt/v), and most preferably from 5% (wt/v) to 20% (wt/v), based on the total volume of the bioink formulation. Depending on the type of the 3D bioprinting technique applied, a person skilled in the art is able to determine suitable mass concentrations of the polymer in the bioink formulation.

[0074] In a preferred embodiment of the present invention, the bioink formulation comprises one or more diluents. Such diluents typically act as solvents and/or dispersants diluting the polymer in the bioink formulation. Preferred diluents are compounds that are liquid at room temperature. Preferred diluents are water, cell culture media such as, for example, DMEM, RPMI and MEM, or water-based buffer systems such as, for example, phosphate buffered saline (PBS), Hank's buffer, Earle's balanced salt solution, Tyrode buffer, HEPES buffer, etc.

[0075] In a preferred embodiment of the present invention, the bioink formulation further comprises one or more selected from crosslinking reagents and crosslinking additives. The crosslinking reagent serves the purpose to crosslink the polymers in the bioink formulation to form a crosslinked three-dimensional hydrogel. The crosslinking additive serves the purpose to react with the polymer in the bioink formulation to form a crosslinked three-dimensional hydrogel.

[0076] Preferred crosslinking reagents are selected from the list consisting of photoinitiators, thermal initiators, basic initiators, oxidative initiators, enzymes

mediating oxidative crosslinking, and agents mediating redox crosslinking.

[0077] Preferred photoinitiators are selected from the list consisting of acetophenone, p-anisil, benzil, benzoin, benzophenone, 2-benzoylbenzoic acid, 4,4'-bis(diethylamino)benzophenone, 4,4'-bis(dimethylamino)benzophenone, benzoin methyl ether, benzoin isopropyl ether, benzoin isobutyl ether, benzoin ethyl ether, 4-benzoylbenzoic acid, 2,2'-bis(2-chlorophenyl)-4,4',5,5'-tetraphenyl-1,2'-biimidazole, methyl 2-benzoylbenzoate, 2-(1,3-benzodioxol-5-yl)-4,6-bis(trichloromethyl)-1,3,5-triazine, 2-benzyl-2-(dimethylamino)-4'-morpholinobutyrophenone, ($\pm$)-camphorquinone, 2-chlorothioxanthone, 4,4'-dichlorobenzophenone, 2,2-diethoxyacetophenone, 2,2-dimethoxy-2-phenylacetophenone, 2,4-diethylthioxanthene-9-one, diphenyl(2,4,6-trimethylbenzoyl)phosphine oxide, 1,4-dibenzoylbenzene, eosin Y, 2-ethylanthraquinone, 1-hydroxycyclohexyl phenyl ketone, 2-hydroxy-2-methylpropiophenone, 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone, 2-isopropylthioxanthone, lithium phenyl(2,4,6-trimethylbenzoyl)phosphinate, 2-methyl-4'-(methylthio)-2-morpholinopropiophenone, 2-isonitrosopropiophenone, 2-phenyl-2-(p-toluenesulfonyloxy)acetophenone, phenylbis(2,4,6-trimethylbenzoyl)phosphine oxide, tris(2,2'-bipyridyl)dichlororuthenium(II) hexahydrate, and a combination of tris(2,2'-bipyridyl)dichlororuthenium(II) hexahydrate and sodium persulfate.

[0078] More preferred photoinitiators are selected from the list consisting of eosin Y, 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone, lithium phenyl-2,4,6-trimethylbenzoylphosphinate, tris(2,2'-bipyridyl)dichlororuthenium(II) hexahydrate, and a combination of tris(2,2'-bipyridyl)dichlororuthenium(II) hexahydrate and sodium persulfate.

[0079] Preferred thermal initiators are selected from the list consisting of tert-amyl peroxybenzoate, 4,4'-azobis(4-cyanovaleric acid), 1,1'-azobis(cyclohexanecarbonitrile), 2,2'-azobisisobutyronitrile (AIBN), 2,2'-azobis(2-methylpropionamidine)dihydrochloride, benzoyl peroxide, 2,2-bis(tert-butylperoxy)butane, 1,1-bis(tert-butylperoxy)cyclohexane, 2,5-bis(tert-butylperoxy)-2,5-dimethylhexane, 2,5-bis(tert-butylperoxy)-2,5-dimethyl-3-hexyne, bis(1-(tert-butylperoxy)-1-methylethyl)benzene, 1,1-bis(tert-butylperoxy)-3,3,5-trimethylcyclohexane, tert-butyl hydroperoxide, tert-butyl peracetate, tert-butyl

peroxide, tert-butyl peroxybenzoate, tert-butylperoxy isopropyl carbonate, cumene hydroperoxide, cyclohexanone peroxide, dicumyl peroxide, lauroyl peroxide, 2,4-pentanedione peroxide, peracetic acid, ammonium persulfate, potassium persulfate, and sodium persulfate.

[0080] More preferred thermal initiators are selected from the list consisting of 4,4-azobis(4-cyanovaleric acid), 2,2'-azobisisobutyronitrile (AIBN), 2,2'-azobis(2-methylpropionamidine)dihydrochloride, benzoyl peroxide, tert-butyl peroxide, ammonium persulfate, potassium persulfate, and sodium persulfate.

[0081] Preferred basic initiators are selected from the list consisting of hydroxides, amines and amides. More preferred basic initiators are selected from the list consisting of MOH, MOR, NH₃, RNH₂, and R₂NM, wherein M is an alkali metal, preferably Li, Na, K; and R is a carbyl moiety, preferably C₁-C₆ alkyl. Most preferred basic initiators are selected from the list consisting of OH⁻, NH₃ and (iPr)₂NLi (LDA).

[0082] Preferred oxidative initiators are selected from the list consisting of hydroxyl (HO[•]) and peroxy (ROO[•]), peroxyxynitrous acid/peroxyxynitrite (ONOOH/ONOO⁻), nitrogen dioxide (NO₂[•]), nitrosoperoxyxynitrite (ONOO⁻CO₂⁻), carbonate (CO₃⁻), and lipid hydroperoxides (LOOH), wherein R is a carbyl moiety, preferably C₁-C₆ alkyl, and L is a lipid moiety.

[0083] Preferred enzymes mediating oxidative crosslinking are selected from the peroxidase families, including horseradish peroxidase, myeloperoxidase, laccase, etc.

[0084] Preferred agents mediating redox crosslinking are selected from the list consisting of peroxides, hypochlorous acid, chloramines, hypobromous acid, bromamines, hypothiocyanous acid, nitroxyl, peroxyxynitrous acid, and other nitrating species.

[0085] In a preferred embodiment of the present invention, the bioink formulation comprises two crosslinking reagents selected from the lists described above.

[0086] In a more preferred embodiment of the present invention, the bioink formulation comprises lithium phenyl-2,4,6-trimethylbenzoylphosphinate.

[0087] In a more preferred embodiment of the present invention, the bioink

formulation comprises a combination of tris(2,2'-bipyridyl)dichlororuthenium(II) hexahydrate and sodium persulfate.

[0088] Preferred crosslinking additives are compounds comprising two or more polymerizable groups with at least one C=C double bond. Preferred polymerizable groups with at least one C=C double bond are selected from the list consisting of acrylate, methacrylate, allyl, styryl and vinyl groups. Alternatively preferred crosslinking additives are compounds comprising two or more thiol groups, preferably three or more thiol groups, more preferably four thiol groups. Examples of this include 4-armed-PEG-SH.

[0089] In a preferred embodiment of the present invention, the concentration of the crosslinking reagents in the bioink formulation is from 0.1 mM to 100 mM, preferably from 0.2 mM to 50 mM, more preferably from 0.5 mM to 40 mM, particularly preferably from 1 mM to 30 mM, even particularly preferably from 5 mM to 25 mM, and most preferably from 15 mM to 25 mM.

[0090] The bioink formulations of the present invention are useful for different 3D bioprinting techniques including DLP-based bioprinting, droplet-based bioprinting, extrusion-based bioprinting, forward transfer bioprinting, inkjet bioprinting, integrated bioprinting, laser-induced bioprinting, stereolithography-based bioprinting, magnetic bioprinting, and volumetric bioprinting.

[0091] In addition, the bioink formulations of the present invention are compatible with several human and non-human cell types and are therefore useful to print various tissue constructs. This is illustrated by the cell lines used in the experimental examples hereinbelow such as, for example, mouse 3T3 fibroblasts, human bone marrow-derived mesenchymal stem cells, etc., which should not be construed as limiting in any way.

[0092] In conclusion, the bioink formulations described herein overcome the disadvantages known from the prior art. In particular, the bioink formulations described herein are suitable for use in 3D bioprinting, they are biocompatible, biodegradable, and show no cell toxicity, they maintain high cell viability and promote cellular functions including, but not limited to, attachment, proliferation, metabolism, differentiation, and extracellular matrix deposition, they show better

printability and crosslinkability to ensure high dimensional fidelity and structural integrity of printed structures created therefrom so to maintain 3D bioprinted structures, and they exhibit beneficial shear thinning properties.

[0093] In a second embodiment of the present invention, a method for producing a three-dimensional object is provided, wherein the bioink formulation according to the present invention is applied to a 3D bioprinting technique to produce said three-dimensional object.

[0094] Similar to thermoplastics commonly used in traditional 3D printing, bioinks can be extruded through printing nozzles or needles into filaments that retain their shape once applied. However, bioinks are sensitive to the processing conditions of conventional 3D printing. Compared to traditional 3D printing materials, bioinks typically require lower printing temperatures (typically 37°C or less) and milder curing conditions to avoid compromising cytocompatibility and bioactivity.

[0095] Extrusion-based bioprinting and digital light processing (DLP)-based bioprinting are both 3D bioprinting techniques commonly used to produce biofabricated three-dimensional objects. In extrusion-based bioprinting, a bioink in solution or semi-solution form is loaded into a cartridge and a mechanical force, usually air pressure or a motor-drive piston or screw, pushes the bioink through a nozzle to form a filament that can be deposited layer by layer to that the desired three-dimensional object is produced. DLP-based bioprinting is also a layer-by-layer process. However, instead of extruding the material through a nozzle, a source of illumination treats each layer with a still image. This image is projected into a vat of light-sensitive liquid, triggering a chemical reaction that causes the liquid to cure in the illuminated area. The printed three-dimensional object is obtained by stacking these cured layers on a build platform.

[0096] Traditional bioprinting techniques involve depositing material layer-by-layer to create the end structure, but recently a new method called volumetric bioprinting was reported (see: P.N. Bernal, P. Delrot, D. Loterie, Y. Li, J. Malda, C. Moser and R. Levato, Volumetric Bioprinting of Complex Living-Tissue Constructs within Seconds, *Adv. Mater.* 2019, 31, 1904209). Volumetric bioprinting occurs when a bioink is placed in a liquid cell and is selectively irradiated by an energy source. This

method will actively polymerize the irradiated material and that will comprise the final structure. Manufacturing biomaterials using volumetric bioprinting of bioinks can greatly decrease the manufacturing time. In materials science, this is a breakthrough that allows personalized biomaterials to be quickly generated.

[0097] Unlike traditional 3D printing materials such as thermoplastics that are essentially fixed once they are printed, bioinks are a dynamic system because of their high-water content and often non-crystalline structure. The shape fidelity of the bioink after filament deposition must also be taken into account. Finally, the printing pressure and nozzle diameter must be taken into account to minimize the shear stresses placed on the bioink and on any cells within the bioink during the printing process. Too high shear forces may damage or lyse cells, adversely affecting cell viability.

[0098] Preferred 3D bioprinting techniques for the method for producing a three-dimensional object according to the present invention are selected from DLP-based bioprinting, droplet-based bioprinting, extrusion-based bioprinting, forward transfer bioprinting, inkjet bioprinting, integrated bioprinting, laser-induced bioprinting, stereolithography-based bioprinting, magnetic bioprinting, and volumetric bioprinting. These 3D bioprinting techniques allow precise placement/positioning in spatially predefined locations within confined 3D structures.

[0099] In a preferred embodiment of the method for producing a three-dimensional object according to present invention, the bioink formulation is subjected to irradiation and/or thermal treatment to crosslink the polymer comprised in the bioink formulation. Such irradiation and/or thermal treatment is typically performed after the bioink formulation has been applied to a 3D bioprinting technique and has been deposited to form a three-dimensional object.

[0100] Preferred irradiation treatment includes IR irradiation, VIS irradiation, and/or UV irradiation. Preferred IR radiation is in the range from 600 nm to 1 mm, more preferably in the range from 750 nm to 900 nm, and most preferably in the range from 770 nm to 790 nm. Preferred VIS radiation is in the range from 400 nm to 700 nm, more preferably in the range from 400 to 500 nm, and most preferably in the range from 400 to 450 nm. Preferred UV radiation is in the range from 100 nm to 400

nm, more preferably in the range from 300 nm to 400 nm, and most preferably in the range from 350 nm to 400 nm. Preferred thermal treatment includes exposure to elevated temperatures up to 120°C, preferably up to 100°C, more preferably up to 60°C. Most preferably, thermal treatment includes exposure to elevated temperatures between 30°C and 40°C. Depending on the type of bioink formulation and the polymer comprised therein, a person skilled in the art is able to determine suitable irradiation and/or thermal treatment conditions.

[0101] The irradiation and/or thermal treatment for crosslinking the polymer in the method for producing a three-dimensional object according to the present invention can be optionally carried out during or after the printing depending on the 3D bioprinting technique used such as, for example, extrusion-based bioprinting or DLP-based bioprinting or any of the other bioprinting techniques mentioned above.

[0102] In a third embodiment of the present invention, a three-dimensional object obtainable or obtained by the method for producing a three-dimensional object according to the present invention is provided. Preferably, said three-dimensional object is for use in tissue engineering, regenerative medicine, cell delivery, drug delivery, drug discovery, wound dressing, biosensors, cosmetics, hygiene products, medical devices, implantable electronics and/or other biomedical applications.

[0103] It is to be understood that a skilled person can freely combine the above-mentioned preferred, more preferred, even more preferred, and most preferred embodiments relating to the bioink formulation and related embodiments of the present invention.

Definitions

[0104] As used herein, the term “synthetic bioink formulation” means bioink formulations that are free from animals, plants, and microorganisms-derived products. The microorganisms include, but are not limited to bacteria, protozoa, algae, and fungi. The synthetic bioink formulations of the present invention are formulated from poly(aspartamide) (PAspAm) derived polymers. Bioinks are materials used to produce engineered/artificial tissue/organs, disease models and organoids using 3D bioprinting. They must meet certain characteristics, including amongst others rheological, mechanical, biofunctional and biocompatible properties. Using bioinks

provides a high reproducibility and precise control over the fabricated constructs in an automated manner. These inks are considered as one of the most advanced tools for tissue engineering and regenerative medicine (TERM).

[0105] As used herein, the term “polymer” includes, but is not limited to, homopolymers, copolymers, for example, block, random, and alternating copolymers, terpolymers, quaterpolymers, etc., and blends and modifications thereof. Furthermore, unless otherwise specifically limited, the term “polymer” shall include all possible configurational isomers of the molecule. These configurations include, but are not limited to isotactic, syndiotactic, and atactic symmetries. A polymer is a molecule of high relative molecular mass, the structure of which essentially comprises the multiple repetition of units (i.e. repeating units) derived, actually or conceptually, from molecules of low relative mass (i.e. monomers).

[0106] As used herein, the term “monomer” refers to a molecule which can undergo polymerization thereby contributing constitutional units (repeating units) to the essential structure of a polymer or an oligomer.

[0107] As used herein, the term “copolymer” generally means any polymer derived from more than one species of monomer, wherein the polymer comprises more than one species of corresponding repeating unit. In one embodiment the copolymer is the reaction product of two or more species of monomer and thus comprises two or more species of corresponding repeating unit. It is preferred that the copolymer comprises two, three, four, five or six species of repeating unit. Copolymers that are obtained by copolymerization of three monomer species can also be referred to as terpolymers. Copolymers that are obtained by copolymerization of four monomer species can also be referred to as quaterpolymers. Copolymers may be present as block, random, and/or alternating copolymers.

[0108] As used herein, the term “block copolymer” refers to a copolymer, wherein adjacent blocks are constitutionally different, i.e. adjacent blocks comprise repeating units derived from different species of monomer or from the same species of monomer but with a different composition or sequence distribution of repeating units.

[0109] As used herein, the term “random copolymer” refers to a copolymer in which the probability of finding a given repeating unit at any given site in the chain is

independent of the nature of the adjacent repeating units. Usually, in a random copolymer, the sequence distribution of repeating units follows Bernoullian statistics.

[0110] As used herein, the term “alternating copolymer” refers to a copolymer consisting of macromolecules comprising two species of repeating units in alternating sequence.

[0111] As used herein, the term “crosslinking reagent” refers to a reagent that provides a reactive species capable of activating a reactive group to react with another reactive group in a crosslinking reaction. This enables the crosslinking of two or more polymer chains that carry reactive groups. Typical crosslinking reagents in the context of the present invention are photoinitiators, thermal initiators, basic initiators, oxidative initiators, enzymes mediating an oxidative crosslinking, and agents mediating a redox crosslinking. The crosslinking reagents mentioned either directly provide a reactive species or must be activated for this purpose, for example by irradiation treatment, thermal treatment, etc.

[0112] As used herein, the term “crosslinking additive” refers to a compound that is capable to react with a reactive group in a crosslinking reaction. For example, the crosslinking additives can react with the reactive groups in the polymer, thereby providing additional crosslinking.

[0113] In the context of the present invention, the term “diluent” as used herein, denotes one or more compounds serving as a solvent, suspending agent, carrier and/or matrix for the polymer and any other component comprised in the bioink formulation. Diluents are typically inert compounds that do not react with said polymers and said other components. Typical diluents are compounds that are liquid at room temperature.

[0114] As used herein, the term “shear thinning” is the non-Newtonian behavior of fluids whose viscosity decreases under shear strain. Bioinks with shear thinning properties enable extrusion at lower extrusion forces thus minimizing damage to cells while bioprinting. The ratio G''/G' of the shear loss modulus (G'') to the shear storage modulus (G') in a viscoelastic material is defined as the $\tan \delta$ (loss tangent), which provides a measure of damping the material. $\tan \delta$ can also be visualized as the tangent of the phase angle δ between the storage and loss modulus. Shear thinning

means that the viscosity decreases with increasing shear rate. This is critical during extrusion-based printing because extruding highly viscous bioink at a relatively high speed (high shear rate) would result in high shear stress, thus killing cells. If the bioink is shear thinning, which means its viscosity is decreasing during extrusion (high shear rate), it lowers the shear stress applied on cells and thereby protecting cells from damage. To some extent, a shear thinning bioink under extrusion (high shear rate) would become less viscous and more liquid, suggesting higher G'' and/or lower G' .

[0115] As used herein, the term “supramolecular interactions” refers to a class of molecular interactions categorized by their non-covalent character. Supramolecular interactions include host-guest interactions or self-assembly interactions based, for example, on van-der-Waals forces, pi-pi stacking, hydrogen bonding, hydrophobic interactions, metal-ligand coordination, and/or electrostatic interactions.

[0116] As used herein, the term “alkyl” refers to a saturated hydrocarbon chain, such as, but not limited to, methyl, ethyl, propyl and butyl. The alkyl group may be straight-chain or branched-chain. For example, as used herein, propyl encompasses both *n*-propyl and *iso*-propyl; butyl encompasses *n*-butyl, *sec*-butyl, *iso*-butyl and *tert*-butyl, and so forth. Divalent alkyl is also referred to as “alkylene” in the present application. Those skilled in the art are familiar with this nomenclature.

[0117] As used herein, the term “allyl” refers to a substituent with the structural formula $R-CH_2-CH=CH_2$, where R is any other group of atoms or H. It consists of a methylene bridge ($-CH_2-$) attached to a vinyl group ($-CH=CH_2$).

[0118] As used herein, the term “vinyl” refers to a substituent with the structural formula $R-C(R')=CH_2$, where R and R' are independently from each other any other group of atoms or H. Preferably, it consists of a vinyl group ($-CH=CH_2$).

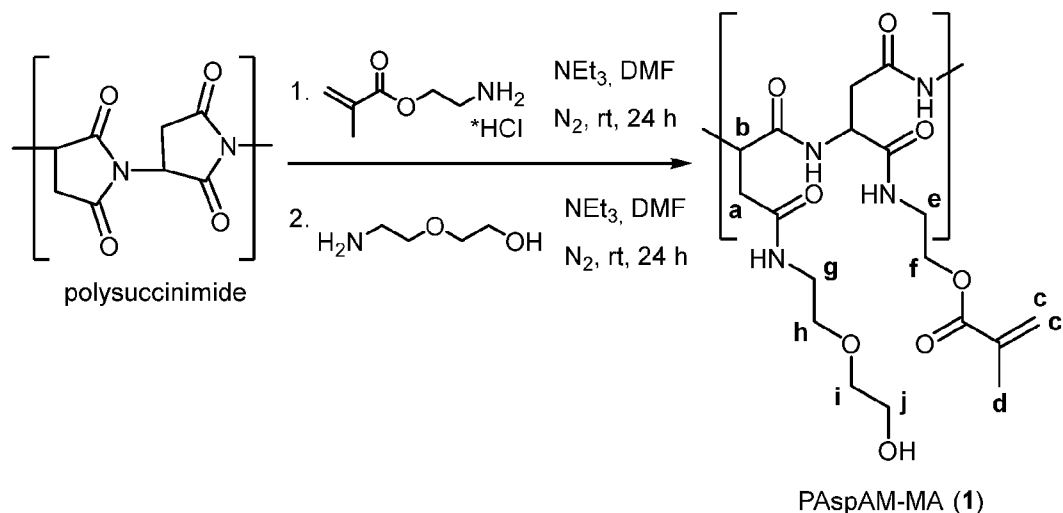
[0119] The present invention is further illustrated by the examples following hereinafter which shall in no way be construed as limiting. A skilled person will acknowledge that various modifications, additions and alternations may be made to the invention without departing from the spirit and scope of the invention as defined

in the appended claims.

Examples

[0120] Example 1

[0121] Synthesis of methacrylated poly(aspartamide) (PAspAm-MA) (**1**)



[0122] PAspAm-MA (**1**) was synthesized by ring opening of polysuccinimide using aminoethyl methacrylate. Polysuccinimide (500 mg) was dissolved in 5 mL anhydrous dimethylformamide (DMF) and aminoethyl methacrylate hydrochloride (237 mg) was added to the solution. The reaction was carried out in the presence of 0.72 mL triethylamine under room temperature for 24 h. Then, an excess amount of 2-(2-aminoethoxy) ethanol (0.79 mL) was added to the reaction mixture and stirred for 24 h at room temperature. Both steps were carried out under a nitrogen atmosphere. The reaction mixture was first precipitated in diethyl ether and then dissolved in deionized water, followed by dialysis for 2-3 days and lyophilization. The degree of substitution of methacrylate groups on polysuccinimide was determined by ^1H NMR (Bruker 500MHz) with D_2O as solvent. All spectra were recorded at room temperature.

[0123] The chemical structure of PAspAm-MA (**1**) was confirmed by ^1H NMR (see Figure 1). The methylene (2H) and methine (1H) protons on the poly(aspartamide) backbone showed peaks at 2.5-2.9 ppm and 4.6 ppm. The acrylic protons (2H) of the methacrylate were located at 5.6 and 6.0 ppm. The peaks at 3.3-3.6 ppm indicated the

methylene protons (2H) in 2-(2-aminoethoxy) ethanol. The degree of substitution of the methacrylate group calculated from the ^1H NMR was 7.2%.

[0124] Rheological properties of PAspAm-MA (1)

[0125] Rheology measurements were performed at 25 °C on a Discovery HR-2 hybrid Rheometer (TA instruments) fitted with a 20 mm diameter plate-plate geometry. The PAspAm-MA (1) was photocrosslinked in-situ, and oscillatory time sweeps were performed under a shear strain of 1% and frequency of 1 Hz for 5 min. The samples were irradiated with visible light (OmniCure LX500, 405 nm, 8.8 mW/cm²) after the time sweep test started for 60 s. About 320 μL PAspAm-MA (1) solutions (5% and 10% wt/v in deionized water) containing 0.25% wt/v lithium phenyl(2,4,5-trimethylbenzoyl)phosphinate (LAP) were used for each test and the geometry gap was set at 1 mm.

[0126] The time sweep test of the shear storage modulus (G') and shear loss modulus (G'') versus time was carried out to determine the gelation behavior during photo-crosslinking. As shown in Figure 2, both 5% and 10% PAspAm-MA (1) solutions can form gel rapidly and their shear moduli can reach a plateau within 30 s and 60 s under light exposure, respectively. The G' and G'' of the cured PAspAm-MA (1) hydrogel increased remarkably with increasing polymer concentration. The 5% PAspAm-MA (1) hydrogel had a storage modulus at 0.1 kPa whereas the 10% PAspAm-MA (1) hydrogel had storage modulus at 3 kPa.

[0127] Cell viability in PAspAm-MA (1) hydrogel

[0128] Mouse 3T3 fibroblasts (ATCC) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Corning) in a humidified atmosphere with 5% CO₂ at 37 °C. PAspAm-MA (1) polymer and LAP were dissolved in phosphate buffered saline (PBS) and filtered through a 0.2 μm filter, then mixed with cell suspension. The final concentrations for each component were 5% and 10% wt/v of PAspAm-MA (1) polymer, 0.25% wt/v of LAP and 2.5 M/mL of mouse 3T3 fibroblasts, respectively. The polymer/cell mixture solution was added to a 96-well plate at 32 μL per well and cured at 405 nm light (8.8 mW/cm²) for 1 min. The cell viability in the cell-laden PAspAm-MA (1) hydrogel was quantified by PrestoBlue® cell viability agent

(Invitrogen) at 1 and 3 days ($n = 4$). A Live/Dead cell staining kit (Live, calcein-AM; Dead, propidium iodide; MilliporeSigma) was used to visualize the cell growth in the PAspAm-MA (1) hydrogel.

[0129] Live/Dead staining and resazurin assay were used to qualitatively and quantitatively evaluate the cell viability in the PAspAm-MA (1) hydrogel over 3 days of culture (see Figure 3). Live/Dead staining images showed the 3T3 fibroblasts in both 5% and 10% PAspAm-MA (1) hydrogels were considerably viable and a small portion of cells started to elongate at day 3 (see Figure 3A). The cell survival rates of the 3T3 fibroblasts inside the PAspAm-MA (1) hydrogels were all above 93% from day 1 to day 3 (see Figure 3B). The PrestoBlue® assay showed the cell metabolism in 10% PAspAm-MA (1) hydrogel was significantly higher than that in 5% PAspAm-MA (1) hydrogel during 3 days of culture (see Figure 3C), which might be because the 5% PAspAm-MA (1) hydrogel network is too loose to entrap all the seeded cells, thus resulting in cell loss into medium. There was no significant difference on cell viability between day 1 and day 3 within each group. The results support the PAspAm-MA (1) has no significant cell toxicity, thus can be potentially used in biomedical applications.

[0130] Preparation of PAspAm-MA (1) bioink formulation

[0131] A bioink is formulated by dissolving 500 mg of PAspAm-MA (1) in 5 mL of PBS buffer. To this solution LAP (25 mg) and tartrazine (2.5 mg) was added and then vortexed to complete dissolution. The solution was sonicated for 30 min to remove air bubbles and filtered through a 0.45 micro filter.

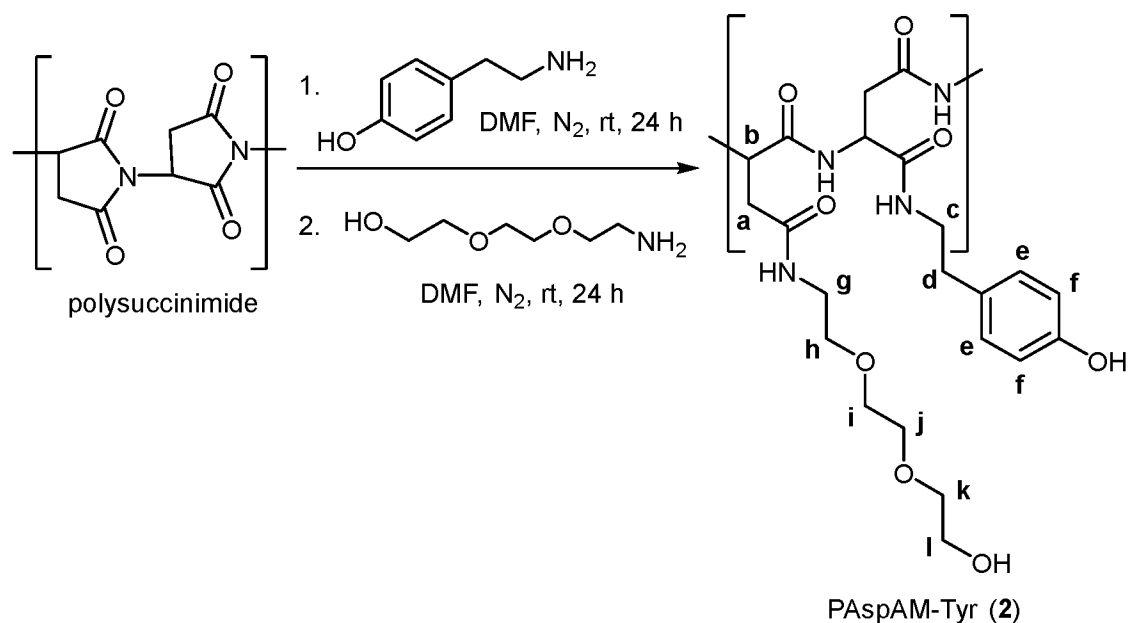
[0132] Printability of PAspAm-MA (1) bioink formulation

[0133] The bioink of the above formulation was printed on a digital light processing (DLP) bioprinter, Lumen X+ (Volumetric Bio and Cellink). The printing was carried out at room temperature under near visible light exposure (405 nm, 20 mW/cm²). A M shape print was obtained at a dimension of 25 mm (L)*13 mm (W)*0.6 mm (H) (see

Figure 4).

[0134] Example 2

[0135] Synthesis of tyramine-functionalized polyaspartamide (PAspAm-Tyr) (**2**)



[0136] PAspAm-Tyr (**2**) was synthesized by derivatizing polysuccinimide with tyramine by ring opening nucleophilic reaction. Polysuccinimide (1,000 mg) was dissolved in 10 mL anhydrous dimethylformamide (DMF). Tyramine (144.2 mg) in 10 mL DMF was first added and stirred at room temperature under N₂ for 24 h. Then an excess amount of 2-[2-(2-aminoethoxy)ethoxy]ethanol (2.67 mL) was added to the reaction mixture and stirring for another 24 h at room temperature under N₂. The reaction mixture was first precipitated in diethyl ether and then dissolved in deionized water, followed by dialysis for 2-3 days and lyophilization.

[0137] The chemical structure of PAspAm-Tyr (**2**) was confirmed by ¹H NMR (see Figure 5). The methylene (2H) and methine (1H) protons on the polysuccinimide backbone showed peaks at 2.5-2.9 ppm and 4.6 ppm. The benzene ring protons (-C₆H₄-) on the grafted tyramine were located at 6.7 and 7.0 ppm, indicating the success of tyramine functionalization. The peaks at 3.3-3.7 ppm indicated the methylene protons (2H) in 2-[2-(2-aminoethoxy)ethoxy]ethanol. The degree of substitution of

the tyramine group calculated from the ^1H NMR was 9.6%.

[0138] Rheological properties of PAspAm-Tyr (2)

[0139] Rheology measurements were performed at 25 °C on a Discovery HR-2 hybrid Rheometer (TA instruments) fitted with a 20 mm diameter plate-plate geometry. The PAspAm-Tyr (2) was photocrosslinked in-situ, and oscillatory time sweeps were performed under a shear strain of 1% and frequency of 1 Hz for 5 min. The samples were irradiated with visible light (OmniCure LX500, 405 nm, 8.8 mW/cm²) after the time sweep test started for 60 s. About 320 μL PAspAm-Tyr (2) solutions (10% wt/v in PBS) containing various amounts of tris(2,2'-bipyridyl)dichlororuthenium(II) hexahydrate (Ru; 1 mM, 0.5 mM, and 0.25 mM) and sodium persulfate (SPS; 20 mM and 10 mM) were used for each test and the geometry gap was set at 1 mm.

[0140] The polymer rheological properties with different amounts of co-photoinitiators were assessed by rheological measurements and plotted in Figure 6. The increasing amounts of SPS and Ru led to an increase in shear storage modulus (G') of the PAspAm-Tyr (2) hydrogels. The highest storage modulus was found in the sample (PAspAm-Tyr-1mM-20mM) crosslinked with 1 mM of Ru and 20 mM of SPS (26.6 ± 0.4 kPa), while the lowest storage modulus was found in the sample (PAspAm-Tyr-0.25mM-10mM) containing 0.25 mM of Ru and 10 mM of SPS (9.0 ± 0.1 kPa) (see Table 1). In addition, when the SPS amount was fixed, the hydrogel full crosslinking time increased with decreased Ru concentrations (see Table 1). The PAspAm-Tyr-0.25mM-20mM needed the longest crosslinking time (73 s) to reach plateau. It is worth noting that when the Ru amount is fixed, the higher amount of SPS would lead to longer hydrogel crosslinking time which might be because of the higher oxidation/reduction efficiency of Ru in the photopolymerization reaction, thus

resulting in higher storage modulus (see Table 1 and Figure 6).

Experiment	Polymer (%)	Ru (mM)	SPS (mM)	G' at plateau (kPa)	Full cross-linking time (s)
PAspAm-Tyr-1mM-20mM	10	1	20	26.6 ± 0.4	43
PAspAm-Tyr-1mM-10mM	10	1	10	11.2 ± 0.1	37
PAspAm-Tyr-0.5mM-20mM	10	0.5	20	25.6 ± 0.2	43
PAspAm-Tyr-0.5mM-10mM	10	0.5	10	10.1 ± 0.9	37
PAspAm-Tyr-0.25mM-20mM	10	0.25	20	23.1 ± 0.6	73
PAspAm-Tyr-0.25mM-10mM	10	0.25	10	9.0 ± 0.1	55

Table 1. Rheological properties of PAspAm-Tyr (**2**) under visible light exposure.

[0141] Preparation of PAspAm-Tyr (**2**) bioink formulation

[0142] Various combination of PAspAm-Tyr (**2**) bioink solutions were formulated to study the effects of Ru (1mM, 0.5 mM, 0.25 mM), SPS (10 mM and 20 mM) and photoabsorber, tartrazine (0.0125% wt/v, 0.025% wt/v, 0.05% wt/v) on the gelation kinetics, printability and the cell viability after bioprinting of those bioinks. The

polymer concentration was remained at 10% wt/v.

[0143] Printability of PAspAm-Tyr (2) bioink formulation

[0144] The formulated PAspAm-Tyr (2) bioinks were printed on a digital light processing (DLP) bioprinter, Lumen X+ (Volumetric Bio and Cellink). The printing was carried out at room temperature under near visible light exposure (405 nm, 20 mW/cm²). A grid shape and a “BIOINK” word were printed at a dimension of 9 mm x 9 mm x 0.5 mm and 34 mm x 7.2 mm x 0.5mm, respectively (see Figure 7).

[0145] Cell printing and cell viability

[0146] Human bone marrow-derived mesenchymal stem cells (hMSCs; ATCC) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (Gibco), 1% penicillin-streptomycin (Corning) and 8 µg/mL fibroblast growth factor-basic human (hBFGF, Sigma) in a humidified atmosphere with 5% CO₂ at 37 °C. PAspAm-Tyr (2) polymer together with the co-photoinitiators, Ru and SPS, and the photoabsorber, tartrazine, were dissolved in PBS and filtered through a 0.2 µm filter, then mixing with the cell suspension. The final concentrations for each component included 10% wt/v of PAspAm-Tyr (2) polymer, 1 mM Ru, 10 mM or 20 mM SPS, 0.0125% tartrazine and 1.5 M/mL of hMSCs, respectively. A 4 x 8 round disk array was printed with each disk dimension at 5 mm (diameter) x 1 mm (height). The light exposure intensity was 20 mW/cm² and body exposure was 8.25 s for each layer (layer thickness at 100 µm) for the 10 mM SPS experiment and 6.25 s for the 20 mM SPS experiment. The obtained gel disks were cultured in ultra-low attachment 24-well plates. The cell viability in the bioprinted PAspAm-Tyr (2) hydrogel was quantified by PrestoBlue® cell viability agent (Invitrogen) at 1, 7 and 14 days (n = 4). A Live/Dead cell staining kit (Live, calcein-AM; Dead, propidium iodide; MilliporeSigma) was used to visualize the cell growth in the PAspAm-Tyr (2) hydrogel.

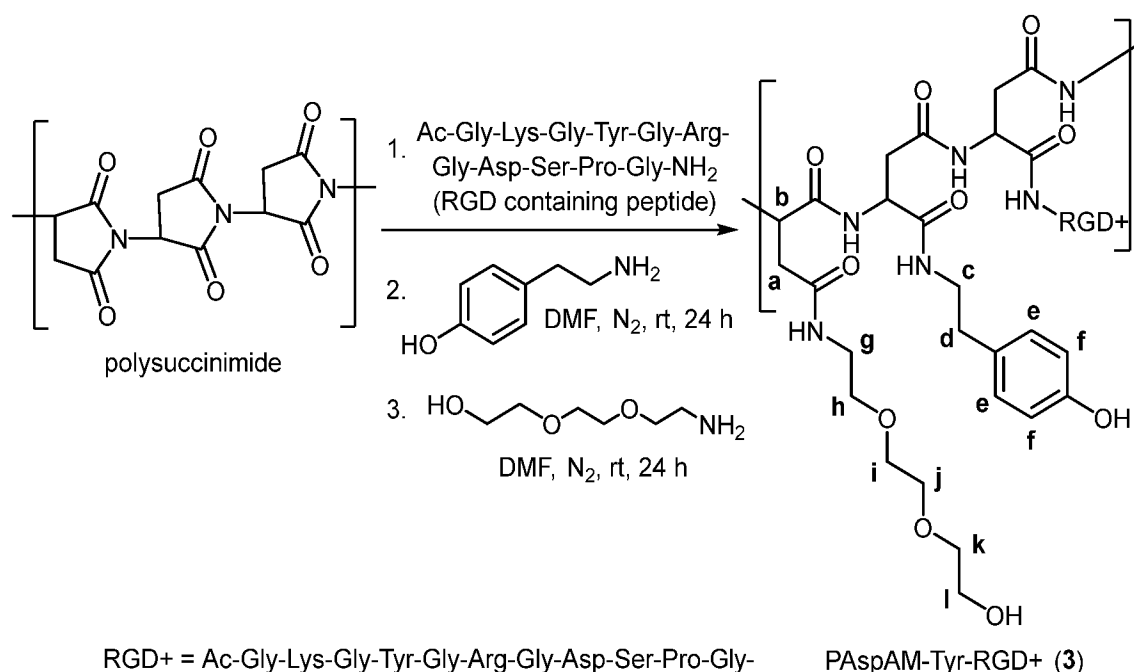
[0147] Live/Dead staining and PrestoBlue® assay were used to qualitatively and quantitatively evaluate the cell viability in the PAspAm-Tyr (2) bioink hydrogel over 14 days of culture (see Figure 8). Live&Dead staining images showed hMSCs in both 10 mM and 20 mM SPS crosslinked hydrogels were considerably viable and showed the trend to form cell aggerates (see Figure 8A). The PrestoBlue® assay showed the

hMSCs in both experiments were proliferating from day 1 to day 14 and the cell metabolism in 20 mM SPS experiment was significantly higher than that in 10 mM SPS experiment (see Figure 8B). Without wishing to be bound by theory, it is believed that this might be due to the higher storage modulus in 20 mM SPS experiment which would be more favorable to the hMSCs growth. The cell survival rates of the hMSCs inside the bioprinted PAspAm-Tyr (**2**) hydrogels were all above 95% in 14 days of culture (see Figure 8C). The results support the PAspAm-Tyr (**2**) bioink has no significant cell toxicity and can support hMSCs proliferation in 14 days of culture, thus having great potential to be applied in biomedical applications.

[0148] Example 3

[0149] Synthesis of PAspAm-Tyr (PAspAm-Tyr-RGD+) (**3**) conjugated with RGD containing peptide

[0150] The subsequent scheme discloses SEQ ID NOS: 9 and 10, respectively, in order of appearance.



[0151] PAspAm-Tyr-RGD+ (**3**) was synthesized by first reacting polysuccinimide with the RGD containing peptide Ac-Gly-Lys-Gly-Tyr-Gly-Arg-Gly-Asp-Ser-Pro-Gly-NH₂ (SEQ ID NO: 9). Polysuccinimide (680 mg) was dissolved in 10 mL anhydrous dimethylformamide (DMF). RGD containing peptide (100 mg) in 10 mL

DMF was first added and stirred at room temperature for 24 hrs. Then, tyramine (107.9 mg) dissolved in 10 mL DMF was added to the reaction mixture and reacted for another 24 h at room temperature. The final step was the addition of an excess amount of 2-[2-(2-aminoethoxy)ethoxy]ethanol (1.82 mL) and stirring for 24 h at room temperature. All the steps were carried out under N₂ atmosphere. The final solution was first precipitated in diethyl ether and then dissolved in deionized water, followed by dialysis for 2-3 days and lyophilization. The degree of substitution of RGD containing peptide and tyramine groups on polysuccinimide backbone were determined by ¹H NMR (Bruker 500MHz) with D₂O as solvent. The molar feeding ratio of RGD containing peptide and tyramine to the succinimidyl rings in the backbone were 0.78% and 10.3%, respectively.

[0152] Preparation of (PAspAm-Tyr-RGD+) (3) bioink formulation

[0153] PAspAm-Tyr-RGD+ (3) bioink was composed of 10% wt/v polymer, 1 mM of Ru, 20 mM of SPS and 0.0125% of tartrazine. The PAspAm-Tyr (2) containing 10% wt/v polymer, 1 mM of Ru, 20 mM of SPS and 0.0125% of tartrazine was used as a comparison. Poly(ethylene glycol) diacrylate (PEGDA; Mn = 6000) was formulated into the bioink with 10% wt/v polymer concentration, 0.5% wt/v photoinitiator, lithium phenyl-2,4,6-trimethylbenzoylphosphine (LAP), and 0.01% tartrazine as the control.

[0154] Cell printing and cell viability

[0155] Mouse 3T3 fibroblasts (ATCC) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Corning) in a humidified atmosphere with 5% CO₂ at 37 °C. The above formulated bioinks were filtered through a 0.2 µm filter, then mixing with the cell suspension. The final cell density was 1 M/mL. A 4 x 8 round disk array was printed and the dimension of each disk was 5 mm (diameter) x 1 mm (height). The light exposure intensity was 20 mW/cm² and body exposure was 8.25 s for each layer (layer thickness at 100 µm) for the PAspAm-Tyr-RGD+ (3) experiment and 6.25 s for the PAspAm-Tyr (2) experiment. The PEGDA(6k) bioink needed longer body exposure at 20 s for each layer. The obtained gel disks were cultured in ultra-low attachment 24-well plates. The cell viability in the bioprinted PAspAm-Tyr

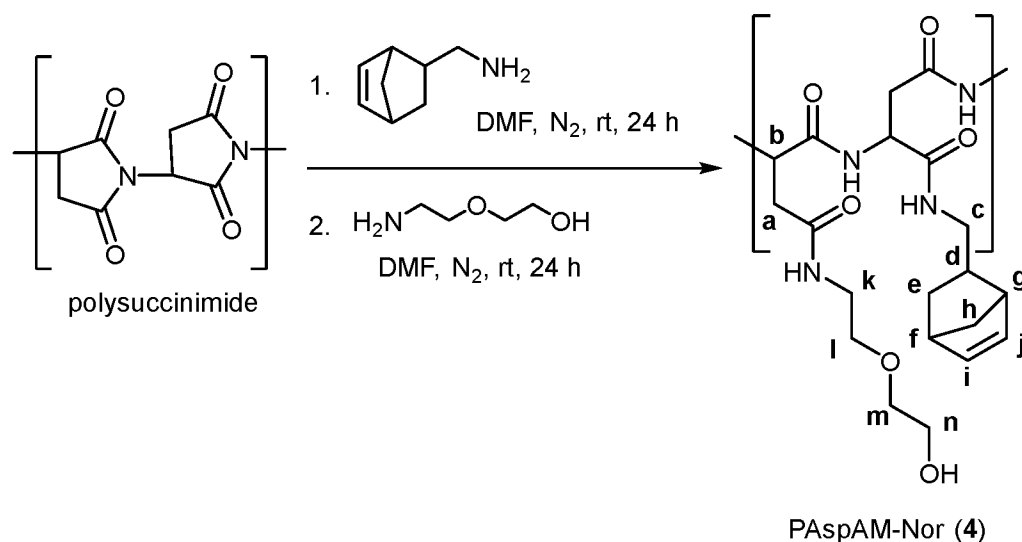
(2) hydrogel was quantified by PrestoBlue® cell viability agent (Invitrogen) at 1, 7, 14 and 21 days (n = 4). A Live/Dead cell staining kit (Live, calcein-AM; Dead, propidium iodide; MilliporeSigma) was used to visualize the cell growth in the PSI-MA hydrogel.

[0156] Live/Dead staining and PrestoBlue® assay were used to qualitatively and quantitatively evaluate the cell viability in the PAspAm-Tyr-RGD+ (3) bioink hydrogel over 21 days of culture (see Figures 9 and 10). Live/Dead staining images showed 3T3 fibroblasts in both PAspAm-Tyr-RGD+ (3) and PAspAm-Tyr (2) hydrogels proliferated vigorously with minimal dead cells (see Figure 9). Cells in the PAspAm-Tyr-RGD+ (3) hydrogel showed elongated shape and secreted their own extracellular matrix. In contrast, cells in the PAspAm-Tyr (2) hydrogel formed numerous cell aggregates. However, a large number of dead cells were observed in PEGDA(6k) hydrogel (see Figure 9). The PrestoBlue® assay showed the 3T3 fibroblasts in both groups were proliferating dramatically from day 1 to day 21 (see Figure 10A). The metabolism activity of the cells in PAspAm-Tyr-RGD+ (3) bioink hydrogel formulation increased by 28 times from day 1 to day 21, while the metabolism activity of the cells in PAspAm-Tyr (2) bioink hydrogel formulation increased by 17 times over 21 days of culture. The metabolic activities in PEGDA(6k) bioink hydrogel formulation were close to 0 since day 1, indicating most of the cells were dead from the first day after printing. The cell survival rates of the 3T3 fibroblasts inside the bioprinted PAspAm-Tyr-RGD+ (3) and PAspAm-Tyr (2) hydrogels were all above 95% in 21 days of culture (see Figure 10B). The results confirmed the high cell affinity of the PAspAm-Tyr-RGD+ (3) bioink due to the RGD+ peptides serving as cell binding sites which can boost cell attachment and proliferation. Compared to the synthetic PEGDA bioink in which majority of 3T3 fibroblasts died at the first day of culture, our synthetic PAspAm-Tyr-RGD+ (3) and PAspAm-Tyr (2) bioinks are cell-supportive and much potential to be used in various

biomedical applications.

[0157] Example 4

[0158] Synthesis of norbornene-functionalized polyaspartamide (PAspAm-Nor) (**4**)



[0159] PAspAm-Nor (**4**) was synthesized by derivatizing polysuccinimide with 5-norbornene-2-methylamine by ring opening nucleophilic reaction (see Figure 11A). Polysuccinimide (1,000 mg) was dissolved in 10 mL anhydrous dimethylformamide (DMF). 5-norbornene-2-methylamine (414.4 mg) in 10 mL DMF was first added and stirred at room temperature under N₂ for 24 h. Then 1.48 mL of 2-(2-aminoethoxy)ethanol was added to the reaction mixture and stirring for another 24 h at room temperature under N₂. The reaction mixture was first precipitated in diethyl ether and then dissolved in deionized water, followed by dialysis for 3 days and lyophilization.

[0160] The chemical structure of PAspAm-Nor (**4**) was confirmed by ¹H NMR (see Figure 11B). The methylene (2H) and methine (1H) protons on the polysuccinimide backbone showed peaks at 2.5-2.9 ppm and 4.6 ppm. The peaks at 5.9 and 6.1 ppm were assigned to the double bond (CH=CH) on the norbornene group. The peaks at 3.3-3.7 ppm indicated the methylene protons (2H) in of 2-(2-aminoethoxy)ethanol. The degree of substitution of the norbornene group calculated from the ¹H NMR was

32%.

[0161] Preparation of PAspAm-Nor (4) bioink formulation

[0162] A bioink (PAspAm-Nor) composed of PAspAm-Nor (4) polymer was formulated by mixing it with a thiol functionalized crosslinking molecule in water. The crosslinking molecule was 4-armed-PEG-SH (MW = 5 kDa). The total mass concentration of the PAspAm-Nor (4) polymer and 4-armed-PEG-SH in water was 10% wt/v. 0.5% wt/v lithium phenyl(2,4,5-trimethylbenzoyl)phosphinate (LAP) and 0.025% tartrazine were included as the photo-initiator and photo-absorber, respectively.

[0163] The crosslinking occurs by click chemistry reaction between the norbornene and thiol groups of PAspAm-Nor (4) polymer and 4-armed-PEG-SH respectively, when irradiated with 405 nm light at ambient condition.

[0164] Preparation of PAspAm-Nor-RGD++ (4a) bioink formulation

[0165] In order to enhance the biological functionality of the PAspAm-Nor (4) bioink, a thiol group-containing RGD-containing peptide with the sequence of H-Gly-Arg-Gly-Asp-Ser-Pro-Cys-OH (SEQ ID NO: 11) (RGD++) was added to the above formulated bioink (*i.e.* pre-crosslinked PAspAm-Nor (4) bioink) with a molar ratio to the number of the norbornene functional groups at 10%.

[0166] The crosslinking occurs by click chemistry reaction between the norbornene of PAspAm-Nor (4) polymer and the thiol groups of 4-armed-PEG-SH and the thiol-group of the RGD containing peptide, when irradiated with 405 nm light at ambient condition as shown in the reaction scheme in Figure 12.

[0167] Rheological properties of PAspAm-Nor (4) and PAspAm-Nor-RGD++ (4a)

[0168] Rheology measurements were performed at 25 °C on a Discovery HR-2 hybrid Rheometer (TA instruments) fitted with a 20 mm diameter plate-plate geometry. The above formulated bioinks with (4) and (4a) were photocrosslinked in-situ, and oscillatory time sweeps were performed under a shear strain of 1% and frequency of 1 Hz for 5 min. The samples were irradiated with visible light (OmniCure LX500, 405 nm, 8.8 mW/cm²) after the time sweep test started for 60 s.

The geometry gap was set at 1 mm.

[0169] The time sweep test of the storage modulus (G') and loss modulus (G'') versus time was carried out to determine the gelation behavior during photo-crosslinking. As shown in Figure 13, both PAspAm-Nor (4) and PAspAm-Nor-RGD++ (4a) bioinks crosslinked into mechanically strong hydrogel rapidly within 2 s. The G' of the cured PAspAm-Nor (4) hydrogel was $34.5 \text{ k} \pm 2.1 \text{ Pa}$, while that of the cured PAspAm-Nor-RGD++ (4a) hydrogel was $32.7 \pm 1.8 \text{ kPa}$, indicating that the addition of RGD++ peptide at 10% molar ratio to the number of the norbornene groups had no significant impact on the hydrogel stiffness.

[0170] Cell printing and cell viability

[0171] Mouse 3T3 fibroblasts (ATCC) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Corning) in a humidified atmosphere with 5% CO_2 at 37 °C. PAspAm-Nor (4) and PAspAm-Nor-RGD++ (4a) bioinks were filtered through a 0.2 μm filter, then mixing with cells at a density of 1 M/mL. A 4 x 8 round disk array was printed with each disk dimension at 5 mm (diameter) x 1 mm (height). The light exposure intensity was 20 mW/cm² and body exposure was 2.25 s for each layer (layer thickness at 100 μm). The obtained gel disks were cultured in ultra-low attachment 24-well plates. The cell viability in the bioprinted PAspAm-Nor (4) and PAspAm-Nor-RGD++ (4a) hydrogels were quantified by PrestoBlue® cell viability agent (Invitrogen) at 1, 7, 14, 21 and 28 days (n = 4). A Live/Dead cell staining kit (Live, calcein-AM; Dead, propidium iodide; MilliporeSigma) was used to visualize the cell growth in the PAspAm-Nor (4) and PAspAm-Nor-RGD++ (4a) hydrogels.

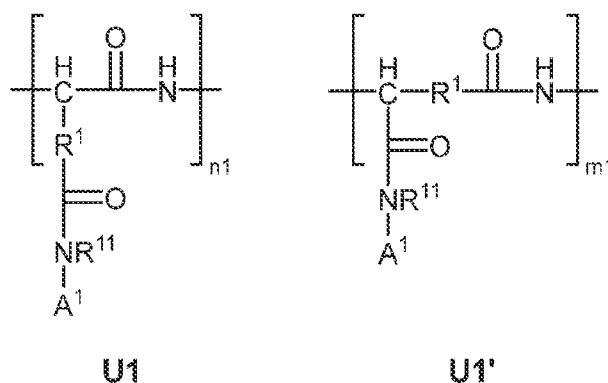
[0172] Live/Dead staining and PrestoBlue® assay were used to qualitatively and quantitatively evaluate the cell viability in the PAspAm-Nor (4) and PAspAm-Nor-RGD++ (4a) bioink hydrogels over 28 days of culture (see Figure 14). Live/Dead staining images showed 3T3 fibroblasts in both PAspAm-Nor-RGD++ (4a) and PAspAm-Nor (4) hydrogels proliferated vigorously with minimal dead cells (see Figure 14A). Cells in the PAspAm-Nor-RGD++ (4a) hydrogel showed elongated shape and secreted their own extracellular matrix. In contrast, cells in the PAspAm-Nor (4) hydrogel remained round. The PrestoBlue® assay showed the 3T3 fibroblasts

in both groups were proliferating dramatically from day 1 to day 28 (see Figure 14B). The metabolism activity of the PAspAm-Nor-RGD++ (4a) group increased by 28 times from day 1 to day 28, while that of the PAspAm-Nor (4) group increased by 5 times over 28 days of culture. The results confirmed the high bioactivity of the PAspAm-Nor-RGD++ (4a) bioink as the RGD containing peptides serving as the cell binding sites which can boost cell attachment and proliferation.

[0173] The examples provided herein are not intended in any way to limit the scope of the invention as set forth in the claims.

We claim:

1. A bioink formulation comprising a polymer, wherein the polymer comprises a repeating unit U1 and a repeating unit U1':



wherein:

R¹ is selected from alkylene, preferably from C₁-C₂₀ alkylene, more preferably from C₁-C₁₀ alkylene, most preferably from methylene, ethylene and propylene;

R¹¹ is selected from hydrogen or alkyl, preferably from hydrogen or C₁-C₂₀ alkyl, more preferably from hydrogen or C₁-C₁₀ alkyl, most preferably from hydrogen, methyl, ethyl and propyl;

A¹ is a moiety imparting hydrophilicity to the polymer;

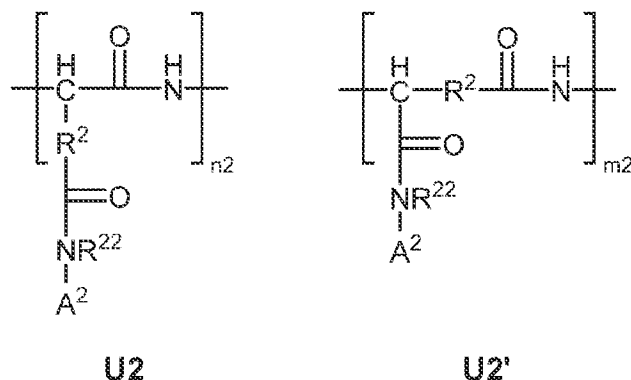
n₁ is an integer from 1 to 5,000, preferably from 10 to 5,000, more preferably from 100 to 5,000; and

m₁ is an integer from 1 to 5,000, preferably from 10 to 5,000, more preferably from 100 to 5,000.

2. The bioink formulation according to claim 1, wherein A¹ comprises one or more groups selected from -OH, -SH, -NH₂, -CO-NH₂ and -CO₂H.

3. The bioink formulation according to claim 1 or 2, wherein A¹ comprises an alkylene unit -(CH₂)_x- and/or an ethylene oxide unit -(CH₂-CH₂-O)_y-, wherein x and y are independently from each other integers from 1 to 2,000.

4. The bioink formulation according to one or more of claims 1 to 3, wherein the polymer further comprises a repeating unit U2 and a repeating unit U2':



wherein:

R^2 is independently from each other selected from alkylene, preferably from $\text{C}_1\text{-C}_{20}$ alkylene, more preferably from $\text{C}_1\text{-C}_{10}$ alkylene, most preferably from methylene, ethylene and propylene;

R^{22} is independently from each other selected from hydrogen or alkyl, preferably from hydrogen or $\text{C}_1\text{-C}_{20}$ alkyl, more preferably from hydrogen or $\text{C}_1\text{-C}_{10}$ alkyl, most preferably from hydrogen, methyl, ethyl and propyl;

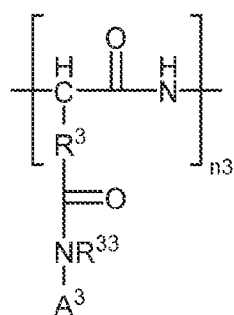
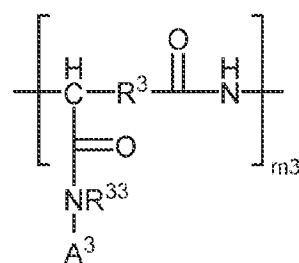
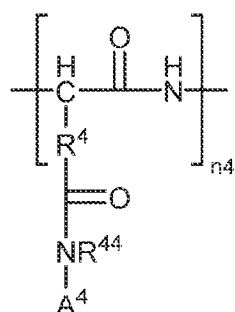
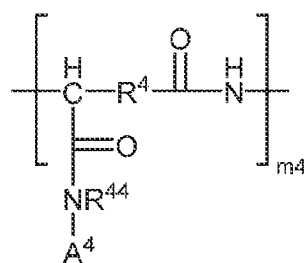
A^2 is a moiety containing at least one reactive group which can be further reacted to crosslink two or more polymer chains;

$n2$ is an integer from 1 to 5,000, preferably from 10 to 5,000, more preferably from 100 to 5,000; and

$m2$ is an integer from 1 to 5,000, preferably from 10 to 5,000, more preferably from 100 to 5,000.

5. The bioink formulation according to claim 4, wherein the reactive group contained in A^2 is selected from the list consisting of groups capable to undergo polymerization crosslinking reactions, groups capable to undergo Michael-type or click chemistry-type crosslinking reactions, groups capable to undergo oxidative crosslinking reactions, and groups capable to undergo redox crosslinking reactions.

6. The bioink formulation according to claim 4 or 5, wherein the reactive group contained in A^2 is selected from groups capable to undergo polymerization crosslinking reactions, wherein preferably said groups contain one or more vinyl or allyl groups.
7. The bioink formulation according to claim 4 or 5, wherein the reactive group contained in A^2 is selected from groups capable to undergo Michael-type or click chemistry-type crosslinking reactions, wherein preferably said groups contain one or more acrylate, methacrylate, n-alkyne, cyclo-alkyne, amine, azide, carboxyl, hydrazide, hydroxyl, maleimide, norbornene, tetrazine and/or thiol groups.
8. The bioink formulation according to claim 4 or 5, wherein the reactive group contained in A^2 is selected from groups capable to undergo oxidative crosslinking reactions, wherein preferably said groups contain one or more aromatic moieties with one or more aromatic hydroxyl groups, wherein more preferably said groups contain one or more mono-phenol, di-phenol, tri-phenol, oligo-phenol and/or poly-phenol moieties, wherein particularly preferably said groups contain a mono-phenol moiety.
9. The bioink formulation according to claim 4 or 5, wherein the reactive group contained in A^2 is selected from groups capable to undergo redox crosslinking reactions, wherein preferably said groups contain one or more thiol and/or amine groups.
10. The bioink formulation according to any one of claims 4 to 9, further comprising a reactive peptide compound capable to react with A^2 , wherein preferably the reactive peptide compound comprises one or more functional groups capable to react with the reactive group contained in A^2 , wherein said functional group is preferably selected from the list consisting of allyl, vinyl, acrylate, methacrylate, maleimide and thiol.
11. The bioink formulation according to one or more of claims 1 to 10, wherein the polymer further comprises (i) a repeating unit $U3$ and a repeating unit $U3'$; (ii) a repeating unit $U4$ and a repeating unit $U4'$; or (iii) a repeating unit $U3$, a repeating unit $U3'$, a repeating unit $U4$ and a repeating unit $U4'$.

**U3****U3'****U4****U4'**

wherein:

R^3 and R^4 are independently from each other selected from alkylene, preferably from C_1 - C_{20} alkylene, more preferably from C_1 - C_{10} alkylene, most preferably from methylene, ethylene and propylene;

R^{33} and R^{44} are independently from each other selected from hydrogen or alkyl, preferably from hydrogen or C_1 - C_{20} alkyl, more preferably from hydrogen or C_1 - C_{10} alkyl, most preferably from hydrogen, methyl, ethyl and propyl;

A^3 is a moiety imparting cellular function to the polymer;

A^4 is a moiety imparting shear thinning properties to the polymer;

wherein in case (i):

$n3$ is an integer from 1 to 5,000, preferably from 10 to 5,000, more preferably from 100 to 5,000;

m3 is an integer from 1 to 5,000, preferably from 10 to 5,000, more preferably from 100 to 5,000; and

the sum of n1, n2, n3, m1, m2 and m3 is greater than 5, preferably greater than 50, more preferably greater than 500; or

wherein in case (ii):

n4 is an integer from 1 to 5,000, preferably from 10 to 5,000, more preferably from 100 to 5,000;

m4 is an integer from 1 to 5,000, preferably from 10 to 5,000, more preferably from 100 to 5,000; and

the sum of n1, n2, n4, m1, m2, and m4 is greater than 5, preferably greater than 50, more preferably greater than 500, or

wherein in case (iii):

n3 is an integer from 1 to 5,000, preferably from 10 to 5,000, more preferably from 100 to 5,000;

m3 is an integer from 1 to 5,000, preferably from 10 to 5,000, more preferably from 100 to 5,000;

n4 is an integer from 1 to 5,000, preferably from 10 to 5,000, more preferably from 100 to 5,000;

m4 is an integer from 1 to 5,000, preferably from 10 to 5,000, more preferably from 100 to 5,000; and

where in case (iii) the sum of n1, n2, n3, n4, m1, m2, m3 and m4 is greater than 7, preferably greater than 50, more preferably greater than 500.

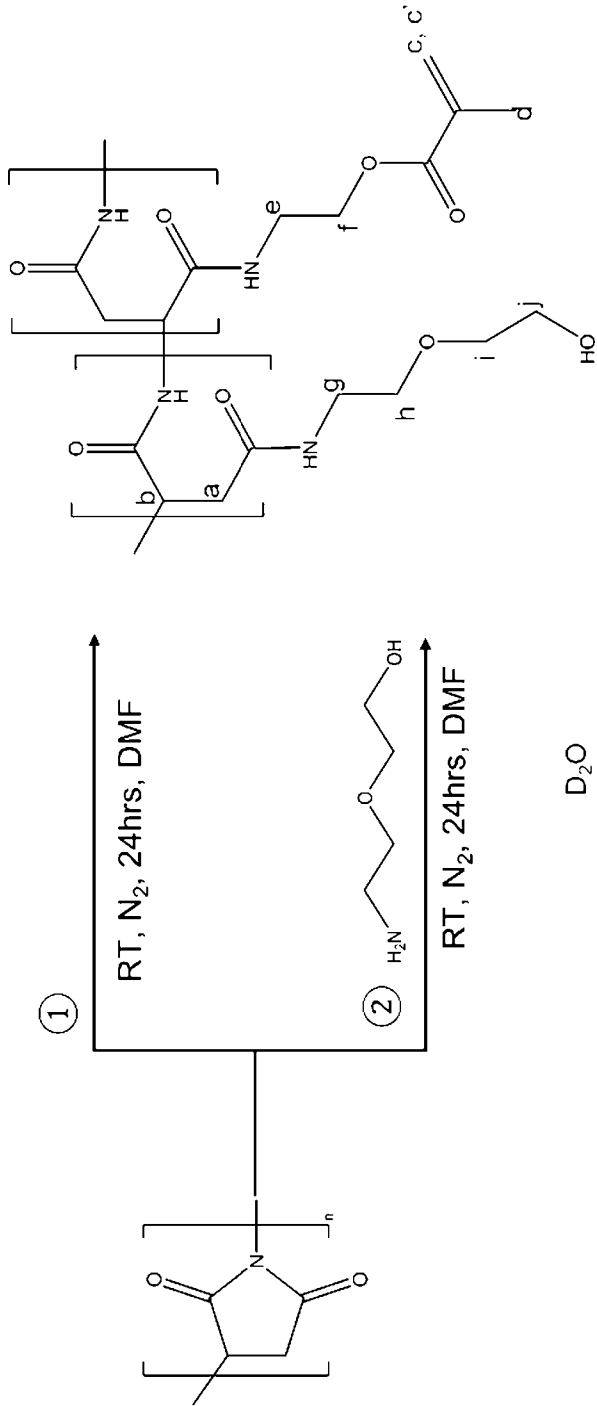
12. The bioink formulation according to claim 11, wherein A³ contains one or more synthetic functional peptide sequences found in native proteins, wherein preferably said native proteins are fibronectin, laminin, collagen, elastin, albumin, immunoglobulin or silk.

13. The bioink formulation according to claim 11 or 12, wherein A^3 contains one or more synthetic functional peptide sequences selected from the list consisting of RGD, YIGSR (SEQ ID NO: 1), IKVAV (SEQ ID NO: 2), DGEA (SEQ ID NO: 3), PHSRN (SEQ ID NO: 4), PRARI (SEQ ID NO: 5), $(GAGAGS)_p$ (SEQ ID NO: 6), $(VPGXG)_n$ (SEQ ID NO: 7), $(GPO)_m$, and Gly-Lys-Gly-Tyr-Gly-Arg-Gly-Asp-Ser-Pro-Gly (SEQ ID NO: 8), wherein p, n and m are independently from each other integers of ≥ 1 .
14. The bioink formulation according to one or more of claims 11 to 13, wherein A^4 is a moiety imparting shear thinning properties due to supramolecular interactions.
15. The bioink formulation according to claim 14, wherein the supramolecular interactions are host-guest or self-assembly interactions, wherein such interactions preferably include interactions between cyclodextrin, adamantane, cucurbit[8]uril, cholesterol, polyethylene glycol and ureidopyrimidinone.
16. The bioink formulation according to claim 14 or 15, wherein A^4 contains one or more cyclodextrin, adamantane, cucurbit[8]uril, cholesterol, polyethylene glycol or ureidopyrimidinone moieties.
17. The bioink formulation according to one or more of claims 1 to 16, wherein the mass concentration of the polymer is from 0.1% (wt/v) to 99% (wt/v), preferably from 1% (wt/v) to 70% (wt/v), more preferably from 2% (wt/v) to 40% (wt/v), particularly preferably from 5% (wt/v) to 30% (wt/v), and most preferably from 5% (wt/v) to 20% (wt/v), based on the total volume of the bioink formulation.
18. The bioink formulation according to one or more of claims 1 to 17, further comprising one or more selected from crosslinking reagents and crosslinking additives.
19. The bioink formulation according to claim 18, wherein the crosslinking reagents are selected from the list consisting of photoinitiators, thermal initiators, basic initiators, oxidative initiators, enzymes mediating oxidative crosslinking, and agents mediating redox crosslinking.

20. The bioink formulation according to claim 18 or 19, wherein the crosslinking additives are compounds comprising two or more polymerizable groups with at least one C=C double bond, wherein preferably said polymerizable groups with at least one C=C double bond are selected from the list consisting of acrylate, methacrylate, allyl, styryl and vinyl groups.
21. The bioink formulation according to claim 18 or 19, wherein the crosslinking additives are compounds comprising two or more thiol groups, preferably three or more thiol groups, more preferably four thiol groups.
22. A method for producing a three-dimensional object, wherein the bioink formulation according to one or more of claims 1 to 21 is applied to a 3D bioprinting technique to produce said three-dimensional object.
23. The method according to claim 22 wherein the bioink formulation is subjected to irradiation and/or thermal treatment to crosslink the polymer comprised in the bioink formulation.
24. A three-dimensional object obtainable by the method according to claim 22 or 23.
25. The three-dimensional object according to claim 24, wherein said three-dimensional object is for use in tissue engineering, regenerative medicine, cell delivery, drug delivery, drug discovery, wound dressing, biosensors, cosmetics, hygiene products, medical devices, implantable electronics and/or other biomedical applications.

Fig. 1

A



B



Fig. 2

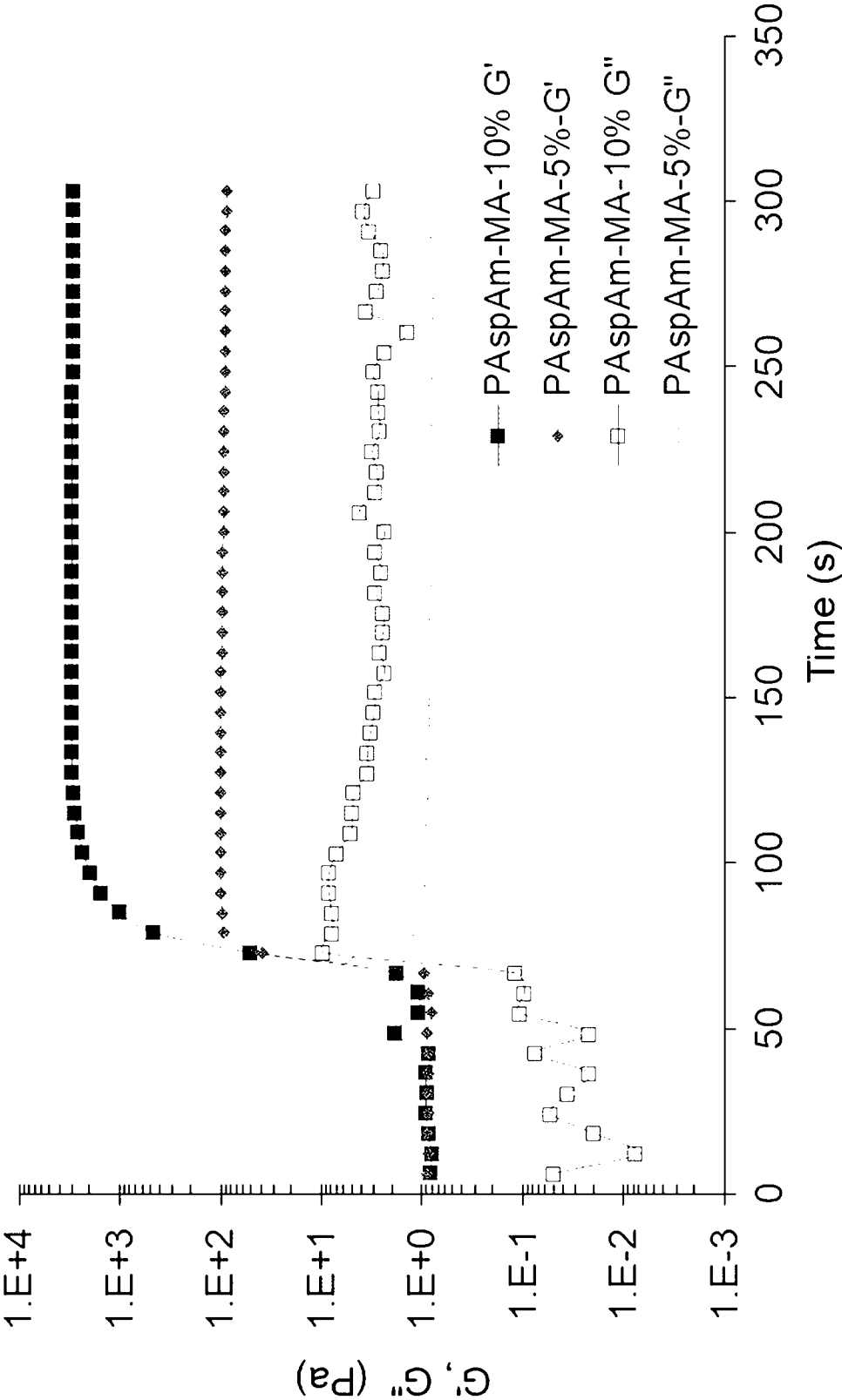
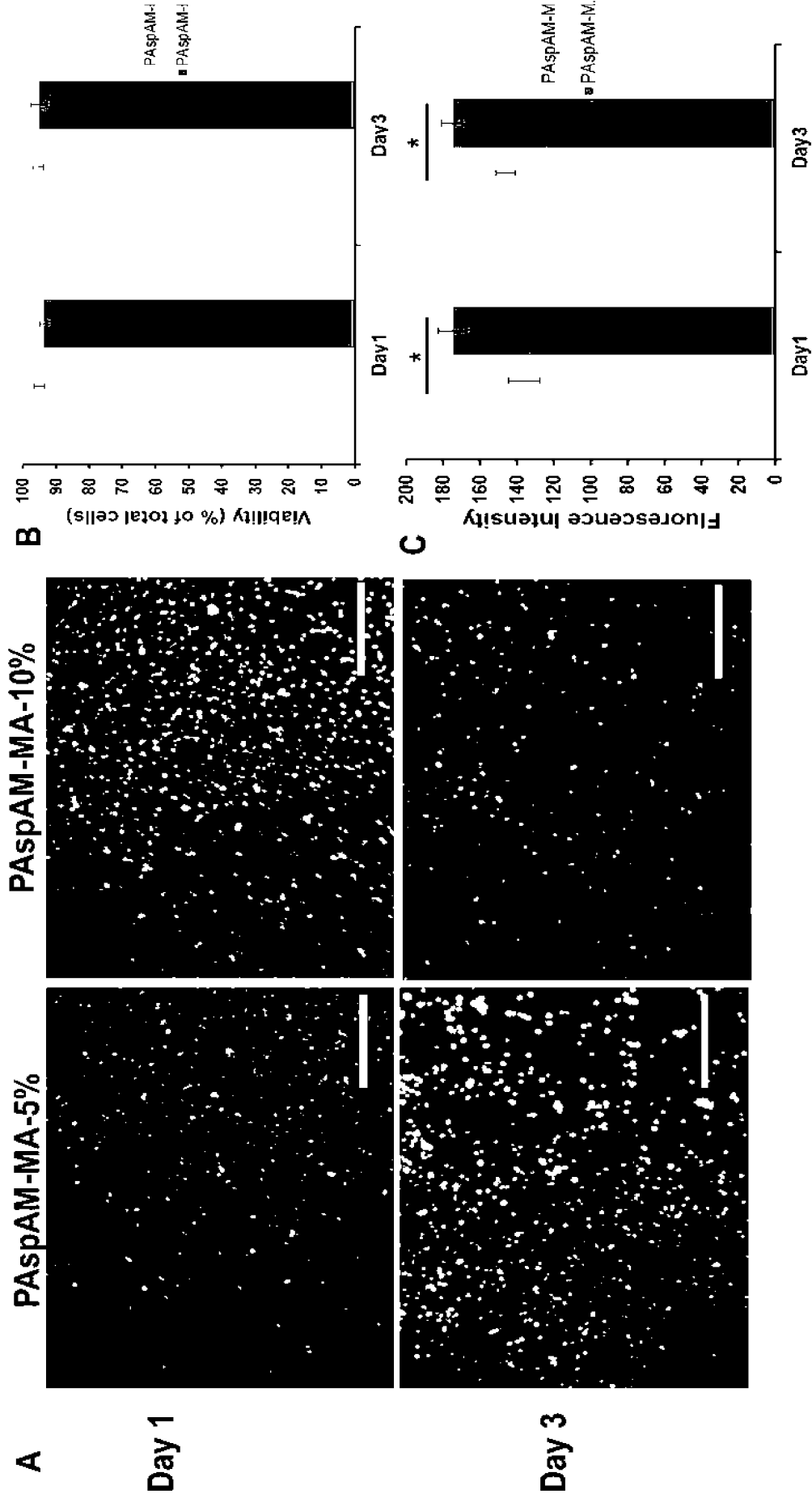


Fig. 3



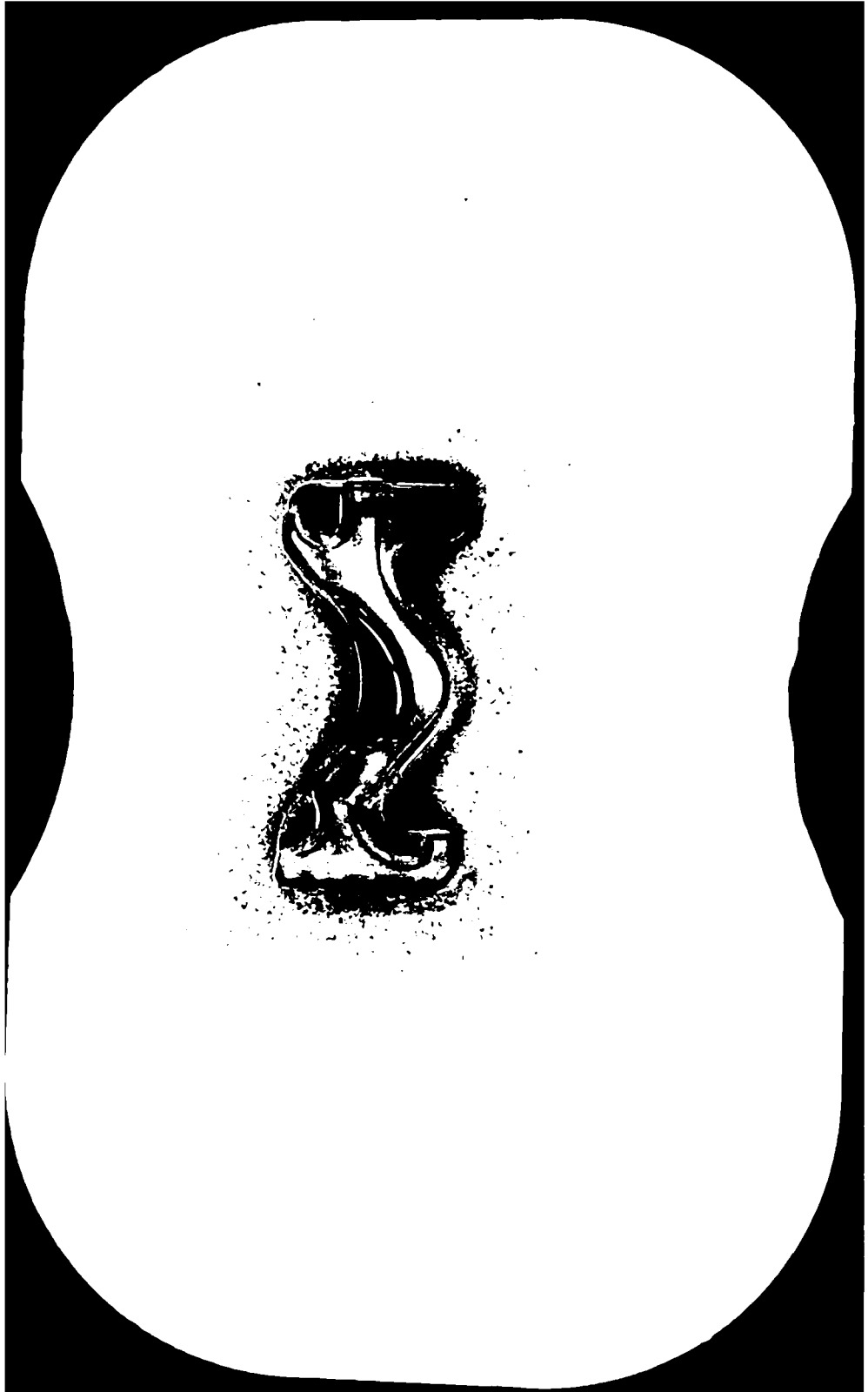


Fig. 4

Fig. 5

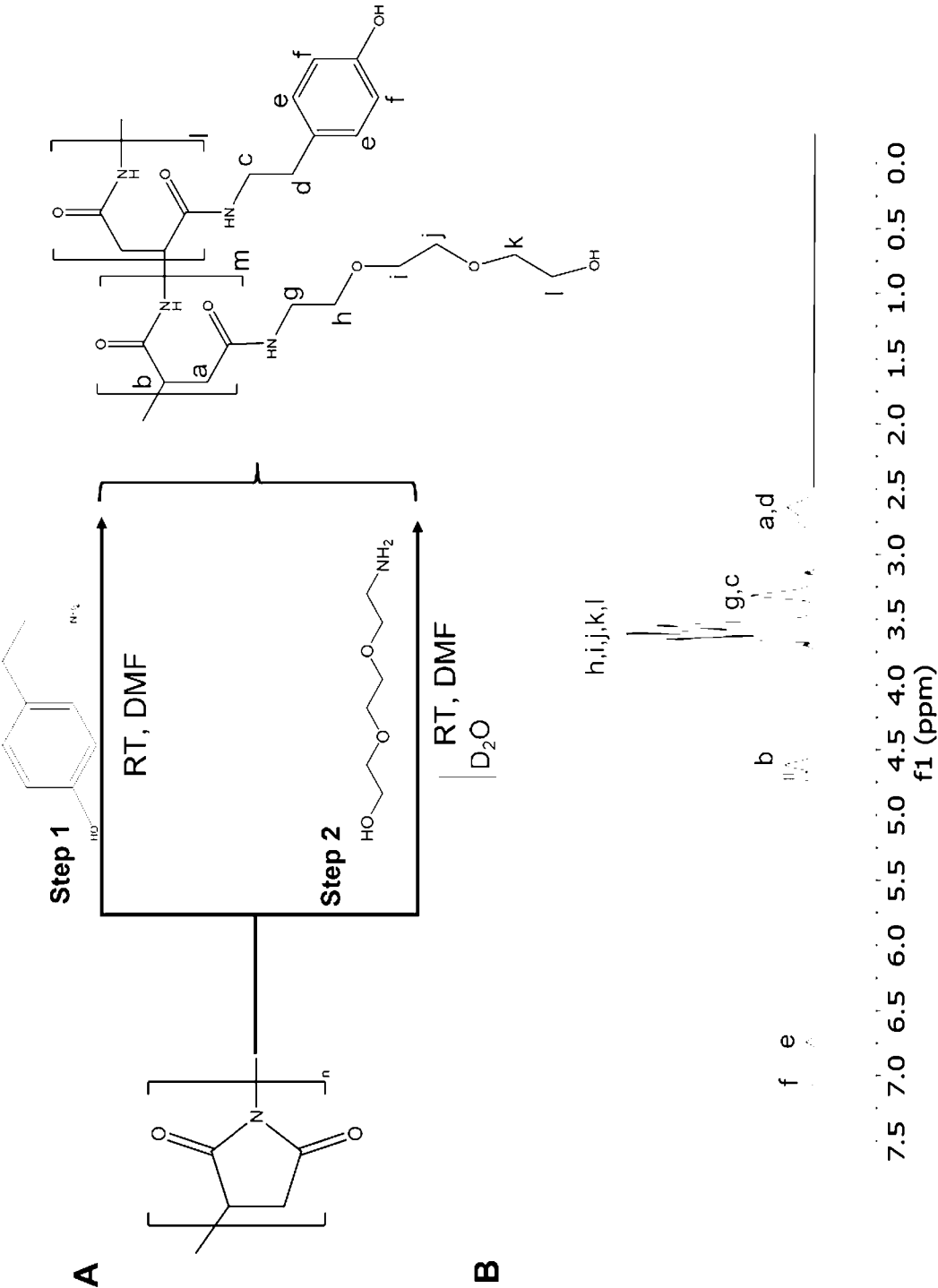
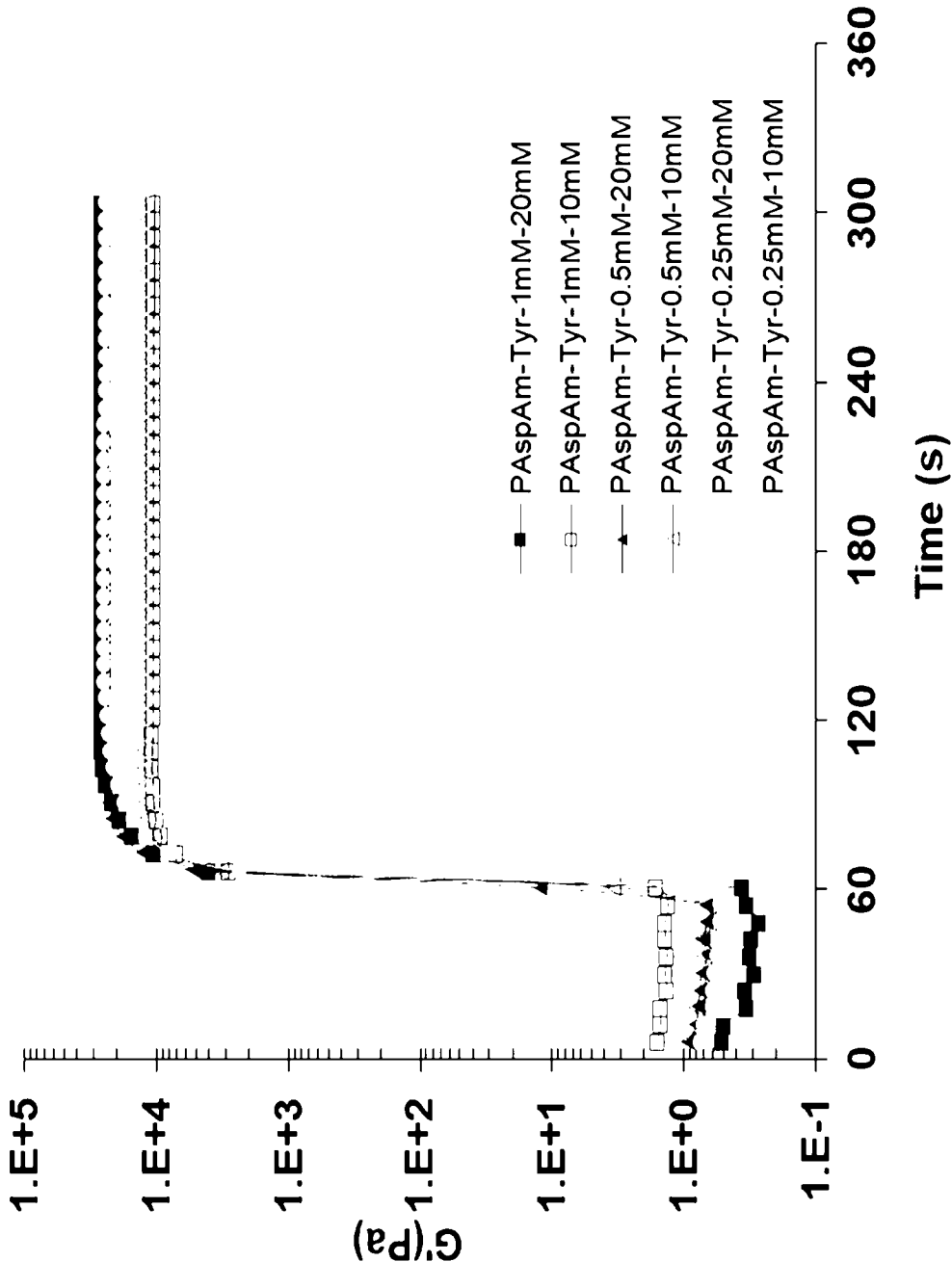
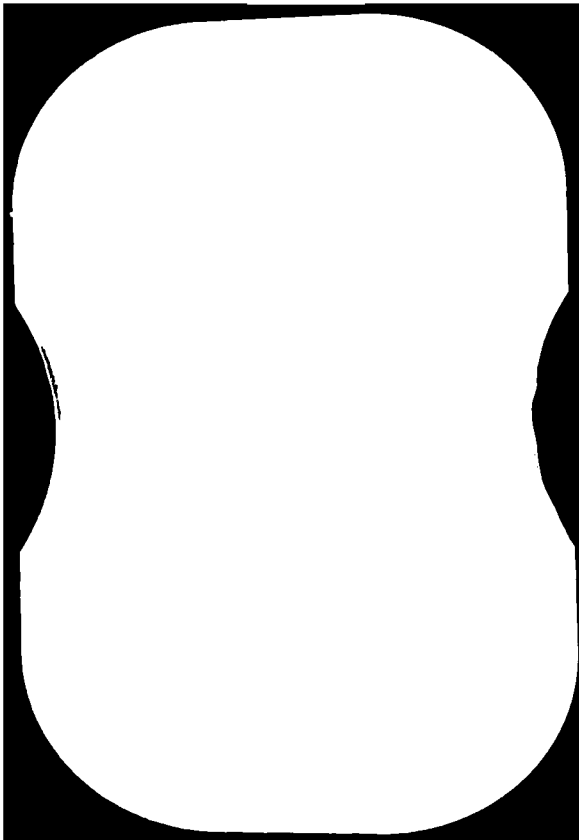
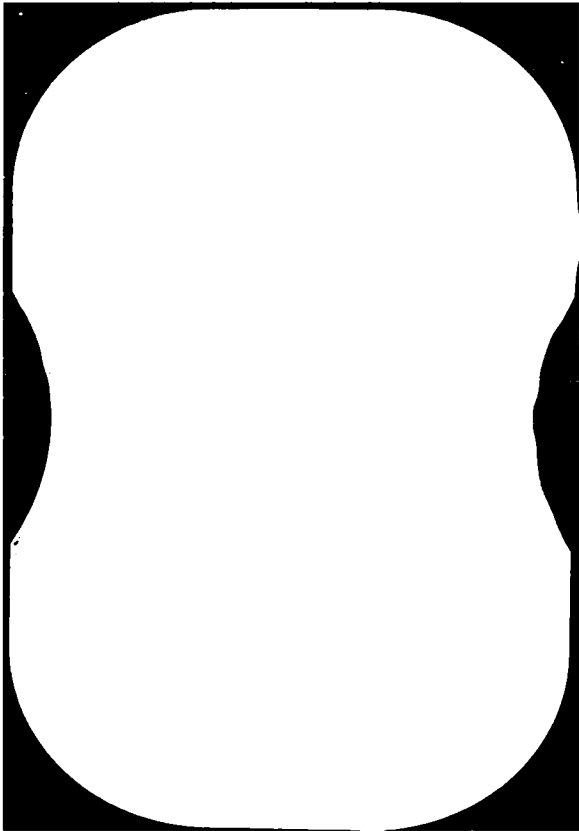


Fig. 6





B



A

Fig. 7

Fig. 8

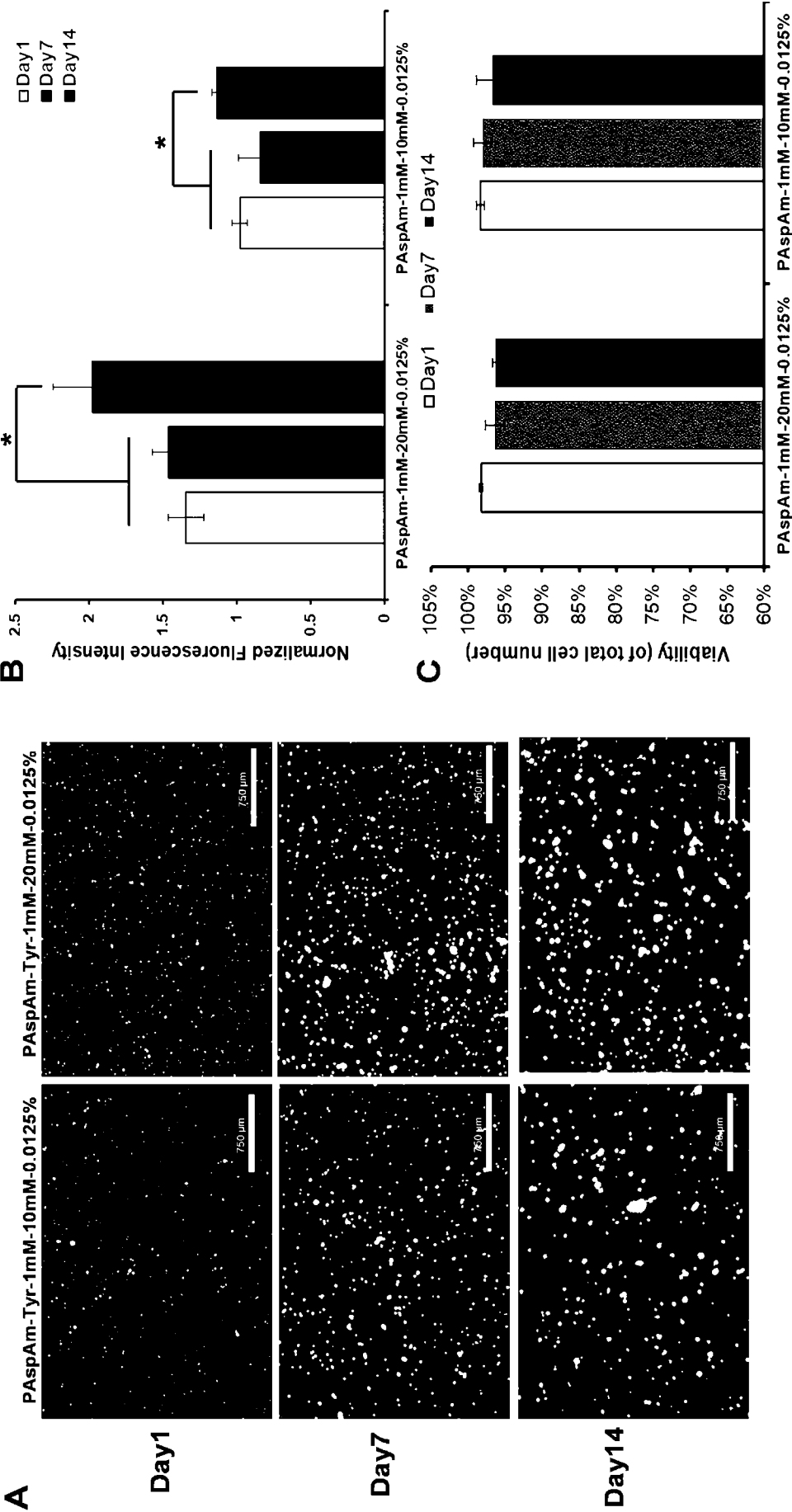


Fig. 9

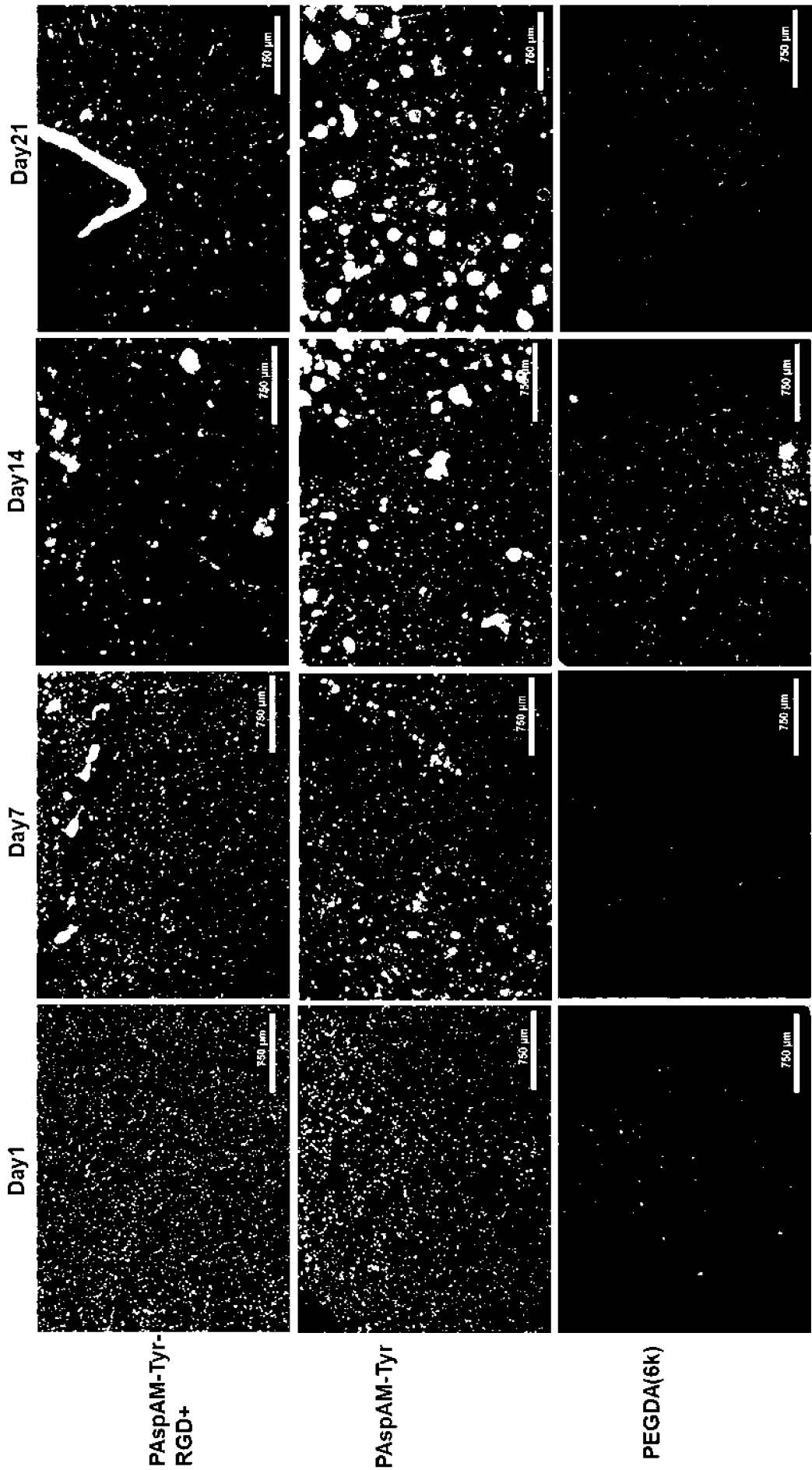


Fig. 10

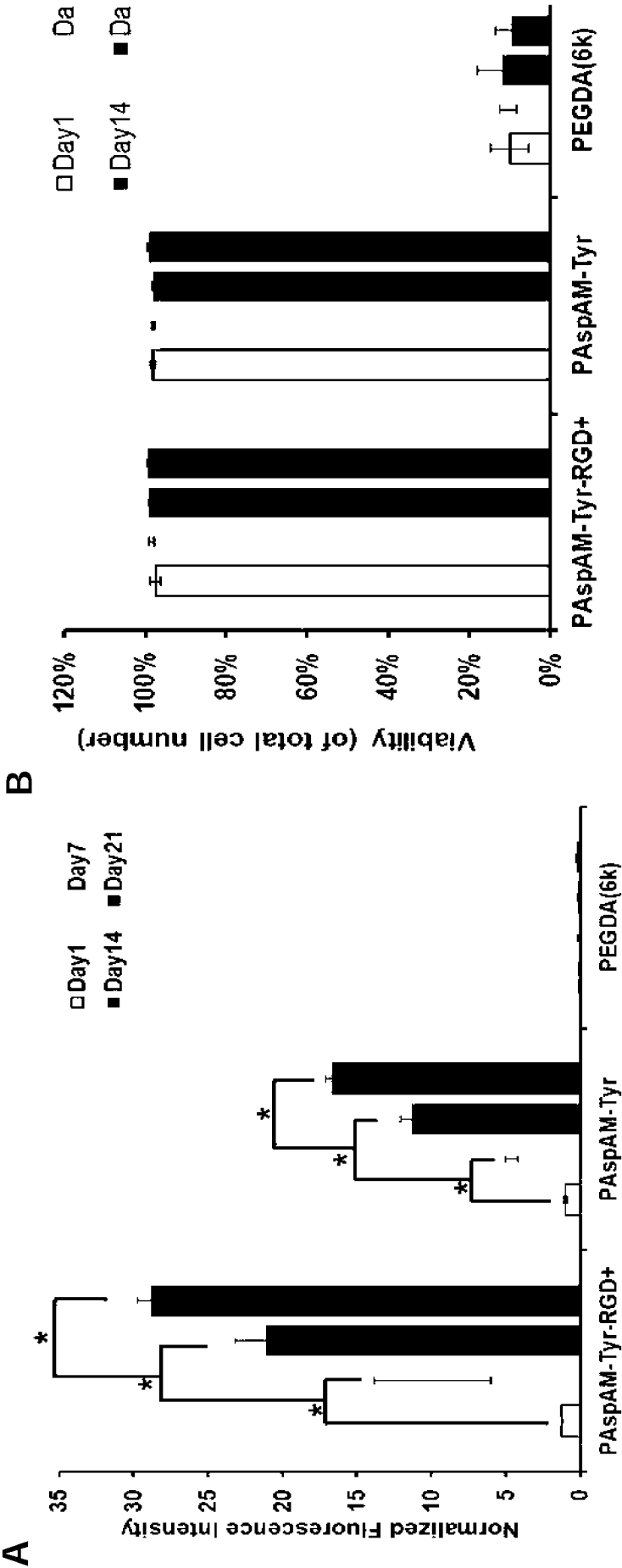


Fig. 11

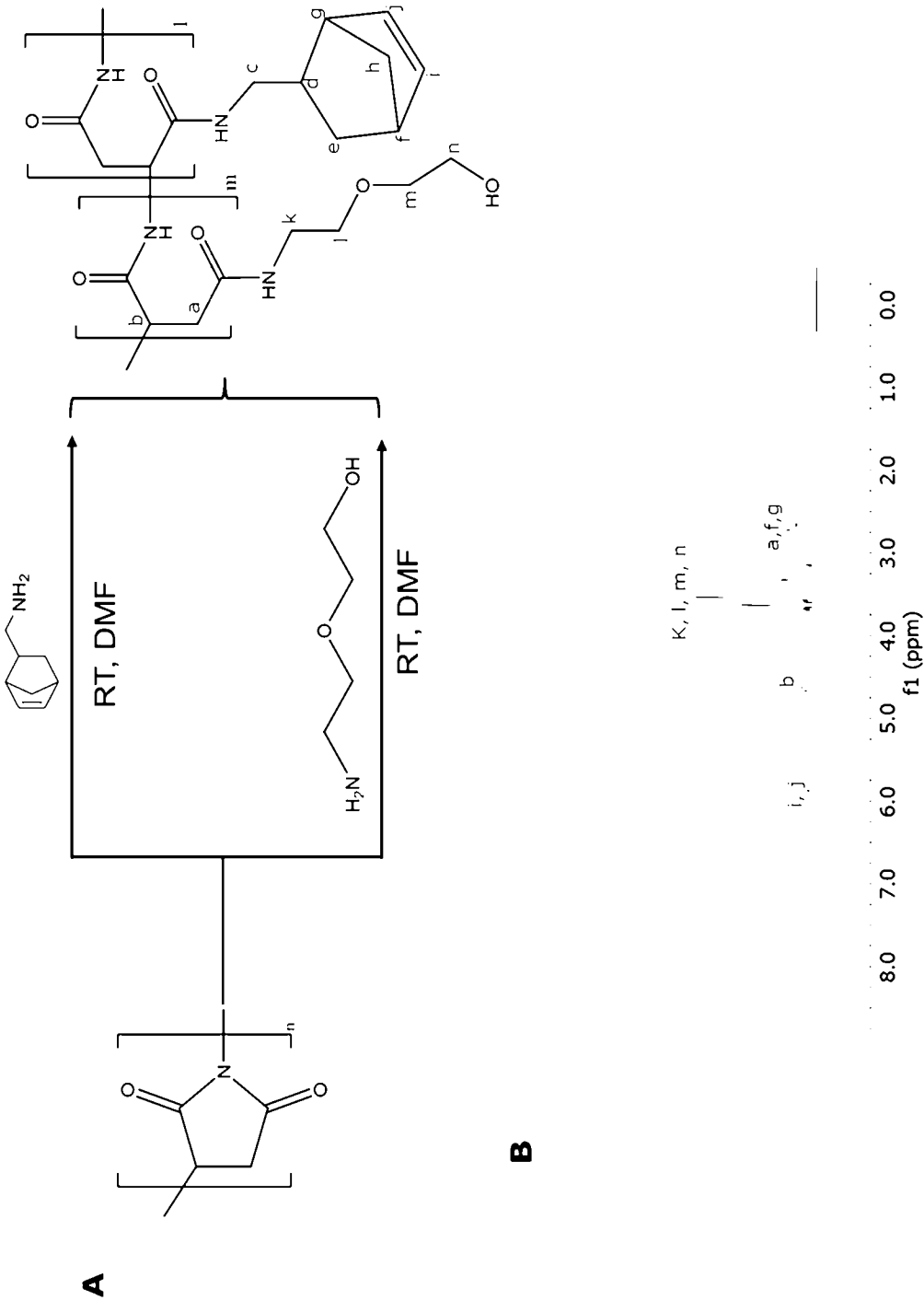
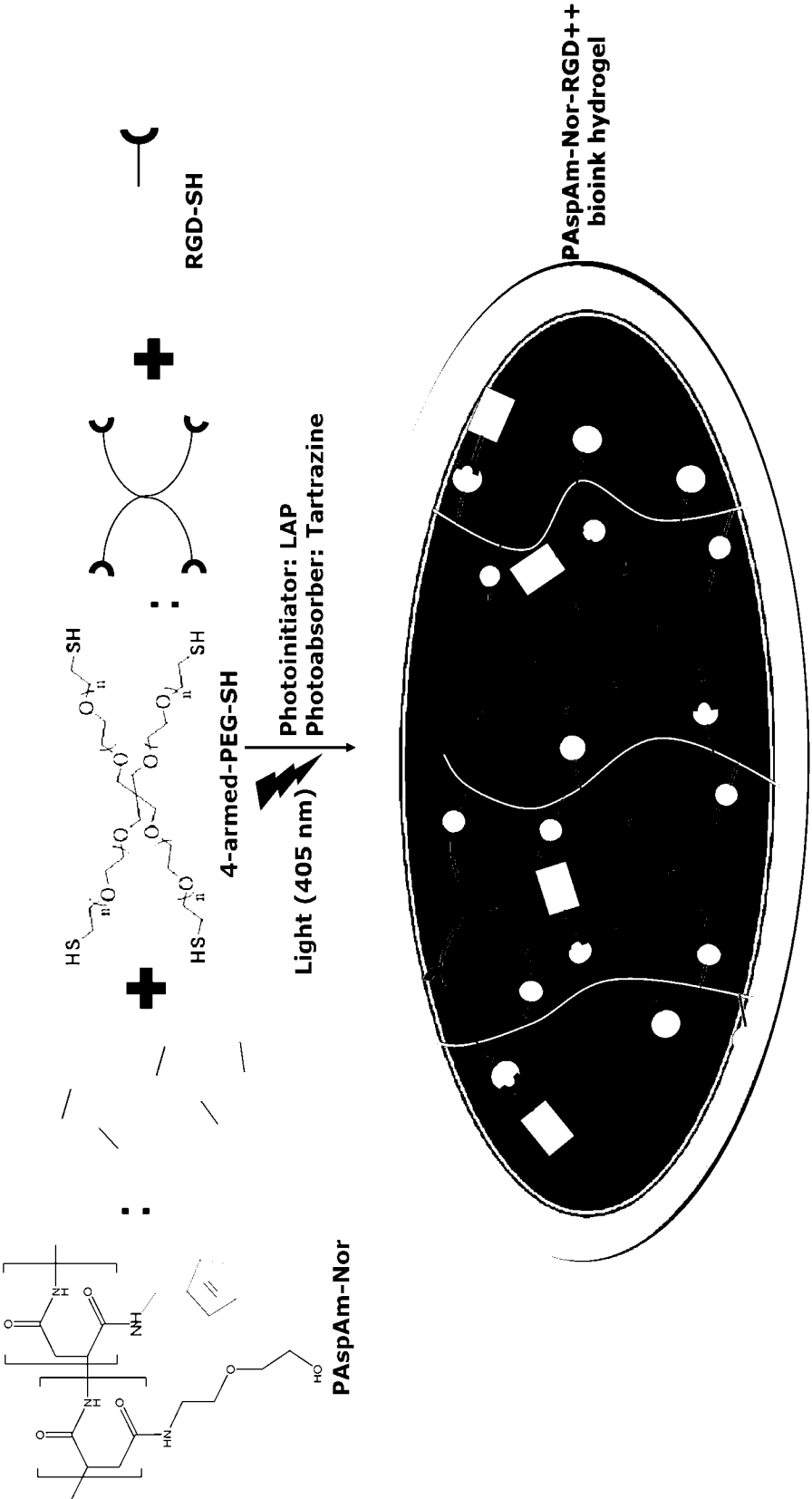


Fig. 12



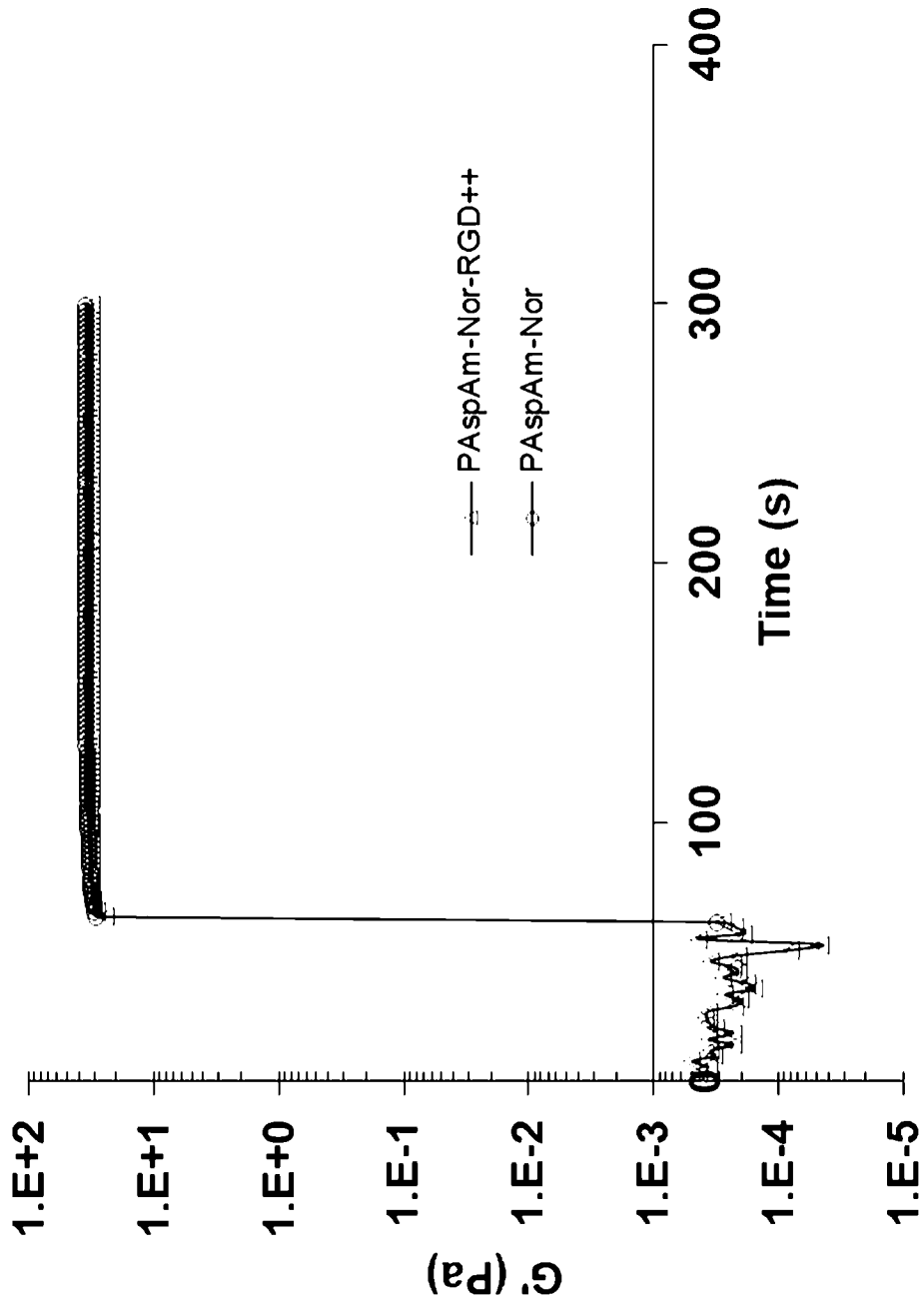
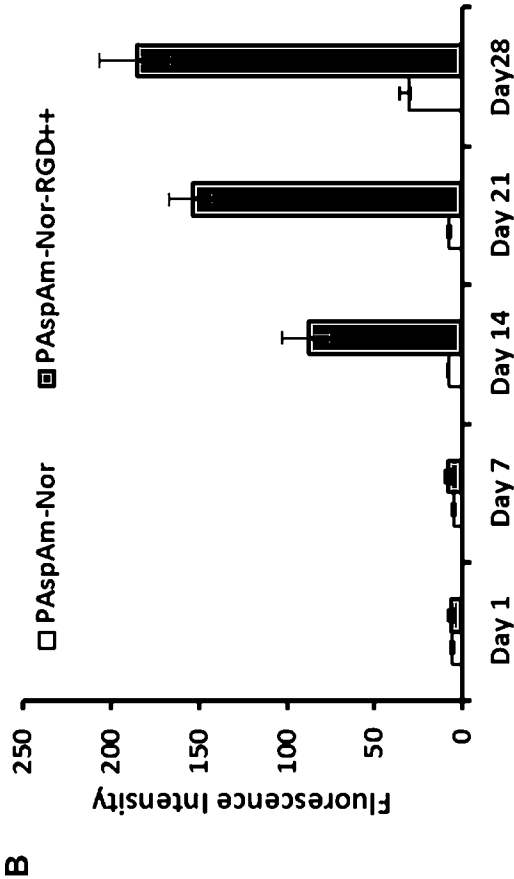
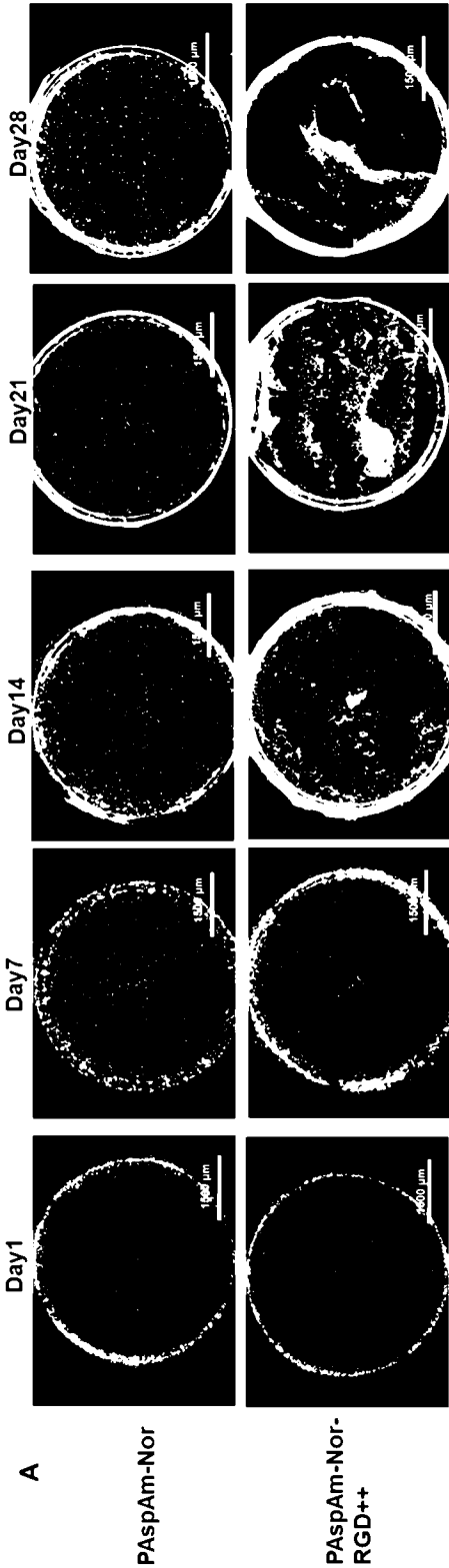


Fig. 13

Fig. 14



INTERNATIONAL SEARCH REPORT

International application No

PCT/US2024/045168

A. CLASSIFICATION OF SUBJECT MATTER

INV. C08G69/10 A61L27/18 A61L27/22 C08G69/40 C08G69/48
C09D11/101 C09D11/102 C09D177/04 C12N5/00

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)

C08G C12N A61L C09D

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, CHEM ABS 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 6 022 401 A (TANG JIANSHENG [US] ET AL) 8 February 2000 (2000-02-08) claims 1-7 column 6; table 1 examples column 9 - column 10 ----- - / - -	1-9, 11, 14



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

13 December 2024

Date of mailing of the international search report

02/01/2025

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

Mader, Margarita

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/045168

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed.
 - b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13ter.1(a)).
☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.
2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2024/045168

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DAVID D N 'DA ET AL: "Carrier-bound Methotrexate. IV. Antiproliferative Activity of Polyaspartamide-MTX Conjugates against Leukemic Lymphoblast Cell Lines", S. AFR. J. CHEM, vol. 59, 30 November 2006 (2006-11-30), pages 135-140, XP055718945, page 136 scheme 1; figure 1 section 3.1.; page 137, column 2, paragraph 2 -----	1-9,11, 14,17
X	PITARRESI G ET AL: "Biodegradable hydrogels obtained by photocrosslinking of dextran and polyaspartamide derivatives", BIOMATERIALS, ELSEVIER, AMSTERDAM, NL, vol. 24, no. 23, 1 October 2003 (2003-10-01), pages 4301-4313, XP004436365, ISSN: 0142-9612, DOI: 10.1016/S0142-9612(03)00332-6 -----	1-9,11, 14-20
Y	abstract section 2.2. and section 2.3.; page 4302 - page 4303 table 6 section 3.2. and section 3.3.; page 4308 - page 4312 -----	10,12, 13,21-25
Y	KR 2022 0031906 A (LEIBNIZ INSTITUT FUER NEUE MAT GEMEINNUETZIGE GMBH [DE]) 14 March 2022 (2022-03-14) claims 1, 4, 13 examples paragraph [0136] - paragraph [0150] -----	10,12, 13,21-25

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2024/045168

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 6022401 A	08-02-2000	CA 2205717 A1	16-01-1998
		US 6022401 A	08-02-2000
		US 6184336 B1	06-02-2001

KR 20220031906 A	14-03-2022	DE 102019117997 A1	07-01-2021
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		JP 2022539551 A	12-09-2022
		KR 20220031906 A	14-03-2022
		US 2022235316 A1	28-07-2022
		WO 2021001203 A1	07-01-2021
