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(54) Title: BIOINK GEL FORMULATIONS COMPRISING NUCLEOLIPID-BASED COMPOUND

(57) Abstract: The present invention relates to nucleolipid-based compounds for use in regenerative medicine. More specifically, the invention relates to the use of nucleolipid-based compounds in bioink gel formulations and a bioprinting method comprising the use of a bioink formulation comprising said nucleolipid-based compounds. The invention further relates to a bioprinted construct comprising nucleolipid-based compound(s) and bioink gel formulations.

BIOINK GEL FORMULATIONS COMPRISING NUCLEOLIPID-BASED COMPOUND

Technical field

5 The present invention relates to nucleolipid-based compounds for use in regenerative medicine. More specifically, the invention relates to the use of nucleolipid-based compounds in bioink gel formulations and to a bioprinting method comprising the use of a bioink formulation comprising said nucleolipid-based compounds. The invention further relates to a
10 bioprinted construct comprising nucleolipid-based compound(s) and bioink gel formulations.

 In the following description, references between brackets ([]) direct to the list of references presented at the end of the description.

15

State of the art

 Three-dimensional (3D) printing is a growing field in regenerative medicine and represents a valuable tool for the construction of complex 3D structures [1,2].

20 In literature, two approaches involving 3D printing are described: first, the use of 3D printing to build an acellular scaffold which is seeded with cells after fabrication (Top-Down approach), and secondly [3], the creation of tissue constructs by directly printing a biological ink or bioink composed of a mixture of cells and biomaterial (Bottom-Up approach). This
25 latter method can be performed through several techniques as: inkjet, laser and extrusion-based bioprintings [4].

 Regarding these different methods, extrusion-based bioprinting (EBB) is part of the most widespread tools used in additive manufacturing, mainly due to its capabilities for building large volume constructs such as
30 tissue or organ equivalents [3,5]. This technique offers a large opportunity in terms of bioink as it can be adaptable to a wide range of materials including scaffold-based (hydrogels, microcarriers, decellularized matrix

components) and scaffold-free (cells aggregates) bioinks [6]. Murphy et al. [1] exposed their concept of “ideal material” which needs to respond to several features: printability, biocompatibility, degradation kinetics and byproducts, structural and mechanical properties, material biomimicry.

5 Among all types of bioinks, hydrogels seem to be a promising biomaterial as they are easy to handle, affordable, and can mimic extracellular matrix [7]. Hydrogels are mainly composed of water entrapped in a 3D network of molecules, which can be covalently (chemically) or physically (ionic, hydrogens bonds or hydrophobic effect)
10 crosslinked [6,7].

 Initial approaches of extrusion based bioprinting used synthetic polymers such as polyethylene glycol (PEG) or methacrylated gelatin (GelMA) as scaffold materials but they suffer from poor biomimicry as their synthetic nature does not provide them a biological activity for their use in
15 cell culture [8]. As an alternative, natural polymers derived from fibrous proteins or peptides have been developed for their original biological activities.

 Among natural polymers, alginate, chitosan, collagen and gelatin are actually the most studied despite their weaknesses. Indeed, these
20 polymers encounter several issues such as low mechanical properties [3], the need of an external crosslinker to form a solid-like structure e.g. reticulation of alginate with calcium cation [9] and variability of collagen samples properties from a batch to another [10]. Moreover, a prerequisite for a bioink is the capacity of the supramolecular hydrogel to be formulated
25 in a cell culture medium, as for cell viability [8,11].

 Physically-crosslinked hydrogels, also known as supramolecular hydrogels [12], represent a promising tool in biological applications as their physical properties vary reversibly with conditions or stimuli (temperature, etc.). For this reason, physical hydrogels formed by low molecular weight
30 hydrogelators (LMWHs) [13,14] developed a great interest in biomedical applications due to their self-assembly abilities and biocompatibility [15]. Among LMWHs, the bioinspired amphiphilic compounds such as

nucleotide lipid (NL) and glycosyl nucleoside lipid (GNL) display promising properties due to their chemical structure and their resulting physico-chemical properties [16-19]. Composed of natural moieties like nucleoside/nucleotide, sugar and aliphatic chain, these low molecular systems allow a better biocompatibility, biostability and biodegradability. Their self-assembling properties in water to form hydrogels demonstrated enhanced rheological properties particularly thixotropy. In addition to their bioinspired structures which favour cell compatibility; these properties highlighted them as promising candidates for EBB.

But not all of bioinspired amphiphilic compounds display suitable properties to be incorporated in bioinks.

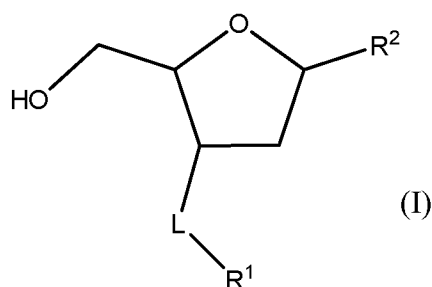
There is therefore a need for a hydrogel that does not only provide a biological activity for its use in cell culture but also high mechanical properties.

Description of the invention

Applicants have thus successfully developed a bioink formulation that fulfils both the need of a biological activity and high mechanical properties.

The invention relates to a use of a nucleolipid-based compound in a bioink formulation.

The invention further relates to the use of a nucleolipid-based compound of formula I:



wherein,

- L represents an oxycarbonyl —O—C(O)— group, a thiocarbamate —O—C(S)—NH— group, a carbonate —O—C(O)—O— group, a

carbamate —O—C(O)—NH— group, an oxygen atom, a phosphate group, a phosphonate group or a heteroaryl group comprising 1 to 4 nitrogen atoms, unsubstituted or substituted by a linear or branched, saturated or unsaturated C₂-C₃₀ hydrocarbon chain,

5 - R¹ represents

- a linear or branched C₂ to C₃₀ hydrocarbon chain, preferably C₆ to C₂₅ and more preferably C₈ to C₂₅, saturated or unsaturated, optionally completely or partially fluorinated, unsubstituted or substituted on the end of the chain by a
10 fluorine atom or by a benzyl or naphthyl ester or ether, or
- a diacylglycerol (or diglyceride) in which each acyl chain is a linear or branched C₂ to C₃₀ hydrocarbon chain, preferably C₆ to C₂₅ and more preferably C₈ to C₂₅, saturated or unsaturated, optionally completely or partially fluorinated,
15 unsubstituted or substituted on the end of the chain by a fluorine atom or by a benzyl or naphthyl ester or ether,

20 - R² represents a purine or pyrimidine base selected from uracil, adenine, guanine, cytosine, thymine, hypoxanthine, or their derivatives, optionally substituted, or a non-natural mono- or bicyclic heterocyclic base each ring of which comprising from 4 to 7 members, preferably guanine, cytosine or thymine and more preferably thymine, and

the compound being optionally in the form of a salt, preferably a metal cation salt and more preferably a metal cation selected from Li⁺, Na⁺, K⁺,
25 Rb⁺ and Cs⁺,
in a bioink formulation.

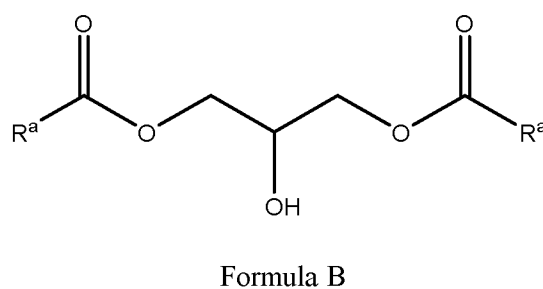
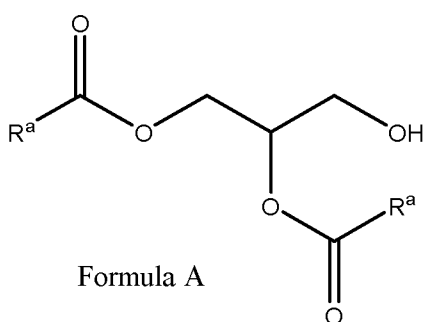
In the context of the invention, it is meant by “non-natural heterocyclic base”, a base different from uracil, adenine, guanine, cytosine, thymine or hypoxanthine and that cannot be found in nature.

30 It is meant by “derivative”, a compound that is derived from a similar compound by a chemical reaction.

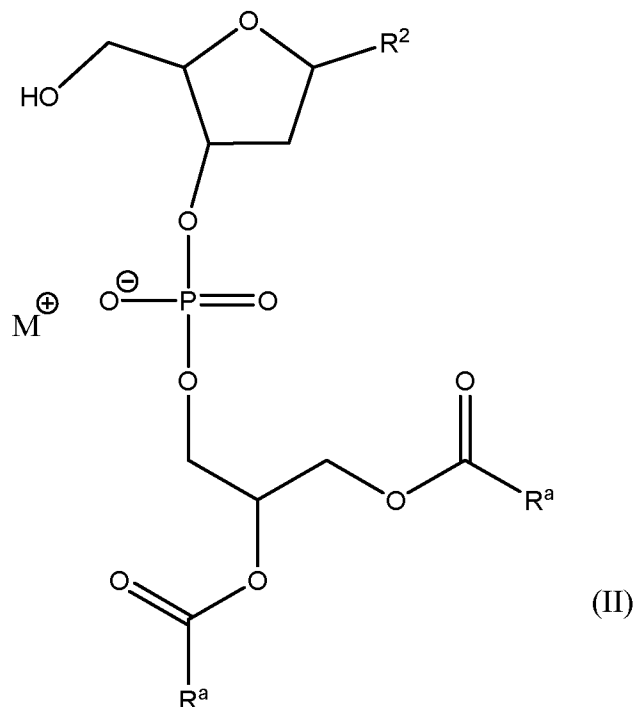
Advantageously, the purine or pyrimidine base, or the non-natural heterocyclic base can be substituted by at least one substituent chosen, for example, from a group comprising a halogen, an amino, a carboxy, a carbonyl, a carbonylamino, a hydroxy, an azido, a cyano, an alkyl, a cyclo alkyl, a perfluoroalkyl, an alkoxy, an oxycarbonyl, a vinyl, an ethynyl, a propynyl or an acyl group.

It is meant by "heteroaryl", a monocyclic or bicyclic, aromatic or partially unsaturated, carbocyclic group containing 5 to 12 atoms, interrupted by 1 to 4 nitrogen atoms, in particular a pyrazole, triazole, tetrazole or imidazole group.

It is meant by "diacylglycerol" or "diglyceride", a glyceride consisting of two acid chains covalently bonded to a glycerol molecule through ester linkages. Two possible forms exist, 1,2-diacylglycerols (formula A) and 1,3-diacylglycerols (formula B):



Advantageously, the nucleolipid-based compound may be of formula II:



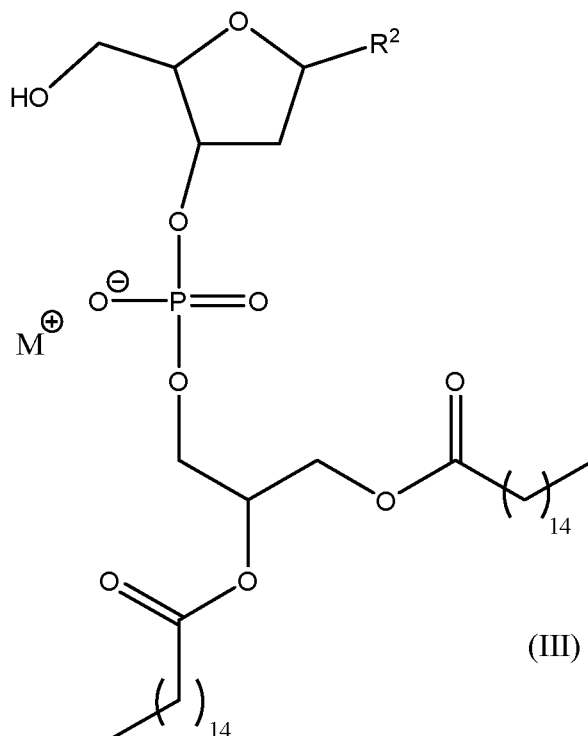
wherein,

- each R^a , identical or different, is a linear or branched C_1 to C_{29} hydrocarbon chain, preferably C_5 to C_{24} and more preferably C_7 to C_{24} , saturated or unsaturated,
- R^2 represents a purine or pyrimidine base selected from uracil, adenine, guanine, cytosine, thymine, hypoxanthine, or their derivatives, optionally substituted, preferably guanine, cytosine or thymine and more preferably thymine, and

M^+ represents a cation, preferably a metal cation, and more preferably selected from Li^+ , Na^+ , K^+ , Rb^+ , Cs^+ .

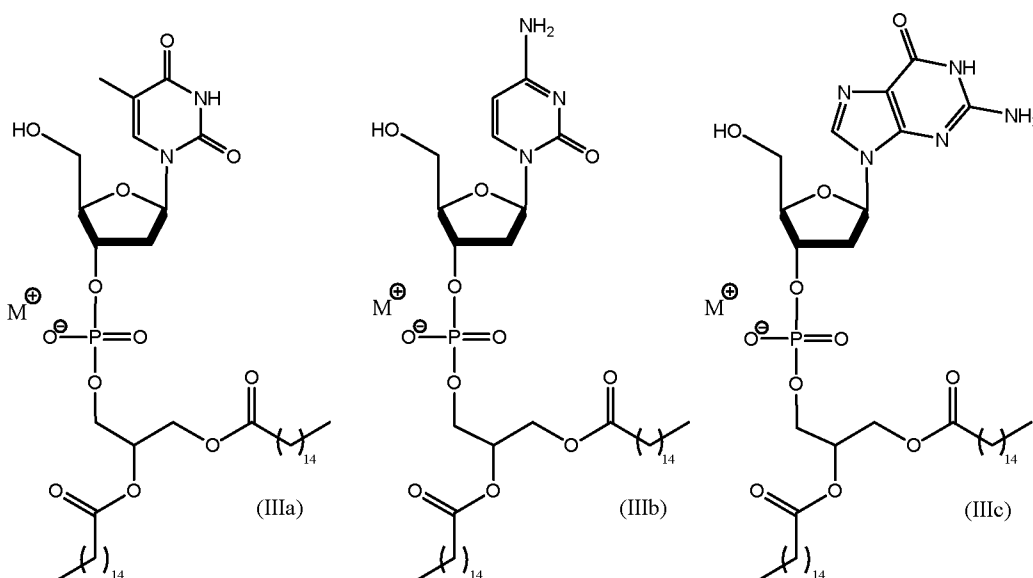
Advantageously, R^a , identical or different, may be a C_{13} to C_{19} linear hydrocarbon chain, preferably a C_{15} linear hydrocarbon chain.

Advantageously, the nucleolipid-based compound may be of formula III:



wherein R^2 represents a purine or pyrimidine base selected from guanine, cytosine and thymine, preferably R^2 represents a pyrimidine base and more preferably R^2 represents thymine and M^+ is as defined above.

5 Advantageously, the nucleolipid-based compound may be selected from compounds of formula IIIa, IIIb and IIIc, preferably of formula IIIa:



M^+ being as defined above.

“Bioprinting or “3D bioprinting” is the process of creating cell patterns in a confined space using 3D printing technologies, where cell function and viability are preserved within the printed construct. Generally, 3D bioprinting utilizes the layer-by-layer method to deposit materials known as Bioinks to create tissue-like structures that are later used in medical and tissue engineering fields. There are currently at least three types of 3D bioprinting technologies: laser printing technology, micro extrusion or extrusion technology and inkjet technology. In addition to these technologies, acoustic printer and hybrid printing technologies are emerging.

“Bioinks” are materials that mimic an extracellular matrix environment to support the adhesion, proliferation, and differentiation of living cells. Depending on the bioprinting technology that is used, the bioink may or may not comprise cells. For instance, micro-droplets of bioink containing both a gel and cells may be projected on a support. As an alternative, the bioink may not contain the cells and then the bioprinter may work with two printheads, one depositing the gel and the other the cells. The cells are pushed through a micro syringe and deposited using a needle. The layers are alternately deposited, a hydrogel layer (water mixture) followed by a layer of cells. The hydrogel is used to structure the assembly of cell layers, similar to scaffolding.

Advantageously, the bioink may be a gel, preferably selected from hydrogels and supramolecular gels.

“Thixotropy” is a time-dependent shear thinning property. A thixotropic fluid is a fluid which takes a finite time to attain equilibrium viscosity when introduced to a steep change in shear rate. Thixotropic fluids can take various periods of time to return to a gel state.

Advantageously, the gels according to the invention show a thixotropic profile in rheology. The gel may return to its initial state after a high shear stress in about less than 5 minutes. For example, in the context of the invention, bioinks take less than 5 minutes to return to their initial

state after a high shear stress, preferably between 5 seconds and 2 minutes.

The “storage modulus” (G'), also called “elastic modulus” or “Young’s modulus”, corresponds to the amount of energy stored and released in each oscillation. The “loss modulus” (G'') or viscous modulus describes the dissipated energy as heat which is lost.

Advantageously, G' values exceed G'' values over the entire frequency range, showing the solid character of these materials. In the case of the present invention, gels may have G' values comprised between 150 Pa and 150 kPa and G'' values comprised between 100 Pa and 20 kPa. For example, before extrusion, G' values may be comprised between 150 and 3 kPa and G'' values may be comprised between 100 Pa and 1.2 kPa. Gels may also have at room temperature (about 25°C) and after extrusion, G' values comprised between 300 Pa and 150 kPa and G'' values comprised between 150 Pa and 15 kPa. Gels may also have at physiological temperature (about 37°C) and after extrusion, G' values comprised between 300 Pa and 20 kPa and G'' values comprised between 150 Pa and 7 kPa.

“Tsol-gel” is the temperature of transition of a sol to a gel at the gel point.

Advantageously, the gels according to the invention have a Tsol-gel comprised between 25 and 45°C, preferably between 35 and 42°C.

Advantageously, the gel may be a hydrogel. It is meant by “hydrogel”, a gel in which the swelling agent is water.

Advantageously, the hydrogel may comprise an aqueous solution. The aqueous solution may comprise a salt chosen in the group comprising lithium, sodium, potassium, rubidium, cesium, or ammonium chloride, preferably sodium chloride. The concentration of lithium, sodium, potassium, rubidium, cesium or ammonium chloride in the aqueous solution may be comprised between 0.5 to 1.5 wt.%, preferably is 0.9 wt. %.

Advantageously, the aqueous solution may comprise a salt chosen in the group comprising lithium, sodium, potassium, rubidium or cesium chloride, preferably sodium chloride. The concentration of lithium, sodium, potassium, rubidium or cesium chloride in the aqueous solution may be comprised between 0.5 to 1.5 wt.%, preferably is 0.9 wt. %.

Advantageously, the aqueous solution may be a 0.9% aqueous sodium chloride solution.

Advantageously, the concentration of compound of formula I, II, III, IIIa, IIIb or IIIc in the gel may be comprised between 0.1 and 10 % w/w, preferably between 2 and 5 % w/w and more preferably 3 % w/w. The gel may comprise one or more compound of formula I, II, III, IIIa, IIIb or IIIc.

Advantageously, the gel may comprise between 0.1 and 10 % w/w concentration of a compound of formula I, II, III, IIIa, IIIb or IIIc and between 90 and 99.9 % of an aqueous solution comprising lithium, sodium, potassium, rubidium, cesium or ammonium chloride. For example, the gel may comprise 1.5 % w/w concentration of compound of formula I, II, III, IIIa, IIIb or IIIc and 98.5 % w/w of a 0.9 % w/w sodium chloride aqueous solution or the gel may comprise 3 % w/w concentration of compound of formula I, II, III, IIIa, IIIb or IIIc and 97 % w/w of a 0.9 % w/w sodium chloride aqueous solution. For example, the hydrogel may comprise between 0.05 and 15 % w/w of an aqueous solution, advantageously between 0.5 and 10% w/w.

Advantageously, the gel may further comprise a cell culture medium. The cell culture medium may be selected from the Dulbecco's Modified Eagle Medium (DMEM), the enriched Dulbecco's Modified Eagle Medium (eDMEM), RPMI (Roswell Park Memorial Institute), MEM (Minimum Essential Media), IMDM (Iscove's Modified Dulbecco's Medium), opti-MEM medium (Reduced Serum Media is a modification of Eagle's Minimum Essential Media, buffered with HEPES and sodium bicarbonate, and supplemented with hypoxanthine, thymidine, sodium pyruvate, L-

glutamine, trace elements, and growth factors) and DMEM/F-12 (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12).

Advantageously, the mass ratio aqueous solution / cell culture medium in the gel may be comprised between 10:90 and 50:50, preferably
5 between 20:80 and 40:60, more preferably the ratio is 30:70.

Advantageously, the gel may comprise between 0.1 and 10 % w/w concentration of a compound of formula I, II, III, IIIa, IIIb or IIIc and between 90 and 99.9 % of a mixture of an aqueous solution comprising lithium, sodium, potassium, rubidium, cesium, or ammonium chloride and a
10 cell culture medium, the mass ratio aqueous solution / cell culture medium in the gel may be comprised between 10:90 and 50:50, preferably between 20:80 and 40:60, more preferably the ratio is 30:70.

Advantageously, the gel may comprise between 0.1 and 10 % w/w concentration of a compound of formula I, II, III, IIIa, IIIb or IIIc, between 90
15 and 99.9 % of a culture medium and a lithium, sodium, potassium, rubidium, cesium, or ammonium chloride salt, said salt final concentration in the gel being comprised between 0.5 to 1.5 wt.%.

Advantageously, the gel may further comprise cells. The cells may
20 be selected from apical papilla stem cells, human bone marrow cells, human skin fibroblast, human gingival fibroblast, human umbilical vein cells, endothelial cells, hematopoietic stem cells (HSC), human induced pluripotent stem cells (HiPSC) and mesenchymal stem cells (MHSC).

Advantageously, the gel may comprise a concentration of cells
25 between 0.5 million to 5 millions of cells/mL of gel, preferably between 1 and to 5 millions of cells/mL of gel and more preferably between 1 and 2 millionscells/mL of gel.

Advantageously, the bioink formulation according to the invention may be suitable for bioprinting. Preferably the bioprinting method is
30 selected from the group comprising inkjet bioprinting, laser bioprinting and extrusion based bioprinting.

The invention further relates to a bioprinting method comprising the use of a bioink formulation comprising a nucleolipid-based compound of formula I, II, III, IIIa, IIIb or IIIc.

5 The invention also relates to a bioprinting method comprising a step of (preferably layer by layer) deposition of a bioink formulation comprising a nucleolipid-based compound of formula I, II, III, IIIa, IIIb or IIIc.

The invention may also relates to nucleolipid-based compounds of formula I, II, III, IIIa, IIIb or IIIc for use in regenerative medicine, preferably
10 for use in the bioprinting of vascular niches for cells, wherein the bioprinting is chosen in the group comprising inkjet bioprinting, laser bioprinting and extrusion based bioprinting.

On another aspect, the invention also concerns bioprinted grid or
15 prototype construct comprising nucleolipid-based compounds of formula I, II, III, IIIa, IIIb or IIIc.

The invention is also linked to a bioink gel formulation, preferably a hydrogel or a supramolecular gel, comprising a compound of formula I, II, III, IIIa, IIIb or IIIc, aqueous lithium, sodium, potassium, rubidium, cesium,
20 or ammonium chloride solution, a cell culture medium and optionally cells.

Advantageously, the invention is also linked to a bioink gel formulation, preferably a hydrogel or a supramolecular gel, comprising a compound of formula I, II, III, IIIa, IIIb or IIIc, aqueous lithium, sodium,
25 potassium, rubidium, or cesium chloride solution, a cell culture medium and optionally cells.

Advantageously, the bioink gel formulation, preferably in the form of a a hydrogel or a supramolecular gel, may comprise between 0.1 and 10 % w/w of a compound of formula I, II, III, IIIa, IIIb or IIIc, a 0.5 to 1.5 wt.
30 % w/w aqueous lithium, sodium, potassium, rubidium or cesium chloride solution, a cell culture medium, and optionally cells, wherein the mass ratio

aqueous solution / cell culture medium is comprised between 10:90 and 50:50.

Advantageously, the bioink gel formulation, preferably a hydrogel or a supramolecular gel, may comprise:

- 5 - between 0.1 and 10 % w/w of a compound of formula I, II, III, IIIa, IIIb or IIIc,
- a 0.5 to 1.5 wt. % aqueous lithium, sodium, potassium, rubidium, cesium, or ammonium chloride solution,
- a cell culture medium, and
- 10 - optionally cells,

wherein the ratio aqueous solution / cell culture medium is comprised between 10:90 and 50:50.

Advantageously, the bioink gel formulation, preferably a hydrogel

15 or a supramolecular gel, may comprise:

- between 0.1 and 10 % w/w of a compound of formula I, II, III, IIIa, IIIb or IIIc,
- a 0.5 to 1.5 wt. % aqueous lithium, sodium, potassium, rubidium or cesium chloride solution,
- 20 - a cell culture medium, and
- optionally cells,

wherein the ratio aqueous solution / cell culture medium is comprised between 10:90 and 50:50.

Advantageously, the bioink gel formulation, preferably a hydrogel

25 or a supramolecular gel, may comprise:

- between 0.1 and 10 % w/w of a compound of formula I, II, III, IIIa, IIIb or IIIc,
- between 0.1 and 10 %w/w of a 0.5 to 1.5 wt. % aqueous lithium, sodium, potassium, rubidium, cesium or ammonium chloride
- 30 solution,
- a cell culture medium, and
- optionally cells,

wherein the ratio aqueous solution / cell culture medium is comprised between 10:90 and 50:50.

Advantageously, the bioink gel formulation, preferably a hydrogel or a supramolecular gel, may comprise:

- between 0.1 and 10 % w/w of a compound of formula I, II, III, IIIa, IIIb or IIIc,
- between 0.1 and 10 %w/w of a 0.5 to 1.5 wt. % aqueous lithium, sodium, potassium, rubidium or cesium chloride solution,
- a cell culture medium, and
- optionally cells,

wherein the ratio aqueous solution / cell culture medium is comprised between 10:90 and 50:50.

Brief description of the figures

Figure 1 represents images of hydrogels formed with (a) diC16-3'-dT in 0.9% sodium chloride aqueous solution at 3% (w/v), (b) diC16-3'-dT in 0.9% sodium chloride enriched DMEM (eDMEM) at 3% (w/v).

Figure 2 represents oscillatory rheological experiments of diC16-3'-dT-based gel in eDMEM (enriched DMEM) at 3% (w/v). (a) Frequency sweep results at ambient ($25 \pm 0.01^\circ\text{C}$) and physiological ($37 \pm 0.01^\circ\text{C}$) temperatures. (b) Step-strain experiment at a fixed angular frequency (6.283 rad/s). The gel was swept from 0.1% to 15% then back to 0.1% strain.

Figure 3 represents bioprinted grids of (a) diC16-3'-dT-0.9% NaCl aqueous solution hydrogel; 1 layer (b) diC16-3'-dT-0.9% NaCl aqueous solution hydrogel; 5 layers (c) diC16-3'-dT in 0.9% sodium chloride enriched DMEM (70/30% v/v) (eDMEM) hydrogel; 1 layer (d) diC16-3'-dT-eDMEM hydrogel; 5 layers.

Figure 4 represents pictures of bioprinted grid pore of (a) diC16-3'-dT-0.9% NaCl aqueous solution-based gel and (b) diC16-3'-dT in 0.9%

sodium chloride enriched DMEM (70/30% v/v) gel (magnification x2.5) (scale bars: 500µm).

Figure 5 represents confocal microscopy images of Live/Dead® tests at different times (magnification x10) (scale bars: 100µm).

5 **Figure 6** represents (a) Alamar Blue® representations of D1, D3 and D7 values against blank and (b) a trend curve of Alamar Blue® values at D1, D3 and D7 against D1.

Figure 7 represents the synthesis scheme of nucleolipids diC16-3'-dG (**1a**) and diC16-3'-dC (**1b**).

10 **Figure 8** represents Oscillatory rheological experiments of diC16-3'-dC -based gel in DMEM/NaCl 123.5 mM. (a) Frequency sweep results at 1.5% (w/v) and 3% (w/v) at ambient temperature (25±0.01°C) (b) destructure/restructure experiment diC16-3'-dC -based gel in DMEM/NaCl 123.5mM at 3% (w/v) at a fixed frequency (1Hz). The gel was
15 swept from 0.1% to 25% then back to 0.1% strain.

EXAMPLES

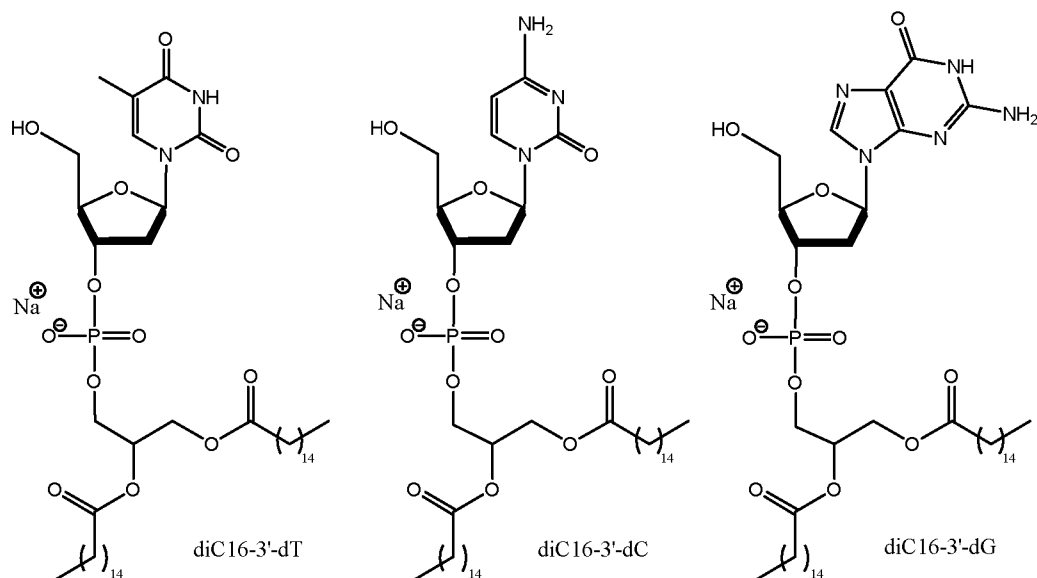
Example 1:

20 Materials

The different aqueous solvents were purchased from the following companies: 0.9% sodium chloride aqueous solution (Fresenius Kabi, Bad Homburg vor der Höhe, Germany); Dulbecco's Modified Eagle Medium (DMEM) with low glucose, GlutaMAX® and pyruvate (Gibco®,
25 ThermoFisher Scientific, Waltham, Massachusetts, USA). Milli-Q® water was obtained by filtration through a PURELAB® ultra ELGA (Veolia Water, Antony, France) system with a 0.2µm filter at the water outlet point.

Nucleolipid-based compounds

30 Nucleolipids (NL) were synthesized according to the procedure described in WO 2009/098404, in particular diC16-3'-dT.



Nucleolipids diC16-3'-dG and diC16-3'-dC were synthesized according to the following procedures.

5 Synthesis of triethylammonium (2*R*,3*S*,5*R*)-5-(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)-2-(hydroxymethyl)tetrahydrofuran-3-yl ((*R*)-2,3-bis(palmitoyloxy)propyl) phosphate (diC16-3'-dG) (compound **1a**)

The synthesis scheme is represented on Figure 7.

10

a) Step 1 (deprotection of isobutyryl group): (2*R*,3*S*,5*R*)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-5-(2-isobutyramido-6-oxo-1,6-dihydro-9*H*-purin-9-yl)tetrahydrofuran-3-yl (2-cyanoethyl) diisopropyl-phosphoramidite (isobutyryl-dG-CE phosphoramidite) **2a** (2 g, 2.38 mmol, 1 equiv.) was dissolved in MeNH₂ (2 M in THF, 23.81 mL, 47.6 mmol, 20 equiv.) at room temperature under argon. After being stirred for 14 h, the mixture was evaporated under reduced pressure. The completion of the reaction was confirmed by TLC (disappearance of the spot corresponds to starting material and appearance of a new spot at slightly lower retardation factor (*R_f*)). The residue was chromatographed on a column of silica gel with 1% MeOH/DCM (v/v) containing 1% TEA and then 3% MeOH/DCM (v/v) containing 1% TEA to give the fractions

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containing the target (2*R*,3*S*,5*R*)-5-(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-tetrahydrofuran-3-yl (2-cyanoethyl) diisopropylphosphoramidite **3a**.

Yield: 1.33 g (72.7 %). The product was utilized for next step without further characterization.

b) Step 2 (coupling with lipid): (2*R*,3*S*,5*R*)-5-(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-tetrahydrofuran-3-yl (2-cyanoethyl) diisopropylphosphoramidite **3a** (product of step 1) (1.2 g, 1.64 mmol, 1 equiv.) and 1,2-dipalmitoyl-*sn*-glycerol (1.21 g, 2.14 mmol, 1.3 equiv.) were dissolved in dry THF (25 mL). A tetrazole solution in acetonitrile (0.45 M, 6.2 mL, 2.8 mmol, 1.7 equiv.) was added, and the reaction mixture was stirred overnight at room temperature followed by oxidation with I₂ solution (0.02 M in THF/Pyridine/H₂O, 140 mL, 2.8 mmol, 1.7 equiv.). After 6 h at room temperature, the solvent was evaporated under high vacuum and the intermediate products were dissolved in ethyl acetate (100 mL) and then washed with saturated Na₂S₂O₃ solution (2 x 25 mL). The fractions were dried over anhydrous sodium sulfate, and the solvent was evaporated. The resulting product was dissolved in DCM (20 mL) and trichloroacetic acid (TCA, 3% in DCM) solution (15 mL) under argon, after 4 h at room temperature, 5 mL of methanol and 25 mL of sodium hydrogen carbonate solution were added. The aqueous phase was extracted with DCM (2 x 30 mL), and then the organic fractions were collected, dried over anhydrous sodium sulfate, and the solvent evaporated. The intermediate product was dissolved 33% trimethylamine (TEA) in DCM (50 mL DCM + 25 mL TEA) and stirred overnight at room temperature (to ensure the complete deprotection of cyanoethyl chain). The solvent was evaporated under high vacuum.

The product **1a** was isolated after purification on silica gel (DCM:methanol:TEA from 98:1:1 to 90:9:1). Yield: 852 mg (32.8 %). *R_f* (product **1a**) = 0.3 (DCM:methanol:TEA 90:9:1).

¹H NMR (300 MHz, CDCl₃): δ in ppm 0.86 (t, 6H, *J*=6.6 Hz, 2CH₃ of palmitoyl chain), 1.23 (s, 48H, 24CH₂ (palmitoyl chain)), 1.33 (t, 9H, *J*=7.2 Hz, 3CH₃ (triethylammonium)), 1.57 (bs, 4H, 2CH₂-CH₂-CO), 2.13-2.40 (m, 4H, 2CH₂-CO), 2.54 (bs, 1H, H2'(sugar)), 2.87 (bs, 1H, H2''(sugar)),
 5 3.11 (q, *J*=7.1, 3CH₂ (triethylammonium)), 3.64-4.49 (m, 8H, 2CH₂ (glycerol), H3' (sugar), H4'(sugar), 2H5'(sugar)), 5.03-5.36 (m, 2H, -CH-glycerol, -OH), 6.16 (t, 1H, *J*=6.8 Hz, H1'), 6.52 (bs, 2H, -NH₂), 7.67 (s, 1H, H-8(base)), 12.42 (s, 1H, -NH(base)).

¹³C NMR (75 MHz, CDCl₃): δ in ppm 8.7 (CH₃, triethylammonium),
 10 14.2 (CH₃ chain), 22.8 (CH₂-CH₃ chain), 25.0 (CH₂-CH₂-C=O), 29.2-29.8 (CH₂ chain), 32.0 (CH₂-CH₂-CH₃ chain), 34.2 (CH₂-C=O chain), 34.4 (CH₂-C=O chain), 45.9 (CH₂ triethylammonium), 62.7(CH₂-O-C=O glycerol), 63.2 (CH₂-O-P=O glycerol), 63.7 (CH₂, C5'-sugar), 70.35 (CH, C3'-sugar), 70.44 (CH glycerol), 86.7 (C, C4'-sugar), 87.7 (CH, C1'-sugar),
 15 118.6 (C, C5-base), 137.0 (CH, C8-base), 149.9 (C, C4-base), 154.0 (C, C2-base), 158.2 (C=O, C6-base), 173.2 (C=O chain), 173.5 (C=O chain).

³¹P NMR (121 MHz, CDCl₃): δ in ppm 1.86. High-resolution ESI MS
 20 [M-H]⁻, theoretical *m/z*=896.55192, observed *m/z*=896.5525 (1 ppm).

Synthesis of triethylammonium (2*R*,3*S*,5*R*)-5-(4-amino-2-oxopyrimidin-1(2*H*)-yl)-2-(hydroxymethyl)tetrahydrofuran-3-yl ((*R*)-2,3-bis(tetradecanoyloxy)propyl) phosphate (diC16-3'-dC) (compound **1b**)

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The synthesis scheme is represented on Figure 7.

a) Step 1 (deprotection of isobutyryl group): (2*R*,3*S*,5*R*)-5-(4-acetamido-2-oxopyrimidin-1(2*H*)-yl)-2-((bis(4-methoxyphenyl)(phenyl)-methoxy)methyl)tetrahydrofuran-3-yl (2-cyanoethyl) diisopropyl-phosphoramidite (Ac-dC-CE phosphoramidite) **2b** (2 g, 2.6 mmol, 1 equiv.) was dissolved in MeNH₂ (2 M in THF, 25.9 mL, 51.8 mmol, 20

equiv.) at room temperature under argon. After being stirred for 2 h, the mixture was evaporated under reduced pressure. The completion of the reaction was confirmed by TLC (disappearance of the spot corresponds to starting material and appearance of a new spot at slightly lower retardation factor (R_f)). The residue was chromatographed on a column of silica gel with 1% MeOH/DCM (v/v) containing 1% TEA and then 3% MeOH/DCM (v/v) containing 1% TEA to give the fractions containing the target (2*R*,3*S*,5*R*)-5-(4-amino-2-oxopyrimidin-1(2*H*)-yl)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)tetrahydrofuran-3-yl (2-cyanoethyl) diisopropylphosphoramidite **3b**.

Yield: 1.68 g (89.2 %). The product was utilized for next step without further characterization.

b) Step 2 (coupling with lipid): (2*R*,3*S*,5*R*)-5-(4-amino-2-oxopyrimidin-1(2*H*)-yl)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-tetrahydrofuran-3-yl (2-cyanoethyl) diisopropylphosphoramidite **3b** (product of step 1) (1.4 g, 1.81 mmol, 1 equiv.) and 1,2-dipalmitoyl-*sn*-glycerol (1.34 g, 2.36 mmol, 1.3 equiv.) were dissolved in dry THF (25 mL). A tetrazole solution in acetonitrile (0.45 M, 6.9 mL, 3.1 mmol, 1.7 equiv.) was added, and the reaction mixture was stirred overnight at room temperature followed by oxidation with I₂ solution (0.02 M in THF/Pyridine/H₂O, 154 mL, 3.1 mmol, 1.7 equiv.). After 6 h at room temperature, the solvent was evaporated under high vacuum and the intermediate products were dissolved in ethyl acetate (100 mL) and then washed with saturated Na₂S₂O₃ solution (2 x 25 mL). The fractions were dried over anhydrous sodium sulfate, and the solvent was evaporated. The resulting product was dissolved in DCM (20 mL) and trichloroacetic acid (TCA, 3% in DCM) solution (15 mL) under argon, after 4 h at room temperature, 5 mL of methanol and 25 mL of sodium hydrogen carbonate solution were added. The aqueous phase was extracted with DCM (2 x 30 mL), and then the organic fractions were collected, dried over anhydrous sodium sulfate, and the solvent evaporated. The intermediate

product was dissolved 33% trimethylamine (TEA) in DCM (50 mL DCM + 25 mL TEA) and stirred overnight at room temperature (to ensure the complete deprotection of cyanoethyl chain). The solvent was evaporated under high vacuum.

5 The product **1b** was isolated after purification on silica gel (DCM:methanol:TEA from 98:1:1 to 90:9:1).

Yield: 653 mg (37.7 %). R_f (product **1b**) = 0.3 (DCM:methanol:TEA 90:9:1).

10 ¹H NMR (300 MHz, CDCl₃): δ in ppm 0.85 (t, 6H, J =6.5 Hz, 2CH₃ of palmitoyl chain), 1.22 (s, 48H, 24CH₂ (palmitoyl chain)), 1.27 (t, 9H, J =7.4 Hz, 3CH₃ (triethylammonium)), 1.55 (bs, 4H, 2CH₂-CH₂-CO), 2.00-2.34 (m, 5H, 2CH₂-CO, H2'(sugar)), 2.51 (bs, 1H, H2''(sugar)), 3.02 (q, J =7.2, 3CH₂ (triethylammonium)), 3.25-4.91 (m, 8H, 2CH₂ (glycerol), H3' (sugar), H4' (sugar), 2H5' (sugar)), 5.21 (bs, 1H, -CH- glycerol), 6.02 (d, 1H, J =6.5 Hz, H-5(base)), 6.12 (t, 1H, J =5.3 Hz, H1'), 8.05 (bs, 1H, H-6 (base)).

15 ¹³C NMR (75 MHz, CDCl₃): δ in ppm 9.0 (CH₃, triethylammonium), 14.2 (CH₃ chain), 22.8 (CH₂-CH₃ chain), 25.0 (CH₂-CH₂-C=O), 29.3-29.8 (CH₂ chain), 32.0 (CH₂-CH₂-CH₃ chain), 34.2 (CH₂-C=O chain), 34.4 (CH₂-C=O chain), 45.9 (CH₂ triethylammonium), 61.4 (CH₂, C5'-sugar), 62.8 (CH₂-O-C=O glycerol), 63.6 (CH₂-O-P=O glycerol), 70.4 (CH glycerol), 74.8 (CH, C3'-sugar), 86.4 (C, C4'-sugar), 95.4 (CH, C5-base), 95.5 (CH, C1'-sugar), 142.1 (CH, C6-base), 155.1 (C=O, C2-base), 162.5 (C, C4-base), 173.2 (C=O chain), 173.6 (C=O chain).

20 ³¹P NMR (121 MHz, CDCl₃): δ in ppm 1.90. High-resolution ESI MS [M-H]⁻, theoretical m/z =856.54577, observed m/z =856.5459 (0 ppm).

Hydrogel bioink formulations

Hydrogels formulations of diC16-3'-dT, diC16-3'-dC, diC16-3'-dG were prepared according to previous work [17–19].

30 In order to evaluate gel fixation (i.e. gelation), samples were turned upside-down; remaining still under its own weight indicated a complete gelation.

Preparation of diC16-3'-dT, diC16-3'-dC and diC16-3'-dG hydrogels

diC16-3'-dT, diC16-3'-dC or diC16-3'-dG was mixed with 0.9% sodium chloride aqueous solution, DMEM and DMEM/0.9% sodium chloride aqueous solution (70/30% v/v) called enriched DMEM (eDMEM) to make 3% (w/v) gels. eDMEM osmolarity was controlled with a Type 15 osmometer (Fisher Scientific SAS, Illkirch Graffenstaden, France). Samples were heated at 55°C and stirred at 1000 rpm for 30 minutes. Samples were then stored at room temperature.

Further diC16-3'-dC based hydrogels were formed in a range of concentration of 1.5% to 3% (w/v) in either aqueous NaCl 154mM or DMEM/NaCl(aq) (70/30 % v/v, 123.5mM NaCl final concentration). A hydrogel at a concentration of 3% into DMEM/ NaCl(S) (123.5mM NaCl final concentration) was also formed.

Example 2:

Characterization

Determination of hydrogels mechanical properties by rheology.

1) diC16-3'-dT

Rheological experiments were performed using a Malvern Kinexus Pro+ rheometer (Malvern Instrument SA, Worcestershire, United Kingdom) with a steel cone-plate geometry (diameter: 20mm; angle: 1°).

A solvent trap was used to prevent solvent evaporation and to control temperature. The low plate was equipped with a Peltier temperature control system. Samples of hydrogels of example 1 were studied at ambient ($25 \pm 0.01^\circ\text{C}$) and physiological ($37 \pm 0.01^\circ\text{C}$) temperatures. Materials were placed on the low geometry under their gel states using a

plastic spatula and allowed to rest 30 minutes before any experiments. All measurements were carried out within the linear viscoelastic regime (LVR). It was determined by amplitude strain sweeps from 0.01 to 100% at an angular frequency of 1Hz (6.283 rad.s⁻¹). Storage modulus (G') and loss modulus (G'') were studied using a frequency sweep test ranging from 0.1Hz to 10Hz.

To determine the transition temperature (T_{sol-gel}), a single frequency stress controlled temperature ramp was performed. This sol-gel transition temperature was determined at 1Hz frequency and 1Pa strain.

The ramp was included between 25 and 90°C (heating rate of 2°C/min).

Thixotropy was assessed by subjecting gels to low/high strain cycles. Gel was first subjected to a low strain (0.1%, into its LVR) for 10 minutes. Then, the sample was suddenly subjected to a higher strain (15%, beyond its LVR) for 2 minutes. Last, the strain came back to the first step value (0.1%, into its LVR) until full recovery of its initial properties.

The values of G' and G'' are compiled in the tables below.

diC16-3'-dT	Before extrusion		After extrusion	
25°C	0.9% sodium chloride	eDMEM	0.9% sodium chloride	eDMEM
G' (Pa)	1945 ± 549	362 ± 176	576 ± 171	2993 ± 1591
G'' (Pa)	732 ± 200	269 ± 144	289 ± 105	784 ± 337

Table 1: Shear moduli (G' and G'') of 3 %w/v diC16-3'-dT-based gels formed in 0.9% sodium chloride aqueous solution or in eDMEM (enriched DMEM) at ambient temperature (25±0.01°C) before and after syringe pump extrusion with a 22 gauge needle, expressed as mean ± standard deviation of the mean.

diC16-3'-dT- eDMEM 37°C	Before extrusion	After extrusion
G' (Pa)	675 ± 417	5222 ± 1144
G'' (Pa)	340 ± 230	1189 ± 176

Table 2: Shear moduli (G' and G'') of 3 %w/v diC16-3'-dT-based gels formed in eDMEM (0.9% sodium chloride aqueous solution enriched DMEM) at physiological temperature (37±0.01°C) before and after syringe

pump extrusion with a 22 gauge needle, expressed as mean $\pm$ standard deviation of the mean.

diC16-3'-dT- eDMEM	25°C	37°C
G' (Pa)	112133 $\pm$ 1305	39220 $\pm$ 1160
G'' (Pa)	14430 $\pm$ 324	5181 $\pm$ 453

Table 3: Shear moduli (G' and G'') of 3 %w/v diC16-3'-dT-based gels formed in eDMEM (0.9% sodium chloride aqueous solution enriched DMEM) at ambient (25 $\pm$ 0.01°C) and physiological (37 $\pm$ 0.01°C) temperatures after syringe pump extrusion with a 22 gauge needle and 21 days stability in a 37°C dried incubator, expressed as mean $\pm$ standard deviation of the mean.

2) diC16-3'-dC

A Malvern Kinexus Pro+ rheometer (Malvern Instrument SA, Worcestershire, United Kingdom) was used to evaluate the rheological properties of the hydrogels.

A cone-plate geometry measuring system (diameter: 20mm, angle: 1°) placed atop an integrated peltier system for temperature control was used with a solvent trap to keep the samples hydrated.

Experiments were performed at 25°C unless otherwise indicated. Samples were deposited under their gel form and allowed to equilibrate for 30 min prior to testing. An amplitude sweep test of strain from 0.01 to 100 % at a frequency of 1Hz was performed to define the linear viscoelastic region (LVR). Then, frequency sweep test from 0.1 Hz to 10 Hz within LVR was used to determine elastic modulus (G') and viscous modulus (G''). In order to evaluate the relative strength of the hydrogels, the viscoelastic moduli were compared at a frequency of 1Hz.

The sol-gel transition temperature was determined by heating the samples from 25 to 90°C at 1Hz frequency and 1Pa strain. The intersection

between viscoelastic moduli G' and G'' represented the sol-gel temperature.

Destructuration/restructuration experiments were performed to check the thixotropic property of hydrogels at fixed frequency (1Hz).

5 Samples were subjected to three strain values consecutively: a strain of 0.1% (within LVR) for 10 min for monitoring the initial state, 25% (above LVER) for 2 min for gel destructuration and the initial strain (0.1%) for restructuration of the gel.

10 In order to check the impact of cell inclusion on rheological properties of hydrogels, frequency sweep test from 0.1 Hz to 10 Hz within LVR was performed to determine elastic modulus (G') and viscous modulus (G'') of both, basic hydrogel and cell laden hydrogel, at physiological temperature (37°C).

15 Mechanical properties of the formed gels were investigated via frequency sweep test conducted within the linear viscoelastic region (LVR) by measuring the viscoelastic moduli. The elastic modulus also called storage modulus (G') represents the stiffness of a viscoelastic material. It describes the amount of energy stored and released at each oscillation; whereas the viscous modulus (G'') is related to the dissipation of energy.
20 For all hydrogels tested, the elastic modulus (G') exceeded the viscous modulus (G'') over the entire frequency range (0.1 Hz to 10 Hz), indicating that the hydrogels behave as solid-like (gel) material.

Moduli of diC16-3'-dC hydrogels are illustrated in Figure 8a.

25 The elastic modulus G' increased with concentration of NL, and was significantly higher ($p < 0.0001$) at a concentration of 3% compared to 1.5 % in both NaCl and DMEM (Figure 8a). When shifting from NaCl 154mM solution to DMEM/NaCl(aq) cell culture media, G' significantly decreased in all gelation media for diC16-3'-dC ($p < 0.0001$).

30 At a concentration of 3% diC16-3'-dC based hydrogel formulated into DMEM/NaCl(s) (123.5mM NaCl final concentration) showed a G' significantly lower (0.7kPa, $P < 0.0001$) compared to diC16-3'-dC based hydrogel formulated into DMEM/NaCl(aq) (123.5mM NaCl final

concentration) at the same concentration. These results suggest that the dilution of DMEM with aqueous NaCl improved the gel mechanical properties.

The diC16-3'-dC gives a thixotropic gel that rapidly (Figure 8b) recovered gel structure.

Hydrogel	% compound	G'(Pa)	G''(Pa)	Thixotropic gel (Y/N)
diC16-3'-dC NaCl (154mM)	1.5	893 ± 95	264 ± 27	Y
	3	2540 ± 255	747 ± 62	Y
diC16-3'-dC DMEM / NaCl(aq) (123.5mM)	1.5	491 ± 40	170 ± 12	Y
	3	1382 ± 137	435 ± 32	Y
diC16-3'-dC DMEM/NaCl(s) (123.5mM)	3	733 ± 44	234 ± 9	Y

Table 4: Shear moduli (G' and G'') of 1.5 or 3 %w/v diC16-3'-dC-based gels formed in 0.9% sodium chloride aqueous solution, DMEM / NaCl(aq) (NaCl final concentration = 123.5mM) or DMEM/NaCl(s) (NaCl final concentration = 123.5mM), at ambient temperature (25±0.01°C).

Hydrogel thermal reversibility was observed with a gel-sol transition temperature at 41°C determined by rheometer and confirmed with differential scanning calorimetry (DSC). Differential scanning calorimetry analyses were performed on a DSC 1 STAR System (METTLER TOLEDO, Singapore). Samples crimped in 100µL aluminium crucibles were heated from 25°C to 60°C at a rate of 2°C/minute then cooled to 25°C at the same rate. Three heating and cooling cycles of all hydrogels were performed. An empty aluminium crucible was used as a reference.

This result show that cells can be readily and homogenously incorporated into the gelling matrix at 40-41°C, which is still compatible with cell optimal viability, and that at physiological temperature 37°C the formulation is at gel state. Furthermore, the concentration of diC16-3'-dC does not affect the sol-gel temperature. Sol-gel transition temperatures for

different diC16-3'-dC concentrations in NaCl and DMEM are reported in Table 5:

	DSC		Rheometer
	Cooling Cycle (°C)	Heating Cycle (°C)	(°C)
diC16-3'-dC 1.5 % (w/v)- NaCl 154mM	41.9± 0.08	39.0± 0.08	41.2
diC16-3'-dC 1.5 % (w/v)- DMEM/NaCl(aq) 123.5mM	40.4± 0.05	37.9± 0.03	39.6
diC16-3'-dC 3% (w/v)- NaCl 154mM	41.4± 0.09	38.9± 0.05	40.4
diC16-3'-dC 3% (w/v)- DMEM/NaCl(aq) 123.5mM	40.9± 0.08	38.5± 0.08	40.3

Table 5: Sol-gel transition of diC16-3'-dC DMEM/NaCl (123.5mM) at 1.5 % and 3% (w/v) measured with DSC and rheometer.

5

Example 3:

Preliminary structural stability

A preliminary structural stability of diC16-3'-dT construction was carried out with a UFE400 heating oven (Memmert GMBH CO KG, Schwabach, Deutschland) for 21 days. Samples were checked up at Day D0, D1, D2, D3, D7, D14 and D21. Two series of the same batch were incubated in glass vials at 37°C to mimic cell culture conditions within 3 days. One series consisted in 6 samples of syringe pump extruded gel. 3 samples per series were put in a 2ml bath of DMEM while the other three were stored without bath.

A semi quantitative assessment was carried out to evaluate degradation and release of diC16-3'-dT into DMEM bath, 6µl of bulk solution were removed for each samples covered up by DMEM and analysed in triplicate with a micro-volumes spectrophotometer mySPEC (VWR International, Fontenay-sous-Bois, France).

Structural stability of hydrogel construct is required for cell culture as it was aimed to have 21 days stability of the scaffold with cell-laden hydrogel after bioprinting. Macroscopic stability was assessed by a syringe

pump extrusion-based construction of diC16-3'-dT-eDMEM hydrogel covered by DMEM.

After 21 days, visual observations showed dehydrated constructs for samples without a bath of DMEM. Gels placed in DMEM visually showed a harder structure after 21 days. These observations were confirmed by rheological experiments carried out at ambient and physiological temperatures. G' modulus increased drastically compared to day 0 (table 3). These moduli were almost height times higher for eDMEM solvent and 35 times higher for 0.9% sodium chloride aqueous solution. G' values were statistically different ($p < 0.0001$) for both conditions i.e. after extrusion and after stability study reflecting a concentration of the gel.

In vitro cell assays - Gingival fibroblasts culture

The human gingival fibroblast (FGh) was chosen as a stromal cell which viability could resist to the extrusion stress. Human gingival fibroblasts cultures were obtained from gingival explants of healthy non-smoker patients with no history of periodontitis. All patients gave their informed consent according to the Helsinki Declaration of 1975, and denied having recently taken drugs that could affect connective tissue metabolism. Briefly, gingival explants were collected from surgical wastes under local anaesthesia and conserved in DMEM completed with 20% foetal calf serum (FCS), 100 UI/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin B (all from Invitrogen).

Explants were put in culture flasks and the medium was changed every 2 days. After the first passage, FGhs were grown in DMEM completed with 10% FCS and used from passages 3 to 5.

In vitro cell viability

Cell viability was controlled at D1, D3, D7, D14 and D21 with a Live/Dead® test (Thermofisher Scientific, Waltham, MA, USA). In living cells, non-fluorescent substrate acetoxymethyl calcein (AMC) is cleaved into green fluorescent calcein by an intracellular esterase. Dead cells which

membranes are compromised allow intercalating agent ethidium homodimer-1 (EDT-1) to pass inside cell cores and so amplifying red fluorescence. The Live/Dead® solution was prepared as follow: an aliquot of AMC and an aliquot of EDT-1 were mixed with 2.5ml of DMEM and
5 heated in a boiling water bath for 15 minutes sheltered from light.

Two series of 5 samples of 100µL of diC16-3'-dT hydrogel in eDMEM were seeded with FGh (2.106 cells/ml) and deposited in a 12-well plate. At each time, sample was sacrificed and incubated (15 minutes, 37°C, 5% CO₂) then washed with DPBS 1X (Gibco®). A confocal
10 microscope (TCS SPE, CTR 6500, Leica, Wetzlar, Germany) was used for observations and wavelengths were fixed at 488 nm for living cells and 561 nm for dead cells.

In vitro cellular activity

15 An Alamar Blue® (ThermoFischer Scientific, Waltham, MA, USA) test was performed to assess cellular activity at D1, D3, D7, D14 and D21. Alamar Blue® reagent is a cellular health indicator using the reducing power of living cells to quantitatively measure the spread of multi lineage cells. Briefly, when cells are alive, they maintain a reduced environment
20 into cytosol. Resazurine, active ingredient of Alamar Blue® reagent, is a non-toxic non-fluorescent cell permeability blue dye. Inside cells, resazurine is reduced into resofurine which is a red fluorescent component. Living cells convert continuously resazurine into resofurine, enhancing the total fluorescence.

25 Two series of 3 samples of 100µl of diC16-3'-dT hydrogels in eDMEM seeded with FGh (2.106 cells/ml) were placed on a 6-well plate and reused for the different times. For each time, hydrogel matrices were washed two times in order to eliminate Alamar Blue® traces. Results were obtained at 590 nm by a spectrophotometer 2030 Multilabel Reader
30 VICTOR X3 (PerkinElmer, Waltham, MA, USA) linked to WorkOut® software.

diC16-3'-dT hydrogel formed in 0.9% sodium chloride aqueous solution already proved minimal inflammatory and fibrosis reactions (19). Cells choice was first headed toward FGh as they were resistant to multiple external aggressions, easy to handle and less expensive than HSCs.

5 Previously studies showed that FGh did not suffer from extrusion, making them a solid layout for preliminary cells assays before testing HSCs.

Confocal microscopy observations with Live/Dead stain showed morphological changes. At Day 1 cells were round, consequence of a possible stress but dead cells were practically non-existent. Some FGhs
10 began to show extents at Day 3 and regained their spindle-shape at Day 7 (fig. 5). Moreover, cell-cell interactions were visible at Day 7 at the matrix edge. Alamar Blue® assessment, triplicated for each time, showed a progressive increase of cellular activity (fig.6a). This activity improved at each time in comparison to Day 1 (Day 3: 120% of Day 1 activity and Day
15 7: 160%) (fig.6b).

Those results proved cell culture compatibility of diC16-3'-dT-eDMEM hydrogel and assumed sufficient porosity of the gel for oxygen and nutrients to reach cells.

Printing of diC16-3'-dT-eDMEM hydrogel was successful and FGh
20 showed spreading and interactions inside the gel.

Statistical analysis

To handle reproducibility issues, experiments were performed in triplicate or more. All data were expressed as mean $\pm$ standard deviation of
25 the mean (SD). XLstat® (Addinsoft, Paris, France) software was used for all statistical analyses. Distribution normality was assessed with a Shapiro-Wilk test. Student's t-test (2-tailed) with a Fisher variance testing was performed to determine differences between groups. Significance was set at $p < 0.05$ or less.

30

Example 4: 3D-extrusion-based bioprinting

Printer compatibility testing

Bioink was prior tested on bench using a simple extrusion test with
5 syringe and small gauge needles tip (18 and 22 gauges). Briefly, bioinks
were prepared as described above but were loaded in syringe under their
liquid states before cooling down or sonication steps. After complete
gelation, they were prepared for a syringe pump (Razel Scientific
Instrument, Fairfax, USA) extrusion at a flow rate near to the one of
10 bioprinter (14.5 ml/h). Next, bioinks prepared using the same protocol were
directly tested on bioprinter.

Extrusion-based bioprinter (EBB)

Multiprint bioprinter was an extrusion-based 3D printer designed by
15 the Fablab Coh@bit of Technological University Institute of Bordeaux
University (IUT). The system was mechanical as material was pushed out
the syringe using a piston handled by an endless screw adapted for glass-
metal syringes (glass body; metal piston) of three different volumes: 1, 3,
5ml. Straight and non-bevelled luer-lock needles of 18 and 22 gauges
20 diameters were used. For computer-aided design (CAD), multiprint was
linked to BioPrinter® software and printing templates were generated by
SketchUp software® (Trimble Inc., Sunnyvale, CA, USA). Two CAD
designs were used: cube and grid with 2x2 square millimeters pores. Each
layer of the construct was composed of approximately 100µL of hydrogel
25 for both patterns.

3D extrusion-based bioprinting

Bioink characteristics should meet mechanicals requirements for
the EBB bioprinting. Before using the Multiprint 3D bioprinter, hydrogels
30 printability was evaluated by manual syringe pump extrusion.

diC16-3'-dT based bioinks were tested in ether Milli-Q® water, 0.9% sodium chloride aqueous solution and DMEM/eDMEM. diC16-3'-dT regained a gel solid-like shape after extrusion.

Interestingly, after extrusion at room temperature with a syringe pump at a controlled rate, diC16-3'-dT/eDMEM hydrogel (3% w/v) at ambient temperature showed an increase of its G' modulus up to ten times higher. Comparable behaviour was found at physiological temperature before and after extrusion with a syringe pump ($p=0.003$) (table 2). Extruded hydrogel at physiological temperature showed a comparable G' to perivascular environment in bone marrow niche (about 3 kPa) allowing spreading of HSCs.

diC16-3'-dT-based hydrogels were then printed using the Multiprint printer into a CAD-grid matrix (fig.3). A pause of 2 minutes was respected between two layers in order to have enough time for the hydrogel reconstruction and prevent grid collapse.

In order to counterbalance the higher viscosity of diC16-3'-dT formed in eDMEM compared to the 0.9% sodium chloride aqueous solution, bioprinting parameters were changed to an increased extrusion coefficient.

Grid is a commonly used matrix in EBB as it showed a perfect compatibility with cells proliferation [3,21]. Cells growth was enhanced by pores created into the structure allowing a greater exchange surface between cells and culture medium. Hydrogels constructs showed pores of 1 mm square in 0.9% sodium chloride aqueous solution and smaller ones (500 μm) in eDMEM (fig.4).

Printing of diC16-3'-dT-eDMEM hydrogel was successful and FGh showed spreading and interactions inside the gel.

Applicants successfully bioprinted a bioinspired supramolecular nucleolipid-based hydrogel in culture medium for the first time. Compounds of the invention are suitable molecules for use in supramolecular hydrogel bioink formulations.

The physico-chemical formulations properties allowed forming a stable and cell compatible gel in a culture medium. Rheological properties highlighted high valuable capacities for EBB allowing cells culture and thixotropy providing a biomaterial free of external crosslinkers.

- 5 These excellent results combined with in vitro compatibility and biodegradability show that use of nucleolipid based bioink according to the invention is a real improvement in the field of bioinks and bioprinting.

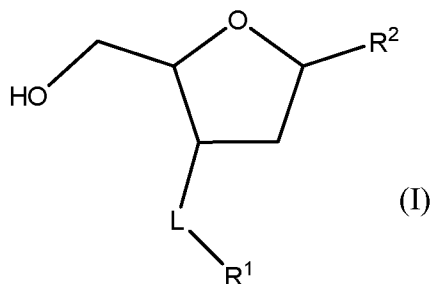
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CLAIMS

1. Use of a nucleolipid-based compound of formula I:



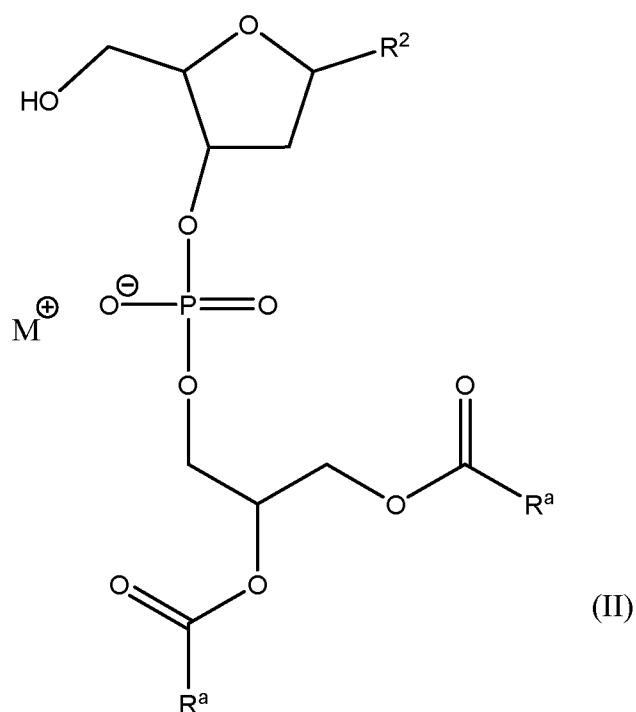
5 wherein,

- L represents an oxycarbonyl —O—C(O)— group, a thiocarbamate —O—C(S)—NH— group, a carbonate —O—C(O)—O— group, a carbamate —O—C(O)—NH— group, an oxygen atom, a phosphate group, a phosphonate group or a heteroaryl group comprising 1 to 4
- 10 nitrogen atoms, unsubstituted or substituted by a linear or branched, saturated or unsaturated $\text{C}_2\text{—C}_{30}$ hydrocarbon chain,
- R^1 represents
 - o a linear or branched C_2 to C_{30} hydrocarbon chain, preferably C_6 to C_{25} and more preferably C_8 to C_{25} , saturated or
 - 15 unsaturated, optionally completely or partially fluorinated, unsubstituted or substituted on the end of the chain by a fluorine atom or by a benzyl or naphtyl ester or ether, or
 - o a diacylglycerol (or diglyceride) in which each acyl chain is a linear or branched C_2 to C_{30} hydrocarbon chain, preferably C_6
 - 20 to C_{25} and more preferably C_8 to C_{25} , saturated or unsaturated, optionally completely or partially fluorinated, unsubstituted or substituted on the end of the chain by a fluorine atom or by a benzyl or naphtyl ester or ether,
- R^2 represents a purine or pyrimidine base selected from uracil,
- 25 adenine, guanine, cytosine, thymine, hypoxanthine, or their derivatives, optionally substituted, or a non-natural mono- or bicyclic heterocyclic base each ring of which comprising from 4 to 7

members, preferably guanine, cytosine or thymine and more preferably thymine, and

- the compound is optionally in the form of a salt, preferably a metal cation salt and more preferably the metal cation may be selected from Li^+ , Na^+ , K^+ , Rb^+ , Cs^+ ,
in a bioink formulation.

2. Use according to claim 1, wherein the nucleolipid-based compound is of formula II:



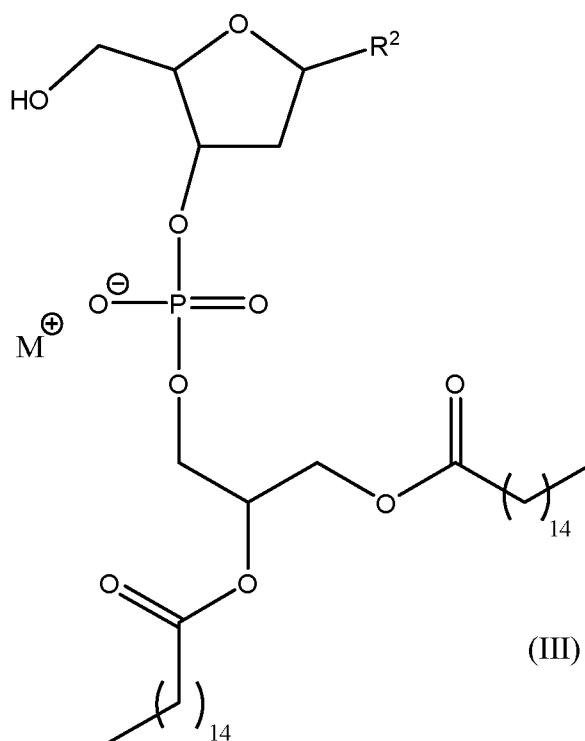
wherein,

- each R^a , identical or different, is a linear or branched C_1 to C_{29} hydrocarbon chain, preferably C_5 to C_{24} and more preferably C_7 to C_{24} , saturated or unsaturated,
- R^2 represents a purine or pyrimidine base selected from uracil, adenine, guanine, cytosine, thymine, hypoxanthine, or their derivatives, optionally substituted, preferably guanine, cytosine or thymine and more preferably thymine, and
- M^+ represents a cation, preferably a metal cation, and more preferably selected from Li^+ , Na^+ , K^+ , Rb^+ , Cs^+ .

3. Use according to claim 2, wherein each R^a , identical or different, is a C_{13} to C_{19} linear hydrocarbon chain, preferably a C_{15} linear hydrocarbon chain.

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4. Use according to any of the preceding claims, wherein the nucleolipid-based compound is of formula III:

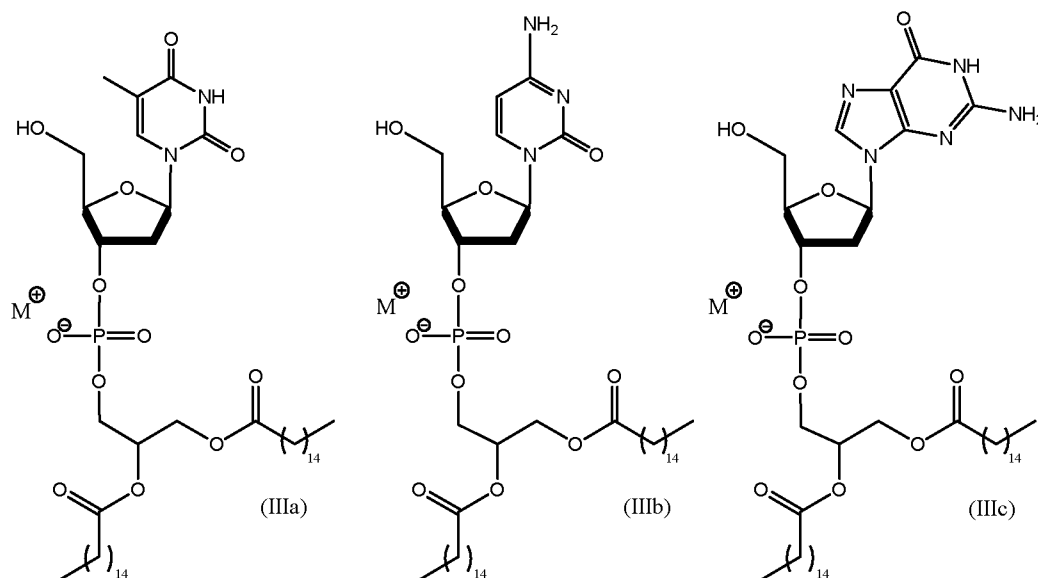


wherein R^2 represents a purine or pyrimidine base selected from guanine, cytosine and thymine, preferably R^2 represents a pyrimidine base and more preferably R^2 represents thymine and M^+ is as defined in claim 2.

10

5. Use according to any of the preceding claims, wherein nucleolipid-based compound is selected from compounds of formula IIIa, IIIb and IIIc, preferably of formula IIIa:

15



M^+ being as defined in claim 2.

6. Use according to any of the preceding claims, wherein the bioink is a gel, preferably selected from hydrogels and supramolecular gels.

7. Use according to the preceding claim, wherein the gel is a hydrogel and further comprises a 0.5 to 1.5 wt. % aqueous lithium, sodium, potassium, rubidium or cesium chloride solution, preferably a 0.9% aqueous sodium chloride solution.

8. Use according to claim 6 or 7, wherein the concentration of compound of formula I, II, III, IIIa, IIIb or IIIc in the gel is comprised between 0.1 and 10 % w/v, preferably 3 % w/v.

9. Use according to any of claims 6 to 8, wherein the gel further comprises a cell culture medium, preferably selected from the Dulbecco's Modified Eagle Medium (DMEM) or the enriched Dulbecco's Modified Eagle Medium (eDMEM), RPMI, MEM, IMDM, opti-MEM medium and DMEM/F-12.

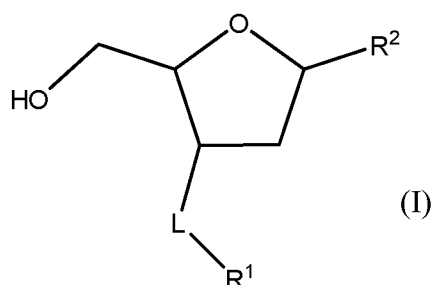
10. Use according any of claims 6 to 9, wherein the mass ratio aqueous solution / cell culture medium is comprised between 50:50 and 10:90, preferably between 40:60 and 20:80, more preferably the ratio is 30:70.

5

11. Use according to any of claims 6 to 10, wherein the gel further comprises cells, preferably the cells are selected from apical papilla stem cells, human bone marrow cells, human skin fibroblast, human gingival fibroblast, human umbilical vein cells, endothelial cells, hematopoietic stem cells (HSC), human induced pluripotent stem cells (HiPSC) and mesenchymal stem cells (MHSC).

12. Use according to any of the preceding claims, wherein the bioink formulation is suitable for bioprinting, preferably bioprinting is selected from comprising inkjet bioprinting, laser bioprinting and extrusion based bioprinting.

13. Bioprinting method comprising the use of a bioink formulation comprising a nucleolipid-based compound of formula I:



20

wherein,

- L represents an oxycarbonyl —O—C(O)— group, a thiocarbamate —O—C(S)—NH— group, a carbonate —O—C(O)—O— group, a carbamate —O—C(O)—NH— group, an oxygen atom, a phosphate group, a phosphonate group or a heteroaryl group comprising 1 to 4 nitrogen atoms, unsubstituted or substituted by a linear or branched, saturated or unsaturated $\text{C}_2\text{—C}_{30}$ hydrocarbon chain,
- R^1 represents

25

- a linear or branched C₂ to C₃₀ hydrocarbon chain, preferably C₆ to C₂₅ and more preferably C₈ to C₂₅, saturated or unsaturated, optionally completely or partially fluorinated, unsubstituted or substituted on the end of the chain by a fluorine atom or by a benzyl or naphthyl ester or ether, or
- a diacylglycerol (or diglyceride) in which each acyl chain is a linear or branched C₂ to C₃₀ hydrocarbon chain, preferably C₆ to C₂₅ and more preferably C₈ to C₂₅, saturated or unsaturated, optionally completely or partially fluorinated, unsubstituted or substituted on the end of the chain by a fluorine atom or by a benzyl or naphthyl ester or ether,
- R² represents a purine or pyrimidine base selected from uracil, adenine, guanine, cytosine, thymine, hypoxanthine, or their derivatives, optionally substituted, or a non-natural mono- or bicyclic heterocyclic base each ring of which comprising from 4 to 7 members, preferably guanine, cytosine or thymine and more preferably thymine, and
- the compound is optionally in the form of a salt, preferably a metal cation salt and more preferably the metal cation may be selected from Li⁺, Na⁺, K⁺, Rb⁺, Cs⁺.

14. Nucleolipid-based compounds of formula I, II, III, IIIa, IIIb or IIIc for use in regenerative medicine, preferably for use in the bioprinting of vascular niches for cells, wherein the bioprinting is chosen in the group comprising inkjet bioprinting, laser bioprinting and extrusion based bioprinting.

15. Bioprinted grid or prototype construct comprising nucleolipid-based compounds of formula I, II, III, IIIa, IIIb or IIIc.

16. Bioink gel formulation, preferably a hydrogel or a supramolecular gel, comprising:

- between 0.1 and 10 % w/w of a compound of formula I, II, III, IIIa, IIIb or IIIc,
- a 0.5 to 1.5 wt. % w/w aqueous lithium, sodium, potassium, rubidium or cesium solution,
- 5 - a cell culture medium, and
- optionally cells,

wherein the mass ratio aqueous solution / cell culture medium is comprised between 10:90 and 50:50.

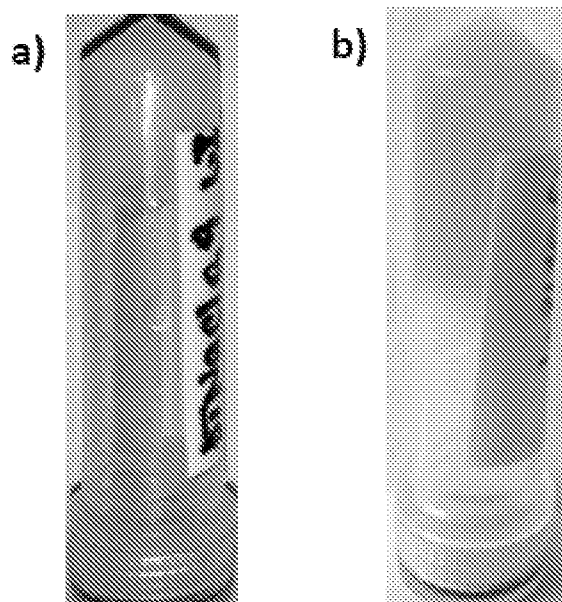


Figure 1

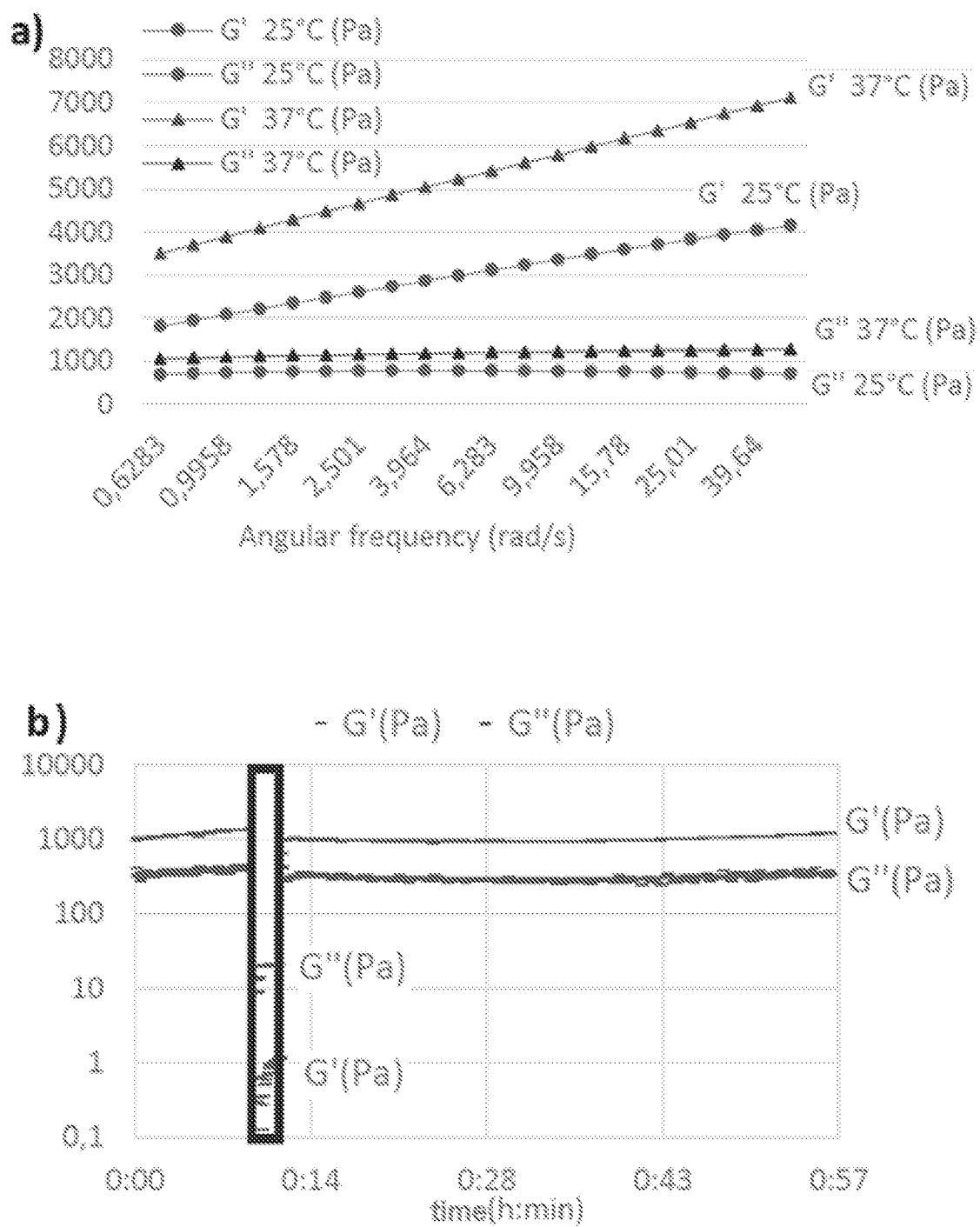


Figure 2

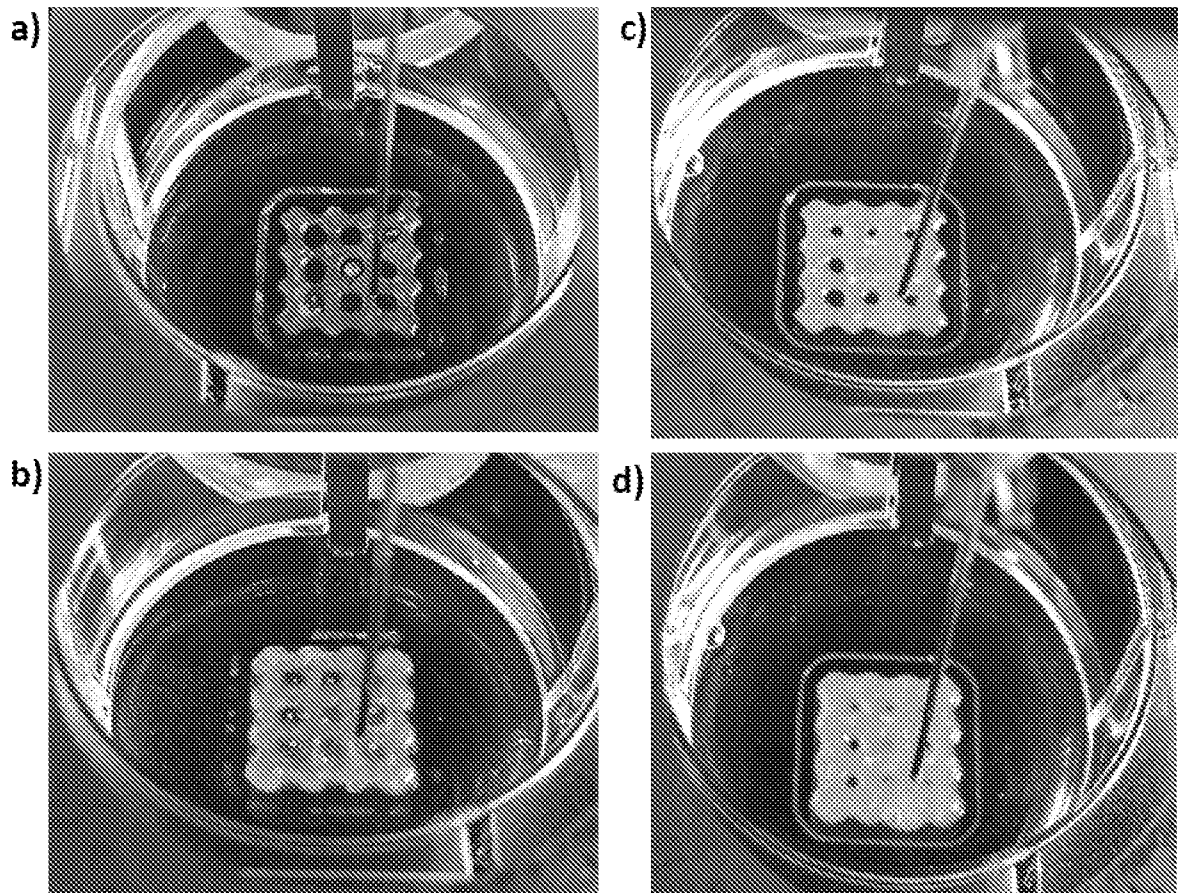


Figure 3

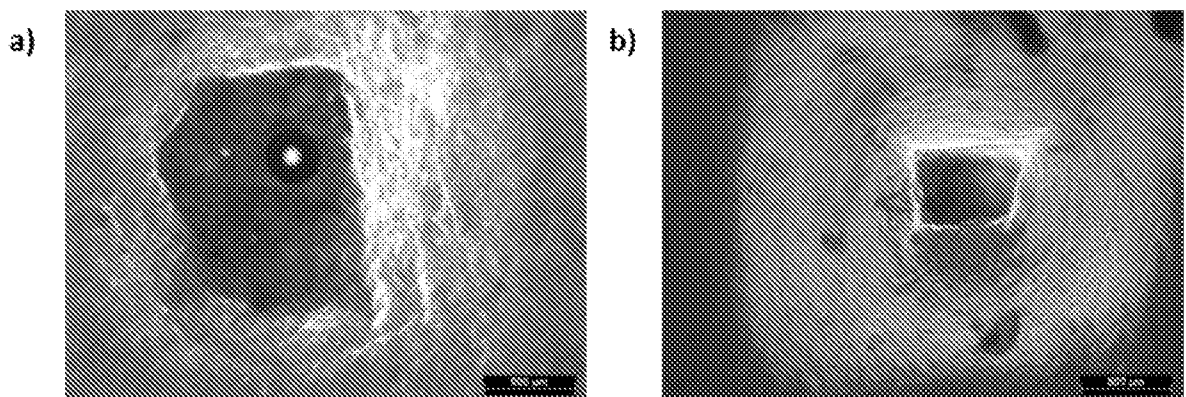


Figure 4

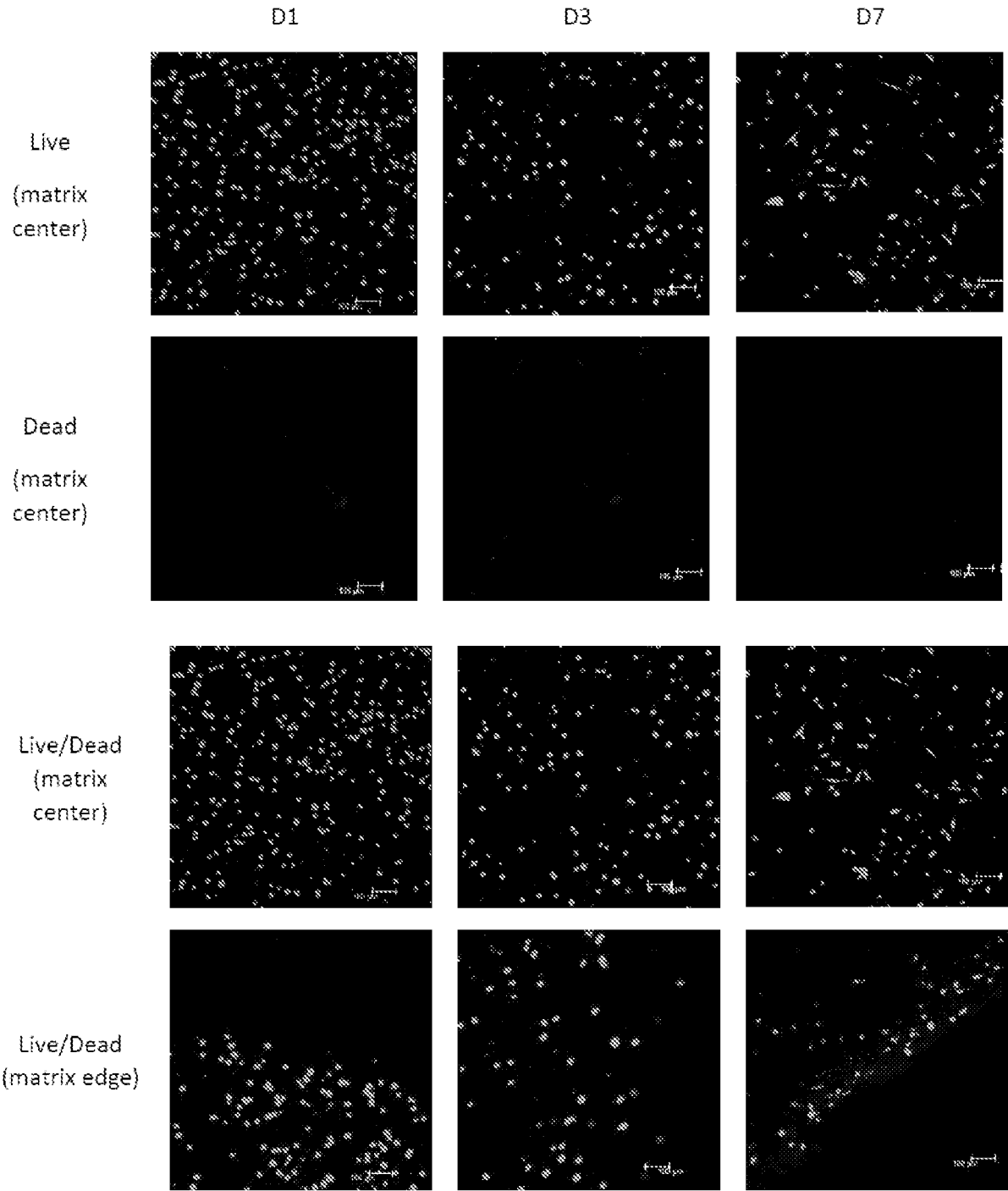
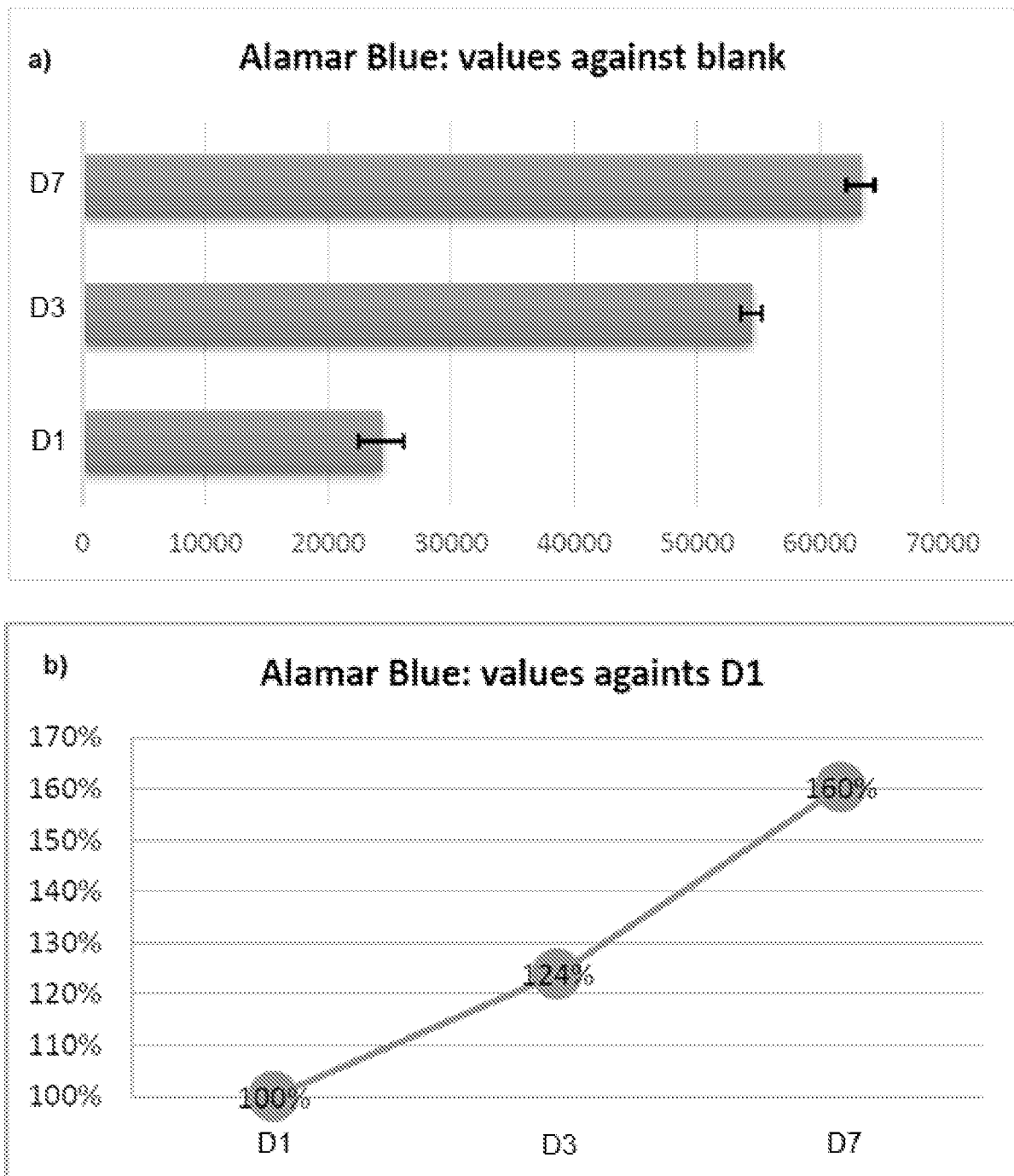


Figure 5

**Figure 6**

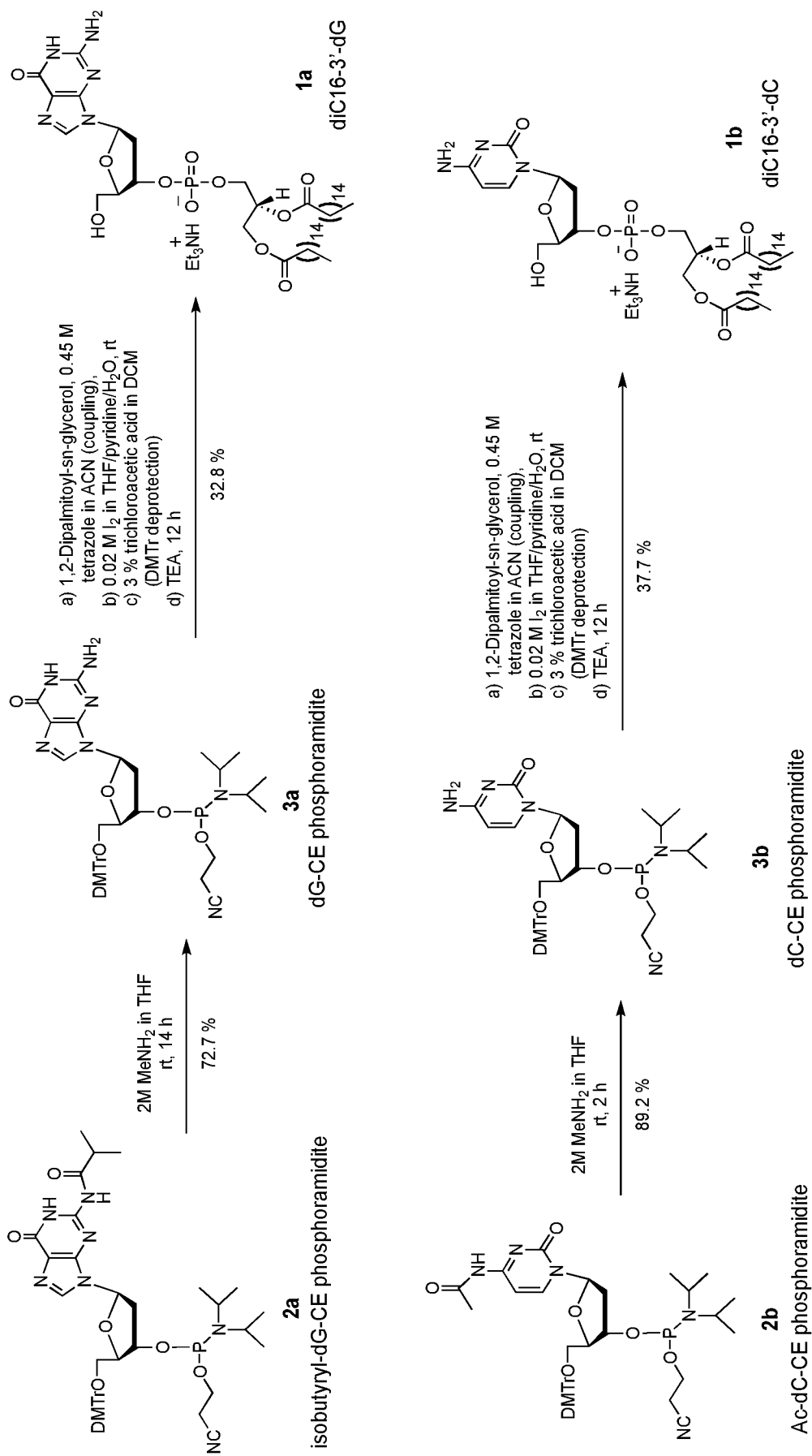


Figure 7

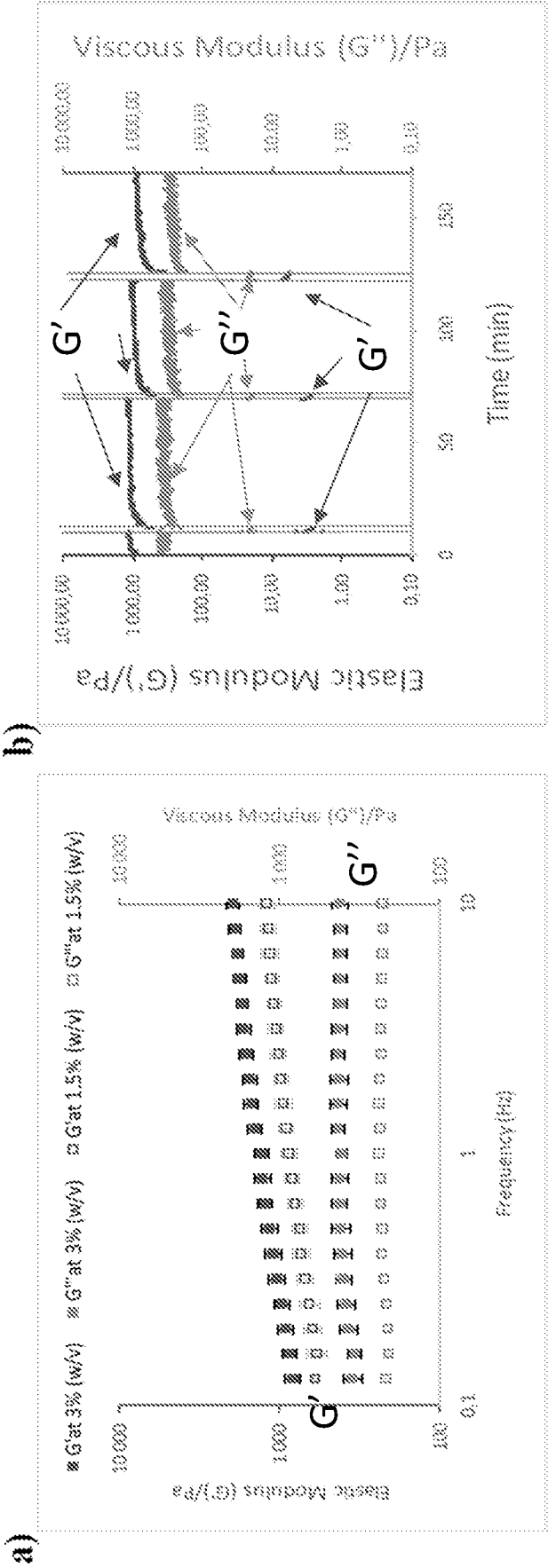


Figure 8

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2018/060651

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K38/17 A61L27/52 A61K31/395 A61K31/712 C07H19/04
C07H19/20 C12N5/071 B33Y80/00 A61K47/54 C08G83/00
C08J3/075

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)

A61K A61L C07H C12N B33Y C08G C08J

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, COMPENDEX

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MICHAEL A. RAMIN ET AL: "Cation Tuning of Supramolecular Gel Properties: A New Paradigm for Sustained Drug Delivery", ADVANCED MATERIALS, vol. 29, no. 13, 2 February 2017 (2017-02-02), page 1605227, XP055484388, DE	1-12, 14-16
A	ISSN: 0935-9648, DOI: 10.1002/adma.201605227 the whole document ----- -/--	13



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

8 March 2019

Date of mailing of the international search report

22/03/2019

Name and mailing address of the ISA/

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Authorized officer

Costantini, Nicola

INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2018/060651

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MICHAEL A. RAMIN ET AL: "Cation Tuning of Supramolecular Gel Properties: A New Paradigm for Sustained Drug Delivery", ADVANCED MATERIALS, vol. 29, no. 13, 1 April 2017 (2017-04-01), page 1605227, XP055499050, DE ISSN: 0935-9648, DOI: 10.1002/adma.201605227	1-12, 14-16
A	the whole document	13
A	KARIN S STALEY ET AL: "Biomaterial design strategies for the treatment of spinal cord injuries", JOURNAL OF NEUROTRAUMA, M.A. LIEBERT, NEW YORK, NY, US , vol. 27, no. 1 26 January 2010 (2010-01-26), pages 1-19, XP002695805, ISSN: 0897-7151, DOI: 10.1089/NEU.2009.0948 Retrieved from the Internet: URL:http://online.liebertpub.com/doi/pdf/10.1089/neu.2009.0948 [retrieved on 2009-08-21] page 7, left-hand column, paragraph 4 - page 13, right-hand column, paragraph 1	14,15
A	CHUANG LI ET AL: "Rapid Formation of a Supramolecular Polypeptide-DNA Hydrogel for In?Situ Three-Dimensional Multilayer Bioprinting", ANGEWANDTE CHEMIE INTERNATIONAL EDITION, vol. 54, no. 13, 5 February 2015 (2015-02-05), pages 3957-3961, XP055489253, ISSN: 1433-7851, DOI: 10.1002/anie.201411383 page 3957, right-hand column, paragraph 2 - page 3958, left-hand column, paragraph 1	1-16
A	WO 2016/064648 A1 (UNIV WAKE FOREST HEALTH SCI) 28 April 2016 (2016-04-28) claim 1 page 13, line 35 - page 15, line 6 figures 2A, 2B, 3A, 3B, 3C, 4A and 4B	1-16
A	US 2015/084232 A1 (RUTZ ALEXANDRA L [US] ET AL) 26 March 2015 (2015-03-26) claims 1,10	1-16

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/IB2018/060651

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2016064648 A1	28-04-2016	AU 2015336283 A1	11-05-2017
		CA 2964926 A1	28-04-2016
		EP 3209718 A1	30-08-2017
		JP 2017537996 A	21-12-2017
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		US 2017307598 A1	26-10-2017
		WO 2016064648 A1	28-04-2016

US 2015084232 A1	26-03-2015	US 2015084232 A1	26-03-2015
		WO 2015048355 A1	02-04-2015
