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Tailor-made functionalized self-assembled peptide (nano)fibers and hydrogels, and methods, uses and kits related thereto

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(54) Title: TAILOR-MADE FUNCTIONALIZED SELF-ASSEMBLED PEPTIDE (NANO)FIBERS AND HYDROGELS, AND METHODS, USES AND KITS RELATED THERETO

(57) Abstract: The invention relates to self-assembling peptide (nano)fibers, hydrogels, and methods, uses and intermediate products and kits relating thereto. Provided is a method for providing peptide-based functionally modified (nano)fibers, comprising (i) providing a fiber forming solution comprising pseudopeptide building blocks of the formula A-Peptide-B, wherein: Peptide is a moiety of 1 to 8 amino acid residues having a sequence that is predisposed to form a one-dimensional array, such as β-sheet fibrils; A is an aromatic moiety carrying two reactive thiol groups; and B is a reactive α-nucleophile; (ii) exposing the fiber forming solution to oxidizing conditions to induce supramolecular self-assembly of the pseudopeptide building blocks into peptide-based (nano)fibers; and (iii) contacting said (nano)fibers with at least one biologically relevant functional group of interest comprising reactivity C forming a reactive pair with B to obtain covalently functionally modified nanofibers.

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Title: Tailor-made functionalized self-assembled peptide (nano)fibers and hydrogels, and methods, uses and kits related thereto.

The invention relates to the fields of material science and tissue engineering. More specifically, it relates to functionalized self-assembling peptide fibrillar hydrogels, and methods, uses, intermediate products and kits relating thereto.

Currently, the field of material science relies on a strategy that involves understanding and mimicking of structures and behaviors found in nature, to provide solutions in areas ranging from medicine and energy to environmental sustainability. In particular, synthetic biomaterials are being explored at a rapid pace, as they may have suitable properties to be used as extracellular matrices for tissue engineering.[1] Functional supramolecular polymers have been viewed as a powerful platform for such application, since they can be constructed from short peptides[2] or peptide-amphiphiles,[3] containing binding sites for biologically functional ligands.[4] Cell adhesion motifs,[5] peptide sequences that can direct cell differentiation[6] and susceptibility to degradation[7] are important ingredients for successful 3D cell growth within synthetic materials.[8] Synthetic biomaterials are advantageous regarding batch-to-batch consistency, enabling the translation of basic research into clinical applications.[9,10]

At the same time, Dynamic covalent chemistry (DCC) has been recognized as a tool to study fundamental questions of living systems,[11] but also to access emergent behaviors of functional materials. For example, self-healing,[12] shape memory, and stimuli-induced stiffness changes are properties that can be achieved by the implementation of reversible bonds into dynamic hydrogels.[13] However, the combination of multiple dynamic orthogonal chemistries has made its way into functional materials only recently, and exciting examples have started to emerge.[12,14]

However, current synthetic hydrogels and their functionalization face a number of practical limitations. For example, a highly specific ligand-functionalized material is typically required in order to promote proliferation of a particular type of cells or differentiation of stem cells. This means that, for each specific outcome, a specific material and corresponding 'building blocks' must be developed. Previously reported approaches do not offer a single and simple methodology for a high diverse set of application.

For example, the hydrogel material developed by the group of Stupp (*Science* 303, 1352 (2004); US2005/0272662) involves the synthesis of a large set of self-assembling charged peptide-amphiphiles comprising three segments: an alkyl tail, a structural peptide and a functional peptide. To obtain a functionalized gel capable of generating a specific type of cell response or behaviour, each peptide-amphiphile building block must be provided with a specific functional peptide, like a biologically relevant signal portion. This approach is cumbersome and time-consuming, and limits a widespread practical applications in the field of tissue engineering.

Therefore, the present inventors set out to provide a novel type of hydrogel that can undergo a fast triggered gelation and is cytocompatible. Ideally, they aimed at an approach that allows for "on-demand" chemical customization of fibrillar scaffold, thereby offering the potential for the application as bioactive platform that can dictate and/or stimulate cell fate processes.

These goals were met by the development of a novel system that uses two dynamic covalent bonds in tandem (e.g. disulfides and hydrazones), for the formation of hydrogels containing biologically relevant ligands. The system is based on the DCC mediated self-assembly of pseudo-peptide based building blocks into a one-dimensional array to yield supramolecular fibers, followed by the post-assembly surface decoration of the fibers with one or more (biologically active) compound(s) of interest. The subsequent addition

of a cross-linker can trigger the formation of a hydrogel, which is not only biocompatible but also supports cell morphology.

Thus, the present invention encompasses the discovery that it is possible to modify self-assembled (nano)fibers by incorporating one or more biological signal(s) that do not participate in gel formation, while still permitting hydrogelation by covalent cross-linking of the modified fibers.

For example, in case of the first DCC reaction involving disulfides and hydrazones, the reversibility of the disulfide bonds allows for fiber formation upon oxidation of a dithiol peptide-based building block, while a second reaction, which is in essence also DCC reaction, between the hydrazide-functionalized peptide and an aldehyde cross-linker results in a gel. The same acyl-hydrazone bond-forming reaction is advantageously exploited for the "decoration" of supramolecular nanofiber assemblies, e.g. by cell-adhesion promoting sequences (RGD and LDV) or peptides promoting cell proliferation and/or cell differentiation (e.g. IPP), which functionalized nanofibers are subsequently able to undergo hydrogelation by cross-linking. This modular approach allows the molecular engineering of tailor-made material on demand. Moreover, it is suitably used to incorporate different ligands at the same time, for example using a first molecule that promotes cell adhesion and a second molecule that promotes cell differentiation.

Accordingly, the invention provides a method for providing peptide-based functionally modified fibers, comprising the steps of :

(i)   providing a fiber forming solution comprising pseudopeptide building blocks of the formula A-Peptide-B, wherein:

       Peptide is a moiety of 1 to 8, preferably 3 to 5, amino acid residue(s) and having a sequence that is predisposed to form a one-dimensional array;

       A is an aromatic moiety carrying two reactive thiol groups;

       B is a reactive α-nucleophile;

(ii) exposing the fiber forming solution containing the pseudopeptide building blocks to oxidizing conditions to induce supramolecular self-assembly of the pseudopeptide building blocks into peptide-based fibers; and

(iii) contacting said nanofibers with at least one functional group of interest comprising reactivity C capable of reacting (i.e. forming reactive pair) with B to obtain covalently functionally modified fibers.

The concept underlying the present invention is not known or suggested in the art. US2005/181973 discloses that self-assembling peptides can be modified by incorporating an additional domain that does not self-assemble, while still permitting assembly of the self-assembling portion. Thus, unlike the present invention, the β-sheet forming domains are modified/functionalized with an additional domain prior to self-assembly. The additional domain is for instance a biologically active peptide motif or a target site for an interaction with a biomolecule. Exemplary self-assembling β-sheet forming domains comprise 2 to 4 copies of the tetrapeptide unit RADA.

Thiol-functionalized peptide building blocks and their self-assembly into fibers have been previously disclosed by the Otto group. For example, Malakoutikhah *et al.* (J. Am. Chem. Soc. 2013, 135, 18406-18417) describes the oxidation of peptide-based dithiol building blocks into macrocyclic structures of different ring sizes, and their assembly into long fibrils held together by β-sheet formation between the peptide chains. Exemplified pentapeptide chains contain the sequence Gly-Leu-Lys-Xaa-Lys, with Xaa having a varying degree of hydrophobicity. See also Sadownik *et al.* (Nature Chemistry volume 8, 264–269 (2016)) and the PhD Thesis by Altay, Y. (Novel peptide replicators from dynamic combinatorial libraries. (2019) University of Groningen).

Van Duc Nguyen *et al.* (Soft Matter, 2016, 12, 432) relates to cross-linking self-assembled fibers based on the pentapeptide Gly-Leu-Lys-Phe-Lys with a

focus on the effects of filament length and crosslink density on the mechanical properties of filamentous networks. The crosslinker used is the triblock copolymer $PSPMA_{25}$-PEO_{230}-$PSPMA_{25}$, which links the fibers together due to ionic (i.e. non-covalent) interaction with the positively charged fibers

However, the art is silent about covalent fiber functionalization, let alone that it suggests the covalent decoration of assembled fibers with one or more biologically active (peptide) compounds prior to hydrogel formation.

Prestwich *et al.* (J. Contr. Release 53 (1998) 93-103) discloses the formation of hyaluronic acid (HA)-hydrogel and post-modification of the hydrogel with a drug comprising a functional group that reacts with the hydrazide group carried by the hydrogel. This system differs from that of the present invention not only in the non-peptidic nature of the gel, but also in the fact that in Prestwich the hydrogels are modified after they have been prepared. In contrast, according to the present invention self-assembled peptide-based nanofibers are modified prior to the formation of a hydrogel.

The present invention is centered around a pseudopeptide building block represented by an amino-acid based moiety herein referred to as "Peptide", which consists of a few (1- 8) amino acids, Peptide being functionalized, preferably at its N-terminus, with reactivity A, i.e. an aromatic moiety carrying two reactive thiol (-SH) groups. Group A is capable of undergoing reversible thiol-disulfide exchange allowing for the formation of macrocyclic structures via dynamic covalent bond formation between thiol groups of A moieties of other A-Peptide-B (or A-Peptide; see below) building blocks. The thiol-groups can be attached directly to a carbon atom of the aromatic moiety, or they can be attached via an aliphatic chain, for example a C_1-C_4 linear alkyl. Preferably, the thiol-groups are attached directly to a carbon atom of the aromatic moiety.

The aromatic moiety is preferably a dithiol-substituted 5- or 6-membered aromatic moiety. The relative position of the thiol-substituents should allow for the formation of macrocyclic structures of A-moieties of a plurality of pseudo-peptide building blocks. In one aspect, it is a 1,3- dithiol-benzene ring, preferably with the peptide moiety attached at the 5-position.

In addition to the two thiol groups, the aromatic ring may contain one or more further substituents that do not interfere with macrocyclic ring formation. Suitable substituents include C_1-C_3 alkyls, such as -CH_3, and halogens, such as -Cl or -F. The further substituent(s) is for example positioned at the 2- and/or 4- position of a 1,3- dithiol-benzene ring. In a specific aspect, A is 1,3-dithiol-2-methyl-benzene, 1,3-dithiol-2-ethyl-benzene, 1,3-dithiol-2-chloro-benzene, 1,3-dithiol-2-fluoro-benzene, 1,3-dithiol-2,4-dimethyl-benzene, 1,3-dithiol-2,4-diethyl-benzene, 1,3-dithiol-2,4-dichloro-benzene, or 1,3-dithiol-2, 4-difluoro-benzene. In another aspect, the aromatic moiety is only substituted with two thiol groups.

Pseudopeptide building block A-Peptide-B is furthermore characterized in the Peptide moiety being functionalized with reactivity or group B, which B reactivity is compatible with, and can form a covalent bond, with both reactivity C (present on the compound of interest) and reactivities D (present on the cross-linking agent; see further herein below). Herewith, the fibrils resulting from pseudo-peptide self-assembly can not only be covalently functionalized with one or more compounds of interest, but they can also be triggered to undergo hydrogelation by covalent cross-linking. The B reactivity can be present at the C-terminus of Peptide, or it can be attached to one or more amino acid side chains. Good results are obtained when the B reactivity is present at the C-terminus of Peptide, preferably in combination with group A being attached at the N-terminus.

According to the invention, B is a reactive α-nucleophile, also known in the art as "α-effect nucleophile". The alpha effect refers to the increased nucleophilicity of an atom due to the presence of an adjacent (alpha) atom

with lone pair electrons. This first atom does not necessarily exhibit increased basicity compared with a similar atom without an adjacent electron donating atom.

5   It will be appreciated by a person skilled in the art that this approach requires a careful selection of the suitable B-C and B-D chemistries. The reaction between the B-C reactive pair and the B-D reactive pair should be compatible with disulfide chemistry. This means that they are orthogonal, i.e. that they do not interfere with each other and they are performed

10 independently. Also, each of the B, C and D reactivities must biocompatible. The B functionality facilitates hydrogelation and must allow for bio-orthogonal bond formation between the fiber-like supramolecular structure and the cross-linker reversible covalent chemistries.

15 For example, a method of the invention relies on the disulfide/hydrazone combination or the disulfide/oxime combination. The reactions that form oxime and hydrazone bonds are among the oldest, most intensively studied bioconjugation reactions. Bioconjugation reactions must be compatible with the biomolecules upon which they act. Hydrazone and oxime bond-forming

20 reactions are biocompatible. They are also versatile, leading to the formation of widely diverse products, with usually rapid reaction rates.
    In oxime and hydrazone bond-forming reactions, an aldehyde or ketone reacts readily with a nucleophilic alkoxyamine (also known as aminooxy or oxyamine) or hydrazine, to yield an oxime or hydrazone, respectively. These

25 reactions are chemoselective. Water forms as a byproduct of the reaction. In general, aldehydes react more rapidly than ketones.

In one embodiment, a method of the invention relies on the disulfide/hydrazone combination. Herein, the B reactivity is or comprises a

30 hydrazine ($-NH-NH_2$) group and the C and D reactivities contain a carbonyl. The resultant reaction leading to a hydrazine bond involves $R-NH-NH_2 + R'(CO)R'' -> R-NH-N=CR'R''$. In a preferred embodiment, B is or comprises a

hydrazine ($-NH-NH_2$) group and reactivity C is an aldehyde or glyoxylyl group and/or reactivity D is an aldehyde group.

In another embodiment, a method of the invention relies on the disulfide/oxime combination. Herein, the B reactivity is or comprises a alkoxy-amine ($-O-NH_2$) group and the C and D reactivities contain a carbonyl. The resultant reaction involves $R-O-NH_2 + R'(CO)R'' \rightarrow R-O-N=CR'R''$. For example, B is or comprises a $-O-NH_2$ group and reactivity C is an aldehyde or glyoxylyl group and/or reactivity D is an aldehyde group. The $-NH-NH_2$ or $-O-NH_2$ group is readily attached to the Peptide, either at the C-terminus or an amino acid side chain. For example, hydrazides of aspartic and glutamic acid can be prepared from corresponding Fmoc-protected amino acid anhydrides (Barnes et al. Chem. Commun., 2021,57, 1006-1009). As another example, non-natural amino acids that are synthetically accessible can be used (see WO2007/079130). In one embodiment, 2-amino-3-(4-(hydrazineylmethyl)phenyl)propanoic acid, 2-amino-3-(4-(hydrazineylmethoxy)phenyl)propanoic acid, or 2-amino-3-(4-(aminoglycyl)phenyl)propanoic acid is used.

In a preferred embodiment, $-NH-NH_2$ or $-O-NH_2$ group is attached to the Peptide's C-terminus. For example, the pseudo-peptide building block is of the formula I, II, III or IV herein below.
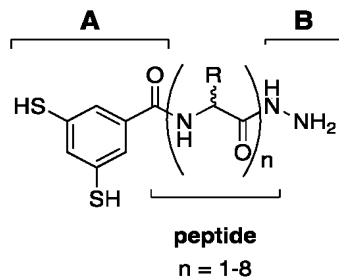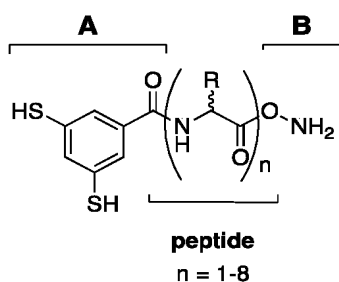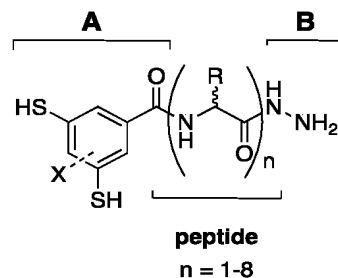
Formula I   Formula II
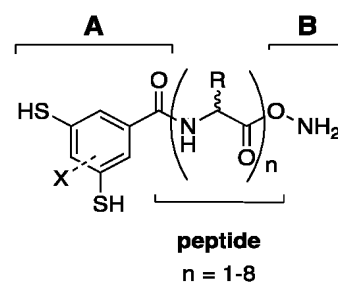
Formula III   Formula IV

Indicated are the A and B reactivities, and the Peptide moiety comprising n amino acids. Preferably, n is 2-8, more preferably 2-6. R is any natural or non-natural amino acid side chain, which can be attached in the D- or L-configuration. Optional substituent X is halogen or C_1-C_3-alkyl. Preferably, X is fluorine (F) or methyl (CH_3). If present, X is preferably at the para-position.

The A and B moieties can be attached by methods known in the art. For example, a dithiol substituted aromatic core is suitably synthesized using procedures adapted from: J. M. A. Carnall *et al.* (Science 2010, 327, 1502–1506), S. Otto *et al.* (Science 2002, 297, 590-593) and L. Field & P. R. Engelhardt (J. Org. Chem. 1970, 35, 3647-3655). A carboxylic substituent on the dithiol core can be reacted with the N-terminus of peptide in the last step of solid phase peptide synthesis (SPPS). The B moiety can be introduced via hydrazinolysis of Peptidyl-Wang-TentaGel resins (C. Bello *et al*, J. Pept. Sci. 2015, 3, 201-207).

Whereas the B reactivities of the building blocks allow for post-assembly functional modification of the nanofibers and subsequent hydrogelation, it is not necessary that all peptide-derived building blocks contain a reactive group B. In fact, the use of a mixture of building blocks of (i) B-containing

5   building blocks of the formula A-Peptide-B and (ii) building blocks of the formula A-Peptide can be advantageous e.g. it allows to fine tune the properties of the resulting functionalized hydrogel. Accordingly, in one embodiment the fiber forming solution further comprises pseudopeptide building blocks of the formula A-Peptide, wherein A and Peptide are as

10   defined herein above. In one aspect, the fiber forming solution comprises a mixture of A-Peptide-B and A-Peptide building blocks in a molar ration of 4:1 to 1:4, e.g. 3:1 to 1:3, 3:1 to 1:1, 2:1 to 1:1, 1:1, 1:1 to 1:2 or 1:1 to 1:3. In one embodiment, A-Peptide is present in molar excess to A-Peptide-B. In another embodiment, A-Peptide-B is present in a molar excess to A-Peptide.

15   In yet another embodiment, the fiber forming solution contains about equal amounts of A-Peptide and A-Peptide -B pseudopeptide building blocks.

It is preferred that the A-moieties of building blocks A-Peptide-B and A-Peptide within a fiber forming solution are the same. However, the Peptide

20   moiety can be the same, or it can be different.
A method of the present invention relies among others on the intermolecular interaction or "stacking" of peptide motifs contained in the pseudopeptide building blocks to form a one-dimensional array, thereby allowing for the formation of fibers. In other words, the peptide-based building blocks, after

25   reacting into disulfides, are capable of assembling together under suitable conditions as a stable supra-molecular structure. In one aspect, the fibers are of nanometric dimensions and referred herein as nanofibers. The term "nanofibers" is well known in the art, and refers to fibres that have a mean diameter of the order of several nanometers, e.g. tens of nanometers, to

30   several hundreds of nanometers.

Peptides can assemble into complex nanostructures through highly specific biomolecular interactions such as hydrogen bonding and hydrophobic interaction. For example, Peptide is a peptide moiety having a sequence that is predisposed to form ß-sheet fibrils.

The beta sheet, (ß-sheet) (also ß-pleated sheet) is a common motif of the regular protein secondary structure. Beta sheets consist of beta strands (ß-strands) connected laterally by at least two or three backbone hydrogen bonds, forming a generally twisted, pleated sheet. Beta-sheet fibrils are highly consistent in their morphology, even when constructed from unrelated peptides or proteins. The fact that there exist so many peptides of varying sequences, lengths, and patterning of polar and nonpolar residues that form ß-sheet fibrils suggests that ß-sheet assemblies can tolerate a variety of appended sequences or other modifications. In many but not all cases, ß-strand formation occurs when hydrophilic and hydrophobic peptide side chains are positioned on opposite sides of a peptide backbone. Accordingly, in one embodiment of the invention the pseudopeptide building block contains a peptide moiety or peptide chain comprising or consisting of alternating hydrophobic and hydrophilic amino acid residues.

Hydrogen bonding between individual strands promotes association into intermolecular ß-sheets that contain both a hydrophilic and a hydrophobic face. In ß-sheets, the backbone N–H and C=O of one strand hydrogen bond with the C=O and N–H of an adjacent strand, respectively, and the side chains project alternatively above and below the plane of the sheet. Although this hydrogen bonding pattern between peptide backbones is the defining characteristic of ß-sheets, the structure is also reinforced by hydrophobic interactions, van der Waals forces, and electrostatic interactions that occur between the side chains and between the side chains and backbone. Most sheets also have a twist of 0–30° between consecutive strands.

It is also known that certain patterns of polar and nonpolar amino acids favor the formation of ß-sheet structures. Peptides with strictly

alternating polar and nonpolar residues, for example, can produce ß-sheet fibrils, and many such peptides have been utilized in the construction of self-assembling biomaterials. However, many ß-sheet fibrillizing peptide sequences that do not strictly adhere to the alternating polar/nonpolar design have been described. In a ß-sheet with alternating polar/nonpolar sequences, all hydrophobic residues are placed on one side of the sheet, and all hydrophilic residues are placed on the opposite side, providing an amphiphilic surface that drives assembly into tertiary structures such as fibrils. Accordingly, in one embodiment the peptide moiety in a building block of the invention comprises alternating polar and nonpolar residues.

Apart from the repetitive sequence pattern, there are few rules governing the formation of ß-strands and sheets, although it is known that amino acids with ß-branched side-chains, e.g., Val (V), Thr (T), and Ile (I), are preferred. Furthermore, large aromatic residues (tyrosine, phenylalanine, tryptophan) and ß-branched amino acids (threonine, valine, isoleucine) are favored to be found in ß-strands in the middle of ß-sheets. Further useful peptide sequences include those referred to in the art as "peptide replicators". See for example Dissertation of Yigit Altay (2019), University of Groningen, ISBN 978-94-034-1802-5; Dissertation of Meniz Altay (2019), University of Groningen, ISBN 978-94-034-1804-9; M. Malakoutikhah *et al.* J. Am. Chem. Soc. 2013 (doi:10.1021/ja4067805); J.M.A. Carnall *et al.* Science 2010 (doi: 10.1126/science.1182767; B. Bartolec *et al.* Chem. Comm. 2018 (doi:10.1039/C8CC06253F). Peptides may be synthesized using standard Fmoc chemistry and purified using high pressure liquid chromatography.

Accordingly, in one embodiment the pseudopeptide building block comprises Peptide of the sequence –(Xaa)n-, wherein n is 1 to 8, and wherein Xaa can be any D- or L-amino acid (natural or non-natural) provided that Peptide as a whole allows for fiber formation.

Suitable peptides include those containing at least 2 or 3 amino acid residues. For example, they consist of 2-8, 2-7, 2-6, 2-5, 2-4, 2-3, 3-8, 3-

7, 3-6, 3-5 or 3-4 amino acids. Specific peptides are dipeptides, tripeptides, tetrapeptides, pentapeptides, hexapeptides, heptapeptides and octapeptides. Very good results can be obtained using dipeptides, tripeptides, tetrapeptides, pentapeptides or hexapeptides. However, nanofibers can also

5   be formed with building blocks containing only a single amino acid, e.g. Lys.

It is preferred that Peptide comprises at least one Lys residue. In a specific aspect, Peptide is Lys or contains a Lys residue at the C-terminus. In one embodiment, Peptide is a dipeptide of the formula Xaa1-Xaa2, for example wherein Xaa1 is p-amino-L-Phe and wherein Xaa2 is Lys. In

10   another embodiment, Peptide is a dipeptide of the formula Tyr-Lys or Lys-Tyr.

In another embodiment, Peptide is a tripeptide of the formula Xaa1-Xaa2-Xaa3, for example wherein Xaa1 and Xaa2 are non-polar amino acids and Xaa3 is a positively charged amino acid. In one aspect, Xaa1 is

15   Gly, Ala, Val, Ile or Phe, preferably Gly; Xaa2 is Gly, Leu, Ala, Val, Ile or Phe, preferably Leu; and Xaa3 is Lys, Arg or His, preferably Lys. Very good results are obtained when Xaa2 is Leu and Xaa3 is Lys. In a particularly preferred embodiment, Peptide is or comprises the sequence Gly-Leu-Lys (GLK), Gly-Ser-Lys (GSK), Gly-Ile-Lys (GIK) or Gly-Ala-Lys (GAK).

20   In yet another embodiment, Peptide is a tetrapeptide of the formula Xaa1-Xaa2-Xaa3-Xaa4, for example wherein Xaa1 and Xaa2 are non-polar amino acids, Xaa3 is a positively charged amino acid and Xaa4 is any non-polar, polar or unnatural amino acid residue. Exemplary tetrapeptides include Gly-Leu-Lys- Ser/Phe/Leu.

25   In yet another embodiment, Peptide is a pentapeptide of the formula Xaa1-Xaa2-Xaa3-Xaa4-Xaa5, for example wherein Xaa1 provides flexibility, e.g. Gly, ß-Ala or γ-aminobutyric acid (GABA), Xaa2 is a non-polar amino acid, Xaa3 is a positively charged amino acid , Xaa4 is any non-polar, polar or unnatural amino acid residue, and Xaa5 is a positively

30   charged amino acid residue. Exemplary pentapeptides include Gly-Leu-Lys-Phe/Ala-Lys

In a still further embodiment, Peptide is a hexapeptide of the formula Xaa1-Xaa2-Xaa3-Xaa4-Xaa5-Xaa6, for example wherein Xaa1 provides flexibility, e.g. Gly, ß-Ala or γ-aminobutyric acid (GABA), Xaa2 is a non-polar amino acid, Xaa3 is a positively charged amino acid , Xaa4 is any

5 non-polar, polar or unnatural amino acid residue, Xaa5 is a positively charged amino acid residue, and Xaa6 is a negatively charged residue, such as Asp or Glu.

Exemplary penta- or hexapeptide sequences for use in the present invention are of the formula Xaa1-Xaa2-Xaa3-Xaa4-Xaa5-Xaa6, wherein

10 Xaa1 is Gly, ß-Ala or γ-aminobutyric acid (GABA)

Xaa2 is Ala, Val, Leu, Ile or Phe, preferably Leu

Xaa3 is Lys, Arg or His, preferably Lys

Xaa4 is Ala, Val, Leu, Ile or Phe

Xaa5 is Lys, Arg or His, preferably Lys

15 Xaa6 is Asp, Glu, or absent.

As mentioned herein above, the invention also related to a method for providing peptide-based functionally modified (nano)fibers, comprising the steps of :

20 (i)   providing a fiber forming solution comprising a mixture of (a) pseudopeptide building blocks of the formula A-Peptide-B and (b) pseudopeptide building blocks of the formula A-Peptide, wherein:

Peptide is a moiety of 1 to 8, preferably 3 to 5, amino acid residues having a sequence that is predisposed to form a one-dimensional

25 array, such as ß-sheet fibrils;

A is an aromatic moiety carrying two reactive thiol groups; and

B is a reactive α-nucleophile;

(ii) exposing the fiber forming solution to oxidizing conditions to induce

30 supramolecular self-assembly of the mixture pseudopeptide building blocks into peptide-based (nano)fibers; and

(iii) contacting said (nano)fibers with at least one functional group of interest comprising reactivity C forming a reactive pair with B to obtain functionally modified nanofibers.

It one embodiment, the Peptide-moieties of building blocks A-Peptide-B and/or A-Peptide within a mixture of building blocks are different with respect to the number of amino acids and/or the nature of the amino acids, provided that the different Peptide moieties allow for the formation of a one-dimensional array. For example, the fiber forming solution comprises a mixture of (i) A-Peptide-B wherein Peptide is (Xaa)n; and (ii) A-Peptide is wherein Peptide is (Xaa)m, wherein n and m are individually selected from 1-5, and wherein n and m are the same or distinct. In one aspect, n is 3-4 and m is 1-4. See example 7 herein below for various representative mixtures of pseudopeptide building blocks with and without the B reactive α-nucleophile.

In a method of the invention, step (ii) comprises exposing the fiber forming solution comprising the (mixture of) pseudopeptide building blocks to oxidizing conditions to induce supramolecular self-assembly of the pseudopeptide building blocks into peptide-based fibers. The oxidizing conditions allow for group A to undergo reversible thiol-disulfide exchange, resulting in the formation of macrocyclic structures via dynamic covalent bond formation. Furthermore, the conditions facilitate, or at least do not interfere with, intermolecular interactions between the peptide moieties of the building blocks.

Suitable oxidizing conditions may involve one or more of the following:
1) exposure to air (slow oxidation) or Na-perborate (fast oxidation);
2) pH between 8 and 8.5 to have thiols deprotonated so they can attack disulfides and promote exchange;
3) temperature between room temperature to about 45°C. An increase in temperature can alter the kinetic of assembly formation.

4) aqueous conditions, such as a borate buffer. High concentration of salt can cause unwanted precipitation of assemblies/precursors or fiber aggregation.

In one embodiment, peptide building blocks are dissolved to a concentration of about 2-10 mM, e.g. 4-6 mM, in an aqueous borate buffer in the range pH 8-8.5, followed by oxidation using perborate, for example a 20-60 mM sodium perborate solution.

Because remaining B groups ought to participate is subsequent hydrogelation, only partial covalent modification of the B groups is desired. For example, if it is intended to modify 10% of B moieties with biologically relevant ligand functionalized with C, it can be calculated what is the 10% of total number of moles of A-peptide-B. Since B and C react in a 1 to 1 stoichiometry, this means that a remaining 90% of free moiety B is available for subsequent hydrogelation. The degree of modification is readily monitored, e.g. by UPLC.

(Nano)fiber formation may involve the self-assembly of one type of pseudopeptide building blocks, or a mixture of two or more types of pseudopeptide building blocks, differing for example in the Peptide sequence, the dithiol-aromatic core A, and/or reactivity B. Also, reactivity B may be absent in some of the building blocks. In case the same building blocks are used, self-assembly results in homogenous (nano)fibers. In case different building blocks are used, self-assembly results in heterogenous (nano)fibers. For example, it has been reported that in mixed systems, in which peptide 1 is already assembled into short fibers, peptide 2 can assemble on top and bottom of the first assembly to result in triblock fibrous copolymers (Pal *et al.* Angewandte Chemie (2015), Vol. 54, Issue 27,7852-7856).

In one embodiment, a Formula I or II building block is used for A-Peptide-B, preferably Formula I. In another embodiment, a Formula III or IV building block is used, preferably Formula III.

In yet another embodiment, a combination of two or more building blocks A-Peptide-B according to Formula I, II, III or IV is used, for example a mixture comprising Formula I and II, Formula I and III, Formula II and III; or Formula II and IV building blocks is used.

5 These embodiments may be combined in a mixed system comprising A-Peptide building blocks.

Following self-assembly, the (nano)fibers are "decorated" or functionalized with one or more (bioactive; biologically relevant) compounds of interest. To

10 that end, step (iii) of a method of the invention comprises contacting the assembled (nano)fibers with at least one functional group of interest comprising reactivity C capable of reacting with B (i.e. B and C form a reactive pair) to obtain functionally modified nanofibers. Thus, reactivity C is selected on the basis of the chemical nature of group B. For example, in

15 case B is or comprises a hydrazine ($-NH-NH_2$) or an alkoxy-amine ($-O-NH_2$) group, the C reactivity suitably comprises a carbonyl moiety to allow for covalent nanofiber functionalization via, respectively, a hydrazine or an oxime bond. In one embodiment, reactivity C is or comprises an aldehyde or glyoxylyl group. In a specific embodiment, reactivity C is a 4-formylbenzoyl

20 group.

A person skilled in the art will understand and appreciate that a "biologically relevant functional group of interest" refers to any molecule, ligand, substance or moiety that is (suspected to be) of use in cell or tissue

25 engineering, e.g. in preparing a cell responsive hydrogel. It can be a proteinaceous or non-proteinaceous substance. It can be any substance capable of directing, supporting or enhancing cell fate, development, morphology, growth or differentiation.

30 Since a method of the invention involves post-assembly modification it is advantageously used to functionally modify the nanofibers with two or more different functional groups of interest. Accordingly, in one aspect step (iii)

comprises contacting said nanofibers with two or more different biologically relevant functional groups of interest, either sequentially or simultaneously i.e. using a mixture of two or more different functional groups of interest in a predetermined ratio. This approach is especially attractive in studies

5   involving the optimization of one or more cell responses in the presence of a functionalized hydrogel.

Exemplary proteinaceous functional groups of interest comprise biologically active peptide motifs that are well known in the art, e.g., a peptide motif

10   found in a naturally occurring protein, or a target site for an interaction with a biomolecule. In certain embodiments of the invention the naturally occurring protein is a component of the extracellular matrix (ECM), e.g., a component of the basement membrane.

Preferably, the peptide of interest is selected from the group

15   consisting of growth factors, differentiation factors, cytokines, chemokines, cell adhesion ligands and protease cleavage sites. For example, the peptide of interest is or comprises a protease-degradation site, such as a substrate for matrix metalloproteinases (MMPs).

In another specific aspect, the biologically relevant peptide of

20   interest comprises or consists of an oligopeptide. For example, the oligopeptide has the amino acid sequence RGD or LDV. These motifs are often employed peptides for stimulated cell adhesion on synthetic surfaces. RGD has been introduced into synthetic biopolymers formed by blending, co-polymerization, chemical or physical treatment, as stable covalent linking of

25   the bioactive signal is a prerequisite for promoting cell spreading. The LDV motif has been applied for its cell adhesive properties, in chemical systems that can inhibit tumor metastasis and soft materials for T-cell activation. As another example, the biologically relevant group of interest is a peptide having an effect on osteoblasts, for instance the tripeptide Ile-Pro-Pro.

30

In a specific aspect, step (iii) of a method of the invention comprises contacting assembled (nano)fibers comprising -NH-NH$_2$ and/or -O-NH$_2$

groups with at least one functional ligand comprising an aldehyde or glyoxylyl-reactivity.

It was found that post-modified fibers remained stable over a period of at least several weeks, and that they can be triggered to undergo rapid hydrogelation upon an external trigger. A further aspect of the invention therefore relates to a method for providing a peptide-based functionalized hydrogel. Hydrogels are in principle three-dimensional (3D) polymeric networks that are filled with water. The water content reaches as high as 90%–99% while it depends on the polymer concentration. This fact explains high hydrophilicity of hydrogels and the ability to safely incorporate biological entities (proteins and cells) without an aggregation. The mechanical behavior of hydrogels is typically viscoelastic which is associated with the water and the movement of polymer networks in fluid.

A method of the invention for providing a peptide-based functionalized hydrogel comprises providing functionally modified peptide-based (nano)fibers according to a method as described herein above, followed by adding a crosslinker comprising a plurality of reactive groups D capable of reacting with B to induce hydrogelation of the functionally modified (nano)fibers. As explained previously, any type of chemistry can be used for the B and D moieties, provided that the cross-linker comprises functionalities complementary to the B groups. Moreover, the conditions required or applied for covalent bond formation between B and D should be compatible, e.g. sufficiently mild, with peptide-based nanofibers. For example, a fiber solution is prepared as above mentioned at a 0.5 to 10 mM concentration (total of A-Peptide-B and A-Peptide units). Cross-linking may involve adding corresponding amount of cross-linker dissolved in a suitable solvent, such as acetone. Preferably, the relative amount/concentration of assembled fibers and the amount/concentration of cross-linker are chosen such that the molar ratio of complementary (remaining) reactivities B and D are about equal.

In one aspect, reactive group D comprises a carbonyl-functionality, preferably an aldehyde. For example, suitable crosslinkers comprising a plurality of D reactivities include di- and trialdehydes. In a specific aspect, the cross-linker is selected from the group consisting of glutaraldehyde, 2,5-thiophenedicarboxaldehyde, terephthaldehyde, isophthaldehyde, succinaldehyde, substituted versions of benzene-1,3 and 1,4 biscarboxaldehydes, and benzene- 1,3,5- tricarboxaldehyde. These cross-linkers are particularly suitable for preparing a hydrogel from functionalized (nano)fibers comprising carbonyl groups as B reactivity.

As is demonstrated herein below, cells grown on hydrogels prepared from nanofibers comprising the GLK tripeptide motif displayed extended morphologies, characteristic for cell spreading and adhesion independent of the ligand used for its functionalization. Herewith, GLK-containing building blocks are advantageously used for (nano)fiber assembly and subsequent hydrogelation. The invention therefore also relates to a method for providing an ECM-mimicking/cell adhesive peptide-based hydrogel, comprising the steps of:

(i)   providing pseudopeptide building blocks of the formula A-Peptide-B, wherein "Peptide" is a peptide moiety of 3 to 8, preferably 3 to 6, amino acid residues comprising or consisting of the sequence GLK, A is an aromatic moiety carrying two reactive thiol groups, and B is a reactive α-nucleophile;

(ii) exposing the pseudopeptide building blocks to oxidizing conditions to induce supramolecular self-assembly of the pseudopeptide building blocks into peptide-based (nano)fibers; and adding a crosslinker comprising a plurality of reactive groups D capable of reacting with B to induce hydrogelation of the (nano)fibers into an ECM-mimicking hydrogel. Prior to hydrogelation, the assembled fibers may be functionally modified with one or more groups of interest, e.g. with further adhesive ligands and/or bioactive signals, as described herein above.

The present invention discloses materials characterized by several properties that potentially make this system a tunable, bioactive scaffold that can stimulate cell fate processes. First, similarly to the extracellular matrix (ECM), the synthetic material possesses a fibrous structure, as it is prepared from one-dimensional assemblies of molecular stacking motifs. Second, the B functionality on the building blocks facilitates hydrogelation between a fiber-like supramolecular polymer and a suitable cross-linker having functionalities D. The same reaction can allow attachment of C-functionalized biologically relevant ligand(s), such as cells adhesion motifs (e.g., RGD, LDV) or short peptide sequences known to direct differentiation of cells, e.g. osteoblasts or stem cells. Third, due to its peptide nature, the material has an excellent cytocompatibility. The invention therefore also relates to the products obtainable or obtained by a method herein disclosed. In one embodiment, it provides peptide-based functionally modified (nano)fibers obtainable by the "post-assembly modification" method. In a further embodiment, it provides a peptide-based functionalized hydrogel obtainable by a method of the invention.

Further aspects of the invention relate to various uses, methods, compositions and applications of peptide-based functionally modified (nano)fibers and/or a peptide-based functionalized hydrogel as provided herein. Exemplary uses include tissue engineering, scaffold synthesis, cell culturing, cell culture substrates, for drug and gene delivery, as wound dressing, as an implant, as an injectable agent that gels in situ, in pharmaceutical or cosmetic compositions, in regenerative medicine, in tissue engineering and tissue regeneration.

One embodiment relates to a composition comprising (i) peptide-based functionally modified (nano)fibers and/or a peptide-based functionalized hydrogel according to the invention and (ii) cells, preferably mammalian cells. The composition may comprise one or more further components, such as bioactive therapeutic(s) that stimulate regenerative processes and/or

modulate the immune response. Preferred compositions include peptide-based functionalized hydrogel according to the invention and mammalian cells.

5   The invention provides uses of and methods for using peptide-based functionally modified (nano)fibers and/or a peptide-based functionalized hydrogel according to the invention in tissue engineering and tissue regeneration. Disclosed is a method for tissue engineering, comprising contacting cells with a peptide-based functionalized hydrogel. Also disclosed

10   is a method of tissue regeneration comprising the steps:
       providing a peptide-based functionalized hydrogel as defined above,
       exposing said hydrogel to cells which are to form regenerated tissue,
       allowing said cells to grow on said hydrogel. In one embodiment, the method as defined above is performed in-vitro or in-vivo. In one embodiment, the

15   method as defined above is performed in vivo, wherein, a hydrogel is provided at a place in a body where tissue regeneration is intended. In one embodiment, the method comprises injecting said hydrogel at a place in the body where tissue regeneration is intended.

20   A further embodiment relates to a tissue-engineering or biomimetic scaffold, that can support the in vivo and/or in vitro growth of cells and facilitate the regeneration of native tissue. In one aspect, the invention provides tissue-engineering or biomimetic scaffold, comprising:
       (a) a microporous scaffold comprising a biocompatible material suitable for

25   use in tissue-engineering scaffolds, wherein said biocompatible material is a biocompatible polymer that comprises a biodegradable polymer; and
       (b) a peptide-based functionalized hydrogel according to the invention.

       Since the concept underlying the present invention relies on the post-

30   assembly functionalization of peptide-based (nano)fibers, it also provides a kit-of-parts that allows for the "on site" functionalization of a peptide-based fiber assembly by the end user. The kit may provide the pre-assembled

(nano)fibers, or it may provide pseudo-peptide based building blocks for
(nano)fiber self-assembly on-site.

Accordingly, the invention provides a kit-of-parts for use in a
method of the invention, comprising (i) first container comprising pseudo-
peptide building blocks of the formula A-Peptide-B, wherein Peptide
consists of 1 to 8, preferably 2 to 8, more preferably 3 to 5, amino acid
residues; A is an aromatic moiety carrying two thiol groups capable of
undergoing reversible thiol-disulfide exchange; B is a reactive α-nucleophile;
and (ii) in a second container one or more functional group(s) of interest
comprising reactivity C capable of reacting with B.

Also provided is a kit-of-parts for providing a functionalized
hydrogel, comprising (i) peptide-based (nano)fibers comprising moiety B
being a reactive α-nucleophile; and (ii) one or more functional group(s) of
interest comprising reactivity C capable of reacting with B. Herein, the B
and C reactivities are as described herein above. Preferably, the kit
comprises two or more distinct biologically relevant groups of interest, such
as a growth factor and an adhesion ligand, each carrying the same reactivity
C. The kit may further comprise a cross-linking agent having functionalities
D.

Analogous to what is described herein above in relation to the building
blocks, B may comprise or consist of a -NH-NH$_2$ or -O-NH$_2$ functionality,
preferably B is -NH-NH$_2$. Reactivity C and D may comprise a carbonyl-
functionality, preferably C is an aldehyde or glyoxylyl group and D is an
aldehyde. The kit may furthermore comprise pseudo-peptide building blocks
of the formula A-Peptide.

LEGEND TO THE FIGURES

Figure 1. a) Mechanism of fiber formation starting from aromatic dithiol
functionalized pseudo-peptide building block **1**. Molecule **1** undergoes
oxidation to form a small dynamic combinatorial library (DCL) consisting of
cyclic disulfides in different oligomeric states. Macrocycle **1**$_5$ self stabilizes

by forming short stacks of rings, shifting the exchange pool towards the formation of **1₅**. Short stacks elongate from both ends, resulting in a supramolecular polymer with a fiber-like structure.

b) Cartoon representation of the stepwise hydrogelation process. Fast triggered gelation is achieved through the acyl-hydrazone formation by the addition of dialdehyde cross-linker to the fiber solution. Here, for simplicity reasons, only cross-linking with dialdehyde is schematically depicted, while it is expected that cross-linking with trialdehyde is achieved identically. Covalent linking interconnects the fibers into a hydrogel, a 3D network that can contain a large amount of water.

c) Di- and trialdehydes tested for cross-linking of **1₅** fibers for hydrogel formation: glutaraldehyde (**2**), aldehyde-PEG-aldehyde, MW 600 (**3**), terephthaldehyde (**4**), isophthaldehyde (**5**), quinolone-based dialdehyde (**6**), benzene – 1,3,5 – tricarboxaldehyde (**7**) and triazine-based trialdehyde (**8**).

d) Hydrogel formation upon the addition of different cross-linkers. Vial inversion test indicates the formation of hydrogels in the presence of di- and trialdehydes containing short and rigid spacer groups between the carbonyls, such as **2**, **4**, **5**, and **7**.

Figure 2 Scanning electron micrographs of a) hydrogel formed upon addition of **2**, b) hydrogel formed upon addition of **4**, c) hydrogel formed upon addition of **5** and d) hydrogel formed upon addition of **7**. The solvent of the hydrogels was removed in vacuo to yield the product as a white powder, which was then spread over a clean silicon wafer for SEM measurement.

Figure 3 i) Gel formation (V = 300 μL, [1] = 4.0 mM) upon addition of terephthalaldehyde; iv) vial inversion test shows undamaged gel in presence of growth medium on day 5; ii) and v) addition of 200 μL of growth medium on gel (day 0 and day 5, respectively); iii) and vi) vial inversion test a few minutes after a new portion of the medium has been added (day 0 and day 5, respectively); before adding a new portion of the medium, the previous layer

of the liquid has been removed. The gel exhibits a good stability for at least several months.

Figure 4 a) Representative transmitted light image and b) representative fluorescence microscopy image of human bone marrow-derived mesenchymal stem cells (hBM-MSCs) on hydrogel-coated glass coverslips in growth medium, stained with calcein-AM (live cells) and propidium iodide (dead cells), after 24 h. Scale bars equal to 150 μm. c) Quantification of cell viability cultured on hydrogel-coated glass coverslips in growth medium for three repetitions of the experiment. Mean ± SD (n=3, three random image fields per experiment).

Figure 5 UPLC analyses of dynamic combinatorial libraries (DCLs) made by oxidation and disulfide exchange of 1 (4.0 mM) in borate buffer (12.5 mM, pH 8.2) stirred at 1200 rpm: a) when dominated by self-assembly 1_5; b) post-assembly functionalization of 1_5 by glyoxylyl-RGD (1_5-RGD) and c) glyoxylyl-LDV (1_5-LDV), both recorded 30 minutes after addition of the corresponding aldehydes. Formed acyl-hydrazones (mixed species) are highlighted in blue. Hydrogel formation upon addition of terphthalaldehyde (CL) to DCLs dominated by 1_5 and post-modified by d) glyoxylyl-RGD (1_5-RGD) and e) glyoxylyl-LDV (1_5-LDV). The vial inversion test indicates the formation of hydrogels within 2 minutes after the addition of CL.

Figure 6 Representative fluorescence microscopy images of human bone marrow-derived mesenchymal stem cells (hBM-MSCs) cultured on hydrogels made from: a) 1_5 fibers; b) 1_5 fibers post-modified with glyoxal-RGD, or c) 1_5 fibers post-modified with glyoxal-LDV, and coated on glass coverslips in the growth medium, stained with calcein-AM (live cells) and propidium iodide (dead cells), after 24 h. Scale bars equal to 150 μm. For clarity, red circles emphasize the presence of dead cells (red). d) Quantification of cell viability cultured on three different types of hydrogel-

coated glass coverslips in growth medium for three repetitions of each type of material. Mean ± SD (n=3, at least three random image fields per experiment, at least 50 counted cells per image field).

Figure 7 Representative fluorescence microscopy images of hBM-MSCs cells cultured on hydrogels prepared from a) – c) 1_5 fibers; d) – f) 1_5 fibers post-assembly functionalized with glyoxylyl-RGD and g) – i) 1_5 fibers post-assembly functionalized with glyoxylyl-LDV, stained with DAPI (blue) and FITC-phalloidin (cytoskeleton), after 24 h. The bottom images represent the merge of the two channels. Scale bars equal to 300 µm.

Figure 8 Representative UPLC traces of DCL prepared from mixed building blocks 1 and 16 (see Example 7A).

Figure 9 Characterization of mixed building block fibers. Panel A: The transmission electron microscopy (TEM) photograph of example 7A following covalent post-assembly modification with IPP, prior to cross-linking. Panel B: Hydrogel formation of cross-linked functionalized nanofibers prepared from mixed building blocks, with or without covalent post-assembly modification.

Figure 10 A synthetic strategy towards mimicking the native extracellular matrix. Acyl-hydrazone bond formation can be employed to achieve: a) cross-linking of peptide-based, fibrous self-assemblies yielding hydrogel formation; b) immobilization of bioactive short peptide sequences within the material; c) decoration of fibrillar scaffold with full-length proteins and growth factors, and/or d) tuning in protease-sensitive peptides as cross-linkers that would facilitate cell-induced enzymatic remodeling of the gel. As fibers bear NH-NH$_2$ groups (reactivity B), biologically relevant ligands comprising reactivity C and cross-linkers comprising reactivity D, e.g. mono- or di- carbonyl-functionalized, form a reactive pair (B-C and B-D). This

unique modular approach allows the molecular engineering of tailor-made material on demand.

EXPERIMENTAL SECTION

Materials

Cross-linkers were purchased from TCI Chemicals, Sigma-Aldrich, Acros Organics, or Creative PEGworks. Boric anhydride (Sigma-Aldrich) and sodium hydroxide (Merck Chemicals) were utilized for buffer preparation and pH adjustments. The concentration of borate buffer is given in $B_4O_7^{2-}$ ions. Acetonitrile (UPLC-MS grade), water (UPLC-MS grade), DMF (HPLC grade), and trifluoroacetic acid (HPLC grade) were purchased from Biosolve BV. Building block **1** was purchased from Cambridge Peptides Ltd. (Birmingham, U.K.) by coupling 3,5-bis(tritylthio)-benzoic acid, which was synthesized via a previously reported procedure[11a] at the N-terminus. Glyoxylyl-RGD and glyoxylyl-LDV were purchased from Cambridge Peptides Ltd. (Birmingham, U.K.) and GenScript Biotech (Piscataway, New Jersey, USA), respectively. Gels were prepared on round, 12 mm diameter, and 0.17 mm thick borosilicate glass coverslips (Fischerbrand). Costar® multiple well plates (24 well, Corning® CellBIND® surface, polystyrene) were purchased from Corning and used for cell culture. Human bone marrow-derived mesenchymal stem cells (hBM-MSCs) were used for cell experiments. The growth medium supplemented with Alpha modified Eagle's medium (Gibco), 10% fetal bovine serum (Gibco), 0.1% ascorbic acid 2-phosphate (Sigma), and 1% penicillin/streptomycin (Gibco) and trypsin (Sigma-Aldrich) were utilized for cell culture and harvesting, respectively. Dyes propidium iodide and calcein-AM were obtained from Sigma-Aldrich and Thermo Fisher Scientific. For immunostaining cells were incubated with a primary antibody for vinculin (clone hVin-1, Sigma), followed by treatment with a secondary antibody Rhodamine Red™-X-

labeled goat-anti-mouse antibody (Jackson Immunolab). The nucleus and
cytoskeleton were stained with DAPI (4′,6-diamidino-2-phenylindole) and
TRITC-phalloidin purchased from Sigma-Aldrich. EM-grade PFA was
purchased from Electron Microscopy Sciences.

5

General methods

Fiber preparation
Building block **1** was dissolved to a concentration of 4.0 mM in borate buffer
10 (12.5 mM in $B_4O_7^{2-}$, pH 8.2) to obtain a fiber forming solution. Due to **1**
being a salt of TFA, the pH value had to be re-adjusted to 8.2 with 1M
NaOH. Subsequently, the solution was oxidized to approximately 40% with
$NaBO_3$ (40 mM). The sample was equilibrated in a UPLC vial (12 x 32 mm)
with a Teflon-coated screw cap. The UPLC vial was equipped with a
15 cylindrical stirring bar (2 x 5 mm, Teflon-coated, purchased from VWR), and
the solution was stirred at 1200 rpm using an IKA C-MAG HS 7 control hot
plate stirrer. The temperature was kept constant at 25 °C using a Thermo
Scientific Compact Digital Dry Bath.

20 *UPLC/MS analysis*
UPLC/MS measurements were performed using a Waters Acquity UPLC H-
class system coupled to Waters Xevo-G2 TOF. The mass spectrometer was
operated in the positive electrospray ionization mode with the following
ionization parameters: capillary voltage: 3 kV, sampling cone voltage: 20 V,
25 extraction cone voltage: 4 V, source gas temperature: 120°C, desolvation gas
temperature: 450°C, cone gas flow (nitrogen): 1 L/h, desolvation gas flow
(nitrogen): 800 L/h.

Atomic force microscopy (AFM)
30 AFM samples were prepared by depositing 100 μL of a sample (diluted to 10
μM concentration of **1** with UPLC water) onto a clean mica surface (Grade

V1, Van Loenen Instruments). Subsequently, the excess solvent was evaporated by blotting into a piece of paper, the surface was washed twice with 100 µL UPLC grade water, blotted into a piece of paper and finally dried in a gentle stream of air. The AFM measurements have been

5 performed using a Bruker Multimode 8 instrument in ScanAsyst-Air imaging mode. Measurements were performed in air at room temperature. As a probe, a ScanAsyst Air (Bruker) silicon tip on a nitride cantilever was used with the following parameters: length: 115 µm, width: 25 µm, resonance frequency: 70 kHz, force constant: 0.4 N/m. The images were

10 recorded with frequencies between 0.5 and 1.5 Hz and analyzed with NanoScope Analysis 1.50 software (Bruker Corporation, 2015).

Post-assembly functionalization of fibers with glyoxylyl-RGD and glyoxylyl-LDV and gel preparation

Self-assembly 1_5 was obtained by oxidizing building block 1 under basic

15 conditions ([1] = 4.0 mM, 12.5 mM borate buffer, pH 8.2). After UPLC/MS analysis confirmed that DCLs are dominated by assembly 1_5, 10 mol% of glyoxal-RGD and glyoxal-LDV was added, and the library was stirred for another 30 min. The reaction between the aldehyde and 1_5 was confirmed by UPLC/MS, where mixed species ($1_41l/1_31l_2$ and $1_41l2/1_31l2_2/1_21l2_3$) were

20 observed. To a fiber solution post-modified with monoaldehyde, a portion of cross-linker 4 was added to achieve hydrogelation. The remaining reactive groups of fibers and cross-linkers were kept constant at a molar ratio of 1:1.

UPLC analysis

25 UPLC measurements were performed on a Waters Acquity UPLC H-class system equipped with a PDA detector. UPLC analyses were performed on an Acquity UPLC Peptide CSH C18 1.7 µm (150 x 2.1 mm) column, purchased from Waters, using UPLC-MS grade water (eluent A) and UPLC-MS grade acetonitrile (eluent B), containing 0.1 V/V % TFA as a modifier. A

flow rate of 0.3 mL/min and a column temperature of 35°C were applied. Spectra were recorded at a detection wavelength of 254 nm.

Preparation of gel-coated cover slips and cell seeding

5   Glass coverslips were plasma treated for 5 min in PlasmaFlecto 10 to enhance the surface's wettability and adhesion. The reactive groups of fibers and cross-linkers were kept constant at a molar ratio of 1:1. The stock solution of cross-linker **CL** was prepared by dissolving the compound in acetone. Gels were prepared by mixing 100 μL of fiber solution (4.0 mM in

10   building block **1**) with 2 μL of cross-linker stock solution, in an Eppendorf. Before gelation occurred, 25 μL (preliminary cytotoxicity experiments) or 50 μL (cytotoxicity of modified gels, cell morphology experiments) of the mixture was placed on top of the coverslip and left undisturbed until gelation was complete (app. 5 min). Coverslips were transferred into a 24-

15   well plate. To each of the wells containing gel-coated coverslip, 250 μL of MiliQ water was added to prevent gel drying for the duration of UV-treatment. Gels were sterilized with UV-light for 15 minutes. After sterilization, cells were seeded on top of hydrogels at the app. 10000 cell/mL of culture medium (V = 1 mL per well), for all experiments. Plates were

20   incubated at 37°C, 5% CO_2, at maximum humidity. Live/dead staining was performed after 24 h, proliferation kinetics was followed for five days (day 1, 3, and 5), while cell morphology when cells are seeded on top or encapsulated was assessed after one day.

25   *Staining for assessment of cell morphology*

For assessment of cell morphology, cells were rinsed with PBS and fixed for 15 min using warm 3.7% EM-grade paraformaldehyde (PFA) solution in PBS after 24h. Afterward, the membranes of the cells were permeabilized with 0.5% Triton-X 100 solution in PBS for 3 min, and non-specific

30   background was blocked with 5% bovine serum albumin (BSA) in PBS for 30 minutes. The nucleus and cytoskeleton of fixed cells were stained with DAPI

and FITC-labelled phalloidin. The working solution was prepared in PBS
containing 1% bovine serum albumin (BSA) by adding FITC-labelled
phalloidin (final concentration = 2 μg/mL) and DAPI (final concentration = 4
μg/mL) and protected from light. The hydrogels were incubated with a
5   sufficient amount of working solution to completely cover the sample (V =
400 μL) in the dark, for an hour, and subsequently visualized by
fluorescence microscopy.

Confocal microscopy and image analysis
10   Cells were imaged with Celldiscoverer 7 with Plan-APOCHROMAT 5x/0.35
air objective (Carl Zeiss AG, Oberkochen, Germany) and TissueFaxs
(TissueGnostics GmbH, Vienna, Austria) microscopes. Cell quantification
was performed in ImageJ. Data are given as mean values ± standard
deviation (SD).

15

EXAMPLE 1: Peptide-based fiber formation

This example describes the use of dynamic combinatorial chemistry for the
formation of fiber-like, supramolecular polymers from pseudo-peptide
20   building blocks. We designed a pseudo-peptide building block **1** (Figure 1a)
of the formula A-Peptide-B, with three distinctive features: (i) a dithiol,
aromatic core to promote thiol-disulfide exchange and stacking via **π-π**
interactions (A); (ii) a short peptide chain that will assist self-assembly and
is biocompatible (Peptide);[9,15] and (iii) a hydrazide-functionalized C-
25   terminus representing further chemical reactivity (B) through a second
dynamic covalent bond.
          Upon exposure to air, pseudopeptide building block **1** underwent
oxidation to form a dynamic combinatorial library (DCL) of differently sized
cyclic disulfides ($\mathbf{1_3}$, $\mathbf{1_4}$ and $\mathbf{1_5}$), which continuously interconvert by the thiol-
30   disulfide covalent exchange. In a such mixture, one of the rings ($\mathbf{1_5}$) can form
stacks with copies of itself, which promotes the process of self-assembly.
This shifts the distribution of the exchange pool towards the synthesis of

more of the self-assembling library member. Growth of the stacks from their ends, combined with breakage of stacks through mechanical agitation, results in an increase in the amount of supramolecular fibrous polymer (Fig. 1a).

The composition of the resulting DCL was monitored with UPLC for 23 days. The progress of 1_5 (fiber) formation was followed by sampling 5 μL of the DCL and diluting it to 50 μL with doubly distilled water. When **1** was depleted, and 1_5 had an abundance of at least 50 % (based on the integration of UPLC chromatograms), the solution was used for gel preparation. The fiber sample was used directly since products of hydrazide hydrolysis were observed upon prolonged incubation.

Atomic force microscopy (AFM) and cryo-electron microscopy (cryo-EM) of DCLs dominated by 1_5 indicated the presence of fibers with lengths ranging from hundreds of nanometers to several micrometers (data not shown).

EXAMPLE 2: Hydrogel formation and characterization

Subsequently, we investigated the capacity of the peptide-based fibers for hydrogel formation upon the addition of a di- or trialdehyde (Figure 1b) as cross-linkers. The selection of cross-linkers (**CLs**) is based on differences in length and flexibility of the spacer group between the aldehyde moieties, and the number of cross-linking groups (Figure 1c). The concentration (4.0 mM in **1**) and the ratio of **1** to **CL** (1:1 in complementary functionalities i.e., NH-NH$_2$ and carbonyls) were kept constant in all experiments. The addition of **CLs** containing short and rigid spacers (**2**, **4**, **5**, and **7**) to the solutions of pre-formed fibers did result in gel formation. Hydrogelation occurs in the span of 2 to 10 minutes, depending on the cross-linking reagent (Figure 1d). In contrast, molecules with a flexible spacer **3**, **6**, and **8** yielded viscous solutions even upon prolonged incubation. We hypothesize that an increase in distance between carbonyl groups and spacer flexibility enables hydrazone formation within a single fiber.

In order to probe the concentration dependence of **1** for gel formation, we prepared a series of samples at different concentrations ranging from 4.0 mM to 0.25 mM, while using glutaraldehyde (**2**) as a model cross-linker. Remarkably, the critical gelation concentration (CGC) was found to be 0.5

5   mM (in **1**) or 0.025 wt%, which is at the low end of the scale for hydrogels reported in the literature.[16] At 0.25 mM, the sample exists as a viscous solution. The same value was found using terephthalaldehyde (**4**) as a cross-linking agent.

To directly visualize the organization of fibers, as a component of the gel

10   scaffold, we used cryo-EM and scanning electron microscopy (SEM) following drying in a high vacuum. The appearances of the samples without and with cross-linkers were remarkably identical, despite the distinct macroscopic differences. Regardless of cross-linker, the samples do not exhibit longer-range order within the hydrogel network (Figure 2).

15

EXAMPLE 3: Hydrogel stability in the presence of culture media

Maintaining bulk stability and properties of the material on the time scale of cell culture experiments is essential, as the migration of cells to the stiff

20   bottom of the culture container could lead to alteration of cell morphology. Such lack of stability would essentially reduce a 3D ECM model (if cells are encapsulated within the material) or 2D soft model (if cells are cultured on top of the soft material) to the traditional and static 2D culture on glass or plastic substrate. Both soft-material models are recognized as significantly

25   more-true to tissue microenvironments[17] since stiffness can influence stem cell lineage specification,[18] morphology, and adhesion.[19]

Therefore, prior to encapsulating cells or seeding them on top of the gels, we tested compatibility between gel material and growth medium. The growth medium used was Alpha modified Eagle's medium, containing

30   10% fetal bovine serum, 0.1% ascorbic acid 2-phosphate, and 1% penicillin/streptomycin.

The gels were prepared as described in Example 2 (Fig. 3-i), and a portion of growth medium was added on top (Figure 3-ii), to mimic an experiment involving cell seeding. The culture medium was found to diffuse inside the gel without any effect on the macroscopical level (Figure 3-iii), as evidenced by the red color of the medium penetrating the gel, while the material remained undamaged. The hydrogel was stable after prolonged incubation with culture medium (Figure 3-vi), indicating its applicability in experiments that require long-term intactness of material.

EXAMPLE 4: Cytocompatablity of Hydrogels

In this example, the potential cytotoxic effect of the hydrogel was evaluated based on viability levels and proliferation rate of cells, as these parameters are good indicators of cell health.

Cell culture hBM-MSCs

Human bone marrow-derived mesenchymal stem cells (hBM-MSCs) from Lonza (passage 2) were cultured in a growth medium supplemented with Alpha modified Eagle's medium (Gibco), 10% fetal bovine serum (Gibco), 0.1% ascorbic acid 2-phosphate (Sigma), and 1% penicillin/streptomycin (Gibco). Cells were incubated at 37°C with 5% CO_2. Every 3 days, the culture medium was refreshed, and cells were passaged or harvested at approximately 80% confluence. The confluent cells were subcultured by trypsinization. hBM-MSCs of passage 4 were used for the next experiments.

Live/dead staining

Live/dead staining was performed using propidium iodide and calcein-AM. Live cells were characterized by enzymatic conversion of cell-permeant, non-fluorescent calcein-AM into strongly fluorescent calcein (green) within live cells. Dead cells were characterized by propidium iodide as it binds to double-stranded DNA by intercalating between the bases but is excluded

from cells with intact plasma membranes. Once the dye is bound, its fluorescence is enhanced 20- to 30-fold, producing a bright red fluorescence in dead cells. The working solution was prepared in serum-free media by adding propidium iodide (final concentration = 3 µM) and calcein-AM (final concentration = 5 µM) and protected from light. Culture media was removed, and hydrogels were washed with warm PBS, to which a sufficient amount of working solution to completely cover the sample (V = 400 µL) was added. Cells were incubated at 37°C for 20 minutes and subsequently visualized by fluorescence microscopy.

In order to quantify the survival of cells, a live/dead analysis was performed using fluorescence microscopy. Human bone marrow-derived mesenchymal stem cells (hBM-MSCs) were seeded on top of the gels prepared by the previously established procedure, incubated for one day, and subsequently stained by two fluorescent dyes, calcein-AM and propidium iodide (PI). After a short-term culture, the hydrogel material showed a minimal negative effect on cell viability, as there are more live cells (green dye) than dead cells (red dye) as shown in Figure 4. Quantification of the number of live cells per unit macroscopic field gave a mean of 91.0 ± 1.0%, among the three repetitions of the experiment.

To follow the change in cell number, staining with 4',6-diamidino-2-phenylindole (DAPI) at different time points, followed by fluorescence imaging was employed, as the dye strongly interacts with AT-rich regions of DNA within the nuclei. Visualization of cells within the population showed an increase in cell density over a period of five days, indicating that the material is suitable for cell survival and division. Furthermore, staining of the cytoskeleton with tetramethylrhodamine (TRITC)-phalloidin revealed that the cells do not display the rounded morphologies typical of apoptosis (data not shown), suggesting that the hydrogel chemistry is not intrinsically toxic.

Overall, the outcome of these *in vitro* cytotoxicity tests (cell viability and cellular proliferation) indicates cytocompatibility of the hydrogels.
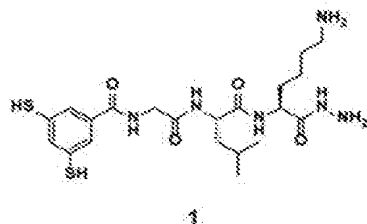
5   **EXAMPLE 5: Post-assembly functionalization**

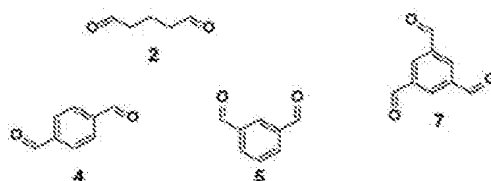**Incorporation of Cell Adhesion Motives Into Fibrillar Scaffold**

As shown in the previous examples, a hydrogel based on fibers of self-
10   assembled building blocks **1** shows sufficient mechanical stability to support cells, as well as the desired stability towards degradation by growth media, and is non-toxic. A further important aspect for application of the hydrogel in cell and tissue engineering relates to an adequate interaction between the hydrogel and cells.

15   This example illustrates the surprising finding that it is possible to incorporate biologically relevant ligands into the material, by post-assembly functionalization of peptide-based (nano)fibers and their subsequent hydrogelation. The known cell adhesion peptide motifs RGD and LDV were used as exemplary biologically active compounds for post-assembly
20   "decoration" of nanofiber assemblies. Scheme 1 shows the chemical structures of the components used. This "on-demand" approach to provide a chemically customized fibrillar scaffold is advantageously used to promote a specific cell response.

5

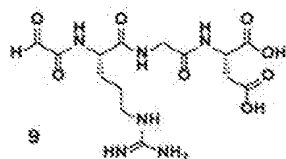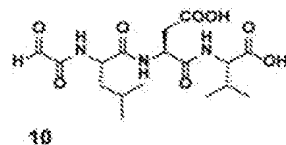Scheme 1: Exemplary Dithiol pseudo-peptide building block (1) used for fiber formation; exemplary di- and trialdehydes used for cross-linking of 1_5 fibers for hydrogel formation: glutaraldehyde (2), terephthaldehyde (4), isophthaldehyde (5), and benzene $-1,3,5-$ tricarboxaldehyde (7); carbonyl-

10   functionalized bioactive peptides: glyoxylyl-RGD (9) and glyoxylyl-LDV (10), and corresponding acyl-hydrazone adducts 11 and 12, represented as a part of a macrocycle.

Functionalization of Fibers with Cell Adhesion Signals and Their Hydrogelation

Supramolecular, self-assembled fibrous structure (1_5) was obtained as
described herein above. After UPLC/MS analysis confirmed that DCLs are
dominated by 1_5 (Figure 5a), the preformed assembly was split into two and
reacted with 10 mol% of glyoxylyl-RGD and glyoxylyl-LDV. Upon short-term
stirring (30 min), UPLC traces of the two libraries showed partial covalent
modification of 1_5 (Figure 5b-c). The reaction appeared to be finished within
30 minutes, as a further change in library composition was not observed
even upon prolonged stirring (20 h; data not shown).

In general, incorporation of both carbonyl-functionalized peptide
sequences led to the formation of mixed self-assemblies ($1_4 1 1$, $1_3 1 1_2$, and
$1_4 1 2$, $1_3 1 2_2$, $1_2 1 2_3$), while 1_5 also remained present. As remaining hydrazide
groups ought to participate in hydrogel formation, partial covalent
modification is desired. Atomic force micrographs of DCLs dominated by
pentamer species functionalized with biologically relevant peptides
demonstrate the presence of fibers, similar to the original library composed
of 1_5 (data not shown).

Next, the possibility for customized, functionalized fibers to undergo
hydrogelation upon the addition of a cross-linker was investigated. Vial-
inversion tests indicate gel formation in 2 minutes, for fibers functionalized
with either glyoxylyl-RGD (Figure 5d) or glyoxylyl-LDV (Figure 5e).

These data show that acyl-hydrazone chemistry allows for a quick and
efficient customization of (nano)fibers by biologically relevant ligands, as
well as their hydrogelation. It demonstrates the unique capacity of the
"post-assembly" decorated peptide-based (nano)fibers of the invention to
undergo hydrogelation. Apparently, the addition of a functional group of
interest does not interfere with the hydrogelation induced by cross-linking.

This approach can find application in the fabrication of versatile materials by simple "decoration" of the fibrillar scaffold.

EXAMPLE 6: Application in cell culture

This example describes the characterization of spreading and organization of the actin cytoskeleton in order to evaluate the effectiveness of hydrogel materials functionalized with RGD or LDV. Furthermore, to assess whether the presence of signaling peptides has an effect on cell viability, live/dead staining was performed on cells in the presence of material based on pseudo-peptide 1_5 and functionalized with glyoxylyl-RGD or glyoxylyl-LDV. For identifying the influence of these peptide motifs on the morphology of the cells, hBM-MSCs were cultured on modified substrates and allowed to attach/spread for 1 day prior to visualization.

Live/Dead Viability Assay of Cells Cultured on hydrogels functionalized with Cell Adhesion Signals

Cytotoxicity of hydrogel material based on 1_5-RGD or 1_5-LDV was evaluated as described herein above. Cells were seeded on the hydrogels and visualized after a one day incubation. The green (live) and red (dead) cells (Figure 6a-c) were manually counted in the three random domains per sample (n=3) per type of material. The results are shown in Figure 6d, demonstrating a high viability of cells in all cases.

Influence on Cell Morphology

To determine the effect of the RGD and LDV cell adhesion motifs on the morphology of human bone marrow-derived mesenchymal stem cells, the cells were cultured for one day on the original material based on either the non-functionalized 1_5 hydrogels, or on the functionalized substrates 1_5-RGD and 1_5-LDV. After that, cells were fluorescently labelled to visualize their

nuclei (DAPI) and cytoskeleton (FITC), (Figure 7a-i). From fluorescent imaging of the cytoskeleton (Figure 7) it was observed that hBM-MSCs exhibit extended morphologies on all three types of materials, without significant discrepancies. The absence of notable effect of cell adhesion

5   peptides on cell morphology, but also of cells with rounded morphology on the original substrate, indicate that peptide sequence of 1, GLK, on its own promotes cell spreading. To the best of our knowledge, this peptide sequence has not been reported as a cell adhesion motif (naturally or synthetically derived) thus far.

10

EXAMPLE 7: Post-assembly functionalization and hydrogel formation of functionalized nanofibers prepared from mixed pseudopeptide building blocks.

15   This example describes DCLs of mixed building blocks (A-Peptide-B and A-Peptide) that are capable of being modified, and then induces to undergo gel formation via dialdehyde cross-linking.

General procedure of dynamic combinatorial library (DCL)
20   **formation:**

A fiber forming solution was prepared by dissolving a mixture of building blocks A-Peptide-B and A-Peptide (see table 1) were dissolved in 12.5 mM in $B_4O_7^{2-}$ borate buffer in different predetermined molar ratios, so that the total building block concentration was 4.0 mM. The pH was adjusted to 8.2

25   with 1.0 M NaOH, and the DCLs were left stirring at 1200 rpm for 20 days at 32°C.

Table 1. Conditions and detected species of dynamic combinatorial libraries of mixed building blocks.

Example	Building block #1 A-Peptide-B	Building block #2 A-Peptide	Molar Ratio	Species formed
A	XGLK-NHNH$_2$ (1)	XGLKFK (16)	3:1, 1:1, 1:3	$1_1 16_2, 1_2 16_1, 1_2 16_2, 1_2 16_3, 1_1 16_4$
B	XGLK-NHNH$_2$ (1)	XGLKAK (17)	3:1, 1:1, 1:3	$1_2 17_2, 1_1 17_2, 1_2 17_1, 1_1 17_7, 1_2 17_6, 1_3 17_5, 1_4 17_4$
C	XGLK-NHNH$_2$ (1)	XGSK (18)	3:1	$1_1 18_3, 1_2 18_2, 1_2 18_3, 1_3 18_2, 1_4 18_1, 1_1 18_2, 1_2 18_1$
D	XGLK-NHNH$_2$ (1)	X(p-NH$_2$-F)K (19)	1:1	$1_1 19_3, 1_2 19_2, 1_3 19_1, 1_1 19_2, 1_2 19_1$
E	XGLK-NHNH$_2$ (1)	XK (20)	3:1	$1_1 20_3, 1_2 20_2, 1_3 20_1, 1_3 20_2, 1_4 20_1, 1_1 20_2, 1_2 20_1$
F	XGLK-NHNH$_2$ (1)	XK-NHNH$_2$ (21)	1:1	$1_1 21_3, 1_2 21_2, 1_3 21_1, 1_1 21_2, 1_2 21_1$
G	XGIK-NHNH$_2$ (13)	XGLKFK (16)	1:1	$13_2 16_2, 13_1 16_3, 13_2 16_3, 13_1 16_4, 13_2 16_1, 13_1 16_2$
H	XGAK-NHNH$_2$ (14)	XGLKFK (16)	1:1	$14_2 16_2, 14_2 16_1, 14_1 16_3, 14_2 16_3, 14_1 16_5, 16_6$
I	X(p-NH$_2$-F)K-NHNH$_2$ (15)	-		15_6

5

Scheme 2. Structures of C-terminal hydrazide building blocks A-Peptide-B (**13-15**) and C-terminal acid building blocks A-Peptide (**16-20**) that formed gels upon cross-linking with dialdehyde **4**.
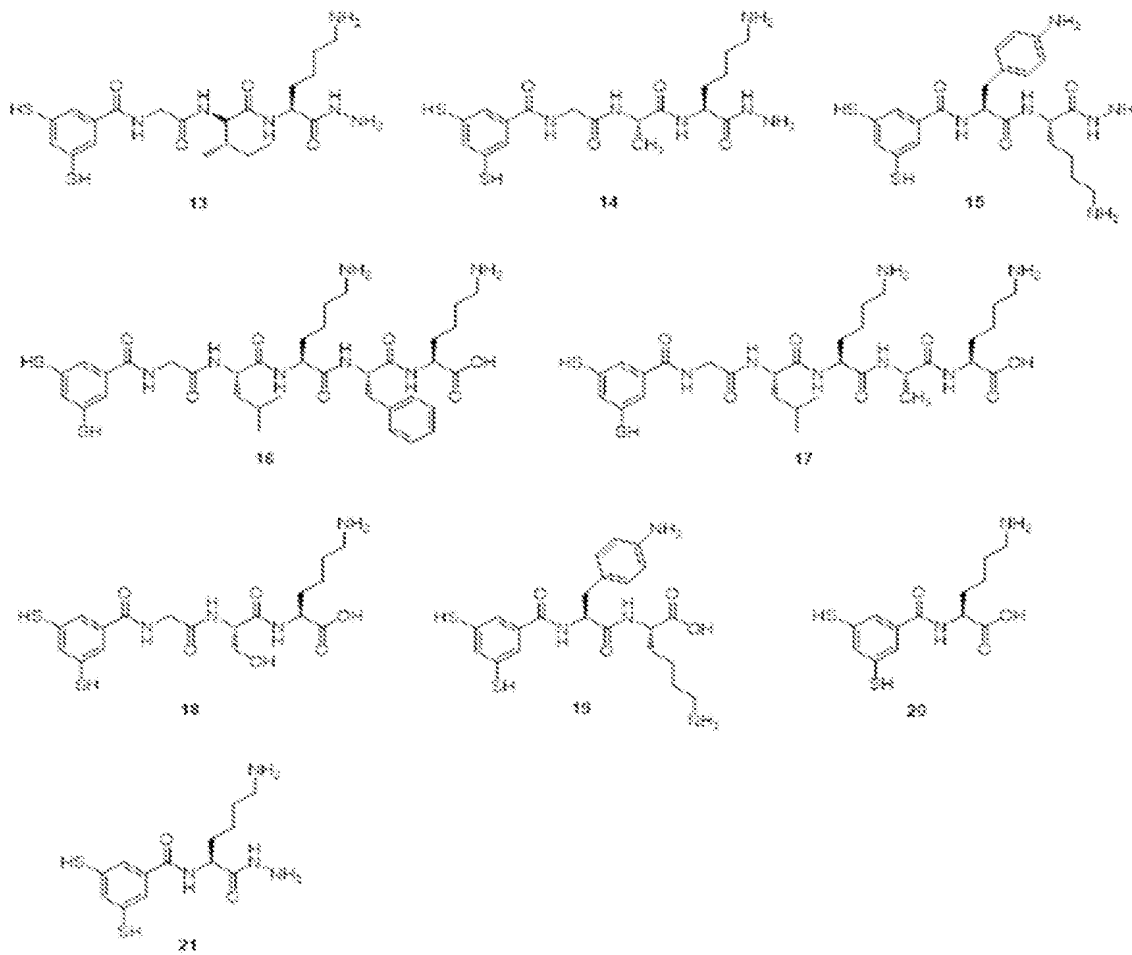
Scheme 3. (4-Formylbenzoyl)-IPP peptide (**22**) and acyl-hydrazone adducts (**23-26**):
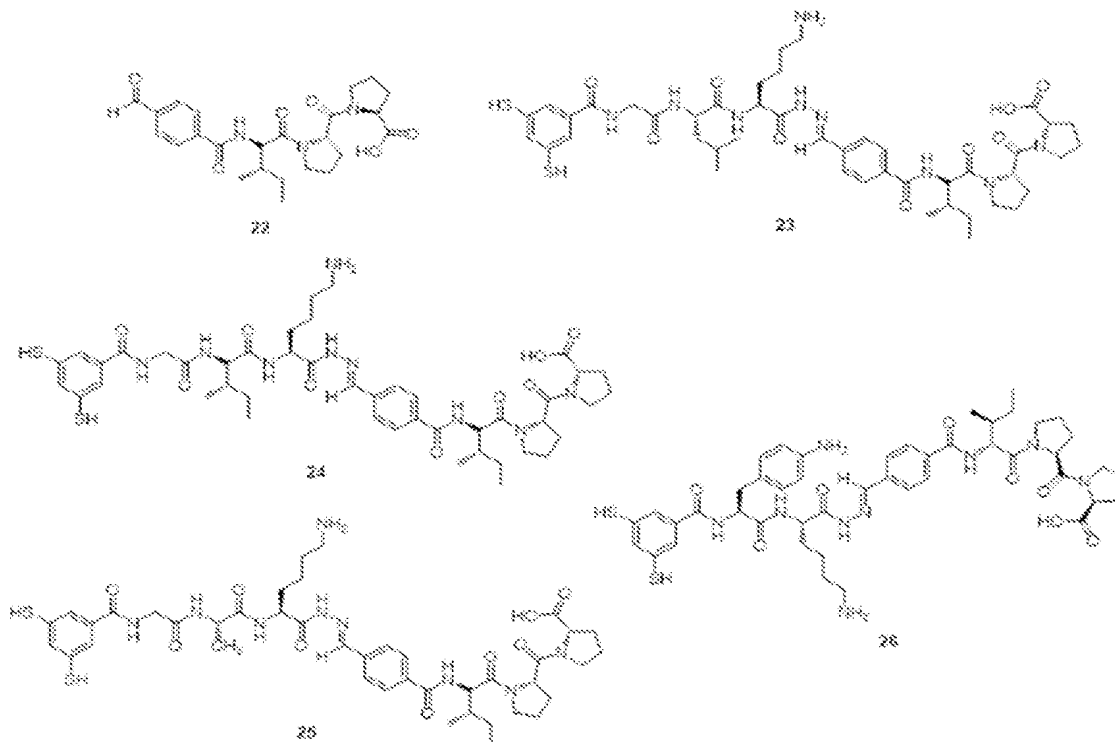
5

General procedure of post-assembly functionalization of fibers from a mixed building block system with biologically active peptide IPP.

Self-assembly of a mixed system of building blocks was obtained according to the general procedure. When the macrocycles interconversion was

10   completed and the DCL composition is stable for several days, 10 mol. % of IPP derivative **22** (4-formylbenzoyl-IPP) was added. The tripeptide Ile-Pro-Pro (IPP) is implicated among others in osteoblast differentiation, which is relevant for biomineralization and bone formation.

The DCL was stirred for 30 minutes at 1200 rpm, and the appearance of

15   macrocycles containing acyl-hydrazone adducts **23-26** (incorporated into the disulfide macrocycles) was confirmed by UPLC/MS. Hydrogels from the IPP-

functionalized samples were obtained according to the general procedure of a hydrogel preparation.

Results

The UPLC traces Figure 8 show the formation of new mixed species between building blocks and their development in time (day 0, 10 and 20) of DCL from representative example 7A. By day 20, the DCL is dominated with mixed pentamer species ($1_2 16_3$ and $1_1 16_4$) that form fibers. Upon functionalization of fibers with addition of biologically active substance **22**, the intensity of $1_2 16_3$ and $1_1 16_4$ peaks decreased. At the same time, the appearance of new broad peaks $1_3 16_1 23$, $1_2 16_2 23$ and $16_4 23$ in area 8.4-8.9 minutes were observed. These peaks are adducts of chemical interaction between **22** and pentamer species $1_2 16_3$, $1_3 16_2$ and $1_1 16_4$, providing evidence of covalent post-modification of fibers.

Table 2 shows the elution times of the formed species after 20 days of stirring of the 1:1 mixture at 1200 rpm.

Table 2

Species	UPLC elution time, min	UPLC/MS elution time, min
1_1	6.44	6.88
16_1	7.80	8.28
$1_2 16_2$	7.77	8.09
$1_2 16_3$	7.91	8.35
$1_1 16_4$	8.02	8.46
$1_2 16_1$	8.28	8.72
$1_1 16_2$	8.41	8.85
16_3	8.58	9.03
IPP-functionalized macrocycles		
$1_3 16_1 23$	8.15	8.60
$1_2 16_2 23$	8.26	8.71

$1_1 16_2 23$	8.32	8.77
$1_1 16_3 23$	8.38	8.82
$16_4 23$	8.45	8.91

The transmission electron microscopy (TEM) picture of Figure 9A demonstrates the fibers formed in DCL from example 7A (after functionalization with IPP and before cross-linking).

Essentially the same results were obtained using the 3:1 or 1:3 mixture of building blocks **1** and **16**, and for the other mixed building block systems of Examples 7B-7H (data not shown).

Example 7I concerns a fiber forming solution with only A-Peptide-B building blocks **15**, wherein Peptide is dipeptide (p-NH2)-F-K, comprising a hydrazide B-moiety. This building block forms fibers, can be functionally modified e.g. with IPP or RGD peptides, and can form a gel (data not shown).

Example 8: Hydrogel formation of functionalized nanofibers prepared from mixed building blocks.

In this example, the IPP-functionalized nanofibers according to examples 7A-7G were cross-linked to undergo hydrogelation. To that end,

5   functionalized fiber solutions were cross-linked with dialdehyde **4**, giving a firm elastic material, which sticks to the walls of a glass vial. Non-functionalized nanofibers were included as control to investigate the effect of post-assembly modification on gelation.

As is shown in Figure 9B, non-functionalized as well as IPP-functionalized

10   nanofibers of representative example 7B gave rise to a solid gel. The same results were obtained using the other mixed building block systems (data not shown).

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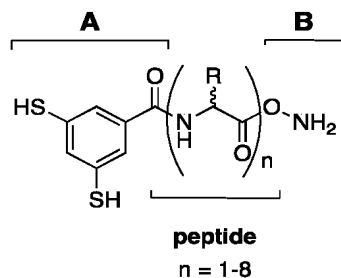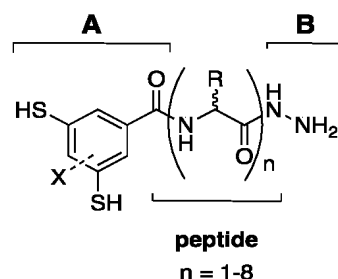
<u>Claims</u>

1.   A method for providing peptide-based functionally modified
      (nano)fibers, comprising the steps of :

(i)   providing a fiber forming solution comprising pseudopeptide building
      blocks of the formula A-Peptide-B, wherein:
            Peptide is a moiety of 1 to 8, preferably 3 to 5, amino acid residues
      having a sequence that is predisposed to form a one-dimensional array,
      such as β-sheet fibrils;
            A is an aromatic moiety carrying two reactive thiol groups; and
            B is a reactive α-nucleophile;

(ii) exposing the fiber forming solution to oxidizing conditions to induce
      supramolecular self-assembly of the pseudopeptide building blocks into
      peptide-based (nano)fibers; and

(iii) contacting said (nano)fibers with at least one biologically relevant
      functional group of interest comprising reactivity C forming a reactive
      pair with B to obtain covalently functionally modified nanofibers.

2.   Method according to claim 1, wherein A comprises a 5- or 6-membered
      thiol-substituted aromatic ring, preferably a thiol-substituted benzene.

3.   Method according to claim 1 or 2, wherein B is or comprises a -NH-NH$_2$
      or a -O-NH$_2$ functionality, preferably wherein B is -NH-NH$_2$.

4.   Method according to claim 3, wherein the pseudopeptide building block
      is of the formula I, II, III or IV
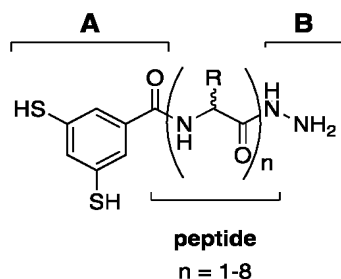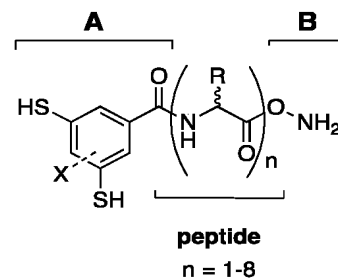
Formula I   Formula II

Formula III   Formula IV

wherein R is any natural or non-natural amino acid side chain

X is halogen, preferably fluorine, or C_1-C_3 alkyl,

preferably CH_3.

5.   Method according to any one of the preceding claims, wherein the fiber forming solution further comprises pseudopeptide building blocks of the formula A-Peptide, preferably wherein the fiber forming solution comprises a mixture of A-Peptide-B and A-Peptide in a molar ratio of 4:1 to 1:4, more preferably 3:1 to 1:3.

6.   Method according to any one of the preceding claims, wherein the Peptide moiety comprises alternating hydrophobic and hydrophilic amino acid residues.

7. Method according to any one of the preceding claims, wherein the Peptide moiety comprises or consists of the formula

(i) Xaa1-Xaa2-Xaa3, wherein

Xaa1 is Gly, Ala, Val, Ile or Phe, preferably Gly;

Xaa2 is Gly, Ala, Val, Ile or Phe; and

Xaa3 is Lys, Arg or His, preferably Lys;

(ii) Xaa1-Xaa2-Xaa3-Xaa4-Xaa5-Xaa6, wherein

Xaa1 is Gly, ß-Ala or γ-aminobutyric acid (GABA)

Xaa2 is Ala, Val, Leu, Ile or Phe

Xaa3 is Lys, Arg or His, preferably Lys

Xaa4 is Ala, Val, Leu, Ile or Phe

Xaa5 is Lys, Arg or His, preferably Lys

Xaa6 is Asp, Glu, or absent.

8. Method according to any one of the preceding claims, wherein reactivity C comprises a carbonyl-functionality, preferably an aldehyde or glyoxylyl group.

9. Method according to any one of the preceding claims, wherein the biologically relevant functional group of interest is a biologically active peptide, preferably selected from the group consisting of growth factors, differentiation factors, cytokines, chemokines, cell adhesion ligands, or wherein the biologically relevant functional group of interest is or comprises a protease-degradation site, such as a substrate for matrix metalloproteinases (MMPs).

10. Method according to any one of the preceding claims wherein step (iii) comprises contacting said (nano)fibers with two or more different biologically relevant functional groups of interest, either sequentially or simultaneously.

11. A method for providing a peptide-based functionalized hydrogel, comprising providing peptide-based functionally modified (nano)fibers according to a method of any one of the preceding claims, followed by adding a crosslinker comprising a plurality of reactive groups D capable of reacting with B to induce hydrogelation of the functionally modified (nano)fibers.

12. Method according to claim 11, wherein reactive group D comprises a carbonyl-functionality, preferably an aldehyde, more preferably wherein the crosslinker is a di- or trialdehyde, most preferably selected from the group consisting of glutaraldehyde, 2,5-thiophenedicarboxaldehyde, terephthaldehyde, isophthaldehyde, succinaldehyde, substituted versions of benzene-1,3 and 1,4 biscarboxaldehydes, and benzene- 1,3,5-tricarboxaldehyde.

13. Peptide-based functionally modified (nano)fibers obtainable by a method according to any one of claims 1-10.

14. A peptide-based functionalized hydrogel obtainable by a method according to claim 11 or 12.

15. The use of peptide-based functionally modified (nano)fibers according to claim 13 and/or a peptide-based functionalized hydrogel according to claim 14 in tissue engineering.

16. A method for tissue engineering, comprising contacting cells with a peptide-based functionalized hydrogel according to claim 14.

17. A composition comprising (i) peptide-based functionally modified (nano)fibers according to claim 13 and/or a peptide-based functionalized hydrogel according to claim 14 and (ii) cells, preferably mammalian cells.

18.   A tissue-engineering scaffold, comprising:

(a) a microporous scaffold comprising a biocompatible material suitable for use in tissue-engineering scaffolds, wherein said biocompatible material is a biocompatible polymer that comprises a biodegradable polymer; and

5   (b) a peptide-based functionalized hydrogel according to claim 14.

19.   A kit-of-parts for use in a method of claims 1-12, comprising

(i) pseudo-peptide building blocks of the formula A-Peptide-B, wherein Peptide comprises 1 to 8, preferably 3 to 5, amino acid residues; A is an

10   aromatic moiety carrying two thiol groups capable of undergoing reversible thiol-disulfide exchange; B is a reactive α-nucleophile; and

(ii)   one or more functional group(s) of interest comprising reactivity C capable of reacting with B.

15   20.   A kit-of-parts for providing a functionalized hydrogel, comprising

(i) peptide-based (nano)fibers comprising moiety B being a reactive α-nucleophile, the (nano) fibers being prepared from pseudo-peptide building blocks of the formula A-Peptide-B, wherein Peptide comprises 1 to 8, preferably 3 to 5, amino acid residues; A is an

20   aromatic moiety carrying two thiol groups capable of undergoing reversible thiol-disulfide exchange; and

(ii) one or more functional group(s) of interest comprising reactivity C capable of reacting with B.

25   21.   Kit-of-parts according to claim 19 or 20, wherein B is or comprises a -NH-NH$_2$ or a -O-NH$_2$ functionality, and wherein C comprises a carbonyl-functionality, preferably wherein C is an aldehyde or glyoxylyl group.

22.   Kit-of-parts according to any one of claims 19-21, further
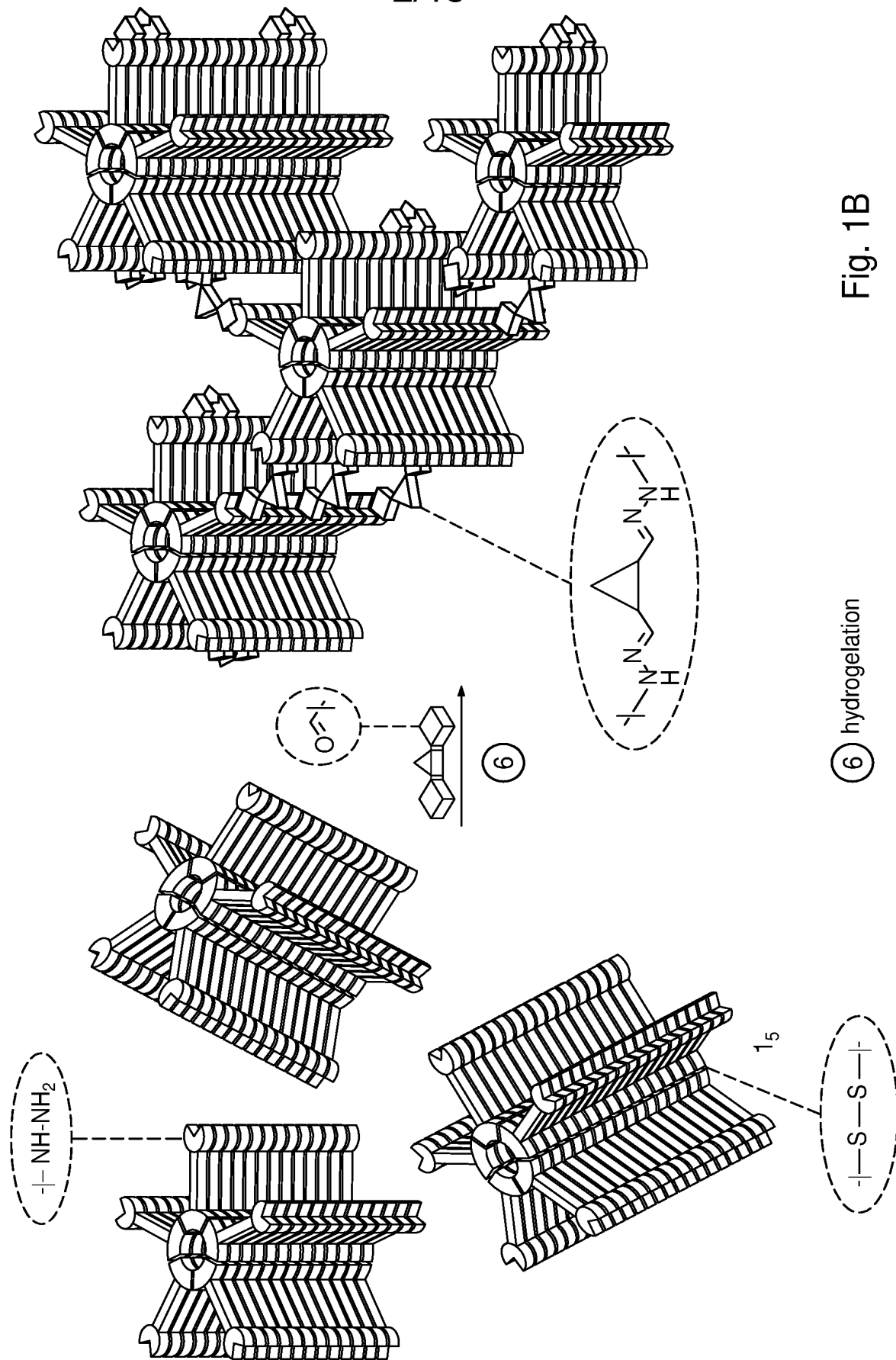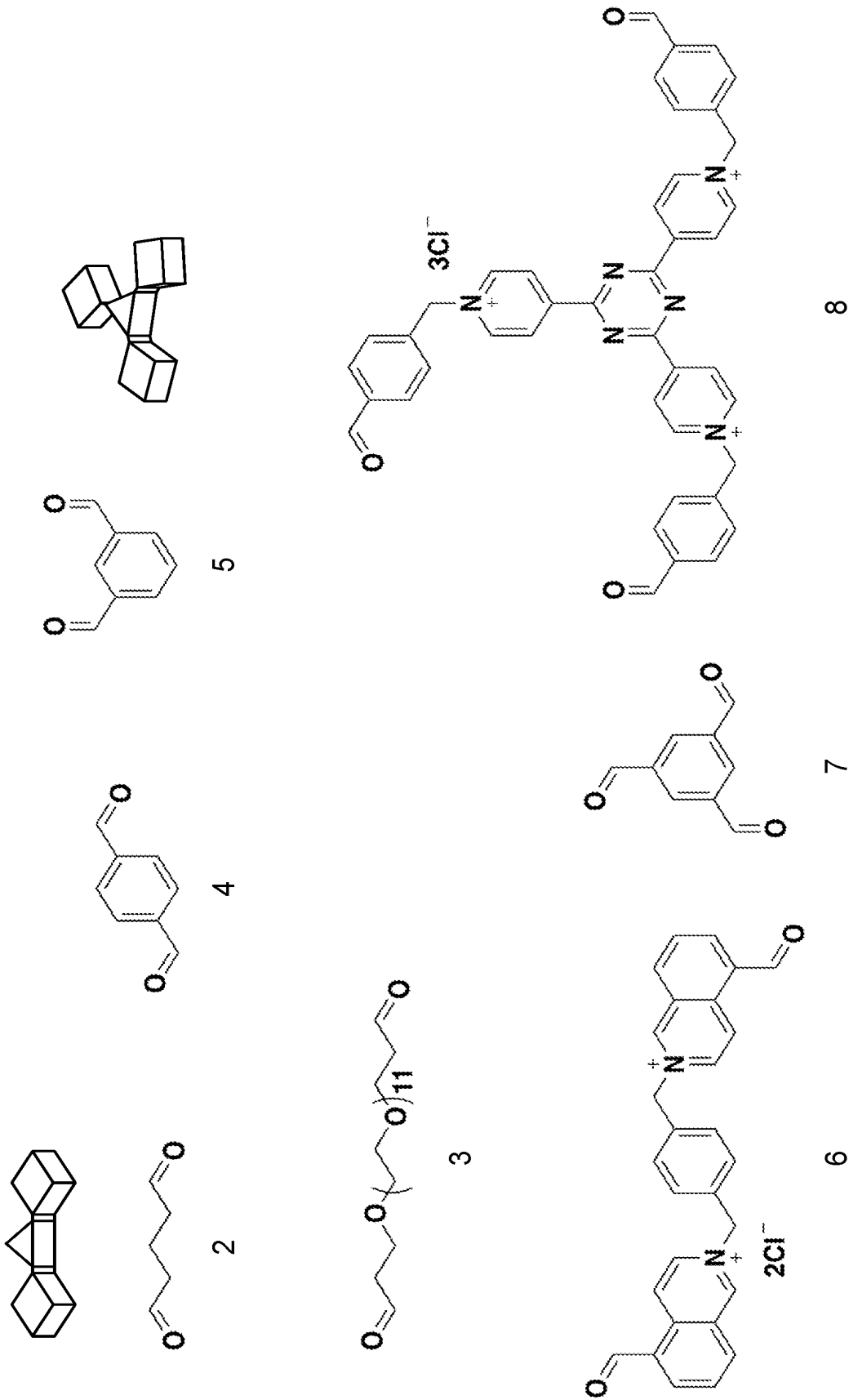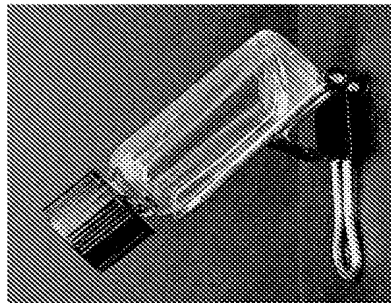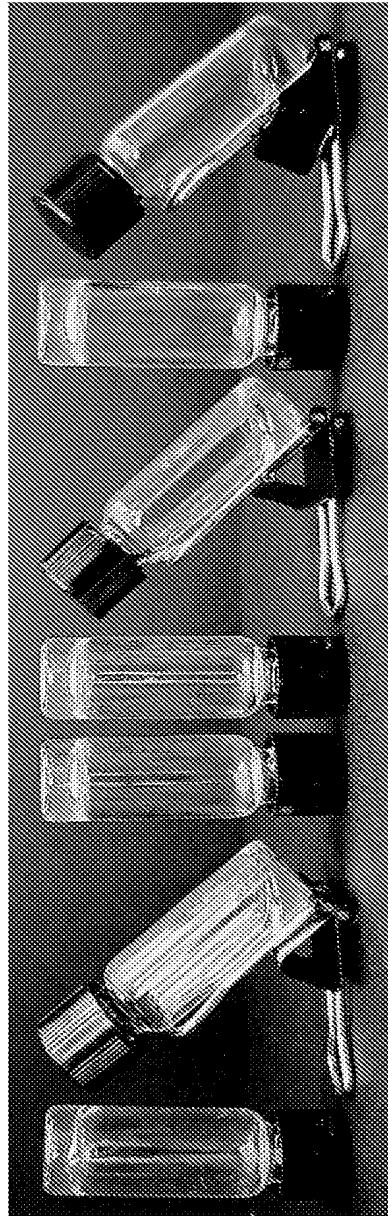
30   comprising a cross-linking agent.

Fig. 1A

Fig. 1B

Fig. 1C

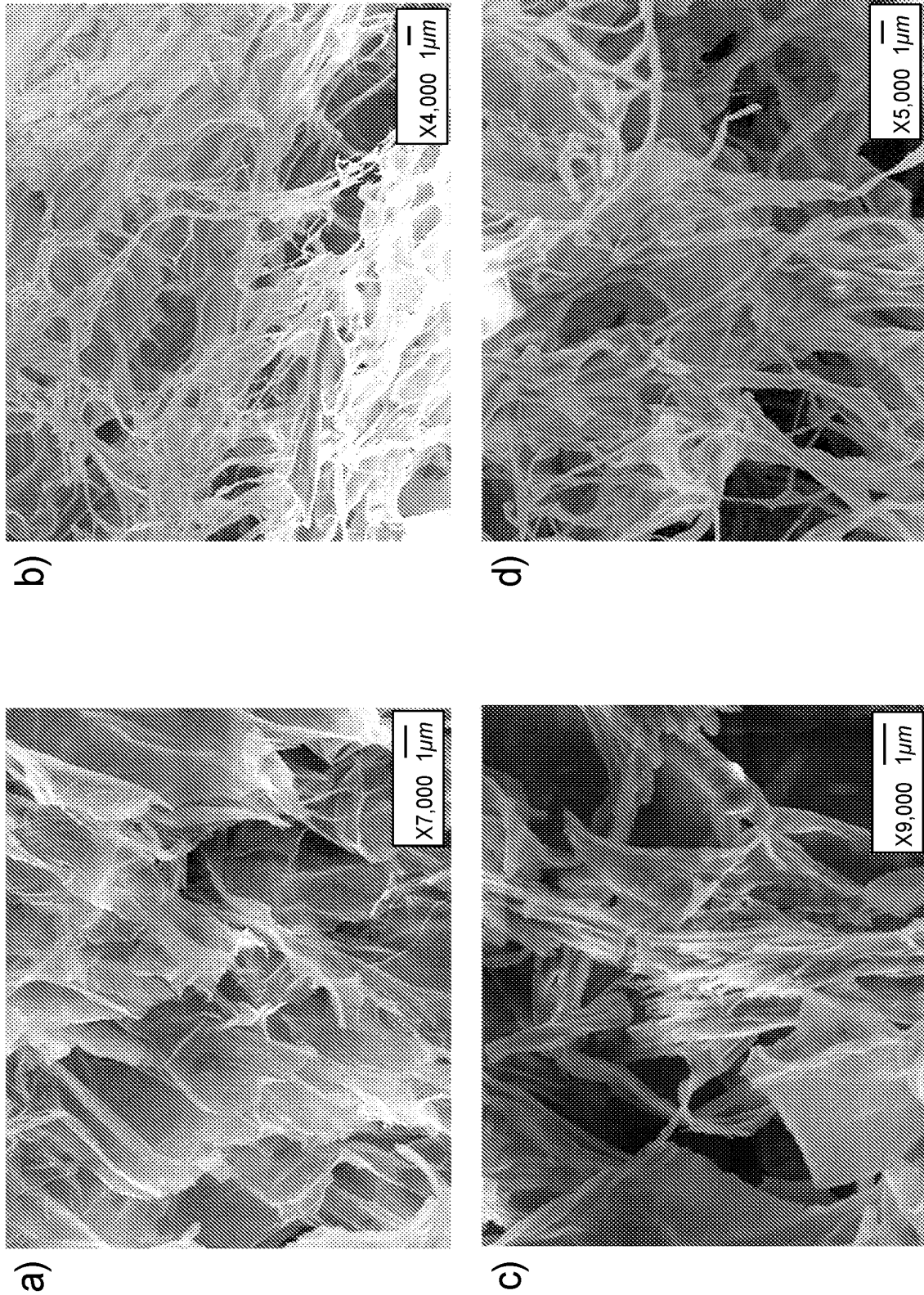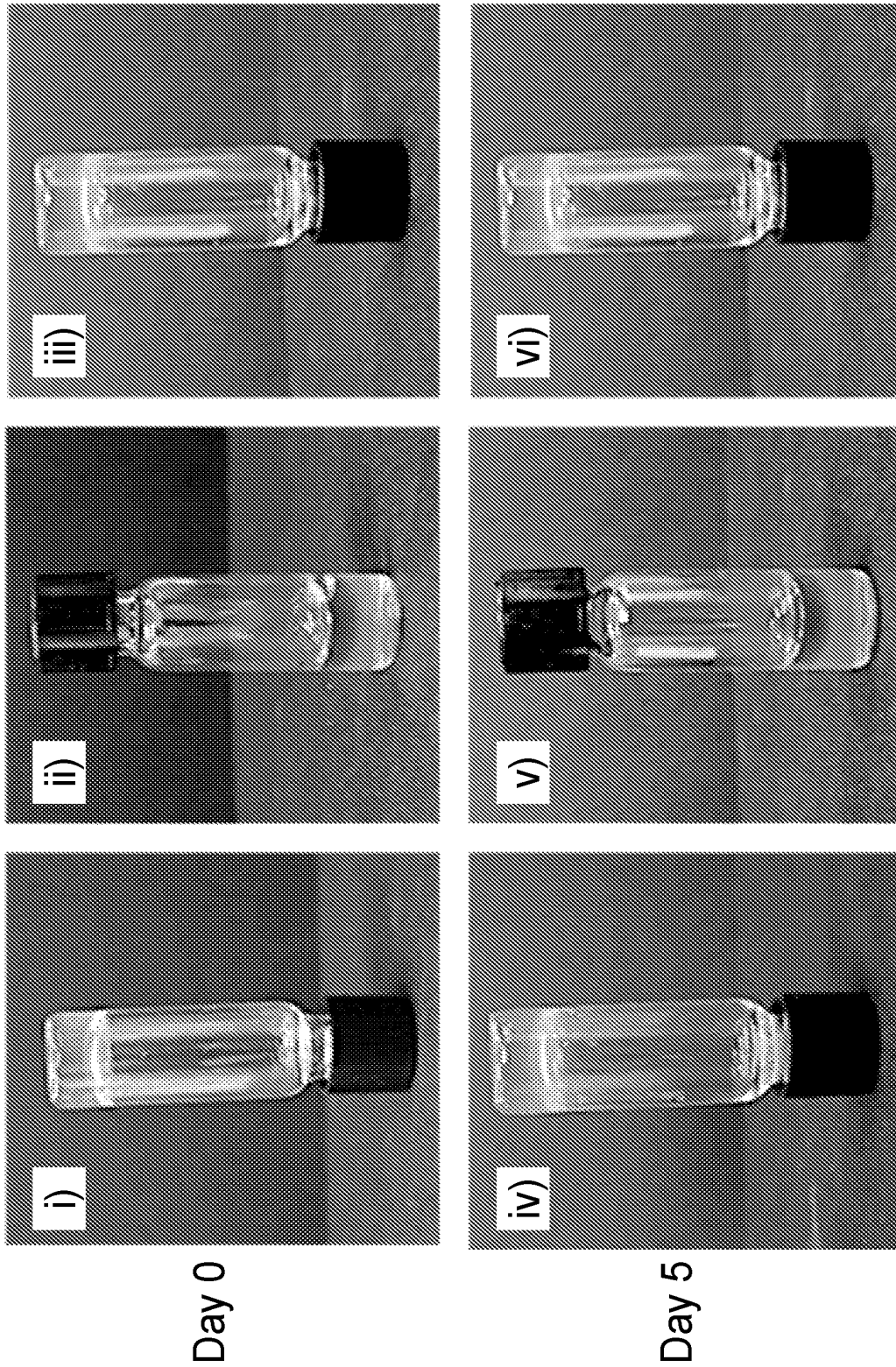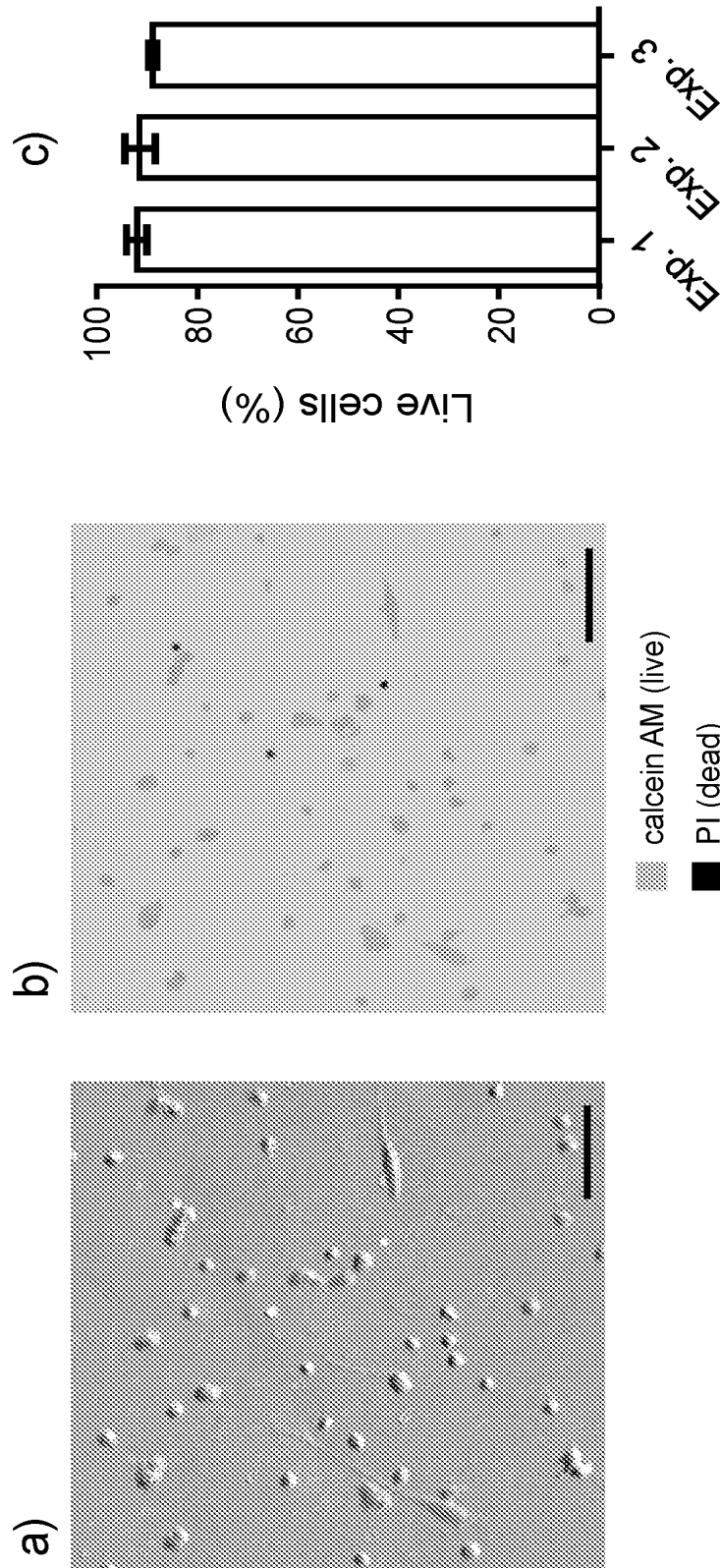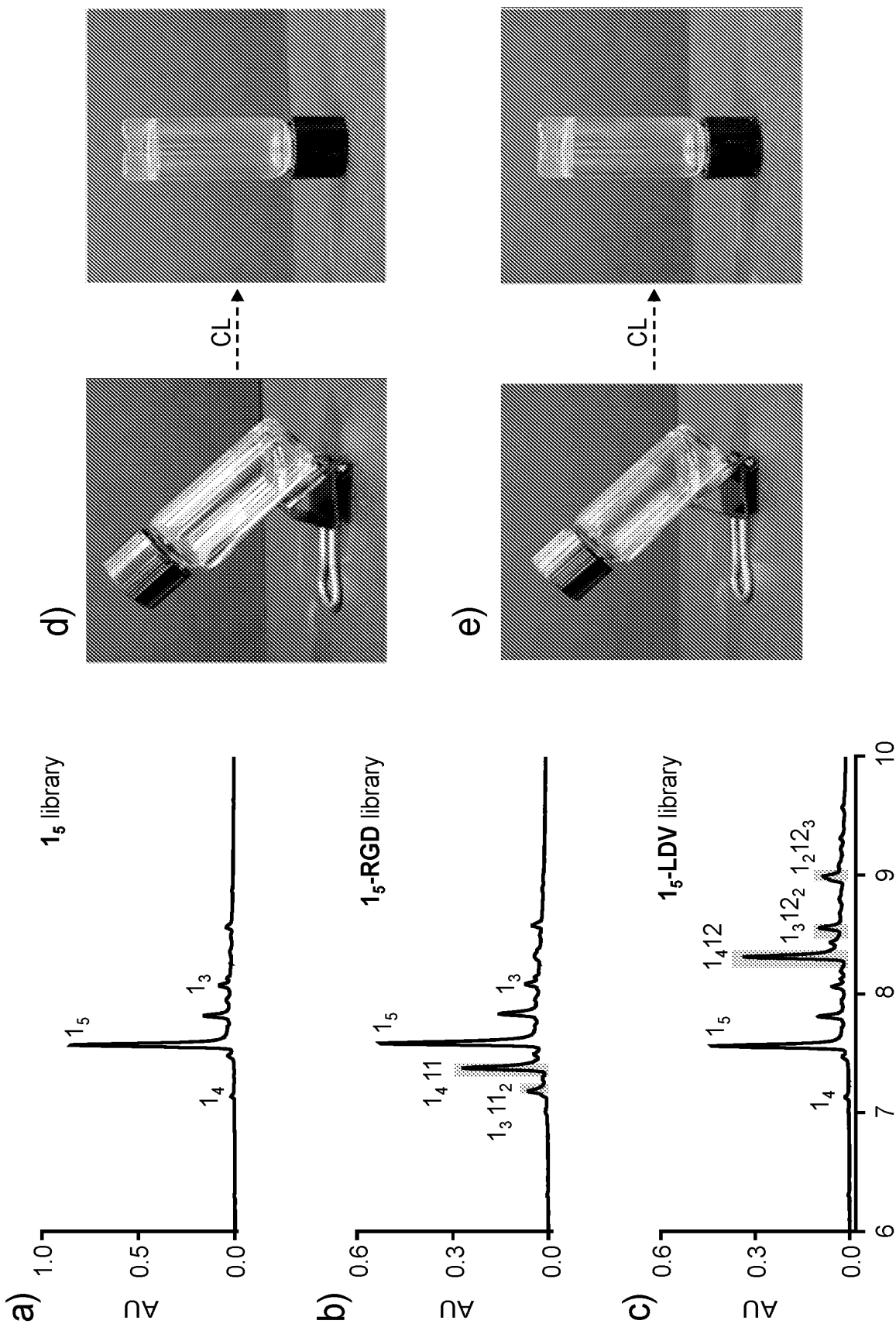| fiber solution	CL	2	3	4	5	6	7	8
eq. of CL		0.5	2	0.5	0.5	2	0.33	1.32
gelation time (min)		10	/	2	2	/	2	/

Fig. 1D

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Fig. 8

Hydrogel from non-functionalized nanofibers

Hydrogel from IPP functionalized nanofibers
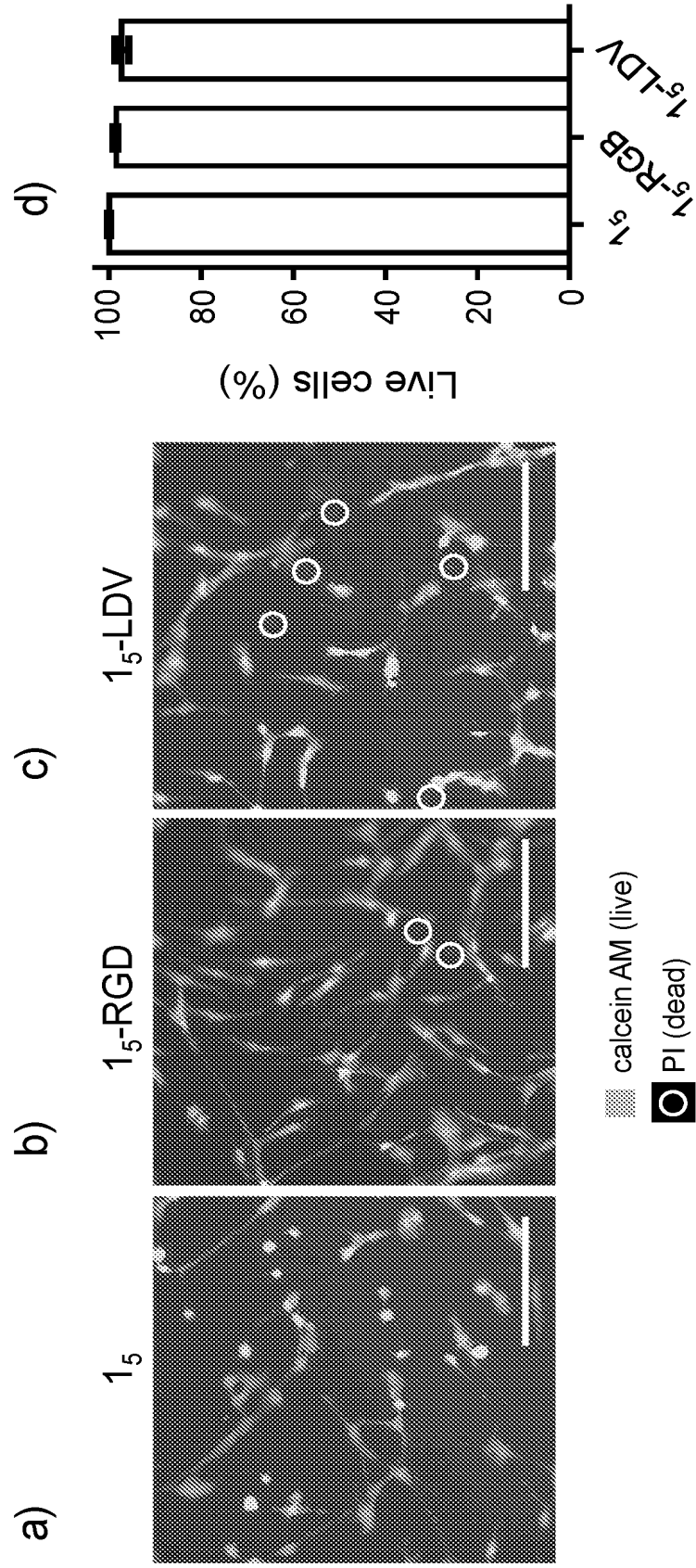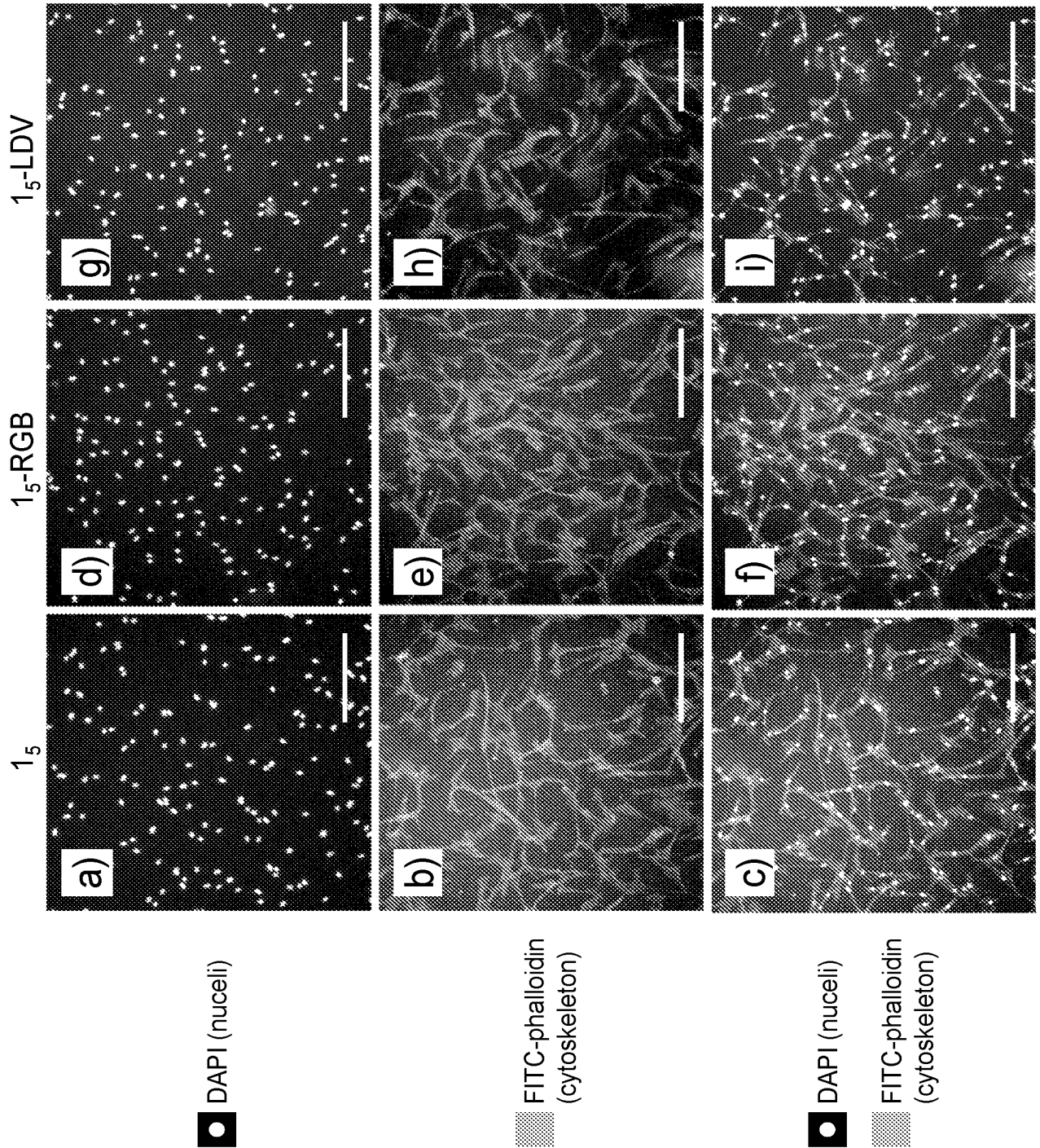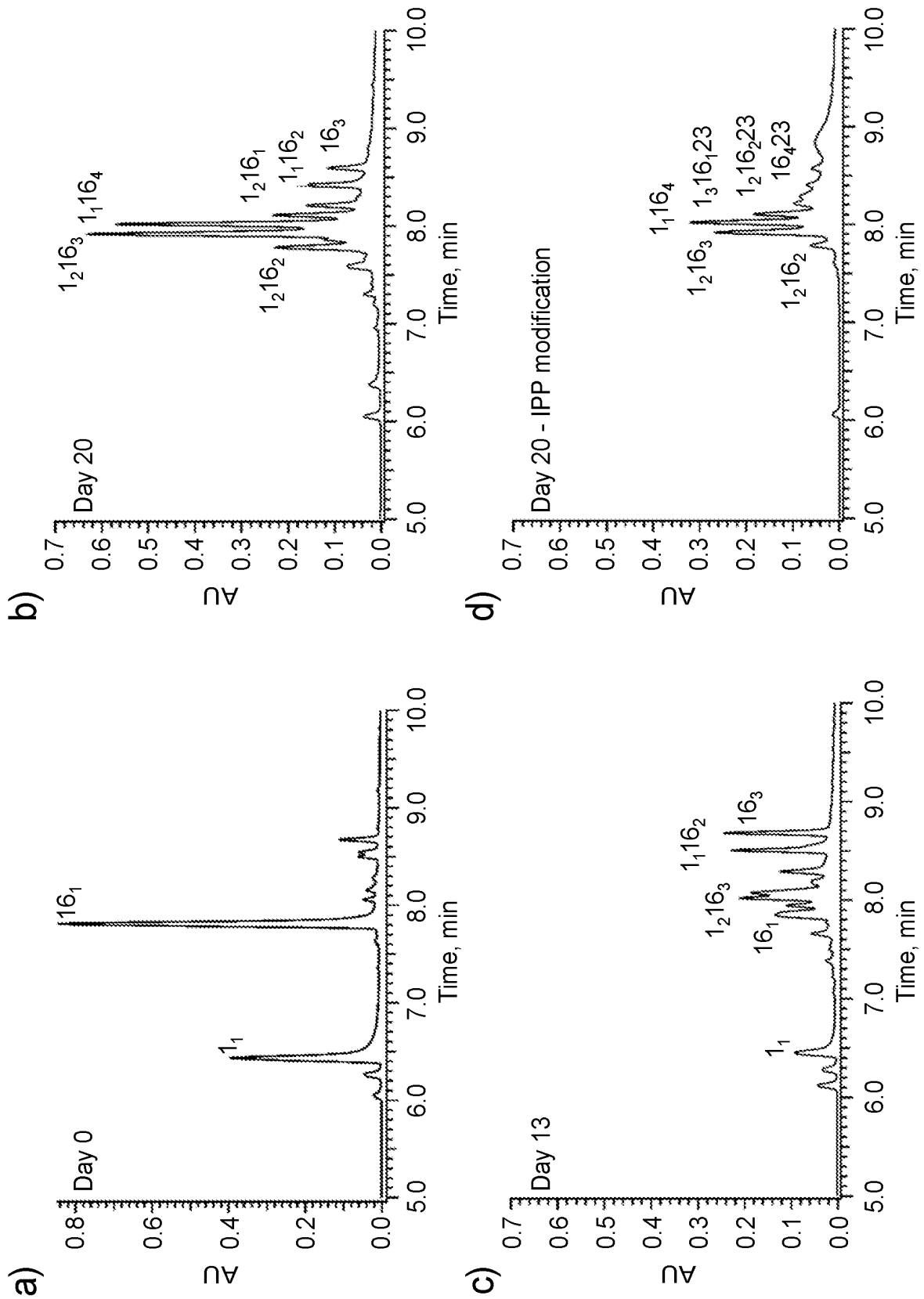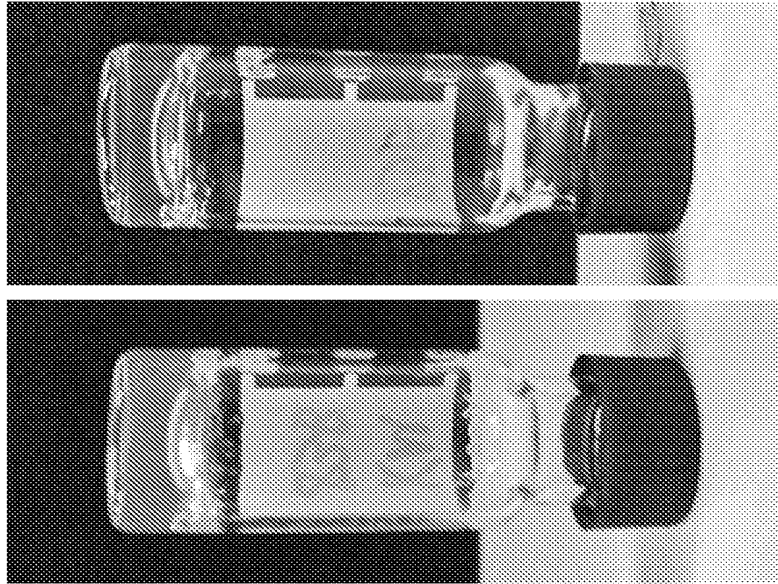
Fig. 9B

Fig. 9A

Fig. 10

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K5/00   A61K38/00   A61L27/52
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)
C07K A61L A61K

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, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NGUYEN VAN DUC ET AL: "Multi-step control over self-assembled hydrogels of peptide-derived building blocks and a polymeric cross-linker", SOFT MATTER (PRINT), vol. 12, no. 2, 12 October 2015 (2015-10-12), pages 432-440, XP055871333, GB ISSN: 1744-683X, DOI: 10.1039/C5SM02088C	1,2,5-15
Y	Abstract;Fig. 1	1-22

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[X] Further documents are listed in the continuation of Box C.

[X] 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

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"O" document referring to an oral disclosure, use, exhibition or other means

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"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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 July 2022	29/07/2022

| Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016 | Authorized officer López García, F |

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SOTIRIOS KOUTSOPOULOS: "Self-assembling peptide nanofiber hydrogels in tissue engineering and regenerative medicine: Progress, design guidelines, and applications : Self-Assembling Peptides in Tissue Engineering and Regeneration", JOURNAL OF BIOMEDICAL MATERIALS RESEARCH PART A, vol. 104, no. 4, 25 January 2016 (2016-01-25), pages 1002-1016, XP055491859, US ISSN: 1549-3296, DOI: 10.1002/jbm.a.35638 Abstract; Fig. 2, 3, 5 -----	1-22
Y	PRESTWICH G D ET AL: "Controlled chemical modification of hyaluronic acid: synthesis, applications, and biodegradation of hydrazide derivatives", JOURNAL OF CONTROLLED RELEASE, ELSEVIER, AMSTERDAM, NL, vol. 53, no. 1-3, 30 April 1998 (1998-04-30), pages 93-103, XP004121260, ISSN: 0168-3659, DOI: 10.1016/S0168-3659(97)00242-3 Abstract; Fig. 1,2 -----	1-22
A	WO 2014/039245 A1 (UNIV CALIFORNIA [US]; MAYNARD HEATHER D [US] ET AL.) 13 March 2014 (2014-03-13) Abstract; Fig. 1 -----	1-22
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Form PCT/ISA/210 (continuation of second sheet) (April 2005)

C(Continuation).   DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MENZIES DONNA J. ET AL: "Tailorable Cell Culture Platforms from Enzymatically Cross-Linked Multifunctional Poly(ethylene glycol)-Based Hydrogels", BIOMACROMOLECULES, vol. 14, no. 2, 11 February 2013 (2013-02-11), pages 413-423, XP055932948, US ISSN: 1525-7797, DOI: 10.1021/bm301652q Title; Abstract; Fig. 1;p. 415, right hand column, "Gelation", last paragraph -----	1-22
X	LIU CHANG ET AL: "Preparation and applications of peptide-based injectable hydrogels", RSC ADVANCES, vol. 9, no. 48, 9 September 2019 (2019-09-09), pages 28299-28311, XP055871511, GB ISSN: 2046-2069, DOI: 10.1039/C9RA05934B Title; Abstract; table 1; p. 28300, left hand column, penultimate sentence -----	1-22
X	MARIC IVANA: "The quest for function in systems with two dynamic covalent bonds: supramolecular self-assembly, self-replication and hydrogels for biomedical applications", UNIVERSITY OF GRONINGEN, 1 January 2021 (2021-01-01), pages 1-287, XP055932684, DOI: 10.33612/diss.167788912 Retrieved from the Internet: URL:https://pure.rug.nl/ws/portalfiles/portal/167788934/Complete_thesis.pdf> the whole document -----	1-22

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			WO 2014039245	A1	13−03−2014