(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
28 August 2003 (28.08.2003)

PCT

(10) International Publication Number
WO 03/070749 A2

(51) International Patent Classification[7]: C07K

(21) International Application Number: PCT/US03/04779

(22) International Filing Date: 18 February 2003 (18.02.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/357,228 15 February 2002 (15.02.2002) US

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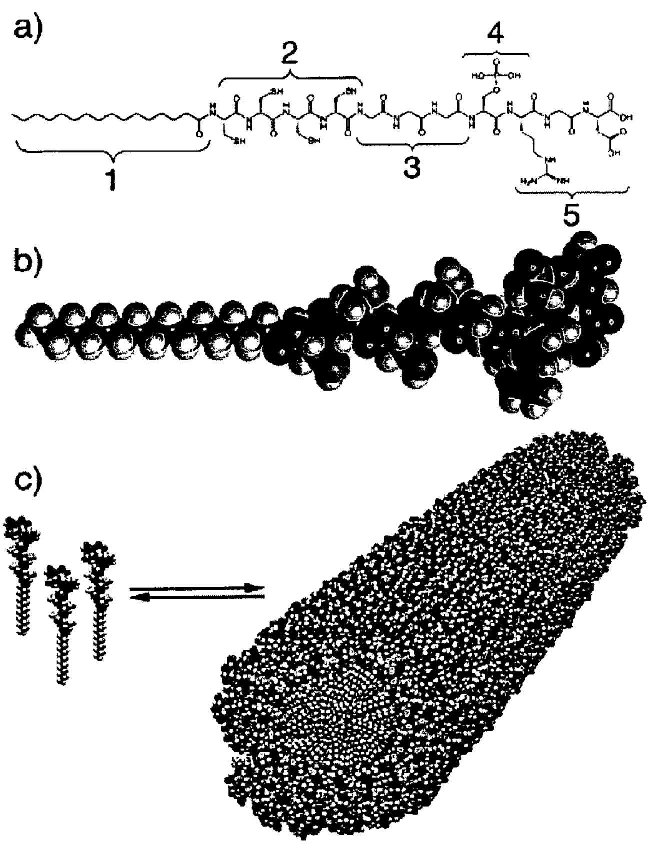
(81) Designated States (*national*): AU, CA, CN, JP.

(84) Designated States (*regional*): European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, SE, SI, SK, TR).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: SELF-ASSEMBLY OF PEPTIDE-AMPHIPHILE NANOFIBERS UNDER PHYSIOLOGICAL CONDITIONS



(57) Abstract: Peptide amphiphile compounds, compositions and methods for self-assembly or nanofibrous network formation under neutral or physiological conditions.

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SELF-ASSEMBLY OF PEPTIDE-AMPHIPHILE NANOFIBERS UNDER PHYSIOLOGICAL CONDITIONS

This application claims priority benefit from U.S. provisional application serial no. 60/357,228 filed February 15, 2002, the entirety of which is incorporated herein by reference.

The United States government has certain rights to this invention pursuant to Grant Nos. DE-FG02-00ER45810/A001, DMR-9996253 and F49620-00-1-0283/P01 from, respectively, the DOE, NSF and AFOSR-MURI to Northwestern University.

Background of the Invention.

Self-assembly and biomineralization are used for fabrication of many composite materials. Natural bone tissue is a particularly complex example of such a composite with multiple levels of hierarchical organization (S. Weiner, H. D. Wagner, *Annu. Rev. Mater. Sci.* **28**, 271-298 (1998)). At the lowest level of this hierarchy is the organization of collagen fibrils with respect to hydroxyapatite (HA) crystals. Collagen fibrils are formed by self-assembly of collagen triple helices while the HA crystals grow within these fibrils in such a way that their *c*-axes are oriented along the long axes of the fibrils (W. Traub, S. Weiner, *Proc. Nat. Acad. Sci.* **86**, 9822-9826 (1989)). The preparation of any material with structure on the nanoscale is a challenging problem. Fabrication of materials that resemble bone, even at the lowest level of hierarchical organization, is even more difficult because it involves two dissimilar organic and inorganic nanophases that have a specific spatial relationship with respect to one another. One approach, using an artificial system, has been to prepare an organic nanophase designed to exert control over crystal nucleation and growth of the inorganic component.

The controlled nucleation and growth of crystals from organic templates has been demonstrated in *in vitro* experiments and in a number of natural biomineralizing systems (S. Mann, J. P. Hannington, R. J. P. Williams, *Nature* **324**, 565-567 (1986); D. D. Archibald, S. Mann, *Nature* **364**, 430-433 (1993); S. L. Burkett, S. Mann, *Chem. Commun.* 321-322 (1996); S. I. Stupp, P. V. Braun, *Science* **277**, 1242-1248 (1997); J. Aizenberg, A. J. Black, G. M. Whitesides, *Nature* **398**, 495-498 (1999); S. R. Whaley, D. S. English, E. L. Hu, P. F. Barbara, A. M. Belcher, *Nature* **405**, 665-

668. (2000); L. Addadi, S. Weiner, *Angew. Chem., Int. Ed. Engl.* **31**, 153-169 (1992); S. Mann, *J. Chem. Soc., Dalton Tran.* 3953-3961 (1997); S. Weiner, L. Addadi, *J. Mater. Chem.* **7**, 689-702 (1997)). These studies on templated crystal growth suggest that nucleation occurs on surfaces exposing repetitive patterns of anionic groups. Anionic groups tend to concentrate the inorganic cations creating local supersaturation followed by oriented nucleation of the crystal. Many groups have investigated the preparation of bone-like materials using three dimensional organic substrates such as poly(lactic acid), reconstituted collagen and many others, and some studies shows a similar correlation between the crystallographic orientation of hydroxyapatite when the organic scaffold is made from reconstituted collagen(G. K. Hunter, H. A. Goldberg, *Biochem. J.* **302**, 175-179 (1994); G. M. Bond, R. H. Richman, W. P. McNaughton, *J. Mater. Eng. Perform.* **4**, 334-345 (1995); J. H. Bradt, M. Mertig, A. Teresiak, W. Pompe, *Chem. Mater.* **11**, 2694-2701 (1999); N. Ignjatovic, S. Tomic, M. Dakic, M. Miljkovic, M. Plavsic, D. Uskokovic, *Biomaterials* **20**, 809-816 (1999); F. Miyaji, H. M. Kim, S. Handa, T. Kokubo, T. Nakamura, *Biomaterials* **20**, 913-919 (1999); H. K. Varma, Y. Yokogawa, F. F. Espinosa, Y. Kawamoto, K. Nishizawa, F. Nagata, T. Kameyama, *J. Mater. Sci.: Mater. Med.* **10**, 395-400 (1999); A. Bigi, E. Boanini, S. Panzavolta, N. Roveri, *Biomacromolecules* **1**, 752-756 (2001); M. Kikuchi, S. Itoh, S. Ichinose, K. Shinomiya, J. Tanaka, *Biomaterials* **22**, 1705-1711 (2001)). However, such results have never been demonstrated in a pre-designed and engineered self-assembling molecular system.

Brief Description of the Drawings.

Figure 1. In accordance with this invention: a) Chemical structure of a preferred peptide amphiphile, highlighting one or more structural features thereof. Region 1 may comprise a long alkyl tail that conveys hydrophobic character to the molecule and combined with the peptide region makes the molecule amphiphilic. Region 2 may comprise one or more (four consecutive, shown) cysteine residues which when oxidized may form disulfide bonds to provide a desired robust self-assembled structure. Region 3 may comprise a flexible linker region of one or more glycine residues, preferably three, or functionally similar such residues or monomers, to provide the hydrophilic head group flexibility from the more rigid crosslinked

region. Region 4 may comprise a single phosphorylated serine residue which is designed to interact strongly with calcium ions and help direct mineralization of hydroxyapatite. Region 5 may comprise cell adhesion ligand RGD. b) Molecular model of an illustrated PA showing the overall conical shape of the molecule going from the narrow hydrophobic tail to the bulkier peptide region. c) Schematic showing the self-assembly of PA molecules into a cylindrical micelle.

Figure 2. a) Negative stain (phosphotungstic acid) TEM of self-assembled nanofibers before covalent capture. Fibers are arranged in ribbon-like parallel arrays. b) Vitreous ice cryo-TEM of the fibers reveals the diameter of the fibers in their native hydrated state to be 7.6±1nm. c) Positive stain (uranyl acetate) TEM of the self-assembled nanofibers after oxidative cross-linking showing electron dense regions due to the stain that localized on the periphery of the fibers. d) Thin section TEM of positively stained (uranyl acetate) nanofibers after oxidative cross-linking and embedding in epoxy resin. Two fibers are observed in cross-section (arrows) clearly showing the lack of staining in the interior of the fiber.

Figure 3. a) TEM micrographs of the unstained, cross-linked peptide-amphiphile fibers incubated for 10 min in $CaCl_2$ and Na_2HPO_4 solution. The fibers arranged in bundles are visible due to the high concentration of inorganic ions on their surface. b) After 20 minutes forming HA crystals (arrows) are observed in parallel arrays on some of the PA fibers. c) After 30 minutes mature HA crystals (arrows) completely cover the PA fibers. d) Electron diffraction pattern taken from a mineralized bundle of PA fibers after 30 minutes of exposure to calcium and phosphate. The presence and orientation of the diffraction arcs corresponding to the 002 and 004 planes indicate preferential alignment of the crystals with their *c*-axes along the long axis of the bundle. e) Plot of intensity versus inverse angstroms reveals that the 002 and 004 peaks of hydroxyapatite are strongly enhanced along the peptide-amphiphile fiber axis. f) EDS profile of mineral crystals after 30 minutes of incubation reveals a Ca/P ratio of 1.67 +/- 0.08 as expected for HA.

Figure 4. Scheme showing possible relationships between peptide-amphiphile fibers and hydroxyapatite crystals in the mineralized bundle. Arrow indicates the direction of the *c*-axes of the crystals.

Figure 5. A tilt pair taken from mineralized PA fibers after 30 minutes of incubation with calcium and phosphate demonstrating the plate shape of the crystals. The crystals that were "edge-on" (electron dense, narrow objects) in the zero degree image lose contrast in the 45 degree rotated image (arrow 1) while the contrast of the crystals that were "face-on" in the zero degree images increase (arrow 2).

Figure 6. a) Nonphosphorylated PA fibers after 20 minutes of incubation with calcium and phosphate shows only amorphous mineral deposit concentrate on the fibers. b) Nonphosphorylated PA after 30 minutes of incubation with calcium and phosphate continue to show only amorphous mineral in contrast with phosphorylated PA which shows heavy crystallization at this time point.

Figures 7-9. TEM micrographs for several cylindrical micelles prepared from PA molecules listed in Table 1. Specifically: **Figure 7**, Top: Molecule #4 containing a C10 alkyl tail. Bottom: Molecule #13 containing a C22 alkyl tail; **Figure 8**, Top: Molecule 8 utilizing a tetra alanine sequence in place of tetra cysteine and containing a C16 alkyl tail. Bottom: Molecule 9 utilizing a tetra alanine sequence in place of tetra cysteine and containing a C10 alkyl tail; and **Figure 9**, peptide-amphiphiles with three different peptide head groups. Top: Molecule 10 with "KGE". Middle: Molecule 14 lacking the phosphoserine group. Bottom: Molecule 15 with "IKVAV."

Figure 10A-B. Chemical structures of PA compositions 21 and 22, with reference to Table 2, below.

Figures 10C-E. Chemical structures of four peptide-amphiphiles used for self-assembly.

Figure 11. Molecule 1 self-assemblies into nanofibers upon drying from a solution at physiological pH.

Figure 12. TEM micrographs of positively stained peptide-amphiphile gels formed by addition of: A) Ca+2 to molecule 2 solution; B) Cd+2 and molecule 2 solution; C) Ca+2 to molecule 4 solution; D) Fe+2 to molecule 1 solution; and e) Zn+2 to molecule 1 solution.

Figure 13. Self-assembly induced by mixing two different peptide amphipiles (21 and 22) containing opposite charges.

Figures 14A-C. TEM images of three different self-assembled peptide-amphiphile nanofibers. 14A: Negatively charged peptide amphiphile 25 assembled with acid. 14B: Positively charged 24 assembled with base. 14C: Nanofibers formed at neutral pH with a mixture of 24 and 25.

Figures 15A-C. FT-IR spectra of the peptide-amphiphile gels. **A.** The fragment of the spectrum of $CaCl_2$ induced gel of the molecule 27, showing the regions of Amide A, Amide I and Amide II bands. **B.** The Amide I and II region of the normalized spectra of the gels of the molecule 27 assemblied by addition of $CaCl_2$ and at low pH. **C.** The Amide I and II region of the normalized spectra of the gels of the molecule 32 assemblied by addition of $CaCl_2$, pH and KCl.

Summary of the Invention.

In light of the foregoing, it is an object of the present invention to provide a nanostructured fiber-like system, or nanostructure providing other shapes such as spherical or oblate, and/or molecular components thereof, together with various methods for assembly and use to recreate or mimic the structural and/or functional interaction between collagen fibrils and hydroxyapatite crystals in bone or an extracellular matrix, thereby providing a nanostructured approach divergent from the prior art. It will be understood by those skilled in the art that one or more aspects of this invention can meet certain objectives, while one or more other aspects can meet certain other objectives. Each objective may not apply equally, in all its respects, to every aspect of this invention. As such, the following objects can be viewed in the alternative with respect to any one aspect of this invention.

It is an object of the present invention to provide a composition which can be used for assembly of a molecular structure having dimensional and functional characteristics biomimetic with collagen fibrils.

It can also be an object of the present invention to provide a nanostructured fibrous system as a template for tissue development.

It can also be an object of the present invention to provide a composite of a mineralized nanofiber structure biomimetic with collagen fibrils and hydroxyapatite crystals in natural bone tissue.

It can also be an object of the present invention to provide a system for the facile self-assembly of nanostructured fibers under a particular pH regime or under substantially neutral or physiological pH conditions, for use in conjunction with one or more of the preceding objectives, such fibers as can be reversibly stabilized to promote structural integrity.

It can also be an object of the present invention to provide peptide amphiphile compositions comprising two or more oppositely charged peptide components, each such component as can further include the same or a differing bioactive epitope sequence, for subsequent biomedical applications including without limitation either in vitro or in vivo drug delivery, cell therapies or tissue engineering.

It can also be an object of the present invention, irrespective of any end use application, to provide one or more peptide amphiphile compositions which are stable at physiological pHs with or without covalent cross-linking, such stability of as can be provided via ionic cross-linking of charged functional groups present within the component amphiphiles of such compositions.

It can also be an object of the present invention to provide a methodology using fibers in a stabilized three-dimensional structure to direct and/or control mineralization and crystal growth thereon.

It can also be an object of the present invention to provide a molecular system for the design and engineering of specific nanofibers and components thereof to target particular cell and/or mineral growth en route to a variety of hard or soft biomimetic materials for biological and non-biological applications, the later including, but not limited to, catalysis, photonics and electronics.

Other objects, features, benefits and advantages of the present invention will be apparent from this summary and its descriptions of various preferred embodiments, and will be readily apparent to those skilled in the art having knowledge of natural biomineralizing systems. Such objects, features, benefits and advantages will be apparent from the above as taken into conjunction with the accompanying examples, data, figures and all reasonable inferences to be drawn therefrom.

With respect to various embodiments, the present invention comprises use of self-assembly techniques, such self-assembly as may be employed in conjunction with mineralization to prepare a nanostructured composite material which recreates or mimics the structural orientation and interaction between collagen and hydroxyapatite observed in bone. A composite may be prepared by self-assembly, covalent capture, and mineralization of one or more peptide-amphiphile (PA) compositions. As evident from the preceding, the peptide-amphiphile (PA) compositions of this invention can be synthesized using preparatory techniques well-known to those skilled in the art -- preferably, by standard solid phase chemistry, with alkylation or other modification of the N-terminus of the peptide component with a hydrophobic moiety. Mono or di-alkyl moieties attached to the N or C termini of peptides may influence their aggregation and secondary structure in water in both synthetic and natural systems. As illustrated in several embodiments, a hydrophobic, hydrocarbon and/or alkyl tail component with a sufficient number of carbon atoms coupled to an ionic peptide having a preference for beta-strand conformations can in certain embodiments be used to create an amphiphile that assembles in water into nanofiber structures. The amphiphile's overall conical shape can also have an effect on such assemblies. (J. N. Israelachvili *Intermolecular and surface forces*; 2nd ed.; Academic: London San Diego, 1992). The hydrophobic tails pack in the center of the assembly with the peptide segments exposed to an aqueous or hydrophilic environment. These cylindrical nanostructures can be viewed as fibers in which the chemistry of the peptide region is repetitively displayed on their surface. Comparably, consistent with this invention, amphiphile molecules can also be designed to provide micelles having structural shapes that may differ from a fiber like appearance.

Without limitation, three structural and/or functional features can be engineered into the peptide region of a PA composition of this invention. First, the prepared fibers are optimally robust and, for this reason, one or more cysteine amino acid residues -- four in some embodiments and/or, optionally, consecutive -- can be incorporated in the sequence for covalent capture of supramolecular nanofibers. (D. Y. Jackson, D. S. King, J. Chmielewski, S. Singh, P. G. Schultz, *J. Am. Chem. Soc.* **113**, 9391-9392 (1991); T. D. Clark, K. Kobayashi, M. R. Ghadiri, *Chem Eur J* **5**, 782-792 (1999); Y. Y. Won, H. T. Davis, F. S. Bates, *Science* **283**, 960-963 (1999); E. R. Zubarev, M. U. Pralle, L. M. Li, S. I. Stupp, *Science* **283**, 523-526 (1999); E. A.

Archer, N. T. Goldberg, V. Lynch, *J Am Chem Soc* **122**, 5006-5007 (2000); F. Cardullo, M. C. Calama, B. H. M. Snellink-Ruel, J. L. Weidmann, A. Bielejewska, R. Fokkens, N. M. M. Nibbering, P. Timmerman, D. N. Reinhoudt, *Chem. Comm.* **5**, 367-368 (2000)). Such residues can be used to form disulfide bonds between adjacent PA molecules upon oxidation to lock the supramolecular structure into place. The formation of the disulfide bonds is reversible, as described elsewhere herein, allowing self correction of improper disulfide bonds or return to the supramolecular structure by treatment with mild reducing agents.

With regard to a second feature, the fibers of various embodiments may be able to nucleate the formation of HA crystals in the proper environment. It is well known that acidic moieties play a key role in biomineralization processes and in the formation of calcium phosphate minerals phosphorylated groups are particularly important. (G. K. Hunter, H. A. Goldberg, *Biochem. J.* **302**, 175-179 (1994); S. Weiner, L. Addadi, *J. Mater. Chem.* **7**, 689-702 (1997)). For example in dentin, the phosphophoryn protein family contains numerous repeats of the sequences Asp-Ser(P)-Ser(P) and Ser(P)-Asp (A. George, L. Bannon, B. Sabsay, J. W. Dillon, J. Malone, A. Veis, N. A. Jenkins, D. J. Gilbert, N. G. Copeland, *J. Biol. Chem.* **271**, 32869-32873 (1996)). These massively phosphorylated proteins are closely associated with the collagen extracellular matrix (ECM) and are known to play an important role in HA mineralization (A. Veis In *Biomineralization: Chemical and Biological Perspectives*; S. Mann, J. Webb, J. R. P. Williams, Eds.; VCH: Weinheim New York, 1989; pp 189-222). Accordingly, at least one phosphoserine residue can be incorporated into the peptide sequence which, after self assembly, allows the fiber to display a highly phosphorylated surface functionally biomimetic to a long peptide segment. This, in part, may be used to simulate a repetitive organization of phosphate groups found in phosphophoryn proteins.

Third, with respect to one or more of the preceding embodiments or others within the scope of this invention, it would be beneficial for biomedical applications to provide fibers promoting surface adhesion and growth of cells. Another collagen associated protein, fibronectin, contains the sequence Arg-Gly-Asp (RGD). As this sequence has been found to play an important role in integrin-mediated cell adhesion, an RGD sequence can also be included in preferred peptide components and/or PA compositions, depending upon end-use application. Collectively, these and other design principles led to preparation of a PA molecule of the type shown in Fig. 1. While the PA compound of Fig. 1 is shown with a bioactive RGD sequence, other epitope sequences can be used, as described elsewhere herein including, but not limited to, the IKVAV sequence.

Notwithstanding the numerous embodiments provided above, broader aspects of the present invention include a peptide amphiphile compound/composition having 1) a hydrophobic component and 2) a peptide or peptide-like component further including a bioactive epitope sequence. In various preferred embodiments, the hydrophobic component of such a compound or composition is of sufficient length to provide amphiphilic behavior and nanofiber assembly/formation in water or another polar solvent system. Typically, such a component may be about a C_6 or greater hydrocarbon moiety, although other hydrophobic, hydrocarbon and/or alkyl components could be used as would be well-known to those skilled in the art to provide similar structural or functional effect. Such hydrophobic components include, without limitation, cholesterol, biphenyl and p-aminobenzoic acid. Regardless, a peptide component of such a composition may include the aforementioned RGD sequence found especially useful for the nanofiber cell adhesion and mineralization described herein. Alternatively, an IKVAV sequence can be incorporated into a PA compound.

Preferred peptide components of such compounds/compositions can also include a phosphoryl-functionalized residue or sequence (as indicated with the corresponding letter code and a parenthetical "P"), as described above. Inclusion of a phosphoserine residue, S(P), has been found especially useful for HA mineralization. Other embodiments can include, for example and without limitation, a phosphotyrosine residue. The peptide component of such compositions can also include a residue or sequence capable of promoting intermolecular bonding and structural stability of the micelles/nanofibers available from such compositions. A sequence of cysteine residues can be used with good effect, providing for the facile intermolecular oxidation/reduction of the associated thiol functionalities.

Peptide components of this invention preferably comprise naturally-occurring amino acids. However, incorporation of known artificial amino acids such as beta or gamma amino acids and those containing non-natural side chains, and/or other similar monomers such as hydroxyacids are also contemplated, with the effect that the corresponding component is peptide-like in this respect. One example already tested includes an amino acid substituted with a thiophene moiety so that polymerization can produce electrically conductive and/or fluorescent materials. Accordingly, such artificial amino acids, hydroxyacids or related monomers can be used to meet the spacer, phosphorylation and/or intermolecular bonding objectives described above.

Various aspects of the present invention can be described with reference to the peptide amphiphile illustrated in Figure 1, but consistent with broader aspects of this invention, other compounds and compositions can be prepared in accordance with this invention and used for the self-assembly of fibrous cylindrical micelles and corresponding nanostructures. See, Table 1, below.

Table 1

PA	N-terminus	Peptide (N to C)	C-terminus
1	C16	CCCCGGGS(P)RGD	H
2	C16	CCCCGGGS(P)	H
3	H	CCCCGGGS(P)RGD	H
4	C10	CCCCGGGS(P)RGD	H
5	C6	CCCCGGGS(P)RGD	H
6	C10	GGGS(P)RGD	H
7	C16	GGGS(P)RGD	H
8	C16	AAAAGGGS(P)RGD	H
9	C10	AAAAGGGS(P)RGD	H
10	C16	CCCCGGGS(P)KGE	H
11	C10	AAAAGGGS(P)KGE	H
12	C16	AAAAGGGS(P)KGE	H
13	C22	CCCCGGGS(P)RGD	H
14	C16	CCCCGGGSRGD	H

Table 1

PA	N-terminus	Peptide (N to C)	C-terminus
15	C16	CCCCGGGEIKVAV	H
16	C16	CCCCGGGS(P)RGDS	H
17	C_n	LLLKK-X	H
18	C_n	LSL-X	H
19	C_n	LSLS-X	H

Depending upon desired cell or mineral growth, a phosphorylated moiety or residue may not be included (see PAs 14 and 15). As discussed above, cellular adhesion or other bio-interaction may be promoted by a particular sequence of the peptide component. With reference to PAs 10-12 and 15, a non-RGD sequence can be utilized depending upon cellular target or end-use application. In particular, the IKVAV sequence has been identified in other contexts as important for neuron growth and development. The YIGSR sequence, as discussed more fully below, can also be used. Accordingly, the amphiphile compositions of this invention can include a peptide component having such a sequence for corresponding use. Other residues and/or bioactive epitope sequences capable of promoting cell adhesion, growth and/or development are known in the art and can be used in conjunction with the present invention, such residues/sequences as can be incorporated into the peptide components and/or PA compositions of this invention using available synthetic techniques or straight-forward modifications thereof. With respect to drug delivery and related end-use applications, various bioactive epitope sequences incorporated into the PA compounds/compositions of this invention can be used to adsorb, bind and/or deliver a number of hydrophilic therapeutic agents, including but not limited to growth factors, related co-factors and/or activators. Conversely, such compounds/compositions can be used to control the delivery rate of various hydrophobic/hydrocarbon therapeutic agents bound and/or encapsulated within the hydrophobic components thereof. With respect to Table 1, it is noted that several PA compounds/compositions do not include cysteine residues: while such a residue or peptide sequence can be used to enhance intermolecular nanofiber stability, it is not required for micelle formation in the first instance. Reference is made to Figures 7-9 for TEM micrographs of several PA compositions identified in Table 1.

Further reference is made to Table 1 and, in particular, PA compositions 17-19. Various other embodiments of this invention may comprise the residues shown, or where optionally X may further comprise one or more of the aforementioned residues as may be utilized for intramolecular structural flexibility, intermolecular stability, mineralization and/or cellular interaction. Accordingly, each such composition can optionally comprise, as desired for end-use application, one or more glycine, cysteine, phosphorylated and/or cell growth, development or adhesion residues. In accordance with the preceding discussion, the amphiphilic nature of such compositions provides for the presence of a suitably hydrophobic component C_n, where n is an integer corresponding to the number of carbon atoms in such a component sufficient to provide sufficient amphiphilic character and/or assembly structure. Without limitation, various embodiments of such compositions comprise a hydrophobic component (e.g., alkyl, biphenyl, cholesterol, etc.) of about C_6- about C_{26}. More specifically and without limitation, a sequence such as that provided by PA composition 17 (or as further comprising residue(s) X) can be utilized to modify and/or enhance the rate of nanostructure self-assembly. Likewise, without limitation, the sequences of PA compositions 18 and 19 (or as further comprising residue(s) X) can be used, as needed, to improve the structural or mechanical properties of the corresponding available nanostructured gel materials. Implementation of such peptides in the PA compositions of this invention do not, of course, preclude use of other residues. For example, PA compositions 17-19 can further comprise one or more glycine, cysteine, phosphorylated and/or cellular interaction residues, in accordance with this invention.

In part, the present invention also provides a sol-gel system including 1) a polar or aqueous solution and/or containing of one or more of the amphiphile compounds or compositions described herein, and 2) a factor or reagent sufficient to induce assembly, agglomeration of gelation under neutral or physiological conditions. Such gelation and/or self-assembly of various PA compositions into micellular nanofibers can be achieved under substantially neutral and/or physiological pH conditions through drying, introduction of a mono- or multivalent metal ion and/or the combination of differently charged amphiphiles. The approach of using differently charged amphiphiles can also be utilized to deliver in the self assembling nanofibrous system two or more bioactive molecules, each bearing different charges and this way combining the gelation technology with the delivery of multiple biological signals. Such facile factors, as described more fully below and in several of the following examples, can extend the sol-gel system and/or methodology of this invention to a variety of medical applications. These and other aspects of the present invention can be described with reference to the PA compositions provided in Table 2, below, with further reference to Figs. 1, 10A-B and Table 1, above.

Table 2

PA	N-terminus	Peptide (N to C)	C-terminus	Net Charge at pH 7
17	C16	CCCCGGGS(P)RGD	COOH	-3
18	C16	AAAAGGGS(P)RGD	COOH	-3
19	C10	AAAAGGGS(P)RGD	COOH	-3
20	C16	CCCCGGGSRGD	COOH	-1
21	C16	CCCCGGGEIKVAV	COOH	-1
22	C16	CCCCGGGKIKVAV	$CONH_2$	+1
23	C16	CCCCGGGS(P)RGDS	COOH	-3
24	C16	AAAAGGGKYIGSR	$CONH_2$	+2
25	C16	AAAAGGGEIKVAV	COOH	-1

The peptide epitopes on molecules 22-25 demonstrate the biomedical potential of the self assembling systems described here. RGD is the well known cell adhesion ligand found in fibronectin while IKVAV and YIGSR are laminin sequences known to interact with mammalian neurons. IKVAV promotes neurite outgrowth in mammalian neurons, while YIGSR plays a related role in neuronal cell-substrate adhesion. While these and other bioactive epitope sequences can be used to effect cell adhesion, proliferation or differentiation and related outcomes, in a broader context, the PA compounds/compositions and related methods of this invention can be used in conjunction with any epitope sequence capable of cellular interaction and/or binding to a cellular membrane receptor. In particular, peptide amphiphiles 23 and 25 have a net negative charge at neutral pH, whereas PAs 22 and 24 have a net positive charge. Electrostatically driven co-assembly between PA compounds 24 and 25 as well as 23 and 22 provide mixed nanofibers that simultaneously present two biological signals in a cellular environment.

Various peptide-amphiphile compositions are shown in Tables 1 and 2, but are provided only by way of illustrating one or more aspects of this invention. It will be understood by those skilled in the art that a range of other compositions are also contemplated. For example, the peptide components can be varied, limited only by functional considerations of the sort described herein. Accordingly, peptide length and/or sequence can be modified by variation in number or identity of amino acid or substituted monomer. Further, it will be understood that the C-terminus of any such sequence can be construed in light of an associated carboxylate functionality or derivative thereof. While the N-terminus of the peptides is illustrated with respect to the referenced conjugated hydrophobic and/or hydrocarbon components, it will be understood that such components can also be varied by length and/or composition, such variation limited only by the functional considerations presented herein. For example, a sixteen carbon (C16) component can comprise but is not limited to a straight-chain alkyl hydrocarbon. As would be understood by those skilled in the art, the structure and/or chemistry of such a component can be varied with regard to a particular, desired functionality of an amphiphile composition or assembly thereof. Likewise, the length of such a component is limited only by way of the degree of hydrophobicity desired for a particular amphiphile composition and/or assembled structure, in conjunction with a given solvent medium.

Consistent with broader aspects of this invention, it was found that representative peptide amphiphiles (for example, PAs 17-19 and 21, Table 2 and those of Table 1), when dissolved at neutral pH were dried onto surfaces, self-assembled into cylindrical micelles (Figure 11). Such a facile approach allows this novel material to be formed in a pH independent manner, as may be important in applications that are particularly sensitive to changes in pH, including the delivery of cells in conjunction with the gelling system and generally avoiding contact between tissues and materials at non-physiological pH.

Illustrating another such factor, the self-assembly of the PA molecules (for example, but not limited to PAs 17, 18 and 20, Table 2 and Table 3) into nanofibers can be induced by addition of metal ions such as, but not limited to K^{+}, Ca^{+2}, Mg^{+2}, Cd^{+2}, Fe^{+2} and Zn^{+2}, and metal ions having higher oxidation states such as but not limited to the trivalent Al^{+3} and Fe^{+3}. Self-supporting gels can be formed upon addition of a stoichiometric excess of such ions, preferably on the order of about 2-3 metal ions per molecule of PA. TEM study of these gels reveals a network of nanofibers that pack into flat bundles, similar to those found in the gels self-assembled by acidification (Figure 12). (See examples 19a-c and Table 3, below.)

More generally, such PA compositions can be prepared with appropriate alkaline, alkaline earth, transitional metal salts or reagents comprising such salts. With further reference to Table 3, while negatively-charged PAs tend to gel in the presence of metal ions, gelation properties may vary depending upon PA composition and metal ion identity. Self-assembly and/or gelation can also be varied through modification of the hydrophobic and hydrophilic portions/regions of the PA compositions, one relative to the other. With regard to the hydrophilic region, choice of amino acid residues can affect gelation depending upon net charge of and/or charge distribution therein.

As yet another factor inducing gelation under physiological conditions, consider two amphiphiles, such as but not limited to PAs 21 and 22 (Table 2), one having a net negative charge at neutral pH and one having a net positive charge at neutral pH -- both dissolved at neutral pH. However, upon mixing such amphiphiles immediately form a gel. Examination by negative stain TEM shows the gel composed of cylindrical micelles (Figure 13).

In one possible working model of the molecular organization of the mixed PA nanofibers, the hydrophobic alkyl tails are hidden in the center of the micelles with the more hydrophilic peptide segments of the molecules in contact with the aqueous environment. The cylindrical structure of this micelle could in part be explained by the tapered shape of individual molecules, but a second driving force might be beta sheet hydrogen bonding among peptide segments down the long axis of the fibers: the parallel β-sheet hydrogen bonding conformation is observed by FT-IR and on the unusual dominance of the cylindrical self-assembly motif across such a broad concentration range (< about 0.001% to > about 10% by weight). (See Example 9, below.)

In the case of the mixed PA fibers, two oppositely charged molecules are believed thoroughly mixed within any given nanofiber as opposed to molecules segregating into mixtures of homomeric fibers. If homomeric fibers formed in spite of the highly unfavorable charge concentrations associated with these structures, it would be expected that the fibers pack into bundles of oppositely charged fibers in order to reduce electrostatic repulsion. However, the same amount or less bundling in the mixed PA fibers is observed as compared to fibers formed from a single PA molecule (Figure 14).

Self-assembly and/or gelation under physiological conditions, as induced by the preceding factors, raise numerous implications regarding end use application and effect. Without limitation, with reference to the preceding, a binary or higher PA mixture makes available a sol-gel system for the formation of micellular nanofibers in a aqueous environment at neutral and/or physiological pH conditions. As discussed elsewhere herein, such a combination of two or more PA compounds can be used to assemble nanofibers with a range of residues providing a corresponding variety of concurrent chemical or biological signals for cell adhesion proliferation, differentiation and the like, yielding enhanced properties with regard to tissue engineering or regenerative applications. Alone, or in conjunction with one or more of the other factors discussed herein, it is contemplated that preferred medical or therapeutic embodiments of such a system or methodology can be implemented upon step-wise introduction and mixing of the subject PA compositions, with in situ gel formation.

Accordingly, such a system can be used in conjunction with a drug, medication or other therapeutic agent, as would be understood by those skilled in the art: the subject drug or therapeutic agent can be provided with or introduced to an appropriate aqueous or polar medium separately or in conjunction with one or more

PA compounds. Introduction of a reagent and/or factor induces nanofiber assembly and/or gelation, incorporating such a drug/agent therein, if hydrophobic, or as bound to or sorbed on the surface thereof, if hydrophilic. Disassembly or solubilization of the nanofibrous network or gel can release or deliver the drug/agent as or where required. As would be understood by those skilled in the art made aware of this invention, a range of both hydrophobic and hydrophilic drugs/agents can be utilized herewith. In particular, with regard to the peptide epitopes thereof, hydrophilic growth factors, co-factors and/or activators can be adsorbed on, delivered with and/or released by the PA compounds/compositions of this invention.

In preferred embodiments, regardless of any such factor or physiological condition, the amphiphile composition(s) of such a system includes a peptide component having residues capable of intermolecular cross-linking. The thiol moieties of cysteine residues can be used for intermolecular disulfide bond formation through introduction of a suitable oxidizing agent. Conversely, such bonds can be cleaved by a reducing agent introduced to the system. The concentration of cysteine residues could be varied to control the chemical and biological stability of the nanofibrous system and therefore control the rate of drug or therapeutic delivery using the nanofibers as the carriers. Furthermore, enzymes could be incorporated in the nanofibers to control biodegradation rate through hydrolysis of the disulfide bonds. Such degradation and/or the concentration of cysteine residues can be utilized in a variety of tissue engineering contexts.

Corresponding to one or more preferred embodiments of such a material, composition or system, the present invention can also include a nanostructured template for mineral crystal and/or cellular growth. Such a template includes a micelle assembly of amphiphile composition(s) of the type described herein, wherein the peptide component thereof may include a residue or sequence capable of intermolecular bond formation. In preferred embodiments, as described above, cysteine residues can be used for intermolecular disulfide bond formation. Various other preferred embodiments can further include one or more phosphorylated residues to promote crystal growth and/or mineralization, depending upon a desired material or tissue target.

In the context of biomimetic hard material or tissue, the present invention can also include an organo-mineral composite having a nanostructured peptide amphiphile template with mineral crystals thereon. As described above, this aspect of the present invention can be illustrated with the present amphiphile compositions, assembled as nanostructured fibers, used to nucleate and grow hydroxyapatite crystals. In preferred embodiments, the amphiphilic compositions include peptide components having one or more residues promoting crystal nucleation and growth. Such preferred peptide components can also include one or more residues capable of intermolecular bonding to stabilize the nanofiber template. While this inventive aspect has been described in conjunction with hydroxyapatite nucleation and growth, the mineral component of this composite can include other inorganic compounds and/or oxides. Such residues, sequences or moieties are of the type described herein, or as would otherwise be understood by those skilled in the art made aware of this invention.

Regardless, the c-axes of the mineral crystals of such composites are aligned with the longitudinal fiber axes, in a manner analogous to the alignment observed between collagen fibrils and HA crystals in natural bone tissue. Accordingly, the present invention can also include a method of using a peptide amphiphile, in accordance with this invention, to promote and control HA crystal growth. The identity of the PA compositions used therewith is limited only by way of those structural considerations described elsewhere herein. Consistent therewith and with the broader aspects of this invention, such a method includes 1) providing an aqueous or other suitable polar medium of one or more peptide amphiphile compositions, 2) inducing assembly thereof into cylindrical micelle structures, 3) optimally stabilizing the structures with intermolecular bond formation, and 4) introducing to the medium reagents suitable for the preparation of HA and crystalline growth thereof on the nanofiber micelle structures.

As provided elsewhere herein, one or more amphiphile compositions can be used to provide fibers with a variety of cell adhesion, mineralization and/or structural capabilities. A combination of such compositions can be used to assemble a nanofibrous matrix with synergistic properties beneficial for a particular crystal and/or cellular development, such compositions as may vary according to peptide component, residue sequence, hydrophobic or hydrocarbon component and/or resulting PA compound or assembly configuration. As described elsewhere herein, a combination of charged PA compositions can be used to effect assembly -- likewise with drying and incorporation of divalent cations.

Examples of the Invention.

The following non-limiting examples and data illustrate various aspects and features relating to the compositions, micelles, composites and/or methods of the present invention, including self-assembly of a peptide-amphiphile nanofiber system, as is available through the methodologies described herein. In comparison with the prior art, the present structures, template designs and related methods provide results and data which are surprising, unexpected and contrary thereto. While the utility of this invention is illustrated through the use of several amphiphiles, biomimetic micelles and resulting organo-mineral composites, it will be understood by those skilled in the art that comparable results are obtainable with various other amphiphiles, nanofibers/micelles and/or composites, as are commensurate with the scope of this invention.

Example 1

After synthesis (see, example 10), the PA of Figure 1 (characterized by ^1H NMR and MALDI-TOF MS: $[M-H]^{-1} = 1333$) was treated with dithiothreitol (DTT) at a pH of 8 to reduce all cysteine residues to free thiols. At this pH the PA was found to be soluble in excess of 50mg/ml in water. However, upon acidification of the solution below pH 4 the material rapidly becomes insoluble. Solutions more concentrated than 2.5 mg/ml form birefringent gels in water that are self-supporting upon inversion of the container. Examination of the gels by cryo-TEM, which preserves the native, hydrated state of the material, revealed a network of fibers with a diameter of 7.6±1nm and lengths up to several microns (Fig. 2B). Negatives were digitized in the Umax PowerLook scanner with resolution 1200 dpi. The average thickness of the PA fibers was determined by two different procedures. First the Fourier transform of the images and following measurements of the distances between the peaks were performed in the NIH Image 1201.1262 program. The second approach applied was averaging technique based on the cross-correlation method, widely used in single particle reconstruction using the EMAN 1201.1202 program. For each data set 1200-1300 individual boxes were selected. (E. Beniash, W. Traub, A. Veis, S. Weiner, *J. Struct. Biol.* **132**, 212-225 (2000). Positively and negatively stained dried fibers were found to have diameters of 6.0±1nm (Fig. 2A,C). TEM using the positive stain uranyl acetate, which preferentially stains acidic groups, revealed increased electron density at the periphery of the fiber. (J. R. Harris, Ed. *Electron Microscopy in Biology, A Practical Approach*; Oxford University Press: New York, 1991). Additionally, gels that were stained, embedded in epoxy resin and sectioned for TEM, showed fibers in cross-section in which donut shaped patterns were observed indicating staining only on the outer portion of the fiber (Fig. 2D). The two positive staining experiments of this example indicate that the hydrophobic alkyl tails pack on the inside of the fiber and the acidic moieties of the peptide are displayed on the surface of the fiber.

Example 2

The formation of the fibers was found to be concentration independent over more than three orders of magnitude (0.01mg/ml to 50mg/ml), however a second level of hierarchy was observed that was concentration dependent. As the concentration of the PA was increased, a larger number of the fibers were observed to pack into flat ribbons of fibers (Fig. 2A,C).

Example 3

Examination of the self-assembled material by FT-IR revealed a bimodal amide I peak with maxima at 1658cm^{-1} (α-helix) and 1632cm^{-1} (β-sheet) along with a N-H stretching peak at 3287cm^{-1} indicating the formation of a hydrogen bonded structure, possibly utilizing a combination of β-sheet and α-helical secondary structure in the fibers (S. Krimm, J. Bandekar, *Adv. Protein Chem.* **38**, 181-364 (1986); W. K. Surewicz, H. H. Mantsch, D. Chapman, *Biochemistry* **32**, 389-394 (1993)). Based on the above data the nanofibers were modeled as cylindrical micelles in which the alkyl tails pack on the inside of the fiber and peptide segments are displayed on the outside containing both β-sheet and α-helical secondary structure. This model results in a fiber with a diameter of 8.5nm, which is within the margin of error of our TEM measurements (Fig. 1C).

With reference to examples 4-9, the following abbreviations are employed for the reagents used. PA: peptide-amphiphile, TEM: transmission electron microscopy, DTT: dithiothreitol, EDT: ethanedithiol, TIS: triisopropyl silane, TFA: triflouroacetic acid, HBTU: (2-(1h-benzotriazole-1-yl) - 1,1,3,3 - tetramethyluronium hexafluorophosphate, DiEA: Diisopropylethylamine. Except as noted below, all chemicals were purchased from Fisher or Aldrich and used as provided. DiEA and Piperidine were redistilled before use. Amino acid derivatives, derivatized resins and HBTU were purchased from Nova Biochem. All water used was deionized with a Millipore Milli-Q water purifier operating at a resistance of 18 MΩ.

Example 4

With reference to examples 5-9, below, the peptide-amphiphiles of Table 2 were prepared on a 0.25mmole scale using standard FMOC chemistry on an Applied Biosystems 733A automated peptide synthesizer. All peptides prepared have a C-terminal carboxylic acid and were made using pre-derivatized Wang resin. After the peptide portion of the molecule was prepared the resin was removed from the automated synthesizer and the N-terminus capped with a fatty acid containing 10 or 16 carbon atoms. The alkylation reaction was accomplished using 2 equivalents of the fatty acid, 2 equivalents HBTU and 6 equivalents of DiEA in DMF. The reaction was allowed to proceed for at least six hours after which the reaction was monitored by ninhydrin. The alkylation reaction was repeated until the ninhydrin test was negative. In general the longer the fatty acid the more repetitions were required to drive the reaction to completion.

Cleavage and deprotection of the peptide-amphiphiles containing cysteine was done with a mixture of TFA, water, TIS and EDT in a ratio of 91:3:3:3 for three hours at room temperature. The cleavage mixture and two subsequent TFA washings were filtered into a round bottom flask. The solution was roto-evaporated to a thick viscous solution. This solution was triturated with cold diethylether. The white precipitation was collected by filtration, washed with copious cold ether and dried under vacuum. Typically, 200mg of the peptide-amphiphile powder was dissolved in 20ml of water with the addition of 1M NaOH to adjust the pH of the solution to 8 and 200mg of dithiothreitol (DTT) to reduce all cysteine amino acids to the free thiol and allowed to stir overnight. The solution was then filtered through a 0.2 μm nylon Acros filter into a new round bottom flask. This 10mg / ml (1% by weight) solution was used for all subsequent manipulations. Work up of peptide-amphiphiles not containing cysteine were performed as above accept that ethanedithiol was omitted from the cleavage reaction and DTT was not used in the preparation of aqueous solutions. Peptide-amphiphiles were characterized by MALDI-TOF MS and were found to have the expected molecular weight.

Example 5

Nanofibers containing two different peptide-amphiphiles can be prepared as follows. Peptide-amphiphile 21 was dissolved in water at pH 7 at a concentration of 5 mg/ml. Peptide-amphiphile 22 was dissolved in water at pH 7 at a concentration of 5mg/ml in a separate vial. Both solutions were slightly cloudy. Upon mixing the two solutions the material formed a gel in less then one second.

Example 6

Several PAs with basic amino acids that can self-assemble at alkaline pH and also mixed systems, in which oppositely charged PAs self-assemble at neutral pH, were prepared. For this purpose PA compounds 22-25 were prepared by standard solid phase peptide synthesis followed by alkylation with the C16 fatty acid, palmitic acid. See Table 2, Fig. 10B (PA 22) and Figs. 10C-E (PAs 23-25). The peptides thus prepared were characterized by electrospray mass spectroscopy and found to correspond to their respective expected masses. All four of the PAs were found to dissolve in water at neutral pH at concentrations of 1mg/ml or less. Molecules 22 and 23 were fully reduced to eliminate disulfide bond crosslinking then used under anaerobic conditions or left in an excess of dithiothreitol. Compounds 24 and 25 could be dissolved at higher concentrations (10mg/ml) but molecules 22 and 24 were cloudy and viscous at this concentration.

Example 7

Solutions of each compound of example 6 were prepared at a concentration of 0.1mg/ml at neutral pH. Molecule 25 was slowly acidified (HCl) and found to precipitate below a pH of 4.5 while increasing the solution's pH (KOH) left the molecule completely dissolved. Conversely, base was slowly added to a solution of molecule 24 and it was found to be soluble until a pH above 9.5 was reached at which point a precipitate formed. At neutral pH both molecules were completely dissolved, however, upon mixing these clear solutions a precipitation formed within seconds with no significant change of pH. At higher concentrations (5mg/ml) mixing the oppositely charged amphiphiles caused the immediate formation of a birefringent gel. Molecules 22 and 23, which can be covalently crosslinked through oxidation of their cysteine residues, behaved in a similar fashion.

Example 8

Samples of the precipitated material in each of the six solutions of examples 6-7, one for each individual PA compound and mixed samples of PAs 24 and 25 and PAs 22 and 23, were examined by negative stain transmission electron microscopy (TEM) and FT-IR. TEM revealed that in all cases the PA had self-assembled into nanofibers with nearly uniform diameters of 7nm +/- 1nm, often many microns long.

Example 9

FT-IR spectra of the preceding solutions showed strong hydrogen bonding based on N-H stretching frequencies between 3276 and 3289cm^{-1}, and all spectra showed significant parallel β sheet character based on the position of the amide I band at 1630cm^{-1}. Additional contributions in this region between 1650 and 1680cm^{-1} indicate that the peptide region also adopts significant α-helix and random coil characteristics. The pH response of the PA compounds, their structure by TEM, and their IR spectra suggest, without limitation, a possible model of self-assembly: at neutral pH molecule 25 has a net negative charge of -1. This charge helps to keep the molecule solubilized through electrostatic repulsion of other negatively charged species despite the large hydrophobic bulk of its fatty acid tail. Similarly, molecule 24 has a net charge of +2 at neutral pH and is soluble. Self-assembly is initiated when these charges are eliminated, as in the pH driven self-assembly, or when the charges are attractive instead of repulsive as in the case when oppositely charged amphiphiles are mixed. The fact that the mixed self-assembly occurs at neutral pH where the individual molecules are soluble strongly suggests that the self-assembly is driven by an electrostatic attraction involving both positively and negatively charged molecules and not simply hydrophobic collapse involving one or the other PA.

Example 10

100μl of 10 mg/ml solution of peptide-amphiphile 20 was treated with 1M $CaCl_2$ (adjusted to pH 6) drop wise in 1 μl increments. The solutions were shaken after each addition of the $CaCl_2$ solution in order to obtain better diffusion of the divalent metal ions. Gelation was immediate. When examined by positive stain TEM the gel formed with calcium ions was found to be composed of nanofibers with the same dimensions as those formed by acid induced self-assembly and by surface drying. This calcium induced self-assembly may be particularly useful for medical applications where formation of a gel at physiological pH is desired. Trivalent metal cations can also be used for gelation.

Example 11

To demonstrate cross-linking, in accordance with this invention, the gels formed above (examples 5 and 6) were treated with 0.05M I_2 which was adjusted to a pH of 3.5. The iodine solution was placed on top of the gel and allowed to slowly diffuse into the gel. After the iodine color had completely penetrated the gel, excess iodine was removed. The gel was then soaked in a bath of deionized water which was periodically changed until the discoloration from the iodine was gone as judged by eye (roughly 48 hours depending on the size of the gel).

Example 12

Illustrating another gelation factor of this invention, self-assembly can occur by simply taking a PA (such as PA 17, Table 2) or a combination of PAs dissolved in water at pH 8 and placing it on a surface which is allowed to dry (for example, directly on a carbon coated TEM grid). Upon examination of this preparation by negative stain TEM we observed clearly the formation of nanofibers. (See, Figure 11.)

Example 13

Samples of the peptide-amphiphiles were prepared for TEM analysis in two different ways. In some cases a small sample of the gel, prepared in bulk as described above, was smeared onto a holey carbon coated TEM grid (Quantifoil). Other samples were prepared directly on the grid by placing 10 ul of 0.01-0.02% solution of PA directly on the grid. The grid was then placed into a sealed chamber with HCl vapors for 10 minutes after which the grids were washed with de-ionized water. Two routine staining techniques, negative staining with PTA (phosphotungstic acid) or positive staining with uranyl acetate, were used in this study[Harris, 1991]. In all cases electron microscopy was performed at an accelerating voltage of 200kV. (See, Figs. 10-13).

Example 14

To investigate the mineralization properties of PA nanofibers of this invention, the material was assembled and mineralized directly on a holey carbon coated TEM grid -- to allow study of the dynamics of the mineralization process while minimizing artifacts of TEM sample preparation. To prepare samples, a drop of aqueous PA (1mg/ml) was mounted on the holey grid and the self-assembly of the PA was induced in an atmosphere of HCl vapor. The grids were immersed in aqueous iodine to oxidize the cysteine thiol groups to disulfides. After rinsing with distilled water, the PA coated grids were treated with 5μl of 10mM $CaCl_2$ on one side and 5μl of 5mM Na_2HPO_4 on the other side. The two solutions are able to mix only by passing through the holes in the carbon support. Grids were examined by TEM at different time intervals (Fig. 3) and after 10 minutes inorganic material was observed to be concentrated around the fibers thus increasing their contrast (a crystalline phase was not detected at this time by electron diffraction). At 20 minutes the fibers begin to be covered with crystalline mineral, although significant amourphous material remains. After 30 minutes plate shaped polycrystalline mineral is visible throughout the surface of the fibers (Fig. 3C and Fig. 5). The mineral was analyzed by EDS (Energy Dispersion X-Ray Fluorescence Spectroscopy) which revealed a Ca/P ratio of 1.67 ± 0.08 which is consistent with the formation of HA with a formula of $Ca_{10}(PO_4)_6(OH)_2$ (Fig. 3F).

Example 15

As controls for the experiment of example 6, carbon coated TEM grids were treated as above but without PA fibers. In this case no mineral deposit was found on the grids. In a second control, a PA was prepared in which phosphoserine was replaced by serine and treated as above with calcium and phosphate. The nonphosphorylated fibers were observed by TEM after 20 and 30 minutes of incubation and in both cases an amorphous mineral deposit around the fibers was observed, but crystals did not form within this time frame (Fig. 6).

Example 16

In order to discern the relative orientation of the HA crystals with respect to PA fibers, several samples of example 6, in which isolated mineralized bundles of PA fibers could be observed, were analyzed by electron diffraction. In all cases preferential alignment of the HA crystallographic *c*-axis with the PA fiber long axis was observed. (Fig. 3D,E).

Example 17

Mineralization experiments show that PA fibers of this invention are able to nucleate hydroxyapatite on their surfaces. Negatively charged surfaces can promote mineralization by establishing local ion supersaturation (S. Weiner, L. Addadi, *J. Mater. Chem.* **7**, 689-702 (1997)). Particularly, the two acidic aminoacids phosphoserine and aspartic acid used here are abundant in the proteins of mineralized tissues proven to initiate crystal growth (L. Addadi, S. Weiner, *Proc. Natl. Acad. Sci.* **82**, 4110-4114 (1985); G. Falini, S. Albeck, S. Weiner, L. Addadi, *Science* **271**, 67-69 (1996); A. George, L. Bannon, B. Sabsay, J. W. Dillon, J. Malone, A. Veis, N. A. Jenkins, D. J. Gilbert, N. G. Copeland, *J. Biol. Chem.* **271**, 32869-32873 (1996); S. Weiner, L. Addadi, *J. Mater. Chem.* **7**, 689-702 (1997)). The fact that the fibers gain extra electron density prior to formation of the crystalline phase suggests the above mechanism may be utilized in our system. More surprising is the observation that the *c*-axes of the HA crystals are co-aligned with long axes of the fibers (Fig. 4). This fact implies that the orientation of crystalline nuclei and the subsequent crystal growth are not random but are controlled by the PA micelles. The exact mechanism of this control is not clear, however in similar systems such control is gained by specific arrangement of acidic groups that promote growth of the crystals in a particular orientation by an epitaxial mechanism (S. Mann, J. P. Hannington, R. J. P. Williams, *Nature* **324**, 565-567 (1986); S. Weiner, L. Addadi, *J. Mater. Chem.* **7**, 689-702 (1997); J. Aizenberg, A. J. Black, G. M. Whitesides, *Nature* **398**, 495-498 (1999)). An analogous mechanism may be employed with PA fibers. Previous *in vitro* studies showing oriented crystal growth from organic templates were done mainly in two dimensional systems. The results of this example show for the first time oriented crystal growth in a synthetic fibrous organic substrate.

Example 18

Several additional representative peptide-amphiphiles of this invention were prepared by manual solid phase peptide synthesis starting from 0.5 mmoles of an FMOC-Asp(tBu)-WANG resin. Deprotection of the initial and subsequent FMOC groups and was accomplished by double treatment with 15ml of 30% piperidine in DMF for 2 minutes and 7 minutes. The resin was then washed with 15ml of DMF 5 times. With the exception of cysteine derivatives, amino acids (4 equivalents) were preactivated with HBTU (3.95 equivalents) and DiEA (6 equivalents) in 10ml of DMF for two minutes. The activated solution was then added to the deprotected resin and allowed to shake for 30 minutes after which a small sample was removed and analyzed with ninhydrin to test for completeness of the reaction. Failed reactions were recoupled in an identical fashion. Cysteine was coupled via the FMOC-Cys(Trt)-OPfp activated ester with the addition of 1 equivalent of DiEA in order to avoid suspected epimerization found using the standard FMOC-Cys(ACM)-OH derivative with the conditions described above. Other amino acid derivatives used included FMOC-Gly-OH, FMOC-Arg(Pbf)-OH and FMOC-Ser(PO(OBzl)OH)-OH. Analogous derivatives available in the art may be used to couple A, S, L, K and other such residues, including those shown in Tables 1 and 2. The corresponding protecting groups and associated chemistries are known in the art and may vary depending upon the subject amino acid residue. The final step was coupling of a fatty acid (palmitic acid) and was accomplished using 2 equivalents of the acid activated with 2 equivalent of HBTU and 3 equivalents of DiEA in 15ml DMF for 2 minutes. The solution was allowed to react with the resin overnight. Likewise, other reagents and starting materials of the sort useful in the preparation of the inventive compositions, but not limited to those shown in Tables 1 and 2, are readily-available and known to those skilled in the art, such reagents/starting materials as may be used for the hydrophobic/hydrocarbon and peptide (residues/monomers) components.

Cleavage and deprotection of the peptide-amphiphiles was done with a mixture of TFA, water, triisopropyl silane (TIS) and ethane dithiol (EDT) in a ratio of 91:3:3:3 for three hours at room temperature. The cleavage mixture and TFA washings were filtered into a round bottom flask. The solution was roto-evaporated and then redissolved in a minimum of neat TFA. This solution was triturated with cold diethylether. The white precipitation was collected by filtration and dried under vacuum.

Example 19a

To determine the relative number of metal ions necessary for gelation of the PAs, titration experiments were performed with PA molecules 26 and 27. These tests show that the minimal amount of Ca^{+2} and Gd^{+3} ions needed for the gelation is or about equal to the number of PA molecules in solution. (See Table 3, below). PAs positively charged without containing negatively charged functional groups did not gel upon addition of metal salts. None of the negatively charged peptide-amphiphiles except molecule 32 formed gels in the presence of KCl at a ratio 1 molecule of PA per 20 molecules of KCl. Further tests on the gelation abilities of monovalent salts showed that 10 mM solutions of compounds 26 and 27 did not gel in the presence of KCl or NaCl at concentrations up to 6M. In contrast to other PAs, an addition of 200 mM of KCl to 10 mM solution of molecules 32 and 33 caused formation of a self-supporting gel.

Example 19b

An additional factor in self-assembly may be exemplified by the structure of PAs 32 and 33. Both these molecules contain the IKVAV sequence at the c-terminus of the peptide segment. This sequence is comprised is of alternating extremely hydrophobic amino acids I and V and more hydrophilic ones such as A, and K. Since the side chains of adjacent amino acids are located on opposite sides of the peptide backbone this sequence is, itself, amphiphilic. As such, PAs 32 and 33 may be considered as double or two dimensional amphiphiles, with one axis of amphiphilicity coinciding with the backbone of the molecule and amphiphilic peptide segment at the c-terminus, in addition to hydrophobic interactions between the alkyl moieties of the molecules.

Example 19c

The gels formed by addition of metal ions are remarkably stable. Such experiments show that they endure very basic conditions up to pH 11, and are stable at pH as low as 4. The gels also survive heating up to 100°C, though they slightly shrink. The gels also remain intact when exposed to a volume of deionized water 10 times greater than the volume of the gel for several days without any visible changes.

Example 20a

When $Cu(ClO_4)_2$ was added to the PA solutions the samples changed from transparent to blue upon gelation. The aqueous solution of copper perchlorate at same concentration was completely transparent. UV-Vis analysis of the gels of molecules 27 and 28 shows significant shift in absorbance compare to the aqueous solution of $Cu(ClO_4)_2$ of same concentration. These results are suggestive of formation of metal PA complexes upon gelation.

Example 20b

A significant absorbance shift in the UV-vis spectra of Cu^{+2} induced gels, compared to aqueous solution of $Cu(ClO_4)_2$, implies formation of the metal-PA complexes. The disappearance of the peak at 1730 cm^{-1} accompanied by decreasing of the depth of minimum between Amide I and Amide II bands in the spectra of metal induced gels hints on the formation of metal-carboxyl ionic bonds. Also, the strength of the polyvalent metal ion induced gels in comparison to the pH driven self-assemblies under wide variety of conditions suggests presence of relatively strong bonds between PA molecules.

Table 3. Peptide-amphiphiles and their gelation properties.

	PA	KCl***	$MgCl_2$	$CaCl_2$	$BaCl_2$	$Cu(ClO_4)_2$	$ZnBr_2$	$GdCl_3$
26	Alkyl*-$C_4G_3S^{(P)}$RGD-COOH (-3)**	Liquid	weak gel	gel	gel	no data	gel	gel
27	Alkyl-$A_4G_3S^{(P)}$RGD-COOH (-3)	Liquid	weak gel	gel	gel	gel	gel	gel
28	Alkyl-$A_4G_3S^{(P)}$KGE-COOH (-3)	Liquid	Viscous liquid	gel	gel	no data	gel	gel
29	Alkyl-C_4G_3SRGD-COOH (-1)	Liquid	Cloudy liquid	gel	gel	no data	gel	gel

	PA	KCl***	$MgCl_2$	$CaCl_2$	$BaCl_2$	$Cu(ClO_4)_2$	$ZnBr_2$	$GdCl_3$
30	Alkyl-A_3G_2EQS-COOH (-2)	Liquid	gel	gel	gel	gel	gel	gel
31	Alkyl-A_4G_3ERGDS-COOH (-2)	liquid	Cloudy liquid	cloudy liquid	cloudy liquid	no data	gel	gel
32	Alkyl-C_4G_3EIKVAV-COOH (-1)	gel	gel	gel	no data	no data	gel	gel
33	Alkyl-C_4G_3KIKVAV-NH_2 (+2)	weak gel	Viscous liquid	viscous liquid	viscous liquid	no data	viscous liquid	viscous liquid

*C_{16} alkyl moieties (as tested) or about C_6 - about C_{26} as referenced elsewhere herein.

**Overall charge of the molecule.

*** KCl concentration was 200 mM for all molecules except molecules 26 and 27 that were examined at KCl concentration up to 6 M. Other salts concentrations were 20 mM. Concentration of the peptide amphiphiles in all cases was 10 mg/ml (roughly 8 mM).

Example 21

One potential application of the peptide-amphiphile self-assembled gels is in the area of tissue engineering, in particular the formation of artificial extracellular matrices and cell delivery systems. The results of this example show PA self-assembly *in situ* and *in vitro* cell culture systems. Body fluids as well as cell culture media contain significant amounts inorganic cations. It was presumed that the peptide amphiphiles would form a gel upon mixing with these liquids. Indeed, gel formation was observed when 10 mM solutions of PAs 27, 28 or 33 in water were mixed with equal amounts of cell culture media. Solutions of PA 27 and 28 were mixed with PBS and Hank's solutions depleted by Ca+2 and Mg+2, and no gel formation was observed -- showing that multivalent metal ions present in the cell culture media induce self-assembly of negatively charged PAs in a cell culture medium.

Example 22a

TEM studies revealed the ultrastructural organization of metal ion induced PA gels. The electron micrographs of positively stained samples and resin embedded section show that the gels are comprised of 3-dimensional network of fibrils 5-6 nm in diameter, consistent with other measurements of dehydrated nanofibers (hydrated fibrils can be ~7.6 nm in diameter). The analysis of TEM data of positively stained material reveals that the uranyl acetate stains only peripheral parts of the fibrils, whereas the core remains unstained. The TEM of the resin embedded gel sections shows the same organization. It is apparent from the micrographs that the fibrils sectioned transversely have a doughnut appearance, with unstained central part and intensively stained outer circle. Since the uranyl acetate stains mainly charged groups and does not react with saturated hydrocarbons, these TEM results suggest that in the presence of polyvalent metal ions PA assemble into cylindrical micelles with their aliphatic tails in the core and the peptide parts comprising exterior layer. This structural organization of the PA nanofibers is similar to nanofibers assembled by a pH induced mechanism.

Example 22b

For TEM studies, positively stained samples of the PA gels were prepared as follows: The small amounts of the gels were mounted on the TEM grid and briefly dipped into deionized water in order to remove the excess of the material. The grids then were blotted against filter paper and placed on droplets of 2% uranyl acetate (EMS) aqueous solution. The samples were stained for 30-40 min in the dark. The grids then were washed in deionized water and dried. The samples were studied in the Hitachi 8100 high resolution TEM at acceleration voltage 200 kV.

For resin embedding, the gels were fixed in 2% glutar aldehyde aqueous solution (EMS). Samples then were washed in deionized water and stained with 2% aqueous uranyl acetate. The samples were dehydrated in ethanol gradient followed by propylene oxide. The samples then were transferred into 1:1 mixture of propylene oxide and epoxy resin (SPIpon, SPI). After 1 day of incubation the samples were transferred into pure epoxy resin. The resin was changed twice in 6 hours. The samples were polymerized for a day at 50°C then at 60°C for another day and at 70°C for two more days. Thin sections of the samples were cut using Leica Ultracut. The samples were studied in the JEOL 100C TEM at acceleration voltage 100 kV.

Example 23a

FT-IR spectroscopy was employed to analyze interactions between the PA molecules in the fibrils. The Amide I band maxima of the PA gel samples, prepared by addition of metal ions, was located at 1630-1640 cm^{-1}. The position of Amide I band maximum in this region suggests that the peptide segments of the molecules adopt β-sheet conformation. In all the spectra studied no secondary peak at 1690 cm^{-1}, characteristic for the anti-parallel structures, has been detected, suggesting that the peptides in the cylindrical micelles form parallel β-sheets -- consistent with a model based on TEM data. Amide A stretching band maximum in all spectra studied appears around 3280 cm^{-1}, indicating a high level of hydrogen bonding in the PA supramolecular assemblies.

The spectra of the PA gels formed by pH induced self-assembly contain a peak in the 1720-1750 cm-1 region -- characteristic of C=O stretching of non ionized carboxyls. In contrast, no evident peak has been detected in this region in the spectra of the polyvalent metal ion induced gels. It has been reported in the literature that the formation of the metal carboxyl complexes of amino acids results in a low frequency shift of this band maximum to the 1560 cm^{-1}-1610 cm^{-1} region. No such peaks were observed in this region in the spectra of ion induced PA supramolecular assemblies, which may be due to the overlap between this peak and neighboring Amide I and Amide II bands. However the fact that the minima between Amide I and Amide II peaks is deeper in the spectra of pH induced self-assemblies compare to ion induced self-assemblies suggests presence of the peak in this region. (Reference is made to Fig. 15.)

Example 23b

For FT-IR studies the gels were quick frozen in liquid N2 and lyophilized. The KBr pellets of the lyophilized gels were analyzed on an FT-IR Biorad spectrometer. 32 scans per spectrum were taken at resolution 2 cm-1. The spectra were analyzed using Origin 6.0 program.

Example 24

The synthesis of the peptide amphiphiles illustrates in examples 19-22 was performed using a standard synthesis scheme described elsewhere, herein, and as further available in the literature. Regarding gelation and with reference to the data of Table 3, 200 μm volumes of PA solutions at concentrations 10 mg/ml (approximately 8 mM), pH 7.5 were mixed with 1 M solutions of NaCl, KCl, $MgCl_2$, $CaCl_2$, $BaCl_2$, $ZnBr_2$, $CdCl_3$, Gd^{+3} and $Cu(ClO_4)_2$. The amount of polyvalent ions added to the solutions varied from 0.5 to 5 metal ions per molecule of PA. For monovalent metal ions the maximum concentration of 6 M of metal ions per 10 mM of PA molecules has been tested. The solutions of the metal salts were added to the PA solutions by micropeppetors, the solutions were stirred briefly, in order to obtain better mixing of the components. The formation of self-supporting gels was examined by flipping of the vials up side down. In order to test the ability of cell compatible solutions to induce a gelation of PAs equal amounts 10 mM PA solutions were mixed with following formulations: MEM-α, DMEM containing 10 % of fetal bovine serum, PBS without Ca^{+2} and Mg^{+2} and Hank's solution without Ca^{+2} and $Mg^{+}2$ (Gibco). The ability of the above solutions to induce formation of the PAs was examined visually by flipping the vials up side down.

Various other amphiphile compositions of this invention can be prepared in analogous fashion, as would be known to those skilled in the art and aware thereof, using known procedures and synthetic techniques or straight-forward modifications thereof depending upon a desired amphiphile composition or peptide sequence.

* * *

As demonstrated by the preceding examples, figures and data, the fact that the hydroxyapatite crystals grow on the bundles of fibers with their *c*-axes oriented along the long axes of the micelles could be of interest for design of new materials for mineralized tissue repair. This nanoscale organization resembles that of hydroxyapatite crystals in mineralized ECM in which the HA crystals also grow in parallel arrays with their *c*-axes co-aligned with long axes of the organic fibers (W. Traub, S. Weiner, *Proc. Nat. Acad. Sci.* **86**, 9822-9826 (1989)). This arrangement is the most important characteristic of the biominerals belonging to the bone family (S. Weiner, H. D. Wagner, *Annu. Rev. Mater. Sci.* **28**, 271-298 (1998)). The organization of the collagen fibers, porosity, mineral-organic ratio vary in different members of this family, yet all of them are built from the collagen fibrils containing parallel arrays of hydroxyapatite crystals (W. Traub, S. Weiner, *Proc. Nat. Acad. Sci.* **86**, 9822-9826 (1989); S. Weiner, W. Traub, *FASEB J.* **6**, 879-885 (1992); W. J. Landis, K. J. Hodgens, J. Arena, M. J. Song, B. F. McEwen, *Microsc. Res. Techniq.* **33**, 192-202 (1996)).

It has been proposed that in the case of the pH triggered self-assembly cylindrical micelles form due to the protonation of acidic groups of the peptide and reduction of the repulsive forces between molecules -- that in the case of pH triggered self-assembly of the PAs the major driving force is hydrophobic interactions between the aliphatic tails of the molecules, rather the interactions between the peptide segments. The results reported herein show that the interactions between peptide segments of the PA via metal bridges or due to amphiphilicity of the sequence, may also play a role in the self-assembly. Gelation under physiological conditions demonstrate such PAs as potentially important materials for biomedical applications, such as tissue engineering or delivery of cells, drugs or other therapeutic agents. Supramolecular assemblies induced by addition of metal ions are stable in wide pH range and provide as potential applications embedding cells in a gel or in situ gelation of a tissue repair material.

While the principles of this invention have been described in connection with specific embodiments, it should be understood clearly that these descriptions are added only by way of example and are not intended to limit, in any way, the scope of this invention. For instance, various peptide amphiphiles have been described in conjunction with specific residues and corresponding cell adhesion, but other residues can be used herewith to promote a particular cell adhesion and tissue growth on the nanostructures prepared therefrom. Likewise, while the present invention has been described as applicable to biomimetic material or tissue engineering, it is also contemplated that gels or related systems of such peptide amphiphiles can be used as a delivery platform or carrier for drugs, cells or other cellular or therapeutic material incorporated therein. Other advantages and features will become apparent from the claims filed hereafter, with the scope of such claims to be determined by their reasonable equivalents, as would be understood by those skilled in the art.

What is Claimed:

1. A sol-gel system comprising a peptide amphiphile compound having a bioactive epitope sequence, a hydrophobic component, and a net charge at substantially physiological pH; and a reagent to induce gelation of said amphiphile compound.

2. The system of claim 1 wherein said reagent comprises a metal ion and said system is a gel of supramolecular nanofibers of said amphiphile compound.

3. The system of claim 2 wherein said ion is selected from the group consisting of Ca, Mg, Cd, Fe and Zn divalent ions, Al, Fe and Gd trivalent ions, and combinations of said ions.

4. The system of claim 3 wherein said metal ion is in stoichiometric excess of said amphiphile compound.

5. The system of claim 1 wherein said peptide component of said amphiphile compound comprises a residue with a functional moiety capable of intermolecular covalent bond formation.

6. The system of claim 5 wherein said residue is cysteine.

7. The system of claim 6 further comprising an oxidizing agent to form a disulfide bond between amphiphile compounds.

8. The system of claim 7 further comprising a subsequent reducing agent to cleave said disulfide bond.

9. The system of claim 1 wherein said peptide amphiphile compound has a net charge, and said reagent comprises an aqueous solution of another peptide amphiphile compound with an opposite net charge at substantially physiological pH, and said system is a network of nanofibers of said amphiphile compounds.

10. The system of claim 9 wherein said peptide components of said amphiphile compounds comprise a residue with a functional moiety capable of intermolecular covalent bond formation.

11. The system of claim 10 wherein said residue is cysteine.

12. The system of claim 11 further comprising an oxidizing agent to form a disulfide bond between amphiphile compounds.

13. The system of claim 12 further comprising a subsequent reducing agent to cleave said disulfide bond.

14. The system of claim 1 wherein said bioactive epitope sequence is selected from RGD, IKVAV and YIGSR.

15. The system of claim 1 wherein said peptide amphiphile compound has a net positive charge, and said reagent provides said system a basic pH, and said system comprises a network of nanofibers of said amphiphile compounds.

16. The system of claim 15 wherein said reagent comprises hydroxide ion.

17. A method of peptide amphiphile nanofiber formation, said method comprising:

providing an aqueous medium including a peptide amphiphile compound having a peptide component with a bioactive epitope sequence, and a hydrophobic component, said hydrophobic component sufficient to afford nanofiber formation of said compound;

placing said medium on a surface; and

removing said aqueous component from said medium.

18. The method of claim 17 wherein said removal is passive drying.

19. The method of claim 17 wherein said amphiphile compound has a net charge at a physiological pH.

20. The method of claim 17 wherein said bioactive sequence is RGD.

21. The composition of claim 14 wherein said bioactive sequence is IKVAV.

22. The method of claim 17 wherein said peptide component further comprises at least one phosphorylated residue.

23. The method of claim 17 wherein said hydrophobic component comprises an alkyl moiety of at least six carbon units.

24. A method of peptide amphiphile nanofiber formation, said method comprising:

providing an aqueous medium comprising a peptide amphiphile compound having a bioactive epitope sequence, a hydrophobic component, and a net charge at substantially physiological pH; and introducing a reagent to said medium to induce nanofiber formation.

25. The method of claim 24 wherein said reagent comprises a metal ion.

26. The method of claim 24 wherein said peptide amphiphile compound has a net charge, and said reagent comprises an aqueous medium of another peptide amphiphile compound with an opposite net charge at substantially physiological pH.

27. The method of claim 24 wherein said peptide amphiphile compound has a net positive charge and said reagent provides said medium a basic pH.

28. A peptide amphiphile composition comprising a first peptide component comprising a hydrophobic moiety and a first amino acid sequence, said sequence further comprising a first bioactive epitope sequence; and a second peptide component comprising a hydrophobic moiety and a second amino acid sequence, said sequence further comprising a second bioactive epitope sequence, said first amino acid sequence having a net charge at a physiological pH, and said second amino acid sequence having a net charge at a physiological pH opposite said net charge of said first amino acid sequence.

29. The composition of claim 28 wherein each of said first and second bioactive epitope sequences is selected from RGD, IKVAV and YIGSR.

30. The composition of claim 28 wherein each of said first and second peptide components comprise a residue with a functional moiety capable of intermolecular covalent bond formation.

31. The composition of claim 30 wherein said residue is cysteine.

32. The composition of claim 28 wherein at least one of said first and second peptide components further comprises at least one phosphorylated residue.

33. The composition of claim 28 having a nanofibrous structured assembly.

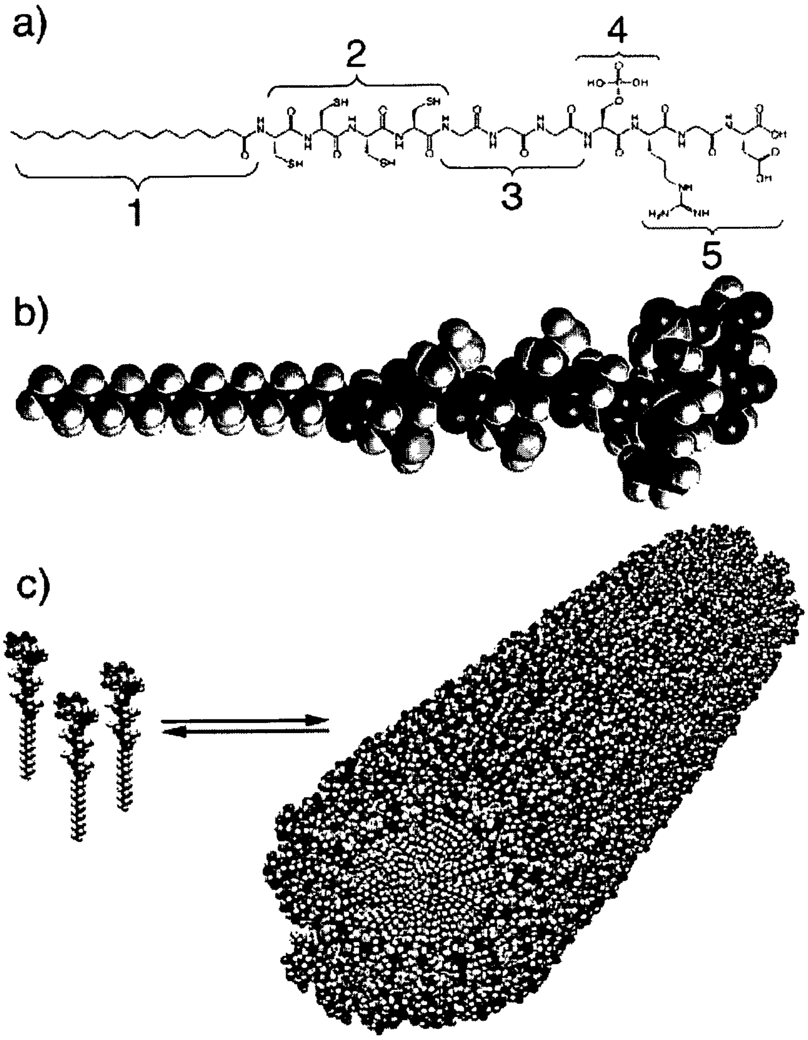


FIGURE 1

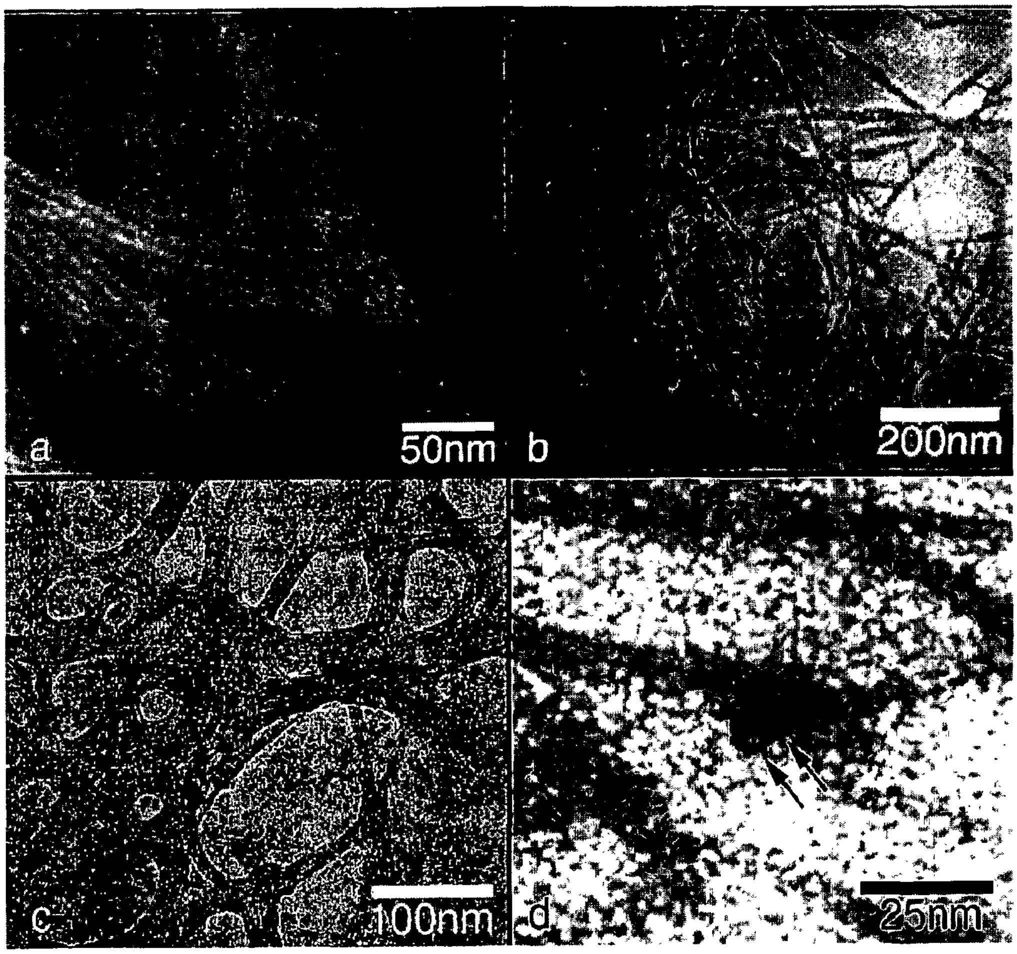
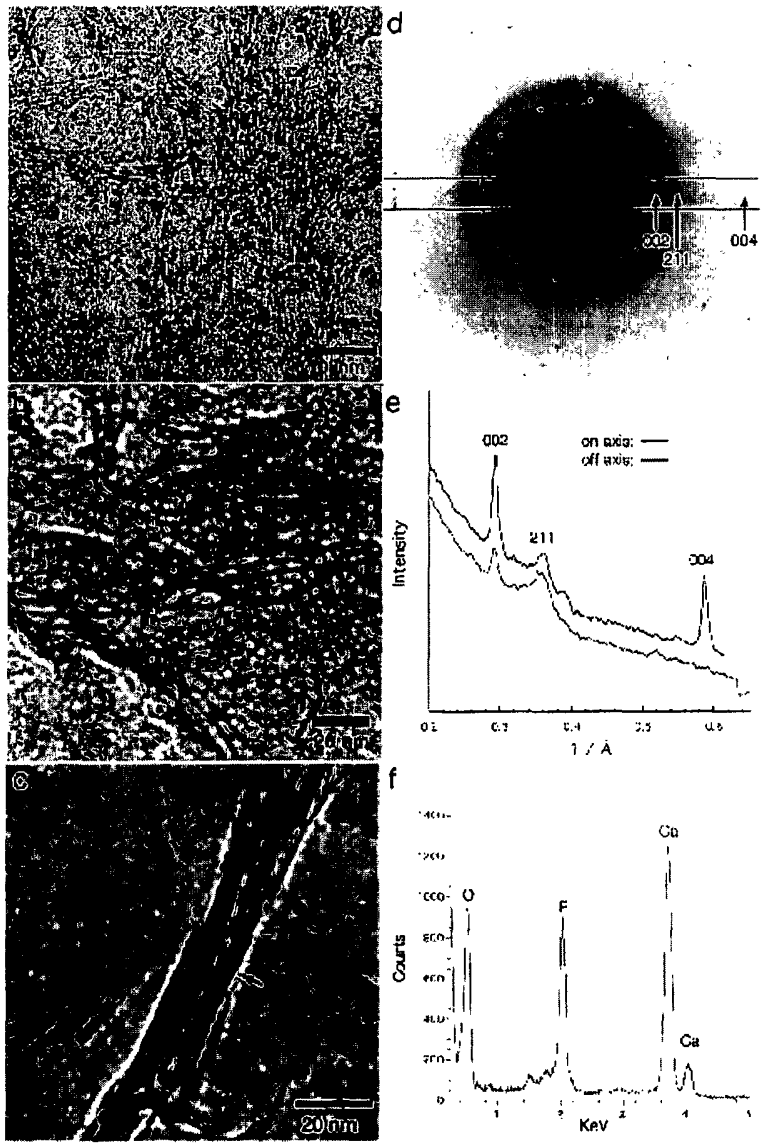


FIGURE 2


d
002
211
004
e
002
on axis:
off axis:
211
004
Intensity
1 / Å
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Cu
O
F
Ca
Counts
Kev
20 nm

FIGURE 3

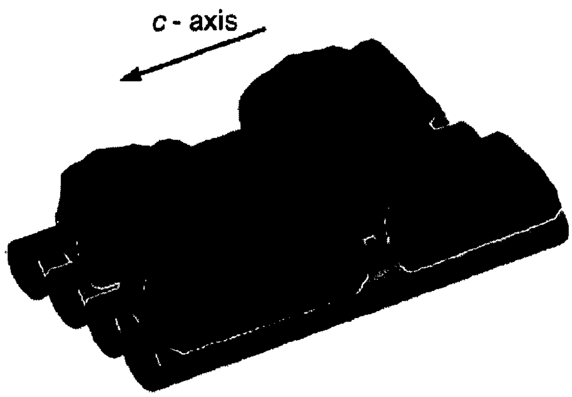


FIGURE 4

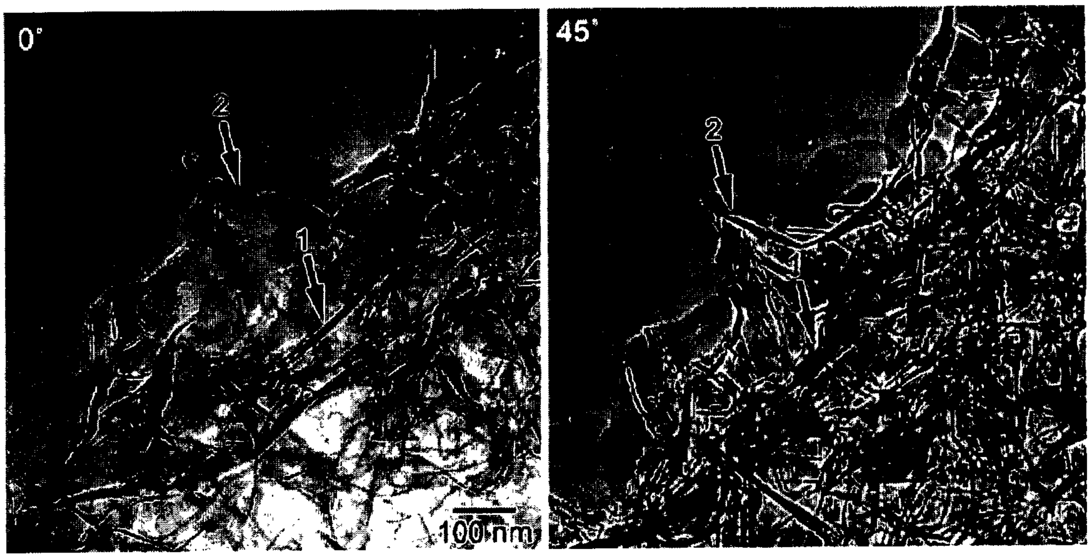


FIGURE 5

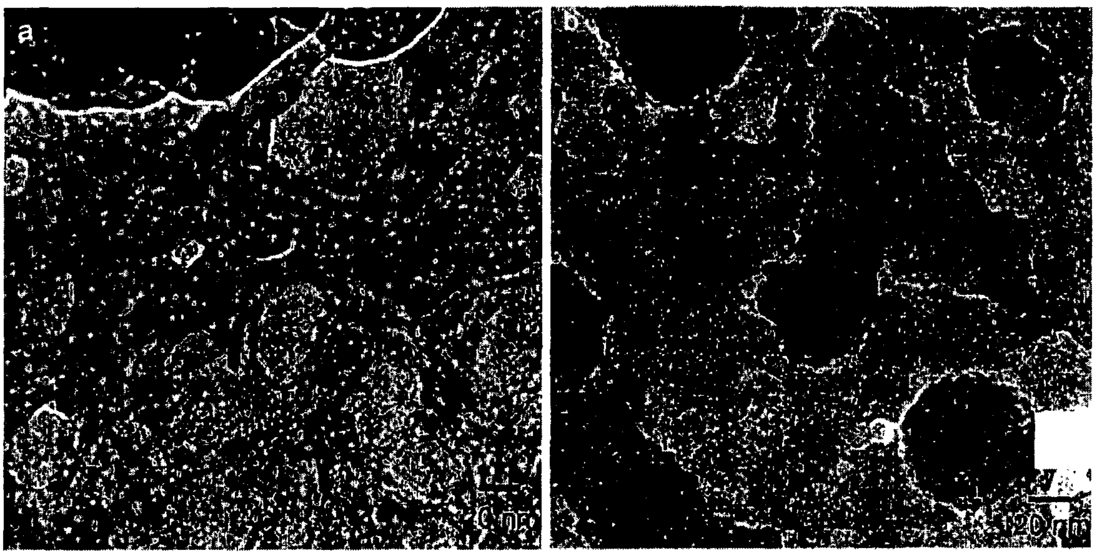


FIGURE 6

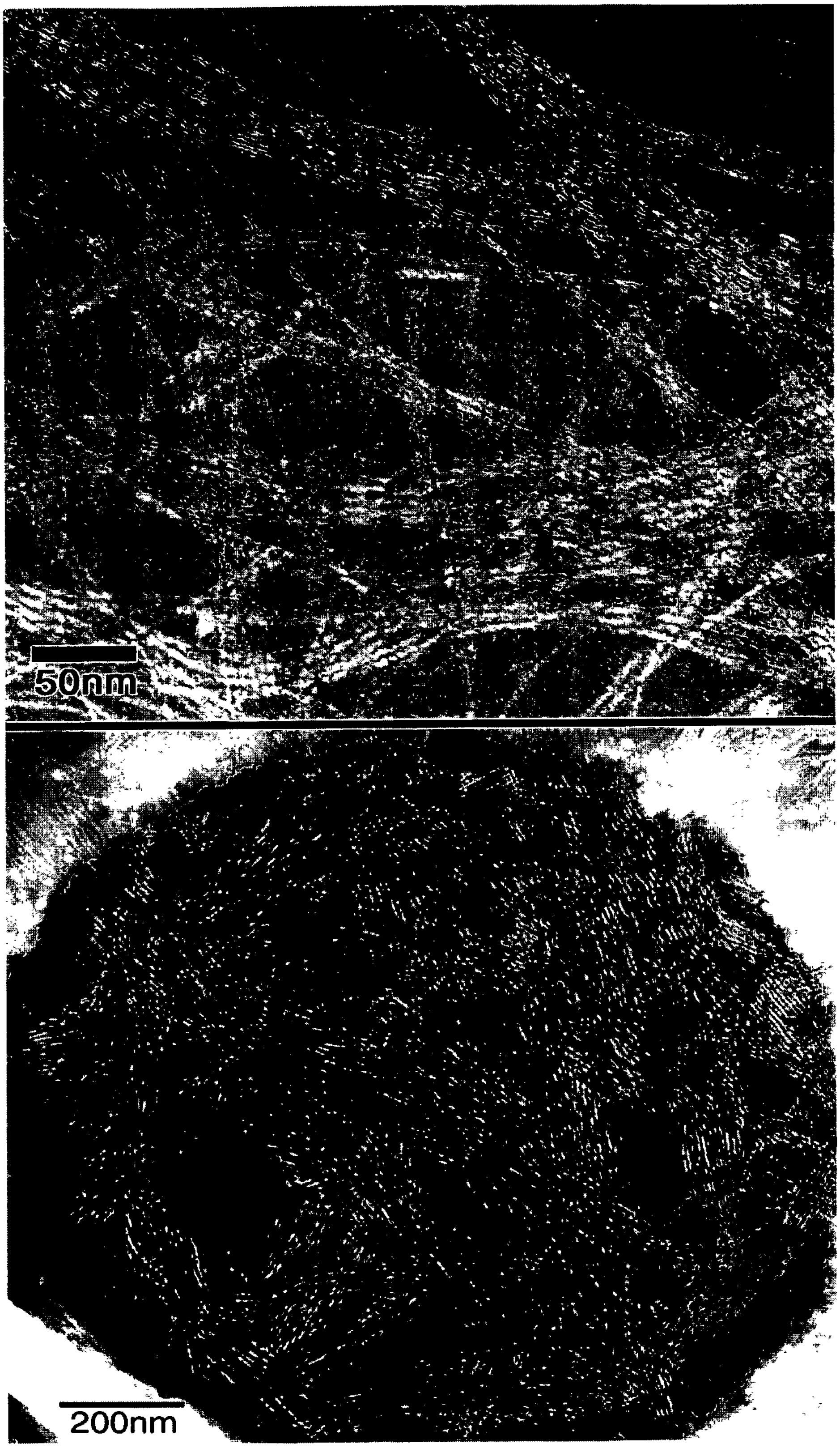


FIGURE 7

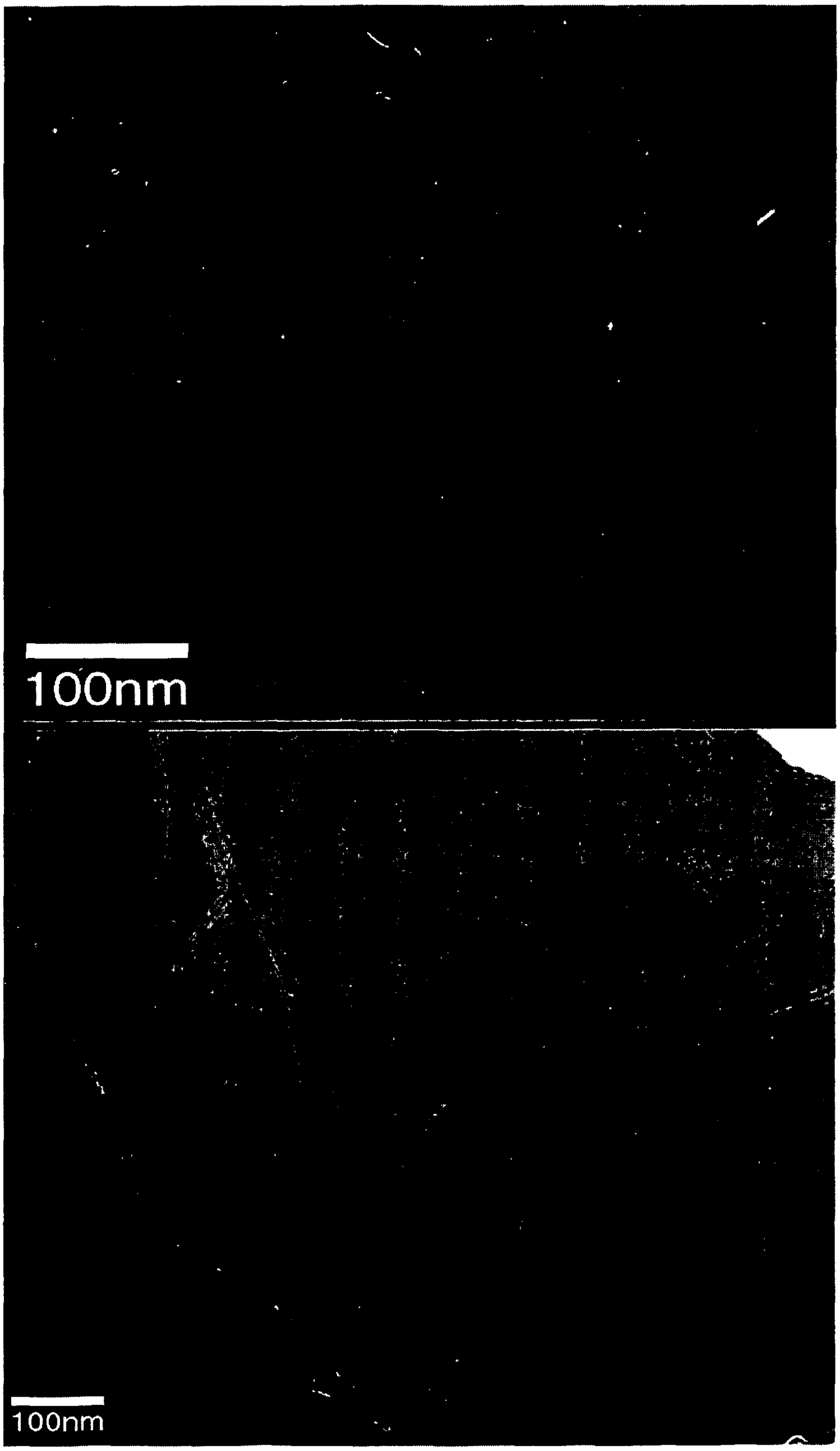


FIGURE 8

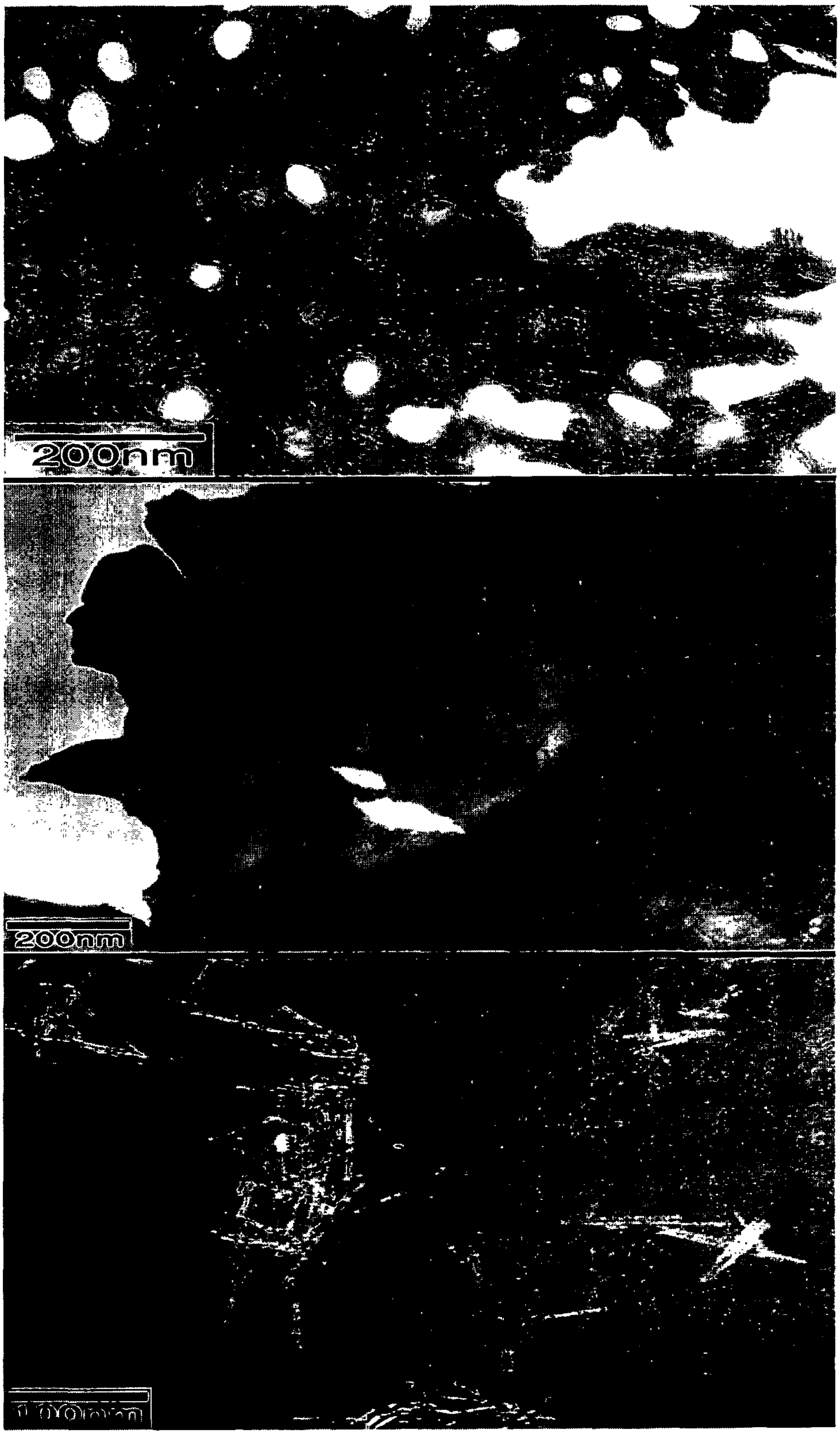


FIGURE 9

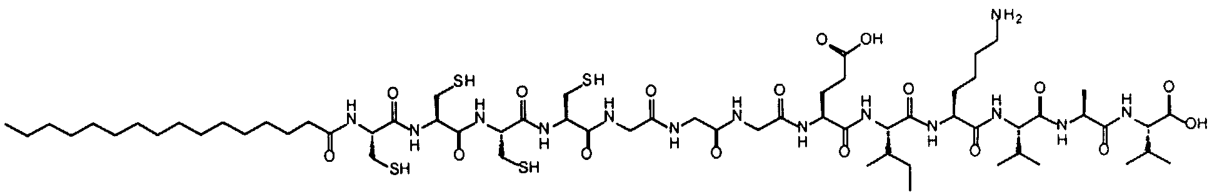
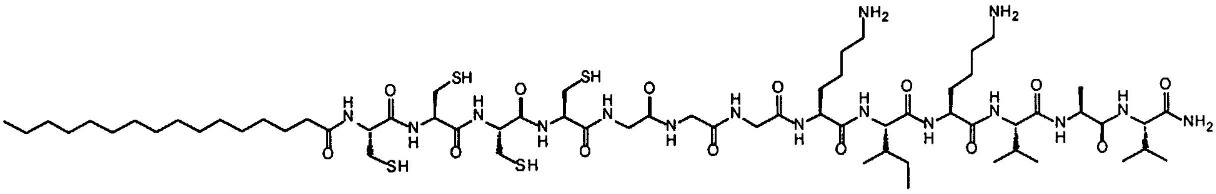


FIGURE 10a

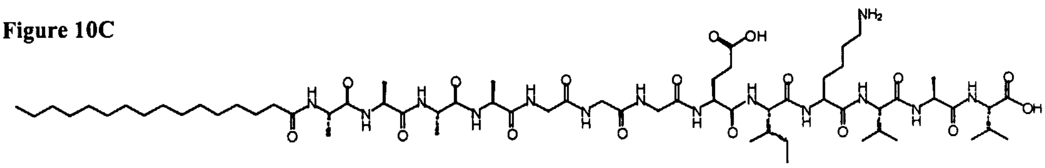
PA 21



PA 22

FIGURE 10b

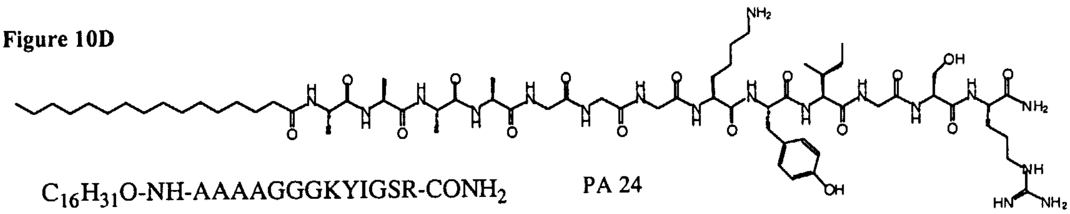
Figure 10C



$C_{16}H_{31}O$-NH-AAAAGGGEIKVAV-COOH

PA 25

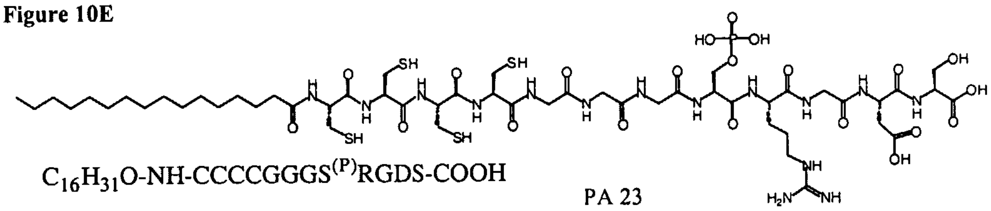
Figure 10D



$C_{16}H_{31}O$-NH-AAAAGGGKYIGSR-$CONH_2$

PA 24

Figure 10E



$C_{16}H_{31}O$-NH-CCCCGGGS$^{(P)}$RGDS-COOH

PA 23

FIGURE 11

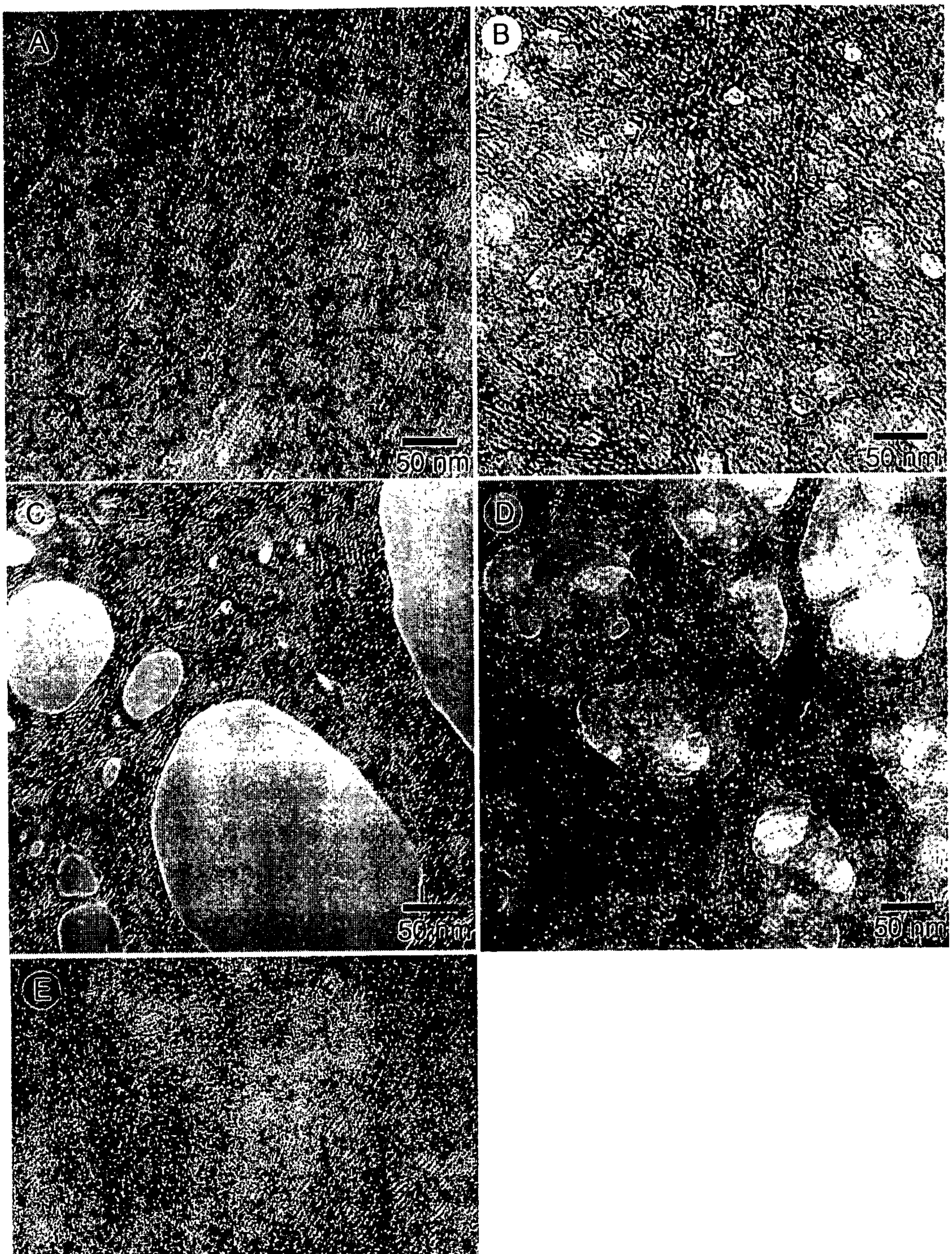


FIGURE 12

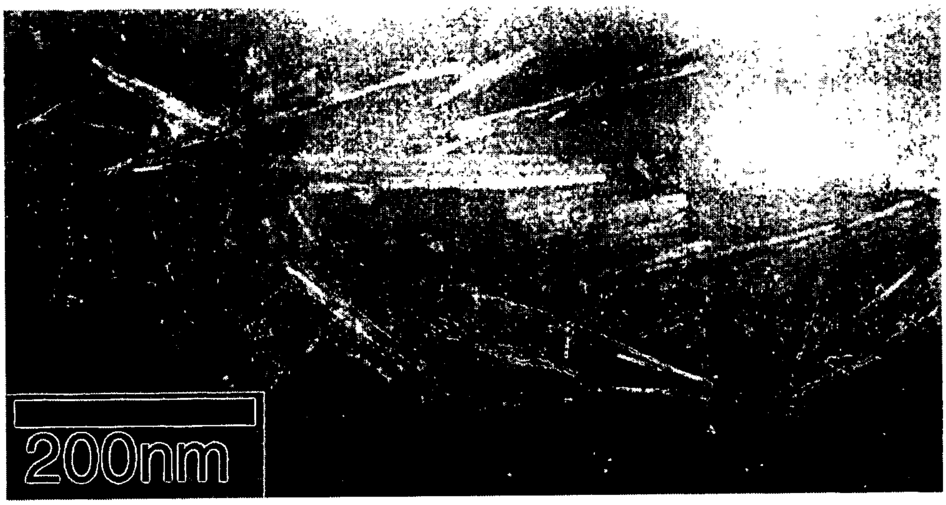


FIGURE 13

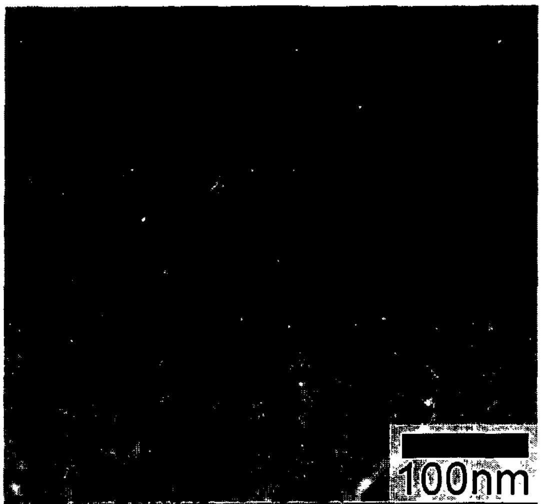


Fig. 14A

Fig. 14B

Fig. 14C

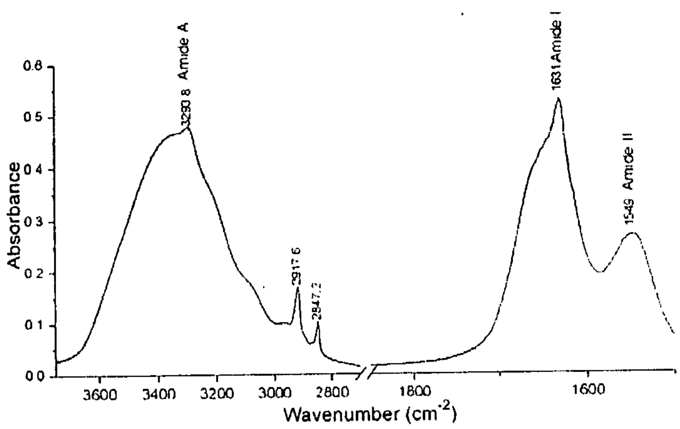


Figure 15A

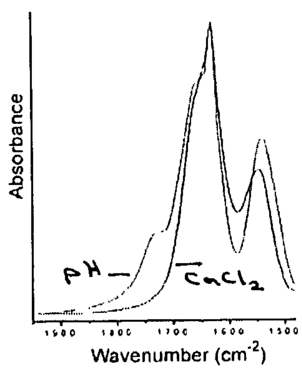


Figure 15B

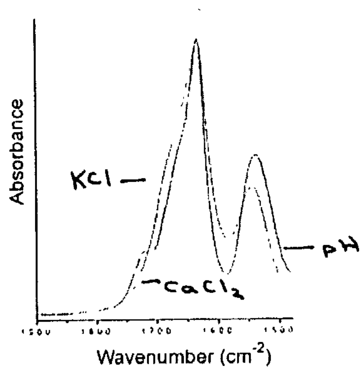


Figure 15C