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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
8 January 2004 (08.01.2004)

PCT

(10) International Publication Number
WO 2004/003561 A1

(51) International Patent Classification[7]: G01N 33/569, 33/68, 33/92

(21) International Application Number: PCT/US2003/020618

(22) International Filing Date: 27 June 2003 (27.06.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/392,619 27 June 2002 (27.06.2002) US

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:
US 60/392,619 (CIP)
Filed on 27 June 2002 (27.06.2002)

(71) Applicant *(for all designated States except US)*: **NORTHWESTERN UNIVERSITY** [US/US]; 2225 North Campus Drive, Evanston, IL 60208 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **STUPP, Samuel, I.** [US/US]; 57 E. Delaware Place, Apartment 2802, Chicago, IL 60611 (US). **CLAUSSEN, Randal, C.** [US/US]; 826 Greenwood Avenue, Wilmette, IL 60091 (US).

(74) Agent: **MILLER, Raymond, A.**; Pepper Hamilton LLP, One Mellon Center, 50th Floor, 500 Grant Street, Pittsburgh, PA 15219 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ (utility model), CZ, DE (utility model), DE, DK (utility model), DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK (utility model), SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search 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: PEPTIDE ROD AMPHIPHILES AND SELF-ASSEMBLY OF SAME

(57) Abstract: Peptide rod amphiphile self-assembly and gelation is described herein. Also described is a method to incorporate the self-assembled peptide rod amphiphile gel in tissue engineering and tissue repair applications. It may also be utilized as three-dimensional scaffold for tissue engineering.

WO 2004/003561 A1

PEPTIDE ROD AMPHIPHILES AND SELF-ASSEMBLY OF SAME

CROSS REFERENCES TO RELATED APPLICATIONS

[0001] This application claims priority from U.S. Provisional Application Serial Number 60/392,619 filed June 27, 2002 the contents of which are incorporated herein by reference in their entirety.

GOVERNMENT INTERESTS

[0002] The United States Government may have certain rights to this invention pursuant to work funded thereby at Northwestern University from the Department of Energy (DOE) under Grant No. DE-FG02-00ER45810.

BACKGROUND OF THE INVENTION

[0003] Self-assembly, self-assembled nanostructures, and templated mineralization are emerging techniques for the fabrication of sensors, composite materials and coatings, and structures useful for the encapsulation and delivery of materials. Biocompatible scaffolds provide viable alternatives to prosthetic materials currently used in prosthetic and reconstructive surgery. These biocompatible materials also hold promise in the formation of tissue or organ equivalents to replaced diseased, defective, or injured tissues. In addition to their use in the biocompatible scaffolds, biodegradable materials may be used for controlled release of therapeutic materials (e.g. genetic material, cells, hormones, drugs, or pro-drugs) into a predetermined area. Polymers currently used to create these scaffolds, such as polylactic acid, polyorthoesters, and polyanhydrides, are generally difficult to mold and are hydrophobic. In addition, these materials generally lack the relevant biological signals or cues that natural systems have which determine tissue regeneration at a site.

SUMMARY

[0004] An embodiment of the present invention is a composition or method of using compositions to form self assembled structures with controlled or tailored chemical functionality on the interior and exterior of the self assembled structures. The chemical functionality of the structures and the molecules which make up the compositions can include biologically relevant signals for cell organization, tissue growth, or mineral formation. The chemical functionality of the molecules can be tailored to bond with a variety of compounds including therapeutic drugs or electronic materials. The size and shape of the self assembled structures may be altered by the design of the molecules which self assemble to form them. The present invention is generally directed to peptide rod amphiphiles and their self assembly to form such materials.

[0005] Embodiments of the present invention preferably include peptide rod amphiphile molecules. The molecules include an amphiphilic peptide segment and a rod segment which is bonded to the peptide amphiphile through a covalent bond.

[0006] One embodiment of the present invention is a composition that includes a peptide rod amphiphile, or its acid or base addition salts, and a reagent that is used to induce self assembly of the peptide rod amphiphiles.

[0007] Another embodiment of the present invention is a composition that includes self assembled peptide rod amphiphiles and or their salts. In the composition, a plurality of peptide rod amphiphiles are operably linked to adjacent peptide rod amphiphiles, or their salts, to form an organized assembly such as a vesicle, a micelle, or nanotube of the peptide rod amphiphiles.

[0008] The peptide rod amphiphiles of the present invention may be used as part of a course of treating a mammalian patient that includes administering a peptide rod amphiphile composition and/or as a carrier agent to the patient. The composition of the present invention

may be utilized to form biocompatible materials or scaffolds that include nanofiber networks of the self assembled peptide rod amphiphiles.

[0009] In another embodiment of the present invention peptide rod amphiphiles are utilized to encapsulate carbon nanofibers, spherical or rodlike therapeutic compounds, oligomers or monomers that can be polymerized within the core of the PRA self assembled structures.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] Various aspects and applications of the present invention will become apparent to the skilled artisan upon consideration of the brief description of the figures and the detailed description of the invention, which follows:

[0011] FIG. 1A illustrates components of a peptide rod amphiphile of the present invention, FIG 1B and FIG. 1C illustrate non-limiting examples of peptide rod amphiphiles of the present invention.

[0012] FIG. 2 illustrates Peptide Rod Amphiphiles (PRAs) 1-10 based upon glutamic acid in accordance with embodiments of the prevent invention;

[0013] FIG.3 illustrated Peptide Rod Amphiphiles 11-14, based upon glutamic acid in accordance with embodiments the prevent invention;

[0014] FIG. 4 illustrates Peptide Rod Amphiphiles 16 and 17, based upon lysine in accordance with embodiments of the prevent invention;

[0015] FIG. 5 illustrates Rod Segments 18-24 formed by solution phase synthesis;

[0016] FIG. 6 illustrates proposed Rod Segments 25-28 for Peptide Rod Amphiphiles;

[0017] FIG.7 illustrates solution phase synthesis of rod segments 18 and 20;

[0018] FIG. 8 Illustrates solution phase synthesis of rod synthesis of rod segments 19, 21, and 22;

[0019] FIG. 9 Illustrates solution phase synthesis of rod segments 23 and 24;

[0020] FIG. 10 Illustrates TEM micrograph of PRA 2;

[0021] FIG. 11 Illustrates TEM micrograph PRA 4;

[0022] FIG. 12 Illustrates TEM micrograph PRA 6;

[0023] FIG. 13 Illustrates TEM micrograph PRA 8;

[0024] FIG. 14 Illustrates TEM micrograph PRA 10;

[0025] FIG. 15 Illustrates TEM micrograph PRA 12; and

[0026] FIG. 16 Illustrates TEM micrograph PRA 14;

[0027] FIG. 17 Illustrates Peptide Rod Amphiphile 29 as an example of incorporating a drug (indomethacin) into a peptide rod amphiphile and Amphiphiles 30-35 based upon cholesterol;

[0028] FIG. 18 Illustrates Peptide Rod Amphiphiles 42-47;

[0029] FIG. 19 Illustrates Peptide Rod Amphiphiles 48-54 based upon 4'-(octyloxy)-biphenyl-4-carboxylic acid;

[0030] FIG. 20 Illustrates Peptide Rod Amphiphiles 55-61 based upon 4'-(octyloxy)-biphenyl-4-carboxylic acid;

[0031] FIG. 21 Illustrates proposed semi-conductive and fluorescent rod segments 62-67 and the bis(biphenyl ester) rod segment 21 of similar size.

DETAILED DESCRIPTION OF THE INVENTION

[0032] Before the present compositions and methods are described, it is understood that this invention is not limited to the particular molecules, compositions, methodologies or protocols described, as these may vary. It is also to be understood that the terminology used

herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

[0033] It must be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to the "peptide amphiphile" is a reference to one or more peptide amphiphiles and equivalents thereof known to those skilled in the art, and so forth. Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods, devices, and materials are now described. All publications mentioned herein are incorporated herein by reference. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

[0034] The peptide rod amphiphiles of the present invention may be used to form self assembled structures such as but not limited to micelles, vesicles, fibrils, or nanotubes with the capability to control the chemical functionality on the interior and exterior of the structures. The chemical functionality of the structures and the molecules which make them up can include biologically relevant signals for cell organization, tissue growth, or material formation. The chemical functionality can be tailored to have optical, electrical or magnetic properties and or to bond with a variety of compounds including therapeutic drugs or electronic materials which may be encapsulated or present on the surface of the structures. The size and shape of the self assembled structures can also be altered by the design of the peptide rod amphiphile molecules which self assemble to form them.

[0035] Embodiments of the present invention include peptide rod amphiphiles or their acid or basic addition salts. For purposes of illustration only, these molecules include an amphiphilic peptide segment and a rod segment of the molecule that is bonded to the peptide amphiphile through a covalent bond. Preferably the amphiphilic peptide segment has a hydrophilic portion and a hydrophobic end portion and the rod segment is bonded to the hydrophobic portion of the peptide amphiphile. A non-limiting illustration of such a peptide rod amphiphile is illustrated schematically in FIG. 1A. The peptide rod amphiphile may be described by different regions, such as the "core chemistry", the "rod", the "spacer" linkage, the hydrophobic and hydrophilic portions of the "peptide amphiphile", and the "peripheral chemistry". Two non-limiting examples of such peptide rod amphiphiles with these features are illustrated in FIG. 1B and FIG. 1C.

[0036] The PRA's of the present invention may include the use of a facially amphiphilic peptide sequence such as the alternating hydrophobic/hydrophilic sequence LKLKLK of PRA 51. The hydrophobe can be found at either the N-terminus of the peptide as in most of the PRAs and the two representations 1B and 1C or at the C-terminus as in PRAs 30, or 44. The attachment of the hydrophobic rod to the C-terminus allows ready synthesis of branching of the peptide as is also illustrated in PRA 44 which has a second generation lysine dendron exposed on the surface of the PRA nanofibers. A mixture of N-terminal and C-terminal PRAs would give antiparallel beta-sheet formation in addition to the parallel beta-sheet formation which is the only arrangement found for a single N-terminal or C-terminal PRA. Peptide rod amphiphiles of the present invention which have been made include the use of D-amino acids as well as L-amino acids as illustrated by PRAs 55 and 56. PRA 56 has three D-

leucines and PRA 55 has a D-leucine adjacent to the KKK region, and they both gel as well as the all-L version.

[0037] A number of hydrophilic and hydrophobic amino acid residues may be used for those respective sections of the peptide amphphile. The more hydrophobic residues include but are not limited to leucine, phenylalanine, glutamic acid benzyl ester, and glycine. The hydrophilic amino acids include but are not limited to lysine, glutamic acid, and histidine. Mixtures of hydrophobic amino acids and mixtures of hydrophilic amino acids may be used to prepare peptides for the respective sections of the PRAs. In some cases hydrophobic amino acids may not be used at all because, for example, the hydrophobic biphenyl unit of the rod alone can serve that role. However, some hydrophilic residues, preferably near the surface exposed end of the PRA, are required for solubility in water. A clearly defined hydrophobic/hydrophilic region may be absent from the peptide amphiphile segment of the PRA. For example, as it has been discovered that six amino acid residues in the amphiphile segment of a PRA that alternate hydrophobic/hydrophilic also self assemble to give fibers. At the pH at which these PRAs gel, no charges remain, so the role of the chargeable residues is to initially solubilize and disperse the molecules.

[0038] As illustrated in FIG. 1A, the peptide amphiphile may have hydrophobic and hydrophilic portions. For purposes of rendering the entire molecule soluble in water or a solution that includes water, amino acids at the hydrophilic end include polar amino acids. As an example, three or four amino acids in the peptide are preferably non-aliphatic amino acids. The amino acid at the hydrophilic end may be charged or uncharged, and may include mixtures of such amino acids. Examples of suitable amino acids in this portion of the peptide amphiphile include but are not limited to KKK and EEEG as shown in FIG. 1B and FIG. 1 C, as well as for

example EDEE or RGD, EE (PRA 48), HHH (PRA 32), G2 lysine dendrons (PRA 44), KIKVAV (PRA 33), EGRDGS (PRA 35), and KRGDS (PRA 34). The last three PRA'sinclude the biologically active epitopes IKVAV and RGDS with an additional charged amino acid for better water solubility. PRA's 34 and 35 are used together as mixed PRA nanofiber and they form a gel upon their mixing at neutral pH, an important consideration in their use in tissue engineering applications. Mixtures of other PRA's may also be used in the practice of this invention.

[0039] The terminal hydrophilic amino acids may be used to provide bioactivity or other chemical functionality on the periphery of the self assembled micelle, vesicle, nanofibril or nanotube. The hydrophilic portion of the peptide amphiphile may be C or N terminal and may be a polar or non-polar amino acid. The peripheral chemistry may be one or more bioactive peptides included in the hydrophilic peptide portion or at the end of the hydrophilic portion of the peptide.

[0040] The hydrophobic portion of the peptide includes neutral, nonpolar, or aliphatic amino acids. Examples of suitable amino acids or mixtures of them include but are not limited to LLL, or LVL. An additional purpose for adding these hydrophobic amino acids is that the formation of secondary structure, such as beta-sheets, may be facilitated, since the linking spacer and the rod itself are hydrophobic. The hydrophobic portion of the peptide amphiphile maybe covalently bonded to a spacing segment and allows the bulky rod segment to best orient itself within the self-assembled structure.

[0041] The peptide rod amphiphiles (PRAs) compositions of the present invention can be synthesized using preparatory techniques well-known to those skilled in the art preferably by standard solid phase chemistry, with alkylation of the N-terminus of the peptide. Mono or di-

alkyl moieties attached to the N or C termini of the peptides can influence their aggregation and secondary structure in water in both synthetic and natural systems. Other compositions may also be used to induce predetermined geometric orientations of the self-assembled peptide rod amphiphile.

[0042] 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. While materials and methods are described herein, methodologies known in the art may be utilized in accordance with the present invention.

[0043] Peptide components of the present invention preferably include naturally occurring amino acids. However, incorporation of 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 as shown for example in PRA 12 where R' is a t-butyl group. Other organic groups may be substituted, such as but not limited to phenyl or alkyl groups, as would be known to those skilled in the art. Preferably the peptide portion of the PRA includes no greater than about 20 amino acids. Amino acids such as but not limited to cysteine, may also be incorporated into the PRA's to provide for covalent capture of the molecules in self assembled structures as disclosed by Hartgerink et al, Science, vol 294, pp 1684, (2001) the contents of which are incorporated herein by reference in their entirety. Amino acids with L or D configuration may also be used in the peptide amphiphiles and rod segments of the present invention.

[0044] The rod segment of the peptide rod amphiphile may be used to impart additional self-assembling characteristics and structural attributes to these molecules. Additionally these mesogens may lead to novel self-assembled structures not readily obtained through amphiphilic behavior alone, and potential novel electronic or photonic behavior that may also be imparted. For example, (PRA 42 which gels- see Table 1) is fluorescent due to its pyrene hydrodrophobe. Figure 22 illustrates potentially electrically conductive and fluorescent rods 62-67 that may also be used in PRA's. While not necessarily characteristic of a rod segment, incorporating a bifunctional hydrophobic rod segment (a non-rod example would be 6-aminohexanoic acid) also allows the preparation of other architectures such as peptide-rod-peptide or oligo(ethylene glycol)-rod-peptide. The rod segment may be used to impart rigidity to all or a portion of the tail group attached to the peptide amphiphile. Examples of such rods include but are not limited to those that have biphenyl or cholesterol units. Variations in the cross sectional area of the rod segment can be used to manipulate the interaction or bonding between amphiphiles as well as adjusting for the size of functional groups in the hydrophilic portion of the molecule. For example, the bulkier PRAs 53, 54, 57, and 58, all form gels, although they are more fragile, more cloudy, and gel at higher concentrations of PRA than those PRA's with leucines, PRA 55 in the regions of phenylalanine or glutamic acid benzyl ester. This illustrates that PRA structure may be used to control the stability of the gels which can be used for controlled release or degradation of self assembled substrates that include therapeutic compounds of act as scaffolds. The PRAs can have semirigid hydrophobes, not truly rigid ones, as for example PRA 11. A narrow bifunctional aliphatic chain or bifunctional aromatic rod unit of various widths, such as biphenyl or naphthalene, may also be used to allow the attachment of peptides, or chemical functional groups to each side of the rod segment.

[0045] The length, shape, and charge distributions within the amphiphile are important characteristics that can be manipulated. The presence of the hydrophobic rod segment of larger cross-sectional area than an aliphatic hydrocarbon chain dictates the use of amino acids of larger size in the peptide amphiphile than might be needed to surround a the fatty acid core of a standard peptide amphiphile. Examples of larger hydrophilic amino acids include but are not limited to glutamic acid, lysine, histidine, arginine, and phosphoserine; examples of larger hydrophobic amino acids include but are not limited to leucine, phenylalanine, tyrosine, valine, tryptophan, and isoleucine. Many of these additional amino acids are also associated with specific bioactivity that can be incorporated into peptide rod amphiphiles to form well-established self assembled structures.

[0046] The linking spacer segment provides a covalent bond between the peptide amphiphile and the rod portion of the molecule. The spacer is hydrophobic and may include but is not limited to: aminoalkylacids, preferably 6-aminohexanoic acid; alkyl dicarboxylic acids, preferably succinic acid; or amino acids, preferably three glycines. The use of a flexible spacing segment allows the bulky rod segment to best orient itself within the self-assembled structure. PRA's without a spacer element are also capable of forming good gels, such as illustrated by PRA's 46-52 and 55-58 in Table 1.

[0047] Preferably the rod segment or hydrophobe is soluble in the coupling media, in which the free acid can be HBTU activated in NMP or DMF, for coupling to the amino terminus of the resin-bound peptide. In the case of larger rods which are not soluble, the rods may be constructed by solid phase methods on the resin. The rods which may be used in the present invention include but are not limited to biphenyls, single aromatic rings as in p-aminobenzoic acid (PABA) or p-hydroxybenzoic acid (PHBA), and cholesterol. Preferably the one or more

aromatic groups lie along the backbone of the rod as for example in PRA10, 44, and 45 and proposed rods in FIG. 21 . Aliphatic segments such as but not limited to succinic acid and beta-alanine can be incorporated into these rods segments to improve reactivity and to prepare free acids that can be coupled. Other considerations include the biocompatibility of the rod. Cholesterol is a biocompatible rod with universal importance in cell membranes and also has important signaling roles in nerve growth. The biphenyl moiety is a very common mesogen, whose 4'-hydroxy-4-carboxylic acid form has been extensively studied as a rod unit either alone or in oligomeric ester form. 4'-Amino-biphenyl-4-carboxylic acid unit, as in 20 in FIG. 5, has been much less studied, but offers the ability to form a more stable amide linkage. Preparing a version of 20 in which the amino group is tethered to the biphenyl group by a methyl or ethyl spacer would greatly enhance the reactivity of the amine, giving in essence a biphenyl version of 24. With the exception of rod segments 18 and 19 in FIG. 5, the other rod units used in these peptide rod amphiphiles are the largest that can be synthesized by solution methods and then coupled to the peptide by solid phase. This makes rod segment 24 of particular value, because a rod segment can be prepared with it in the desired size by solid phase methods. In addition, it is potentially biocompatible, being comprised of naturally occurring beta-alanine and PABA, which is otherwise known as vitamin Bx and is found in the body, although in small quantities. The beta-alanine-PABA repeat for a rod is a hydrogen-bondable semirigid rod. Cholesterol has been coupled to both glycine and 6-aminohexanoate, with subsequent conversion of the terminal amino groups to amides with succinic anhydride. This renders a free carboxylic acid that can be coupled to the peptide by solid phase methods. 6-Aminohexanoate is also known as the trademarked drug Amicar. The possibilities for additional rod segments include but are not limited to those shown in Figure 21. These may include triphenyl units, naphthalene units,

oligothiophene segments, and oligo(phenylene vinylene) segments illustrated by bifluorenyl rod 62, tetrathiophene rod 63, tetraphenyl rod 64, ter(phenylene ethynylene) rod 65, and ter(phenylene vinylene) rods 66 and 67. Dendritic segments may be useful both in the rod portion and in the peptide portion of the PRA's as illustrated by PRA 44.

[0048] Core chemistry, as illustrated schematically in FIG. 1A, is controlled by the functional group at the rod terminus distal from the peptide amphiphile segment. This chemical functional group would be sequestered at the core of the self-assembled cylindrical micelle, or would form the inner wall of a cylindrical vesicle. The core chemistries may include but are not limited to simple hydrophobic hydrocarbon chains (2, 4, 8, 12, and 16), free phenols (6 and 10), and aromatic ammonium salts (17), as well as tetra(ethylene glycol) segments. These various terminal groups demonstrate that a wide variety of functionalities are compatible within the self-assembled structure, which could be of importance for interacting with specific materials, or drugs that are encapsulated by the self-assembled structure. Other core chemistries may prepared as would be known to one skilled in the art for interacting with molecules or membranes. This includes hydrophilic components such as a tetraethylene glycol segment as well as the aromatic ammonium salt group of PRA 17, that might otherwise be perceived as confusing the amphiphilic character of the molecule. It is also envisioned that drug molecules can be bonded, for example but not limited to ionic or covalent bonds, to the core chemistry functional group of the peptide rod amphiphile. This has been demonstrated with the anti-inflammatory drug indomethacin in PRA 29, illustrated in FIG. 17. Possibilities for manipulations of the core chemistry include the incorporation of longer oligo (ethylene glycol) chains, short peptide sequences, or polymerizable segments. The polymerizable segment may include an oligomer of cysteine, which has previously been used for the covalent capture of

peptide amphiphile nanofibers. The use of peptides such as oligo(serine) or of oligo(ethylene glycol) could allow for the mineralization of the core if the appropriate metal ions are trapped in the core of a micelle or within the pore of a tubular vesicle. The appropriately designed peptide rod amphiphile may also be used to encapsulate carbon nanotubes, which can alter their behavior or enhance their processing.

[0049] One embodiment of the present invention is a composition that includes peptide rod amphiphiles , or its salts, and a reagent which is used to induce self assembly of the peptide rod amphiphiles. Mixtures of different peptide rod amphiphiles may also be present in the composition. Heterogeneous mixtures of PRAs, or of PRA's with fatty acid flexible hydrophobe PAs can all be self assembled to give fibers. The reagent may be added or combined with the PRAs to form a gel in a mold or the PRA's may be combined with a solution such as a bodily fluid from a mammalian subject, to form self assembled structures or gels. Self assembly of various peptide rod amphiphiles may be determined by formation of a gel and through SEM and TEM analysis of the gels for self assembled structures such as nanofibers. Different modes of self-assembly of the peptide-amphiphile molecules into organized structures are possible. This self-assembly generally occurs at percent by weight concentrations of the PRA to form a self-supporting gel. Changes in pH may be used to effect self assembly by exposing the peptide amphiphiles to fluids including but not limited to carbon dioxide, ammonia, and hydrochloric acid. Addition of polyvalent metal ions can induce self assembly of negatively charged peptide-amphiphiles at physiological conditions. Negatively charged peptide-amphiphiles can be self-assembled into nanofibers by addition of polyvalent metal ions such as Ca^{+2}, Mg^{+2}, Zn^{+2}, Cd^{+2}, Fe^{+2}, Gd^{+2}. Addition of charged peptide amphiphiles and complexation may also be used. Preferably the concentration of peptide rod amphiphile or mixtures of them is in the range of

from about 0.01% to about 10 % by weight of the mixture. The pH for self assembly may range from about 1 to about 12, and ion concentration, which may be a cation, anion or charged peptide, may range from about 2 micromolar to about 10 micromolar. Some PRAs are designed to form cylindrical micelles and are made soluble in water or other liquids by the formation of charged carboxylate or ammonium groups at the peptide terminus. The solubility of an ammonium salt containing PRA is lowered upon raising the pH and the solubility of the carboxylate containing PRA is lowered upon lowering the pH, resulting in the gelation of the dilute solution (0.3 to 2 wt %) of self-assembled structures. The synthesis of peptide rod amphiphiles includes a hydrophobic rigid rod segment linked to the end of an amphiphilic peptide such as LLLEEEG or LLLKKK, through a GGG or 6-aminohexanoate (6AHJ) spacer segment. These PRA's can self-assembles into a cylindrical micelle under pH control. Basic solutions of 0.3 to 2 wt % of the carboxylate salt-based peptide rod amphiphiles (PRAs) in water gel when the pH is lowered by the introduction of HCI vapor, whereas ammonium salt-based PRAs in water gel when the pH is raised by the introduction of ammonia.

[0050] Cells may be included with the reagent or formed structures of the present invention which are suitable for injection or implantation. These include but are not limited to hepatocytes and bile duct cells, islet cells of the pancreas, parathyroid cells, thyroid cells, cells of the adrenal-hypothalmic-pituitary axis including hormone-producing gonadal cells, epithelial cells, nerve cells, heart muscle cells, blood vessel cells, lymphatic vessel cells, kidney cells, intestinal cells, cells forming bone, cells forming cartilage, cells forming smooth muscle and cells forming skeletal muscle.

[0051] Compositions of the present invention include self assembled peptide rod amphiphiles. In these compositions a plurality of peptide rod amphiphiles are operably bonded

to adjacent peptide rod amphiphiles, or their salts, to form an organized assembly such as but not limited to vesicles, micelles, fibrils, or nanotubes. The peptide rod amphiphile may be operably bonded with each other in the self assembled structures by bonds including but not limited to van der Waals forces, hydrogen bonds, covalent bonds, dipoles, and ionic bonds.

[0052] The peptide rod amphiphiles of the present invention are designed to self-assemble using the principles of amphiphilic phase-separation phenomenon between hydrophilic and hydrophobic sections of a molecule or oligomer. Preferably self assembly results in structures which are cylindrical micelles, nanotubes, fibrils, and cylindrical vesicles, although the peptide rod amphiphile can be altered to form spherical or lamellar architectures as well. The use of peptides as the backbone of the material is advantageous because of the well-established solid-phase synthetic technology that allows one to automate the synthesis of these materials. Peptides constructed from the naturally occurring amino acids present a wide range of potential functional groups to both aid and enhance the self-assembly as well as to take advantage of their natural bioactivity on the surface of these structures. Examples of such relevant amino acid sequences in the peptide amphiphile include but are not limited to RGDS or IKVAV.

[0053] The present invention is directed to various modes of self-assembly and controlled self-assembly of peptide rod amphiphiles. The formation of a self-supporting matrix or solid comprised of these nanofibers under physiological conditions affords the opportunity to utilize this material alone or in conjunction with other materials for a wide range of purposes, including many in situ applications such as tissue growth and regeneration.

[0054] Biocompatible and or biodegradable gels from self assembled PRA's can be useful as a means of delivering isolated cells into a patient to create an organ equivalent or tissue such as cartilage. These gels may promote engraftment and provide three-dimensional templates

for new cell growth. The resulting tissue can be generally similar in composition and histology to naturally occurring tissue.

[0055] The peptide rod amphiphiles of the present invention may be used a part of a course of treating a mammalian patient that includes administering a peptide rod amphiphile composition and a carrier agent to the patient. The carrier agent preferably includes water although other pharmaceutical and cosmetic carriers known to those skilled in the art may also be used. The composition of the present invention may be utilized to form biocompatible materials or scaffolds that include nanofiber networks.

[0056] Cells can be suspended in a self-assembling peptide rod amphiphile solution and injected directly into a site in a patient, where the peptide rod amphiphile gels and organizes into a matrix having cells dispersed therein. Cells can also be suspended in a self-assembled peptide rod amphiphile composition which is poured or injected into a mold having a desired anatomical shape. The composition then organize to form a matrix having cells dispersed therein which can be implanted into a patient. Preferably the self-assembled peptide rod amphiphile gel degrades, such as by the action of enzymes, leaving only the resulting tissue. Other compounds may be incorporated into or encapsulated by the self assembled peptide amphiphile cores which make up the coating. These compounds may enhance in-growth of blood vessels following implantation or delivery of the nanofiber coated secondary substrate to the body. Nutrients, growth factors, inducers of differentiation or de-differentiation, immunomodulators, inhibitors of inflammation, regression factors, biologically active compounds which enhance or allow in-growth of the lymphatic network or nerve fibers, and drugs can also be incorporated into the self assembled peptide amphiphile nanofiber coating. A number of agents that affect cell proliferation have been tested as pharmacological treatments for stenosis and restenosis in an attempt to slow or inhibit

proliferation of smooth muscle cells. These compositions may include heparin, coumarin, aspirin, fish oils, calcium antagonists, steroids, and prostacyclin. Such agents may be systemically encapsulated in fiber or may additionally be delivered on a more local basis using a drug delivery catheter. In particular, biodegradable peptide amphiphile nanofiber matrices containing one or more pharmaceuticals may be implanted at a treatment site. As the nanofiber degrades, the pharmaceutical is released directly at the treatment site.

[0057] This technology can be used for a variety of purposes. For example, custom-molded cell implants can be used to reconstruct three dimensional tissue defects, e.g., molds of human ears could be created and a chondrocyte-self-assembled peptide amphiphile gel replica could be fashioned and implanted to reconstruct a missing ear. Cells can also be transplanted in the form of a three-dimensional structure that could be delivered via injection.

[0058] The resulting self assembled PRA structure is capable of carrying bioactive peptide sequences at its periphery with the appropriate exchange of amino acids. An important consequence of the incorporation of aromatic rod segments at the core may be the formation of a pore in the center of the micelle. This pore might be capable of encapsulating a nanostructure such as a carbon nanotube. The pore could form nanochannels for the delivery of drugs or ions into cells or reagents into a nanoreactor. The choice of cholesterol as the rod allows for the packaging of this material at the core of the micelle, which is of interest for tissue engineering applications.

[0059] An advantage of the peptide rod amphiphiles of the present invention is that they permit control of both the chemistry at the core of the nanofiber and also permit control of the size of the pore that is formed.

[0060] One existing technology for self assembly of peptide amphiphiles is to use flexible, aliphatic fatty acid hydrophobic segments which orient at the core of the self assembled structure. PRAs using rigid-rod segments based upon biphenyl, cholesterol, and alternating sequences of succinic acid or beta-alanine and 4-amino or 4-hydroxybenzoic acid moieties have shown gelation behavior, and those gels studied by TEM have shown the formation of nanofibers. The rod segments may further be extended to oligomeric benzoic acid, biphenyl, or naphthalene rods in both ester and amide forms. The larger of these rods may lead to the formation of hollow channels at the core, or give rise to novel self-assembling behaviors that would not be observed in the aliphatic hydrophobe containing PAs.

[0061] Therapeutic pharmaceutical compositions, carbon nanotubes, spherical or rod-like structures can be encapsulated by the peptide rod amphiphiles. More particularly, encapsulated nanotubes, methods of encapsulating nanotubes, methods of making encapsulated nanotubes and uses of the encapsulated nanotubes utilizing peptide rod amphiphiles may be made by the methods described in U.S. Pat. Application Serial Number yet to be assigned entitled "ENCAPSULATION OF NANOTUBES VIA SELF-ASSEMBLED NANOSTRUCTURES " and related to U.S. Application Serial Number 10/418,474 filed April 18, 2003, the contents of which are incorporated herein by reference in their entirety. The process includes providing a mass of single wall carbon nanotubes that can include bundles of single walled carbon nanotube fibers held in close association by van der Waals forces, encapsulating the SWCNT with peptide rod amphiphiles to surround the single wall carbon nanotubes to produce solutions, and recovering the encapsulated single wall carbon nanotubes from the solution or suspension. The PRA's may have core chemistry designed to favorably interact with carbon nanotubes or functionally derivatized carbon nanotubes.

[0062] The biphenyl rods of the present invention may be used as a model system for nanowire or conduction materials. The biphenyls are not conductive, but their rigid rod like character more or less mimics the character of the more interesting oligo(phenylene vinylene), oligo(thiophene) rods. Replacement of the biphenyl rod units in such PRA's with these or similar short oligomers of the semiconductive materials may lead to conductive self assembled materials. Alternatively, monomers such as thiophene or its derivatives could be loaded into the core or pore of a self assembled PRA (not covalently attached to the rod) and could then be polymerized within the core. Here the biphenyls, as aromatic hydrophobes, may offer better encapsulation of the small monomers. The formation of fibers from the larger rods of PRA's 8 and 10 suggests that rigid rod units based on, for example, ter(phenylene vinylene) or terthiophene, would also form nanofibers due to the similarity in their structures. The process of self-assembly may align these semiconductive rods at the cores of the nanofibers in a manner that may lead to a conductive core, and hence something like nanowires would result.

[0063] Three types of hydrophobes - flexible palmitic acid aliphatic, rigid aromatic biphenyl, and rigid aliphatic cholesterol - trap the dye Nile Red and terthiophene (trimer of thiophene) at their cores as determined by their fluorescence. Nile red was used as a model drug whereas terthiophene as a model monomer.

[0064] The present invention will be further understood by reference to the following non-limiting examples.

EXAMPLE 1

[0065] This example illustrates synthesis of rod segments. *Materials*. Abbreviations: 6AH, 6-aminohexanoic acid or 6-aminohexanoate; Bn, benzyl; t-Bu, *tert*-butyl; BOC, *tert*-butyloxycarbonyl; BOC2O, di tert butyl dicarbonate; Chol, cholesteryl; DIEA, *N,N*-

diisopropylethylamine; DMAP, 4-(dimethylamino)-pyridine; DMF, *N,N*-dimethylformamide; DPTS, 4 (dimethylamino) pyridine p toluenesulfonic acid salts; EDCI, 1-3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride; Fmoc, 9-fluorenylmethoxycarbonyl; Glu (E), L-glutamic acid; Gly (G), glycine; HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1 - hydroxybenzotriazole hydrate; Leu (L), L-leucine; PABA, p aminobenzoic acid; PHBA p hydroxybenzoic acid; Lys (K), L-lysine; Pd/C, 10% palladium on activated carbon, Degussa type El0l NE/W (ca. 50% water by weight); TEA, triethylamine; TFA, trifluoroacetic acid; Val (V), L-valine. The starting materials, reagents, and solvents were obtained from Bachem, Fisher Scientific, Novabiochem and Sigma-Aldrich and were used without further purification. Anhydrous DMF was stored over activated 4 Å molecular sieves and TEA and DIEA were stored over KOH.

[0066] Reactions were performed by stirring at room temperature (rt) in capped flasks unless noted otherwise. Solutions were concentrated by rotary evaporation followed by drying at 0.3 torr, and chromatography was performed over 230-400 mesh Merck flash silica gel, grade 9385. Transmission electron microscopy (TEM) was carried out on a Hitachi H8100 instrument at the Electron Probe Instrumentation Center at Northwestern University using negative staining with phosphotungstic acid. The gels based on L lysine were formed by dissolving the TFA salt peptides in distilled water at concentrations of 0.1 to 2.0% by wt in a 5 ml vial, which was then placed in a chamber containing a vial of ammonium hydroxide for 4 to 12 hrs. The gels based on glutamic acid were formed by dissoving the peptides in 0.1 N KOH at concentrations of 1-2 % by wt in a 5 mL vial, and placing these solutions in a chamber containing a vial of concentrated hydrochloric acid for 8 to 20 hrs. Gelation usually occurred in 20 min to 3 hrs.

[0067] General Procedure for Solid Phase Peptide Synthesis. The peptides described in this disclosure were prepared from HMPB-BHA resin preloaded with Fmoc-Gly or from Fmoc-protected Rink Amide MBHA resin. The resin was swollen with DMF by shaking for 30 min with DMF, then the first Fmoc group was cleaved by shaking twice with 30% piperidine in DMF (10 mL), followed by washing with DMF. The amino acids were then coupled and Fmoc-deprotected in the designed sequences, terminating with the rod segment. The coupling protocol is the standard HBTU coupling method in which 4 equivalents of the amino acid, 3.95 equivalents of HBTU, and 6 equivalents of DIEA are used in DMF (10-20 mL) for 30 min to 12 hour, depending upon the amino acid coupled. Equivalents are based upon substitution level of the resin or exposed amino groups for coupling. The amino acid was preactivated with 1 min of shaking in a 20 mL vial prior to adding this solution to the shaker vessel. After each coupling, the resin was washed well with DMF. After the final coupling, the resin was additionally washed well with CH_2C1_2. For some of the rod segments, such as 24, the amounts used in the couplings were halved to waste less material. Ninhydrin tests were performed to ensure complete conversion after every coupling. Cleavage of Fmoc groups before the next coupling was accomplished by twice shaking the resin with 30% piperidine in DMF (10 mL) for 10-20 mm. Cleavage of the peptide from the HMPB-BHA resin was accomplished with 3% TFA in CH_2C1_2 for 10 min, to give 1, 3, 5, 7, 9, and 11. The completely deprotected peptide rod amphiphiles 2, 4, 6, 8, 10, 12, and 14, were prepared by treatment with 95% TFA, 2.5% H_20, and 2.5% TIS for 3 h. Cleavage of the peptide rod amphiphiles 16, and 17, from the appropriately Rink Amide MBHA resin bound peptides was achieved by shaking for 3 h with 95% TFA, 2.5 H_20, and 2.5% TIS. The peptides were obtained by concentrating the cleavage solutions, triturating the peptide with Et_20, and drying at 0.3 torr overnight.

[0068] Cholesteryl N-BOC-6-aminohexanoate. A 500 mL round-bottom flask equipped with a magnetic stirring bar was charged with *N*-BOC-6-aminohexanoic acid (12.4 g, 53.6 mmol), cholesterol (17.3 g, 44.7 mmol), DMAP (2.4 g, 19.6 mmol), and CH_2C1_2 (150 mL). The mixture was cooled in an ice bath, then EDCI (10.43 g, 54.4 mmol) was added and the solution was stirred at 8 °C for 2 h. After stirring at rt overnight, the mixture was diluted with CH_2C1_2 (300 mL) and extracted with water (2 x 200 mL). The organic phase was dried over $MgSO_4$, filtered, and concentrated to a residue that was chromatographed, eluting with 3% MeOH in CH_2C1_2, to yield 26.14 g (43.6 mmol, 97%) of a white solid.

[0069] Cholesteryl 6-aminohexanoate hydrochloride. A250 mL round-bottom flask was charged with cholesteryl *N*-BOC-6-aniinohexanoate (15.37 g, 25.62 mmol) and HC1 (4M in dioxane, 45 mL). The mixture was capped and swirled to dissolve the solid, then sat at rt for 10 min with frequent venting. The resulting opaque solution/solid was diluted with CH_2C1_2 (20 mL) and after 2 h was concentrated to a solid that was triturated with Et_20 (2 x 10 mL) to give 13.25 g (24.7 mmol, 96%) of a hydroscopic crystalline powder. The remainder of the product was recovered from the ether washings.

[0070] Cholesteryl 6-aminohexanoate semisuccinamide (18). A 100 mL round-bottom flask equipped with a magnetic stirring bar was charged with cholesteryl 6-aminohexanoate hydrochloride (1.68 g, 3.13 mmol), succinic anhydride (0.314 g, 3.14 mmol), CH_2C1_2 (40 mL), and DIEA (1.1 mL, 6.3 mmol). After stirring at rt for 5 h, the mixture was diluted with CH_2C1_2 (150 mL) and extracted with 5% citric acid (2 x 100 mL). The organic phase was dried over $MgSO_4$, filtered, and concentrated to 1.88 g (3.13 mmol, 100%) of a white solid.

[0071] N-BOC-4-Bromoaniline. A 100 mL round-bottom flask equipped with a magnetic stirring bar was charged with 4-bromoaniline (8.60 g, 50.0 mmol), BOC_2O (13.27 g,

60.8 mmol), TEA (14.3 g, 103 mmol), and CH_2Cl_2 (80 mL). After stirring at rt for 2d, the mixture was diluted with CH_2Cl_2 (300 mL) and extracted with water (2 x 200 mL) and 5% citric acid (2 x 200 mL). The organic phase was dried over $MgSO_4$, filtered, and concentrated to a residue that was chromatographed, eluting with 100% CH_2Cl_2, to yield 4.0 g (14.7 mmol, 29%) of a white solid.

[0072] N-BOC-4'-Amino-biphenyl-4-carboxylic acid (20). A 50 mL round-bottom flask was charged with N-BOC-4-bromoaniline (1.10 g, 4.04 mmol), 4-carboxyphenylboronic acid (0.67 g, 4.04 mmol), K_2CO_3 (0.73 g, 5.28 mmol), $Pd(PPh_3)_4$ (0.19 g, 0.16 mmol), and 50 mL of a 4:1 mixture of water:diglyme. The mixture was degassed, flushed with N_2, and heated to 90°C overnight. The solution was diluted with CH_2Cl_2 (150 mL) and extracted with 5% citric acid (2 x 80 mL). The solid that formed in the organic phase was filtered off to give 0.43 g (1.34 mmol, 33%) of the product as a white powder. The filtrate was chromatographed, eluting with 10% MeOH in CH_2Cl_2, to give an additional 0.46 g (1.47 mmol, 36%) of the product as a gray powder.

[0073] Ethyl-4'-tert-butoxy-biphenyl-4-carboxylate. A 1 L glass bottle equipped with a large magnetic stirring bar was charged with ethyl-4'hydroxy-biphenyl-4-carboxylate (18.2 g, 75.1 mmol), concentrated sulfuric acid (2 mL), and CH_2Cl_2 (400 mL). The mixture was cooled in an ice bath, then isobutylene (120 mL) was added and the solution was tightly capped and stirred vigorously at rt overnight. The mixture was directly chromatographed, eluting with 100% CH_2Cl_2, to yield 19.7 g (66 mmol, 88%) of the product as a white solid and the remainder of the starting material (2.2 g, 9.1 mmol, 12%).

[0074] 4'-tert-Butoxy-biphenyl-4-carboxylic acid (19). A 500 mL round-bottom flask equipped with a magnetic stirring bar and a reflux condenser was charged with ethyl-4'-tert-

butoxy-biphenyl-4-carboxylate (1.92 g, 6.42 mmol), NaOH (1N in water, 100 mL) and EtOH (100 mL). The mixture was refluxed for 3 h, then was diluted with 5% citric acid (250 mL) and extracted with CH_2C1_2 (2 x 200 mL). The organic phase was dried over MgSO4, filtered, and concentrated to 1.72 g (6.36 mmol, 99%) of a white solid.

[0075] Dimethyithexylsilyl 4'-hydroxy-biphenyl-4-carboxylate. A 100 mL round-bottom flask equipped with a magnetic stirring bar was charged with 4'-hydroxy-biphenyl-4-carboxylic acid (9.54 g, 44.5 mmol), dimethylthexylsilyl chloride (9.2 mL, 46.8 mmol), morpholine (8.8 mL, 101 mmol), and CH_2C1_2 (80 mL). The solution was stirred at rt overnight, then was diluted with CH_2C1_2 (200mL) and extracted with 5% citric acid (3 100 mL). The organic phase was dried over MgSO4, filtered, and concentrated to 15.7 g (44.0 mmol, 99%) of a clear, colorless oil which crystallized upon sitting.

[0076] Dimethyithexylsilyl 4'-tert-butoxy-bis(biphenyl ester)-4-carboxylate. A 250 mL round-bottom flask equipped with a magnetic stirring bar was charged with 4'-teri-butoxy-biphenyl-4-carboxylic acid (0.66 g, 2.44 mmol), dimethylthexylsi lyl 4' -hydroxybiphenyl-4-carboxylate (0.87 g, 2.44 mmol), DPTS (0.75 g, 2.55 mmol), and CH_2C1_2 (100 mL). EDCI (0.54 g, 2.82 mmol) was added and the mixture was stirred overnight. The solution was diluted with CH_2C1_2 (200 mL) and extracted with 5% citric acid (3 x100 mL) and brine (100 mL). The organic phase was dried over MgSO4, filtered, and concentrated to a residue that was chromatographed, eluting with 100% CH_2C1_2, to give 0.95 g (1.56 mmol, 64%) of a white solid.

[0077] Dimethylthexlsiyl -4 '-('octyloxy,)-bis(biphenyl ester) -4-carboxylate. A 250 mL round-bottom flask equipped with a magnetic stirring bar was charged with 4'- (octyloxy)-biphenyl-4-carboxylic acid (2.76 g, 8.45 mmol), dimethylthexylsilyl-4 ' -hydroxy-biphenyl-4-carboxylate (3.0 g, 8.41 mmol), DPTS (2.57 g, 8.73 mmol), and CH_2C1_2 (200 mL). EDCI (1.82

g, 9.49 mmol) was added and the mixture was stirred for 8 h. The solution was diluted with CH_2C1_2 (200 mL) and extracted with water (3 x 100 mL) and brine (100 mL). The organic phase was dried over $MgSO_4$, filtered, and concentrated to a residue that was chromatographed, eluting with 100% CH_2C1_2, to give 4.54g (6.83 mmol, 81%) of a white solid.

[0078] 4 '-tert-Butoxy-bis(biphenyl ester) -4-carboxylic acid (22). A 250 mL round-bottom flask equipped with a magnetic stirring bar was charged with dimethylthexylsilyl-4'-tert-butoxy-bis(biphenyl ester)-4-carboxylate (0.60 g, 0.99 mmol) and THF (100 mL). The solution was stirred under N2 at -78°C, then TBAF (IM in THF, 1.5 mL, 1.5 mmol) was added. After an additional 45 mm at -78°C, acetic acid (0.25 mL) was added and the solution was warmed to rt. The solution was diluted with CH_2C1_2 (200 mL) and extracted with water (2 x 100 mL) and brine (100 mL). The organic phase was dried over $MgSO_4$, filtered, and concentrated to 0.92 g of a residue that contained both the product and the silyl fluoride byproduct.

[0079] 4 -(Octyloxy)-bis(biphenyl ester)-4-carboxylic acid (21). A 250 mL round-bottom flask equipped with a magnetic stirring bar was charged with dimethyithexylsilyl-4'-(octyloxy)-bis(biphenyl ester)-4-carboxylate (0.95 g, 1.43 mmol) and THF (100 mL). The solution was stirred under N_2 at -78°C, then TBAF (lM in THF, 2.0 mL, 2.0 mmol) was added. After an additional 45 mm at -78°C, acetic acid (0.5 mL) was added and the solution was warmed to rt. The product was collected by filtration.

[0080] N-Fmoc-β-Alanyl-PABA-benzyl ester. A 100 mL round-bottom flask equipped with a magnetic stirring bar was charged with N-Fmoc-β-alanine (3.12 g, 10.0 mmol), benzyl 4-aminobenzoate (1.14 g, 5.02 mmol), and CH_2C1_2 (35 mL). The mixture was cooled in an ice bath, then DMAP (0.08 g, 0.66 mmol) and EDCI (1.99 g, 10.4 mmol) were added and the mixture was stirred over ice for 2 h and at rt overnight. The mixture was diluted with CH_2C1_2

(150 mL) and extracted with water (100 mL), 5% citric acid (2 x 100 mL), 5% $NaHCO_3$ (2 x 100 mL), and brine (100 mL). The organic phase was dried over $MgSO_4$, filtered, and concentrated to a residue that was chromatographed, eluted with 5% MeOH in CH_2C1_2, to yield 2.44 g (4.69 mmol, 93%) of a white solid.

[0081] alanyl-PABA-benzyl ester. A 100 mL round-bottom flask equipped with a magnetic stirring bar was charged with N-Fmoc-β-alanyl-PABA-benzyl ester (0.54 g, 1.04 mmol), piperidine (1 mL), and CH2C12 (30 mL). The solution was stirred at rt for 3 h, then was concentrated to a residue that was triturated with Et_20 (2 x 10 mL) to give 0.30 g (1.01 mmol, 97%) of the product as a white solid.

[0082] 4-(Octyloxy)-PHBA-β-alanyl-PABA-benzyl ester. A 250 mL round-bottom flask equipped with a magnetic stirring bar was charged with β -a1anyl-PABA-benzyl ester (0.149 g, 0.499 mmol), 4-(octyloxy)benzoic acid (0.253 g, 1.01 mmol), DPTS (0.299 g, 1.02 mmol), EDCI (0.336 g, 1.75 mmol), and CH_2C1_2 (20 mL). After stirring the solution at rt overnight, the mixture was directly chromatographed, eluting with 7% MeOH in CH_2C1_2, to give 0.26 g (0.49 mmol, 98%) of a white solid.

[0083] 4-(Octyloxy)-PHBA-β-alanyl-PA BA free acid (23). A 100 mL round-bottom flask equipped with a magnetic stirring bar was charged with 4-(octyloxy)-PHBA-β-alanyl-PABA-benzyl ester (0.22 g, 0.415 mmol), Pd/C (0.056 g), CH_2C1_2 (40 mL), and MeOH (40 mL). The flask was fitted with an H_2 balloon and the mixture was stirred vigorously at rt for 45 min, then was filtered through celite and concentrated to give 0.16 g (0.363 mmol, 88%) of a white solid.

[0084] Dimethyithexylsilyl 4-aminobenzoate. A 1 L round-bottom flask equipped with a magnetic stirring bar was charged with 4-aminobenzoic acid (27.5 g, 200.5 mmol), CH_2Cl_2

(200 mL), morpholine (40 mL, 459 mmol), and dimethylthexylsilyl chloride (43 mL, 219 mmol). The mixture was stirred at rt overnight, then was extracted with water (2 x 100 mL). The organic phase was dried over $MgSO_4$, filtered, and concentrated to 61.41 g (220 mmol, 110%) of a pale yellow oil that solidified upon cooling in the refrigerator.

[0085] Dimethylthexylsilyl N-Fmoc-β-alanyl-4-aminobenzoate. A 100 mL round-bottom flask equipped with a magnetic stirring bar was charged with N-Fmoc-β-alanine (10.2 g, 32.8 mmol), crude dimethylihexylsilyl-4-aminobenzoate (9.92 g, 30 mmol), and CH_2C1_2 (100 mL). The mixture was cooled in an ice bath, then DMAP (0.15 g, 1.23 mmol) and DIPC (4.9 mL, 31.3 mmol) were added and the mixture was stirred over ice for 2 h and at rt overnight. The mixture was diluted with CH_2C1_2 (250 mL) and extracted with water (200 mL), and brine (200 mL). The organic phase was dried over $MgSO_4$, filtered, and concentrated to a residue that was chromatographed, eluting with 5% MeOH in CH_2C1_2, to yield 9.9 g (17.3 mmol, 53%) of a white solid.

[0086] N-Fmoc-β-Alanyl-4-aminobenzoic acid (24). A 500 mL round-bottom flask equipped with a magnetic stirring bar was charged with dimethylthexylsilyl N-Fmoc-β-alanyl-4-aminobenzoate (5.74 g, 10.0 mmol) and THF (150 mL). The solution was cooled under N_2 to -78°C then TBAF (1M in THF, 11 mL, 11 mmol) was added. After an additional 45 mm at -78°C, acetic acid (2.5 mL) was added and the solution was warmed to rt. The mixture was diluted with CH_2C1_2 and extracted with 5% citric acid (150 mL). The organic phase was filtered to collect 3.66 g (10.2 mmol) of a white solid.

EXAMPLE 2

[0087] This example illustrates synthesis of PRA's from the rods of Example 1 and the formation and characterization of self assembled structures with them. The PRA's synthesized

to date include 1-10, in FIG. 2; 11-14, in FIG. 3; and 16 and 17 in FIG. 4. The molecules 1-14, were assembled by solid phase methods on the acid sensitive HMPB-BHA resin from commercially available resin preloaded with Gly, in which the sequences LLLE(OtBu)E(OtBu)E(OtBu), GGGLLLE(OtBu)E(OtBu)E(OtBu), and 6AH-LLLE(OtBu)E(OtBu)E(OtBu) were grown using HBTU activated coupling of commercially available amino acids and piperidine cleavage of the Fmoc protecting groups. Similarly, the PRAs 16 and 17, were prepared from the sequence 6AH-LLLK(BOC)K(BOC)K(BOC) on a Rink Amide MBHA resin. The rod segments that require solution phase synthesis are outlined in FIG. 5, with their synthesis shown in FIGs. 7-9. Proposed additional rod targets are shown in FIGs. 6 and 21. These are coupled to the appropriate resin bound peptide by HBTU activation. Rod segment 4'-(octyloxy)-biphenyl-4-carboxylic acid used for 1-4, is commercially available, but the other rod segments were synthesized by solution methods, as outlined in FIG. 7-9. Cholesterol-6-aminohexanoate semisuccinamide 18 was prepared in three steps and N-BOC-4'-amino-biphenyl-4-carboxylic acid 20 was prepared in two steps, as shown in FIG. 7. Biphenyl ester rods 21 and 22 were prepared in three steps from commercially available 4'-hydroxyl-biphenyl-4-carboxylic acid; rod segment 19 is prepared in two steps from commercially available ethyl-4'-hydroxyl-biphenyl-4-carboxylic acid as shown in FIG. 8. The potentially biocompatible aromatic/aliphatic rods 23 and 24 were prepared in several steps as outlined in FIG. 9. One advantage of using 24 is that the rigid PABA unit can be incorporated into the rod segment of varying lengths by SPPS, whereas 23, with an un-reactive hydrocarbon terminus, can only be used as an end cap for the PRA. Use of the HMPB-BHA resin rather than the traditional Wang resin allowed for the preparation of side chain protected peptides 1, 3, 5, 7, 9, 11, and 13, upon cleavage from the resin with 3% TFA. They were then fully deprotected by treatment with

neat TFA or a mixture of 95% TFA, 2.5% water, and 2.5% triisopropylsilane, to give PRAs 2, 4, 6, 8, 10, 12, and 14, although these could also have been formed directly by cleavage from the resin with nearly neat TFA. Rink Amide MBHA resin was used for target PRAs 16 and 17, because this gives a terminal primary amide rather than a terminal carboxylic acid. Preliminary results on the gel-forming characteristics of the PRA's include the observations made in Table 1:

Table 1.

(PRA)	Wt%	Gelation Behavior	Clarity
2	1.0	self-supporting gel	cloudy
4	1.0	self-supporting gel	clear
6	1.0	self-supporting gel	translucent
8	1.0	self-supporting gel	translucent
	0.5	weak gel	translucent
10	1.0	self-supporting gel	translucent
	0.25	weak gel	cloudy
12	0.7	self-supporting gel	clear
	0.25	weak gel	clear
14	2.0	self-supporting gel	translucent
16	1.0	self-supporting gel	clear
17	1.0	self-supporting gel	clear
29	1.5	self-supporting gel	translucent
30	3	weak gel	clear
31	1.0	self-supporting gel	clear

(PRA)	Wt%	Gelation Behavior	Clarity
32	1.0	weak gel	translucent
33	1.0	self-supporting gel	translucent
34	2.0	self-supporting gel	translucent
35	2.0	self-supporting gel	translucent
42	0.5	self-supporting gel	clear
44	1.0	self-supporting gel	clear
	0.5	weak gel	clear
45	1.0	self-supporting gel	clear
46	1.0	self-supporting gel	clear
47	1.0	self-supporting gel	translucent
48	1.0	self-supporting gel	translucent
49	1.0	self-supporting gel	translucent
50	0.5	self-supporting gel	clear
51	0.5	self-supporting gel	clear
52	0.5	self-supporting gel	clear
53	0.5	self-supporting gel	clear
54	1.0	fragile gel	translucent
55	0.5	self-supporting gel	clear/translucent
56	0.75	self-supporting gel	clear
57	1.0	self-supporting gel	translucent
58	2.0	self-supporting gel	opaque

[0088] The TEM of PRA's 2, 4, 6, 8, 10, 12, and 14 show the presence of nanofibers in the 0.5-2 wt % gels as negatively stained with phosphotungstic acid, and are shown in FIGs. 10-16, respectively. The fiber width varies according to the size of the hydrophobe. For example, most of the PRAs with a single biphenyl rod core such as PRA 6, have diameters of 6-7 nm, whereas the larger cholesterol rod of PRA 14 gives a fiber with an 8-9 nm diameter. The bisbiphenyl ester rod PRAs 8 and 10, have diameters of approximately 8 nm and 7 nm, respectively; for PRA 4, the diameter is approximately 5 nm.

EXAMPLE 3

[0089] This example illustrates encapsulation of molecules by the peptide rode amphiphile of the present invention. The tests for encapsulation utilized Nile Red and terthiophene. Nile Red may be considered a model for the encapsulation of hydrophobic drugs and terthiophene may be considered to be a model polymerizable monomer that can be encapsulated by the PRA's. Sonicating 2 mg of Nile Red or terthiophene with 1 wt % solutions of PRAs 16 and 31 resulted in the formation of dark red solutions containing Nile Red and fluorescent solutions of the terthiophene, in behavior typical of surfactants. Terthiophene is not soluble in water and the dye Nile Red only gives a red color in hydrophobic environments, indicating their encapsulation within micelle-like structures present in the PRA solutions. This red color and fluorescent behavior is maintained for the Nile Red and terthiophene, respectively, when the PRA solutions are gelled by exposure to NH_3, in which these materials are presumably encapsulated within the hydrophobic core of the nanofibers.

[0090] All of the embodiments disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the structure of the peptide

rod amphiphiles, their self assembled structure, methods and condition for making them, the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents that are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention. The self-assembled material has potential applications as biomaterial scaffolds for tissue engineering or drug delivery, coatings for carbon nanotubes for improved manipulation, or as templates for the growth of inorganic minerals.

[0091] 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 rod 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 with reference to examples of peptides and pendant groups, it is also contemplated that gels or related systems of other peptide rod amphiphiles can be self assembled and 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.

CLAIMS

WHAT IS CLAIMED IS:

1. A peptide rod amphiphile composition comprising: an amphiphilic peptide or salt thereof having a hydrophobic end portion; and a rod segment linked thereto via a covalent bond.

2. The composition of claim 1, wherein said rod segment includes a oligomer or a polymer.

3. The composition of claim 1, wherein said rod segment is linked to said peptide amphiphile portion through a spacer segment.

4. The composition of claim 3, wherein said spacer segment is chosen from the group consisting of non-polar amino acid peptides, aminoalkylacids and alkyl dicarboxylic acids.

5. The composition of claim 3, wherein said spacer segment selected from the group consisting of GGG, succinic acid, and aminohexanoate.

6. The composition of claim 1, wherein said rod segment includes a cholesterol moiety.

7. The composition of claim 1 wherein said rod segment includes one or more aromatic moieties.

8. The compound of claim 1 wherein said rod is attached to said hydrophobic end at a proximal portion and is attached to a core chemical portion at the distal end.

9. The composition of claim 1, wherein said peptide-amphiphile includes a residue with a functional moiety capable of intermolecular covalent bond formation.

10. The composition of claim 8, wherein said residue is cysteine.

11. A composition comprising:

    a peptide rod amphiphile, or acid addition salts thereof;

    and a reagent to induce self assembly of said peptide rod amphiphiles.

12. A self assembled peptide rod amphiphile composition comprising:

    a plurality of peptide rod amphiphiles, said peptide rod amphiphiles operably bound to adjacent peptide rod amphiphiles and forming an organized assembly of said peptide rod amphiphiles, said peptide rod amphiphiles including an amphiphilic peptide or acid addition salt thereof, said amphiphilic peptide having a hydrophobic end portion and a rod segment linked thereto via a covalent bond.

13. The composition of claim 12 wherein said peptide amphiphile includes a functional peripheral portion attached to said hydrophilic end.

14. The composition of claim 12, wherein said rod is attached to a core chemical portion at the distal end.

15. The composition of claim 12 further comprising cells.

16. The composition of claim 12 wherein said organized assembly includes one or more nanotubes formed by said peptide rod amphiphiles.

17. The composition of claim 12 further comprising cells.

18. The composition of claim 12 further comprising semiconducting oligomers.

19. The composition of claim 12 further comprising a therapeutic composition encapsulated by said organize assembly.

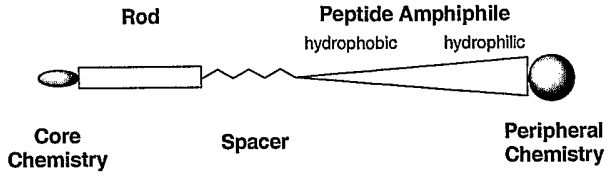
20. A method of treating a patient comprising:

administering a composition including peptide rod amphiphile to said patient.

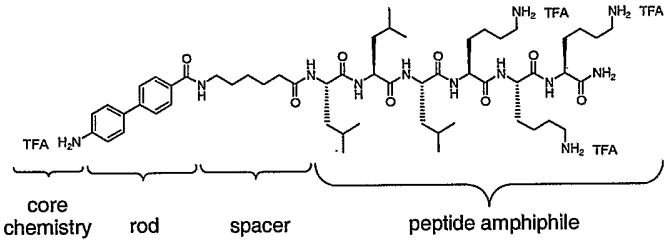
21. The method of claim 20 wherein said composition further comprises a carrier agent selected from the group consisting of a pharmaceutically acceptable carrier, a cosmetically acceptable carrier, and a tissue engineering carrier.

22. The method of claim 20 wherein said composition is a gel of self assembled peptide rod amphiphiles.

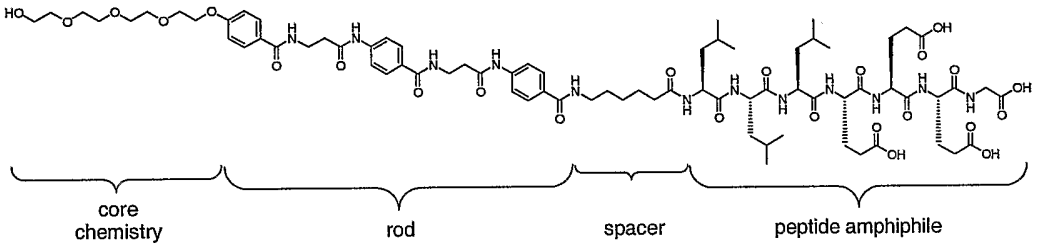
1A

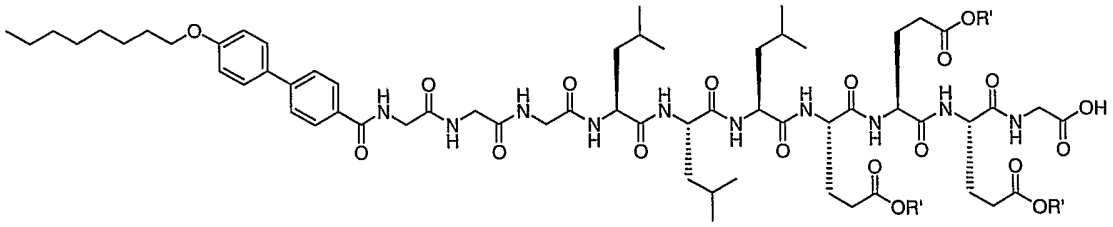

Rod
Peptide Amphiphile
hydrophobic
hydrophilic
Core Chemistry
Spacer
Peripheral Chemistry

1B

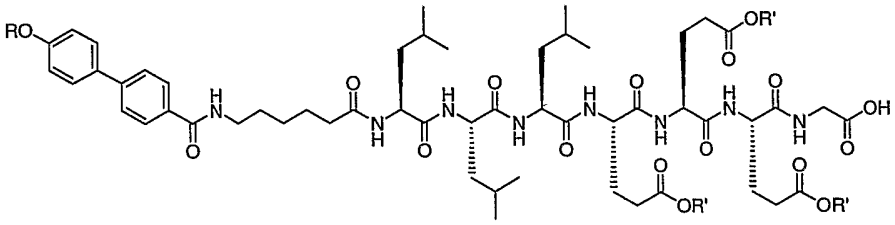

TFA H2N
NH2 TFA
NH2 TFA
NH2
NH2 TFA
core chemistry
rod
spacer
peptide amphiphile

1C

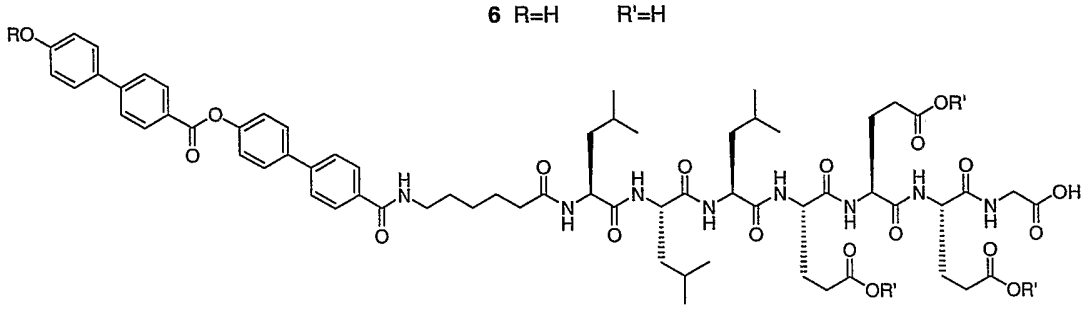

HO
OH
OH
OH
OH
core chemistry
rod
spacer
peptide amphiphile



1 R'=tBu
2 R'=H

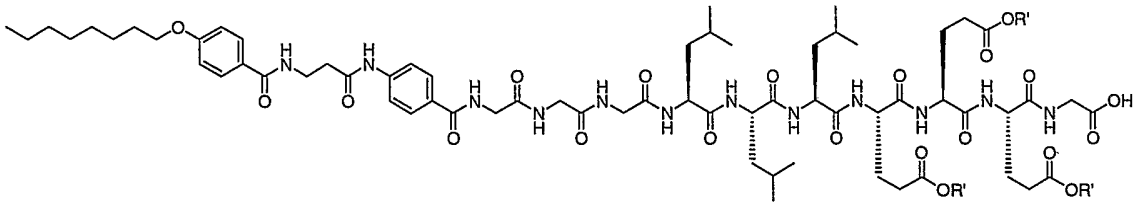


3 R=octyl R'=tBu
4 R=octyl R'=H
5 R=tBu R'=tBu
6 R=H R'=H

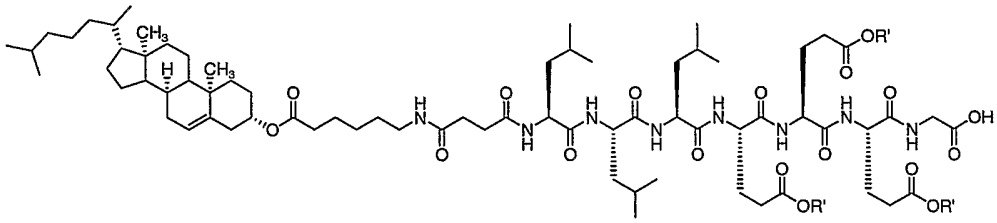


7 R=octyl R'=tBu
8 R=octyl R'=H
9 R=tBu R'=tBu
10 R=H R'=H

FIG. 2

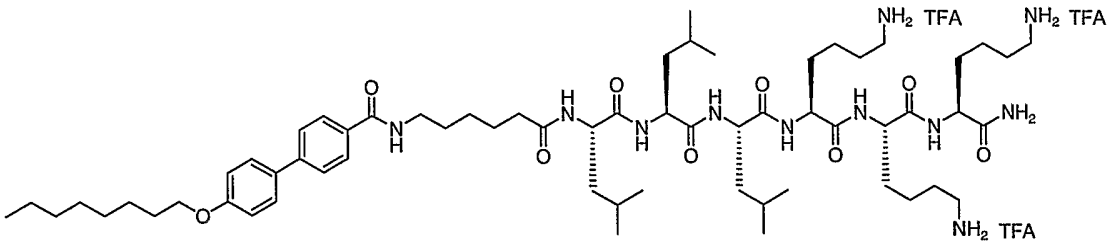


11 R'=H
12 R'=tBu

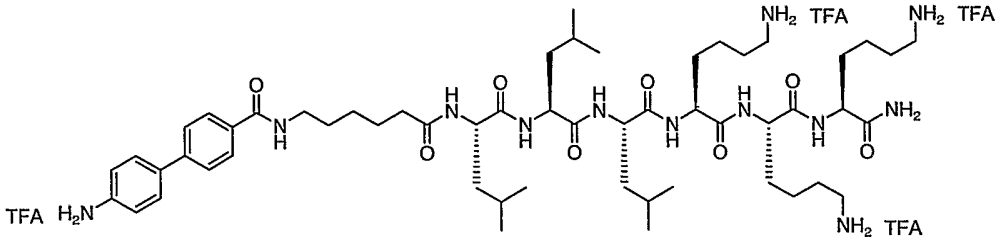


13 R'=H
14 R'=tBu

FIG. 3

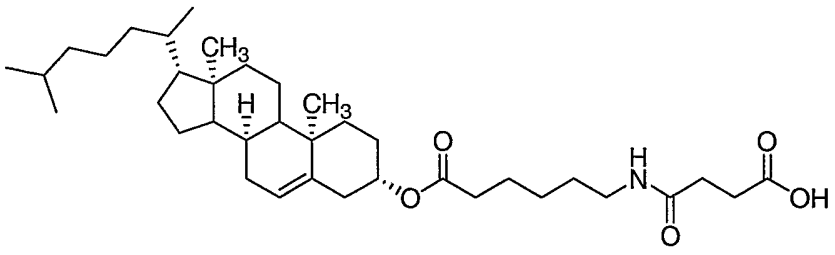


16

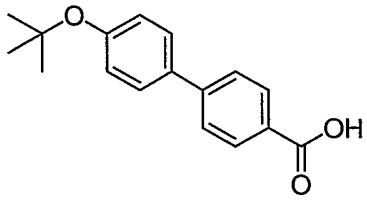


17

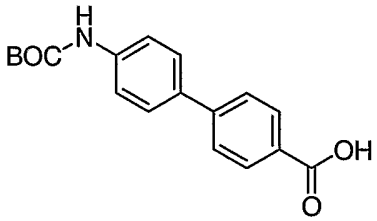
FIG. 4



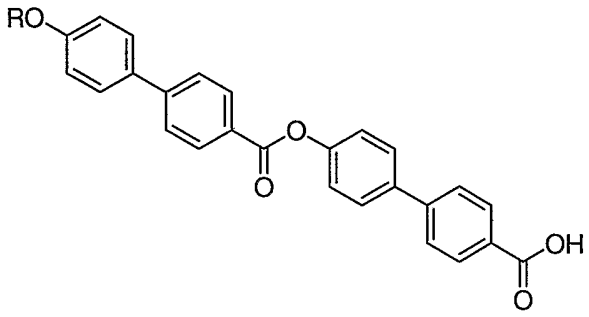
18



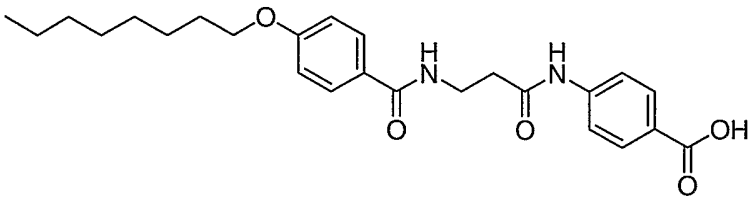
19



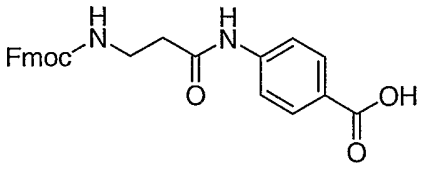
20



21 R=octyl
22 R=tBu

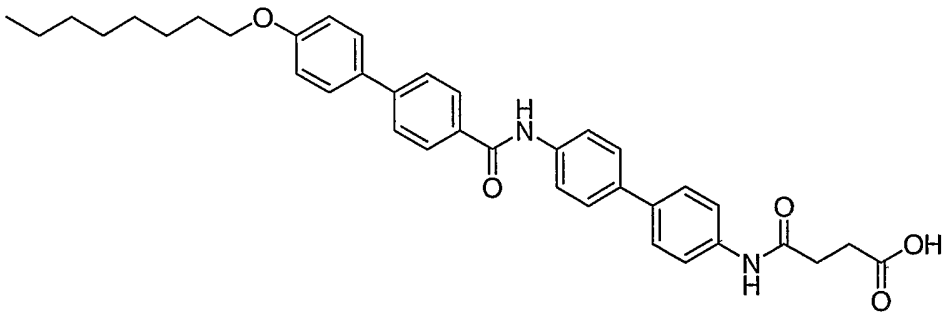


23

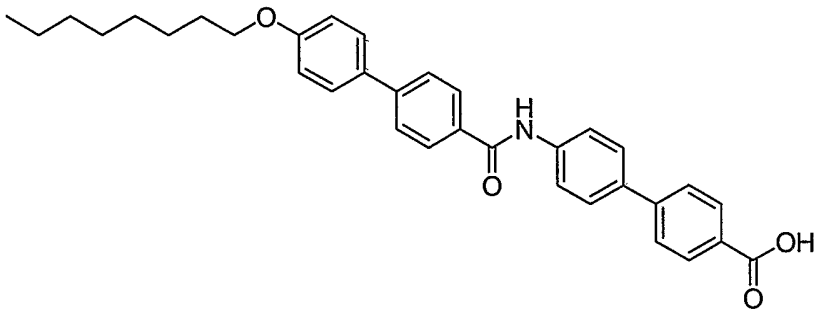


24

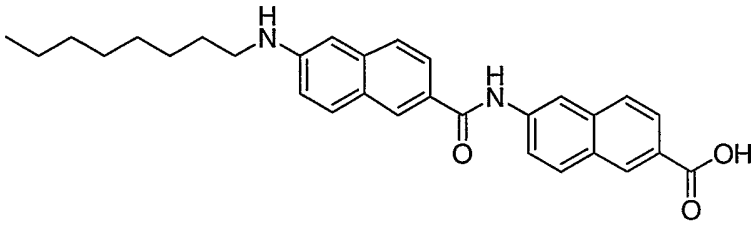
FIG. 5



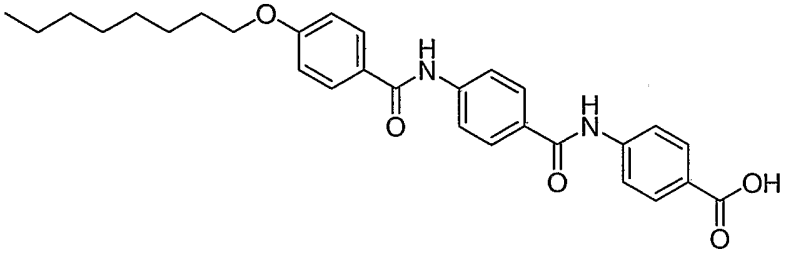
25



26



27



28

FIG. 6

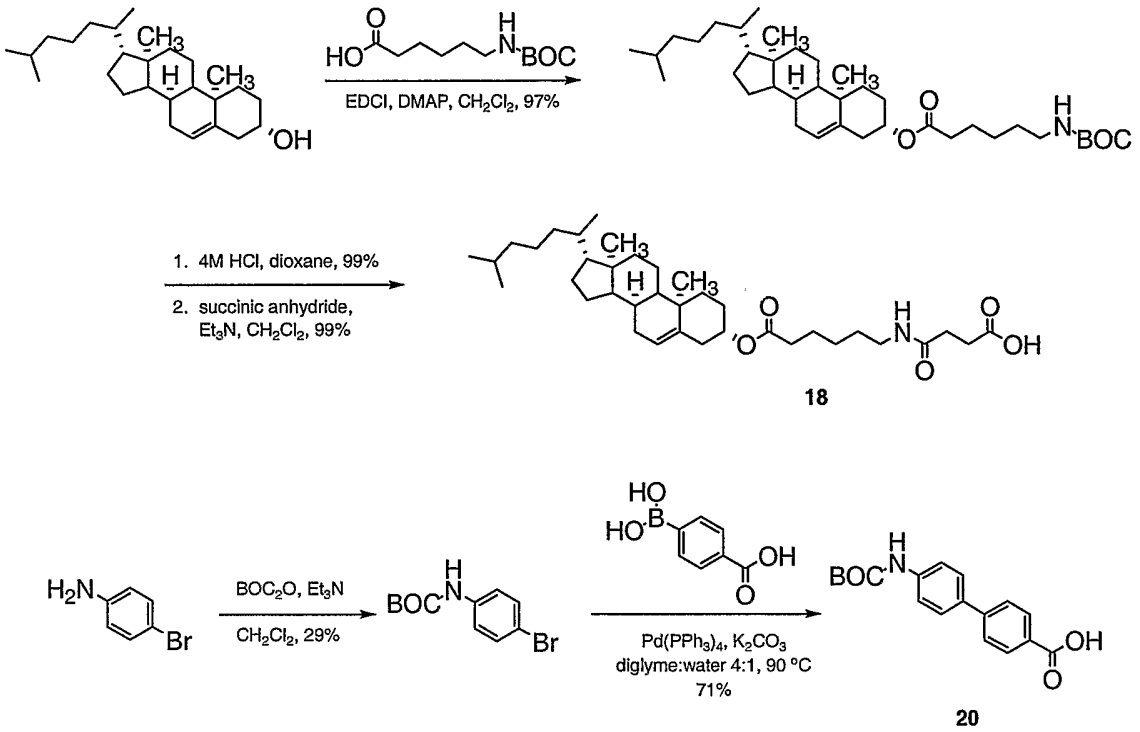

EDCI, DMAP, CH2Cl2, 97%
1. 4M HCl, dioxane, 99%
2. succinic anhydride, Et3N, CH2Cl2, 99%
18
BOC2O, Et3N
CH2Cl2, 29%
Pd(PPh3)4, K2CO3
diglyme:water 4:1, 90 °C
71%
20

FIG. 7

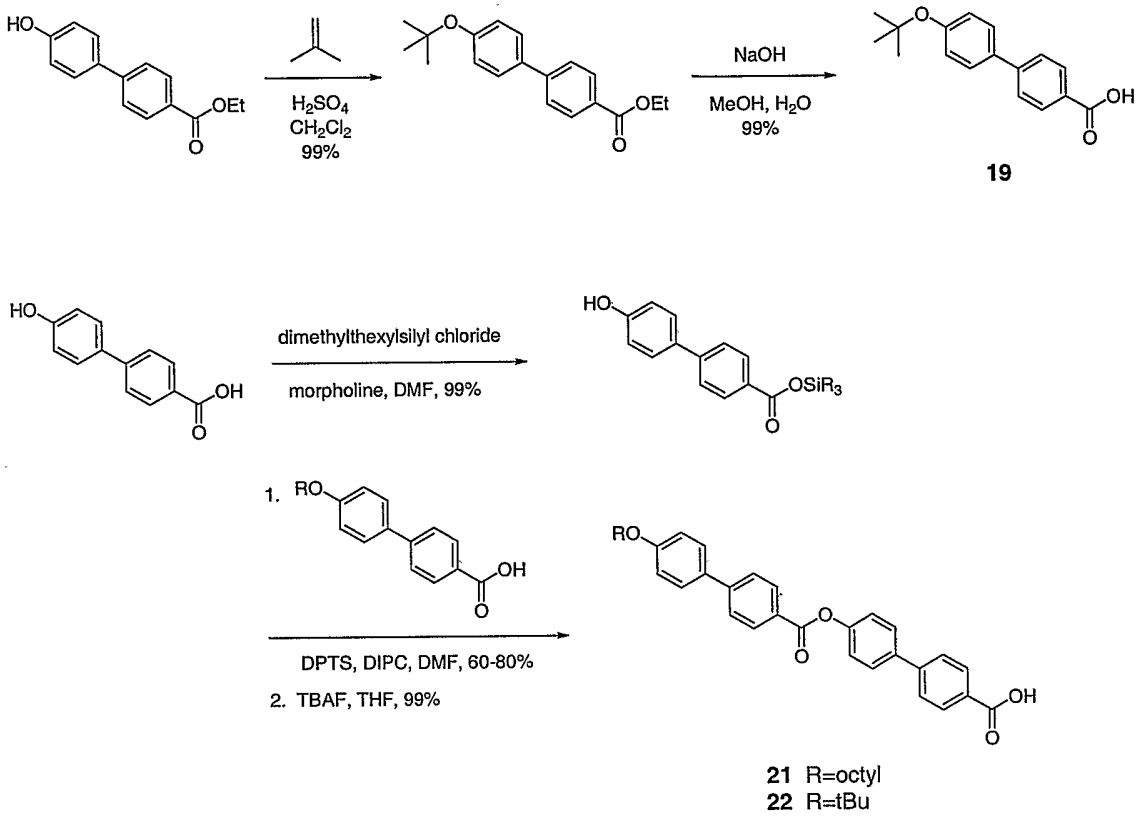

HO
OEt
O
H2SO4
CH2Cl2
99%
O
OEt
O
NaOH
MeOH, H2O
99%
O
OH
O
19
HO
OH
O
dimethylthexylsilyl chloride
morpholine, DMF, 99%
HO
OSiR3
O
1. RO
OH
O
DPTS, DIPC, DMF, 60-80%
2. TBAF, THF, 99%
RO
O
O
OH
O
21 R=octyl
22 R=tBu

FIG. 8

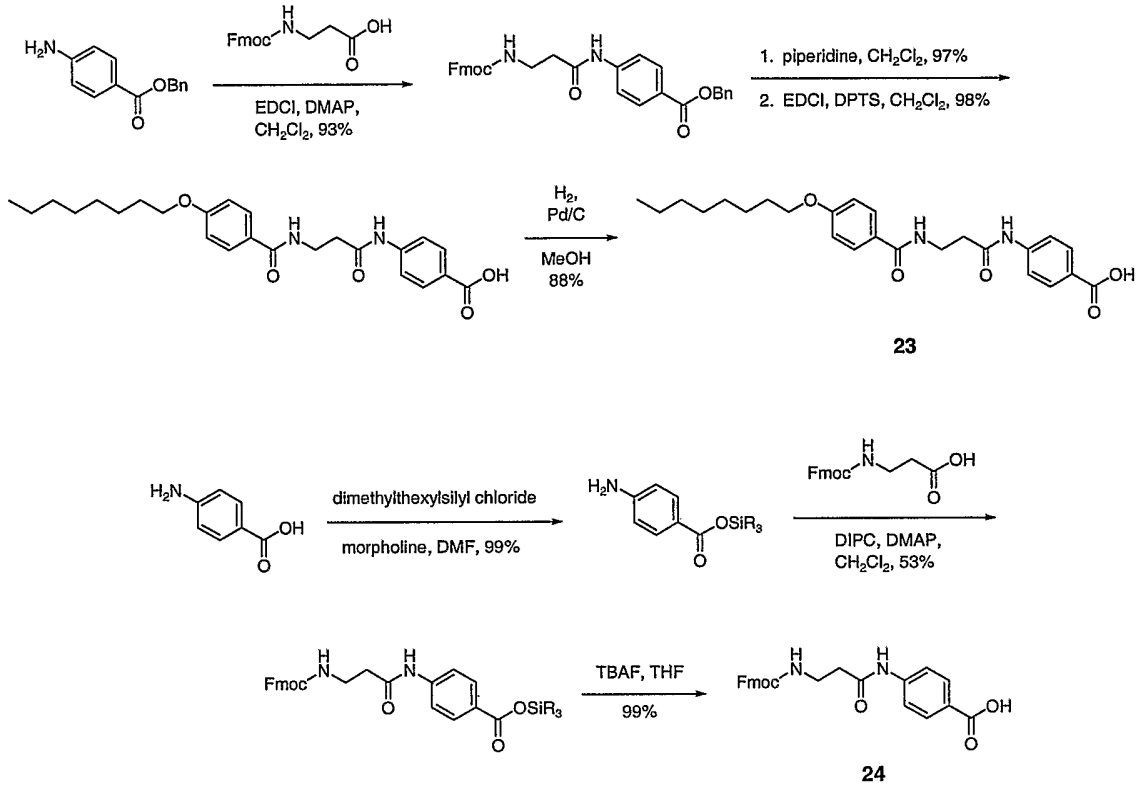

EDCI, DMAP,
CH_2Cl_2, 93%
1. piperidine, CH_2Cl_2, 97%
2. EDCI, DPTS, CH_2Cl_2, 98%
H_2,
Pd/C
MeOH
88%
23
dimethylthexylsilyl chloride
morpholine, DMF, 99%
DIPC, DMAP,
CH_2Cl_2, 53%
TBAF, THF
99%
24

FIG. 9

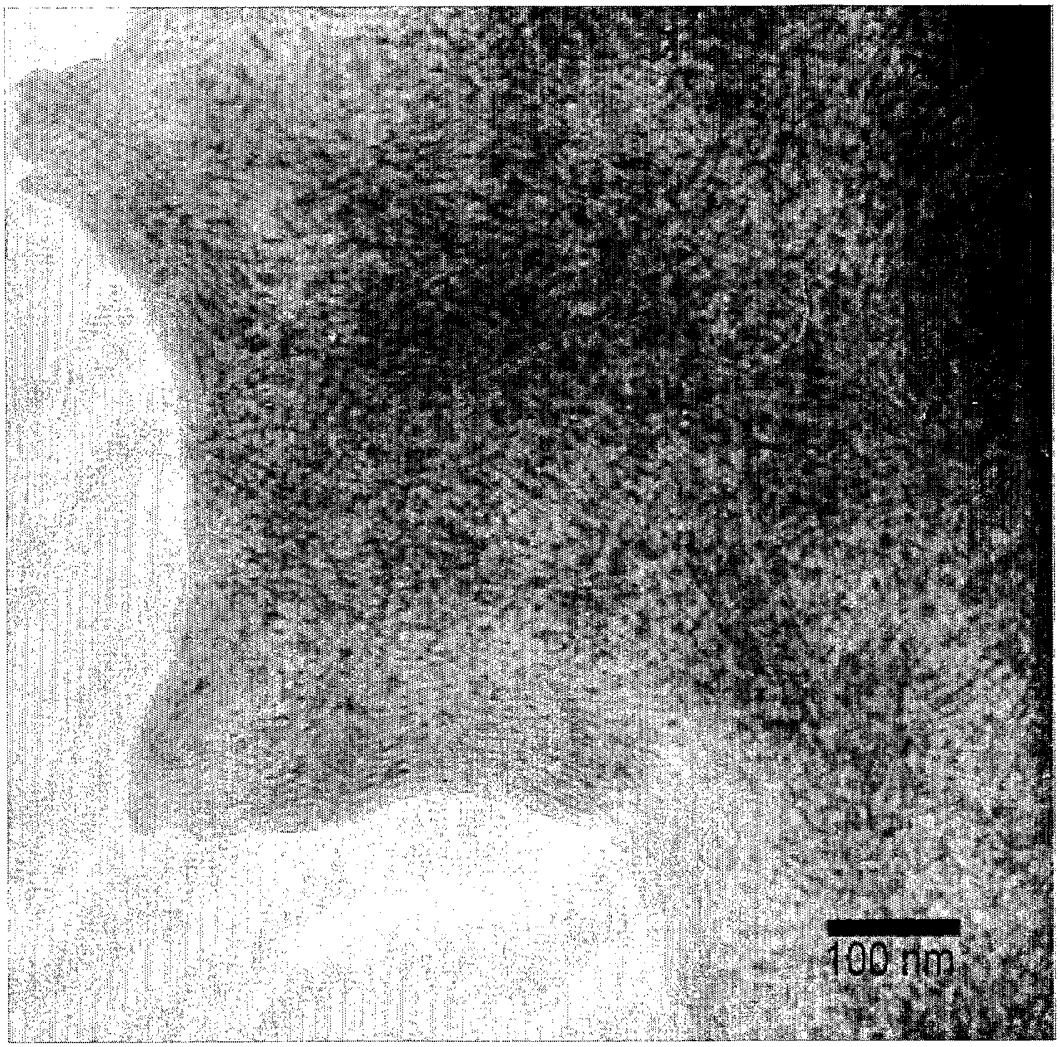


FIG. 10

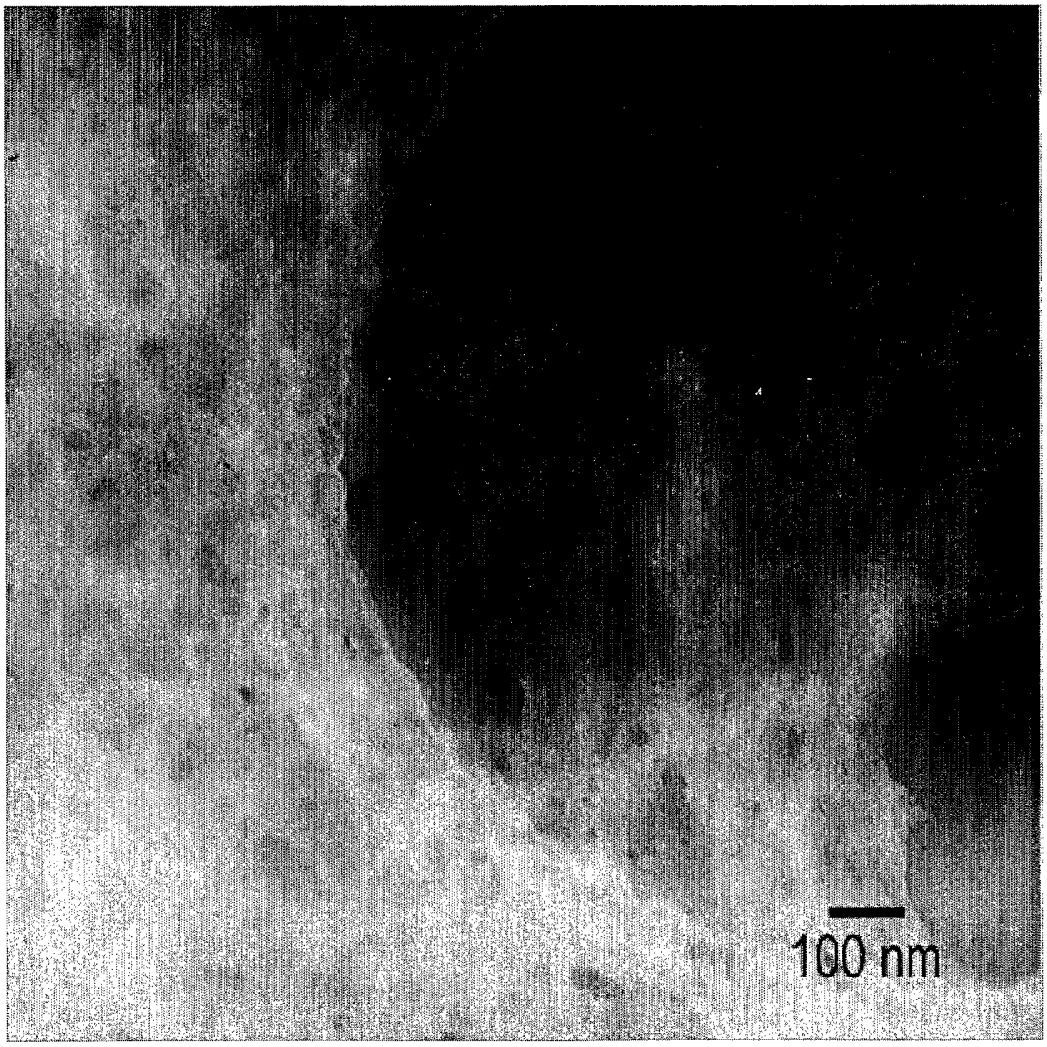


FIG. 11

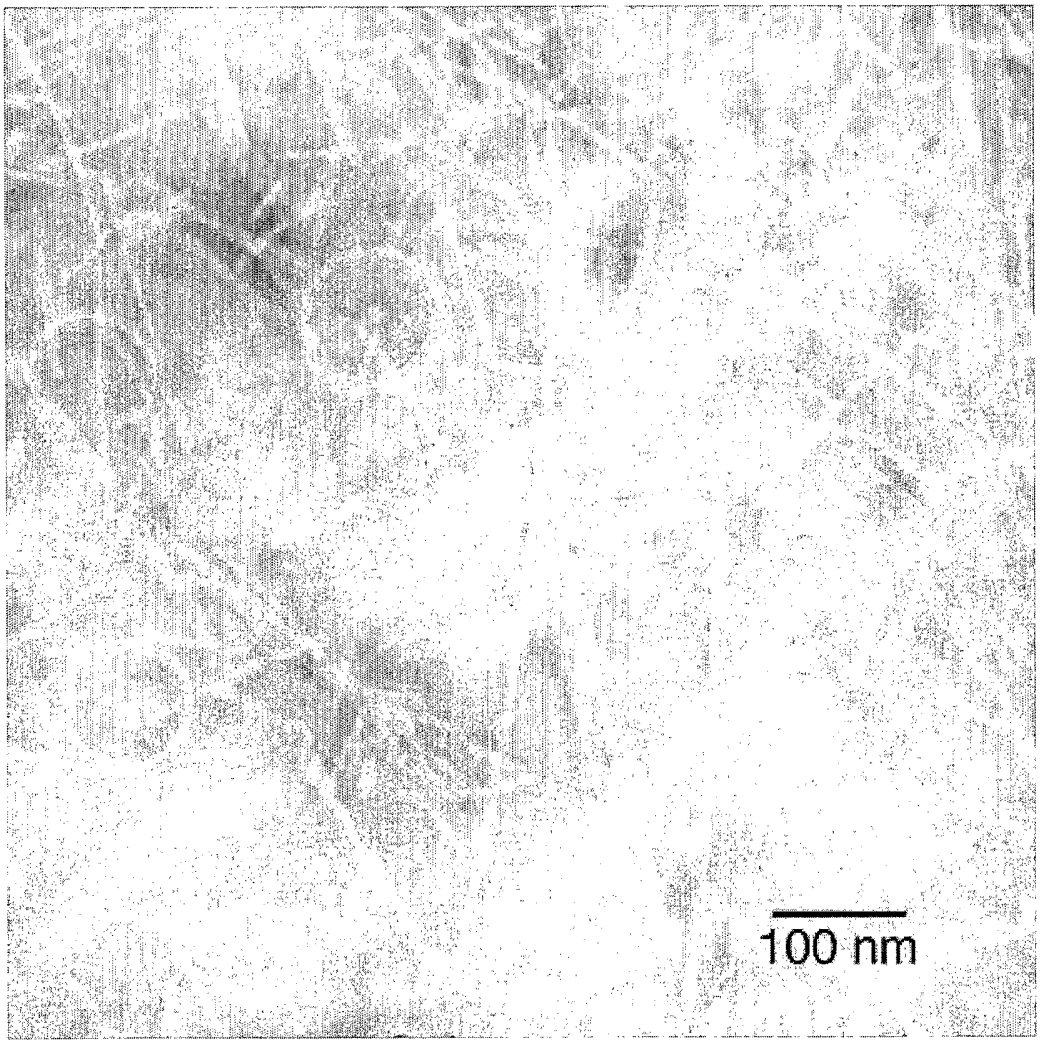


FIG. 12

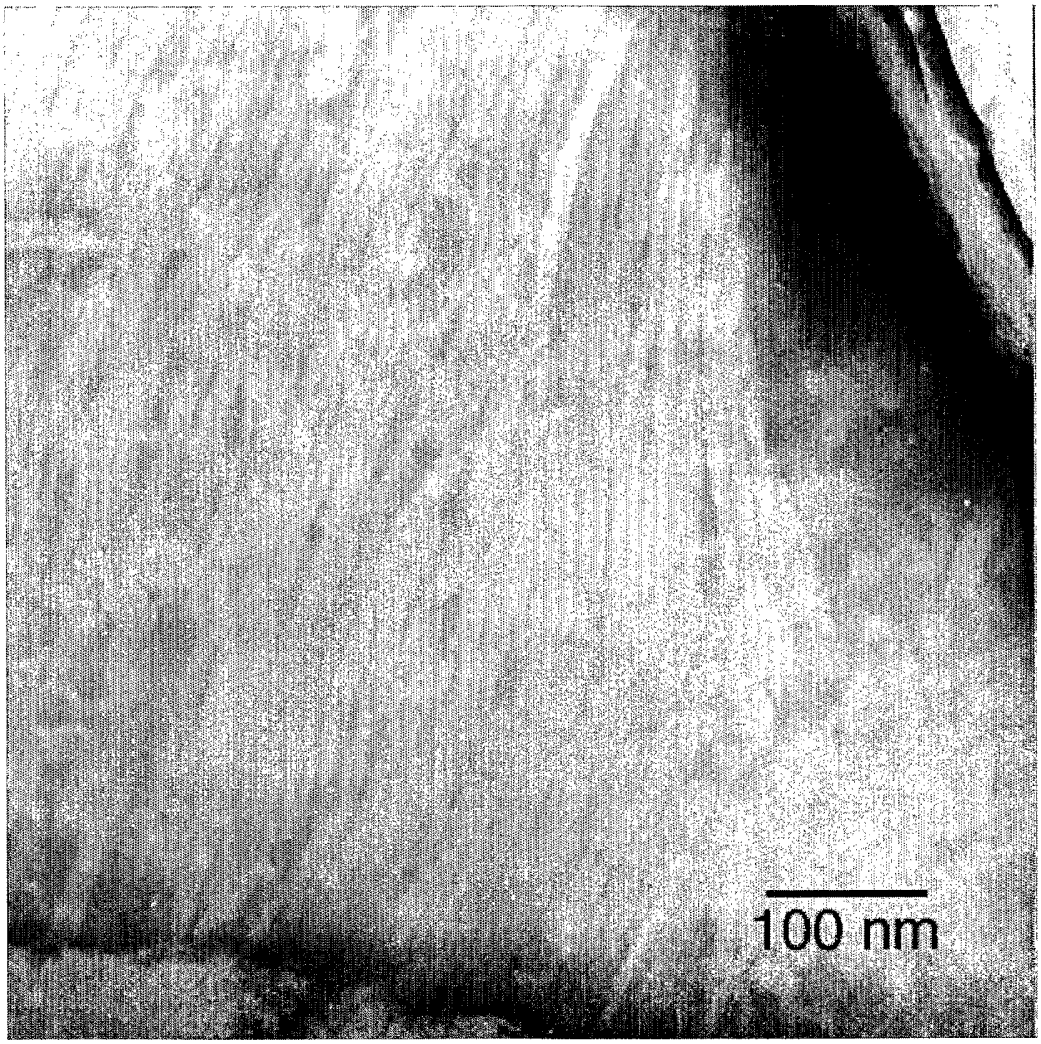


FIG. 13

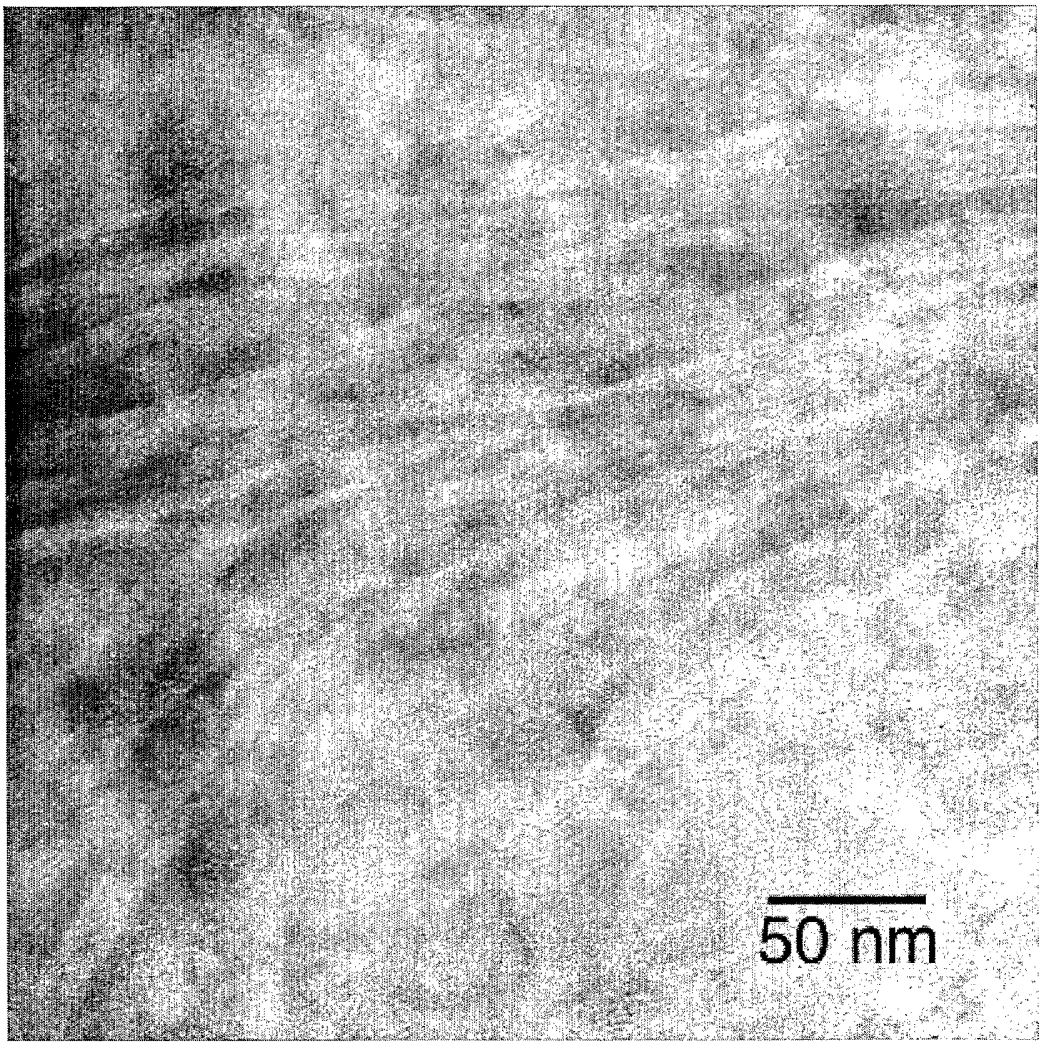


FIG. 14

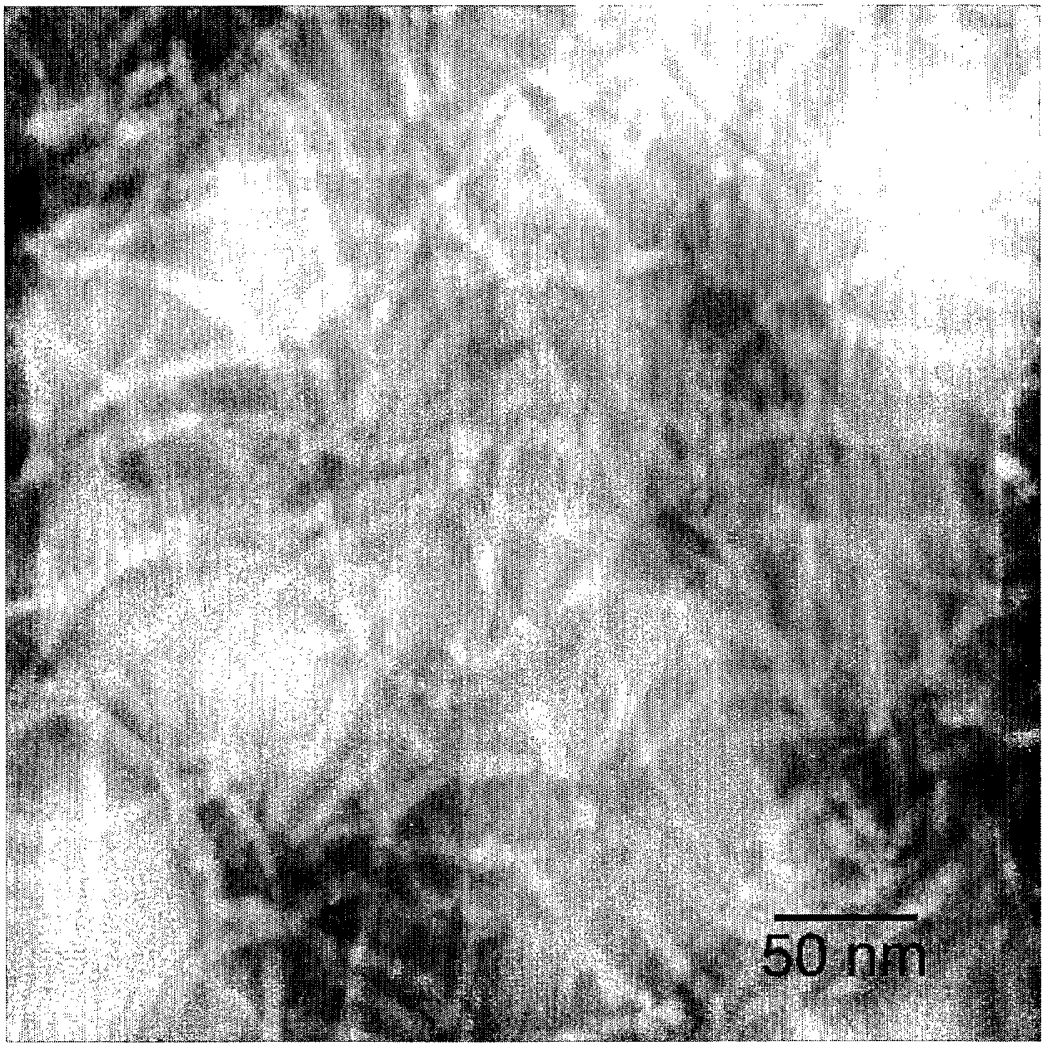


FIG. 15

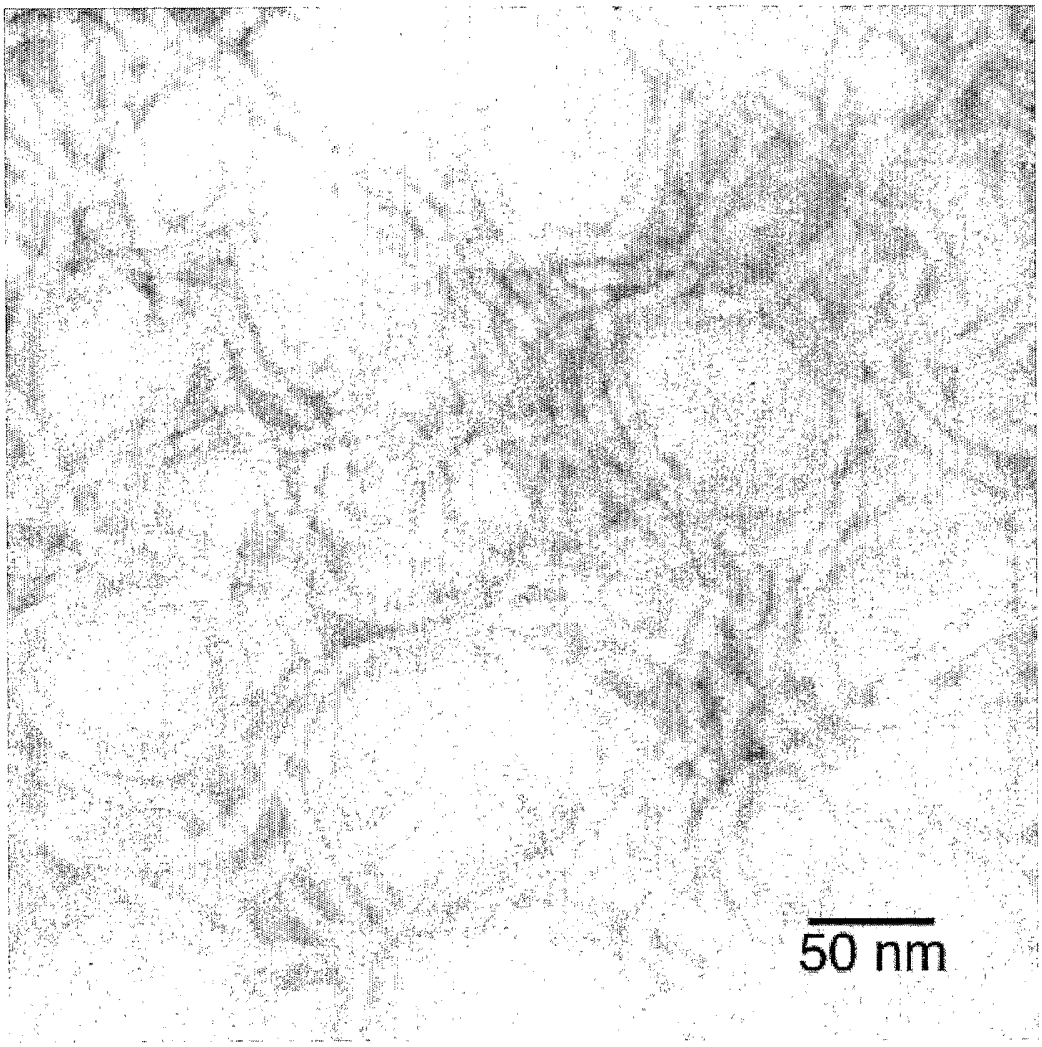


FIG. 16

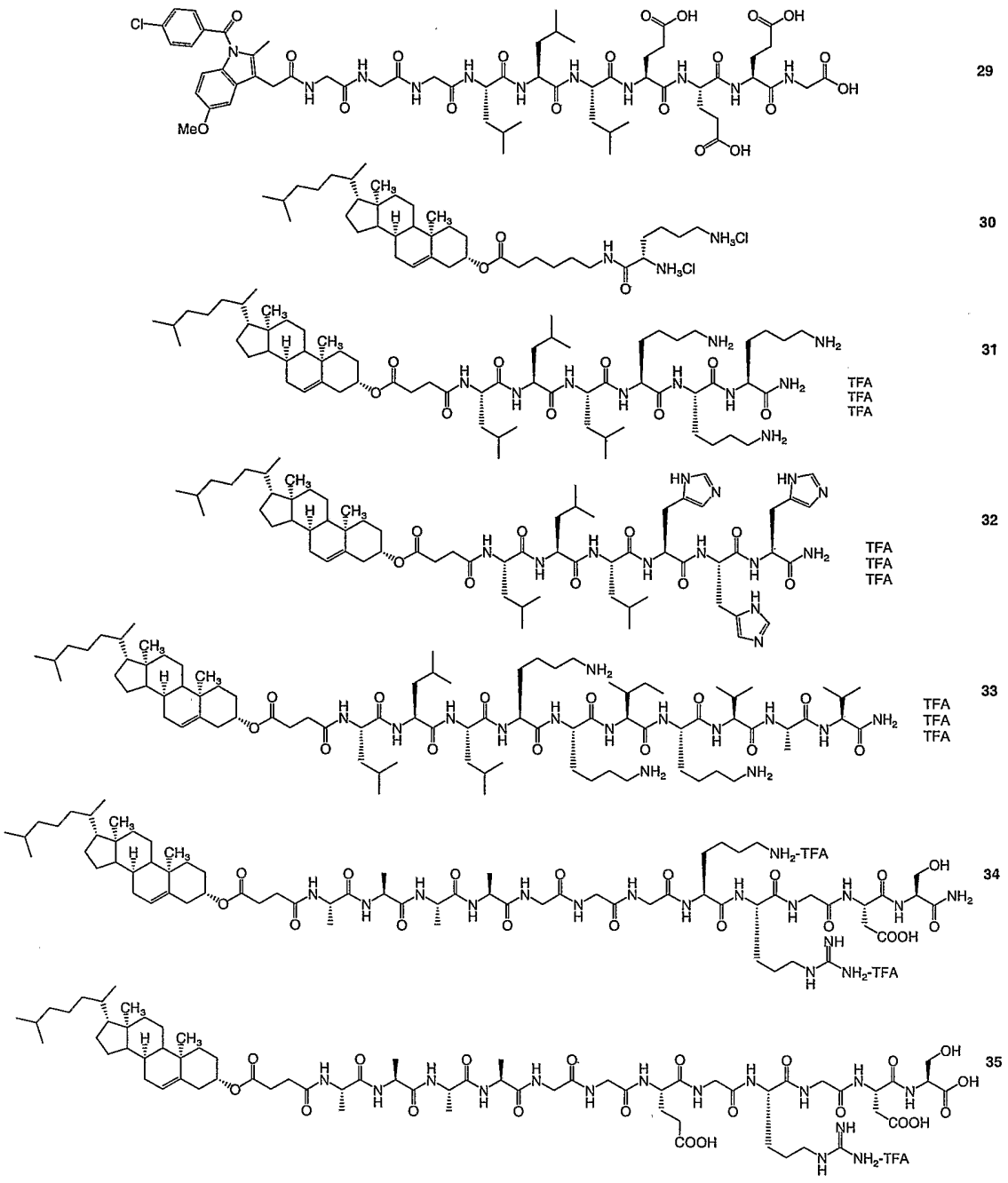
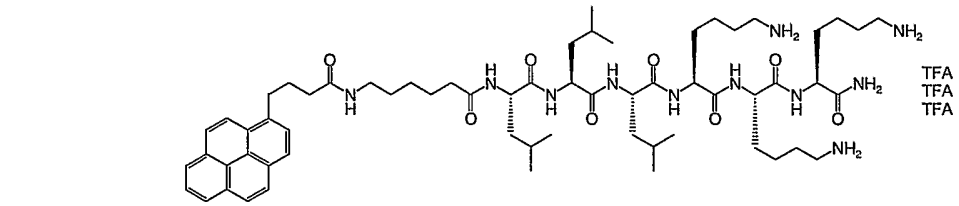
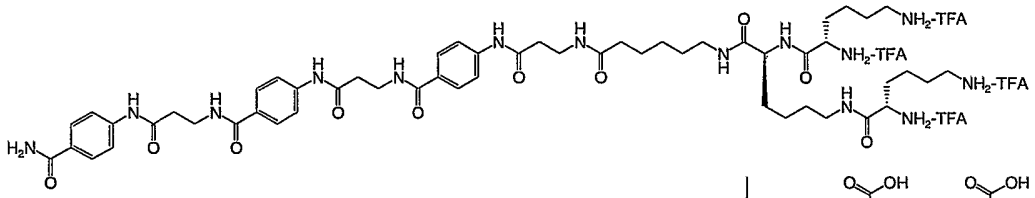


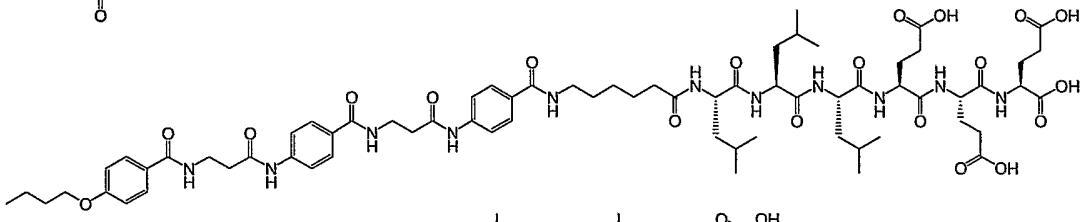
FIG. 17



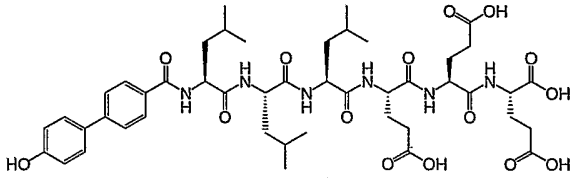
42



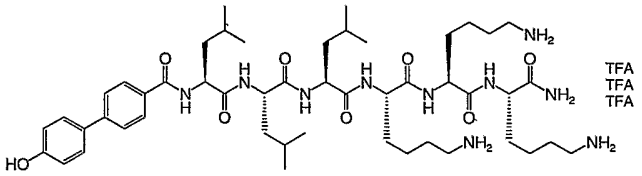
44



45



46



47

FIG. 18

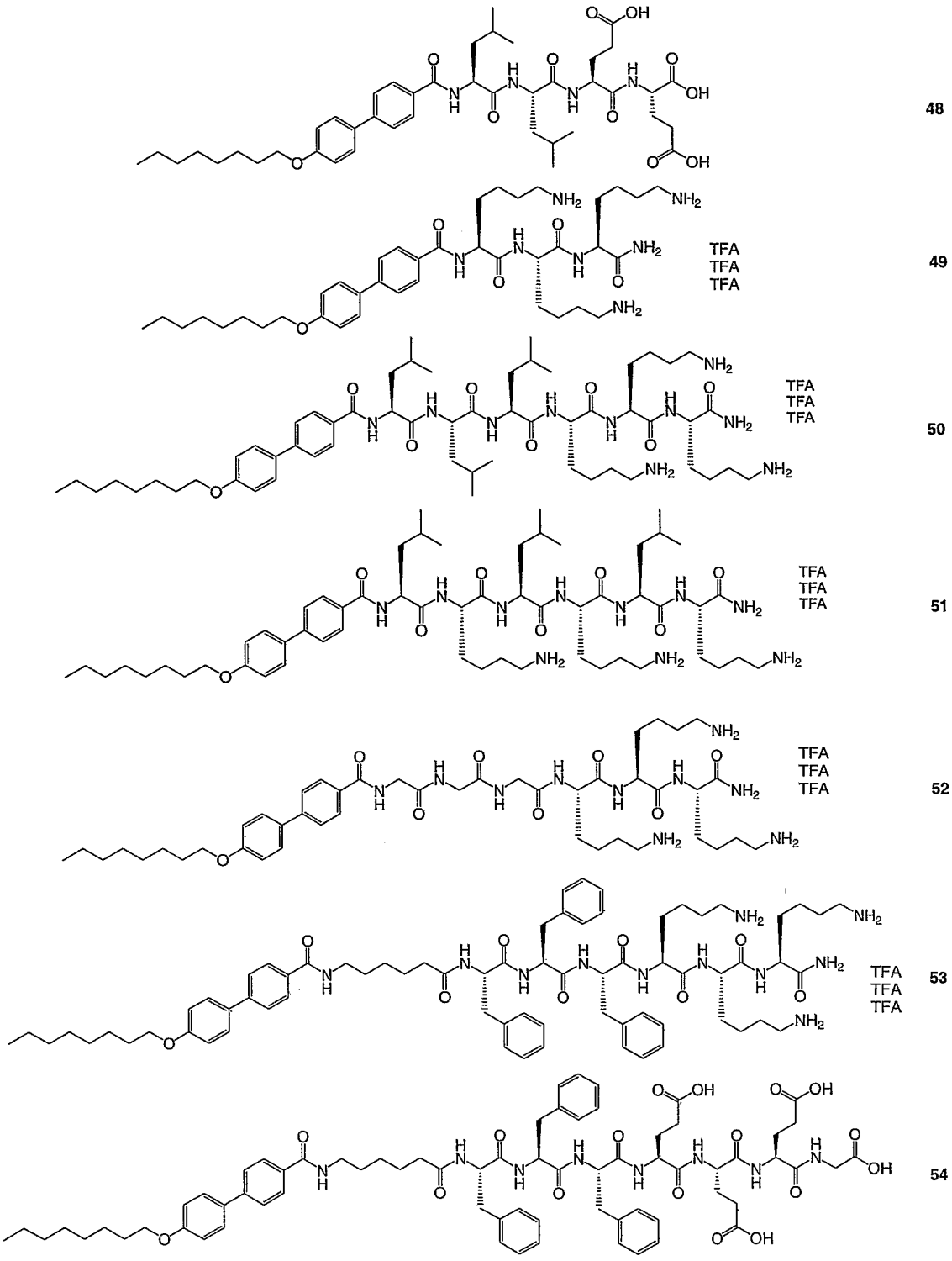
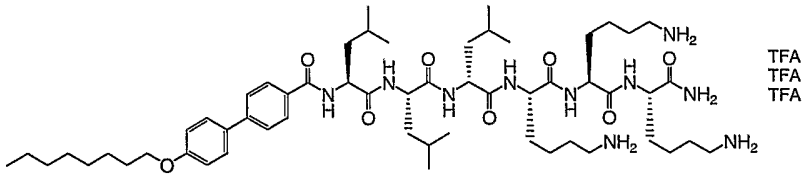
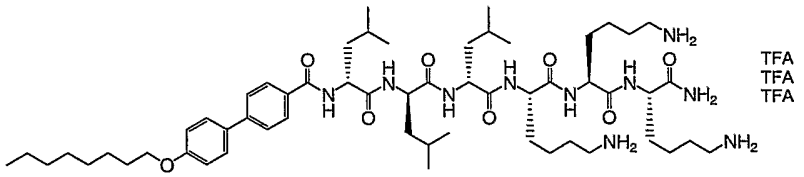

48
49
TFA
TFA
TFA
50
TFA
TFA
TFA
51
TFA
TFA
TFA
52
TFA
TFA
TFA
53
TFA
TFA
TFA
54

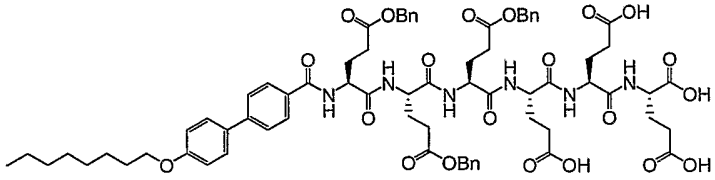
FIG. 19



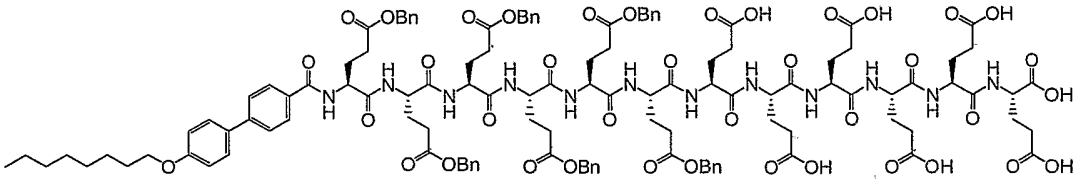
55



56

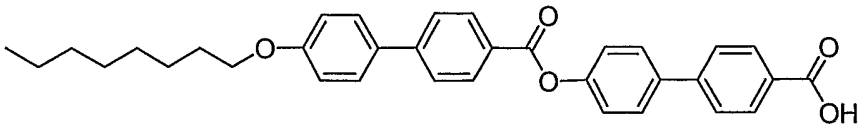


57

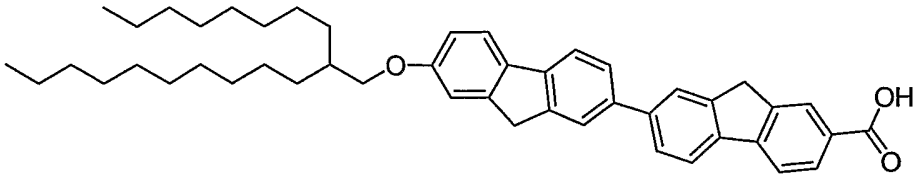


58

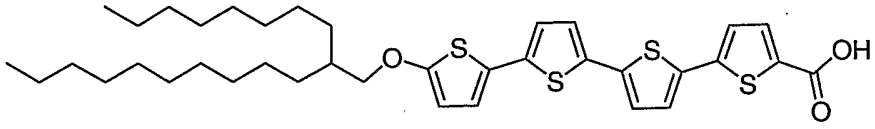
FIG. 20



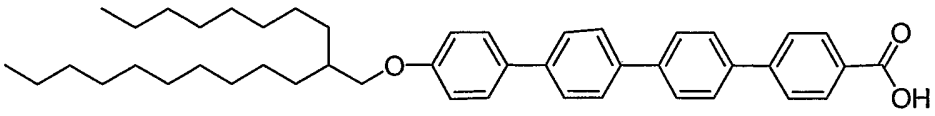
21



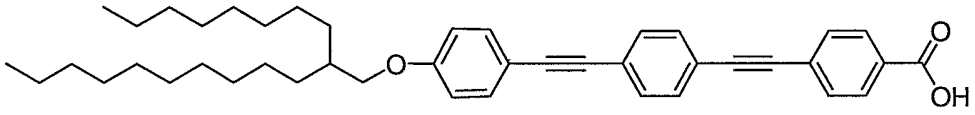
62



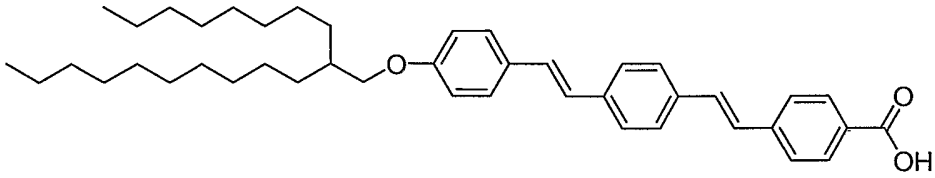
63



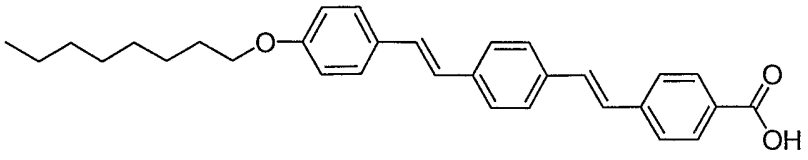
64



65



66



67

FIG. 21

SEQUENCE LISTING

<110> Sammuel I. Stupp; Randal C. Claussen

<120>Peptide Rod Amphiphiles & Self Assembly of Same

<130> Attorney Docket Number 126481.801

<141> June, 27, 2003

<160> NUMBER OF SEQUENCE ID NOS

<210> SEQ ID NO 1

<211> LENGTH: 10

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<400> SEQUENCE: 1

Gly Gly Gly Leu Leu Leu Glu Glu Glu Gly
1 5 10

<210> SEQ ID NO 2

<211> LENGTH: 7

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<400> SEQUENCE: 2

Leu Leu Leu Glu Glu Glu Gly
1 5

<210> SEQ ID NO 3

<211> LENGTH: 6

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<400> SEQUENCE: 3

Leu Leu Leu Lys Lys Lys
1 5

<210> SEQ ID NO 4

<211> LENGTH: 6

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<400> SEQUENCE: 4

Leu Leu Leu His His His
1 5

<210> SEQ ID NO 5

<211> LENGTH: 9

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<400> SEQUENCE: 5

Leu Leu Leu Lys Ile Lys Val Ala
1 5

<210> SEQ ID NO 6

<211> LENGTH: 12

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<400> SEQUENCE: 12

Ala Ala Ala Ala Gly Gly Gly Lys Arg Gly Asp Ser
1 5 10

<210> SEQ ID NO 7

<211> LENGTH: 12

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<400> SEQUENCE: 7

Ala Ala Ala Ala Gly Gly Glu Gly Arg Gly Asp Ser
1 5 10

<210> SEQ ID NO 8

<211> LENGTH: 6

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<400> SEQUENCE: 8

Leu Leu Leu Glu Glu Glu
1 5

<210> SEQ ID NO 9

<211> LENGTH: 4

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<400> SEQUENCE: 9

Leu Leu Glu Glu
1

<210> SEQ ID NO 10

<211> LENGTH: 3

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<400> SEQUENCE: 10

Lys Lys Lys
1

<210> SEQ ID NO 11

<211> LENGTH: 6

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<400> SEQUENCE: 11

Leu Lys Leu Lys Leu Lys
1 5

<210> SEQ ID NO 12

<211> LENGTH: 6

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<400> SEQUENCE: 12

Gly Gly Gly Lys Lys Lys
1 5

<210> SEQ ID NO 13

<211> LENGTH: 6

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<400> SEQUENCE: 13

Phe Phe Phe Lys Lys Lys
1 5

<210> SEQ ID NO 14

<211> LENGTH: 7

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<400> SEQUENCE: 14

Phe Phe Phe Glu Glu Glu Gly
1 5

SEQUENCE LISTINGS for given PRAs .

PRA	SEQUENCE
1,2	GGGLLLEEEG
3,4	Ahx-LLLEEEG
5,6	Ahx-LLLEEEG
7,8	Ahx-LLLEEEG
9,10	Ahx-LLLEEEG
11,12	GGGLLLEEEG
13,14	Ahx-Succ-LLLEEEG
16	Ahx-LLLKKK
17	Ahx-LLLKKK
29	GGGLLLEEEG
30	Ahx-K
31	Succ-LLLKKK
32	Succ-LLLHHH
33	Succ-LLLKIKVAV
34	Succ-AAAAGGGKRGDS
35	Succ-AAAAGGEGRGDS
42	Ahx-LLLKKK
44	b-A-Ahx-G2 Lys dendron
45	Ahx-LLLEEE
46	LLLEEE
47	LLLKKK
48	LLEE
49	KKK
50	LLLKKK
51	LKLKLK
52	GGGKKK
53	FFFKKK
54	FFFEEEG
55	L-D-LLKKK
56	D-L-D-L-D-LKKK
57	E(OBn)E(OBn)E(OBn)EEE
58	E(OBn)E(OBn)E(OBn) E(OBn)E(OBn)E(OBn)EEE

Ahx = 6-aminohexanoic acid; Succ = succinate; b-A = beta-alanine

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/20618

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : G01N 33/569, 33/68, 33/92
US CL : 435/6, 7.1, 7.2, 7.7, 7.71, 7.72, 7.91, 7.92, 973; 436/63, 501, 518, 534

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)
U.S. : 435/6, 7.1, 7.2, 7.7, 7.71, 7.72, 7.91, 7.92, 973; 436/63, 501, 518, 534

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)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GALLI, Monica et al., Kaolin clotting time and dilute Russell's viper venom time distinguish between prothrombin-dependent and beta2-glycoprotein I-dependent antiphospholipid antibodies. Blood, 15 July1995, Vol.86, No.2, pages 617-623, see entire document.	1-41 and 43-161
Y	TRIPLETT, D.A., Antiphospholipid antibodies: Proposed mechanism of action. American Journal of Reproductive Immunology,1992, Vol.28, pages 211-215, see entire	1-41 and 43-161
Y	WO 96/03655 A1 (BOEHRINGER MANNHEIM GMBH) 08 February 1996, see entire document.	1-41 and 43-161
Y	IZUMI et al., The role of prothrombin and beta 2-glycoprotein I in the generation of abnormal waveform parameters in the prothrmbin times from patients with antiphospholipid antibodies., Blood, Vol.98, No.11, Part 2, page 77b, see entire document.	162-184
A	POPOV et al., Compaison of DRVVT and PTT-based testing for the detection of lupus anticoagulants, Clinical Chemistry, 2001, Vol.47, No.6, page A171, see entire document.	1-41 and 43-184
A	CARUSO et al., Preganancy outcome in relation to uterine artery flow velocity waveforms and clinical characteristics in women with antiphospholipid syndrome. Obstetrics & Gynecology, 1993, Vol.82, No.6, pages 970-976.	1-41 and 43-184

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
04 August 2003 (04.08.2003)	08 SEP 2003
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703)305-3230	Authorized officer Lisa V. Daniels-Cook Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

PCT/US02/20618

INTERNATIONAL SEARCH REPORT

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,981,180 A (CHANDLER et al.) 09 November 1999 (09.11.1999), see entire document.	1-41 and 43-184

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/20618

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claim Nos.: 42
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
The claim dependency is not recited.

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

PCT/US02/20618

Continuation of B. FIELDS SEARCHED Item 3:
East and West Patent Database Search
STN- biosis, caplus, embase, medline, cancerlit, japio