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(71) Applicant (for all designated States except US): **NORTH-
WESTERN UNIVERSITY** [US/US]; 633 Clark Street,
Evanston, IL 60208 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **STUPP, Samuel,
I.** [US/US]; 57 E. Delaware Place, Apt. 2802, Chicago,
IL 60611 (US). **DONNERS, Jack, J., J., M.** [NL/US];
800 Hinman Avenue, #315, Evanston, IL 60202 (US).
SILVA, Gabriel, A. [CA/US]; 933 West Van Buren,
#814, Chicago, IL 60607 (US). **BEHANNA, Heather, A.**
[US/US]; 1456 West Addison Street, Chicago, IL 60613
(US). **ANTHONY, Shawn, G.** [US/US]; P.O. Box 214,
New Stanton, PA 15672 (US).

(74) Agents: **DEKRUIF, Rodney, D.** et al.; Reinhart Boerner
Van Deuren s.c., Attn: Linda Gabriel-Kasulke, 1000 North
Water Street, Suite 2100, Milwaukee, WI 53202 (US).

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(54) Title: SELF-ASSEMBLING PEPTIDE AMPHIPHILES AND RELATED METHODS FOR GROWTH FACTOR DELIVERY

(57) Abstract: Amphiphilic peptide compounds comprising one or more epitope sequences for binding interaction with one or more corresponding growth factors, micellar assemblies of such compounds and related methods of use.



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SELF-ASSEMBLING PEPTIDE AMPHIPHILES AND RELATED METHODS FOR GROWTH FACTOR DELIVERY

This application claims priority benefit from application serial no. 60/527,504 filed December 5, 2003, the entirety of which is incorporated herein by reference.

The United States government has certain rights to this invention pursuant to Grant No. DE-FG02-00ER54810 from the Department of Energy to Northwestern University.

Background of Invention.

Growth factors play an important role in cell specialization and are promising factors for controlling stem cell differentiation or reactivating dormant biological processes *in vivo*, both of which may lead to the regeneration of nonfunctional tissues. Growth factors are quickly degraded and rapidly diffuse away from a cellular site or injury. Accordingly, an architecture or scaffold would be useful to retain the growth factor at such a site for release to the surrounding cells in a controlled fashion.

Materials designed molecularly for tissue regeneration are becoming of great interest in advanced medicine. Scaffolds of synthetic polymers, including polymers based on L-lactic or glycolic acid, and biopolymers including collagen, fibrin or alginate have been studied. Growth factors have been physically entrapped in hydrogels as such polymers, covalently linked or bound electrostatically to either anionic polymers or structures such as heparin. Drawbacks to these and related systems relate either to non-specificity of bound growth factors or required degradation of covalent bonds to achieve desired effect. More recently, both natural and synthetic scaffolds have been modified to contain peptides found in extracellular proteins that promote receptor based interactions with cells and have been used to promote cell adhesion or differentiation.

Advances in self-assembly offer new opportunities in molecular design of biomaterials. Amphiphilic molecular building blocks can be assembled in aqueous environments to form scaffolds with well defined and diverse

chemical structures. Various classes of peptide-based amphiphiles have been reported in the literature, including amphiphilic peptides and peptides functionalized with hydrophobic components (*e.g.*, alkyl tails) on one or both termini. Amphiphilic peptides have been shown to form a variety of supramolecular structures like nanotapes, ribbons, fibers, and twisted ribbons. These structures originate from β -sheet formation among the amphiphilic molecules. A special case is that of amphiphilic peptide block copolymers that form gels with properties strongly dependent on the secondary structure of the individual peptide blocks. An example of peptide amphiphiles with alkyl tails on one terminus include amphiphiles derived from peptide motifs found in collagen which result in the formation of triple helical units that form spheroidal or disc-like micellar structures depending on the tail length and number of tails. Another class has two tails, one per terminus. This class of amphiphiles displays amyloid-like behavior in that, upon increasing the concentration, they undergo a transition from a random coil to a β -sheet type conformation that leads to fibrillar structures. There are reports of purely peptidic nanostructures with antiparallel arrangements, and one report of two modified peptide amphiphiles that aggregate in an antiparallel arrangement driven by an unnatural alkylated quaternary ammonium salt.

A class of peptide amphiphiles (PAs) is disclosed in one or both of two co-pending applications comprising a linear hydrophobic tail coupled to a peptide block that includes β -sheet forming segments, charged residues for solubility, and biological epitopes. The alkyl tail is attached to the N-terminus of the peptide, and the epitope segment is placed at the C-terminus. Upon application of a trigger such as a change in pH or ion concentration, these PA molecules can self-assemble in an aqueous medium into nanofibers. The alkyl chains are in the core of the fibers, with the epitopes displayed on the periphery for cell interaction. Epitopes that have been incorporated into the PA molecules mimic extracellular matrix proteins and promote cell adhesion or differentiation through cell signaling. It has also been shown that two different PA molecules with different epitopes and complementary charge can be co-

assembled into the same nanofiber. *See*, co-pending application serial number 10/368,517 filed February 18, 2003 (international publication number WO 03/070749) and application serial number 10/294,114 filed November 14, 2002 (international publication number WO 03/054146), each of which are incorporated herein by reference in their entirety.

However, such PA compounds are typically prepared via solid phase synthesis with the peptide component generated from the C-terminus to the N-terminus. Coupling a hydrophobic component to the N-terminus provides a PA compound with either a free acid or amide group on the periphery of the resulting micellar nanofiber assembly as various epitope or peptide sequences often require a free N-terminus for bioactivity, such PA compounds would be ineffective for growth factor interaction.

Summary of the Invention.

In light of the foregoing, it is an object of the present invention to provide the amphiphilic peptide compounds, assembled compositions thereof and/or related methods for use thereof to affect bioavailability of a range of growth factors, thereby overcoming various deficiencies and shortcomings of the prior, including those outlined above. 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 amphiphilic peptide compounds comprising peptide components coupled at the N-terminus thereof to one or more of a range of epitope sequences capable of non-covalent interaction with one or more growth factors, such epitopes as can comprise and be derived from the recognition product of a phage display process.

It is another object of the present invention to provide a composition comprising an assembly of one or more of the aforementioned amphiphilic peptide compounds for presentation of the epitope binding sequence(s) coupled

thereto, such compositions as can comprise other amphiphilic peptide compounds absent such an epitope sequence.

It can also be an object of this invention to provide one or more of the aforementioned compositions further comprising one or more growth factors corresponding to a coupled epitope sequence, such compositions capable of self-assembly for growth factor delivery and release.

It is also an object of the present invention to provide one or more compositional assemblies of the aforementioned amphiphilic peptide compounds and use thereof to affect growth factor bioavailability and/or stem cell differentiation.

Other objects, features, benefits and advantages of the present invention will be apparent from this summary and the following descriptions of certain embodiments, and will be readily apparent to those skilled in the art having of peptide composition and scaffolds or architectures for growth factor delivery. 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, alone or in consideration with the references incorporated herein.

In part, this invention can be directed to an amphiphilic peptide compound comprising a peptide component and a hydrophobic component, the peptide component comprising a growth factor recognition product of a phage display process. One or more such recognition products can be coupled to or bonded directly with the peptide component at, about or proximate to the N-terminus of the peptide component, with the hydrophobic component coupled to or bonded directly with the peptide component at, about or proximate to the C-terminus thereof. Such a recognition product can be selected from epitope sequences providing one or more binding interactions with a growth factor, such products/sequences and corresponding growth factors including but not limited to those discussed more fully below. The compounds of this invention and/or peptide components thereof can be substantially linear or branched, such branched configurations and the

preparation thereof as disclosed in co-pending application "Branched Peptide Amphiphiles, Related Epitope Compounds and Self-Assembled Structures Thereof," filed concurrently herewith, on December 6, 2004, the entirety of which is incorporated herein by reference.

In part, this invention can also be directed to compositions comprising a plurality of such amphiphilic peptide compounds, with the peptide component of each such compound having a net charge at a physiological pH. Such compositions can further comprise a plurality of amphiphilic peptide compounds having a complementary net charge at a physiological pH, such compounds absent a growth factor recognition product, such that the amino acid sequence of such compounds is of shorter length or less in number than that corresponding to a compound comprising a recognition product. As a result, micellar assemblies of such compounds, in an appropriate medium, provide an enhanced presentation of one or more recognition products, extending beyond the general periphery of the micellar assembly. Such enhanced presentation can be used to affect and/or control the bioavailability of one or more growth factors. As such, various compositions of this invention and methods relating thereto can be used in a stem cell environment for non-covalent interaction or binding with one or more growth factors, whether the growth factor is produced by the stem cell, or delivered to the cellular environment in conjunction with one or more of the aforementioned compositions.

Regardless of compositional use or application, the peptide amphiphiles of this invention can comprise a peptide component of varied length or sequence depending upon desired flexibility, charge and/or capacity of intermolecular interaction or binding. The hydrophobic component of such compounds can also be varied (e.g., ranging from about C₆ to greater than about C₂₂ alkyl or substituted alkyl, saturated or unsaturated, etc.), such components limited only by resulting amphiphilic character and affect on compositions or assemblies of such compounds.

One embodiment of the present invention is a composition that includes a mixture of peptide amphiphiles, each having a binding epitope, and filler peptide amphiphiles without a binding epitope. The mixture is capable of forming self assembled nanofibers or other micelles which consist of the filler peptide amphiphiles and the self assembled peptide amphiphiles having the binding epitope. The peptide amphiphiles in the composition have a alkyl tail portion, a beta sheet portion, and a charged portion. Preferably the epitope on the peptide amphiphile having the binding epitope has a sequence derived by a phage display process and is longer than the filler peptide amphiphile.

Another embodiment of the present invention is a composition or system of self assembled nanofibers or other self assembled micelles including peptide amphiphiles having a binding epitope and filler peptide amphiphiles. The peptide amphiphiles with the binding epitope preferably longer than the filler peptide amphiphiles. In the self assembled structures the peptide amphiphiles with the binding epitope preferably protrude from the surface of the self assembled nanofiber or micelle. Preferably the peptide amphiphile with the binding epitope is capable of interacting with growth factors or other peptides, amino acids, or nucleic acids through non-covalent interaction, even more preferably the non-covalent interaction between the growth factors and the epitopes does not compete with binding of the growth factors to extracellular receptors.

Compositions of self assembled nanofibers or micelles can comprise hydrogels that may further include a growth factor non-covalently bonded to peptide amphiphiles of the nanofiber having the bonding epitope. Such growth factors may be added *in vitro* or may be those at the site of an injury of a patient *in vivo*. The growth factors interacting with the self assembled peptide amphiphiles may include but are not limited to bone morphogenetic proteins, transforming growth factor vascular endothelial growth factor, neurotrophins, and mitogenic factors like FGF-2, Sonic hedgehog and Wnt-proteins. The composition may include cells such as but not limited to stem cells inside the nanofiber hydrogels. Preferably the growth factors non covalently interacting

with the self assembled peptide amphiphiles are released to the surrounding tissue or cells through interaction with the extracellular receptors or by degradation of the nanofiber matrix.. In addition to the cells, other therapeutic compounds may be encapsulated in or bonded to the hydrogel. These compounds may include but are not limited to anti-inflammatories, chemotherapeutics, or combinations of these inside the nanofiber hydrogels. Preferably the growth factors non covalently interacting with the self assembled peptide amphiphiles are released to the surrounding tissue or cells through interaction with the extracellular receptors or by degradation of the nanofiber matrix.

Another embodiment of the present invention is a method of making self assembled peptide amphiphile nanofibers or micelles that includes self-assembling by mixing or combining peptide amphiphiles having a binding epitope with filler peptide amphiphiles, wherein said peptide amphiphiles with binding epitopes are longer in length than said filler peptide amphiphiles. Nanofibers or micelles maybe formed by addition of multivalent ions, addition of complementary charged peptide amphiphiles, or by dehydration, or addition of an acid or base to the peptide amphiphiles. Where hydrogels are formed, preferably the micelles or nanofibers are formed by addition of multivalent ions or ions already present in cell media or bodily fluids. Preferably the peptide amphiphile having the binding epitope has a sequence derived by a phage display process.

Another embodiment of the present invention is a method of treating a tissue which includes administering to a site on a patient in need of regenerating a tissue, nanofibers or other self assembled micelles including peptide amphiphiles with epitopes for non-covalently bonding to growth factors. The nanofibers and their hydrogels may include cells such as but not limited to stem cells. In addition to cells, other therapeutic compounds may be encapsulated in or bonded to the hydrogel. These compounds may include but are not limited to anti-inflammatories, chemotherapeutics, or combinations of these. Preferably the growth factors noncovalently bonded to self assembled

structure are released to the site by interaction with the extracellular receptors or degradation of the matrix. Preferably the self assembled micelles or nanofibers containing growth factors and/or stem cells are administered to a site on a patient having injuries such as but not limited to damaged bone, cartilage, spinal cord, brain tissue, nerves, or a combination of these.

Another embodiment of the present invention is a peptide amphiphile that includes an alkyl tail portion coupled to a first end of beta sheet forming peptide portion also having a second end. An epitope peptide portion of the peptide amphiphile is coupled to the second end of the beta sheet forming peptide portion such that the epitope is available for non-covalent interaction with other molecules or proteins. Preferably the epitope has a sequence that is derived by a phage display process. The peptide amphiphile may alternatively include a charged portion peptide having two ends and coupled between the beta sheet forming portion second end and the epitope peptide portion.

Another embodiment of the present invention comprises an assembly or system for releasing growth factors to cells that includes a scaffold prepared from self assembled peptide amphiphiles, at least a portion of the peptide amphiphiles including peptide epitopes for non-covalently bonding with growth factors, and wherein the growth factor binding epitopes protrude above the nanofiber surface. The amino acid sequence of the epitope is derived from a phage display process. Such a scaffold, may also include stem cells, and can be used in a method for growing tissue, regenerating tissue, or supporting the transplantation of tissue in a patient. In addition to cells, other therapeutic compounds may be encapsulated in or bonded to the hydrogel of the scaffold. These compounds may include but are not limited to anti inflammatories, chemotherapeutics, or combinations of these. The method includes inserting a scaffold to a site on a patient or forming it *in vivo*. The scaffold includes self assembled peptide amphiphiles having peptide epitopes for non-covalent bonding with growth factors and where the epitopes protrude above the nanofiber surface of the scaffold or

are made by a phage display process, and releasing growth factors from the scaffold to the surrounding cells from said scaffold.

The described growth factor binding hydrogels have several applications in the field of regenerative and transplant medicine. The compositions, methods of making, and methods of using them can readily be expanded to other epitopes on the peptide amphiphiles for other growth factors and hence to the regeneration of a wide variety of tissues or support of transplanted tissues in a patient. For example, since BMP-2 and TGF- β 1 play an important role in osteoblastic and chondrogenic differentiation of mesenchymal stem cells, respectively, the self assembled micelles of the present invention can be used for the regeneration of bone and cartilage. Second, BMP-2 also plays an important role in the formation of the brain and the dorsal spinal cord. Therefore, the gels can also be used in combination with neural stem cells for the regeneration of damaged spinal cord and/or to repair damaged brain areas in the case of stroke.

As discussed above, In certain embodiments, this invention utilizes an extension of the mixed fiber approach to incorporate a PA that is capable of binding growth factors by selective non-covalent interactions. In this way, the bio-availability of the growth factors can be modulated by tuning the binding strength and number of binding sites. The initial concentration of growth factor will be either raised or decreased depending whether the localizing effect of binding or whether the effect of binding rendering the growth factors inactive is more dominant. In either case, release of growth factors can be sustained over a longer period. To promote binding epitope recognized by a growth factor, a PA can be made such that the epitope is extending from the fiber surface. The required amino acid sequence of the binding epitope can be determined using phage display. For purpose of illustration, growth factors chosen include bone morphogenetic protein-2 (BMP-2) and transforming growth factor β 1 (TGF- β 1). BMP-2 is, amongst others, involved in the formation of bone and the development of the brain and the dorsal spinal cord, whereas TGF- β 1 is implicated in the formation of cartilage and the

differentiation of smooth muscle cells. The corresponding gels will be used to control the differentiation of mesenchymal stem cells. Mesenchymal stem cells have been shown to differentiate *in vitro* and *in vivo* into a variety of lineages like bone, cartilage, fat, muscle cells and myocardium. The differentiation into the bone lineage under the influence of BMP-2 is shown below.

The gels are superior to the addition of growth factors to media in controlling the differentiation of mesenchymal stem cells into the expected osteogenic lineage. Moreover, the binding gels initially suppress the differentiation into all other lineages. This behaviour is thought to result from either reduced bio-availability of the growth factor or from a more gradual exposure of the cells to these growth factors in the binding gels. Eventually, some α -smooth muscle expression is found in the binding gels as well. This suggests that prolonged exposure to BMP-2 or the absence of factors that drive differentiation to completion might have negative effects on homogeneity of the population. Finally, the endogeneously produced BMP-2 levels seem sufficient when the binding sequence is present to moderate its bioavailability.

The successful co-assembly of the growth factor binding PAs with the regular filler PAs allows the creation of hydrogels that are capable to bind growth factors, retain them in the gels and modulate their bio-availability. More homogeneous populations of specialized cells are obtained initially when mesenchymal stem cells are differentiated in gels with binding PAs compared to those differentiated in the absence of binding PAs. Other filler PAs may be desired to vary the physical properties of the gels depending on its applications and other binding sequences might be desired to optimize binding strengths. In addition, the approach demonstrated by this invention allows the incorporation of multiple signals by simple mixing of multiple binding PAs with a filler. Most likely, multiple growth factors will be required to further optimize the differentiation of stem cells. The differentiation of mesenchymal stem cells exposed to multiple growth factors is currently being investigated. Potentially, temporal release in the multiple growth factors systems can be achieved by selecting binders with different association constants for the growth factors. In

addition, *in vivo* studies of the systems described here are being performed. Finally, the methodology presented here can readily be extended to any growth factor or protein implicated in cell recruitment, specialization or maintenance, thereby making it a highly promising approach for regenerative medicine.

Brief Description of the Drawing.

Figure 1 illustrates representative PAs comprising epitope sequences and complementary PAs for use in assembly therewith.

Detailed Description of Certain Embodiments.

The invention describes a system based on self-assembling peptide amphiphiles (PAs) that is capable of binding growth factors through specific non-covalent interactions. Growth factors play an important role in cell signaling and are therefore promising factors for controlling the differentiation of stem cells, supporting the incorporation of transplanted tissue, and or specific molecular or physiological, or re-activating dormant biological processes *in vivo* which may lead to the regeneration of non-functional tissues. Growth factors are rapidly degraded *in vivo* and rapidly diffuse away from the injury site. Therefore, a scaffold is needed that retains the growth factor at the injury site and releases the growth factors to the surrounding cells in a controlled fashion. Self assembled peptide amphiphiles based hydrogels have been shown to be able to fulfill several functions of an extracellular matrix (ECM), i.e. promoting cell adhesion and controlling differentiation. The present invention concerns a modification of these ECM substitutes with peptide sequences that are capable of binding growth factors, which include but are not limited to bone morphogenetic protein-2 (BMP-2), transforming growth factor β 1 (TGF- β 1), VEGF, and IL-6 in a specific fashion. These growth factor peptide sequences may be obtained through phage display techniques, coupled to peptide amphiphiles to form binding peptide amphiphiles. The binding peptide amphiphiles and filler peptide amphiphiles maybe self assembled to form a hydrogel which can be molded into a shape and used as a scaffold for tissue repair. Alternatively binding peptide amphiphiles and filler peptide amphiphiles may be introduced *in vivo* or *in*

vitro to a sample of cells or tissue and self assembled to a hydrogel in the sample.

As discussed above, the peptide amphiphiles of this invention can comprise an alkyl tail, a β -sheet forming peptide sequence, and a bio-active peptide sequence. The first two blocks result in the formation of micellar fibers, such as but not limited to nanofibers, which under the proper conditions (neutralization of addition of multivalent ions) form hydrogels or other solvent filled wet gels. Preferably the bioactive epitope is partially charged for solubility, but this charge can be varied over a wide range of sequences like integrin-binding sequences, neuro-active sequences etc. Furthermore, peptide amphiphiles of complementary charge co-assemble to form mixed fibers. This principle may be used to prepare nanofibers consisting of regular PAs and longer PAs with growth factor binding epitope sequences, in which the binding sequences are extending from the fiber surface. The desired growth factor binding sequences are obtained through phage display using randomized peptide libraries, which leads to strong binding sequences using combinatorial selection processes. Release of the growth factors will be induced by degradation of the matrix, binding to an extracellular receptor, or a combination of these.

Existing technologies entrap the growth factors in hydrogels, link them covalently with polymeric tethers (WO 03/040336 and U.S. Pat. Pub. No. 0020007217) or bind them electrostatically to either anionic polymers (WO 0/13710) or heparin (WO 00/64481). The disadvantage of the tethered system is the complex preparation and the possibility that the growth factors cannot reach the extracellular receptors. Entrapped growth factors and the growth factors complexed to the anionic polymer are released relatively quick and control over sustained release is poor. For example, the growth factors bound to the anionic polymer are released within 8 hours. The heparin-binding system is non-specific (e.g. non-desired binding of factors present in the serum is possible) and is limited to a subset of growth factors. Advantageously, the present invention is designed such that only the desired growth factor is

complexed with a binding strength that is high enough to retain the growth factor in a bound state but is weak enough that it does not compete with binding to the extracellular receptors.

One aspect of the invention described herein is that it may be used for the derivation of peptide sequences that bind growth factors using phage display techniques and the subsequent preparation of peptide amphiphiles bearing these binding sequences. Growth factors prepared by the phage technique may include the bone morphogenetic proteins, transforming growth factor $\beta 1$, the neurotrophins, and the mitogenic factors FGF-2, Sonic Hedgehog and Wnt-3a. These growth factor proteins are coupled to shorter peptide amphiphile to form longer peptide amphiphiles bearing the growth factor epitope. These growth factor epitope bearing PAs, or binding peptide amphiphiles, are co-assembled with shorter filler or complementary filler PAs to generate nanofibers with the binding epitopes extending from the surface, which can subsequently form hydrogels upon the addition of multivalent ions, or a change in pH. The resulting hydrogels can then be used, either with cells, such as but not limited to stem cells, encapsulated inside the hydrogel or solely as a delivery vehicle, for tissue regeneration *in vivo*. In addition to cells, other therapeutic compounds may be encapsulated in or bonded to the hydrogel. These compounds may include but are not limited to anti-inflammatories, chemotherapeutics, or combinations of these.

Phage display may be used to generate molecular probes against specific targets and for the analysis and manipulation of protein-ligand interactions. Phage display is used to determine the amino acid sequences, peptides, or proteins that will bind to the target molecules such as but not limited to amino acids, peptides, growth factors, enzymes, and various nucleic acids. Phage display may be performed on target molecules like growth factors physisorbed to microtiter well plates using a commercial library. Preferably the library consists of phages in which the N-terminus of the G3 coat protein has been extended with 1 to about 13 or more randomized amino acids in such a way that every possible combination of amino acids is present. The library is

exposed to the target molecules such as growth factors and subsequently non-specifically bound phages are eluted with a detergent solution. After retrieval of the bound phages, this population is amplified in for example *E. Coli* and the process is repeated two more times with increasingly stringent elution conditions. The final population is strongly enriched towards binding phages, and the DNA of several of the clones can be sequenced, allowing the identification of the amino acid sequence displayed on these phages. Subsequently, an ELISA assay may be performed to estimate the relative binding strengths of the isolated clones. Solid state synthesis is used to make the peptides for making the binding peptide amphiphiles using the identified amino acid sequences.

Peptide components in the filler PAs and the binding epitope peptide amphiphiles of the present invention may include naturally occurring amino acids and artificial amino acids. 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 and self assemble to form micelles such as nanofibers.

Various peptide amphiphiles of the present invention can be synthesized using preparatory techniques well-known to those skilled in the art, including those disclosed in the aforementioned incorporated publication WO 03/054146 and modifications of those originally described by Hartgerink, *et al.* (See e.g., J.D. Hartgerink, E. Beniash and S.I. Stupp, *Science* 294, 1683-1688, 2001), which is also incorporated in its entirety by reference. Also contemplated in the practice of this invention are branched peptide amphiphiles with which the synthetic methods and epitopes of the present invention may be used. Branched peptide amphiphile may be made by the using the methods and compositions as disclosed in the forementioned co-pending application filed concurrently herewith on December 6, 2004, the contents of which are incorporated herein by reference in their entirety. Such a branched peptide amphiphile with a binding epitope could be self assembled into a nanofiber. The branched

peptide amphiphile would have an alkyl tail portion coupled to the first end of a beta sheet forming peptide portion and at a second end to an epitope peptide portion that includes one or more peptide branches. The peptide includes branching amino acids and a charged peptide portion. The branched epitope peptide portion may be coupled to a second end of the beta sheet forming peptide portion of the peptide amphiphile. The binding epitope sequence for the branched peptide may be derived from a phage display process. The synthetic schemes set forth in these references may be applied to the present invention. Peptide amphiphiles may be in their fully protonated form, partially protonated form, or as acid or basic addition salts. Generally such peptide amphiphiles can be made by standard solid-phase peptide chemistry, as described in the aforementioned incorporated references or as provided herein. Modifications of these synthetic methods can be made 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.

Filler peptides, of the sort shown in Figure 1, are preferably shorter in length than the peptide amphiphiles with the binding epitopes so that self assembled nanofibers from a combination of these peptide amphiphiles results in protrusion of the growth factor binding epitope from the surface of the nanofiber. The complementary filler peptide amphiphiles have side groups from the amino acids which interact with those of the filler peptide via acid base or other types of bonding.

The peptide sequences derived from the phage display process may be used as a binding site or epitope on a peptide amphiphile for non-covalently bonding to peptides and molecules that include but are not limited to growth factors, enzymes, nucleic acids (RNA, DNA), and amino acids. While phage display is preferred for deriving the epitope sequences, other complementary methods for identifying and determining the sequence of binding epitopes can be used, such as but not limited to yeast hybrid systems. These binding peptides or epitopes may be coupled to the free end of the beta sheet forming

peptide, the free end of the charged peptide, or the free end of the spacer peptide.

A peptide amphiphile design can comprise a simple hydrophobic tail which serves to create the slender portion of the molecule's conical shape for self assembly of the peptide amphiphiles. Preferably the hydrophobic tail is an alkyl chain that can be a variety of sizes but is preferably be greater than 6 carbon atoms in length. The alkyl tail is covalently bonded to the beta sheet forming structural segment of the peptide amphiphile.

The beta sheet peptide structural segment may be used to covalently link the tail group to the phage peptide; or the charged peptide, spacer peptide, and phage peptide; or the charged peptide and phage peptide. The beta sheet peptide structural segment is covalently bonded at one end to the tail and at its other end to an amino acid sequence of the various peptides. If cross-linking is desired, cysteine amino acids maybe utilized in any of the segments, but preferably in the structural beta sheet segment. If cross-linking is not desired, other hydrophobic amino acids such as but not limited to alanine, serine, or leucine may be used in this region (e.g. SLSL or AAAA as described in more detail herein). This cysteine-free system may be more appropriate for *in vivo* biological applications to control the degradation rate of the nanofiber matrix. The SLSL modification to the system may be expected to lead to a slower self assembly of the nano fibers which may be used to control *in vivo* assembly of scaffolds. Without wishing to be bound by theory, it is believed that the bulky leucine side chains may require more time to pack into the fiber. A slowed self-assembly may also have greater applications in a functional, in situ environment such as an operating room, where it may be advantageous to have delayed formation of the nano-fibers. The structural beta sheet forming segment may also include a flexible linker composed of glycine or other amino acids. When the structural segment includes hydrophobic amino acids, it and the alkyl tail may be considered a hydrophobic segment. Where the structural segment includes hydrophilic amino acid, it and the hydrophilic head group may be considered as a hydrophilic segment.

The β -sheet forming unit preferably includes those hydrophobic amino acids which can interact to form beta sheets and which help form the overall conical shape of the peptide amphiphile. For the protruding peptide amphiphile, the number of amino acid in this unit may be chosen to provide a peptide amphiphile that is longer than filler peptide amphiphiles used to form the nanofibers and provide accessibility to the peptide on the bonding peptide amphiphile. There may be from about 4 to about 10 amino acids in this segment and most preferably about 6 amino acids. For a β -sheet forming segment suitable amino acid may include but are not limited to glycine, alanine, valine, leucine, and isoleucine, and other non-naturally occurring amino acids which may be used in a similar chemical and structural manner in the peptide amphiphile. A charged segment is present in the binding peptide amphiphile which provides for solubility of the peptide amphiphile in an aqueous environment, and preferably at a site on a patient. The charged peptide segment may include those amino acids and combinations thereof which provide this solubility and permit self assembly and is not limited to polar amino acids such as E or K and combinations of these for modifying the solubility of the peptide amphiphile. There may be from about 2 to about 7 amino, and preferably there are about 3 or 4 amino acids in this segment. This segment is attached at a first end to the structural peptide and its second end used for bonding to the peptide derived from the phage display process. A spacer group peptide may also be included into the peptide amphiphile. The spacer may include amino acids such as but not limited to S and G, and the spacer may include from 1 to about 6 amino acids. Where the displayed peptides on the phage have a free N-terminus, the classical synthetic scheme for peptide amphiphiles may be amended to provide a free N-terminus. This may be achieved by synthesizing an aspartic acid derivative with which the side chain can be functionalized with dodecyl amine. Coupling of this amino acid to the Wang-resin allowed the completion of the PA synthesis through classical solid state Fmoc-chemistry without further need to functionalize the N-terminus. The free PA may be obtained through treatment with 95%

TFA/2.5% water/2.5% triisopropylsilane. Residual TFA can be removed by dissolving the PA in 3 mM HCl, equilibrating for 1 hr at room temperature followed by lyophilization. The successful synthesis of these molecules may be confirmed using electrospray mass spectrometry.

The peptide amphiphiles with the growth factor epitopes may be self assembled at a site on a patient *in vivo* using ions present in bodily fluids and or added ions/reagents to promote self assembly. Alternatively a composition of suitable peptide amphiphiles with growth factor binding epitopes is poured into a mold and self assembly used to form a scaffold in the shape of a tissue or bone to be replaced or regenerated. The molded scaffold may be inserted into the patient at the site in need of the repair or regenerative treatment. In the case of tissue transplant, the peptide amphiphiles with the growth factor binding epitopes may be formed into a support structure or matrix in a mold and used as a support in the patient for the transplanted tissue. The peptide amphiphile nanofibers or scaffolds thereof may include cells such as but not limited to stem cells. Other therapeutic compounds may be encapsulated in or bonded to the hydrogel. These compounds may include but are not limited to anti inflammatories, chemotherapeutics, or combinations of these.

Co-assembly of peptide amphiphiles with growth factor binding epitopes with the shorter beta sheet structural peptide-charged peptide acid-terminated filler peptide amphiphiles may be used to prepare nanofibers and scaffolds of the present invention. Without limitation one or more peptide amphiphiles with different growth factor binding epitopes may be used, and a variety of filler peptide amphiphiles may be used to prepare the scaffolds and nanofibers of the present invention. The amount of peptide amphiphile with growth factor binding epitopes compared to the filler peptides amphiphiles may be varied without limitation in the preparation of the scaffolds and nanofibers of the present invention. The self assembled micelles and nanofibers may be characterized by NOE and FT-IR spectroscopy, circular dichroism; nanofiber fiber networks can be visualized using transmission electron microscopy.

The peptide amphiphile with the binding epitope may be made by choice of a peptide sequences for the epitope that is capable of interacting with growth factors or other peptides such as but not limited to, amino acids, or nucleic acids, through non-covalent interaction. The degree of non-covalent interaction between the growth factors or other peptides and the peptide amphiphiles with the binding epitopes is chosen such that the epitope does not compete with or has less binding affinity for the growth factors or other peptides as compared to extracellular receptors. Passive release experiments may be used to characterize and subsequently modify the affinity of various nanofibers having growth factor binding epitopes. This characterization may be performed on self assembled nanofiber gels containing growth factors in pre-blocked microtiter plates. These gels may be prepared by first mixing 2% solutions of PAs in the appropriate ratio, followed by a 1:1 dilution with TBS. Growth factors may then be added followed by the addition of 2 equivalents multivalent ions to induce gelation and self assembly. The supernatant may be assayed for its growth factor content to access the growth factor binding capability of such gels.

While it is preferable for tissue growth and other treatments that the binding epitope have less affinity for various peptides than the extracellular receptors of nearby cells or tissues, it is contemplated that it may be desirable the binding epitope of the peptide amphiphile strongly chemically bond to peptides, growth factors, enzymes, or nucleic acids or other molecules from a fluid or sample of cells. In this case binding peptide amphiphiles with suitable epitopes can be self assembled and immobilized on the surfaces to form sensor coatings or removal media. Hydrogels formed from self assembly of these strongly binding peptide amphiphiles could be molded for insertion into a site on a patient or for use in a filtration system. The hydrogels could be used to remove target peptides like HGF or VEGF from a site such as a joint or tumor on a patient or from a fluid in a patient.

Examples of the Invention

The following non-limiting examples and data illustrate various aspects and features relating to the compounds, compositions and/or methods of the present invention, including the synthesis of a range of amphiphilic peptide compounds, with or without epitope sequences capable of non-covalent interaction with growth factors, self-assembly of such compounds or compositions and the use thereof to affect growth factor bioavailability and stem cell differentiation. In comparison with the prior art, the present compounds, compositions and/or methods are surprising, unexpected and contrary thereto. While the utility of this invention is illustrated through the use of several compounds/compositions and assemblies, it will be understood by those skilled in the art that comparable results are obtainable with various other compounds, compositions and/or assemblies, as are commensurate with the scope of this invention.

All resins and Fmoc-L-amino acids were obtained from Novabiochem (San Diego, CA). All reagents for solid phase synthesis were of synthesis grade and obtained from Applied Biosystems (Foster City, CA). All other reagents were obtained from Aldrich Chemical Co. (Milwaukee, WI) and were used as received. Solvents for solid phase peptide synthesis were acquired from Applied Biosystems and were peptide synthesis grade. Other solvents were obtained from Fisher Scientific and were used as received unless stated otherwise.

PAs were synthesized using an Applied Biosystems 433A automated peptide synthesizer. NMR spectra were acquired on a Varian Inova 500 MHz spectrometer at room temperature. Electro Spray mass spectra were collected on a Micromass Quattro II Triple Quadrupole HPLC/MS/MS Mass Spectrometer. CD spectra were recorded on a Jasco J-715 spectropolarimeter with a Jasco PTC-348WI peltier-effect temperature controller. FT-IR spectra were run on a BioRad FTS-40 FT-IR machine, from 400-4000nm with a 2 cm^{-1} resolution.

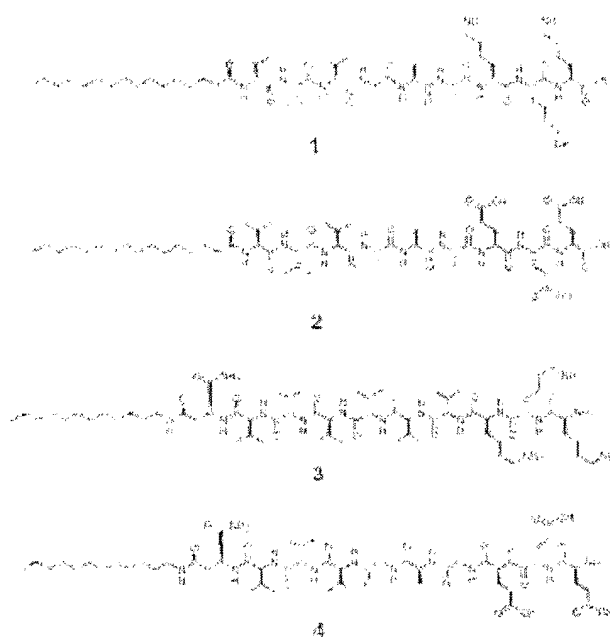
Circular Dichroism Spectroscopy. Quartz cells with a 0.1cm path length were used for all experiments. Each spectrum was recorded from 300 to

190 nm at a scan speed of 100 nm/min, a response time of 2 seconds and a band width of 1 nm and averaged over five scans. Samples were prepared at a concentration of 0.1 mg/mL in water unless stated otherwise.

Acid-Base titrations. pKa titrations were performed on PAs 1-4 in the range of pH 2-10 with a Fisher Accumet pH meter at a concentration of 3.5 mg/mL in 100 mM KCl. For the acidic PAs 2 and 4, 0.1N KOH was added in 1-5 μ L increments, starting at low pH, whereas for the basic PAs 1 and 3, 0.1N HCl was added in 1-5 μ L increments, starting at a high pH.

NMR NOE spectroscopy. PAs 1-6 were dissolved in d_6 -DMSO at concentrations of 5 mg/mL. NOESY spectra were measured in D_2O with a mixing time of 0.1 s and 128 scans at a concentration of 10 or 15 mg/mL of each PA, at a 1:1 molar ratio. For FT-IR studies, 2% by weight samples were lyophilized from water and then pressed into KBR pellets.

Scheme 1. Chemical structures of peptide amphiphiles

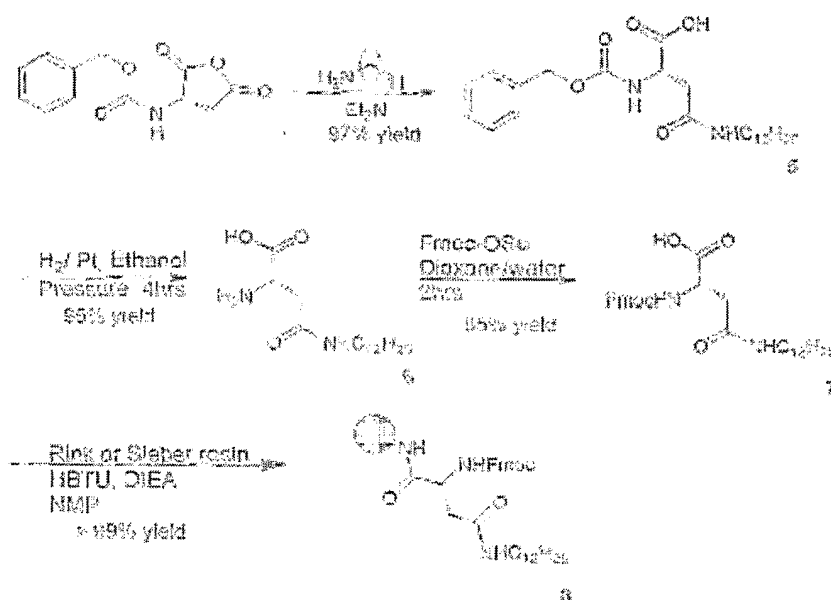


Example 1

Four PAs, two with a triple lysine sequence (1,3) and two with a triple glutamic acid sequence (2,4), were prepared (Scheme 1). PAs 1 and 2 were prepared by standard Fmoc solid-phase peptide techniques using a preloaded

Wang resin followed by alkylation with palmitic acid with 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) as a coupling reagent. The amphiphile was cleaved from the resin with a mixture of 95% trifluoro-acetic acid (TFA), 2.5% water and 2.5% tri-isopropylsilane (TIS). For the synthesis of peptide amphiphiles 3 and 4, amino acid 7 was synthesized according to Scheme 2. N-Carbobenzyloxy-L-aspartic anhydride was reacted with dodecylamine, yielding fatty acid amino acid 5. The CBz group was then removed by catalytic hydrogenation to yield 6, followed by Fmoc-protection of the amine. This synthesis proceeds readily on a 5 gram scale. Product 7 was then coupled to a rink resin using HBTU as a coupling reagent. Subsequently, the remaining amino acids were added to 8 using standard Fmoc solid phase techniques. Standard cleavage conditions yielded PAs with reverse structure as previously described. ^1H NMR and electrospray ionization mass spectrometry are consistent with the expected structures.

Scheme 2. Synthesis of fatty acid amino acid



Example 1a

N-dodecyl-2-carbobenzyloxyamino-succinamic acid (5).

N-Carbobenzyloxy-L-aspartic anhydride (1mmol) was dissolved in 50 mL

methylene chloride, followed by the addition of 1.05 eq of dodecylamine and 1.1 eq of triethylamine. The reaction was capped to prevent evaporation, and stirred for 12 hours. When no trace of starting material could be detected by thin layer chromatography (TLC) (CH_2Cl_2 , 5% MeOH), the reaction was quenched with 20mL 1N hydrochloric acid followed by extraction with chloroform (5x). The organic layer was dried over magnesium sulfate, and 7 was obtained as a white solid (yield 97%).

^1H NMR (CDCl_3): δ 0.8 (t, $J=8.5\text{Hz}$, 3H, tail CH_3), 1.18 (s, 18H C_9H_{18} tail), 1.48 (br-s, 2H, CONHCH_2), 2.68 (s, 2H, $\text{CONHCH}_2\text{CH}_2$), 3.15 (br-s, 2H, Asp H_β), 4.49 (m, 1H, Asp H_α), 5.12 (s, 2H, PhCH_2O), 7.28 (s, 5H, Ph). ^{13}C (DMSO-D_6) δ 14.6, 22.8, 29.4, 29.6, 29.7, 32.0, 52.2, 56.7, 66.1, 128.4, 129.0, 137.7, 156.5, 171.1, 172.5. ESI MS: m/z : 435.4 (MH^+).

Example 1b

2-amino-N-dodecyl-succinamic acid (6). In 100 mL ethanol, 100 mmol of 7 was dissolved and transferred to a reaction vessel containing Pd and C (10% by weight). The vessel was then placed under hydrogen (35 Torr) for 3 hours. The reaction mixture was filtered over celite, and the product obtained as a white solid after evaporation to dryness under reduced pressure. Yield 95%.

^1H NMR (CD_3CN): δ 1.15 (m, 23H, aliphatic tail), 1.3 (m, 2H, $\text{CONH}_2\text{CH}_2\text{CH}_2$), 2.37 (br-s, 2H, CONH_2CH_2), 3.55 (m, 3H, Asp H_β), 4.6 (m, 1H, Asp H_α). ESI MS: m/z : 301.2 (MH^+).

Example 1c

N-dodecyl-2-Fmoc-amino-succinamic acid (7). Into 200mL of a water/dioxane (1:1 v:v) mixture, 6.6 mmol of 6 were dissolved with 1.3 mL (1.5 eq.) of triethylamine followed by 1 eq. of Fmoc-OSuccinimide (Fmoc-OSu). The reaction was monitored by TLC (CH_2Cl_2 , 10% MeOH) and after 2-3 hours, all of the Fmoc-OSu was consumed. The reaction was quenched with acid resulting in a white precipitate that was collected by filtration. Yield 85%.

^1H NMR ($\text{d}_6\text{-DMSO}$): δ 0.84 (t, $J=8$ Hz, 3H, terminal aliphatic CH_3), 1.19 (s, 18H, aliphatic CH_2), 1.34 (s, 2H, $\text{NH}_2\text{CH}_2\text{CH}_2$), 2.61 (br-s, 2H, CONHCH_2), 3.08 (s, 2H, Asp H_β), 4.24 (m, 3H, Asp H_α + Fmoc CH_2CONH + Fmoc CH), 7.3 (t, $J=9$ Hz, 1H, FmocH), 7.39 (t, $J=9$ Hz, 1H, FmocH), 7.68 (d, $J=8.5$ Hz, 1H, FmocH), 7.85 (d, $J=9$ Hz, 1H, FmocH). ^{13}C (DMSO-D_6) δ 14.6, 22.8, 29.4, 29.5, 29.7, 32.0, 46.1, 47.3, 52.2, 6.7, 120.8, 126.0, 127.7, 128.3, 141.5, 144.5, 156.5, 171.1, 172.5. ESI MS: m/z 524 (MH^+).

Example 1d

PA synthesis and purification. PAs 1-2 were prepared as described in ref. 3. For PA's 3-6, the standard rink resin was placed in a reaction vessel and deprotected three times with 30% piperidine in NMP, and then coupled to 2 eq. of 7 overnight. Coupling was repeated until a ninhydrin test showed negative results. This modified resin 8 was then loaded onto the automated synthesizer, and peptide synthesis proceeded as for PAs 1-2. When the automated synthesis was complete, the PA was cleaved from the resin as described in ref. 3.

Example 1e

$\text{C}_{15}\text{H}_{31}\text{CONHVal-Val-Val-Ala-Ala-Ala-Lys-Lys-Lys-COOH}$ (1)

^1H NMR (d_6DMSO): δ 0.80- 0.84 (m, 21H, Val_γ + tail CH_3), 1.22 (br-s, 28H, C_{14} aliphatic tail), 1.36 (m, 6H, Ala H_β), 1.51 (m, 11H, Lys H_γ + tail $\text{CH}_2\text{CH}_2\text{CONH}$), 1.62 (m, 6H, Lys H_β), 1.91 (m, 9H, Val H_β + Lys H_δ), 2.16 (m, 2H, tail CH_2CONH), 2.73 (s, 6H, Lys H_ϵ), 4.10-4.23 (m, 9H, H_α), 7.6 – 8.1 (Amide NH). ESI MS ($\text{MeOH:H}_2\text{O}$ 1:1 v:v): m/z = 1151 (MH^+).

Example 1f

$\text{C}_{15}\text{H}_{31}\text{CONHVal-Val-Val-Ala-Ala-Ala-Glu-Glu-Glu-COOH}$ (2)

^1H NMR (d_6DMSO): δ 0.81 (br-s, 21H, Val_γ + tail CH_3), 1.21 (br-s, 31H, C_{14} aliphatic tail), 1.45 (s, 2H, Ala H_β), 1.75 (m, 6H Glu H_β), 1.94 (m, 9H, Val β + Glu H_β), 2.24 (s, 6H, Glu H_γ), 4.2 (s, 9H, H_α), 7.6-8.1 (Amide NH). ESI MS ($\text{MeOH:H}_2\text{O}$ 1:1 v:v): m/z = 1177 (MNa^+).

Example 1g

Asp(CONHC₁₂)-Val-Val-Val-Val-Val-Val-Lys-Lys-Lys-NH₂ (3)

¹H NMR (d₆DMSO): δ 0.82 (br-s, 39H, Val H_γ + tail CH₃), 1.22 (br-s, 20H, C₁₀ aliphatic tail), 1.34-1.53 (m, 6H, Lys H_γ), 1.69 (m, 6H, Lys H_β), 1.93 (m, 12H, Lys H_δ + Val H_β), 2.74 (m, 2H, tail CH₂NH), 2.98 (br-s, 6H, Lys H_ε), 4.09-4.44 (m, 9H, H_α), 7.02-8.25 (Amide NH). ESI MS (MeOH:H₂O 1:1 v:v): *m/z* = 1279 (MH⁺).

Example 1h

Asp(CONHC₁₂)-Val-Val-Val-Ala-Ala-Ala-Glu-Glu-Glu-NH₂ (4)

¹H NMR (d₆DMSO): δ 0.82 (br-s, 21H, Val H_γ + aliphatic tail CH₃), 1.22 (s, 20H, tail C₁₀), 1.32 (m, 9H, Ala H_β), 1.74 (m, 3H, Glu H_β), 1.94 (m, 6H, Glu H_β + Val H_β), 2.25 (m, 6H, Glu H_γ), 2.97 (d, J= 6 Hz, 2H, tail CH₂CH₂CONH), 4.10-4.44 (m, 9H, H_α), 7.23-8.2 (Amide NH). ESI MS (MeOH:H₂O 1:1 v:v): *m/z* = 1197 (MH⁺).

Example 2

When dispersed in aqueous media in the presence of suitable stimuli, PAs typically self-assemble into high aspect ratio cylindrical nanofibers. Based on previous work, fibers consisting of either one PA molecule or a mix of two PA molecules would be expected to show secondary structures with β-sheet-like hydrogen bonding. It was anticipated that mixing of the negatively charged 2 or 4 with oppositely charged 1 or 3, respectively, would result in parallel β-sheet-like hydrogen-bonding arrangements, while mixing 1 or 2 with amine terminated oppositely charged 4 or 3, respectively, could result in antiparallel arrangements.

Example 3

At a concentration of 0.1 mM, the CD spectra of 2-4 revealed peptide segments with predominantly random coil character. This most likely results from electrostatic repulsion among the highly charged molecules. Upon a change of pH to neutralize the charges or upon addition of calcium ions, all PAs exhibited β-sheet signatures. In contrast, 1 exhibits a β-sheet signature

under any pH. 1 may be less charged than the others, since the acid terminus can neutralize the charge of one of the lysine residues, giving the molecule a formal net charge of +2. This lower overall charge may reduce repulsion and allow β -sheet hydrogen bonding within the fibers to take place. Conversely, 4 would have a formal net charge of -2, as the amine terminus would neutralize the charge of one of the glutamic acid residues, allowing for the β -sheet interactions to occur. However, 4 still exhibited a disordered CD signature. To resolve this apparent contradiction, the actual charge state of and apparent pKa of the aggregates at pH 7 was determined to better understand the driving forces for self-assembly of these various systems.

Example 4

pKa titrations of aggregates of molecules 1-4 were carried out at a concentration of 3 mM. All titrations were started at a pH where the molecules were already in their aggregated state in order to avoid kinetic effects due to self-assembly. Only the pKa titration of 3 showed sharp transitions, correlating to two apparent pKa's, one attributed to the deprotonation of the more solvent accessible amine terminus, and the other originating from one or more of the ϵ lysine amines. Aggregates of PAs 1, 2 and 4 show complex curves with transitions occurring over wide ranges, implying that the protonation/deprotonation of these supramolecular objects occurs slowly, with variations of acidity due to the local microenvironments within the nanofibers. It is clear from these results that aggregation changes the apparent pKas of the acid and amine groups, consistent with recent reports in the literature.

Example 5

Co-assembly of charge complementary PA molecules should lead to fibers containing a mix of the two components with β -sheet hydrogen bonding arrangements. When 2 is mixed with the triple lysine amphiphiles 3 or 1 (abbreviated as 2/3 or 2/1), the CD-spectrum obtained corresponds to a pure β -sheet. The fact that the observed β -sheet signature is not merely a superposition of individual CD spectra of the two components strongly suggests the formation of mixed nanofibers in which two molecules form a

single aggregate structure. PA 1 mixed with the triple glutamic acid PAs 4 or 2 (1/4 or 1/2) exhibit similar behavior. When mixing two PAs of similar charge, the 1/3 lysine mixture shows a β -sheet, whereas the 2/4 glutamic acid mixture shows disordered conformation, possibly due to greater charge repulsion among the glutamic acid residues.

Example 6

In order to further demonstrate co-assembly of the molecules within the fibers, nuclear overhauser (NOE) spectroscopy was performed on a 1.5% by weight gel made up of the charge complementary PAs. A representative NOESY of 2 and 3 (2/3) shows close contacts ($<3\text{\AA}$) observed between the Glu- H_β protons of 2 and the Lys- H_ϵ and Val- H_δ protons of 3, respectively. Several other possible intermolecular contacts were detected but could not be attributed unambiguously to 2/3 contacts. These results provide additional evidence that the two PA molecules are co-assembled within the same nanofiber.

Example 7

Once the successful co-assembly of the amphiphiles was established, the gelation behavior of the systems from both single and multiple PAs was investigated. One-weight percent solutions of 1-4 were slightly opaque and could be gelled by the addition of acid (1,3) or base (2,4), respectively. Transmission electron microscopy reveals the formation of nanofibers with average diameters of 6.5 nm and average lengths of several hundred nanometers, similar to those observed previously in other PAs.

Example 8

Phage display is typically used to find receptor-blocking peptides, *i.e.* sequences with high binding constants. To transfer a growth factor from the PA binding site to the cell receptor, an extremely high binding constant is not required and might even be unfavorable. Therefore, the 7-mer commercial phage library was elected over the 13-mer library, aiming at intermediate binding strengths. Growth factors were physisorbed onto 96-well plates and subsequently exposed to the phage library. After incubation at room

temperature for 60 minutes, the unbound phage was removed by rinsing with detergent solution. Bound phage was subsequently eluted by incubating with a growth factor solution for 60 minutes. The recovered phages were amplified for 4 h in *E. Coli* at 37 °C and the panning was repeated two more times. In subsequent panning rounds, the stringency was increased by raising the detergent concentration and reducing the exposure time to the library. The final phage population was plated and the DNA of 10 clones of colonies containing only one phage type were sequenced. Next, ELISA screening was used to test for false positives and determine relative binding strengths of the selected clones. Utilizing this protocol, the best binding epitope for rh-BMP-2 was found to be YPVHPST and for rh-TGF- β 1 LPLGNSH. (See Table 1, below.)

Example 8a

Phage display was performed with the Ph.D. 7 kit (New England Biosystems). Fifty microliters of diluted M13-phage library (containing 2×10^9 phages and all possible 7-mer sequences) were added to 96-well microtiter plates, which had been exposed to 50 μ l 10 μ g/ml growth factor (peprotech) solution for 18 h at 0 °C. The plates were blocked for 1 h then incubated at room temperature for 60 minutes with gentle agitation. Unbound phage was removed by rinsing with TBS/Tween-20 (0.1% v/v Tween-20). Bound phage was subsequently eluted by incubating with 50 μ l growth factor solution for 60 minutes. The presence of binding phage was determined by serial dilution of the phages and subsequent plating with *E. Coli* on agar plates. The number of plaques formed can be related to the number of phages in the original mixture. The phages were subsequently amplified for 4 h in *E. Coli* at 37 °C. In subsequent panning rounds, the concentration of Tween-20 was raised to 0.5% and the binding and elution times were decreased and increased, respectively, to increase the stringency of the selection process. After three rounds of panning, the recovered phage mixture is diluted and plated with bacteria on agar. Subsequently, plaques are isolated containing a single DNA sequence. After purification, ten clones were sequenced. Next, ELISA

screening was used to eliminate false positives and determine relative binding strengths of the selected clones. Each clone was incubated for 1 h on growth factor coated microtiter plates, the plates were rinsed with TBS/Tween-20 and subsequently HRP-conjugated anti-M13 antibody (Amersham Biosciences) was incubated for 1 h. After addition of ABTS, the absorbance at 450 nm was measured after 30 minutes to determine the relative binding strengths of the isolated clones. The strongest binder was subsequently selected.

Table 1: Binding sequences for BMP-2 and TGF- β 1 derived using phage display

BMP-2:		TGF- β 1	
YPVHPST (1)	LHYPFMT (3)	LPLGNSH (1)	RTTSPTA (3)
KVPPANT (3)	QQTQAQH (4)	LRNYSHS (3)	GKYPPTS (3)
KQALTQT (3)	PIQPDER (2)	VYRHLPT(2)	AWKSVTA (3)
WPALFTH (3)	PFDPPVR (2)	RVSTWDT (3)	LPSPIQK (2)
PGPTVQG (2)	DVSPAYH (3)	PAPRWIH (3)	

The numeric coding illustrates the relative binding strengths of the selected clones in decreasing binding strength from highest (1) to lowest (4). All sequences bind significantly.

Example 9

Since the peptide sequences as displayed on the phage had a free N-terminus, it was not possible to use a typical synthesis route for the PAs. Therefore, an aspartic acid whose side chain was modified with dodecylamine was reacted with a Rink amide resin. (See, Scheme 2, above.) Subsequently, the remainder of the peptide could be synthesized using regular Fmoc-peptide synthesis techniques, yielding a PA with reverse polarity. The elected peptide sequences were V₆K₃SG₃YPVHPST (9) for BMP-2 and V₃A₃K₃SG₃LPLGNSH (10) for TGF- β 1, respectively (Scheme 3). The K₃ segment was chosen to co-assemble with the charged E₃ head groups of the filler 2. The SG₃ sequence is a spacer unit and is the same spacer as was present on the phage. Binding constants of BMP-2 with, respectively, YPVHPST and KQALTOT were

$K_a = 1.9 \pm 0.9 \cdot 10^6 \text{ M}^{-1}$ and $2.1 \pm 0.85 \cdot 10^5 \text{ M}^{-1}$ as determined by fluorescence depolarization, illustrating the tunability of binding strength.

Example 9a

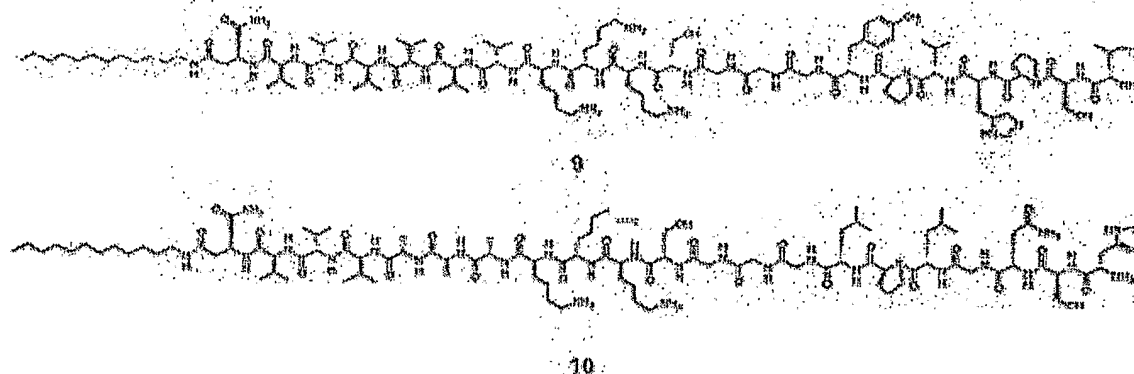
Asp(CONHC₁₂)-Val-Val-Val-Val-Val-Val-Lys-Lys-Lys-Ser-Gly-Gly-Gly-Tyr-Pro-Val-His-Pro- Ser-Thr (9)

¹H NMR (d₆DMSO): δ 0.82 (br-s, 39H, Val H_γ + tail CH₃), 1.22 (br-s, 20H, C₁₀ aliphatic tail), 1.53 (m, 6H, Lys H_γ), 1.69 (m, 6H, Lys H_β), 1.93 (m, 16H, Lys H_δ + Val H_β + Pro H_γ), 2.37 (m, 4H, Pro H_β), 2.74 (br-s, 6H, Lys H_ε), 2.98 (m, 2H, tail CH₂NH), 3.45 (m, 8H, Pro H_δ + His H_β + Tyr H_β), 3.72 (m, 3H, Thr H_α + Thr H_β), 4.1-4.6 (m, 13H, H_α + Ser H_β), 6.81 (m, 5H, Tyr Aromatic Hs + His H), 7.02-8.25 (Amide NH + His H). ESI MS (H₂O): 773.9 (M + 3H)⁺³.

Example 9b

Asp(CONHC₁₂)-Val-Val-Val-Ala-Ala-Ala-Lys-Lys-Lys-Ser-Gly-Gly-Gly-Leu-Pro-Leu-Gly-Gln-Ser-His-NH₂ (10)

¹H NMR (d₆DMSO): δ 0.82 (br-s, 21H, Val H_γ + aliphatic tail CH₃), 1.22 (br-s, 20H, tail C₁₀), 1.32 (m, 9H, Ala H_β), 1.53 (m, 6H, Lys H_γ), 1.64 (m, 6H, Lys H_β), 1.84 (m, 4H, Leu H_β), 1.93 (m, 16H, Lys H_δ + Val H_β + Pro H_γ), 2.37 (m, 2H, Pro H_β), 2.74 (br-s, 6H, Lys H_ε), 2.97 (m, 2H, tail CH₂CH₂CONH), 3.45 (m, 4H, Pro H_δ + His H_β), 3.72 (m, 3H, His H_α + Ser H_β), 4.10-4.61 (m, 9H, H_α), 6.81 (m, 2H, His H), 7.02-8.25 (Amide NH + His H). ESI MS (MeOH:H₂O 1:1 v:v): $m/z = 1086.7$ (M + H)⁺².

Scheme 3:Example 10

Co-assembly of PA 9 with the filler PA 2 could be demonstrated utilizing circular dichroism (CD) spectroscopy. At a concentration of 0.13 mM, the CD spectrum of the individual PA nanofibers at this concentration have predominantly random-coil character. When mixed together, however, the CD-spectrum reflects a pure β -sheet, illustrating that the PAs co-assemble within one fiber rather than form individual homo-fibers consisting of only one PA molecule. Co-assembly is energetically favored since the electrostatic repulsions are reduced when the KKK and EEE part form a complex. Additional evidence for co-assembly was obtained using Nuclear Overhauser Effect spectroscopy on 1.5 wt% solutions. Close contacts ($< 3 \text{ \AA}$) were observed between the E_β and K_ϵ protons and between the E_β and V_δ protons and this distance would be significantly larger between homo-fibers. Other possible intermolecular contacts could not be attributed unambiguously.

Finally, Fourier Transform infrared spectroscopy on lyophilized samples and 1 wt% solutions showed the presence of the 1630 cm^{-1} peak typical of β -sheets. Similar results were obtained for 10/2 mixtures. Annealing of the samples for 24 h at $37\text{ }^{\circ}\text{C}$ led to a significant increase in the strength of the CD effect. This increase is attributed to an increase in the size of the domain of perfectly coupled chromophores. Presumably, the initial mixing leads to the kinetic entrapment of a non-perfect mixed system and the annealing allows for the subsequent reorganization of amphiphiles to the thermodynamically most stable state. Even prolonged heating at $80\text{ }^{\circ}\text{C}$, well above the expected melting temperatures for the hydrogen bonding network and the aliphatic tails, did not lead to any noticeable loss of supramolecular chirality, illustrating the high stability of the nanofibers.

Example 11

In order to test the physiological significance of this approach, mesenchymal stem cells were cultured in gels containing different levels of growth factors. For these cell experiments, the binding epitopes were diluted to a 50:1 (filler:binder) ratio to ensure recognition by the protein. Gels were prepared by mixing $50\text{ }\mu\text{l}$ of pre-mixed PA (2% by weight) containing the desired amount of growth factor and $50\text{ }\mu\text{l}$ mesenchymal stem cell suspension (100,000 cells) on glass cover slips, followed by the addition of $10\text{ }\mu\text{l}$ 30 mg/ml CaCl_2 solution. The gels were allowed to solidify for 1.5 hours after which 1 ml of mesenchymal stem cell media was added. In this way, gels were prepared consisting either of 9/2 or 2 alone and containing 0, 1 or 50 ng/ml of BMP-2. As a further control, 10,000 stem cells were plated on glass cover slips with BMP-2 added to the media in identical concentrations. The media was refreshed every fourth day and triplicate samples were isolated after 7 and 21 days of culture. Subsequently, the protein expression of lineage-specific markers osteocalcin (osteoblast), adiponectin (adipocytes), collagen II and aggrecan (chondrocytes) and α -smooth muscle actin and desmin (smooth muscle cells) was assessed by western blot.

Example 11a

Protocol for cell experiments: Human mesenchymal stem cells (Cambrex technologies) were plated at a density of 5,000 cells/cm² in four T75 culture flasks in 15 ml of mesenchymal stem cell media (Cambrex Technologies) and incubated at 37 °C, 5% CO₂, 90% humidity. At 90% confluency, the cells were trypsinized with 2.5 ml 0.25% trypsin/EDTA for 5 minutes. Subsequently, 8 ml of media was added to quench the protease activity. The cell suspensions were combined and centrifuged at 850 rpm for 8 minutes. The cell pellet was re-suspended in 1 ml of media and 10 µl was used for cell counting. The cell suspension was diluted with media to a concentration of 2,000,000 cells/ml.

PAs 1 and 3 were dissolved at a concentration of 2 wt%. Subsequently, 50 µl of 1 was mixed with 1250 µl of 3. The mixture was split in 3 portions of 410 µl and in addition 3 portions of 410 µl 3 (2wt%) was prepared. Then, 20 µl of 2µg/ml BMP-2 (Peprotech) was added to one portion of 1/3 and 1 to a concentration of 100 ng/ml BMP-2. Similarly, 41 µl of 20 ng/ml BMP-2 was added to a final concentration of 2 ng/ml BMP-2.

Gels were prepared in triplo by mixing 50 µl PA solution with 50 µl cell suspension on a microscope glass cover slip. Then, 10 µl 30 mg/ml CaCl₂ was added and the gels were allowed to solidify at 37 °C, 5% CO₂, 90% humidity for 90 minutes. The resulting concentrations are 1 wt% PA and 0, 1 or 50 ng/ml in BMP-2. Next, 1 ml media was added. Controls with growth factor in solution were prepared by placing 5 µl cell suspension on a glass cover slip followed by the addition of 1 ml of media. Then, 25 µl 2 µg/ml BMP-2 and 0.5 µl 2 µg/ml BMP-2 were added to obtain BMP-2 concentrations of 50 ng/ml and 1 ng/ml in solution, respectively. Half the media was refreshed every fourth day

Example 12

Cell viability assays showed that the viability of the cells was > 95% in all the gels after 3 weeks of culture. Expression of endoglin could not be detected by western blot at either time point, indicating that all cells had started

to differentiate. No expression of adiponectin and aggrecan could be detected at any point either. The expression of osteocalcin showed a marked difference between the gels and the controls. Whereas no osteocalcin could be detected in the controls, all gels expressed significant levels of osteocalcin at 7 days already rather than the usual 2-3 weeks (figure 3a), but did not increase significantly at the 3 week time point (figure 3b). The comparable expression levels at 1 and 3 weeks then imply that the expression levels of osteocalcin are constant during the culture period. Surprisingly, the expression seems to be independent on the concentrations of BMP-2 and the ratio is 1:2 between binding and no binding PA-containing gels. Moreover, the gels to which no BMP-2 was added show similar levels. Apparently, the endogenous BMP-2 levels produced by the cells themselves are enough to stimulate osteoblastic differentiation, but the capability of the binding gels to bind some of the BMP-2 lowers the amount of expressed osteocalcin slightly. The expression of osteocalcin could also be visualized with immunocytochemistry.

In contrast, α -smooth muscle actin was not detected in gels containing 9, whereas it was expressed in the gels without the binder. However, it was expressed in either case after 3 weeks (figure 3b), but the expression in 9/2 gels was still slightly less than in the 2 gels and significantly lower than the controls. Furthermore, no desmin expression could be detected, which is the marker of terminally differentiated smooth muscle cells.

Example 12a

Western Blot protocol: After removal of the media, the gels were rinsed with 1 ml phosphate buffer saline and subsequently, the cells were lysed in 200 μ l 2% sodium dodecyl sulfate, 0.08 M Tris, 10% glycerol. The three lysates were combined and protein concentrations were measured by mixing 10 μ l lysate with 200 μ l BCA protein assay reagent (Pierce) and reading the absorbance at 562 nm against a BSA standard series after 30 min. incubation at 37 °C. Subsequently, 10 μ g of protein, 3 μ l β -mercapto-ethanol, 3 μ l bromophenolblue were mixed and water was added to a total volume of 36 μ l. The mixture was boiled for 5 minutes and loaded onto either a 4% (endoglin,

aggrecan) or a 10-20% (osteocalcin, α -smooth muscle actin, adiponectin, desmin) Novex tris(glycine) gel (Invitrogen). The gels were run for 90 minutes in tris(glycine) running buffer (Invitrogen) at a constant voltage of 130 V. Then, the proteins were transferred to a nitrocellulose membrane (Bio-Rad) at a constant current of 190 mA for 90-120 minutes.

The membranes were blocked for 1 h in 5% non-fat milk (Bio-Rad) and probed for the respective markers at a 1:500 dilution in 1% milk for 2 h using the monoclonal antibodies for osteocalcin (clone 190125, R&D systems) endoglin (clone 166709, R&D systems), adiponectin (clone 166126, R&D systems), α -smooth muscle actin (clone 1A4, R&D systems) and polyclonal aggrecan (AF1220, R&D systems). The membranes were rinsed 3x for 15 minutes with tris buffer saline/0.1% tween-20 (TTBS). Then, secondary antibodies were equilibrated for 1 h at a 1:3,000 dilution in 1% milk followed by 3 rinsing steps with TTBS. The membranes were developed by 1 minute equilibration with ECL western blot analysis solution (Amersham Biosciences) and exposed to ECL hyperfilm (Amersham Biosciences).

Example 12b

Cells were fixed for 20 mins with 2% paraformaldehyde/0.2% glutaraldehyde/0.2M sodium cacodylate at 4°C. After rinsing with PBS (2x), the samples were incubated for 5 mins with 0.1% Triton-X in PBS. Subsequently, the samples were rinsed with PBS (2x). Next, human osteocalcin monoclonal antibody (R&D systems, 1:200) in 1% BSA/PBS was added and the samples were incubated overnight at 4 °C. After triple rinsing with PBS, FITC-conjugated secondary antibody in 1% BSA/PBS was added and incubated for 2h. The samples were visualized after triple PBS rinsing on a Nikon Eclipse TE2000 microscope at 10x magnification.

Example 13

A series of experiments were performed as for PA 10, binding TGF- β 1, BMP-2 experiments, except that the highest TGF- β 1 concentration is 20 ng/ml rather than 50 ng/ml. Samples were isolated after 10 days. Culturing in the

presence of TGF- β 1 leads to differentiation into the smooth muscle and cartilage lineages. Collagen II expression is higher for the binding gels and seems to increase with increasing TGF- β 1. Surprisingly, osteocalcin and collagen X are expressed, which indicates that maturation to the stage of hypertrophic chondrocytes occurs, which is unexpected at this early time point. Similarly, α -smooth muscle actin expression increases with TGF- β 1 concentration but is higher in the binding gels than in the nonbinding gels. It's even higher in the controls, but the expression levels of desmin show that these are mainly immature muscle cells.

Example 14

Numerous binding sequences were determined for vascular endothelial growth factor (VEGF), both with 7- and 12-mer phage display kit, basic fibroblast growth factor (FGF-2), neurotrophin-3 (NT-3) and laminin-5; * denotes the strongest binders:

VEGF	
<i>7-mer</i>	<i>12-mer</i>
WPTWVNN	PTPLKVRLHSYN*
YYTVHHM	VSILSTIPNSMT*
WHWSLNH	PLTPSALLPIFE*
SWWAPFH	LPQKNTIQYEKM
FTEPLAS	
THAFRVM	
ASLFSSN	
LLTVSSY	
LPYPHYH	

FGF-2

PMHHHKH
 AQVRSGD
 KHPPTNW
 AMLSHLS
 DFIQPYQ
 VYWSRIE
 AMPQRPL
 HSRHFHH
 RMTQVPL
 LSTPPLR

NT-3

HTTEILH
PSNYQTS
SYFPSSA
EARQSYS
DEPQKAH
TLGLGLH
YMRRSLS
VVLYLPL

Laminin-5

SKLNTKS
PTYHHRH
LRHKSLH
RYHPHLH*
GRYHHYLH

As illustrated by the preceding examples, this invention provides a synthetic strategy for peptide amphiphile molecules with free N-termini compatible with standard solid phase methodology. When mixed with free C-terminus PAs, these molecules self-assemble into nanofibers containing highly thermally stable β -sheet structures which appear to be more stable than co-assemblies of PAs with identical polarity. The new opportunity to create assemblies with free N-termini on their surfaces enables the design of bioactive nanofibers not accessible previously with nanostructures that expose the C-terminus of the peptide sequence.

Although the present invention has been described in considerable detail with reference to certain preferred embodiments thereof, other versions are possible. For example peptide or protein sequences derived from a phage display process may be used to form binding peptide amphiphiles that bind and retain proteins other than growth factors. Enzymes could be coupled to form binding peptide amphiphiles which can be self assembled and immobilized on the surfaces of nanofiber hydrogels. Such hydrogels may be used as a coating for sensor applications. Alternatively, the binding interaction of the phage display derived peptide coupled to the peptide amphiphile could be selected to strongly bind peptides such as HGF (hepatocyte growth factor) or VEGF and treat various diseases and conditions

by removing them. Hydrogels formed from self assembly of these binding peptide amphiphiles could be molded for insertion into a site on a patient or for use in a filtration system. The hydrogels could be used to remove target peptides like HGF or VEGF from a site such as a joint or tumor on a patient or from a fluid in a patient.

We claim:

1. An amphiphilic peptide compound comprising a peptide component and a hydrophobic component, said peptide component comprising a growth factor recognition product of a phage display process, said recognition product coupled to said peptide component at about the N-terminus thereof, said hydrophobic component coupled to said peptide component at about the C-terminus thereof.
2. The compound of claim 1 wherein said recognition product is selected from epitope sequences providing binding interactions with growth factors BMP-2, TGF- β 1, VEGF, FGF-2, NT-3 and laminin-5.
3. The compound of claim 2 wherein said binding epitope sequence comprises YPVHPST.
4. The compound of claim 3 wherein said sequence is in a non-covalent binding interaction with growth factor BMP-2.
5. The compound of claim 2 wherein said binding epitope sequence comprises LPLGNSH.
6. The compound of claim 5 wherein said sequence is in a non-covalent binding interaction with growth factor TGF- β 1.
7. The compound of claim 1 wherein said peptide component has a net charge at a physiological pH.
8. The compound of claim 7 comprising residues selected from alanine, glycine, leucine, cysteine, valine and serine.
9. The compound of claim 2 wherein said hydrophobic component comprises an alkyl moiety ranging from about C₆ to about C₂₂.
10. The compound of claim 1 wherein said peptide component is substantially linear.
11. The peptide composition comprising a plurality of a first amphiphilic peptide compound, each said compound comprising a growth factor recognition product of a phage display process coupled to a peptide component of said compound, said peptide component having a net charge at a physiological pH and coupled to a hydrophobic component at about the

C-terminus thereof.

12. The composition of claim 11 further comprising a plurality of a second amphiphilic peptide compound, each said compound absent a growth factor recognition product and having a net charge at a physiological pH complementary to said net charge of said first compound.

13. The composition of claim 12 wherein the amino acid sequence of the peptide component of said second compound has a length shorter than the length of said first compound peptide amino acid sequence.

14. The composition of claim 13 comprising a micellar assembly in an aqueous medium.

15. The composition of claim 14 comprising at least one growth factor non-covalently interacting therewith.

16. The composition of claim 15 wherein said growth factor is selected from BMP-2, TGF- β 1, VEGF, FGF-2, NT-3, laminin-5 and combinations thereof.

17. The composition of claim 16 contacting a stem cell.

18. The composition of claim 11 further comprising a plurality of a second amphiphilic peptide compound, said compound comprising a peptide amino acid sequence having a length shorter than the length of said first compound peptide amino acid sequence, said second compound absent a growth factor recognition product and having a net charge at a physiological pH complementary to said net charge of said first compound.

19. The composition of claim 18 comprising a micellar assembly in an aqueous medium.

20. The composition of claim 19 contacting stem cells and comprising a growth factor non-covalently interacting with said composition.

21. A method of using an amphiphilic peptide compound to affect bioavailability of a growth factor, said method comprising:

providing a micellar assembly comprising a plurality of a first amphiphilic compound, each said compound comprising a growth factor recognition product of a phage display process coupled to a peptide component

of said first compound, said peptide component having a net charge at a physiological pH and coupled to a hydrophobic component at about the C-terminus thereof, and a plurality of a second amphiphilic peptide compound comprising a peptide amino acid sequence having a length shorter than the length of first compound peptide sequence, said second compound absent a growth factor recognition product and having a net charge at a physiological pH complementary to the net charge of said first compound; and

contacting a stem cell with said assembly.

22. The method of claim 21 wherein said growth factor is produced by said stem cell.

23. The method of claim 21 wherein said growth factor is selected from BMP-2, TGF- β 1, VEGF, FGF-2, NT-3, laminin-5 and combinations thereof.

24. The method of claim 23 wherein said recognition product is selected from epitope sequences providing non-covalent interaction with one of growth factors BMP-2 and TGF- β 1.

25. The method of claim 24 wherein said epitope sequence comprises YPVHPST non-covalently interacting with growth factor BMP-2.

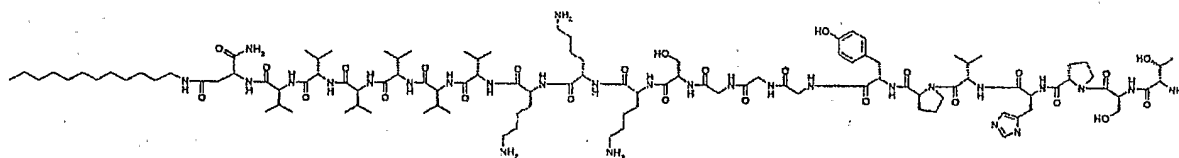
26. The method of claim 24 wherein said epitope sequence comprises LPLGNSH non-covalently interacting with growth factor TGF- β 1.

27. The method of claim 21 wherein said contact is sufficient for stem cell differentiation.

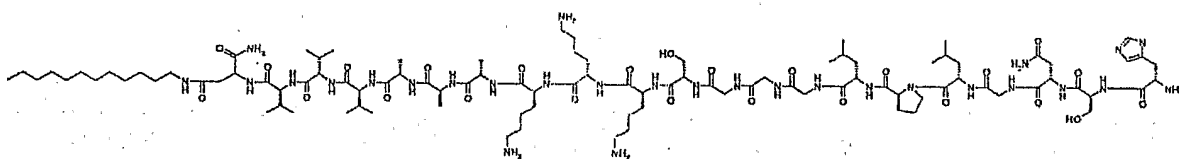
28. The method of claim 27 wherein said assembly comprises growth factor BMP-2 and said recognition product comprises an epitope sequence non-covalently interacting with said growth factor.

29. The method of claim 28 wherein said stem cell is a mesenchymal stem cell.

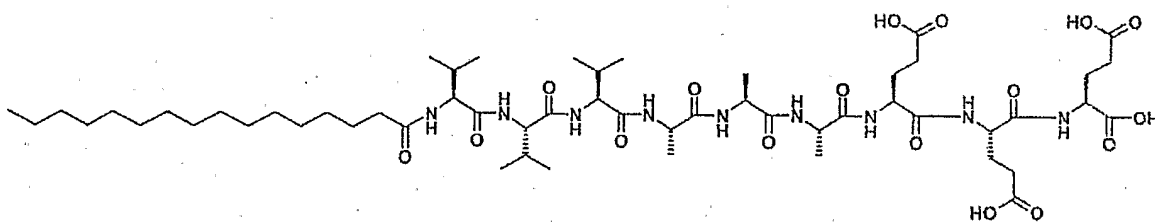
30. The method of claim 29 wherein said stem cell differentiates into bone cells.



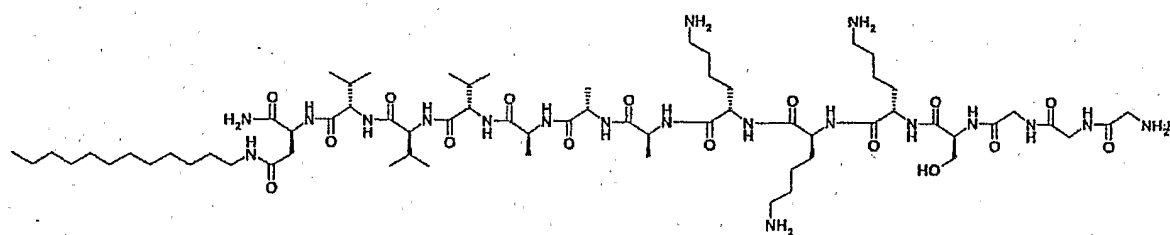
BMP-2 binding peptide amphiphile



TGF-β1 binding PA



Filler PA



Complementary filler

Figure 1

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US04/40550

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 38/00

US CL : 514/14

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. : 514/14

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)

STN EAST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2002062969 (SEMINO et al) 15 August 2003 (15.08.2003)	1-30
A	US 2002/0160471 (KISIDAY et al) 31 October 2002 (31.10.2002)	1-30
P	Donners et al. Growth factor binding self-assembling nanofiber networks for tissue regeneration. March 28-April 1, 2004. Abstracts of Papers, 227th ACS National Meeting, Anaheim, CA, American Chemical Society. BIOT-023.	1-30
P	Shawn et al. Self-assembling nanofiber matrix for bone regeneration. March 28-April 1, 2004, Abstracts of Papers, 227th ACS National Meeting, Anaheim CA, American Chemical Society. BIOT-340	1-30
Y	Anthony. Injectable Biomaterials for Bone Tissue Engineering. http://www.nuance.northwestern.edu/downloads/Anthony%20Murphy%20Report%20Spring%202003.pdf (Spring 2003) Accessed online 4/28/05. pages 1-12. See entire document, e.g., pages 3-7 and figures 1-2.	1-30

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

* Special categories of cited documents:

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"E"	earlier application or patent published on or after the international filing date	"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
"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)	"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
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		

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Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Facsimile No. (703) 305-3230

Authorized officer

Marcela M Cordero Garcia

Telephone No. (571) 272-1600

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US04/40550

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Wang, Lin-Fa. Epitope Identification and Discovery Using Phage Display Libraries: Applications in Vaccine Development and Diagnostics. Current Drug Targets, 2004, Vol. 5, No. 1, pages 1-15.	1-30