



(51) International Patent Classification:

A61K 38/18 (2006.01) C07K 14/50 (2006.01)
A61K 47/48 (2006.01) C07K 17/08 (2006.01)
A61P 17/02 (2006.01)

(21) International Application Number:

PCT/US2016/030510

(22) International Filing Date:

3 May 2016 (03.05.2016)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/157,194 5 May 2015 (05.05.2015) US
62/270,170 21 December 2015 (21.12.2015) US

(71) Applicant: **THE REGENTS OF THE UNIVERSITY OF CALIFORNIA** [US/US]; 1111 Franklin Street, Oakland, CA 94607-5200 (US).

(72) Inventors: **MAYNARD, Heather, D.**; 13328 Chalon Rd., Los Angeles, CA 90049 (US). **NGUYEN, Thi**; 6847 Chimineas Avenue, Reseda, CA 91335 (US). **DECKER, Caitlin**; 10744 Lawler St., Apt. 9, Los Angeles, CA 90034 (US).

(74) Agent: **BAKER, Jean, C.**; Quarles & Brady LLP, 411 E. Wisconsin Avenue, Milwaukee, WI 53202 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— with international search report (Art. 21(3))

(54) Title: FIBROBLAST GROWTH FACTOR POLYMERS, METHODS FOR MAKING THE SAME AND APPLICATIONS THEREOF



Fig. 1

(57) Abstract: AN FGF multimer comprising two or more FGF monomers, each of the two or more FGF monomers comprising one linking amino acid residue; and a linker molecule, wherein the linker molecule links the two or more FGF monomers through the linking amino acid residues is disclosed. Site-specific chemical dimerization of fibroblast growth factor 2 (FGF2) with the optimal linker length resulted in a FGF2 homodimer with improved granulation tissue formation and blood vessel formation at exceptionally low concentrations. Homodimers of FGF2 were synthesized through site-specific linkages to both ends of different molecular weight poly (ethylene glycols) (PEGs). The optimal linker length was determined by screening dimer- induced metabolic activity of human dermal fibroblasts and found to be that closest to the inter- cysteine distance, 70 Å, corresponding to 2 kDa PEG.

FIBROBLAST GROWTH FACTOR POLYMERS, METHODS FOR MAKING THE SAME AND APPLICATIONS THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority from US Provisional Application No. 62/157,194, filed on May 5, 2015 and US Provisional Application No. 62/270,170, filed on December 21, 2015, both of which are entitled “bFGF-Polymer Conjugates and Methods for Making the Same” and which are hereby incorporated by reference herein in their entirety.

STATEMENT REGARDING FEDERALLY-SPONSORED RESEARCH

[0002] This invention was made with Government support under EB013674, awarded by the National Institutes of Health and 0809832, awarded by the National Science Foundation. The Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

[0003] Healing in chronic wounds including venous, arterial, diabetic, and pressure ulcers is impaired due to decreased growth factor production, keratinocyte and fibroblast proliferation and migration, granulation tissue formation, and angiogenesis. Treatment of chronic wounds costs over 9.5 billion U.S. dollars annually, worldwide. Diabetes alone is projected to affect 439 million adults (ages 20-79) by 2030 globally, and 15% of diabetic patients develop chronic foot ulcers. Thus, viable treatment of chronic wounds represents a significant challenge to the medical community.

[0004] Fibroblast growth factors (FGFs) are important players in wound healing. For example, fibroblast growth factor 2 (FGF2), a growth factor whose expression is impaired or is enzymatically degraded in both diabetic and pressure ulcers, moderates cell proliferation, differentiation and migration of multiple cell types. FGF2 is critical in wound healing, angiogenesis, bone regeneration, neuroregeneration, and can even result in scarless healing. As a result, FGF2 released from gels alone or in combination with other proteins and/or heparin has been employed as a strategy to increase angiogenesis, ischemic heart repair, nerve regeneration, etc. *in vivo*. While FGF2 alone appears to be a good candidate for the treatment of chronic

wounds, and is approved in Japan (Fiblast or Trafermin) for skin ulcers, US and European clinical trials (Phase II for treatment of peripheral arterial and coronary disease and Phase III for neuropathic diabetic foot ulcers, respectively) have shown minimal effectiveness. Thus, increasing the effectiveness of FGF2 is an important endeavor. This has been undertaken by a variety of approaches, including adding peptides or proteins that bind to both FGF2 and its receptor, by truncating the FGF2 sequences, mutating specific amino acid residues, covalent modification, or by utilizing FGF receptor peptide agonists as an alternative.

[0005] FGF2 activity is dependent on the formation of a tetrameric complex, consisting of two FGF2 proteins and two FGF receptors (FGFR1). Many proteins, like FGF2, exist or self-assemble into homodimers or multimers in their native or active state and these structures are often required for protein activity. Synthetic routes to protein dimerization are pursued in the scientific community as a means to study protein interactions and to create superagonist growth factor therapeutics. Preorganization of dimeric ligands is known to increase the effective local concentrations, thereby facilitating activation of receptors. Heparin or oligoheparins are mimics of membrane-bound heparin-sulfates that are known to facilitate FGF2 receptor binding and dimerization. It has been shown that adding heparin, or oligoheparins is important for FGF2 activity, and it has been proposed that the heparin molecules promote FGF2 dimerization in heparanoid complexes. Nonspecific chemical crosslinking of FGF2 lysine side-chains through reaction with short (11.4 Å) tethers such as bis(sulfosuccinimidyl) suberate has been performed as a means to study the interaction of FGF2 with heparin oligomers. Towards the same end FGF2 has also been oligomerized through biotin-streptavidin binding. In addition, recombinant expression of a dimeric FGF2 has led to enhanced biological activity compared to FGF2 alone. While these prior reports nicely demonstrate the importance of dimerization of FGF2, we hypothesized that much more significant activity could be obtained by 1) conjugating site-selectively to residues spatially separated from both the heparin-binding domain and receptor binding sites and 2) probing the ideal length of the dimerizer.

[0006] Site-specific conjugation is imperative in the development of protein homodimers in order to avoid the formation of protein multimers or complex protein-polymer networks. In addition, polymer conjugation at or near an active site, or the addition of multiple polymers to a protein therapeutic can shut down protein activity. Therefore targeting a single reactive site is important to maintain protein activity. Cysteine is an ideal target for site-specific protein

modification due to its low abundance and nucleophilicity. FGF2 contains two surface-exposed free cysteines (Cys-78 and Cys-96). The mutation of either cysteine, as shown by Lappi et al, is not detrimental to protein activity, and Kang et al observed a retention of activity after PEGylation at both surface-exposed cysteines with a 5 kDa PEG. Therefore Applicants chose to install the genetic modification cysteine to serine at amino acid 78 (C78S), resulting in an FGF2 containing a single surface-exposed cysteine, Cys96 (shown in red, Fig. 1).

[0007] In addition, there have been several PEG-FGF2 monoconjugates prepared that have improved circulation life times and other favorable *in vivo* features such as enhanced penetration into the injured spinal cord.

[0008] Needed in the art is development of FGF multimers, including dimers, such as FGF2 dimers, as superagonists that could be used to create a more fertile wound healing bed.

SUMMARY OF THE INVENTION

[0009] In one aspect, the present invention discloses an FGF multimer. The FGF multimer comprises two or more FGF monomers, each of the two or more FGF monomers comprising one linking amino acid residue; and a linker molecule, wherein the linker molecule links the two or more FGF monomers through the linking amino acid residues, and wherein the linker molecule is a polymer.

[0010] In one embodiment, the FGF multimer is an FGF dimer. In one embodiment, the FGF monomers are naturally existing FGF2 monomers or modified FGF2 monomers.

[0011] In one embodiment, the FGF monomers are modified FGF2 monomers.

[0012] In one embodiment, the modified FGF2 monomers comprise a mutation of a cysteine. In one embodiment, the modified FGF2 monomers comprise a mutation of C78S. In one embodiment, the linking amino acid residue is a cysteine. In one embodiment, the linking amino acid residue is Cys96.

[0013] In one embodiment, the linker molecule is a poly(ethylene glycol) (PEG). In one embodiment, the PEG is a PEG with its molecular weight (MW; in g/mol) in the range of about 0.1kDa to about 20kDa. In one embodiment, the PEG is a PEG with its molecular weight about 2 kDa.

[0014] In one aspect, the present invention discloses a pharmaceutical composition for treating or healing chronic wound. The pharmaceutical composition comprises an FGF multimer

comprising two or more FGF monomers, each of the two or more FGF monomers comprising one linking amino acid residue and a linker molecule, wherein the linker molecule covalently links the two or more FGF monomers through the linking amino acid residues, and wherein the linker molecule is a polymer.

[0015] In one embodiment, the FGF multimer is an FGF dimer. In one embodiment, the FGF multimer is an FGF2 dimer.

[0016] In one embodiment, the pharmaceutical composition further comprises one stabilizing agent.

[0017] In one embodiment, the pharmaceutical composition further comprises one delivering agent.

[0018] In one aspect, the present invention discloses a method for treating or healing a wound in a subject. The method comprises administering to the subject a therapeutically effective amount of a composition, the composition comprising an FGF multimer comprising two or more FGF monomers, each of the two or more FGF monomers comprising one linking amino acid residue and a linker molecule, wherein the linker molecule covalently links the two or more FGF monomers through the linking amino acid residues, and wherein the linker molecule is a polymer, wherein the wound is treated or healed.

[0019] In one embodiment, the FGF multimer is an FGF dimer such as an FGF2 dimer.

[0020] In one embodiment, the wound is a chronic wound.

[0021] In one embodiment, the FGF monomers are modified FGF2 monomers.

[0022] In one embodiment, the linker molecule is a PEG with its molecular weight about 2 kDa.

[0023] In one embodiment, the composition further comprises at least one stabilizing agent.

[0024] In one embodiment, the composition further comprises at least one delivering agent.

[0025] In one embodiment, the method further comprises at least one advanced wound care therapy selected from the group consisting of the use of collagen, growth factors, bioengineered skin, gene and stem cell therapy, silver products, ozone oxygen therapy, and negative pressure wound therapy.

[0026] In one aspect, the present invention discloses a kit for treating or healing a wound in a patient. The kit comprises a composition comprising an FGF multimer comprising two or more FGF monomers, each of the two or more FGF monomers comprising one linking amino acid residue and a linker molecule, wherein the linker molecule covalently links the two or more FGF monomers through the linking amino acid residues, and wherein the linker molecule is a polymer.

[0027] In one embodiment, the FGF multimer is an FGF dimer such as an FGF2 dimer.

[0028] In one embodiment, the kit further comprises another composition for sanitizing the wound before the treatment.

[0029] In one aspect, the present invention discloses a medical device for treating or healing a wound in a patient. The medical device comprises a substrate comprising an FGF multimer comprising two or more FGF monomers, each of the two or more FGF monomers comprising one linking amino acid residue and a linker molecule, wherein the linker molecule covalently links the two or more FGF monomers through the linking amino acid residues, and wherein the linker molecule is a polymer; and wound covering means for releaseably covering the wound of the patient, wherein after the wound of the patient is covered, the substrate controllably releases the FGF multimer into the wound.

[0030] In one embodiment, the FGF multimer is an FGF dimer such as an FGF2 dimer.

DESCRIPTION OF THE DRAWINGS

[0031] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0032] Fig. 1 is a systematic diagram showing the active tetrameric complex consisting of two FGF2s (gold) two FGFRs (silver) with Cys96 in red and poly(ethylene glycol) represented in blue. The diagram was modified from PDB 1CVS using Chimera software.

[0033] Fig. 2 is a graph showing western blot of purified conjugates. Lane 1: Protein Ladder; Lane 2: FGF2; Lane 3: FGF2-VS-FGF2; Lane 4: mPEG2k-FGF2; Lane 5: FGF2-PEG2k-FGF2; Lane 6: mPEG5k-FGF2; Lane 7: FGF2-PEG6k-FGF2; Lane 8: mPEG20k-FGF2; Lane 9: FGF2-PEG20k-FGF2.

[0034] Figs. 3A and 3B are a set of graphs showing screening of PEG linker-length effect on HDF metabolic activity and migration. Fig. 3A and Fig. 3B show percent metabolic activity of HDFs (mean $\pm$ SD). All samples are normalized to blank media per treatment set (n=6). In Fig. 3B, bars for 0.1, 1, and 10 ng/mL are duplicated from Fig. 3A. Fig. 3C shows migration scratch assay of HDFs treated with FGF2 or FGF2-PEG2k-FGF2 for 18 hours. Each treatment was repeated 5-7 times. The distance across the scratch was measure in ImageJ in pixels and the % increased HDF migration was calculated as $100 - \text{distance at T} / \text{distance at T}_0 * 100$. Statistical analysis was carried out using a student's t-test (Fig. 3C) or ANOVA + Tukey (Fig. 3A) and (Fig. 3B). # = statistically greater than FGF2 control at that concentration, $p < 0.05$. * or *** = statistically greater than both FGF2 and mPEG-FGF2, $p < 0.05$ and $p < 0.001$, respectively at that concentration.

[0035] Figs. 4A, 4B and 4C are a set of graphs and images showing exemplary use of FGF2 and FGF2-PEG2k-FGF2. Fig. 4A: Metabolic activity of HUVECs at various concentrations of FGF2 and FGF2-PEG2k-FGF2. Each treatment was repeated 5-6 times and the entire study repeated twice. Fig. 4B: Migration scratch assay of HUVECs treated with FGF2 or FGF2-PEG2k-FGF2 for 18 hours. Each treatment was repeated 4-7 times and the entire study repeated twice (n=9-12 total). The distance across the scratch was measured using ImageJ in pixels and the % increased migration was calculated as $100 - \text{distance at T} / \text{distance at T}_0 * 100$. For Fig. 4A and Fig. 4B all samples were normalized to blank media per treatment set and recorded as mean $\pm$ SD. Statistical analysis was carried out using a student's t-test. * = $p < 0.05$. *** = $p < 0.001$. Fig. 4C: Representative images of HUVECs (5x magnification) at 18 hours and 0 hours after scratching and treatment with 0.1 ng/mL of each sample.

[0036] Figs. 5A, 5B, 5C and 5D are a set of graphs and images showing effect of FGF2 and FGF2-PEG2k-FGF2 at various concentrations on angiogenesis through the co-culture of HDFs and HUVECs (n=3). Fig. 5A: Representative images of select sample sets, cord-like structures stained. Fig. 5B: Comparison of the number of nodes per condition / sample set. Fig. 5C: Comparison of the number of cord-like structures per condition/sample set. Fig. 5D: The average total cord-like structure length per image (pixels) per condition / sample set (mean $\pm$ SD). Statistical analysis was carried out using a student's t-test. * = $p < 0.05$.

[0037] Figs. 6A, 6B, 6C, 6D and 6E are a set of graphs and images showing *in vivo* assessment of FGF2-PEG2k-FGF2 in diabetic wounds. Sterile wounds (8 mm punch biopsy)

were performed on TallyHo/JngJ diabetic mice and the wounds were covered (Tegaderm) and treated daily for 5 days with 0.02 μ g of FGF2, FGF2-PEG2k-FGF2 or D-PBS control. Fig. 6A: Representative photographs of the skin wounds in treated diabetic wounds. Fig. 6B: Mean wound area $\pm$ s.e.m. Fig. 6C: Representative histology (H&E stained) at low magnification (left panels) and high magnification of the boxed dotted area (right panels) with black arrows indicating wound edges and white arrows indicating blood vessels. Quantitative analysis of granulation tissue area (Fig. 6D) and blood vessel density (Fig. 6E). * p <0.05, ** p <0.01 FGF2-PEG2k-FGF2 versus FGF2 or D-PBS (n =5-10/group).

[0038] Figs. 7A and 7B are a set of graphs showing MALDI-MS of unmodified FGF2 (Fig. 7A) or FGF2-PEG2k-FGF2 (Fig. 7B) conjugates. Fig. 7A: m/z = 17.2 kDa (FGF2), 34.5 kDa (naturally occurring disulfide dimer). Fig. 7A: m/z = 17.2 kDa (FGF2), 34.5 kDa (naturally occurring disulfide dimer). Fig. 7B: m/z = 17.2 kDa (FGF2), 18.2 kDa (FGF2-PEG2k some cleavage), 19.3 (FGF2-PEG2k), 36.5 kDa (FGF2-PEG2k-FGF2), 38.4 kDa (PEG2k-FGF2-PEG2k with one additional nonspecific polymer addition).

[0039] Fig. 8 is a graph showing $^1\text{H-NMR}$ (CDCl_3) of mPEG2k-VS.

[0040] Fig. 9 is a graph showing $^1\text{H-NMR}$ (CDCl_3) of mPEG5k-VS.

[0041] Fig. 10 is a graph showing $^1\text{H-NMR}$ (CDCl_3) of mPEG20k-VS.

[0042] Fig. 11 is a graph showing $^1\text{H-NMR}$ (CDCl_3) of VS-PEG2k-VS.

[0043] Fig. 12 is a graph showing $^1\text{H-NMR}$ (CDCl_3) of VS-PEG6k-VS.

[0044] Fig. 13 is a graph showing $^1\text{H-NMR}$ (CDCl_3) of VS-PEG20k-VS.

[0045] Fig. 14 is an image showing an example of cord length and node identification according to one embodiment of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

[0046] IN GENERAL

[0047] Before the present materials and methods are described, it is understood that this invention is not limited to the particular methodology, protocols, materials, and reagents 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 any later-filed nonprovisional applications.

[0048] As used herein and in the appended claims, the singular forms “a,” “an,” and “the” include plural reference unless the context clearly dictates otherwise. As well, the terms “a” (or “an”), “one or more” and “at least one” can be used interchangeably herein. The terms “comprising,” “including,” and “having” can be used interchangeably.

[0049] 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 and materials are now described. All publications and patents specifically mentioned herein are incorporated by reference for all purposes including describing and disclosing the chemicals, instruments, statistical analysis and methodologies which are reported in the publications which might be used in connection with the invention. All references cited in this specification are to be taken as indicative of the level of skill in the art. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

[0050] DEFINITIONS

[0051] Before the composition and related methods are described, it is to be understood that this invention is not limited to the particular methodology, protocols, materials, and reagents described, as these may vary. 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 any later-filed non-provisional applications.

[0052] The term "amino acid," as used herein, refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, e.g., hydroxyproline, γ -carboxyglutamate, and O-phosphoserine.

[0053] The term "amino acid analogs," as used herein, refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, i.e., an a carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, e.g., homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs may have modified R groups

(e.g., norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid.

[0054] The term "amino acid mimetics," as used herein, refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but functions in a manner similar to a naturally occurring amino acid.

[0055] The term "unnatural amino acid," as used herein, refers to the "D" stereochemical form of the twenty naturally occurring amino acids. It is further understood that the term unnatural amino acid may include homologues of the natural amino acids, and synthetically modified forms of the natural amino acids. The synthetically modified forms may include, but are not limited to, amino acids having alkylene chains shortened or lengthened by up to two carbon atoms, amino acids comprising optionally substituted aryl groups, and amino acids comprised halogenated groups, preferably halogenated alkyl and aryl groups.

[0056] In one embodiment, the synthetically modified amino acid may be an amino acid including a linker group or moiety that allows the amino acid to connect with another compound.

[0057] The term "linker group or moiety," as used herein, refers to any functional group or moiety that forms linkage between a first compound and a second compound.

[0058] In one embodiment, a synthetically modified amino acid may be an amino acid including a functional group of an azide, such as azido alanine, para-azidophenylalanine, etc.

[0059] A synthetically modified amino acid may be an amino acid including a functional group of alkyne, such as propynyl glycine, alkyne functionalized proline, etc.

[0060] A synthetically modified amino acid may be an amino acid including a functional group of tetrazine, e.g., tetrazine-phenyl-2-aminopropanoic acid, etc.

[0061] A synthetically modified amino acid may be an amino acid including a functional group of maleimide, e.g., AEG.

[0062] A synthetically modified amino acid may be an amino acid including a functional group of dehydroalanine.

[0063] A synthetically modified amino acid may be an amino acid including a functional group of ketone, e.g., p-acetylphenylalanine.

[0064] A synthetically modified amino acid may be an amino acid including a functional group of aminooxy, such as diphenyl-p-tolyl-methyl)-aminooxy]-N-[(phenylmethoxy)carbonyl]-serine and so on.

[0065] A synthetically modified amino acid may be an amino acid including a functional group of aldehyde.

[0066] A synthetically modified amino acid may be an amino acid including a functional group of alkene, e.g., 6-N-ally-loxycarbonyl-L-lysine, O-allyltyrosine and so on.

[0067] A synthetically modified amino acid may be an amino acid including a functional group of tetrazole, e.g., tetrazolyl glycine, tetrazolyl alanine, etc.

[0068] A synthetically modified amino acid may be an amino acid including a functional group of transcyclooctene, e.g., lysine-transcyclooctene.

[0069] A synthetically modified amino acid may be an amino acid including a functional group of cyclooctyne, e.g., lysine-transcyclooctyne.

[0070] A synthetically modified amino acid may be an amino acid including a functional group of Norbornene, e.g., 5-norbornene-2-yloxy carbonyl-L-lysine and others.

[0071] The term "fibroblast growth factors" or "FGFs," as used herein, refers to a family of growth factors, with members involved in angiogenesis, wound healing, embryonic development and various endocrine signaling pathways. The FGFs may be heparin-binding proteins and interactions with cell-surface-associated heparan sulfate proteoglycans have been shown to be essential for FGF signal transduction. FGFs may be key players in the processes of proliferation and differentiation of wide variety of cells and tissues.

[0072] In humans, 23 members of the FGF family have been identified, all of which are *structurally* related signaling molecules, including members FGF1 through FGF10 all bind fibroblast growth factor receptors (FGFRs). FGF1 is also known as acidic, and FGF2 is also known as basic fibroblast growth factor.

[0073] Members FGF11, FGF12, FGF13, and FGF14, also known as FGF homologous factors 1-4 (FHF1-FHF4), have been shown to have distinct functions compared to the FGFs. Although these factors possess remarkably similar sequence homology, they do not bind FGFRs and are involved in intracellular processes unrelated to the FGFs. This group is also known as "iFGF".

[0074] Human FGF18 may be involved in cell development and morphogenesis in various tissues including cartilage. Human FGF20 was identified based on its homology to *Xenopus* FGF-20 (XFGF-20). FGF15 through FGF23 were described later and functions are still being characterized. FGF15 is the mouse ortholog of human FGF19 (there is no human FGF15)

and, where their functions are shared, they are often described as FGF15/19. In contrast to the local activity of the other FGFs, FGF15/19, FGF21 and FGF23 have systemic effects.

[0075] In one embodiment, members of the fibroblast growth factor family of proteins, including FGF1, FGF2, FGF3, FGF4, FGF5, FGF6, FGF7, FGF8, FGF15, FGF16, FGF17, FGF18, FGF19, FGF20, FGF21, FGF22, and FGF23, may be required to be in the dimer form for FGF receptor activation. In another embodiment, although FGF9 may be able to form a dimer *in-vivo*, it is possible that the pre-dimerization of FGF9 would enhance protein activity. The present invention is applicable to all these FGF members and others.

[0076] The term "modified FGF," as used herein, refers to an FGF having at least one mutated amino acid while the FGF may still retain its substantial activity. For example, at least one amino acid of an FGF may be mutated into another amino acid so that the modified FGF may or may not be linked to another molecule through the other amino acid.

[0077] The term "basic FGF" or "bFGF," as used herein, refers to any basic fibroblast growth factor, also called fibroblast growth factor 2.

[0078] The term "linking amino acid residue," as used herein, refers to an amino acid residue on an FGF which may be linked to a non-FGF molecule such as a linker molecule. In one embodiment, the linking amino acid residue is a surface-exposed amino acid on an FGF. In one embodiment, the linking amino acid residue is a cysteine residue.

[0079] In one embodiment, the linking amino acid residue is a Lysine residue or N-terminal amine.

[0080] In one embodiment, the linking amino acid residue is a histidine residue.

[0081] In one embodiment, the linking amino acid residue is a tyrosine residue.

[0082] In one embodiment, a modified FGF may have at least one surface exposed linking amino acid residue. In one preferred embodiment, a modified FGF may have only one surface exposed linking amino acid residue.

[0083] In one embodiment, Applicants envision that the surface exposed linking amino acid residue may include any amino acid, which is capable of linking the modified FGF to another molecule such as a linker, linker molecule, or linking molecule.

[0084] In one specific embodiment of the present invention, the mutated amino acid may be a cysteine.

[0085] The term "fibroblast growth factor receptor" or "FGFR," as used herein, refers to receptors to fibroblast growth factors. The mammalian fibroblast growth factor receptor family has 4 members, FGFR1, FGFR2, FGFR3, and FGFR4. The FGFRs consist of three extracellular immunoglobulin-type domains (D1-D3), a single-span trans-membrane domain and an intracellular split tyrosine kinase domain. FGFs interact with the D2 and D3 domains, with the D3 interactions primarily responsible for ligand-binding specificity. Heparan sulfate binding is mediated through the D3 domain. A short stretch of acidic amino acids located between the D1 and D2 domains has auto-inhibitory functions. This 'acid box' motif interacts with the heparan sulfate binding site to prevent receptor activation in the absence of FGFs. Alternate mRNA splicing gives rise to 'b' and 'c' variants of FGFRs 1, 2 and 3. Through this mechanism seven different signaling FGFR sub-types may be expressed at the cell surface. Each FGFR binds to a specific subset of the FGFs. Similarly most FGFs can bind to several different FGFR subtypes. FGF1 is sometimes referred to as the 'universal ligand' as it is capable of activating all 7 different FGFRs. In contrast, FGF7 (keratinocyte growth factor, KGF) binds only to FGFR2b (KGFR).

[0086] The term "FGF multimer" or "FGF polymer," as used herein, refers to two or more FGFs or modified FGFs, which are connected through linker molecule[s]. In one embodiment, FGF multimer are FGF2 multimer including two or more FGF2s or modified FGF2s connected through linker molecule[s].

[0087] The terms "polymer" and "polymeric," unless otherwise indicated, refer broadly to homopolymers and block/random copolymers having a chain of at least three or more monomer structural units formed by polymerization reactions (e.g., condensation or ring-opening polymerization).

[0088] The term "FGF dimer," as used herein, refers to two FGFs or modified FGFs which are connected through a linker molecule. In one specific embodiment, FGF dimers of the present invention are FGF2 dimers including two FGF2s or modified FGF2s connected through a linker molecule.

[0089] The term "linker," "linker molecule" or "linking molecule," as used herein, refers to a molecule which can link two or more FGFs. In one embodiment, a linker, linker molecule, or linking molecule may include specific binding moieties at its two ends. The specific binding moieties can covalently bind FGFs through linking amino acid residues. The example of linker, linker molecule or linking molecule may include peptides, polymers, macromolecule or

biomolecule. In one embodiment, the linker, linker molecule or linking molecule is a polymer. In one specific embodiment, the linker, linker molecule or linking molecule is a poly(ethylene glycol) (PEG) or its derivative.

[0090] The term "poly(ethylene glycol)" or "PEG," as used herein, refers to a polymer made of repeating units of compounds containing $-(O-CH_2-CH_2)-$ but having molecular weights in the range of about 200 Daltons to about 5000 kDa.

[0091] The term "PEG derivative," as used herein, relates to a PEG which is modified by the addition of one or more linker groups or moieties. An exemplary PEG or PEG derivative is a PEG or PEG derivative (further) substituted with one or more linker groups or moieties including, but not limited to, acid (carbonic acid, sulphonic acid), CN, OH, OR, SH, SR, NH₂ or NHR, wherein R = C₁ to C₄ chain, alkyne, triarylphosphine, trialkylphosphine, bistranscyclooctene, bisnorbornene, biscyclopropene, or other reagents for Diels-Alder reactions, thiol, aminoxy, aldehyde, bisaldehyde, ketone, diketone or other reagents for oxime or any type of imine or reductive amination reactions, alkene or other reagents for thiol-ene reactions, alkyne or other reagents for thiol-yne reaction, tetrazine (e.g., 1,2,4,5-tetrazines) or bis-tetrazine for tetrazine ligation, dialkene or other reagents for tetrazole-alkene reactions, bi-azides for azide alkyne reactions, and so on.

[0092] In one embodiment, when linking amino acid residues are naturally existing amino acids such as cysteine, lysine, histidine, tyrosine and so on, PEG derivatives may be PEGs including good leaving groups.

[0093] The term "leaving group," as used herein, refers to a portion of a compound that is cleaved from the compound in a reaction. The leaving group may be an atom (or a group of atoms) that is displaced as stable species taking with it the bonding electrons. Typically the leaving group may be an anion (e.g., Cl⁻) or a neutral molecule (e.g., H₂O). Useful leaving groups may include, but are not limited to, halides, sulfonic esters, oxonium ions, alkyl perchlorates, sulfonates, e.g., arylsulfonates, ammonioalkanesulfonate esters, and alkylfluorosulfonates, phosphates, carboxylic acid esters, carbonates, ethers, and fluorinated compounds (e.g., inflates, nonaflates, tresylates). Exemplary leaving groups may include a halogen, B(OR¹)(OR²), OC(O)R¹, OP(O)R¹R², OS(O)R¹, OSO₂R¹, SR¹, (R¹)₃P⁺, (R¹)₂S⁺, P(O)N(R¹)₂(R¹)₂, P(O)R³R¹R⁴R¹ in which each R¹ and R² is independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted aryl, substituted or unsubstituted

heteroaryl and substituted or unsubstituted heterocycloalkyl. R^3 and R^4 are each either S or O. When the leaving group is a boric acid ester, it is optionally a cyclic boronic acid ester.

[0094] In one embodiment, PEG derivatives may include divinyl sulfone-PEG-divinyl sulfone, PEG-bismethacrylate, PEG-bismaleimide, diallyl-PEG, divinyl-PEG, Pyridyl disulfide-PEG-Pyridyl disulfide, PEG-bis(iodoacetamide) and others.

[0095] In one embodiment, PEG derivatives may include PEG-bisaldehyde, PEG-bis(NHS-ester), PEG-bisisocyanate, PEG-bisithiocyanate and others.

[0096] In one embodiment, PEG derivatives may include PEG-bissulfone.

[0097] In one embodiment, PEG derivatives may include PEG-bis(diazonium salt).

[0098] The term "multi-Arm PEG," as used herein, refers to PEGs that are formed around a core molecule permitting multiple PEG molecules to be covalently bonded to the core. A multi-arm PEG includes any PEG having multiple PEGs attached to a core molecule.

[0099] The term "multi-Branch PEG," as used herein, refers to a single PEG polymer having in-chain epoxide moieties attached thereto. Multi-branched PEGs may be characterized by having a particular ratio of epoxide:ethylene oxide moieties. A fully derivatized multi-branch PEG will have an epoxide:ethylene oxide ratio of 2. However, it should be understood that multi-branch PEGs may have epoxide:ethylene oxide ratios of less than 2, and that the ratio, on average, need not be integral in a plurality of PEG molecules.

[00100] As used herein, the term "organic group" is used for the purpose of this invention to mean a hydrocarbon group that is classified as an aliphatic group, cyclic group, or combination of aliphatic and cyclic groups (e.g., alkaryl and aralkyl groups). In the context of the present invention, suitable organic groups for FGF multimers such as dimers or PEG of this invention are those that do not interfere with the FGF multimers (such as dimers) or PEG activity. In the context of the present invention, the term "aliphatic group" means a saturated or unsaturated linear or branched hydrocarbon group. This term is used to encompass alkyl, alkenyl, and alkynyl groups, for example.

[00101] The terms "hydroxy" and "hydroxyl" refer to the group -OH.

[00102] The term "oxo" refers to the group =O.

[00103] The term "carboxylate" or "carboxyl" refers to the group -COO^- or -COOH .

[00104] The term "cyano" refers to the group -CN.

[00105] The term "nitro" refers to the group -NO_2 .

- [00106]** The term “amino” refers to the group -NH_2 .
- [00107]** The term “acyl” or “aldehyde” refers to the group -C(=O)H .
- [00108]** The term “amido” or “amide” refers to the group -C(O)NH_2 .
- [00109]** The term “aminoacyl” or “acylamino” refers to the group -NHC(O)H .
- [00110]** The term “thiol” refers to the group -SH .
- [00111]** The term “thioxo” refers to the group =S .
- [00112]** The term “sulfinyl” refers to the group -S(=O)H .
- [00113]** The term “sulfonyl” refers to the group $\text{-SO}_2\text{H}$.
- [00114]** The term “sulfonylamido” or “sulfonamide” refers to the group $\text{-SO}_2\text{NH}_2$.
- [00115]** The term “sulfonate” refers to the group SO_3H and includes groups having the hydrogen replaced with, for example a C_{1-6} alkyl group (“alkylsulfonate”), an aryl (“arylsulfonate”), an aralkyl (“aralkylsulfonate”) and so on. C_{1-3} sulfonates are preferred, such as for example, SO_3Me , SO_3Et and SO_3Pr .
- [00116]** The terms “alkyl,” “alkenyl,” and the prefix “alk-,” as used herein, refer to straight chain groups and branched chain groups and cyclic groups, e.g., cycloalkyl and cycloalkenyl. Unless otherwise specified, these groups contain from 1 to 20 carbon atoms, with alkenyl groups containing from 2 to 20 carbon atoms. In some embodiments, these groups have a total of at most 10 carbon atoms, at most 8 carbon atoms, at most 6 carbon atoms, or at most 4 carbon atoms. Cyclic groups can be monocyclic or polycyclic and preferably have from 3 to 10 ring carbon atoms. Exemplary cyclic groups include cyclopropyl, cyclopropylmethyl, cyclopentyl, cyclohexyl, adamantyl, and substituted and unsubstituted bornyl, norbornyl, and norbornenyl.
- [00117]** Unless otherwise specified, “alkylene” and “alkenylene” are the divalent forms of the “alkyl” and “alkenyl” groups defined above. The terms, “alkylenyl” and “alkenylenyl” are used when “alkylene” and “alkenylene”, respectively, are substituted. For example, an arylalkylenyl group comprises an alkylene moiety to which an aryl group is attached.
- [00118]** The term “aryl” as used herein includes monocyclic or polycyclic aromatic hydrocarbons or ring systems. Examples of aryl groups include phenyl, naphthyl, biphenyl, fluorenyl and indenyl. Aryl groups may be substituted or unsubstituted. Aryl groups include aromatic annulenes, fused aryl groups, and heteroaryl groups. Aryl groups are also referred to herein as aryl rings.

[00119] The term "heteroalkyl," as used herein, refers to, unless otherwise stated, a stable straight or branched chain, or cyclic hydrocarbon, or combinations thereof, consisting of the stated number of carbon atoms and at least one heteroatom selected from the group consisting of O, N, Si and S, and wherein the nitrogen and sulfur atoms may optionally be oxidized and the nitrogen heteroatom may optionally be quaternized. The heteroatom(s) O, N and S and Si may be placed at any interior position of the heteroalkyl group or at the position at which the alkyl group is attached to the remainder of the molecule. Examples may include, but are not limited to, $-\text{CH}_2\text{-CH}_2\text{-O-CH}_3$, $-\text{CH}_2\text{-CH}_2\text{-NH-CH}_3$, $-\text{CH}_2\text{-CH}_2\text{-N(CH}_3\text{)-CH}_3$, $-\text{CH}_2\text{-S-CH}_2\text{-CH}_3$, $-\text{CH}_2\text{-CH}_2\text{-S(O)-CH}_3$, $-\text{CH}_2\text{-CH}_2\text{-S(O)}_2\text{-CH}_3$, $-\text{CH=CH-O-CH}_3$, $-\text{Si(CH}_3\text{)}_3$, $-\text{CH}_2\text{-CH=N-OCH}_3$, and $-\text{CH=CH-N(CH}_3\text{)-CH}_3$. Up to two heteroatoms may be consecutive, such as, for example, $-\text{CH}_2\text{-NH-OCH}_3$ and $-\text{CH}_2\text{-O-Si(CH}_3\text{)}_3$. Similarly, the term "heteroalkylene" by itself or as part of another substituent, refers to a hydrocarbon derived from heteroalkyl, as exemplified, but not limited by, $-\text{CH}_2\text{-CH}_2\text{-S-CH}_2\text{-CH}_2\text{-}$ and $-\text{CH}_2\text{-S-CH}_2\text{-CH}_2\text{-NH-CH}_2\text{-}$. For heteroalkylene groups, heteroatoms may also occupy either or both of the chain termini (e.g., alkyleneoxy, alkylenedioxy, alkyleneamino, alkylenediamino, and the like). Still further, for alkylene and heteroalkylene linking groups, no orientation of the linking group is implied by the direction in which the formula of the linking group is written. For example, the formula $-\text{CO}_2\text{R}'$ represents both $-\text{C(O)OR}'$ and $-\text{OC(O)R}'$.

[00120] The terms "cycloalkyl" and "heterocycloalkyl," as used herein, refer to cyclic versions of "alkyl" and "heteroalkyl", respectively. Additionally, for heterocycloalkyl, a heteroatom may occupy the position at which the heterocycle is attached to the remainder of the molecule. Examples of cycloalkyl may include, but are not limited to, cyclopentyl, cyclohexyl, 1-cyclohexenyl, 3-cyclohexenyl, cycloheptyl, and the like. Further exemplary cycloalkyl groups may include steroids, e.g., cholesterol and its derivatives. Examples of heterocycloalkyl may include, but are not limited to, 1-(1,2,5,6-tetrahydropyridyl), 1-piperidinyl, 2-piperidinyl, 3-piperidinyl, 4-morpholinyl, 3-morpholinyl, tetrahydrofuran-2-yl, tetrahydrofuran-3-yl, tetrahydrothien-2-yl, tetrahydrothien-3-yl, 1-piperazinyl, 2-piperazinyl, and the like.

[00121] The terms "halo" or "halogen," as used herein, refer to, fluorine, chlorine, bromine, or iodine atom. Additionally, terms such as "haloalkyl," are meant to include monohaloalkyl and polyhaloalkyl. For example, the term "halo(C1-C4)alkyl" is meant to include,

but not be limited to, trifluoromethyl, 2,2,2- trifluoroethyl, 4-chlorobutyl, 3-bromopropyl, and the like.

[00122] The term "aryl," as used herein, refers to a polyunsaturated, aromatic, substituent that can be a single ring or multiple rings (preferably from 1 to 3 rings), which are fused together or linked covalently. The term "heteroaryl," as used herein, refers to aryl groups (or rings) that contain from one to four heteroatoms selected from N, O, S, Si and B, wherein the nitrogen and sulfur atoms are optionally oxidized, and the nitrogen atom(s) are optionally quaternized. A heteroaryl group may be attached to the remainder of the molecule through a heteroatom. Non-limiting examples of aryl and heteroaryl groups may include phenyl, 1 -naphthyl, 2-naphthyl, 4-biphenyl, 1-pyrrolyl, 2-pyrrolyl, 3-pyrrolyl, 3-pyrazolyl, 2-imidazolyl, 4-imidazolyl, pyrazinyl, 2-oxazolyl, 4-oxazolyl, 2-phenyl-4-oxazolyl, 5-oxazolyl, 3-isoxazolyl, 4-isoxazolyl, 5-isoxazolyl, 2-thiazolyl, 4-thiazolyl, 5-thiazolyl, 2-furyl, 3-furyl, 2-thienyl, 3- thienyl, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2-pyrimidyl, 4-pyrimidyl, 5-benzothiazolyl, purinyl, 2-benzimidazolyl, 5-indolyl, 1-isoquinolyl, 5-isoquinolyl, 2-quinoxaliny, 5-quinoxaliny, 3- quinolyl, and 6-quinolyl. Substituents for each of the above noted aryl and heteroaryl ring systems are selected from the group of acceptable substituents described below.

[00123] Unless otherwise indicated, the term "heteroatom" refers to the atoms O, S, or N.

[00124] When a group is present more than once in any formula or scheme described herein, each group (or substituent) is independently selected, whether explicitly stated or not. For example, for the formula $-C(O)NR_2$ each of the two R groups is independently selected.

[00125] The invention is inclusive of the compounds described herein (including intermediates) in any of their pharmaceutically acceptable forms, including isomers (e.g., diastereomers and enantiomers), tautomers, salts, solvates, polymorphs, prodrugs, and the like. In particular, if a compound is optically active, the invention specifically includes each of the compound's enantiomers as well as racemic mixtures of the enantiomers. It should be understood that the term "compound" includes any or all of such forms, whether explicitly stated or not (although at times, "salts" are explicitly stated).

[00126] The term "pharmaceutically acceptable," as used herein, means that the compound or composition or carrier is suitable for administration to a subject to achieve the treatments described herein, without unduly deleterious side effects in light of the necessity of the treatment.

[00127] The term "therapeutically effective amount" or "pharmaceutically appropriate dosage", as used herein, refers to the amount of the compounds or dosages that will elicit the biological or medical response of a subject, tissue or cell that is being sought by the researcher, veterinarian, medical doctor or other clinician.

[00128] The term "pharmaceutically-acceptable carrier," As used herein, refers to any and all dry powder, solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic agents, absorption delaying agents, and the like. Pharmaceutically-acceptable carriers may be materials, useful for the purpose of administering the compounds in the method of the present invention, which are preferably non-toxic, and may be solid, liquid, or gaseous materials, which are otherwise inert and pharmaceutically acceptable, and are compatible with the compounds of the present invention. Examples of such carriers include, various lactose, mannitol, oils such as com oil, buffers such as PBS, saline, polyethylene glycol, glycerin, polypropylene glycol, dimethylsulfoxide, an amide such as dimethylacetamide, a protein such as albumin, and a detergent such as Tween 80, mono- and oligopolysaccharides such as glucose, lactose, cyclodextrins and starch.

[00129] As used herein, the term "patient" or "subject" refers to an animal, typically a human (i.e., a male or female of any age group, e.g., a pediatric patient (e.g., infant, child, adolescent) or adult patient (e.g., young adult, middle-aged adult or senior adult) or other mammal, such as a primate (e.g., cynomolgus monkey, rhesus monkey); other mammals such as rodents (mice, rats), cattle, pigs, horses, sheep, goats, cats, dogs; and/or birds, that will be or has been the object of treatment, observation, and/or experiment. When the term is used in conjunction with administration of a compound or drug, then the patient has been the object of treatment, observation, and/or administration of the compound or drug.

[00130] The term "treating," "treat," or "treatment," as used herein, refers to partially or completely inhibiting or reducing the condition from which the subject is suffering. In one embodiment, this term may refer to an action that occurs while a patient is suffering from, or is diagnosed with, the condition, which reduces the severity of the condition, or retards or slows the progression of the condition. Treatment may not result in a complete cure of the condition; partial inhibition or reduction of the condition is encompassed by this term. Treatment may be intended to encompass prevention or prophylaxis.

[00131] The term "administering" or "administration," as used herein, refers to providing the compound or pharmaceutical composition of the invention to a subject suffering from or at risk of the diseases or conditions to be treated or prevented.

[00132] The term "systemic delivery", as used herein, refers to any suitable administration methods which may delivery the compounds in the present invention systemically. In one embodiment, systemic delivery may be selected from the group consisting of oral, parenteral, intranasal, inhaler, sublingual, rectal, and transdermal administrations.

[00133] A route of administration in pharmacology and toxicology is the path by which a drug, fluid, poison, or other substance is taken into the body. Routes of administration may be generally classified by the location at which the substance is applied. Common examples may include topical, oral and intravenous administration. Routes can also be classified based on where the target of action is. Action may be topical (local), enteral (system-wide effect, but delivered through the gastrointestinal tract), or parenteral (systemic action, but delivered by routes other than the GI tract), via lung by inhalation.

[00134] The term "stabilizing agent," as used herein, refers to any agents which stabilize FGF multimers such as dimers or other biologically active compound(s).

[00135] The term "delivering agent," as used herein, refers to any agents which enhance the release or solubility (e.g., from a formulation delivery vehicle), diffusion rate, penetration capacity and timing, uptake, residence time, stability, effective half-life, peak or sustained concentration levels, clearance and other desired intranasal delivery characteristics (e.g., as measured at the site of delivery, or at a selected target site of activity such as the bloodstream or central nervous system) of FGF multimers such as dimers or other biologically active compound(s). Enhancement of intranasal delivery can thus occur by any of a variety of mechanisms, for example by increasing the diffusion, transport, persistence or stability of FGF multimers such as dimers, increasing membrane fluidity, modulating the availability or action of calcium and other ions that regulate intracellular or paracellular permeation, solubilizing mucosal membrane components (e.g., lipids), changing non-protein and protein sulfhydryl levels in mucosal tissues, increasing water flux across the mucosal surface, modulating epithelial junctional physiology, reducing the viscosity of mucus overlying the mucosal epithelium, reducing mucociliary clearance rates, and other mechanisms.

[00136] The term "chronic wound," as used herein refers to a clinical condition having characteristic symptoms wherein the wounds that do not heal for a prolonged time and frequently have a strong tendency to recurrence. The chronic wounds may be associated with states in which the normal wound healing ability is weakened, such as diabetes, patients in their older age, patients with limited ability of movement, or patients during steroid treatment. Thus, patients with such badly healing wounds are those suffering from diabetic ulcers, including diabetic foot ulcers, diabetic neuropathic ulcers, including neuropathic forefoot ulcers, diabetic pressure ulcers or diabetic venous ulcers, as well as patients with limited movement ability suffering from bed sores. Bed sores may affect peoples who stay in one place for prolonged time for any reason.

II. THE INVENTION

[00137] POLYMERS, DIMERS, COMPOSITIONS AND FORMULATIONS

[00138] In one aspect, the present invention discloses FGF multimers and compositions and formulations including such FGF multimers.

[00139] Applicants developed a method for making FGF multimers with controllable distance among monomers by controlling the length of linker molecule(s).

[00140] In one embodiment, FGF multimers are FGF dimers.

[00141] In one embodiment, the present invention discloses FGF dimers with controllable distance among monomers by controlling the length of linker molecule(s).

[00142] In one embodiment, an FGF multimer comprises two or more FGF monomers and a linker molecule. Each of the two or more FGF monomers comprises one linking amino acid residue. The linker molecule links the two or more FGF monomers through the linking amino acid residues. In one embodiment, the linker molecule is a polymer.

[00143] In one embodiment, FGF multimers are FGF dimers.

[00144] In one embodiment, an FGF dimer comprises two FGF monomers, each of the two FGF monomers comprising one linking amino acid residue; and a linker molecule, wherein the linker molecule links the two FGF monomers through the linking amino acid residues.

[00145] In some embodiments, FGF2 dimer is used as a specific example of the present invention. As can be appreciated, any other FGF multimer may be made according to the present invention.

[00146] In one embodiment, naturally existing FGF monomers such as FGF2 monomers may be used as the FGF monomers.

[00147] In another embodiment, modified FGF monomers such as modified FGF2 monomers may be used as the FGF monomers.

[00148] In one embodiment, the linking amino acid residue of FGF or FGF2 monomers may include naturally existing amino acids such as cysteine, lysine residue or N-terminal amine, histidine, tyrosine and others.

[00149] In one specific embodiment, the linking amino acid residue of FGF or FGF2 monomers may be a cysteine.

[00150] As shown in the Examples, Applicants found there are two surface-exposed free cysteine residues existing in the naturally existing FGF2 monomer. These two surface-exposed free cysteine residues include Cys78 and Cys96.

[00151] In the Examples, Applicants demonstrated a method of mutating one of the surface-exposed free cysteine residues to form a modified FGF2 monomer. For example, the modified FGF2 monomers comprise a mutation of C78S. As such, only one surface-exposed free cysteine residue of Cys96 remains in the modified FGF2 monomer.

[00152] In one embodiment, the linking amino acid residue of FGF monomers may be a synthetically modified amino acid. The synthetically modified amino acid may include functional groups through which a linker molecule is connected with the FGF monomers.

[00153] Table 1 shows some exemplary function groups of synthetically modified amino acid in respect to exemplary peg-derivatives and exemplary orthogonal chemistry.

[00154] For example, a synthetically modified amino acid may be an amino acid including a functional group of azide, such as azido alanine, para-azidophenylalanine, etc.

[00155] A synthetically modified amino acid may be an amino acid including a functional group of alkyne, such as propynyl glycine, alkyne functionalized proline, etc.

[00156] A synthetically modified amino acid may be an amino acid including a functional group of tetrazine, e.g., tetrazine-phenyl-2-aminopropanoic acid, etc.

[00157] A synthetically modified amino acid may be an amino acid including a functional group of thiol-ene or Michael acceptor, e.g., maleimide, allyl-etherified eugenol (AEG) and vinyl sulfone.

[00158] A synthetically modified amino acid may be an amino acid including a functional group of dehydroalanine.

[00159] A synthetically modified amino acid may be an amino acid including a functional group of ketone, e.g., p-acetylphenylalanine.

[00160] A synthetically modified amino acid may be an amino acid including a functional group of aminooxy, such as diphenyl-p-tolyl-methyl)-aminooxy]-N-[(phenylmethoxy)carbonyl]-serine and so on.

[00161] A synthetically modified amino acid may be an amino acid including a functional group of aldehyde or ketone.

[00162] A synthetically modified amino acid may be an amino acid including a functional group of alkene, e.g., 6-N-allyl-oxycarbonyl-L-lysine, O-allyltyrosine and so on.

[00163] A synthetically modified amino acid may be an amino acid including a functional group of tetrazole, e.g., tetrazolyl glycine, tetrazolyl alanine, etc.

[00164] A synthetically modified amino acid may be an amino acid including a functional group of transcyclooctene, e.g., lysine-transcyclooctene.

[00165] A synthetically modified amino acid may be an amino acid including a functional group of cyclooctyne, e.g., lysine-transcyclooctyne.

[00166] A synthetically modified amino acid may be an amino acid including a functional group of Norbornene, e.g., 5-norbornene-2-yl-oxycarbonyl-L-lysine and others.

[00167] In one embodiment, the linker molecule may include any biocompatible polymer.

[00168] The term "biocompatible polymer," as used herein, refers generally to a polymer that is tolerated when placed within the body without causing significant adverse reactions (e.g., toxic or antigenic responses).

[00169] A wide variety of polymers may be used as the biocompatible polymer, including many biologically compatible, water-soluble and water dispersible, cationic or anionic polymers. Due to an absence of water diffusion barriers, favorable initial biodistribution and multivalent site-binding properties, hydrophilic polymer components are typically useful for enhancing nanoparticle distribution in tissues. Any combination of any biocompatible polymer may be included in accordance with the present invention, while still realizing benefits of the present invention. Some non-exhaustive examples of biocompatible polymers include proteins, peptides, carbohydrates, glycoproteins, polyamides, polycarbonates, polyalkylenes, polyalkylene glycols,

polyalkylene oxides, polyalkylene terephthalates, polyvinyl alcohols, polyvinyl ethers, polyvinyl esters, polyvinyl halides, polyvinylpyrrolidone, polyglycolides, polysiloxanes, polyurethanes and copolymers thereof, alkyl cellulose, hydroxyalkyl celluloses, cellulose ethers, cellulose esters, nitro celluloses, polymers of acrylic and methacrylic esters, methyl cellulose, ethyl cellulose, hydroxypropyl cellulose, hydroxy-propyl methyl cellulose, hydroxybutyl methyl cellulose, cellulose acetate, cellulose propionate, cellulose acetate butyrate, cellulose acetate phthalate, carboxylethyl cellulose, cellulose triacetate, cellulose sulphate sodium salt, poly(ethylene glycol), poly(ethylene oxide), poly(ethylene terephthalate), poly(vinyl alcohols), poly(vinyl acetate), poly vinyl chloride, polystyrene, polyhyaluronic acids such as hyaluronan, casein, gelatin, gluten, polyanhydrides, polyacrylic acid, alginate, chitosan, any copolymer thereof, and any combination of any of these.

[00170] In one embodiment, biocompatible polymers may include poly(N-vinyl pyrrolidone) (PVP), poly(glycerol) (PG), poly(N-(2-hydroxypropyl) methacrylamide) (pHPMA), polyoxazolines (pOZs), poly(N-acryloylmorpholine) (pNAcM), poly(zwitterions), polycarbonates, Poly(glutamic acid), dextrin, polysialic acid, hyaluronic acid, hydroxyethyl starch and others.

[00171] Other biocompatible polymer may include heparin-mimicking polymers as Applicants previously disclosed in PCT/US2012/066905.

[00172] In one preferred embodiment, the linker molecule is a poly(ethylene glycol) (PEG) or a PEG derivative. Specifically, a PEG or PEG derivative of the present invention may include linker groups which connect two or more FGF monomers. The term "linker groups," as used herein, refers to any functional group which is capable of covalently or non-covalently bonding with a natural existing amino acid or a synthetically modified amino acid of FGF monomers.

[00173] In one embodiment, a PEG or PEG derivative of the present invention may include at least two linker groups.

[00174] In one embodiment, a PEG or PEG derivative of the present invention may have a linear structure with two ends. Both ends of the PEG or PEG derivative may include linker groups. When a PEG or PEG derivative with linker groups at its both ends is used, an FGF dimer may form.

[00175] In one embodiment, a PEG or PEG derivative of the present invention may have a multi-branch structure with multiple ends. In one specific embodiment, at least two ends of the PEG or PEG derivative may include linker groups. In another specific embodiment, all the multiple ends may include linker groups.

[00176] When a PEG or PEG derivative with linker groups at its multiple ends is used, a multimeric FGF will form. If a PEG or PEG derivative with linker groups at two ends and FGF with two reactive groups is used, other polymers such as a polymer of FGF-polymer-FGF-polymer may form.

[00177] An exemplary PEG or PEG derivative may be a PEG or PEG derivative substituted with one or more linker groups or moieties including, but not limited to, acid (carbonic acid, sulphonic acid), aldehyde, CN, OH, OR, SH, SR, NH₂ or NHR, wherein R= C₁ to C₄ chain, alkyne, triarylphosphine, trialkylphosphine, bistranscyclooctene, bisnorbornene, biscyclopropene, or other reagents for Diels-Alder reactions, thiol, aminoxy, aldehyde, bisaldehyde, ketone, diketone or other reagents for oxime reactions, alkene or other reagents for thiol-ene reactions, alkyne or other reagents for thiol-yne reaction, tetrazine (e.g., 1,2,4,5-tetrazines) or bis-tetrazine for tetrazine ligation, bi-alkene or other reagents for tetrazole-alkene reactions, bi-azides for azide alkyne reactions, and so on.

[00178] Table 1 shows exemplary PEG or PEG derivative with linker groups or moieties with corresponding exemplary functional groups of synthetically modified amino acid, and Exemplary Orthogonal Chemistry.

Table 1. Exemplary functional groups of synthetically modified amino acid, Exemplary PEG-derivatives and Exemplary Orthogonal Chemistry.

Exemplary functional groups of synthetically modified amino acid	Exemplary PEG or PEG-derivatives	Exemplary Orthogonal Chemistry
Azide (e.g. Azido alanine, Para-azidophenylalanine, etc)	Alkyne-PEG-alkyne OR PEG-bis(triarylphosphine ester)	Azide-alkyne or Staudinger Ligation
Alkyne (e.g. Propynyl glycine, Alkyne functionalized proline, etc)	Azide-PEG-azide	Azide-alkyne
Tetrazine (e.g. Tetrazine-phenyl-2-aminopropanoic acid etc)	PEG-bistranscyclooctene, PEG-bisnorbornene, PEG-biscyclopropene	Tetrazine Ligation
Maleimide (e.g. AEG,	SH-PEG-SH	Michael addition thiolene

Dehydroalanine	SH-PEG-SH	Michael addition thiolene
Ketone (e.g. p-acetylphenylalanine)	Aminooxy-PEG-Aminooxy	Oxime
Aminooxy (Diphenyl-p-tolyl-methyl)-aminooxy]-N-[(phenylmethoxy)carbonyl]-serine	PEG-bisaldehyde or PEG-bisketone	Oxime
Aldehyde	Aminooxy-PEG-Aminooxy	Oxime
Alkene (e.g. 6-N-allyloxycarbonyl-L-lysine, O-allyltyrosine)	SH-PEG-SH	Thiolene
Tetrazole (e.g. tetrazolyl glycine, tetrazolyl alanine etc)	PEG-bisalkene	Tetrazole-alkene
Transcyclooctene (e.g. lysine-transcyclooctene)	PEG-bistetrazine	Tetrazine-trans-cyclooctene (TCO)
Cyclooctyne (e.g. lysine-transcyclooctyne)	Azide-PEG-azide	Azide-alkyne
Norbornene (e.g. 5-norbornene-2-yloxycarbonyl-L-lysine)	PEG-bis tetrazine	Tetrazine ligation

[00179] Table 2 shows Exemplary naturally-existing amino acids, Exemplary PEG or PEG-derivatives and Exemplary Conjugation Chemistry.

Table 2. Exemplary naturally-existing amino acids, Exemplary PEG or PEG-derivatives and Exemplary Conjugation Chemistry.

Exemplary naturally-existing amino acids	Exemplary PEG or PEG-derivatives	Exemplary Conjugation Chemistry
Cysteine	Divinyl sulfone-PEG-Divinyl sulfone, PEG-bismethacrylate, PEG-bismaleimide, diallyl-PEG, divinyl-PEG, Pyridyl disulfide-PEG-Pyridyl disulfide, PEG-bis(iodoacetamide)	Michael addition thiolene, thiolene, disulfide exchange, or SN2
Lysine or N-terminal amine	PEG-bisaldehyde, PEG-bis(NHS-ester), PEG-bisisocyanate, PEG-bis(isothiocyanate)	Reductive amination, amidation; with only one free lysine/amine or with controllable terminal amine pKa by using acidic

		conditions
Histidine	PEG-bissulfone	Mutation of two or more histidines into the protein at close proximity
Tyrosine	PEG-bis(diazonium salt)	electrophilic substitution

[00180] In one embodiment, specific PEG or PEG derivatives may be used in respect to specific surface-exposed linking amino acid residues of FGF monomers - i.e., naturally existing amino acids or synthetically modified amino acids of FGF monomers.

[00181] For example, when surface-exposed linking amino acid residues of FGF monomers are synthetically modified amino acids, PEG or PEG derivatives with specific linker groups may be used.

[00182] In one specific embodiment, when the surface-exposed linking amino acid residue of FGF monomers is an amino acid including a functional group of azide, such as azido alanine, para-azidophenylalanine and the like, PEG or PEG derivatives with alkynes or triarylphosphine groups may be used. A reaction of azide-alkyne or Staudinger Ligation may be used to form FGF multimers such as dimers.

[00183] When the surface-exposed linking amino acid residue of FGF monomers is an amino acid including a functional group of alkyne, such as propynyl glycine, alkyne functionalized proline and the like, PEG or PEG derivatives with azido groups may be used. A reaction of azide-alkyne may be used to form FGF multimers such as dimers.

[00184] In another specific embodiment, a synthetically modified amino acid is an amino acid including a functional group of tetrazine, e.g., tetrazine-phenyl-2-aminopropanoic acid, etc. PEG or PEG derivatives with bistranscyclooctene, bisnorbornene, biscyclopropene, or the like may be used. A reaction of Tetrazine Ligation may be used to form FGF multimers such as dimers.

[00185] In another specific embodiment, a synthetically modified amino acid may be an amino acid including a functional group of thiol-ene or Michael acceptor, e.g., maleimide, allyl-etherified eugenol (AEG) and vinyl sulfone. PEG or PEG derivatives with thiol group(s) may be used. A reaction of Michael addition or thiol-ene may be used to form FGF multimers such as dimers.

[00186] In another specific embodiment, a synthetically modified amino acid may be an amino acid including a functional group of dehydroalanine. PEG or PEG derivatives with thiol

group(s) may be used. A reaction of Michael addition or thiol-ene may be used to form FGF multimers such as dimers.

[00187] In another specific embodiment, a synthetically modified amino acid may be an amino acid including a functional group of ketone, e.g., p-acetylphenylalanine. PEG or PEG derivatives with aminooxy group(s) may be used. A reaction involved oxime may be used to form FGF multimers such as dimers.

[00188] In another specific embodiment, a synthetically modified amino acid may be an amino acid including a functional group of aminooxy, such as diphenyl-p-tolyl-methyl)-aminooxy]-*N*-[(phenylmethoxy)carbonyl]-serine and so on. PEG or PEG derivatives with aldehyde, bisaldehyde, ketone or bisketone may be used. A reaction involved oxime may be used to form FGF multimers such as dimers.

[00189] Similarly, when a synthetically modified amino acid may be an amino acid including a functional group of aldehyde, PEG or PEG derivatives with aminooxy group(s) may be used. A reaction involved oxime may be used to form FGF multimers such as dimers.

[00190] In another specific embodiment, a synthetically modified amino acid may be an amino acid including a functional group of alkene, e.g., 6-*N*-ally-loxycarbonyl-1-lysine, *O*-allyltyrosine and so on. PEG or PEG derivatives with thiol group(s) may be used. A reaction of thiol-ene may be used to form FGF multimers such as dimers.

[00191] In another specific embodiment, a synthetically modified amino acid may be an amino acid including a functional group of tetrazole, e.g., tetrazolyl glycine, tetrazolyl alanine, etc. PEG or PEG derivatives with bisalkene group(s) may be used. A reaction of Tetrazole-alkene may be used to form FGF multimers such as dimers. Other similar reactions may include reductive amination or reaction with hydrazine, etc.

[00192] In another specific embodiment, a synthetically modified amino acid may be an amino acid including a functional group of transcyclooctene, e.g., lysine-transcyclooctene. PEG or PEG derivatives with bistetrazine group(s) may be used. A reaction of Tetrazine-TCO may be used to form FGF multimers such as dimers.

[00193] In another specific embodiment, a synthetically modified amino acid may be an amino acid including a functional group of cyclooctyne, e.g., lysine-transcyclooctyne. PEG or PEG derivatives with azido group(s) may be used. A reaction of Azide-alkyne may be used to form FGF multimers such as dimers.

[00194] In another specific embodiment, a synthetically modified amino acid may be an amino acid including a functional group of Norbornene, e.g., 5-norbornene-2-ylloxycarbonyl-L-lysine and others. PEG or PEG derivatives with bis tetrazine group(s) may be used. A reaction of Tetrazine-Ligation may be used to form FGF multimers such as dimers.

[00195] Other reactions suitable for the present invention may include tetrazine ligation and diels alder reaction. Other functional group suitable for the present invention may include bicyclealkane such as bicyclopentene.

[00196] In one embodiment, surface-exposed linking amino acid residues of FGF monomers are naturally existing amino acids, PEG or PEG derivatives with other specific linker groups may be used.

[00197] In one specific embodiment, a naturally existing amino acid may be cysteine. PEG or PEG derivatives with divinyl sulfone or the like, bisacrylate or the like, bismaleimide or the like, or diallyl or the like, divinyl or the like, bispyridyl disulfide or the like, bis(iodoacetamide) or the like group(s) may be used. A reaction of Michael addition thiol-ene, thiol-ene, disulfide exchange or nucleophilic reactions may be used to form FGF multimers such as dimers.

[00198] In one specific embodiment, a naturally existing amino acid may be Lysine or N-terminal amine. PEG or PEG derivatives with bisaldehyde or the like, bis(NHS-ester) or the like, bisisocyanate or the like, or bisisothiocyanate or the like group(s) may be used. A reaction of reductive amination, amidation may be used to form FGF multimers such as dimers. In one embodiment, FGF dimers may form when there is only free lysine or amine or when the terminal amine pKa is controlled by using acidic conditions.

[00199] In one specific embodiment, a naturally existing amino acid may be tyrosine. PEG or PEG derivatives with bis(diazonium salt) or the like group(s) may be used. A reaction of electrophilic aromatic substitutions (EAS) may be used to form FGF multimers such as dimers.

[00200] In one embodiment of the present invention, the length of the linker molecule of FGF multimers such as dimers, e.g., PEG or PEG derivatives, may be controllable. As such, the distance among the FGF monomers in the polymers or dimers.

[00201] In one specific embodiment, the length of the linker molecule of FGF multimers such as dimers, e.g., PEG or PEG derivatives, may be controlled by changing molecular weight of the linker molecule, e.g., PEG or PEG derivatives.

[00202] For example, the PEG or PEG derivatives of present invention may have their molecular weight (MW, in g/mol or kDa) in the range of about 0.1kDa to about 200kDa, preferably about 0.1kDa to about 20kDa, more preferably about 0.6kDa to about 10kDa.

[00203] In one specific embodiment, the PEG or PEG derivatives of present invention may have their molecular weight (MW, in g/mol or kDa) in the range of about 0.7kDa to about 8kDa, preferably about 0.9kDa to about 6kDa, more preferably about 1.1kDa to about 4.5kDa.

[00204] In one preferred embodiment, the PEG or PEG derivatives of present invention may have their molecular weight in the range of about 1.3kDa to about 3.5kDa, preferably about 1.5kDa to about 2.5kDa, more preferably about 2.0kDa.

[00205] As shown in Fig. 1 and the Examples, Applicants determine the optimal linker length for a FGF2 dimer by screening dimer-induced metabolic activity of human dermal fibroblasts and found to be that closest to the inter-cysteine distance, 70 Å, corresponding to 2 kDa PEG.

[00206] In one preferred embodiment, FGF multimers such as dimers of the present invention include a FGF2 dimer. For example, the FGF2 dimer may be FGF2-PEG-FGF2.

[00207] In one embodiment, the FGF2 dimer of FGF2-PEG-FGF2 may include PEG with a controllable molecular weight so that the distance between the two FGF2 monomers may be controlled.

[00208] In one embodiment, the FGF2 dimer of FGF2-PEG-FGF2 may include FGF2-PEG0.1k-20k-FGF2 wherein the PEG has its molecular weight in the range of about 0.1kDa to about 20kDa.

[00209] In another embodiment, the FGF2 dimer of FGF2-PEG-FGF2 may include FGF2-PEG-FGF2 wherein the PEG has its molecular weight in the range of about 0.7kDa to about 8kDa, preferably about 0.9kDa to about 6kDa, more preferably about 1.1kDa to about 4.5kDa.

[00210] In another embodiment, the FGF2 dimer of FGF2-PEG-FGF2 may include FGF2-PEG-FGF2 wherein the PEG has its molecular weight in the range of about 1.3kDa to about 3.5kDa, preferably about 1.5kDa to about 2.5kDa, more preferably about 2.0kDa.

[00211] The Examples show different FGF2 dimers including FGF2-PEG2k-FGF2, FGF2-PEG6k-FGF2 and FGF2-PEG20k-FGF2. The Examples also show that FGF2-PEG2k-FGF2 is the preferred dimer for FGF2.

[00212] Applicants envision that FGF polymers or FGF multimers such as FGF2 multimers including more than two FGF2 monomers can be made when multi-branched linker molecule (e.g., multi-branched PEG or PEG derivatives) is used. For example, Applicants envision that multiarm (star) PEG vinyl sulfone could be employed to conjugate FGF monomers such as FGF2 monomers, where the number of arms would dictate the number of FGF monomers linked together. Alternatively, native FGF2 monomers that contains two free cysteines could be used in combination with a linker molecule such as the PEG bis vinyl sulfone to prepare multimeric FGF2 polymer with FGF2-PEG-FGF2-PEG, etc., construct.

[00213] As discussed above, the length of the linker molecule (e.g., PEG or PEG derivatives) of such FGF polymers may be controlled by changing molecular weight of the linker molecule, e.g., PEG or PEG derivatives.

[00214] In one aspect, the present invention discloses pharmaceutical compositions or formulations including FGF multimers such as dimers.

[00215] In one embodiment, the pharmaceutical compositions or formulations of the present invention may be used to treat and prevent neurodegenerative disease and for promoting nerve regeneration and spinal chord repair.

[00216] In another embodiment, the pharmaceutical compositions or formulations of the present invention may be used for treatment of disorders associated with myocardial infarction, congestive heart failure, hypertrophic cardiomyopathy and dilated cardiomyopathy.

[00217] In another embodiment, the pharmaceutical compositions or formulations of the present invention may also be useful for limiting infarct size following a heart attack, for promoting angiogenesis and wound healing following angioplasty or endarterectomy, for developing coronary collateral circulation, for revascularization in the eye, for complications related to poor circulation such as diabetic foot ulcers, for stroke (as described above), following coronary reperfusion using pharmacologic methods and other indications where angiogenesis is of benefit. The pharmaceutical compositions or formulations of the present invention may be useful for improving cardiac function, either by inducing cardiac myocyte neogenesis and/or hyperplasia, by inducing coronary collateral formation, or by inducing remodeling of necrotic myocardial area.

[00218] In another embodiment, the pharmaceutical compositions or formulations of the present invention may also be useful the treatment of nervous system diseases. As used herein,

the term "nervous system disease," refers to a disease involving one or more nerve cells, which may be a disease of the central nervous system or of the peripheral nervous system. Diseases or disorders of the central nervous system may include but are not limited to Pathophysiologic complications such as herniations and cerebral edema; Malformations and developmental diseases such as neural tube defects and syringomyelia and hydromyelia; Perinatal brain injury such as cerebral palsy; Trauma such as parenchymal injuries (concussion, etc.), traumatic vascular injury (e.g., hematoma and traumatic subarachnoid hemorrhage and traumatic intraparenchymal hematoma), and spinal cord injury; Cerebrovascular Disease such as hypoxia, ischemia and infarction, nontraumatic intracranial hemorrhage, vascular malformations, hypertensive cerebrovascular disease; Infections such as meningitis, chronic meningoencephalitis (e.g., tuberculous and chronic meningitis, neurosyphilis, lyme disease), viral encephalitis, spongiform encephalopathies, fungal infection; Demyelinating diseases such as multiple sclerosis and acute disseminated encephalomyelitis; Degenerative diseases such as Alzheimer's disease, Pick's disease, Parkinsonism, Huntington's disease, Friedreich's ataxia; Genetic diseases of metabolism (affects CNS); Toxic and acquired metabolic diseases such as vitamin deficiencies; and Neurocutaneous syndromes such as neurofibromatosis (NF1, NF2), tuberous sclerosis and Von Hippel-Lindau disease.

[00219] In one embodiment, the pharmaceutical compositions or formulations of the present invention is used for treating or healing a wound such as a chronic wound in a patient.

[00220] A pharmaceutical composition or formulation for treating or healing a wound such as a chronic wound comprises an FGF multimer such as a dimer. Any of FGF multimers such as dimers as discussed above may be used in the pharmaceutical composition or formulation.

[00221] Specifically, an FGF multimer such as a dimer FGF multimer such as a dimer comprises two or more FGF monomers, each of the two or more FGF monomers comprising one linking amino acid residue; and a linker molecule, wherein the linker molecule covalently links the two or more FGF monomers through the linking amino acid residues. In one embodiment, the linker molecule is a polymer.

[00222] In one embodiment, the FGF monomers may be naturally existing FGF monomers or modified FGF monomers. In one specific embodiment, the FGF monomers may be naturally existing FGF2 monomers or modified FGF2 monomers.

[00223] In one specific embodiment, the FGF monomers may be the modified FGF2 monomers as discussed above. Specifically, the modified FGF2 monomers comprise a mutation of a cysteine. More specifically, the modified FGF2 monomers comprise a mutation of C78S.

[00224] As discussed above, the linking amino acid residue of the modified FGF2 monomers is a cysteine. Specifically, the linking amino acid residue is Cys96.

[00225] In one embodiment, the linker molecule is any of the biocompatible polymers as discussed above.

[00226] In one specific embodiment, the linker molecule is a PEG or PEG derivative.

[00227] More specifically, the PEG or PEG derivative is modified to include linker groups such as thiol to covalently bond with the linking amino acid residues of the FGF2 monomers.

[00228] In one embodiment, the length of the linker molecule of FGF multimers such as dimers, e.g., PEG or PEG derivatives, may be controlled by changing molecular weight of the linker molecule, e.g., PEG or PEG derivatives.

[00229] For example, the PEG or PEG derivatives of present invention may have their molecular weight (MW, in g/mol or kDa) in the range of about 0.1kDa to about 200kDa, preferably about 0.1kDa to about 20kDa, more preferably about 0.5kDa to about 10kDa.

[00230] In one preferred embodiment, the PEG or PEG derivatives of present invention have molecular weight about 2 kDa.

[00231] In one embodiment, the pharmaceutical compositions or formulations of the present invention may further comprise one stabilizing agent. For example, an approach for stabilizing solid protein compositions or formulations of the invention is to increase the physical stability of purified, e.g., lyophilized, protein of FGF multimers such as dimers. This will inhibit aggregation via hydrophobic interactions as well as via covalent pathways that may increase as proteins unfold. Stabilizing compositions or formulations in this context may often include polymer-based formulations, for example a biodegradable hydrogel formulation/delivery system. The critical role of water in protein structure, function, and stability is well known. Typically, proteins are relatively stable in the solid state with bulk water removed. However, solid therapeutic protein compositions or formulations may become hydrated upon storage at elevated humidities or during delivery from a sustained release composition or device. The stability of proteins generally drops with increasing hydration. Water may also play a significant role in solid protein aggregation, for example, by increasing protein flexibility resulting in enhanced

accessibility of reactive groups, by providing a mobile phase for reactants, and by serving as a reactant in several deleterious processes such as beta-elimination and hydrolysis.

[00232] As such, an effective method for stabilizing proteins against solid-state aggregation for oral or topical delivery may be to control the water content in a solid composition formulation and maintain the water activity in the formulation at optimal levels. This level depends on the nature of the protein, but in general, FGF multimers such as dimers maintained below their “monolayer” water coverage will exhibit superior solid-state stability.

[00233] A variety of additives, diluents, bases and delivery vehicles may be provided within the invention that effectively control water content to enhance FGF multimers such as dimers stability. These reagents and carrier materials effective as anti-aggregation agents in this sense may include, for example, polymers of various functionalities, such as polyethylene glycol, dextran, diethylaminoethyl dextran, and carboxymethyl cellulose, which significantly increase the stability and reduce the solid-phase aggregation of peptides and proteins admixed therewith or linked thereto. In some instances, the activity or physical stability of FGF multimers such as dimers may also be enhanced by various additives to aqueous solutions of the peptide or protein drugs. For example, additives, such as polyols (including sugars), amino acids, proteins such as collagen and gelatin, and various salts may be used.

[00234] Certain additives, in particular sugars and other polyols, may also impart significant physical stability to dry, e.g., lyophilized proteins such as FGF multimers such as dimers. These additives may also be used within the invention to protect the proteins such as FGF multimers such as dimers against aggregation not only during lyophilization but also during storage in the dry state. For example sucrose and Ficoll 70 (a polymer with sucrose units) exhibit significant protection against peptide or protein aggregation during solid-phase incubation under various conditions. These additives may also enhance the stability of solid proteins embedded within polymer matrices. In one embodiment, Heparin may be used as stabilizer of FGF multimers such as FGF2 multimers.

[00235] Yet additional additives, for example sucrose, stabilize proteins such as FGF multimers such as dimers against solid-state aggregation in humid atmospheres at elevated temperatures, as may occur in certain sustained-release formulations of the invention. Proteins such as gelatin and collagen also serve as stabilizing or bulking agents to reduce denaturation and aggregation of unstable proteins in this context. These additives can be incorporated into

polymeric melt processes and compositions within the invention. For example, polypeptide microparticles can be prepared by simply lyophilizing or spray drying a solution containing various stabilizing additives described above. Sustained release of unaggregated peptides and proteins such as FGF multimers such as dimers can thereby be obtained over an extended period of time.

[00236] Various additional preparative components and methods, as well as specific compositions or formulation additives, are provided herein which yield formulations for mucosal delivery of aggregation-prone peptides and proteins, wherein the peptide or protein such as FGF multimers such as dimers is stabilized in a substantially pure, unaggregated form using a solubilization agent. A range of components and additives are contemplated for use within these methods and formulations. Exemplary of these solubilization agents are cyclodextrins (CDs), which selectively bind hydrophobic side chains of polypeptides. These CDs have been found to bind to hydrophobic patches of proteins in a manner that significantly inhibits aggregation. This inhibition is selective with respect to both the CD and the protein involved. Such selective inhibition of protein aggregation may provide additional advantages within the delivery methods and compositions of the invention.

[00237] Additional agents for use in this context include CD dimers, trimers and tetramers with varying geometries controlled by the linkers that specifically block aggregation of peptides and protein such as FGF multimers such as dimers. Yet solubilization agents and methods for incorporation within the invention involve the use of peptides and peptide mimetics to selectively block protein-protein interactions. In one aspect, the specific binding of hydrophobic side chains reported for CD multimers may be extended to proteins via the use of peptides and peptide mimetics that similarly block protein aggregation. A wide range of suitable methods and anti-aggregation agents may be available for incorporation within the compositions and procedures of the invention.

[00238] In another embodiment, the pharmaceutical composition or formulation may further comprise one delivering or delivery-enhancing agent.

[00239] Suitable delivering or delivery-enhancing agents may include a suitable carrier or vehicle for topical or transdermal delivery. As used herein, the term “carrier” refers to a pharmaceutically acceptable solid or liquid filler, diluent or encapsulating material. A water-containing liquid carrier can contain pharmaceutically acceptable additives such as acidifying

agents, alkalizing agents, antimicrobial preservatives, antioxidants, buffering agents, chelating agents, complexing agents, solubilizing agents, humectants, solvents, suspending and/or viscosity-increasing agents, tonicity agents, wetting agents or other biocompatible materials. A tabulation of ingredients listed by the above categories, may be found in the *U.S. Pharmacopeia National Formulary*, 1857-1859, (1990).

[00240] Some other examples of the materials which may serve as pharmaceutically acceptable carriers are sugars, such as lactose, glucose and sucrose; starches such as corn starch and potato starch; cellulose and its derivatives such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients such as cocoa butter and suppository waxes; oils such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; glycols, such as propylene glycol; polyols such as glycerin, sorbitol, mannitol and polyethylene glycol; esters such as ethyl oleate and ethyl laurate; agar; buffering agents such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen free water; isotonic saline; Ringer's solution, ethyl alcohol and phosphate buffer solutions, as well as other non toxic compatible substances used in pharmaceutical formulations. Wetting agents, emulsifiers and lubricants such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the compositions, according to the desires of the formulator.

[00241] Formulations of the disclosed FGF multimers such as dimers suitable for oral or topical administration may be presented as discrete units such as capsules, cachets or tablets each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous or non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be administered as a bolus, electuary or paste.

[00242] A tablet is made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder, lubricant, inert diluent, preservative, surface active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered active ingredient moistened with an inert liquid diluent. The tablets may optionally be coated or scored

and optionally are formulated so as to provide slow or controlled release of the active ingredient therefrom.

[00243] The amount of active ingredient of FGF multimers such as dimers that may be combined with the carrier material to produce a single dosage form will vary depending upon the host treated and the particular mode of administration. For example, a time-release formulation intended for oral or topical administration to humans may contain approximately 1 to 1000 mg of active material compounded with an appropriate and convenient amount of carrier material which may vary from about 5 to about 95% of the total compositions (weight:weight). The pharmaceutical composition can be prepared to provide easily measurable amounts for administration. For example, an aqueous solution intended for intravenous infusion may contain from about 3 to 500 μ g of the active ingredient per milliliter of solution in order that infusion of a suitable volume at a rate of about 30 mL/hr can occur.

[00244] Effective dose of active ingredient depends at least on the nature of the condition being treated, toxicity, whether the compound is being used prophylactically (lower doses) or against an active viral infection, the method of delivery, and the pharmaceutical formulation, and will be determined by the clinician using conventional dose escalation studies. It can be expected to be from about 0.0001 to about 100 mg/kg body weight per day; typically, from about 0.01 to about 10 mg/kg body weight per day; more typically, from about 0.01 to about 5 mg/kg body weight per day; most typically, from about 0.05 to about 0.5 mg/kg body weight per day. For example, the daily candidate dose for an adult human of approximately 70 kg body weight will range from 1 mg to 1000 mg, preferably between 5 mg and 500 mg, and may take the form of single or multiple doses.

[00245] In one embodiment, a formulation for delivery of the present invention may be a topical cream that can biodegrade and/or slowly release the FGF2 including widely used current dressings. The dressings may include carboxymethylcellulose (CMC, Aquacel® Hydrofiber® Wound Dressings, SoloSite® Wound Gel and Comfeel® Plus Ulcer), hyaluronic acid (HA, Hylase® Wound Gel, Hyalomatrix®), and alginate (Kaltostate®, Tegagen®, and Sorbsan®). Additionally, fibrin may be utilized as a glue in wound healing and cardiothoracic treatments (Evicil®, Tisseel™ and Evarrest® fibrin glues). Some dressings (for example CMC - Regranex® and fibrin) have been utilized for growth factor delivery. The dressings may include Tegaderm™ which consists of a copolymer of 96% isooctyl acrylate and 4% acrylamide; and the

acrylamide side chains or similar products that are modified to also contain heparin so the protein can be simultaneously stabilized.

[00246] METHODS AND KITS

[00247] In another aspect, the present invention discloses a method for treating or healing a disease condition in a subject.

[00248] In one embodiment, the disease condition may include any disease as discussed above.

[00249] In one embodiment, the disease condition is a wound, specifically a chronic wound.

[00250] In one embodiment, the method for treating or healing a wound comprises administering to the subject a therapeutically effective amount of a composition so that the wound is treated or healed.

[00251] The composition may be any of the compositions or formulations as discussed above. In one embodiment, the composition comprises an FGF multimer such as a dimer. Any of FGF multimers such as dimers as discussed above may be used in the pharmaceutical composition or formulation.

[00252] Specifically, an FGF multimer such as a dimer comprises two or more FGF monomers, each of the two or more FGF monomers comprising one linking amino acid residue; and a linker molecule, wherein the linker molecule covalently links the two or more FGF monomers through the linking amino acid residues. In one embodiment, the linker molecule is a polymer.

[00253] In one embodiment, the FGF monomers may be naturally existing FGF monomers or modified FGF monomers. In one specific embodiment, the FGF monomers may be naturally existing FGF2 monomers or modified FGF2 monomers. In one specific embodiment, the FGF monomers may be the modified FGF2 monomers as discussed above. Specifically, the modified FGF2 monomers comprise a mutation of a cysteine. More specifically, the modified FGF2 monomers comprise a mutation of C78S. As discussed above, the linking amino acid residue of the modified FGF2 monomers is a cysteine. Specifically, the linking amino acid residue is Cys96.

[00254] In one embodiment, the linker molecule is any of the biocompatible polymers as discussed above. In one specific embodiment, the linker molecule is a PEG or PEG derivative. More specifically, the PEG or PEG derivative is modified to include linker groups such as thiol to covalently bond with the linking amino acid residues of the FGF2 monomers.

[00255] In one embodiment, the length of the linker molecule of FGF multimers such as dimers, e.g., PEG or PEG derivatives, may be controlled by changing molecular weight of the linker molecule, e.g., PEG or PEG derivatives. For example, the PEG or PEG derivatives of present invention may have their molecular weight (MW, in g/mol or kDa) in the range of about 0.1kDa to about 200kDa, preferably about 0.1kDa to about 20kDa, more preferably about 0.5kDa to about 10kDa. In one preferred embodiment, the PEG or PEG derivatives of present invention have molecular weight about 2 kDa.

[00256] The composition may further comprise at least one stabilizing agent and at least one delivering agent as discussed above.

[00257] Application envision that the present method may be combined with other methods or treatments to achieve better results.

[00258] For example, a method for treating or healing a wound may further comprise at least one advanced wound care therapy selected from the group consisting of the use of collagen, growth factors, bioengineered skin, gene and stem cell therapy, silver products, ozone oxygen therapy, and negative pressure wound therapy.

[00259] One or more of the disclosed agents (herein referred to as the active ingredients) and the composition are administered by any route appropriate to the condition to be treated. Suitable routes include oral, rectal, nasal, topical (including buccal and sublingual), vaginal and parenteral (including subcutaneous, intramuscular, intravenous, intradermal, intrathecal and epidural), and the like. It will be appreciated that the preferred route may vary with for example the condition of the recipient. An advantage of the compounds of this invention is that they are orally bioavailable and can be dosed orally.

[00260] In another embodiment, one of the more disclosed agents and the composition is administered during surgery, through topical application to desired areas, intrathecal administration, bathing of tissues in the disclosed agents, or surgical placement of a slow release device, gel, or matrix.

[00261] As shown in the Examples, FGF2-PEG2k-FGF2 induced greater fibroblast metabolic activity than FGF2 alone, all other dimers, and all monoconjugates, at each concentration tested, with the greatest difference observed at low (0.1 ng/mL) concentration. FGF2-PEG2k-FGF2 further exhibited superior activity compared to FGF2 for both metabolic activity and migration in human umbilical vein endothelial cells, as well as improved angiogenesis in a coculture model *in vitro*. Efficacy in an *in vivo* wound healing model was assessed in diabetic mice. FGF2-PEG2k-FGF2 increased granulation tissue and blood vessel density in the wound bed compared to FGF2. The results suggest that this rationally designed construct may be useful for improving the fibroblast matrix formation and angiogenesis in chronic wound healing.

[00262] In another aspect, the present invention discloses a kit for treating or healing a wound in a patient. The kit comprises a composition as discussed above.

[00263] Specifically, the composition for the kit comprises an FGF multimer such as a dimer. Any of FGF multimers such as dimers as discussed above may be used in the kit. Specifically, an FGF FGF multimer such as a dimer comprises two or more FGF monomers, each of the two or more FGF monomers comprising one linking amino acid residue; and a linker molecule, wherein the linker molecule covalently links the two or more FGF monomers through the linking amino acid residues. In one embodiment, the linker molecule is a polymer.

[00264] In one embodiment, the kit further comprises another composition for sanitizing the wound before the treatment.

[00265] Examples of the disinfectant or sanitizer may include any main components, which are known for their wound disinfection, such as Glutaral Concentrate, benzalkonium, benzethonium, chlorohexidine, cresol, ethanol, isopropyl ethanol, povidone-iodine, silver nitrate, or salts thereof.

[00266] In yet another aspect, the present invention discloses a medical device for treating or healing a wound in a patient.

[00267] The medical device comprises a substrate and wound covering means for releaseably covering the wound of the patient.

[00268] The substrate comprises an FGF multimer such as a dimer. Any of FGF multimers such as dimers as discussed above may be used in the medical device. Specifically, an FGF multimer such as a dimer comprises two or more FGF monomers, each of the two or more

FGF monomers comprising one linking amino acid residue; and a linker molecule, wherein the linker molecule covalently links the two or more FGF monomers through the linking amino acid residues. In one embodiment, the linker molecule is a polymer.

[00269] After the wound of the patient is covered, the substrate controllably releases the FGF multimer such as a dimer into the wound so that the wound is treated.

EXAMPLES

[00270] Site-specific chemical dimerization of fibroblast growth factor 2 (FGF2) with the optimal linker length resulted in a FGF2 homodimer with improved granulation tissue formation and blood vessel formation at exceptionally low concentrations. Homodimers of FGF2 were synthesized through site-specific linkages to both ends of different molecular weight poly(ethylene glycols) (PEGs). The optimal linker length was determined by screening dimer-induced metabolic activity of human dermal fibroblasts and found to be that closest to the inter-cysteine distance, 70 Å, corresponding to 2 kDa PEG. FGF2-PEG2k-FGF2 induced greater fibroblast metabolic activity than FGF2 alone, all other dimers, and all monoconjugates, at each concentration tested, with the greatest difference observed at low (0.1 ng/mL) concentration. FGF2-PEG2k-FGF2 further exhibited superior activity compared to FGF2 for both metabolic activity and migration in human umbilical vein endothelial cells, as well as improved angiogenesis in a coculture model *in vitro*. Efficacy in an *in vivo* wound healing model was assessed in diabetic mice. FGF2-PEG2k-FGF2 increased granulation tissue and blood vessel density in the wound bed compared to FGF2. The results suggest that this rationally designed construct may be useful for improving the fibroblast matrix formation and angiogenesis in chronic wound healing.

[00271] In addition to site-selective dimerization, the length of the tether is also imperative to protein activity. Linker length is essential to receptor activation for similar growth factors. Based on the crystal structure of the tetrameric FGF2:FGFR1 complex, Applicants hypothesized that a flexible linker with a length close to the inter-cysteine distance of 70Å would induce the greatest activity.

[00272] Poly(ethylene glycol) (PEG) was chosen as the linker based on the ease of modification. PEG is also known to improve pharmacokinetics through stabilization and improved circulation time, and many FDA-approved, PEGylated therapeutic agents are on the market.

MATERIALS AND METHODS

[00273] Materials

[00274] All chemicals and reagents were purchased from Sigma-Aldrich and used as received unless otherwise indicated. Enzyme-linked immunosorbent assay (ELISA) Development DuoSet kit was purchased from R&D Systems. Normal Human Dermal Fibroblasts (HDFs), HUVECs, and cell media were purchased from ATCC.

[00275] HDF Metabolic activity Assay

[00276] HDF cells (passage 4 - ATCC) were suspended in UltraCULTURE™ (Lonza) serum-free medium supplemented with 2 mM L-Glutamine, 100 µg/ml penicillin, and 100 µg/ml streptomycin, and plated at 2000 cells/well in a 96-well plate. The cells were allowed to adhere for 16 hours at 37 °C, 5% CO₂. After 16 hours the medium was removed by aspiration and replaced with 100 µL of unmodified FGF2, mPEG-FGF2, or FGF2-PEG-FGF2, diluted in UltraCULTURE™ medium (original concentration determined by ELISA). The cells were incubated with the samples for 72 hours at 37 °C, 5% CO₂ without media exchange, after which cell metabolic activity was assessed using a CellTiter-Blue® assay. All experimental groups were normalized to the control group, which received only blank medium. Each group was done with six replicates.

[00277] HUVEC Metabolic activity Assay

[00278] HUVEC cells (passage 5 - ATCC) were suspended in EGM™ (-BBE) medium supplemented with 100 ug/ml penicillin and 100 µg/ml streptomycin, and plated at 1000 cells/well in a 96-well plate. The cells were allowed to adhere for 16 hours at 37 °C, 5% CO₂. After 16 hours the medium was removed by aspiration and replaced with 100 µL of unmodified FGF2, mPEG-FGF2, or FGF2-PEG-FGF2 (protein concentration determined by ELISA) diluted in epidermal growth medium without bovine brain extract (EGM™ -BBE). The cells were incubated with the samples for 72 hours at 37 °C, 5% CO₂ without media exchange, after which

cell metabolic activity was assessed using a CellTiter-Blue[®] assay. All experimental groups were normalized to the control group, which received only blank medium. Each group was done with six replicates

[00279] HDF Migration Assay

[00280] Two horizontal lines were drawn on the back of each well of a 0.2% gelatin-coated 24-well plate. HDFs from ATCC (P4) were seeded in fibroblast growth medium with full supplements at 70,000 cells/well (400 μ L/well) and allowed to incubate for 24 hours at 37 °C, 5% CO₂. The media was then removed, and the wells rinsed with warm D-PBS 2x, followed by the addition of starvation media, DMEM + 2% FBS and the cells incubated for 24 hours. A vertical scratch was then made using a P200 pipette tip. The wells were then rinsed 2x with warm D-PBS, and then the diluted samples of FGF2 or FGF2-PEG2k-FGF2 (in DMEM + 2% FBS) were added into 4 wells per concentration ($n_{\max}=8$) as well as 4 wells containing blank media. The cells were then incubated for 18 hours. Bright field images were taken just above and just below each drawn line, and analyzed using ImageJ, by measuring the distance (pixels) of the cell-free area at T=0 and T=18. The study was blinded before image analysis. Cell images were taken in bright field with a Zeiss Axioscope equipped with an AxioCam MRm at 5x magnification.

[00281] HUVEC Migration Assay

[00282] Two horizontal lines were drawn on the back of each well of a 0.2% gelatin-coated 24-well plate. HUVECs (P5) were seeded in complete Epidermal Growth Media (EGM), at 60,000 cells/well (400 μ L/well) and allowed to incubate for 24 hours at 37 °C, 5% CO₂. The media was then removed, and the wells rinsed with warm D-PBS 2x, followed by the addition of starvation media, DMEM + 2% FBS and the cells incubated for 24 hours. A vertical scratch was then made using a P200 pipette tip. The wells were then rinsed 2x with warm D-PBS, and then samples diluted in DMEM + 2% FBS, were added at each concentration into 4 separate wells, as well as 4 wells of blank media. The cells were then incubated for 18 hours. Pictures were taken at 5X magnification at T=0 and T=18 hours. Bright field images were taken just above and just below each drawn line, and analyzed using ImageJ, by measuring the distance (pixels) of the cell-free area at T=0 and T=18. One of the two full repeats was blinded before image analysis.

[00283] *In vitro* Angiogenesis Co-Culture Assay

[00284] Methods for angiogenesis co-culture and cord-like structure staining were adapted from literature procedures[57, 58]. HDFs (P3) were plated at 12,500 cells/well in a 48-well plate, in endothelial growth medium (EGM) with full supplement and incubated at 37°C for 72 hours or until cells reached confluency. The cells were then starved for 18 hours with EGM (-BBE) minus epidermal growth factor (-EGF). HUVECs (P4) were trypsonized and resuspended in EGM (-BBE) (-EGF). Starvation media was aspirated and HUVECs were plated on top of the HDF monolayer at 10,000 cells/well in EGM (-BBE) (-EGF) followed by sample (either FGF2 or FGF2-PEG2k-FGF2) diluted in EGM (-BBE) (-EGF). Final concentrations of FGF2 and FGF2-PEG2k-FGF2 were 10 ng/mL, 5 ng/mL, 1 ng/mL, 0.5 ng/mL, and 0.1 ng/mL. After 72 and 144 hours the sample solutions were refreshed with samples EGM (-BBE) (-EGF) at the appropriate concentrations. Ten days after HUVEC and the first sample addition, the medium was removed and each well fixed with 70% EtOH (at -20°C) for 30 minutes. After fixing, the wells were rinsed with 0.5 mL of 1% BSA in D-PBS three times. Next, endogenous alkaline phosphatase was removed by incubation with 0.3% H₂O₂ in MeOH at room temperature for 15 minutes. The H₂O₂ solution was removed once the solution was cloudy. The wells were washed 3 times with 1% BSA. The wells were then incubated with primary antibody (mouse anti-human PECAM1/CD31, R&D Systems) at 1 ug/mL in 1% BSA for 60 minutes at 37°C. The wells were then rinsed 3 times with 1% BSA. The wells were then incubated with secondary antibody (goat anti-mouse IgG – alkaline phosphatase, Sigma Aldrich) at 3 ug/mL in 1% BSA for 60 minutes at 37 °C and then wells were rinsed 3x5 minutes with milliQ water. Next, the wells were incubated with BCIP/NBT solution (one tablet dissolved in 10 mL milliQ water, filtered) at room temperature for 6-15 minutes. After the cord-like structures were visually stained, the BCIP/NBT (Life Technologies) was removed and the wells were washed 3 times with milliQ water and allowed to dry. Plates were stored for up to 60 days at -80°C. To assess the extent of angiogenesis, 5 bright field images were captured per well at 5x magnification. Number and length of the cord-like structures as well as number of nodes were calculated manually using NIH ImageJ Software. The values for each of 5 images per well were summed and then the sums were averaged across 3 wells. The sample identities remained blinded during analysis and until after results were calculated. An example of how the structures were counted is provided in the Supporting Information.

[00285] *In vivo* Assessment of Wound Healing in Diabetic Mice

[00286] Eight to nine-week-old male TallyHo/JngJ mice (Jackson Laboratories, Bar Harbor, ME) were used as a model of type II diabetes. Briefly, 1 day prior to wounding, dorsal hair was shaved and depilated and mice were housed individually. Full-thickness wounds were created on the dorsum of mice (8 mm punch biopsy; Acuderm, Inc., Lauderdale, FL). The wound bed was covered with TegadermTM (3M, St. Paul, MN) and treated with 20 μ l of FGF2 or FGF2-PEG2k-FGF2 (0.02 μ g total) or D-PBS control (-Mg²⁺, -Ca²⁺) by injecting through the covering into the wound bed once daily for 5 days. The covering was removed on day 7. Digital photographs taken on day 0, 1, 2, 3, 4, 7, 10, and 14 and wound area was measured using ImageJ software. The wound beds were excised en bloc with the surrounding soft tissue and fixed with 10% formalin solution and histologic analysis was performed on day 14. The granulation tissue area and blood vessel density were measured as previously described[59]. The procedures were conducted blinded.

[00287] Results**[00288]** Conjugate Synthesis

[00289] FGF2 was genetically engineered (C78S) to contain a single surface-exposed free cysteine, Cys96, to facilitate site-specific polymer conjugation and therefore stoichiometric homodimerization. This mutation was made on a pET29c(+)hFGF-2 plasmid provided by the Helmholtz Centre for Infection Research, Braunschweig, Germany[60]. Employing *E. coli* host BL21(DE3), the mutant was expressed and purified as previously described[60]. The activity of the mutant was confirmed in an *in vitro* metabolic activity assay in HDFs, and no significant difference was observed as compared to natural FGF2. Pure mutant protein (hereby called simply “FGF2”) was obtained as visualized by Western Blot (Fig. 2, lane 2) with a molecular weight of 17.2 kDa measured by MALDI mass spectrometry (Fig. 7). The mutation was further verified by trypsin-digestion followed by liquid chromatography-tandem mass spectrometry, and the expected peptide (GVVSIKGVSANR) showing the C78S mutation was found.

[00290] PEG linker lengths were chosen based on the inter-cysteine distance (Cys96 of one FGF2 to Cys96 in the second FGF2) in the 2:2 FGF2:FGFR1 tetrameric complex (Fig. 1), which is approximately 70 Å. Based on prior work[61-63], we hypothesized that in the active

tetrameric complex the PEG would be stretched slightly from its normal random coil configuration. As a guide we considered Flory's Radius ($R_F = aN^{0.6}$) where a = monomer unit length and N = # monomers or degree of polymerization, to estimate the random coil length of various PEGs. We also used the equation for a fully-extended polymer chain of length $L = aN$ to estimate the maximum possible length. We hypothesized that the best candidate would be a PEG with an $R_F < 70$ Å and an $L > 70$ Å (both 2 and 6 kDa PEG fit this description, Table 3). For comparison we also chose a small molecule linker, divinyl sulfone (DVS), where both R_F and $L \ll 70$ Å and a large 20 kDa PEG control where both R_F and $L \gg 70$ Å (Table 3).

Table 3. Crosslinker length based on Flory's radius of gyration and fully extended chain length.

Crosslinker	MW*	$a^\dagger$	N	$R_F^\dagger$	$L^\dagger$
DVS	118	<10	1	<10	<10
VS-PEG2k-VS	2000	3.5	45	35	160
VS-PEG6k-VS	6000	3.5	136	67	480
VS-PEG20k-VS	20000	3.5	454	138	1600

*Molecular weight (MW) in g/mol; † in Å

[00291] Site-specific conjugation can be achieved through Michael addition of nucleophilic amino acid residues to vinyl sulfone (VS) groups. By maintaining a pH between 7 and 9, cysteine residues can be targeted preferentially compared to the reaction to lysine or N-terminal amines[64], and FGF2 is stable between pH 5 and 9[65]. Therefore PEGs (2, 6, and 20 kDa) were modified at both ends with VS according to literature procedures[66]. Since PEGylated proteins often exhibit enhanced activity due to increased stabilization or conversely can decrease activity due to steric hindrance, monoconjugated controls were synthesized. Towards that end, poly(ethylene glycol methyl ether) (mPEGs) with molecular weights of 2, 5, and 20 kDa were modified on one end with VS.

[00292] In order to synthesize the conjugates, FGF2 was incubated with 0.5 eq. of polymer (2, 6, or 20 kDa VS-PEG-VS, 2, 5, or 20 kDa mPEG-VS) or small molecule crosslinker DVS in 100 mM Tris buffer, pH 9 at room temperature for 12 hours. A range of buffer types, pHs, polymer equivalents, times, and addition of organic solvents were screened, and in all cases the maximum yield of the desired conjugate (monoconjugate or homodimer) was obtained using

the conditions described below. Although excess polymer is typically used to maximize monoconjugate yield, excess polymer resulted in multiple polymer additions, which were difficult to remove. Kang et al. also observed up to four polymer additions for monoconjugations and hypothesized that with increased time the two internal cysteines are also able to react[48]. This was avoided by using 0.5 equivalents of polymer incubated with the protein at 73 μ M concentration in 100 mM Tris, pH 9, 5 mM EDTA for 12 hours. Conjugates were purified by fast protein liquid chromatography (FPLC). Pure monoconjugates and homodimers were obtained for each length PEG as visualized by western blot (Fig. 2, procedure as discussed below). For FGF2-PEG20k-FGF2 (Fig. 2, lane 9) the homodimer appears as a smear, which is often observed with larger polymer-conjugates.

[00293] Conjugate Screening in Metabolic activity and Migration Assays

[00294] Efficacy of FGF2 in wound healing is often first assessed *in vitro* by fibroblast and endothelial cell metabolic activity (proliferation), migration, vasculogenesis, and angiogenesis[14, 67-70]. Human dermal fibroblast (HDF) metabolic activity was chosen to screen and compare the activity of all PEG-linked dimers, monoPEGylated controls, the small molecule control, and FGF2 alone. HDFs were serum and growth factor starved, followed by treatment with samples for 3 days.

[00295] FGF2-PEG2k-FGF2 induced significantly greater metabolic activity as determined by the standard CellTiter Blue assay in HDFs than all other linker lengths, all monoPEGylated controls, and free FGF2 at every concentration tested (Fig. 3A). FGF2-PEG2k-FGF2 induced the most marked difference in metabolic activity at low (0.1 ng/mL) concentrations where the dimer induced 169% compared to blank media (set to 100%) while FGF2 alone and monoconjugate were only 118% and 131%, respectively. PEG is known to increase the stability of FGF2 thereby increasing *in vitro* activity[53, 54, 56], and likely the 2 kDa PEG increased the stability of FGF2 in the monoconjugate. Yet the activity of the dimer was significantly higher than the monoconjugate demonstrating that dimerization, as opposed to increased stability alone, was the important factor. FGF2-PEG2k-FGF2 induced the highest metabolic activity overall, 181% compared to blank media, at 1 ng/mL while FGF2 alone induced only 144% metabolic activity at 1 ng/mL. The increase in metabolic activity compared to blank plateaued at 1 ng/mL for FGF2-PEG2k-FGF2 but even with this plateau, the increased

metabolic activity is greater than all other conjugates and FGF2 alone. The small molecule linked homodimer, FGF2-VS-FGF2, did stimulate significantly greater growth than FGF2 at low concentrations (0.1 ng/mL). However this increase was small, likely due to the steric restrictions of such a short linker. The fact that there was increased activity indicates that there is some flexibility in the 2:2 FGF2:FGFR tetrameric complex and indeed other modes of dimerization have been shown to be effective[45]. While FGF2-PEG6k-FGF2 did induce greater metabolic activity than FGF2 at low concentrations (0.1 and 1 ng/mL), it did not significantly increase metabolic activity beyond that of its monoPEGylated control (mPEG5k-FGF2) indicating that the increase in activity might be due solely to the added stability induced by PEG. Again, the enhanced activity was only slightly increased (14 and 13%, respectively) compared to FGF2 at the two lower concentrations. For FGF2-PEG20k-FGF2, a decrease in activity as compared to FGF2 was observed. FGF2-PEG20k-FGF2 induced 13% and 18% less metabolic activity than FGF2 at 1 and 10 ng/mL, respectively. While we expected the longer linker to be less effective than the shorter linkers, decreased activity as compared to FGF2 was not expected. Taken together, these results supported our hypothesis that the optimal linker length was: R_F (35 Å) < inter-cysteine distance (70 Å) and L (160 Å) > the inter-cysteine distance. We then tested a greater number of concentrations (0.01, 0.05, 0.1, 1, 2, 5, 10 ng/mL) of FGF2 and FGF2-PEG2k-FGF2 on HDF metabolic activity, to assess the lowest effective concentration (Figure 3, b). Even at 0.01 ng/mL FGF2-PEG2k-FGF2 induced a 139% increase in HDF metabolic activity as compared to 105% for FGF2. Thus, FGF2-PEG2k-FGF2 exhibited superagonist activity in the metabolic activity of HDFs, even at extremely low concentrations, making it a good target for further study.

[00296] To further assess the viability of FGF2-PEG2k-FGF2, HDF migration was assessed in a scratch assay (Fig. 3C). A scratch was made through a confluent layer of growth factor starved cells followed by the application of sample and incubation for 18 hours. Incubation time was restricted to 18 hours to ensure that differences were not due to proliferation. FGF2-PEG2k-FGF2 induced a slight, but significantly greater migration at the lowest concentration tested (0.05 ng/mL) resulting in 131% increase in HDF migration compared to 117% for FGF2 alone. While both FGF2 and FGF2-PEG2k-FGF2 induced significantly greater migration as compared to blank at all higher concentrations, there was no significant

difference between the two. These results indicate that the FGF2 dimer does not greatly enhance migration of HDFs and that metabolic activity is more strongly influenced in this cell type.

[00297] Next the *in vitro* metabolic activity and migration of human umbilical vein endothelial cells (HUVECs) was assessed with FGF2-PEG2k-FGF2 and compared to free FGF2 (Fig. 4). Since the most drastic improvements in activity occurred at lower concentrations in HDFs, we focused our test on low concentrations for HUVEC metabolic activity. FGF2-PEG2k-FGF2 significantly increased metabolic activity compared to FGF2 at low concentrations and exhibited similar activity at 1 ng/mL (Figure 4a). Specifically, FGF2-PEG2k-FGF2 increased metabolic activity as compared to FGF2 by 28%, 21% and 21% at 0.01, 0.05 and 0.1 ng/mL, respectively. Compared to blank media, FGF2-PEG2k-FGF2 increased metabolic activity most effectively at 0.1 ng/mL, to 148% compared to blank media. HUVEC migration was investigated over a range of concentrations in a scratch-assay using the same protocol as described for HDFs (Figs. 4B and 4C). FGF2-PEG2k-FGF2 significantly increased migration into the scratch compared to FGF2 at 0.01, 0.05, 0.1, and 1 ng/mL. The greatest increase in migration was observed at 0.1 ng/mL, where HUVEC migration was increased by 163% compared to blank media for FGF2-PEG2k-FGF2 while for FGF2 it was increased by only 108%. These results further indicated that FGF2-PEG2k-FGF2 was a good candidate for additional study at low concentrations.

[00298] FGF2-PEG2k-FGF2 Increases Angiogenesis *In Vitro*

[00299] FGF2-PEG2k-FGF2 was also tested for increased angiogenesis. A rudimentary co-culture assay of HDFs and HUVECs was utilized[57, 58]. HDFs were first allowed to grow to confluency before HUVECs were added with the respective experimental sample. Sample solutions were refreshed at days 3 and 6. After 10 days the cells were fixed and cord-like structures stained for CD31. FGF2-PEG2k-FGF2 induced a significant increase in the number of nodes, cord-like structures, and total length of cord-like structures compared to FGF2 at low concentrations (0.1 and 0.5 ng/mL) and performed as well as FGF2 at higher concentrations (1, 5, and 10 ng/mL) (Fig. 5). Specifically, at 0.1 ng/mL, cells treated with FGF2-PEG2k-FGF2 increased the average number of nodes by 97, cord-like structures by 186, and increased the total length of cord-like structures to 9 times that of FGF2. At 0.5 ng/mL, FGF2-PEG2k-FGF2 increased the average number of nodes by 120, cord-like structures by 229, and the total length

of the cord-like structures was increased to 4 times that of FGF2. Thus at these low concentrations, FGF2-PEG2k-FGF2 improved all evaluated aspects of angiogenesis compared to free FGF2.

[00300] FGF2-PEG2k-FGF2 Increases Granulation Tissue and Blood Vessel Density in Wounded, Diabetic TallyHo Mice

[00301] Since FGF2-PEG2k-FGF2 significantly enhanced *in vitro* metabolic activity, migration, and co-culture cord-like structures, the efficacy of FGF2-PEG2k-FGF2 was investigated *in vivo* in a wounded mouse model. TallyHo/JngJ mice, which develop a type II diabetic phenotype, were wounded (8 mm punch biopsy) and then treated daily for 5 days with 0.02 µg of FGF2, FGF2-PEG2k-FGF2 or D-PBS control. The wound closure was measured at days 0, 1, 4, 7, 10, and 14. There was no significant difference in wound closure among the cohorts of mice (Figs. 6A and 6B) as is common for this animal model and wound size[73]. In contrast, the granulation tissue area and the blood vessel density in the wound bed were both significantly greater in the FGF2-PEG2k-FGF2 treated mice than either FGF2 treated mice ($p < 0.05$) or D-PBS control ($p < 0.01$) (Figs. 6C, 6D and 6E). These results indicate that the low concentration of FGF2-PEG2k-FGF2 was highly effective in promoting granulation tissue and angiogenesis in the wound bed.

[00302] Discussion

[00303] Chronic wounds represent a widespread and increasing socioeconomic problem. Depressed levels of growth factors and their receptors, including FGF2, are a main factor in non-healing wounds. Currently, advanced wound care therapies for non-healing ulcers include the use of collagen, growth factors, bioengineered skin, gene and stem cell therapy, silver products, ozone oxygen therapy, and negative pressure wound therapy[74]. We sought to improve upon the activity of FGF2 for its potential use alone or in combination with advanced wound care therapies. Our studies show that FGF2-PEG-FGF2, where the tether length is 2 kDa, represents a promising candidate for increasing the granulation tissue and blood vessel density during wound healing.

[00304] Utilizing available knowledge of FGF2 activity and its crystal structure with its receptor and theoretical investigation, we were able to rationally design a protein more active

than the native one in promoting fibroblast metabolic activity and endothelial cell metabolic activity and migration. Mutation of the protein was undertaken in order to obtain a single reactive cysteine for modification, spatially separate from residues required for receptor binding and protein activity. Homodimerization of the protein was achieved through Michael addition to bis-reactive linear PEG tethers, varying the length based on both the spatial restrictions informed by the active protein-receptor crystal structure, as well as taking in to account PEG flexibility and stretch. The best linker-length in this work was determined to be 2 kDa PEG. Analytic calculations based on a Gaussian coil model of the tether suggest that shortening the tether down to this distance should be beneficial. At such short distances, however, a reasonable model must account for steric interactions between the ligands and between the chain and the ligands – see Data in Brief [ref], for details. The simplicity of synthesis and purification as well as the effectiveness of the current model represents a viable candidate for further studies.

[00305] FGF2-PEG2k-FGF2 showed enhanced metabolic activity, migration, and formation of cord-like structures in an angiogenesis co-culture assay *in vitro*, as well as enhanced granulation tissue and blood vessel density in diabetic mice *in vivo*, all at low effective concentrations as compared to unmodified FGF2. Proliferation of HDF and HUVEC cells is often utilized as a first test to determine the *in vitro* efficacy of FGF2[14, 67-70] and the dimer performed far better than the native protein in the cell metabolic assay. Migration is another measure of the ability of the growth factor to potentially elicit a response in wound healing since migration of cells into a wound is an important factor. The dimer produced much higher migration of HUVEC cells in a scratch assay. In addition, the dimer produced a greater number of cord-like structures with longer lengths and more nodes than the native protein in a basic *in vitro* co-culture angiogenesis assay. These assays, although simple in nature, have limitations. Two-dimensional assays are useful to analyze the proliferation, migration, and migration of single cells[77]. However, these assays have been shown to have significant differences compared to three dimensional systems and therefore are not necessarily relevant to wound healing *in vivo*, which involve numerous processes occurring simultaneously[78]. The co-culture assay is simple to carry out, but does not provide information about tubular structures or perfusion through the structures as does the more sophisticated assays. Thus, *in vivo* experiments were carried out in a wounded diabetic mouse model. The results show that granulation tissue formation and blood vessel number is increased compared to FGF2. Since

granulation tissue results from fibroblast proliferation and blood vessel number increase is likely due to angiogenesis[79], the *in vivo* results confirm the *in vitro* ones.

[00306] A previous report demonstrated that a recombinantly engineered dimeric FGF2 had higher proliferation activity in smooth muscle cells and was able to partially rescue activity in a serum starved media at a slightly lower concentration than the monomer alone[45]. Our data is not directly comparable to this reference for several reasons. First a different cell type was used for the proliferation (cell metabolic activity) assay and for HUVEC cells we utilized much lower concentrations. In addition for the latter, although both examples were serum starved, we added the protein to EGF. So we see enhancement above normal activity, not a partial rescue of activity as in the case of the recombinant dimer. Yet in our case the approach is likely simpler because it is chemically dimerized rather than requiring a new recombinant synthesis and folding methodology.

[00307] While increased rate of closure was not observed *in vivo*, this is likely due to the extremely low concentrations tested and the animal model used (diabetic mice heal by contraction in addition to reepithelialization). This has been observed in other studies: For instance, Orgill and coworkers tested wound closure in C57BL/KsJ db+/db+ mice, and found no increase in wound closure when 1.5 cm² wounds were treated with 10 µg of PDGF each day for five days, but did see an increase in granulation tissue[73]. Hubbell and coworkers tested genetically modified superagonists of vascular endothelial growth factor (VEGF), PDGF, and bone morphogenetic protein-2 at low concentrations (200 ng/wound) and found that the modified growth factors increased granulation tissue formation compared to no treatment, while unmodified growth factors induced no increase at this concentration[80]. Therefore increased granulation tissue area and angiogenesis indicates better blood supply and a more fertile wound bed preparation, both favorable properties in wound healing. This could translate to faster wound healing in higher order animals, and this will be tested in future work.

[00308] This work does show that PEG dimerization of the FGF2 results in enhanced activity at low concentrations. Low concentrations are advantageous to avoid superphysiologic doses often used for growth factors, that can significantly increase the costs of a therapeutic [75]. However, growth factors, particularly FGF2, are known to be unstable [81, 82]. Although PEG does increase stability of proteins including FGF2[53, 54, 56], further stabilization of the protein through release from a heparinized matrix or by using a heparin mimicking polymer as a tether

would be advantageous. We recently reported a heparin mimicking polymer conjugate that stabilizes FGF2 to therapeutically relevant conditions, including elevated temperatures and treatment with proteolytic enzymes[83]. What was learned from that work could be readily combined with the dimer strategy reported herein to produce a superagonist growth factor effective at lower concentrations that is also stable to a variety of stressors, and this work is ongoing.

[00309] Conclusions

[00310] We have developed a superagonist FGF2 by site-specific dimerization utilizing 2 kDa PEG. The ideal linker length was determined empirically, with the most effective linker being that with a Flory's radius closest to the FGF2:FGFR tetramer inter-cysteine distance. FGF2-PEG2k-FGF2 exhibited superior activity compared to FGF2 for both metabolic activity and migration in a scratch assay in fibroblasts and human umbilical vein endothelial cells as well as exhibiting improved cord-like structures in a co-culture of the two cell lines, all at very low concentrations. Finally, FGF2-PEG2k-FGF2 induced increased granulation tissue and blood vessel density in the wound bed of diabetic mice. Overall, the data suggests that this FGF2 dimer is a promising candidate for increasing the quality of the wound bed in wound healing.

[00311] Analytical Techniques

[00312] NMR spectra were obtained on an Avance 500 MHz DRX spectrometer. Proton NMR spectra were acquired with a relaxation delay time of 10 seconds. MALDI-MS was performed on an Applied Biosystems Voyager DE-STR system and operated in linear mode with external calibration and sinapinic acid MALDI matrix. GPC was conducted on a Shimadzu HPLC system equipped with a refractive index detector RID-10A, one Polymer Laboratories PLgel guard column, and two Polymer Laboratories PLgel 5 μ m mixed D columns. LiBr (0.1 M) in dimethylformamide (DMF) at 40°C was used as an eluent (flow rate: 0.60 mL/min). Calibration was performed using near-monodisperse poly(methyl methacrylate) (PMMA) standards from Polymer Laboratories. Fast protein liquid chromatography (FPLC) was performed on a Bio-Rad BioLogic DuoFlow chromatography system equipped with a GE Healthcare Life Sciences Superdex 75 10/300 column. Protein concentration was determined using the Human FGF basic Duo ELISA kit from R&D Systems.

[00313] Mutagenesis

[00314] A pET29c(+)hFGF-2 plasmid from the Helmholtz Centre for Infection Research, Braunschweig, Germany, as used by Chen et. al., was graciously provided[84]. Plasmid sequence determination, plasmid mutagenesis, and protein expression were all performed at the UCLA PETC (University of California, Los Angeles, Protein Expression Technology Center, UCLA/DOE Institute for Genomics and Proteomics).

[00315] Mutations were introduced into the plasmid by PCR-amplification with Pfu Turbo polymerase (Agilent, Santa Clara, CA) using the Quikchange protocol (Agilent, Santa Clara, CA) and mutagenic primers (Valuegene, San Diego, CA) incorporating the specific mutations (C78S). PCR products were treated with DpnI enzyme (New England Biolabs, Ipswich, MA) to digest the parental plasmid template, transformed into competent *E. coli* DH5alpha cells (New England Biolabs, Ipswich, MA) and plated on LB plates containing kanamycin. Putative positive plasmids were sequenced (South Plainfield, Piscataway, NJ) to confirm that the proper mutation had been introduced.

[00316] Based on the DNA sequence of the plasmid, the amino acid sequence of the mutant (with cysteines in bold and the mutated amino acid in red) is:

MAAGSITTLP ALPEDGGSGA FPPGHFKDPK RLYCKNGGFF LRIHPDGRVD
GVREKSDPHI KLQLQAEERG VVSIKGVSAN RYLAMKEDGR LLASKCVTDE
CFFFERLESN NYNTYRSRKY TSWYVALKRT GQYKLGSKTG PGQKAILFLP MSAKS

[00317] Protein Expression and Purification:

[00318] Protein expression in *E. coli* BL21(DE3) was performed as previously described[1] using a BioEngineering NLF22 fermentor on a scale of 12 liters. After lysis, the supernatant was purified with a sartobind S75 absorber (cationic), a heparin column (5 mL) and a sartobind Q75 absorber (anionic) to remove endotoxin. The protein was eluted from the heparin column using a salt gradient and fractions, which eluted at 2 and 2.5 M NaCl were collected and concentrated for further analysis and use. The final yield of protein was 0.47 mg purified protein per 1 gram of cell pellet. The final endotoxin level was determined using the ToxinSensor Chromatogenic LAL Endotoxin Assay Kit from Genscript to be ≤ 0.002 EU/ μ g. According to

European Pharmacopoeia, $< 5 \text{ EU/kg} \cdot \text{hr}$ is accepted for clinical application. The purified mutant protein contained 3 cysteine residues, verified by Ellman's assay.

[00319] After protein expression and purification, the mutant protein FGF2(C78S) was digested with trypsin followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using an Applied Biosystems Q-STAR Elite Quadropole-TOF Hybrid LC-MS System at the UCLA Molecular Instrumentation Center (MIC). The peptide was identified with 78% sequence coverage and a Mascot sequence search score of 54, which is considered significant.

[00320] Modification of poly(ethylene glycol) and poly(ethylene glycol methyl ether) with vinyl sulfone

[00321] The end-group alcohol(s) of PEG (2, 6, and 20 kDa) or mPEG (2, 5, and 20 kDa) were modified with VS, according to literature procedures[85, 86], resulting in bis (VS-PEG-VS) and mono (mPEG-VS) reactive PEGs, respectively. As an example, the synthesis of 2 kDa VS-PEG-VS is described below.

[00322] Briefly, sodium hydride (12.5 mmol, 25 eq.) was weighed into an oven-dried round-bottom flask under inert atmosphere. The flask was cooled in an ice-bath, and 100 mL of dry dichloromethane (DCM) was added. PEG, 2 kDa, (0.5 mmol, 1 eq.), previously freeze-dried from benzene to remove water, was dissolved in 50 mL dry DCM. The PEG-alkoxide was then added dropwise to the cooled slurry of sodium hydride and DCM, to prevent gelation of the PEG. It was imperative that the solution was dilute (3 mM for PEG in this case) to prevent gelation, and this was especially important for smaller PEGs (2 and 6 kDa). After hydrogen evolution (30 minutes stirring), divinyl sulfone (30 mmol, 60 eq.) was added quickly. The solution was allowed to come to room temperature and stir for 3 days under argon. The solution was then neutralized with acetic acid, concentrated, and purified by precipitation into cold ether five times.

[00323] $^1\text{H-NMR}$ (CDCl_3) was used to approximate the percentage end-group modification by comparing the integration of the new vinylic protons (6.0, 6.3, and 6.7 ppm, 1H) to the main-chain methylene protons (3.4-3.9 ppm) (Figs. 8-13). For VS-PEG2k-VS, 2000 Da / 44 Da per $(-\text{CH}_2\text{CH}_2\text{O}-)$ repeat = 45.45 repeats $\times 4 \text{ H} / \text{repeat} = 181.8 \text{ H's}$ in the backbone at 3.4-3.9 ppm (Fig. 11). The integration for the vinylic H at 6.3 ppm is set to 1. For monofunctionalized mPEG-VSs, percent functionalization was calculated by:

- [00324]** Integration from 3.4-3.9 ppm (expected / observed) *100
- [00325]** For bisfunctionalized VS-PEG-VS, percent functionalization was calculated by:
- [00326]** Integration from 3.4-3.9 ppm (expected / (observed * 2)) * 100
- [00327]** % Functionalization per polymer by ^1H -NMR:
- [00328]** mPEG2k-VS: $181.8/181.46*100 = 100\%$ functionalization
- [00329]** mPEG5k-VS: $454.5/536.81*100=85\%$ functionalization
- [00330]** mPEG20k-VS: $1818.2/3088.95*100 = 59\%$ functionalization
- [00331]** VS-PEG2k-VS: $181.8/(94.43*2)*100 = 96\%$ bis-functionalization
- [00332]** VS-PEG6k-VS: $545.5/(330.01*2)*100 = 83\%$ bis-functionalization
- [00333]** VS-PEG20k-VS: $1818.2/(1071.67*2)*100 = 85\%$ bis-functionalization

[00334] Conjugation of FGF2 to mPEG-VS and VS-PEG-VS

[00335] Each polymer, mPEG (2, 5, or 20 kDa) or VS-PEG-VS (2, 6, or 20 kDa) was dissolved in 100 mM Tris, pH 9, 5 mM EDTA. 0.5 equivalents of each polymer were added into a separate tube containing 1.3 mg (77 nmol) FGF2 in an Eppendorf Lobind tube, so that the final concentration of protein was 73 μM .

[00336] The solutions were placed on a rotating plate at room temperature for 12 hours. The solutions were then concentrated / solvent exchanged into D-PBS + 10 mM DTT using Centriprep MWCO 10,000 for 2, 5, and 6 kDa PEG conjugates, and using MWCO 30,000 for 20 kDa PEG conjugates, at 6 x 10 min cycles. 16.1 rcf (13.2 rpm). Ultracentrifugation resulted in the removal of unreacted FGF2 from mPEG20k-FGF2 and removal of all unreacted FGF2 and monoconjugate from FGF2-PEG20k-FGF2. All other conjugates required purification by fast protein liquid chromatography (FPLC).

[00337] Each vial was concentrated to a final volume of 200 μL , and 100 μL was loaded onto a size exclusion column (Superdex 75 10/300 GL) at 0.4 mL/min in D-PBS, 10 mM DTT, pH 7.4. Fractions were collected every 0.25 mL, for a total of 15 mL. After concentration by ultracentrifugation, SDS-PAGE gel electrophoresis was run to assess which fractions contained the respective desired product. Fractions containing the desired conjugate were then combined, concentrated by ultracentrifugation, and buffer exchanged into D-PBS ($-\text{Mg}^{2+}$ $-\text{Ca}^{2+}$) for use in cell or animal studies. The fractions in which each product was found is: mPEG2k-FGF2 (13.5-

14 mL), FGF2-PEG2k-FGF2 (11-11.25 mL), mPEG5k-FGF2 (11.5-12 mL), FGF2-PEG6k-FGF2 (10-10.5 mL), FGF2-PEG20k-FGF2 (9-9.75 mL)

[00338] Western Blot

[00339] All reagents for western blot were purchased from Bio-Rad except as noted. 4-20 ng of FGF2 or conjugate was diluted in Laemmli buffer, 0.65 M DTT (Sigma) and heated to 95 °C for 6 minutes before loading onto a mini-ProTEAN TGX any-Kd gel. Precision Plus Protein™ WesternC™ Standards (6 µL) were loaded into the first lane of the gel. The gel was run in Tris/Glycine/SDS buffer at 200 V for 28 minutes, then transferred onto nitrocellulose in Tris/Glycine buffer at 100 V for 2 hours. The nitrocellulose membrane was blocked overnight with Tris-buffered Saline Tween 20 (TBST) + 5% fat-free dry milk, 1% BSA (Sigma) (blocking buffer). The membrane was then incubated with the primary antibody (rabbit-antiFGF2, “FGF Antibody (Center)”, Abgent) diluted 1:100 in blocking buffer for 16 hours at 4 °C on a rotating plate. The membrane was then washed with TBST 3 x 5 minutes, and incubated with secondary antibody (goat anti-rabbit IgG-HRP conjugate) diluted 1:1000 in blocking buffer as well as the secondary antibody for the protein standards (Strep-Tactin HRP conjugate) diluted 1:10,000 at room temperature on a rotating plate for 30 minutes. The membrane was then washed with TBST 3 x 5 minutes, and incubated with Clarity™ Western ECL Substrate for 5 minutes. Chemiluminescence was imaged on an Alpha Innotec FluorChem® FC2 Imaging System at the UCLA-DOE Biochemistry Instrumentation Core Facility.

[00340] Details of Co-Culture Assay Counting.

[00341] Number and length of cord-like structures were manually counted as previously reported[87, 88]. Images were captured using a low magnification and analysis was performed using ImageJ software by NIH. Cord-like structure length was measured by identifying each cord-like structure and manually drawing a line in the software along the cords. The length of the lines was measured by the software in pixels. Cord and node number were counted using a cell counter function in the software and were measured at every junction of two or more cord like structures. An example of cord length and node identification is shown below.

[00342] Other embodiments and uses of the invention will be apparent to those skilled in the art from consideration from the specification and practice of the invention disclosed herein.

All references cited herein for any reason, including all journal citations and U.S./foreign patents and patent applications, are specifically and entirely incorporated herein by reference. It is understood that the invention is not confined to the specific reagents, formulations, reaction conditions, etc., herein illustrated and described, but embraces such modified forms thereof as come within the scope of the following claims.

References

- [1] Brem H, Tomic-Canic M. Cellular and molecular basis of wound healing in diabetes. *J. Clin. Invest.* 2007;117:1219-22.
- [2] Jiang L, Dai Y, Cui F, Pan Y, Zhang H, Xiao J, et al. Expression of cytokines, growth factors and apoptosis-related signal molecules in chronic pressure ulcer wounds healing. *Spinal Cord.* 2014;52:145-51.
- [3] Demidova-Rice TN, Hamblin MR, Herman IM. Acute and impaired wound healing: pathophysiology and current methods for drug delivery, part 1: Normal and chronic wounds: biology, causes, and approaches to care. *Adv. Skin Wound Care.* 2012;25:304-14.
- [4] Shaw JE, Sicree RA, Zimmet PZ. Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Res. Clin. Prac.* 2010;87:4-14.
- [5] Bikfalvi A, Klein S, Pintucci G, Rifkin DB. Biological roles of fibroblast growth factor-2. *Endocr. Rev.* 1997;18:26-45.
- [6] Akita S, Akino K, Hirano A. Basic fibroblast growth factor in scarless wound healing. *Adv. Wound Care.* 2013;2:44-9.
- [7] Bhang SH, Kim JH, Yang HS, La WG, Lee TJ, Sun AY, et al. Combined delivery of heme oxygenase-1 gene and fibroblast growth factor-2 protein for therapeutic angiogenesis. *Biomaterials.* 2009;30:6247-56.
- [8] Chen WC, Lee BG, Park DW, Kim K, Chu H, Kim K, et al. Controlled dual delivery of fibroblast growth factor-2 and Interleukin-10 by heparin-based coacervate synergistically enhances ischemic heart repair. *Biomaterials.* 2015;72:138-51.
- [9] Cui Y, Lu C, Meng D, Xiao Z, Hou X, Ding W, et al. Collagen scaffolds modified with CNTF and bFGF promote facial nerve regeneration in minipigs. *Biomaterials.* 2014;35:7819-27.
- [10] Nillesen ST, Geutjes PJ, Wismans R, Schalkwijk J, Daamen WF, van Kuppevelt TH. Increased angiogenesis and blood vessel maturation in acellular collagen-heparin scaffolds containing both FGF2 and VEGF. *Biomaterials.* 2007;28:1123-31.
- [11] Lederman RJ, Mendelsohn FO, Anderson RD, Saucedo JF, Tenaglia AN, Hermiller JB, et al. Therapeutic angiogenesis with recombinant fibroblast growth factor-2 for intermittent claudication (the TRAFFIC study): A randomised trial. *Lancet.* 2002;359:2053-8.

[12] Simons M, Annex BH, Laham RJ, Kleiman N, Henry T, Dauerman H, et al. Pharmacological treatment of coronary artery disease with recombinant fibroblast growth factor-2: double-blind, randomized, controlled clinical trial. *Circulation*. 2002;105:788-93.

[13] Olympus Biotech Corporation. The TRAfermin in neuropathic diabetic foot ulcer study - southern Europe the TRANS-south study. In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2000-[cited 2015 Dec 04]. Available from: <https://www.clinicaltrials.gov/ct2/show/NCT01217463> NLM Identifier: NCT0121746

[14] Grazul-Bilska AT, Luthra G, Reynolds LP, Bilski JJ, Johnson ML, Adbullah SA, et al. Effects of basic fibroblast growth factor (FGF-2) on proliferation of human skin fibroblasts in type II diabetes mellitus. *Exp. Clin. Endocrinol. Diabetes*. 2002;110:176-81.

[15] Aviles RJ, Annex BH, Lederman RJ. Testing clinical therapeutic angiogenesis using basic fibroblast growth factor (FGF-2). *Br. J. Pharmacol.* 2003;140:637-46.

[16] Turner N, Grose R. Fibroblast growth factor signalling: from development to cancer. *Nat. Rev. Cancer*. 2010;10:116-29.

[17] Benjamin H, Chai L, Findeis MA, Goodwin W, Hundal A, Israel DI, et al. (Praecis Pharmaceuticals Inc., USA). Peptide compounds useful for modulating FGF receptor activity. WO Patent PCT/US97/21070, Publication date May 22, 1998.

[18] Ballinger MD, Shyamala V, Forrest LD, Deuter-Reinhard M, Doyle LV, Wang JX, et al. Semirational design of a potent, artificial agonist of fibroblast growth factor receptors. *Nat. Biotechnol.* 1999;17:1199-204.

[19] Tassi E, Al-Attar A, Aigner A, Swift MR, McDonnell K, Karavanov A, et al. Enhancement of fibroblast growth factor (FGF) activity by an FGF-binding protein. *J. Biol. Chem.* 2001;276:40247-53.

[20] Yayon A, Rom E, Chumakov I, Blumenstein S. (Prochon Biotech Ltd., USA). FGF-2 variants having N-terminal deletions and increased receptor selectivity and uses thereof. US Patent Number 12/443,638, Publication date February 24, 2015.

[21] Springer BA, Pantoliano MW, Sharp CM. (3-Dimensional Pharmaceuticals, inc., USA). Analogs of human basic fibroblast growth factor mutated at one or more of the positions glutamate 89, aspartate 101 or leucine 137. US Patent Number 09/220,077, Publication date August 14, 2001.

[22] Bogin O, Yayon A. (ProChon Biotech Ltd., USA). FGF variants and methods for use thereof. WO Patent Number PCT/IL2003/000379, Publication date November 20, 2003.

[23] Davis JC, Venkataraman G, Shriver Z, Raj PA, Sasisekharan R. Oligomeric self-association of basic fibroblast growth factor in the absence of heparin-like glycosaminoglycans. *Biochem. J.* 1999;341:613-20.

[24] Venkataraman G, Shriver Z, Davis JC, Sasisekharan R. Fibroblast growth factors 1 and 2 are distinct in oligomerization in the presence of heparin-like glycosaminoglycans. *Proc. Natl. Acad. Sci. U.S.A.* 1999;96:1892-7.

[25] Platonova N, Miquel G, Chiu L-Y, Taouji S, Moroni E, Colombo G, et al. Dimerization capacities of FGF2 purified with or without heparin-affinity chromatography. *PLoS One.* 2014;9:e110055.

[26] Rudenko O, Tkach V, Berezin V, Bock E. Effects of FGF receptor peptide agonists on animal behavior under normal and pathological conditions. *Neurosci. Res.* 2010;68:35-43.

[27] Li S, Christensen C, Kohler LB, Kiselyov VV, Berezin V, Bock E. Agonists of fibroblast growth factor receptor induce neurite outgrowth and survival of cerebellar granule neurons. *Dev. Neurobiol.* 2009;69:837-54.

[28] Plotnikov AN, Schlessinger J, Hubbard SR, Mohammadi M. Structural basis for FGF receptor dimerization and activation. *Cell.* 1999;98:641-50.

[29] Schlessinger J, Plotnikov AN, Ibrahimi OA, Eliseenkova AV, Yeh BK, Yayon A, et al. Crystal structure of a ternary FGF-FGFR-heparin complex reveals a dual role for heparin in FGFR binding and dimerization. *Mol. Cell.* 2000;6:743-50.

[30] Marianayagam NJ, Sunde M, Matthews JM. The power of two: Protein dimerization in biology. *Trends Biochem. Sci.* 2004;29:618-25.

[31] Matthews J, Sunde M. Dimers, Oligomers, Everywhere. Matthews J, editor. In: *Protein dimerization and oligomerization in biology*: Springer New York; 2012. p. 1-18.

[32] Krueger AT, Kroll C, Sanchez E, Griffith LG, Imperiali B. Tailoring chimeric ligands for studying and biasing ErbB receptor family interactions. *Angew. Chem. Int. Ed.* 2014;53:2662-6.

[33] Kuhl PR, Griffith-Cima LG. Tethered epidermal growth factor as a paradigm for growth factor-induced stimulation from the solid phase. *Nat. Med.* 1996;2:1022-27.

[34] Thomas TP, Shukla R, Kotlyar A, Liang B, Ye JY, Norris TB, et al. Dendrimer-epidermal growth factor conjugate displays superagonist activity. *Biomacromolecules*. 2008;9:603-9.

[35] Jeppesen C, Wong JY, Kuhl TL, Israelachvili JN, Mullah N, Zalipsky S, et al. Impact of polymer tether length on multiple ligand-receptor bond formation. *Science*. 2001;293:465-8.

[36] Kramer RH, Karpen JW. Spanning binding sites on allosteric proteins with polymer-linked ligand dimers. *Nature*. 1998;395:710-13.

[37] Green NS, Reisler E, Houk KN. Quantitative evaluation of the lengths of homobifunctional protein cross-linking reagents used as molecular rulers. *Protein Sci*. 2001;10:1293-304.

[38] Ornitz DM, Yayon A, Flanagan JG, Svahn CM, Levi E, Leder P. Heparin is required for cell-free binding of basic fibroblast growth-factor to a soluble receptor and for mitogenesis in whole cells. *Mol. Cell. Biol*. 1992;12:240-7.

[39] Ishihara M, Ono K. Structure and function of heparin and heparan sulfate; Heparinoid library and modification of FGF-activities. *Trends in Glycosci. Glyc*. 1998;10:223-33.

[40] Ishihara M, Tyrrell DJ, Stauber GB, Brown S, Cousens LS, Stack RJ. Preparation of affinity-fractionated, heparin-derived oligosaccharides and their effects on selected biological-activities mediated by basic fibroblast growth factor. *J. Biol. Chem*. 1993;268:4675-83.

[41] Herr AB, Ornitz DM, Sasisekharan R, Venkataraman G, Waksman G. Heparin-induced self-association of fibroblast growth factor-2 - Evidence for two oligomerization processes. *J. Biol. Chem*. 1997;272:16382-9.

[42] Moy FJ, Safran M, Seddon AP, Kitchen D, Bohlen P, Aviezer D, et al. Properly oriented heparin-decasaccharide-induced dimers are the biologically active form of basic fibroblast growth factor. *Biochem*. 1997;36:4782-91.

[43] Waksman G, Herr AB. New insights into heparin-induced FGF oligomerization. *Nat. Struct. Biol*. 1998;5:527-30.

[44] Safran M, Eisenstein M, Aviezer D, Yayon A. Oligomerization reduces heparin affinity but enhances receptor binding of fibroblast growth factor 2. *Biochem. J*. 2000;345:107-13.

- [45] Kwan C-P, Venkataraman G, Shriver Z, Raman R, Liu D, Qi Y, et al. Probing fibroblast growth factor dimerization and role of heparin-like glycosaminoglycans in modulating dimerization and signaling. *J. Biol. Chem.* 2001;276:23421-9.
- [46] Pelegri-O'Day EM, Lin E-W, Maynard HD. Therapeutic protein-polymer conjugates: advancing beyond PEGylation. *J. Am. Chem. Soc.* 2014;136:14323-32.
- [47] Spicer CD, Davis BG. Selective chemical protein modification. *Nat. Commun.* 2014;5: 4740.
- [48] Kang CE, Tator CH, Shoichet MS. Poly(ethylene glycol) modification enhances penetration of fibroblast growth factor 2 to injured spinal cord tissue from an intrathecal delivery system. *J. Controlled Release.* 2010;144:25-31.
- [49] Lappi DA, Matsunami R, Martineau D, Baird A. Reducing the heterogeneity of chemically conjugated targeted toxins: homogeneous basic FGF-saporin. *Anal. Biochem.* 1993;212:446-51.
- [50] Alconcel SNS, Baas AS, Maynard HD. FDA-approved poly(ethylene glycol)-protein conjugate drugs. *Polym. Chem.* 2011;2:1442-8.
- [51] Knop K, Hoogenboom R, Fischer D, Schubert US. Poly(ethylene glycol) in Drug delivery: pros and cons as well as potential alternatives. *Angew. Chem. Int. Ed.* 2010;49:6288-308.
- [52] Huang Z, Ye C, Liu Z, Wang X, Chen H, Liu Y, et al. Solid-phase N-terminus PEGylation of recombinant human fibroblast growth factor 2 on heparin-sepharose column. *Bioconjugate Chem.* 2012;23:740-50.
- [53] Wu XP, Li X, Zeng YY, Zheng Q, Wu S. Site-directed PEGylation of human basic fibroblast growth factor. *Protein Express. Purif.* 2006;48:24-7.
- [54] Hadadian SS, D. N.; Mirzahoseini, H.; Shokrgozar, M. A.; Bouzari, S.; Sepahi, M. Stability and biological activity evaluations of PEGylated human basic fibroblast growth factor. *Adv. Biomed. Res.* 2015;4:176.
- [55] Wu X, Liu X, Xiao Y, Huang Z, Xiao J, Lin S, et al. Purification and modification by polyethylene glycol of a new human basic fibroblast growth factor mutant-hbFGF(Ser25,87,92). *J. Chromatogr. A.* 2007;1161:51-5.

[56] Zhu G, Chen G, Shi L, Feng J, Wang Y, Ye C, et al. PEGylated rhFGF-2 conveys long-term neuroprotection and improves neuronal function in a rat model of Parkinson's Disease. *Mol. Neurobiol.* 2015;51:32-42.

[57] Hetheridge C, Mavria G, Mellor H. Uses of the in vitro endothelial–fibroblast organotypic co-culture assay in angiogenesis research. *Biomedical Soc. Trans.* 2011;39: 1597-600.

[58] Bishop ET, Bell GT, Bloor S, Broom IJ, Hendry NK, Wheatley DN. An in vitro model of angiogenesis: Basic features. *Angiogenesis.* 1999;3:335-44.

[59] Avitabile S, Odorisio T, Madonna S, Eyerich S, Guerra L, Eyerich K, et al. Interleukin-22 promotes wound repair in diabetes by improving keratinocyte pro-healing functions. *J. Invest. Dermatol.* 2015;135: 2862-70.

[60] Chen R, John J, Lavrentieva A, Müller S, Tomala M, Zhao Y, et al. Cytokine production using membrane adsorbers: Human basic fibroblast growth factor produced by *Escherichia coli*. *Eng. Life Sci.* 2012;12:29-38.

[61] Linegar KL, Adeniran AE, Kostko AF, Anisimov MA. Hydrodynamic radius of polyethylene glycol in solution obtained by dynamic light scattering. *Colloid J.* 2010;72:279-81.

[62] Emilsson G, Schoch RL, Feuz L, Höök F, Lim RY, Dahlin AB. Strongly stretched protein resistant poly(ethylene glycol) brushes prepared by grafting-to. *ACS Appl. Mater. Interfaces.* 2015;7:7505-15.

[63] Lu Y, Harding SE, Turner A, Smith B, Athwal DS, Grossmann JG, et al. Effect of PEGylation on the solution conformation of antibody fragments. *J. Pharm. Sci.* 2008;97:2062-79.

[64] Morpurgo M, Veronese FM, Kachensky D, Harris JM. Preparation and characterization of poly(ethylene glycol) vinyl sulfone. *Bioconjugate Chem.* 1996;7:363-8.

[65] Vemuri S, Beylin I, Sluzky V, Stratton P, Eberlein G, Wang YJ. The stability of bFGF against thermal denaturation. *J. Pharm. Pharmacol.* 1994;46:481-6.

[66] Lutolf MP, Hubbell JA. Synthesis and physicochemical characterization of end-linked poly(ethylene glycol)-co-peptide hydrogels formed by Michael-type addition. *Biomacromolecules.* 2003;4:713-22.

[67] Singer AJ, Clark RAF. Cutaneous wound healing. *N. Engl. J. Med.* 1999;341:738-46.

[68] Makino T, Jinnin M, Muchemwa FC, Fukushima S, Kogushi-Nishi H, Moriya C, et al. Basic fibroblast growth factor stimulates the proliferation of human dermal fibroblasts via the ERK1/2 and JNK pathways. *Br. J. Dermatol.* 2010;162:717-23.

[69] van Horssen R, Galjart N, Rens JAP, Eggermont AMM, ten Hagen TLM. Differential effects of matrix and growth factors on endothelial and fibroblast motility: Application of a modified cell migration assay. *J. Cell. Biochem.* 2006;99:1536-52.

[70] Giraux J-L, Matou S, Bros A, Tapon-Bretonnière J, Letourneur D, Fischer A-M. Modulation of human endothelial cell proliferation and migration by fucoidan and heparin. *Eur. J. Cell Biol.* 1998;77:352-9.

[71] Mohammadi M, Olsen SK, Goetz R. A protein canyon in the FGF-FGF receptor dimer selects from an a la carte menu of heparan sulfate motifs. *Curr. Opin. Structural Biol.* 2005;15:506-16.

[72] Mohammadi M, Olsen SK, Ibrahimi OA. Structural basis for fibroblast growth factor receptor activation. *Cytokine Growth Factor Rev.* 2005;16:107-37.

[73] Chan RK, Liu PH, Pietramaggiore G, Ibrahim SI, Hechtman HB, Orgill DP. Effect of recombinant platelet-derived growth factor (Regranex®) on wound closure in genetically diabetic mice. *J. Burn Care Res.* 2006;27:202-5.

[74] Yazdanpanah L, Nasiri M, Adarvishi S. Literature review on the management of diabetic foot ulcer. *World J. Diabetes.* 2015;6:37-53.

[75] Greer N, Foman N, Dorrian J, et al. Advanced wound care therapies for non-healing diabetic, venous, and arterial ulcers: A Systemic Review. Department of Veterans Affairs, Washington DC, November 2012.

[76] Papanas D, Maltezos E. Benefit-risk Assessment of becaplermin in the treatment of diabetic foot ulcers. *Drug Saf.* 2010;33:455-61.

[77] Safferling K, Sutterlin T, Westphal K, Ernst C, Breuhahn K, James M, et al. Wound healing revised: a novel reepithelialization mechanism revealed by in vitro and in silico models. *J. Cell Biol.* 2013;203:691-709.

[78] Ali N, Hosseini M, Vainio S, Taieb A, Cario-Andre M, Rezvani HR. Skin equivalents: skin from reconstructions as models to study skin development and diseases. *Br. J. Dermatol.* 2015;173:391-403.

[79] Häkkinen L, Larjava H, Koivisto L. Granulation tissue formation and remodeling. Oral Wound Healing: John Wiley & Sons, Ltd.; 2012. p. 125-73.

[80] Martino MM, Briquez PS, Güç E, Tortelli F, Kilarski WW, Metzger S, et al. Growth factors engineered for super-affinity to the extracellular matrix enhance tissue healing. Science. 2014;343:885-8.

[81] Edelman ER, Mathiowitz E, Langer R, Klagsbrun M. Controlled and modulated release of basic fibroblast growth-factor. Biomaterials. 1991;12:619-26.

[82] Whalen GF, Shing Y, Folkman J. The fate of intravenously administered bFGF and the effect of heparin. Growth Factors. 1989;1:156-64.

[83] Nguyen TH, Kim SH, Decker CG, Wong DY, Loo JA, Maynard HD. A heparin-mimicking polymer conjugate stabilizes basic fibroblast growth factor. Nat. Chem. 2013;5:221-7.

[84] R. Chen R, J. John A. Lavrentieva, S. Müller M. Tomala, Y. Zhao, et al. Cytokine production using membrane adsorbers: Human basic fibroblast growth factor produced by Escherichia coli. Eng. Life Sci. 12 (2012) 29-38.

[85] M. P. Lutolf, J. A. Hubbell, Synthesis and physicochemical characterization of end-linked poly(ethylene glycol)-co-peptide hydrogels formed by Michael-type addition. Biomacromolecules 4 (2003) 713-22.

[86] M. Morpurgo, F. M. Veronese, D. Kachensky, J. M. Harris, Preparation and characterization of poly(ethylene glycol) vinyl sulfone. Bioconjugate Chem. 7 (1996) 363-8.

[87] D. Donovan, N. J. Brown, E. T. Bishop, C. E. Lewis, Comparison of three *in vitro* human 'angiogenesis' assays with capillaries formed *in vivo*. Angiogenesis 4 (2001) 113-21.

[88] C. Hetheridge, G. Mavria, H. Mellor, Uses of the *in vitro* endothelial-fibroblast organotypic co-culture assay in angiogenesis research. Biochem. Soc. Trans. 39 (2011) 1597-600.

CLAIMS

WE CLAIM:

1. An FGF multimer, the FGF multimer comprising:
two or more FGF monomers, each of the two or more FGF monomers comprising one linking amino acid residue; and
a linker molecule, wherein the linker molecule links the two or more FGF monomers through the linking amino acid residues, and wherein the linker molecule is a polymer.
2. The FGF multimer of claim 1, wherein the FGF multimer is an FGF dimer.
3. The FGF multimer of claim 1, wherein the FGF monomers are naturally existing FGF2 monomers or modified FGF2 monomers.
4. The FGF multimer of claim 3, wherein the FGF monomers are modified FGF2 monomers.
5. The FGF multimer of claim 4, wherein the modified FGF2 monomers comprise a mutation of a cysteine.
6. The FGF multimer of claim 5, wherein the modified FGF2 monomers comprise a mutation of C78S.
7. The FGF multimer of claim 1, wherein the linking amino acid residue is a cysteine.
8. The FGF multimer of claim 1, wherein the linking amino acid residue is Cys96.
9. The FGF multimer of claim 1, wherein the linker molecule is a poly(ethylene glycol) (PEG).

10. The FGF multimer of claim 9, wherein the PEG is a PEG with its molecular weight (MW; in g/mol) in the range of about 0.1kDa to about 20kDa.
11. The FGF multimer of claim 109, wherein the PEG is a PEG with its molecular weight about 2 kDa.
12. A pharmaceutical composition for treating or healing chronic wound, comprising an FGF multimer comprising:
 - two or more FGF monomers, each of the two or more FGF monomers comprising one linking amino acid residue; and
 - a linker molecule, wherein the linker molecule covalently links the two or more FGF monomers through the linking amino acid residues, and wherein the linker molecule is a polymer.
13. The pharmaceutical composition of claim 12, wherein the FGF multimer is an FGF dimer.
14. The pharmaceutical composition of claim 12, wherein the pharmaceutical composition further comprises one stabilizing agent.
15. The pharmaceutical composition of claim 12, wherein the pharmaceutical composition further comprises one delivering agent.
16. A method for treating or healing a wound in a subject, the method comprising:
 - administering to the subject a therapeutically effective amount of a composition, the composition comprising
 - an FGF multimer comprising:
 - two or more FGF monomers, each of the two or more FGF monomers comprising one linking amino acid residue; and

a linker molecule, wherein the linker molecule covalently links the two or more FGF monomers through the linking amino acid residues, and wherein the linker molecule is a polymer,
wherein the wound is treated or healed.

17. The method of claim 16, wherein the FGF multimer is an FGF dimer.
18. The method of claim 16, wherein the wound is a chronic wound.
19. The method of claim 16, wherein the FGF monomers are modified FGF2 monomers.
20. The method of claim 16, wherein the linker molecule is a PEG with its molecular weight about 2 kDa.
21. The method of claim 16, wherein the composition further comprises at least one stabilizing agent.
22. The method of claim 16, wherein the composition further comprises at least one delivering agent.
23. The method of claim 16, the method further comprising at least one advanced wound care therapy selected from the group consisting of the use of collagen, growth factors, bioengineered skin, gene and stem cell therapy, silver products, ozone oxygen therapy, and negative pressure wound therapy.
24. A kit for treating or healing a wound in a patient, the kit comprising:
a composition comprising:
an FGF multimer comprising:
two or more FGF monomers, each of the two or more FGF monomers comprising one linking amino acid residue; and

a linker molecule, wherein the linker molecule covalently links the two or more FGF monomers through the linking amino acid residues, and wherein the linker molecule is a polymer.

25. The kit of claim 24, wherein the FGF multimer is an FGF dimer.
26. The kit of claim 21, wherein the kit further comprises another composition for sanitizing the wound before the treatment.
27. A medical device for treating or healing a wound in a patient, comprising:
 - a substrate comprising
 - an FGF multimer comprising:
 - two or more FGF monomers, each of the two or more FGF monomers comprising one linking amino acid residue; and
 - a linker molecule, wherein the linker molecule covalently links the two or more FGF monomers through the linking amino acid residues, and wherein the linker molecule is a polymer; and
 - wound covering means for releaseably covering the wound of the patient,
 - wherein after the wound of the patient is covered, the substrate controllably releases the FGF multimer into the wound.
28. The medical device of claim 27, wherein the FGF multimer is an FGF dimer.
29. The medical device of claim 27, wherein the FGF multimer is an FGF2 dimer.



Fig. 1

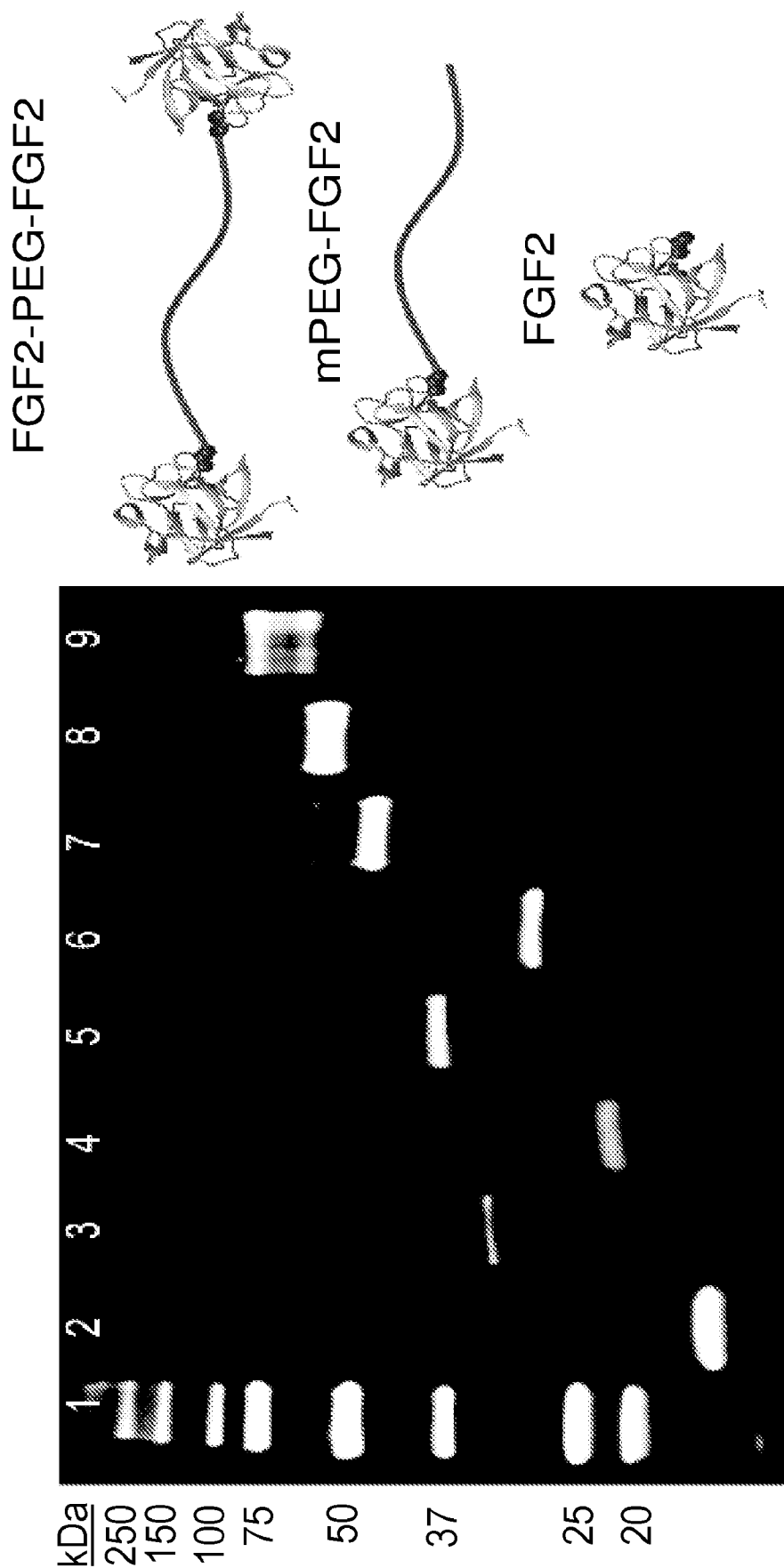


Fig. 2

Fig. 3 A.

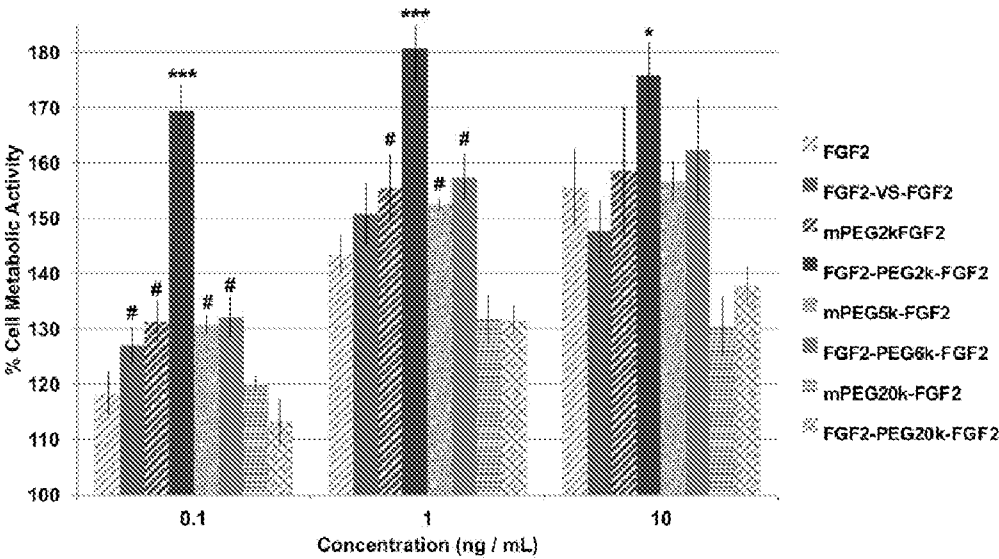


Fig. 3 B.

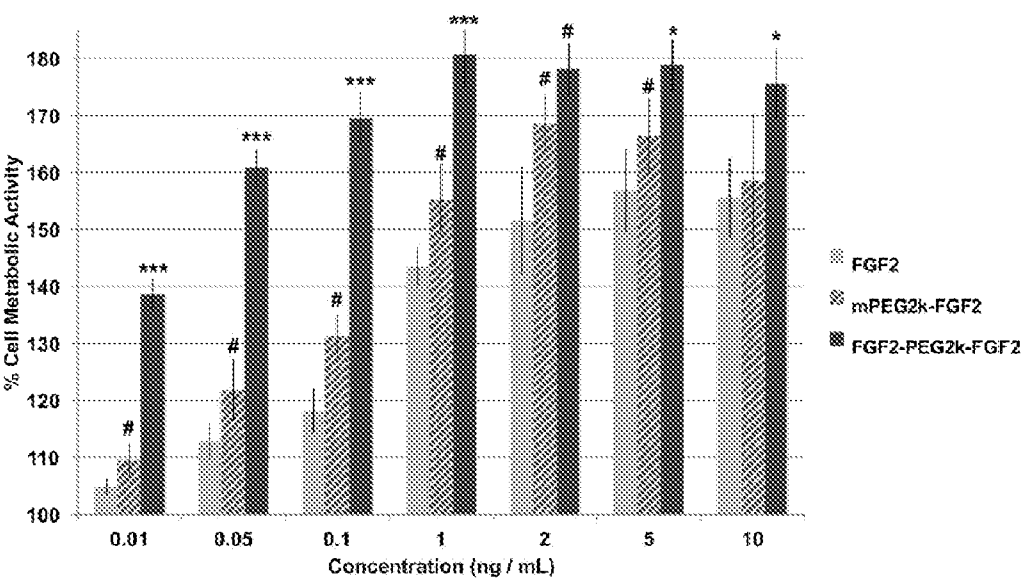


Fig. 3 C.

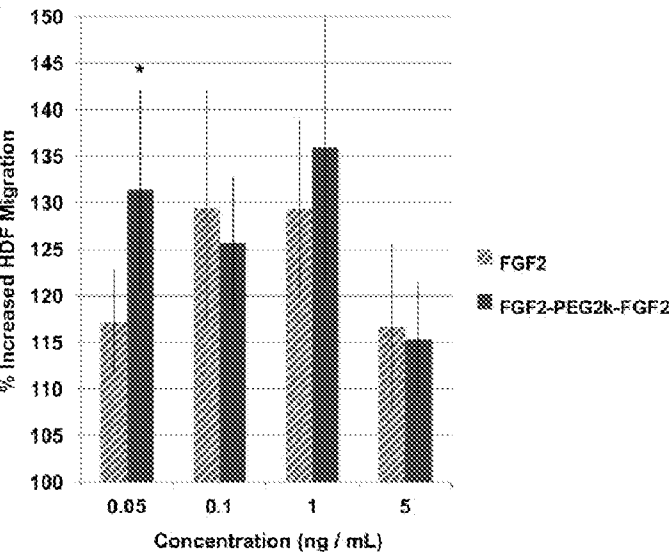


Fig. 3A-C

Fig. 4 A.

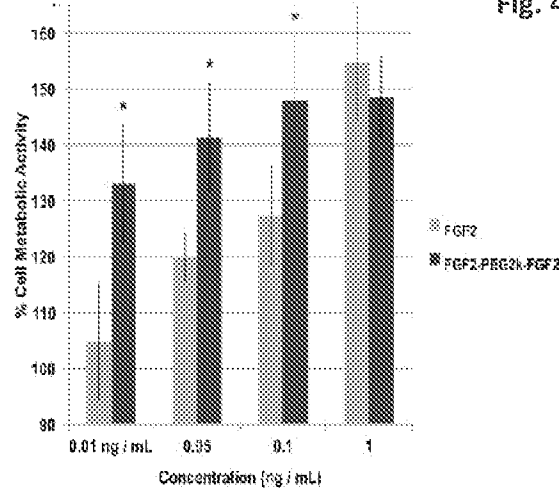


Fig. 4B.

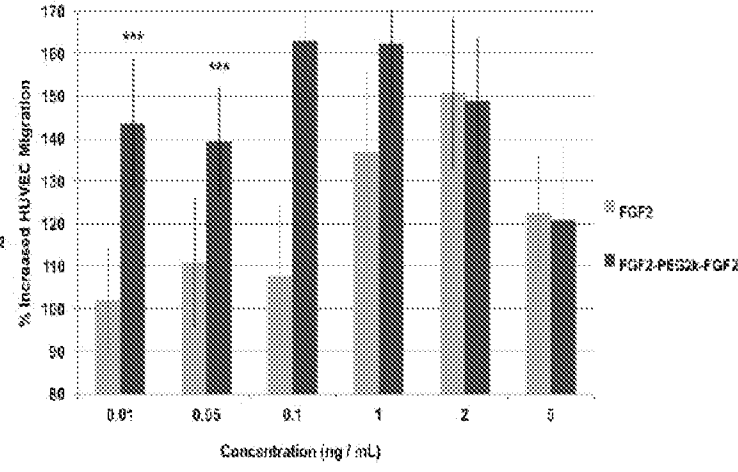


Fig. 4 C.

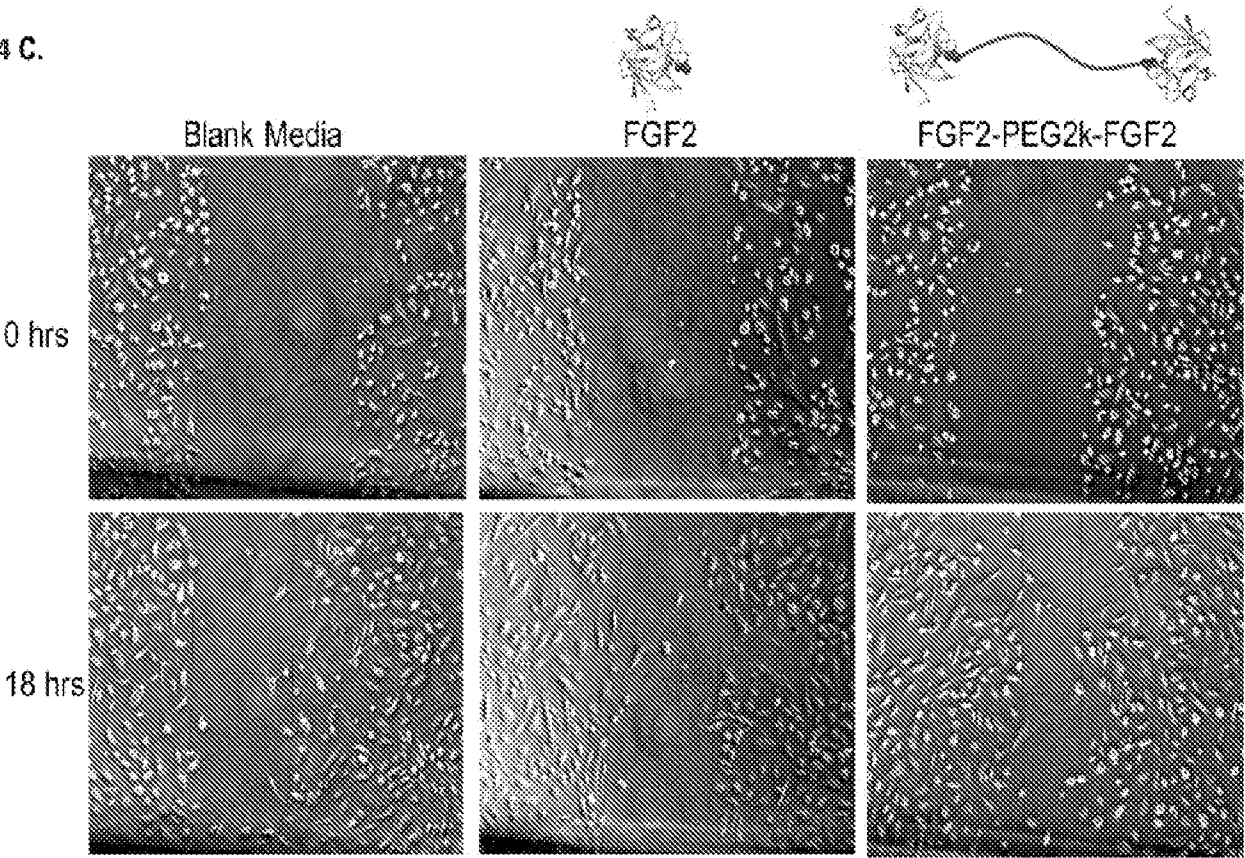


Fig. 4A-C

Fig. 5 A.

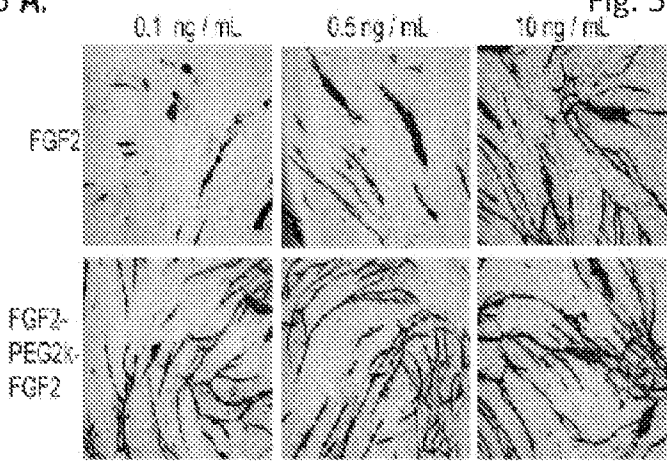


Fig. 5B.

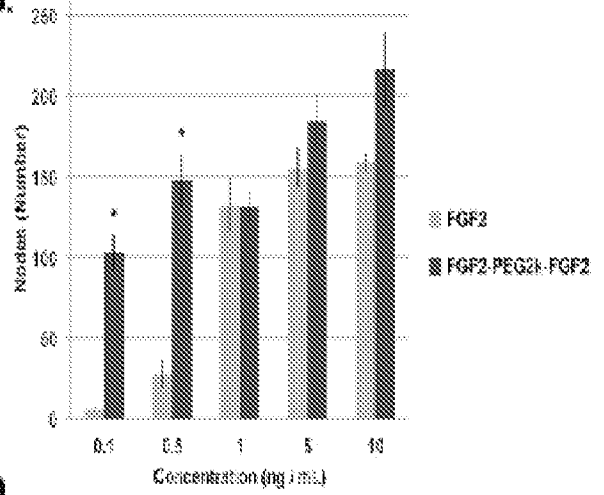


Fig. 5 C.

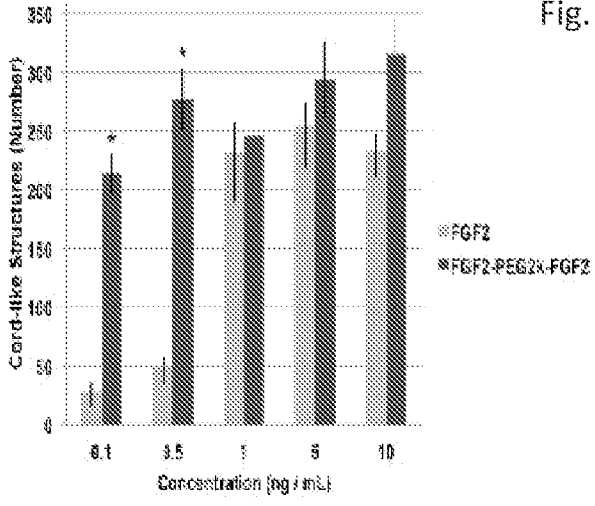


Fig. 5D.

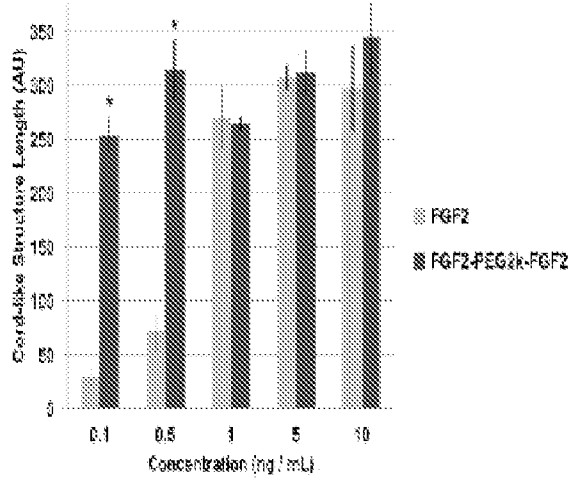
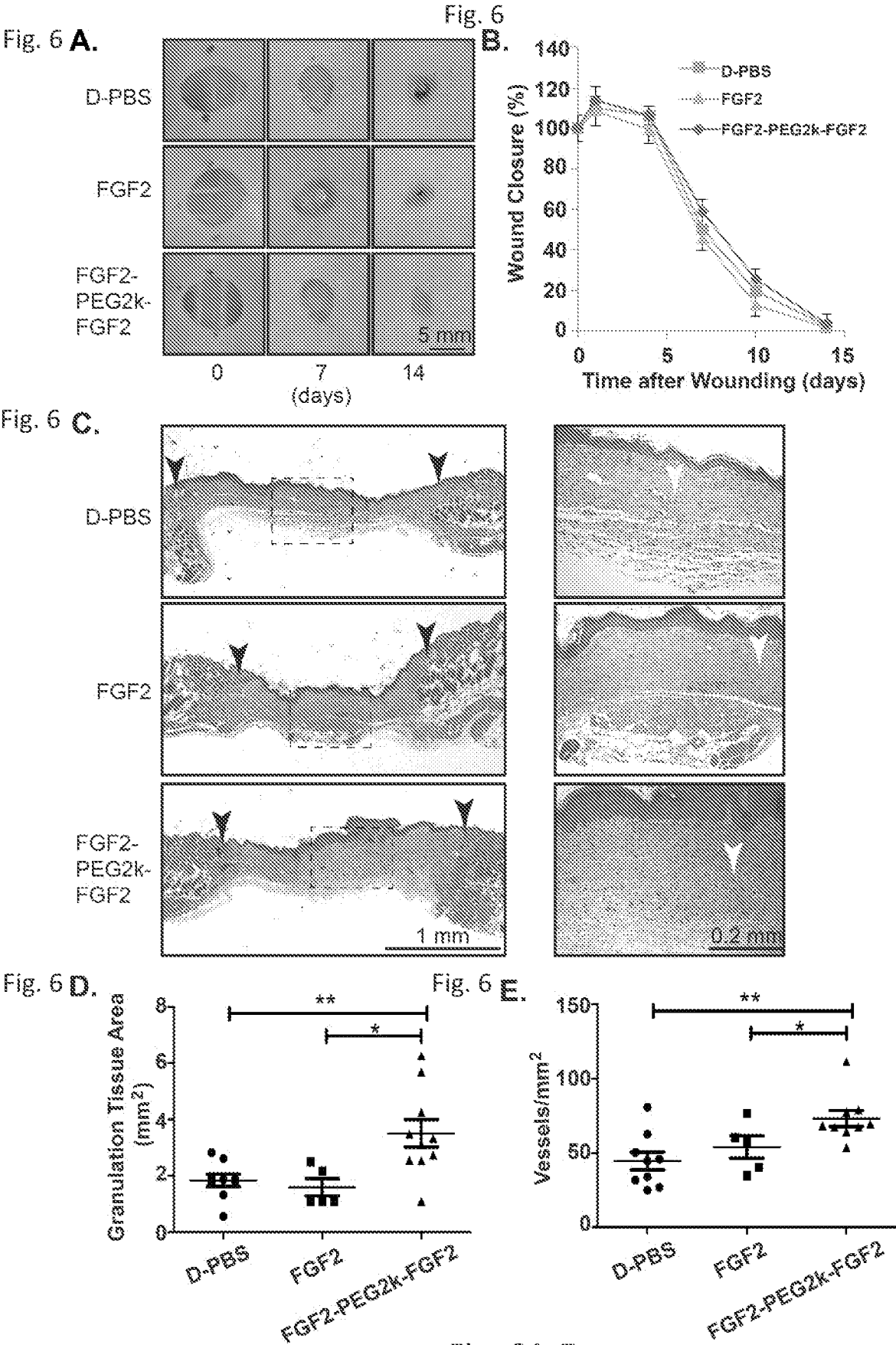


Fig. 5A-D



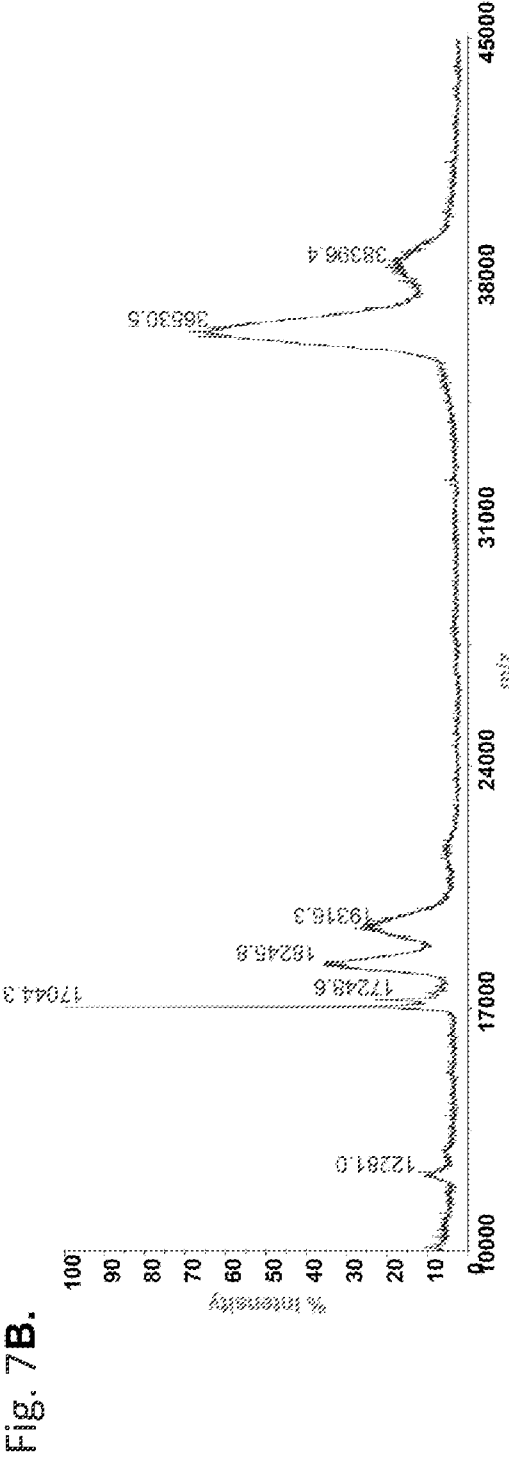
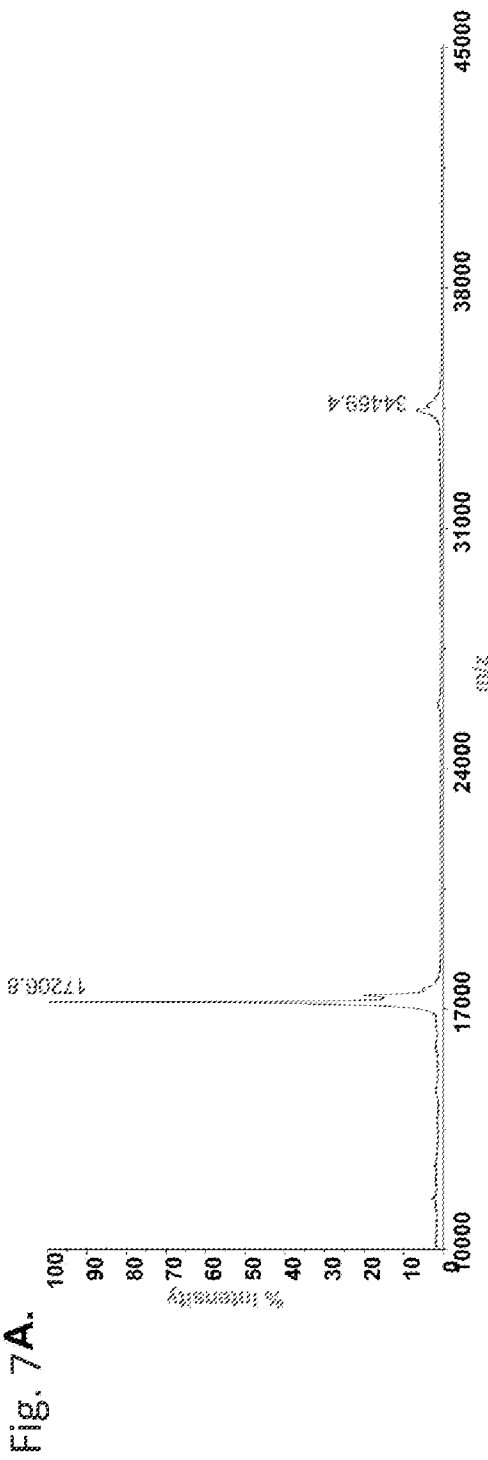


Fig. 7 A-B

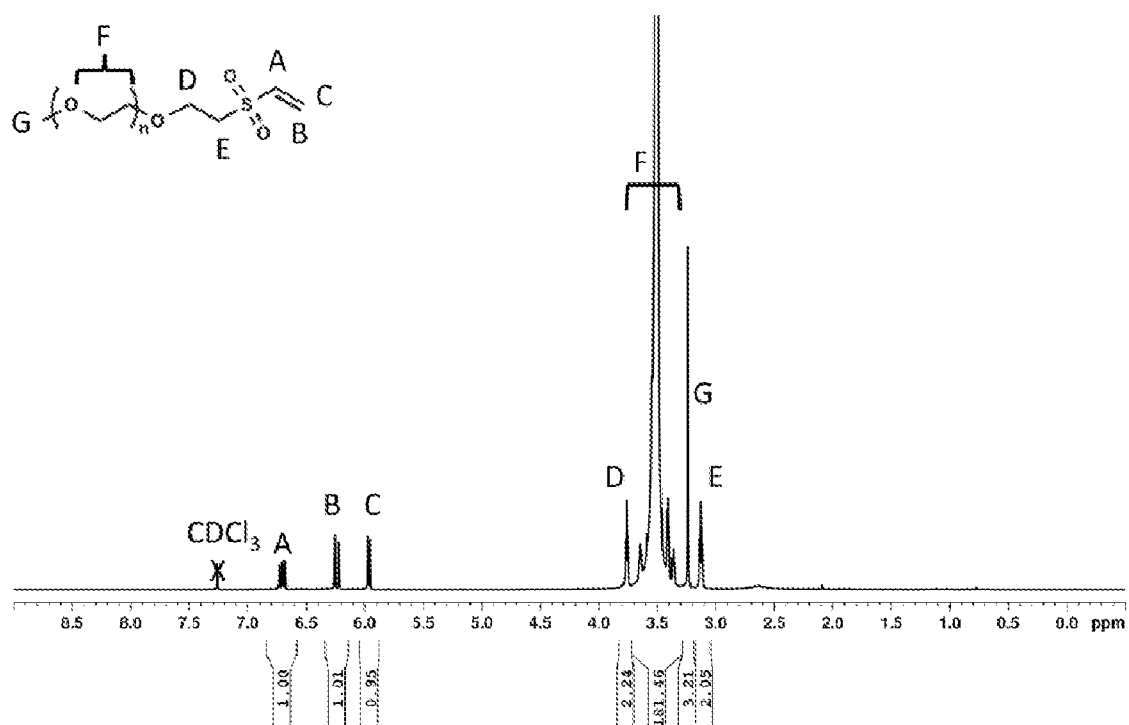


Fig. 8

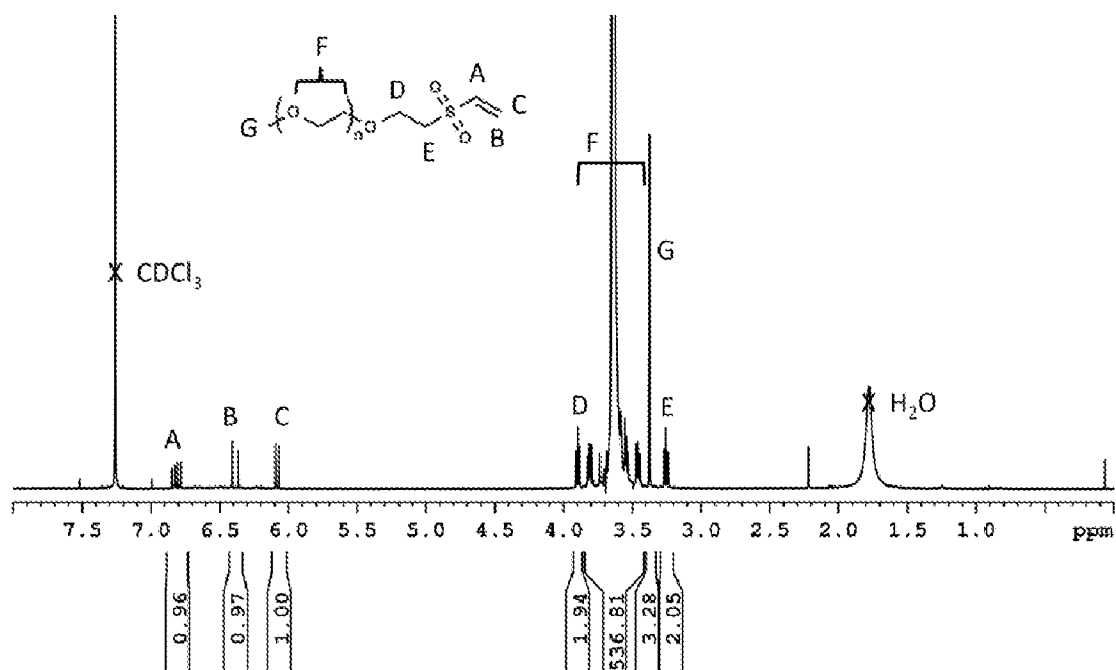


Fig. 9

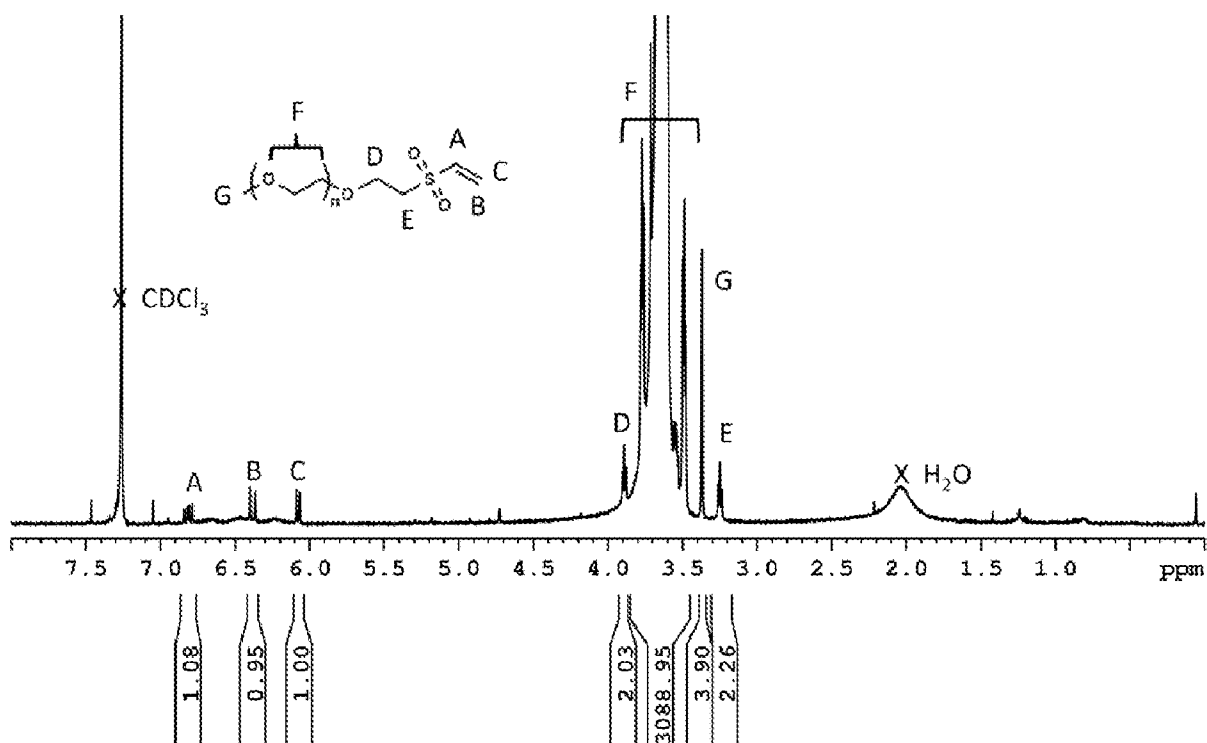


Fig. 10

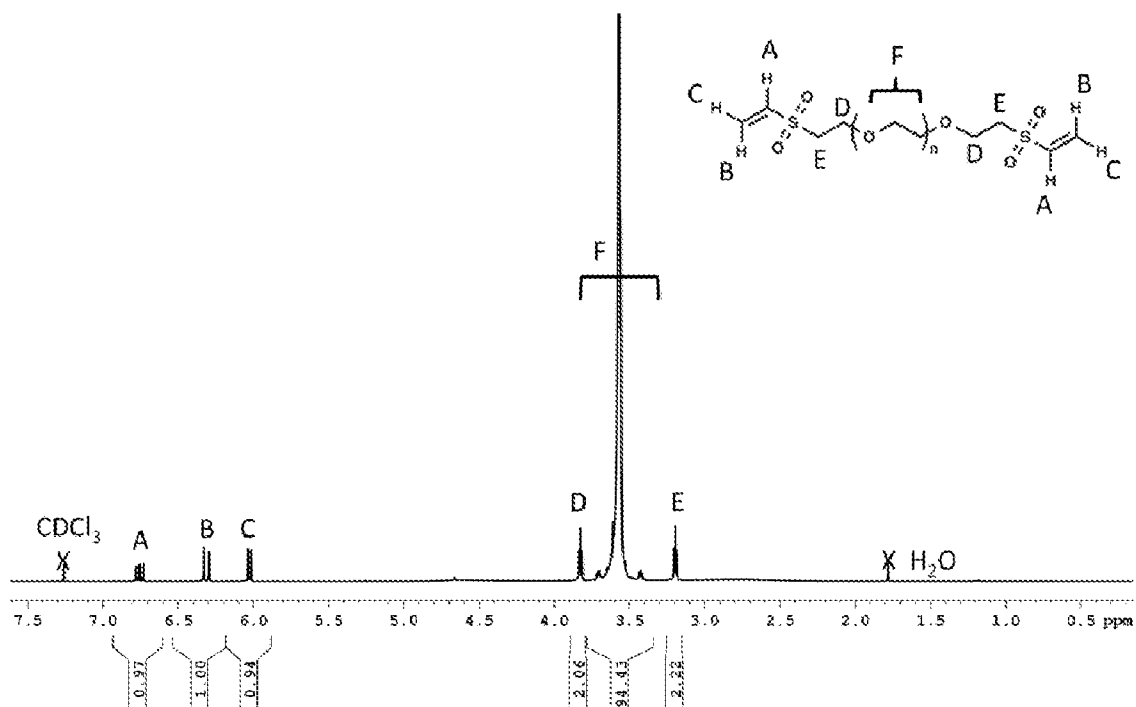


Fig. 11

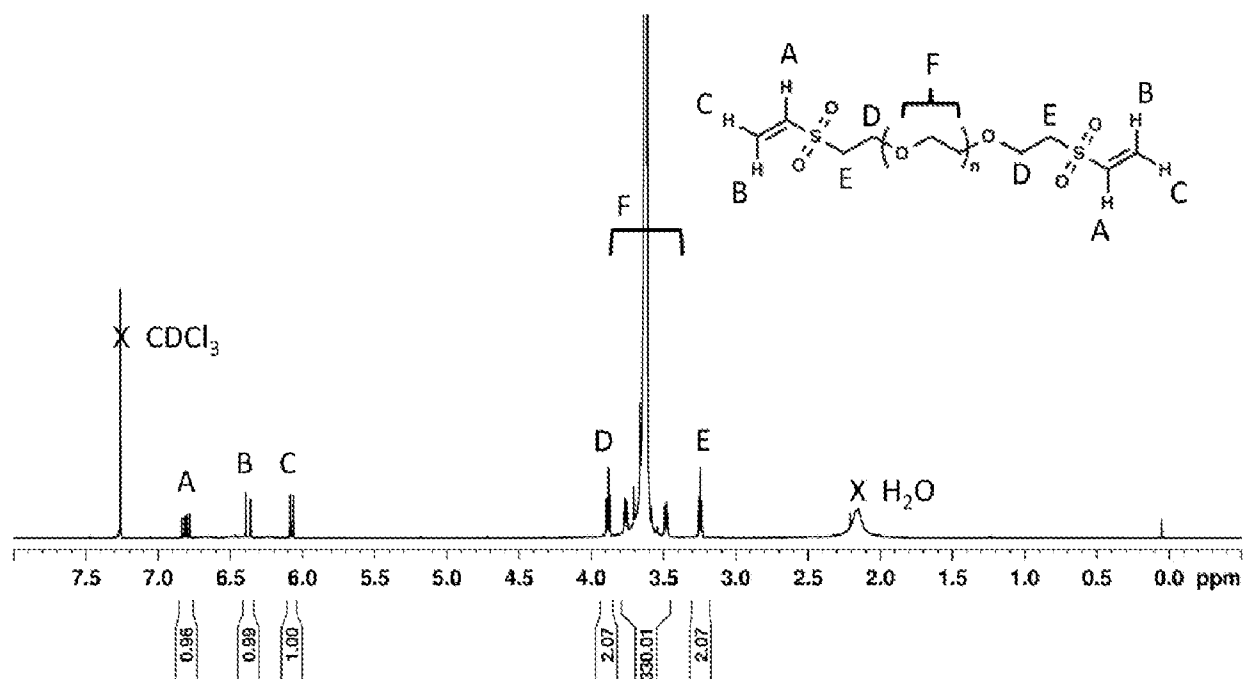


Fig. 12

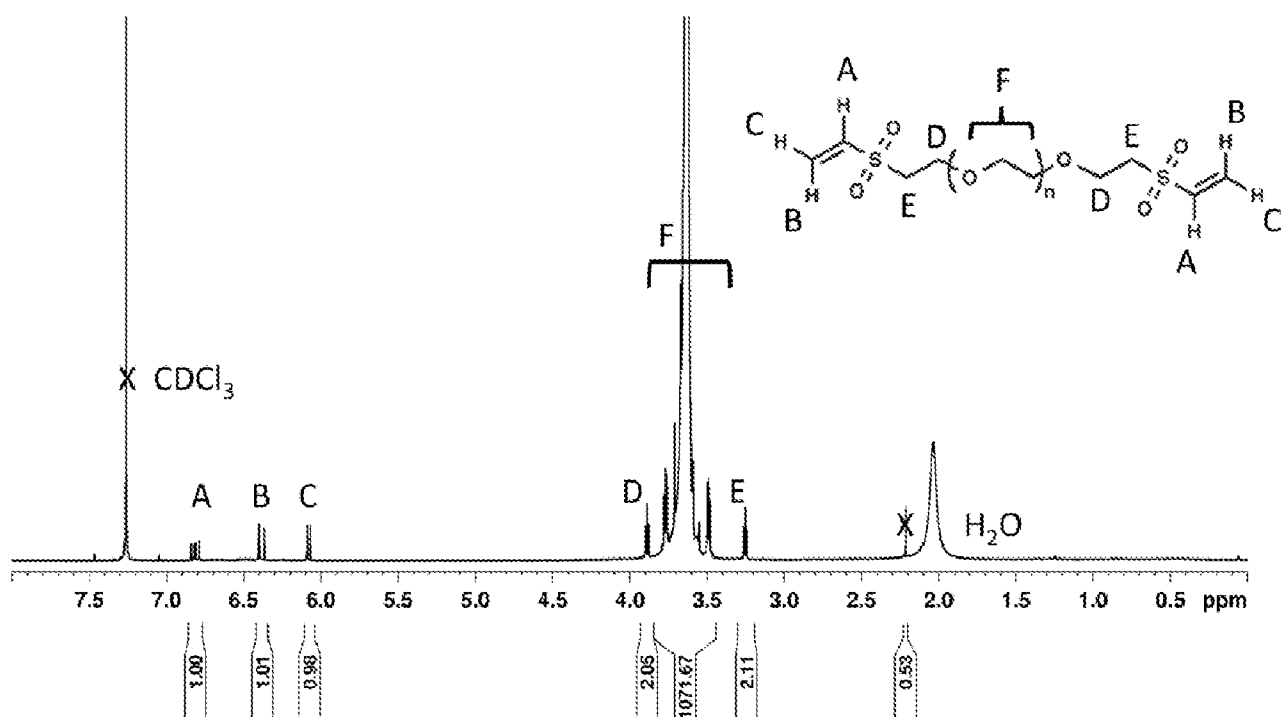


Fig. 13



Fig. 14

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2016/030510

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 38/18; A61K 47/48; A61P 17/02; C07K 14/50; C07K 17/08 (2016.01)

CPC - A61K 38/1825; A61K 47/48; A61K 47/48215; C07K 14/50; C07K 14/503 (2016.05)

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)

IPC - A61K 38/18; A61K 47/48; A61P 17/02; C07K 14/50; C07K 17/08

CPC - A61K 31/795; A61K 38/00; A61K 38/1825; A61K 47/48; A61K 47/48215; C07K 14/50; C07K 14/503

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC - 514/9.1; 514/9.4; 514/19.1; 530/399 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PatBase, Google Patents, PubMed

Search terms used: (Fibroblast growth factor "2") OR FGF2 OR (FGF "2") OR BFGF OR FGFB OR HBGF2 OR (HBGF "2") OR (FGF beta) OR FGFbeta OR (basic fibroblast growth factor%) dimer% OR multimer% OR oligomer%

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2002/077199 A2 (MASSACHUSETTS INSTITUTE OF TECHNOLOGY) 03 October 2002 (03.10.2002) entire document	1-8, 12-15, 24-29
X -- Y	US 2014/0335045 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 13 November 2014 (13.11.2014) entire document	1, 9, 10, 16-19, 21-23 ----- 11, 20
Y	WO 2006/050247 A2 (NEOSE TECHNOLOGIES, INC.) 11 May 2006 (11.05.2006) entire document	11, 20
A	US 6,010,999 A (DALEY et al) 04 January 2000 (04.01.2000) entire document	1-29
A	US 2011/0195895 A1 (WALKER et al) 11 August 2011 (11.08.2011) entire document	1-29
A	CN 102329395 B (WENZHOU MEDICAL COLLEGE et al) 12 June 2013 (12.06.2013) entire document; see machine translation	1-29

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

* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

21 July 2016

Date of mailing of the international search report

18 AUG 2016

Name and mailing address of the ISA/

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, VA 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Blaine R. Copenheaver

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774