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[Continued on nextpage]

(54) Title: COMPLEX COACERVATE FOR CONTROLLED RELEASE AND RELATED METHODS

(57) Abstract: Provided herein are coacervate compositions including cytokines, and methods of making and using the same. The coacervate can be formed by the mixing of an act-ive agent, such as a drug or protein with the polyanion, such as heparin or heparan sulfate, and a custom-made polycation (e.g., PEAD or PELD). The coacervates can be used in the treatment of diseases and disorders where targeted treatment is desired, for example in treatment of cancers.

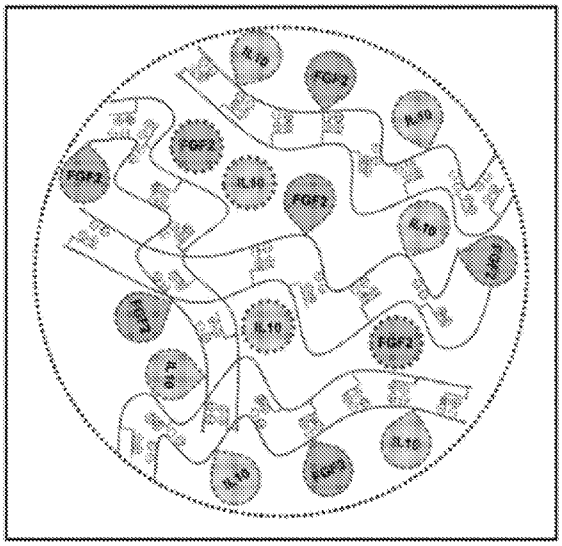

Fig. 1A

WO 2017/106333 A1

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

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COMPLEX COACERVATE FOR CONTROLLED RELEASE AND RELATED METHODS

STATEMENT REGARDING FEDERAL FUNDING

(0001) This invention was made with government support under Grant Nos DMR1005766 and IIP1444774, awarded by the National Science Foundation, The government has certain rights in the invention.

CROSS REFERENCE TO RELATED APPLICATIONS

(0002) This application claims the benefit of United States Provisional Patent Application No, 62/266,896, filed December 14, 2015, which is incorporated herein by reference in its entirety.

[0003] Provided herein are compositions useful for drug delivery, ior example for delivery of cytokines, and methods of use of those compositions, 6 r example for treatment of coronary heart disease and cancer.

f0004] Cytokines are produced by a broad range of cell types and serve as sol uble modulators of immune function in vii.ro and in vivo. In. the cancer setting, interleukins (IL), such as IL-2 and 1L-12, and interferons (IFN), such as IFN-α and iFN-γ, are capable of promoting protective anti-tumor immunity- in patients with solid 6 rms of cancer. However, systemic delivery of high-doses of these agents over prolonged periods of time has resulted in severe tox icities, and even patient deaths.

[0005] Coronary heart disease (CHD) affects 15.4 million Americans and is the most common type of heart disease, CHD alone accounts for 385,000 death s and costs an. estimated total of $1(18.9 billion annually (direct and indirect) in theUnited States, CHD caused by pathological blockage of the coronary circulation may lead to prol onged ischemia which in turn resul ts in permanent cardiomyopathy and/or myocardial infarction (MI). Mi causes death of cardiac myocytes and triggers local inflammatory responses and the compensatory scar formation, leading to pathological remodeling and ultimately heart failure (HE). Recent experimental therapies for cardiac repair primarily focus on revascularization and regeneration of impaired myocardium. However, to break the vicious cycle of MI-to-HF, not only is revascularization of the ischemic tissue desirable, but also modulation or the over-activated and prolonged inflamrnation following myocardial injury.

[0f 06] A safe and effective method of delivery of cytokines is needed for treatment of patients, for example with coronary heart disease and cancer.

.SUMMARY

[0007] Systemic toxicities of cytokines -can be avoided by the directed deiiveiy of these cytokines in a local manner, i.e. into the treatment site. Provided herein is a controlled deliver y coacervate made of a .combination of a polyanion, such as heparin or heparan .-sulfate and a synthetic polycationic copolymer. The coacervate is formed .by the mixing of an active agent, such as a drug or protein with the polyanion, such as heparin or heparan sulfate, and a custom-made polycatioji -(e.g., PEAD or PELD). Complex eoaeervates are fanned by mixing oppositely charged polyelectrolytes resulting in spherical droplets of organic molecules held together noncovalently and apart from the surrounding liquid. The coacervate system provides a Higher level of control over the release of drugs from a delivery system. Embedding a drug in coacervate compositions leads to the release of the drug over days to months. Slow release of drags also is desirable when timing of delivery impacts treatment - for example, to prevent release of a large bol us of cytokines to a patient and to normalize delivery of cytokines over a much longer time period than has been possible using conventional deiiveiy vehicles such as saline.

[0008j Provided is a composition comprising a complex or coacervate of a polycationie polymer, a polyanionic polymer, such as heparin or heparan sulfate and a cytokine selected from an interferon and/or an interleiikin. In one aspect, the polycationie polymer is- PEAD or PELD, or polymer composition comprising at least one moiety selected from the following:

(a) [-OC(O)-CH(NHY)-CH_2-C(O)O-CH_2-CH(O-R1)-CH_2-O-CH_2-CH_2-O-CH_2-C» (O-R2)-CH_2-]r ,

(b) [-OC(O)-C¾ -CH(NHY)-C(O)O-CH_2-CH(O-R1)-CH_2-O-CH_2-CH_2-O-CH_2-CH(O-R2)-CH_2-]n ,

(c) [-OC(O)-CH(NHY)-CH_2-C¾-C(O)O-CH_2-CH(O-R1)-CH_2-O-CH_2-CH_2-O-C¾-CH(0-R2>CH2-3n, and/or

(d) [-OC(O)-CH2-CH2-CH(NHY)-C(O)O-CH2-CH(O-R1)-C¾-0 -C¾ -e H_2-O-C!¾-CH(Q-R2)-C¾-Jn,

wherein Y is -C(Q)-CH(NH_3^+)-(C¾)3-NiH-C(NH_2)$_2^+$ or -C(O)-CH(NH_3^+)-(CH_2)^4-(NH_3)$^+$, and R1 and R2 are the same or different and are independently selected from -the group consisting of hydrogen, a carboxy-containing group, a C_{1-6} alkyl group, an amine-eontaining group, a quaternary .ammoai um coataining group, and a peptide. In. one aspect, the cytokine is immunomodulatory. In another aspect, the cytokine is 1L,-12. in another, the cytokine is IL-10, and the composition optionally further comprises an angiogenic growth factor such as FGF2. In another aspect; die composition is embedded in a hydrogeS.

|0009| Also provided are methods of treatment of coronary heart disease, such as a myocardial infarction comprising administering to a patient in need thereof an effective amount of tbe composition described above, and herein, e.g., inducting effective amounts of IL-10 and FGF2, Further, a method 'of treatment of a cancer is provided, comprising administering to a patient in need thereof an effective amount of the composition described above and herein, e.g., including an effective amount of an IL-12.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] Figures 1A-IF, Characterization of FGF2/IL-10 coacervate. (Figure 1A) Schematic illustration of putative FGF2/IL-10 coacervate structure fPEAD in blue in original, heparin in green in original, FGF2 in yellow in original, and IL-10 in orange in original). Please note that FGF2 and IL-10 molecules bound to heparin are circled with green dashed lines while FGF2 and IL-10 molecules physically entrapped within the coacervate structure (no affinity-based binding) are circled with dashed line, (Figure 1B) Analysis of coacervate droplet size with polydispersity index (PDI). (Figure 1C) The release profile of FGF2/IL-10 coacervate in vitro for 3 weeks. Equal amount of FGF2 and IL-10 were first combined and then mixed with heparin, foliowed by the: addition of .PEAD to form coacervate, with a mass ratio of PEAD:heparin:FGF2 :IL-J0=500: 100;1:1. Supematants were collected on Day 0, 0.5, L 4, "7, 10, 14, and 21 and then replenished with PBS..containing-fresh.0..5 U/mL heparinase II in order to simulate the release of cargo proteins in vivo (N=4 per time point). The amount of released FGF2 and IL-10 was measured by ELISA, Data are presented as percent cumulative release (normalized to the original load). Error bars indicate means ± SD. (Figure 1D) Incorporation of fluorescently labeled FGF2 (green in original) and IL-10 (red in original.) into coacervate droplets (scale bar ~ 25 μm) (Figure 1E) High-magnification enlargement of the coacervate droplet in the dotted; area in (Figure 1D) showing fluorescently labeled FGF-2 and IL-10 molecules evenly distributed within the droplet (scale bar = 5 μη). (Figure 1F) Triply labeled heparin (red in original), FGF2 (green in original) and iL-10 (blue in original) showing nearly homogeneous structure of FGF-2/1L-10 coacervate (scale bar ~ 10 μm). Please note that due o the limitation of imaging resolution, larger coacervate droplets were chosen for high-magnification imaging.

[0011] Figure 2. The spontaneous degradation of free IL-10 in vitro. The degradation profile of free IL-10 (1 μg/ηιL) in PBS containing 0,5 U/mL heparinase II was measured for 7 days, Supematants were collected on Day 0, 1, 2, 3, 5, and 7 and stored at -80¾ for simultaneous ELISA analysis (N=4 per time point). A spontaneous loss of free IL-10 was detected at an average rate of 1.59% per day.

f0012j Figure 3, Bioactivtty **of FGF-2**/IL-10 coacervate on eardi ac stromal cell proliferation in vitro. ·Bioactivity of FGF2/IL-10 coacervate on cardiac stromal cel! proli feration was tested in a non-contact transwell system for 72 hours with human umbilical vein endothelial cells (HUVECs), human cardiac Fibroblasts (hCFs), and human heart pericytes (HHPs), and mouse cardiac fibroblasts **(mCFs)** cultured at bottom wells and treatment solutions loaded into suspended transwelis. (A) Under the simple serum-deprived condition, Free-F/I-500/100, Coa-F-500, Coa-F/I-500/100 and Coa-F/I-500/500 had siaiiflcanilv hiaher HUVEC proliferation than the no-treatment control and Free-F/I-500/500. Coa-F/I-500/500 had significantly lower mCF proliferation than the no-treatment control, Free-F/1-500/1 00, and Free-F/I-500/500 whi le Coa-FVI-500/100 had significantly lower mCF proliferation than Free-**F**/1-500/100 and Free-F/I-500/500. Free-F/I-500/100, Free-F/I-500/500, and Cqa-F/--5OO/500 marginally promoted hHP proliferation (all p>0.05). (B) Under the seiiim-deprived condition with inflammatory stress (10 ng/ffilof TNF-α for HUYEGs and 100 ng/ml of TNF-α for all other cell types), Coa-F-500, Coa-F/I~500/1(K), and Coa-F/1-500/500 had significantly higher HliVEC proliferation than the no-treatment control. Coa-F-500, Coa-F/I-S0O!00, and Coa-F/f -500/5 00 had significantly lower hCF prolié ration than Pree~F/f-500/100. Additionally, Coa-F/I~5D0/500 had significantly lower mCF proliferation than the no-treatment control, Free-F/I-500/100, and Free~F/i-50Q/5CM) while Coa-F-500 and Coa-F/1-500/100 had significantly lower mCF proliferation than the no-treatment control and Free-F/I-500/100. Error bars indicate means ± SD. Statistical differences between groups were analysed by one-way AN0 VA with Bonferroni post-hoc analysis. {*p≤0,05, †p< 0.01, §p<0.005, #p<0.001 |« all graphs) (From left-to-right in each group in Figure; No-Tc Ctrl; basal medium; Vehicle; empty [PBAD:heparin] coacervate; Free-F/I-50O!o0: naked mixture of 500 ng FGF2 and 100 ng IL-10; Free-F/I-500/500:; naked mixture of 500 ng FGF2 and 500 ng IL-10; Coa-F-500; coacervate loaded with 500 ng FGF2; Coa-F/I-5Q0/t00: coacen¾te loaded with 500 ng FGF2 and 100 ng IL-10; Coa-F/f-500/500: coacervate loaded with 500 ng FGF2 and 500 ng IL-10)

[0013] Figures 4A-4E. Sntramyoeardlal injection of FGF2/IL-10 coacervate improves long-term cardiac contractility and ameliorates adverse remodeling. Echocardiographie analyses (N=8 per group except Free-P/I~5O0/500, N=?) were .repeatedly performed at 5 days, 2 and 6 weeks post-surgery. Statistical differences in overall treatment effect were analyzed by two-way repeated ANOVA with Bonferroni multiple comparison test. The results revealed substantial improvement in LV contractility following intramyocardial injection of either €oa-F/I-500/100 or Coa-F/l-500/500, as indicated by the higher (Figure **4A)** tractiona! shortening

(FS), (Figure 4B) ..fractional area change (FAQ, and (Figure 4C) ejection fraction (EF) (* p<O,05, †p<(l01, §p30.005, #p≤0.001 in all graphs; FS: Coa-F/l-500/100 and Coa~F/l-5O0/500 vs. Saline, Free-F/I-500/500, and Coa-F-500; FAC and EF: Coa-F/ESOO/500 vs. all groups, Coa-F~500 and Coa~F/i-500/100 vs. Saline and Free~P/I~500/500, Free-F/I~S00/50O vs. Saline), Significant reductions of (Figure 4D) end-diastolic area (EDA) and (Figure 4E) end-systolic area (ESA) of LV were observed in hearts treated wife Coa-F/I-500/ 100 or Coa-F/i-500/500 (*p≤0.05, †p≤0i)I, §p≤0.005, #p≤0.001 in all graphs; EDA; Coa-E/i-S00/500 vs. Saline, Free-F/I-500/500, and Coa-F-500; Coa-F/I-S00/100 vs. Saline and Eree~F/F5OO/50O; ESA: Coa-F/l-500/500 vs. Saline, Free~F/l~500/50O, and Coa-F-500; Coa-F-500 and Coa-F/I-500/100 vs. Saline and Free-F/I-500/500), Error bars indicate means ± SD. Please note that lime points on the X-axis (time) In all graphs are not scaled to actual experimental duration.

10014] Figures 5Λ and 5B, FGF~2/IL-10 coacervate amends elasticity of infarcted myocardium. (Figure 5A) Representative axial strain maps laid over B-mode images (4 x 6 mm) showing the axial strain distribution of the normal (left panel) and untreated MI control (right panel, mid-iniaret level) left ventricles respectively. For normalization purpose, the infarct area was designated as B, and the non-infaret area was designated as A. (Figure. 5B) Normalized strain was obtained by dividing spatially averaged: axial strain of B by thai of A (B/A). Coa-F-500, Coa-F/l-500/100, and Coa-F/1-500/500 showed markedly greater normalized strains than the saline control and Free-F/1-500/500. Free-F/1-500/500 also showed notably higher normalized strains than the saline control (#n<0,001, †p≤0.0 1; N=3 per group), Error bars indicate means ± SD, Statistical differences between groups were analyzed by one¬ way ANdVA with BonfeiToni post-hoc analysis.

|001S| Figure 6. B-mode images of normal and untreated Ml control hearts without strain maps and ROis.

|0016) Figures 7A and 78. FGF-2/II.-10 coacervate increases long-term endothelial cell density. Endothelial cell (EC) density at the infarct and peri-inSaret border zone was revealed by immunohistochemical detection of CD3 1+ ECs at 6 weeks post-infarction. (Figure 7A) Representative images of CD31+ ECs (red in original) and αSMA+ cells (green in original) within the infarct and peri-infarct areas at the mid-infarct level, with dotted areas enlarged. Please note that vascular smooth muscle cells (VSMC) were defined as perivascvilar/peri-CD3 1 αSMA+ cells. Nuclei were stained with. DAPI in bine (in original), (scale bars = 100 urn) (Figure 7B, left) Quantitative analyses of CD31+ EC density within the infarct area revealed that Coa~F/l-5O0/500 had significantly higher EC density than the saline control and Free-F/i-

500/500 while Coa-F/i-S00/100 had significantly higher EC density than the saline control (*p<0.05, †p<0.01; N=4 per group). (Figure **78,** right) Within the peri-infarct area, Coa~F/I~ 500/500 had significantly higher EC density than the saline control, Free-F/I-S00/500, and Coa-F-500 while Coa-F/I-500/100 had significaMly higher EC density than the saline control and Free~F/l-50O/50O (#p<0.001, *p<0.Q5; N=4-per group). Error bars indicate means-. ± SD. Statistical differences between groups were analyzed by one-way ANOVA with Bonferroni post-hoe analysis.

[0017] Figure 8. FGF-2/IL-10 coaeervate enhances long-term vascular stromal cell density. Vascular stromal cell density at the infarct and peri-mtarct border zone was revealed by immunohistochernical detection of vascular smooth muscle cells (VSMC', defined as pettvasc«lar/peri-CD3 1 αSMA+ cells; representative images shown in Figure **7**(A)).at **6** weeks post-infarction . (left) Quantitative analyses of perivascular αSMA+ VSMC density within the infarct area .revealed that Coa-F/I-500/500 had significantly higher VSMC density than the saline control, Free-F/I-500/S00, and Coa-F-500 (*$p \leq 0.05$; N=4 per group), (right) Coa-F/I-500/100 and Coa-F/I-500/500 had marginally higher VSMC density than the saline control Free-M-500/500, and Coa-F-500 within the peri-inferct area (all $p > 0.05$; N=4 per group). Error bars indicate means ± SD. Statistical differences between groups were analyzed by one¬ way ANOVA with Bonferroni post-hoc analysis.

[0018] Figure 9. FGF-2/ïE-10 coaeervate reduces myocardial fibrosis. Masson's tricbrdme histological staining was employed to reveal left ventricular (LV) myocardial fibrosis at ▷ weeks postinfarction, (A) Representative images of myocardial fibrosis at the mid-infarct level (transverse sections of hearts). Collagen deposition (fibrosis/scar) was -stained in blue/purple while cardiac muscle was stained in red (scale bars « 1 mm). (B) Quantification of the LV fibrotic area fraction. Coa-F/I -500/500 exhibited significantly reduced LV fibrotic area fraction (#p≤0.001, vs. saline; N=4 per group). Healthy heart (Normal) served as a negative control. (G) Measurement of **LV** wall thickness at the center of the infarct. Coa-F/I-500/5Q0 had significantly thicker infarct wall than all other groups (*p<0-OS, vs. all poops; N=4 per group). Error bars indicate means ± SD. Statistical differences between groups were analyzed by one¬ way *ANOVA* with Bonferroni post-hoc analysis.

[0019] Figure 10. FGF~2/IL~10 coaeervate inhibits chronic phagocytic ceil infiltration The effect of FGF-2/IL-10 coaeervate on chronic inflammatory responses was evaluated by the number of focally infiltrating CD68+ phagocytic cells within the infarct region at 6 weeks post¬ infarction. When compared with the saline control, Coa-F-500, Coa-F/I-500/HK), and Coa~F/**Ir**

500/500 had significantly reduced numbers of infiltrated CD68+ phagocytic ceils within the infarct area. Error bars indicate means ± SD. -Statistical differences between groups were analyzed by one-way AMOVA with Bonferroni post-hoc analysis. (*$p \leq 0.05$, †$p \leq 0.01$, vs, saline; N=4 per group).

[0020] Figure 11. Estimation of the duration of coacervate treatment in vivo. Multi-photon excitation (MPE) .imaging was employed to detect intramyocardially injected free (Free-Rho), hepariii-boond (Hep-Rho), or eoaeervate-bourid rbodamine (Coa-Riio). Collagen iibers (blue in original) were revealed by second harmonic generation (SHG) signals. Quantification of the fluorescence volume of Free-Rho, Hep-Rho, and Coa-Rho at 5, 14, and 28 days post-injection. Error bars indicate means ± SD. Statistical differences between groups were analyzed by one-way AMOVA with Bonferroni post-hoc analysis. (*/.?<0.05, vs. Free-Rho and Hep-Rho; N=3 per group).

£0021] Figure 12 depicts postulated mechanisms of FGF-2/IL-10 coacervate-mediated cardiac repair and functional recovery.

[0022] Figure 13 is a .graph depicting suryival proportions as. described in Example 5.

{0023} Figure 14 provides graphs showing a summary of results for Example 5 (lL-2).

[0024] Figures 15A-15C are graphs showing the results for individual mice for Example 5 (IL,-2).

[0025] Figure 16 provides graphs showing a summary of results for Example 5 (!L-12).

£0026] Figures 17A-17C are graphs showing the results for individual mice for Example 5 (1L-12).

DETAILED DESCRIPTION

|0O27j The use of numerical values in the various ranges specified in this application, unless expressly indicated otherwise, are stated as approximations as though the minimum and maximum values within the stated ranges are both preceded by the word "about". In this manner, slight variations above and below the stated ranges can be used to achieve substantially the same results as values within the ranges. Also, unless indicated otherwise, the disclosure of these ranges is intended as a continuous range including: every value between the minimum and maximum values. For definitions provided herein, those definitions -refer to word forms, cognates and grammatical variants of those words or phrases,

£0028] A composition is provided herein to control the delivery rate of cytokines, for example, interfeukins (IL), such as fL-2 and IL-12.(e.g., lL-12 p70), and interferons (IFN), such as IFN-y. The controlled delivery .system comprises a recently developed heparin-based eoacervaie.

Complex coacervates are formed by mixing oppositely charged polyelectroiytes resulting in spherical droplets of organic molecules held together noncovalently and apart f om the surrounding liquid and have shown potential in sustained protein delivery; One application of the coacervate is to control the release of cytokines and interf o ns. Methods of making and using the composition also ae .provided.

[0029) As used herein, the term "polymer composition" is a composition comprising one or more polymers. As a class, "polymers" includes homopoiymers, beteropolymers, co-polymers, block polymers, block co-polymers and can be both natural and synthetic, Homopolymers contain one type of building block, or monomer, whereas co-polymers contain more than one type of monomer.

[0030) As used herein, the terms "comprising," "comprise" or "comprised ,* and variations thereof, are meant to be open ended. The terms "a" and "an" are intended to refer to one or more.

[0031] As used herein, the term "patient" or "subject" refers to members of the animal kingdom including but not limited to human beings.

[0032) A "coacervate" refers o herein as a reversible aggregation of compositions in a .liquid, for example, as described herein, for example, resulting from the aggregation of oppositely-charged polyionic compositions. Exemplary coacervates are illustrated in the examples below with the aggregation of the polycation, polyanion, and active agent(s), as described herein, for example with the aggregation of PBAD, Heparin, and IL-12, or IL-10 cotnbined with FGF2. A "complex" is a non-eovalent aggregation of two or more compositions.

|0033] The terra "alky!" refers to both branched and straight-chah saturated aliphatic hydrocarbon groups. These groups can have a stated number of carbon atoms, expressed as C_{x-y}, where x and y typically are integers. For example, C_{5-10}, includes C_5, $C_{3/4}$, C_7, C_8, C_9, and C_{10}. Alkyl groups include, without limitation: methyl, ethyl, propyl, isopropyl, n-, s- and t-hutyi, n- and s-pentyl, hexyl, heptyi, octyl, etc. Alkenes comprise one or more double bonds and alkynes comprise one or more triple bonds. These groups include groups that have two or more points of attachment (e.g., aikylene). Cydoaikyl groups are saturated ring groups, such as eyciopropy!, cyclobutyi, or cyclopentyi As used herein, "halo" or "halogen" refers to fluoro, chloro, bromo, and iodo.

[0034] A polymer "comprises" or is "derived from" a slated monomer if that monomer is incorporated into the polymer. Thus, the incorporated monomer that the polymer comprises is not. the. same as the monomer prior to incorporation into a polymer, in that at the very least, certain terminal groups are incorporated into the polymer backbone. A polymer is said to

comprise a specific type of linkage, such as an ester, or methane linkage, if that linkage is present in the polymer.

[0035] Certain polymers described herein, such as heparin and PEAD, are said to be hioerodible or biodegradable, By that, it is meant that the polymer„ once implanted and placed in contact with bodily fluids and tissues, or subjected to other environmental conditions, such as composting, will degrade either partially or completely through chemical reactions, typically and often preferably over a time period of hours, days, weeks or months. Mon-limiting examples of such chemical reactions include acid/base reactions, hydrolysis reactions, and enzyme catalyzed bond scission. Certain polymers described herein contain labile ester linkages. The polymer or polymers may be selected so that it degrades over a time period. Non-limiting examples of useful *in situ* degradation rates include between 12 hours and **5** years, and increments of hours, days, weeks, months or years therebetween.

[0036] A drug delivery composition is provided, comprising, a coaeervate of a polycationic polymer, a polyanionic polymer, and an active agent. In certain aspects, the polycationic polymer described herein comprises the structure (that is, comprises the moiety: [-OC(O)-B-CH(OR1)-B-]$_n$ or –[OC(O)-B-C(O)O-CH_2-CH(O-R1)-CH_2-B'-CH_2-CH(O-R2)-CH_2-]$_n$, in which B and 8' are the same or different and are organic groups, or B' is not present, including, but not limited to: alkyl, ether, tertiary amine, ester, amide, or alcohol, and can be linear, branched or cyclic, saturated or unsaturated, aliphatic or aromatic, and optionally comprise one or more protected active groups, such as, without limitation, protected amines and acids, and R_1 and R2 are the same or different and are hydrogen or a fonctional group (e.g., as described herein). As seen below, the composition exhibits low polydispersity, with a polydispersity index of less than 3.0, and in many cases less than *2.0*. These compositions are described in United States Patent No, 9,023,972, which is incorporated by reference in its entirely.

[003?] In one aspect, the polycationic polymer is a polymer composliiofi comprising at least one moiety selected from the following in. which **B** and B' are residues of aspartie acid or glutamic acid, which are optionally further derivatized with an amine-containing group, for example, the amines of the aspartic acid or glutamic acid are further derivatized with lysine or arginine:

(a) [-OC(O)-CH(NHY)-C¾ -C(O)O-CH_2-CH(O-R1)-CH_2-O-CH_2-CH_2-O-CH2~CH(0-R2)-CH3-]n,

(b) [-OC(O)-C¾ -CH(NHY)-C(O)O-CH_2-CH(O-R1)-CH_2-O-CH_2-CH_2-O-C¾-CH(0-R2)-CH $_2$-]$_n$,

(c) [-OC(O)-CH(NHY)-CH_2-C¾ -C(O)O-CH2-CH(O-R1)-CH_2-O-CH_2-C¾-O-C¾-CH(O-R2)-Ce3-]$_n$, and/or

(d) [-OC(O)-CH_2-CH_2-CH(NHY)-C(O)O-CH2-CH(O-R1)-CH_2-O-CH_2-CH2-0-CH 2-CH(O-R2)-CH_2-|»,

wherein Y is -C(O)-CH(NH_3^+)-(CH_2)$_3$-NH-C(NH_2)$_2^+$ or -C(O)-CH(NH_3^+)-(CH_2)$_4$-(NH_3)$^+$, and R1 and R2 are the same or different and are independently selected from the group consisting of hydrogen, a carboxy-containiag group, a C1-¾ alkyl group, an amine-containing group, a quaternary ammonium containing. group, and a peptide.

£0038} The polymers described herein can be fonciiona!ized, e.g., at B, B', R l and R2, meaning they comprise one or more groups with an .activity, such as a biological activity. For example and without limitation, as shown herein, the polymer may be functionalized with an acetylehoime-like group or moiety, a cross-linking agent (Cfoss-Iinking agents contain at least two reactive groups that are reactive towards numerous groups, including sulfhydryls and amines, and create chemical oovalent bonds between two or more molecules, functional groups that can be targeted with cross-linking agents include primary amines, earboxy!s, su!ihydry!s, carbohydrates and carboxyHc acids. A large number of such agents are available commercially from, e.g., Thermo fisher Scientific (Pierce) and Sigma.

[0039] Other functions that can be provided by or enhanced by addition of functional groups include: increased hydrophobicity, for instance by functionalizing with a superhydrophobfc moiety, such as a perfluoroalkane, a perflnoro(alkylsilane), and/or a siloxane; increased hydropkiiicity, 6 r instance by f metionalé mg with polyethylene glycol (PEG); or antimicrobial, for instance, by functionalizing with a quaternary amine. The polymer can he functionalized with a tag, such as a fluorescent tag (e.g., FITC, a cyanine dye, etc). The polymer can be funetionalized by linking to additional synthetic or natural polymers, including, without limitation: synthetic polymers, such as a polymer derived from an alpha-hydroxy acid, a polyfaetide, a poly(laetide-co-giycolide), a poly(L-lactide-co-caprolactone), a polyglycolic acid, a poly(dl-lactide-co-glycolide), a poly(l-Iaciide-ccvdi-lactide), a polymer comprising a lactone monomer, a polycaprolacione, a polymer comprising carbonate linkages, a polycarbonate, a polyglyconate, a poly(giyColide-co-trimethyiene carbonate), a poiy(glyeoIide~eo-trimethylene ca.rbonate-co-d.ioxanone), a polymer comprising urethane linkages, a polyurethane, a po.ly(ester urethane) nrea, a. poly(ester urethane) urea elastomer, a polymer comprising ester linkages, a polyalkanoate, a pelyhydroxyhuiyrate, a polyhydroxyvalerate, a

poSydioxanone, a polygalact a, or natural polymers, such as chitosan, collagen, gelatin, elastin, alginate, cellulose, hyaluronic acid and other glyeosaminoglycans .

|0o40] The compositions may be functionalized with organic or morganic moieties to achieve desired physical attributes (*e.g.*, hardness, elasticity, color, additional chemical reactivity, *etc:*), or desired functionality. For example, the polymer composition may be derivattzed with maleic acid or phosphate.

[0041] The composition is formed into a coaeervaie with active agents or polyanionic or polycationic groups for sequestering active agents for controlled -delivery in vivo. Drug products comprising the coaeervaie described herein may be delivered to a patient by any suitable route of delivery *(e.g.* oral or parenteral), or as an implantable device for slow release of the active agent.

|0042j The functional groups may vary as indicated above. For example, in certain embodiments, Ri and R2 are the same or different and are independently selected irom the group consisting of hydrogen, a carboxy-containing group, a *C*\-6 alkyl group, an amine-containing group, a quaternary ammonium containing group, and a peptide, In one embodiment, one or more of B, B', R1 and R2 are charged such that it is possible to bind various water insoluble organic or morganic compoimds to the polymer, such as magnetic inorganic compounds. As above, in one embodiment, one or more of B, B', R1 and R2 are positively charged. In one embodiment, one or both of Ri and R2 are functionalized with a phosphate group. In another -embodiment, the composition is attached non-eovakutly to a calcium phosphate (including as a group, for example and without limitation: hydroxyapatite, apatite, tricalcium phosphate, octacalcium phosphate, calcium hydrogen phosphate, and calcium dihydrogen phosphate), In yet another embodiment, R.1 and R2 are independently one Ile-Lys-V¾l-Ala-Val (!KVAV) (SEQ ID HQ: 1), Arg~Gly~Asp (ROD), Arg-GIy~Asp~Sei (RODS) (SEQ ID NO: 2), Ala-Gly-Asp (AGD), I,ys-Gln-Aia-Giy~Asp-Vai **(KQAGDV)** (SEQ ID HQ: 3), Vai~Ala-Pro-Gly-Val-GIy (VAPGVG) (SEQ ID NO: ¾ APGVGV (SEQ ID HQ: 5), PGVGVA (SEQ ID NO: 6), VAP, GYGVA (SEQ ID NO: 7), VAPG (SEQ ID NO: 8), VGVAPG (SEQ ID HO: 9), VGVA. (SEQ ID HO: 10), VAPGY (SEQ ID NO: 11) and GVAPGV (SEQ ID NO: 1.2».

}0043j In forming the composition (e.g., coaeervaie), the cationic polycationic polymer is complexed with a polyanionic polymer, such as heparin or heparan sulfate, which is further complexed with an active agent, such as a growth factor, small molecule, cytokine, drug, a biologic, a protein or polypeptide, a chemoattractant, a binding reagent an antibody or antibody fragment, a receptor or a receptor fragment, a ligand, or an antigen and/0? an epitope. Specific

examples of active agents include interleukins (IL), such as IL-2 -and 1L-12 (e.g., IL-12 p70), and interferons- (IFN), such as IFN-γ. In one aspect, the composition comprises a coaeervaie of a polycationic polymer composing one or more of moieties (a), (b), (c), and/or (d), as described above, and further comprising heparin or heparin sulfate complexed (that is non-covalently bound) with the a first active agent, such as IL-2, IL-12 (e.g., IL-12 p70), and/or IFN-γ, in any combination. The composition is formed, for example, by mixing in a suitable solvent, such as an aqueous solution, such as water, saline (e.g. norma! saline), or PBS, the polyaniome. polyeationic, and active agent constituents of the composition,

[0044] Additional acti ve agents that may be incorporated into the coaeervaie include, without limitation, anti-milanmatories, such as, without limitation, NSAIDs (non-steroidal anti-inflamniatory drags) such as salicylic acid, indomethaein, sodium indomethacin txihydrate, 8alicylamide, naproxen, colchicine, fenoprofen, sulindac, diflunlsal, diclofenac, mdoprofen sodium salieylamide, anti-inflammatory cytokines, and anti-inflammatory proteins or steroidal anti-inf ammatory agents); antibiotics; anticiotting factors such as heparin, Pebac, enoxaparin, aspirin, hirudin, plavix, hivalirudin, prasugrel, idraparinux, warfarin, Coumadin, clopidogrel, PPACK, GGACK, tissue plasminogen activator, urokinase, and streptokinase; growth factors. Other active agents include, without limitation: (1) immua bsuppressants; glucocorticoids such as hydrocortisone, betamethasone, dexamethasone, ilunetbasone, isof upredone, methylprednisoione, prednisone, prednisolone, and triamcinolone aeetonide; (2) antiangiogenies such as fluorouraei!, paeliiaxel, doxorubicin, cisplatin, mediotrexate, cyclophosphamide, etoposide, pegaptanib, lucentis, tryptophanyl-tRNA synthetase, retaane, CA4P, AdPBDP, VEGF~TRAP~EYE, AG-103958, Avastm, JSM6427, TG100801, ATG3, OT-551, cndostatin, thalidomide, bevacizumab, neovastat: (3) anti-proliferatives such as sirohrnns, paelitaxel, perillyl alcohol, farnesyl-transferase inhibitors, FPTIII, L744, anti¬proliferative factor, Van 10/4, doxorubicin, 5~FU, Daunomycin, Mitomycin, dexamethasone, azathiopr ne, chlorambucil, cyclophosphamide, methotrexate, mofetil, vasoactive intestinal polypeptide, and PA.CAP; (4) antibodies; drugs acting on immunophilins, such as cyclosporine, zotarolimus, everolimus, tacrolimus and sirolio us irapamyein), interferons, TNF binding-proteins; (5) taxanes, such: as paelitaxel and docetaxel; statins, such as aiorvastatin, lovastatin, simvastatin, pravastatin, fiuvastatin and rosuvastatin; (6) nitric oxide donors or precursors, such as, without limitation, Angeli's Salt, L-Argininc, Free Base, nitrates, nitrites, Diethylamine NONOate, Diethyiamine NONOate/AM, Glyeo-SNAP-i, Glyco-SNAP-2, (,+-.)-S-Nitroso-N-acetylpenicillamine, S-Miitosogiutathione, NOC-5, NOC-7, NOC-9,NOC-12, NOC-18, NOR-1, NOR-3, SfN-l:, Hydrochloride, Sodium Nitropmsside, Dihydrate, Spermine 'NONpate,

Streptozotocin; and (7) antibiotics, such as, without limitation: acyclovir, ofloxacin, ainpieillin, amphotericin B, atovaquoiie, azithromycin, ciprofloxacin, clarithromycin, clindamycin, clofazimine, dapSone, dielazuril, doxycycline, erythromycin, ethambutol, fluconazole, iluoroquinolones, foseamet, ganciclovir, gentamicin, itraconazole, isonia¾id, ketoconazole, levofloxacin, lincomycin, miconazole, neomycin, norfloxacin, ofloxacin, paromomycin, penicillin, pentamidine, polymyxin B, pyrazinamide, pyrimethamine, rifabutin, rifampin, sparfioxacin, streptomycin, sulfadiazine, tetracycline, tobramycin, tr fluorouridine, trimethoprim sulfate, Zn-pyrlthione, and silver salts such as chloride, bromide, iodide and periodate,

[0045] Further examples of additional active agents include: basic fibroblast growth factor (bFGF or FGF-2), acidic fibroblast growth factor (aFGF), nerve growth factor (NGF), vascular endothelial growth factor (VEGF), hepatoeyte growth factor (HGF), transforming growth factor-beta pleiotrophia protein, midkine protein, platelet-derived growth factor. (PDGF) and angiopoleiin-1 (Ang-1). Active agents are included in the delivery system described herein, and are administered in amounts effective to achieve a desired end-point,- such as angiogenesis, tissue growth, inhibition of tissue growth, or any other desirable end-point.

[0f 46 j According to one aspect, complex structures are provided that comprise the coacervate described herein mixed with, distributed within, or otherwise combined with another composition, such as a hydrogel , a polymer, an inorganic substrate, a medical implant or device such as a prosthetic, a dosage form, a woven or non-woven mesh, etc.. According to one aspect, the. coacervate is combined with a hydrogel, *for* example by embedding the coacervate in a hydrogel. Such a structure is useful for providing complex release profiles for active agents, for instance for promoting specific tissue growth or as a timed-release dosage form, In such an aspect, one or more active agents are distributed by any method in the coacervate and in the hydrogel so as to cause a defined degradation and release pattern. One useful aspect would be to embed the coacervate having a first active agent into a hydrogel, having a second active agent, to provide a complex release profile. In. any aspect, the active agentfs) can be any effective active agent(s), for example as described above. As an example, factor A is embedded into a hydrogel, e.g. a fibrin gel, for early release and factor B is contained within the eoacervate, for delayed release. For each indicated purpose it is noted that appropriate relative amounts of the coacervate and hydrogel may be used, as well as including effective amounts of the active agents for the intended purpose, respectively in the eoacervate and hydrogel. Appropriate and effective amounts of each component can be determined in the ordinary course by a person of Skill in the art.

{0047] Examples of useful active agents and coBibinafions of agents for incorporation into the described eoacervate for treatment of cancers include: 1L2, IL-12, and IFNy and combinati ons thereof. Also described herein is a method of treatment of myocardial infarction, using the combination of 1L-10 and -fibroblast: growth factor-2 (FGF-2) in the described -eoacervate.

{(1048] The .eoacervate composition is delivered *in* any manner useful for treatment of a condition in a patient, such as for treatment of cardiovascular disease or cancer, such as by enteral, parenteral, or topical routes, for example and without limitation by: intravenous (IV), local injection, intramuscular, intracerebral, subcutaneous, orally, inhalation, topically, enema, intravaginai, intrauterine, ocular, or otic routes.

{0049] Suitable exeipients or carriers are employed for delivery of the eoacervate composition, though the exeipients are consistent with maintenance of the eoacervate complex. Suitable exeipients are broadly-known in the pharmaceutical arts, and include: solvents, such as water, phosphate-buffered saline (PBS), saline; buffers; salts; acids; bases; rheology modifiers; chelating agents; colorants; flavorings; penetration enhancers; and preservatives. The eoacervate composition is provided in a suitable vessel for storage, distribution and/or use of the composition. In one aspect, the eoacervate composition is provided in a tube, a medical syringe, an IV bag. In another aspect, the eoacervate composition is delivered to a patient in an amount effective to treat a myocardial infarction, for example by direct injection of the eoacervate composition comprising IL-10 and FGF-2 into the heart, e.g., the myocardium at or adjacent to an: infarct

{005e] An "amount effective" for treatment of a condition is an amount of an active agent or dosage form, such as the eoacervate composition described herein, effective to achieve a determinable end-point. The "amount effective" is preferably safe - at least to the extent the benefits of treatment outweighs the detriments and/or the detriments are acceptable to one of ordinary skill and/or to an appropriate regulatory agency, such as the U.S. Food and Drug Administration, In the context of cancers, the end point may be increased survival, reduction in tumor mass, or any other objectlvely-determinable indicator of improvement in a patient's condition or symptoms, Using the teachings of the present disclosure, a person of ordinary skill in.the arts- can prepare the eoacervate composition described herein, and titrate the effect on any objeetively-determinable end-point, such as tumor mass or survival, for instance first in an animal model and later in humans, As shown in the Examples below, -an example of an "amount effective'* is indicated.

{0051] The eoacervate composition may be administered continually for a period of time, or at intervals, ranging from hourly, weekly, monthly, or yearly, including increments therebetween,

such as from one to six times per day, daily, every other day, weekly, bi-weekly, -monthly, bi-monthly, quarterly, etc. An appropriate dosing schedule can be -determined by a person of ordinary skill, such as a physician, and can also be tailored to disease progression and severity in a patient (e.g., staging and/Or grading) and/or the type of cancer, or improvement *in* cardiac output or repair.

{0052) In use, according to one aspect, the coacervate composition is delivered to a patient in an amount effective to treat a cancer or hyperplasia in a patient. Cancers or hyperplasia particularly suited for-treatment in this manner include solid tumors, that is, a mass or masses of cancerous cells, such as melanoma, or other hyperplasia. The composition is delivered, for example by injection, at or adjacent to a mass. In one aspect, the composition is delivered to a patient at or adjacent to a mass, such as a tumor, the composition comprising IL-12, The composition is administered in an amount effective to treat the cancer, that is to improve one or more clinically-relevant markers, such as to reduce mass size, to destroy the mass, to reduce the cancer .grade, and/or to improve patient survival

{0053) In another -aspect, the coacervate composition is delivered to a patient in an amount effective. -to treat a cardiovascular disease, such as coronary heart disease, including treatment of ischemic conditions, such as myocardial infarction. In one aspect, the composition is delivered to a patient's myocardium at or adjacent to an infarct, the composition comprising an antiinflammatory immunomodulatory cytokine, such as IL-10, and an amount of an angiogenic growth factor, such as FGF-2, The cytokine and angiogenic growth factor are administered in an amount effective to treat the infarct, that is to improve one or more clinically-relevant markers, such as to improve cardiac function parameters such as myocardial elasticity, to reduce infarct size, to increase revascularization of the infarct, to reduce scarring of the myocardium, and/or stimulate repair of the myocardium. Other conditions, such as myocardial reperfusion injury and peripheral artery disease may be treated in the same manner.

Example 1 ~ Synthesis and testing of PEAD

10054) Synthesis and testing of PEAD, PEAD-heparin, and PBAD FGF2 are described in United States Patent No. 9,023,972, which is incorporated by reference in its entirety. Briefly, for synthesis of PEAD - t-BOC protected aspartic acid (t-BOC Asp), t-BOC protected arginine (t-BOC-Arg) (HMD Chemicals, NJ), ethylene glycol dlgiycidyl ether (EGDE), trifluoroacetic acid (TFA) (TCI America, OR), anhydrous 1,4-dioxane and tetra-n-hutylamraoniura bromide (XBAB) (Acros organics, Geel, Belgium), dicyclohexylearbodiimide (DCC), N-hydroxysuecimmide (NHS) (Alia Aesar, MA) and 4-dimeihylammopyiidine (DMAP) (Avocado Research Chemicals Ltd, Lancaster, UK) were used for PEAD synthesis without

purification. The synthesis of PEAD is performed as follows. EGDE and t~BOC Asp were polymerized in 1,4~dioxane under the catalysis of TBAB, t~BOC protection was later removed by TEA to generate primary amine. t~BOC-Arg was conjugated by DCC/NHS/DMAP coupling followed by the second de-protection to yield PEAD. The chemical structure was confirmed using NMR and FT~IR. The molecular weight of PEAD was measured by PL-GPC 50 Plus- RI equipped with a PD 2020 light scattering detector (Variaft, MA). Two MesoPore 300x7.5 mm columns and 0,1% of LiBr in DMF were used as solid phase and mobile phase, respectively. In one example, the weight-average -molecular weight (Mw) is 30,337 Da with polydispersity index (PD!) 2.28.

[0055] Since PEAD is a positively-charged molecule, addition of PEAD into heparin solution should neutralize the negative charge ofheparin and forms PEAD/heparin complex. To test the binding ability of PEAD to heparin, zeta potential measurement was performed and the zeta potential of the complex shifted from negatively-charged- (-45 mV) at ratio 1 to positively-charged {+23.2 mV) at ratio 10. Continuing adding more PEAD did not change the zeta potential and +23.2 .mV -is close to the zeta potential of PEAT) itself Data suggested that for the described PEAD preparation after ratio 10 the complex was all covered by PEAD, Besides it also shows at ratio 5 PEAD almost neutralized all negative charges of heparin. From the macroscopic observation, below ratio 5 the addition of PEAD let the heparin solution became more turbid and precipitate was seen after a few minutes. Whereas the ratio was over 5, the addition of PEAD would let the solution become clear again.

[0056] Further confirming the binding-ability, different amounts of PEAD to heparin solutions were mixed and then precipitated by cenirifugation.. Because the neutralization of the negative-charged heparin favors the formation of precipitate, we measured the amount ofheparin left in the supernatant was measured to determine the binding affinity between PEAD and heparin. For this assay, a heparin binding dye, dimethylmethylene blue (DMB) was applied to detect free heparin by measuring the- absorption of DMB at 520nm. The result shows the amount of heparin in the supernatant was gradually lowered with the addition of PEAD. When the ratio of PEAD to heparin is over 3, > 90 % of heparin was precipitated through eentrifiigation. At the ratio 5, that would be > 99 % of heparin. This resul t has a good correlation with that of zeta potential measurement because both experiments suggest at ratio 5 PEAD and heparin has the maximum interaction.

[0057] It is understood that a variety of growth factors can bind to heparin with the dissociation constant (Kd) from μM to nM, The loading efficiency of growth factors to PEAD/heparin eomplex was studied. lOOng or 500ng of fibroblast growth factor-2 (FGF-2) plus ^{125}I-labeled

FGF-2 used as a tracer were mixed with heparin then added into PEAD solution. After staying at room temperature for 2hr, centrifugation was used to precipitate PEAD/heparin/FGF-2. The amount of unloaded FGF-2 remaining in the supernatant can be determined by a gamma counter. The; result showed PEAD/heparin loaded ~ 68 % of EGF-2 for both high and low amounts o:fFGF-2. The other growth: factor, NOP, the release is:.faster. Th einitial burst reached almost 20%. The release sustained till day 20 and reached a plateau corresponding to ~ 30% of the loaded NGF.

Example 2 – Cytokine delivery and **treatment** of **cancer**

[0058J The following represents use of the compositions described herein in the eancer setting using coaeervates integrating the immmostimulattny cytokines IL-2, IL-12, and IFN-γ as loeoregional therapies against murine melanomas.

Methods;

|(f059] *Generation of QC of Coacervaies:* Coaeervates containing cytokines will be prepared according to the following. Briefly, poly(ethylene argininylaspartate diglyceride) (PEAD) and clinical-grade heparin (Scientific Protein Labs, Waunakee, WI, USA) will be separately dissolved in 0.9% normal saline (Baxter Healthcare. Deerfieki IE, USA) at 10 mg/ml and sterilized by passing through a 0.22 μm syringe filter. A 5:1 ratio of PEAD and heparin by weight will be used to maintain electric neutrality (that is, the coaeervate has a neutral charge where the rado of the polycationie polymer to the polyanionic polymer is such that the overall positive charge of the polycationie polymer equals or approximates the overall negative charge of the polyanitmic polymer in the coaeervate). Heparin will be first complexed with a pre¬ determined, equal amount of recombinant murine IL-2, IL-12p70, or IFN-γ (all irons PeproTech, Rocky Hill, NJ, USA) and mixed well. PEAD will subsequently be added into the solution containing [hepar mcytokine] complexes. Self-assembly of PEAD and [heparimeytokine] will immediately precipitated the ternary complex out of solution to form the cytokine coacen-ates, Precipitation of coaeervate complexes will immediately increase turbidity in solution. Coaeervates will be freshly-prepared immediately before all *in vitro* and *in vivo* experiments- to avoid aggregation, Coaeervate droplet sizes will be measured using a Zetasizer Nana 2 S90 (Malvern, Worcestershire, UK) and reported as the mean with poiydispersity index (PD1) from 25 measurements. Results will then be averaged from measurements of three independent coaeervate samples for each cytokine cohort. PD1 in the area of light scattering will depict the droplet size distribution,

[0060] The cytokine .release profile of the prepared coacervate s will be determined *in vitro* as previously described (Chen WC, *el al.*, Controlled dual delivery of fibroblast growth factor-2 and Inе rleukin-10 by heparin-based coacervate synergisiically enhances ischemic heart repair, *Biomaterials*. 2015;72:138-51. PMID; 26370927; PMCiD: PMC4617784). To simulate release of cargo molecules, phosphate-buffered saline (PBS) supplemented with 0.5 U/mL heparinase II will be added to each sample to bring up the final volume to 200 μL. Four independent samples were then placed statically in a humidified cell culture incubator at 37 °C. At Day 0, 0.5, 1, 4, 7, 10, 14, and 21, samples will be pelleted by eentrifisgation (12,100 g for 10 rain), followed by the collection ©f supernatants. Samples xviil then be replemshed with fresh solution and well mixed before being returned to the incubator. Solutions wiilbe stored at -80 °C prior to analysis using cytokine-specific ELISA (BD Biosciences).

[0061] *Tumor Therapy Experiments:* C57BL/6 mice will be injected s.e. in their right flank with 1-2 x 10^5 syngeneic melanoma cells (BRA P^{WT} B16 or BRAP ^{V600E} BP) and tumors allowed to establish for 7-10 days. Tumor-bearing mice will then be randomized into cohorts of 5 nice/giOup, with each group exhibiting similar mean tumor siz.es (based on the product of orthogonal measurements in mm^2). Cohorts of mice will then be injected iatra-tumorally (i t.) with 50 microliters of i.) PBS (control), if) a. eytokine-free coacervate (control), iii.) eoaeervates containing rmIL-2, iv.) eoaeerv at.es containing rmIL~12, v.) eoaeervates containing rmIFN- γ, vi.) rmIFN-γ (control), vii.) rmIL-2 (control), viti.) tmlL-12 (control), and/or ix.) rmIFN-y (control). Combinations of cytokines also are- tested essentially as indicated above. If *in yitro* QC analyses suggest abbreviated release of a given incorporated cytokine, individual cohorts of mice may be retreated with an identical i.t injection (PBS, control coacervate or cyiokine-containtng coacervate) based on. the kinetic profile of cytokine release. Mice will be monitored for tumor size over time, as well as, iime~to-euthanasia as a measure of survival. Animals will be euthanized if melanomas exceed a size of 400 mm^1 or if they become openly ulcerated. It is to be expected that animals undergoing a protective immune response will exhibit inflammation at tumor sites, hence the reddening of lesions may be reflective of an ongoing local immune response and will not he. .grounds for euthanasia. Mice will also be euthanized if they exhibit signs of discomfort or behavioral abnormalities (i.e. hunching, labored breathing, fur raffling), or if they exhibit a >20 % weight-loss on protocol. Experiments will be performed at least twice for both the B16 and BP melanoma models.

[0062] Second-level analyses of eoacervates thai mediate statistically-significant therapeutic benefits to raelanoiua-bearing mice will be evaluated in 2-site (s.e, right flank + s.e, left fiank) melanoma models, where only tumor on the right flank will be treated by i.t delivered

coacervates (vs. PBS). **This** will allow us **to** discern systemic immune benefits resulting from treatment on both **directly-treated** tumors (right **flank) vs.. untreated** lesions (left flank), **allowing** us to interpret therapy efficacy against disseminated disease. At time of euthanasia, we will harvest the lungs of tumor-bearing mice to enumerate pulmonary metastases (i.e. both B16 **and BP** melanomas spontaneously **metastasize to.** the lungs) as we have **previously** described [3*]. If > 1 cytokme-containmg **coacervate** mediates **anti-melanoma** efficacy in **vivo, we.** plan to determine whether combination of such species is capable of providing improved treatment **outcome.** Such studies would involve cohorts (n =—5 each) of melanoma-bearing animals treated with i) **PBS,** ii.**) eytokine-free coacervate,** hi.) **cytokine** 1 **coacervate,** iv.) cytokine 2 coacervate, v.) cytokine **1** coacervate + cytokine 2 coacervate. Combinations of cytokines also are tested essentially as **indicated** above,

[0063] In **both** single and 2-site **melanoma** models we may include additional animals per cohort to allow for immune monitoring. in particular, 1-2 additional mice/e atment cohort would allow **for** us to harvest spleens, tumor-draining lymph nodes (TDLNs) and tumors for analysis of anti-melanoma **CDS*** T cell **.frequencies** (after stimulation with melanoma antigen [MARTI, gp100, TRP2]-derived peptides as monitored in IFN-γ **ELISPOT** assays) and total lymphocyte subset counts (i.e. $CD4^+$ **T** cells, $CD8^+$ **T** cells, **NKp46*** NK. cells, **CD4*Foxp3*** Treg, and $CD11b^+Gr1^+$ MDSC as determined **by** flow cytometry). It would be anticipated that **cytokine-containing** coacervates mediating **treatment benefits** would promote increased anti-specific $CD8^+$ T cell frequencies and reduced presence of Treg/MDSC based on our past **experience** with effective immunotlieraples in murine melanoma models,

{©064) *Siativics:* Non-parametric tests **will be** used **for** the comparison **of different** groups **of** in vitro experiments. Mixed effect models will **be** fit to the log scale tumor volume to compare the growth **curve** of different treatment groups to **controls.** Time-to-euthanasia will be summarized by the Kaplan-Meier method, and log-rank tests will **be** used **to** compare the **survival** curves between different treatment groups.

Example 3 - Cytokine delivery and treatment of myocardial infarctioa (MI)

[0065] Myocardial infarction **(MI)** causes myocardial necrosis, triggers **chronic** inflammatory responses, and leads to pathological remodeling. Controlled delivery of a combination of angiogenic and immunoregulatory proteins may **be** a **promising therapeutic** approach ior MX **We** investigated **the** bloaetivity and therapeutic potential of an injectable, **heparin-based** coacervate co-delivering an angiogenic factor, fibroblast growth **factor-2** (FGF2), and an **anti¬inflammatory** cytokine, Interleukin-10 (IL-10) **m** a spatially and temporally **controlled** manner.

Coacervate delivery of FGF2 and IL-10 preserved their bioactivMes on cardiac stromal cell proliferation in vitro. Upon intramyocardiai injection into a mouse MI model, echocardiography revealed that FGF2/1L-10 coacervate treated groups showed significantly improved long-term LV contractile function and ameliorated LV dilatation. FGF2/IL-10 coacervate substantially augmented LV myocardial elasticity. Additionally, FGP2/IL-10 coacervate notably enhanced long-term revascularization* especially at the infarct area. In addition, coacervate. loaded with 500 ng FGF2 and 500 rig 11,-10 significantly reduced LV fibrosis, considerably preserved infarct wall thickness, and markedly inhibited chronic inflammation at the infarct area. These results indicate that FGF2/IL-10 coacervate has notably greater therapeutic potential than coaeervaie containing only FGF2. Overall, our data suggest therapeutically synergistic effects of FGF-2/IL-10 coacervate, particularly coaeervaie with FGF2 and 500 ng IL-10, for the treatment of ischemic heart disease.

Example 4 – Controlled **Dual** Delivery of Fibroblast **Growth** Facte **r**-2 and **Inte**r**leu**kin-**10** by **He**par**in-base**t**l** Coacervate Syncrgistiealiy Enhances **Ischemic Heart** Repair

{0066} We recently developed a controlled delivery system that utilizes the charge interaction between a biodegradable polycation, poly(ethylene arginioylaspartate dlglyceride) (PEAD), and a natural polyanion, heparin, to form coacervate. This heparin-based coaeervaie delivery platform protects and steadily releases heparin-binding growth facto rs, including fibroblast growth factor-2 (FGF2) (See, e.g., U.S. Patent No. 9,023,972, which is incorporated herein by reference in its entirety), nerve growth factor (NGF), heparin-binding epidermal growth factor-like growth factor (HB-EGP), stromal cell-derived factor (SDF)-1*a*, and bone morphogenetic protein-2 (Chu, H., *et al., A [polycation: heparin} complex releases growth factors with enhanced hioacimty.* Journal of Controlled Release, 2011. 1.50(2): *p*. 157-163; Johnson, N.IL and Wang, Y., *Controlled delivery--ofheparin-binding EGF-like growth factor yields fast and comprehensive-wound healing.* Journal of Controlled Release, 2013. 166(2): p. 124-129; Li, R , *et a/., Sustained Release of Bone Morphogenetic Protein 2 via Coacervate Improves the Osteogenic Potential ofMwcle-Derived Stem Cells.* Stem Cells Translational Medicine, 2013, 2(9): p. 667-77; and Li, 1l., *et «/., Sustained Release of Bone Morphogeneiic Protein 2 via Coacervate Improves the Osteogenic Potential of Muscle-Derived Stem- Cells.* Stem Cells Translationa! Medicine, 2013. 2(9): p. 667-77). In addition, heparin-based coacervate has been shown to efficiently deliver H8-EGF hi a mouse model of skin wound healing, accelerating keratinocyte migration and wound closure, and FGF2 in a mouse model of subcutaneous injection, promoting local neoangiogenesis and blood vessel maturation (Johnson, MR. and Wang, Y., Journal of Controlled Release, 2013. 166(2); p. 1.24-129 and Chu, R , *et al,*

Injectable fibroblast growth factor-2 coacervate for persistent angiogenesis. Proceediigs of the National Academy of Sciences, 201 1. 108(33): p. 13444-1 3449. Furthermore, in a murine *MI* model, coaeervate containing 500 ng FGF2 has been proven effective in augmenting functional angiogenesis and blood vessel stabilization, reducing catdiorayoeyie death and peri-infarct fibrosis, and improving cardiac function (Chn, H., *et al., The effect of a heparin-based coaeervate of fibroblast growth factor-2 on scarring in the infarcted myocardium.* Biomaterials, 2013. 34(6): p. 1747-1756). Utilizing the versatile protein-binding capacity of heparin, we theorized that dual delivery of FGF2 and an anti-inflammatory agent by coaeervate can be therapeutically more effective than the delivery of FGF2 alone,

[0067] Interleukin-10 (lL-10) is a p!eiotropie cytokine that exhibits broad immunoregulatory and anti-inflammatory activities. Human lx.-10 binds to heparin with high affinity at pH 7.4 (Kd=54 ± 7 nM). The role of IL- 1.0 in the cardiac milieu has been investigated in recent years. In congestive HF patients, higher plasma levels of anti-infiamniatory mediators such as IL-10 notably correlates with augmented contractile function of the left ventricle (LV). Daily subcutaneous- injections of recombinant human IL,-10 (rhIL-1O, 75 gg/kg-day) for 4 weeks in a rat model of acute MI (AMI) resulted in. significantly reduced productions of proinflammatory cytokines, diminished myocardial macrophage infiltration, and augmented LV function (Stumpf C., *et al., Interleukm-10 improves left ventricular function in rats with heart failure subsequent to myocardial infarction.* European Joun al of Heart Failure, 2008. 10.(8): p. 733-739). Nonetheless, due to its short half-lite (2,7 to 4,5 hours) after subcutaneous injection, it typically requires repeated -administrations of high-dose lL-10 to achieve therapeutic potency, leading to increasing risks of side-effects and high treatment cost Given its high heparin-binding affinity, coaeervate may serve as an. ideal vehicle for sustained, localized delivery of IL-10.and further reduce the required therapeutic dosage.

[0068] Controlled co-delivery of two trophic factors to promote tissue repair has lately been explored, In particular, sustained delivery of FGF2 and hepatoeyte growth factor (HGF) via cross-linked albuniin-alginate microcapsules augmented angiogenic and arteriogenic responses, improved cardiac perfusion and function, and. attenuated cardiac hypertrophy and fibrosis (Banquet, *$,f et al., Arteriogenic Therapy by Inimmyomrdial Susiained Delivery of a Novel Growth Factor Combination Prevents Chronic Heart Failure.* Circulation, 201 I. 124(9): p, 1059-1069). Co-delivery of angiogenic FGF-2 and arteriogenic platelei-derived growth factor (PDGF)-BB with self-assembling peptides resulted in reduced infarct size, stable vessel formation, and improvernent of cardiac function (Kim, X.H., *et al., The enhancement of mature vessel formation and cardiac fimetion in infareied hearts using dual growth factor delivery*

with self-assembling peptides. **Biomaterials,** 2011. 32(26): p. 6080-6088), Using a poly(D,L-lactic-co-glycolic acid) micro sphere/alginaie **hydrogel hybrid** system, combined: delivery of vascular **endothelial** growth factor (VEGF) and angiopoietin-I synergisEcally enhanced **vascular** Maturation and attenuated muscle degeneration **at** the ischemic site in an **murine** model hind-limb ischemia, more **effective** than single **factor delivery** (Shin, S.-H., *et «/., Co-delivery of Vaseular Endothelial Growth Factor and Angiopoietin-l Using Injectable Microsphere/Hydrogel Hybrid* ***Systems*** *far Therapeutic Angiogenesis.* **Pharmaceutical** Research, 2013, 30(8)' p. 2157-2165). Do the other hand, our group recently demonstrated **that** heparin-based coacervate is capable of **incotporatiag and sustaining** the **release of VEGF** and **HGF** for at least three weeks (Awada, H.K., Johnson, N.R., and Wang, Y., Dual Deliver)'- of Vascular **Endothelial Growth Factor** and **Hepatoeyte** Growth Factor **Coacervate** Displays Strong Angiogenic Effects. *Macromolecular Bioscience,* 2014. 14(5); p. 679-686). Dual delivery of VEGF and HGF by coacervate showed stronger angiogenic effects on endothelial cell proliferation and tube **formation** in vitro than free or coacervate **delivery** of individual factor (Awada, H,K., Johnson, N.R., and Wang, Y., *Macromoleeular Bioscience,* 2014, **14(5):** p. 679-686).

[0069] It was hypothesize that dual delivery of FGF2 and !L-10 synergistically enhances their angiogenic and/or cardioprotective potency in the ischemic heart. Here, the characterized FGF2/IL-10 **coacervate is** chaa cterized and its bioaeiivity **on cardiac** stromal **cells** in vitro **is** investigated. The therapeutic efficacy of FGF2/1L-10 coacervate was evaluated in a **mouse** AMI model. The data suggest that controlled release of FGF2 and IL-10 by heparin-based **coacervate** exerts synergistic **effects** in improving long-term cardiac **function,** augmenting **myocardial** elasticity, promoting revascularisation, ameliorating **myocardial** fibrosis, and **inhibiting** chronic inflammation,

Material and Methods

[0070] Preparation of PGF-2/IL-10 Coacervate: Poly(ethylene argininylaspartate diglyceride) (PEAD) was synthesized as previously described. PEAD and clinical-grade heparin **(Scientific, Protein** Labs, Waunakee, **WI,** USA) **were separately** dissolved in 0.9% **normal** saline (Baxter Healthcare, Deerfield, IL, USA) **at 10** mg mi^{-1} and sterilized by passing through 0.22 μη syringe filter. A 5:1 ratio of PEAD and heparin by weight was used to maintain electric neutrality **as** previously described [7]. Heparin was first eomplexed with a **predetermined,** equal **amount of recombinant** human FGF-2 (rhFGF-2; 17.2 kDa protein consisting of 154 amino acid residues) and IL-10 (rhIL-10; 18,6 kDa protein of consisting of 161 amino acid residues) (both from PeproTech, Rocky Hill **NJ,** USA) and mixed well. PEAD

was subsequently added into the solution containing [heparin:FGF-2/IL-10] complexes. Self-assembly of PEAD and [hep&rin:FGP-2/lL-10 j immediately precipitated the ternary complex out of solution to form the FGF-2/IL-10 eoacervate. Precipitation of eoacervate complexes immediately increased opaque turbidity in solution, Coacervate was freshly prepared immediately before all ***in viiro*** **and *in viva*** experiments to avoid aggregation. Coacervate droplet size was. measured by Zetasizer Nano ZS90 (Malvern, Worcestershire, UK) and reported as the mean with polydispersity index (PDI) from 25 measurements. Results were then averaged trora measurements of three independent eoacervate saniples. PDI in the area of light scattering depicts the droplet size distribution.

[0071] Fluorescent labeling of FGF-2/IL-iii **Coacervate:** To fluoreseently visualize the incorporation of PGP2 and IL-10 in eoacervate complexes, amine-e act ve dyes (Thermo Scientific, Waltham, MA, USA) were utilized to label FGF2 and IL-10 molecules, following the. manufacturer's instructions. Briefly, FGF2- and IL-iO solutions were added into vials containing concentrated NHS ester-activated derivatives of DyLig!it 488 and DyLight 594 respectively and reacted at room temperature for 1 hour. A spin desalting column was used o remove unreacted dyes. To triply label biological components in FGF-2/IL-10 eoacervate, FGF2 and IL10 were first labelled with NHS-DyLight 488 and NHS-DyLight 405 individually. FGF2-DI.488 and IL-10-DL405 were then mixed well with heparin before rhodamine conjugated *ukx eiropaeus* agglutinin I.(UEA-1) was applied to label heparin. PEAD was then added into the solution containing [heparin-f iodarmne: FGF2-DL488/IL-10-DL405] complexes to form coaeervate.

[0072] ***In vitro*** **Release** Profile of FGF-2/IL-10 **Coaeervate:** The release profile of FGF2/IL-10 coaeervate was determined ***in*** *vitro* as previously described. Briefly, **FGF2/IL-10** eoacervate was freshly prepared with a mass ratio of PlSAD:heparln:FGF2;iL- 10^500; 100:1;!, using 100 ng each of FGF2 and lL-10. To simulate .release of cargo molecules ***in vivo,*** addit onal phosphate-buffered saline (PBS?) supplemented with 0,5 U/mL heparinase 11 was added to each sample to bring up the final volume to 200 µt . Four independent samples were then placed statically in a humidified cell culture incubator at 3? °C. At Day 0, 0.5, 1, 4, 7, 10, 14, and 21, samples were pelleted by eentrifugaiion (12,100 g for 10 min), followed by the collection of supematants. Samples were then replenished with fresh solution and well mixed before being returned to the Incubator. Solutions were stored at -80°C for future analysis. After the final collection on Day 21, samples were replenished with PBS supplemented with 2 U/mL heparinase II and incubated at **37** *°C* overnight in order to dissociate the remaining eoacervate.

The amount of FGF2 and IL-10 released into the supernatant was quantified by enzyme-linked immunosorbent assay (BUSA) for FGF2 or IL-10 respectively (both from Abeam, Cambridge, iVlA, USA), following tide manufacturer's instructions. Supernatants collected from four samples at all-time points were analyzed simultaneously,- The absorbarice was recorded by SynergyMX (Biotek, Winooski, VT, USA) or Infinite 200 PRO plate reader (Tecan, Mannedorf, Switzerland). Results were averaged. The loading efficiency was determined from the -first collection immediately after the initial resaspension (Day 0).

[0073] **Primary Cell Isolation and Culture:** Single donor-derived human umbilical vein endothelial cells (HUVECs) and human cardiac fibroblasts (hCFs) were purchased from Lonza (Allendale, NJ, USA) and respectively expanded in complete endothelial cell growth medium 2 (EGM-2, Lonza) and DMEM high glucose supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S) (all from Life Technologies, Grand island, NY, USA), Mouse cardiac fibroblasts (naCFs) were isolated as previously described (Balasubramaniam S., *et* ff/., β3 Integral in Cardiac Fibroblast Is Critical for Extracellular Matrix Accumulation during Pressure Overload Hypertrophy in Mouse. *PLoS ONE,* 2012. 7(9): p. e45076) and expanded in. DMEM high glucose with 10% FBS and 1% P/S. Human heart pericytes (hHPs) were isolated and purified by flow cytometry as we previously reported (Chen, W.C.W., *et al.*, Human Myocardial Pericytes: Multipotent Mesodermal Precursors Exhibiting Cardiac Specificity. *STEM CELLS*, 2015, 33(2); p. 557-573). hHPs were expanded in DMEM high glucose with 20.% FBS and 1% P/S. Primary cells at passage 5*7 were used in subsequent experiments,

[0074] **Measurement of Cell Proliferation** *in vitro:* HUVECs, hCFs, MIPs, and mCFs were tiypsinizedand plated in triplicate (1.3 x 10^3 cells/well) overnight with 100 µl complete culture media in bottom wells of a UTS Transwell-96 well permeable support system (Corning, Tewksbury, MA, USA), Immediately before the transwell support was assembled, all bottom wells were first filled up with 135 µl fresh serum-free basal media (EBM-2 for HUVECs and DMEM for hCFs, hHPs, and mCFs; both supplemented with 1% P/S) with or without 10 ng/ml (for HUVECs) or 100 ng/ml (for all other cell types) of tumor necrosis factor alpha (TNF-α). Free 500 ng FGF2 combined with either 100 ng or 500 ng IL-10 and coaervate containing a fixed load of 500 ng FGF2 alone or combined with either 100 ng or .500 ng IL-10 were resuspended in 75 µl serum-free basal media and then added into transwells. Control transwells were added with plain basal media with or without empty coacervate vehicle. The final concentration of serum was approximately 33% .of that in complete culture media in each well. Plates were assembled and subsequently incubated for 72 hours under ambient conditions.

After washing all wells, CellTiter 96* AQueous One Solution Cell Proliferation Assay (MI S) reagent (Promega, Madison, WI, **USA)** in DMEM was added, The plate was incubated in 5% CO_2 at 37 °C for 3 hrs, at which point the absorban.ee at 490 nm {with reference at 650 ran) was read with Infinite 200 PRO plate reader (Tecan, Männedorf, Switzerland). A11experiments were independently repeated 3 times. Results were individually normalized to each experimental control and then averaged.

[0075] Experimental Animals: A Total of 77 male BALB/cJ mice at 9-12 weeks old (Jackson Laboratory, Bar harbor, ME, USA) were used for this -study.

[0076] Intramyocardial Administration of FGF-2/IL-10 Coacervate in a Mouse Model of Acute **Myocardial** Infarction **(AMI):** After the induction of anesthesia with 4% isoflurane gas, mice were intubated and inhalationally anesthetized with 2% isoflurane gas throughout the surgery. The induction of myocardial infarction (Ml) and intramyocardiai injection have been performed -as previously reported (Chu, H., *ei al., Biomateriais*, 2013. 34(6); p. 1747-1756 and Chen, C.-W., *et ai.*, Human Pericytes for Ischemic Heart Repair, *STEM CELLS*, 2013. 31(2): p. 305-316). In brief, MÏ was -microscopically induced by permanent ligation of the lef anterior descending coronary artery (I A D). Mice were then randomly assigned to one of the five groups: saline control, FGF2 500 ng coacervate (Coa-F-500), Free FGF2/IL-10 500/500 ng (Free-F/I-500/500), FGF2/IL-10 500/100 ng eoaeervate (Coa-F/i-500/ 100), or F6 F2/IL-10 500/500 ng. coacervaie (Coa-P/I-500/500). Five minutes after the induction of Ml free or coacervate FGF2/IL-i0 diluted in 30 µi of sterile *0,9%* norma! saline were injected at three sites of the ischemic myocardium (center: and two borders of the infarct). Control mice received injections of 30 µï saline,

[0077] Echocardiography: iichocardiographie studies were performed repeatedly before surgery and at 5 - days, 2 and 6 weeks post-surgery to assess the cardiac function as we previously described (Chu, H., *ei al., Biomaieriais*, 2013. 34(6): p. 1747-1756 and Chen, €,-W., *et al.*, *STEM CELLS,* 2013. 31(2): p. 305-316). Briefly, mice were anesthetized with 2% isoflurane gas and **immobilized** on a heated stage equipped with electrocardiography. Heart and respiratory rates were continuously monitored while the body temperature was -maintained at 37°C. Bchoeardiographic parameters were measured using a high-resolution echocardiography system (Vevo 2100) equipped with a high-frequency linear probe (MS400, 30 MHz) (FUJIFILM VisualSonics, Toronto, Ontario, Canada). Three hundred B-mode frames were acquired at a frame rate of 40 Hz -during each scan. End-systolic dimension (BSD) and end-diastolic dimensions (EDD) were determined from the short axis images of the LV and measured from. 1.0 consecutive beats using the M-mode tracing. End-systolic area (ESA) and

end-diastolic area (EDA) were- measured from short-axis images of the .LV. All echocardiographie measurements were taken at the. mid-in&rct level in LV. Functional parameters, .including LV fractional shortening (LVFS), LV fractional area change (LVFAC), and LV ejection fraction (LVEF), were determined as previously described (Manning, **W.J.,** *et al.*, In vivo assessment of LV mass in mice using high-frequency cardiac ultrasound: necropsy validation. *American Journal of Physiology - Heart and Circulatory Physiology,* 1994, 266(4): *p*. H1672-H1675; Polliek, C., Hale, S.L,, and Kloner, R.A., Echocardiographie and cardiac doppler assessment **of** -Slice. *Journal of the American Society of Echocardiography,*. 1995. 8(5, Part. 1): p. 602-610; and Wandt, B., *el ah,* Echocardiographie assessment of ejection fraction in left ventricular hypertrophy. *Heart*, 1999. 82(2); p, 192-198). Mice died or sacrificed for histological analysis prior to 6 weeks post-injection were not included in the echocardiographie study.

[0078] Ultrasonic Analysis of Myocardial Elasticity: The. ultrasound in~phase and quadrature (lQ) data were separately acquired at 6 weeks post-Mi during the echocardiography scanning described in the above section (N~ 3 per group). The lQ data were then converted to the radio frequency (RF) data using standard quadrature sampling algorithms and subsequently analyzed by a blinded investigator. Briefly, pixels were selected in the lateral (infareted region) and anterior medial (non-infare. region); walls of I V in the first B~node frame. The 2D phase-sensitive speckle tracking was then applied o the RF data to obtain frame-to-frame axial displacements (direction along the ultrasound beam) of the selected pixels **(O'**Donnell, ML, *et al.*, Internal displacement and strain imaging using ultrasonic speckle tracking. *Ultrasonics, Ferroelectrics, and Frequency Control, IFEE Tramactiam on,* 1994. 41.(3); p. 314-325 and Lubhski, **M.A.,** *ei aL,* Speckle tracking methods for ultrasonic elasticity imaging using short-time correlation. *Ultrasonics, Ferroelcctrim, and Frequency Control, IFEE Transactions on,* 1999. 46(1): p. 82-96). Axial displacements were accumulated during each cardiac cycle (from diastole to systole). Axial strains in LV wall were obtained by derivative of the accumulated axial displacements. To unbiasedly estimate myocardial elasticity, two regions of interest (ROI) in the axial strain map were respectively selected in the infareted and non-infaret LV walls. Axial strains in these ROIs were spatially averaged and then normalized by dividing the averaged strata of the infareted ROI by that of the non-infarct ROI.

[0079] Histology and Immunohistochemistry: Mice were sacrificed at 6 weeks post-surgery. intraventricular injection of 1M potassium chloride (KC1) was performed to arrest hearts in diastole. For histology and immunohistoehemistry', harvested hearts were flash frozen in 2-methylbutane (Sigma-Aldrich, St. Louis, MO, USA) pre-cooled in liquid nitrogen, preserved

at -80°C, and then processed as formerly described (Chu, H., *et al.*, *Biomaterials*, 2013. 34(6): p. 1747-1756 and Chen, C.-W., *et al.*, *STEM CELLS*, 2013. 31(2): p. 305-316). Briefly, frozen hearts were serially cryosectioned at 6-8 μm thickness from apex to the ligation level (approximately 0,5 *mm* in length). Each series contained 18-21 heart sections and was collected on one glass slide. Hematoxylin and eosin (H&E) staining was performed following the standard protocol. For immunohistochemistry, sections were fixed *in* a pre-cooled (-20°C) mixture *of* methanol and acetone (1:1) for 5 min or in 4% paraformaldehyde for 8 min prior to staining. Non-specific antibody binding was blocked with 10% donkey or goat serum for 1-2 hours at room temperature (RT), and, if necessary, with the Monse-on-Mouse antibody staining kit (Vector Laboratories, Burlingame, CA, USA). Sections were incubated overnight at 4 °C with the following primary antibodies (all diluted with 5% donkey or goat serum in PBS): rat anti-mouse CD31 antibody (diluted at 1:100; Becton-Dickinson Biosciences, Franklin Lakes, NJ, USA), mouse anti-mammalian alpha-smooth muscle actin (αSMA)-FITG (diluted at 1:100; Sigma-Aidrich, St. Louis, MO, USA), and/or rat anti-mouse CD68 antibody (diluted at 1:200; Abeam, Cambridge, MA, USA). Sections were then incubated at RT for 1 hour with the following fluoroehroiiie-corijugaied antibodies: donkey anti-rat-Alexa594 IgG or goat anti-rat-Alexa488 IgG (both diluted at 1:250; Jackson Laboratory, Bar Harbor, ME, USA). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPi) (1:1000, Life Technologies, Grand island, NY, USA) at RT for 5 min. Immunofluorescent images were taken by Nikon Eclipse T1 fluorescence microscope equipped with NIS-Elsments AR imaging software (both from Nikon, Tokyo, Japan).

[008β] Measurement of Cardiac Fibrosis and Infarct Wall Thickness: Masson's trichrome staining kit (IMEB, San Marcos, CA, USA) was used to reveal collagen deposition on heart serial cross-sections, following the manufacturer's instruction. The area of collagen deposition (representing fihrosis/scar) and the area of the entire left ventricular cardiac tissue (including septa! area but excluding right ventricle and void space in the chamber cavity) were separately measured using a digital image analyzer (Image **J,** National Institutes of Health, Bethesda, **MD,** USA). Fibrotic area fraction was estimated as the ratio of lett ventricular fibrotic tissue to the entire left ventricular tissue. Results were averaged from 6 randomly selected sections at comparable infarct levels per heart. Left ventricular wall thickness at the center of the infarct was estimated as the mean of 3 adjacent measurements (0.25 mm apart) and was averaged from 6 randomly selected sections at comparable infarct levels per heart.

[0181] Quantification of Chronic **Inflammation** and **Revascularization:** To evaluate chronic inflammation within the infarct region, immunofluorescent staining of phagocytic cell

marker CD68 was performed on serial eryoseetions as described above. The infiltration index,, represented by the number of CD68+ phagocytic cells per mun^2, was subsequently computed by a blinded investigator from 6-8 randomly selected images of the infarct region of each heart at the mid-infarct. level, using image J, To quantify revasctilarixalion post-MI, immunoftuorescent staining-of endothelial cell (EC) marker CD31 and .vascular smooth muscle cell (VSMC) marker αSMA was sequentially performed on serial eryoseetions. The capillary density, represented by the number of GD3 I+ capillary £Cs per mm^2, was subsequently computed by a blinded investigator from 6 randomly selected images of the infarct or peri-infarct area of each heart at the mid-infarct level, using Image I as described previously (Chu, H., *at al*., *Biomaterials*, 2013. 34(6): p. 1747-1756 and Chen, C.-W., *et al*., *STEM CELLS*, 2013. 31(2): p. 305-316). The VSMC density, represented by the number of perivascular (i.e. adjacent to CD31+ ECs and/or surrounding vascular structures) αSMA+ cells per mm^2, was subsequently computed fo m 6 randomly selected images of the Infarct region or peri-infarct area of each heart at the niid-infaret level using Image J.

|**0082**j Multi-photon Excitation Imaging: For multi-photon -excitation (MPE) imaging, rhodaraine tagged with UEA-1 (2 μg) was -mixed well with heparin before PEAD-was added into the solution containing [heparin:rhoxlamine] complexes to form coaeervate. Intramyocardiai injection of free or heparin-bound rhodamme-OKA-l (2 μg) or rhodamine-UEA-1 coaeervate (all diluted in 30 μl of sterile 0.9% normal saline) was performed alter the induction of Ml as. described above. Hearts were harvested at 5, 14, and 28 days postinfection, washed 3 times in PBS, and immediately fixed in fresh 4% paraformaldehyde overnight. Hearts wee then washed in PBS twice and subsequently immersed in SeaieVtew-A2 optical clearing agent (Olympus Scientific Solutions Americas, Walfham, MA, USA) at. 4°C for 7-10 days. Processed hearts were block-sectioned at 1 mm thickness to-obtain cross-sections from apex to ligature immediately before performing MPE imaging on an Olympus multiphoton microscope at the Center for Biologic Imaging, University of Pittsburgh,

[0083] Statistical Analysis: All measured data are presented as mean ± standard deviation (SD)), Kaplan-Meier survival curve estimation with log-rank test was performed to compare the animal survival rate between treatment groups. Statistical differences between groups were analyzed by one-way ANOVA (multiple groups) or two-way repeated ANOVA (for repeated echocardiographic measurements) with 95% confidence interval. Statistical significance was set at $p \leq 0.05$,- Bonferroni multiple comparison test was performed for ANOVA post-hoc

analysis. Statistical analyses were performed with SigmaStat3.S (Systat Software) and SPSS21 (IBM) statistics software.

Results

[0084] Characterization of FGF~2/lL-i0 coaeervate; FGF-2 and IL-10 both have high heparin-bmding affinity (FGF-2: K_d≈74 nM [34J; IL-10: ¾ ≈54 nM). A mixture of FGF2 and IL-10 is first comp!exed with heparin and subsequently incorporated into the ternary [PFA D;heparin:FGF2/lL-10] coaeervate droplets by adding FEAD. We .have-theorized that the four structural components of FGF-2/IL- 10 coaeervate (PEAD, heparin, FGE2, and IL-10) are evenly distributed when the coaeervate forms, following affinity-based binding of FGF2 and IL-10 to heparin, charge interactions between FEAD and heparin, and physical entrapment. of FGF2 and IL-10 within the complex coaeervate (Figure 1A). FGF2/IL-10 coaeervate droplets had an average si¾e of 432.6 ±42. l nm.in diameter, smaller than the sizes of coaeervate droplets containing only FGF2 (60S,3 ± 96.3 a n) or IL-10 (502.6 ± 101 .5 nm) (Figure IB),

[0085] To simulate the release of cargo proteins *in vivo*, the amount of FGF2 and IL-10 released from FGF2/IL-10 coaeervate was measured by ELiSA after immersion in PBS supplemented with heparinase .11 (0.5 U/mL) for 0, 0.5, l, 4, 7, 10, 14, and 2.1 days (N=4). The loading efficiency of FGF2 and IL-10 was approximately 98.0 ± 1.6% and 97.9 ± 0.5% .respectively (Figure 1C). Cumulatively,. FG.F2/IL-10 epacervaie released roughly 16.l ± 3.8% and 12.5 ± 2.4% FGF2 and IL-10 respecti vely dining the first 12 hours and approximately 28.7 ± 5,0% and 14.8 ± 2,3% respectively by 24 hours (Figure 1C). The total release of FGF2 and IL-10 from coaeervate was estimated to be 86.8 ± 7.1% and 28,2 ± 3.6% respectively over the 21-day duration (Figure IC). Final digestion with 2 U/nsL heparinase II showed that at least nearly 3% FGF2 and 15% 1L-10 remained in residual coaeervate. However, these data did not take into account the spontaneous degradation of free IL-10, on average i.59% per day, in PES supplemented with 0.5 U/mL heparinase II {Figure 2).

[0086] To further demonstrate that FGF2 and IL-10 have been evenly incorporated into coaeervate droplets, we ftuorescent!y labeled FGF2 (DyLight 488, green in original) and IL-10 (DyLight 594, red in original). Spherical droplets of different sizes containing FGF-2 and IL-10 -were observed following -coaeervate formation- (Figure 1D). High-magnification eonfoeal microscopy showed an even distribution of FGF-2 and IL-10 molecules within a coaeervate droplet (Figure- IE), By triply labeling heparin (rhodamine, red in original FGF2 (DyLight 488, green in original) and IL-10 (DyLight 405, blue in original), the nearly homogeneous structure of EGF~2/lL~10 coaeervate was further revealed (Figure 1F).

{(1087] Bioaetivity of FGF-2/IL-10 coaeervate *in vitrot* The bioactivity of FGF2/IL-10 coacervate on cardiac stromal cell proliferation was tested in a non-contact -release system to avoid direct ingestion of coacervate particles by cells. Cells were cultured at bottom wells with treatment solutions in suspended transwells. Human umbilical vein endothelial cells (HUVEGs), human cardiac fibroblasts (hCFs), and human heart pericytes (bHPs), and mouse cardiac fibroblasts (mCFs) were used in this assay. Cells were seeded in complete culture media overnight and then maintained in diluted media throughout the experiment to simulate nutrient .starvation following coronary artery blockage. Based on previous study work, we selected a fixed load of FGF2 (500 ng) alone or combined with a low (100 ng) or high (500 ng) load of !L-10 for coacervate delivery (designated as Coa-F-501), Coa-F/I-S00/100, and Coa-F/I-500/500 respectively). Free 500 ng FGF2 combined with either 100 ng or 500 ng IL,-10 served as positive controls (designated as Free-F/I-500/ 1C0 and Free-F/1-500/500 respectively). Mo treatment (plain or DMEM basal medium) and empty coacervate vehicle; groups served as negative controls,

{0088) After incubation tor 72 hours, Free-F/I-500/100, Coa-F-500, Coa-F/I-500/100 and Coa-F/i-500/500 significantly increased HUVEC proliferation when compared with the no-treatment control and Free-F/I-500/500 (Figure 3A, all $p<0.01$). Coa-F/I-500/100 and Coa-F/I-500/500 showed trends of reducing hCF proliferation and notably inhibited mCF proHieratiotv when compared with Free-F/I-500/100 and Free-F/I-500/500 (Figure 3A, both $p<0.05$). Coa-F/I-500/500 demonstrated the most significant inhibition of mCF proliferation when compared with the fo-treatment control ($p=0.006$). On the other hand, Free-F/I-500/100, Free-F/I-500/500, and Coa-F/i-500/500 slightly promoted hHP proliferation (Figure 3A, all $p>0.05$). No significant difference was observed between no-treatment control and empty coacervate vehicle group in all four cell types- (Figure 3(A), $p>0.05$).

{0089] To further simulate inflammatory stress following ischemic insult, 10 ng/ml of XNF-α for HUVECs and 100 ng/ml of TNF-α for all other cell types were added into bottom wells immediately before the start of the experiment. After incubation for 72 hours, Coa-F-500, Coa-F/I-500/100 and Coa-F/I-500/500 signifieantly promoted HUVEC. proliferation when compared with the no-treatment control (Figure 3(B), all $p<0.05$). All three coacervate groups exhibited reduced hCF proliferation and significantly inhibited mCF proliferation when compared with the no-treairnent control and Free-F/I-500/!O0 (Figure 3(B), all $p<0,01$). Similarly* Coa-F/I-500/500 showed the most striking inhibition of mCF growth when compared with all non-coacervate groups (all $p<0.01$). All treatment groups maintained hHP

growth under **inflammatory** stress (all $p>0.05$). There was no notable difference between **no-**treatment and empty vehicle groups under **inflammatory** stress in all tested cell **populations** (Figure 3(B), $p>0.05$). Altogether these results suggest that FGF2/IL-10 **eoacervate** supports **HOVEC** growth under nutrient deprivation while inhibiting the proliferation of CFs, especially **under inflammatory stress.**

£0090) Intramyoeardial codelivery of FGF-2/IL-10 eoacervate synergisti eally improves cardiac function: We selected **500 ng. FGF2 combined with** a low (100 og) or **high** (500 **ng)** dose of IL-10 for eoacervate- based codelivery (Coa-F/L 50O/100 and Coa-F/I-500/500 respectively) **and examined** the therapeutic efficacy of F6F-2 /**1L**-10 eoacervate *in viva*. **lntramyocardial** injection of saline, **eoacervate** containing only 500 ng FGF2 (Coa-F-500), or free **FGF-2** 500 ng **combined** with free **IL-10** 500 ng (Free-F I-500/50O) sew ed as controls. The mortality rate was around **15%** during and **immediately -after me- Surgery.** Among all mice which recovered from the surgery, two died in each: **of the** following groups: Saline, Free-F **/I-500/500,** and **Coa-F-500**, and one died **in** each of the **following** groups: **Coa-F/I-500/l.00** and **Coa-F/I-500/500**, before the terminal time point. **Most** of these deaths **occurred within** the first week post-surgery. These **mice** were excluded from functional studies. No significant **difference** in animal survival rate was noted.

f0091] Cardiac **function** was assessed by M~ and B-mode echocardiography performed repeatedly at the **mtd*tm¾rct** level before (baseline) and **after-surgery at 5** days, 2 weeks, and 6 weeks (N=8 per group except Free~F/i~S00/500, N=7; data **analysed** by two-way repeated ANQVA). By analyzing the treatment effect, both FGF2/1L-10 eoacervate groups exhibited substantially higher LVFS (Figure 4A). **LVFA.C**(Figure **413), and LVEF** (Figure **4C) than** Saline **(all** $p<0.001$) and Free-F/i-500/500 (all $p<0.05$**),** indicating better **LV contractility** following controlled release of FGF2 and **1L-10.** However, only Coa-F/l~50O/5OO, but not **Goa-F**/I-500/100, showed **significant improvement in a**ll three contractile parameters when compared with Coa-F-500 **(all** $p<0.005$**)**. Moreover, Coa-F/I-500/500 had significantly bete **LVFAC** and LVEF **than** Coa-F/I-500/100 **(both** $p<0.05$**), suggesting** a role of **IL-**10 dosage **in** prompting **a** notable **synergistic** effect for LV contractility.

[0092] In addition, ischemic hearts treated with either **Coa-F/I~500/100** or Coa-F/I-500/500 had markedly reduced LVEDA (Figure **4D)** and LVESA **(Figure 4E)** than Saline (all $p<0.001$) and **Free-F/I-500/500** (all $p<0.005$**)**, suggesting **amelioration** of progressive LV dilatation by FGF2/1L-10 **eoacervate treatment.. Similarly,** only **.Coa-F/I~500/50O,** but not Coa-F71-500/100, showed significant **diminution** in both dilatation **parameters** when compared **with-** Coa-F-500 (all $p<0.001$), suggesting a role of **IL-10** dosage in ameliorating **LV** remodeling. Overall, our

results indicate **that** the **intramyocardial administration** of FGF2/IL-10 **eoacervate, regardless** of the IL-10 dose, **significantly** improved **the** LV contractile function and reduced the LV **dilatation.** These data farther suggest the **importance** of **JL-10** dosage in the synergistic **therapeutic** effect induced by FGF2/1L-10 eoaeervate.

[0093] FGF~2/IL-10 eoacervate amends elasticity of the infareted myocardium: To further assess the **effect** of FGF2/1L-10 eoacervate on myocardial **elasticity, we** performed ultrasonic **strain estimation** at 6 weeks post~ML The axial **strains wer determined in.** the infarcied (area B) and non-infarct (area A) **LV** walls during **a** cardiac cycle using the 2D **correlation** based speckle tracking. Figure 5A shows the axial strain **maps of normal** (left panel) and untreated MI control (right panel) hearts laid over **B**-mode images **reconstructed** from IQ data respectively (negative **strains** hi blue color **and** positive strains in red), The elasticity of the infected myocardium was estimated from spatially averaged axial strains in **B** and **subsequently normalized by** dividing the averaged strain of B **by** that of A (B/A) (Figure 5A). **B-mode** images of normal and untreated *M l* control hearts without strain maps and ROis are included in Figure **6** for comparison. While all groups had similar averaged axial **strains i n** A, Coa-F-500 (both p<0.0**1),** Coa-F/i-500/1X) (both p<0.0*Ql),* and Coa-F/I-500/500 (both px 0.0Oi) had **significantly** greater normalized strains than the saline control and Free-F/I-**500/500** (Figure 4B, N=3 per group). I-fee-F/I-500/50(i also **exhibited** notably higher **normalized** strains than **the** saline **central** (p=0.004) (Figure **4B).** No significant difference was observed **between the** three eoacervate **treatment** groups (Figure 4B, all p>0,0$}, These data indicate the efficacy of **FGF2/IL-10** eoacervate in sustaining the long-term LV myocardial elasticity.

f0094j FGF-2/IL-10 eoacervate promotes long-term revascularisation: The potency of **intramyocardial** administration of **FGF2/IL-** 10 eoacervate on **long-term** revascularization was **investigated .** Immunohistochemistry **revealed the presence** of CD31+ endothelial cells (ECs; mostly located at niicrovascuiatore/eapillary) (Figure 7A**)** and vascular smooth muscle cells **(VSMCj** mostly surrounding larger blood vessels) (Figure 7A) in the **infarct -and** peri-infarct areas at *6* weeks post-infarction. **The number** of CD31+ **ECs was** subsequently **quantified** in the infarct (Figure **7.B,** left) and **peri-infarct** (Figure 7B, right) areas (N=4 per group). Within the infarct area, Coa-F/1-500/500 had higher CD31÷ EC density when compared with the saline **control .(p<0.001),** Free-F/l-500/500 (p=0.02), and Coa-F-500 *(p >0.05)* (Figure **7B, left),** Coa-F/I-500/100 had higher CD31+ **EC** density when compared with the **saline** control (p=0.007) and **Free-F/i-500/5OO** (p> 0.**05**) (Figure 7B, left). Within the **peri-infarct** area, **Coa-F/!-500/500** exhibited the highest CD31+ EC density when compared with the saline control **(p<0.001),**

Free-F/[-500/500 (p<0.001), Coa-F-500) (p=0,001), and Coa-F/l-500/100) (p>0M) *(Figure 7B,* right). Coa~F/i-500/lQ0 also had higher CD31+ EC density when compared with the saline control (p=0.008), Free~F/i~500/500 (p=0.012), and Coa~F-500 (p>0.05) (Figure 7B, right). The number of VSM€s and/or pericytes (i.e. perivascular a SMA+ ceils) was also quantified in the infarct (Figure 8, left) and peri-infarct (Figure 8, right) areas (N=4 per group) at 6 weeks post-infarction. The results showed that Coa-F/l-500/500 had significantly higher VSMC density when compared with the .saline control (p=0,008), Free~F/i-5Q0/5t)0 (p=0.038), and Coa~F-500 (p=0.031) within the infarct area (Figure 8, kit). In addition, hearts treated with Coa-F/l-500/100 and Coa-F/l- 500/500 exhibited trends of increased VSMCs within the peri-iniarct area (Figure 8, right). Altogether our nasults suggest FGF2/1L~10 coacervate treatment promotes Long-term revascularization at 6 weeks post-infarction, especially with Coa-F/I-500/500 treatment. Additionally, these data imply that the revascularzing effect of FGF2/IL-10: eoaceryate is positively correlated with the- dose pf IL-10.

[0095] FGF-2 **/IL-10** coacervate reduces myocardial fibrosis: The effect of FGF2/IL-10 coacervate on long-term LV myocardial .fibrosis was evaluated using Masson's trichrome histological staining (collagen deposition stained in bine/purple). At 6 weeks post-infarction, Coa-F/l-500/100 and Coa-F/I-S00/500 appeared to have reduced infarct size and scar formation at the mid- infarct level when compared with the saline control and Free- F/l-500/500 (Figure 9(A)). Quantitative analysis revealed that Coa-F/l-500/500 exhibited notably smaller LV scar fraction than the saline control (p=0.001) and all other treatment groups (all p>0.05) (Figure 9(B), N=4 per group). Analysis of the LV wall thickites s at the infarct center further showed that Coa-F/l-500/500 had a significantly thicker wall than the saline control (p<0.001) and all other test groups (all p<0.05) (Figure 9(C), N~4 per group). These data suggest the efficacy of coacervate containing higher dose of IL-10 in ameliorating the formation of myocardial fibrosis and preserving wall thickness post-infarction .

[0096] FGF-2/IL-10 coacervate inhibits chronic phagocytic cell infiltration: To investigate the underlying mechanism for the amelioration of myocardial fibrosis, we examined the anti¬inflammatory effeet of FGF2/3L-10 coacervate. Phagocytic cells within the infarct area were detected by anti-CD68 immunohistoehemistry at 6 weeks post-infarction. All three coacervate groups showed significantly decreased numbers of infiltrated CD68+-phagocytic cells within the infarct area when compared with the saline control (Figure 10, N=4 per group; Coa-F-500, p-0.037; Coa-F/i~500/100, p=0.007; Coa-F/i-500/500, p=0.002). In particular, Coa-F/f-500/100 and Coa-F/I-500/500 exhibited substantia! 47.2% and 59.9% reduction ofCD68+ cells

respectively when compared with Free-F/I-500/500 (Figure 10, both $p>Q,Q5$). Although there is no statistical significance in the number of CD68+ cells between all three eoacervaie groups, comparing with Coa-F-500, Coa-F/I-S00/100 and Coa-F/I-500/500 displayed notable 23.9% and 42.3% diminution of CD68+ ceils respectively, Together these results suggest that eoacervate delivery of FGF.2 and IL-10 increases their long-term potency for immunoreguiatioa, and the addition of 1L~10 *in* FGF2 eoacervate augments the Inhibition of chronic inflammation.

|0097| Estimation of the duration of eoacervate treatment *in vivo*: To estimate the duration of eoacervate treatment post-Mi, we employed multi-photon excitation (MPE) imaging to detect intramyoeardiaily injected rhodanime-tagged eoacervate (Coa-Rho). Collagen fibers were identified by second harmonic generation (SHG) signals. At 5 days after injection, Coa-Rho exhibited robust fluorescent signals within the infarct area while weak: signals were detected in free (Free-Rho) or heparin-bound (Hep-Rbo) rhodamine injected hearts. At 2 and 4 weeks post-Mi, fluorescent signals were only detected in hearts injected with Coa-Rho, but not with Free-Rho- or Hep-Rho. No signal was detected in any group at 6 weeks post-Mi. Quantification of the: fluorescence: volume found that at 5 days post-injection, Coa-Rho had 28.9 * 11.1 and 7.1: ± 2,7 folds higher signals than that of Free-Rho and Hep-Rho respectively (Figure 11, N=3 per group, $p<0.05$). Moreover, when compared with the signal on Day 5, Coa-Rho had roughly 65.9% and 25.7% of residual fluorescence volume at *2* and 4 weeks post-injection respectively (Figure 11,, N=3 per time point). These results suggest a temporal distribution and progressive degradation of eoacervate for at least 4 weeks *in situ* in infarcted hearts,

!Diseussion

{0098} Molecular therapy using trophic factors to promote cardiac repair and regeneration has been widely investigated. To promote revascularization in the ischemic myocardium, angiogenic GFs such as FGF2 and VEGF have been successfully tested in preclinical models of MI. However, clinical attempts using angiogenic GFs have demonstrated mixed results (Segers, V.M. and Lee, R., Protein Therapeutics for Cardiac Regeneration after Myocardial Infarction. *Journal of Cardiovascular Translational Research* 2010. 3(5): p, 469-477). One major obstacle of molecular therapy with exogenous GFs and/or cytokines is the short *in vivo* half-life of most biological factors. In addition, the bioavailability of systemically delivered tropliic faetor(s) in the target tissue/organ varies dramatically, highly dependent on the availability of local vasculature. These shortcomings have led to common administrations of

large, repetitive doses of GFs in order to achieve therapeutic efficacy, thus increasing the risk of on-target and/or off-target side effects. For example, VEGF can induce nitr c oxide-mediated hypotension when a dose over 50 ng/kg/min is administered by iniraeotonary infusions in. patients with myocardial ischemia. (Henry, **T.D.,** *et al.*, Iniraeorouary administration of recomMnant human vascular endothelial growth factor to patients, with coronary artery disease. *American Heart Journal*, 2001. 142(5): p. 872-880). To effectively augment the local bioavailability and potency of exogenous trophic factor(s) and minimize the required therapeutic dosage in the context of ischemic insult, a sisitabie vehicle for sustained, localized delivery is critically needed.

[0Oэ¼*) Here, nearly even incorporation and homogeneous distribution of FGF2 and **IL-IO** is shown within coacervate droplets, FGP2/IL-10 coaeervate not only had high loading efficiencies for FGF2 and !L-10 (approximately 98% for both) but also exhibited low initial releases of around **16.1%** FGF2 and 12,5% ft- 10 in the presence of heparinase during the first 12 hours and relatively linear releases of both factors thereafter throughout 21 days. The seemingly low cumulative release of **IL-IO** was primarily due to the spontaneous degradation of released 1L,-10 and molecules trapped in residual coaeervate. Coaeervate delivery of FGF2 and !L-10 preserved their bioaciivities on cardiac stromal cell proliferation *in vitro*. FGF2/IL-10 coaeervate sustained iHUVEC and HHP po liferaiion while reducing CF proliferation in general especially under the inflammatory stress condition,

[00100] Hearts treated with PGP2/IL~10 coaeervate, Coa-F/i-500/500 in particular, exhibited significantly improved long-term LV contractile function and ameliorated LV dilatation, suggesting the syhergistically therapeutic efficacy by controlled delivery of FGF2 and IF-10. FGF2/IL-10 coaeervate, especially €oa-F/I~500/500, augmented .long-term revascularization ,particularly at the infarct area. The data-provided in this .Example also imply a positive correlation of revascul arizing: effects with the dose of 1L,-10. in addition, coaeervate Containing FGF2 and 500 ng **IL-1O** reduced LV fibrosis, preserved infarct wall thickness, and inhibited chronic phagocytic cell infiltration at the infarct area, more effective than coaeervate loaded with FGF2 alone. These results further suggest the synergistic effects of coaeervate with FGF2 and IL~10 in anti- fibrosis and anti-inllammafion,

[00101**]** Moreover, Coa-F/I-500/100 and Coa-F/I-500/500 had substantially augmented long-term LV niyocardial elasticity maintaining around 80% of the normal myocardial strain. Interestingly, Coa-F-500 treated hearts also exhibited significantly- increased niyocardial elasticity. These data suggest the primary effect of controlled, localized delivery of FGF2 on niyocardial elasticity; largely independent of IL-10 mediated anti-inllammaiory benefits. This

is likely attributed to the enhanced functional revascularization and reduced cardiomyocyte death mediated by controlled release of FGF2.-Putative mechanisms involved in FGF2/IL~1.0 eoaeervate mediated ischemic heart repair arc summarized in Figure 12.

|00102J The estimation of the duration of eoaeervate treatment post-Mi by MFE imaging indicates that injected coacervate had a temporal distribution of at least 4 weeks *in situ*. Consequently; FGF2, IL-10, or other therapeutic proteins with high heparin affinity will likely persist within eoaeervate and increase their long-term bioavailability in the local tissue.

[00103] Overall, Coa-M-500/500 exhibited the highest therapeutic potential among all treatment groups. This warrants the pre-elinieal translation of coacervate delivery of FGF2 in combination with IL-10 in large animal models, In addition, the application of FGF2/IL~10 coacervate for the treatment of other ischemic conditions such as myocardial reperfnsion injury and peripheral artery disease demands future investigation. Currently we are investigating the dose-dependent effect(s) and the precise meehauisrn(s) of anti-inflammation and immunomodulaiion mediated by controlled release of IL-10,

In summary, heparin-based eoaeervate represents a promising vehicle tor localized, controlled delivery of a combination of angiogenic and anti-inflammatory proteins, A single coacervate treatment with 500 ng each of FGF2 and IL-10 resulted in long-term synergistic benefits in a mouse MI model Future study in pre-clinical large animal models is warranted to evaluate its therapeutic potential for the treating ischemic heart disease. Given that heparin binds a wide range of trophic factors, coacervate delivery of single or multiple therapeutic proteins can be further expanded to applications in different pathological conditions.

Example 5 - Treatment of Melanoma

f00l04ji Twenty four (24) C57BL/6 female mice were divided into three treatment groups: 1) protein coacervate; 3 doses; 2) Blank coacervate, and PBS (phosphate-buficred saline), n=4 for each group. Ten days prior to the first treatment, the mice were each inoculated with cells in saline at two sites, one in each leg. Mice were randomized into groups and treated with 1st injection in only one leg (primary site). A second treatment was given seven (7) days subsequent to the first treatment, 50 µL inj ection per mouse per treatment consists of 25 pL of IL-2 or IL~12, and 25 µL of 3.75 rag coacervate. IL-2 (interleukin-2, purchased from PeproTech, Rocky Hill, NT) in 10 mM sodium citrate buf er with 0.1% BSA was dosed at 0,01 pg, 0.1 pg, and 1 pg per dose and mixed with 1,78 µL of 150 mg/mL heparin in 0.9% saline and 23.22 pL of 150 mg/mL PEAD in 0.9% saline. IL-12 (interleukin-12, purchased from PeproTech, Rocky Hill NX) in 1,5x PBS with 0.!% BS.A was dosed at 1 pg, 10 pg, and 30 pg

per dose **and mixed with heparin and** PBAD **in the same manner** as 11.-2 **coacervates**. **Tumor** size **was determined for** each **time point,** and **blood was** collected for **analysis** (TBD).

[00105] As depicted in **Figure** 13, *IL*-12 **in** mid- to high-doses prolong survival in mice. One mouse from the IL-12, 30μg group died early (04), which is believed to he due to the **cancer** overpowering the mouse **before treatment** had **a chance** to work. There is minimal difference across the IL-2 groups and controls.

[00106] As shown **in** Figures 14 and 15A-15C, **no significant difference** was observed in tumor size across all groups, for both the primary and **contralateral** sites. It is noted that the drops in average tumor size were due to mice dying, and therefore an **artifact**

[00107J As shown **in** Figures *16* and 17A-17C, the highest dose (30ug) yields **minimal tumor** growth for 3 **out** of **4** mice. As indicated above, one mouse died early **on D4.** The mid dose (1Oug) **.yields** some effect but not as pronounced as the high dose; one: mouse survived **until** D24 even though die size of the tumor grew **up "to** *57(mm²*. The low dose shows the least **difference** between the experimental and control groups

|00108| While several examples and embodiments of the methods are described hereinabove **in** detail, other examples and embodiments will be apparent to, and readily made **by,** those skilled in the art without departing from the scope and spirit of the **invention.** For example, it is to be understood that this disclosure contemplates that, to the extent possible, one or more features of any embodiment can he combined with one or more features of any other embodiment. Accordingly, the foregoing description is intended to be illustrative rather than **restrictive.**

[001©9| The following clauses **illustrate** various aspects of the invention.

1. A composition comprising a complex or coacervate of a polycationic polymer, a **polyanionic.** polymer, and a cytokine selected from an **interferon** and/or an interieukiu.

2. The composition of clause i, wherein the polyaniome is a heparin or heparan sulfate.

X The composition of clause 1, wherein the poiycationic polymer is a polymer composition **comprising** at least one moiety **selected** from the following:

(a) **[-(X')(0)-CH(NHY)¾ -C(O)O-CH_2-CH(O-R1)-CH_2-O-CH_2-CH_2-O-CH_2-CH(O-R2)-CH_2-]$_n$,**

(b) [-OC(O)-C¾ -C¾ NHY)-C(O)O-CH_2-CH(O-R1)-CH_2-O-CH_2-CH_2-0 -CH2-CH{(O-R2)-C¾ -]$_n$,

(c) [-OC(O)-CH(NHY)-CH_2-CH_2-C(O)O-CH2-CH(O-R1)-CH_2-O-CH_2-CI¾-Q-C1¾-CH(0-R2)~C¾~],,, and/or

(d) [-OC(O)-CH_2-CH_2-CH(NHY)-C(O)O-CH2-CH(O-R1)-CH_2-O-CH_2-CH_r O-G¾-e H(0 ~R2)-C¾ -]m,

wherein Y is -C(O)-CH(NH_3^+)-$(CH_2)_3$-NH-$C(NH_2)_2^+$ or -C(O)-CH(NH_3^+)-$(CH_2)_4$-$(NH_3)^+$, and R1 and R2 are the same or different and are independently selected from the group consisting of hydrogen, a carboxy-containiag group, a C1¾ alkyl group, an amine-containing group, a quaternary ammonium containing. group, and a. peptide,

4. The composition of clause 3, in which the polycatioiiic polymer is a polymer composition comprising at least one moiety selected from formulae (a) or (b).

5. The composition of clause 3, in which the polycationic: polymer is a polymer composition comprising at least one moiety selected from formulae (e) or ***id).***

6. The composition of any one of clauses 3-5, wherein the polycationic polymer has a polydisperstty index of less than 3.0.

7. The composition of any one of clauses 3-5, wherein the: poiycationic polymer has a polydisperstty index of less than .2.0,

8. The composition of any one of clauses 3-5, in which R1 and R2 are selected from the group consisting of lle~Lys-Va!-Ala-Val (IKVAV) (SEQ **ID** NO: 1**),** Arg~Gly-Asp (EGD), Arg-Gly-Asp-Ser (RODS) (SEQ **ID** NO: 2), Ala-Giy-Asp (AGD), Lys-Gln-AIa-GIy~Asp-Vat (RQAGDV) (SEQ ID NO: 3), Val-Aia-Pro-Gly-Val-Gly (VAPGVG) (SEQ ID NO: 4), APGVGV (§EQ ID NO: 5), **PGVGVA** (SEQ ID NO; 6), VAP, GVOVA (SEQ ID NO: 7), **VAPG** (SEQ ID NO: 8), VGVAPG (SEQ ID NO: 9), VGVA (SEQ ID NO: 10), VAPGV (SEQ **ID** NO: 11) and **GVAPGV** (SEQ ID NO: **12).**

9. The composition of any one of clauses 3-5, in which the polycationic polymer is complexed with heparin or heparan sulfate.

10. The composition of any one of clauses 3-5, in which one or "both of R1 and R2 are ma!eate or phosphate.

11. The composition of any one of clauses 3-5, wherein Y is -C(Q)-CH(NH_3^+)-$(CH_2)_4$-$(NH_3)^+$.

12. The composition of any one of clauses 3-5, wherein **Y** is -C(O)-CH(NH_3^+)-$(CH_2)_3$-NH-$C(NH_2)_2^+$.

13. The composition of any one of clauses 3-5, in which R1 is hydrogen .

14. The composition of any one of clauses 3-5, in which one or both of R1 and R2 are charged.

15. The composition of any one of clauses 1-14, in which the ratio of the polycationie polymer to the polyanionic polymeria the composition results in a neutral charge,

1.6. The composition of any one of clauses 1-14, in which the ratio of the polycationie polymer to the polyanionic polymer in the composition results in a negative charge.

17. The composition of any one of clauses 1-14, in which the ratio of the polycationie polymer to the polyanionic polymer in the composition results in a positive charge.

18. The composition of any one of clauses 1-17, wherein the cytokine is one or more cytokines selectee! from the group consisting of an IL-2 (inter!eukin-2), an IL-12 {interleukin-12, e.g., IL-12 p70), and/or an IFN-γ (interferon gamma), in any combination.

19. The composition of any one of clauses 1.-17, wherein the cytokine is an IL-12.

20. The composition of any one of clauses 1-17, wherein the cytokine is an immunomodulatory cytokine .

21. The composition of clause 20, wherein the cytokine is Ï.-10, and the composition further comprises an angiogenic growth factor.

22. The composition of clause 21, wherein the angiogenic growth facto* is FGF2.

23. The composition of any one of clauses 1-22, embedded in a hydrogel.

24. A method of delivering an interferon and/or an interleukin to a patient in need thereof, comprising administering the composition of any of clauses .-23 to the patient.

25. The method of clause 18, wherein the composition is delivered by enteral, parenteral, or topical routes, for example and without limitation by: intravenous (IV), local injection, intramuscular, .intracerebral, subcutaneous, orally, inhalation, topically, enema, iniravaginal, intrauterine, ocular, or otic routes.

26. A method of treating a cancer in a patient, comprising, delivering to the patient, e.g. by enteral, parenteral, or topical routes, for example and without limitation by: intravenous (IV), local injection, intramuscular, intracerebral, subcutaneous, orally, inhalation, topically, enema, intravaginal, intrauterine, ocular, or otic routes, the composition of any of clauses 1-20,

27. The method of clause 26, wherein the cytokine is IL-12.

28. The method of clause 27 or 28, wherein the cancer is melanoma.

29, A **method of** treating a myocardial infarct **in** a patient, **comprising, delivering to the -myocardium** at or adjacent **to** the infarct a **composition of any** one of clauses 20-23,

30. The method of clause 2¾ wherein the composition comprises FGF2 and (1.-10.

We claim:

1. A composition comprising a complex or eoacervate of a polycationic polymer, a polyanionie polymer, and a cytokine selected from an interferon and/or an interleukin.
2. The composition of claim 1, wherein the polyanionie is a heparin or heparan sulfate.
3. The composition of claim 1, wherein the poiycaiionic polymer is a polymer composition comprising at least one moiety selected from the following:

(a) [-OC(O)-CH(NHY)-CH_2-C(O)O-CH_2-CH(O-R1)-CH_2-O-CH_2-CH_2-O-CH_2-CH(O-R2)-CH_2-]n,

(b) [-OC(O)-C¾-CH(NHY)-C(O)O-CH_2-CH(O-R1)-CH_2-O-CH_2-CH_2-O-CH_2-CH(O-R2)-CH_2-]n,

(c) [-OC((¾-CH(NHiY)-CH_2-CH_2-C(O)O-CH2-CH(O-R1)-CH_2-O-CH_2-CH_2-O.CH_2-CH(O-R2)-CH_2-]n, and/or

(d) [-OC(O)-C¾ -CH_2-CH(NHY)-C(O)O-CH2-CH(O-R1)-CH_2-O-CH_2-C¾ rO-CH_2-CH_{2}-CH_{0}-R2)-CH_2-]n,

wherein Y is -C(O)-CH(NH_3^+)-(CH_2)3-NH-C(NH_2)$_2^+$ or -C(O)-CH(NH_3^+)-(CH_2)4-(NH_3)$^+$, and R1 and R2 are the same or different and are independently selected from the group consisting of hydrogen, a earboxy-contaming group, a C_{1-6} alkyl group, an amine-eontaining group, a quaternary ammonium containing group, and a peptide.

4. The composition of claim 3, wherein the poiycaiionic polymer has a polydispersity index of less than 3.0.

5. The composition of claim 3, in which R1 and R2 are selected from the group consisting of Iie-Eys~Val~Ala~Val (IKVAV) (SEQ ID NO: 1), Arg-Gly~Asp (RGB), Arg-Gly~Asp~Ser (RGDS) (SEQ ID NO: 2), Aia-Gly-Asp (AGD), Lys-Gm~Aia~Giy-Asp-Val (KQAGDV) (SEQ ID NO: 3), Val-Ala-Pro-Gly-Val-Gly (VAPGVG) (SEQ ID NO: 4), APGVGV (SEQ ID NO: 5), PGVGVA (SEQ ID NO: 6¼ .VAP, GVGVA (SEQ ID NO: ?), VAPG (SEQ ID NO: 8), VGVAPG (SEQ ID NO: *9),* VGVA (SEQ ID NO: 10), VAPGV (SEQ 3D NO: 11) and GVAPGV (SEQ ID NO: 12).

6. The composition of claim 3, wherein Y is -C(O)-CH(NH_3+)-(CH_2)4-(NH_3)+.

7. The composition of claim 3/wherein Y is -C(O)-CH(NH_3+)-(CH_2)3-NH-C(NH_2)2+.

8. The composition of claim I, in which the ratio of the poiycaiionic polymer to the polyanionie polymer in the composition results in a neutral, negative, or positive charge.

9. The composition of claim 1, wherein the cytokine is an IL-12 (interleukin-12).

10. The composition of claim 1, wherein the cytokine is 11,-10.

11. The composition of claim 10, wherein the composition further comprises an angiogenic growth factor, such as-FGFZ

12. The composition of any of claims 1-11, embedded in a hydrogel.

13, A method of delivering an irjiederon and/or an interknlcin to a patient in need thereof, comprising administering the composition of any one of claims 1-1.2 to the patient.

14. A method of treating a cancer in a patient, comprising, delivering to. the patient the composition of any one of claims J-10.

15, The method of claim 14, wherein the cytokine is IL-12 and/or die cancer is melanoma.

16, A method of treating a myocardial infarct in a patient, comprising, delivering to the myocardium at or adjacent to the infarct a composition of any one of claims 1-8, 10, and 11, wherein the composition optionally comprises FGF2 and IL-!O.

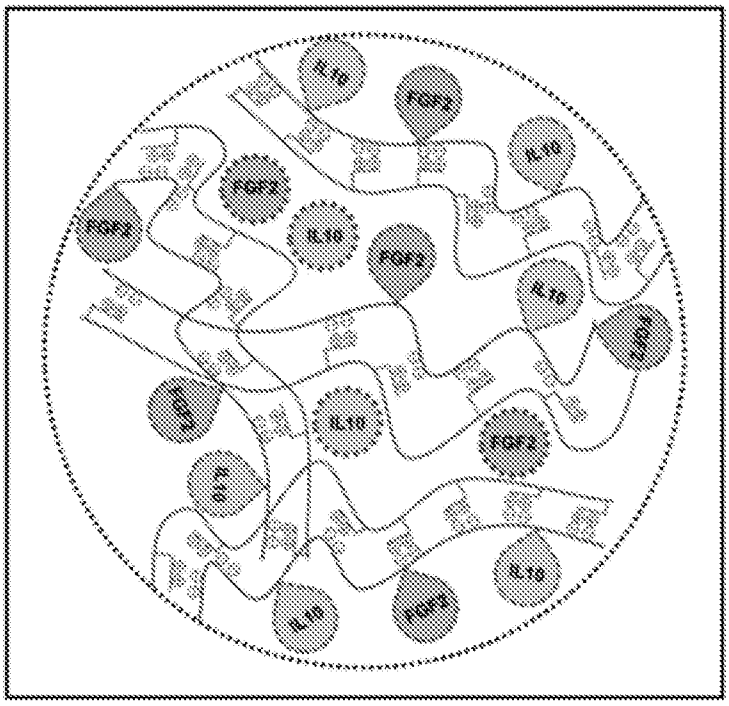
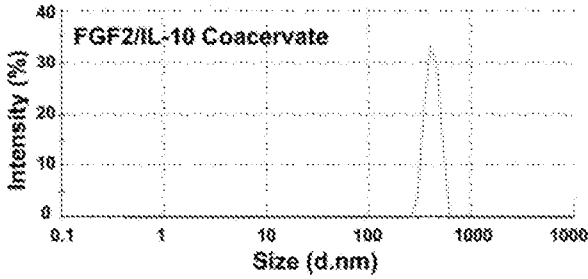


Fig. 1A



Composition	Diameter (nm)	PDI
FGF2 Coacervate	608.3 ± 96.3	0.05
IL-10 Coacervate	502.6 ± 101.5	0.21
FGF2/IL-10 Coacervate	432.6 ± 42.1	0.18

Fig. 1B

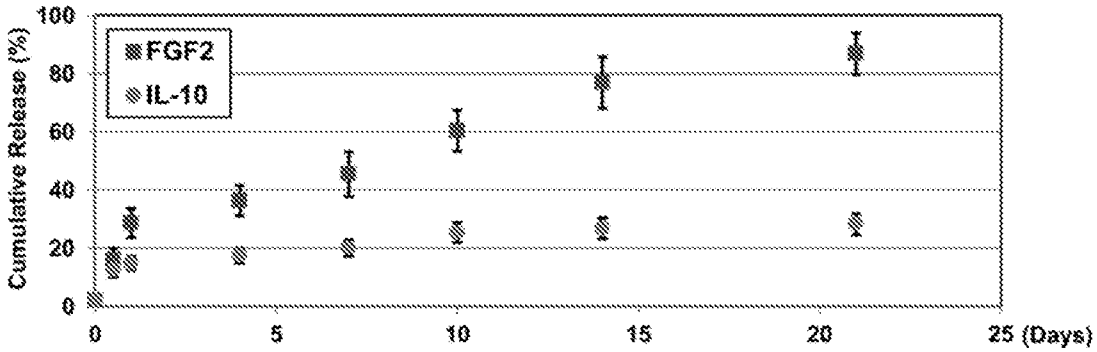


Fig. 1C

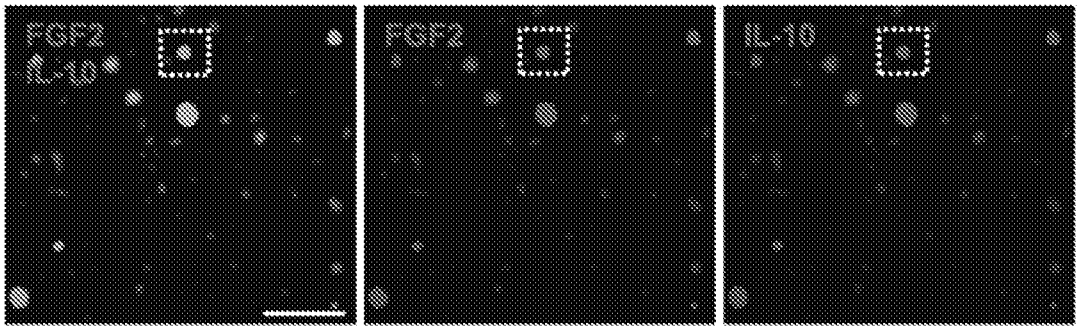


Fig. 1D

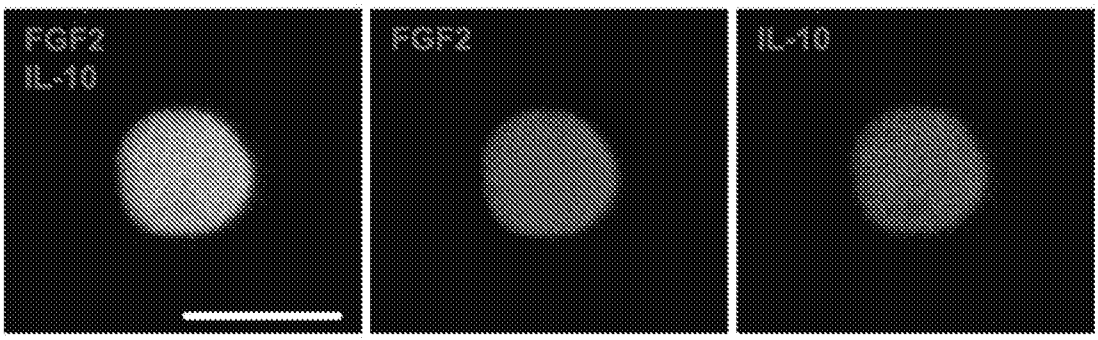


Fig. 1E

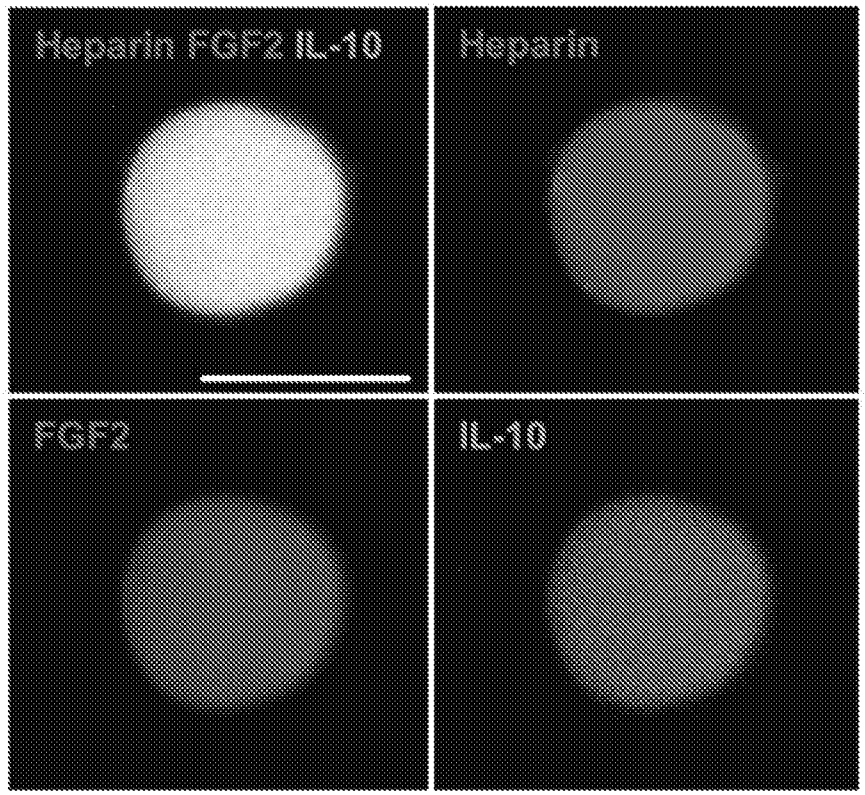


Fig. 1F

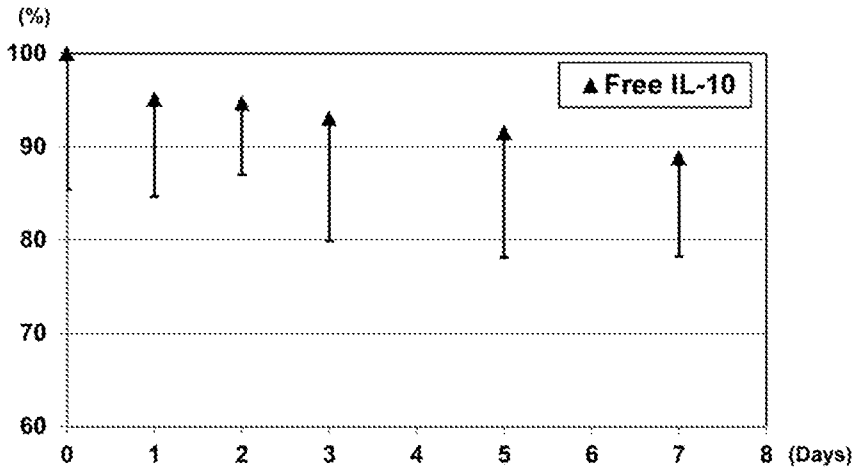


Fig. 2

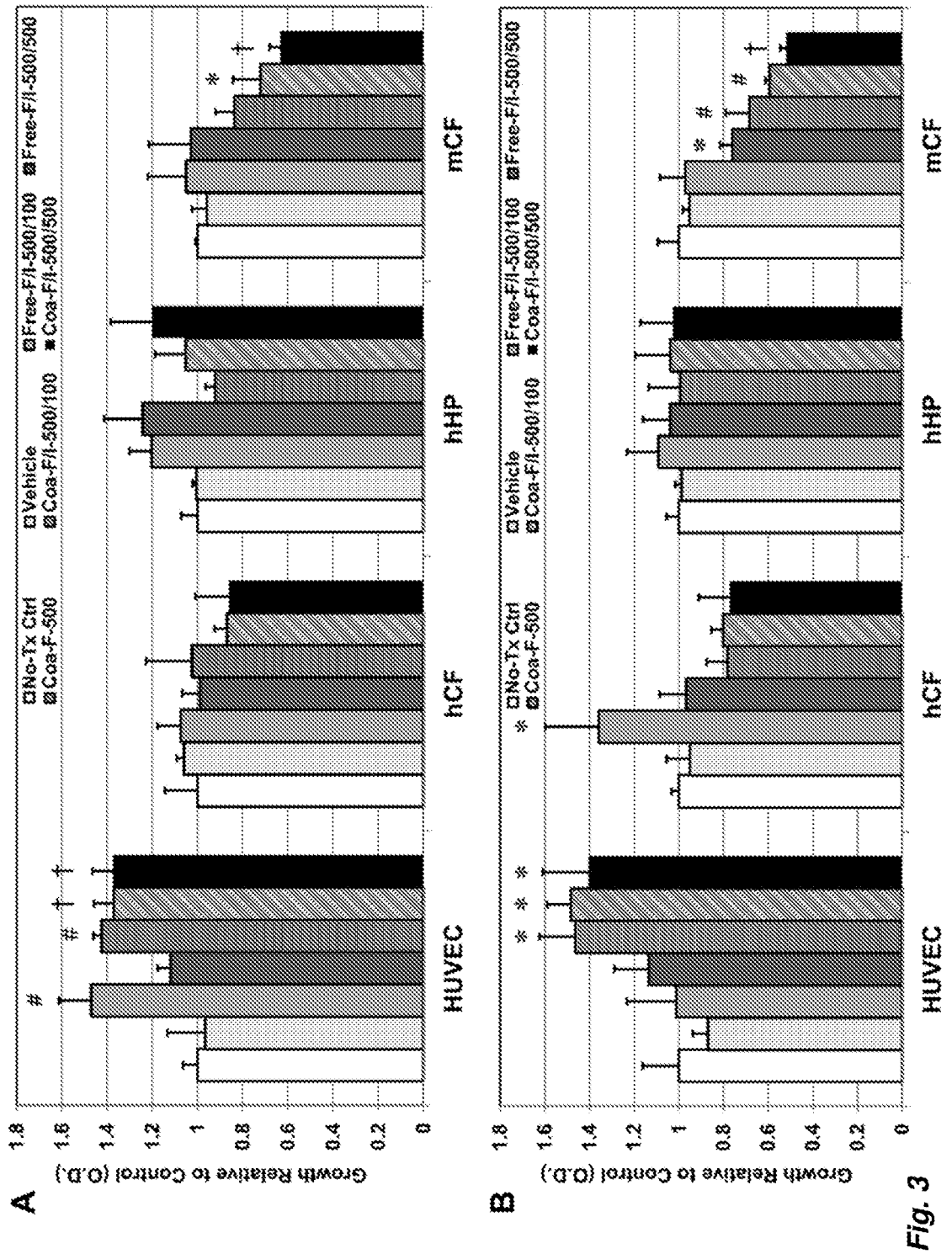

A
B
No-Tx Ctrl
Coa-F-500
Vehicle
Coa-F/I-500/100
Free-F/I-500/100
Coa-F/I-500/500
Free-F/I-500/500
Growth Relative to Control (O.D.)
HUVEC
hCF
hHP
mCF
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8

Fig. 3

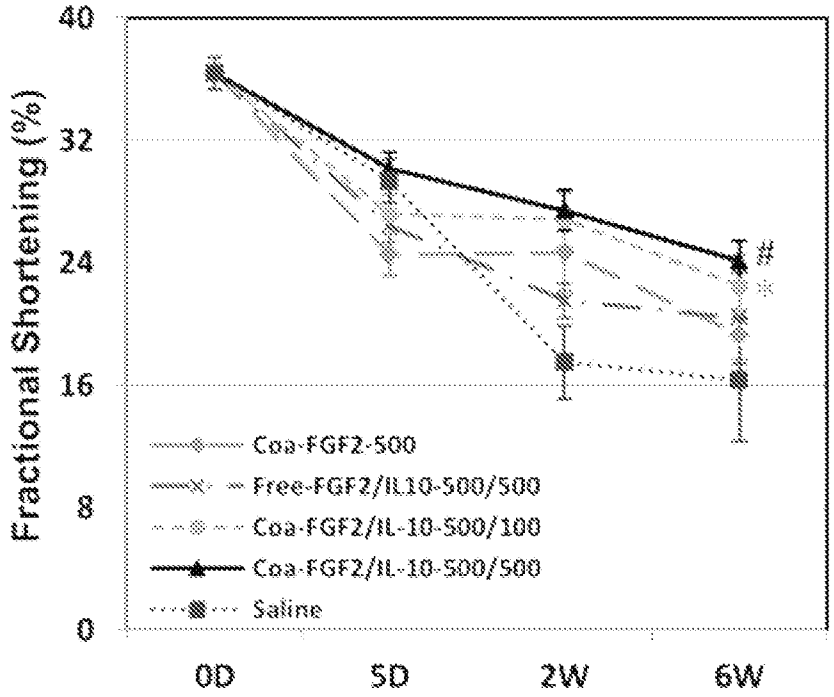

Fractional Shortening (%)
40
32
24
16
8
0
0D
5D
2W
6W
Coa-FGF2-500
Free-FGF2/IL10-500/500
Coa-FGF2/IL-10-500/100
Coa-FGF2/IL-10-500/500
Saline
#

Fig. 4A

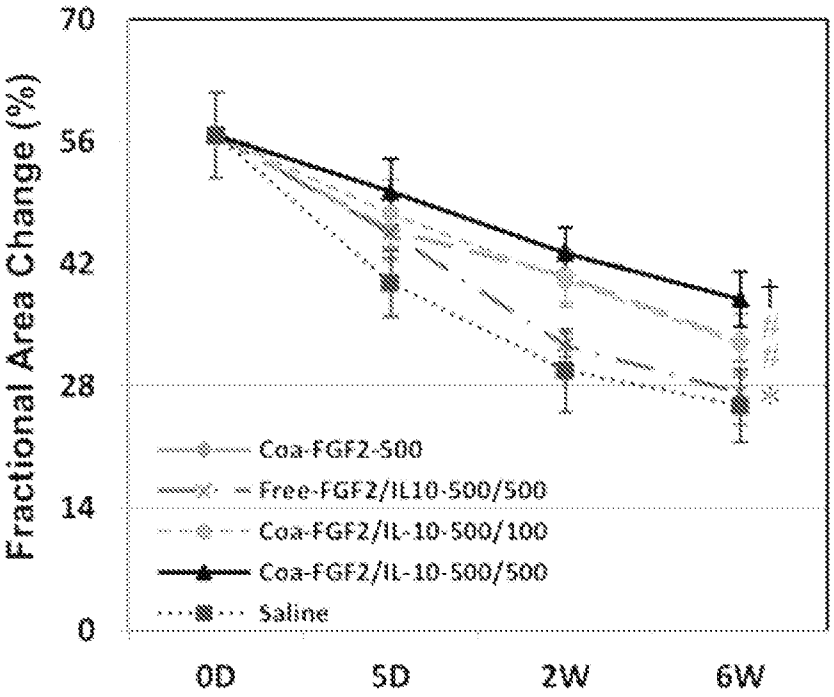

Fractional Area Change (%)
70
56
42
28
14
0
0D
5D
2W
6W
Coa-FGF2-500
Free-FGF2/IL10-500/500
Coa-FGF2/IL-10-500/100
Coa-FGF2/IL-10-500/500
Saline
†

Fig. 4B

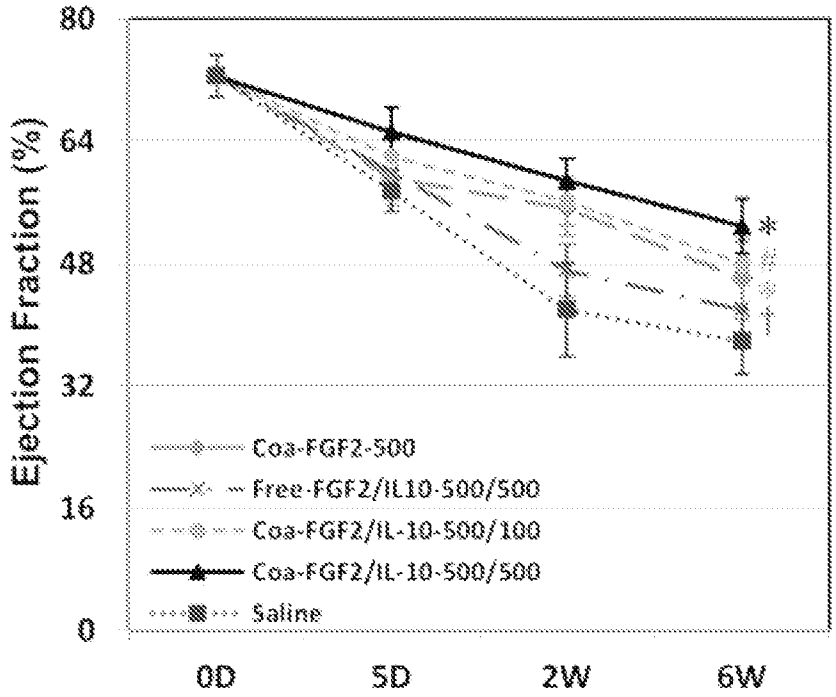

Ejection Fraction (%)
80
64
48
32
16
0
0D
5D
2W
6W
Coa-FGF2-500
Free-FGF2/IL10-500/500
Coa-FGF2/IL-10-500/100
Coa-FGF2/IL-10-500/500
Saline
*
#
†

Fig. 4C

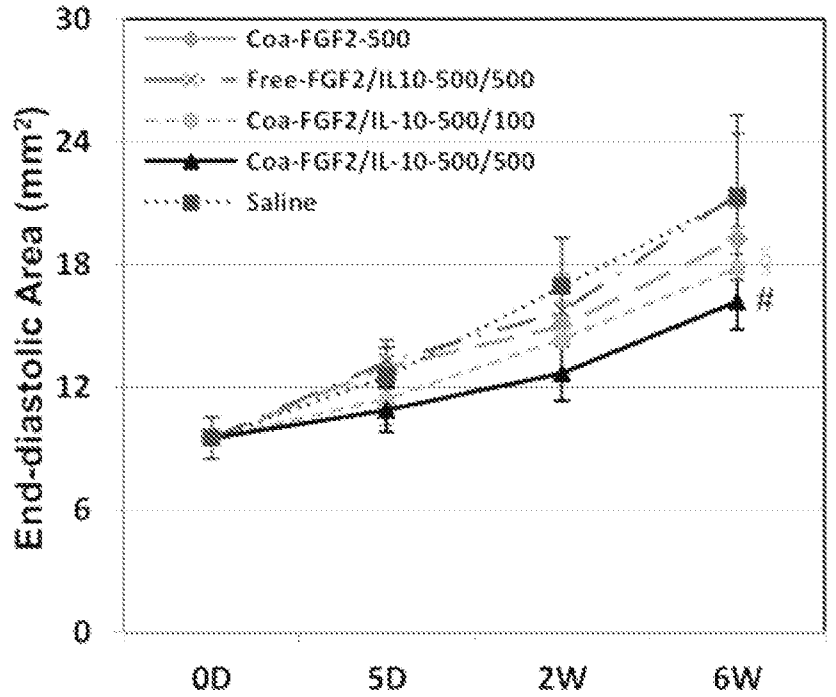

End-diastolic Area (mm²)
30
24
18
12
6
0
0D
5D
2W
6W
Coa-FGF2-500
Free-FGF2/IL10-500/500
Coa-FGF2/IL-10-500/100
Coa-FGF2/IL-10-500/500
Saline
#
†

Fig. 4D

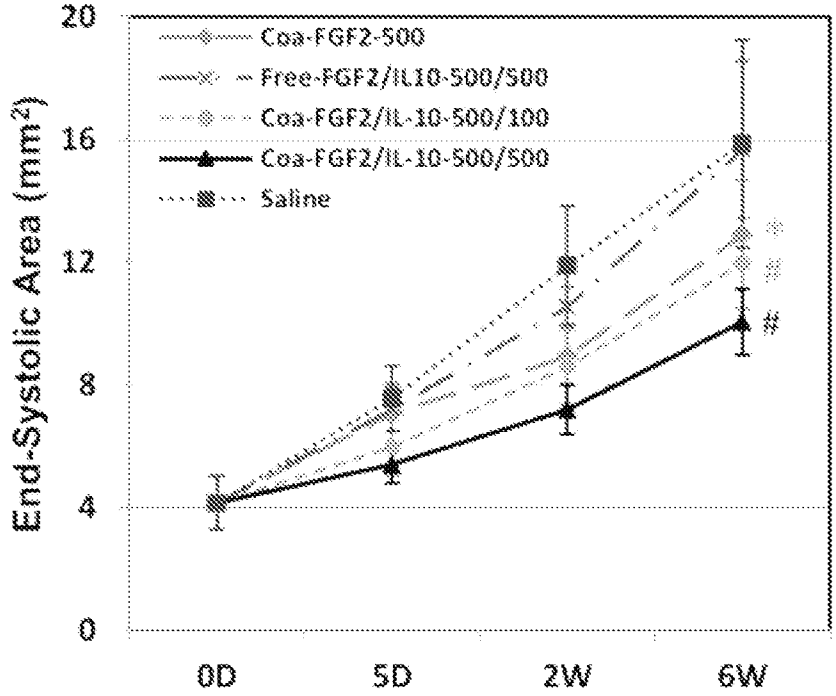

End-Systolic Area (mm²)
20
16
12
8
4
0
Coa-FGF2-500
Free-FGF2/IL10-500/500
Coa-FGF2/IL-10-500/100
Coa-FGF2/IL-10-500/500
Saline
0D
5D
2W
6W

Fig. 4E

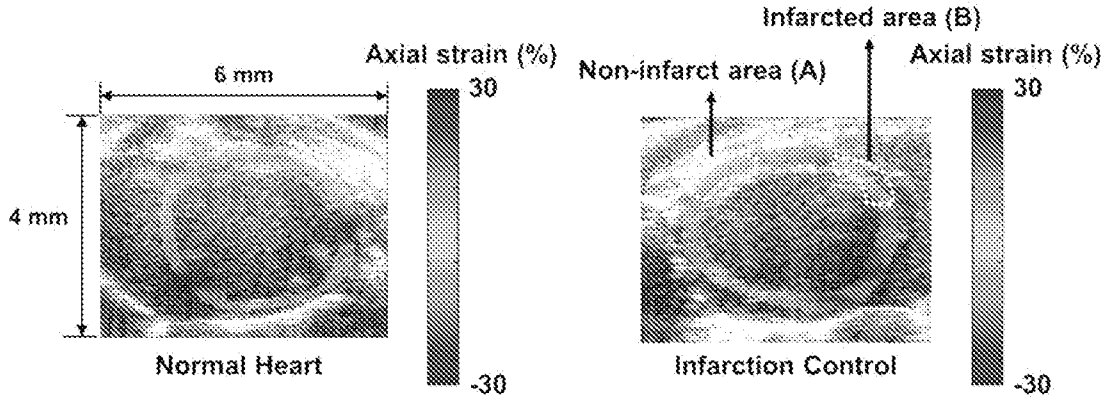

6 mm
4 mm
Axial strain (%)
30
-30
Normal Heart
Infarcted area (B)
Non-infarct area (A)
Axial strain (%)
30
-30
Infarction Control

Fig. 5A

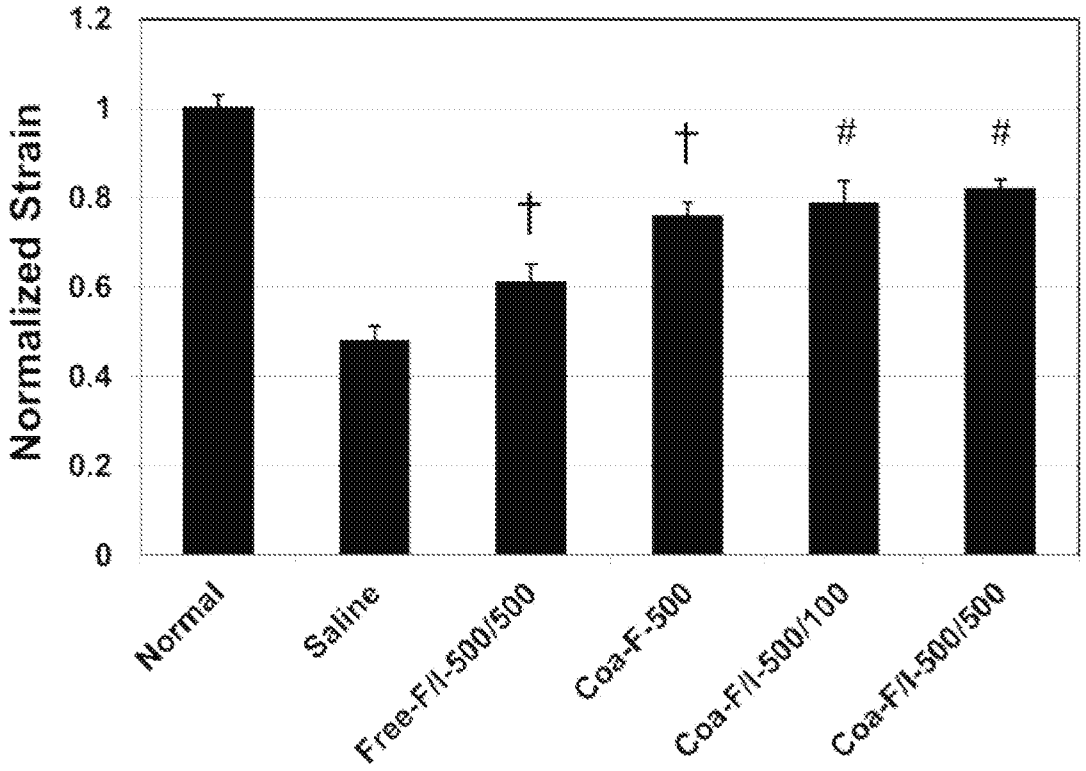

Normalized Strain
1.2
1
0.8
0.6
0.4
0.2
0
†
†
#
#
Normal
Saline
Free-F/I-500/500
Coa-F-500
Coa-F/I-500/100
Coa-F/I-500/500

Fig. 5B

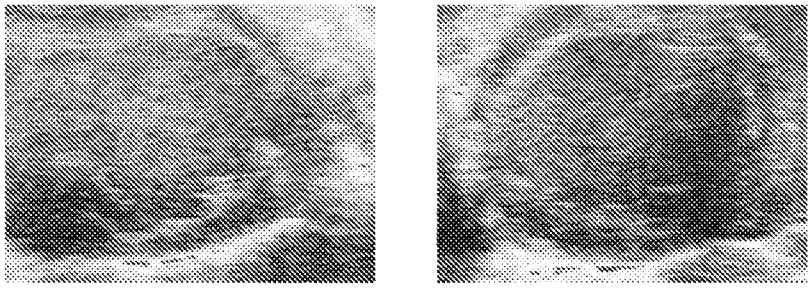

Normal Heart
Infarction Control

Fig. 6

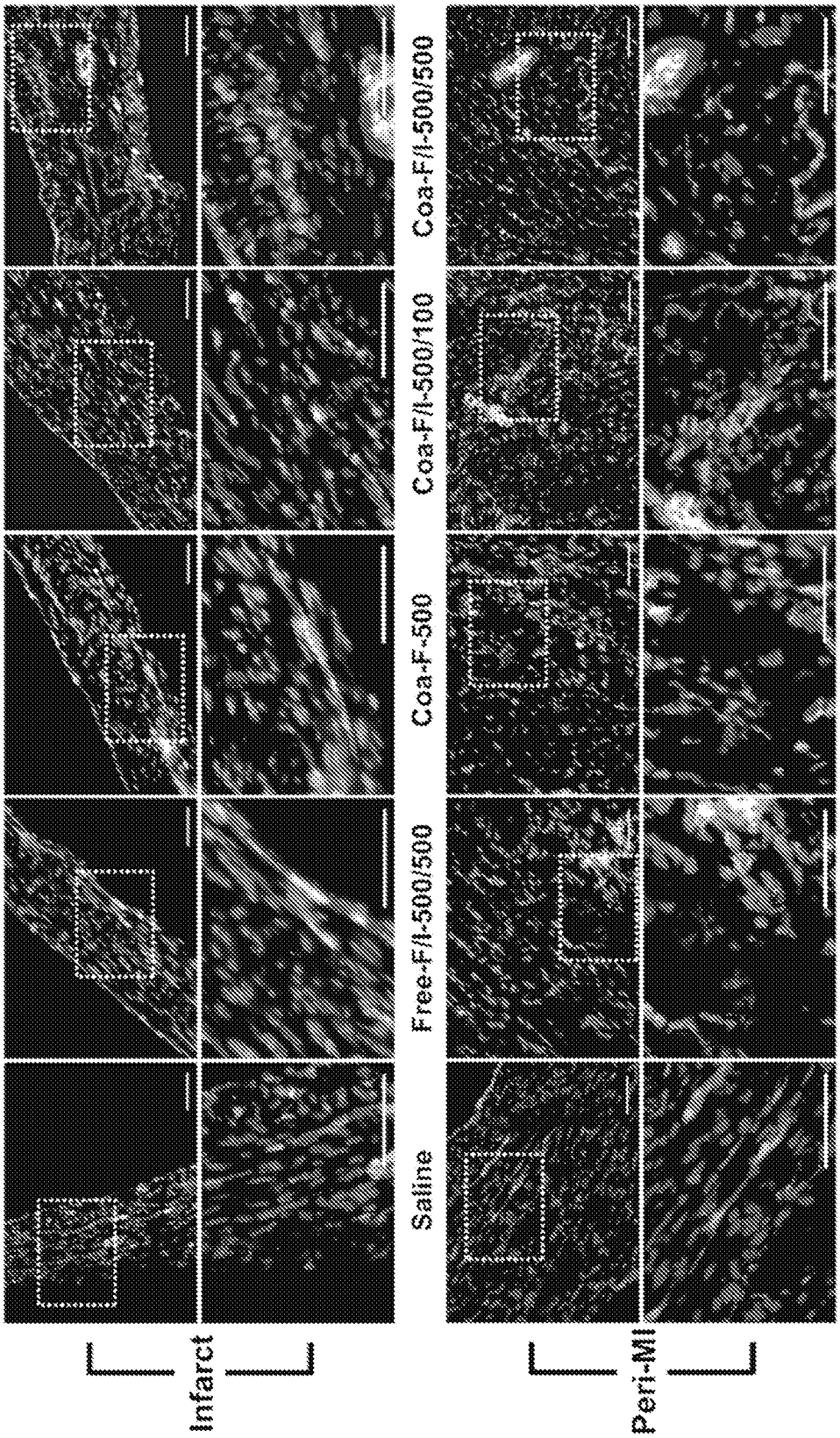

Saline
Free-F/I-500/500
Coa-F-500
Coa-F/I-500/100
Coa-F/I-500/500
Infarct
Peri-MI

Fig. 7A

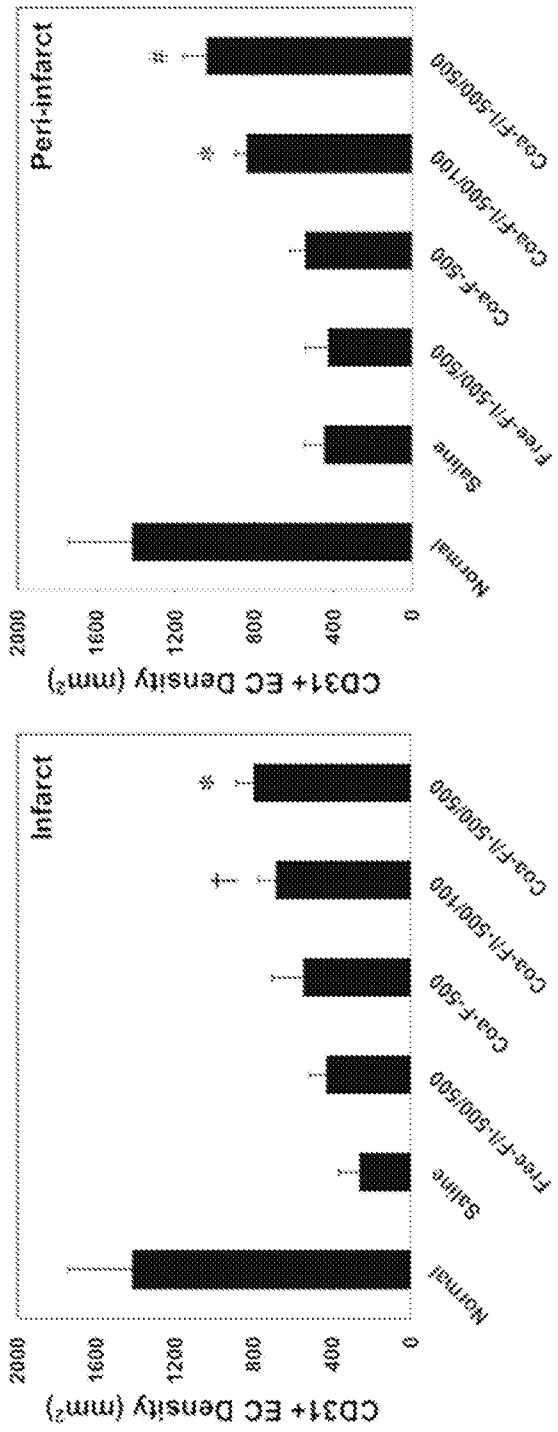

Peri-infarct
CD31+ EC Density (mm²)
2000
1600
1200
800
400
0
Normal
Saline
Free-FI-500/500
Coa-F-500
Coa-FI-500/100
Coa-FI-500/500
Infarct
CD31+ EC Density (mm²)
2000
1600
1200
800
400
0
Normal
Saline
Free-FI-500/500
Coa-F-500
Coa-FI-500/100
Coa-FI-500/500

Fig. 7B

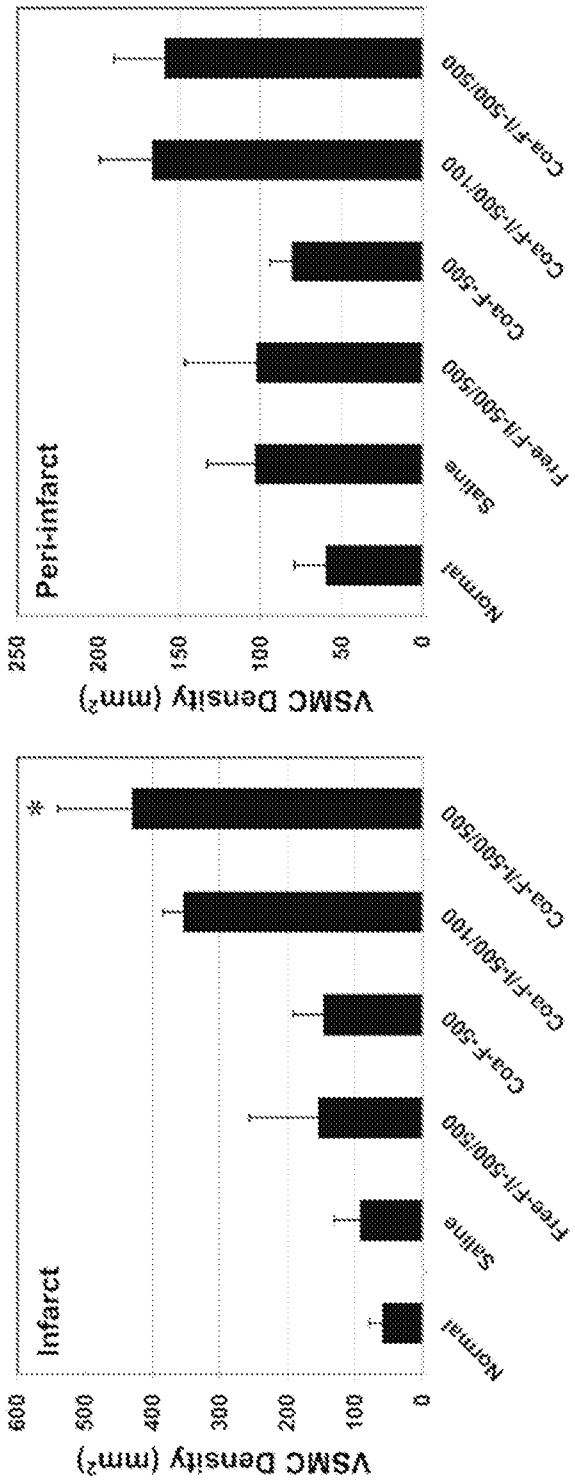

Peri-infarct
VSMC Density (mm²)
0
50
100
150
200
250
Normal
Saline
Free-Ff-500/500
Coa-F-500
Coa-Ff-500/100
Coa-Ff-500/500
Infarct
VSMC Density (mm²)
0
100
200
300
400
500
600
*
Normal
Saline
Free-Ff-500/500
Coa-F-500
Coa-Ff-500/100
Coa-Ff-500/500

Fig. 8

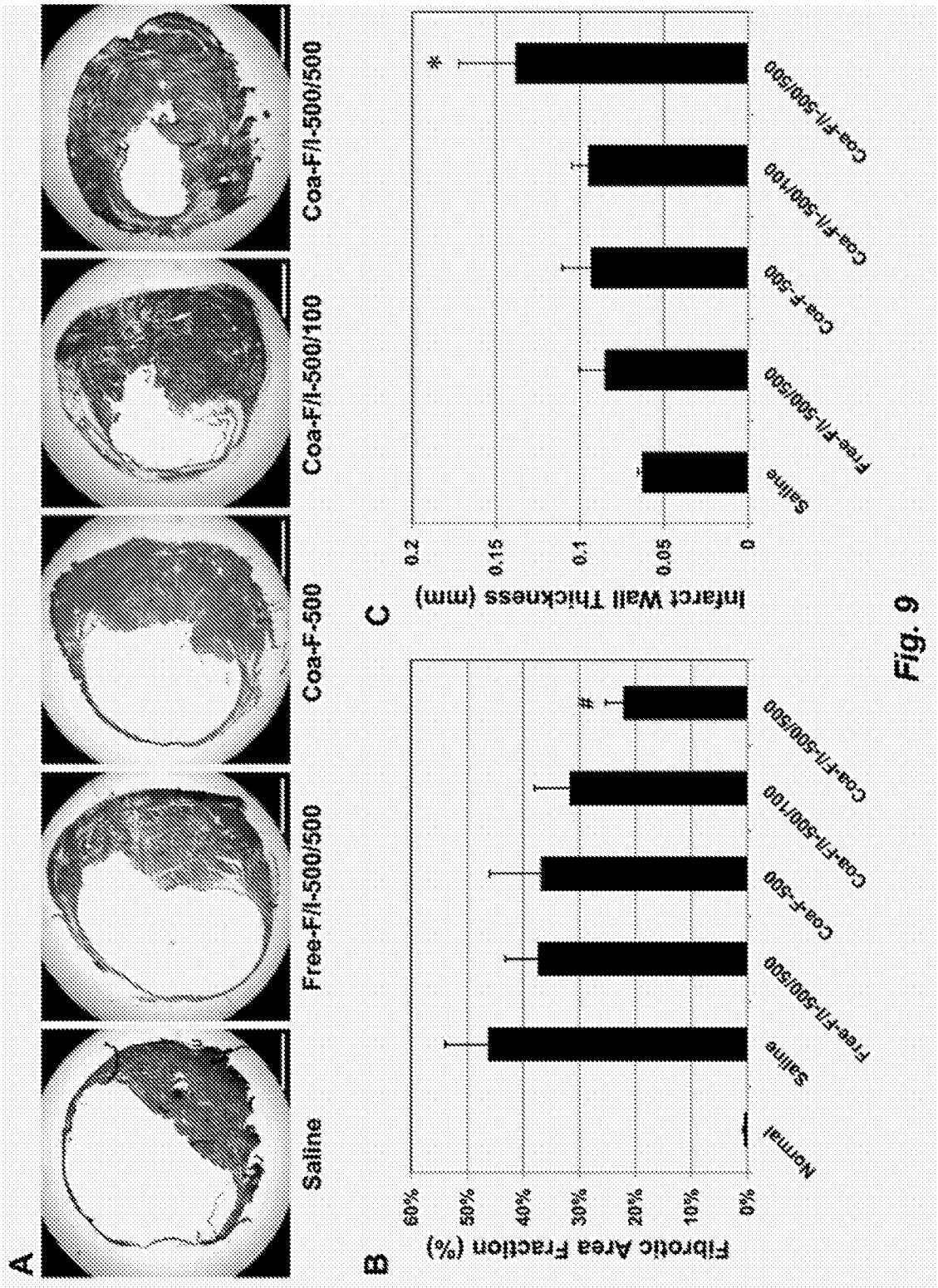

A
Saline
Free-F/I-500/500
Coa-F-500
Coa-F/I-500/100
Coa-F/I-500/500
B
Fibrotic Area Fraction (%)
60%
50%
40%
30%
20%
10%
0%
Normal
Saline
Free-F/I-500/500
Coa-F-500
Coa-F/I-500/100
Coa-F/I-500/500
#
C
Infarct Wall Thickness (mm)
0.2
0.15
0.1
0.05
0
Saline
Free-F/I-500/500
Coa-F-500
Coa-F/I-500/100
Coa-F/I-500/500
*

Fig. 9

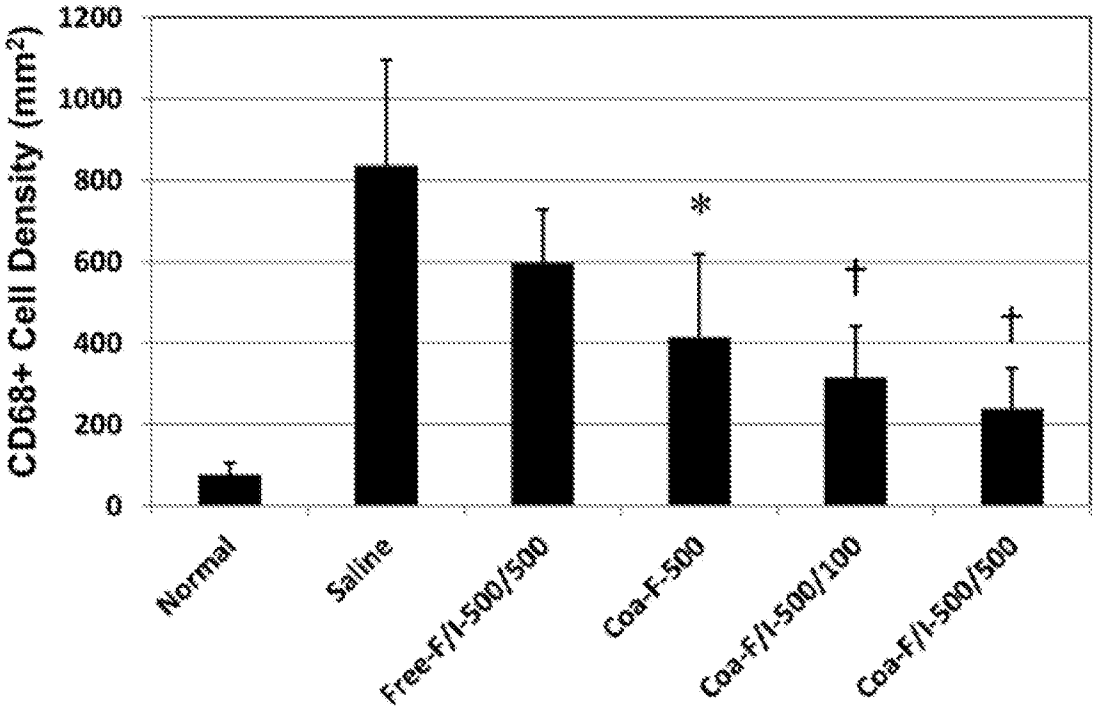

CD68+ Cell Density (mm²)
1200
1000
800
600
400
200
0
*
†
†
Normal
Saline
Free-F/I-500/500
Coa-F-500
Coa-F/I-500/100
Coa-F/I-500/500

Fig. 10

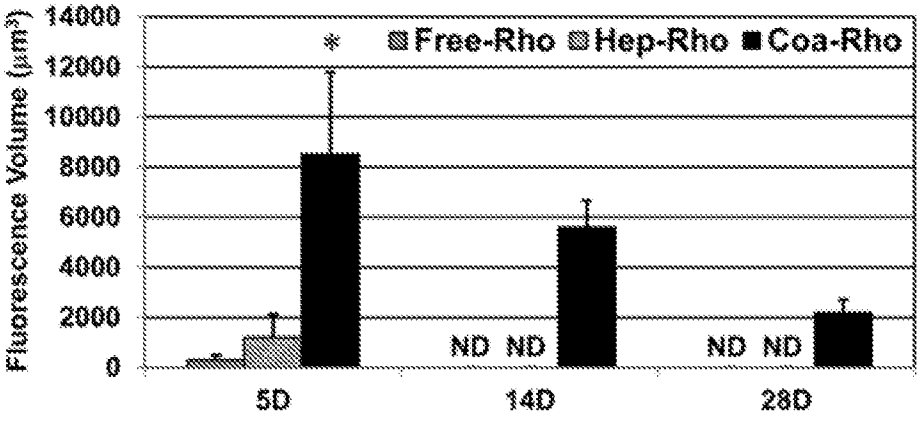

Fluorescence Volume (μm³)
14000
12000
10000
8000
6000
4000
2000
0
*
Free-Rho
Hep-Rho
Coa-Rho
ND ND
ND ND
5D
14D
28D

Fig. 11

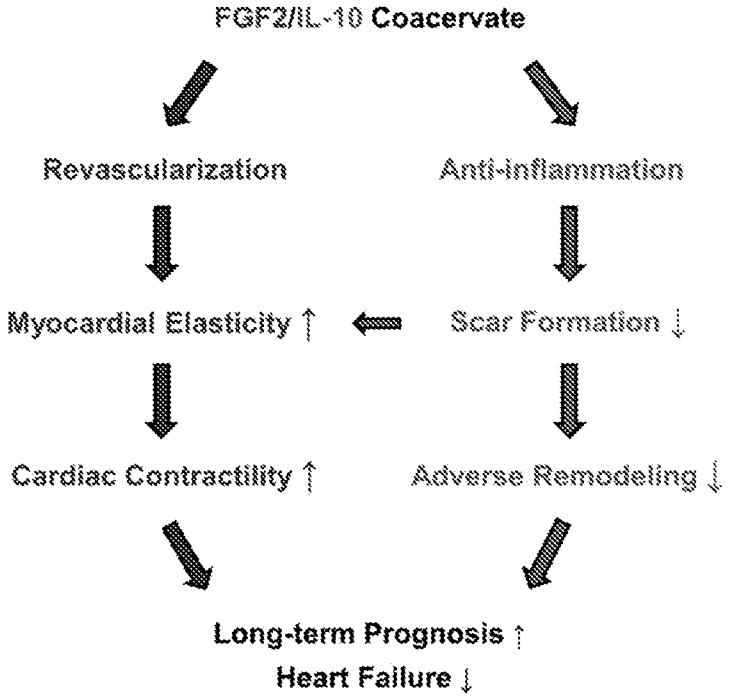

FGF2/IL-10 Coacervate
Revascularization
Anti-inflammation
Myocardial Elasticity ↑
Scar Formation ↓
Cardiac Contractility ↑
Adverse Remodeling ↓
Long-term Prognosis ↑
Heart Failure ↓

Fig. 12

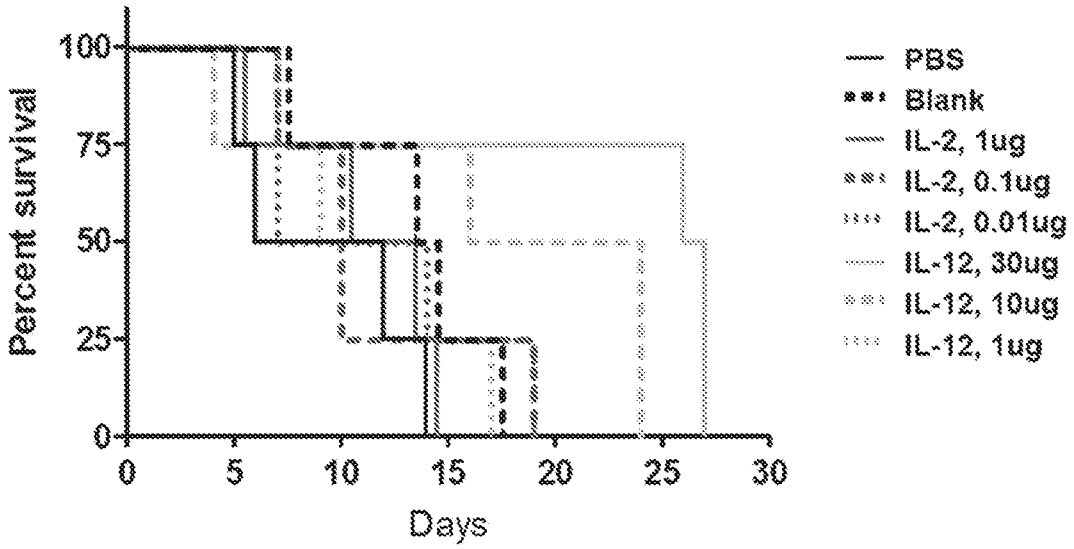
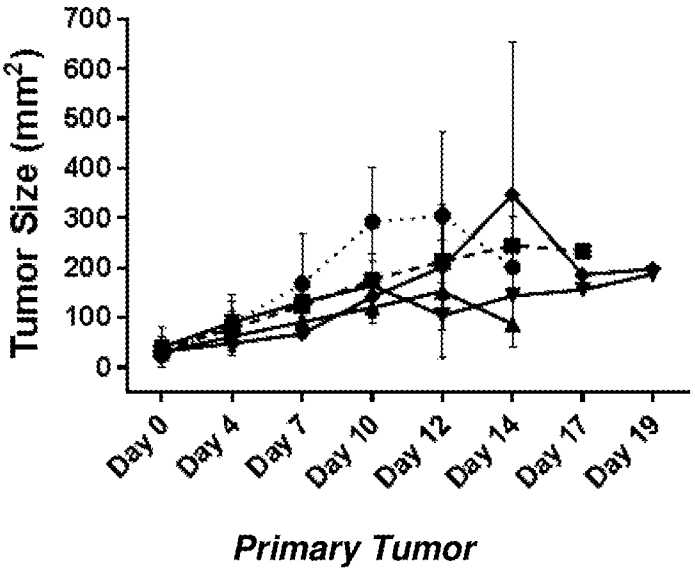

Percent survival
100
75
50
25
0
0
5
10
15
20
25
30
Days
PBS
Blank
IL-2, 1ug
IL-2, 0.1ug
IL-2, 0.01ug
IL-12, 30ug
IL-12, 10ug
IL-12, 1ug

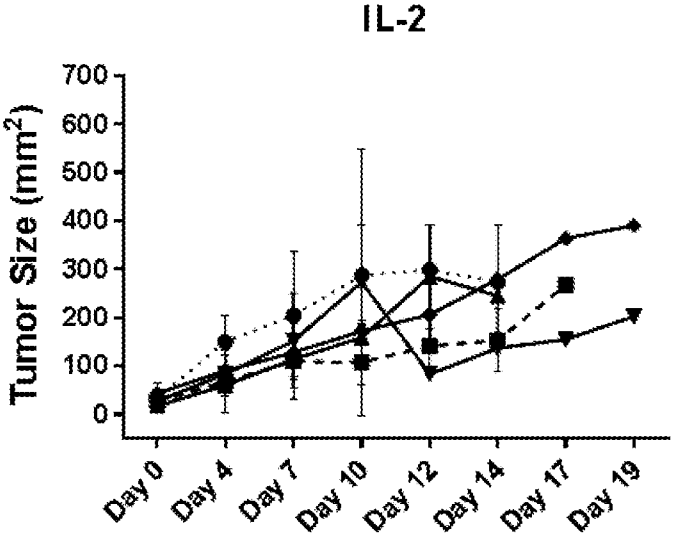
Fig. 13

A



Primary Tumor

B



Contralateral Tumor

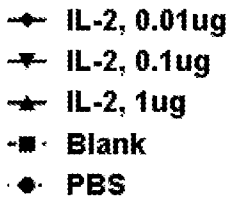


Fig. 14

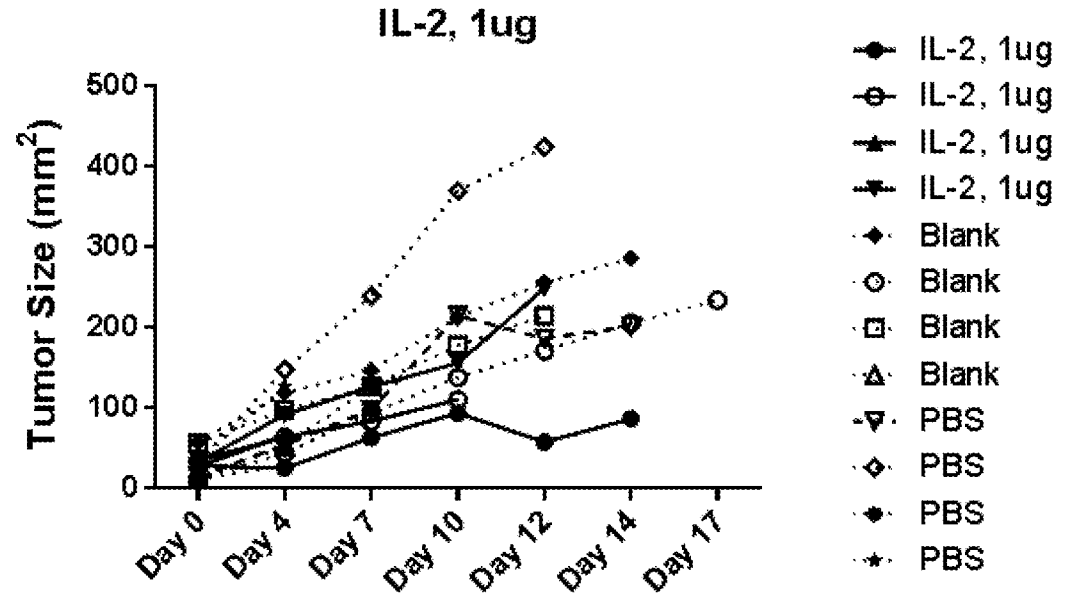


Fig. 15A

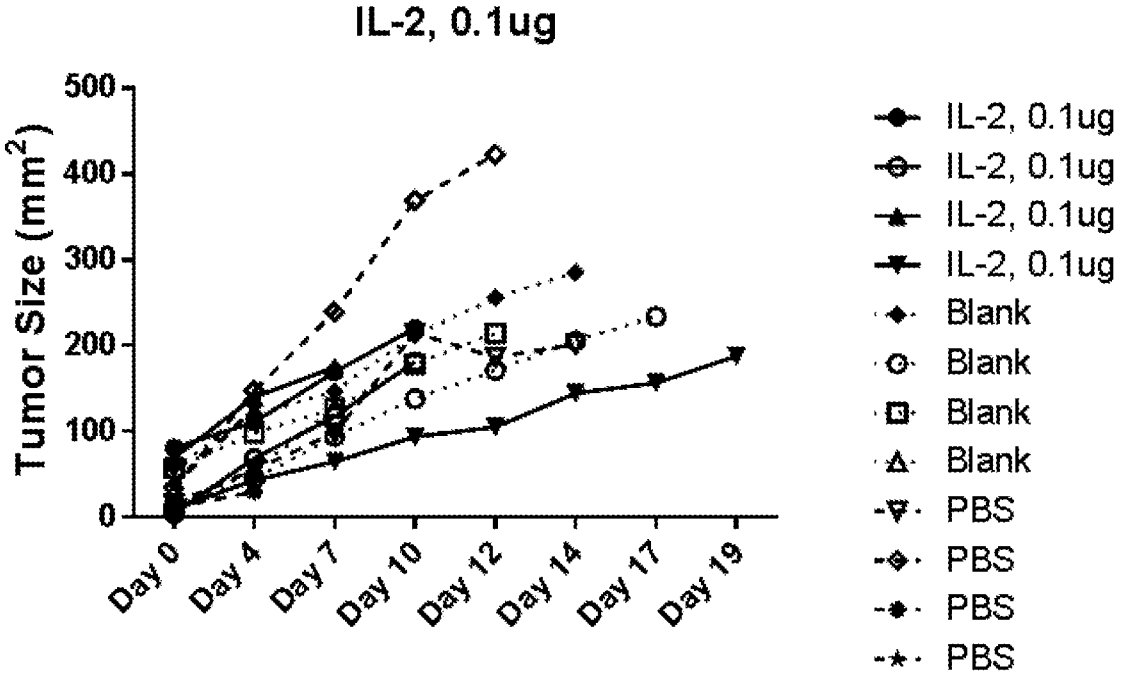


Fig. 15B

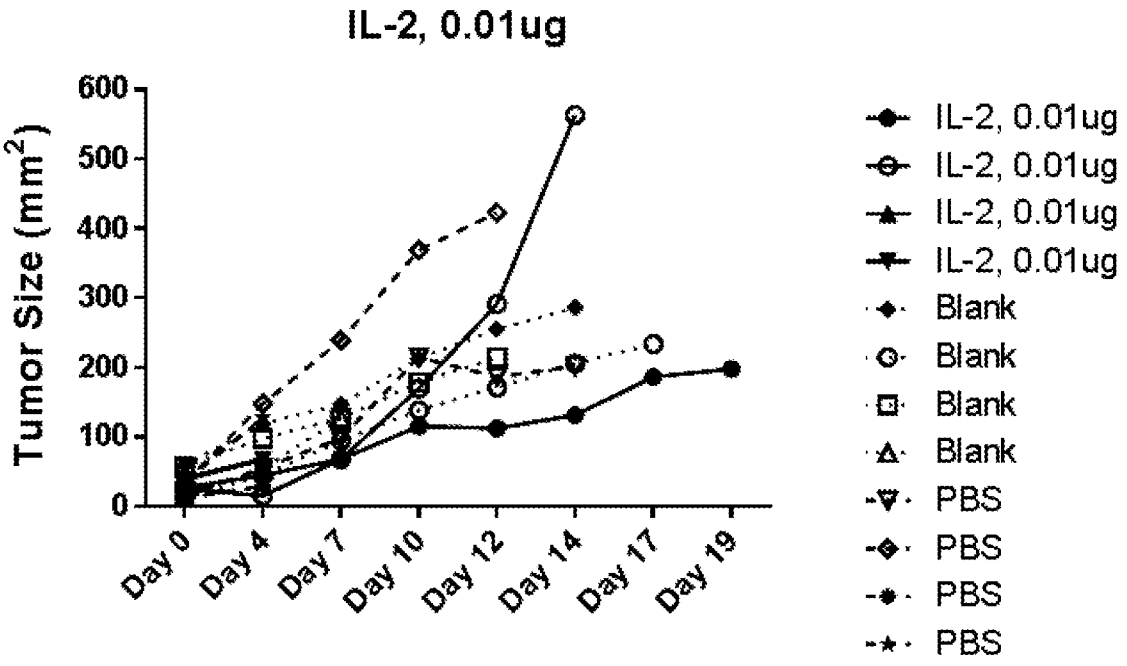
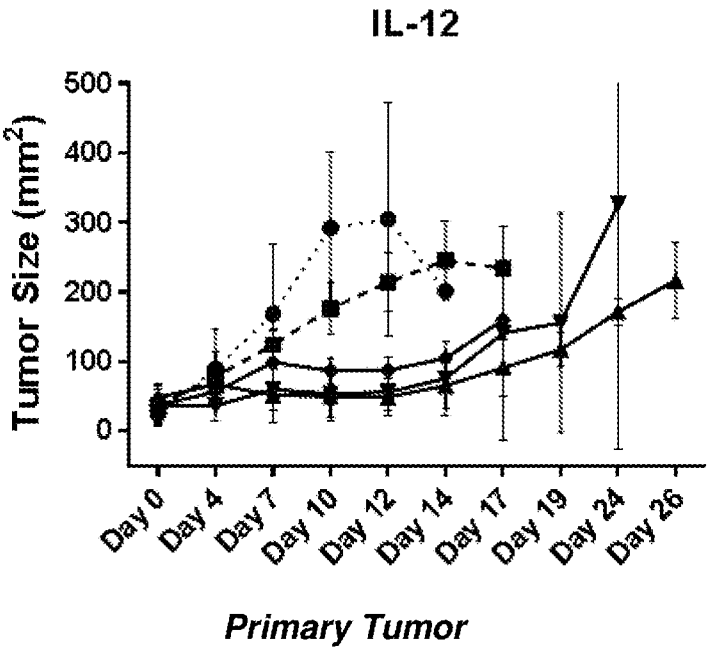

IL-2, 0.01ug
Tumor Size (mm2)
600
500
400
300
200
100
0
Day 0
Day 4
Day 7
Day 10
Day 12
Day 14
Day 17
Day 19
IL-2, 0.01ug
IL-2, 0.01ug
IL-2, 0.01ug
IL-2, 0.01ug
Blank
Blank
Blank
Blank
PBS
PBS
PBS
PBS

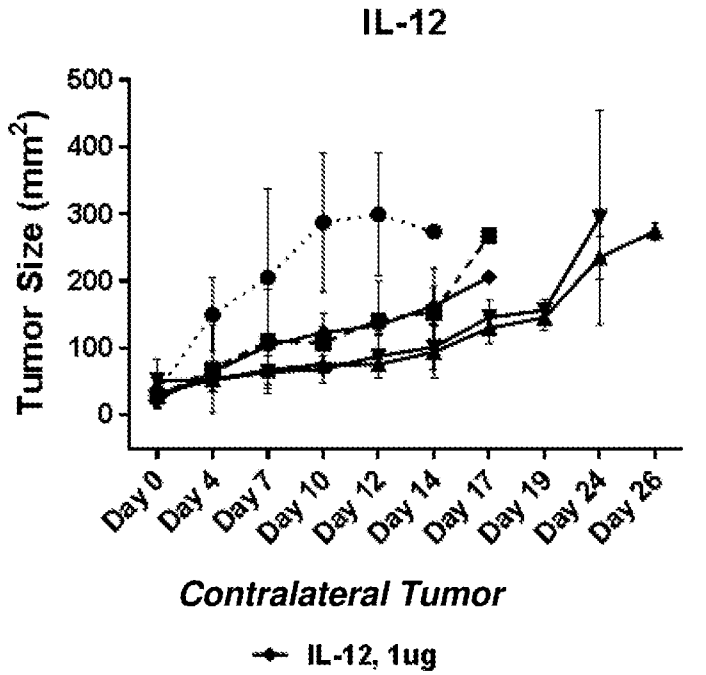
Fig. 15C

A



Primary Tumor

B



Contralateral Tumor

IL-12, 1ug
IL-12, 10ug
IL-12, 30ug
Blank
PBS

Fig. 16

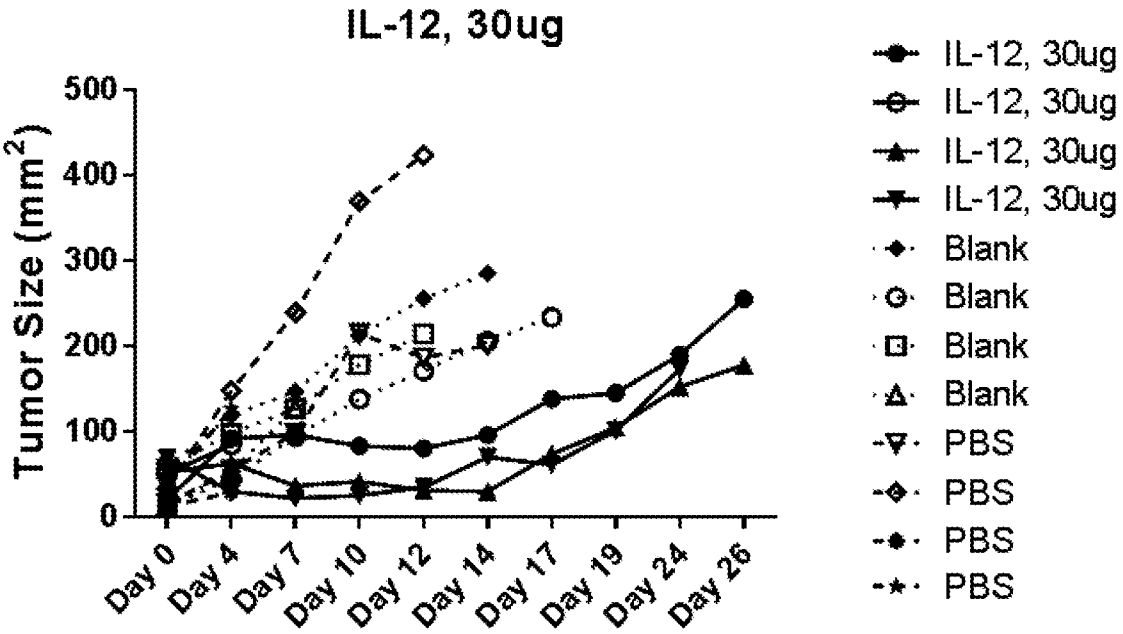


Fig. 17A

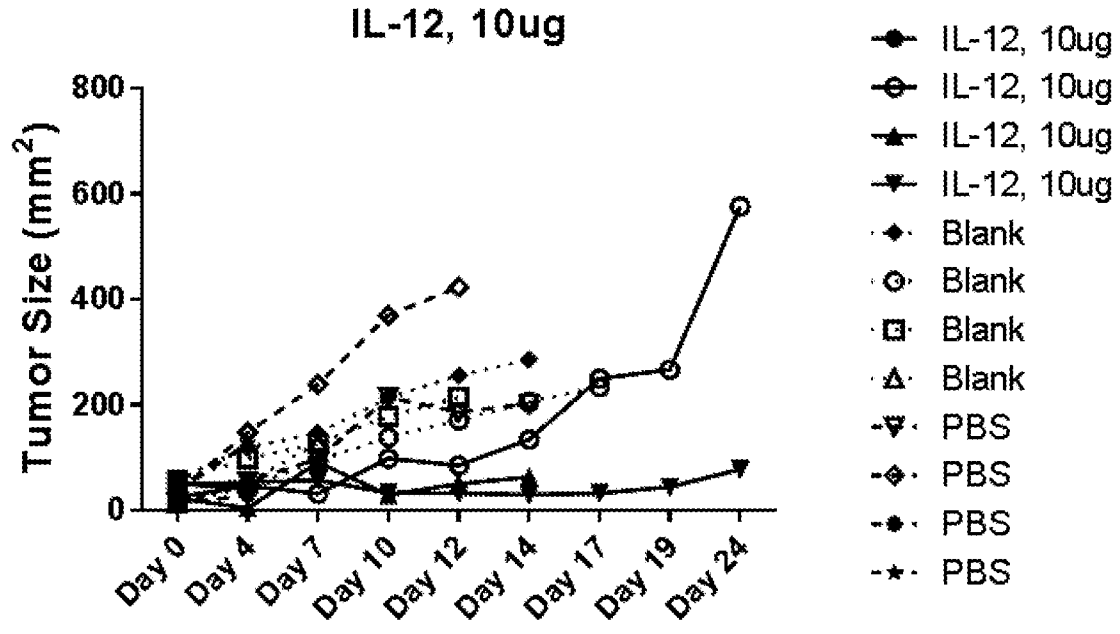


Fig. 17B

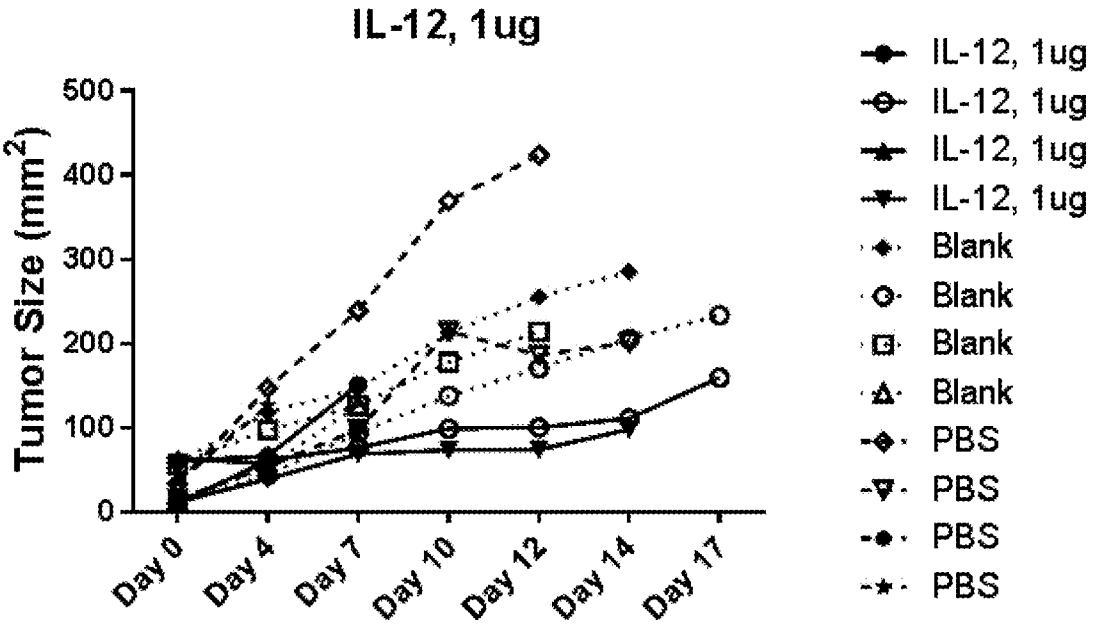

IL-12, 1ug
Tumor Size (mm2)
500
400
300
200
100
0
Day 0
Day 4
Day 7
Day 10
Day 12
Day 14
Day 17
IL-12, 1ug
IL-12, 1ug
IL-12, 1ug
IL-12, 1ug
Blank
Blank
Blank
Blank
PBS
PBS
PBS
PBS

Fig. 17C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/066640

A. CLASSIFICATION OF SUBJECT MATTER

A61K 9/10(2006.01)i, A61K 9/127(2006.01)i, A61K 47/30(2006.01)i, A61K 47/36(2006.01)i, A61K 38/20(2006.01)i, A61K 38/21(2006.01)i, A61K 38/18(2006.01)i

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)
A61K 9/10; A61K 9/14; A61K 47/34; A61K 31/74; C08G 63/00; A61K 38/48; A61K 47/48; A61K 9/127; A61K 47/30; A61K 47/36; A61K 38/20; A61K 38/21; A61K 38/18

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: polycationic polymer, polyanionic polymer, cytokine, IL-12, ILIO, PEAD, heparin

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 9023972 B2 (CHU, H. et al.) 05 May 2015 See abstract ; columns 18, 19, 55; and claims 1-13 .	1–12
A	CHU, H. et al., 'A [polycation:heparin] complex releases growth factors with enhanced bioactivity' Journal of Controlled Release , 2011 , Vol. 150 , pp. 157-163 See the whole document .	1–12
A	AWADA, H. K. et al., 'Dual delivery of vascular endothelial growth factor and hepatocyte growth factor coacervate displays strong angiogenic effect s' Macromolecular Bioscience , 2014 , Vol. 14, No. 5, pp. 679-686 See the whole document .	1–12
A	US 2007-0110813 Al (INGENITO, E. P. et al.) 17 May 2007 See the whole document .	1-12
A	US 9095619 B2 (ABBOTT CARDIOVASCULAR SYNTEMS INC.) 04 August 2015 See the whole document .	1-12

☐ 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	Date of mailing of the international search report
28 March 2017 (28.03.2017)	**28 March 2017 (28.03.2017)**

Name and mailing address of the ISA/KR
International Application Division
Korean Intellectual Property Office
189 Cheongsa-ro, Seo-gu, Daejeon, **35208,** Republic of Korea
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Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/066640

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

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

1. ☒ Claims Nos.: 13-16
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 13-16 pertain to methods for treatment of the human body by therapy, and thus relate to a subject matter which this International Searching Authority is not required, under PCT Article 17(2)(a)(i) and PCT Rule 39.1(iv), to search.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 13
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. Ill Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

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

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

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

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2016/066640

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 9023972 B2	05/05/2015	US 2013-0071930 Al	21/03/2013
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		wo 2007-123872 A2	01/11/2007
		wo 2007-123872 A3	13/03/2008