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(54) **Title:** PROTECTION AND DELIVERY OF MULTIPLE THERAPEUTIC PROTEINS

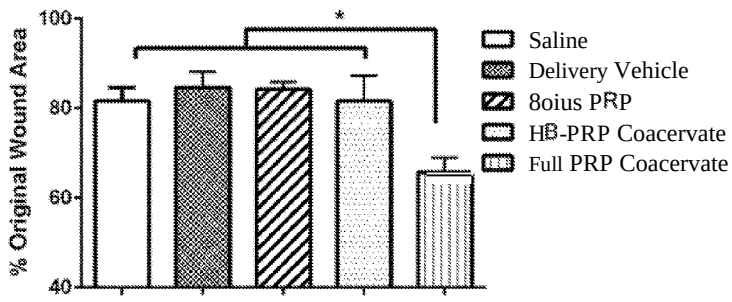


Fig. 5B

(57) **Abstract:** Compositions are provided herein comprising a coacervate of a polycationic polymer, a polyanionic polymer, and platelet-rich plasma and/or serum, or a fraction or concentrate thereof. The composition is useful in wound healing. Compositions also are provided that comprise a hydrogel comprising TIMP-3; and a complex of a polycationic polymer, a polyanionic polymer, FGF-2 and SDF-1a embedded in the hydrogel, which is useful in treating a myocardial infarction.

WO 2017/152039 A1

PROTECTION AND DELIVERY OE MULTIPLE THERAPEUTIC PROTEINS

STATEMENT REGARDING FEDERAL FUNDING

[0001] This invention was made with government support under Grant No, HP!444774, awarded by the National Science Foundation and Grant No, and IR01NR0i6436-0i, awarded by the National Institutes of Health. 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 Nos. 62/303,591, filed March 4, 2016, and 62/460,449, filed February i7, 20i7, each of which is incorporated herein by reference in its entirety,

[0003] Wound healing (and tissue repair) within the human body is governed by the expression of many cytokines, growth factors, and therapeutic proteins. Many researchers in the past have attempted to improve upon this healing response by administering protein therapies such as Vascular Endothelial Growth Factor (VFXJP) and Keratinoeyte Growth Factor (KGF), However, these proteins are expensive and have half-lives in the range of minutes, requiring multiple: large doses of proteins to show any improvements. This is expensive and has been linked to several adverse side effects. Therefore patients need access to more affordable therapeutic proteins that will not lead to damaging side effects.

[0004] Protein therapies have high potential to improve clinical outcomes in the field of tissue regeneration. These proteins, particularly growth factors and cytokines, relay signals between cells and their extracellular environment, providing cues for cells to proliferate, differentiate, migrate or secrete extracellular matrix (ECM) proteins. Supplying exogenous growth factors to. injured tissues has the potential :to-accelerate healing and regeneration of tissues; however, they must be applied in a sustained fashion to show efficacy. Due to very short half-lives, this is not possible with free growth factors, while targe doses aro cost-prohibitive and often result In undesirable off-target effects. The ideal regenerative therapy would involve a delivery system to extend the bioaetive half-lives of naturally-derived exogenous proteins rather than expensive recombinant proteins. Several approaches have been proposed to meet this clinical need, but few have demonstrated success in a clinical setting.

[0005] Cutaneous wound healing requires the coordination of many complex processes such as cell proliferation, migration, angiogenesis, and ECM deposition (S,A. Ernirig, *ei al.*, Wound repair and regeneration: mechanisms, signaling, and translation, *Sci Transl Med*, 6 (2014) 265sr266) . Each of these processes relies on a specific set of signaling cytokines and growth factors. Wounds are typically able to resolve themselves and close over time, however comorbidities such as diabetes and obesity can impair the healing response and result in a chronic non-healing wound. Chronic wounds are often the result of deficient growth factor signaling or reduced sensitivity to growth factors and require the use of advanced therapies. This contributes to the expanding $15 billion market for wound care products In

the United States. Even the most advanced wound care options currently available require many treatments, are often unsuccessful and result: in 185,000 limb amputations performed in the United States each year as a result of noh~healmg- wounds. New approaches addressing the deficient growth factor regulation of chronic wounds are urgen tly needed.

[0006] Growth factors have been assessed for therapeutic benefit in wound applications for more than forty years. Despite thorough evaluation of more than a dozen different growth factors, only human-platelet-derived growth factor (PDGF) has received approval for clinical treatment of diabetic ulcers. Under the trade name REGRANEX®, the PDGF gel promotes epithelial cell and fibroblast migration and a meta-analysis of 6 clinical trials reveals that overall the treatment is efficacious (X.H. Zhao, *et al.*, Efficacy of topical recombinant human platelet-derived growth factor for treatment of diabetic lower-extremity ulcers: Systematic review and meta-analysis, *Metabolism: clinical ana' experimental*^ 63 (2014) 1304-1313). However, this has not necessarily translated to significant clinical usage, in. part due to its high cost and poor reputation among clinicians (N, Papanas, *et al.*, Becapiermin gel .in the treatment of diabetic neuropathic foot ulcers, *Clinical imervemiom in aging*, 3 (2008) 233-240). Other notable candidates that have shown moderately positive results in clinical trials include vascular endothelial growth factor (VEGF) for neuropathic foot ulcers (J.R. fianft, *et al.*, Phase I trial on the saf ty of topical rhV EOF on chronic neuropathic diabetic foot ulcers. *Journal of wound care*, 17 (2008) 30-32, 34-37), fibroblast growth factor (FGF-1) for deep burns (B. Ma, *et al.*, Randomized, multicenter, doable-blind, and placebo-controlled trial using topical recombinant human acidic fibroblast growth factor for deep partial-thickness burns and skin graft donor site, *Wound Repair Regen*, 15 (200?) 795-799), and transforming growth factor-beta 3 (TGP-β3) for sear prevention (M.W. Ferguson, *et al.*, Prophylactic adnrinistraiion of avotermin for improvement of skin scarring: three double-blind, placebo-controlled, phase t/ll studies, *Lancet*, 373 (2009) 1264-1274), among others, though none have progressed further, presumably over concerns of a similar late to REGR.ANEX*. The multi-faceted etiology of chronic wounds suggests that exogenous application of a single factor may not have a dramatic comprehensive effect on healing outcomes.

[0007] Thus, there is a need for improved compositions -and methods for use in wound healing, and, more generally for use in regenerative medicine. There also is- a need for compositions and methods for repairing myocardial infarcts.

SUMMARY

[0008] A eoaeervaie protein delivery system is used to extend the half-lives of proteins and. deliver them to the site of injury. Described herein is the deli very of multiple growth factors and proteins, .such as growth factors and proteins obtained in one aspect from a very inexpensive and accessible source, platelet-rich plasma, or other protein-rich solutions-, such as solutions obtained from or prepared from products of ceils, tissues, organs, organ systems, or organisms, e,g., blood serum. The solution can be from cells, tissues, organs, organ systems, or organisms (natural or genetically altered, e.g. recombinant or transgenic). This advancement: allows delivery of a complex cocktail of therapeutic proteins^ that

cannot be made- synthetically. Platelet-Rich Plasma is already FDA-approved for clinical use; however, studies show that it is ineffective wiibout a protein delivery system. Therefore this innovation improves upon an appro ved therapy for wound healing by requiring less doses, reducing cost, and healing wounds more quicfcly. Other uses include treatment of myocardial infarction, cartilage regeneration, treatment of burns, and treatment of surgical trauma.

|0009| The compositions described herein use both synthetic and natural polymers to protect and deliver proteins over several weeks, extending their half-lives from just minutes, and reducing the quantity of protein needed by at least an order of magnitude, Thisis due to the natural interaction present between an anionic polymer, such as a natural polymer, e.g., heparin or heparan sulfate, and the proteins to be delivered. This forms an affinity-based protein delivery system, In addition, the ability to encapsulate and deliver many proteins simultaneously provides a less-expensive protein source, requiring less processing before implantation. The complex array of proteins this eoaeervate system is capable of delivering cannot be replicated using recombinant protein sources, and certain described natural protein sources (e.g., platelet-rich plasma, "PRP") are already FDA-approved.

BRIEF-DESCRIPTION OF THE DRAWINGS

[0010] The drawings provided herein are for illustrative purposes. Certain photomicrographs are color in their original, and are presented herein in grayscale unless otherwise noted. Original colors are indicated in the following descriptions,

[0011) Figure 1. Outline of the wounding procedure . (A) Combining PEAD with PRP-loaded heparin neutralizes the charged molecules, forming a complex eoaeervate. Treatments are applied via sterile pipet to a collagen foam before being applied directly to a wound. **(B)** Forced randomization of treatments: accounts. for differences in the healing response based on location.

10012) Figures 2A and 2B. Heparin-binding proteins preferentially load into the eoaeervate, (Figure **2A)** PDGF and VEGF exhibit loading efficiencies over 8 times greater than total protein loading due to their affinity to heparin. (Figure 2B) In vitro release of VEGF and PDGF as represented ve PRP proteins show a sustained release over 3 weeks. Reported as mean ± SD,

[0013) Figure 3, Full PRP eoaeervate accelerates.- reepithelializatioo of -wounds. Wound sections showing coverage by keratinoeyies 10 days after treatment with **(A)** saline, (**B**) delivery vehicle, (C) free PRP, (D) HB-PRP Coacervate, -or (E) **FRF** Coacervate, (F) Wound coverage at ten days is shown relative- to the 10-day wound length. Wounds treated with PRP exhibit a 35% increase in reepithelialization compared to saline. Error bars indicate mean ± SEM. Scale bars are *2mm,*

£0014) Figure 4, Full PRP eoaeervate improves vascular maturity at the wound margin, (A) Staining with vWF for endothelial cells shows a high vascularity within all wounds with the exception of full PRP eoaeervate when imaged at the wound margin, (B) As wounds exhibit higher vascularity than healthy tissue raid-wound at 10 days. (€) When imaged at the wound margin, wound receiving full PRP eoaeervate have similar vascular density to .healthy tissue while all other wounds exhibit a significant increase in vascularity. Scale bars = 100μm,

[0015] Figures 5A and *SB.* Controlled release reduces overall wound .size. (Figure SA) Representative wound images show overall wound health at 10 days. All treatment groups other than full PRP coacervate exhibit incomplete filling of the wound bed at this time point Wounds receiving full PRP coacervate were consistently a healthy pink color (in original) and smaller in area than other treatment groups. Tick marks are urn (Figure SB) full PRP coacervate shows significantly smaller wound size compared to all other treatment 10 days after wounding,

[0016] Figure 6. Full PRP coacervate aligns collagen deposition. MTS images of tissues taken mid-wound show increased collagen alignment when treated with M l PRP coacervate. Less gaps are also present as a result of this treatment Scale bars are Sfiim,

[0017] Figure 7»Design and protein release kinetics of fibrin gel-eoaeervate composite, (A) The release system was comprised of a fibrin gel embedding T1MP-3. aimed for early release; and FGF-2/SDF-1a-loaded coacsrvates distributed within the same gel aimed for late release. The coacervate was formed through electrostatic interactions by combining FGF-2 and SDF-1α with hepair n then with PEAD poiycaiion. (S) The release system described achieved sequential quick release of H MP-3 by one week followed by a sustained release of FGF-2 and SDF- la up to six weeks. Data are presented as means * SD (n~3).

[001.8] Figure; 8. Cumulative release profile; of complementary proteins. The cumulative release plot of the complementary proteins shows the total percentage amount of each protein released with time. The plot shows quick release of T!MP~3 by one week followed by a sustained release of FGF-2 and S.DF-1« up to six weeks. Data are presented as means-* SD (r_~3-}.

[0019] Figure 9, Effect of controlled protein release on cardiac function and LV dilation. (A) Traces of ESA and EDA areas from short-axis B-mode images of the left ventricle using echocardiography. (B) FAC values show differeaces between groups after Ml at multiple rime points, with- significantly higher FAC value of controlled release (CR) compared to saline and free proteins from two weeks onward. (C) Saline and free proteins groups show increasing ESA values, which wee :reduced in CR group. (D) Saline and free proteins groups show increasing EDA values, which were reduced in CR group. Data are presented as means ± SD (n- 9-10 per group). (E) Traces of ESA and EDA areas from short-axis view images of the LV using cardiac MRI. (F) BP values show differences between groups after *Ml* at -eight weeks, with significantly higher EF% of the CR group compared to saline and free proteins. (G) Saline and free proteins groups show .increasing ESV value at eight weeks, which was significantly reduced in CR, (H) Saline and tree proteins groups show increasing EDV value at eight weeks, which was reduced in CR. Data are presented as means ± SD (n.^5-8 per group). * $p<0.05$ vs saline, ≠ $p<0.05$ vs free proteins, ψ $p<0.0S$ vs sham.

[0020] Figures 1OA and 10B. Effect of controlled -protein release on myocardial strain levels. (Figure 1OA) Strain of an infarcted sample was estimated by nbrmalizmg the estimated peak radial strain in the infarcted area to that of the average of four non-infarct. areas in LV walls during a cardiac cycle, (Figure !0B) Saline and free proteins groups show decreasing radial strain at eight weeks, which was

significantly higher in CR group. Data are presented as means ± SD (n=5 per group), * p<0.05 vs saline, ψ p<0.05 vs sham,

[0021] Figure 11. Effect of controlled protein release on left ventricle wall thinning, MMP activity, and fibrosis. (A) Representative H&E images showed left ventricle (LV) wall thinning with damaged cardiac muscle surrounded by scar tissue in saline and free proteins groups at two and eight weeks. However, these damages were apparently alleviated in the CR group. Scale bar=100μm. (B) Quantitative analysis shows reduced ventricular wall thinning by CR at two and eight weeks over saline and free proteins groups. Data are presented as means * SD (n=3-4/group at two wks, n=4-6 at eight wks). (C) MMP-2/9 activity assay showed high levels of activity in infarct groups at eight weeks, but was significantly reduced at CR compared to saline. Data are presented as means ± SD (n=3-4 per group). (D) Representative picrosirius red staining images (red in original) show the dense collagen deposition along the left ventricle wall and infarct zone in saline, followed by the free proteins group, whereas it was limited to the infarct region in CR at eight weeks, (E) Quantitative analysis shows that collagen deposition was not different in infarct groups: at two weeks but was significantly less in CR compared to saline and tree groups at eight weeks. Data are presented as means * SD (n=3-5/group at two wks, m=4~? at eight wks). * p<0.05 vs saline, ≠ p<0.05 vs free proteins, ψ p<0.05 vs sham.

[0022] Figure 12, Effect of spatiotemporal protein delivery on collagen deposition at 2 weeks. Representative picrosirius red staining images (red in original) show the extent of collagen deposition in the different groups at two weeks after Mi.

[0023] Figure 13, Effect of controlled protein release on Inflammation. (A) Representative images of the different groups showing co-staining of F4/80 (red in original), a paii-macrophage marker, and CD163, an M.2 macrophage marker (green in original) at two weeks. Co-localisation of the two markers shows the color as yellow (in original), (B) The CR group shows a reduced number of non-MZ macrophages compared to saline and free proteins, but not statistically significant. (C) CR shows a significantly increased presence of M2 macrophages compared to saline. Data are presented as means ± SD (n=3-4 per group at two wks). * p<0.05 vs saline, ψ p<0.05 vs stem.

[0024] Figure 14. Effect of spatiotemporal protein delivery on. cytokine secretion levels at 8 weeks. Quantitative ELISA analysis shows (A) significant reduction in IL-1β levels in delivery and free proteins groups compared to saline, (8) no difference in 1L-6 levels among the infarct groups, arid (D) significant reduction in TNF-α level in delivery -group compared to both saline and free proteins. Data are presented as means * SD (n~3-4/group at eight wks), * p<0.05 vs saline, ≠ p<0.05 vs free proteins, ψ p<0.05 vs sham.

[0025] Figure 15, Effect of spatiotemporal protein delivery on. cardiac muscle viability at two weeks. Representative images of the different groups showing staining of viable cardiac muscle by cardiac troponin I (cTnI) (green in original.) at two weeks after MI.

[0026] Figure 16. Effect of controlled protein release on cardiomyocyte survival and apoptosis. (A) Representative images of the different groups showing staining of viable cardiac muscle by cardiac

troponin ! (cTnI) (green in original). Reduced viable muscle can be observed in all infarct groups, with belter preservation of the muscle in the CR group at eight weeks, (B) Quantitative analysis shows no differences between infarct groups at two weeks, but demonstrates the CR group's significant preservation of cardiac muscle viability at eight weeks compared to saline. Data are presented as means ± SD (n=3-5/giOup at two wks, n=*S-6* at eight wks), (C) Representative *western* blot images of the expression levels of p-ERK, p-Akt and cleaved caspase-3 in study .groups at eight weeks. (D) Intensity band analysis of cleaved caspase-3 shows significant reduction of expression level in CR compared to saline and free proteins groups. (E) Analysis of p-ERK 1/2 shows significant increase of expression level in CR compared to saline and free proteins groups, with free showing significance over saline as well. (F) Analysis of ρ-Akt shows significant of expression level in CR compared to saline-.and free groups. Data are presented as means * SO (n~3/gro«p at. 8 wks), * p<0,O5 vs saline, ≠ p<C>.05 vs free proteins, ψ p<0,05 vs sham.

[0027] Figure 17. Effect of spatiotemporal protein delivery on angiogenesis at two weeks. Representative images of the infarct groups showing co-staining of vWF (red in original), an endothelial marker,- and a-SMA (green in original), a pericyte marker at two weeks after M L

[0028] Figure 18. Effect of controlled protein release on angiogenesis, (A) Representative images of the different groups showing co-staining of vWF (red in original}, an endothelial marker, and a-SMA (green in original), a pericyte marker at eight weeks. (B) CR shows a significantly greater number of vWF+ vessels compared to saline at two weeks and compared ɒ saline and free proteins at eight weeks. (OCR shows a significantiy greater nuntber of vWF⁺ α-SMA⁺ vessels than .saline and free proteins groups at eight weeks but not at two weeks. Data are presented as means ± SD- (n=3-4/group at two wks, n=5-6 at eight wks). * p<0,05 vs saline, ≠ p<0.05 vs foe proteins.

[0029] Figures 19A and 19B. Effect of controlled protein release on stem cell homing. (Figaro 19A) Representative images of the different groups showing staining of c-Kit⁺ stem cells (green in original) at eight weeks, (Figure 19B) Quantitative analysis shows significantly greater number of c-Kit⁺ stem cells in CR compared- to saline and. free proteins groups. Data are presented as means ±.SD (n~5/grotsp at eight wks),. * p<0,05 vs saline, ≠ p<0.OS vs free proteins, ψ p<0,0S vs sham,

[0030] Figure 20. Effect of spatiotemporal protein delivery on secretion levels of relevant proteins at eight weeks. Quantitative ELISA analysis shows that (A) Free proteins and CR groups significantly increased IGF-I levels, (B) CR significantly increased VEGF levels compared to saline, (C) CR significantiy increased Shh levels compared to saline, and (D) Free proteins group significantly decreased TGP~β1 levels compared to saline, but delivery group significantly decreased TGF~β1 levels compared to both saline and free proteins. Data are presented as means ± SD (n=3~4/gronp at eight wks), * p<0.05 vs saline, ≠ p<0,05 vs free proteins, ψ p<0,05 vs. sham,

DETAILED DESCRIPTION

[0031] The use of numerical values in the various ranges specified in this application, unless expressly indicated otherwise, are stafed 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 staled ranges can be used to achieve-substantially the same results as values within the ranges. Also, unless indicated otherwise, the disclosure of these ratiges is intended as a continuous range including every value between the minimum and maximum values. l¾r definitions provided herein, those definitions refer to word forms, cognates and grammatical variants of those words or phrases.

|0032| As used herein, the term "polymer composition" is a composition comprising one or more polymers. As a class, "polymers" includes homopoiymers, heteropo!yniers, co-polymers, block polymers, block co-polymeirs and can be both natural and synthetic. Homopoiymers contain one type of building block, or monomer, whereas co-polymers contain more than one type of monomer.

[0033} 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.

[0034] As used herein, the term "patient" or "subject" refers to members of the animal kingdom including- hut not limited to human beings.

[0035] A ¾oaeervate" refers to herein as a reversible aggregation of compositions in a liquid, for example, as described herein, for example, resulting from the aggregation of oppositely-charged po!yiorsic compositions. Exemplary eoaeervates are illustrated in the examples below with the aggregation of the polycation, polyamiors, and active agent(s), as described herein, for example with the aggregation of PBAD, heparin, and PRP, or a combination of FCiF-2 and SDF-1α. A "complex" is a non-eovalent aggregation of two or more compositions,

|0036] The terra "alkyl" refers to both branched and straight-chain 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_{5-15} includes C_5, C_6, C_7, C_8, C_9, and C_{10}. Alkyi groups include, without limitation: methyl, ethyl, propyl, isopropyh n-, s- and t-butyl, n- and s-pentyt hexyl, hepiyt, oetyl, etc. Aikenes comprise one or more double bonds and aSkynes comprise one or more triple bonds. These groups include groups that have two or more points of attachment (e.g., alkylene). Gycloalkyl groups are saturated ring groups, such as cyclopropyl, cyclobutyl, or cyclopentyJ. As used herein, "halo" or "halogen" refers to fluoro. ehloro, bromo, and iodo.

|0037] A polymer "comprises" or is "derived from" a staled 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 or atoms are incorporated into the polymer backbone or are excised, A polymer is said to comprise a specific type of linkage, such as an ester, or urethane linkage, if that linkage is present in the polymer.

(0038] According to one aspect of the invention, a composition is provided comprising a complex, e.g. a coacervate of a polyaiiionie polymer, a polycationic polymer, and platelet-rich plasma or -serum, including concentrations thereof, according to any aspect described herein. The platelet-rich plasma or serum, including concentrations thereof, is mixed with the polyanionie polymer composition to form a complex, and the resulting complex is then mixed with a polycationic polymer composition to form a

composition, e.g., a eoacervate. The charges of the polycation arid polyamon are generally approximately equal to form a ehatge-rieutral complex, e.g., coacervate.

[0039] Suitable polyaiiionic polymers include as a. class sulfated and/or sulfamated polymer or oligomers, such as sulfated polysaccharides or .sulfated giyeosiaminoglyeans. Sulfated and/or sulfamated polymer or oligomers include sulfated and/or sulfamated polysaccharides. Synthetic and natural sulfated and/or sulfarnated -polysaccharides or oligosaccharides include, for example and without limitation, -sulfated glycosaminoglyeam or sulfated galsctans, ulvans and fueans (Jiao, G., *et at.* Chemical Structures and Bioactivities of Sulfated Polysaccharides from Marine Algae (2011) Mar. Drugs 9:196-223). Non-limiting examples of sulfated and/or sulfamated polysaccharides include, pentosan polysul&tes, dermatan sulfate, keratan -sulfates, chondroitin sulfates, sulfated agarans.(e.g ., porphyrans), and carageenans. in another aspect, the sul&ted at d/or sul&mated polymer or oligomer is a sulfated and/or sulfamated synthetic polymer, such as a polyurethane, polyester, polyarea, polyamide-ester, polyether, polycarbonate, ppiyanvide, or poSyolefin, or .copolymers thereof, as are broadly-known in the polymer arts. By "sulfated" , it is meant thai the polymer comprises a plurality of pendant sulfate **(-OSO_3)** groups, though many such compositions -also are "sulfaniated" - comprising a plurality of pendant sutfamate (-NSO3) -groups. Examples of suitable polysaccharides include, without limitation: a sulfated polysaccharide, a .sulfamated polysaccharides, a sulfated and/or .sulfamated polydisaccharide, a sulfated glycosammoglycan, heparin, and heparan sulfate.

[0040] "Platelet-rich plasma" or "PRP" in its broadest sense is blood plasma with an enriched platelet content, where "enriched" is in reference to normal blood of a patient. Typically platelet content is enriched at least two-fold, and -often at least five-fold or ten-fold. Platelet-rich plasma is typically prepared by centrifugation of anti-coagu$ase-treated blood obtained .from one or more patients, and can be autologous. Four forms of P.RP are -commoniy-ava-iiabje: Pure Platelet-Rich Plasma: (P-PRP) or leucocyte-poor P.RP products are preparations without leucocytes and with a low-density fibrin network after activation; Leneocyte-PRP fL-PRP) products are preparations with leucocytes and with a low-density fibi n network after activation. This is the most common commercial PRP product; Pure platelet-rich fibrin (P-PRF) or leucocyte-poor platelet-rich fibrin preparations are without leucocytes and with a high-density fibrin network; and Leucocyte- arid platelet-rich fibrin (L-PRF) or second-generation PRP products are preparations with leucocytes and with a high-density fibrin network (Dhurat *et at.* Principles and Methods of Preparation of Platelet-Rich Plasma; A Review and Author's Perspective. *Journal of Cutaneous and Aesthetic Surgery.* 2014;7(4):189-197).

[0041] A number of methods are broadly-known for preparation of PRP. in one instance, blood is collected in tubes containing anticoagulants. A platelet layer, a buffy coat layer, and a red blood cell (SBC) layer are produced. For production of P-PR.P, the platelet layer ad only the superficial buffy coat layer are transferred to a clean tube. For preparation of 1,-PRP, the platelet layer and *huffy* coat layer are transferred to a clean tube. The second tube is spun in a centrifuge resulting in a soft platelet pellet at the bottom of the tube. A portion, e.g. 2/3, of the platelet-poor top volume is.removed, and the

platelet pellet is **then** dispersed, e.g. **homogenized, in** the **remaining** plasma, An **alternative** method is **known** as the "huffy coat" method in which whole blood is ceivtrifuged at a high speed to **.form** a tighter **huffy** coat as **compared** to **the PRP** method above,. PRP, **and** in general platelets, can be activated by addition of calcium and thrombin as is broadly-known, or by any other useful means.

[0042] "Serum" is blood that is allowed to coagulate, and the clot and cellular constituents are **then removed.** Fractions **of serum** or platelet-rich plasma may be employed. By "fractions", it is meant a portion of the **serum** or platelet-rich plasma prepared by any suitable method, including by precipitation, solvent extraction, filtration, centrifugation, or any other suitable method - so long as the fractionated product is **not. reduced to a** single purified compound, **such as** a single protein, **glycoprotein,** polysaccharide, or other composition found in the platelet-rich plasma or serum. **Concentrates** are solutions *in* which a portion of the **solvent,** e.g. **water in the** case of a blood product, is removed thereby increasing the **concentration** of compounds **present in** the solution, such as proteins, glycoproteins, polysaccharides, or **other** desirable compositions found in the platelet-rich plasma or serum.

[0043] FGF-2 is Fibroblast **growth** factor 2 (HGNG: 3676, Bntrez Gene: 2247, Ensembi: ENSG00000138685, OMIM: 134920, UniProtKB: P09038), having the sequence, for example:

MVGVGGGDVEDVTPRPGGC QISGRGARGCNG IPGAAAWEAALPRRRPRE
HPSVNPRSRAAGSPRTRGRRTEERPSGSRLGDRGRGRALPGGRLGGRGR
GRAPERVGGRGRGRGTf APRAAPAARGSRPGPAGTMAAGS ITTLPALPED
GGSGAFPPGHFKDPKRLYCKNGGFFLRIHPDGRVDGVREKSDPHIKLQLQ
AEERGWS IKQVCANRYLAMKEDGRLLASKCVTDSCEFFERLESNNYNTY
RSRKYTSWYVALKRTGQYKLGSKTGPGQKAILFLPMSAKS (SEQ ID NO: !)

fO044] SDF-1α, Stromal Cell-Derived Factor 1, is **the** geane product of CXCL12 gene in humans (HGNC: 10672, **Entrez** Gene: 6387, Ensembi: ENSG00000107562, OMIM: 600835, UniProtKB: P48061), and having an **exemplary** sequence:

MNAKVVVLV LVLTALCLSD GKPVSLSYRC PCRFFESHVA RANVKHLKIL
NTPNCALQIV ARLKNNNRQV CIDPKLKWIQ EYLEKALNKR FKM (SPQ ID NO: 2).

[0045] TIMP-3 is **TJMP** is Tissue Inhibitor of Metaiioproteinase 3 **(HGNC:** 10672, Entrex Gene: 6387, Ensembi: ENSG00000107S62, OMIM: **600835,** UniProtKB: P48061), having an exemplary **amino** acid sequence;:

MTPWLGLIVLLGSWSLGDWGAEACTCSPSHPQDAFCNSDIVIRAKVVGKKLVKEGPFGTL
VYTIRQMKMYRGFTKMPEVQYIHTEASESLCGLKLEVNKYQYLLTGRVYDGKMYTGLCNF
VERWDQLTLSQRKGLNYRYHLGCNCKIKSCYYLPCFVTSKNECLWTDMLSNFGYPGYQSK
HYAGXRQKGGYCSWYRGWAPPDKSIINAYDP (SEQ ID N0: 3}.

{0046] Certain polymers described herein, **such** as heparin and PEAD, are said to be bioerodible 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, Non-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.

[0047] A drag delivery composition is provided, comprising, a eoaeervate of a polycationic polymer, a potyanionie polymer, and an active agent. In certain aspects, the polycationic polymer described herein comprises the structure (that is, comprises the moiety: {-OC(0)-B'-CH(OR1)-B-3« Of ~[OC(0)-B-C(O)O-CH_2-CH(O-R1)-CH_2-B'-CH_2-CH(O-R2)-CH_2-}« in which B and B' are the same or different and are organic groups, or B' is not present, including, but not limited to: alky!, ether, tertiary amine, ester, amide, or alcohol, and can be linear, branched or cyclic, saturated or unsaturated, aliphatic or aromatic, arid optionally comprise one or more protected active groups, such as, without limitation, protected amines and acids, and R1 and R2 are the same or different and are hydrogen or a functional group (e.g., as described herein). As seen below, the composition exhibits low poiydispersity, with a poiydispersity index of less than 3.0, and in many cases less than 2.0. These compositions: are described in United Slates Patent No, 9,023,972, which is incorporated by reference in its entirety.

[0048] In one aspect, the polycationic polymer is a polymer composition comprising at least one moiety selected from the following in which B and B' are residues of aspartic acid or glutamic acid, which. are optionally further derivatissed with an ainine-containing group, for example, the amines of the aspartic acid or glutamic acid are further derivatized with lysine or arginine:

(a) (-DC(0)-CH(NHY)-CBrC(Q)0-Cl-irCi I(O-R1)-Ci¾-O-C¾ -CBr O-€ ¾ -CH(0 -R2)-CH_2-j$_n$,

(b) [-OC(0)-CH3-CH(NHY)-C(0)0 -CH2-CH(0 -R1)-CH_2-O-CH_2-CH_2-O-CH_2-CH(0 -R2)-CH_2-]$_n$,

(c) [-OC(O)-CH(NHY)-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)-CH_2-CH_2-CH(NHY)-C(O)O-CH2-CH(O-R1)-CH_2-O-CH_2-CH_2-O-CS¾-CH.(0-R2)-CH3-)$_n$,

wherein Y is -C(O)-Ch(NHi$^+$)-(CH_2)j-NH-C(NHj)$_2^+$ or -C(0)-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-containing group, a quaternary ammonium containing group, and a peptide,

[0049] The polymers described herein can be functiona!ized, e.g., at B, B', R1 and R2, meaning they comprise one or more groups with an activity, such as a biological activity. For example and without iimitation, as shown herein, the polymer may be functionalized with an acetyleholine-ifke group or

moiety, a cross-linking agent (cross-linking agents contain at least two reactive groups that are reactive towards numerous groups, including sulfhydryls and amines, and create chemical covaient bonds between two or more molecules, functional groups that can be targeted with cross-Unking agents include primary amines, cartoxyls, sulfhydryls, carbohydrates and carhoxylic acids, A large number of such agents are available commercially from, e.g., Thermo tlsher Scientific (Pierce) and Sigma),

[0050] Other functions that can be provided by or enhanced by addltion of functional groups include: increased hydrophobielty, for instance by funetioualizing with a superhydrophobie moiety, such as a perflnoroai.fcane, a perfluon (alkyisiiane), and/of a siloxane; increased hydrophl!ieity, for instance by functionalizing with polyethylene glycol (PEG); or antimicrobial, for instance, by functionalizing with a quaternary amine. The polymer can be functionalized with a tag, such as a fluorescent tag (e.g.,FITC, a cyanine dye, etc.). The polymer can be functionalized by linking to additional- synthetic: or natural polymers, including, without limitation: synthetic polymers, such as a polymer derived from an alpha-hydroxy acid, s polylaetide, a poly(lactide-co-glyeolide), a poly(L-lactide-c0-caprolaetone), a polyglycoitc acid, apo!yidl-Sactide-co-giycolide), a poIy(l-lactide-co"dI-lactide), a polymer comprising a lactone monomer, a polycaproiactcme, a polymer comprising carbonate linkages, a polycarbonate, a poiygtyconate, a pol><gtycolide-co-trimethyiene carbonate), a poiy(glyeolide-co-tri:methylene carbonate-'co-dioxanorte), a polymer comprising urethane linkages, a poSyurethane, a poly(esier urethane) urea, a poiy(ester urethane} urea elastomer, a polymer comprising ester linkages, a poiyaikanoate, a. po!yhydroxybutyrate, a polyhydroxyvalerate, a poiydioxanone, a poiygalaetin, or natural polymers, such as chltosan, collagen, gelatin, elasiin, alginate, cellulose, hyaluronic acid and other -glycosaminoglycans ,

[0051] The compositions may be functionalized with organic or inorganic moieties to achieve desired physical attributes *{e.g.,* hardness, elasticity, color, additional chemical reactivity, *etei),* or desired functionality. For example, the polymer composition may be derivatized with ma Sie acid or phosphate.

(0052] Further to the above, functional groups may vary as indicated above. For example, in certain aspects, ill and R2 are the same or different and are independently selected from the group consisting -of hydrogen, a earboxy-eontaining group, a C_{1-6} aikyl group, au-amitie-containing group, a quaternary ammonium containing group, and a peptide. In one aspect, one or more of B, B', R1 and R2 are charged such that it is possible to bind various water insoluble organic or inorganic compounds to the polymer, such as magnetic Inorganic compounds. As above, in one aspect, one or more of B, B', Rl and R2 are positively charged. In one aspect, one or both of R1 and R2 are functionalized with a phosphate group. In another aspect, the composition is attached non-covalently-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, R1 and R2 are independently one of Ile-Lys-Val-Ala-VaJ (IKVAV) (SBQ ID NO: 4), Arg-Gly-Asp (ROD), Arg-Gly-Asp-Ser (RODS) *(SBQ* 10 NO: 5), Ata-Gly-Asp (AGD), Lys-Gln-Ala-Gly-Asp-Val (KQAGDV) (SEQ ID NO; 6), Vai-Ala-Pro-Gly-Val-Gly (VAPGVG) *(SEQ* ID NO: 7),

APGVGV (SEQ ID NO: 8). PGVGVA (SEQ ID NO: 9), **VAP, GVGVA** (SEQ ID NO: 10), **VAPG** (SEQ ID NO: 11), **VGVAPG** (SEQ ID NO: 12), VGVA (SEQ ID **NO:** 13), **VAPGV** (SEQ ID NO: 14) and. **GVAPGV** (SEQ ID NO: 15).

[0053] The composition is formed into a coacervate with active agents or **polyanionic** or polycationic groups for sequestering active agents for controlled **delivery** *in vivo.* Drug products comprising the coacervaie described herein **maybe** 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.**

[0054] In forming the composition (e.g., coacervaie), .the cationie polycationie polymer is compiexed with a poiyanionic polymer, **such** as heparin or hepaan **sulfate,** which is complexed with an active agent, such as a growth **factor-, small molecule,** cytokine, **drug, a. biologic,** a. protein or polypeptide, a ehetnoattractant, a binding reagent, an **antibody** or antibody fragment, a receptor or a receptor fragment, **a** ligand, an antigen and/or **an** epitope, PRP, **or a composition obtained from an organism or cultured** cells, tissues or organs and **containing** a native, complex mixture of proteins and/or growth factors. Specific examples of active agents include **interieukins** (S.L), such as **IL-2** and il,-12 (e.g., **IL-12** *p70*), and interferons (IFN), such as IFN-γ. In one aspect, the composition comprises a coacervaie of a polycationic polymer comprising one or more of moieties **(a),** (b), (e), and/or (d), as described above, and further comprising heparin or heparin sulfate compiexed (that is non-covalently bound) with **FGP-**2 and **SDF-1α**. The composition is **formed, for** example, **by** mixing in a suitable solvent, such as an aqueous **solution, such as** water, saline (e.g. **normal saline), or PBS, the. poiyanionic,** polycationie, **and** active agent constituents of the composition.

[0055] Additional active **agents** that **may** be incorporated into **the coacervate. include,** without Sim.iia.tion, antiinflammatories, such as, without limitation, NSAIDs (non-steroidal anti-inflammatory drags) such as **salicylic** acid, indoniethacin, sodium indomethacin trihydrate, saSieylaraide, naproxen, **colchicine,** fenoprofen, su!indac, diilunisai, diclofenac, indoprofcn sodium salieyiamide, anti-inflammatory cytokines, and anti-inflammatory **proteins** or steroidal anti-inflammatory agents); antibiotics; antic-lotting factors such as **heparin, Pebae, enoxaparin,.** aspirin, hirudin, plavix, bivalirudin, prasugrel, **idraparinux**, warfarin, **Coumadin, clopidogrel,** PPACK, **GGACK,** tissue **plasminogen** activator, urokinase, and streptokinase; growth footers. Other active agents **include,** without limitation: **(!) immunosuppressants;** glucocorticoids such as hydrocortisone, betamethasone, dexamethasone, flumethasone, isofiupredone, .raeihylpred.nisolo.ne, prednisone, prednisolone, and triamcinolone acetonide; (2) antiangioge-mcs such as tluorouracit, pae!itaxet, doxorubicin, cispiatin, methotrexate, **cyclophosphamide,** etoposide, **pegaptanib,** hieeiitis, tryptophanyl-tRNA synthetase, retaane, CA4P, AdPEDP, VEGF~TRAP-EYfi, **AG-1039S8, Avastin,** JSM6427, **TG10080!,** ATG3, OT-SS1, **-endostatin, thalidomide, bevacizttmab,.** neovastai; (3) anii-proiiferatives such as sirolimns, pae!itaxei, periliyi alcohol, faroesyl. transferase inhibitors:, FPT!il, **L744, anti-proliferative.** factor. Van 10/4, doxorubicin, 5-FU, Daunomycin, **Mitomycin,** dexamethasone, azathioprine, chlorambucil, **cyclophosphamide ,** methotrexate, **mofetil,** vasoactive intestinal polypepii.de, and PACAP; (4)

antibodies; drugs acting on immnnophiSins, such as cye!osporine, zotarolhnus, evero!imus, tacrolimus and siroiimus Crapamyein), interferons, TNF binding proteins; (5) -taxanes, such as paclitaxei and docetaxel; statins, such as atorvastathi, lovastatin, simvastatin, pravastatin, fluvastatm and rosuvastatin; (6) nitric oxide donors or precursors, such as, without limitation, Angeli's Salt,. L-Arginine, free Base, nitrates, nitrites, Diethyl amine NONOate, Diethylamine NONOate/AM, GSyco-SNAP-1, Glyco-SNAP-2, <+.)-S-Nitroso-N-acetylpenicillamine, S-Nitrosogluiathioue, NOC-5, NOC-7, NOC-9, NOC-12, NGC-18, NOR-1, NOE-3, SIN-1, Hydrochloride, Sodium Nitroprusside, Dihydrate, Spermine NONOate, Streptoxotocin; and (7) antibiotics, such as, without limitation: acyclovir, ofloxacin, anipiciiiin, amphotericin B, atovaquone, azithromycin, ciprofloxacin, clarithromycin, clindamycin, clotazimine, dapsone, dic iazuril, doxycycline, erythromycin, ethambutol, fluconazole, fluoroqumolon.es, fbsearnet, ganciclovir, gentamicin, itraconazole* isoniazid, ketocortazole, ievofloxacin, Iineomycin, miconazole, neomycin, norfloxacin, ofloxacin, paromomycin, penicillin, pentamidine, polymyxin B, pyraxmamide, pyrimethamine, rifabutin, rifampin, sparfloxacin, streptomycin, .sulfadiazine, tetracycline, tobramycin, irifluorouridine, trimethoprim sulfate, 2n-pyrithione, and silver salts such as chloride, bromide, iodide and periodate,

|β©56| 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), hepatocyte growth facto r (HSGF), transforming growth factor-beta pfeiotrophin protein, midkine protein, platelet-derived growth factor fP.DGF) and angiopoietio-l (Aog-I). Active agents are included in the delivery system described herein, and are administered in amounts effective to achieve a desired end-point, such as augiogenesis, tissue growth, inhibition of tissue growth, repair of tissue (e.g. an infarct) or any other desirable end-point.

|0057| 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, arid/or an inorganic substrate, and can.be combined with 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 as fibrin.. Such a structure is nsefui 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 ageni(s) can he 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 coacervate, 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

coacervate and hydrogel. Appropriate and effective amounts of each component can be-determined in the ordinary coarse by a person of skill in the art

[0058] Therefore, according to one aspect of the invention, a composition is provided comprising a complex, e.g. a coacervate of a poiyanionic polymer, a polycaisonie polymer, and one or more active agents, that is embedded in a hydrogel, such as a .fibrin hydrogel, which contains an active agent for faster release than active agent(s) complexed in. the coacervate, In one aspect, the active agent -is PRP, serum* or a complex mixture of proteins and/or growth factors produced by cells, tissue, or an organism. In one aspect, the active agents complexed in the coacervate are FGF-2 and SDF-1α by first mixing with the polyanionic polymer, followed by mixing with the polycationic polymer to form the eoaeervate. The charges of the poiycaiion .and polyanion are generally approximately equal to form a charge-neutral complex, e.g., coacervate. TlMF-3 and the coacervate are then mixed into a hydrogel. composition, e.g. prior to or during formation of the hydrogel. In one aspect, each active agent is present in amounts effective to treat a myocardial infarct, by application of the composition at or near a myocardial infarct, e.g., by injection.

[0059] Examples of useful active agents and combinations of agents for incorporation into the described coacervate for treatment of a myocardial infarct include: TS.MP-3, FGF-2, and SDF-1α, Also described herein is a method of treatment of myocardial infarction, using the combination of TIMP-3 in a hydrogel, and a eoaeervate as described comprising FGF-2 and SDF-1«..

[0060] The coacervate composition according to any aspect described herein is delivered in any manner use-fid and appropriate -for treatment of it condition in a patient, sueh as for treatment of a wound, cardiovascular disease, or an infarct, such as by enteral, parenteral, or topical routes, for example and without limitation by: intravenous (IV), local injection, intramuscular, intracerebral, subcutaneous, oral, inhalation, topical, enema, intravaginal, intrauterine, ocular, or otic routes. Typically, due to the nature of wounds and myocardial infarcts, the composition is typically applied either topically or by injection at or near the site of the wound or infarct, as opposed to systemieally.

[0061] Suitable excipients Or .carriers are employed tor delivery of the coacervate composit on, though the excipients are consistent with maintenance of the coacervate complex. Suitable excipients are broadly-known in the pharmaceutical arts, and include: solvents, such as water, phosphate-buffered saline (PBS), saline: buffers; salts; acids; bases; theology modif ers; chelating agents; colorants; flavorings; penetration enhancers; and preservatives. The coacervate composition is provided in a suitable vessel for storage, distribution and/or use of the composition. In one aspect, the eoaeervate composition is provided in a tube, a -medical syringe, an IV bag. In another aspect, the coacervate composition is delivered to a patient in an amount effective to treat a myocardial infarction, for example by direct injection of the -eoaeervate composition comprising TiMP-3, FOF-2, and SDF-1α into the heart, e.g., the myocardium at or adjacent to an infarct

[0062] An "amount effective" for treatment of a condition is an amount of an active agent or dosage form, such as the coacervate composition described herein, effective to achieve a determinable end-

point. The "amount effective" is preferably safe - at feast to the extent the benefits of treatment outweighs the detriments and/or the detriments are acceptable to one i&f ordinary ski!l and/or to art appropriate regulatory agency, such as the U.S. Food and Drug Administration. In the context of wound healing, the end point may be decreased wound size, in a myocardial infarction, the end point may be improved cardiac output or improvement in the infarcted tissue, or in both instances any other ©bjecttvely-detennmable 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 coacervate composition described herein, and titrate the effect on any objectively-deierminabie end-point, 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.

[0063] The coacervate 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-weekiy, monthly, bi-mon thly, 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 wound or infarct severity *in* a patient, or improvement in wound healing, cardiac output or infarct repair.

[0064] In use, according to One aspect, the coacervate composition is delivered to a patient in an amount effective to treat a wound in a patient. For treating a wound, the coacervate is formed in the presence of platelet-rich plasma or serum according to any aspect described herein. The composition is delivered, for example by injection, at or adjacent to a wound, or application as a solution, gel, or as applied to any medical device or wound dressing. In one aspect, the composition is delivered to a patient at or adjacent to a wound by injection, topical application, spraying (spay or aerosol), swabbing, or any effective means of transferring the composition to the wound location. In another aspect, the composition is applied to a wound dressing, such as a bandage, a suture, a surgical mesh, or a non-woven material as are broadly-known in the medical arts. In yet another aspect, the composition is applied to an implanted medical device, such as a prostheses, so that the composition facilitates integration of the device into the local tissue and/or healing .of wound surrounding the device as a result of trauma, disease, or the process of inserting the device, such as a heart valve,

[0065] 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 a hydrogek such as a fibrin hydrogel, comprising TiMP-3, and a complex or coacervate of a polycationic polymer, a polyanionic polymer, FGF-2 and SDF-1α embedded in the hydrogel. The composition and respective amounts of TfMP-3, FGF-2, and SDF-1a are administered in amounts 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.

[0066] In another aspect, a composition is provided comprising a coacervate of a poiyeationic polymer, a polyanionic polymer, and a composition obtained from an organism or cultured ceils, tissues or organs andconiaining a native, complex mixture of proteins and/or growth factors. A "native, complex mixture of proteins and/or growth factors" refers to a composition produced by a living source, and though it may proceed through one or more purification steps, as in the case of PRF as described herein to remove blood cells, activate platelets, and optionally to remove -fibrin and to concentrate the proteins, it is not an isolated or purified single constituent, but includes a plurality of compounds and proteins, such as growth factors, essentially in amounts and proportions found in, or produced .by the cells or organism. Examples of suitable sources of the native, complex- mixture of proteins and/or growth factors include; a bodily fluid such as blood - including plasma or serum, or processed plasma or serum, conditioned medium from a ceil, tissue or organ -culture, or a cell or tissue- lysate or homogenate.

[0067] As used herein "Oondft-ioned media" is media prepared: by the culture of cells or other living tissue therein, wherein the cells or living tissue are natural.or genetically-modified. The conditioned media .includes proteins and other compositions representative of the secretorae of the living material grown therein. The cell secretome refers not only to the collection of proteins that contain a signal peptide and are processed via the endoplasmic reticulum and Golgi apparatus through the classical secretion pathway, but encompasses proteins shed from the cell surface and intracellular proteins released through non-classical secretion pathway or exosomes. These secreted proteins may be enzymes, growth factors, cytokines, hormones, and/or other soluble mediators. The medium used to produce the conditioned media- by growth of living ceils or tissue therein may be any medium suitable for growth of the living cells or tissue, A large variety of media is available commercially, and one of ordinary skill in the art could determine a useful or optima! medium for use in this context. In one aspect, the .media is serum-free. Various other fractionation processes, sueh as precipitation, centrifugation, affinity separation, or filtration may be applied to, clean up, to remove harmful compounds, or to otherwise fractionate the conditioned media.

[0068] The coacervate compositions* according to any aspect described herein are formulated into medically- and pharmaceutieally-acceptabe dosage forms or devices, such as a liquid, a gel, a spray, an aerosol, or a wound dressing or medical device, such as a non-woven, a bandage, a suture, a mesh, a prosthetic, or an implantable/implanted medical device. The composition may comprise any useful excipient, or inactive ingredient, such as water, saline, phosphate-buffered saline, and effective -amounts of any non-interfering active agent, such as, without limitation: an antibiotic, an. anti-inflammatory, or an analgesic, or any other useful active agent:, as are broadly-known in the pharmaceutical arts, A person of ordinary skill in the medical and pharmaceutical arts can readily fashion any of these products.

Example 1 - Synthesis and testing of PEAID

[0069] Synthesis and testing of PEAR PEAD-heparin, and PEAD FGF2: are described in United States **Pateat** No, 9,023,972, which is incorporated by reference in its entirety. Briefly, for **synthesis** ofPEAD - **t-BOC** protected aspartic acid (t-BOC Asp), t-**BOC** protected arginiue (t-BOC-Arg) (EMD **Chemicals,** NJ), ethylene glycol diglyeidyl ether (EGDE), trifiuoroacetic acid (TFA) {TCI Ameriea, OR), **anhydrous** 1**,4-dioxane** and tetra-n-butylammonium **bromide (TBAB)** (Aeros **orgaaics,** GeeL **Belgium), dicyclohexytcarbodiimide** (DCC), N-**hydroxysuccinimide** (NHS) (Alfa **Aesar,** MA) **aad** *4*-dimethylaminopyridine (DMA?) (Avocado Research Chemicals Ltd, Lancaster, UK) were used for PEAD synthesis without **purification.** The synthesis **ofPEAD** 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 **TFA** to **generate** primary amine. t-BOG-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 FEAD was measured by PL-GPC **50** Plus- ¾l equipped with a PD 2020 Sight **scattering** detector (Varan, MA), Two MesoFore 300x7.5 mm columns and 0.1% of LiBr in **DMP** were **used** as solid phase **and** mobile phase, **respectively.** I*n* **one** example, **the** weight-average molecular weight (Mw) is **30,337 Da** with **polydispersiry index (PDI)** 2.28.

[0070] Since PEAD is a **positively-charged** molecule, addition of PEAD into heparin solution should **neutralize** the negative charge of heparin and forms PEAD/heparin complex. To test the binding ability of PEAD **to** heparin, zeta potential measurement was perf rmed and **the zeta** potential of the complex shifted **from** negatively-charged (-45 m*V)* 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 PEAD itself. Data suggested that for the described READ preparation after ratio 10 the complex was all covered by FBAD, 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.

[0071] Further **confirming** the **binding** ability, different amounts of PEAD to heparin solutions were mixed and then precipitated by centrifugation. Because the neutralization of the negative-charged heparin favors the formation of precipitate, we measured the **amount of heparin** 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 defect free heparin fey measuring the absorption of DMB at 52()nm, 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 X > 90 % of heparin was precipitated **through** centrifugafion. At the ratio 5, that would be > **99 % of heparin**. This **result** has a good correlation with that **©f ¾eta** potential measurement because both experiments suggest at ratio 5 FEAD and heparin has the maximum interaction,

{0072} it is understood that a variety of growth factors can bind ɒ heparin with the dissociation constant (Kd) from μM to nM. Ths loading efficiency of growth factors ɒ PEAD/heparip complex was studied, 10Ong or 5GOng 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 -2.hr, centrifug&tion was used to precipitate PEAD/hepari.n/FGF-2, The amount of unloaded FGF-2 remaining in the supernatant can be-determined by a gamma counter. The result showed PEAD/hepar n leaded -- 68 % of FGF-2 for both high and low amounts of FGF-2, The other growth factor,. NGF, the release is faster. The initial burst reached almost 20%. The release sustained till day 20 and reached a plateau corresponding ɒ ~ 30% of the loaded NGF.

{0073} PRP is a blood product containing many therapeutic growth factors, It is used -clinically although its true efficacy for wound healing- is debated throughout the Held due to a lack of systematic studies concerning its use. PRP is theoretically advantageous due to the complex mixture of therapeutic proteins present, but the short half-life- of these proteins could render them useless within minutes.

Example 2 - Fuil-Thiekness Excisional Wound Pig Model

{0074} Platelet-rich plasma (PRP) is widely used for many clinical indications including wound healing due io the high concentrations of growth factors. However, the shor half-life of these therapeutic proteins requires multiple large doses, and their efficacy is highly debated among clinicians; Here we report a method of protecting these proteins and releasing them in a controlled manner via a heparin-based coacervate delivery vehicle to Improve wound healing in a porcine model. Platelet-derived proteins incorporated into the coacervate were protected and slowly released over 3 weeks in vitro. In a porcine model, PRP coacervate significantly accelerated the healing response over 10 days, in part by increasing the rate of wound reepitheiiaibation by 35% compared to control. Additionally, PRP coacervate doubled the: rate of wound contraction compared to: all other treatments, including that of naked PRP proteins. Wounds treated with PRP coacervate exhibited increased collagen alignment and an advanced state of vascularity compared ɒ control treatments. These results suggest that this preparation of PRP accelerates healing of cutaneous wounds only as a controlled release formulation,- The coacervate delivery vehicle is a simple and effective tool to improve the therapeutic efficacy of platelet-derived proteins for wound healing.

{0β75} One alternati ve to mono-therapy Is ɒ harness the mixture of growth factors produced by the patient themself (autologous) or from a healthy allogenic donor in the form of platelet-rich plasma (PRP). PRP- is a fraction of blood plasma containing many therapeutic growth factors released from aipha~granni.es opon platelet activation (F. Mussano, *et al.*, Cytokine, ehemokine, and growth factor profile of platelet-rich plasma, Platelets, (2016) 1-5). This protein cocktail has high potential ɒ stimulate an accelerated healing. -response since it contains numerous factors known to play different vital roles in the natural healing progression .-Autologous PRP avoids the risk of an immune response during treatment, although allogenic PRP has also been used safely in a clinical setting without serious adverse effects as long asthe platelets were removed (Z.Y. Zhang, *et ui.*, The potential use of allogeneic

piatelet-rich plasma for large bone defect treatment: immunogenieity and defect healing efficacy, *Cell Tr&mplant,* 22 (2013) 175-187). PRP is currently approved for use in orthopedic **applications** and is under **investigation** for several others **.including** woand healing (Z, **Y.** Zhang, *et al.*, The potential use of allogeneic platelet-rich plasma for large bone defect treatment; immunogenicity and defect healing efficacy, *Cell Transplant,* 22 (2013) 175-187; V.R. Driver, *.ea' al.*, G. Autologei Diabetic Foot Uicer Study, A prospective, randomised, controlled trial of **autologous platelet-rich** plasma **gel** for the **treatment** of diabetic **Foot ulcers,** *Ost&ny/woimd management,* **52** (2006) 68-70, 72, 74 passim; K.K, Middietoii., *et al.*, Evaluation of the efieets of platelet-rich plasma (PRP) therapy involved in the healing of sports-related soft tissue injuries, *Iom Orihop J* 32 (2012); **T.D. Vu**, *et aL,* An **autologous platelet-rich plasma hydrogel** compound restores left ventricular **structure,** function and ameliorates adverse remodeling in a minimally invasive large animal myocardial restoration model: a translationai approach:. **Vu** and Pal. "Myocardial Repair: PRP, Hydrogel and **Supplements",** *Biomateriafs,* 45 (2015) 27-35 150-163; and H.**S.** Yang, *el aL,* Enhanced **skin wound** healing by a sustained release **of growth** factors contained **.in** platelet-ti ch piasma, *Exp Mol Meet,* 43 (201 **1**) 622-629), There are many ways to prepare **-.FRF** though very **f** w **studies have** utilized consistent methodology, **wound types,** or patient demographies, **which has led to conflicting data regarding** its **efficacy. Numerous studies** report **that** PRP improves the wound healing response (V.R, Driver, *et al., Oslomy/wound management,* 52 (2006) 68-70, 72, **74** passim; *U.S.* Yang, *et al., Exp Moi Med,* 43 (201 1) 622-629; and MJ, **Martinez-Zapata,** *et al.*. Autologous **platelet-rich** piasma for treating chronic **wounds,**. *The. Cochrane database of systematic reviews,* 10 (2012) Cd006899), while numerous others **found** it to have no significant effect **on healing** outcomes (Mi. Mariinez-Zapata, *et al., The Cochrane database of systematic reviews,* **10** (20 12) Cd006899). Since each study **involves*** different **formulation,** h is difficult to determine the true potential **of FRF** as a wound **healing** therapy. **As** with individual growth factor therapies, the proteins **found** in. PRP **also have** short **halt-lives, limiting** their **efficacy** without repeated **administrations** (K, Lee, *et al.,* Growth factor **delivery-based tissue** engineering: **general approaches** and a review of recent **developments,** *Journai of the Royal Society Interface / the Royal Society,* 8 (201 1) 153-170and Hi.S, Yang, *et aL, Exp Moi Med,* 43 (201 1) 622-629),

[0076] Complex:coacervates form when a eationie polymer solution is mixed with: an anionic polymer solution that leads to charge neutralization and phase separation of a polymer-rich phase **from** the bulk water, Here, we use heparin as the anionic polymer. Many therapeutic **growth** factors such as VEGF, heparin-binding EGF-like growth factor (HB-EGF), and hepatocyte growth factor (HGF) have a natural affinity for **heparan** sulfate, a glycosaniinoglyean (GAG) found in the ECM, Heparin has **simitar** structure and **functionality** as **heparan sulfate,** with the ability to protect growth **factors** from proteolytic degradation and present them to ceil receptors in **biomimetic** fashion, A synthetic **polycation, poly(ethylene arginitnylasp¾rtate digiyceride)** (PEAD), interacts with the anionic heparin via polyvalent charge attraction, forming **a** complex **coaeervate** containing **high concentrations** of the **polymers while** the bulk water phase has **little** polymer **left** Thus/this system loads hepari.n-bin.ding growth **factors with**

high efficiency. The final delivery system consists coaeervate droplets suspended within the aqueous phase. The droplets- range from 10 to 500nm in diameter and remain stable for at least one month *in vivo*. Previous studies have shown that this system delivers- growth factors for weeks and significantly extends their half-lives, .Coaeervate delivery of growth iactors can improve cardiac function after myocardial infarction and accelerate wound healing, see Example 3. Here we- utilise our eoscervate system with PRP in a pig model of wound healing. Pig skin is very similar to human skin and thus provides an appropriate pre-clinical indication for potential success in human patients. Since PRP is widely available and inexpensive, its validation as a growth factor source for-therape¾tic,apptication ia a large animal model takes this .technology one step closer to clinical translation.

Materials and Methods

£0077] *PRP **Preparation** and Protein -**Quantification:*** Patient samples were obtained from the- Central Blood Bank of Pittsburgh. Fresh plasma was obtained within four hours of Collection from the patient to maintain high bioactivity of -growth factors and cytokines. To isolate PRP, the plasma was then centtimged at 2,Q00xg for 15 minutes, and the bottom half of the solution was taken ss PRP, Thrombin (Sigma- Aldrich, St. -Louis, MO) was added at 1,0QU/mL and $CaCl_2$ added at 10% (w/v) with gentle agitation to activate the platelets for one hour. Platelets released their therapeutic proteins into the surrounding plasma upon activation, and the f brin clot was removed by eentrifugation. A 3 kDa MWCO centrifugal filter unit (EM D Mil!ipore, Bil!erica, MA) was used to further concentrate the proteins in solution. Total protein content was measured by Pierce 660nm protein assay (Thermo Fisher Scientific, Waitham, MA), and individual growth factor concentrations were quantified using sandwich ELISA kits per manufacturer instructions (PeproTech, Rocky Hill, NJ). Concentrated PRP was stored at -80°C until later use.

[0078] *Platelet-Derived Proiem Loading and Release*: The loading and release of platelet-derived proteins from the coaeervate -delivery system over 4 weeks *in vitro* was measured. Polyethylene argimnyiaspartate diglyceride) (PEAD) was synthesized as previously described (H. Chu, *el. at,* A {polyeatiomheparin} complex reieases growth, iactors with enhanced bioactivity. *Journal of controiled release: official Journal of the Controlled Release Society*, 150 (2011) 157-163), e.g., as described in Example 3. To form the coaeervate, 200uL PRP was combined with 1.6mg heparin (porcine intestine, Scientific Protein Labs, Waunakee, WI), allowing heparin-binding proteins to bind. The coaeervate then -self-assembled upon the addition of 8mg PEAD- in a total volume of 80QuL.

[0079] The coaeervate was pelleted by eentrifugation at 12,100xg for 10 minutes and initial growth factor loading was determined by measuring the concentration in the supernatant and comparing to the concentration prior to coaeervate formation. Fresh 0.9% saline was added to resuspend the coaeervate and samples were incubated at 37°C, At predefined time points extending to 21 days, the pelleting procedure was repeated and the supernatant collected for analysis. Fresh saline was then added to resuspend the coaeervate.

{0080} *Animals*: Two 3-ruonth old female Yorkshire pigs were used in this study, The pigs were fed standard lab diet: twice per day with unrestricted access to water and their .health was monitored at least twice daily for any signs of pain or distress, Following surgeries, pigs were housed individually to avoid perturbation of the wound sites.

1.0081 j *Wounding Procedure** All surgical procedures were conducted under .supervision of the Division of Laboratory Animal Resources (DLAR) at the University of Pittsburgh, Sedation was induced using an IM injection of ketamine (20mg/kg) and xylazine (2mg/kg) and anesthesia was maintained following intubation with 1-3% isofluorane. Twenty-two fuSI-thiekuess excisional wounds were created on the back of each pig using 2e.ro diameter biopsy punches to ensure consistent wound size (Shoney Scientific !nc, Waukesha, Wi). The punch was driven into the fat layer underlying the dermis, and scissors were used to cut along the underlying fat and remove the skin section. Constant pressure was applied with sterile gauze to stop bleeding, using hemostatic collagen (Davol fee, Warwick, RI) or epinephrine as needed.

[00s 2] Once the bleeding was stopped, Avitene Ultrafoam (Dsvol Ine, Warwick, RI) cut to f t the wound was applied, and group-specific treatment solution was atkied via sterile pipet. Upon addition of these solutions the collagen foam swelled to form a gel. and retained the treatments within the wound site. Collagen 6 ams have previously been used hi these healing models and are used clinically to facilitate healing (D. Brett, A Review of Collagen and Collagen-based Wound Dressings, *Wounds : a compendium of clinical research and practice*, 20 (200.8) 347-356), One of five treatments was applied to each wound: (!) Saline, (2) unloaded eoacervate, (3) bolus PRP, (4) full PRP eoaeervate, or (5) the heparin-bindsng fraction of PRP eoaeervate (HB-PRP) (n« 8-10). Full PRP eoaeervate was formed by combining 400µ1PRP with 3.2mg filter-steri lized heparin and 16mg filter-sterilized. PEA D, To isolate the HB-PRP eoaeervate, the full PRP eoaeervate was pelleted by eentrifugation, the supernatant was aspirated and discarded, and the pellet was resuspended in fresh DI water. Wound treatments were assigned by forced randomization to account for any differences in the skin based on location, and each wound was assumed o be independent of other wounds and treatments (Figure 1). Ai! wounds received 5rag ciprofloxacin administered topically to prevent infection.

|0083] Large Tegaderm. bandages ;(3M, St. Paul, MN) were used o cover wound sites followed by Opsite transparent films (Smith & Nephew, London, UK) around the perimeter, forming a watertight dressing. A surgical pad sprayed with siiieone-based medical adhesive (Holiister tec, Libertyville, *VL)* was then applied on top of the entire wound area to protect the wounds and bandages, followed by a custom-fit jacket (Loniir Biomedical Ine, Malose, MY), Baytril (2,5mg/kg) was administered IM once per day for seven days following surgery and amoxicillin (7mg/kg) was admin.isie.red orally twice per day tor the remainder of the study to prevent infection. Carprofen (2mg/kg) was administered for pain twice daily for five days following surgery. At days 3 and % the bandages were changed under brief sedation with ketamine (20mg,*g) and xyhazine (2mg/kg).

[0084] *Tissue Harvesting and Processing:* Ten days after **wounding** the animals were sacrificed with an overdose: of sodium **pentobarbital** {100mg/kg} **administered intravenously. Wounds** were photographed **for** analysis before explant No **signs of infection were** present in **any wound.** The wounds **were** harvested along with at least 1 cm of surrounding healthy tissue at the depth **of** the muscle feseia. Each wound was then **cat** in half sagittaliy prior to processing. For histology **measurements** and iffiinuBosiainiiig of cytokeratin, tissues were fixed in 2% **(w/v)** paraformaldehyde **for** 2 hours and then **transferred to a 30%** (w/v) sucrose solution **for** eryoproteetion for 24 hours. Tissues **used for immunostaining** of von Willebrand Factor (vWF) remained unfixed. Alt tissue; samples were then **embedded in optimal** cutting **temperature** (OCT) media and frozen **in** liquid **a.itfogen-cooled** 2-**t«ethylb«tane.** Tissues were **then .eryosectioned at 6um 6** r further analysis.

[0085] *Measuring Overaii Wound Contraction:* Overall wound **size was** measured using images taken **of** the **wound during the wounding procedure and** after sacrifice. **Wound area was measured** using **an** automated filter and **measurement** macro **in** Image! and compared to the **original** wound area,

[0086] *Histology:* Tissue **sections** were stained with hematoxylin and eosin **(H&E)** for gross morphology and qualitative wound healing parameters such as thickness **of** granulation tissue **and** the **formation of** healthy skin structures, Masson's **trichrome** stain (MTS) was used **to determine** qualitative collagen deposition and alignment within the granulation tissue.

[0087] *Immunostaining Tissue Sections:* **tmmuwfluorescent** staining of tissue sections was used *to* **determine** the effect of each treatment on angiogeoesis and reepitheSia!i¾atian. A rabbit **polyclonal** von Willebrand Factor **(vWF)** antibody (1**:400** dilution, US Abeam, **Cambridge,** MA) **followed** by an Alexa Fluor 594 **goat** anti-rabbit antibody (Invitrogen, Carlsbad, CA) **was used to detect** endothelial **cells** within the tissue. Since healing is delayed in the **.center of** the wound, images were taken at both the wound edge and the center for quantification. The number **of** vWF+ cells was counted automatically using N1S Elements software (Nikon, Tokyo, Japan) and is reported as blood vessels per mm^3 area.

[0088] Reepitheliahzation **of a wound reestablishes** a **functional barrier between the wound and its** environment and is essential in preventing infection of the underlying tissue. **Reepetheiiaiization** was quantified using a rabbit polyclonal cytokeratin antibody (1:100 **dilution,** US Abeam, Cambridge, MA) followed by an Alexa Fluor 594 goat anti-rabbit antibody. The length of the epidermal tongue was measured and reported as **a** percentage of the total wound length. All images were taken Using a Nikon Eclipse Ti inverted microscope.

[0089] *Statistics:* Ail parameters were tested for significant differences between treatment groups using one-way independent analysis of variance **(ANOVA)** followed by Gabriel's post hoc testing **with** a significance value $p < 0.05$. Analysis was performed using SPSS 22.0 software.

.Results

[0090] *Coacervate system preferentially hack and releases hepemn-hindmg growth factors:* PEAD **carries two** positive **charges** per **repeat** unit. The **polycation** forms **a** complex coacervate **when** mixed with anionic heparin, visible as a turbid solution. The natural aff nity between many therapeutic **growth**

factors and heparin allows these proteins to preferentially load into the coacervate system. Although only 7% of total PRP proteins were loaded, heparin-binding VEGF and PDGF each exhibit a loading efficiency exceeding 60% (Figure 2A), These proteins exhibited a hurst release in the first day, followed by a nearly linear release over the following three weeks (Figure 2B),

[0091] ***Full PRP coacervaie accelerates pig wound closure:*** Porcine skin, lite that of humans, heals primarily by reepethelialization, Ima unofluoreseent deiection of cytokeratin for dermal epithelial cells showed a significantly increased reepithelialization rate (35% relative to saline) 10 days after wounding compared to saline and delivery vehicle alone (Figure 3). No significant ^differences existed between other treatment groups. Wounds treated with full PRP coacervate also exhibited a thicker epidermis adjacent to the wound margin compared to other groups, Positive cytokeratin staining w¾s also observed in .sebaceous glands and hair follicles of healthy tissue as expected.

[0092] ***Full FRF coacervate modulates vascular density:*** The vascular density of the wounds was measured at day 10 using vWF immunofluorescence to identify endothelial cells, In the wound center, vascular density was significantly higher than in healthy tissue indicating an influx of blood vessels into the wound bed, and «o significant di-Terences between treatment groups were observed (Figure 4 (A,B)). However, at the wound edges, blood vessel density of wounds treated with full PRP coacervate resembled that of uninjured tissue (Figure 4 (A)), Furthermore, the blood vessel density in full PRP coacervate treated wounds was significantly lower than all other treatment groups (Figure 4 (A,C)), Since wounds heal more quickly at the edges* these data suggest that wounds treated with full PRP coacervate were at an advanced stage of healing compared to other treatments.

[0093] ***FRF*** *coacervate decreases wound size:* Wound closure was also analyzed macroseopically o confirm measurements made histologically. Automated measurements: of wound area -showed- a significant"decrease- in wound size after ten days compared to all other treatments (65% original wound size for full PRP coacervaie treated Wounds compared to 83% when treated with saline). The remaining treatments did not cause. a significant reduction in wound size (Figure 5b). Further, wounds that did not receive full PRP coacervate treatment were visibly deeper and dark red in color, indicating unhealthy granulation tissue formation (Figure 5a)(J,E. Grey, *et al.*, Wound assessment, *BMJ*, 332 (2006) 285-288).. The light pink color and raised appearance of granulaiion tissue seen in the full PRP coacervaie wounds indicate healthy granulation. No signs of infection were present in any wound,

{0094] ***PRP*** *Coacervate* ***accelerates granulation*** *tissue maturafktn*: Granulation tissue provides a temporary matrix- for cells to infiltrate and repair the wound bed after injury. As expected, H&E staining showed high eellularity and vascularity in the granulation tissue of all wounds relative to surrounding healthy tissue ***(not shown).*** Collagen deposition by fibroblasts had begun within 10 days in all wounds as seen by MTS ***(not shown)***. Upon careful evaluation it was evident that full PRP coacervate resulted in increased deposition of aligned collagen compared to other treatment groups where the collagen fibers were more randomly oriented (Figure 6). The granulation tissue was thin in saline-treated control wounds, extending only partially to the normal skin surface.

.Discussion

|0095| The widespread use of PRP as a therapy remains highly debated in medicine, Autologous PRP is typically highly variable because of inherent differences between patients, methods of preparation, and whether it is used as a liquid or a thromhin-indueed hydrogei. This leads to inconsistent results between patients and studies when used clinically as an autologous therapy. PRP activated by thrombin can be applied as a gel and has been described as a controlled release system to improve the efficacy of PRP. However, these studies have been restricted to small animal models or large animals models without suff cient characterization of protein release, and most have not been injectable (Y. Yan, ***ei al.,*** Acceleration of Full-thickness Wound Healing in Porcine Model by Autologous Platelet Gel, Wounds: a compendium of clinical research and practice, 19 (2007) 79-86). Provided herein is a systematic study of the effects of PRP in a porcine model and the benefits of controlled release tor wound healing.

£0096) Appropriate controlled delivery systems are crucial to achieve high therapeutic efficacy of growth factors. Short halt-lives, adverse off-target effects, and poor spatio-temporal control are common issues of bolus application which can be solved using controlled release systems. One approach is to harness the native properties of ECM rn.olecui.es which sequester growth factors, prevent their degradation and promote their bioactivity (O.S. Schultz, *etal* f interactions between extracellular matrix and growth factors in wound healing, ***Wound*** *Repair Regen,* 17 (2009) 153-162). Several different types of delivery platforms have been developed along this theme, utilizing ECM molecules vitronectin, fibronectin, and heparin, In one such platform, vitronectin complexes with insulin-like growth factor (IGF) and epidermal growth factor (EOF) demonstrated wound healing efficacy in large animals and safety in humans, In another approach, synthetic f bronectin-like peptides were developed to deliver several different Iseparin-bindtng growth factors and cytokines and evaluated in small animal studies. Our lab has characterized- a heparin-based platform which takes the form of liquid coaeervaie droplets that load and release heparin-binding proteins (IT Chu, ***ei*** «/., A [polycation:heparin] complex releases growth factors with enhanced bioactivity, ***Journal of controlled*** *release :* ***officialjournal*** *of the Controlled Release* ***Society**** 150 (2011) 157-163 and N.R, Johnson, ***ei*** *al.,* Lysine-based polycationfhepartn coaeervaie for controlled protein delivery, Acta biomaterialia, 10 (20:14) 40-46). HB-EGF delivered by the coaeervaie system accelerated healing in. both diabetic and nOn-diabetic rodent wound -models (N.R. Johnson, *et al* , Controlled delivery of heparin-binding EGF-like growth faclor yields fast and comprehensive wound healing. ***Journal of controlled .release :*** ***officialjournal of*** *the Controlled Release Society,* 166 (2013) 124-129 and N.R. Johnson, & «/,, Coaeervaie delivery of !TB-EGF accelerates healing of type 2 diabetic wounds, ***Wound*** *Repair Regen,* 23 (2015) 591-600).

[0097] The use of multiple growth factors with the eoaeervate system has been explored previously to stimulate angiogenesis with VEGF and hepatocyte growth factor (HOF) **(H.**K. Awada, *ei* ***al„*** Dual delivery of vascular endothelial growth factor and hepatocyte growth factor eoaeervate displays strong angiogenic effects , Maeromolecufar bioscience, 14 (2014) 679-686). The use of two growth factors exhibited a near-linear release profile over-three weeks .with no initial burst release. In comparison, both

PDGF and **VEGP** from the PRP eoacervate exhibit a 50% burst release within a day of eoacervate formation (Figure *2b)* followed by a nearly linear release profile over 3 weeks. **Interactions** between heparin and **growth factors** are primarily charge-driven. When using small doses of proteins as **was** done previously, this interaction has **negligible** effects on the PEAD;lieparin charge-based Interaction. However, this study utilizes high protein concentrations; as more proteins bind to a **heparin** molecule, fewer **sulfate** groups are available to bind the PEAD polycation. Thus the eoacervate formed is less **stable** than those formed with small **protein** doses. This allows **the** eoacervate **to** dissociate at a faster rate, which likely **explains** the high initial release of proteins shown here.

|0098) Heparin **has** been **used in. many** growth **factor therapies since the heparin;grmvth factor complex** is stable and resistant **to** proteolysis. Additionally, **heparin** potentiates the bioactivity of the proteins by facilitating their reactions with **cell** surface **receptors,** lts use in our delivery system provides the **advantage** of preferentially **loading heparin-binding growth factors for sustained** release. **Although less** than 10% of the total protein **content** of PRP was incorporated into the coacervate, heparin-binding proteins such as PDGF and VBGF exhibited a loading efficiency exceeding 60%, To our knowledge this is the f rst **repor of a** PRP delivery **system** that is **able to** load and release **heparin-binding** growth **factors in a** sustained **manner.** We **suspect that** the non-heparin binding **fraction** of the platelet released factors provide important acute **signaling** for healing the tissue. Therefore, we tested the 'full PRP eoacervate" **along with** the heparin-binding fraction only, **namely** the "HB-PRP eoacervate".

[0©99) Prior studies **-utilizing** heparin have largely involved its covalent immobilization to the **surface of a polymeric** scaffold or within a: hydregeh thereby endowing the biomateria! with growth factor affinity (S.E. Sakiyama-Eibert, incorporation of heparin into **biomaterials.** ***Acta Biomater***, 10 **(2014)** 15SI-1587). However, covalent linkage may partially or fully inhibit the activity of heparin, reducing growth factor loading efficiency or creating a sterie selectivity for heparin-binding protein **of certain** sizes or **conformations.** The eoacervate **.platform** employs heparin in **.free-form which** maintains its native **functionality** and **consistently provides high** loading **capacity. Furthermore, .the** heparin-binding complexes are mobile and able to interact with ceil surface receptors **and.** thereby potentiate growth factor bioactivity. in comparison, immobilized heparin may hold **growth** factors to a surface or deep within a scaffold that cells must inf ltrate to access. A. system using free heparin amphiphi!es has also been reported, but their growth factor release has not been reported beyond 10 days (S.S. Lee, ***et al.***, Bone regeneration with **low** dose BMP-2 **amplified** by biomimetic supramolecuSar nanofibers within collagen **scaffolds,** *Biomaterials*, 34 (2013) 452-459 and K, Rajangam, *el a!.*, **Heparin** binding nanostructures to promote growth of blood vessels, Nano Lett, 6 (2006) 2086~2090).The advantages of the **eoacervate** system **are** owed to **the** unique **liquid-liquid phase** separated structure held together by **electrostatic-** interactions of heparin and the novel caiionic **polymer** PEAD.

[OO[O0l **A** porcine **model of full-thickness** exeisional wounds is one **of the** best pre-eiinical wound healing models as pig skin heals by similar mechanisms to human patients. Here we demonstrate that PRP itself is **unable** to Improve wound healing; however the controlled release of its therapeutic

proteins can accelerate the **wound** healing process. **Wound vascularization** and **collagen** fiber **deposition and** alignment in the granulation tissue **indicated** improved wound maturation h full PRP eoaeervaie-treated wounds. Additionally, smaller gross **wound** size and **enhanced** reepiihelializaiion shows **that** wound closure was also accelerated. Furthermore, we found **that** removal of noH-hepa.rin-binding **proteins from** PRP alter coacervaie **formation** reduced its **efficacy.** Interestingly, these data suggest that the non-heparin-hmding PRP **fraction** plays a vital role in the healing response. The use of full PRP coacervaie essentially creates two stages of release: an initial **diffusion** of **all** unincorporated PRP proteins followed by a sustained release of the heparin-bindmg growth **factors-.** Sequential release of **individual** growth **factors by** the coacervaie **has** been **demonstrated to improve** the therapeutic efficacy (H.K. **Awada,** *et at.* Sequential delivery **of angiogenic growth** factors **improves revascularization** and heart **function** after **myocardial** infarction. *Journal of controlled release : -official journal of the Controlled Release Society,* 207 (2015) 7-17 and H.K. Awada, *et al.* factorial Design of Experiments to Optimize Multiple Protein Delivery for Cardiac Repair, *ACS Biomaterials Science & Engineering,* 2 (2016) 879-886). The coacervaie creates **an** analogous situation **in** the present study **where** high initial concentrations of **unincorporated** proteins induce a **strong** acute response **followed** by the sustained release of heparin-bindmg proteins which enact long-term effects. The **removal** of **unincorporated** proteins (as in the HB-PRP coaeerate) reduces efficacy which could he due to a weaker acute response.

[00101] Angiogenesis is a vital component of the wound healing process. The formation of leaky **capillaries allows -cells -and** proteins to infiltrate the damaged tissue and begin healing. A high **capillary-density** was **observed** in the center of **all** wounds assessed in this study, indicating the normal angiogenic process was not significantly disturbed. Since **wounds** heal **from** the edge, granulation tissue near the wound edge matures faster than the center of the wound where granulation tissue forms last. Therefore we are able to see a progression of the angiogenic **process moving. Iron)** the edge to the **interior of** the wound. The vessel density of all treatment groups was Sower at the wound perimeter **compared** to the center, indicating that the wounds had progressed through the peak of angiogeiiesis and were returning to the levels of native skin. The difference arises in wounds heated with full PRP coacervaie, where vessel density is **similar to** other wounds at the center but is significantly lower at the wound margin. This suggests that controlled PRP release accelerates the angiogenic phase of healing, **returning** vessel density to near-normal levels **ai** a faster rate and exhibiting **a** more advanced stage of healing. This has significant relevance when comorbidities are present such as peripheral arterial disease or diabetes,

[00102] This study demonstrates the therapeutic benefits of controlled PRP release for wound healing **applications.** A quick and simple assembly method allows adaptability for either rapid autologous preparation at the bedside or an allogenic sottrced **off-the-shelf product.** The liquid **nature** of our coacervaie **system** allows it **to** be injected directly info the wound bed or incorporated into a substrate for application. **The controlled release** mechanism allows treatment to **be** applied **once** every few weeks rather than daily as seen **in- traditional protein** therapies. Given the **ability** of this therapy to

capture many proteins, this study suggests that the eoaeervate vehicle can be adapted to other therapeutic applications in large animal models,

[00103] In conclusion, this Example evaluated the efficacy of traditional PRP treatment and the advantage of a controlled release PRP formulation on cutaneous wound healing, It is shown dial the sustained release of PRP proteins is able to significantly improve wound closure within 10 days of wounding, while ê e clinical standard of naked PRP proteins demonstrate no significant benefit. This is accompanied by a. significant alteration in vascularity of the wound edge, returning vessel density to near-healthy levels. These results suggest that the controlled delivery of PRP proteins using the eoaeervate vehicle encourages accelerated healing of cutaneous wounds in a porcine model. The widespread use of PRP in humans combined with the clinical relevance of the porcine wound healing model emphasize the impact of this study on the -translations! potential to enhance autologous procedures with an easy-to-use protein delivery platform.

Example 3 - Functional Recovery of the Infarcted Myoeardium by a Single Injection of Three Proteins

[00104] After a heart attack, the infarcied myocardium undergoes pathological remodeling instead of repair and regeneration. Protein signaling plays a pivotal role in tissue regeneration. With multiple pathologies developing after myocardial infarction (MI), treatment using several complementary proteins is expected to address these range of pathologies more effectively than a single-agent therapy. Three complementary factors are combined in one injection: tissue inhibitor of nietaiSoproteinases 3 (TIMP-3) was embedded in a fibrin gel. for signaling in. the initial phase of the treatment, while basic fibroblast growth factor (FGF-2) and stromal cell-derived factor . alpha (SDF-1α) were embedded in a hepar in-based eoaeervate and distributed within the same fibrin gel to exert their effects over a longer period of time. The spatiotempora!ly controlled release .of these proteins counters excessive inflammation, extracellular matrix (ECM) degradation, and cell death post Ml in rats. The contractility of the treated hearts stabilizes and slightly improves after a drop in the first two weeks whereas all the controls kept deteriorating. Accompanying the functional .-restoration are reductions in dilation, inflammation, fibrosis, and ECM degradation. Revascularization, cardiomyocyte survival, stem cell homing, and preservation of myocardial strain levels likely all "contribute to the repair. This study demonstrates the potential of this multifactorial therapeutic approach in Ml.

[00105] Myocardial infarction (Mi) affects 7.6 million Americans with approximately 720,000 experiencing a heart attack each year. *MI* leads to defects in- the contractile function .of cardiomyocytes and alterations in the extracellular matrix (ECM) and ventricle geometry. As a consequence of the maladaptation, a non-contracting scar tissue forms, a significant portion of which results in congestive heart failure. Current treatments such as reperfusion, ^-blockers, and angiotensin converting enzyme (ACE) inhibitors, reduce -damage but do not restore function. Therefore, therapies that can prevent or reverse the multiple pathologies caused by MI, regenerate the myocardium, and restore cardiac function are urgently needed.

[0106] To treat **multiple pathologies resulted** from MI, we set out to explore the use of multiple therapeutic proteins. Our recent study using a statistical fractional **factorial** design of experiment focused our **effort** into the **controlled** and timed release of a **combination of** complementary proteins that are relatively distinct in their roles in **cardiac function:** tissue inhibitor of metalloproteinases 3 (TIMP-3), basic fibroblast growth factor (FGF-2), and stromal cell-derived factor 1 **alpha** (5DF~1«) (H. K. Awada, *et al.*, Factorial Design of **Experiments** to Optimize Multiple Protein: **Delivery** for Cardiac Repair, ***ACS Biomaterials Science & Engineering*** **2**, 879-886 (2016), TIMP-3 inhibits the activity of matrix metalloproteinases **(MMPs)** which cleave **EGM** components. FGF-2 plays a chief role in formation of neovaseuSature. SDF-1α is a potent cSemotactic factor that can recruit stem cells to the infarct region.

[0107] TiMP-3 reduces EGM degradation soon after MI, FGF-2 and SDF-!a promote augiogenesis **and recruit** progenitor **cells to the infarct** region, which **are** events that require **prolonged** signaling. We designed a composite hydrogel compi sed of fibrin gel and heparin-hased coacervaies to achieve the sequential release of TIMP-3 followed by **FGP-2** and SDF-iα. To achieve this **controlled release,** T.5MP-3 was encapsulated in fibrin gel to offer early release, **while** FGF-2 and SDF-1α were encapsulated in heparin-based coacervaies and distributed in the same fibrin gel to offer sustained release (Fig, 1A). Complex coacervaies **form spontaneously** by electrostatic **interactions** between the aqueous solutions **of** a polycation and a pdlyanion. A synthetic **polycation** poiy(ethylene argiuinyiaspartate diglycer de) (PEAD), **heparin,** and hepar n-binding proteins were used to form protein-loaded coacervaies that have been shown to encapsulate proteins with high **efficiency** and sustain their release in vivo and in vitro.

[00108] In this study, the efficacy of the spatiolempofal **delivery** of TiMP-3, PGF-2, and 8DF-1«.on cardiac function, ventricular dilation and wail thinning, myocardial strain levels, **MM?** activity, fibrosis, inflammation, cardiomyoeyte survival, angiogenesis, stem cell homing, protein signaling, and cell apoptosis. **The** first report of controlled delivery of complementary proteins mitigates the MI **injury** and initiates a robust cardiac **repair** process, giving **hope of a higher** level of functional and **structural** recovery **of the** snfarcted **heart**

Results

[00109] *Sequential protein release?* **abilility** of a f brin gel-coacervate composite **to** release TIMP-3 early followed by a sustained release of FGF-2 and **SDF-** 1α by an in vitro release assay was **tested** (Fig. 7 (A)). The loading efficiencies were 85% for TIMP-3, 97% for FGF-2, and 98% for **SDF-**1α (Fig. 8). By day one, approximately 40% **of loaded TIMP-3** was released, reaching 90% total release by one week (Fig. **8}** translating into relatively higher **concentrations of TIMP-3-** reaching **a** maximum **of** . ug/ml during the first week and decreasing thereafter (Fig. 7 <**B**)), We observed **a** longer sustained release for FGF-2 and SDF-1α with concentrations between 0.25-0.5: ag/mSthat **tasted** for >six weeks **due** to their encapsulation within the **coacervaies** inside **the gel** (Fig. **7** (B)). **By.** one week, **only** 2*1%* of FGF-2 and 28% of SBF-1α were released, reaching 55% and 48% total release respectively **by** six

weeks (Fig. 8). Thus the composite coacervate gel achieved quick release of TIMP-3 after Ml to reduce ECM degradation and inflammation, while: providing FGF-2 and SOP-1et in a sustained manner for triggering a robust neoyascuiaiure formation process and stem eel! recruitment

[00116J *improved cardiac function and reduced ventricular dilation;* The: effect of spatioiemporal delivery of TfMP-3, FGF-2, and SDF-1α was evaluated in a rat Ml model using sham, saline, and free proteins as controls. We evaluated changes in left ventricle (LV) contractility as a measure of heart function was evaluated. Using echocardiography, fractional area change (FAC) was computed from end-systolic area (ESA) and end diastolic area (EDA) values (Fig, 9 (A)}, Sham group maintained an FAC value of approximately 55% at all time points posi-Ml, significantly higher than all infarct groups (Fig, 9 (S)). 1-week post-infarction, FAC values of saline, free protein, and controlled release (CR) groups "dropped: significantly, however, both CR and free proteins had significantly higher FAC than saline (p<0.01). This suggests that the three-proteins significantly improved cardiac function within one week after Ml, At two weeks, CR group diverged from the negative controls and improved function significantly (p« i Q01). Although free proteins was significantly better than saline (p<0.001) tip to five weeks, function in both groups kept dropping, In contrast, functional improvement of CR group continued and displayed increasingly larger differences relative to the controls. At eight weeks, the last time point of the study, CR led to a 48% FAC, which was 87% that of the normal FAC value and represented a 74% improvement over saline. The two control groups, saline and free proteins, no longer showed any statistical difference at eight weeks (p>0.G5) (Fig. 9 (B)).

f00111] To evaluate the therapy's effect on ventricular dilation, changes in EDA and ESA values were assessed. The CR group showed significant reduction or trend towards lower EDA and ESA after Ml compared to saline (Fig. 9 (CD)). On the other hand, saline and free protein groups showed progressively higher ESA and EDA at all time points after Ml, with no .statistical differences between them (pX).05) (Fig. 9 (C,D)).

[00112] The echocardiography results were consistent with MRI measurement at eight weeks, End-systohe volume (iSV). and end-diastolic volume (EDV) were computed and ejection traction (EF) was calculated (Fig, 9 (E)), EF in CR group was 58·%, which was at 84% of the sham group (69%) and significantly higher (p<0.G01) than saline (41%) and free proteins (46%), The two negative controls showed no difference between each other (p>0,0S) (Fig, 9 (F)). Correspondingly, the left ventricle of the CR group was less dilated with a significantly smaller ESV than saline (p<0.01) and not significantly different from sham (Fig. 9 (G)). CR also showed a trend towards lower EDV compared with saline and free protein groups (Fig. 9 (H)).

[00113j *Preserved myocardial elasticity:* Myocardial strain analysis at eight weeks post-Mi to evaluate the changes in the radial strain levels of the myocardium with respect to the various treatments by normalizing the peak strain of the infareted region to the average peak strain in tour non-infaret regions (Fig. 10A). The radial strain, defined as the percent change in myocardial wall thickness, was measured. The CR group exhibited a radial myocardial -strain similar to that of sham control (p>0.0S),

and was significantly higher than the saline group (p<0.01) (Fig. 10B). The free proteins group is similar to saline (p>0,05) and significantly less than sham (p<0.05)(Fig, 10B). This result suggests thai controlled delivery of TIMP-3, FGF-2, and SDF-1α protects myocardial elasticity after Ml, The prevention of ventricular wall stiffening helps to maintain the heart's ability to contract and dilate properly,

[00114] *Reduced left ventricle wall thinning, MMP-activity, andfibrosis:* In order to understand tissue level changes that contributed to the functional improvement, ventricular wall thickness, MMP activity, and fibrosis were investigated at two and eight weeks. M&E stained hearts showed increased granulated scar tissue areas with thinner left ventricle- walls in the infarct zone and borderzone that exacerbated with time in saline and free proteins groups but to a less extent: in CR-group (Fig- 11(A)). CR significantly prevented ventricular wall thinning at two weeks compared to saline (p<0,05) (Fig. 11 (B)). In contrast, left ventricle wall thickness decreased considerably in saline and free proteins groups as early as two weeks. At eight weeks, there were no statistical differences in wail thickness between saline, free proteins, and CR; although CR clearly maintained a thicker wall average (Fig. 1l (B)). CR wall thickness was not different f om sham at both time points (p>0.05),

[00115] At eight weeks, we evaluated the activity of MMPs in the heart samples. MMP-2 andMMP-9 are important -players implicated in many cardiovascular diseases and EGM degradation (T. Eton, *et al.*, Myocardial and interstitial matrix metaUoproteinase activity after acute myocardial infarction in pigs, *American journal of physiology. Heart and circtlatf ry p hysiology* 281,.H987-994 (2001)). All infarct groups showed a high level of MMP-2/9 activity (Fig. 1. (Q). However, CR:showed significantly lower MMP activity compared to saline (p<0.01) and also lower activity than free proteins group but not to a significant level (p>0.05) (Fig. 11 (C)). MMP activity *in* CR was not statistically different from sham (p>0,05). The enhanced reduction of MMP activity by the CR group is likely due to the controlled delivery of TiMP-3 within the fibrin gel-coacervate composite, where Ti.MP-3 can form tight complexes with MMP-2 and MMP-9 to prevent their acti vation, and thereby reducing ECMi degradation and ventricular -dilation and remodeling.

[00116] Interstitial fibrosis develops at the infarct region and extends to nou-infarct areas due to the excessive and uncontrollable collagen deposition that takes place in later stages after Ml, This increased collagen deposition leads to increased stiff Hess- in the myocardium, leading to contractile dysfunction. The extent of fibrosis was assessed using pierosirius red staining which stains collages fibers (Fig, 11 (D». The saline group, and to a lesser degree the. free proteins group, showed extensive amount of fibrosis that extended from the infarct to non-infarct regions, while CR showed far less fibrosis that seemed limited to the infarct area at two weeks (Fig, 12*)* and at eight weeks (Fig. 11 (D)). Collagen deposition was quantified as a positive fraction of the heart area and no statistical differences were found between the infarct groups at two weeks despite a clear reduction in the CR group (p>0.05) (Fig. 11(E». At eight weeks, collagen deposition increased in all infarct groups, but it was found to be

significantly less in CR (11%) compared to both saline (23%) (p<0.01) and free proteins (18%) groups (p<0.01) (Fig. 11 (E)). Sham had significantly less collagen than all groups at both time points,

[00117] *Reduced inflammation:* Modulating the inflammatory response after MI in which certain-harmful aspects of inflammation, are prevented, can the very beneficial for the treatment of the infarcted myocardium, In this study, inflammation by co-staining for F4/80, a pan-macrophage cell surface marker, and CD163, an M2 macrophage marker (Fig, 13 (A)) were assessed. Non-M2 macrophages, namely MI, promote inflammation, whereas M2 macrophages contribute to tissue repair and anti-inflammation J. M. Lambert, E, F. Lopez, M, L. Lindsey, Macrophage roles following myocardial infarction. *International journal of cardiology* 130, 147-158 (2008). At two weeks post-MI, CR showed a trend towards decreasing. the presence of non-M2 macrophages, while they were present in high numbers in saline and free proteins groups (p>0,05) (Fig. 13 (A,B)). On the other hand, CR significantly increased the presence of the beneficial M2 macrophages compared saline (p<0.01) (Fig, 13. (A3))· Saline and free proteins showed no statistical differences- in their- M2 macrophage numbers (p>0.05j (Fig. 13 (A3)}. The sham control showed minimal presence of macrophages.

[00118] The effect of the treatment on the secretion of -pro-inflammatory cytokines was then investigated. Tissue lysates were tested at eight weeks for interteukin 1β (IL-Iβ), interlenkin 6 (IL-6), and tissue necrosis factor a (TNF-«) (Fig. 14). Quantitative analysis by ELISA showed significantly lower levels of IL-1β in CR and free proteins groups (p<0.05) compared to saline (Fig. 14 (A)), There was no statistical differences in the levels of IL-6 between the groups (p>0,05) (Fig. 14 (B)). Finally, CR significantly reduced the levels of TNF-α compared to saline and free proteins (p<0,01), while the free proteins- group was statistically indifferent to saline (p>O.05) (Fig. 14 (C)). These results indicate the efficacy of the controlled release of IL MP-3, FGF-2. and SDF-1α at reducing the detrimental effects of an excessive -inflammatory environment -post-MI and at promoting tissue healing through polarization toward M2 macrophages.

[00119) *Increased cardiomyocyte survival and reduced apoptosis:* The viability of the cardiac muscle is crucial for the proper function of the heart. Cardiomyocytes are responsible for imparting proper and synchronized contraction of the heart. As MI and the pathologies developed afterward trigger massive death of cardiomyocytes, ii is beneficial to increase their survival, prevent their apopiosis, and trigger the regeneration of a viable myocardium, A major loss -of viable myocardium was observed in the saline group, followed by the free proteins group, theft by the CR group that apparently preserved the live cardiomyocytes to a larger extent at two weeks (Fig, 15) and at eight weeks (Fig. 16 (A)). Quantitative analysis of the area fraction of the viable cardiac muscle demonstrated a reduction in the amount of survived cardiomyocytes is all infarct groups at two weeks, with no statistical differences between them (p>0,05) (Fig. 16 (8)). At eight weeks, the viability of the cardiac muscle was reduced more in the saline group (.(54% viabie muscle), followed by the free proteins group (75%) with -no significant differences between them (p>0.05). In contrast, CR was able to maintain the survival of the cardiac muscle (83%) -significantly better than -saline- at eightweeks (p<0.0i) (Fig. 16 (B)).

f0l)120j A number of molecular pathways play important roles in promoting survival or inducing apoptosis of cells. The activated (phosphoryiated) MAPK/ERK and Akt pathways have been shown to be cardioprotective after ischemia and preventive of apoptosis (A. Kis, *ei al.*. Second window of protection following myocardial preconditioning: aft essential role for PI3 kinase and p70$6 kinase. *Journal qf mslecular anil cellular cardiology* 3S, 1063-107 l (2003); A. Tsang, *ei al.*, Postconditioniog; a form of "modi tied reper fusion" protects the myocardium by activating the phosphatidylinositol 3-kmase-Akt pathway. Circulation research 95, 230-232 (2004); and Y. Wang, Mitogen-activ&ted protein kinases in heart development and diseases. Circulation 116, 1411-3423 (2007)). To analyze the effect of our treatment, we quantified the expression levels of cleaved caspase-3, a pro-apoptosis mediator, and pro-survival markers p-ERKi/2 and p-Akt at. 8 weeks by western blotting .(Fig. 16 (C)}, Among infarcted animals, the CR group had the lowest level of cleaved caspase-3 and the highest levels of p-ERK1/2 and p-Akt (Fig. 16 (C)). The f ee proteins group displayed significantly higher p-ERK1/2 expression than saline (p<0.0i) (Fig. 16(D)). However, CR significantly reduced the expression of cleaved caspase-3 and increased the expression of p-ERKl/2 and p-Aki compared to both saline (p<0.001) and free proteins (ρ<0,0i) groups (Fig. 16 (D,E,F». CR was statistically indifferent to sham in all three cases. Taken together, these results demonstrate the effectiveness of the approach described herein at supporting the long-terra survival of cardiomyocytes, preventing their apoptosis, and providing overall eardioprotsetion after *MI* through activation of the Akt and ERK1/2 signaling pathways and the suppression of caspase-3 mediated apoptosis,

((0 121.f *Enhanced angiogenesis:* The revasetdarization of the ischemic myocardium is key o tissue regeneration- and functional recovery. New blood vessel formation can help restore the blood, nutrient, and oxygen flow to the damaged myocardial regions, and thereby enhance the survival of cardbmyoeytes, and reduce the risk of chronic heart failure. To investigate the process of angiogenes is, tissue slices were co-stained for vWF and a-SMA at two weeks (Fig. 1:7) and eight weeks (Fig. 18 (A)), Angiogenesis was evaluated only in the infarct groups, and not in sham since angiogenesis happens after infection and not in healthy hearts. A higher number of neovessels was observed in the CR group compared to saline and free proteins (Fig, 18 (A)). Quantitative analysis of infarct groups showed significantly higher number of vWF-positive vessels in CR compared to saline at two weeks (p<0,05) (Fig. 18 (8)). At eight weeks, CR showed a significantly higher number of vWF-positive vessels than both saline and free proteins groups (p<0.01) (Fig. 18 (B>).

{0122} Co-localization of vWF and a-SMA was used- as markers of mature neovessels, and no significant differences was found among the infarct groups at two weeks (p>0.()5) (Fig, I8 (C)). However, at eight weeks, CR showed significantly higher presence of mature neovessels than saline and free proteins groups (p<0.00 l) (Fig. !8 (C)), Our results demonstrate the ability of our treatment to induce robust angiogenesis with stable and mature neovasculature. This enhanced revascularization in the CR group is likely due to the sustained presence of the potent angiogenic factor PGF-2 being provided by the heparin-based coacervate within our composite gel.

[00123] *Greater stem cell homing to the myocardium;* Stem cells recruited to the infareted myocardium have the potential to differentiate into functional ceils of cardiac lineages such as cardiomyocytes, vascular endothelial, and mural cells. Stem ceils can also impart beneficial paracrine effects that activate repair and regeneration signaling (K. Maliiaras, ***ei*** *al.*, Cardiomyocyte proliferation vs progenitor cells in myocardial regeneration: The debate -continues. *Global* ***cardiology*** *science & practice* 2013, 303-315). To examine the homing of stem ceils to the infareted myocardium, e-Kit. a stem cell marker was stained for (Fig. 19A). At eight weeks after Ml, saline and free proteins groups showed no significant differences in. the number of e-Kit-positive cells present st the horderzone ($p>0.05$) (Fig. 19B). In contrast, the CR group showed a significantly greater presence of e-Kit~positive cells at the boixteone compared to both saline and free proteins groups ($p<0.01$) (Fig, 19B), The sham control showed very lew stem cells in the area where- an infarct would have been induced, suggesting their limited presence in absence of an Ml injury. These results indicate the efficacy of the controlled release approach at recruiting se m cells to the infarct region to potentially contribute: in the, repair and regeneration of the myocardium. The enhanced and long-term presence of stem cells; in the CR group is likely due to the sustained availability of the powerful chemoattraetant SDF-1α within the composite gel.

[00124] *Secreiion of key signaling proteins;* Certain proteins are involved hi triggering cardiac repair mechanisms and others are implicated hi advancing pathological changes post infarction. Therefore, regulation of the secretion levels of such proteins represents an important aspect of effective therapies. The presence of proteins such as the-ones in the complementary -combination, TIMP-3, FGF-2, and SDF-1α, described herein likely affect the signaling and secretions levels of other proteins in the heart after ML To investigate the effect of our treatment on the levels of relevant proteins, tissue iysates were tested for the levels of insulin-like growth faetor-I (1GF--1), vascular endothelial growth factor (VEGF), sonic hedgehog (Shh), and transforming growth factor-pi (TGP-β1) at eight weeks (Fig. 20). Quantitative analysis by ELISA showed significantly higher levels of 1GF-I, an anti-apoptotic factor, in CR ($p<0.001$) and free proteins ($p<0.01$) groups compared to saline (Fig. 20 (A)). Moreover, CR significantly increased the levels of VEGF, a potent angiogenic factor, and Shh, a master cardiac morphogen, over saline ($p<0.05$), while the free proteins group was statistically indifferent to saline Cp>0.O5) (Fig. 20 (B,C». Lastly, CR significantly decreased the levels of TGF-β1, a pro-tlbrotie factor, compared to saline ($p<0,001$) and free proteins ($p<O,0S$) groups (Fig. 20 (D)}. The free proteins group also significantly decreased the levels of I GF-β ($p<0.05$) (Fig. 20 (0)).

fO0125] Ml results in multiple pathologies and maladaptive remodeling of the heart. Numerous efforts toward cardiac repair and regeneration are underway. Stem cell-related technology can provide new csrdiomyocytes via direct reprogramming and paracrine signaling via cell injection. Proteins and nucleic acids can alter the composition- of local signaling molecules and enhance repair and regeneration (H. B. Sager, ***ei al.,*** RNAi targeting multiple ceil adhesion molecules- reduces immune cell recruitment and vascular inflammation after myocardial infarction. *Science Transiatkmal Medicine* 8, 342ra380-

342ra380 (2016).. Tissue-engineered patches can combine cells, growth factors, and mechanical signal to provide comprehensive cues to restore structure and functions of the heart (8. M. Ogle, *et al.*. Distilling- complexity to advance cardiac tissue engineering. *Science Transiatiofial -Medicine* 8, 342ps313-342ps313 (2016)). Proper spatial and temporal signals of proteins can benefit all 3 approaches (B. M. Ogle, *et al., Science Translational Medicine* 8, 342ps3i3-342ps313 (2016)), hi the present example, the concept: of sequential delivery of TlMP-3, FGF-2, and SDF-i α on countering the multitude of pathologies post-Mi was explored in a rat model. These three proteins impart significant benefit p n cardiac function after M L The results, presented herein, demonstrate the ability of the fibria gel-coacervate composite to provide early release of TlMP-3 by one week, followed by a sustained release of FGF-2 and SDF-l α that: lasted at least six weeks,

£001261 The eff cacy of spatiotemporal release of T!MF-3, FGF-2, and SDF- la f-om the .fibrin gel-coacervate composite was tested in a rat Ml model and was compared to sham, saline, and free proteins groups. The CR. group's significant potential to improve cardiac function and trigger repair mechanisms after infarction was demonstrated bringing it close to the normal case of the sharn control *in* many evaluations. In most cases, CR showed significant differences compared to saline group, and to free proteins group in many cases. The free-proteins group, although showing some potential and trends of improvement in different evaluations, -was not able to induce significant repair as CR did compared to saline. This was indicative of the importance of controlled and timed release of T1MP-3, FGF-2, and SDF-!α. Many protein therapies tail to prove long-term efficacy for Ml treatment because of the shortcomings of proteins applied in free form. Including very short-half lives, low retention at the target site, high doses required, and lack of spatio-temporal cues (P. Tayaiia, *et al.,* Con trolled growth factor delivery for tissue engineering. *Advanced materials* 21, 3269-3285 (2009)). A recent study concluded that bolus injections of a cocktail of four important proteins: FGF-2, SDF-!α, IGF-!, and hepatocyte growth factor (HGF), did not improve cardiac function, reduce infarct size, or promote stable rnicrovasculature (If. -Hwang, *et* «/., The combined administration of multiple soluble factors in the repair of chronically infarcted rat myocardium. *Journal of can liovascuhr pharmacoiog}-* 57, 282-286 (201.3)). The study's resiilts might be attributed to the absence of controSed release because without properly protecting the therapeutic proteins and delivering them spatiotemporally, a therapy might prove ineffective at cardiac repair. The delivery approach provided herein offers a solution to these challenges, by protecting the proteins within the fibrin gel-coacervate composite, local king their presence at target tissue, and releasing them spatiotemporal!y.

|0012?J The CR group significantly improved the heart contractile function as early as one week after Ml and lasted up to eight weeks in comparison to saline and free proteins .groups, which had the cardiac function continuously drop over the period tested, measured by echocardiography and further confirmed by cardiac *MM* at eight weeks. Λ cardiac function improvement of -60-75% above non-treated infarcted hearts is reported. This effectively reduced the risk of MI progressing to heart failure. Significant reductions in. ventricular dilation, ventricular wall thinning, myocardial stiffness, and *MM*P

activity. These assessments are interrelated and linked to adverse remodeling and early ECM degradation. The reductions we show in these evaluations might be attributed to the vital role of early TIMP-3 release from the delivery system. TSMP-3 is an ECM-boond enzyme that forms tight non-covaleni and stable complexes with the non-activated latent form of MMPs (p.ro-MMP), blocking the MMP's catalytic domain and preventing its access to substrates (R. Visse, *et al.,* Maim metallopfoteinases and tissue inhibitors of nietailoproteiiiases: structure, -function, and biochemistry. ***Circulation*** *research* 92, 827-839 (2003); *M.* D. SternSicht, *et ai.*, How matrix metalloproteiiiases regulate ceil behavior. ***Annual*** *review of cell and developmental biology* 17, 463-5 16 (2001); and f Yu, *et* ***aL,*** TIMP-3 binds ɒ sulfated glycosaminogiyeans of the extracellular matrix. The Journal of biological chemistry 275, 31226-31232 (2000)). This effectively inhibits activation of MMPs, responsible for clea ving and hydrolyzing many components of the ECM including elastin, fibroneetin, collagen, and proteoglycans (R. Visse, *et al.*, *Gradation research* 92, 827-839 (2003); M. D. Sternlicht, *et aL Annual review of cell* ***and developmental*** *biology* 17, 463-516 (200 1); and T, It V», *et al.* Matrix fTjeialloproieinases; effectors of development and normal physiology, ***Genes*** *& development* 14, 2123-2133 (2000)}.

{00i28J This feature of **TIMP-3**, being able ɒ reduce ECM degradation, likely contributed to mitigating LV adverse remodeling, wall thinning, and dilation; thereby reducing the risk of cardiac rupture and contractile dysfunction. Other studies have shown the importance of TIMP-3 in cardiac diseases. .Deficiency in TIMP-3 has been reported ɒ lead ɒ cardtac dilation, dysfunction, rapture, and mortality. Cell-based TIMP-3 gene delivery improved heart function and reduced cardiac expression and activity of MMP-2 and -9 (H. Tian, *et al.* Inhibiting matrix metal loprotemase by cell-based timp-3 gene- transfer effectively treats acute and chronic ischemic cardiomyopathy. ***Celt transp k ntation*** 21, 1039-1053 (2012)). TIMP-3 delivered by collagen or- hyaluronic gels was able to improve ejection {Taction and reduce ventricular dilation and infarct size in rat and pig models (S. R. Eckbouse, *et «/.*, Local hydrogel release of recombinant TIMP-**3** attenuates adverse left ventricular remodeling after experimental myocardial infarction. ***Science*** *in mslational medicine* 6, 223ra221 (2014) and A. Uchmaka* ***et al*** , Tissue inhibitor of metalloproteinase- 1and -3 improves cardiac function in an ischemic cardiomyopatby model rat. *Tissue engineering. Part A* 20, 3073-3084 (2014)).

{00129} The unregulated and excessive collagen deposition in the infarct, and later non-infaret regions, leads to interstitial fibrosis that increases myocardial -stiffness and risk of contractile dysfunction. Fibrosis arises; as a result of an imbalance in ECM structure and increased -production of collagen by different cells, mainly .myofibroblasts. Myofibroblasts contribute to adverse remodeling and are heavily influenced by the signaling of p.m~fih.rotie factors such as TGF-fi In the present example, it is demonstrated that the spatiotemporal delivery of TIMP-3, FGF--2, and $DF-1α prevented the development of interstitial fibrosis and the expansion of scar and granulation tissue to a large extent. Our CR group proved very effective at decreasing the levels of TGF-βi, a main promoter of fibrosis post-infarction. Therapies that aimed to antagonize TGF-β and.reduee fibrosis proved beneficial .for the

heart recovery (K. E, Porter, *et al.*, Simvastatin reduces human atrial myofibroblast proliferation independently of cholesterol lowering via inhibition of RhoA. *Cardiovascular research* 61, 745-735 (2004); Y. Sun, *et al.*, Angiotensin II, transforming growth factor-beta 1 and repair in the infarcted heart. *Jourml ofmokcu!ar anci cellular cardhlogy* 30, 1559-1.569 (1998); N. A. Turner, *et a*/., Chroniobeia2-adrenergic receptor stimulation increases proliferation of human cardiac fibroblasts via an autocrine mechanism. *Cardiovascular research* 57, 784-792 (2003); C. M. Yti, *et al.*, Effects of combination of angfoteasm-converting enzyme inhibitor and angiotensin receptor antagonist on inflammatory cellular infiltration and myocardial interstitial fibrosis after acute myocardial infarction. *Journal of the American College of Cardiology* 38, 1207-121 5 (2001)). These results like ly helped in the preservation of myocardial elasticity as witnessed in the CR group. Therefore, the efficacy of the spatiotemporal delivery .approach at preventing the excessive deposition of fibrillary collagen reduced the risk of stiffening the ventricular wall, its loss of contractile ability, and progression to heart failure. An excessive inflammatory response can have detrimental effects after Ml, Large amounts of -reactive oxygen species :{ROS) produced by inflammatory cells invading the infarcted -myocardium can cause massive e e ls death. The spatiotemporai delivery approach employed herein proved effecti ve at reducing inflammation and promoting tissue repair. The results revealed the CR's reduction of non-M2 macrophages, which contain M1 macrophages that exacerbate inflammation and ECM degradation. An increase in M2 macrophages, which contribute to reconstruction of the ECM and anti-inflammatory effects is also reported herein.. TlMP-3, provided by this delivery approach, can exert antiinflammatory effects by inhibiting rN.F-a-con.vert.ing enzyme (TACE), the.: enzyme activator of TNP-α. TNF-α is a pro-inflammatory factor which increases in heart failure and is involved in. inducing inflammatory cell Invasion of the infarcted myocardium, MMP production, and cell apoptosis. it is demonstrated that the CR groups helps reduce the levels of pro-inflammatory cytokines 1.L-1β, IL-6, and TNF-α. The strategy reduced the potentially deleterious impact of excessive inflammation by preventing the infil tration of harmful macrophages into the infarcted myocardium or possibly forcing a change in the pbenotype of present ones to become of M2 pbenotype involved in tissue-repair,

}0013(} As Ml causes the death of mill ions of cardiomyocytes and puts millions more at risk, it is an indispensable task to support the survival of cardiomyocytes after Ml and prevent their apoptosis. OR showed remarkable ability to preserve the viability of the cardiac: muscle, activate pro-survival molecular pathways ERK I/2 and A kt, inhibit apoptosis mediated by easpase~3, and increase expression of anti-apoptorie factor iGF~L Studies have proved the important role of activating the PI3K/Akt and Ras-Raf-MEK-ERK pathways to inhibit apoptosis and provide eardioprotection (A, Kis, *et* if/., Second window of protection foiiowing myocardial preconditioning: an essential role for PI3 kinase and p70S6 kinase. *Joun al of molecular and cellular cardiology* 35, 1063-1071 (2003); A, Tsang, *et al.*, Posteonditioning; a form of "modified reperfusion" protects the myocardium by activating the phosphatidylisositoi 3-kmase-Akt. pathway. *Circulation research* 95, 230-232 (2004); Y. Wang, Mitogen-aettvated protein kinases in heart development and diseases. *Circulation* 116, 1413-1423

(2007)). The complementary **proteins** in the **system. described** herein, T!MP-3, FGF-2, and SDF-1α **have** ail been reported to prevent caMiornyocyte apoptosis. Moreover, CR **induced** higher secretions levels of IGF-1 **and** Shh. IGF-I is a well-studied potent cardioprotective and anti-apoptotic factor that **activates** the PI3K/Aft pathway and prevents eardiomyocyte apoptosis. Shh also reduces cardiomyocyte apoptosis through increased expression of **pro-survival** markers and reduced expression of apoptotic markers, **as** we and other groups Slave shown.

£09131} In addition, the CR **group improved** revascularization of **the** infarcted myocardium, **triggering** a robust angiogeiies is process that led to the fomia.ti.on of mature neovessels with potential **of participating in** blood **flow** and perfusion. The triggers behind the .formation of mature neovaseulature **in** the **borderzones of** the. in farct **region** can. be finked mainly to FGF-2 and, to **a** lesser degree SDF-1α, present in the described protein combination and delivered. in a sustained manner by **the-coacervate.** As **a strong** angiogenic factor, FGF-2 induces **endothelial** cell proliferation and sprouting leading to **the** formation of tube-like structures that evolve into neovesseis with **lumens**. Protein **signaling** results shows that the delivery group upregulates **VEGF** expression, **which** is an eftdothelial-speeific factor that is important for angiogenesis **and** vasodilation Ail these **indications** support our **finding** that spatioternporal delivery **of T!MP-3**, FGF-2. and SDF-1α leads to the formation of new, mature, and stable blood vessels, necessary to the repair of **Ml.** injury.

109132.) **Stem-cell** recruitment to the infarct region is another **important** aspect of an effective **therapy** because of the **potential** of stem **cells** to ultimately differentiate into cardiac and vascular cells **and/or** support the repair by paracrine signaling, thereby supporting the survival of remaining **cardiomyocytes** and regeneration of a viable **myocardium** that **replaces** the lost **damaged** one. Our CR group showed **significant ability at** homing stem cells to the bord;erzones of the **infarct. This** is likely due to the sustained **bioavailability** of SDF-3 **a** provided by the **coacervate within** our composite, SDF-.a is a **powerful** chemoattractant that can mobilise different **types of progenitor cells** such as **endothelial** progenitor cells (EPCs), hematopoietic stern cells (BSC), **mesenchymal** stem ceils (MSCs), and cardiac **stem** cells (CSCs) to the infareted **myocardium**

{00133} There are some limitations o this study. Although an ischemia-reperfusiori mode! would have been **more clinically-relevant**, a permanent ligation MI model was used in the rats of this study in order to induce **-more-.severe-damages** o the **myocardium,** thus enabling the detection of bigger differences between comparison groups due **to different treatments.** Due to limited number of animals **at** the two-week time point, only histology experiments were performed. **Important assessments** such as MMP activity, **although** performed at eight weeks, would be evaluated at earlier time points in **future** experiments. **Small** animal **models as** the one employed in this study provide significant insight in protein therapy , **However,** a large **animal** model such as a pig **MI** model is the **logical** next step **because adult** pigs present similar anatomy, response to ischemic insult, and expansion of an **infarct to. humans. Another** area **of improvement** is to achieve **catheter** delivery **to** reduce **surgical** invasiveness. **Viscosity** and gelation parameters need to be optimised so that gelation doesn't occur while the injectable material

is still in the catheter, or too late after injection where the therapeutic cargo would diffuse away from the target site.

Materials slid Methods

[00134] *Rationale and study design.* The approach here was to inject a fibrin-coaeervate composite gel loaded with TIMP-3, FGF-2, and SDF-1α for spatiotemporal release in the infarcted hearts of rats. The effects of this therapeutic approach on cardiac function, dilation, and myocardial strain levels were assessed at different .time points after MI *by* mttltimodality imaging (echocardiography, MRI), Tissue-level changes were evaluated at two and/or eight weeks using histology, immunohistochemistry,.western blot, and ELISA.

[00135] *Power analysis.* In a recent study, MR] was for assessment of cardiac function in a rat Mi model at four weeks (6}. From the results of this cardiac assessment, we had a standard deviation (SD) of σ=3%. Based on this value, a power analysis calculation carr ed out with Minitab statistical software estimates that in. order to he able to detect an effect size of 6% (~2 SD) between EF% of treatment and sham, with a significance value of 5% and a power of 80%, at least n~6 animals per group were required.

100136! The treatment assignments were randomized at the time of surgery. A total of 56 rats were used in this study for four groups; Sham, saline, free proteius, and CR with evaluations performed at. two weeks (n- 17 rats; 4-5 per group) and eight weeks (n=39; 9-10 per group). Sample sizes for each experimental measue ment are provided in the figure legend or text, as appropriate.

[00137] ***Re-lease*** *assay of* ***complementary*** *prneins:* PEAD was synthesized as previously described (1-1. Chu, ***et*** *til.* Design, synthesis, and bioeompatibiihy of an argioirse-based polyester. *Biotechnology'progress* 28, 257-264 (2012)). The release assay was performed as previously described and further detailed herein (H, K. Awada, *ef ah* Sequential delivery of angiogenic growth factors improves revascularization and. heart function after myocardial infarction. *Journal* ***of*** *controlled release:* ***official-*** *joun al of the Controlled Release Society* **207, 7-17** (2015)).

[00138] *Rat* ***acute-*** *Ml modek* The induction of Ml was performed as previously described (H. K. Awada, *et al.*. Factorial Design of Experiments to Optimize Multiple Protein Delivery for Cardiac Repair. *ACS Biomaterials Science S. Engineering* 2, 879-886: (2016); H. K. Awada, *et al. Journal-* ***of*** *controlled release: official journal of the Controlled Release Society* 207, 7-17 (2015)) and further detailed herein. Four groups (n~56 rats) were evaluated: sham, saline, free proteins, and CR, Empty vehicle (empty fibrin gei-coacervate compos its) was not. tested as a -control in this study as it has shown no difference to saline in our previous work (H. K. Awada, *et ah Journal of controlled release: official journal* ***of*** *the Controlled Release* ***Society*** *207,* **747** (2015))..

[00139] *Ecinocardiography and cardiac MRI* Echocardiography and cardiac MRI were performed to compute ESA, EDA, FAC, ESV, EDV, and EF as previously described. *{6, 18*) and further detailed in Supplementary Methods.

[0114] *Myocardial strain level measurements.* The B-mode frames of LV short-axis view acquired at eight weeks post-MI were analyzed (n=5 rats per group) using a strain analysis algorithm (VevoStrain™, Vevo2100). Five regions of interest (ROI) were selected along the LV mid-wall including one ROI in the anterior lateral (infected area) and four ROIs in the anterior medial, septal, posterior, posterior lateral (unaffected areas) walls of the LV. The peak strain in the infected area was normalized to the average peak strains of the four ROIs in unaffected LV walls during full cardiac cycles. The radial strain, defined as the percent change in myocardial wall thickness, was computed.

[00141] *Histology:* At two weeks (n=4-5 per group) and eight weeks (n=5-7 per group) post-infarction, rats were sacrificed by injecting 2ml of saturated potassium chloride (KG) solution (Sigma Aldrich, St. Louis, MO) in the LV to arrest the heart in diastole. Hearts were harvested, fixed in 2% paraformaldehyde (fisher Scientific, Fair Lawn, Hi) for 1-2 hours, deposited in 30% sucrose solution (w/v) overnight, frozen in O.C.T compound (Fisher Healthcare, Houston, TX), and stored at -20X. Specimens were cryoseciioned at 8 μm thickness from apex to the ligation level with 500 μm intervals.

[00142] Hematoxylin and eosin (H&E) staining was performed for general evaluation. H&E stained slides were selected and the ventricular wall thickness in the infarct zone (n=3-4 per group at two wks, n==4-6 at eight wks) was measured near the mid-section level of the infarct tissue using NIS Elements AR imaging software (Nikon Instruments, Melville, NY).

[00143] For assessment of interstitial fibrosis, picrosirius red staining was used to stain collagen fibers and image under polarized light The fraction area of collagen deposition in the cross-Sectional area of the whole heart was measured by NIS software near the mid-section level of the infarct tissue (n=3-5 per group at two wks, n=4-7 at eight wks). An object count tool was used to include RGB pixels specific to the stained collagen fibers in the heart area by defining a proper threshold value.

[00144] *Jmmunohistochemistry:* For evaluation of inflammation, a rabbit polyclonal antibody F4/80 (1:100, Santa Cruz Biotechnology, Dallas, TX), a pan-macrophage surface marker, was used followed by an Alexa fluor 594 goat anti-rabbit antibody (1:200, Invitrogen, Carlsbad, CA). Slides were also co-stained by a mouse anti-rat GD163 (1:150, Bio-Rad Laboratories, Hercules, CA), an M2 macrophage phenotype marker, followed by an Aiexa fluor 48$ goat anti-mouse antibody (1:200, Invitrogen, Carlsbad, CA). Slides were last eonnterstaitled with 4',6-diamidino-2-pheiylindo)e (DAP!) (Invitrogen, Carlsbad, CA). For quantification near the mid-section level of the infarct tissue, F4/80-positive and CD163-positive cells were counted in two opposite regions of the infarct border zone, averaged, and reported per mm³ areas (n=3-4 rats per group at two wks),

[00145] For evaluation of cardiac muscle viability, a rabbit polyclonal cardiac troponin I (eTnl) antibody (1:20Q, US Abeam, Cambridge, MA) was used followed by an Alexa fluor 488 goat anti-rabbit antibody (1:2i)0, !nvitrogen, Carlsbad, CA), Slides were countersiained with DAPL The fraction area of viable eardiae muscle in the cross-sectional area of the whole heart was measured by NIS Elements AR software near the mid-section level of the infarct tissue (n=3-5 per group at two wks, n=5-6 at eight

wks). An object count tool was used to include RGB pixels specific to the staked viable cardiac muscle in the heart area by defining a proper threshold -value,

[00146] For evaluation of angrogenesis, endothelial cells (EC's) were detected by a rabbit polyclonal von Willebrand.-factor (vWF) antibody (1:200, US Abeam, Cambridge, MA.) followed by an Aiexa fluor 594 goat anti-rabbit antibody (1:200). Mural ceils were detected by a PITC-conjugated anti-α-smooth muscle actk (α-SMA) monoclonal antibody (1:500, Sigma. Aldrich, St., Louis, *MO).* Slides were last eouuterstamed with DAPI. For quantification near the mid-section level of the infarct tissue, vWP-posit»ve vessels (defined as those with lumen) and a-SMA-positive vessels were counted in two opposite regions of the infarct border zone, averaged, -and reported per mm² areas (n~3-4 .rats per group at two wks, n=5-6.per group at eight wks).

[00147] For evaluation of stem cell homing, stem/progenitor cells were detected by a rabbit polyclonal c-Kit antibody (I; 100, Santa Cruz Biotechnology, Dallas, TX) followed by an Alexa fluor 488 goat anti-rabbit antibody (1:200), Slides were eounterstained with DAPL For quantification near the mid-section level of the infarct tissue, e-Kit-positive cells were counted in two opposite regions of the infarct border zone, averaged, and reported per mm² areas (n-5 rats per group at eight wks).

[00148] *Molecular markets expression by western blot:* Rat hearts (n=15) were harvested and rapidly stored at - 80°C for western blotting. For protein extraction, myocardial specimens weighing approximately 100 mg were excised from the LV generating a composite material comprising a spectrum between normal, infarct, and borderzone tissue. The tissues were then homogenized at .0.2 µg/ml in a modified lysis RIPA buffer (50mM Xris-HCl, 1% NP-40, 20 mM-DTT, 150 mM NaCl, pH=7,4) with protease and phosphatase inhibitors. The complex was then eentrifuged at 12,100 g for 10 mm, and the supernatant was collected and stored at -8Q°C until use,

[00149] For total protein content, the extracts above were quantified with Pierce 660 *nm* Protein Assay (Thermo Fisher Scientific,. Waitham, MA). The equivalent of 100 µg protein was separated using 11.5% gel and then transferred onto a PVDF membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was blocked with 5% BSA in TBS with 0,05% Tween 20 for 1h, then incubated with following antibody solutions: AKT, p-AKT, ERK1/2, p-BRK1/2 (all at 1:300, Santa Cruz Biotechnology, Dallas, TX), cleaved caspase~3 (1:1,000, Cell Signaling Technology, Boston, MA), and GAPDH (1:5000, OS Abeam, Cambridge, MA). The membranes were washed with TBS three times and incubated with secondary antibodies for 2h at room temperature. Signals were visualized using the ChemiDie ™ XRS + Imaging System (Bio-Rad Laboratories, Hercules, CA), and band densities were quantified using NIK linage.! (n~3 per group).

[00150] *Myocardial proiein secretion levels by ELISA:* The tissue lysates acquired in the western blot section (n=3-4 rats per group) were used for detecting the levels of insuSin-iike growth factor-! (IGF-I), vascular endothelial growth factor (VEGF), sonic hedgehog (Shh), transforming growth factor-β1 (TGF-β1), a terleukin 1β.(!L-1β), 11,-6, and tissue necrosis factor-α (TNF-a) in the LV myocardium. Sandwich ELISA kits (PeproTech, Rocky Hill, NJ) were used per the manufacturer's

instructions with lysate dilations for VEGF (i:20), IGP-I (1:50), IL-1β (S.:i5). !L-6 (1:15), and TNF-α (1:15). .For Shh and TGF-β1, indirect ELISAs were run using rabbit.polyclonal antibodies against -Shh and TGF-β1 (both at 1:30,.Santa Cruz Biotechnology, Dallas, TX) followed by a secondary biotinylated goat anti-rabhit IgG (1:10Q..Santa Cruz Biotechnology, Dallas, TX). Lysates were diluted 1:15 for Shh and 1:25 for TGP-β1. The absOrbance at. 450/540nm was measured by a SyrasrgyMX plate reader. Results were corrected to-account for differences in total protein content of samples.

[0151] *MMP-2/9 activity assay:* The tissue lysates acquired in the western blot section (n=3-4 rats per group) were used for detecting the activity of MMP-2/9 in the L.V myocardium. The Caibiochem InnoZyme™ Geiatinase activity assay fluorogenic kit (EM.D Millipore, Belieriea. MA) was followed per the manufacturer's instructions, Briefly, lysate. samples (diluted 1:2 in activation buffer) were incubated with a fluorogenie substrate solution that is highly selective for MMP-2 and MMP-9. Gelaiinases in the sampie lysates of the myocardium cleave the substrate, resulting in an increase in I uorescent signal measured at an excitation wavelength of 320nm and an emission wavelength of 405nm by a SynergyMX plate reader. The gelatinase control, activated similarly, was used at serial dilutions to create a standard -curve for converting the fluorescence values of MMP activity to concentrations (ng/ml),

[00152] *Statistical analysis:* Results are presented as means ± standard deviations (SO). GraphPad Prism 5.0 software (La Jolla, CA) was used for statistical analysis. Statistical differences between groups were analyzed by one-way ANOVA (multiple groups) or two-way repeated ANOVA (repeated echoeardiographie -measurernents) with 95% confidence interval. Bonferrorii multiple comparison test was performed for ANOVA post-hoc analysis. Statistical significance was set at $p<0.05$.

[0015]3| *Release assay of complementary proteins:* The: release .assay was performed using 10Ong of each of TIMP-3 (R&D Systems, Minneapolis, MN), FGF-2, and SDF-1α (PeproTech, Rocky Hill, *H3).* All solutions were prepared in 0.9% sterile-saline, FGF-2 and SDF-ia eoacervates were made and mixed with a fibrinogen solution containing TiMP-3, followed by thrombin (Sigma-AIdrieh, St. Louis, MO) to induce gelation, resulting in a 1OOμI fibrin gel-coacervate composite. FGF-2 and SDF-1o eoacervates were made by mixing 1μl of i00ng/μI for each of FGF-2 and SDF-i« with 2μl of 5mg/ml heparin first (Scientific Protein Labs, Waunakee, Wl), then with 2μl of 2Smg/ml of PEAD at PEAD:hepafin.:protein mass ratio of 250:50:1. This formed δμl of FGF-2/SDF-ltt eoacervates. Fibrin gel-coacervate composite was made by mixing 82μl of 20mg/ml fibrinogen (Sigma-Aldrich, St. Louis, MG), 1μl of 5mg/mi heparin, 1μl of 10Ong/μI of TIMP-3; then the 6μi PGF-2/SDF-1α eoacervates were added, followed by 5μl of img/mi aprotonin (Sigma-AIdrich, St Louis, MO). Lastly, 5μI of 1ng/ml thrombin (Sigma-AWvich, St. Louis, MO) was added to-induce gelation, .resulting in a 100μl fibrin gel-coacervate composite.

[00154] A 1OOμI of 0.9% saline was deposited on top of the gel composite to be collected at 1h, 16h, 1, 4, 7, 14, 28; and 42 days. The samples (n=J) were incubated at 37°C. After eetrtrifugaiion at

12,100g for 10 nrin, supernatant was collected and stored at -80°C to detect amount of released proteins by sandwich enzyme-linked immunosorbent assay (ELISA) kite (PeprbTech, Rocky Hill, NJ) (R&D Systems, Minneapolis, MN), The absofbanee at 4S0/54Onm was measured by a SynergyMX plate reader (Biotek, Winooski, VT). Standard solutions (n===3) that contained 100ng of each of the proteins in free form in 10Oμl of 0.9% saline were prepared to create standard curves and determine total release,

100155] *Detailed method of mi Ml model:* Six o seven week old (i 75~225g) male Sprague-Daw!ey rats -(Charles River Labs, Wilmington, MA) were anesthetized first then maintained with 2% isofiurane at 0.31./mi« (Butler Sehein, Dublin, OH), -Intubated, and connected to a mechanical ventilator to support breathing during surgery. The body temperature was. maintained at 37°€ by a hot pad. The ventral side was shaved and. a small incision was made through the skin. Forceps, scissors,, and q-tips were used to dissect through the skin, muscles, and ribs. Once the heat was visible, the pericardium was torn. Ml was induced by permanent ligation of the left anterior descending (LAD) coronary artery using a 6-0 polypropylene suture (Elhieon, Bndgewater, NJ). lufaret was confirmed by macroscopic Observation of a change in color from bright e d to light pink in the area below the ligation suture. Five minutes after the induction of MI, different treatment and control solutions- were injected intramyocardiaily at three equidistant points around the infarct aone using a 31-gauge needle (BD, Franklin Lakes, NJ). Four groups (n=56 rats) were evaluated; sham, saline, free proteins, and delivered proteins. Empty vehicle (empty f brin gel-e-oaeervate composite) was not tested as a control in this study as it has shown no difference to-saline in our previous work,

[00156] The .Sham group (n- 13) underwent the surgery in which the heart was exposed and pericardium was torn, then chest was closed and rat recovered. The saline group (n~ l4) underwent the surgery in which MI was induced and I00μl of 0,9% sterile saline was injected around the infarct region. The free proteins group (n~14) underwent the surgery in which Ml was Induced and 100μl of 0.9% sterile saline containing 3μg each of free TfMP-3, FG.P-2-, and SDP-1α was injected around the infarct region. The delivered proteins group (n=-T5) underwent the surgery in which Ml was induced and 100μi of fibrin gel-coacervate. composite was injected around the infarct region. The fibrin gel-coacervate composite was prepared briefly as follows: 18μ1eoaeervate solution containing 3μg each of FGP-2 and SDF-1α, 67μ| of 20rng/ml fibrinogen, 6μ1of solution containing heparin and 3μg of TIMP-3, 5μ1of 1rng/mi aprotonin (Sigma-Aldrich, Si. Louis, MO). Lastly, 4μi of ..Smg/ml thrombin (Sigma- Aidrich, St. Louis, MO) was added and the total solution was injected shortly before- gelation occurred, approximately 40 seconds after mixing, All solutions were prepared in 0.9% sterile saline. The chest Was closed and the rat was allowed to recover. At multiple time points, rats were imaged using echocardiography. At eight weeks, a subset was imaged using cardiac MRI. Alter 2 (n=17) or 8 weeks (n-39), animate were sacrificed and hearts were harvested tor histological, immunohistoehemical, and western blot evaluations.

10157] *Echocardiography:* At pre-Ml, one, two, five and eight weeks post-Ml, rats (n=9-1f> per group) were anesthetized then maintained with 1-1.5% isoilurarse gas throughout the

ec!iocaixitographic study. Rats were placed in the supine position, immobilized on a heated stage equipped with echocardiography, arid the hair in the abdomen was removed. The body temperature was maintained at 37°C. Short-axis videos of the- LV by B-a ode were obtained using a high-resolution in small animal imaging system (Vev© 2100, Visual Sonics, Ontario, Canada) equipped with a high-frequency linear probe (MS400, 30 MHz) (FUJIFILM VisuaiSonies, Canada). End-systolic (ESA) and etid-diastolic (EDA) areas were measured using NIH ImageJ and fractional area change (FAC) was -calculated as: [(EDA-ESA)/EDA]×100%. Percent improvements of one group over another were calculated as the difference between the % drops in FAC vaiy.es of the first and second groiips divided by the higher % drop of the two groups.

f00158J *Cardiec MRI:* Cardiac MRI was used to measure LV volumes and ejection traction (EF) from infarcted rat hearts at eight weeks (n-5-S per group). MR*I* was preformed using a Broker Biospec 4.7-Tesla 40-cm scanner equipped with a 12-cm shielded gradient set a 72mm transmit R.F coil (Bruker Biospin, Billerica MA), and a four-channel rat cardiac receive array (Rapid MR International, Columbus, Off). Rats were induced with isoflurane, intubated, and ventilated at 1mL/100g of body weight and maintained at 2% isoflurane in 2:1 O2:N2O gas mixture at 60 BPM. During the MR! procedure rats were continually monitored and rectal temperature was maintained at 37°C with warm air (SA Instruments, Stony Brook, NY). Following pilot scans, rats were imaged using a self-gated cine FLASH sequence (IntraGate) with the following parameters: TR/TE= 9,0/3.0 ms, 40x40mm FOV, 256x256 matrix, FA=10", and 200 repetitions. 10-12 slices were collected to cover the area between the heart apex to tire mitral valves with i.5r»m slice thickness with common navigator slice. End-systolic and end-diastdlic phases were identified for each subject and the LV cavity .manually traced using NIH Image! to determine LV end-systolic (ESV) and end-diastolic (EDV) volumes. These volumes were used to compute ejection fraction as EF% = [(EDV-ESV)/EDV]×100%. Percent imptovements of one group over another were calculated as the difference between the % drops in EF values of the.first and second groups divided by the higher % drop of the two groups.

[00159] The following numbered clauses provide illustrative examples of various aspects of the invention.

1. A composition comprising a eoaeervate of a polyeationic polymer, a poiyanionic polymer, and platelet-rich -plasma and/or serum, or a fraction or concentrate thereof

2. The composition of clause 1, comprising platelet-rich plasma and wherein platelets of the platelet-rich plasma are .activated to produce a fibrin clot, and the fibrin clot is optionally removed from the platelet-rich plasma,

3. The composition of clause 1, comprising pure plateiet-rich fibrin (P-PRF) or leukocyte-rich PRF, with or without the fibrin clot removed,

4. The composition of clause 2 or 5, wherein the fibrin clot is removed and the protein content of the PRP is concentrated (for example, as compared to the solution phase of activated PRP, in. which the fibrin is removed, but prior to concentration, e.g., by use of a centrifugal filter unit).

5. A composition comprising a coacervate of a polycationic polymer, a polyanionic polymer, and a composition obtained from an organism or cultured cells, tissues or organs and containing a (complex, e.g.) mixture of proteins and/or growth factors produced by the organism or cultured cells, tissues or organs (that is, the composition is obtained from a living source, and though it may proceed through *am* or more fractionation and/or purification steps not limited to activation or fractionation, e.g., precipitation, chromatography, and/or affinity separation:, as in the case of activated PRP as decribed herein in which the platelets of the PRP are activated, and optionally, the resultant clot is removed and the composition is optionally concentrated, it is not an isolated or purified single constituent, but includes coststuents, such as, for example, a plurality (at least two, e.g., three or more or four or mere) of proteins and/or growth factors, essentially in relative amounts and/or a ratio found in, or produced by the cells or organism).

6. The composition of clause 5, wherein the composition containing a complex mixture of proteins and/or growth factors is prepared from a bodily fluid of aft organism, a cell or tissue lysate, or conditioned media in which cells or a tissue- is grown.

7. The composition of clause 5, wherein the ceils, tissues, or organism are genetically-modified.

8. A composition comprising:

a. a hydrogel comprising TIMP-3; and

b. a complex or coacervate of a poiycationic polymer, a polyanionic polymer, FGF-2 and SDF-1et embedded in the hydrogel.

9. The composition of any one of clauses; 1-8, wherein the polyanionic polymer is a sulfated -or suifarnated polysaccharide.

10. The composition of any one of clauses 1-8, wherein the polyanionie is a heparin or heparan sulfate.

11. The composition of any one of clauses 1-10, wherein the poiycationic polymer is a polymer composition comprising at least one moiety selected from the following;

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

(c) [-OC(O)-CH(NHY)-CH_2-CH_2-C(O)O-CH2-CH(O-R1)-CH_2-O-CH_2-CH_2-O-CHr CH(0-R2)-CH_2-]$_s$, and/or

(d) [-OC(O)-CH_2-CH_2-CH(NHY)-C(O)O-CH2-CH(O-R1)-CH_2-O-CH_2-CH_2-O-CH_2-CH(O-R2)-CH_2-]$_m$,

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~contaming group, a C i-¾aiky l -group, an amine-containing group, a quaternary ammonium containing -group, and a peptide,

12. The composition of clause 11, wherein the polyeationic polymer has a polydispersity Index of less than 3.0.

13. The composition of clause 11, wherien R1 and R2 are selected from the group consisting of lle-Lys-Val-Ala-Vai (IKVAV) (SEQ ID NO: 4), Arg-Gly-Asp (ROD), Arg-Gly-Asp-Ser (RODS) (SEQ ID NO: 5), Ala-Oiy-Asp (AGD), Lys-Gln-Ala-Gly-Asp-Vai (KQAGDV) (SEQ ID NO: 6), Vai-Ala-Pro-Gly-Val-Giy (VAPGVG) (SEQ ID NO: ?), Ai¾VGV (SEQ ID NO: 8), PGVG3VA (SEQ ID NO: 9), VAP, GiVGVA (SEQ ID NO; 10), VAPG (SEQ ID NO; 11), VGVAPG (SEQ ID NO: 12), VGVA (SEQ ID NO: 13), VAPGV (SEQ ID NO: 14) and GVAPGV (SEQ ID NO: 15).

14. The composition of clause 11, wherein Y is -C(O)-CH(NH_3+)-(CH_2)$_4$-(NH_3)* .

15. The composition of clause 11, wherein Y is -C(O)-CH(NH_3+)-(CH_2)$_3$-NH-C(NH_2)$_2$+.

16. The composition of any one of clauses 1-15, wherein the ratio of the polyeationic polymer to the poiyanionle polymer in the composition results in a neutral, negative, or positive charge in the coacervate.

17. The composition of claused, wherein the hydrogel comprises fibrin.

18. The composition of any one of clauses 8-17, wherein the amounts of TlMP-3, FGF-2 and/or SDF-1« its the compositions are -amounts effective o treat a myocardial infarct in a patient (that is, an amount effecti ve to improve one or more clinically-relevant- measures of the myocardial infarct |n the patient.

19. A method of treating a patient having a myocardial infarct, comprising administering the composition of any one of clauses 8-18 to a patient at or near a site of a myocardial infarct in the patient. in an amount effective to treats myocardial infarct in a patient

20. A method of treating a wound in a patient comprising administering a composition according to any one of clauses 1-7 at or adjacent to a wound in the patient in an amount effective to treat a wound in a patient.

21. The method of clause 20, wherein the composition is administered to the patient more than once,

22. A method of preparing a therapeutic composition for use in treating a -wound in a patient, comprising:

a. mixing a poiyanionic polymer with platelet-rich plasma, serum, a fraction thereof, a concentrate thereof, or platelet-rich plasma (PRP) in which the platelets have been activated to produce a fibrin clot; and

b. mixing the PRP and poiyanionic polymer mixture with a polyeationic polymer,

23. The method of clause 22, wherein the poiyanionic polymer is a sulfated or suiamated polysaccharide, such as heparin or heparan sulfate.

24. The method of clause 22 or 23, wherein the polycationie polymer **is** a polymer **composition** comprising at least one moiety selected from the following:

(a) [-OC(0 }-CH(NHY)-CH$_2$-C(0)0 -CH$_2$-CiH(0 -R 1)-C¾ -C>C¾ -CH$_2$-0 -C¾ - CH(O-R2)-CH$_2$-]$_n$,

(b) [-OC(O)-CH$_2$-CH(NHY)-C{0)O-CH2-CH(O-R I)-0 H$_2$-O-CH$_2$-CH$_2$-O-CH$_2$-C¾ O-R2)-C¾ -]$_n$,

(c) [-OC(O)-CH(NHY)-CH$_2$-CH$_2$-C(O)O-CH2-CH(O-R1)-CH$_2$-O-CH$_2$-CH$_2$-O-**G¾-C¾-i(G-ll2)-Gf** l$_2$-]$_m$, **and/or**

(d) [-OC(O)-CH$_2$-CH$_2$-CH(NHY)-C(O)O-CH2-CH(O-R1)-CH$_2$-O-CH$_2$-CH$_2$-O-CH$_2$-CB(0 -R2)-CH$_2$~]$_m$,

wherein Y **is** -C(O)-CH(NH$_3$$^+$)-(CH$_2$)i-NH-C(NH$_2$)$_2$$^+$ or -C(O)-CH(NHi$^+$)-(CH$_2$)$_4$-(NH$_3$)$^+$, and R1 **and** R2 are the same or **different** and are **independently** selected trom the group **consisting** of hydrogen, a earboxy-contaiiving group, a. C$_{1-6}$ **alkyl** group, an amine-containing group, a quaternary ammonium containing group, and a peptide,

25. The method of clause 24, **wherein the polycationie polymer** has a **polydispersity** index of less than 3.0.

26. The method of clause 24, wherein R1 and R2 are selected from the group consisting of Ile-Lys-Val-Ak-Vai **(IKVAV)** (SEQ ID **NO:** 4),. Arg-Gly-Asp **(ROD),** Arg-G!y-Asp-Ser (RODS) **(S.EQ** ID **NO:** 5), Ala-Gly-Asp **(AGD),** Lys-G1n~AIa-Gly-Asp~Va1 **(KQAGDV).** (SEQ **ID NO:** 6), Vai-Ala-Pro-Gly-Val-Gly (VAPGVG) (SEQ **ID NO:** ?), APGV'GV (SEQ ID NO: 8), PGV'GVA (SEQ ID NO: 9), **VAP,** GVGYA (SEQ ID NO; 10**),** VAPG (SEQ ID NO: 11), VGVAPG (SEQ I**D.NO:** 12**), VGVA** (SEQ ID NO: i3), **VAPGV (SEQ: ID** NO: 14) and; GVAPG V (SEQ **ID** NO: 15),

27. The: **method** of clause 24, wherein Y is ~C(0)-CM(NI-i:$_2$$^+$)-(CH$_2$)$_4$-(NH$_3$)$^+$,

28. The method of clause 24, wherein Y is -C(O)-CH(N¾ +)-(CH$_2$)$_3$-NH-C(N¾)$_2$+.

29. The method of any one of clauses 22-28, wherein the ratio of the polycationie polymer to the polyanionic polymer in the **composition** results in a neutral, negative, or positive charge **in** the coacervate.

30. The method **of** any one of **clauses** 22-29, wherein the **platelet-rich** plasma, **serum,** a fraction thereof, a concentrate **thereof, or** platelet-rich plasma (PRP) in which the platelets have been activated **to** produce **a** fibrin clot is **autologous** to a patient to be treated.

31. The method of any one **of clauses** 22-30, wherein the platelet-rich plasma, **serum,** a fraction **thereof,** a.concentrate thereof, or PRP **in** which the platelets have been activaied to produce a fibrin clot **PRP** in **which the** platelets have been activated is PRP in **which** the platelets have bees activated **to** produce a fibrin clot PRP in which the platelets have been activated that is further processed to remove the fibrin clot from the PRP prior **to** mixing with **the poiyanionic** polymer.

32. The method of clause 31, further comprising concentra ting the proteins in the PRP.

33. The method of *my* one of clauses 22-32, further **comprising** applying the **therapeutic** composition to a medical device or wound dressing,

34, The **method of clause** 33, wherein the medical device or wound **dressing** is a **bandage,** suture, surgical mesh, limb or joint prosthesis, or a noo-woven material,

35, A medical device or wound **dressing** comprising a **composition** according to any one of clauses **1**-7.

36. **The medical device** or **wound** dressing of **clause** 35, wherein **the** medical device or wound dressing is a **bandage,** suture, surgical mesh, limb or joint prosthesis, or a nors-woven materia!.

{0 0 t 6 0} While the **present invention** is described with reference to several distinct **embodiments, those** skilled in the art may make moditlcatiosis and alterations withotit **departing *from*** the scope and spirit. **Accordingly,** the above detailed **description** is **intended** to be illustrative rather than restrictive.

We claim:

1. A composit ion eornprisi ng a coacervate of a polycatioo ic polymer, a polyan sonic polymer, and platelet-rich plasma **and/or** serum, or **a** fraction or **concentrate** thereof.

2. The composition of claim 1, comprising platelet-rich plasma and wherein **platelets** of the platelet-rich plasma are acts vated to produce a fibrin clot, and the **fibrin** clot is optionally removed **from the** platelet-rich plasma.

3. **The** composition **of** claim 1, **comprising pure** plateiet-i eh **fibrin (P-PRF) or leukocyte-rich** PRF, with: or without the fibrin clot removed.

4. The composition of claim. 2, wherein the fibrin clot **is ..removed and the** protein content of the **P.RP** is. **concentrated.**

5. A composition comprising a coacervate of a polycatidnie polymer, **a** polyamOnic polymer, and a **composition obtained from an** organism **or** cultured ceils, **tissues or organs and containing** a mixture of proteins and/or growth factors produced by the organism or **cultured** ceils, tissues or organs.

6. **The** composition of claim 5, **wherein** the composition containing a mixture of proteins **and/or** growth **factors** is **prepared from** a **bodily fluid of an** organism, a **cell** or tissue lysate, **or** conditioned media in which cells or a tissue is grown.

7. The composition of claim 5, wherein the cells, tissues, or organism are **genetically-modified.**

8. A composition comprising:

a. a hydrogel comprising TiMP-3; and

b, a **complex** or coacervate **of a** polycationic polymer, a polyanionie polymer, FGF-2 and **SDF-1**α embedded in the hydrogel,

9. The composition of any one of claims 1-7, wherein **the"** polyanionic polymer is a sulfated or suliamated polysaccharide, such **as** heparin or heparan **sulfate.**

10. The composition of any one of claims 1-S, wherein the **polycationic** polymer is a polymer composition comprising at least one moiety selected from the following:

(a) **[~Oe** (0)-CH(NHY)-C]H$_2$-C{0 }0 -C}H$_2$-CH{O-R1)-CH$_2$-O-CH$_2$-CH$_i$-0 -CH2-CH(O-R2)-CH$_2$-]$_n$,

(b) [-OC(O)-CH$_2$-CH(NHY)-C(O)O-CH$_2$-CH(O-R1)-CH$_2$-O-CH$_2$-CH$_2$-O-CH$_2$-CH(O-R2)-CJ¾ $_n$,

(c) [-OC(0 }-CH(NHY)-CH$_2$-CH$_2$-C(0)0 -CH2-CHfO-R1)-CH$_2$-O-CH$_2$-CH$_2$-O-CH$_2$-CH(O-R2)-CH$_2$-]$_n$, and/or

(d) [-OC{O}-CH$_2$-CH$_2$-CH(NHY)-C(0)0 -CH2-CH(O-R1)-CH$_2$-0 -C¾-CH $_2$-O-CH$_2$-CH(O-R2)-CH$_2$-]$_n$,

wherein **Y** is -C(**0**)-CH(N.H$_3^+$)-(CH$_2$)$_3$-NH-C(NH$_2$)$_2^+$ or -C(O)-CH(NH$_3^+$)-(Cf)$_4$-iNH$_3$)$^+$, and R! 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-containing group, a quaternary ammonium containing group, and a peptide,

11. The composition of claim 10, wherein the polycationic polymer has a polydispersity index of less than. 3.0.

12. The composition of claim 10, wherein R1 and R2 are selected from the group consisting of Ile-Lys-Val-Ala-Vai (IKVAV) (SEQ ID NO: 4), Arg-Gly-Asp (RGD), Arg-Gly-Asp-Ser (RGDS) (SEQ ID NO: 5), Aia-Gty-Asp (AGD), Lys-Gln-Afa-Gly-Asp-Vai (KQAGDV) (SEQ ID NO: 6), Vai-Ala-Pro-Gly-Vai-Gly (VAPGVG) (SEQ ID NO: ?), APGVGV (SEQ ID NO: 8), K3YGVA (SEQ ID NO: 9), VAP, OVOVA (SEQ ID NO; 10), VAPG (SEQ ID NO; 1i), VGVAPG (SEQ ID NO: 12), VGVA (SEQ ID NO: 13), VAPGV (SEQ ID NO: 14) *mid* GVAPGV (SEQ ID NO: 15).

13. The composition of claim I or 8, wherein the ratio of the. polycationic polymer to the polyankmic polymer in the composition results in a neutral charge in the coaeervate.

14. The composition of claim 8, wherein the hydrogel comprises fibrin.

15. The composition of claim 8, wherein the amounts of T1MP-.3,. FGF-2 and/or SDF-1α in the compositions are amounts effective to treat a myocardial infarct in a. patient.

16. A method of treating a patient having a myocardial infarct, comprising administering the composition of claim. 8 to a patient at or near a site of a myocardial infarct in the patient, in an amount effective to treat a myocardial infarct: in a patient.

17. A method of treating a wound in a patient comprising administering a composition according to any oile of claims 1-7 at or adjacent to a wound in the patient, man amount effective to treat a wound in a patient,

18. The method of claim 17- wherein the composition is admin istered to the patient more than once.

19. A method of preparing a therapeutic composition for use in treating a wound in a patient, comprising;

a, mixing a polyaniooie:polymer with platelet-rich plasma, serum, a fraction thereof a concentrate thereof, or platelet-rich -plasma (FRF) in which the platelets have been activated to produce a fibrin clot; and

b. mixing the PRP and polyaniouie polymer mixture with a polycationic polymer.

20. The method of -claim 19, wherein the polyanionie polymer is a sulfated or sulfamated polysaccharide, such as heparin or heparan sulfate.

21. The method of claim 19 or 20, wherein the polycatiomc polymer is a polymer composition comprising at least one moiety Selected from the. following;

(a) [-Oe (0)-CH(NHY)-CH_2-C(Q)Q.CI-b-CIi(0~R 1)-CH_2-O-CH_2-C¾-0 -C¾ - CH(O-R2)-CH_2-]$_f$.

(b) [-OC(O)-CH_2-CH(NHY)-C(O)O-CH_2-CH(O-R1)-CH_2-O-CH_2-CH_2-O-CH_2-CH(O-R2)-C¾~]«.

(c) [-OC{0)-CH(NHY)-CH2-CH$_2$-C(0)0 -CH2-CH(O-R !)-CH$_2$-0 -CH$_2$-CH$_2$-0 ~ GH$_2$-CW(0-R2}-Clfe-]«, and/or

(d) [~OC(0)-CH$_2$-CHr CH(NHY)-C(O)O-CH2-CH(O-R1)-CH$_2$-O-CH$_2$-CH$_2$-O-CH$_2$-CH(O-R2)-CH$_2$-]$_n$,

wherein Y is -C(O)-CH(NH$_3^+$)-(CH$_2$)$_4$-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-containtRg** group, a C$_{1-8}$ alkyi group, an amine-containing group, a **quaternary-** ammonium containing group, and a peptide,

22. The method **of claim** 21, wherein the **poiycationic -polymer** has a polydispersity index of less than 3.0,

23. The method of **claim** 21, wherein R1 and R2 are selected **from** the group **consisting** of Ile-Lys-Va!-Ala-Val (IKVAV) (SEQ ID NO: **4), Arg-Gly-Asp (ROD), Arg-Gly-Asp~Ser** (**RGDS**) (SEQ ID NO: 5), Ala-Gly-**Asp** (AGD), Lys-Gln-Ala-Gly-Asp~Vai (KQAGDV) (SEQ **ID** NO: 6), Val-Ala-Pro-Gly-Val-Gly (VAPGVQ) (SEQ ID **NO:** 7), **APGVGV** (SEQ ID NO: **8),** PGVGVA (SEQ ID NO: **9),VAP** , **GVGVA** (SEQ ID **NO:** 10), **VAPO** (SEQ ID NO: 1 **I), VG**VAPO (SEQ **ID** NO: 12), **VGVA** (SEQ **ID** NO: 13), **VAPGV** (SEQ ID **NO:** 14) and **GVAPGV** (SEQ ID NO: 15).

24. The method **of claim** 19, **wherein** the **.ratio** of the polycatiomc-**pdiymer** to the polyanionie polymer in the composition **results** in a neutral charge in the **coacervate.**

25. The method of claim 19, wherein the platelet-rich plasma, serum, a {taction thereof, a concentrate thereof, or platelet-rich plasma (PRP) in which the platelets have been **activated** to produce **a** fibrin clot is autologous to a patient to **be** treated.

26.: The method of claim 19, wherein the platelet-rich plasma, serum, a **traction** thereof, a concentrate thereof, or PRP in which the platelets have been activated to produce a fibrin clot PRP *in* which the platelets have been **activat e d** is **PRP** in **which** the platelets have been activated **to** produce **a** fibrin clot PRP in which the platelets have been activated that is **further** processed to remove the fibrin clot **from** the PRP prior to mixing with the **polyanionic** polymer.

27. The method of claim 26, further comprising concentrating the proteins in the PRP.

28. The method of any one **of claims** 19, iitrther comprising applying the therapeu tic **composition** to a medical device or wound dressing, optionally wherein the medical device or wound dressing is a bandage, suture, surgical mesh, limb or joint prosthesis* or a non-woven material,

29. **A. medical** device or wound dressing comprising a composition according to **claim** 1, wherein the medical device or wound dressing optionally is a bandage, **suture,** surgical mesh, limb or joint prosthesis, or a non-woven material.

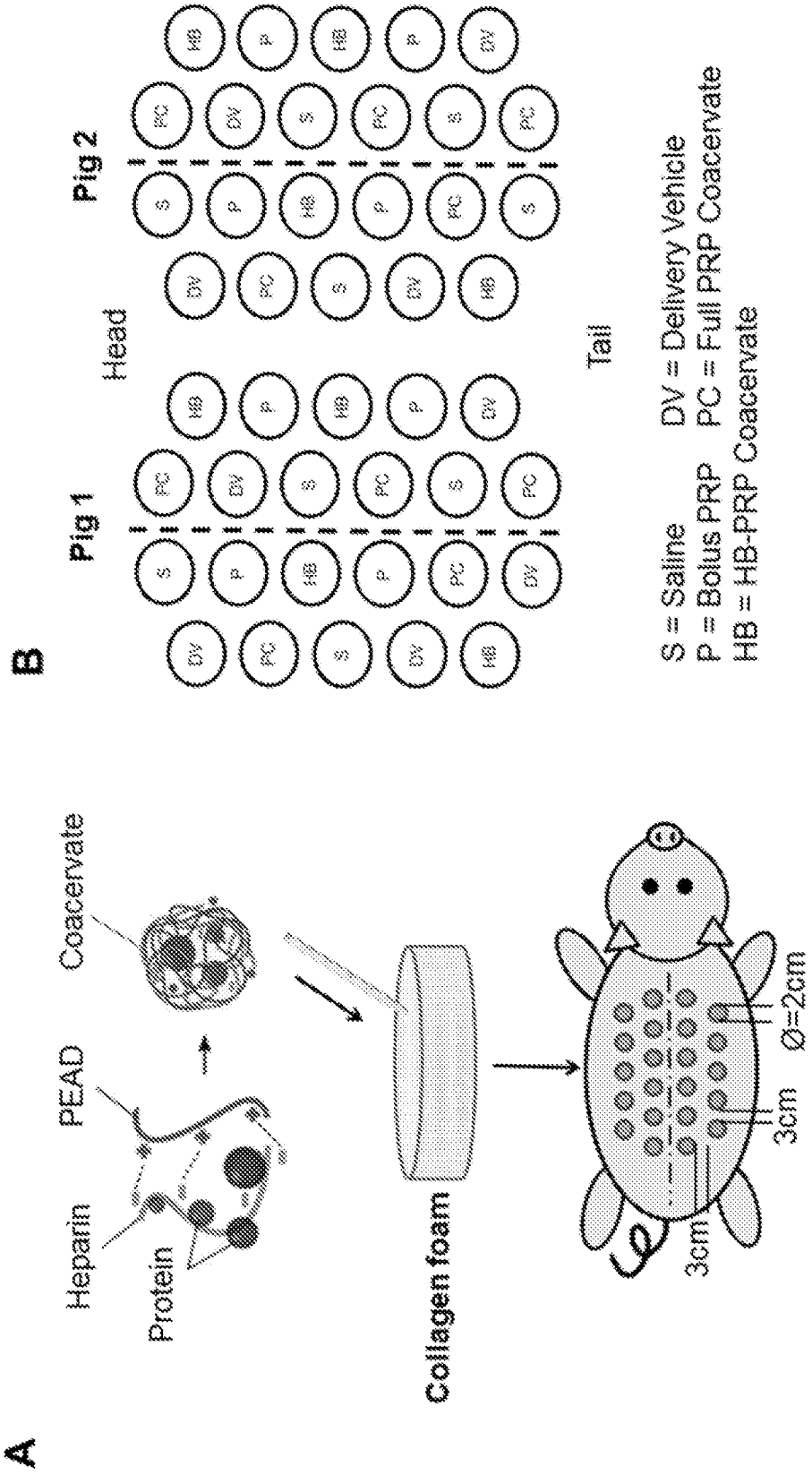

A
Heparin
PEAD
Protein
Coacervate
Collagen foam
3cm
3cm
Ø=2cm
B
Pig 1
Pig 2
Head
Tail
S = Saline
P = Bolus PRP
HB = HB-PRP Coacervate
DV = Delivery Vehicle
PC = Full PRP Coacervate

Fig. 1

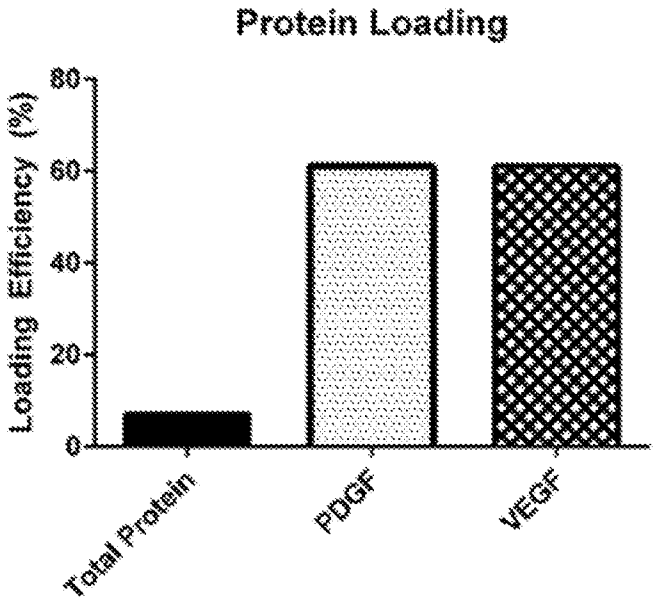

Protein Loading
Loading Efficiency (%)
80
60
40
20
0
Total Protein
PDGF
VEGF

Fig. 2A

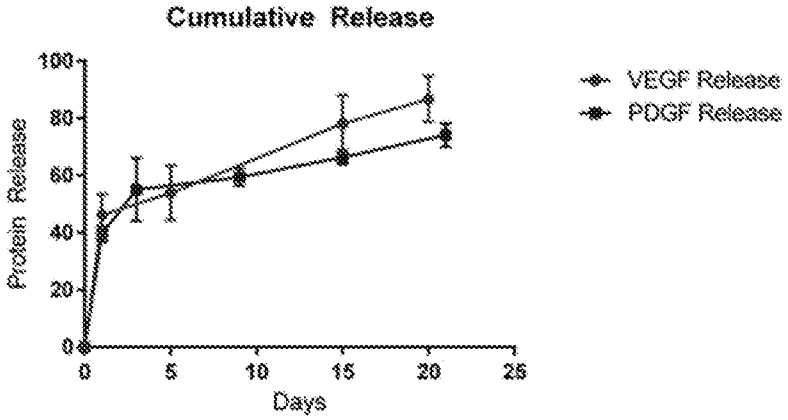

Cumulative Release
Protein Release
100
80
60
40
20
0
0
5
10
15
20
25
Days
VEGF Release
PDGF Release

Fig. 2B

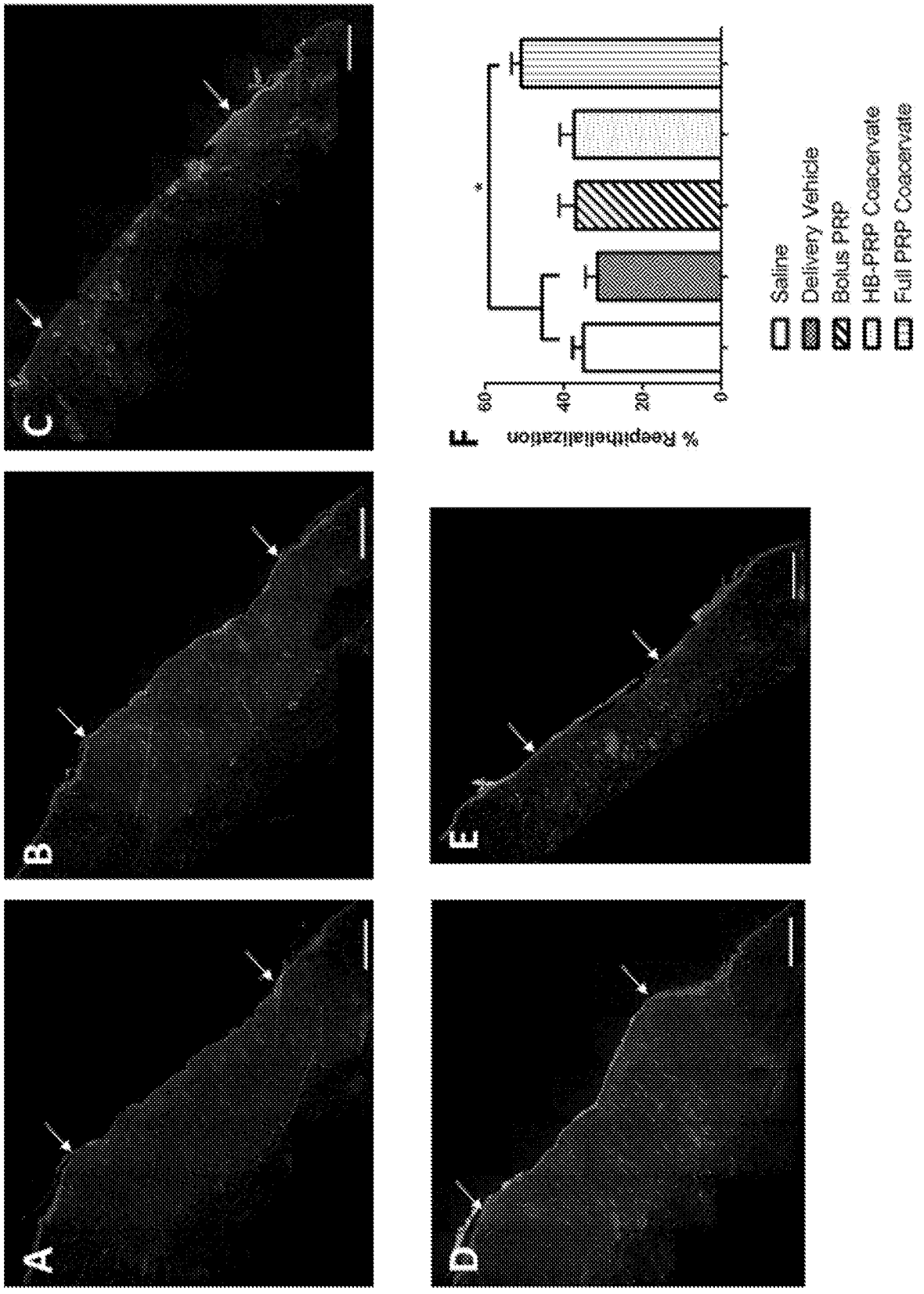

A
B
C
D
E
F
% Reepithelialization
60
40
20
0
*
Saline
Delivery Vehicle
Bolus PRP
HB-PRP Coacervate
Full PRP Coacervate

Fig. 3

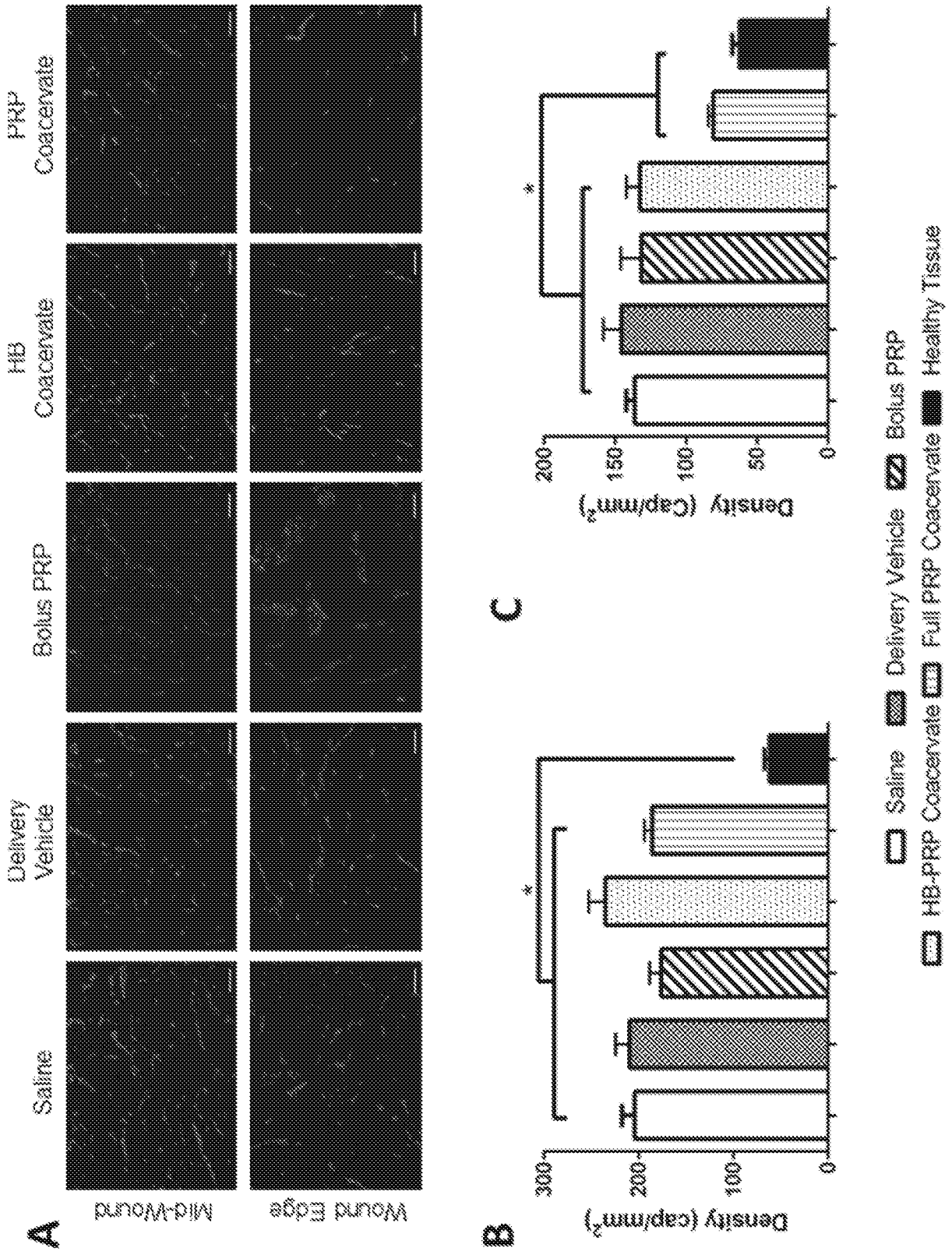

A
Saline
Delivery Vehicle
Bolus PRP
HB Coacervate
PRP Coacervate
Mid-Wound
Wound Edge
B
Density (cap/mm²)
300
200
100
0
*
C
Density (Cap/mm²)
200
150
100
50
0
*
Saline
Delivery Vehicle
Bolus PRP
HB-PRP Coacervate
Full PRP Coacervate
Healthy Tissue

Fig. 4

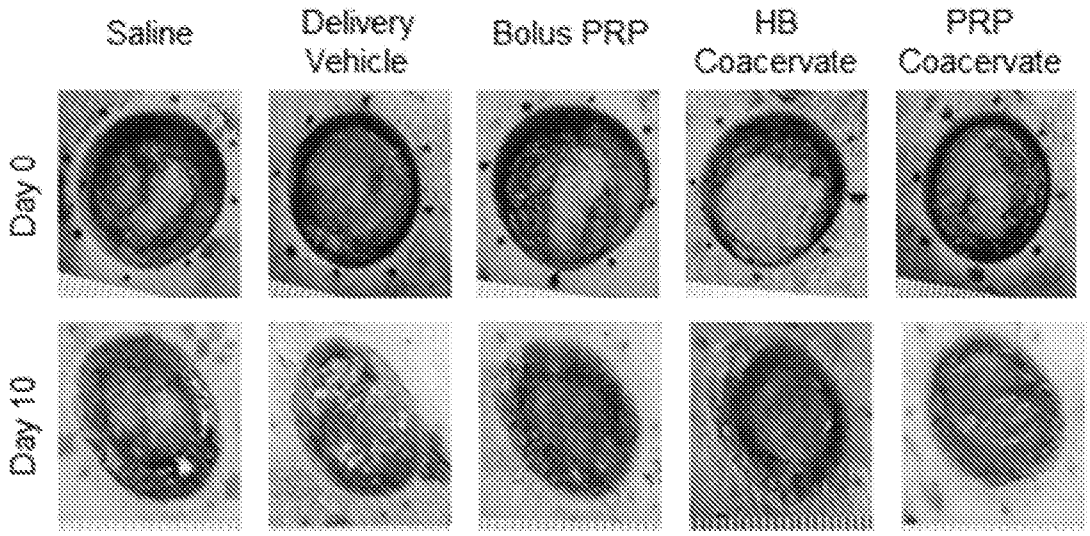

Saline
Delivery Vehicle
Bolus PRP
HB Coacervate
PRP Coacervate
Day 0
Day 10

Fig. 5A

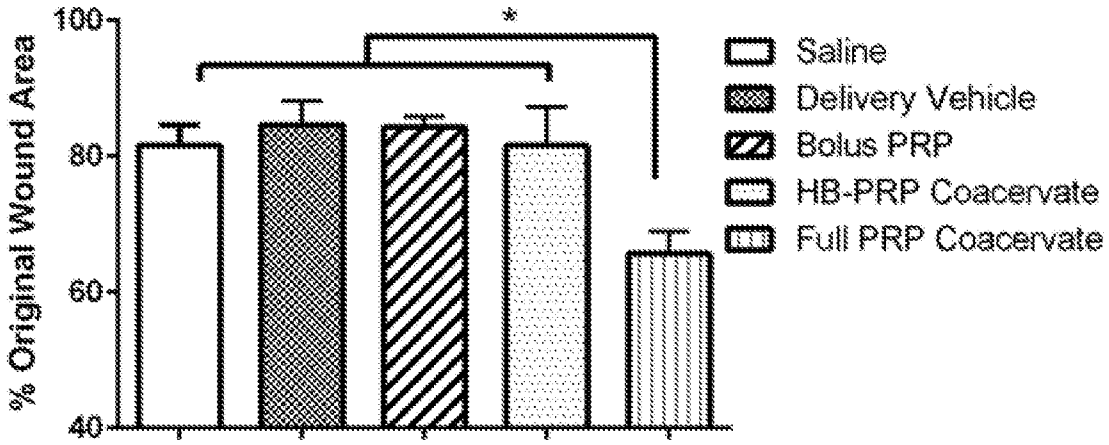
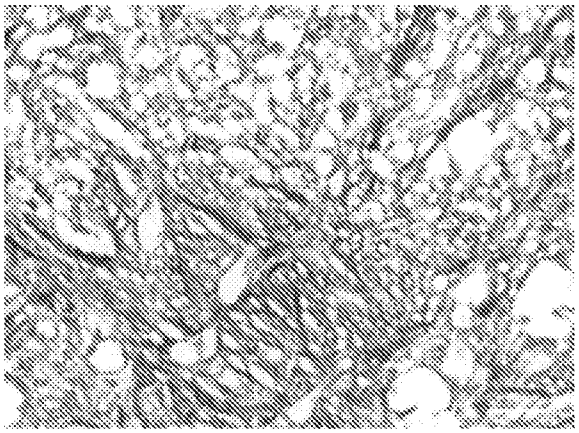
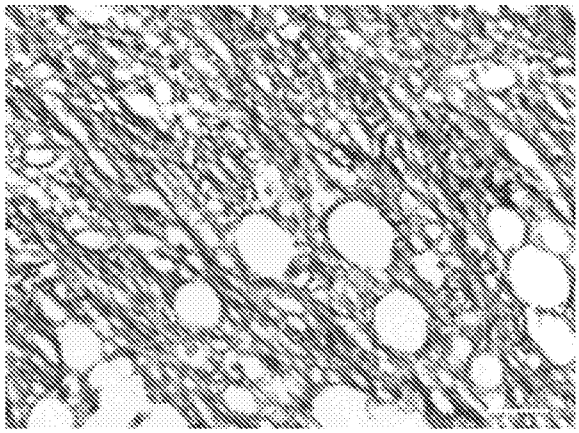

% Original Wound Area
100
80
60
40
*
Saline
Delivery Vehicle
Bolus PRP
HB-PRP Coacervate
Full PRP Coacervate

Fig. 5B

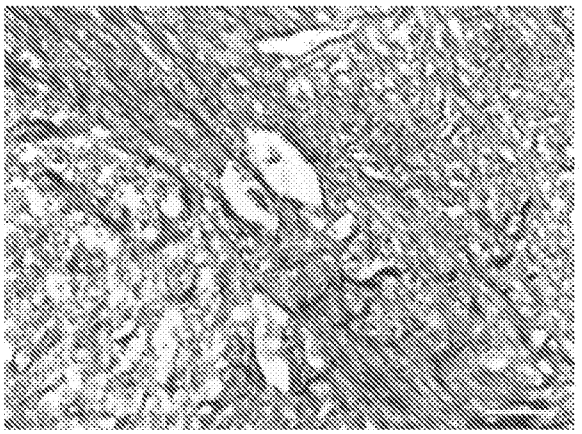
Saline



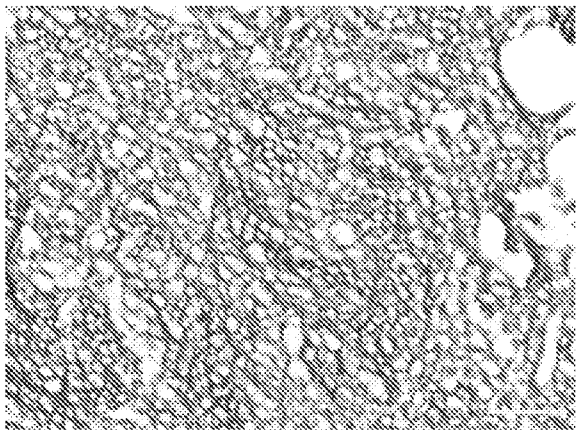
Delivery Vehicle



Bolus PRP



HB-PRP Coacervate



Full PRP Coacervate

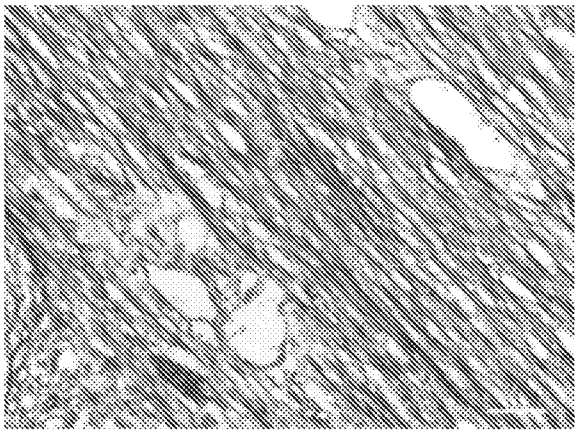
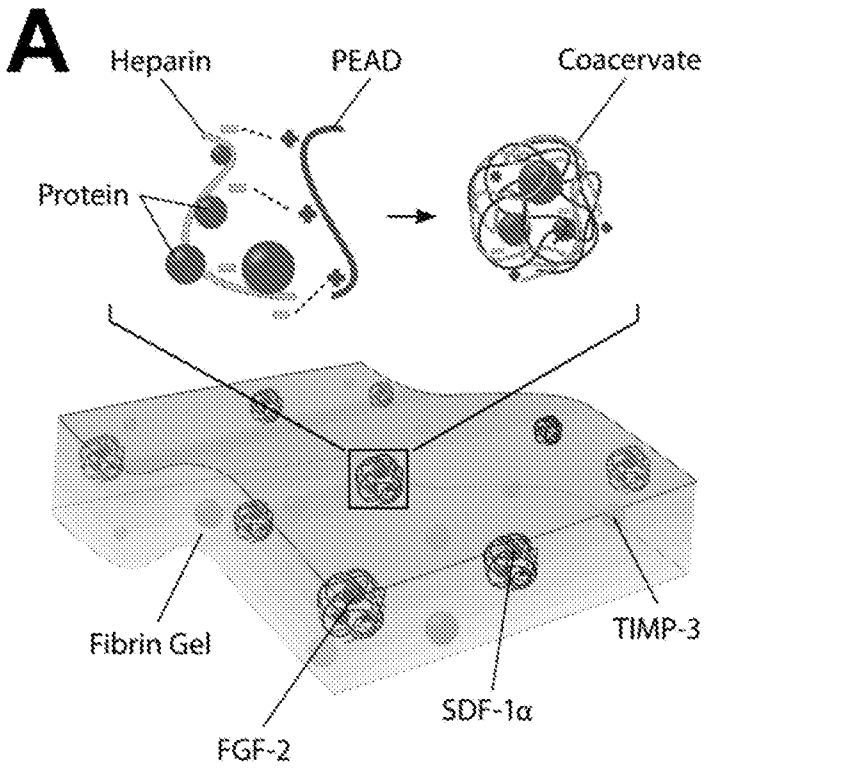


Fig. 6


A
Heparin
PEAD
Coacervate
Protein
Fibrin Gel
FGF-2
SDF-1α
TIMP-3

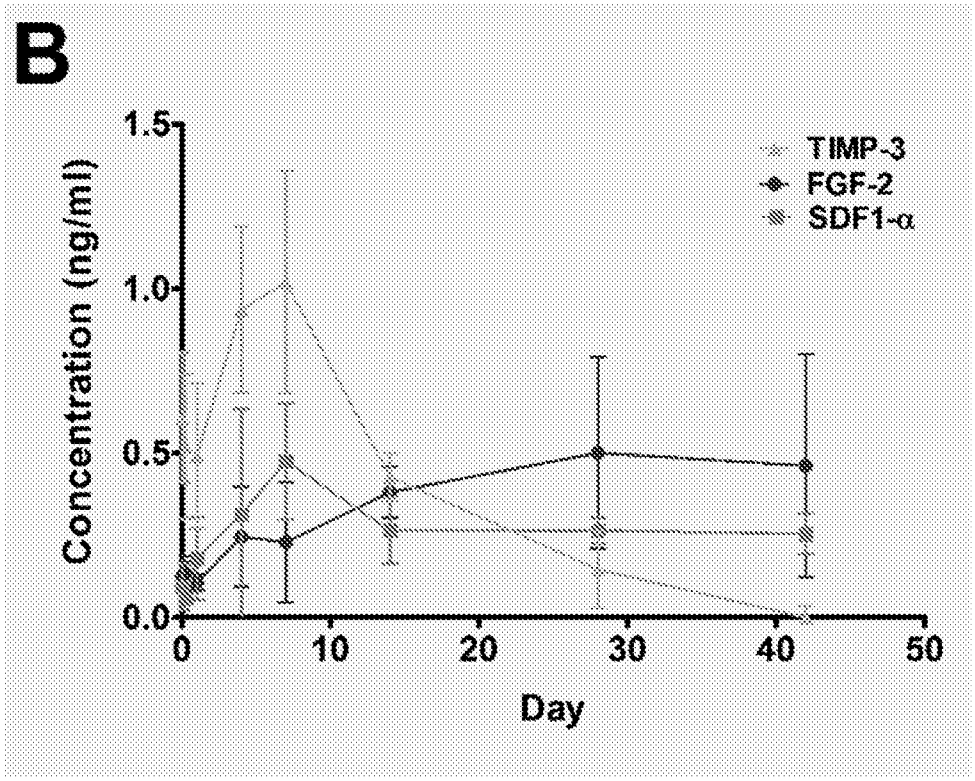

B
Concentration (ng/ml)
1.5
1.0
0.5
0.0
0
10
20
30
40
50
Day
TIMP-3
FGF-2
SDF1-α

Fig. 7

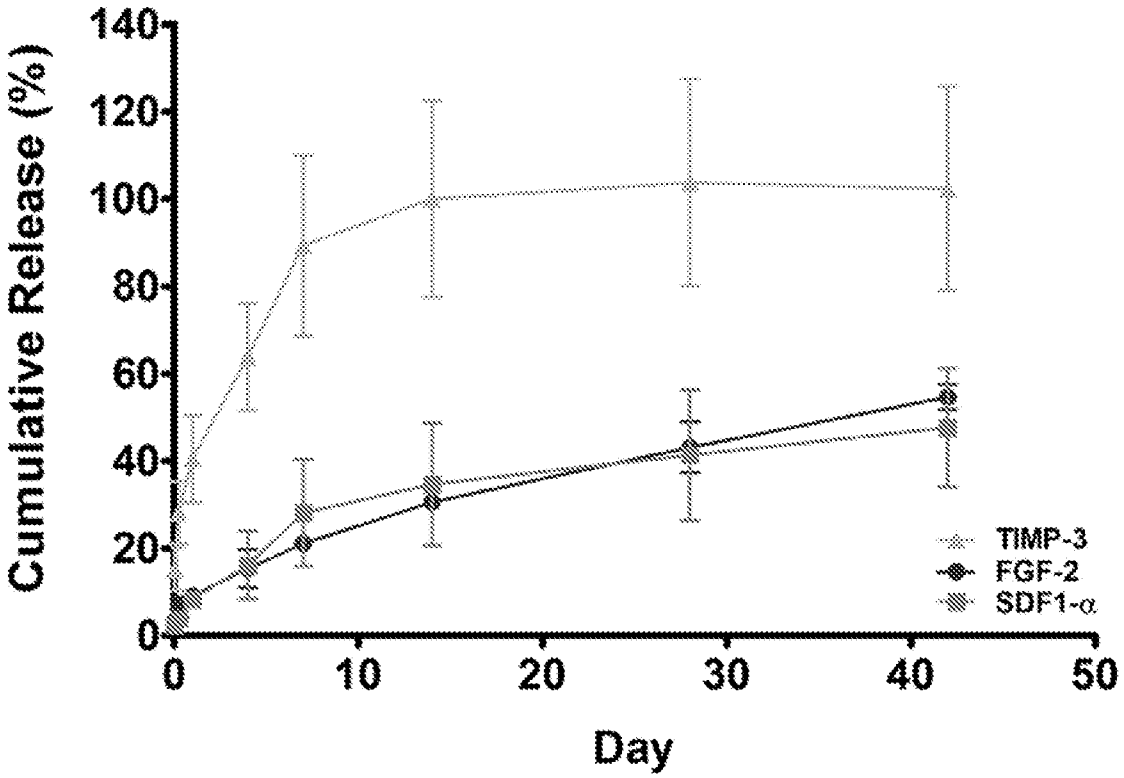

Cumulative Release (%)
140
120
100
80
60
40
20
0
0
10
20
30
40
50
Day
TIMP-3
FGF-2
SDF1-α

Fig. 8

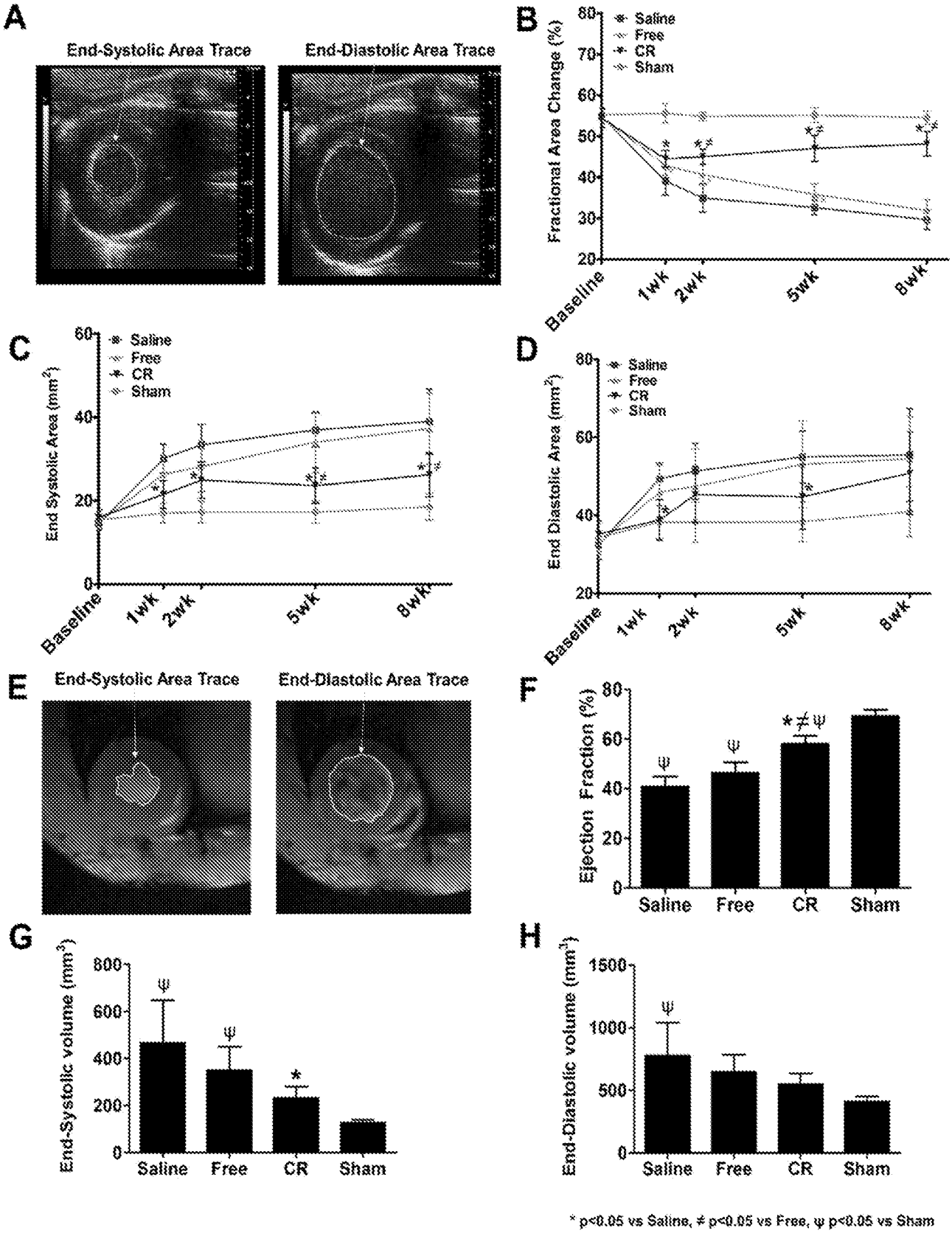

A
End-Systolic Area Trace
End-Diastolic Area Trace
B
Saline
Free
CR
Sham
Fractional Area Change (%)
Baseline
1wk
2wk
5wk
8wk
C
End Systolic Area (mm²)
D
End Diastolic Area (mm²)
E
End-Systolic Area Trace
End-Diastolic Area Trace
F
Ejection Fraction (%)
G
End-Systolic volume (mm³)
H
End-Diastolic volume (mm³)
* p<0.05 vs Saline, ≠ p<0.05 vs Free, ψ p<0.05 vs Sham

Fig. 9

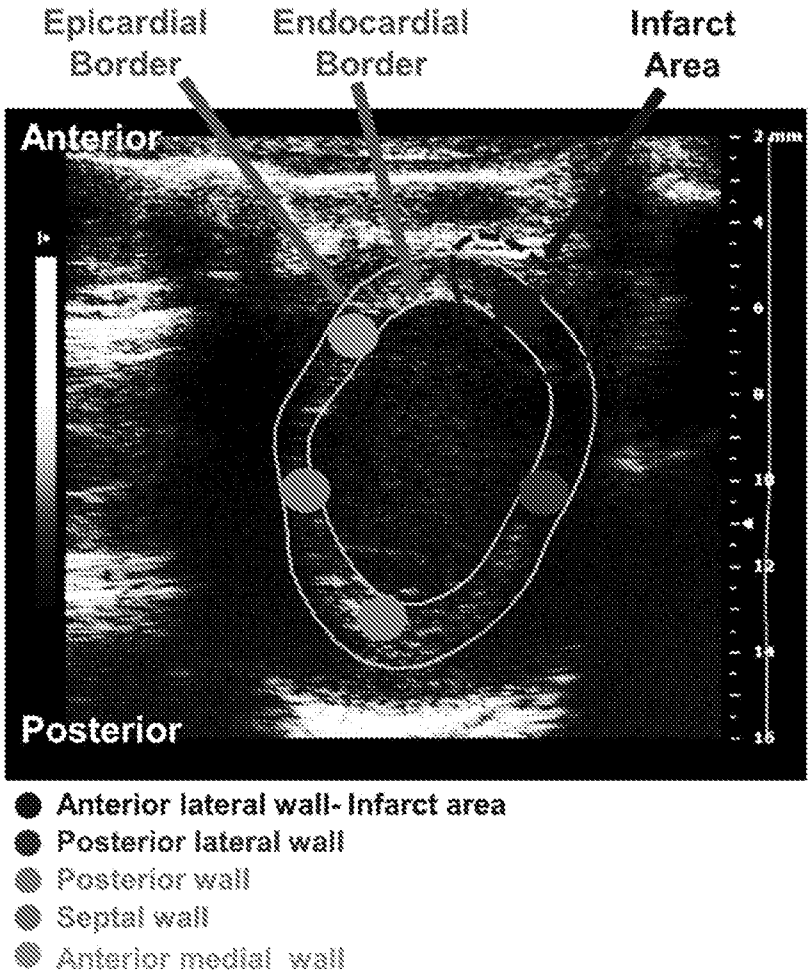
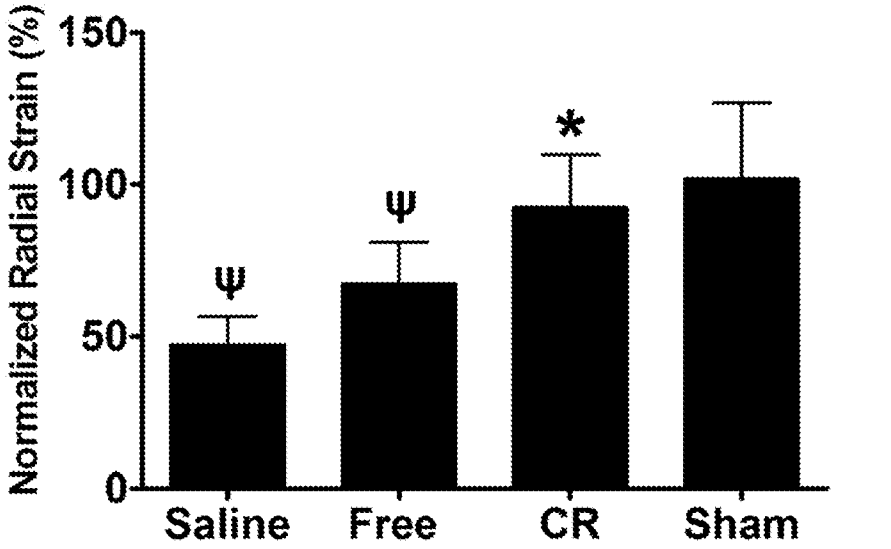


Fig. 10A



* p<0.05 vs Saline, ψ p<0.05 vs Sham

Fig. 10B

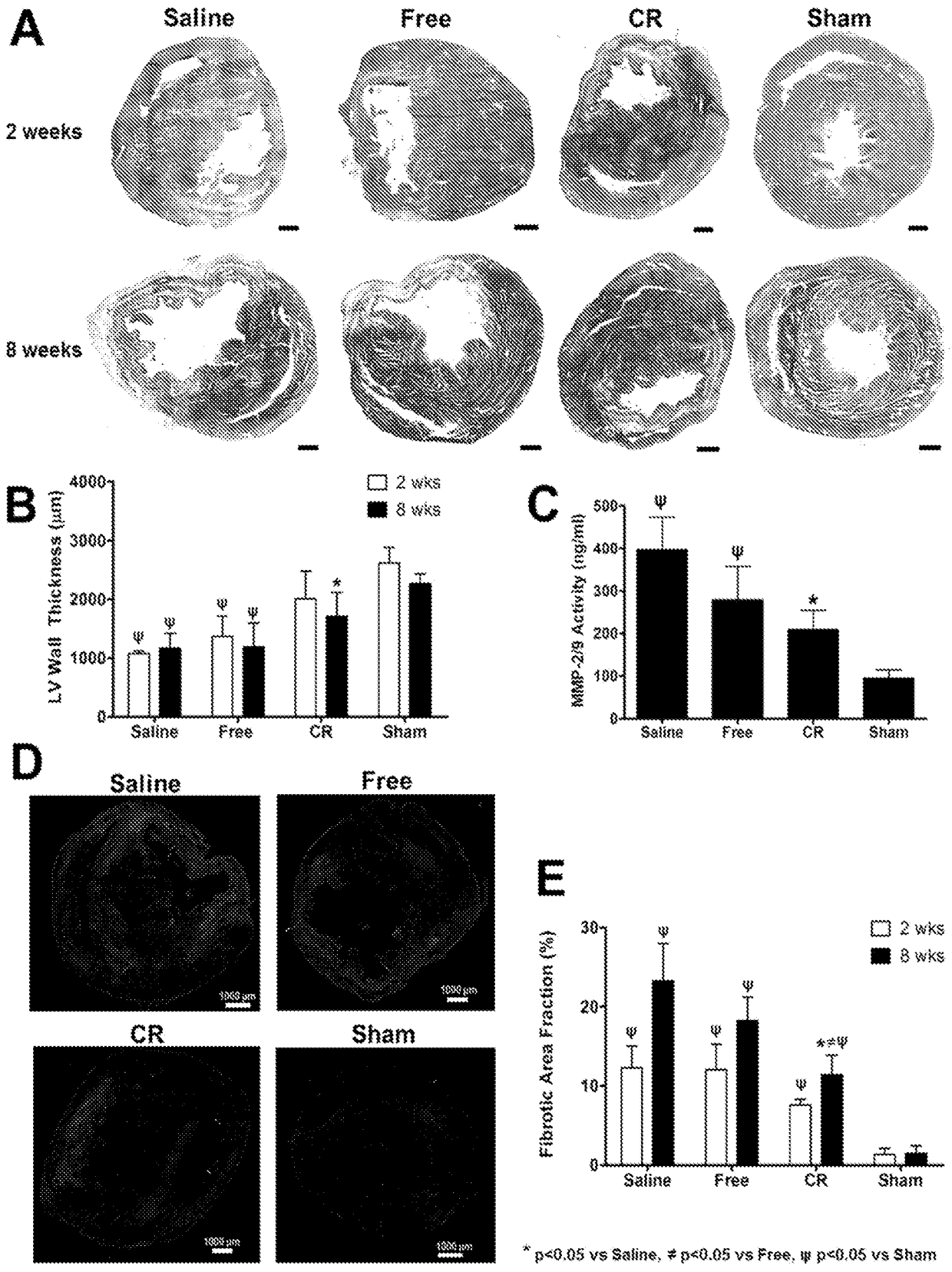

A
Saline
Free
CR
Sham
2 weeks
8 weeks
B
LV Wall Thickness (μm)
4000
3000
2000
1000
0
2 wks
8 wks
Saline
Free
CR
Sham
C
MMP-2/9 Activity (ng/ml)
500
400
300
200
100
0
D
Saline
Free
CR
Sham
E
Fibrotic Area Fraction (%)
30
20
10
0
2 wks
8 wks
* p<0.05 vs Saline, ≠ p<0.05 vs Free, ψ p<0.05 vs Sham

Fig. 11

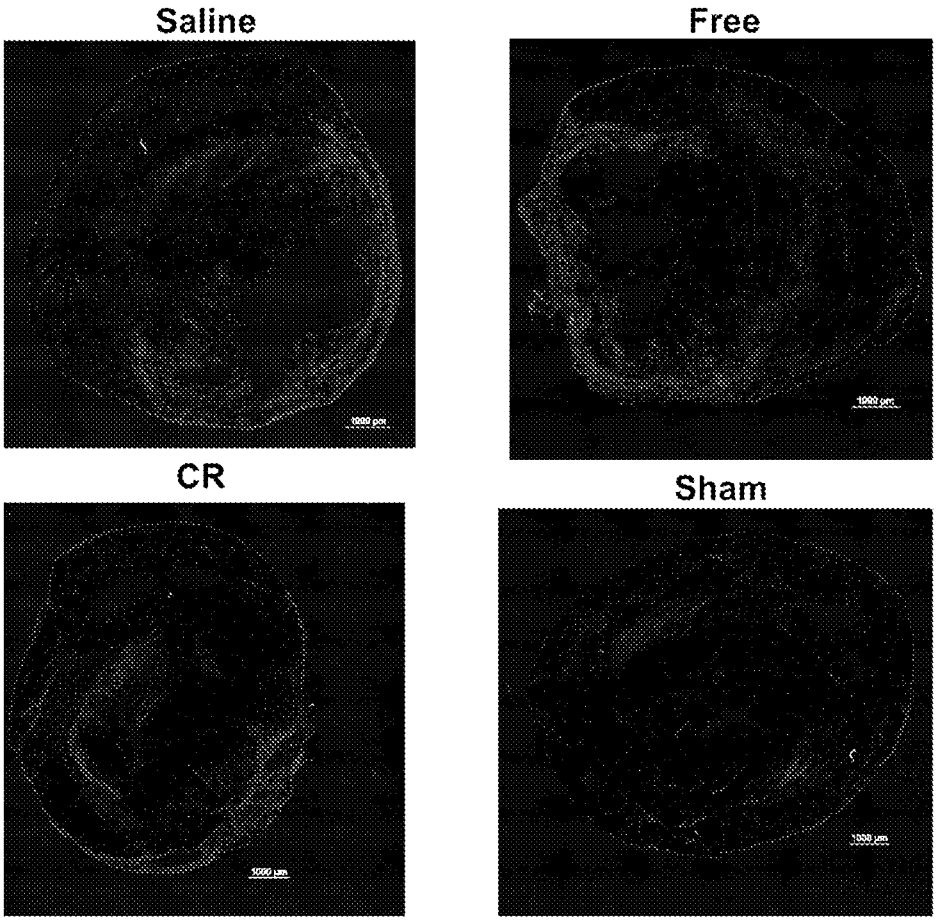


Fig. 12

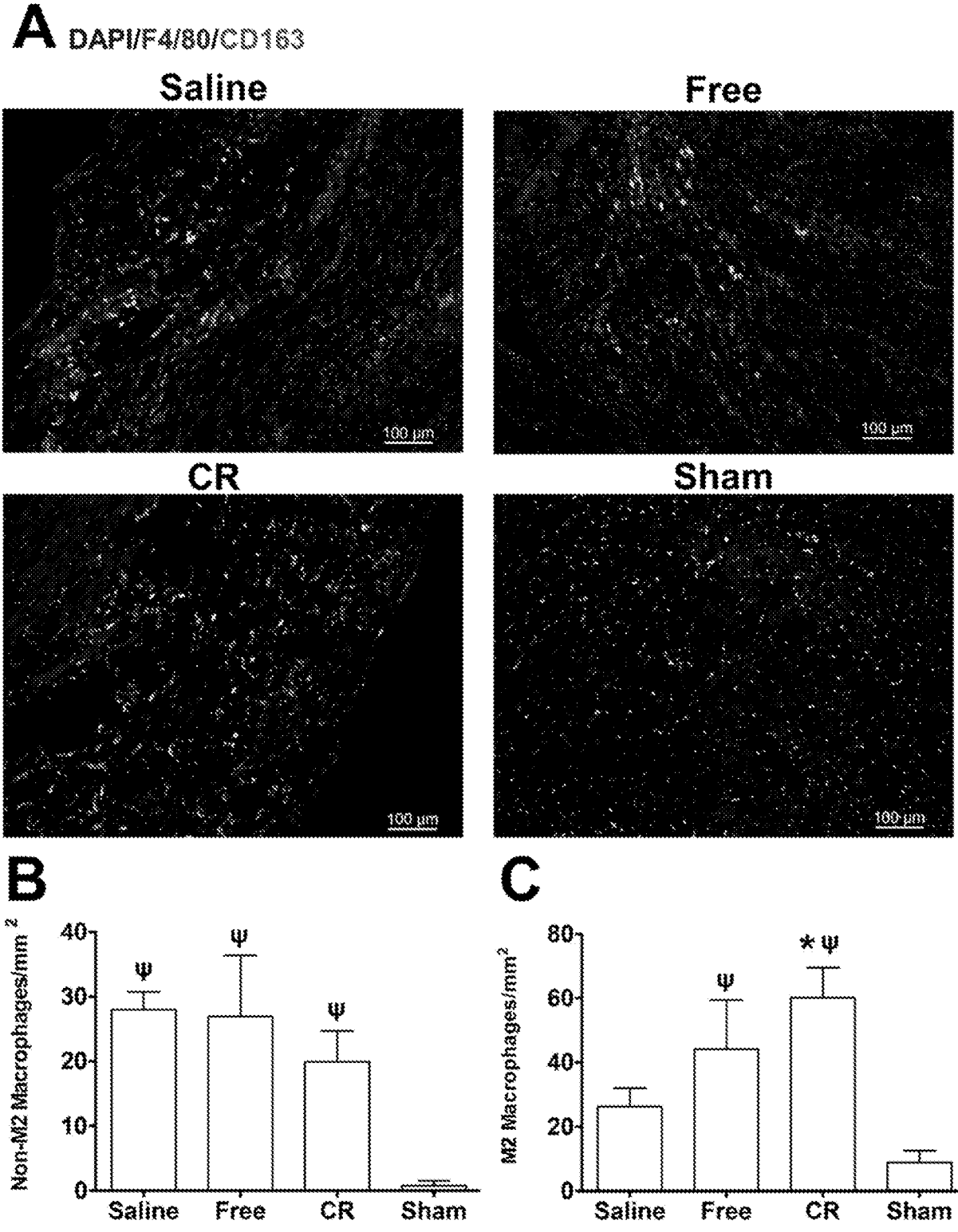
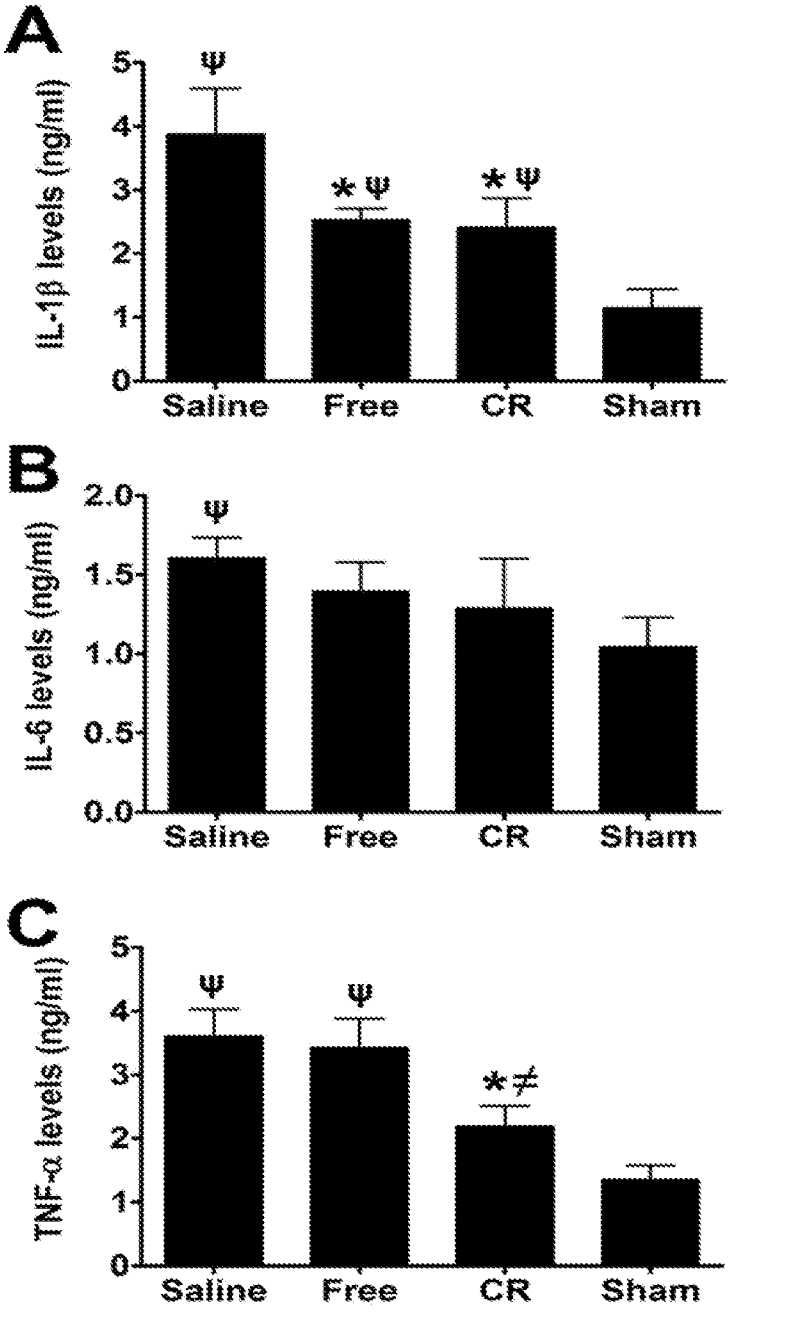

A DAPI/F4/80/CD163
Saline
Free
CR
Sham
100 μm
B
Non-M2 Macrophages/mm 2
40
30
20
10
0
ψ
ψ
ψ
Saline
Free
CR
Sham
C
M2 Macrophages/mm2
80
60
40
20
0
* ψ
ψ
Saline
Free
CR
Sham
* p<0.05 vs Saline, ψ p<0.05 vs Sham

Fig. 13



* p<0.05 vs Saline, ≠ p<0.05 vs Free, ψ p<0.05 vs Sham

Fig. 14

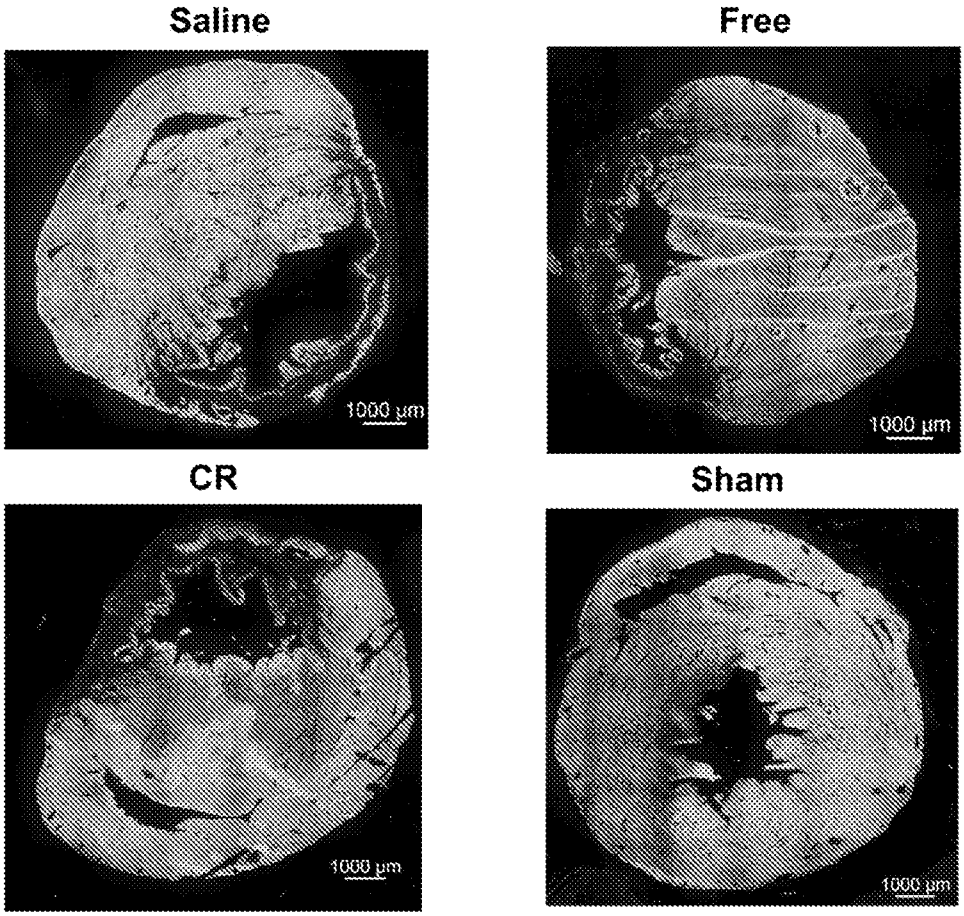
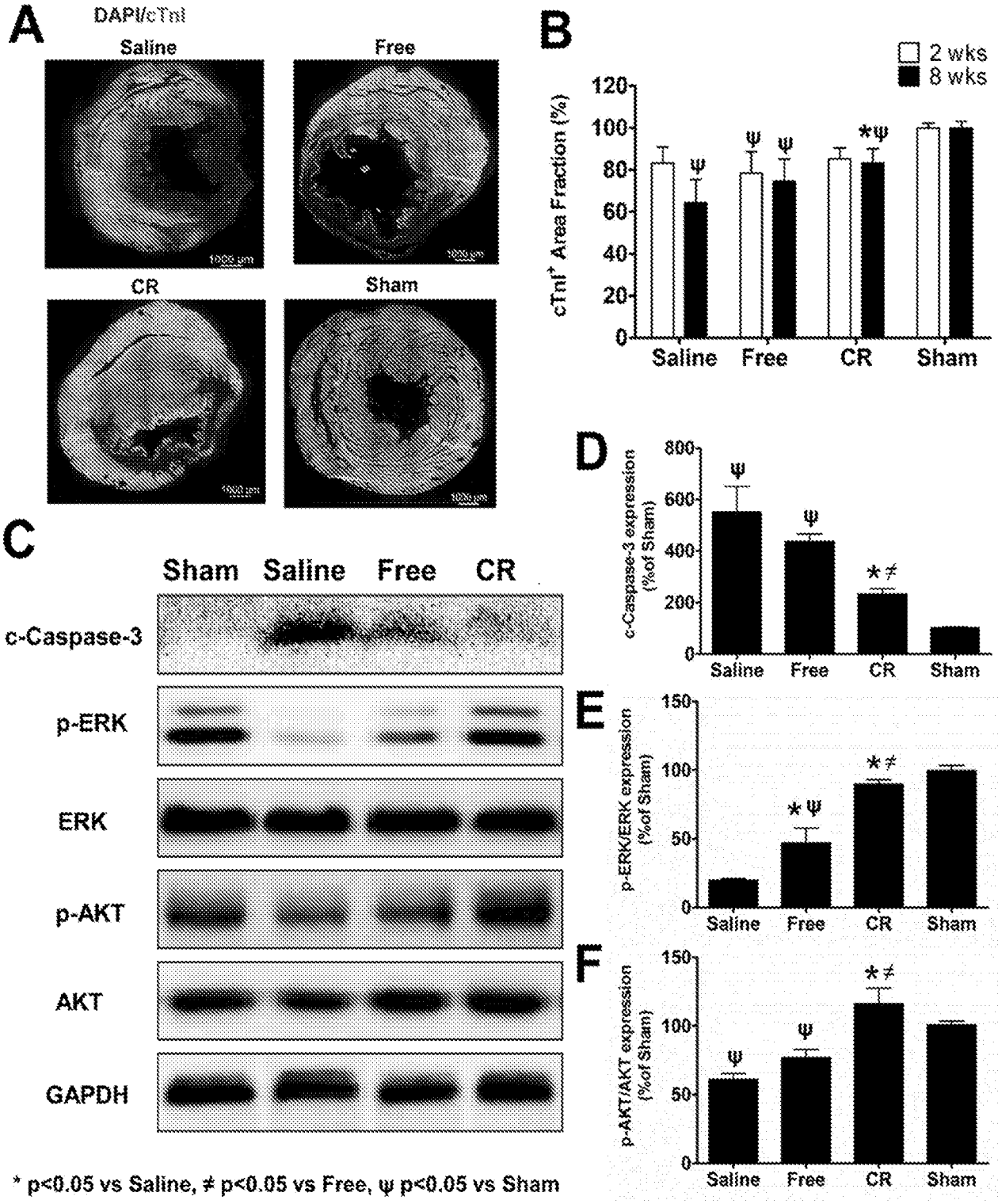


Fig. 15



* p<0.05 vs Saline, ≠ p<0.05 vs Free, ψ p<0.05 vs Sham

Fig. 16

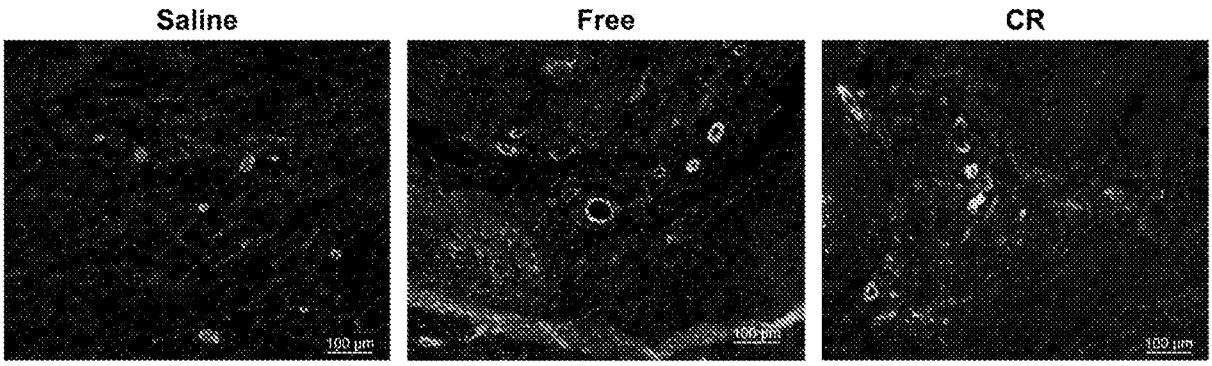
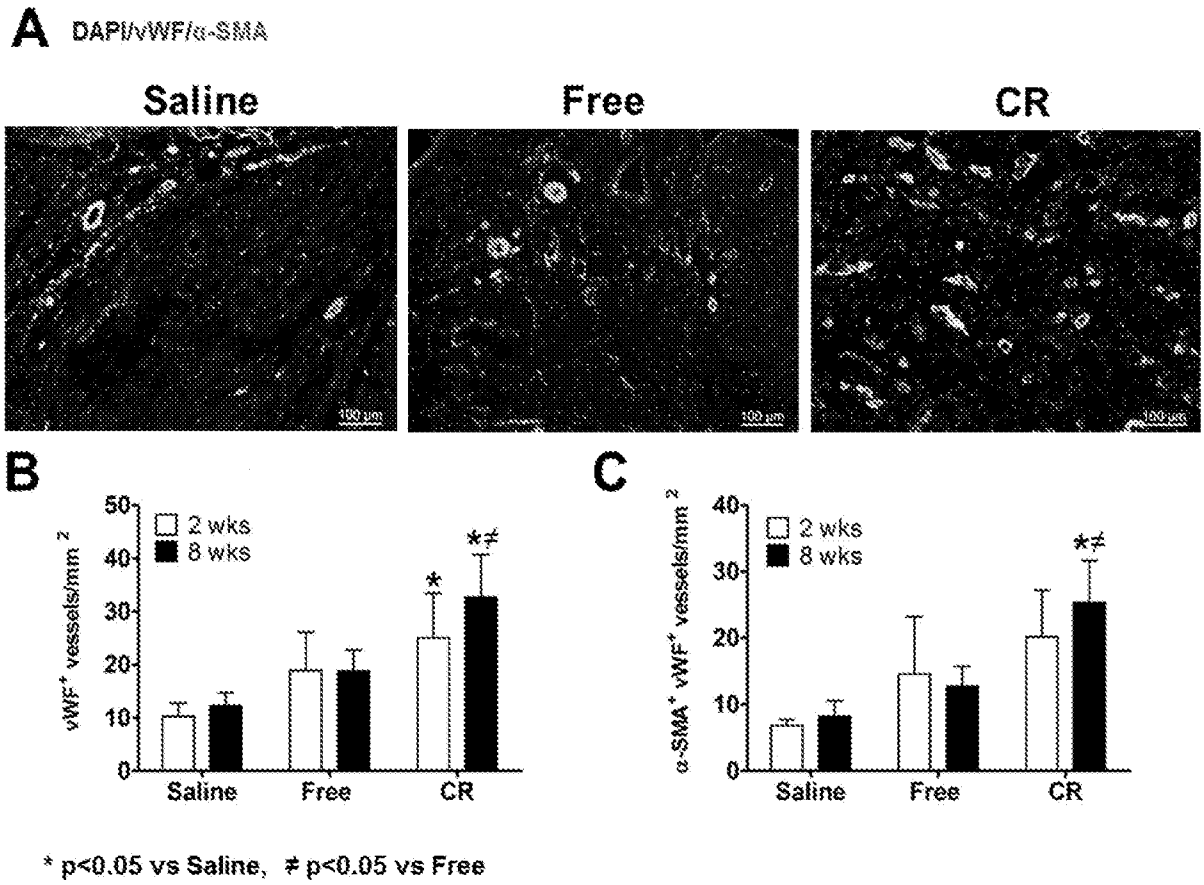


Fig. 17



* p<0.05 vs Saline, ≠ p<0.05 vs Free

Fig. 18

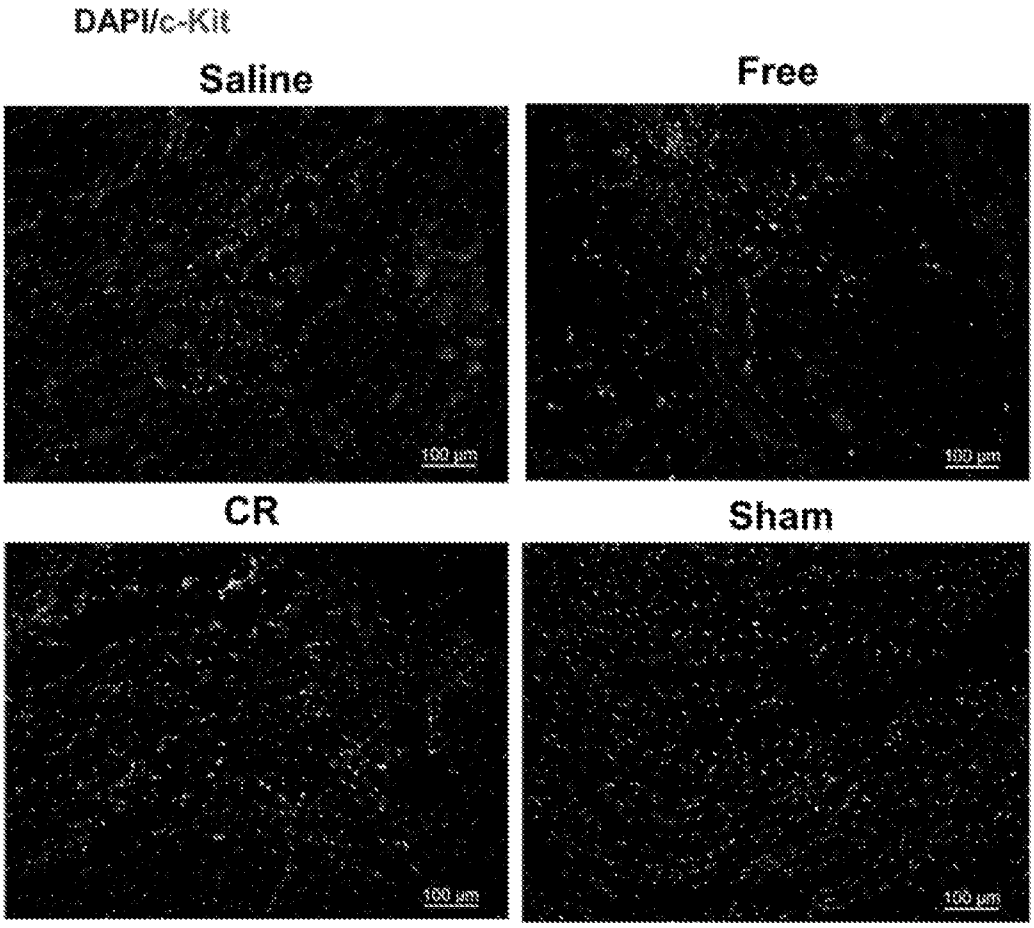
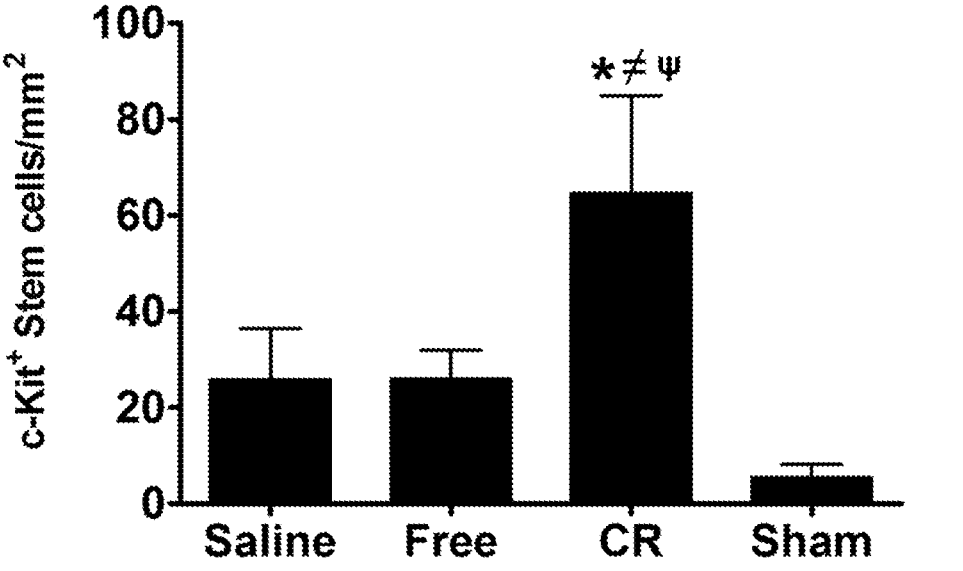
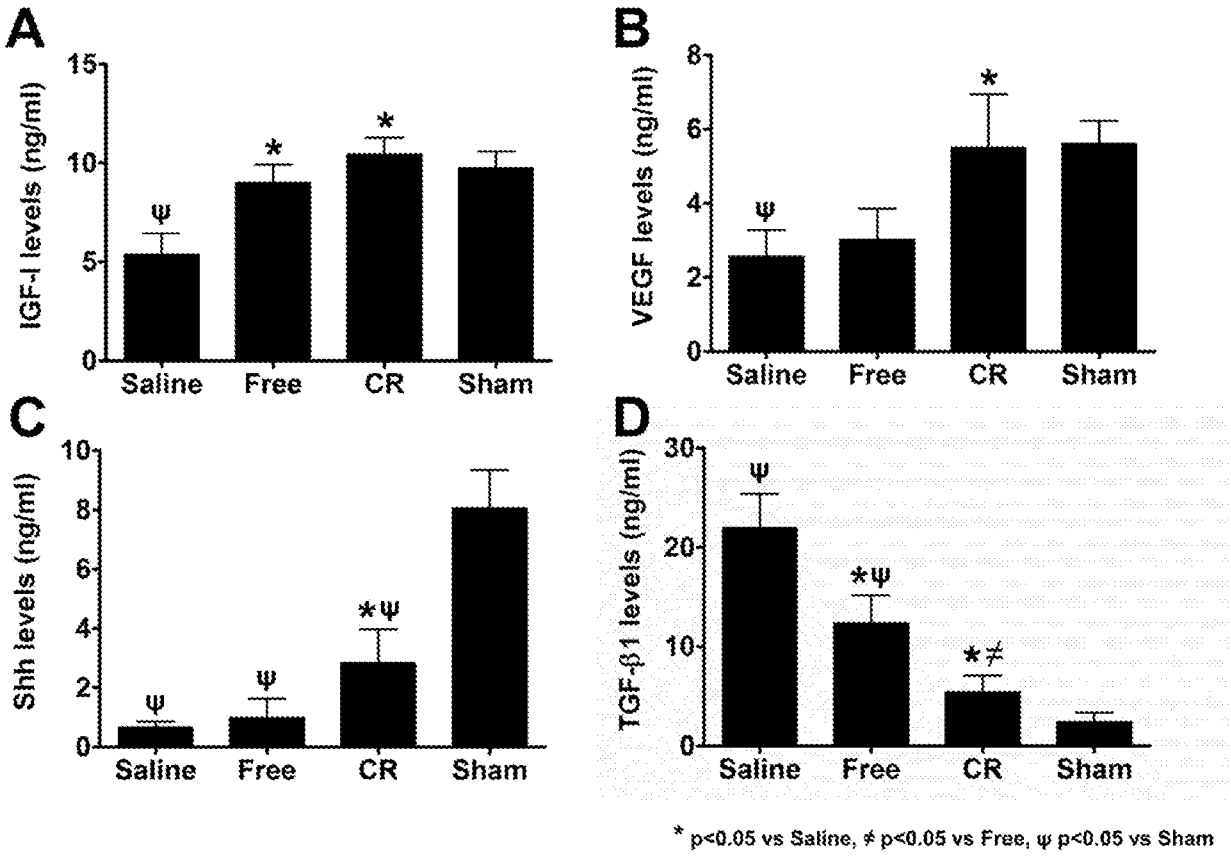


Fig. 19A



* p<0.05 vs Saline, ≠ p<0.05 vs Free, ψ p<0.05 vs Sham

Fig. 19B



* p<0.05 vs Saline, ≠ p<0.05 vs Free, ψ p<0.05 vs Sham

Fig. 20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/020642

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.: 16-18
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 16-18 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.:
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

International application No.
PCT/US2017/020642

A. CLASSIFICATION OF SUBJECT MATTER

A61K 9/10(2006.01)i, A61K 9/06(2006.01)i, A61K 47/30(2006.01)i, A61K 35/19(2014.01)i, A61L 15/22(2006.01)i, A61L 15/44(2006.01)i, A61L 15/26(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 47/34; A61K 47/42; A61K 38/57; C08G 63/00; A61K 9/06; A61K 47/30; A61K 35/19; A61L 15/22; A61L 15/44; A61L 15/26

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: coacervate, PEAD, heparin, platelet rich plasma, TMP-3, FGF-2, SDF-Iα, myocardial infarction

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AWADA, H. K. et al., 'Sequential delivery of angiogenic growth factors improves revascularization and heart function after myocardial infarction' Journal of Controlled Release, 2015, Vol.207, pages 7-17 See abstract; page 8; Fig. 1.	1-7, 9-11, 13, 19-22, 24-29
Y		8, 12, 14-15, 23
Y	US 9023972 B2 (CHU, H. et al.) 05 May 2015 See abstract; claims 1, 7.	12, 23
Y	US 2014-0148395 A1 (BURDICK, J. A. et al.) 29 May 2014 See paragraph [0033]; claims 1-3, 6, 10, 15.	8, 14-15
A	YANG, H. S. et al., 'Enhanced skin wound healing by a sustained release of growth factors contained in platelet-rich plasma' Experimental and Molecular Medicine, 2011, Vol.43, No.11, pages 622-629 See the whole document.	1-15, 19-29
A	KISHIMOTO, S. et al., 'Novel experimental and clinical therapeutic uses of low-molecular-weight heparin/protamine micropartícles' Pharmaceutics, 2012, Vol.4, pages 42-57 See the whole document.	1-15, 19-29

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

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
05 June 2017 (05.06.2017)	**07 June 2017 (07.06.2017)**

Name and mailing address of the ISA/KR
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/020642

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	AWADA, H. K. et al., 'Factorial design of experiments to optimize multiple protein delivery for cardiac repair' ACS Biomaterials Science and Engineering, 18 April 2016, Vol.2, pages 879-886 See abstract ; Figures 1-2.	1-11, 13-15, 19-22, 24-29
PX	AWADA, H. K. et al., 'A single injection of protein-loaded coacervate-gel significantly improves cardiac function post infarction' Biomaterials, E-pub. 17 February 2017, Vol.125, pages 65-80 See abstract ; Fig. 1.	1-11, 13-15, 19-22, 24-29

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2017/020642

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
		US 2015-0307655 Al	29/10/2015
		¥0 2011-091411 A2	28/07/2011
		¥0 2011-091411 A3	22/12/2011
US 2014-0148395 Al	29/05/2014	¥0 2011-163069 A2	29/12/2011
		¥0 2011-163069 A3	22/03/2012