



(51) International Patent Classification:

A61F 2/00 (2006.01) *A61F 13/00* (2006.01)
A61J 3/00 (2006.01) *A61K 47/48* (2006.01)

(21) International Application Number:

PCT/US2014/049774

(22) International Filing Date:

5 August 2014 (05.08.2014)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

61/862,297 5 August 2013 (05.08.2013) US

(71) Applicant (for all designated States except US): **UNIVERSITY OF ROCHESTER** [US/US]; 601 Elmwood Avenue, Box URV, Rochester, NY 14642 (US).

(72) Inventors; and

(71) Applicants (for US only): **HOVE, Amay, Van** [US/US]; 242 Oxford Street, #2, Rochester, NY 14607 (US). **BEN-OIT, Danielle** [US/US]; 130 Sunset Drive, Rochester, NY 14618 (US).(74) Agents: **HAAS, Dennis** et al.; Riverside Law, LLP, 300 Four Falls Corporate Center, Suite 710, 300 Conshohocken State Road, West Conshohocken, PA 19428 (US).

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

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

Published:

- with international search report (Art. 21(3))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: COMPOSITIONS AND METHODS FOR STIMULI-RESPONSIVE RELEASE OF A THERAPEUTIC AGENT

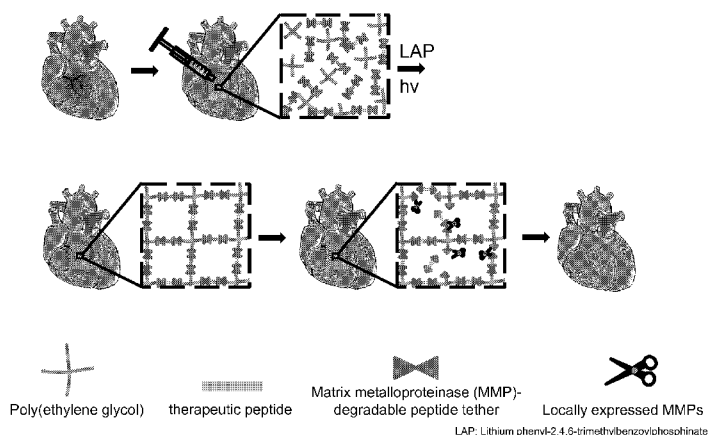


Figure 1

(57) Abstract: Compositions and methods for stimuli-responsive treatment are described. For example, in certain embodiments, the compositions and methods provide for the stimuli-responsive release of a therapeutic agent, wherein the release is dependent upon the local environment of a treatment site. In one embodiment, the composition comprises a prodrug comprising at least one therapeutic domain and at least one cleavage domain, such that stimuli-induced cleavage releases a therapeutic agent.

COMPOSITIONS AND METHODS FOR STIMULI-RESPONSIVE RELEASE OF A THERAPEUTIC AGENT

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application claims priority to U.S. Provisional Patent Application No. 61/862,297 filed August 5, 2013, the contents of which are incorporated by reference herein in their entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR 10 DEVELOPMENT

 This invention was made with government support under R01 AR064200, awarded by the National Institutes of Health (NIH). The government has certain rights in the invention.

15 BACKGROUND

 Peptide drugs have been identified for a variety of applications including to promote vascularization (Finetti et al., 2012, Biochem Pharm., 84(3):303-11; Santulli et al., 2009, J of Trans Med. 7:41), reduce inflammation (Akeson et al., 1996, J Biol Chem. 271(48):30517-23; Schultz et al., 2005, Biomaterials 26(15):2621-30), and as
20 cancer therapeutics (Yang et al., 2003, Cancer Res. 63(4):831-7; Selivanova et al., Nat Med. 1997, 3(6):632-8). Peptides typically mimic the bioactivity of larger proteins or growth factors, and offer many advantages over traditional protein delivery. Due to small sizes, peptides can be produced synthetically and delivered at concentrations higher than whole proteins while maintaining high specificity to targets, resulting in high potencies of
25 action and relatively few off-target effects. Additionally, some peptides often do not require complex tertiary structures for bioactivity, resulting in lower susceptibility to denaturation and degradation *in vivo* (Finetti et al., 2012, Biochem Pharm., 84(3):303-11). As peptides often do not fully recapitulate protein bioactivities, some reports indicate peptides may need to be delivered at higher doses to achieve therapeutic results (Ben-
30 Sasson et al., 2003, Blood. 102(6):2099-107), while other studies indicate that at equivalent molar doses of peptides and proteins offer similar bioactivities (Santulli et al.,

2009, J of Trans Med. 7:41). Similar to proteins, however, peptides suffer from rapid clearance and poor pharmacokinetics, thus motivating the development of controlled release systems (Craik et al., 2013, Chem Biol Drug Des. 81(1):136-47).

Numerous methods for systemic delivery of peptide drugs have been
5 developed, including the use of liposomes (Camelo et al., 2007, Mol Vis. 13(256):2263-74), poly(lactide-co-glycolide) (PLGA) microparticles (Aguado et al., 1992 Immunobiology 184(2-3):113-25) and chitosan nanoparticles (Kim et al., 2008, Biomaterials. 29(12):1920-30). However, these drug delivery systems do not target specific tissues, and methods to achieve localized peptide delivery are poorly developed
10 (Du et al., 2014, Biomacromolecules 15(4):1097-114). Bolus injection of peptides is often used (Hardy et al., 2008, Biochem Pharm. 75(4):891-9; Hardy et al., 2007, Peptides. 28(3):691-701), necessitating repeat injections to achieve longitudinal therapeutic concentrations. Osmotic pumps can be used to achieve prolonged peptide delivery (Santulli et al., 2009, J of Trans Med. 7:41); however, the pump must be
15 removed after it has delivered its payload, resulting in revision surgery to remove the device. Diffusion-mediated peptide release can be achieved using polymers such as Hydron (Ben-Sasson et al., 2003, Blood. 102(6):2099-107; Failla et al., 2008, Blood. 111(7):3479-88) or hydrogels such as ReGel (PLGA-b-PEG-b-PLGA) (Choi et al., 2004, Pharm Res. 21(5):827-31), pluronics (Santulli et al., 2009, J of Trans Med. 7:41) or
20 Matrigel (Van Slyke et al., 2009, Tissue Eng Pt A. 15(6):1269-80). However, delivery using these approaches occurs over pre-dictated, and not necessarily therapeutically-relevant, timeframes. To more closely meet tissue demands, stimuli-responsive drug delivery can be dictated by the local tissue microenvironment (Phelps et al., 2010, P Natl Acad Sci USA 107(8):3323-8). However, stimuli-responsive materials have not yet been
25 developed for peptide drug delivery (Du et al., 2014, Biomacromolecules 15(4):1097-114). The fields of tissue engineering and regenerative medicine have made amazing strides towards *de novo* tissue/organ formation. However, there is one critical factor hindering this progress: vascularization of new tissue. Additionally, a plethora of ischemic tissue disorders exist which could benefit from localized pro-angiogenic drug
30 delivery. One such disorder is cardiac ischemia, the leading cause of death worldwide in 2008, killing more than 7.2 million people (Mathers and Loncar, 2006, PLoS Medicine,

3(11)). Cardiac ischemia often occurs as a result of myocardial infarction, and current treatments are unable to adequately repair ischemic cardiac tissue, with up to one-third of patients ultimately developing heart failure. Other ischemic tissue disorders, which could benefit from localized pro-angiogenic drug delivery, including peripheral vascular
5 ischemia and diabetic ulcers, are characterized in part by a decrease in angiogenesis seen at the wound site.

Commonly, pro-angiogenic proteins, such as vascular endothelial growth factor (VEGF) or platelet derived growth factor (PDGF), are delivered via simple injection into tissue. However, incomplete recovery is observed using such approaches.

10 For example, this approach is limited as proteins have poor stability *in vivo* and simple injection is not sufficient to locally deliver relevant protein concentrations over therapeutic timescales. Treatments with large biomolecules such as VEGF are difficult as they require the protein to have a specific 3D structure to remain bioactive. Additionally, they have a high production cost as the proteins must be grown up in *E. coli.* or yeast. A
15 further limitation is the extended delivery of these biomolecules required to achieve stable vessel formation.

Thus, there is a need in the art for improved compositions and methods for effective drug delivery. The present disclosure satisfies this unmet need.

20

SUMMARY

In one aspect, provided herein is a composition comprising a plurality of synthetic monomers, and a plurality of prodrugs. In one embodiment, each prodrug comprises at least one therapeutic domain and one or more cleavable domains, wherein the cleavable domains couples the prodrug to one or more of the plurality of synthetic
25 monomers. In one embodiment, the ratio of prodrug to synthetic monomer in the composition is greater than about 1:1. In one embodiment, the ratio of prodrug to synthetic monomer in the composition is greater than about 2:1.

In one embodiment, the prodrug comprises a therapeutic peptide coupled to a cleavable peptide sequence at its amino terminus and a cleavable peptide sequence at
30 its carboxy terminus.

In one embodiment, the synthetic monomer comprise poly(ethylene glycol) (PEG). In one embodiment, the PEG is functionalized with terminal norbornene groups.

5 In one embodiment, the composition is a polymerized matrix. In one embodiment, polymerization of the matrix is induced by UV light. In one embodiment, the composition is a hydrogel. In one embodiment, the composition is a solution.

10 In one embodiment, the at least one cleavage domain comprises a matrix metalloproteinase (MMP) -sensitive cleavage domain. In one embodiment, the MMP-sensitive cleavage domain comprises the amino acid sequence of SEQ ID NO: 1. In one embodiment, an increased expression, activity, or combination thereof, of a MMP induces cleavage of the MMP-sensitive cleavage domain.

15 In one embodiment, the therapeutic domain comprises a pro-angiogenic amino acid sequence. In one embodiment, the therapeutic domain comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 5, and SEQ ID NO: 6.

20 In one embodiment, cleavage of the cleavage domains releases a released therapeutic agent. In one embodiment, the released therapeutic agent comprises a fragment of a first cleavage domain, wherein the fragment is positioned at a first terminal end of the released therapeutic agent. In one embodiment, the released therapeutic agent comprises a fragment of a second cleavage domain, wherein the fragment is positioned at a second terminal end of the released therapeutic agent. In one embodiment, the released therapeutic agent comprises a first fragment of a first cleavage domain, wherein the first fragment is positioned at a first terminal end of the released therapeutic agent, and a second fragment of a second cleavage domain, wherein the second fragment is positioned at a second terminal end of the released therapeutic agent. In one embodiment, the released therapeutic agent is a peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 8, SEQ ID NO: 10, and SEQ ID NO: 12. In one embodiment, the composition provides localized and stimuli-induced release of the released therapeutic agent.

30 Also provided herein is a method of delivering a therapeutic agent to a subject in need thereof, comprising administering to a subject a composition described

herein. In one embodiment, the method comprises administering a composition comprising a plurality of synthetic monomers, and a plurality of prodrugs. In one embodiment, each prodrug comprises at least one therapeutic domain and one or more cleavable domains, wherein the cleavable domains couples the prodrug to one or more of the plurality of synthetic monomers. In one embodiment, the ratio of prodrug to synthetic monomer in the composition is greater than about 1:1. In one embodiment, the ratio of prodrug to synthetic monomer in the composition is greater than about 2:1.

In one embodiment, the prodrug comprises a therapeutic peptide coupled to a cleavable peptide sequence at its amino terminus and a cleavable peptide sequence at its carboxy terminus.

In one embodiment, the synthetic monomer comprise poly(ethylene glycol) (PEG). In one embodiment, the PEG is functionalized with terminal norbornene groups.

In one embodiment, the composition is a polymerized matrix. In one embodiment, polymerization of the matrix is induced by UV light. In one embodiment, the composition is a hydrogel. In one embodiment, the method comprises implanting the hydrogel into the subject at a treatment site.

In one embodiment, the composition is a solution. In one embodiment, the method comprises polymerizing the composition ex vivo. In one embodiment, the method comprises the step of administering UV light to the subject in vivo, thereby polymerizing the composition to form a matrix.

In one embodiment, the at least one cleavage domain comprises a matrix metalloproteinase (MMP) -sensitive cleavage domain. In one embodiment, the MMP-sensitive cleavage domain comprises the amino acid sequence of SEQ ID NO: 1. In one embodiment, an increased expression, activity, or combination thereof, of a MMP induces cleavage of the MMP-sensitive cleavage domain.

In one embodiment, the therapeutic domain comprises a pro-angiogenic amino acid sequence. In one embodiment, the therapeutic domain comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 5, and SEQ ID NO: 6.

In one embodiment, cleavage of the cleavage domains releases a released therapeutic agent. In one embodiment, the released therapeutic agent comprises a fragment of a first cleavage domain, wherein the fragment is positioned at a first terminal end of the released therapeutic agent. In one embodiment, the released therapeutic agent comprises a fragment of a second cleavage domain, wherein the fragment is positioned at a second terminal end of the released therapeutic agent. In one embodiment, the released therapeutic agent comprises a first fragment of a first cleavage domain, wherein the first fragment is positioned at a first terminal end of the released therapeutic agent, and a second fragment of a second cleavage domain, wherein the second fragment is positioned at a second terminal end of the released therapeutic agent. In one embodiment, the released therapeutic agent is a peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 8, SEQ ID NO: 10, and SEQ ID NO: 12. In one embodiment, the composition provides localized and stimuli-induced release of the released therapeutic agent.

In one embodiment, the subject has a condition associated with reduced vascularization, wherein the therapeutic domain comprises a pro-angiogenic amino acid sequence. In one embodiment, the condition is selected from the group consisting of cardiac ischemia, coronary heart failure, peripheral vascular ischemia, peripheral arterial disease, ischemic bowel disease, bone fracture healing, and diabetic ulcers. In one embodiment, the subject comprises an implanted engineered tissue in need of vascularization. In one embodiment, the subject is a human.

BRIEF DESCRIPTION OF THE DRAWINGS

The following detailed description of embodiments will be better understood when read in conjunction with the appended drawings. It should be understood, however, that the embodiments are not limited to the precise arrangements and instrumentalities of the embodiments shown in the drawings.

Figure 1 is a schematic detailing the development of pro-angiogenic biomaterials aimed at coordinating tissue vascularization. As an example, ischemic cardiac tissue develops as a result of arterial disease or myocardial infarction. The hydrogel precursors, comprising of norbornene-functionalized poly(ethylene glycol)

macromers and angiogenic peptides connected to MMP-degradable tethers, are injected using minimally-invasive laparoscopic techniques, and polymerized *in situ* via UV polymerization. The MMP-degradable tether is cleaved, resulting in a prolonged release of the angiogenic peptide into the ischemic tissue. This simultaneously degrades the hydrogel network, meaning that ultimately nothing remains except revascularized, functionally recovered host tissue. While schematically depicted for the treatment of ischemic cardiac tissue by delivery of pro-angiogenic peptides, this material could be used for the delivery of any number of therapeutic peptides, including anti-apoptotic, anti-inflammatory, or chemotherapeutic.

Figure 2 is a schematic depicting thiol-ene photopolymerization of the matrix. (A) Upon exposure to the UV light, the photoinitiator is cleaved in two and abstracts a hydrogen atom from a thiol on a cysteine amino acid. (B) The resulting thiyl radical propagates across the norbornene carbon-carbon double bond. (C) This produces a norbornene radical, (D) which abstracts a hydrogen from another thiol, (E) completing the thio-ether bond formation, and propagating the reaction.

Figure 3 depicts the results of experiments demonstrating the influence of PEG structure/molecular weight on hydrogel properties.

Figure 4 depicts representative MALDI-ToF results, which shows correct synthesis of Qk(2T). Correct synthesis of each peptide was confirmed by the observed and expected molecular weight peaks coinciding.

Figure 5 depicts the results of experiments demonstrating that all peptides except for T7 increase HUVEC proliferation in their native form. * $p < 0.05$ vs. basal media by two-way t-test; $n=12$; error bars represent SEM.

Figure 6 depicts the results of experiments examining the effect of peptides in their two-tailed (2T) form on HUVEC proliferation. * $p < 0.05$ vs. basal media by two-way t-test; $n=12$; error bars represent SEM.

Figure 7 is a graph depicting the percent increase in proliferation over control media treated cells from within the same plate. Effect of native and 2T forms of peptides is shown. * $p < 0.05$ vs. basal media by two-way t-test; $n=12$; error bars represent SEM.

Figure 8 is a graph demonstrating that all peptides except for Ten2 increase HUVEC tube formation in their native form. * $p < 0.05$ vs. basal media by two-way t-test; $n=9$; error bars represent SEM.

Figure 9 is a graph demonstrating that Qk, SPARC₁₁₃ and SPARC₁₁₈ significantly increase HUVEC tube formation in their 2T form. * $p < 0.05$ vs. basal media by two-way t-test; $n=9$; error bars represent SEM.

Figure 10 is a set of images depicting select representative fluorescent images of tube formation. All peptide treatments were given at 100 nM, VEGF at 1 nM. Scale bars = 200 μ m.

Figure 11 is a graph demonstrating the quantification of tube formation, as depicted in Figure 10. Fold increase in tube length over control media treated HUVECs from the same plate was calculated. Effect of native and 2T forms of the peptides is shown. * $p < 0.05$ vs. control media by two-way t-test; $n=9$; error bars represent SEM.

Figure 12 depicts a summary of the effects of the native and 2T forms of the tested peptides in the HUVEC proliferation and HUVEC tube formation assays described herein.

Figure 13, comprising Figure 13A and Figure 13B, depict the results of experiments demonstrating hydrogel degradation and peptide release. Hydrogel degradation (Figure 13A) and peptide release (Figure 13B) was quantified upon incubation in at 37 °C in PBS or PBS containing 250 μ g/mL collagenase. * $p < 0.05$ vs. 118(2NDL) in PBS by two-way ANOVA with Bonferroni post-hoc testing; $n=3$; error bars represent SEM.

Figure 14 is a schematic demonstrating enzymatically responsive release of therapeutic peptides from poly(ethylene glycol) hydrogels. PEG hydrogels are formed via thiol-ene photopolymerization between norbornene functionalized PEG macromers and thiol groups on cysteine amino acids on either end of the crosslinking peptide sequence. Peptide drugs are flanked with the enzymatically degradable sequence IPES↓LRAG (SEQ ID NO: 1), to achieve enzymatically responsive hydrogel degradation and peptide release. ↓ indicates cleavage site.

Figure 15, comprising Figure 15A and Figure 15B, depicts the results of experiments demonstrating the effects of residual “tails” on peptide bioactivity. (Figure

15A) Fold increase in HUVEC tube length over control media with peptide treatment and (Figure 15B) percent increase in HUVEC proliferation, quantified by measuring DNA content, over control media after 3 days of peptide treatment. White bars indicate “N” peptide; grey bars indicate “2T” peptide; black bars indicate control groups. * $p < 0.05$ vs. treatment with control media. $n=9$ for tube formation, $n=12$ for proliferation; error bars represent SEM.

Figure 16, comprising Figure 16A through Figure 16G, is a set of illustrations depicting the predicted structures for peptides (Figure 16A) the degradable linker (DL) alone, (Figure 16B) SPARC₁₁₈(DL), (Figure 16C) SmPho(DL), (Figure 16D) SPARC_{3X}(DL), (Figure 16E) SPARC₁₁₃(DL), (Figure 16F) Scrambled (DL), and (Figure 16G) Qk(DL). Arrowheads on α -helix ribbons point to C-termini.

Figure 17, comprising Figure 17A through Figure 17D, depicts the results of experiments characterizing the physical properties of hydrogels and examining hydrogel degradation. (Figure 17A) Bulk images of hydrogels, scale bar = 1 cm, (Figure 17B) hydrogel swelling ratios, (Figure 17C) % peptide incorporated into hydrogels, and (Figure 17D) % peptide not forming crosslinks. horizontal bars indicate groups that are statistically equivalent ($p > 0.05$), * $p < 0.05$, & $p < 0.01$, \$ $p < 0.001$. $n=12$; error bars represent SEM.

Figure 18, comprising Figure 18A and Figure 18B, depicts the results of experiments examining hydrogel degradation and peptide release. (Figure 18A) Time to complete degradation (study ended at 10 days), and (Figure 18B) amount of “2T” peptide released upon complete hydrogel degradation. a-f indicates groups that are statistically equivalent ($p > 0.05$), \$ $p < 0.001$, # $p < 0.0001$. $n=6$; error bars represent SEM (some obscured by symbol).

Figure 19, comprising Figure 19A through Figure 19E, depicts is a set of graphs demonstrating the relationship between peptide size ((Figure 19A) molecular weight, (Figure 19B) sequence length) and hydrophobicity (Figure 19C) percent hydrophobic amino acids, (Figure 19D) Kyte-Doolittle, (Figure 19E) Hopp-Woods scale) and hydrogel degradation (left axis, black circles and solid black line) and “2T” peptide release (right axis, open grey squares and dashed grey line). n.s. $p > 0.05$, & $p < 0.01$, # $p < 0.0001$. $n=6$; error bars represent SEM (some obscured by symbol).

Figure 20, comprising Figure 20A and Figure 20B, depicts the results of experiments investigating the in vitro efficacy of peptide-releasing hydrogels. (Figure 20A) Representative images of and (Figure 20B) fold increase in tube length over control media upon treatment with degraded peptide-releasing hydrogels. HUVECs were treated with $\sim 1/7,000^{\text{th}}$ of a degraded gel, corresponding to 100 nM of released SPARC₁₁₈(2T). * $p < 0.05$, & $p < 0.01$ vs. treatment with control media. Scale bar = 250 μm ; $n=9$; error bars represent SEM.

Figure 21 is a set of illustrations depicting the predicted structures for various “N” and “2T” peptides. Comparing peptides that lost (KRX-725 and SPARC_{3X}) and retained (SPARC₁₁₃ and Qk) bioactivity upon inclusion of the “2T”, no clear change in structure between “N” and “2T” form was observed. Arrowheads on α -helix ribbons point to C-termini.

Figure 22, comprising Figure 22A through Figure 22F, is a set of graphs demonstrating the stability of released peptides. 0.8 μmol (Figure 22A) SPARC₁₁₈(2T), (Figure 22B) SmPho(2T), (Figure 22C) SPARC_{3X}(2T), (Figure 22D) SPARC₁₁₃(2T), (Figure 22E) Scrambled(2T), or (Figure 22F) Qk(2T) in 1mL buffer or buffer containing 10 nM MMP2 were incubated at 37 °C, and the amount of peptide remaining in its “2T” form tracked over time using HPLC. All six peptides remained stable in both solutions, indicating they are not degraded after release from the hydrogel network. $n=1$.

Figure 23, comprising Figure 23A through Figure 23F, depicts the results of MALDI-ToF mass spectrometry analysis of degraded hydrogels. (Figure 23A) SPARC₁₁₈(DL), (Figure 23B) SmPho(DL), (Figure 23C) SPARC_{3X}(DL), (Figure 23D) SPARC₁₁₃(DL), (Figure 23E) Scrambled(DL) and (Figure 23F) buffer alone. SPARC₁₁₈(DL) gels were degraded in Brij-free buffer for mass spectrometry, as the Brij signal obscured the “2T” form of the peptide. All degraded gels have a clear single peak at the expected “2T” peptide molecular weight, indicating peptides are not being further cleaved by MMP2 after release from the hydrogel networks. An increase of 1 or 2 Da indicates the released peptide is protonated. No clear spectra could be obtained for degraded Qk(DL) gels.

Figure 24, comprising Figure 24A through Figure 24C, is a set of graphs depicting the results of experiments examining the in vitro mass loss (left), swelling ratio

(center), and “2T” peptide release from (right) enzymatically responsive hydrogels. Gels were incubated in buffer alone (solid symbol and line) for 24 hours, at which point 10 nM MMP-2 was added (open symbol, dashed line). (Figure 24A) SPARC₁₁₃(DL), (Figure 24B) SPARC₁₁₈(DL), and (Figure 24C) Scrambled(DL) were investigated. * $p < 0.05$, & $p < 0.01$, \$ $p < 0.001$, # $p < 0.0001$ vs. gel in buffer alone at same time point. $n=6$ except for the swelling data where gel degradation reduced sample size at later timepoints; error bars represent SEM (some are obscured by symbol).

Figure 25, comprising Figure 25A through Figure 25E, depicts the results of experiments examining the in vitro diffusive release of (Figure 25A) Qk(2T), (Figure 25B) SPARC₁₁₃(2T), (Figure 25C) SPARC₁₁₈(2T), and (Figure 25D) Scrambled(2T) out of non-degradable PEG gels. (Figure 25E) Data was fit to the Fickian diffusion model, and the diffusion coefficient for each peptide calculated. $n=6$; error bars represent SEM (some are obscured by the symbol). There was no significant difference between the diffusion coefficients for the three peptides ($p > 0.05$ by one-way ANOVA,).

Figure 26, comprising Figure 26A through Figure 26C, depicts the results of experiments examining the effect of peptide concentration on tube formation in cultures treated with native or 2T forms of Qk (Figure 26A), SPARC₁₁₃ (Figure 26B), or SPARC₁₁₈ (Figure 26C). $p < 0.05$, & $p < 0.01$ vs. 0 μM treatment. $n=6$; error bars represent SEM.

Figure 27, comprising Figure 27A through Figure 27D, depicts the results of experiments examining tube formation induced upon treatment with varying amounts of degraded hydrogel. (Figure 27A) Tube formation images (at 1/7,000th of a gel per well). (Figure 27B – Figure 27D) Dose response curves for degraded hydrogel products, in terms of fraction of a gel per well, (Figure 27B) Qk, triangles, (Figure 27C) SPARC₁₁₃, diamonds and (Figure 27D) SPARC₁₁₈, circles. The scrambled peptide releasing gels (squares) did not affect tube formation at any concentration. * $p < 0.05$, & $p < 0.01$, \$ $p < 0.001$ vs. media alone. $n=9$; error bars represent SEM.

Figure 28 is a graph depicting tube formation dose response curves for degraded hydrogel products, in terms of “2T” peptide dose per well. The scrambled peptide releasing gels did not affect tube formation at any concentration. * $p < 0.05$, & $p < 0.01$, \$ $p < 0.001$ vs. 0 μM treatment. $n=9$; error bars represent SEM.

DETAILED DESCRIPTION

Described herein are compositions and methods for stimuli-responsive release of a therapeutic agent. The compositions and methods described herein are at least partially based upon the discovery that therapeutic agents can be incorporated into a hydrogel matrix, where the therapeutic agents can be induced to be released through the cleavage of a cleavage domain within the matrix. For example, in certain embodiments, a composition comprises a prodrug comprising at least one cleavage domain and at least one therapeutic domain. In certain instances, cleavage of the cleavage domain is dependent upon the localized environment at a treatment site. For example, in one embodiment, cleavage is dependent upon the expression and/or activity of a matrix metalloproteinase (MMP), whose expression and/or activity are known to be enhanced in tumors, ischemic tissue, and regions in need of vascularization.

For example, in certain embodiments, a method to promote angiogenesis or vascularization comprises the stimuli-induced release of pro-angiogenic therapeutic agents from an implanted polymerized matrix. In one embodiment, the method may be used to promote vascularization of a tissue engineered construct in vivo. In one embodiment, the method may be used to promote vascularization of a tissue engineered construct ex vivo.

In one embodiment, a method to treat a tumor comprises the stimuli-induced release of anti-tumor therapeutic agents, including for example a chemotherapeutic agent or apoptotic agent, from an implanted matrix at a treatment site. In certain embodiments, a method to treat inflammation comprises the stimuli-induced release of an anti-inflammatory agent from an implanted matrix at a treatment site.

In one embodiment, polymerization of a hydrogel matrix is initiated via administration of UV light, which, in some embodiments, results in the thiol-ene polymerization of the matrix. For example, in one embodiment, the matrix comprises PEG functionalized with terminal norbornene groups, which upon administration of UV-light, binds to thiol-containing groups, which thereby allows for easy and effective incorporation of prodrugs into the matrix. In one embodiment, the composition comprises a matrix that is polymerized ex vivo, which is later implanted at a treatment site.

In another embodiment, the composition comprises a solution of synthetic monomers and prodrugs, which can be injected at treatment site and polymerized in vivo through the application of UV light.

5 Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the compositions and methods.

10 As used herein, each of the following terms has the meaning associated with it in this section.

The articles “a” and “an” are used herein to refer to one or to more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, “an element” means one element or more than one element.

15 “About” as used herein when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of $\pm 20\%$ or $\pm 10\%$, or $\pm 5\%$, or $\pm 1\%$, or $\pm 0.1\%$ from the specified value, as such variations are appropriate to perform the disclosed methods.

The term “abnormal” when used in the context of organisms, tissues, cells
20 or components thereof, refers to those organisms, tissues, cells or components thereof that differ in at least one observable or detectable characteristic (e.g., age, treatment, time of day, etc.) from those organisms, tissues, cells or components thereof that display the “normal” (expected) respective characteristic. Characteristics which are normal or expected for one cell or tissue type, might be abnormal for a different cell or tissue type.

25 As used here, “biocompatible” refers to any material, which, when implanted in a mammal, does not provoke an adverse response in the mammal. A biocompatible material, when introduced into an individual, is not toxic or injurious to that individual, nor does it induce immunological rejection of the material in the mammal.

A “disease” is a state of health of an animal wherein the animal cannot maintain homeostasis, and wherein if the disease is not ameliorated then the animal’s health continues to deteriorate.

5 In contrast, a “disorder” in an animal is a state of health in which the animal is able to maintain homeostasis, but in which the animal’s state of health is less favorable than it would be in the absence of the disorder. Left untreated, a disorder does not necessarily cause a further decrease in the animal’s state of health.

10 A disease or disorder is “alleviated” if the severity of a symptom of the disease or disorder, the frequency with which such a symptom is experienced by a patient, or both, is reduced.

An “effective amount” or “therapeutically effective amount” of a compound is that amount of compound which is sufficient to provide a beneficial effect to the subject to which the compound is administered. An “effective amount” of a delivery vehicle is that amount sufficient to effectively bind or deliver a compound.

15 As used herein, the term “gel” refers to a three-dimensional polymeric structure that itself is insoluble in a particular liquid but which is capable of absorbing and retaining large quantities of the liquid to form a stable, often soft and pliable, but always to one degree or another shape-retentive, structure. When the liquid is water, the gel is referred to as a hydrogel. Unless expressly stated otherwise, the term “gel” will be
20 used throughout this application to refer both to polymeric structures that have absorbed a liquid other than water and to polymeric structures that have absorbed water, it being readily apparent to those skilled in the art from the context whether the polymeric structure is simply a “gel” or a “hydrogel.”

“Homologous” refers to the sequence similarity or sequence identity
25 between two polypeptides or between two nucleic acid molecules. When a position in both of the two compared sequences is occupied by the same base or amino acid monomer subunit, e.g., if a position in each of two DNA molecules is occupied by adenine, then the molecules are homologous at that position. The percent of homology between two sequences is a function of the number of matching or homologous positions
30 shared by the two sequences divided by the number of positions compared X 100. For example, if 6 of 10 of the positions in two sequences are matched or homologous then the

two sequences are 60% homologous. By way of example, the DNA sequences ATTGCC and TATGGC share 50% homology. Generally, a comparison is made when two sequences are aligned to give maximum homology.

“Isolated” means altered or removed from the natural state. For example, a nucleic acid or a peptide naturally present in a living animal is not “isolated,” but the same nucleic acid or peptide partially or completely separated from the coexisting materials of its natural state is “isolated.” An isolated nucleic acid or protein can exist in substantially purified form, or can exist in a non-native environment such as, for example, a host cell.

As used herein, an “instructional material” includes a publication, a recording, a diagram, or any other medium of expression which can be used to communicate the usefulness of a composition, peptide, method, and the like in a kit for stimuli-induced release of a therapeutic agent, as recited herein. Optionally, or alternately, the instructional material can describe one or more methods of providing stimuli-induced release. The instructional material of the kit can, for example, be affixed to a container which contains the identified composition or precursors thereof or be shipped together with a container which contains the composition or precursors thereof. Alternatively, the instructional material can be shipped separately from the container with the intention that the instructional material and the composition be used cooperatively by the recipient.

As used herein, the terms “peptide,” “polypeptide,” and “protein” are used interchangeably, and refer to a compound comprised of amino acid residues covalently linked by peptide bonds. A protein or peptide must contain at least two amino acids, and no limitation is placed on the maximum number of amino acids that can comprise a protein’s or peptide’s sequence. Polypeptides include any peptide or protein comprising two or more amino acids joined to each other by peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides and oligomers, for example, and to longer chains, which generally are referred to in the art as proteins, of which there are many types. “Polypeptides” include, for example, biologically active fragments, substantially homologous polypeptides, oligopeptides, homodimers, heterodimers, variants of polypeptides, modified

polypeptides, derivatives, analogs, fusion proteins, among others. The polypeptides include natural peptides, recombinant peptides, synthetic peptides, or a combination thereof.

As used herein, the term “polymer” refers to a molecule composed of repeating structural units typically connected by covalent chemical bonds. The term “polymer” is also meant to include the terms copolymer and oligomers.

As used herein, the term “polymerization” refers to at least one reaction that consumes at least one functional group in a monomeric molecule (or monomer), oligomeric molecule (or oligomer) or polymeric molecule (or polymer), to create at least one chemical linkage between at least two distinct molecules (e.g., intermolecular bond), at least one chemical linkage within the same molecule (e.g., intramolecular bond), or any combination thereof. A polymerization reaction may consume between about 0% and about 100% of the at least one functional group available in the system. In one embodiment, polymerization of at least one functional group results in about 100% consumption of the at least one functional group. In another embodiment, polymerization of at least one functional group results in less than about 100% consumption of the at least one functional group.

As used herein, the term “patient,” “subject,” “individual,” and the like are used interchangeably herein, and refer to any animal, or cells thereof, whether *in vitro* or *in situ*, amenable to the methods described herein. In certain non-limiting embodiments, the patient, subject or individual is a mammal, non-limiting examples of which include a primate, dog, cat, goat, horse, pig, mouse, rat, rabbit, and the like, that is in need of tissue vascularization. In some embodiments, the subject is a human being. In such embodiments, the subject is often referred to as an “individual” or a “patient.” The terms “individual” and “patient” do not denote any particular age

As used herein, the phrase “a site in need of vascularization” refers to any site or region within a subject which, for any reason, is in need vascularization or angiogenesis. For example, in certain embodiments, the site is a region of ischemic tissue.

A “therapeutic” treatment is a treatment administered to a subject who exhibits signs of pathology, for the purpose of diminishing or eliminating those signs.

As used herein, "treating a disease or disorder" means reducing the frequency with which a symptom of the disease or disorder is experienced by a patient. Disease and disorder are used interchangeably herein.

5 The phrase "therapeutically effective amount," as used herein, refers to an amount that is sufficient or effective to prevent or treat (delay or prevent the onset of, prevent the progression of, inhibit, decrease or reverse) a disease or condition, including alleviating symptoms of such diseases.

Ranges: throughout this disclosure, various aspects can be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 10 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 2.7, 3, 4, 5, 5.3, and 6. This applies regardless of the breadth of the range.

Description

Described herein are compositions and methods for stimuli-responsive treatment. For example, in certain embodiments, the compositions and methods provide 20 for the stimuli-responsive release of a therapeutic agent, wherein the release is dependent upon the local environment of a treatment site. In one embodiment, release of the therapeutic agent is dependent upon enzymatic activity present at the treatment site, where the enzymatic activity is associated with the need for treatment. The compositions and methods provide for the controlled, local, and stimuli-responsive release of a 25 therapeutic agent that can be used to treat any type of disease or disorder. That is, the particular therapeutic agent, and the stimuli-responsive release of the therapeutic agent, can be altered depending upon the type of disease being treated and the need at the treatment site.

30 In one embodiment, compositions and method for stimuli-responsive release of pro-angiogenic therapeutic agents are provided. The compositions and methods

can be used for the controlled and local delivery of pro-angiogenic therapeutic agents for the treatment of diseases and disorders including, but not limited to cardiac ischemia, coronary heart failure, peripheral vascular ischemia, peripheral arterial disease, ischemic bowel disease, bone fracture healing, and diabetic ulcers.

5 In certain embodiments, the controlled and local delivery of pro-angiogenic therapeutic agents is used to promote the vascularization of engineered tissue. The compositions and methods could also be used to enhance vascularization of transplanted cells and tissues, increasing viability of the tissue and the therapeutic efficacy of the treatment. Particularly, tissue engineering and regenerative medicine
10 strategies of tissues could benefit from the angiogenic therapy provided by the compositions and methods described herein, to promote host vascularization, because simple diffusion is insufficient to deliver nutrients and remove waste from these constructs.

 In one embodiment, a composition for stimuli-responsive treatment is
15 provided herein. In certain embodiments, the composition comprises a plurality of synthetic monomers and a plurality of prodrugs. In one embodiment, the prodrugs are cleaved to release a therapeutic agent from a polymerized matrix when the local environment of the composition dictates that release is needed. In one embodiment, the therapeutic agent is a therapeutic peptide. In one embodiment, the prodrug comprises a
20 stimuli-responsive releasing element. For example, in one embodiment, the prodrug comprises a stimuli-responsive cleavable domain, which when cleaved releases the therapeutic agent. For example, in one embodiment, the prodrug comprises a therapeutic peptide coupled to a cleavable peptide sequence at its amino terminus and a cleavable peptide sequence at its carboxy terminus.

25 In certain embodiments, the ratio of prodrug to synthetic monomer in the composition is greater than about 1:1. In one embodiment, the ratio of prodrug to synthetic monomer in the composition is greater than about 2:1. In one embodiment, the ratio of prodrug to synthetic monomer in the composition is greater than about 4:1. In one embodiment, the ratio of prodrug to synthetic monomer in the composition is greater than
30 about 8:1. In one embodiment, the ratio of prodrug to synthetic monomer in the composition is greater than about 16:1.

The released therapeutic agent may comprise a peptide, protein, nucleic acid, small molecule, and combinations thereof.

In one embodiment, the matrix comprises a prodrug comprising at least one therapeutic domain and at least one cleavable domain. In certain embodiments, the
5 peptide comprises a therapeutic domain positioned between two cleavable domains.

The therapeutic domain of the prodrug may comprise a peptide, protein, nucleic acid small molecule, and combinations thereof.

The cleavable domain of the prodrug may comprise a peptide, protein, nucleic acid small molecule, and combinations thereof.

10 In one embodiment, the prodrug is a peptide.

In one embodiment, the at least one cleavable domain of the prodrug is a peptide, while the at least one therapeutic domain is a small molecule drug.

In certain embodiments, the cleavable domain is cleaved by a particular enzyme, whose activity at a given treatment site regulates the release of the therapeutic
15 agent. For example, in certain embodiments, the expression, activity, or both of the enzyme is increased at the time and location where the therapeutic agent is needed. In certain embodiments, the composition is a hydrogel comprising polymerized matrix. In another embodiment, the composition is a solution comprising matrix materials or precursors, including for example non-polymerized synthetic monomers and prodrugs.

20 In certain embodiments, the composition comprises poly(ethylene glycol) (PEG). For example, in one embodiment, the composition comprises PEG monomers. In one embodiment, the composition comprises a polymerized matrix comprising PEG. In certain embodiments, the PEG functionalized with terminal norbornene groups, which allows for incorporation of the prodrug into a matrix through the use of thiol-containing
25 groups. For example, in certain embodiments, one or more arms of PEG is linked or conjugated to norbornene groups, thereby allowing for the linkage of the PEG-norbornene to a thiol-containing group of the prodrug. In one embodiment, the composition comprises a matrix comprising PEG bound or tethered to a prodrug, wherein the prodrug comprises at least one therapeutic domain and at least one cleavable domain.
30 In one embodiment, the at least one cleavable domain is an amino acid sequence cleaved by a MMP, which is upregulated at a treatment site. In one embodiment, the at least one

cleavable domain comprises the amino acid sequence IPESLRAG (SEQ ID NO: 1), which when cleaved forms the fragments IPES (SEQ ID NO: 2) and LLAG (SEQ ID NO: 3). In certain embodiments, the therapeutic domain comprises a pro-angiogenic amino acid sequence. The pro-angiogenic amino acid sequence may be selected from the group consisting of Qk (KLTWQELYQLKYKGI; SEQ ID NO: 4), SPARC₁₁₃ (TLEGTKKGHLHLDY; SEQ ID NO: 5), and SPARC₁₁₈ (KKGHK; SEQ ID NO: 6).

In one embodiment, the prodrug comprises a therapeutic domain positioned between two cleavable domains. For example, upon cleavage of the cleavable domains, a therapeutic agent is released from the matrix. Therefore, in certain embodiments, the released therapeutic agent comprises the therapeutic domain, as well as a fragment of a first cleavable domain located at a first terminal end of the therapeutic agent and a fragment of the second cleavable domain located at a second terminal end of the therapeutic agent.

For example, in one embodiment, the released therapeutic agent is a released therapeutic peptide which comprises SEQ ID NO: 3 at the N-terminus. In one embodiment, the released therapeutic peptide comprises SEQ ID NO: 2 at the C-terminus. In some instances, the released therapeutic peptide is referred to herein as the “two-tailed” or “2T” version. As demonstrated herein, the compositions and methods described herein are partly based upon the discovery that the 2T version of pro-angiogenic peptides retain their therapeutic activity, and thus can be incorporated into the stimuli-responsive composition. In certain instances, the therapeutic agent may be released from the matrix, but still bound to matrix materials (e.g. PEG), and still retain bioactivity.

25 Matrix

Various compositions and methods of the application employ a matrix. In various embodiments, the matrix materials are formed into a 3-dimensional scaffold. The scaffold can contain one or more matrix layers. For example, the scaffold can contain at least two matrix layers, at least three matrix layers, at least four matrix layers, at least five matrix layers, or more.

Matrix materials of various embodiments are biocompatible materials. In certain embodiments, the matrix is biodegradable. For example, in certain embodiments, the matrix is degraded through the cleavage of cleavage domains located throughout the matrix. The matrix can be fabricated into structural supports, where the geometry of the structure (e.g., shape, size, porosity, micro- or macro-channels) is tailored to the application.

Suitable matrix materials are discussed in, for example, Ma and Elisseeff, ed. (2005) *Scaffolding in Tissue Engineering*, CRC, ISBN 1574445219; Saltzman (2004) *Tissue Engineering: Engineering Principles for the Design of Replacement Organs and Tissues*, Oxford ISBN 019514130X.

In one embodiment, at least two matrix materials are used to fabricate a tissue module described herein. The at least two matrix materials can be homogeneously mixed throughout the scaffold, heterogeneously mixed throughout the scaffold, or separated into different matrix layers of the scaffold.

Matrices can be produced from proteins (e.g. extracellular matrix proteins such as fibrin, collagen, and fibronectin), polymers (e.g., polyvinylpyrrolidone), polysaccharides (e.g. alginate), hyaluronic acid, or analogs, mixtures, combinations, and derivatives of the above.

The matrix can be formed of synthetic polymers. Such synthetic polymers include, but are not limited to, poly(ethylene) glycol, bioerodible polymers (e.g., poly(lactide), poly(glycolic acid), poly(lactide-co-glycolide), poly(caprolactone), polyester (e.g., poly-(L-lactic acid), polyanhydride, polyglactin, polyglycolic acid), polycarbonates, polyamides, polyanhydrides, polyamino acids, polyortho esters, polyacetals, polycyanoacrylates), polyphosphazene, degradable polyurethanes, non-erodible polymers (e.g., polyacrylates, ethylene-vinyl acetate polymers and other acyl substituted cellulose acetates and derivatives thereof), non-erodible polyurethanes, polystyrenes, polyvinyl chloride, polyvinyl fluoride, polyvinyl pyrrolidone, poly(vinylimidazole), chlorosulphonated polyolifins, polyethylene oxide, polyvinyl alcohol (e.g., polyvinyl alcohol sponge), synthetic marine adhesive proteins, teflon®, nylon, or analogs, mixtures, combinations (e.g., polyethylene oxide-polypropylene glycol

block copolymer; poly(D,L-lactide-co-glycolide) fiber matrix), and derivatives of the above.

The matrix can be formed of naturally occurring polymers or natively derived polymers. Such polymers include, but are not limited to, agarose, alginate (e.g.,
5 calcium alginate gel), fibrin, fibrinogen, fibronectin, collagen (e.g., a collagen gel), gelatin, hyaluronic acid, chitin, and other suitable polymers and biopolymers, or analogs, mixtures, combinations, and derivatives of the above. Also, the matrix can be formed from a mixture of naturally occurring biopolymers and synthetic polymers.

The matrix can comprise a composite matrix material comprising at least
10 two components described above. As an example, a composite matrix material can comprise at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least ten, or more, components. The plurality of components can be homogenously mixed throughout the scaffold, heterologously mixed throughout the scaffold, or separated into different matrix layers of the scaffold, or a combination
15 thereof.

In some embodiments, one or more matrix materials are modified so as to increase biodegradability. For example, PCL is a biodegradable polyester by hydrolysis of its ester linkages in physiological conditions, and can be further modified with ring opening polymerization to increase its biodegradability.

20 In certain embodiments, the matrix comprises a plurality of PEG monomers, including for example a plurality of PEG macromers. As would be understood by those skilled in the art, any suitable PEG macromer may be incorporated into the matrix. The particular type of PEG macromer utilized may be dependent upon the particular application of the matrix/scaffold and the desired properties of the
25 matrix/scaffold. In one embodiment, the matrix comprises an 8-arm 10kDa PEG macromer. However, as would be understood by those skilled in the art, alternative forms of PEG, including forms of differing size or structure, can be utilized in the matrix. For example, in certain embodiments, the PEG macromer is a multi-arm macromer comprising 4, 8, 16, or more arms.

30 In certain embodiments, the PEG macromer is functionalized with terminal norbornene groups, which in certain instances allow for linking of the PEG

macromer to a thiol-containing prodrug. For example, in certain embodiments, one or more arms of PEG is linked or conjugated to norbornene groups, thereby allowing for the linkage of the PEG-norbornene to a thiol-containing group of the prodrug. For example, the norbornene functionalized PEG macromers can be linked to a peptide using thiol-

5 norbornene chemistry, making use of thiol-containing amino acids. Therefore, the norbornene functionalized PEG macromers allows for easy incorporation of a peptide into the matrix. However, the matrix is not limited to this particular method of matrix crosslinking. Rather, any type of crosslinking chemistry can be used in formation of the matrix.

10 In one embodiment, the matrix comprises a prodrug linked to the PEG macromer. For example, in certain embodiments, the matrix comprises PEG macromers that are crosslinked together via the incorporation of a prodrug between two PEG macromers. The prodrug of the matrix is degradable or cleavable, which promotes the degradation of the matrix. As described herein, the cleavage of the prodrug to release a

15 therapeutic agent is dependent upon a given stimulus, for example, the activity of a specific enzyme. In one embodiment, the prodrug comprises at least one therapeutic domain and at least one cleavable domain. In certain embodiments, cleavage of the at least one cleavable domain, releases a therapeutic agent from the matrix.

In one embodiment, the at least one cleavable domain is an amino acid

20 sequence which is cleaved by a MMP. Thus, in certain embodiments, the cleavage domain is an MMP-sensitive cleavage domain. The MMP-sensitive cleavage domain may be cleaved by any known MMP, including but not limited to MMP1, MMP2, MMP3, MMP4, MMP5, MMP6, MMP7, MMP8, MMP9, MMP10, MMP11, MMP12, MMP13, MMP14, MMP15, MMP16, MMP17, MMP18, MMP19, MMP20, MMP21, MMP22,

25 MMP23, MMP24, MMP25, MMP26, MMP27, MMP28, and the like. In one embodiment, the at least one cleavable domain comprises the amino acid sequence IPESLRAG (SEQ ID NO: 1), which when cleaved forms the fragments IPES (SEQ ID NO: 2) and LRAG (SEQ ID NO: 3). In certain instances the cleavage domain comprising SEQ ID NO: 1 is optimized for cleavage by MMP14, but is also susceptible to cleavage

30 by MMP1, MMP2, MMP3, MMP7, and MMP9.

It should be understood by those skilled in the art, that the composition of encompasses a cleavable domain that is cleaved by a specific stimulus. For example, the composition is not limited to MMP-sensitive cleavage domains. Rather, in certain embodiments, the cleavage domain is a substrate of a known enzyme with lytic activity.

5 For example, in one embodiment, the cleavage domain is cleaved via the activity of cathepsins.

The at least one therapeutic domain of the prodrug may comprise any bioactive molecule which demonstrates therapeutic activity, including but not limited to, a protein, isolated nucleic acid, antibody, small molecule, isolated peptide, and
10 conjugates thereof.

In certain embodiments, the therapeutic domain comprises a pro-angiogenic peptide. In one embodiment, the amino acid sequence of the pro-angiogenic peptide may be selected from the group consisting of Qk (KLTWQELYQLKYKGI; SEQ ID NO: 4), SPARC₁₁₃ (TLEGTKKGHLHLDY; SEQ ID NO: 5), and SPARC₁₁₈
15 (KKGHK; SEQ ID NO: 6).

In one embodiment, the prodrug comprises a therapeutic domain positioned between two cleavable domains. In one embodiment, the prodrug comprises one or more therapeutic domains positioned between two cleavable domains. That is, in certain embodiments, the prodrug comprises one or more repeats of the therapeutic
20 domain, where all of the repeats are positioned between two cleavable domains. In another embodiment, the prodrug comprises alternating repeats of the cleavable domain and therapeutic domain.

In certain embodiments, upon cleavage of the cleavable domains, a therapeutic agent is released from the matrix. Therefore, in certain embodiments, the
25 released therapeutic agent comprises the at least one therapeutic domain, as well as a fragment of a first cleavable domain located at the first terminal end of the therapeutic agent and a fragment of the second cleavable domain located at the second end of the therapeutic agent.

For example, in one embodiment, the released therapeutic agent is a
30 released therapeutic peptide, where the released therapeutic peptide comprises SEQ ID

NO: 3 at the N-terminus. In one embodiment, the released therapeutic peptide comprises SEQ ID NO: 2 at the C-terminus.

In one embodiment, the prodrug of the matrix is a peptide comprising the amino acid sequence of IPESLRAGKLTWQELYQLKYKGIPELRAG (SEQ ID NO: 7), such that the released therapeutic peptide comprises the amino acid sequence of LRAGKLTWQELYQLKYKGIPE (Qk(2T); SEQ ID NO: 8).

In one embodiment, the prodrug of the matrix is a peptide comprising the amino acid sequence of IPESLRAGTLEGTKKGHLHLDYIPESLRAG (SEQ ID NO: 9), such that the released therapeutic peptide comprises the amino acid sequence of LRAGTLEGTKKGHLHLDYIPES (SPARC₁₁₃(2T); SEQ ID NO: 10).

In one embodiment, the prodrug of the matrix is a peptide comprising the amino acid sequence of IPESLRAGKKGHKIPESLRAG (SEQ ID NO: 11), such that the released therapeutic peptide comprises the amino acid sequence of LRAGKKGHKIPES (SPARC₁₁₈(2T); SEQ ID NO: 12).

In one embodiment, the prodrug of the composition comprises terminal cysteine residues, which in certain instances, allow for linkage to the norbornene functionalized PEG. For example, in certain embodiments, the prodrug comprises the amino acid sequence of CIPESLRAGKLTWQELYQLKYKGIPELRAGC (SEQ ID NO: 13), CIPESLRAGTLEGTKKGHLHLDYIPESLRAGC (SEQ ID NO: 14), or CIPESLRAGKKGHKIPESLRAGC (SEQ ID NO: 15).

The matrix can be formed by any method known in the art. For example, in certain embodiments, the matrix is constructed from the polymerization of monomer, polymer, or macromer matrix materials. In one embodiment, the matrix is formed through the polymerization of PEG macromers. In certain embodiments, the peptides described herein crosslink PEG macromers. In one embodiment, polymerization of the matrix is achieved using “click” chemistry. For example, in one embodiment, polymerization of the matrix is performed using thiol-norbornene chemistry. As described elsewhere herein, in certain embodiments the PEG macromer is functionalized with terminal norbornene groups. In some instances, the thiol-norbornene chemistry used to construct the matrix allows for rapid formation under cytocompatible conditions. This cross-linking occurs via step-by-step polymerization, creating homogenous networks and

allows for easy incorporation of prodrugs through the use of thiol-containing groups. In certain embodiments, initiation of the polymerization of the matrix occurs via the administration of UV light. For example, in one embodiment, the polymerization of the matrix makes use of lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP)

5 photoinitiator. This allows for the matrix to be formed in either an in vitro, ex vivo, or in vivo embodiment. For example, in one embodiment, the matrix is polymerized ex vivo, and is later implanted into a target treatment site. In another embodiment, a solution comprising matrix materials is administered to a target treatment site

In one embodiment, the composition is a hydrogel comprising the matrix,
10 as described herein. Hydrogels can generally absorb a great deal of fluid and, at equilibrium, typically are composed of 60-90% fluid and only 10-30% polymer. In a preferred embodiment, the water content of hydrogel is about 70-80%. Hydrogels are particularly useful due to the inherent biocompatibility of the polymeric network (Hill-West, et al., 1994, Proc. Natl. Acad. Sci. USA 91:5967-5971). Hydrogel biocompatibility
15 can be attributed to hydrophilicity and ability to imbibe large amounts of biological fluids (Brannon-Peppas. Preparation and Characterization of Cross-linked Hydrophilic Networks in Absorbent Polymer Technology, Brannon-Peppas and Harland, Eds. 1990, Elsevier: Amsterdam, pp 45-66; Peppas and Mikos. Preparation Methods and Structure of Hydrogels in Hydrogels in Medicine and Pharmacy, Peppas, Ed. 1986, CRC Press: Boca
20 Raton, Fla., pp 1-27). In certain embodiments, the hydrogels can be prepared by crosslinking hydrophilic biopolymers or synthetic polymers. In certain embodiments, construction of hydrogels comprises the polymerization and/or copolymerization of monomers, macromers, polymers and the like. For example, in one embodiment hydrogel formation comprises copolymerization of two or more types of biopolymers and/or
25 synthetic polymers.

Examples of the hydrogels formed from physical or chemical crosslinking of hydrophilic biopolymers, include but are not limited to, hyaluronans, chitosans, alginates, collagen, dextran, pectin, carrageenan, polylysine, gelatin or agarose. (see.: W. E. Hennink and C. F. van Nostrum, 2002, Adv. Drug Del. Rev. 54, 13-36 and A. S.
30 Hoffman, 2002, Adv. Drug Del. Rev. 43, 3-12). These materials consist of high-molecular weight backbone chains made of linear or branched polysaccharides or

polypeptides. Examples of hydrogels based on chemical or physical crosslinking synthetic polymers include but are not limited to (meth)acrylate-oligolactide-PEO-oligolactide-(meth)acrylate, poly(ethylene glycol) (PEO; PEG), poly(propylene glycol) (PPO), PEO-PPO-PEO copolymers (Pluronics), poly(phosphazene), poly(methacrylates),
5 poly(N-vinylpyrrolidone), PL(G)A-PEO-PL(G)A copolymers, poly(ethylene imine), PEG monoethylether methacrylate, etc. (see A. S Hoffman, 2002 Adv. Drug Del. Rev, 43, 3-12). Hydrogels closely resemble the natural living extracellular matrix (Ratner and Hoffman. Synthetic Hydrogels for Biomedical Applications in Hydrogels for Medical and Related Applications, Andrade, Ed. 1976, American Chemical Society: Washington,
10 D.C., pp 1-36). Hydrogels can also be made degradable in vivo by incorporating PLA, PLGA or PGA polymers. Moreover, hydrogels can be modified with fibronectin, laminin, vitronectin, or, for example, RGD for surface modification, which can promote cell adhesion and proliferation (Heungsoo Shin, 2003, Biomaterials 24:4353-4364; Hwang et al., 2006 Tissue Eng. 12:2695-706). Indeed, altering molecular weights, block structures,
15 degradable linkages, and cross-linking modes can influence strength, elasticity, and degradation properties of the instant hydrogels (Nguyen and West, 2002, Biomaterials 23(22):4307-14; Ifkovits and Burkick, 2007, Tissue Eng. 13(10):2369-85).

Hydrogels can also be modified with functional groups for covalently attaching a variety of proteins (e.g., collagen) or compounds such as therapeutic agents.
20 Therapeutic agents which can be linked to the matrix include, but are not limited to, analgesics, anesthetics, antifungals, antibiotics, anti-inflammatories, anthelmintics, antidotes, antiemetics, antihistamines, antihypertensives, antimalarials, antimicrobials, antipsychotics, antipyretics, antiseptics, antiarthritics, antituberculosics, antitussives, antivirals, cardioactive drugs, cathartics, chemotherapeutic agents, a colored or
25 fluorescent imaging agent, corticoids (such as steroids), antidepressants, depressants, diagnostic aids, diuretics, enzymes, expectorants, hormones, hypnotics, minerals, nutritional supplements, parasympathomimetics, potassium supplements, radiation sensitizers, a radioisotope, sedatives, sulfonamides, stimulants, sympathomimetics, tranquilizers, urinary anti-infectives, vasoconstrictors, vasodilators, vitamins, xanthine
30 derivatives, and the like. The therapeutic agent can also be other small organic molecules, naturally isolated entities or their analogs, organometallic agents, chelated metals or

metal salts, peptide-based drugs, or peptidic or non-peptidic receptor targeting or binding agents. It is contemplated that linkage of the therapeutic agent to the matrix can be via a protease sensitive linker or other biodegradable linkage. Molecules which can be incorporated into the hydrogel matrix include, but are not limited to, vitamins and other
5 nutritional supplements; glycoproteins (e.g., collagen); fibronectin; peptides and proteins; carbohydrates (both simple and/or complex); proteoglycans; antigens; oligonucleotides (sense and/or antisense DNA and/or RNA); antibodies (for example, to infectious agents, tumors, drugs or hormones); and gene therapy reagents.

In certain embodiments, the hydrogel comprises the prodrug described
10 elsewhere herein. For example, in one embodiment, the hydrogel comprises crosslinked PEG macromer, wherein the PEG macromers are linked to prodrug comprising at least one cleavage domain and at least one therapeutic domain.

PEG hydrogels have been used to deliver therapeutic molecules such as peptides and proteins, and offer numerous advantages as controlled release systems
15 (Slaughter et al., 2009, *Adv Mater.* 21(32-33):3307-29). PEG hydrogels are highly hydrophilic, inert, and biocompatible. Additionally, due to their synthetic nature, PEG hydrogels have highly tunable degradation profiles and mechanical properties (Lin et al., 2009, *Pharm Res.* 26(3):631-43). Moreover, PEG hydrogels can be formed using a number of synthetic schemes that are compatible with peptide incorporation. For
20 example, norbornene-functionalized PEG (PEGN) can be reacted with thiol-containing crosslinkers to form hydrogel networks via step-growth photopolymerizations (Fairbanks et al., 2009, *Adv Mater.* 21(48):5005-10). This polymerization strategy is cytocompatible, produces homogeneous PEG-peptide networks, and allows for facile incorporation of peptides that include cysteine (thiol R-group) amino acids (Fairbanks et al., 2009, *Adv*
25 *Mater.* 21(48):5005-10). Thiol-ene based PEG hydrogels can also be rendered enzymatically-degradable through the incorporation of proteolytically-responsive peptide crosslinkers (Patterson et al., 2010, *Biomaterials.* 31(30):7836-45). Enzymatically-responsive peptide sequences have been used to control the temporal availability of the cell adhesion peptide RGD within non-degradable PEG hydrogels (Salinas et al., 2008,
30 *Biomaterials.* 29(15):2370-7), and to provide responsive release of tethered vascular endothelial growth factor (VEGF) (Phelps et al., 2010, *P Natl Acad Sci USA*

107(8):3323-8). When employed to treat a mouse model of hindlimb ischemia, enzymatically-responsive VEGF delivery results in greater vascular reperfusion compared to bolus injection of VEGF, presumably due to extended therapeutic protein delivery (Phelps et al., 2010, P Natl Acad Sci USA 107(8):3323-8).

5 In certain embodiments, one or more multifunctional cross-linking agents may be utilized as reactive moieties that covalently link biopolymers or synthetic polymers. Such bifunctional cross-linking agents may include glutaraldehyde, epoxides (e.g., bis-oxiranes), oxidized dextran, p-azidobenzoyl hydrazide, N-[α -maleimidoacetoxy]succinimide ester, p-azidophenyl glyoxal monohydrate, bis-[β -(4-
10 azidosalicylamido)ethyl]disulfide, bis[sulfosuccinimidyl]suberate, dithiobis[succinimidyl]proprionate, disuccinimidyl suberate, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) and other bifunctional cross-linking reagents known to those skilled in the art. It should be appreciated by those in skilled in the art that the mechanical properties of the hydrogel are greatly influenced by the cross-
15 linking time and the amount of cross-linking agents.

In another embodiment utilizing a cross-linking agent, polyacrylated materials, such as ethoxylated (20) trimethylpropane triacrylate, may be used as a non-specific photo-activated cross-linking agent. Components of an exemplary reaction mixture would include a thermoreversible hydrogel held at 39°C, polyacrylate
20 monomers, such as ethoxylated (20) trimethylpropane triacrylate, a photo-initiator, such as eosin Y, catalytic agents, such as 1-vinyl-2-pyrrolidinone, and triethanolamine. Continuous exposure of this reactive mixture to long-wavelength light (>498 nm) would produce a cross-linked hydrogel network.

In one embodiment, the hydrogel comprises a UV sensitive curing agent
25 which initiates hydrogel polymerization. For example, in one embodiment, a hydrogel comprises the photoinitiator 4-(2-hydroxyethoxy)phenyl-(2-hydroxy-2-propyl)ketone. In one embodiment, polymerization is induced by 4-(2-hydroxyethoxy)phenyl-(2-hydroxy-2-propyl)ketone upon application of UV light. Other examples of UV sensitive curing agents include 2-hydroxy-2-methyl-1-phenylpropan-2-one, 4-(2-hydroxyethoxy)phenyl
30 (2-hydroxy-2-phenyl-2-hydroxy-2-propyl)ketone, 2,2-dimethoxy-2-phenyl-acetophenone 1-[4-(2-Hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propane-1-one, 1-

hydroxycyclohexylphenyl ketone, trimethyl benzoyl diphenyl phosphine oxide and mixtures thereof. In one embodiment, the polymerization of the hydrogel is induced by the lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) photoinitiator.

5 The stabilized cross-linked hydrogel matrix may be further stabilized and enhanced through the addition of one or more enhancing agents. By “enhancing agent” or “stabilizing agent” is intended any compound added to the hydrogel matrix, in addition to the high molecular weight components, that enhances the hydrogel matrix by providing further stability or functional advantages. Suitable enhancing agents, which are admixed with the high molecular weight components and dispersed within the hydrogel matrix, 10 include many of the additives described earlier in connection with the thermoreversible matrix discussed above. The enhancing agent can include any compound, especially polar compounds, that, when incorporated into the cross-linked hydrogel matrix, enhance the hydrogel matrix by providing further stability or functional advantages.

Preferred enhancing agents for use with the stabilized cross-linked 15 hydrogel matrix include polar amino acids, amino acid analogues, amino acid derivatives, intact collagen, and divalent cation chelators, such as ethylenediaminetetraacetic acid (EDTA) or salts thereof. Polar amino acids are intended to include tyrosine, cysteine, serine, threonine, asparagine, glutamine, aspartic acid, glutamic acid, arginine, lysine, and histidine. The preferred polar amino acids are L-cysteine, L-glutamic acid, L-lysine, and 20 L-arginine. Suitable concentrations of each particular preferred enhancing agent are the same as noted above in connection with the thermoreversible hydrogel matrix. Polar amino acids, EDTA, and mixtures thereof, are preferred enhancing agents. The enhancing agents can be added to the matrix composition before or during the crosslinking of the high molecular weight components.

25 The enhancing agents are particularly important in the stabilized cross-linked bioactive hydrogel matrix because of the inherent properties they promote within the matrix. The hydrogel matrix exhibits an intrinsic bioactivity that will become more evident through the additional embodiments described hereinafter. It is believed the intrinsic bioactivity is a function of the unique stereochemistry of the cross-linked 30 macromolecules in the presence of the enhancing and strengthening polar amino acids, as well as other enhancing agents.

In one embodiment, a method comprising manufacture of a hydrogel comprising the prodrug described herein is provided. Manufacture of a hydrogel may comprise any known methods or techniques known in the art. For example, in certain embodiments, the method comprises forming a solution comprising the prodrug and
5 optionally, one or more suitable biopolymers or synthetic polymers. In certain embodiments, the manufacture of the hydrogel comprises administering of a crosslinker to a hydrogel solution. In some embodiments, the hydrogel is fabricated by emulsification, photolithography, microfluidic synthesis, micromolding, or micro-electrospinning, or a combination thereof. The hydrogel can have a structure, e.g.,
10 including one or more of a film, pad, cylinder, tube, micro thin film, a micro pad, a micro thin fiber, a nanosphere or a microsphere. The hydrogel may be formed to be of any size or geometry as needed by its application. For example, the hydrogel may be formed with a pre-determined size and geometry, or alternatively may be cut into a desired size and geometry after formation. The hydrogel may be formed to comprise any suitable amount
15 of the prodrug. For example, the concentration of the prodrug within the hydrogel may be altered by increasing or decreasing the amount of prodrug added to a hydrogel solution. The desired amount of the prodrug comprised in the hydrogel depends on the activity of the released therapeutic agent, the disease state of the patient, and the drug delivery characteristics of the released therapeutic agent. In certain embodiments, the hydrogel is
20 formulated to provide sustained release of the therapeutic agent. For example, in certain embodiments, the hydrogel provides sustained release of the therapeutic agent for at least 1 hour, 1 day, 3 days, 1 week, 2 weeks, 1 month, 3 months, 6 months, 1 year, 5 years, 10 years, or more. In one embodiment, the release rate of the therapeutic agent is dependent on the type of cleavage domain and the stimuli which cleave the cleavage domain, as
25 described elsewhere herein. For example, in certain embodiments, the release characteristics of the therapeutic agent are dependent on the expression, activity, or both of MMPs within the target tissue.

In certain embodiments, the hydrogel is modified to improve its functionality. For example, the hydrogel may be coated with any number of compounds
30 in order enhance its biocompatibility, reduce its immunogenicity, enhance stability, enhance degradation, and/or enhance drug delivery.

The hydrogel may be shaped into any number of desirable configurations to satisfy any number of overall system, geometry or space restrictions. For example, the matrix or hydrogel may be shaped to conform to the dimensions and shapes of the whole or a part of the tissue. The hydrogel may be shaped in different sizes and shapes to
5 conform to the organs of differently sized patients. The matrix or hydrogel may also be shaped in other fashions to accommodate the special needs of the patient.

In certain embodiments, the hydrogel comprises one or more cells. For example, in certain embodiments, the hydrogel comprises one or more cells embedded within the hydrogel. In one embodiment, the hydrogel comprises one or more cells on the
10 hydrogel surface.

In one embodiment, the hydrogel is seeded with one or more populations of cells. The cells may be autologous, where the cell populations are derived from the subject's own tissue, or allogenic, where the cell populations are derived from another subject within the same species as the patient. The cells may also be xenogenic, where
15 the different cell populations are derived from a mammalian species that is different from the subject. For example the cells may be derived from organs of mammals such as humans, monkeys, dogs, cats, mice, rats, cows, horses, pigs, goats and sheep.

Cells may be isolated from a number of sources, including, for example, biopsies from living subjects and whole-organ recover from cadavers. The isolated cells
20 are preferably autologous cells, obtained by biopsy from the subject intended to be the recipient. For example, a biopsy of skeletal muscle from the arm, forearm, or lower extremities, or smooth muscle from the area treated with local anesthetic with a small amount of lidocaine injected subcutaneously, and expanded in culture. The biopsy may be obtained using a biopsy needle, a rapid action needle which makes the procedure
25 quick and simple.

Cells may be isolated using techniques known to those skilled in the art. For example, the tissue or organ may be disaggregated mechanically and/or treated with digestive enzymes and/or chelating agents that weaken the connections between
neighboring cells making it possible to disperse the tissue into a suspension of individual
30 cells without appreciable cell breakage.

Preferred cell types include, but are not limited to, mesenchymal cells, especially smooth or skeletal muscle cells, endothelial cells, endothelial progenitor cells, myocytes (muscle stem cells), fibroblasts, chondrocytes, adipocytes, fibromyoblasts, and ectodermal cells, including ductile and skin cells, hepatocytes, Islet cells, cells present in
5 the intestine, and other parenchymal cells, osteoblasts and other cells forming bone or cartilage. In some cases, it may also be desirable to include nerve cells. In other cases, it may be desirable to include stem cells, including for example, embryonic stem cells, adult stem cells, mesenchymal stem cells, hematopoietic stem cells, induced pluripotent stem cells, cord blood derived stem cells, and the like.

10 Isolated cells may be cultured in vitro to increase the number of cells available for coating the biocompatible scaffold. The use of allogenic cells, and more preferably autologous cells, is preferred to prevent tissue rejection. However, if an immunological response does occur in the subject after implantation of the artificial organ, the subject may be treated with immunosuppressive agents such as, cyclosporin or
15 FK506, to reduce the likelihood of rejection. In certain embodiments, chimeric cells, or cells from a transgenic animal, may be coated onto the biocompatible scaffold.

Isolated cells may be normal or genetically engineered to provide additional or normal function. Methods for genetically engineering cells with retroviral vectors, polyethylene glycol, or other methods known to those skilled in the art may be
20 used. These include using expression vectors which transport and express nucleic acid molecules in the cells. (See Goeddel; Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, Calif. (1990).

DNA or RNA may be introduced into cells via conventional transformation or transfection techniques. Suitable methods for transforming or
25 transfecting host cells can be found in Sambrook et al. (Molecular Cloning: A Laboratory Manual, 3rd Edition, Cold Spring Harbor Laboratory press (2012)), and other laboratory textbooks.

Seeding of cells onto the matrix or hydrogel may be performed according to standard methods. For example, the seeding of cells onto polymeric substrates for use
30 in tissue repair has been reported (see, e.g., Atala, A. et al., J. Urol. 148(2 Pt 2): 658-62 (1992); Atala, A., et al. J. Urol. 150 (2 Pt 2): 608-12 (1993)). Cells grown in culture may

be trypsinized to separate the cells, and the separated cells may be seeded on the hydrogel. Alternatively, cells obtained from cell culture may be lifted from a culture plate as a cell layer, and the cell layer may be directly seeded onto the hydrogel without prior separation of the cells. In certain embodiments, a seeded hydrogel is formed by adding one or more isolated cells to a hydrogel precursor solution, such that polymerization of the precursor solution results in a polymerized hydrogel with the isolated cell or cells encapsulated or embedded within the hydrogel.

In order to facilitate cell growth on the scaffold, the hydrogel may be coated with one or more cell adhesion-enhancing agents. These agents include but are not limited collagen, laminin, and fibronectin. The hydrogel may also contain cells cultured on the hydrogel to form a target tissue substitute. The target tissue that may be formed using the scaffold may be an arterial blood vessel, wherein an array of microfibers is arranged to mimic the configuration of elastin in the medial layer of an arterial blood vessel.

Peptides

In certain embodiments, the composition includes an isolated peptide. For example in one embodiment, the composition comprises a prodrug, comprising at least one cleavage domain and at least one therapeutic domain. In one embodiment the at least one cleavage domain, the at least one therapeutic domain, or both comprise a peptide. In one embodiment, the prodrug is an isolated peptide comprising at least one cleavage domain and at least one therapeutic domain. In one embodiment, cleavage of the peptide at the one or more cleavage domains releases a therapeutic peptide comprising the therapeutic domain from the composition. In one embodiment, the at least one cleavable domain comprises the amino acid sequence IPESLRAG (SEQ ID NO: 1), which when cleaved forms the fragments IPES (SEQ ID NO: 2) and LRAG (SEQ ID NO: 3). In certain embodiments, the therapeutic domain comprises a pro-angiogenic amino acid sequence. The pro-angiogenic amino acid sequence may be selected from the group consisting of Qk (KLTWQELYQLKYKGI; SEQ ID NO: 4), SPARC₁₁₃ (TLEGTKKGHLHLDY; SEQ ID NO: 5), and SPARC₁₁₈ (KKGHK; SEQ ID NO: 6).

As described elsewhere herein, in certain embodiments, upon cleavage of the cleavable domains, a therapeutic peptide is released from the matrix. Therefore, in certain embodiments, the released therapeutic peptide comprises at least therapeutic domain, as well as a fragment of a first cleavable domain located at the N-terminus of the therapeutic peptide and a fragment of the second cleavable domain located at the C-terminus of the therapeutic peptide. For example, in one embodiment, the released therapeutic peptide comprises SEQ ID NO: 3 at the N-terminus. In one embodiment, the released therapeutic peptide comprises SEQ ID NO: 2 at the C-terminus.

In one embodiment, the peptide of the matrix comprises the amino acid sequence of IPESLRAGKLTWQELYQLKYKGIPESLRAG (SEQ ID NO: 7), such that the released therapeutic peptide comprises the amino acid sequence of LRAGKLTWQELYQLKYKGIPES (Qk(2T); SEQ ID NO: 8).

In one embodiment, the peptide of the matrix comprises the amino acid sequence of IPESLRAGTLEGTKKGHKLHLDYIPESLRAG (SEQ ID NO: 9), such that the released therapeutic peptide comprises the amino acid sequence of LRAGTLEGTKKGHKLHLDYIPES (SPARC₁₁₃(2T); SEQ ID NO: 10).

In one embodiment, the peptide of the matrix comprises the amino acid sequence of IPESLRAGKKGHKIPESLRAG (SEQ ID NO: 11), such that the released therapeutic peptide comprises the amino acid sequence of LRAGKKGHKIPES (SPARC₁₁₈(2T); SEQ ID NO: 12).

In one embodiment, the peptide of the matrix comprises terminal cysteine residues, which in certain instances, allow for linkage to the norbornene functionalized PEG. For example, in certain embodiments, the peptide of the matrix comprises the amino acid sequence of CIPESLRAGKLTWQELYQLKYKGIPESLRAGC (SEQ ID NO: 13), CIPESLRAGTLEGTKKGHKLHLDYIPESLRAGC (SEQ ID NO: 14), or CIPESLRAGKKGHKIPESLRAGC (SEQ ID NO: 15).

The peptide also encompasses peptide variants. The variants of the peptides may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, (ii) one in which there are one or more modified amino acid residues,

e.g., residues that are modified by the attachment of substituent groups, (iii) one in which the peptide is an alternative splice variant of the peptide, (iv) fragments of the peptides and/or (v) one in which the peptide is fused with another peptide, such as a leader or secretory sequence or a sequence which is employed for purification (for example, His-tag) or for detection (for example, Sv5 epitope tag). The fragments include peptides
5 generated via proteolytic cleavage (including multi-site proteolysis) of an original sequence. Variants may be post-translationally, or chemically modified. Such variants are deemed to be within the scope of those skilled in the art from the teaching herein.

As known in the art the “similarity” between two peptides is determined
10 by comparing the amino acid sequence and its conserved amino acid substitutes of one peptide to a sequence of a second peptide. Variants are defined to include peptide sequences different from the original sequence, preferably different from the original sequence in less than 40% of residues per segment of interest, more preferably different from the original sequence in less than 25% of residues per segment of interest, more
15 preferably different by less than 10% of residues per segment of interest, most preferably different from the original protein sequence in just a few residues per segment of interest and at the same time sufficiently homologous to the original sequence to preserve the functionality of the original sequence and/or the ability to bind to ubiquitin or to a ubiquitylated protein. The compositions described herein includes amino acid sequences
20 that are at least 60%, 65%, 70%, 72%, 74%, 76%, 78%, 80%, 90%, or 95% similar or identical to the original amino acid sequence. The degree of identity between two peptides is determined using computer algorithms and methods that are widely known for the persons skilled in the art. The identity between two amino acid sequences is
25 preferably determined by using the BLASTP algorithm [BLAST Manual, Altschul, S., et al., NCBI NLM NIH Bethesda, Md. 20894, Altschul, S., et al., J. Mol. Biol. 215: 403-410 (1990)].

The peptides can be post-translationally modified. For example, post-translational modifications include signal peptide cleavage, glycosylation, acetylation, isoprenylation, proteolysis, myristoylation, protein folding and proteolytic processing,
30 etc. Some modifications or processing events require introduction of additional biological machinery. For example, processing events, such as signal peptide cleavage and core

glycosylation, are examined by adding canine microsomal membranes or *Xenopus* egg extracts (U.S. Pat. No. 6,103,489) to a standard translation reaction.

The peptides may include unnatural amino acids formed by post-translational modification or by introducing unnatural amino acids during translation. A variety of approaches are available for introducing unnatural amino acids during protein translation. By way of example, special tRNAs, such as tRNAs which have suppressor properties, suppressor tRNAs, have been used in the process of site-directed non-native amino acid replacement (SNAAR). In SNAAR, a unique codon is required on the mRNA and the suppressor tRNA, acting to target a non-native amino acid to a unique site during the protein synthesis (described in WO90/05785). However, the suppressor tRNA must not be recognizable by the aminoacyl tRNA synthetases present in the protein translation system. In certain cases, a non-native amino acid can be formed after the tRNA molecule is aminoacylated using chemical reactions which specifically modify the native amino acid and do not significantly alter the functional activity of the aminoacylated tRNA. These reactions are referred to as post-aminoacylation modifications. For example, the epsilon-amino group of the lysine linked to its cognate tRNA (tRNA_{LYS}), could be modified with an amine specific photoaffinity label.

A peptide may be conjugated with other molecules, such as proteins, to prepare fusion proteins. This may be accomplished, for example, by the synthesis of N-terminal or C-terminal fusion proteins provided that the resulting fusion protein retains the functionality of the peptide.

In one embodiment, the composition comprises cyclic derivatives of the peptides or chimeric proteins. Cyclization may allow the peptide or chimeric protein to assume a more favorable conformation for association with other molecules. Cyclization may be achieved using techniques known in the art. For example, disulfide bonds may be formed between two appropriately spaced components having free sulfhydryl groups, or an amide bond may be formed between an amino group of one component and a carboxyl group of another component. Cyclization may also be achieved using an azobenzene-containing amino acid as described by Ulysse, L., et al., J. Am. Chem. Soc. 1995, 117, 8466-8467. The components that form the bonds may be side chains of amino acids, non-amino acid components or a combination of the two. In an embodiment, cyclic peptides

may comprise a beta-turn in the right position. Beta-turns may be introduced into the peptides by adding the amino acids Pro-Gly at the right position.

It may be desirable to produce a cyclic peptide which is more flexible than the cyclic peptides containing peptide bond linkages as described above. A more flexible peptide may be prepared by introducing cysteines at the right and left position of the peptide and forming a disulphide bridge between the two cysteines. The two cysteines are arranged so as not to deform the beta-sheet and turn. The peptide is more flexible as a result of the length of the disulfide linkage and the smaller number of hydrogen bonds in the beta-sheet portion. The relative flexibility of a cyclic peptide can be determined by molecular dynamics simulations.

In other embodiments, the subject peptide therapeutic agents are peptidomimetics of the peptide. Peptidomimetics are compounds based on, or derived from, peptides and proteins. The peptidomimetics typically can be obtained by structural modification of a known peptide inhibitor sequence using unnatural amino acids, conformational restraints, isosteric replacement, and the like. The subject peptidomimetics constitute the continuum of structural space between peptides and non-peptide synthetic structures; peptidomimetics may be useful, therefore, in delineating pharmacophores and in helping to translate peptides into nonpeptide compounds with the activity of the parent peptides.

Moreover, as is apparent from the present disclosure, mimetopes of the subject peptide can be provided. Such peptidomimetics can have such attributes as being non-hydrolyzable (e.g., increased stability against proteases or other physiological conditions which degrade the corresponding peptide), increased specificity and/or potency, and increased cell permeability for intracellular localization of the peptidomimetic. For illustrative purposes, peptide analogs can be generated using, for example, benzodiazepines (e.g., see Freidinger et al. in *Peptides: Chemistry and Biology*, G. R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gamma lactam rings (Garvey et al. in *Peptides: Chemistry and Biology*, G. R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988, p123), C-7 mimics (Huffman et al. in *Peptides: Chemistry and Biology*, G. R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988, p. 105), keto-methylene pseudopeptides (Ewenson et al. (1986) *J Med*

Chem 29:295; and Ewenson et al. in Peptides: Structure and Function (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, Ill., 1985), β -turn dipeptide cores (Nagai et al. (1985) Tetrahedron Lett 26:647; and Sato et al. (1986) J Chem Soc Perkin Trans 1:1231), β -aminoalcohols (Gordon et al. (1985) Biochem Biophys Res Commun 126:419; and Dann et al. (1986) Biochem Biophys Res Commun 134:71), diaminoketones (Natarajan et al. (1984) Biochem Biophys Res Commun 124:141), and methyleneamino-modified (Roark et al. in Peptides: Chemistry and Biology, G. R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988, p134). Also, see generally, Session III: Analytic and synthetic methods, in in Peptides: Chemistry and Biology, G. R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988)

In addition to a variety of side chain replacements which can be carried out to generate the peptidomimetics, the compositions specifically contemplate the use of conformationally restrained mimics of peptide secondary structure. Numerous surrogates have been developed for the amide bond of peptides. Frequently exploited surrogates for the amide bond include the following groups (i) trans-olefins, (ii) fluoroalkene, (iii) methyleneamino, (iv) phosphonamides, and (v) sulfonamides.

Moreover, other examples of mimetopes include, but are not limited to, protein-based compounds, carbohydrate-based compounds, lipid-based compounds, nucleic acid-based compounds, natural organic compounds, synthetically derived organic compounds, anti-idiotypic antibodies and/or catalytic antibodies, or fragments thereof. A mimetope can be obtained by, for example, screening libraries of natural and synthetic compounds for compounds capable of binding to the peptide inhibitor. A mimetope can also be obtained, for example, from libraries of natural and synthetic compounds, in particular, chemical or combinatorial libraries (i.e., libraries of compounds that differ in sequence or size but that have the same building blocks). A mimetope can also be obtained by, for example, rational drug design. In a rational drug design procedure, the three-dimensional structure of a compound can be analyzed by, for example, nuclear magnetic resonance (NMR) or x-ray crystallography. The three-dimensional structure can then be used to predict structures of potential mimetopes by, for example, computer modelling, the predicted mimetope structures can then be produced by, for example,

chemical synthesis, recombinant DNA technology, or by isolating a mimetope from a natural source (e.g., plants, animals, bacteria and fungi).

A peptide, or chimeric protein, may be synthesized by conventional techniques. For example, the peptide inhibitors or chimeric proteins may be synthesized
5 by chemical synthesis using solid phase peptide synthesis. These methods employ either solid or solution phase synthesis methods (see for example, J. M. Stewart, and J. D. Young, Solid Phase Peptide Synthesis, 2nd Ed., Pierce Chemical Co., Rockford Ill. (1984) and G. Barany and R. B. Merrifield, The Peptides: Analysis Synthesis, Biology editors E. Gross and J. Meienhofer Vol. 2 Academic Press, New York, 1980, pp. 3-254 for solid
10 phase synthesis techniques; and M Bodansky, Principles of Peptide Synthesis, Springer-Verlag, Berlin 1984, and E. Gross and J. Meienhofer, Eds., The Peptides: Analysis, Synthesis, Biology, suprs, Vol 1, for classical solution synthesis.)

Peptides may be developed using a biological expression system. The use of these systems allows the production of large libraries of random peptide sequences and
15 the screening of these libraries for peptide sequences that bind to particular proteins. Libraries may be produced by cloning synthetic DNA that encodes random peptide sequences into appropriate expression vectors. (see Christian et al 1992, J. Mol. Biol. 227:711; Devlin et al, 1990 Science 249:404; Cwirla et al 1990, Proc. Natl. Acad, Sci. USA, 87:6378). Libraries may also be constructed by concurrent synthesis of overlapping
20 peptides (see U.S. Pat. No. 4,708,871).

The peptides and chimeric proteins may be converted into pharmaceutical salts by reacting with inorganic acids such as hydrochloric acid, sulfuric acid, hydrobromic acid, phosphoric acid, etc., or organic acids such as formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, succinic acid, malic
25 acid, tartaric acid, citric acid, benzoic acid, salicylic acid, benzenesulfonic acid, and toluenesulfonic acids.

Therapeutic Methods

Described herein is a method for treating a disease or disorder comprising
30 a stimuli-induced delivery of a therapeutic agent. The methods are not limited to the treatment or prevention of any particular disease or disorder. Rather, the methods

encompass the treatment of any disease or disorder treatable the activity of a particular therapeutic agent. For example, the compositions described elsewhere herein may be designed and constructed to comprise any prodrug comprising at least one therapeutic domain and at least one cleavage domain. In certain embodiments, the cleavage of the cleavage domain induces the release of a therapeutic agent. In certain embodiments, the cleavage of the cleavage domain is dependent upon a particular stimulus associated with the disease or disorder to be treated. In one embodiment, the stimulus is present specifically at the treatment site and at the time in which the therapeutic agent is needed. Thus, a method for stimuli-induced treatments is provided herein.

In one embodiment, a method for promoting vascularization at a treatment site is provided, comprising the stimuli-induced release of a pro-angiogenic agent. For example, in certain embodiments, a method for treating a disease or disorder associated with reduced vascularization or blood flow is provided. Exemplary disorders treatable by the method includes, but is not limited to, cardiac ischemia, coronary heart failure, peripheral vascular ischemia, peripheral arterial disease, ischemic bowel disease, bone fracture healing, and diabetic ulcers.

In certain embodiments, the controlled and local delivery of pro-angiogenic therapeutic agents by way of the method is used to promote the vascularization of engineered tissue. The method could also be used to enhance vascularization of transplanted cells and tissues, increasing viability of the tissue and the therapeutic efficacy of the treatment. In one embodiment, the method can be used to promote angiogenesis or vascularization of a tissue engineered construct ex vivo prior to implantation of the construct. In one embodiment, the method may be used to promote angiogenesis or vascularization of a tissue engineered construct in vivo during or after implantation. Particularly, tissue engineering and regenerative medicine strategies of tissues could benefit from the angiogenic therapy provided by the methods described herein, to promote host vascularization, because simple diffusion is insufficient to deliver nutrients and remove waste from these constructs.

In one embodiment, a method for treating cancer in a subject is provided comprising the stimuli-induced release of an anti-cancer or anti-tumor agent. For example, in certain instances MMPs or other cleavage enzymes are upregulated in the

tumor microenvironment. Thus, the method provides for the stimuli-induced release of an anti-cancer agent from a matrix in order to treat cancer. Exemplary cancers treatable by way of the method include, but are not limited to carcinoma, blastoma, and sarcoma, and certain leukemia or lymphoid malignancies, benign and malignant tumors, and
5 malignancies *e.g.*, sarcomas, carcinomas, and melanomas. Adult tumors/cancers and pediatric tumors/cancers are also included.

In one embodiment, a method for treating inflammation in a subject is provided, comprising the stimuli-reduced release of an anti-inflammatory agent. For example, the method may be used to treat acute or chronic inflammation, an autoimmune
10 disease, or other inflammatory disorders.

In one embodiment, the method comprises forming a matrix described herein. For example, in one embodiment, the method comprises polymerizing matrix materials, including for example a plurality of synthetic monomers and a plurality of prodrugs, into a matrix. In certain embodiments, the polymerization of the matrix
15 comprises the use of thiol-norbornene chemistry to crosslink the matrix. In one embodiment, polymerization of the matrix is induced by administration of UV-light. Matrix formation may be performed in an *ex vivo* or *in vivo* environment. The relative amount or concentration of the prodrug may be varied depending on the particular application, treatment site, disease severity, and the like.

The composition and method described herein allows for a high density of prodrug to be incorporated into a polymerized matrix. For example, in one embodiment, the ratio of prodrug to synthetic monomer in the composition is greater than about 1:1. In one embodiment, the ratio of prodrug to synthetic monomer in the composition is greater than about 2:1. In one embodiment, the ratio of prodrug to synthetic monomer in the
20 composition is greater than about 4:1. In one embodiment, the ratio of prodrug to synthetic monomer in the composition is greater than about 8:1. In one embodiment, the ratio of prodrug to synthetic monomer in the composition is greater than about 16:1.
25

In one embodiment, the method comprises administering a hydrogel comprising a polymerized matrix to a treatment site. In one embodiment, the method
30 comprises forming a suitable hydrogel *ex vivo* and then administering the hydrogel to a desired location in a patient or subject in need. In another embodiment, the method

comprises administering a solution comprising hydrogel matrix material or precursors to a location within the patient or subject, followed by inducing the polymerization of the hydrogel in vivo.

In certain embodiments, the hydrogel composition is administered to an
5 ischemic treatment site, or a site in need of angiogenesis or vascularization. As described elsewhere herein, in regions of ischemia, the expression, activity, or both, of MMPs are enhanced, which cleave the cleavage domain and release the therapeutic agent from the hydrogel. Thus, the therapeutic agent is released only in regions of ischemia and is thereby able to promote angiogenesis at the treatment site. In some instances, the
10 hydrogel may also be embedded with one or more additional factors that are released from the hydrogel upon either hydrogel degradation or prodrug cleavage. Such factors include, but are not limited to, growth factors, hormones, immunosuppressive agents, antibiotics, bacteriocides, fungicides, proteins, and the like. In some embodiments, the hydrogel is embedded with cells, including for example, endothelial cells, progenitor
15 cells, and the like, that would aid promoting angiogenesis at the site.

The hydrogel may be administered to a patient or subject in need in a wide variety of ways. Modes of administration include intravenous, intravascular, intramuscular, subcutaneous, intracerebral, intraperitoneal, soft tissue injection, surgical placement, arthroscopic placement, and percutaneous insertion, e.g. direct injection,
20 cannulation or catheterization. The methods described herein result in localized administration of the therapeutic agent comprising hydrogel to the site or sites in need of treatment. Any administration may be a single application of a composition or multiple applications. Administrations may be to single site or to more than one site in the individual to be treated. Multiple administrations may occur essentially at the same time
25 or separated in time.

Although the description of compositions provided herein is principally directed to compositions which are suitable for administration to humans, it will be understood by the skilled artisan that such compositions are generally suitable for administration to animals of all sorts. Modification of compositions suitable for
30 administration to humans in order to render the compositions suitable for administration to various animals is well understood, and the ordinarily skilled veterinary

pharmacologist can design and perform such modification with merely ordinary, if any, experimentation. Subjects to which administration of the pharmaceutical compositions is contemplated include, but are not limited to, humans and other primates, mammals including commercially relevant mammals such as non-human primates, cattle, pigs, horses, sheep, cats, and dogs.

EXPERIMENTAL EXAMPLES

The compositions and methods are further described in detail by reference to the following experimental examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. Thus, the compositions and methods should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the compositions and practice the claimed methods. The following working examples therefore are not to be construed as limiting in any way the remainder of the disclosure.

The materials and methods employed in these experiments are now described.

Peptide synthesis

Peptides (Table 1 and Table 3) were synthesized on Fmoc-Gly-Wang resin (EMD) using a Liberty1 automated peptide synthesizer with UV monitoring (CEM). Amino acids (AAPPTec) were prepared at 0.2 M in N-methylpyrrolidone (NMP). 5% piperazine (Alfa Aesar) in dimethylformamide (DMF, Fisher Scientific) was used for deprotection, 0.5 M O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluorophosphate (HBTU, AnaSpec) in DMF was used as the activator, and 2 M diisopropylethylamine (DIEA, Alfa Aesar) in NMP was used as the activator base, except for Qk(DL), SPARC₁₁₃(DL), and SPARC_{3X}(DL) where 0.5 M diisopropylcarbodiimide (DIC, Chem-Impex International) in DMF was used as the activator and 1 M

hydroxybenzotriazole (HOBt, Advanced ChemTech) in DMF used as the activator base. Peptides were cleaved from resin in a cleavage cocktail composed of 92.5 vol% trifluoroacetic acid (TFA, Alfa Aesar) and 2.5 vol% each triisopropylsilane (Alfa Aesar), 3,6-Dioxa-1,8-octanedithiol (Alfa Aesar), and distilled, deionized water (ddH₂O) for 2-3
5 hours. For peptides containing arginine, 2.5 vol% thioanisole (Alfa Aesar) was added to the cleavage cocktail and cleavage time increased to 4-5 hours to aid in the removal of Pbf protecting groups. After cleavage, resin was removed by vacuum filtration and peptide solutions were precipitated in ice cold diethyl ether. Peptides were collected by centrifugation, and washed four times in diethyl ether. Expected peptide molecular
10 weights were confirmed using a Bruker AutoflexIII Smartbeam Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-ToF) mass spectrometer, using 50/50 ddH₂O/acetonitrile with 0.1% TFA as the solvent, and α -Cyano-4-hydroxycinnamic acid (Tokyo Chemical Industry) as the matrix, with calibrations performed using the Care peptide standards (Bruker 206195). Peptides were dialyzed in ddH₂O (1-500 or 1000
15 MWCO tubing, Spectrum Laboratories) overnight and collected by lyophilization. Final peptide concentrations were assessed via absorbance at 205 nm using an Evolution 300 UV/Vis detector (Thermo Scientific) (Anthis et al., 2013, Protein Sci. 22(6):851-8). Solid peptide and stock peptide solutions in PBS were stored at -20 °C until use.

20 Norbornene functionalization of poly(ethylene glycol)

8-arm 10 kDa , 8-arm 20 kDa and 4-arm 10 kDa PEG (JenKem Technologies USA) were functionalized with terminal norbornene groups as previously described (Fairbanks et al., 2009, Adv Mater, 21: 5005-5010). PEG was dissolved in dichloromethane (DCM) containing 5 molar excess (to PEG hydroxyl groups) pyridine
25 and 0.5 molar excess 4-(dimethylamino)pyridine (DMAP), and stirred at room temperature until completely dissolved. In a separate reaction vessel, 5 molar excess N,N'-dicyclohexylcarbodiimide (DCC) was combined with 5 molar excess 5-norbornene-2-carboxylic acid in DCM and stirred at room temperature for 30 minutes, where formation of a white precipitate (dicyclohexylurea) indicated formation of dinorbornene
30 carboxylic acid anhydride. The PEG, pyridine, DMAP solution was then added drop-wise to the norbornene-containing reaction vessel. The reaction vessel was then covered with a

rubber septa pierced with a needle and stirred overnight at room temperature. The mixture was then filtered via vacuum filtration, and the filtrate precipitated in ice-cold diethyl ether. The product was then collected by filtration, dissolved in chloroform, and re-precipitated in diethyl ether twice. Functionalization efficiency was assessed with ¹H-NMR with a Bruker Avance 400 MHz spectrometer, using deuterated chloroform as the solvent, by comparing the peaks from the double bond on the norbornene (5.9-6.2 ppm) and those from the PEG backbone (3.6 ppm).

4-arm 10 kDa PEG (JenKem Technologies USA) was functionalized with norbornene as previously described (Fairbanks et al., 2009, Adv Mater. 21(48):5005-10). Functionalization was determined (> 95%) with ¹H-NMR using a Bruker Avance 400 MHz spectrometer, and deuterated chloroform as the solvent (δ = 6.25 - 5.8 ppm (8 H/molecule, norbornene vinyl protons, multiplet), 4.35 – 4.05 ppm (8 H/molecule, -COOCH₂-, doublet), 3.9 – 3.35 ppm (892 H/molecule, -CH₂CH₂O-, multiplet)). The final product was dialyzed overnight in ddH₂O, using 1000 MWCO dialysis tubing and collected by lyophilization. Functionalized PEG was stored at -20 °C until use.

Human umbilical vein endothelial cell culture

Cells and cell culture materials were obtained from Lonza unless otherwise noted. HUVECs were cultured in Endothelial Growth Media 2 (EGM-2; Endothelial Basal Media-2 (EBM-2) containing EGM-2 SingleQuots) for at least two passages after thawing from cryostorage before use. Cells were maintained at 37 °C with 5% CO₂, split 1:4, and used before passage 10. EBM-2 media with 2.5% fetal bovine serum (Atlanta Biologicals), 100 U/mL penicillin, 100 U/mL streptomycin, and 250 ng/mL amphotericin B (Thermo Scientific) was used as control media for the angiogenic assays employed.

HUVEC tube formation assay

Reduced growth factor Matrigel (BD Biosciences) was thawed overnight on ice at 4 °C, diluted to 7.8 mg/mL with control media, and polymerized via incubation at 37 °C for 30 minutes (150 μ L per well of a 48-well plate). Cells (1.2x10⁵ cells/mL) were suspended in either control media alone, or control media containing peptide drugs

as well as negative and positive controls. Cell solutions (200 μ L/well) were placed on the polymerized Matrigel. Cells were incubated at 37 °C for 8 hours before fluorescent imaging (0.5 μ L/mL calcein AM, Invitrogen) using a temperature and humidity-controlled chamber (Pathology Devices) on a Nikon Eclipse Ti 2000 inverted light microscope (Arnaoutova et al., 2010, Nat Protoc. 5(4):628-35). Fluorescent images were converted to 16-bit greyscale and inverted in ImageJ for quantification using the image analysis software Angioquant (Niemisto et al., 2005, Ieee T Med Imaging. 24(4):549-53). Each plate contained a control media group to account for plate-to-plate variability.

10 HUVEC proliferation assay

HUVECs were suspended in control media at $1.0\text{-}2.0 \times 10^4$ cells/mL. 0.5 mL of the cell solution was seeded in each well of a 24-well plate. To achieve uniform cell adhesion, cells were allowed to adhere at room temperature for 1 hour before being transferred to the incubator (Ryan JA. 2012 Corning guide for identifying and correcting common cell growth problems: Corning Incorporated Life Sciences). Sixteen (16) hours later, cells were washed twice with PBS and treated with 0.5 mL of either control media with or without peptide drugs or controls. A preliminary dose screening study was conducted to identify concentration at which each “N” peptide induced proliferation, and that concentration was used for both the “N” and “2T” treatments. Media was changed daily, and after 72 hours cells were washed twice with PBS, and lysed via sonication in 1x TE buffer (10 mM Tris, 1 mM EDTA in ddH₂O, pH 7.5). DNA content was quantified using the Quant-iT picoGreen dsDNA quantification assay (Invitrogen) on a Tecan infiniteM200 microplate. Treatment with 10nM VEGF (PeproTech) was used as a pro-proliferative positive control (Silva et al., 2010, Biomaterials. 31(6):1235-41) and treatment with Sulforophane (EMD Milipore) (Asakage et al., 2006, Angiogenesis. 9(2):83-91) or scrambled peptide was used as an anti-proliferative negative control for both *in vitro* assays. Each plate contained a control media group to account for plate-to-plate variability.

30 Peptide property predictions

Peptide structure was predicted using the Pepfold 1.5 *de novo* structure prediction server, and displayed in cartoon mode color-coded by group (Thevenet et al., 2012, Nucleic Acids Res. 40(W1):W288-W93). Peptide characteristics (charged/polar uncharged/hydrophobic) were calculated using classifications from Lehninger Principles of Biochemistry (Lehninger et al., 2000, Lehninger principles of biochemistry. 3rd ed. New York: Worth Publishers), as well as the Kyte-Doolittle (Kyte et al., 1982, J Mol Biol. 157(1):105-32) and Hopp-Woods (Hopp et al., 1981, P Natl Acad Sci-Biol. 78(6):3824-8) hydropathy protein characterization methods.

10 Hydrogel formation

Peptide-crosslinked PEG hydrogels were formed via thiol-ene photopolymerizations. Cysteine-terminated peptides and 10 kDa PEGN (4-arm or 8-arm) were dissolved in PBS, with the exception of Qk(DL), which was dissolved in a 50/50 mixture of ddH₂O and acetonitrile, in a 1:1 thiol:ene ratio to form a precursor solution containing 10 wt% PEG and 0.05 wt% lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP, synthesized as previously described (Fairbanks et al., 2009, Biomaterials. 30(35):6702-7)). 40 μ L of hydrogel precursor solution was then injected into a custom cylindrical mold and exposed to 365 nm UV light for 10 minutes (intensity $\sim$ 2.5 mW/cm²), resulting in hydrogel polymerization (final gel diameter $\sim$ 5 mm, height $\sim$ 2 mm). For “2T” peptide diffusive release studies, “2T” peptide was added to the precursor solution at equal molar amounts to the crosslinking peptide NDL.

Hydrogel characterization

Hydrogels were swollen in buffer (50 mM Tricine (Acros Organics), 50 mM NaCl, 10 mM CaCl₂, 50 μ M ZnCl₂ (Alfa Aesar), and 0.05 wt% Brij35 (Alfa Aesar) in ddH₂O, pH 7.4) at 37 °C for 24 hours to achieve equilibrium swelling before characterization. Hydrogel images were collected using a Canon EOS Rebel T2i digital camera. Mass swelling ratio was determined by measuring equilibrium swelling and dry gel mass after lyophilization. The efficiency of peptide incorporation into the hydrogels was measured by collecting the buffer solution and quantifying the amount of peptide released into buffer via absorbance at 205 nm. The amount of peptide incorporated into

the gel but not fully crosslinked was quantified by incubating gels in 1 mL of 0.35 mg/mL Ellman's reagent in PBS for 30 minutes, then measuring absorbance at 405 nm on a Tecan infiniteM200 microplate reader, and comparing to a standard curve generated using peptide alone (Ellman GL. 1959, Arch Biochem Biophys. 82(1):70-7).

5

Hydrogel degradation studies

After formation, hydrogels were stored in buffer 1 mL buffer (10 mM CaCl₂, 50 mM NaCl, 50 μ M ZnCl₂ (Alfa Aesar), 50 mM Tricine (Acros Organics), and 0.05 wt% Brij35 (Alfa Aesar) in ddH₂O, pH 7.4) at 37 °C for 24 hours, at which point solutions were changed to either fresh buffer, or buffer containing 10 nM recombinant human MMP2 (PeproTech). As MMP2 inactivates over time (Patterson et al., 2010, Biomaterials. 31(30):7836-45), MMP2 solutions were collected and replaced with fresh MMP2 every 48 hours. For consistency, buffer solutions were collected and replaced every 48 hours as well. At predetermined time points hydrogel bathing solutions were collected and gels were removed from solution and wet mass measured. Gels were then frozen, lyophilized, and the dry mass measured. Hydrogel swelling ratio was calculated by dividing hydrogel wet mass by dry mass ($Q=M_{\text{wet}}/M_{\text{dry}}$). Hydrogel incubation solutions were stored at -80 °C for subsequent peptide release quantification. Gels were inspected daily until complete degradation occurred, for a maximum of 10 days.

20

Quantification of peptide release

The amount of "2T" peptide released into solution was quantified by High Performance Liquid Chromatography (HPLC, Shimadzu Prominence) with a Kromasil Eternity C18 column (4.6 x 50 mm). Water and acetonitrile containing 1% TFA were used as the mobile phases and samples were run using gradients from 5% to 95% acetonitrile at 0.5 mL/minute. Peptide elution was monitored using a UV/Vis detector (SPA-20AV, Shimadzu Prominence) at 214 nm, and concentrations were determined by integrating peak area and comparing to standard curves generated using the "2T" form of the peptides. The amount of peptide incorporated into the hydrogels was determined by fully degrading the gels in 1 M NaOH prior to HPLC analysis.

30

Formation and hydrogel subcutaneous implantation

To aid in hydrogel localization after *in vivo* degradation, gels were polymerized within a non-degradable silicone sheath. Briefly, reactors were cut from platinum-cured silicone tubing (Thermo Scientific) using a custom cutting mold (outer diameter = 7.94 mm (5/16"), inner diameter = 4.76 mm (3/16"), height = 2.25 mm). 40 μ L hydrogel precursor solution (10 wt% PEGN, 1:1 thiol:ene, 0.05 wt% LAP, 2.8 mM RGD) in PBS was injected into silicone reactors in a custom holder, and polymerized by 10 minute exposure to 365 nm UV light. For diffusive release studies, gels were formed using the Scrambled(DL) peptide as a crosslinker and equimolar amounts of the treatment peptide.

6-8 week old female BALB/c mice were obtained from Taconic (Hudson, NY). Mice were given 1.6 mg/mL acetaminophen in water from 1 day pre- to 3 days post-surgery as analgesia. Anesthesia (60 mg/kg ketamine and 4 mg/kg xylazine) was administered via intraperitoneal injection. Two subcutaneous pockets were formed on each side of the mouse dorsal flank, and one hydrogel implant placed in each pocket with the open circular face of the reactor in contact with the underlying tissue. After various time points, mice were sacrificed by CO₂ inhalation, and the reactors dissected from surrounding tissue for analysis.

Hemoglobin quantification

Hydrogel material and invading tissue was removed from explanted reactors and weighed. Implants were manually homogenized and sonicated in 1 mL Drabkin's reagent (RICAA chemical). Samples were then centrifuged at 14,000 g for 20 minutes and the supernatant filtered through 0.45 μ m polyvinylidene fluoride (PVDF) filters (PerkinElmer), to remove particulates. The hemoglobin concentration in each sample was determined by measure absorbance at 540 nm, and compared to hemoglobin standards (Alfa Aesar) (Barcelos et al., 2004, Inflamm Res, 53: 576-584).

Statistical analysis

Data assembly, normalization, and calculations were performed in Microsoft Excel 2010 v14.0. Figures were produced and statistical analysis performed in

Graphpad Prism 5.04. Peptide bioactivity data was evaluated with two-sample t-tests (Mann-Whitney U when data was non-normal), due to ANOVA overprotecting α and potentially rejecting significance simply due to the large number of peptides being investigated. Gel characterization was analyzed using a one-way ANOVA with Tukey's
5 post-hoc testing. Hydrogel degradation and "2T" peptide release was analyzed using a two-way ANOVA with Bonferroni post-hoc testing. Relationships between peptide characteristics and hydrogel behavior were analyzed using linear regression, and the value of the slope compared to 0 using an F-test. $p < \alpha = 0.05$ was considered significant for all analyses. $n=6-12$ as specified in figure legends; data is presented as mean $\pm$ standard
10 error of measurements (SEM) for all figures and reported values.

Example 1: Hydrogels designed to provide sustained, stimuli-responsive release of pro-angiogenic peptides.

To address the limitation of current drug delivery methods, experiments
15 were conducted to develop and evaluate responsive hydrogels, which provide extended, stimuli-responsive delivery of pro-angiogenic peptides to ischemic tissue (Figure 1). As matrix metalloproteinase (MMPs) are up-regulated in ischemic tissues, peptides can be controllably delivered by linking them to hydrogels through MMP-degradable linkers. Peptide sequences that mimic the function of larger pro-angiogenic proteins were
20 identified and synthesized using solid phase synthesis techniques. The peptides were tested using two *in vitro* angiogenic assays: the human umbilical vein endothelial cell (HUVEC) proliferation and tube formation assays.

The results of the experiments are now described.
25

As described herein, three peptides significantly increased both proliferation and tube formation in both their native form and in the form they would be released from the hydrogel networks: Qk (mimicking VEGF), SPARC₁₁₃, and SPARC₁₁₈ (mimicking the secreted protein acidic and rich in cysteine, SPARC). SPARC₁₁₈ was then
30 incorporated into poly(ethylene glycol) hydrogel networks via MMP-degradable tethers,

and enzymatically-responsive hydrogel degradation and peptide release was demonstrated.

Poly ethylene glycol was chosen as the basis for this biomaterial as its hydrophilic nature makes PEG resistant to protein adsorption, providing it with its inert, biocompatible nature. Additionally, as PEG hydrogels are synthetic rather than naturally derived, they can be easily modified to control properties such as stiffness and degradation rate. Moreover, hydrogels are commonly used for regenerative medicine applications. While there are a number of chemistries which can be used to crosslink PEG hydrogels, a thiol-norbornene chemistry was chosen. This allows for rapid formation of hydrogels under cytocompatible conditions. This crosslinking occurs via step-growth polymerization, creating homogeneous hydrogel networks, and allows for easy incorporation of peptides through the use of thiol-containing cysteine amino acids.

Instead of delivering large angiogenic proteins, the constructs designed and developed herein are used to deliver low molecular weight peptides that mimic the angiogenic potential of these proteins. This allows higher concentrations of more stable angiogenic factors to be delivered to the ischemic tissue.

The use of a stimuli-responsive tether to link the peptides into the hydrogel allows for the extended, controlled release of the peptides to host tissue. This is important as extended delivery of biomolecules has been shown critical in the formation of mature, stable vessels in vivo. Additionally, while the hydrogel provides initial structural support to the infarcted tissue, hydrogel degradation is ultimately desired as even a relatively inert material like PEG can elicit a foreign body response if allowed to remain in the body indefinitely.

The specific MMP-responsive tether used in these experiments was IPESLRAG (SEQ ID NO: 1), where the cleavage site is in between the "S" and "L" residues, thereby, when cleaved producing an IPES (SEQ ID NO: 2) fragment and a LLAG (SEQ ID NO: 3) fragment. This sequence has been optimized for cleavage by MMP-14, but is also susceptible to MMPs 1, 2, 3, 7 & 9. It should be noted that the sequence is cleaved in the center; this means that when the angiogenic peptides are released from the hydrogel, residual amino acids will remain on either side of the pro-angiogenic peptides. As many of the MMPs that this sequence is susceptible to are

expressed at increased level in ischemic tissues, the use of the tether facilitates peptide delivery in an ischemia-dependent manner.

Hydrogels were formed as follows. First, poly (ethylene glycol) (PEG) was functionalized with terminal norbornene groups (PEGN) as previously described (Fairbanks et al., 2009, Adv Mater, 21(48): 5005-5010). PEG was reacted with 4-
5 (dimethylamino)pyridine, pyridine, N,N'-dicyclohexylcarbodiimide, and 5-norbornene-2-carboxylic acid in dichloromethane overnight. The mixture was then filtered and precipitated in diethyl ether. Functionalization efficiency was confirmed with ¹H-NMR.

As shown in Figure 2, hydrogels were formed using thiol-ene
10 photopolymerization (Fairbanks et al., 2009, Adv Mater, 21(48): 5005-5010). 10 wt% PEGN was combined with crosslinking peptides in a 1:1 thiol:ene ratio to form a precursor solution also containing 0.05 wt% lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) photoinitiator. 40 µL of hydrogel precursor solution was then injected into a cylindrical mold and exposed to 365 nm UV light for 10 minutes.

15 Experiments were conducted to evaluate the mechanical properties of hydrogels constructed with various forms of PEG. It is important to match hydrogel stiffness to that of the target tissue, as implanting a material that is significantly stiffer than the host tissue can cause tissue damage. As cardiac tissue has a stiffness varying between 10-20 kPa at the beginning of diastole, and 200 kPa at the end, it was desired to
20 identify the conditions required to form hydrogels with a stiffness of approximately 20 kPa. It was examined whether increasing the number of arms on the PEG macromer from 4 to 8 while keeping arm length constant would increase hydrogel stiffness. Further, it was investigated whether halving the length of each arm would cause a further increase in stiffness. Hydrogels were synthesized using these three PEG precursors (Figure 3) and a
25 non-degradable crosslinking peptide. All hydrogels were produced at 10 wt% PEG, with a 1:1 thiol:ene ratio, and allowed to swell in PBS for 48 hours before mechanical testing. Mechanical testing demonstrated that by increasing the number of arms while keeping arm length constant, the hydrogel stiffness was increased from 1.4 to 9.4 kPa. Further halving the length of each PEG arm further increased the hydrogel stiffness to 17.6 kPa,
30 within the target range for cardiac tissue (Figure 3).

The next step in producing these stimuli-responsive hydrogels was to identify peptides for incorporation. Ten pro-angiogenic peptides were identified as being angiogenic in vitro, in vivo, or both (Table 1). These peptides are derived from a variety of sources, including angiogenic proteins and extracellular matrix molecules.

Peptides were synthesized in both their “native” (as-published) form, and in their “two-tailed” form (2T) - that is, the form they will be released from the hydrogel in, with the residual LLAG amino acids on the N terminus, and IPES amino acids on the C terminus. A scrambled control was also synthesized. Table 2 depicts several of the angiogenic peptides in their 2T form, as well as in a degradable (containing the MMP-sensitive cleavage sequence at both ends), and in a nondegradable control sequence. Peptides were synthesized in house using standard microwave-assisted solid phase peptide synthesis, and correct synthesis confirmed using mass spectrometry. Figure 4 depicts the MALDI-ToF plot demonstrating the correct synthesis of the 2T version of the QK peptide.

Sequence*	Name	Source	Reference
HHHRHSP (SEQ ID NO: 16)	T7	Tie-2 binding region of Angiopoietin	US2011/0097300
KLTWQELYQLKYKGI (SEQ ID NO: 4)	QK	α -helix region of VEGF	Santulli et al., 2009, JoTM
TLEGTKKGHLHLDY (SEQ ID NO: 5)	SPARC ₁₁₃	Basic region of SPARC	Jendraschak and Sage, 1996, Cancer Biology
KKGHK (SEQ ID NO: 6)	SPARC ₁₁₈	²⁺ Cu binding region of SPARC	Jendraschak and Sage, 1996, Cancer Biology
KKGHKKGHKKGHK (SEQ ID NO: 17)	SPARC _{3X}	Triplet of SPARC ₁₁₈	Jendraschak and Sage, 1996, Cancer Biology
MFRKPISTVKA (SEQ ID NO: 18)	Col4-1	Digested collagen IV	Demidova-Rice et al, 2011, Wound Repair and Regen
GEETEVTVEGLEP (SEQ ID NO: 19)	Ten2	Digested tenascin X	Demidova-Rice et al, 2011, Wound Repair and Regen
DINE(S)EIGAPAGEETEV TVEGLEP (SEQ ID NO: 20)	Comb1	Digested fibrillin 1 & tenascin X	Demidova-Rice et al, 2011, Wound Repair and Regen
NYLTHRQ (SEQ ID NO: 21)	Pep12	Mimics the Ig-like domain II of VEGFR-1	Soro et al., 2008, Blood
MRPYDANKR (SEQ ID NO: 22)	KRX-725	Sphingosine 1-phosphate mimic	Licht et al., 2003, Blood
GLKEQSPRKHRL (SEQ ID NO: 23)	Scrambled	Scrambled peptide	
* in certain instances, sequence may have a carboxy terminal glycine residue, which is product of peptide synthesis.			

Table 1: Ten angiogenic peptides used, in their native form.

Name	Peptide Form	Peptide Sequence *
Q _K	Naked (N)	KLTWQELYQLKYKGI (SEQ ID NO: 4)
	Two-Tailed (2T)	LRAGKLTWQELYQLKYKGIPES (SEQ ID NO: 8)
	MMP degradable (DL)	CIPESLRAGKLTWQELYQLKYKGIPESLRAGC (SEQ ID NO: 13)
	MMP nondegradable (NDL)	CKGKKLTWQELYQLKYKGKGC (SEQ ID NO: 24)
SPARC ₁₁₃	N	TLEGTKKGHKHLHDY (SEQ ID NO: 5)
	2T	LRAGTLEGTKKGHKHLHDYIPES (SEQ ID NO: 10)
	DL	CIPESLRAGTLEGTKKGHKHLHDYIPESLRAGC (SEQ ID NO: 14)
	NDL	CKGKTLEGTKKGHKHLHDYKGC (SEQ ID NO: 25)
SPARC ₁₁₈	N	KKGHK (SEQ ID NO: 6)
	2T	LRAGKKGHKIPES (SEQ ID NO: 12)
	DL	CIPESLRAGKKGHKIPESLRAGC (SEQ ID NO: 15)
	NDL	CKGKKKGHKKGC (SEQ ID NO: 26)
SPARC _{3X}	N	KKGHKKGHKKGHK (SEQ ID NO: 17)
	2T	LRAGKKGHKKGHKKGHKGIPES (SEQ ID NO: 27)
Comb1	N	DINE(S)EIGAPAGEETEVTVEGLEP (SEQ ID NO: 20)
	2T	LRAGDINE(S)EIGAPAGEETEVTVEGLEPGIPES (SEQ ID NO: 28)
Pep12	N	NYLTHRQ (SEQ ID NO: 21)
	2T	LRAGNYLTHRQIPES (SEQ ID NO: 29)
KRX-725	N	MRPYDANKR (SEQ ID NO: 22)
	2T	LRAGMRPYDANKRIPES (SEQ ID NO: 30)

Scrambled	-	GLKEQSPRKHRL (SEQ ID NO: 23)
NDL		CKCKC (SEQ ID NO: 31)
DL		CIPESLRAGC (SEQ ID NO: 32)
* in certain instances, the peptides used during the present studies comprise a c-terminal glycine residue, which is product of peptide synthesis.		

Table 2: Angiogenic peptides investigated. Standard amino acid abbreviations are used: Alanine A, Arginine R, Asparagine N, Aspartic Acid D, Cysteine C, Glutamine Q, Glutamic Acid E, Glycine G, Histidine H, Isoleucine I, Leucine L, Lysine K, Methionine M, Phenylalanine F, Proline P, Serine S, Threonine T, Tryptophan W, Tyrosine Y, Valine V. Functionally distinction portions of the peptide are separated by spaces, with the pro-angiogenic portion indicated in *italics*. (S) indicates serine amino acids used in place of cysteine to prevent tethering of the angiogenic sequence to hydrogel networks. (N) indicates peptides in their “naked” (as-published) form; (2T) indicates peptides containing the residual amino acid “tails” that will be present upon their release from hydrogel networks; (DL) indicates peptides containing the MMP-responsive sequence and (NDL) indicates peptides containing a control non-degradable sequence.

The pro-angiogenic efficacy of these peptides was then assessed using two in vitro models of angiogenesis: the human umbilical vein endothelial cell (HUVEC) proliferation and tube formation assay.

In the proliferation assay, HUVECs are seeded in basal media (EBM-2) at 10,000 cells/well in 24-well plates and allowed to adhere overnight. Cells are then washed with PBS, and treated with either basal media alone, or basal media supplemented with a treatment factor. VEGF and the chemotherapeutic drug sulforaphane were used as positive and negative controls, respectively. Media is changed daily for 3 days, at which point cells are again washed with PBS, lysed, and their DNA quantified using the pico green dsDNA incorporation assay. Figure 5 depicts the results of the proliferation assay for all ten of the angiogenic peptides, in their native form, listed in Table 1, while Figure 6 and depicts the results for the ten peptides in their 2T form. It is observed that all peptides, except for T7, increase proliferation in the naked form (Figure 5), while all peptides, except for KRX-725, increase proliferation in the 2T form

(Figure 6). Treatment with positive control VEGF caused the expected dose-dependent increase in cell proliferation, while the scrambled peptide had no significant impact on proliferation. Treatment with the chemotherapeutic inhibitor of angiogenesis, sulforaphane, significantly decreased cell proliferation.

5 Figure 7 depicts the results of peptides Qk, SPARC₁₁₃, SPARC₁₁₈, and Pep12, both in their naked and 2T forms, along with positive and negative controls. Treatment with Qk, SPARC₁₁₃, SPARC₁₁₈, and Pep12 in their naked forms caused statistically significant increases in proliferation over control media alone. While slight changes in bioactivity were observed upon inclusion of MMP-responsive peptide
10 remnants, all four peptides significantly increased proliferation in their two-tailed form in at least one concentration investigated.

 Focusing upon SPARC₁₁₈, Figure 6 and Figure 7 depict the results of the HUVEC proliferation assay with cells treated with the naked and 2T versions of SPARC₁₁₈. Treatment with naked SPARC₁₁₈ caused a significant increase in cell
15 proliferation. Upon inclusion of the residual amino acids which would be present upon release of the peptide from the hydrogel, the efficacy of the peptide was slightly reduced, but even in the two-tailed form the peptide was able to significantly increase HUVEC proliferation.

 The peptides were next evaluated in the HUVEC tube formation assay. In
20 this assay, Reduced growth-factor Matrigel was polymerized in 48-well plates. 2.4×10^5 cells/well in control media or media containing various treatments were seeded onto Matrigel. Cells were incubated for 8 hours before imaging at 4x using a Nikon Eclipse Ti inverted light microscope. Fluorescent images were obtained using the live cell stain Calcein AM and quantified using the image analysis program Angioquant. Figure 8
25 depicts the results of the tube formation assay for all ten of the angiogenic peptides, in their native form, listed in Table 1, while Figure 9 depicts the results for the ten peptides in their 2T form. It is observed that all peptides, except Ten 2, increase HUVEC tube formation in the naked form (Figure 8), while only Qk, SPARC₁₁₃, and SPARC₁₁₈ significantly increase HUVEC tube formation in the 2T form (Figure 9).

30 Figure 10 depicts images of tube formation in cells treated with either the naked or 2T forms of QK or SPARC₁₁₈. Figure 11 depicts the quantification of tube

length in cells treated with either the native or 2T forms of Qk, SPARC₁₁₃, SPARC₁₁₈, and Pep12.

Focusing upon SPARC₁₁₈, Figure 10 depicts images of tube formation of HUVECs treated with naked or 2T SPARC₁₁₈, compared to positive control (VEGF) and negative control (Basal media). Treatment with the positive control VEGF caused the formation of smooth, fully connected honeycomb like tube networks, as compared to the basal media alone, where minimal tube network formation is seen, and the networks are punctuated and disconnected. Treatment with SPARC₁₁₈ in both the naked and 2T form caused the formation of smooth tube networks more similar to that of the positive control than basal media alone. Quantification, as depicted in Figure 11, reflected the qualitative observations of the tube networks: the positive control VEGF significantly increased tube length, as did SPARC₁₁₈ in both its naked and two-tailed form. Treatment with the scrambled peptide did not significantly affect tube network formation, while the inhibitor of angiogenesis sulforaphane significantly decreased tube length.

Of the ten pro-angiogenic peptides studied, three significantly increased both HUVEC proliferation and tube formation in both the naked and two-tailed forms, qualifying for hydrogel incorporation: Qk, SPARC₁₁₃ and SPARC₁₁₈. A summary of the observed effects of the native and 2T forms of all ten peptides are shown in Figure 12.

To examine release of a peptide from a hydrogel, hydrogels made from 8-arm PEG, were formed with either the MMP-degradable peptide SPARC₁₁₈(2DL) or the non-degradable SPARC₁₁₈(2NDL). Gels were then incubated at 37 °C in either PBS alone, or PBS containing 250 µg/mL collagenase. Hydrogels were removed, frozen at -80 °C, and lyophilized to track hydrogel dry mass over time. As shown in Figure 13A, when SPARC₁₁₈ was tethered to the hydrogel via the non-degradable linker and incubated in either PBS alone or the collagenase solution, no significant change in hydrogel mass was observed. Similarly, hydrogels formed with SPARC₁₁₈ tethered via the MMP-degradable sequence were stable when incubated in PBS alone. It was only when the hydrogels produced with the MMP-degradable sequence were incubated in the collagenase solution that rapid degradation of the hydrogels was observed, with hydrogels fully degrading in 24 hours. This confirms that enzymatically-responsive hydrogel degradation can be achieved using the MMP-responsive, peptide-releasing sequence.

In order to confirm that peptide degradation, the hydrogel-bathing solutions were also collected and stored at -80 °C until peptide quantification by High Performance Liquid Chromatography. As can be seen in Figure 13B, release of the pro-angiogenic peptide SPARC₁₁₈ mirrored hydrogel degradation, with only the

5 SPARC₁₁₈(2DL) hydrogels in collagenase solution resulting in release of peptide. While full peptide release was not observed, this was not unexpected as only peptide released in its two-tailed form was included in quantification, and it is likely that some peptides remained tethered at one end to the multi-arm PEG macromers upon release.

The studies presented herein demonstrated that pro-angiogenic peptides

10 can be screened in common angiogenic assays: HUVEC proliferation and tube formation. Based on these assays, it is shown herein that Qk, SPARC₁₁₃, and SPARC₁₁₈ all retain their pro-angiogenic activity in their two-tail form, the form comprising additional amino acid at its N-terminus and C-terminus corresponding to the cleaved portions of the MMP-sensitive cleavable peptide. Further, it is shown that SPARC₁₁₈ can be incorporated into

15 the hydrogel network via the MMP-degradable peptide sequence IPESLRAG (SEQ ID NO: 1), and stimuli-responsive hydrogel degradation and peptide release was achieved. The present data demonstrates that stimuli-responsive delivery of pro-angiogenic peptides can be achieved from PEG hydrogel networks. Experiments are conducted to examine the efficacy of hydrogel released peptides in both in vitro and in vivo models.

20

Example 2: Development of enzymatically-responsive poly(ethylene glycol) hydrogels for the delivery of therapeutic peptides

Despite the recent expansion of peptide drugs, delivery remains a challenge due to poor localization and rapid clearance upon bolus injection. To overcome

25 this limitation, described herein is the development of a hydrogel-based platform technology that controls and sustains peptide drug release via matrix metalloproteinase (MMP) activity. Specifically, hydrogels were developed using step-growth photopolymerizations, exploiting thiol (cysteine)-functionalized peptide drugs flanked with enzyme substrates as crosslinkers to react with norbornene-functionalized

30 poly(ethylene glycol). First, the bioactivity of six previously-described peptide drugs including residual amino acids from enzyme substrate (e.g., enzymatically-released

forms) was investigated *in vitro*. Of these, three peptides retained bioactivity: Qk (from Vascular Endothelial Growth Factor), SPARC₁₁₃, and SPARC₁₁₈ (from the Secreted Protein Acidic and Rich in Cysteine). Degradation of and peptide release from enzymatically-responsive PEG hydrogels upon treatment with MMP2 showed hydrogels containing Qk, SPARC₁₁₃, and SPARC₁₁₈ degraded in ~ 6.7, ~ 6 and ~ 1 days, and released ~ 5, ~ 8 and ~ 19% of peptide, respectively. Peptide drug size controlled hydrogel swelling and degradation rate, while hydrophobicity impacted peptide release. While pro-angiogenic peptides were the focus of this study, the design parameters detailed allow for adaptation of hydrogels to control peptide release for a variety of therapeutic applications.

Experiments were designed to develop poly(ethylene glycol) hydrogels that provide sustained, enzymatically-responsive release of peptide drugs (Figure 14). To test the impact of substrate “tails” left on the peptide drugs upon release from the hydrogels (LRAG-peptide-IPES; “2T”), six bioactive peptide sequences were identified from literature as candidates for incorporation into enzymatically-responsive hydrogels. Pro-angiogenic peptides were a focus due to therapeutic potentials and well-defined *in vitro* assays available (Auerbach et al., 2003, Clin Chem. 49(1):32-40). First, the effects of residual enzyme substrate amino acid “tails” on the peptide drugs after enzyme-mediated release were assessed using the human umbilical vein endothelial cell (HUVEC) tube formation and proliferation assays. Three peptide drugs retained bioactivity in released forms. These and two additional peptides encompassing a range of peptide sizes and hydrophobicities were incorporated into PEG hydrogels via enzymatically responsive linkers (C- IPESLRAG-peptide-IPESLRAG C; degradable linker, “DL”). The enzymatically responsive sequence IPESLRAG (SEQ ID NO: 1) was utilized as it is susceptible to matrix metalloproteinases (MMPs) 1, 2, 3, 7, 9 & 14 (Cantley et al., 2001, Nat Biotechnol. 19(7):661-7), many of which are expressed at increased levels in diseased or regenerating tissues. The resulting hydrogels were characterized, and enzymatically-responsive hydrogel degradation and “2T” peptide release upon treatment of gels with MMP2 was investigated.

The results obtained from the experiments are now described.

Effect of enzyme substrate residues (“tails”) on peptide bioactivity

Six *a priori* identified bioactive peptides (Table 3) were selected from literature to determine the effect of the residual peptide “tails” left on peptides upon enzymatically-responsive hydrogel release. Bioactive peptide selection was restricted to pro-angiogenic peptides to allow for objective comparison of the impact of the “tails” on all peptides using well-established *in vitro* assays (Auerbach et al., 2003, Clin Chem. 49(1):32-40). Peptides were chosen with a variety of sizes and hydrophobicity (Table 4), to investigate if these characteristics provided predictive power for the effect of “tails” on bioactivity. Peptides were synthesized both in their as-published, “native” (“N”) sequence alone, and in their “two-tailed” (“2T”) form (Table 3). The “2T” form mimics incorporation of the MMP2 degradable substrate, IPESLRAG (SEQ ID NO: 1), on both the N- and C- termini of the peptide drug for enzymatically-responsive hydrogel formation, and subsequent substrate cleavage and peptide release. Peptide bioactivities and the impact of the “tails” were compared using two *in vitro* models of angiogenesis: the HUVEC tube formation and proliferation assays (Figure 15) (Auerbach et al., 2003, Clin Chem. 49(1):32-40). For clarity, tube formation data is reported as fold-increase and proliferation data is reported as % increase, with both sets of data normalized to control media.

20

Peptide Drug	Sequence	Notes
SPARC ₁₁₈	KKGHK (SEQ ID NO: 6)	Cu ²⁺ binding region of SPARC (Jendraschak et al., 1996, Semin Cancer Bio. 7(3):139-46; Iruelaarispe et al., 1995, Mol Biol Cell. 6(3):327-43); Small, hydrophilic peptide
SmPho	KLVPLA (SEQ ID NO: 33)	Model of small, hydrophobic peptide
KRX-725	MRPYDANKR (SEQ ID NO: 22)	The second intracellular loop of Sphingosine 1-phosphate 3 (Ben-Sasson et al., 2003, Blood. 102(6):2099-107)
SPARC _{3X}	KKGHKKGHKKGHK (SEQ ID NO: 17)	Triplet of SPARC ₁₁₈ ; Large, hydrophilic peptide; 3 repeats of SPARC ₁₁₈
SPARC ₁₁₃	TLEGTKKGHKLHLDY (SEQ ID NO: 5)	Basic region of SPARC (Jendraschak et al., 1996, Semin Cancer Bio. 7(3):139-46; Iruelaarispe et al., 1995, Mol Biol Cell.

Qk	KLTWQELYQLKYKGI (SEQ ID NO: 4)	6(3):327-43) 17-25 α -helix region of VEGF ₁₆₅ (Finetti et al., 2012, Biochem Pharm., 84(3):303-11; Santulli et al., 2009, J of Trans Med. 7:41); Large, hydrophobic peptide
Comb1	DINE(S)EIGAPAGEETE VTVEGLEPG (SEQ ID NO: 20)	Combination of EGF-like domain from Fibrillin 1 and Fibronectin III-like domain of Tenascin X (Demidova-Rice et al., 2011, Wound Repair Regen. 19(1):59-70)
Scrambled	GLKEQSPRKHRLG (SEQ ID NO: 23)	Scrambled control peptide
"N" form	peptide drug	
"2T" form	LRAG-peptide drug-IPES	
"DL" form	C IPESLRAG-peptide drug- IPESLRAG C	
	<u>except</u> Qk(DL): EEEE C IPESLRAG KLTWQELYQLKYKG IPESLRAG C EEEE (SEQ ID NO: 34)	
*in certain instances, the peptides used for the present studies may have a C-terminal glycine residue, which is product of peptide synthesis. No additional Gly added to C-termini of Comb1(N) or Scrambled. No additional Ile added to C-temini of Qk(2T).		

Table 3: Peptide drugs investigated. Standard amino acid abbreviations are used. (S)

indicates serine amino acids used in place of cysteine to prevent intersequence peptide drug tethering within hydrogel networks. Peptide drugs are demarcated by italics.

5

Peptide	Molecular Weight (Da)	Sequence Length (# amino acids)	Hydropathy		
			# Average (%)	K-D Score	H-W Score
SPARC ₁₁₈	597	5	20%	-15.3	8.5
SmPho	640	6	67%	8.1	-2.6
KRX-725	1150	9	33%	-19.1	8.1
Scrambled	1449	12	25%	-22.2	11.4
SPARC _{3X}	1498	13	23%	-38.4	19.5
SPARC ₁₁₃	1740	15	40%	-17.2	5.5
Qk	1911	15	53%	-10.9	-3.2
Comb1	2543	25	44%	-13.7	14.3
DL	842	8	50%	-0.7	2.2

Table 4: Peptide characteristics. Hydrophobic amino acids are G, A, V, L, I, M, F, Y, W (Lehninger et al., 2000, Lehninger principles of biochemistry. 3rd ed. New York: Worth

Publishers). # Average indicates number average, by amino acid type. In the Kyte-Doolittle (K-D) index, positive values indicate hydrophobicity (Kyte et al., 1982, J Mol Biol. 157(1):105-32); in the Hopp-Woods (H-W) index, negative values indicate hydrophobicity (Hopp et al., 1981, P Natl Acad Sci-Biol. 78(6):3824-8). Sequences were
 5 analyzed as the sequence alone, without any linker, “tails”, or C-terminal Gly amino acid used for synthesis.

All six “N” peptides significantly increased HUVEC tube length over control media; however, only three retained this bioactivity in “2T” forms, specifically
 10 Qk, SPARC₁₁₃, and SPARC₁₁₈ (Figure 15A). Qk(N) and Qk(2T) increased average tube length to ~ 2.1- and ~ 2.4-fold that of control media. SPARC₁₁₈(N) and SPARC₁₁₈(2T) increased tube length ~ 2.0 and ~ 1.8-fold, while SPARC₁₁₃(N) and SPARC₁₁₃(2T) increased tube length ~ 1.3 and ~ 1.7-fold. Comb1(N) significantly increased average tube length ~ 1.5-fold, but Comb1(2T) did not significantly affect tube length (~ 1.3-
 15 fold). SPARC_{3X} was also inactivated by the “tails”, with the “N” form significantly increasing tube length ~ 1.5-fold, and the “2T” form resulting in a statistically insignificant ~1.4-fold increase. KRX-725(N) significantly increased tube length ~ 1.2-fold, while KRX-725(2T) resulted in a statistically insignificant ~1.1-fold increase. The scrambled peptide did not significantly affect relative tube length (~ 0.9-fold), indicating
 20 that the observed results are due to specific peptide drugs.

A preliminary dose screening study did not identify a singular concentration at which all “N” peptides resulted in HUVEC proliferation. Therefore, the dose used to investigate the effect of the peptide “tails” on HUVEC proliferation was varied between peptide types, but kept constant between the “N” and “2T” forms of each
 25 peptide. All except for KRX-725 retained their “N” proliferative capacity upon inclusion of the “2T” (Figure 15B). Qk(N) and Qk(2T) at 100 nM both significantly increased HUVEC proliferation ~ 17%, SPARC₁₁₃(N) and SPARC₁₁₃(2T) at 1 nM significantly increased HUVEC proliferation ~ 12 and ~ 18%, and SPARC_{3X}(N) and SPARC_{3X}(2T) at 100 nM significantly increased HUVEC proliferation ~ 11 and ~ 14%. Comb1 at 100 nM
 30 increased proliferation ~ 21% in the “N” form and ~ 14% in the “2T” form. SPARC₁₁₈ at 1 nM exhibited similar behavior, with the “N” form increasing HUVEC proliferation ~

28%, while the “2T” form increased proliferation ~ 14%. KRX-725 completely lost bioactivity upon inclusion of the “2T”, with KRX-725(N) significantly increasing proliferation ~ 26%, and KRX-725(2T) decreasing proliferation ~ 3% below that of control media, both at 1 nM. The scrambled peptide did not affect proliferation (~ 3%), again indicating that the observed results are sequence-specific. Based on these results, three peptides were identified that retained bioactivities in released form from enzymatically-responsive hydrogels: Qk, SPARC₁₁₃, and SPARC₁₁₈. These peptides have varying sizes and hydrophobicities (Table 4), and no clear property was identified that could predict if peptide would remain bioactive with residual enzyme substrate ‘tails’.

Similarly, peptide structure predictions were unable to provide insight as to which peptide retained or lost bioactivity (Figure 21).

Prediction of crosslinking peptide structure

To investigate the versatility of the enzymatically-responsive peptide delivery system, release of five peptides was investigated. This included four of the previously-investigated pro-angiogenic peptide drugs: Qk, SPARC₁₁₃, SPARC₁₁₈, and SPARC_{3X} as well as a scrambled control. These peptides represent a variety of peptide types, from small and hydrophilic (SPARC₁₁₈) to large and very hydrophobic (Qk) (Table 4). As none of the peptides investigated in the bioactivity study were small and hydrophobic, a model peptide drug (SmPho) was also investigated. All five peptides were synthesized in the full enzymatically-responsive and crosslinkable forms (“DL” form, Table 3).

To further investigate differences in peptide properties, prediction software was used to investigate differences in peptide structure (Asakage et al., 2006, Angiogenesis. 9(2):83-91). Figure 16 illustrates the predicted peptide structures for all five peptide-releasing sequences investigated, as well as the degradable linker IPESLRAG (SEQ ID NO: 1). The degradable linker was predicted to form an α -helix with ~ 1.5 turns, a structure maintained in all five of the peptide-releasing sequences. Sequences designed to release large peptides (SPARC_{3X}, SPARC₁₁₃, Scrambled, and Qk) all exhibited increased number or length of α -helices above those contributed by the “DL”s alone (Figure 16D – Figure 16G), while the smaller peptide drugs (SmPho and

SPARC₁₁₈) had either equal or reduced α -helix length (Figure 16B and Figure 16C). The central region of Qk(DL) and SPARC₁₁₃(DL) was predicted to have ~ 1.5 and ~ 0.5 turns, respectively. SPARC_{3X}(DL) was predicted to have a longer, ~ 3.5 -turn C-terminal α -helix, but no central α -helix. Scrambled(DL) similarly had an extended ~ 2.5 turn N-terminal α -helix, and both the N- and C- terminal α -helices on Qk(DL) were also extended to ~ 2.5 turns. SmPho(DL) and SPARC₁₁₈(DL) were not predicted to have any additional α -helices, and SPARC₁₁₈ had a slightly shorter N-termini α -helix. This structure prediction method has been validated against 37 linear peptides, and shown to deviate from NMR structures by only 3 Å (Thevenet et al., 2012, Nucleic Acids Res, 40(W1): W288). Additionally, the predicted structure of the central peptide drug of Qk(DL) in particular is consistent with prior investigations of Qk that measured α -helix conformation using circular dichroism (D'Andrea et al., 2005, P Natl Acad Sci USA. 102(40):14215-20), thereby adding confidence to the predicted structures herein.

15 Formation and characterization of enzymatically-responsive hydrogels

In certain instances, the Qk(DL) peptide initially designed (C IPESLRAG *KLWQELYQLKYKGI* PESLRAG C (SEQ ID NO: 13)) was not sufficiently soluble in aqueous solution to allow for incorporation into PEG hydrogels. Therefore, the sequence was modified to enhance solubility by inclusion of four additional hydrophilic Glu (E) amino acids on both ends (Table 3). Even with these additional hydrophilic amino acids, the peptide required the use of a water/acetonitrile co-solvent to form hydrogels. All other (DL) peptides were soluble in buffer at the necessary concentrations for hydrogel formation.

Hydrogels described here were constructed from 10%wt 4-arm 10kDa PEG. Macroscopically, the Qk(DL) gels were somewhat opaque, while all other gels were transparent (Figure 17A). The hydrogels containing the smaller peptide drugs, SPARC₁₁₈(DL) and SmPho(DL), had significantly higher mass swelling ratios than the other four gels, specifically ~ 27 and ~ 28 mg/mg, as compared to ~ 15 , ~ 16 , ~ 13 , and ~ 15 mg/mg for the SPARC_{3X}(DL), SPARC₁₁₃(DL), Scrambled (DL), and Qk(DL) gels, respectively (Figure 17B). While there were significant differences in peptide incorporation between the gels, all had nearly complete incorporation of the crosslinking

peptide (97% or greater, Figure 17C). Only the Qk(DL) gels had any detectable amount of peptide incorporated into the gel not actively forming crosslinks, with ~ 9% of the incorporated peptide retaining a free thiol (Figure 17D).

5 Degradation and “2T” peptide release from enzymatically-responsive hydrogels

Only the SmPho(DL) gels degraded when incubated in the buffer alone (~ 9.7 days), with all other gels remaining intact over the course of the degradation study (10 days, Figure 18A). Qualitative decreases in stiffness of the SPARC₁₁₈(DL) and SmPho(DL) in buffer was observed, while all other gels were stable over this timeframe.

10 All six gel types fully degraded in the presence of 10 nM MMP2, and all degraded significantly faster than in buffer (Figure 18A). In the presence of MMP2, the SPARC₁₁₈(DL) and SmPho(DL) gels degraded the fastest, in ~1 day, followed by the Scrambled (DL) and SPARC_{3X}(DL) gels which degraded in ~ 3 days and ~ 3.5 days, respectively. The SPARC₁₁₃(DL) and Qk(DL) gels degraded the slowest, over ~ 6 and ~

15 6.7 days, respectively. All gel types released undetectable amounts of “2T” peptide in buffer alone, and all released significantly more “2T” peptide in MMP2 containing buffer (Figure 18B), demonstrating the enzymatically-responsive nature of these gels. Upon reaching the reverse gelation point, the Qk(DL), SPARC₁₁₃(DL), SmPho(DL), SPARC₁₁₈(DL), Scrambled(DL), and SPARC_{3X}(DL) gels released ~ 5, ~ 8, ~16, ~19, ~

20 22, and ~42% of peptide in its “2T” form, respectively. Tracking of “2T” peptides in solution over time demonstrated all peptides remain stable in both buffer and MMP-containing buffer (Figure 22), indicating differences in “2T” peptide release are not due to further degradation of released peptides. Mass spectrometry on degraded

25 SPARC₁₁₈(DL), SmPho(DL), SPARC_{3X}(DL), SPARC₁₁₃(DL), and Scrambled(DL) hydrogels showed singular peaks at the expected “2T” peptide molecular weight, further confirming that the MMP is cleaving the peptides at the expected sites and that peptides are not nonspecifically degraded upon release from the hydrogels (Figure 23).

30 Relationship between peptide drug properties and enzymatically responsive hydrogel behaviors

To determine if any relationship existed between peptide drug properties and behavior of enzymatically-responsive hydrogels, time to complete hydrogel degradation and amount of “2T” peptide release upon degradation were plotted as a function of various measures of peptide size and hydrophobicity, and linear regression was utilized to investigate if a relationship existed (Figure 19). Two measures of peptide size, molecular weight and sequence length, exhibited a significant linear relationship with the rate of hydrogel degradation, but not with the amount of “2T” peptide released from hydrogels. Three measurements of peptide hydrophobicity, % hydrophobic amino acids, Kyte-Doolittle Average, and Hopp-Woods Average, exhibited linear relationships with the amount of “2T” peptide released from hydrogels, but none had a significant linear relationship with time to complete hydrogel degradation. Differences in units between the peptide characteristics investigated (Da, #, %, etc.) prevented any meaningful comparison between the slopes of the linear fits obtained.

15 In vitro efficacy of degraded hydrogels

The enzymatically-responsive gels releasing peptides found to be bioactive in their “2T” form, SPARC₁₁₈(DL), SPARC₁₁₃(DL), and Qk(DL), as well as the scrambled peptide releasing gel Scrambled(DL) were degraded with MMP2. Subsequently, the HUVEC tube formation assay was used to assess the pro-angiogenic potential of degraded hydrogel products and released peptide drugs. Filtered, degraded hydrogel solutions were diluted in media and assessed for bioactivity using the tube formation assay such that the concentration of SPARC₁₁₈(2T) present was 100 nM, the concentration utilized in the efficacy screening study, equating to ~ 1/7,000th gel/well. The gel/well ratio was kept constant across all hydrogels investigated, but variations in the amount of “2T” peptide present in the degraded hydrogel solutions caused variation in the amount of “2T” peptide present (4.5 nM Qk(2T), 130 nM SPARC₁₁₃(2T) and 240 nM Scrambled(2T)). Degraded SPARC₁₁₈(DL), SPARC₁₁₃(DL) and Qk(DL) hydrogels significantly increased HUVEC tube formation to 2.8, 1.7 and 3.1-fold that of control media (Figure 20). The degraded Scrambled(DL) gels did not significantly affect tube length (0.8-fold) indicating that the observed results are due to specific peptide drugs released from the hydrogels.

Stimuli-responsive delivery of peptide drugs from hydrogels

The study presented herein details the development of a platform technology for the stimuli-responsive delivery of peptide drugs from hydrogels. The effect of residual peptide “tails” on peptide drugs upon release from enzymatically-responsive hydrogels on the bioactivity of six peptides with varying physical properties was investigated and three peptides were identified that retained bioactivity in released forms. No property was identified that predicted which peptide would retain bioactivity. Nonetheless, peptide release from enzymatically-degradable hydrogels was achieved through the use of MMP-substrate crosslinkers. Testing revealed the impact of the peptide drug on hydrogel degradation and peptide release; peptide drug size was found to affect hydrogel swelling and rate of hydrolytic and enzymatic degradation, while peptide hydrophobicity affected the extent of peptide fully released in its “2T” form. Upon MMP-mediated degradation, degraded peptide-releasing hydrogels were able to induce HUVEC tube formation, indicating these hydrogels release bioactive components upon degradation.

Therapeutic peptides are often delivered via injection (Hardy et al., 2008, Biochem Pharm. 75(4):891-9; Hardy et al., 2007, Peptides. 28(3):691-701), osmotic pumps (Santulli et al., 2009, J of Trans Med. 7:41) or diffusional release of from polymeric particles (Ben-Sasson et al., 2003, Blood. 102(6):2099-107; Failla et al., 2008, Blood. 111(7):3479-88) or gels (Santulli et al., 2009, J of Trans Med. 7:41; Choi et al., 2004, Pharm Res. 21(5):827-31; Van Slyke et al., 2009, Tissue Eng Pt A. 15(6):1269-80). However, many of these approaches do not offer controlled, sustained release of therapeutic peptides, and delivery occurs over pre-dictated timescales and not in response to the local tissue microenvironment. Previous studies have shown superiority of tissue-dictated release of drugs. For example, persistent vascularization of hydrogels *in vivo* was attributed to tissue-responsive cleavage of VEGF resulting in sustained release, in contrast to typical burst release of diffusion-controlled drug release systems or bolus delivery (Phelps et al., 2010, P Natl Acad Sci USA 107(8):3323-8). While enzymatically-responsive growth factor release has shown promising results (Phelps et al., 2010, P Natl Acad Sci USA 107(8):3323-8; Zisch et al., 2003, Faseb J. 17(13):2260-+), delivery of

peptide drugs in this manner has not yet been demonstrated. The enzymatically-responsive material presented here is designed to deliver peptide drugs in a sustained and tissue-dependent manner, as the MMPs to which the substrate is susceptible are expressed at increased levels in ischemic (Muhs et al., 2003, J Surg Res. 111(1):8-15; 5 Phatharajaree et al., 2007, Can J Cardiol. 23(9):727-33) and inflammatory tissues (Lawrance et al., 2001, Hum Mol Genet. 10(5):445-56; Kontinen et al., 1999, Ann Rheum Dis. 58(11):691-7), and in tumor microenvironments (Kurahara et al., 1999, Head Neck-J Sci Spec. 21(7):627-38; Heppner et al., 1996, Am J Pathol. 149(1):273-82). These hydrogels are a promising candidate for minimally-invasive treatment as they can be 10 polymerized *in situ* using cytocompatible UV light (Fairbanks et al., 2009, Adv Mater. 21(48):5005-10). Additionally, degradation circumvents secondary surgeries necessary to remove drug delivery systems.

The impact of residual enzyme substrate “tails” on the efficacy of six *a priori* identified pro-angiogenic peptide sequences was investigated. Some peptides 15 (Qk, SPARC₁₁₃ and SPARC₁₁₈) were unaffected by the presence of residual amino acid residues, with “2T” results very similar to “N” results (Figure 15). Others exhibited decreased (SPARC_{3X} and Comb1) or entirely lost (KRX-725) ability to induce HUVEC proliferation and tube formation in their “2T” form. Some peptides only lost bioactivity in one of the two assays (Comb1 and SPARC_{3X}); however, differential effects between 20 outcomes are not completely unexpected or unprecedented, as proliferation and tube formation are different cell processes (Hardy et al., 2007, Peptides. 28(3):691-701; Demidova-Rice et al., 2011, Wound Repair Regen. 19(1):59-70). In addition to identifying three peptides likely to be bioactive after release from enzymatically-responsive hydrogels (Qk, SPARC₁₁₃, and SPARC₁₁₈), these data demonstrate a 25 limitation of the enzymatically-responsive delivery system developed. The same peptide “tails” have very different impacts on peptide bioactivity, and neither size, hydrophobicity, or structure could be used to predict which peptides retain bioactivity (Figure 15, Figure 21, and Table 4). These findings are consistent with previous work that demonstrates that residual amino acid residues and drug properties can affect 30 bioactivity. For example, VEGF containing a PEG linker retains similar bioactivity to the native protein (Zisch et al., 2003, Faseb J. 17(13):2260-+). However, PEG is a

hydrophilic, unstructured molecule with few similarities to polypeptide chains. Similar to our approach, though, by altering the cleavable peptide spacer conjugating the chemotherapeutic Mitomycin C to a polymeric carrier, the rate of drug release and the off-target hematopoietic toxicity of the released drug is affected (Soyez et al., 1997, J Control Release 47(1):71-80). Additionally, the N- versus C- terminal position of peptide “tails” strongly affects bioactivity of CD95L fusion proteins (Watermann et al., 2007, Cell Death Differ. 14(4):765-74). These prior results, along with our findings, indicate that the therapeutic agent being used and the chemical composition and position of residual “tails” can impact therapeutic agent bioactivity upon release. These data also suggest the possibility of restoring bioactivity of peptide drugs inactivated by the “tails” left after degradation by changing the amino acid residues of the linker or incorporating additional amino acids prior to the degradable linker substrate (Cantley et al., 2001, Nat Biotechnol. 19(7):661-7).

The three promising pro-angiogenic peptide drugs Qk, SPARC₁₁₃, SPARC₁₁₈, as well as SPARC_{3X}, and the model peptide drug SmPho, were incorporated into enzymatically-responsive hydrogels. The large, hydrophobic crosslinking peptide Qk produced opaque gels, while all other gels were transparent (Figure 17A), as is typical for PEG hydrogels. This is likely a result of increased intermolecular interactions between the Qk α -helix (Figure 16G), and is consistent with previous findings where α -helix containing peptides self-assembled into opaque hydrogels (Kisiday et al., 2002, P Natl Acad Sci USA. 99(15):9996-10001). The swelling ratio of the gels is inversely related to sequence length, with smaller peptides (SPARC₁₁₈ and SmPho) having larger swelling ratios than gels formed with larger peptides (Qk, SPARC₁₁₃, Scrambled, and SPARC_{3X}, Figure 17B). As larger crosslinking peptides make up a higher weight percent of the hydrogel dry mass (~48% for Qk(DL) gels, compared to ~23% for SPARC₁₁₈(DL) gels), intermolecular interactions between crosslinking peptides are more probable. It is also possible that the increased number and length of α -helices found within the larger peptides (Figure 16) increases non-covalent intermolecular peptide interactions within these hydrogels, effectively increasing the crosslinking density of the gels. The lower crosslinking efficiency in Qk(DL) hydrogel properties could be due to the peptide structure and hydrophobicity decreasing cysteine availability, or to the evaporation of

acetonitrile co-solvent during polymerization, which effectively increases peptide crowding, leading to less efficient crosslinking. Taken together, these results demonstrate the impact the size of the crosslinking peptide can have on hydrogel physical properties.

The incorporation of reactive enzymatically-responsive tethers

5 IPESLRAG (SEQ ID NO: 1) into pro-angiogenic peptides resulted in hydrogels that were degraded by MMP2. Previous work using IPESLRAG (SEQ ID NO: 1) directly as a hydrogel crosslinker results in PEG hydrogels that degrade in 2 days in MMP2, but are stable for over 10 days in MMP1 (Patterson et al., 2010, *Biomaterials*. 31(30):7836-45). Degradation over similar timescales was expected here, as the previously studied “DL”
10 gels were smaller, had a lower crosslinking density, and used a higher concentration of MMP2, but only had one “DL” per crosslink. All five gels exhibited enzymatically-responsive degradation and peptide release, as demonstrated by accelerated degradation and release of “2T” peptide only in the presence of MMP2 (Figure 18). As the same degradable substrates were used for every peptide investigated, it was expected that all
15 hydrogels would degrade at similar rates and release similar amounts of “2T” peptide. However, testing revealed that peptide drug has a significant effect on both behaviors. Further investigation revealed that the rate of enzymatically-responsive hydrogel degradation is related to peptide drug size, while “2T” peptide release is related to peptide drug hydrophobicity (Figure 19). Numerous other studies have been conducted
20 varying the rate of enzymatically-responsive hydrogel degradation by altering the degradable sequence employed, particularly the ~ 4 amino acids immediately adjacent to the cleavage site (Fairbanks et al., 2009, *Adv Mater*. 21(48):5005-10; Patterson et al., 2010, *Biomaterials*. 31(30):7836-45; Miller et al., 2010, *Biomaterials*. 31(13):3736-43); to our knowledge no study has investigated the role of linker-adjacent peptides in
25 hydrogel degradation and peptide release. Vessillier et al. found that addition of hydrophilic amino acids flanking a peptide cleavage substrate increases substrate sensitivity (Vessillier et al., 2004, *Protein Eng Des Sel*. 17(12):829-35), but the contributions of hydrophilicity and size of the flanking sequences were not specifically isolated. Particularly for the release of Qk from the developed system, where additional
30 glutamic acid residues were included to improve solubility, the alteration in peptide may have contributed to the decreased substrate cleavage and “2T” release. As MMP activity

requires a zinc-bound water molecule activated for nucleophilic attack, and glutamic acid residues shuttle protons and allow for peptide bond cleavage (Diaz et al., 2008, J Phys Chem B. 112(28):8412-24), it is possible that modifying the MMP substrate with additional glutamic acids may have also affected proton transfer. Introduction of
5 alternative hydrophilic amino acids and/or hydrophilic linkers may allow for additional Qk(2T) release. With the rate of degradation affected by glutamic acid residues notwithstanding, Qk-based hydrogels are observed to degrade in an MMP2-dependent manner.

By incorporating the therapeutic molecule as the crosslinking agent of the
10 enzymatically-responsive hydrogels, higher concentrations of peptide can be loaded into gels. However, this also inherently links peptide release to hydrogel degradation. To achieve hydrogel degradation in this system, peptide crosslinkers need only to be cleaved at one "DL" site. However, both "DL" sites need to be cleaved for "2T" peptide release (Figure 14). It is likely that crosslinking peptide not detected in "2T" forms after
15 hydrogel degradation remained tethered to PEG molecules, rather than being further degraded upon release. This is supported by the stability of all "2T" peptides in both buffer and MMP2 (Figure 22), and the single peak observed in the mass spectrometry of degraded hydrogels (Figure 23). As hydrogel degradation is predicted by peptide size (Figure 19), it can be inferred that the size of the peptide drug affects the rate of cleavage
20 of one "DL". Interestingly, the SPARC_{3X} peptide, which is 3 repeats of the SPARC₁₁₈ peptide, directly highlights the impact of peptide drug size on degradation. Whereas hydrogels formed with SPARC₁₁₈ degraded in ~ 1 day in the presence of MMP2, SPARC_{3X}-releasing hydrogels required ~ 3.5 days to reach the reverse gelation point (Figure 18). The mesh size of these gels varied from 30 ± 4 nm (Qk(DL)) to 63 ± 2 nm
25 (SmPho(DL)) (Zustiak et al., 2010, Biomacromolecules. 11(5):1348-57), but should have minimal hindrance to diffusion of MMP2 (~ 2.6 nm) within all gels (Erickson et al., 2009, Biol Proced Online. 11(1):32-51), indicating that the observed differences in degradation rate are not due to diffusional limitations. Additionally, the hydrolytic degradation observed for the SPARC₁₁₈(DL) and SmPho(DL) gels is likely a minor
30 contributor to accelerated enzymatic degradation, as there is a substantial difference in timescales (~ 1 versus ~ 10 days) of hydrolytic and enzymatic degradation for

SmPho(DL) gels. Similar to previous reports, the hydrogels hydrolytically degraded due to the presence of ester bonds between PEG and norbornene groups (Roberts et al., 2013, Biomaterials. 34(38):9969-79; Shih et al., 2012, Biomacromolecules. 13(7):2003-12).

Modifications to the present platform could employ an alternate chemistry for norbornene functionalization of PEG where esters are replaced with amide bonds, preventing hydrolytic degradation (Roberts et al., 2013, Biomaterials. 34(38):9969-79).

Hydrophobicity is strongly related with “2T” release (Figure 19), implying that once the first “DL” is cleaved, hydrophobic peptides decrease the cleavage rate of the second “DL”. Differences in peptide structure may also impact the rate of “DL” cleavage, as the

more slowly degrading gels have a larger proportion of α -helices (Figure 16). While the degradation and “2T” release data provides interesting information on how the peptide drug affects the relative rates of “DL” cleavage, it is unable to elucidate any difference in cleavage rate or order between the N- and C- terminal “DL”, or if the differences in cleavage rate are due to changes in cleavage site accessibility or substrate cleavage kinetics.

The degradation and release data here provide valuable insight for development of similar enzymatically-controlled peptide release systems: large, hydrophilic peptide drugs are predicted to produce gels that slowly degrade and fully release a large fraction of the peptide, while small, hydrophobic peptide drugs are expected to produce gels that degrade slowly and release only a modest amount of peptide drug. As linear relationships were found between two measures of peptide size (molecular weight and sequence length) and hydrogel degradation, and between three measures of peptide hydrophobicity (% hydrophobic amino acids, Kyte-Doolittle average, and Hopp-Woods average) and “2T” peptide release, it is likely these underlying peptide drug characteristics control hydrogel behavior.

Despite various amounts of peptide released in “2T” form, all three peptide drug releasing hydrogels significantly increased HUVEC tube formation. Degraded SPARC₁₁₈(DL) and SPARC₁₁₃(DL) hydrogels significantly increased HUVEC tube length 2.8 and 1.7-fold, demonstrating that these hydrogels release bioactive components upon MMP mediated degradation. The degraded Qk(DL) gels significantly increased HUVEC tube length 3.1-fold control media, despite releasing substantially less

peptide in “2T” form than the SPARC₁₁₈(DL) and SPARC₁₁₃(DL) hydrogels. Indeed, degraded Qk(DL) hydrogels induced tube formation at lower “2T” levels (4.5 nM) than those previously investigated in the screening study (100 nM). However, the 100 nM dose previously used was not verified to be the minimum effective dose, and it is possible that “2T” peptide alone is also able to induce tube formation at this lower concentration. Additionally, as PEG is bio-inert, it is possible that peptides released from the hydrogel in PEG-tethered forms retain their bioactivity (Zisch et al., 2003, *Faseb J*, 17(13): 2260). However, additional studies would be required to separate the contributions of peptide fully released from the hydrogel in “2T” form from those of peptide still tethered to a PEG molecule. Nevertheless, this study demonstrates that the SPARC₁₁₈(DL), SPARC₁₁₃(DL), and Qk(DL) hydrogels release bioactive components upon MMP-mediated degradation.

A critical consideration for responsive delivery systems is therapeutic impact. The hydrogels investigated here contain ~ 0.8 μmol peptide per 40 μL gel. Omitting temporal considerations, with the ~ 8 and ~ 19% “2T” release achieved for SPARC₁₁₃(DL) and SPARC₁₁₈(DL) gels and *in vitro* efficacy at 100 nM, one gel should release enough “2T” drug to reach target concentrations in ~ 1.5 L of tissue. The decreased “2T” release from the Qk(DL) gels means these gels only release enough “2T” drug to reach 100 nM in ~ 0.4 L of tissue. Despite differences in “2T” peptide release, SPARC₁₁₈(DL), SPARC₁₁₃(DL), and Qk(DL) gels were all able to induce tube formation at the same gel dilution ratios. This indicates that the all three gels should be able to reach therapeutic levels in similar volumes of target tissue. Only one concentration of degraded gel was assessed for *in vitro* bioactivity, and it is possible that the enzymatically-responsive gels developed here are bioactive at even lower concentrations and could achieve therapeutically relevant concentrations in even larger volumes of tissue.

A further consideration is the timescale over which the gels degrade and release peptide drug. Smaller peptide drugs resulted in hydrogel degradation over ~ 1 day, while gels releasing larger peptide drugs degraded in ~ 1 week (Figure 18). While indicative of enzymatic-response, this is not expected to be predictive of degradation timescales *in vivo*, as supra-physiological concentrations of MMP2 were used (Spinale et al., 2000, *Circulation*. 102(16):1944-9). Additionally, the presence of other MMPs in an

in vivo environment (Muhs et al., 2003, J Surg Res. 111(1):8-15; Phatharajaree et al., 2007, Can J Cardiol. 23(9):727-33; Lawrance et al., 2001, Hum Mol Genet. 10(5):445-56; Konttinen et al., 1999, Ann Rheum Dis. 58(11):691-7; Kurahara et al., 1999, Head Neck-J Sci Spec. 21(7):627-38; Heppner et al., 1996, Am J Pathol. 149(1):273-82) will likely alter the rate of hydrogel degradation and peptide release. Degradation timescales could be altered by adjusting hydrogel crosslinking density, with increases in density causing a decrease in degradation rate (Lutolf et al., 2003, P Natl Acad Sci USA. 100(9):5413-8). Degradation time could also be altered by changing the specific degradable substrate used (Patterson et al., 2010, Biomaterials. 31(30):7836-45; Cantley et al., 2001, Nat Biotechnol. 19(7):661-7); however, this would also alter the “tails” left on the peptide, likely affecting bioactivity based on the data observed here.

The peptide drugs investigated here were restricted to pro-angiogenic peptides; however, controlled release any therapeutic peptide is feasible, including anti-inflammatory (Akeson et al., 1996, J Biol Chem. 271(48):30517-23; Schultz et al., 2005, Biomaterials 26(15):2621-30) and chemotherapeutic (Yang et al., 2003, Cancer Res. 63(4):831-7; Selivanova et al., Nat Med. 1997, 3(6):632-8) peptides. This approach also has promise for use in cell-based tissue engineering approaches; for example, the peptide SPARC₁₁₈ was recently described to increase mesenchymal stem cell (MSC) production of pro-angiogenic factors (Jose et al., 2014, Acta Biomater), suggesting the possibility to enhance the therapeutic effect of transplanted or host MSCs (Hoffman et al., 2013, Biomaterials. 34(35):8887-98) using SPARC₁₁₈(DL) hydrogels. Additionally, release of multiple peptide drugs could be achieved by using a mixture of crosslinking peptides during gel formation or, as illustrated here, the differences in release among peptide drugs could be exploited to deliver two drugs over different timeframes using one delivery system. For example, sequential delivery of pro-angiogenic and pro-maturation growth factors improves vessel formation and stability *in vivo* (Brudno et al., 2013, Biomaterials. 34(36):9201-9). However, as the peptide drug delivered affects the rate of hydrogel degradation and peptide release, achieving well-controlled sequential release of multiple factors is non-trivial. Altogether, these results demonstrate the development of a novel method for enzymatically-responsive delivery of peptide drugs, with potential application in a variety of disease states and tissue engineering applications.

With the goal of developing a hydrogel-based platform technology for enzymatically-responsive delivery of peptide drugs, this study compared the relative *in vitro* efficacies of six pro-angiogenic peptides and the impact residual peptide “tails” had on peptide bioactivity. Qk, SPARC₁₁₃, and SPARC₁₁₈ were found to retain their
5 bioactivity in their released form, but no clear peptide property was identified to *a priori* predict which peptides retain bioactivity in the released form. Five peptides with varying properties were incorporated into PEG hydrogels via the enzymatically-responsive linker IPESLRAG (SEQ ID NO: 1), achieving enzymatically-responsive hydrogel degradation and peptide release. Linear regression analysis indicated peptide drug size controls the
10 rate of hydrogel degradation, while peptide drug hydrophobicity affects the amount of peptide fully released from the PEG macromers. This represents a novel method to controllably deliver peptide drugs in an enzymatically-responsive manner, with potential applications to aid in development of tissue engineered constructs, or for the treatment of tissue disorders such as ischemia, chronic inflammation, and tumors.

15

Example 3: Dose-dependent behavior of pro-angiogenic peptides

Experiments were first conducted to characterize the enzymatically responsive hydrogels comprising either SPARC₁₁₃(DL), SPARC₁₁₈(DL) or scrambled(DL) peptides. Hydrogels were formed as previously described and evaluated
20 for *in vitro* mass loss, swelling ratio, and “2T” peptide release (Figure 24). Gels were incubated in buffer for 24 hours at which point either 10nM MMP2 was added (open symbols) or the gels were left in buffer alone (closed symbols).

Gel degradation in terms of hydrogel dry mass and swelling ratios was tracked over time (Figure 25). All three gels were stable in buffer alone, but degraded in
25 the presence of MMP2, showing the enzymatically responsive nature of the gels. Differences in the peptide drug being released was associated with differences in the rate of hydrogel degradation, but all degraded via bulk degradation as evident by the increase in swelling ratio over the degradation time period for all three gel types, in the MMP2 solution. Tracking of “2T” peptide drug released from the hydrogels over time showed
30 that all gels release the drug in a stimuli-responsive manner, releasing “2T” peptide only in the presence of the MMP2 containing solution, while not releasing any in the buffer

alone condition. There were also variations in the amount of “2T” peptide released over the course of the study, depending on the drug being delivered from the hydrogel.

Experiments were also conducted to examine the diffusive release of peptides out of non-degradable PEG gels (Figure 25). It was observed that there was no significant difference between the diffusion coefficients of the examined peptides (SPARC₁₁₃ and SPARC₁₁₈). This illustrates that differences in peptide release are due to differences in rates of cleavage of the degradable linker, and not due to differences in the rate of diffusion of the peptides out of the gel after release from the network.

Tube network formation was assessed upon treatment with both the “N” and “2T” forms of Qk, SPARC₁₁₃, and SPARC₁₁₈ in a dose escalation study. Data is shown in Figure 26 – Figure 28, and statistical analyses are summarized in Table 5. Qk(N) significantly increased relative tube length to ~ 2.2-, ~ 2.3-, ~ 2.9-, ~ 2.6-, and ~ 2.9-fold that of control media at 0.01, 0.1, 1, 10, and 100 μ M, respectively, while Qk(2T) significantly increased relative tube length ~ 2.5-, ~ 3.1-, ~ 2.8-, and ~ 2.5-fold that of control media at 0.01, 0.1, 1, and 10 μ M, respectively. The amino acid “tails” increase overall pro-angiogenic peptide hydrophobicity, making Qk(2T) insoluble at 100 μ M; thus, this condition was eliminated in dose response experiments. SPARC₁₁₃ significantly increased tube length at 0.01, 0.1, 1, and 10 μ M (~2.5-, ~2.8-, ~3.0-, and ~2.5-fold, respectively) in its “N” form, but only at 100 μ M in the “2T” form (~ 2.6-fold, Figure 26). SPARC₁₁₈ significantly increased tube length at every concentration in its “N” form (~1.8-, ~2.0-, ~1.9-, ~2.0-, and ~2.2-fold at 0.01, 0.1, 1, 10, and 100 μ M, respectively), but only at 0.01 μ M in the “2T” form (~ 1.9 fold, Figure 26). The ability of Qk to induce tube network formation was significantly affected by peptide concentration, but not by the presence of amino acid “tails” (“N” vs “2T”). SPARC₁₁₃ was significantly affected both by concentration and by the amino acid “tails”, and there was a significant interaction between these two factors. The ability of SPARC₁₁₈ to induce tube network formation was significantly affected by peptide concentration and the presence of the amino acid “tails”, but no interaction existed between these two factors (Table 5).

Peptide	Source of Variation	P value	Significance
SPARC ₁₁₃	Interaction	0.0218	*

	Concentration	< 0.0001	#
	Tails	0.0269	*
SPARC ₁₁₈	Interaction	0.3208	ns
	Concentration	< 0.0001	#
	Tails	0.0105	*
Scrambled	Concentration	0.8007	ns

Table 5: Results of statistical analysis performed for Figure 26. Results for the three pro-angiogenic peptides were analyzed using a two-way ANOVA to determine the effect of the peptide dose and presence of amino acid “tails” on the peptide’s ability to induce tube network formation. Results for the scrambled peptide were analyzed using a one-way ANOVA. ns p>0.05; * p<0.05; # p<0.0001.

Further investigation into the bioactivity of the three most promising peptides provides additional information regarding the effect of the “2T” on peptide bioactivity. The ability of Qk to induce tube network formation was equivalent in the “N” and “2T” forms (Figure 26A, Table 5), but it lost “2T” efficacy at 1 nM in the proliferation assay. Qk, a VEGF mimic, has been shown to induce cellular processes such as proliferation, survival, and migration by binding to the tyrosine kinase receptor VEGFR-2 and inducing activation of ERK1/2, Akt and eNOS (Finetti et al., 2012, Biochem Pharm, 84: 303-311). The present results, combined with these previous reports, imply that the presence of the “2T” only slightly affects the ability of Qk to bind VEGFR-2 and cause observed downstream effects of proliferation and tube formation (D’Andrea et al., 2005, P Natl Acad Sci USA, 102: 14215-14220). Qk, in both “N” and “2T” forms, was able to significantly increase relative tube length at every concentration investigated, and exhibited dose-dependent behavior, with the response plateauing upon treatment above 1 μ M, (Figure 26A). Previous work by Finetti et al. demonstrated a more linear dose-dependency when using Qk in the HUVEC proliferation assay *in vitro* and the DIVAA assay *in vivo*; however, their study focused on much lower treatment concentrations (~ 2 to 50 nM) (Finetti et al., 2012, Biochem Pharm, 84: 303-311). Therefore, it is possible that the VEGF receptors become saturated upon treatment with 1 μ M of Qk, causing the observed plateau in response. When degraded Qk(DL) hydrogels were investigated in the tube formation assay, degraded hydrogel products were able to

significantly increase average tube length at every dose investigated (Figure 27 and Figure 28). This demonstrates that the Qk(DL) hydrogels are indeed able to release bioactive components upon degradation, demonstrating the therapeutic potential of this peptide delivery system.

5 The ability of SPARC₁₁₃ to induce tube network formation was significantly affected by the incorporation of “2T”, as well as the concentration of peptide used, with a significant interaction occurring between these two factors (Figure 26, Table 5). SPARC₁₁₃(N) exhibited increasing tube lengths from 0.01 to 1 μ M, before decreasing and losing statistical significance as concentration was increased to 100 μ M. Conversely, 10 SPARC₁₁₃(2T) exhibited an increase in relative tube length from 0.01 to 100 μ M, and only reached significance at the highest concentration investigated. However, when tube formation was investigated using SPARC₁₁₃(DL), degraded from gels, it was observed that SPARC₁₁₃ significantly increased tube length at 1/70,000th, 1/7,000th, 1/700th, and 1/70th fraction of a gel (Figure 27 and Figure 28) As these gel dilutions produce 15 significant increases in tube formation lower released SPARC₁₁₃(2T) concentrations lower than the minimum effective concentration using the “2T” drug (~0.01 μ M vs. 100uM) alone, this indicates that the peptide remaining bound to the PEG molecule but not fully released in its “2T” form retains bioactivity and is able to induce tube network formation. (Figure 26 and Figure 28) However, in the proliferation assay, SPARC₁₁₃ 20 increased HUVEC proliferation at 1 nM and not at 100 nM, in both the “N” and “2T” form. Together, these results indicate that the “2T” adversely affects the effect of SPARC₁₁₃ on the intracellular signaling associated with tube formation, but not with proliferation. Differential effects between these two assays upon treatment with the same drug is not unexpected as they are distinct cell processes, and previous studies have also 25 reported differences in HUVEC proliferation and tube formation upon treatment with the same drug (Hardy et al., 2007, Peptides, 28: 691-701; Demidova-Rice et al., 2011, Wound Repair Regen, 19: 59-70). SPARC₁₁₃ has previously been shown to induce cord formation of bovine aortic endothelial (BAE) cells after 7-10 days of *in vitro* culture at 125 μ M (Lane et al., 1994, J Cell Biol, 125: 929-943), and to increase vessel formation in 30 the chicken chorioallantoic membrane (CAM) assay at 10-500 μ M, with a maximum increase in capillary density occurring at 50 μ M (Iruela-Arispe et al., 1995, Mol Cell Biol,

6: 327-343). While the concentration ranges and assays differ from those investigated here, the observed trend of increasing then decreasing response with increasing doses is consistent with previous results.

SPARC₁₁₈(2T) was only able to increase relative tube length at the lowest
5 concentration, in contrast to the “N” version of the peptide which significantly increased tube length at every concentration investigated (Figure 26). While both the presence of the tails and the concentration of peptide used affected tube length, there was no interaction between these two factors (Table 5). However, when tube formation was investigated using SPARC₁₁₈(DL), degraded from gels, it was observed that SPARC₁₁₈
10 significantly increased tube length at only the highest gel fraction (1/70th; Figure 27) corresponding to the highest released peptide concentration (10 µM; Figure 28). This discrepancy in bioactive concentration between “2T” and degraded gel released “2T” concentration indicates that the effective PEGylation of peptides that occurs when they are released from gels, but not fully released from a PEG molecule affects peptide
15 bioactivity, and alters the dose response curve observed. Additionally, in the HUVEC proliferation assay, the same trend was observed between SPARC₁₁₈ “N” and “2T”: in both forms, 1 nM SPARC₁₁₈ increased HUVEC proliferation to a higher level than 100 nM, with the “2T” form being less effective than the “N” form at both concentrations. These results indicate that the “2T” adversely affects, but does not abrogate, the
20 activation of intracellular signaling induced by SPARC₁₁₈. The similar peptide SPARC₁₁₉ (KGHK rather than KKGHK) causes dose-dependent increases in capillary density in the CAM assay as peptide concentration is increased from 10 to 5,000 µM (Iruela-Arispe et al., 1995, Mol Cell Biol, 6: 327-343), similar to the relatively steady increase in tube length upon treatment with increasing concentrations of SPARC₁₁₈(N) observed here.
25 SPARC₁₁₉ also induces cord formation of BAE cells when used at 125 µM (Lane et al., 1994, J Cell Biol, 125: 929-943). Increases in capillary density (Iruela-Arispe et al., 1995, Mol Cell Biol, 6: 327-343) and the increase in tube length with increasing dose observed here suggest that if higher concentrations of SPARC₁₁₈(N) and (2T) been investigated, larger increases in relative tube length may have been observed.

30

The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety. While the compositions and methods have been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations may be devised by
5 others skilled in the art without departing from the true spirit and scope. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

CLAIMS

What is claimed:

1. A composition comprising
a plurality of synthetic monomers, and
a plurality of prodrugs, wherein each prodrug comprises at least one therapeutic domain and one or more cleavable domains,
wherein the cleavable domains couples the prodrug to one or more of the plurality of synthetic monomers,
and wherein the ratio of prodrug to synthetic monomer in the composition is greater than about 1:1.
2. The composition of claim 1, wherein the ratio of prodrug to synthetic monomer in the composition is greater than about 2:1.
3. The composition of claim 1, wherein the prodrug comprises a therapeutic peptide coupled to a cleavable peptide sequence at its amino terminus and a cleavable peptide sequence at its carboxy terminus.
4. The composition of claim 1, wherein the synthetic monomer comprise poly(ethylene glycol) (PEG).
5. The composition of claim 4, wherein the PEG is functionalized with terminal norbornene groups.
6. The composition of claim 1, wherein the composition is a polymerized matrix.
7. The composition of claim 1, wherein the composition is a solution.
8. The composition of claim 1, wherein the at least one cleavage domain comprises a matrix metalloproteinase (MMP) -sensitive cleavage domain.

9. The composition of claim 8, wherein the MMP-sensitive cleavage domain comprises the amino acid sequence of SEQ ID NO: 1.

10. The composition of claim 8, wherein an increased expression, activity, or combination thereof, of a MMP induces cleavage of the MMP-sensitive cleavage domain.

11. The composition of claim 1, wherein the therapeutic domain comprises a pro-angiogenic amino acid sequence.

12. The composition of claim 11, wherein the therapeutic domain comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 5, and SEQ ID NO: 6.

13. The composition of claim 1, wherein cleavage of the cleavage domains releases a released therapeutic agent.

14. The composition of claim 13, wherein the released therapeutic agent comprises a fragment of a first cleavage domain, wherein the fragment is positioned at a first terminal end of the released therapeutic agent.

15. The composition of claim 13, wherein the released therapeutic agent comprises a fragment of a second cleavage domain, wherein the fragment is positioned at a second terminal end of the released therapeutic agent.

16. The composition of claim 13, wherein the released therapeutic agent comprises a first fragment of a first cleavage domain, wherein the first fragment is positioned at a first terminal end of the released therapeutic agent, and a second fragment of a second cleavage domain, wherein the second fragment is positioned at a second terminal end of the released therapeutic agent.

17. The composition of claim 13, wherein the released therapeutic agent is a peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 8, SEQ ID NO: 10, and SEQ ID NO: 12.

18. The composition of claim 1, wherein the composition provides localized and stimuli-induced release of the released therapeutic agent.

19. The composition of claim 6, wherein polymerization of the matrix is induced by UV light.

20. The composition of claim 1, wherein the composition is a hydrogel.

21. A method of delivering a therapeutic agent to a subject in need thereof, comprising administering to a subject the composition of any one of claims 1-20.

22. The method of claim 21, wherein the subject has a condition associated with reduced vascularization, wherein the therapeutic domain comprises a pro-angiogenic amino acid sequence.

23. The method of claim 22, wherein the condition is selected from the group consisting of cardiac ischemia, coronary heart failure, peripheral vascular ischemia, peripheral arterial disease, ischemic bowel disease, bone fracture healing, and diabetic ulcers.

24. The method of claim 21, wherein the subject comprises an implanted engineered tissue in need of vascularization.

25. The method of claim 21, wherein the subject is a human.

26. The method of claim 21, comprising polymerizing the composition *ex vivo*.

27. The method of claim 21, wherein the composition comprises a hydrogel, and wherein the method comprises implanting the hydrogel into the subject at a treatment site.

28. The method of claim 21, wherein the method comprises the step of administering UV light to the subject in vivo, thereby polymerizing the composition to form a matrix.

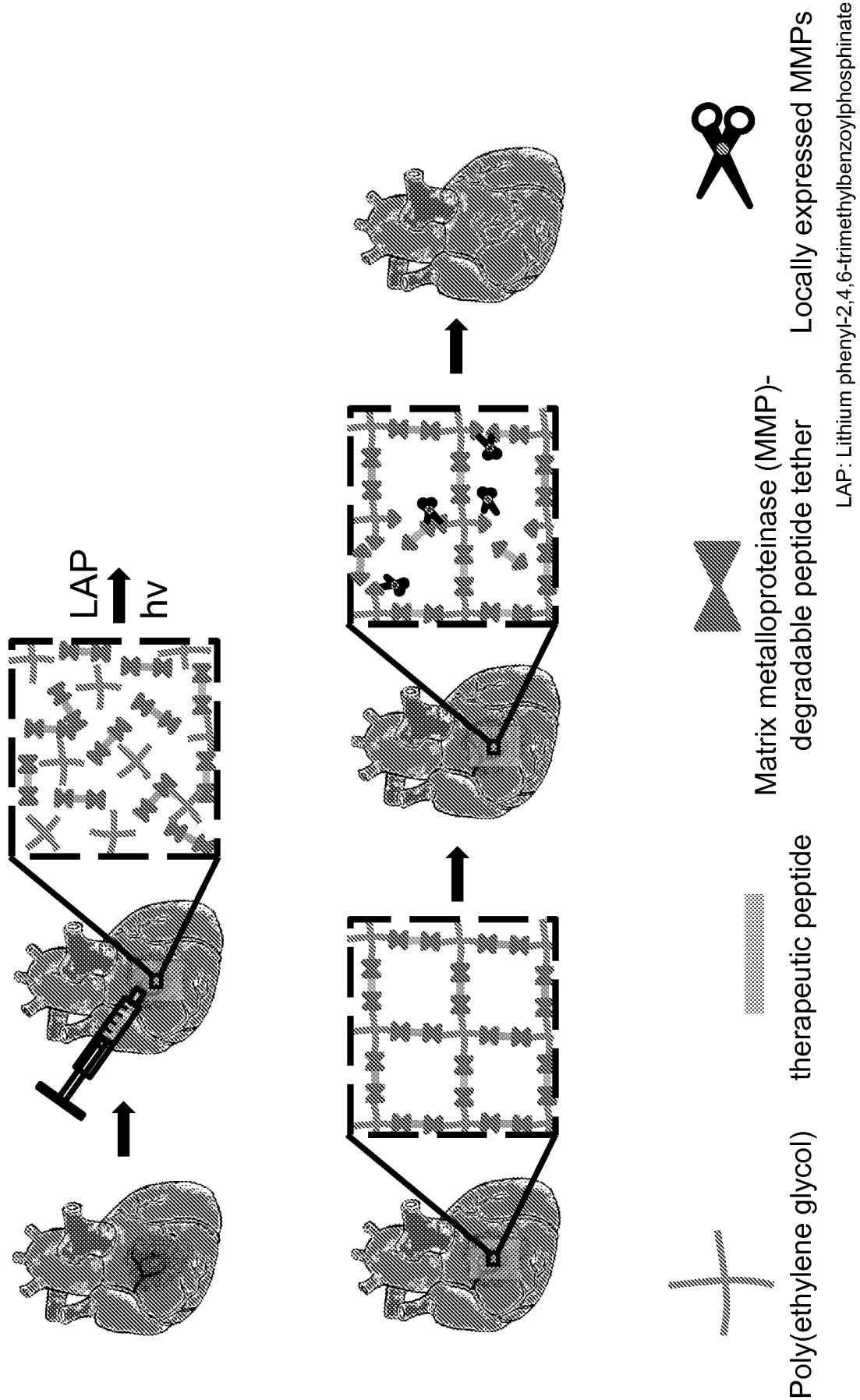


Figure 1

2/29

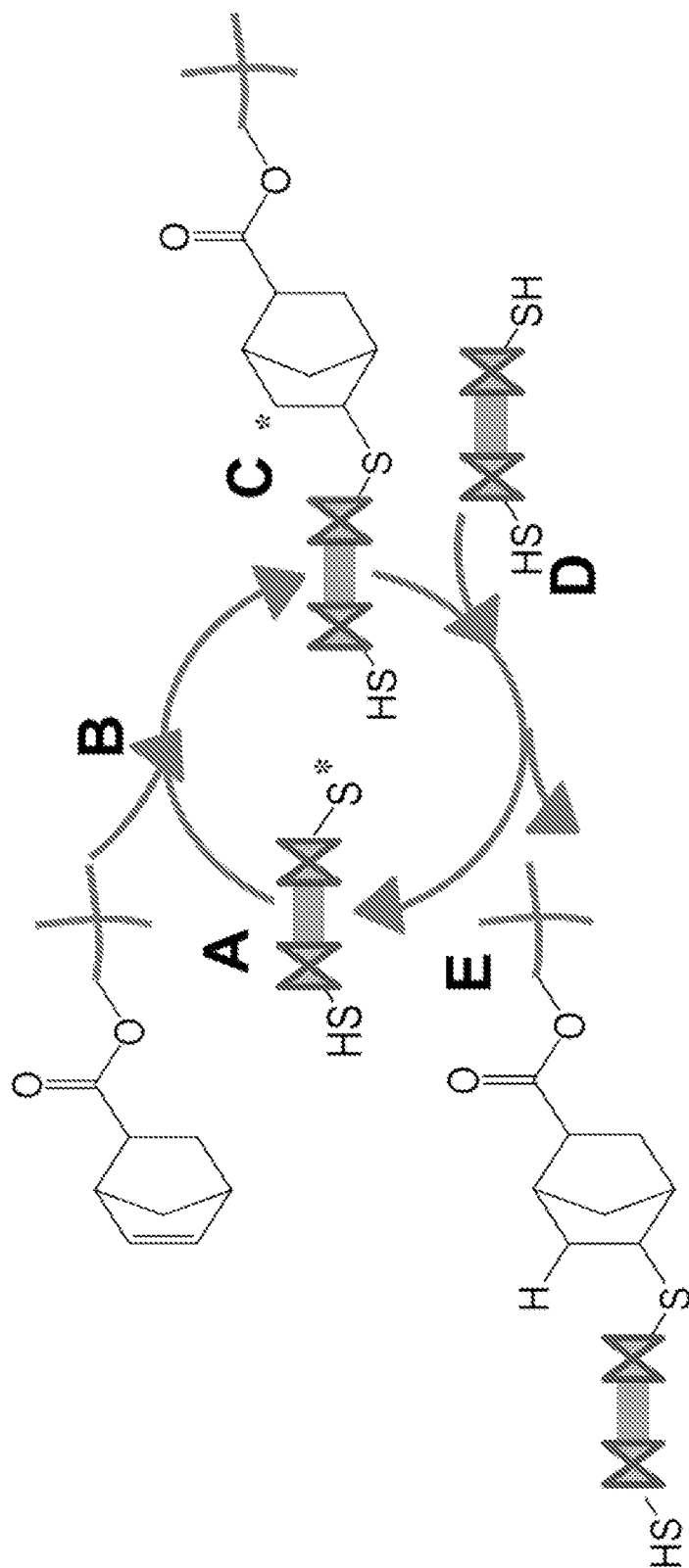


Figure 2

3/29

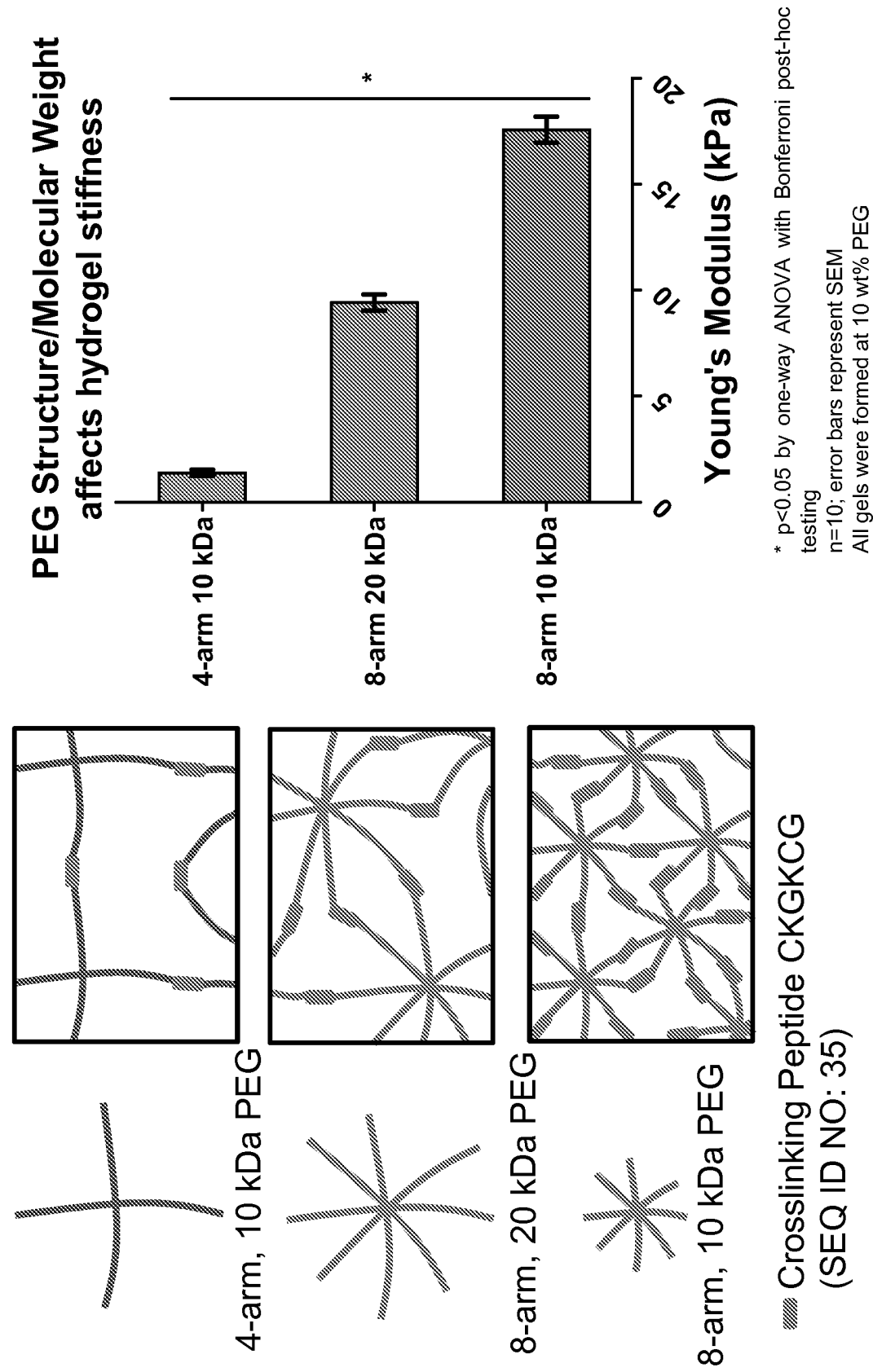


Figure 3

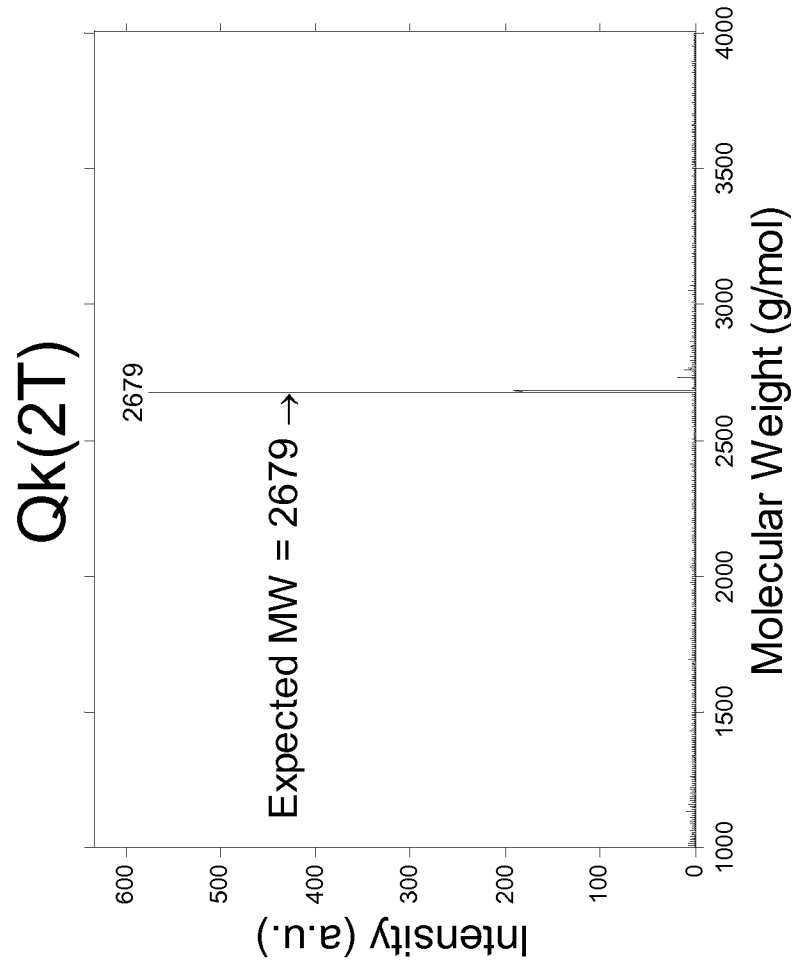
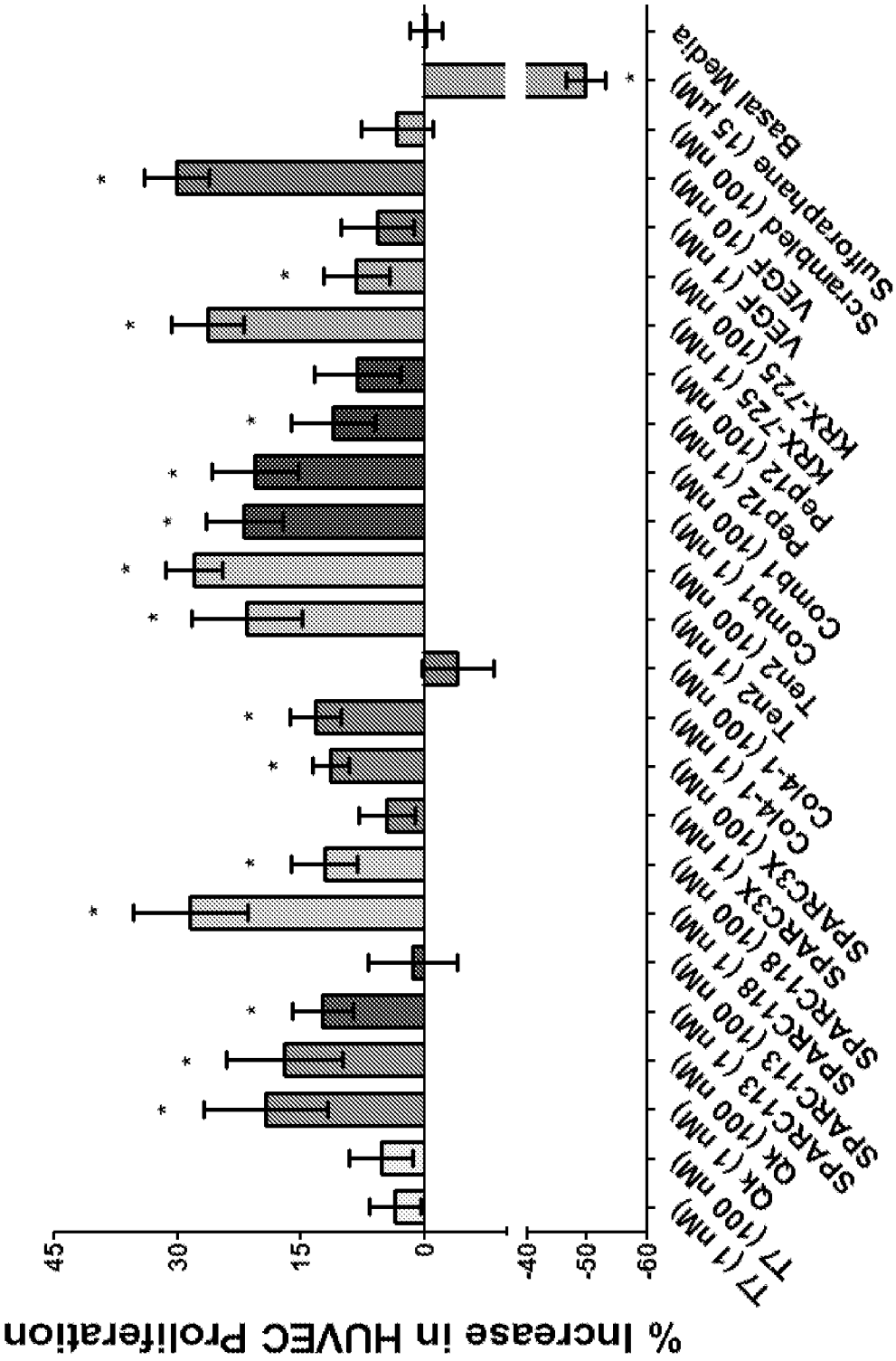


Figure 4

5/29



n=12; error bars = SEM;
* p<0.05 vs. basal media

Figure 5

6/29

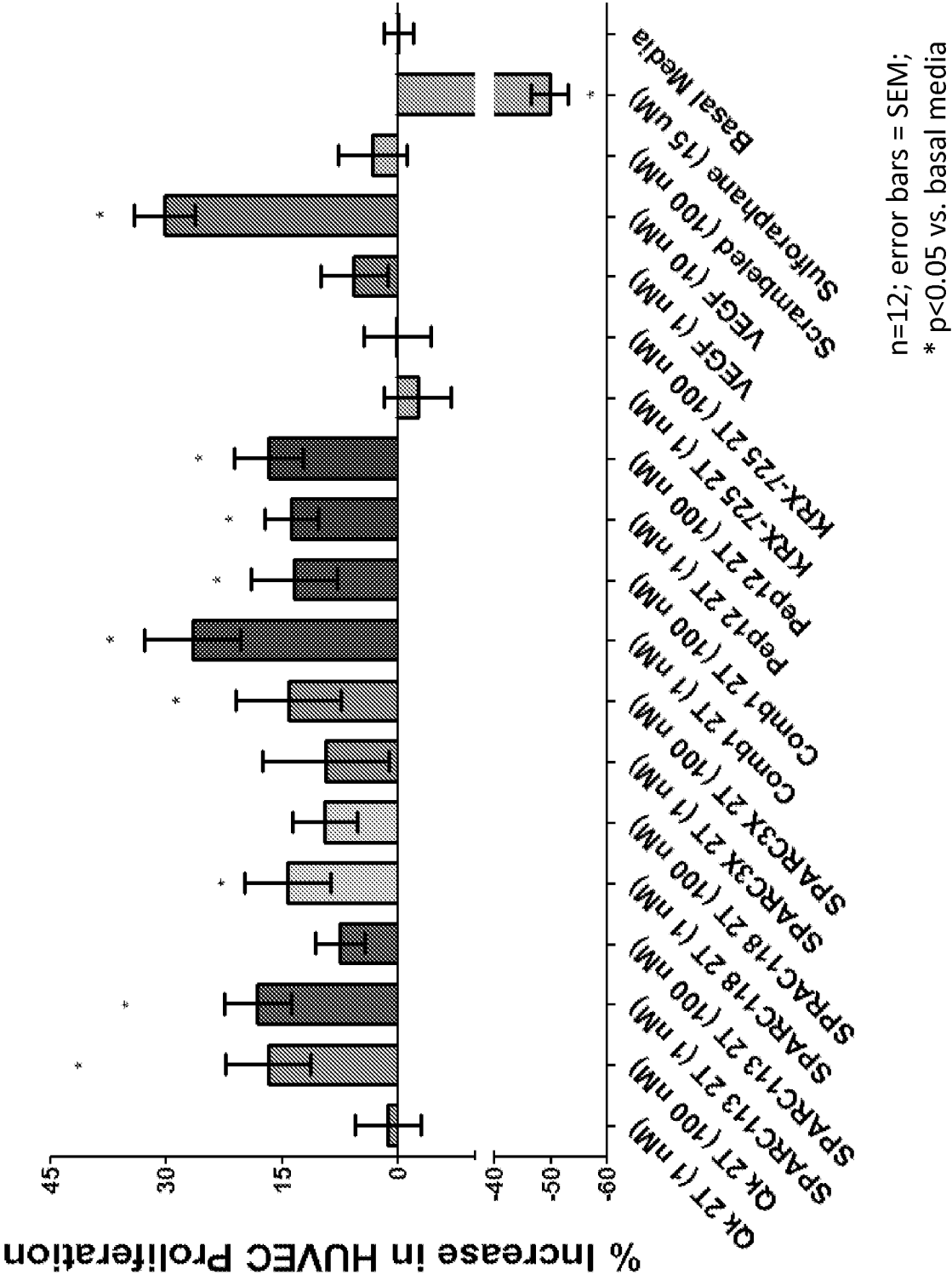


Figure 6

7/29

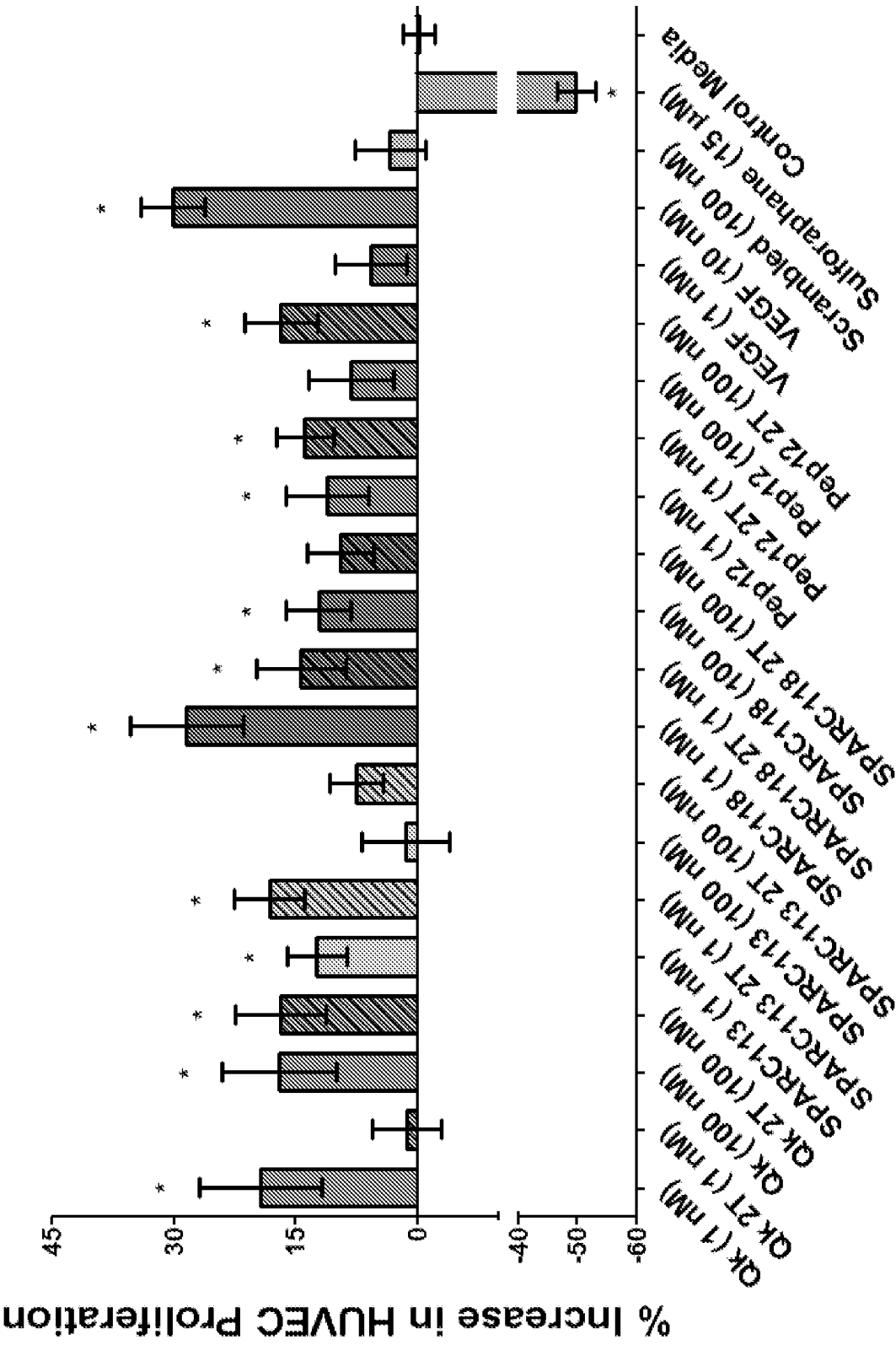
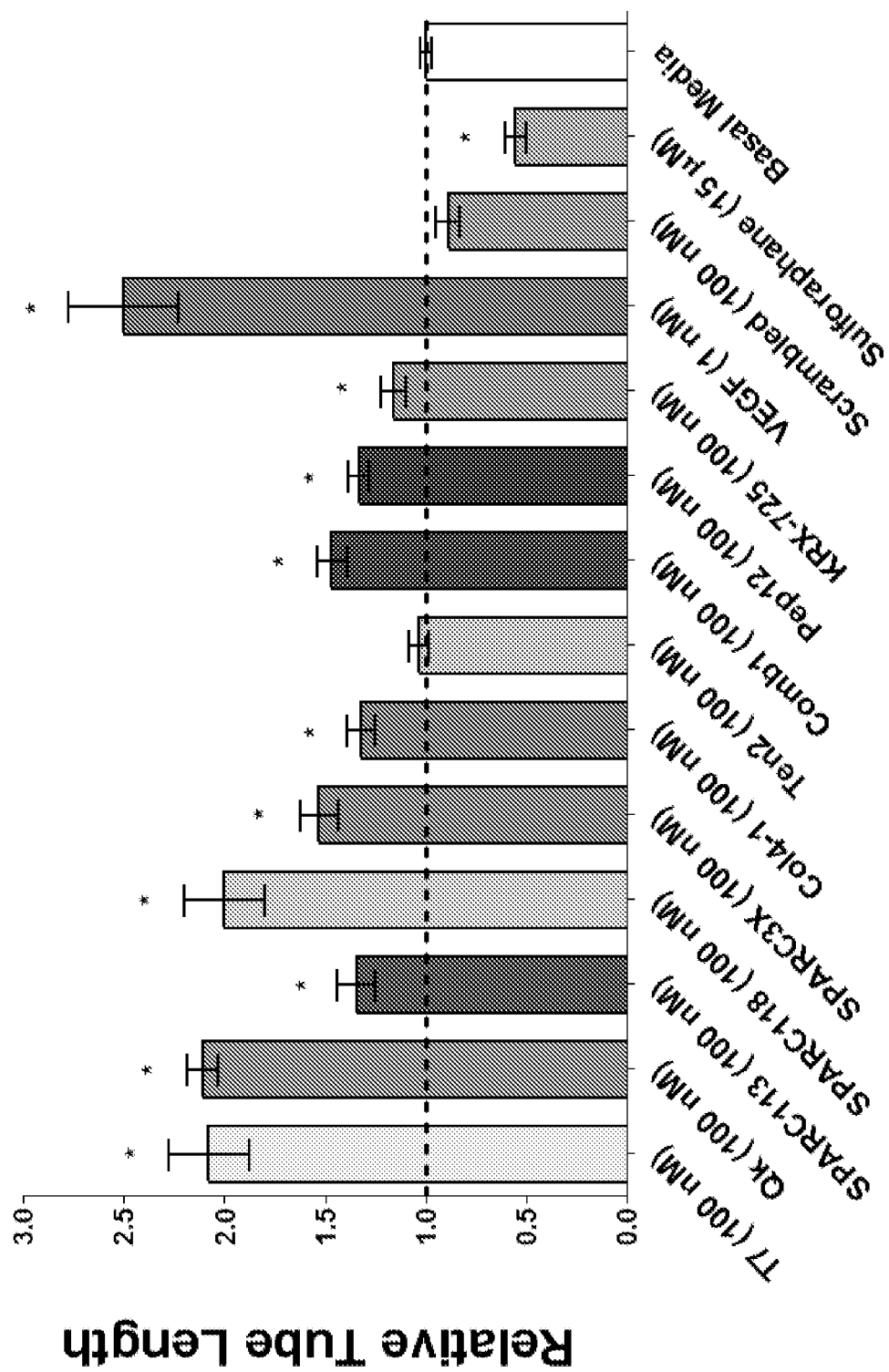


Figure 7

8/29



n=9; error bars = SEM;
* p<0.05 vs. basal media

Figure 8

9/29

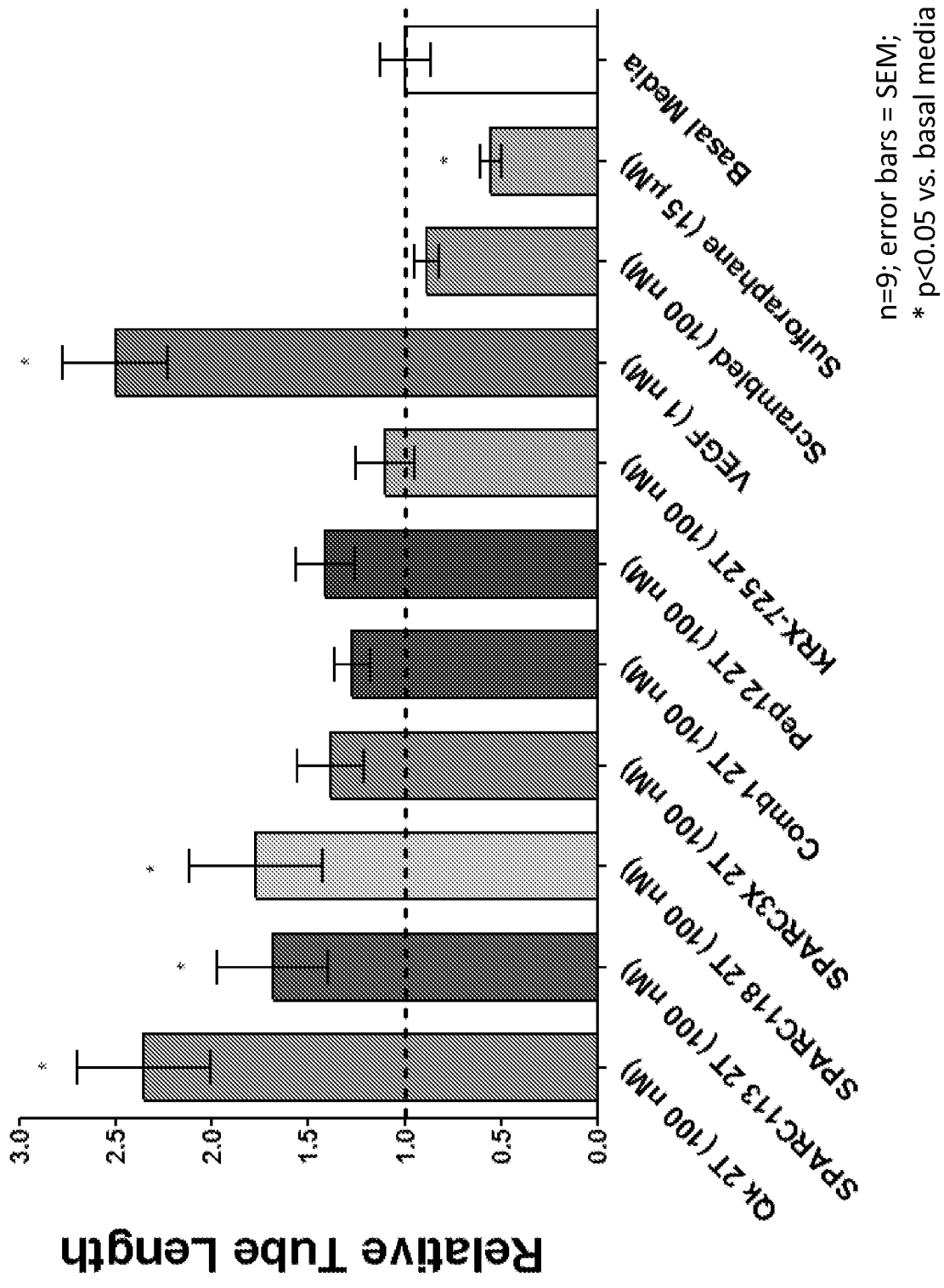


Figure 9

10/29

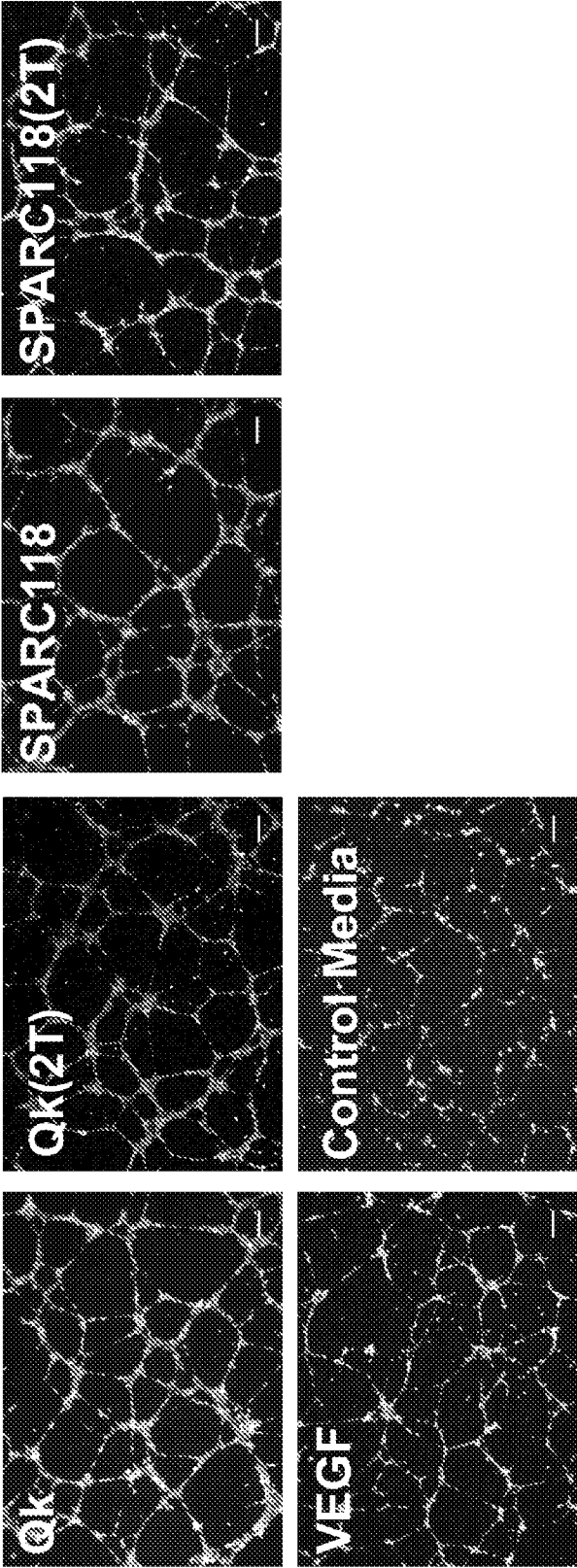


Figure 10

11/29

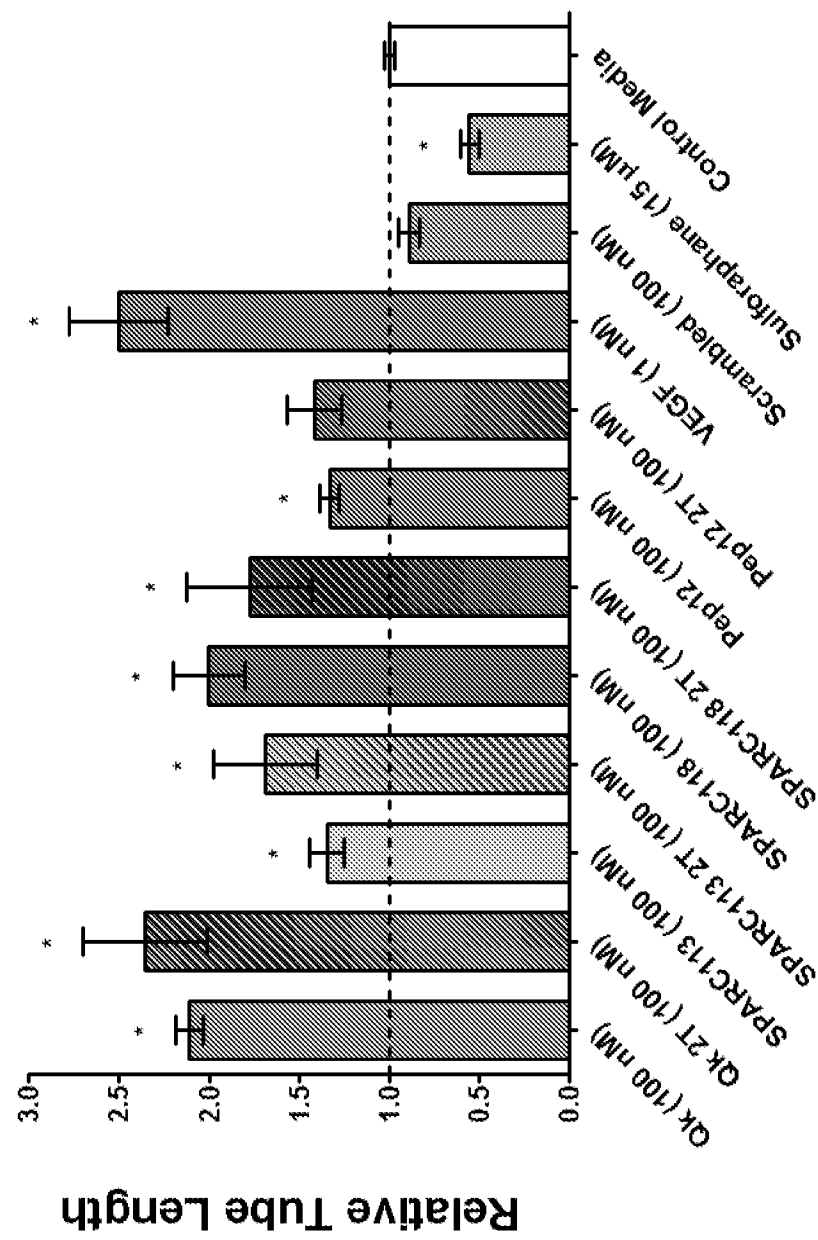
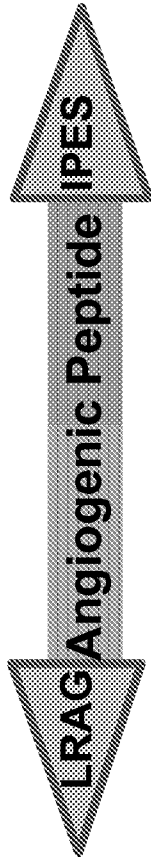


Figure 11

12/29



Peptide	Naked Form			2T Form		
	HUVEC Proliferation	HUVEC Tube Length	Qualify for 2T Testing?	HUVEC Proliferation	HUVEC Tube Length	Hydrogel Incorporation?
T7	n.s.	↑	No	-	-	No
QK	↑	↑	Yes	↑	↑	Yes
SPARC ₁₁₃	↑	↑	Yes	↑	↑	Yes
SPARC ₁₁₈	↑	↑	Yes	↑	↑	Yes
SPARC _{3X}	↑	↑	Yes	↑	n.s.	No
Col4-1	↑	↑	No	-	-	No
Ten2	↑	n.s.	No	-	-	No
Comb1	↑	↑	Yes	↑	n.s.	No
Pep12	↑	↑	Yes	↑	n.s.	No
KRX-725	↑	↑	Yes	n.s.	n.s.	No
Scrambled	n.s.	n.s.	-	-	-	-

↑ significantly increased over basal media ($p < 0.05$); **Figure 12**
 n.s. not statistically significant; - not tested

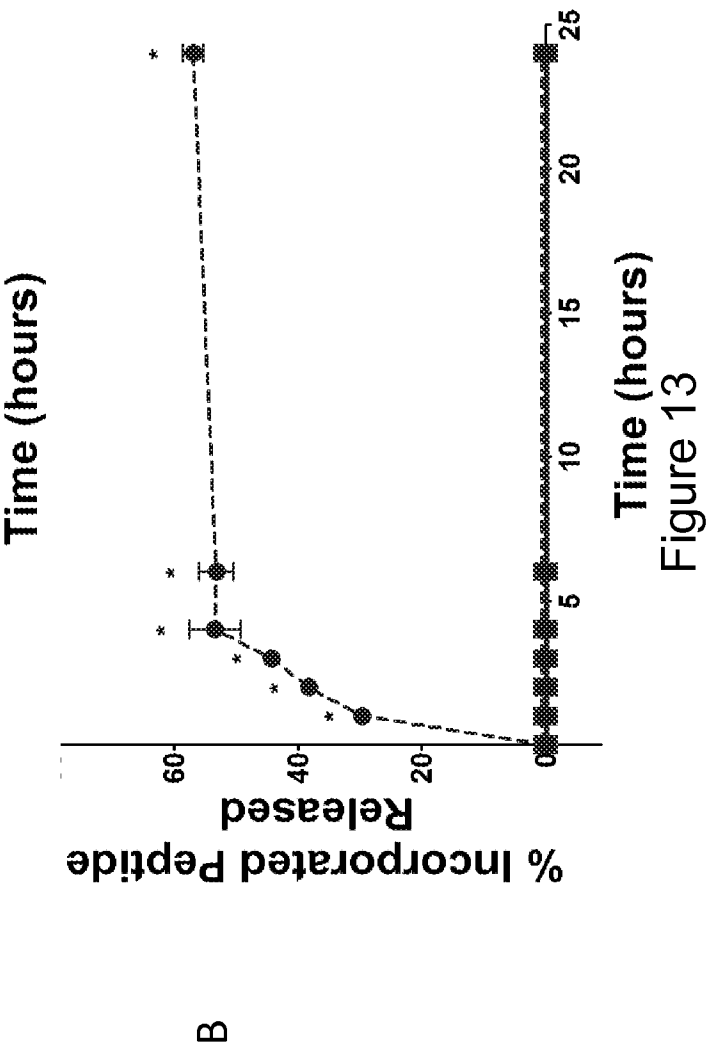
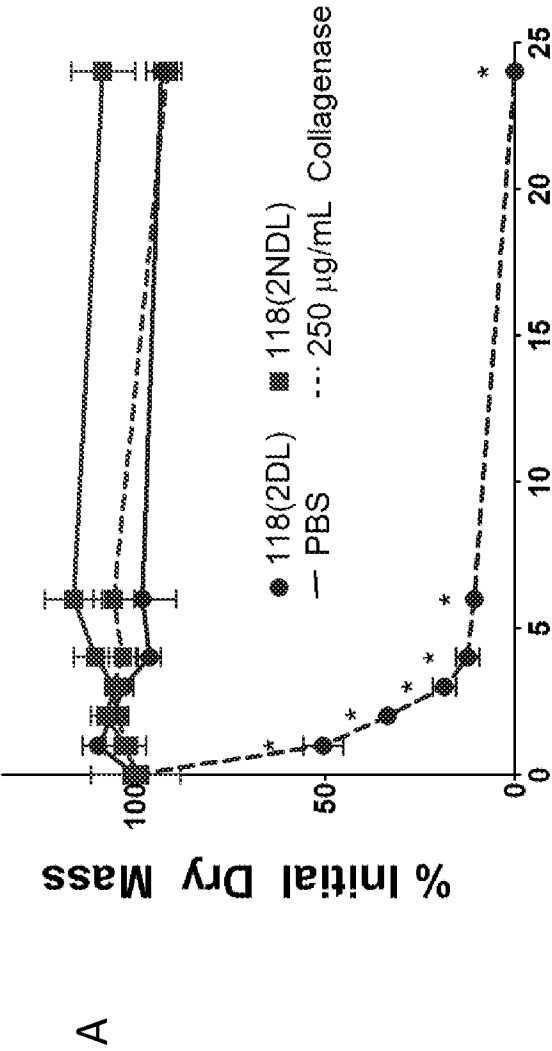


Figure 13

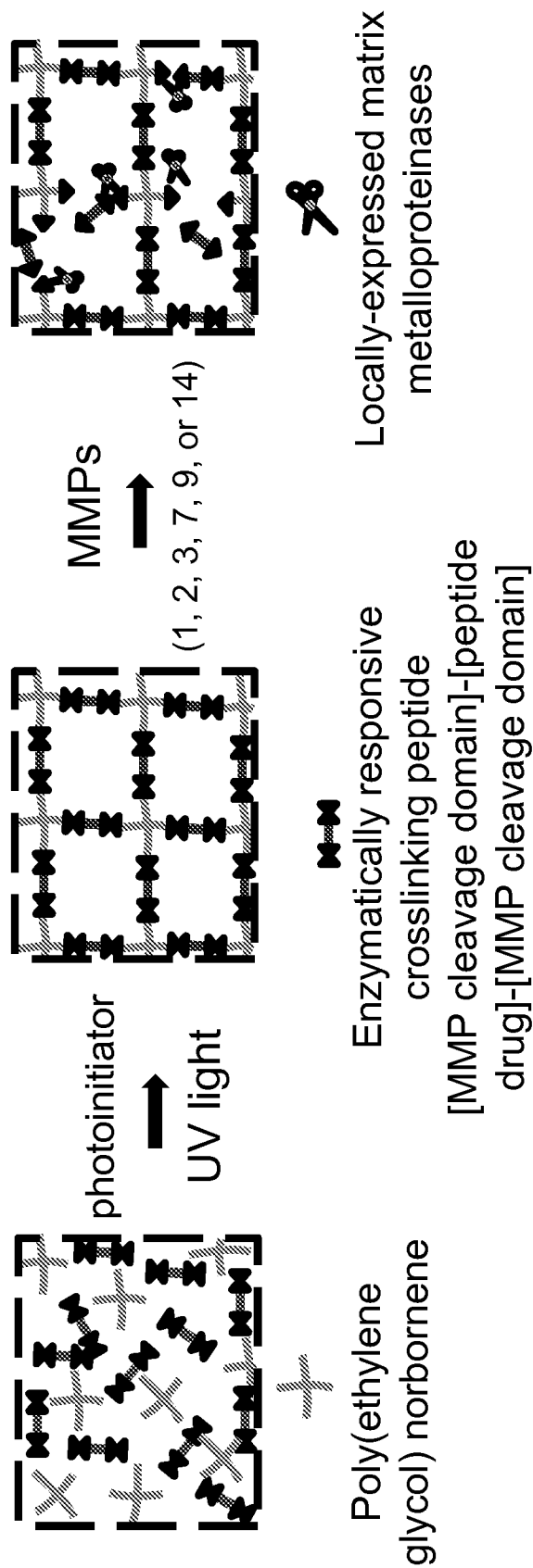


Figure 14

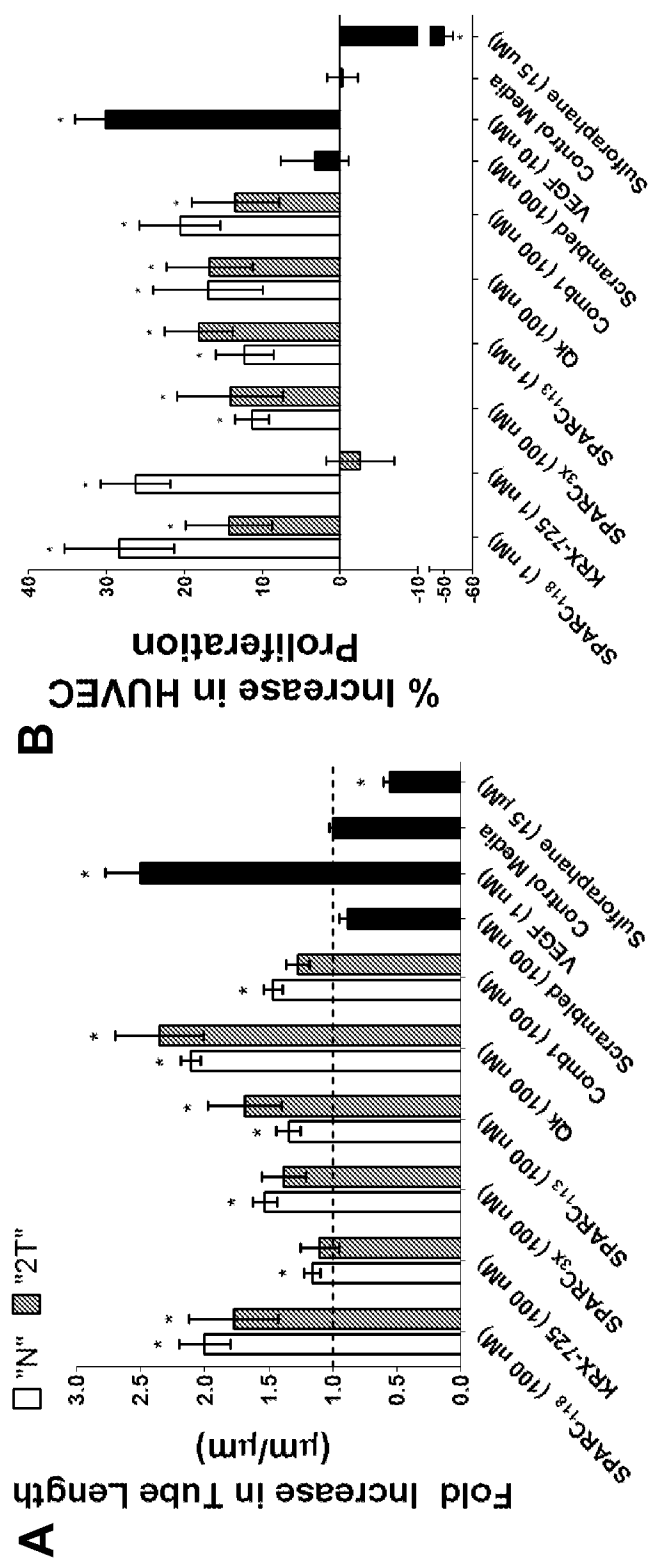


Figure 15

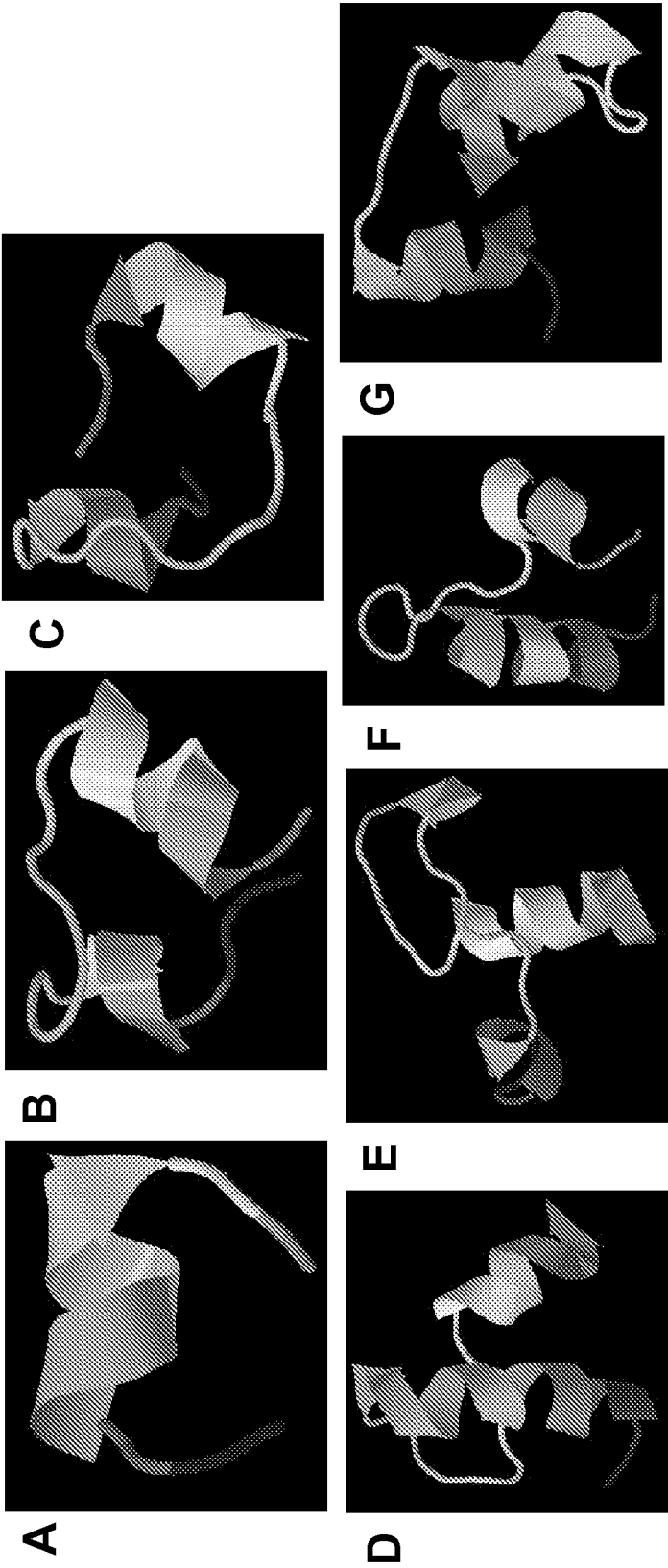


Figure 16

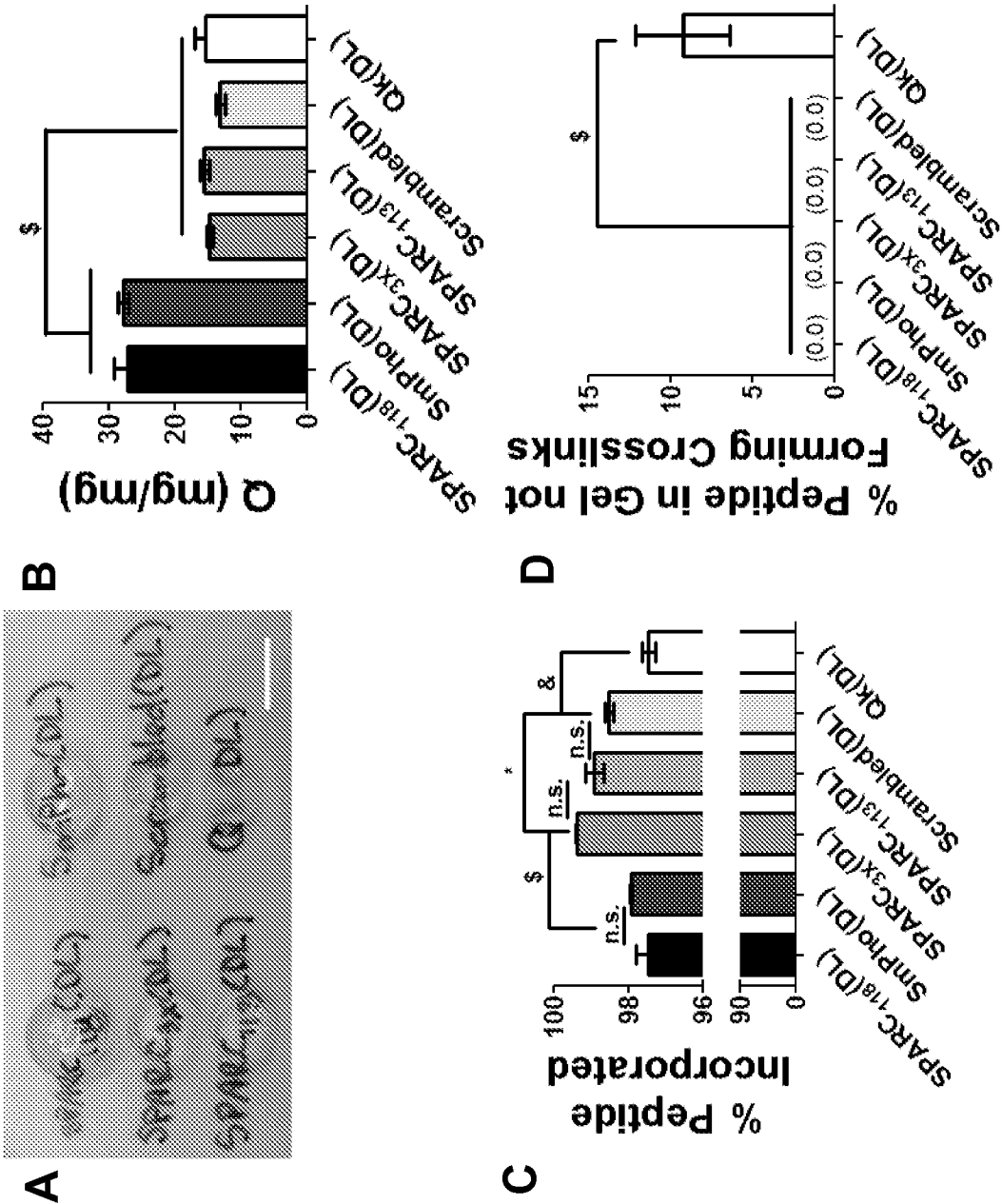


Figure 17

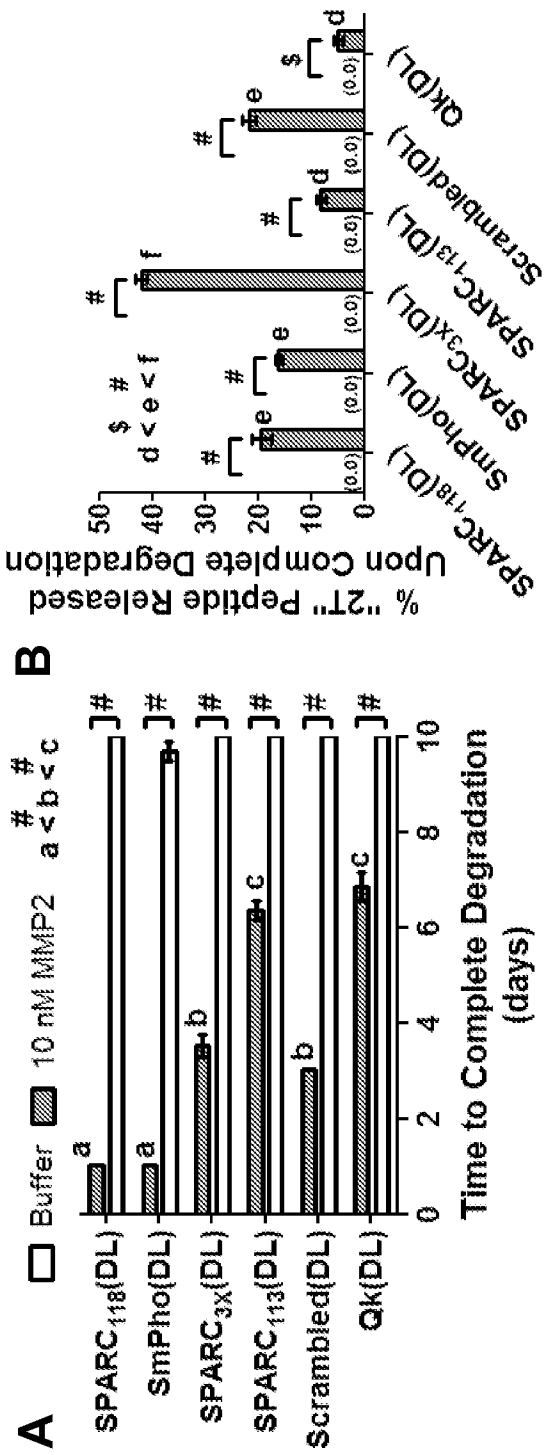


Figure 18

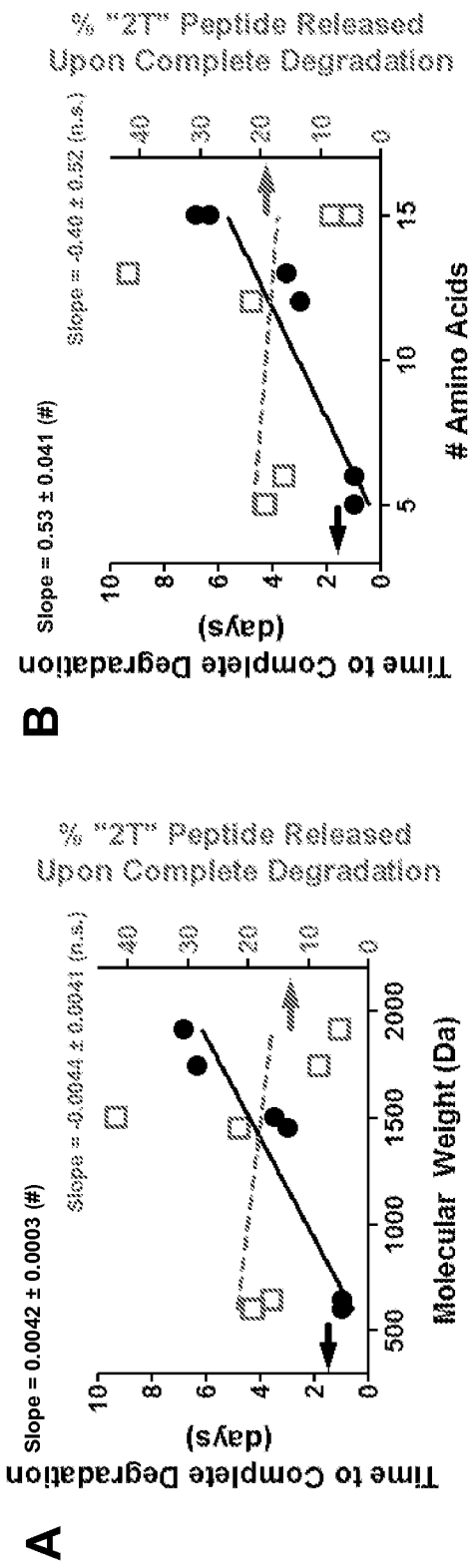


Figure 19A – Figure 19B

20/29

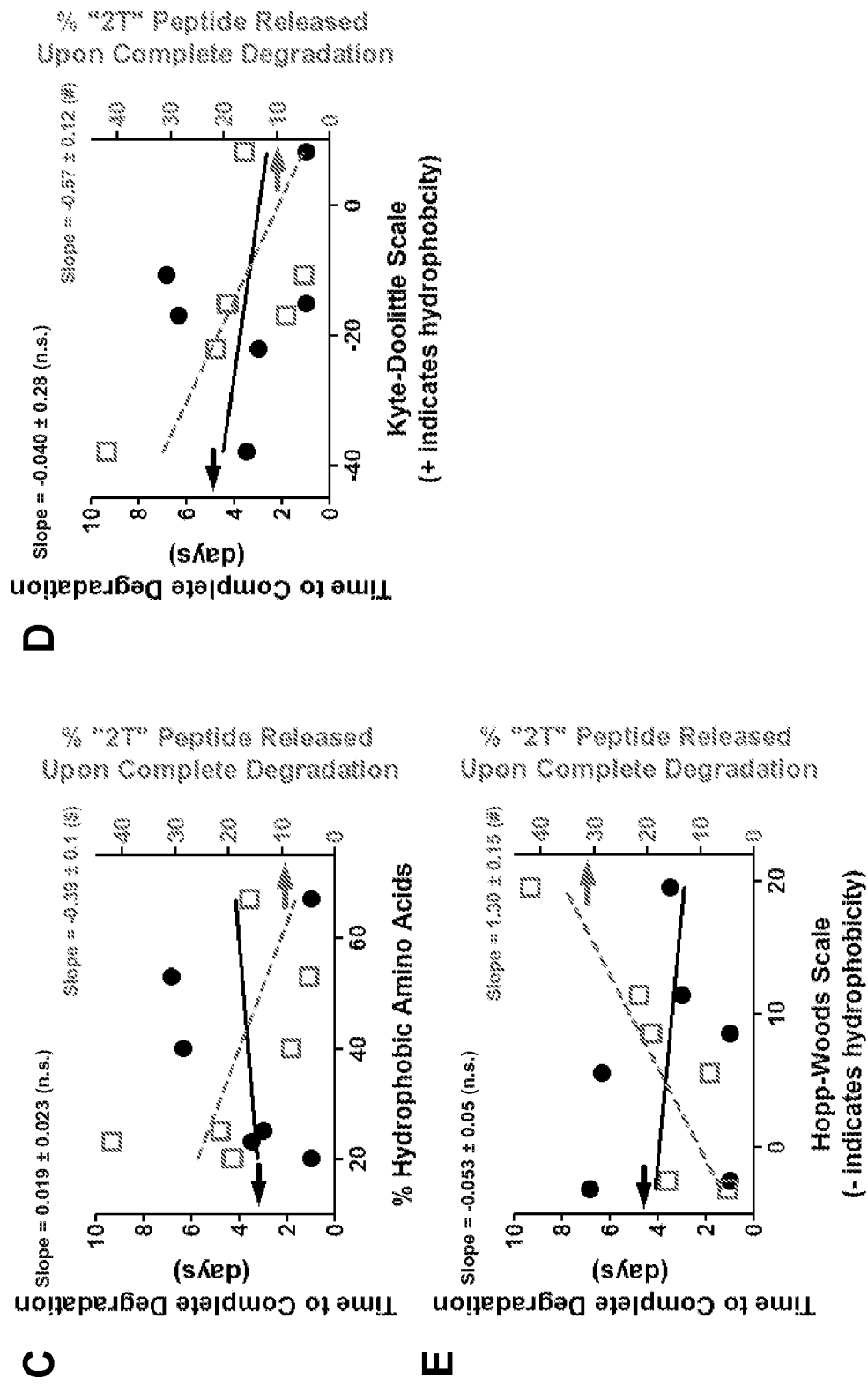


Figure 19C – Figure 19E

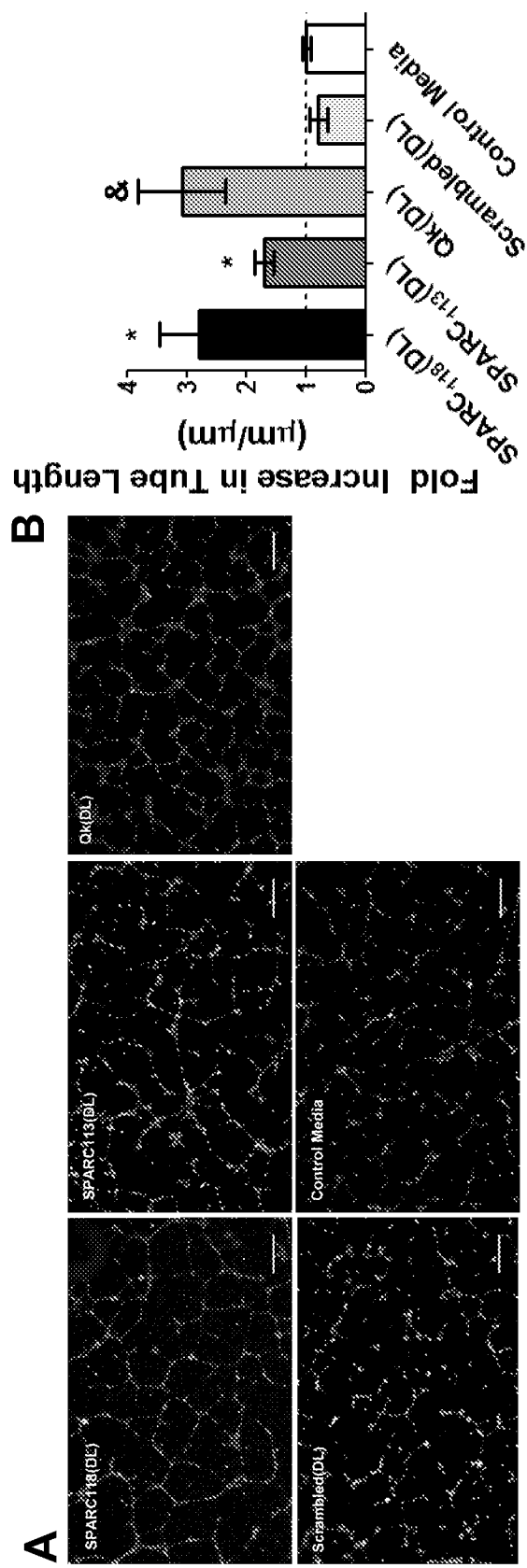


Figure 20

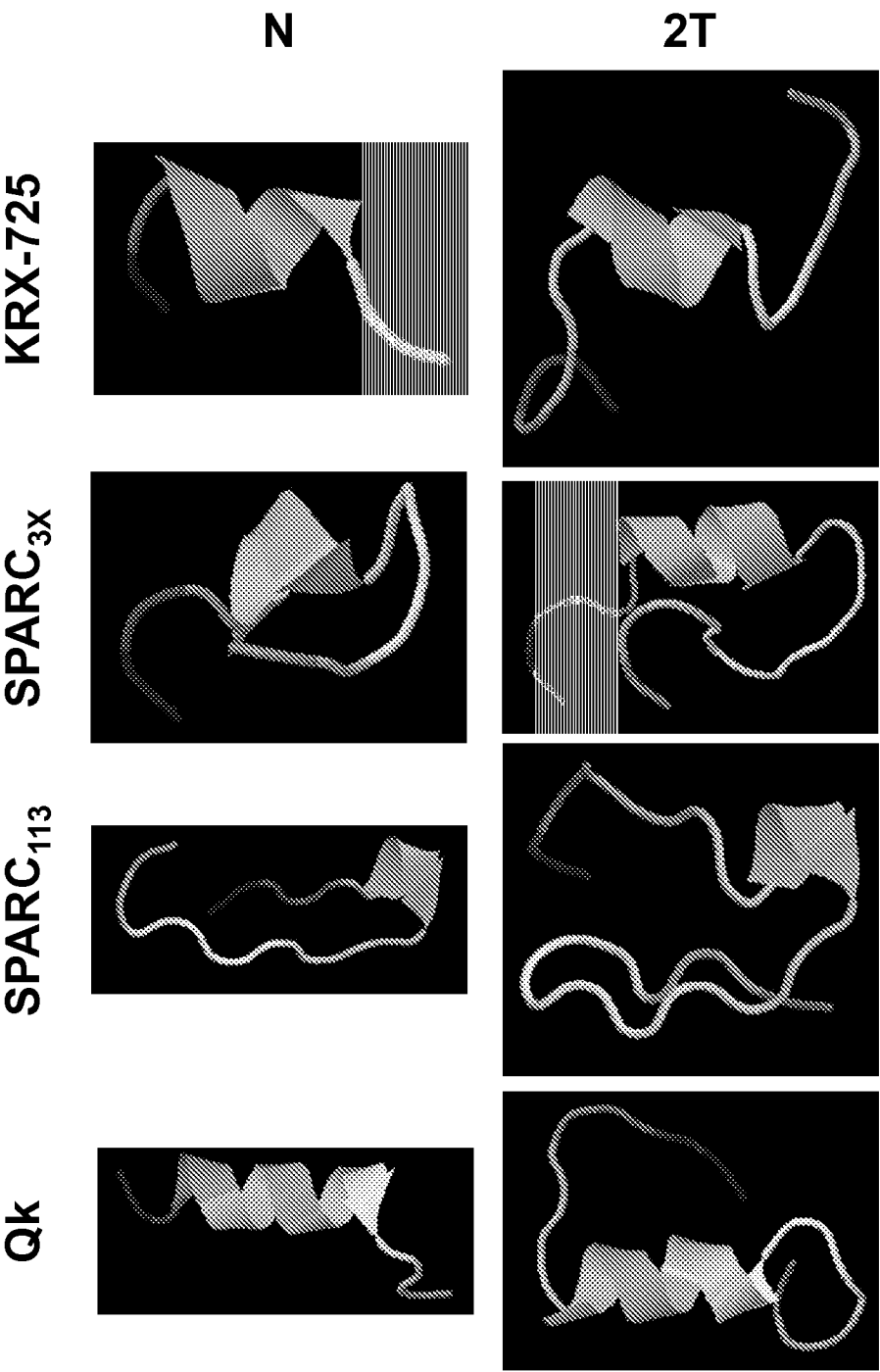


Figure 21

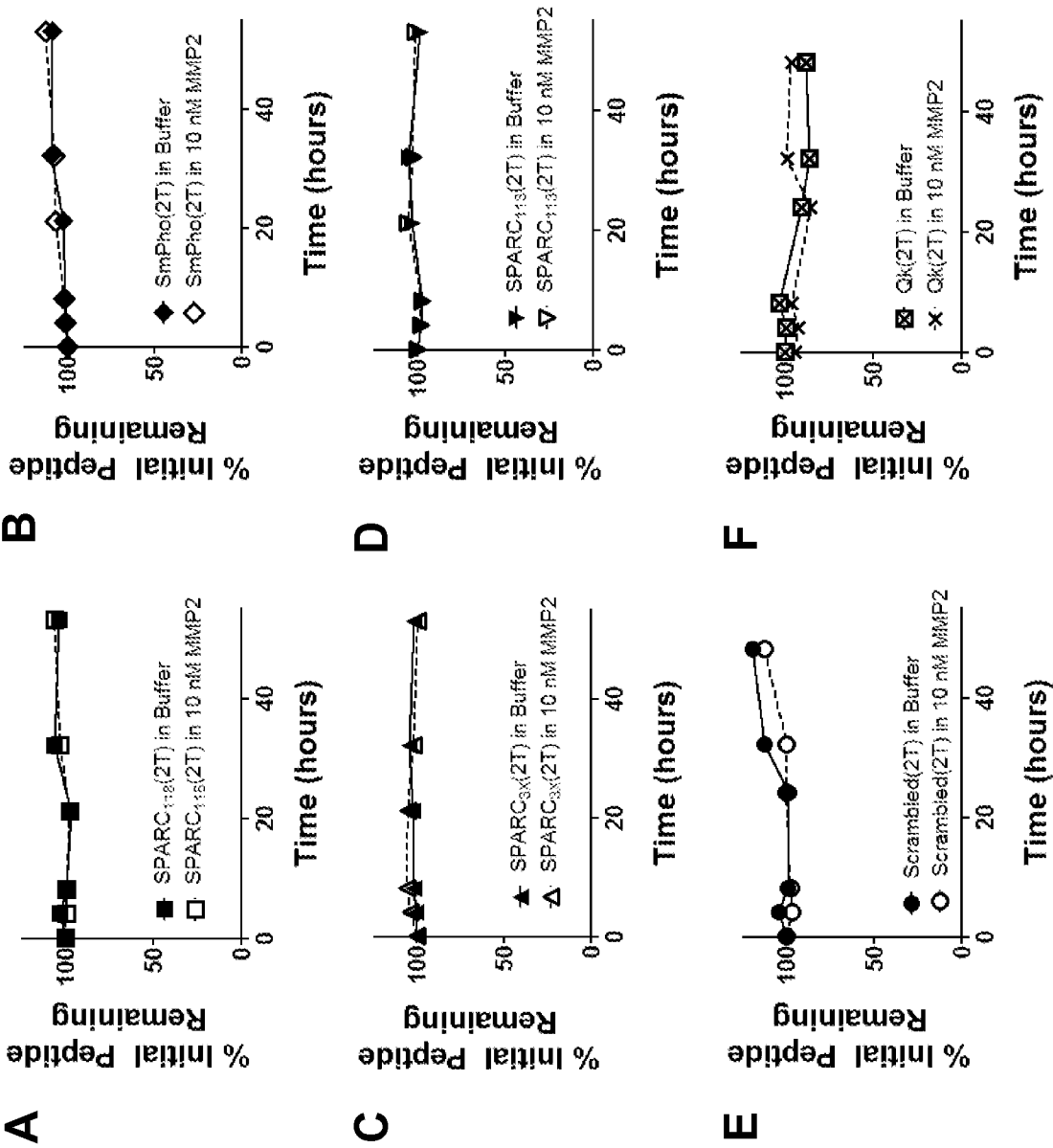


Figure 22

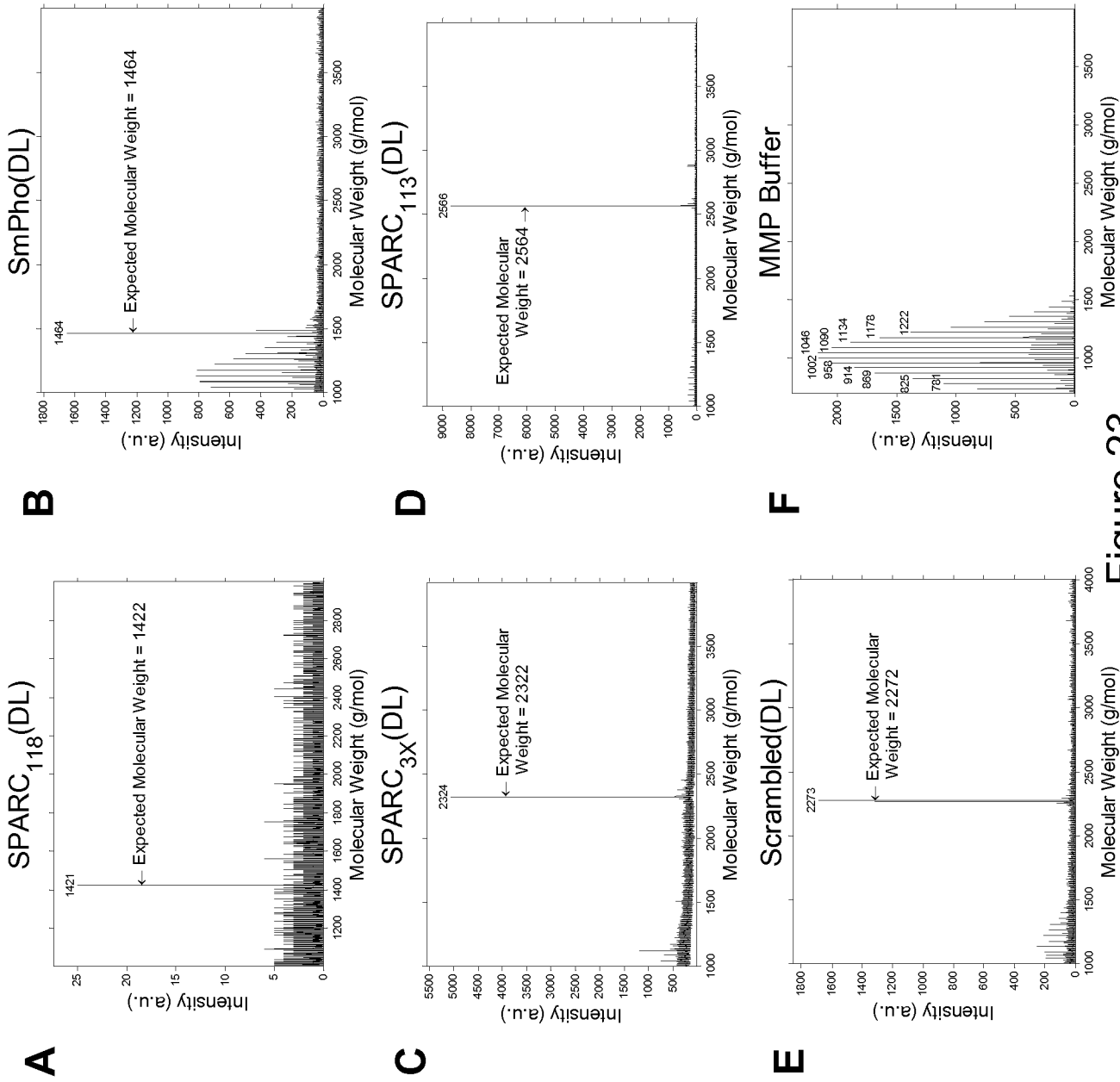


Figure 23

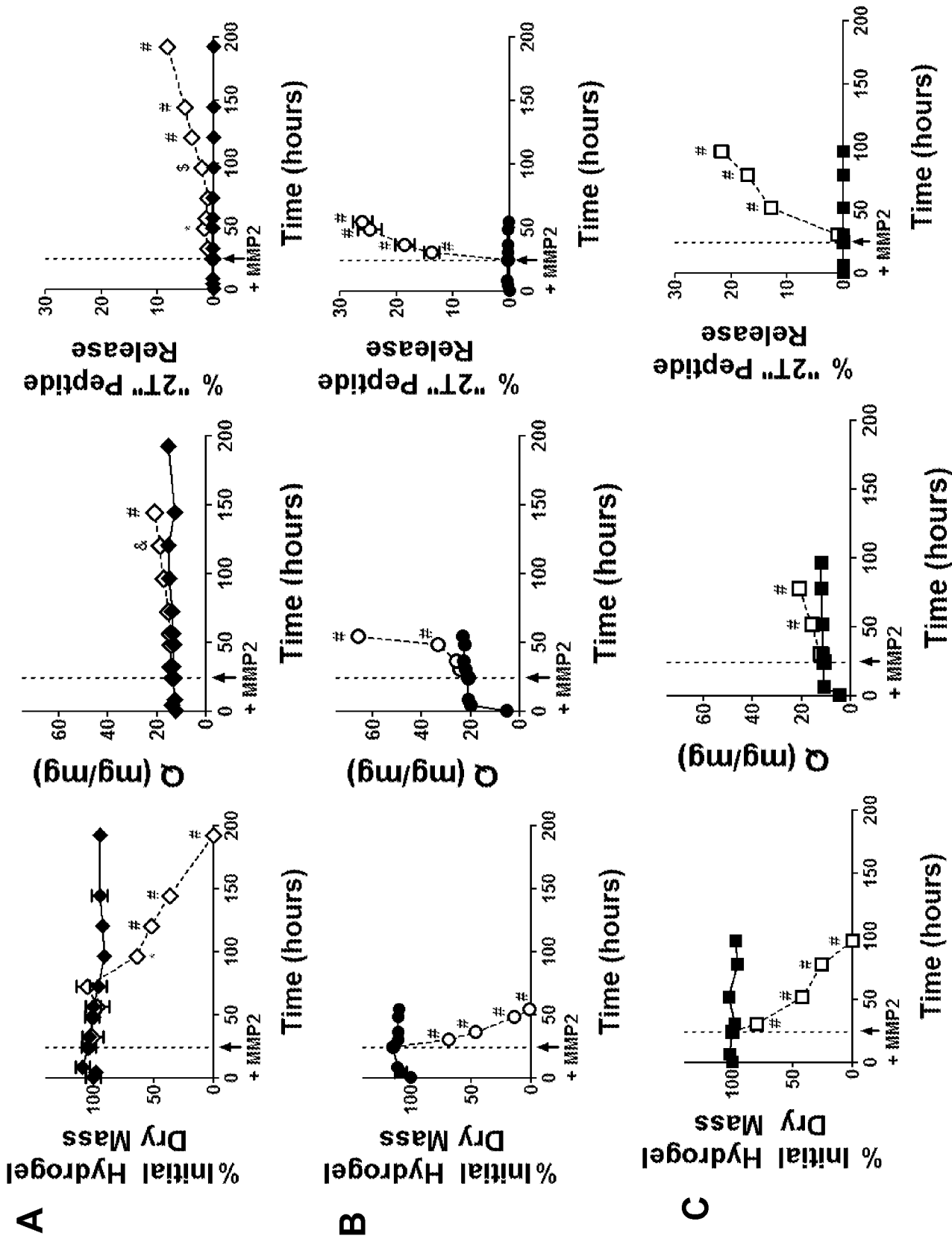


Figure 24

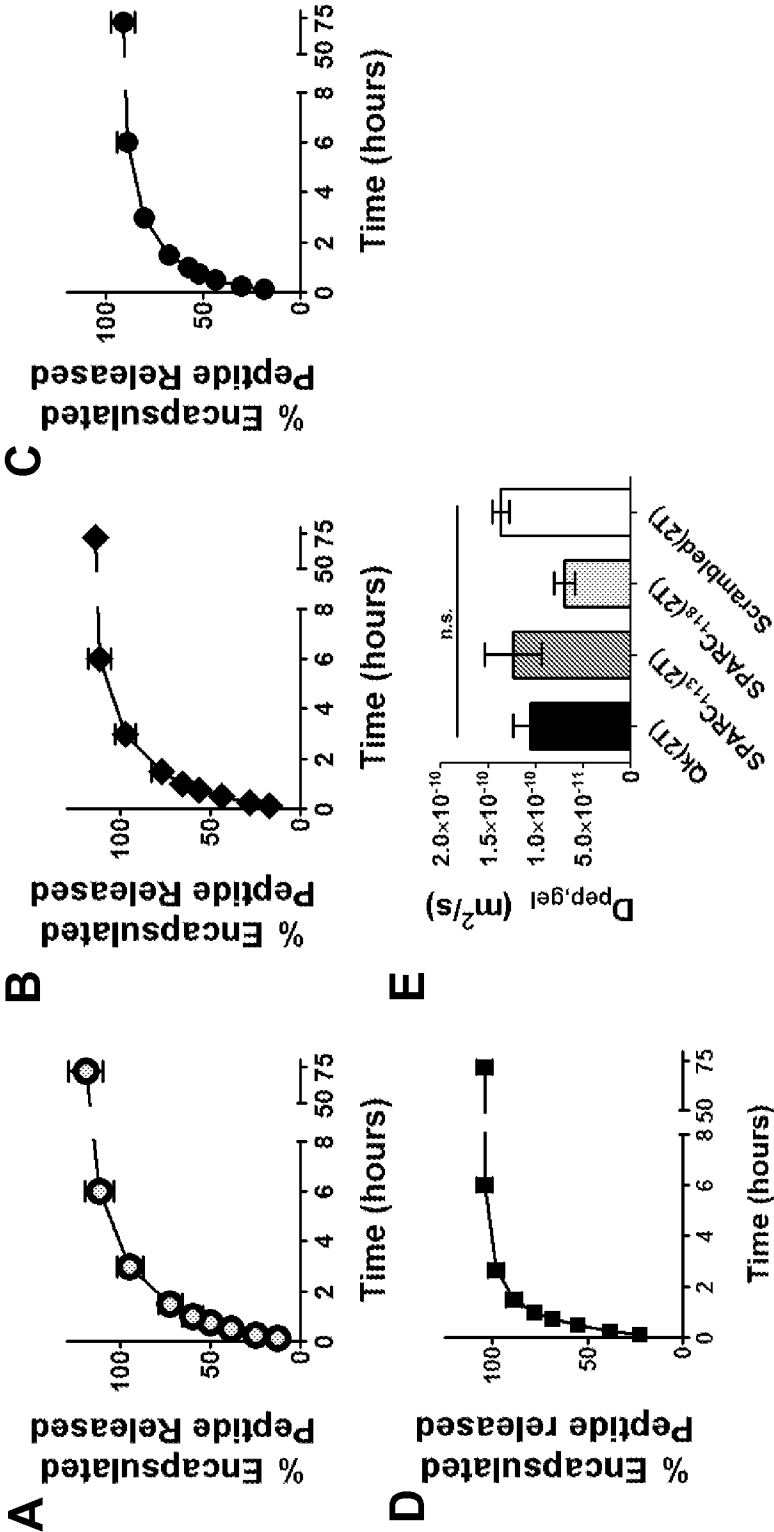


Figure 25

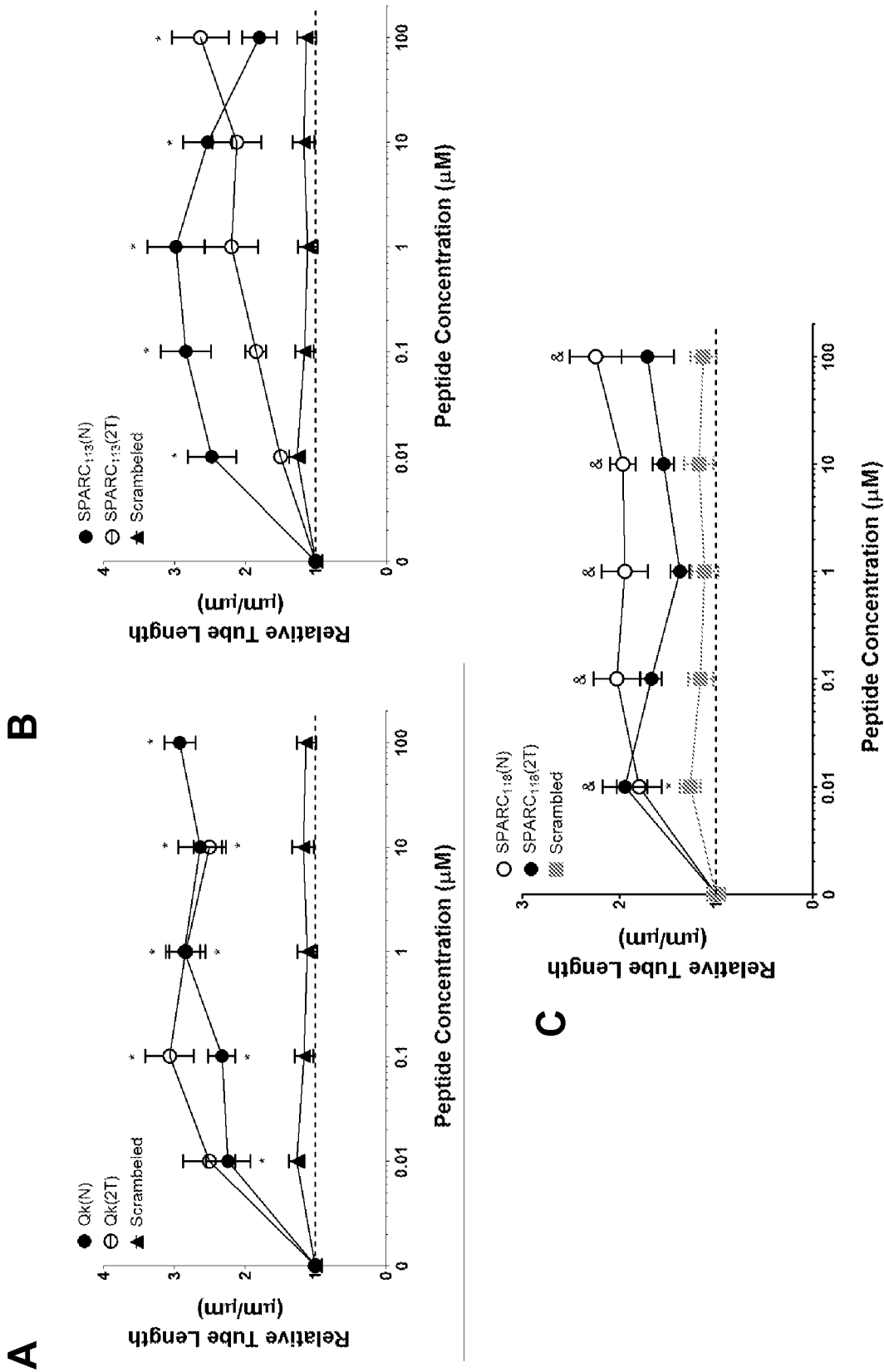


Figure 26

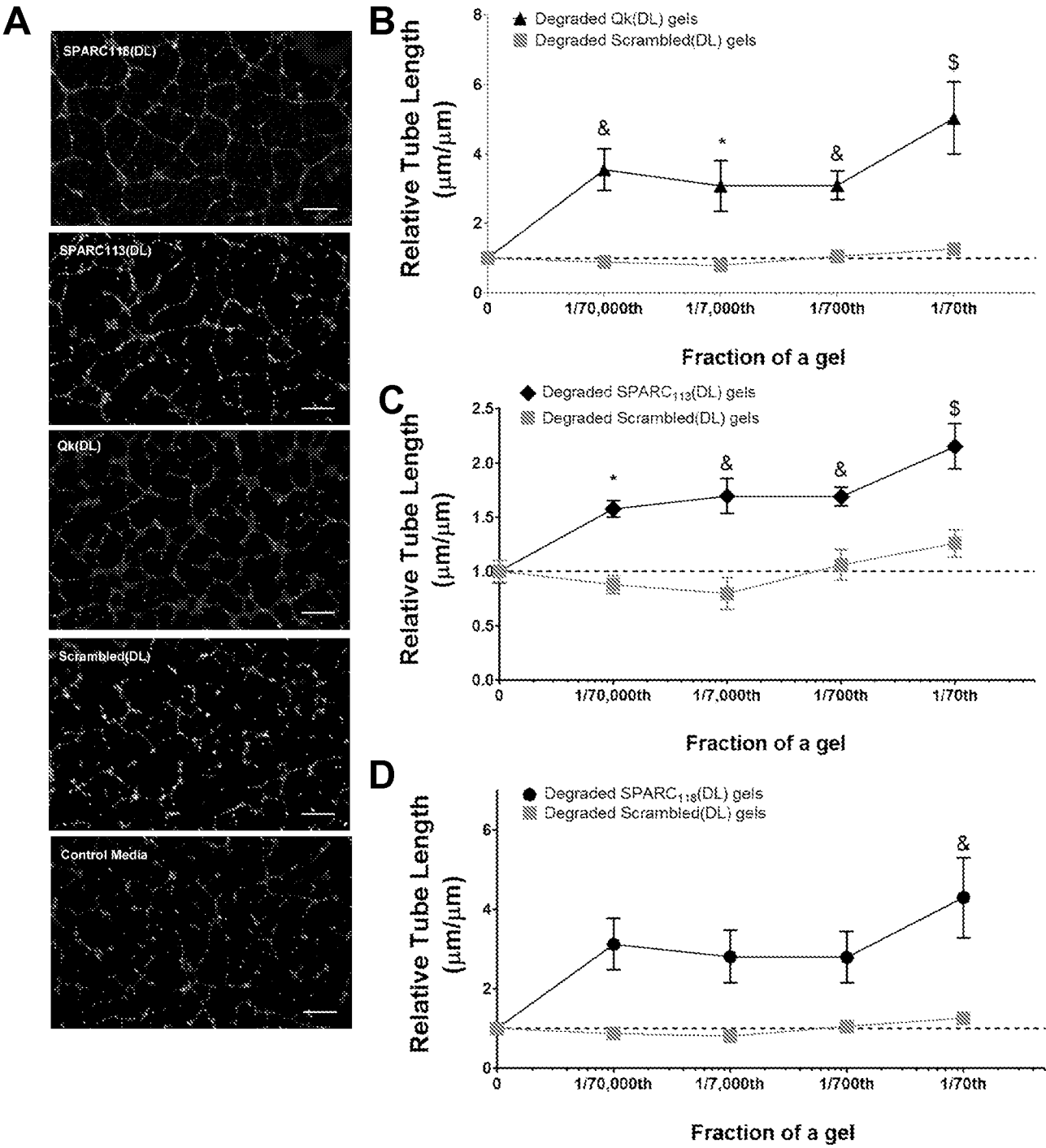


Figure 27

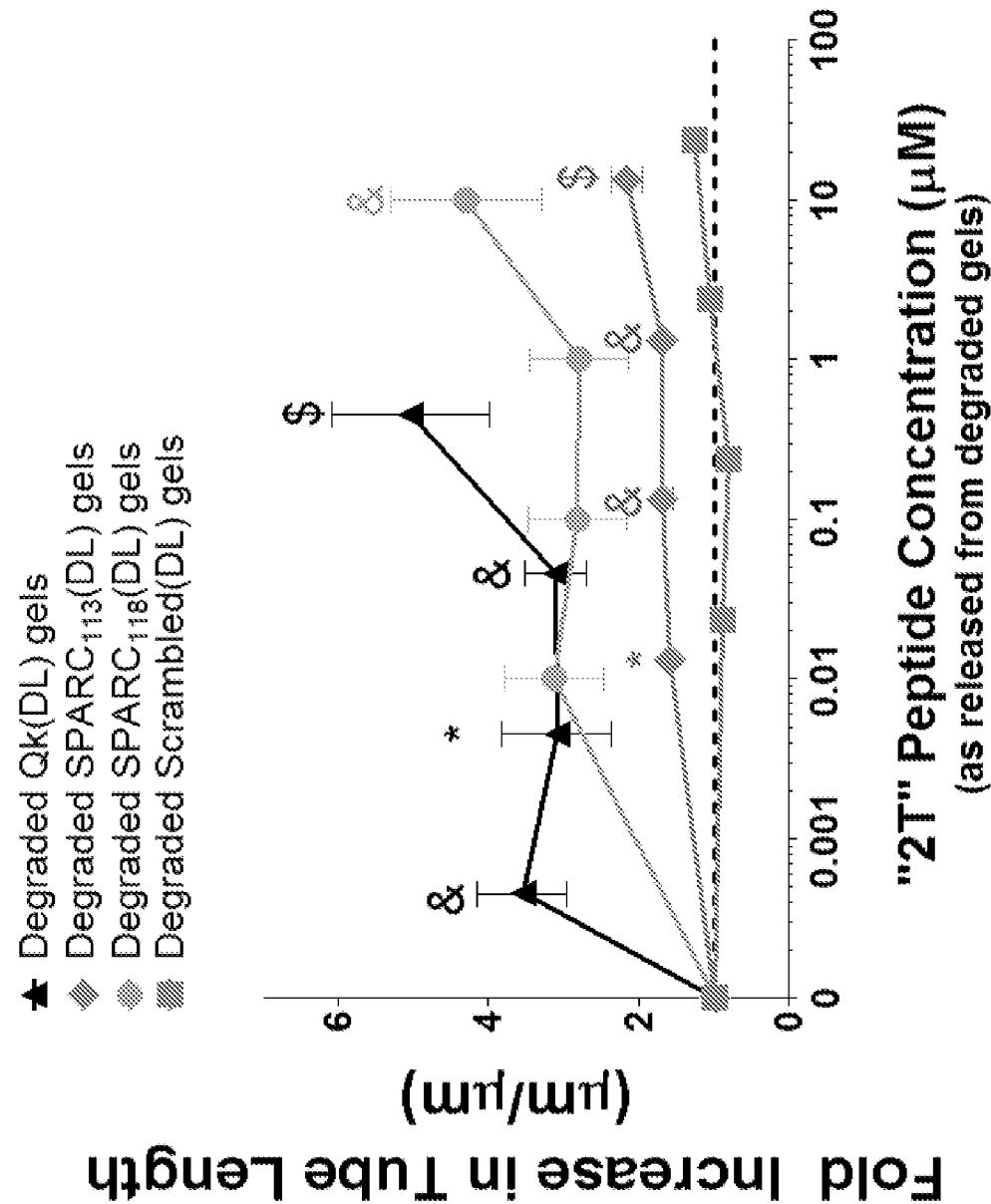


Figure 28

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 14/49774

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61F 2/00, A61J 3/00, A61F 13/00, A61K 47/48 (2014.01)

CPC - A61K 9/0024, A61J 3/005, A61K 38/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8): A61F 2/00, A61J 3/00, A61F 13/00, A61K 47/48 (2014.01)

CPC: A61K 9/0024, A61J 3/005, A61K 38/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 424/426, 427/2.22, 424/422, 525/54.1; 424/94.67, 435/375; 428/402Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST, PatBase, Google Patents, Google Scholar: Prodrug, drug, therapeutic, ratio, cleav*, monomer, synthetic, PEG, poly(ethylene glycol), polyethylene glycol, norbornene, polymeriz*, matrix, MMP, matrix metalloproteinase, angiogen*, terminal, UV, light, tissue, engineer*, ex vivo, in vivo

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2012/0165296 A1 (KRATZ et al.) 28 June 2012 (28.06.2012); para [0013], [0020], [0022], [0023], [0025], [0028], [0057],	1-16, 18-20, (21-28)/1-16,18-20)
A		17, (21-28)/17
Y	US 2011/0040113 A1 (WU et al.) 17 February 2011 (17.02.2011); para [0097], [0103], [0104]	1-16, 18-20, (21-28)/1-16,18-20)
A		17, (21-28)/17
Y	WO 1998/043615 A1 (PEPPAS et al.) 8 October 1998 (08.10.1998); p 5, ln 12-21	6, 19, (21-28)/(6,19)
Y	US 2011/0165244 A1 (NEFF) 7 July 2011 (07.07.2011); SEQ ID NO: 7, para [0045], [0087]	9, (21-28)/9
Y	US 8,367,639 B2 (KILCK et al.) 5 February 2013 (05.02.2013); col 2, ln 54-59; col 39, ln 12-40	11-12, 21/(11-12), (22-24)/(1-16,18-20), (25-28)/(11-12)
Y	US 7,022,343 B2 (PHILBROOK et al.) 4 April 2006 (04.04.2006); col 12, ln 20-23; col 12, 56-col 13, ln 10	26/(1-16,18-20), 28/(1-16,18-20)

☒ Further documents are listed in the continuation of Box C.

* 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

9 October 2014 (9.10.2014)

Date of mailing of the international search report

06 NOV 2014

Name and mailing address of the ISA/US

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

Facsimile No. 571-273-3201

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 14/49774

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2013/0072650 A1 (TEW et al.) 21 march 2013 (21.03.2013); para [0156]	5, (21-28)/5
Y	US 2013/0101628 A1 (WEBBER et al.) 25 April 2013 (25.04.2013); SEQ ID NO: 3; para [0023]	17, (21-28)/17
Y	US 2005/0090437 A1 (NULTALL et al.) 28 April 2005 (28.04.2005); SEQ ID NO: 69	17, (21-28)/17
Y	B8MAUS, Fumarylacetoacetate hydrolase, putative, Uniprot Submission number TSTA_115790, 03 March 2009 [online]. [Retrieved on 9 October 2014]. Retrieved from the internet <URL: http://www.uniprot.org/uniprot/B8MAU5 >	17, (21-28)/17