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Cell-Demanded VEGF Release via Nanocapsules Elicits Different Receptor Activation Dynamics and Enhanced Angiogenesis

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Abstract

Although the delivery of vascular endothelial growth factor (VEGF) with extended release profiles has consistently shown beneficial therapeutic effects compared with bolus delivery, it remains unclear if the reason is solely due to the physical availability and the reduced degradation of the protein. Here we studied the activation of VEGF receptor 2 (VR-2) by sustained released VEGF compared with bolus delivered VEGF to unveil that sustained delivery system alters the dynamics of receptor activation and affects the actions of cells between sprouting and proliferation. We utilized a protein nanocapsule delivery strategy that releases VEGF as mediated by extracellular proteases. These protein nanocapsules were synthesized through an aqueous assembly of a nanogel-peptide shell around the protein, leading to one to two proteins encapsulated per nanocapsule. Receptor activation studies revealed differential dynamics of receptor activation for slowly released VEGF compared with bolus delivered VEGF. As expected sustained released VEGF *via* nanocapsules resulted in enhanced vascular sprouting *in vitro* and *in vivo*. These studies demonstrate the physical presentation of VEGF, in this case of a slow release with time, can affect its molecular mechanism of actions and cause alterations in cellular responses and therapeutic outcomes.

Keywords

Growth factor; Sustained release; Protease-sensitive; Wound repair

INTRODUCTION

Since the discovery of insulin in the 1920s, protein-based therapeutics have been investigated for suitable routes of delivery and extended biological half-life.¹² Growth factors (GFs) are attractive candidates because of their unique roles in signaling cells and regenerating tissues.^{14,19,25} The challenges of controlled release dosing and reducing the frequency of administration have driven formulations of sustained delivery.^{1,10,11,16,17,26,32}

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ELECTRONIC SUPPLEMENTARY MATERIAL

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However, little has been studied on the receptor activation and potentially signaling alterations of cells that are exposed to these sustained delivered growth factors.

Vascular endothelial growth factor (VEGF) is a widely studied growth factor to induce revascularization of ischemic tissues. Despite several clinical trials of recombinant protein or gene transfer of VEGF,^{9,23} the delivery has not been successfully translated into approved therapies. It is now increasingly clear that the mode of delivery is critical for VEGF-induced angiogenesis, as the dose and timing are critical for the success of revascularization.^{8,22,28} Thus, bioengineering approaches to tune the delivery of VEGF to achieve sustained release have been extensively investigated. These approaches have shown enhanced therapeutic benefit, but the effects of sustained release are poorly understood on the molecular and cellular levels.

We previously reported on the aqueous-based synthesis of a polymer shell around proteins,^{29,30} and by incorporating opposite chiralities of enzyme substrates, protein nanocapsules capable of controlled release rates could be generated.³³ By introducing peptides as crosslinkers to the polymer shell, cell-demanded release of the protein is realized *via* the catalytic hydrolysis of peptides by proteases in the extracellular matrices. Herein, we utilize these VEGF nanocapsules to study the molecular and cellular differences between a sustained delivery and a bolus delivery. In addition, we demonstrate our nanocapsule delivery approach can be tuned to deliver VEGF at a rate that promotes angiogenesis within granulation tissue to improve tissue healing.

MATERIALS AND METHODS

All chemicals were purchased from Sigma Aldrich unless otherwise noted.

Synthesis of Protein Nanocapsules

The formation of growth factor nanocapsules, n(GF), was designed and synthesized such that the protein was encapsulated without covalent modifications. Buffering vascular endothelial growth factor-A (VEGF-A 165, from here on VEGF, Genentech) with positively charged monomer *N*-(3-aminopropyl) methacrylamide, neutral monomer acrylamide, and bisacrylated KNRVK peptide crosslinkers (China Tech Peptides) enabled electrostatic and hydrogen-bonding interactions to enrich the monomers and crosslinkers around the protein. *In-situ* free-radical polymerization initiated by ammonium persulfate and tetramethylethylenediamine formed a thin polymer layer around the protein. Protein nanocapsules were dialyzed against 10 mM phosphate buffer (pH 7.4) in dialysis units (MWCO 10,000, Thermo Scientific). The concentration of the VEGF content in n(VEGF) was assayed using a protein quantification kit NanoOrange (Life Technologies) following manufacturer's instructions.

Characterizations of n(VEGF)

Transmission Electron Microscopy (TEM)—n(VEGF) suspension was transferred to a copper grid and stained with 2% (vol/vol) phosphotungstic acid for 1 min. Samples were imaged with T12 cryo-electron microscope and images were quantified with ImageJ to measure the size of nanocapsules.

Enzyme-Linked Immuno Assay (ELISA)—n(VEGF) (of a VEGF content 50 ng) was treated with plasmin (0.25 μ g, activity of 6.6 units/mg, BD Biosciences) or collagenase IV (Worthington Biochemical) for specific amounts of time. Subsequent proteolytic activity of plasmin was inhibited with aprotinin. Treated samples were diluted to 3 ng/mL in 1% BSA for plating in ELISA (R&D Systems) following the manufacturer's instructions. A series of known concentrations of VEGF were quantified as the calibration curve.

The Thermal Stability of n(VEGF)—In comparison to VEGF, n(VEGF) was studied where 300 μ g/mL of samples were incubated at 37 °C for 10 days and assayed with human umbilical endothelial cells (HUVECs, Lonza) followed by western blot analysis (see “Receptor Activation Assays” section).

HUVEC Bead Assay

Early passages (p2-4) of HUVECs were harvested and incubated with 2500 autoclaved Cytodex beads at a density of 1 million cells in 1.5 mL of media for 4 h with gentle shaking every 20 min to allow for coating of cells on the surface of beads. Bead/cells were transferred to a 25-cm² cell culture flask and cultured overnight in 5 ml of EGM-2 media (Lonza). Fibrin matrices with bead/cells incorporated were formed with fibrinogen (at a final concentration of 2 mg/mL), aprotinin (37.3 μ g/mL), penicillin/streptomycin (0.1% v/v), bead/cell (500 beads/mL), thrombin (20 U/mL), CaCl₂ (2 mM Ca²⁺) and VEGF samples in Lab-Tek chambered cover glass for clear imaging facileness. Four conditions were tested (i) VEGF added to fibrinogen mixture during gelation process at 200 ng/mL (ii) no VEGF added, (iii) plasmin-sensitive n(VEGF) added to fibrinogen mixture during gelation at 200 ng/mL, and (iv) non-degradable n(VEGF) added to fibrinogen mixture during gelation at 200 ng/mL. Human dermal fibroblasts, previously cultured in DMEM media supplemented with 10% FBS and penicillin/streptomycin (0.1% v/v), were harvested and resuspended in VEGF-withdrawn EBM-2 media at a density of 40,000 cells/mL. 20,000 fibroblasts were added on top of fibrin matrices for every 250 cell-beads in a gel. The medium was refreshed every 2 days where VEGF-withdrawn medium was supplemented with different amounts or no VEGF as indicated in the Results. Nanocapsules of VEGF were added in the fibrin matrices from day 0. Bright field images were acquired with Zeiss microscope. Three different gels of each condition were evaluated, and each gel was analyzed by averaging the measurements from five different beads within the same gel.

Receptor Activation Assays

HUVECs (p5-7) were used for incubation with n(VEGF) or un-encapsulated VEGF at 50 ng/mL, followed by western blotting analysis with details previously described.³ Briefly, HUVECs were starved in serum-free media for 6 h before exposure to n(VEGF) or VEGF for different amounts of time. Treated cells were lysed in mRIPA lysis buffer (supplemented with fresh aprotinin, leupeptin and PMSF) and collected for supernatants. After performing Dc protein quantification assay (Biorad), 5 μ g protein mass of cell lysates were run in NuPAGE 4–12% gradient bis–Tris gel for electrophoresis (100 min at 150 V) and transferred to nitrocellulose membranes (2 h at 400 mA at 4 °C). Western blotting analysis was performed according to manufacturer's instructions with primary antibodies (1:1000, Cell Signaling Technology) and horseradish peroxidase (1:5000, Invitrogen) for visualization

(with ECL detection reagents, GE Healthcare, Piscataway, NJ) on a Typhoon scanner (GE, Amersham Biosciences, Piscataway, NJ). Protein-loading control was assessed with corresponding antibodies (Cell Signaling Technology). Images of three biological samples were analyzed in ImageJ. PathScan phospho-VEGFR-2 (Tyr 1175) Sandwich ELISA antibody pair was also used in the analysis of cell lysates according to manufacturer's instructions.

Splinted Mouse Wound Model

This protocol was approved by the Animal Research Committee (ARC) of University of California Los Angeles and all animal care was in accordance with guidelines. This is an established protocol of splinted wound healing model previously published by others.⁷ Briefly, ten to 12-week old female Balb/c mice (Charles River Laboratories) were acclimated to the environment for at least 1 week upon delivery prior to the procedure. Mice were individually anesthetized with 4% isoflurane and maintained at 1.5–2% isoflurane during surgery. Buprenorphine (four dosages of 60 μ L each of 0.015 mg/mL per 20 g of mouse weight administered before, 8, 20 and 28 h after surgery) was injected subcutaneously. The dorsal surface was shaved with an electric clipper, depilated by Nair (1 min) and sterilized with betadine and ethanol before surgery on an aseptic pad atop warming heat pads. Using sterile biopsy punches (4 mm wide), two clean, well-defined wounds side-by-side were created slightly above the middle of the animal's back. Fibrin matrices (of fibrinogen at 10 mg/mL, thrombin at 2 U/mL, factor XIII at 4 U/mL, calcium at 5 mM and test agents of interest) of 30 μ L were injected on wounds. Aseptic silicon ring splints formed prior to surgery had a 6-mm wide window that was double-sided with Tegaderm. Mastisol liquid adhesive was added on the splints which were placed on a wound. Eight interrupted sutures around each splint were made as a secondary means to hold down splints. Animals were monitored until awakening and housed individually in cages. 7 days post surgery, mice were injected with 5-bromo-2'-deoxyuridine intraperitoneally twice at 4 and 2 h before euthanization. After euthanization, wound tissues were fixed with 1% paraformaldehyde (PFA) for 16–18 h at 4 °C and paraffin embedded for slicing into 5- μ m thickness. Three slides (50–100 μ m apart) and each with three fields of views were imaged using a Zeiss fluorescent microscope with fixed exposure times after standard immunohistochemistry (IHC) staining procedures. Specifically, rat anti-mouse CD31 antibody (1:100, BD Pharmingen) was used with goat anti-rat Alexa 568 (1:200, Invitrogen) and 4',6-diamidino-2-phenylindole, DAPI, nuclear stain (1:500). Controls which omitted primary antibody were included to confirm the absence of fluorescent antibody binding. BrdU antibody was used after denaturation of DNA *via* acid treatment according to common IHC procedures. Nine images of each wound were quantified for CD31 positive signals and averaged to generate one data point, and at least four wounds per condition were quantified for statistical analysis.

Statistical Analysis

For *in vitro* and *in vivo* studies, statistical analyses were carried out using GraphPad Prism. Data is reported as mean $\pm$ standard error of the mean (SEM) of at least three independent subjects. Statistical significance was determined by performing a one-way analysis of variance (ANOVA) followed by a Tukey's test, unless otherwise noted. *P* values less than

0.05 were considered significant. For *in vivo* quantification, statistical significance was determined by performing a Kruskal–Wallis test followed by Dunn's test, where *P* values less than 0.05 were considered significant.

RESULTS

Plasmin-Labile Nanocapsules for Selective Release of Active VEGF

Protease labile VEGF nanocapsules, n(VEGF), was assembled by enriching the surface of VEGF with charged acrylamide-based monomers and bisacryl plasmin-labile peptide (Ac-KNR↓VK-Ac, ↓ indicates plasmin cleavage site), followed by *in situ* radical polymerization in forming an plasmin-degradable nanogel shell around VEGF²⁹ (Fig. 1a). Alternatively, *D*-chiral enantiomer of the peptide crosslinker, Ac-K_DN_DR_DV_DK_D-Ac, was added to form shell cross-links with *D*-chirality. The *D*-chiral enantiomer has lower degradation kinetics compared to the *L*-chiral peptide^{15,18}; thus, n(VEGF) synthesized with an increasing *D*-chiral peptide content will have a slower degradation rate, and thereby a slower release rate of VEGF.³³ The selection of this plasmin-sensitive peptide sequence as the crosslinker is such that VEGF can be released when proteolytic and fibrinolytic proteases are present from endothelial cells (Fig. 1b). Transmission electron microscope (TEM) images of purified n(VEGF) showed an average diameter of 22 ± 3 nm (Fig. 1c). Incubation for 5 min of plasmin-degradable n(VEGF) with plasmin but not with collagen IV (Col-IV) resulted in a significant release of VEGF (Fig. 1d). At later time points (20 and 30 min), Col-IV-incubated n(VEGF) released VEGF likely due to the crude tryptic activity in Col-IV (Supplementary Fig. S1).

Plasmin-Degradable n(VEGF) Supports EC Sprouting via Cell-Demanded VEGF Release

An endothelial cell (EC) sprouting assay²¹ was used to confirm that n(VEGF) releases VEGF in a sustained manner, inducing significant EC sprouting compared to bolus delivery controls. VEGF was added either as one dose at $t = 0$ or at four different times at $t = 0, 2, 4, 6$ days and at different concentrations 200 or 2.5 ng/mL. One-time delivery at $t = 0$ day of naked VEGF at 200 ng/mL (bolus delivery) caused insignificant endothelial sprouting and tube formation by 7 days (Fig. 2). Delivery of VEGF at 200 ng/mL at $t = 0, 2, 4, 6$ days resulted in a thick layer of cells without sprouting around the bead (Fig. 2). Thus, deliveries of 200 ng of VEGF/mL either as one dose or at four doses did not promote sprouting. In contrast, delivery of VEGF mimicking sustained released doses of low levels of VEGF led to significant sprouting by 7 days (Fig. 2). It should be noted that VEGF must be delivered throughout the 7-day incubation at a low dose in order to have EC sprouting.^{20,21} We next delivered n(VEGF) at $t = 0$ at 200 ng/mL concentration to demonstrate n(VEGF) would be degraded slowly for sustained, low-dose release of VEGF. A one-time delivery at $t = 0$ of n(VEGF) resulted in a significant sprouting and tube formation for 7 days, indicating that n(VEGF) can be degraded to release VEGF at a rate that enables endothelial cell sprouting over 7-days. Importantly, non-degradable n(VEGF) comprising methylene bisacrylamide as the crosslinker, or nanocapsules comprising of 100% *D*-chiral peptide as the crosslinker, denoted n(VEGF)_{0%}, resulted in no EC sprouting (Supplementary Figs. S2 and S3).

Cell-Mediated VEGF Release from Nanocapsules Alters Receptor Phosphorylation Dynamics

VEGF solution and plasmin-degradable n(VEGF) were compared side by side to activate endothelial cells (ECs). In a 2-h time course, native VEGF (50 ng/mL) strongly stimulated the phosphorylation of VEGFR-2 (pVR-2) after 5 min of incubation, but the phosphorylation decreased rapidly thereafter. In contrast, n(VEGF) (50 ng/mL) stimulated sustained pVR-2 throughout the first 30 min, which decreased slowly afterward (Fig. 3a). As a control, VEGF from 1 ng/mL up to 20 ng/mL increasingly stimulated receptor phosphorylation after 5 min incubation (Fig. 3b). Next, VEGF solution (50 ng/mL) and n(VEGF) solution (50 ng protein content/mL) were compared in their individual, repetitive 30-min incubations with fresh sets of HUVECs. Specifically, a test solution was added to treat a first set of HUVECs for 30 min at 37 °C. Subsequently, the test solution was aspirated from the first set of HUVECs and transferred to another set of untreated HUVECs for a second 30-min incubation. The process was repeated until the test solution had a total of four incubations (30-min each) with four sets of HUVECs, and each set of HUVECs was collected as soon as the test solution was removed therefrom for analysis of receptor activation. When compared in the same number of cycling, n(VEGF) induced statistically significantly greater activation of the receptors than VEGF in the first set of HUVECs (*, $P < 0.05$, t test) and in the third set of HUVECs (**, $P < 0.01$, t test) (Fig. 3c). This difference in the stimulation of the first set of HUVECs is consistent with the result from the 2-h incubation study (Fig. 3a), where n(VEGF) was more effective in inducing receptor phosphorylation than VEGF at 30 min. Although n(VEGF) also appeared to be more effective than VEGF in the second and the fourth set of HUVECs, they were not statistically different. When compared across the four cycles for each test solution, n(VEGF) stimulated the most receptor phosphorylation in the third set of HUVECs, which was statistically significantly more than that in the first and the second sets (++ , $P < 0.01$, ANOVA). Soluble VEGF also stimulated more receptor phosphorylation in the third set of HUVECs than in the first set (ζ, $P < 0.05$, ANOVA), but they were both significantly lower than that stimulated by n(VEGF). It is not clear why VEGF was more effective in the third cycle than in the first cycle, but this observation is suggestive of the “depletion” of available receptors for ligand-mediated phosphorylation at the 60- and 120-min time points, rather than the “depletion” of ligand. Nevertheless, the significantly greater stimulation by n(VEGF) than soluble VEGF even in the third cycle indicates that n(VEGF) can slowly release VEGF in the presence of proteases secreted by new HUVECs. As another control, adding a small amount of fresh VEGF at 5 min before each activation analysis time point led to a substantial stimulation of receptor phosphorylation, even for the 60- and 120-min time points (Fig. 3d). Hence, it suggests the persistent activation profile seen in n(VEGF)-treated HUVECs at 60 and 120 min in Fig. 3a is likely due, at least in part, to the released VEGF from n(VEGF) close to these time points. Altogether, the data collectively reinforces our hypothesis that HUVECs can continuously degrade nanocapsules to release small amounts of active VEGF, prolonging the receptor phosphorylation.

The thermal stability of n(VEGF) was determined by its ability to activate VEGFR-2 after incubation at 37 °C for days. While native VEGF after 10 days of incubation could activate VEGFR-2 only to 50% of that normally achieved with fresh VEGF, n(VEGF) stored at

similar conditions induced VEGFR-2 phosphorylation to the same level as fresh VEGF did (Fig. 2e). Nanocapsules that contain 25% *L*-chiral peptide and 75% *D*-chiral peptide, denoted n(VEGF)_{25%}, even led to higher levels of phosphorylation at 10 and 14 days compared to 100% *L*-chiral peptide n(VEGF) capsules.

VEGF Nanocapsule Delivery from Hydrogels Enhances Vascularization in Skin Wounds

To investigate the therapeutic benefit of n(VEGF), a humanized full-thickness skin wound Balb/c mouse model was splinted in order to prevent skin contraction—the primary mode of healing in mice. By doing so, wound closure in human is better simulated because closure is promoted through vascularized granulation tissue (Fig. 4a).⁴ Prior to splinting, a factor XIII supplemented fibrin dressing containing one of the following components was injected into wound beds *in situ*: (i) no exogenous VEGF, (control), (ii) 200 ng of VEGF, (iii) a combination of 100% *L*-peptide crosslinked n(VEGF) (100 ng) and 75% *D*-peptide crosslinked n(VEGF) (100 ng), denoted n(VEGF) slow, (iv) a combination of 100% *L*-peptide crosslinked n(VEGF) (100 ng) and 50% *D*-peptide crosslinked n(VEGF) (100 ng), denoted n(VEGF) medium, and (v) 200 ng of 100% *L*-peptide crosslinked n(VEGF), denoted n(VEGF) fast. At 7 days post wounding, the histology of different wounds was compared for the amount of granulation tissue in wound beds (Fig. 4b). Additionally, angiogenesis in the granulation tissue, as well as proliferating cells at wound beds, was compared *via* immunohistochemical staining (Fig. 4c). The amount of granulation tissue at wound sites that were treated with either slow- or medium-releasing nanocapsules of VEGF was significantly greater than the one in control wounds (Fig. 4d); these slow-releasing nanocapsules also led to significantly larger density of vessels per mm² compared with medium-, fast-releasing, or naked VEGF or control (Fig. 4e). All nanocapsule groups showed high numbers of proliferating cells, although statistically comparable to the VEGF group and the control group (Fig. 4f).

DISCUSSION

Although sustained VEGF delivery has been consistently reported as a superior mode of delivery for improved therapeutic outcome in the treatment of ischemic tissues, the mechanism by which this improvement occurs is poorly understood. Ligand-receptor binding is the first step in the cascade of biological events, and we believe the receptor activation is closely related to the manner of released proteins and could begin to explain improved therapeutic outcomes. We believe that the sustained delivery of small doses of VEGF will induce differential dynamics of receptor activation and responses of endothelial cells compared to the bolus delivery of VEGF. Our previous work with bound VEGF has already demonstrated that bound VEGF convey differential signaling responses to endothelial cells.⁶ In those studies, we also showed that bolus VEGF delivery saturates all the available receptors at the surface of endothelial cells,² which suggests to us that no new receptor activation could occur because there were no receptors at the cell surface to phosphorylate. Here, we validate that this observation of persistent receptor activation is also true with sustained released VEGF, which is realized either by pipetting in small quantities of naked VEGF over time or through the use of bio-engineered nanocapsules. This is in contrast to bolus delivery where phosphorylation peaks early in the time course (5 min) and

subsidies. Further, we show that our n(VEGF) delivery strategy can be utilized *in vitro* for sustaining endothelial sprouting and lumenization, or further engineered with *D*-chiral peptides to modulate *in vivo* release rates to enhance vascularization at wound sites.

VEGF nanocapsules built with a plasmin-sensitive peptide sequence were cleavable for the release of VEGF by isolated plasmin, tryptic activity in crude Col-IV, or ECs. ECs in culture and *in vivo* contain proteolytic and fibrinolytic potential by producing urokinase-type and tissue-type plasminogen activators as well as plasminogen activator inhibitor-1 into the extracellular environment.³¹ Released VEGF from nanocapsules in the EC environment activates receptors in a different dynamics than bolus delivered VEGF, suggesting of a changed ligand/receptor interaction, and/or receptor availability at the cell surface. Since similar sustained activation was observed by pipetting small quantities of VEGF, through n(VEGF) exposure and through n(VEGF) recycling, we conclude that n(VEGF) can be continually degraded by endothelial cells and that the released VEGF can activate nearby receptors.

In the event of recapitulating angiogenesis *in vitro*, previous reports have shown replenished VEGF at a low dose resulted in the optimal three-dimensional sprouting for ECs *in vitro*.²¹ Although the rapid diffusion of VEGF and the cell-laden, protease rich environment may directly necessitate the replenishments of fresh VEGF to support endothelial tubule formation, they are not the entire explanation. Delivery of high doses of VEGF (200 ng/mL) every 2 days does not result in robust sprouting; rather it leads to “uncontrolled” proliferation of ECs at the bead surface. This phenomenon further demonstrates the importance of VEGF presentation (high or low dose, immediate or continued) on EC activation. Importantly, a single dose of n(VEGF) nanocapsules (all 100% *L*-chiral degradable) incorporated into the cell-laden matrice from day 0 was able to induce EC sprouting and to support lumenization. Due to the presence of aprotinin, i.e., a plasmin inhibitor, in this fibrin matrix, n(VEGF)_{0%} comprising 100% *D*-chiral peptide crosslinker was not effectively degraded to release VEGF, therefore did not induce EC sprouting.

Microenvironmental concentrations and a balanced distribution of exogenous VEGF are critical factors in determining normal angiogenesis *in vivo*.^{22,28} Therefore the delivery of VEGF in a cell-demanded and adaptive way would be particularly beneficial. The amount and kinetics of released VEGF from nanocapsules is related to the amount and activity of proteases secreted by endothelial cells, the physiopathological environment, and the sensitivity (i.e., sequence and the *L*-to-*D* enantiomer ratio) of the peptide crosslinkers of the shell of nanocapsules to protease cleavage. In the sprouting assay *in vitro*, the concentration of the released VEGF from nanocapsules resulted in a level of sprouting and long tube formation similar to that achieved by soluble VEGF delivered at the optimal concentration as previously reported.²¹ Hence, our data suggests the release from nanocapsules in the sprouting assay is equivalent to that delivered in the soluble condition.

To evaluate if VEGF nanocapsules can deliver VEGF at a rate necessary for a therapeutic benefit, n(VEGF) with *D*-chiral peptides was tested in a splinted skin wound healing model. By modulating the release rate of VEGF, the formation of granulation tissue and vascularization were significantly different at 7 days post wounding. The release rate from

nanocapsules in this damaged environment can be inferred in a similar study, where we demonstrated 100 ng of n(Protein)_{100%} was mostly released by day 3 and 100 ng of n(Protein)_{25%} was mostly released from day 3 to day 6.³³

The stability of protein molecules determines the availability of delivered VEGF, and encapsulating the protein inside *in situ* formed nanocapsules improved its biostability. While naked proteins are highly sensitive to thermal deactivation and proteolytic degradation as shown by others^{13,27} and our receptor activation assay, VEGF nanocapsules preserved the bioactivity of the protein against thermal denaturation even after 2 weeks of incubation at an elevated temperature. Furthermore, substituting the *L*-peptide crosslinker with *D*-peptide crosslinker improved the stability of VEGF nanocapsule. While other methods have extended the half-life of protein biologics including PEGylation and mixture of stabilizing factors (i.e. heparin),²⁴ they either covalently altered the structure of proteins or had to resort to alternate splicing variants.

Looking forward, the enhancement in angiogenesis would further benefit from a pro-maturation factor such as platelet derived growth factor or angiopoietin-2.⁵ Nevertheless, the protease-modulated single-protein nanocapsule system shown here identifies the molecular and cellular ramifications of a cell-demanded and adaptive VEGF delivery strategy, providing a means to the mechanistic investigation of growth factor therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Single-protein nanocapsules for cell-secreted plasmin-triggered release of VEGF. (a) The synthesis of VEGF nanocapsules by enriching monomers and crosslinkers around individual VEGF molecules and subsequent *in situ* polymerization. The monomers are acrylamide (neutral) and *N*-(3-aminopropyl)methacrylamide (positive charge). The crosslinkers include either bisacryl *L*-peptide Asn-Arg-Val (NRV) (degradable substrate of plasmin) or bisacryl *D*-peptide NRV, or a combination of both. (b) When plasmin secreted by endothelial cells slowly degrades the nanogel and releases the VEGF, the receptor is activated with a persistent dynamics. (c) A TEM image of n(VEGF), scale bar = 50 nm. (d) An ELISA readout for the free or released VEGF, or degraded n(VEGF), as a percentage of total n(VEGF) that was treated with either collagenase IV or plasmin.

FIGURE 2.

Single dose plasmin-degradable n(VEGF) supports endothelial sprouting and tubule formation. (a) Microscopic images of endothelial cell (EC)-coated beads with EC sprouts (outlined) after 7 days incubation in fibrin matrices that were incorporated with either 2.5 ng/mL VEGF at days 0, 2, 4, and 6 (VEGF low, 4 doses, 4×), 200 ng/mL VEGF at day 0 (VEGF high, 1 dose, 1×), 200 ng/mL VEGF at days 0, 2, 4, and 6 (VEGF high, 4 doses, 4×), or 200 ng/mL n(VEGF) comprising plasmin degradable crosslinker at day 0 (n(VEGF) 1 dose, 1×), Scale bar 5 100 μ m. (b) Quantifications of 15 beads from 3 groups of gels were measured for each condition.

FIGURE 3.

The dynamics of VEGF/VEGF receptor-2 (VR-2) interactions is compared when VEGF is administered in a bolus manner or through cell-mediated slow release from n(VEGF). (a) Western blot analysis ($n = 3$) of VR-2 after incubating endothelial cells (ECs) with VEGF or n(VEGF) at 50 ng/mL for 5, 10, 15, 30, 60 and 120 min. The time point with the greatest phosphorylation/total VR-2 for the VEGF treatment (i.e. 5-min) and that for the n(VEGF) treatment (i.e. 15-min) were considered as 100% to normalize the pVR-2/VR-2 data of all other time points for the same treatment. (b) Western blot analysis of VR-2 after incubating ECs with different concentrations of VEGF for 5 min. (c). Phospho-ELISA readout of the (remaining) activities of VEGF or n(VEGF) (at 50 ng/mL) that was consecutively applied

and reapplied to fresh ECs for four times and 30 min in each time; $n = 3$. (t test, comparing VEGF vs. n(VEGF) within each cycle, * $P < 0.05$, ** $P < 0.01$; one-way ANOVA, comparing VEGF at different cycles, ζ $P < 0.05$; one-way ANOVA, comparing n(VEGF) at different cycles, + $P < 0.05$, ++ $P < 0.01$) (d) Phospho-ELISA readout of a prolonged phosphorylation of VR-2 over 2 h induced by an initial bolus VEGF (50 ng/mL) followed by repetitive additions of VEGF (5 ng/mL) at 5, 10, 25, 55 and 115 min; $n = 3$. (e) Western blot analysis of phosphorylation of VR-2 after a 5-min incubation of ECs with n(VEGF) or VEGF, where the n(VEGF) or VEGF has been previously stored at 37 °C for 10 days (10-d) and 14 days (14-d) in comparison to fresh VEGF (as a positive control).

FIGURE 4.

Wound repair and angiogenesis at day 7 in splinted mouse dermal excisional wounds. (a) Splinted 4-mm wound model prevented loose skin from contraction and allows healing by the formation of granulation tissue. (b) Images of hematoxylin and eosin stained, full-length wounds 7-day post surgery (the border of granulation tissue of a dense cell population was separated from the center of the wound with dashed lines). Wounds were dressed at day 0, prior to splinting, with *in situ* crosslinked fibrin matrices containing (i) no exogenous VEGF (control), (ii) 200 ng VEGF (VEGF), (iii) 100 ng n(VEGF)_{100%} combined with 100 ng n(VEGF)_{25%} (slow), (iv) 100 ng n(VEGF)_{100%} combined with 100 ng n(VEGF)_{50%} (medium), or (v) 200 ng n(VEGF)_{100%} (fast). (c) Fluorescent images of angiogenesis (CD31+) and proliferating cells (BrdU+) in wounds. Scale bars = 20 μ m (CD31 staining), and 10 μ m (BrdU staining). (d–f) Quantifications of the area of granulation tissue (d, representative images shown in panel b), density of capillaries (e, representative images

shown in the upper row of panel c), and proliferating cells (f, representative images shown in the lower row of panel c). $N=4$ (3 for control), with each data point being the average of nine view of images representing three sections (50–100 μm apart) of each wound. $*P < 0.05$, Kruskal–Wallis test with Dunns post test.