Injectable fibroblast growth factor-2 coacervate for persistent angiogenesis

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Enhancing the maturity of the newly formed blood vessels is critical for the success of therapeutic angiogenesis. The maturation of vasculature relies on active participation of mural cells to stabilize endothelium and a basal level of relevant growth factors. We set out to design and successfully achieved robust angiogenesis using an injectable polyvalent coacervate of a polycation, heparin, and fibroblast growth factor-2 (FGF2). FGF2 was loaded into the coacervate at nearly 100% efficiency. In vitro assays demonstrated that the matrix protected FGF2 from proteolytic degradations. FGF2 released from the coacervate was more effective in the differentiation of endothelial cells and chemotaxis of pericytes than free FGF2. One injection of 500 ng of FGF2 in the coacervate elicited comprehensive angiogenesis in vivo. The number of endothelial and mural cells increased significantly, and the local tissue contained more and larger blood vessels with increased circulation. Mural cells actively participated during the whole angiogenic process: Within 7 d of the injection, pericytes were recruited to close proximity of the endothelial cells. Mature vasculature stabilized by vascular smooth muscle cells persisted till at least 4 wk. On the other hand, bolus injection of an identical amount of free FGF2 induced weak angiogenic responses. These results demonstrate the potential of polyvalent coacervate as a new controlled delivery platform.

Angiogenesis is a physiological process involving the formation of nascent vasculature from existing blood vessels. The complex interactions between endothelial cells and mural cells including vascular smooth muscle cells and pericytes are highly coordinated by various signals (1, 2). Therapeutic angiogenesis is promising in treating many human diseases, especially coronary and peripheral ischemia (3, 4). Among various approaches to therapeutic angiogenesis, delivery of growth factors is the most simple and direct because it does not need viral vectors in gene therapy or cells in cell therapy. Direct injection of free growth factors failed to demonstrate efficacy in clinical trials (5). Therefore, appropriate controlled delivery strategy for growth factor is highly desirable and extensively studied. However, loading capacity and long-term efficacy still present significant challenges to growth factor delivery.

In the human body, most secretory growth factors are associated with extracellular matrix usually through interactions with glycosaminoglycans. Glycosaminoglycans are negatively charged linear polysaccharides that can have different composition, function, and distribution in the body (6). Together with other extracellular matrix molecules, glycosaminoglycans provide a substratum for cell attachment (7). Furthermore, their interaction with growth factors is critical in many biological processes such as development (8, 9) and cancer progression (10, 11). Heparin and heparan sulfate are well-studied glycosaminoglycans for their high affinity to a variety of growth factors including heparin-binding EGF (HB-EGF), FGF, and VEGF families (12). Heparin can modulate the conformation of the growth factors (13, 14), protect them from proteolytic cleavage (15), and potentiate their bioactivity (16). Incorporation of heparin in a delivery vehicle is therefore a promising approach to preserve the bioactivity of the delivered growth factors (17, 18).

The FGF family is well known for its high affinity to heparin, which modulates the interaction between FGFs and their receptors. As revealed by the crystal structure (Fig. 1A), the heavily anionic heparin brings the cationic sequences on FGF and its receptor closely together and stabilizes the ternary complex largely through polyvalent ionic interactions (19). In order to mimic the interactions between these three components, we use a synthetic polycation to substitute the heparin-binding sequence of the FGF receptor and form a ternary complex containing the polycation, heparin, and FGF (Fig. 1A). We designed a polycation, PEAD, with excellent biocompatibility to conjugate heparin and a potent angiogenic factor, fibroblast growth factor-2 (FGF2) (20, 21). The complex of heparin and FGF2 is soluble in water and not amenable to controlled local delivery. The addition of PEAD neutralizes the excess negative charge of heparin and immediately induces the formation of [PEAD:heparin:FGF2] coacervates. This anchors FGF2 and enables its controlled release. We utilize charge interaction between PEAD and heparin instead of covalent modification of heparin in an effort to minimize the perturbation of the functions of heparin. As a result, FGF2 released from the coacervate induces much more potent angiogenic responses than free FGF2 in mice: Mural cell participation significantly increases and the neovasculature is mature and persists to at least 4 wk.

Results

Interactions Among PEAD, Heparin, and FGF2. This research uses PEAD that consists of aspartic acid, arginine, and diglyceride moieties (Fig. 1B). The amino and guanidine groups are positively charged under physiological conditions and enable PEAD to interact strongly with heparin, the most negatively charged glycosaminoglycan. We employ this charge interaction to load heparin-binding growth factors into the delivery vehicle. PEAD lowers the solubility of [heparin:FGF2] complex in water by forming a coacervate through charge interactions (Fig. 1C). [PEAD:heparin:FGF2] coacervates aggregate over time and settle at the bottom of the vessel as an oil droplet after 24 h of standing. The droplet is easily resuspended by agitation, which returns the solution to the turbid state. Scanning electron microscopy reveals that the morphology of the [PEAD:heparin:FGF2] mainly consists of globular domains that can fuse with each other to form strands (Fig. 1D).

An effective delivery vehicle should have high loading efficiency in addition to controlled release of the cargo. We tested the loading efficiency of the delivery vehicle by Western blot. Following the centrifugation of the coacervate, the amounts of FGF2 in the supernatant and the coacervate were compared to the amount in the original loading solution. Comparison of the

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Fig. 1. (A) Design of a coacervate delivery matrix. The crystal structure of [FGF2:heparin:FGFR] complex indicates that heparin actively participates in the interaction of FGF and its receptor whose heparin-binding domains are labeled yellow and pink, respectively (Top Left). (Top Right) Scheme represents the design of the coacervate where a synthetic polycation replaces the heparin-binding domain of FGFR and forms a complex with heparin and FGF. (B) Chemical structure of poly(ethylene arginylaspartate diglyceride) (PEAD). The backbone of PEAD composed of aspartic acid and ethylene glycol diglyceride is linked by ester bonds (arrows). The conjugation of arginine provides the polymer with two cationic groups per repeating unit: ammonium and guanidinium. (C) The three components dissolve well in water individually as represented here by heparin. Adding FGF2 induces no apparent changes in solubility. Upon addition of PEAD, the solution turns cloudy. Charge neutralization between the polycation and heparin forms the [PEAD:heparin:FGF2] coacervate, which is insoluble in the aqueous solution, enabling local delivery of FGF2. Upon standing for 24 h, the coacervate aggregates to the bottom of the tube. (D) SEM micrograph revealed that [PEAD:heparin:FGF2] mainly consisted of globular domains that fuse together. The globular nature of the coacervate is more distinguishable at higher magnification. Scale bars: 10 μ m (low magnification) and 1 μ m (high magnification). (E) The loading efficiency is >95% for FGF2 (500 μ g PEAD, 100 μ g heparin, FGF2 range tested: 100–1,000 ng). Western blot demonstrated that the intensity of the coacervate and the loading solution is the same. S: FGF2 in the supernatant after centrifugation. C: FGF2 in the settled coacervates. L: total amount of FGF2 in the loading solution. (F) Protection from proteolysis by the coacervate. FGF2 and trypsin (mass ratio 1:200) was incubated for 30 min or 2 h at 37 $^{\circ}$ C. The results indicated that free FGF2 (I) was completely degraded within 0.5 h. On the other hand, heparin (II) and the coacervate (III) protected FGF2 from degradation for at least 2 h. (G) The coacervate localized FGF2 release in fibrin gel. Fibrin gels were prepared with free FGF2 or FGF2-containing coacervate. The amount of FGF2 in the medium was determined by Western blot. Less FGF2 was present in the medium of the coacervate group, suggesting that FGF2 was localized better in the fibrin gel than the free FGF2 group. (H) Endothelial tube formation in fibrin gels. HUVECs mixed with free FGF2 (50, 250, or 500 ng/mL) or the same amount of FGF2 in the coacervate were encapsulated in the fibrin gel. After incubation of 3 d, the coacervate induced extensive tube network formation at 250 and 500 ng/mL of FGF2. On the contrary, FGF2 alone induced sparse tube formation at all growth factor concentrations. Scale bar: 100 μ m. (I) Chemotaxis of pericytes by the coacervate. After incubation for 12 h, migrated pericytes were stained by PicoGreen. Quantitative comparison suggested that the coacervate induced significantly higher extent of chemotaxis than free FGF2 (254 ± 44 vs. 108 ± 14 per mm^2 , $p < 0.05$, Student's t test). Scale bar: 100 μ m.

band intensity of Western blot using National Institutes of Health (NIH) ImageJ found no FGF2 in the supernatant. Furthermore, the amount of FGF2 in the coacervate when 100, 500, and 1,000 ng of FGF2 was used (Fig. 1E) is statistically the same as the loading solution suggesting nearly 100% loading efficiency. We expect the loading efficiency will also be near 100% for higher amount of growth factors because of the large excess of heparin relative to growth factor. We ended the test at 1,000 ng because the subsequent *in vivo* examination used only 500 ng FGF2.

Most free growth factors degrade quickly *in vivo*; therefore, bolus injection has very low efficacy. Because heparin can protect growth factors from proteolytic cleavage, we examined if heparin in the coacervate retains its protective capability. When treated with a broad spectrum protease, trypsin, all free FGF2 was degraded within 0.5 h (Fig. 1F, column I). On the other hand, heparin protected FGF2 from trypsin digestion (Fig. 1F, column II) and so did the coacervate (Fig. 1F, column III). An average of 86.1% of FGF2 was present in the coacervate 2 h after trypsin treatment, demonstrating that heparin retained its protective effect.

In order to examine the ability of the coacervate to localize FGF2 in the tissue, we used fibrin gel to mimic the *in vivo* environment. The gel was overlaid with medium and the amount of FGF2 in the medium was determined after 24 h by Western blotting. A significantly lower amount of FGF2 was detected in the medium of the coacervate group, indicating that the coacervate can effectively localize FGF2 (Fig. 1G). To investigate potential endothelial response to FGF2 released from the coacervate, human umbilical vein endothelial cells (HUVECs) were embedded in the fibrin gel as previously described (22). Different concentrations of FGF2 were tested for their ability to induce tube formation. After 3 d, free FGF2 did not induce tube formation at any concentration (Fig. 1H, column I). Most cells were round with a few spread-out cells scattered throughout the gel. On the other hand, HUVECs in the coacervate groups formed a clearly visible network of nascent endothelial tubes at 250 and 500 FGF2 ng/mL FGF2 (Fig. 1H, column II). This indicated that the FGF2 coacervate may induce more potent localized angiogenesis *in vivo* than free FGF2.

We further examined the bioactivity of FGF2 using mural cells that are important in the stabilization of blood vessels. Pericytes are actively involved in early angiogenesis, and their recruitment is critical to stabilize the newly formed vessels. Chemotaxis assays using pericytes revealed that both free FGF2 and the coacervate had higher chemotactic activities than the control groups, which were basal medium and the delivery vehicle. More importantly, pairwise comparison between the coacervate and free FGF2 yielded a p value lower than 0.05 revealing a higher chemotactic activity of FGF2 released from the coacervate (Fig. 1I).

[PEAD: Heparin: FGF2] Promotes More Potent Angiogenesis Than Free FGF2.

In order to examine the *in vivo* efficacy of the coacervate, we injected the coacervate containing 500 ng of FGF2 subcutaneously in the back of male BALB/cJ mice and compared its angiogenic capability to that of saline, delivery vehicle, and 500 ng of free FGF2, respectively. Macroscopic observation of the coacervate group revealed extensive formation of blood vessels at the injection site, whereas the contralateral site showed no difference from normal tissue (Fig. 2A). Hematoxylin and eosin staining revealed that the gross appearance of the saline and delivery vehicle groups are indistinguishable, suggesting the delivery vehicle itself had no angiogenic effect (Fig. 2B). Free FGF2 induced aggregation of nucleated cells, but few blood vessels were identified. On the contrary, the coacervate showed significant blood vessel formation with closed circles of nucleated cells surrounded by muscle bundles (arrow). The lumen of vessel was filled with red cells further supporting the function of the nascent blood vessels.

Fig. 2. (A) Macroscopic observation of subcutaneous tissue showed that the coacervate clearly induced new blood vessel formation at the injection site (2-wk pictures from the same mouse). The injection site was marked by a circle. (B) Hematoxylin and eosin staining of subcutaneous tissues after 4 wk. For the saline, delivery vehicle, and free FGF2 groups, there was no clear growth of vasculature in the subcutaneous region. The coacervate group, on the contrary, revealed the feature of blood vessel that had a closed inner layer of nucleated cells surrounded by smooth muscle bundles (arrow). Scale bar: 50 μ m. (C) Hemoglobin quantification compared the extent of angiogenesis between different groups. The result suggested that the coacervate group had a higher amount of hemoglobin 2 wk postinjection, whereas free FGF2 did not have statistical difference between the saline and delivery vehicle groups. This difference lasted at least for 4 wk (mean $\pm$ SD, $n = 4$ –8 for each condition). Normalized to the saline group. One-way ANOVA followed by Bonferroni correction was applied for multiple comparisons. * $p < 0.05$, ** $p < 0.01$. (D) The ratio of hemoglobin at the injection sites and the contralateral sites. For the coacervate, the ratio was significantly higher than that of the free FGF2 group. The result explained that FGF2 was well localized at the injection site by the delivery vehicle. Student's t test was used as a statistical tool. * $p < 0.05$, ** $p < 0.01$.

As an indirect measure of blood vessel density, we quantified the amount of hemoglobin in the tissue at the injection site at 1-, 2-, and 4-wk postinjection using a previously published method (23). All the groups had no significant difference at week 1. The coacervate group had higher amounts of hemoglobin than any other groups at weeks 2 and 4 (Fig. 2C). On the other hand, bolus injection of free FGF2 showed no statistical difference in hemoglobin content from saline and delivery vehicle groups at any time point. After 4 wk, the coacervate group still exhibited a significantly higher amount of hemoglobin than all control groups, suggesting the long-term stability of the newly formed blood vessels and the bioactivity of FGF2 released from the coacervate. The ratio of hemoglobin at the injection and contralateral site revealed that angiogenic responses were spatially controlled for the coacervate, whereas the free FGF2 group showed no difference (Fig. 2D). This correlated well with the macroscopic observation that the angiogenic activity of the coacervate was localized at the injection site. For the free FGF2 group, the ratio was close to 1 at every time point and was significantly lower than that of the coacervate after 2 wk. This combined with the insignificant change of hemoglobin from saline control suggested that bolus injection of 500 ng FGF2 had little angiogenic potency. Overall, the histological examination demonstrated the high efficacy of

the coacervate in promoting angiogenesis and the spatial control of FGF2 release from the coacervate.

[PEAD:Heparin:FGF2] Stimulates Proliferation of Endothelial and Mural Cells. The more potent angiogenesis induced by the coacervate warranted further study on the effects of the released FGF2 on cell functions. We investigated two specific markers for cells closely associated with angiogenesis: CD31 for endothelial cells and α -smooth muscle actin (α -SMA) for mural cells (pericytes or smooth muscle cells). Both the coacervate and free FGF2 promoted more endothelial cells (CD31-positive) than saline and the delivery vehicle qualitatively 1 wk postinjection (Fig. 3A). This was attributed to the proliferation of endothelial cells stimulated by FGF2. On the other hand, only a small number of mural cells (α -SMA-positive) were present in all groups. After 2 wk, a higher number of endothelial cells can still be found at the presence of the coacervate and free FGF2. However, the coacervate also induced a significant amount of mural cells. More importantly, the blood vessels in the coacervate group were well organized into circles of endothelial cells surrounded by mural cells. This difference was even more pronounced after 4 wk. Compared to the coacervate, the other three groups were similar in that most endothelial cells lacked support by mural cells. Higher magnifications of the vessels in the coacervate group showed distinctive structure of blood vessels with aligned endothelial cells closely associated and surrounded by mural cells (Fig. 3B).

Quantification of the endothelial and mural cells was consistent with higher angiogenic potency of the coacervate. Six low magnification (200 $\times$) fields for each group were chosen to quantify the number of endothelial and mural cells. The result suggested that the coacervate increased the number of endothelial cells by 120%, 95%, and 47% relative to that of the saline, delivery vehicle, and free FGF2, respectively (Fig. 3C). All the comparisons were statistically significant with p values lower than 0.01 supporting the coacervate induced more proliferation of endothelial cells. The free FGF2 group had more CD31-positive cells than the saline group ($p < 0.01$), whereas the delivery vehicle group showed no difference from the saline group ($p = 0.81$) revealing that the delivery vehicle itself had little effect to the proliferation of endothelial cells. More striking difference was the number of mural cells. A significant amount of mural cells were observed in the coacervate group. The quantification of mural cells demonstrated that the coacervate group was 6.71, 3.39, and 2.02 folds higher than that of the saline, delivery vehicle, and free FGF2 groups, respectively ($p < 0.01$ for all comparison). Again, the free FGF2 group induced more mural cells than saline ($p < 0.05$), but the delivery vehicle had no difference from saline ($p = 0.89$).

Furthermore, we compared the number and the size of the blood vessels in free FGF2 and coacervate groups by counting CD31-positive blood vessels and measuring their area according to a previously published procedure (24). The average number of blood vessels in the coacervate group was higher than that of the free FGF2 group (63.5 ± 3.6 vs. 46.0 ± 7.7 per mm^2 , $p < 0.05$). This is consistent with the above data that showed the coacervate group induced more vascular cell proliferation and the injection site contains more hemoglobin (Figs. 2C and 3D). Comparison of the size of the blood vessels revealed that the coacervate group had more blood vessels per mm^2 for all three size groups including $<400 \mu\text{m}^2$ (34.1 vs. 28.6), 400 – $1,000 \mu\text{m}^2$ (20.6 vs. 15.9), and $>1,000 \mu\text{m}^2$ (8.7 vs. 1.6). It is worth noting that blood vessels with areas larger than $1,000 \mu\text{m}^2$ all had abundant α -SMA expression and are likely arterioles and venules. Collectively, the immunohistochemical analysis strongly supported higher angiogenic potency of the FGF2 coacervate.

[PEAD:Heparin:FGF2] Coacervate Promotes Neovascularization Maturation by Recruiting Mural Cells. The maturity of newly formed blood

Fig. 3. [PEAD:heparin:FGF2] coacervate induces more potent angiogenesis *in vivo*. (A) Representative confocal micrographs showed the distribution of blood-vessel associated markers CD31 (endothelial cell, red) and α -SMA (mural cell, green) of each group at three time points. Both the free and coacervate FGF2 groups revealed a higher quantity of endothelial cells than saline control after 1 wk, but only the coacervate induced an increase of α -SMA expression after 2 wk. The circular vessel-like structures were observed in the field. After 4 wk, more endothelial and mural cells were present in the coacervate group demonstrating the long-term efficacy of the FGF2 coacervate. Scale bar: 50 μ m. (B) High magnification revealed the maturation of the blood vessels induced by the coacervate. The endothelial tubes were clearly surrounded by mural cells. Scale bar: 50 μ m. (C) Comparison of CD31 and α -SMA expression in the four injected groups. The number of endothelial cells in the coacervate group was higher than those of the control groups by 47% to 120%. More significantly, the number of mural cells in the coacervate group was 2.02 folds of that in the free FGF2 group. One-way ANOVA followed by Bonferroni correction, $*p < 0.05$; $**p < 0.01$. (D) Comparison of the number of blood vessels in a given size range between free and coacervate FGF2 groups as previously described; the value represents the cumulative number of all the slides examined (24). The coacervate induced more blood vessel formation than free FGF2. Furthermore, the coacervate group contained more large vessels ($>1,000 \mu\text{m}^2$, likely associated with arterioles and venules).

vessels is critical for their stability and function. Thus, we studied the maturation of neovasculature induced by the coacervate from early to late stages of angiogenesis. For the early angiogenic process, CD31 was costained with a pericyte specific marker, platelet derived growth factor receptor- β (PDGFR- β) (25). We observed many CD31-positive endothelial cells clustered with PDGFR- β -positive cells in the coacervate group 1 wk postinjection (Fig. 4A). On the contrary, no association of pericytes and endothelial cells was observed in the free FGF2 group. This difference again demonstrated the efficacy of the coacervate in maintaining the bioactivity of FGF2.

For long-term vessel maturity, we examined markers associated with vascular functions. Von Willebrand factor (VWF), an important protein involved in hemostasis, was stained to show the potential of the nascent blood vessels to participate in hemostasis. We found that the coacervate induced strong expression of VWF (Fig. 4B). The overlap of CD31 and VWF signals indicated that the nascent endothelial cells were functional. Desmin, a component of intermediate filament expressed in mural cells, is a widely used marker to study perivascular structure. We observed desmin coexpressed in α -SMA-positive blood vessels (Fig. 4C). Addition-

ally, α -SMA-negative but desmin-positive blood vessels were also found in the field. This likely reflected the distribution of vessel sizes and the heterogeneity of pericytes that had low α -SMA expression at capillary levels (26). Calponin mediate contractile responses of vascular smooth muscle cells, and we costained this important marker with α -SMA to evaluate the functionality of nascent blood vessels. The result showed again that more and larger blood vessels were induced by the coacervate than by free FGF2 (Fig. 4D). Overall, the results of immunohistochemical analysis demonstrated that more mature vasculature was induced by the coacervate, which correlated well with the higher local hemoglobin concentration at the injection site.

Discussion

Currently, polymer microspheres and foams, especially those made of poly(lactic-co-glycolic acid) (PLGA) and hydrogels including nanofibrous peptides, are main platforms adopted for controlled delivery of growth factors. PLGA is a highly biocompatible material and has been approved by the US Food and Drug Administration for several medical applications. It has been applied in a very creative combination of foam and microspheres to release dual growth factors for over a month and shown to have more effective angiogenesis than bolus injection of free growth factors (24). However, foams would require implantation and will be difficult to use minimally invasively. Usually the incorporation of growth factors into PLGA microspheres requires organic solvents that have high tendency to denature growth factors. The loading efficiency is usually below 50% for PLGA (27). Hydrogels composed of biological materials, such as gelatin (28) and alginate (29), or synthetic materials, such as poly(ethylene glycol) (30), can embed growth factors and allow a diffusion-dependent release to activate surrounding cells. However, the release profile is not easily controlled with initial burst reaching as high as 50% in certain cases (31). Peptide nanofibers have exhibited promising *in vivo* results (32), but the high cost for peptide synthesis may pose a challenge for clinical translation.

Our goal is to develop an injectable platform that is easily administered even in a basic clinic. Thus we developed a coacervate formed by simple mixing of three water soluble components: a biocompatible polycation, heparin, and a heparin-binding growth factor. The resultant coacervate has very low viscosity and is injectable via a 31 G needle (outer diameter: 0.26 mm) or a catheter. The loading efficiency of FGF2 is nearly 100% according to the results of both ELISA and Western blot (21). The coacervate delivery platform is highly effective with a low dosage of 500 ng FGF2. In many test results reported here, there are insignificant differences between free FGF2, saline, and the delivery vehicle. We attribute this to the 500-ng dosage, which is much lower than what is commonly used in the literature. The injectability combined with high loading efficiency and efficacy of the coacervate may enable previously undescribed opportunities of growth factor treatment for certain diseases such as cardiac infarct where only a small volume can be injected and wound must be minimal.

Therapeutic angiogenesis via exogenous growth factors including FGF2, VEGF, and PDGF has been examined extensively to treat human ischemic diseases (33, 34) that resulted in huge progress in understanding of growth factor signaling and its interaction with the host. However, there has yet to be a clear demonstration of clinical benefits (5). Typically, the treatment group showed improvement at the early stage but had no significant difference from the placebos in long-term observation (35). This has been attributed to the lack of stability of the nascent blood vessels. The current approach to solve this challenge is codelivery of multiple growth factors to boost the long-term stability of the neovasculature (36, 37). Often, a growth factor can stimulate a range of cells to accomplish physiological events such as embryonic development, angiogenesis, vasculogenesis, and wound healing (38–40). FGF2 binding to FGF receptors dimerizes the

Fig. 4. Enhanced maturity of the nascent blood vessels by the coacervate. (A) The colocalization of CD31-positive and PDGFR- β -positive cells suggested the coacervate quickly recruited pericytes to interact with endothelial cells in the nascent vessels. This phenomenon was absent in the free FGF2 group. (B) Significant colocalization (green + red = yellow) of VWF- and CD31-positive cells suggested that the endothelial cells in the nascent vessels can potentially participate in hemostasis. (C) Both α -SMA and desmin are markers for mural cells. Their expression pattern revealed that larger vessels coexpressed these markers, whereas smaller vessels were dominated by the expression of desmin (arrows). (D) Calponin, a calmodulin associated with vascular smooth muscle cells contraction, was costained with α -SMA to examine the potential functionality of the new blood vessels. The result indicated that the blood vessels in the coacervate group had abundant expression of calponin. In addition, the blood vessels were much larger in the coacervate group than those in the free FGF2 group. Scale bars indicate 50 μ m for both low and high magnification.

receptors, activates their tyrosine kinases, and triggers the downstream signaling pathways (41). For endothelial cells, FGF2 is a potent mediator that can promote their proliferation, migration, and differentiation and stimulate the expression of VEGF to initiate angiogenesis (42). In addition, FGF2 can recruit pericytes to newly formed blood vessels (43) and promote the survival and proliferation of vascular smooth muscle cells (44). Both pericytes and smooth muscle cells substantially enhance the stability of the neovasculature. Consequently, mature vasculature is achievable if the bioactivity of FGF2 is well maintained. The challenge is that the delivery system must maintain the bioactivity of the growth factors and release them with appropriate spatiotemporal control. By designing the delivery system *de novo*, we developed a coacervate that maintained the bioactivity of FGF2 well and enabled formation of mature vasculature with the controlled release of a single growth factor.

The current study evaluates the angiogenic activity of a heparin-based coacervate using a rodent model. Only one injection of the coacervate is required for sustained angiogenesis. Furthermore, the blood vessels formed are mature and stable to at least 4 wk. The FGF2 coacervate has a much higher angiogenic efficacy than free FGF2. Further investigations include the effectiveness of this platform in ischemic animal models and *in vivo* imaging of the blood vessels to monitor flow and potential vasoreactivity. Because the polyvalent charge interaction is critical to the formation and stability of the coacervate, we expect efficient control of growth factor release by tailoring charges and

size of PEAD as we demonstrated in a related polycation (20). We expect this previously undescribed delivery platform will be useful in the controlled release of many heparin-binding growth factors that control important biological functions.

Materials and Methods

Delivery Vehicle Preparation and Scanning Electron Microscopy. PEAD was synthesized as previously described (21). To prepare the delivery vehicle, PEAD was mixed with heparin under constant stirring, and the solution immediately became cloudy as the delivery vehicle formed. The complex was dropped on an aluminum stub, lyophilized, and sputtered with gold, and the morphology was examined by a Jeol 6335 field emission gun SEM (Jeol).

Analysis of FGF2 Loading Efficiency by Western Blotting. PEAD and heparin were each dissolved in normal saline to obtain 10 mg/mL solutions. For preparation of the coacervate, 100, 500, or 1,000 ng of FGF2 (PeproTech) was first mixed with 10 μ L of the heparin solution and then 50 μ L of the PEAD solution. The coacervate was equilibrated at room temperature for 15 min followed by centrifugation at 12,100 $\times$ g for 10 min. The supernatant and the pellet were mixed with the sample buffer and denatured at 95 $^{\circ}$ C for 5 min. SDS-PAGE was utilized for separation followed by protein blotting on a PVDF membrane. A rabbit anti-human FGF2 polyclonal antibody (PeproTech) was applied for recognition followed by a peroxidase conjugated anti-rabbit IgG antibody (Sigma). The intensity of the individual band was determined by NIH ImageJ software.

Protection from Proteolysis by the Coacervate. Trypsin digestion of FGF2 was evaluated as previously described (45). Briefly, FGF2 (100 ng) alone, heparin-bound FGF2 (100 μ g of heparin and 100 ng of FGF2), or the coacervate (500 μ g of PEAD, 100 μ g of heparin and 100 ng of FGF2) was incubated with 2 μ g of trypsin at 37 $^{\circ}$ C for 30 min or 2 h. The digested solution was mixed with the sample buffer and denatured at 95 $^{\circ}$ C for 5 min. Western blotting was utilized to examine the amount of intact FGF2.

Bioactivity of FGF2 in Fibrin Gel. The fibrin gel (400 μ L) was formed by mixing fibrinogen (4 mg/mL) solution with either 500 ng free FGF2 or the coacervate containing 50 μ g PEAD, 10 μ g heparin and 500 ng of FGF2, and thrombin in the basal medium. The gel was incubated at 37 $^{\circ}$ C for 30 min and overlaid with 600 μ L basal medium. After a 24-h incubation, FGF2 in the medium was precipitated by addition of trichloroacetic acid (TCA) and centrifuged at 12,100 $\times$ g for 10 min. Ice-cold acetone was utilized to wash out residual TCA. The pellets were dissolved in sample buffer for Western blotting.

Endothelial tube formation was investigated at three FGF2 dosing levels (50, 250, and 500 ng/mL) using fibrin gel assay as described above. HUVECs (Lonza) were maintained in Endothelial Cell Growth Medium-2 (Lonza) supplemented with growth factors according to the supplier's instruction. The experimental procedures of tube formation followed a published protocol (19). Briefly, 8×10^4 cells (passage 7) were mixed with fibrinogen solution containing FGF2 (50, 250, or 500 ng) or the same amount of FGF2 in the coacervate. After addition of thrombin, the whole solution was gelled at 37 $^{\circ}$ C for 30 min. The gel was last overlaid with 600 μ L of the basal medium to provide the basic nutrient. After incubation for 3 d, the phase contrast images were taken by a microscope.

Chemotaxis of Pericytes Induced by Coacervate. Human pericytes were isolated following the established method (46). For the chemotaxis experiment, 1.0×10^4 pericytes (passage 7) were added in Transwell® inserts (pore size of 8 μ m) and placed in a 24-well plate containing blank solution, delivery vehicle, 100 ng of FGF2, or the coacervate having 100 ng of FGF2. After incubation for 12 h, nonmigrating cells were removed with cotton swabs and migrating cells were stained by Quant-iT™ PicoGreen® (Invitrogen). The fluorescent images were taken by an inverted microscope Eclipse Ti (Nikon).

Animal Care and Subcutaneous Injection. Male BALB/cJ mice (Jackson Laboratory) with an average age of 6–7 wk were used and cared for in compliance with a protocol approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Under isoflurane anesthesia, 65 μ L of saline, delivery vehicle (500 μ g of PEAD and 100 μ g of heparin), free FGF2 (500 ng of FGF2), or the coacervate (500 μ g of PEAD, 100 μ g of heparin, and 500 ng of FGF2) was injected in the left back of the mice through a 31G insulin needle. The right back, which did not receive injection, served as the contralateral control. All groups contained four to eight mice.

The animals were sacrificed at postinjection weeks 1, 2, and 4. The subcutaneous tissue (1.5 cm $\times$ 1.5 cm) was harvested at the injection site and the contralateral site. The hemoglobin in the harvested tissue was extracted

by the hemolysis buffer containing 17 mM of Tris-HCl (pH 7.4) and 0.75 wt % ammonium chloride. The absorbance at 410 nm corresponding to the hemoglobin Soret band was recorded by a SynergyMX plate reader (Biotek) (47). All values were normalized to that of the saline injection.

Immunofluorescent Staining. The subcutaneous tissue was embedded and frozen in Tissue-Tek OCT compound (Sakura Finetek USA). Sections of 5- μ m thickness were cut with a cryomicrotome and stored at -80°C . The following antibodies were applied per supplier instructions: rat anti-mouse CD31 monoclonal antibody (BD Biosciences), Cy3-conjugated anti-rat IgG antibody (Invitrogen), FITC-conjugated anti- α -SMA monoclonal antibody (Sigma), goat anti-mouse PDGFR- β polyclonal antibody (R&D Systems), Alexa Fluor 488-conjugated anti-goat IgG antibody (Invitrogen), FITC-conjugated anti-vWF antibody (US Biological), rabbit anti-desmin polyclonal antibody (Sigma), Alexa Fluor 594-conjugated anti-rabbit IgG antibody (Invitrogen), and rabbit anti-mouse calponin-1 monoclonal antibody (Millipore). All slides were counterstained with DAPI (Invitrogen). The fluorescent images were taken by a Fluoview 500 Confocal microscope (Olympus).

Quantitative Analysis of Immunofluorescent Staining. Six low magnification (200 $\times$) fields containing the highest number of CD31- or α -SMA-positive cells were selected for each group following a previously published criteria (48). The number of CD31- or α -SMA-positive cells in the field was counted and confirmed by DAPI-positive nuclei. The value was divided by the area of the tissue and normalized to that of the saline group. For comparison of the number and the size of blood vessels, CD31-positive blood vessels in three low magnification (200 $\times$) fields were measured by NIS-Elements software (Nikon).

Statistical Analysis. Student's *t* test was used for pair comparison. ANOVA followed by post hoc Bonferroni test was utilized to compare the number of CD31- and α -SMA-positive cells between all conditions for multiple comparison. Data are presented as mean $\pm$ standard deviations. **p* value < 0.05; ***p* value < 0.01.

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