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Scale-up synthesis of a polymer designed for protein therapy

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ABSTRACT

Here we report optimization and scale-up of a biodegradable polycation, poly(ethylene argininylaspartate diglyceride) (PEAD) for the purpose of clinical translation. This polycation complexes heparin to form a coacervate. The resultant complex coacervate acts as a vehicle for controlled delivery of therapeutic proteins and through the bioactivity of heparin, greatly increases the half lives of many therapeutic proteins. In an effort to translate this drug delivery platform commercially and clinically, it is necessary to consider the scale of the synthesis, the environmental impact and the cost of the production. Therefore, we substituted the more expensive and hazardous solvents in the reactions, optimized the reaction parameters for a higher yield, scaled up the synthesis capacity up to hundreds of grams per batch and reduced the polymer cost by nearly 90%. The resultant PEAD bears more positive charges than the counterpart made using our previous method, and thus less polymer is needed to complex heparin for coacervation. The material demonstrated good cytocompatibility with human umbilical vein endothelial cells at concentrations up to 5 mg/ml, nearly 500-times more cytocompatible than the commercially resourced polyethyleneimine. Basic fibroblast growth factor was loaded in the coacervate with loading efficiency above 99%. The cargo steadily released approximately 1% over 37 days *in vitro* without an initial burst, indicating a highly stable coacervate for sustained release. This study paves the way for the translation of this delivery platform and may inform the scale-up of similar polymers.

1. Introduction

Therapeutic proteins, e.g., growth factors, play significant roles in biology and medicine [1,2]. According to a latest Research and Markets report, the therapeutic protein market is expected to reach approximately $316 billion by 2025 [3]. However, the half lives of many proteins, particularly growth factors, are very short (minutes to hours) [4]. Scientists have been seeking an efficient delivery platform to extend the half lives *in vivo* for decades [4–6]. Heparin binds more than 400 proteins and peptides [7]. The binding stabilizes these proteins significantly. Many of them are growth factors and cytokines with important biological functions including cell migration, proliferation and differentiation [8,9]. To exploit the natural heparin affinity to proteins, we designed a polycation, poly(ethylene argininylaspartate diglyceride) (PEAD), to complex heparin by polyvalent interactions to form a coacervate as a controlled delivery vehicle [10,11]. The coacervate controls the release of various heparin-binding proteins spatially and temporally. We have demonstrated the utility of this platform for the delivery of vascular endothelial growth factor, basic fibroblast growth factor, nerve growth factor, heparin-binding EGF like growth factor, stromal cell-derived factor-1α, bone morphogenetic protein, and hepatocyte growth factor, among others [11–19]. Each demonstrated a distinct therapeutic application. The released heparin-binding proteins are stable at least for 2 h even in the presence of a protease [13,20]. *In vivo*, this protein controlled release system is still present after 4 weeks [21]. This demonstrated the benefit of this platform [22]. Thus, this coacervate vehicle can be very useful in the therapeutic protein markets.

The key step for clinical translation of this delivery platform is to scale up the synthesis of PEAD. We already established a mature benchtop protocol to make the PEAD in gram quantities [10]. However, when scaling up in hundreds of grams per batch, the reaction parameters need further optimization. Moreover, the solvents such as dichloromethane and diethyl ether are more hazardous and expensive to dispose of in large scale manufacturing. Therefore, the main objectives of this study are to substitute the expensive and hazardous solvents in the reactions, optimize the reaction parameters for a higher yield, scale up the synthesis capacity and reduce the polymer cost.

This manuscript reports the scale-up of PEAD synthesis in hundreds of grams per batch with improved reaction parameters and conditions. The physicochemical and biological properties of the resultant PEAD and the PEAD/heparin coacervate are re-evaluated for its effectiveness

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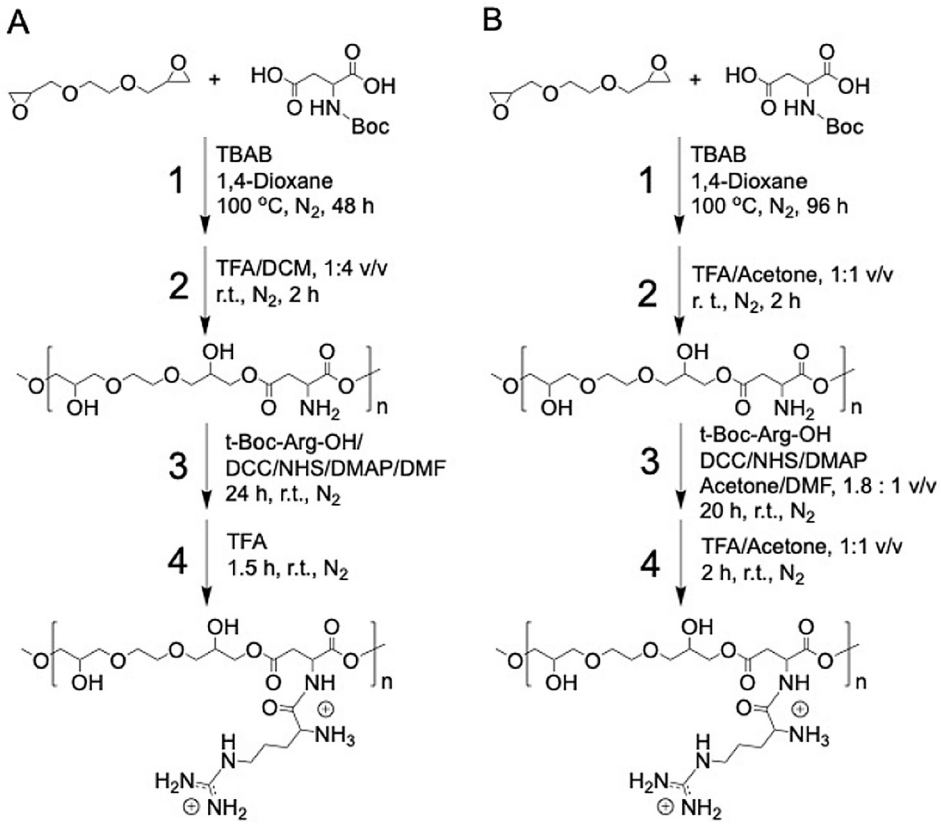


Fig. 1. Comparison of the synthesis protocols of (A) benchtop and (B) scale-up. (1) Ring opening polymerization to obtain a Boc-PED precursor; (2) deprotection of Boc group to obtain the intermediate PED; (3) coupling Boc-Arg-OH with amine groups on the PED; (4) deprotection of Boc group to yield the resultant PEAD.

as drug delivery vehicles. These include the characterization of polymer molecular weight, chemical properties and zeta potential, coacervation with heparin, protein loading and controlled release profile, and *in vitro* cytocompatibility.

2. Result and discussion

2.1. Scale-up synthesis of PEAD

We have successfully developed a new protocol to scale up the synthesis of PEAD (Fig. 1). The key step is to polymerize ethylene glycol diglycidyl ether (EGDE) and N-Boc-L-aspartic acid (Boc-Asp-OH) to yield a Boc-protected poly(ethylene aspartate diglyceride) (Boc-PED) precursor polymer (Step 1). Compared to the benchtop protocol (Fig. 1A), our studies revealed that when the molar quantities of the monomers are increased up to 1.2 mol per batch (80-times increase), just doubling the reaction time could yield a desirable Boc-PED precursor. Whereas, other reaction parameters such as the reactant concentration, ratio and reaction temperature could remain the same (Fig. 1A, B, Step 1). The Boc-PED precursor is then deprotected in acetone and trifluoroacetic acid (TFA) at appropriate ratio to yield the PED intermediate (Fig. 1B, Step 2). Notably, the deprotection could be completed in 2 h with approximately 96% deprotection efficiency (Fig. S1). Here, we successfully replaced the more toxic dichloromethane (DCM) with acetone in the deprotection reaction, reduced the ratio of TFA to Boc-PED from 1.3:1 in the old protocol to 0.82:1 (ml/g) in the new deprotection reaction and achieved a similarly high deprotection efficiency without increasing the reaction time. The efficient removal of Boc- protection groups is crucial to generate the PED intermediate with free amine groups for coupling reaction in the next step. Using this new protocol, approximately 180 g of the PED intermediate can be made per batch with a yield of ca. 96%. The yield of the PED is comparable to the benchtop protocol [10].

In the next two steps, the PED intermediate is conjugated with N-Boc-Arginine (Boc-Arg-OH) followed by deprotecting to obtain the resultant PEAD. In our benchtop protocol, we used N,N-dimethylformamide (DMF) as solvent in the coupling reaction (Fig. 1A, Step 3). DMF is a hydrophilic aprotic solvent with high boiling point that is used to dissolve the reactants for reaction in this step. However, the DMF is difficult to remove which leads to a low yield of the final PEAD in our benchtop protocol [10]. Therefore, we have replaced the majority of DMF with acetone in the new protocol, but a portion of DMF is still needed to dissolve the Boc-Arg-OH in the coupling reaction (Fig. 1B, Step 3). In addition, we further optimized the reactant concentration and procedure for the conjugation (See experimental section). With these improvements, approximately 77 ± 6.3 mol.% of Boc-Arg-OH can be conjugated with the amine groups on the PED within 20 h, 4 h less than the benchtop protocol (Step 3). After the insoluble dicyclohexylurea byproduct from the DCC/NHS coupling reaction is removed by filtration, the polymer solution is condensed by rotary evaporation to remove most of the acetone solvent. In this way, a high yield of Boc-protected PEAD (Boc-PEAD) could be collected by precipitating in hexanes and ethyl acetate. The Boc-PEAD sediment is then deprotected in acetone/TFA for 2 h (Fig. 1B, Step 4). Notably, the ratio of TFA to Boc-PEAD for deprotection in this step reduced from 11.3:1 in the old protocol to 1.9:1 (ml/g) in the new protocol. Combined with the deprotection in step 2, the new method significantly reduced the unit quantity of TFA in the reactions while achieving a similar deprotection efficiency [10]. After deprotection, the PEAD solution is condensed by rotary evaporation and repeatedly solvated and precipitated in hexanes and ethyl acetate to obtain the resultant PEAD with a yield of ca. 93%, nearly 3 times increase to that of the benchtop protocol [10].

As noted, we substituted diethyl ether by hexanes and ethyl acetate for precipitation of the polymer at each step. Diethyl ether tends to

Table 1
The main differences between the benchtop and scale-up protocols.

Protocol	Benchtop	Scale-up
Polymerization (1)	48 h	96 h
Deprotection (2, 4)	DCM or DMF + TFA	Acetone + TFA
Conjugation (3)	DMF, 24 h	Acetone + DMF, 20 h
Precipitation (1 – 4)	Diethyl ether	Hexanes + Ethyl Acetate
Capacity/Batch	1 g	150 g
Overall Yield	31%	89%

accumulate explosive peroxides in either storage or reaction, which makes it very hazardous and expensive to dispose of in large scale manufacturing [23]. After optimizing these reaction parameters and processing conditions, we can now synthesize PEAD approximately 150 g per batch with an overall yield as high as 89%. The production capacity is increased by ca. 150 times per batch and the overall yield is increased by ca. 3 times to that of the benchtop protocol [10]. As a result, the polymer cost is reduced by ca. 90%. Table 1 lists the main differences between the new and old protocols.

2.2. Characterization of the scaled-up PEAD

Gel permeation chromatography (GPC) is used to examine the molecular weight and the mass distribution of the resultant PEAD. The PEAD has weight average molecular weight (M_w) of 47,100 Da, which is comparable to the counterpart made by the benchtop protocol (30,330 Da) [10]. However, the polydispersity is increased up to 8.78, indicating a relatively wide range of mass distribution. The GPC spectrum shows a side band along with the main band (Fig. 2), indicating the existence of a portion of low molecular weight oligomers in the PEAD product. These oligomers could not be efficiently removed by precipitation processes which further broadened the mass distribution. We observed a significant increase of viscosity upon the polycondensation in step 1 (Fig. 1). Such viscosity increase leads to a non-uniform mixing of the reactants in the reaction vessel, particularly those far from the stirring center. Thus, the reaction is prone to generate some low molecular weight PEAD and oligomers, which result in increase of the polydispersity of the polymer. Such phenomenon was also seen in other large scale polycondensations [24]. We expect using a more effective mechanical stirring tool could make the polymerization proceed more uniformly and consequently narrow down the mass distribution.

The chemical structure and composition of the PEAD are analyzed by proton NMR spectroscopy. The chemical shifts (δs) are correspondingly labeled and listed in Fig. 3. For example, δs at 3.12, 2.81, and 3.51 ppm are contributed by the protons of H_a, H_d and H_g. They are

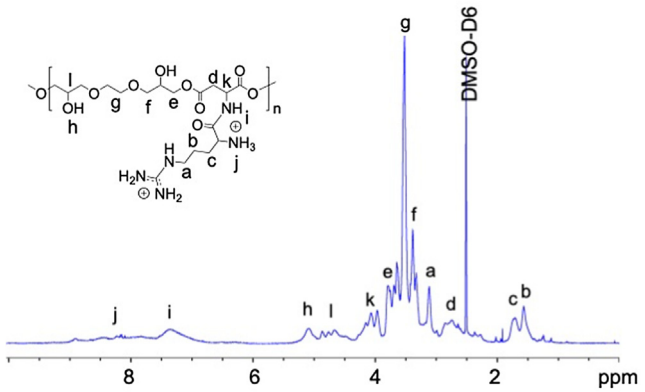


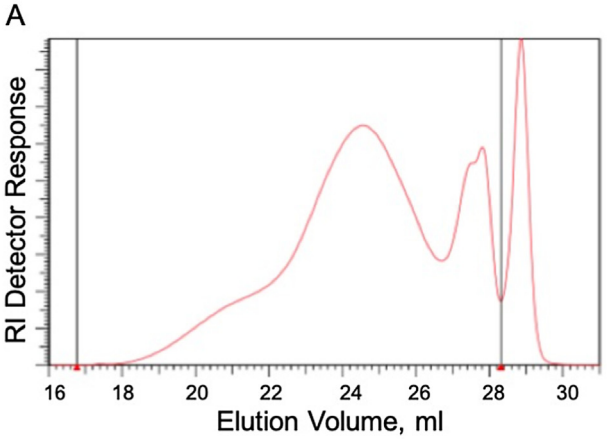
Fig. 3. Proton NMR analysis of the resultant PEAD. The resultant PEAD is further purified by dialysis against deionized water for NMR analysis. The integral area ratio of H_a to H_d is used to calculate the actual content of argininyl conjugated on PED backbone (Fig. S2).

respectively assigned to the protons from argininyl, aspartate and ethylene glycol diglyceride components in the PEAD. To evidence the argininyl pendants being conjugated on the PED backbone, the NMR spectrum also shows the δ at 7.31 ppm from the amide proton (H_i), indicating the efficient conjugation between Arg-OH and amine on the PED. The actual argininyl conjugation is determined by the integral area ratio of H_a to H_d to be approximately 77 ± 6.3 mol.% (Fig. S2). These NMR data confirmed the desired chemical structure of the PEAD. The actual argininyl content in this new PEAD is higher than the counterpart from the benchtop protocol [10], indicating a higher conjugation efficiency using the new protocol.

The argininyl pendants are responsible for imparting the positive charges to the PEAD. The positive charges together with the polymer molecular weight and mass distribution will eventually dominate the coacervation of PEAD with polyanion and the coacervate stability for use. In the following sections, we will examine the zeta potential, coacervation with heparin, drug loading and controlled release, and cytocompatibility of the new PEAD and PEAD/heparin coacervate.

2.3. Zeta potential and coacervation with heparin

Because the new PEAD has a higher argininyl content than the previous one, we re-optimized the ratio of PEAD to heparin to make a coacervate with neutral charge for use. Zeta potential measurement shows +29.4 ± 3.4 mV of the new PEAD versus +17.0 ± 2.6 mV of the old one at the same concentration ($p = 0.0017$, Fig. 4A). The



B

	Molecular Weight, Da
M_n	5370
M_w	47100
M_p	15300
PDI	8.78

Fig. 2. (A) GPC spectrum of the scaled-up PEAD. (B) Molecular weight and polydispersity (PDI). The main elution band followed by a side band indicates the existence of a portion of low molecular weight oligomers in the resultant PEAD. The band between the elution volume of 28–30 is from solvent.

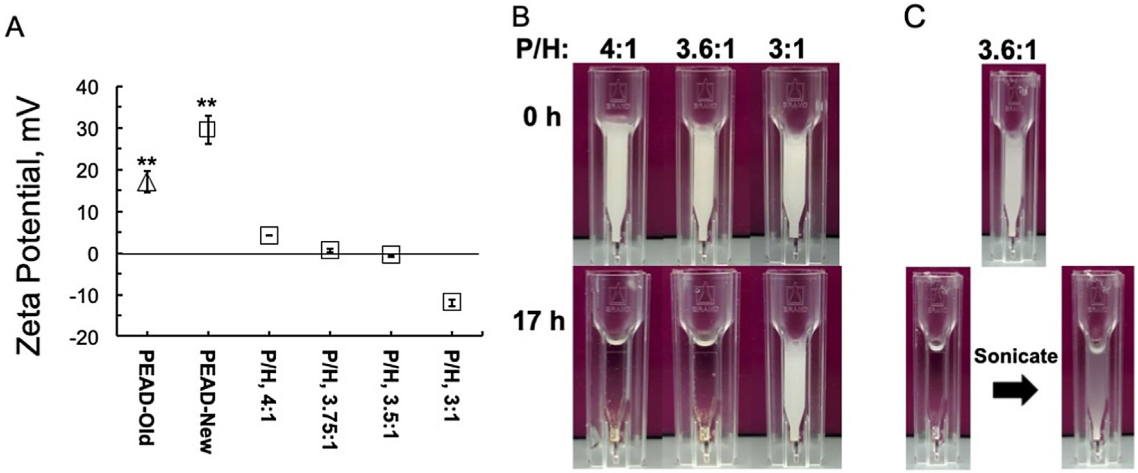


Fig. 4. (A) Zeta potential measurements of the PEAD solutions and coacervates with different P/H weight ratio. All PEAD solutions and coacervate suspensions are freshly prepared at 10 mg/ml in deionized water for measurements. (Triangle) the old PEAD from the benchtop protocol; (Square) the new PEAD from the scale-up protocol and the coacervates with different P/H ratio. The coacervate with neutral charge is formed at P/H ratio of approximately 3.6:1. Unpaired *t* test is performed for statistical analysis. $^{**}p = 0.0017$; A p value < 0.05 is considered significant different. Data represent as mean ± SD ($n \geq 3$). (B) 10 mg/ml coacervates with different P/H ratio are compared against settlement. The coacervates with P/H ratios at 4:1 and 3.6:1 are sedimented to the bottom after 17 h and difficult to re-disperse by sonication, indicating strong polyvalent interactions in the sediments; (C) 5 mg/ml coacervate is similarly settled for 17 h. The coacervate is partially re-dispersed by a 2-min sonication (the bottom row, right image).

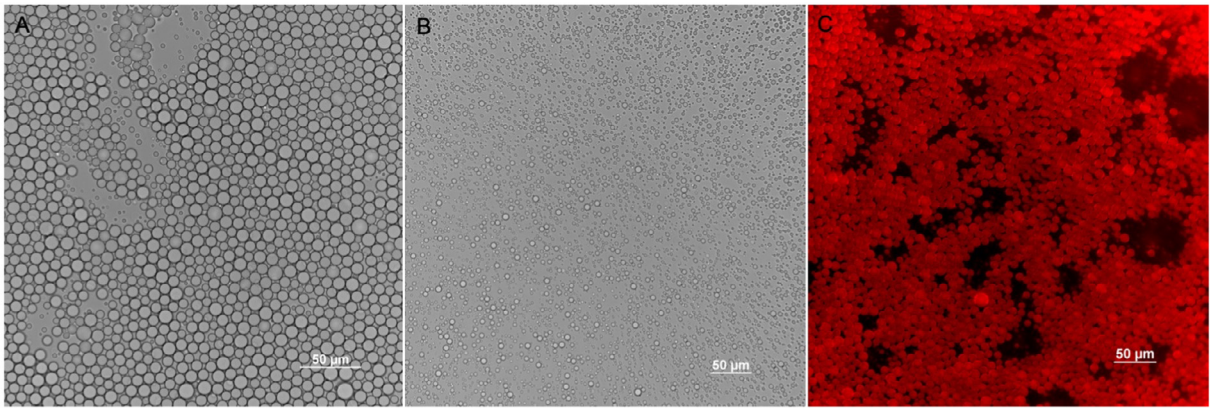


Fig. 5. Microscopic images of the coacervate suspensions with and without protein loading. (A) 10 mg/ml coacervate, (B) 5 mg/ml coacervate, and (C) 10 mg/ml coacervate loaded with fluorescence-labeled bovine serum albumin (BSA-647). The BSA to heparin to PEAD ratio is 1:100:360 by weight. Scale bar, 50 μm. Both the 10 mg/ml and 5 mg/ml coacervates are spherical droplets. The 10 mg/ml coacervate forms more and denser droplets with a larger diameter, which is easier to settle down and merge into bulk sediments compared to the 5 mg/ml one.

increase of zeta potential further confirmed the higher arginine conjugation efficiency using the new protocol. When the PEAD complexes heparin, the zeta potential accordingly changes from positive to negative as the heparin ratio increases (Fig. 4A). We therefore determine the new PEAD to heparin ratio (P/H) at approximately 3.6:1 to reach an isoelectric point. The coacervate formed at this ratio bears neutral charge for use because either positive or negative charges will be more cytotoxic than the neutral form. In contrast, our old PEAD complexes heparin at P/H ratio of 5:1 to reach the isoelectric point [11]. Therefore, less new PEAD is needed to complex heparin for a neutral coacervation because of the higher positive charges.

After knowing the zeta potential properties, we further examine the coacervation between the new PEAD and heparin and the coacervate stability. The coacervation between them not only depends on the electrostatic interactions, but the polymer concentration and the P/H ratio also play significant roles. We first compared the coacervation and coacervate stability at 10 mg/ml with different P/H ratio (Fig. 4B). At all of the P/H ratios tested, PEAD solution can immediately complex heparin solution to form coacervate suspensions upon mixing them, but the stabilities of the coacervate suspensions are different as the P/H ratio varies. As noted, the coacervate suspensions with P/H ratio ≥ 3.6:1 are easier to aggregate and precipitate after settlement (Fig. 4B). The precipitates are difficult to re-disperse by sonication, indicating very strong polyvalent interactions in the PEAD/heparin aggregates. However, the coacervate with P/H ratio at 3:1 is more stable to suspend in the saline for at least 17 h. This is likely due to the net negative charges to repulse the coacervate droplets each other and thus prevent the aggregation. In contrast, the coacervate at 5 mg/ml with P/H ratio at 3.6:1 can be partially re-dispersed by sonicating for several minutes after a similar settlement (Fig. 4C). This result indicates that the lower polymer concentration could generate somehow loosely bound coacervates between the PEAD and heparin chains so as to be reversibly dispersible. However, there are also some sediments which are relatively difficult to be re-dispersed by sonication. We speculate they are likely formed between the large molecular weight PEAD and heparin. For comparison, our old PEAD/heparin coacervate even at 10 mg/ml could be easily re-dispersible by pipetting or sonicating [11].

The above data indicate that the new PEAD bears more positive charges with stronger polyvalent interactions with heparin compared to our old PEAD. At a higher concentration, more polymers participate in

coacervation to form more complex droplets than that at a lower concentration. The coacervate at 10 mg/ml yields more spherical droplets with a much larger diameter than the 5 mg/ml coacervate (Fig. 5A and B). Both 10 and 5 mg/ml coacervate droplets can merge into larger ones upon contact during settlement (see supporting videos 1 and 2). The sedimentation process is quicker in the 10 mg/ml coacervate because of the larger diameter and denser coacervate droplets. Therefore, once the suspension is sedimented, the 10 mg/ml coacervate tends to merge into bulk aggregates that are not as easy to re-disperse as the 5 mg/ml coacervate. Additionally, the new PEAD has a wide range of mass distribution (PDI = 8.78, Fig. 2). In the 5 mg/ml coacervate, the portion of low molecular weight PEAD is prone to loosely complex heparin that are reversibly dispersible, whereas the high molecular weight PEAD and heparin complexes are not easy to re-disperse due to the stronger polyvalent interactions between them. This is why we observed only part of the 5 mg/ml coacervate was easily re-dispersed by the sonication for approximately two minutes (Fig. 4C, bottom row, right).

To visualize the morphology of a protein-loaded coacervate, a fluorescently labeled bovine serum albumin (BSA-647) is complexed with heparin followed by adding PEAD to form a BSA-loaded coacervate (Fig. 5C). The fluorescent micrograph shows an efficient entrapment of the BSA in the spherical droplets. Here we examined the loading of the coacervate using a PEAD/heparin/BSA weight ratio of 360:100:1. The result demonstrated a strong coacervation and protein loading capability at the ratio tested.

Through the above discussion, we can control over the coacervate morphologies and stabilities through adjusting the concentrations of the PEAD and heparin and their ratios for coacervation. In the following study, we will focus on the coacervate with neutral charge for protein delivery and examine the growth factor loading and controlled release profile.

2.4. Protein loading and controlled release study

We have accordingly updated the protocol to load proteins in the new PEAD/heparin coacervate for controlled release. Generally, a desired amount of proteins is first mixed with heparin solution, followed by adding a suitable amount of PEAD solution to form a protein-loaded coacervate for injection. Because of the strong polyvalent interactions between the new PEAD and heparin, the coacervate can be prepared between 10 and 5 mg/ml for utility (Fig. 5). The PEAD/heparin/protein (P/H/P) weight ratio can be ranged from 3600:1000:1 to 36:10:1 for protein loading and manipulating the protein release kinetics. We have demonstrated an efficient loading of relatively large molecular weight protein (BSA, 66.5 kDa) in the coacervate at P/H/P ratio of 360:100:1 (Fig. 5C). Now we examine the growth factor loading and its release kinetics for utility. We would like to note that when using the new PEAD to make the coacervate at a high concentration, e. g., 10 mg/ml, the coacervate should be freshly prepared for use within hour because it is relatively easy to sediment which likely causes a dosing error.

To compare with our previous release profile [11], we load the growth factor FGF2 in the new coacervate at 10 mg/ml with P/H/P ratio at 3600:1000:1 to examine the release kinetics. All loading parameters and experimental conditions are remained the same, except the new P/H ratio changed to 3.6:1 (5:1 in our old coacervate). The loading efficiency can reach above 99%, identical to our previous data [11]. Notably, only ca. 1% of the FGF2 is steadily released from the new coacervate at 37 °C over 37 days without a burst release (Fig. 6); whereas our old coacervate released ca. 40% over 42 days with an initial burst of ca. 10% in the first day [11]. The new coacervate is apparently more stable and strongly bound with the heparin/FGF2 complexes, significantly reducing the release rate. We anticipate that when the coacervate is injected *in vivo*, enzyme-catalyzed hydrolysis and oxidation will increase PEAD degradation rate and facilitate the coacervate dissociation and the release of the cargo. The enzymes which could promote biomaterial degradation have been well investigated

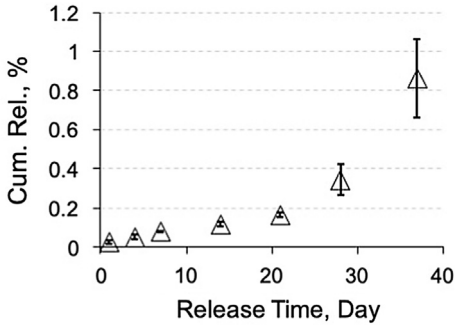


Fig. 6. Cumulative release of basic fibroblast growth factor (FGF2) from the new PEAD/heparin coacervate over 37 days at 37 °C in 0.9% saline. Data represent the mean ± SD (n = 4).

both *in vitro* and *in vivo*, including lipase, esterase and myeloperoxidase, among others [25–28]. PEAD is a cationic polyester that is biodegradable as previously demonstrated in animal models [13,16,29,30]. The new PEAD and PEAD/heparin coacervate might undergo slightly different *in vivo* degradation compared with our old PEAD and coacervate because of the different arginine content, molecular weight and PEAD/heparin ratio. But we do not expect the overall *in vivo* degradation will change significantly.

It has been well established that heparin binds growth factors to form a stabilized structure and significantly extend the half lives against denaturation and proteolysis [13,20,31]. In our case, the PEAD/heparin/growth factor coacervate possesses the same stabilized feature as we have shown using Western blotting assay in our prior work [13]. Although the new PEAD complexes heparin more tightly than the old PEAD, as we observed before, the merging process between the coacervate droplets continues after precipitating to a substrate (Supporting videos 1–4). Such droplet merging phenomenon is a typical liquid phase characteristic of a coacervate [32]. On the other hand, although the affinity between the new PEAD and heparin is stronger, the interaction between the heparin and growth factors within the coacervate stays the same, heparin will continue to stabilize the growth factors.

2.5. In vitro cytocompatibility

Because the new PEAD is more positively charged than our previous one, we therefore re-evaluated the cytocompatibility using human umbilical vein endothelial cells (HUVECs). Although the new PEAD shows the presence of some low molecular weight oligomers (Fig. 2) and miniscule organic solvent residues (Fig. S4), for typical applications, we do not need further purification. Therefore, we expect the following *in vitro* assays represent the lower limit of cytotoxicity. After the HUVECs are incubated with the PEAD solutions ranged from 0 to 10 mg/ml for 24 h, the MTT, Live/Dead and lactate dehydrogenase activity (LDH) assays are performed to evaluate the impacts on the cellular metabolism, viability and membrane integrity (Fig. 7A).

Compared to the TCPS control (0 mg/ml, cell medium alone), the MTT, Live/Dead and LDH assays demonstrate no impacts of the PEAD on the cellular metabolism, viability and membrane integrity as the PEAD concentrations are below 5 mg/ml. When the PEAD concentration reaches 5 mg/ml, the cells show a statistically significant reduction in both the cellular metabolism and the viability, but no impacts on the membrane integrity. The microscopic images of the Live/Dead cells also demonstrate similar cellular morphologies, attachment and spread on the substrate with PEAD concentration up to 5 mg/ml compared to the control (Fig. 8A–C). However, the PEAD at 10 mg/ml demonstrates high cytotoxicity and severe membrane damages, which is reflected by significantly reducing the MTT and Live/Dead values and increasing the LDH value (Fig. 7A). Few HUVECs could survive and proliferate on the TCPS substrate (Fig. 8D). These data indicate that the new PEAD below

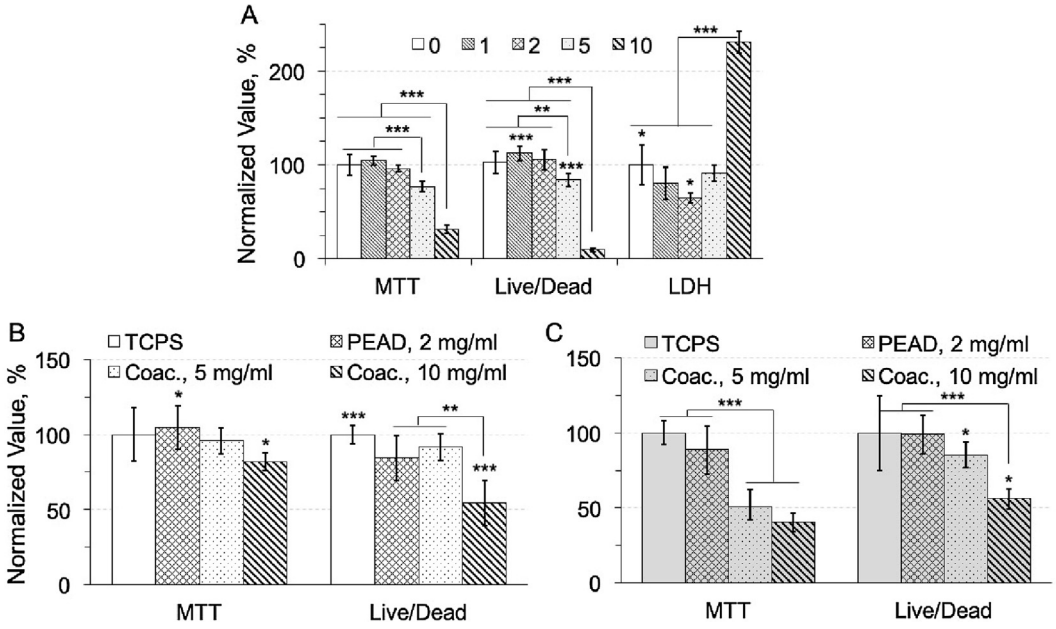


Fig. 7. (A) *In vitro* cytocompatibility of the new PEAD on HUVECs with concentration ranged from 0 to 10 mg/ml. MTT, Live/Dead and LDH assays are performed to evaluate the cellular metabolism, viability and membrane integrity respectively. At concentrations of 2 mg/ml or below, PEAD shows no toxicity. This is well above the concentration we use PEAD *in vivo*. As the polymer concentration increases to 5 mg/ml, the normalized values of the MTT and Live/Dead assays are reduced to 77 ± 5.7% and 84 ± 7.0% respectively, demonstrating statistically significant cytotoxicity. However, the LDH assay remains similar to the TCPS control (0 mg/ml), indicating no impact on the cell membrane integrity. PEAD at 10 mg/ml increased the LDH value to 231 ± 11.5%, indicating membrane damage; the MTT and Live/Dead normalized values reduced to 31.6 ± 4.2% and 9.7 ± 1.8% respectively, showing signs of cytotoxicity. $^{***}p < 0.0001$. (B and C) MTT and Live/Dead assays are used to further evaluate the cytotoxicity of 5 and 10 mg/ml coacervates by culturing with the HUVECs for 24 and 48 h. TCPS control and PEAD at 2 mg/ml are used for comparison. At 24 h, only 10 mg/ml coacervate (Coac., 10 mg/ml) significantly reduced the cell viability to 54.6 ± 15.1%, but no significant impacts on the cellular metabolism. After culturing for 48 h, both the 5 and 10 mg/ml coacervates significantly inhibited the cellular metabolism. The 10 mg/ml coacervate also significantly reduced the live cell numbers. $^{*}p = 0.0285$ and $^{***}p < 0.0001$ in Fig. 7B and C. Data represent the mean value ± SD. A p value < 0.05 is considered significantly different.

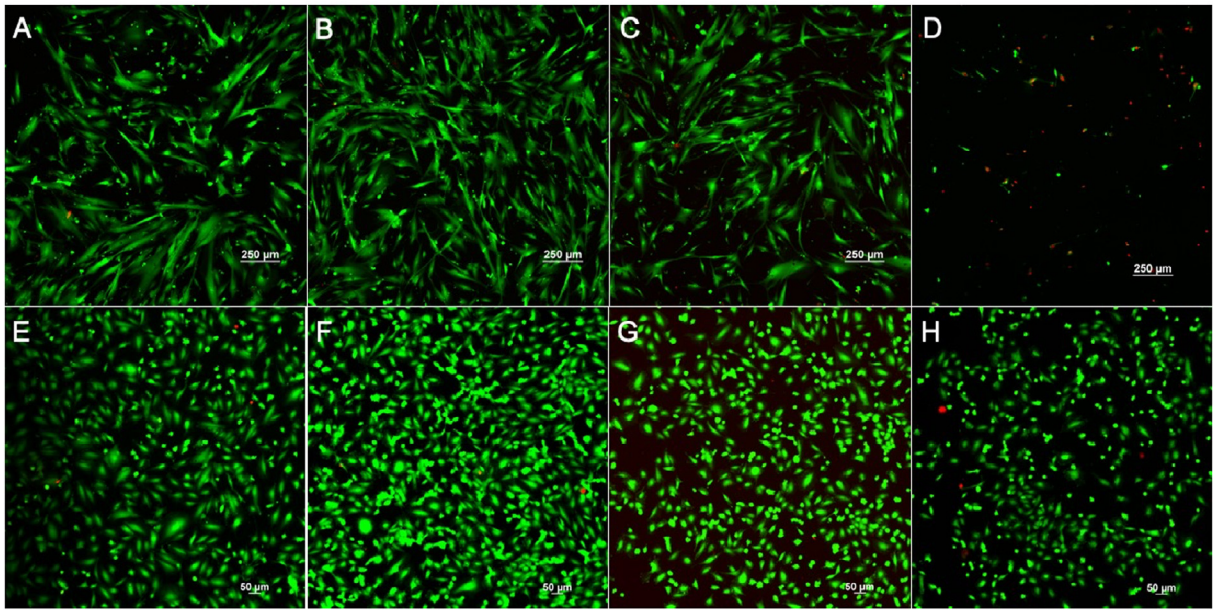


Fig. 8. Representative fluorescent microscopic images of Live/Dead HUVEC cells (green/red stains). (A–D) Cells are incubated with different PEAD concentrations for 24 h in a 96-well plate. (A) TCPS control, (B) 2 mg/ml PEAD, (C) 5 mg/ml PEAD, (D) 10 mg/ml PEAD. Scale bar, 250 μm. (E–H) HUVECs are incubated with a PEAD solution and different coacervate suspensions for 48 h in a 96-well plate. (E) TCPS control, (F) 2 mg/ml PEAD, (G) 5 mg/ml coacervate, (H) 10 mg/ml coacervate. Scale bar, 50 μm. When culturing with the PEAD solutions, only 10 mg/ml PEAD elicits severe cell death. When culturing with the coacervates, the live cell density decreases as the coacervate concentration increases. No severe cell death is observed even at 10 mg/ml coacervate. The cell morphologies, attachment and spread remain similar among them. Passage 7 and 4 HUVEC cells were respectively used for the tests with PEAD solutions (A–D) and coacervates (E-H). Therefore, the cell morphologies and spread are different between the two groups. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

5 mg/ml exhibits good cytocompatibility. For comparison, a widely used polycation, PEI, showed significant cytotoxicity at a concentration as low as 0.01 mg/ml as we previously examined on multiple cell lines including the HUVECs [10,33]. Therefore, the new PEAD is nearly 500-times cytocompatible compared to the commercially resourced PEI for biomedical applications.

It is worth noting that the above cytocompatibility is evaluated in PEAD solution, not PEAD/heparin complex coacervate. We typically make PEAD/heparin coacervate at the isoelectric point (P/H ratio at 3.6:1) for use. We therefore further evaluated the cytocompatibility of the coacervates at 5 and 10 mg/ml respectively. After injecting 100 μl of the coacervate to each cell-seeded well, the HUVECs were cultured with the coacervates for 24 and 48 h and examined by MTT and Live/Dead assays (Fig. 7B and C). TCPS control and PEAD solution at 2 mg/ml are used for comparison. Consistent with the results in Fig. 7A, the PEAD at 2 mg/ml like the TCPS control demonstrates no cytotoxicity at both 24 and 48 h. After culturing for 24 h, only the 10 mg/ml coacervate (Coac., 10 mg/ml) induces a significant reduction of the cell viability to 54.6 ± 15.1% normalized value, but no significant impact is observed in the cellular metabolism compared to the TCPS control (Fig. 7B). At 48 h, the 10 mg/ml coacervate significantly reduces the MTT value to 40.2 ± 6.2% and the viability to 56.2 ± 6.7% (Fig. 7C). Interestingly, the 5 mg/ml coacervate demonstrates no impacts on both the cellular metabolism and viability at 24 h (Fig. 7B). However, a significant reduction is observed in MTT assay at 48 h, but no significant impacts on the cell viability (Fig. 7C). Such cytotoxicity phenomena are different with the free PEAD solutions demonstrated (Fig. 7A). The Live/Dead cell images also show a cell density decrease as the coacervate concentration increases, but no severe cell death is observed even in the 10 mg/ml coacervate group (Fig. 8E–H). Additionally, compared to the TCPS control, the cellular morphologies and attachment are similar in the presence of the coacervates, except the cell density. These data indicate that the coacervate above 5 mg/ml could to some extent affect the cellular activities including the metabolism and proliferation, and thus reduce the cell viability. However, the cytotoxicity is much lower than the free PEAD solution at 10 mg/ml demonstrated.

We observed that the coacervate droplets merged into larger ones upon contacting one another and sedimented on the TCPS plate after incubating at 37 °C for 3 h (supporting videos 3 and 4). Such process is more notable in the well with 10 mg/ml coacervate. As a result, the sedimented coacervate droplets likely covered the seeded cells and occupied many non-seeded substrate surface so as to inhibit the cell spread and proliferation, particularly in the 10 mg/ml coacervate group (Fig. S5B). Thus, the 10 mg/ml coacervate treated cells significantly reduced both the cellular metabolism and viability (Fig. 7C), but did not cause severe cell death as the free PEAD solution at 10 mg/ml did (Fig. 8D and H). On the other hand, although the 5 mg/ml coacervate also sedimented in the well, the coacervate droplets did not severely merge into larger ones and just sparsely deposited on the substrate (Fig. S5A). It appears the cell spread and proliferation were not significantly hindered at the first 24 h according to the MTT and Live/Dead assays (Fig. 7B). However, after culturing for 48 h, the deposited coacervate droplets also significantly inhibited the cellular metabolism but only with a slight reduction of the cell viability (Fig. 7C).

Overall, the above *in vitro* cytotoxicity analyses indicate that the neutral coacervate is much more cytocompatible than the free PEAD when the concentration is above 5 mg/ml. According to our previous studies, we typically injected approximately 50–100 μl of 10 mg/ml coacervate to deliver proteins per animal [13,16,21,30]. When using the new PEAD, we can make a coacervate at concentration between 10 and 5 mg/ml for utility (Fig. 5). Therefore, the amount of the coacervate in our application will be well tolerated as we have previously observed in many studies. Thus, the material cytotoxicity will not be a concern for practical applications.

3. Conclusion

We have successfully scaled up the production of PEAD at 150 g each batch. The new synthesis route significantly improves the production safety, capacity and yield, and significantly reduces the cost relative to our previous method that yields gram quantities. It is worth noting that in the scale-up protocol, the reaction time and agitation of the reaction mixture dominate the polymer molecular weight and mass distribution. In addition, this new protocol could produce the PEAD bearing more positive charges due to a higher arginine conjugation efficiency and thus a stronger complexing ability with heparin for coacervation. Because of the increased polydispersity and higher positive charges, we re-evaluated the physicochemical and biological properties of the resultant PEAD for utility. The new PEAD demonstrated good cytocompatibility at concentrations ≤5 mg/ml, nearly 500-times more cytocompatible than the commercially available PEI. The as-formed PEAD/heparin coacervate possessed a more stable structure and thus demonstrated a slower release of proteins compared to the counterpart from the benchtop protocol. These should expand the release window of the controlled delivery system. The successful scale-up makes it easier to commercialize this polycation for biomedical applications. This work may also inform scale-up efforts of similar polymers.

4. Experimental section

4.1. Scale up the synthesis of PEAD

The PEAD was synthesized in four steps based on our benchtop protocol [10]. The steps 1 and 2 were to synthesize a Boc-protected poly (ethylene aspartate diglyceride) (Boc-PED) precursor and deprotect to yield the PED intermediate (Fig. 1). Specifically, in step 1, 0.600 mol of ethylene glycol diglycidyl ether (EGDE, 104.53 g) (Pfaltz&Bauer) and 0.600 mol of N-Boc-L-aspartic acid (Boc-Asp-OH, 139.94 g) (Bachem Americas Inc.) with 1.2 mmol of tetrabutylammonium bromide as catalyst (TBAB, 0.386 g) (98 + %, Alfa Aesar) were mixed in 160 ml of 1,4-dioxane anhydrous (≥99.8%, Alfa Aesar) in a 2 L three neck round bottom flask. The reaction flask was settled in a heating mantle set above a magnetic stirring plate, and connected with a condenser and a Schlenk line. The reaction solution was purged with nitrogen gas for 1 h with magnetic stirring and then heated to 100 °C for polymerization. The polycondensation was continued for 72 h, 84 h, and 96 h under vigorous magnetic stirring with mild nitrogen gas purge. At each time point, about 1 ml of the Boc-PED solution was taken and precipitated in hexanes. The molecular weight of the collected Boc-PED samples was monitored by GPC analysis and 96 h was determined to be suitable time to obtain the Boc-PED precursor with desired molecular weight. After cooling down to room temperature, 200 ml acetone (HPLC, Pharmco-Aaper) was added to dilute the viscous Boc-PED solution. The Boc-PED solution was then precipitated in about 2.5 L hexanes (99.9%, Pharmco-Aaper) in a 3 L one neck round bottom flask with magnetic stirring overnight. The supernatant was decanted and the viscous Boc-PED residue was rotary-evaporated at 50 °C and ca. 140 mbar for 15 min to further remove some solvent residue.

In step 2, the Boc-PED precursor in the 3 L flask was then dissolved in 200 ml acetone (dried with molecular sieves before use), followed by slowly adding 200 ml trifluoroacetic acid (TFA, ≥99.5% for HPLC, Alfa Aesar). The deprotection reaction was performed at room temperature for 2 h under nitrogen atmosphere with magnetic stirring. The reaction solution was then rotary-evaporated at 60 °C and 80–100 mbar for 2 h to obtain a condensed PED solution. The viscous PED solution was dispensed into two 4 L beakers for precipitation. To each beaker, 1.5 L hexanes was first added for precipitation with vigorous magnetic stirring for ca. 20 min, followed by adding 1.5 L ethyl acetate (HPLC, Pharmco-Aaper) (hexanes/ethyl acetate, 1:1 v/v). The precipitation was magnetically stirred for ca. 20 h. The supernatant was decanted and

the PED residue was dissolved in ca. 100 ml acetone in each beaker. The PED solvation and precipitation were performed additionally twice in same volumes of hexanes and ethyl acetate using the same procedure. The PED residue was then dried at 55 °C in a vacuum oven for 24 h to obtain ~180 g of a yellow solid PED intermediate with a yield of ca. 96%.

Step 3 and 4 were to conjugate N-Boc-L-arginine (Boc-Arg-OH) with the PED amine groups to yield a Boc-protected poly(ethylene argininylaspartate diglyceride) (Boc-PEAD) and deprotect to yield the resultant PEAD product. Acetone was dried with molecular sieves before use. Specifically, in step 3, 108.86 g of PED (0.354 mol based on the repeat unit) was dissolved in 225 ml acetone in a round bottom flask. 80.20 g of N,N'-Dicyclohexylcarbodiimide (DCC, 0.389 mol) (99%, Alfa Aesar) was dissolved in 300 ml acetone in a flask. In another round bottom flask, 97.10 g of Boc-Arg-OH (0.354 mol) (Bachem Americas Inc.), 40.74 g of N-hydroxysuccinimide (NHS, 0.354 mol) (> 98.0%, TCI AMERICA) and 0.433 g of 4-(Dimethylamino)pyridine (DMAP, 3.54 mmol) (≥99%, Alfa Aesar) were dissolved in 300 ml N,N-Dimethylformamide anhydrous (DMF, ≥99.9%, Alfa Aesar) with magnetic stirring and purged with nitrogen gas. The DCC in acetone solution was then slowly added to the Boc-Arg-OH/NHS/DMAP in DMF solution and vigorously stirred for 15 min to activate the carboxylic acid in Boc-Arg-OH. Then the PED in acetone solution was slowly added to couple with the activated Boc-Arg-OH. The reaction was remained at room temperature for 20 h with magnetic stirring under nitrogen atmosphere to yield the Boc-PEAD. The reaction solution was filtrated to isolate the insoluble byproduct and the dicyclohexylurea solid was washed thrice with 75 ml acetone per wash. The clear solution was collected in a 3 L round bottom flask and condensed by rotary evaporation to remove most acetone (ca. 400–600 ml). Then 1.4 L hexanes and 1.4 L ethyl acetate (1:1, v/v) were subsequently added to the condensed Boc-PEAD solution with magnetic stirring overnight for precipitation. After decanting the supernatant, the viscous Boc-PEAD sediment was washed twice with same volumes of hexanes and ethyl acetate and stirred overnight per wash to extract as much DMF residue as possible. After decanting the supernatant, the Boc-PEAD residue was briefly dried by rotary evaporation for 10 min at 60 °C and 100 mbar.

In step 4, the Boc-PEAD was then deprotected by adding 400 ml acetone, followed by slowly adding 400 ml TFA (100 ml per portion) with vigorously magnetic stirring. The deprotection reaction was remained for 2 h at room temperature with magnetic stirring under nitrogen atmosphere to yield a clear PEAD solution. The PEAD solution was condensed by rotary evaporation at 60 °C and 100–120 mbar for 2 h. The viscous PEAD solution was dispensed into two 4 L beakers for precipitation. To each beaker, the condensed PEAD solution was precipitated by subsequently adding 1.2 L hexanes and 2.4 L ethyl acetate (1:2, v/v) with vigorously magnetic stirring for ca. 17 h. The supernatant was decanted. The viscous PEAD sediment in each beaker was dissolved in ca. 100 ml methanol and precipitated again in same volumes of hexanes and ethyl acetate. Such solvation and precipitation were repeated additionally twice with magnetic stirring overnight per precipitation. The PEAD precipitates were then dried at 55 °C in a vacuum oven for 24 h to obtain ca. 153 g of a yellow solid PEAD with yield of ca. 93%.

Proton NMR analysis was performed to examine the chemical structure and composition of the resultant PEAD. 1 g of the dried PEAD was further dialyzed against 3 L of deionized water for 48 h using a dialysis tubing with molecular weight cutoff of 1 k Da. Deionized water was replaced per 24 h. The dialyzed PEAD was freeze-dried and 15 mg was dissolved in 0.75 ml of DMSO-D_6 solvent for NMR (Bruker 500 Hz) analysis.

4.2. Molecular weight analysis

Gel permeation chromatography (PG07 GPC instrument) analysis was performed by PSS-USA Inc. to determine the average molecular weight and mass distribution of the PEAD. PSS NovemaMax columns (particle size 10 μm, G, 30, 2x1000 Å, ID 8.0 mm × 300 mm) were used as stationary phase and 0.1 M NaCl + 0.1 vol% TFA was used as mobile phase with flow rate at 1.00 ml/min. Pullulan standard polymers were used for the molecular weight calibration. The PEAD sample solution was prepared at 3.00 mg/ml and filtrated through a PTFE membrane with pore size of 1 μm. The column temperature was set at 35 °C and the eluent was detected by PSS SECcurity 1260 differential refractometer RID. The PSS WinGPC UniChrom Version 8.3 software was used for calibrating the average molecular weight and polydispersity.

4.3. Zeta potential measurement

PEAD samples from the benchtop protocol (PEAD-Old) and the scale-up protocol (PEAD-New), and heparin solution were prepared in deionized water with all concentrations at 10 mg/ml. All solutions were filtered through a 0.2 μm syringe filter. 0.7 ml of each PEAD solution was loaded in a polystyrene cuvette for zeta potential measurements (Malvern Zetasizer Nano ZS90). Separately, fresh PEAD/heparin coacervate suspensions with PEAD/heparin (P/H) ratios at 4:1, 3.75:1, 3.5:1 and 3:1 were prepared. 0.7 ml of each coacervate suspension was accordingly transferred to a new polystyrene cuvette for zeta potential measurements. The measurements were replicated for at least three times for each sample.

4.4. Coacervation with heparin

PEAD and heparin solutions were respectively prepared in 0.9% saline with concentration at 10 mg/ml. Then 0.20 ml heparin solution was transferred to each polystyrene cuvette, followed by mixing with 0.80, 0.72 and 0.60 ml PEAD solution to form coacervate at 10 mg/ml with PEAD/heparin (P/H) ratio at 4:1, 3.6:1 and 3:1. The coacervate suspensions were settled for 17 h to record the sedimentation status. The completely sedimented coacervates were sonicated for 3 min to evaluate the re-dispersion ability. Similarly, the PEAD and heparin solutions at 5 mg/ml were prepared to make a coacervate with P/H ratio at 3.6:1 for comparison.

To observe the original coacervate morphologies in saline, a house-made glass reservoir was made by mounting a punched silicone rubber layer on a glass slide with 0.76 mm in thickness and 8 mm in diameter. Both 10 and 5 mg/ml coacervate suspensions were freshly prepared and 10 μl of each suspension was measured and dropped on the center of the reservoir. The drop was immediately covered with a cover slide to avoid solvent evaporation during microscopic observation from the cover slide side (Fig. S3).

To visualize the morphology of a protein-loaded coacervate, Alexa Fluor™ 647 conjugated bovine serum albumin (BSA-647, ThermoFisher Scientific) was loaded in the 10 mg/ml coacervate for microscopic observation. Specifically, 1 μl of 10 mg/ml BSA-647 was mixed with 100 μl of 10 mg/ml heparin in saline, followed by adding 360 μl of 10 mg/ml PEAD in saline to form the BSA-loaded coacervate. Then 10 μl of the BSA-loaded coacervate suspension was transferred on the house-made glass reservoir for microscopic observation. The fluorescent microscopic images were obtained using Nikon ECLIPSE Ti2 microscope.

4.5. Growth factor loading and controlled release test

PEAD and heparin solutions in 0.9% saline were prepared at 10 mg/ml respectively and filtrated through 0.2 μm syringe filter before use. The controlled release test was replicated for four times. To each Eppendorf tube, 4 μl of 50 ng/μl basic fibroblast growth factor (FGF2) (PEPROTECH, USA) was mixed with 20 μl of the heparin solution, followed by adding 72 μl of the PEAD solution and gently mixing by pipetting to form a FGF2-loaded coacervate suspension. The suspension was centrifuged at 10,000*g* for 1 min and the supernatant was collected

by pipette. The FGF2 in the supernatant was used to determine the unloaded portion and thus the loading efficiency was calculated. To the coacervate sediment, 500 µl of 0.9% saline was added for controlled release test at 37 °C. At day 1, 4, 7, 14, 21, 28 and 37, the tube was centrifuged at 10,000*g* for 1 min, and the supernatant was collected and replaced with 500 µl of fresh saline until the controlled release was complete. The unloaded and released FGF2 samples were quantified using a standard ELISA assay kit (PEPROTECH, USA) according to the manufacturer's instructions. The absorbance was recorded using a SpectraMax M3 microplate reader (Molecular Devices, LLC. USA). According to the unloaded FGF2 quantity, the loading efficiency was determined to be 99.98%.

4.6. In vitro cytocompatibility

In vitro cytocompatibility of the new PEAD was evaluated with human umbilical vein endothelial cells (HUVECs). HUVECs (Passage 7) were cultured in EGM™ −2 Basal Medium supplemented with EGM™-2 MV Microvascular Endothelial SingleQuots™ Kit (Lonza, USA) at 37 °C with 5% CO_2 until sufficient cell quantities were obtained.

Lactate dehydrogenase (LDH) assay (n = 4), Live/Dead assay (n = 6) and MTT assay (n = 6) were performed to evaluate the cell membrane integrity, viability and metabolic activity. For these assays, 5000 cells per well were seeded in 96-well plates one day before the experiments. The PEAD solutions were prepared by dissolving in the cell medium with concentrations of 10, 5, 2, 1 mg/ml and filtrated through 0.2 µm syringe filter before use. The cell medium alone on tissue culture treated polystyrene (TCPS) was used as the control (0 mg/ml).

After 24 h incubation, LDH activity in the culture medium was measured using a CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit (Promega, USA). Cell metabolic activity was determined using a Vybrant™ MTT Cell Proliferation Assay Kit (Invitrogen, USA). Live/dead assay was performed using a LIVE/DEAD™ Viability/Cytotoxicity Kit (Invitrogen, USA). The absorbance and fluorescence were recorded using the SpectraMax M3 microplate reader.

To evaluate the cytotoxicity of coacervate, heparin and PEAD solutions at 5 and 10 mg/ml were prepared in cell medium respectively, and filtered through 0.2 µm syringe filter before use. Then, 5 and 10 mg/ml coacervates were freshly prepared by mixing PEAD and heparin solutions at P/H weight ratio of 3.6:1 to form the coacervates for cytotoxicity test. HUVEC cells (Passage 4) were cultured in cell medium until sufficient cell quantities were obtained. To a 96-well plate, 5000 cells in 100 µl cell medium were seeded in each well and incubated at 37 °C for about 2 h. Then 100 µl of the coacervate was added to each cell well and incubated for 24 and 48 h. MTT and Live/Dead assays were performed to examine the cellular metabolism and viability like the above procedure (n = 6). The Live/Dead cell images were obtained using Nikon ECLIPSE Ti2 microscope.

To monitor the coacervate sedimentation and merged morphologies in the cell medium under incubation, 100 µl of the freshly prepared coacervate was injected into each well which already contained 100 µl of cell medium but without cell seeding. After incubating for 3 h at 37 °C, the coacervate morphologies were recorded by the microscope.

4.7. Statistical analysis

The results of *in vitro* cytocompatibility assays were analyzed by ANOVA with *post hoc* Bonferroni correction. A p value < 0.05 was considered significant. Data represent the mean ± standard deviation (SD).

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Data Availability

The raw data and processed data required to reproduce these findings are available by contacting with the corresponding author.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.eurpolymj.2019.05.032.

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