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Microfluidic Synthesis of Biodegradable Polyethylene-Glycol Microspheres for Controlled Delivery of Proteins and DNA Nanoparticles

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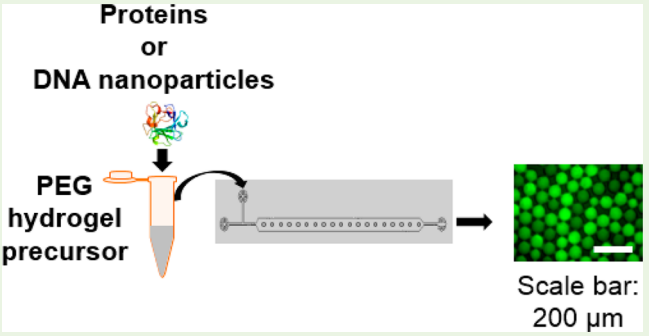
Supporting Information

ABSTRACT: Polymeric microspheres represent an injectable platform for controlling the release of a variety of biologics; microspheres may be combined in a modular fashion to achieve temporal release of two or more biomolecules. Microfluidics offers a versatile platform for synthesizing uniform polymeric microspheres harboring a variety of biologics under relatively mild conditions. Poly(ethylene glycol) (PEG) is a bioinert polymer that can be easily tailored to encapsulate and control the release of biologics. In this study, we report the microfluidic synthesis of biodegradable PEG-based microparticles for controlled release of growth factors or DNA nanoparticles. Simple changes in microfluidic design increased the rate of microparticle formation and controlled the size of the microspheres. Mesh size and degradation rate were controlled by varying the PEG polymer weight percent from 7.5 to 15% (w/v), thus tuning the release of growth factors and DNA nanoparticles, which retained their bioactivity in assays of cell proliferation and DNA transfection, respectively. This platform may provide a useful tool for synthesizing microspheres for use as injectable carriers to achieve coordinated growth-factor or DNA nanoparticle release in therapeutic applications.



KEYWORDS: *controlled release, drug delivery, biodegradable, microspheres, hydrogels*

INTRODUCTION

In tissue regeneration and repair, soluble cues such as growth factors play an important role in guiding cell fate; this activity requires well-orchestrated dosing and timing. To achieve such temporal release in vivo, polymeric microspheres have been developed that contain various biologics, such as protein or DNA nanoparticles, with well-tuned release kinetics. Homogeneous polymer matrices and microsphere uniformity are fundamental for reliably achieving the desired release kinetics. Changes in the physical and chemical characteristics of the employed polymer, as well as changes in microsphere size and morphology, may be exploited to tune the release of biological cargos.

Among the microfabrication approaches for synthesizing polymeric microparticles, microfluidics offers a relatively simple and consistent method for generating uniform microparticles, including droplet microfluidics[1−3] and flow lithography.[2] Although each approach offers unique advantages, droplet microfluidics facilitates fabrication of spherical microparticles (i.e., microspheres) or microparticles with complex chemical compositions,[3] and potentially enables higher throughput synthesis.[4] Polymeric microspheres containing multiple reagents and biologics are synthesized by adding polymer and biomolecules to the precursor solution and polymerizing the resulting microparticles.[1−3] In droplet microfluidics, microsphere size and morphology can be controlled by adjusting the input flow rates or the microchannel dimensions.[5] Prior reports have demonstrated the ability of applying microfluidic-based platforms for encapsulating multiple types of biologics[3,5,6] and cells.[7] Microspheres synthesized using microfluidic-based methods generally yield microspheres with higher homogeneity in size and drug release kinetics than microspheres generated via simple water-in-oil emulsions, and microsphere size has been shown to affect drug-release characteristics.[5,8] Other

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methods for controlling drug release include the use of core–shell structures[9,10] and stimuli-responsive microgels.[10,11] While prior reports have demonstrated the utility of such microspheres in encapsulating and release of small molecules,[5,6,9,10] applying microfluidic-synthesized microspheres for tunable release of proteins or DNA nanoparticles remain to be demonstrated, which are commonly used biological cues for controlling cell fates in situ or guiding tissue regeneration. Given such biologics are sensitive to chemical microenvironments, it is important to identify appropriate hydrogel formulations that are bioinert while supporting retention of biological activity of the encapsulated cargo during encapsulation and release process. Previous reports on releasing proteins using microparticles have generally employed polysaccharide-based formulations and used a model protein (such as bovine serum albumin, BSA) without assessing bioactivity after release.[6,12] As polysaccharide-based hydrogels are usually associated with heterogeneous polymer network, employing polysaccharide-based microparticles to achieve consistent and prolonged protein release remains unsatisfactory and a great challenge.[5,6,12]

Poly(ethylene glycol) (PEG) is a well-established hydrophilic bioinert polymer, and has been widely used to form hydrogels for a broad range of biomedical applications due to its tunable chemical and physical properties. Various approaches have explored PEG's utility as a drug-delivery platform including direct physical entrapment,[13] increasing affinity of cargo using a protein-binding molecule such as heparin sulfate,[14] or covalently linking the protein to the polymer network.[15] Physical entrapment is a low-cost and straightforward approach that generally relies on the encapsulation of a protein with a diameter comparable to or smaller than the diameter of the mesh size of the PEG hydrogel network; degradation of the PEG network releases the protein cargo. PEG matrices that have these characteristics can be polymerized via step-growth or condensation reactions using PEG precursors that have been functionalized with a hydrolytically degradable moiety. Step growth-polymerized PEG matrices can display more homogeneous network structures, which may lead to more consistent and reliable release kinetics and may have less renal toxicity because of smaller-molecular-weight degradation products.[16] Furthermore, degradation rates can be tuned by simply varying PEG concentration, which can effectively tune release kinetics.[17]

In the present investigation, we used a microfluidic device to synthesize biodegradable, PEG-based microspheres for the controlled release of growth factors or DNA nanoparticles. We have chosen PEG given its bioinert nature and well-established biocompatibility, which allows effective protection of sensitive biological cargos. We hypothesized that the release of these cargos from PEG hydrogels could be tuned by using microspheres with controlled diameters and by varying the polymer concentration to change mesh size and degradability. We therefore designed various microfluidic devices for the rapid synthesis of microparticles of different sizes. The effects of varying microsphere size and PEG concentration on release kinetics were then assessed. The biological activity of released basic fibroblast growth factor (bFGF) was assessed by assaying the protein's effect on the proliferation of adipose-derived stem cells (ADSCs), and transfection of HEK293T cells was used to evaluate the activity of DNA nanoparticles encapsulated as cargo.

EXPERIMENTAL SECTION

Materials and Methods. *Microfluidic Device Fabrication.* Microfluidic devices were fabricated using standard soft lithography and micromolding techniques in the Stanford Microfluidics Foundry clean room. A transparency mask was printed using a high-resolution printer (Fineline Imaging, USA). Master molds were fabricated with one layer of negative SU8 photoresist (MicroChem, USA) on a silicon wafer (100 μm). The microfluidic device was fabricated using polydimethylsiloxane (Sylgard 184, USA) micromolding against the master mold. Sharpened needles (20 gauge) were used to punch inlets and outlets. The molded polydimethylsiloxane layer was plasma-bonded to a glass slide that was coated with an additional layer of polydimethylsiloxane.

PEG Functionalization. Eight-arm-PEG-norbornene and 8-arm-PEG-mercaptoacetic acid were synthesized as previously described.[18] Briefly, 8-arm-PEG-mercaptoacetic acid was synthesized by dissolving 5 g of 8-arm PEG (Jenkem Technology, USA) in 150 mL of toluene (Sigma-Aldrich, USA), followed by the addition of 34 mg p-toluenesulfonic acid (Sigma-Aldrich). After adding 1.74 mL of 3-mercaptopropionic acid (Sigma-Aldrich), the solution was refluxed overnight. The azeotropic mixture was collected periodically. After cooling the solution to room temperature, the product was precipitated in ice-cold diethyl ether. Eight-arm PEG-norbornene was synthesized by reacting 8-arm PEG with norbornene acid (Sigma-Aldrich) following the procedure used for 8-arm-PEG-mercaptoacetic acid.

Microparticle Synthesis. Microparticles were synthesized using a t-junction droplet breakup microfluidic device designed with input channels of size 50, 100, 150, and 200 μm. The discontinuous phase consisted of 8 arm-PEG-norbornene and 8-arm-PEG-mercaptoacetic acid in a 1:1 stochiometric ratio, which were dissolved at 7.5% w/v, 10% w/v, 12.5% w/v, and 15% w/v). The final solutions incorporated 0.1% (w/v) lithium arylphosphanate in the discountinuous phase, which was the photoinitiator for PEG polymerization. Lithium arylphosphanate was synthesized as previously described.[19] The continuous phase consisted of 2% (w/w) PEG-perfluoropolyether-PEG in Novec 7500 (3M, USA). The PEG-perfluoropolyether-PEG triblock surfactant was synthesized as previously described.[20] The solutions were run through the microfluidic channels at ~1.0 psi/ ~ 1.0 psi (water phase/oil phase). Synthesized microspheres were collected in an oil bath with the same composition as the continuous phase positioned under a broad-spectrum, high-intensity UV lamp. Novec 7500 was extracted by mixing with Novec 7000 (5 mL per wash) (3M, USA), then removing the oil phase after each wash cycle. This process was repeated five times. Residual Novec 7000 was allowed to evaporate in a water bath at 37 °C. The resulting microparticles were diluted in 2 mg/mL L-cysteine (Sigma-Aldrich) and then lyophilized. Prior to use, microparticles were resolubilized in PBS at 2.5% (w/v).

Swelling Ratio and Mesh Size. The equilibrium degree of swelling for each hydrogel was acquired in order to calculate the PEG mesh size. PEG hydrogels (100 μL) were polymerized under UV light and their mass after swelling was measured. The hydrogels were then lyophilized and their dry mass was measured. The mass swell ratio (Q_M) was determined by calculating the ratio of the swollen hydrogel mass to dry hydrogel mass for hydrogels composed of 7.5% w/v, 10% w/v, 12.5% w/v, and 15% w/v PEG.

Hydrogel mesh size (ξ) was calculated from Q_M using the Flory–Rehner calculations as previously described.[21] First, Q_M was converted to a volumetric swell ratio (Q_V) using eq 1

$$Q_V = 1 + \frac{\rho_p}{\rho_S}(Q_M - 1) \quad (1)$$

where ρ_p is the density of the dry hydrogel (1.12 g/cm^3 for PEG) and ρ_s is the density of the solvent (1 g/cm^3 for water).

Second, the molecular weight between cross-links (M_C) was calculated using eq 2

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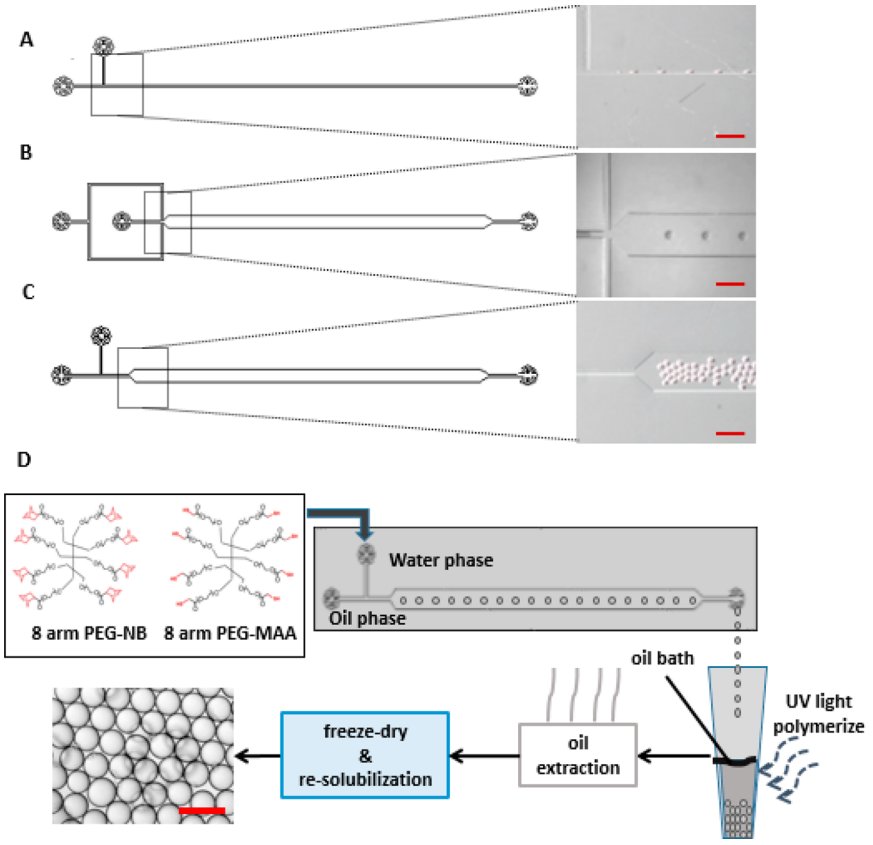

Figure 1. Schematics of microfluidic chip design for microsphere synthesis. (A–C) Design of three droplet microfluidic chips. (A, C) Two t-junction designs with differing output channel widths. (B) Flow-focusing device with a wide output channel. Snapshots of microsphere formation are shown on the right. Scale bar: 500 μm. (D) Schematic of the fabrication of PEG-based microspheres. 8-arm PEG-norbornene (PEG-NB) and 8-arm PEG-mercaptoacetic acid (PEG-MAA) were added to the continuous phase in a 1:1 stochiometric ratio. The final image shows microsphere formation after resolubilizing (scale bar: 200 μm).

$$\frac{1}{\overline{M}_c} = \frac{2}{\overline{M}_n} - \frac{\frac{\bar{v}}{V_1}(\ln(1-\nu_2)+\nu_2+\chi_1\nu_2^2)}{\nu_2^{1/3}-\frac{\nu_2}{4}} \quad (2)$$

where $\overline{M}_n$ is the number-average molecular weight of the uncross-linked hydrogel (the molecular weight of the polymer), V_1 is the molar volume of the solvent (18 cm^3/mol for water), ν_2 is the polymer volume fraction in the equilibrium swollen hydrogel ($1/Q_V$), $\bar{v}$ is the specific volume of the polymer (ρ_s/ρ_p), and χ_1 is the polymer–solvent interaction parameter (0.426 for PEG-water).[21]

Last, the mesh size was calculated using eq 3

$$\xi = \nu_2^{1/3} l C_n^{1/2} \left(2\frac{\overline{M}_C}{M_r}\right)^{1/2} \quad (3)$$

where l is the average PEG bond length (0.146 nm),[12] C_n is the characteristic ratio of PEG (4.0), and M_r is the molecular weight of the PEG repeat unit (44).

Microsphere Diameter Measurements. The average microsphere diameter was measured via image processing. Microspheres were synthesized using input channels of size 50 μm, 100 μm, 150 μm, and 200 μm. Images were taken at 10× magnification and analyzed with MatLab R2010a (The MathWorks, USA) using custom code. Briefly, images were converted to black and white based upon contiguous pixels. Microsphere area was determined by quantifying the number of pixels per microsphere and converting that value to micrometers based on the Zeiss program standards. Sphere diameter was then calculated. Ten images per group were assessed.

Protein Encapsulation and Release. Protein encapsulation was assessed by adding FITC-BSA (0.1 mg/mL, Life Technologies, USA) or bFGF (1.0 mg/mL, Peprotech, USA) to the discontinuous phase prior to microparticle synthesis. To assess the efficiency of protein encapsulation, the total pool of encapsulated FITC-BSA was collected by dissolving the microparticles with 0.1 N NaOH and balancing the pH with 0.1 N HCl and PBS. To determine the release kinetics of bFGF, we plated microparticles in the upper well of a transwell plate (Corning, USA) with 1.0 mL of PBS containing 1 mg/mL BSA in the lower well, which was the collection medium. bFGF released into the lower well was collected at various time intervals over 10 days in a 37 °C incubator, and the volume was replenished with fresh collection medium. The amount of bFGF released was quantified via enzyme-linked immunosorbent assay (PeproTech, USA).

Cell Proliferation. The bioactivity of bFGF released from microparticles was determined by assessing the mitogenic response of ADSCs exposed to these molecules. ADSCs were isolated from the abdominal fat of a female patient who had undergone a free flap breast reconstruction surgery at Stanford University, as previously described.[22] All procedures were approved and guided under the Stanford Institutional Review Board protocol. ADSCs were plated in a 96-well plate at a seeding density of 10 000 cells per well. After 24 h, the culture medium (Dulbecco's Modified Eagle Medium [DMEM], Invitrogen, USA, containing 10% fetal bovine serum [FBS], Invitrogen) was replaced with 100 μL of medium collected after bFGF was released from microparticles at various time points, mixed with 50 μL of DMEM containing 2% FBS per well, and cultured for another 48 h in a humidified chamber at 37 °C. Cell proliferation was assessed with the CellTiter AQueous One Solution Cell Proliferation Assay (Promega, USA).

DNA Nanoparticle Encapsulation and Transfection. DNA nanoparticles encapsulated in microspheres were generated by preparing DNA nanoparticles in PEG precursor solution, which was run through the microfluidic device to synthesize microspheres. DNA

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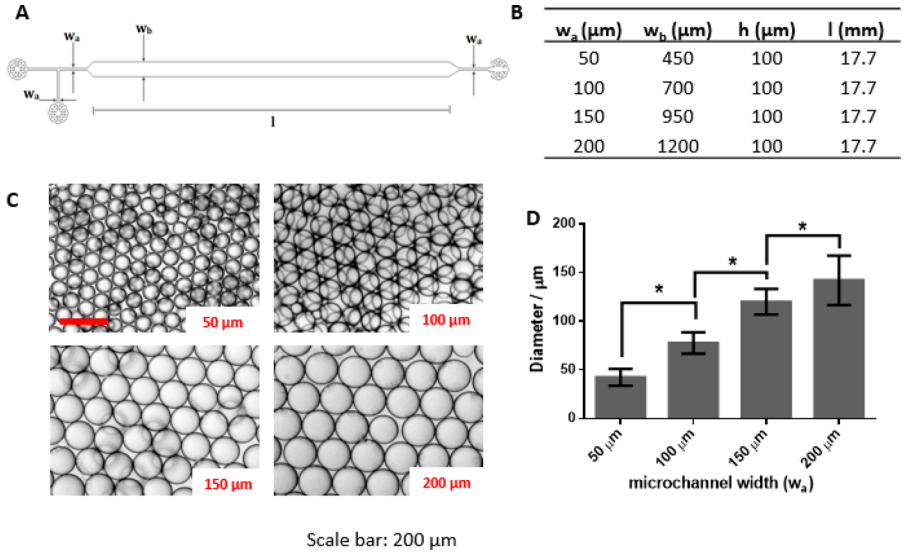

w_a (µm)	w_b (µm)	h (µm)	l (mm)
50	450	100	17.7
100	700	100	17.7
150	950	100	17.7
200	1200	100	17.7

Figure 2. Controlling microsphere size by adjusting microchip input channel dimensions. (A) Schematic of the t-junction open-channel design. (B) Dimensions of the design in A. (C) Microspheres synthesized based on the four values of w_a in B. Scale bar: 200 μm. (D) Mean diameters (n = 100) of microspheres synthesized using microfluidics chip with varying microchannel width. *p < 0.05. Scale bar: 200 μm.

nanoparticles were prepared by solubilizing in-house synthesized D32−122 PBAE polymer in 1 M NaAc as previously described,[23] then adding plasmid DNA (see below) to the resulting solution at a ratio of 20:1 PBAE polymer weight to DNA weight. After incubating the mixture for 10 min, the DNA nanoparticles were added to a PEG solution at a final DNA concentration 200 μg/mL. Microspheres were synthesized as described above and resolubilized at 3.5% w/v (70 μg/mL DNA).

Transfection was assessed by coincubating DNA-nanoparticle encapsulated microspheres with HEK293T cells in a transwell. DNA nanoparticles harboring pGluc (encoding *Gaussia* luciferase, Elim Biopharmaceuticals, USA) or pBLAST-VEGF (encoding VEGF, Aldeveron, USA) were used to assess transfection. Microspheres (200 μL with 14 μg DNA) were added to the upper well of the transwell and HEK293T cells (70 000 cells per well) were plated in the lower well cultured in DMEM containing 10% FBS and 10 ng/mL bFGF. Culture medium was collected every 48 h for up to 10 days and assayed for transfection. Luciferase transfection was assessed using the BioLux Gaussia Luciferase Assay Kit (New England Biolabs, USA). VEGF transfection was assessed with the Human VEGF Standard ELISA Development Kit (PeproTech, USA).

Statistical Analysis. GraphPad Prism v6.01 (Graphpad Software, USA) was used to perform all statistical analyses. One-way analysis of variance with Tukey's multiple comparison test as a posthoc test was performed to compare all experimental groups and to determine statistical significance of p < 0.05. All quantitative data were expressed as mean ± standard deviation.

RESULTS

Synthesis of Biodegradable PEG Microspheres. Three droplet microfluidic designs were evaluated for their ability to efficiently synthesize PEG microspheres with controllable diameter in a repeatable and consistent fashion (Figure 1). Essentially, the designs consisted of two main parts: a water-in-oil junction (either a t-junction or a flow-focusing design) and an extended output channel. This latter part was either a straight channel with the same dimensions as the input channels (Figure 1A), or an open channel that widened to roughly 7× the dimension of the input channels (Figure 1B, C). Compared to the t-junction straight channel (Figure 1A), the volume of spheres synthesized per hour in the t-junction open channel (Figure 1C) increased nearly 10-fold (input channel width of 100 μm and input pressures ∼1.0 psi/∼1.0 psi, water/oil) (Table S1 in the Supporting Information). To determine whether increasing the height of the channel further increased synthesis rates, we compared microchannels 100 μm in height with microchannels 150 μm in height; the increased height led to jetting and did not reliably generate droplets (data not shown). When comparing the two open channel designs of flow-focusing (Figure 1B) and t-junction (Figure 1C), we noted that increasing the input pressures in both designs sped the rate of microsphere formation (Table S1 in the Supporting Information). Increasing the input pressures in the flow-focusing design also increased the microsphere size, whereas attempts to generate small microspheres largely slowed the rate of microsphere formation (Table S1 in the Supporting Information). Because we wished to increase the speed of microsphere formation while minimizing the effects on microsphere size, we chose to use the t-junction open channel design in Figure 1C for subsequent experiments. In practice, the t-junction design was also easier to use and reuse.

The procedure for synthesizing monodisperse PEG-based microspheres is shown in Figure 1D and fully described in the Experimental Section. Briefly, end-functionalized 8-arm PEG polymers were solubilized in water to generate the discontinuous phase. Droplets formed via the t-junction open channel microfluidic design (Figure 1C) were collected into an oil bath and polymerized under UV light. The synthesized microspheres were oil-extracted by diluting the microspheres in a lower-molecular-weight miscible oil, which was evaporated at 37 °C. Microspheres were resolubilized in an L-cysteine solution to prevent microsphere flocculation (covalent bond formation between microspheres). The resulting solution was freeze-dried and then resolubilized in phosphate-buffered saline (PBS) prior to use.

Controlling Microsphere Diameter. To control the microsphere diameter, we fabricated four microfluidic devices based on the same t-junction design (Figure 1C) but with varying input-channel dimensions (50, 100, 150, and 200 μm) (Figure 2A, B). Using these four dimensions and running the microfluidic device with input pressures of ∼1.0 psi/∼1.0 psi (water pressure/oil pressure), microspheres with the following

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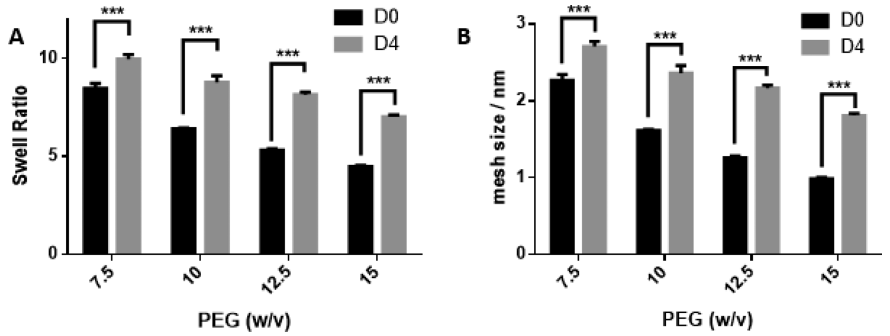

Figure 3. Increasing the PEG polymer weight percent decreased (A) the mass swelling ratio and (B) the calculated theoretical mesh size. After 4 days of incubation in serum-free cell culture medium, the mean swelling ratio and mesh size increased relative to day 0, indicating hydrogel degradation. ***$p < 0.001$, $n = 3$.

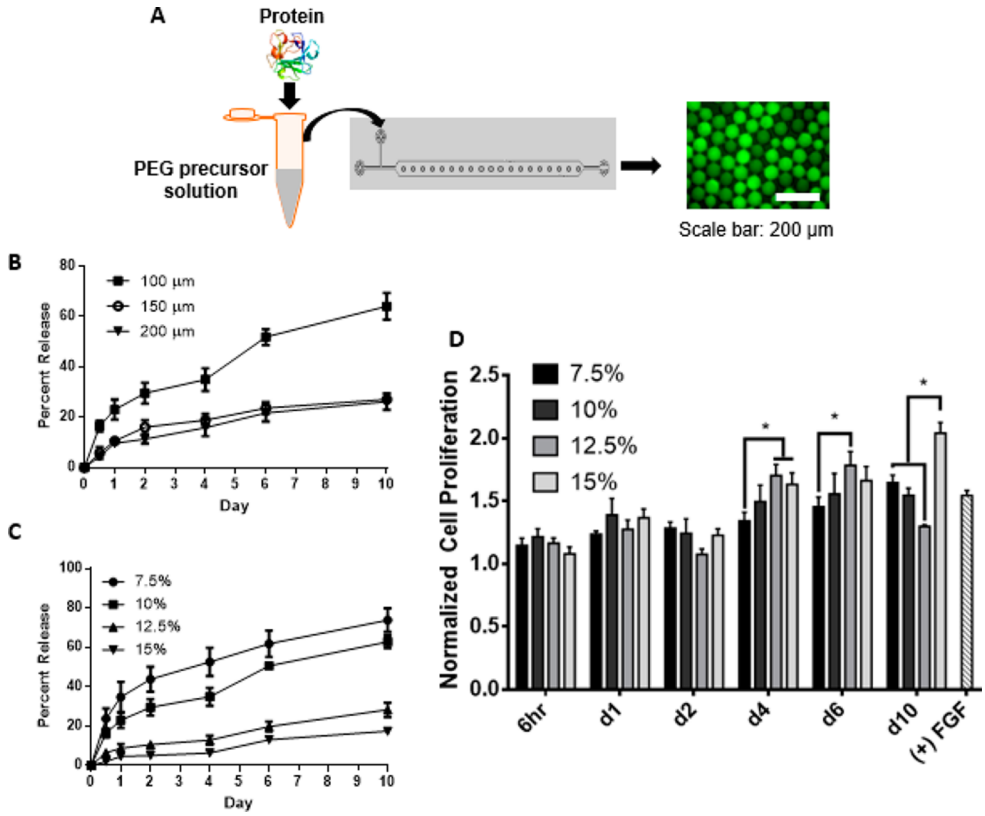

Figure 4. Encapsulation, release kinetics, and bioactivity of bFGF released from biodegradable PEG microspheres of varying size and polymer weight percent (w/v). (A) Schematic of the addition of bFGF (protein) to the PEG precursor solution and subsequent microsphere formation. FITC-BSA (green) was used to visualize protein encapsulation. Scale bar: 200 μm. (B) Microspheres with smaller sizes exhibited faster protein release (mean ± SD, $n = 3$). (C) Decreasing PEG polymer concentration led to faster protein release over 10 days (mean ± SD, $n = 3$). (D) Released bFGF over 10 days exhibited retained bioactivity, as indicated by a cell proliferation assay using ADSCs. Data was reported as fold of cell proliferation relative to cells cultured without bFGF (− control). Cells cultured with fresh bFGF (+) was included as positive control. *$p < 0.05$ (mean ± SD, $n = 4$).

mean diameters were synthesized: 42.4 ± 8.6, 77.6 ± 11.1, 120.1 ± 13.1, and 142.1 ± 25.6 μm (Figure 2C, D). The synthesized microspheres were homogeneous and their size was easily controlled by varying the dimensions of the input channel (Figure 2). Additional tuning of microsphere diameter could be achieved by varying the input pressures or flow rates (Table S1 in the Supporting Information).[24]

Controlling Microsphere Mesh Size and Degradation. To control microsphere mesh size and degradation, we varied the PEG concentration in the precursor solution from 7.5% w/v to 15% w/v (Figure 3). Two types of end-functionalized 8-arm PEG polymers with ester-containing functional groups were used. Polymerization occurred via a thiol−ene reaction, which proceeds as a step-growth reaction. Increasing the PEG concentration caused a progressive decrease in the swell ratio (Figure 3A), which corresponded with calculated theoretical mesh sizes of ~2.2−0.9 nm (Figure 3B). To confirm degradation of the PEG matrices, we measured the swell ratio after incubating for 4 days in serum-free cell culture medium. After 4 days of incubation of the PEG microspheres in serum-free cell culture medium, the swell ratio of each PEG hydrogel increased (Figure 3A), which is consistent with the bulk degradation process expected for hydrolytically degradable hydrogels. At these swell ratios, the theoretical mesh size for

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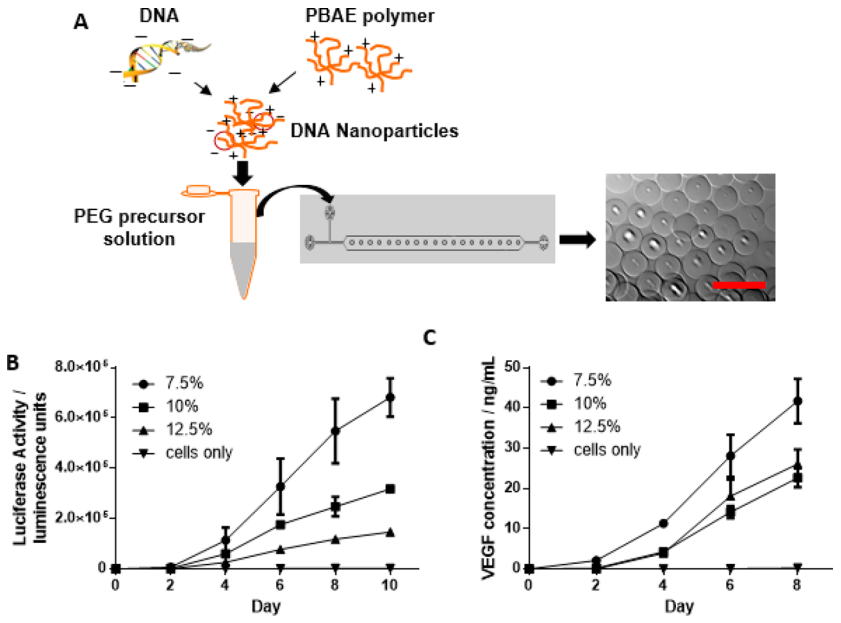

Figure 5. Bioactivity of DNA nanoparticles encapsulated and released from biodegradable PEG microspheres of varying polymer weight percent (w/v). (A) Schematic illustrating the electrostatic formation of PBAE/DNA nanoparticles in a PEG precursor solution prior to microsphere synthesis. Scale bar: 200 μm. (B) Luciferase activity in medium from the culture of HEK293T cells transfected with DNA nanoparticles encoding luciferase (mean ± SD, n = 3). (C) VEGF in medium from the culture of HEK293T cells transfected with DNA nanoparticles encoding VEGF (mean ± SD, n = 3).

each group would be expected to increase above ~2.2 nm to ~2.7 nm, except for that of 15% (w/v) PEG. As many growth factors have hydrodynamic diameter of ~2−3 nm, many encapsulated biologics should readily diffuse through these PEG matrices by day 4. The hydrodynamic diameter of bFGF has been reported to be ~3 nm (unit cell of a = 3.04 nm, b = 3.34 nm, c = 3.59 nm),[25] and the size of the polymer/DNA nanoparticles are ~100−200 nm.[26] Given these dimensions, FGF is more likely diffuse out of the PEG matrix upon degradation. On the other hand polymer/DNA nanoparticles possibly create large defects within the PEG matrix, and upon degradation they likely move through interconnected defects.

Protein Encapsulation, Release, and Bioactivity. Protein encapsulation and release was assessed using fluorescein isothiocyanate-conjugated BSA (FITC-BSA) or bFGF (Figure 4). To encapsulate protein into PEG microspheres, we added FITC-BSA or bFGF to the PEG precursor solution, then ran it through the microfluidic device (Figure 4A). FITC-BSA encapsulation was confirmed by fluorescence microscopy (Figure 4A). Qualitative differences in the amount of FITC-BSA incorporated into each microsphere was noted, and was likely due to the aggregation of BSA in solution.[27] Encapsulation efficiency was assessed using bFGF and was found to be ~80% (Figure S1 in the Supporting Information). Growth-factor release kinetics were assessed using bFGF and varying microsphere sizes and PEG concentrations (Figure 4B, C). Protein release kinetics was determined by collecting supernatant containing released bFGF over time, and measuring the amount of released protein by ELISA. Microspheres fabricated with the 100 μm channel exhibited the fastest rate of protein release, with over 60% of encapsulated growth factors released by day 10. In contrast, microspheres fabricated with 150 or 200 μm channels showed comparable and slowed protein release, with ~20% protein released by day 10 (Figure 4B). Attempts to synthesize microspheres in usable amounts using the 50 μm channels were largely unsuccessful because the rate of microsphere formation was slow and the channels tended to clog after 1 h of run time (data not shown).

Decreasing the PEG concentration generally resulted in more rapid protein release, with 7.5% (w/v) PEG-based microspheres leading to ~70% accumulated protein release by day 10 (Figure 4C). In general, smaller microspheres and those with lower weight percentages released their contents most quickly. Because of the presence of bulk degradation, we monitored our system for potential bursts in release. Around day 4, we noted subtle increases in release rates in some of the release curves. These increases were associated with the increase in swell ratio by day 4 (Figure 3). Release of bFGF persisted beyond this time point, as indicated by the release curves (Figure 4).

To confirm that the bFGF released from the microspheres remained bioactive, we incubated bFGF-containing microspheres in PBS. Supernatants were collected over multiple time points and applied to ADSCs. Cell proliferation was assayed over 10 days and results were reported as fold of proliferation relative to day 1 (Figure 4D). Compared to ADSCs with no bFGF treatment, ADSCs treated with supernatant collected from all time points containing bFGF released from the microspheres, displayed increased proliferation that was comparable to that of positive controls treated with 10 ng/mL bFGF. Consistent with our observation of a small increase in bFGF release at day 4 (Figure 4B, C), ADSC proliferation also increased around this time point (Figure 4D), providing further evidence that bFGF release was delayed before hydrogel swelling, and that the molecule remained bioactive during microsphere processing and release. Interestingly, although there was a higher amount of FGF released from 6 h to day 1, cell proliferation at day 1 remains comparable to the 6 h time point. We speculate that this seeming discrepancy may be caused by the varying degree of association of bFGF with PEG molecules/hydrogels at different time points of release. For example, bFGF released during early dissolution from the PEG

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microgel may remain partially associated or caged within PEG molecules/hydrogel structure, thereby reducing bioactivity of released bFGF. At later time points, PEG microgel is more thoroughly hydrolytically degraded, thereby facilitating higher amount of bioactivity of released bFGF.

DNA Nanoparticle Encapsulation and Transfection. To extend the possible range of therapeutic applications of our microsphere system for delivering biologics, we synthesized microspheres encapsulating polymer/DNA nanoparticles by mixing nanoparticles within PEG precursor solutions of various concentrations and flowing the resulting solution through the microfluidic chip (Figure 5A). DNA nanoparticles containing plasmids encoding luciferase or vascular endothelial growth factor (VEGF) were prepared using D32−122, an in-house synthesized poly(β-amino ester) (PBAE)-based transfection reagent at a polymer-to-DNA weight ratio of 20:1. After microsphere synthesis and resolubilization, the DNA nanoparticle concentration was 70 μg/mL of microspheres. To evaluate the bioactivity of DNA released from microspheres, we coincubated microspheres loaded with 14 μg DNA with HEK293T cells in transwell culture (microspheres in the upper well and HEK293T cells in the lower well of 2 cm^2 cell growth area). With direct delivery of PBAE DNA nanoparticles, optimal transfection is generally achieved using 5 μg of DNA per 2 cm^2 cell growth area.[28] It is important to note that DNA dosage reported in this study is not used for one time transfection, and only a fraction of the total loaded DNA nanoparticles are delivered at each time point upon controlled release. As such, the total amount of loaded DNA needs to be higher to allow prolonged release of DNA with doses high enough for efficient transfection. Culture medium was collected over 8 days, and the ability of released nanoparticles to transfect cells was validated by monitoring luciferase activity or VEGF release from HEK293T cells for up to 10 days. Our results confirmed that nanoparticles released from all microsphere formulations led to successful transfection, as shown by luciferase activity (Figure 5B) and VEGF secretion (Figure 5C) over 10 days. Furthermore, microspheres with lower PEG weight percent generally led to a higher amount of protein production by the transfected cells, indicating that gene delivery efficiency can be controlled by tuning the PEG concentration of the microspheres. Thus, tunable DNA nanoparticle release could be achieved using our microsphere platform, and released DNA remained bioactive during microsphere encapsulation and release processes.

DISCUSSION

Here we report the microfluidic synthesis of biodegradable, PEG-based microspheres for the controlled delivery of growth factors and DNA nanoparticles with tunable release kinetics. Because of the uniformity of the microspheres diameter and the homogeneity of the PEG matrices, this approach may serve as a reliable and consistent method for preparing injectable growth-factor delivery depots. Importantly, controlling microsphere properties using our strategies was relatively simple and robust, and the encapsulated biologics remained bioactive during microsphere processing and after release (Figures 4D, 5B, 5C). We controlled two microsphere properties here: the mesh size of the hydrogel (by varying the PEG concentration in the precursor solution) and the microsphere diameter (by using microfluidic channels of differing dimensions). Varying these two parameters considerably affected the rate of release of growth factors and DNA nanoparticles. Given the bioinertness of PEG and the mildness of the processing conditions, this platform may be useful for encapsulating and releasing a broad range of growth factors and other biological molecules.

The microsphere mesh size and degradation rate were easily controlled by varying the PEG concentration in the precursor solution, which affected the rates of cargo release, consistent with previous studies.[16] Implementing this strategy in a microsphere formulation would facilitate controlled delivery of multiple biologics with varying release profiles, in which separate microspheres containing different cargos could be designed with different PEG formulations. Furthermore, the mixed microsphere formulations are injectable, which can be delivered in a minimally invasive manner. We found that lower PEG concentrations led to increased release of both protein and DNA nanoparticles, whereas higher PEG concentrations prompted the opposite response. These findings demonstrate the potential for generating mixed microsphere formulations to control the release of multiple biologics, which has been challenging to achieve using bulk hydrogel formulations.[29]

PEG has many advantages over other polymers because it is easily chemically modified, providing another level of control over biologic encapsulation and release.[16] Thiol−ene chemistry enables the facile incorporation of multiple functionalities via the incorporation of biomolecules containing a free thiol. For example, L-cysteine was used in this investigation in order to harness the spontaneous covalent cross-linking that results between neighboring microparticles due to unreacted thiol groups, highlighting the potential for modifying microsphere surfaces by resolubilizing them with any other molecule containing a thiol, such as a biofunctional peptide. Similarly, functionality can be incorporated within the PEG microparticle by adding a functional peptide or biomolecule containing a thiol to the precursor solution. Thus, protein release could be further modulated by incorporating protein-binding biomolecules or even by covalently linking the growth factor directly to the microparticle.

With regards to the homogeneity of microsphere size, microfluidic set up allows robust synthesis of uniform polymeric microspheres.[2,3] Measures to ensure microsphere homogeneity include maintenance of input pressures, clear microchannels for uninhibited flow, and use of an effective surfactant. This was well observed in this study. However, addition of biologics with complex character (i.e., amphilicity, ionic charge, pH dependency, etc.), such as DNA nanoparticles, and protein, may disrupt microsphere stability.[1] To overcome this issue, we employed highly efficient polymerization (i.e., 8-arm PEG thiol−ene polymerization) and an effective water-in-fluorocarbon surfactant to facilitate rapid encapsulation of biologics such as bFGF and polymer/DNA nanoparticles.[20] Although the addition of such biologics may lead to a slight increase in the deviation of diameter, the resulting microspheres remained largely uniform, thereby demonstrating the ability to use such an approach to encapsulate complex biologics into homogeneous microspheres.

We demonstrated that simple modifications to the design of the microfluidic devices used to manufacture microspheres increased the rate of synthesis and enabled more robust control over microsphere size. We decreased the resistance of the output channel after droplet formation by expanding its width, increasing the rate of microsphere formation by more than 10-fold. To control microsphere size, we designed multiple microfluidic chips with varying input-channel dimensions. Compared to adjusting the input flow rates, varying the

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ACS Biomater. Sci. Eng. 2015, 1, 157−165

channel dimensions was more robust because relying on input flow rates requires maintaining the pressure difference between the water and oil channels throughout the whole run. Although this accuracy can be easily achieved with a syringe pump, a pneumatic controller may be more efficient when running multiple microfluidic devices in parallel, which would subject them to subtle changes in air pressure. Our approach rapidly synthesizes PEG-based microspheres of varying sizes, which may support the use of the microfluidic device as a manufacturing platform for biologics for therapeutic use. Although the present study focuses on using PEG as a polymer formulation, the reported microfluidic set up can be broadly applicable for rapid synthesis of microspheres based on other biopolymers, as long as the precursor solution is within a certain range of viscosity and can be cross-linked in an efficient manner.

CONCLUSION

In summary, here we report a droplet microfluidics platform that allows the easy synthesis of biodegradable PEG-based microspheres for the controlled release of proteins and DNA nanoparticles. The one-component approach used in this study has remarkable simplicity, which offers an advantage for downstream translation. In addition, the authors adopted a microsphere-based delivery system, which is especially suitable for subcutaneous and intramuscular administration, while allowing for more precise controls over variousformulation parameters such as loading and release. Tuning the width of the microfluidics channel enabled us to synthesize microspheres with tunable diameter, and microsphere compositions were modulated by varying the PEG concentrations of the precursor solutions. Protein cargos were released gradually from our PEG-based microspheres; these release kinetics were further tuned by varying the PEG composition and microsphere size. Given the bioinertness of PEG, our platform may be useful for encapsulating and releasing a broad range of proteins and DNA nanoparticles. Further chemical modification of PEG may yield another level of control over biologic encapsulation and release that can be easily incorporated into the present system. To achieve temporal release of multiple biologics, we could combine microparticles with different release kinetics or loaded with different cargos to provide an injectable drug delivery system for treating a broad range of diseases and enhancing tissue regeneration.

ASSOCIATED CONTENT

Supporting Information

The following file is available free of charge on the ACS Publications website at DOI: 10.1021/ab500051v.

bFGF encapsulation efficiency (Figure S1) and microsphere formation rate (Table S1) (PDF)

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Notes

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ABBREVIATIONS

PEG, polyethylene glycol; basic fibroblast growth factor, bFGF; adipose-derived stem cells, ADSCs; FITC-BSA, fluorescein bovine serum albumin; PBAE, poly(β-amino esters); pGluc, plasmid gaussia luciferase; VEGF, vascular endothelial growth factor

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