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Microgels produced using microfiuidic on-chip polymer blending for controlled released of VEGF encoding lentivectors

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Abstract

Alginate hydrogels are widely used as delivery vehicles due to their ability to encapsulate and release a wide range of cargos in a gentle and biocompatible manner. The release of encapsulated therapeutic cargos can be promoted or stunted by adjusting the hydrogel physiochemical properties. However, the release from such systems is often skewed towards burst-release or lengthy retention. To address this, we hypothesized that the overall magnitude of burst release could be adjusted by combining microgels with distinct properties and release behavior. Microgel suspensions were generated using a process we have termed on-chip polymer blending to yield composite suspensions of a range of microgel formulations. In this manner, we studied how alginate percentage and degradation relate to the release of lentivectors. Whereas changes in alginate percentage had a minimal impact on lentivector release, microgel degradation led to a 3-fold increase, and near complete release, over 10 days. Furthermore, by controlling the amount of degradable alginate present within microgels the relative rate of release can be adjusted. A degradable formulation of microgels was used to deliver VEGF-encoding lentivectors in the chick chorioallantoic membrane assay and yielded a proangiogenic response in comparison to the same lentivectors delivered in suspension. The utility of blended microgel suspensions may provide an especially appealing platform for the delivery of lentivectors or similarly sized therapeutics.

Keywords

alginate hydrogels; microgels; controlled delivery; sustained gene delivery; regenerative medicine; therapeutic angiogenesis

INTRODUCTION

Polymeric delivery systems have played an important role in modern medicine by addressing limitations associated with the systemic delivery of therapeutic agents including short half-

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DISCLOSURES

None.

lives, nonspecific tissue distribution, adverse side effects and toxicity levels.[1] In particular, hydrogel systems represent an especially appealing class of delivery vehicle as they can be introduced into the body with minimally invasive techniques and are highly biocompatible.[2] Several naturally occurring hydrogels have been successfully tested as therapeutic-releasing vehicles, including alginate.[3] Alginate is a block copolymer of (1,4)-linked β-D-mannuronate (M) and α-L-guluronate (G) residues that undergoes a gelation process that ionically crosslinks neighboring polymer chains when exposed to divalent cations such as calcium.[4] The gentle nature of the ionic alginate gelation is suitable for the encapsulation of sensitive bioactive therapeutics without harm including cells,[5] proteins,[6, 7] and genetic vectors.[8] However, the appropriate design of alginate hydrogels is required to gain control of the spatial and temporal release of these encapsulated therapeutics.

Typically, the release rate of therapeutic cargos from alginate hydrogels can be adjusted by controlling three different parameters, including 1) the hydrogel mesh size, 2) the presence of affinity interactions between alginate polymer chains and the therapeutic cargo, and 3) hydrogel degradation.[9] The hydrogel mesh serves to slow diffusive release through steric hindrance, and accordingly the degree of crosslinking and swelling can be used to alter the release of small molecule drugs and proteins.[10–12] Similarly, diffusive release can be slowed through the presence of affinity interactions, and alginate hydrogels have demonstrated affinity for cationic and heparin-binding drugs.[6, 13, 14] Conversely, hydrogel degradation can promote release of therapeutics that are sufficiently entrapped by steric hindrance and/or strong affinity interactions.[15, 16] These mechanisms provide avenues for manipulating release rates, yet achieving steady release can be challenging. Release is commonly characterized by an initial burst followed by periods of prolonged retention.[9] This is especially true for larger therapeutics such as genetic vectors where release from hydrogels is heavily dependent on degradation.[8, 17, 18] In some applications, it can be desirable to provide both up-front burst-like release in addition to steadier and continued therapeutic delivery. For instance, cellular turnover in diseased hypoxic tissue may motivate this pattern of delivery for proangiogenic genes in therapeutic angiogenesis.

Alginate microgels may provide a tool for tailoring release profiles by combining distinct formulations into a composite injectable suspension. In comparison to the uniform content of macroscopic hydrogels, this may provide a sum of parts approach to manipulate the overall release from a microgel suspension. While to our knowledge such composite suspensions have not yet been described, we recently reported on strategies for generating alginate microgels of controlled size and morphology using microfluidic templating.[17] These microgels were used to encapsulate lentivectors, a viral vector that is effective for gene delivery because of wide tropism, infectivity for both dividing and non-dividing cells, and low immunogenicity.[19–21] The large particle size of lentivectors limits their release from alginate hydrogels, and consequently, modifications in the physiochemical properties of alginate are needed to further promote release.[8, 17] Here, we hypothesize that alginate hydrogel physiochemical properties can be tailored to promote lentivector release with steadier profiles being achievable though creating composite suspensions of distinct microgels.

In this study, we describe a strategy for generating a suspension of microgels composed of linear gradients of different alginate formulations. We then verify how this approach can modulate the release profile of encapsulated lentivectors. First, we demonstrate how a microfluidic gelation system can be used to blend two distinct alginate formulations on-chip and in real time to create a composite suspension of microgels. This blending system was then employed to assess the effect of two key variables on lentivector encapsulation and release, namely alginate content and degradability. We then demonstrate the capacity of degradable microgels to deliver lentivectors encoding vascular endothelial growth factor (VEGF) and promote vascularization. In doing so, we have established the utility of blended microgel suspensions in controlled release applications and, specifically, we have produced a degradable alginate microgel formulation capable of promoting a proangiogenic response.

MATERIALS AND METHODS

Microfluidic device fabrication

Microfluidic devices were fabricated using standard soft lithography as previously described. [17, 22, 23] Briefly, polydimethylsiloxane (PDMS) elastomer (Sylgard, Auburn, MI, USA) was poured over SU-8 master molds prepared on 4-inch silicon wafers (Microchem, Westborough, MA, USA), degassed, and cured for 1 hour at 70 °C. Microfluidic channels were created by bonding individual PDMS replicas to glass slides using O_2 plasma surface treatment. The hydrophobicity of the microfluidic channels was assured through the application of Aquapel™ solution for 30 seconds. For all microgel generation experiments, the flow focusing channel dimensions (H × W) were 100 × 100 μm.

Lentivectors

The plasmids pCHMWS-eGFP-T2A-FLuc (transfer vector driving Green Fluorescent Protein (GFP) and luciferase (Luc) reporter genes from a universal immediate early CMV promoter, psPAX2 and pCMV-VSV-G (Addgene, Cambridge, MA, USA) were used to produce the lentivectors. Viral vector production, concentration, and titration were performed following established protocols.[19] In brief, lentivectors were produced in human embryonic kidney (HEK-293T) (ATCC, Manassas, VA, USA) cells cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Waltham, MA, USA) with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin-streptomycin (Invitrogen) (DMEMc) at 37 °C and under 5% CO_2. HEK-293T cells were transfected using the calcium phosphate co-precipitation method. To determine the viral titer, which we express herein as lentiviral TU/mL, HEK-293T cells were transduced with different concentrations of lentivectors. After 3 days, the HEK-293T cells transduced with lentivector expressing GFP were counted (> 10,000 events analyzed) using a FACScan cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and the data was analyzed using FlowJo software (TreeStar Inc., Ashland, OR, USA).

Binary molecular weight alginate formulations

For all studies, alginate hydrogels and microgels were prepared from a consistent bimodal molecular weight distribution of low and high molecular weight polymers combined at a mass ratio of 75:25. PRONOVA™ ultrapure MVG alginate (Novamatrix, Sandvika,

Norway) was used as the high molecular weight component. Low molecular weight alginate was obtained by (Y)-irradiation of MVG alginate as previously described.[6, 24] Alternatively, Protanal LF10/60 and LF20/40 (Novamatrix) alginates were substituted for use as the low and high molecular weight components for fluorescently-labelled studies and were oxidized for degradation studies. The polymers used in these studies are comparable in molecular weights (HMWs~250 kDa and LMWs~120–150 kDa) and relative guluronic acid and mannuronic acid content.[25] Alginate solutions were prepared fresh for each experiment by dissolving overnight in 0.9% NaCl at room temperature.

Preparation of degradable alginate via partial oxidation

Alginates (LF10/60, LF 20/40) were partially oxidized (5% of the sugar residues) to generate a hydrolytically degradable polymer by following a previously described protocol.[15] Briefly, 1% alginate solutions (2.0 g, 10.3 mmol uronate) were oxidized with sodium periodate (Sigma Aldrich, St. Louis, MO, USA) by keeping reaction in the dark for 17 hours at room temperature. The reaction was stopped by adding an equimolar amount of ethylene glycol (Fisher Scientific, Hampton, NH, USA) for 30 minutes. at room temperature. Sodium chloride (5 g) (Sigma Aldrich) was added to the solution followed by precipitation with an excess amount of ethyl alcohol (KOPTEC). Alginate was redissolved in deionized water and again precipitated with ethyl alcohol. Finally, the precipitate was redissolved in deionized water and purified *via* ultrafiltration in Amicon Stirred Cells (Millipore, Burlington, MA, USA) against deionized water. The extent of oxidation could be quantified by measuring the number of aldehydes in these polymers using a t-butyl carbazate (Sigma Aldrich), as previously described.[26] The partially oxidized alginate, which we now refer to as degradable alginate, was then filtered (0.22 km, Millipore), lyophilized, and stored at 20 °C until use.

Fluorescent labelling of alginate

Alginates (LF10/60, LF 20/40) were labelled with either Alexa Fluor® 350 hydrazide (AF350) or Alexa Fluor® 555 hydrazide (AF555) (Thermo Fisher, Waltham, MA, USA), using aqueous carbodiimide chemistry. For both, a 1% solution of alginate was prepared in 0.1 M MES buffer (Sigma Aldrich) to which 1-ethyl-(dimethylaminopropyl) carbodiimide (EDC) (Sigma Aldrich), N-hydroxysulfosuccinimide (Sulfo-NHS) (Thermo Fisher) and the fluorescent label were added and stirred in the dark for 18 hours at room temperature. Unconjugated label was removed by ultrafiltration against deionized water. The purified alginate-AF350 and alginate-AF555 conjugates were then filtered (0.22 km, Millipore), lyophilized, and stored at 4 °C until use.

Rheologic characterization of alginate hydrogels

Rheological measurements were performed using a Discovery HR2 hybrid rheometer (TA instruments, New Castle, DE, USA) with an 8-mm parallel-plate geometry in strain sweep mode. Hydrogel disks crosslinked with calcium chloride ($CaCl_2$) (Sigma Aldrich) were prepared by casting alginate solutions into a mold (8 mm diameter, 1.5 mm height) sandwiched between dialysis membranes (SpectrumLabs, 3 kDa MWCO, Rancho Dominquez, CA, USA). The alginate molds were immersed in $CaCl_2$ (100 mM) overnight to allow for full crosslinking, washed twice in phosphate buffered saline (PBS) (Invitrogen),

and swollen in PBS for 6 hours before use. All measurements were obtained within the linear viscoelastic region which was determined via frequency and strain sweep analysis of fully crosslinked hydrogels ($n = 8$).

Swelling and degradation of alginate hydrogels

For swelling studies, alginate disks were prepared as described above. Excess PBS was blotted from the hydrogels and the wet masses were recorded. Hydrogels were then frozen at −20 °C for 24 hours and lyophilized after which the dry masses were obtained. The degree of swelling, Q, was defined as the reciprocal of the polymer volume fraction in the hydrogel (v_2):

$$v_2 = \frac{1}{\rho_P}\left(\frac{Q_m}{\rho_w} + \frac{1}{\rho_P}\right)^{-1} \quad (1)$$

$$Q = {v_2}^{-1} \quad (2)$$

Here, ρ_P is the density of alginate (1.515 g cm^{-3}), ρ_w is the density of water, and Q_m is the swelling ratio of wet mass over dry mass for the microgels ($n = 6$).[24]

For degradation studies alginate disks were prepared as described above but cast under sterile conditions within molds of 1.6 cm diameter and 0.5 cm height. These disks were collected and washed in PBS. Hydrogel disks were then immersed and incubated in DMEMc at 37 °C and 5% CO_2. At 0, 1, 3, 5, and 10 days the hydrogels were lyophilized and their dry weights were recorded ($n = 3$ per timepoint).

Generation of alginate microgels

Alginate microgels were prepared using external gelation of monodisperse w/o emulsions generated using a microfluidic flow-focusing device as previously detailed. [17] Flow focusing was achieved using light mineral oil (Sigma Aldrich) with 2% SPAN 80 (Sigma Aldrich) as an immiscible carrier phase. Downstream of the emulsifying junction, $CaCl_2$ infused mineral oil replaced the carrier phase. Flow rates were 1.5, 3, and 9 kL/min for the aqueous, mineral oil, and calcium infused mineral oil, respectively. The microgels were then collected by centrifugation at (10 rcf) for 3 minutes. For the studies involving incubation at 37 °C and/or lentivector encapsulation, all solutions were sterile filtered and the entire process was carried out in sterile conditions. Microgels were imaged using fluorescent and phase contrast microscopy (Zeiss Axio Vert A.4, Oberkochen, Germany), and the resulting images were analyzed using Image J (NIH, Rockville, MD, USA).

Microgels from on-chip polymer blending

Blending of polymers on-chip was achieved by pumping two distinct alginate solutions into separate aqueous inlets under the control of individual syringe pumps. The two inlet flows pass through a short mixing channel prior to emulsification. The total aqueous flowrate is held constant per a simple summation, $Q_T = Q_1 + Q_2$, where Q_T is total flow rate and Q_1 and Q_2 are the flow rates of the first and second alginate streams respectively. The range of

possible microgel formulations that can be produced therefore spans the two extremes of alginate solution mixing (from $Q_T = Q_1$ through $Q_T = Q_2$). To create gradient blends of microgels one syringe pump was ramped up from 0 to 1.5 μL/min while the opposing syringe pump was ramped down from 1.5 to 0 μL/min.

On-chip microgel blending with fluorescently labelled alginates

Two fluorescently labelled alginate polymers (i.e. alginate-AF350 and alginate-AF555) were reconstituted to 1% solutions and used for on-chip polymer blending to create microgels with distributions of these two fluorophores. Alginate-AF350 was ramped down from 1.5 to 0 μL/min and alginate-AF555 was ramped up from 0 to 1.5 μL/min over a microgel collection period of 2 hours. The fluorescent intensities of the microgels were quantified via fluorescent and phase-contrast image analysis. The 8-bit intensity values were normalized to maximum and minimum values, and their distribution was analyzed using linear regression.

Blending of alginate concentration for lentivector release

Alginate-AF350 and alginate-AF555 were reconstituted to 1 and 2% solutions respectively. These solutions were then used for on-chip polymer blending to create microgels with distributions of both alginate concentration and the two fluorophores. 1% alginate-AF350 was ramped down from 1.5 to 0 μL/min and 2% alginate-AF555 was ramped up from 0 to 1.5 μL/min over a microgel collection period of 2 hours. The microgels were collected and swollen in PBS for 3 hours at 37 °C. The fluorescent intensities and volumes of the resulting gels were then quantified from fluorescent and phase-contrast images and their distribution was analyzed using linear regression. Alternatively, lentivectors were encapsulated within microgels of either 1%, 2%, or 1–2% blended alginate concentration. Lentivectors were mixed into 1% alginate and 2% alginate precursor solutions. These lentivector-loaded alginates were formed into microgel suspensions of purely 1% or 2% alginate using constant flow rates of these respective solutions. A lentivector-loaded microgel suspension containing a blend of 1 to 2% alginate concentration were prepared using the on-chip blending procedure.

Blending of alginate degradative behavior for lentivector release

Two alginate formulations were reconstituted overnight as 2% w/v solutions under sterile conditions. These included alginate-AF350 which was termed as nondegradable, and 5% oxidized alginate which was termed as degradable. These solutions were then used for on-chip polymer blending to create microgels with distributions of both degradable alginate content and AF350 fluorescence in the resulting suspension. Microgel suspensions of purely nondegradable and purely degradable formulations were prepared alongside the degradation blended microgel suspensions. These three microgel suspensions were then incubated at 37 °C and samples were taken at 0, 3, 5, and 10 days. Suspension samples were imaged using fluorescent and phase contrast microscopy and the resulting images were analyzed using Image J. For each time-point, the fluorescent intensities of more than 400 microgels were obtained and normalized using the average intensity of the nondegradable microgels and the average intensity of the image backgrounds. Alternatively, lentivectors were encapsulated within microgels of either nondegradable, degradable, or degradation-blended alginate concentration. Lentivectors were mixed into nondegradable alginate and degradable

alginate precursor solutions. These lentivector-loaded alginates were formed into microgel suspensions of purely nondegradable or degradable alginate using constant flow rates of these respective solutions. Lentivector-loaded microgel suspensions containing a blend of degradable alginate content were prepared using the on-chip blending procedure.

Quantification of Lentivector release by ELISA

For lentivector release studies, lentivector-loaded microgels were prepared as described above. The resulting suspensions were aliquoted into DMEMc samples (500 μL) containing microgels (5 μL, total TU=4×10^5) and incubated at 37 °C and 5% CO_2 to allow for release ($n = 3$). At 1, 5, and 10 days, microgels were pelleted *via* centrifugation and the supernatant was collected and stored at −20 °C. A fresh aliquot of medium was then used to resuspend the microgels. The lentivector concentrations of the supernatant samples were determined in terms of the p24 capsid protein as measured by a HIV p24 Antigen ELISA kit (Zeptomatrix Co, Buffalo, NY, USA) per the manufactures guidelines.

Transduction efficiency of lentivector-loaded alginate microgels

Transduction studies were carried out using a consistent number of HEK-293T cells seeded in 24-well plates and cultured in DMEMc for 24 hours 37 °C and 5% CO_2 prior to exposure to lentivector-loaded microgels. Lentivectors were encapsulated in microgels formed from degradable alginate, nondegradable alginate, or a linear blend of these solutions (final concentration of 8×10^4 TU μL^{-1}). Microgels (10 μL) were suspended in DMEMc (250 μL) (total multiplicity of infectio$n = 25$) and added to the wells of HEK-293T cells ($n = 5$). After 12 hours, an additional DMEMc (750 μL) was added to each well. Following 48 hours of culture time the microgels were collected, pelleted by centrifugation, resuspended in DMEMc (250 μL) and added to a fresh monolayer of HEK-293T cells. The transduction efficiency for each set of cells was determined qualitatively via fluorescent microscopy and quantitatively using flow cytometry (ThermoFisher Attune Nxt) to count GFP-positive cells (> 25,000 events analyzed/experimental condition).

Chick Chorioallantoic Membrane (CAM) Assay

The *in vivo* activity of VEGF lentivectors released from alginate microgels or in suspension was assessed via a modified open-shell CAM assay.[25, 27] Fertilized hyline white leghorn chicken eggs (E0) purchased from the UC Davis Avian Facility were incubated horizontally at controlled temperature and humidity (37.8 °C with 55–65% humidity) and six rotations per day for 3 days. After 3 days (E3), the eggs were released from their shells into 88.9 × 88.9 mm weigh boats (Fisher Scientific). Under aseptic conditions, the embryos were then incubated at 37.8 °C and 60% humidity. On day 11 (E11), treatments and blank groups were placed on each CAM within silicone rings of 8 mm inner diameter. VEGF-GFP lentivector treatments (TU=6.25×10^5) were applied either in suspension, or encapsulated within microgels composed of a single formulation of nondegradable and degradable alginate mixed at a 10:90 ratio ($n = 5$). Recombinant human VEGF (R&D) was applied in suspension (100 ng/mL) as a positive control ($n=2$). Pictures were taken of the region inside the ring at days 11 and 14 with a 12-megapixel camera (Galaxy S7 edge; Samsung Inc.). The angiogenic effects were quantified by manually assessing the number of blood vessels within this 4mm radius region as previously described.[28] The percent change between day

3 and day 0 in the number of blood vessels over 24 hours was calculated for each CAM and was normalized to blank controls. A two-tailed paired Student's t-test was used for the *in vivo* CAM assay to determine if each treatment factor led to an increase in vasculature.

Statistical analysis

All statistical analyses were performed using Student t-tests (two-tail comparisons) or one-way analysis of variance (ANOVA) with post hoc Tukey's test unless stated otherwise, and analyzed using Prism 7 software (Graphpad, La Jolla, CA, USA). Differences between conditions were considered significant if $P < 0.05$. Linear regression analysis was performed using Prism 7 software.

RESULTS

Microgels from on-chip blending of alginate formulations

In this study, we generated suspensions of alginate microgels with gradient compositions of two distinct polymer formulations. This was achieved by the on-chip blending of alginate formulations upstream of emulsification and gelation (Fig. 1). Two distinct alginate solutions were pumped through a microfluidic flow-focusing device with flow rates that were inversely and linearly ramped using programmable syringe pumps (Fig. 1A). Over the time course of blending and microgel production, the formulation of the microgels being produced shifted from that of the first alginate to the second.

The feasibility of this blending scheme was first validated by blending 1% solutions of alginate polymers labelled with two distinct fluorophores Alexa Fluor® 350 hydrazide (alginate-AF350) and Alexa Fluor® 555 hydrazide (alginate-AF555). Fluorescent images of the mixing lane acquired over the timespan of blending depict how the linear ramps in flow rate lead to gradual replacement of alginate-AF555 with alginate-AF350 (Fig. 1B). The microgels produced are monodisperse in volume (coefficient of variation, (CV)=0.22, CV=0.16 coalescences excluded) (Fig. 1C, E). Decisively, the blended scheme produced a suspension of microgels spanning a range of formulation as revealed by the spectrum of red (alginate-AF555) to blue (alginate-AF350) microgel fluorescent intensities (Fig. 1D). We plotted AF350 vs. AF555 fluorescence to determine if an even and linear distribution was present in the suspension. The alginate-AF555 and alginate-AF350 fluorescent intensities are linearly related with a slope near −1 which reflects the linear blending scheme ($Y = -0.94X + 0.84$) (Fig. 1F). There is an even distribution of fluorescent intensities within each microgel suggesting that sufficient mixing is occurring prior to full gelation (Supplemental Fig. 1). However, to further promote sufficient and even mixing of the two alginates it is possible to extend the length of the mixing channel prior to droplet formation and exposure to $CaCl_2$.

Blending of alginate content and lentivector release

We varied the alginate content of hydrogels and microgels from 1 to 2% to adjust mechanical properties and observe any effects of these adjustments on lentivector release. First, the effect of alginate percentage on hydrogel mechanical properties was demonstrated for disks of 1, 1.5, and 2% alginate. Increasing alginate content led to stronger hydrogels

with increased elastic behavior. Rheological characterization of these hydrogel disks yielded storage moduli of 9.0±2, 23±6, and 36±10 kPa for 1, 1.5, and 2% alginate respectively (Fig. 2A). Similarly, degrees of swelling of 58±6, 45±4, and 39±1 for hydrogel disks of 1, 1.5, and 2% alginate indicate a greater extent of crosslinking with increasing alginate content (Fig. 2B). From the swelling and storage modulus data the mesh size of these hydrogels is estimated to be 35 nm, 24 nm, and 22 nm for 1, 1.5, and 2% alginate.[17, 29–32]

These adjustments in mechanical properties using alginate percentage were then applied to microgels. A suspension of microgels was produced using on-chip blending of a 1% solution of alginate-AF350 and a 2% solution of alginate-AF555. The resulting microgels therefore contained fluorescent signatures in the blue and red wavelengths that were coupled to alginate content (Fig. 2C). Similar swelling behavior to macroscopic hydrogels was observed for microgels over this range of polymer content. Raising alginate content led to microgels with lower swollen volumes as shown by the inverse correlation between volume and AF555 fluorescent intensity. Conversely, increased volumes for lower alginate content microgels were observed in the direct correlation between volume and AF350 fluorescent intensity (Fig. 2D).

Lastly, lentivectors were encapsulated within microgel suspensions of 1%, 2%, or an on-chip blend of 1 to 2% alginate. Despite the marked difference in material properties between 1 and 2% alginate, a relatively small difference was observed in the profiles of lentivector release over the course of ten days. A total release of 40±10% of lentivectors were released from 1% alginate microgels whereas 32±4% and 32±8% were released from the 2% and on-chip 1–2% blended microgels respectively (Fig. 2E).

Blending of alginate degradability and lentivector release

To promote the release of encapsulated lentivectors we constructed degradable hydrogels and microgels. An alginate susceptible to hydrolysis was prepared via partial oxidation of the polymer backbone, and here we refer to this polymer as degradable alginate. Increasing the content of degradable alginate in hydrogels had a marked impact on the initial mechanical integrity. 2% alginate hydrogels composed of nondegradable alginate, degradable alginate, or a 50:50 mixture of these two alginates yielded storage moduli of 36±10, 2.1±0.3, and 14±2 kPa respectively (Fig. 3A). From swelling and storage modulus data the mesh size of these hydrogels is estimated to be 82 nm, 33 nm, and 22 nm for degradable, 50:50, and nondegradable alginate.[17, 29–32] Even a small fraction of nondegradable alginate led to a marked increase in hydrogel mechanical properties as disks composed of a 10:90 ratio of nondegradable to degradable alginate exhibited a storage modulus of 7.2±0.7 kPa (Supplemental Fig. 1A). Furthermore, the degradation behavior of hydrogel disks correlated with the content of degradable alginate. Alginate hydrogels prepared with degradable alginate lost polymer weight over time when incubated in culture media with the most significant weight loss occurring in hydrogels of purely degradable alginate (Fig. 3B, Supplemental Fig. 1B). In contrast, nondegradable hydrogels showed no weight loss over 10 days. Indeed, a marginal increase in weight was observed and is attributable to adsorption/entrapment of protein from the DMEMc incubation media within the alginate disks.

Microgels were successfully generated using both nondegradable alginate (alginate-AF350) and degradable alginate (non-fluorescent). Additionally, an on-chip blend of these two alginates was produced and yielded a range of formulation from fluorescent and nondegradable to non-fluorescent and degradable as visualized by AF350 fluorescence and brightfield imaging (Fig. 3C–F). Degradable microgels were visibly falling apart after 1 day, and no intact microgels remained after 10 days. In comparison, microgels of the solely nondegradable suspension persisted intact for 10 days. For the blended suspension, there was initially a wide distribution of microgel fluorescent signatures, however after 10 days only microgels with some measurable amount of fluorescence persisted. Of these, the microgels with lower amounts of fluorescence could be seen in the process of falling apart (Fig 3E). Analysis of the fluorescent intensity frequency distribution of the microgel suspensions showed that, over 5 days, there was a decrease in the amount of microgels persisting intact within the lower extremes of fluorescent intensity (Fig. 3F).

Increasing the degradable alginate content of microgels drastically promoted lentivector release. Lentivectors were encapsulated within microgel suspensions of nondegradable, degradable or an on-chip blend of these two alginates. After 1 day, degradable and blended microgels had released significantly more of the total encapsulated lentivectors (72±20% and 49±7%) than nondegradable microgels (22±3%) as measured by the p24 capsid protein (Fig. 4A). Furthermore, while the nondegradable microgel release plateaued following the first day, the degradable and blended suspensions provided continued release out to 5 days. The blended suspension released a further 29% between days 2 and 4, whereas the degradable suspension released 16%. After 10 days, the degradable suspension had released 89±9%, the blended suspension 80±10%, and the nondegradable suspension 27±1% of their total encapsulated contents. The released lentivectors retained functionality as seen by GFP transduction in HEK-293T cells exposed to microgel suspensions (Fig. 4B). Microgel suspensions were continuously moved to freshly seeded cells every 2 days capture the ability of released lentivectors to transduce GFP. Here, the percentage of transduced HEK-293T cells at each time point reflects the trends in lentivector release quantified by ELISA (Fig. 4C). While degradable microgels produced the highest levels of GFP expression in the first 2 days of release, the blended microgels produced higher levels of expression for the remaining timeframe of release. Again, the incorporation of small fractions of nondegradable alginate led to a reduction in GFP expression in comparison to purely degradable microgels or a continuous blend (Supplemental Fig. 1C–D).

Angiogenesis induced by degradable microgels releasing VEGF-GFP lentivector

Lastly, the proangiogenic potential of microgels loaded with lentivectors encoding for VEGF was probed via a CAM assay. Here, the results of blending degradation behavior were used to identify a formulation of microgels that would degrade within the time window of the CAM assay. The results of Figure 3 identified a transition to larger amounts of degradation for the bottom 20% of nondegradable alginate content. Consequently, and to ensure degradation, a uniform suspension of 90:10 microgels was used to provide suitable lentivector release rates for the 3-day long assay. Overall, microgels loaded with VEGF lentivectors demonstrated a significant increase of 21±5% blood vessel formation in comparison to blank control groups, and the magnitude of this response was similar to the

positive control group of rhVEGF (22±6%). Interestingly, VEGF lentivectors in suspension produced no significant increase in blood vessel formation (0±5%) compared to blank treatments.

DISCUSSION

Microgels present an opportunity to more precisely control therapeutic release in comparison to macroscopic hydrogels of uniform composition. The breakup of a macroscopic, microliter-scale, volume of hydrogel into picoliter-scale parts allows a bottom-up approach for engineering an overall release profile. Microgels with varying release behavior can be generated by identifying and manipulating hydrogel parameters that significantly affect therapeutic release from encapsulation. These microgels can then serve as building blocks, where an overall profile of release is constructed by the summed release behavior of each individual microgel. In this study, we sought to develop a simple strategy for creating microgels of varied composition and then applied this approach towards the delivery of lentivectors. The results of this study demonstrate how multiple formulations of alginate microgels can be combined in a single suspension to obtain control over lentivector release kinetics for gene delivery or to probe how a given hydrogel parameter affects release. We used the blending procedure to identify a formulation of microgels specifically suitable for the CAM assay and demonstrated how this approach can promote the delivery of therapeutically relevant genes.

We have described how the blending of two alginate formulations upstream of gelation in a microfluidic templating system, a process we have termed as on-chip polymer blending, can produce a range of alginate microgels in a single suspension. This process has corollaries to previous studies that have produced hydrogels with gradients of encapsulated particles,[33] biochemical cues,[34] and mechanical properties.[35, 36] We employed on-chip polymer blending with a simple mixing scheme where the total flow rate of alginate gradually transitions from one alginate formulation to a second. Set up in this manner, this process creates a continuous blend of microgel compositions between two distinct microgel composition extremes. Alternatively, this process could be employed to mix discrete populations of microgel formulations all within a single run. The output of this blending procedure can be visualized using two alginates labelled with spectrally distinct fluorophores. The fluorescent label is not expected to have any appreciable impact on alginate gelling properties,[37, 38] and accordingly, a uniform suspension of microgels is produced that vary only in their fluorescent signatures. Of note, for a given microgel produced with this blending strategy the concentration of the first fluorophore is inversely related to that of the second fluorophore and the composition of microgels that can be produced is limited by the linear relationship of the blending scheme. Nevertheless, this system provides a straightforward approach to creating spectrums of microgel composition and assessing how a given parameter of interest will relate to release while not significantly increasing the technical complexity of the microgel fabrication process.

In general, material-guided lentivector delivery can be designed to transduce cells either internally or externally to the material system.[2, 39, 40] There are inherent strengths and limitations to both approaches. For internal cellular transduction, cells are seeded within the

biomaterial system along with lentivectors and, typically, the biomaterial is designed to promote host cellular infiltration.[39, 41–45] These systems can achieve tight control over where transduction takes place, however the logistical/technical complexity of the carrier system is increased and transduction is limited to transplanted cells or cells that are capable of infiltration.[46, 47] In the alternative external transduction approach pursued here, the technical complexity is reduced, but controlling the profile of release becomes important for delivering the spatially and temporally correct amount lentivectors to surrounding tissue.

Alginate content and polymer degradability are two parameters that have documented effects on hydrogel mechanical properties and release behavior.[48, 49] While degradable alginate formulations are well described for macroscopic hydrogels, our knowledge a process for generating degradable alginate microgels has not established from these same principles.[15, 50, 51] We designed the on-chip blending process to examine how these parameters affect microgel character lentivector release behavior. We first created microgels composed of alginate of 1 to 2% alginate solids content. Interestingly, decreasing alginate concentration produced microgels of larger volume. We interpret this change in volume as evidence of decreased mechanical strength and increased swelling for lower alginate content microgels. However, the size of the droplet template produced in the flow focusing microfluidics could also play a role. Alginate viscosity is heavily dependent on concentration,[48] and for flow-focusing devices viscosity is an important parameter in the balance of forces that dictate droplet size.[52] It is therefore plausible that the droplet template size and microgel swelling can together explain the trends in volume observed. Regardless, only a small increase in release was achieved by lowering alginate concentration and, overall, a limited amount of lentivectors were released from microgels spanning 1 to 2% of alginate concentration. The increased swelling obtained with lower alginate concentration is not sufficient to expand the mesh size to an extent that significantly increases lentivector release. Encouragingly, this nondegradable formulation of alginate can effectively act as a low, base level of release as, predominantly, lentivectors remain sterically entrapped.

Microgel degradation provides a means of augmenting lentivector release above the base level achieved with nondegradable formulations. We compared 2% alginate, the strongest hydrogel with the most limited release, to a 2% degradable alginate to highlight the impact of degradability. Decisively, the degradable alginate, which was obtained *via* partial oxidation, produced hydrogels that were both comparatively weak initially, presented a larger initial mesh size, and degraded in solution over time. These microgels degraded rapidly and completely in comparison to macroscopic hydrogels of the same formulation. This was likely due to increased overall surface area and decreased distances for the breakdown products of the alginate backbone to diffuse out of the hydrogel.[9] However, on-chip polymer blending revealed that the incorporation of even small fractions of nondegradable alginate has a drastic effect on the breakdown of microgels during the timeframes monitored. Varying the relative portions of nondegradable and degradable alginate provides a simple means to adjust the overall proportion of slow-retentive behavior and fast burst-like behavior. Blended microgels achieved a steadier profile of lentivector release and transduction of HEK-293T cells over the first four days and resulted in increased transduction from 2 days onwards in comparison to degradable or nondegradable uniform suspensions. Finally, while we only experimented with a linear blend spanning the entire

spectrum from nondegradable to degradable formulations, it is possible to envision the formulation of microgel blends that skew towards quicker or slower release by adjusting the relative fractions of microgel composition. This flexibility may provide a simple and robust strategy for engineering lentivector release rates on a case-by-case basis.

We highlighted how the composite suspension system can be adapted to the requirements of a given situation by employing a degradable formulation identified in our blending experiments for quick release and proangiogenic response in the CAM assay. Purely degradable microgels, or a 100:0 formulation, were observed to degrade too rapidly. Furthermore, their weak initial strength lowers the yield in recovering microgels from emulsification. Accordingly, we used the results from our degradation studies to select a formulation that provided strength for collection, and degradation over the 3-day window of the assay. The CAM assay is a well-documented and accessible pre-clinical platform for probing vascular development and response to pro- and anti-angiogenic therapies.[53] Previous work has established that VSVg pseudotyped lentivectors are capable of delivering transgenes to the CAM of chick embryos.[54] Additionally, hyaluronic modified fibrin hydrogels have been used to deliver non-viral plasmid DNA encoding VEGF for proangiogenesis.[55] However, to our knowledge this is the first demonstration of therapeutic angiogenesis achieved by the controlled delivery of a lentivector from alginate hydrogels. Interestingly, there was a marked difference in the proangiogenic response between encapsulated and free lentivectors. Possible explanations for this difference include the potential for harm to the CAM at high concentration of lentivectors which may be minimized using controlled delivery to maintain concentrations at tolerable and beneficial levels. Alternatively, lentivector dissemination may affect the magnitude of angiogenic response in the region of interest, as the delivery of lentivectors in suspension has previously been shown to produce transgene expression in a wide range of somatic tissues throughout the chicken embryo.[54] It is conceivable that controlled delivery may limit dissemination of the lentivectors and localize transgene expression to the area of interest. In future studies, it will be on interest to investigate the effects of controlled lentivector release on the immune response and duration of transgene expression.

CONCLUSIONS

In this work, we designed and validated a simple, yet powerful, process for creating suspensions of alginate microgels with gradient compositions. The results of this study highlight the potential of creating blends of microgels for controlled release applications and, specifically, that alginate percentage and degradability are parameters that can tune release and retention of microgel-encapsulated lentivectors. We have demonstrated how polymer oxidation leads to alginate microgels that degrade over the course of days in solution. This strategy of composite suspensions may be advantageous for the delivery of lentivectors as it can provide greater control in the spatiotemporal control of gene delivery. Furthermore, this process can be used to screen across different formulations to identify compositions of microgels suitable for a given application. Finally, we highlighted the proangiogenic potential of controlled delivery of VEGF lentivectors from degradable microgels. Future work will be needed to validate this system on a clinically relevant animal model such as the murine hindlimb ischemia model. To our knowledge this is the first

demonstration of a bottom-up approach in the design of hydrogel systems for controlled viral vector release. Moreover, the concept of on-chip polymer blending can be extended for utility in the controlled delivery of other cargos including small molecules, proteins, or cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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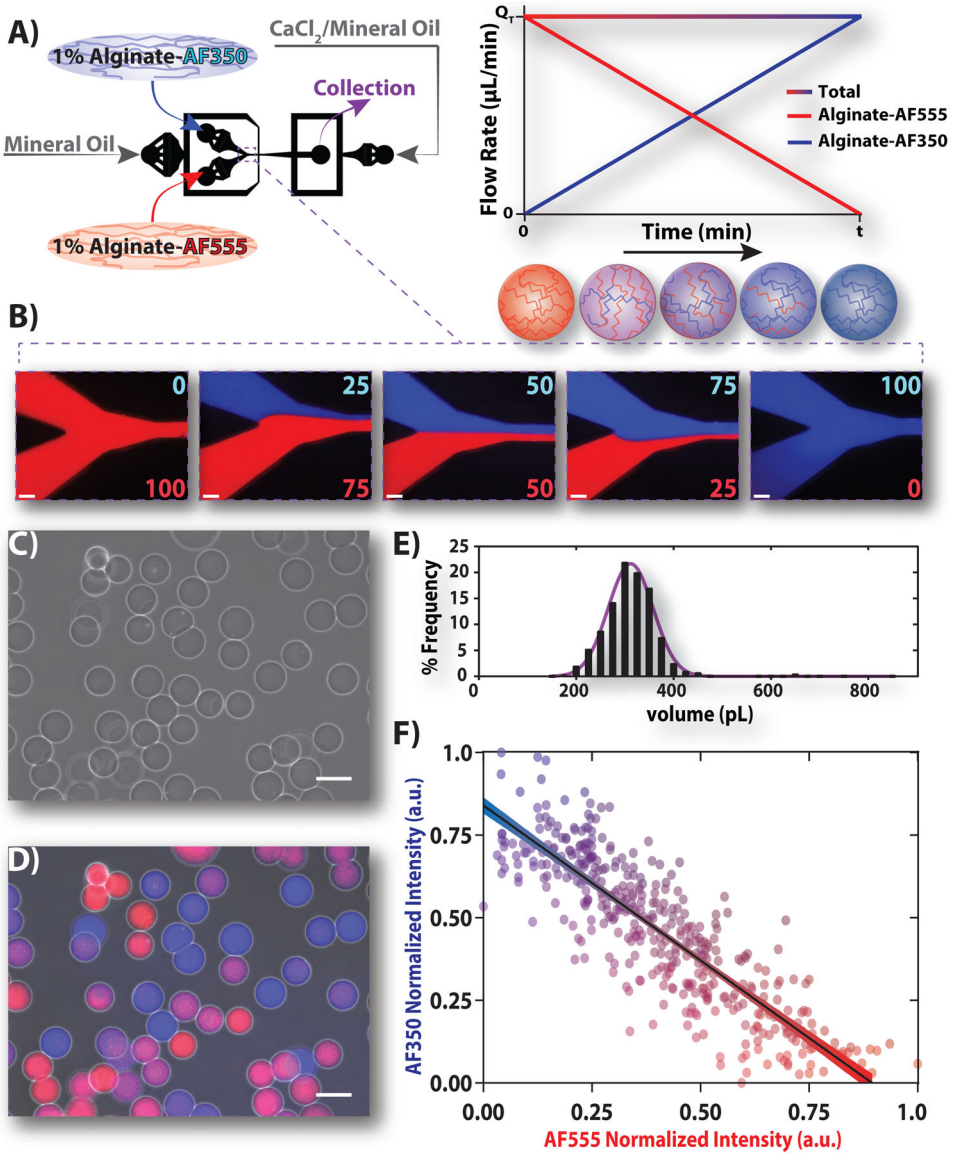

Figure 1: Gradient microgel suspension generated via microfluidic alginate blending.

Two distinct polymer formulations are pumped through a microfluidic droplet generator with flow rates that are inversely and linearly ramped to generate a blended suspension of microgels (A). The gradual replacement of alginate-AF555 with alginate-AF350 is visualized using composite fluorescent images of the microfluidic mixing lane over the time course of blending (B). Representative phase-contrast (C) and composite fluorescent (D) images of the resulting suspension of blended-alginate microgels. The overall distribution of microgel volume collected from the blending process (E). Distribution of AF555 (red) and AF350 (blue) fluorescent intensities for the resulting microgels shows a linear correlation (F, $n = 414$, trendline indicates linear regression fit and shaded area the 95% confidence interval). Scale bars represents 100 µm.

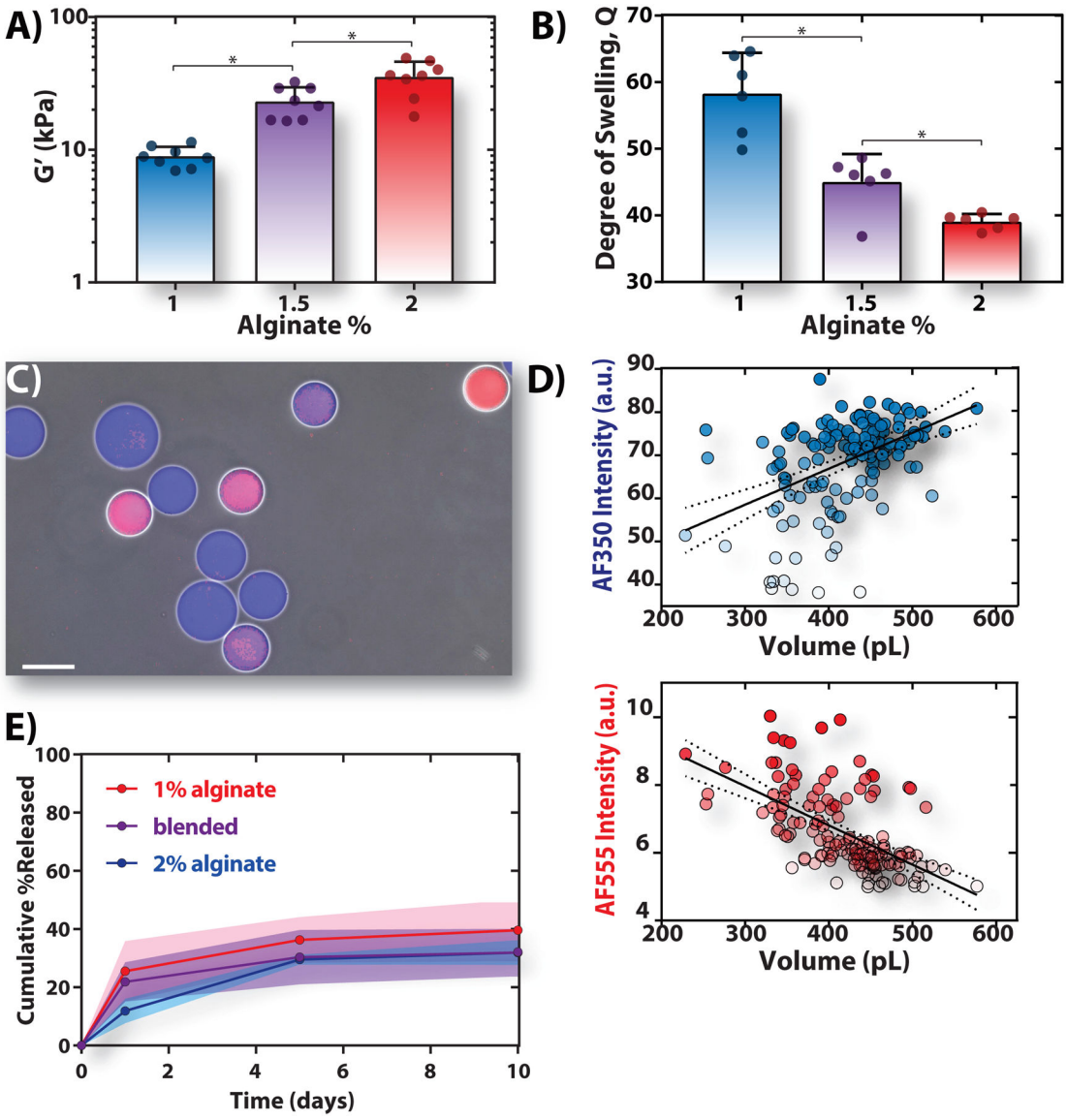

Figure 2: Microfluidic blending of alginate percentage.

Alginate hydrogels and microgels are generated using solutions of 1 and 2% alginate. Hydrogel disks of 1% and 2%, or disks created from a 50:50 mixture to yield 1.5%, display an increase in storage modulus (A, $n = 8$) and a decrease in swelling (B, $n = 6$) with increasing alginate content. A linear blend of microgels from 1 to 2% was generated using 1% alginate-AF350 and 2% alginate-AF555 solutions (C, scale bar represents 100 μm). The volumes of the resulting microgels were directly related to AF350 and inversely related to AF555 fluorescent intensities (D, $n = 145$, plots indicate linear regression fit and 95% confidence interval). For 1%, 2%, and blended 1–2% suspensions of microgels the release profile of encapsulated lentivectors was evaluated in terms of p24 capsid protein over the course of ten days (E, $n = 3$). Bars represent mean, scatter dot plots display individual measurements and error bars represents standard deviation. Shaded areas represent standard

deviation. Asterisk (*) indicates statistically significant differences ($P < 0.05$) between conditions.

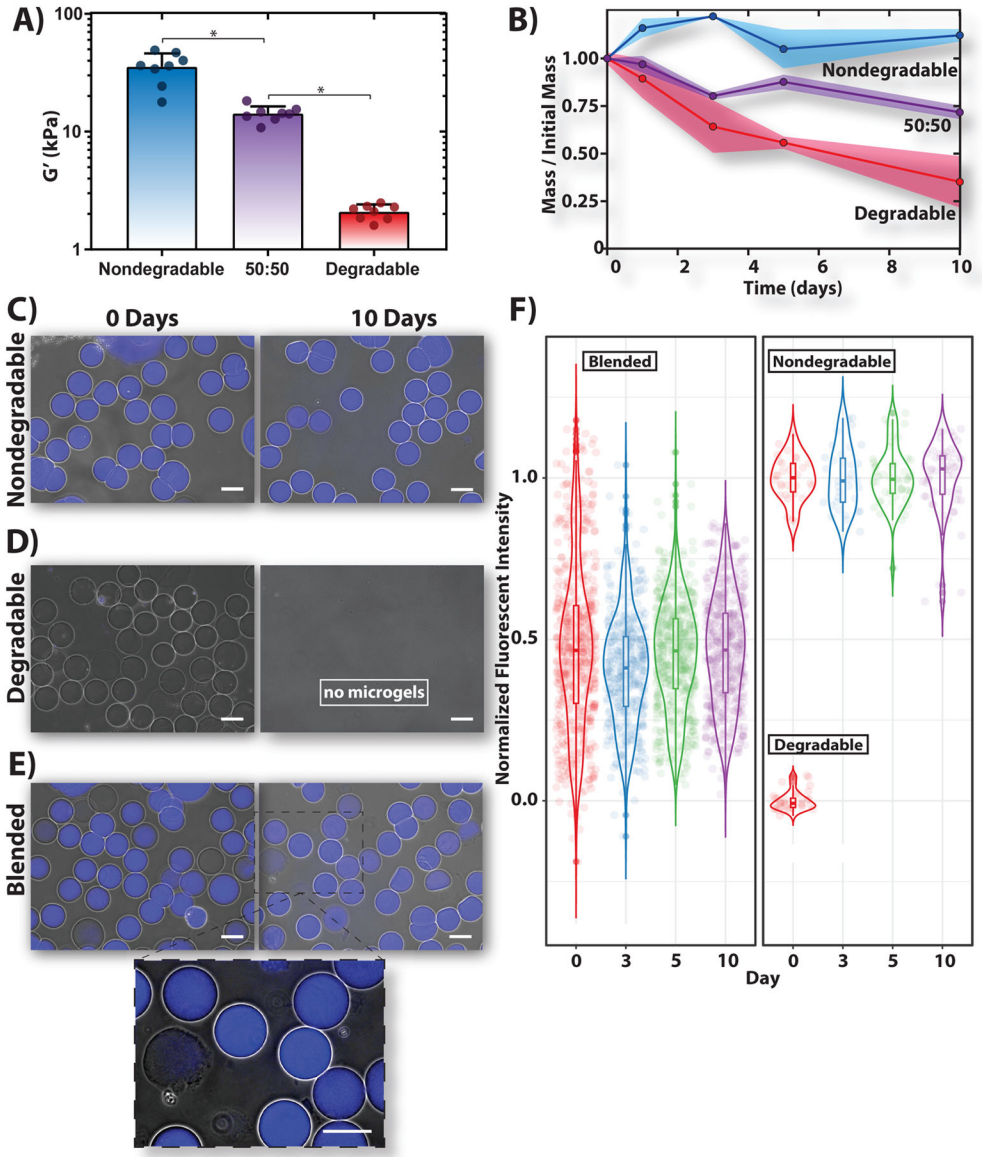

Figure 3: Microfluidic blending of alginate degradative behavior.

Alginate hydrogels and microgels are generated using 2% solutions of nondegradable, fluorescently labeled alginate and non-labelled, degradable alginate. Increasing the content of degradable alginate in hydrogel disks leads to lower storage moduli (A, $n = 8$) and increased degradation over 10 days at 37 °C (B, $n = 3$). Microgels were prepared as non-degradable, degradable, and a linear blend of these two formulations, and a comparison of these suspensions reveals a range of degradative behavior over 10 days at 37 °C (C, D, E, scale bars represent 100 μm). The distribution of microgel fluorescent intensities demonstrates a loss of non-fluorescent, degradable microgels over the course of 10 days within the blended suspension and purely degradable suspension (F, n > 400 for each timepoint). The proportion of microgels with a particular fluorescent intensity is proportional to violin-plot width. Box-plots within the violin-plot show median, inter-quartile range, minimum and maximum intensities and individual measurements are

represented by scatter points. Bars represent mean and error bars represents standard deviation. Shaded areas represent standard deviation. Asterisk (*) indicates statistically significant differences ($P < 0.05$) between conditions.

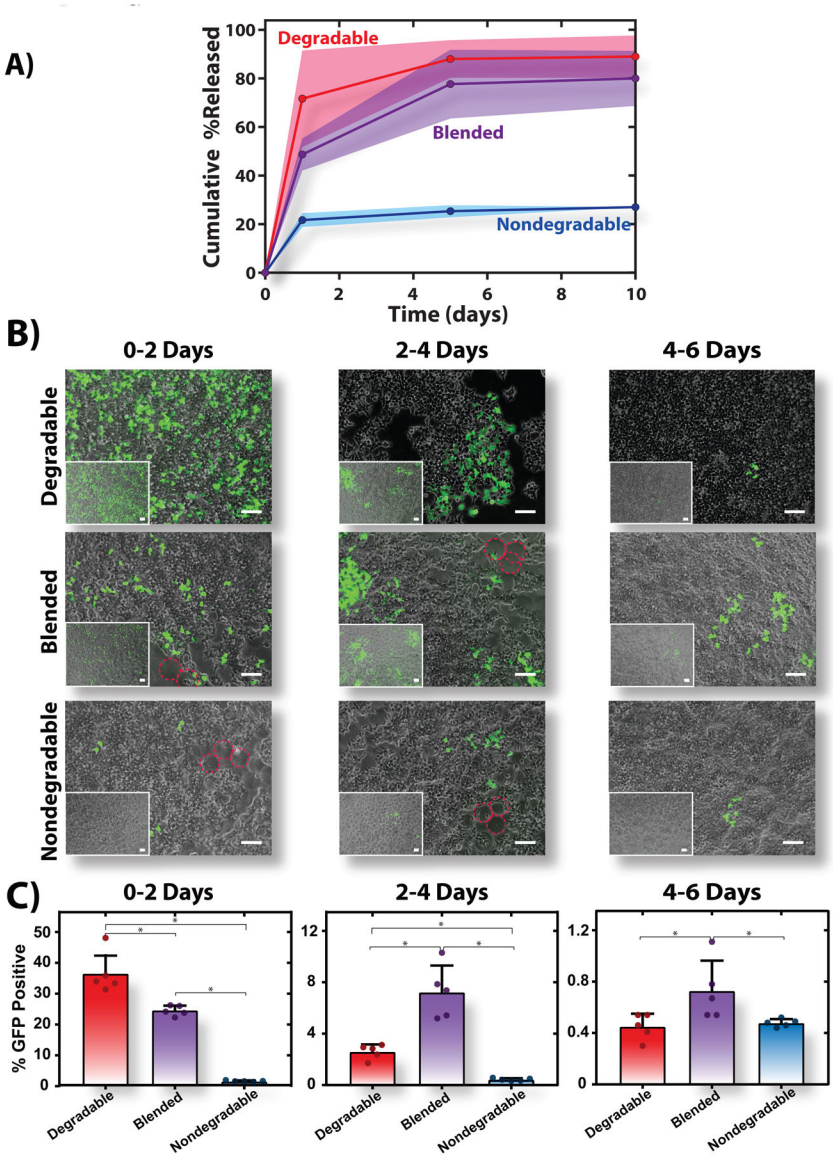

Figure 4: Lentivector release from degradable microgel blends.

Microgels were prepared from nondegradable alginate, degradable alginate, and a blend of these two alginate formulations. The incorporation of degradable alginate led to large amounts of lentivector release in comparison to nondegradable alginate, and the blended suspension displayed a more gradual, but still substantial, release (A, $n = 3$). Released lentivectors retained their functionality and promoted GFP expression in HEK-293T cells as observed by merged phase-contrast/fluorescent photomicrographs of cells in contact with microgels (representative microgels in red outline) for 2 day timespans (B, inlets contain zoomed out images, all scale bars are 100μm). During the first two days of release degradable microgels yielded higher percentages of GFP positive cells, whereas the blended suspension yielded higher transduction efficiency over the following four days for the subsequent sets of cells (C, $n = 5$). Bars represent mean and error bars represents standard

deviation. Shaded areas represent standard deviation. Asterisk (*) indicates statistically significant differences ($P < 0.05$) between conditions.

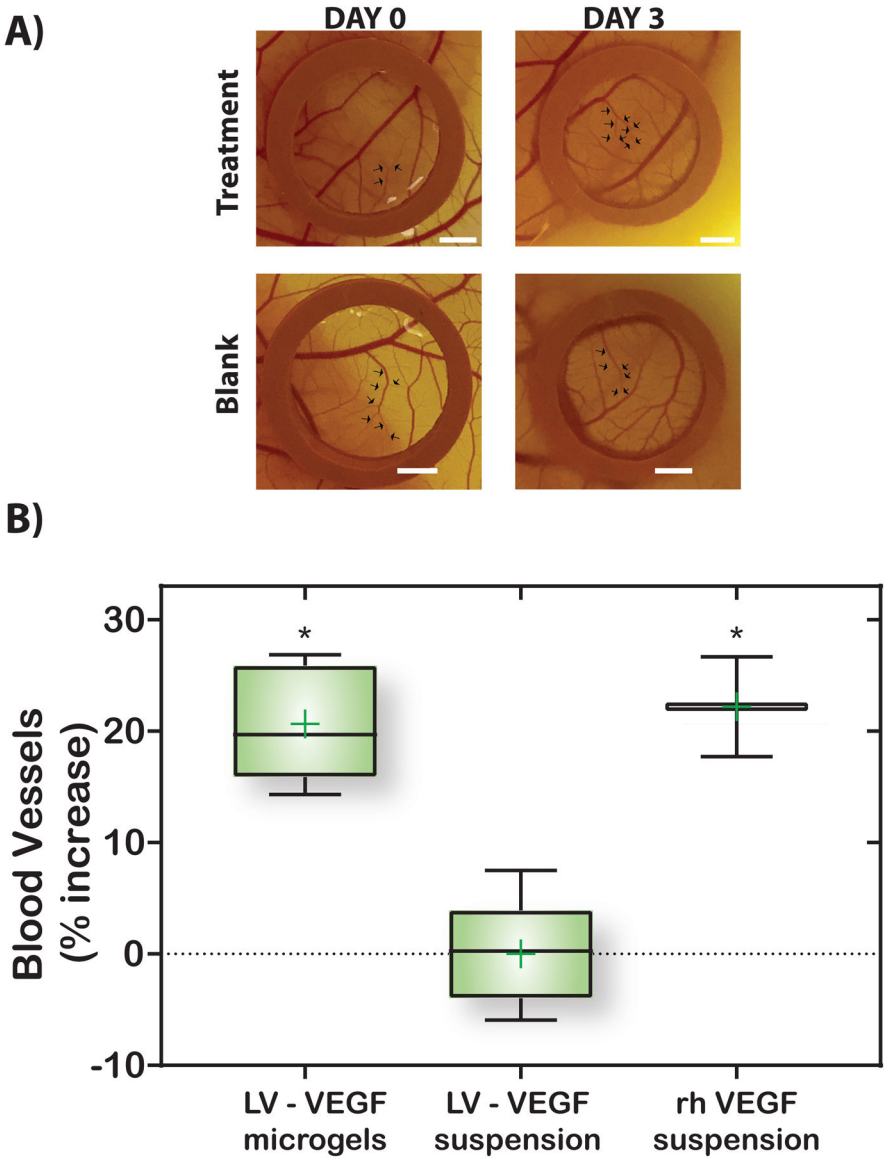

Figure 5: Controlled delivery of VEGF lentivectors induced blood vessel formation in a CAM assay.

Controlled delivery of VEGF lentivectors induced blood vessel formation in a CAM assay. Representative images of the CAM treatment and blank regions at 0 h and after 3 days of incubation are shown (A). Arrows point to representative vessels. Scale bars are 2 mm. Quantified blood vessel development showed that VEGF-lentivectors released from degradable microgels and rhVEGF suspensions resulted in statistically significant blood vessel development (B). VEGF-lentivectors in suspension showed no difference from blank treatment controls. The horizontal line within the box indicates the median, boundaries of the box indicate the 25th- and 75th -percentile, and the whiskers indicate the highest and lowest values of the results. The "+" marked in the box indicates the mean. The asterisks indicate statistically significant differences ($P < 0.05$) in comparison to the blank treatment alone. (n=2−5).