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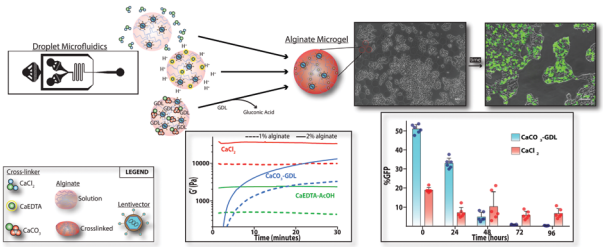

Microfluidic generation of alginate microgels for the controlled delivery of lentivectors

Justin L. Madrigal, Roberta S. Stilhano, Christian Siltanen, Kimberly Tanaka, Sabah Rezvani, Ryan Morgan, Alexander Revzin, Sang W. Han and Eduardo A. Silva*

Microgels fabricated through distinct microfluidic procedures encapsulate and release functioning lentivectors in a controlled manner.

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Microfluidic generation of alginate microgels for the controlled delivery of lentivectors†

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Lentivectors are widely used for gene delivery and have been increasingly tested in clinical trials. However, achieving safe, localized, and sufficient gene expression remain key challenges for effective lentivectoral therapy. Localized and efficient gene expression can be promoted by developing material systems to deliver lentivectors. Here, we address the utility of microgel encapsulation as a strategy for the controlled release of lentivectors. Three distinct routes for ionotropic gelation of alginate were incorporated into microfluidic templating to create lentivector-loaded microgels. Comparisons of the three microgels revealed marked differences in mechanical properties, crosslinking environment, and ultimately lentivector release and functional gene expression *in vitro*. Gelation with chelated calcium demonstrated low utility for gene delivery due to a loss of lentivector function with acidic gelation conditions. Both calcium carbonate gelation, and calcium chloride gelation, preserved lentivector function with a more sustained transduction and gene expression over 4 days observed with calcium chloride gelated microgels. The validation of these two strategies for lentivector microencapsulation may provide a platform for controlled gene delivery.

Introduction

Gene therapy has been envisioned to treat a wide range of diseases and disorders.[1] Viral-based strategies, in particular, predominate therapeutic usage due to their higher efficiency of gene expression relative to their non-viral counterparts.[2,3] Lentivectors pseudotyped with VSV-g protein ae extensively used because of wide tropism, infectivity for both dividing and non-dividing cells, and low immunogencity.[4–6] Typically, lentivectors are administered either *ex vivo* or *via* bolus injections.[1] However, these routes of administration can provide a challenge to obtaining safe, localized, and sufficient expression.[7] Therefore, there is a need to develop novel delivery systems that guide the timing, dosage, and localization of lentivectors, which may improve the safety and efficiency by minimizing off-target expression and regulating vector concentrations in target tissue.[2,8–10]

Biomaterial systems could be designed to locally present lentivectors in a sustained manner. More specifically, hydrogels are an especially attractive class of biomaterials for controlled delivery strategies due to their high-water content, ease of administration, and chemical and structural versatility.[11–15] These hydrogels can be designed to provide a localized depot of lentivectors that can be released in a spatio-temporal manner for cellular transduction.[11,14] Recently, the advent of droplet microfluidic technologies has allowed for the formation of hydrogels in discrete volumes with characteristic dimensions on the order of micrometers.[16] These microgels are generated through emulsion templating, where precursor polymers are emulsified through a flow focusing junction prior to gelation, leading to controllable and monodisperse size.[17,18] In contrast to macroscopic hydrogels, microgels exhibit reduced characteristic lengths and increased surface area, which provides potential for manipulation as a delivery platform. These strategies have been used to promote cellular encapsulation,[19,20] control drug release,[21,22] and have formed a platform for responsive materials.[23–26] Microgel encapsulation may hold advantage for the delivery of lentivectors,[27–29] but adapting these microfluidic techniques for producing lentivector-compatible microgels represents an engineering challenge.

Alginate has been widely studied for encapsulation in both macro- and micro-hydrogels because of its inherent biocompatibility and mild crosslinking chemistry with divalent cations.[30] Recent work demonstrated the capability of degradable alginate hydrogels to deliver lentivectors to the murine hindlimb.[14]

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Alginate-delivered lentivectors led to a prolonged gene expression in comparison to a bolus injection, but there was an initial delay in expression which can be was connected with a slow lentivector release. The release kinetics could be modified by altering alginate microgels fabrication methods, but suitable microfluidic strategies for encapsulating lentivectors remain to be identified.[31] While early work produced alginate microgels through the extrusion of alginate droplets into crosslinking baths,[32–34] recent studies have applied droplet microfluidics to create more monodisperse alginate microgels.[35–39] Several strategies for achieving gelation within an emulsion template have been investigated, including internal and external gelation. For internal gelation, a sequestered source of divalent cations is dispersed throughout the alginate solution and released after emulsification. In contrast, external gelation involves infusing the carrier phase with a water-soluble divalent cation source which then partitions into the alginate emulsions. The distinction between internal and external gelation impacts the cross-linking time, mechanical structure, and homogeneity of the resulting hydrogel.[40] Importantly, the type of gelation strategy applied can decisively affect the cargo biofunctionality after encapsulation.[37,41] Nevertheless, a side-by-side comparison of the suitability of these techniques for the encapsulation and release of sensitive therapeutic cargos such as lentivectors has not been reported.

To our knowledge, the delivery of lentivectors from alginate microgels generated with microfluidic templating has not been evaluated. Here, for the first time we are employing microfluidics to encapsulate lentivectors within alginate microgels in order to create a controlled delivery system. To identify a suitable microgel for lentivector delivery, we have directly compared three microfluidic gelation strategies in terms of the mechanical properties, encapsulation conditions, and gene delivery potential. In doing so we have validated two platforms for lentivector delivery from alginate microgels that hold potential for improving administration routes for gene therapy.

Experimental

Microfluidic device fabrication

Microfluidic devices were fabricated using standard soft lithography where polydimethylsiloxane (PDMS) elastomer (Sylgard) was degassed and cured for 1 hour at 70 °C after pouring over SU-8 master molds prepared on 4-inch silicon wafers (Microchem). Individual PDMS replicas were bonded to glass slides using O_2 plasma surface treatment. Finally, microfluidic channels were made hydrophobic through the application of Aquapel™ solution for 30 seconds. Flow focusing channel dimensions ($H \times W$) were 100×100 μm for all droplet generation procedures.[18,20]

Lentivector

The pCCLc-MNDU3-luciferase-PGK-EGFP-WPRE construct was kindly obtained from the UC Davis/CIRM Institute for Regenerative Cures (Prof. Jan Nolta).[42] The viral titer, which we express herein as lentiviral transducing units per mL (TU per mL), was determined as previously reported.[14] Briefly, human embryonic kidney (HEK-293T) (ATCC) cells were transduced with different concentrations of lentivectors in the presence of 8 μg mL^{-1} of Polybrene (Sigma). After 3 days, the HEK-293T cells transduced with lentivector expressing GFP were counted (>10 000 events analyzed) using an Attune flow cytometer (Thermo Fisher) and the data was analyzed using FlowJo software (TreeStar Inc.). Additionally, the viral titer was determined in terms of the concentration of p24 capsid protein as measured by ELISA (ZeptoMetrix Co) according to the manufacturer's guidelines and protocol. A conversion factor of 25 pg per TU was measured which indicates efficiency in viral packaging.[43,44]

Generation of alginate microgels

Alginate hydrogels were prepared in three distinct manners at room temperature to yield micrometer dimension hydrogels, including (1) internal gelation with calcium carbonate and glucono delta-lactone ($CaCO_3$–GDL),[45] (2) internal gelation with ethylenediaminetetraacetic acid (EDTA)-chelated calcium (CaEDTA–AcOH),[41] and (3) external gelation with calcium chloride ($CaCl_2$).[46] All three procedures used a common precursor polymer solution composed of a binomial mixture of low and high molecular weight alginates mixed at a 3 : 1 ratio (LF10/60 and LF20/40, NovaMatrix). Alginate solutions were prepared fresh for each experiment by dissolving alginates in 0.9% NaCl overnight at room temperature. For studies including lentivectors, an appropriate volume of lentivector suspension was mixed into precursor alginate solutions prior to droplet formation and gelation. Microgels were imaged using a Zeiss Axio Vert A.4 phase contrast microscope and morphology was quantified with Image J.

To prepare $CaCO_3$–GDL microgels, an alginate solution containing suspended $CaCO_3$ nanoparticles (SkySpring Nanomaterials) and a second alginate solution containing freshly dissolved GDL (Sigma Aldrich) were mixed on-chip prior to flow focusing and emulsification.[45] Flow focusing was achieved using HFE-7500 oil (3 M) with 2% v/v PEGylated fluorosurfactant (RAN Biotech) as an immiscible carrier phase. The aqueous and oil flow rates used were 10 μL min^{-1} and 50 μL min^{-1} respectively. $CaCO_3$–GDL microdroplets were collected and incubated at room temperature for 1 hour to allow for gelation. To collect the microgels, the droplet emulsion was destabilized using 20% (v/v) perfluorooctanol (Sigma Aldrich) in HFE-7500 and washed (DMEMc) (Dubelco's Modified Eagle Medium (DMEM) (Invitrogen) containing 1% penicillin/strepamycin (Invitrogen) and 10% fetal bovine serum (FBS)(Invitrogen)).

CaEDTA–AcOH microgels were prepared using the same microfluidic process as stated above but differing in the gelation strategy.[41] Here, chelated calcium was first prepared by mixing $CaCl_2$ (Sigma Aldrich) and EDTA (Sigma Aldrich) at equimolar ratios at a pH of 7.5. Gelation was initiated immediately after droplet collection by washing the emulsion with HFE-7500 containing 17.4 mM acetic acid (Sigma Aldrich). Microgels were then collected from the emulsion as stated above. For both internal gelation strategies, the ratio between calcium ions and carboxyl moieties on the alginate backbone was kept constant

at 0.36. For $CaCO_3$–GDL gelation a constant ratio between $CaCO_3$ and GDL of 0.5 was maintained for pH balance.

$CaCl_2$ microgels were prepared using a second microfluidic chip design (Fig. 1) that includes a second inlet for a calcium infused carrier phase. $CaCl_2$ infused mineral oil was prepared by sonication of a 1 : 9 volumetric mixture of 0.7 g mL^{-1} $CaCl_2$ and light mineral oil (Sigma Aldrich) with 1.2% SPAN 80 (Sigma Aldrich). Here, flow focusing was achieved using light mineral oil with 1.2% SPAN 80 as an immiscible carrier phase. Downstream, $CaCl_2$ infused mineral oil was used to replace the carrier phase. Flow rates were 2, 6, and 15 μL min^{-1} for the aqueous, mineral oil, and calcium infused mineral oil, respectively. The microgels were then collected by centrifugation at (10 rcf) for 3 minutes.

Rheologic characterization of microgels

Measurements of storage and loss shear moduli were performed using a Discovery HR2 hybrid rheometer (TA instruments) with an 8 mm parallel-plate geometry in time sweep and strain sweep modes.[47] Pre-hydrogel solutions were mixed and crosslinked within the parallel plate geometry. The linear viscoelastic region was determined *via* strain sweep analysis of fully crosslinked hydrogels. Time sweeps were then performed within the linear viscoelastic regime at a strain of 0.3%, and a frequency of 1 rad s^{-1}, and a temperature of 23 °C to monitor the development of hydrogel crosslinking. Gelation crossover time was measured as the point where $\tan(\delta) = 1$. Geometry edges were coated with sunflower seed oil to prevent evaporation during measurement.

Equilibrium swelling behavior of microgels

Microgels were prepared and incubated on top of cell strainers (30 μm mesh size, BD) in phosphate buffered saline supplemented with 0.1 g L^{-1} $CaCl_2$ and 0.1 g L^{-1} $MgCl_2$ (PBS^{++}) (Invitrogen) at 37 °C for 24 hours. The excess PBS^{++} was strained and thoroughly blotted from around the microgels and the wet weight of the microgels was measured. Microgels were then frozen at −20 °C for 24 hours and lyophilized for an additional 24 hours at which point the dry weights of the microgels were measured. The degree of swelling, Q, was defined as the reciprocal of the polymer volume fraction in the hydrogel (ν_2):

$$\nu_2 = \frac{1}{\rho_P}\left(\frac{Q_m}{\rho_w} + \frac{1}{\rho_P}\right)^{-1} \quad (1)$$

$$Q = \nu_2^{-1} \quad (2)$$

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.[48]

Mesh size calculation

Mesh size for the hydrogels was estimated using swelling and rheometry data.[49–51] The molecular weight between crosslinks (M_c) was calculated from the shear modulus elastic component (G', Pa) using the following equation:

$$M_c = C_p RT/G' \quad (3)$$

where C_p is the concentration of alginate, R is the gas constant, and T is the temperature at which the measurement was taken. From here, a characteristic mesh size (ξ) was calculated as follows:

$$\xi = \nu_2^{-1/3} l \left(2\frac{M_c}{M_r}\right)^{1/2} C_n^{1/2} \quad (4)$$

where M_r is the molecular weight of the repeating unit (194 g mol^{-1}), l is the length of the repeating unit (5.15 Å) and C_n is the characteristic ratio $C_n = 0.021\ M_n + 17.95 = 21.1$.[52]

Monitoring of pH during microgel gelation

A pH probe (Mettler Toledo) was immersed within alginate solutions and gelation was initiated to form a hydrogel around the probe. pH measurements were recorded once per minute for three hours ($n = 2$). Additionally, hydrogels were prepared in the same manner and the pH was measured after one hour ($n = 3$).

Effect of pH and encapsulation on lentivector functionality

Lentivector suspensions were pre-incubated in media with adjusted pH values. For pH treatment, media was equilibrated with acetic acid to pH levels of 5.0, 6.0, 6.5, and 7.5. Lentivector suspension containing 6×10^4 TU were incubated within treated media, and following times of 10, 30, and 60 minutes, the lentivector suspension was diluted with DMEMc and applied to HEK-293T cells. Alternatively, 7.5×10^4 TU of lentivectors were encapsulated within alginate microgels, released by hydrogel enzymatic digestion with alginate lyase[53] (Sigma Aldrich), and then applied to HEK-293T cells. For all experiments the transduction efficiency of the lentivectors was quantified by flow cytometry (>10 000 events analyzed).

Quantification of lentivector release from alginate microgels

Alginate microgels (1% alginate) were prepared using the $CaCl_2$ and $CaCO_3$–GDL procedures described above and loaded with 8250 TU per μL. 12 μL of microgels were collected and immersed within DMEMc in microcentrifuge tubes at 37 °C and 5% CO_2 to allow for lentivector release (n = 2–3). At selected time points spanning 10 days, the supernatant was collected and stored at −20 °C (until sample concentration was determined), and a fresh aliquot of medium was added over each disk. Alternatively, lentivectors were immediately recovered from a separate 12 μL of microgels by digestion with 10 units per mL of alginate lyase[53] (n = 3). The concentrations of lentivectors in supernatant and digestion samples were determined in terms of the p24 capsid protein. The p24 concentrations were quantified using an HIV p24 Antigen ELISA Kit (ZeptoMetrix Co) according to the manufacturer's guidelines and protocol as has previously described.[14,54]

Transduction efficiency of lentivector-loaded alginate microgels

In all the experiments detailed below, 3×10^4 HEK-293T cells were seeded per well in 24-well plates and cultured in DMEMc for 24 hours at 37 °C and 5% CO_2 prior to any contact with lentivectors. Alginate microgels (1% alginate) were prepared

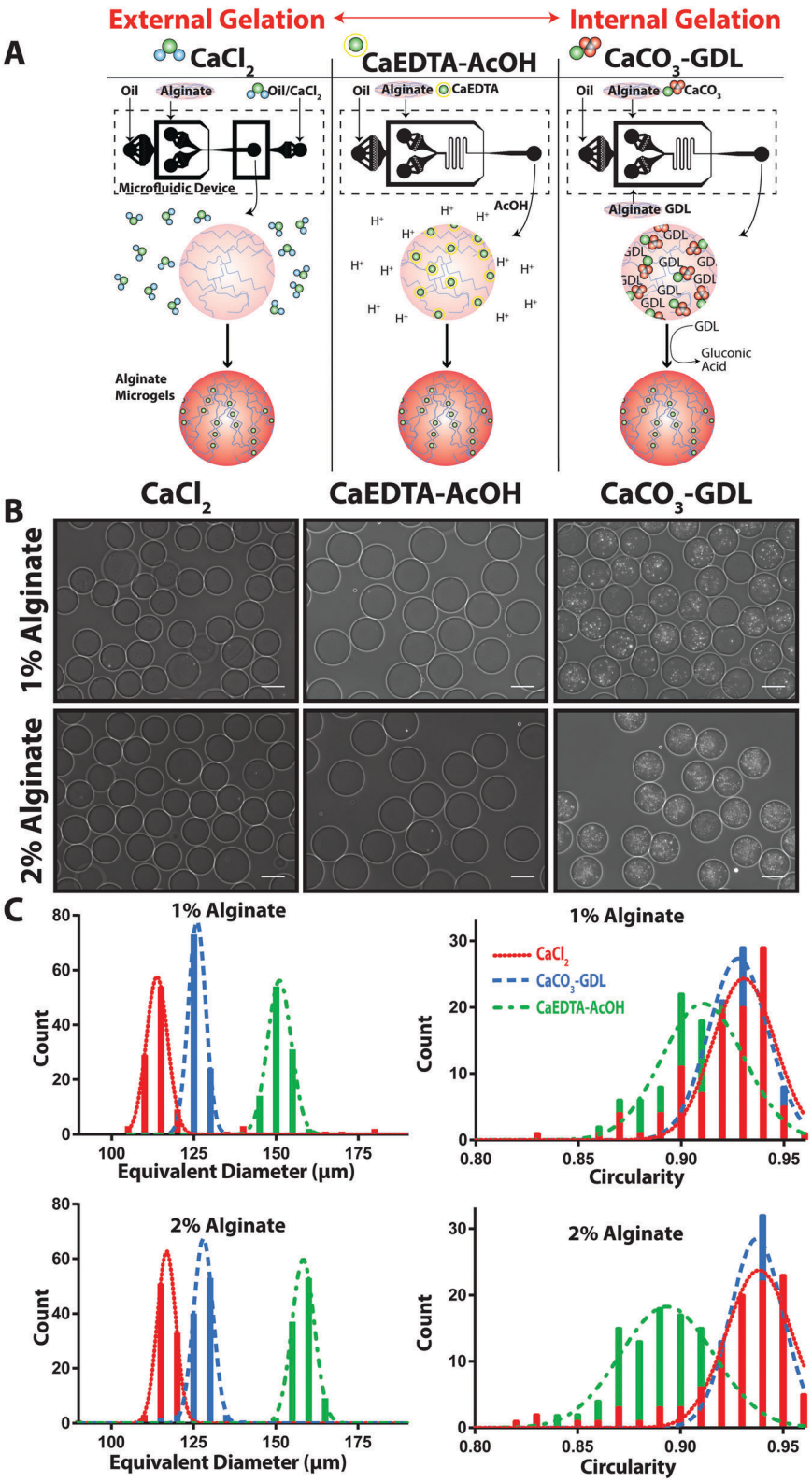

Fig. 1 Morphological characterization of alginate microgels. Illustration of the formulation of three distinct microgels fabricated from microfluidics devices (A). Representative phase-contrast photomicrographs of alginate microgels fabricated using three different gelation techniques (B). Microgels with controlled dispersity in microgel diameter (C) and circularity (D) were obtained by use of microfluidic templating (n = 103 microgels). Calibration bar represents 100 μm.

using the $CaCl_2$ and $CaCO_3$–GDL procedures as described above and loaded with 8250 TU per μL of lentivectors. 12 μL of microgels (total MOI = 3.5) were suspended in 250 μL DMEMc. The medium of HEK-293T was then replaced with

the microgel suspension. After 12 hours, an additional 750 μL of DMEMc was added to each well and incubated for an additional 12 hours. Following this first day, the media containing the microgel suspension was collected and replaced with DMEMc and the cells were cultured for an additional 24 hours to allow for GFP expression. The microgels were then recovered from the collected media by centrifugation (30 rcf) for 3 minutes and were resuspended in 250 μL of fresh DMEMc. This microgel suspension was then added to HEK-293T cells which had been seeded the prior day and the cycle was repeated for a total of 4 days. The transduced HEK-293T cells from each time point were incubated for 48 hours for determination of transduction efficiency as previously described.[55,56] In the negative control wells only DMEMc was added, and in the positive control wells DMEMc with lentivector (3×10^5 TU per well) was added. The transduction efficiency was assessed both qualitatively and quantitatively *via* fluorescent microscopy (Axio Vert.A1; Zeiss) and flow cytometry counting GFP-positive cells (>10 000 events analyzed/experimental condition).

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 6 software (Graphpad). Differences between conditions were considered significant if $P < 0.05$.

Results

Characterization microgel dimensions and morphology

In this study, we investigated the utility of microfluidic strategies for the encapsulation of lentivectors within alginate microgels. Three distinct gelation mechanisms were tested including two strategies employing internal gelation and one using an external gelation approach (Fig. 1A). One internal gelation strategy used calcium carbonate, which releases Ca^{2+} upon the slow hydrolysis and acidification of GDL ($CaCO_3$–GDL).[40] Here, a stoichiometric ratio of $CaCO_3$ to GDL is maintained in order to minimize the acidification of the crosslinking environment. Furthermore, the crosslinking proceeds in a gradual and controlled manner. The other internal gelation strategy takes advantage of the preferential affinity of EDTA for divalent cations at basic pH (CaEDTA–AcOH).[41] Here, chelated Ca^{2+} is loaded into alginate and released by an abrupt drop in pH. The resultant crosslinking is rapid, which minimizes the duration of, but does not avoid, potentially harmful acidic gelation conditions. Distinctively, the external gelation approach infused the carrier oil with calcium chloride and allowed partitioning into the aqueous alginate phase to initiate gelation.[45] Again, the crosslinking is rapid, but no associated drop in pH is to be expected. Importantly, all three strategies yielded microgels with approximately spherical morphology and micrometer equivalent diameters (Fig. 1B, C and Table 1). Microgels fabricated using either the calcium chloride bath or EDTA caged calcium produced transparent hydrogels in comparison to the calcium carbonate method. Calcium carbonate precipitate remains visible after 1 hour of gelation. With extended gelation times these microgels became transparent indicating that for full calcium dissolution lengthened times may be necessary.

Mechanical testing of hydrogels

To analyze the mechanical properties of hydrogels generated by the three gelation strategies, both macroscopic hydrogel disks and microgels were fabricated for testing. Rheological testing identified the time at which the storage modulus surpassed the loss modulus, and provided the maximum storage modulus of macroscopic disks (Fig. 2). For CaEDTA–AcOH and $CaCl_2$ hydrogels, respective plateaus in storage modulus of 2.3 ± 0.7 kPa (2% alginate), 0.5 ± 0.2 kPa (1% alginate) and 9.5 ± 1.3 kPa (1% alginate) and 35.3 ± 9.3 kPa (2% alginate) were reached within the first minute after the addition of either acetic acid or $CaCl_2$ respectively. In comparison, 2.5 minutes passed for gelation and more than 30 minutes was required to reach a plateau in storage modulus for the slow Ca^{2+} releasing $CaCO_3$–GDL method (Table 1 and Fig. 2A). The final storage modulus of $CaCO_3$–GDL gels plateaued at values of 4.0 ± 0.6 kPa (1%) and 18.7 ± 3.5 kPa (2%). For all three formulations, there was an increase in storage modulus when alginate concentration increased from 1 to 2% (Fig. 2B). While gelation kinetics are expected to differ between microgel and macro-scale disks, our rheological data correlated closely with swelling behavior observed in microgels. Here, CaEDTA–AcOH microgels exhibited greater swelling ratios, 57.1 ± 2.2 (1% alginate) and 40.7 ± 5.9 (2% alginate), than $CaCl_2$ microgels, 40.5 ± 6.6 (1% alginate) and 24.1 ± 1.8 (2% alginate), and $CaCO_3$–GDL microgels, 29.5 ± 1.3 (1% alginate) and 24.8 ± 1.6 (2% alginate) with a similar dependence on polymer content as was seen with storage modulus (Fig. 2C). In addition, the mesh size was calculated from the measured storage modulus and swelling data and yielded values of 18 ± 5 nm and 9.9 ± 2 nm for 1% and 2% CaEDTA–AcOH microgels, 4.8 ± 0.4 nm and 2.9 ± 0.3 nm for 1% and 2% $CaCO_3$–GDL microgels, and 3.4 ± 0.3 nm and 2.1 ± 0.3 nm for 1 and 2% $CaCl_2$ microgels (Fig. 2D).

Particle encapsulation efficiency

To determine the ability to encapsulate and recover cargo, microgels were loaded with fluorescently labeled polystyrene

Table 1 Summary of microgel production and morphological characterization

			Crossover time (seconds)		Equivalent diameter (μm)		Circularity	
Microgel	Production rate (μL min^{-1})	Ca:COOH	1%	2%	1%	2%	1%	2%
$CaCl_2$	2	NA	<5	<5	104 ± 8.4	98.5 ± 16	0.91 ± 0.045	0.91 ± 0.049
CaEDTA–AcOH	10	0.36	<5	<5	152 ± 13	159 ± 11	0.91 ± 0.020	0.90 ± 0.021
$CaCO_3$–GDL	10	0.36	153 ± 21	138 ± 43	126 ± 2.5	127 ± 5.0	0.92 ± 0.017	0.93 ± 0.018

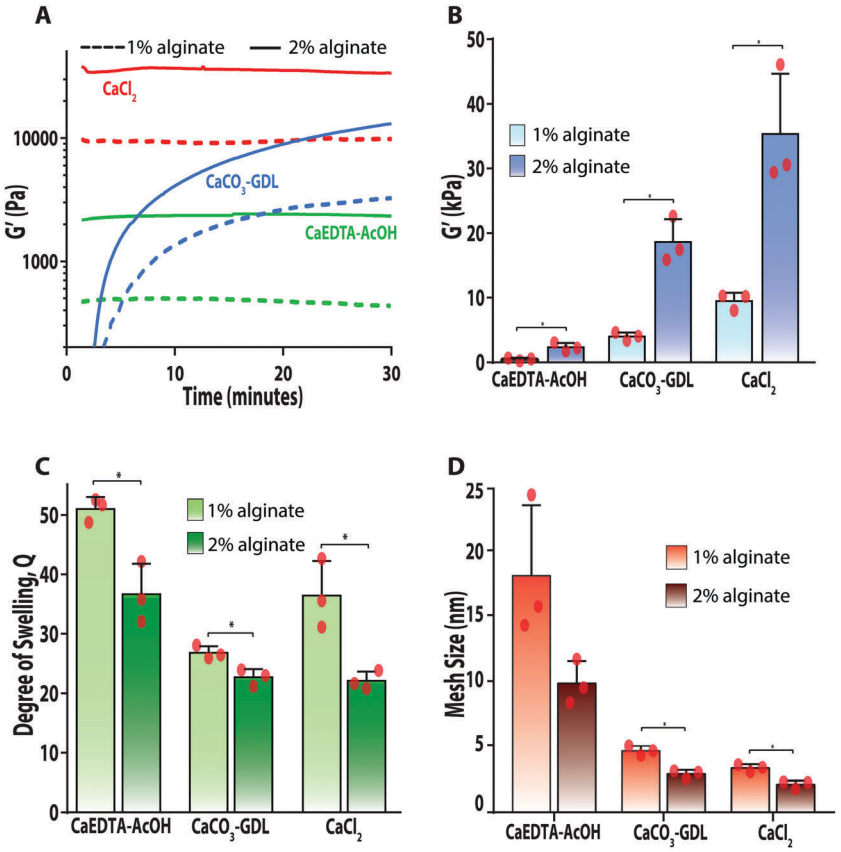

Fig. 2 Structural characterization of alginate hydrogels. Alginate hydrogels gelated *via* $CaCO_3$–GDL display an evolution of storage modulus in comparison to hydrogels gelated *via* either $CaCl_2$ or CaEDTA–AcOH (A). The plateau storage modulus of hydrogels (B) and equilibrium swelling behavior of microgels (C) incubated at 37 °C in PBS indicate increased crosslinking with the use of $CaCl_2$ or $CaCO_3$–GDL. Mesh sizes of hydrogels calculated from collected swelling and rheological data (D). * indicates statistically significant differences ($P < 0.05$). Bar represent mean, scatter dot plots displays individual measurements and error bars represents standard deviation. (A–D, $n = 3$).

particles with diameters on the same order of magnitude as lentivectors (100 nm). The encapsulation was visualized by the localized fluorescence within microgels and all three formulations demonstrated a near full recovery (>90%) of the loaded particles after enzymatic digestion of the microgels (Fig. S1, ESI†).

Effect of pH and encapsulation on lentivector functionality

We measured pH levels during gelation of macroscopic hydrogels by each of the three strategies (Fig. 3A). The pH remained constant during the $CaCl_2$ gelation strategy. For $CaCO_3$–GDL, pH decreased slowly from 7 to 5.8 over the course of three hours. In practice, we found these microgels were sufficiently crosslinked after only one hour (pH 6.3), at which point they are washed into buffered media (Fig. 3B). In contrast, CaEDTA–AcOH resulted in an abrupt drop in pH to 4.3, but only 5 minutes is required before washing into buffered media.

Subsequently, to assess lentivector sensitivity to pH we studied the capability of lentivectors to transduce HEK-293T after exposure to acidified media (Fig. 3C). Lentivectors pre-incubated with acidified media had a diminished ability to transduce HEK-293T cells. This harmful effect was exaggerated as the duration of pre-incubation was lengthened and was statistically significant compared to neutral media for all pH tested after one hour of pre-incubation. At a pre-incubation pH of 6.5 no effect was observed for the first 30 minutes, however at 1 hour a marginal drop to 89 ± 3.2% of the control transduction efficiency occurred. At a pH of 6.0 a drop to 91 ± 1.0% and then 82 ± 1.4% was observed after 30 minutes and 1 hour respectively. For a pH of 5.0 the transduction efficiency dropped to 78 ± 0.6% after 30 minutes and was reduced to 73 ± 1.7% after 1 hour.

Next, lentivectors were encapsulated within the different microgels and the viability of the lentivectors was tested (Fig. 3D). Immediately following encapsulation, lentivectors were released by enzymatic digestion of the microgels and the functionality of the released lentivectors was analyzed. No significant reduction in transduction efficiency was observed after encapsulation in either $CaCl_2$ or $CaCO_3$–GDL microgels. However, for CaEDTA–AcOH a dramatic drop in transduction efficiency to 4.3 ± 0.5% of the control was observed. Accordingly, CaEDTA–AcOH microgels were not considered in the following studies.

Loading and release of lentivectors from microgels

We next studied the encapsulation efficiency and release profile for lentivectors encapsulated within $CaCO_3$–GDL and $CaCl_2$

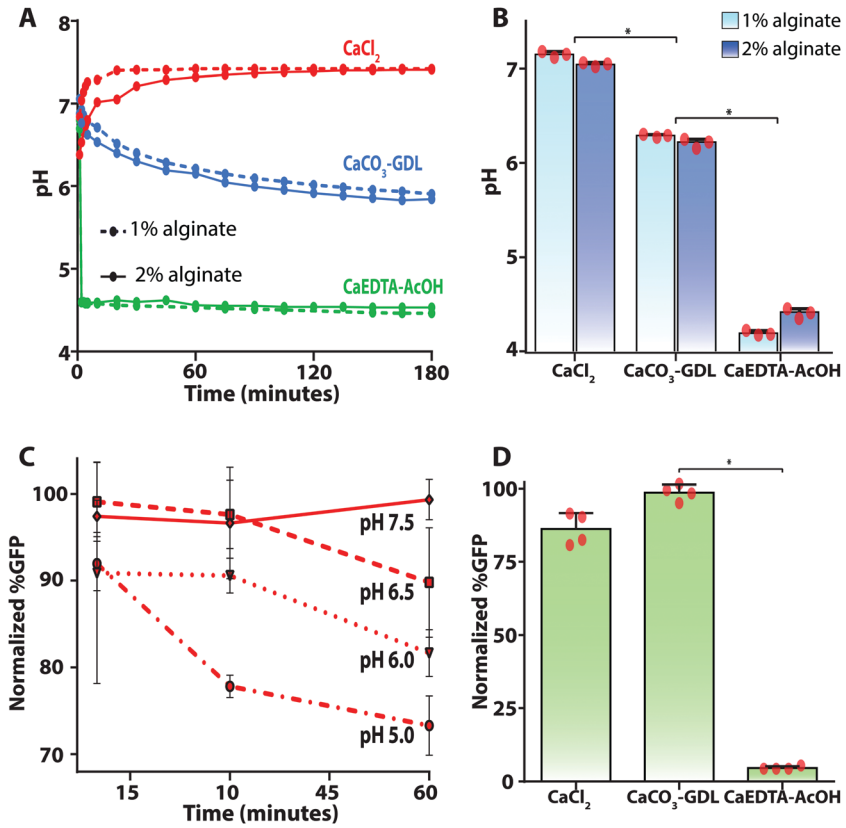

Fig. 3 Lentivector sensitivity to hydrogel gelation environment. The pH profile during hydrogel gelation was monitored in macroscopic gels (A) and the pH after 1 hour demonstrates a minimal pH drop for $CaCO_3$–GDL hydrogels (B). Lentivectors demonstrated a loss of function after pre-incubation for increasing amounts of time in acidified media prior to transduction of HEK-293T cells (C). Lentivectors were encapsulated within alginate microgels and immediately released *via* hydrogel digestion and the transduction potential of the lentivector was analyzed *via* flow cytometry (D). * indicates statistically significant differences ($P < 0.005$). Bar represent mean, scatter dot plots displays individual measurements and error bars represent standard deviation. (A, $n = 2$; B, $n = 3$; C and D, $n = 4$).

microgels (1% alginate). For both formulations, the total amount of p24 capsid protein recovered from digested microgels was close to the initial amount loaded with encapsulation efficiencies of 1.3 ± 0.4 and 1.1 ± 0.1 for the $CaCO_3$–GDL and $CaCl_2$ microgels respectively (Fig. 4A). In parallel to the encapsulation efficiency, the release of lentivectors was measured over the course of 10 days (Fig. 4B). Here, a more gradual release was observed for the $CaCl_2$ microgels compared with a faster initial release observed with the $CaCO_3$–GDL microgels. After 6 hours, 7.3% of the encapsulated p24 capsid protein was released from $CaCl_2$ microgels compared with 36.1% released from the $CaCO_3$–GDL microgels. However, following this initial burst, the release from $CaCO_3$–GDL microgels plateaued. Consequently, over the course of 10 days, both formulations had released approximately 60% of the loaded lentivectors.

Lentivector transduction from alginate microgels

Finally, we evaluated the potential and efficiency of the alginate microgels to serve as a controlled release system for lentivectors that retain their transduction activity over time (Fig. 5). Lentivector-loaded microgels were suspended above HEK-293T cell monolayers over the course of four days. A new monolayer of cells was exposed to the lentivector-loaded microgels every 24 hours. As a positive control, lentivectors in suspension were used and demonstrated efficient transduction (95%). Successful GFP expression was observed in cells brought into contact with both the $CaCO_3$–GDL and $CaCl_2$ microgels. The gene expression as assessed *via* fluorescence microscopy (Fig. 5A) demonstrates the preservation of lentivector function and ability to escape from the microgel meshwork. In parallel, quantification of GFP expression *via* flow cytometry revealed a difference in the relative profiles of transduction brought about by the two formulations (Fig. 5B). For both formulations, transduction efficiency was highest during the first 24 hours of lentivector release. However, this initial burst release was greater for $CaCO_3$–GDL microgels. For cells exposed to the first 24 hours of lentivector release there was significantly higher transduction from the $CaCO_3$–GDL microgels (51%) compared with the $CaCl_2$ microgels (18.9%). Following this high level of transduction, the percentage of cells transduced by the $CaCO_3$–GDL microgels dropped to 33% and then 4.8% for cells in contact with microgels during release times of 24–48 hours and 48–72 hours respectively. Little transduction was observed on subsequent days (<1%). In comparison, the $CaCl_2$ microgels sustained a more consistent level of transduction with 7.1% at 24–48 hours, 10.3% at 48–72 hours, 5.9% at 72–96 hours, and 6.7% at 96–120 hours.

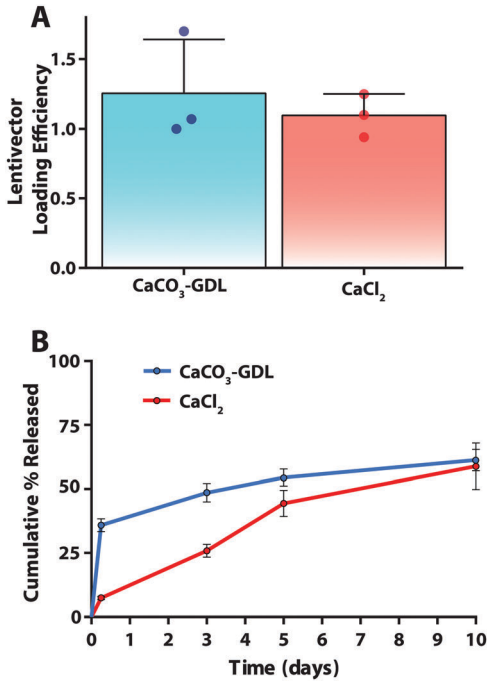

Fig. 4 Lentivector encapsulation efficiency and release profiles. Efficient loading of lentivectors within microgels was measured for microgel formulations (A). The release profile of encapsulated lentivectors was evaluated in terms of p24 capsid protein over the course of ten days indicating a more controlled release with the $CaCl_2$ microgels (B). On A, bar represent mean, scatter dot plots display individual measurements and error bars represent standard deviation. (A, n = 3; B, n = 2–3).

Discussion

The results of this study demonstrate the capability of alginate microgels, fabricated using microfluidic technology, to successfully encapsulate and release functional lentivectors for gene delivery *in vitro*. Three alginate gelation strategies were successfully combined with microfluidic templating to generate microgels. While these procedures yielded monodisperse, spherical structures with high loading efficiencies, discernable differences between the three in overall mechanical properties, lentivector release, and cellular transduction were observed. Together, these results highlight microgel crosslinking and pH control as key variables for efficient delivery of lentivectors, and identify two potential platforms for an alginate-microgel lentivector delivery system.

The main objective of this work was to identify suitable strategies for encapsulating lentivectors in alginate microgels for controlled release and cellular transduction. We have adapted three microfluidic gelation strategies towards this goal.[41,45,46] Alginate is emulsified into droplet templates and crosslinked using either internal or external ionotropic gelation which yields, for all three strategies, microgels with tight control over size and morphology. While successfully generating microgels, the three gelation strategies are distinct in the mechanical properties they impart on the final hydrogel, which will in turn affect the expected release profiles of encapsulated cargo. The diameter of the microgels varied slightly between

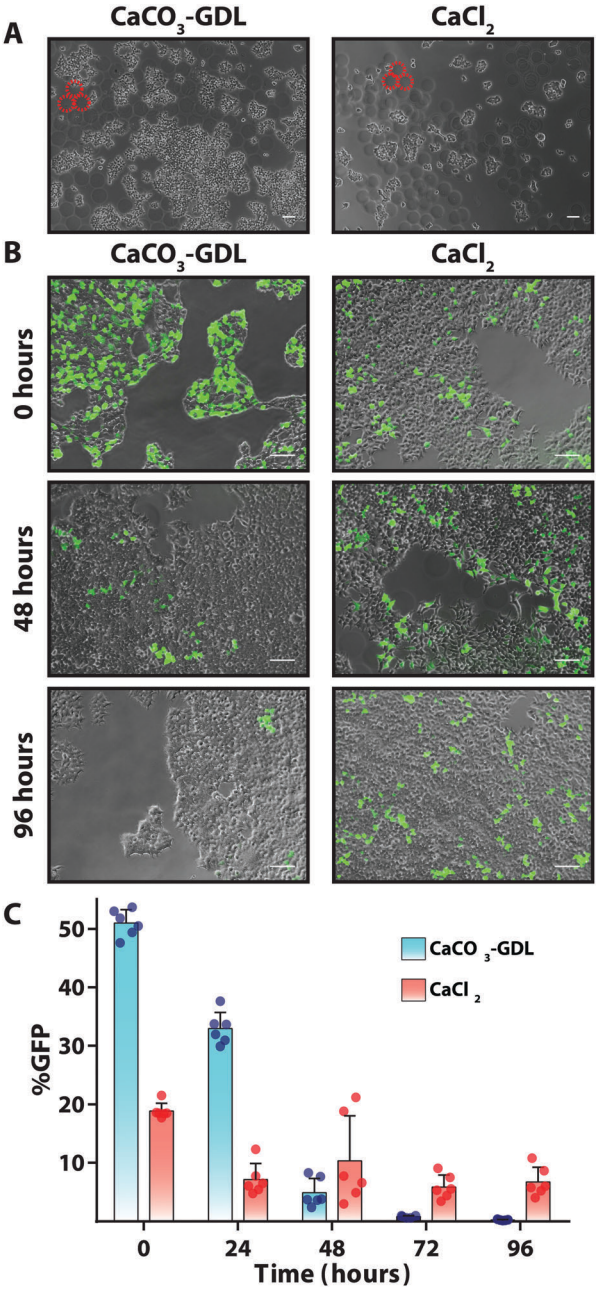

Fig. 5 *In vitro* transduction over time *via* lentivectors released from alginate microgels. Phase-contrast images of lentivectors-loaded microgels in contact with HEK-293T cells with three representative microgels outlined in red-dashed line (A). Representative merged phase-contrast/fluorescent photomicrographs of HEK-293T cell monolayers after exposure to lentivector-loaded microgels encoding for encoding for GFP at 0, 2 and 4 days after encapsulation (B). Lentivector-loaded microgels were placed in contact with a series of HEK-293T monolayers (contact time 24 hours) and the GFP expression of these cells was then analyzed by flow cytometry (C). Calibration bar represents 100 μm. Bar represent mean, scatter dot plots displays individual measurements and error bars represents standard deviation. (C, n = 6).

the three gelation schemes despite flow focusing occurring in all cases through a 100 × 100 μm junction. The final size of the microgels depends on the size of the emulsion template and

the degree of crosslinking. In practice, the size of the emulsions can be tuned by altering the relative flow rates of the oil and aqueous phases or changing the size of the flow focusing junction (data not shown). External gelation produced the smallest diameter microgels, highest storage moduli, and lowest degree of swelling. In macroscopic systems, and likely here in the microfluidic system, external gelation creates a gradient of polymer and crosslinking concentration and makes control over total calcium content and gelation rates difficult to achieve.[40,57,58] In comparison, internal gelation allows for a consistent calcium to carboxyl molar ratio to be set.[40] The resulting microgels exhibited larger diameters, diminished storage moduli, and higher degrees of swelling in comparison to external gelation. Specifically, these differences were significantly pronounced for CaEDTA–AcOH in comparison to $CaCO_3$–GDL. This discrepancy may be due to gelation kinetics and the extent to which the sequestered calcium is fully released. Alginate crosslinked at slower rates produces hydrogels of increased mechanical integrity which has been attributed to an increased order of the network structure.[31,40,59] $CaCO_3$–GDL hydrogels undergo gradual crosslinking, as was evident here by the evolution of the storage modulus over time, due to the gradual hydrolysis of GDL to gluconic acid and concomitant gradual release of calcium from the carbonate salt.[40,45,60] In comparison, CaEDTA–AcOH gelation occurred rapidly alongside a drop in pH, and it is possible that the reversible nature of calcium–EDTA interactions may inhibit gelation to some extent.

In addition to differences in mechanical properties the three gelation strategies impart different encapsulation environments. These environmental conditions (temperature, pH, ionic strength, shear stress, *etc.*) to which lentivectors will be exposed can dramatically affect stability and infectivity.[61] Previous studies have shown a severe reduction in the half-life of VSV-G pseudotyped retroviral vectors at a pH of 6.[62] Similarly, ecotropic moloney murine leukemia virus has a narrow window of infectivity between a pH of 5.5 and 8.0 with irreversible damage outside of this range.[63] Here, the need for acid-mediated cation release for both internal gelation strategies becomes an important consideration for developing a viable encapsulation strategy. While both internal gelation strategies require an acid, the pH of the $CaCO_3$–GDL is controlled by setting a stoichiometric ratio of GDL to $CaCO_3$. The pH drop is minimal as GDL is hydrolyzed to gluconic acid and then further neutralized by reaction with calcium carbonate. Conversely, dissociation of calcium–EDTA complexes is dependent upon a sharp drop in pH.[64] A marked loss of transduction efficiency was observed for lentivectors encapsulated within CaEDTA–AcOH microgels, and accordingly this system was removed from further consideration as a viable lentivector delivery system for this study.

The control of lentivirus release can be governed based on three key aspects of a hydrogel system: (i) the diffusivity of the vector within the hydrogel; (ii) the degradability of the hydrogel; and (iii) the affinity between the polymers used and the vector.[11] However, the exact mechanism for lentivector release from alginate hydrogels remains to be elucidated. Diffusion of lentivectors through an alginate meshwork will be severely diminished due to the relative size of lentivectors ($\sim$100 nm)[65] and alginate mesh size ($\sim$10 nm).[66–68] Nevertheless, we have observed release of lentivectors from both macroscopic alginate hydrogels[14] and microgels. For microgels it is possible that increased surface area contributes to the amount of lentivectors released. Additionally, the reported mesh sizes may not completely prevent the transport of such particles through the mesh.[54,69,70] This measurement provides only an approximation of an idealized meshwork structure, and in reality does not account for network imperfections such as closed polymer loops, dangling ends, and slipping chain entanglements.[71]

Interestingly, the profiles of lentivector release and cellular transduction suggest that the mode of gelation and extent of crosslinking may be important parameters in regulating lentivector delivery from microgels. While a large burst release was observed for $CaCO_3$–GDL microgels, a more gradual and sustained released was achieved using $CaCl_2$ microgels. The calculation of mesh sizes revealed a more compact network for the tightly crosslinked $CaCl_2$ microgels. In addition to a tighter network, the gradient crosslinking that occurs with external gelation has been correlated to decreased surface permeability.[72] Together, these phenomena may explain the slower release and sustained transduction that was observed with $CaCl_2$ microgels, but further studies within one gelation strategy will be needed to elucidate the role that these parameters play in lentivector release. Encouragingly, these studies provide a foundation for creating microgels with different kinetics of lentivector release. As such, future studies can aim to generate systems where multiple patterns of lentivector delivery can be incorporated into a single mixture of microgels.

Conclusions

In summary, we have for the first time investigated microfluidic templating as a strategy to encapsulate lentivectors within alginate microgels. We directly compared three processes and have identified that internal gelation with $CaCO_3$–GDL and external gelation with $CaCl_2$ are suitable for creating lentivector compatible microgels. The profiles of lentivector release from these microgels correlated with measured mechanical properties and suggest that the overall crosslinking density and homogeneity of alginate microgels can be used as tunable parameters to adjust the release of lentivectors. This strategy can be advantageous for the controlled delivery multiple vectors as it becomes possible to engineer suites of microgels with desired release rates. Given the flexibility of genes that can be transduced with lentivectors, the engineering of such delivery systems may provide benefit across a wide range of therapeutic applications.

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Disclosures

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