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Injectable alginate hydrogel for enhanced spatiotemporal control of lentivector delivery in murine skeletal muscle

Roberta S. Stilhano [a, 1], Justin L. Madrigal [b, 1], Kevin Wong [b], Priscilla A. Williams [b], Priscila K.M. Martin [a], Fabio S.M. Yamaguchi [c], Vivian Y. Samoto [a], Sang W. Han [a, *], Eduardo A. Silva [b, **]

[a] *Department of Biophysics, Federal University of São Paulo, São Paulo, Brazil*
[b] *Department of Biomedical Engineering, University of California, Davis, CA, USA*
[c] *Department of Internal Medicine, Irmandade da Santa Casa de Misericórdia de São Paulo, São Paulo, Brazil*

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ABSTRACT

Hydrogels are an especially appealing class of biomaterials for gene delivery vehicles as they can be introduced into the body with minimally invasive procedures and are often applied in tissue engineering and regenerative medicine strategies. In this study, we show for the first time the use of an injectable alginate hydrogel for controlled delivery of lentivectors in the skeletal muscle of murine hindlimb. We propose to alter the release rates of lentivectors through manipulation of the molecular weight distribution of alginate hydrogels. The release of lentivector was tested using two different ratios of low and high molecular weight (MW) alginate polymers (75/25 and 25/75 low/high MW). The interdependency of lentivector release rate and alginate degradation rate was assessed *in vitro*. Lentivector-loaded hydrogels maintained transduction potential for up to one week *in vitro* as demonstrated by the continual transduction of HEK-293T cells. Injection of lentivector-loaded hydrogel *in vivo* led to a sustained level of transgene expression for more than two months while minimizing the copies of lentivirus genome inserted into the genome of murine skeletal muscle cells. This strategy of spatiotemporal control of lentivector delivery from alginate hydrogels may provide a versatile tool to combine gene therapy and biomaterials for applications in regenerative medicine.

1. Introduction

Gene therapy is the transfer of genetic material into cells with the aim of altering the course of a medical condition or disease [1]. The delivery of genetic cargo to a target tissue allows for the endogenous production of tissue inductive factors which can bypass limitations in timing and dose that are encountered with protein delivery. In addition, proteins produced endogenously are subjected to post-translational modifications that can increase biological activity and minimize immune response [2]. Viral vectors remain the most clinically used approach for gene delivery [3], and in particular, lentivectors have been used in an increasing amount of clinical trials [4,5]. Lentivirus is a genus of viruses of the Retroviridae family that has the ability to transduce both dividing and non-dividing cells, accept large genes in their construction, and integrate the delivered genes into host chromosomes to enable long-term expression [6–8]. Key challenges to clinical translation revolve around achieving safety, sufficient expression, and controlling the presentation of lentivectors to cells allow for regulation of lentivector integration. However, current delivery strategies rely upon simple injections that do not allow for control in lentivector presentation. By designing biomaterial systems for lentivector delivery, control over the timing and localization can be improved.

Polymeric systems that allow localized and sustained presentation of lentivectors may enable one to address limitations of current delivery strategies [9]. In particular, hydrogels are an especially appealing class of delivery vehicle, as they can be introduced into the body with minimally invasive procedures, and are often highly biocompatible due to their high water content. Recent studies have demonstrated the utility of both synthetic and naturally occurring hydrogels including poly(ethylene glycol), fibrin, and collagen for the delivery of lentivectors and transduction of cells [10–12]. In these studies, cellular transduction within the confines of the hydrogel was promoted. Exogenous cells seeded within the hydrogel, or endogenous cells that infiltrated *in vivo* were efficiently transduced, with better efficacy demonstrated when lentivectors were retained within the hydrogel. However, the reliance on either exogenous cellular sources or cellular infiltration is not always desirable. *Ex-vivo* manipulation adds technical and safety complications, and cellular infiltration can include off-target cell populations such as macrophages and dendritic cells [13]. One alternative strategy could be to transduce cell populations located in the immediate vicinity of the hydrogel.

Alginate hydrogels have been extensively used in the controlled release of different therapeutics including genetic cargos [14–16]. Alginate is a naturally occurring polysaccharide, derived from brown algae, composed of polyguluronate and polymannuronate repeating blocks [17]. Alginate demonstrates low immunogenicity, mild

* Corresponding author.
** Corresponding author at: Department of Biomedical Engineering, University of California, 1 Shields Ave. Davis, CA 95616, USA.
Email addresses: sang.han@unifesp.br (S.W. Han); esilva@ucdavis.edu (E.A. Silva)
[1] These authors contributed equally to the current study.

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crosslinking chemistry, and gentle protein and cell encapsulation, but does not support cellular infiltration without modification [17–19]. In the absence of infiltration, the delivery of therapeutics to surrounding cells becomes dependent on release from the hydrogel. This release can be governed by diffusion and hydrogel degradation. Alginate is not enzymatically digested by mammals, and without modification, alginate hydrogels will only degrade in an uncontrolled manner as divalent ions are exchanged with surrounding media [20]. However, extensive work has established several strategies for achieving and controlling the degradation of alginate hydrogels, including mismatching the cross-linking junction size, crosslinking with hydrolytically labile linkers, oxidation of the polymer backbone, and alteration of polymer molecular weight distribution [21–25].

Here, we investigated the encapsulation of lentivectors within alginate hydrogels as a simple strategy to achieve localized and sustained release and subsequent transduction of local endogenous cells. We hypothesized that the mild gelation process would preserve the activity of the virus and that modulation of vector release would be possible through control of hydrogel degradation. We have combined polymer backbone oxidation and a binary molecular weight formulation to produce hydrogels with preserved biocompatibility and release kinetics influenced by hydrogel degradation. Vector encapsulation, release, and activity were studied *in vitro* and the extent and the duration of transgene expression after injection of lentivector-loaded hydrogel *in vivo* was compared to bolus delivery in a murine hindlimb. This strategy for lentivector delivery may prove useful for a wide range of gene delivery applications as the gene sequence within the viral vector can be easily changed without the need for redesigning the delivery system.

2. Materials and methods

2.1. Lentivector production

The plasmids M107 (transfer vector containing Green Fluorescent Protein (GFP), M334 (Gag-Pol), REV, and M5 (VSV-G) envelope used for the lentivector construction were kindly provided by Professor M.D. V. Laer (Department of Hygiene, Microbiology, Social Medicine Medical University). The plasmid pLenti CMV V5-LUC Blast (21,474) was purchased from Addgene. Viral vector production, concentration, and titration were performed following a protocol established by Naldini et al. [6]. In brief, lentivectors were produced in human embryonic kidney (HEK-293T) (ATCC) cells cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin-streptomycin (Invitrogen) 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 in the presence of 8 μg/mL of Polybrene (Sigma). After 3 days, the HEK-293T cells transduced with lentivector expressing GFP were counted (> 10,000 events analyzed) using a FACScan cytometer (Becton Dickinson – BD) and the data was analyzed using FlowJo software (TreeStar Inc.). The HEK-293T cells transduced with lentivector expressing luciferase were selected for using blasticidine (6 μg/mL) after 15 days in culture. The cell colonies were stained using Brilliant Blue G (Sigma) for positive identification. 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 29 ± 3.5 pg/TU was measured which indicates efficiency in viral packaging [26,27].

2.2. Alginate hydrogel formulations and in vitro degradation

Unary and bimodal alginate hydrogels were formulated using both low and high molecular weight (MW) alginate polymers. Ultrapure alginate (Pronova UP MVG; FMC) was utilized as the high molecular weight (~ 250 KDa) (HMW) component. Low molecular weight alginate (~ 50 KDa) (LMW) was obtained by gamma (γ)-irradiating the HMW alginate polymer at a γ-dose of 3.0 Mrad as specified by Silva et al. [28]. Unary hydrogels were composed of unoxidized, 100% HMW alginate polymer. In the case of bimodal hydrogels, both low and high molecular weight alginate polymers were oxidized (1% of the sugar residues in the polymer chains) with sodium periodate (Sigma) as previously described [28]. Two different alginate polymer formulations were ionically crosslinked to create bimodal hydrogels, including either a ratio of 75/25 or 25/75 (LMW/HMW). To prepare hydrogels, sterile lyophilized alginates were reconstituted to 2% (w/v) and stirred overnight. Gelation occurred when the alginate solution was mixed with 0.21 g/mL calcium sulfate (Sigma) slurry (25:1 alginate to calcium solution volume ratio) *via* a syringe connector. Subsequently, the alginate gel was cast between glass plates and allowed to gel for 30 min at room temperature. After this time elapsed, a dermal biopsy punch (Acuderm) was used to create alginate hydrogel disks (10 mm diameter, 1 mm thick). These disk specifications were used for all *in vitro* studies unless otherwise indicated. The degradation rate of the two binary MW alginate hydrogels was evaluated by the dry mass loss (as percentage of initial dry mass) following incubation in a saline solution at 37 °C for various amounts of time (n = 4/time point/condition). At each time point, the alginate disks were lyophilized and an analytic scale was used to measure their weight (Mettler Toledo). During these experiments, alginate disks were maintained under sterile conditions to prevent bacterial and fungal contamination. When appropriate, lentivectors (2.55×10^6 TU/mL) were loaded into the alginate solution prior to gelation. All alginate solutions were made fresh for each experiment and were maintained on ice or at 4 °C during experimental procedures.

2.3. Quantification of lentiviral transduction in vitro

For all of the experiments detailed below, a consistent number of HEK-293T cells were seeded in 24-well plates (3×10^4 cells/well) and cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin for 24 h at 37 °C and 5% CO_2 prior to any contact with lentivectors. Alginate disks loaded with 2.5×10^5 TU of lentivectors were placed in 24-well plates that were previously seeded with HEK-293T cells and cultured for 6 h in order to capture the initial levels of release (burst release). After 6 h, the alginate disks were transferred to a new set of 24-well plates with HEK-293T cells and incubated for an additional 18 h. Subsequently, the alginate disks were transferred to a new set of 24-well plates with HEK-293T cells every 24 h over a period of 6 days. During each transfer of hydrogel disks, the media surrounding the disk was also added to a fresh monolayer of cells. HEK-293T cells from each time point, including those that were in contact with the disk or disk-media, were incubated for 48 h in total prior to quantification of transduction efficiency as is consistent with previous studies [29,30]. Positive and negative controls were used in these studies for appropriate experimental validation. In the negative control wells only supplemented DMEM was added. In the positive control wells supplemented DMEM with lentivector in suspension (2.5×10^5 TU) 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 using the FACScan cytometer and analyzed using FlowJo (> 10,000 events/ experimental condition).

2.4. Real time PCR

Total genomic DNA (gDNA) was extracted from HEK-293T cells ($3–5 \times 10^5$ cells) using the Qiamp Kit (Qiagen) and a quantitative RT-PCR assay was performed using QuantiFast SYBR Green RT-PCR Kit (Qiagen) in a Mastercycler RealPlex (Eppendorf). All preparation and collection of samples were conducted according to the manufacturer's protocols. Two different primers were used including, WPRE_F: 5′-CGC TGC TTT AAT GCC TTT GT-3′, WPRE_R: 5′-GGG CCA CAA CTC CTC ATA AA-3′. The number of copies per cell was calculated as previously described [30].

2.5. Quantification of lentivector release from alginate hydrogel disks

Alginate hydrogel disks were individually immersed within DMEM supplemented with 10% FBS in single wells of a 24-well plate and incubated at 37 °C and 5% CO_2 to allow for lentivector release. At selected time points, 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. After 6 days, the concentrations of lentivector within collected supernatant samples were quantified using an HIV p24 Antigen ELISA Kit (ZeptoMetrix Co) according to the manufacturer's guidelines and protocol. Lentivector encapsulation efficiency was determined by immediately digesting hydrogel disks with 10 units/mL of alginate lyase (Sigma Aldrich) and comparing the digest and precursor solution lentivector concentrations by ELISA.

2.6. In vivo transduction by lentivectors released from alginate hydrogels

All animal work described here was performed in full compliance with the institutional guidelines and was approved by the Research Ethics Committee of the Universidade Federal de São Paulo, Brazil (Approval number: CEP 0347/12). Female Balb/c (24–26 g; 12 weeks old purchased from the Instituto Nacional de Farmacologia, São Paulo, Brazil) were housed with *ad libitum* access to normal diet and water, and maintained on a 12 h light/dark cycle.

For all *in vivo* studies, 8.8 x 10^7 TU were used in each animal and 2% (w/v) alginate solutions (75% LMW, 1% oxidized; 25% HMW, 1% oxidized) crosslinked with calcium sulfate (0.21 g/mL, ratio of 25:1 alginate to $CaSO_4$) were used. Luciferase gene carrying lentivectors were either loaded into alginate hydrogels or suspended in DMEM (8.8×10^7 TU in 50 μL) and subsequently injected into the left hindlimb (*Gastrocnemius*). Local bioluminescence expression was monitored using an IVIS imaging system (PerkinElmer). For imaging, animals were anesthetized with ketamine (100 mg/kg body weight) and xylazine (10 mg/Kg body weight) and D-Luciferin (150 mg/kg body weight, 20 mg/mL in PBS; Promega) was injected intraperitoneally (IP). These animals were placed in a light-tight chamber and bioluminescence images were acquired (every 2 min for a total of 20 min) until the peak light emission was reached. Gray scale and bioluminescence images were superimposed using the Living Image software (PerkinElmer). A consistently sized region of interest was drawn over the scaffold implantation site and at another site on the top of the animal to serve as background. The signal intensity was reported as an integrated light flux (photons/s) subtracting background, which was determined by IGOR software (WaveMetrics). After 7 days and 77 days post lentivector injection, mice were humanely euthanized prior to D-luciferin injection and several organs were removed and imaged including the heart, liver, hindlimb muscle and control hindlimb muscle, kidneys, spleen, and lungs.

2.7. PCR from genomic DNA

The Qiamp kit (Qiagen) was used to extract the gDNA from the collected organs. The PCR reaction was performed using the Taq Platinum DNA polymerase (Life Technologies) and primers for luciferase (Luciferase_F: 5'CAA CTG CAT AAG GCT ATG AAG AGA3' and Luciferase_R: 5′ ATT TGT ATT CAG CCC ATA TCG TTT3'). Murine Glyceraldehyde 3-phosphate dehydrogenase primers (GAPDH_F: 5'ACC ACA GTC CAT GCC ATC AC3' and GAPDH_R: 5'TCC ACC ACC CTG TTG GTG TA3') were used as an endogenous control as previously described [31].

2.8. 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 $P < 0.05$.

3. Results

3.1. In vitro lentivector release from degradable alginate hydrogels

Alginate hydrogels with predicted degradation rates can be formulated by combining high and low MW polymers that have been partially oxidized resulting in the formation of the polymer chains liable to hydrolysis [21,28]. The application of such bimodal alginate hydrogels has been shown to be particularly useful in obtaining spatiotemporal control of release of pro-angiogenic growth factors [28,32], but the utility to deliver genes has not yet been reported to our knowledge. We first assessed the ability of these degradable alginate hydrogels to serve as delivery vehicles for sustained lentivector release *in vitro* (Fig. 1). The degradation rates of two distinct binary molecular weight alginate gels were evaluated by dry weight loss over time (Fig. 1A). The increase of LMW alginate content resulted in a rapid and significant increase of mass loss. By day 7, the 75/25 (LMW/HMW) hydrogel displayed a dry mass loss of ~ 50%. In contrast, the dry mass of the 25/75 (LMW/HMW) hydrogel only dropped by ~ 20%. To confirm the expected correlation between gel degradation rate and lentivector release rate, lentivectors were loaded into alginate hydrogels and its release was monitored over time *via* an ELISA for the p24 antigen. All three formulations successfully encapsulated the lentivectors with comparable efficiencies of 77%, 84%, and 82% for 75/25, 25/75, 0/100 (LMW/HMW) formulations respectively. Following an initial burst, lentivector was released in a sustained fashion over ~ 1 week (Fig. 1B). The 75/25 (LMW/HMW) alginate hydrogels released approximately 65% of the total lentivector loaded within that period of time as compared to the slower release (~ 45% of total) displayed by the 25/75 (LMW/HMW) hydrogel formulation. Finally, the unoxidized unary formulation of HMW alginate displayed the slowest release profile as compared with the binary formulations. In all subsequent experiments we decided to use the 75/25 (LMW/HMW) alginate hydrogels due to the more complete re-

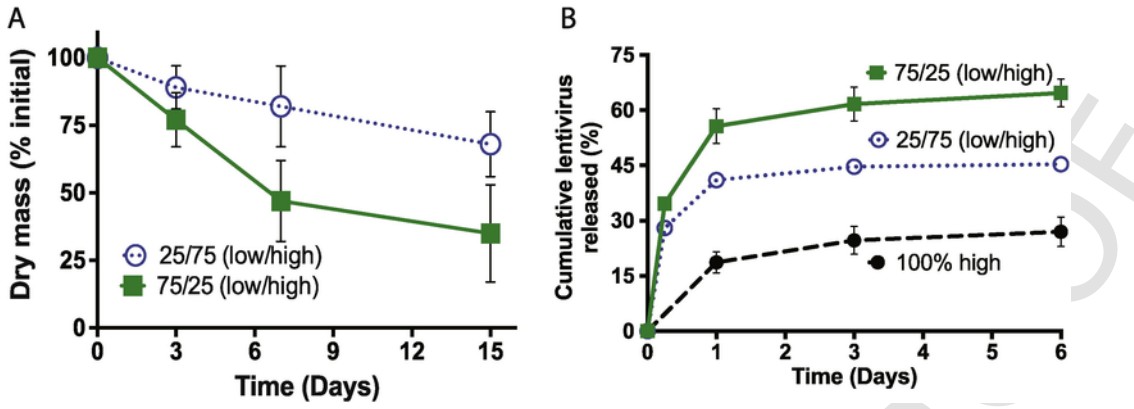

Fig. 1. *In vitro* degradation and lentivector release from binary MW alginate hydrogels. The dry mass losses (as percentage of initial dry mass) of 75/25 low/high MW (1% oxidized) (■) and 25/75 low/high MW (1% oxidized) (○) formulations were monitored *in vitro* over the time (A, n = 4). The 75/25 low/high MW (1% oxidized) alginate hydrogels (■) displayed faster lentivector release kinetics as compared to the 25/75 low/high MW (1% oxidized) gels (○), and unoxidized unary high MW (●) displayed the slowest release (B, n = 3). Values represent mean and standard deviation.

lease observed and we broadly refer to this formulation as alginate hydrogels for clarity.

3.2. In vitro lentivector transduction from alginate hydrogels

We next evaluated the potential and efficiency of the alginate hydrogels to serve as an injectable system to deliver lentivectors that retain their transduction activity over time (Fig. 2). HEK-293T cells were placed either in direct contact with alginate hydrogels loaded with lentivectors encoding for GFP or with the media surrounding these hydrogels. Successful GFP transduction was continuously observed for 6 days as assessed *via* fluorescence microscopy (Fig. 2A) demonstrating the ability of the lentivectors to escape from the hydrogel matrix while retaining infectivity. Further quantification of GFP transduction *via* flow cytometry confirmed the sustained release of the lentivector over the course of 6 days as indicated by continued transduction of fresh HEK-293T monolayers (Fig. 2B). During the initial 6 h, approximately 15% of the HEK-293T cells cultured with the lentivector-loaded alginate disks were GFP positive. Sustained transduction over time was then observed with GFP positive cells comprising 13% of HEK-293T at day 1, 8% at day 2, and 6% at day 3. In addition, the number of copies of lentivector genome per cell was also evaluated *via* PCR (Fig. 2C). As expected, the trends observed for the number of copies/cell correlated with the fluorescent expression and the percentage of GFP positive cells.

3.3. In vivo lentivector transduction from alginate hydrogels

We next compared the functionality of the injectable alginate hydrogel *versus* bolus suspension to promote gene delivery *in vivo* in the hindlimb muscle of mice (Fig. 3). Expression at the injection site was continuously monitored for 77 days in a non-invasive fashion using the IVIS system. Two days after injection, animals treated with bolus lentivector displayed bioluminescence in the injected hindlimb muscle (Fig. 3A). In contrast, bioluminescent signal was not present at day 2 for lentivector-loaded hydrogel delivery but was later observed from day 7 onwards. A difference in temporal response was noted between the two experimental groups. The maximal transgene expression for the lentivector suspension group was observed at day 14 post injection while that of the alginate + lentivector group was observed at day 21 (Fig. 3B). Strikingly, the animals injected with the alginate hydrogel loaded with lentivector displayed a sustained bioluminescence signal for 42 consecutive days (from day 21 to day 63). In contrast, the animals injected with bolus lentivector suspension exhibited a gradual decrease of transgene expression after the day 14 peak, returning to background levels by around day 49. No transgene expression was observed *via* IVIS for any animal group at day 77.

Finally, to investigate the levels of transgene expression in different organs, the bioluminescent signal was evaluated post-mortem in the liver, lungs, heart, kidneys, spleen, and both the control (non-injected) and injected hindlimb muscles at day 7 and day 77 (Fig. 4). No transgene expression was detected for off target organs for both early and late time points (Fig. 4A). Only the hindlimb muscles injected with alginate hydrogel loaded with lentivector harbored bioluminescence at day 77 (Fig. 4B). Hindlimb muscles treated with bolus lentivector displayed no signal increase compared to the background signal obtained from the control hindlimb muscle. Although luminescence signal was not observed with the IVIS imaging system in the remaining organs of interest, there still exists the possibility of lentivector integration in the genome of these organs. To analyze the genomic integration, PCR using specific primers for the Luciferase gene was performed (Fig. 4C). PCR confirmed that the viral genome was only present within the hindlimb muscles injected with either alginate + lentivector or bolus lentivector. Further quantification demonstrated that the hindlimb muscles of the animals treated with bolus lentivector presented a significantly higher number of lentivector genome copy number as compared with the animals treated with alginate hydrogels loaded with lentivector (Fig. 4D). No presence of lentivector genome was found in any other organs of interest.

4. Discussion

The results of this study indicate that lentivectors delivered from injectable alginate hydrogels are capable of promoting long-term gene expression in cells located in the murine hindlimb muscle. Alginate hydrogel degradation can be tuned by combining partial oxidation and a binary MW distribution. Altering the MW composition modulated the *in vitro* release kinetics of the lentivectors. As opposed to bolus delivery, the lentivectors delivered from an injectable alginate hydrogel induced noticeable gene expression even after 11 weeks post *in vivo* injection. Furthermore, the results of this study suggest the utility of alginate hydrogels to promote localized and safe delivery of lentivector as demonstrated by the lower copies of viral genome specific to the injected hindlimb in comparison with bolus injection.

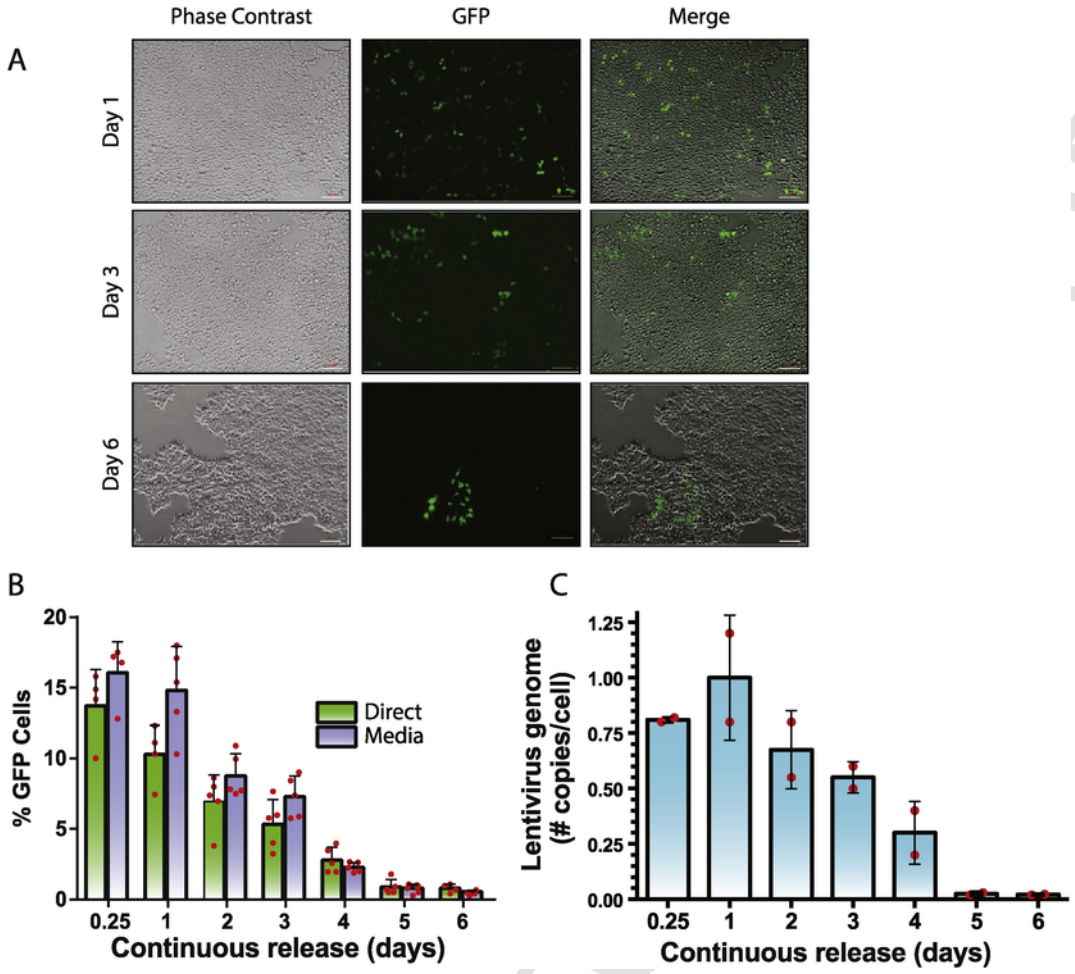

Fig. 2. *In vitro* release of lentivector expressing GFP from alginate hydrogels over the time. Representative phase-contrast, fluorescent and merged photomicrographs of HEK-293T cell monolayers placed in direct contact with alginate hydrogels loaded with lentivectors encoding for GFP 1, 3 and 6 days after transduction (A). GFP expression quantified for HEK-293T monolayers after their exposure to alginate-released lentivectors by either co-incubation with hydrogel disks or incubation with the surrounding disk media (B). The number of copies present on these HEK-293T cells was directly quantified *via* real time PCR (C). Calibration bar represents 100 μm. Bars represent mean, scatter dot plots display individual measurements and error bars represents standard deviation (B, n = 4–5; C, n = 2).

The main objective of this work was to demonstrate the utility of injectable alginate hydrogels as lentivector delivery vehicles. Alginate hydrogels have been extensively developed as vehicles for growth factor and/or cell delivery; however, comparatively few studies have extended this strategy to viral vectors [33,34]. Alginate microspheres have been used to deliver a β-galactosidase adenovirus despite vector-specific immune presence [35]. Similarly, the delivery of an oncolytic adenovirus *via* alginate hydrogel diminished the dissemination into off-target tissue and extended the biological activity of the vector [36]. In one study lentivector delivery from PLG microporous scaffolds with alginate-filled pores was investigated and shown to promote a minimal amount of transduction [13]. However, this study focused on retaining lentivectors within a scaffold for the transduction of infiltrating cells and alginate was used as a control due to the minimal amount of cellular infiltration induced by this polymer. These studies all employed alginate formulations with uncontrolled and slow degradation kinetics. Here we have investigated degradable alginate hydrogels as controlled release vehicles for lentivectors. To our knowledge, degradable alginate hydrogels have not been employed for the delivery of viral vectors including lentivectors.

The results of this study demonstrate that altering the molecular weight distribution of partially oxidized alginate can impact hydrogel degradation kinetics which in turn affects lentivector release. The mechanism for this control in hydrogel degradation has been previously reported. Partial oxidation of alginate produces hydrolytically labile acetal-like groups in the polymer backbone, allowing for chain scission and renal clearance [24]. This chain scission is faster for lower initial MW polymers [22]. By formulating binary oxidized hydrogels the degradation rate can be adjusted according to the LMW content due to faster chain breakage coupled with increased separation of crosslinked domains [21,22]. Here, we confirm that binary alginate hydrogels with a majority of low MW polymers imposed faster degradation as compared to a majority of high MW polymers. This increase in degradation correlated with more rapid release kinetics of lentivectors. While the profile of release is similar for both binary formulations, a significantly larger cumulative release was observed with higher concentrations of LMW over 6 days. As expected, hydrogels formulated with unoxidized HMW alginate, where degradation is comparatively slow and uncontrolled, showed a diminished release rate compared to both binary oxidized formulations.

Key characteristics of this system may contribute to the relationship between lentivectors release and hydrogel degradation. The nanostructure present in alginate hydrogels, with pores ranging from 5 to 200 nm [20,37], will facilitate the diffusion of small molecules

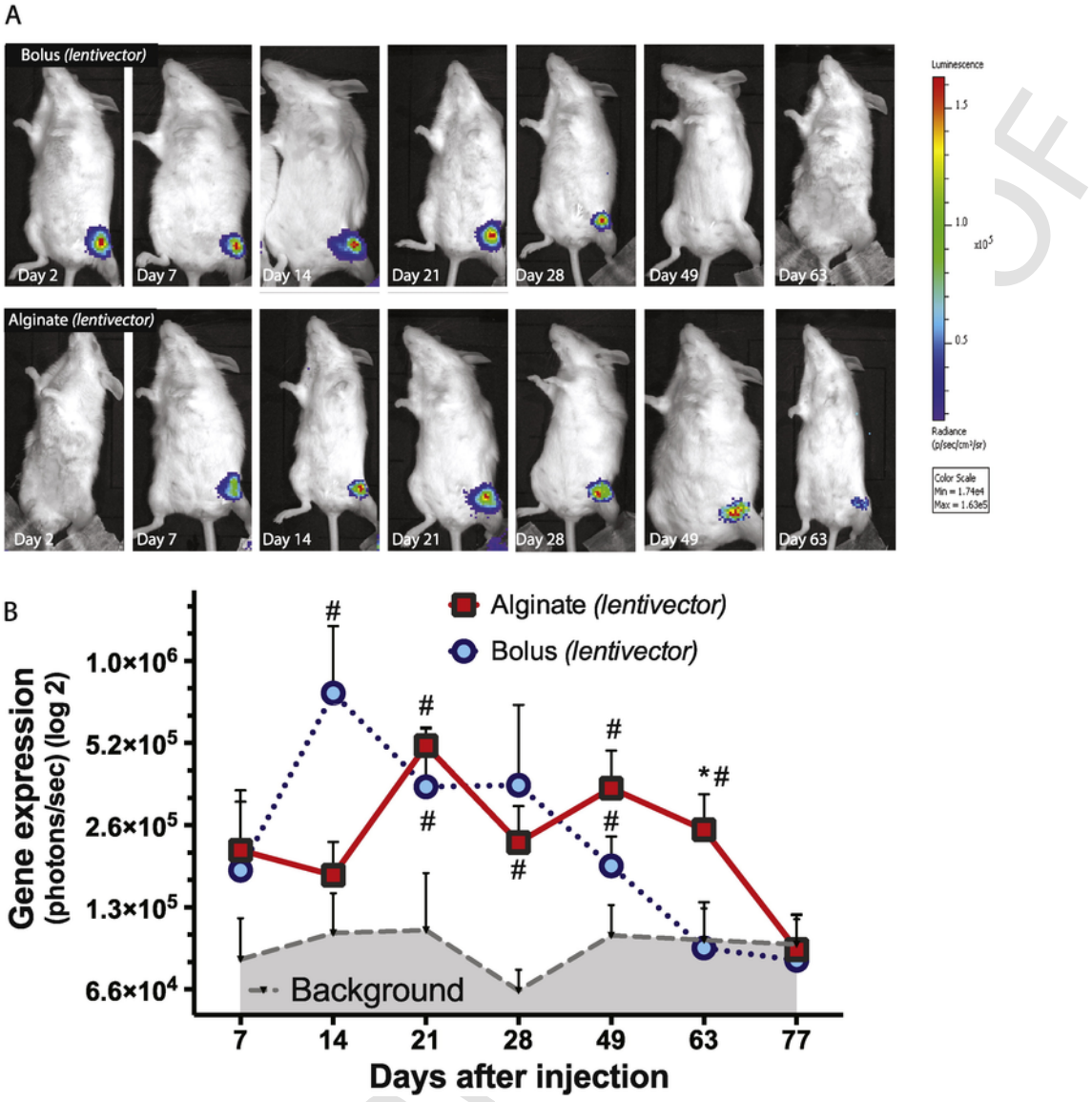

Fig. 3. Injectable alginate hydrogels loaded with lentivector expressing luciferase enabled long transduction and expression in the murine hindlimb muscle. Representative bioluminescence imaging of transgene expression for both the bolus lentivector and the lentivector-loaded into alginate hydrogels (A). Quantification of the light emission from the hindlimb regions was monitored over the time for animals treated with bolus lentivector suspension (○), alginate hydrogel loaded with lentivector (■) and control mice (background) (B). Values represent mean and standard deviation (n = 4–5). # indicates statistically significant differences ($P < 0.05$) in comparison to Background. * indicates statistically significant differences ($P < 0.05$) in comparison to bolus lentivector.

and particles more readily than larger cargo such as lentivectors (100–200 nm in diameter) [38]. Furthermore, diffusion is also regulated by vector-polymer interactions and ionically cross-linked alginate hydrogels have been reported to reversibly bind to proteins that display heparin affinity, slowing their diffusion [33,39]. Indeed, this direct correlation between heparin binding and alginate binding is likely to play a role on the rate of vector release from the alginate hydrogels. The vesicular stomatitis virus (VSV-G) proteins present in the vector envelope have a known affinity for heparin [40,41], which may slow the release from the hydrogels and increase the dependency of vector diffusion on the alginate degradation rate.

Binary alginate hydrogels led to prolonged levels of gene expression when injected *in vivo*. The bolus injection of lentivector into the hindlimb induced a rapid transgene expression which peaked at 14 days, but gradually decreased over the following 2 weeks. For the animals injected with alginate hydrogels loaded with lentivector, the peak of transgene expression was observed at day 21, but in contrast with the bolus treatment a long-term lentivector expression was then continuously observed for a total of two-months. A possible explanation for this observation can be related to an interdependency between vector release and hydrogel degradation rate. This observation is partially supported by the *in vitro* degradation and release results, despite the initial burst observed *in vitro*. Further, the timing of expression *in vivo* seems to align with the timing of hydrogel degradation where significant degradation had occurred by two weeks *in vitro*. The complex nature of the *in vivo* environment, in contrast with controlled *in vitro* conditions, may account for differences in release and increase the dependence of transduction on hydrogel degradation. Interestingly, the sustained activity of lentivector after an initial delay suggests that encapsulation within a hydrogel may serve to prolong lentivector activity.

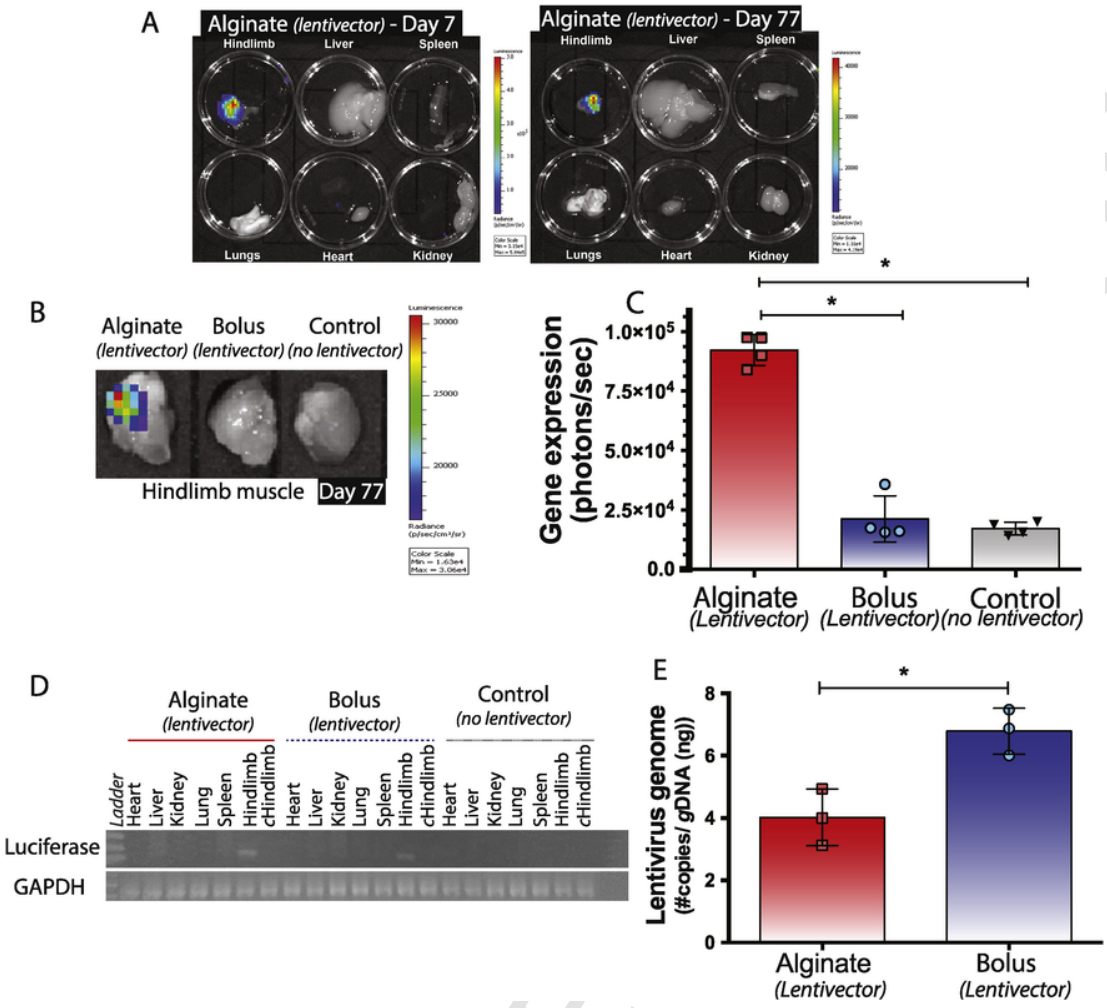

Fig. 4. Quantification of luciferase expression in different organs after 7 and 77 days post lentivector injections. Bioluminescent signal was present only in the alginate-injected hindlimb with no signal in select off-target organs of interest (A). Representative images of the hindlimb muscles denoting expression of the animals treated with alginate hydrogel loaded with lentivector 7 and 77 days post-treatment (B). Quantification of the bioluminescence levels present at the hindlimb muscle, 77 days post-treatment (C). PCR gel of the viral genome present in the different organs of interest including heart, liver, kidney, lungs, spleen, injected hindlimb muscle, non-injected control hindlimb muscle and a negative control without DNA (last line on the right) (D). Quantification *via* Real time PCR of the relative luciferase copy number found in genomic DNA (gDNA) present in injected hindlimb muscles from the animals treated with lentivector suspension and alginate hydrogel loaded with lentivector (E). Bars represent mean, scatter dot plots displays individual measurements and error bars represents standard deviation (C, n = 4; E, n = 3). * indicates statistically significant differences ($P < 0.05$) between conditions.

Previous studies using hydrogels loaded with lentivector displayed a decrease of luminescent signal after 1 month post *in vivo* application [12,13,42]. Here, we observed a sustained luminescent signal for a total of 42 days induced by the lentivector-loaded alginate hydrogel. Importantly, this study describes a sustained luciferase expression with a lower copy number of lentivector integrated compared with bolus injection. Achieving this sustained expression with a lower copy number may be significant for decreasing the risks of oncogenesis and silencing of lentiviral sequences by methylation as these risks increase with copy number [43–45]. Similar long-term luciferase expression has been previously observed using PLG scaffolds [46], but in this case the delivery vehicle was loaded with both cells and lentivectors. In contrast, the lentiviral delivery strategy described in this study targets transgene expression of endogenous cells thereby avoiding the safety and technical limitations associated with the use of exogenous cells within the vehicle. Lastly, previous approaches aimed at delivering lentivectors have been tested on either subcutaneous implantations or intraperitoneal injections [12,13,47,48]. In this study we test the function of our delivery system in skeletal muscle, which is a desirable target tissue due to the wide variety of pathologies, including peripheral artery disease and muscle fibrosis, that can be treated with muscle-based gene transfer [1,49].

5. Conclusions

In summary, this study demonstrates the capability of locally delivering functional lentivectors from alginate hydrogels. Further, it provides a proof of concept for the transduction of murine cells *via* a degradable injectable hydrogel system in the absence of cellular infiltration. The spatiotemporal vector bioavailability provided by this system led to a prolonged transduction in murine hindlimbs, and this system may also be broadly useful for the delivery of multiple genes. The engineering of such delivery systems can be used in a wide variety of applications to enhance current gene delivery potential.

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Disclosures

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2016.06.047.

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