Acta Biomaterialia 9 (2013) 4487–4495

Contents lists available at SciVerse ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Functionalized, biodegradable hydrogels for control over sustained and localized siRNA delivery to incorporated and surrounding cells

Khanh Nguyen [a], Phuong Ngoc Dang [a], Eben Alsberg [a,b,*]

[a] Department of Biomedical Engineering, Case Western Reserve University, Cleveland, OH 44106, USA
[b] Department of Orthopaedic Surgery, Case Western Reserve University, Cleveland, OH 44106, USA

ARTICLE INFO

Article history:
Received 7 June 2012
Received in revised form 8 August 2012
Accepted 8 August 2012
Available online 16 August 2012

Keywords:
Dextran
Injectable biomaterials
Controlled delivery
Photopolymerization
RNA interference

ABSTRACT

Currently, the most severe limitation to applying RNA interference technology is delivery, including localizing the molecules to a specific site of interest to target a specific cell population and sustaining the presentation of these molecules for a controlled period of time. In this study, we engineered a functionalized, biodegradable system created by covalent incorporation of cationic linear polyethyleneimine (LPEI) into photocrosslinked dextran (DEX) hydrogels through a biodegradable ester linkage. The key innovation of this system is that control over the sustained release of short interference RNA (siRNA) was achieved, as LPEI could electrostatically interact with siRNA to maintain siRNA within the hydrogels and degradation of the covalent ester linkages between the LPEI and the hydrogels led to tunable release of LPEI/siRNA complexes over time. The covalent conjugation of LPEI did not affect the swelling or degradation properties of the hydrogels, and the addition of siRNA and LPEI had minimal effect on their mechanical properties. These hydrogels exhibited low cytotoxicity against human embryonic kidney 293 cells (HEK293). The release profiles could be tailored by varying DEX (8 and 12% w/w) and LPEI (0, 5, 10 μg/100 μl gel) concentrations with nearly 100% cumulative release achieved at day 9 (8% w/w gel) and day 17 (12% w/w gel). The released siRNA exhibited high bioactivity with cells surrounding and inside the hydrogels over an extended time period. This controllable and sustained siRNA delivery hydrogel system that permits tailored siRNA release profiles may be valuable to guide cell fate for regenerative medicine and other therapeutic applications such as cancer treatment.

Published by Elsevier Ltd. on behalf of Acta Materialia Inc.

1. Introduction

RNA interference is an efficient method to post-transcriptionally turn off the expression of specific proteins and transcription factors using small interfering RNA (siRNA) for cancer therapeutics or tissue engineering applications [1–8]. Naked siRNA bears negative charges which limit its ability to passively diffuse across cell membranes and it is easily degraded by ribonucleases [1,2]. To overcome these limitations, a number of siRNA delivery systems, including liposomes, lipoplexes, nanoparticles and microparticles, have been developed to deliver siRNA to treat a wide range of diseases [1]. Unfortunately, these systems can be easily dispersed in vivo on account of their small size, making it difficult to locally target sites of interest for a prolonged period of time [1,2]. Localized and sustained delivery is a promising strategy for siRNA delivery in vivo, which may enhance its clinical applicability. For example, localized delivery would permit targeted siRNA exposure to non-malignant tumors or sites of tumor resection, which may lower the dose required for efficacy and potentially reduce effects on non-target cells [7]. siRNA delivery at a specific location in the body may also permit regulation of transplanted or host cell gene expression to aid in the regeneration of damaged or diseased tissues [8]. In addition, sustained delivery of siRNA may provide a silencing effect over an extended period of time. There are currently no injectable hydrogel systems that provide temporal control over the local delivery of siRNA to incorporated and surrounding cells.

To address the issues of rapid dispersion and transient delivery of siRNA from the aforementioned nano- or microparticle systems, we previously demonstrated sustained and localized siRNA delivery from macroscale, biodegradable polymer hydrogels [2]. Hydrogels, highly hydrated three-dimensional (3-D) networks of crosslinked hydrophilic polymer chains, have been widely explored for use as bioactive agent delivery vehicles and tissue engineering scaffolds [9–12]. Injectable hydrogels are valuable for these applications because polymer solutions can be easily mixed with bioactive agents and/or cells, administered via a minimally invasive method at desired sites and triggered to form hydrogels in situ [9]. Our previous work using photocrosslinked alginate, calcium crosslinked alginate and collagen hydrogels demonstrated that sustained and localized siRNA release could be achieved, and that the

* Corresponding author. Tel.: +1 216 368 6425; fax: +1 216 368 4969.
E-mail address: eben.alsberg@case.edu (E. Alsberg).

1742-7061/$ - see front matter Published by Elsevier Ltd. on behalf of Acta Materialia Inc.
http://dx.doi.org/10.1016/j.actbio.2012.08.012

release profiles were material dependent [2]. However, siRNA release from those hydrogels could not be easily controlled [2].

The goal of this work was to engineer the first hydrogel system that would permit controlled, localized and sustained delivery of siRNA to cells incorporated within and surrounding the biomaterial. To achieve controlled siRNA release profiles, we designed photocrosslinked dextran (DEX) hydrogels covalently functionalized with cationic LPEI molecules via a biodegradable ester linkage. It was hypothesized that siRNA could be retained within the hydrogels via electrostatic interactions between the negatively charged siRNA and positively charged LPEI, and degradation of the ester linkages would permit tunable, controlled release of siRNA/LPEI complexes. The release profiles could be tailored by regulating the degree of these interactions and controlling the degradation rate of hydrogels. For this purpose, the hydrogels were photocrosslinked from solutions of DEX methacrylate containing various concentrations of methacrylated linear polyethyleneimine (LPEI). We tested whether LPEI modification affects the hydrogel physical properties, such as swelling, degradation profiles and mechanical properties, and the viability of human embryonic kidney 293 cells (HEK293) cultured near and within the hydrogels. Hydrogels containing varying DEX, LPEI and siRNA concentrations were examined to determine the role of these parameters on siRNA release profiles. Bioactivity of released siRNA and its ability to transfect cells inside the hydrogels were also investigated to demonstrate the utility of this system with tunable delivery profiles.

2. Experimental

2.1. Materials

DEX from Leuconostoc mesenteroides (average molecular weight of 40,000 g mol^{-1}), 4-(dimethylamino)pyridine (DMAP), glycidyl methacrylate (GMA, 97% pure), 2-hydroxyethyl methacrylate (HEMA), 1,1′-carbonyldiimidazole (CDI), dimethyl sulfoxide (DMSO), chloroform, deuterium oxide (D_2O) and Irgacure D-2959 were purchased from Sigma Aldrich (St Louis, MO, USA). Linear polyethyleneimine (LPEI, 25,000 g mol^{-1}) was purchased from Polysciences Inc. (Warrington, PA). 2-hydroxylethyl methacrylate imidazolylcarbamate (HEMA-IC) was synthesized as previously reported [13]. CellTiter 96 Aqueous One Solution, which contains 3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxy-phenyl]-2-[4-sulfophenyl]-2H-tetrazolium (MTS-tetrazolium), was purchased from Promega Corp. (Madison, WI). Dulbecco's modified Eagle medium with 4.5 g l^{-1} glucose (DMEM-HG) and fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT). Accell eGFP Control siRNA, Accell green cyclophilin B control and Accell Delivery Media (ADM serum-free) were obtained from Thermo Scientific Dharmacon (Lafayette, CO). Accell siRNA can enter cells without the use of a transfection reagent. HEK293 cells stably transfected with destabilized GFP (deGFP) were a generous gift from Piruz Nahreini, PhD (University of Colorado Health Sciences Center). Falcon Transwell inserts were obtained from Becton Dickinson (Franklin Lakes, NJ). Nuclease-free water was purchased from Ambion (Austin, TX). Dialysis membrane (MWCO 3500) was obtained from Spectrum Laboratories Inc. (Rancho Dominguez, CA).

2.2. Synthesis of DEX methacrylate and LPEI methacrylate macromers

DEX methacrylate (DEX-HEMA) was synthesized via the reaction of HEMA-IC to the hydroxyl groups of the DEX main chain as previously described [13]. Briefly, to synthesize DEX with 7% theoretical methacrylation, DEX (10 g) and DMAP (2 g) were dissolved in DMSO (90 ml) in a dry 250 ml round bottom flask. After complete dissolution, HEMA-IC (0.93 g) was added. The reaction occured for 4 days at room temperature, followed by dialysis (MWCO 3500) against ultrapure deionized water (diH_2O) for 3 days and lyophilization. White DEX-HEMA power (9 g) was obtained after lyophilization. LPEI methacrylate (LPEI-GMA) modified at a theoretical degree of 5% was synthesized via the ring opening reaction of epoxy groups of GMA with amine groups of LPEI. LPEI (0.5 g) and GMA (105 μl) were dissolved in chloroform (30 ml) in a 250 ml round-bottom flask for 1 h in a 60 °C silicon oil bath. The chloroform was then completely evaporated under vacuum and the mixture was reconstituted in ultrapure diH_2O (30 ml) at pH 6.0. The LPEI-GMA was purified by dialysis against ultrapure diH_2O at pH 6.0 (MWCO 3500) for 3 days, filtered using a 0.22 μm filter and lyophilized. The final yield was 0.32 g. DEX-HEMA and LPEI-GMA were characterized by proton nuclear magnetic resonance (^{1}H-NMR) in D_2O using a Varian Unity-300 (300 MHz) NMR spectrometer (Varian Inc., Palo Alto, CA). Peaks a and e in Fig. 1b and peak b and the peaks from 2-ethyl-2-oxazoline in LPEI in Fig. 1c were used to determine the actual degree of modification of DEX-HEMA and LPEI-GMA, respectively.

2.3. Photocrosslinking

DEX-HEMA (8 or 12% w/w) was dissolved in phosphate buffered saline (PBS) with 0.05% w/v photoinitiator (Irgacure D-2959) and different LPEI-GMA concentrations (0, 5 and 10 μg/100 μl gel). The polymer solutions (100 μl) were placed into the wells of a 96-well plate and hydrogels were formed by photocrosslinking with 320–500 nm UV light at 3.5 mW cm^{-2} for 85 s using an Omnicure S1000 UV Spot Cure System (Lumen Dynamics Group, Mississauga, Ontario, Canada).

2.4. Swelling and in vitro degradation

To determine the swelling profiles of these photocrosslinked DEX hydrogels, their dry and wet weights were determined at various time points over 17 days. The prepared photocrosslinked DEX hydrogels were lyophilized and their initial dry weights (W_d initial) were measured. Each dried hydrogel sample was immersed in 5 ml PBS at pH 7.4 and incubated at 37 °C. The PBS was changed every three days. At predetermined time points, samples were removed and rinsed with diH_2O, and the weights of the swollen hydrogel samples (W_s) were measured. The swelling ratio (Q) was calculated by $Q = W_s/W_d$ initial.

To determine the degradation profiles, each dried hydrogel sample was immersed in 5 ml of PBS and incubated at 37 °C. The PBS was replaced every three days. At predetermined time points, samples were removed, rinsed with diH_2O and lyophilized. The dry weights of the samples at different time points (W_d) were measured. The percentage mass loss was calculated by $(W_i - W_d)/W_i \times 100$, where W_i is the dry weight of the hydrogel samples at the initial time point. The samples ($N = 3$ for each time point) were prepared and tested at the same time.

2.5. Mechanical properties

Rheological properties of photocrosslinked DEX hydrogels (12% w/w) with various LPEI concentrations with or without encapsulated siRNA (26.6 μg/100 μl hydrogel solution) were measured using a strain-controlled AR-2000ex rheometer (TA Instruments, New Castle, DE) with stainless steel parallel plate geometry (plate diameter of 8 mm, gap of 0.75 mm). Hydrogels were made by pipetting solutions between two glass plates separated by two 0.75 mm spacers and photocrosslinked as previously described. Photocrosslinked hydrogel disks were punched out with an 8 mm biopsy punch to match the diameter of the parallel plates. G′ and G″ of each hydrogel were measured by performing a dynamic

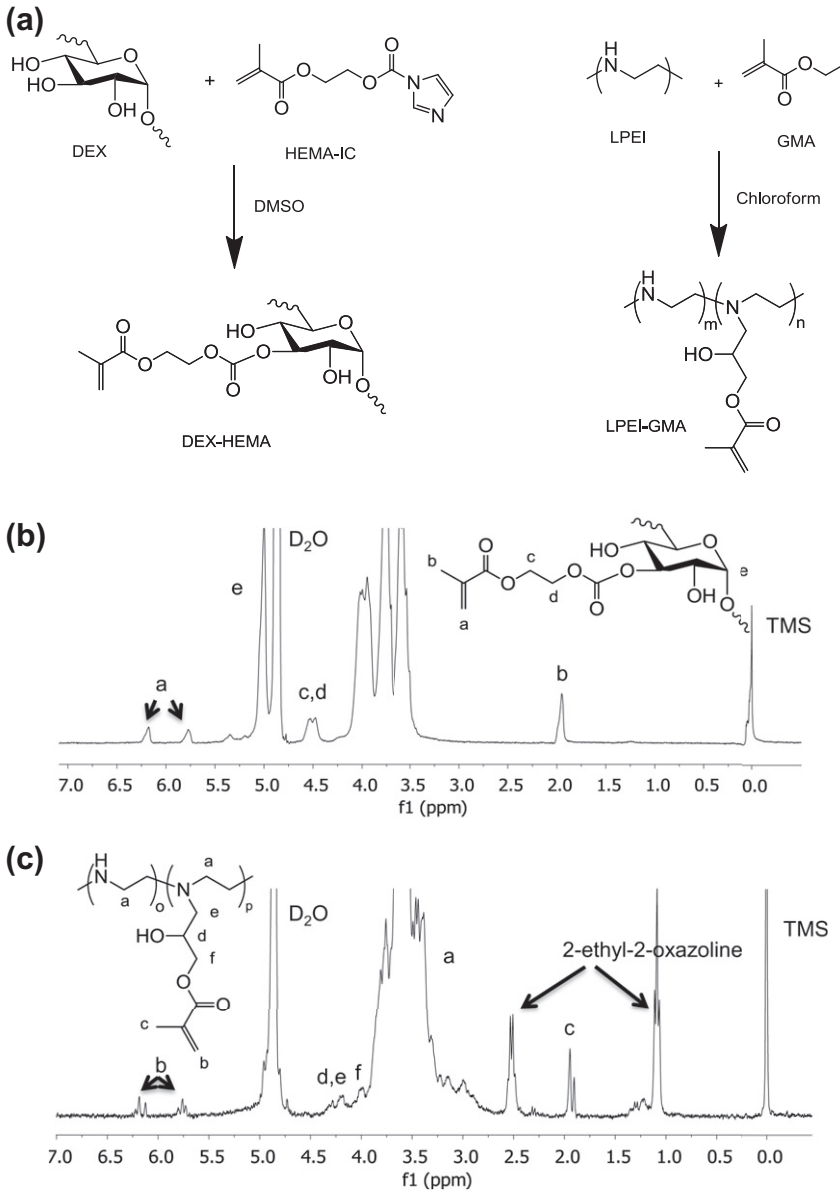

Fig. 1. (a) Synthesis of DEX-HEMA (left) and LPEI-GMA (right), and ^{1}H-NMR spectra of (b) DEX-HEMA and (c) LPEI-GMA in D_2O.

frequency sweep test in which a sinusoidal shear strain of constant peak amplitude (0.1%) was applied over a range of frequencies (0.1–10 Hz). The samples ($N = 3$ per condition) were prepared and tested at the same time.

2.6. Cell viability

Photocrosslinked DEX hydrogels with various concentrations of LPEI were formed in cell culture inserts above a monolayer culture of HEK293 cells and cell viability was assessed using an MTS assay used according to the manufacturer's instructions. Briefly, HEK293 cells (passage 25) were seeded in a 24-well plate at 2×10^5 cells per well in 0.5 ml of DMEM-HG containing 10% FBS and cultured at 37 °C and 5% CO_2 in a humidified incubator for 24 h. The DEX-HEMA solutions with various LPEI-GMA concentrations (100 μl, 12% w/w) were photocrosslinked in cell culture inserts (8 μm pore size), and then placed into the wells containing cells. Cells were cultured in parallel without the hydrogel or insert as a control. Media were changed every 3 days. After 2 and 7 days, media and inserts were removed, cells were rinsed with PBS, and a 20% CellTiter 96 Aqueous One Solution in PBS (0.5 ml) was added to each well. After 90 min incubation at 37 °C, absorbance was measured at 490 nm on a plate reader (SAFIRE, Tecan, Austria). Cell viability was calculated by normalizing the absorbance of samples at 490 nm to that of the control.

To evaluate the viability of cells encapsulated in hydrogels of various LPEI concentrations, 2×10^5 HEK293 cells (passage 27) were mixed into 100 μl of 12% w/w dextran solutions containing different LPEI concentrations. The cell/polymer mixtures were photocrosslinked in a 48-well plate and the cell-encapsutated hydrogels were cultured in 0.5 ml of DMEM-HG containing 10% FBS. The media was replaced every 3 days. After 7 days, media were removed and the hydrogels were rinsed with PBS. The cell-encapsulated hydrogels were homogenized for 30 s using a TH homogenizer (Omni International, Kennesaw, GA) prior to measuring absorbances at 490 nm of an MTS assay as described above. The samples ($N = 3$ per condition) were prepared and tested at the same time.

2.7. siRNA release kinetics

Accell green cyclophilin B control siRNA, which is fluorescently labeled with fluorescein isothiocyanate (FITC), was used to examine its release kinetics from DEX hydrogels (8 and 12% w/w). Hydrogels (8 and 12% w/w) with different methacrylated LPEI concentrations (0, 5, 10 μg), containing 13.3 or 26.6 μg of the siRNA, were fabricated in a 96-well plate. For example, to prepare hydrogels (12% w/w) with 13.3 μg of siRNA, first, 10 μl of siRNA (100 μM) was mixed with 17 μl of LPEI-GMA (pH 6.0), and the resulting mixture was allowed to sit for 30 min at room temperature. Second, 73 μl of DEX-HEMA solution (16.4% w/w) was added to the mixture. Then, the 100 μl mixture was pipetted into a 96-well plate, followed by exposure to UV light for 85 s. The formed hydrogel was transferred into a well of a 24-well plate containing 1 ml PBS. At predetermined time points, the PBS was removed and replaced. Separate standard curves were made for each condition by preparing matched hydrogels as described above and homogenizing in 1 ml PBS. The resulting solutions were then diluted to various concentrations (6, 2, 0.5, 0.05, 0 μg ml^{-1}). All the released siRNA and the standards were measured at pH 12 to dissociate the complexes using a plate reader (fmax, Molecular Devices, Inc., CA) set at excitation 485/emission 538 [14,15]. The samples ($N = 3$ per condition) were prepared and tested at the same time.

2.8. Bioactivity of released siRNA

100 μl hydrogel mixtures (12% w/w) were prepared as described above and then photocrosslinked in Transwell membranes (8 μm pore size) in 24-well plates. ADM (0.5 ml) was pipetted into the gel containing wells. The released siRNA in ADM was collected and refreshed at days 3, 7 and 14. HEK293 cells stably transfected with deGFP were seeded in 24-well plates at 1.5×10^5 cells per well in media containing DMEM-HG and 10% FBS. After 1 day of incubation at 37 °C and 5% CO_2 in a humidified incubator, the media were removed and replaced with ADM containing the released siRNA from each time point above. The cells were collected and deGFP knockdown was determined by flow cytometry (EPICS XL-MCL, Beckman Coulter, Fullerton, CA) after two days of incubation. The samples ($N = 3$ per condition) were prepared and tested at the same time.

2.9. Transfection of cells encapsulated inside hydrogels

HEK293 cells were mixed with the hydrogel solutions (12% w/w) at a concentration of 20×10^6 cells ml^{-1}. The cells/hydrogel solutions were transferred onto Transwell membrane inserts (0.4 μm pore size), and then crosslinked as described above. The cells/hydrogel constructs were initially cultured in ADM alone and the media were replaced with ADM + 1% FBS at days 1, 3, 5, 7 and 9. Transfection was assessed using confocal microscopy (LSM510, Zeiss, Jena, Germany) on day 3, 7 and 11. Images were taken every 5 μm in the z-direction for 100 μm from the top of the hydrogels and compiled into a single 3-D projection. The samples ($N = 2$ per condition) were prepared and tested at the same time.

2.10. Statistical analysis

All data are expressed as mean ± standard deviation. Statistical significance was determined using Turkey–Kramer Multiple Comparisons Test with one-way analysis of variance (ANOVA) using InStat sofware (GraphPad Software, La Jolla, CA). $P < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Synthesis and characterization of the polymer macromers, and hydrogel formation

Methacrylated DEX (DEX-HEMA) and methacrylated LPEI (LPEI-GMA) were synthesized by coupling HEMA-IC to the hydroxyl groups of the DEX main chain [13] and by the reaction of the epoxy group of the GMA with amine groups of the LPEI, respectively, as shown in Fig. 1a. ^{1}H-NMR spectra of the synthesized DEX-HEMA and LPEI-GMA in D_2O are presented in Fig. 1b and c. The signals corresponding to the methacrylate groups of both DEX-HEMA and LPEI-GMA appeared at 5.76 and 6.17 ppm. The peaks appearing at 4.48–4.55 ppm in Fig. 1b indicated the successful conjugation of HEMA-IC to DEX. Peaks at 4.00, 4.18 and 4.30 ppm in Fig. 1c confirm the ring opening reaction of epoxy group of GMA with amine groups of LPEI. From the ^{1}H-NMR spectra, the actual degrees of methacrylation of DEX and LPEI were 4.2% and 1.2%, respectively. DEX-HEMA macromers were photocrosslinked with various concentrations of LPEI-GMA macromers to form hydrogels for controlled, localized and sustained delivery of siRNA to inhibit gene expression of surrounding or encapsulated cells (Fig. 2).

3.2. Swelling kinetics and degradation of the photocrosslinked hydrogels

A photocrosslinked hydrogel system was formed by combining neutral DEX-HEMA and cationic LPEI-GMA macromers capable of forming electrostatic interactions with siRNA. DEX-HEMA, previously reported by Hennink [13], is a biodegradable and biocompatible polymer [16,17], The covalent crosslinks formed following photopolymerization under low level UV light contain ester linkages that can degrade in aqueous media. Biomaterial swelling and degradation rate are important for (1) the transport of oxygen and nutrients to and the removal of waste products from incorporated cells, (2) providing space for new tissue formation in tissue engineering applications, and (3) controlling the release of bioactive molecules [18,19] such as siRNA. These parameters can be tailored by varying the degree of methacrylation [20,21], size of crosslinker [22] and hydrogel macromer concentration [17]. Swelling ratios of photocrosslinked DEX hydrogels (8 and 12% w/w) with 0, 5 and 10 μg LPEI/100 μl gel (DEX-only, DEX + 5PEI and DEX + 10-PEI, respectively) in phosphate buffered saline (PBS) over the course of 9 and 17 days are shown in Fig. 3a and b. The DEX hydrogels with various LPEI concentrations displayed similar swelling profiles, indicating that the LPEI did not affect this hydrogel property at the concentrations investigated. The 8% w/w DEX hydrogels achieved maximal swelling after 5 days, whereas the 12% w/w DEX hydrogels reached maximal swelling after 12 days.

Mass loss (%) of photocrosslinked DEX hydrogels with various LPEI concentrations was examined by measuring their dry weights over time (Fig. 3c and d). The 8% w/w DEX hydrogels completely degraded by day 9, while the 12% w/w DEX hydrogels did not completely degrade until day 17 due to the higher crosslinking density of the hydrogels formed with higher macromer concentration. At both concentrations of DEX in the hydrogels, there was no significant difference in degradation profiles with various LPEI contents.

3.3. Rheology

To investigate whether the addition of LPEI or siRNA alters the mechanical properties of the hydrogels, storage (G') and loss (G'') moduli were measured using a rheometer. G' and G'' represent the elastic and viscous behavior, respectively, of the hydrogels. Fig. 4 shows G' and G'' of the DEX hydrogels (12% w/w) with different LPEI

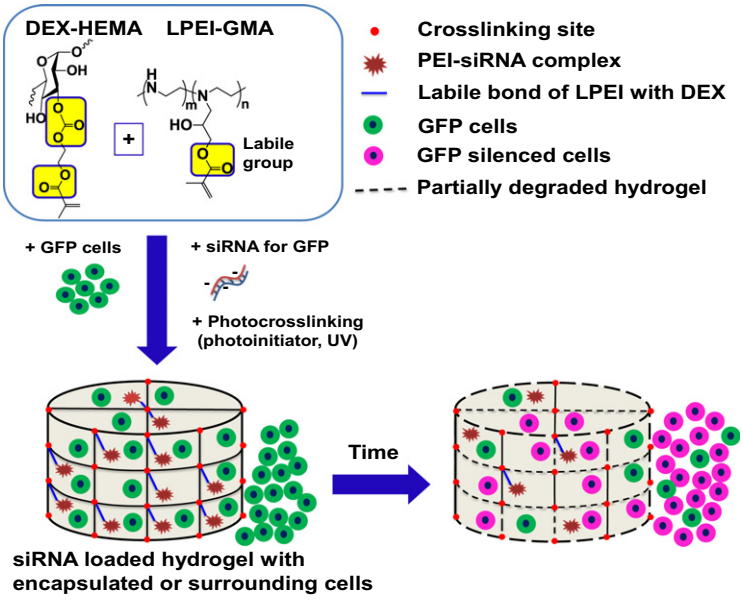

Fig. 2. Schematic of hydrogel formation for delivery of siRNA to subsequently silence gene expression of encapsulated and surrounding HEK293 cells.

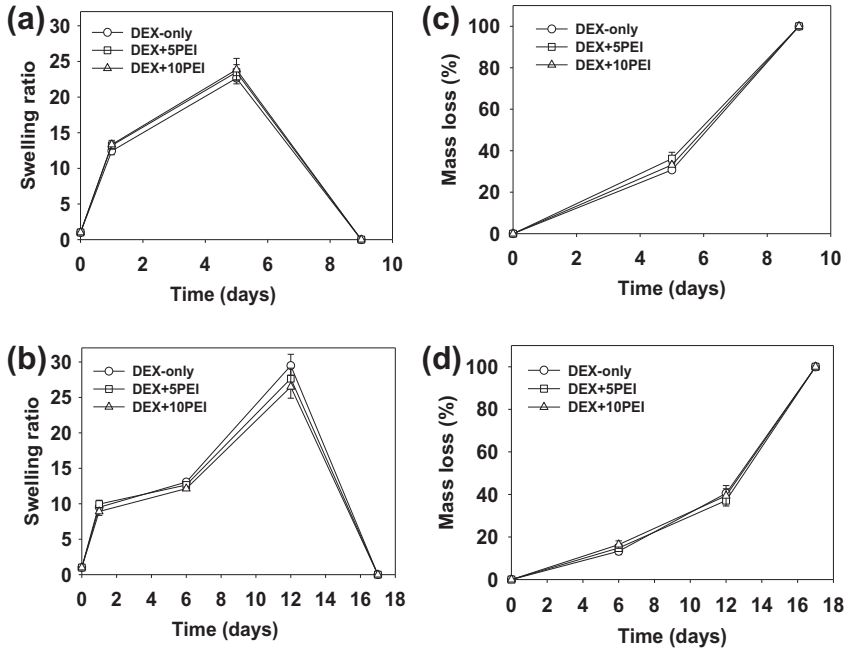

Fig. 3. In vitro (a, b) swelling and (c, d) degradation of (a, c) 8% w/w and (b, d) 12% w/w DEX hydrogels with different LPEI concentrations.

contents with and without incorporated siRNA. G' of the DEX + 5PEI and DEX + 10PEI hydrogels was slightly greater than that of the DEX-only hydrogel. Hydrogels with siRNA exhibited a trend towards higher G' than those without siRNA. In addition, G' was greater than G'' for all frequencies tested (0.1–10 Hz), indicating that elastic behavior of the hydrogels dominates in this range [23]. The increased hydrogel G' with LPEI likely resulted from increased crosslinking density of the hydrogels, and the increase in G' with siRNA may be a result of higher density of the hydrogel constructs.

3.4. Cell viability

Hydrogels for use in biological molecule delivery or tissue engineering applications must be cytocompatible. LPEI, a cationic synthetic polymer, was modified with GMA to make it photocrosslinkable with DEX-HEMA. Since LPEI can be cytotoxic to cells, we tested the viability of deGFP expressing HEK293 cells cultured in the presence of photocrosslinked hydrogel degradation products. These cells were used because they constitutively express deGFP, which gets turned off by the released siRNA in our bioactivity evaluation experiments. Photocrosslinked DEX hydrogels (12% w/w) with different LPEI concentrations were prepared in cell culture inserts above a monolayer culture of HEK293 cells and cell viability was determined using an MTS assay, which measures mitochondrial metabolic activity, and normalized to wells containing cells and culture medium only (Fig. 5a). After 2 days in culture, cell viability in the presence of DEX hydrogels with various LPEI concentrations (DEX-only, DEX + 5PEI and DEX + 10PEI)

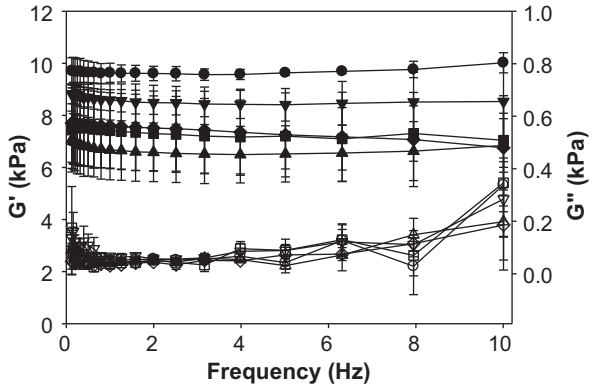

Fig. 4. Storage (G') and loss (G'') moduli of 12% w/w DEX hydrogels with different LPEI concentrations with or without siRNA (26.6 μg/100 μl gel solution) ($N = 3$). Filled and unfilled symbols represent G' and G'', respectively. Circle, DEX + 10PEI with siRNA; triangle down, DEX-only with siRNA; diamond, DEX + 10PEI; square, DEX + 5PEI; triangle up, DEX-only.

was 95.42% ± 0.96, 94.09% ± 8.44 and 93.56% ± 3.03, respectively, that of the control wells. After 7 days in culture, they remained highly viable at 97.63% ± 1.73, 97.00% ± 1.70 and 96.00% ± 1.52, respectively, compared to the controls. No significant differences were found between experimental conditions and the control or between time points.

Cell viability of HEK293 cells encapsulated within 12% w/w DEX hydrogels containing various LPEI concentrations was also determined by the MTS assay to examine survival of the cells during photocrosslinking formation of the hydrogels and in 3-D culture (Fig. 5b). Since photocrosslinked hydrogels formed with DEX-HEMA have been previously shown to be cytocompatible [24], we compared MTS assay absorbances at 490 nm of cells in the DEX + 5PEI and DEX + 10PEI hydrogels to that of cells in the DEX-only control. There was no significant difference between the absorbances of the samples (1.21 ± 0.04 for DEX-only, 1.165 ± 0.022 for DEX + 5PEI, and 1.124 ± 0.135 for DEX + 10PEI) after 7 days in culture, which indicates that the addition of

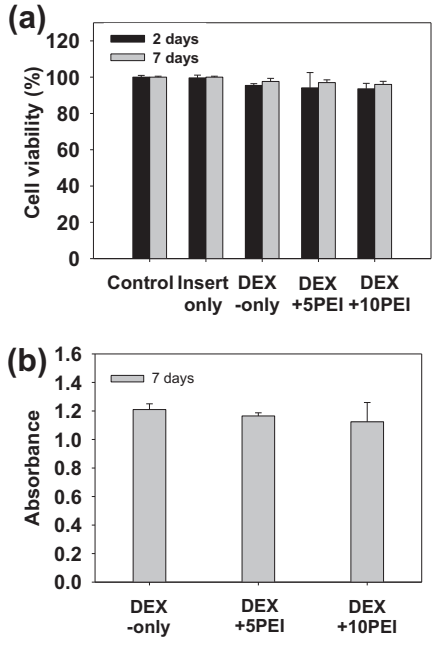

Fig. 5. Viability of (a) cells surrounding and (b) encapsulated within 12% w/w DEX hydrogels with various LPEI concentrations as measured using MTS assay.

LPEI-GMA to the system did not affect cell viability. While in monolayer culture HEK293 cells may require cell adhesion for survival, this is not the case in 3-D culture as our group [2] and others [25,26] have shown that HEK293 cells remain viable when cultured within hydrogels that do not promote cell adhesion, such as alginate.

3.5. siRNA release

PEI is capable of complexing with and condensing siRNA into nanoparticles which can protect siRNA from denaturation by ribonucleases [1] and enhance cellular uptake [27]. For example, siRNA/PEI complexes have been reported to silence VEGF expression to reduce tumor growth [28] and siRNA/PEI conjugates physically trapped in scaffolds were released to suppress fibroblast proliferation and knockdown type 1 collagen mRNA expression [29]. In this study, LPEI was utilized because of two of its important properties: (1) its capacity to be chemically modified via amine groups on its backbone and (2) its ability to form stabilized complexes with siRNA. To examine whether the covalent incorporation of LPEI into the DEX hydrogels during photopolymerization could delay and control the release of siRNA over a prolonged time period, the temporal release of siRNA from 8 and 12% w/w hydrogels with various LPEI contents was compared (Fig. 6). The release was sustained and delayed as the LPEI amount was increased from 5 to 10 μg. For example, on the first day, the cumulative release of siRNA from the DEX-only hydrogels (8 and 12% w/w) with siRNA loading amount of 13.3 μg was 67.22% and 65.59% whereas the release from the DEX + 5PEI hydrogels was significantly less at only 36.92% and 32.61%, respectively (Fig. 6a and b). Additionally, even less siRNA (19.63% and 16.54%) was released from the DEX + 10PEI hydrogels (8 and 12% w/w, respectively) on the first day. With the siRNA loading amount of 26.6 μg, a similar trend was observed at the first day (Fig. 6c). These results show that the significant initial burst release resulting from the hydrogels without LPEI (DEX-only) decreased markedly by the covalent conjugation of a small amount of LPEI (DEX + 5PEI and DEX + 10PEI). Insets show the siRNA release rate (μg day^{-1}) from the corresponding hydrogels.

The release was also controlled by the hydrogel concentration. For example, the 8% w/w hydrogels released the siRNA over the course of 9 days as the hydrogels degraded completely (Fig. 6a). Similarly, siRNA was released from the 12% w/w hydrogels until they completely degraded by day 17 (Fig. 6b). siRNA was released from the DEX-only hydrogels predominantly via simple diffusion in addition to the degradation of the hydrogels at later time points, resulting in a substantial initial burst release. The addition of free PEI into hydrogels does not allow for control over the release because the siRNA/PEI complexes simply diffuse out of the hydrogels [29]. Therefore, to achieve control over the release, LPEI was modified with GMA, which could covalently bind to the hydrogels via free radical photopolymerization and form hydrolysable ester-containing crosslinks. Importantly, the interactions were independently controlled by adding defined amounts of LPEI-GMA and siRNA prior to photocrosslinking gelation. The mechanism of siRNA release from the LPEI-containing hydrogels at later time points is thus regulated predominantly by controlled degradation of the hydrogels and the ester linkages between the LPEI and DEX, followed by diffusion of the siRNA/LPEI complexes from the hydrogels.

3.6. Bioactivity evaluation

This study would determine whether the UV light used in the photocrosslinking process, the photoinitiator, the electrostatic interactions of siRNA and methacrylated LPEI, and the subsequent

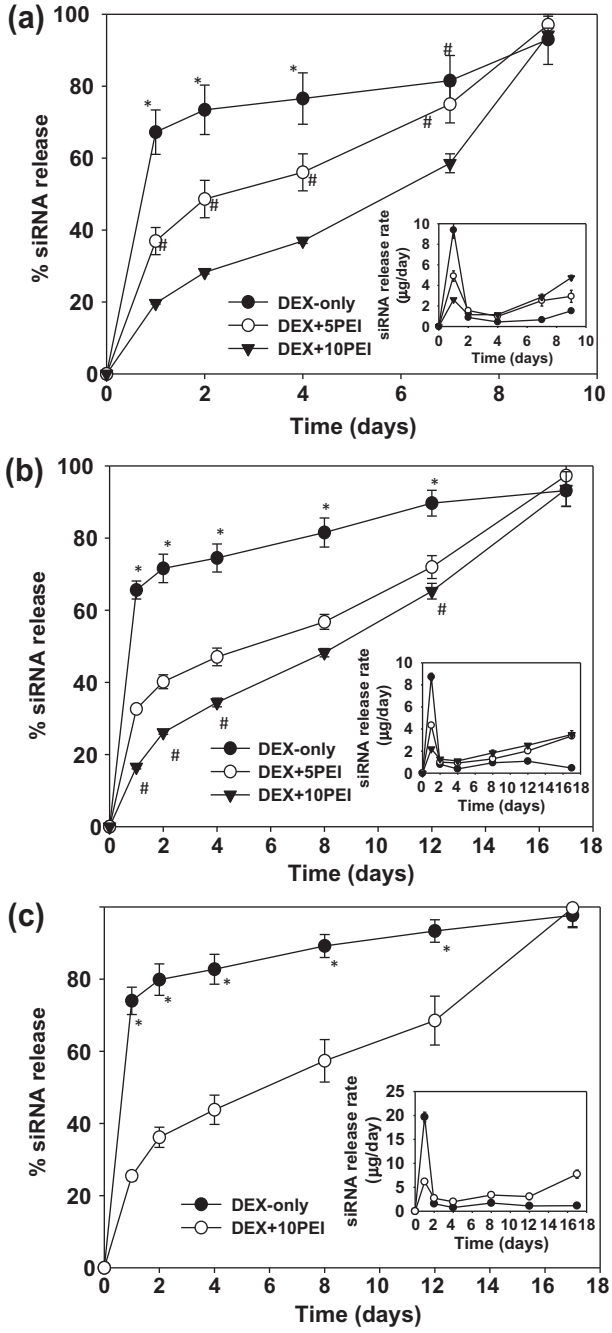

Fig. 6. siRNA release profiles from (a) 8% w/w DEX hydrogels with siRNA loading of 13.3 μg ($^{*}P < 0.05$ compared with DEX + 5PEI and DEX + 10PEI, $^{\#}P < 0.05$ compared with DEX + 10PEI), (b) 12% w/w DEX hydrogels with siRNA loading of 13.3 μg ($^{*}P < 0.05$ compared with DEX + 5PEI and DEX + 10PEI, $^{\#}P < 0.05$ compared with DEX + 5PEI), and (c) 12% w/w DEX hydrogels with siRNA loading of 26.6 μg ($^{*}P < 0.05$ compared with DEX + 10PEI). Insets indicate siRNA release rate (μg day^{-1}) from the corresponding hydrogels.

release of siRNA from the hydrogels affects the gene silencing ability of the released siRNA or siRNA/LPEI complexes. siRNA against deGFP (13.3 and 26.6 μg gel^{-1}) was released in ADM from 12% w/w DEX hydrogels (DEX-only, DEX + 5PEI and DEX + 10PEI), which degraded completely after 14 days of culture, and collected at days 3, 7 and 14. HEK293 cells plated in tissue culture plastic one day earlier were then exposed to collected siRNA release samples. deGFP expression of these cells was measured by flow cytometry after 48 h treatment with the released siRNA.

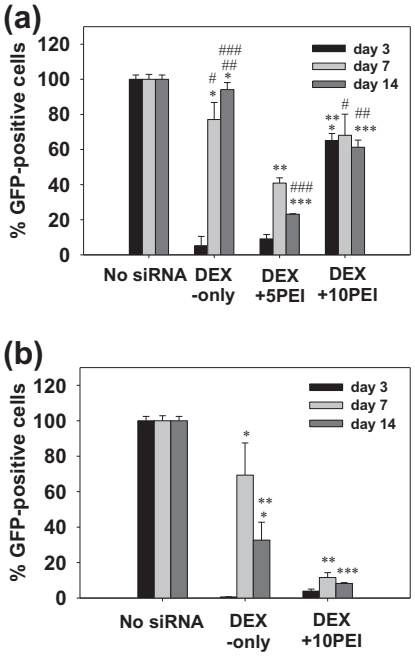

Fig. 7. Percentage of positive deGFP HEK293 cells after exposure to released media from 12% w/w DEX-LPEI hydrogels originally containing (a) 13.3 μg ($^{*}P < 0.05$ compared with day 3 of DEX-only, $^{**}P < 0.05$ compared with day 3 of DEX + 5PEI, $^{***}P < 0.05$ compared with day 14 of DEX-only, $^{\#}P < 0.05$ compared with day 7 of DEX + 5PEI, $^{\#\#}P < 0.05$ compared with day 14 of DEX + 5PEI, and $^{\#\#\#}P < 0.05$ compared with day 14 of DEX + 10PEI), or (b) 26.6 μg siRNA ($^{*}P < 0.05$ compared with day 3 of DEX-only, $^{**}P < 0.05$ compared with day 7 of DEX-only, $^{***}P < 0.05$ compared with day 14 of DEX-only). Samples are normalized to release samples from hydrogels without any siRNA. GFP expression of cells exposed to all release samples (except those exposed to release samples from DEX-only by day 14 in Fig. 7a) was significantly different compared with the controls (no siRNA) at corresponding time points.

Hydrogels (12% w/w) displayed a sustained silencing of deGFP expression only when LPEI was covalently incorporated within the hydrogels (Fig. 7a and b). deGFP expression of cells exposed to siRNA (13.3 μg original mass) released from DEX-only hydrogels was silenced to 5.23% of control samples with releasates from day 3, but increased to 77.10% and 94.13% with releasates from days 7 and 14, respectively (Fig. 7a). In contrast, the percentage of deGFP-positive cells treated with siRNA released from DEX + 5PEI hydrogels was 9.71, 40.89 and 23.16% with releasates from days 3, 7 and 14, respectively, and ~ 70% for all three time points with siRNA from DEX + 10PEI hydrogels. These results indicate more sustained knockdown when LPEI was coupled to the hydrogels. When the siRNA amount in the hydrogels was increased to 26.6 μg, deGFP knockdown increased (Fig. 7b). Importantly, deGFP expressing cells were reduced to less than 11.56% for releasates from all time points from the DEX + 10PEI hydrogels. These results confirm that the siRNA and siRNA/LPEI complexes released from the hydrogels retained their capacity to substantially knock down deGFP expression in HEK293 cells cultured on tissue culture plastic. The magnitude and duration of knockdown were dependent on the amount of siRNA and coupled LPEI present in the hydrogels.

3.7. Transfection of cells incorporated into hydrogels

Hydrophilic, biocompatible and biodegradable 3-D hydrogel networks can serve as temporary matrices for cell growth and new tissue formation in regenerative medicine applications when implanted or injected at a defect or damaged tissue site [23,30–32]. Therapeutics incorporated into hydrogels can be retained

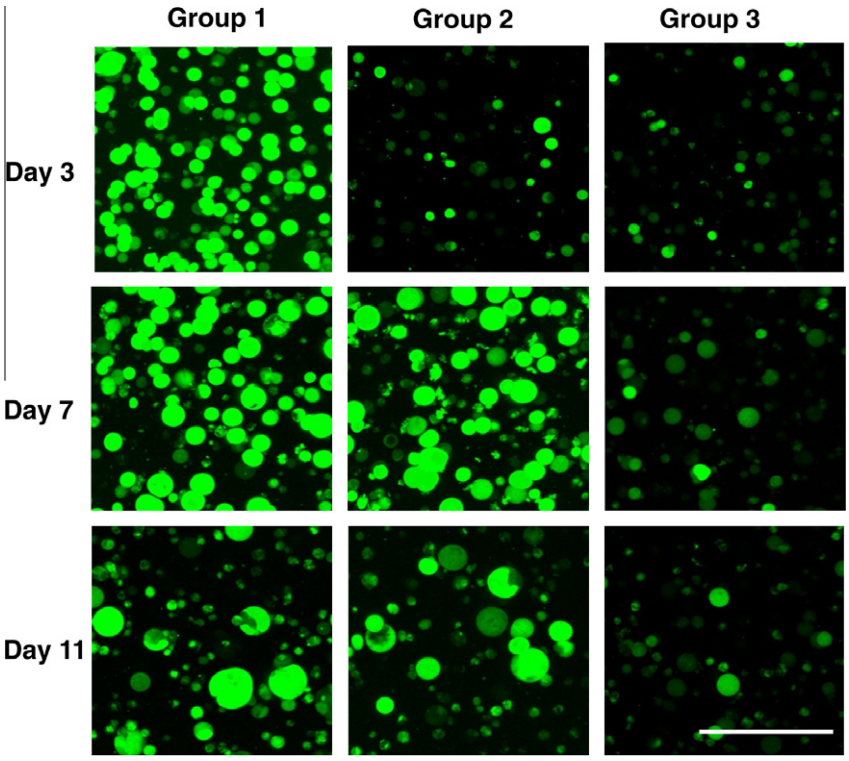

Fig. 8. Confocal fluorescent photomicrographs of deGFP HEK293 cells encapsulated in 3-D hydrogels with or without siRNA (Group 1: DEX-only without siRNA, Group 2: DEX-only with siRNA, Group 3: DEX + 10PEI with siRNA). Control hydrogels without siRNA treatment contained cells with strong deGFP expression at all time points. deGFP expression of cells in the DEX-only hydrogels with siRNA was decreased at day 3, but strong expression returned at day 7 and day 11. Substantial knockdown of deGFP expression was observed at all time points for cells in the DEX + 10PEI hydrogels. The scale bar indicates 200 μm.

and then locally released to transplanted cells within the hydrogels and to host cells at specific sites to treat diseases or promote healing of damaged tissues [9,18,32,33]. Therefore, we examined the ability of this system to knock down deGFP expression of cells cultured within the hydrogels. Three groups of 12% w/w DEX hydrogels were used: (1) DEX-only hydrogels without siRNA (control); (2) DEX-only hydrogels with 26.6 μg siRNA; (3) DEX + 10PEI hydrogels with 26.6 μg siRNA. Photomicrographs depicting the fluorescence activity of cells in the hydrogels as a measure of transfection are shown in Fig. 8. deGFP expression of cells in the hydrogels with siRNA (groups 2 and 3) was substantially reduced at day 3, whereas that of cells in the hydrogels without siRNA (group 1) was not. Compared to strong deGFP expression of cells in groups 1 and 2 at time points of 7 and 11 days, cells in group 3 exhibited substantial deGFP silencing, even at these later time points. This demonstrates that the coupled LPEI in group 3 was acting to retain bioactive siRNA within the hydrogels for a longer period of time to affect incorporated cells.

Cells in group 1 strongly expressed deGFP at all time points because they were not transfected with siRNA (Fig. 8). deGFP expression of cells in group 2 that were transfected with siRNA was significantly reduced at day 3, but increased at days 7 and 11 (Fig. 8) since most of the siRNA had been released from the hydrogels by these time points (Fig. 6c). In contrast, cells in group 3, which were encapsulated in hydrogels containing LPEI which acted to retard siRNA release, exhibited sustained deGFP knockdown at days 3, 7 and 11 in culture. Due to the swelling of the hydrogels, the density of cells imaged on the confocal microscope on day 11 in all groups decreased for all conditions. At later time points, large areas of green represent cell clusters that arise due to cell proliferation and merging. Their morphology is round as would be expected within a material that does not support cell adhesion. We previously reported photocrosslinked alginate hydrogels without coupled LPEI that silenced deGFP expression of incorporated cells at day 3, but at a later time point (day 6) deGFP expression increased significantly [2]. This result is similar to our current findings with cells incorporated into hydrogels in group 2. In group 2, siRNA was released from the hydrogel by simple diffusion or degradation. In contrast, in group 3 the release of siRNA was governed by a small amount of covalently coupled LPEI molecules, resulting in a subsequent extended deGFP knockdown.

4. Conclusion

In this work, we have designed biodegradable hydrogels using methacrylated DEX and methacrylated LPEI that were photocrosslinked with exposure to UV light. The swelling and degradation were controlled by varying the hydrogel concentration and thus the crosslinking density. The low concentration of LPEI used did not affect the swelling and degradation properties of the hydrogels. LPEI and siRNA slightly increased the mechanical strength. The photocrosslinked hydrogels showed low cytotoxicity against HEK293 cells. Importantly, this is the first report of hydrogels covalently functionalized with cationic polymeric molecules (LPEI-GMA) for the tunable, controlled release of bioactive siRNA. The release of siRNA from the hydrogels was tailored over a prolonged time period by regulating the degree of the electrostatic interactions between siRNA and cationic molecules, and was a result of degradation of the ester linkages and diffusion of siRNA/LPEI complexes. While in this study release profiles were controlled by varying the concentration of LPEI, siRNA and DEX, varying the degree of DEX methacrylation would likely also regulate release. The released siRNA and siRNA/LPEI complexes retained high biological activity. Moreover, this system allowed for controlled and sustained knockdown of protein expression in cells incorporated into

the hydrogels. This macroscopic hydrogel system permitting tunable, controlled release of bioactive siRNA over an extended time period may have great utility for a variety of gene therapy and regenerative medicine applications.

Acknowledgements

The authors gratefully acknowledge funding from the National Institutes of Health (R21DE022376) and Biomedical Research and Technology Transfer Grant 08-081 from the Ohio Department of Development.

Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figs. 2, and 8, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at http://dx.doi.org/10.1016/j.actbio.2012.08.012.

References

[1] Krebs MD, Alsberg E. Localized, targeted, and sustained siRNA delivery. Chem Eur J 2011;17:3054–62.
[2] Krebs MD, Jeon O, Alsberg E. Localized and sustained delivery of silencing RNA from macroscopic biopolymer hydrogels. J Am Chem Soc 2009;131:9204–6.
[3] Monaghan M, Pandit A. RNA interference therapy via functionalized scaffolds. Adv Drug Deliv Rev 2011;63:197–208.
[4] Whitehead KA, Langer R, Anderson DG. Knocking down barriers: advances in siRNA delivery. Nat Rev Drug Discov 2009;8:129–38.
[5] Kim W, Kim S. Efficient siRNA delivery with non-viral polymeric vehicles. Pharm Res 2009;26:657–66.
[6] Perkel J. RNAI therapeutics: a two-year update. Science 2009;326:454.
[7] Akhtar S, Benter IF. Nonviral delivery of synthetic siRNAs in vivo. J Clin Invest 2007;117:3623–32.
[8] Yao Y, Wang C, Varshney R, Wang D-A. Antisense makes sense in engineered regenerative medicine. Pharm Res 2009;26:263–75.
[9] Nguyen MK, Lee DS. Injectable biodegradable hydrogels. Macromol Biosci 2010;10:563–79.
[10] Coviello T, Matricardi P, Marianecci C, Alhaique F. Polysaccharide hydrogels for modified release formulations. J Controlled Release 2007;119:5–24.
[11] Shu XZ, Ahmad S, Liu Y, Prestwich GD. Synthesis and evaluation of injectable, in situ crosslinkable synthetic extracellular matrices for tissue engineering. J Biomed Mater Res Part A 2006;79A:902–12.
[12] Nguyen KT, West JL. Photopolymerizable hydrogels for tissue engineering applications. Biomaterials 2002;23:4307–14.
[13] van Dijk-Wolthuls WNE, Tsang SKY, Kettenes-van den Bosch WE, Hennink JJ. A new class of polymerizable dextrans with hydrolyzable groups: hydroxyethyl methacrylated dextran with and without oligolactate spacer. Polymer 1997;38:6235–42.
[14] Mountziaris P, Sing D, Chew S, Tzouanas S, Lehman E, Kasper F, et al. Controlled release of anti-inflammatory siRNA from biodegradable polymeric microparticles intended for intra-articular delivery to the temporomandibular joint. Pharm Res 2011;28:1370–84.
[15] Moret IS, Esteban Peris J, Guillem VM, Benet M, Revert F, Dasi F, et al. Stability of PEI-DNA and DOTAP-DNA complexes: effect of alkaline pH, heparin and serum. J Controlled Release 2001;76:169–81.
[16] Cadée JA, van Luyn MJA, Brouwer LA, Plantinga JA, van Wachem PB, de Groot CJ, et al. In vivo biocompatibility of dextran-based hydrogels. J Biomed Mater Res 2000;50:397–404.
[17] van Dijk-Wolthuis WNE, van Steenbergen MJ, Underberg WJM, Hennink WE. Degradation kinetics of methacrylated dextrans in aqueous solution. J Pharm Sci 1997;86:413–7.
[18] Van Tomme SR, Hennink WE. Biodegradable dextran hydrogels for protein delivery applications. Expert Rev Med Devices 2007;4:147–64.
[19] Singh A, Suri S, Roy K. In-situ crosslinking hydrogels for combinatorial delivery of chemokines and siRNA–DNA carrying microparticles to dendritic cells. Biomaterials 2009;30:5187–200.
[20] Jeon O, Bouhadir KH, Mansour JM, Alsberg E. Photocrosslinked alginate hydrogels with tunable biodegradation rates and mechanical properties. Biomaterials 2009;30:2724–34.
[21] Bencherif SA, Srinivasan A, Horkay F, Hollinger JO, Matyjaszewski K, Washburn NR. Influence of the degree of methacrylation on hyaluronic acid hydrogels properties. Biomaterials 2008;29:1739–49.
[22] Kong HJ, Alsberg E, Kaigler D, Lee KY, Mooney DJ. Controlling degradation of hydrogels via the size of crosslinked junctions. Adv Mater 2004;16:1917–21.
[23] Lei Y, Segura T. DNA delivery from matrix metalloproteinase degradable poly(ethylene glycol) hydrogels to mouse cloned mesenchymal stem cells. Biomaterials 2009;30:254–65.
[24] De Groot CJ, Van Luyn MJA, Van Dijk-Wolthuis WNE, Cadee JA, Plantinga JA, Otter WD, et al. In vitro biocompatibility of biodegradable dextran-based hydrogels tested with human fibroblasts. Biomaterials 2001;22:1197–203.
[25] Workman VL, Dunnett SB, Kille P, Palmer DD. Microfluidic chip-based synthesis of alginate microspheres for encapsulation of immortalized human cells. Biomicrofluidics 2007;1:014105–14109.
[26] Visted T, Furmanek T, Sakariassen P, Foegler WB, Sim K, Westphal H, et al. Prospects for delivery of recombinant angiostatin by cell-encapsulation therapy. Hum Gene Ther 2003;14:1429–40.
[27] Günther M, Lipka J, Malek A, Gutsch D, Kreyling W, Aigner A. Polyethylenimines for RNAi-mediated gene targeting in vivo and siRNA delivery to the lung. Eur J Pharm Biopharm 2011;77:438–49.
[28] Kim SH, Jeong JH, Lee SH, Kim SW, Park TG. Local and systemic delivery of VEGF siRNA using polyelectrolyte complex micelles for effective treatment of cancer. J Controlled Release 2008;129:107–16.
[29] Takahashi H, Wang Y, Grainger DW. Device-based local delivery of siRNA against mammalian target of rapamycin (mTOR) in a murine subcutaneous implant model to inhibit fibrous encapsulation. J Controlled Release 2010;147:400–7.
[30] Rocha FG, Sundback CA, Krebs NJ, Leach JK, Mooney DJ, Ashley SW, et al. The effect of sustained delivery of vascular endothelial growth factor on angiogenesis in tissue-engineered intestine. Biomaterials 2008;29:2884–90.
[31] Kong HJ, Liu J, Riddle K, Matsumoto T, Leach K, Mooney DJ. Non-viral gene delivery regulated by stiffness of cell adhesion substrates. Nat Mater 2005;4:460–4.
[32] Patterson J, Siew R, Herring SW, Lin ASP, Guldberg R, Stayton PS. Hyaluronic acid hydrogels with controlled degradation properties for oriented bone regeneration. Biomaterials 2010;31:6772–81.
[33] Zhao X, Kim J, Cezar CA, Huebsch N, Lee K, Bouhadir K, et al. Active scaffolds for on-demand drug and cell delivery. Proc Natl Acad Sci 2011;108:67–72.