Biomaterials 34 (2013) 9657–9665

Contents lists available at ScienceDirect

Biomaterials

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

Modulating polymer chemistry to enhance non-viral gene delivery inside hydrogels with tunable matrix stiffness

Michael Keeney [a], Sheila Onyiah [b], Zhe Zhang [d], Xinming Tong [a], Li-Hsin Han [a], Fan Yang [a,c,*]

[a] Department of Orthopaedic Surgery, Stanford University, Stanford, CA 94305, USA
[b] Department of Mechanical Engineering, Stanford University, Stanford, CA 94305, USA
[c] Department of Bioengineering, Stanford University, Stanford, CA 94305, USA
[d] Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

ARTICLE INFO

Article history:
Received 6 June 2013
Accepted 19 August 2013
Available online 5 September 2013

Keywords:
Hydrogel
Gene therapy
Mechanical properties
In-vitro test

ABSTRACT

Non-viral gene delivery holds great promise for promoting tissue regeneration, and offers a potentially safer alternative than viral vectors. Great progress has been made to develop biodegradable polymeric vectors for non-viral gene delivery in 2D culture, which generally involves isolating and modifying cells *in vitro*, followed by subsequent transplantation *in vivo*. Scaffold-mediated gene delivery may eliminate the need for the multiple-step process *in vitro*, and allows sustained release of nucleic acids *in situ*. Hydrogels are widely used tissue engineering scaffolds given their tissue-like water content, injectability and tunable biochemical and biophysical properties. However, previous attempts on developing hydrogel-mediated non-viral gene delivery have generally resulted in low levels of transgene expression inside 3D hydrogels, and increasing hydrogel stiffness further decreased such transfection efficiency. Here we report the development of biodegradable polymeric vectors that led to efficient gene delivery inside poly(ethylene glycol) (PEG)-based hydrogels with tunable matrix stiffness. Photocrosslinkable gelatin was maintained constant in the hydrogel network to allow cell adhesion. We identified a lead biodegradable polymeric vector, E6, which resulted in increased polyplex stability, DNA protection and achieved sustained high levels of transgene expression inside 3D PEG-DMA hydrogels for at least 12 days. Furthermore, we demonstrated that E6-based polyplexes allowed efficient gene delivery inside hydrogels with tunable stiffness ranging from 2 to 175 kPa, with the peak transfection efficiency observed in hydrogels with intermediate stiffness (28 kPa). The reported hydrogel-mediated gene delivery platform using biodegradable polyplexes may serve as a local depot for sustained transgene expression *in situ* to enhance tissue engineering across broad tissue types.

1. Introduction

Non-viral gene delivery holds great promise for treating a wide range of diseases by directly regulating cell fate via genetic engineering and offers a safe alternative to the commonly used viral vectors for transporting genetic material to the intracellular environment. Cationic polymers or lipids have been widely used to condense negatively-charged DNA cargo to form nanoparticles by an electrostatic force-driven self-assembly process. Compared to the use of naked DNA alone, the use of polymeric vectors may protect DNA from being degraded by environmental nucleases and enhance cellular uptake. Various cationic vectors have been developed for such purposes, including the most commonly used polyethylenimine (PEI) and Lipofectamine 2000. However, most of the previously developed polymeric vectors are non-degradable and often suffer from low transfection efficiency. Furthermore, unbound free cationic polymers have been shown to cause cell shrinkage, vacuolization of the cytoplasm and a reduced number of mitoses [1].

To facilitate clinical translation of non-viral based gene therapy, biodegradable cationic polymers with high transfection efficiency and minimal cytotoxicity would be highly desirable. Towards this end, combinatorial synthesis and high-throughput screening of polymer libraries with diverse chemical structures offers a potential tool for rapidly discovering novel polymeric vectors for efficient gene delivery. Poly(β-amino)esters (PBAEs), a family of hydrolytically degradable polymers, has attracted particular interests for

* Corresponding author. 300 Pasteur Drive, Edwards R105, Stanford, CA 94305-5341, USA. Tel.: +1 650 725 7128; fax: +1 650 723 9370.
E-mail addresses: mkeeney@stanford.edu (M. Keeney), sonyiah@stanford.edu (S. Onyiah), zhezhang@mit.edu (Z. Zhang), xinming@stanford.edu (X. Tong), hanl5@stanford.edu (L.-H. Han), fanyang@stanford.edu (F. Yang).

0142-9612/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2013.08.050

combinatorial studies given their ease of synthesis and easily tunable chemical structure. High-throughput screening studies have identified lead PBAE structures that resulted in markedly enhanced gene delivery efficiency across a broad range of cell types both *in vitro* and *in vivo* [2–6]. We have previously reported the use of PBAE-based polyplexes to program stem cell *in vitro*, followed by transplantation of such non-viral engineered stem cell for *in situ* production of angiogenic factors. Using such strategies, we demonstrated significantly enhanced blood reperfusion and enhanced tissue regeneration both in a mouse hindlimb ischemia model [7] and a mouse excisional wound healing model [8].

Despite the great promise of biodegradable polyplex-mediated gene therapy for tissue regeneration, broad clinical translation of such therapy still faces challenges including the need for cell isolation and cell modification *in vitro*, and subsequent transplantation back to the patient. Such multi-step processes are associated with increased cost and pose a higher barrier for regulatory approval. In addition, *ex vivo* transfection using polyplexes is a one-time bolus delivery in the medium and only leads to transient up-regulation of target genes, ultimately limiting their applications for diseases in which prolonged transgene expression are necessary. Furthermore, bolus delivery cannot be used in applications where *in situ* spatial patterning of gene expression is needed. Therefore, there is a strong need to develop novel methods that would support *in situ* delivery of nucleic acids using polymeric vectors in a prolonged and spatially controlled manner.

Scaffold-mediated gene delivery offers a promising solution to the above challenges, in which polyplexes can be immobilized to a substrate surface or encapsulated in three-dimensional scaffolds for prolonged release over time. [9] Hydrogels are particularly attractive scaffolds given their tissue-like water content, injectability and tunable biochemical and biophysical properties. Surface deposition of polyplexes on hydrogel films has been used for both *in vitro* and *in vivo* delivery [10,11]. While surface-mediated release of polyplexes provides a 2D surface for cell attachment, cells *in vivo* reside in a 3D extracellular matrix. To better mimic the physiological scenario and for ease of clinical application, various attempts have been made to encapsulate polyplexes inside 3D hydrogels for sustained gene delivery, which can be co-encapsulated with transplanted cells or used to directly modulate endogeneous cells [12–16]. However, studies have shown that transfection inside 3D hydrogels is much more difficult to achieve than transfection in 2D [14,17]. Unlike the 2D environment, cells in 3D hydrogels often showed limited proliferation due to space constraint, and polyplexes need to make their way to the recipient cells for successful transfection to occur. As non-viral gene delivery relies partly on cell proliferation for nucleus uptake, reduced proliferation often leads to decreased transfection efficiency [14]. To enhance gene delivery to cells inside hydrogels, recent studies have introduced macroporosity inside 3D hydrogels to facilitate cell proliferation and enhance gene delivery within the macropores [18,19] but platforms that allow gene delivery homogeneously in bulk hydrogels remain lacking. Furthermore, recent studies have demonstrated that transfection efficiency of polyplex-mediated gene delivery further decreases dramatically as the hydrogel stiffness increases, and minimal transfection can be obtained when hydrogel stiffness exceeds 1 kPa, which limits its applications only for soft tissues [14,17].

The purpose of this study is to develop biodegradable polymeric vectors that can lead to efficient gene delivery inside 3D hydrogels with tunable matrix stiffness, which can serve as a local gene delivery depot for sustained transgene expression. We have chosen PBAEs as polymeric vectors given their biodegradable nature, tunable chemical structure and demonstrated high transfection efficiency. While the effects of varying PBAE chemical structure on their ability for gene delivery in 2D culture has been studied extensively, the use of PBAE/DNA polyplexes in hydrogel-mediated gene delivery for encapsulated cells has not been previously explored. We hypothesize that transfection efficiency of PBAE/DNA polyplexes inside 3D hydrogels can be enhanced by modulating the polymer chemistry of PBAE and the stability of these biodegradable polyplexes. To test our hypothesis, we synthesized 3 PBAEs and 3 poly(amido amine)s (PAAs) with different backbone chemistry, and examined the effects of varying PBAE chemistry on the resulting polyplex stability and their ability to transfect cells in 2D using Human Embryonic Kidney 293 (HEK293) cells as a model cell type. Using luciferase or GFP encoding DNA as a reporter, lead polyplexes with prolonged stability and high transfection efficiency in 2D were subsequently encapsulated in 3D photocrosslinkable PEG hydrogels with tunable matrix stiffness ranging from 2 kPa to 175 kPa. The extent and duration of gene expression were monitored using luciferase assays and fluorescence microscopy.

2. Materials and methods

2.1. Polymer synthesis

Acrylate-terminated PBAEs and PAAs were synthesized as described previously [3]. 1,4-Butanediol diacrylate (E4) and 1,6-Hexanediol diacrylate (E6) were purchased from Sigma Aldrich (St. Louis, MO) and 1,10-Bis(acryloyloxy)decane (E10) was purchased from VWR (Radnor, PA). N,N′-Methylene-bis-acryl (A1) and N,N′-Bis(acryloyl)cystamine (As) were obtained from Fisher Scientific (San Jose, CA) and N,N′-Hexamethylenebisacrylamide (A6) was obtained from Polysciences (Warrington, PA). The first step of polymer synthesis produced six acrylate-terminated polymers with different backbone chemistry utilizing Michael addition between monomers with diacrylate or amine end groups with an excess of diacrylate monomer (e.g. X-32-R) (Fig. 1). The 6 diacrylate monomers used in the synthesis are shown in Fig. 1C. An amine capping step was then performed to attach tetraethyleneglycoldiamine (122) (Molecular Biosciences, Boulder, CO), yielding six different polymers: E4-32-122, E6-32-122, E10-32-122, A1-32-122, A6-32-122, As-32-122. For abbreviation purposes, final polymers were referred to based on their backbone chemistry in the rest of the paper and figures (e.g. E6-32-122 is referred to as E6 for convenience).

2.2. Polyplex formation

Polyplex formation was induced by mixing plasmid DNA solution (120 μg/ml) and polymer solution (3.6 mg/ml) in 25 mM sodium acetate (Thermo Fisher Scientific) followed by a 10s vortex. PEI (Branched, 25 kDa, Sigma Aldrich) based polyplex were formed by mixing a plasmid DNA solution (24 μg/ml) and PEI (0.15 mM) in MES-HEPES buffered saline (50 mM MES hydrate, 50 mM HEPES, 75 mM NaCl in H2O; adjust pH to 7.2) (all products from Sigma Aldrich) and incubated at room temperature for 10 min. Plasmids encoding Gaussia Luciferase or Green Fluorescent Protein (New England Biolabs, Ipswich, MA) were used as reporters in this study. Polyplexes were allowed to self-assemble for 10 min and then added to cell culture media containing 10% serum for transfection. Alternatively, polyplexes were diluted in 1:1 vol/vol with sucrose (30 mg/ml in water) (Sigma Aldrich), frozen at −80 °C, and then lyophilized for 24 h. Lyophilized polyplexes were stored at −20 °C until used. To determine optimal transfection conditions, each polymer was complexed with DNA at varying weight ratios (polymer:DNA) including 10:1, 15:1, 20:1 and 30:1. Luminescence units were measured (Fig. S1) to determine the optimal weight ratio used for all following studies.

2.3. Determining stability of polyplexes

We next assess the stability of polyplexes formed using polymers with varying chemistry and incubated for different durations (0, 1, 2, 3, 4 and 5 days) in medium containing 10% serum. Polyplexes were synthesized each day over a 5 day period and analyzed altogether on the final day. During the incubation, polyplexes were stored in Dulbecco's Modified Eagles Medium (DMEM) (Life Technologies, Grand Island, NY) containing 10% fetal bovine serum (FBS) (Life Technologies) at 37 °C until analysis on the final day. The stability of polyplexes was analyzed via electrophoresis on a 1.2% agarose gel (Life Technologies) and a PicoGreen DNA intercalation assay (Life Technologies). The DNA intercalation assay involved the addition of 200 μl PicoGreen dye to 50 μl of polyplexes and quantifying the resulting fluorescence on a plate reader (Spectramax M2e, Molecular Devices, Sunnyvale, CA). To determine the degree of polyplex formation for DNA protection, 1 U of DNAse (Life Technologies) was added to 50 μl of polyplexes and incubated at 37 °C for 30 min followed by the addition of 100 μl PicoGreen dye. The detectable fluorescence intensity correlates

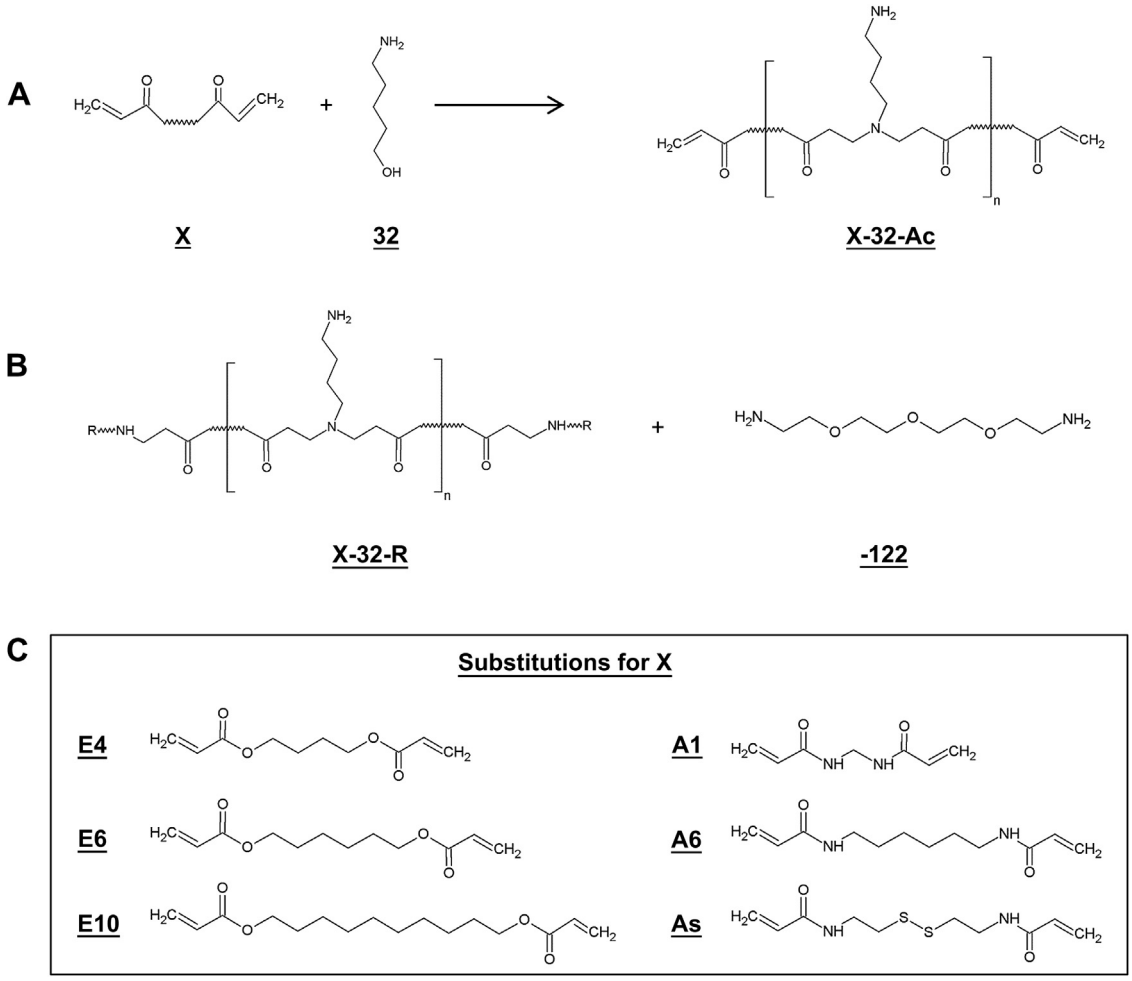

Fig. 1. (A) Synthesis of biodegradable poly(β-amino)esters and poly(β-amino)amides by 1.2:1.0 diacrylate:amine polymerization. (B) End-modification of acrylate-terminated polymers to introduce tetraethyleneglycoldiamine (122) end group to all polymers. (C) Chemical structures of six different backbone monomers used for synthesis.

with un-condensed DNA, and fluorescence signals with or without DNAse treatment was measured using a plate reader.

2.4. Cell culture

Human embryonic kidney (HEK293) cells, a commonly used model cell type for gene delivery, were used throughout this study. HEK293 cells were grown in DMEM supplemented with 10% FBS, and 1% penicillin/streptomycin (Life Technologies). Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2. Medium was changed every 2–3 days.

2.5. Transfection in 2D culture

The transfection efficiency of polymers with varying backbone chemistry was first evaluated in 2D culture to examine the effects of varying polymer backbone chemistry on their ability to transfect. Cell culture plates (96-well) were coated with gelatin (0.1% w/v) for 45 min prior to cell seeding to aid cell attachment. HEK293 cells were seeded at a concentration of 75,000 cells/ml (200 μl/well) and cultured overnight at 37 °C and 5% CO_2. Transfection was performed by replacing cell culture medium with 200 μl of polyplex-containing medium (0.8 μg plasmid DNA per well). All transfections were performed in cell culture medium containing 10% FBS. After 4 h of transfection, polyplex-containing medium was removed and replaced with regular cell culture medium.

2.6. Transfection quantification

Luciferase protein production was measured using the Gaussia Princeps Luciferase assay kit (New England Biolabs). Supernatant from the each well (2 μl) was diluted in 98 μl PBS in an opaque 96 well plate before adding 50 μl luciferase substrate (1×). Luciferase expression was immediately quantified by measuring luminescence with a plate reader. Cells transfected with plasmids encoding for GFP were imaged using a fluorescent microscope (Axio Observer Z1, Zeiss, Thornwood, NY).

2.7. Hydrogel material synthesis

Polyethylene Glycol dimethacrylate (PEG-DMA) was synthesized in house by reacting PEG diol (3 kDa) with methacryloyl chloride catalyzed by potassium carbonate and potassium iodide in dichloromethane overnight. All reagents used here were purchased from Sigma Aldrich. Further purification was performed by dialyzing synthesized polymers against DI water with cellulose dialysis tubing of 1 kDa cut-of-molecular weight (Fisher Scientific) for 2 days and freeze dried before use. Gelatin methacrylate (Gelatin-MA) was synthesized as previously described [20]. Briefly, methacrylic anhydride was reacted with Type-B gelatin (Sigma Aldrich) under stirring at 50 °C for 3 h. Gelatin-MA was extracted in acetone and purified by dialysis. Nuclear magnetic resonance (NMR) was performed on PEG-DMA and Gelatin-MA by dissolving the materials in deuterated chloroform and deuterated water respectively before obtaining 1H NMR spectra on a Varian 400 MHz and Inova 300 MHz NMR spectrometers (Agilent Technologies, Santa Clara, CA) respectively.

2.8. Transfection in 3D hydrogels

Hydrogels were formed by dissolving PEG-DMA and Gelatin-MA in DMEM. For quantifying transfection in 3D over time, hydrogels were made using 10% (w/v) of PEG-DMA and 3% (w/v) of Gelatin-MA. To examine the effects of varying hydrogel stiffness on the transfection efficiency inside 3D hydrogels, hydrogels with varying stiffness were made by varying PEG-DMA concentrations (6%, 8%, 10%, 12%, 14%, and 16%) (w/v) while keeping Gelatin-MA constant at 3% (w/v). To encapsulate polyplexes and cells in hydrogels, lyophilized polyplexes containing 100 μg DNA were dissolved in 990 μl of hydrogel solution, and then used to suspend HEK293 cells (5×10^6 cells/ml). Finally, 10 μl of Irgacure D2959 (5% w/v in 70% ethanol)

(Ciba Specialty Chemistry, Basel, Switzerland) was added to bring the total volume to 1000 μl. The gel solution was placed in a Teflon mold (50 μl/sample) and exposed to UV light (365 nm, 4 mw/cm^2, 5 min) for gelation to occur. After crosslinking, the hydrogels were transferred to a 48 well plate and washed twice with DMEM. Finally, 500 μl of DMEM containing 10% FBS and 1% penicillin/streptomycin was added to each well and the plate was kept in an incubator at 37 °C. Supernatant containing secreted luciferase protein was collected every 2 days up to 12 days and stored at −20 °C until further analyses.

2.9. Proliferation in 3D hydrogels

Cell proliferation inside 3D hydrogels were measured using a CellTiter 96® AQueous One Solution Cell Proliferation assay (Promega, Madison, WI) at multiple time points (day 2, 6 and 12). Briefly, regular culture medium was removed from each sample and replaced with 120 μl assay solution (mixture of 100 μl DMEM and 20 μl Aqueous One Solution). All hydrogels were incubated at 37 °C for 60 min after which the assay solution was removed and frozen at −20 °C. To collect entrapped Aqueous One Solution from 3D hydrogels, 120 μl of 1% (w/v) sodium dodecyl sulfate was added to each gel and incubated at room temperature overnight. The sodium dodecyl sulfate containing solution was then combined with the previously collected supernatant and absorbance was measured at 490 nm using a plate reader.

2.10. Mechanical testing

The compression modulus of hydrogels was characterized using unconfined compression tests as we previously described [21]. Briefly, hydrogels without cells or polyplexes were formed in a cylindrical mold (6.5 mm in diameter and 1.5 mm thick), and placed in serum containing media for 24 h prior to mechanical testing. Unconfined compression tests were conducted using an Instron 5944 materials testing system (Instron Corporation, Norwood, MA) fitted with a 10 N load cell (Interface Inc., Scottsdale, AZ). All tests were conducted in PBS solution at room temperature. Before each test, a preload of approximately 2 mN was applied. The upper platen was then lowered at a rate of 1% strain/sec to a maximum strain of 30%. Load and displacement data were recorded at 100 Hz. The compressive modulus was determined for strain ranges of 0%–10%, 10–20%, and 20–30% from linear curve fits of the stress vs. strain curve in each strain range.

2.11. Statistics

Minitab™ (Minitab Inc., USA) software was used for statistical analysis. One-way analysis of variance (ANOVA) with a Tukey's post-hoc analysis was used to determine statistical significance between groups while a paired *T*-test was used to directly compare two groups. A value of $p < 0.05$ was considered significant.

3. Results and discussion

Scaffold-mediated gene delivery is a promising tool for enhancing tissue repair by allowing sustained delivery of nucleic acids *in situ* in a spatially controlled manner. Furthermore, it offers a tool to directly transfer genetic materials to cells *in situ* without the need of cell isolation and manipulation *in vitro*. This mitigates costs, time and lowers the regulatory barrier for clinical translation. Hydrogels are particularly attractive scaffolds as local depot for gene delivery due to their high water content and injectable nature. However, achieving non-viral gene delivery from hydrogel-based scaffolds has proven very challenging; in addition, platforms that allow efficient non-viral gene delivery inside 3D hydrogels across stiffnesses that represent a broad range of tissue types remain lacking. This study provides a solution to the aforementioned challenges by providing a hydrogel-mediated, non-viral based gene delivery platform that enables efficient gene delivery in hydrogels with tunable stiffness. Specifically, we have chosen PBAEs and PAAs as polymeric vectors given their biodegradable nature and tunable chemical structures. Previous high-throughput studies have identified lead PBAE structures for efficient gene delivery in 2D bolus delivery, but the ability of PBAE-based polyplexes to work in 3D hydrogels remain largely unknown. We have chosen PEG-based hydrogels as the scaffold given their wide applications in tissue engineering, and tunable physical properties to mimic tissues with varying stiffness without altering biochemical cues. Given the high water content present in hydrogels and the fact that PBAEs are hydrolytically degradable, controlling the degradation rate of PBAE will likely affect polyplex stability in hydrogels. By controlling polyplex stability we can avoid premature degradation of the polyplexes before they reach the target cells. To increase polyplex stability and evaluate the effects of varying PBAE chemistry on gene delivery in 3D hydrogels, we first synthesized 3 PBAEs and 3 PAAs with varying backbone chemistry and varying degrees of degradation. Our results showed that transfection efficiency was directly influenced by modulating polymer chemistry, and polyplexes with high transfection efficiency in 2D may not achieve high efficiency 3D hydrogels. We identified 2 PBAE structures (E4 and E6) with similarly high transfection efficiency in 2D, however an 89-fold difference in accumulated transgene protein production was observed in 3D over 12 days. We identified a lead PBAE structure (E6) with slower degradation and enhanced stability of the resulting polyplexes, which resulted in highest transfection efficiency in 3D hydrogels across a broad range of stiffness. Hydrogels synthesized at the 28 kPa intermediate stiffness region, proved to have the highest transfection efficiency. Furthermore, our leading polymer resulted in a 7-fold increase in transfection efficiency inside 3D PEG hydrogels over the current gold standard, PEI.

3.1. Varying polymer structure significantly affects transfection efficiency in 2D

The polymer backbone was modified by incorporating monomers with varying rates of degradation. Polymers E4, E6 and E10 contained 4, 6 and 10 consecutive carbon molecules respectively in the backbone, leading to increasing hydrophobicity and decreasing degradation. Polymers A1 and A6 were similar in structure, however the ester groups were replaced with amides, hence decreasing the degradation rate of the polymer. Polymer As contained 2 sets of carbon molecules in the backbone connected by a disulfide bond. The presence of disulfide bonds has been previously shown to stabilize polyplexes in the extracellular environment. Once engulfed by the cell, the high intracellular concentration of glutathione has been shown to reduce the disulfide linkage, thus destabilizing the polyplex and releasing DNA [22]. Using luciferase and GFP DNA as model plasmids, our transfection results showed that polymer E4 and E6 resulted in 2–3 fold higher transfection efficiency compared to PEI (Fig. 2A and B). Increasing hydrophobicity from E4 to E6 led to increased transfection efficiency, however a further increase to E10 leads to a significant loss in transfection efficiency. Previous results have also shown that performance of PBAE-based non-viral gene delivery is highly sensitive to small changes in chemical structures [3]. One possible explanation for such rapid loss of transfection efficiency may be that increased hydrophobicity of E10 prevents the dissociation of DNA from polyplexes, hence reducing transfection efficiency. Replacing esters with amides can further slow down the degradation rate of polymers and affect transfection efficiency [23–25]. The presence of amides in the polymer backbone significantly reduces degradation rate of the polymer, therefore bioreducible disulfide linkages are often incorporated into the polymer to trigger polyplex dissociation upon intracellular stimuli such as high concentrations of glutathione. Despite increasing polymer stability, transfection appeared sacrificed as all three polymers containing amide or disulfide linkages tested in our study (A1, A6 and As) showed minimal transfection efficiency in 2D transfection. To maximize gene delivery efficiency without inducing significant cytotoxic, we performed a transfection study to determine the optimal polymer:DNA weight ratios for each polymer by measuring luciferase production (Fig. S1) and cell viability (Fig. S2). Our lead polymers, E4 and E6, reached highest transfection efficiency at lowest polymer:DNA weight ratio (10:1) and all 6 polymers demonstrated increased viability relative to PEI under optimal transfection conditions. Polymers A1, A6 and As

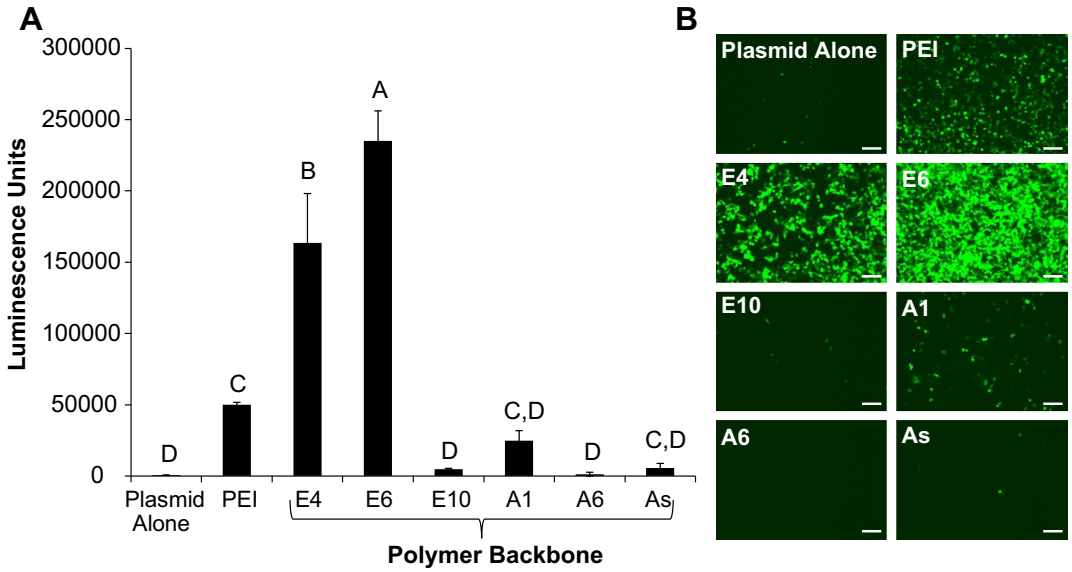

Fig. 2. (A) Quantitative luciferase protein production in HEK293 cells at day 2 post-transfection using polyplexes formed by different polymer structures. Cells were transfected with plasmid DNA encoding luciferase. Data is presented as mean ± standard deviation. (B) Fluorescent images of HEK293 cells at day 2 post-transfection using DNA encoding green fluorescent protein (GFP) complexed with different polymer structures. Scale bar = 200 μm. Bars with shared letters are not statistically different from each other.

generally only reached ~10% of transfection efficiency of E4 and E6, and further increases in polymer:DNA ratio did not increased transfection efficiency (Fig. S1). Given the results from 2D transfection, we have chosen to focus on the two PBAEs (E4 and E6) that outperformed PEI for further characterization and 3D studies.

3.2. *Polymer structure significantly affects polyplex stability and DNA protection*

One advantage of using polymeric vectors for gene delivery is to condense DNA into polyplexes, thereby protecting the DNA from degradation by environmental nucleases. To examine the effects of varying polymer structures on polyplex stability and DNA protection over time, we performed two complementary assays, electrophoresis and picogreen assay, by incubating polyplexes at one day interval over 5 days in medium containing 10% FBS. In gel electrophoresis, only free or released DNA can migrate in the gel whereas condensed DNA in polyplexes will stay trapped in the loaded wells. Fig. 3A showed that E4 complexed DNA tightly on day 0, as shown by the bright band trapped in the loading well. Starting from day 1, no polyplexes were detected in the wells and a smear of migrating bands showed up at the base of the gel, indicating E4-based polyplexes have degraded and released DNA (Fig. 3A). In contrast, polymer E6 remained stable throughout 5 days with strong signals in the wells. We included PEI, a non-degradable polymer as a control. As expected, no free DNA was observed over the 5-day incubation period. Also only faint bands were detected in the loading wells for PEI-based polyplexes, suggesting that PEI formed a much tighter polyplexes. We further confirmed the results observed in gel electrophoresis by PicoGreen assay, and our results generally showed the same trends in a more quantitative manner (Fig. 3B). In this assay, stable polyplexes would emit a low fluorescence, as the fluorescence intensity correlates with released free DNA. At day 0, all three polymers (E4, E6 or PEI) resulted in 80–90% decrease in fluorescence signal compared to plasmid alone, indicating successful polyplex formation and DNA protection (Fig. 3B). E4-based polyplexes showed a gradual increase in fluorescence intensity until day 3, indicating degradation of polyplexes and release of plasmid DNA. The presence of free plasmid DNA is confirmed by treatment with DNAse, which caused a decrease in fluorescence. At day 4 and 5, the plasmid DNA released from E4 polyplexes degraded in the serum containing medium, as shown by a further decrease in fluorescence signal intensity, hence the presence of a smear towards the base of the electrophoresis gel. Unlike E4, polymer E6-based polyplexes remained stable throughout the 5-day incubation as shown by the relatively stable, low level picogreen signal over 5 days. Treatment with DNAse only causes minimal changes in fluorescence intensity, indicating that no free DNA was released from E6-based polyplexes over 5 days. As expected, the non-degradable PEI-based polyplexes showed the lowest fluorescence signals and highest stability over time. Together, our results showed that E6-based polyplexes are relatively stable in serum containing conditions, whereas E4-based polyplexes degraded rapidly beyond one day.

To further determine the effects of polyplex stability on transfection, we pre-incubated polyplexes in serum containing medium up to 48 h before applying them for transfection. Although PEI offered the highest level of DNA protection as shown by Fig. 3, the ability of PEI-based polyplexes to transfect was rapidly lost after 12 h of incubation in serum-containing medium (Fig. 4). This might be due to the aggregation of PEI-based polyplexes in serum and causing an increase in particle size. In contrast, both E4 and E6 demonstrated increased transfection efficiency following 12 h of pre-incubation, suggesting these polymers are more suitable for gene delivery in serum containing conditions. E6-based polyplexes preincubated for 36 h showed comparable transfection efficiency as freshly prepared polyplexes (0 h), while a rapid loss in transfection efficiency was observed with E4-based polyplexes preincubated for 24 h or beyond. These results indicate that E6 would be most suitable for hydrogel-mediated gene delivery where more stable polyplexes are desirable. Likewise, E6 may be more suitable for in-vivo delivery, where polyplexes may reside in the extracellular matrix or travel through the bloodstream for an extended period of time before cell up-take. The increased stability of E6 is likely due to its slower degradation rate relative to E4 given the increased number of carbon linkages located in the polymer backbone.

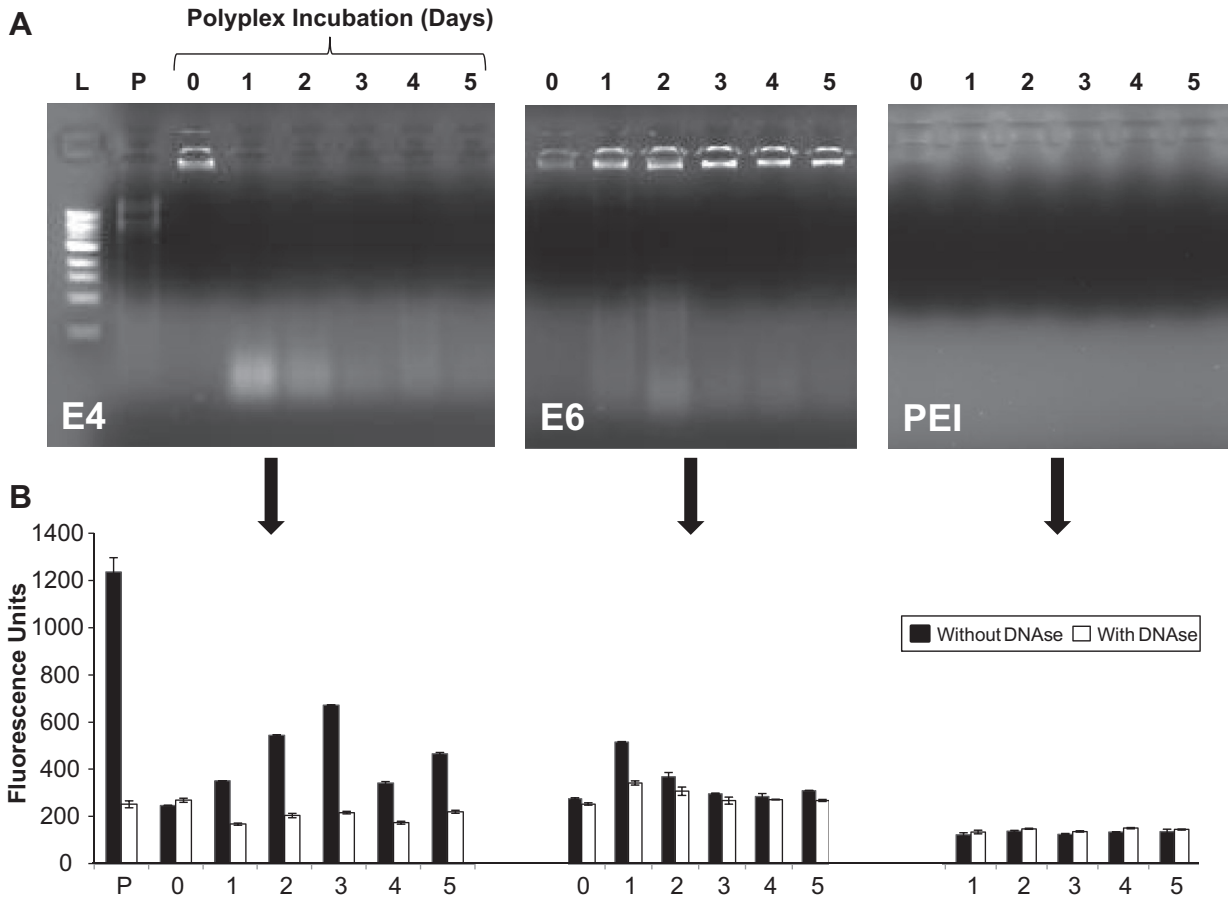

Fig. 3. The stability of polyplexes and effects of polymeric vectors on DNA protection, as measured by electrophoresis and PicoGreen assay. Plasmid DNA was complexed with different polymers (E4, E6 or PEI) and pre-incubated in serum containing medium for different time (0, 1, 2, 3, 4 or 5 days). (A) Gel electrophoresis showed rapid polyplex degradation and free DNA released after day 1 in E4-based polyplexes, and E6-based or PEI based polyplexes remained stable over 5 days. Intact polyplexes remain entrapped within the upper well whereas free DNA plasmid showed up as migrated bright bands in gel electrophoresis. L = Ladder (1 kB) and P = Free plasmids. (B). Plasmid complexed with polymers resulted in a sharp decrease in PicoGreen signal compared to free plasmid control, suggesting successful polyplex formation and reduced accessibility to free DNA. A decrease in fluorescence signal following DNAse treatment indicates degradation of free plasmid released from the polyplexes. Data is presented as mean ± standard deviation.

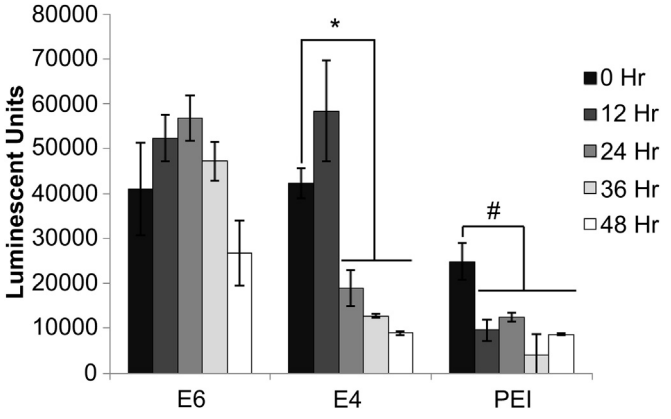

Fig. 4. The effects of polyplex stability on transfection as shown by luciferase protein production in HEK293 cells 2 days after initial transfection. Polyplexes were prepared using various polymers (E6, E4 or PEI) and pre-incubated in serum containing medium for varying periods (0, 12, 24, 36 and 48 h) before used for transfection. Data is presented as mean ± standard deviation. *or #: $p < 0.05$ compared to freshly prepared polyplexes (0 h) within each group.

3.3. Gene delivery inside 3D hydrogels over time

Previous studies have shown that gene delivery within 3D hydrogels is difficult to achieve. To examine if the polyplexes developed in the current study were suitable for 3D transfection, we encapsulated polyplexes (E4, E6 and PEI-based) and HEK cells in 3D hydrogels composed of 10% PEG-DMA and 3% Gelatin-MA. Gelatin-MA was included to provide cell adhesion sites and PEG-DMA was used to control matrix stiffness (Fig. 5A). NMR spectra confirmed successful modification of PEG and Gelatin into PEG-DMA and Gelatin-MA. (Fig. S3) We chose PEG-DMA as the scaffold in our study given its wide applications in tissue engineering, blank slate nature and easily tunable properties [26–28]. Remarkably, E6 demonstrated an 89-fold increase in accumulated luciferase production over 12 days compared to E4-based polyplexes (Fig. 5B).

The substantial increase in transfection efficiency is likely due to increased stability of E6- over E4-based polyplexes leading to greater DNA protection and prolonged availability of functional polyplexes as demonstrated in Figs. 3 and 4. We also confirmed that such differences in gene delivery are not caused by increased or decreased cell proliferation among groups. The CellTiter assay confirmed that similar proliferation rates were observed in all groups: E4, E6 or PEI (Fig. S4). Previous reports have shown that achieving gene delivery inside hydrogels is challenging using multiple non-viral vectors such

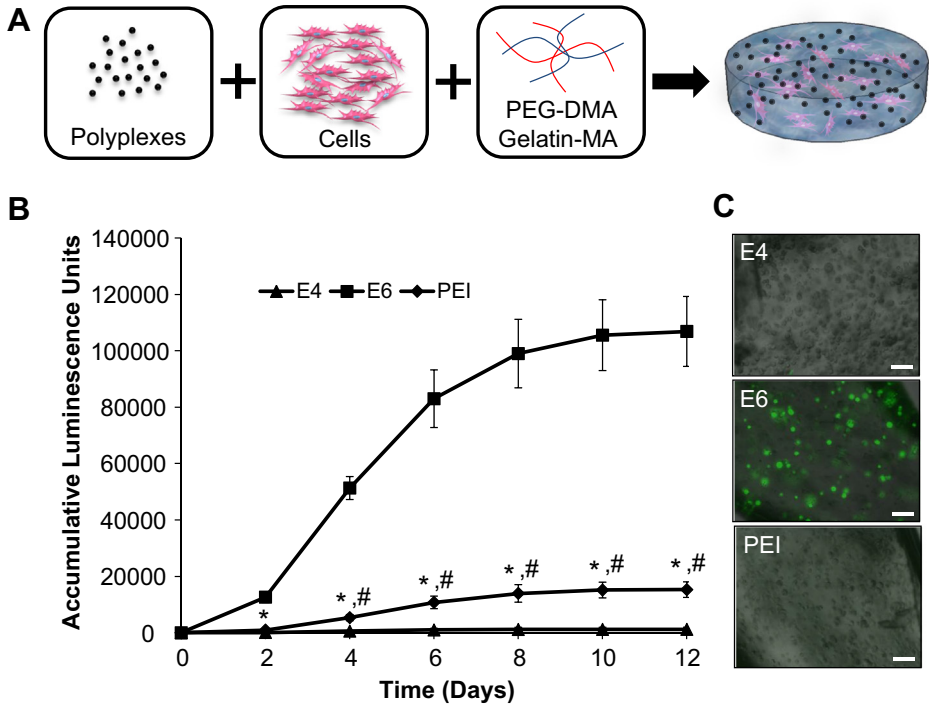

Fig. 5. (A) Schematic illustrating the co-encapsulation of cells and polyplexes within a PEG-DMA/Gelatin-MA crosslinked hydrogel. (B) Luminescence quantification of luciferase protein production in HEK293 encapsulated in 3D hydrogels composed of 10% PEG-DMA and 3% Gelatin-MA. Cells were co-encapsulated inside hydrogels containing polyplexes formed using E4, E6 or PEI complexed with DNA encoding luciferase. Data is reported as accumulative luminescence over 12 days. (C) Fluorescent microscope images of HEK293 cells at day 4 co-encapsulated inside hydrogels containing polyplexes formed using E4, E6 or PEI complexed with DNA encoding GFP. The fluorescent images are overlaid with the corresponding bright field images. Scale bar = 100 μm. Data is presented as mean ± standard deviation. * and #: $p < 0.05$ compared to E6 and E4 respectively.

as PEI and Lipofectamine 2000 [14,17,29]. Similarly, we observed low level of transgene expression inside hydrogels using PEI-based polyplexes in our study. We also examined the 3D transfection using a GFP DNA reporter, which allowed direct visualization of positively transfected cells and their distribution inside 3D hydrogels. Consistent with the luciferase results, strong and homogeneous GFP signal was observed in hydrogels containing E6-polyplexes, whereas minimal GFP expression was detected in hydrogels containing E4- or PEI-based polyplexes (Fig. 5C). Together, our results confirmed E6 as an efficient biodegradable polymeric vector for sustained gene delivery inside 3D hydrogels.

3.4. *Lead PBAE resulted in efficient gene delivery inside hydrogels with tunable stiffness*

Matrix stiffness varies across a broad range for different tissue types, and has been shown to directly influence cell differentiation, proliferation, morphology and transfection efficiency [14,17,30–32]. The above studies were performed in hydrogels with an intermediate stiffness of 28 kPa. We then further examine the ability of E6-based polyplexes for gene delivery inside hydrogels with tunable stiffness. By varying the concentration of PEG-DMA from 6% to 16%, we obtained hydrogels with stiffness levels ranging from 2 kPa to 175 kPa at a strain of 10%–20%. Similar to reports by previous studies, we observed highest cell proliferation in softest gels (2 kPa), and increasing hydrogel stiffness led to decreased cell proliferation (Fig. 6) [14]. Such decrease in cell proliferation is likely caused by a more restrictive 3D environment in hydrogels with higher crosslinking density. With regards to the effects of matrix stiffness on transfection efficiency, it has been shown previously that transfection efficiency rapidly decreases as the matrix stiffness increased beyond 1 kPa, which is speculated to be caused by decreased cell spreading and proliferation [14,17]. In our study, we were able to achieve high transfection efficiency across a broad range of stiffness ranging from 2 kPa to 175 kPa, with the highest gene delivery efficiency obtained in hydrogels with intermediate stiffness at 28 kPa. Further decrease or increase in hydrogel stiffness led to a decrease in

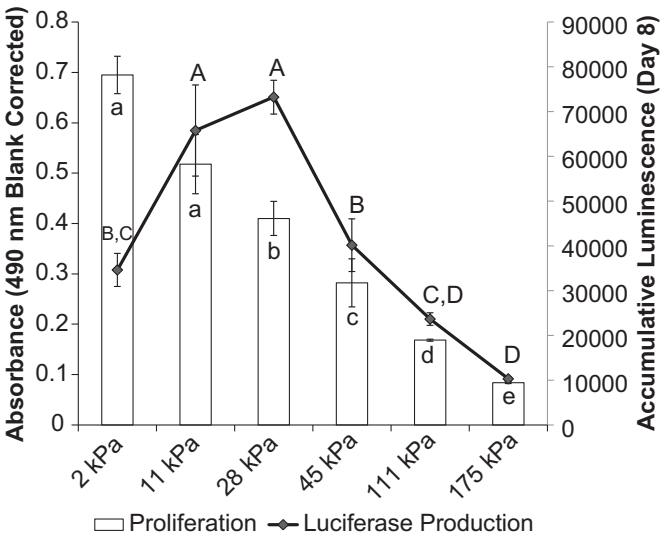

Fig. 6. Cell proliferation and corresponding luciferase production of HEK293 cells by day 8 after being encapsulated within PEG-DMA/Gelatin-MA hydrogels of varying stiffness containing polyplexes formed using polymer E6. Hydrogels with varying stiffness range (2–175 kPa) were obtained by varying PEG-DMA concentration (6–16%) while keeping Gelatin-MA concentration constant at 3%. Data is presented as mean ± standard deviation. Bars or points with shared letters are not statistically different from each other. Note: uppercase letters apply to luciferase production.

gene delivery efficiency. Previous studies have shown gene delivery is achievable inside hydrogels with storage modulus in a soft tissue range (0.1–1.7 kPa) using PEI-mediated polyplexes, and increasing hydrogel stiffness resulted in rapid decrease in the transfection efficiency. E6-based polyplexes also substantially outperformed PEI-based polyplexes inside 3D hydrogels, with 7–8 fold higher transfection efficiency achieved inside hydrogels at 28 kPa. Using our newly identified biodegradable E6 polymer, we were able to extend substantially the range of hydrogel-mediated non-viral gene delivery to mimic tissue stiffness across a broader range.

It is interesting to note that the gene delivery efficiency trend in our study did not correlate inversely with cell proliferation, suggesting that hydrogel stiffness may influence cell behavior in other manners. Recent studies suggest that matrix stiffness alone can influence various cell behaviors such as stem cell differentiation both in 2D and 3D culture [30,33], and different optimal stiffness ranges have been identified for promoting differentiation towards different lineages. In the current study, we observed that transfection efficiency for HEK cells reached a peak in 3D hydrogels with an intermediate stiffness (~28 kPa), and lower or higher stiffness both resulted in decreased transfection efficiency. It is possible that mechanotransduction plays an important role in modulating cell fate in hydrogels with varying stiffness, which may be partially responsible for the trend observed. Our results also highlight the importance to take into account matrix stiffness when designing non-viral gene delivery system for 3D culture. Future research on elucidating the mechanisms underlying how matrix stiffness regulates cell fate may also aid in optimizing the design of gene delivery platforms for 3D transfection.

4. Conclusion

In this study, we examined the effects of varying polymer chemistry on polyplex stability, DNA protection and gene delivery efficiency in 2D and inside 3D hydrogels. By synthesizing and characterizing polymers with varying backbone chemical structures, we identified a lead biodegradable polymeric vector, E6, with increased stability and achieved a sustained high level of transgene expression inside 3D hydrogels for at least 12 days. Furthermore, we demonstrated that E6-based polyplexes allowed efficient gene delivery inside hydrogels with tunable stiffness ranging from 2 to 175 kPa, which spans across a broad range of tissue types, with the peak transfection efficiency observed in hydrogels with intermediate stiffness (28 kPa). To our knowledge, this is the first study that identified biodegradable polymeric vectors that allow efficient gene delivery inside 3D hydrogels across a broad range of stiffness, which may provide a powerful tool for achieving hydrogel-mediated non-viral gene delivery in various tissue engineering applications.

Acknowledgments

The authors would like to thank the Donald E. and Delia B. Baxter Foundation Faculty Award, the McCormick Faculty Award and the Stanford Bio-X Interdisciplinary Initiative grant for funding.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2013.08.050.

References

[1] Lv H, Zhang S, Wang B, Cui S, Yan J. Toxicity of cationic lipids and cationic polymers in gene delivery. J Control Release 2006;114:100–9.
[2] Lynn DM, Langer R. Degradable poly(β-amino esters): synthesis, characterization, and self-assembly with plasmid DNA. J Am Chem Soc 2000;122:10761–8.
[3] Sunshine J, Green JJ, Mahon KP, Yang F, Eltoukhy AA, Nguyen DN, et al. Small-molecule end-groups of linear polymer determine cell-type gene-delivery efficacy. Adv Mater 2009;21:4947–51.
[4] Sunshine JC, Akanda MI, Li D, Kozielski KL, Green JJ. Effects of base polymer hydrophobicity and end-group modification on polymeric gene delivery. Biomacromolecules 2011;12:3592–600.
[5] Eltoukhy AA, Siegwart DJ, Alabi CA, Rajan JS, Langer R, Anderson DG. Effect of molecular weight of amine end-modified poly(beta-amino ester)s on gene delivery efficiency and toxicity. Biomaterials 2012;33:3594–603.
[6] Zugates GT, Peng W, Zumbuehl A, Jhunjhunwala S, Huang YH, Langer R, et al. Rapid optimization of gene delivery by parallel end-modification of poly(beta-amino ester)s. Mol Ther 2007;15:1306–12.
[7] Yang F, Cho SW, Son SM, Bogatyrev SR, Singh D, Green JJ, et al. Genetic engineering of human stem cells for enhanced angiogenesis using biodegradable polymeric polyplexes. Proc Natl Acad Sci U S A 2010;107:3317–22.
[8] Nauta A, Seidel C, Deveza L, Montoro D, Grova M, Ko SH, et al. Adipose-derived stromal cells overexpressing vascular endothelial growth factor accelerate mouse excisional wound healing. Mol Ther 2013;21:445–55.
[9] Pannier AK, Segura T. Surface- and hydrogel-mediated delivery of nucleic acid polyplexes. Methods Mol Biol 2013;948:149–69.
[10] Katz JM, Roth CM, Dunn MG. Factors that influence transgene expression and cell viability on DNA–PEI-seeded collagen films. Tissue Eng 2005;11:1398–406.
[11] Bielinska AU, Yen A, Wu HL, Zahos KM, Sun R, Weiner ND, et al. Application of membrane-based dendrimer/DNA complexes for solid phase transfection in vitro and in vivo. Biomaterials 2000;21:877–87.
[12] Chu C, Kong H. Interplay of cell adhesion matrix stiffness and cell type for non-viral gene delivery. Acta Biomater 2012;8:2612–9.
[13] des Rieux A, Shikanov A, Shea LD. Fibrin hydrogels for non-viral vector delivery in vitro. J Control Release 2009;136:148–54.
[14] Gojgini S, Tokatlian T, Segura T. Utilizing cell-matrix interactions to modulate gene transfer to stem cells inside hyaluronic acid hydrogels. Mol Pharm 2011;8:1582–91.
[15] Lei Y, Rahim M, Ng Q, Segura T. Hyaluronic acid and fibrin hydrogels with concentrated DNA/PEI polyplexes for local gene delivery. J Control Release 2011;153:255–61.
[16] Li Z, Yin H, Zhang Z, Liu KL, Li J. Supramolecular anchoring of DNA polyplexes in cyclodextrin-based polypseudorotaxane hydrogels for sustained gene delivery. Biomacromolecules 2012;13:3162–72.
[17] Shepard JA, Huang A, Shikanov A, Shea LD. Balancing cell migration with matrix degradation enhances gene delivery to cells cultured three-dimensionally within hydrogels. J Control Release 2010;146:128–35.
[18] Li Y, Yang C, Khan M, Liu S, Hedrick JL, Yang YY, et al. Nanostructured PEG-based hydrogels with tunable physical properties for gene delivery to human mesenchymal stem cells. Biomaterials 2012;33:6533–41.
[19] Shepard JA, Virani FR, Goodman AG, Gossett TD, Shin S, Shea LD. Hydrogel macroporosity and the prolongation of transgene expression and the enhancement of angiogenesis. Biomaterials 2012;33:7412–21.
[20] Han LH, Lai JH, Yu S, Yang F. Dynamic tissue engineering scaffolds with stimuli-responsive macroporosity formation. Biomaterials 2013;34:4251–8.
[21] Nii M, Lai JH, Keeney M, Han LH, Behn A, Imanbayev G, et al. The effects of interactive mechanical and biochemical niche signaling on osteogenic differentiation of adipose-derived stem cells using combinatorial hydrogels. Acta Biomater 2013;9:5475–83.
[22] Kakizawa Y, Harada A, Kataoka K. Glutathione-sensitive stabilization of block copolymer micelles composed of antisense DNA and thiolated poly(ethylene glycol)-block-poly(L-lysine): a potential carrier for systemic delivery of antisense DNA. Biomacromolecules 2001;2:491–7.
[23] Piest M, Lin C, Mateos-Timoneda MA, Lok MC, Hennink WE, Feijen J, et al. Novel poly(amido amine)s with bioreducible disulfide linkages in their diamino-units: structure effects and in vitro gene transfer properties. J Control Release 2008;130:38–45.
[24] Lin C, Zhong Z, Lok MC, Jiang X, Hennink WE, Feijen J, et al. Linear poly(amido amine)s with secondary and tertiary amino groups and variable amounts of disulfide linkages: synthesis and in vitro gene transfer properties. J Control Release 2006;116:130–7.
[25] Chen J, Wu C, Oupicky D. Bioreducible hyperbranched poly(amido amine)s for gene delivery. Biomacromolecules 2009;10:2921–7.
[26] Burdick JA, Anseth KS. Photoencapsulation of osteoblasts in injectable RGD-modified PEG hydrogels for bone tissue engineering. Biomaterials 2002;23:4315–23.
[27] Bryant SJ, Anseth KS. Controlling the spatial distribution of ECM components in degradable PEG hydrogels for tissue engineering cartilage. J Biomed Mater Res A 2003;64:70–9.
[28] Ashley GW, Henise J, Reid R, Santi DV. Hydrogel drug delivery system with predictable and tunable drug release and degradation rates. Proc Natl Acad Sci U S A 2013;110:2318–23.
[29] Bhise NS, Gray RS, Sunshine JC, Htet S, Ewald AJ, Green JJ. The relationship between terminal functionalization and molecular weight of a gene delivery polymer and transfection efficacy in mammary epithelial 2-D cultures and 3-D organotypic cultures. Biomaterials 2010;31:8088–96.

[30] Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. Cell 2006;126:677–89.
[31] Yeung T, Georges PC, Flanagan LA, Marg B, Ortiz M, Funaki M, et al. Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion. Cell Motil Cytoskeleton 2005;60:24–34.
[32] 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.
[33] Khetan S, Guvendiren M, Legant WR, Cohen DM, Chen CS, Burdick JA. Degradation-mediated cellular traction directs stem cell fate in covalently crosslinked three-dimensional hydrogels. Nat Mater 2013;12:458–65.