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PEGylated poly(ethylene imine) as a copolymer for gene delivery from hyaluronic acid hydrogels

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PEGylated poly(ethylene imine) as a copolymer for gene delivery from hyaluronic acid hydrogels

A thesis submitted in partial satisfaction

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in Chemical Engineering

by

Shayne Nicholas Siegman

2014

ABSTRACT OF THE THESIS

PEGylated poly(ethylene imine) as a copolymer for gene delivery from hyaluronic acids hydrogels

by

Shayne Nicholas Siegman

Master of Science in Chemical Engineering

University of California, Los Angeles, 2014

Professor Tatiana Segura, Chair

The effective delivery of DNA locally could increase the applicability of gene therapy in tissue regeneration and therapeutic angiogenesis. One promising approach is through use of porous hydrogel scaffolds that incorporate and deliver DNA in the form of nanoparticles to the affected sites. Although the Segura group has previous reported the ability to load DNA nanoparticles within porous HA hydrogels at high concentrations, gene delivery and transfection levels remain too low for therapeutic application. In this study, we report two alternative approaches to polyplex presentation that attempt to increase transgene expression levels. The first approach attempts to reduce polyplex aggregation by utilizing polyethylene glycol modification to mitigate charge-charge interactions between polyplexes and the scaffold during gelation. The second approach utilizes surface coated polyplex presentation is increase cell-particle interaction that in

not present with encapsulated presentation. sPEG-PEI polymer formed a smaller, less toxic, and more stable polyplex that exhibited little to no aggregation within HA gels when compared to the traditionally used LPEI polymer. Furthermore, sPEG-PEI retained transfection abilities comparable to LPEI in 3D, with no significant difference at 14 days. Surface coated polyplex presentation resulted in transgene expression levels that were three orders of magnitude greater than levels produced by encapsulated presentation. These results demonstrate a significant improvement in the porous HA gel system utilized by the Segura group, and hold promise for successful future studies in tissue engineering therapies.

The thesis of Shayne Nicholas Siegman is approved.

Tatiana Segura

Harold Monbouquette

James Liao

University of California, Los Angeles

2014

Dedication

I dedicate this work to my parents Tim and Pia Siegman. Thank you for always being awesome!

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Acknowledgements

I would like to thank Professor Segura for her support in this project and continued guidance over the past 5 years. Without her support, my graduate work would not be possible.

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1. Introduction

Chronic wounds present a serious health burden and are a source of staggering health care costs in the United States[1]. It is estimated that 6 million people in the U.S. are affected by chronic wounds, which results in roughly $20 billion in annual costs to the health care system[2]. The wound healing response in tissues is a complex process that involves numerous interactions between cells[3]. The mechanisms underlying the wound healing cascade include: (i) inflammatory response via mediators and growth factors; (ii) cell-cell and cell-ECM interactions that govern proliferation, migration, and differentiation; (iii) epithelialization and angiogenesis; (iv) wound contraction; and (v) remodeling[4]. This process typically results in the compromised integrity of the restored tissue, and is often complimented with the formation of scar tissue that further inhibits restored tissue function[3]. Cellular ingrowth and vascularization to the site of the wound must occur for proper tissue regeneration to take place. Without vascularization, lack of oxygen and nutrient diffusion to the wound prevents cellular infiltration and survival, resulting in impaired repair and tissue formation[5]. Furthermore, it has been suggested that chronic wound fluid (CWF) at the site of wounds inhibits endothelial cell and keratinocyte growth due to high concentrations of matrix metalloproteinases that break down growth factors necessary for tissue repair [6-8]. This wound healing cascade is typically incomplete or absent in patients that suffer from *chronic* wound healing; therefore, addressing this problem through tissue engineering therapies can improve the state suffering patients.

A current approach to restoring the microenvironment of a wound site is through the implantation of biocompatible three-dimensional scaffold materials that replace the structural and functional characteristics of damaged tissues[9]. 3-D systems more accurately recreate the interaction between biological and mechanical signals that affect cell action relative to 2-D

systems[10]. Moreover, some physiological and pathological cellular processes have been demonstrated to occur exclusively when cells are organized in a 3-D fashion[11]. A general approach to designing a scaffold for tissue engineering is to choose a material that mimics the extracellular matrix and provides structural support that can resist tensile and compressive stresses[11]. Cells can sense and respond to mechanical properties of their environment due to tight connections between their cytoskeleton and the extracellular matrix. Therefore, use of a material that interacts and responds to these cell environments can prove beneficial in tissue engineering applications.

Aside from using biomimicry as a design strategy to synthesize materials, molecular cues can also be incorporated into scaffolds to induce a therapeutic cellular response. The molecular cues that define the ECM consist of soluble macromolecules, insoluble factors, and proteins presented on the surface of cells [12]. These cues can be encapsulated within the scaffold and released during scaffold degradation, can electrostatically interact with the surface of the scaffold and be released during mechanical stress, or can be covalently linked to the scaffold and released upon cell cleaving. Incorporation of molecular cues that initiate or enhance the wound-healing cascade would serve beneficial in improving the wound-healing response in deficient patients.

Hydrogels are hydrophilic polymer networks that can self-assemble or be crosslinked into 3D structures[12], and are a current scaffold utilized in tissue engineering. These water-swollen polymer networks may contain an array of functional groups that can be chemically modified to attach desired cues such as small molecules, peptides, or proteins. Scaffolds can be synthesized from naturally occurring or synthetically produced materials. Naturally occurring biomaterials (i.e. collagen, hyaluronic acid) are advantageous due to their low immunogenicity and thorough integration with tissues at the site of implantation[13], while synthetic materials (i.e

poly(ethylene glycol), poly(lactic acid)) are advantageous due to their reproducibility and flexibility in property control [12]. Hyaluronic acid (HA) is an anionic, non-sulfated polysaccharide that is widely distributed throughout the ECM of connective tissues[14]. HA is an exciting hydrogel scaffold candidate for wound-healing therapies due to its high biocompatibility and low immunogenicity[15-17], but more importantly, HA chains have been known to promote angiogenesis and up-regulate MMP expression[18-20]. Specifically, HA interacts with CD44, RHAMM, and ICAM-1 surface receptors that contribute to cell proliferation and migration, which are processes that are necessary for the wound-healing cascade[21,22]. Hyaluronic acid can be crosslinked to form a hydrogel via an array of methods, including photopolymerization, thermosensitive crosslinking, and chemical crosslinking. Photopolymerization utilizes UV light to initiate a radical reaction that is quick and easily controlled, but free radicals pose issues with toxicity. Thermosensitive crosslinking allows for minimally invasive delivery to precise locations *in vivo*, but release profiles from these hydrogels are not well defined due to the undefined shape of the hydrogel. Chemical crosslinking allows for *ex vivo* synthesis of a hydrogel with defined properties (i.e crosslink density, polymeric weight percent, porosity, etc.). The Segura group, among others, has previous demonstrated the ability to chemically modify the HA backbone with functional acrylate groups that can be crosslinked into a hydrogel with matrix metalloproteinase (MMP)-degradable peptides via Michael addition chemistry[23].

Aside from providing mechanical support to the site of the wound to promote angiogenesis, HA hydrogels can be manipulated to include genes that further stimulate vascularization[24]. The major aim of gene therapy is to effectively deliver genes to cells inducing genetic modification that results in functional repair[25]. An approach to improving the wound healing cascade in suffering patients is to deliver pro-angiogenic genes (i.e. vascular

endothelial growth factor [VEGF] and platelet-derived growth factor [PDGF]) to the site of a wound that promote vascularization in order to increase nutrient flow and improve tissue repair. Poor delivery efficiency of naked DNA is often experienced, though, due the plasmid's negative charge and large hydrodynamic radius that result in plasmid degradation or ineffective internalization and trafficking by the cell[16]. For this reason, research efforts have focused on developing viral and chemical agents that can be used to condense DNA into nanoparticles for more efficient transport to the nucleus of cell[26]. While viral agents exhibit high levels of transfection they are limited by issues of immunogenicity and insertion mutagenesis[27]. For this reason, non-viral agents (i.e. chemical/polymer) have gained popularity in recent years.

Cationic polymers are a promising non-viral agent for successful DNA delivery. Cationic polymers are able to complex DNA through electrostatic interactions of cationic molecules on the polymer backbone with negatively charged phosphate groups on the DNA backbone, resulting in charge neutralization and compaction of the nucleotide fragment[28] Polyethyleneimine (PEI) is one such cationic polymer that currently serves as the gold standard for gene transfection. PEI was first utilized as a transfection agent in 1995 by the Behr group[29], and since then has been studied extensively in its ability to efficiently deliver DNA to the nucleus of cells. Positively charged amine groups on the backbone of PEI interact with the negatively charged phosphate groups of DNA, condensing the DNA into a tight polymer-DNA complex (polyplex) that can more easily be internalized by a cell due to its small size[30,31] and more neutral charge[28].

Although the Segura group has demonstrated the ability to deliver DNA-PEI polyplexes from HA gels, direct polyplex encapsulation resulted in aggregation when concentrations exceeding 0.2 μg/μL. To mitigate this issue, Lei *et. al*. developed a caged nanoparticle

encapsulation (CnE) technique that utilized neutral sacchrides (sucrose) and polysacchrides (agarose) to protect the polyplexes from inactivation and aggregation during lyophilization and hydrogel formation, respectively[32]. This CnE technique allowed for the incorporation of polyplexes in HA hydrogels at a concentration up to 2.5μg/μL without observing aggregation. This technique was coupled with the introduction of micron-sized pores within gels by Tokatlian *et. al*. in an attempt to promote increased cell migration and infiltration to the scaffold. Porosity in hydrogels has previously been shown to promote cell migration *in vitro*[33,34] and hydrogel integration/vascualarization *in vivo*[35,36]; moreover, porous scaffold serve as non-viral gene carriers by coating or encapsulating bioactive signals onto or within the gel[9,37,38]. Although gene delivery was achieved both in vitro[39] and in vivo[13] from encapsulated polyplexes, transgene expression levels remained too low for therapeutic application. We hypothesize the CnE and acetone processing during micron size pore hydrogel formation causes an increase in gel stiffness and reduces pore size, resulting in a slower rate of gel degradation and polyplex release.

Herein, we present two approaches to prevent polyplex aggregation within HA hydrogels, which either introduce DNA polyplexes *during* or *after* hydrogel formation. The first approach utilizes polyethylene glycol modification to mitigate charge-charge interactions between polyplexes and the scaffold during gelation. It has been shown that complexing DNA with PEGylated PEI results in a less toxic and more stable polyplex that aggregates less than PEI polyplexes[40-45]. The second approach utilizes surface coated polyplex presentation *after* porous gel formation to increase cell-polyplex interaction. Its been shown that surface coated polyplex presentation results in transgene expression levels that are an order of magnitude

greater than levels produced by encapsulated polyplexes[38], therefore exploring this technique may prove useful to improving our HA system.

2 Materials and Methods

2.1 *Materials*

Peptides Ac-GCRDGPQGIWGQDRCG-NH_2 (HS-MMP-SH) and Ac-GCGYGRGDSPG-NH_2 (RGD) were purchased from Genscript (Piscataway, NJ). Sodium hyaluronan (HA) was a gift from Genzyme Corporation (60 kDa, Cambridge, MA). High molecular weight linear poly(ethylene imine) (LPEI, 25kDa) and low molecular weight linear poly(ethylene imine) (LMW-PEI, 2.5kDa) were purchased from Polysciences (Warrington, PA). 8-arm poly(ethylene glycol) succinimidyl carboxyl methyl ester (PEG-SCM, 10kDa) was purchased from Creative PEGWorks (Winston Salem, NC). Vectors for the mammalian expression of Gaussia luciferase (pGluc) and secreted embryonic alkaline phosphatase (pSEAP) were purchased from New England Biolabs (Ipswich, MA) and BD Biosciences (San Jose, CA), respectively. Both vectors were expanded using a Giga Prep kit from Qiagen (Valencia, CA) per manufacturer's protocol. All other chemical were purchased from Fisher Scientific (Pittsburgh, PA) unless otherwise noted.

2.2 *Methods*

2.2.1 Hyaluronic acid modification

Sodium hyaluronan was modified to contain acrylate function groups as previously described. Briefly, 2.0 g of hyaluronic acid (5.28 mmol, 60 kDa) was reacted with 36 g (211 mmol) of adipicdihydrazide (ADH) and 4 g (20 mmol) of 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) overnight at pH 4.75. The reaction mixture was then purified by dialysis (8000 MWCO) in deionized (DI) water for 2 days, and the purified product (HA-ADH) was lyophilized for NMR analysis. A small sample was taken and analyzed using ^{1}H-

NMR (D_2O) and indicated a 54.67% modification of the carboxyl groups on the HA backbone to ADH groups, which was determined by taking the ratio of peaks at δ = 1.6 and 2.3 corresponding to the eight hydrogens of the methylene groups of the ADH to the singlet peak of the acetyl methyl protons in HA (δ = 1.88). HA-ADH (1.9 g) was reacted with N-acryloxysuccinimide (NHS-Ac) (1.33 g, 4.4 mmol) in HEPES buffer (10 mM HEPES, 150 mM NaCl, 10 mM EDTA, pH 7.2) overnight and was purified by dialysis in DI water for 2 days prior to lyophilization. ^{1}H-NMR (D_2O) spectroscopy confirmed 11.42% acrylate modification (HA-Ac) by taking the ratio of the multiplet peak at δ = 6.2 corresponding to the cis and trans acrylate hydrogens to the singlet peak of the acetyl methyl protons in HA (δ = 1.88).

2.2.2 Synthesis of sPEG-PEI

LMW-PEI was conjugated to the 8-arm PEG-SCM to obtain a more water soluble PEI polymer complex. 190.8mg (0.07632 mmol) LMW-PEI was dissolved in MES buffer (100uM, pH 5.5), and once fully dissolved the pH was increased to 7.4. After the PEI had fully dissolved, 50 mg of PEG-SCM (0.00477mmol) was dissolved in separate MES buffer pH 7.4. The PEG-SCM solution was drop wise added to the PEI solution while maintaining a constant pH, and the mixture was allowed to react overnight. The product was purified by dialysis (3,500 MWCO) to get rid of an unreacted PEI, and the product was analyzed using ^{1}H-NMR (D_2O). ^{1}H-NMR (D_2O) spectroscopy indicated attachment of PEI to every arm of the 8-arm PEG-SCM by assessing the ratio of the PEI peak (δ = 3.0) to the PEG-SCM peak (δ = 3.6).

2.2.3 Cell culture

HEK293T cells were a kind gift from Lonnie Shea of Northwestern, and HEK293T-MMP2 cells were a kind gift from Jeffrey Smith from the Burnham Institute for Medical Research. Mouse-bone-marrow-derived mesenchymal stem cells (D1, CRL12424) were purchased from ATCC (Manassas, VA). HEK293T, HEK293T-MMP2, and D1 cells and cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Grand Islands, NY) supplemented with 10% bovine growth serum (BGS, Hyclone, Logan, UT) and 1% penicillin/streptomycin (Invitrogen) at 37°C and 5% CO_2. The cells were passaged using trypsin following standard cell culture protocols every 2-3 days.

2.2.4 Polyplex formation and characterization

To form polyplexes, 3 μg of plasmid DNA was diluted in 150 μl of nuclease free water and the desired amount of either LPEI or sPEG-PEI, depending on the required N/P ratio (ratio of the number of nitrogen groups on the polymer to the number of phosphate groups on the DNA backbone), was diluted into a separate 150 μl of nuclease free water. For polyplexes formed at N/P 7, 4 μg sPEG-PEI was used and 2.73 μg LPEI was used. For polyplexes formed at N/P 12, 6.87 μg sPEG-PEI was used and 4.69 μg LPEI was used. The PEI solution (either LPEI or sPEG-PEI) was drop wise added to the DNA while vortexing, and each sample was incubated at room temperature for 15 min. 150mM NaCl or PBS was then added to each polyplex solution and the size and ζ-potential of the polyplexes were determined by photon correlation spectroscopy (Malvern Zetasizer, Malvern Instruments Ltd., U.K.). The measurements were performed at 25°C.

2.2.5 Agarose gel retardation assay

An agarose hydrogel retardation assay was performed in order to assess the N/P ratio at which the polyplexes are fully condensed. Polymer/pDNA complexes were prepared at N/P ratios 1-7 in nuclease free water per the aforementioned protocol, with naked DNA containing no polymer as the control. The polyplexes were electrophoresed through a 1% (w/v) agarose hydrogel containing a 1/10,000 dilution of SYBR® Safe DNA Stain (Life Technologies, Grand Island, NY) in 1X Tris-acetate-EDTA (TAE) buffer at 80 V for 30 min. The hydrogel was then analyzed on a Hydrogel Doc EZ Imager (Bio Rad, Hercules, CA) to observe the fluorescence of each polyplex relative to naked DNA.

2.2.6 Polyplex lyophilization by caged nanoparticle encapsulation (CnE)

For CnE, plasmid DNA (8.3 μg) and either sPEG-PEG or LPEI (13.4 μg or 9.1 μg, respectively) were mixed in 3.5 mL water in the presence of 3.5mg (0.01 mmol) of sucrose (Ultrapure, MP Biomedicals, Santa Ana, CA) and incubated at room temperature for 15 min. Low-melting point agarose (0.1 mg, Ultrapure Agarose, T – 34.5-37.5°C, Invitrogen, Grand Islands, NY) in 150 mL water was added before lyophilization. Each aliquot was intended for a 10 μL hydrogel.

2.2.7 Porous hydrogel design template using PMMA microspheres

Chemically sintered microsphere templates were prepared as previously described[5]. Briefly, polymethyl methacrylate (PMMA) microspheres (53-63 μm, Cospheric, Santa Barbara, CA) were suspended in sintering solution (70% ethanol, 1% acetone) at a concentration of 0.4444mg μL^{-1}, and 75 μL of this bead solution was then added to every well of flexiPERM

molds (Sigma-Aldrich, St. Louis, MO) adhered to Sigmacote (Sigma-Aldrich, St. Louis, MO) glass slides. The molds were sintered at 37°C for 2 hours before use.

2.2.8 Porous and nanoporous hydrogel formation

Hydrogels were formed by Michael-addition of acrylate functionalized HA (HA-Ac) with bis-cysteine containing MMP peptide crosslinkers at pH 7.6-7.8. Prior to the reaction, a hydrogel precursor solution was made by mixing HA-Ac with a lyophilized aliquot of the cell adhesion peptide RGD for 30 min at 37°C. After incubation, HA-RGD was mixed with the remaining HA-Ac and 0.3M triethanolamine (TEOA, pH 8.8), for a final hydrogel concentration of 3.5% weight/volume% HA and 100uM RGD. Finally, lyophilized aliquots of the crosslinker (0.8mg HS-MMP-SH) were diluted in 16 μL of TEOA buffer, pH 8.2, immediately before addition to the hydrogel precursor solution. This hydrogel precursor solution was then mixed with either lyophilized (CnE) or fresh (direct encapsulation) DNA/polymer polyplexes for hydrogels containing polyplexes. For direct encapsulation, DNA and sPEG-PEI or LPEI were mixed according to the aforementioned protocol. For nanoporous hydrogels (n-pore), 20uL of the hydrogel solution was added between 2 slides with a 1mm spacer to separate the two slides. Hydrogels were incubated at 37°C for 30 min, then hydrated in phosphate-buffered saline (PBS) and left in PBS until used. For the micro-porous (μ-pore) hydrogels, 20 μL of the hydrogel solution was then added directly on top of a PMMA microsphere template and perfused into the template by centrifugation at 500 g for 15min at 4°C. The template was then incubated at 37°C for an additional 20 min to induce complete polymerization. Once complete, the hydrogels were removed from the flexiPERM molds and placed directly into 100% acetone fro 48 h to dissolve

the PMMA microsphere template. The acetone solution was replaced 3 times a day for the 48 h wash. The hydrogels were then serially hydrated in PBS, and left in PBS until used.

2.2.9 Characterization of HA hydrogel mechanical properties

The storage and loss modulus of nanoporous and microporous hydrogels were measured with a plate-to-plate rheometer (Physica MCR, Anton Paar, Ashland, VA) using a 8 mm plate under a constant strain of 0.1% and frequency ranging from 0.1 to 10 rad/s. Nanoporous and microporous hydrogels were synthesized according the aforementioned protocol and cut to 8 mm using an 8 mm biopsy punch. To prevent the hydrogel from drying, a humidity hood was utilized and the stage was set to 37°C.

2.2.10 DNA loading efficiency

In order to determine the amount of DNA loaded on the surface of porous hydrogels, plasmid DNA was radiolabeled with ^{32}P-dCTP (250uCi, PerkinElmer, Waltham, MA) using a Nick translation kit (Roche, Indianapolis, IN) per the manufacturer's protocol. Briefly, an equimolar mixture of dATP, dGTP, dTTP, and ^{32}P-dCTP was prepared and added to the DNA (1.25 μg) solution. Once the enzyme solution was added to the mixture, the final solution (225 μL) was gently mixed and incubated for 35 min at 15°C. The reaction was stopped by the addition of 10 μL 0.2 M EDTA (pH = 8.0) and heating at 65°C for 10 min. The DNA was purified using the Mini Prep kit from Qiagen (Valencia, CA) following the manufacturer's instructions. The 1.25 μg radiolabeled DNA was then mixed with 498.75 μg unradiolabeled DNA to make a 0.25% "hot" DNA solution, and with a final total DNA concentration of 2.22 μg/μL. In order to determine the extent of release of the surface coated polyplexes, hydrogels

swollen in PBS were biopsy-punched using a 4mm biopsy and placed in individual 1.5 mL tubes. Each gel was incubated with 50 μL of a 0.2ug/ul pDNA/polymer polyplex solution for 2 hr at room temperature, flicking every 20 min. Hydrogels were then extensively washed with PBS to remove any unbound polyplexes, then placed in 2 mL of Bio-Safe II scintillation cocktail (Research Products International Corp., Mt. Prospect, IL) and measured using a scintillation counter at the UCLA chemistry facility. The readout was analyzed using a standard curve.

2.2.11 DNA release from surface coated hydrogels

In order to determine the overall the extent of release of surface coated polyplexes, gels were formed and surface coated using the aforementioned protocols with 0.25% radiolabeled DNA polyplexes. Gels were then placed in 200 μL of either 1 U/mL collagenase type 1 solution (Col I) (Worthington Biochemical, Lakewood, NJ) or PBS (control). The solutions were collected and replenished daily, and DNA concentrations were measured using a scintillation counter at the UCLA chemistry facility. The readouts were analyzed using a standard curve.

2.2.12 Polyplex visualization

Polyplexes were visualized within nanoporous and porous gels to determine if sPEG-PEI polyplexes aggregate less than LPEI polyplexes. Prior to the formation of polyplexes, plasmid DNA was tagged with a 500 nm tag using a PromoFlour Nick Translation kit (Promokine, Heidelberg, Germany) per the manufacturer's protocol. Briefly, 1.5 μg DNA was mixed with 2 μL 10x NT labeling buffer, 2 μL PromoFluor labeling mix, 2 μL enzyme mix, and 16 μL water. The mixture was incubated at 15°C for 90 min, and the reaction was stopped by the addition of 5 μL of stop buffer. The DNA was purified using the mini Prep kit from Qiagen (Valencia, CA)

following the manufacturer's instructions. sPEG-PEI or LPEI/tagged-DNA polyplexes were form per the aforementioned protocol, and incorporated into CnE, direct encapsulation, or surface coated hydrogels per the aforementioned protocols. Hydrogels were imaged over a constant z-plane using a Nikon C2+ confocal microscope (Nikon Instruments Inc., Melville, NY).

2.2.13 Cytotoxicity of sPEG-PEI and LPEI polyplexes

An MTT assay (CellTiter 96^{R} AQ$_{ueous}$ One Solution Cell Proliferation Assay, Promega, Madison, WI) was used to quantify the metabolic activity of cells exposed to sPEG-PEI or LPEI polyplexes in order to correlate metabolic activity to cell viability. 12,000 D1 cells were cultured in a 96-well plate for 16 hours, then transfected with 20 μL of either sPEG-PEI or LPEI polyplexes at DNA concentrations ranging from 0-0.2 μg/μL and N/P ratios 7 and 12. After a 2 day incubation, the media in each well was aspirated and cells were then incubated with 20 μL of MTT reagent and 100 μL of DMEM for 2 hours at 37°C. Following the 2 hour incubation, 25 μL of 10% sodium dodecyl sulfate was added to each well to stop the reaction, the solutions were transferred to a new plate, and the absorbance was measured at 490 nm using a standard plate reader. The readings taken from cells not exposed to any polyplexes were used as a control.

2.2.14 In vitro 2-D bolus transfection

To assess the tranfection efficiency of sPEG-PEI compared to LPEI, pGluc or pSEAP polyplexes of N/P ratios 7 and 12 were created using both polymers. D1 or HEK293T-MMP2 cells were seeded on a 48-well plate at a density of 40,000 cells/well in 500 μL of media, and allowed to incubate for 16 hours. Following this incubation, the media from each well was

removed and replaced with fresh media. 50 μL of polyplex solution (0.02 μg DNA/μL) was added drop wise to each well, the plate was swirled to evenly distribute the polyplexes within each well, and finally NaCl was added to each well to a resulting concentration of 150 mmol. The cells were allowed to incubate for 2 days, after which the media was collected and frozen until assayed. To quantify secreted Gaussia luciferase levels in the media, the samples were thawed on ice and assayed using a BioLux Gaussia Luciferase Assay Kit (New England Biolabs, Ipswich, MA) as per the manufacturer's protocol. Briefly, 20 μL of each sample were mixed with 50 μL of substrate solution, pipetted for 2 to 3 seconds to mix, and read for luminescence with a 5 s integration time using a Modulus Fluorometer (Turner BioSystems, South San Francisco, CA). Background was determined with media from cells that did not contain any DNA polyplexes and values were expressed as relative light units (RLU). To quantify secreted embryonic alkaline phosphatase levels in the media, the samples were thawed on ice and assayed using a pSEAP Assay Kit (Life Technologies, Grand Island, NY). Briefly, 100 μL of sample was incubated with 200 μL of dilution buffer and incubated at 65°C for 30 min. 100 μL of the diluted sample was then mixed with 100 μL of assay buffer and incubated for 20 min. 100 μL of reaction buffer was then added to each sample, incubated for 20 min, and samples were read for luminescence with a 1 s integration time. Background was determined with media from cells that did not contain any DNA polyplexes and values were expressed as RLU.

2.2.15 3D transfection from surface coated porous HA hydrogels

To assess sPEG-PEI vs. LPEI transfection efficiency in 3-D, 3.5% HA porous hydrogels were synthesized via the aforementioned protocol. After fully hydrating the hydrogels in PBS, each hydrogel was cut to a diameter of 4 mm using a 4-mm biopsy punch and placed in

individual 1.5 mL tubes. 50 μL of a 0.2ug/ul pGluc/polymer polyplex solution was added to each tube and incubated for 2 hr at room temperature, flicking every 20 min. Hydrogels were then washed three times with PBS to remove any unbound polyplexes. Hydrogels were then surface coated with 40,000 D1 in 250 μL of media cells for 3 hr with flicking every 20 min, and washed to remove any unbound cells. At 2, 4 and 7 days, conditioned medium was collected from all samples and Gaussia luciferase expression was determined for both polymers via the aforementioned protocol.

2.2.16 Two-gene delivery system from porous hydrogels

In order to demonstrate a dual gene delivery system, pGluc polyplexes were synthesized per the aforementioned protocol and incorporated into the hydrogel precursor solution at a concentration of 0.2 μg/μL. Porous gels were then formed with this hydrogel precursor solution per the aforementioned protocol. After the PMMA beads had dissolved and the hydrogels had been swelled, pSEAP polyplexes were synthesized per the aforementioned protocol. Each gel was surface coated with 50 μL of 0.2 μg/μL polyplex solution per the aforementioned protocol. Finally, 40,000 HEK293T-MMP2 cells in 250 μL media were surface coated on the porous gels per the aforementioned protocol. Media was collected at days 2, 4, 7, 10, and 14 and assayed for pGluc and pSEAP expression as per the aforementioned protocols.

2.2.17 Statistical analysis

Statistical analyses were performed using Prism (GraphPad, San Diego, CA). All data sets except for polyplex size were analyzed using a one-way analysis of variance (ANOVA) test followed by a Tukey posed hoc test. The results are presented as mean ± SD. Polyplex size data

was analyzed using a t-test, and the results are presented as mean ± SD. Single, double, and triple asterisks represent $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively. A p value of < 0.05 was considered statistically significant.

3 Results

3.1 HA Modification and sPEG-PEI synthesis

Acrylates were conjugates onto the HA backbone through a two-step process, as previously described[46]. Briefly, HA was first modified with adipic acid dihydrazide (ADH) suing EDC coupling and the resulting hydrazide group was then modified with NHS acrylate to obtain acrylamide functionalities. Analysis by NMR showed and ~11.5% of the carboxylic acids were modifies with acrylates, resulting in ~18 acrylates per HA chain. RDG adhesion peptides were incorporated through Michael-type addition of the cysteine side chain in the peptide to the acrylate groups of the HA backbone, followed by the addition of an MMP-degradable peptide cross-linker to form the final hydrogels. Porous hydrogels were synthesized around a PMMA microsphere template that was formed via a chemical scintering method as previously described [5], and the PMMA template was finally dissolved away using acetone leaving behind a porous network within the hydrogel (Figure 1A).

sPEG-PEI was synthesized following the scheme as depicted in Figure 2. Due to insoluble nature of PEI, LMW-PEI was first dissolved in acidic conditions and once fully dissolved the pH of this solution was slowly increased to 7.4 in order to prevent precipitation. LMW-PEI was conjugated to the ends of the 8-armed PEG-SCM through NHS chemistry via the secondary amines on the LMW-PEI backbone. The extent of conjugation was calculated through NMR by comparing the observed peaks of LMW-PEI and PEG-SCM at δ=3.05 and δ=3.65, respectively, and indicated that an LMW-PEI group had attached to each arm of the 8-armed PEG-SCM.

3.2 Polyplex Characterization

Polyplexes of either sPEG-PEI or LPEI with plasmid DNA were formed under physiological salt concentrations at a N/P ratio of 7 and 12 to study the influence of polymer composition on particle size and stability. After the addition of salt, sPEG-PEI polyplexes doubled in size but showed no drastic increase in size thereafter until day 5 (Figure 3A). Conversely, LPEI polyplexes experienced a significant 7-fold increase after the addition of salt, and showed a 130-fold increase from the initial size just after day 1 (Figure 3B). These results indicate sPEG-PEI forms a smaller, more stable polyplex when complexed to pDNA compared to LPEI. To assess polyplex zeta potential, sPEG-PEI and LPEI polyplexes were synthesized in either 150 mmol NaCl or PBS solutions. The average charge of sPEG-PEI polyplexes N/P 7 in 150 mmol NaCl and PBS was 10.47 mV and 2.16 mV, respectively, and for polyplexes N/P 12 in 150 mmol NaCl and PBS was 22.17 mV and 5.68 mV, respectively (Figure 3D). The average charge of LPEI polyplexes N/P 7 in 150 mmol NaCl and PBS was 25.00 mV and 11.03 mV, respectively, and for polyplexes N/P 12 in 150 mmol NaCl and PBS was 30.43 mV and 12.83 mV, respectively (Figure 1D). The charges of each LPEI polyplexes were higher than their relative sPEG-PEI counterpart, which can be attributed to the higher number of nitrogen groups on the LPEI backbone compared to the sPEG-PEI backbone (25.25 nmol N/1 μg LPEI, 15.63 nmol N/1 μg sPEG-PEI).

The efficiency of the polymer/DNA interactions was evaluated by determining the amount of conjugate required to retard the migration of DNA through an agarose gel over a range of N/P ratios 0-7. As shown in Figure 3C, complete retardation of sPEG-PEI polyplexes was observed at an N/P ratio of 6 while complete retardation of LPEI polyplexes was observed at an N/P ratio of 4. We attribute the ability of LPEI to more tightly complex DNA due to the

higher number of nitrogen groups on the LPEI backbone that can interact with the phosphate groups on the DNA backbone. Moreover, the presence of PEG groups on sPEG-PEI interferes with complexation due to steric hindrance.

3.3 Gel Characterization Studies

In order to measure the mechanical stiffness of each hydrogel condition, the storage (*G'*) and loss moduli (*G"*) of 3.5% HA porous and nanoporous hydrogels were measured at 37°C using a plate-to-plate rheometer with an 8 mm geometry. Results showed that the *G'* and *G"* did not cross at any measured frequency (0.1 – 10 Hz) and were frequency-independent for both porous and nanoporous hydrogels (Figure 4A), both of which are consistent with typical hydrogel characteristics. Porous and nanoporous gels had an average *G'* of 518 Pa and 209 Pa, respectively (Figure 4B). This difference in mechanical stiffness was statistically significant between the two gel types.

To measure the amount of DNA that remained electrostatically bound to the surface of porous gels after surface coating, polyplexes formed with 0.25% radiolabeled DNA were coated on the surface of the porous hydrogels and read using a scintillation counter. After analysis with a standard curve, readouts showed that 34% of sPEG-PEI polyplexes N/P 7 and 35% of LPEI polyplexes N/P 7 remained bound to the hydrogel (Figure 4C). Furthermore, 50% of sPEG-PEI polyplexes N/P 12 and 44% of LPEI polyplexes remained bound to the hydrogel (Figure 4C). HA is a negatively charged polymer therefore it is expected that a higher percent of polymer polyplexes N/P 12 would remain bound to the hydrogel when compared to polyplexes of N/P 7 due to the increased charge of the N/P 12 polyplexes. There was no statistical significance between any of the 4 conditions.

The release of surface bound polyplexes due to enzymatic degradation of the hydrogel was assessed by submerging polyplex-bound porous gels in solutions of Col I or PBS (control). Data indicates a sustained release of polymer polyplexes in the presence of Col I over a 14 day period, with less than 25% released in PBS (Figure 4D). sPEG-PEI polyplexes of N/P's 7 and 12 released more quickly than their LPEI polyplex counterpart, which we attribute to weaker electrostatic interactions with the hydrogel due to the more neutral charge of sPEG-PEI polyplexes. For gels suspended in Col I solution, sPEG-PEI and LPEI polyplexes N/P 7 exhibited 90% release by days 5 and 7, respectively. Furthermore, sPEG-PEI polyplexes N/P 12 exhibited 90% release by day 12, while LPEI polyplexes N/P 12 did not achieve 90% release by the end of the study at 14 days.

To assess PEG's ability to prevent polyplex aggregation within HA gels, sPEG-PEI and LPEI polyplexes were encapsulated within nanoporous and porous gels both with and without CnE, or were surface coated onto the pores of porous gels containing no CnE (Figure 4E-N). Images show large aggregates for LPEI polyplexes with little to no aggregates for sPEG-PEI polyplexes in nanoporous and porous gels both with and without CnE (Figure 4E-L). Furthermore, sPEG-PEI polyplexes remained even distributed through the gel while LPEI polyplexes exhibited uneven distribution. For surface coated gels, sPEG-PEI polyplexes demonstrated no aggregation on pores and present a clear defined porous structure. LPEI polyplexes formed large aggregates on the surface of pores and appear to have reduced the interconnected pore diameter (Figure 4M, N).

3.4 Cell Characterization Studies

An MTT assay was performed to compare the toxicity of sPEG-PEI to LPEI polyplexes (Figure 5A). Since the concentrations used in this study were high for *in vitro* culture systems, some toxicity was expected. Cells exposed to sPEG-PEI polyplexes N/P 7 show no decrease in metabolic activity up to 200 ng/μL, and cell exposed to sPEG-PEI polyplexes N/P 12 show no decrease in metabolic activity up to 100 ng/μL. For cells exposed to LPEI polyplexes N/P 7 we observe no decrease in metabolic activity up to 100 ng/μL, and for cells exposed to LPEI polyplexes N/P 12 we observe no decrease in metabolic activity up to 50 ng/μL. This indicates the toxicity of sPEG-PEI polyplexes are half that of LPEI polyplexes at corresponding N/P ratios. When comparing the metabolic activities of cells exposed to sPEG-PEI polyplexes N/P 7 to cells exposed to LPEI N/P 7, we observe a significant difference at concentrations of 150 ng/μL and 200 ng/μL. When comparing the metabolic activities cells exposed to sPEG-PEI polyplexes N/P 12 to cells exposed to LPEI N/P 12, we observe a significant difference at concentrations of 100 ng/μL, 150 ng/μL, and 200 ng/μL. These results indicate sPEG-PEI polymer can serve as a less toxic alternative to LPEI polyplexes in systems where polyplex toxicity presents issues.

A bolus transfection using the Gaussia luciferase vector was performed to compare the transfection abilities of sPEG-PEI and LPEI polyplexes to D1 cells in 2D. Results indicate sPEG-PEI polyplexes N/P 7 and 12 transfected an average RLU of 5,801 and 65,917, respectively (Figure 5B) . Results indicate LPEI polyplexes N/P 7 and 12 transfected an average RLU of 266,887 and 1,697,562, respectively (Figure 5B).

To compare polymer transfection abilities in 3D, 3.5% HA gels were first surface coated with polyplexes followed by coating of 40,000 D1 cells. Results indicate sustained transfection

over the period of 14 days for sPEG-PEI and LPEI polyplexes at N/P 7 and 12, with the highest individual levels of transfection occurring at day 2 for all conditions (Figure 5C). When analyzing the total cumulative transfection over 14 days, we observe that LPEI polyplexes N/P 12 transfected the highest with a total cumulative expression of 1.995 x 10^7 RLU (Figure 5D) Comparably, sPEG-PEI polyplexes N/P 12 exhibited a total cumulative expression of 1.289 x 10^7 RLU (Figure 5D), which is not statistically significant in comparison to LPEI polyplexes N/P 12. This same conclusion is met when comparing the total cumulative expression of LPEI and sPEG-PEI polyplexes N/P 7, which resulted in cumulative expressions of 2.474 x 10^6 and 9.010 x 10^6 RLU, respectively. These results, when coupled with the toxicity data presented in Figure 5A, suggest that sPEG-PEI can serve as an alternative transfection polymer to LPEI that is less toxic and still retains high levels of expression over 14 days.

The delivery of multiple genes from a single system can have applications in tissues engineering and regenerative medicine[47-49], which inspired the creation of a two-gene delivery system. pGluc plasmid was encapsulated within the nanoporous region of porous gels, and pSEAP was surface coated onto the pores of the porous gel (Figure 1C). HEK293T-MMP2 cells that over express MMP2 were surface coated onto the pores of the hydrogel at a concentration of 40,000 cells/hydrogel, and were used instead of D1 cells in order to ensure hydrogel degradation and subsequent polyplex release. Because pGluc and pSEAP are different plasmids that result in varying transfection levels at comparable DNA concentrations, a 2D transfection was performed to normalize the expression levels of the two vectors in this two-gene delivery assay (data not shown). By assessing Figure 5E, we observed sustained transfection over 14 days of both DNA plasmids from all four polyplex conditions. Analogous to the 3D surface-coated transfection results, there was no significant difference in total expression of

surface-coated pSEAP at 14 days between sPEG-PEI and LPEI polyplexes N/P 7 or sPEG-PEI and LPEI polyplexes N/P 12.

4. Discussion

It has been demonstrated that HA hydrogels serve as a promising scaffold for local gene delivery due to their easily manipulated properties, low immunogenicity, and thorough integration with the ECM of cells. Gene delivery from hydrogels via viral agents results in high gene expression[50] but poses issues of immunogenicity and mutagenesis[27]. Therefore, non-viral vectors such as polymers have been investigated as less harmful transfection alternatives. Poly(ethylene imine) is a non-viral cationic polymer that has been shown to successfully transfect cells in 2D[51], and has been studied by the Segura group as a transfection agent from nanoporous hydrogels in 3D. Although the Segura group has coupled a previously developed CnE technique with gel porosity to improve DNA delivery to cells, transgene expression levels remain low and can further be improved. We hypothesize that CnE and acetone processing during hydrogel formation causes an increase in gel stiffness, resulting in a slower rate of gel degradation and polyplex release. This study aims to bypass CnE processing through use of PEGylated-PEI as a transfection polymer, and further investigates surface coated polyplex presentation as an alternative to encapsulated presentation to increase transgene expression.

To address aggregation issues, PEG was grafted to PEI to make a more water soluble PEI conjugate. PEGylation creates a hydrophilic exterior that reduces polyplex interaction with plasma proteins and salts, thus preventing aggregation. A linear form of PEI (compared to a branched form) was used in PEGylation in order to keep our studies comparable with the previous findings of the Segura group. A paper published by the Kim group[52] reported the easy conjugation of low molecular weight PEI to the arms of a 6-armed PEG-NHS, and served as an initial inspiration to this approach. LMW linear PEI (MW 2.5kDA) and 8-armed PEG-SCM were utilized because a complete reaction would yield 8 PEI groups per 1 PEG molecule

resulting in a total PEI molecular weight of 20kDA, which is similar in size to the 25kDA PEI utilized by the Segura group. LMW-PEI was reacted in excess with PEG-SCM at a 16:1 molar ratio because preliminary attempts at lower ratios resulted in the formation of a gel and a high PEI-PEG ratio would ensure conjugation of PEI onto each arm of the 8-armed PEG-SCM. NMR confirmed the successful attachment of 8 PEI groups to every PEG arm of 8-armed PEG-SCM.

The sizes, condensation abilities, and zeta potentials of sPEG-PEI and LPEI polyplexes were characterized prior to utilization within a hydrogel scaffold (Figure 3). sPEG-PEI polymer formed a smaller, more stable polyplex after the addition of salt when compared to the LPEI polymer, and retained its size over a 3 day period (Figure 3A, B). In contrast, LPEI polyplexes exhibited a dramatic 7-fold increase in size after the addition of salt, with a 130-fold increase by day 1. The small size of sPEG-PEI polyplexes is the result of a steric barrier that is formed by PEG, which provides protection against interactions with proteins and salts[45,53,54]. LPEI polyplexes lack the ability to form this steric barrier therefore the presence of charged species can facilitate interactions between LPEI polyplexes resulting in aggregation[55]. This data correlates to work previously done with PEGylated PEI[52,55-57]. The charges of sPEG-PEI polyplexes N/P 7 and 12 were less than that of LPEI polyplexes N/P 7 and 12, respectively. This data, coupled with the size data we report, further indicates that hydrophilic PEG chains shield the positive charge of PEI/DNA complexes, which is supported by previous work[58]. Agarose gel retardation results show full plasmid condensation at N/P 6 and 4 for sPEG-PEI and LPEI polymers, respectively. The need of higher N/P ratios for sufficient sPEG-PEI/DNA complex formation implies that PEGylation exerts hindering effects on DNA complexation, which is in correlation with previously studies[41,59].

Polymer polyplexes were surface coated onto porous gels and placed into solution to assess release in the presence or absence of enzymes (Figure 4D). Studies indicate sustained polyplex release over 14 days in the presence of Col I, and less than 25% percent release in the presence of PBS (control). Release in Col I solution was expected due to enzymatic breakdown of the HA backbone to which polyplexes were adhered, resulting in polyplex release. When analyzing polyplex release in PBS we see minimal release of about 1.7% per day for all 4 conditions, which may be due to mechanical stresses caused by pipetting during collection. Polyplexes remain bound because the ionic concentration of the salt solution is too weak to displace the strong electrostatic interactions between positively charged sPEG-PEI/LPEI polyplexes and negatively charged carboxyl groups on the HA backbone. The sustained release of surface coated DNA polyplexes is an significant improvement over the burst release of surface coated naked DNA from porous PEG gels[37], with over 50% DNA release by day 3 in PBS, yet more rapid compared to encapsulated polyplex release from porous fibrin[38] and HA gels[39].

Polymer polyplexes were encapsulated within nanoporous and porous gels or surface coated onto the pores of porous gels to assess polyplex aggregation. The PEG group present on sPEG-PEI further demonstrated the ability to mitigate charge-charge interactions during hydrogel formation as evidenced by the lack of aggregates in all three picture types (Figure 4E-N). Conversely, LPEI formed large, micron-sized aggregates even with utilization of the CnE technique. These images indicate that sPEG-PEI polymer can be used instead of coupling PEI and CnE techniques to prevent polyplex aggregation within gels. Future studies should aim to optimize sPEG-PEI polyplex loading concentrations within gels in order to increase transgene expression levels.

After characterizing polyplex and gel properties, the two systems were combined to characterize their effects on cell behavior. Cytotoxicity studies indicate that sPEG-PEI polyplexes are significantly less toxic then their corresponding LPEI polyplexes at DNA concentrations greater than 50 ng/μL. Free PEI can cause cell death *prior* to cellular internalization through interactions with the cell membrane that cause destabilization, or *after* internalization through interactions with chromosomal DNA that prevent transcription[60]. The presence of hydrophilic PEG groups produces a more neutrally charged polyplex that can still interact with the cell membrane without eliciting a toxic effect. Transfection studies were performed in 2D and 3D to compare the transfection ability of sPEG-PEI with LPEI. For 2D studies, transgene expression for LPEI polyplexes was greater than sPEG-PEI (Figure 5B), which is attributed to the presence of PEG groups that hinder the first step of intracellular trafficking[61]. Although this same conclusion is observed when analyzing *per day* transgene expression in 3D from surface coated porous gels (Figure 5C), the difference in *total* transgene expression between the two polymers is insignificant over 14 days in both a single (Figure 5D) and dual gene delivery system (Figure 5E). These results, when coupled with the less toxic, more stable, and non-aggregating nature of sPEG-PEI, suggest that sPEG-PEI can be used as a suitable alternative to LPEI in long term studies.

The effect of polyplex presentation on cell transfection was assessed using two methods: (1) coated presentation on the surface of pores, and (2) encapsulated presentation within the n-pore region of the hydrogel (Figure 5E). Transgene expression levels of the surface coated pSEAP vector were 3 orders of magnitude greater than expression levels of encapsulated pGluc vector, which is attributed to the increased concentration of polyplexes that cells are in direct contact with[62]. Release of encapsulated polyplexes is limited to the rate at which cells degrade

the hydrogel. Therefore, transgene levels typically remain low. The introduction of pores within the hydrogel mitigates this problem by increasing the surface area to which polyplexes and cells can bind, thus increasing the cell-polyplex interaction. It is important to note that although the expression levels between surface coated and encapsulated presentations are significantly different, the dual system can be utilized together for angiogenic therapies. It is widely accepted that the molecular mechanisms controlling the formation of mature vasculature involve several factors, each playing a distinct role[63,64]. It has been shown that dual delivery of the pro-angiogenic proteins VEGF and PDGF from porous collagen scaffolds resulted in the highest density of subcutaneous blood vessels in rats[65,66]. Moreover, findings by Richardson et. al. showed that *sequential* delivery of VEGF followed by PDGF results in dense, mature vasculature in rats: VEGF initiates rapid *formation* of blood vessels, while PDGF initiates *maturation* of these blood vessels[67]. Our proposed dual gene delivery assay can be utilized to improve vascularization within hydrogel constructs keeping in mind the aforementioned findings. Plasmid encoding for VEGF can be surface coated onto the pores of hydrogels to initiate rapid blood vessel formation, which sustained release of encapsulated plasmid coding for PDGF can promote vessel maturation.

5. Conclusion

This study aimed to overcome polyplex aggregation issues and improve DNA delivery from porous HA gels through use of sPEG-PEI copolymer and surface coated polyplex presentation, respectively. sPEG-PEI formed a smaller, more neutral, and less toxic polyplex when complexed to DNA compared to LPEI. Furthermore, sPEG-PEI prevented polyplex aggregation when encapsulated within nanoporous and porous gels, while LPEI experienced high aggregation and formed large polyplex clusters. Although transgene expression levels were higher for LPEI than sPEG-PEI polyplexes in 2D, no significant difference was observed in 3D over 14 days. Furthermore, we demonstrated the ability to utilize our porous HA gel system to simultaneously deliver multiple vectors, which holds promise for future successful tissue engineering treatments.

6. Appendix

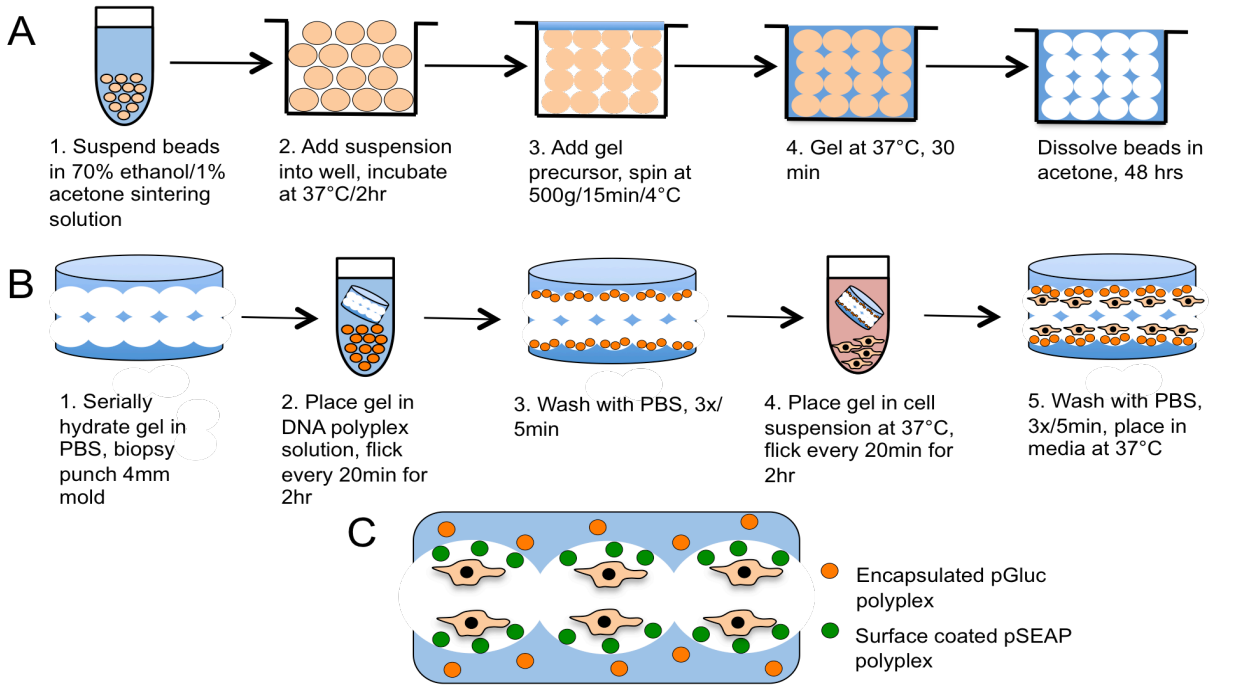

Figure 1: Schematic representation of porous hydrogel design template and hydrogel formation (A). Schematic representation of DNA surface coating process onto porous gels (B). Schematic representation of 2-gene delivery assay with encapsulated pGluc and surface coated pSEAP vectors (C).

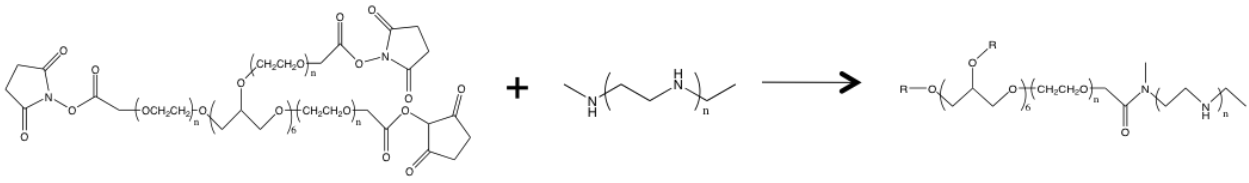

Figure 2: Schematic representation of sPEG-PEI synthesis from PEG-SCM and low molecular weight linear PEI polymers.

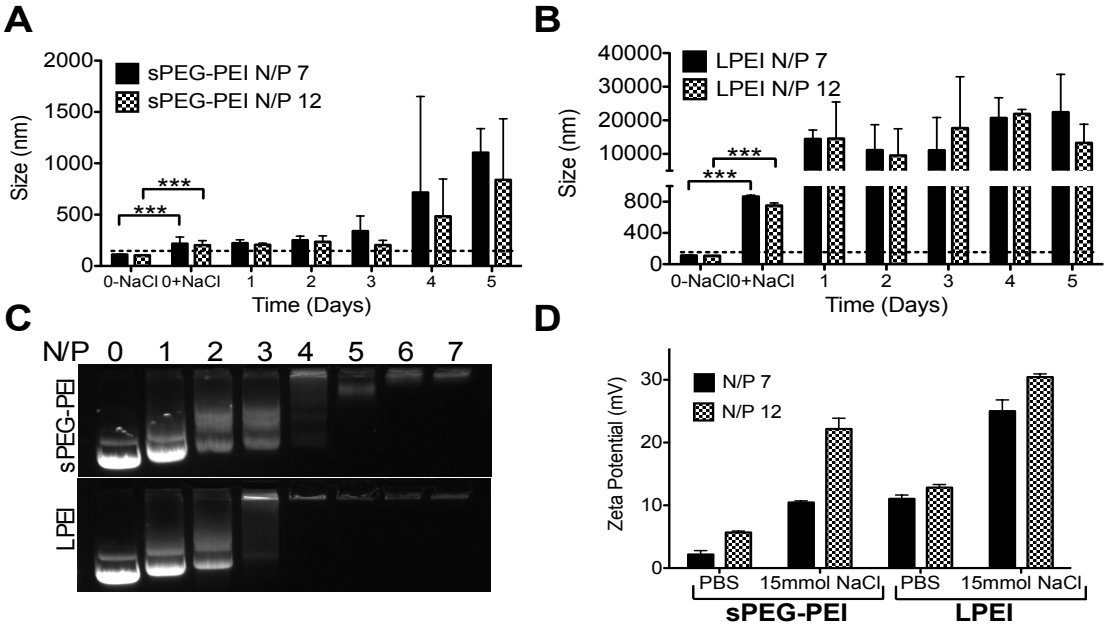

Fig. 3. Size and stability of sPEG-PEI polyplexes (A) and LPEI polyplexes (B) over 5 days. Agarose gel retardation assay to assess the condensation abilities of sPEG-PEI and LPEI polymers with pDNA (C). Average zeta potential of sPEG-PEI and LPEI polyplexes formed at N/P 7 and 12 in 150 mmol NaCl and PBS (D).

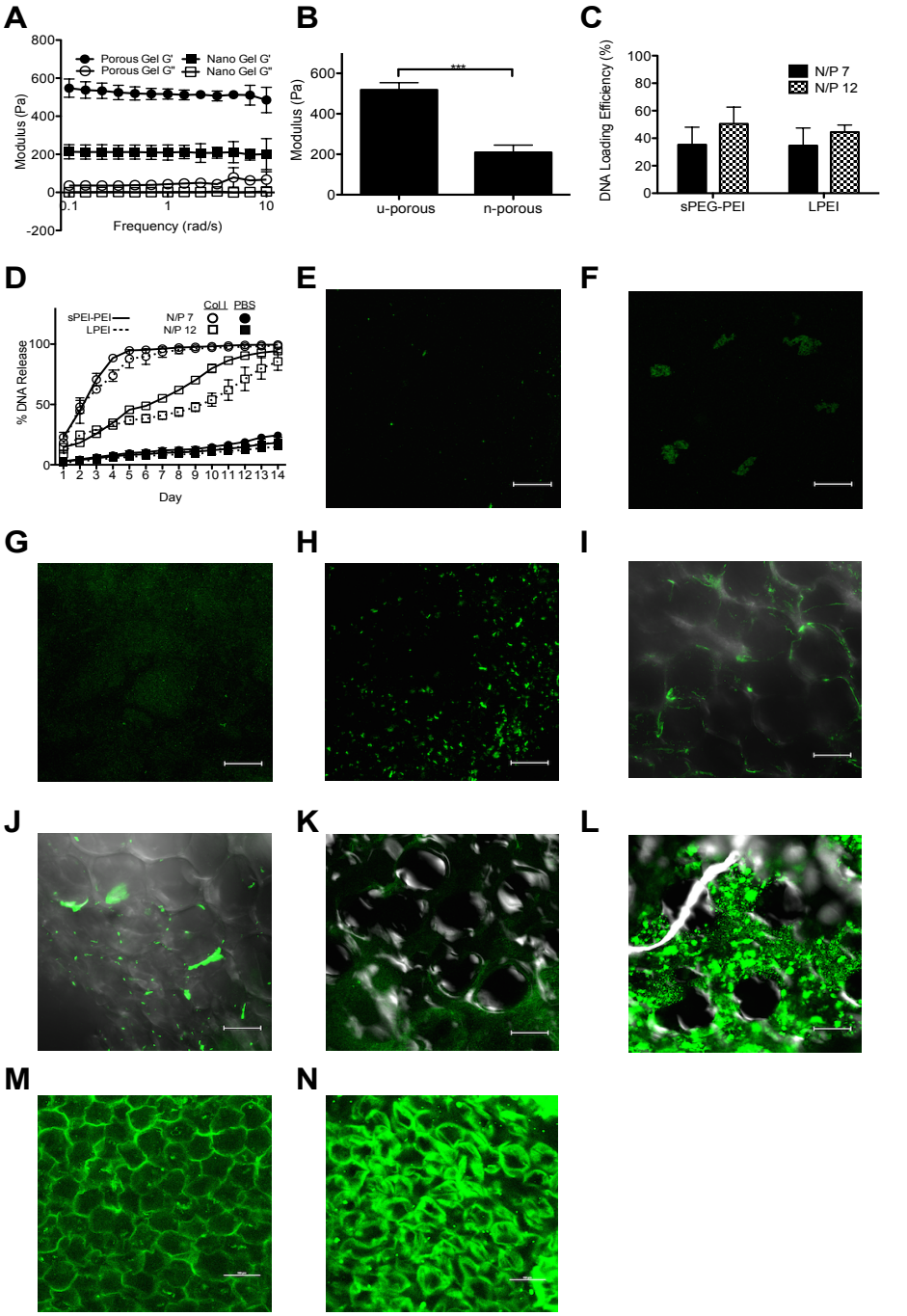

Fig. 4. *G'* and *G"* of porous and nanoporous gels over frequency range 0.1 – 10 rad/s (A). Average *G'* of porous and nanoporous gels (B). DNA loading efficiency of surface coated sPEG-PEI and LPEI polyplexes (C). DNA release of surface coated polyplexes (D). Images of fluorescently tagged sPEG-PEI (E) and LPEI (F) polyplexes in nanoporous gels without CnE, and with CnE (G, H). Images of fluorescently tagged sPEG-PEI (I) and LPEI (J) polyplexes in porous gels without CnE, and with CnE (K, L). Images of surface coated sPEG-PEI (M) and LPEI (N) polyplexes in porous gels. All images were taken at 40x magnification and all scale bars represent 50 μm. Porous gel images include DCI images overlayed with fluorescent images

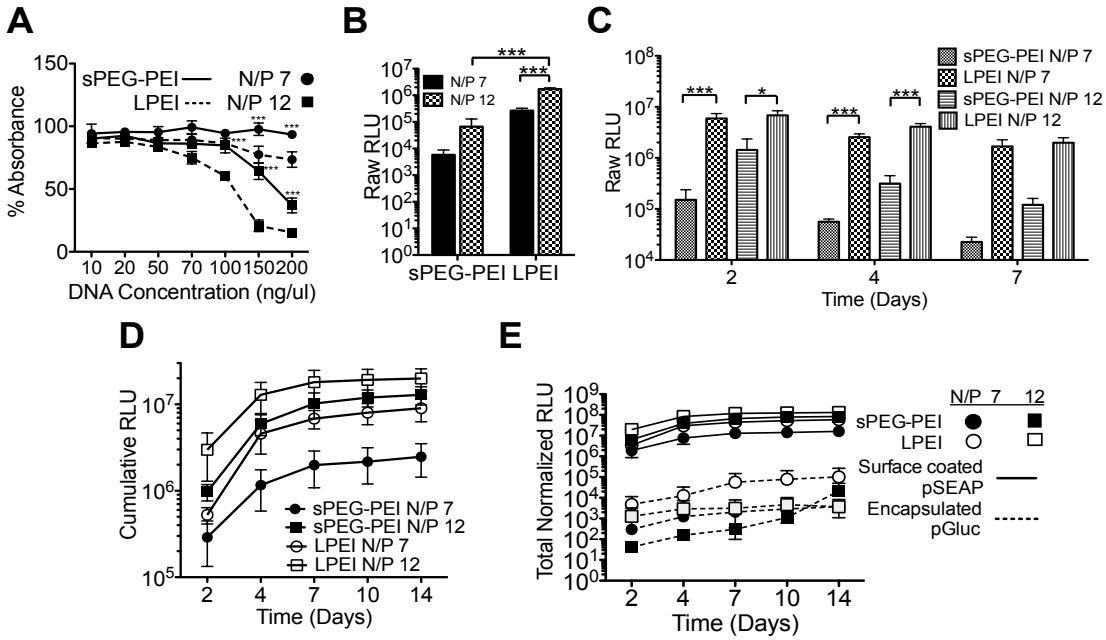

Fig. 5. MTT assay assessing toxicity of sPEG-PEI and LPEI polymers (A). 2D bolus transfection with sPEG-PEI and LPEI polymers (B). 3D transfection from surface coated gels with sPEG-PEI and LPEI polymers (C). Cumulative 3D transfection profile of sPEG-PEI and LPEI polymers over 14 days (D). 2 gene delivery assay with surface coated pSEAP and encapsulated pGluc vectors (E).

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