2010

Incorporation of Active DNA/Cationic Polymer Polyplexes into Hydrogel Scaffolds

Yuguo Lei
University of Nebraska-Lincoln, ylei14@unl.edu

Suxian Huang
University of California, Los Angeles

Pooria Sharif-Kashani
University of California, Los Angeles

Yong Chen
University of California, Los Angeles

Pirouz Kavehpour
University of California, Los Angeles

See next page for additional authors

Published in final edited form as:
Biomaterials. 2010 December ; 31(34): 9106–9116. doi:10.1016/j.biomaterials.2010.08.016.

Incorporation of Active DNA/Cationic Polymer Polyplexes into Hydrogel Scaffolds

Yuguo Lei[1], **Suxian Huang**[2], **Pooria Sharif-Kashani**[2], **Yong Chen**[2], **Pirouz Kavehpour**[2], and **Tatiana Segura**[1,*]

[1]University of California, Los Angeles, Chemical and Biomolecular Engineering Department

[2]University of California, Los Angeles, Mechanical and Aerospace Engineering Department

Abstract

The effective and sustained delivery of DNA and siRNAs locally would increase the applicability of gene therapy in tissue regeneration and cancer therapy. One promising approach is to use hydrogel scaffolds to encapsulate and deliver nucleotides in the form of nanoparticles to the disease sites. However, this approach is currently limited by the inability to load concentrated and active gene delivery nanoparticles into the hydrogels due to the severe nanoparticle aggregation during the loading process. Here, we present a process to load concentrated and un-aggregated non-viral gene delivery nanoparticles, using DNA/polyethylene imine (PEI) polyplexes as an example, into neutral polyethylene glycol (PEG), negatively charged hyaluronic acid (HA) and protein fibrin hydrogels crosslinked through various chemistries. The encapsulated polyplexes are highly active both *in vitro* and *in vivo*. We believe this process will significantly advance the applications of hydrogel scaffold mediated non-viral gene delivery in tissue regeneration and cancer therapy.

Introduction

The effective delivery of DNA and siRNAs locally would increase the applicability of gene therapy in tissue regeneration and cancer therapy. For tissue regeneration, localized gene delivery can be used to promote the expression of tissue inductive factors to be used as signals to promote tissue formation [1,2]. For cancer therapy, localized gene delivery can be used to induce cancer cell apoptosis [3]. Local gene delivery using hydrogel scaffolds has been studied for nearly a decade primarily through the encapsulation of naked DNA during hydrogel formation. Naked DNA has been successfully incorporated inside collagen [4], pluronic-hyaluronic acid [5], PEG-poly(lactic acid)-PEG [6], alginate [7], oligo(polyethylene glycol) fumarate [8] and engineered silk elastin [9]. Although naked DNA has shown gene expression and ability to guide regeneration *in vivo* [4,10], limitations with low gene transfer efficiency and rapid diffusion of the DNA from the hydrogel scaffold motivated the use of DNA nanoparticles instead of naked DNA. Both viruses (viral delivery) and chemical agents (non-viral delivery) can be used to condense DNAs or siRNAs into nanoparticles that transport the nucleotides into cells [11]. Since tissue regeneration and

*Corresponding Author, Tatiana Segura, 420 Westwood Plaza, 5531 Boelter Hall, Los Angeles, CA, 90095, tsegura@ucla.edu, Phone: 310-206-3980, Fax: 310-206-4170.

Current address: Yuguo Lei, Assistant Professor, Chemical & Biomolecular Engineering, University of Nebraska-Lincoln, ylei14@unl.edu

cancer therapy only require temporal up- or down-regulation of genes, non-viral delivery becomes greatly attractive. In addition, the non-viral approach has the following advantages (1) large number of non-viral particles can be more readily produced than large number of viruses, (2) repeat injections of non-viral particles are possible since they do not elicit severe immune responses, (3) there is little risk of insertion mutagenesis and (4) large genes can be condensed and delivered. DNA condensed either with cationic peptides, lipids, or polymers has been introduced into fibrin [12–16], enzymatically degradable PEG hydrogels [17] and PEG-hyaluronic acid [13] hydrogels. Among these agents, poly(ethylene imine) (PEI), a cationic polymer that has been widely utilized for non-viral gene delivery, is considered a gold standard when developing other non-viral vectors and designing delivery systems due to its success *in vitro* and *in vivo.* PEI is able to condense DNA through electrostatic interactions between the positively charged amines on the PEI and the negatively charged phosphates on the DNA, forming nanoparticles (polyplexes) in the range of 50 to 200 nm [18]. DNA/PEI polyplexes enter cells through endocytosis and are believed to escape the endosome through endosomal buffering (the proton sponge hypothesis, [19–21]). PEI has been successfully used *in vivo* delivering DNA or siRNA to the brain [22,23], lungs [24–27], abdomen [28], and tumors [29–31]. Although DNA nanoparticles have been introduced into various hydrogels, the current methodologies cannot load high concentrations of active DNA nanoparticles because of the severe aggregations. Due to the soft, loose and charged structures, non-viral gene delivery nanoparticles such as polyplexes and lipoplexes tend to aggregate at high particle concentrations during their incorporation into hydrogels. Others and we could only incorporate low amounts of DNA nanoparticles into hydrogels (for instance, our previous results showed no more than 30μg DNA could be introduced into 100μL hydrogel) and shown low levels of gene transfer *in vitro* [12–16]. This motivated us to develop effective approaches for introducing concentrated and un-aggregated non-viral gene delivery nanoparticles into hydrogel scaffolds.

Methods

Materials

Peptides GCRDGPQGIWGQDRCG (HS-MMP-SH) and Ac-GCGWGRGDSPG-NH2 (RGD) were purchased from Genscript; Human fibrinogen, Bovine plasma thrombin and Linear poly(ethylene imine) (25 kDa, PEI) were bought from Enzyme Research Laboratories, Sigma and Polysciences, respectively. Vectors expressing mammalian secreted alkaline phosphatase (pSEAP) and green fluorescent protein-luciferase (pEGFP-LUC) were obtained from Genlantis and BD Biosciences, respectively. Vectors expressing vascular endothelial growth factor (pVEGF) and beta-galactosidase (p-β-gal) were gifts from Prof. Lonnie Shea's laboratory (Northwestern University). All plasmids were expanded using a Giga Prep kit from Qiagen. Four-arm PEG-vinyl sulfone (PEG-VS) was synthesized from four-arm PEG-OH (20kDa, Nektar) as previously described [17]. Sodium hyaluronan (HA) was a kind gift from Genzyme Corporation (60 KDa MW, Cambridge, MA). All other chemicals were purchased from Fisher Scientific unless otherwise noted.

Hyaluronic acid modification

Acrylated hyaluronic acid (HA-AC) was prepared using a two-step synthesis. Hyaluronic acid (1.0g, 0.017mmole, 60kDa) was reacted with 18.0 g (105.5mmole) adipic dihydrazide at pH 4.75 in the presence of 2.0g (10.41mmole) 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) overnight and purified through dialysis (8000 MWCO) in DI water for 1 week. The purified intermediate (HA-ADH) was lyophilized and stored at −20°C until used. 38.8% of the carboxyl groups were modified with ADH based on the TNBSA assay. HA-ADH (1.0g, 0.014mmole) was reacted with N-Acryloxysuccinimide

(0.75g, 4.4mmole) in HEPES buffer (pH 7.2) overnight and purified through dialysis in DI water for 1week. All the primary amines were acrylated based on the TNBSA assay.

Polyplex lyophilization

Plasmid DNA (1~500μg) and PEI or jetPEI or lipofectamine (1.95~978μg) were mixed in 3.5 mL water in the presence of 35 mg (0.10mmole) of sucrose (Ultra pure, MP Biomedicals) and incubated at room temperature for 15 min. Agarose (1.0mg, UltraPureTM Agarose, Tm = 34.5~37.5°C, Invitrogen) in 1.5 mL water was added before lyophilization. Morphologies of lyophilized sucrose, agarose, polyplexes and their combinations were studied with scanning electronic microscope (SEM, Hitachi S-4500) after gold sputtering.

Hydrogels synthesis and characterization

To make PEG and HA hydrogels, lyophilized polyplexes were reconstituted with 100μL PEG-VS or HA-AC solution (in 0.3 M TEOA, pH=8.0) containing crosslinker (HS-MMP-SH) or photo initiator 2,2-Dimethoxy-2-phenylacetophenone (Sigma, 18mM) and gelled either through incubating at 37°C for 30 min or shining with 365 nm UV for 1-min. To make 100μL fibrin hydrogels, fibrinogen solution in PBS (5 mg/mL) was mixed with lyophilized polyplexes and initiated to form gel at 37°C for 20 min with thrombin (2 U/mL). All hydrogels were swelled in PBS for 2-hrs. The relative light translucency was recorded with black field microscope (Observer Z1, Zeiss) under the same light intensity and analyzed with Image J. To visualize the polyplexes' distribution swelled gels were stained with ethidium bromide (12 μM) for 2-hrs before imaging with confocal microscope (Leica TCS SP MP). The storage and loss modulus were measured with a plate-to-plate rheometer (AR2000, TA Instruments) under the constant strain of 0.05 and frequency ranging from 0.1 to 10 rad/s. The viscosity of gel precursor solution was obtained using the same rheometer with shear rate varying from 0.1 to 1000 S^{-1}. DNA released from hydrogels in PBS or recovered from hydrogels after degradation in 0.25% trypsin was quantified with HOECHST dye (H33258, [17]). Activities of lyophilized polyplexes or polyplexes recovered from the degraded hydrogel were tested through bolus transfection of HEK293 and compared with freshly prepared polyplexes. Gels were dried in gradient ethanol solution (25, 50, 75, 100%, each for 10min) and imaged with SEM. To test cell viability, 150,000 D1 cells (CRL12424, ATCC) were encapsulated inside a 5 μL fibrin clot (2 mg/mL fibrinogen and 2 U/mL thrombin) and placed in a 6.5% MMP degradable PEG hydrogel modified with 200 μM RGD. At day 15, the gel was stained with the LIVE/DEAD kit (Molecular Probes) to access the cell viability. To test gene transfer in 3D fibrin hydrogel, 100,000 NIH 3T3 cells were co-encapsulated with lyophilized polyplexes containing pSEAP in 100μL hydrogel. Medium was collected at predetermined time points and the amount of SEAP protein was quantified using Phospha-lightTM SEAP Reporter Gene Assay (Applied Biosystems) as previously described [17].

Choriallantoic Membrane (CAM) angiogenesis assay

Fertilized chicken eggs were released into tissue culture dishes after 3-days of incubation in a 38°C ventilated, humid egg incubator and were incubated in a sterile humid incubator at 37°C for another 10 days. Hydrogels with polyplexes were placed on the CAM away from major veins and incubated for 3 additional days. Gross pictures were recorded (Stemi 2000-C, Zeiss) before the embryo was infused with 1 mL of FITC-dextran (0.5 mg/mL in PBS, Sigma). The CAM with hydrogel was cut and fixed in 4% paraformaldehyde for 2-hrs before imaging with a fluorescent microscope (Observer Z1 Zeiss). Fixed CAMs were immersed in 37°C X-gal solution (5mM Potassium ferricyanide, 5mM Potassium ferrocyanide, 1mM $MgCl_2$, 1mg/mL X-Gal (Gold Biotechnology) in PBS) for 24-hrs to visualize the β-Gal expression, which was recorded with a color camera attached to a dissecting microscope (Stemi 2000-C, Zeiss)

Results

DNA/PEI Polyplexes Lyophilization and Characterization

A process was developed to load concentrated and un-aggregated polyplexes into various hydrogles. DNA/PEI polyplexes were prepared in the presence of sucrose and agarose, lyophilized and reconstituted with the gel precursor solution (Fig. 1A). The activity of PEI/DNA polyplexes after lyophilization was studied as a function of the concentration of sucrose used during lyophilization. It was found that adding sucrose to polyplexes to a final concentration of 20 mM was enough to protect the polyplex stability during lyophilization (Fig. 1B), with lyophilized polyplexes resulting in the same transgene expression as polyplexes that were formed immediately prior to transfection (Fresh). Adding more sucrose (up to 500mM) did not result in any additional benefit or detriment to the efficiency of transgene expression, with all sucrose concentrations resulting in the same level of transgene expression as fresh polyplexes. For all subsequent lyophilized polyplexes, a sucrose concentration of 20 mM was used. It was our hypothesis that increasing the viscosity of the hydrogel precursor solution would result in slowing the motion of polyplexes inside the hydrogel precursor solution, which would prevent their aggregation during gelation even when a high concentration of polyplexes was used. Agarose was used to increase the viscosity of the hydrogel precursor solution. Using plate-to-plate rheometry with a shear rate ranging from 0.1 to 1000 S^{-1} at 25°C, we found the presence of agarose together with the sucrose increased the viscosity of the hydrogel precursor solution from ~0.3 to ~73Pa.s at low shear rate (Fig. 1C).

The morphology of lyophilized polyplex powder was studied using SEM (Fig. 2). Sucrose and sucrose plus polyplexes exhibited a smooth and crystalline morphology (Fig. 2A,D), while agarose and agarose plus polyplexes exhibited fibrilar morphology (Fig. 2B,E). The combination of sucrose and agarose or sucrose, agarose and polyplexes resulted in the formation of microfibers and microspheres (Fig. 2C,F). The morphology was not affected by different concentrations of polyplexes or the N/P ratio used to form the polyplexes, with 100, 300 and 500 μg DNA (Fig. 2F–H) or N/P at 15 and 25 (Fig. 2F,K) resulting in similar morphologies. Likewise, the morphology of the lyophilized three-component powder was not affected by the increase of either sucrose or agarose, with 35 mg (20mM) and 70 mg (40mM) sucrose (Fig. 2F,I) and 1 mg (0.02%) and 2 mg (0.04%) agarose (Fig. 2F,J) resulting in similar morphologies. The morphology of a PEI/DNA loaded hydrogel was analyzed through the serial dehydration of the hydrogel in ethanol. The hydrogel exhibited a smooth surface with little texture (Fig. 2L).

In vitro characterization of high DNA loaded hydrogels

DNA/PEI polyplexes were encapsulated inside hydrogels by mixing either freshly prepared polyplexes (referred as regular polyplex encapsulation) or the lyophilized powder of the polyplexes (referred as Caged Nanoparticle Encapsulation or CnE, and the resulting hydrogels were called high DNA loaded hydrogels) with the gel precursors prior to crosslinking. Four types of gel precursors were used (i) vinyl sulfone modified four arm PEG and a dicysteine containing matrix metalloproteinase (MMP) labile peptide (PEG-VS/MMP), (ii) PEG-diacrylate and a photoinitiator (PEG-AC/UV), (iii) acrylate modified hyaluronic acid (HA-AC) and a dicysteine containing MMP labile peptide (HA-AC/MMP), and (iv) fibrin. The distribution of polyplexes inside the hydrogel was characterized using fluorescently stained polyplexes and confocal microscopy. The hydrogel scaffolds had limited auto-fluorescence (data shown for PEG-VS/MMP, Fig. 3A). The encapsulation of freshly prepared polyplexes containing 100 μg DNA at N/P ratios of 15 and 25 into PEG-VS/MMP hydrogels resulted in severe polyplex aggregation, with some aggregates being visible to the naked eye (Fig. 3B,C). Similarly the encapsulation of lyophilized powder

consisting of sucrose plus 100 μg DNA polyplexes into PEG-VS/MMP hydrogels resulted in severe aggregation of the polyplexes (Fig. 3D). In contrast, when lyophilized power consisting of sucrose and agarose plus either 50, 100, 300 or 500 μg DNA polyplexes was encapsulated into PEG-VS/MMP hydrogels the polyplexes were un-aggregated (Fig. 3E–I). The polyplex distribution inside PEG-VS/MMP hydrogels was not affected when different percent PEG was used, with both 6.5% and 5% PEG resulting in similar distribution (Fig. 3G, 6.5%, Fig. 3J, 5%). Introduction of lyophilized powder consisting of sucrose, agarose and 100 μg DNA polyplexes in PEG-AC (Fig. 3K), HA/MMP (Fig. 3L) and fibrin (Fig. 3M) hydrogels resulted in un-aggregated polyplexes with similar distribution as in PEG-VS/MMP hydrogels. In addition, jetPEI/DNA and Lipofectamine/DNA nanoparticles can be encapsulated into hydrogel through CnE without significant aggregation (Fig. 3N,O). The distribution of the polyplexes inside the hydrogels was not completely homogeneous, with more polyplexes localizing into some microdomains. However, both inside and outside these micro-domains, only un-aggregated polyplexes were observed. The translucency of the hydrogels decreased linearly as the concentration of polyplexes increased, indicating that the mass introduced to the hydrogel was not aggregated (Fig. 4A).

For DNA/PEI polyplexes encapsulated inside PEG-VS/MMP hydrogels no release was observed in PBS until trypsin was added at 180 hours, indicating that the polyplexes were not free to diffuse inside the hydrogel. Trypsin addition resulted in the burst release of the DNA due to the rapid degradation of the hydrogel (Fig. 4B). The mechanical properties of the PEG hydrogel were affected by the encapsulation of polyplexes. Adding lyophilized sucrose and agarose without polyplexes slightly increased the storage modulus of the hydrogels (Fig. 4C, D). The incorporation of 50 μg DNA resulted in the storage modulus a 6.5% PEG hydrogel increased from 400 Pa to 730 Pa and was further increased to 830 and 920Pa when 100 and 300μg DNA were encapsulated respectively (Fig. 4C, D). However, the mechanical properties of the DNA polyplex containing hydrogels could mimic those of empty hydrogels by changing the percent of the hydrogel. The storage of a 4.5% PEG hydrogel loaded with 100 μg of DNA polyplexes was the same as a 6.5% PEG hydrogel without DNA loading. The finding that the G' and G" did not cross at any measured frequency (0.1 to 10 Hz) and that the storage and loss modulus were frequency independent were consistent with hydrogel rheology. The loss tangent values (ratio of G" to G') were lower than 0.006 for all the hydrogels tested indicating that the hydrogels were highly elastic.

Cellular characterization in vitro

The DNA loading efficiency, which was defined as the ratio of DNA recovered from hydrogel after degrading with trypsin to the original DNA loaded was found to be above 85% for 50, 100, 300 and 500 μg DNA encapsulated (Fig. 5A). To test the activity of the polyplexes after CnE encapsulation, polyplexes were recovered from PEG-VS/MMP hydrogels with 100μg and 300μg loading after trypsin degradation. The recovered polyplexes were then used to perform a bolus transfection to HEK293 cells and the transfection level was normalized to that obtained with freshly prepared polyplexes. We found the encapsulated polyplexes remained about 65% of their original activity (Fig. 5B). Using the LIVE/DEAD assay it was observed that mesenchymal stem cells were able to survive and spread inside PEG-VS/MMP hydrogels loaded with 100 μg DNA polyplexes without significant cell death (Fig. 5C). The activity of encapsulated polyplexes was further tested *in vitro* through co-encapsulating polyplexes and NIH/3T3 cells inside fibrin hydrogels. Gene transfer to NIH/3T3 cells was found to be a function of the DNA loading and the time of cell incubation. Increasing DNA loading resulted in increasing transgene expression reaching 20 ng of SEAP protein produced over an 11-day period for the highest

amount of DNA used (Fig. 5D). The amount of transgene expression each day increased linearly with increase dose of DNA loaded into the hydrogel (Fig. 5D).

In vivo characterization of high DNA loaded PEG-VS/MMP hydrogels

The ability of DNA loaded PEG-VS/MMP hydrogels through CnE to mediate gene transfer *in vivo* was assessed using a chorionic chick embryo (CAM) assay. PEG-VS/MMP hydrogels loaded with DNA encoding for β-galactosidase and containing different percentages of PEG-VS were placed on top of the CAM. Regular encapsulation of polyplexes (Fig. 6B,C) or the encapsulation of DNA encoding for VEGF (Fig. 6D,G) did not result in β-galactosidase gene expression determined through X-gal staining. The degree of β-galactosidase gene expression depended on the percent hydrogel used, with lower percent gels (5%, Fig. 6E,F) resulting in more robust staining than high percent gels (6.5%, Fig. 6H,I). The N/P ratio used did not seem to affect the transgene expression with N/P equal to 15 (Fig. 6E,H) showing the same transgene expression as N/P equal to 25 (Fig. 6F,I).

The delivery of DNA encoding for VEGF was able to induce blood vessel formation in the CAM assay. Gross evaluation of the blood vessels around the implanted hydrogels showed that hydrogels with no DNA (Fig. 7A), regular encapsulation of DNA (Fig. 7B,C) and hydrogels loaded with DNA polyplexes encoding for β-galactosidase (Fig. 7D) did not result in enhanced blood vessel formation. However, all hydrogels containing stabilized DNA encoding VEGF through CnE resulted in enhanced blood vessel formation around the implant site (Fig. 7E–J, arrow heads). The robustness of the angiogenic response observed was dependent on the percent hydrogel used, lower percentage hydrogels (Fig. 7E–H) resulted in a more robust angiogenic response than higher percentage hydrogels (Fig. 7I,J). However, the N/P ratio or increasing the dose of DNA did not increase the level of angiogenesis around the implant (Fig. 7E–H). Evaluation of the blood vessels underneath/inside the hydrogel scaffold after perfusion showed enhanced micro-vessel formation for all hydrogels that contained stabilized DNA/PEI polyplexes encoding for VEGF through CnE (Fig. 7O–R, arrows). Hydrogels with no DNA, or regularly encapsulated DNA resulted in no micro-vessel formation (Fig. 7L–N). The native CAM is shown in Fig. 7K.

Discussion

The effective delivery of DNAs or siRNAs locally would increase the applicability of gene therapy in tissue regeneration and cancer therapy. One approach to deliver drugs locally is to use hydrogels to retain a high concentration of the drug at the target site [32]. These approaches have been effective to deliver small molecular drugs, viruses, naked plasmids and proteins to areas such as solid tumors [9,33,34] and cartilage defects [35] and to deliver progenitor cells locally [36]. However, the delivery of DNA/cationic polymer nanoparticles (polyplexes) using hydrogels has not been successful due to aggregation and inactivation of the polyplexes inside hydrogel scaffolds. The incorporation of polyplexes is challenging due to the polyplex charge and instability in salt containing solutions. Further, different hydrogel chemistries pose unique problems to the incorporation of DNA polyplexes; for example the direct mixing of DNA/PEI polyplexes with HA results in the decomplexation of the polyplexes since HA is highly negatively charged (data not shown). In this study, we found a strategy to introduce unaggregated and highly active DNA/PEI polyplexes to hydrogel scaffolds. In particular, we have found that, as observed with other published reports [37], lyophilization of the polyplexes in the presence of sucrose retained their activity after reconstitution (Fig. 1). However, sucrose did not prevent aggregation during hydrogel formation, with polyplexes showing significant aggregation after hydrogel formation (Fig. 3D). Increasing the viscosity of the gel precursor solution with agarose, however, did prevent the aggregation during hydrogel formation (Fig. 3) and resulted in hydrogels that can deliver genes *in vitro* (Fig. 5) and *in vivo* (Fig. 6 and 7).

The tendency of DNA polyplexes to aggregate at high concentrations of DNA has complicated their incorporation into hydrogel materials. We have previously shown that the incorporation of low amounts of DNA polyplexes (< 30μg DNA/100μL gel) into PEG-VS/MMP scaffolds could be achieved without significant aggregation. However, incorporation of larger amounts of DNA resulted in severely aggregated polyplexes ([17], Fig. 3B–C). In order to avoid polyplex aggregation a method that allowed the formation of the polyplexes in a dilute solution, the lyophilization of the polyplex solution to obtain a powder, and the resuspension of the powder in the desired volume was investigated. We used sucrose to protect the polyplexes during the lyophilization process [37]. As expected the activity of the polyplexes was retained for those polyplexes lyophilized in the presence of sucrose but not for those lyophilized in the absence of sucrose (Fig. 1B). However, sucrose alone could not prevent polyplex aggregation during hydrogel formation (Fig. 3D). The introduction of agarose along with sucrose to the dilute polyplex solution prior to freeze-drying resulted in polyplexes that were un-aggregated after hydrogel formation with up to 500 μg of DNA being incorporated. The incorporated polyplexes were not completely uniform inside the hydrogel scaffolds displaying microdomains with higher concentration of polyplexes (Fig. 3). Inside the microdomains un-aggregated polyplexes were found. We hypothesize that these microdomains are a result of incomplete dissolution of the microspheres and microfibers during the formation of hydrogles (Fig. 2), which keep the polyplexes trapped inside the microspheres. This is to our knowledge the first report of the incorporation of high concentration, un-aggregated polyplexes into hydrogel scaffolds.

Although the role of agarose in preventing the aggregation of polyplexes inside the hydrogel scaffolds is clear, its exact mechanism of action is unknown and was not studied in detail in the present report. However, we can hypothesize that agarose act through slowing polyplex motion or anisotropic caging in the gel precursor solution. The aggregation rate of nanoparticles is proportional to their motion and their motion is inversely proportional to the viscosity of the solution in which they are suspending [38]. Our data showed that agarose, along with sucrose, increased the viscosity of the gel precursor solution from ~0.3Pa to ~72Pa at low shear rate, which represent the condition for making the gels (Fig. 1C). The presence of agarose may also “cage” the particles as has been previously suggested for 68 nm latex nanoparticles diffusing in polyacrylamide solutions [39]. Caging the polyplexes would result in less polyplex to polyplex-polyplex collisions, which is inversely proportional to the aggregation.

DNA/PEI polyplexes were successfully loaded into three different hydrogels scaffolds formed with neutral synthetic polymers (PEG), highly negatively charged natural polymers (HA) and protein polymers (Fig. 3A–O). Further, three different types of gelation chemistries were shown to be compatible with the polyplex encapsulation approach, Michael addition, UV initiated radical polymerization, and enzymatic gelation. These hydrogel building blocks and gelation chemistries are widely used in biomedical applications. Thus, we believe that the method described here for the encapsulation of active polyplexes is widely applicable to other polymers and chemistries. In addition, our results showed that the process can be used to encapsulate jetPEI/DNA and lipofectamine/DNA nanoparticles (Fig. 3N,O).

Polyplexes encapsulated inside the hydrogel through the CnE process described here were found highly active both *in vitro* and *in vivo*. The polyplexes recovered from MMP degradable PEG hydrogel were about 65% of its original activity (Fig. 5B). When polyplexes were co-encapsulated with NIH/3T3 cells, up to 10 ng of SEAP were produced daily (Fig. 5D). The SEAP gene expression increased linearly with time (Fig. 5D), indicating that the polyplexes are distributed throughout the scaffold since the cells can internalize and process the polyplexes at similar rates through time. *In vivo* protease

degradable PEG hydrogels were able to deliver DNA to CAM cells as shown by β-galactosidase expression and enhance angiogenesis. Two main mechanisms are hypothesized to contribute to the gene transfer from the DNA loaded hydrogel scaffolds to the CAM, polyplex release and cellular infiltration. DNA/PEI polyplexes released from the hydrogel scaffold after hydrogel degradation transfect cells surrounding the hydrogel as shown by the β-galactosidase staining around the hydrogels (Fig. 6D–I). As cells from the CAM infiltrate the scaffold they encounter the entrapped DNA polyplexes and become transfected leading to β-galactosidase expression inside the hydrogel area (Fig. 6D–I). *In vivo*, the transgene expression was also a function of the crosslinking density of the hydrogel, with hydrogels with less crosslinks resulting in more β-galactosidase transgene expression (Fig. 6D–I). To ensure that the expressed transgene was present at sufficient concentration to induce an angiogenic response in and around the hydrogel, plasmid DNA encoding for VEGF was entrapped within the hydrogel using the method described. An angiogenic response was observed in all CAMs that contained a hydrogel with pVEGF/PEI polyplexes entrapped through CnE. The angiogenic response extended out in a radial orientation toward the CAM (Fig. 7E–J, arrow heads), suggesting that the expressed VEGF diffused out of the hydrogel area and created a VEGF gradient with the highest concentration near the hydrogel. In contrast, the vessels observed at the hydrogel area are highly branched neovessels without preferred orientations (Fig. 7O–R, arrows), suggesting that the VEGF concentration at the gel area was high and relatively constant.

Hydrogels capable of effectively delivering DNA or siRNA to cells at the disease site would have wide applications in tissue regeneration [1], cancer treatment [29–31], stem cell differentiation and *in vivo* cell reprogramming [40]. Ideally, these hydrogels would (1) be composed of materials that are safe and non-immunogenic, (2) be able to achieve prolonged gene expression, and (3) be able to target delivery to a particular cell type or tissue. In this report, we demonstrated the incorporation of DNA/PEI polyplexes into hydrogel materials that are currently used in the clinic, PEG, HA and fibrin. Further, non-viral gene delivery is considered safe due to its low immunogenicity and limited chance for insertion mutagenesis. Due to the relative low transfection efficiency and transient expression profile of non-viral vectors, the ideal hydrogels for the delivery of non-viral DNA should be able to keep delivering active DNA nanoparticles through the tissue formation process. Thus, hydrogels with high DNA loading of active polyplexes are essential. Since the expressed gene is likely needed throughout the tissue formation process the ideal hydrogels would release the DNA nanoparticles through the action of cell secreted proteases that are present at the various stages of tissue morphogenesis or adult wound healing. Through making the hydrogels degradable to MMPs or plasmin we were able to demonstrate one approach of cell-triggered release. However, one could envision that through the use of different degradable crosslinkers, degraded by different proteases, cell or tissue specific release could be achieved since different tissue/cells express different types of proteases.

Conclusion

In summary, we successfully developed a process to load concentrated polyplexes into hydrogels. With this process: (1) high DNA loading (up to 5μg DNA polypexes/μL of hydrogel) can be achieved without significant aggregation, (2) the encapsulated polyplexes remain active to transfect cells both *in vitro* and *in vivo*, and (3) various non-viral gene delivery nanoparticles can be loaded to neutral, charged or protein hydrogels formed through various chemistries. We believe this process will advance the application of the hydrogel scaffold mediated gene delivery to tissue regeneration and cancer treatment.

Acknowledgments

The authors would like to thank Quinn Ng (UCLA) and Talar Tokatlian (UCLA) for the helpful discussions and the National Institute of Health (R21EB007730) and the National Science Foundation (CAREER 0747539) for funding the research.

References

1. De Laporte L, Shea LD. Matrices and scaffolds for DNA delivery in tissue engineering. Adv Drug Deliv Rev 2007;59:292–307. [PubMed: 17512630]
2. Cheema SK, Chen E, Shea LD, Mathur AB. Regulation and guidance of cell behavior for tissue regeneration via the siRNA mechanism. Wound Repair Regen 2007;15:286–295. [PubMed: 17537114]
3. Krebs MD, Jeon O, Alsberg E. Localized and sustained delivery of silencing RNA from macroscopic biopolymer hydrogels. J Am Chem Soc 2009;131:9204–9206. [PubMed: 19530653]
4. Bonadio J, Smiley E, Patil P, Goldstein S. Localized, direct plasmid gene delivery in vivo: prolonged therapy results in reproducible tissue regeneration. Nat Med 1999;5:753–759. [PubMed: 10395319]
5. Chun KW, Lee JB, Kim SH, Park TG. Controlled release of plasmid DNA from photo-cross-linked pluronic hydrogels. Biomaterials 2005;26:3319–3326. [PubMed: 15603827]
6. Quick DJ, Anseth KS. DNA delivery from photocrosslinked PEG hydrogels: encapsulation efficiency, release profiles, and DNA quality. J Control Release 2004;96:341–351. [PubMed: 15081223]
7. Kong HJ, Kim ES, Huang YC, Mooney DJ. Design of biodegradable hydrogel for the local and sustained delivery of angiogenic plasmid DNA. Pharm Res 2008;25:1230–1238. [PubMed: 18183476]
8. Kasper FK, Jerkins E, Tanahashi K, Barry MA, Tabata Y, Mikos AG. Characterization of DNA release from composites of oligo(poly(ethylene glycol) fumarate) and cationized gelatin microspheres in vitro. J Biomed Mater Res A 2006;78A:823–835. [PubMed: 16741980]
9. Megeed Z, Haider M, Li D, O'Malley BW Jr, Cappello J, Ghandehari H. In vitro and in vivo evaluation of recombinant silk-elastinlike hydrogels for cancer gene therapy. J Control Release 2004;94:433–445. [PubMed: 14744493]
10. Jang JH, Rives CB, Shea LD. Plasmid delivery in vivo from porous tissue-engineering scaffolds: transgene expression and cellular transfection. Mol Ther 2005;12:475–483. [PubMed: 15950542]
11. Bleiziffer O, Eriksson E, Yao F, Horch RE, Kneser U. Gene transfer strategies in tissue engineering. J Cell Mol Med 2007;11:206–223. [PubMed: 17488473]
12. Lei P, Padmashali RM, Andreadis ST. Cell-controlled and spatially arrayed gene delivery from fibrin hydrogels. Biomaterials 2009;30:3790–3799. [PubMed: 19395019]
13. Wieland JA, Houchin-Ray TL, Shea LD. Non-viral vector delivery from PEG-hyaluronic acid hydrogels. J Control Release 2007;120:233–241. [PubMed: 17582640]
14. Saul JM, Linnes MP, Ratner BD, Giachelli CM, Pun SH. Delivery of non-viral gene carriers from sphere-templated fibrin scaffolds for sustained transgene expression. Biomaterials 2007;28:4705–4716. [PubMed: 17675152]
15. Trentin D, Hall H, Wechsler S, Hubbell JA. Peptide-matrix-mediated gene transfer of an oxygen-insensitive hypoxia-inducible factor-1 alpha variant for local induction of angiogenesis. Proc Natl Acad Sci U S A 2006;103:2506–2511. [PubMed: 16477043]
16. Trentin D, Hubbell J, Hall H. Non-viral gene delivery for local and controlled DNA release. J Control Release 2005;102:263–275. [PubMed: 15653151]
17. Lei YG, Segura T. DNA delivery from matrix metalloproteinase degradable poly(ethylene glycol) hydrogels to mouse cloned mesenchymal stem cells. Biomaterials 2009;30:254–265. [PubMed: 18838159]
18. Lungwitz U, Breunig M, Blunk T, Gopferich A. Polyethylenimine-based non-viral gene delivery systems. Eur J Pharm Biopharm 2005;60:247–266. [PubMed: 15939236]

19. Akinc A, Thomas M, Klibanov AM, Langer R. Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis. J Gene Med 2005;7:657–663. [PubMed: 15543529]
20. Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, et al. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc Natl Acad Sci U S A 1995;92:7297–7301. [PubMed: 7638184]
21. Sonawane ND, Szoka FC Jr, Verkman AS. Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine-DNA polyplexes. J Biol Chem 2003;278:44826–44831. [PubMed: 12944394]
22. Abdallah B, Hassan A, Benoist C, Goula D, Behr JP, Demeneix BA. A powerful nonviral vector for in vivo gene transfer into the adult mammalian brain: Polyethylenimine. Hum Gene Ther 1996;7:1947–1954. [PubMed: 8930654]
23. Wang S, Ma N, Gao SJ, Yu H, Leong KW. Transgene expression in the brain stem effected by intramuscular injection of polyethylenimine/DNA complexes. Mol Ther 2001;3:658–664. [PubMed: 11356070]
24. Wiseman JW, Goddard CA, McLelland D, Colledge WH. A comparison of linear and branched polyethylenimine (PEI) with DCChol/DOPE liposomes for gene delivery to epithelial cells in vitro and in vivo. Gene Ther 2003;10:1654–1662. [PubMed: 12923564]
25. Kichler A, Chillon M, Leborgne C, Danos O, Frisch B. Intranasal gene delivery with a polyethylenimine-PEG conjugate. J Control Release 2002;81:379–388. [PubMed: 12044576]
26. Rudolph C, Schillinger U, Plank C, Gessner A, Nicklaus P, Muller RH, et al. Nonviral gene delivery to the lung with copolymer-protected and transferrin-modified polyethylenimine. Biochim Biophys Acta 2002;1573:75–83. [PubMed: 12383945]
27. Gautam A, Densmore CL, Golunski E, Xu B, Waldrep JC. Transgene expression in mouse airway epithelium by aerosol gene therapy with PEI-DNA complexes. Mol Ther 2001;3:551–556. [PubMed: 11319917]
28. Segura T, Schmokel H, Hubbell JA. RNA interference targeting hypoxia inducible factor 1alpha reduces post-operative adhesions in rats. J Surg Res 2007;141:162–170. [PubMed: 17561118]
29. Iwai M, Harada Y, Tanaka S, Muramatsu A, Mori T, Kashima K, et al. Polyethylenimine-mediated suicide gene transfer induces a therapeutic effect for hepatocellular carcinoma in vivo by using an Epstein-Barr virus-based plasmid vector. Biochem Biophys Res Commun 2002;291:48–54. [PubMed: 11829460]
30. Aoki K, Furuhata S, Hatanaka K, Maeda M, Remy JS, Behr JP, et al. Polyethylenimine-mediated gene transfer into pancreatic tumor dissemination in the murine peritoneal cavity. Gene Ther 2001;8:508–514. [PubMed: 11319617]
31. Coll JL, Chollet P, Brambilla E, Desplanques D, Behr JP, Favrot M. In vivo delivery to tumors of DNA complexed with linear polyethylenimine. Hum Gene Ther 1999;10:1659–1666. [PubMed: 10428211]
32. Bhattarai N, Gunn J, Zhang M. Chitosan-based hydrogels for controlled, localized drug delivery. Adv Drug Deliv Rev 2010;62:83–99. [PubMed: 19799949]
33. Tauro JR, Gemeinhart RA. Matrix metalloprotease triggered delivery of cancer chemotherapeutics from hydrogel matrixes. Bioconjug Chem 2005;16:1133–1139. [PubMed: 16173790]
34. Wang Y, Liu S, Li CY, Yuan F. A novel method for viral gene delivery in solid tumors. Cancer Res 2005;65:7541–7545. [PubMed: 16140915]
35. Capito RM, Spector M. Collagen scaffolds for nonviral IGF-1 gene delivery in articular cartilage tissue engineering. Gene Ther 2007;14:721–732. [PubMed: 17315042]
36. Schmidt JJ, Rowley J, Kong HJ. Hydrogels used for cell-based drug delivery. J Biomed Mater Res A 2008;87:1113–1122. [PubMed: 18837425]
37. Huang YC, Connell M, Park Y, Mooney DJ, Rice KG. Fabrication and in vitro testing of polymeric delivery system for condensed DNA. J Biomed Mater Res A 2003;67A:1384–1392. [PubMed: 14624526]
38. Friedlander, SK. Smoke, dust, and haze : fundamentals of aerosol dynamics. 2nd ed.. New York: Oxford University Press; 2000.

39. Dunstan DE, Stokes J. Diffusing probe measurements of polystyrene latex particles in polyelectrolyte solutions: deviations from Stokes-Einstein behavior. Macromolecules 2000;33:193–198.

40. Zhou Q, Brown J, Kanarek A, Rajagopal J, Melton DA. In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. Nature 2008;455:627–632. [PubMed: 18754011]

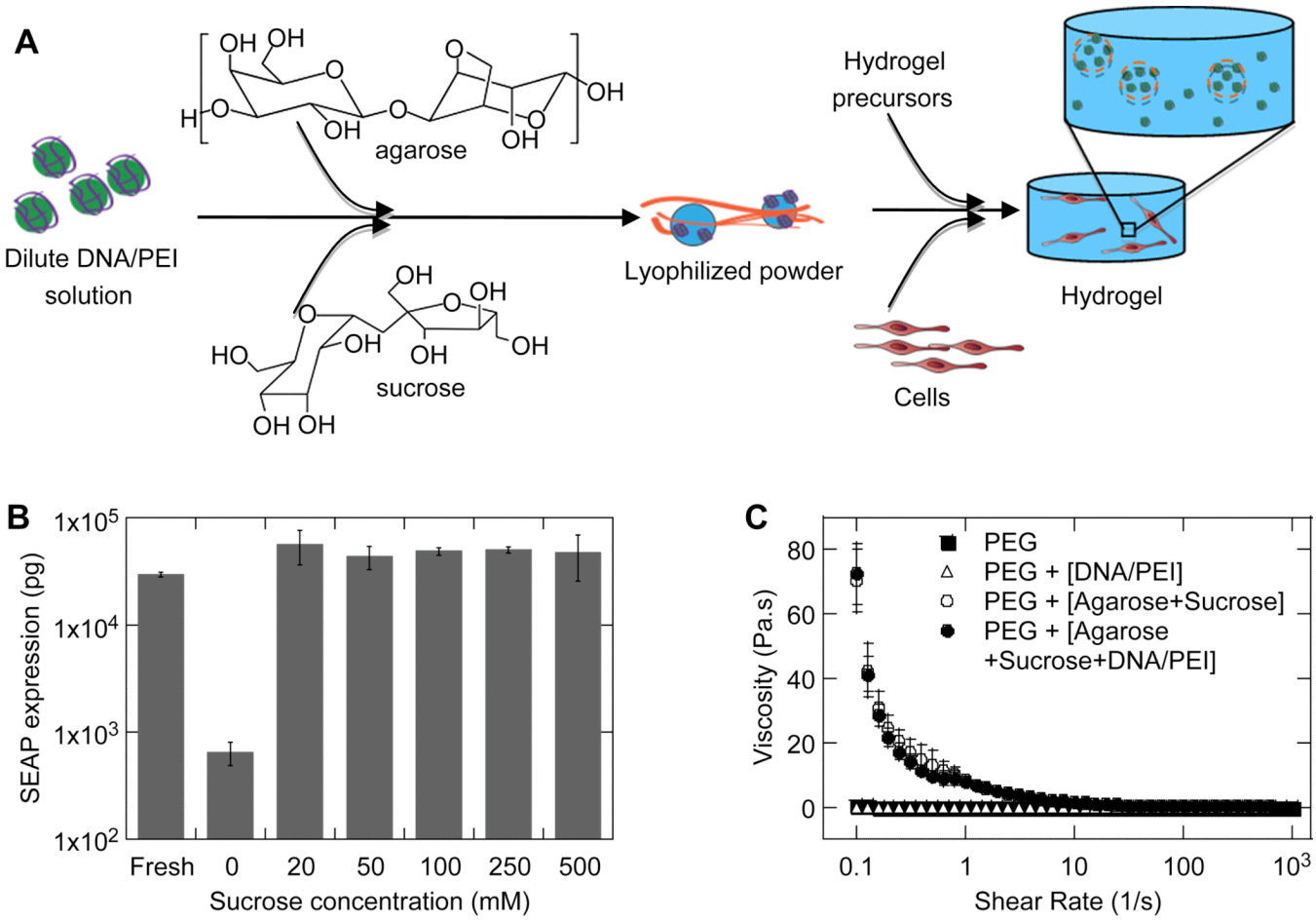

Figure 1.

Caged nanoparticle encapsulation (CnE) process. (A) Schematic illustration of the CnE process. pDNA and PEI are mixed in dilute solution in the presence of sucrose and agarose. This solution is lyophilized and the dry powder is reconstituted with hydrogel precursor solution with or without cells before crosslinking to form a hydrogel. (B) Activity of DNA/PEI polyplexes post lyophilization is maintained by the presence of sucrose. Polyplexes containing pSEAP were lyophilized in the presence of sucrose at different concentrations. The lyophilized polyplexes were used to transfect HEK293 cells and the transgene expression was compared with that of the freshly prepared polyplexes (Fresh). (C) Agarose significantly increases the viscosity of the hydrogel precursor solution. The viscosity of PEG hydrogel precursor solution with different compositions was measured using a rheometer with shear rate ranging from 0.1 to 1000Hz. PEG means a 6.5% PEG (20,000 Da) solution, + [DNA/PEI] means adding lyophilized polyplexes (100μg DNA, N/P15), + [Agarose + Sucrose] means adding lyophilized (1mg agarose + 35mg sucrose), + [Agarose + Sucrose + DNA/PEI] means adding lyophilized (1mg agarose + 35mg sucrose + polyplexes (100μg DNA, N/P15). Precursor solution volume is 100μL.

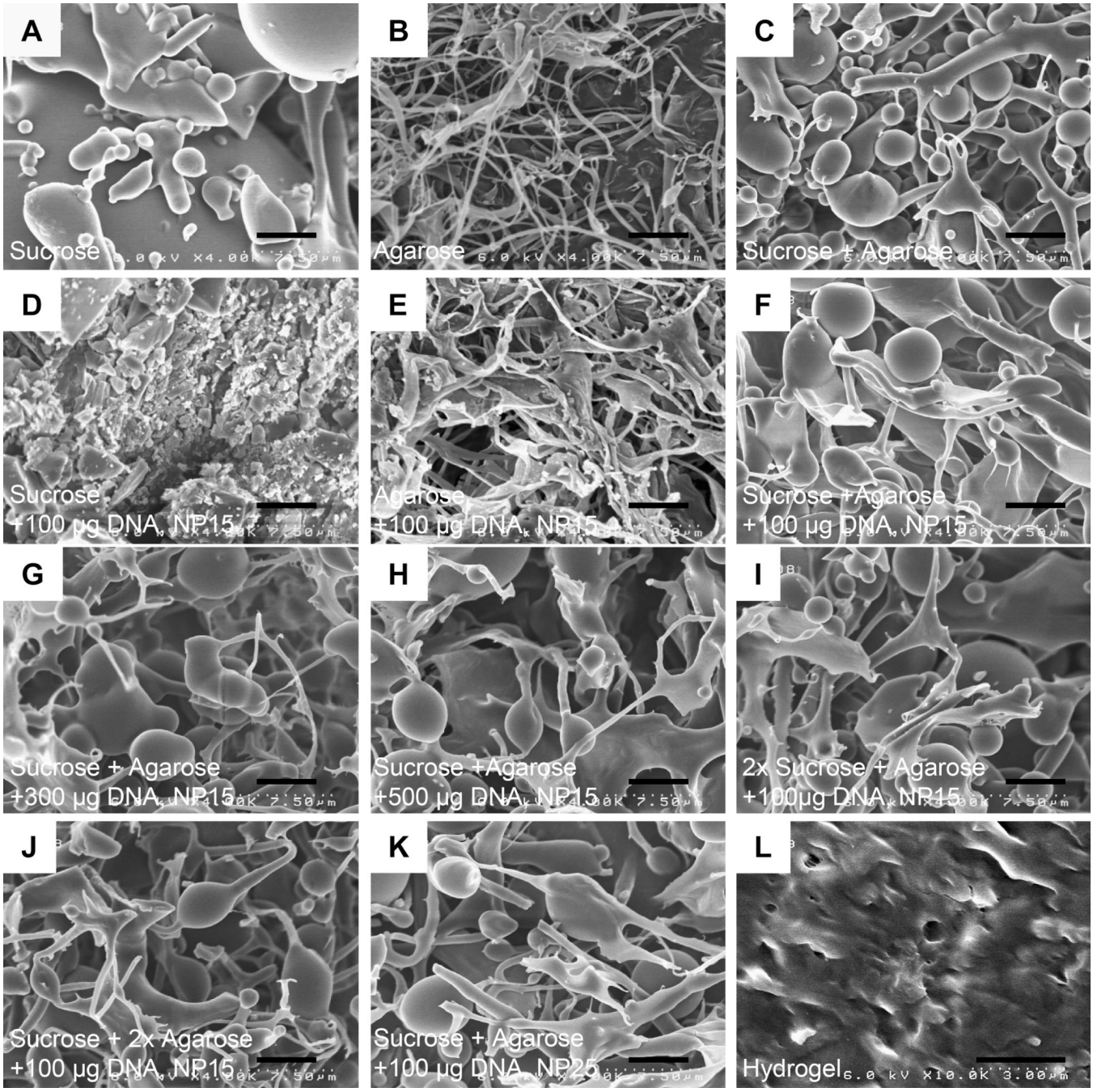

Figure 2.

Combination of sucrose and agarose results in different powder morphology by SEM. Lyophilized powders containing different components were coated with gold and imaged with SEM. (A) Sucrose (35 mg, 20mM). (B) Agarose (1 mg, 0.02% solution). (C) Agarose and sucrose, (D) DNA/PEI polyplexes and sucrose. (E) DNA/PEI polyplexes and agarose. (F–K) DNA/PEI polyplexes, sucrose and agarose. (L) PEG hydrogel with agarose, sucrose and polyplexes. 2x sucrose and 2x agarose indicate 40mM and 0.04% solutions respectively. Scale bar: A–K: 5 µm; L: 3 µm

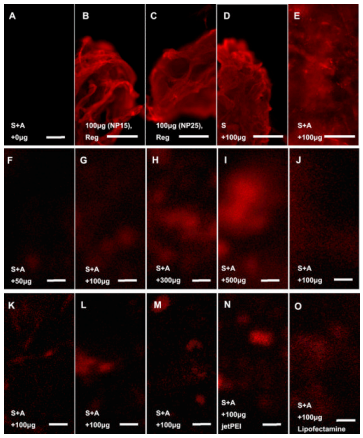

Figure 3.

Confocal micrographs of the distribution of polyplexes inside 100 μL hydrogel scaffolds. (A–I, N–O) 6.5% PEG-VS/MMP, (J) 5% PEG-VS/MMP, (K) 6.5% PEG-AC/UV, (L) 3% HA-AC/MMP, and (M) 5mg/mL fibrin hydrogels. Agents used to condense DNA: A–M: L-PEI; N: jetPEI; O: lipofectamine, Reg means regular polyplex encapsulation, where freshly prepared polyplexes were encapsulated during hydrogel formation. S means sucrose, A means agarose; x μg means DNA/PEI polyplex containing 0, 50, 100, 300 or 500 μg DNA at N/P=15 as indicated. Scale bar is 500μm for B–E, 500nm for A, F–O.

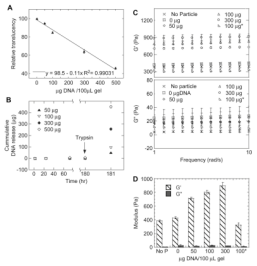

Figure 4.

In vitro characterization of high DNA loaded hydrogels. (A) Light translucency of high DNA loaded PEG-VS/MMP hydrogel. Lyophilized (sucrose + agarose + polyplexes (50~500μg DNA, N/P = 15)) were encapsulated in 100 μL 6.5%PEG-VS/MMP hydrogel. Light passed through the hydrogels was recorded and normalized to a gel without polyplexes. (B) DNA release from high DNA loaded PEG-VS/MMP hydrogels. Lyophilized (sucrose + agarose + polyplexes (50–500μg DNA, N/P = 15)) were encapsulated in 100 μL of 6.5% PEG-VS/MMP hydrogel and incubated in PBS (pH = 7.4) for 180-hrs before being degraded with 0.25% trypsin. DNA was quantified with HOECHST dye. (C, D) Storage (G') and loss (G") modulus of various PEG-VS/MMP hydrogels. Lyophilized (sucrose + agarose + polyplexes (0~300μg DNA, N/P = 15)) were encapsulated in 100μL 6.5% PEG-VS/MMP hydrogel (except one sample with 4.5% PEG as indicated with a *). G' and G" were measured with rheometer and compared to the hydrogel without sucrose, agarose and polyplexes (labeled as No particle). The overall G', G" (C) and the G', G" over the entire frequency (D) were shown.

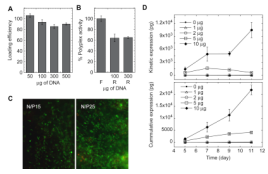

Figure 5.

Sucrose and agarose allow for the introduction of active DNA/PEI polyplexes to hydrogels. (A) DNA loading efficiency. PEG hydrogels with 50 to 500 μg DNA through CnE process were degraded with 0.25% trypsin. The ratio of recovered DNA over the DNA used for making the hydrogel times 100 was defined as loading efficiency. (B) Polyplex's activity after CnE encapsulation. Polyplexes recovered from PEG-VS/MMP hydrogels with 100 (100 R) or 300μg DNA (300 R) were used to transfect HEK293 cells. The activities were normalized to that of the freshly prepared polyplexes (F). Transfections were done using 1μg DNA. (C) LIVE/DEAD cell staining for mMSCs at day 15, green indicates live cells and red indicates dead cells. Lyophilized polyplexes containing 50 μg DNA at N/P = 15 or 25 were encapsulated into 100μL PEG-VS/MMP hydrogel using CnE. (D) Polyplex's activity in fibrin hydrogels after CnE. NIH/3T3 (100,000 cells/100μL gel) and lyophilized polyplexes (N/P = 15) encoding for SEAP were co-encapsulated into fibrin (5mg/mL) hydrogels. The expression of SEAP protein was recorded with time and plotted as a kinetic or cumulative expression.

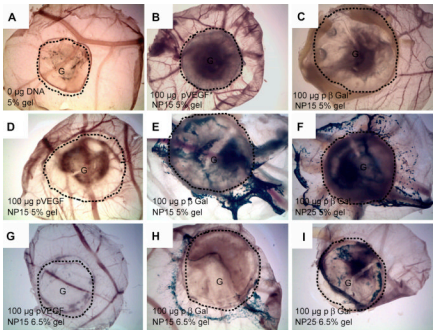

Figure 6.

DNA loaded hydrogels using CnE resulted in gene transfer *in vivo*. PEG-VS/MMP hydrogels loaded with polyplexes through regular polyplex encapsulation (B,C) or CnE encapsulation (A, D–I) were placed on top of CAM for 3 days. The gels with CAM were cut, fixed and stained with x-gal solution for 24hrs. Positive β-galactosidase expression resulted in blue color. x μg DNA means 0 or 100 μg DNA as indicated, pVEGF or pβgal indicate the plasmid used to for DNA/PEI polyplexes, NP15 or NP25 indicate NP = 15 or 25 as indicated and 5% or 6.5% indicate the percent PEG-VS/MMP hydrogel used. The symbol * indicates the polyplexes encapsulated using the regular encapsulation approach. The dash line outlines the edge of the hydrogel and G indicates the gel area.

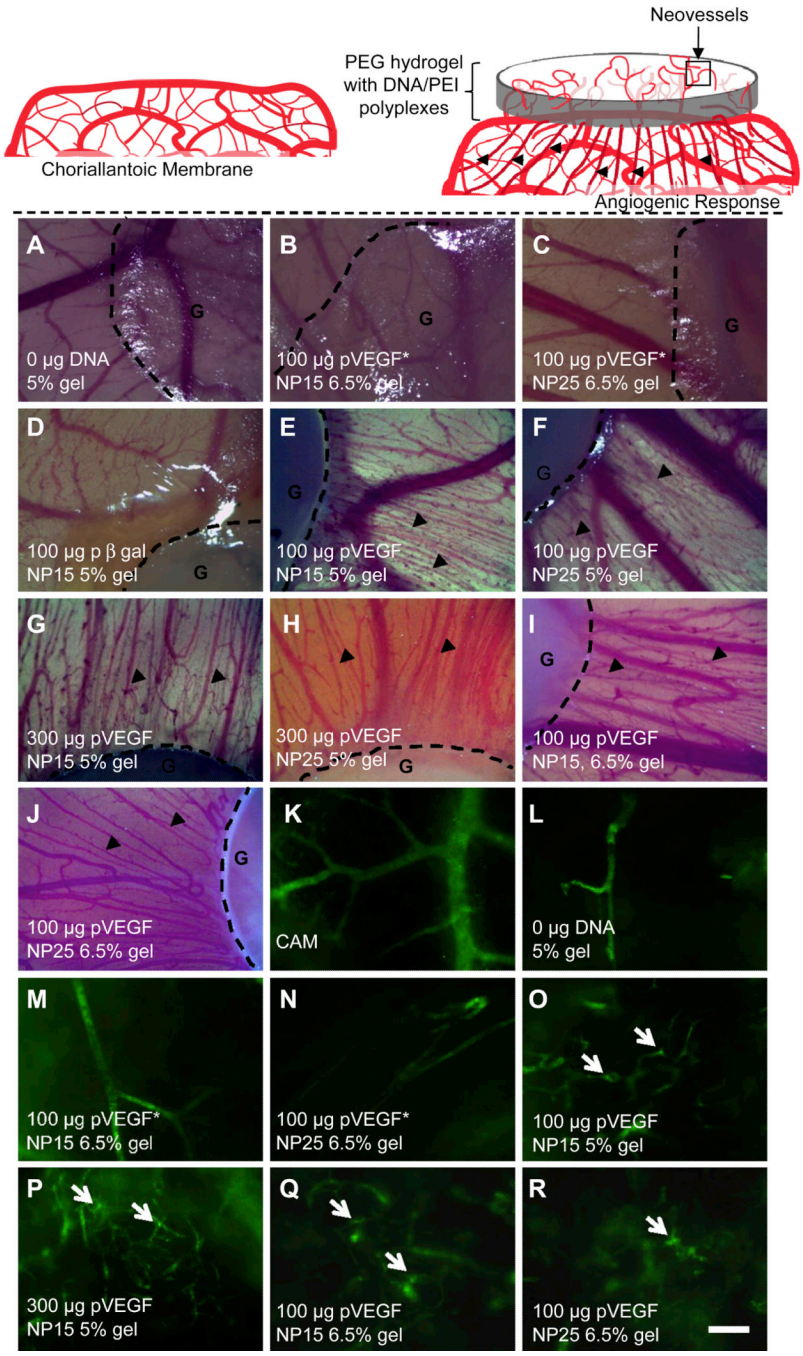

Figure 7.

DNA loaded hydrogels using CnE resulted in enhanced angiogenesis in a CAM model. The top panel illustrates the blood vessel network of the CAM (left). PEG-VS/MMP hydrogels with polyplexes containing pVEGF were placed on top of the CAM for 3 days (right). pVEGFs were transferred to cells and produced a high concentration of VEGF at the gel area, which resulted in hyperbranced neovessels (arrow). VEGFs diffusing out the gel created a decreasing VEGF gradient around the gel and led to radial neovessels around the gel (arrowheads). Gross pictures on the gel edges (A–J) were recorded before the CAMs were infused with FITC-dextran for fluorescent imaging (K–R) at the gel area. Induced neovessels were found both around the gels (E–J, arrow heads) and at the gel area (O–R,

arrows) with pVEGFs through the CnE process, which were not found in the negative controls (A–D, K–N). x μg DNA means 0, 100 μg or 300 μg DNA as indicated, pVEGF or pβgal indicate the plasmid used to for DNA/PEI polyplexes, NP15 or NP25 indicate NP = 15 or 25 as indicated and 5% or 6.5% indicate the percent PEG-VS/MMP hydrogel used. The symbol * indicates the polyplexes encapsulated using the regular encapsulation approach. The dash line outlines the edge of the hydrogel and G indicates the gel area. Scale bar: K–R: 200μm