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Multilayer mediated forward and patterned siRNA transfection using linear-PEI at extended N/P ratios

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Abstract

Gene delivery from a substrate depends, in part, on the vector-nucleic acid complex that is bound to the surface and the cell adhesive properties of the surface. Here, we present a method to deliver patterns of small interfering RNA (siRNA) that capitalize on a forward transfection method (transfection by introducing siRNA-transfection reagent complexes onto plated cells); herein denoted as multilayer mediated forward transfection (MFT). This method separates the substrate-mediated delivery from the cell adhesive properties of the surface. pH responsive layer-by-layer (LbL) assembled multilayers were used as the delivery platform and microcontact printing technique (μ CP) was used to pattern nanoparticles of transfection reagent-siRNA complexes onto degradable multilayers. Efficient MFT depend on optimal formulation of the nanoparticles. 25 kDa linear polyethylenimine (LPEI) was optimized as the siRNA transfection reagent for normal forward transfection (NFT) of the nanoparticles. A broad range of LPEI-siRNA nitrogen/phosphate (N/P) ratios (ranging from 5 to 90) was evaluated for the relative amounts of siRNA incorporated into the nanoparticles, nanoparticle size and NFT efficiencies. All the siRNA was incorporated into the nanoparticles at N/P ratio near 90. Increasing the amount of siRNA incorporated into the nanoparticles, with increasing N/P ratio correlated with a linear blue-shift in the ultraviolet/visible (UV/vis) absorbance spectrum of the LPEI-siRNA nanoparticles. NFT efficiency greater than 80% was achieved with minimal cytotoxicity at N/P ratio of 30 and siRNA concentration of 200 nM. Similarly, MFT efficiency $\geq 60\%$ was achieved for LPEI-siRNA nanoparticles at N/P ratios greater than 30.

Keywords

Patterned siRNA transfection; Forward transfection; Layer-by-layer (LbL) assembly; Linear polyethylenimine (LPEI)-siRNA nanoparticles; N/P ratio; UV/vis blue shift

1. Introduction

RNA interference (RNAi) is a sequence-specific post-transcriptional gene silencing process triggered through small interfering RNAs (siRNAs) [1,2] which serves as a powerful

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therapeutic tool [3,4] in gene therapy. An important aspect of gene therapy for regenerative medicine and organized tissue formation is to manipulate the location of transfected cells, requiring the generation of gene expression patterns in spatially controlled environments [5, 6]. Patterned delivery of DNA has been demonstrated with cells seeded onto modified surfaces, where vector-DNA complexes were immobilized onto chemically modified surfaces, including self-assembled monolayers (SAMs), using different patterning techniques [6–8]. Delivery of patterned siRNA from a substrate to adherent cells for high-throughput functional genetic analysis has been demonstrated with reverse transfection-based RNAi microarrays [9,10]. Reverse transfection plates the cells at the time of transfection [10], whereas forward transfection plates the cells to allow them to first attach and grow, prior to transfection. Reverse transfection-based approaches for gene delivery or RNAi microarrays requires that the cells must be able to adhere to the surface containing the expression vector, or the substrates must be chemically modified to immobilize the non-adherent cell lines [11]. Gene delivery from a substrate depends, in part, on the vector-nucleic acid complex that is bound to the surface [6]. Various parameters such as surface charge, hydrophobicity/hydrophilicity [8], rigidity of the cell adhesion substrates [12] all contribute to the molecular interactions between the vector and the polymer on the surface. Any chemical modification of the substrates that may enhance cell adhesion could adversely affect the release of the vector-nucleic acid complexes from the surface and thus interfere with cellular internalization of the polymer-nucleic complexes [6], and efficient gene delivery. The present study describes a method for forward transfection of siRNA, yielding micron-sized patterns of transfected mammalian cells. With this method, the cells are cultured separately from a degradable LbL assembled multilayer arrayed with the siRNA, thereby separating the two issues, the complex release from, and the cell adhesion on the substrate.

The layer-by-layer (LbL) assembly method introduced by Decher and co-workers [13,14] for multilayer thin film formation is an attractive approach for controlled release of biomolecules from surfaces [15]. LbL thin films provide flexibility in terms of their choice of substrate and constituent components, surface patterning techniques, fabrication conditions, and tunable structural properties [16]. Other advantages include their ease of preparation and cost-effectiveness. Different patterning techniques can be employed to conjugate biomolecules, such as nucleic acids, to multilayer structures. Soft-lithographic micro-contact printing (μ CP) [17,18] is one such technique, which has emerged as a platform of choice for biochips and drug delivery applications [19]. Various “inks”, including, proteins, DNA, RNA, and polyelectrolytes have been used in μ CP to pattern surfaces without the need for dust-free environments and harsh chemical treatments [20].

A previous method of in vitro localized transfection of cultured cells from multilayer thin films did not involve patterned delivery of DNA from these films [21]. Nonetheless, LbL thin film application of reverse transfection of DNA to form cell microarrays have been previously demonstrated [22], but the method is not easily applied to siRNA due to the enhanced susceptibility of siRNAs to degradation as compared to DNAs [3,23]. Approaches to embed the polymer and nucleic acid alternatively to form LbL films [24] or embed the polymer-nucleic acid complexes within the multilayers [25] have not involved patterned delivery. Thus, spatially controlled delivery of siRNA based on thin film chemistries has yet to be realized.

Here, we describe the application of a LbL assembled degradable multilayer film for patterned delivery of siRNA using a forward transfection approach. The transfection process involved the following steps (Fig. 1). (1) pH controlled, biocompatible and degradable multilayers were fabricated using LbL assembly under acidic conditions [26]. (2) Nanoparticles of vector-siRNA complexes prepared at physiological pH conditions were used as “ink” in μ CP to form patterns on multilayer substrates. (3) Multilayer substrate containing patterned nanoparticles laid on top of cells at physiological pH conditions degraded the multilayer and formed patterns of

transfected cells. This method is a variation of the forward transfection technique; herein denoted as the multilayer mediated forward transfection (MFT) method. Quantification of MFT efficiencies with linear polyethylenimine (LPEI)-siRNA nanoparticles found nitrogen/phosphate (N/P) ratios ≥ 30 gave significant transfection ($\geq 60\%$). MFT of patterned siRNA provides an efficient and simple approach to spatially controlled siRNA delivery for tissue engineering applications. This method also provides a proof-of concept study towards the eventual development of a forward transfection-based cell microarray.

Efficient MFT is contingent on the formulation of the vector-siRNA nanoparticles. Normal forward transfection (NFT) was evaluated for a suitable range of nanoparticle formulations for MFT, using 25 kDa LPEI as the non-viral gene delivery vector. Among the polymeric vectors, PEI is the gold standard for gene delivery, but its high transfection efficiency is often associated with high cytotoxicity [27,28]. In addition, despite the superficial similarities of siRNAs and DNAs, their distinct characteristics, such as molecular weight and topography [3,23,29], results in significant differences in their optimal delivery formulation with transfection reagents [29]. Therefore, delivery vehicles and transfection conditions must be designed and optimized to cater to their individual requirements for efficient delivery. The use of PEI [23,27,28,30–38] as a polymeric DNA transfection reagent has been studied extensively, with PEI-DNA complexes analyzed for a broad range of N/P ratios [22,30–32,35–37], up to a N/P ratio of 135 [31]. However, to our knowledge, reported studies with siRNA transfection has been limited to narrow ranges of PEI-siRNA compositions (mostly limited to N/P ratio of 10) [23,33,34, 37,38], until recently [39]. A recent study characterized ketal modified and unmodified branched PEI (BPEI)-siRNA complexes up to a N/P ratio of 100 [39], but a similar N/P ratio analysis has not been performed with LPEI. 22 kDa LPEI was reported unable to provide in vitro siRNA transfection, while 25 kDa BPEI successfully mediated siRNA transfection (with 200 nM siRNA) at N/P ratios up to 8 [33]. However, BPEI is more cytotoxic than LPEI [27, 33]. Thus, there is a need for a less toxic form of PEI that can provide siRNA silencing (preferably, better silencing), at similar or lower siRNA concentrations than used previously with BPEI.

Therefore, in this study we evaluated a broad range of LPEI-siRNA N/P ratios (ranging from 5 to 90) using 25 kDa LPEI as the transfection reagent. For comparison, 25 kDa BPEI was also evaluated as a transfection reagent. Our results indicated LPEI to be a better siRNA transfection reagent than BPEI using siRNA concentration of 200 nM. We characterized the LPEI-siRNA nanoparticles for this range of N/P ratios. We found that: (1) complete incorporation of the siRNA was achieved at N/P ratio near 90, which produced transfection efficiency greater than 90% and nanoparticles ~ 50 nm in size, (2) partial incorporation of the siRNA was observed at N/P ratios between 30 to 75, which produced transfection efficiencies greater than 80%. The nanoparticle size was ~ 150 nm for N/P ratios between 30 to 60 and less than 100 nm for N/P ratio of 75, and (3) further reduction in the incorporation of siRNA was observed at N/P ratios of 5 and 15, no transfection was observed at N/P ratio of 5 and $\sim 40\%$ transfection was observed at N/P ratio of 15. The nanoparticles size was greater than 200 nm for N/P ratio of 5 and ~ 150 nm for N/P ratio of 15. Finally, N/P ratio of 30 for LPEI-siRNA nanoparticles was optimal for achieving high NFT ($\sim 85\%$) transfection efficiency with minimum cytotoxicity.

2. Materials and methods

2.1. Materials

Poly(acrylic acid, sodium salt) solution (PAA, M_w 15,000; catalog no. 416037), poly(ethylene glycol) (PEG, M_w 10,000; catalog no. P6667), and branched polyethylenimine (BPEI, M_w 25,000; catalog no. 408727) were purchased from Sigma-Aldrich Chemical (Milwaukee, WI). The linear polyethylenimine (LPEI, M_w 25,000; catalog no. 23966) was obtained from Polysciences, Inc. (Warrington, PA). Poly(dimethylsiloxane) (PDMS) from the Sylgard 184

silicone elastomer kit (Dow Corning, Midland, MI) was used to prepare stamps. Barnstead Nanopure Diamond (Barnstead International, Dubuque, IA) purification system with a resistance of $> 18.2 \text{ M}\Omega \text{ cm}$ was used as a source for deionized (DI) water. Solution pH was adjusted using HCl or NaOH. Layer-by-layer (LbL) assembled multilayer films were made on quartz slides (Technical Glass Products, OH) that were cut to fit 12-well tissue culture polystyrene (TCPS) plates (Costar, Corning, NY). Lipofectamine 2000 (LF2k) transfection reagent (1 mg ml^{-1}), OptiMEM reduced serum medium (catalog no. 31985), Dulbecco's Modified Eagle Medium (DMEM), penicillin, streptomycin, were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Biomedica Corp (Foster City, CA). HeLa cells were purchased from American Type Culture Collection (Rockville, MD). Custom synthesized siRNA with sense sequence 5'-GGUGAAGGUAGAUCAAAGAdTdT-3' and anti-sense sequence 5'-UCUUUGAUCUACCUUCACcdTdT-3', targeting human double-stranded RNA-dependent protein kinase (PKR) mRNA was purchased from Dharmacon (Chicago, IL), and diluted to a concentration of $80 \text{ }\mu\text{M}$. Fluorescein and Alexa Fluor-555 conjugated double-stranded RNA (dsRNA) oligomers (Block-iT Fluorescent and Block-iT Alexa Fluor Red Fluorescent Oligos) (Invitrogen, Carlsbad, CA) were used to demonstrate patterned delivery in MFT.

2.2. Cell culture

HeLa cells were maintained in DMEM with 10% FBS and 100 U ml^{-1} penicillin plus $100 \mu\text{g ml}^{-1}$ streptomycin (P/S) at 37°C and 10% CO_2 . For transfection and cytotoxicity studies, HeLa cells were cultured and grown to complete confluency in 12-well plates in 1.2 ml of P/S free DMEM supplemented with 10% FBS.

2.3. Degradable layer-by-layer (LbL) multilayer fabrication

Hydrogen (H)-bonded (PAA/PEG) $_{n.5}$ multilayers were fabricated at a deposition pH of 2.0. PAA and PEG polymer solutions used to fabricate multilayer assemblies were prepared in DI water to final concentrations of 1 mg ml^{-1} . The pH of both solutions was adjusted to 2.0 and filtered with a $0.22 \text{ }\mu\text{m}$ cellulose acetate filter (Corning, NY). DI water adjusted to pH 2.0 was used as the wash solution. PAA consists of COO^- terminal groups, which due to their acidification forms H-bond with PEG molecules at low pH conditions [26]. These H-bonded multilayers degrade upon immersing into physiological pH conditions [26]. Different numbers of bilayers of PAA and PEG were prepared with PAA as the terminating layer in each case. Quartz slides were cleaned in piranha solution (7:3; concentrated sulfuric acid: 30% hydrogen peroxide), dried under N_2 gas and further cleaned using a plasma cleaner (Harrick Scientific Corporation, NY) for 10 min at 0.15 torr and 50 sccm flow of O_2 . A Carl Zeiss slide stainer was used to deposit multilayers on quartz substrates. Quartz substrates were immersed in 100 mM LPEI solution (pH ~ 7.4) for 30 min to provide an initial positive charge and then rinsed in DI water for 3 min with agitation. Subsequently, the substrates were dipped into PAA solution for 15 min followed by 3 min in wash solution with agitation. The substrates were then dipped into PEG solution for 15 min followed by 3 min in wash solution with agitation to create one bilayer. The dipping cycle was repeated to form multilayer films. Multilayer films are abbreviated as (PAA/PEG) $_{n.5}$, where n is the number of PAA and PEG bilayers and the ".5" indicates an additional, single terminating layer of PAA.

2.4. PDMS preparation

Patterned PDMS stamps were created by curing degassed prepolymer and initiator (10:1) mixture against a microfabricated silicon master in an oven overnight at 60°C , as described elsewhere [17]. The masters consisted of features: parallel lines from 200 to $250 \text{ }\mu\text{m}$ and squares from 200 to $700 \text{ }\mu\text{m}$. Non-patterned PDMS stamps were prepared against a plane silicon wafer

as the master. PDMS stamps were cut to the size of multilayer substrate in order to obtain uniform transfer of nanoparticles with minimum loss during the stamping process.

2.5. PEI-siRNA nanoparticle formation

100 mM LPEI and 1 mM BPEI stock solutions (molarities with respect to repeat units of the polymer) were prepared in DI water and adjusted to pH 7.2. Volumes of LPEI or BPEI mixed with siRNA were calculated for the different nitrogen/phosphate (N/P) ratios (the ratio of protonable amine groups on PEI to phosphates on siRNA). OptiMEM was used as a buffer to prepare nanoparticles in all cases, except for the zeta (ζ)-potential analysis. N/P ratios of 5, 15, 30, 45, 60, 75 and 90 were used for LPEI-siRNA and N/P ratios of 5, 10 and 15 were used for BPEI-siRNA formulations. Solutions of PEI and siRNA were prepared separately in OptiMEM buffer (at physiological pH) at the calculated concentrations (at room temperature), and mixed within 5 min after their preparation. For agarose gel electrophoresis assay, UV/vis, ζ -potential, DLS, NFT and cytotoxicity studies; PEI and siRNA solutions were prepared in separate volumes of 100 μ l, giving 200 μ l of mixed volume. For MFT, ζ -potential analysis of the nanoparticles released from the multilayers, SEM and AFM; PEI and siRNA solutions were prepared in separate volumes of 12.5 μ l and mixed to give 25 μ l of nanoparticles. PEI-siRNA mixture was kept at room temperature for 30 min prior to use in transfection or characterization studies, unless specified otherwise.

2.6. Nanoparticle stamping (microcontact printing) onto multilayers

After multilayer and nanoparticle formation, the next step of MFT was stamping of the nanoparticles onto the multilayer using PDMS as the stamping elastomer. PDMS stamps were plasma treated for 2 min and drop-coated with nanoparticles. The plasma treatment facilitated spreading of the nanoparticle ink, enabling temporary electrostatic interactions between the nanoparticles and the SiO^- groups on the PDMS. To minimize loss of the nanoparticles, the stamps were air-dried for 45 min rather than dried with N_2 assistance. As the nanoparticle ink dried, the PDMS recovered partial hydrophobicity causing the dried nanoparticles on the surface to have weaker interactive forces with the PDMS. Nanoparticles were transferred to $(\text{PAA/PEG})_{n,5}$ multilayers through μ CP. PAA ($pK_a \sim 5$) when incorporated into a multilayer assembly remains partially ionized even at the pH of 2.0 [40]. During stamping, the weak binding of the nanoparticles to the PDMS facilitated their transfer to the partially negative PAA terminated multilayer. Non-patterned stamps were used for quantifying the transfection efficiency.

2.7. Normal forward transfection (NFT) and multilayer mediated forward transfection (MFT)

HeLa cells were transfected with different nanoparticle formulations for 48 h and the cell-culture medium was not changed post-transfection.

2.7.1 NFT—LPEI-siRNA nanoparticle solution (200 μ l) was added to the cultured cells in 1 ml of fresh cell culture medium. For NFT, the final concentration of siRNA was 200 nM, unless specified otherwise. For comparison, LF2k (2 μ g) and BPEI were also used as transfection reagents, with 40 pmol ($\equiv$ 33 nM) and 240 pmol ($\equiv$ 200 nM) final concentration of siRNA, respectively.

2.7.2 MFT—Multilayer quartz substrates containing the LPEI-siRNA nanoparticles were sterilized under UV light for at least 15 min. Prior to transfection the cell culture medium was removed, and the quartz substrate was placed top-down onto the cultured cells. 1.2 ml of fresh culture medium was added. For MFT, 240 pmol of siRNA ($\equiv$ 200 nM final concentration in NFT) was used, unless specified otherwise. For comparison, LF2k (2 μ g) with 40 pmol of siRNA ($\equiv$ 33 nM final concentration in NFT) was also evaluated.

2.8. Characterization

2.8.1. Agarose gel electrophoresis assay—Relative amount of free siRNA in the LPEI-siRNA nanoparticle preparation at each N/P ratio was evaluated by a gel retardation assay. Nanoparticles were prepared as described above at a constant siRNA concentration of 200 nM at each N/P ratio. A 15 μ l aliquot of the samples with 3 μ l of loading buffer (Bio-Rad, CA) was loaded on a 0.8% agarose gel prepared in 1X Tris-boric acid-EDTA (TBE) buffer (Bio-Rad, CA). Electrophoresis of the LPEI-siRNA nanoparticles was run in 1X TBE buffer at 110V for 30 min. siRNA bands were visualized using SYBR gold nucleic acid gel stain (Invitrogen) and a UV transilluminator.

2.8.2. Ultraviolet/visible (UV/vis) absorbance—LPEI-siRNA nanoparticle solutions prepared from 750 nM final concentration of siRNA at the different N/P ratios were diluted in 1.2 ml of OptiMEM buffer (at physiological pH). Higher siRNA concentration was used to obtain measurable absorbance values which did not alter the calculation of the amount of siRNA incorporated into the nanoparticles. UV/vis peaks were measured in a 10 mm path length quartz cuvette at 25°C and with a wavelength interval of 1 nm using SPECTRAmax Plus 384 (Molecular Devices, CA).

2.8.3. Scanning electron microscopy (SEM)—Constant siRNA amount of 240 pmol ($\equiv$ 200 nM final concentration in NFT) was used with varying concentrations of PEI. SEM images of air-dried nanoparticles were collected with a field emission JEOL 6300F electron microscope.

2.8.4. Dynamic light scattering (DLS)—LPEI-siRNA nanoparticle solutions prepared from 750 nM final concentration of siRNA at the different N/P ratios were diluted to 3.0 ml of total volume in OptiMEM buffer (at physiological pH). Hydrodynamic particle size of LPEI-siRNA nanoparticles was determined by DLS at 25°C with a 90Plus/BI-MAS multi-angle particle size analyzer (Brookhaven Instruments Corp., NY). The wavelength of the 15 mW solid laser used was 660 nm, and the scattering angle used was 90°. A dust filter value of 30 was used. Refractive index for particles in each sample was taken as $1.50 + 0i$. Refractive index and viscosity of the aqueous suspension used was 1.33 and 0.89 cP, respectively. Measurements were performed within 30 min of mixing the LPEI and siRNA for nanoparticle preparation. Each measurement was performed for 10 runs per sample, each run of 2 min. Intensity-weighted size distribution in the multimodal size distribution (MSD) analysis mode (based on non-negatively constrained least squares (NNLS) algorithm in MAS OPTION software from Brookhaven) showed bimodal distribution of particles, with a primary population in a size range less than 500 nm and a second population in a range greater than 5 μ m. PEI-nucleic acid complexes tend to form aggregates over time in the buffer solutions [36,41]. These aggregates were detected in the second population of particles. Since PEI-siRNA complexes greater than 150 nm, which is the size limit for non-specific cellular uptake through clathrin-coated pits [42], have been reported unable to mediate gene silencing [33], only the primary population size of the particles are reported. Mean and standard deviation were plotted by the number-weighted size distribution in MSD analysis mode. Particle sizes were corroborated by scanning electron and atomic force microscopy.

2.8.5. Zeta (ζ)-potential—LPEI-siRNA nanoparticles were prepared in nuclease-free DI water, as explained above. siRNA concentration was kept constant at or equivalent to 750 nM. A higher amount of siRNA was used to obtain detectable count rates [33]. ζ -potential values of “direct complexes” (i.e., nanoparticle complexes immediately after their formation and diluted to final volume of 1.5 ml of nuclease free DI water) and “multilayer released complexes” (i.e., nanoparticle complexes released from multilayers in a volume of 1.5 ml

nuclease free DI water) were measured in polystyrene cuvettes at 25°C using ZetaPALS, zeta potential analyzer (Smoluchowski model) (Brookhaven Instruments Corp., NY).

2.8.6. Fluorescence analysis of patterned delivery—Confocal laser scanning microscopy (CLSM) images were obtained using Olympus Fluoview 1000 laser scanning confocal microscope. Conventional fluorescence images were collected using Leica DM IL inverted microscope (Bannockburn, IL) equipped with SPOT RT color camera (Diagnostics Instruments, MI).

2.8.7. Real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)—Total RNA was extracted from cells with RNeasy mini kit (Qiagen, Valencia, CA) and depleted of contaminating DNA with RNase-free DNase (Qiagen). Equal amounts of total RNA (1 µg) were reverse-transcribed using an iScript cDNA synthesis kit (Bio-RAD). The first-strand cDNA was used as a template. The primers used for qRT-PCR analyses of human PKR (5'-CCTGTCCTCTGGTTCTTTTGCT-3' and 5'-GATGATTCAGAAGCGAGTGTGC-3') [43], and human GAPDH (5'-AACTTTGGTATCGTGAAGGA-3' and 5'-CAGTAGAGGCAGGGATGATGT-3') were synthesized by Operon Biotechnologies, Inc. (Huntsville, AL). RT-PCR was performed in 25 µl reactions using 1/10 of the cDNA produced by reverse transcription, with 0.2 µM of each primer, 1 X SYBR green supermix from Bio-RAD, and at an annealing temperature of 60°C for 40 cycles. Each sample was assayed in three independent RT reactions and triplicate reactions were performed and normalized to GAPDH expression levels. The cycle threshold (C_T) values corresponding to the PCR cycle number at which fluorescence emission in real time reaches a threshold above the base-line emission were determined using MyIQ™ Real-Time PCR Detection System (Bio-RAD).

2.8.8. Cytotoxicity tests—HeLa cells were cultured with different nanoparticle formulations (with 200 nM of siRNA) for 48 h and the supernatant was collected and stored at -80°C until analysis. Cells were washed with phosphate buffered saline (PBS) and kept in 1% triton-X-100 in PBS for 24 h at 37°C and 10% CO₂. Cell lysate was collected, vortexed and centrifuged at 500 rcf for 10 min. Cytotoxicity was measured using a cytotoxicity detection kit (Roche Applied Science, Indianapolis, IN) as the fraction of lactate dehydrogenase (LDH) released into the medium, normalized to the total LDH (released + lysed).

2.9. Statistical analysis

All experiments were performed at least three times, and representative results are shown. All data, unless specified, are shown as the mean ± S.D. for indicated number of experiments. Student t-test was used to evaluate statistical significances between different treatment groups. Statistical significance was set at $p < 0.01$, unless specified otherwise.

3. Results and discussion

A broad range of N/P ratios (ranging from 5 to 90) of the LPEI-siRNA nanoparticles was characterized for their degree of siRNA incorporation into the nanoparticles. Concomitantly, we find a blue shift in the UV/vis absorbance of the polymer-nucleic acid complexes with increasing siRNA incorporation into the nanoparticles, as a function of N/P ratio. These nanoparticles were further characterized for their zeta (ζ)-potential, sizes, normal forward transfection (NFT) efficiencies and cytotoxicities. LPEI as the delivery vector provided high transfection efficiencies with 200 nM siRNA as compared with ~33 nM, a standard concentration used with Lipofectamine 2000 (LF2k) [44], and therefore 200 nM was used to evaluate the LPEI formulations. Subsequently, the LbL assembled multilayer mediated

forward transfection (MFT) of siRNA was demonstrated, and the transfection efficiencies quantified and compared with LPEI and LF2k as the transfection reagents.

3.1. Physical property evaluation of LPEI-siRNA nanoparticles at different N/P ratios

3.1.1. Agarose gel electrophoresis—To determine the degree of incorporation of siRNA into the nanoparticles at varying N/P ratios, we performed agarose gel electrophoresis of LPEI-siRNA nanoparticles ranging from N/P ratio of 5 to 90, at a constant siRNA concentration of 200 nM (Fig. 2). At 200 nM siRNA and no LPEI (N/P = 0), the siRNA is completely unbound (i.e., all free siRNA) (lane 1); at 200 nM siRNA and 31 $\mu\text{g ml}^{-1}$ LPEI (N/P = 90), the siRNA appears to be completely bound, i.e., no free siRNA appears in the gel (lane 8). Therefore the gel suggests that all the siRNA incorporated into the nanoparticles at N/P ratio greater than 75 and near 90.

3.1.2. Ultraviolet/visible (UV/vis) spectroscopy—We observed a maximum peak for LPEI in DI water at 240 nm for all the concentrations used in this study, without siRNA or any additional components (Fig. 3a). Note that this is different from previous spectrophotometric methods that detected for PEI, where the PEI was either complexed with copper [45], or a protein-based assay was used [46]. We observed a LPEI peak at 244 nm when suspended in OptiMEM buffer (a buffer used for transfection studies), without siRNA or any additional components (Fig. 3b). The difference in the LPEI peak in water vs. in buffer could be due to the presence of salts in the buffer solution, and the resulting change in effective dielectric function [47] of the LPEI-buffer solution from that of the LPEI-water solution.

We measured the UV/vis absorbance of the LPEI-siRNA nanoparticles as a function of increasing N/P ratios. The peak absorbance wavelength of the LPEI-siRNA nanoparticles underwent a linear blue shift away from 260 nm (characteristic peak of nucleic acid, Fig. 3c inset) as the LPEI concentration increased. The peak gradually shifted towards lower wavelengths with increasing N/P ratios; shifting to 256 nm at N/P ratios of 5, and 244 nm at N/P ratio of 75 and higher (Fig. 3c,d). The plasmon band of the nanoparticles at N/P ratio of 75 (and higher ratios) was the same as the plasmon band for LPEI without siRNA in OptiMEM buffer solution. Indeed this gradual UV shift has been observed with bimetallic “core-shell” and alloy nanoparticles. Mallik et al. [48] showed that progressive covering or encapsulation of gold particles by silver layers resulted in a UV/vis blue shift. As the silver covered the gold, the plasmon band was silver dominated. Similarly, core-shell type silver-gold alloy nanoparticles showed a red shift with a single intermediate absorbance peak as concentration of the gold in the nanoparticles increased [49]. Their results indicated that the peak shift depended on the ratio of the two metals. Based upon these literature results and our gels results, we suggest that the gradual shift in the UV peak correlate with the increasing incorporation of the siRNA into the nanoparticles, resulting in less free or naked siRNA with increasing amount of LPEI at the higher N/P ratios (at a constant siRNA concentration), reaching complete incorporation of the siRNA at N/P ratio greater than 75.

3.1.3. Particle size analysis—Scanning electron microscopy (SEM) images were taken for N/P ratios from 5 to 90 (Fig. 4a). Several of the sizes were further confirmed with atomic force microscopy (AFM) (Supplementary information, Fig. S1). Overall, the size of the nanoparticles decreased with increasing N/P ratios, with significant changes in size for N/P ratios in the range of 5 to 15 and 60 to 75. The size of the nanoparticles was greater than 200 nm at N/P ratio of 5, decreased to ~150 nm at N/P ratios between 15 and 60, decreased further to less than 100 nm at N/P ratio of 75, and ~50 nm at N/P ratio of 90. The hydrodynamic sizes of these nanoparticles were measured using dynamic light scattering (DLS) (Fig. 4b), and found to be similar to the sizes obtained with SEM and AFM.

3.1.4. Zeta (ζ)-potential analysis— ζ -potential was measured to assess the relative charge of the nanoparticles at the different N/P ratios. As more siRNA was incorporated into the nanoparticles, the ζ -potential shifted to more positive values (Fig. 5). A negative ζ -potential value was obtained at N/P ratio of 5 (~ -10 mV) with its magnitude less than that of pure naked siRNA (~ -30 mV). This suggests that most of the siRNA remain in free form at low N/P ratio of 5, which is corroborated by the agarose gel (Fig. 2). N/P ratio of 15 shifted the ζ -potential from negative to positive, suggesting more siRNA incorporated into the nanoparticles than at N/P ratio of 5. Further increase in N/P ratios to between 30 and 75 increased the ζ -potential to 20–25 mV, suggesting enhanced siRNA incorporation. At N/P ratio of 90 the ζ -potential reached ~ 30 mV, similar to the ζ -potential of pure LPEI at an equivalent LPEI concentration to N/P ratio of 90. The ζ -potential measurements corresponded with the UV/vis and agarose gel electrophoresis results indicating complete incorporation of the siRNA at N/P ratio near 90.

ζ -potential was also measured immediately after the stamped nanoparticles were released from the multilayers at 37°C. ζ -potential values were negative for N/P ratios ≤ 60 , and positive for N/P ratios of 75 and 90. In addition to the nanoparticles, PAA and PEG were released upon multilayer degradation, presenting the possibility of further interaction of PAA or PEG with the released nanoparticles. The presence of PAA itself in the solution or PAA coating of the nanoparticles, or PEG followed by PAA coating of the nanoparticles, could result in negative ζ -potential at the lower N/P ratios (5 to 60). Interestingly, enhanced gene silencing was observed with MFT, as compared to NFT, for N/P ratio of 5 (Fig. 7), which may be indicative of further PAA or PEG interaction with the released nanoparticles (discussed further in Section 3.5). Such an increase in MFT efficiency was absent at N/P ratios of 15 to 90, suggestive of the presence of PAA or PEG in solution with minimal interaction with the nanoparticles. These N/P ratios (5 to 60) gave negative ζ -potentials. The positive ζ -potential values at the higher N/P ratios of 75 and 90, albeit slightly lower than that obtained with the “direct complexes”, suggested minimal coating, if any, of these nanoparticles or that the higher LPEI concentration minimized the effect of PAA and PEG in solution.

3.2. Multilayer mediated forward transfection (MFT) for patterned siRNA delivery, and effect of the number of bilayers

For MFT, the multilayer substrate containing the nanoparticles was positioned over a confluent monolayer of cultured mammalian cells. The physiological pH of the culture medium resulted in the disintegration of the multilayer and release of the nanoparticles from the multilayer and delivery to the cells. Figs. 6 and S2 (Supplementary information) show the patterned delivery to cells via MFT. Placing the substrate on top of cells was not detrimental to the health of the cell. This was evident from the cell images in Figs. 6, S2, S3, and from the high yields of total RNA extracted from the cells after transfection (see qRT-PCR characterization in Section 3.3 for RNA extraction process; RNA yield data not shown). A similar procedure of placing the substrate over the cultured cells has also been previously demonstrated by Lynn and co-workers, where the authors show the non-patterned localized DNA delivery from a LbL assembly [21].

The degradation kinetics of PAA/PEG multilayers reported by Ono and Decher [26] showed that less than 7 bilayers of PAA/PEG did not release the upper films as self-standing, floating films; however bilayers greater than 7 released the upper films within 30 min. Here, we find the release of the nanoparticles for patterned delivery and transfection efficiencies were independent of the number of bilayers. We evaluated patterned delivery and quantified MFT efficiencies (quantification discussed in Section 3.3) for 6.5 bilayers (Figs. 6 and 7b) and 30.5 bilayers of multilayers (Figs. 7b and S2) and found they were similar. Since stamping of the nanoparticles onto (PAA/PEG)_{n,5} multilayers formed only an additional monolayer of particles

(instead of a complex film), this could explain the thickness-independent release of the nanoparticles from the multilayers.

To confirm that the transfection is due to the release of the complexes upon degradation of the film and subsequent cellular uptake of the complexes, we evaluated a plasma-treated bare quartz substrate (negatively charged) which should be similar to a non-degrading multilayer; both are non-degrading and bind the complexes to their surfaces. We stamped the complexes onto the plasma-treated quartz substrate, and observed no transfection after 48 h, suggesting that the complexes were not released and did not penetrate into the cell merely through cell/complex interaction (Fig. S3).

3.3. Evaluation of transfection efficiency: real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis

To evaluate the transfection efficiencies of MFT, non-patterned PDMS stamps were used to stamp nanoparticles containing custom-designed siRNA targeting the double-stranded RNA-dependent protein kinase (PKR) gene. MFT efficiencies with LPEI (at different N/P ratios) and LF2k as transfection reagents were compared with those of NFT using LPEI, BPEI (at different N/P ratios) and LF2k as transfection reagents (Fig. 7a,b).

NFT efficiency using LF2k with 33 nM siRNA gave more than 80% silencing. Gene silencing was not observed for LPEI with 33 nM of siRNA (at the highest N/P ratio of 90). In support, a previous study demonstrated that to achieve sufficient silencing, 200 nM of siRNA was required with BPEI at N/P ratios of 6 and 8 [33]. Therefore, we selected a siRNA concentration of 200 nM for the gene knockdown evaluations with LPEI. There was no NFT observed with LPEI at N/P ratios of 5, 40% silencing at N/P ratio of 15, and more than 80% silencing at N/P ratios of 30 and higher (Fig. 7a). BPEI, similar to LPEI, showed no silencing at a N/P ratio of 5; however, its transfection efficiency was better than LPEI for higher N/P ratios. BPEI at N/P ratio of 10 gave similar while BPEI at N/P of 15 gave higher level of silencing as compared with LPEI at N/P ratio of 15. Conversely, BPEI was more cytotoxic than LPEI at these N/P ratios (see Section 3.4).

MFT efficiencies were lower than NFT at similar N/P ratios for the LPEI-siRNA nanoparticles. This reduction in efficiency may be due to either the loss of nanoparticles during the stamping process, with some of the nanoparticles sticking to the stamp and not transferring to the multilayer, or potential dissociation of the nanoparticles, i.e., LPEI sticking to the PDMS and exposing the nucleic acids. This reduced efficiency was also evident with the stamping of the LF2k-siRNA nanoparticles (used 40 pmol siRNA $\equiv$ 33 nM in NFT, a standard concentration used with LF2k [44]) in the MFT process (Fig. 7a,b). To assess whether the preparation time was a factor, the NFT efficiency of LF2k-siRNA complexes (typical NFT) was evaluated after 20 min (standard preparation time of LF2k-siRNA complexes [44]) and 2.5 h (average process time of MFT), and found to be similar. The large standard deviations associated with MFT may be attributed to the experimental variation introduced through the manual (non-automated) transfer of the nanoparticle particles by the μ CP process. MFT of siRNA can be performed with any transfection reagent, as long as the nanoparticles remained stable and functional throughout the stamping process. MFT can be performed on any cell type, in addition to the ones described here.

MFT efficiencies were $\geq 60\%$ for LPEI-siRNA nanoparticles at N/P ratios ≥ 30 . When compared to NFT efficiencies, there was an unexpected increase observed in MFT efficiency for nanoparticles at N/P ratio of 5. This may be attributed to interaction of the larger nanoparticles (greater than 200 nm) with the PEG and PAA upon multilayer degradation in the culture medium, enhancing transfection at N/P ratio of 5 (discussed further in Section 3.5).

The small amount of silencing observed with “LPEI (N/P 45) Only” mock sample in Fig. 7a,b is essentially noise in the qRT-PCR characterization.

3.4. Cytotoxicity evaluation: transfection reagent and N/P ratio optimization

Cytotoxicity measurements along with transfection efficiencies were used to determine the optimum N/P ratio and transfection reagent for NFT and MFT. A good transfection reagent must provide high transfection efficiency with minimal cytotoxicity. LDH release was used to assess the cytotoxicities of the LPEI-siRNA, BPEI-siRNA and LF2k-33nMsiRNA complexes during NFT (Fig. 8).

The cytotoxicity level for BPEI-siRNA N/P ratio of 10 was much higher than for LPEI-siRNA nanoparticles at N/P ratios from 5 to 30. Increasing the BPEI-siRNA N/P ratio to 15 increased cytotoxicity (Fig. 8) as well as transfection efficiency (Fig. 7a), but the transfection efficiency was still lower than for LPEI-siRNA nanoparticles at N/P of 30. Within a narrow range of N/P ratio (less than 10) evaluated previously [33], BPEI was reported to provide higher transfection efficiency than LPEI. Our results are similar to previous findings at the low N/P ratio range; however, at a high N/P ratio of 30, LPEI provided higher transfection efficiency and lower cytotoxicity than BPEI at the low N/P ratio of 10 (Figs. 7 and 8). Therefore, LPEI at N/P ratio of 30 was deemed a better choice over BPEI, based on transfection efficiencies and cytotoxicities. However, LPEI if added to cells at very high concentrations, even in the absence of siRNA, is toxic to the cells. Therefore, as the N/P ratios increases greater than 45, i.e., as more LPEI is added, the observed toxicity increases, as expected.

The cytotoxicity of LPEI-siRNA nanoparticles at N/P ratio of 30 was found to be comparable to that obtained with the LF2k-33nMsiRNA complexes (Fig. 8). However, LPEI at N/P ratio of 30 was a better choice than LF2k for MFT, since lower siRNA amounts cannot provide high MFT (Fig. 7b), and to increase the siRNA amounts in the LF2k-siRNA complexes would require higher amounts of LF2k, which would further enhance the toxicity (Fig. 8).

3.5. Relationships between degree of siRNA incorporation, ζ -potential, size and transfection efficiencies of LPEI-siRNA nanoparticles

Based on the UV/vis and agarose gel electrophoresis results, the plasmon peak shifted to the blue region, as the concentration of LPEI increased, and reached that of pure LPEI when all the siRNA incorporated into nanoparticles and no free siRNA remained. The average nanoparticle size decreased as the N/P ratio increased. Nanoparticle size greater than 200 nm with negative ζ -potential may be inferred to have less siRNA incorporated given the large amount of unbound siRNA in lane 1 of the agarose gel (Fig. 2) and the maximum UV/vis peak was at 256 nm (Fig. 3c). This was associated with no gene silencing (N/P ratio 5). Nanoparticle size ~150 nm with positive ζ -potential between 20 and 25 mV may be inferred to have more siRNA incorporated since lanes 2–7 of the gel show less unbound siRNA and the position of the UV/vis peak shifted to intermediate wavelengths. Complete siRNA incorporation into nanoparticles may be inferred at nanoparticle sizes ≤ 50 nm with ζ -potential ≥ 30 mV (similar to the ζ -potential of pure LPEI at an equivalent concentration) since lane 8 of the gel shows no free siRNA and the UV/vis peak shifted to 244 nm, which is the maximum peak for LPEI. A possible explanation for the decreasing size of the nanoparticles could be that at increasing amounts of LPEI and a fixed amount of siRNA (i.e., increasing N/P ratios), there may be more electrostatic repulsion between the LPEI molecules at a given level of electrostatic attraction between LPEI and siRNA, thus contributing to smaller sized particles.

Correspondingly, minimal silencing was observed at N/P ratio of 15 and greater than 80% gene silencing was observed for N/P ratios ranging from 30 to 75. Transfection efficiency was increased further at N/P ratio 90. Therefore, the smaller size nanoparticles, with 25kDa LPEI,

provided more efficient NFT efficiencies. Particle size of ~150 was observed to be the cut-off size for efficient cellular uptake, which agreed with previously reported size limit of 150 nm for non-specific cellular uptake through clathrin-coated pits [42]. N/P ratio of 15 was an exception: despite their particle size of 150 nm, they gave lower transfection as compared with the higher N/P ratios from 30 to 60 (Fig. 7a). However, the smaller blue shift and low ζ -potential at N/P ratio of 15 could explain, in part, the reduced transfection efficiency.

A possibility exists that some of the small nanoparticles observed at the high N/P ratios are aggregates of LPEI alone in solution. However, since we observed high level of silencing at these N/P ratios, this coupled with the fact that nanoparticles larger than 150 nm are reported unable to provide siRNA silencing [33], we believe the small size LPEI nanoparticles contain siRNA molecules and are not likely aggregation of LPEI.

The lower ζ -potential values for the nanoparticles released from the multilayers suggest that the released PAA or PEG from the film may also interact with LPEI-siRNA nanoparticles. Increase in transfection efficiencies at N/P ratio of 5 is observed for MFT as compared to NFT. Amino and carboxylic acid pendant PEG chain coating on PEI/DNA complexes have been shown to increase their transfection efficiencies, even at negative ζ -potentials [50]. Also, PEG is known to reduce nanoparticle aggregation [41,51], modulating the complex properties (i.e., surface charge, size, and complex-cell interactions) leading to improved transfection efficiencies in some cases [51,52]. These previous studies support the possibility that PAA or PEG could alter the properties of larger nanoparticles (greater than 200nm) in solution to enhance the MFT efficiency at N/P ratio of 5.

4. Conclusions

The MFT method provided a forward transfection approach that used degradable multilayers for patterned siRNA delivery to cultured cells. Forward transfection provides an advantage over reverse transfection in terms of tuning the substrate chemistry, such as the multilayers for polymer-siRNA immobilization, separately from the substrate modification that may be required for cell adhesion. Lower siRNA amounts (~40 pmol $\equiv$ 33 nM siRNA in NFT) did not provide sufficient MFT efficiencies, whereas higher siRNA amounts (~240 pmol $\equiv$ 200 nM siRNA in NFT) provided $\geq 60\%$ transfection efficiencies of LPEI-siRNA nanoparticles at N/P ratios greater than 30. Therefore, based on the cytotoxicities and transfection efficiencies, LPEI at N/P ratio of 30 was a better choice than LF2k for MFT, even though they both gave comparable NFTs. We selected and characterized N/P ratios ranging from 5 to 90 for their UV/vis spectra, relative amounts of siRNA incorporated into the nanoparticles, ζ -potentials and nanoparticle sizes. UV/vis shift to 244 nm, ζ potential of 30 mV, and complete siRNA incorporation was achieved at N/P ratio near 90. NFT efficiencies increased with decreasing nanoparticle size and increasing N/P ratio. 25 kDa LPEI was found to be a highly efficient transfection reagent at N/P ratio of 30, providing greater than 80% transfection efficiencies at siRNA concentration of 200 nM, better than BPEI. Finally, MFT provides a method for forward transfection of siRNA, yielding micron-sized patterns of transfected mammalian cells, and may be a potential approach for developing cell microarrays based on forward transfection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Diagram illustrating the multilayer mediated forward transfection (MFT) of cationic vector complexed siRNA for patterned delivery.

Fig. 2. Agarose gel electrophoresis of LPEI-siRNA nanoparticles at various N/P ratios with 200 nM siRNA concentration. Numbers indicate the N/P ratios (0 N/P ratio indicates the naked or free siRNA).

Fig. 3.

Ultraviolet/visible (UV/vis) absorption spectra of LPEI suspended at varying concentrations in: (a) DI water, or (b) OptiMEM buffer, without siRNA, showed maximum peaks at 240 nm and 244 nm, respectively. (“LPEI concentration ($\equiv$ N/P ratio)” corresponds to the concentration of LPEI for a given N/P ratio calculated for siRNA final concentration of 750 nM.) Same peak positions were observed for all the other LPEI concentrations used in the range for nanoparticle fabrication. (c) UV/vis absorption spectra of LPEI-siRNA nanoparticles show a blue shift with increasing N/P ratios. (Inset: Maximum UV/vis peaks of siRNA and LPEI in OptiMEM are 260 nm and 244 nm, respectively.) (d) Plot of the wavelengths corresponding to the absorbance peak of LPEI-siRNA nanoparticles as a function of the N/P ratio.

(a)

(b)

Fig. 4.

Variation in the size of LPEI-siRNA nanoparticles as a function of N/P ratio. (a) Scanning electron microscopy (SEM) images of the nanoparticles and their sizes as a function of N/P ratio. Scale bar represents 500 nm, and the error bars indicate the standard deviations of at least ten measurements performed on three different scanned areas per sample. (b) Histograms of DLS determined nanoparticle sizes for N/P ratios of 5, 45, 60 and 75 show a decrease in the average particle size with increasing N/P ratio.

Fig. 5.

Zeta-potential values of LPEI-siRNA nanoparticles as a function of N/P ratio, immediately after they were formed (direct complexes) and released upon multilayer degradation (multilayer released complexes) in deionized water, measured at 25°C. “LPEI (N/P90) only” corresponds to the amount of LPEI for N/P ratio of 90 without siRNA. Error bars indicate the standard deviation of three measurements of ten runs per sample.

Fig. 6.

Fluorescent images demonstrating patterned siRNA delivery to HeLa cells with multilayer mediated forward transfection (MFT) using (PAA/PEG)_{6,5} multilayer assembly, fluorescent dsRNA oligomers (100 pmol) and Lipofectamine 2000 (LF2k, 5 μ g). Nanoparticles and HeLa cell patterns transfected with: (a) Alexa Fluor 555-labeled oligomers, (b) Fluorescein and Alexa Fluor 555-labeled oligomers (overlaid images). Top panel: CLSM images of LF2k-fluorescent oligomer nanoparticles arrayed onto multilayer. Middle and bottom panels: HeLa cell patterns transfected with fluorescent oligomers and their corresponding phase contrast images acquired using CLSM (middle panel) and conventional fluorescence microscopy (bottom panel). Scale bar represents 500 μ m.

(a)

(b)

Fig. 7.

qRT-PCR quantified expression levels of PKR gene knockdown in HeLa cells 48 h post-transfection of siRNA delivery using 25 kDa LPEI or 25kDa BPEI at different N/P ratios, or Lipofectamine 2000 (LF2k, 2 μ g) with: (a) normal forward transfection (NFT) and (b) multilayer mediated forward transfection (MFT). Amount of siRNA was 240 pmol ($\equiv$ 200 nM final concentration in NFT) and transfection reagent was LPEI, unless specified otherwise. Numbers in parentheses denote the amount of LPEI corresponding to the noted N/P ratio of 45, but without siRNA. BLs denote the number of bilayers. Error bars indicate the standard deviations of RT-PCR reactions on three independent samples. #, ## $p < 0.01$ as compared with “cells only” (i.e. cells without any transfection reagent or siRNA). ### $p < 0.05$ as compared with “cells only”. * $p < 0.05$ compared with “N/P 5 in Fig. 7a”.

Fig. 8.

Cytotoxicity levels in HeLa cells 48 h post-NFT of siRNA delivery using 25 kDa LPEI or 25 kDa BPEI at different N/P ratios, or Lipofectamine 2000 (LF2k, 2 μ g). Concentration of siRNA was 200 nM and transfection reagent was LPEI, unless specified otherwise. Numbers in parentheses denote the amount of LPEI or BPEI corresponding to the amount of PEI at the noted N/P ratio but without siRNA. Error bars indicate the standard deviations of %LDH release of three independent samples. * $p < 0.01$ compared with “N/P 45” and “BPEI-N/P 10”.