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Reductively Responsive siRNA-Conjugated Hydrogel Nanoparticles for Gene Silencing

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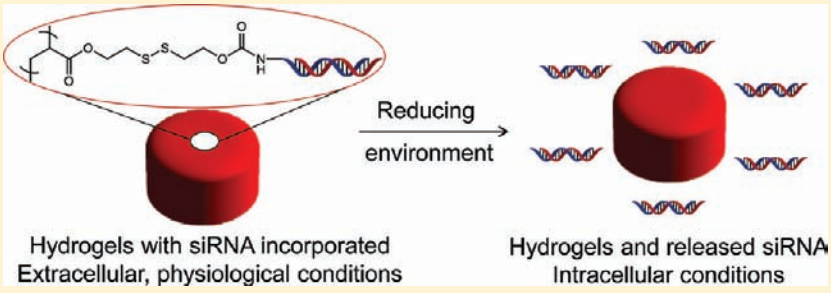
Supporting Information



ABSTRACT: A critical need still remains for effective delivery of RNA interference (RNAi) therapeutics to target tissues and cells. Self-assembled lipid- and polymer-based systems have been most extensively explored for transfection with small interfering RNA (siRNA) in liver and cancer therapies. Safety and compatibility of materials implemented in delivery systems must be ensured to maximize therapeutic indices. Hydrogel nanoparticles of defined dimensions and compositions, prepared via a particle molding process that is a unique off-shoot of soft lithography known as particle replication in nonwetting templates (PRINT), were explored in these studies as delivery vectors. Initially, siRNA was encapsulated in particles through electrostatic association and physical entrapment. Dose-dependent gene silencing was elicited by PEGylated hydrogels at low siRNA doses without cytotoxicity. To prevent disassociation of cargo from particles after systemic administration or during postfabrication processing for surface functionalization, a polymerizable siRNA pro-drug conjugate with a degradable, disulfide linkage was prepared. Triggered release of siRNA from the pro-drug hydrogels was observed under a reducing environment while cargo retention and integrity were maintained under physiological conditions. Gene silencing efficiency and cytocompatibility were optimized by screening the amine content of the particles. When appropriate control siRNA cargos were loaded into hydrogels, gene knockdown was only encountered for hydrogels containing releasable, target-specific siRNAs, accompanied by minimal cell death. Further investigation into shape, size, and surface decoration of siRNA-conjugated hydrogels should enable efficacious targeted in vivo RNAi therapies.

INTRODUCTION

Gene silencing via RNA interference (RNAi)[1,2] has demonstrated great potential for the treatment of diseases[3,4] by halting the production of target proteins. The major challenge in realizing the potential of RNAi therapies resides in delivering small interfering RNA (siRNA) effectively to the cytoplasm of a target cell. With a highly negatively charged backbone and a

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molecular weight of ca. 13 kDa, siRNA is unable to effectively cross cell membranes without assistance. Additionally, siRNA is susceptible to degradation by ubiquitous RNases in serum. A suitable carrier is required to enhance stability and facilitate delivery to the cytoplasm of cells. Exemplar carriers include oligonucleotide conjugates,[5−14] polyplexes,[8,15−18] and lipoplexes.[19−22] After systemic administration, the siRNA carrier encounters several biological hurdles en route to the target tissue and cell such as clearance by the reticuloendothelium system, protein fouling, and size requirements to reach particular tissues. Designing delivery vehicles with surface decorations including stealthing (e.g., polyethylene glycol, PEG[23]) and targeting (e.g., peptide[13]) ligands may promote prolonged circulation and passive delivery to tissues of interest followed by actively targeting cell surface receptors for internalization by desired cells.

Hydrogels and nanogels have been explored as delivery vector candidates for transfection of cells with siRNA. Effective gene knockdown in kidney, epithelial, ovarian, and hepatoma cell lines has been achieved by siRNA-associated hydrogels and nanogels.[24−28] Due to their robust and tunable mechanical properties, hydrogel micro- or nanoparticles may enable delivery of siRNA to a wide range of tissues in vivo including delivery to circulating cells. Bottom-up approaches for encapsulating siRNA in nanogels through electrostatic attraction postfabrication may result in dynamic association of cargo, uncontrollable cargo release, and modification of particle surface properties. Particle replication in nonwetting templates (PRINT) technology allows for fabrication of hydrogels with control over size, shape, composition, surface chemistry, and modulus such that delivery properties may be tuned to particular applications.[29−36] Encapsulation of siRNA in hydrogels may be engineered with PRINT technology, which allows for direct physical entrapment or covalent incorporation of siRNA during particle fabrication.

RESULTS AND DISCUSSION

Transfection of Cancer Cells with siRNA Electrostatically Entrapped in Hydrogels. Initially, a highly cationic, moderately cross-linked hydrogel composition (Table S1, Supporting Information (SI)) was pursued to allow for physical entrapment and electrostatic association of siRNA within cylindrical (diameter [d] = 200 nm; height [h] = 200 nm) PRINT particles prepared by a film-split technique. To promote cytocompatibility and dispersibility of highly cationic hydrogel nanoparticles in aqueous media, amine handles on hydrogels were reacted with succinimidyl succinate monomethoxy PEG_{2K} (Figure 1a). After PEGylating the hydrogels, a concomitant decrease in the ζ-potential was observed (Table S2 (SI)), resulting in a low surface charge that would be minimally toxic to cell membranes. Steady release of siRNA from the hydrogel particles in PBS at 37 °C was observed over time, reaching maximum concentration around 48 h (Figure 1b). By gel electrophoresis, siRNA loading was determined to be about 1.4 wt %, and encapsulation efficiency of siRNA in hydrogels was determined to be ca. 28% (1.4 wt % final siRNA loading in particles relative to the 5 wt % siRNA charged into the composition of the preparticle solution). Scanning electron microscopy (SEM) analysis of the hydrogel particles demonstrates their cylindrical shape and dimensions (Figure 1c).

To evaluate the transfection potential of particles loaded with siRNA, a stably transfected, luciferase-expressing human cervical cancer (HeLa/luc) cell line was utilized for in vitro

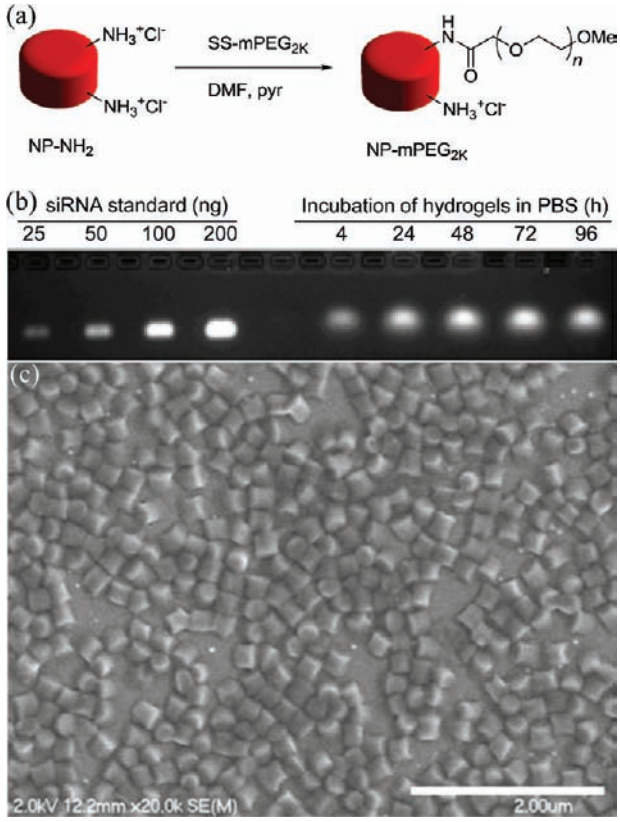

Figure 1. (a) Reaction scheme for PEGylation of hydrogels with succinimidyl succinate monomethoxy PEG_{2K} (SS-$mPEG_{2K}$), (b) time-dependent release of siRNA from particles incubated at 2 mg/mL and 37 °C in PBS, and (c) scanning electron micrograph (SEM) of particles illustrating their 200 × 200 nm cylindrical dimensions (scale bar = 2 μm).

studies. Particles were dosed on HeLa cells for 4 h followed by 72 h incubation. Because of the positive charge of the particles, the PRINT hydrogel particles were readily internalized into the HeLa cells (Figure 2a) as determined by flow cytometry. Dose-dependent knockdown of luciferase expression (Figure 2b) was observed for HeLa cells incubated with the antiluciferase siRNA-charged particles with a half-maximal effective concentration (EC_{50}) of ca. 6 nM siRNA. Conversely, PRINT hydrogel particles charged with a control siRNA sequence did not elicit gene knockdown, implying that transfection was sequence-specific (Figure 2b). Additionally, both particles were found to be cytocompatible with HeLa cells (Figure S1 (SI)). Confocal microscopy of HeLa cells dosed with PEGylated particles (Figure 2c,d) shows the internalization of fluorescent particles and their distribution throughout the cellular cytoplasm as well as the perinuclear region. Even though the desired cell activity was achieved with these particles, subsequent efforts to modulate in vivo behavior by conjugation of targeting ligands to the particle surface resulted in extensive premature release of siRNA. For example, 50% loss of encapsulated cargo was observed during a sequence of reactions to add targeting moieties to the particle surface (Figure S2 (SI)).

siRNA Prodrug for Triggered Release from Hydrogels. In order to combat the premature release issues with the PRINT hydrogel particles described above, an alternative prodrug strategy was employed, which involved covalently conjugating the siRNA directly to the PRINT hydrogel

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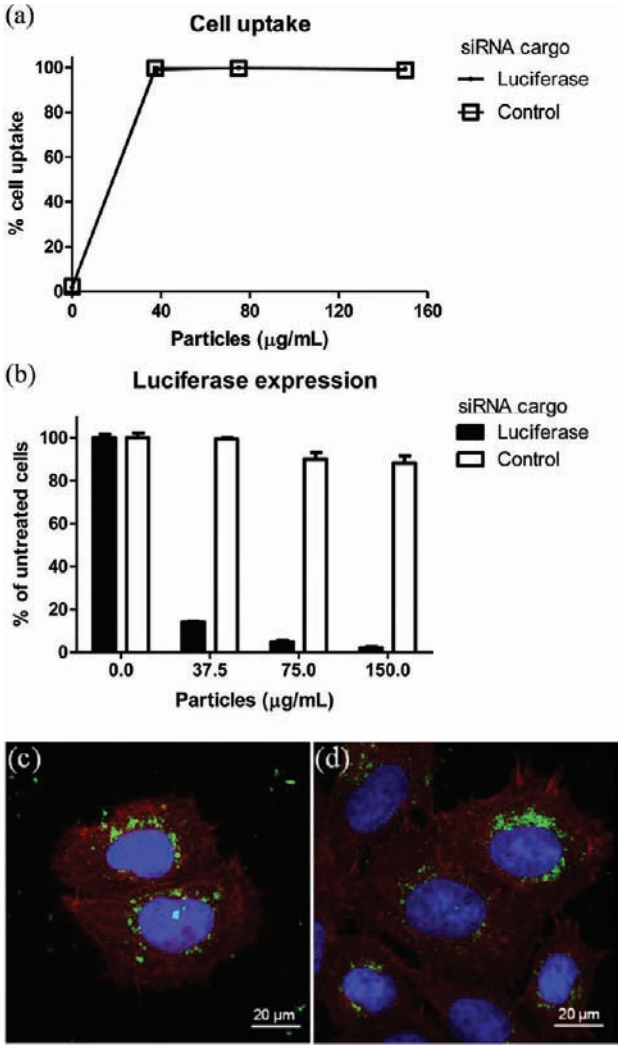

Figure 2. siRNA delivery with PEGylated cationic hydrogels to luciferase-expressing human cervical cancer (HeLa/luc) cells. (a) Cellular uptake. HeLa/luc cells were dosed with particles for 4 h followed by trypan blue treatment and flow cytometry analysis. (b) Luciferase expression. HeLa/luc cells were dosed with particles for 4 h followed by removal of particles and 72 h incubation in media. Data are representative of at least three independent experiments. The error bars represent standard deviation from triplicate wells in one experiment. (c,d) Confocal micrographs. HeLa/luc cells were dosed with 50 μg/mL of particles containing (c) luciferase or (d) control siRNA cargos for 4 h. Cellular actin cytoskeleton was stained with phalloidin (red) and nuclei with DAPI (blue), while particles (green) were labeled with the fluorescent monomer fluorescein *O*-acrylate during particle fabrication.

particles. Acrydite DNA oligonucleotides have been incorporated into hydrogels for nucleic acid hybridization assays,[37,38] while a CpG oligonucleotide methacrylamide has been copolymerized into acid-degradable microparticles to invoke immune responses.[39,40] For the triggered release of therapeutic conjugates from particles or delivery vectors under biologically relevant conditions, acid-labile,[41−43] enzymatically degradable,[44,45] and redox-sensitive linkages[46] have been examined. Glutathione and reducing enzymes are present at high concentrations inside cells relative to the extracellular space in normal and cancer cell lines.[47] Considering the serum stability of disulfide conjugates,[46,48] cleavage of disulfide linkages in therapeutic conjugates may selectively occur in the intracellular reducing environment. Disulfide−siRNA conjugates to polymers and lipids have been previously reported[6,7,10−12] as reductively labile systems.

In this work, siRNA was derivatized with a photopolymerizable acrylate bearing a degradable disulfide linkage for reversible covalent incorporation into the PRINT hydrogel nanoparticles (Figure 3a). In the designed "pro-siRNA

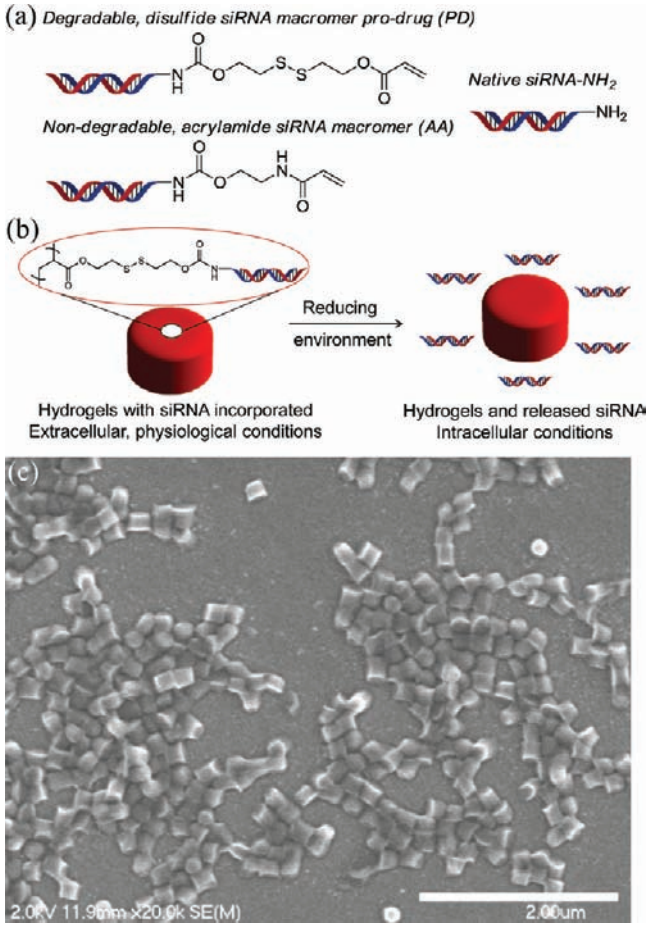

Figure 3. (a) Structures of degradable and nondegradable siRNA macromers as well as native siRNA, (b) illustration of pro-siRNA hydrogel behavior under physiological and intracellular conditions, and (c) SEM micrograph of pro-siRNA, 200 × 200 nm cylindrical nanoparticles (scale bar = 2 μm).

hydrogels", it was envisioned that the siRNA cargo would be retained in the particle until entry of the particle into the cytoplasm of a cell, where the disulfide linkage would be cleaved in the reducing environment, allowing for release and delivery of the siRNA (Figure 3b). A water-based preparticle composition containing the disulfide siRNA pro-drug and a higher content of hygroscopic, liquid monomers (Table S3 (SI)) was applied to fabricate cylindrical (d = 200 nm; h = 200 nm) loosely cross-linked cationic PRINT hydrogel particles using a film-split technique. SEM micrographs confirmed the dimensions and shape of cylindrical siRNA-containing particles (Figure 3c).

To study the response of pro-siRNA hydrogels to a reducing environment, nondisulfide acrylamide siRNA-based hydrogel particles and native siRNA-complexed particles were prepared as controls (Figure 3a). Release of native siRNA−NH_2 occurred rapidly from these porous, loosely cross-linked

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hydrogels (Figure S4 (SI)). Figure S5 (SI) showed that different siRNAs were loaded into hydrogel particles and remained associated with particles directly after harvesting. Unconjugated siRNA was found in hydrogels charged with native siRNA or siRNA macromers due to incomplete conversion; therefore, in the following experiments all particles were extensively washed with 10× PBS to remove the sol fraction containing free siRNA. The time-dependent release of the siRNA from the pro-drug PRINT hydrogel particles was evaluated under physiological and reducing conditions (Figure 4a). The siRNA was retained in the hydrogel particles over 48 h

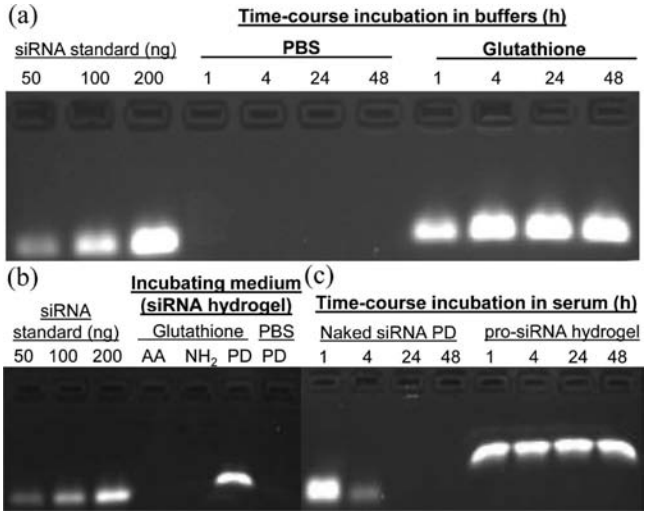

Figure 4. Release profiles and stability of siRNA in 30% AEM-based hydrogels. All hydrogels were washed with 10× PBS buffer to remove the sol fraction containing unconjugated siRNA before release studies were performed. (a) Time-dependent incubation of pro-siRNA hydrogels (1 mg/mL) in PBS and under reducing conditions (glutathione, 5 mM) at 37 °C. (b) Selective release of the disulfide-coupled siRNA prodrug (PD) from hydrogels under reducing conditions compared to the acrylamide (AA) macromer and native siRNA (NH_2). Hydrogels were incubated in 10× PBS with or without 5 mM glutathione for 4 h at 1 mg/mL and 37 °C. (c) Retention of siRNA integrity when conjugated to hydrogels after exposure to 10% fetal bovine serum (FBS) over time. Naked siRNA PD macromer was incubated at 36 μg/mL in 10% FBS for given times, proceeded by storage of solution. Pro-siRNA hydrogels were incubated at 1.2 mg/mL in 10% FBS at 37 °C for given times followed by incubation in 10× PBS (5 mM glutathione) for 4 h at 1.2 mg/mL and 37 °C to release siRNA. Differences in siRNA migration observed in gels among the standards and samples that were released from hydrogels incubated in PBS and 10× PBS may arise from the differences in salt concentrations of sample solutions.

at 37 °C in PBS while the siRNA was quickly released from hydrogels when incubated in a reducing environment (5 mM glutathione), reaching maximum concentration around 4 h (Figure 4a).

Moreover, the siRNA conjugate did not even leak out of the hydrogel particles when they were exposed to high salt concentration buffer (10× PBS) at 37 °C for 4 h (Figure 4b). Incubation of the nondisulfide, nondegradable acrylamide control siRNA hydrogel particles did not result in release of the siRNA under reducing conditions as expected. Stability of the siRNA covalently conjugated to the PRINT hydrogel particles was tested by incubation of the particles in serum (10% FBS) as a function of time. Over 48 h, the siRNA in the pro-drug PRINT hydrogel particles could be protected from degradation by RNases when incubated in serum, while siRNA in the form of the simple macromonomer in the absence of the particle to protect it was rapidly degraded in serum under the same conditions (Figure 4c).

Pro-siRNA Hydrogels for Gene Silencing. The PRINT particles were designed to have a positive zeta potential to facilitate cell internalization and endosomal escape by including an amine monomer (AEM, 2-aminoethylmethacrylate hydrochloride). It is known that excessive amine content in hydrogels may disrupt and destroy the plasma membrane, eliciting cell death. Conversely, an insufficient amine content may not enable efficient cell uptake and endosomal escape for transfection. To optimize cytocompatibility and gene silencing efficiency of pro-siRNA hydrogels, the AEM content was varied from 5 to 50 wt % (Table S3 (SI)). Pro-siRNA hydrogels were not PEGylated as were siRNA-complexed hydrogels whereby the cytocompatibility of bare particles would be determined. For systemic delivery of particles in vivo, sheddable coatings that reveal bare particles are attractive to enable targeting and endosomal escape for effective gene delivery.[49,50]

ζ-potentials of cationic hydrogels increased with amine content and the *Z*-average diameters (D_z) of the resultant particles ranged from 250 to 350 nm (Table 1). Encapsulation

Table 1. Zetasizer Analysis of Pro-siRNA Hydrogels with Variable Amine Content

amine content (wt %)	ζ-potential (mV)	D_z (nm)
5% AEM	+18.2 ± 0.5	350.2 ± 5.4
20% AEM	+22.6 ± 0.1	281.6 ± 1.9
25% AEM	+27.1 ± 0.3	307.4 ± 6.6
30% AEM	+27.9 ± 1.5	324.3 ± 5.6
40% AEM	+30.6 ± 1.0	281.3 ± 6.0
50% AEM	+34.1 ± 0.4	253.7 ± 3.4

of the siRNA in the hydrogel PRINT particles reached a roughly constant value once the amine content was greater than or equal to 20 wt % (Figure S6 (SI)). The encapsulation efficiency was determined to be ca. 35% for AEM contents ≥20 wt %, while when only using 5% AEM, the encapsulation was lower (ca. 15%). When the pro-drug, disulfide-containing siRNA hydrogel PRINT particles were dosed onto luciferase-transfected HeLa cells (HeLa/luc) for 5 h followed by 48 h incubation at 37 °C, dose-dependent knockdown of the luciferase expression was observed (Figure 5a) for hydrogels with amine contents greater than 5 wt % AEM. Cytocompatibility was maintained at the lower amine contents and dosing concentrations (Figure 5b). It appeared that the 30% AEM-containing PRINT hydrogel particles provided the ideal combination of gene silencing efficiency (EC_{50} ~ 20 nM siRNA) and cytocompatibility (even at high dosing concentrations).

To further investigate the in vitro gene knockdown efficacy of the PRINT hydrogel particles, the 30% AEM-based hydrogel composition was utilized with four different cargos: (1) native luc siRNA, (2) degradable disulfide luc siRNA, (3) nondegradable, acrylamide luc siRNA, and (4) degradable disulfide control siRNA. Zetasizer analysis of the hydrogel PRINT particles indicated that their size and charge were similar (Table S4 (SI)), and gel electrophoresis (Figure S7 (SI)) allowed for confirmation of the release of the various cargos. After dosing the particles on cells and incubating for 48 h, cell viability was

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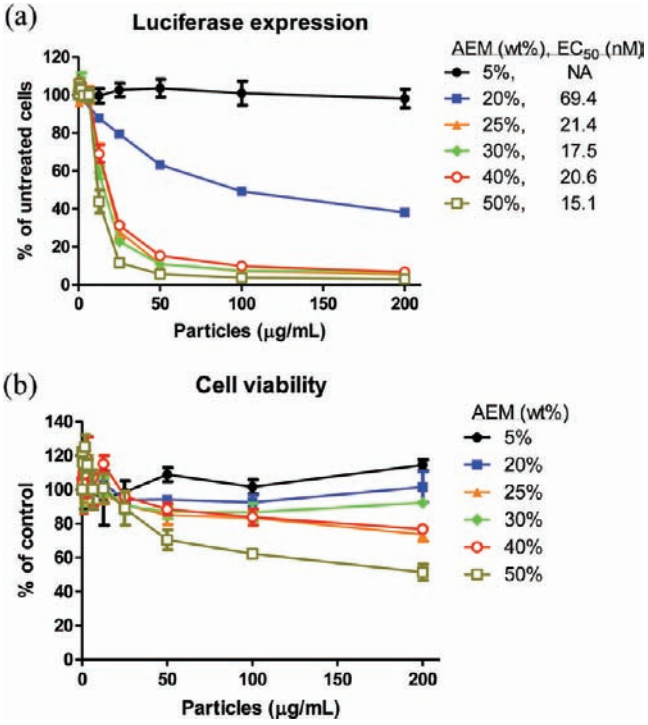

Figure 5. (a) Luciferase expression and (b) viability of HeLa/luc cells dosed with cationic pro-siRNA hydrogels fabricated with different amine (AEM) contents. Cells were dosed with particles for 5 h followed by removal of particles and 48 h incubation in media. Data are representative of two independent experiments. The error bars represent standard deviation from triplicate wells in the same experiment. Half maximal effective concentrations (EC_{50}) of siRNA (nM) for luciferase gene knockdown are listed in the legend. EC_{50} was not available (NA) for hydrogels prepared with 5 wt % AEM because of the absence of dose-dependent luciferase knockdown.

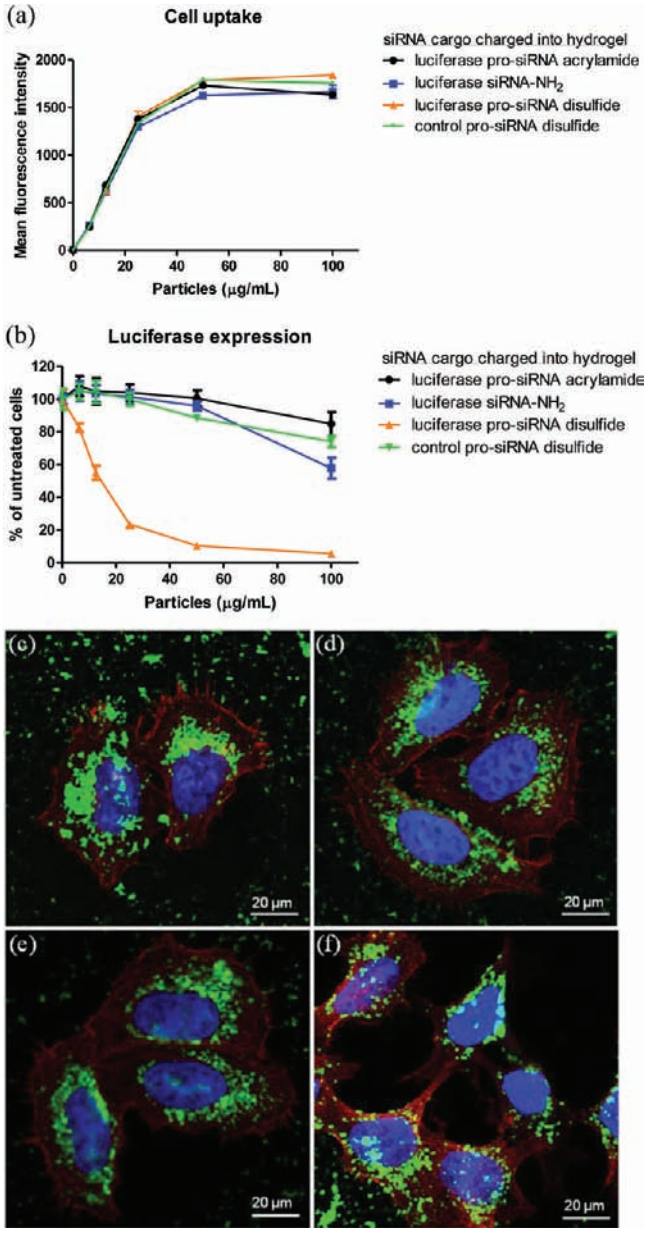

Figure 6. 30% AEM-based hydrogel particles charged with different siRNA cargos for transfection of HeLa cells. (a) Cellular uptake. HeLa/luc cells were dosed with particles for 4 h followed by trypan blue treatment and flow cytometry analysis. (b) Luciferase expression. HeLa/luc cells were dosed with particles for 4 h followed by removal of particles and 48 h incubation in media. Data in (a) and (b) represent one of two independent experiments. The error bars represent standard deviation from triplicate wells in the same experiment. Note that all hydrogels were thoroughly washed after fabrication to remove nonconjugated siRNA in the sol fraction. (c–f) Confocal micrographs. HeLa/luc cells were dosed with 50 μg/mL of hydrogels containing (c) luc PD, (d) luc siRNA-NH_2, (e) luc acrylamide, and (f) control PD siRNA cargos for 4 h. Cellular actin cytoskeleton was stained with phalloidin (red) and nuclei with DAPI (blue), while particles (green) were labeled with the fluorescent monomer, DyLight 488 maleimide.

maintained above 80% for all of the samples across all dosing concentrations, except for the particles charged with the free siRNA (Figure S8 (SI)). Uptake of all of the hydrogel particles approached saturation at around 50 μg/mL of particle concentration (Figure 6a). Dose-dependent silencing of luciferase expression was elicited notably for the pro-drug, disulfide-based siRNA-containing hydrogel particles, while the control particles did not elicit significant gene knockdown (Figure 6b).

The transfection efficiency between siRNA-complexed, PEGylated particles and pro-siRNA hydrogels is comparable, which can be explained by differences in release characteristics. PEGylated particles release siRNA slowly in PBS, while pro-siRNA hydrogels rapidly release siRNA under intracellular conditions. Confocal microscopy images of HeLa cells dosed with particles (Figure 6c–f) illustrates uptake of the particles, which accumulated largely in the cytoplasm and perinuclear region. Particles appear significantly brighter in Figure 6c–f compared to Figure 2c,d because of the higher fluorescence intensity emitted by DyLight 488 compared to fluorescein. Given the internalization of cationic particles by HeLa cells and distribution throughout the cytoplasm and perinuclear area, potential exists for the encapsulation of other therapeutic nucleic acids in hydrogel nanoparticles to transfect diseased cells.

■ CONCLUSIONS

siRNA may be physically or electrostatically entrapped within hydrogel nanoparticles to provide effective gene knockdown. Nevertheless, to retain cargo during particle functionalization

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and under physiological conditions, a covalent incorporation approach was deemed necessary. To our knowledge, this is the first work to polymerize siRNA in hydrogel particles for controlled intracellular delivery. The siRNA cargo was found to be adequately protected by the particles and only released upon reaching the reducing environment of the cytoplasm for effective gene knockdown. Physical dimensions and surface functionalization of pro-siRNA hydrogel nanoparticles with targeting and stealthing ligands are currently under investigation to enable in vivo systemic delivery of siRNAs for the treatment of diseases.

EXPERIMENTAL SECTION

Materials. 2,2′-Dithiodiethanol, acryloyl chloride, PEG_{700} diacrylate, disuccinimidyl carbonate (DSC), 2-aminoethyl methacrylate hydrochloride (AEM), 1-hydroxycyclohexyl phenyl ketone (HCPK), fluorescein *O*-acrylate, tetraethylene glycol, and Irgacure 2959 were purchased from Sigma Aldrich. Poly(vinyl alcohol) 75% hydrolyzed MW ≈ 2 kDa was obtained from Acros Organics. SS-$mPEG_{2K}$ and PEG_{1K} dimethacrylate were from Polysciences, Inc. $mPEG_{5K}$ acrylate and SCM-PEG_{2K}-biotin were from Creative PEGWorks. Antihuman CD71 (transferrin receptor) biotinylated OKT9 monoclonal antibody was purchased from eBioscience. UltraAvidin was obtained from Leinco Technologies. Tetraethylene glycol monoacrylate (HP_4A) was synthesized in-house and kindly provided by Dr. Matthew C. Parrott, Dr. Ashish Pandya, and Mathew Finniss. PRINT molds were graciously supplied by Liquidia Technologies. siRNAs were purchased as duplexes from Dharmacon, Inc. Sense sequence of amine-modified and native antiluciferase siRNA: 5′-N6-GAUUAUGUCCGGUUAUGUAUU-3′; antisense: 5′-P-UACAUAACCGGACAUAAUCUU-3′. Sense sequence of amine-modified and native control siRNA: 5′-N6-AUGUAUUGGCCUGUAUUAGUU-3′; antisense: 5′-P-CUAAUACAGGCCAAUACAUU-3′. All other reagents were obtained from Thermo Fisher Scientific, Inc. in molecular biology grade or RNase-free when available.

Synthesis of siRNA Macromers. *Degradable Disulfide Macromer Precursor.* 2,2′-Dithiodiethanol (15 mL, 0.12 mol) was dissolved in anhydrous DMF (250 mL) in a 500-mL round-bottomed flask containing NEt_3 (20.5 mL, 1.2 equiv) under a N_2 blanket to which acryloyl chloride (11.0 mL, 1.1 equiv) was added dropwise and allowed to react for 8 h. Crude product was extracted into dichloromethane against 5% LiCl and purified via silica gel chromatography (EtOAc/hexanes) to provide monoacrylate-substituted 2,2′-dithiodiethanol (63% yield). 2-((2-hydroxyethyl)disulfanyl)ethyl acrylate (10 g, 48 mmol) was dissolved in anhydrous acetonitrile (100 mL) in a N_2-purged 250-mL round-bottomed flask, followed by addition of disuccinimidyl carbonate (14.8 g, 1.2 equiv). The reaction proceeded for 8 h, and product was purified by silica gel chromatography (EtOAc/hexanes 4:1) to afford 2-*N*-hydroxysuccinimide, 2′-acryloyl-dithiodiethanol as a clear, viscous liquid (82% yield): ^{1}H NMR (600 MHz, $CDCl_3$) δ = 6.46 (dd, J = 17.4 Hz, 1H), δ = 6.2 (dd, J = 10.7 Hz, 6.8 Hz, 1H), δ = 5.90 (dd, J = 10.7 Hz, 1H), δ = 4.59 (t, J = 6.5 Hz, 2H), δ = 4.45 (t, J = 6.5 Hz, 2H), δ = 3.05−3.00 (m, J = 6.8 Hz, 4H), δ = 2.87 (s, 4H).

Nondegradable siRNA Conjugate Precursor. *N*-Hydroxyethyl acrylamide (15 mL, 0.14 mol) was dissolved with DSC (51.9 g, 1.4 equiv) in ACN/DMF 4:1 (250 mL) and reacted for 16 h. Afterward, ACN was removed via rotary evaporation, and product was extracted into EtOAc against 5% LiCl. Product was concentrated and purified by silica gel column chromatography (EtOAc/hexanes 4:1) to provide 2-(succinimidyl carbonate)ethyl acrylamide (80% yield) as a fine white solid: ^{1}H NMR (600 MHz, $CDCl_3$) δ = 6.50 (br, 1H, *NH*), δ = 6.34 (dd, J = 17.1 Hz, 1H), δ = 6.19 (dd, J = 10.3 Hz, 6.4 Hz, 1H), δ = 5.71 (dd, J = 10.3 Hz, 1H), δ = 4.46 (t, J = 5.0, 2H), δ = 3.71 (m, J = 5.5 Hz, 2H), δ = 2.87 (s, 4H).

siRNA Macromers. siRNA-NH_2 (2 mg, 148 nmol, antiluciferase siRNA or control sequence) was dissolved in DEPC-treated PBS (200 μL) in a 1.5-mL RNase-free Eppendorf tube. Separately, 2-*N*-hydroxysuccinimide, 2′-acryloyl-dithiodiethanol (5.2 mg, 100 equiv), or 2-(succinimidyl carbonate)ethyl acrylamide (3.8 mg, 100 equiv) was dissolved in RNase-free DMF (150 μL) and added to the solution of siRNA. The reaction was allowed to proceed for 36 h, where additional 100 equiv of the acrylate or acrylamide were added to the reaction mixture every 12 h. 5 M NH_4OAc (50 μL) and EtOH (1.1 mL) were added to the reaction mixture, which was vortexed for 15 s. The sample was incubated in a −80 °C freezer for 4 h followed by centrifugation (14 krpm, 4 °C, 20 min) to pellet the siRNA. The supernatant was decanted, and the pellet was washed twice with 70% EtOH (ice-cold) to provide siRNA prodrug (79% yield). HR-ESI-MS: m/z found for siRNA sense strand $[M - H]^-$ = 6832.366; m/z calc. for disulfide macromer $[M - H]^-$ = 7067.676; found $[M - H]^-$ = 7067.855; m/z calc. for siRNA acrylamide macromer $[M - H]^-$ = 6974.506; found $[M - H]^-$ = 6974.871. Characterization of siRNA prodrug precursors was carried out on a 600 MHz Bruker NMR spectrometer equipped with a Cryoprobe, and siRNA macromonomers were analyzed by an IonSpec Fourier transform mass spectrometer FTMS (20503 Crescent Bay Drive, Lake Forest, CA 92630) with a nano-electrospray ionization source in combination with a NanoMate (Advion, 19 Brown Road, Ithaca, NY 14850) chip-based electrospray sample introduction system and nozzle operated in the negative ion mode as well as reversed phase high-performance liquid chromatography (Figure S9 (SI)).

Fabrication of Hydrogels via PRINT Process. Preparticle solutions were prepared with listed compositions at 2.5 wt % in RNase-free DMF (for physically entrapped siRNA) or DEPC-treated water containing 0.01% sodium dodecyl sulfate (for prodrug siRNA, where all remaining steps were conducted in a humidity room maintained at 70% relative humidity). Specifically, for each composition, solid and liquid monomers were dissolved in respective preparticle solution solvent at a total weight percent of 2.5%. The film-split technique for preparing particles was performed as follows: using a #5 wire wound rod (R.D.S.), 150 μL of preparticle solution was cast at 6 ft/min on a sheet of poly(ethylene terephthalate) (PET), followed by brief evaporation of solvent with heat gun to yield a transparent film (delivery sheet). 200 × 200 nm cylindrical Fluorocur-patterned PRINT molds (Liquidia Technologies) were laminated against the delivery sheet with moderate pressure (40 psi) and then gently delaminated. The filled mold was laminated against corona-treated PET and subsequently cured in a UV chamber (λ_{max} = 365 nm, 90 mW/cm^2) for 5 min. After photocuring, the mold was removed to reveal an array of particles on PET. Particles were harvested off PET with water mechanically using a cell scraper (1 mL/48 in^2). Particles were washed via centrifugation (15 min, 14 krpm, 4 °C), removal of supernatant, and resuspension in water. Particle yield was determined by thermogravimetric analysis (Q5000IR, TA Instruments). Pro-siRNA hydrogels were washed repeatedly with 10× PBS containing 0.05% PVA 2 kDa to remove the sol fraction.

Hydrogels with electrostatically entrapped siRNA were PEGylated by reacting particles with SS-$mPEG_{2K}$ (2 wt equiv, weight equivalents relative to particle mass) using pyridine (2 wt equiv) in DMF at 2 mg/mL particle concentration for 6 h at room temperature followed by washing particles with water. For postfunctionalization of particles with ligands, SCM-PEG_{2K}-biotin (2 wt equiv) was reacted with particles in DMF at 2 mg/mL for 2 h along with pyridine (4 wt equiv). NHS-$mPEG_{12}$ (2 wt equiv) was added to quench nonbiotinylated amines with pyridine (4 wt equiv) in DMF at 2 mg/mL for 30 min. Particles were centrifuged, isolated through removal of supernatant, and washed with PBS. UltraAvidin (0.25 wt equiv) was conjugated to biotinylated particles in PBS at 2 mg/mL for 20 min, followed by centrifugation, washing with PBS, and adding biotinylated OKT9 antibody (20 wt mequiv) for another 20 min at 2 mg/mL, after which particles were washed with PBS to remove unconjugated antibody and resuspended in PBS at 2 mg/mL.

Particle Characterization. Scanning electron microscopy (SEM) enabled imaging of hydrogels that were dispersed on a glass slide and

coated with 2 nm of Au/Pd (Hitachi S-4700). ζ-Potential measurements were conducted on 20 μg/mL particle dispersions in 1 mM KCl using a Zetasizer Nano ZS particle analyzer (Malvern Instruments Inc.).

Analysis of siRNA by Gel Electrophoresis. 2.5% agarose gel in TBE buffer was prepared with 0.5 μg/mL of ethidium bromide. For studying release of siRNA from hydrogels, aliquots of particle dispersions were centrifuged (15 min, 14 krpm, 4 °C) for recovery of the supernatant at various time points and frozen. Similarly, aliquots of siRNA prodrug incubated in 10% FBS at 37 °C were taken at various time points and frozen for storage. Twelve microliters of sample (supernatants from particle dispersions, siRNA solutions, or particle dispersions) was mixed with 3 μL of 6× loading buffer (60% glycerol, 0.12 M EDTA in DEPC-treated water) and loaded into the gel. 70 V/cm was applied for 25 min, and the gel was then imaged with ImageQuant LAS 4000 (GE). Analysis of siRNA band intensity was conducted with Image J software for quantification. siRNA loading was calculated by comparing the maximum amount of siRNA released to the particle mass. Encapsulation efficiency of siRNA was calculated by comparing wt % of final siRNA loading to the siRNA charged into the preparticle composition (5 wt % of particle).

Cell Culture. Luciferase-expressing HeLa cell line (HeLa/luc) was from Xenogen. HeLa/luc cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) high glucose supplemented with 10% FBS, 2 mM L-glutamine, 50 units/mL of penicillin and 50 μg/mL of streptomycin, 1 mM sodium pyruvate and nonessential amino acids. All media and supplements were from GIBCO except for FBS, which was from Mediatech, Inc.

In Vitro Cell Uptake Analysis. HeLa/luc cells were plated in 96-well plates at 10 000/well and incubated overnight at 37 °C. Cells were dosed with fluorescently tagged particles in OPTI-MEM at 37 °C (5% CO_2) for 4 h or indicated time for cell uptake studies. Fluorescently tagged particles were prepared by incorporation of fluorescent monomers (fluorescein *O*-acrylate for PEGylated particles and DyLight 488 maleimide for pro-siRNA particles) in the particle composition and copolymerization of these monomers into particles matrix. After incubation, cells were washed and detached by trypsinization. After centrifugation, cells were resuspended in a 0.4% trypan blue (TB) solution in Dulbecco's phosphate buffered saline solution (DPBS) to quench the fluorescein fluorescence from particles associated to the cell surface. Cells were then washed and resuspended in DPBS or fixed in 1% paraformaldehyde/DPBS, and analyzed by CyAn ADP flowcytometer (Dako). Cell uptake was represented as percentage of cells that were positive in fluorescein fluorescence.

In Vitro Cytotoxicity and Luciferase Expression Assays. HeLa/luc cells were plated in 96-well plate at 10 000/well and incubated overnight at 37 °C. Cells were dosed with particles or Lipofectamine 2000/siRNA mix in OPTI-MEM at 37 °C (5% CO_2) for 4 or 5 h, then particles were removed, and complete grow medium was added for another 48 h incubation at 37 °C. Cell viability was evaluated with Promega CellTiter 96 AQ_{ueous} one solution cell proliferation assay, and luciferase expression level was evaluated with Promega Bright-Glo luciferase assay according to manufacturer's instructions. Light absorption or bioluminescence was measured by a SpectraMax M5 plate reader (Molecular Devices). The viability or luciferase expression of the cells exposed to PRINT particles was expressed as a percentage of that of cells grown in the absence of particles. Half-maximal effective concentration (EC_{50}) of siRNA required to elicit gene knockdown was determined by applying the dose-dependent gene knockdown data to a log(inhibitor) vs response variable slope nonlinear function in GraphPad Prism software for given siRNA concentrations (calculated from particle concentration and siRNA loading).

Confocal Laser Scanning Microscopy. HeLa/luc cells were plated in 8-well chamber slides (BD) at 10 000/well and incubated overnight at 37 °C. Cells were dosed with green fluorescent particles (fluorescein or DyLight 488 labeled) in OPTI-MEM at 37 °C (5% CO_2) for 4 h. Cells were then washed three times with PBS, fixed with 4% paraformaldehyde for 10 min, and permeabilized with 0.5% saponin for 30 min at room temperature. Cells were stained with 100 nM phalloidin-AlexaFluor 555 for actin for 1 h at room temperature, and then counterstained with 30 μM DAPI for 15 min. Images were collected with LSM710 confocal laser scanning microscope (Carl Zeiss).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Compositions of PRINT particles and additional Zetasizer measurements in Tables S1−S4. Viability of HeLa cells dosed with PEGylated hydrogels and pro-siRNA hydrogels containing different siRNA cargos, transfection of HeLa cells with different siRNAs using Lipofectamine, gel electrophoresis of siRNA loaded into and released from different hydrogels, and analysis of siRNAs by HPLC in Figures S1−S9. Completed author list for abbreviated references. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): Joseph DeSimone is a founder, member of the board of directors, and maintains a financial interest in Liquidia Technologies. Liquidia was founded in 2004 to commercialize PRINT technology and other discoveries of Professor Joseph DeSimone and colleagues at the University of North Carolina, Chapel Hill.

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