

Figure 2. Micelle structure and properties. (A) Schematic representation of polymeric micelle structure and key components. (B) Size distribution histogram of cRGD-2IT-95 micelles determined by dynamic light scattering showing narrowly dispersed particles. (C) Atomic force microscopy image of cRGD-2IT-95 micelles showing spherical structures. (D) Enhancement of micelle stability by 2IT modification of PEG-*b*-PLL at 150 mM and 600 mM NaCl with or without dithiothreitol (a disulfide reducing agent) as determined by light scattering intensity analysis. Micelles prepared with cRGD-2IT-95 were cross-linked prior to analysis.

TABLE 2. Micelle Properties

polymer	polymer/siRNA (moles)	size (d, nm) ^b	PDI ^b	ζ-potential ^c	free thiol content (%) ^d
2IT-0	1.2	194 ± 15	0.45 ± 0.09	ND	ND
cRGD-2IT-0	1.2	160 ± 15	0.44 ± 0.06	ND	ND
2IT-95 ^e	7.6	45 ± 2	0.09 ± 0.03	-1.99 ± 0.55	1.7 ± 0.9
cRGD-2IT-95 ^e	7.6	45 ± 3	0.09 ± 0.03	-0.55 ± 0.91	2.0 ± 0.7

^a Micelles cross-linked before analysis. ^b Determined by dynamic light scattering. ^c Determined by laser Doppler electrophoresis. ^d Determined by Ellman's assay.

at the specific molar ratio of polymer/siRNA of 1.2 (also near the charge-neutral point) for unmodified polymers, while 2IT modified polymers formed micelles with siRNA over a broad range of polymer/siRNA molar ratios until a plateau in SLI was observed at the polymer/siRNA molar ratio of ~8 (Table 2 and Supporting Information). Formation of micelle structures with siRNA in the presence of higher molar ratios of polymer compared to the parent PEG-*b*-PLL is a characteristic of the 2IT-95 polymer and is consistent with our previous reports.^{15,16} The shift in the polymer/siRNA molar ratio for micelle formation with the 2IT-95 polymer reflects the presence of cyclic N-substituted 2-iminothiolane structures in the siRNA binding segment of the block copolymer. The imine groups contained in the N-substituted 2-iminothiolane ring has a lower pK_a than amidines (~6–7 vs ~11–12), which results in reduced polymer charge at pH 7.4.²³ This heterocyclic functional group also contains features that could allow for other non-electrostatic binding modes with siRNA,

allowing micelle formation over a broad range of polymer/siRNA ratios. Specifically, N-substituted 2-iminothiolane rings are more hydrophobic than unmodified lysines, contain a dipole moment between C–S and C–N bonds, and also contain an imine nitrogen. These characteristics could lead to enhanced van der Waals and dipole–dipole interactions as well as hydrogen bonding capability with siRNA or with other polymer chains associated within micelle structures. On the other hand, the optimal polymer/siRNA molar ratio for particle formation with PEG-*b*-PLL containing unmodified lysine residues was very sensitive to the amount of polymer, suggesting that excess cationic charge interferes with micelle formation. 2IT-95 (both with and without cRGD) polymer generally produced higher quality micelles with smaller size and narrow size dispersion, demonstrating improved control of micelle assembly (Figure 2B).

On the basis of these light scattering results, the optimal micelle formation conditions were defined as the molar ratio of polymer/siRNA that resulted in the

maximum SLI, indicating formation of multimolecular micelle structures. Micelles were prepared at the polymer/siRNA molar ratios shown in Table 2 and, in the case of 2IT modified polymers, cross-linked before use. Micelle cross-linking was achieved by dialysis in HEPES buffer containing DMSO, which is a mild oxidant of free thiols.²⁴ Reduced polymer thiol content following the oxidation procedure confirmed that disulfide formation occurred (Table 2). Direct observation of cross-linked micelles by atomic force microscopy (AFM) revealed spherical structures approximately 15–20 nm in size, which is smaller than the hydrodynamic diameter determined by light scattering measurement and probably more indicative of the micelle core size (Figure 2C). Calculation of the theoretical micelle diameter assuming that the AFM image represents the micelle core results in a value similar to DLS results, as the height of a 12K PEG in the mushroom configuration is ~ 9 nm, thus, ~ 20 nm + $(\sim 9$ nm \times 2) = ~ 38 nm.²⁵ Furthermore, the micelle diameters determined by light scattering measurement were expressed as z-averaged diameters. Conversion of DLS data to the number-averaged diameter yields ~ 32 nm, which is in good agreement with the theoretical diameter calculated from the AFM image. Analysis of particle ζ -potentials demonstrated that micelle structures were nearly neutral in charge, which is consistent with the formation of core-shell micelle structures (Table 2).

Modification of PEG-*b*-PLL with 2IT increased the stability of micelles prepared with siRNA, as evidenced by SLI measurement of micelle solutions at different ionic strengths (Figure 2D). Increased ionic strength is expected to shield the charge of siRNA and disrupt electrostatic interactions, leading to dissociation of unstable micelle structures and decreased SLI. cRGD-2IT-0 micelles nearly completely dissociated at 150 mM NaCl (physiological ionic strength), whereas cRGD-2IT-95 micelles remained largely intact at both 150 and 600 mM NaCl. Addition of the disulfide reducing agent dithiothreitol (DTT) to solutions of cRGD-2IT-95 micelles resulted in a decrease in SLI at 600 mM NaCl, showing that disulfide cross-linking contributed to micelle stability. However, $\sim 60\%$ of the SLI intensity was retained for cRGD-2IT-95 micelles in the presence of DTT at 600 mM NaCl, suggesting that the iminothiolane ring structure is also important for micelle stability. It should be noted that a polymer similar to cRGD-2IT-95 containing only amidines and thiols in the polymer structure, and lacking N-substituted 2-iminothiolane rings, nearly completely dissociated under the same conditions in the presence of DTT.¹⁶ Additionally, the presence of the cRGD peptide did not affect micelle stability in this assay (Supporting Information). For all experiments described hereafter, micelle formulations were prepared for each polymer at the optimal polymer/siRNA molar ratio indicated in Table 2 and, in the

case of 2IT-95 and cRGD-2IT-95 formulations, cross-linked before use.

In Vitro Gene Silencing. Polymeric micelles were analyzed for their ability to inhibit protein expression in cultured HeLa cells using a luciferase assay. HeLa cells express $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors that can bind the cRGD peptide, and thus are appropriate for use in this study to determine the effects of the cRGD peptide on micelle performance.^{26,27} Micelle encapsulation of siRNA within stable structures containing cRGD is expected to increase the internalization of siRNA into cells and improve gene silencing efficacy. Micelles prepared with siRNA-targeting luciferase mRNA for degradation were administered to HeLa cells stably expressing GL3 luciferase (HeLa-luc), and cell luminescence was measured over time. Polymeric micelle formulations lacking the cRGD peptide were ineffective at decreasing cell luminescence; however, micelles prepared from polymer modified with both 2IT and cRGD showed a marked decrease in cell luminescence after 50 h incubation (Figure 3A). Further analysis of cells treated with cRGD-2IT-95 micelles showed that luminescence intensity gradual decreased over the entire 50 h of the experiment, suggesting that siRNA was slowly released into the site of activity in the cell cytoplasm (Figure 3B). No reduction in luminescence occurred when off-target siRNA was contained in cRGD-2IT-95 micelles, suggesting that reduced luminescence observed for cells treated with target siRNA was due to sequence-specific gene silencing (Figure 3A,B). cRGD-2IT-95 micelles were less effective than the commercial transfection reagent RNAiMax employed as the positive control. However, this lipid-based siRNA delivery agent achieves high efficacy *in vitro* by directly transporting siRNA across the exterior cell membrane, thus avoiding cell uptake and trafficking barriers encountered with PEGylated micelles.²⁸

The chemical structure of the siRNA binding segment in the block copolymer used to prepare micelles was critical to realize performance gain due to the cRGD peptide, as only the cRGD-2IT-95 formulation showed activity. This demonstrates the importance of stable micelle structures, which may allow for multiple cRGD peptides to be presented in a single particle and improve binding to cell-surface integrin receptors. The importance of cRGD density was further investigated by preparing cRGD-2IT-95 micelles with reduced cRGD content. cRGD-2IT-95 micelles prepared at the polymer/siRNA molar ratio of 4 or by mixing cRGD-2IT-95 and 2IT-95 polymers before micelle formation with siRNA (polymer/siRNA molar ratio = 7.6) were less effective at reducing gene silencing (Figure 3C), which suggests that high cRGD content was necessary for effective micelle formulations.

Analysis of micelle toxicity following incubation in HeLa-luc cells showed that micelles were well tolerated, as cell metabolism was maintained at high levels

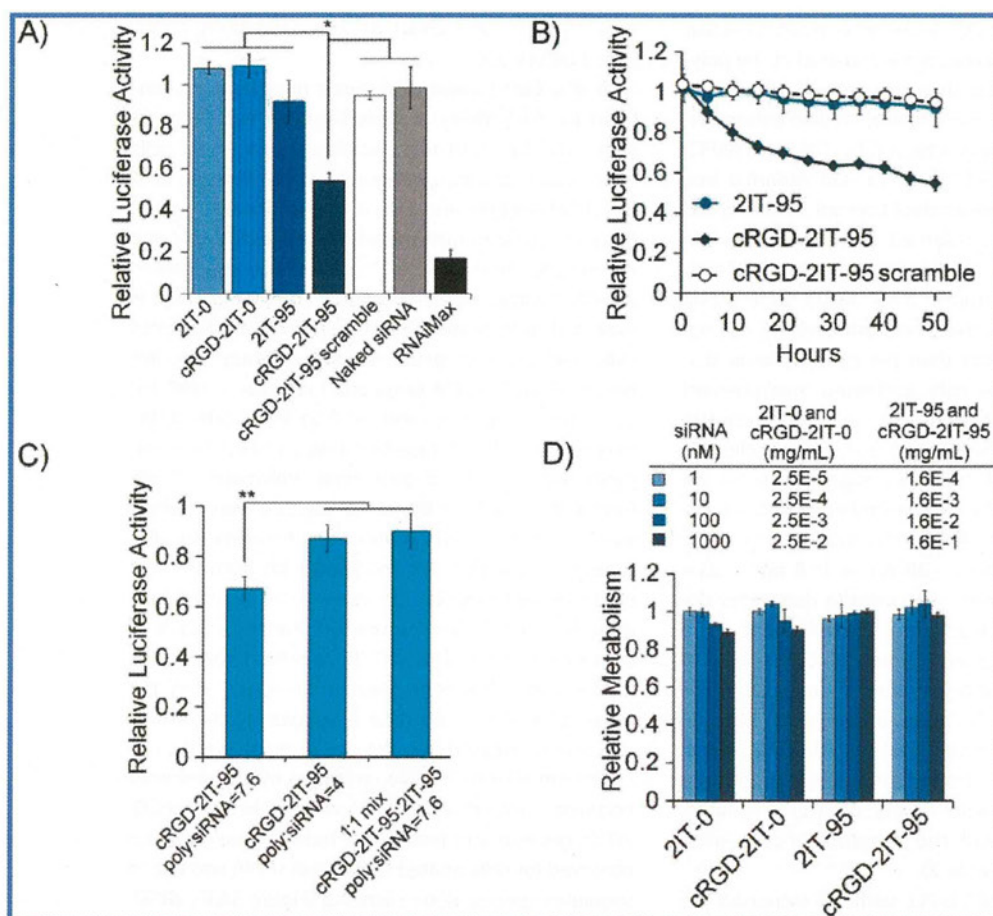


Figure 3. Gene silencing ability of siRNA micelles and tolerance in HeLa-luc cancer cells. (A) Quantification of luciferase luminescence following incubation with micelles containing antiluciferase siRNA (or scramble siRNA as noted in legend). Micelles were prepared at the optimal polymer/siRNA ratio shown in Table 2 and administered at a siRNA concentration of 200 nM, while RNAiMax was administered at a concentration of 10 nM. All samples were incubated for 50 h, $n = 4$, $*p < 0.001$. (B) Time dependence of luminescence reduction for cRGD-2IT-95 micelles, 200 nM siRNA, $n = 4$. (C) Activity of cRGD-2IT-95 micelles prepared at non-optimal conditions. Micelles were prepared at the polymer/siRNA molar ratio of 4 or mixed 1:1 with 2IT-95 polymer before micelle formation with siRNA (polymer/siRNA molar ratio = 7.6) and subsequent cross-linking. All samples were incubated for 50 h, 200 nM siRNA, $n = 4$, $**p < 0.005$. (D) Toxicity of micelles toward HeLa-luc cells (48 h) at different concentrations. The concentrations of siRNA and polymer are denoted in the legend.

for all polymeric micelle formulations even at concentrations 5 times higher than that used for gene silencing experiments (Figure 2D). These results further support that reduced luminescence observed for the cRGD-2IT-95 micelle formulation was due to the specific inhibition of luciferase expression and not an artifact of reduced cell luminescence due to cytotoxicity.

Cell Uptake and Trafficking of Micelles. Initial screening of polymeric micelle formulations *in vitro* revealed that high micelle stability and high cRGD content were linked to improved gene silencing activity. One possible reason for the improved activity observed for cRGD-2IT-95 micelles is higher cell uptake due to the improved interactions with the cell surface. PEGylation of nanoparticles in general creates a unique dilemma, as both benefits and detriments result. PEG reduces nonspecific interactions between particles with each other and components in biological milieu, preventing

aggregation or nonspecific binding to off-target species. However, this also reduces interactions with cell surfaces, leading to lower uptake. In this study, installation of the integrin-targeting cRGD peptide was aimed to increase cell uptake due to improved cell-surface interactions.

Cellular uptake of micelles was determined by flow cytometric analysis of HeLa-luc cells following incubation with micelles prepared with fluorescent-labeled siRNA (Figure 4). No improvement in cell uptake due to the cRGD peptide was observed for cRGD-2IT-0 micelles, while the cRGD-2IT-95 micelle formulation showed a shift in the cell population toward higher fluorescence intensity compared to 2IT-95 micelles, demonstrating increased uptake of siRNA. This corroborates well with *in vitro* gene silencing results, where 2IT-0 formulations showed no difference in gene silencing between (+) and (-) cRGD formulations.

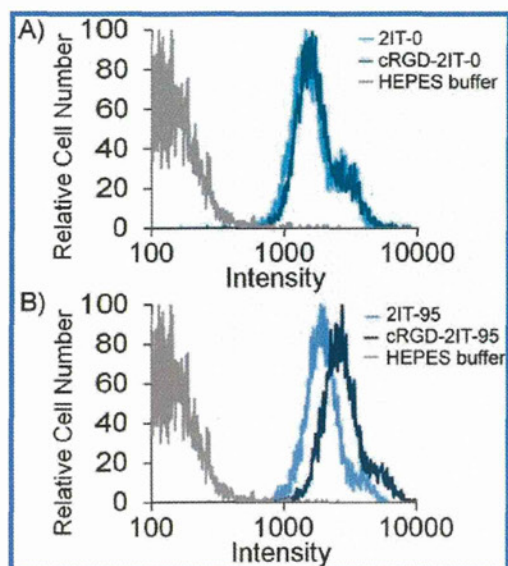


Figure 4. Cellular uptake of micelles in HeLa-luc cells. Cells were incubated with micelles prepared with fluorescent siRNA for 2 h (300 nM siRNA) prior to analysis by flow cytometry.

Increased cell uptake may be due to the ability of stable micelle structures to present multiple cRGD peptides on the nanoparticle surface. The presence of multiple cRGD peptides in close proximity has been shown to increase integrin binding efficiency in other studies.^{29,30} Specifically, the binding constant of a cyclic decapeptide functionalized with cRGD increased 10-fold upon increasing the cRGD valency from one to four.³¹ Similarly, a cRGD-functionalized PAMAM dendrimer showed a 100-fold increase in integrin binding affinity by increasing the number of cRGD peptides per dendrimer from one to eight.³² Our results showing that stable micelle particles were needed to realize a benefit from cRGD targeting seems consistent with other reports that show more cRGD peptides in close proximity generally improves performance. However, in the case of the polymeric micelles described here, there seems to be a critical density of cRGD needed for improved performance to be realized.

In addition to improving cell uptake, the cRGD peptide may affect subcellular trafficking of attached cargo. Micelle structures (and generally macromolecules, as well) must be actively internalized into cells by endocytosis, which results in encapsulation within membrane-bound organelles (endosomes and lysosomes). We were specifically interested to see if the micelles used here were trafficked to lysosomes, as these subcellular vesicles can be a “dead end” for nucleic acids due to their function of degrading substances internalized within the cell.³³ Thus, we investigated the subcellular distribution of fluorescent-labeled siRNA contained within cRGD-2IT-95 or 2IT-95 micelles (samples that showed a difference in gene silencing ability and cell

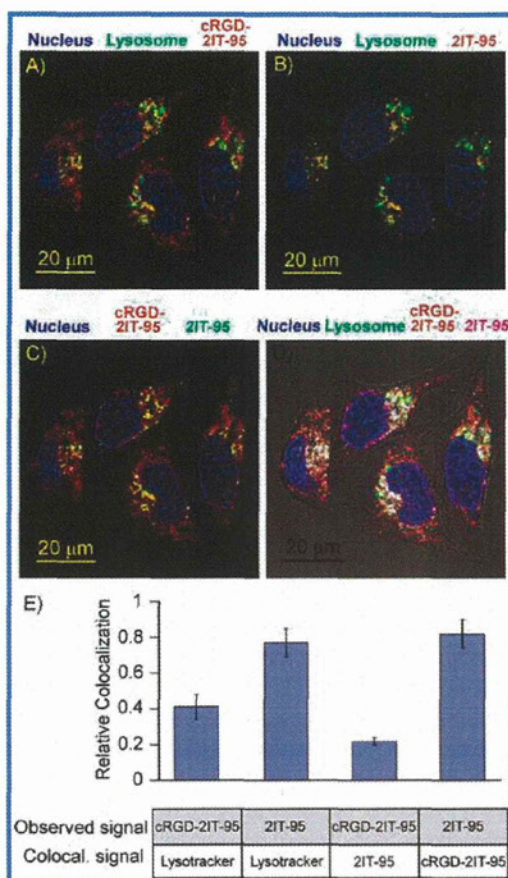


Figure 5. Subcellular distribution of 2IT-95 and cRGD-2IT-95 micelles following coincubation in HeLa-luc cells for 4 h (500 nM siRNA for each micelle formulation). Lysosomes were stained with LysoTracker green and micelles were prepared with Cy3- or Cy5-labeled siRNA for cRGD-2IT-95 and 2IT-95 formulations, respectively. (A–C) Cell images showing individual fluorescence signals. (D) Overlay of all fluorescent signals onto the transmitted image. (E) Quantification of fluorescence signal colocalization ($n = 8$ cells from two different images).

uptake). Lysosome compartments were stained with LysoTracker Green, which is specific marker for these acidic organelles. Micelle subcellular distribution was markedly different between the two formulations when co-incubated with HeLa-luc cells (Figure 5A–D). cRGD-2IT-95 micelles exhibited a broader cell distribution, and less than 50% of micelles were localized within lysosome compartments (Figure 5E), which is consistent with our previous report describing decreased lysosomal accumulation of pDNA polyplexes containing the cRGD peptide.²¹ In contrast, 2IT-95 micelles were mostly confined to lysosome compartments (~80%). Further analysis of the fluorescent signals showed that cRGD-2IT-95 micelles were able to co-accumulate with 2IT-95 micelles within lysosomes, but 2IT-95 micelles were unable to colocalize with cRGD-2IT-95 micelles in nonlysosomal regions (Figure 5E). This result demonstrates that the cRGD peptide affects the subcellular fate of micelles,

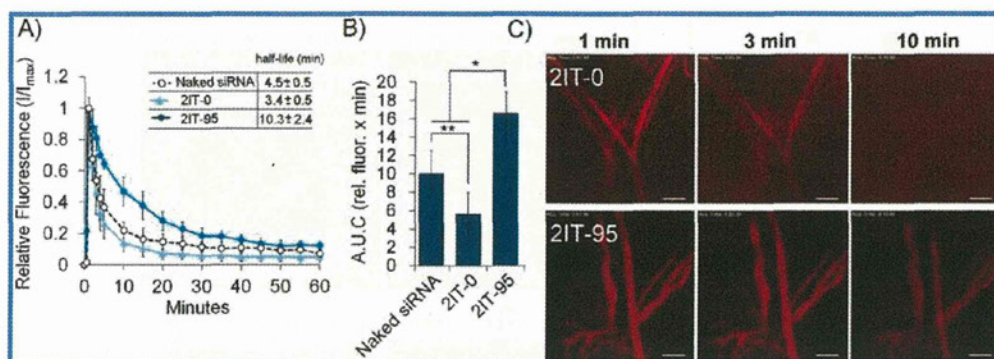


Figure 6. Blood circulation properties of micelles following tail-vein injection (24 μg siRNA). (A) Fluorescence signal over time and calculated half-lives, $n = 3$. (B) Area under the curve values 60 min after injection, $n = 3$, * $p < 0.05$, ** $p = 0.1$. (C) Ear-lobe dermis snap-shots at 1, 3, and 10 min following micelle injection; siRNA fluorescence is shown as red, scale bar = 100 μm .

resulting in broader subcellular distribution in nonlysosomal regions.

It is also interesting to note that internalization of cRGD conjugates may also facilitate their transport into the cell cytoplasm. All nucleic-acid-based therapeutics must gain access to the cytoplasm (and further import into the nucleus for pDNAs) in order to be effective. Due to their large size and highly charged nature, nucleic acids cannot readily diffuse across membranes, whether cell surface, endosomal, or lysosomal in nature. There is building evidence, including the current work, showing that incorporation of cRGD into nucleic acid delivery systems improves their efficacy, even when delivery systems do not contain programmed endosome/lysosome escape functionality.^{21,34} In those studies, the nucleic acid cargo associated with the cRGD peptide is shown to have a broader subcellular distribution and in some cases avoids a high degree of colocalization with lysosomal compartments, as was observed in our current study. This could be a characteristic of the internalization route itself; that is, particles are not trafficked to lysosomes, or accumulation in lysosomes is delayed, or that particles escape endocytotic vesicles before or after transport to the lysosome. A previous study regarding the subcellular trafficking of adenovirus vectors showed that RGD motifs at the penton base improved endosomal escape of the virus, even when lysosome acidification was prevented with ammonium chloride (preventing another pH-dependent viral escape mechanism from occurring).²⁷ Thus, the cRGD peptide may be associated with a natural mechanism that facilitates cytoplasmic entry. Altogether, our data showed that improved cell uptake and broader subcellular distribution was correlated with higher RNAi activity in cultured HeLa-luc cells treated with cRGD-2IT-95 micelles, but the exact mechanism of siRNA cytoplasmic entry remains unclear and needs further investigation. We plan to investigate the subcellular trafficking of siRNA delivered with cRGD-2IT-95 micelles in more detail using blockers of specific cellular internalization pathways

(e.g., filipin for caveole pathways, chlorpromazine for clatherin-dependent pathways, and amiloride for macropinocytosis), advanced reagents available for imaging endocytotic vesicles (e.g., CellLite reagents for preparing fluorescent protein-labeled early and late endosomes), and also reagents known to disrupt lysosome structures (e.g., chloroquine).

Blood Stability of Micelles. In order to achieve tumor targeting by IV injection, micelles must remain in the bloodstream long enough to reach the tumor mass. Micelle stability is related to the core structure, and thus the effect of 2IT modification on blood stability was assayed. Blood circulation behavior was determined using intravital real-time confocal laser scanning microscopy (IVRTCLSM) imaging of micelles prepared with fluorescent-labeled siRNA. IVRTCLSM is a non-invasive technique that allows quantification of fluorescence intensity in the ear-lobe dermis of mice and also observation of physical behavior (such as aggregation) of micelles in the bloodstream.^{35,36}

Fluorescence intensity curves, calculated half-lives, area under the fluorescence intensity curves (AUCs), and representative ear snap-shots of micelle formulations following injection are shown in Figure 6. Calculation of the circulation half-lives and AUC values showed that micelles prepared with 2IT modified polymer increased the blood residence time of siRNA (Figure 6A,B), demonstrating the importance of micelle stability for improved blood circulation. Specifically, 2IT-95 micelles showed an over 2-fold improvement of circulation half-life and 70% increase in total fluorescence over 60 min compared to naked siRNA. On the other hand, 2IT-0 micelles were quickly and completely removed from the bloodstream, similar to naked siRNA. None of the samples showed aggregation following injection, and fluorescent signal remained homogeneous without visible aggregates throughout the entire observation period (Figure 6C).

Biodistribution of siRNA Administered in Micelle Formulations. Accumulation of siRNA within tumors is critical for effective RNAi-based cancer treatment. The ability of

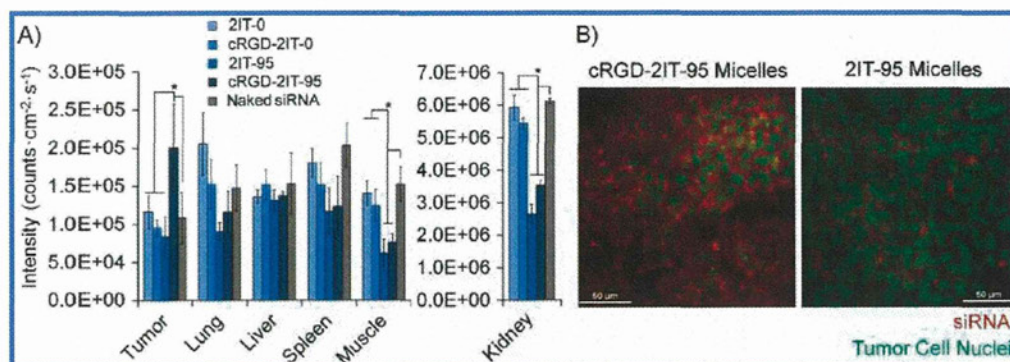


Figure 7. Biodistribution of siRNA administered in micelle formulations 24 h post-injection (24 μ g siRNA injected). (A) Quantification of Cy5.5-siRNA fluorescence by IVIS, $n = 4$, $*p < 0.05$. (B) IVRTCLSM image of H2BGFP-HeLa tumors in live mice 24 h after injection of micelles prepared with Cy5-siRNA (24 μ g Cy5-siRNA injected).

micelle formulations to deliver siRNA to subcutaneous HeLa tumors was estimated using IVIS imaging of fluorescent siRNAs 24 h post-injection. This time point was chosen because *in vitro* results revealed that the onset of gene silencing was gradual and thus micelles must accumulate and persist in the tumor tissue for an extended time in order to facilitate siRNA release. Enhanced tumor targeting was achieved for cRGD-2IT-95 micelles, with $\sim 2\times$ accumulation compared to naked siRNA, 2IT-0, and cRGD-2IT-0 formulations (Figure 7A). Furthermore, the cRGD peptide was critical for enhanced tumor accumulation, as 2IT-95 micelles lacking the cRGD peptide showed signal intensities similar to naked siRNA, 2IT-0, and cRGD-2IT-0 formulations. In contrast, cRGD-2IT-0 micelles showed no enhancement of tumor accumulation due to the cRGD peptide, which corroborates well with *in vitro* cell uptake results shown in Figure 4. In addition to increasing tumor accumulation, stable micelles prepared with 2IT modified polymer showed decreased accumulation at off-target sites such as the lung, spleen, muscle, and kidney compared to naked siRNA and 2IT-0 micelles. This demonstrates that stable micelle structures can allow more control of siRNA distribution following injection to favor enhanced accumulation within the target site of activity.

Overall, the highest fluorescence signal for all micelle formulations was found in the kidneys. This is interesting because the kidney filters components present in the bloodstream based on size, and it is generally accepted that the filtration cutoff is on the order of $\sim 5\text{--}10$ nm.³⁷ This suggests that 2IT-0 micelles dissociated in the bloodstream, allowing lower molecular structures or free siRNA to enter and accumulate within this blood filtration organ. The fact that 2IT-95 and cRGD-2IT-95 micelles are better at evading kidney accumulation further supports the fact that they form stable structures. On the basis of these findings, it appears that the primary excretion route of the micelles presented here is glomerular filtration, which is an advantage compared to other nanoparticles with prolonged circulation, which often show high liver

accumulation. This property could reduce potential side effects such as hepatotoxicity.

Tumor accumulation was also observed using IVRTCLSM imaging of tumors in live mice 24 h post-injection of cRGD-2IT-95 micelles or 2IT-95 micelles, and results were in good agreement with IVIS results. In this experiment, H2BGFP-HeLa cells were used, which allows identification of HeLa cancer cells by the green fluorescent protein (GFP) signal located in the cell nucleus.³⁸ Fluorescence signal was higher and more broadly distributed for cRGD-2IT-95 micelles containing the cRGD ligand, showing that tumor uptake was improved by the cRGD peptide (Figure 7B). It is also important to note that the fluorescent dye observed is attached to siRNA and not the polymer carrier. Thus, improved tumor accumulation for cRGD-2IT-95 micelles suggests that siRNA is associated with micelle structures at the time of cellular entry. In the current study, only \pm cRGD micelles were compared for tumor-targeting properties and no control peptide with a different amino acid sequence was used. However, specificity of the cRGD sequence has already been demonstrated, as changing the peptide sequence to cRAD abolishes tumor-targeting ability.³⁹

The micro distribution of siRNA within HeLa H2BGFP tumors, the region surrounding tumors, and blood vessels distant from tumors were further investigated IVRTCLSM in live mice 24 h after injection of cRGD-2IT-95 or 2IT-95 micelle formulations (Figure 8). Higher fluorescence signal in tumor cells and also enhanced accumulation of siRNA within blood vessels in close proximity (several hundred micrometers) to the tumor mass was observed for cRGD-2IT-95 micelles. Endothelial cells that are activated by tumor growth signals are known to express more integrin receptors that specifically bind to the cRGD peptide.⁴⁰ For cRGD-2IT-95 micelles, blood vessels containing fluorescent siRNA were abundant and easy to locate in the region surrounding the tumor mass and in vessels directly entering the tumor. In contrast, blood vessels could not be located in tumors treated with

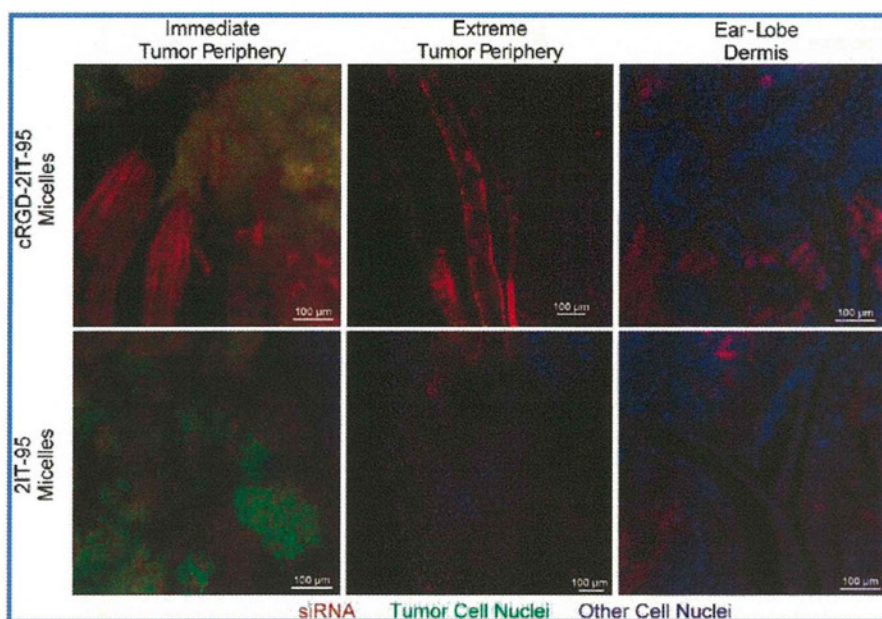


Figure 8. Micro distribution of siRNA 24 h post-injection of micelles. Micelle solutions prepared with Cy-5 siRNA were injected at a dose of $24 \mu\text{g}$ siRNA. Extreme tumor periphery corresponds to $500\text{--}1000 \mu\text{m}$ outside of the tumor mass.

2IT-95 micelles lacking the cRGD peptide, even after extensive searching. Blood vessel targeting was specific to the tumor region, as blood vessels in the ear-lobe dermis showed no siRNA accumulation. These findings are consistent with another report showing the accumulation of cRGD-containing particles in blood vessels associated with tumors.⁴¹ Altogether, the finding that cRGD-2IT-95 micelles showed enhance accumulation in both the tumor mass and also tumor-associated blood vessels suggests that both sites are accessible targets for therapeutic siRNA treatment.

Therapeutic Activity of siRNA Administered in Micelle Formulations. Polymeric siRNA micelles were evaluated for their ability to suppress the growth of subcutaneous HeLa tumors using an antiangiogenic treatment strategy. Angiogenesis is the process in which new blood vessels are formed within the region of a growing tumor in order to enhance the supply of nutrients to the cancer cells. Vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor-2 (VEGFR2) are central to the process of new blood vessel formation in growing tumors, and their effects are reviewed in detail elsewhere.^{42,43} VEGF is a protein excreted by tumor cells in order to initiate the angiogenic process, and expression can be up regulated in response to hypoxia in ischemic tissues (*i.e.*, poorly vascularized tumors). VEGFR2 controls the majority of downstream effects of VEGF and is expressed in blood vessel endothelial cells, with expression heightened in the region of growing tumors. Upon activation by VEGF, endothelial cells respond by increasing the microvascular permeability and also show increased proliferation and migration to form new blood vessels.⁴²

Antiangiogenic therapy with siRNAs contained in cRGD-functionalized siRNA nanoparticles was first described by Schiffelers *et al.* to inhibit ocular neovascularization as well tumor growth of a N2A murine neuroblastoma cancer model and has since been verified by another group as well.^{44–46} Thus, the previous precedent of this strategy for suppression of tumor growth seemed appropriate to confirm the *in vivo* efficacy of our micelle siRNA delivery system. In the above-mentioned studies, siRNAs targeting vascular endothelial growth factor receptors 1 and 2 (VEGFR1 and VEGFR2) were used. We chose to target VEGFR2 and also VEGF itself, in order to disrupt both the transmitting and receiving ends of the angiogenesis signal network. This strategy was chosen following observation of micelle accumulation in both tumor-associated blood vessels as well as the tumor mass. By simply changing the siRNA sequences used the specific target site of siRNA activity can be modulated to target both types of tissue showing siRNA accumulation, VEGFR2 siRNA targets blood vessel endothelial cells while VEGF siRNA targets the tumor mass.

Micelles were prepared incorporating VEGF or VEGFR2 siRNAs in separate micelles. Micelles containing the different siRNA sequences were injected ($24 \mu\text{g}$ siRNA/injection) separately on consecutive days, followed by two days with no injection, and this cycle was repeated three times. The 2 day gap in the injection cycle was chosen based on *in vitro* results that showed gene silencing gradually increased over 50 h, thus a properly timed dosing schedule could potentially prolong the RNAi benefit.

Growth curves of HeLa-luc tumors treated with micelle formulations are shown in Figure 9A. No tumor growth inhibition was observed for naked siRNA,

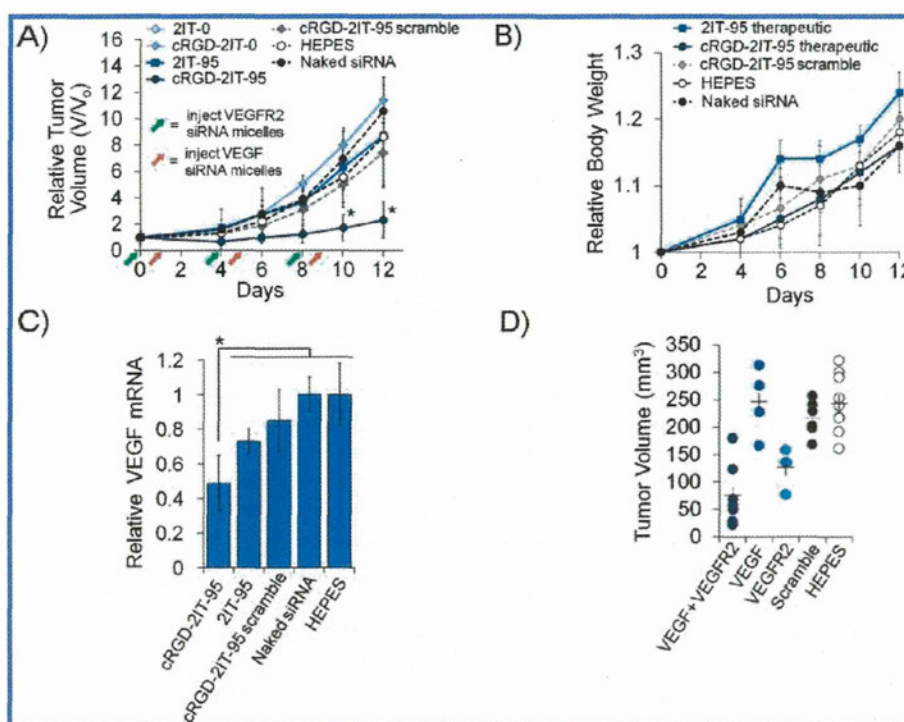


Figure 9. Treatment of subcutaneous HeLa-luc tumors with micelles containing antiangiogenic siRNA. (A) Tumor growth following treatment with micelles containing antiangiogenic siRNA, $n = 4$, $* p < 0.05$ relative to HEPES control tumors. (B) Mouse body weight during micelle treatment. (C) Quantification of VEGF mRNA in tumor tissue following treatment with micelles, $n = 4$, $* p < 0.05$. (D) Size of individual tumors following treatment with cRGD-2IT-95 micelles containing different siRNA sequences (day 12), $n = 4-8$, average tumor size is indicated by cross symbols.

2IT-95 micelles lacking the cRGD peptide, or cRGD-2IT-0 and 2IT-0 formulations. This is consistent with *in vitro* results, which also revealed that these formulations were ineffective at reducing luciferase expression in cultured HeLa-luc cells. In contrast, marked tumor growth inhibition was achieved for cRGD-2IT-95 micelles, which corroborates well with *in vitro* data showing that this formulation exerts RNAi activity (Figure 3A) and also with biodistribution results that demonstrated increased tumor accumulation for this formulation (Figure 7). No therapeutic effect was observed for cRGD-2IT-95 micelles prepared with scramble siRNA, demonstrating that sequence specificity was achieved. Additionally, no significant change in mouse body weight or overall health was noted following treatment with cRGD-2IT-95 micelles despite multiple injections, which suggests that there is no immediate or severe toxicity associated with micelle treatment (Figure 9B). Direct evidence of the RNAi effect in the tumor mass was found following reverse transcription polymerase chain reaction (RT-PCR) analysis of VEGF mRNA levels. The mechanism of siRNA activity results in degradation of target mRNA, which can be quantified by RT-PCR. Thus, this analysis provides molecular proof of the RNAi effect. Tumors treated with cRGD-2IT-95 micelles showed a clear reduction (~50%) of VEGF mRNA levels, whereas tumors treated with 2IT-95 micelles lacking the cRGD peptide were less effective,

and cRGD-2IT-95 micelles containing scramble siRNA showed no difference compared to naked siRNA and untreated tumors (Figure 9C). Together, these results confirm that cRGD-2IT-95 micelles can reduce protein expression in the tumor mass by the RNAi mechanism and that the cRGD peptide is critical for maximizing the siRNA activity of this formulation.

Attempts to quantify VEGFR2 mRNA levels in tumors were unsuccessful, as results were inconsistent and contained large errors despite repeated analysis (data not shown). This is likely due to the relatively low number of endothelial cells contained in the excised tumors, making reference to house-keeping genes difficult as a result of inhomogeneous cell populations. As a result, we qualitatively assessed the activity of individual siRNA sequences contained in cRGD-2IT-95 micelles in a different group of mice. Tumor growth inhibition was achieved for micelles containing VEGFR2 siRNAs, confirming that endothelial cell targeting was successful (Figure 9D). Interestingly, tumor growth was not inhibited following treatment with VEGF siRNA alone, although gene silencing was confirmed by RT-PCR experiments. This suggests that partial knockdown of VEGF in HeLa-luc tumors is ineffective to inhibit this fast growing tumor model. Overall, tumors treated with the combination of VEGF and VEGFR2 siRNAs grew the least, probably due to a synergistic effect when both types of siRNAs are used together.

In summary, we have found a multifunctional block copolymer that can be used to form polymeric micelles with siRNA that elicit therapeutic activity toward solid tumors following intravenous injection. Control of the chemistry contained in synthetic block copolymers improved micelle formation with siRNA and also the stability of resulting structures. Nearly complete amine modification of PEG-*b*-PLL with 2IT resulted in a unique and versatile polycation that formed stable micelle structures with siRNA without sensitivity to precise molar ratios of polymer and siRNA. High micelle stability was achieved through a combination of covalent disulfide cross-links in the micelle core and non-covalent interactions due to 2-iminothiolane ring structures in the polymer structure.

The cRGD peptide was crucial for realization of RNAi both *in vitro* and *in vivo*. On the cellular level, this is quite interesting because the cRGD-2IT-95 polymer used for micelle formation in this work does not have designed endosome escape functionality, as evidenced by the lack of activity for micelles without the cRGD peptide. Currently, many siRNA delivery systems are specifically designed to facilitate site-specific release of siRNAs from endosome and lysosome compartments, often exploiting the change in pH following accumulation in these subcellular vesicles. However, delivery efficacy can be improved by utilizing other biological pathways that change the subcellular trafficking and distribution of siRNA. This could prove to be a fundamental new design strategy for next-generation siRNA delivery vehicles that can avoid rapid accumulation in lysosomes.

The combination of improved blood stability and tissue-targeting ability of the cRGD peptide enhanced the accumulation of micelles in both tumor blood vessels and the tumor mass, allowing two tissue types to be utilized for antiangiogenic therapy. Herein lies a distinct advantage of siRNA therapeutics, as disease targets can be expanded by simply changing the nucleotide sequence of siRNA while utilizing the same carrier. This is often not the case for small molecule delivery systems where the chemistry of the carrier is matched with a specific drug (*e.g.*, doxorubicin hydrazone conjugates) and site-specific activity depends on site-specific targeting ability of the carrier.

Furthermore, the choice of siRNA sequences is critical for therapeutic benefit to be realized. Here, we found that VEGFR2 was more effective than VEGF for HeLa-luc tumor growth inhibition, although VEGF mRNA knock-down was confirmed.

We will continue to explore ways to expand upon this siRNA delivery platform and increase its efficacy and scope of treatable targets. Modulating the types of siRNA contained in the micelle core can easily be achieved once new target sequences are identified. Additionally, conjugation of different peptides to the polymer could provide specificity for different disease sites or further alter the subcellular trafficking of micelles. The chemistry used to couple the cRGD peptide can be extended to any peptide containing an N-terminal cysteine, thus providing a convenient synthetic handle for polymer conjugation. Development of effective siRNA therapies continues to be a major focus in our lab and in the field of drug delivery. The cRGD-2IT-95 polymeric micelle siRNA delivery system described in this work demonstrates the feasibility of cancer treatment by intravenous injection of therapeutic siRNAs and represents a significant step forward for development of this therapeutic modality toward practical application.

CONCLUSIONS

In this study, we identified a polymeric micelle formulation that is capable of delivering siRNA to solid tumors and exerting a therapeutic benefit by RNAi activity. PEG-*b*-PLL served as the core polymer structure for micelle formation, but modification of lysine amines with 2-iminothiolane was needed for increased stability, and installation of the cRGD peptide on the PEG terminus was necessary for improved biological activity including: RNAi activity, improved cell uptake, and broader subcellular distribution. Stable and targeted micelle structures improved siRNA accumulation within tumors and their associated blood vessels following IV injection, and effective tumor treatment was realized by targeting angiogenic proteins for knock-down at both sites of micelle accumulation. These results demonstrate that polymeric micelles can be used for siRNA cancer therapies administered by intravenous injection.

METHODS

General. *N*-Methyl-2-pyrrolidinone (NMP, 99.5% anhydrous), LiCl (>99%), diisopropylethylamine (DIPEA, 99.5%), 2 N HCl solution, D₂O (99.9%), tetramethylsilane (TMS, 99.5%), and DCI (35% in D₂O) were obtained from Sigma Aldrich (St. Louis, MO) and used without further purification. 2-Iminothiolane hydrochloride (2IT), diethyl ether (99+%), dithiothreitol (DTT, molecular biology grade DNase and RNase free), ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA, 99.5%), sodium dihydrogen phosphate · 2H₂O (99–102%), disodium hydrogen phosphate · 12H₂O (99+%), glutathione (reduced form), and

sodium chloride (99+%) were supplied by Wako Pure Chemical Industries (Osaka, Japan). Ellman's reagent [5,5-dithio-bis-(2-nitrobenzoic acid)] and slide-a-lyzer dialysis cassettes (MWCO = 3.5 kDa) were obtained from Thermo Scientific (Rockford, IL). Sterile HEPES (1 M, pH 7.3) was purchased from Amresco (Solon, OH). Spectra/Por dialysis tubing (6–8 kDa MWCO) was acquired from Spectrum Laboratories (Rancho Dominguez, CA). The cyclo-[RGDFK(C-ε-Acp) peptide (cRGD peptide) was purchased from Peptide Institute Inc., Osaka, Japan.

siRNAs were synthesized by Hokkaido System Science Co., Ltd., and sequences used are as follows: (1) Firefly GL3 luciferase:

5'-CUU ACG CUG AGU ACU UCG AdTdT-3' (sense), 5'-UCG AAG UAC UCA GCG UAA GdTdT-3' (antisense); (2) scramble: 5'-UUC UCC GAA CGU GUC ACG UdTdT-3' (sense), 5'-ACG UGA CAC GUU CGG AGA AdTdT-3' (antisense); (3) VEGF (human): 5'-GGA GUA CCC UGA UGA GAU CdTdT-3' (sense), 5'-GAU CUC AUC AGG GUA CUC CdTdT-3' (antisense); (4) VEGFR2 sequence 1 (mouse): 5'-AUG CGG CGG UGG UGA CAG UdTdT-3' (sense), 5'-ACU GUC ACC ACC GCC GCA UdTdT-3' (antisense); (5) VEGFR2 sequence 2 (mouse): 5'-AGC UCA GCA CAC AGA AAG AdTdT-3' (sense), 5'-UCU UUC UGU GUG CUG AGC UdTdT-3' (antisense). Fluorescent firefly GL3 luciferase siRNA contained the fluorophore (Cy3, Cy5, or Cy5.5) attached to the sense strand.

¹H NMR analysis of cRGD-PEG-*b*-PLL and 15–45% 2IT modified polymers was conducted in D₂O containing 0.05% v/v tetramethylsilane at 22 °C using a 300 MHz spectrometer (EX 300, JEOL, Tokyo, Japan). cRGD-2IT-95 and 2IT-95 polymers were analyzed in D₂O containing 0.05% v/v tetramethylsilane and 3 μL/mL DCl solution (35% DCl in D₂O) at 22 °C using a 300 MHz spectrometer (EX 300, JEOL, Tokyo, Japan). Static and dynamic light scattering measurements were performed at 25 °C on a ZetaSizer Nano ZS instrument (Malvern Instruments Ltd., Malvern, UK) equipped with a He–Ne laser ($\lambda = 633$ nm) as the incident beam with samples (16 μL) loaded into a Zen 2112 low-volume cuvette. Absorbance and fluorescence measurements were performed with NanoDrop ND-1000 and ND-3300 instruments (NanoDrop Technologies Inc., Rockland, DE), respectively.

The luciferase-expressing human cervical cancer cell line, HeLa-luc, was purchased from Caliper LifeScience (Hopkinton, MA). Dulbecco's modified Eagle's medium (DMEM) was obtained from Sigma Aldrich (St. Louis, MO). Fetal bovine serum was provided by Dainippon Sumitomo Pharma Co. (Osaka, Japan). Falcon Easy-Grip 35 × 10 mm vacuum gas plasma-treated polystyrene tissue culture dishes were obtained from BD Biosciences (San Jose, CA). Luciferin was purchased from Summit Pharmaceutical International (Tokyo, Japan). Luciferase bioluminescence in HeLa-luc cells was measured using an ATTO Kronos Dio photon countable incubator (ATTO Corp., Tokyo, Japan).

All animal experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals as stated by the National Institutes of Health.

Block Copolymer Synthesis. Acetal-PEG-*b*-PLL block copolymer was synthesized as previously described, comprising a 12 000 MW PEG segment and a 45 amino acid PLL segment, $M_w/M_n = 1.2$, and all modified polymers were prepared from the same polymer stock.²⁶ Attachment of cRGD and modification of PLL amines was performed sequentially in aqueous and organic conditions, respectively.

cRGD was coupled to the terminus of acetal-PEG-*b*-PLL by reaction of the N-terminal cysteine contained on cRGD with the aldehyde generated in acetal-PEG-*b*-PLL following incubation at acidic pH. cRGD peptide (26.2 mg, 5 equiv relative to acetal-PEG-*b*-PLL) was dissolved in 1 mL of 10 mM phosphate, pH 7.4, followed by addition dithiothreitol (5 mg, 1 equiv relative to cRGD). The solution was stirred at 25 °C for 30 min to reduce any disulfides present. Acetal-PEG-*b*-PLL (125 mg, 1 equiv) was separately dissolved in 0.2 M sodium acetate buffer, pH 4.0 (5 mL), and then reduced cRGD peptide solution was added dropwise while stirring. The reaction continued for 4 days at 25 °C with continuous stirring. After the reaction period, crude product was transferred to dialysis tubing (SpectraPor, 6–8K MWCO) and dialyzed against 10 mM PBS, pH 7.4, for 2 days, then distilled water for 2 days. Purified cRGD-PEG-*b*-PLL solution was passed through a 0.22 μm filter then frozen and lyophilized. Yield: 112 mg, ~86%, white powder. The amount of cRGD conjugated to the polymer was estimated by ¹H NMR, using the integration ratio of phenyl CH proton peaks of cRGD to PEG CH₂ backbone peaks. The cRGD introduction rate was typically 70–80%.

2-Iminoethanol modification was achieved by reaction of primary amines in PLL with 2IT according to our previously described method.¹⁵ First, cRGD-PEG-*b*-PLL (55 mg, 0.12 mmol amine, 1 equiv) was added to 5 mL of *N*-methyl pyrrolidinone (NMP) containing 5 wt % LiCl, and the reaction vessel was

purged with Ar, capped with a septum, and stirred. The polymer solution was stirred vigorously at 50 °C for 30 min to completely dissolve all solids. Next, diisopropylethylamine (DIEA, 252 μL, 5 equiv relative to lysine amines) was added to the polymer solution under Ar through the septum. 2IT·HCl (106 mg) was separately dissolved in NMP containing 5% LiCl (7.86 mL) and DIEA (134 μL, 1 equiv relative to 2IT) under argon to yield a 96 mM solution. 2IT solution (3 mL, 2.4 equiv relative to Lys amines) was added to the polymer solution dropwise while stirring under Ar atmosphere, and the reaction continued for 18 h at 25 °C. After 18 h, the reaction was terminated by precipitation into a 10-times volume excess of dry diethyl ether. Precipitated product was washed several times with ether and dried under vacuum to a constant mass. Crude product was dissolved in PBS buffer, pH 6.0, and then dialyzed (SpectraPor7, 10 kDa MWCO) against PBS pH 6.0 for 1 day and distilled water for 1 day with frequent media changes. Dialyzed product solution was passed through a 0.2 μm filter and then lyophilized. Yield: 61 mg (~85%), white powder. The degree of IM introduction was determined from the ¹H NMR spectrum by the peak intensity ratio of the β , γ , δ -methylene protons of Lys ((CH₂)₃, $\delta = 1.3$ –1.9 ppm) to the protons of trimethylene units of mercaptopropyl groups (HS-(CH₂)₃, $\delta = 2.1$ –2.8 ppm). The calculated 2IT introduction rate was ~95%. The cRGD content of polymers did not change following the 2IT modification procedure.

Analysis of Thiol Content. Polymer solutions (5 mg/mL) were incubated in 10 mM HEPES buffer containing 5 mM EDTA and 15 mM DTT for 30 min at room temperature to reduce any disulfides present. The reduced polymer solution was placed on ice and handled in a timely manner at 0–4 °C until the addition of Ellman's reagent.⁴⁷ After reduction, DTT was removed from the polymer solution using a NanoSep centrifugation device (3000 MWCO). Samples were subjected to three successive concentration/rinsing cycles with 10 mM HEPES containing 5 mM EDTA as the rinsing buffer. After the final centrifugation cycle, concentrated polymer solution was collected and diluted to its original volume. The final flow-through fraction was also collected and diluted in the same manner as the polymer-containing fraction to determine the amount of DTT remaining in the sample. Polymer and flow-through samples were subjected to Ellman's assay according to the manufacturer's protocol, and sample absorbance was measured at 412 nm. Free thiol content of solutions were determined from a standard curve generated with reduced glutathione. Polymer thiol content was obtained by subtracting the thiol content in the flow-through fraction to correct for residual DTT. PEG-*b*-PLL was analyzed as a negative control in a similar fashion, except the polymer solution (5 mg/mL) was used directly without DTT incubation. Thiol content in cross-linked micelles was determined similarly as described above, except that micelle solutions were added directly to Ellman's reagent solution without additional treatment as described for polymer samples.

Micelle Preparation. Polymer samples were dissolved in 10 mM HEPES buffer (pH 7.4) at a concentration of 5 mg/mL. For cRGD-2IT-0 and 2IT-0 micelles, polymer stock solution was further diluted to the polymer/siRNA molar ratio of 1.2 and mixed with siRNA solution (15 μM in HEPES, pH 7.4). The solution was vortexed briefly to yield micelles. For cRGD-2IT-95 and 2IT-95 micelles, DTT was added (30 mg/mL) to polymer stock solution, and the polymer was incubated for 30 min at room temperature to cleave any disulfides present. After DTT reduction, polymer solution was added to siRNA solution (15 μM in HEPES, pH 7.4) and the mixture was vortexed. Micelle solution was then transferred to a slide-a-lyzer cassette (MWCO = 3.5 kDa) and dialyzed against HEPES (pH 7.4) containing 0.5% DMSO for 2 days, followed by dialysis against HEPES (pH 7.4) for 2 days. Micelles were recovered from the slide-a-lyzer cassette and passed through a 0.2 μm filter before use.

Micelle Characterization. Micelles were analyzed by static and dynamic light scattering (DLS) to determine scattered light intensity (SLI) and size/PDI, respectively. Size distributions were determined by cumulant and histogram analysis of DLS data using the software provided by the manufacturer. Results are shown as the z-average diameter (cumulant mean) with the polydispersity index (PDI) (defined in the ISO standard