Targeted Polymeric Micelles for siRNA Treatment of Experimental Cancer by Intravenous Injection

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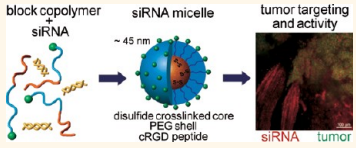
ABSTRACT Small interfering ribonucleic acid (siRNA) cancer therapies administered by intravenous injection require a delivery system for transport from the bloodstream into the cytoplasm of diseased cells to perform the function of gene silencing. Here we describe nanosized polymeric micelles that deliver siRNA to solid tumors and elicit a therapeutic effect. Stable multifunctional micelle structures on the order of 45 nm in size formed by spontaneous self-assembly of block copolymers with siRNA. Block copolymers used for micelle formation were designed and synthesized to contain three main features: a siRNA binding segment containing thiols, a hydrophilic nonbinding segment, and a cell-surface binding peptide. Specifically, poly(ethylene glycol)-*block*-poly(L-lysine) (PEG-*b*-PLL) comprising lysine amines modified with 2-iminothiolane (2IT) and the cyclo-Arg-Gly-Asp (cRGD) peptide on the PEG terminus was used. Modification of PEG-*b*-PLL with 2IT led to improved control of micelle formation and also increased stability in the blood compartment, while installation of the cRGD peptide improved biological activity. Incorporation of siRNA into stable micelle structures containing the cRGD peptide resulted in increased gene silencing ability, improved cell uptake, and broader subcellular distribution *in vitro* and also improved accumulation in both the tumor mass and tumor-associated blood vessels following intravenous injection into mice. Furthermore, stable and targeted micelles inhibited the growth of subcutaneous HeLa tumor models and demonstrated gene silencing in the tumor mass following treatment with antiangiogenic siRNAs. This new micellar nanomedicine could potentially expand the utility of siRNA-based therapies for cancer treatments that require intravenous injection.



KEYWORDS: siRNA delivery · block copolymer · micelle · cRGD · cancer therapy

Small interfering ribonucleic acid (siRNA) therapeutics have great potential for treatment of disease through inhibition of protein expression, but transfer of this technology to the clinic has proven to be a great challenge. siRNA-based therapies block the expression of aberrant proteins using a natural subcellular pathway that degrades messenger RNA (mRNA) based on the nucleotide sequence contained in the siRNA molecule, a process known as RNA interference (RNAi).[1] siRNA is a 20−23 base pair degradation product generated from longer double-stranded RNA and represents the smallest double-stranded RNA deliverable that can gain access to the RNAi pathway. Potent inhibition of gene expression can be achieved due to the catalytic nature of RNAi, which results in formation of an activated RNA-induced silencing complex (RISC) that can degrade multiple strands of mRNA that are complementary to the loaded siRNA strand.[2]

The molecular machinery necessary for RNAi is located in the cell cytoplasm, thus therapeutic siRNA must also localize in the cytoplasm to exert the desired effect of protein knockdown. However, siRNA is a large water-soluble polyion (~13 000 MW) that has unique delivery obstacles not typically encountered with small hydrophobic molecules such as anticancer drugs. Due to its large size and anionic nature, siRNA cannot readily diffuse across cell membranes and requires active internalization into cells by endocytosis. Furthermore, administration of siRNA by systemic injection is challenging due to the immunogenicity of naked siRNA, degradation into inactive fragments by enzymatic activity, high renal clearance, and overall poor accumulation at target sites.[3−6] Efforts to overcome the above-mentioned obstacles has resulted in development of next-generation siRNA delivery strategies including chemically modified siRNAs that increase stability and suppress immune system activation, as well

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as development of many types of particle-based systems that reversibly encapsulate siRNA. Particle-based systems are often designed to exploit the large polyanionic structure of siRNA to form polyion complexes which assemble into aggregates, vesicles, or micelles. Such particles have been prepared from cationic lipids, lipidoids, peptides, as well as synthetic polymers and block copolymers and are typically several tens to hundreds of nanometers in size. The most sophisticated delivery systems to date include features such as a PEG coating to control particle morphology and reduce nonspecific interactions with biological components, reversible cross-links for site-specific release of siRNA, environment-sensitive polymers or peptides to assist subcellular trafficking, and cell-targeting moieties to improve cell uptake and specificity. Several excellent reviews describing siRNA delivery systems in detail have recently been published.[7−14]

In this report, we introduce a nanomedicine capable of encapsulating siRNA and then delivering it through the bloodstream to tumor models in mice. This micellar siRNA delivery system was prepared using a block copolymer containing features to improve micelle stability and biological activity. Block copolymer chemistry was chosen based on our previous work with poly(ethylene glycol)-*block*-poly(L-lysine) (PEG-*b*-PLL) containing lysine amines modified with 2-iminothiolane (2IT). We found that this polymer formed nanosized micelle structures with siRNA, which prolonged blood circulation; however, RNAi activity was low.[15,16] Here, we further improved the performance of micelles by incorporating a short peptide on the micelle surface to enhance cell uptake and distribution of siRNA on the subcellular and whole organism levels. Specifically, we used the cyclo-arginine-glycine-glutamic acid (cRGD) peptide, which binds to integrin receptors that are displayed on the surface of several types of tumors and also endothelial cells associated with growing tumors.[17−20] Addition of the cRGD peptide to the micelle structure resulted in a targeted nanoparticle capable of directing siRNA to the site of activity, improving tumor accumulation and cell uptake following intravenous injection. We found that the cRGD-containing micelles improved siRNA activity both *in vitro* and *in vivo* through a combination of improved cell uptake, broadened subcellular distribution, blood stability, and tumor accumulation.

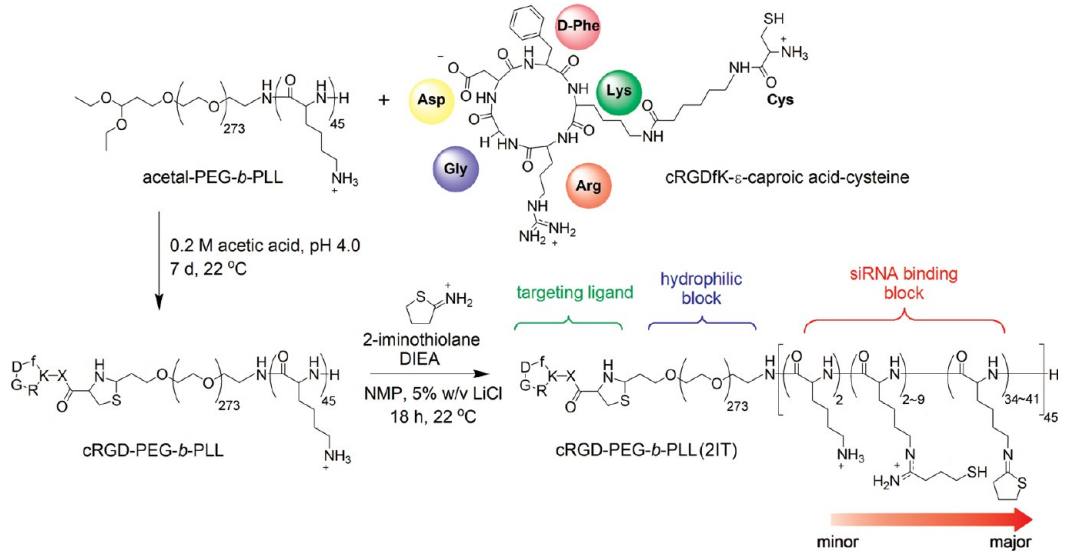
RESULTS AND DISCUSSION

Design and Synthesis of Block Copolymers. Formation of micelles with siRNA requires a material capable of binding a large polyanion and also directing assembly into higher ordered multimolecular structures. Such necessities can be fulfilled by block copolymers, which contain regions of distinctly different chemistries tailored to meet functional demands. Block copolymers used to facilitate micelle formation with siRNA in this work were designed and synthesized to contain three basic features: (i) a cationic segment with thiol groups, (ii) a hydrophilic and biologically benign segment, and (iii) a cell-surface-targeting moiety. This polymer design facilitates micelle formation following electrostatic interaction between oppositely charged macromolecules, resulting in charge neutralization and self-assembly into micelle structures with siRNA contained in the core which is surrounded by a PEG shell. Since polymers and siRNA self-assemble into core−shell micelle structures, modification of the distal end of PEG allows presentation of bioactive moieties, such as the cRGD peptide, on the micelle surface. Chemically, these features were produced from an acetal-PEG-*b*-PLL block copolymer, with the PLL segment serving as the siRNA binding region and also the point of chemical modification by reaction with lysine amines. Acetal functionality contained on the PEG terminus provided a protected aldehyde that was regenerated at low pH to provide the site of attachment for the cRGD peptide by reaction with an N-terminal cysteine residue.[21]

In our previous work, we found that PEG-*b*-PLL alone does not form stable micelle structures with siRNA, so we further modified PEG-*b*-PLL amines with 2IT to introduce amidines and free thiols into the lysine segment of the block copolymer. This modification was aimed to improve the stability of micelle structures through disulfide cross-linking in the core, which can also provide environment-sensitive stabilization of micelle structures following reduction of the covalent disulfide cross-links by free thiols in solution. Disulfide reduction is likely to occur faster within cells than in the bloodstream due to higher glutathione concentrations inside of cells, thus offering site-specific siRNA release functionality.[22] While 2IT modification of PEG-*b*-PLL resulted in the introduction of thiol groups, it also resulted in the formation of cyclic N-substituted 2-iminothiolane ring structures in the lysine side chains, which also showed a micelle-stabilizing effect.[16]

The overall synthesis scheme for preparation of modified polymers starting from acetal-PEG-*b*-PLL (PEG M_w = 12 000, PLL degree of polymerization = 45) is shown in Scheme 1. First, acetal-PEG-*b*-PLL was reacted with excess cRGD peptide at pH 4.0 to produce the peptide−polymer conjugate *via* thiazolidine ring formation between the aldehyde generated on PEG and the N-terminal cysteine residue contained on the cRGD peptide. Analysis of the peptide−polymer conjugate by ^{1}H NMR showed that the reaction was successful, with the appearance of cRGD benzyl protons (D-phenyl alanine) observed (Figure 1A). Further analysis of the integration values determined that ~80% of polymer chains contained conjugated cRGD.

Next, cRGD-PEG-*b*-PLL or acetal-PEG-*b*-PLL was reacted with 2IT to produce cRGD-PEG-*b*-PLL(2IT) and PEG-*b*-PLL(2IT), respectively. For simplicity, polymers



Scheme 1. Modification of PEG-*b*-PLL with cRGD and 2-iminothiolane.

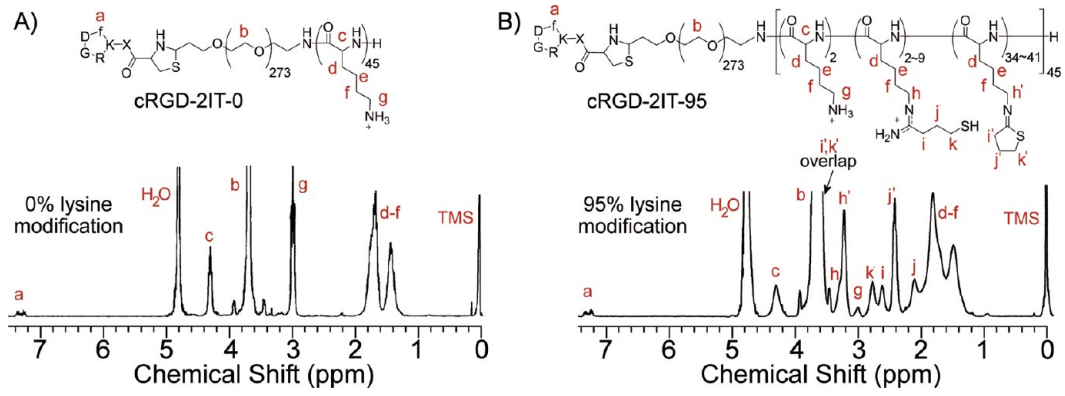


Figure 1. Characterization of polymer products. (A) ^{1}H NMR spectrum of cRGD-PEG-*b*-PLL recorded in D_2O. (B) ^{1}H NMR spectrum of cRGD-PEG-*b*-PLL(2IT) recorded in D_2O containing 3 μL/mL 35% DCl. Polymer composition was estimated based on the ^{1}H NMR spectrum and also results of Ellman's assay shown in Table 1.

are denoted as *X*2IT-*Y*, with "*X*" indicating the presence of the cRGD peptide and "*Y*" indicating the degree of 2IT modification. Polymer modification proceeded *via* nucleophilic reaction of lysine amines with 2IT in the presence of an organic base, with ~2.4 molar equiv of 2IT needed for nearly complete conversion of lysine amines. The degree of 2IT modification was determined to be 95% by ^{1}H NMR analysis, using the ratio of integration values of lysine β, γ, and δ-methylene protons ($(CH_2)_3$, δ = 1.3−1.9 ppm) to values corresponding to 2IT moieties (Figure 1B). Reaction of lysine amines with 2IT generated both 1-(4-mercaptobutyl)-amidine and cyclic iminothiolane functional groups in the PLL segment of the block copolymer, and cyclic iminothiolanes were the major product (~80%) under the reaction conditions used here. Ellman's assay for free thiols revealed a polymer thiol content much lower than the degree of lysine modification, which is consistent with iminothiolane ring formation as this functional group lacks free thiols.

TABLE 1. Summary of Polymer Compositions

polymer	MW[a]	2IT modified lysines (%)[a]	free thiol content (% side chains)[b]
2IT-0	19500	0	0.08 ± 0.04
cRGD-2IT-0	20300	0	0.5 ± 0.07
2IT-95	21800	95	4.6 ± 1.6
cRGD-2IT-95	22500	95	5.1 ± 0.7

[a] Calculated from ^{1}H NMR integration values. [b] Determined by Ellman's assay.

Preparation of Micelles. Micelle structures formed spontaneously upon mixing block copolymer with siRNA, and the theoretical configuration of the particle showing siRNA in the core, a PEG shell, and the cRGD peptide on the micelle surface is depicted in Figure 2A. Micelle formulations are named based on the polymer used for preparation (Table 2.) Micelle formation behavior was different between 2IT modified and unmodified polymers, as scattered light intensity (SLI) analysis showed that micelle structures only formed

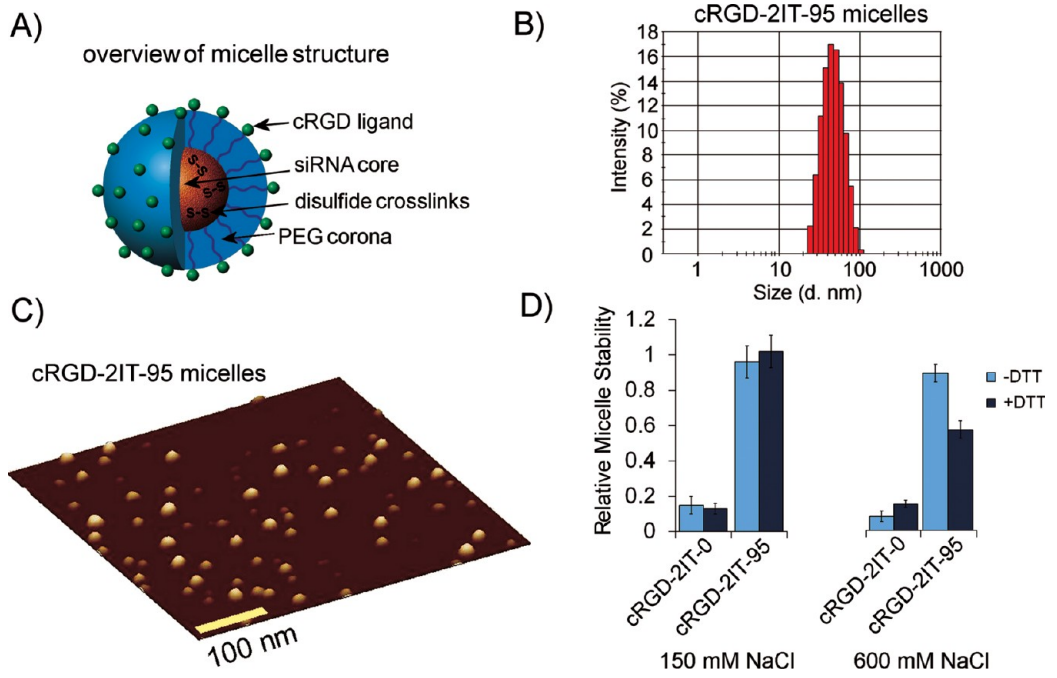


Figure 2. Micelle structure and properties. (A) Schematic representation of polymeric micelle structure and key components. (B) Size distribution histogram of cRGD-2IT-95 micelles determined by dynamic light scattering showing narrowly dispersed particles. (C) Atomic force microscopy image of cRGD-2IT-95 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[a]	7.6	45 ± 2	0.09 ± 0.03	−1.99 ± 0.55	1.7 ± 0.9
cRGD-2IT-95[a]	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 + (∼9 nm × 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, ∼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

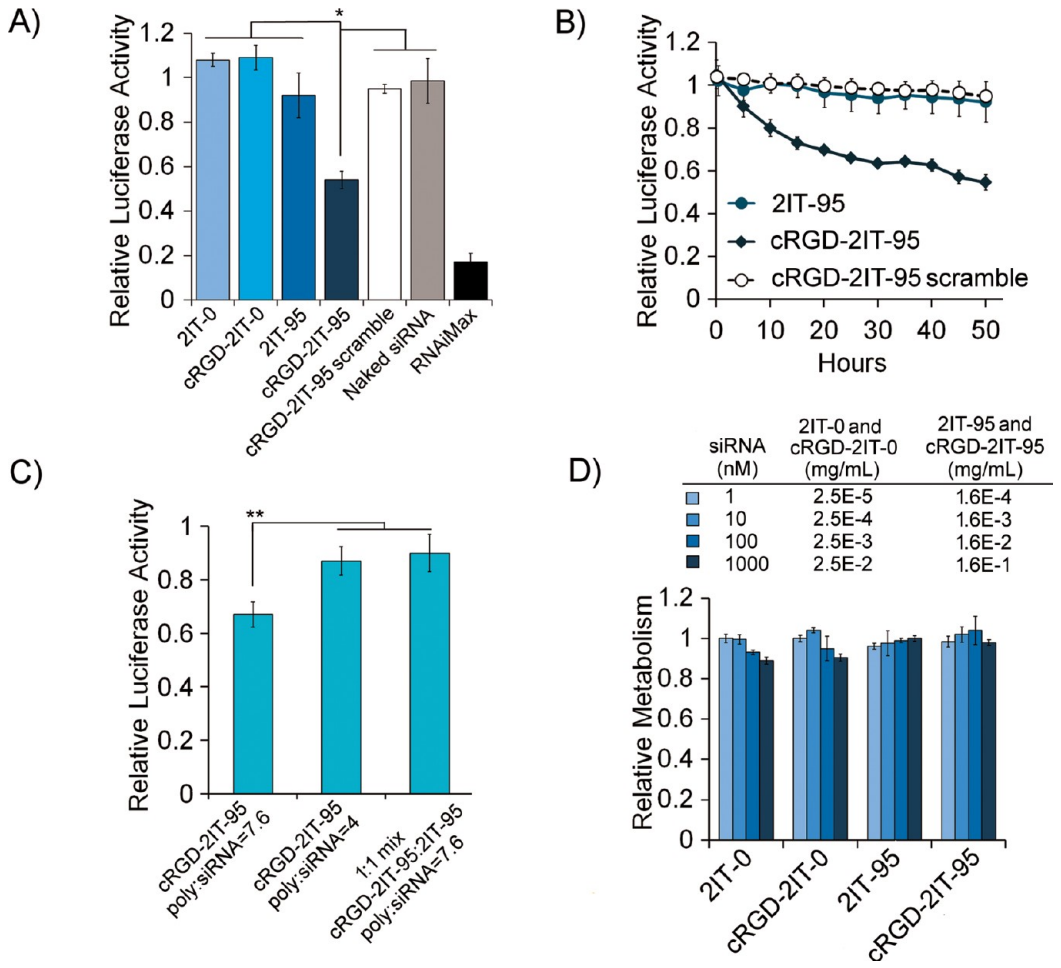


Figure 3. Gene silencing ability of siRNA micelles and tolerance in HeLa-luc cancer cells. (A) Quantification of luciferase luminescence following incubation with micelles containing antiluciferase siRNA (or scramble siRNA as noted in legend). Micelles were prepared at the optimal polymer/siRNA ratio 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.

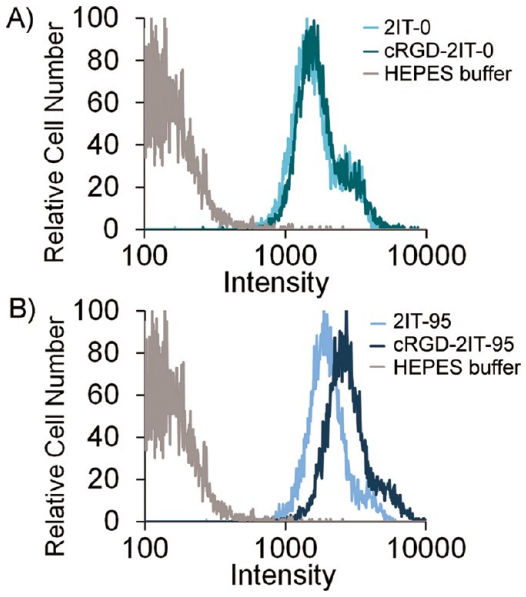


Figure 4. Cellular uptake of micelles in HeLa-luc cells. Cells were incubated with micelles prepared with fluorescent siRNA for 2 h (300 nM siRNA) prior to analysis by flow cytometry.

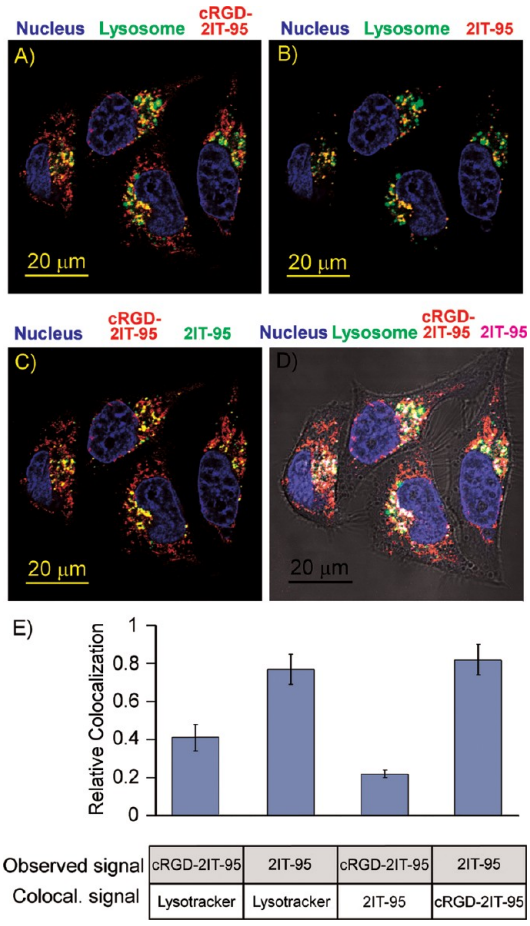


Figure 5. Subcellular distribution of 2IT-95 and cRGD-2IT-95 micelles following coincubation in HeLa-luc cells for 4 h (500 nM siRNA for each micelle formulation). Lysosomes were stained with LysoTracker green and micelles were prepared with Cy3- or Cy5-labeled siRNA for cRGD-2IT-95 and 2IT-95 formulations, 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).

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 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,

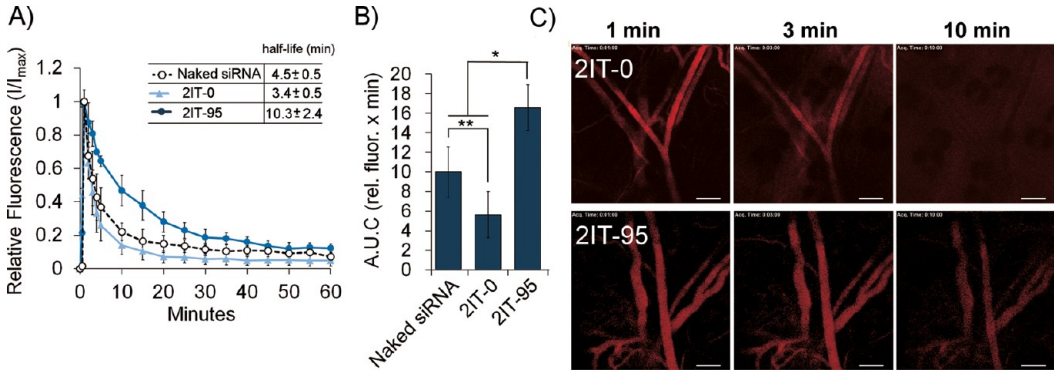


Figure 6. Blood circulation properties of micelles following tail-vein injection (24 μg siRNA). (A) Fluorescence signal over time and calculated half-lives, $n = 3$. (B) Area under the curve values 60 min after injection, $n = 3$, * $p < 0.05$, ** $p = 0.1$. (C) Ear-lobe dermis snap-shots at 1, 3, and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

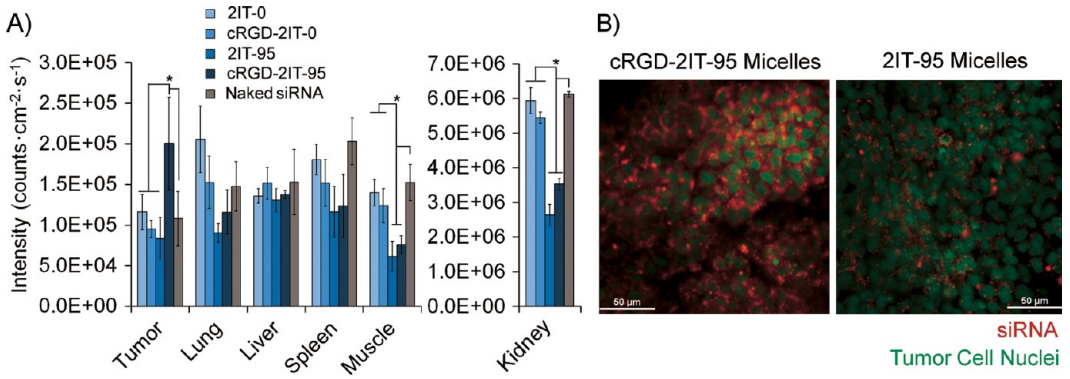


Figure 7. Biodistribution of siRNA administered in micelle formulations 24 h post-injection (24 μg siRNA injected). (A) Quantification of Cy5.5-siRNA fluorescence by IVIS, $n = 4$, *$p < 0.05$. (B) IVRTCLSM image of H2BGFP-HeLa tumors in live mice 24 h after injection of micelles prepared with Cy5-siRNA (24 μg 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 ~2× 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 ~5−10 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 +/− 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

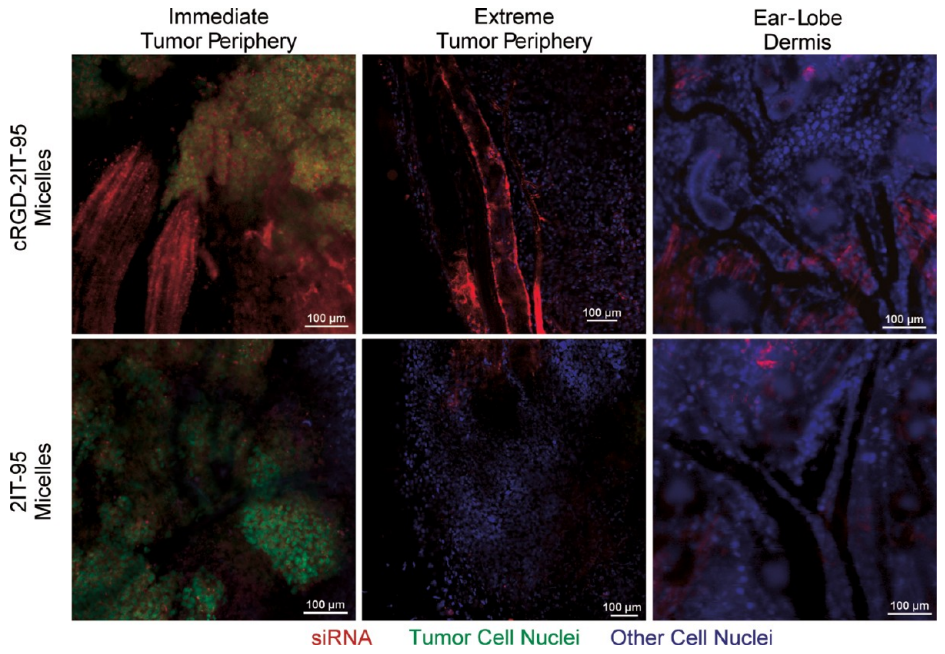


Figure 8. Micro distribution of siRNA 24 h post-injection of micelles. Micelle solutions prepared with Cy-5 siRNA were injected at a dose of 24 μg siRNA. Extreme tumor periphery corresponds to 500−1000 μm outside of the tumor mass.

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 μg 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,

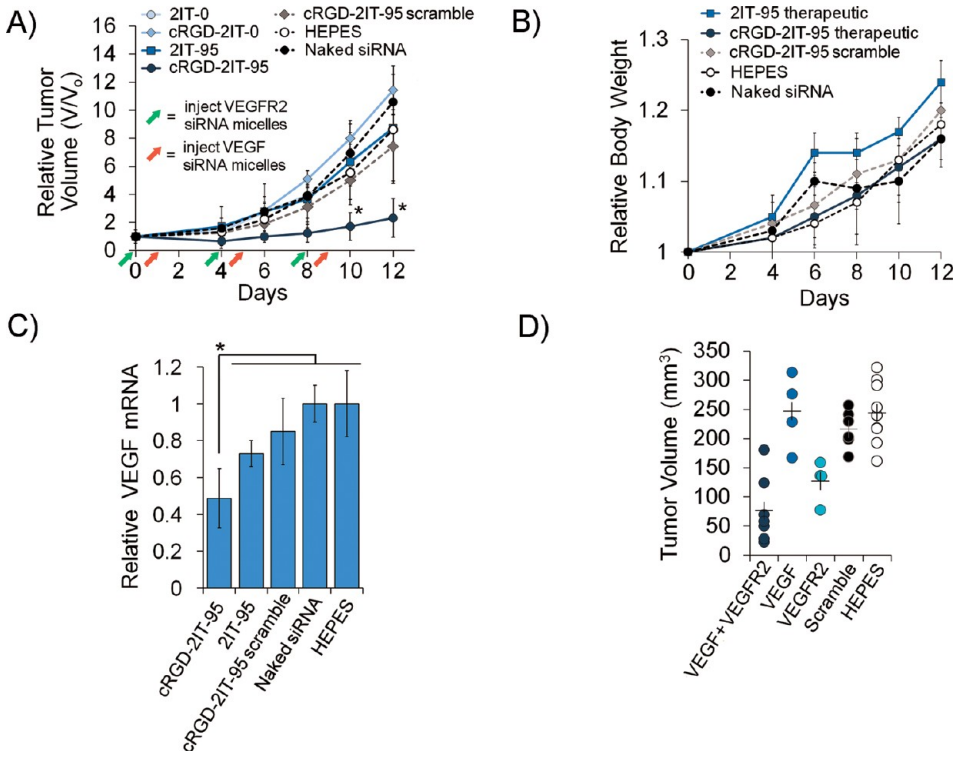


Figure 9. Treatment of subcutaneous HeLa-luc tumors with micelles containing antiangiogenic siRNA. (A) Tumor growth following treatment with micelles containing antiangiogenic siRNA, $n = 4$, * $p < 0.05$ relative to HEPES control tumors. (B) Mouse body weight during micelle treatment. (C) Quantification 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 noncovalent 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 knockdown 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 knockdown 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, D_2O (99.9%), tetramethylsilane (TMS, 99.5%), and DCl (35% in D_2O) 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·$2H_2O$ (99−102%), disodium hydrogen phosphate·$12H_2O$ (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.

^{1}H NMR analysis of cRGD-PEG-*b*-PLL and 15−45% 2IT modified polymers was conducted in D_2O 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 D_2O containing 0.05% v/v tetramethylsilane and 3 μL/mL DCl solution (35% DCl in D_2O) 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 (λ = 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 ^{1}H NMR, using the integration ratio of phenyl CH proton peaks of cRGD to PEG CH_2 backbone peaks. The cRGD introduction rate was typically 70−80%.

2-Iminothiolane 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 ^{1}H NMR spectrum by the peak intensity ratio of the β, γ, δ-methylene protons of Lys ($(CH_2)_3$, δ = 1.3−1.9 ppm) to the protons of trimethylene units of mercaptopropyl groups (HS-$(CH_2)_3$, δ = 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

document 13321:1996) and histogram of size distribution. The ζ-potentials of micelles were measured by laser Doppler electrophoresis in 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl at 37 °C. All samples were equilibrated to the defined temperature for 2 h prior to measurement. Atomic force microscopy (AFM) imaging of the siRNA complexes was carried out in air using a Bruker AXS MMAFM Nanoscope V (Madison, WI) operated by Scan Assist with a standard silicon probe on the freshly cleaved mica substrate. Raw AFM images were processed by flattening to remove the background slope of the substrate surface.

***In Vitro* Micelle Stability.** Micelle stability was measured in the presence of 150 and 600 mM NaCl in the presence or absence of the disulfide reducing agent DTT. Micelle samples were diluted 1:1 with NaCl solution at desired concentrations and incubated at 37 °C for 24 h. Samples subjected to disulfide reducing conditions were diluted in the same fashion as above, however, with NaCl solutions containing 200 mM DTT. After the 24 h incubation period, samples were measured by static and dynamic light scattering as described in the micelle characterization section. Relative micelle stability was determined by dividing the scattered light intensity of treated samples with the scattered light intensity of samples diluted in the same fashion with only HEPES buffer.

***In Vitro* Gene Silencing.** HeLa human cervical cancer cells stably expressing luciferase (HeLa-luc) were seeded onto 35 mm Petri dishes (25 000 cells/dish) and allowed to attach for 24 h. After cell attachment, the medium was removed and replaced with media (2 mL) containing 100 μM luciferin and cross-linked micelles (200 nM siRNA). For each analysis, control samples were prepared by addition of media diluted with HEPES instead of micelle solution. The total dilution of media after addition of luciferin and micelle solution was less than 200 μL additives per 10 mL of media. Samples were placed into a Kronos real-time photon countable incubator, and the luminescence intensity was measured periodically over a 50 h time period, with the temperature and CO_2 maintained at 37 °C and 5%. Relative luminescence was determined by dividing the average luminescence intensity of treated samples by the average luminescence intensity of control samples, $n = 4$. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and antiobiotics during the experiment.

Cytotoxicity. HeLa-luc cells were seeded onto 24-well plates (10 000 cells/well) and allowed to attach for 24 h under standard cell culture conditions. Next, the medium was removed and replaced with fresh media supplemented with cross-linked micelles at the desired siRNA concentration. Cells were further incubated with micelle-containing media for 48 h. Metabolism was assessed following 1 h incubation with cell counting kit 8 solution (1 μL/10 μL media) (CCK 8, Dojindo Laboratories, Kumamoto, Japan) followed by absorbance measurement of extracellular media at 450 nm. All data are expressed relative to untreated control cells, $n = 6$.

Cell Uptake. HeLa-luc cells were seeded onto 6-well plates (100 000 cells/well) and allowed to attach for 24 h. After the attachment period, medium was exchanged with fresh media containing micelles prepared with Cy3-siRNA (300 nM). Cells were incubated with micelle-containing media for 2 h under standard cell culture conditions then rinsed four times with PBS and harvested by trypsinization. Harvested cells were subjected to flow cytometric analysis using a BD LSR II instrument (BD Biosciences, San Jose, CA) equipped with appropriate excitation/emission filter combinations, and forward and side-scatter gates were set to exclude debris. A total of 10 000 events were recorded for each analysis, and data were analyzed using BD FACSDiva software (BD Biosciences).

***In Vitro* Confocal Microscopy.** HeLa-luc cells (50 000 cells) were seeded onto a 35 mm glass bottom dish (Iwaki, Tokyo, Japan) and allowed to attach for 24 h. Micelles were prepared with Cy5 siRNA (2IT-95) or Cy3 siRNA (cRGD-2IT-95) and introduced simultaneously to cells (500 nM siRNA each) followed by incubation for 4 h. After incubation with micelles, the medium was exchanged with fresh media containing LysoTracker Green (Invitrogen Molecular Probes, Eugene OR) and Hoechst 33342 (Dojindo Laboratories, Kumamoto, Japan), and the cells were incubated for an additional 15 min. Cells were rinsed with PBS containing Ca^{2+} and Mg^{2+} and imaged in this buffer. Confocal laser scanning microscopy was performed in live cells using a LSM 510 (Carl Zeiss, Oberlochen, Germany) with a C-Apochromat 63× objective (Carl Zeiss) and appropriate excitation sources and emission filters. Data were analyzed using LSM imaging software (Carl Zeiss). Colocalization was determined with respect to Cy3, Cy5, and LysoTracker using the following equation: relative colocalization = $\Sigma pixels_{colocalized}/\Sigma pixels_{total}$.

Blood Circulation. Micelle stability in the blood compartment was evaluated using intravital confocal laser scanning microscopy (IVRTCLSM) in live mice. All picture/movie acquisitions were performed using a Nikon A1R confocal laser scanning microscope system attached to an upright ECLIPSE FN1 (Nikon Corp., Tokyo, Japan) equipped with a 20× objective, 640 nm diode laser, and a band-pass emission filter of 700/75 nm. The pinhole diameter was set to result in a 10 μm optical slice. Eight-week-old female BALB/c nude mice (Oriental Yeast Co., Ltd., Tokyo, Japan) were anesthetized with 2.0−3.0% isoflurane (Abbott Japan Co., Ltd., Tokyo, Japan) using a Univenter 400 anaesthesia unit (Univentor Ltd., Zejtun, Malta). Mice were then subjected to lateral tail vein catheterization with a 30 gauge needle (Dentronics Co., Ltd., Tokyo, Japan) connected to a nontoxic, medical grade polyethylene tube (Natsume Seisakusho Co., Ltd., Tokyo, Japan). Anesthetized mice were placed onto a temperature-controlled pad (Thermoplate; Tokai Hit Co., Ltd., Shizuoka, Japan) integrated into the microscope stage and maintained in a sedated state throughout the measurement. Ear-lobe dermis was observed without surgery following fixation beneath a coverslip with a single drop of immersion oil. Data were acquired in video mode for 3 min (30 frames/s), followed by snap-shots every 1 min thereafter. 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.

Micelles prepared with Cy5-labeled siRNA were injected (200 μL of 9.2 μM siRNA, ∼24 μg total siRNA) *via* the tail vein 10 s after the start of video capture. Video data were analyzed by selecting regions of interest (ROIs) within blood vessels or extravascular skin tissue, and the average fluorescence intensity per pixel for each time point was determined using the Nikon NIS-Elements C software provided by the manufacturer. To produce the blood retention profiles shown in Figure 6, vein fluorescence data were expressed relative to the maximum observed value (typically 45 s to 1 min.). First, the background fluorescence intensity was determined from video captured during the 10 s before sample injection. This background value was subtracted from the average pixel intensities measured after micelle injection to create background-corrected intensities for each time point. Next, relative fluorescence intensities were determined by dividing the average fluorescence intensity at each time point by the maximum observed fluorescence intensity. Each experiment was performed in triplicate in separate animals. A detailed description of the microscope apparatus and mouse positioning for IVRTCLSM, as well as examples of data workup showing ROIs, can be found in our previously published report.[35] Half-lives were calculated by plotting the natural log of intensity *versus* time for the initial period of signal decay (10 min for naked siRNA and 2IT-0, 15 min for 2IT-95). The slope of these plots were determined and applied to the half-life equation: $T_{1/2} = 0.693/(-\text{slope})$. Areas under the curve values were calculated using the trapezoid rule.

Biodistribution. HeLa-luc tumors were prepared by *in vivo* passage of solid tumor fragments. Donor tumors were prepared by injecting HeLa-luc cells (2.6×10^6 cells) under the skin in the right rear flank of BALB/c nude mice and allowed to mature for 2 weeks. After 2 weeks, donor tumors were excised and cut into 3 × 3 mm pieces. Tumor fragments were transplanted under the skin into the rear flank of 6 week old female BALB/c nude mice, and the wound was closed with a suture. Tumors were allowed to mature for 8 days, then mice were randomly assigned into treatment groups ($n = 4$ per group). Mice were fed alfalpha-free chow for 2 weeks before injecting micelle samples. Micelles prepared with Cy5.5 siRNA were injected (200 μL of 9.2 μM siRNA, ∼24 μg total siRNA) *via* the tail vein.

After 24 h, mice were sacrificed and individual organs were excised and rinsed with PBS. Organs and tumors were imaged using an IVIS instrument in fluorescence mode with appropriate excitation and emission filters. Data were analyzed using Living Image software by drawing ROIs around whole organs to determine the total photon counts and signal area in square centimeters. The fluorescence intensity was normalized to sample area and irradiation time using the following equation: intensity = total photons/[illumination time (s) × area (cm^2)].

***In Vivo* Confocal Microscopy.** Micelles prepared with Cy5 siRNA were injected (200 μL of 9.2 μM siRNA, ~24 μg total siRNA) *via* the tail vein into mice bearing subcutaneous HeLa-H2BGFP tumors. HeLa-H2BGFP cells express green fluorescent protein (GFP) in the cell nucleus, which allows identification of tumor cells. After 24 h, mice were anesthetized and Hoechst 33342 dye (8 mg/kg in PBS, Lonza Group Ltd., Basel, Switzerland) was used to stain the nuclei of cells present in circulation and the perivascular space. Next, the tumor was exposed by a series of dorsal cuts in the skin surrounding the tumor to create a hinged skin flap with the tumor attached to the skin, while leaving blood vessels feeding the tumor intact. The exposed tumor was mounted under a coverslip and imaged using the IVRTCLSM equipped with a 40 or 60× objective. Hoechst, GFP, and Cy5 signals were detected simultaneously using 405, 488, and 640 nm excitation lasers and band-pass emission filters of 450/50, 525/50, and 700/75 nm, respectively. Images were analyzed using the Nikon NIS-Elements C software provided by the manufacturer.

Tumor Growth Inhibition. HeLa-luc tumors were prepared by *in vivo* passage of solid tumor fragments. Donor tumors were prepared by injecting HeLa-luc cells (2.6×10^6 cells) under the skin in the right rear flank of mice and allowed to mature for 2 weeks. After 2 weeks, donor tumors were excised and cut into 3 × 3 mm pieces. Tumor fragments were transplanted under the skin into the rear flank of 6 week old female BALB/c nude mice, and the wound was closed with a suture. Tumors were allowed to mature for 4 days, then mice were randomly assigned into treatment groups ($n = 4$ per group).

Micelle formulations were screened for *in vivo* efficacy using a combination therapy of VEGFR2 and VEGF siRNAs. Micelles were prepared to contain either type of siRNA in different micelle formulations and were injected separately. Micelle treatment began on day four following tumor implantation. Micelles containing VEGFR2 siRNA were injected on the first day of treatment, micelles incorporating VEGF siRNA were injected on day two, followed by two days with no injection. This sequence was repeated three times for a total of three injections of each micelle formulation and six total injections of micelles. For each injection, 200 μL of micelle solution (9.2 μM siRNA, ~24 μg total siRNA) was administered. The negative control sample for cRGD-2IT-95 formulation was prepared with scramble siRNA and was administered in the same fashion as described above. Tumor size was monitored over time by caliper measurement, and tumor volumes were calculated using the following equation: volume = $^1/_2 a \times b^2$, where a is the long axis and b is the short axis measured.

For determining the effect of each siRNA sequence individually (delivered by cRGD-2IT-95 micelles), subcutaneous tumors were prepared by *in vivo* passage as described above and the injection sequence used was the same, except for samples delivering only one type of siRNA. For groups receiving only one type of siRNA (VEGF or VEGFR2), micelles containing scramble siRNA were injected on the second day to keep the total amount of siRNA micelles injected into each mouse constant. Each group consisted of four mice, with additional data from other groups (HEPES, scramble, and VEGF+VEGFR2 treatment) combined in Figure 9.

PCR Analysis. Tumors were excised from mice 72 h after the last injection of micelles, and ~20 mg of non-necrotic tissue was selected from the tumor mass ($n = 4$ tumors, each tumor was analyzed in triplicate). Tumor fragments were sonicated for 10 s in lysis buffer and then centrifuged to remove excess debris. RNA was extracted from the supernatant using a RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Extracted RNA samples were normalized to the same 260 nm absorbance value, and genomic DNA was eliminated, followed by RNA transcription to cDNA using a QuantiTect reverse transcription kit (Qiagen, Valencia, CA). RNA was quantified following conversion to cDNA and amplified using real-time PCR. Primers used for human actin and human VEGF were synthesized by Hokkaido System Science (Hokkaido, Japan), and the sequences used were as follows: CCAACCGCGAGAAGATGA (actin forward); CCAGAGGCGTACAGGGATAG (actin reverse); AGTGGTCCCAGGCTGCAC (VEGF forward); TCCATGAACTTCACCACTTCGT (VEGF reverse).

Statistical Analysis. All data are expressed as the average value ± the standard deviation. The p values were determined by the Student's t test using a two-tailed distribution and two-sample unequal variance with the T.Test function of Microsoft Excel. The p values of less than 0.05 were considered as statistically significant.

Conflict of Interest: The authors declare no competing financial interest.

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Supporting Information Available: Light scattering intensity of polymer and siRNA mixtures at different molar ratios, and stability of cRGD-2IT-95 and 2IT-95 micelles at 600 mM NaCl is provided. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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