Smart Multilayered Assembly for Biocompatible siRNA Delivery Featuring Dissolvable Silica, Endosome-Disrupting Polycation, and Detachable PEG

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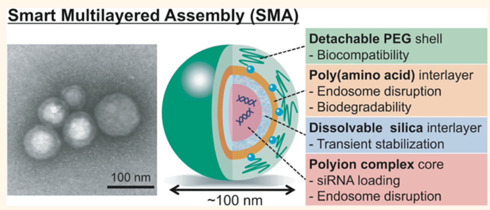
ABSTRACT Multifunctional delivery systems of small interfering RNA (siRNA) are needed to overcome the intrinsic biological barriers toward efficient gene silencing in the cell cytoplasm. In this report, a smart multilayered assembly (SMA) was fabricated by a layer-by-layer method with polyionic materials. The SMA was designed to feature a siRNA-loaded core, a transiently core-stabilizing silica interlayer, an endosome-disrupting polycation interlayer, and a biocompatible poly(ethylene glycol) (PEG) shell with reductive environment-responsive detachability. The SMA was confirmed to be approximately 160 nm in size with narrow distribution and spherical morphology by DLS and TEM analyses. The PEG detachability of the SMA based on disulfide cleavage was also confirmed by the increase in both ζ-potential and size due to the exposure of the polycation interlayer and the compromised colloidal stability. The silica interlayer rendered the SMA highly tolerant to dissociation induced by anionic lipids, while after 24 h dialysis siRNA release from the SMA was clearly observed, presumably due to gradual dissolution of the silica interlayer based on the equilibrium shift to silicate ions. The entrapment ratio of siRNA delivered by the SMA within the endosome was significantly lower than that by nondisulfide control (NDC) without PEG detachability, suggesting the improved endosomal escape of SMA with the exposed, endosome-disrupting interlayer after PEG detachment. SMAs induced significantly higher gene silencing efficiency in various cultured cells, compared to NDC, without associated cytotoxicity. The systemic administration of SMAs for subcutaneous tumor-bearing mice achieved significant endogenous gene silencing in tumor tissue without hematological toxicity.



KEYWORDS: siRNA delivery · polyion complex · silica · poly(ethylene glycol) · layer-by-layer

Small interfering RNA (siRNA) has generated tremendous research interest in therapeutic applications for the treatment of various intractable diseases caused by aberrant gene expression, such as cancer.[1,2] This interest stems from the highly specific and potent gene silencing ability inherent to siRNA, termed RNA interference (RNAi). In order to attain therapeutic benefit, siRNA needs to overcome several biological hurdles, such as rapid renal clearance, enzymatic decomposition, distribution in nontarget tissues, inefficient cellular uptake, and endosomal/lysosomal entrapment. To date, various types of nanoparticle formulations have been developed as a delivery vehicle of siRNA, *e.g.*, polymer-based complexes (polyplexes),[3−12] lipid-based complexes (lipoplexes),[13−15] silica nanoparticles,[16,17] calcium phosphate nanoparticles,[18,19] gold nanoparticles,[20,21] and also their hybrid systems. Nevertheless, further improvement of siRNA vehicles is still demanded to gain better therapeutic efficacy for their translation into pharmaceutical agents.

Several properties required for the ideal siRNA vehicle are apparently conflicting, *i.e.*, high stability for siRNA protection in extracellular conditions *vs* smooth payload release within the cytoplasm and reduced nonspecific interactions with biomacromolecules (or biocompatibility) *vs* efficient cellular uptake (endocytosis) and subsequent endosomal escape in target cells. One promising solution to these conflicts is to design several smart materials, which exert the desired function in response to the specific biological environment, and then to integrate those component materials into one formulation toward multifunctionalities. For this purpose, construction of multilayered polyion complexes (PICs) by a layer-by-layer (LbL) technique allows the facile integration of a variety of charged components with different functionality as well as negatively charged nucleic acids into one nanoparticle.[22−24] A few recent

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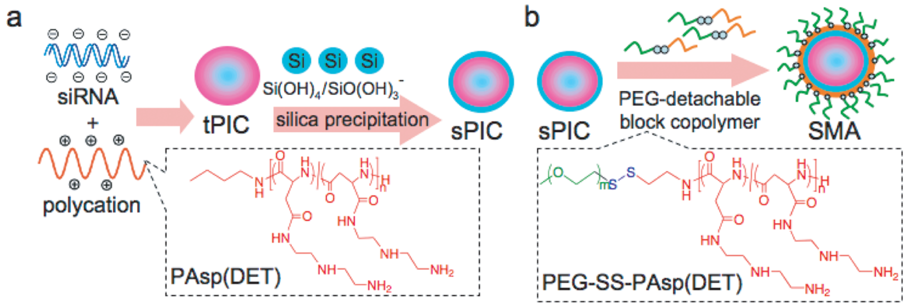

Figure 1. Preparation schemes of a smart multilayered assembly (SMA) by the layer-by-layer method. (a) Preparation of silica-coated PIC (sPIC) by silica-coating of the template PIC (tPIC). (b) Preparation of the SMA (PEG-SS-PAsp(DET)/silica-coated PAsp(DET)/siRNA PIC) by additional coating with PEG-block-polycation.

studies reported that the LbL technique successfully prepared siRNA-incorporating nano/microparticles from gold nanoparticle templates or mesoporous silica microparticle templates with several charged components for enhanced siRNA delivery.[25−27]

For transient stabilization of PIC structures, the covering of their surface with a silica layer is one of the promising approaches, because the silica layer (i) can be easily prepared on the surface of cationic PICs, (ii) is negatively charged, thus available as a substrate for additional adsorption of the oppositely charged components, and (iii) substantially stabilizes the PIC, while gradually dissolving for payload release under highly dilute conditions based on the equilibrium shift to silicate ions.[28,29] Indeed, our previous study revealed that the plasmid DNA-carrying PIC was successfully coated with a silica layer by simple incubation with sodium silicate solutions and was reversibly stabilized in a silicate concentration-dependent manner, leading to enhanced gene expression in cultured cells.[29] Meanwhile, for the preparation of biocompatible nanoparticles, installation of poly(ethylene glycol) (PEG) on their surface, termed PEGylation, is one of the most promising strategies. PEGylated nanoparticles are sterically stabilized by highly flexible and hydrated PEG chains, preventing the nanoparticles from secondary aggregate formation and nonspecific interactions with biomacromolecules, such as serum components and blood cells.[30−32] However, in the case of nucleic acid delivery, such PEG shielding has concurrently compromised the membrane-disrupting (or endosomal escape) functionality of nanoparticles, resulting in lower transfection efficiency (termed PEG dilemma).[33] To bypass this dilemma, several previous studies, including ours, demonstrated the utility of PEG-detachable systems, which are constructed by smart block copolymers of PEG and an endosome-disrupting polycation tethered by cleavable linkers in the endosome/lysosome, *e.g.*, a reductive environment-responsive disulfide bond[33,34] and an acidic pH-responsive hydrazone.[35] The PEG detachment within the endosome can expose the endosome-disrupting polycation on the nanoparticle surface for facilitated endosomal escape.

In the present study, a smart multilayered assembly (SMA) was developed with the PIC featuring a siRNA-loaded core, a transiently core-stabilizing silica interlayer, an endosome-disrupting polycation interlayer, and a detachable PEG shell, altogether aimed toward biocompatible and mutifunctional siRNA delivery (Figure 1). The distinctive feature of this formulation was that SMAs were constructed with soft materials, *i.e.*, hydrophilic polymers and nonannealed silica. They can be metabolized more rapidly in the body compared to previous formulations prepared from iron, gold, or annealed mesoporous silica nanoparticles, thereby providing a great advantage for multiple-dose administration. As the endosome-disrupting polycation, a polyaspartamide derivative bearing two repeating units of aminoethylene (termed PAsp(DET)) was selected and further tethered with PEG through a disulfide bond (−SS−), which can be cleaved by reducing enzymes on the cellular membrane or in the endosome/lysosome.[36,37] PAsp(DET) has been extensively shown to induce strong membrane disruption selectively at endosomal acidic pH (∼5.5) for efficient, less toxic endosomal escape, probably due to the distinctive change in the protonated structure in the side chain, *i.e.*, the monoprotonated state at extracellular pH 7.4 and the diprotonated state at acidic pH.[38−42] The utility of SMAs was physicochemically and biologically investigated in comparison with the control without PEG detachability. In particular, the feasibility of SMAs for siRNA-based cancer therapy was examined with regard to endogenous gene silencing efficiency in a subcutaneous tumor model and also hematological toxicity after systemic administration. Ultimately, we demonstrate that SMAs may be promising formulations for successful siRNA delivery.

RESULTS AND DISCUSSION

Preparation and Physicochemical Characterization of the SMA. A SMA, that is, PEG-SS-PAsp(DET)/silica-coated PAsp(DET)/siRNA PIC, was prepared as illustrated in Figure 1. At first, the PAsp(DET)/siRNA-based template PIC (tPIC) was prepared by mixing siRNA with PAsp(DET) in a buffer solution (Figure 1a). A residual molar ratio of amines in PAsp(DET) to phosphate in siRNA = 3, corresponding to a residual molar ratio of protonated amines in PAsp(DET) to phosphates in siRNA of 1.5,[12] was selected to obtain the positively charged tPIC with a minimal amount of unbound polycations for effective silica-coating. The formation of tPICs with a hydrodynamic size of 115 nm, a polydispersity index (PDI) of less than 0.1, and a positive ζ-potential (∼20 mV) was confirmed with the Zetasizer (Table 1 and Figure 2a). It should be noted that PAsp(DET) was selected as a component polycation because of its considerably low cytotoxicity and excellent endosome-disrupting capability.[38−42] In detail, the protonation state of the side chain of PAsp(DET) changes from a monoprotonated state to a diprotonated state in response to the endosomal acidification. The membrane-disrupting activity of PAsp(DET) is quite low in the monoprotonated state, but is significantly augmented in the diprotonated state for effective endosome disruption. Despite the excellent endosome-disrupting capability of PAsp(DET), the low stability of PAsp(DET)/siRNA PICs in serum-containing media has substantially limited their gene silencing efficiency,[12,43,44] thereby requiring additional stabilization strategies for successful siRNA delivery.

According to the previously reported protocol,[29] silica-coating was performed by incubation of tPIC with sodium silicates in 10 mM HEPES buffer (pH 7.3) for 24 h at room temperature, then characterized with the Zetasizer. In the range of sodium silicate concentrations above 3 mM, the obtained PICs showed almost the same sizes (∼130 nm) and negative ζ-potentials (∼−20 mV) (Supporting Figure 2). Through incubation with silicates, the PIC size was considerably increased, and the ζ-potential was converted from the positive to the negative, well consistent with the formation of the anionic silica layer on the PIC surface. It should be noted that no leakage of siRNA from PICs was observed after silica-coating (Supporting Figure 3). The silica-coated PIC (sPIC) prepared at 3.5 mM sodium silicate was selected for the following experiments, as it exhibited the smallest size with relatively less silicates; at 3.5 mM sodium silicate sPICs had a hydrodynamic size of 125 nm, and a relatively narrow size distribution (PDI = 0.08) for a polymer-based self-assembly, a negative ζ-potential (−19 mV), and spherical morphology, as shown in dynamic light scattering (DLS) results (Table 1 and Figure 2a and b) and transmission electron microscopy (TEM) images (Figure 2d and Supporting Figure 5a).

Next, a smart polymer, PEG-SS-PAsp(DET), was utilized for the additional covering of sPIC for construction of the SMA (Figure 1b). Prior to addition of the polymer, the sPIC solution was applied to ultrafiltration (3000*g*, molecular weight cutoff: 300 000 Da) to remove the unbound silica species. Then, the purified sPIC solution was mixed with the polymer solution with varying concentrations, followed by 1 h incubation at room temperature. In the mixed solution, large aggregates (several μm in diameter) were formed in lower polymer concentrations (<250 μM in polymer amine) (Supporting Figure 4), probably due to the diminished electrostatic repulsion between the sPICs through charge neutralization of the surface silica by polymer binding. With further increase in polymer concentration, the PIC size was gradually decreased, presumably because of effective PEG-shielding, and consequently the size change leveled off at around a residual amino group concentration of 400 μM (equivalent to 5 times the siRNA phosphates in the solution). Considering the

TABLE 1. Size and Polydispersity Index (PDI) of PICs in 10 mM HEPES Buffer (pH 7.3), Determined by Dynamic Light Scattering

	diameter (nm)	PDI
tPIC	115 ± 8	0.08 ± 0.01
sPIC	125 ± 13	0.08 ± 0.01
SMA	159 ± 8	0.10 ± 0.03
NDC	159 ± 10	0.08 ± 0.03
NPC	143 ± 15	0.14 ± 0.04

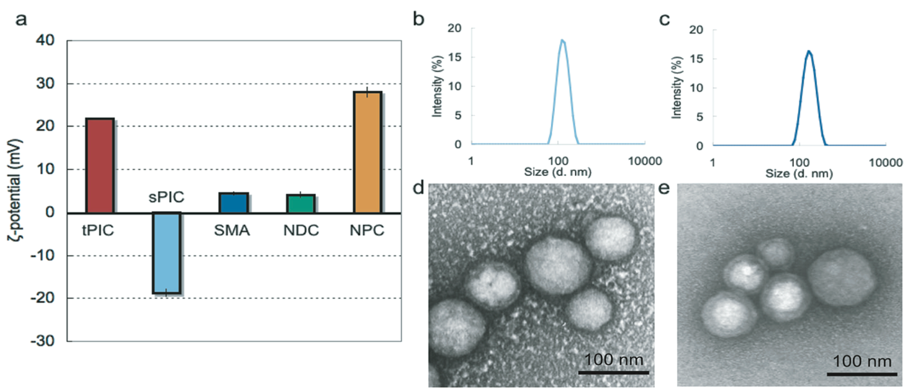

Figure 2. Physicochemical characterization of nanoparticle formulations. (a) ζ-Potential of PICs in 10 mM HEPES buffer (pH 7.3). (b, c) Intensity-based histograms of PICs determined by DLS: (b) sPIC and (c) SMA. (d, e) TEM images of PICs: (d) sPIC and (e) SMA (scale bar: 100 nm). PIC samples (2 μM siRNA) were applied onto a copper TEM grid with carbon-coated collodion film and stained with uranyl acetate solution (2% w/v).

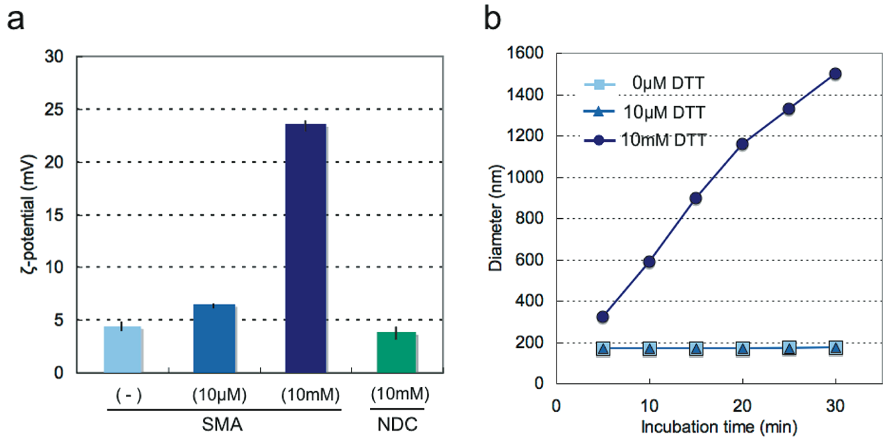

Figure 3. Reductive environment responsiveness of the SMA. (a) ζ-Potentials of the SMA and NDC after 30 min incubation in 10 mM HEPES buffer (pH 7.3) with or without DTT (0, 10 μM and 10 mM DTT). Results are expressed as mean and standard deviation (n = 3). (b) Time-dependent change in size of the SMA in 10 mM HEPES buffer containing 150 mM NaCl (solid squares); in 10 mM HEPES buffer containing 150 mM NaCl and 10 μM DTT (solid triangles); and in 10 mM HEPES buffer containing 150 mM NaCl and 10 mM DTT (solid circles), measured by DLS.

fact that an excess increase in polymer concentration may generate free (or uncomplexed) polymer in the solution, the sample prepared at a residual amino group concentration of 400 μM was selected for further studies.

The hydrodynamic size and ζ-potential of a series of siRNA PICs are summarized in Table 1 and Figure 2a. A non-PEGylated control (NPC), which was prepared by mixing sPIC with PAsp(DET) homopolymer, had a hydrodynamic size of 143 nm with a PDI of 0.14 and a strongly positive ζ-potential of 28 mV, suggesting the formation of a cationic PAsp(DET) outer layer. In contrast, the SMA as well as the nondisulfide control (NDC), which was prepared from PEG-PAsp(DET), had a hydrodynamic size of 159 nm with the PDI of ~0.10 and a ζ-potential close to neutral (~4 mV). The larger size (P < 0.05 for NPC) and the almost neutral ζ-potential of the SMA and NDC, compared with NPC, suggest that the sPIC should be successfully covered with a neutral PEG outer layer *via* the electrostatic interaction between anionic silica and the cationic PAsp(DET) segment of the block copolymers. Furthermore, the DLS and TEM histograms (Figure 2c and Supporting Figure 5b, respectively) and TEM images (Figure 2e and Supporting Figure 6) of the SMAs revealed that the relatively narrow size distribution and spherical morphology of sPICs were maintained. Note that the size distribution histograms obtained from TEM images were comparable to those from the number statistics of DLS (Supporting Figure 5).

Reductive Environment Responsiveness of the SMA. In order to examine the reductive environment-responsive PEG detachment from the SMA, the ζ-potential of the SMA was examined in 10 mM HEPES buffer (pH 7.3) containing different amounts of a reducing agent, dithiothreitol (DTT) (0, 10 μM and 10 mM). Note that 10 μM and 10 mM DTT were chosen to mimic the extracellular and the intracellular reducing potentials, respectively, generated by glutathione.[36] In 10 mM HEPES buffer (pH 7.3), the SMA was observed to have a ζ-potential close to neutral (~4 mV), suggesting the presence of a PEG shell that covers the nanoparticle. In contrast, after 30 min incubation in 10 mM HEPES buffer (pH 7.3) containing 10 mM DTT, the SMA exhibited an appreciably increased ζ-potential (24 mV), comparable to NPC, whereas a much lower ζ-potential was observed in the 10 μM DTT condition (Figure 3a). The appreciably increased ζ-potential of the SMA in the stronger reductive condition is well consistent with the exposure of the positively charged PAsp(DET) layer due to the PEG detachment based on the disulfide cleavage. The pivotal role of the disulfide bond for PEG detachment in the SMA is also suggested by the result that the NDC maintained the neutral ζ-potential even in the stronger reductive conditions (Figure 3a). The PEG detachability in the SMA was further examined from the standpoint of the colloidal stability of the nanoparticles. DLS measurement was conducted to monitor the size change of SMAs as well as NDCs and NPCs in 10 mM HEPES buffer (pH 7.3) containing 150 mM NaCl. First, the NDC and NPC were compared to estimate the effect of PEG on the size change. The size of the NPCs clearly increased over the incubation period, reaching μm dimensions in 30 min, presumably due to secondary aggregate formation under the physiological salt conditions, which attenuates the electrostatic repulsion between the charged nanoparticles (Supporting Figure 7). In contrast, this secondary aggregate formation was dramatically suppressed in the NDC formulation (Supporting Figure 7), demonstrating the enhanced colloidal stability of PICs with PEG palisades. Next, the SMAs were similarly evaluated by DLS in the same buffer with or without DTT (Figure 3b). At 0 and 10 μM DTT, no size change of the SMAs was observed following 30 min incubation, similar to NDCs, indicating that the colloidal stability of

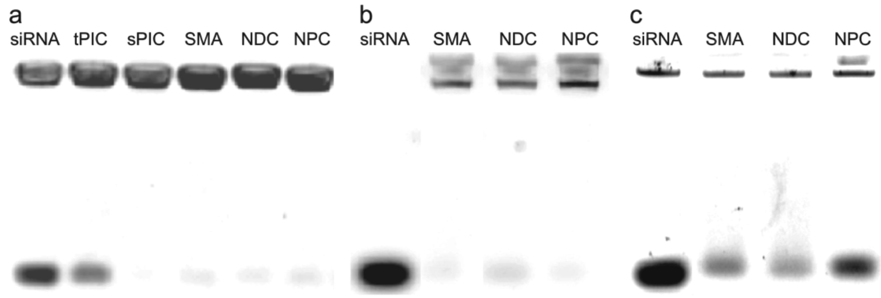

Figure 4. Tolerability of PICs against dissociation triggered by anionic lipids. siRNA release from PICs was evaluated by agarose gel electrophoresis. (a) Each PIC was incubated in 10 mM HEPES buffer (pH 7.3) containing anionic lipids, DOPS, for 48 h at a molar ratio of carboxyl groups in DOPS to phosphate groups in siRNA of 32. (b, c) Each PIC solution was dialyzed (molecular weight cutoff: 5000 Da) against a 1000-fold excess of 10 mM HEPES buffer (pH 7.3) for (b) 3 h and (c) 24 h. Then, each PIC solution was mixed with DOPS and incubated for 24 h, followed by electrophoresis.

the SMA was maintained even in mild reductive conditions. In sharp contrast, in the conditions containing 10 mM DTT, the increase in size of the SMA was clearly observed over the incubation period, similar to NPC (Supporting Figure 7), indicating the significantly compromised colloidal stability presumably due to the PEG detachment. Altogether, the PEG palisade in the SMA may sterically stabilize siRNA PICs in extracellular conditions, but can be removed on the cellular surface and/or in the endosome/lysosome with higher reducing potentials,[36,37] to expose the endosome-disrupting PAsp(DET) layer.

Stability Shift in the SMA with a Dissolvable Silica Interlayer. When administered in the body, siRNA PICs encounter abundant charged biomolecules, such as serum proteins and cell membranes, which may nonspecifically bind to the PICs, possibly leading to undesirable dissociation. Thus, high resistance toward such dissociation is a prerequisite for *in vivo* applications of siRNA PICs. In addition, once delivered into the cytoplasm, siRNA must also be released from PICs to allow association with RNAi-related proteins. Integration of a silica layer onto siRNA PICs should be a promising solution to overcome this conflicting requirement, because the silica layer stabilizes the siRNA PICs while also dissolving in a time-dependent manner in dilute conditions based on the equilibrium shift in the silicate ions.[29,45]

To investigate the stability shift induced by silica dissolution, the SMA was challenged by an anionic lipid, 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine sodium salt (DOPS),[46] before and after dialysis against a 1000-fold volume of 10 mM HEPES buffer (pH 7.3) for 3 and 24 h for removal of generated free silicates. Note that DOPS was selected as a model anionic molecule in this assay, as phosphatidylserine is one of the major components of cell membranes that can interact with siRNA PICs in intracellular conditions, thereby is useful for estimation of the feasibility of siRNA release from PICs in cells. Each sample was incubated with DOPS (the mixing ratio was set at a molar ratio of carboxyl groups in DOPS to phosphate groups in siRNA of 32), followed by agarose gel electrophoresis. As shown in Figure 4a, the band corresponding to naked siRNA was clearly observed for the tPIC sample incubated with DOPS, indicating PIC dissociation. In contrast, siRNA release was significantly suppressed for the SMA formulation as well as sPICs before dialysis. Thus, it was confirmed that the silica layer rendered siRNA PICs highly tolerant to counteranion-triggered dissociation. On the other hand, siRNA was considerably released from SMAs after dialysis for 24 h (Figure 4c), whereas release was still limited after dialysis for 3 h (Figure 4b). Note that a similar tendency was also observed for NDCs and NPCs featuring a silica layer (Figure 4a–c). These results demonstrate that the dissolvable silica layer in siRNA PICs can induce a stability shift for smooth release of siRNA in the cytoplasm.

Gene Silencing and Cell Viability Assays. The efficiency of gene silencing was evaluated using a luciferase assay against HuH7-Luc, a human hepatoma cell stably expressing luciferase. A linear polyethyleneimine-based transfection reagent (ExGen 500) was used as a positive control in this assay. As shown in Figure 5a, the SMA as well as NDC and NPC appreciably enhanced the gene silencing efficiency at higher siRNA concentrations, compared to tPIC and sPIC, suggesting that PAsp(DET)-based polymers bound onto a sPIC surface improved the siRNA delivery efficiency. Although there was no significant difference between SMAs and NDCs at the lowest siRNA concentration tested, the SMAs exerted significantly higher gene silencing efficiency than NDCs at higher siRNA concentrations ($P < 0.01$ at 200, 300, and 400 nM siRNA), suggesting that the enhanced efficiency of the SMA should be due to the PEG detachability. The exposure of the PAsp(DET) layer after the PEG detachment may improve the endosomal escape efficiency of siRNA PICs. On the other hand, the gene silencing efficiency of the SMAs was lower than that of NPCs as well as ExGen 500 (Figure 5a), probably due to more efficient cellular uptake of the non-PEGylated PICs, as shown in the next section. Despite lower gene silencing efficiency, the SMA formulation with PEG palisades is more suitable for systemic administration *in vivo*, because the much lower colloidal

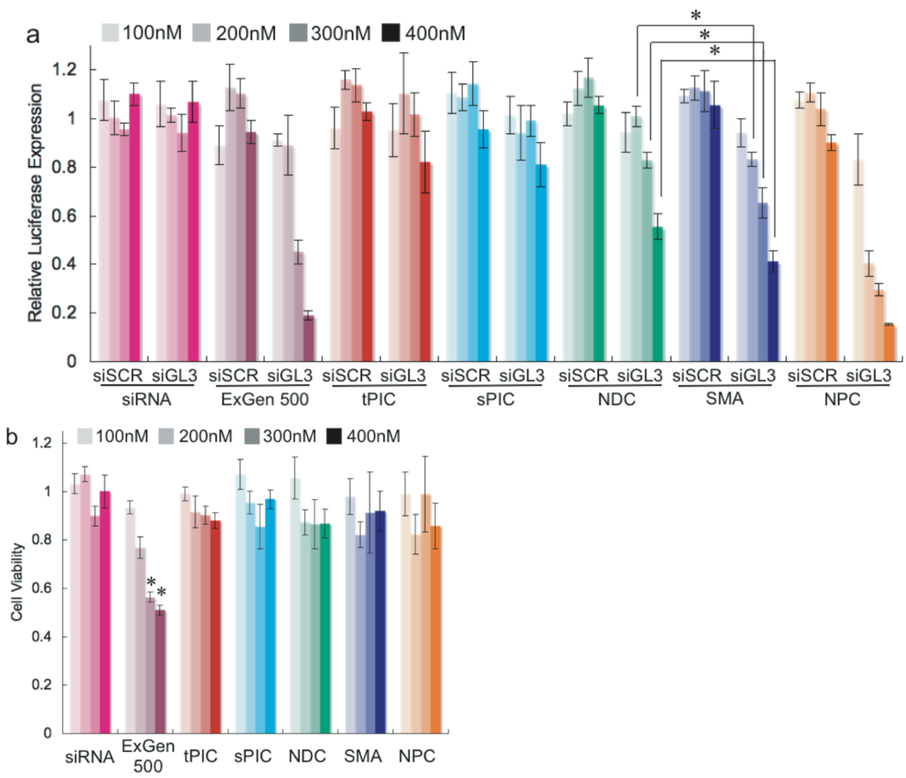

Figure 5. (a) Gene silencing activity against HuH7-Luc cells. Scramble siRNA (siSCR) was used as a control sequence for GL3 luciferase siRNA (siGL3). HuH7-Luc cells were treated with each PIC for 48 h (siRNA concentrations from the left: 100, 200, 300, and 400 nM) and subjected to the luciferase assay. Relative luciferase expression was calculated as the ratio of luciferase expression relative to nontreated cells. Results are expressed as mean and standard deviation ($n = 4$). *$P < 0.01$ for NDC. (b) Cell viability of HuH7-Luc cells in the same conditions as the gene silencing study was determined with the Cell Counting Kit-8 assay, with the cell viability of the nontreated cells set to 1. Results are expressed as mean and standard deviation ($n = 8$). *$P < 0.01$ compared to other samples. In both experiments, ExGen 500 was used according to the manufacturer's protocol as a commercially available positive control.

stability and strong positive ζ-potential of the non-PEGylated PICs would likely lead to the formation of secondary aggregates through nonspecific interactions with biomacromolecules during blood circulation.[32] Note that a similar profile in gene silencing efficiency was also observed in other luciferase-expressing cell lines, human lung cancer (A549-Luc) and human ovarian cancer (SKOV3-Luc) (Supporting Figure 8). Meanwhile, all the PAsp(DET)-based PICs (tPIC, sPIC, NDC, SMA, and NDC) did not show substantial toxicity in cultured cells, which was in sharp contrast to ExGen 500 (Figure 5b). Reduced cytotoxicity of PAsp(DET)-based formulations is consistent with the previous reports of nucleic acid delivery using PAsp(DET), which features a monoprotonated state in each side chain at an extracellular neutral pH with appreciably less cytoplasmic membrane damage compared to other polycations, such as polyethyleneimine.[12,40−42] Thus, the SMA is demonstrated to enhance the siRNA delivery efficiency without increasing the cytotoxicity.

Cellular Uptake and Intracellular Distribution Studies. The mechanism of the enhanced gene silencing achieved by the SMA was further investigated in comparison with the controls (NDC and NPC as well as sPIC). To quantify the cellular uptake of each siRNA PIC by HuH7-Luc cells, the cells were incubated with the PICs prepared from Cy3-labeled siRNA (Cy3-siRNA) for 24 h and then subjected to flow cytometric analysis. As shown in Figure 6, the cellular uptake of Cy3-siRNA PICs was increased in the polymer/silica-coated systems (SMA, NDC, and NPC), compared to sPIC. In detail, the Cy3-siRNA uptake of the SMA was significantly higher than that of sPIC ($P < 0.05$) and significantly less than that of NPC ($P < 0.01$), whereas no statistical difference was observed between PEGylated systems (SMA and NDC) ($P > 0.05$). The observed cellular uptake profile was apparently correlated with the surface charge of PICs; higher fluorescence (or cellular uptake) was obtained by the PICs showing a higher ζ-potential (Figure 2a), possibly due to the fact that the positively charged surface should facilitate an association with the anionic cytoplasmic membrane.[47,48] It should be noted that NPCs are likely to form secondary aggregates in the cell culture medium due to their lower colloidal stability (Supporting Figure 7), possibly affecting the cellular association and uptake. The significantly limited uptake efficiency observed for the SMA, compared to NPC, suggests that the PEG shell in the SMA might be effectively maintained during

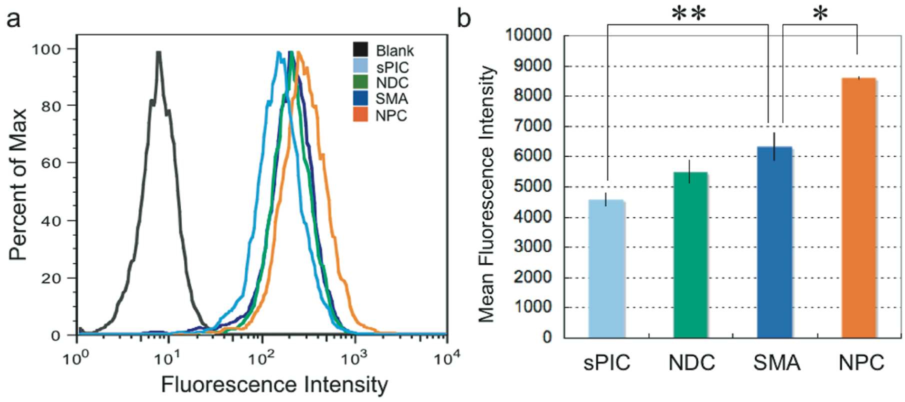

Figure 6. Cellular uptake of Cy3-siRNA PICs evaluated by flow cytometric analysis. HuH7-Luc cells were incubated with each PIC (siRNA concentration: 400 nM) for 24 h. (a) Flow cytometric data shown in the histogram. (b) Flow cytometric data shown in the bar graph. Results are expressed as mean and standard deviation ($n = 3$). *$P < 0.01$ for NPC. **$P < 0.05$ for sPIC.

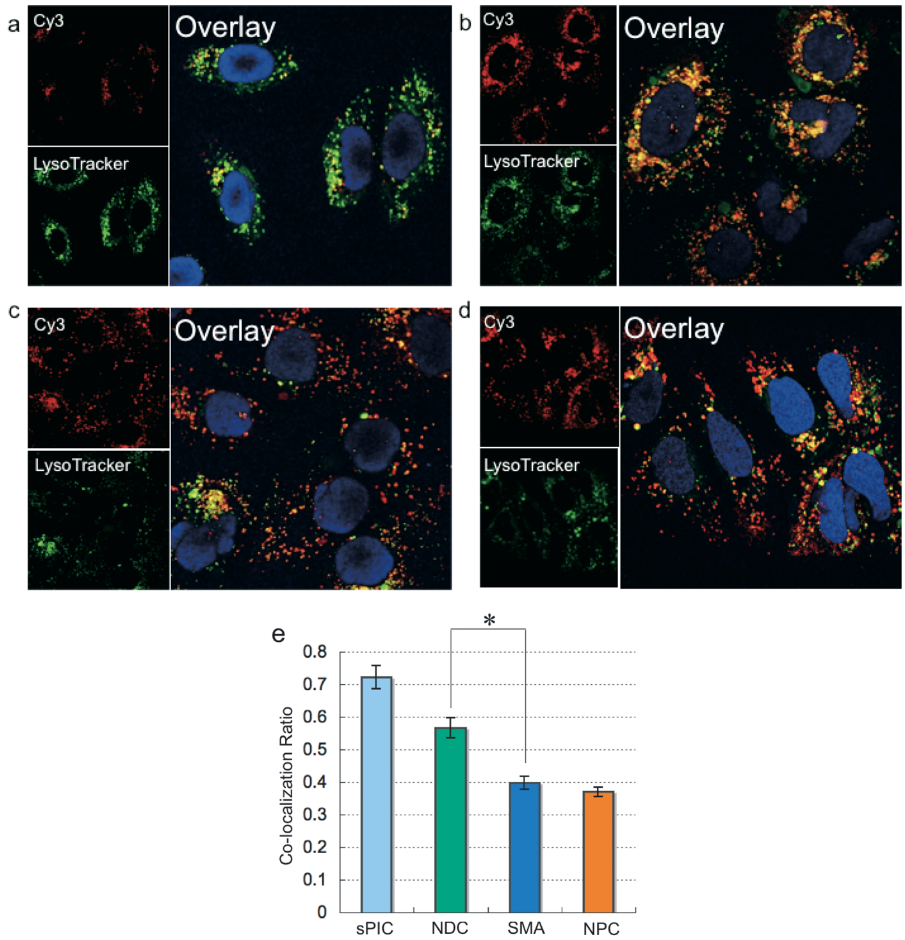

Figure 7. Intracellular distribution of Cy3-siRNA PICs. (a–d) CLSM images of (a) sPIC, (b) NDC, (c) SMA, and (d) NPC (red: Cy3-siRNA, green: LysoTracker Green, blue: Hoechst33342). HuH7-Luc cells were incubated with each PIC at 400 nM siRNA for 24 h. (e) Co-localization ratio of Cy3-siRNA with the late endosome/lysosome marker (LysoTracker Green) calculated from the number of pixels in the obtained CLSM images. Results are expressed as mean and standard deviation ($n = 20$). *$P < 0.01$ for NDC.

incubation in the cell culture medium. The facilitated cellular uptake in the polymer/silica-coated systems, especially in NPC, might contribute to their enhanced gene silencing efficiency (Figure 5a). Nevertheless, the similar uptake efficiency between the SMA and NDC also suggests that there is another crucial factor to explain their significantly different gene silencing efficiency (Figure 5a), as demonstrated by the following confocal laser scanning microscopic (CLSM) analysis.

Once endocytosed by cells, siRNA PICs were generally transported to the late endosome/lysosome, which is the digestive organelle. Thus, the efficient endosomal escape of PICs is critical for successful siRNA delivery into the cytoplasm. Herein, the intracellular distribution of Cy3-siRNA PICs was observed using CLSM after 24 h incubation with the cells. In the obtained CLSM images, the signals from Cy3-siRNA, LysoTracker Green, and Hoechst33342 are shown in red, green, and blue, respectively (Figure 7a–d). The endosomal entrapment efficiency was further estimated by calculating the number ratio of the pixels of Cy3-siRNA co-localizing with the late endosome/lysosome marker (yellow) to all the pixels of Cy3-siRNA (yellow and red). Obviously, the co-localization ratios in the polymer/silica-coated systems (SMA, NDC, and NPC) were lower than that in sPIC (Figure 7e), indicating that efficient endosomal escape should be induced by the coating of sPIC with PAsp(DET)-based polymers. Notably, the SMA featuring the detachable PEG shell showed a significantly lower co-localization ratio than NDC ($P < 0.01$), suggesting that reductive environment-responsive PEG detachment (Figure 3) might accelerate the endosomal escape of the SMA for the significantly enhanced gene silencing efficiency (Figure 5a).

Therapeutic Gene Silencing *in Vitro* and *in Vivo*. To further examine the potential utility of the SMA formulation for siRNA-based cancer therapy, the gene silencing ability of SMAs was investigated *in vitro* and *in vivo* for a therapeutic gene targeting the human renal cancer cell line OS-RC-2. Treatment of renal cancer with siRNA is a particularly attractive therapeutic target, as highly effective clinical anticancer drugs are currently unavailable. Vascular endothelial growth factor (VEGF) was selected as the target gene, because VEGF is a proangiogenic molecule that is overexpressed in a wide variety of cancer cells to stimulate angiogenesis and tumor growth.[49] To date, several previous studies demonstrated that VEGF gene silencing in tumor tissues significantly inhibits tumor growth.[34,50−53] Thus, the gene silencing ability of SMAs containing siRNA targeted toward VEGF (siVEGF) was examined in cultured OS-RC-2 cells by real-time reverse transcriptional PCR (RT-PCR). As shown in Figure 8a, SMAs containing siVEGF significantly reduced the VEGF mRNA level (∼57%), compared to control SMAs containing scramble siRNA (siSCR).

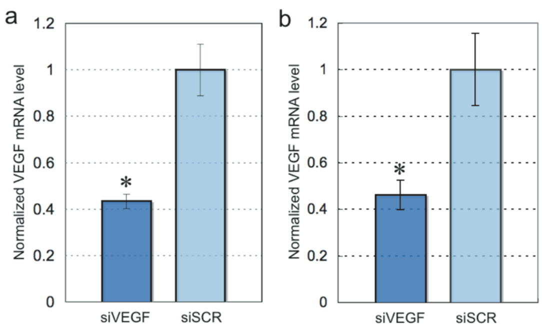

Figure 8. (a) *In vitro* VEGF gene silencing activity of SMAs in OS-RC-2 cells determined by RT-PCR. OS-RC-2 cells were incubated with SMAs for 48 h (siRNA concentration: 200 nM) prior to RT-PCR analysis. (b) *In vivo* VEGF gene silencing activity of SMAs in subcutaneous OS-RC-2 tumors. SMAs were intravenously injected into mice (1.25 mg siRNA/kg mouse) at days 1 and 2, and at day 3 subcutaneous tumors were excised and total RNA was extracted. In both *in vitro* and *in vivo* experiments, the level of VEGF mRNA was normalized to actin mRNA, and scramble siRNA (siSCR) was used as a control sequence for VEGF siRNA (siVEGF). Results are expressed as mean and standard error of the mean (n = 4). *P < 0.05 compared to siSCR.

Next, the *in vivo* gene silencing activity of siRNA delivered in SMAs was investigated using a subcutaneous OS-RC-2 tumor model following systemic administration by tail vein injection (1.25 mg siRNA/kg mouse). Prior to the gene silencing assay, several hematological parameters of mice treated with SMAs containing siSCR were monitored 24 h after systemic administration as indices of *in vivo* toxicity. Analysis of hematological parameters revealed that systemically administered SMA induced no problematic hematological toxicity (Table 2). For *in vivo* gene silencing assays, each mouse was injected twice at days 1 and 2, and at day 3 subcutaneous tumors were excised and RNA was extracted for RT-PCR. Figure 8b clearly shows that systemic administration of SMAs containing siVEGF induced significant reduction in the VEGF mRNA level (∼50%) in the tumor tissue, compared to the control containing siSCR ($P < 0.05$), demonstrating the sequence-specific gene silencing ability of siRNA delivered by SMAs. Altogether, these results demonstrate the strong potential of SMAs for systemic siRNA delivery to tumor tissues without adverse side effects.

CONCLUSIONS

In this study, a smart multilayered assembly was prepared by a layer-by-layer method with PICs to feature a siRNA-loaded core, a transiently core-stabilizing silica interlayer, an endosome-disrupting PAsp(DET) interlayer, and a detachable PEG shell for

TABLE 2. Hematological Parameters of Mice Treated with SMA[a]

	siRNA dose (mg/kg)	ALT (U/L)	AST (U/L)	RBC ($\times 10^5/\mu$L)	WBC ($\times 10^2/\mu$L)	hemoglobin (g/dL)
buffer control	0	48 ± 4	54 ± 2	100 ± 2	27 ± 4	14.4 ± 0.3
SMA	1.25	43 ± 5	49 ± 4	97 ± 4	30 ± 4	14.4 ± 0.5

[a] Blood samples (n = 4) were collected 24 h after systemic administration. ALT: alanine aminotransferase; AST, aspartate aminotransferase; RBC, red blood cells; WBC, white blood cells.

multifunctional siRNA delivery. The successful preparation was confirmed by the change in size and ζ-potential of the PICs as well as their TEM images. The PEG detachability of the SMA in response to the reductive conditions was also confirmed by the change in size (or colloidal stability) and ζ-potential. The silica interlayer in the SMA substantially improved the tolerability of siRNA PICs to the dissociation triggered by the anionic lipids, while significant siRNA release was induced after the dialysis for removal of generated free silicates, indicating the dissolvable nature of the silica interlayer. SMAs significantly enhanced gene silencing for not only a reporter gene but also an endogenous therapeutic gene (VEGF) in cultured cancer cells without increased cytotoxicity. The major mechanism for the enhanced gene silencing in SMA was probably the facilitated endosomal escape of siRNA PICs through endosome disruption by the exposed PAsp(DET) layer after PEG detachment. Ultimately, systemic administration of SMAs into subcutaneous tumor-bearing mice resulted in significant VEGF gene silencing in the tumor tissue without problematic hematological toxicity. These results demonstrate the potential utility of the SMA formulation for systemic siRNA delivery aimed toward cancer therapy.

MATERIALS AND METHODS

Materials. α-Methoxy-ω-mercapto poly(ethylene glycol) (PEG-SH, M_n = 10 000) and α-methoxy-ω-amino poly(ethylene glycol) (PEG-NH_2, M_n = 12 000) were obtained from NOF Co. (Tokyo, Japan). β-Benzyl L-aspartate *N*-carboxy anhydride (BLA-NCA) was purchased from Chuo Kaseihin Co., Inc. (Tokyo, Japan). Methanol (MeOH), 2-aminoethanethiol, benzene, hexane, ethyl acetate, *N*,*N*-dimethylformamide (DMF), dichloromethane (DCM), diethylenetriamine (DET), *N*-methyl-2-pyrrolidone (NMP), *n*-butylamine, and dithiothreitol (DTT) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). DMF, DCM, NMP, *n*-butylamine, and DET were distilled before use. Dulbecco's modified Eagle's medium (DMEM) was purchased from Sigma Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Dainippon Sumitomo Pharma Co, Ltd. (Osaka, Japan). Sterile HEPES (1 M, pH 7.3) was purchased from Amresco (Solon, OH, USA). The luciferase assay system was purchased from Promega Co. (Madison, WI, USA). All the RNA molecules, including 5′-Cy3-labeled RNA, were synthesized by Hokkaido System Science (Hokkaido, Japan). The sequences are as follows: GL3 luciferase siRNA (sense: 5′-(Cy3-)CUU ACG CUG AGU ACU UCG AdTdT-3′, antisense: 5′-UCG AAG UAC UCA GCG UAA GdTdT-3′), human VEGF siRNA (sense: 5′-GAU CUC AUC AGG GUA CUC CdTdT-3′, antisense: 5′-GGA GUA CCC UGA UGA GAU CdTdT-3′), and scramble siRNA (sense: 5′-UUC UCC GAA CGU GUC ACG UdTdT-3′, antisense: 5′-ACG UGA CAC GUU CGG AGA AdTdT-3′).

Synthesis of PEG-SS-NH_2. α-Methoxy-ω-dithioamino poly(ethylene glycol) (PEG-SS-NH_2) was synthesized as previously described.[33] In brief, PEG-SH (3.75 g, 0.375 mmol) was dissolved in methanol (400 mL) containing 28% sodium methoxide. 2-Aminoethanethiol (4.23 g, 37.3 mmol) was added to the PEG-SH solution, and the mixture was stirred for 5 days at room temperature. Then, the reaction solution was neutralized with cold 5 N HCl (8.15 mL, 40.8 mmol), followed by dialysis against distilled water for 1 day. The dialyzed solution was further purified through the ion-exchange resin (SP-Sephadex C-50, solute: H_2O), followed by lyophilization to obtain PEG-SS-NH_2 as a chloride salt form. Finally, PEG-SS-NH_3Cl dissolved in deionized water was dialyzed against 0.1% NH_3 solution for 1 day to deprotonate the amino group of the PEG-SS-NH_2. Then, the sample (PEG-SS-NH_2) was lyophilized and collected (2.76 g, 0.276 mmol).

Synthesis of PEG-SS-PBLA, PEG-PBLA, and PBLA. Poly(ethylene glycol)-disulfide-poly(β-benzyl L-aspartate) (PEG-SS-PBLA), poly(ethylene glycol)-poly(β-benzyl L-aspartate) (PEG-PBLA), and poly(β-benzyl L-aspartate) (PBLA) were synthesized by the ring-opening polymerization of BLA-NCA initiated by PEG-SS-NH_2, PEG-NH_2, and *n*-butylamine, respectively, according to the previously described method.[33] The typical synthetic procedure is briefly shown for PEG-SS-PBLA. PEG-SS-NH_2 (0.40 g, 0.04 mmol) and BLA-NCA (1.08 g, 4.32 mmol) were dissolved in DCM (6.0 mL) and DMF (1.5 mL), respectively. The solution containing BLA-NCA was added to the PEG-SS-NH_2 solution and stirred at 35 °C under an argon atmosphere. After 48 h, the reaction solution was precipitated in hexane/ethyl acetate (6:4 v/v) and dried overnight under reduced pressure to obtain PEG-SS-PBLA (1.12 g, yield 87%). In order to determine the molecular weight distribution (M_w/M_n) of the obtained polymer, size exclusion chromatography (SEC) was performed using a TOSOH HLC-8820 equipped with TSK gel columns (SuperAW4000 and SuperAW3000 × 2, TOSOH, Japan) and an internal refractive index detector at a flow rate of 0.3 mL min^{-1} at 40 °C. NMP with 10 mM LiBr was used as an eluent. A narrow M_w/M_n (= 1.10) was confirmed from the SEC (data not shown). PEG-PBLA and PBLA were synthesized in a similar manner; *e.g.*, BLA-NCA (840 mg, 3.36 mmol) and PEG-NH_2 (370 mg, 0.03 mmol) for PEG-PBLA (926 mg, yield 93%); and BLA-NCA (1.40 g, 4.86 mmol or 1.21 mg, 4.20 mmol) and *n*-butylamine (6.00 mL, 0.0607 mmol or 2.93 μL, 0.0296 mmol) for PBLA with different degrees of polymerization (DPs) (888 mg, yield 77% or 910 mg, yield 91%), respectively. The DPs of the PBLA segment in PEG-SS-PBLA and PEG-PBLA were calculated to be 87 and 92, respectively, from the peak intensity ratio of the PEG protons to the benzyl protons ($C_6H_5CH_2-$, δ = 5.1 and 7.3 ppm) at the side chain, and the DPs of two PBLAs were determined to be 92 and 225 from the peak intensity ratio of the butyl protons ($CH_3CH_2CH_2CH_2-$, δ = 0.8−1.5 ppm) at the α-chain end to the benzyl protons ($C_6H_5CH_2-$, δ = 5.1 and 7.3 ppm) at the side chain in the ^{1}H NMR spectrum (polymer concentration: 10 mg/mL, solvent: dimethyl sulfoxide-d_6, temperature: 80 °C) (data not shown).

Synthesis of PEG-SS-PAsp(DET), PEG-PAsp(DET), and PAsp(DET). Introduction of a *N*′-[*N*-(2-aminoethyl)-2-aminoethyl] moiety into the polyaspartamide side chain was performed by the aminolysis reaction of benzyl groups of the PBLA segment with DET as previously described.[33] Briefly, a typical synthetic procedure is shown for poly(ethylene glycol)-disulfide-poly{*N*′-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide} (PEG-SS-PAsp(DET)). PEG-SS-PBLA (200 mg, 7 μmol) lyophilized from a mixed solution of DCM (5 mL) and benzene (20 mL) was dissolved in NMP (10 mL) under an argon atmosphere. DET (3.44 mL, 32 mmol, 50 equiv to benzyl group in PEG-SS-PBLA) was dissolved in NMP (3.44 mL). The PEG-SS-PBLA solution was then added to the cooled DET solution and stirred at 4 °C for 1 h under an argon atmosphere. The reaction solution was added dropwise into cold 5 N HCl_{aq} (19.2 mL, 96 mmol) for neutralization and subsequently dialyzed against 0.01 M HCl_{aq} for 24 h and deionized water for an additional 24 h using a dialysis membrane (molecular weight cutoff: 6000−8000 Da). The dialyzed solution was lyophilized to obtain PEG-SS-PAsp(DET) as a hydrochloride salt (170 mg, yield 70%). Poly(ethylene glycol)-poly{*N*′-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide} (PEG-PAsp(DET)) and poly{*N*′-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide} (PAsp(DET)) were synthesized in a similar manner, *e.g.*, PEG-PBLA (103 mg, 3.20 μmol) and DET (1.5 mL, 14.7 mmol) for PEG-PAsp(DET) (98.0 mg, yield 85%); and PBLA (149 mg, 7.90 μmol) and DET (3.9 mL, 38.2 mmol) for PAsp(DET) (182 mg, yield 91%). The quantitative conversion of PBLA to PAsp(DET) in PEG-SS-PAsp(DET) and PEG-PAsp(DET) was confirmed from the peak intensity ratio of the protons of the PEG chain ($-(CH_2)_2$-O$-$, δ = 3.7 ppm) to those of the ethylene unit in the 1,2-diaminoethane ($H_2N(CH_2)_2NH(CH_2)_2NH-$, δ = 3.1$-$3.5 ppm) moieties in the side chain of PAsp(DET) in the ^{1}H NMR spectra (polymer concentration: 10 mg/mL, solvent: D_2O, temperature: 80 °C) (Supporting Figure 1a and b, respectively). In the case of PAsp(DET), the quantitative conversion was confirmed from the peak intensity ratio of the protons of the butyl group at the α-chain end ($CH_3CH_2CH_2CH_2-$, δ = 0.8$-$1.5 ppm) to the ethylene protons in the 1,2-diaminoethane ($H_2N(CH_2)_2NH(CH_2)_2NH-$, δ = 3.1$-$3.5 ppm) moieties in the side chain of PAsp(DET) in the ^{1}H NMR spectra (polymer concentration: 10 mg/mL, solvent: D_2O, temperature: 80 °C) (Supporting Figure 1c and d).

Preparation of a Series of PICs. PAsp(DET) (DP = 225) was dissolved in 10 mM HEPES buffer (pH 7.3) at a concentration of 5 mg/mL. Then, the PAsp(DET) solution was mixed with 15 μM siRNA (10 mM HEPES buffer, pH 7.3) to obtain an siRNA-incorporating PIC (final siRNA concentration: 2 μM). The residual molar ratio of amines in PAsp(DET) to phosphates in siRNA in the PIC solution was set to 3 to obtain a slightly positive PIC without an excess amount of polycations. After 30 min incubation at 4 °C, varying concentrations of sodium silicate solutions (10 mM HEPES buffer (pH 7.3)) were added to the PIC solution (final siRNA concentration: 1 μM). After 24 h incubation at room temperature, the mixed solution was purified by ultrafiltration (3000*g*, molecular weight cutoff: 300,000 Da) to remove the unbound silica species. Then, the purified solutions were mixed with the solution containing PEG-SS-PAsp(DET), PEG-PAsp(DET), or PAsp(DET) (DP = 92) (final siRNA concentration: 2 μM), followed by incubation at 25 °C for 1 h to obtain polymer/silica-coated PICs.

Size and ζ-Potential Measurements. The size and ζ-potential of each PIC were measured using a Zetasizer Nano-ZS instrument (Malvern Instruments, Malvern, UK) equipped with a He$-$Ne ion laser (λ = 633 nm) as an incident beam at a detection angle of 173° and at a temperature of 25 °C. In DLS, each PIC solution (20 μL, 1 μM siRNA, 10 mM HEPES buffer (pH 7.3) with or without 150 mM NaCl) was added into a low-volume cuvette (Malvern Instruments, Malvern, UK) for the measurements. The cumulant method was used to analyze the data obtained from the decay in the photon correlation function to obtain the hydrodynamic diameters and polydispersity indices. The results were shown as mean and standard deviation of the mean obtained from eight samples.

For ζ-potential measurement, each sample (700 μL, 1 μM siRNA, 10 mM HEPES buffer (pH 7.3)) was put into a folded capillary cell (Malvern Instruments, Malvern, UK). The obtained electrophoretic mobility was converted to the ζ-potential by applying the Smoluchowski equation: $\zeta = 4\pi\eta\nu/\varepsilon$ (η: viscosity of the solvent, ν: electrophoretic mobility, ε: dielectric constant of the solvent). The results were shown as mean and standard deviation obtained from three samples.

Transmission Electron Microscopic Observation. TEM observation was conducted using an H-7000 electron microscope (Hitachi, Tokyo, Japan) operated at 75 kV acceleration voltage. Copper TEM grids with carbon-coated collodion film were glow-discharged for 10 s using an Eiko IB-3 ion coater (Eiko Engineering Co. Ltd., Japan). The grids were dipped into PIC solution (2 μM siRNA), which was mixed with uranyl acetate solution (2% w/v) for 60 s. After removal of excess solution with a filter paper, the sample grids were allowed to dry in air and then TEM observation was performed.

Agarose Gel Electrophoresis. siRNA release from PICs caused by an anionic lipid, 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine sodium salt, was evaluated by agarose gel electrophoresis for estimation of the PIC stability. Each PIC solution (2 μM siRNA) was mixed with DOPS solution (2 mg/mL) at a molar ratio of carboxyl groups in DOPS to phosphate groups in siRNA of 32. After 48 h incubation at room temperature, the mixed solutions were subjected to electrophoresis by 0.9 wt % agarose gel in the TAE buffer (pH 7.4). siRNA in the gel was stained by ethidium bromide and analyzed by an FX molecular imager (BIO-RAD) equipped with Quantity One software (BIO-RAD).

Gene Silencing Assay. The gene silencing efficiency of the PICs was evaluated from the luciferase-based luminescence intensity of luciferase-expressing human hepatoma cells, HuH7-Luc. The cells were plated on a 48-well plate at a cell density of 5000 cells/well in DMEM supplemented with 10% FBS and incubated for 24 h. Then, the old medium was replaced with fresh medium, and PICs were added at a concentration of 100, 200, 300, and 400 nM siRNA. After 48 h incubation, the PIC-containing medium was removed. After washing with 100 μL of PBS, the cells were lysed with 100 μL of the cell culture lysis buffer (Promega). The luciferase expression in the lysate was measured using a luciferase assay system (Promega) and a luminescence microplate reader (Mithras LB 940, Berthold Technologies, Bad Wildbad, Germany). The relative luciferase expression of the cells treated with PICs was calculated as a ratio of the expression of nontreated cells. The results were expressed as mean and standard deviation obtained from four samples.

Cell Viability Assay. HuH7-Luc cells were plated on a 48-well plate at a cell density of 5000 cell/well in DMEM supplemented with 10% FBS. After incubation for 24 h, the old medium was replaced with fresh medium, and the PICs were applied at a concentration of 100, 200, 300, and 400 nM siRNA. After 48 h incubation, the cell viability assay was performed using Cell Counting Kit-8 with WST-8, a soluble tetrasolium salt, according to the manufacturer's protocol (Dojindo, Japan). The absorbance was measured using a microplate reader with a filter of 450 nm (model 680, BIO-RAD). The cell viability was determined as a percentage of the absorbance of nontreated cells. The results were expressed as mean and standard deviation obtained from eight samples.

Flow Cytometric Analysis. HuH7-Luc cells were plated on a 12-well plate at a cell density of 25 000 cells/well in DMEM supplemented with 10% FBS and incubated for 24 h. The PICs prepared from Cy3-siRNA were applied to each well at a concentration of 400 nM siRNA. After 24 h incubation, cells were washed three times with PBS, treated with a trypsin-EDTA solution, and suspended in PBS. The fluorescence intensity of the suspended solutions was measured using a BD LSR II flow cytometer (BD Biosciences). The results were expressed as mean and standard deviation obtained from three samples.

Confocal Laser Scanning Microscopic Observation. HuH7-Luc cells were plated on a 35 mm glass-based dish (Iwaki, Tokyo, Japan) at a density of 50 000 cells/well in DMEM supplemented with 10% FBS and incubated for 24 h. The old medium was replaced with fresh medium, and each sample prepared from Cy3-siRNA was applied at 400 nM siRNA. After 24 h treatment with PICs, the transfection medium was removed and then staining with LysoTracker Green (Molecular Probes, Eugene, OR, USA) and Hoechst 33342 (Dojindo, Japan) in the medium was performed. The CLSM imaging was conducted using a LSM 510 (Carl Zeiss, Oberlochen, Germany) equipped with a C-Apochromat 63× objective (Carl Zeiss). The LysoTracker Green, Cy3-siRNA, and Hoechst 33342 were excited at 488 nm (Ar laser), 543 nm (He$-$Ne laser), and 710 nm (MaiTai laser for two-photon imaging), respectively.

The intracellular distribution of Cy3-siRNA was quantitatively evaluated by calculating the co-localization ratio of Cy3-siRNA pixels with LysoTracker Green pixels.[12,42] The calculation was conducted as follows:

$$\text{Co-localization ratio } (\%) = 100 \times \text{number of yellow pixels} / \text{number of yellow and red pixels}$$

The results are expressed as mean and standard deviation obtained from 20 cells.

RNA Recovery from Cultured and Subcutaneous OS-RC-2. Endogenous gene silencing efficiency of SMAs was evaluated using a human renal cell carcinoma, OS-RC-2 (RIKEN Bioresource Center, Tsukuba, Japan). For *in vitro* experiments, the cells were seeded onto a 12-well plate at a cell density of 125 000 cells/well in RPMI-1640 containing 10% FBS. After 24 h incubation, the medium was exchanged, and SMAs containing siVEGF or siSCR were added at 200 nM siRNA. After 48 h incubation, the cells were lysed in 1 mL of Isogen (Nippon Gene, Tokyo, Japan), followed by RNA extraction by a conventional method with chloroform and 2-propanol. For *in vivo* experiments, Balb/c nude mice (male, 6-weeks-old) were subcutaneously inoculated with OS-RC-2 cells (10^7 cells/mouse), and tumors were allowed to grow for 1 week before sample injection. SMAs prepared at 2 μM siVEGF or siSCR were concentrated to 10 μM siRNA using a Vivaspin column (Sartorius Stedim Biotech, Bohemia, NY, USA) and then injected *via* the tail vein two times at days 1 and 2 (25 μg of siRNA in 200 μL per injection). At day 3, each tumor was excised and lysed in 0.5 mL of Isogen with sonication, followed by RNA extraction.

Real-Time RT-PCR. After RNA extraction, the RNA concentration in each sample was adjusted to ~20 μg/mL. Genomic DNA elimination and cDNA synthesis were performed using a ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan) according to the manufacturer's protocol. Real-time RT-PCR was performed using an ABI 7500 Fast real-time RT-PCR system (Applied Biosystems, Foster City, CA, USA) with QuantiTect SYBR Green PCR Master Mix (Qiagen, Valecia, CA, USA). Human actin was used as a housekeeper gene (internal standard), and obtained data were normalized before statistical analysis. VEGF primer (forward: AGTGGTC-CCAGGCTGCAC, reverse: TCCATGAACTTCACCACTTCGT) and actin primer (forward: CCAACCGCGAGAAGATGA, reverse: CC-AGAGGCGTACAGGGATAG) used for RT-PCR were obtained from Hokkaido System Science (Hokkaido, Japan). The results were expressed as mean and standard error of the mean from four samples.

Hematological Toxicity Assay. SMAs were intravenously injected into the tail vein of Balb/c mice (male, 7-weeks-old), similar to the *in vivo* gene silencing assay (25 μg of siRNA in 200 μL per injection). HEPES buffer (10 mM, pH 7.4) containing 150 mM NaCl was used as a control solution. After 24 h, the mice were anesthetized and their blood was collected from the postcaval vein. The collected blood was analyzed to determine the level of ALT and AST with DRI-CHEM 7000i (Fuji Film, Tokyo, Japan) and the level of RBC, WBC, and hemoglobin with pocH-100iV Diff (Sysmex, Hyogo, Japan) according to the manufacturer's protocol. The results were expressed as mean and standard error of the mean from four samples.

Data Analysis. The experimental data were analyzed by Student's *t*-test. $P < 0.05$ was considered statistically significant.

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

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Supporting Information Available: ^{1}H NMR spectra of PEG-SS-PAsp(DET) (Figure 1a), PEG-PAsp(DET) (Figure 1b), PAsp(DET) with DP = 92 (Figure 1c), and PAsp(DET) with DP = 225 (Figure 1d). Diameter and ζ-potential of silica-coated siRNA PICs prepared at varying sodium silicate concentrations (Figure 2). Agarose gel electrophoresis of siRNA PICs (Figure 3). Diameter of PEG-SS-PAsp(DET)/silica-coated siRNA PICs prepared at varying concentrations of PEG-SS-PAsp(DET) amine (Figure 4). Size distribution histograms of sPICs and SMAs determined by TEM images and the number statistics of DLS (Figure 5). TEM image of SMAs obtained with JEM-1400 (JEOL Ltd., Tokyo, Japan) (Figure 6). Time-dependent change in size of NPC and NDC in 10 mM HEPES buffer containing 150 mM NaCl (Figure 7). Gene silencing efficiency in A549-Luc and SKOV3-Luc cells (Figure 8). These materials are available free of charge *via* the Internet at http://pubs.acs.org.

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