Engineered Proteinticles for Targeted Delivery of siRNA to Cancer Cells

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Considering the problems of small interfering RNA (siRNA) delivery using traditional viral and nonviral vehicles, a new siRNA delivery system to enhance efficiency and safety needs to be developed. Here human ferritin-based proteinticles are genetically engineered to simultaneously display various functional peptides on the surface of proteinticles: cationic peptide to capture siRNA, tumor cell targeting and penetrating peptides, and enzymatically cleaved peptide to release siRNA inside tumor cell. In the in vitro treatment of poly-siRNA-proteinticle complex, both of the tumor cell targeting and penetrating peptides are important for efficient delivery of siRNA, and the red fluorescent protein (RFP) expression in RFP-expressing tumor cells is notably suppressed by the delivered siRNA with the complementary sequence to RFP mRNA. It seems that the human ferritin-based proteinticle is an efficient, stable, and safe tool for siRNA delivery, having a great potential for application to in vivo cancer treatment. The unique feature of proteinticles is that multiple and functional peptides can be simultaneously and evenly placed and also easily switched on the proteinticle surface through a simple genetic modification, which is likely to make proteinticles appropriate for targeted delivery of siRNA to a wide range of cancer cells.

1. Introduction

RNA interference is a highly conserved biological mechanism whereby double-stranded RNA (dsRNA) interferes with the expression of genes with a complementary sequence. With increasing knowledge of the molecular mechanisms of RNA interference,[1–4] small interfering RNAs (siRNAs) have recently emerged as an innovative nucleic acid drugs for the treatment of various diseases including viral infections[5,6] and cancer.[7–9] The use of siRNA as a therapeutic agent has the advantage of utilizing a naturally occurring and biologically conserved phenomenon, and furthermore siRNA is highly specific and can target any genes (or mRNAs) without a limit.[10–12] In addition, compared to other nucleic acid-based therapeutics aimed at post-transcriptional gene silencing, such as antisense oligodeoxynucleotides, siRNA molecules achieve greater magnitude and duration of gene silencing at significantly lower doses. The siRNA-baed gene silencing effect continues for 3–7 days in rapidly dividing cells or even for several weeks in nondividing cells.[13,14] However, the application of siRNA to the treatment of disease poses several significant challenges that must be overcome. One of such challenges is that naked siRNA is subject to being rapidly degraded by endogenous nuclease enzymes. In addition, siRNA is too large and negatively charged to effectively cross cellular membranes and penetrate into cytoplasm.[13,15] Therefore, the success of siRNA-based therapy is highly dependent on the design of delivery vehicles that should be able to protect siRNA from in vivo degradation and specifically deliver siRNA to cytosol of target cells.

Reportedly, to deliver siRNA to cytosol of target cells, two major delivery systems have been developed, viral and nonviral vehicle-based systems. As regards to viral delivery systems employing viral vectors that express short hairpin RNA (shRNA) and subsequently produce siRNA after transfected into target cells,[16–20] large scale preparation of viral vectors are difficult, and there is a significant concern about safety issues including side effects such as mutagenesis and carcinogenesis.[21–23] Nonviral siRNA delivery systems that are mostly based on liposomes and lipids,[24–27] cationic polymers,[28] cholesterol conjugates,[29] and cationic polypeptides[30,31] have been reported to be less efficient than viral delivery systems and have dose-dependent toxicity problems.[32,33] Recently, the new delivery systems based on RNA nanoparticles have been reported.[34–37] Considering the great therapeutic potential of siRNA, therefore a new siRNA delivery system with enhanced efficiency and decreased toxicity needs to be developed.

Recently, proteinticles, i.e., nano-scale protein particles that are self-assembled inside cells with constant 3D structure and surface topology (e.g., diverse human-, bacteria-, and virus-derived proteinticles) have been used for a variety of in vitro bioassays and in vivo medical applications.[38–42] Proteinticles

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can be synthesized cost-effectively in *E. coli* with uniform size distribution and have much enhanced biocompatibility compared to synthetic nanomaterials. More importantly, they can be easily engineered to simultaneously display multiple and different peptides (e.g., cationic peptide for siRNA conjugation, cell-targeting and cell-penetrating peptides in this case) on their surface through simple genetic modification of the N- or C-terminus or internal region of subunit protein.[38–46] To solve the traditional problems (inefficiency of siRNA delivery and toxicity or side effects of viral- and nonviral vehicles), here we designed proteinticle-based delivery systems through surface engineering of human ferritin: on the surface of proteinticle consisting of 24 human ferritin heavy chains, we genetically displayed cationic peptides for tightly interacting with siRNA as well as cell-targeting and cell-penetrating peptides and specific peptide sequence for enzymatic cleavage to effectively release siRNA inside cytoplasm of target cells. In this study, the tight and stable complexes between the surface-engineered proteinticle and siRNA were successfully formed and were effectively delivered to target cancer cells, followed by efficient gene silencing.

2. Results and Discussion

2.1. Synthesis of Tumor-Specific Proteinticles to Enhance Cell Targeting and Penetration and siRNA Delivery

Owing to self-assembly activity inside cells, human ferritin heavy chain (hFTH) forms homopolymer (24-mer), i.e., spherical proteinticle with a diameter of about 12 nm, when expressed in *E. coli*.[44] Both for the effective capture of siRNA and for the targeted delivery of siRNA to tumor cells, here we engineered human ferritin-based proteinticles to simultaneously display various functional peptides on the surface of proteinticles: cationic peptide (CAP) to capture siRNA, tumor cell targeting and penetrating peptides (CTP and CPP, respectively), and enzymatically cleaved peptide (ECP) to release siRNA inside tumor cell. That is, we genetically fused the CAP, CTP, CPP, and ECP to the C-terminus of hFTH (**Figure 1**A), and the modified hFTHs were expressed and self-assembled to form the spherical proteinticles with uniform size (about 18 nm in diameter) in *E. coli* (Figure 1B, and Figure S1, Supporting Information). The surface of individual proteinticle displays 24 peptide composites, each of which is NH_2-ECP-CAP-CPP-CTP-*COOH* (Figure 1A).

The CAP used in this study is derived from human protamine, a chromatin-compacting sperm component. Protamine that is a natural arginine-rich cationic polypeptide and condenses negatively charged nucleic acids has been used as an efficient gene/siRNA delivery carrier.[47,48] Also, the protamine-siRNA complexes did not bring any noticeable increase in inflammatory cytokines (e.g., interferon-α, interleukin-12, etc.) in serum, indicating negligible immunostimulatory effect.[48]

For the tumor cell-specific delivery of siRNA, CTP in the peptide composite (NH_2-ECP-CAP-CPP-CTP-*COOH*) should be changed in accordance with target moiety on tumor cell. Here we employed two different peptides as CTP, i.e., vimentin- and human epidermal growth factor receptor

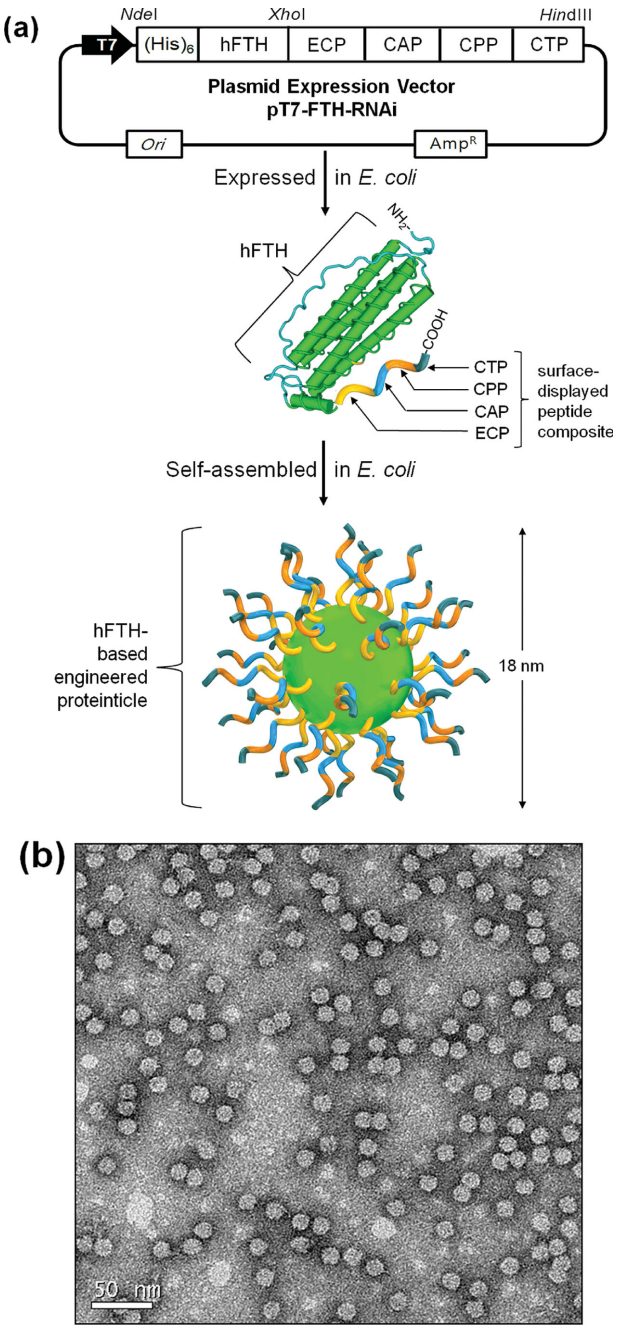

Figure 1. A) A schematic showing genetic modification of human ferritin heavy chain (hFTH) and biosynthesis of hFTH-based engineered proteinticles. B) TEM image of hFTH-based engineered proteinticles used for siRNA delivery.

(EGFR)-recognizing peptides (named VRP and ERP, respectively) for the targeted delivery of siRNA to the tumor cells with different target moieties on their surface, i.e., vimentin and EGFR. Vimentin is a protein that is expressed on the surface of various cancer (human prostate, colon,[49] hepatocellular,[50] and gemcitabine-resistant pancreatic cancers,[51] and the tumor stromal cells in human colorectal tumors[52] and epithelial cells during angiogenesis.[53] The up-regulation of vimentin is associated with the epithelial-to-mesenchymal

transition, which is important for motility as well as metastasis in the tumors above.[53] EGFR is an important target receptor in many types of cancers, including nonsmall cell lung cancer, skin cancer, breast cancer, small cell carcinoma of head and neck, and prostate cancer.[54–56] Overexpression of EGFR is shown to be correlated with poor prognosis and increased metastatic potential.[57,58] As demonstrated here, the CTP on the surface of proteinticle can be easily switched to other one, depending on the cancer cell type, which is a great advantage of proteinticle-based siRNA delivery.

The CPP used here is derived from the basic motif of human immunodeficiency virus type 1 (HIV-1) transactivator of transcription (TAT) that possesses a unique membrane-transduction property.[59] The Arg-rich basic region (G48-Q60) of TAT is called protein transduction domains or cell-penetrating peptides and have been widely exploited as vehicles for intracellular delivery of macromolecules (e.g., oligonucleotides, peptides, low-molecular-mass drugs, nanoparticles, liposomes, etc.).[60,61] It is presumed that the short basic peptide leads to the effective penetration of macromolecules into anionic cell surface with anionic membrane proteins and phospholipids.[59] Also we included ECP in the peptide composite on the engineered proteinticle surface. The ECP is specifically recognized and cleaved by lysosomal protease cathepsin B that is known to play an important role in growth, migration, invasion, and metastasis of various cancers.[62] Once taken up by the target cells, siRNAs are released from the complexes by enzymatic cleavage in the cytoplasm and enter the RNAi pathway.[63]

2.2. Formation and Characterization of Engineered Proteinticle-siRNA Complexes

To form stable and condensed complex between the surface-engineered proteinticles and siRNA, siRNAs were polymerized to poly-siRNA through disulfide-linkage between thiol groups attached at 5′ end of each siRNA. Recently, we reported that poly-siRNA with higher molecular mass and anionic charge can easily form a condensed structure with cationic polymers, resulting in increased stability and efficient delivery to cancer.[64] More importantly, the poly-siRNA could be spontaneously reduced as functional monomeric siRNA molecules in cytosol to lead successful gene silencing in vitro and in vivo. After chemical polymerization of thiolated siRNA, the poly-siRNA showed the ladder-like migration pattern in the 8% polyacrylamide gel electrophoresis (PAGE), indicating successful polymerization with molecular weight distributions between 20 to above 300 bps (**Figure 2**B). Then, to make poly-siRNA-proteinticle complex, freshly prepared poly-siRNA was simply added to the surface-engineered proteinticle solution at a molar ratio of 24:1 (poly-siRNA:proteinticle) with gentle shaking (PBS, pH 7.4). Interestingly, as shown in Figure 2A, poly-siRNA tightly bound to CAP on the proteinticles, resulting in the formation of compact and raspberry-like poly-siRNA-proteinticle complex, which is also confirmed through gel retardation assay (Figure 2B) showing that the majority of poly-siRNAs form high-molecular-mass complex when is mixed with the engineered proteinticles. Dynamic light scattering (DLS) analysis shows that the average size of poly-siRNA-proteinticle

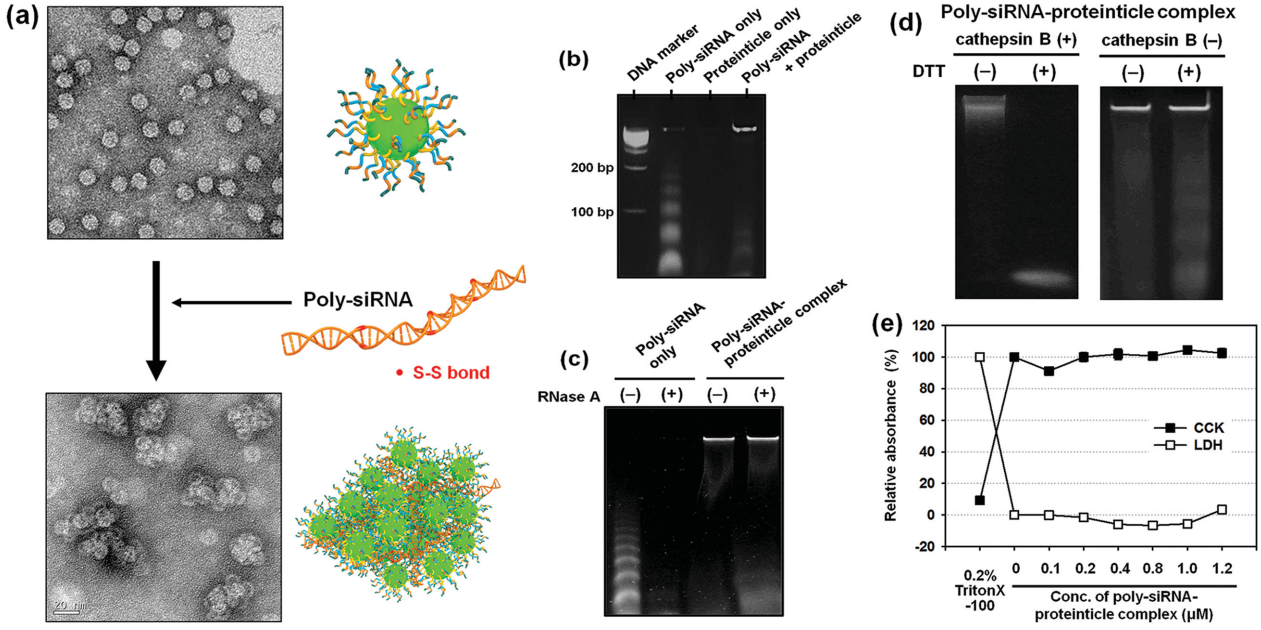

Figure 2. A) TEM images and B) result of gel retardation assay, both of which show the formation of condensed structure of poly-siRNA-proteinticle complex. C) Result of gel retardation assay showing that siRNAs in the poly-siRNA-proteinticle complex are effectively protected from endonuclease due to a shielding effect of proteinticles. D) Result of agarose gel electrophoresis showing the release of siRNA monomers from the poly-siRNA-proteinticle complex by cathepsin B protease in the presence of reducing agent (dithiothreitol/DTT). E) Cytotoxicity of poly-siRNA-proteinticle complex determined by CCK (solid square) and LDH (empty square) assays [Murine melanoma cells (B16F10) was incubated with different concentrations of poly-siRNA-proteinticle complex).

complex is about 53 nm (Figure S2, Supporting Information). The hFTH-based native proteinticle without any peptides of CAP, CTP, CPP, and ECP seldom forms the poly-siRNA-proteinticle complex (Figure S3, Supporting Information). Table S1 (Supporting Information) shows that the pI value of native proteinticles is 5.76, while the engineered proteinticles with CAP have the much higher pI values at around 10.0, implying that the engineered proteinticles have positive net charge at pH 7.4 and can easily form the complexes with poly-siRNA. Furthermore, siRNAs in the condensed structure of poly-siRNA-proteinticle complex were effectively protected from endonuclease (RNase A) due to a shielding effect of proteintlcies (Figure 2C). Figure 2D also shows that poly-siRNA in the complex was successfully separated into siRNA monomers in the presence of reducing agent (dithiothreitol, DTT) when cathepsin B enzyme was added to cleave ECP on the proteinticle surface. Since the tumor cells (B16F10) used in this study produce the intracellular cathepsin B enzyme (Figure S4, Supporting Information), this demonstrates that poly-siRNA-proteinticle complex can effectively release free siRNA monomers under the reductive condition of cytosol through the ECP cleavage by lysosomal cathepsin B after penetrated into cytoplasm of tumor cell.

Reportedly, the liposome-based siRNA delivery systems that have been intensively studied for last decade suffer from a substantial toxicity due to the high charge density and limited biodegradability, leading to contraction, mitotic inhibition, cytoplasmic vacuole formation in cells, and toxic damage to cells that lack proteoglycans. This limitation poses a significant barrier on in vivo application of liposome.[63,65–67] Similarly, the synthetic polymers such as poly lactic-co-glycolic acid (PLGA) and polyethylenimine (PEI) that have been widely investigated also have cytotoxicity problems.[68] In particular, PEI causes cellular necrosis and apoptosis and thereby cell death in a variety of cell lines.[69,70] The results of lactate dehydrogenase (LDH) and Cell Counting Kit-8 (CCK-8) assays evidently demonstrate no or little cytotoxicity of poly-siRNA-proteinticle complex although the concentration of poly-siRNA-proteinticle complex increased up to 1.2×10^{-6} M in the culture of murine melanoma (B16F10) cells (Figure 2E). Recently, Li et al.[42] have verified the safety of hFTH particles through both in vitro and in vivo tests, that is, they observed no adverse effect on cell viability even with a high dose (6×10^{-6} M) of hFTH particles, also indicating that hFTH-based proteinticles used in this study are free of cytotoxicity with high biocompatibility.

2.3. Tumor-Specific Cell Targeting of Proteinticle-siRNA Complexes and Subsequent Gene Silencing in RFP-Expressing Tumor Cells

We synthesized two different types of proteinticles, each one of which displays a specific CTP (ERP or VRP) for targeting a particular tumor cell: one proteinticle with ERP for targeting the EGFR-expressing human breast cancer cells (MDA-MB-468) and the other one with VRP for targeting vimentin-expressing murine melanoma cells (B16F10). The efficiency of tumor cell targeting of the poly-siRNA-proteinticle complexes was estimated using fluorescent dye(Cy5.5)-labeled proteinticles (Cy-proteinticles) in the cell culture of B16F10 (**Figure 3**A) or MDA-MB-468 (Figure 3C). The poly-siRNA-proteinticle complexes containing Cy-proteinticle with CTP (i.e., ERP or VRP) were incubated with the tumor cells for 2 h. In Figure 3A,C, stronger fluorescence signal was observed in the cells treated with poly-siRNA-proteinticle complexes containing Cy-proteinticle with CTP, compared to the cells incubated with poly-siRNA-proteinticle complexes containing CTP-free Cy-proteinticles, which suggested the importance of CTP for targeting tumor cells. It is also noticeable from Figure 3A,C that when CPP was removed from the proteinticles, the uptake level of poly-siRNA-proteinticle complexes by tumor cells was significantly lowered in spite of the presence of CTP on the proteinticles. (The results of quantitative analysis of fluorescence intensity per cell in Figure 3A,C were also presented in Figure S5, Supporting Information.) The time-course analysis of the delivery of poly-siRNA-proteinticle complexes into the cytoplasm of MDA-MB-468 or B16F10 cells show that the complexes initially gain access to the cell surface and ultimately move into the cytoplasm (Figure 3B,D). When incubated with human breast cancer cells (MCF-7) that express vimentin and EGFR at very low level, the poly-siRNA-proteinticle complexes were far less effectively taken up by MCF-7 cells (Figure S6, Supporting Information) compared to the results of Figure 3, indicating that the uptake of poly-siRNA-proteinticle complexes is highly dependent on the surface target molecule on tumor cell line. Through many previous studies,[71–76] it has been concluded that targeting receptor (e.g., vimentin and EGFR in this case) or using TAT or TAT-derived peptides leads to enhanced cellular entry of proteins via endocytosis rather than through direct cytosolic delivery by plasma membrane translocation. These results indicate that both of CTP and CPP are important for enhancing the efficiency of tumor cell targeting of siRNA. More importantly, both of CTP and CPP can be easily placed on the surface of proteinticle through a simple genetic modification of proteinticles, and also CTP can be easily changed to other one in accordance with target moiety on cancer cell. The release of free siRNAs from the poly-siRNA-proteinticle complexes inside cell was also monitored in B16F10 cells using confocal microscopy, wherein siRNA was labeled with green fluorescence dye (YOYO-1), and proteinticle was labeled with Cy5.5. The green spots shown in Figure 3E indicate the siRNAs were successfully released and localized in cytosol of cancer cells after the fast cellular uptake of poly-siRNA-proteinticle complexes. It looks likely that this unique feature of proteinticles makes proteinticles appropriate for targeted delivery of siRNA to a wide range of cancer cells.

Finally, we evaluated the efficacy of poly-siRNA-proteinticle complex in RFP (red fluorescent protein) gene silencing in the RFP-expressing B16F10 cells (RFP-B16F10). The siRNA-based gene silencing is a fundamental RNAi pathway in eukaryotic cells, by which sequence-specific siRNA is able to target and cleave complementary mRNA.[13] After poly-siRNA-proteinticle complex (100×10^{-9} M siRNA) was added to the culture of RFP-B16F10 and subsequently incubated for 1 day, the red fluorescence of tumor cells was monitored using fluorescence microscopy. As shown in **Figure 4**A, the treatment of poly-siRNA-proteinticle complex dramatically reduced the red fluorescence signals from RFP-B16F10 cells, compared to the untreated controls. The result of RFP gene silencing was similar to that of

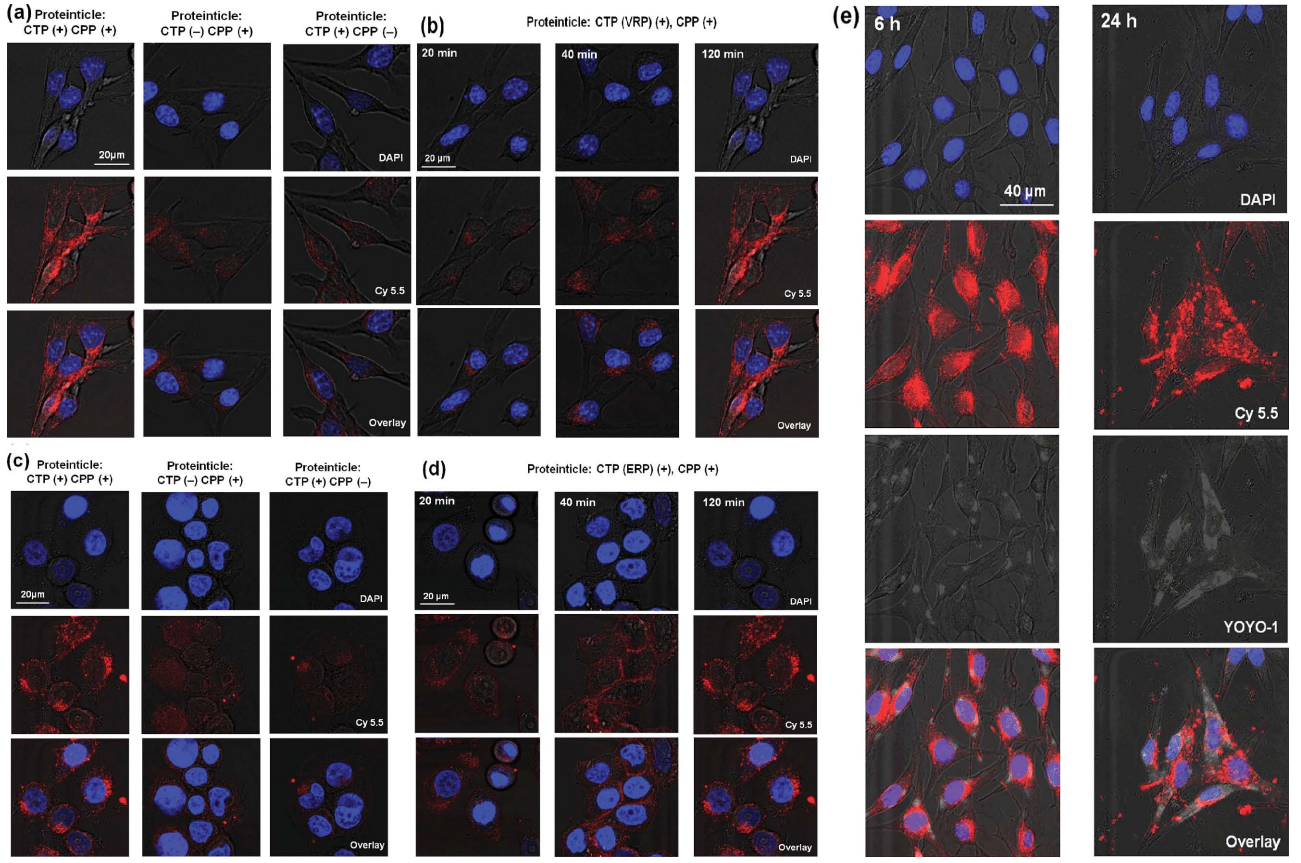

Figure 3. Cellular images after the poly-siRNA-proteinticle complex were incubated in the vimentin-expressing B16F10 A,B) and EGFR-expressing MDA-MB-468 C,D) cells for 2 h. The results of time-course analysis in the uptake of poly-siRNA-proteinticle complex by B16F10 B) and MDA-MB-468 D) cells are also presented. E) Results of cellular images showing localization and release of siRNA in cytosol of B16F10 cell after cellular uptake of poly-siRNA-proteinticle complex. (Before incubation with the tumor cells, the engineered proteinticles with VRP A,B) or ERP C,D) were labeled by Cy5.5 and poly-siRNA were labeled by YOYO-1 E). They subsequently used to form the poly-siRNA-proteinticle complex. Nuclei were counterstained with DAPI (blue).)

poly-siRNA delivery using lipofectamine 2000 (LF), a commercial transfection reagent. As a negative control, the cells were treated with the proteinticles alone without siRNA, and the red fluorescence level of tumor cells was comparable to the case of untreated control. In addition, sc-siRNA-proteinticle complex [containing scrambled siRNA (sc-siRNA) with noncomplementary sequence to RFP gene] did not decrease the red fluorescence of RFP-B16F10 cells, indicating that the suppression of RFP gene expression in the RFP-B16F10 cells was caused by the sequence-specific siRNA that was delivered in the form of poly-siRNA-proteinticle complex. As shown in Figure 4B, the RFP gene silencing in the RFP-B16F10 cells was also confirmed through the analysis of real-time reverse transcription-polymerase chain reaction (RT-PCR) that monitors the degradation of the RFP mRNA: the poly-siRNA-proteinticle complex effectively silenced RFP gene expression: approximately 60% reduction in RFP mRNA was observed, compared to the untreated control cells.

Conclusively, the siRNA delivery using the proteinticles that were genetically engineered to enhance siRNA capture, tumor-specific cell targeting followed by penetration into tumor cell cytoplasm, and intracellular release of siRNA was highly effective in gene silencing in tumor cells. It seems that the siRNA delivery system using human ferritin-based proteinticles is an efficient, stable, and safe tool for siRNA delivery and has a great potential for application to in vivo cancer treatment.

3. Experimental Section

Biosynthesis and Characterization of Engineered Proteinticles for siRNA Delivery:Through PCR amplification using the appropriate primers, three gene clones were prepared, encoding NH_2-*Nde*I-H_6-hFTH-ECP-*Xho*I-*COOH*, NH_2-*Xho*I-CAP-*Eco*RI-*COOH*, and NH_2-*Eco*RI-CAP-CPP-CTP-*Hin*dIII-*COOH*. hFTH gene was cloned using a previously cloned expression vector,[44] and the CAP, CPP, CTP, and ECP were cloned using overlap and extension PCR method. Each gene clone was sequentially ligated into a pT7–7 plasmid to construct the expression vector: pT7-FTN-RNAi. The CAP(NH_2-MARYRCCRSQSRSRYYRQRQRSRRRRRRSCQ TRRRAMRCCRPRYRP RCRRH-*COOH*) is derived from human protamin, a component of sperm chromatin. The CTP (NH_2-VNTANST-*COOH* or NH_2-VDNKFNKEMWAAWEEIRNLPNLNGW QMTAFIASLVDDPSQSANL LAEAKKLNDAQAPK-*COOH*) specifically recognizes vimentin or human epidermal growth factor receptor I (EGFR), respectively, that is expressed on the surface of tumor cells. The CPP is derived from the basic motif (NH_2-GRKKRRQRRRPPQ-*COOH*) of the human immunodeficiency virus type 1 transactivator of transcription (HIV-1 TAT), which has a unique membrane-transduction property. The ECL (NH_2-GRRGKGG-*COOH*) is specifically recognized and cleaved by lysosomal protease cathepsin

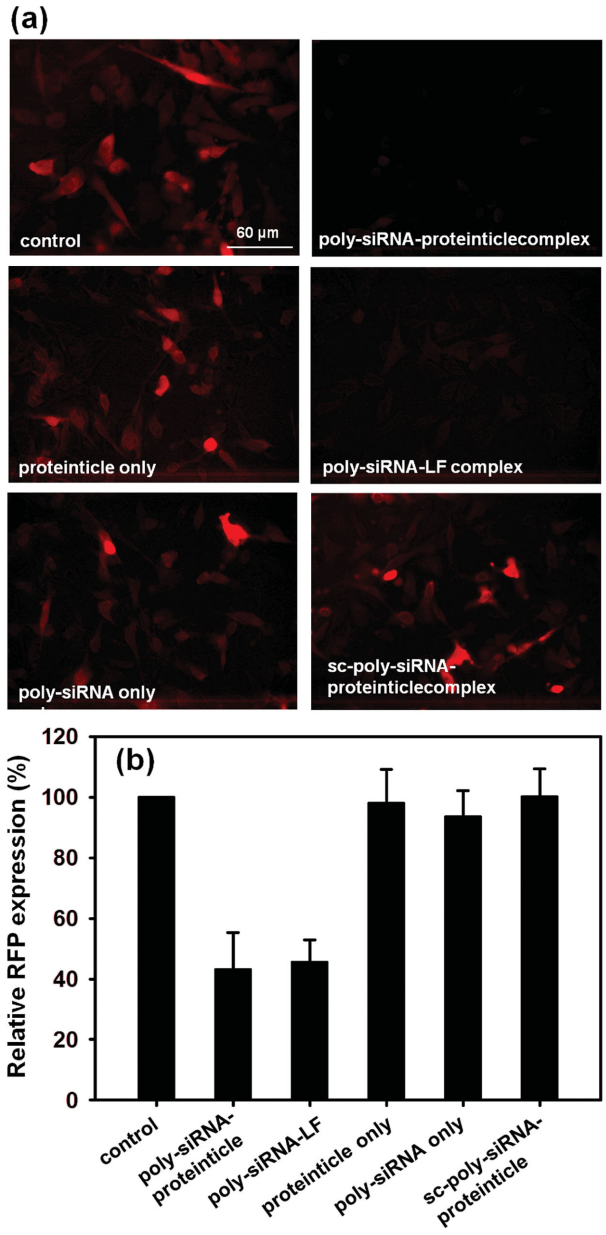

Figure 4. RFP gene silencing by poly-siRNA-proteinticle complex (100×10^{-9} M siRNA) in RFP-B16F10 cells. (The sequence of siRNA in poly-siRNA-proteinticle complex is complementary to mRNA of RFP.) (Control: RFP-B16F10 cells only.) A) Fluorescence microscopic images. B) Results of real-time RT-PCR showing mRNA level of RFP in RFP-B16F10 cells. (RFP mRNA level was quantified by normalizing with β-actin expression).

B, which is known to play an important role in growth, migration, invasion, and metastasis of various cancers. After complete sequencing of expression vector, *E. coli* (BL21(DE3) [F-ompThsdSB(rB-mB-)]) was transformed with the aforementioned expression vector, and ampicillin-resistant transformant was finally selected. The detailed procedures for recombinant gene expression and purification of synthesized proteinticles are well described in our previous report.[45] Transmission electron microscopy (TEM) images of purified proteinticles were obtained according to the previously described procedure,[45] and the particle size of proteinticles was measured using DLS (NanoBrook 90Plus, Brookhaven Instruments, NY, USA).

Preparation of Poly-siRNA and Formation and Characterization of Poly-siRNA-Proteinticle Complex: The siRNA with the complementary sequence to gene of RFP (red fluorescent protein) and the scrambled (sc) siRNA that is not complementary to RFP gene were synthesized and annealed by Bioneer (Daejeon, Korea). The sequences of siRNA and sc-siRNA are as follows: 5′-UGUAGAUGGACUUGAACUCdTdT-3′ (RFP sense strand), 5′-GACUUCAAGUGCAACUUCAdTdT-3′ (RFP antisense strand), 5′-UGAAGUUGCACUUGAAGUCdTdT-3′ (sc-siRNA for RFP sense strand), and 5′-GACUUCAAGUGCAACUUCAdTdT-3′ (sc-siRNA for RFP antisense strand). The 5′ end of siRNA was thiol-modified for the self-polymerization of siRNA under mild oxidative conditions. The detailed procedures for the polymerization of siRNA are well described in our previous report.[64] To form poly-siRNA-proteinticle complex, poly-siRNA (20 μg) was added to the surface-engineered proteinticle at a molar ratio of 24:1 (poly-siRNA:proteinticle), followed by incubation in diethyl pyrocarbonate (DEPC)-treated phosphate buffered saline (PBS) (pH 7.4) for 1 h at room temperature. All the solutions for preparation of poly-siRNA were prepared in RNase-free distilled water and autoclaved prior to being used. The complex formation was verified using a gel retardation assay (8% polyacrylamide gel) in a Tris-borate/ethylene diamine tetraacetic acid (TBE) buffer. For the estimation of size and morphology of the poly-siRNA-proteinticle complex, DLS and TEM analyses were performed, respectively, as described above. To estimate the effect of proteinticles on the protection of poly-siRNA from endonucleases, poly-siRNA-proteinticle complex or naked poly-siRNA was incubated with 0.3 U of RNase A at 37 °C for 3 h, followed by gel retardation assay. The siRNA released from poly-siRNA-proteinticle complex in the presence of DTT (50×10^{-3} M) and cathepsin B (10×10^{-9} M) (37 °C for 12 h) were detected using gel retardation assay. Cytotoxicity of poly-siRNA-proteinticle complex was evaluated in RFP-expressing B16F10 murine melanoma cells. Briefly, the seed culture of B16F10 cells (1×10^4 cells mL^{-1}) was added to 96-well plates and allowed to grow for 24 h in RPMI1640 media supplemented with 10% (w/v) fetal bovine serum (FBS) and penicillin G (100 U mL^{-1})/streptomycin (100 μg mL^{-1}) at 37 °C, 5% CO_2. Then the poly-siRNA-proteinticle complex (0.1×10^{-6}–2.0×10^{-6} M) was added to each well. After 24 h of incubation, cell viability was estimated by using CCK-8 assay (Dojindo Laboratories, Kumamoto, Japan) that measures mitochondrial succinate dehydrogenase activity: 10 μL of 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) solution was added to each well and incubated for 4 h at 37 °C, followed by measuring the amount of produced formazan dye at 450 nm using a microplate reader (Infinite M200 PRO, TECAN, Austria). The absorbance (450 nm) measured from the WST-1-free well was used as a control to correct each absorbance value measured. Also, to estimate cell damage by cytotoxicity, the activity of lactate dehydrogenase (LDH) (absorbance at 490 nm) was measured in the in vitro tumor cell culture containing poly-siRNA-proteinticle complex using LDH assay kit (Roche Diagnostics, Indianapolis, IN, USA). The relative percentage of cell damage was calculated as follows: [LDH activity with poly-siRNA-proteinticle complex—LDH activity without poly-siRNA-proteinticle complex]/[LDH activity with 0.5% Triton X-100—LDH activity without poly-siRNA-proteinticle complex] × 100.

In Vitro Cellular Uptake of Poly-siRNA-Proteinticle Complex: To estimate the cellular uptake of poly-siRNA-proteinticle complex, the N-hydroxysuccinimide (NHS) ester groups of Cy5.5 dye were chemically conjugated to the amine groups of lysine residues on the exterior surface of proteinticles as described in our previous report[33] and YOYO-1 (dimeric cyanine nucleic acid dye) was used for labeling poly-si-RNA.[77] Each of EGFR-expressing human breast cancer (MDA-MB-468) and vimentin-expressing murine melanoma (B16F10) cell cultures was added to a 6-well plate (1×10^4 cells per well). After 24 h of incubation, Cy5.5-labeled poly-siRNA-proteinticle complex or Cy5.5- and YOYO-1-labeled poly-siRNA-proteinticle complex in serum-free media was added to each cell culture and incubated at 37 °C. As negative control, the complex of poly-siRNA-proteinticle without CPP or CTP was added and incubated under the same condition. Finally, after cell fixation, the nuclei were stained with diamidino-2-phenylindole (DAPI) and the fluorescence microscopic images were acquired using fluorescence microscopy (Leica, DM IL, Germany).

Gene Silencing by Poly-siRNA-Proteinticle Complex Delivered into RFP-Expressing Tumor Cells: The efficacy of in vitro gene silencing by the poly-siRNA-proteinticle complex was evaluated using RFP-expressing B16F10 cells. At 24 h after cells was initially added to a 6-well plate (1×10^4 cells per well), the serum-free transfection media containing poly-siRNA-proteinticle or sc-siRNA-proteinticle complexes (equivalent to 100×10^{-9} M siRNA) were added and subsequently incubated for 4 h. The complex of poly-siRNA and LF (poly-siRNA-LF) was prepared according to the manufacturer's protocol and utilized as a positive control. Then the transfection media was removed and replaced with fresh RPMI1640 media containing 10% FBS, followed by an additional 24-h incubation at 37 °C. After cell fixation, red fluorescence from the cells was examined using fluorescence microscope (Axioskop 2 FS plus microscope; Carl Zeiss, Thornwood, NY). Real-time reverse transcription-polymerase chain reaction (RT-PCR) was also performed to analyze the RFP-gene silencing efficiency of poly-siRNA-proteinticle complex within the RFP-expressing B16F10 cell. After the total RNA from the lysed cells was extracted according to the manufacturer's protocol by using the RNeasy Mini Kit (QIAGEN, Valencia, CA), reverse transcription was performed using the MultiScribe Reverse Transcriptase [High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA)] and the following PCR primers (Bioneer, Daejeon, Korea): 5′-AGAGGGAAATCGTGCGTGAC-3′ and 5′-CAATAGTGATGACCTGGCCGT-3′ (forward and reverse primers for the synthesis of β-actin cDNA, respectively) and 5′-GCTGCTTCATCTACAAGGT-3′ and 5′-CGTCCA CGTAGTAGTAGCC-3′ (forward and reverse primers for the synthesis of RFP cDNA, respectively). Using these primers, 30 ng of cDNA per reaction was amplified in 20 cycles of PCR. The level of amplified RFP cDNA was normalized relatively against the level of amplified β-actin cDNA.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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