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Transferrin-Dressed Virus-like Ternary Nanoparticles with Aggregation-Induced Emission for Targeted Delivery and Rapid Cytosolic Release of siRNA

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Abstract

Viruses have evolved to be outstandingly efficient at gene delivery, but their use as vectors is limited by safety risks. Inspired by the structure of viruses, we constructed a virus-mimicking vector (denoted as TR4@siRNA@Tf NCs) with virus-like architecture and infection properties. Composed of a hydrophilic peptide, an aggregation-induced emission (AIE) luminogen, and a lipophilic tail, TR4 imitates the viral capsid and endows the vector with AIE properties as well as efficient siRNA compaction. The outer glycoprotein transferrin (Tf) mimics the viral envelope protein and endows the vector with reduced cytotoxicity as well as enhanced targeting capability. Because of the strong interaction between Tf and transferrin receptors on the cell surface, the Tf coating can accelerate the intracellular release of siRNA into the cytosol. Tf and TR4 are

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ASSOCIATED CONTENT

Supporting Information

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Characterization of TR4, fluorescence spectra, transmission electron microscopy, colocalization experiment, dynamic light scattering, and CLSM images (PDF)

The authors declare no competing financial interest.

eventually cycled back to the cell membrane. Our results confirmed that the constructed siRNA@TR4@Tf NCs show a high siRNA silencing efficiency of 85% with significantly reduced cytotoxicity. These NCs have comparable transfection ability to natural viruses while avoiding the toxicity issues associated with typical nonviral vectors. Therefore, this proposed virus-like siRNA vector, which integrates the advantages of both viral and nonviral vectors, should find many potential applications in gene therapy.

Graphical abstract

Keywords

gene delivery; virus-like vectors; transferrin; active targeting; aggregation-induced emission

INTRODUCTION

Gene therapy based on small interfering RNAs (siRNAs) has been considered as a powerful approach for treating a wide spectrum of diseases, such as cancer, viral infections, and hypercholesterolemia, by specifically silencing the functional gene in eukaryotic cells.^{1–4} Because naked siRNA cannot easily cross the cellular membrane and is at risk of degradation by enzymes, gene vectors that can protect the siRNA from degradation and convey it to the cytosol of target cells are highly desired.^{5–7} Vectors based on natural viruses have the advantage of efficient delivery owing to their precisely programmed infection properties but are hampered by some shortcomings including native cell tropism, immunogenicity, carcinogenesis, and difficulties with fabrication.^{8–11} In contrast, nonviral vectors, such as polycations,^{12,13} lipoplexes,^{14,15} and peptides,^{16,17} are faced with severe challenges of significant cytotoxicity and poor transfection capability despite the fact that they are safer and easier to synthesize.^{18,19} Therefore, constructing novel nonviral vectors with virus-like gene transfer properties is a promising approach for realizing high silencing efficiency as well as low cytotoxicity.

Great efforts have been made to construct a series of virus-mimicking vectors by self-assembly techniques.^{20–22} Shen and co-workers developed a pH-sensitive viral-mimicking nanocapsule to accelerate free DNA release and enhance transfection efficiency.²³ The nanocapsule can efficiently condense DNA under acidic conditions and unpack it in a neutral environment. Moreover, the nanocapsule is degradable in the acidic environment, which further accelerates the DNA release. Once the nanocapsule is internalized by the cells, efficient gene transfection can be achieved. To realize programmed gene delivery and

enhance gene transfection efficiency, Gu and coworkers designed a virus-mimicking DNA vector that uses a reduction-controlled hierarchical unpacking strategy.²⁴ In the tumor environment, first-stage deshielding will expose the positive charge and enhance the cellular uptake of the vectors. After the vectors are internalized by the cell, second-stage unpacking will accelerate the DNA release. Although these vectors can efficiently deliver genes and enhance transfection efficiency to some extent, various problems still exist that hinder their progress toward further applications. These include the complicated synthetic procedures, the poor repeatability of polymer synthesis, and the uncertain fate of the vectors during the transfection process.

For a deep insight into the transfection mechanism, it is necessary to monitor the fate of gene vectors, including the cellular internalization pathway and the spatial-temporal interaction between the vectors and siRNA.²⁵ A widely used strategy for visualizing vectors is fluorophore tagging through conjugation with fluorescein (FITC), cyanine dyes, and so forth.^{26,27} However, this raises concerns about the changed physicochemical properties and internalization behaviors of the vectors upon labeling as well as false information generated by the dissociated fluorophores. Alternatively, fabrication of gene vectors with self-indicating properties is a feasible way to obtain more information about the gene transfer process.²⁸ Aggregation-induced emission (AIE) molecules, a novel library of fluorophores, are nonemissive in the molecular state but highly fluorescent in the aggregated state.²⁹ AIE luminogens (AIEgens) exhibit distinguishing features of superior photostability and low background signals compared to the conventional fluorophores.^{30–33} Thus, gene vectors containing AIE moieties as a self-indicating “beacon” can facilitate real-time monitoring throughout the transfection process.

Inspired by these issues, we aimed to construct a virus-like nonviral gene vector from molecules carrying AIE moieties to facilitate self-tracking along with active targeting, low cytotoxicity, and high transfection efficiency. In our previous work, we synthesized a peptide derivative, TR4, based on a hydrophilic tetra-arginine peptide modified by the hydrophobic AIEgen tetraphenylethylene (TPE) and palmitic acid (PA) (Figure 1B).^{34,35} Herein, TR4 was adapted for use as a virus-like nonviral vector for siRNA delivery by dressing the TR4@siRNA binary complexes with transferrin (Tf) instead of a viral envelope to form TR4@siRNA@Tf nanocomplexes (TR4@siRNA@Tf NCs). The as-prepared TR4@siRNA@Tf NCs showed inherent AIE properties and allowed spatial-temporal real-time monitoring throughout the transfection process (Figure 1A). The negatively charged Tf corona was able to shield the positive charge of the TR4@siRNA NCs, resulting in lower cytotoxicity. The Tf coating also facilitated targeted cellular internalization and gene delivery via the Tf receptor (TfR). In addition, our results showed that the as-prepared Tf-dressed TR4@siRNA@Tf NCs accelerated the release of siRNA in the cytosol compared with that of TR4@siRNA NCs. Consistent with the virus-like transfection behavior of the ternary vectors, the majority of TR4 and Tf were located on the cell membranes, and the siRNA silencing efficiency was as high as 85%. Thereby, the as-prepared TR4@siRNA@Tf NCs possessed some notable properties, including virus-like transfection behavior, low cytotoxicity, targeting delivery, and inherent AIE for self-tracking, which suggest that this kind of constructed virus-like nonviral gene vector may find many potential applications in gene therapy.

RESULTS AND DISCUSSION

Characterization of TR4 for siRNA Transfer

The synthesis and purification of TR4 were developed from our previous methods.³⁵ MALDI-TOF MS and HPLC were used to verify the molecular weight and purity, respectively, of TR4 (Figure S1). The intrinsic fluorescence of TR4 with different concentrations in aqueous solution was tested by a fluorescence spectrophotometer. The fluorescence of TR4 was very weak at low concentrations ($<200 \mu\text{M}$), indicating the good solubility of TR4 in aqueous solution. With increasing concentration of TR4 ($200 \mu\text{M}$ and above), the fluorescence was turned on, indicating the aggregation of TR4 (Figure S2). Thus, low concentrations ($<200 \mu\text{M}$) of TR4 can be used for the following experiments. It is a well-known fact that siRNA has rigid structure and low charge density that lead to low encapsulation efficiency. Here, the TR4 that we designed not only contains positively charged arginine agents that specifically bind siRNA but also has two hydrophobic agents, TPE and PA, that further enhance the stability of the vectors.^{36,37} To evaluate the ability of TR4 to transfer siRNA, we studied the siRNA condensation capability and gene silencing efficiency. First, the siRNA condensation capability of TR4 was checked by agarose gel electrophoresis, DLS, and TEM. As shown in Figure 1C, the siRNA was completely retarded by TR4 when the N/P ratio was 4:1. At this N/P ratio, the zeta potential of TR4@siRNA NCs was positive, and the size was much smaller than that when the N/P ratio was 2:1. When the N/P ratio was increased above 2:1, the zeta potentials and sizes of the TR4@siRNA NCs only changed slightly (Figure 1D and E). When the N/P ratio was 10:1, the morphology of the TR4@siRNA NCs was mainly spherical (Figure S3). Second, we evaluated the cytotoxicity and silencing efficiency of TR4@siRNA NCs in Luc-HeLa cells, which stably express luciferase and overexpress TfR.^{38,39} At low N/P ratios, the cytotoxicity of TR4@siRNA NCs was negligible, but the cytotoxicity increased at higher N/P ratios (Figure 1F). At an N/P ratio of 10:1, the cell viability was no more than 63%. Gene transfection experiments showed that TR4@siRNA NCs effectively silenced the expression of luciferase at N/P ratios of 4:1 and above (Figure 1G). Although higher silencing efficiency could be achieved at higher N/P ratios, more severe cytotoxicity was also inevitable. Thus, considering both the cytotoxicity and silencing efficiency, an N/P ratio of 10:1 was chosen for the following study. Last, the RNA digestion assay using RNase A was performed to investigate the stability of the TR4@siRNA NCs. As shown in Figure 3B, the naked siRNA was digested immediately upon addition of RNase A, whereas the siRNA in TR4@siRNA complexes (N/P ratio of 10:1) remained intact after exposure to RNase A for 30 min, indicating that the TR4@siRNA NCs can perfectly protect siRNA from RNase A digestion. In summary, the amphiphilic TR4 is similar to the “capsid” of a virus. It can not only effectively package the siRNA at a relatively low N/P ratio and protect siRNA from RNase A digestion but can also achieve high silencing efficiency.

Construction and Characterization of TR4@siRNA@Tf NCs

Enveloped viruses have natural tropism toward certain cells because they can identify and bind to specific receptors.^{40,41} It was previously shown that a Tf shell contributes to longer circulation in blood and targeted uptake by cells that overexpress the transferrin receptor (TfR).⁴² Here, the transferrin (Tf) was introduced to the TR4@siRNA NCs via the

electrostatic interaction to facilitate the vector with reduced cytotoxicity as well as enhanced targeting capability. As shown in Figure 2, the zeta potentials and sizes of the as-prepared TR4@siRNA@Tf NCs decreased as the amount of Tf increased. TR4@siRNA@Tf-30 NCs had a negatively charged surface, which would reduce nonspecific interactions with negative macromolecules in vivo and contribute to selective internalization of the vector by the corresponding cells. The diameter of the TR4@siRNA@Tf-30 NCs was 107.5 nm, and the spherical morphology was confirmed by TEM (Figure 2C).

A colocalization experiment was carried out by confocal laser scanning microscopy (CLSM) to verify the presence of Tf in the vector. The aggregated TR4 exhibited blue fluorescence, Cy5-siRNA showed red fluorescence, and the Tf (labeled with FITC) showed green fluorescence. As shown in Figure 2D, the signals from the three components of the vector were perfectly merged, indicating that the TR4@siRNA@Tf-30 NCs were successfully constructed. Subsequently, the stability of the TR4@siRNA@Tf-30 NCs was tested by size stability and RNA digestion assay. As shown in Figure 3A, the sizes of TR4@siRNA NCs were rapidly increased after 30 min incubation in 10% FBS medium, whereas the sizes of TR4@siRNA@Tf-30 NCs were almost constant all the time. These results indicated that the TR4@siRNA@Tf-30 NCs had good stability in 10% FBS medium, which was mainly attributed to the coating of negatively charged Tf. The RNA digestion assay using RNase A was also carried out to confirm that the TR4@siRNA@Tf-30 NCs could protect RNA from nuclease digestion after the incorporation of Tf. As shown in Figure 3B, the naked siRNA was digested immediately upon addition of RNase A, whereas the siRNA in TR4@siRNA and TR4@siRNA@Tf-30 complexes remained intact after exposure to RNase A for 30 min. These results indicated that the incorporation of the negatively charged Tf did not affect the stability and siRNA condensation capability of nanovectors. As a negative control for subsequent experiments, we also constructed TR4@siRNA@BSA-30 NCs, which cannot target TfR. The three components of TR4@siRNA@BSA-30 NCs were also completely merged (Figure S4). The zeta potential and diameter of the TR4@siRNA@BSA-30 NCs were also tested. As shown in Figure S5, they were also negatively charged (-8.35 mV) and had a diameter of 112.2 nm. Thus, we confirmed the construction of negatively charged virus-like nonviral TR4@siRNA@Tf NCs with good stability and targeting potential.

Cytotoxicity and Silencing Efficiency of the TR4@siRNA@Tf NCs

After these structural investigations, we next evaluated the cytotoxicity and silencing efficiency of the TR4@siRNA@Tf NCs in Luc-HeLa cells. As shown in Figure 4A, TR4@siRNA@Tf NCs were much less cytotoxic than TR4@siRNA NCs due to the presence of the Tf coating. The viability of the cells treated with TR4@siRNA@Tf-30 NCs was approximately 86%, which was comparable to that of cells treated with TR4@siRNA@BSA-30 NCs and Lipo 2000. The gene silencing efficiency of the complexes was evaluated by luciferase assay (Figure 4B). Compared to TR4@siRNA NCs with silencing efficiency of 76%, the gene silencing efficiency of TR4@siRNA@Tf-30 NCs was dramatically enhanced by up to 85%, which is higher than with Lipo 2000 (74%). In contrast, the silencing efficiency of TR4@siRNA@BSA-30 NCs was much lower than that of TR4@siRNA@Tf-30 NCs, which can be attributed to the higher cellular uptake of TR4@siRNA@Tf-30 NCs mediated by the positive targeting ability of Tf. For a better

understanding of the role of Tf, cells in another control group were pretreated with free Tf before transfection to block the TfR, which resulted in much lower silencing efficiency (Figure 4B). Taken together, these results show that the Tf coating on the TR4@siRNA@Tf NCs decreased the initial cytotoxicity of TR4 while simultaneously inducing a dramatic enhancement of their silencing efficiency.

Investigations on the Virus-like Transfection Behaviors of TR4@siRNA@Tf NCs

After demonstrating the attractive transfection properties of TR4@siRNA@Tf NCs, we considered the cellular uptake and intracellular distribution of the complexes to better understand the behavior of TR4@siRNA@Tf NCs during transfection. The outer Tf shell was expected to endow the TR4@siRNA@Tf NCs with higher cellular uptake via interaction with TfR, and this was verified in Luc-HeLa cells that overexpress TfR.⁴³ As shown in Figure 5A and B, a much weaker fluorescence signal was detected in the cells incubated with TR4@siRNA@BSA-30 than with TR4@siRNA@Tf-30 NCs, suggesting higher cellular uptake efficiency of TR4@siRNA@Tf-30 NCs. The cellular uptake of TR4@siRNA@Tf-30 NCs was substantially decreased in the cells pretreated with free Tf to block TfR. These lines of evidence verified the Tf-mediated endocytosis pathway of TR4@siRNA@Tf-30 NCs. Correspondingly, as shown in Figure 4B, the higher gene silencing efficiency of the TR4@siRNA@Tf-30 NCs over TR4@siRNA@BSA-30 NCs can be attributed to the higher endocytosis efficiency of TR4@siRNA@Tf-30 NCs. Despite the Tf-TfR mediated endocytosis of TR4@siRNA@Tf-30 NCs, the cellular accumulation of these NCs was significantly lower than the accumulation of positively charged TR4@siRNA NCs (Figure 5B). However, the gene silencing efficiency of TR4@siRNA@Tf-30 NCs was higher than that of TR4@siRNA NCs (Figure 4B). For deeper insight into the gene silencing efficiency of TR4@siRNA@Tf-30 NCs being higher than that of TR4@siRNA NCs, spatial-temporal monitoring of the intracellular gene transfer process was carried out by CLSM. TR4, which contains the AIEgen TPE, was clearly distinguished by its blue fluorescent emission. The siRNA and protein (Tf or BSA) shell were labeled with Cy5 and FITC, respectively, and could clearly be localized within the cell by their red (Cy5) and green (FITC) fluorescence under appropriate excitations. As shown in Figure S6, at the initial stage (4 h) of gene transfection, obvious fluorescent signals were detected only in the cell membranes of the vector-treated cells. The fluorescent signals from the different channels overlapped well, indicating no dissociation of components from the vector complexes. It was notable that the fluorescent signals from the cells incubated with TR4@siRNA@BSA-30 NCs were much weaker than the signals from the cells treated with the TR4@siRNA@Tf-30 NCs, which suggested higher cell membrane adhesion of TR4@siRNA@Tf-30 NCs via the Tf-TfR interaction. This in turn contributes to the higher gene silencing efficiency of TR4@siRNA@Tf-30 NCs.

When the incubation time was extended to 12 h, most of the vectors were endocytosed into cells and were located in the cytoplasm, as shown in Figure 5C. For the cells treated with TR4@siRNA or TR4@siRNA@BSA-30 NCs, the red signals (Cy5/siRNA) overlapped well with the blue fluorescence (TPE/TR4), indicating the presence of compact complexes without siRNA release. We believe that this is because of the tight interaction between TR4 and siRNA, which might lead to overcondensed complexes and inadequate siRNA release. It

was noticeable that the cells incubated with TR4@siRNA@BSA-30 NCs showed the weakest fluorescence, which was attributed to the inefficient cellular uptake of TR4@siRNA@BSA-30 NCs. In contrast, when the cells were treated with TR4@siRNA@Tf-30 NCs, the red signals from siRNA were distinct from the blue (TR4) and green (Tf) fluorescence on the dark field (DF) merged image. The mismatch between the red signals and the others suggested that the siRNA was successfully released from the TR4@siRNA@Tf-30 NCs into the cytoplasm. Blue and green signals were observed on the cell membrane. These results show that the TR4@Tf complex adhered to the cell membrane, and the siRNA was released into the cytoplasm. Meantimes, the colocalization of TR4@siRNA@Tf-30 NCs and lysosome compartments at 12 h was observed using CLSM. As shown in Figure S7, the majority of the red colored points did not merge with lysosomes, indicating that the released siRNA from the TR4@siRNA@Tf-30 NCs was mainly located in cytosol. Thus, the TR4@siRNA@Tf-30 NCs have virus-like transfection behavior. We hypothesized that this virus-like behavior of TR4@siRNA@Tf-30 NCs might be due to the specific binding and strong interaction between Tf on the surface of the vector and TfR in the cell membrane. A series of experiments were carried out to investigate the mechanism in more detail. As shown in Figure 5C, when the cells were preincubated with free Tf and then treated with TR4@siRNA@Tf-30 NCs, few separate red signals were observed in the cytoplasm on the DF merged image, which suggests that no siRNA was released from the complexes. The interactions of TR4 with siRNA and Tf were further characterized by fluorescence spectroscopy. Because the AIE fluorescence intensity of TR4 is closely correlated with the compactness of the complexes, we compared the fluorescence of free TR4, TR4@siRNA, and TR4@Tf under the same conditions.⁴⁴ As shown in Figure 6A, TR4@Tf showed that a much more dramatic increase in fluorescence was achieved than for TR4@siRNA. This implied that the interactions between TR4 and Tf were much stronger than those between TR4 and siRNA. Thus, we believe that Tf draws TR4 onto the membranes of cells that overexpress TfR so that the siRNA is released from TR4 into the cytosol. Taken together, our results suggest that the presence of Tf endows the TR4@siRNA@Tf NCs with virus-like transfection behaviors, including adherence of the viral capsid-like TR4@Tf NCs to the cell membrane and release of siRNA into the cytosol.

To investigate the advantageous properties of this virus-like gene vector, we further compared the gene silencing abilities of the TR4@siRNA@Tf-30 NCs and the commercial Lipo 2000 at various transfection time points. As shown in Figure 6B, the silencing efficiency of TR4@siRNA@Tf-30 NCs was 57% at 12 h, whereas for Lipo 2000 it was only 21%. This indicated that the accelerated release of siRNA from TR4@siRNA@Tf-30 NCs contributed to accelerated siRNA silencing. Therefore, the Tf-dressed virus-like vector not only exhibited TfR-specific endocytosis but also facilitated the release of siRNA into the cytosol and accelerated gene silencing in a virus-like manner.

CONCLUSIONS

In brief, we successfully developed novel virus-like siRNA vectors via a sequential self-assembly strategy. The as-prepared TR4@siRNA@Tf NCs were able to specifically deliver siRNA in a way that could be visualized and showed high gene silencing efficiency with low cytotoxicity. In the presence of a Tf shell, TR4@siRNA@Tf NCs efficiently deliver siRNA

and realize gene silencing in a virus-like manner, where the siRNA was released into the cytosol and the TR4@Tf NCs adhered to the cell membrane like virus capsids. Our results revealed that TR4@siRNA@Tf NCs accelerated the release of siRNA into the cytosol and thus achieved a higher gene silencing efficiency than Lipo 2000 in a short time. With advantageous properties of low cytotoxicity and efficient gene silencing, these virus-like nonviral TR4@siRNA@Tf NCs hold great potential in the area of gene therapy. This concept of vector construction has the potential to open up new ways of preparing virus-like vectors by sequential self-assembly.

EXPERIMENTAL SECTION

Materials and Methods

TR4 was prepared as reported previously.¹⁷ Transferrin and BSA were purchased from Sigma-Aldrich. The two types of siRNA used in this work, anti-Luc siRNA and Cy5-labeled siRNA, were supplied by Guangzhou IGE Biotechnology Ltd. The sequences of the anti-Luc siRNAs were: sense, 5'-CCCUAUUCUCCUUCUUCGCdTdT-3'; antisense, 5'-GCGAAGAAGGAGAAUAGGGdTdT-3'; and the sequences of the Cy5-labeled siRNAs were sense strand, 5'-UUCUCCGAACGUGUCACGUDtTdT-3'; antisense strand, 5'-ACGUGACACGUUCGGAGAAAdTdT-3'. The 5' end of the sense strand was labeled with the fluorophore Cy5. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were bought from Wisent, Inc. (Multicell, Wisent, Inc., St. Bruno, Quebec, Canada). Antibiotic solution (penicillin and streptomycin) and trypsin-EDTA (0.25%) were purchased from Invitrogen (Invitrogen, Carlsbad, CA).

Preparation of TR4@siRNA@Tf NCs

The TR4@siRNA@Tf NCs were prepared by a sequential self-assembly strategy. Equal volumes of TR4 and siRNA diluted in 5% glucose were mixed together. After incubation for 30 min, the binary complexes were coated by Tf with the same method. For TR4@siRNA@Tf-30 NCs, they consist of 1 μ g of siRNA, 10.5 μ g of TR4, and 30 μ g of Tf. The TR4@siRNA@BSA-30 NCs were also obtained by the same method.

Characterization of TR4@siRNA@Tf NCs

The siRNA condensation capability was evaluated by the agarose gel electrophoresis assay. Ten microliters of TR4@siRNA NCs containing 1 μ g of siRNA was mixed with 2 μ L of 6 $\times$ loading buffer, and then, electrophoresis was carried out at a voltage of 120 V for 15 min. The photos were acquired using an image master VDS thermal imaging system (Bio-Rad, CA). The sizes and zeta potentials of binary and ternary complexes were measured via dynamic light scattering (DLS). Samples containing 1 μ g of siRNA were diluted in 1 mL of PBS buffer. The morphology of the complexes was characterized with a transmission electron microscope (TEM, HT7700, Hitachi) using the negative-staining technique with uranyl acetate solution.

Cell Culture

The Luc-HeLa cells were kindly provided by Prof. Zicai Liang at the Institute of Molecular Medicine, Peking University. Luc-HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS and 1% antibiotic solution at 37 °C in 5% CO₂.

Cell Viability

The effects of TR4@siRNA and TR4@siRNA@Tf NCs on cell viability were assessed by MTT assay. First, the Luc-HeLa cells were seeded into 96-well plates at 4×10^3 cells/well for 24 h, and then the binary and ternary complexes were added at the siRNA concentration of 0.2 μg /well for an additional 24 h. The viability assessment was carried out with an Infinite M200 (TECAN, Mannedorf, Switzerland) after incubation of the cells with MTT and DMSO.

Gene Silencing

The gene silencing efficiencies of the binary and ternary complexes were evaluated by the luciferase assay. Luc-HeLa cells were seeded into 24-well plates at 4×10^4 cells/well for 24 h, and then the cells were incubated with binary and ternary complexes (containing 0.8 μg of siRNA for each well) diluted in Opti-MEM for 4 h. The transfection solutions were replaced by 0.5 mL complete DMEM for an additional 44 h. The luminescence intensity of the cells was measured using a fluorometer (Synergy HT, BioTek, USA) after 10 μL of cell lysate was reacted with 50 μL of luciferase detection agent (Promega Co., Madison, WI). Total proteins were quantified using a BCA protein assay kit (Pierce, USA).

Internalization of the Nanocomplexes

In this experiment, the transfection procedure was the same as for gene silencing (above). The cells were seeded into 12-well plates at 8×10^4 cells/well. Cy5-labeled siRNA was used to transfect the cells (Cy5-siRNA: 1 μg /well). After 12 h transfection, the cells were washed three times with cold PBS and subsequently analyzed by flow cytometry (Life Technologies, Carlsbad, CA).

Colocalization of TR4@siRNA@Tf NCs

The colocalization of TR4, Cy5-labeled siRNA, and FITC-labeled Tf was observed using confocal laser scanning microscopy (CLSM). Luc-HeLa cells were seeded in 35 mm dishes at 8×10^4 cells/dish and grown for 36 h before the experiment. The cells were incubated with ternary complexes containing 1.2 μg of Cy5-siRNA, 12.6 μg of TR4, and 36 μg of Tf (containing 12 μg of FITC-Tf) for 4 h. The cells were then washed three times with PBS and directly observed by CLSM to collect the images for the 4 h time point. For the 12 h time point, the cells were incubated for an additional 8 h in complete DMEM and then imaged by CLSM.

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Figure 1.

(A) Schematic illustration of the gene delivery process of TR4@siRNA@Tf NCs. (B) Chemical structure of TR4. (C) Agarose gel electrophoresis retardation assay of TR4@siRNA NCs at different N/P ratios (N/P = 0 means the naked siRNA). (D) Sizes and (E) zeta potentials of the TR4@siRNA NCs at different N/P ratios. (F) Cytotoxicity and (G) silencing efficiency of the TR4@siRNA NCs at different N/P ratios in Luc-HeLa cells. The naked siRNA served as a negative control.

Figure 2.

(A) Sizes and (B) zeta potentials of the TR4@siRNA@Tf NCs with different amounts of Tf.

(C) Morphology of the TR4@siRNA@Tf-30 NCs characterized by TEM. Scale bar = 50 nm.

(D) Colocalization of the TR4@siRNA@Tf-30 NCs imaged by CLSM. Blue: TR4; red: Cy5-siRNA; green: FITC-Tf; white: merged image of TR4, Cy5-siRNA, and FITC-Tf fluorescent signals. Scale bar = 10 μ m.

Figure 3.

Stability of the TR4@siRNA and TR4@siRNA@Tf-30 NCs. (A) Size stability of TR4@siRNA and TR4@siRNA@Tf-30 NCs in 10% FBS. The concentration of siRNA was 1 $\mu\text{g}/\text{mL}$. (B) RNA digestion assay using RNase A. The amount of siRNA in each well was 0.7 μg . After the incubation of RNase A, all of the samples were exposed to 1.5% SDS for 1 h at 4 $^{\circ}\text{C}$ to denature the RNase A and release the siRNA. The RNase A concentration of each sample was 66.7 $\text{ng}/\mu\text{L}$.

Figure 4.

(A) Cytotoxicity and (B) gene silencing efficiency of the TR4@siRNA@Tf NCs in Luc-HeLa cells. For the TR4@siRNA@Tf-30 + Tf media group, cells were pretreated with free Tf (100 $\mu\text{g}/\text{mL}$) for 30 min before transfection. TR4@siRNA@BSA-30 NCs and Lipo 2000 served as controls.

Figure 5.

(A, B) Cellular uptake and (C) intracellular distributions of different TR4@siRNA preparations in Luc-HeLa cells. For the TR4@siRNA@Tf-30 + Tf media group, the cells were pretreated with free 100 $\mu\text{g}/\text{mL}$ Tf for 30 min to block the TfR sites. (A) Flow cytometry results showing cellular uptake of different TR4@siRNA preparations after 12 h incubation. (B) Quantification of the flow cytometry results. *** $P < 0.001$. (C) CLSM images showing the intracellular distributions of the different vectors at 12 h. The red arrows indicate the free siRNA in the cytoplasm; white arrows indicate the merged FITC-Tf/FITC-

BSA (green) and TR4 (blue) signals. Blue, TR4; red, Cy5-siRNA; green, FITC-Tf/FITC-BSA. Scale bar = 20 μm .

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Figure 6.

(A) Fluorescence spectra of free TR4, TR4@siRNA, and TR4@Tf in aqueous solution. The TR4 concentration was 25 μ M for each sample. The inset shows aqueous solutions of (1) TR4, (2) TR4@siRNA, and (3) TR4@Tf at an excitation wavelength of 330 nm. (B) Gene silencing efficiency of the TR4@siRNA@Tf-30 NCs in Luc-HeLa cells after 8, 12, 24, and 48 h of transfection with Lipo 2000 as the control.