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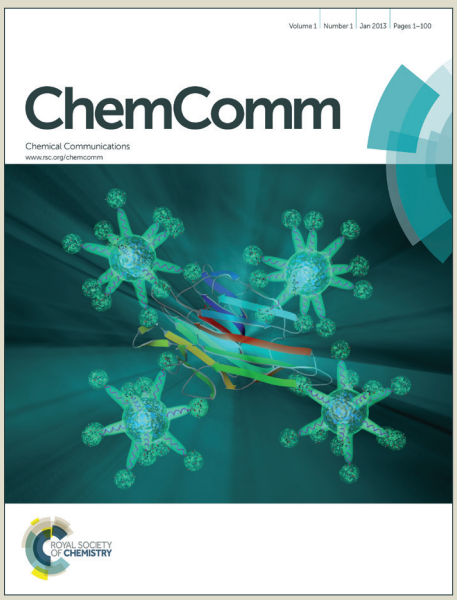

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Biocompatible, Chimeric Peptide-Condensed Supramolecular Nanoparticles for Tumor Cell-Specific siRNA Delivery and Gene Silencing

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Chimeric peptides composed of arginine-rich RNA-binding and tumor cell-targeting motifs have been employed to condense siRNA to form supramolecualr nanoparticles *via* electrostatic associations and deliver siRNA agents in a tumor cell-specific manner.

The targeted delivery of siRNA-based agents to appropriate cells holds great promise not only as a research tool to suppress the expression of a target gene but also as a strategy for the treatment of various disorders, including viral infections and cancers.[1] However, these applications have been hampered by the physicochemical characteristics of siRNA, such as its low stability against serum nuclease degradation, polyanionic charge, and low permeability across biological membranes. To realize the broad potential of siRNA-based therapeutics, it is urgently necessary to develop safe and effective delivery systems.[2] At present, a variety of siRNA delivery vehicles are under exploration to address this challenge, including cationic polymers,[3] lipid nanoparticles,[4] antibodies,[5] inorganic materials[6] and viral platforms.[7] While many creative materials have shown promise, there are significant concerns regarding their safety and biocompatibility when used for human therapy.[8] In addition, the procedures required to prepare these vehicles are laborious and time-consuming.

The self-assembly of biocompatible materials with siRNA therapeutics into nanoscale packages is an efficient way to address these problems. In particular, nanoparticles can preferentially accumulate within solid tumors as a result of enhanced permeability and retention (EPR) effect. Arginine-rich cationic peptides are known to complex with polyanionic siRNA to form stable nanoparticles via electrostatic interactions.[9] Although the methods using these cationic peptides for siRNA delivery are effective, they lack cell- or tissue-specificity. We envisioned that strategies using targeting motifs to deliver siRNA into the appropriate diseased cells *via* cell-surface receptors without harming normal cells would tremendously enhance the utility of this technology. To confer targeting capabilities, Kumar et al. demonstrated that a neuronal cell-targeting short peptide derived from a rabies virus glycoprotein (RVG) fused to nine arginine residues can deliver siRNA to the brain.[10] Therefore, in spite of several successful examples, the development of vehicles capable of malignant tumor cell-specific siRNA delivery by arginine-rich cationic peptides remains a great challenge.[11]

Hepatocellular carcinoma (HCC), one of the most common types of malignant tumors worldwide, is not susceptible to current conventional chemotherapies.[12] Therefore, the development of alternative siRNA-based therapeutics by active targeting strategies is desirable. Herein, we take advantage of a simple two-component, polypeptide-condensed siRNA supramolecular nanoparticle to achieve HCC-specific delivery of siRNA payloads (Figure 1). Furthermore, we demonstrated the feasibility of this approach for suppressing endogenous target gene expression in a cell-specific manner. The simplicity and versatility of this approach and the biocompatibility of the peptidic vehicle make this a potential in vivo siRNA delivery system for cancer-targetable therapeutic applications.

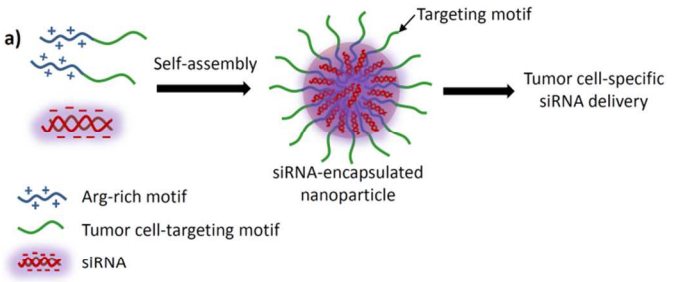

Figure 1. a) Self-assembly of polypeptides with siRNA payloads to form nanocomplexes. b) The HCC-specific peptide SP94 (green) was fused to the N-terminus of nine D- or L-arginine residues *via* a four-glycine linker.

As a proof of concept, we chose the SP94 peptide as a targeting motif, which was originally identified by phage display and conjugated to various nanostructures to enhance their affinities toward human HCC cells.[13] Thus, three chimeric peptides (**1**-**3**) composed of two segments comprising SP94 ligands and cationic nona-arginine residues were prepared by standard solid-phase peptide synthesis. The siRNA-binding capabilities of the designed

peptides were first tested by a gel retardation assay. As shown in Figure 2a and Figure S1, SP94-dR **1** and **3** were able to bind siRNA in a dose-dependent manner. At the molar ratio of 20:1 (amine-to-siRNA phosphate, N/P=4.3/1), almost complete retardation of the siRNA band was observed, consistent with the previous brain-targeting RVG-dR peptide.[10] The disappeared siRNA band could be recovered in the presence of heparin, a polyanionic complex-destabilizer (Figure S2). As a comparison, peptide **2**, in which the consecutive D-arginine was replaced by L-arginine, was not able to complex the siRNA until the molar ratio reached 40:1 (Figure S3). These data indicate that the ability of D-arginine residues to complex siRNA is greater than that of L-arginine.

The hydrodynamic sizes of the chimeric peptide/siRNA nano-complexes were investigated by dynamic light scattering (DLS). At a 20:1 molar ratio, the SP94-dR 1/siRNA complex was determined to be approximately 190 nm (Figure 2d, Table S1), and the surface was positively charged (ξpotential=25.9±2.6). Unfortunately, we could not obtain the hydrodynamic size of SP94-R **2**/siRNA by DLS analysis, even though they formed complexes from the gel retardation assay (Figure S3). On the other hand, direct visualization of the SP94-dR **1**/siRNA by transmission electron microscopy (TEM) revealed spherical nanoparticles with diameters of 30-100 nm (Figure 1e).

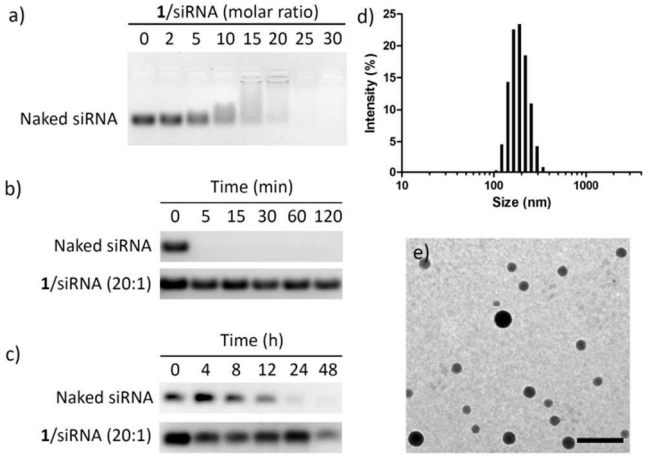

Figure 2. Characterization of SP94-dR **1**/siRNA supramolecular nanoparticles. a) Agarose gel (2%) retardation assay at different molar ratios of SP94-dR to siRNA. b) Stability of naked siRNA and siRNA complexed with **1** at a 20:1 molar ratio against enzymatic degradation after incubation with RNase A at indicated time points. c) Serum stability after incubation in 50% mouse-serum solution. d) DLS suggested that the nanoparticles exhibited an average hydrodynamic diameter of ca. 190 nm. e) TEM image of the dried nanoparticles counterstained with 4% uranyl acetate. Scale bar = 200 nm.

The protection of siRNA agents against nucleases and other enzymes when circulating in the bloodstream is critical for siRNA-based therapies. We thus assessed whether complexation with SP94-dR **1** could enhance the stability of siRNA when incubated with RNA nucleases and mouse serum. In these experiments, after incubation at indicated time points, heparin was added to release the intact siRNA, and the samples were subjected to agarose gel electrophoresis. In the RNase degradation test, encapsulation within the nanoparticles significantly improved the siRNA stability. There was no visible decrease in the siRNA band at 2 h or even over the course of the 54 h experimental period (Figure 2b and Figure S4). In sharp contrast, naked siRNA was degraded within 5 min under identical conditions. For serum stability, siRNA complexed with SP94-dR **1** was found to remain relatively intact after a 48 h incubation, whereas naked siRNA was significantly degraded after 12 h (Figure 2c). Hence, the siRNA molecules within the nanoparticles were effectively protected against degradation by nucleases. This long-term stability could be beneficial for in vivo siRNA delivery and could enhance its efficacy.

In addition to stability, targetability is another critical element of a suitable siRNA delivery vehicle for *in vivo* utilization. In our design, the HCC-specific SP94 peptide was expected to be displayed on the surfaces of the nanoparticles multivalently, which could enable the recruitment of nanoparticles to HCC cells. We therefore verified the *in vitro* specificity by flow cytometry using SP94-dR **1** nanoparticles loaded with Cy3-labeled siRNA. After a 6 h transfection in serum-free Opti-MEM media, the cells were further cultured for 12 h with freshly replaced DMEM growth media. Flow cytometry analysis showed that the Huh7 human HCC cells have a strongly positive fluorescence signal (42.5%), whereas the H1299 lung cancer cells showed negligible levels of reactivity (Figure 3a).

To examine the mechanism of internalization, live-cell confocal fluorescence microscopy was used to observe the cellular uptake. The siRNA cargo was found to be colocalized with the endosomal compartment, which was evidenced by the significant overlap between the fluorescence signals derived from the Cy3-siRNA and FITC-labeled Dextran (Figure 3b). In contrast, the SP94-dR **1**/siRNA nanoparticles did not target the H1299 cells (Figure S6), consistent with the results of flow cytometry. Furthermore, although the use of scrambled SP94 **3** also assembled siRNA to form nanocomplexes, almost no detectable siRNA internalization was observed in the Huh7 cells (Figure S7). These results demonstrated that the siRNA cargoes were internalized *via* endocytosis mediated by the affinity between the SP94 peptide ligand and unknown cell-surface receptor(s) expressed by the HCC cells.[13]

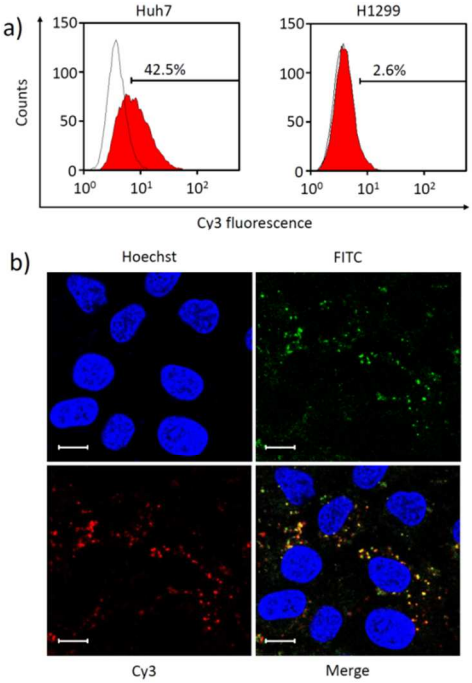

Figure 3. The SP94 motif-functionalized nanoparticle specifically delivers siRNA to hepatocellular carcinoma. a) Flow cytometry evaluation of Cy3-labeled siRNA complexed with SP94-dR **1** on Huh7 (left) and H1299 (right) cells. b) Representative confocal fluorescence microscopy images of Huh7 cells that were treated with Cy3-labeled siRNA (50 nM) complexed with the

SP94-dR **1** peptide. The nuclei and the endosomes/lysosomes were stained with Hoechst 33342 (blue) and FITC-labeled Dextran (green, endo/lysosome tracker), respectively. All scale bars = 10 μm.

Next, we assessed whether the SP94-displaying nanoparticles loaded with siRNA would be able to achieve HCC-specific gene silencing in vitro. The Foxo3a gene, which is overexpressed in tumor cells as a potential target for cancer therapy,[14] was targeted. Two cell lines, Huh7 and H1299, were initially assessed by treating with Foxo3a siRNA (siFOX)-encapsulated SP94-dR **1** nanoparticles at 50 nM for 6 h in serum-free media. After 24 h, the levels of Foxo3a mRNA expression were quantitatively determined by reverse transcription polymerase chain reaction (qRT-PCR) analysis (Figure 4a). Obviously, the SP94-dR **1**/siFOX nanoparticles significantly reduced the Foxo3a mRNA expression in the Huh7 cells (~85% reduction), but not in the H1299 cells (Figure 4a). As a comparison, Lipofectamine 2000 (Invitrogen) induced the gene-silencing of Foxo3a in both of the tested cell lines due to its non-targeting feature. Although the increased cellular uptake of the Cy3-labeled siRNA using lipofectamine was observed by flow cytometry analysis (Figure S5), the SP94-dR **1**/siFOX nanoparticles exhibited improved gene-silencing effects, indicating that greater endosomal escape and siRNA release in the cytoplasm was achieved upon internalization. The HCC-specific gene-silencing was also confirmed by western blot analysis as shown in figure 4b, and the efficacy of RNAi with our targetable delivery system was superior to that of the conventional transfection reagent Lipofectamine in the Huh7 cells. In contrast, negative control siRNA failed to induce a Foxo3a RNAi response (Figure S8 and S9). To further demonstrate that our strategy can cause highly specific RNAi toward HCC, other four cell lines were evaluated for SP94-dR **1**-mediated gene-silencing. As shown in Figure S9, Foxo3a expression was effectively abolished by SP94-dR **1**/siFOX nanoparticles in both of HCC cells, but not in breast cancer cells.

Dose-dependent knockdown experiments showed that the silencing was highly effective and plateaued at below 12.5 nM siRNA in the Huh7 cells (Figure 4c). In contrast, only a slight reduction in Foxo3a expression was observed in lung cancer cells at concentrations of up to 200 nM siRNA (Figure 4d). Notably, the SP94-dR **1**/siFOX nanoparticles showed comparable Foxo3a gene-silencing even in the presence of 80% serum in the transfection media (Figure S10). This behavior is quite different from that of Lipofectamine, whose gene silencing was significantly impeded by serum co-incubation.[15]

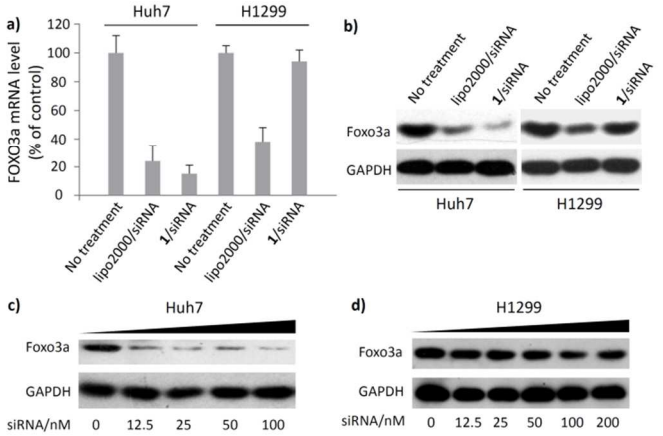

Figure 4. Cell-specific gene silencing by SP94-dR **1**/siRNA nanocomplexes *in vitro*. a) Quantification of gene expression in the Huh7 and H1299 cell lines. The data represent the means±SD and are representative of three independent experiments. b) Western blotting also showed that endogenous foxo3a gene expression was specifically reduced in the Huh7 cells, but not in the H1299 cells. c-d) siRNA concentration-dependent gene knockdown in Huh7 and H1299 cells, respectively.

Finally, we evaluated whether SP94-dR **1** could specifically deliver nucleic acid cargoes into hepatocellular carcinoma in vivo. We established an orthotopic human MHCC-97H (an invasive HCC cell line) xenograft model in BALB/c nude mice to test the nanoparticle targetability after systemic administration. The siRNA mimic oligo-deoxynucleic acid (ODN) was labeled with the near-infrared fluorescent (NIRF) probe Cy5.5 and complexed to either SP94-dR **1** or a randomized peptide **3**. After intravenous injection of the nanoparticles at 4 h and 24 h, the mice were sacrificed and the major organs were visualized. The NIRF images showed that the Cy5.5 signal was more highly distributed in the liver tumor after administration of the SP94-dR **1** compared to the non-targetable peptide **3**-derived nanoparticles (Figure 5 for 4 h and S11 for 24 h). These results clearly indicated that SP94-dR **1** enables the intravenous delivery of nucleic acid cargo to orthotopic HCC in vivo.

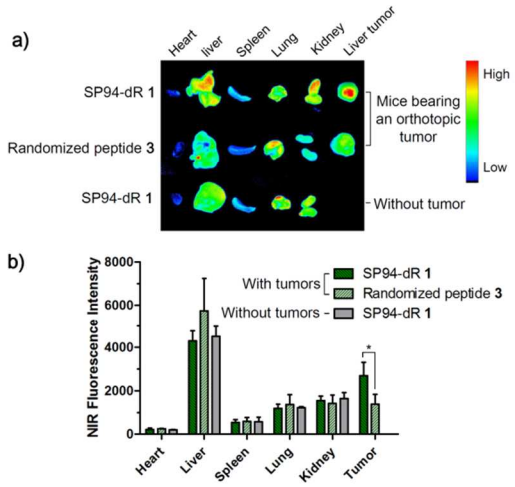

Figure 5. In vivo distribution of peptide-encapsulated Cy5.5-ODN nanoparticles in orthotopic MHCC-97H hepatocellular carcinoma mice model. (a) Representative images of SP94-dR **1**-complexed and randomized peptide **3**-complexed Cy5.5-ODN nanoparticles at 4 h after intravenous injection. Mice without tumors were also injected with SP94-dR **1**/ Cy5.5-ODN nanoparticles for comparison. Ex vivo NIRF images of the major organs. (b) NIR fluorescence intensity in major organs at 4 h after injection of **1** and **3**/ Cy5.5-ODN nanoparticles (n=3). * indicates $p < 0.05$.

Conclusions

We developed a targetable siRNA delivery system based on the self-assembly of a "two-in-one" chimeric peptide with siRNA therapeutics. Upon mixing, the dense cationic charges of the chimeric peptide condense the siRNA into nanoparticles, facilitating the multivalent display of cancer cell-specific targeting ligands. Both *in vitro* and *in vivo* studies demonstrated that this strategy features high specificity toward the targeted HCC cells. Although the scope of the current example was limited to HCC-specific targeting, many practically relevant siRNA delivering nanoparticles targeting other tumors could be designed because a variety of peptide ligands are available[16] and can be easily synthesized. Therefore, this targetable platform provides potential utility for human therapy. Further studies of the specific delivery of siRNA *in vivo* as well as in cancer therapy are now under investigation in our laboratories.

Notes and references

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