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Multifunctional hierarchically-assembled nanostructures as complex stage-wise dual-delivery systems for coincidental yet differential trafficking of siRNA and paclitaxel

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Abstract

Development of multifunctional nanostructures that can be tuned to co-deliver multiple drugs and diagnostic agents to diseased tissues is of great importance. Hierarchically-assembled theranostic (HAT) nanostructures based on anionic cylindrical shell crosslinked nanoparticles and cationic shell crosslinked knedel-like nanoparticles (cSCKs) have recently been developed by our group to deliver siRNA intracellularly, and to undergo radiolabeling. In the current study, paclitaxel, a hydrophobic anticancer drug, and siRNA have been successfully loaded into the cylindrical and spherical components of the hierarchical assemblies, respectively. Cytotoxicity, immunotoxicity and intracellular delivery mechanism of the HAT nanostructures and their individual components have been investigated. Decoration of nanoparticles with F3-tumor homing peptide was shown to enhance the selective cellular uptake of the spherical particles, whereas the HAT nanoassemblies underwent an interesting disassembly process in contact with either OVCAR-3 or RAW 264.7 cell lines. The HAT nanostructures were found to “stick” to the cell membrane and “trigger” the release of spherical cSCKs templated onto their surfaces intracellularly, while retaining the cylindrical part on the cell surface. Combination of paclitaxel and cell-death siRNA (siRNA that induces cell death) into the HAT nanostructures resulted in greater reduction in cell viability than siRNA complexed with Lipofectamine and the assemblies loaded with the individual drugs. In addition, a shape-dependent immunotoxicity was observed for both spherical and cylindrical nanoparticles, with the latter being highly immunotoxic. Supramolecular assembly of the two nanoparticles into the HAT nanostructures significantly reduced the immunotoxicity of both cSCKs and cylinders. HAT nanostructures decorated with targeting moieties, loaded with nucleic acids, hydrophobic drugs, radiolabels, fluorophores, with control over their toxicity, immunotoxicity and intracellular delivery might have great potential for biomedical delivery applications.

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Details of experimental procedures and characterizations are included in the supporting information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Keywords

Multifunctional shell crosslinked nanoparticles; siRNA; paclitaxel; combinational therapy; theranostics; intracellular delivery mechanisms; immunotoxicity; nanoparticle shape; cylindrical nanoparticles

The development of multifunctional nanoparticles for the co-delivery of multiple drugs and diagnostics is of great importance.^{1–9} The term “multifunctional” means to incorporate more than one cargo or smart feature (*e.g.* passive and active targeting) into the same nanocarrier to maximize the therapeutic benefits, minimize toxicity and enhance the efficiency of diagnosis and treatment of diseases.¹⁰ For example, theranostic nanoparticles, or “nanotheranostics”, involve the use of nanoparticles loaded with therapeutic drugs and imaging probes for the combined therapy and diagnosis.^{3,5,11,12} Although the synthetic procedures to prepare polymeric units that are capable of binding more than one drug and carry other functionalities for targeting and/or imaging become more complicated, investigation towards the development of multifunctional nanoparticles has been gaining wide interest. Park and coworkers have developed magnetic nanoparticles that were conjugated *via* disulfide linkage to siRNA (labeled with Cy5), and poly(ethylene glycol) (PEG) functionalized with a cyclic Arg-Gly-Asp (RGD) peptide at the distal end.⁵ The RGD peptide on the distal end of the PEG binds specifically to $\alpha_v\beta_3$ integrin, which is overexpressed in metastatic tumor cells and tumor endothelial cells, the PEG provides stabilization for the nanoparticles and can potentially prolong the *in vivo* circulation time, and Cy5 can be utilized for fluorescent imaging. In addition, Lavasanifar and coworkers have developed multifunctional poly(ethylene oxide)-*block*-poly(ϵ -caprolactone) polymeric micelles that could be chemically modified to electrostatically complex siRNA and chemically conjugate doxorubicin.³ The shell of the nanoparticles was decorated with RGD peptide and TAT peptide for cancer-specific targeting and enhancing the intracellular delivery of nanoparticles, respectively. The nanoparticles were also labeled with near-infrared fluorescent probes, and siRNA was labeled with a Dy677 fluorescent probe. This platform was designed to overcome the multidrug resistance of tumor cells by delivering siRNA (that inhibits P-glycoprotein) and doxorubicin specifically to tumor cells that overexpress $\alpha_v\beta_3$ integrin. Although promising data were obtained with these multifunctional platforms, the procedures involved in the construction of these nanoparticles are complicated as modification of numerous parameters is required in a single construct to tune the composition and to optimize their performance. In addition, the steps involved in loading various drugs (*e.g.* loading hydrophobic drugs usually utilize organic solvents) might affect the stability of other drugs (*i.e.* proteins and nucleic acids). Alternatively, preparation of individual nanoparticles loaded with various therapeutics and/or diagnostics, followed by hierarchical supramolecular assembly into higher order nanostructures may provide fine-tuned control over the adjustment of the composition of the assemblies and ease in incorporation of multifunctionality *via* simple iterations. Our group has developed a new strategy, based on controlled polymerization chemistry and supramolecular assembly, to synthesize and construct nanoparticles of various sizes, morphologies, dimensions, surface chemistries, including multicompartamental nanoparticles and nanocages.^{13–18} In particular, shell crosslinked nanoparticles have been utilized regularly, by our group and others, due to their higher kinetic stability, lower toxicity, and ability to withstand the harsh biological conditions experienced *in vivo*.^{1,19–22} In this study, shell crosslinked spherical cationic nanoparticles were templated onto shell crosslinked anionic cylinders to form hierarchically-assembled nanostructures, with the two components being prepared, optimized and loaded with various therapeutic, diagnostic and/or targeting moieties before the supramolecular assembly process. The purposes of this design were to have better control on the optimization of every compartment with the maximum stability and drug-loading before

supramolecular assembly, and to take the advantage of utilizing the expanded morphologies as they were associated with longer *in vivo* blood circulation time.²³ Another added advantage is that an individual compartment (spheres or cylinders) might be utilized, for instance, to administer siRNA/cSCKs complexes to reduce tumor resistance to a chemotherapeutic agent, followed by the HAT nanostructures loaded with siRNA and chemotherapeutic agent. The particular HATs studied here were observed to exhibit unique and enhanced characteristics beyond those of the individual components. Therefore, this method is expected to expand the potential of nanomaterials in biomedical delivery applications and to facilitate the clinical translation of multifunctional nanocarriers.

Results and discussion

Preparation and characterization of hierarchically-assembled nanostructures

The targeted and dual siRNA- and paclitaxel-loaded complex nanomaterials were designed based upon an earlier demonstration of the hierarchical assembly of two different nanoparticle modules, one loaded with siRNA and the other with sites to carry imaging agents, which was previously achieved by templating cationic shell crosslinked knedel-like nanoparticles (cSCKs) that can electrostatically bind negatively-charged siRNA, onto anionic shell crosslinked rods (SCRs), the core of which provides sites for radiolabeling.²⁴ The cSCKs were prepared in a two-step process, first by direct dissolution of poly(acrylamidoethylamine)₁₆₀-*block*-polystyrene₃₀ (PAAE₁₆₀-*b*-PS₃₀) to obtain a micellar solution, followed by selectively crosslinking 5% of the amines within the hydrophilic shell domain by employing a diacid crosslinker.²⁵ Similarly, anionic cylinders were prepared by aqueous self-assembly and shell crosslinking of approximately 10% of the acrylic acid residues by amidation of the amphiphilic block copolymer poly(acrylic acid)₁₄₀-*b*-poly(*para*-hydroxystyrene)₅₀ (PAA₁₄₀-*b*-PpHS₅₀), that consisted of PAA as the hydrophilic shell segments and PpHS as the hydrophobic core domain. The hierarchical assemblies were prepared by mixing cationic spheres with anionic cylinders in aqueous medium at amine-to-carboxylate (N/C) ratio of 10:1. Characterizations of the size and zeta-potential of the HATs and the individual components have previously been performed by dynamic light scattering (DLS) and transmission electron microscopy (TEM) and were determined to be *ca.* 20 nm and 15 nm (*n* = 100), respectively and a zeta potential value of *ca.* 35 mV, for the cSCKs. The SCR had diameters of *ca.* 10 nm and lengths of *ca.* 1.5 μ m (*n* = 100) and a zeta potential of -30 mV. The surfaces of the nanorods were confirmed to be coated with the spheres, as demonstrated by TEM imaging and by the reduced value of the net zeta potential value of *ca.* 25 mV.²⁴ These HAT nanostructures were able to complex siRNA onto the PAAE shell of the spheres and carry ⁷⁶Br radiolabels in the PpHS core of the cylinders. In this study, the ability of these novel nanostructures to carry more than one cargo (paclitaxel in their cores and siRNA in the shell) and present F3 tumor homing peptide at their surfaces was studied (Scheme 1). In addition, *in vitro* toxicity, immunotoxicity and intracellular delivery mechanisms of these nanoparticles were investigated.

Paclitaxel, a highly hydrophobic anti-cancer drug was loaded into the rod-shaped nanostructures by a post-assembly method to obtain paclitaxel-loaded SCRs (PTX-SCRs). To an aqueous solution of SCRs, PTX (10 *wt* %) was added slowly from a stock solution in DMF and stirred overnight to allow for PTX to migrate into the core. The solution was dialyzed against nanopure water for three days to remove DMF and free drug to afford PTX-SCRs. The PTX concentration in the SCRs was determined to be 1.3 *wt* % by HPLC, as reported previously.²⁶ Tumor-homing F3 peptide was conjugated to the cSCKs to target nucleolin, overexpressed at tumor cell surfaces and tumor vasculature.^{27–32} F3 peptide (KDEPQRRSARLSAKPAPPKPEPKPRKAPAKK) has several lysine units that can undergo reaction with the activated ester of the bifunctional reagent to install maleimide groups along the peptide backbone. F3 underwent reaction with a 10-fold excess of

bifunctional coupling agent (succinimidyl-*N*-maleimidoethyl carboxylate) containing both a NHS ester and maleimide. Maleimide-functionalized F3 peptides, and their scrambled analogs, were subjected to reactions with amine functionalized cSCKs at pH *ca.* 8.0 overnight (Scheme S1) to afford the targeted and non-targeted (scrambled control) cSCK samples, respectively. The nanoparticle conjugates were purified with Sephadex G-75 columns in order to remove unbound peptide and unreacted small molecules.

The sizes and charges of the cSCKs were determined using dynamic light scattering and zeta potential measurements. The micelles and cSCKs were *ca.* 21 nm as measured by DLS prior to conjugation of the peptides and *ca.* 25 nm post-peptide conjugation. The sizes of the particles remained the same *ca.* 21–25 nm, within the experimental error, across the series. The zeta potential values of the micelles and cSCKs, *ca.* 30 mV (at pH 5.5, in nanopure water), confirmed the positive surface charge density. After covalent conjugation to peptides, the zeta potential values were found to be *ca.* 43 mV (at pH 5.5, in nanopure water), perhaps due to incorporation of several lysine units present on the peptide backbone that contributed to the increase in cationic character of the peptide-cSCK conjugates.

The cytotoxicity of cylinders, cSCKs and HATs of two different compositions (HAT-A (10 *wt%* cylinders) and HAT-B (4 *wt%* cylinders)) was evaluated in RAW 264.7 mouse macrophages and compared to Lipofectamine, a commonly used, commercially available transfection reagent (Table S1, Supporting Information). The IC₅₀ values of cylinders could not be determined because high cell-viabilities were observed at the range of the tested concentrations (1–100 µg/mL). Lipofectamine induced lower cytotoxicity than cSCKs and HATs. The cytotoxicities of cSCKs and HATs of both compositions were similar. A slightly higher IC₅₀ was observed for HAT-A than HAT-B. In addition, a better siRNA binding affinity was observed for HAT-A, as compared to HAT-B, as confirmed by the gel retardation assay (Figure S1, Supporting Information). Hence, HAT-A was selected to perform other *in vitro* assays, and will be designated as “HATs” in rest of the manuscript.

The immunotoxicities of Lipofectamine, cSCKs, cylinders and HATs were studied by measuring the release of cytokines from cells treated with the nanoparticles, which is commonly utilized as an indication of possible immunotoxicity of nanoparticles and their immunomodulatory effects.^{9,33–35} The structure and shape of nanoparticles can greatly impact their ability to interact with the various components of the immune system, where unintentional immunomodulation can be serious and cause severe adverse reactions and complications.^{36,37} For instance, we have recently shown that incorporation of histamine into the shell composition of cSCKs (comprised of primary amines) can significantly reduce the *in vitro* immunotoxicity, as compared to the unmodified nanoparticles.²⁵ The immunotoxicities of Lipofectamine, cSCKs, HATs (5 µg/mL) and cylinders (0.5 µg/mL) were studied by measuring the levels of 23 cytokines upon treatment of RAW 264.7 mouse macrophages with the nanoparticles for 24 h (Figure S2, Supporting Information and Figure 1). Generally, the highest secretion of cytokines was observed from cells treated with the cylinders, followed by Lipofectamine and cSCKs, whereas the HATs were found to be relatively the least immunotoxic. The strong induction of cytokine release from cells upon treatment with anionic cylinders (0.5 µg/mL) (which had no cytotoxicity up to a concentration of 100 µg/mL) was surprising and might be explained by similarities to bacterial dimensions and surface properties, as recently reported.³⁵ A dose-dependent increase in the release of cytokines from cells upon treatment with the cylinders was also observed (data not shown). The immunotoxicity induced by cSCKs might be explained by non-specific binding to the cell membrane or similarity to viral dimensions and has frequently been reported for cationic carriers.^{35,36} The HAT nanostructures induced lower release for most of the tested cytokines as illustrated in Figure 1. Templating the cationic spheres around the anionic cylinders may reduce cytokine release by masking the negative

charge of the bacterial-like nanoparticles. In addition, immobilization of the spheres from freely moving and binding to biomolecules and cell membranes might explain the lower immunotoxicity of the HATs compared to the cSCKs. Another contributing factor could be the partial neutralization of positive charges on the cSCKs by binding to the cylindrical anionic surface. It has been demonstrated several times that both the size and dimensions of nanoparticles influence the strength of immune responses, and hence the extent of cytokine release upon treatment with nanoparticles.^{35,38–40} For example, RNA/protamine nanoparticles elicited viral-like response, while microparticles of the same composition triggered bacterial-like immune response in human peripheral blood mononuclear cells, probably due to the similarity in dimensions to viruses and bacteria.³⁵

The cellular uptake of Cy3-labeled siRNA mixed with cylinders or complexed to cSCKs and HATs at nitrogen-to-phosphorus (N/P) ratio of 5 was studied by laser scanning confocal microscopy and flow cytometry and compared to Lipofectamine transfection of siRNA (Figure 2 and 3). Both cSCKs and HATs were efficiently taken up by human ovarian adenocarcinoma (OVCAR-3) cells and RAW 264.7 mouse macrophages as indicated by the distribution of the red color (Cy3-siRNA) in the two- and three-dimensional images collected by the confocal microscopy (Figure 2). Lower uptake was observed for Lipofectamine transfection, while no uptake was observed for siRNA mixed with the cylinders (data not shown). The mean fluorescence intensities (MFI) of RAW 264.7 mouse macrophages treated with fluorescein-siRNA either naked or mixed with nanoparticles were measured by a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA) (Figure 3). Negligible uptake was observed with the free siRNA or the siRNA mixed with the cylinders. The MFI of cells treated with cSCKs and HATs were significantly higher than those treated with Lipofectamine ($p < 0.05$). The fluorescence intensity was slightly higher for HATs than for cSCKs, although the difference was not significant.

The abilities of the fully assembled paclitaxel- and cell-death siRNA-loaded HATs to affect cell viability were determined, with comparisons made against each of the individual cSCK and SCR components, various combinations of HATs, cSCKs and SCRs, and Lipofectamine controls (Figure 4). We have previously demonstrated that HAT nanostructures have higher transfection efficiency than cSCKs in both OVCAR-3 and RAW 264.7 mouse macrophages, while cylinders were not able to increase the uptake of siRNA, or induce any gene silencing, as expected, due to the absence of a cationic surface that is required for efficient complexation and cellular uptake.²⁴ In the current study, the effect of N/P ratio on the transfection efficiency and cytotoxicity of AllStars death-siRNA complexed to cSCKs and HATs was studied in OVCAR-3 cells. Complexes prepared at N/P ratio of 10 showed only slightly higher transfection than a N/P ratio of 5 and exhibited similar cytotoxicity (Figure S3). Therefore, the complexes prepared at N/P ratio of 5 were utilized for further characterizations. As indicated in Figure S3, the cell-death siRNA complexed to cSCKs or HATs was incapable of inducing cell death to the extent as observed for Lipofectamine, even at N/P ratio of 10, which might be related to the higher stability of the complexes that partially retard the release of siRNA intracellularly.^{24,41} In order to impart therapeutic potential in two modes, a combined effect of siRNA and paclitaxel in the same nanocarrier on the cell viabilities was studied (Figure 4). The IC_{50} values of paclitaxel-loaded HATs and SCRs were measured in OVCAR-3 and RAW 264.7 mouse macrophages, and were found to be $0.007 \mu M$ and $0.004 \mu M$ in OVCAR-3, respectively. In RAW 264.7 cells, the paclitaxel-loaded HATs had an IC_{50} of $0.007 \mu M$, whereas, surprisingly, no cytotoxicity was observed for the paclitaxel-loaded SCRs. Hence, further studies were conducted in OVCAR-3 cells. Due to the hydrophobicity of paclitaxel, it is possible that it may diffuse from the cylinders (in SCRs or HATs) laying on the cell surface and being internalized and induce cytotoxicity. To assess the synergistic effect of combining siRNA and paclitaxel, the latter was utilized at a concentration that demonstrated little cytotoxicity in the absence of siRNA (cell viabilities

of 72.5 ± 8.1 and 86.7 ± 14.7 , for the paclitaxel-loaded SCRs and HATs, respectively). As expected, the combination of siRNA/cylinder mixture with the paclitaxel-loaded SCRs or HATs did not result in significant cytotoxicity. In general, siRNA- and paclitaxel-loaded HAT nanostructures were the most efficient in reducing cell viability and were the only nanostructure to reduce cell viability more than Lipofectamine transfection ($p < 0.05$). In addition, the paclitaxel-loaded HAT assemblies had a greater effect when combined with siRNA/HAT complexes than when combined with siRNA/cSCKs. Throughout the experiments, the concentrations of nanoparticles utilized to deliver paclitaxel at this low drug concentration ($0.002 \mu\text{M}$) are too low to induce any changes in viability. Combinational therapy (*i.e.* including more than one drug into the same nanocarrier) is aimed at synergistically increasing the efficacy of the treatment. For example, targeted poly(ethylene oxide)-*block*-poly(ϵ -caprolactone)-based nanoparticles loaded with siRNA and doxorubicin induced higher cytotoxicity *in vitro* than did the same nanoparticles but loaded with the individual drugs (*i.e.* doxorubicin or doxorubicin/scrambled siRNA).³ In another study, the tumor growth in mice was significantly inhibited upon treatment with PEGylated lipidic nanoparticles decorated with tumor-homing peptide (NGR), and loaded with doxorubicin and c-myc siRNA. The inhibition of tumor growth was higher than for the nanoparticles loaded with the individual drugs.² It has been reported in literature several times that combination of paclitaxel (same drug utilized in this study) with siRNA targeting specific genes that are essential for cell survival results in synergistic effect and induces high cytotoxicity.^{4,42–44} The synergetic effect is mostly explained by co-delivering the two drugs into the same cell.

The effect of various inhibitors of different endocytic pathways on the transfection efficiency of the complexes was studied by pre-treating cells with sodium azide (NN, 50 nM), chlorpromazine (CP, $10 \mu\text{g/mL}$), nystatin (NS, $10 \mu\text{g/mL}$), cytochalasin D (CD, $5 \mu\text{g/mL}$), and Dynasore (DN, $40 \mu\text{M}$) for 30 min before transfection and continued during the 3 h transfection (Figure 5, **upper panel**). The effect of Bafilomycin A1 (BA, 100 nM), which inhibits the endosomal acidification, and thus inhibiting the endosomal escape of nanoparticles *via* the “proton-sponge effect”, was also studied.^{25,45} The effect of these inhibitors on the cellular uptake of the same complexes also was studied by flow cytometry (Figure 5, **lower panel**). Relatively low concentrations of inhibitors were used because higher concentrations resulted in cytotoxicity (data not shown), and hence, complete inhibition of transfection may not have been achieved at these lower concentrations. The greater inhibition by Dynasore as compared to sodium azide is due to the lower concentration of the sodium azide utilized, because the latter was toxic to cells at higher concentrations. In addition, careful review of the literature reveals that all these endocytotic pathways affected by those inhibitors are interchangeable and depend on the cell line and characteristics of the nanomaterials and that one inhibitor might affect more than one endocytic pathway.^{1,46,47} However, this study was carried out to predict the possible intracellular delivery mechanisms involved in the uptake of these nanostructures. The highest reduction in the cell viability induced by siRNA/Lipofectamine complexes was achieved by pre-incubation with CD and DN, thus indicating a possible involvement of macropinocytosis and/or a combination of dynamin, clathrin and caveolin-mediated endocytosis in Lipofectamine transfection. Uptake of siRNA following Lipofectamine transfection was inhibited by CD, DN, and CP. The overall data suggest that several endocytic pathways are involved in the uptake and intracellular delivery of siRNA during Lipofectamine transfection, which is supported by available literature.^{47,48} The reduction in gene silencing activity of the siRNA/cSCKs complexes was unclear for all the inhibitors used, probably due to the low transfection of the cSCKs themselves, and hence, it was difficult to observe the reduction in transfection. A slight increase in transfection of cSCKs upon pre-treatment with CP was observed (not significant, $p > 0.05$). A significant reduction of cellular uptake of siRNA/cSCKs complexes was induced by CD and DN. A similar

pattern was observed for the HAT nanostructures, except upon pretreatment with CP, where the transfection of siRNA/HAT complexes was significantly enhanced ($p < 0.05$). The flow cytometric analyses, together with the cell viability data, suggest caveolae and macropinocytosis pathways to be major pathways for delivery of siRNA complexed to the HAT nanostructures. Blocking the uptake *via* the clathrin pathway by incubation with CP^{46,47,49} might result in increased uptake *via* other pathways (*i.e.* caveolae and/or macropinocytosis) which are usually associated with higher transfection, as compared to the clathrin pathway.⁵⁰

When the uptake of nanoparticles was investigated by confocal microscopy (Figure 2), it was observed during construction of the three-dimensional images that the thicknesses (where fluorescence can be detected) of untreated cells and cells treated with complexes of siRNA/Lipofectamine and cSCKs were *ca.* 17 μM , whereas cells treated with siRNA/HATs had thicknesses of *ca.* 21 μM . This observation was consistent, reproducible and confirmed in both OVCAR-3 cells and RAW 264.7 mouse macrophages. One possible explanation is that HAT structures might be still laying on cell surface, while spherical cSCKs gradually detach from cylinders after being sequestered into cells *via* electrostatic binding to cell membrane and subsequent endocytosis. To study this hypothesis, cells were pre-treated with Lipofectamine, cSCKs or HATs (no siRNA) for 3 h, followed by extensive washing and addition of 500 nM naked siRNA, followed by further washing. There was not any uptake or binding observed for untreated cells after addition of the naked siRNA (Figure 6). Cellular binding/uptake was observed for Lipofectamine, but was more significant for cSCKs and HATs, and might occur due to the remaining carriers that are associated with cell membranes and can bind the added siRNA. The same difference in thickness of the constructed three-dimensional images was also observed (*ca.* 17 μM for cells treated with siRNA/Lipofectamine or cSCKs and 21 μM for cells treated with siRNA/HATs). Interestingly, upon washing cells with excess heparin, most of the Cy3-siRNA was washed out and the thickness of the cells treated with all complexes was reduced to 17 μM (Figure 6). The same trend was observed in both OVCAR-3 cells and RAW 264.7 mouse macrophages. For further confirmation, the uptake of cylinders labeled with Alexa Fluor[®] 633 and cSCKs labeled with Alexa Fluor[®] 488, either separately or assembled into HAT nanostructures, was studied by confocal microscopy (Figure S4, Supporting Information). Clearly, cSCKs either free or templated onto cylinders (*i.e.* HATs) were taken up by cells, whereas no uptake was observed for cylinders. Decoration of cSCKs with F3 peptides increased the uptake and transfection of siRNA/cSCKs, as compared to cSCKs decorated with F3 scrambled sequence (Figure S5 and S6, Supporting Information). However, decoration of HAT nanostructures with F3 peptides did not result in significant uptake by cells (Figure S6, Supporting Information) and did not induce any change in cell viability when complexed with cell-death siRNA (data not shown). To test if the surface modification with F3 peptide altered the siRNA binding affinity to HAT structures, a gel shift assay was performed for cSCKs and HATs with or without F3 peptide decoration (Figure S7, Supporting Information). Decoration with F3 peptides did not alter the binding affinity of cSCKs and HATs to siRNA.

The ability of nanoparticles to impart stability to the Cy3-labeled siRNA in a highly concentrated serum-containing medium (87% *v/v* fetal bovine serum) was studied (Figure S8, Supporting Information). Both cSCKs and HATs conferred high stability to the complexed siRNA as compared to the non-complexed siRNA and the siRNA complexed to Lipofectamine over 72 h, which indicate their capacity to shield siRNA from the nucleases and provide prolonged stability to the siRNA. In the confocal microscopy study of the cellular uptake, washing with heparin reduced the thickness of the cells treated with the HATs, whereas the thickness was not reduced when cells were washed with culture media. If the increased thickness is due to sedimentation of the nanoparticles, it would not be

reduced upon washing with heparin. Hence, it is postulated that the HAT nanostructures “stick” to cell membranes, and then upon electrostatic interactions between cSCKs and cell membranes and subsequent endocytosis, the cylinders “trigger release” of the cSCKs intracellularly while still residing on the cell surface (Figure 7). The presence of F3 peptides on the surface of HAT structures may prevent the direct electrostatic interactions between cSCKs and the cell surface, hindering the “stick and trigger” intracellular delivery. To the best of our knowledge, this is the first report of a new intracellular delivery mechanism that might be involved in the cellular uptake of higher order complicated nanostructures.

In summary, multifunctional hierarchical nanoassemblies have been constructed based on a bottom-up approach from individual cSCKs and cylindrical nanoparticles. A precise control over the morphology, toxicity, immunotoxicity, transfection, and intracellular trafficking pathway could be attained. Simultaneous loading of nucleic acids, hydrophobic drugs, fluorescent probes and targeting ligands could be achieved, creating a HAT complex that demonstrated increased efficacy *in vitro* than any of the individual components. In addition, a new “stick and trigger” intracellular delivery mechanism was proposed for the HAT nanostructures. Moreover, combined with our previous work, radiolabels could be added in the future, and the entire system can be applied with feasibility of adding or removing some of the built-in functionalities to fit a particular therapeutic application. Although the initial studies here have focused on targeting of cancer cells, we are also pursuing the use of the F3 peptide in HAT nanostructure designs that can alternatively be employed toward treatment of inflammation, such as occurs in lung injury. These controlled supramolecular assemblies are expected to have great potential and open new avenues for combinational therapy and biomedical delivery applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Heat map of the relative expression of the following mouse cytokines - interleukin (IL)-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (P40), IL-12 (P70), IL-13, IL-17, Eotaxin, granulocyte-colony-stimulating factor (G-CSF), granulocyte macrophage-colony-stimulating factor (GM-CSF), interferon- γ (IFN- γ), keratinocyte-derived chemokine (KC), monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1 α , MIP-1 β , RANTES (regulated upon activation normal T-cell expressed and presumably secreted) and tumor necrosis factor- α (TNF- α) - following the treatment of RAW 264.7 cells with medium (control), cylinders, HATs, cSCKs, and Lipofectamine for 24 h.

Figure 2.

Laser scanning confocal microscopy images of OVCAR-3 cells and RAW 264.7 mouse macrophages treated with only media (control) (**A**), Cy3-labeled siRNA (200 nM) complexed with Lipofectamine (**B**), cSCKs (**C**) and HATs (**D**) for 3 h. The nuclei of both cell lines were stained with DRAQ5 nuclear stain. The N/P ratio of siRNA complexes to cSCKs or HATs was fixed at 5. Two-(left panel) and three-dimensional (right panel) images were collected for the cells. On the two-dimensional images, the nuclei were stained with DRAQ5 nuclear stain (blue panel), whereas Cy3-labeled siRNA appears in red. The transmitted light-images and merged images are also presented.

Figure 3.

The mean fluorescence intensity of RAW 264.7 mouse macrophages that were either untreated (control) or incubated with fluorescein-labeled siRNA (200 nM) free, mixed with cylinders, or complexed to Lipofectamine, cSCKs and HATs at N/P ratios of 5, as measured by FacsCalibur flow cytometer. The values are presented as the means $\pm$ standard deviations of at least triplicate, and at least 1000 cells were counted *per* experiment.

Figure 4.

Transfection efficiency of cell-death siRNA mixed with cylinders or complexed with Lipofectamine, cSCKs or HATs into OVCAR-3 cells at N/P ratio of 5. The siRNA-loaded nanoparticles were mixed with paclitaxel-loaded SCR and HATs to test the synergistic effect of combining paclitaxel and siRNA on the cell viability of transfected cells. Values are given as percentage of viability of cells treated with comparable preparations using negative control-siRNA complexes (means $\pm$ SD). The percentage viability of control cells that do not receive any treatment is also presented.

Figure 5.

Transfection efficiency of cell-death siRNA (upper panel, OVCAR-3 cells) and the mean fluorescence intensity of fluorescein-labeled siRNA (200 nM, lower panel, RAW 264.7 mouse macrophages) complexed with Lipofectamine, cSCKs or HATs at a N/P ratio of 5. The effect of various inhibitors was studied by pre-treating cells with sodium azide (NN, 50 nM), bafilomycin A1 (BA, 100 nM), chlorpromazine (CP, 10 μ g/mL), nystatin (NS, 10 μ g/mL), cytochalasin D (CD, 5 μ g/mL), and Dynasore (DN, 40 μ M) for 30 min before transfection and continued during the 3 h of transfection.

Figure 6.

Laser scanning confocal microscopy images of RAW 264.7 mouse macrophages pre-treated with only media (no pre-treatment), 3 μ g of Lipofectamine, cSCKs or HATs for 3 h, followed by a series of washing and addition of naked Cy3-siRNA (500 nM) for 30 min, and further washing. The nuclei of cells were stained with DRAQ5 nuclear stain. Two-(left panel) and three-dimensional (right panel) images were collected for cells treated with Lipofectamine, cSCKs and HATs. On the twodimensional images, the nuclei were stained with DRAQ5 nuclear stain (blue panel), whereas Cy3-siRNA appears in red. The transmitted-light images and merged images are also presented. The last panel presents the confocal microscopy image of cells pre-treated with 3 μ g of HATs for 3 h, and treated in the same way as for the other images, but the final washing was carried out using a heparin solution. Thicknesses of the constructed three-dimensional images are indicated below the images.

Figure 7.

Illustration of the possible intracellular delivery mechanisms of the spherical cSCKs and cSCKs-SCRs assemblies (HATs) that are either decorated or not with F3 targeting peptide. The first step (1) is a demonstration for untreated cell of *ca.* 17 μM thickness that remains the same after being treated (2) with cSCKs but becomes thicker (*ca.* 21 μM) upon treatment with HATs. The difference in thickness remains the same even after the intracellular delivery (3) of the complexed siRNA (appears in red in the confocal images, whereas the nuclei appear in blue). On the right side is the intracellular delivery of F3-targeted cSCKs and HATs. In the latter, F3 peptide-surface decoration hinders the multivalent electrostatic binding of assemblies to cell surface, and thus impeding the “stick and trigger” intracellular delivery.

Scheme 1.

Construction of HAT nanostructures as a template for the co-delivery of siRNA and paclitaxel: (A) Electrostatic complexation of PAAE₁₆₀-*b*-PS₃₀ cSCKs and nucleic acids (*e.g.* siRNA) and decoration of the surface of nanoparticles with targeting ligands (*e.g.* F3 peptides); (B) Loading of hydrophobic drugs (*e.g.* paclitaxel) into SCR composed of PAA₁₄₀-*b*-PpHS₅₀. (C) Hierarchical-assembly of cSCKs and SCR to form the HAT nanoassemblies and the cross-sectional view of the multifunctional HAT nanostructures.