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Synthesis of Biomolecule-Modified Mesoporous Silica Nanoparticles for Targeted Hydrophobic Drug Delivery to Cancer Cells

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Abstract

Synthetic methodologies integrating hydrophobic drug delivery and biomolecular targeting with mesoporous silica nanoparticles are described. Transferrin and cyclic-RGD peptides are covalently attached to the nanoparticles utilizing different techniques and provide selectivity between primary

and metastatic cancer cells. The increase in cellular uptake of the targeted particles is examined using fluorescence microscopy and flow cytometry. Transferrin-modified silica nanoparticles display enhancement in particle uptake by Panc-1 cancer cells over that of normal HFF cells. The endocytotic pathway for these particles is further investigated through plasmid transfection of the transferrin receptor into the normal HFF cell line, which results in an increase in particle endocytosis as compared to unmodified HFF cells. By designing and attaching a synthetic cyclic-RGD, selectivity between primary cancer cells (BT-549) and metastatic cancer cells (MDA-MB 435) is achieved with enhanced particle uptake by the metastatic cancer cell line. Incorporation of the hydrophobic drug Camptothecin into these two types of biomolecular-targeted nanoparticles causes an increase in mortality of the targeted cancer cells compared to that caused by both the free drug and nontargeted particles. These results demonstrate successful biomolecular-targeted hydrophobic drug delivery carriers that selectively target specific cancer cells and result in enhanced drug delivery and cell mortality.

1. Introduction

The integration of hydrophobic drug delivery using nanoparticles with biomolecular targeting agents, such as peptides and proteins, demonstrates a new material that can carry effective cancer therapeutics and deliver them selectively to the cells that require treatment. Although this combination of drugs with biomolecules for nanoparticle-based drug delivery systems is simple in concept, it is difficult to accomplish on account of the incompatible conditions required for both drug molecules and targeting groups to function correctly and independently. In order to integrate these components, four major objectives must be achieved simultaneously. They are: 1) a fluorescent dye, for optical monitoring, and the targeting group must both be attached to the nanoparticle; 2) the linker used to attach the targeting agent to the particle must react selectively with the targeting group and the particle surface; 3) the targeting group must be attached to the particle in such a way as to be recognized and bind with the cell's receptor; and 4) the imaging and cell-recognition motifs must not interfere with the loading and release of the anticancer drug. In this paper, demonstration of the successful integration of biomolecular targeting with hydrophobic drug delivery to generate a new hybrid system that is both selective for and effective against a variety of different cancer cells is reported.

In seeking pharmaceutical agents to treat cancer, many different hydrophobic drugs, such as Paclitaxel, have been found to be very effective at killing cancer cells. However, when used in vivo, these drugs are found to have limited application because of their insolubility in aqueous systems. At present, about 40% of small-molecule drugs in the pipelines of pharmaceutical companies have low water solubility and therefore cannot be administered intravenously or, in some cases, at all.^[1] One specific example is Camptothecin (CPT), which has been shown^[2,3] to be effective against various carcinomas, but its clinical application has not been possible to date because of the poor water solubility of the drug. Modifications to the drug that change its physicochemical characteristics in order to formulate water-soluble salts of CPT for intravenous injection have been pursued. These changes lead to loss of antitumor activity and significant alterations in the toxicological profile.^[2,4,5] Nanoparticle technology is being investigated actively for its ability to deliver these hydrophobic compounds into cancer cells.^[6–12] One material which shows great promise as a viable platform for delivering hydrophobic drugs is mesoporous silica nanoparticles (MSNs).^[13–15] The silica nanoparticles are effective since they have a large internal volume for drug loading, a robust and defined structure for the containment of hydrophobic molecules, and the ability to release the drug under specific conditions.^[7,16–20] Cytotoxicity of these nanoparticles has been investigated in a variety of different ways. We, as well as others, have consistently observed that MSNs are nontoxic at relatively low silica

concentrations ($<100 \mu\text{g mL}^{-1}$).^[16–24] Furthermore, a recent investigation by us demonstrated the superior biocompatibility of MSNs at concentrations adequate for pharmacological applications.^[21] Different amounts of MSNs (up to as much as 200 mg kg^{-1}) injected into animals showed toxic effects to the animals only at extremely high levels of nanoparticles.^[21–24] The question of biodegradability and excretion is an important issue in the clinical use of MSNs, and these points are currently being investigated.^[25,26] As studies progress to make the system more efficient in vitro and in vivo, the delivery of hydrophobic drugs needs to be targeted to cancer cells selectively and the uptake of the particles by these cancer cells must be maximized to decrease the required dosage of the drug delivery system. To date, MSN drug delivery systems have only been targeted to cancer cells using small nutrient molecules such as mannose or folic acid.^[27–29] Other recent work in nanotechnology has placed a great deal of interest on attaching different biomolecular targeting agents onto various nanoarchitectures, including polymers, liposomes, viruses, and inorganic nanoparticles, to target cancer cells with heavily overexpressed transmembrane receptors.^[30–40] The protein transferrin,^[41] which interacts with the upregulated transferrin receptor on cancer cell plasma membranes, and the RGD peptide (arginine-glycine-aspartic acid) that interacts with the $\alpha\text{v}\beta\text{3}$ integrin receptor, are of special interest because the transferrin receptor is known to be highly overexpressed in various human cancers, while the integrin is highly overexpressed in metastatic cancers.^[42–44] By altering the surface chemistry of the MSNs, this drug delivery vehicle can be utilized to target different stages of cancers, such as primary cancers and metastatic cancers.

In this paper, we describe how synthetic methodologies have been devised to achieve these goals successfully and demonstrate a significant enhancement of particle uptake into cancer cells and an increase in hydrophobic drug delivery capability using the silica nanoparticle platform. By bonding either transferrin (Tf) or cyclic-RGD to fluorescently modified mesoporous silica nanoparticles (FMSNs), an up to 10-fold increase in particle uptake by pancreatic cancer cells (PANC-1, Tf-FMSNs), premetastatic breast cancer cells (BT-549, Tf-FMSNs), and metastatic breast cancer cells (MDA-MB 435, RGD-FMSNs) is observed as compared to healthy cells (Human Foreskin Fibroblast cells, HFF) that express low levels of the targeted surface receptors. In addition, through the use of the RGD peptide, particle recognition and selectivity between cancer cell lines is made possible, with a large enhancement in particle uptake specifically by the MDA-MB 435 metastatic breast cancer cell line. The transferrin receptor gene was then transfected into a control cell line (HFF) to overexpress the receptor, resulting in the enhancement of Tf-FMSN uptake, proving that the observed uptake by the cells is a result of the specific, functional targeting agent on the surface of the MSNs. Finally, by loading the particles with the hydrophobic drug Camptothecin, increased drug delivery efficiency of the targeted MSNs was achieved, resulting in enhanced cancer cell death when compared with systems without the Tf or cyclic-RGD targeting. These targeted silica systems show advantages over other biomolecular targeted silica systems for their multifunctionality, which integrates 1) the controlled release of difficult to deliver, but highly effective therapeutic agents with 2) the selectivity shown by the targeting species for specific cell surface receptors.

2. Results and Discussion

2.1. Design and Synthetic Challenges

To generate an effective drug delivery system, the synthetic challenges of independent molecular integration, organosilane linker selectivity, biorecognition of the surface targeting group, and drug-release capability must be taken into account for both the Tf and RGD systems. It is desirable that the particles contain a fluorescent molecule for imaging purposes and a surface modification that can bind the biological targeting agent.^[21,45–47]

Furthermore, the nanoparticle's targeting agent must be recognized by the receptor and the drug utilized must not interact with any of these molecules.

The most important reaction in the construction of this biological signaling motif for nanoparticle-based enhancement is the silica surface modification and its selective interaction with the targeting agent. This modification can be approached from two sides: the surface linker can be chosen so that its reactivity is selective towards the biological agent of interest, or the biological targeting agent can be modified to react selectively with a desired linker on the particle surface. In this work, both approaches are used to solve this synthetic challenge.

Lastly, the ability for the nanoparticles to contain the drug and release it effectively inside cancer cells to induce cell apoptosis must be confirmed, otherwise the entire system is not effective at its primary objective of drug delivery. Previous research has shown^[7,16] that MSNs are effective hydrophobic drug carriers that release upon cell endocytosis. However, the question arises, will the biological surface modifications interfere with this function, or will the drug interfere with the biological function of the targeting agent? Utilizing the methodologies reported herein, cell viability assays demonstrate that hydrophobic drug delivery results in an enhanced drug delivery system with both sensitivity and selectivity.

To satisfy all of these synthetic challenges, the following general approach to generate a viable nanovector was taken (Scheme 1). Firstly, the silica nanoparticles were synthesized by standard sol-gel techniques.^[48,49] Fluorescein was chosen for its emission at 520 nm, which does not overlap with the emission from other fluorophores used during experimentation. Then, targeting agent-specific organosilane surface modifications were grafted onto the nanoparticle surface for later reaction with either the Tf protein or the cyclic-RGD peptide. Camptothecin was then loaded into the nanoparticle's mesoporous network by diffusion in *N,N'*-dimethylformamide (DMF). These conditions prevent reaction between the drug, solvent, surface modification, and fluorophore (drug-loading omitted for nanoparticles used in cellular-uptake studies). Finally, the drug-loaded nanoparticles were reacted with the targeting agent in buffer and washed to remove unreacted or adsorbed materials. By delaying the attachment of the targeting agent until the last step, any unfavorable interactions between the sensitive targeting groups and the reagents used for nanoparticle preparation are avoided.

To prove that the surface modification of MSNs can be achieved for targeting primary cancer cells, transferrin conjugation was chosen first of all. For the Tf modification, specific choices were made to tailor the system for the most effective drug delivery when utilizing a biologically synthesized protein. Firstly, a phosphonate coating was used during the FMSN synthesis. Next, the linker 3-glycidypropyltrimethoxysilane (3-GPTMS) was condensed onto the nonphosphonated portions of the nanoparticles (pore openings) in toluene. This epoxide linker was chosen for its strong reactivity with amines.^[50] The loading of CPT into epoxide-modified FMSNs in DMF did not induce an epoxide ring-opening reaction, thereby binding the Tf effectively after drug loading. Finally, Tf was utilized as a targeting agent for both pancreatic and breast cancer cells since it has been shown to interact successfully with the Tf receptor to induce receptor-mediated endocytosis.

Other than targeting primary cancers by conjugating with transferrin, it is possible to make MSNs specifically target metastatic cancer cells by surface modification with a RGD peptide, which binds to the plasma membrane integrin. Unlike the bottom-up approach used for Tf targeting, the RGD can be modified synthetically for attachment to the surfaces of MSNs. In work developed by Kessler,^[52] a cyclic, five-amino-acid form of RGD was shown to be one of the most selective and active antagonists of the $\alpha_v\beta_3$ integrin receptor.^[53–55] By

utilizing an analog of this design, cyclo(Arg-Gly-Asp-d-Phe-Cys) (c[RGDfC]) was synthesized^[56–58] so that a free thiol as part of the cysteine could be included without diminishing its binding capability. This allows for a peptide-to-particle attachment via a disulfide bond to 3-mercaptopropyltrimethoxysilane modified particles. A disulfide bond was chosen for the following reasons: 1) disulfide bonds have been utilized for therapeutic applications;^[59] 2) in order that the attachment could be made in mild reaction conditions; 3) to render the design widely applicable to different biological targeting agents, and; 4) to allow for the reducing environment of the cell to cleave the targeting group to make drug release as efficient as possible. The peptide was prepared by Fmoc-based solid-phase synthesis of the protected linear peptide, followed by macrocyclization, deprotection, reverse-phase high-performance liquid chromatography (RP-HPLC) purification and characterization (Supporting Information (SI), Scheme 1 and Figure S1–S3).

2.2. Synthesis and Characterization of Tf–FMSN and RGD–FMSN

The structure of Tf–FMSN was characterized using transmission electron microscopy (TEM), scanning electron microscopy (SEM), X-ray diffraction (XRD), dynamic light scattering (DLS), and IR spectroscopy. TEM images of FMSN before Tf modification shows that individual nanoparticles, ranging in size from 50 to 150 nm in diameter, were produced (Figure 1--1). SEM indicates that the material is relatively uniform in size and shape (SI, Figure S4). Low-angle XRD shows that after surfactant extraction the nanoparticles are mesoporous with a 2θ of 2.16, and a d -spacing of 4.1 nm (SI, Figure S5). Infrared spectroscopy, after the solvent extraction, shows the absence of the C–H stretches of CTAB at 2900–3000 cm^{-1} , thus verifying that CTAB is removed (SI, Figure S6). DLS shows that particles in nanofiltered water have an approximate hydrodynamic radius of about 190 nm (SI, Table S1). After transferrin attachment to produce the Tf–FMSN, characterization was initially performed using TEM (Figure 1). The Tf modification resulted in nanoparticles that have dark spots, which is likely an effect of the surface modification by Tf. Dispersibility of the nanoparticles was studied using DLS, which resulted in an increase to an average of 230 nm (SI, Table S2). Verification of the protein surface modification was carried out using a protein staining technique and UV–vis spectroscopy (Figure 2A and SI, Figure S10–S12). The presence of the Tf on the nanoparticles was confirmed by protein staining with Coomassie Blue and analyzing for the absorbance at 595 nm (a modified Bradford assay). If the amount of nanoparticles is increased, then the absorbance at 595 nm increases proportionally. Nanoparticles without protein do not shift the absorbance maximum to 595 nm, as observed for the Tf–FMSNs. This result leads to the conclusion that the transferrin attached to the nanoparticle surface causes the shift to 595 nm. A CPT loading assay for Tf–FMSNs gave a value of 1.3% CPT by weight (SI, Figure S13).^[27,51]

To prepare the RGD–FMSNs, the FMSN synthesis was first performed. The surface of the nanoparticle was then modified with 3-mercaptopropyltrimethoxysilane by condensation in toluene, followed by thiol exchange with 2,2'-dithiopyridine to activate the nanoparticle surfaces to attach the cysteine-based cyclic-RGD peptide. Attempts to target phosphonated nanoparticles were unsuccessful, an observation which is attributed to the inaccessibility of the cyclic-RGD to the integrin receptor because of the presence of the phosphonate coating on the nanoparticle exterior. Therefore, nonphosphonated nanoparticles were used. Camptothecin was then loaded into the mesoporous network of the FMSNs by diffusion in DMF (drug loading omitted for nanoparticles used in cellular uptake studies).

The structure of the RGD–FMSNs was characterized using TEM, XRD, DLS, and IR spectroscopy. TEM images before cyclic-RGD attachment to the nanoparticles show that individual particles, ranging in size from 50 to 150 nm in diameter, were produced (SI, Figure S7). Low-angle XRD shows that the nanoparticles, after extraction, are mesoporous

with a 2θ of 2.20 and a d -spacing of 4.0 nm (SI, Figure S8). Infrared spectroscopy after the solvent extraction shows the absence of the C–H stretches of CTAB at 2900–3000 cm^{-1} , thus verifying that CTAB is removed. (SI, Figure S9). DLS shows that the nanoparticles in nanofiltered water have an approximate hydrodynamic radius of about 178 nm after extraction (SI, Table S2). After attachment of the RGD to complete the synthesis of RGD–FMSNs, the presence and effectiveness of the RGD targeting agent was investigated using flow cytometry by comparing the MDA-MB 435 cellular uptake of particles with cyclic-RGD to those without (Figure 2B). The presence of attached RGD resulted in up to 10 times greater nanoparticle uptake by the MDA-MB 435, indicating that the targeting agent is present and interacting actively with the integrin to undergo endocytosis. A drug-loading assay of RGD–FMSNs gave a value of 0.4% CPT by weight (SI, Figure S13)^[27,51]

2.3. Enhanced Endocytosis with Tf–FMSN and RGD–FMSN In Vitro

Once the Tf–FMSNs and RGD–FMSNs were completely characterized, in-vitro studies were carried out beginning with an investigation of the cellular uptake of transferrin-modified silica nanoparticles to show selective uptake by cancer cells. The effect of transferrin derivatization of mesoporous silica nanoparticles on the uptake of the particles by cells with and without transferrin receptors was studied in vitro using cancer and normal human cell lines. The expression of transferrin receptor (TfR) by the human pancreatic cancer cell line PANC-1, and the human breast cancer cell line BT-549, is significantly higher than TfR expression in the normal human foreskin fibroblast cell line HFF (Figure 3a). When control FMSNs were incubated with these cell lines, fluorescence microscopy showed FMSNs (green fluorescence) were taken up by all three cell lines (red fluorescence, WGA-Alexa Fluor 594 stain), in agreement with previous reports (Figure 3c, upper panels).^[21–23] When the Tf–FMSNs were incubated with these cells and compared to FMSNs, there was an apparent difference in cellular uptake and selectivity. The Tf modification on the FMSNs significantly increased the nanoparticle uptake by the PANC-1 and BT-549, but not by the HFF (Figure 3c, upper panels). These results corroborated the importance of the overexpression of TfR on the PANC-1 and BT-549 cancer cells, a mechanism which facilitates the recognition and binding of the Tf–modified FMSNs, and therefore increases the intracellular uptake of FMSNs. These results also indicate that nanoparticles will concentrate inside cancer cells to a much greater extent than inside healthy cells. These results were the first indication that enhancement of FMSN uptake was possible using the specific synthetic assembly strategies.

To confirm that the increased uptake of Tf–FMSNs by human cancer cells was indeed a result of the overexpression of the specific receptors on the cell plasma membrane, the Tf receptor gene was transfected into HFF cells with a TfR plasmid (pAcGP67A-TfR, Addgene). The enhanced expression of TfR in HFF cells after transfection was confirmed by Western blot analysis (Figure 4a). Next, the uptake of FMSNs or Tf–FMSNs in these HFF cells overexpressing TfR was examined. Although overexpression of TfR in HFF cells did not change the uptake of FMSNs (Figure 4b), many more Tf–FMSNs accumulated in the HFF cells that were transfected to express the TfR when compared to that in untransfected HFF cells. These results indicate that the enhanced uptake of Tf–FMSNs in cells overexpressing TfR is mediated by TfR.

Interestingly, even greater uptake of the Tf–FMSNs was observed in the breast cancer cells, BT-549, by enhancing the expression of TfR in these cells through plasmid transfection. The same plasmid transfection with Lipofectamine used in the HFF cells was carried out to enhance expression of TfR in BT-549. The intracellular uptake of FMSNs, with or without modification of transferrin, in cells with or without transfection, was then examined using fluorescence microscopy. Although transfection with TfR did not significantly change the intracellular uptake of the unmodified FMSNs in BT-549 cells (Figure 4c, based on

approximate particle-per-cell concentrations), the amount of fluorescent nanoparticles in BT-549 cells transfected with TfR plasmid was enhanced, nearly fully occupying the cytoplasm space of the cells. These results further confirm that the increased uptake of Tf-FMSNs is a result of the enhanced expression of TfR.

Experiments to investigate the cellular uptake of the RGD-FMSNs were carried out to target metastatic cancer cells selectively. Using this nanoparticle design, selectivity for the integrin $\alpha_v\beta_3$ receptor was expected, thereby enhancing nanoparticle uptake into cancer cell lines that are metastatic in nature.

To correlate the cyclic-RGD modification with cellular uptake, the breast cancer cell lines MDA-MB 435 (metastatic) and MCF-7 (nonmetastatic) were tested for the expression of integrin $\alpha_v\beta_3$ through Western blot analysis (Figure 3a). The metastatic cancer cell line MDA-MB 435 showed high expression of both subunits of the integrin, while the non-metastatic cell line MCF-7 had low expression of the α_v subunit and no detectable expression of β_3 subunit. Based on these results, the two cell lines were used to compare cellular uptake of unmodified FMSNs and RGD-FMSNs. To obtain a quantitative measurement of RGD-FMSN uptake enhancement, flow cytometry was performed after incubation with the nanoparticles. The flow cytometry profiles demonstrate that MDA-MB 435 cells, which express integrin $\alpha_v\beta_3$, take up RGD-FMSNs 7–10 times more efficiently than untargeted FMSNs (Figure 3b). Fluorescence microscopy analysis demonstrated that both cell lines take up FMSNs and RGD-FMSNs, while the uptake of RGD-FMSNs is enhanced in MDA-MB 435 (Figure 3c, lower panels). These results suggest a high degree of selectivity for the RGD-FMSNs by meta-static cancer species mediated through the $\alpha_v\beta_3$ integrin.

2.4. Enhanced Delivery of Hydrophobic Chemotherapeutics Using Tf-FMSN and RGD-FMSN

The above results show that nanoparticle uptake can be enhanced drastically by the presence of the biological signaling agents transferrin and RGD. In addition, by using TfR expression as a model system, the results show that the enhancement is a result of the specific interaction between the surface receptors and the nanoparticle surface mediation.

The enhancement in cell killing of the Tf-FMSNs and RGD-FMSNs is demonstrated through cell viability assays. The enhanced cellular uptake of Tf-FMSNs by cancer cells suggests that preferential delivery of anticancer drugs to cancer cell lines that overexpress TfR should occur. It has been shown previously that mesoporous silica nanoparticles can deliver hydrophobic drugs into human cancer cells.^[7] To verify that delivery was possible with the Tf-FMSNs, the cytotoxicity of these nanoparticles loaded with CPT in both PANC-1 (Figure 5) and BT-549 (Figure 6) cancer cells was explored. FMSNs and Tf-FMSNs without CPT are not cytotoxic. However, growth inhibition of PANC-1 cells was observed with CPT-loaded FMSNs at concentrations higher than $10 \mu\text{g mL}^{-1}$, showing that the hydrophobic drug was delivered into the cell by the FMSNs. There was a large increase in the cytotoxicity of Tf-modified CPT-loaded FMSNs to PANC-1 cells compared to that of untargeted CPT-loaded nanoparticles, which correlated with the enhanced nanoparticle intracellular uptake. The results obtained indicate that, with Tf-FMSN, cell mortality begins with dosages 3 orders of magnitude lower than nanoparticles without the Tf-modification, with much higher rates of cell mortality up to the maximum tested dosage of $100 \mu\text{g mL}^{-1}$ nanoparticles. These results indicate that the increased nanoparticle uptake, caused by Tf modification of the FMSNs, delivers more drug to the cancer cells that overexpress TfR and is therefore more cytotoxic to these cells than to normal cells.

It is important to investigate if the observed growth inhibition of cancer cells by the Tf-FMSNs loaded with anti-cancer drugs was indeed caused by the apoptosis-inducing ability of CPT, and whether or not the enhanced expression of TfR on cancer cells by plasmid transfection would facilitate more cell killing by increased delivery of drugs into the cells. The FMSNs or Tf-FMSNs loaded with CPT were incubated with two varieties of BT-549, one further overexpressing TfR by plasmid transfection and one with standard levels of TfR expression. Both samples were incubated with the Tf-FMSNs for 48 h before examining cell apoptosis. Nuclear fragmentation and chromatin condensation were observed in most of the cells treated with CPT-loaded FMSNs (Figure 5b), and similarly in the cells treated with CPT-loaded Tf-FMSNs. An increase in cell death for cells over-expressing TfR treated with CPT-loaded Tf-FMSNs was observed by lower cell survival rates and more nuclear fragmentation. These results indicate that the enhanced expression of TfR in human cancer cells further increased the efficacy of drug delivery.

To show that RGD-FMSNs are also a viable drug delivery system, both RGD-FMSNs and FMSNs were loaded with CPT and incubated with MDA-MB 435 to observe the difference in cell viability when the RGD-targeted silica nanoparticles are used over the untargeted nanoparticles (Figure 6). First, the control nanoparticles (both the RGD-FMSNs and the FMSNs do not contain CPT loaded into the pore structure) were incubated with the cells and appear to be nontoxic at this dosing ($\approx 4 \mu\text{g mL}^{-1}$ of nanoparticles). Then, MDA-MB 435 cells were incubated with free drug (10 ng mL^{-1} in DMSO), CPT-loaded FMSNs and CPT-loaded RGD-FMSNs (at $\approx 4 \mu\text{g mL}^{-1}$ of particles with 0.4% by weight loaded CPT, yielding $\approx 10 \text{ ng mL}^{-1}$ CPT for both CPT-loaded FMSNs and CPT-loaded RGD-FMSNs), resulting in cell mortality rates of 14, 29 and 49%, respectively. The nanoparticles that have the cyclic-RGD surface peptide show a 20% increase in cell death when compared to nanoparticles without the cyclic-RGD modification and a 35% increase in cell death when compared to the free drug at a concentration of 10 ng mL^{-1} of CPT. This observation indicates that the surface modification, which generated an increase in intracellular nanoparticle concentration, also causes a significantly increase in cell killing not only compared to the free drug, but compared to nanoparticles that do not contain a biomolecular targeting agent.^[60]

3. Conclusion

Mesoporous silica nanoparticles with covalently bonded biological signaling agents that induce selective uptake via receptor-mediated endocytosis enhance the uptake efficiency and thereby efficacy of the mesoporous silica hydrophobic drug delivery system. By attaching Tf onto the surface of the nanoparticles, an increase is observed in nanoparticle uptake and cell killing in PANC-1 and BT-549 at very low doses of nanoparticles when compared to particles that are not Tf-modified. By utilizing the selectivity of RGD for $\alpha_v\beta_3$ integrin, mesoporous silica was targeted specifically to metastatic cancer cell lines resulting in a 10-fold enhancement in nanoparticle uptake. To show that these results are an effect of the interaction between targeting and upregulated receptors, the Tf receptor was overexpressed in HFF cells, inducing particle endocytosis into cells that previously were insensitive to the targeted nanoparticles. Finally, the presence of a targeting agent enhanced cell killing of PANC-1, BT-549 and MDA-MB 435 when compared to systems not utilizing these targeting group. Hydrophobic drug delivery has been successfully integrated with biomolecular signaling utilizing both protein- and peptide-based receptor mediated endocytosis.

A hallmark of this research is that it can be concluded that a wide array of benefits will result from the use of the targeted mesoporous silica platform to enhance the effectiveness of hydrophobic drug delivery in vivo. These techniques will allow for a wide variety of

different biomolecules to be integrated onto the nanoparticle surface for greater selectivity in various different types of cancer treatments. Additionally, these surface modifications may further enhance in-vivo studies by enhancing the biodistribution and particle circulation lifetime, as has been observed for small molecule surface modification of silica nanoparticles.^[11] Lastly, these synthetic strategies for surface modification can be integrated onto more complex nanoparticle delivery systems to enhance their efficiency at cell specific particle uptake for hydrophobic drug delivery.

4. Experimental Section

RGD Peptide Synthesis

A fritted glass reaction vessel was charged with 2-chlorotrityl resin (8.33 g, 10 mmol, Novabiochem). The resin was derivatized by gently bubbling N₂ through the resin with a solution of Fmoc-Gly-OH (2.47 g, 8.31 mmol, 0.83 equiv) in 20% 2,4,6-collidine/CH₂Cl₂ (ca. 20 mL) for 18 h. The solution was then drained using nitrogen pressure and the resin was washed with CH₂Cl₂ (3× ca. 100 mL). After the loading step, unreacted sites on the resin were capped using a solution of 2,4,6-collidine/MeOH/CH₂Cl₂ (1/2/17, v/v, 3× 40 mL) for ca. 1 h. The reaction vessel was then drained, and the resin was washed with DMF (3× ca. 100 mL), and then with CH₂Cl₂ (3× ca. 100 mL). The Fmoc group was removed by adding a solution of DBU-HOBt-DMF (1.2 mL, 1.0 g, 88 mL, 3× 40 mL) to the resin followed by gentle agitation (N₂). The solution was drained, and the resin was washed with DMF (3× ca. 100 mL), and then with CH₂Cl₂ (3× ca. 100 mL).

Elongation of the protected linear peptide was accomplished by preactivating the appropriate Fmoc-protected amino acid (3.0 equiv) with HCTU (3.0 equiv) in 20% 2,4,6-collidine-DMF (ca. 10 mL) and CH₂Cl₂ (ca. 10 mL). The resultant coupling solution was then added to the resin and gently agitated for 4 h. The Kaiser^[51] test was used to determine if the couplings were complete. The solution was then drained and the coupling procedure was repeated until elongation was complete. After final Fmoc deprotection, the protected linear peptide was cleaved from the resin using a solution of HFIP-CH₂Cl₂ (1/4, v/v) for 3.5 min. The peptide solution was drained from the resin and concentrated to a solid under vacuum to obtain 1.9 g of crude protected linear peptide.

Cyclization was accomplished by dissolving the above protected linear peptide (1.90 g, 1.66 mmol) and 4 equiv of HCTU (2.48 g, 6.0 mmol) in DMF (100 mL). The resultant solution was added dropwise over a period of 4 days into a stirring solution of 2% 2,4,6-collidine-CH₂Cl₂ (500 mL). After washing the reaction mixture with 0.2 M HCl (aq), the solution was concentrated under vacuum to obtain ca. 1.0 g of crude protected cyclized peptide as a yellow solid. Global deprotection of the side chain protection groups was carried out in a 250 mL round-bottomed flask equipped with nitrogen inlet adaptor and a stir bar using a 50 mL solution of TFA/TIS/H₂O (8/1/1, v/v) for 5 h. TFA was removed under vacuum and the resultant slurry was partitioned between H₂O (ca. 100 mL) and Et₂O (ca. 100 mL). The organic layer was extracted with Et₂O (2×) and the combined aqueous layers were concentrated under vacuum to afford peptide **1** as an off-white solid (0.54 g) (SI, Scheme S1).

Purification of peptide **1** was accomplished with preparative RP-HPLC (water-MeCN with 0.1% TFA) using an Agilent C₁₈ column. The analytical HPLC spectrum of the crude peptide **1** shows two major peaks, one is associated with 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate (HCTU) and the other was identified as the product (SI, Scheme S1). Pure fractions (>95%) were combined, the MeCN was removed under vacuum, and the remaining aqueous solution was frozen and lyophilized to afford a fluffy white powder. Purification of 0.54 g of crude material yielded ca. 100 mg of

analytically pure product: ^1H NMR, (600 MHz, D_2O , 298 K) δ 7.43–7.40 (m, 2 H), 7.37–7.31 (m, 3 H), 4.78 (t, $J = 7.8$ Hz, 0.67 H), 4.73 (t, $J = 7.8$ Hz, 0.67 H), 4.66 (t, $J = 7.2$ Hz, 0.33 H), 4.60 (t, $J = 7.5$ Hz, 0.33 H), 4.45 (*app. t*, $J = 7.0$ Hz, 0.33 H), 4.36 (dd, $J = 9.0$, 5.4 Hz, 0.67 H), 4.34 (dd, $J = 9.0$, 5.4 Hz, 0.33 H), 4.25 (d, $J = 15$ Hz, 0.67 H), 4.25 (dd, $J = 7.2$, 5.4 Hz, 0.67 Hz), 4.09 (d, $J = 15.6$, 0.33 H), 3.68 (d, $J = 15$ Hz, 0.33 H), 3.55 (d, $J = 15$ Hz, 0.67 H), 3.28–3.17 (m, 2.33 H) 3.14–3.05 (m, 1.67 H), 2.95–2.70 (m, 4 H), 1.98–1.83 (m, 1.0 H), 1.78–1.55 (m, 3 H); ESI-MS m/z for $\text{C}_{24}\text{H}_{35}\text{N}_8\text{O}_7\text{S}$ $[M+\text{H}]^+$ calcd 579.23, found 579.31.

Preparation of Phosphonated FMSNs for Tf–FMSNs

FITC-modified MCM-41: In a 10 mL round-bottomed flask, fluorescein isothiocyanate (FITC, Sigma, 90%, 5.5 mg) was dissolved in EtOH (3 mL) with stirring. 3-Aminopropyltriethoxysilane (3-APTES, Aldrich, 98%, 12 μL) was added to solution and allowed to react with the FITC for 2 h under nitrogen. Tetraethyl orthosilicate (TEOS, Aldrich, 98%, 2.5 mL) was added and allowed to mix into the solution. In another 250 mL round-bottomed flask, cetyl trimethyl ammonium bromide (CTAB, Aldrich, 0.5 g) was added to deionized H_2O (240 mL). 2 M NaOH (Fisher, 1.75 mL) was added to the solution causing the pH to increase to approximately 12.4, inducing the complete dissolution of CTAB. The solution was heated to 80 $^\circ\text{C}$ while stirring to create a homogenous solution. The fluorescein solution was added to the basic CTAB solution rapidly inducing particle condensation. 15 min after mixing the two previous solutions, 3-trihydroxysilylpropylmethylphosphonate (Gelest, 42% in H_2O , 0.63 mL) was added slowly. This reaction mixture was allowed to stir for an additional 2 h at about 80 $^\circ\text{C}$. Particles were collected by filtration. The filtered cake was then washed with MeOH. The particles were extracted by suspending them in MeOH (100 mL) and slowly adding 12 M HCl (5 mL). The generated particle suspension was refluxed overnight to remove the CTAB template. Particles are then separated by filtration and washed with methanol.

Particle Surface Modification for Tf Attachment

FITC modified MCM-41 particles (100 mg) were suspended in PhMe (10 mL), sonicated and then stirred. 3-Glycidoxypropyltrimethoxysilane (Gelest, 98%, 4 μL) was added slowly to this suspension. The solution was then placed under nitrogen and refluxed overnight. Particles were then separated from solution by centrifugation, washed with PhMe and *i*-PrOH, and vacuum dried overnight.

Drug Loading for Tf–FMSNs

Camptothecin (CPT, Sigma, 95%, 5 mg) was dissolved in anhydrous DMF (5 mL). The CPT solution was added directly to 50 mg of modified particles and stirred in a round-bottomed flask under nitrogen for 4 h. Particles were collected by centrifugation, the supernatant completely removed, and the particles dried for two days under vacuum to remove DMF. The presence of the CPT was confirmed by taking a small sample of the dried particles and suspending them in water with sonication and vortexing, followed by centrifugation. This process was repeated several times. These materials were kept in the water solution overnight, collected again by centrifugation and then suspended in 3 mL of Me_2SO and examined using UV–vis Spectroscopy.

Protein Modification

For this study, apo-human transferrin (Tf) (Sigma) was attached covalently to the FITC modified MCM-41 particles. Protein solution was prepared by generating 2 mg mL^{-1} concentration of Tf in HEPES buffer (0.05 M, Sigma). Particles were suspended in HEPES buffer (1 mL) and dispersed by vortexing and sonicating. After the particle suspension

appeared evenly dispersed into the buffer, 1 mL of 2 mg mL⁻¹ of the protein solution was added and allowed to react at room temperature overnight. Tf samples were wrapped to avoid adverse effects from light exposure. The particles were collected by centrifugation with supernatant collected and removed for analysis. Particles were then washed several times with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer and sonicated in order to remove any adsorbed protein and then suspended in PBS buffer.

Production of Nonphosphonated FMSN for RGD–FMSN

In a 10 mL round-bottomed flask, fluorescein isothiocyanate (FITC, Sigma, 90%, 5.5 mg) was dissolved in EtOH (3 mL) by stirring. 3-APTES (Aldrich, 98%, 12 μ L) was added to solution and allowed to react with the FITC for 2 h under nitrogen. Tetraethyl orthosilicate (TEOS, Aldrich, 2.5 mL) was added and allowed to mix into the solution. In another 250 mL round-bottomed flask, CTAB (Aldrich, 0.5 g) was added to deionized H₂O (240 mL). 2 M NaOH (1.75 mL) was added to the solution, causing the pH to increase to approximately 12.4, inducing the complete dissolution of CTAB. The solution was heated to 80 °C while stirring to create a homogenous solution. The fluorescein solution was added to the basic CTAB solution rapidly, inducing particle condensation. This reaction mixture was allowed to stir for an additional 2 h at about 80 °C. Particles were collected by filtration. The filtered cake was then washed with MeOH. The particles were extracted by suspending the particles MeOH (100 mL) and slowly adding 12 M HCl (5 mL). The generated particle suspension was allowed to reflux overnight to remove the CTAB template. Particles were then separated by filtration and washed with MeOH.

Particle Surface Modification for RGD

FITC modified MCM-41 particles (50 mg) were suspended in 5 mL of PhMe (5 mL), sonicated and then left stirring. 3-Mercaptopropyltrimethoxysilane (3-MPTMS, Gelest, 98%, 4 μ L) was added slowly to this suspension. The solution was then placed under nitrogen and allowed to reflux overnight. Particles were then separated from solution by centrifugation, washed with PhMe twice and allowed to remain suspended in PhMe for one week. 2,2'-dithiopyridine (Aldrich, 25 mg) was dissolved in EtOH (7.5 mL) containing acetic acid (Glacial, 100 μ L). 3-MPTMS-modified FMSN (25 mg) were suspended EtOH (5 mL) and added slowly to the 2,2'-dithiopyridine solution during 15 min while stirring. Reaction was allowed to proceed overnight under inert atmosphere. Particles were isolated by centrifugation and washed two times with EtOH. These particle are herein referred to as pyridine disulfide modified FMSNs.

Drug Loading for RGD–FMSNs

Camptothecin (CPT, Sigma, 95%, 1.5 mg) was dissolved in anhydrous DMF (5 mL). The CPT solution was added directly the modified particles (50 mg) and stirred in a round-bottomed flask under nitrogen for 4 h. Particles were collected by centrifugation, the supernatant completely removed, and the particles dried for 2 days under vacuum to remove DMF. The presence of the CPT was confirmed by taking a small sample of the dried particles and suspending them in water with sonication and vortexing followed by centrifugation. This process was repeated several times. These materials were kept in the water solution overnight, collected again by centrifugation and then suspended in 3 mL of Me₂SO and examined using UV–vis Spectroscopy.

Reaction of Pyridine Disulfide Modified FMSN with Cyclic RGD

The cyclic peptide (4 mg) was dissolved in EtOH (7.5 mL) containing acetic acid (Glacial, 100 μ L). Pyridine disulfide modified FMSN (15 mg) were suspended in 5 mL of ethanol. The particle solution was added to the peptide solution during 15 min while stirring. The

reaction was allowed to proceed overnight under inert atmosphere. The product, RDG-FMSNs, was then washed two times in 0.05 M aqueous HEPES buffer (Sigma) and left suspended in HEPES.

Cell Culture

Human pancreatic cancer-cell line PANC-1 and breast cancer cell line BT-549 were obtained from the American Type Culture Collection. Human foreskin fibroblast cells were a gift from Dr. Peter Bradley's laboratory at UCLA. MCF-7 and MDA-MB-435 cells were a gift from Dr. Neil O'Brien at UCLA. All cells, except MDA-MB-435, were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% fetal calf serum (Sigma), 2% l-glutamine, 1% penicillin, and 1% streptomycin stock solutions. MDA-MB 435 cells were maintained in RPMI-1640 medium (Cellgro) supplemented with 10% fetal calf serum (Sigma). The media for all the cells was changed every three days, and the cells were passaged by trypsinization before confluence.

Fluorescent Microscopy

The fluorescence of the nanoparticles at an excitation wavelength of 488 nm was used to confirm the cellular uptake of the FMSNs. The cells were incubated in an 8-well Lab-Tek chamber slide system (Nalge Nunc International) with the nanoparticles and then washed with PBS to remove the NPs that did not enter the cells. The cells were then stained with DAPI solution for nuclear and WGA-Alexa Fluor 594 for plasma membrane before being monitored using the fluorescence microscope.

Western Blot Analysis

Cell lysate was separated by gel electrophoresis on a polyacrylamide gel containing sodium dodecyl sulfate and then transferred to nitrocellulose membranes. The membranes were blocked with Tris-buffered saline (TBS) containing 5% (w/v) skimmed milk. After being washed with TBS containing 0.1% Tween 20 (Sigma), the membranes were incubated overnight at 4 °C with primary antibody (anti-human CD71 antibody from Sigma, Cat# C2063 or anti-integrin α v from Cell Signaling, Cat# 4711S) diluted with TBS. After being washed, the membranes were incubated for 2 h at room temperature with the second antibody (Santa Cruz Biotechnology). Bands were detected with an ECL system (Amersham Pharmacia Biotech.) After detection, the membrane that was incubated with integrin α v antibody was stripped, washed with TBS-T and incubated with integrin β 3 antibody (cell signaling, Cat# 4702) overnight at 4 °C. Incubation with secondary antibody was repeated as well as detection with the ECL system.

Flow Cytometry

Cells were seeded in a six-well plate at a confluency of 1×10^5 cells per well overnight. Cells were incubated with $5 \mu\text{g mL}^{-1}$ of FMSN or RGD-FMSN for 24 h. After incubation, cells were washed with PBS, trypsonized, washed with 0.05% trypan blue solution to decrease the background fluorescence and washed two more times with PBS. Flow cytometry was performed measuring the green fluorescence inside the cells, which corresponds to the fluorescence of the FMSNs.

Cell Death Assay

The cytotoxicity assay was performed by using a cell-counting kit from Dojindo Molecular Technologies, Inc.^[7] Cells were seeded in 96-well plates (5000 cells per well) and incubated in fresh culture medium at 37 °C in a 5% CO₂/95% air atmosphere for 24 h. The cells were then washed with PBS and the medium was changed to a fresh medium containing the nanoparticles, with or without drug loaded at the indicated concentrations. After 24 h, the

cells were washed with PBS to remove FMSNs that were not taken up by the cells, and the cells were then incubated in fresh medium for an additional 48 h. The cells were washed with PBS and incubated in DMEM with 10% WST-8 solution for another 2 h. The absorbance of each well was measured at 450 nm with a plate reader. Since the absorbance is proportional to the number of viable cells in the medium, the viable cell number was determined by using a previously prepared calibration curve (Dojindo Co.).

Apoptosis Assay

Cell death was also examined by using the propidium iodide and Hoechst 33342 double-staining method. The cells were stained with propidium iodide/Hoechst 33342 (1:1) for 5 min and then examined with fluorescence microscopy.

Plasmid Transfection

Transferrin receptor plasmid pAcGP67A-TfR was purchased from addgene. Cell transfection was carried out with Lipofectamine. Briefly, 1.5×10^5 cells were seeded in a 24-well plate with fresh media at 37 °C and 5% CO₂ for overnight until the cells reach 40–80% confluent. The mixtures of 0.8 or 1.6 µg of plasmid DNA dissolved in TE buffer with 50 µL cell growth medium containing no serum or antibiotics and 5 µL of Lipofectamine were incubated for 10 min at room temperature. The mixtures were then added to cells. The cells were incubated with the complexes at 37 °C and 5% CO₂ for 24 h to allow for gene expression. The cells were then harvested for gene expression assay or further cell experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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60. With this system it is interesting to note that there was a 10-fold increase in particle uptake by MDA-MB 435 as compared to MCF-7 cancer cells, but only a 20% increase in cell killing over the passively uptaken phosphonated nanoparticles. This difference may be because of the difference in experimental design, as particle uptake does not always directly correlate to its observed cytotoxic effect.

Figure 1.
TEM images of Tf-FMSNs. A) TEM images of the FMSNs before (1) and after (2) surface modification with Tf.

Figure 2.

a) Coomassie Blue analysis of particles after Tf modification to monitor protein attachment. UV-vis spectroscopy shows an increase in absorbance at 595 nm for Tf-FMSNs, indicating the presence of protein. This spectroscopic difference is apparent in the particle color, as the particles with the protein are stained blue while the particles without the protein maintain the standard green dye color. b) Analysis of targeting of the RGD modification. Since the smaller peptide is more expensive and harder to detect, particle uptake using flow cytometry is utilized to confirm the presence and activity of the cyclic RGD.

Figure 3.

a) Western blots showing expression of the different cell surface receptors for different cells being investigated. b) Enhancement of cellular selectivity and particle uptake when RGD is attached (red trace) versus particles without the targeting agent (green trace) as shown using flow cytometry. c) Fluorescence microscopy images of cells exposed to either Tf-modified FMSNs (upper) or RGD-modified FMSNs (lower), showing the correlation between cell surface receptor expression (a) and particle uptake (green/yellow fluorescence).

Figure 4.

Effect of enhanced cellular expression of cell-surface receptors on the uptake of Tf-FMSNs. a) Western blot analysis of expression of TfR on HFF cells transfected with TfR plasmid. Cells transfected with Lipofectamine, but not containing TfR, is indicated as the control ('HFF'). Different ratios of Lipofectamine to plasmid DNA were tested. b) The effect of uptake of FMSNs in the HFF cells (green fluorescence) transfected with TfR plasmid. Cell plasma membranes were stained with WGA (red fluorescence). c) The same experiments were conducted with breast cancer cell BT-549. Uptake of Tf-FMSNs in BT-549 was increased (concentration per cell) by transfection with TfR plasmid. Blue fluorescence shows the nucleus (stained with 4',6-diamidino-2-phenylindole (DAPI)).

Figure 5.

Cell proliferation assays were carried out with PANC-1 and BT-549 (fluorescence microscopy images) to observe the effectiveness of the Tf nanoparticle drug delivery enhancement. a) PANC-1 cells were treated for 48 h with FMSNs (Tf- CPT-), Camptothecin-loaded FMSNs (Tf- CPT+), nanoparticles modified with transferrin (Tf+ CPT-), or Camptothecin-loaded nanoparticles modified with transferrin (Tf+ CPT+). The enhanced uptake of Tf-FMSNs by PANC-1 cells led to an increase in the cytotoxicity. b) BT-549 cells were treated for 48 h with $10 \mu\text{g mL}^{-1}$ of the same particle types as described above. The enhanced uptake of Tf-FMSNs by BT-549 cells led to an increase in the cytotoxicity. c) Enhanced expression of TfR on BT-549 led to more apoptosis in these cancer cells. Nuclear fragmentation and chromatin condensation were observed in most of the cells treated with CPT-loaded FMSNs and similarly in the cells treated with CPT-loaded Tf-FMSNs. However, a large increase in cell death for cells overexpressing TfR treated with CPT-loaded Tf-FMSNs was observed, shown by a lower number of surviving cells and more nuclear fragmentations.

Figure 6. MDA-MB 435 cell proliferation assays were carried out to observe the effectiveness of RGD-based nanoparticle drug delivery efficacy. RGD-FMSN loaded with CPT are more effective than the free drug.

Scheme 1.

Methods for particle drug loading and attachment of the protein or peptide to the particles (see Experimental Section for details). In part (a) a general overview for each major step in the synthetic scheme is displayed. Specifically, to attach the protein transferrin (b), the mesoporous silica particle is first modified with 3-GPTMS, loaded with CPT in dimethylsulfoxide (DMSO) and then reacted with the Tf to provide the particle–cell signaling and uptake enhancement. To attach the RGD cyclic peptide (c), the surface was thiol-modified with 3-mercaptopropyltrimethoxysilane, reacted with 2,2'-dithiopyridine (2,2'-DTP), CPT loaded in DMF and then allowed to react with the peptide to bind it to the particle covalently. PBS refers to phosphate buffered saline.