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Study of Mesoporous Silica Nanoparticles' (MSNs) intracellular trafficking and their application as drug delivery vehicles

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Author
Yanes, Rolando Eduardo

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Study of Mesoporous Silica Nanoparticles' (MSNs) intracellular trafficking and their application as drug delivery vehicles

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Microbiology, Immunology, and Molecular Genetics

by

Rolando Eduardo Yanes

2013
ABSTRACT OF THE DISSERTATION

Study of Mesoporous Silica Nanoparticles’ (MSNs) intracellular trafficking and their application as drug delivery vehicles

by

Rolando Eduardo Yanes

Doctor of Philosophy in Microbiology, Immunology, and Molecular Genetics

University of California, Los Angeles, 2013

Professor Fuyuhiko Tamanoi, Chair

Mesoporous silica nanoparticles (MSNs) are attractive drug delivery vehicle candidates due to their biocompatibility, stability, high surface area and efficient cellular uptake. In this dissertation, I discuss three aspects of MSNs' cellular behavior. First, MSNs are targeted to primary and metastatic cancer cell lines, then their exocytosis from cancer cells is studied, and finally they are used to recover intracellular proteins. Targeting of MSNs to primary cancer cells is achieved by conjugating transferrin on the surface of the mesoporous framework, which resulted in enhancement of nanoparticle uptake and drug delivery efficacy in cells that overexpress the transferrin receptor. Similarly, RGD peptides are used to target metastatic cancer cell lines that over-express integrin αvβ3. A circular RGD peptide is bound to the surface of MSNs and the endocytosis and cell killing efficacy of camptothecin loaded nanoparticles is significantly improved in cells that express the target receptor. Besides targeting, I studied the
ultimate fate of phosphonate coated mesoporous silica nanoparticles inside cells. I discovered that the nanoparticles are exocytosed from cells through lysosomal exocytosis. The nanoparticles are exocytosed in intact form and the time that they remain inside the cells is affected by the surface properties of the nanoparticles and the type of cells. Cells that have a high rate of lysosomal exocytosis excrete the nanoparticles rapidly, which makes them more resistant to drug loaded nanoparticles because the amount of drug that is released inside the cell is limited. When the exocytosis of MSNs is inhibited, the cell killing efficacy of nanoparticles loaded with camptothecin is enhanced. The discovery that MSNs are exocytosed by cells led to a study to determine if proteins could be recovered from the exocytosed nanoparticles. The procedure to isolate exocytosed zinc-doped iron core MSNs and identify the proteins bound to them was developed. This serves as a foundation to use MSNs as protein harvesting tools and investigate protein expression in cancer cells.
The dissertation of Rolando Eduardo Yanes is approved

Jeffrey I. Zink

Sherie L. Morrison

Wenyuan Shi

Fuyuhiko Tamanoi, Committee Chair

University of California, Los Angeles

2013
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VITA

2006  B.S., Microbiology, Immunology and Molecular Genetics
      University of California, Los Angeles
      Los Angeles, California

2007  NSF Alliance for Graduate Education and the Professoriate Grant

2007  Eugene Cota-Robles Award
      University of California, Los Angeles

2008, 2009  Teaching Assistant (MIMG 185A)
             Department of Microbiology, Immunology and Molecular
             Genetics

2008-2010  NIH Biotechnology Training in Biomedical Sciences and
           Engineering Program

PUBLICATIONS

Tamanoi F. Involvement of Lysosomal Exocytosis in the Excretion of Mesoporous Silica
Nanoparticles and Enhancement of Drug Delivery Effect by Exocytosis Inhibition. Small 9(5),

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Synthesis of Biomolecule-Modified Mesoporous Silica Nanoparticles for Targeted Hydrophobic

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PRESENTATIONS


2009  **Yanes RE**, Ferris DP, Zink J I, Tamanoi F. *Targeting Mesoporous Silica Nanoparticles (MSNs) to Metastatic Cancer Cells*. Biomedical Training in Biomedical Sciences and Engineering 3rd Annual Symposium. UCLA, Los Angeles, California. (Poster)
Chapter I

Introduction
Preface

Cancer is one of the most prevalent diseases in the world and is projected to become the major cause of death in the next decade. This increase in cancer occurrences, along with the limitations of current cancer therapy, has led to the development of new technologies aimed to improve the survival outcome. Current cancer therapy consists of surgery, chemotherapy, and radiation. Surgery is the most invasive method to remove the tumor, but in most cases complete removal of cancer cells cannot be achieved resulting in cancer recurrence. Furthermore, in some types of cancers surgery presents a high risk, particularly when the tumor is present in vital organs. Surgery is most efficient at early stages of the disease before metastasis has taken place.

In instances when surgery is not possible or not sufficient to completely eradicate the tumor, chemotherapy and radiation are utilized to kill cancer cells. Chemotherapy consists in the use of compounds that kill rapidly dividing cells. These compounds are usually administered intravenously, resulting in broad distribution throughout the body. This broad biodistribution leads to side effects that result in damage to normal tissue. In order to reduce side effects, the development of drug delivery vehicles has received much attention because they prevent the drug from eliciting its effect in the bloodstream and only when the drug is released, preferably inside the cells, does the drug become active. Advances in the nanotechnology field have led to the engineering of nanoparticles that can be used to deliver cargo to specific tissues. There is a broad variety of nanoparticles that can be used for cargo delivery. Nanoparticles composed of polymers, lipids, gold, nanodiamond, silicon and mesoporous silica, among others, have been used for cancer therapy and imaging.
Nanoparticles

Nanoparticles are molecules with a size in the nanometer range that can encapsulate a guest molecule. Nanoparticles can be divided into two categories that are composed of organic or inorganic materials. Organic nanoparticles can be composed of lipids, polymers, or proteins and due to their organic nature can be broken down inside the cell or organism with very low toxicity. One of the drawbacks of organic nanoparticles is their relative instability and low circulation time. Many changes in nanoparticle formulations have improved these aspects of the nanoparticles leading to their clinical applications for drug delivery. Liposomes are composed of lipid bilayers encapsulating an aqueous core where the cargo can be enclosed. When creating liposomal formulations for therapeutic delivery the physical and biochemical properties of the lipids, such as, the stability, size, charge, and hydrophobicity must be taken into account to achieve the desired delivery efficacy.\textsuperscript{[1-5]} A number of liposomes have been developed that are capable of delivering small compounds or nucleic acids to cells.\textsuperscript{[6]} Different ligands or antibodies have been attached to the surface of liposomes to target them to specific cells with relative success.\textsuperscript{[7,8]}

Another type of organic nanoparticles are polymeric micelles which have been shown to be effective drug delivery and nucleic acid vehicles. There are a number of polymers that can be used to synthesize nanoparticles and these include poly(lactic-co-glycolic acid) (PLGA),\textsuperscript{[9]} poly(D,L-lactic acid) (PLA),\textsuperscript{[10]} chitosan,\textsuperscript{[11]} gelatin,\textsuperscript{[12]} and poly-\varepsilon-caprolactone (PCL)\textsuperscript{[13]} among others. Polymer micelles are composed of amphiphilic co-polymers that can self-assemble in aqueous environments and range in size from 10 to 100 nm in diameter.\textsuperscript{[14]} Genexol PM, a paclitaxel-containing PEG-PLA micelle, has shown promising results in a phase II clinical trial.
on 41 patients with metastatic breast cancer.\textsuperscript{[15]} The patients had an overall response rate of 58.5\% with a treatment of 300 mg/m\(^2\) every 3 weeks. To date one of the most successful polymer micelles for siRNA delivery is CALAA-01 which consist of 10,000 cyclodextrin polymer (CDP) molecules, 2000 siRNA molecules, 4000 adamantane-polyethylene glycol (AD-PEG) molecules and 100 adamantane-polyethylene glycol-transferrin (AD-PEG-Tf).\textsuperscript{[16]} These nanoparticles are 70 nm in diameter and have been used to deliver siRNA against ribonucleotide reductase subunit 2 (RRM2) with successful results. A phase I clinical trial was performed by administering CALAA-01 containing RMM2 siRNA to human melanoma patients resulting in reduction of both RMM2 mRNA and RMM2 protein levels in tumor tissue.\textsuperscript{[17]} These were the first micellar nanoparticles to show siRNA delivery in humans and mechanistic evidence of RNAi in humans.

There are many materials from which inorganic nanoparticles can be synthesized and these include gold, nanodiamond, silicon, and mesoporous silica among others. For the majority of inorganic nanoparticles the cargo must be covalently attached to the outer surface of the particle. Gold nanoparticles have the capability of generating heat through the plasmon resonance effect, which can result in cell death. By conjugating antibodies that are specific to receptors over-expressed on cancer cells to the surface of gold nanoparticles, preferential killing of cancer cells has been achieved by the plasmon resonance effect.\textsuperscript{[18-20]} Nanodiamond (ND) particles have also shown potential application as delivery vehicles. ND particles are smaller than 10 nm and can be generated using the detonation technique.\textsuperscript{[21]} This material is chemically inert and has displayed low toxicity in several cell lines.\textsuperscript{[22]} They have been shown to be efficient carriers of doxorubicin and facilitate cellular uptake of the drug.\textsuperscript{[23]} Porous silicon nanoparticles are one of the few inorganic particles that are biodegradable and their luminescent
properties allows them to be used for imaging. They have been shown to be able to deliver
doxorubicin to cells in vitro and animal studies have demonstrated no significant toxicity and
biodegradation. In this dissertation, Mesoporous Silica Nanoparticles (MSNs) were studied to
assess their efficacy as drug delivery vehicles to primary and metastatic cancer cell lines and to
learn about their interaction and behavior inside the cells.

Mesoporous Silica Nanoparticles (MSNs)

Procedures describing the synthesis of mesoporous silica were generated and patented in
the late 1960s but it wasn't until the 1990s that researchers in Japan and later at Mobil
Corporation laboratories were able to synthesize particles with this material. This material
received the name of Mobil Crystalline Material (MCM-41). MCM-41 is a solid material that
has a porous structure and cargo can be stored within the pores. The biomedical application of
this material is usually in the form of nanoparticles for drug delivery or imaging. Mesoporous
silica nanoparticles (MSNs) can be synthesized in different sizes and shapes. This size tunability
makes them good candidates for a variety of applications since the size and shape can affect their
function and biodistribution. For example, Mesoporous silica micro-meter sized spheres were
first synthesized by Unger, Stucky and Zhao and their goal was to use them for
chromatographical applications. Years later the synthesis of nanometer sized mesoporous
silica nanoparticles was developed which prompted their biomedical application.

MSNs are synthesized using the sol-gel method. For the synthesis of MSNs, TEOS is
used as the silica source, cetyltrimethylammonium bromide (CTAB) is the cationic surfactant
used as the structure directing agent, water is the solvent, and sodium hydroxide is used as a
morphological catalyst. The synthesis consists of the surfactant (CTAB) forming micelles upon
which the silica condensates to form the nanoparticle structure. After the nanoparticle has formed, the surfactant is removed by solvent extraction (HCl in methanol) to generate the pores. Fluorophores, such as, FITC can be covalently incorporated into the structure of the nanoparticles to give them fluorescent properties and allow them to be tracked. By adjusting the concentrations of TEOS or surfactant, or by adjusting the synthesis conditions, nanoparticles with different characteristics can be produced. For example the nanoparticle size can be tuned from 50 to 300 nm and the pore size can be adjusted from 2 to 6 nm in diameter.\textsuperscript{31} Previous studies have shown that for drug delivery to cells the cellular uptake of the nanoparticles depends on their size and shape as nanoparticles greater than 150 nm are not endocytosed as efficiently as particles with a diameter of 110 nm or lower.\textsuperscript{32} In our studies we use nanoparticles that have a diameter between 100-150 nm and have pores that are 2 nm in diameter. MSNs have a very rigid and stable framework that is relatively resistant to heat, pH, and mechanical stress. The high surface area and large pore volume allows high loading of drug molecules. The fact that there are two surface areas, the outer surface area of the particle and the inner surface area of the pore, allows for differential surface modifications that allow for the attachment of targeting moieties on the outside and the attachment of controlled drug release molecules on the pore surface. This results in multifunctional nanoparticles displaying the capacity of targeting cells that express a specific receptor and of on-command drug release.

During the early stage of MSNs application in the biological field, the aggregation of nanoparticles was a very serious issue that reduced the efficacy of nanoparticle uptake by the cells. The aggregation of the particles was caused by hydrogen bonding between the surface silanol groups and the amine groups resulting in the formation of large silica bodies that would not be taken up by cells. In order to overcome this problem, researchers began modifying the
outer surface of the particles by attaching charged groups. One of these modifications was the attachment of trihydroxysilylpoly methylphosphonate (THMP, referred as phosphonate in this dissertation), which has a net negative charge and increased the electrostatic repulsion between the nanoparticles (Scheme 1.1). This modification reduced nanoparticle aggregation and improved nanoparticle uptake by cells.

**MSNs as drug delivery vehicles**

Our laboratory was one of the first ones to demonstrate that hydrophobic drugs can be packaged into MSNs and delivered to cells. In our studies, nanoparticle endocytosis was demonstrated by fluorescent microscopy and staining of the lysosomes demonstrated that the majority of the nanoparticles co-localized with these organelles. Loading the hydrophobic drug Camptothecin (CPT) into the pores of the nanoparticles is achieved by passive diffusion. Camptothecin is a topoisomerase inhibitor that is very effective at inducing cancer cell death, but its hydrophobic nature prevents it from being effective in biological conditions. When MSNs and CPT are mixed in an aqueous environment, CPT goes into the pores of the nanoparticles. Under UV light, CPT has blue fluorescence which allows for the measurement of the amount of drug that has been loaded into the nanoparticles through UV-vis and for the co-localization of the drug and the FITC labeled nanoparticles under the fluorescent microscope. When the CPT loaded MSNs are incubated with cells for 24 hrs, cell killing can be achieved. The cell killing is more efficient with CPT loaded MSNs than by free CPT. This suggests that the drug is able to exit the nanoparticles once inside the cell and go to the nucleus where it can inhibit topoisomerase activity. Other studies delivering doxorubicin and paclitaxel have been done demonstrating that these drugs can also be delivered to cells by MSNs. Furthermore all of these
studies have shown that in cell culture, the empty MSNs do not have any significant cytotoxicity and cell killing is only observed when the nanoparticles are loaded with a drug.

**Nanomachine-equipped MSNs**

The high stability of Mesoporous Silica Nanoparticles allows for the attachment of movable molecules that can respond to specific stimuli, resulting in the development of nanomachines that could revolutionize drug therapy. The discovery that pseudorotaxanes can be conjugated to a silica solid support, created the foundation to the development of nanomachines. Since then, more sophisticated machinery has been attached to MSNs that has allowed for the controlled release of cargo upon chemical or biological triggers. This has led to development of nanovalves, nanopinstons, snap-tops and nanoimpellers which can respond to pH, enzyme activation, or light activation.\[34\]

Nanovalves that are responsive to low pH represent a type of nanomachine that responds to biological triggers and could lead to the efficient delivery of hydrophilic drugs with minimal leakage outside the cell. Nanovalves consist of a gatekeeper that is located close to the pore opening of the nanoparticles obstructing the exit of the cargo, upon the correct stimuli, the gatekeeper either moves away from the pore opening or completely dissociates from the nanoparticle allowing for the release of the cargo. In the case of pH nanovalves, the activating trigger are protons and in a low pH environment, as is the lysosomal environment, the valve opens and the guest molecule exits the nanoparticle. This design prevents the release of the drug in the extracellular environment or when the nanoparticle is in the circulation; only when the nanoparticle has been endocytosed will the cargo be released.
There are different pH nanovalve designs that have demonstrated proper function. For example, nanovalves consisting of trisammonium stalks threaded on a curcubit[6]uril (CB[6]) ring\textsuperscript{[35]} remain closed at neutral pH but open at low pH. This nanovalve is very attractive because the pH at which the nanovalve opens can be adjusted by changing the pKa of the anilinium nitrogen that is present in the nanovalve stalk. Another nanovalve design in which the gatekeeping molecule completely dissociates from the stalk was synthesized using β-cyclodextrin (β-CD) as the cap and N-menthylbenzimidazole (MBI) as the stalk.\textsuperscript{[36]} This valve was designed so that the β-CD will interact with the stalk at pH 7.4, but will dissociate from it at pH 6 or lower. This mechanized MSN was shown to efficiently encapsulate and release Hoechst dye in a pH dependent manner. There are other pH responsive structures called nanopistons that are composed of β-CD rings attached to the orifices of the pores and rhodamine B/benzidine stalks that can move in and out of the ring in response to pH changes.\textsuperscript{[37]} These nanomachine equipped nanoparticles can deliver both small and large molecules and the release of each can be adjusted for different pH, which could be important for dual drug therapy. The small molecules can go through the β-CD ring when the stalk moves away from the pore and the large molecules can exit the nanoparticle when the ring dissociates from the nanoparticle at a specific pH.

Enzyme activated snap-top nanomachines are other types of structures that can achieve controlled drug release in specific environments. One type of snap-top system consists of [2]rotaxanes composed of tri(ethylene glycol) chains threaded by α-CD tori that are held in place by cleavable stoppers.\textsuperscript{[38]} The stoppers are tethered to the surface of the nanoparticles and are made of ester-linked adamantyl. In the presence of esterase enzymes, which are present inside cells, the ester link is cleaved and the guest molecule can diffuse out of the nanoparticle. A similar approach was used for the development of a redox sensitive [2]rotaxane machine\textsuperscript{[39]} in
which the disulfide bonds in the [2]rotaxanes can be reduced, leading to the breakdown of the stalk and release of the gatekeeping molecule. The use of disulfide bonds is important because they are stable outside the cells, but once reaching the intracellular environment they can be reduced by glutathione. This allows the nanomachine to operate autonomously utilizing the biochemical processes already taking place within the cell to induce drug release.

Controlled drug release inside cells is an important contribution to drug therapy but it is not limited to activation by intracellular triggers, on-demand cargo release by external stimuli has been accomplished by light or magnetic activation. A nanoimpeller consisting of azobenzene derivatives conjugated to the inner surface of the nanopore has been developed. The azobenzene prevents the diffusion of the cargo out of the nanoparticle, but upon photoexcitation, the azobenzene fluctuates between cis and trans conformations causing the cargo molecules to exit the nanoparticles. This nanomachine has been shown to release dyes and anticancer drugs in a time and light intensity dependent manner. A magnetically activated nanomachine was developed by combining mesoporous silica nanoparticles with zinc-doped iron oxide nanocrystals (ZnNCs). The nanovalve in this nanoparticles is thermally responsive and upon application of an alternating magnetic field to the nanoparticles the nanocrystals generate local internal heating resulting in the disassembly of the molecular machines.

**Biocompatibility of MSNs**

Biocompatibility of drug delivery vehicles is required for their application in clinical studies. The nanoparticles must have no cytotoxicity at concentrations that provide effective drug delivery results. Studies have demonstrated that MSNs ranging from 50 nm to 300 nm in diameter display no major cytotoxicity at concentrations below 100 μg/mL,
making them particularly good candidates for drug delivery. It is important to note that the cellular uptake of the nanoparticles depends on their size and shape. Nanoparticles that have a diameter greater than 150 nm are not endocytosed as efficiently as particles with a diameter of 110 nm or lower.\textsuperscript{[47]} The shape of the nanoparticles also plays an important role in the cellular uptake of the material.\textsuperscript{[48]} In our studies we use spherical MSNs that have a diameter between 100-130 nm at concentrations between 5-40 μg/mL which display efficient cellular uptake with no significant cytotoxicity.

The promising results with low cytotoxicity of MSNs in cell culture experiments led to studies in animals to determine their biocompatibility \textit{in vivo}. Early studies investigating the biocompatibility of MSNs resulted in discouraging outcomes considering that work by Hudson \textit{et al} \textsuperscript{[49]} suggested that intraperitoneal or intravenous injections were lethal to mice but subcutaneous injections displayed no toxicity. In this study 1.2g/kg of nanoparticles were injected into mice, which is a very high dose, and large nanoparticles were used. Such a high dose of nanoparticles is not necessary to achieve effective drug delivery to tumors since a large payload of drug can be loaded into MSNs. In our laboratory, the biocompatibility of the nanoparticles was examined by performing a dose escalating study of phosphonate-MSNs (100-130 nm). This study demonstrated that repeated doses of phosphonated MSNs ranging from 3 mg/kg to 50 mg/kg in mice did not result in significant toxicity as determined by body weight, histology, and serological and hematological studies.\textsuperscript{[50]} Furthermore, there were no adverse effects in short-term treatments of 5 injections in 14 days or a long-term treatment of 18 injections in 68 days. As mentioned before, phosphonate MSNs display much better dispersibility than non-surface modified MSNs, so aggregation of the nanoparticles might have contributed to the lethality observed in the study by Hudson and co-workers along with the high
dosage concentration. As long as doses lower than 50 mg/kg phosphonate-MSNs can achieve efficient cargo delivery to the target site, these nanoparticles should be safe to use as delivery vehicles. Just as phosphonate affects the surface properties of the nanoparticles, further studies are needed to determine the effects that different surface modifications have on the biocompatibility of the nanoparticles as these modifications could affect the interaction of the nanoparticles with different organs and tissues.

Dissertation

In this dissertation I describe three projects that expand our understanding of the intracellular trafficking of MSNs, develop novel targeting approaches to primary and metastatic cancer cells, and create the foundation for new applications of MSNs as protein harvesting tools. First, I address targeting of cancer cells by attaching either transferrin or the arginine-glycine-aspartic acid (RGD) peptide to the surface of the mesoporous silica nanoparticles. I study the uptake and drug delivery efficacy of these nanoparticles to a cancer cell lines that over-expresses the target receptors. The results of this project are presented along with the methods used and a discussion of the significance of this approach.

Secondly, I set out to investigate the fate of the nanoparticles after they have been endocytosed by cells by using fluorescently labeled MSNs and monitoring their presence inside cells. While many studies have investigated the application of MSNs as drug delivery vehicles, there are still many basic questions about the interaction of this material with cells that have not been addressed. I discover that MSNs are exocytosed from cells with no apparent degradation. Cellular exocytosis mechanisms are studied to determine their role in the transport of
nanoparticles. I present the results of this project along with its importance in drug delivery efficacy to cancer cells.

Finally I set out to develop a new application for MSNs that takes advantage of their exocytosis from cells. I investigate if the exocytosed MSNs carry proteins out of the cells and we try to identify the proteins that come out with the nanoparticles. I also test MSNs with different surface modifications to determine if the surface properties play a role in the type of proteins that they bind.

**Specific Aims of the Dissertation**

1. Attach transferrin or the RGD peptide to the surface of MSNs and demonstrate that these nanoparticles are preferentially endocytosed by cancer cell lines that over-express transferrin receptor 1 (TfR1) or integrin αvβ3 respectively.

   In Chapter II of this dissertation, the synthesis and attachment of transferrin or the RGD peptide on the surface of MSNs is described. I demonstrate that cancer cells line that over-express TfR1 or integrin αvβ3 endocytose Tf-MSNs or RGD-MSNs more efficiently than a cancer cell lines that do not express the target receptors. Furthermore, the targeted nanoparticles loaded with CPT are more efficient at killing the target cancer cells than non-targeted MSNs loaded with CPT.

2. Investigate the ultimate fate of MSNs after they are endocytosed by cells. Determine if they accumulate or are exocytosed from cells.
In Chapter III, I show conclusive evidence that phosphonated MSNs (P-MSNs) do not accumulate inside of cells but are exocytosed and the rate of MSN exocytosis differs among different cell lines. I present data that shows that the exocytosed MSNs are intact with no apparent degradation.

3. Identify the exocytosis mechanism responsible for the exocytosis of MSNs and determine if the rate of MSN exocytosis has any effect on the drug delivery efficacy of the nanoparticles.

In Chapter III, I demonstrate that the majority of phosphonated MSNs are exocytosed through lysosomal exocytosis. The rate of lysosomal exocytosis determines the MSN drug delivery efficacy in different cell lines, cell lines that exocytose the nanoparticles faster are more resistant the CPT loaded MSNs. By slowing down lysosomal exocytosis a higher level of cell killing is achieved by CPT loaded MSNs.

4. Determine if exocytosed MSNs have proteins attached to their surface and identify those proteins through Mass Spectrometry. Determine if the surface modification of the MSNs plays a role on the type of proteins that are attached to the nanoparticle.

In Chapter IV, I use zinc-doped iron core MSNs and a magnet to isolate exocytosed nanoparticles from cancer cells. Gel electrophoresis and silver staining is performed on the isolated nanoparticles and protein bands are detected suggesting that the nanoparticles are able to carry proteins out of the cell. The amount of nanoparticles that can be isolated using a magnet is
quantified to determine the nanoparticle isolation efficacy. Finally Mass Spectrometry is performed to identify the proteins that are bound to the nanoparticles.
**Figures**

**Figure 1.1** Characterization of MSN. SEM (left) and TEM (right) images of FMSN. Reprinted with permission from Lu *et al.* [51] Copyright (2012) Elsevier.
Scheme 1.1 Phosphonate surface modification of MSNs. A) Unmodified MSNs aggregate due to hydrogen bonding between the silanols and amines on the surface of the particles. B) The attachment of THMP causes electrostatic repulsion between the particle and reduces aggregation.

Chapter II

Targeting MSNs to Cancer Cell Lines Using the RGD Peptide and Transferrin
Introduction

The idea of targeting cancer cells with nanoparticles by attaching different targeting moieties to the particle surface has been tested by various groups.\[^{51-54}\] The goal is to increase the endocytosis of targeted nanoparticles by cancer cells so that a lower dose of the drug would be needed to achieve successful results and reduce the possibility of side effects. The first MSN drug delivery systems were targeted to cancer cells using small nutrient molecules such as mannose or folic acid.\[^{51,53,54}\] Other recent work in nanotechnology has placed a great deal of interest on attaching different biomolecular targeting agents onto various nano-architectures, including polymers, liposomes, viruses and inorganic nanoparticles, to target cancer cells with heavily overexpressed transmembrane receptors.\[^{18-20,55-61}\] The transferrin receptor and integrin $\alpha\nu\beta_3$ are two cell surface molecules that are over-expressed by primary cancer cells and metastatic cancer cells respectively.\[^{62}\] The transferrin receptor binds to transferrin which is an iron binding blood plasma glycoprotein. Integrin $\alpha\nu\beta_3$ binds to components of the extracellular matrix by interacting with the RGD (arginine-glycine-aspartic acid) sequence. By altering the surface modifications of the MSNs and attaching targeting molecules, such as Transferrin or RGD peptides, this drug delivery vehicle can be utilized to target primary cancer cells and metastatic cancers which are very aggressive and have a high mortality rate.

Iron homeostasis is a very important cellular process that regulates the iron concentration inside the cell. Iron serves as a prosthetic group for proteins involved in respiration, DNA synthesis, and oxygen transport.\[^{63}\] Due to its important role in cellular metabolism and biological processes, iron is taken up by cells through the ubiquitously expressed Transferrin receptor 1 (TfR1).\[^{64}\] There is another transferrin receptor (TfR2) that is only expressed by
hepatocytes. TfR1 is a type II receptor that resides on the cell membrane and cycles into acidic endosomes in a clathrin/dynamin dependent manner. After iron is delivered into the cell, TfR1 recycles back to the cell membrane. TfR1 is expressed at low levels in most normal human tissues but is over-expressed in malignant cells and its expression can be correlated with tumor stage and cancer progression. This expression profile has led to the development of targeting agents that are specific for this receptor. The conjugation of active agents (drugs) to TfR1 targeting moieties (Tf, antibodies, peptides) has been used to induce cell death in malignant cells that over-express this receptor. In this chapter, we conjugate Tf to the outer surface of MSNs to improve nanoparticle uptake by cancer cell lines and increase the cytotoxicity of drug loaded Tf-MSNs. We also generate a different targeting approach by attaching the RGD peptide to MSNs which binds to a different receptor, integrin αvβ3, to enhance nanoparticle uptake by metastatic cancer cells.

Metastasis is one of the most difficult obstacles in cancer therapy accounting for 90% of cancer related deaths. Metastasis is the spread of cancer cells from the primary tumor site to distant organs where they form secondary tumors. The dissemination of cancer is particularly lethal when vital organs are invaded by the metastases and the propagation of the disease is impossible to eliminate successfully. The progression of tumors to the metastatic stage is facilitated by a number of cellular events that allow the transition of endothelial cells to the mesenchymal phenotype. During this transition, a number of genes are expressed that change the phenotype and migrating ability of the cells. The first change in cancer cells that allows them to metastasize to other tissues is the loss of cellular adhesion to the primary tumor. This allows them to detach from the original tumor and, by expressing genes that increase their mobility and invasiveness, they are able to move away from the tumor and travel to other tissues. The move
to distant organs is generally aided by the circulatory system, either by entering the blood
circulation or the lymphatic system, metastatic cancer cells are able to reach distant sites. Once
metastatic cells have reached the desired location, they must exit the circulation by expressing
adhesion molecules that allow them to bind to endothelial cells and extravasate into the tissue.
Once out of the circulation, the metastatic cells must migrate deeper into the tissue and colonize
the new site. The journey of metastatic cells requires a number of different cell surface receptors
to be expressed at different times throughout this process. One family of receptors that allow
metastatic cells to migrate away from the primary tumor are integrins. Integrins are a family of
heterodimeric adhesion molecules that bind to the extracellular matrix and immunoglobulin
superfamily molecules. There are at least 24 integrin heterodimers that are formed by the
combination of 18 α subunits and 8 β subunits. They play an important role in cancer
progression and proliferation. One integrin in particular, integrin ανβ3, participates in the arrest
of tumor cells in the blood stream, enhancing their extravasation to target tissues and also in
tumor cell migration by allowing cells to bind and move through the extracellular matrix.
Signaling through integrin ανβ3 activates focal adhesion kinases (FAK) and SRC family
kinases leading to activation of ERK, NFκB, JUN and AKT, resulting in tumor cell
proliferation and survival.

Integrin ανβ3 is vastly up-regulated on metastatic cells, the tumor vasculature and
primary tumor cells with low expression on resting endothelial cells and normal tissue. The
expression of this integrin has been reported in melanoma, breast, prostate, pancreatic, ovarian,
cervical cancers, and glioblastoma. It has been well established that this integrin binds strongly
to the peptide sequence Arginine-Glycine-Aspargic Acid residues of proteins. In addition, the
common morphology of these sequences in biological systems is a cyclic ring structure that fits strongly into the binding site of the integrin.

**Cell Targeting Peptides**

Cell-targeting peptides (CTPs) have emerged as effective tools for targeting cancer cells that express or over express certain receptor proteins that recognize and internalize CTPs by receptor mediated endocytosis. CTPs have been designed to consist of the shortest amino acid sequence while maintaining the highest affinity and specificity to a given receptor. One of the most extensively studied CTP is the RGD tri-peptide (Arg-Gly-Asp) that targets the \( \alpha_v\beta_3 \) integrin receptor. This integrin receptor plays an important role in angiogenesis of solid tumors and in metastatic activity.\[81\] The RGD sequence has been found to have higher affinity and selectivity towards \( \alpha_v\beta_3 \) integrin when the sequence is extended to a penta-peptide and when peptide is in a cyclic form.\[82\]-\[84\] One variant of the RGD motif developed by Kessler,\[85\] cyclo[Arg-Gly-Asp-D-Phe-Val] (c[RGDfV]) has been shown to be one of the most selective and active antagonists to the \( \alpha_v\beta_3 \) integrin receptor.\[86\]-\[88\]

Multivalence is another well accepted approach for increasing peptide ligand-peptide interactions.\[89\]-\[92\] Studies *in vivo* of dendrimers using RGD peptide ligands demonstrate that binding increases in the order from the monomer, to the dimer, and have the greatest effect in the tetramer.\[92\] A similar multivalence effect should be obtained from RGD peptide functionalized surfaces of mesoporous silica nanoparticles.

In this chapter, I describe our approach to drug delivery using two targeting moieties, transferrin and RGD peptides (Scheme 2.1). By taking advantage of the known targeting ability of transferrin and RGD peptides, cargo capacity and multivalence effects associated with
mesoporous silica frameworks we have established a drug delivery system that targets primary cancer cells or metastatic cancer cells. Since the nature of the cargo is independent of the targeting and release methodologies, this system offers the flexibility of being able to easily change both the targeting moiety and the cargo. Thus, with a general targeting drug delivery system, it may be possible to target a wide range of cells using different peptides, proteins, or even nucleic acids.

The work presented in this chapter was published in *Small* (2011) 7(13), pp 1816-26. My contribution to this work consisted in performing the experiments concerning the enhanced nanoparticle uptake and drug delivery efficacy of RDG targeted MSNs. Dr. Jie Lu performed the experiments concerning transferrin targeted MSNs. Dr. Daniel P. Ferris synthesized the nanoparticles used in these studies and Dr. Christopher Gothard synthesized the RGD peptides.

**Results**

**Synthesis of the cyclic RGD peptide**

The RGD targeting peptide was designed to resemble the sequence *cyclo*[Arg-Gly-Asp-D-Phe-Val] (c[RGDfV]), as developed by Kessler because it is one of the most active and selective antagonists to the \( \alpha_\text{v}\beta_3 \) integrin receptor. Further structure–activity studies indicated that exchanging the valine residue to a lysine residue did not significantly reduce selectivity or activity. Unlike valine, the side chain of lysine contains an amino functional group at the \( \varepsilon \) position that can be covalently modified and used as a tether position for the attachment of signaling moieties or others types of cargo. Other amino acids such as glutamic and aspartic acid contain carboxylic acid side chains and have similar advantages. The amino acid cysteine is
unique in that it contains a sulfhydryl (SH) group on its side chain which has the added advantage of orthogonal reactivity with respect to other amino acid side chains and upon oxidation with another sulfhydryl group, a disulfide bond is formed. The reversibility of this bond is an attractive design feature for drug delivery systems.\textsuperscript{[97]}

RGD peptide 1, cyclo(Arg-Gly-Asp-D-Phe-Cys), was synthesized by Fmoc-based solid-phase synthesis of the protected linear peptide, followed by macrocyclization, deprotection, and RP-HPLC purification. The linear peptide was prepared on chlorotrityl resin with 3 equiv of protected amino acid, HCTU coupling reagent, 2,4,6-collidine base, and 2-4 h coupling times. After final Fmoc-deprotection, the protected linear peptide was cleaved from the resin using hexafluoroisopropanol (HFIP) in CH\textsubscript{2}Cl\textsubscript{2} and cyclized at tenth millimolar concentrations with HCTU, 2,4,6-collidine, and a 24 h reaction time.

Analytical HPLC studies established that the purity of the peptide 1 after the cyclization step was generally high and that the unpurified compound is compatible with standard reverse-phase chromatography (RP-HPLC). Following preparative RP-HPLC, peptide 1 is isolated as the trifluoroacetic salt and after lyophilization affords an easy to handle white fluffy solid. A typical synthesis starting with 5 mmol of chlorotrityl resin afforded approximately 100 mg of analytically pure (~97\%) macrocycle.

**Synthesis and Characterization of RGD-FMSN**

To prepare the RGD-FMSNs, the FMSNs synthesis was performed first of all. The surface of the nanoparticle was then modified with 3-merceptopropyltrimethoxysilane 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, non-phosphonated 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. TEM images before cyclic-RGD attachment to the nanoparticles shows that individual particles, ranging in size from 50 to 150 nm in diameter, were produced (Figure 2.1 A). 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 (Figure 2.1 B). 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. (Figure 2.1 C). DLS shows that the nanoparticles in nano-filtered water have an approximate hydrodynamic radius of about 178 nm after extraction (Table 2.1). Drug loading assay of RGD-FMSNs gave a value of 0.4% CPT by weight (Figure 2.1 D).[^98]

### Synthesis and Characterization of Tf-FMSN

For the Tf conjugation, some modifications to the nanoparticle design had to be made to allow for the attachment of a biologically synthesized protein. A phosphonate coating was used during the nanoparticle synthesis and the linker 3-glycidoxypropyltrimethoxysilane (3-GPTMS) was condensed onto the non-phosphonated portions of the nanoparticles (pore openings) in toluene. This epoxide linker was chosen for its strong reactivity with amines.[^99] 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.
The structure of Tf-FMSN was characterized using TEM, SEM, XRD, DLS and IR. TEM images of FMSN before Tf modification show that the nanoparticles produced had a size that ranged from 50 to 150 nm in diameter (Figure 2.2 A). The SEM images indicate that the material is relatively uniform in size and shape (Figure 2.2 B). The pore distribution of the nanoparticles is analyzed by low-angle XRD demonstrating that the nanoparticles after surfactant extraction are mesoporous with a 2θ of 2.16, and a d-spacing of 4.1 nm (Figure 2.2 C). Infrared spectroscopy, after the solvent extraction, verifies that CTAB is removed by showing the absence of the C-H stretches of CTAB at 2900-3000 cm⁻¹ (Figure 2.2 D). DLS shows that particles in nano-filtered water have an approximate hydrodynamic radius of about 190 nm (Table 2.2). After transferrin attachment to produce the Tf-FMSN, characterization was initially performed using TEM (Figure 2.2 A). The Tf modification resulted in nanoparticles that have dark spots, which are likely an effect of the surface modification by Tf. Dispersibility of the nanoparticles was studied using Dynamic Light Scattering (DLS) which resulted in an increase to an average of 230 nm (Table 2.3). To confirm that the transferrin protein was conjugated to the surface of the nanoparticles, a protein staining technique and UV-Vis spectroscopy was carried out (Figure 2.3A). Tf-FMSNs were treated with Coomassie Blue and their absorbance at 595 nm was analyzed (modified Bradford assay). Coomassie Blue stains proteins, so if the transferrin is attached to the nanoparticles the absorbance at 595 nm should increase when compared to the unmodified nanoparticles. The results demonstrate that when the amount of nanoparticles is increased, the absorbance at 595 nm increases proportionally. Nanoparticles without protein do not shift the absorbance maximum to 595 nm from 650 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.
Enhanced RGD-FMSN uptake by cell lines that over-express integrin αvβ3

The breast cancer cell line MCF-7 and the metastatic melanoma cell line MDA-MB 435 were tested for the expression of integrin αvβ3 through western blot analysis. MDA-MB 435 showed high expression of both subunits of the receptor, while MCF-7 had low expression of the αv subunit and no detectable expression of β3 subunit (Figure 2.4 A). It is important to note that the αv subunit can interact with other β subunits to form a different integrin molecule and this could account for the detection of αv in MCF-7 cells but no β3 subunit. Based on these results, these two cell lines were used to compare their cellular uptake of FMSN and RGD-FMSN. The cells were incubated with FMSN or RGD-FMSN at a concentration of 5μg/mL for 24 hours. Fluorescent microscopy analysis demonstrated that both cell lines uptake FMSN and RGD-FMSN, but the uptake of RGD-FMSN is higher in MDA-MB 435 (Figure 2.4 B). To obtain a more 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 αvβ3, take up RGD FMSNs more efficiently than non-targeted FMSNs (Fig. 2.4C). In addition, cells that do not express integrin αvβ3, such as MCF-7, do not exhibit an increase in RGD FMSN uptake. These results suggest that the uptake of RGD FMSN is mediated by integrin αvβ3.

Enhanced Tf-FMSN uptake by cell lines that over-express TfR1

The endocytosis of transferrin modified mesoporous silica nanoparticles was studied in vitro using cancer and normal human cell lines, which have differential expression profiles of the transferrin receptor (TfR1). The expression of TfR1 by the different cell lines was monitored by western blot analysis. The human pancreatic cancer cell line, PANC-1, and the human breast cancer cell line BT-549 have a significantly higher expression of TfR1 than the normal human
foreskin fibroblast cell line HFF (Figure 2.5A). The uptake of the nanoparticles by the cells was studied by fluorescent microscopy, the cellular plasma membrane was stained with WGA-Alexa Fluor 594 to confirm that the nanoparticles were inside the cell. When non-targeted FMSNs (control) 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 2.5B upper panel).[33,52,100] When the Tf-FMSNs were incubated with these cells and compared to non-targeted FMSNs, there was a noticeable difference in cellular uptake and selectivity. The attachment of Tf on the FMSNs increased significantly the nanoparticle uptake by the PANC-1 and BT-549, but not by the HFF (Figure 2.5B-upper). These results corroborated the importance of the over-expression of TfR1 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 mesoporous silica nanoparticles uptake was possible using the specific synthetic assembly strategies.

To confirm that TfR1 mediates the uptake of Tf-FMSNs, the TfR1 gene was transfected into HFF cells with a TfR1 plasmid (pAcGP67A-TfR1, Addgene). Western blot analysis was performed to confirm the enhanced expression of TfR1 in HFF cells after transfection (Figure 2.6A). Next, the uptake of non-targeted FMSNs or Tf-FMSNs in these HFF cells over-expressing TfR was examined. Overexpression of TfR1 in HFF cells did not change the uptake of non-targeted FMSNs (Figure 2.6B) but it did increase the endocytosis of Tf-FMSNs. The fluorescent microscopy results show that Tf-FMSNs accumulated in the transfected HFF much more than in
the untransfected cells. These results indicate that the enhanced uptake of Tf-FMSNs in cells over-expressing TfR1 is mediated by the transferrin receptor.

Interestingly, transfection of the TfR1 into the breast cancer cell line BT549, which already expressed the receptor, augmented the uptake of the Tf-FMSNs. The same plasmid transfection with Lipofectamine that was used in the HFF cells was carried out to enhance expression of TfR in BT-549. The endocytosis of non-targeted FMSNs and Tf-FMSNs, in cells with or without transfection, was examined using fluorescence microscopy. The transfection with TfR did not significantly change the intracellular uptake of non-targeted FMSNs in non-transfected BT-549 cells (Figure 2.6C, based on approximate particle per cell concentrations), but the amount of transferrin modified nanoparticles in BT-549 cells transfected with TfR plasmid was significantly increased. These results further confirmed that the increased uptake of Tf-FMSNs is a result of the increased expression of TfR.

**Enhanced cell killing of MDA-MB 435 by CPT loaded RGD-FMSNs**

The increase in RGD FMSN uptake by MDA-MB 435 cells results in a higher drug delivery efficacy by these nanoparticles than by the non-targeted FMSNs. It has been shown previously that mesoporous silica nanoparticles can deliver hydrophobic drugs into human cancer cells.\(^{31,33,45}\) To confirm that drug delivery was possible with RGD-FMSNs, the cell killing of CPT loaded nanoparticles was measured by cell viability assay. MDA-MB 435 cells were treated with empty FMSNs and empty RGD-FMSNs and no toxicity was detected. This suggests that there is no inherent toxicity by the nanoparticles and the cell killing should be done by the drug. The treatment with free CPT and CPT loaded FMSNs resulted in approximately 15% and 29% of cell killing respectively. When the cells were treated with CPT loaded RGD
FMSNs the cell killing improved to approximately 49%. These results agree with the nanoparticle uptake results in which integrin αvβ3 expressing cells had a higher RGD FMSN endocytosis than non-targeted MSNs.

**Enhanced killing of TfR1 expressing cells by CPT loaded Tf-FMSNs**

The enhancement in cell killing of the Tf-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. To demonstrate that CPT delivery was possible with the Tf-FMSNs, the cytotoxicity of these nanoparticles loaded with Camptothecin (CPT) in both PANC-1(Figure 2.8A) and BT-549 (Figure 2.8B) 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 μg/mL, 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 μg/mL of 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 them than to normal cells.

**Discussion**

In this chapter of the dissertation we demonstrate the generation of two targeted MSNs one with Tf and the other one with the RGD peptide attached to their surface. The conjugation
of Tf onto the surface of MSNs presented a particular challenge because the size of the protein could block the pore openings where the drug would be released. The attachment of Transferrin, a 80 kDa protein with 679 amino acids serves as a proof of principle study that demonstrates that large proteins can successfully be conjugated to the surface of MSNs enhancing their endocytosis by target cells. We also show the preferential nanoparticle uptake by MDA-MB 435 cells which over-express integrin ανβ3. The increase in RGD-FMSN by MDA-MB 435 cells enhances the killing of these cells by CPT loaded RGD-MSNs. Targeting metastatic cancer cells for drug delivery is a very important step since it can reduce the aggressiveness of the tumor and in turn reduce cancer mortality. A successful treatment that could inhibit cancer metastasis would greatly reduce cancer mortality and turn it into a chronic, non-lethal disease.

The results in this chapter demonstrate 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 medication. The stability and large surface area of MSNs allow for a variety of surface modifications, which as demonstrated here can lead to the use of different linkers to conjugate a variety of targeting moieties. This increases the arsenal of weapons that can be used specifically for different types of cancers depending on the receptors the cells express. The possibility of successfully targeting primary and metastatic cancer cells will greatly improve cancer therapy outcome at different stages of the disease.
Materials and Methods

RGD Peptide Synthesis.

A fritted glass reaction vessel was charged with 2-chlorotriyl 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 x 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 x 40 mL) for ca. 1 h. The reaction vessel was then drained, and the resin was washed with DMF (3 x ca. 100 mL), and then with CH₂Cl₂ (3 x 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 x 40 mL) to the resin followed by gentle agitation (N₂). The solution was drained, and the resin was washed with DMF (3 x ca. 100 mL), and then with CH₂Cl₂ (3 x ca. 100 mL).

Elongation of the protected linear peptide was accomplished by pre-activating 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 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 drop-wise over a period of 4 days into a stirring solution of 2% 2,4,6-collidine-CH$_2$Cl$_2$ (500 mL). After washing the reaction mixture with 0.2 N 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-H$_2$O (8/1/1, v/v) for 5 h. TFA was removed under vacuum and the resultant slurry was partitioned between H$_2$O (ca. 100 mL) and Et$_2$O (ca. 100 mL). The organic layer was extracted with Et$_2$O (2×) and the combined aqueous layers were concentrated under vacuum to afford peptide 1 as an off-white solid (0.54 g).

Purification of peptide 1 was accomplished with preparative RP-HPLC (water-MeCN with 0.1% TFA) using an Agilent C$_{18}$ column. The analytical HPLC spectrum of the crude peptide 1 shows two major peaks, one is associated with HCTU and the other was identified as the product (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.

**Production of non-phosphonated 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-Aminopropyltriehtoxysilane (3-APTES, Aldrich, 98%, 12µL) 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, cetyl trimethylammonium bromide (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 (100mL) 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 µL) 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, 25mg) was dissolved in EtOH (7.5 mL) containing acetic acid (Glacial, 100 µL). 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 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 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 µL). 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.

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%, 12µL) 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 H₂O (240 mL). 2 M NaOH (Fisher, 1.75mL) 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. 15 min after mixing the two previous solutions, 3-trihydroxysilylpropylmethyolphosphonate (Gelest, 42% in H₂O, 0.63 mL) was added slowly. 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 them in MeOH (100 mL) and slowly adding 12 M HCl (5mL). 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 (100mg) 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 iPrOH, 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 Me₂SO 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 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 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 HEPES buffer and sonicated in order to remove any adsorbed protein and then suspended in PBS buffer.

**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.

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 αν 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 αν antibody was stripped, washed with TBS-T and incubated with integrinβ3 antibody (cell signaling, Cat# 4702) overnight at 4°C. Incubation with secondary antibody was repeated as well as detection with the ECL system.

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 eight-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. After incubation, the cells were fixed with 70% ethanol for 2 hours at 4°C and the plasma membrane was stained red with WGA for 10 min. A mounting solution containing DAPI was used to stain the nucleus blue. Pictures were taken using a ZEISS Imager Z1 microscope with an Axiocam MRm.

**Flow Cytometry**

Cells were seeded in a six-well plate at a confluency of 1X10^5 cells per well overnight. Cells were incubated with 5ug/mL of FMSN or RGD-FMSN for 24 hours. After incubation, cells were washed with PBS, trypsinized, and washed with 0.05% tryphan blue solution to decrease the background fluorescence. Flow cytometry was performed measuring the green fluorescent 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. Cells were seeded in 96-well plates (5000 cells/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.).
Plasmid Transfection.

Transferrin receptor plasmid pAcGP67A-TfR was purchased from Addgene. Cell transfection was carried out with Lipofectamine. Briefly, 1.5X10^5 cells were seeded in a 24 wells plate with fresh media at 37 °C and 5% CO_2 for overnight until the cells reach 40–80% confluent. The mixtures of 0.8 µg or 1.6 µg of plasmid DNA dissolved in TE buffer with 50 L 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% CO_2 for 24 h to allow for gene expression. The cells were then harvested for gene expression assay or further cell experiments.
Scheme 2.1 Methods for particle drug loading and attachment of the protein or peptide to the particles. 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-glycidoxypropyltrimethoxysilane, loaded with CPT in anhydrous DMF 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’-DTP, CPT loaded in DMF and then allowed to react with the peptide to bind it to the particle covalently.
Figure 2.1 Characterization of RGD-FMSNs. A) TEM of unphosphonated particles before and after modification with RGD. Because of the smaller size of the peptide the two TEMs look fairly similar. B) XRD pattern after the extraction of the CTAB template from the unphosphonated particle structure using acidic methanol solution. C) The IR spectrum of FITC unphosphonated with CTAB template extracted from the pores of the particle. The absence of the C-H peak at ~2900 cm\(^{-1}\) indicates the removal of the surfactant.
Figure 2.2 Characterization of Tf modified MSNs. A) TEM images of the FMSNs before (1) and after (2) surface modification with Tf. B) SEM images of FITC-modified mesoporous silica nanoparticles. The consistent particle size of about 100 – 150 nm shows that the bulk of the synthesized particles used in this study are relatively monodispersed. C) XRD pattern after the extraction of the CTAB template from the particle structure using acidic methanol solution. Retention of the XRD pattern indicates that the bulk material is still mesoporous and capable of containing cargo within the template. D) The IR spectrum of FITC phosphonate with CTAB template extracted from the pores of the particle. The absence of the C-H peak at ~2900 cm\(^{-1}\) indicates the removal of the surfactant.
Figure 2.3 Coomassie Blue analysis to confirm Tf conjugation onto MSNs. UV-Vis spectroscopy shows an increase in absorbance at 595 nm for Tf-FMSNs, indicating the presence of protein.
Figure 2.4 Integrin receptor expression, RGD-FMSN fluorescent microscopy and flow cytometry of particle uptake into cancer cells. In A) the expression of integrin from gel
chromatograph is displayed showing that MDA-MB 435 over-expresses the integrin when compared to MCF-7. Confocal images in B) show the uptake of RGD-FMSNs as compared to non-targeted FMSN in MCF 7 and MDA-MB 435 cells. When RGD particles are compared with untargeted FMSN in C), the uptake of RGD-FMSN is approximately 7 fold greater than the unmodified particles.
**Figure 2.5** Enhanced Tf-FMSN uptake by cells expressing the transferrin receptor.  A) Western blots showing expression of the Transferrin receptor for the different cell lines being investigated. B) Fluorescence microscopy images of cells exposed to Tf–modified FMSNs (upper), showing the correlation between cell surface receptor expression (A) and particle uptake (green fluorescence).
Figure 2.6. Effect of enhanced cellular expression of transferrin receptor 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 2.7 Enhanced cell killing by RGD-FMSNs loaded with drug. 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 shown to be more effective than the free drug, but more notably, are more effective at delivering the drugs into the MDA-MB 435 cells than our past particle designs with phosphonated coating by 20%.
Figure 2.8 Enhanced cell killing by Tf-FMSNs loaded with drug. Cell proliferation assays were carried out with (a) PANC-1 and (b) BT-549 (fluorescent microscope 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 µg/ml of 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 BT-549 cells led to an increase in the cytotoxicity.
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Table 2.1 Dynamic light scattering data of the CTAB-templated, extracted unphosphonated nanoparticles after RGD modification is complete (no CPT). Hydrodynamic radius of the RGD modified mesoporous silica nanoparticles was determined using a DLS. The mean particle diameter was determined to be 178.0 nm with a standard deviation of 36.54 nm.

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Table 2.2 Dynamic light scattering data of the CTAB-templated, extracted phosphonated nanoparticles. The mean particle diameter was determined to be 190.0 nm with a standard deviation of 35.41 nm.
Table 2.3 Dynamic light scattering data of the CTAB-templated, extracted phosphonated nanoparticles with Tf covalently bound (no CPT). The mean particle diameter was determined to be 233.5 nm with a standard deviation of 39.71 nm.

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Chapter III

Involvement of Lysosomal Exocytosis in the Excretion of Mesoporous Silica Nanoparticles in Cancer Cell Lines
Introduction

The use of MSNs as delivery vehicles depends on their successful uptake by cancer cells and a number of studies have demonstrated that MSNs are endocytosed by cells and can serve as anti-cancer drug delivery vehicles \[^{52,53,93,101,102}\]. In one of our previous studies we had shown that the uptake of MSNs by cancer cells is mediated by energy-dependent endocytosis.\[^{100}\]

While nanoparticle uptake is very important for drug delivery applications, questions about the ultimate fate of the particles after they enter the cells had not been addressed. The co-localization of MSNs with the endo/lysosome compartments of the cell has been shown by a number of groups, \[^{33,103-106}\] but where the nanoparticles go after this step is not known. Do they go to other organelles? If they do, how are they transported? Do they escape the lysosomes? Do they accumulate inside the cell or do they eventually exit the cell? All of these questions are important in order to understand the interaction of these inorganic nanoparticles with cells and determine the nanoparticle properties that can improve drug delivery or discover novel biological applications for the nanoparticles. The exocytosis of MSNs from cells was recently reported but that study was done with nanoparticles that don't have any surface modifications.\[^{107}\] Various surface modifications have been done to MSNs in order to improve nanoparticle dispersibility and circulation time, to attach different targeting moieties, or to electrostatically attract the cargo onto their surface. Considering that coating the outer surface of MSNs with 3-(trihydroxysilyl)propyl methylphosphonate has been shown to improve nanoparticle dispersibility, and that MSNs used for cancer therapy usually have targeting moieties attached on their surface, exocytosis needs to be studied using surface modified MSNs. In this chapter I investigate the mechanism of phosphonate-MSN (P-MSN) exocytosis and its importance in drug delivery efficacy.
In order for the MSNs to be excreted out of the cell, they must be packaged into vesicles that fuse with the plasma membrane and release their contents out of the cell. There are a number of exocytosis mechanisms used by cells to export proteins and other cellular components and among these are: the classical secretory pathway from the Golgi apparatus, and lysosomal exocytosis (Scheme 3.1). The secretory pathway from the Golgi Apparatus is the primary exocytosis mechanism used by all cells. This pathway starts at the Endoplasmic Reticulum (ER) where newly synthesized proteins are folded and transported to the Golgi apparatus. In the Golgi, proteins are packaged into vesicles that are transported to different organelles or to the cell periphery where they fuse with the plasma membrane. Lysosomal exocytosis is the fusion of the lysosomal membrane with the plasma membrane, resulting in the release of the lysosome components out of the cell. This mechanism has been observed in a variety of cells and is thought to be involved in membrane repair,[108] acquisition of metastatic potential in cancer cells,[109] and resistance to autophagic induced cell death.[110] The fusion of lysosomes with the plasma membrane has been reported in hepatocytes, fibroblasts, epithelial cells,[111] and cancer cells.

In this chapter, I provide convincing evidence that P-MSNs are exocytosed out of cells, that lysosomal exocytosis is important for P-MSN excretion and that the resident time inside the cells of drug loaded P-MSNs affects their drug delivery efficacy. MSNs with different surface modifications display different exocytosis rates from cells resulting in longer retention inside the cells, suggesting that surface properties change the behavior of the nanoparticles inside the cells and could affect their intracellular localization. I further investigated the exocytosis of P-MSNs and found a wide difference in the rate of nanoparticle exocytosis among different human cancer cell lines. Interestingly, this difference correlates with the rate of lysosomal exocytosis,
suggesting that the lysosomal exocytosis plays a major role in the transport of MSNs out of the cell. In support of this idea, inhibition of lysosomal exocytosis but not classical protein secretion pathway from the Golgi apparatus resulted in the decrease of exocytosed P-MSNs. By inhibiting P-MSN exocytosis we achieve higher cell killing with camptothecin loaded MSNs, suggesting that the drug delivery by MSNs is improved. I discover that cell lines that exocytose P-MSNs faster are more resistant to cell killing induced by drug loaded P-MSNs. This is because the resident time of the MSNs inside the cells allows the drug to be released inside the cell and improve the cytotoxicity of the drug. This is important because it could affect the drug delivery efficacy of the nanoparticles to tumors of different origin.

The work presented in this chapter was published in Small (2013) 9(5), pp. 697-704.[112] I performed all of the cell studies, designed the experiments, and analyzed the data. The growth of stem cells was aided by Dr. Shaun S. Sherman. The various nanoparticles used in these studies were synthesized by Derrick Tarn, Angela A. Hwang, Dr. Daniel P. Ferris and Dr. Courtney R. Thomas.

Results:

**MSNs are excreted from cancer cells**

We have previously shown that P-MSNs labeled with FITC are quickly taken up into human cancer cells. The nanoparticles are prepared by sol-gel method and were surface modified by the addition of phosphonate (Figure 3.1A). FITC was included during the synthesis to provide fluorescent feature to enable tracking of MSNs. The particles have an average
diameter of 130 nm and contain more than a thousand pores each having a diameter of 2-3 nm. The fluorescent MSNs were endocytosed by the human lung cancer cell line A549 (Figure 3.1B) and the majority was found to be co-localized with the lysosomes. The green fluorescence of P-MSNs can be detected inside the cells 2 hours after the addition of P-MSNs into the media. The fluorescence largely overlaps with the staining of LAMP-1, suggesting that they are localized in the lysosomes.

When performing the P-MSN endocytosis experiment, it was observed that the fluorescent signal inside the cell decreased upon further incubation (Figure 3.1D). I further investigated this phenomenon by first incubating A549 cells with P-MSNs for 2 hours and then washing the cells with PBS two times before adding fresh medium for further incubation for 6, 24 and 48 hours. The cells were subjected to flow cytometry analysis to quantitate the amount of fluorescence in the cells. The fluorescence inside the cells dramatically increased after incubation with the P-MSNs for 2 hours due to nanoparticle uptake, but this fluorescence significantly decreased after 6 hours and reached almost basal level after 24 and 48 hours suggesting that the particles have exited the cells (Figure 3.1C). There was over 80% decrease in fluorescence inside the cells after 24 hrs and over 90% decrease after 48 hrs. These results were confirmed with ICP-OES, in which the silicon concentration inside the cells was measured after P-MSN endocytosis and compared to the silicon concentration in the cell media after 24 hrs for detection of exocytosed P-MSNs. A total mass of [5 μg] of silicon was endocytosed by 4 x 10^5 cells after 2 hr treatment with P-MSNs, and 24 hrs later [4.7 μg] of silicon were exocytosed and present in the cell media (Fig 3.1E).
MSNs are exocytosed from cells in intact form

Concerns about the degradation of MSNs that could account for the decrease in fluorescence observed inside the cells led me to examined the exocytosis of P-MSNs containing zinc-doped iron oxide cores. The purpose of this experiment was to collect the exocytosed nanoparticles with a Neodymium (NdFeB) magnet and study their structure by TEM. These nanoparticles are prepared by forming a silica shell around and zinc-doped iron oxide nanoparticle core. They have the same size and surface modification (phosphonate attachment) as the P-MSNs used in the previous exocytosis experiments. The magnetic properties of these nanoparticles makes them easy to be manipulated and captured with a magnet. They were endocytosed and were excreted from A549 cells in a manner similar to that seen with P-MSNs without zinc-doped iron oxide core. The zinc-doped iron core MSNs had 90% exocytosis after 24 hours (Fig. 3.2A). The exocytosed zinc-doped iron core P-MSNs were collected from the medium using a NdFeB magnet. TEM analysis of the exocytosed zinc-doped iron oxide core P-MSNs showed an intact spherical structure with organized pores, similar in appearance with the MSNs added to the medium (Figure 3.2B). In the TEM pictures we can observe that not all the nanoparticles added to the cells had a zinc-doped iron oxide core, but the majority of the nanoparticles collected with the magnet had the iron oxide core. This suggests that the isolated nanoparticles were collected due to the magnetic properties of the iron oxide core. These results provide convincing evidence that the complete P-MSNs are excreted from cells and not degraded pieces.
Different rate of P-MSN exocytosis among different cell lines

The first step in the identification of the exocytosis mechanism responsible for P-MSN exocytosis was to determine if the exocytosis rate was different among different cell lines. By identifying cell lines with high and low P-MSN exocytosis we could compare different cellular mechanism among the cell lines and find candidate pathways that could be involved in nanoparticle exocytosis. A number of human cancer cell lines were tested to examine whether the rate of MSN exocytosis differs among them. In addition to the lung cancer cell line A549, we tested breast cancer cell lines MDA-MB231, MCF-7, melanoma cell line MDA-MB435, as well as a pancreatic cancer cell line PANC-1. These cells were incubated with fluorescent P-MSNs for 2 hours to allow for the uptake of nanoparticles by the cells. After the nanoparticle uptake, the cells were washed with PBS to remove remaining P-MSNs. The decrease of fluorescence inside the cells 24 hours after changing to fresh media without P-MSNs was examined by flow cytometry. The results demonstrate that there are variations in the rate of P-MSN exocytosis among different human cancer cell lines. The order of exocytosis efficiency starts with A549 cells having the highest P-MSNs excretion with 87% followed by MDA-MB231 (81%), PANC-1 (75%), MCF-7 (61%) and MDA-MB 435 (36%) (Fig 3.3A).

Besides cancer cell lines, the exocytosis of P-MSNs from the human embryonic stem cell line H9 was also investigated. Previous studies have reported that MSNs can be used to label stem cells\textsuperscript{[113,114]} and that they can be tracked for several days after transplantation into mice.\textsuperscript{[115]} This is consistent with our observation that stem cells have very slow exocytosis. H9 cells were grown in feeder free media and treated with P-MSNs for 2 hours and then the growth media was removed along with nanoparticles not taken up by cells. Fresh growth medium was added to the
cells and incubated for 24 hrs. Flow cytometry analysis demonstrated that the majority of P-MSNs remained inside the cells after 24 hrs (Fig 3.3A).

**Surface modified MSNs are excreted from cells at different rates**

Very few groups continue using non-modified MSNs for drug delivery applications. Nowadays, the majority of MSNs are surface modified to reduce nanoparticle aggregation, improve circulation time, or attach targeting moieties. Since the surface properties of the nanoparticles could affect their behavior inside the cells, I investigated if surface modifications with different moieties (Figure 3.3C) would affect the rate of MSN exocytosis. MSNs modified with either polyethyleneimine (PEI) or folate on their surface were incubated with A549 cells and the amount of nanoparticles that had been exocytosed after 6 hrs was compared to MSNs modified with phosphonate by ICP-OES. PEI gives a net positive charge to the MSNs while folate allows the nanoparticles to bind to the folate receptor, which is over-expressed on cancer cells and enhances cellular uptake. Both PEI and folate coated nanoparticles are exocytosed by cells but at a slower rate than phosphonated MSNs. After 6 hrs of incubation, 84% of the phosphonated MSNs had been exocytosed from the cells, compared to 66% and 49% of the Folate-MSNs and PEI-MSNs respectively (Fig 3.3B). This suggests that different surface modifications could affect the exocytosis rate of MSNs. I decided to further investigate the exocytosis of phosphonated MSNs as these are exocytosed faster than the other surface modified MSNs.

**Disruption of the Golgi apparatus does not affect P-MSN exocytosis**

P-MSNs are too big to cross the plasma membrane. In order to be transported out of the cells they must be packaged into vesicles that fuse with the plasma membrane. The vesicle
factory of cells is the Golgi apparatus. If the nanoparticles follow the same exocytosis pathway as newly synthesized proteins, then disruption of the Golgi apparatus would prevent P-MSNs exocytosis. To test this hypothesis, cells were treated with Exo 1 and Brefeldin A, two compounds known to inhibit protein exocytosis by causing collapse of the Golgi Apparatus.\(^{[116-119]}\) Cells were treated with P-MSNs for two hours to allow cellular uptake of the nanoparticles, then the growth media was changed to fresh media with Exo1 (50 μM) or Brefeldin A (10 μM). Flow cytometry was performed 6 hrs later to monitor if treatment with these compounds had any effect on MSN exocytosis (Fig 3.4A). The results indicate that disruption of the Golgi Apparatus does not have a significant effect on MSN excretion by cells. The cells were able to exocytose MSNs as efficiently as the control cells, even though the Golgi had collapsed by treatment with Brefeldin A and Exo1. Anti-golgin 97 staining and fluorescence microscopy was used to confirm that the Golgi structure is disrupted by treatment with these compounds (Fig 3.4B).

**Correlation of MSN Exocytosis with Lysosomal Exocytosis**

Since the Golgi apparatus does not seem to be involved in P-MSN exocytosis we decided to study lysosomal exocytosis which is the other candidate exocytosis pathway. Lysosomal exocytosis can be detected by measuring the amount of β-hexosaminidase, an enzyme that resides inside lysosomes, released from the cells into the culture media. Enzyme assays for the release of β-hexosaminidase revealed that A549 cells and MDA-MB 231 cells have a relatively high rate of lysosomal exocytosis with 51% and 44% of the enzyme being released after 24 hours of incubation respectively, while the cell line PANC-1 had 25%, and MCF-7, MDA-MB 435, and H9 had less than 10% enzyme secretion (Fig 3.5A). The order of these cells to secrete β-hexosaminidase appears to correlate well with the order of excretion of P-MSNs. The
correlation between β-hexosaminidase release and P-MSN exocytosis was determined by plotting the values for each assay and calculating the correlation coefficient (Figure 3.5B). The correlation coefficient had a value of 0.943 indicating that there is a strong correlation between the ability of cells to carry out lysosomal exocytosis and the rate at which they excrete P-MSNs.

**Disruption of lysosomal exocytosis impedes P-MSN exocytosis**

MSNs are known to reside in the lysosomes and a number of studies have demonstrated that lysosomes are capable of fusing with the plasma membrane releasing their contents outside the cell.\[108-111\] Actin polymerization and microtubule formation are required for transport of the lysosomes to the periphery and fusion with the plasma membrane.\[120-122\] Signaling through the lipid kinase PI3K leads to an increase in cytosolic Ca\(^{2+}\) resulting in the fusion of lysosomes with the plasma membrane.\[^{110}\] In various cell lines, lysosomal exocytosis is a method to reduce the volume of acidic compartments inside the cells and make the cells more resistant to autophagic induced death. Inhibition of lysosome acidification can inhibit lysosomal exocytosis. Taking into account the importance of these cellular events for lysosomal exocytosis, inhibitors for these pathways were used namely: Nocodazole, Cytochalasin D, Bafilomycin A1 and LY294002. Nocodazole inhibits microtubule formation, while Cytochalasin D inhibits actin polymerization, Bafilomycin A1 inhibits the vacuolar H\+(ATPase) in the lysosomal membrane preventing lysosome acidification, and LY294002 inhibits PI3 kinase signaling. It was found that all of these inhibitors slowed down MSN exocytosis with Bafilomycin A having the biggest effect and LY294002 the lowest (Figure 3.6A). To confirm that these inhibitors were in fact blocking lysosomal exocytosis, the secretion of the enzyme β-hexosaminidase, which resides in the lysosomes, was monitored by performing a β-hexosaminidase enzyme assay on the growth
media of cells treated with each inhibitor and comparing it with the amount of enzyme inside the cells. The control cells secreted approximately 23% β-hexosaminidase after 6 hrs incubation whereas the cells treated with LY294002 and Nocodazole secreted approximately 16% and 14% respectively. In cells treated with Cytochalasin D and Bafilomycin A1 the secretion was less than 5% (Figure 3.6B).

Another important characteristic of lysosomal exocytosis is its regulation by calcium. We found that an increase in intracellular calcium enhances MSN exocytosis. The fusion of the lysosomal membrane with the plasma membrane depends on synaptotagmin VII. Synaptotagmin VII is a transmembrane protein that upon binding calcium undergoes a conformational change that allows it to bind to the SNARE complex on the plasma membrane, facilitating membrane fusion. We studied the role of calcium in MSN exocytosis by increasing the intracellular calcium concentration. Cells were treated with ionomycin, an ionophore that transports calcium into the cells. The increase in intracellular calcium accelerated MSN exocytosis (Figure 3.6C) with 80% of the nanoparticles out of the cells after only two hours of incubation. This corresponded to the increase in lysosomal exocytosis caused by the intracellular calcium increase (Figure 3.6D).

**Effect of U18666A on lysosomal and P-MSN exocytosis**

U18666A is a compound that is a class 2 amphiphile that results in accumulation of cholesterol in the lysosomes and disrupts calcium homeostasis in these organelles. We pre-treated A549 cells with 10 μM U18666A for 18 hrs and then added MSNs for two hours. After rinsing the cells with PBS, the cells were incubated for an additional 6 hours. Treatment with U18666A markedly decreased MSN exocytosis in A549 cells in a concentration dependent
manner (Figure 3.7A). The cells treated with this compound also displayed a reduction in lysosomal exocytosis as evidenced by low β-hexosaminidase release (Figure 3.7B). To demonstrate that U1866A disrupted cholesterol trafficking, we stained the cholesterol inside the cells with Filipin and found that treatment with this compound reduced the amount of cholesterol on the cell membrane when compared to the control (Figure 3.7C).

Enhanced cell killing by inhibiting exocytosis of CPT loaded MSNs

The amount of time that the MSNs remain inside the cells could affect the drug delivery efficacy of the nanoparticles, as longer retention time would allow for more drug to be released inside the cells and increase cytotoxicity. We treated A549 cells with camptothecin (CPT) loaded MSNs and Bafilomycin or U18666A for 24 hrs and a cell killing assay was performed. We discovered that by inhibiting MSNs exocytosis with these compounds, the cytotoxicity of the CPT loaded MSNs increased by 10% with Bafilomycin A and by 15% with U18666A (Figure 3.8A). These results suggest that longer retention time inside the cells enhances intracellular drug release leading to more cell killing. To support these results, we studied the cell killing efficacy of CPT loaded P-MSNs in different cell lines. We discovered that the cell lines that had previously been determined to have low P-MSN (MDA MB 231 and MDA MB 435) exocytosis were killed more efficiently than the A549 cell line which has high nanoparticle exocytosis (Figure 3.8B).

Discussion

In this study, I present convincing evidence that MSNs are exocytosed from cells after intracellular uptake. The exocytosis of MSNs is demonstrated by measuring the FITC fluorescence inside the cells through flow cytometry and by fluorescence microscopy. In both
cases there was a significant decrease in fluorescence as early as 6 hours after uptake. After 48 hours, the fluorescence had decreased over 90%. Conclusive evidence for the MSN exocytosis was obtained by using iron oxide core MSNs. These nanoparticles were easily captured by the use of a magnet. TEM analysis showed that exocytosed particles similar in shape and appearance can be recovered from the cell culture media.

The exocytosis of nanoparticles may depend on the surface property of the particles. I compared the exocytosis rates of MSNs coated with phosphonate, PEI and folate. The phosphonated MSNs have a net negative charge that helps decrease the aggregation and improves dispersability in solution.[33] PEI coated MSNs have a net positive charge and have been used for siRNA delivery to cells, while Folate-MSNs have been used for targeted drug delivery to cancer cells, as the folate receptor is over-expressed in these cells.[53] I discovered that PEI-MSNs and Folate-MSNs are also exocytosed by cells but slower than phosphonated-MSNs. After 6 hrs incubation, 84% of the phosphonated-MSNs have been exocytosed, compared to 66% and 49% of the Folate-MSNs and the PEI-MSNs respectively. This suggests that different surface modifications can alter the behavior of MSNs inside the cells.

Different cell lines showed different MSN exocytosis efficiency and this correlates with their efficiency to carry out lysosomal exocytosis. This exocytosis pathway is triggered by an increase in intracellular free calcium and requires signaling through PI3 kinase and transport of the lysosomes to the cell periphery through the actin cytoskeleton and microtubules. It has been suggested that during the acidification process of the endosomes, calcium is pumped out of the late endosome into the cytoplasm as protons are pumped in, to keep a charge balance.[108] This process might contribute to the rise in cytoplasmic free calcium. I showed that calcium plays an
important role in MSN exocytosis by treating the cells with ionomycin and accelerating the rate of MSN exocytosis. This acceleration correlated to the increase in lysosomal exocytosis induced by this calcium ionophore. Inhibition of lysosomal exocytosis by disruption of the actin cytoskeleton, microtubule formation, PI3 kinase signaling and lysosome acidification impeded MSN secretion. In contrast, we found that secretion from the Golgi apparatus does not play a major role in MSNs excretion, as two compounds, Exo 1 and Brefeldin A that disrupt the Golgi Apparatus, did not affect MSN exocytosis.

To understand the trafficking mechanism responsible for MSN exocytosis, I treated cells with U18666A, a type 2 amphipile known to alter different intracellular trafficking pathways. Treatment of cells with U18666A mimics the cellular effects of Niemann-Pick disease type C1 (NPC1), a neurodegenerative disease characterized by excess accumulation of cholesterol in the lysosomes.\textsuperscript{123-125} This compound can alter the calcium concentration in lysosomes\textsuperscript{124} and inhibit the function of NPC1, a transmembrane protein with a cholesterol sensing domain responsible for cholesterol trafficking. U18666A also causes accumulation of tyrosinase in the lysosomes of melanocytes\textsuperscript{126} and inhibition of neurotransmitter release in human neuroblastoma cells.\textsuperscript{125} I found that treatment of cells with this compound decreased the exocytosis of MSNs, suggesting that altering transport from the lysosome to the plasma membrane decreases MNS exocytosis.

While lysosomal exocytosis appears to be a major pathway for MSN exocytosis, there could be other mechanisms. It is important to note that inhibition of lysosomal exocytosis does not completely block MSN excretion. Even strong inhibitors of lysosomal exocytosis, such as, cytochalasin D and Bafilomycin A, cannot completely inhibit MSN exocytosis. This suggests
that a portion of the nanoparticles that are taken up by cells are excreted through different pathways. In fact, detection of a minor population of MSNs that do not co-localize with the lysosomes has been observed by other groups.[105,113,127] This agrees with reports that suggest the escape of MSNs from lysosomes. The fact that some cell lines carry out lysosomal exocytosis slower than others could provide enough time for the nanoparticles to escape the lysosome, but in cell lines with faster lysosomal exocytosis the escape could be minimized. Further studies in the intracellular localization of MSNs are needed to determine what other pathways contribute to the transport of MSNs out of the cell.

MSNs have become great candidates for drug delivery because of their stability and biocompatibility, but the lack of biodegradation presents a challenge for drug release. Drug release from MSNs is based on the diffusion of the cargo from the nanopores to the outside environment and this takes time. If the MSNs are excreted out of the cells before enough drug has been released to induce apoptosis, then the cell killing efficacy is decreased. Furthermore, the fact that some cell lines carry out MSN exocytosis faster than others, suggests that such cells could be more resistant to MSN drug delivery. I demonstrate that by slowing down MSNs exocytosis with Bafilomycin A or U18666A we can enhance cell killing with CPT loaded MSNs. Furthermore, cancer cell lines that normally carry out MSN exocytosis slower, such as, MDA MB 231 and MDA MB 435 are more sensitive to drug loaded MSNs than A549 cells that have a high rate of nanoparticle exocytosis.

A previous study by a different group had investigated MSNs exocytosis with nanoparticles that were not surface modified, but their results were limited to demonstrating that the nanoparticles exit the cells and that they bind proteins.107 The cellular pathway responsible
for MSN exocytosis was not identified and the exocytosis of surface modified MSNs was not studied. Identification of the MSN exocytosis pathway is important in order to understand the intracellular localization of the nanoparticles, which could help develop new applications for the nanoparticles. Moreover, by elucidating the nanoparticle exocytosis pathway, its role in MSN drug delivery efficacy can be evaluated and ways to manipulate it can be developed. The fact that surface modified MSNs were not used in that study is a major limitation considering that most of the MSNs used as delivery vehicles are modified with molecules that improve their dispersibility or that provide targeting capabilities. The work presented in this chapter expands our knowledge of nanoparticle exocytosis by identifying the exocytosis pathway of phosphonated MSNs, determining that MSNs with different surface modifications are exocytosed from cells at different rates, and inhibiting phosphonate MSN exocytosis to improve drug delivery efficacy.

In this chapter, I identify lysosomal exocytosis as the major pathway used by cancer cells to excrete phosphonated MSNs. The excretion of MSNs by cells relies on the ability of the lysosomes to fuse with the plasma membrane and inhibition of this process causes retention of the MSNs inside cells. I demonstrate that PEI coated and Folate conjugated MSNs are also exocytosed from cancer cells but at slower rates than phosphonated MSNs. By inhibiting exocytosis of drug loaded phosphonated MSNs, the cell killing efficacy is enhanced demonstrating that manipulation of this pathway could have an impact in the medical application of these nanoparticles.
Materials and Methods:

Synthesis of Phosphonated, PEI, and Folate-Coated fluorescently-Labeled MSNs.

In a typical synthesis, cetyltrimethylammonium bromide (CTAB, 250 mg, 0.7 mmol) was mixed with NaOH solution (875 μL, 2 M) and H₂O (120 mL). The mixture solution was heated to 80 °C. Fluorescein isothiocyanate (2.7 mg) was dissolved in absolute EtOH (1.5 mL) and mixed with 3-aminopropyltriethoxysilane (APTES, 6 μL) for 2 h. After the temperature had stabilized, tetraethyl orthosilicate (1.2 mL) was mixed with the ethanolic FITC-APTES solution and added to the CTAB solution. For phosphonate-coated nanoparticles, 3-(trihydroxysilylpropyl)methylphosphonate (315 μL) was added to the solution after 15 min. The solution was stirred vigorously at 80 °C for 2 h. The synthesized nanoparticles were collected by centrifugation and washed with MeOH. For PEI coating, as-synthesized nanoparticles (30 mg) were suspended in a 3 mL solution of PEI (weight 0.8 kDa, 2.5 mg/mL) and ethanol. Particles were stirred for 30 minutes and washed three times with ethanol to remove excess PEI. For folate modified MSNs, folic acid (50 mg) was first dissolved in 5 mL DMSO and APTES (250 μL) was added to the solution. Next, N-hydroxysuccinimde (0.15 mg) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.25 mg) were added to the mixture and stirred for 2 hours. The previous solution was then added to a suspension of MSNP (100 mg) and toluene (20 mL), and stirred for 20 h at room temperature. Materials were washed twice with toluene.

Synthesis of Phosphonated, Fluorescent Iron-Core MSNs

As previously reported by C.R. Thomas et al. J. Am. Chem. Soc. 2010,¹²⁸ magnetic nanoparticles with Zn ion doped were synthesized using the method developed by Jang et al.¹²⁹
A typical synthesis to produce Zn0.4Fe2.6O4 nanoparticles is as follows: ZnCl$_2$ (30 mg), FeCl$_2$ (40 mg), and Fe(acac)$_3$ (353 mg) were placed in a 50 mL three-neck round-bottom flask in the presence of surfactants (oleic acid and oleylamine) in octyl ether. The reaction mixture was heated at 300 °C for 1 h and the reaction products were cooled to room temperature. Upon addition of ethanol, a black powder precipitated and was isolated by centrifugation. The isolated nanoparticles were dispersed in toluene. Nanocrystals have 15 nm size with narrow size distribution ($\sigma < 5\%$). Zinc-doped iron oxide nanocrystals were dissolved in chloroform at a concentration of 50 mg/mL. One milliliter of the iron oxide nanocrystals in chloroform was added to a solution of 100 mg cetyl trimethylammonium bromide (CTAB, Aldrich, 95%) in 5 mL of water. The mixture was sonicated and the chloroform was boiled off from the solution with rapid stirring. The aqueous CTAB stabilized zinc-doped iron oxide nanocrystals were added to an 80 °C solution of 43 mL distilled water with 350 µL of 2.0 M NaOH, and 500 µL tetraethyl orthosilicate (TEOS, Aldrich, 98%) was slowly added. After 15 minutes, 125 µL 3-(hydroxysilylpropyl) methylphosphonate was added. Following two hours of rapid stirring at 80 °C, the magnetic-core silica nanoparticles were collected by centrifugation and washed with ethanol and water. The CTAB was removed by dispersing the as-synthesized materials in a solution containing 133.3 mg ammonium nitrate (Fisher) and 50 mL 95% ethanol. This mixture was heated to 60 °C for 15 minutes, then the particles were collected by centrifugation and washed with ethanol. The fluorescent functionality for optical monitoring of the nanoparticles in cells, fluorescein isothiocyanate, was attached to the mesoporous silica framework. 3 mg fluorescein isothiocyanate (FITC, Sigma, 90%) was dissolved in 1 mL ethanol, and 12 µL 3-aminopropyltriethoxysilane (3-APTES, Aldrich, 98%) was added. This solution was reacted under nitrogen for 2 hours, then added to the 80 °C solution of aqueous sodium hydroxide. After
10 minutes, the CTAB-ZnNC solution was added, and the procedure followed in the same manner as above.

**Cell Culture.**

The human cancer cell line A549 was obtained from American Type Culture Collection and was maintained in Dulbecco’s modified Eagle’s medium (DMEM; GibCO) supplemented with 10% fetal bovine serum (Sigma), 1% L-glutamine, 1% penicillin and 1% streptomycin stock solutions. Human cancer cell lines MDA-MB 231 and MDA-MB 435 were a gift from Dr. Neil O’Brien at UCLA and were maintained RPMI-1640 medium (Cellgro) supplemented with 10% fetal bovine serum (Sigma). The media were changed every three days, and the cells were passaged by trypsinization before confluence. The human embryonic stem cell line H9 was cultured as previously described. Briefly, human embryonic stem cells were cultured in DMEM/F-12 medium supplemented with 20% Knock-out serum replacement (Gibco), 1% non-essential amino acids, 0.5% L-glutamine, 1% penicillin-streptomycin, 100 μM β-mercaptoethanol, and 4 ng/ml bFGF. hESC cultures were maintained on feeder layers of inactivated murine embryonic fibroblasts (MEFs). For nanoparticle uptake experiments hESCs were plated on Matrigel (BD Biosciences) and fed with MEF-conditioned medium.

**Iron Core MSN Transmission Electron Microscopy (TEM).**

Cells were seeded in a 6-well plate at a confluency of $4 \times 10^5$ cells per well overnight. The next day, cells were treated with 20 μg/mL P-MSNs for two hours and then the medium was changed with fresh growth medium. After 24 hours incubation, the media from the six wells were collected and mixed in a culture flask. A NdFeB magnet was placed on the wall of the flask to immobilized the nanoparticles, the media was removed, the nanoparticles were washed
with PBS and resuspended in a small volume of PBS. TEM was performed on the isolated nanoparticles.

**Fluorescent Microscopy for Lysosome Staining and Golgi Apparatus Staining.**

The fluorescence of nanoparticles at an excitation wavelength of 488 nm was used to confirm cellular uptake of P-MSNs. The cells were incubated in an eight-well Lab-Tek chamber slide system (Nalge Nunc International) with the nanoparticles and then washed with PBS to remove the nanoparticles that did not enter cells. The cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton X-100 in PBS for 10 min, and blocked with 1% BSA in PBS for 1 hr. Anti-LAMP1 (Abcam ab24170) antibody was added (2 μg/mL) and incubated overnight at 4° C. Staining with secondary antibody tagged with Dylight 594 (Abcam ab96885) was done for 1 hr at room temperature. The cells were then stained with DAPI solution for nuclear staining and pictures were taken using a fluorescent microscope.

For Golgi apparatus staining, cells were seeded in a four-well chamber slide and treated with Exo 1 (50 μM) and Brefeldin A (5 μM) for 6 hours. Cells were prepared similar to the lysosome staining procedure previously described. Anti-golgin 97 antibody (Invitrogen CDF4) was used for staining (5 μg/mL). Staining with secondary antibody tagged with FITC was done for two hours at room temperature.

**MSN Exocytosis Measurement by Flow Cytometry.**

Cells were seeded in a six-well plate at a confluency of 4 x 10^5 cells per well overnight. Cells were incubated with 20 μg/mL of P-MSN (or iron core MSN) for 2 hrs, then washed with PBS and either incubated in growth media for different time points or treated with inhibitors for 6 hrs (LY294002 250 nM, Nocodazole 10 μM, Cytochalasin D 20 μM, Bafilomycin A1 100
nM). After incubation, cells were trypsinized, 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 of the cells, which corresponds to the fluorescence of the P-MSNs.

**ICP-OES Analysis**

A549 cells were seeded in a 6-well plate at a confluency of 4 x 10⁵ cells per well. Cells were treated with 40 μg/mL P-MSNs for two hours and then the medium was removed and the cells washed with PBS two times. Fresh growth medium was added to the cells and incubated for 24 hrs. The cell media were collected, the cells washed with 2 mL of PBS, and the PBS wash was combined with the cell media for ICP-OES analysis. In a typical ICP-OES run, cell culture washes were digested by adding an equal volume of 70% nitric acid (Aldrich), and heated at 80°C for 3 hours. Digested solutions were sonicated for 15 minutes with stirring to ensure sample homogeneity and diluted to a 5% nitric acid concentration. Runs were standardized through serial dilutions of a stock solution of 1000 μg/mL silicon in 5% nitric acid (Fisher). Samples were run on a Thermo Jarrell Ash IRIS 1000 ICP-OES instrument, and silicon concentration determined through monitoring the emission of the 251.611 nm line.

**β-Hexosaminidase Assay.**

Cells were seeded in a twelve-well plate at a confluency of 3 x 10⁵ cells per well overnight. The medium was removed from the wells, the cells were washed with PBS and fresh medium was added to the wells. The cells were incubated for 6 hours with or without inhibitors (LY294002 250 nM, Nocodazole 10 μM, Cytochalasin D 20 μM, Bafilomycin A1 100 nM). The media (supernatant) were collected and the cells were lysed with 0.1% Triton X 100 in DMEM.
The β-hexosaminidase assay was performed in a 96-well plate by mixing 50 μL of 2 mg/mL 4-nitrophenyl N-acetyl-β-D-galactosaminide in 0.1 M citrate buffer (pH 4.5) with 75 μL of supernatant or cell lysate and incubating for 1 hour at 37° C. After the incubation, 100 μL of 0.2 M borate buffer (pH 9.8) was added to the mixture to stop the reaction. The absorbance was read at 405 nm using a plate reader. Percentage values were obtained by dividing the reading from the supernatant with that of the cell lysate.

**Cell Killing Assay.**

Cells were seeded in a 96-well plate at a confluency of 5 x 10³ cells per well overnight. The next day, cells were treated with Bafilomycin A1 (12.5 nM), U1866A (2.5 μM), CPT-loaded P-MSNs, and the combinations of P-MSN-CPT and Bafilomycin A1 or U18666A for 24 hrs. Cells were washed with PBS and incubated in DMEM with 10% WST-8 solution (Dojindo Co) for 3 hrs. The absorbance of each well was measured at 450 nM in a plate reader.
Scheme 3.1 MSN endocytosis and possible mechanisms of exocytosis. Endocytosed P-MSNs mainly co-localize to the lysosome. We examined two exocytosis pathways that could be responsible for MSN exocytosis, excretion through the Golgi apparatus and lysosomal exocytosis.
A) 100 nm

B) MSNs   lysosomes

C) % P-MSN Fluorescence in A549 cells

Key
- Control
- P-MSN 20μg/mL (2hrs)
- P-MSN 20μg/mL (2hrs) + 24hrs incubation

0 hrs 6 hrs 24 hrs 48 hrs
Figure 3.1 Exocytosis of P-MSNs from A549 cells. A) TEM pictures of phosphonate modified MSNs (P-MSNs) used for exocytosis experiments. B) Fluorescent microscopy analysis detecting the P-MSNs fluorescence inside the cells after 2 hrs treatment with the nanoparticles and staining of lysosomes with lyso-Tracker Red. MSNs (green), Lysosomes (red), Nucleus (blue). C) Flow cytometry analysis of A549 cells treated with P-MSNs for 2 hrs, washed with PBS, and incubated for an additional 6, 24, and 48 hrs. Fluorescence is quantitated as shown in the lower panel. D) Fluorescence microscopy demonstrates the uptake of fluorescent P-MSNs after 2 hr nanoparticle treatment but the amount of nanoparticles inside the cells decreases after 24 hr incubation post-treatment. E) ICP-OES analysis of P-MSNs endocytosed by A549 cells after 2 hrs (left) as well as P-MSN exocytosis after 24 hrs (right).
**Figure 3.2** Recovery of exocytosed Zinc-Doped iron core P-MSNs. A) Flow cytometry analysis to measure the endocytosis and exocytosis of zinc-doped fluorescently labeled P-MSNs. B) TEM pictures of Zinc-Doped iron core P-MSNs before being added to cells (left) and collected with a magnet after being exocytosed by cells (right).
Figure 3.3 P-MSN exocytosis differs among cell lines and among surface modified MSNs. A) The exocytosis of nanoparticles in different cell lines is determined through flow cytometry analysis. B) ICP-OES analysis to measure the extent of exocytosis of phosphonated, PEI-coated, and folate modified MSNs. C) Scheme of the molecules attached to the surface of the modified nanoparticles
Figure 3.4 Disruption of the Golgi apparatus does not disrupt MSN exocytosis. A) Measurement of P-MSN exocytosis by flow cytometry of A549 cells treated with Brefeldin A (10 μM) and Exo1 (50 μM). B) Immunofluorescence demonstrates that Brefeldin A and Exo1 disrupt the Golgi apparatus (stained with green anti-golgin 97).
Figure 3.5 Efficiency of cells to excrete P-MSNs correlates well with secretion of β-hexosaminidase. A) Exocytosis of P-MSNs is based on flow cytometry analysis in different cell lines. Release of β-hexosaminidase is a measurement of lysosomal exocytosis. B) Plot of MSN exocytosis vs β-hexosaminidase release and measurement of the correlation coefficient (R).
Figure 3.6 Regulation of lysosomal exocytosis affects P-MSN exocytosis. A) A549 cells were treated with the following inhibitors: Nocodazole (20 μM), Cytochalasin D (20 μM), Bafilomycin A1 (100 nM), and LY294002 (2 μM). These inhibitors impede lysosomal exocytosis as evidenced by β-Hexosaminidase release assay. B) These inhibitors also impede P-MSN exocytosis as demonstrated by flow cytometry analysis. C) Acceleration of lysosomal exocytosis can be achieved by treating cells with ionomycin. D) Acceleration of lysosomal exocytosis results in acceleration of P-MSN exocytosis.
**Figure 3.7** Effect of U18666A on cholesterol trafficking from the lysosomes to the plasma membrane, lysosomal exocytosis and P-MSN exocytosis. A) U18666A inhibits P-MSN exocytosis in a concentration dependent manner. B) Treatment of A549 cells with U18666A results in a decrease in lysosomal exocytosis. C) The effect that U18666A on nanoparticle and lysosomal exocytosis is due to inhibition of cholesterol trafficking from the lysosome to the plasma membrane, staining of cholesterol with filipin demonstrates that the cells treated with the compound have reduced cholesterol on their membrane.
Figure 3.8 Importance of P-MSN exocytosis on drug delivery efficacy. A) Inhibition of exocytosis of drug loaded P-MSNs by bafilomycin or U18666A increases cell killing efficacy in A549 cells. B) Different cell lines are more sensitive to CPT loaded P-MSNs and this sensitivity correlates to the rate of P-MSNs exocytosis. Cells with lower nanoparticle exocytosis (MDA-MB 231 and MDA-MB 45) are more sensitive than cells with high nanoparticle exocytosis (A549).
Chapter IV

Identification of proteins bound to MSNs that are exocytosed by cancer cells
Introduction

The discovery that P-MSNs are exocytosed from cells opens the possibility of developing new applications for the nanoparticles that could take advantage of this property. One potential application is to recover molecules that might have attached to the surface of the nanoparticles. Some groups have reported the binding of a protein corona on the surface of nanoparticles depending on the functional group on the surface of the particles.\[^{131-133}\] In these studies the binding of proteins occurs outside the cell, when the nanoparticle is in solution. Another study showed that unmodified MSNs could be used to harvest proteins from cells. We wanted to determine if surface modified MSNs were able to bind proteins inside the cell and carry them out of the cell, when they are exocytosed. Furthermore, we wanted to discover if the conjugation of different functional groups onto the surface of the particles affects the type of proteins that the nanoparticles bind. By analyzing the proteins that are bound to the nanoparticles, we can determine the intracellular localization of the nanoparticles and identify the trafficking mechanism used to transport MSNs. Furthermore, depending on the proteins recovered, the nanoparticles could be used as a diagnostic tool to learn more about the proteins expressed by different cancer cells. As mentioned in Chapter III, we observed a difference in the rate of nanoparticle exocytosis depending on the surface modification of the particles, this would suggest that MSNs with different surface properties are excreted out of the cell by different exocytosis mechanisms. By comparing the proteins that are bound to nanoparticles with different surface modifications we could obtain clues about the cellular mechanisms that are used to excrete the different nanoparticles out of the cell.
In this chapter we use mesoporous silica nanoparticles that have a zinc-doped iron oxide core (Mag@MSN) for exocytosis experiments. The exocytosed Mag@MSNs are isolated from the cell media with a neodymium magnet. The binding of proteins to the exocytosed nanoparticles is determined by polyacrylamide gel electrophoresis and silver staining. Identification of the proteins bound to the nanoparticles is performed by mass spectrometry. Nanoparticles with two different surface modifications are used, phosphonate modified and PEI coated Mag@MSNs. We analyze the proteins bound to the two types of nanoparticles by Mass Spectrometry to determine if the surface modification affects the type of proteins the nanoparticles bind.

Results

Isolation of Mag@MSN from cell media

A549 cells were treated with zinc-doped iron oxide core MSNs for 2 hrs to allow the cells to endocytose the nanoparticles. Then the growth media was removed and the cells were washed two times with PBS to remove excess nanoparticles that were not taken up by cells. Fresh growth media was added to the cells and they were incubated for 36 hrs. After the incubation period, the media from the wells was collected into a single tube. A neodymium magnet was placed on the bottom of a cell culture flask and the media was collected from the cells and placed inside the flask. Once the magnetic nanoparticles were attracted to the magnet, the media was removed leaving the nanoparticles inside flask. The nanoparticles were washed with PBS two times using the same procedure of magnetic attraction. After the washes, the nanoparticles were suspended in PBS and subjected to TEM or ICP-OES analysis. TEM images of the exocytosed
magnetic nanoparticles show that the nanoparticles can be successfully isolated from the media (Figure 4.1 A). ICP-OES was used to measure the concentration of silicon (Si) and iron (Fe) recovered from the media. One sample of exocytosed nanoparticles was not subjected to the magnetic isolation procedure and was used as a control to quantify the total amount of Si and Fe that was exocytosed by the cells. The ICP-OES results for this sample were compared to the results for the nanoparticles that were isolated with the magnet. Based on these values, we were able to calculate the percentage of Si and Fe that was isolated using the neodymium magnet. For the phosphonated Mag@MSN, 33.3% of the total Si exocytosed by cells was recovered by the magnet isolation procedure, similarly 39.5% of the total Si was isolated for PEI coated Mag@MSN (Figure 4.1 A). The measurements for iron concentration show that 58% of exocytosed iron was recovered from phosphonated magnetic MSNs and 51.3% was recovered from PEI coated nanoparticles (Figure 4.1B).

**Exocytosed iron core MSNs carry proteins out of the cell**

Following the magnetic isolation procedure of exocytosed nanoparticles, we set out to determine if the isolated nanoparticles had proteins attached to them. For this purpose, the isolated nanoparticles were treated with Laemmli buffer and incubated at 95°C for 5 min to dissociate the proteins from the nanoparticles. The samples were then run in SDS polyacrylamide gels (SDS-PAGE) to separate the proteins by size. The gels were subjected to silver staining to visualize the proteins (Figure 4.2). The control lane shows the sample from cell media without nanoparticle addition, and as expected no protein is visible in this sample. The phosphonate modified iron core MSNs that were exocytosed from the A549 cells did have proteins attached to them as evidenced in the gel. There are many bands that are visible in this
sample suggesting that there is a variety of proteins that are bound by these nanoparticles. There were no bands visible for the exocytosed PEI coated nanoparticles, but this might be due to low level of protein bound to the nanoparticles.

**Identification of proteins bound to Mag@MSNs**

In order to identify the proteins that were bound to the exocytosed nanoparticles, mass spectrometry was employed. The exocytosed nanoparticles were magnetically isolated from the growth media and subjected to trypsin digestion. The peptides were removed from the nanoparticles and ran on a nano ultra-performance liquid chromatography (nanoUPLC) Waters nanoACQUITY system (Manchester, UK). Protein identification analysis was carried out using ProteinLynx Global Server 2.5.2 (PLGS) and searched against the UniProtKB-SwissProt *Homo sapiens* (Human) database. Three independent experiments were carried out for each type of nanoparticles (phosphonated and PEI coated). There were over twenty different proteins that were identified from the phosphonated Mag@MSN samples, the eleven proteins that were reproducible on the three independent experiments are presented in Table 4.1 along with the intracellular localization of each protein. For PEI coated MSNs there were much less proteins, as shown in Table 4.2 there were only five proteins identified from these samples. Amine modified nanoparticles were also studied and, as shown in Table 4.3, they were found to bind fifteen proteins, most of which are localized to the cytoplasm of the cell.

**Discussion**

The isolation of exocytosed magnetic nanoparticles from the cell media was achieved successfully, but improvements can be made to increase the amount of nanoparticles that can be collected. The discrepancy in the ICP-OES measurements of recovered Si and iron (Figure 4.1)
from the cell media might be due to fact that not all the MSNs synthesized have an iron core. The synthesis of zinc-doped iron core MSNs relies on the co-precipitation of silica on the iron core, but some MSNs can form without an iron core. When the nanoparticles are added to the cells for exocytosis experiments, the nanoparticles that don't have an iron core are lost during the magnetic isolation procedure. This explains why the percentage of Si recovered from exocytosed MSNs is lower than the percentage of iron. For phosphonated MSNs, only 33.3% of Si was recovered compared to 58% of iron. For PEI coated MSNs, 39.5% of Si and 51.3% of iron was recovered. In both cases a significant amount of Si and Fe is lost during the isolation procedure. The significant loss of nanoparticles limits our ability to identify proteins that might be present in low amounts in the samples. The method of nanoparticle isolation could be improved by the development of a fluidic mechanism that could isolate magnetic nanoparticles. By enhancing the collection of exocytosed nanoparticles, more nanoparticle-bound proteins can be collected and improve the identification of proteins.

Based on the silver stain results, it seems that the nanoparticles do not bind a significant amount of serum proteins that are present in the cell growth media. Excess nanoparticles that were not endocytosed by the cells after the 2 hr incubation period, were isolated from the cell growth media to determine if they had bound to any serum proteins. The last two lanes on the right hand side of the silver stained gel (Figure 4.2) demonstrate that there are no protein bands visible in these samples. This indicates that the proteins detected on the exocytosed nanoparticles were bound when the nanoparticles were inside the cells and also suggests that the intracellular environment might create changes on the surface of the nanoparticles that allow them to bind proteins. The acidic environment of the lysosome might modify the surface properties of the particles making them more prone to interact with proteins. Some groups have
reported that the adsorption of serum proteins onto the surface of nanoparticles creates a protein corona that affects the cellular uptake of the nanoparticles.\textsuperscript{[134-135]} It has been shown that the serum proteins reduce the interaction of the nanoparticles with the cell membrane reducing the endocytosis of the nanoparticles. In the case of actively targeted nanoparticles, the protein corona reduces the targeting capability by blocking the active sites of the targeting ligand. The fact that our nanoparticles display minimal or no serum protein binding is an advantage because the adverse effect of the protein corona on nanoparticle uptake is minimized.

The Mass Spectrometry results show that there is a different number of proteins bound by phosphonated (Table 4.1), PEI (Table 4.2), and Amine (Table 4.3) modified iron core MSNs. Phosphonated MSNs are known to co-localize with the lysosomes and as discussed in Chapter III of this dissertation, the majority of them are excreted from cells through lysosomal exocytosis. This would suggest that the majority of the proteins that are bound by these nanoparticles are in the lysosomes. The lysosome is a degradative compartment where many proteins are broken down, this would suggest that the nanoparticles are binding to protein fragments that are located in the lysosome. This would explain the high number of proteins identified by Mass Spec. The majority of proteins identified by Mass Spec are localized in the cytoplasm of the cell and the probability of the nanoparticles encountering all of those proteins in such a big area is pretty low.

PEI coated MSNs have been used for siRNA delivery with successful gene knock-down results. These nanoparticles have a net positive charge and interact with the negatively charged siRNAs. In order to achieve efficient gene knock-down, the siRNAs must exit the lysosome and enter the cytoplasm of the cell to induce mRNA degradation. The positive charge of the nanoparticles could result in a proton sponge effect that might disrupt the lysosome and allow
either the nanoparticle or the free siRNA to enter the cytoplasm. If the PEI coated nanoparticles exit the lysosome and are localized to different areas within the cell than phosphonated MSNs, then they might be able to bind to different proteins. To investigate this, we collected exocytosed PEI iron core MSNs and identified the proteins that were bound to them. Surprisingly, there were only five proteins identified from the PEI coated particles compared to the more than thirteen proteins identified in the phosphonate nanoparticle samples. The only protein in common between phosphonated and PEI coated MSNs is Histone 2A, all the other proteins are different for each nanoparticles. This suggests that the PEI coated nanoparticles go to different parts of the cell than phosphonated particles or that the different charges on the surface of the nanoparticles have an effect on the type of proteins that can be bound.

Amine modified MSNs have positively charged amine groups on their surface. Both PEI coated and amine modified MSNs have a net positive charge, but the amine groups are much smaller than PEI and they are covalently attached to the surface of the nanoparticle. As seen in Table 4.3, fifteen proteins are identified from exocytosed amine modified MSNs. There are five proteins in common between exocytosed phosphonated and amine MSNs, namely, Actin cytoplasmic 1, tubulin alpha 1, profilin 1, pyruvate kinase isoenzymes M1/M2, and retinal dehydrogenase are found in both samples. Interestingly, eight of the proteins identified in exocytosed amine MSNs are enzymes that are either involved in cellular metabolism (Glyceraldehyde-3-phosphate dehydrogenase, Retinal dehydrogenase 1, L-lactate dehydrogenase, Pyruvate kinase isozymes M1/M2, Alpha enolase, Aldehyde dehydrogenase mitochondrial), cellular differentiation (Mitogen-activated protein kinase 12), or protein translation (Elongation factor 1-alpha 2). The fact that these enzymes can be recovered with amine MSNs, suggests that the delivery of inhibitors for these enzymes would have a strong
effect on cellular function. These nanoparticles could also be used to monitor the expression of these enzymes in different type of cells.

The binding of proteins by exocytosed MSNs was first reported by Slowint et al.\textsuperscript{107} but in their study only three proteins were identified using MSNs that were not surface modified. They detected α-actinin-4, cytoplasmic actin 1, and annexin A2 bound to MSNs that were exocytosed from HUVEC cells. In their experiments, they ran the samples in SDS-PAGE, stain with coomasie blue, and cut the visible bands for mass spec. In our experiments, more proteins are detected because the samples are not run on a gel in order to do mass spec. By only doing mass spec analysis on the visible bands on the gel, proteins that are present in the sample, but their concentration is too low to be detected in the gel, are ignored. In our experiments, the whole sample that is collected after the magnetic isolation is run on the mass spec, so that the high sensitivity of the instrument allows us to detect all the proteins present.

The use of MSNs as protein harvesting tools could lead to many important applications both as diagnostics or research tools. The ability to collect proteins from cells without the need of lysing them could result in the use of nanoparticles \textit{in vivo} to collect proteins from tumors in order to determine what biomarkers they express and develop personalized therapies.

Materials and Methods

Synthesis of Phosphonated and PEI Fluorescent Iron-Core MSNs

As previously reported by C.R. Thomas et al. J. Am. Chem. Soc. 2010,\textsuperscript{128} magnetic nanoparticles with Zn ion doped were synthesized using the method developed by Jang et al.\textsuperscript{129} A
A typical synthesis to produce Zn$_{0.4}$Fe$_{2.6}$O$_4$ nanoparticles is as follows: ZnCl$_2$ (30 mg), FeCl$_2$ (40 mg), and Fe(acac)$_3$ (353 mg) were placed in a 50 mL three-neck round-bottom flask in the presence of surfactants (oleic acid and oleylamine) in octyl ether. The reaction mixture was heated at 300 °C for 1 h and the reaction products were cooled to room temperature. Upon addition of ethanol, a black powder precipitated and was isolated by centrifugation. The isolated nanoparticles were dispersed in toluene. Nanocrystals have 15 nm size with narrow size distribution (σ < 5%). Zinc-doped iron oxide nanocrystals were dissolved in chloroform at a concentration of 50 mg/mL. One milliliter of the iron oxide nanocrystals in chloroform was added to a solution of 100 mg cetyl trimethylammonium bromide (CTAB, Aldrich, 95%) in 5 mL of water. The mixture was sonicated and the chloroform was boiled off from the solution with rapid stirring. The aqueous CTAB stabilized zinc-doped iron oxide nanocrystals were added to an 80 °C solution of 43 mL distilled water with 350 μL of 2.0 M NaOH, and 500 μL tetraethyl orthosilicate (TEOS, Aldrich, 98%) was slowly added. After 15 minutes, 125 μL 3-(hydroxysilylpropyl) methylphosphonate was added. Following two hours of rapid stirring at 80 °C, the magnetic-core silica nanoparticles were collected by centrifugation and washed with ethanol and water. The CTAB was removed by dispersing the as-synthesized materials in a solution containing 133.3 mg ammonium nitrate (Fisher) and 50 mL 95% ethanol. This mixture was heated to 60 °C for 15 minutes, then the particles were collected by centrifugation and washed with ethanol. The fluorescent functionality for optical monitoring of the nanoparticles in cells, fluorescein isothiocyanate, was attached to the mesoporous silica framework. 3 mg fluorescein isothiocyanate (FITC, Sigma, 90%) was dissolved in 1 mL ethanol, and 12 μL 3-aminoethyltriethoxysilane (3-APTES, Aldrich, 98%) was added. This solution was reacted under nitrogen for 2 hours, then added to the 80 °C solution of aqueous sodium hydroxide. After
10 minutes, the CTAB-ZnNC solution was added, and the procedure followed in the same manner as above. For PEI coating, as-synthesized nanoparticles (30 mg) were suspended in a 3 mL solution of PEI (weight 0.8 kDa, 2.5 mg/mL) and ethanol. Particles were stirred for 30 minutes and washed three times with ethanol to remove excess PEI. For the Amine surface modification, the solvent extracted MSNs (250 mg) were suspended in ethanol. Then \(N-(2\text{-aminoethyl})3\text{-aminopropyl})\text{trimethoxysilane} \ (110 \mu\text{L}, 0.5 \text{mmol})\) was added, and the mixture was refluxed under nitrogen for 24 h. The amine modified MCM-41s were collected by centrifugation, washed with ethanol, and dried under vacuum.

**Cell Culture**

The human cancer cell line A549 was obtained from American Type Culture Collection and was maintained in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO) supplemented with 10% fetal bovine serum (Sigma), 1% L-glutamine, 1% penicillin and 1% streptomycin stock solutions.

**Mag@MSN exocytosis experiment and nanoparticle isolation**

Cells were seeded at a confluency of \(5 \times 10^5\) cells per well in 6-well plates and incubated overnight. Iron core MSNs (phosphonated or PEI coated) were added to the cells at a concentration of 80 μg/mL and incubated for two hours. The growth media was removed from the cells and collected in a tube for nanoparticle isolation. The cells were washed with PBS two times and fresh growth media was added to the cells. The cells were incubated for 24 hrs to allow the nanoparticles to be exocytosed. After 24 hrs, the growth media was collected in a tube and nanoparticle isolation using a magnet was carried out. The cell media of three 6-well plates was collected in a single 50 mL conical tube for a total of 36 mL of collected media.
The iron core MSNs magnetic isolation was carried out by placing a neodymium magnet on the bottom of a 25 mL cell culture flask. From the 38 mL of media collected from the cells, 6 mL were placed inside the cell culture flask and 1 hour was given for the nanoparticles to be attracted to the bottom of the flask by the magnet. After 1 hour, the media was carefully removed avoiding disturbance on the side where the magnet was placed. After the media was removed, another 6 mL of the collected media from the cells were added to the flask and again 1 hr was given for magnetic attraction of the nanoparticles and subsequent media removal from the flask. This process was repeated until the total 36 mL of media collected from the cells were subjected to the magnetic isolation of nanoparticles. The attraction from the magnet would keep the nanoparticles in the flask while the media was being removed. The nanoparticles were washed with PBS two times inside the flask to remove any proteins that were not bound to the nanoparticles. The nanoparticles were suspended in 1 mL of PBS and another wash was carried out using a magnetic rack to separate the nanoparticles from the PBS. After all the washes, the nanoparticles were suspended in 50 μL of PBS and either subjected to SDS PAGE, ICP-OES or Mass Spectrometry.

**SDS PAGE and silver staining of Mag@MSN bound proteins**

Isolated iron core MSNs were treated with Laemmli buffer and heated at 95° C for 5 min to induce protein unfolding and dissociation from the nanoparticles. The samples were loaded onto a 10% SDS polyacrylamide gel and run at 120 V for 1 hr. After SDS PAGE, the gel was silver stained using the Invitrogen SilverQuest staining kit.
ICP-OES Analysis of Mag@MSN

Solutions of magnet isolated Mag@MSN (2 mL or 0.5 mL) were diluted with HNO₃ (70%, equal volume of HNO₃ added to each sample). The acidic solution was then digested by sonicating (15 min) in a sealed polyethylene tube before heating at 70°C for 24 hours. After the digestion steps, the solutions were diluted with portions of ddH₂O to reach a final nitric acid concentration of 5%. Samples were run for qualitative element detection on a Shimadzu ICPE-9000 instrument to determine a proper standard concentration range for both Si and Fe. Standard curves for both Fe and Si were generated by running a set of standards made by subsequent serial dilutions of a stock solution (1000 ppm, 5% HNO₃, Sigma-Aldrich). Each sample was plotted against the standard curve to quantify concentrations of both iron and silicon.

Trypsin Digest of Nanoparticle-Bound Proteins.

Nanoparticle (NP) samples are placed into a magnetic rack to isolate protein-bound NPs, and PBS solution is removed. NPs are washed twice with ultrapure H₂O. A reduction reaction to reduce cysteine residues is conducted by the addition of 200 mM ammonium bicarbonate (ABC) in 0.1% deoxycholic acid (DCA), 30 mM dithiothreitol (DTT), and subsequent incubation at 95°C at 300 rpm. The high temperature ensures that proteins are efficiently unfolded thus allowing trypsin sufficient access for digestion. After a 30-minute reduction period, 100 mM iodoacetamide (IAN) is added to the solution and incubated in the absence of light at 37°C for 1-hour. This serves to block reduced cysteine residues, ensuring the proteins remain unfolded. A small amount of 30 mM DTT is added to the solution to quench the reaction. Additional ABC-DCA solution is added to the reaction, trypsin (Promega Corporation;
Madison, WI) is added in a ratio of 1 μg trypsin : 100 μg protein, and the digestion reaction incubates overnight at 37°C.

After the digestion is complete, NPs must be removed prior to mass spectrometry analysis. NPs are not compatible with liquid chromatography tandem mass spectrometry (LC-MS/MS) as they are not ionizable by electrospray ionization (ESI). Digest samples with NPs in solution are placed into a magnetic rack, and the digest solution is pipetted into a fresh vial, leaving behind the NPs. DCA is subsequently removed as it is a detergent that interferes with mass spectrometry analyses. This is achieved by organic extraction methods, where ethyl acetate and formic acid are added to the solution, the mixture is vortexed, and centrifuged with the top (organic) layer removed as it contains extracted DCA. This process is repeated twice. Residual aqueous solution is evaporated, leaving a digested protein pellet that is then resuspended in 0.5% formic acid.

**Mass Spectrometry Analysis of Trypsin Digest of Nanoparticle-Bound Proteins.**

Samples are run on a nano ultra-performance liquid chromatography (nanoUPLC) Waters nanoACQUITY system (Manchester, UK) equipped with a nanoACQUITY UPLC Symmetry C18 180 μm x 20 mm trap column and a nanoACQUITY UPLC BEH C18 75 μm x 150 mm reversed phase (RP) analytical column. An aqueous mobile phase (phase A) consists of 0.1% formic acid in water, and an organic mobile phase (phase B) consists of 0.1% formic acid in acetonitrile (ACN). For each sample, 5 μL are injected onto the trap column, washed with 97% A and 3% B for 5 minutes, and subsequently injected onto the RP column with 3% B. Peptides are eluted from the column with a gradient of 3-50% B over 70 minutes at 5 μL/min, followed by
a gradient of 50-95% B over 3 minutes. A 15 minute rinse with 95% B is followed by a 20 minute re-equilibration period with 97% A.

The nanoACQUITY system is interfaced with a Waters Xevo quadrupole time-of-flight (qToF) mass spectrometer. All data acquisitions were conducted in positive mode over a range of 100-8000 m/z. Peptides were fragmented in a data-independent mode (MS$^E$) in order to maximize protein identification. It is non-selective and continuous, alternating between low and high collisional energies every second, which allows for observation of resulting precursor and associated fragment ion spectra.

All data were processed in ProteinLynx Global Server 2.5.2 (PLGS) and searched against the UniProtKB-SwissProt *Homo sapiens* (Human) database for protein identifications. Parameters were set to a minimum of 7 peptides per protein identification, two missed cleavages, 4% false positive rate, fixed carabimidomethyl modification of cysteine residues, and variable modifications that included: oxidation of methionine residues, deamidation of asparagine and glutamine residues, and acetylation of lysine residues as well as the N-terminus.
Figure 4.1 Recovery of Mag@MSNs with magnetic isolation procedure. A) TEM pictures of exocytosed zinc-doped iron core MSNs recovered with a neodymium magnet. B) Measurement of Si recovered from exocytosed nanoparticle using a neodymium magnet. C) Measurement of Fe recovered from exocytosed nanoparticles using a neodymium magnet.
Figure 4.2 Exocytosed Mag@MSNs carry proteins out of the cell. Iron core MSNs (Phosphonated or PEI coated) exocytosed from A549 cells were isolated from the cell growth media and subjected to SDS-PAGE and silver staining. The bands represent proteins that were bound to the nanoparticles.
### Table 4.1 Proteins bound by phosphonated iron core MSNs from A549 cells.

The exocytosed magnetic nanoparticles were isolated from the cell growth media and mass spectrometry was performed to identify the bound proteins.

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Intracellular localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histone H2A type 1</td>
<td>Nucleus</td>
</tr>
<tr>
<td>Actin cytoplasmic 1</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>Tubulin alpha 1C</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>Beta actin like protein 2</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>Ubiquitin 60S ribosomal protein L40</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>Actin cytoplasmic 2</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>Profilin 1</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>Annexin A1</td>
<td>Membranes</td>
</tr>
<tr>
<td>Pyruvate kinase isozymes M1 M2</td>
<td>Cytoplasm, Nucleus</td>
</tr>
<tr>
<td>POTE ankyrin domain family member E</td>
<td>Unknown</td>
</tr>
<tr>
<td>Retinal dehydrogenase 1</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>Protein Names</td>
<td>Intracellular Localization</td>
</tr>
<tr>
<td>--------------------------------------------------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>Histone H4</td>
<td>Nucleus</td>
</tr>
<tr>
<td>Histone 2A</td>
<td>Nucleus</td>
</tr>
<tr>
<td>Isoform 2 of Pre mRNA Splicing Factor 18 OS</td>
<td>Nucleus</td>
</tr>
<tr>
<td>Isoform 3 of Polycystic kidney disease 2 like 1 protein</td>
<td>Membranes</td>
</tr>
<tr>
<td>Zinc finger HIT domain containing protein 2</td>
<td>unknown</td>
</tr>
</tbody>
</table>

**Table 4.2** Proteins bound by PEI coated iron core MSNs from A549 cells. The exocytosed magnetic nanoparticles were isolated from the cell growth media and mass spectrometry was performed to identify the bound proteins.
<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Intracellular Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin cytoplasmic 1</td>
<td>cytoplasm</td>
</tr>
<tr>
<td>Isoform 2 of Actin gamma-enteric smooth muscle</td>
<td>cytoplasm</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>cytoplasm</td>
</tr>
<tr>
<td>Profilin 1</td>
<td>cytoplasm</td>
</tr>
<tr>
<td>POTE ankyrin domain family member F</td>
<td>Unknown</td>
</tr>
<tr>
<td>Tubulin alpha 1</td>
<td>cytoplasm</td>
</tr>
<tr>
<td>Retinal dehydrogenase 1</td>
<td>cytoplasm</td>
</tr>
<tr>
<td>L-lactate dehydrogenase</td>
<td>cytoplasm</td>
</tr>
<tr>
<td>Pyruvate kinase isozymes M1/M2</td>
<td>cytoplasm</td>
</tr>
<tr>
<td>Alpha enolase</td>
<td>cytoplasm/cell membrane</td>
</tr>
<tr>
<td>Histone H1.4</td>
<td>Nucleus</td>
</tr>
<tr>
<td>Mitogen-activated protein kinase 12</td>
<td>Cytoplasm/nucleus</td>
</tr>
<tr>
<td>Aldehyde dehydrogenase mitochondrial</td>
<td>Mitochondrion Matrix</td>
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<tr>
<td>Elongation factor 1-alpha 2</td>
<td>Nucleus</td>
</tr>
<tr>
<td>Protein OSCP1</td>
<td>Cell membrane</td>
</tr>
</tbody>
</table>

**Table 4.3** Proteins bound by Amine modified iron core MSNs from A549 cells. The exocytosed magnetic nanoparticles were isolated from the cell growth media and mass spectrometry was performed to identify the bound proteins.
Chapter V

Conclusion
Targeted drug delivery to cancer cells

The projects described in this dissertation have contributed to the advancement of the nanotechnology field by developing novel targeting approaches that improve drug delivery to cancer cells using MSNs, studying the interaction between MSNs and cells, and setting the foundation for the use of MSNs as tools to recover proteins from cells. The targeting of MSNs to specific cancer cells has the potential to revolutionize cancer therapy by improving nanoparticle uptake by malignant cells and reducing side effects to normal tissue. The enhancement in targeted MSNs uptake could result in a lower dosage necessary to achieve successful results, which could lower the toxicity of anticancer drugs in vivo. The fact that whole proteins or small peptides can be conjugated to mesoporous silica frameworks gives us the flexibility of using different targeting molecules; this improves our ability to generate personalized therapies. The heterogeneity of tumors and the different surface receptors that cancer cells express requires a flexible approach so that the disease can be fought from different angles. MSNs provide a solid structure upon which many different surface modifications can result in drug delivery vehicles with different targeting properties.

Characterization of MSNs exocytosis

In this work we address the question about the ultimate fate of mesoporous silica nanoparticles in cells. The results presented demonstrate that the nanoparticles do not accumulate inside the cell but are excreted. This is an important finding as the drug delivery efficacy of the nanoparticles depends on the amount of time they remain inside the cell to allow drug release. The fact that lysosomal exocytosis was identified as an important pathway involved in nanoparticle exocytosis allows us to carry experiments to determine which types of
cells are more resistant to drug loaded MSNs. Cells that have a higher rate of lysosomal exocytosis are able to excrete the nanoparticles fast, minimizing the amount of drug that is released inside the cell. In our results we demonstrate that surface modifications affect the amount of time nanoparticles remain inside the cells. Nanoparticles that were coated with polyethyleneimine (PEI) took longer to be exocytosed from cells than phosphonate coated MSNs. This suggests that different types of nanoparticles might use different exocytosis pathways to exit cells. Further studies are needed with different types of nanoparticles to identify how nanoparticles with different surface properties are excreted from cells. There is the possibility of discovering new cellular pathways that mediate the exocytosis of MSNs with different surface modifications. By identifying these pathways we could manipulate them to improve drug delivery efficacy or we could develop new applications for the nanoparticles that take advantage of their intracellular localization.

**MSNs as protein harvesting tools**

In this dissertation we demonstrate that MSNs bind to proteins when they are inside the cells and these proteins can be recovered and identified from exocytosed nanoparticles. This project is in the preliminary stage and more experiments are needed to confirm the results and determine if the surface properties of the nanoparticles affect the type of proteins that are bound. This approach could help determine if MSNs with different surface properties (charge, surface modification, nanomachines) are exocytosed by cells using different exocytosis pathways and track their intracellular localization. The proteins that are bound to the exocytosed nanoparticles serve as a footprint of the intracellular location where the nanoparticles have been. By discovering the intracellular location of the different MSNs, new applications could be
developed or a better understanding of what type of cargo should be delivered by the nanoparticles could be achieved. For example if it is determined that a particular type of MSN goes to a particular organelle, inhibitors to an enzyme that is located in that organelle should be delivered to achieve the desired effect.

The use of MSNs to recover proteins from cells could serve as a diagnostics tool for cancer. The prospect of injecting nanoparticles into a patient and after a certain amount of time collect the nanoparticles from a blood sample and look for cancer biomarkers that are bound to the nanoparticles, could provide valuable information about specific proteins that the tumor expresses. The advantage lies in the ability of the nanoparticles to enter cells and carry out proteins that are usually not secreted by the cell. This would allow for the recovery of proteins that are typically not detected in blood samples. By attaching molecules on the surface of nanoparticles that bind to specific proteins, it would be possible to fish out proteins of interest that could provide information about the stage of the tumor or possible targets for therapy.

The results presented in this dissertation serve as the beginning of a promising novel application for MSNs. Further studies are needed to achieve the potential impact that MSNs could have on cancer therapy and diagnostics. Different surface modifications could improve the protein binding properties of the nanoparticles or affect their intracellular localization so that target proteins could be recovered. Animal studies need to be performed to determine if protein recovery from cells can be achieved in vivo. The isolation of the nanoparticles from blood samples might require a stronger magnetic field or the development of a fluidics instrument to recover the maximum amount of exocytosed nanoparticles. The combination of a stable drug
carrier, nanomachine, and protein harvesting tool, make MSNs a very valuable tool that could revolutionize medicine.
References


