Rational surface functionalization on mesoporous silica nanoparticles for targeted and controlled drug delivery

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**RATIONAL SURFACE
FUNCTIONALIZATION ON
MESOPOROUS SILICA
NANOPARTICLES FOR TARGETED
AND CONTROLLED DRUG
DELIVERY**

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SCHOOL OF PHYSICAL AND MATHEMATICAL SCIENCES

2016

**RATIONAL SURFACE
FUNCTIONALIZATION ON
MESOPOROUS SILICA NANOPARTICLES
FOR TARGETED AND CONTROLLED
DRUG DELIVERY**

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TABLE OF CONTENTS

Acknowledgements	i
Table of Contents	ii
Abstract	v

Chapter 1: Introduction

1.1 Nanoparticles and its role in drug delivery.....	1
1.2 Properties of MSNPs for drug delivery.....	7
1.3 Varying MSNPs functional groups and cargoes for biological applications.....	11
1.3.1 Targeting moieties.....	12
1.3.2 Stimuli- controlled cargo release.....	15
1.3.3 Stoppers for cargo retention within pores of MSNPs.....	21
1.3.4 Common anti- cancer cargoes.....	27
1.3.5 Sensor molecules for biological detection.....	31
1.4 Common Characterisation techniques for MNSPs.....	34
1.5 Research objectives.....	39
1.6 References.....	41

Chapter 2: Targeted Delivery of Mesoporous Silica Nanoparticles into neuronal cells

2.1 Introduction.....	57
2.2 Experimental.....	60
2.2.1 Synthesis of organic compounds.....	62
2.2.2 Synthesis of MSNPs.....	65
2.2.3 In vitro experiments.....	67
2.3 Results and Discussion.....	68
2.3.1 Effect of size on MSNPs uptake into neuronal cells.....	68
2.3.2 Effect of targeting ligand conjugated MSNPs uptake into neuronal cells.....	70
2.4 Conclusion.....	74
2.5 References.....	75

Chapter 3: An iGlu Receptor Antagonist and Its Simultaneous Use with an Anticancer Drug for Cancer therapy

3.1 Introduction.....	79
3.2 Experimental.....	82
3.2.1 Synthesis of MSNPs.....	83
3.2.2 Synthesis of Compound D.....	85
3.2.3 Synthesis of Compound 2.....	86
3.2.4 Amine quantity test.....	87
3.2.5 Procedure for in vitro studies.....	88
3.2.6 Drug loading and release studies.....	90
3.3 Results and Discussion.....	91
3.3.1 Synthesis and characterization of functionalized MSNPs.....	91
3.3.2 Drug loading and release studies.....	96
3.3.3 Endocytosis and anticancer efficiency towards B16F10.....	99
3.3.4 Anticancer efficiency of MSNPs-4-Dox.....	102
3.3.5 Inhibitory effects and intracellular drug release of MSNPs-6-Dox.....	103
3.4 Conclusion.....	106
3.5 References.....	107

Chapter 4: Drug Encapsulation and Release by Mesoporous Silica Nanoparticles: The Effect of Surface Functional Groups

4.1 Introduction.....	112
4.2 Experimental.....	115
4.2.1 Synthesis of Organic compounds.....	116
4.2.2 Synthesis of functional MSNPs.....	126
4.2.3 Drug loading and release studies.....	129
4.2.4 Procedure for in vitro studies.....	131
4.3 Results and Discussion.....	132
4.3.1 Synthesis and characterization of functionalized MSNPs.....	132
4.3.2 Effect of ligand length on drug loading and release.....	135
4.3.3 Effect of terminal groups on drug loading and release.....	137
4.3.4 Effect of different amount of ligands on the surface of MSNPs on drug loading and release.....	141
4.4 Conclusion.....	145
4.5 References.....	145

Conclusion	149
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List of Publications	151
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ABSTRACT

Mesoporous silica nanoparticles (MSNPs) have been widely used in many applications such as gas storage, catalysis and to serve as absorbants for various chemicals. In recent years, there has been a growing trend for the use of these MSNPs in the area of drug delivery. MSNPs have large surface areas for appending various molecules on the surface or within the pores and tunable pore sizes and volumes to allow excellent storage of various drug molecules. Owing to this, this dissertation focuses on improving the drug delivery efficiency by altering the functional moieties on the surface of MSNPs.

Although MSNPs are widely utilized in cancer therapy, the use of MSNPs for the treatment for other disease has often been neglected. Lately, there has been a surge in the number of patients suffering from brain related disease worldwide. Hence, in Chapter 2 we aim to utilize MSNPs to target this range of brain diseases. By taking advantage of the easy synthesis and functionalisation of MSNPs, a targeting ligand can be conjugated to the MSNPs surface to test its uptake into neuronal cells. From the results obtained, the targeting specificity towards neuronal cells can be enhanced by using targeting ligands and by synthesising small MSNPs.

According to previous literatures, the neuronal targeting ligand used in Chapter 2 could have the potential of serving as a targeting ligand for certain cancer cell lines. Hence, in chapter 3, we focused on the use of this ligand together with the use of anticancer drug, Doxorubicin (DOX), to study their simultaneous effect on B16F10 melanoma cancer cell line and NIH3T3 normal cell line. The design of the drug

delivery system involves conjugating the ligand to the surface of the MSNPs and storing the drug within the mesopores by trapping it with a redox cleavable capping group on the MSNPs surface. In vitro studies revealed that the ligand showed targeting properties as well as therapeutic properties. When the ligand was used with DOX, a synergistic effect was observed for the anticancer treatment. This demonstrated the feasibility of employing a neuroprotective drug for the enhancement of cancer therapy.

In addition to altering the different targeting moieties on the surface, the drug loading and release mechanism of MSNPs can also be improved. In Chapter 3, we adopted a catalysis screening method for analyzing the effects of chain length, terminal group and density of disulfide-appended functional ligands on the surface of MSNPs on drug-loading capacity and glutathione-triggered drug-release kinetics. The ligand with intermediate length (5 carbon atoms) and a bulky terminal group cyclohexyl that complexes with β -cyclodextrin showed the highest drug loading capacity and good release kinetics. Decreasing the surface coverage of this ligand on the MSNPs also led to an enhancement in drug release. This screening method serves as a general guide for developing more effective MSNPs systems for drug delivery.

In conclusion, the above methods have a common goal of improving the drug delivery efficiency of MSNPs for the treatment of more diseases.

Chapter 1

Introduction

1.1 Nanoparticles and its role in drug delivery

In recent years, the area of drug delivery and biological imaging for cancer therapy has garnered much attention and many research groups have embarked on the journey in search of the best drug delivery systems for cancer therapy. Drug delivery systems are designed with the intention of providing efficient cancer therapy with minimal side effects¹. These systems are designed to protect the drug against degradation, allow selective release of drug at the target site and contain targeting properties². Currently, many of these systems are designed for cancer therapy and have proven to be effective in retarding cancer growth.

One of the most common drug delivery system makes use of organic-based nanoparticles, particularly using polymer in the form of liposomes, micelles, polymersomes and polymer based nanoparticles for the delivery of drug molecules³ (Figure 1). Liposomal based nanocarriers comprise of a lipid bilayer that separates the drug containing aqueous core from the surrounding aqueous environment⁴. Currently several FDA approved drugs, such as Doxil[®], Ambisome and Daunorubicin, have adopted the use of the liposomal systems for more efficient drug delivery⁵. The use of liposomal systems for drug delivery was established many years ago with one example being the use of liposomes for the delivery of adriamycin into metastatic liver cells⁶. Micelles are another class of polymer based nanocarriers that are formed by the aggregation of the hydrophilic head towards the aqueous solution with the

hydrophobic regions forming the micelle center⁷. Hydrophobic drugs and therapeutic agents that are insoluble in aqueous solutions are often loaded into the core of the micelle to enhance the solubility of the drug and improve its therapeutic efficiency⁸. Torchilin and co-workers utilised polyethylene glycol-phosphatidylethanolamine conjugates for the delivery of poorly water soluble drug, taxol, into lung carcinoma cell model. The micelles were also conjugated with targeting agents such as mAb 2C5 for increased therapeutic efficiency. The results successfully demonstrated good targeting properties as well as enhanced water solubility of the anti cancer drug⁹.

Figure 1. Organic-based nanoparticles commonly used for the delivery of drug molecules. Targeting and stimuli-responsive properties can be incorporated into these nanoparticle systems. (Reproduced with permission from ref 3c. Copyright 2007 Nature Publishing Group.)

Another common class of polymer based nanoparticles involve supramolecular self-assembly polymers. This class of polymers make use of supramolecular self assembly techniques to encapsulate the drugs within the polymer network for the purpose of drug delivery¹⁰. Supramolecular self-assembly involves the use of weak and reversible bonds between different molecules¹¹. One supramolecular based polymer system that our group has employed is the use of two different polyarctic acid chains containing β -cyclodextrin and adamantane groups respectively. Together with the use of polyethylene glycol side chains, a folic acid targeting moiety and anticancer drug, Doxorubicin (Dox), drug containing polyarctic acid nanoparticles were synthesised and in vitro and in vivo experiments were conducted. The in vitro experiments showed good targeting efficiency with the cancer cell lines, MDA-MB231 and B16-F10, having lower cell viability than that of the normal cell line, HEK293. In vivo experiments also indicated the same selectivity whereby the polyarctic acid nanoparticles containing folic acid showed a slower tumor growth as compared to that of the folate negative nanoparticles¹². Besides the use of supramolecular self assembled nanoparticles, some groups have also made use of protein based nanoparticles that form microspheres via a spray-drying technique or by emulsification. These nanoparticles are usually synthesised from naturally occurring protein sources such as albumin, collagen, gelatin, soy protein and whey protein¹³.

Besides the use of organic-based nanoparticles for drug delivery, inorganic nanoparticles have also been extensively used for the purpose of drug delivery and imaging. Gold nanoparticles are commonly used for imaging purposes, photothermal properties, selective release of drug in the presence of glutathione

as well as to enhance photodynamic efficiency of photosensitizers¹⁴. Owing to its small size (diameter 5-200 nm) and the presence of a conduction band, gold nanoparticles exhibit surface plasmon when irradiated with light at certain frequencies¹⁵. This surface plasmon can be harnessed for the use in two-photon luminescence for the imaging of cancerous tissues¹⁶. In a work by Ben-Yakar and co-workers, gold nanorods were used for deep tissue imaging with two-photon luminescence. In order to obtain targeted imaging, anti-epidermal growth factor antibodies were conjugated to the surface of the gold nanorods (Figure 2). These targeted nanorods were then introduced into A431 skin cancer cells that were cultured in a collagen matrix. The results revealed sufficient image brightness at varying depths of 15 μm , 35 μm , 55 μm and 75 μm ¹⁷.

Figure 2. Gold nanorods used for two photon imaging of A431 cancer cells. (A) TEM image of gold nanorods. (B) Two photon imaging at different cell depths of the collagen matrix. a) Two-photon auto fluorescence of unlabelled cells and b) Two-photon luminescence of nanorod labelled cells. (Reproduced with permission from ref 17. Copyright 2007 American Chemical Society.)

In addition to gold nanoparticles, iron oxide nanoparticles have also been commonly used as an imaging agent as well as for drug delivery. Iron oxide

nanoparticles are superparamagnetic and this makes them useful candidates for magnetic resonance imaging. In addition to their use as an imaging agent, iron oxide nanoparticles can be functionalised with different drug molecules and can also serve as drug delivery vehicles¹⁸. In a recent work by Jon and co-workers, polymer coated superparamagnetic iron oxide nanoparticles (TCL-SPIONs) were used as magnetic resonance contrast agents as well as for the delivery of Dox into tumor bearing mice. The results obtained reflected slow tumour growth and low retention of TCL-SPIONs within other organs¹⁹.

Apart from the above inorganic nanoparticles, silica-based nanoparticles are another large class of inorganic nanoparticles that have been widely used in many applications. They can be divided into a few classes namely, Stöber silica nanoparticles, mesoporous silica nanoparticles and hollow mesoporous silica nanoparticles²⁰. This class of inorganic nanoparticles, unlike their organic-counterparts, are much easier to synthesise, functionalise and modify and are very stable in different environments²¹. Stöber silica nanoparticles were the first type of silica nanoparticles synthesized and consists of a solid core with silanol groups on the surface for further functionalisation. When these nanoparticles are used for drug delivery applications, a dye molecule can be included in the core of the nanoparticles for in-vitro imaging and drugs can be functionalised on the outer surface for therapeutic purposes²². As the surface area of Stöber silica nanoparticles is small, the resulting drug payload of the nanoparticles is proportionately low and this results in a low therapeutic efficiency. Owing to this, modified Stöber silica nanoparticles containing pores were synthesised and this type of nanoparticles are now commonly known as mesoporous silica nanoparticles. Mesoporous silica nanoparticles (MSNPs)

were first synthesised around 1970s and it quickly gained popularity due to its remarkable properties. In the 1990s, the first MSNPs were synthesized by researchers in Japan. Later they were also produced by Mobil Crystalline laboratories and were named Mobil Crystalline Materials (MCM-41). A few years later, the University of California, Santa Barbara, synthesized MSNPs with larger pores and they were labelled as SBA-15. These MSNPs were initially synthesized to serve as molecular sieves but were later used in other applications such as catalysis, energy or gas storage and drug delivery.

MCM-41 MSNPs are more commonly used in drug delivery applications and this is due to its easy synthesis and functionalisation. MSNPs also contain large surface areas, high pore volume and tunable pore sizes²³. The large surface area of these MSNPs allows different moieties to be functionalised on the surface and this renders new properties to the MSNPs. The large pore volume allows a considerable amount of cargo to be loaded into the pores of the MSNPs and the tunable pore size enables the transportation of cargoes with various sizes. Owing to the above properties, MSNPs have been widely used in many applications such as catalysis, gas capture and storage and most importantly in the area of drug delivery and biological imaging²⁴. Another class of porous silica material is the hollow mesoporous silica nanoparticles (HMSNPs). These nanoparticles have a larger pore volume as compared to their non-hollow counterparts and hence are able to attain a higher drug payload. However the disadvantage of the hollow mesoporous silica nanoparticles is the need for complete blockage of the pores.

Owing to this, MSNPs are still the preferred choice in the area of drug delivery and biological imaging. Many research groups have utilised its unique

properties for delivery of therapeutic molecules to cancerous tissue to improve the therapeutic efficiency. The most common types of MSNPs used for therapeutic purposes are the Mobil Cystralline Materials (MCM-41 nanoparticles). MCM-41 nanoparticles have cylindrical mesopores that are arranged regularly with each pore having a diameter of 2-10 nm. They are also known to have a high specific surface area and pore volume of around $1500 \text{ m}^2 \text{ g}^{-1}$ and 1.3 mL g^{-1} and high thermal stability.

Although MSNPs have large surface area and volume and are easy functionalization, the drawback of MSNPs for drug delivery purposes is its inability to be biodegradable. Many groups have been optimising these MSNPs and some have managed to prove its ability to be excreted. This can be done by adding polyethylene glycol (PEG) moieties to improve its solubility. In addition, small MSNPs are also adopted to prevent its accumulation within the liver and promote excretion of the MSNPs. Owing to the ability to easily customise the MSNPs, they can still serve as good candidates for drug delivery.

1.2 Properties of MSNPs for drug delivery

MCM-41 nanoparticles are known to have small cylindrical pores that are parallel and run across the length of the nanoparticle. The synthesis of these nanoparticles firstly involves the use of a surfactant for the formation of the micelles. Thereafter the micelles will align to form hexagonal arrays and a base as well as the silica precursors will be added into the solution. Upon addition, the silica precursors will hydrolyse and interact with the micelles to form a silica layer. The silica layer then continues forming over the initial layer to

form the nanoparticles^{23a, 25} (Figure 3). After the reaction is complete, the nanoparticles will be obtained by centrifugation.

Figure 3. Schematic illustration of template assisted synthesis of MCM-41 mesoporous silica nanoparticles. (Reproduced with permission from ref 23a. Copyright 2014 The Royal Society of Chemistry.)

The MSNPs that are synthesised via this method contains silanol groups on the surface which can be further used for surface modification with different functional groups. This surface modification process is commonly known as grafting and involves the use of silane precursors²⁶. These precursors consist of three ethoxy or methoxy groups which will form bonds with the silanol groups present on the MSNPs and an alkyl containing functional moiety eg. chloride, amine, thiol etc. A work by Lin and co-workers revealed that different functional groups that were grafted on the surface of mesoporous silica nanoparticles affected the extent of nanoparticle uptake into Hela cells²⁷. By grafting different functional moieties on the surface, various molecules can be attached to the surface of the MSNPs. These molecules can be categorized into targeting agents, end-cap groups to keep drugs within the pores of the MSNPs, water soluble functional groups and labelling dyes. In addition to grafting, another method that is commonly used for introducing functional moieties is

the co-condensation method. This method involves the use of the silane precursors during the synthesis of the MSNPs²⁸. The MSNPs synthesised via this method contains specific functionalities throughout the silica network which can again be used for conjugation with other molecules. Uniform dye-doped mesoporous silica nanoparticles synthesised by Hyeon and co-workers employed the co-condensation method for the incorporation of fluorescein into the silica network²⁹.

Apart from controlling the different surface functionalities on the MSNPs, the size of the MSNPs can also be controlled for different purposes. In a recent work reported by Zhao and co-workers, the biodistribution of polyethylene glycol (PEG) coated mesoporous silica nanoparticles of size 48 nm, 72 nm and 100 nm were tested and the results (Figure 4) showed that PEG coated mesoporous silica nanoparticles of size 48 nm showed the highest accumulation in tumor tissue and lowest retention in the liver and spleen³⁰. This result was further confirmed by a work conducted by Tamanoi and co-workers whereby mesoporous silica nanoparticles with size of about 45 nm were observed in the liver, kidney and urinary bladder a few hours post injection and were successfully excreted via the renal route³¹.

Figure 4. Various sizes (48 nm, 72 nm, 100 nm) of Polyethylene glycol mesoporous silica nanoparticles containing folic acid were synthesised (PEG-MSNPs). Biodistribution of these nanoparticles in the liver, spleen, kidney, heart, lung and tumor were analysed at various times intervals of a) 24 h, b) 48 h and c) 72. d) Accumulation of these MSNPs at different time intervals. (Reproduced with permission from ref 30. Copyright 2013 John Wiley and Sons.)

The size of the MSNPs can be controlled by changing the type of surfactant used, varying the ratio of silica precursors as well as changing the temperature and pH during the synthesis of the MSNPs. Mou and co-workers reported that a low pH of the synthesis solution will result in a smaller particle size³². A similar effect was observed in another work by Haynes and co-workers whereby varying sizes of mesoporous and nonporous silica nanoparticles can be obtained by varying the ratio of NH_4OH and ethanolic tetraethyltriethoxysilane, temperature and pH³³. Small nanoparticles are known to be useful in the excretion and biodegradation process of these materials but are also known to have low retention in the targeted site of treatment. On the contrary, larger nanoparticles have higher retention at the target site of treatment but are less biodegradable and harder to excrete³⁴. Owing to the above problems, many groups have been in search of the optimum sized MSNPs for drug retention and

excretion. The optimum sized nanoparticles exhibit therapeutic properties by its ability to enter the intratumoral matrix as well as by blocking the blood vessels that supply nutrients to the cancer tissues. Due to the lack of nutrients, the cancer cells are unable to survive and will eventually die. This process is commonly known as angiogenesis and is used in many types of cancer therapies. In a work by Tseng and co-workers, varying sizes of Dox-encapsulated supramolecular magnetic nanoparticles were synthesized and injected into DLD-1 xenografted mice. The results showed that the 70 nm nanoparticles were largely accumulated in the tumor which further confirmed tumor specific uptake of the nanoparticles³⁵.

The pore size of the MSNPs can also be altered to cater to cargoes of varying sizes. This can be done during the initial synthesis of the MSNPs by changing the surfactant used or by post treatment of the pores³⁶. MSNPs synthesised using cetyltrimethylammonium bromide (CTAB) have a pore size of around 2-3 nm which are able to store small drug molecules such as Dox and cisplatin³⁷. Other large cargoes such as proteins and DNA can only enter larger pores of around 8 nm or 17 nm which can be synthesised by using surfactant template mixtures or by Pluronic F127 respectively³⁸. These cargoes are loaded within the pores of the MSNPs for the delivery to the target site and exhibit therapeutic properties when released into the cancerous cells.

1.3 Varying MSNPs functional groups and cargoes for biological applications

As mentioned in the previous section, different functional moieties can be attached to the surface of the MSNPs with the use of different alkyl triethoxysilane or trimethoxysilane molecules. The functionalisation of these molecules enhances the usefulness of the MSNPs for drug delivery purposes.

1.3.1 Targeting moieties

The earliest method of enhancing therapeutic efficiency of MSNPs is to use the passive targeting technique. Passive targeting employs the use of nanoparticles that have an appropriate size, surface potential and hydrophobicity^{3c, 39}. As mentioned in the previous section, these nanoparticles are able to enter the tumour either by the increased permeability to the tumour blood vessels or are able to block blood vessels that supply nutrients to the cancerous cells via the enhanced permeability and retention (EPR) effect, all of these will result in tumour size reduction (Figure 5). In a recent work by Kim and co-workers, the various sizes of PEGylated MSNPs were investigated for their ability to be retained at the tumor site. Based on the results obtained, the optimum PEGylated MSNPs size for passive tumour targeting is around 100-150 nm⁴⁰. In another work by Nel and co-workers, 50 nm Dox loaded MSNPs functionalised with polyethyleneimine-polyethylene glycol co-polymer was found to have the best tumor uptake with minimal liver and renal injury⁴¹. Although this method of targeting has proven to be successful in many instances, the therapeutic efficiency of the MSNPs can be further improved.

Figure 5. Schematic illustration of passive and active targeting. Passive targeting occurs through the enhanced permeability of the tumour vasculature and the EPR effect. Active targeting on the other hand occurs by functionalizing the surface of the nanoparticles with cell specific ligands for enhanced uptake. (Reproduced with permission from ref 3c. Copyright 2007 Nature Publishing Group.)

In recent years, many groups have begun to employ small molecules or macromolecules as targeting moieties for cancer therapy. This mode of targeting is commonly known as active targeting (Figure 5). One example of a small molecule that has targeting properties is folic acid⁴². As cancer cells grow uncontrollably, their demand for folic acid is higher as compared to the normal cells. Owing to this, there is an over-expression of folate receptors on the surface of cancerous cells⁴³ and this has helped many drug delivery vehicles achieve targeting properties. The targeting mechanism occurs by the binding of the folic acid to the folate receptors which then results in the endocytosis of the drug delivery vehicles⁴⁴. Zhao and co-workers loaded anticancer drug, Dox, into hollow mesoporous silica nanoparticles and the drug was kept within the pores by a disulphide bond that is linked to an α -CD rotaxane capped with folic acid at the end. Good targeting efficiency of these nanoparticles was seen from

the flow cytometry data whereby normal endothelial cells showed higher cell viability as compared to the Hela cancer cells⁴⁵. Similar results were also seen in vivo when targeted and non-targeted nanoparticles were used. In another work by Kros and co-workers, MSNPs were again functionalised with a β -CD rotaxane capped with folic acid at the end. However, in this work, the release of drug was induced by an enzyme known as porcine liver esterase (PLE) and the anticancer drug employed is camptothecin. In vitro experiments on human osteosarcoma cells (U2Os) showed that the presence of folic acid gave rise to higher cell death which proved the efficacy of targeted drug delivery⁴⁶.

Another receptor that has been highly expressed in some cancer cell lines would be the integrin receptor. Integrin receptors are important for various cell functions and are also vital in the progression and adhesion of some metastatic tumor cell lines⁴⁷. Hence, some cancer therapies have also focused on the use of integrin receptors as targets for selective uptake of drug delivery vehicles⁴⁸. A simple molecule that has been employed for binding to integrin receptors would be arginylglycylaspartic acid (RGD peptide). RGD peptide binds to the integrin receptors of cells and the uptake of nanoparticles occurs by endocytosis⁴⁹. In a recent work by Zhang and co-workers, the surface of the MSNPs was functionalised with disulfide groups and anticancer drug Dox was kept within the pores by the RGD peptide (Figure 6). In vitro cell experiments on $\alpha_v\beta_3$ -integrin-positive cell line (U87 MG) and $\alpha_v\beta_3$ -integrin-negative cell line (COS 7) were then conducted and it was proven that MSNPs containing the RGD peptide showed enhanced selectivity towards U87 MG cell line as compared to COS 7 cell line⁵⁰.

Figure 6. Endocytosis of the nanoparticles when functionalised with RGD peptide on the surface of the MSNPs. (Reproduced with permission from ref 50. Copyright 2015 The Royal Society of Chemistry.)

Zink and co-workers also made use of the interaction between integrin proteins and cyclic RGD peptide for the delivery of chemotherapeutic drug camptothecin. Cyclic RGD peptide was conjugated to the surface of the MSNPs and these MSNPs showed 7-10 times enhancement in uptake in the metastatic cancer cells (MDA-MB 435) as compared to the MSNPs without the cyclic RGD peptide⁵¹.

From the above mentioned works, the use of targeting ligands has proven to improve the anticancer efficiency of drug delivery systems.

1.3.2 Stimuli-controlled cargo release

The use of targeting groups towards cancer cells is one of the methods of improving therapeutic efficiency. Another common method would be to functionalise the surface of the MSNPs with bulky groups to keep the therapeutic molecules within the pores of the MSNPs and selectively releasing

these molecules upon a stimulus⁵². A useful stimulus for the release of therapeutic molecules would be a condition whereby a molecule or an ion is over-expressed in cancer cells but not in normal cells⁵³. Owing to this, the therapeutic molecules can only be released in the cancerous cells while having only a mild effect on the normal cells. One common stimulus used for the release of drug from the MSNPs is glutathione. Glutathione (GSH) is highly expressed in cancer cells due to the high metabolic rate of the cells⁵⁴. Owing to this, many research groups have made use of this form of thiol responsive drug release for the selective release of drugs within the cancerous cells.

Figure 7. (A) Schematic diagram of the as-synthesised MSNPs whereby a glutathione cleavable bond was functionalised on the surface of the MSNPs with moieties to retain drugs within the MSNPs as well as dyes to serve as a sensor for the drug release. (B) Comparison of fluorescence microscopy images for cells containing high and low amounts of GSH. Top panel: excitation at 405 nm; Bottom panel: merged images with FITC channel at 488 nm excitation. (Reproduced with permission from ref 55. Copyright 2013 American Chemical Society.)

Lee and co-workers employed the use of the excess glutathione present in cancer cells for detection of glutathione as well as for drug release (Figure 7A).

A fluorescence resonance energy transfer (FRET) based sensor system was designed with an initial green emission. In the presence of glutathione (Figure 7B), the FRET donor and acceptor are separated and a blue emission is observed with the release of anticancer drug Dox⁵⁵. In another work by Kim and co-workers, a thiol containing β -cyclodextrin was used to block the pores to prevent the leeching of Dox from the MSNPs. At a concentration of 0.01 mM there was minimal release of Dox, however when 1 mM and 0.1 mM of GSH was used a significant increase was observed. From the results obtained, a GSH concentration dependent release of Dox from the as-synthesised MSNPs system was observed⁵⁶. Griebenow and co-workers also made use of glutathione for the release of a model enzyme carbonic anhydrase (CA) from the pores of the MSNPs. Insignificant amount of enzyme was released in the absence of GSH

and in the presence of 1 μ M of GSH. However, when 10 mM of GSH was used all the enzyme was released after 20 days⁵⁷.

Another common stimulus of drug release would be to employ the acidic environment present in cancerous cells. Many cancer cells lines are known to have an acidic extracellular environment of pH 6.5 and an intracellular environment of pH 5. Normal cell lines, on the other hand, have both intracellular and extracellular environment of pH 7.4⁵⁸. Owing to this, many drug delivery systems have made use of this property for the selective release of drug within cancerous cells. A few years ago, Zink and co-workers attached a rotaxane on the surface of the MSNPs to serve as a plug for drug leakage. The surface of the MSNPs were first functionalised with β -cyclodextrin and cargo, 2,6-naphthalenedisulfonic acid disodium, was loaded and capped within the pore with a rhodamine B/benzidine conjugate. In acidic environment, this conjugate is protonated and is unable to bind to the β -cyclodextrin on the surface of the MSNPs. This causes 2,6-naphthalenedisulfonic acid disodium to be released and it was detected by fluorescence measurements⁵⁹. In a more recent work by Zhao co-workers, pH release of drug was used in supramolecular polymer based nanoparticles. It was proposed that the anticancer drug Dox remained in the core of the polymer by forming an iminium bond as well as non-covalent electrostatic interaction with the polymer. Owing to this, in the presence of acid, the iminium bond is cleaved off and the drug is released. These two ways of drug release employs either the direct protonation of the drug or the indirect protonation of a capping moiety to cause the release of drug¹².

Another less common way of drug release from these MSNPs employs the use of enzymes for specific bond cleavage. Enzymes are known to have binding

pockets which are able to cleave specific bonds in molecules. Hence many drug delivery systems have made use of such enzymes to release drugs via the breakage of bonds⁶⁰. Payá and co-workers conjugated a peptide sequence to the surface of the MSNPs to retain the model drug, $[\text{Ru}(\text{bipy})_3]^{2+}$, in the pores of the MSNPs. The work investigated the release of model drug in the absence and presence of peptidase and also in the presence of acid. From the results obtained, only the condition involving the peptidase enzyme showed good release and this reflects the feasibility of the as-designed system⁶¹. In another work by Ju and co-workers, human telomerase enzyme was used to cleave off a DNA sequence that was used to trap the model cargo, fluorescein, within the pores of the MSNPs (Figure 8A). Telomerase enzyme is known to be highly expressed in cancer cells due to their ability to undergo unlimited proliferation. In this work, a turn-on fluorescence of the cargo was expected during its release. Hence when HeLa cells were incubated with these MSNPs (Figure 8B), green fluorescence was observed in the cytoplasm after 90 min and this represents the successful release of the drug⁶².

Figure 8. (A) MSNPs conjugated with a DNA chain (O1) for the blockage of pores. A black hole fluorescence quencher was immobilised within the pores of the MSNPs and the pores were loaded with fluorescence dye, fluorescein. Telomerase enzyme reacts with the telomerase primer (TP) to release the DNA chain O1 as well as the fluorescein. **(B) a)** Time dependent confocal images to monitor the release of the fluorescein (green fluorescence) within the cytoplasm of cells. **b)** TEM images to identify the internalization

of MSNPs HeLa cervical cancer cell line. (Reproduced with permission from ref 62. Copyright 2013 American Chemical Society.)

Thiol, pH and enzyme responsive release of drug from the MSNPs make use of the intrinsic properties of the cellular environment. Many research groups have also made use of external stimuli, such as light, temperature, magnetic fields and ultrasound as the trigger for drug release from MSNPs⁶³. Light triggering systems often involve the use of photoswitchable molecules such as azobenzenes which are capable of changing conformation under different wavelengths of light^{37b, 64}. In addition, some systems use light to induce a temperature change in the MSNPs⁶⁵. This causes the breakage of bonds on the surface of the MSNPs, thus releasing the drug molecules from the MSNPs pores. Cho and co-workers also made use of Au/Ag hollow nanoshells to induce the release of Dox by near-infrared (NIR) light as well as for photothermal cancer therapy. From the release profile, there was an increase in drug release when NIR light was applied to the nanoparticles. The photothermal effect was also investigated by measuring the rise in temperature of the nanoparticle suspension after NIR laser irradiation⁶⁶.

Magnetic field triggered drug release also indirectly employs heat for the release of drug from MSNPs. Magnetic nanoparticles are used concurrently with MSNPs and these magnetic nanoparticles produce heat when an alternating magnetic field is applied⁶⁷.

In addition to the above external stimulus, ultrasound triggered drug release has also gained popularity in recent years⁶⁸. Ultrasound is often either used to produce heat, acoustic cavitation or acoustic radiation forces⁶⁹. Du and co-workers designed a polymer, comprising of polyethylene oxide, 2-(diethylamino)ethyl methacrylate and (2-tetrahydrofuranyloxy)ethyl

methacrylate, that is ultrasound and pH responsive. Dox was encapsulated within the vesicles by dropwise addition of Dox and polymer solution into water. This encapsulated drug was then tested at different sonication times as well as different pH and the results obtained showed the positive effect of these two stimulus⁷⁰.

The use of appropriate stimuli for the selective release of drugs will improve the therapeutic efficiency as well as reduce the toxic side effects of the anti-cancer drugs.

1.3.3 Stoppers for cargo retention within pores of MSNPs

As mentioned in the previous section, the conjugation of stimuli responsive release moieties on the surface of the MSNPs improves the therapeutic efficiency of the MSNPs. However, in order to retain the cargoes within the pores of the MSNPs, large or moderately bulky groups have to be employed. The bulky groups can include large molecules like cyclodextrin⁷¹, proteins⁷², polymers⁷³, DNA⁷⁴ or even the use of other types of inorganic nanoparticles^{52b}.

Cyclodextrin has been widely used in many publications for the capping of drug molecules in MSNPs. This is due to its ease in modification and its low price. Cyclodextrin consists of 5 or more sugar molecules that are bound together in a ring and the sugar units are linked at the 1 and 4 carbon. There are three common types of cyclodextrins namely the α -cyclodextrin (α -CD), β -cyclodextrin (β -CD) and γ -cyclodextrin (γ -CD) and they consist of 6, 7 and 8 - membered sugars respectively. Due to their cyclic structure, cyclodextrins have

a hydrophilic exterior and hydrophobic interior (Figure 9A). The hydrophobic interior has the ability to bind to different organic molecules via van der Waals interaction and this binding is known as supramolecular self-assembly⁷⁵. This phenomena encompasses host-guest chemistry whereby the cyclodextrin serves as a host and the organic molecule that resides in the hydrophobic interior is the guest⁷⁶ (Figure 9B). Due to the different size of the cyclodextrins, each cyclodextrin has the ability to bind to molecules of varying sizes and polarities.

Figure 9. (A) Three types of cyclodextrin (α -CD, β -CD and γ -CD) with their respective dimensions. (B) Host-guest complexation of cyclodextrins with guest molecules in different ratios. (Reproduced with permission from ref 75a. Copyright 2014 American Chemical Society.)

In some early publications, α -CD commonly forms a pseudorotaxane with polyethylene glycol (PEG)⁷⁷ and β -CD with polypropylene glycol (PPG)⁷⁸. In

later publications, β -CD was used together with adamantane in the formation of a host-guest complex⁷⁹. The binding of adamantane and β -CD is known to be strong and this can be used for retention of drug within the pores of the MSNPs. This phenomenon can be seen in a work published by Zhang and co-workers⁸⁰. MSNPs with β -CD on the surface was synthesised and an adamantane moiety containing the targeting ligand RGD was included in the hydrophobic β -CD cavity. Hence from this work, it can be seen that by using a simple host-guest complex, different molecules can be added to the surface of the MSNPs. This serves as an easier method of imparting more properties such as, targeting and water solubility, to the MSNPs. Apart from using β -CD for the blocking of MSNPs pores, other cyclodextrins have also been employed for the same purpose. In a work by Kim and co-workers, various cyclodextrins were employed for its threading with PEI to block the pores of the MSNPs. It was observed that α -CD and γ -CD gave the best threading with β -CD being the less favourable due to its inability to form a stable polypseudorotaxane⁸¹. Another work by Zink and co-workers employed the use of the interaction of α -CD with azobenzene, with adamantane as the stopper, to prevent the α -CD from leaving the surface of the MSNPs. Initially trans-azobenzene is favoured due to it being thermally stable, however, upon the irradiation of light, a cis-azobenzene is formed. The change of the azobenzene from trans to cis causes the α -CD to shift in position from the azobenzene and this successively cause the release of drugs from pores of the MSNPs⁸².

In order to ensure that there is appropriate binding to the different cyclodextrins, researchers have employed a few ways to determine the host-guest ratio as well as the host-guest binding constant. The host-guest ratio is

determined by the job plot and the host-guest binding constant is derived by the Benesi-Hildebrand method. The two common ways to obtain a job plot and Benesi-Hildebrand plot is to use UV-vis spectroscopy or NMR⁸³. By noticing the shift of the peaks (Figure 10A), the change can be obtained and this can be correlated to the concentration or the ratio and the Hildebrand and job plot can be obtained respectively⁸⁴ (Figure 10B). By obtaining the binding ratio and binding constant of the host-guest complex, the ability of the cyclodextrin-guest complex to retain molecules within the pores of the MSNPs can be confirmed.

Figure 10. (A) NMR spectra obtained by varying the different host guest concentrations. The shift in the peaks reflect the binding of the host guest molecules. (B) Job plot obtained by plotting the amount of guest molecules with respect to the $\Delta\delta$ in chemical shift. (Reproduced with permission from ref 84a. Copyright 2014 The Royal Society of Chemistry.)

Apart from the use of cyclodextrins to block the pores of the MSNPs, other large molecules such as polymers have also been employed. As many polymers are long, they are able to cover the MSNPs pores to retain the cargoes. In a recent work by Lu and co-workers, a visible light-triggered hollow mesoporous silica nanoparticle system was employed for controlled drug release and cell imaging. After the loading of Dox, the photo-degradable amphiphilic co-polymer was coated on the surface of the MSNPs by self-assembly to retain the drugs within the hollow MSNPs. These hollow MSNPs were tested for cytotoxicity and observed under fluorescence microscopy. From the results

obtained, the cancer cells that were incubated with polymer coated MSNPs together with visible light irradiation showed significantly lower cell viability after 6 hrs. This reflected the good retention and release of drug by the use of a polymeric stopper⁸⁵.

Proteins and DNA have also been useful candidates for the retention of therapeutic molecules within the MSNPs pores. Besides the use of polymers, biological molecules such as proteins and DNA can also be used for pore blockage. Proteins have a size range of around 1-100 nm and hence are large and bulky⁸⁶. DNA on the other hand is usually long and they bind in a similar manner as polymers. Due to their large size and length, these macromolecules are able to cover the surface of the MSNPs and prevent the drug from releasing. Qu and co-workers employed a unique DNA sequence for the blockage of the MSNPs pores with rhodamine B as the model drug. The DNA sequence was conjugated on the surface of the MSNPs via a “click” reaction and the drugs were released by the denaturation of DNA by heat⁸⁷. In another work by Wang and co-workers, magnetic silica nanoparticles were firstly loaded with Dox, then Poly(D,L-lactic-co-glycolic acid) (PLGA) was coated over it, thereafter paclitaxel, another anticancer drug, was loaded and finally the MSNPs was grafted with transferrin. It was observed that by using a mixture of PBS with Tween® 80, release of Dox and paclitaxel was observed⁸⁸.

In addition to the above examples, there are many other groups that make use of nanoparticles to block the pore of the MSNPs. These nanoparticles are often much smaller than the MSNPs but are larger than the pores of the MSNPs. In order for successful blockage of the MSNPs pores, the common route employed is to graft thiol or amine functional groups on the surface of the MSNPs. As

these functional groups tend to bind to these small nanoparticles, the nanoparticles are able to adhere to the MSNPs surface and this will then facilitate the retention of drug within the MSNPs. Jiang and co-workers employed MSNPs containing silver nanoparticles for antibacterial activity. Figure 11 shows that the synthesis of the combined nanoparticle system adopted the use of triethoxysilane groups that contain amine functional groups chelated to Ag^+ ions. Thereafter, the Ag nanoparticles were synthesised by adding formalin to the Ag^+ containing MSNPs⁸⁹.

Figure 11. Schematic illustration of the synthesis of Ag nanoparticles on MSNPs by firstly chelating the Ag^+ ions with an ethylene diamine moiety before the formation of the MSNPs. (Reproduced with permission from ref 89. Copyright 2014 American Chemical Society.)

In a similar work by Qu and co-workers, MSNPs supported gold nanoparticles were synthesised for antibacterial properties. Amine functional groups were co-condensed into the pores of the MSNPs and gold nanoparticles were synthesised within it without any external stabilizing agent⁹⁰. Some groups also made use of other types of nanoparticles, like quantum dots, for the capping of MSNPs and release of drugs within the intracellular environment. Fu and co-workers employed MSNPs surface functionalised with graphene quantum dot (GQD) for drug delivery and imaging. The drug loading process occurs by the physical mixing of the drug with the MSNPs, thereafter the capping of the MSNPs occurs by an acylation reaction of the amine group on the MSNPs with

the GQDs. The drug release process of such a system employs the use of a low pH for the cleavage of the acetal bond⁹¹.

From the different ways of cargo retention and release, various types of cargoes can then be employed for therapeutic purposes.

1.3.4 Common anti-cancer cargoes

Apart from the design of the MSNPs, the cargoes loaded within the pores of the MSNPs are of utmost importance. It will determine the properties of the MSNPs and its therapeutic efficiency. The most common cargo that has been employed is the anticancer drug, Dox. Dox has proved to be effective for the treatment of a wide range of cancers and is now widely used in hospitals all around the world. Its molecular structure consists of an aromatic and sugar group whereby the aromatic group favours the intercalation to DNA (Figure 12). This disrupts the DNA replication process, thus retarding the growth of cancer cells⁹².

Figure 12. The intercalation of Dox with DNA (Reproduced with permission from ref 92. Copyright 2013 Nature Publishing Group.)

In addition to its good therapeutic properties, Dox has red fluorescence and is very water soluble. Hence, this makes it easy to monitor its loading and release

from the MSNPs⁹³. In a work by Nel and co-workers, various size MSNPs were synthesised and polyethyleneimine-polyethylene glycol copolymer was coated on the surface of MSNPs. Dox was loaded into the pores of the MSNPs via the interaction between the copolymer and the co-condensed phosphonate groups in the MSNPs. In vivo experiments revealed the presence of Dox in the tumor tissue by histological staining with its visualisation under fluorescence microscope⁴¹. In another work by the same group, Dox was loaded into the pores of the MSNPs and siRNA was coated on the MSNPs surface. This siRNA is able to restore Pgp knockdown in Dox resistant cell lines and hence restore the Dox sensitivity in these cell lines. This positive result was observed from the confocal microscopy images of the KV-V1 cells whereby red fluorescence was seen in the cytoplasm as well as in the nucleus. The detection of Dox in the nucleus further confirms its ability to chelate with DNA⁹⁴.

Figure 13. Mechanism for the interaction of Camptothecin that results in the irreversible arrest of the replication process. (Reproduced with permission from ref 95. Copyright 2006 John Wiley and Sons.)

Camptothecin is another common anticancer drug that is used as a cargo for MSNPs. Its mechanism of action towards cancer cells involves binding to the DNA complex and preventing the re-ligation of the DNA which then causes apoptosis⁹⁵ (Figure 13). Unlike Dox, camptothecin has poor water solubility

and does not fluoresce. Owing to this, many methods have been adopted to increase its solubility and this is most commonly done by loading camptothecin into various nanoparticles. For example, MSNPs can be functionalised with various water soluble groups and the drug can be easily delivered to the target site. This is seen in a work by Tamanoi and co-workers whereby the surfaces of the MSNPs were modified with trihydroxysilylpropyl methylphosphonate for the loading of camptothecin. Due to the insolubility of camptothecin in water, no release was observed when the drug loaded MSNPs were soaked in aqueous solution. When these MSNPs were incubated with PANC-1 cells, camptothecin was released in hydrophobic parts of the cells. As camptothecin does not fluoresce, absorbance is often used to monitor its release⁹⁶.

Apart from the above organic molecules, some inorganic molecules have also shown to possess anticancer properties and one such drug is cisplatin. In the presence of water, cisplatin forms an unstable complex and this intermediate readily interacts with DNA to prevent cell division and mitosis⁹⁷. The detection of cisplatin in vitro usually involves the use of inductively coupled plasma optical emission spectroscopy (ICP-OES) or inductively coupled plasma mass spectrometry (ICP-MS)⁹⁸. As cisplatin contains platinum as the main component, the cells containing cisplatin will be digested and tested for its platinum concentration. This mode of characterization of cisplatin was modified in a work by Kim and co-workers which involves the use of FITC labelled Pt(IV). This design aids in the detection of cisplatin release and its localization in cells⁹⁹.

Besides employing small molecules as drug cargoes, some groups have also investigated other cargoes like proteins and DNA. These cargoes are larger in

size and are usually not loaded into the pores of the MSNPs. Instead, they are immobilized on the surface of the MSNPs and are released upon a specific stimulus after the MSNPs are uptaken into the cells¹⁰⁰. In a work by Nel and co-workers, Dox was loaded into the pores of the MSNPs and siRNA that targets P-glycoprotein (Pgp) was attached to the surface via its interaction with a polyethyleneimine-polyethylene glycol (PEI-PEG) polymer chain¹⁰¹. Another work by Du and co-workers made use of proteins to block the pores of the MSNPs. In order to achieve this, the MSNPs surface were conjugated with ligands containing a mannose moiety at the end and the cargoes were kept within the nanoparticle pore by the interaction of mannose and concanavalin A (Con A)¹⁰². In addition to anticancer drugs and biological cargoes, another class of cargoes that are frequently loaded into the pores of MSNPs are photosensitizers. These cargoes exhibit anticancer properties by converting triplet oxygen to cytotoxic singlet oxygen in the presence of light and this process is known as photodynamic therapy (PDT)²⁵. Lo and co-workers made use of Pd-porphyrin (PdTPP) for photodynamic therapy and covalently conjugated them to the inner pores of the MSNPs (Figure 14A). Thereafter, the authors used a 532 nm laser operating at around 250 mW cm^{-2} , irradiated for 5 s with a total energy of 1.2 J per well for in vitro studies. From the results obtained (Figure 14B), significant cytotoxicity was observed when MSNPs containing the targeting ligand and PdTPP was incubated with human glioblastoma cells (U87 MG)¹⁰³. This result showed promise for the use of photodynamic therapy in cancer treatment.

Figure 14. (A) Synthesis of tri-functionalized MSNPs containing PdTPP, a photosensitizer in the pores of the MSNPs and a cRGD, a targeting moiety, on the surface of the MSNPs. (B) Cell viability of a) U87 MG and b) MCF-7 cells in the presence and absence of 532 nm NIR laser. (Reproduced with permission from ref 103. Copyright 2010 The Royal Society of Chemistry.)

1.3.5 Sensor molecules for biological detection

In addition to the use of various functional moieties on the surface or within the silica network for drug delivery purposes, the internal silane network can also be modified to achieve better diagnostic applications. Currently, many nanoparticle systems are able to achieve good diagnostics for the detection of various biological molecules such as glutathione, proteins, enzymes and many others^{55, 104}. These works commonly employ Förster resonance energy transfer

(FRET), photoinduced electron transfer (PET) and photoinduced charge transfer (PCT) for detection¹⁰⁵. MSNPs that serve as ratiometric sensors make use of the above methods to induce a change in the sensor system and this change can then be detected via fluorescence. Ratiometric sensors involve the use of two dye molecules with one being sensitive to the stimulus and the other being inert to the stimulus or both being sensitive to the stimulus but with opposite effect¹⁰⁶. In a work by Han and co-workers, the presence of acid quenched fluorescein while rhodamine 6G lactam exhibited fluorescence at 552 nm (Figure 15A). Owing to this, the group employed these two dyes for a ratiometric sensing of lysosomal activity¹⁰⁷ (Figure 15B).

Figure 15. (A) Synthesis of dual coloured MSNPs containing rhodamine 6G and fluorescein as the ratiometric sensor pair. (B) Changes in fluorescence intensity at various pH using the as-synthesised MSNPs (a) Dual wavelength excitation at FITC $\lambda_{\text{ex}} = 488$ nm and R6G-amide $\lambda_{\text{ex}} = 532$ nm. (b) Single wavelength excitation at $\lambda_{\text{ex}} = 504$ nm.

(Reproduced with permission from ref 107. Copyright 2011 The Royal Society of Chemistry.)

Zhang and co-workers also employed a rhodamine derivative, rhodamine 101, as the inert dye and used 8-aminoquinoline was used as the sensor dye. During the synthesis of the MSNPs, rhodamine 101 was introduced into the solid silica core, later a layer of mesoporous silica containing 8-aminoquinolin was coated over it. When the MSNPs (10^{-5} M) was tested with different ions, Zn^{2+} ions showed a threefold increase in I/I_0 value as compared to other cations and this reflected the selectivity of the as-synthesised MSNPs¹⁰⁸. Although these systems are useful for detection, many of them have a high detection limit or are only able serve the sole purpose of detection. In order to compensate for these disadvantages, the area of theranostics, which involves the combination of therapy and diagnostics in one nanoparticle system, has been gaining popularity¹⁰⁹. Theranostics gives nanoparticles a new lease of life by allowing them to be multifunctional and to achieve a “lab-on-chip” approach. In a recent work by Chen and co-workers, RGD coated MSNPs were synthesised with $[\text{Ru}(\text{phen})_2\text{p-MOPIP}](\text{PF}_6)_2 \cdot 2\text{H}_2\text{O}$ (RuPOP) loaded within the pores of the MSNPs (Figure 16A). RuPOP is a cytotoxic complex made of multiple pyridyl groups and have strong autofluorescence (Figure 16B). Owing to this, RuPOP has proven to be a good anticancer and diagnostic agent¹¹⁰. Another approach involving MSNPs for theranostic applications was performed by Wei and co-workers. Their work involved the use of an aggregation induced emission dye, An18, which is a derivative of 9,10-distyrylanthracene with an alkoxy end group. This dye was introduced into the surfactant core by means of van der Waals interaction during the MSNPs synthesis and the MSNPs showed good anticancer properties as well as in vivo imaging¹¹¹.

Figure 16. (A) Synthesis of MSNPs containing RuPOP as the drug and RGD as a targeting moiety. (B) Uptake of MSNPs within the cells at various time intervals of 0, 0.5, 1, 2, 4, 6, 8, 12 h with green fluorescence indicating higher uptake efficiency. (Reproduced with permission from ref 110. Copyright 2014 John Wiley and Sons.)

1.4 Common characterisation techniques for MSNPs

Apart from functionalising and loading the MSNPs with different types of molecules, there are some characterization techniques to determine the regularity of the MSNPs as well as the changes in surface functionalities of the MSNPs. A very common characterization technique to determine the shape and size of nanomaterials is by the use of an electron microscope. By applying the de Broglie postulate, the wavelength of a fast moving electron is much smaller compared to the wavelength of light. Hence, the images produced from an electron microscope have much higher resolution than that of an optical microscope and this helps in the study of the morphology of nanomaterials. There are two different types of electron microscope, the transmission electron microscope (TEM), and the scanning electron microscope (SEM). The two methods differ in the way their images are captured. When using the TEM for characterization, the regions with the nanoparticles scatter the electrons away and only the transmitted electrons are captured. This results in a dark two-dimensional image. In comparison, when SEM is used for characterization, the image that is captured results from the scattered electrons of the sample, hence

providing a three-dimensional image¹¹². Due to the operation principles of electron microscopy, materials containing atoms with high atomic number would provide better electron scattering and thus a clearer image. Besides using the electron microscope to view various morphologies, some electron microscopes are also equipped with the function of elemental analysis such as energy dispersive X-ray spectroscopy (EDX or EDS)¹¹³ or electron energy loss spectroscopy (EELS)¹¹⁴.

Although electron microscope gives information about the morphology of the MSNPs, the details within the MSNPs still have to be determined by other methods. From the TEM images, the pores of the MSNPs can usually be seen, however in order to accurately quantify the pore size of the MSNPs, powder X-ray diffraction (pXRD) have to be employed¹¹⁵. pXRD is often used to study the crystallinity of materials, hence the samples have to be grinded during the pXRD sample preparation. This grinding process provides the possibility of all different crystalline orientations in the powdered sample. Thereafter, the sample is mounted and X-ray is irradiated onto the powdered samples which then diffracts the X-rays at a few characteristic angles (θ) based on the Bragg's Law.

As MSNPs contain highly ordered hexagonal mesoporous structures, they are suitable materials that can be measured using pXRD (Figure 17A). MSNPs have a pore size of around 2.4 nm with 100 and 200 facets, hence small angle XRD is often used to measure the MSNPs pore size¹¹⁶.

Figure 17. (A) X-ray diffraction pattern for MSNPs. (Reproduced with permission from ref 110. Copyright 2013 American Chemical Society.) (B) Nitrogen gas absorption/desorption isotherms and (C) Barret-Joyner-Halenda pore distribution of the as-synthesised MSNPs. (Reproduced with permission from ref 37a. Copyright 2012 John Wiley and Sons.)

Besides measuring the pore size of the MSNPs, the pore volume and surface area of the MSNPs can also be measured by nitrogen gas (N_2) adsorption and desorption^{37a, 117}. The use of gas molecules for the detection of surface area and pore volume can be explained by the Brunauer-Emmet-Teller (BET) theory. Before a gas adsorption and desorption experiment is carried out, the sample has to undergo outgassing which is the removal of gas or vapours from the pores of the MSNPs. This is to ensure that there are no gas molecules that cover the surface of the MSNPs which might then cause an inaccurate determination of surface area. During the gas adsorption experiment, liquid nitrogen or other gases is adsorbed onto the surface of the MSNPs to form a monolayer. This causes the instrument to generate a plot between relative pressure of N_2 versus the amount of N_2 adsorbed. During the desorption experiment, the gas removed is measured with respect to the decrease in pressure. The graph combining the adsorption and desorption plots is known as the isotherm plot (Figure 17B) and there are a total of 5 types of isotherm plots (Type I to V) that are commonly used to characterise the different types of pores¹¹⁸ (Figure 18).

Figure 18. The five different types of isotherms for measuring the properties of porous materials. (Reproduced with permission from ref 119. Copyright 2014 Springer)

Type I isotherms occur for microporous compounds, Type II on the other hand occurs for non-porous powders, Type III isotherm occurs for materials whereby the adsorbate interacts more with the adsorbed layer than the material layer and Type IV and V is commonly seen in materials with pore size of 1.5 – 100 nm¹¹⁹. Using the BET theory, surface area and volume of the MSNPs can be calculated from the adsorption curve of the isotherm plot. In addition, using another model the Barrett-Joyner-Halenda (BJH) theory, pore size distribution of the MSNPs can be obtained (Figure 17B).

Besides the few characterisation techniques that probe the intrinsic characteristics of the MSNP, there are other techniques that probe the different surface groups or different functionalities within the MSNPs. One common qualitative technique is by infra-red (IR) spectroscopy whereby various functional groups can be observed based on their wavenumber (Figure 19A). When different molecules are grafted or co-condensed within the MSNPs network, measuring the IR of these solid MSNPs will reveal the various functional groups within it¹²⁰. The only drawback of this method of characterization is the overlap of the O-H peak that arises from the surface silanol groups, with other functional groups appearing in the same region for example N-H peaks and sp² C-H peaks. Another method that is used for the characterization of certain functional groups on MSNPs is the use of solid nuclear magnetic resonance (NMR). As compared to solution NMR, solid state NMR usually gives rise to broad peaks. This is due to the chemical shielding anisotropy, dipole-dipole coupling and quadruple coupling. Carbon-13 NMR is usually used for the characterization of the different functional groups on the

surface or within the MSNPs network and its analysis is similar to that with the solution NMR¹²¹ (Figure 19B).

Figure 19. (A) Infrared spectroscopy of MSNPs-SH, MSNP-COOH and MSNPs-Pt. (B) Solid carbon-13 NMR of the different MSNPs and changes in functional groups after conjugation of maleic anhydride. (Reproduced with permission from ref 121a. Copyright 2014 Elsevier)

In order to test the surface elements of the MSNPs, X-ray photoelectron spectroscopy (XPS) is often adopted. XPS makes use of X-ray to irradiate 1-10 nm of the sample surface and this causes the electrons of the different atoms on the sample to get ejected as photoelectrons. In order for the electrons to be ejected, they have to consist of a lower binding energy as compared to the x-ray source. The ejected electrons then travel towards a detector and the kinetic energy of the electron is detected¹²². This is translated to binding energy by taking the kinetic energy and subtracting it from the incident energy of X-ray. By matching the binding energy obtained from the X-ray photoelectron spectrum to a database, the corresponding element can be identified (Figure 20A). Besides the identification of the elements, XPS can also give an estimate of the percentage of each element within the sample¹²³.

Figure 20. (A) X-ray photoelectron spectroscopy of graphite oxide, graphene oxide, chemically reduced and electrochemical reduced graphene oxide. (B) Thermogravimetric analysis of the various MSNPs with epoxy, azido and photoresponsive polymer chains. (Reproduced with permission from ref 124. Copyright 2013 John Wiley and Sons.)

Apart from XPS that provides an estimate of the elemental composition of the sample, thermogravimetric analysis (TGA) also provides a quantitative value of the amount organic material within the sample. TGA is unable to identify each element or functional group but is able to identify the amount of organic moieties within each sample. It functions by measuring the weight loss of the sample in relation to the temperature of the heated sample. As the temperature increases, some components of the sample decompose and this causes the weight to decrease¹²⁴ (Figure 20B).

1.5 Research objectives

From the above sections, it can be concluded that nanoparticles play a very important role in drug delivery due to its ability to be multifunctional. MSNPs, in particular, are very easy to functionalise and its large pore volume and surface area widens its scope for various applications. Although MSNPs have proven to be very useful in drug delivery applications, there is still room for improvement for this system. Many of the MSNPs system have been focused on the delivery of therapeutic cargoes into cancerous cells usually by using

passive or active targeting. If active targeting is employed, the usual candidates will include folic acid and RGD. In addition, the optimizations of the amount of cargo that can be loaded within the different MSNPs systems are not widely investigated.

The objective of this study is to widen the application of MSNPs and adopt new ways to improve the drug delivery efficiency for the treatment of cancer and neuronal diseases. As MSNPs have been very commonly used for cancer therapy, the emphasis for the treatment of other diseases has been neglected. Owing to this, Chapter 2 describes the delivery of MSNPs into neuronal cells with the aid of a targeting ligand. By using MSNPs as drug delivery vehicles, we aim to deliver therapeutic cargoes specifically to neuronal cells in the brain to treat brain related diseases such as Parkinson and Alzheimer disease.

From previous reports, ligands that were similar to the neuronal targeting ligand used in Chapter 2, showed targeting properties towards cancer cells. This claim was investigated in Chapter 3 by conjugating the same ligand on the surface of MSNPs and loading the pore of the MSNPs with Dox to test their simultaneous therapeutic efficiency. As there is always a demand for better targeting molecules, this study would be beneficial for future drug delivery applications. In this work, the ligand was conjugated on the surface of the MSNPs and Dox was loaded and capped within the pores of the MSNPs via a disulfide bridged adamantane/ β -cyclodextrin (β -CD) stopper.

The use of disulfide capping agents has been a very common mode of drug loading and release with MSNPs. However, during the drug loading process, there are many parameters to consider and this often results in low drug loading

of the MSNPs. As there are only a few screening methods for this, drug loading studies are often known to be an arduous task. In Chapter 4, a new screening method for the loading and release of Dox from MSNPs was investigated. This method was adopted from organic chemistry research particularly in the area of catalysis. A catalysis type screening method of different parameters were investigated by varying each condition individually and working towards the achieving a higher drug loading capacity and release. This systemic approach gives us a quick and fast method to obtain good loading MSNPs.

In summary, this dissertation focuses on improving the applicability of MSNPs and improving its drug delivery efficiency with little emphasis on the synthesis of MSNPs.

1.6 References

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Chapter 2

Targeted Delivery of Mesoporous silica nanoparticles into Neuronal Cells

2.1 Introduction

From first chapter, it can be seen that MSNPs have been commonly used for the delivery of therapeutic cargoes into cancer cells. However, few works have employed MSNPs for the treatment of other diseases. Hence, in this chapter we would like to focus on the uptake of MSNPs into neuronal cells

In recent decades there has been a sudden rise in the number of patients with brain related diseases such as Alzheimer's disease¹, Parkinson's disease², epilepsy³ and many others. Many of these diseases are age related and arise due to neurodegeneration⁴. As neuronal cells are unable to regenerate after they die⁵, the ability to prolong their survivability is of utmost importance.

Acetylcholine is important for transmitting messages between nerve cells. Hence, patients with Alzheimer's disease contain low levels of acetylcholine in the nerve cells or have low numbers of nerve cells⁶. Drugs, such as donepezil, rivastigmine and galantmine, that treat Alzheimer's disease, prevent the breakdown of acetylcholine in the brain by inhibiting the enzyme acetylcholinesterase. This increases the concentration of acetylcholine, allows better signal transmission between nerve cells and the delay the onset of Alzheimer's disease⁷. Another drug that has been commonly used for the treatment of Alzheimer's disease is memantine. When the brain cells are damaged, a high concentration of glutamate

will be released within the brain cells⁸. This causes the neighboring brain cells to be further damaged due to the influx of Ca^{2+} ions⁹. Hence, by using memantine, the N-methyl-D-aspartate (NMDA) receptors are blocked and this prevents the prolonged influx of Ca^{2+} ions. This reduces the neuronal excitotoxicity and reduces cell death¹⁰.

Besides treating Alzheimer's disease, parkinson's disease is another age related disease that requires attention. Parkinson's disease is related to the disorder of movement and is caused by the loss of brain cells that produce dopamine¹¹. Dopamine is a chemical that relays messages between different parts of the brain to control movement in various parts of the body. A common drug that is used in the treatment of Parkinson's disease is L-DOPA. Its therapeutic efficiency involves its decarboxylation to produce dopamine¹². Other drugs that have been used for the treatment of Parkinson's disease include dopamine agonists and monoamine oxidase B (MAO-B) inhibitors. However, the newest drug used for the treatment of Parkinson's is amantadine. This drug works by acting as a weak antagonist of N-methyl-D-aspartate (NMDA) glutamate receptor¹³. It increases the dopamine release and reduces dopamine uptake which helps to regulate the amount of dopamine within the brain cells. This helps to prevent neuronal excitotoxicity and prevents the deterioration of the brain cells¹⁴.

There are two main classes of glutamate receptors, the ionotropic and the metabotropic glutamate receptors. Ionotropic glutamate (iGlu) receptors are faster in transmitting information and comprises of the α -amino-3-hydroxy-5-methyl-4-isoxalepropionic acid (AMPA), kainate and NMDA receptors. On the other hand,

metabotropic glutamate (mGlu) receptors deals with prolonged stimulus and encompasses the mGlu_n, where n is 1 to 8¹⁵. In both types of brain diseases, drugs that target the glutamate receptors play an important role. Hence there is an increasing focus for the search of drugs that are able to interact with iGlu¹⁶.

As mentioned above, one of the class of molecules that interact with iGlu are memantine and amantadine. Both of these molecules have a similar structure of an adamantane and binds as an uncompetitive antagonist at the open channel of the receptor. Another molecule that has been gaining popularity in its use as an uncompetitive antagonist of NMDA is dizocilpine¹⁷. Dizocilpine binds inside of the ion channel and prevents the influx of Ca²⁺ ions into the neuronal cells¹⁸. Although this drug has been widely used in many publications, further tests have to be done in order for it to be an FDA approved medication. One other class of iGlu antagonists that has been recently discovered is the polyamine toxins¹⁹. Polyamine toxins are small molecules and are commonly found in the venom of insects. They block the iGlu receptors by serving as an uncompetitive inhibitor and show anticonvulsant property in animal models²⁰.

Owing to this, the current work employs the use of a polyamine toxin that is known to have binding towards NMDA receptors. This work aims to use mesoporous silica nanoparticles (MSNPs) together with a neuronal targeting ligand for therapeutic purposes. The purpose of a targeting ligand is for the specific delivery of drugs to neuronal cells and if this is successful, the mesoporous silica nanoparticles can be used for future neuronal drug delivery applications. The use of MSNPs in this work widens its scope to allow for future drug delivery applications.

As MSNPs are known to be porous and easy to functionalize²¹, they serve as ideal drug carriers. Many groups have employed them by functionalizing the surface with targeting ligands and molecules for stimuli-controlled cargo release²². In addition, the MSNPs pores have been used for the storage of anti-cancer cargoes²³ and sensor molecules²⁴ for the purpose of detection.

Although many targeting ligands are available, polyamine ligand, N-(4-hydroxyphenylacetyl) spermine, was chosen for neuronal targeting due to its easy synthesis. In this work, the polyamine ligand, N-(4-hydroxyphenylacetyl) spermine, was labeled as Ligand 1 (**L1**) and it was reported that it acts as an NMDA receptor antagonist in humans. An alkyne moiety and BOC protecting groups were added to **L1** for its conjugation onto the MSNPs and for its easy synthesis and this molecule was labeled as compound **D**. In addition, fluorescein isothiocyanate was co-condensed within the MSNPs network to track the MSNPs location within the neuronal cells. Two different sizes of MSNPs were synthesised to test its uptake efficiency before proceeding to test the effect of the targeting ligand.

Figure 1. Structure of N-(4-hydroxyphenylacetyl) spermine which is the Ligand 1 (L1**) conjugated on the surface of MSNPs. It is known to act as an antagonist of human NMDA receptor.**

2.2 Experimental

Solvents and reagents were obtained from commercial sources and were used as received. Ultrapure water (18.2 $\Omega\cdot\text{cm}$ resistivity at 25°C and < 10 ppb Total Organic Carbon) was obtained from Satorius Ultrapure Water and used in all experiments. 10X Phosphate buffer saline (PBS) was used as purchased and diluted 10 times to obtain a 1X PBS. All nanoparticles were centrifuged at 10,000 rpm for 10 min. Merck Gerduran® Si 60 silica gel with particle sizes from 40 to 63 μm was employed for flash column chromatography. The silica gel was mixed with the eluting solvent and loaded into the column as slurry. ^1H and ^{13}C NMR spectra were obtained from Bruker AMX 400 spectrometer at ambient temperature. All ^1H NMR spectra were reported as chemical shift δ in units of parts per million (ppm) downfield from TMS (δ 0.0 ppm) or relative to deuterated solvents (7.26 ppm for CDCl_3). Multiplicities were provided as: s (singlet), d (doublet), t (triplet), q (quartet) and m (multiplet), and J coupling constants were expressed in Hz. The number of protons (n) for a given resonance was reported as nH. ^{13}C NMR spectra were reported as chemical shift (δ) in ppm with reference to residual solvent signal (77.0 ppm for CDCl_3). High resolution mass spectrometry (HR-MS) was carried out on Waters Q-ToF Premier MS. FT-IR data were obtained from SHIMADZU IR Prestige-21 spectrophotometer. JOEL JEM 1400 thermionic emission electron microscope was used to obtain transmission electron microscope (TEM) images at an acceleration voltage at 100kV. Zeta potential measurements were obtained from Zetasizer (Malvern Instrument Ltd., Worcestershire UK). Brunauer-Emmett-Teller (BET) nitrogen adsorption/desorption isotherms were obtained from an autosorp-IQ instrument from Quantachrome Instruments Corporation. Confocal

images were obtained using Zeiss LSM510 Meta or Zeiss LSM710 confocal microscopes.

2.2.1 Synthesis of organic compounds

2.2.1.1 Synthesis of Compound D (derivative of L1)

Scheme 1. Synthetic scheme of molecule D.

Methyl 2-(4-hydroxyphenyl)acetate (A): According to previously reported procedures²⁵, briefly, 4-hydroxyphenylacetic acid (1.0 g, 6.58 mmol) was dissolved in methanol (100 mL) and concentrated sulphuric acid (35 μ L) was then added. The mixture was refluxed for 5 h and the solution was concentrated in vacuo. The resulting residue was partitioned between ethyl acetate and water, and the aqueous layer was extracted 3 times with ethyl acetate. After the extraction, the organic solvent was removed under reduced pressure to obtain a white solid **A** (2.1 g, 64%). ¹H NMR (400 MHz, CDCl₃): δ 7.10 (d, J = 8.28 Hz, 2H), 6.74 (d, J = 8.40 Hz, 2H), 3.70 (s, 3H), 3.56 (s, 2H). ¹³C NMR (400 MHz, CDCl₃): δ 173.3, 155.1, 130.4, 125.5, 115.6, 52.3, 40.3. HRMS (H₂O/MeOH): m/z : calcd: 167.0708 [M]⁺; found: 167.0703. IR (KBr) $\bar{\nu}/\text{cm}^{-1}$: 3396.6, 3024.4 2953.0, 2918.3, 2845.0, 1712.8, 1614.4, 1597.1, 1518.0, 1438.9, 1228.7, 1012.6, 827.5.

Methyl 2-(4-(prop-2-ynyloxy)phenyl)acetate (B): Compound **A** (1.0 g, 6.02 mmol) was dissolved in THF (5 mL), and K₂CO₃ (1.7g, 12.0 mmol) was added to the solution. Thereafter, propargyl bromide (1.06 mL, 12.0 mmol) was added and the mixture was refluxed overnight. The solution was then concentrated under reduced pressure and the residue was partitioned between water and CH₂Cl₂. The organic layer was removed and the aqueous layer was extracted 3 times with CH₂Cl₂. The combined organic layers were concentrated in vacuo and the residue was purified by column chromatography by gradient elution (hexane/ethyl acetate 95:5 to 80:20) to give a brown oil **B** (0.93 g, 76%). ¹H NMR (400 MHz, CDCl₃): δ 7.20 (d, *J* = 8.32 Hz, 2H), 6.93 (d, *J* = 8.28 Hz, 2H), 4.66 (s, 2H), 3.67 (s, 3H), 3.56 (s, 2H), 2.52 (s, 1H). ¹³C NMR (400 MHz, CDCl₃): δ 172.2, 156.7, 130.3, 127.1, 115.0, 78.6, 75.6, 55.8, 52.0. HRMS (H₂O/MeOH): *m/z*: calcd: 205.0865 [M]⁺; found: 205.0865. IR (KBr) $\bar{\nu}$ /cm⁻¹: 3286.7, 3001.2, 2953.0, 2121.7, 1735.9, 1587.4, 1512.2, 1436.9, 1219.0, 1028.1, 819.8.

2-(4-(Prop-2-ynyloxy)phenyl)acetic acid (C): To a solution of **B** (1.0 g, 4.90 mmol) in THF (15 mL) was added NaOH (3.25M, 15 mL), and the resulting mixture was stirred at room temperature overnight. Thereafter, THF was evaporated and the resulting mixture was acidified and extracted with ethyl acetate 3 times. The organic layers were combined and concentrated in *vacuo* to yield a white solid **C** (0.87g, 93%). ¹H NMR (400 MHz, CDCl₃): δ 7.22 (d, *J* = 8.68 Hz, 2H), 6.94 (d, *J* = 8.272 Hz, 2H), 4.68 (d, *J* = 2.40 Hz, 2H), 3.60 (s, 2H), 2.51 (t, *J* = 2.38 Hz, 1H). ¹³C NMR (400 MHz, CDCl₃): δ 176.9, 156.9, 130.5, 126.3, 115.1, 78.5, 75.6, 55.9, 40.0. HRMS (H₂O/MeOH): *m/z*: calcd: 191.0708 [M]⁺; found:

191.0715. IR (KBr) $\bar{\nu}/\text{cm}^{-1}$: 3280.9, 3037.9, 2966.5, 2960.0, 2870.1, 2117.8, 1693.5, 1587.4, 1516.1, 1419.6, 1402.3, 1226.7, 1031.9, 806.3. **Compound (D):** Compound **C** (0.20 g, 1.08 mmol) was dissolved in THF (15mL) containing DCC (0.24 g, 1.19 mmol) and NHS (0.14 g, 1.19 mmol). The above mixture was then added dropwise to spermine (0.98 g, 4.85 mmol) dissolved in THF (30 mL). The reaction was stirred at room temperature overnight, and then the mixture was filtered. Di-*tert*-butyl-dicarbonate (3.26 g, 14.9 mmol) was added to the filtrate and the mixture was allowed to react overnight. THF was evaporated in vacuo and the crude residue was purified by silica chromatography by gradient elution (hexane/ethyl acetate 90:10 to 0:100) to obtain a pale yellow oil **D** (0.22 g, 54%). ^1H NMR (400 MHz, CDCl_3): δ 7.14 (d, $J = 7.84$ Hz, 2H), 6.85 (d, $J = 8.32$ Hz, 2H), 4.58 (s, 2H), 3.40 (s, 2H), 3.09 (m, 15H), 2.48 (s, 1H), 1.57 (m, 5H), 1.35 (m, 36H). HRMS ($\text{H}_2\text{O}/\text{MeOH}$): m/z : calcd: 675.4333 $[\text{M}]^+$; found: 675.4335. IR (KBr) $\bar{\nu}/\text{cm}^{-1}$: 3309.8, 3057.2, 2976.2, 2931.8, 2870.1, 2119.8, 1685.8, 1510.3, 1419.6, 1249.9, 1166.9, 1030.0, 868.0.

2.2.2 Synthesis of MSNPs

Scheme 2. Synthesis of route of MSNPs containing targeting ligand, L1.

Synthesis of MSNPs-60 / MSNPs-180: The MSNPs were synthesized according to previous reports²⁶. Fluorescein isothiocyanate (FITC, 4 mg) was added to a round bottom flask and it was purged with nitrogen. (3-Aminopropyl)triethoxysilane (APTES, 44 μL) in EtOH (1 mL) was then added and the resulting solution was stirred overnight at room temperature. The next day, cetyltrimethylammonium bromide (CTAB, 0.1 g) was dissolved in NaOH (0.35 mL, 2M) and distilled water (50 mL) in a separate round bottom flask. The resulting mixture was heated to 80°C before the addition of tetraethyl orthosilicate (TEOS, 0.5 mL) and the as-prepared FITC-APTES solution (50 μL). The solution was stirred for 1 min followed by the addition of ethyl acetate (0.5 mL). The mixture was further stirred for 2 h and the final yellow precipitate was centrifuged (10 000 rpm, 10 min) and washed twice with distilled water and ethanol. The precipitate was then dried under vacuum to afford the as-synthesized MSNPs-180.

The above synthesis route gives rise to MSNPs of 180 nm. In order to synthesize MSNPs with size 60 nm, the volume of 2M NaOH was decreased to 0.22 mL to obtain MSNPs-60. The remaining synthesis steps were similar for both sizes of MSNPs.

Synthesis of MSNPs-60-C / MSNPs-180-C: Anhydrous toluene (120 mL) was added to MSNPs-60 / MSNPs-180 (1.5 g) before the addition of 3-chloropropyltrimethoxysilane (375 μ L) and 2-[methoxy(polyethyleneoxy)-propyl] trimethoxysilane (650 μ L). The resulting mixture was refluxed overnight under inert conditions and the precipitate was collected by centrifugation (10 000 rpm, 10 min) and washed with once with toluene and twice with EtOH to yield MSNPs-60-C / MSNPs-180-C.

Synthesis of MSNPs-60-Cl / MSNPs-180-Cl: MSNP-60-C / MSNPs-180-C (1.0 g) was dispersed in methanolic solution (160 mL methanol and 9 mL HCl) and the mixture was refluxed overnight. The precipitate was collected by centrifugation (10 000 rpm, 10 min) and washed with MeOH to yield MSNPs-60-Cl / MSNPs-180-Cl depending on the initial size used.

After the investigating the effect of MSNPs sizes, it was determined that MSNPs-60-Cl showed better uptake as compared to MSNPs-180-Cl. Hence, subsequent synthesis employed only MSNPs-60-Cl.

Synthesis of MSNPs-60-N₃: MSNPs-60-Cl (1.3 g) was dispersed in DMF (15 mL), and sodium iodide (217 mg) was added to the solution. The suspension was stirred for 5 min before the addition of NaN₃ (94.3 mg). Thereafter, the mixture was

stirred at room temperature overnight and the precipitate was isolated by centrifugation (10000 rpm, 10 min). The precipitate was washed once with DMF and twice with EtOH to yield MSNPs-60-N₃.

Synthesis of MSNPs-60-D: Compound **D** (0.4 g) was dissolved in DMF (3 mL), and aqueous solution (1 mL) containing CuSO₄ (28.0 mg) and ascorbic acid (44.4 mg) was added to the mixture. MSNPs-60-N₃ (0.5 g) was added after stirring for 30 min and the mixture was allowed to stir at room temperature overnight. The precipitate was obtained by centrifugation (10000 rpm, 10 min) and washed extensively with water and MeOH to yield MSNPs-60-D.

Synthesis of MSNPs-60-L1: MSNPs-60-D (0.3 g) was added to dichloromethane (2 mL) containing trifluoroacetic acid (TFA, 2 mL). The solution was allowed to stir at room temperature overnight and the precipitate was obtained by centrifugation (10 000 rpm, 10 min). The precipitate was washed with MeOH and dried under vacuum to yield MSNPs-60-L1.

2.2.3 In vitro experiments

In vitro cell experiments were carried out by our collaborator in School of Biological Sciences, Nanyang Technological University. The experimental procedure to culture the primary neuronal cells was obtained from previous procedures published by our collaborator²⁷.

2.2.3.1 Fluorescence microscopy imaging

Poly-L-lysine treated glass coverslips were used concurrently with a monolayer of rat glial cells in order to culture primary neuronal cells obtained from E15.5 mouse embryonic cortices. Thereafter, the respective MSNPs were added to the solution to give a final MSNPs concentration of 25 μ M. The cells were incubated for 1 hr before the coverslips were stained with DAPI (4',6-diamidino-2-phenylindole) and viewed under the confocal microscope.

2.3 Results and Discussion

2.3.1 Effect of size on MSNPs uptake into neuronal cells

We begin the study by first synthesizing MSNPs of two different sizes and this was done by varying the amount of NaOH used for the reaction. Fluorescein isothiocyanate was co-condensed within the silica network in order to track the position of the MSNPs. The as-synthesised MSNPs were characterized by transmission electron microscopy (TEM) and the results obtained revealed the spherical morphology of the MSNPs with well defined pores of size around 3 nm for both sets of MSNPs. The smaller MSNPs (MSNPs-60) was measured to be around 60-70 nm (Figure 2a) while the bigger MSNPs (MSNPs-180) was measured to have a diameter of around 180-200 nm (Figure 2b).

Figure 2. a) TEM image of MSNPs-180-Cl b) TEM image of MSNPs-60-Cl.

These MSNPs were then grafted with (3-chloropropyl)triethoxysilane and 2-[methoxy(polyethyleneoxy)-propyl] trimethoxysilane. CTAB was then removed from the pores of the MSNPs and without further characterization, these two sets of MSNPs, MSNPs-60-Cl and MSNPs-180-Cl, were incubated with the neuronal cells. Due to the co-condensation of FITC within the MSNPs network, the observation of green fluorescence within the cells is an indication of MSNPs uptake. Figure 3 revealed that both sizes of MSNPs showed positive uptake into neuronal cells with the MSNPs largely localized in the nucleus and in some areas of the dendrites. In order to locate the nucleus of the neuronal cells, a blue dye, which is commonly known as DAPI (4',6-diamidino-2-phenylindole), was used as a nuclear staining agent. The overlap of the green and blue fluorescence is an indication of MSNPs uptake into the nucleus of the cells. Neuronal cells incubated with MSNPs-60-Cl showed lesser clumps in the surrounding environment as compared to MSNPs-180-Cl. Owing to the small size, the MSNPs-60-Cl was better dispersed and this

improves its uptake into the neuronal cells. Therefore, MSNPs-60-Cl was chosen as the ideal size for further modification for enhanced uptake into neuronal cells.

Figure 3. Confocal fluorescence microscope image of neuronal cells to reflect the uptake of a) MSNPs-60-Cl and b) MSNPs-180-Cl with green fluorescence indicating positive uptake. Purple fluorescence was attributed to Alexa Fluor 647 for the complete staining of the neuronal cells.

2.3.2 Effect of targeting ligand on uptake into neuronal cells

Further modification of the MNSPs-60-Cl involved substituting the chloride moiety with azide groups. Later, compound **D**, the protected form of **L1**, was conjugated with the azide groups via a “click” reaction followed by acid deprotection of the BOC groups on compound **D**. The newly functionalized MSNPs are known as MSNPs-60-L1. These MSNPs were then further characterized by IR (Figure 4a) to identify changes in the surface functional groups. After the substitution of the azido moieties on the surface of the MNSPs, a very small peak at around 2129 cm^{-1} was observed. After the conjugation of compound

D with these azido groups, very distinct peaks at 2980 cm^{-1} and 2966 cm^{-1} were seen and these peaks belong to the C-H alkyl stretch of the BOC protecting groups that are present on compound **D**. Comparing the IR spectrum of compound **D** (Figure 4b) and MSNPs conjugated with compound **D** (Figure 4a), both spectrums reveal a similarly-shaped large peak at the 2900 cm^{-1} and this reflects the successful conjugation of compound **D** to the MSNPs. Upon deprotection of the BOC groups on the compound **D**, the IR alkyl C-H stretch from the BOC groups disappeared to give the final Ligand **1** on the surface of the MSNPs.

Figure 4. a) IR spectrum of the various MSNPs synthesized b) IR spectrum of Compound D for comparison of MSNPs before and conjugation with compound D.

Besides employing IR for the detection of the deprotection reaction on the surface of the MSNPs, zeta potential was also used as an indication of successful deprotection. Table 1 shows that a negative zeta potential after the conjugation of compound **D**. This is possibly attributed to the various silanol groups present on the surface of the MSNPs. The remaining molecules functionalized on the surface of the MSNPs are known to be neutral thus they do not contribute to any charge to the MSNPs. However, after the deprotection of the BOC groups to yield the amine functionalites, a positive zeta potential was measured and this is due to the protonation of the amine functional groups on the surface. The change in zeta potential from negative to positive indicates the successful deprotection of the amine functional groups.

MSNPs	Zeta Potential	Standard Deviation
MSNPs-60-D	-21.4	5.20
MSNPs-60-L1	31.6	5.25

Table 1. Changes in zeta potential of MSNPs-60-D and MSNPs-60-L1 shows the successful conjugation and deprotection.

In order to measure the pore volume and surface area of the as-synthesized MSNPs, an adsorption-desorption isotherm plot was obtained and the Brunauer–Emmett–Teller (BET) analysis was applied. The adsorption-desorption isotherm showed a type IV isotherm (Figure 5a) which is a characteristic of MSNPs, a pore volume of 1.47 mL g⁻¹ and surface area of 1348.7737 m² g⁻¹. The pore volume and surface area is slightly larger the reported literatures due to the difference in size of the MSNPs synthesised. By applying the BJH pore size model, the average pore size

was obtained to be 2.79 nm which is close to that obtained from the TEM image (Figure 5b).

Figure 5. a) Absorption/desorption type 4 isotherm plot of MSNPs-60-Cl. b) BJH pore size of MSNPs-60-Cl determined by the isotherm plot.

Upon confirming the presence of the ligand on the surface of the MSNPs, MSNPs-60-L1 was incubated with the neuronal cells to observe its uptake. From the confocal images, MSNPs-60-L1 showed good uptake into the neuronal cells with the nucleus being stained green and the dendrites having a slight uptake (Figure 6). When comparing Figure 3 and 6, both MSNPs-60-L1 and MSNPs-60-Cl showed significant uptake into the nucleus. However, in the case of MSNPs-60-L1, a brighter green fluorescence was observed in the nucleus of the neuronal cells which indicates a higher concentration of MSNPs within the nucleus and better uptake efficiency. In addition, the dendrites are more visible when MSNPs-60-L1 was used. These observations indicate that the presence of the Ligand 1 promotes the uptake of the MSNPs as compared to MSNPs without the ligand.

Figure 6. Confocal fluorescence microscope image of neuronal cells to monitor the uptake of MSNPs-60-L1 with green fluorescence indicating positive uptake. Purple fluorescence was attributed to Alexa Fluor 647 for the complete staining of the neuronal cells.

2.4 Conclusion

In conclusion, when comparing the use of MSNPs-CI with different sizes, the smaller MSNPs-60-CI showed better uptake with less background interference as compared to that of MSNPs-180-CI. Further experiments were then carried out with MSNPs-60-CI by conjugating a targeting ligand to the MSNPs surface. The MSNPs were characterized and fluorescence microscopy images revealed that an enhanced uptake was observed for MSNPs-60-L1. This shows the feasibility of the as-synthesized MSNPs for future use neuronal drug delivery applications.

2.5 References

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Chapter 3

An iGlu Receptor Antagonist and Its Simultaneous Use with an Anticancer Drug for Cancer Therapy

3.1 Introduction

In the previous chapter it was identified that glutamate receptors play an important role in the proper functioning of neuronal cells. Glutamate plays a very important role in the synaptic transmission of the mammalian central nervous systems.¹ Owing to this fact, glutamate receptors are commonly known to be highly expressed on the membrane of neuronal cells.² In the neuronal cells, glutamate binds to the ionotropic glutamate (iGlu) receptors and regulates the amount of calcium ions in the postsynaptic cleft.³ Overstimulation of these receptors causes an overload of Ca^{2+} ions in cells, which could result in the cell damage and subsequent cell death.⁴ The iGlu receptors are essential in memory and learning. The dysfunction of such receptors are related to many neurogenerative disorders,⁵ such as epilepsy, lateral sclerosis, Parkinson's and Alzheimer's diseases⁶. Hence, by designing MSNPs that were small as well as by conjugating targeting molecules on its surface, the MSNPs can be more easily uptaken by the neuronal cells.

In recent years, it was observed that some cancer cell lines also contain glutamate receptors.⁷ Owing to this, there has been a growing trend in the usage of glutamate receptor antagonists for the treatment of cancer.⁸ Ikonomidou et al. observed that iGlu antagonists, dizocilpine and GYKI5246, were able to prevent the proliferation of colon adenocarcinoma, astrocytoma, breast and lung carcinoma, as well as

neuroblastoma cells.^{8e} Another work by Yu and co-workers reported the use of various types of isoquinolines, which are known to be non-competitive inhibitors of AMPA, for the treatment of five human cancer cell lines in vitro.⁹ Although the results of such treatments seem to be effective in cancer therapy,¹⁰ reports for the simultaneous use of a neuroprotective drug and an anticancer drug on a metastatic cancer model are still scarce. Thus, in this study, we adopted the same neuroprotective drug in Chapter 2, which is an iGlu receptor antagonist, and investigated its therapeutic effect on a metastatic melanoma cancer model (B16F10).^{7, 11} As mentioned in the previous Chapter, although there are a couple of iGlu receptor antagonists available, we selected N-(4-hydroxyphenylacetyl) spermine (**L1**) for its easy synthesis and functionalization. To extend the scope of this research, an anticancer drug, Dox, was employed along with the prepared neuroprotective drug to investigate their simultaneous therapeutic efficiency.

To allow these two drugs to be concurrently delivered to the B16F10 cells, mesoporous silica nanoparticles (MSNPs) were employed as the delivery vehicles (Scheme 1).¹² MSNPs have been commonly used in the drug delivery due to their large surface area and easy functionalization.¹³ Furthermore, their large volume allows a high drug-loading capability within the mesopores, which can be released upon a specific stimulus.¹⁴ In this work, multifunctional MSNPs were designed by conjugating the iGlu receptor antagonist, N-(4 hydroxyphenylacetyl) spermine, onto the surface of MSNPs through a “click” reaction (Scheme 2).¹⁵ On the other hand, Dox was loaded within the mesopores of MSNPs and capped by disulfide-bridged adamantane/ β -cyclodextrin (β -CD) stopper.¹⁶ As cancerous cells express a

high concentration of glutathione (GSH), the successful delivery of the multifunctional MSNPs into the cancerous cells enables GSH to react with the disulfide bond, thereby releasing the loaded Dox from the mesopores.¹⁷ Simultaneous delivery and therapeutic efficiency of these two drugs for the treatment of B16F10 cells were investigated in vitro.

Scheme 1. Schematic illustration of the different components used for the preparation of multifunctional MSNPs as the drug carriers with subsequent therapeutic application in vitro.

Scheme 2. Synthetic route for the preparation of MSNPs-6.

3.2 Experimental

Solvents and reagents were obtained from commercial sources and were used as received. Ultrapure water (18.2 $\Omega\cdot\text{cm}$ resistivity at 25°C and < 10 ppb Total Organic Carbon) was obtained from Satorius Ultrapure Water and used in all experiments. 10X Phosphate buffer saline (PBS) was used as purchased and diluted 10 times to obtain a 1X PBS. All nanoparticles were centrifuged at 10,000 rpm for 10min. Merck Gerduran® Si 60 silica gel with particle sizes from 40 to 63 μm was employed for flash column chromatography. The silica gel was mixed with the eluting solvent and loaded into the column as slurry.

^1H and ^{13}C NMR spectra were obtained from Bruker AMX 400 spectrometer at ambient temperature. All ^1H NMR spectra were reported as chemical shift δ in units of parts per million (ppm) downfield from TMS (δ 0.0 ppm) or relative to deuterated solvents (7.26 ppm for CDCl_3). Multiplicities were provided as: s (singlet), d (doublet), t (triplet), q (quartet) and m (multiplet), and J coupling constants were expressed in Hz. The number of protons (n) for a given resonance was reported as nH. ^{13}C NMR spectra were reported as chemical shift (δ) in ppm with reference to residual solvent signal (77.0 ppm for CDCl_3). High resolution mass spectrometry (HR-MS) was carried out on Waters Q-Tof Premier MS. FT-IR data were obtained from SHIMADZU IR Prestige-21 spectrophotometer. UV-Vis spectroscopy experiments were conducted using a SHIMADZU UV-3600 UV-VIS-NIR spectrophotometer. Drug release profiles were carried out with Varian Cary Eclipse fluorescence spectrophotometer equipped with water circulated

temperature controller. JOEL JEM 1400 thermionic emission electron microscope was used to obtain transmission electron microscope (TEM) images at an acceleration voltage at 100kV. Scanning electron microscope (SEM) images were recorded on JOEL JSM-6340F field emission electron microscope operating at an acceleration voltage at 30 kV. Zeta potential measurements were obtained from Zetasizer (Malvern Instrument Ltd., Worcestershire UK). Powder X-ray diffraction (XRD) measurements were carried out on SHIMADZU XRD-6000 Labx diffractometer at 40 kV and 30 mA using Cu K α radiation ($\lambda = 1.5418 \text{ \AA}$). X-ray photoelectron spectroscopy (XPS) measurements were performed with a Phiobos 100 spectrometer using Mg X-ray radiation source (SPECS, Germany) for the wide-range and high resolution scans at 12.53 kV. The XPS samples were coated on a conductive carbon tape, which was then affixed onto an aluminum XPS sample holder. The Si/N ratios were achieved from the wide-range scan XPS measurements. Brunauer-Emmett-Teller (BET) nitrogen adsorption/desorption isotherms were obtained from an autosorp-IQ instrument from Quantachrome Instruments Corporation.

3.2.1 Synthesis of MSNPs

Synthesis of MSNPs-0: The MSNPs were synthesized according to previous reports.¹⁸ Fluorescein isothiocyanate (FITC, 4 mg) was added to an oven-dried round bottom flask purged with N₂. The solid was treated with (3-aminopropyl)triethoxysilane (APTES, 44 μ L) in ethanol (1 mL) in the dark at room temperature for 24 h. The next day, cetyltrimethylammonium bromide (CTAB, 0.1

g) was dissolved in distilled water (50 mL), and NaOH (0.35 mL, 2M) was added to the solution. The mixture was then heated to 80°C followed by addition of tetraethyl orthosilicate (TEOS, 0.5 mL) and the above FITC-APTES solution (50 μ L). The resulted solution was stirred for 1 min before the addition of ethyl acetate (0.5 mL). The mixture was further stirred for 2 h, and the obtained yellow solid was filtered and washed three times with distilled water and MeOH. The precipitate was then dried under vacuum to afford the as-synthesized MSNPs-0.

Synthesis of MSNPs-1: MSNPs-0 (1.5 g) was added to anhydrous toluene (120 mL), and 3-chloropropyltrimethoxysilane (375 μ L) was added to the solution. The resulting mixture was heated at reflux under N₂ overnight. Thereafter, the precipitate was collected by centrifugation (10 000 rpm, 10 min) and washed with toluene and EtOH to yield MSNPs-1.

Synthesis of MSNPs-2: MSNPs-1 (1.3 g) was added to N,N-dimethylformamide (DMF, 15 mL), and sodium iodide (217 mg) was added to the solution. The solution was stirred for 5 min and NaN₃ (94.3 mg) was added thereafter. The mixture was stirred at room temperature overnight and the precipitate was isolated by centrifugation (10000 rpm, 10 min). The precipitate was washed with DMF and EtOH to yield MSNPs-2.

Synthesis of MSNPs-3: MSNP-2 (1.0 g) was suspended in anhydrous toluene (80 mL), and 2-[methoxy(polyethyleneoxy)-propyl] trimethoxysilane (0.65 mL) and 3-mercaptopropyltrimethoxysilane (1.5 mL) were added to the solution. The resulting solution was heated at reflux under N₂ overnight. The precipitate was obtained by

centrifugation (10000 rpm, 10 min) and washed with toluene and EtOH to yield MSNPs-3.

Synthesis of MSNPs-4: MSNP-3 (1.0 g) was added to a flame-dried round bottom flask, and methanol (160 mL) and HCl (9 mL) were added. The mixture was heated at reflux overnight, and the precipitate was collected by centrifugation (10 000 rpm, 10 min) and washed with MeOH to yield MSNPs-4.

Synthesis of MSNPs-5: Compound **D** (0.4 g) was dissolved in DMF (3 mL), and then an aqueous solution (1 mL) containing CuSO₄ (28.0 mg) and ascorbic acid (44.4 mg) was added to the mixture. Thereafter, MSNPs-4 (0.5 g) was added and the mixture was allowed to stir at room temperature overnight. The precipitate was obtained by centrifugation (10000 rpm, 10 min) and washed extensively with water and MeOH to yield MSNPs-5.

Synthesis of MSNPs-6: MSNPs-5 (0.3 g) was added to dichloromethane (2 mL), and then trifluoroacetic acid (TFA, 2 mL) was added. The solution was allowed to stir at room temperature overnight, and the resulting precipitate was obtained by centrifugation (10 000 rpm, 10 min). The precipitate was washed with MeOH and dried under vacuum to yield MSNPs-6.

3.2.2 Synthesis of Compound **D**:

Compound **D** is a derivative of L1. The synthesis of molecule **D** is adopted from Chapter 2.

3.2.3 Synthesis of compound 2:

Scheme 3. Synthetic scheme of compound 2, which binds with β -CD to prevent the leakage of drug from the pores of the MSNPs.

3-(pyridin-2-yl)disulfanylpropanoic acid (**E**): According to previously reported procedures¹⁹, briefly, 2,2'-dipyridyldisulphide (3.75 g, 17.0 mmol) was dissolved in ethanol (10 mL) with glacial acetic acid (0.4 mL). The solution was stirred vigorously, and a solution of 3-mercaptopropanoic acid (739 μ L, 8.48 mmol) in ethanol (5 mL) was added dropwise. The reaction mixture was stirred overnight and then ethanol was removed by evaporation. The resulting yellow oil was dissolved in a solvent mixture (10 mL) of DCM:EtOH (3:2, v/v), which was applied to 75 mL Al_2O_3 column equilibrated with the same solvent system. The column was eluted with the same solvent system until the yellow fraction was eluted. The desired product was then eluted with a mixture (100 mL) of DCM/EtOH (3:2, v/v) containing acetic acid (4 mL). The solvent and acetic acid were then removed under low pressure to yield viscous pale yellow oil **E** (1.56 g, 86%). ^1H NMR (400 MHz, CDCl_3): δ 10.21 (s, 1H), 8.47 (d, J = 4.80 Hz, 1H), 7.69-7.64 (m, 2H), 7.14-7.11 (m, 1H), 3.04 (t, J = 6.94 Hz, 2H), 2.79 (t, J = 6.94 Hz, 2H). ^{13}C NMR (400 MHz, CDCl_3): δ 176.1, 159.6, 149.4, 137.6, 121.2, 120.4, 33.9, 33.7. HRMS ($\text{H}_2\text{O}/\text{MeOH}$): m/z : calcd: 216.0153 $[\text{M}]^+$; found: 216.0512. IR (KBr) $\tilde{\nu}/\text{cm}^{-1}$: 3078.4, 2877.8, 2590.4, 1728.2, 1581.6, 1562.3, 1448.5, 1423.5, 1188.2, 771.5.

Compound **2**: To a solution of compound **E** (1.00 g, 4.65 mmol) in THF (10 mL), N,N-dicyclohexylcarbodiimide (1.25 g, 6.05 mmol) and N-hydroxysuccinimide (0.70 g, 6.05 mmol) were added. The mixture was stirred for 1 h before the addition of triethylamine (1.00 mL, 7.2 mmol) and adamantanamine hydrochloride (1.31 g, 6.98 mmol). The solution was stirred overnight and the resulting precipitate was filtered away. The filtrate was evaporated to dryness and was purified by column chromatography by gradient elution (hexane/ethyl acetate 95:5 to 60:40) to give a pale yellow solid **2** (1.21 g, 75%). ¹H NMR (400 MHz, CDCl₃): δ 8.47 (d, *J* = 4.84 Hz, 2H), 7.68-7.62 (m, 2H), 7.12-7.09 (m, 1H), 3.05 (t, *J* = 6.80 Hz, 2H), 2.51 (t, *J* = 6.82 Hz, 2H), 2.07 (s, 3H), 2.01 (s, 6H), 1.68 (s, 6H). ¹³C NMR (400 MHz, CDCl₃): δ 169.7, 159.9, 149.6, 137.1, 120.9, 120.1, 52.2, 41.7, 36.8, 36.4, 34.9, 29.4. HRMS (H₂O/MeOH): *m/z*: calcd: 349.1408 [M]⁺; found: 349.1400. IR (KBr) $\tilde{\nu}/\text{cm}^{-1}$: 3053.3, 2914.4, 2850.8, 1664.6, 1577.8, 1545.0, 1450.5, 1417.7, 1357.9, 1199.7, 771.5.

3.2.4 Amine quantity tests

When XPS was used as the method for the determination of successful ligand conjugation, the difference in nitrogen content before and after the conjugation of compound **D** was obtained. When ninhydrin was used for the quantification of amine amount on the surface of MSNPs, a standard curve was prepared with different concentrations of aminopropanol in the presence of ninhydrin (4.5 mM) before any measurements were made. Thereafter, a ninhydrin solution (4.5 mM)

containing MSNPs-6 (2 mg) and another solution without MSNPs-6 were measured. The difference in absorbance wavelength at 496 nm was used to calculate the amount of compound **D** with respect to the amount of MSNPs.

3.2.5 Procedure for *in vitro* studies

3.2.5.1 Materials for *in vitro* experiments

Mouse melanoma cells (B16F10) and mouse fibroblast cells (NIH3T3) were obtained from ATCC and the cells were cultured at 5% CO₂ at 37°C. Dulbecco Minimum Essential Medium (DMEM) added with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS) was used as the medium for all *in vitro* experiments. FBS and DMEM containing sodium pyruvate and L-glutamine were purchased from PAA laboratories. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Alfa Aesar and the MTT solution (5 mg mL⁻¹) was prepared by dissolving the MTT crystal in 1X PBS. Tecan Infinite 200 PRO microplate reader was used to obtain cell cytotoxicity results. Fluorescence images were obtained using a Nikon TE2000-E Confocal Fluorescence microscope with a Nikon TE2-PS100W high-pressure mercury lamp.

3.2.5.2 Fluorescence microscopy imaging

To observe the uptake of nanoparticles in the cells, B16-F10 cells and NIH3T3 cells were cultured separately in 35 mm glass-bottomed dishes with an initial seeding density of 2X10⁵. The cells were maintained in the growth medium for 24

h and were subsequently incubated with MSNPs-4 and MSNPs-6 to achieve a final concentration of 0.1 mg mL^{-1} for each MSNPs. After 4 h, the medium was removed and the cells were washed thrice with 1X PBS (2 mL). Fresh medium was then added and the cell culture was incubated for another 24 h. The medium was removed and the cells were washed three times with 1X PBS and fixed with 10% formaldehyde in 1X PBS for 20 min at 37°C . The formaldehyde solution was removed and the cells were again washed with 1X PBS three times. The cells were then incubated with 4',6-diamidino-2-phenylindole (DAPI) solution in 1X PBS (1 mL, $1 \text{ } \mu\text{g } \mu\text{L}^{-1}$) for 20 min, and were later washed three times with 1X PBS. The cells were immersed in 1X PBS (2 mL) and were ready for fluorescence microscopy imaging using Nikon Eclipse TE2000-E confocal fluorescence microscope with Nikon TE2-PS100W high-pressure mercury lamp. The above procedure was repeated to obtain the fluorescence microscopy images for MSNPs-4-Dox and MSNPs-6-Dox at nanoparticle concentrations of 0.23 mg mL^{-1} and 0.10 mg mL^{-1} , respectively.

3.2.5.3 Cells viability assays

A typical cell viability assay was carried out by seeding the respective cell lines into a 96-well plate at a density of 1×10^4 cells per well and incubated with Dulbecco's Modified Eagle's medium (DMEM, 100 μL) for 24 h. The respective sample solutions containing various MSNPs (10 μL) were then added to each well with water serving as the negative control, and these plates were incubated for 4 h. Thereafter, the medium was removed and the cells were washed once before fresh medium (100 μL) was added into the wells. The cells were incubated for another

24 h, and the medium was replaced with medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (0.5 mg mL⁻¹) before a further incubation of 4 h. Then, the medium was removed and dimethyl sulfoxide (DMSO, 100 µL) was added to dissolve the purple crystals. Absorbance reading at 562 nm was performed on a Tecan Infinite 200 PRO microplate reader with the percentage cell viability determined by $[A_{\text{sample}}]/[A_{\text{control}}]$, in which $[A_{\text{sample}}]$ and $[A_{\text{control}}]$ are the average absorbance of the test and control samples. Each sample was performed with 8 replicates and the results are expressed as mean values with error bars representing the standard deviation of the set of readings.

3.2.6 Drug loading and release studies

3.2.6.1 Drug loading

MSNPs-4 (20 mg) was added to distilled water (1 mL) containing Dox (4 mg mL⁻¹), and the mixture was allowed to stir overnight. In the next day, water was evaporated and EtOH (1 mL) containing compound 2 (43.2 mg) was added to the mixture. In the following day, EtOH in the mixture was evaporated and replaced with water (1 mL) containing β-CD (50 mg). The mixture was allowed to stir overnight, and the nanoparticles were obtained by centrifugation (10000 rpm, 10 min) and washed extensively with distilled water to yield drug loaded MSNPs-4-Dox. This procedure was repeated with MSNPs-6 in order to obtain the drug loaded MSNPs-6-Dox.

3.2.6.2 Loading capacity

In order to calculate the loading capacity, a standard Dox curve was prepared via UV-Vis spectroscopy. The loading percentage of Dox within the pores of MSNPs was determined by measuring the UV-Vis absorption of Dox before and after the introduction of MSNPs. The difference in the intensity at the absorption maxima ($\lambda_{\text{abs}} = 480 \text{ nm}$) was then obtained, which reflects the amount of Dox loaded into the nanoparticles.

3.2.6.3 Drug release studies

The β -CD capping agent was attached to the surface of MSNPs via binding to the adamantane group that was bridged by a cleavable disulfide bond. MSNPs-4-Dox (4 mg) was placed in a cuvette and was carefully filled with distilled water (3 mL, pH 7.4). GSH was then added to the cuvette to give a final concentration (5 mM), and fluorescence readings at 593 nm ($\lambda_{\text{ex}} = 480 \text{ nm}$) under 5 min intervals were recorded until there was no more increase in the fluorescence intensity. The same procedure was performed for MSNPs-6-Dox to obtain its release profile.

3.3 Results and discussion

3.3.1 Synthesis and characterization of functionalized MSNPs

The use of MSNPs as drug delivery vehicles has shown a lot of promise in the treatment of many types of cancers²⁰. Due to the easy synthesis and functionalization, in the present work, MSNPs were adopted as a platform for the

dual delivery of the iGlu receptor antagonist **L1** and Dox. The integration of these two drugs in a single nanoparticle system enhances the efficiency for the cancer treatment. Briefly, the MCM-41-type silica nanoparticles were synthesized through a template-directed co-condensation method²¹ before grafting 3-chloropropyltriethoxysilane onto the surface of the obtained MSNPs. The chloride moiety was then substituted with an azide group followed by grafting 3-mercaptopropyltriethoxysilane and 2-[methoxy(polyethyleneoxy)-propyl]trimethoxysilane. The surfactant was later removed to yield MSNPs-4. To anchor iGlu receptor antagonist **L1** onto the surface of MSNPs-4, an alkyne moiety was introduced onto **L1** to yield molecule **D**. A “click” reaction was then performed between molecule **D** and MSNPs-4 to yield MSNPs-5. The final deprotection of the tert-butyl groups from the molecule **D** on MSNPs-5 afforded MSNPs-6.

Figure 1. (a) BET isotherms for MSNPs-0 without CTAB and for MSNPs-6. (b) XRD measurement for the surfactant removed MSNPs-0 (without CTAB).

To characterize the surface area and pore volume of the MSNPs-0, the surfactant was removed and Brunauer–Emmett–Teller (BET) analysis was performed. A type

IV isotherm (Figure 1a) was obtained for the MSNPs with a pore volume of 1.123 mL g⁻¹ and a surface area of 1084.843 m² g⁻¹. The pore size was determined to be 2.89 nm by using the Barret–Joyner–Halenda (BJH) model (Figure 1b). After the functionalization with different moieties on the surface to obtain MSNPs-6, the pore volume and surface area decreased to 0.296 mL g⁻¹ and 543.903 m² g⁻¹ respectively with a pore size remaining almost the same at 2.95 nm. Powder X-ray diffraction (XRD) spectrum of MSNPs-6 also showed a characteristic hexagonal and well-ordered porous structure with a pore size of 4.02 nm. As the calculation of the pore size from the XRD analysis considers the thickness of the silica wall around the pore, the pore size value obtained from the XRD measurement was slightly higher than that obtained from the BJH model. MSNPs-6 was further characterized by both transmission electron microscope (TEM) and scanning electron microscope (SEM), showing a spherical morphology with a diameter around 200 nm (Figure 2a and b). Based on these results, the presence of mesoporous structure for the as-synthesized nanoparticles was confirmed.

Figure 2. a) TEM and b) SEM images of MSNPs-6.

The changes in the surface functionalities of MSNPs were determined by FTIR spectroscopy and X-ray photoelectron spectroscopy (XPS). The examination of the FTIR spectra revealed that MSNPs-2 contains an azide peak at 2029 cm^{-1} , due to the azide substitution that was performed on the chloride moiety of MSNPs-1. After the conjugation of molecule **D** onto the surface of MSNPs-4, a strong carbonyl peak at 1571 cm^{-1} for MSNPs-5 was observed, which was attributed to the tert-butyl carbamate functional group of molecule **D**. The deprotection of the tert-butyl carbamate protecting group led to a weak carbonyl signal for MSNPs-6, indicating the successful deprotection from MSNPs-5 (Figure 3).

Figure 3. FTIR spectra of MSNPs-1–6.

The data obtained from XPS also exhibited similar trends, in which an increase in the nitrogen content was observed after the conversion of chloride to the azide moiety as well as after the conjugation of molecule **D** onto the surface of MSNPs-4 (Figure 4). Comparing the XPS data between MSNPs-1 and MSNPs-2, the

disappearance of the Cl 2p peak at 200.47 eV from MSNPs-1 and the appearance of the N 1s peak at 398.52 eV in MSNPs-2 indicated the successful conversion of chloride to azide. In addition, the increase in the nitrogen content from 3.1% to 5.9% for MSNPs-4 and MSNPs-5 showed the successful conjugation of molecule D to the surface of MSNPs-5. Apart from the increase in the nitrogen content that was observed for different MSNPs, the appearance of an S 2p peak at 163.52 eV in MSNPs-3 revealed the successful grafting of 3-mercaptopropyltriethoxysilane.

Figure 4. XPS data of the various MSNPs synthesized.

The changes of different surface functionalities were also confirmed by zeta potential measurements (Figure 5a). Based on the data obtained, MSNPs-4 gave a positive zeta potential, which is probably attributed to the protonation of the azide group. After the treatment of molecule **D** with MSNPs-4, the azide moiety formed a 1,2,3-triazole ring, leading to the zeta potential shift to a negative value. The increase in the zeta potential values from MSNPs-5 to MSNPs-6 was likely caused

by the protonation of the amine groups on the surface, further demonstrating the successful deprotection of the tert-butyl carbamate groups.

Figure 5. (a) Zeta potential of MSNPs-4, 5, 6. (b) Ninhydrin test for the detection of the amine functionalities on the surface of MSNPs-6.

Ninhydrin detection of primary amines was used to trace the deprotection of the tert-butyl carbamate group (Figure 5b). The addition of MSNPs-6 to ninhydrin showed a drastic increase in absorbance at 496 nm. This absorbance increase was attributed to the byproduct formation when the deprotected form of molecule **D** reacted with ninhydrin. The deprotection of the tert-butyl carbamate group afforded the amine unit, which reacted with ninhydrin in solution to give a series of byproducts having the combined absorbance at 496 nm. After calculations, the amount of ligand **L1** on the surface of the MSNPs-6 was determined to be 0.08 μmol per mg of MSNPs-6.

3.3.2 Drug loading and release studies

After the characterization of the MSNPs, the drug loading and release capabilities with the anticancer drug Dox were investigated. Dox was loaded within the mesopores of the MSNPs capped with the disulfide bond-linked adamantane/ β -CD complex. Firstly, Dox (4 mg mL^{-1}) was loaded into the pores of the MSNPs-6 (20

mg) in water. Thereafter, water was evaporated, and molecule **2** dissolved in ethanol was added to the mixture. Lastly, ethanol was evaporated and an aqueous solution of β -CD was added to the mixture. The resulting MSNPs-6-Dox were then collected by centrifugation and washed extensively with water. The drug loading capacity of MSNPs-6-Dox was determined to be 2.1%. The relatively low loading capacity is due to the multiple functionalities present on the surface of the MSNPs, which hinder the drug-loading process. To compare the effects of **L1** for in vitro cancer therapy, MSNPs-4 was also loaded with Dox to serve as a control. It was found that MSNPs-4-Dox had a drug loading capacity of 2.4 %. As MSNPs-4 does not contain **L1**, the hindrance present on the surface of MSNPs-4 is lower, resulting in a slightly higher loading capacity.

Thereafter, the Dox release behavior from MSNPs-4-Dox and MSNPs-6-Dox was investigated (Figure 6). The fluorescence of Dox was measured at 593 nm ($\lambda_{\text{ex}} = 480$ nm), and the concentration of Dox released with respect to the amount of MSNPs was calculated with reference to a standard curve. In the absence of the reducing agent, release of the drug was not observed in the two sample solutions. However, when 5 mM and 10 mM GSH was added to both MSNPs-6-Dox and MSNPs-4-Dox, a significant increase in the Dox concentration was observed (Figure 6). The use of 10 mM GSH gave a higher Dox release than that of 5 mM GSH. Further comparison of two release profiles at 5 mM GSH revealed that the release of Dox from MSNPs-6-Dox was almost two times higher than that of MSNPs-4-Dox. This large difference in the Dox release could be due to the interaction of the carboxylic acid groups on GSH with the amino groups on the

surface of the MSNPs. This interaction might allow GSH to easily access the disulfide bond and cause its cleavage. The above results indicate the successful loading and release of drugs by MSNPs-4 and MSNPs-6.

(a)

(b)

Figure 6. Dox release profiles for (a) MSNPs-4–Dox and (b) MSNPs-6–Dox without and with GSH at 10 and 5 mM.

3.3.3 Endocytosis and anticancer efficiency towards B16F10

To verify the targeting ability and toxicity of polyamine toxin **L1** towards the B16F10 melanoma cell line, cell viability assays of MSNPs-6 and MSNPs-4 were evaluated. As **L1** is a glutamate receptor antagonist and can be adopted for the treatment of cancer, it was hypothesized that MSNPs-6 might have the ability to inhibit the progression of B16F10 cell line. This hypothesis was tested by adding different concentrations of MSNPs-4 and MSNPs-6 (0.2, 0.1, 0.05, 0.025 mg mL⁻¹) into cultured B16F10 cells (1X10⁴) followed by the incubation for 4 h. Comparing the effects of the two types of MSNPs on B16F10 cell line revealed that MSNPs-6 gave 50% cell viability, which is significantly lower than the 70% cell viability of MSNPs-4 (Figure 7). There may be two main reasons for this trend. Firstly, the polyamine groups on **L1** being positively charged in phosphate buffered saline (PBS) might disrupt some cell membranes, causing the cell death. Secondly, the presence the polyamine tail on **L1** allows it to bind and antagonize Ca²⁺ permeable iGlu receptors. The binding of **L1** results in a voltage-dependent block of inward current, which decreases the cell division and enhances the cell death. Thus, **L1**-containing MSNPs-6 exhibits some cytotoxic effect towards the B16F10 cell line, which is consistent with our hypothesis as well as the literature reports.²²

Figure 7. Cell viability of MSNPs-4 and MSNPs-6 using cancerous cells (B16F10) and non-cancerous cells (NIH3T3).

To ensure that the nanoparticles exhibit less cytotoxic effects to normal cells, the same experiment was conducted on mouse embryonic fibroblast NIH3T3 cells, a normal cell line, using the same concentrations of MSNPs-4 and MSNPs-6 (Figure 7). Based on the results obtained, both samples gave a cell viability of 70% with a negligible difference in the cytotoxic effects. Comparing the results with the B16F10 cell line, the data obtained were surprising, as it indicates that **L1**-containing MSNPs-6 has a selective cytotoxic effect to B16F10 melanoma cell line over the NIH3T3 normal cell line. As B16F10 cell line is overexpressed with iGlu receptors as compared to the NIH3T3 cell line,²² there was selective uptake of MSNPs-6 by the B16F10 cell line. An insignificant difference in the cytotoxicity of MSNPs-4 towards both cell lines was observed (Figure 7), whereas MSNPs-6 showed a marked difference in cytotoxicity to B16F10 over NIH3T3 cells. This comparison further supports the above observation that **L1**-containing MSNPs-6 is therapeutically selective towards the B16F10 cell line.

Figure 8. Fluorescence microscopy images of B16F10 and NIH3T3 cell lines with MSNPs-4 and MSNPs-6. A) B16F10 cell line incubated with MSNPs-6; B) B16F10 cell line incubated with MSNPs-4; C) NIH3T3 cell line incubated with MSNPs-6; D) NIH3T3 cell line incubated with MSNPs-4. The nucleus was stained with DAPI, and green fluorescence was attributed to the FITC-labeled MSNPs-4 and MSNPs-6. Scale bar: 20 μm .

Fluorescence microscopy images were then obtained to qualitatively determine the amount of MSNPs endocytosed by the cells (Figure 8). To determine the localization of the MSNPs within the cells, fluorescein isothiocyanate (FITC) was incorporated into the nanoparticles during the synthesis. B16F10 and NIH3T3 cells were incubated with MSNPs-4 and MSNPs-6 (0.2 mg mL^{-1}), respectively, for 4 h before fixing and staining with 4',6-diamidino-2-phenylindole (DAPI). When B16F10 cells were incubated with MSNPs-4, there was a substantially low amount of MSNPs-4 uptake into the cells, indicated by the weak and non-localized green fluorescence. When MSNPs-6 was used, significant green fluorescence was

observed in the cytoplasm of the cells, but not in the nucleus. MSNPs-4 and MSNPs-6 were also investigated using NIH3T3 cell line, and the cell images showed minimal green fluorescence in both cases. The fluorescence microscopy images further confirmed the selective uptake of **L1** containing MSNPs-6 by the B16F10 melanoma cells over the NIH3T3 normal cells. The uptake of these nanoparticles occurs by endocytosis²³ after the binding of the **L1** ligand to the cell surface. Since B16F10 cells contain more iGlu receptors,¹¹ the uptake of MSNPs-6 is proportionally higher, leading to the significant green fluorescence observed.

3.3.4 Anticancer efficiency of MSNPs-4–Dox

After screening the therapeutic efficiency of MSNPs-4 and MSNPs-6, Dox was loaded into MSNPs-4 to obtain MSNPs-4–Dox for investigating its anticancer property. Cell viability (Figure 9) showed that there was a decrease of the cell viability for both cell lines when increasing the Dox concentrations of MSNPs-4–Dox. The results revealed a lower cell viability ($p < 0.05$) for B16F10 cancer cells as compared with NIH3T3 normal cells. This is due to the up-regulation of GSH²⁴ within cancerous cells, which causes more disulfide bonds to be cleaved for increased drug release. This observation was further supported by fluorescence microscopy images (Figure 10), whereby the cells were incubated with MSNPs-4–Dox at a Dox concentration of $1.25 \mu\text{g mL}^{-1}$ for 4 h. Red fluorescence from Dox was localized in B16F10 cells but not in NIH3T3 cells, indicating the selective

drug release of MSNPs-4-Dox within the cancerous cells as compared with the non-cancerous cells.

Figure 9. Cell viability of MSNPs-4-Dox with B16F10 cancerous cells and non-cancerous NIH3T3 cells.

Figure 10. Fluorescence microscopy images of B16F10 and NIH3T3 cell lines with MSNPs-4-Dox. A) B16F10 cells incubated with MSNPs-4-Dox and B) NIH3T3 cells incubated with MSNPs-4-Dox. The nucleus was stained with DAPI, green fluorescence was attributed to the FITC-labeled MSNPs-4-Dox, and red fluorescence was derived from anticancer drug Dox. Scale bar: 20 µm.

3.3.5 Inhibitory effects and intracellular drug release of MSNPs-6-Dox

To probe the therapeutic effect of the **L1** ligand together with Dox, MSNPs-6 was loaded with Dox and then capped with a disulfide-bridged adamantane/ β -CD complex. Encapsulating Dox within the nanoparticles enables us to study the dual effects of anticancer and neuroprotective drugs. MSNPs-6–Dox was incubated with both B16F10 and NIH3T3 cell lines for 4 h before the cell viabilities of both cell lines were tested. The data in Figure 11a showed that an increase in the Dox concentration of MSNPs-6–Dox led to a decrease of the cell viability for both B16F10 and NIH3T3 cell lines. The cell viability of the B16F10 cell line was significantly lower ($p < 0.01$) than that of the NIH3T3 normal cell line. This significant difference is attributed to the presence of both glutamate receptor antagonist as well as Dox. The glutamate receptor antagonist provides a targeting ability along with some therapeutic properties. On the other hand, the selective release of Dox from MSNPs-6–Dox was realized by the cleavage of the disulfide bond by GSH.

Figure 11. a) Cell viability of MSNPs-6–Dox with cancerous B16F10 cell line and normal NIH3T3 cell line; b) Comparison of the cell viability data between MSNPs-4–Dox and MSNPs-6–Dox when incubated with B16F10 and NIH3T3.

To compare the targeting and therapeutic efficiency between MSNPs-6-Dox and MSNPs-4-Dox on both cell lines (Figure 11b), a Student's *t*-test was performed to show the difference in the cell viability. Student's *t*-test is a statistical method used to determine if two sets of data are significantly different from each other and they are applied if the test group follows a normal distribution. The results revealed a significant difference in the Student's *t*-test values between two cell lines using MSNPs-6-Dox, and the Student's *t*-test value of MSNPs-6-Dox was 5-fold lower than that of MSNPs-4-Dox. This observation further confirms the anticancer property of **L1**. With the dual selectivity, a synergistic effect consisting of better targeting and therapeutic properties was achieved using MSNPs-6-Dox.

This therapeutic effect was also supported from the fluorescence microscopy images, in which a positive uptake was observed from B16F10 cells but not from NIH3T3 cells when incubated with MSNPs-6-Dox at a Dox concentration of $1.25 \mu\text{g mL}^{-1}$ (Figure 12). The intense-green fluorescence, caused by FITC co-condensed within the MSNPs, was visible in B16F10 cells but not in NIH3T3 cells. This observation is consistent with the result obtained from MSNPs-4-Dox, reflecting the uptake of the MSNPs within the cells. The red fluorescence within the B16F10 cells was attributed to the successful release of Dox from MSNPs-6-Dox into the intracellular environment. In addition, the red fluorescence was also observed in the nucleus of the B16F10 cells, indicating that released Dox entered the nucleus to intercalate with the DNA of the cells. This intercalation process inhibits the proper cellular functions and causes apoptosis. Based on the cell viability data and fluorescence microscopy images, it could be concluded that

MSNPs-6-Dox contains both targeting and therapeutic properties, presenting a promising potential for cancer therapy.

Figure 12. Fluorescence microscopy images of B16F10 and NIH3T3 cell lines with MSNPs-6-Dox. Fluorescence microscopy images of A) B16F10 cell line incubated with MSNPs-6-Dox, and B) NIH3T3 cell line incubated with MSNPs-6-Dox. The blue fluorescence observed was due to the DAPI staining of the nucleus, green fluorescence was derived from FITC-labeled MSNPs-6-Dox, and red fluorescence was derived from anticancer drug Dox. Scale bar: 20 μ m.

3.4 Conclusion

A new type of MSNPs-based delivery system has been prepared, with the simultaneous use of a neuroprotective drug (**L1**) and an anticancer drug (Dox), for the treatment of metastatic melanoma cancer. The MSNPs system consisting of the neuroprotective drug, water-soluble polyethylene glycol chain, and a thiol-cleavable capping group on the surface presents synergistic effects in the cancer therapy. The selective functionalization of the neuroprotective drug on the surface of the MSNPs has shown unexpected targeting as well as anticancer properties. The functionalized MSNPs loaded with Dox and capped with thiol cleavable groups have demonstrated selective drug release to cancer cells over normal cells. When both the neuroprotective drug and the selective release of Dox have been

integrated onto the MSNPs, targeted and enhanced therapeutic efficacy has been observed in cancer cells. This work has successfully demonstrated the feasibility that the drugs used for the treatment of neuronal diseases could also be employed in combination with other anticancer drugs for the treatment of cancer.

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Chapter 4

Drug Encapsulation and Release by Mesoporous Silica

Nanoparticles: The Effect of Surface Functional Groups

4.1 Introduction

Mesoporous silica nanoparticles (MSNPs) have shown various potential biomedical applications, particularly as nanocarriers for drug delivery.¹ Their ease of synthesis and tunable properties make MSNPs highly customizable according to the desired purpose.² Furthermore, its high surface area allows the grafting of a large number of functional groups on the surface so as to impart various interesting properties to MSNPs.³ In Chapter 2 and 3, the attachment of targeting molecules on the surface of the MSNPs showed good uptake of the MSNPs. In addition to the use of targeting moieties, the release of drug under a specific stimulus can also contribute to therapeutic selectivity of these MSNPs. MSNPs can be functionalized with stimuli responsive bonds or end capping groups which can be cleaved or have a change in conformation in the presence of the stimulus. Stimulated cargo release has been widely utilized as a key strategy for the specific release of drugs in a controlled manner⁴ in order to reduce side effects brought about by the use of anticancer drugs for chemotherapy. One common stimulation method is redox activation based on glutathione (GSH) in cancer cells,⁵ as it has been shown that some cancer cells express a significant amount of intracellular GSH compared to healthy cells.⁶ Under this strategy, the surface of MSNPs are bridged with capping agents through a disulfide linkage.⁷ In this way, intracellular GSH would induce

reductive cleavage of the disulfide bond, thereby removing the capping agents and releasing the loaded drugs. In a study by Zink and co-workers,⁸ the effect of complexation of α -cyclodextrin (α -CD) with aniline units on hollow MSNPs on drug release was investigated. Amors and co-workers, on the other hand, investigated the relationship between different alkyl chain lengths and drug release behavior.⁹ In a recent work by Gaberscek and co-workers, redox-responsive systems with different level of hindrance on the disulfide linkage were studied, showing that an increase in hindrance results in slower release.¹⁰

Although some studies based on GSH-triggered drug release in vitro and in vivo have been reported,¹¹ the effect of terminal group, chain length, and amount of disulfide unit on the MSNP surface on drug loading and release is still not well understood.

(a)

Figure 1. a) Synthesis of different types of functional MSNPs under different conditions. First set of conditions screened involved the use of ligands with different chain lengths. The second set of conditions tested was the effect of the terminal groups with different sizes. Lastly, different amounts of the same ligand were conjugated onto the surface of MSNPs to investigate the effects towards the surface coverage, drug loading capacity and release kinetics. *m* indicates the volume (mL) of 3-mercaptopropyltrimethoxysilane (MPS) used per 1 g of MSNPs. b) Synthetic representation for the release mechanism from β -CD-capped and Dox-loaded MSNPs triggered by GSH.

Herein, we report a catalysis screening method to study the effect of chain length, terminal group, and disulfide amount on the surface of MSNPs on drug-loading capacity and release kinetics (Figure 1). Supramolecular complexes involving β -cyclodextrin (β -CD) and the terminal groups of different functional ligands were employed in this study owing to the ease of functionalizing the surface of MSNPs. A systemic approach was adopted, whereby ligands with different chain lengths were first tested. Thereafter, the chain length that gave the highest release capability was used for the following tests that involved different types of terminal units. Then, the effect of varying the amount of disulfide unit on the surface was investigated by using the functional ligand that showed the highest release ability. Finally, in vitro drug-delivery experiments were conducted on a melanoma cell line (B16-F10) by using the functionalized MSNPs screened. We here selected Dox as the cargo within MSNPs, as Dox is a common anticancer drug that is loaded into MSNPs for drug-delivery applications.

4.2 Experimental

All solvents and reagents were of analytical grade and used directly upon received from commercial sources. All reactions were performed at room temperature (23°C) and atmospheric pressure (1.0 atm) unless otherwise stated. Ultrapure water (18.2 Ω -cm resistivity at 25°C and < 10 ppb Total Organic Carbon) was used directly upon dispensing from Sartorius Ultrapure Water dispenser system.

Compound purification by flash chromatography was performed with Merck Geduran® Si 60 silica gel with particles size of 40 to 63 μ m. Column was stacked with the slurry mixture of the silica gel and the eluting solvent, and the pressure was applied on the column with an aquarium pump.

^1H and ^{13}C NMR measurements were carried out on a Bruker AMX 300 MHz and 400 MHz NMR at ambient temperature. Chemical shifts δ from ^1H NMR spectra were reported in unit of parts per million (ppm) downfield from TMS (δ 0.00 ppm) or relative to the deuterated chloroform (δ 7.26 ppm for CDCl_3) used. All multiplicities were reported as: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublet), dt (doublet of triplet) or m (multiplet). Coupling constants (J values) were reported in Hz and the number of equivalent protons for a given resonance was reported as nH. Chemical shifts δ from ^{13}C NMR spectra were reported in units of ppm relative to the residual signals from the deuterated chloroform (δ 77.0 ppm for CDCl_3) used.

High resolution mass spectrometry (HRMS) was performed with Waters Q-tof Premier MS. Fourier transform infrared (FTIR) spectroscopy were carried out on a SHIMADZU IR Prestige-21 spectrophotometer. UV-Vis spectroscopy was carried

out on a SHIMADZU UV-3600 UV-VIS-NIR Spectrophotometer using a 10 mm by 10 mm 3.5 mL quartz cuvette. Fluorescence emission spectra were recorded using a Varian Cary Eclipse fluorescence spectrophotometer, equipped with water circulated temperature controller. Samples were contained in a 10 mm by 10 mm 3.5 mL four sided transparent quartz cuvette. Transmission electron microscope (TEM) images were recorded on JEOL: JEM 1400 thermionic emission electron microscope operating at an acceleration voltage at 100 kV. X-Ray photoelectric spectroscopy was carried out on a Phoibos 100 spectrometer and a monochromatic Mg X-ray radiation source (SPECS, Germany) was used for the wide-range and high resolution S 2p scans at 12.53 kV. N₂ adsorption/desorption isotherms were measured on a Micromeritics ASAP 2020M automated sorption analyzer at a temperature of 192 K. Pre-treatment of the MSNPs was performed by degassing the powder samples at 100 °C for 12 h prior to the analysis. Thereafter, specific surface areas were calculated from the adsorption data in low pressure range using the Brunauer-Emmett-Teller (BET) model and pore size was determined using the Barret-Joyner-Halenda (BJH) model. Powder X-ray diffraction (XRD) patterns were measured using the PANalytical X'Pert diffractometer equipped with Cu K α radiation ($\lambda = 1.5405980 \text{ \AA}$).

4.2.1 Synthesis of organic compounds

Scheme 1. General scheme for the synthesis of *N*-alkyl-(*n*+2)-(pyridin-2-yl)disulfanyl carboxylate amide.

3-(pyridin-2-yl)disulfanylpropanoic acid

Scheme 2. Synthesis scheme for 3-(pyridin-2-yl)disulfanylpropanoic acid.

The molecule was synthesis was adopted from Chapter 3, Section 3.2.3 Synthesis of compound 2.

N-cyclohexyl-3-(pyridin-2-yl)disulfanyl-propioamine

Scheme 3. Synthesis scheme for *N*-alkyl-3-(pyridin-2-yl)disulfanyl-propioamine.

To a stirring solution of 3-(pyridin-2-yl)disulfanylpropanoic acid (1 g, 4.65 mmol) in THF (30 mL) was added with dicyclohexylcarbodiimide (1.25 g, 6.05 mmol) and *N*-hydroxysuccinimide (0.70 g, 6.05 mmol). The resulting turbid solution was

stirred for a period of 3 h before cyclohexylamine (0.60 g, 6.05 mmol) was added. The reaction was allowed to proceed overnight before removing the precipitate by filtration. The solvent of the filtrate was removed by rotary evaporation and the residual mixture was purified by flash column chromatography using hexane/ethyl acetate mixture as the eluent to afford *N*-cyclohexyl-3-(pyridin-2-yl)disulfanylpropioamine as a white solid (0.92 g, 63%). ¹H NMR (400 MHz, CDCl₃): δ 8.46 (dt, *J* = 4.82 Hz, 1.23 Hz, 1H), 7.64 (d, *J* = 4.40 Hz, 2H), 7.16-7.08 (m, 1H), 6.14 (s, 1H), 3.85-3.72 (m, 1H), 3.07 (t, *J* = 6.75 Hz, 2H), 2.55 (t, *J* = 6.28 Hz, 2H), 1.96-1.91 (m, 2H), 1.71 (dt, *J* = 13.3 Hz, 3.54 Hz, 2H), 1.43-1.30 (m, 2H), 1.23-1.09 (m, 3H). ¹³C NMR (400 MHz, CDCl₃): δ 169.7, 149.6, 137.1, 121.0, 120.3, 48.4, 36.1, 35.2, 33.3, 25.6, 24.9. HRMS (H₂O/MeOH): *m/z*: calcd: 297.1095 [M]⁺; found: 297.1086. IR (KBr) $\bar{\nu}$ /cm⁻¹: 3250.1, 3070.7, 2941.4, 2926.0, 2854.7, 1635.6, 1558.5, 1446.6, 1415.8, 765.7.

4.2.1.1 General procedure for the synthesis of *N*-alkyl-(*n*+2)-bromocarboxylate amide

Scheme 4. Synthetic scheme for *N*-alkyl-(*n*+2)-bromocarboxylate amide.

To a stirring solution of *n*-bromocarboxylic acid (1 mol equiv.) in THF was added with dicyclohexylcarbodiimide (1 mol equiv.) and *N*-hydroxysuccinimide (1 mol equiv.). The resulting turbid solution was stirred for a period of 3 h before *N*-alkylamine (1.2 mol equiv.) was added. The reaction was allowed to proceed

overnight before removing the precipitate by filtration. The solvent of the filtrate was removed by rotary evaporation and the residual mixture was purified by flash column chromatography using hexane/ethyl acetate mixture as the eluent to afford *N*-alkyl-*n*-bromocarboxylamide.

***N*-adamantyl-5-bromocarboxylate amide**

Scheme 5. Synthetic scheme for *N*-adamantyl-5-bromocarboxylate amide.

To a stirring solution of 5-bromovaleric acid (1.20 g, 6.67 mmol) in THF (10 mL) was added with dicyclohexylcarbodiimide (1.40 g, 6.67 mmol) and *N*-hydroxysuccinimide (0.77 g, 6.67 mmol). The resulting turbid mixture was stirred for a period of 3 h before 1-adamantamine hydrochloride (1.00 g, 5.34 mmol) and Et₃N (0.93 mL) were added. The reaction was allowed to proceed overnight before removing the precipitate by filtration. The solvent of the filtrate was removed by rotary evaporation and the residual mixture was purified by flash column chromatography using hexane/ethyl acetate mixture as the eluent to afford *N*-alkyl-*n*-bromocarboxylate amide (0.94 g, 56%). ¹H NMR (400 MHz, CDCl₃): δ 5.09 (s, 1H), 3.41 (t, *J* = 6.64 Hz, 2H), 2.11 (t, *J* = 7.26 Hz, 2H), 2.07 (s, 3H), 1.99 (s, 6H), 1.93 (m, 2H), 1.78-1.73 (m, 2H), 1.67(t, *J* = 5.80 Hz, 6H). ¹³C NMR (400 MHz, CDCl₃): δ 171.3, 51.9, 41.7, 36.6, 36.4, 33.4, 32.1, 29.4, 24.3. HRMS (H₂O/MeOH): *m/z*: calcd: 314.1120 [M]⁺; found: 314.1114. IR (KBr) $\bar{\nu}$ /cm⁻¹: 3265.5, 2910.6, 2895.2, 2852.7, 1629.9, 1558.5, 1454.3.

4.2.1.2 General procedure for the synthesis of *N*-alkyl-(*n*+2)-mercaptocarboxylate amide

Scheme 6. Synthetic scheme for *N*-alkyl-(*n*+2)-mercaptocarboxylate amide.

In a typical synthesis, *N*-alkyl-*n*-bromocarboxylate amide (1 mol equiv.) and thiourea (1.5 mol equiv.) were charged into a three neck flask, equipped with a reflux condenser. The flask was evacuated in vacuum before purging it with N₂. The process was repeated for two more cycles before anhydrous ethanol (3 mL) was injected into the flask. The mixture was brought to reflux under stirring for 24 h before the addition of 4 M NaOH (4.5 mL). The reaction was continued for another 4 h. Thereafter, the solvent was removed by rotary evaporation and the mixture was acidified with 1 M HCl. The aqueous mixture was extracted multiple times with CH₂Cl₂ and the combined organic layer was dried over anhydrous Na₂SO₄. The solvent was then removed by rotary evaporation and the residual mixture was purified by flash column chromatography using hexane/ethyl acetate mixture as the eluent to afford *N*-alkyl-*n*-mercaptocarboxylate amide.

4.2.1.3 General procedure for the synthesis of *N*-alkyl-(*n*+2)-(pyridin-2-yl)disulfanyl-carboxylate amide

Scheme 7. Synthetic scheme for *N*-alkyl-(*n*+2)-(pyridin-2-yl)disulfanyl-carboxylate amide.

A stirring solution of 2,2'-dipyridyl disulfide (2 mol equiv.) in EtOH (10 mL) and acetic acid (0.4 mL) was added dropwise with *N*-alkyl-*n*-mercaptocarboxylate amide (1 mol equiv.) in EtOH (5 mL). The resulting yellow solution was further stirred for 24 h before the solvent was removed by rotary evaporation. The residual yellow syrup was purified by flash column chromatography using hexane/ethyl acetate mixture as the eluent to afford *N*-alkyl-*n*-(pyridin-2-yl)disulfanyl-carboxylate amide.

4.2.1.4 Characterisation of compounds synthesised in part 4.2.1.2 and 4.2.1.3

N-cyclohexyl-5-bromocarboxylate amide

Yield: 1.52 g, 80 %. ^1H NMR (300 MHz, CDCl_3): δ 5.95 (s, 1H), 3.72-3.59 (m, 1H), 3.33 (t, J = 6.54 Hz, 2H), 2.11 (t, J = 7.21 Hz, 2H), 1.86-1.51 (m, 9H), 1.33-1.17 (m, 2H), 1.15-0.99 (m, 3H). ^{13}C NMR (300 MHz, CDCl_3): δ 171.4, 48.1, 35.6, 33.3, 33.1, 32.1, 25.5, 24.9, 24.4. HRMS ($\text{H}_2\text{O}/\text{MeOH}$): m/z : calcd: 262.0807 $[\text{M}]^+$; found: 262.0802. IR (KBr) $\bar{\nu}/\text{cm}^{-1}$: 3300.2, 2935.7, 2854.7, 1633.7, 1550.8, 1446.6.

N-butyl-5-bromocarboxylate amide

Yield: 0.39 g, 53 %. ^1H NMR (400 MHz, CDCl_3): δ 6.71 (s, 1H), 3.27 (t, J = 6.60 Hz, 2H), 3.05 (q, J = 6.68 Hz, 2H), 2.10 (t, J = 7.34 Hz, 2H), 1.79-1.70 (m, 2H), 1.65-1.57 (m, 2H), 1.37-1.30 (m, 2H), 1.21-1.15 (m, 2H), 0.75 (t, J = 7.32 Hz, 3H). ^{13}C NMR (400 MHz, CDCl_3): δ 172.8, 39.2, 35.3, 33.3, 32.1, 31.5, 24.3, 20.0, 13.7. HRMS ($\text{H}_2\text{O}/\text{MeOH}$): m/z : calcd: 236.0650 $[\text{M}]^+$; found: 236.0645. IR (KBr) $\bar{\nu}/\text{cm}^{-1}$: 3327.2, 2956.9, 2931.8, 2874.0, 1626.0, 1577.8, 1458.2.

***N*-cyclohexyl-7-bromocarboxylate amide**

Yield: 0.72 g, 65 %. ^1H NMR (400 MHz, CDCl_3): δ 5.40 (s, 1H), 3.78-3.69 (m, 1H), 3.38 (t, J = 6.72 Hz, 2H), 2.12 (t, J = 7.52 Hz, 2H), 1.90-1.80 (m, 4H), 1.70-1.58 (m, 5H), 1.47-1.38 (m, 2H), 1.35-1.28 (m, 4H), 1.14-1.04 (m, 3H). ^{13}C NMR (400 MHz, CDCl_3): δ 172.0, 48.1, 36.8, 33.9, 33.2, 32.5, 28.3, 27.8, 25.6, 25.5, 24.9. HRMS ($\text{H}_2\text{O}/\text{MeOH}$): m/z : calcd: 290.1120 $[\text{M}]^+$; found: 290.1119. IR (KBr) $\bar{\nu}/\text{cm}^{-1}$: 3302.1, 2933.7, 2854.7, 1629.9, 1550.8, 1444.7.

***N*-adamantyl-5-mercaptoparboxylate amide**

Yield: 0.37 g, 86 %. ^1H NMR (400 MHz, CDCl_3): δ 2.41 (q, $J = 7.16$ Hz, 2H), 1.99 (t, $J = 7.06$ Hz, 2H), 1.94 (s, 3H), 1.87 (s, 6H), 1.59-1.48 (m, 10H), 1.27 (t, $J = 7.48$ Hz, 1H). ^{13}C NMR (400 MHz, CDCl_3): δ 172.3, 51.8, 41.4, 36.8, 36.3, 33.3, 29.3, 24.4, 24.2. HRMS ($\text{H}_2\text{O}/\text{MeOH}$): m/z : calcd: 268.1735 $[\text{M}]^+$; found: 268.1726. IR (KBr) $\bar{\nu}/\text{cm}^{-1}$: 3304.1, 2908.7, 2850.8, 1645.3, 1548.8, 1454.3.

***N*-cyclohexyl-5-mercaptocarboxylate amide**

Yield: 0.23 g, 45 %. ^1H NMR (300 MHz, CDCl_3): δ 3.84-3.72 (m, 1H), 2.54 (q, $J = 7.19$ Hz, 2H), 2.15 (t, $J = 7.20$ Hz, 2H), 1.96-1.90 (m, 2H), 1.78-1.61 (m, 8H), 1.46-1.32 (m, 3H), 1.23-1.06 (m, 3H). ^{13}C NMR (400 MHz, CDCl_3): δ 171.5, 48.1, 36.3, 33.5, 33.3, 25.5, 24.9, 24.4, 24.3. HRMS ($\text{H}_2\text{O}/\text{MeOH}$): m/z : calcd: 216.1422 $[\text{M}]^+$; found: 216.1417. IR (KBr) $\bar{\nu}/\text{cm}^{-1}$: 3300.2, 2931.8, 2856.6, 1633.7, 1545.0.

***N*-butyl-5-mercaptocarboxylate amide**

Yield: 75.2 mg, 31 %. ^1H NMR (400 MHz, CDCl_3): δ 5.80 (s, 1H), 3.19 (q, $J = 6.67$ Hz, 2H), 2.49 (q, $J = 7.25$ Hz, 2H), 2.14 (t, $J = 7.34$ Hz, 2H), 1.73-1.66 (m, 2H), 1.63-1.56 (m, 2H), 1.47-1.40 (m, 2H), 1.34-1.27 (m, 3H), 0.88 (t, $J = 7.30$ Hz, 3H). ^{13}C NMR (400 MHz, CDCl_3): δ 172.6, 39.2, 36.0, 33.5, 31.7, 24.4, 24.3, 20.1, 13.7. HRMS ($\text{H}_2\text{O}/\text{MeOH}$): m/z : calcd: 190.1266 $[\text{M}]^+$; found: 190.1266. IR (KBr) $\bar{\nu}/\text{cm}^{-1}$: 3296.4, 2958.8, 2933.7, 1647.2, 1556.6, 1458.2.

***N*-cyclohexyl-7-mercaptocarboxylate amide**

Yield: 0.20 g, 59 %. ^1H NMR (400 MHz, CDCl_3): δ 5.96 (d, $J = 7.88$ Hz, 1H), 3.66-3.57 (m, 1H), 2.39 (q, $J = 7.37$ Hz, 2H), 2.03 (t, $J = 7.54$ Hz, 2), 1.79-1.75 (m, 2H), 1.61-1.45 (m, 8H), 1.32-1.12 (m, 8H), 1.07-0.97 (m, 3H). ^{13}C NMR (400 MHz, CDCl_3): δ 172.1, 48.0, 36.7, 33.7, 33.1, 28.6, 28.0, 25.7, 25.5, 24.9, 24.4. HRMS ($\text{H}_2\text{O}/\text{MeOH}$): m/z : calcd: 244.1735 $[\text{M}]^+$; found: 244.1731. IR (KBr) $\bar{\nu}/\text{cm}^{-1}$: 3302.1, 2928.0, 2852.7, 1631.8, 1550.8, 1444.7.

***N*-adamantyl-3-(pyridin-2-ylidisulfanyl)-pentanamide**

Yield: 0.44 g, 78 %. ^1H NMR (400 MHz, CDCl_3): δ 8.39 (d, $J = 4.84$ Hz, 1H), 7.68 (d, $J = 8.08$ Hz, 1H), 7.60 (dt, $J = 7.72$ Hz, 1.73 Hz, 1H), 7.04 (dd, $J = 7.26$ Hz, 1), 2.74 (t, $J = 6.82$ Hz, 2H), 2.07-2.00 (m, 6H), 1.92 (s, 6H), 1.67-1.65 (m, 4H), 1.61 (s, 6H). ^{13}C NMR (400 MHz, CDCl_3): δ 171.7, 160.4, 149.5, 137.1, 120.6, 119.6, 51.8, 41.6, 38.4, 36.9, 36.3, 29.4, 28.3, 24.5. HRMS ($\text{H}_2\text{O}/\text{MeOH}$): m/z : calcd: 377.1721 $[\text{M}]^+$; found: 377.1712. IR (KBr) $\bar{\nu}/\text{cm}^{-1}$: 3269.3, 2916.4, 2850.8, 1664.6, 1577.8, 1543.1, 1450.5, 1417.7, 771.5.

***N*-cyclohexyl-3-(pyridin-2-ylidisulfanyl)-pentanamide**

Yield: 62.0 mg, 44 %. ^1H NMR (300 MHz, CDCl_3): δ 8.45 (d, $J = 4.83$ Hz, 1H), 7.72-7.61 (m, 2H), 7.07 (t, $J = 6.03$ Hz, 1H), 5.30 (s, 1H), 3.80-3.68 (m, 1H), 2.79 (t, $J = 8.38$ Hz, 2H), 2.12 (t, $J = 8.34$ Hz, 2H), 1.91-1.86 (m, 2H), 1.75-1.57 (m, 8H), 1.42-1.29 (m, 2H), 1.20-1.02 (m, 3H). ^{13}C NMR (300 MHz, CDCl_3): δ 171.3, 160.4, 149.6, 137.0, 120.6, 119.7, 48.1, 38.4, 36.4, 33.2, 28.4, 25.5, 24.9, 24.6. HRMS ($\text{H}_2\text{O}/\text{MeOH}$): m/z : calcd: 325.1408 $[\text{M}]^+$; found: 325.1400. IR (KBr) $\bar{\nu}/\text{cm}^{-1}$: 3462.2, 2931.8, 2854.7, 1635.6, 1573.9, 1558.5, 1446.6, 1417.7, 760.0.

***N*-butyl-3-(pyridin-2-yl)disulfanyl-pentanamide**

Yield: 0.10 g, 87 %. ^1H NMR (400 MHz, CDCl_3): δ 8.43 (d, $J = 4.64$ Hz, 1H), 7.70 (d, $J = 8.08$ Hz, 1H), 7.65-7.60 (m, 1H), 7.07 (dd, $J = 4.87$ Hz, 1.11 Hz, 1H), 5.59 (s, 1H), 3.21 (q, $J = 7.08$ Hz, 2H), 2.80-2.76 (m, 2H), 2.15-2.12 (m, 2H), 1.75-1.70 (m, 4H), 1.48-1.41 (m, 2H), 1.36-1.28 (m, 2H), 0.89 (t, $J = 7.28$ Hz, 3H). ^{13}C NMR (400 MHz, CDCl_3): δ 172.4, 160.4, 149.6, 137.0, 120.6, 119.7, 39.2, 38.4, 36.2, 31.7, 28.4, 24.5, 20.1, 13.7. HRMS ($\text{H}_2\text{O}/\text{MeOH}$): m/z : calcd: 216.1422 $[\text{M}]^+$; found: 216.1417. HRMS ($\text{H}_2\text{O}/\text{MeOH}$): m/z : calcd: 299.1252 $[\text{M}]^+$; found: 299.1247. IR (KBr) $\bar{\nu}/\text{cm}^{-1}$: 3252.9, 2956.9, 2872.0, 2829.6, 1653.0, 1575.8, 1558.5, 1446.6, 1417.7, 760.0.

***N*-cyclohexyl-3-(pyridin-2-yl)disulfanyl-heptanamide**

Yield: 0.14 g, 64 %. ^1H NMR (300 MHz, CDCl_3): δ 8.41 (d, $J = 4.84$ Hz, 1H), 7.68 (d, $J = 8.08$ Hz, 1H), 7.63-7.58 (m, 1H), 7.04 (dd, $J = 7.32$ Hz, 1.05 Hz, 1H), 5.51 (d, $J = 7.40$ Hz, 1H), 3.75-3.66 (m, 1H), 2.74 (t, $J = 7.28$ Hz, 2H), 1.87-1.83 (m, 2H), 1.69-1.61 (m, 4H), 1.60-1.52 (m, 3H), 1.40-1.32 (m, 3H), 1.29-1.24 (m, 6H), 1.15-1.02 (m, 3H). ^{13}C NMR (300 MHz, CDCl_3): δ 172.0, 160.6, 149.5, 137.0, 120.5, 119.6, 48.1, 38.8, 36.8, 33.2, 28.7, 28.1, 25.6, 25.5, 24.9. HRMS ($\text{H}_2\text{O}/\text{MeOH}$): m/z : calcd: 353.1721 $[\text{M}]^+$; found: 353.1720. IR (KBr) $\bar{\nu}/\text{cm}^{-1}$: 3307.9, 3068.8, 2927.9, 2848.9, 1629.9, 1573.9, 1545.0, 1450.5, 1415.8, 754.2.

4.2.2 Synthesis of functional MSNPs

4.2.2.1 Synthesis of MSNPs for the conjugation of different ligands on the surface

Scheme 8. General synthetic scheme for MSNPs-4(1)-1D/1E/1F/1B/1K.

Synthesis of MSNPs-1

To a round bottom flask, CTAB (0.1 g) was dissolved in distilled water (50 mL), and then NaOH (0.35 mL, 2 M) was added to the solution. The mixture was then heated to 80°C followed by the addition of TEOS (0.5 mL) and FITC-APTES (50 µL). The resulting solution was stirred for 1 min before the addition of ethyl acetate (0.5 mL). The mixture was then left to stir for 2 h and the resulting white solid was filtered and washed with distilled water and MeOH for three times. The precipitate was dried under vacuum to obtain the as-synthesized MSNPs-1.

Synthesis of MSNPs-2(1)

MSNPs-1 (0.5 g) was added to anhydrous toluene (80 mL), and 3-mercaptopropyltrimethoxysilane (0.5 mL, 5.38 mmol) was then added to the solution. The resulting mixture was refluxed under N₂ overnight. Thereafter, the precipitate was collected by centrifugation (10000 rpm, 10min) and washed with toluene and ethanol to yield MSNPs-2(1).

Synthesis of MSNPs-3(1)

MSNPs-2(1) (0.4 g) was added to a round bottle flask, and methanol (64 mL) together with HCl (3.6 mL) were added next. The mixture was refluxed overnight and the precipitate was collected by centrifugation (10000 rpm, 10 min) and washed with methanol to yield MSNPs-3(1).

Synthesis of MSNPs-4(1)

MSNPs-3(1) (25 mg) was added to a round bottle flask, and ethanol (1 mL) together with respective *N*-alkyl-(*n*+2)-(pyridin-2-yl)disulfanyl-carboxylate amide (0.135 mmol) were added subsequently. The mixture was stirred at room temperature overnight to yield white precipitate. The solid was collected by centrifugation (10000 rpm, 10 min) and washed with methanol to yield MSNPs-4(1)-1D/ 1E/ 1F/ 1B/ 1K depending on *N*-alkyl-(*n*+2)-(pyridin-2-yl)disulfanyl-carboxylate amide added to the solution.

4.2.2.2 Synthesis of different amounts of thiols on the surface of MSNPs

Scheme 9. General synthetic scheme for MSNPs-4(m)-1E.

Synthesis of MSNPs-2(m)

MSNPs-1 (0.5 g) was added to anhydrous toluene (80 mL), and 3-mercaptopropyltrimethoxysilane ($m = 2$, 1 mL, 5.38 mmol) or ($m = 0.5$, 0.25 mL, 1.35 mmol) was added to the solution. The resulting mixture was refluxed under N_2 overnight. Thereafter, the precipitate was collected by centrifugation (10000 rpm, 10 min) and washed with toluene and ethanol to yield MSNPs-2(m).

Synthesis of MSNPs-3(m)

MSNPs-2(m) (0.4 g) was added to a round bottle flask, and methanol (64 mL) together with HCl (3.6 mL) were added subsequently. The mixture was refluxed overnight and the precipitate was collected by centrifugation (10000 rpm, 10 min) and washed with methanol to yield MSNPs-3(m).

Synthesis of MSNPs-4(m)-1E

MSNPs-3(m) (25 mg) was added to a round bottle flask, and ethanol (1 mL) together with *N*-cyclohexyl-3-(pyridin-2-yl-disulfanyl)-pentanamide ($m = 2$, 43.6 mg, 0.269 mmol) or ($m = 0.5$, 10.9 mg, 0.067 mmol) were then added. The mixture was stirred at room temperature overnight to yield white precipitate. The solid was collected by centrifugation (10000 rpm, 10 min) and washed with methanol to yield MSNPs-4(m)-1E.

4.2.3 Drug loading and release studies

4.2.3.1 Drug loading

A stock solution of Dox (4 mg/mL) was prepared, and respective MSNPs-4 (20 mg) was added to 1 mL of it. The mixture was allowed to stir overnight and then β -CD (mass was calculated based on the moles of thiol group on the surface) was added to the stirring mixture. The mixture was stirred overnight again and the functional MSNPs were obtained by centrifugation (10000 rpm, 10 min) and washed extensively with distilled water to yield the drug loaded MSNPs-4.

4.2.3.2 Determination of drug loading percentage

In order to determine the loading percentage of different MSNPs-4, the absorbance of the Dox stock solution was performed after 40 times dilution. After the functional MSNPs were successfully loaded with Dox and capped with β -CD, the MSNPs were centrifuged down and washed 4 times with distilled water. The supernatant was obtained and diluted 10 times, and its absorbance was measured. The drug loading capacity was then calculated with the following formula:

$$\text{Loading capacity (\%)} = \frac{[Dox\ initial] - [Dox\ final]}{\text{Weight of MSNPs}} \times 100\%$$

Where [Dox initial] = the absorbance obtained from the Dox stock solution (40X dilution)

[Dox final] = the absorbance obtained from the supernate after the MSNPs were centrifuged and washed 4 times (10X dilution)

The above experiments were repeated 3 times and the values were averaged.

4.2.3.3 Release kinetics

Dox loaded MSNPs-4 (1 mg) was placed in a cuvette, which was then carefully filled with distilled water (1 mL). Glutathione (GSH) was added to the cuvette to give a final concentration of 10 mM, and fluorescence readings at 593 nm (λ_{ex} = 480 nm) were taken at 10 min intervals until no more increase in fluorescence intensity was observed.

4.2.4 Procedure for *in vitro* studies

4.2.4.1 Materials for *in vitro* experiments

B16-F10 mouse melanoma cells were obtained from ATCC and the cells were maintained at 37°C and 5% CO₂. Dulbecco Minimum Essential Medium (DMEM) added with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS) was used as the medium for all *in vitro* experiments. FBS and DMEM containing sodium pyruvate and L-glutamine were purchased from PAA laboratories. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Alfa Aesar and the MTT solution (5 mg mL⁻¹) was prepared by dissolving the MTT crystal in 1X PBS. The cell cytotoxicity results were obtained with Tecan Infinite 200 PRO microplate reader. Fluorescence images were obtained using a Nikon TE2000-E Confocal Fluorescence microscope with a Nikon TE2-PS100W high-pressure mercury lamp.

4.2.4.2 Cell viability

The cell viability studies were performed on a 96 well plate with a seeding density of 1X10⁴ cells per well maintained with growth medium (100 µL). The cells were incubated for 24 h before the addition of respective MSNPs solution (10 µL) or water (the negative control). The plates were then incubated for 4 h and the culture medium was replaced with fresh medium (100 µL) thereafter. The cells were again incubated for another 24 h before the medium was replaced and an additional MTT solution (10 µL) was added to each well. Thereafter, the plates were incubated for another 4 h before the medium was removed and replaced with DMSO (100 µL) to

dissolve the purple crystals. Absorbance readings of the wells were performed at 562 nm with the TECAN microplate reader. The percentage cell viability was obtained by taking the percentage sample readings divided by the readings of the negative control. Each sample contains 8 replicates and the results were presented as mean values with error bars representing standard deviation.

4.2.4.3 Fluorescence microscopy images

The fluorescence microscopy studies were performed in a confocal dish with a seeding density of 2×10^5 cells maintained with growth medium (2 mL). After the cells were incubated for 24 h, respective MSNPs (200 μ L, 0.21 mg mL⁻¹) were added into each dish, which was returned to the incubator for a period of 1 h. Thereafter, the medium was removed and the cells were washed twice with 1X PBS. The cells were suspended in 1X PBS (2 mL) and ready for fluorescence microscopy imaging.

4.3 Results and discussion

4.3.1 Synthesis and characterization of functionalized MSNPs

We begin the studies by first synthesizing MCM-41 MSNPs by using a surfactant-directed technique with hexadecyltrimethylammonium bromide (CTAB) as the surfactant. The as-synthesized MSNPs were characterized by transmission electron microscopy (TEM), and the results showed that the obtained MSNPs were spherical in shape with diameter of 70–80 nm (Figure 2a). The Brunauer–Emmett–

Teller (BET) measurements showed a type IV adsorption/desorption isotherm, which is a characteristic feature of mesoporous materials. The BET measurements (Figure 2b) also show that the MSNPs possessed a high BET surface area of $921 \text{ m}^2\text{g}^{-1}$ with a Barrett Joyner–Halenda (BJH) pore size of 3.03 nm (Figure 2c), which is slightly larger than the pore size (3.56 nm) measured using powder X-ray diffraction (XRD) (Figure 2d). These characterizations confirmed the successful synthesis of the MSNPs.

Figure 2. a) TEM image of MSNPs-3(1). b) Adsorption and desorption isotherms using the BET model of MSNPs-1, MSNPs-1 no CTAB and MSNPs-3(1). c) BJH pore size distribution obtained from the adsorption and desorption isotherm for MSNPs-3(1). d) XRD pattern of MSNPs-3(1).

After grafting the surface with a mercaptan group, X-ray photoelectron spectroscopic (XPS) analysis of mercaptan-grafted MSNPs, that is, MSNPs-3(1),

shows the presence of an S 2p peak at 163.6 eV, indicating successful conjugation of the mercaptan group onto the surface. The FT-IR spectrum presents clear alkyl C-H peaks, corresponding to the propyl groups of the grafted 3-mercaptopropyltrimethoxysilane (Figure 3).

Figure 3. IR spectrum of MSNPs-1, MSNPs-2(1), MSNPs-(3)-1.

The next step was the synthesis of a series of thiol-activated ligands (Figure 4) for conjugating with the mercaptan-grafted MSNPs. Thus, the conjugation of the *n*-bromo-terminated carboxylic acids of different chain lengths with a variety of alkyl (R) amines was carried out, followed by the conversion of the bromide groups into the thiol groups. The obtained ligands were then coupled onto the surface of MSNPs prior to Dox loading and complexed with β -CD. The complexation of β -CD with these (R) ending groups could keep the loaded drug within the mesopores without leakage. Thereafter, the Dox loading capacity and release profiles of functionalized MSNPs were measured accordingly.

Figure 4. Ligands conjugated onto the surface of MSNPs for investigating the effect of ligand length and bulkiness.

4.3.2 Effect of ligand length on drug loading and release

The first set of conditions tested was the effect of ligand length on the drug-capping and release efficiency. Ligands with varying lengths (3, 5, and 7 carbon atoms) and cyclohexyl terminal group were used. After conjugation of the respective ligands onto the surface of MSNPs, XPS was adopted to measure the amount of functional ligands on the surface of MSNPs, that is, MSNPs-4(1)-1D, MSNPs-4(1)-1E, and MSNPs-4(1)-1F. The results (Table 1) show that the sulfur content of MSNPs-3(1), MSNPs-4(1)-1D, MSNPs-4(1)-1E are similar, ca. 12.6–14.9 %. However, MSNPs-4(1)-1F showed a slightly higher sulfur content (ca. 15.3%) upon conjugation of ligand 1F to the surface of MSNPs-3(1). As ligand 1F is longer and thus having a lower steric effect, more ligand would conjugate onto the surface of MSNPs-3(1).

(A1)

MSNPs	% Si	% S ^[a]	Loading capacity
MSNPs-3(1)	87.4	12.6	-
MSNPs-4(1)-1D	85.1	14.9	12.2 ± 0.79
MSNPs-4(1)-1E	85.3	14.7	13.9 ± 0.28
MSNPs-4(1)-1F	84.6	15.4	13.2 ± 0.86

(A1) Table 1. XPS data indicating the sulfur content after the conjugation of respective ligands with different chain lengths on the surface of MSNPs. In addition, the Dox loading capacity with standard deviation was obtained for MSNPs-4(1)-1D, MSNPs-4(1)-1E, and MSNPs-4(1)-1F. ^[a] The sulfur content corresponds to the amount of ligands being conjugated onto the surface of MSNPs.

(B5) Figure 5. Dox release kinetics for MSNPs-4(1)-1D, MSNPs-4(1)-1E, and MSNPs-4(1)-1F when GSH (10 mM) was added at the 10th minute.

Thus, the MSNPs-4(1) series was employed for evaluating drug-loading capacity. Table 1 summarizes the Dox loading capacity of these functionalized MSNPs. The order of Dox loading capacity was MSNPs-4(1)-1E>MSNPs-4(1)-1F>MSNPs-4(1)-1D. This observation was surprising as we initially expected that MSNPs-4(1)-1F would have the highest drug-loading capacity owing to it having the largest number of ligands functionalized on the surface of MSNPs. The results, however, showed that ligand 1E, having 5 carbon atoms, gave the highest Dox loading capacity, 13.9 %. The reason for such a high loading capacity may be that ligand 1E possesses the ideal length for the drug to enter into the mesopores and the ability to retain the drug within the mesopores after capping with β -CD. MSNPs-4(1)-1F exhibited a loading capacity of 13.2 %. After capping ligand 1F with β -CD, β -CD is a bit far away from the surface of MSNPs, thus not being able to

efficiently block the mesopores for drug storage. The slight leakage of the loaded drug during the washing process may in turn explain the lower drug-loading capability of MSNPs 4(1)-1F compared with MSNPs-4(1)-1E. Although MSNPs-4(1)-1D has approximately the same amount of ligands on the surface as MSNPs-4(1)-1E, the cyclohexyl group for MSNPs-4(1)-1D is much closer to the mesopores of MSNPs. This makes the drug-loading process difficult and thus results in a low drug-loading capacity compared with MSNPs-4(1)-1E.

Then, the release profiles were obtained to investigate the effect of chain length. Figure 5 shows that MSNPs-4(1)-1E gave the highest release followed by MSNPs-4-1F and lastly MSNPs 4(1)-1D. These results are consistent with the Dox loading capacity, indicating that the interaction of GSH with the disulfide bond on the MSNP surface leads to the uncapping of the β -CD complexes for drug release. The complexation of β -CD did not have an obvious influence on the cleavage of the disulfide bond. Because MSNPs-4(1)-1E showed the highest level of release, this system was chosen for the next set of experiments.

4.3.3 Effect of terminal groups on drug loading and release

After the effect of chain length was investigated, we varied the terminal groups for complexation with β -CD. As the cyclohexyl end group was used in ligand 1E, two other end groups, adamantyl and *n*-butyl, were selected for comparison studies. The adamantyl end group serves as a more bulky counterpart of the cyclohexyl group, while the *n*-butyl group only consists of 4 carbon atoms. To investigate the

effect of these terminal groups on the Dox loading capacities and release profiles, their binding constants involving complexation with β -CD were determined. Table 2 shows the average binding constants obtained from the Hildebrand plot of ^1H NMR chemical shifts of the different terminal groups with β -CD. As expected, the adamantyl group gave the highest binding constant with β -CD, followed by the cyclohexyl group. Because the R^2 value of the binding constant between *n*-butylamine and β -CD was very small, the binding constant was considered to be very low. The binding constants obtained in this work were compared with the binding constants of molecules with similar chemical structures. It was observed that although the absolute binding constants were different, a similar trend was observed when the same method of obtaining association constants was adopted¹².

Capping group	Binding constant Region 1 (R^2 value)	Binding constant Region 2 (R^2 value)	Average
Adamantamine	2044 (0.9725)	3072 (0.9981)	2873
Cyclohexylamine	265 (0.9973)	322 (0.9727)	293
<i>n</i> -Butylamine	1545 (0.7989)	1766 (0.7339)	_[a]

Table 2. Binding constants for the different terminal groups with β -CD. ^[a] Binding constant of *n*-butylamine with β -CD was also determined. Since the R^2 value was very small, the binding constant was considered to be very low.

After obtaining the different binding constants, the conjugation of the different ligands (1B, 1E, 1K) onto the surface of MSNPs was studied using XPS. The XPS data of functionalized MSNPs show different sulfur contents, thus representing different amounts of ligands on the surface of functionalized MSNPs. MSNPs-4(1)-1K exhibits the highest sulfur content followed by MSNPs-4(1)-1B and lastly

MSNPs-4(1)-1E (Table 3). This order is due to the differences in the flexibility and size of the end groups. The small and flexible *n*-butyl group facilitates the conjugation of ligand 1K to the MSNP surface, resulting in the highest sulfur content. The adamantyl and cyclohexyl groups, on the other hand, are much larger than the *n*-butyl group, thus leading to a low density of functional groups on the surface. The rigid adamantyl group may be able to arrange more neatly on the surface of MSNPs as compared with the cyclohexyl group, leading to conjugation of more ligand 1B on the surface than ligand 1E. Thus, the structural nature of these ligands could be used to explain the results obtained in Table 3.

(A3)

MSNPs	% Si	% S ^[a]	Loading capacity
MSNPs-3(1)	87.4	12.6	-
MSNPs-4(1)-1B	85.4	15.6	11.7 ± 0.22
MSNPs-4(1)-1E	85.3	14.7	13.9 ± 0.28
(B6) MSNPs-4(1)-1K	83.1	16.9	13.1 ± 0.05

(A3) Table 3. XPS data indicating the sulfur content upon conjugation of different ligands on the surface of MSNPs. In addition, the Dox loading capacity was obtained for MSNPs-4(1)-1B, MSNPs-4(1)-1E, and MSNPs-4(1)-1K. ^[a] The sulfur content corresponds to the amount of ligands being conjugated onto the surface of MSNPs.

(B6) Figure 6. Release kinetics for MSNPs-4(1)-1B, MSNPs-4(1)-1E, and MSNPs- 4(1)-1K when GSH (10 mM) was added at the 10th minute.

The order of loading capacity for this series of functionalized MSNPs was MSNPs-4(1)-1E>MSNPs-4(1)-1K>MSNPs-4(1)-1B (Table 3). The loading capacity was mainly dependent on two factors: firstly, the amount of the ligand on the surface of MSNPs in relation to access to the mesopores, and secondly, the binding of the ligand with β -CD to prevent the loaded drug from escaping. MSNPs-4(1)-1B gave the lowest loading capacity, although XPS showed that there was a substantial amount of the ligand on the surface of MSNPs. The low level of loading capacity might be due to the steric effect of the adamantyl group, which prevents the drug from entering the mesopores of MSNPs during the loading process. Compared with MSNPs-4(1)-1K with the *n*-butyl end group, MSNPs-4(1)-1E showed higher loading capacity on account of the higher binding constant of the cyclohexyl end group with β -CD to better keep the loaded drug within the mesopores. The Dox release behavior of these systems was then tested, indicating that the trend of the release profiles matches the trend of the Dox loading capacities (Figure 6). The observation further supports that the interaction of GSH with the disulfide bond was consistent for all the functionalized MSNPs. By comparing the release profiles for this set of conditions, MSNPs-4(1)-1E again gave the highest level of release and was chosen for the last set of experiments.

4.3.4 Effect of different amount of ligands on the surface of MSNPs on drug loading and release

The last set of conditions to be explored would be the effect of different amount of the ligands on the surface towards the Dox loading capacity and release efficiency. Firstly, XPS was again used to quantify the amount of thiol units as well as the final ligand on the surface of MSNPs. From the XPS data in Table 4, an increase in the sulfur content was observed when more mercaptan groups were grafted on the surface, giving the following order in terms of the sulfur content: MSNPs-3(2)>MSNPs-3(1)>MSNPs-3(0.5). After conjugation of the final ligands onto the surface through disulfide bond formation, the same order in sulfur content was observed: MSNPs-4(2)-1E>MSNPs-4(1)-1E>MSNPs-4(0.5)-1E. The increase in sulfur content after conjugation was proportional to the type of MSNPs, making the comparison of the drug-loading capacity easier. The loading capacity of various functionalized MSNPs is presented in Table 4, and the results reveal that the loading capacity of MSNPs-4(1)-1E is higher than that of MSNPs-4(2)-1E and MSNPs-4(0.5)-1E. Although MSNPs-4(2)-1E showed the highest amount of ligands on the surface of MSNPs, its loading capacity was lower, as the excessive amount of cyclohexyl groups on the surface could block the mesopores for drug loading. MSNPs-4(0.5)-1E also gave a low loading capacity because having less ligands on the surface cannot well retain a large amount of Dox within the mesopores. Thus, MSNPs-4(1)-1E has a reasonable amount of ligands on the surface to allow sufficient loading of Dox into the mesopores without obvious leakage. The level of drug release of these different samples gave the following

trend: MSNPs-4(0.5)-1E>MSNPs-4(1)-1E>MSNPs-4(2)-1E (Figure 7). This order was unexpected because the loading capacity was proportional to the release profile in above two sets of conditions tested. In this experiment, although MSNPs-4(0.5)-1E did not give the highest loading capacity, the amount of Dox released from MSNPs-4(0.5)-1E was the most significant. This is probably because the low amount of ligands on the surface enables GSH to easily interact with the disulfide bond, thus removing the capping agents for drug release. This explanation could be supported by the release profile of MSNPs-4(2)-1E, where the lowest amount of release was observed. The surface of MSNPs-4(2)-1E is highly hindered owing to the presence of a large amount of ligands capped with β -CD, thus preventing GSH from reacting with the disulfide bond.

(A4)

MSNPs	% Si	% S ^[a]	Loading capacity
MSNPs-3(0.5)	88.1	11.9	-
MSNPs-4(0.5)-1E	86.9	13.1	11.2 $\pm$ 0.07
MSNPs-3(1)	87.4	12.6	-
MSNPs-4(1)-1E	85.3	14.7	13.9 $\pm$ 0.28
MSNPs-3(2)	85.6	14.4	-
MSNPs-4(2)-1E	82.7	17.3	11.3 $\pm$ 0.68

(A4) Table 4. XPS data indicating the sulfur content after grafting different amount of mercaptan groups on the surface of MSNPs as well as after further conjugation of different amount of ligand 1E on the surface. In addition, the Dox loading capacity was obtained for MSNPs-4(0.5)-1E, MSNPs-4(1)-1E, and MSNPs-4(2)-1E. ^[a] The sulfur content corresponds to the amount of ligands being conjugated onto the surface of MSNPs.

(B7) Figure 7. Release kinetics for MSNPs-4(0.5)-1E, MSNPs-4(1)-1E, and MSNPs-4(2)-1E when GSH (10 mM) was added at the 10th minute.

To investigate the applicability of these functional MSNPs, in vitro drug-delivery experiments were carried out on the B16-F10 melanoma cell line. B16-F10 cells were incubated with MSNPs-4(0.5)-1E, MSNPs-4(1)-1E, and MSNPs-4(2)-1E, respectively, and the cell viability data as well as fluorescence microscopy images were then obtained. The cell viability (Figure 8a) involving these functional MSNPs at concentrations of 41.7 mg mL^{-1} and 20.8 mg mL^{-1} exhibited a proportional relationship with the corresponding release profiles (Figure 7). This trend is reasonable because a higher level of drug released would translate to higher cell death, thus causing lower cell viability. At concentrations lower than 20.8 mg mL^{-1} , the difference between the three types of functional MSNPs became insignificant. Therefore, we employed the concentration of 20.8 mg mL^{-1} for in

vitro imaging studies. From the fluorescence images shown in Figure 8b, it was observed that MSNPs-4(0.5)-1E gave the strongest red fluorescence intensity compared to the other two samples. This red fluorescence is attributed to Dox, and the intensity observed is proportional to the amount of Dox released into the cells. Hence, it could be concluded that MSNPs-4(0.5)-1E showed the highest level of Dox release followed by MSNPs-4(1)-1E and lastly MSNPs-4(2)-1E. This observation once again supports the results obtained from the cell viability assay as well as the release profile studies.

(a)

(b)

Figure 8. a) Percentage cell viability of B16-F10 after incubation with MSNPs-4(0.5)-1E, MSNPs-4(1)-1E, and MSNPs-4(2)-1E for 4 h before changing the culture media. b) Fluorescence microscopy images of the B16-F10 cell line after incubation with MSNPs-4(0.5)-1E, MSNPs-4(1)-1E, and MSNPs-4(2)-1E.

4.4 Conclusion

In conclusion, this work has adopted a supramolecular approach in analyzing the effects of the nature of functional groups on the surface of MSNPs on drug-loading capacity and release kinetics. A screening method has been employed by first varying chain length, then terminal group, and lastly the amount of thiol units on the surface. The ligand with an intermediate length (5 carbon atoms) and a bulky terminal group (cyclohexyl) shows the highest drug loading and release capacity under the same conditions. To achieve a high drug release capability, the surface coverage of the ligands should be relatively low. This observation has been further supported by in vitro studies, proving the applicability of the screening method. Guided by the results obtained from this work, better drug delivery systems may be designed for more efficient cancer treatment.

4.5 References

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CONCLUSION AND OUTLOOK

MSNPs have shown much promise in drug delivery and biomedical applications. Hence, it is of utmost importance to fully maximize its potential to achieve better therapeutic efficiency.

In this dissertation, MSNPs that are conventionally used for anticancer therapy was employed for the use with neuronal cells. Neuronal cells were first incubated with large and small MSNPs with the latter showing better cell uptake. Later a targeting ligand was conjugated on the surface of the small MSNPs and its targeting efficiency was shown to be enhanced. In order to further explore the use of the neuronal targeting ligand, the MSNPs containing the ligand was examined on cancer cells. The ligand showed targeting effects as well as therapeutic effects. When utilized concurrently with anti cancer drug DOX, the anticancer properties were synergistic and showed increased therapeutic efficiency with cancer cells.

Besides the probing the effects of various targeting moieties, stimuli controlled drug release from these MSNPs also serve as a form of selective release of anti-cancer drug at the targeted site. Hence, a catalysis screening method was adopted to analyze the effect of drug loading and release based on the different surface functionalities. The results obtained showed that ligand with intermediate chain length (5 carbons) and a terminal moderately bulky group (cyclohexyl) that complexes with β -cyclodextrin gave the best loading and release from the MSNPs. Decreasing the number of these ligands on the surface of these MSNPs led to an improvement in drug release and this was also proven in B16F10 melanoma cancer

cell lines. This method of screening is an easy and straightforward way of obtaining the best conditions with different stimuli controlled drug release.

In conclusion, this dissertation highlights the customizable properties of MSNPs whereby it can be used in the treatment of different diseases by altering the surface functional groups. Various diseases can be targeted and different medical conditions can be addressed concurrently. In addition, its large volume gives a high drug payload which makes it an excellent drug delivery tool. Future work could be conducted to improve the biodegradability of MSNPs in order for it to enhance its biocompatibility. Herewith, the use of MSNPs as drug delivery vehicles is only at its infancy, there is still a huge potential that has yet to be discovered which would benefit future drug delivery research.

List of Publications

1. Chung Yen Ang, Si Yu Tan, Shaojue Wu, Qiuyu Qu, Mun Fei Eddy Wong, Zhong Luo, Pei-Zhou Li, Subramanian Tamil Selvan, Yanli Zhao*, “A Dual Responsive “Turn-On” Fluorophore for Orthogonal Selective Sensing of Biological Thiols and Hydrogen Peroxide” *J. Mater. Chem. C* **2015**, DOI: 10.1039/c5tc01465d.
2. Si Yu Tan, Chung Yen Ang, Zhong Luo, Peizhou Li, Kim Truc Nguyen, Yanli Zhao,* “An iGlu receptor antagonist and its simultaneous use with anticancer drug for cancer therapy” *Chem. Eur. J.* **2015**, *21*, 6123-6131.
3. Chung Yen Ang, Si Yu Tan, Yunpeng Lu, Linyi Bai, Menghuan Li, Peizhou Li, Quan Zhang, Subramanian Tamil Selvan, Yanli Zhao,* “Turn-on fluorescence probe integrated polymer nanoparticles for sensing biological thiol molecules” *Sci. Rep.* **2014**, *4*, 7057-
4. Si Yu Tan, Chung Yen Ang, Peizhou Li, Qi Ming Yap, Yanli Zhao,* “Drug encapsulation and release by mesoporous silica nanoparticles: The effect of surface functional groups” *Chem. Eur. J.* **2014**, *20*, 11276-11282.
5. Chung Yen Ang, Si Yu Tan, Yanli Zhao,* “Recent advancements of biocompatible nanocarriers for delivery of chemotherapeutic cargoes towards cancer therapy” *Org. Biomol. Chem.* **2014**, *12*, 4776-4806.
6. Wangqiao Chen, Si Yu Tan, Yanli Zhao*, Qichun Zhang*, “A concise method to prepare novel fused heteroaromatic diones through double Friedel-Crafts acylation” *Org. Chem. Front.* **2014**, *1*, 391-394.
7. Chung Yen Ang, Si Yu Tan, Xiaoling Wang, Quan Zhang, Majad Khan, Linyi Bai, Subramanian Tamil Selvan, Xing Ma, Liangliang Zhu, Kim Truc Nguyen, Nguan Soon Tan,* Yanli Zhao,* “Supramolecular nanoparticle carriers self-assembled from cyclodextrin- and adamantane-functionalized polyacrylates for tumor-targeted drug delivery” *J. Mater. Chem. B* **2014**, *2*, 1879-1890.
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heteroacene” *Chem. Asian J.* **2014**, 9, 779-783.

9. Liangliang Zhu, Chung Yen Ang, Xin Li, Kim Truc Nguyen, Si Yu Tan, Hans Ågren, Yanli Zhao,* “Luminescent color conversion on cyanostilbene functionalized quantum dots via in-situ photo-Tuning” *Adv. Mater.* **2012**, 24, 4020–4024.
10. Rongrong Liu, Weiling Teo, Si Yu Tan, Huajun Feng, Parasuraman Padmanabhan, Bengang Xing,* “Metallic nanoparticles bioassay for *Enterobacter cloacae* P99 β -lactamase activity and inhibitor screening” *Analyst.* **2010**, 135, 1031-1036.