Stimuli-responsive drug delivery systems based on mesoporous silica nanoparticles

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**NANYANG
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**STIMULI-RESPONSIVE DRUG DELIVERY SYSTEMS
BASED ON MESOPOROUS SILICA NANOPARTICLES**

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**SCHOOL OF PHYSICAL AND MATHEMATICAL
SCIENCES**

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Abstract

With the development in Nanotechnology, Nano-sized drug carrier using Mesoporous Silica Nanoparticles (MSNPs) as a platform has been heavily investigated and it has demonstrated its effectiveness as a potential drug carrier system for anticancer drugs, such as Doxorubicin. This is attributed to the flexibility in modifying the properties of MSNPs to the desired design and parameters in mind. Besides that, surface functionalisation of MSNPs with stimuli responsive trigger has shown remarkable enhancement to the system as this added feature allows the release of drug selectively under specific environment and condition.

In this dissertation, effectiveness of intracellular stimuli responsive ligands, such as disulfide bond and acetal linkage with less than 5 synthetic steps, will be investigated. The systems will be tested in vitro to determine the sensitivity of the ligand under specific conditions, followed by cellular and confocal studies using HeLa cells to demonstrate the toxicity and effectiveness as a drug delivery system.

With this goal in mind, redox-responsive will be presented in chapter 2, along with dual responsive pH and redox responsive ligand in chapter 3. After which, doxorubicin hydrochloride (DOX) will be loaded into MSNPs as a chemotherapeutic drug before functionalisation of respective ligands onto MSNPs surface. Next, a series of characterization work was carried out to investigate the properties and demonstrate the efficacy of the system.

In conclusion, the systems above have demonstrated the effectiveness of intracellular stimuli responsive ligand through the selective release of drug under redox or acidic environment whilst minimising premature release leakage under physiological condition.

With this result, it can be concluded that the above systems are capable to serve as drug delivery carrier by minimising side effects from DOX and enhance toxicity to the cancerous cells.

Chapter 1: Introduction

1.1 Cancer and Nanoparticles

Cancer is second in place in its liability for causing deaths worldwide and has emerged as a major public health problem, with approximately 14 million new cases in 2012 and a worldwide mortality of 8.8 million deaths in 2015. It is characterized by abnormal and uncontrollable cell growth. With malignant phenotypic behaviour such as metastasis and invasion, cancer adversely affects different parts of the body¹. In a human body, cells have a limited lifespan and regenerations via cell division is necessary. In the case of cancer, it is a situation whereby cells in a body mutate and start to multiply rapidly. These mutated cells crowd and congregate to form a lump called tumour. Metastasis then occurs in which tumour cells invade other organs in the body and thus interferes with bodily functions that are necessary to live, allowing cancer to kill its host. In-depth research on the treatment of cancers has been done for decades, such as chemotherapy which have been proven effective. However, patients who survived experienced side-effects of the chemotherapy due to lack of specificity of the drug targeting. Thus, it is paramount that a suitable cure for cancer is found. In recently years, research on nanomedicine has made enormous breakthroughs in its capability to target the affected organs. This reduces the side effects of the cancer treatment²⁻³.

Over the years, extensive research and studies has been carried out to propose new directions for cancer treatment which is safe and effective. Of which, the development of nanomedicine from the advancement in bioengineering has open a new approach for drug delivery. In this application, nanomedicine utilises the inconsistency surface on the tumour formed from the congregation of irregularly shaped tumour cells where it enters and accumulates in the tumour through enhanced permeation and retention effect (EPR)⁴⁻⁶,

leading to the death of cancer cells. In addition, it has been determined that the characteristics of this nanomedicine, such as the size of the carrier, has great impact on the effectiveness in this approach. Thorough research has been carried out and investigated, from fabrication of new nanocarriers using different materials to modification of the carriers, to enhance the effectiveness and minimise side effects from the drug. Today, both organic and inorganic based nanocarriers have been heavily studied.

1.2 Organic-based drug delivery system

Organic-based nanoparticles such as liposomes and dendrimer, and inorganic-based nanocarriers like carbon nanotubes are most commonly used in drug delivery systems. Liposomes are one of the most common and well-investigated organic nanocarriers. Lipid molecules forming the bilayer structure consists of a hydrophilic head and a hydrophobic tail, allowing self-assembly into spherical structure with the versatility of fine-tuning the properties by changing the solvents used in self-assembly synthesis⁷. The characteristics of the lipid bilayer is similar to a typical cell membrane in the body system, thus the rate of phospholipid elimination is greatly retarded and it effects in prolonged circulation period in the body. Attributed to this trait, it also indicates that the lipid bilayer carrier is non-toxic. In addition, the nature of this amphiphilic design allows the loading of both hydrophobic and hydrophilic drug easily while the delivery of the drug is through fusion with the cancer cell membrane, before the release of encapsulated drug. Han and co-workers design a liposome based drug delivery carrier with doxorubicin embedded within the hydrophilic layer and cypate in hydrophobic region. In this system, liposome network will be disrupted upon the entry to cancer cells. This leads to the release of doxorubicin as well as cypate, which initiate chemotherapeutic effect and cell imaging with a wavelength of 765nm⁸.

Besides the use of liposomes, dendrimers are robust and well-defined polymeric structures with organized branching network where the number of generations for the branches can be controlled effectively. Due to this feature, drugs can be loaded to the system through conjugation with the terminal group of the branches⁹, thereby enhancing drug loading.

In this system, monomers such as amidoamine, propylene imine and esteramine, are frequently used as branches due to their ability to display ideal characteristics of a drug carrier such as water solubility and biocompatibility after polymerisation. These monomers polymerise to form the skeletal structure of the carrier which enhances the overall stability of the system. In addition, payload such as anticancer drug and contrasting agent, can be coupled within the branched network and the end of the polymer can be further functionalized with other functional groups to boost biocompatibility.¹⁰ For example, PAMAM dendrimers with methyl poly (ethylene glycol) (M-PEG) grafts were designed by Kojima and co-workers due to their biocompatibility of its resulting hydrophilic shell. M-PEG grafts good water solubility and demonstrated biocompatibility and studies have also shown that carriers PEG coated carrier reduces have a longer circulation time compared to non-PEG coated carriers¹¹. Frechet and co-workers exploit the good water solubility and high biocompatibility of the PEG and took advantage of its properties in increasing the dendrimers' biocompatibility¹².

1.3 Inorganic-based nanoparticles

Besides the organic-based nanocarriers, inorganic-based nanocarriers such as carbon nanotubes (CNTs) are also utilized extensively as drug carriers. CNTs are made up of carbon that is aligned in the shape of a tube¹³. They possess large surface area and can perform imaging capability. Single-walled CNTs (SWNTs) have shown capability in Raman scattering and NIR photoluminescence application, thus allowing photodynamic

therapy (PDT) via IR irradiation for anticancer treatment, on top of chemotherapeutic drug treatment. This demonstrates that CNTs are very versatile and are also a potential drug delivery carrier.

The extensive research on CNTs has proven its usefulness in applications such as composite materials, nano-electronics, field-effect emitters and hydrogen storage¹⁴. In recent years, CNTs have also proven to show capability of raman and NIR, demonstrating efficient in its role as a drug carrier for targeted delivery¹⁵⁻¹⁶. Wang and co-workers successfully developed a SWNT-PEI/DOX/NGR delivery system along with the characteristics of NIR and pH-responsiveness, sustained-release, and tumour-targeting. DOX delivered by SWNT-PEI/DOX/NGR indicates significant tumour growth inhibition in vitro and in vivo, demonstrating that the delivery system has potential to be applied in chemo-photothermal therapy¹⁷.

1.4 Mesoporous Silica Nanoparticles (MSNPs)

1.4.1 Properties of MSNPs

Apart from the above inorganic nanoparticles, Mesoporous silica nanoparticles MSNPs gained tremendous attention in the research field owing to its excellent biocompatibility and unique pore structure that enables cargo loading¹⁸⁻²⁰.

In addition, the fabrication of MSNPs can be fully customized in terms of the framework within the particle and functionalization of its surface, thereby enhancing the drug delivery efficacy. This carrier, with an average particle diameter between 2 to 200nm²¹, along with organized nanopore structure of pore diameter between 2 to 6nm²²⁻²³ is ideal for drug loading. This results in effective encapsulation of highly toxic chemotherapeutic drug, thereby decreasing the side effects and increasing the efficacy of cancer treatment²⁴.

Different sizes of MSNPs can be synthesized. However, the uptake efficiency to the cells has shown to decrease with an increase in particle size. Hoekstra and co-workers has conducted a study on the degree of cell internalization of particles through confocal along with FACS and results has demonstrated that cell internalization was observed with particle range of 50nm to 500nm in diameter, while negligible uptake for particles that is above 500nm in diameter. At the same time, the authors also observed a difference in the rate of uptake, with 50nm being internalized into the cells faster than 500nm. From these results, it demonstrates that the size of nanoparticles will greatly affect the uptake within the cells, which will greatly impact drug delivery²⁵.

One of the trademark of MSNPs are that it consists of large internal surface area and pore volume, allowing various sizes of drugs to be encapsulated, including the possibility to fine-tune the particle morphology, from the particle itself to the pore size. Despite the flexibility in modifications, the rigid matrix structure of MSNPs, which comprises of silica and oxygen bonding in an orderly fashion, contribute to the robustness of the material. Thus, this material is able to withstand harsh conditions, such as changes in pH or mechanical stress, without the need of the additional linkers²⁶⁻²⁹.

Due to the abundance of silanol group on the surface, which has affinity with phospholipids of the cell membrane, MSNPs has the tendency to interact with cell membranes of the red blood cells, leading to hemolysis¹³. In addition, the loaded drug has the tendency to leak out due to the opening of the pores. As a result, this decreases the efficiency of drug delivery and the purpose of drug carrier¹³.

Despite that, researchers have utilized this abundance of silanol group on the surface as active sites for surface modification with organic functional groups. This functionalisation

has not only minimized the possibility of hemolysis by reducing the number of free silanol active sites, it has also enhanced the efficacy of the drug carrier.³⁰

1.4.2 Synthesis of MSNPs

Among the methods of MSNPs synthesis, sol-gel formation is the preferred option due to its straightforward and simple synthesis, as well as template free integration. This method of synthesis revolves around 2 main components, starting with the hydrolysis of silica precursor in acidic or basic condition, followed by the condensation in neutral condition³¹⁻³². Generally, surfactants are normally used to serve as a temporary template for the silica precursor to etch on. Being amphiphilic in nature, surfactants such as hexadecyltrimethylammonium bromide (CTAB) molecules will self-assemble into micelles in water, where the hydrophobic tail will be cluster in the core and the hydrophilic head on the surface¹³. Next, silica precursor, such as tetraethylorthosilicate (TEOS), is introduced to the solution. This silica precursor does not only stabilize the micelle assembly but also condense and interact with the surface of CTAB through physical interactions like hydrogen bonding and electrostatic interaction, thus giving rise to orderly silica framework.

After which, the surfactant is removed through processes such as calcination or extractions to yield mesoporous silica structure consisting of porous channels with hexagonal cross section. After numerous research, it was found that highly controlled monodispersed MSNPs can be synthesized using sol-gel method. In addition, the framework of MSNPs can be customized to the desired conditions by changing the content composition, such as the silica precursor and surfactant, concentration, as well as the temperature³³⁻³⁴. For instance, a change in the surfactant, from hexadecyltrimethylammonium bromide (C₁₆TAB), to dodecyltrimethylammonium bromide (C₁₂TAB), will change the framework

of the pore channels, leading to a change in the efficiency of loading and drug delivery^{23, 29, 35-36}.

Beck and co-workers has first studied the impact of surfactant to pore width of MSN. In this study, the authors first synthesized MSN with a variation of surfactant (C₈TAB to C₁₆TAB) and carried out several characterizations, such as XRD and BET. From XRD results, it has demonstrated that pore width increases with longer surfactant chain length, with C₈TAB having a pore width of 1.8nm as compared to C₁₆TAB having a pore width of 3.7nm. Similarly, benzene sorption analysis on the materials has also demonstrated similar results, which showed an increase in benzene uptake with longer surfactant chain length, with C₈TAB loaded with 16 wt% of benzene as compared to C₁₆TAB loaded with 64 wt% of benzene³⁷.

Another group, Vallet-Regi and co-workers has also carried out similar studies on the impact of surfactant. In this group, the authors first synthesized two variants of MSN using C₁₂TAB and C₁₆TAB. Next, they carried out XRD characterization, which indicates that there is an increase in pore width with an increase in surfactant chain length, with C₁₂TAB having a pore width of 3.3nm and C₁₆TAB having a pore width of 3.6nm. Next, the authors proceed to load ibuprofen and drug release analysis was performed in stimulated body fluid. Form the results, it showed that there is an increase of drug content in C₁₆TAB (68%) compared to C₁₂TAB (55%)²⁴.

In these studies, they have demonstrated that surfactant does not only has impact on the pore width of the material but also affects the loading and release of drug content. Thus, it is one of the major component that will affect the efficiency of drug delivery system.

1.4.3 Surface modifications of MSNPs

1.4.3.1 Co-condensation method

There are two ways for surface modifications, which are co-condensation and post grafting. Co-condensation synthesis involves the incorporation of silica precursor with organosilanes and desired functional group in the presence of surfactants during the formation of silica framework. The advantage of this method is that this modification is direct and organosilanes will be distributed evenly within the silica network, thereby offering consistent active sites in the whole carrier. However, the downside of this modification is the importance of ratio between the silica precursor and organosilanes, which can alter the morphology of the structure. As a result, the mesoporous network will be reduced when the ratio between the silica precursor and organosilanes are not optimize. When this occurs, this will reduce the capability of MSNPs as a drug delivery carrier^{22, 32, 38}.

1.4.3.2 Post grafting method

On the other hand, post grafting involves the modification of MSNPs surface after the formation of the silica network. Similarly, the downside of this method is the obstruction in the pores opening, thus hindering the loading and release of drugs³⁹. In order to obtain desired MSNPs, both functionalisation methods were utilized in this research.

1.5 Stimuli responsive moieties

Apart from that, surface functionalisation was carried out to further minimize the possibility of drug leakage and decrease the rate of clearance by reticuloendothelial system (RES). To achieve this, various responsive ligands are developed with the key idea to block the opening of the pores, minimizing the possibility of the loaded drug to be leaked out during its transition to the targeted site. Upon entering the tumour, the ligands will be

triggered, either through external stimuli such as light or internal stimuli present within the tumour, such as redox^{33, 40-42}, pH^{18, 22, 38, 42-45} or heat⁴⁶, and release loaded drugs.

1.5.1 External stimulus responsive moiety

In light responsive stimulus, the ligand is developed using the approach of PDT, which utilises photosensitizer to serve as the gatekeeper. This works by taking advantage of photosensitizer in terms of the change in conformation upon the exposure of light. Jia and co-workers demonstrated this idea using MSNPs as the base and surface functionalised with hematoporphyrin. Under light irradiation of wavelength 650nm, the change in conformation of hematoporphyrin result to the cleaving of ligand on the surface, thus releases loaded DOX within the pores⁴⁷.

Aside from that, Wu and co-workers functionalised MSNPs surface with mercaptopropyl as the light responsive ligand. Under visible light irradiation, mercaptopropyl will be degraded, detaching the ligands from MSNPs surface, thereby releases loaded DOX within the pores⁴⁸.

1.5.2 Internal stimulus responsive moiety

On the other hand, internal stimuli responsive ligands take advantage of the physiological differences between cancer and healthy cells environment to serve as triggers that releases the drug. For instance, by making use of the difference in pH and concentration of reducing agent between normal and cancer cells.

1.5.2.1 pH responsive moiety

The process of tumour formation, where irregular blood vessels network and poor lymphatic drainage system arises due to the rapid assembly of cancer cells, has led to acute hypoxia. This happens due to the ineffectiveness of the tumour to excrete lactate, from the process of glycolysis in time leading to low pH environment, hence explains why pH in

tumour is around 5 while the normal tissue is around 7⁴⁹. With this property, various acid sensitive ligands have been developed, where the ligand will only be triggered under acidic environment and remain intact under neutral environment, thus minimizing the possibility of drug leakage. Chen and co-workers design a delivery system that uses modified dextrin with pH sensitive Schiff's base as a linker to block the pore opening. Upon acidic condition, modified dextrin is degraded to expose the pores, thus releasing the drug⁵⁰. Yao and co-workers designed another acid linker, consisting of modified zinc with polyethylene glycol and acid sensitive cis-aconitic anhydride, functionalized on the surface of MSN-Histidine. Upon acid environment, the acid sensitive cis-aconitic anhydride, will degrade and expose the pores⁵¹. Another group, Zhao and co-workers developed acid-sensitive ligand utilizing chitosan as the active site and lactobionic acid as the targeting site. In this design, the authors covalently conjugated this ligand onto MSN-COOH surface and demonstrated the sensitivity of this ligand by studying the release profile of the cargo⁵².

1.5.2.2 Redox responsive moiety

Aside from the difference in pH level within tumour and healthy cells, the concentration of glutathione (GSH) are also different, where the concentration of GSH is between 2mM to 10mM and around 10.0 μ M^{40, 53-55} in tumour cells and healthy cells respectively. This indicates that redox responsive ligands, which comprises of disulfide bonds, can be incorporated into the chain of ligands to induce redox responsive trigger and be cleaved when the system is exposed to redox environment⁵⁶. Chen and co-workers demonstrated the efficiency of redox responsive trigger by designing gadolinium-based bovine serum albumin complex (BSA-Gd) surface functionalised onto MSNPs surface through disulfide linkage. Under redox environment, disulfide bond is cleaved and BSA-Gd was detached, releasing loaded DOX in the pores, while BSA-Gd is activated and function as cellular

imaging⁵⁷. Another group, Lin and co-workers, utilised iron oxide as the gatekeeper and is functionalised onto MSNPs surface through disulfide linkage. In this work, the authors performs active targeting to the tumour region through the magnetic properties from the iron oxide. Upon cell internalisation and exposure to redox environment, disulfide bond will be cleaved thereby releasing loaded DOX within the pores⁵⁸. Luo and co-workers also demonstrates the sensitivity by surface functionalisation of collagen and lactobionic acid to perform as gatekeepers as well as active targeting, through disulfide linkage. In this study, the authors determine the effectiveness of the system through the release of loaded fluorescein, when the system is exposed to reducing environment within the cells, thereby functioning as cellular imaging⁵⁹.

1.6 Research Objectives

From the above sections, it has proven that nanoparticles has greatly enhance the efficacy of drug delivery into cancerous cells as compared to free drugs. In particular, MSNPs has greatly demonstrated to be one of the most popular drug delivery carrier due to its wide array of applications as well as the ease of functionalisation of the morphology and the surface. Aside from that, research has also shown that surface functionalisation with stimuli responsive ligands has further enhance the selectivity in releasing loaded drugs in the pore channels as well as minimising the probability of premature release.

In this dissertation, Mesoporous silica Nanoparticles (MSNPs) is chosen as the material for drug delivery along with surface functionalisation of stimuli responsive ligands, with the aim to increase the efficacy of drug delivery directly to cancer cells. MSNPs was first synthesized in both projects, followed by surface functionalisation with redox responsive ligands and dual responsive ligands in chapter 2 and 3 respectively. Following that,

anticancer drug, Doxorubicin, was chosen to be loaded into the system to assess the efficiency as drug delivery system.

In these two projects, the morphology of the material was analysed using multiple characterization techniques followed by the investigation of stimuli responsive ligands efficacy as a gatekeeper as well as the sensitivity upon exposure to the respective triggered environment. After that, intracellular analysis such as MTT and confocal were carried out to examine the uptake of the system and the effectiveness as a drug delivery medium as compared to free DOX.

Chapter 2: Redox responsive stimuli triggered Mesoporous Drug Delivery System

2.1 Introduction

Since the start of 20th century, researchers have been developing new methods to deliver anticancer drugs such as Doxorubicin (DOX), to the tumour cells more effectively and minimising the side effects of the drug. During that period of time, Dr Paul Ehrlich envisaged the fabrication of an ideal drug delivery system, where this system is stable under normal condition and selective release loaded drug in tumour environment.

He proposed magic bullet concept where he envisage the drugs to enter to the target cell structure, which attack the desired pathogens while remaining harmless to healthy tissues. Through his research in this direction, he has successfully applied concepts in chemistry, biology as well as medicine into one system, in search for anticancer therapy⁶⁰.

With this concept in mind, he develops a conceptual drug delivery system where drug is loaded into the system with multi-target inhibitor around the system as a targeting effect. Through his discovery and contribution in this area of research, research has been ongoing where researchers utilise the concept to develop and enhance drug delivery in hope to minimise side effects from the drug as well as maximises the efficacy of the drug to the targeted area. With this attribute, selective targeting is enhanced along with a reduction of side effects from the drug^{43, 60}.

With the development in nanoscience and technology, in-depth research has been carried out with the aim to open up new alternative method in a wide array of applications such as in biomedical imaging and gene delivery⁶¹⁻⁶². Several types of drug delivery systems such as liposomes⁶³⁻⁶⁴, dendrimers⁶⁵, carbon nanotubes¹⁶, gold and iron oxide nanoparticles⁶⁶

and silica particles¹⁸ were developed. Among the various types of drug delivery system, nanomaterials such as mesoporous silica nanoparticles (MSNPs), is one of the material that are being heavily investigated on due to the unique characteristics, as this material offers several distinct properties, such as high surface area and large pore volume, along with the ease of morphology and size control^{40, 49, 67} to the particles. By maximising the advantages and characteristics of MSNPs, it has provided a new approach in biomedical application as a delivery medium^{18, 33, 68}, in vivo imaging and cell labelling⁶⁹⁻⁷⁰.

In addition, MSNPs are well known for its ability to be chemically modifiable at the surface due to the presence of silanol group^{30, 45, 71-72}. From a scan of literature, it was shown that modification of MSNPs surface improves its efficiency in delivering of anticancer drugs such as doxorubicin (DOX)⁴⁴, where surface modified MSNPs displayed a longer retention time in the blood circulation system compared to unmodified MSNPs, as surface functionalised MSNPs minimises the rate of removal by Mononuclear Phagocyte System⁷³. Not only that, results have shown that MSNPs has the tendency to accumulate in tumour cells by enhanced permeation and retention effect⁷⁴. This mode of targeting, known as passive targeting effect, rely on simple diffusion concept, through the poor alignment of endothelial cells in the tumour tissue, thereby minimising the interaction of the particles with the healthy cells.

By making use of the ease of surface modification, intensive research was carried out in tuning and customising the properties of MSNPs, such that the particles are modified according to the desired purpose in mind. In stimuli-responsive drug delivery, particles can be modified such that the loaded drug will only be released under certain conditions, such as light, temperature, magnetism, pH and redox^{40, 45, 49}. In redox type stimulus-responsive drug delivery system where drugs will only be released upon intracellular reduction⁴⁰, linkers such as disulphide bond modification⁴² is commonly chosen to attain

this attribute. Numerous research has been carried out in investigating the level of GSH concentration in the tumour and normal cell and it was observed that GSH concentration is present in a relatively high amount in tumour cells (1-10mM) as compared to normal cells (about 2 μ M)⁵³⁻⁵⁵. This observation indicates that disulphide bond modified MSNPs will be stable in the plasma and it will only be reduced in tumour cells, leading to a reduction of disulphide bonds which releases the drug. With this observation, it allows the system to be more specific and selective in its function and greatly reduce the side effects of the drugs to the healthy cells. Park and co-workers demonstrated the sensitivity of disulfide bond by designing a system that is based on MSNPs and surface functionalised with cystamine modified carboxymethyl cellulose (CMC) through disulfide bond, where CMC served as gatekeeper after the loading of 5-FU. In this work, the results indicate that CMC is intact under physiological condition. Upon redox environment in cancerous cells, it was observed that CMC is detached, indicating the cleavage of disulfide bond, thus the commencement of drug release⁷⁵.

Herein, we design a redox responsive ligand that will be functionalised onto Mesoporous Silica Nanoparticles (MSNP), through surface functionalisation, to induce the release of loaded drug upon the exposure to intracellular environment in cancer cells. In this work, beta cyclodextrin (β -CD) was chosen to achieve strong complexation with adamantane on the ligand, to enhance the hydrophilicity of the system. Doxorubicin hydrochloride (DOX) was chosen to be the drug loaded into the system for this work. The efficacy of this carrier was then investigated by in vitro analysis to determine the sensitivity of the system in drug release under redox, acidic and combination of redox and acidic environment. Followed by cellular studies using HeLa cells to determine the toxicity effect of the system.

Figure 1 Schematic illustration of MSNPs surface functionalised with redox responsive ligand followed by the release of DOX under GSH condition

2.2 Experimental

2.2.1 Materials and methods

All solvents used in this project were of analytical reagent (AR) grade and HPLC grade, with a purity of more than 95% purity, which are purchased commercially and used directly without any further purification unless otherwise stated. Dry Tetrahydrofuran was achieved by drying HPLC grade of tetrahydrofuran over sodium metal and kept in a flask with activated molecular sieve of 4Å from sigma Aldrich. Next, all reagents were obtained from commercial sources and used directly without any further purifications. Ultrapure water (18.2 $\Omega \cdot \text{cm}$ resistivity at 25°C with less than 10 ppb Total Organic Carbon) was collected from Satorius Ultrapure Water system and used in the projects. 10 times Phosphate buffer saline (PBS) was used directly from purchase and use after dilution to 0.1X PBS concentration with ultrapure water. Unless otherwise stated, all nanoparticles synthesized are purified by centrifugation at a spin rate of 10,000 rpm for a duration of 10 minutes. Flash column chromatography was carried out with Merck Gerduran® Si 60 silica gel with particle sizes from 40 to 63 μm . Wet loading technique was utilized to load silica gel in to the column, using the eluent as solvent to turn it into a slurry.

2.2.2 Characterisations

^1H and ^{13}C NMR analysis were carried out using Bruker AMX 400 spectrometer at 25°C. For ^1H NMR spectra, X axis is reported as chemical shift (δ) in units parts per million (ppm) and downfield from TMS (δ 0.0 ppm) or relative to deuterated solvents (7.26 ppm for CDCl_3 and 3.31 ppm for CH_3OD). 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. For ^{13}C NMR spectra were reported as chemical shift (δ) in ppm with reference to residual solvent signal (77.0 ppm for CDCl_3). Chemical shifts, δ , of ^{13}C NMR spectra were reported in units of ppm. Fourier transform infrared (FTIR) spectroscopy were carried out using 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. Dynamic light scattering and 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. 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 samples were coated on a conductive carbon tape and product was concentrated in the middle with reference with the aluminium foil, which

was then attached onto an aluminium XPS sample holder. The Si/N ratios were achieved from the wide-range scan XPS measurements.

HeLa cell line were used in this project. Cells were incubated in the incubator with a temperature of 37.0°C along with a 5% concentration of carbon dioxide. Full media was prepared using Dulbecco's Modified Eagle Medium (DMEM with L-glutamine and sodium pyruvate, Serana) with the addition of 10% fetal bovine serum (Serana) and 1% penicillin streptomycin (Life Technology). Confocal microscopy images were taken with confocal microscope ((CLSM, LSM 510 Meta, Zeiss Co., Germany). Absorbance for the MTT cytotoxicity assay was analysed with an Infinite 200 PRO multimode reader. DAPI (4', 6-diamidino-2-phenylindole) was used to stain the nucleus of the cells during confocal microscopy.

2.2.3 Synthesis of organic ligand

2.2.3.1 Overall synthesis scheme of redox responsive ligand

2.2.3.2 Synthesis of 2,2,5-trimethyl-1,3-dioxane-5-carboxylic acid⁷⁶

To a 100ml round bottom flask, 2, 2 Bis(hydroxymethyl) propionic acid (37.3mmol, 5.00g) was first added, followed by the addition of acetone (20.0ml). Next, catalytic amount of p-toluenesulphonic acid monohydrate (2.00mmol, 0.355g) was added before the addition of 2, 2-dimethoxypropane (55.9mmol, 5.82g), followed by the addition of sodium sulphate (2.00mmol, 0.284g) were added. The reaction was then allowed to stir for 24 hours.

Upon completion, potassium carbonate (2.00mmol, 0.277g) was added and the mixture was allowed to stir for about 10 minutes to neutralize the reaction. Next, the mixture was filtered through vacuum filtration. The filtrate was collected, and solvent was evaporated through rotary evaporation to yield white solid. (5.71g, 90.0%). ^1H NMR (400 MHz, CDCl_3) δ 6.33 (b, 1H), 4.18 (d J = 11.6Hz, 2H), 3.72 (d, J = 12.0Hz, 2H), 1.44 (d, 7.2Hz, 3H), 1.41 (s, 3H), 1.20 (s, 3H). ^{13}C NMR (400 MHz, CDCl_3) δ 190.9, 156.0, 133.8, 131.2, 122.4, 38.7, 36.4, 27.9. m/z: calculated: 174 $[\text{M}]^+$; found: 197. IR (KBr) ν/cm^{-1} : 3097, 3061, 2995, 1724, 1381.

2.2.3.3 Synthesis of 2-(pyridin-2-yl)disulfaneyl)ethan-1-aminium⁷⁷

To a 100ml round bottom flask, 2, 2-dithiopyridine (36.4mmol, 8.00g) was added, followed by the addition of methanol (42.0ml). Next, 2, mercaptoethylamine HCl (18.0mmol, 2.08g) dissolved in methanol (10.0ml) was added into the mixture and the resultant mixture was allowed to stir and react for 24 hours in ambient temperature.

Upon completion, the product was dry over vacuo to yield yellow oil. This oil was then added into diethyl ether (150ml) to induce precipitation. The precipitate was left to settle and recover through Buchner filtration. This process was repeated until white precipitate

is obtained. (3.115g, 92.4%). ^1H NMR (400 MHz, MeOD) δ 8.56 (d, J = 4.4Hz, 1H), 7.82 (m, 1H), 7.67 (d, J =8.0Hz, 1H), 7.35 (m, 1H), 3.33 (m, 4H), 3.15 (t, J = 6.0Hz, 2H). ^{13}C NMR (400 MHz, MeOD) δ 158.01, 149.6, 137.73, 121.91, 37.59, 35.61. m/z : calculated: 184 $[\text{M}]^+$; found: 187. IR (KBr) ν/cm^{-1} : 2642, 1988, 1600, 1575, 1449, 1417.

2.2.3.4 Synthesis of 2-((2-(pyridin-2-yl)disulfaneyl) ethyl) amino)-1-(2, 2, 5-trimethyl-1, 3-dioxan-5-yl) ethan-1-one

To a 100ml round bottom flask, 2, 2, 5-trimethyl-1, 3-dioxane-5-carboxylic acid (1.00mmol, 174mg) was dissolved in dry THF (12.0ml) and the solution was cooled to 0°C. Next, hydroxybenzotriazole (HOBt) (1.20mmol, 183mg) was added to the solution and allowed to stir for 10 minutes. After which, 2-(pyridin-2-yl)disulfaneyl) ethan-1-aminium (1.00mmol, 187mg) and 4-dimethylaminopyridine (2.50mmol, 305mg) were added consecutively to the mixture and the mixture was allowed to stir for another 20 minutes before the addition of N-ethylcarbodimide hydrochloride (1.20mmol, 474mg). The resulting mixture was then allowed to stir in ambient temperature for 24 hours.

Upon completion, the reaction was filtered through Buchner filtration and the supernatant was collected and dry over vacuo. Column purification was carried out with Hexane and Ethyl acetate as the eluent. This purified product obtained yield colourless viscous liquid (298.8mg, 87.24%). ^1H NMR (400 MHz, CDCl_3) δ 8.50 (s, 1H), 7.72 (s, 1H), 7.62 (s, , 2H), 7.11 (t, J = 4.4Hz, 1H), 3.98 (d, J = 12.4Hz, 2H), 3.77 (d, J = 12.4Hz, 2H), 3.61 (d, J = 6.0z, 2H), 2.96 (d, J = 5.6Hz, 2H), 1.47 (d, J = 7.2Hz, 6H) 1.05 (s, 3H). ^{13}C NMR (400 MHz, CDCl_3) δ 175.1, 159.6, 149.9, 137.0, 121.0, 119.9, 98.7, 67.3, 40.6, 38.8, 37.8, 28.2,

19.3, 18.1. m/z: calculated: 342 [M]⁺; found: 343. IR (KBr) ν/cm^{-1} : 2989, 1720, 1645, 1531, 1417

2.2.3.5 Synthesis of 4-hydroxy-3-(hydroxymethyl)-3-methyl-1-((2-(pyridin-2-ylidisulphaneyl) ethyl) amino) butan-2-one

To a 100ml round bottom flask, 2- ((2-(pyridine-2-ylidisulphaneyl)ethyl)amino)-1-(2,2,5-trimethyl-1,3-dioxane-5-yl)ethan-1-one (4.00mmol, 1.26g), Methanol (4.00mmol, 8.00ml) and 1.0M HCl (4.00mmol, 8.00ml) were added accordingly and the mixture was allowed to react in ambient temperature for 24 hours.

Upon completion, the reaction was neutralized with sodium hydroxide (1.00M). Methanol was then removed through rotary evaporator and the crude product was then washed with ethyl acetate thrice followed by brine solution twice. Organic layer was collected, dry over sodium sulphate and solvents was removed through rotary evaporator, to yield white solid. (0.904g, 71.4%). ¹H NMR (400 MHz, CDCl₃) δ 8.51 (s, 1H), 7.89 (s, 1H), 7.62 (dd, J= 8.0Hz, 2H), 7.15 (t, J= 5.6Hz, 1H), 3.80 (d, J= 6.4Hz, 2H), 3.77 (d, J = 6.4Hz, 2H), 3.59 (t, J = 4.4Hz, 2H), 2.96 (t, J = 6.4Hz, 2H), 1.15 (s, 3H). ¹³C NMR (400 MHz, CDCl₃) δ 204.3, 176.8, 163.7, 149.9, 137.0, 121.4, 68.5, 51.2, 48.0, 38.8, 37.6, 18.3. m/z: calculated: 302 [M]⁺; found: 303. IR (KBr) ν/cm^{-1} : 3346, 3286, 2926, 1627, 1556, 1446, 1417.

2.2.3.6 Synthesis of 2-((((1s, 3s)-adamantane-1-carbonyl) oxy) methyl)-2-methyl-3-oxo-4-((2-(pyridin-2-yl)disulfaneyl) ethyl) amino) butyl (3r, 5r, 7r)-adamantane-1-carboxylate

To a 100 ml round bottom flask, 4-hydroxy-3-(hydroxymethyl)-3-methyl-1-((2-(pyridin-2-yl)disulfaneyl) ethyl) amino) butan-2-one (1.00mmol, 0.308g) was added, followed by the addition of dichloromethane (14.0 ml). The solution was then left to cool to 0°C for 15 minutes. Next, triethylamine (3mmol, 1.55ml) was added and allowed to stir for another 15 minutes, before the addition of 1-Adamantanecarbonyl chloride (2.20 mmol, 437mg). The mixture was then allowed to stir in ambient temperature for 24 hours.

Upon completion, Buchner filtration was first performed to the mixture. The supernatant was collected and dry over vacuo. The crude product obtained was dissolved in dichloromethane and washed with water followed by brine before drying the organic layer over sodium sulphate and the solvents were removed through rotary evaporator. Flash column purification was then carried out using Hexane and Ethyl Acetate. ¹H NMR (400 MHz, CDCl₃) δ 8.52 (s, 1H), 7.71 (s, 1H), 7.62 (m, 1H), 7.60 (m, 1H), 7.15 (m, 1H), 4.38 (d, J= 10.8Hz, 1H), 4.15 (d, J = 12.0Hz, 2H), 3.64 (s, 2H), 3.57 (s, 2H), 2.92 (m, 2H), 2.02 (s, 3H), 1.90 (s, 6H), 1.71 (s, 6H). ¹³C NMR (400 MHz, CDCl₃) δ 178.0, 175.5, 174.7, 150.1, 137.2, 121.4, 121.0, 110.8, 66.4, 65.9, 47.6, 43.8, 41.0, 39.0, 38.7, 37.5, 36.4, 17.6. M/z: calculated: 628 [M]⁺; found: 650. IR (KBr) ν/cm⁻¹: 3280, 2924, 2904, 1728, 1641, 1548, 1448, 1421, 1230, 1180.

2.2.4 Synthesis of MSNPs

2.2.4.1 Overall synthesis scheme for MSNPs

2.2.4.2 Synthesis of MSNPs-S1²⁶

To a 500ml round bottom flask, Cetyltrimethylammonium bromide (0.5 g) was dissolved in ultrapure water (240 mL), followed by the addition of sodium hydroxide (1.75 mL, 2.00M). The mixture was left to heat to 80°C for 2 hours, before the addition of tetraethylorthosilicate (2.50ml) and (3-Mercaptopropyl)trimethoxysilane (200 μ l in 1.00 ethanol) followed by the addition of ethyl acetate (2.50ml). The resulting mixture was then heated to 80°C and left to maintain for 2 hours. Upon 2 hours, the resulting mixture was neutralized with 1M hydrochloric acid and left to cool before purifying it over centrifugation (10000 rpm, 10 minutes) and washed with 3 times water, followed by 3 times ethanol, before drying it over in vacuum oven at 65°C for 24 hours, to yield MSNPs-S1.

2.2.4.3 Synthesis of MSNPs-S2

To a 2 neck 100 ml round bottom flask, MSNPs-S1 (0.25g) was added and condenser was attached to one opening while the other is sealed with a rubber septum. Next, gas exchange was carried out 3 times before the addition of anhydrous toluene (40.0ml) and (3-Mercaptopropyl) trimethoxysilane (0.50ml) through the rubber septum. Gas exchange was carried out 2 times after the addition. The resulting mixture was then refluxed for 24 hours

under nitrogen environment. Upon completion, the mixture was purified by centrifugation (10000 rpm, 10 minutes) and washed with 3 cycles of ethanol before drying it over in vacuum oven at 65°C for 24 hours, to yield MSNPs-S2.

2.2.4.4 Synthesis of MSNPs-S3

To a 100ml round bottom flask, MSNPs-S2 (1.00g) was added, followed by the addition of anhydrous methanol (160ml) and 12.1N hydrochloric acid (9.00ml). The mixture was left to reflux for 24 hours under nitrogen environment. Upon completion, the mixture was purified by centrifugation (10000 rpm, 10 minutes) and washed with 3 cycles of ethanol before drying it over in vacuum oven at 65°C for 24 hours, to yield MSNPs-S3.

2.2.4.5 Synthesis of MSNPs-S4

To a 50ml round bottom flask, MSNPs-S3 (0.10g) was added, followed by the addition of absolute ethanol (20.0ml). Next, 2-((((1s, 3s)-adamantane-1-carbonyl oxy) methyl)-2-methyl-3-oxo-4-((2-(pyridin-2-yl)disulfaneyl) ethyl) amino) butyl (3r, 5r, 7r)-adamantane-1-carboxylate (0.10g) was added to the solution dropwise to ensure complete reaction. The mixture was left to stir for 24 hours. Upon completion, the mixture was purified by centrifugation (10000 rpm, 10 minutes) and washed with 3 cycles of ethanol before drying it over in vacuum oven at 65°C for 24 hours, to yield MSNPs-S4.

2.2.4.6 Synthesis of MSNPs-S5

To a 100ml round bottom flask, MSNPs-S4 (0.05g) was added, followed by the addition of excess beta cyclodextrin. Next, ultrapure water (2.00ml) was added and the reaction was left to stir for 24 hours. Upon completion, the mixture was purified by centrifugation (10000 rpm, 10 minutes) and washed with 3 cycles of ethanol before drying it over in vacuum oven at 65°C for 24 hours, to yield MSNPs-S5.

2.2.5 Drug loading

To an Eppendorf tube (2.00ml), doxorubicin hydrochloride (5.00mg) was dissolved in 0.1X PBS (1.00ml) followed by the addition of MSNPs-S4 (20.0mg). The mixture was then sonicated for 1 hour, vortex, before placing it in shaker (1600rpm, 37°C) for 24 hours in the dark. Upon completion, the mixture was centrifuged, washed with ethanol thrice before drying in vacuum oven at 65°C for 24 hours. The supernatant was collected which will be used for drug loading analysis. After 24 hours, excess beta cyclodextrin was dissolved in ultrapure water and this solution (1.00ml) was added into the Eppendorf tube with DOX loaded MSNPs-S4. The resultant mixture was sonicated and vortex for 1 hour to disperse the particles followed by shaking in the shaker (1600rpm, 37°C) for another 24 hours in the dark. Upon completion, the mixture was centrifuged, and washed with 3 times before drying in vacuum oven at 65°C and under – 1.00 atm for 24 hours. The supernatant was collected which will be used for drug loading determination. This process is repeated 3 times.

Drug loading was determined by UV-Vis spectroscopy. Briefly, the absorbance of each stage of supernatant was determined through UV-Vis spectroscopy and the obtained absorbance were converted to the absolute concentration through a calibration curve. Next, the exact drug loading into the carrier can be determined indirectly from the difference between the concentration of DOX present in the respective supernatants and the initial loading.

2.2.6 Drug release

For GSH release studies, DOX loaded MSNPs-S5 were dispersed in 0.1X PBS buffer (1.00ml) with the absence of GSH. The mixtures were sonicated for 5 minutes before loading to the shaker with parameters of 37°C and 1600rpm. These mixtures were then

allowed to shake for a specific period before they were removed and centrifuged (14800rpm, 5 minutes) while the supernatants were collected. A new set of fresh buffers was added, sonicated, and loaded to shaker and the process is repeated twice. From the third cycle onwards, buffer containing respective concentration of GSH (1.00mM, 5.00mM and 10.0mM) were added into the respective centrifuge tubes to mimic the redox concentration present in the body. This cycle was then repeated for a period of 40 hours with the collection of supernatant at specific interval. Collected supernatants were then analyzed by fluorescence instrument, and the concentration of DOX present for each supernatant were determined by the intensity signal detected. These concentrations were then utilized to plot the cumulative release of DOX against the specific time interval of collected supernatant.

2.2.7 In vitro analysis

2.2.7.1 MTT assay

Hela cells were cultured with Dulbecco's modified eagle medium (DMEM) with the addition of 10% concentration of fetal bovine serum (FBS) and 1.00% of penicillin. The surrounding environment for incubation is set to be 37°C with 5.00% of carbon dioxide. Subculture were carried out every 48 hours, when the confluence is about 70.0%, along with a fresh media (10.0ml) replaced each time. During each subculture, initial media present in the incubation flask will be removed, followed by cleansing with 1X PBS (10.0ml) before the addition of trypsin to lift the cells off the surface of the flask. Next, full media (4.00ml) was added to quench trypsin before performing subculture. The desired amount of cells are kept in the incubation flask, followed by the addition of full media (10.0ml) and allowed to incubate for another 48 hours, before the subculture process repeats.

For MTT assay, Hela cells were seeded on a 96 well plates with a density of around 10,000 cells per well and they were then allowed to incubate for 24 hours. Next, respective samples (MSNPs-S5, Free DOX and DOX loaded MSNPs-S5) were added into the respective wells (10.0 μ L) with various concentrations, leaving a row of wells as a control. 6 replicates were conducted per concentration and they were then allowed to incubate for 24 hours. Upon 24 hours, the media was removed and fresh full media, together with the addition of MTT (0.5mg/ml) was added. The cells were then allowed incubate for 4 hours before removing the media. The crystals that are formed at the bottom will be dissolved by the addition of Dimethyl sulphoxide (100 μ L) in each well respectively. These intensities were then obtained by the measurement of the absorbance at 590nm. The intensity of the dissolved solution are directly proportional to cell survivability. The average of six replicates for each respective concentrations were determined along with the standard deviation and error bar. These data will then be used to illustrate the cell viability against the concentration of the sample.

2.2.7.2 Confocal microscopy

For confocal studies, HeLa cells first were seeded on a 6 well plate, with glass slips, with cell density of 200,000 cells per well with full media (2.00ml), and were incubated for 24 hours. After 24 hours incubation, DOX loaded MSNPs-S5 were added into the wells at specific time interval. After that, full media was removed in the wells and 1X PBS (1.00ml) was added to wash the wells 3 times. Next, formaldehyde (4.00%, 1.00ml) was added to each well to affix the cells to the cover slip for 10 minutes followed by the removal and 1X PBS (1.00ml) was added to wash the wells 3 times. Next, DAPI (4',6-diamidino-2-phenylindole, 300nM) was added and allowed to incubate for 3 minutes for nucleus staining, followed by removal and 1X PBS (1.00ml) was added to wash the wells 3 times.

Upon completion, the cover slip was removed from the wells and mounted onto glass slips for analysis.

2.3 Results and Discussions

In this project, mesoporous silica nanoparticles (MSNPs) was synthesized under basic condition, with the addition of sodium hydroxide to water. CTAB was first introduced to the solution and stirred for an hour at 80.0°C. Next, (3-Mercaptopropyl) trimethoxysilane and TEOS were added into the solution dropwise and the mixture was allowed to stir for another 2 hours. Under basic condition, both TEOS and (3-Mercaptopropyl) trimethoxysilane will be deprotonated into negatively charged hydroxyl and carboxyl group respectively, which will be bonded to the positively charged ammonium group on CTAB template through electrostatic interaction. This leads to the formation of organized silica network through condensation, giving rise to structured mesopore channels. After which, surface grafting was carried out with (3-Mercaptopropyl) trimethoxysilane followed by the removal of CTAB. Finally, surface functionalisation of redox responsive ligand was carried out through disulfide bonding conjugation, before the loading of DOX and capping with beta cyclodextrin.

2.3.1 Characterisations of MSNPs

Figure 2 TEM images and DLS histogram for MSNPs-S2 to MSNPs-S4

MSNPs were first characterized with Transmission electron microscopy (TEM) to investigate the shape and size of the particles. From the images, it can be determined that spherical MSNPs with a mean diameter of about 150 nm were synthesized. In addition, the particle size remained consistent even after multiple surface functionalisation, thus indicating the stability of the silica network as there is no destruction or modification of the morphology. Aside from that, pore width with around 3.50 nm is clearly visible in MSNPs-3 and MSNPs-4, indicating that the removal of CTAB template is successful. Despite having multiple cycles of surface functionalisation, these modifications are not reflected in TEM images. This is due to the low amount of electron density from organic chains present as compared to the electron density of silica, thus detection of ligands via TEM is not successful.

Dynamic light scattering (DLS) was also conducted to further evaluate the size of the MSNPs. From the histogram, it shows that the size of MSNPs was approximately 190 nm for MSNPs-S1 and a slight decrease in size to about 142 nm for MSNPs-S3 with an increment to about 164 nm for MSNPs-S4. These results indicate the successful removal of CTAB template which can be observed between MSNPs-S1 and MSNPs-S3, followed

by the success in conjugation of redox responsive ligand onto MSNPs surface, which is reflected by the increment of particle size between MSNPs-S3 and MSNPs-S4.

Figure 3 XRD plot of MSNPs-S1, MSNPs-S3 and MSNPs-S4, showing characteristic spectrum belonging to MCM-41 type mesoporous material, indicating the particles to be mesoporous.

Powder X-ray diffraction was conducted to investigate the pore structure of MSNPs. From the graph, characteristic diffraction peaks of around 2.169° along with broad peak around 3.5° to 4.5° corresponding to plane of (100), (110) and (200) respectively, were observed at each stage. This characteristic observation at low angle 2θ region resembles highly ordered hexagonal pore structure of MCM-41⁷⁸ type mesoporous materials. Apart from that, pore widths of the channels were calculated to be around 2.31nm with an error of $\pm 0.13\text{nm}$, which is within the range of mesoporous⁷⁹. With this result, it can be determined that MSNPs, which was synthesized, belong to the class of MCM-41 type mesoporous materials.

Figure 4 Adsorption and desorption isotherms plots of MSNPs-S2 to MSNPs-S4, indicating Type IV isotherm pattern.

Next, nitrogen adsorption-desorption analysis was carried out to examine the interior morphology of MSNPs, such as surface area, pore volume and pore width distribution. From the isotherm plots, type IV isotherm⁶⁷ nitrogen adsorption-desorption pattern was observed at each stage, indicating that the synthesized MSNPs consist of uniform and organized mesoporous structure. Thus, BET analysis parameters was narrowed down to a relative pressure of 0.05cc/g to 0.35cc/g, which provides both Density Functional Theory (DFT) data and pore volume of MSNPs

	Pore volume (cc/g)
MSNPs-S2	0.533
MSNPs-S3	0.696
MSNPs-S4	0.554

Table 1 Summary of MSNPs-S2 to MSNPs-S4 pore volume

From the table above, an increase in pore volume of 0.163cc/g was observed between MSNPs-S2 and MSNPs-S3, from 0.533cc/g to 0.696cc/g, indicating the removal of CTAB template within the pore channels. Slight decrease in pore volume of 0.142 cc/g was also observed between MSNPs-S3 and MSNPs-S4, from 0.696cc/g to 0.554 cc/g, attributing to the success of surface functionalisation with redox responsive ligand, where the ligand leads to the decrease in pore width by obstructing the pore opening.

Figure 5 DFT pore size distribution obtained from BET isotherm for MSNPs-S2 and MSNPs-S4

DFT analysis was carried out to examine the width of the pore channels in the material. From the plot of $d(V)$ against half-pore width, the half pore width of MSNPs-S2 and MSNPs-S4 was determined to be 10.57 Å and 11.56 Å respectively. This suggests that the pore width of MSNPs obtained is 2.11nm and 2.31nm respectively, with an average of 2.21nm with an error of ± 0.14 nm. This data obtained is consistent with the results observed in TEM and XRD, where there is an increment in pore volume and pore width of MSNPs

after the removal of CTAB template followed by a reduction after surface functionalization with redox responsive ligand.

From the data obtained using XRD and BET, a discrepancy of about 1.87nm in pore width is observed. This difference is due to the different methods in pore width determination between XRD and BET, in which XRD calculates based on the depth reading between the lattice structures of the particle while BET calculated indirectly through the filling of nitrogen gas molecules into the pore channels of the particle. With this understanding, the difference of 1.87nm is determined to be the thickness of the particles.

With the results obtained above, it confirms that the synthesized MSNPs are of MCM-41-type mesoporous material with spherical morphology and diameter of about 150nm, with pore width of approximately 4.00nm. The isotherm plot points out to be a type IV isotherm plot⁷⁸⁻⁷⁹, which further confirmed that this MSNPs is MCM-41 type.

Figure 6 FTIR spectrum for MSNPs-S1 to MSNPs-S5

Surface functionalisation on MSNPs surface was first examined with FTIR. From the combine spectrum, Si-O-Si absorption peaks at around 1074cm^{-1} and 802 cm^{-1} , along with OH absorption peak at around 1635 cm^{-1} and 3410 cm^{-1} , can be observed in all 5 materials. These characteristic peaks of MSNPs functional group suggest that MSNPs is synthesized^{59, 80}. Next, surface functionalisation of each stage was investigated. To begin, surface functionalisation of MSNPs-S1 with (3-Mercaptopropyl) trimethoxysilane, followed by the removal of CTAB template, is successful as the intensity of C-H alkyl stretch around the region of 2850 cm^{-1} to 2950 cm^{-1} decreases without any changes in the intensity of Si-O-Si region. At the same time, CTAB C-H peak at 1467cm^{-1} region is absent. These observations suggest the absence of CTAB template and thus indicate the success of template removal.

In MSNPs-S4, a new peak at around 1740 cm^{-1} which belongs to aromatic and carbonyl group, along with the intensified C-H peak were observed. From this data, it can be determined that surface functionalization with redox responsive ligand has been successful. After complexation with β -CD, peaks around hydroxyl and C-H alkyl regions were intensified, which reflects the success of complexing β -CD with adamantane.

Figure 7 XPS spectrum for MSNPs-S1, MSNPs-S3 and MSNPs-S4

XPS analysis was also carried out to further examine the surface functionalisation of MSNPs. From the combined survey spectrum of MSNPs, peaks corresponding to Silicon, Carbon and Oxygen elements at 103eV, 285eV and 532eV respectively were present in all 4 samples which correspond to MSNPs and thus, it can be further confirmed that the desired MSNPs have been synthesized. Nitrogen peak at around 402eV, which is attributed to the presence of the CTAB template, is reflected in MSNPs-S1 and not reflected in both MSNPs-3 and MSNPs-4, indicating the success of CTAB removal from the MSNPs. Aside from that, the appearance of Sulphur peak at around 164 eV was observed in MSNPs-S3 and MSNPs-S4, but absent in MSNPs-S1, revealing the success in surface modification of (3-Mercaptopropyl) trimethoxysilane to the surface. In addition, it can be observed that slight shift in $S_{2p}^{3/2}$ and $S_{2p}^{1/2}$ region are present between MSNPs-S3 and MSNPs-S4. According to literature library, this shift reflects the different types of sulfur bonding,

where MSNPs-S3 consist of free SH bond while MSNPs-S4 consist of disulfide bond and these indicate the success in surface functionalisation through disulfide bonding.

	Zeta Potential	Standard Deviation
MSNPs-S2	20.77	0.874
MSNPs-S3	-23.93	1.100
MSNPs-S4	-21.3	0.436

Table 2 Summary of Zeta Potential surface charge on MSNPs-S2 to MSNPs-S4

Next, zeta potential analysis was carried out to investigate success of surface functionalisation, through the change in surface charge of MSNPs. From the table, it can be observed that the surface potential for each stage is significantly different, which suggests that the functional groups on the surface of the MSNPs is different, thus signifying that surface functionalisation was successful.

With the results obtained from FTIR, XPS and Zeta potential, it can be deduced that surface functionalisation with redox responsive ligand had been successful without any disruption or modification of MSNPs morphology.

2.3.2 Drug release under stimulus condition

After the confirmation of redox responsive ligand attachment, drug loading and release capability was investigated. Absorption and fluorescence analysis were used to characterize loading and release of Dox because Dox exhibit an excitation wavelength of 480nm and emission wavelength of 558nm. Through UV-Visible Spectrophotometer and Fluorometer, the amount of Dox present in the supernatant can be determined.

With this quantification of unloaded Dox present in the supernatant, the amount of Dox loaded within the system can be determined from the difference between initial concentrations of Dox added with the concentration of Dox detected from the supernatant.

MSNPs was first dispersed in DOX solution (5.00mg/ml) and was placed in shaker (1600rpm, 37°C) in the dark for 24 hours. The mixture was centrifuged, and the supernatant was collected. The particles were then dispersed in β -CD solution to initiate complexation with adamantane for another 24 hours, before centrifuging, washing, and collecting the supernatant. The supernatants at each stage were then analysed using UV-Visible Spectrophotometer ($\lambda_{\text{ex}} = 480\text{nm}$) to determine the concentration of DOX present.

The actual amount of DOX loaded within the pores were determined indirectly, from the difference of DOX present at the initial stage with the amount of DOX present in the supernatants. From the calculation, DOX loading efficiency was 10.1%. This relatively low loading could be owing to the presence of ligand which obstruct the pore opening, thereby reduces the ease of DOX diffusion to the channels. This observation can also be supported by the BET analysis, which also indicate a decrease in pore volume of MSNPs after surface modifications, from 0.696cc/g to 0.554cc/g.

Figure 8 Release profile of DOX under the influence of 0mM, 1mM and 10mM of GSH for a period of 40 hours

Next, the efficiency of redox responsive ligand was investigated through the release of DOX in the pores. In this study, DOX loaded MSNPs-S5 (2.00mg) was first dispersed in a centrifuged tube containing pH 7.5 PBS buffer (1.00ml) with GSH (0mM, 1.00mM and 10.0mM), followed by shaking for a specific time interval at 37°C and 1600rpm. Thereafter, the mixture was centrifuged and the supernatant was separately collected, before the addition of fresh buffer with the same concentration of GSH. The amount of DOX present in each batch of supernatant was then determined using fluorescence of $\lambda_{em} = 588\text{nm}$ ($\lambda_{ex} = 480\text{nm}$). From the result, negligible amount of DOX (7.26%) was detected throughout the analysis, indicating that the ligand is very effective in blocking the pores and minimizing premature release under normal physiological condition. On the other hand, significant increase in DOX released was observed upon the addition of GSH. This is due to the cleavage of the disulphide bond that anchored the ligand to MSNPs surface, thereby releasing the drug. From the results, it was observed that the release profile of 1.00nM and 10.0nM, 36% and 41% respectively, are similar. This indicates that the sensitivity of disulfide bond in this ligand is very sensitive, suggesting that the disulfide bond is not hindered.

2.3.3 In vitro cellular analysis

2.3.3.1 MTT assay

Figure 9 Cell viability assay for MSNPs-S5, Free DOX and DOX loaded MSNPs-S5

Moving on, in vitro cell viability assay was carried out using (3, 4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay with HeLa cells. In this study, HeLa cells were first seeded on a 96 well plates with a density of around 10,000 cells per well and they were then allowed to incubate for 24 hours. Next, respective samples (MSNPs-S5, Free DOX and DOX loaded MSNPs-S5) were added into the respective wells (10.0µL) with specific concentrations, leaving a row of wells as a control. 6 replicates were conducted per concentration and they were then allowed to incubate for 24 hours. Upon 24 hours, the media was removed and fresh full media, together with the addition of MTT (0.5mg/ml). The cells were then incubated for 4 hours before removing the media. The

crystals that are formed at the bottom will be dissolved by the addition of Dimethyl sulphoxide (100 μ L) in each well respectively. These intensities were then obtained by the measurement of the absorbance at 590nm with microplate reader. The intensity of the dissolved solution is directly proportional to cell survivability. The average of six replicates for each respective concentration were determined along with the standard deviation and error bar. These data will then be used to illustrate the cell viability against the concentration of the sample.

In this project, comparison of cell viability for DOX-loaded MSNPs-S5, free DOX and MSNPs-S5 was carried out to examine the toxicity of this drug carrier system. From the results, it can be observed that the cell viability decreases as the concentration of free DOX increases. Besides that, DOX-loaded MSNPs-S5 also displayed a decrease in cell viability as the concentration increased. However, the decrease is significantly larger as compared to free DOX administration, up to a concentration of 200 μ g. This indicates that the carrier is internalized by the cells, thereby reflecting the enhancement of drug localization in the cells. In addition, bare MSNPs-S5 shows minimal toxicity in the cells, with cell viability maintaining at around 73% at a concentration of 400 μ g. This implies that the carrier is highly biocompatible and poses insignificant toxicity to the cells, which also indicates that the toxicity level for DOX-loaded MSNPs-S5 is solely due to DOX. From this MTT assay, it indicates that the system is capable of internalization to cells and provides localized drug delivery within the cancerous cells. On top of that, this result also demonstrates that the carrier is non-toxic to the cells, which is a very promising result for drug delivery carrier.

2.3.3.2 Confocal microscopy

Figure 10 Confocal microscopy images of HeLa cells after incubated with DOX loaded MSNPs-S5. DOX fluorescence and DAPI staining from nucleus can be observed individually as well as in the merged images

Next, confocal laser scanning microscopy analysis was carried out to further investigate internalization of the carrier into the cells. In this study, HeLa cells were seeded to a 6

wells plate with cell density of approximately 250 thousand cells per well and incubated for overnight. Next, DOX loaded MSNPs-S5 was added to the well at specific time interval (120 minutes, 90 minutes, 60 minutes, 45 minutes, 30 minutes, and 15 minutes) and incubated. After which, the cells were affixed with 4% formaldehyde followed by the staining of cell nucleus with DAPI before analysing through confocal. From the images obtained, minimal DOX fluorescence was observed at the initial stage and the intensity of fluorescence increased as the time interval increased. This suggests that the cleavage of disulphide bond for drug release increases over a span of 120 minutes, indicating that the redox responsive ligand is sensitive within the cellular conditions. Apart from that, it can also be observed that DOX fluorescence is more intense around the nuclear region compared to the other parts of the cells. This indicates that the released DOX migrated and localized to the nucleus, killing the cells through interactions with DNA⁸¹. From this time-dependent analysis, it demonstrates that the system is capable to internalize into the cells and the disulphide linkage is sensitive to be cleaved in the reducing environment within the cells, thereby releasing DOX. Besides that, movement and localization of DOX from the cytoplasm to the nucleus can also be observed, indicating that the encapsulation of DOX within the system maintains the efficacy of the drug.

2.4 Conclusion

In conclusion, redox responsive mesoporous silica nanoparticles drug delivery system has been successfully developed. From the above characterization analysis, it shows that the MSNPs synthesized belong to MCM-41 mesoporous material with redox responsive ligand functionalized on the surface. The efficiency of redox responsive ligand was demonstrated in solution as well as in the HeLa cells. From the results obtained, it is indicative that the ligand is capable of preventing premature release of DOX in the carrier under normal physiological conditions as well as the ability to release DOX under redox environment. Apart from that, both MTT and confocal analysis also demonstrated the enhancement of cell toxicity through the internalization and localization of the system into the cells as compared to free DOX. In addition, confocal microscopy also demonstrated the different stages of internalization of carrier, as well as the release of DOX, within the cell. Therefore, it can be concluded that this system is promising as a drug delivery carrier for future chemotherapeutic purposes.

Chapter 3: Dual responsive stimuli triggered Mesoporous Drug Delivery System

3.1 Introduction

Cancer, a disease caused by the tremendous growth and mutation from normal cells, is one of the leading cause of death in many countries². Various types of treatment, such as surgery, radiotherapy and chemotherapy, are commonly utilised to remove the tumour and minimise the probability of recurrence. Despite the effectiveness of the treatments, most of the cancer patients will experience side effects from chemotherapy. With the advancement in technology, the development of drug delivery system has improved chemotherapeutic treatment dramatically⁸²⁻⁸⁴.

Chemotherapy, one of the treatment for cancer patients, is a form of treatment where common anticancer drugs, such as paclitaxel, doxorubicin, or vinblastine⁸⁵⁻⁸⁶, are administrated to decelerate the growth rate of cancer cells, leading to the death of cancer cells. Among the anticancer drugs, doxorubicin (DOX) is one of the popular anticancer drug as it is applicable to a wide range of cancer. It kills cancer cells by inhibiting topoisomerase II and enhance the formation of free radicals⁸⁶. However, side effects of this treatment are relatively high due to the poor physiochemical properties of the anticancer drug, with DOX interacting with both the normal and tumour cells due to the absence of targeting properties. As such, it requires a drug delivery system (DDS) to house the anticancer drug, where this system delivers anticancer drug into cancer cells without any premature release along the way, thereby reducing the rate of drug exposure to normal cells which minimises side effects occurrence from the drug.

DDS, which comprises of Nano-sized carrier, allows the encapsulation of chemotherapeutic drugs like Doxorubicin within the carrier, followed by the delivery of the drug to cancer cells. This decreases the exposure of toxic chemotherapeutic drug to the surrounding healthy cells, thereby minimises side effects as a result of the drug⁸⁷. Among the various types of DDS, mesoporous silica nanoparticles (MSNPs) has been intensively researched on since its discovery in 1992 by a mobile oil corporation⁶⁷ due to its straightforward synthesis along with the modulation of the properties to the desired outcome. Aside from synthetic flexibility advantage, surface of MSNPs consist of numerous silanol functional group. This allows further conjugation of stimuli responsive ligands, such as extracellular and intracellular responsive ligands, onto the surface of MSNPs, thereby further improving the efficiency of MSNPs as a drug delivery carrier.

Among the various types of stimuli ligands such as light, pH, redox and temperature, pH and redox are commonly selected as intracellular stimulus responsive ligand^{22, 32, 40, 44-45, 59, 75}. Numerous research has been carried out to investigate the concentration of GSH in the tumour and normal cell and it was observed that GSH concentration is present in a relatively high amount in tumour cells (1-10mM) as compared to normal cells (about 2 μ M)⁵³⁻⁵⁵. Apart from the difference in GSH concentration, it was also found that the environment in cancer cells are generally acidic (pH 4.5 to pH 5.5) as compared to normal cells (pH 7.5). This observation indicates that linkers such as disulfide bond and acetal or hydrazine bond⁴², which respond to redox and acidic condition respectively, will be effective in inducing drug release within the cancerous cells while preventing drug leakage in normal cells.

In a study by Zhou and co-workers, they investigated the efficiency of dual responsive trigger by designing a system based on MSN and surface functionalised with poly(glycidyl methacrylate (PGMA), followed by the addition of 2,2'-Diaminodiethyl disulfide

dihydrochloride to induce cross coupling polymerisation. In this design, disulfide bond function as a redox trigger while PGMA functions as gatekeeper as well as pH responsive trigger. With this system, the authors demonstrated the intensified released of loaded DOX under both redox and acidic environment, showing the advantage of this new ligand⁸⁸.

Similarly, Lin and co-workers, investigate the sensitivity of dual responsive pH and redox ligand by designing a system based on MSNPs and surface functionalised with hyaluronic acid consisting of disulfide and hydrazine bonding. In this design, both disulfide and hydrazine bond is embedded within hyaluronic acid. Upon degradation of hyaluronic acid by Hyaluronidases enzyme in cancerous cells, stimuli responsive ligands will be exposed and cleaved, leading to drug release. In this study, the authors observed an increment of about 20.0% DOX released in pH and redox condition as compared to single responsive condition, thereby showing the enhanced sensitivity of drug release⁸⁹.

Herein, we design a pH and redox responsive ligand that will be functionalised onto Mesoporous Silica Nanoparticles (MSNPs) through surface functionalisation to induce the release of loaded drug upon the exposure of intracellular environment in cancer cells. In this work, beta cyclodextrin (β -CD) was chosen to achieve strong complexation with adamantane on the ligand, to enhance the hydrophilicity of the system. Doxorubicin hydrochloride (DOX) was chosen to be the drug loaded into the system for this work. The efficacy of this carrier was then investigated by in vitro analysis to determine the sensitivity of the system in drug release under redox, acidic and combination of redox and acidic environment. Followed by cellular studies using HeLa cells to determine the toxicity effect of the system.

Figure 11 Schematic illustration of MSNPs surface functionalised with dual responsive ligand followed by the release of DOX under pH and GSH condition

3.2 Experimental

3.2.1 Materials and methods

All solvents used in this project were of analytical reagent (AR) grade and HPLC grade, with a purity of more than 95% purity, which are purchased commercially and used directly without any further purification unless otherwise stated. Dry Tetrahydrofuran was achieved by drying HPLC grade of tetrahydrofuran over sodium metal and kept in a flask with activated molecular sieve of 4Å from sigma Aldrich. Next, all reagents were obtained from commercial sources and used directly without any further purifications. Ultrapure water (18.2 $\Omega \cdot \text{cm}$ resistivity at 25°C with less than 10 ppb Total Organic Carbon) was collected from Satorius Ultrapure Water system and used in the projects. 10 times Phosphate buffer saline (PBS) was used directly from purchase and use after dilution to 0.1X PBS concentration with ultrapure water. Unless otherwise stated, all nanoparticles synthesized are purified by centrifugation at a spin rate of 10,000 rpm for a duration of 10 minutes. Flash column chromatography was carried out with Merck Gerduran® Si 60 silica gel with particle sizes from 40 to 63 μm . Wet loading technique was utilized to load silica gel in to the column, using the eluent as solvent to turn it into a slurry.

3.2.2 Characterisations

^1H and ^{13}C NMR analysis were carried out using Bruker AMX 400 spectrometer at 25°C. For ^1H NMR spectra, X axis is reported as chemical shift (δ) in units parts per million (ppm) and downfield from TMS (δ 0.0 ppm) or relative to deuterated solvents (7.26 ppm for CDCl_3 and 3.31 ppm for CH_3OD). 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. For ^{13}C NMR spectra were reported as chemical shift (δ) in ppm with reference to residual solvent signal (77.0 ppm for CDCl_3). Chemical shifts, δ , of ^{13}C NMR spectra were reported in units of ppm. Fourier transform infrared (FTIR) spectroscopy were carried out using 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. Dynamic light scattering and 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. Powder X-ray diffraction (XRD) measurements were carried out on SHIMADZU XRD-6000 Labx diffractometer at 40 kV and 30 mA using Cu $K\alpha$ 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 samples were coated on a conductive carbon tape and product was concentrated in the middle with reference with the aluminium foil, which

was then attached onto an aluminium XPS sample holder. The Si/N ratios were achieved from the wide-range scan XPS measurements.

HeLa cell line were used in this project. Cells were incubated in the incubator with a temperature of 37.0°C along with a 5% concentration of carbon dioxide. Full media was prepared using Dulbecco's Modified Eagle Medium (DMEM with L-glutamine and sodium pyruvate, Serana) with the addition of 10% fetal bovine serum (Serana) and 1% penicillin streptomycin (Life Technology). Confocal microscopy images were taken with confocal microscope ((CLSM, LSM 510 Metanlo, Zeiss Co., Germany). Absorbance for the MTT cytotoxicity assay was analysed with an Infinite 200 PRO multimode reader. DAPI (4', 6-diamidino-2-phenylindole) was used to stain the nucleus of the cells during confocal microscopy.

3.2.3 Synthesis of organic ligand

3.2.3.1 Overall synthesis scheme of dual responsive ligand

3.2.3.2 Synthesis of 4-formylphenyl adamantane-1-carboxylate

To a 50ml round bottom flask, 4-hydroxybenzaldehyde (10.0mmol, 1.24g) was dissolved in Tetrahydrofuran (10.0ml), followed by the addition of triethylamine (10.0mmol, 1.53ml). The mixture was then stirred for 20 minutes while allowing to cool to 0oC. Next, 1-adamantanecarbonyl chloride (15.0mmol, 3.03g) was dissolved in minimal tetrahydrofuran and added to the mixture dropwise to ensure homogenous reaction. This resulting mixture was then allowed to react under ambient temperature for 24 hours.

Upon completion, the product is concentrated using rotary evaporation and the product was dissolve in diethyl ether and extracted with sodium carbonate (1.00M). Organic layer was collected, dry over sodium sulphate and the solvents are removed through rotary evaporation to yield white solid (2.47g, 86.7%). ¹H NMR (400 MHz, CDCl₃) δ 9.99 (s, 1H), 7.90 (d J = 8.4Hz, 2H), 7.23 (d, J = 8.4Hz, 2H), 2.10 (s, 3H), 2.07 (s, 6H), 1.78 (s, 6H). ¹³C NMR (400 MHz, CDCl₃) δ 191.0, 156.0, 133.8, 131.2, 122.4, 38.7, 36.4, 27.9. M/z: calculated: 284 [M]⁺; found: 285. IR (KBr) ν/cm⁻¹: 3071, 2904, 1743, 1693, 1600, 1506, 1454, 1226, 1211, 1058.

3.2.3.3 Synthesis of 4-(5-(hydroxymethyl)-5-methyl-1, 3-dioxan-2-yl) phenyl adamantane-1-carboxylate

To a 100ml round bottom flask, 1, 1, 1-tris(hydroxy)ethylethane (1.00mmol, 120.15mg) was dissolved in dry tetrahydrofuran (10.0ml), followed by the addition of p-toluenesulphonic acid monohydrate (0.100mmol, 19.0mg). Next, 4-formylphenyl adamantane-1-carboxylate was dissolved in dry tetrahydrofuran (2.00ml) and loaded into a dropping funnel. 4-formylphenyl adamantane-1-carboxylate solution was then added to the solution

mixture dropwise over a period of 2 hours. After which, the resulting mixture was stirred under ambient temperature for 24 hours.

Upon completion, the mixture was neutralized with ammonia solution (5.00ml) and solvents was removed through rotary evaporation. The resulting crude product was then dissolved in dichloromethane and extracted with brine. The organic layer was collected, dry over sodium sulphate and solvents removed over rotary evaporation. This crude product was then purified with flash column chromatography using hexane and ethyl acetate as the eluents. The product obtained was colourless liquid. (0.904g, 71.4%). ^1H NMR (400 MHz, CDCl_3) δ 7.47 (d, $J = 8.4\text{Hz}$, 2H), 7.04 (d, $J = 9.2\text{Hz}$, 2H), 5.43 (s, 1H), 4.306 (d, $J = 11.2\text{Hz}$, 2H), 3.89 (s, 2H), 3.65 (d, $J=11.6\text{Hz}$, 2H) 2.08 (s, 3H), 2.05 (s, 6H), 1.77 (s, 6H), 0.81 (s, 3H),. ^{13}C NMR (400 MHz, CDCl_3) δ 176.1, 151.6, 143.3, 135.6, 127.2, 121.4, 101.3, 73.4, 65.7, 38.8, 36.5, 27.9, 16.9. m/z: calculated: 386 $[\text{M}]^+$; found: 387. IR (KBr) ν/cm^{-1} : 3487, 2906, 1745, 1608, 1510, 1454, 1215, 1193.

3.2.3.4 Synthesis of 3-(pyridin-2-yl)disulfaneyl)propanoic acid⁹⁰

To a 100ml round bottom flask, 2-2' dithiopyridine (1.00mmol, 215mg) in ethanol followed by the addition of catalytic amount of acetic acid. Next, 3-mercaptopropionic acid was added to the mixture and allow the mixture to stir for 2 hours.

Upon completion, the solvents were removed using rotary evaporator and the crude product was purified using basic alumina column by flashing the column using dichloromethane: ethanol [3:2] initially to remove pyridine that appears in yellow. After which, the column was then flushed with dichloromethane: ethanol: acetic acid in the ratio of [60:40:4] to elute the desired product. The solvent was removed over vacuo and pale-

yellow oil was obtained. (0.904g, 71.4%). ^1H NMR (400 MHz, CDCl_3) δ 8.40 (m, 1H), 7.82 (m, 2H), 7.24 (m, 1H), 3.04 (m, 2H), 2.93 (m, 1H), 2.71 (m, 2H). ^{13}C NMR (400 MHz, CDCl_3) δ 173.7, 160.0, 149.2, 137.9, 121.0, 119.9, 33.0, 19.3. m/z: calculated: 215 $[\text{M}]^+$; found: 216. IR (KBr) ν/cm^{-1} : 2906, 1754, 1573, 1417, 1217.

3.2.3.5 Synthesis of 4-(5-methyl-5-(((3-(pyridin-2-yl)disulfaneyl) propanoyl) oxy) methyl)-1,3-dioxan-2-yl)phenyl-adamantane-1-carboxylate

To a 100ml round bottom flask, dissolved 3-(pyridin-2-yl)propanoic acid in tetrahydrofuran and cool 0°C . Next, 1-hydroxybenzotriazole was added and the mixture was allowed to stir for 15 minutes. Next, 4-(5-(hydroxymethyl)-5-methyl-1,3-dioxan-2-yl)phenyl adamantane-1-carboxylate was added followed by 4-dimethylaminopyridine and the resulting mixture was allowed to stir for another 20 minutes before the addition of N-ethylcarbodiimide hydrochloride. The mixture was then allowed to react for 2 days in room temperature.

Upon completion, Buchner filtration was first performed to the mixture. The supernatant was collected and dry over vacuo. The crude product obtained was dissolved in dichloromethane and washed with water followed by brine before drying the organic layer over sodium sulphate and the solvents were removed through rotary evaporator. Flash column purification was then carried out using Hexane and Ethyl Acetate. ^1H NMR (400 MHz, CDCl_3) δ 8.48 (d, $J = 6.4\text{Hz}$, 1H), 7.67 (m, 2H), 7.48 (d, $J = 8.8\text{Hz}$, 1H), 7.11 (m, 1H), 7.05 (d, $J = 8.8\text{Hz}$, 2H), 5.40 (s, 1H), 3.89 (d, $J = 5.2\text{Hz}$, 2H), 3.83 (d, $J = 4.8\text{Hz}$, 2H), 3.07 (t, $J = 5.6\text{Hz}$, 2H), 2.83 (t, $J = 7.6\text{Hz}$, 2H), 2.07 (s, 3H), 2.05 (s, 6H), 1.76 (s, 6H), 1.31

(s, 3H),. ^{13}C NMR (400 MHz, CDCl_3) δ 176.2, 171.3, 159.6, 151.6, 149.8, 137.1, 135.4, 127.2, 121.4, 101.1, 73.4, 41.0, 38.6, 36.5, 33.7, 27.9, 18.9. m/z: calculated: 583 $[\text{M}]^+$; found: 584. IR (KBr) ν/cm^{-1} : 2490, 1714, 1570, 1448, 1419, 1244.

3.2.4 Synthesis of MSNPs

3.2.4.1 Overall synthesis scheme for MSNPs²⁶

3.2.4.2 Synthesis of MSNPs-S1

To a 500ml round bottom flask, Cetyltrimethylammonium bromide (0.5 g) was dissolved in ultrapure water (240 mL), followed by the addition of sodium hydroxide (1.75 mL, 2.00 M). The mixture was left to heat to 80°C for 2 hours, before the addition of tetraethylorthosilicate (2.50ml) and (3-Mercaptopropyl)trimethoxysilane (200 μl in 1.00 ethanol) followed by the addition of ethyl acetate (2.50ml). The resulting mixture was then heated to 80°C and left to maintain for 2 hours. Upon 2 hours, the resulting mixture was neutralized with 1M hydrochloric acid and left to cool before purifying it over centrifugation (10000 rpm, 10 minutes) and washed with 3 times water, followed by 3 times ethanol, before drying it over in vacuum oven at 65°C for 24 hours, to yield MSNPs-S1.

3.2.4.3 Synthesis of MSNPs-S2

To a 2 neck 100 ml round bottom flask, MSNPs-S1 (0.25g) was added and condenser was attached to one opening while the other is sealed with a rubber septum. Next, gas exchange was carried out 3 times before the addition of anhydrous toluene (40.0ml) and (3-Mercaptopropyl) trimethoxysilane (0.50ml) through the rubber septum. Gas exchange was carried out 2 times after the addition. The resulting mixture was then refluxed for 24 hours under nitrogen environment. Upon completion, the mixture was purified by centrifugation (10000 rpm, 10 minutes) and washed with 3 cycles of ethanol before drying it over in vacuum oven at 65°C for 24 hours, to yield MSNPs-S2

3.2.4.4 Synthesis of MSNPs-S3

To a 100ml round bottom flask, MSNPs-S2 (1.00g) was added, followed by the addition of anhydrous methanol (160ml) and 12.1N hydrochloric acid (9.00ml). The mixture was left to reflux for 24 hours under nitrogen environment. Upon completion, the mixture was purified by centrifugation (10000 rpm, 10 minutes) and washed with 3 cycles of ethanol before drying it over in vacuum oven at 65°C for 24 hours, to yield MSNPs-S3

3.2.4.5 Synthesis of MSNPs-S4

To a 50ml round bottom flask, MSNPs-S3 (0.10g) was added, followed by the addition of absolute ethanol (20.0ml). Next, 4-(5-methyl-5-(((3-(pyridin-2-yl)disulfaneyl) propanoyl) oxy) methyl)-1, 3-dioxan-2-yl) phenyl-adamantane-1-carboxylate (0.10g) was added to the solution dropwise to ensure complete reaction. The mixture was left to stir for 24 hours. Upon completion, the mixture was purified by centrifugation (10000 rpm, 10 minutes) and washed with 3 cycles of ethanol before drying it over in vacuum oven at 65°C for 24 hours, to yield MSNPs-S4.

3.2.4.6 Synthesis of MSNPs-S5

To a 100ml round bottom flask, MSNPs-S4 (0.05g) was added, followed by the addition of excess beta cyclodextrin. Next, ultrapure water (2.00ml) was added and the reaction was left to stir for 24 hours. Upon completion, the mixture was purified by centrifugation (10000 rpm, 10 minutes) and washed with 3 cycles of ethanol before drying it over in vacuum oven at 65°C for 24 hours, to yield MSNPs-S5.

3.2.5 Drug loading

To an Eppendorf tube (2.00ml), doxorubicin hydrochloride (5.00mg) was dissolved in 0.1X PBS (1.00ml) followed by the addition of MSNPs-S4 (20.0mg). The mixture was then sonicated for 1 hour, vortex, before placing it in shaker (1600rpm, 37°C) for 24 hours in the dark. Upon completion, the mixture was centrifuged, washed with ethanol thrice before drying in vacuum oven at 65°C for 24 hours. The supernatant was collected which will be used for drug loading analysis. After 24 hours, excess beta cyclodextrin was dissolved in ultrapure water and this solution (1.00ml) was added into the Eppendorf tube with DOX loaded MSNPs-S4. The resultant mixture was sonicated and vortex for 1 hour to disperse the particles followed by shaking in the shaker (1600rpm, 37°C) for another 24 hours in the dark. Upon completion, the mixture was centrifuged, and washed with 3 times before drying in vacuum oven at 65°C and under – 1.00 atm for 24 hours. The supernatant was collected which will be used for drug loading determination. This process is repeated 3 times.

Drug loading was determined by UV-Vis spectroscopy. Briefly, the absorbance of each stage of supernatant was determined through UV-Vis spectroscopy and the obtained absorbance were converted to the absolute concentration through a calibration curve. Next, the exact drug loading into the carrier can be determined indirectly from the difference

between the concentration of DOX present in the respective supernatants and the initial loading.

3.2.6 Drug release

To study the release efficiency of DOX loaded MSN-S5 under pH and/or redox influence, in vitro stimuli responsive analysis was conducted. For pH release studies, DOX loaded MSNPs-5 were dispensed in 0.1X PBS buffer (1.00ml) with various pH (7.5, 6.5, 5.5 and 4.5) to mimic cellular environment within the body. Next, the mixtures were sonicated for 5 minutes before loading to the shaker at 37°C and 1600rpm. These mixtures were then allowed to shake for a specific period before they were removed and centrifuged (14800rpm, 5 minutes). The supernatants were collected. A new set of fresh buffers was added, sonicated, and loaded to shaker and the process is repeated for a period of 40 hours with the collection of supernatant at specific interval. Collected supernatants were analyzed by fluorescence instrument, and the concentration of DOX present for each supernatant were determined by the intensity signal detected. These concentrations were then utilized to plot the cumulative release of DOX against the specific time interval of collected supernatant.

For GSH release studies, DOX loaded MSNPs-S5 were dispersed in 0.1X PBS buffer (1.00ml) with the absence of GSH. The mixtures were sonicated for 5 minutes before loading to the shaker with parameters of 37°C and 1600rpm. These mixtures were then allowed to shake for a specific period before they were removed and centrifuged (14800rpm, 5 minutes) while the supernatants were collected. A new set of fresh buffers was added, sonicated, and loaded to shaker and the process is repeated twice. From the third cycle onwards, buffer containing respective concentration of GSH (1.00mM, 5.00mM and 10.0mM) were added into the respective centrifuge tubes to mimic the redox concentration present in the body. This cycle was then repeated for a period of 40 hours

with the collection of supernatant at specific interval. Collected supernatants were then analyzed by fluorescence instrument, and the concentration of DOX present for each supernatant were determined by the intensity signal detected. These concentrations were then utilized to plot the cumulative release of DOX against the specific time interval of collected supernatant.

For dual responsive pH and GSH release studies, DOX loaded MSNPs-S5 were dispensed in 0.1X PBS buffer (1.00ml) with various pH (7.5, 6.5, 5.5 and 4.5) along with GSH (1.00mM, 5.00mM and 10.0mM) to mimic physiological condition within the body. Next, the mixtures were sonicated for 5 minutes before with parameters of 37°C and 1600rpm. These mixtures were then allowed to shake for a specific period before they were removed and centrifuged (10000rpm, 5 minutes) while the supernatants were collected. A new set of fresh buffers was added, sonicated, and loaded to shaker and the process is repeated for a period of 40 hours with the collection of supernatant at specific interval. Collected supernatants were analysed by fluorescence instrument, and the concentration of DOX present for each supernatant were determined by the intensity signal detected. These concentrations were then utilized to plot the cumulative release of DOX against the specific time interval of collected supernatant.

3.2.7 In vitro analysis

3.2.7.1 MTT assay

Hela cells were cultured with Dulbecco's modified eagle medium (DMEM) with the addition of 10% concentration of fetal bovine serum (FBS) and 1.00% of penicillin. The surrounding environment for incubation is set to be 37°C with 5.00% of carbon dioxide. Subculture were carried out every 48 hours, when the confluence is about 70.0%, along with a fresh media (10.0ml) replaced each time. During each subculture, initial media

present in the incubation flask will be removed, followed by cleansing with 1X PBS (10.0ml) before the addition of trypsin to lift the cells off the surface of the flask. Next, full media (4.00ml) was added to quench trypsin before performing subculture. The desired amount of cells are kept in the incubation flask, followed by the addition of full media (10.0ml) and allowed to incubate for another 48 hours, before the subculture process repeats.

For MTT assay, Hela cells were seeded on a 96 well plates with a density of around 10,000 cells per well and they were then allowed to incubate for 24 hours. Next, respective samples (MSNPs-S5, Free DOX and DOX loaded MSNPs-S5) were added into the respective wells (10.0 μ L) with various concentrations, leaving a row of wells as a control. 6 replicates were conducted per concentration and they were then allowed to incubate for 24 hours. Upon 24 hours, the media was removed and fresh full media, together with the addition of MTT (0.5mg/ml) was added. The cells were then allowed incubate for 4 hours before removing the media. The crystals that are formed at the bottom will be dissolved by the addition of Dimethyl sulphoxide (100 μ L) in each well respectively. These intensities were then obtained by the measurement of the absorbance at 590nm. The intensity of the dissolved solution is directly proportional to cell survivability. The average of six replicates for each respective concentration were determined along with the standard deviation and error bar. These data will then be used to illustrate the cell viability against the concentration of the sample.

3.2.7.2 Confocal microscopy

For confocal studies, HeLa cells first were seeded on a 6 well plate, with glass slips, with cell density of 200,000 cells per well with full media (2.00ml), and were incubated for 24 hours. After 24 hours incubation, DOX loaded MSNPs-S5 were added into the wells at specific time interval. After that, full media was removed in the wells and 1X PBS (1.00ml)

was added to wash the wells 3 times. Next, formaldehyde (4.00%, 1.00ml) was added to each well to affix the cells to the cover slip for 10 minutes followed by the removal and 1X PBS (1.00ml) was added to wash the wells 3 times. Next, DAPI (4',6-diamidino-2-phenylindole, 300nM) was added and allowed to incubate for 3 minutes for nucleus staining, followed by removal and 1X PBS (1.00ml) was added to wash the wells 3 times. Upon completion, the cover slip was removed from the wells and mounted onto glass slips for analysis.

3.3 Results and Discussions

In this project, mesoporous silica nanoparticles (MSNPs) was synthesized under basic condition, through the addition of sodium hydroxide to water. To begin, CTAB was first introduced to the solution and was stirred for an hour at 80.0°C. Next, (3-Mercaptopropyl) trimethoxysilane and TEOS were added into the solution dropwise and the mixture was allowed to stir for another 2 hours. Under basic condition, both TEOS and (3-Mercaptopropyl) trimethoxysilane will be deprotonated into negatively charged hydroxyl and carboxyl group respectively, which will be bonded to the positively charged ammonium group on CTAB template through electrostatic interaction. This leads to the formation of organized silica network through condensation, giving rise to structured mesopore channels. After which, surface grafting was carried out with (3-Mercaptopropyl) trimethoxysilane followed by the removal of CTAB. Finally, surface functionalisation of redox responsive ligand was carried out through disulfide bonding conjugation, before the loading of DOX and capping with beta cyclodextrin.

3.3.1 Characterisations of MSNPs

Figure 12 TEM images and DLS histogram for MSNPs-S1, MSNPs-S3 and MSNPs-S4

MSNPs was first characterized with Transmission electron microscopy (TEM) to investigate the shape and size of the particles. From the images, it can be determined that spherical MSNPs with a mean diameter of about 160nm were synthesized. In addition, the size of the particles remain consistent even after multiple surface functionalisation, thus indicating that there are no destruction or modification of the morphology. Aside from that, pore channels, with pore width around 2.38nm, are clearly visible in MSNPs-S3 and MSNPs-S4, indicating that the removal of CTAB template is successful. Despite having surface multiple cycles of surface functionalisation, these changes are not reflected in TEM images. This is due to the low amount of electron density of organic chains present as compared to the electron density of silica, thus detection of ligands is not successful.

Dynamic light scattering (DLS) was also conducted to further evaluate the size of MSNPs. From the result, it shows that the size of MSNPs was around 200nm for MSNPs-S1 and MSNPs-S2 and a slight decrease in size to about 164nm for MSNPs-S3 to MSNPs-S5, which is attributed to the removal of CTAB template. Despite the small difference in size determined by DLS, the results obtained is similar to the size observed from TEM of around 160nm.

Figure 13 XRD plot of MSNPs-S1 to MSNPs-S5, showing characteristic spectrum belonging to MCM-41 type mesoporous material, indicating the particles to be mesoporous.

Powder X-ray diffraction was conducted to investigate the pore structure of MSNPs. From the graph, characteristic diffraction peaks of around 2.2° along with broad peak around 3.5° to 4.5° corresponding to plane of (100), (110) and (200) respectively, were observed at each stage. This characteristic observation at low angle 2θ region resembles highly ordered hexagonal pore structure of MCM-41⁷⁸ type mesoporous materials. Apart from that, pore width of the channels were calculated to be around 4.01nm, which is within the range of mesoporous⁷⁹. With this result, it can be determined that MSNPs, which was synthesized, belong to the class of MCM-41 type mesoporous materials.

Figure 14 Adsorption and desorption isotherms plots of MSNPs-S2 to MSNPs-S4, indicating Type IV isotherm pattern.

Next, nitrogen adsorption-desorption analysis was carried out to examine the interior morphology of MSNPs, such as surface area, pore volume and pore width distribution. From the isotherm plots, type IV isotherm⁶⁷ nitrogen adsorption-desorption pattern was observed at each stage, indicating that the synthesized MSNPs consist of uniform and organized mesoporous structure. With this identification, BET analysis parameters was narrowed down to a relative pressure of 0.05cc/g to 0.35cc/g, which provides both DFT data and pore volume of MSNPs.

	Pore volume (cc/g)
MSNPs-2	0.453
MSNPs-3	0.661
MSNPs-4	0.218

Table 3 Summary of MSNPs-S2 to MSNPs-S4 pore volume

From the table above, an increase in pore volume of 0.208cc/g was observed between MSNPs-S2 and MSNPs-S3, from 0.453cc/g to 0.661cc/g, indicating the removal of CTAB template within the pore channels. Next, a significant decrease in pore volume of 0.443 cc/g was observed between MSNPs-S3 and MSNPs-S4, from 0.661cc/g to 0.218 cc/g, attributing to the success of surface functionalisation with dual responsive ligand, where the ligand caused a decrease in pore width by obstructing the pore opening.

DFT analysis was carried out to examine the width of the pore channels in the material. From the plot of $d(V)$ against half pore width, half pore width of MSNPs-S2, MSNPs-S3 and MSNPs-S4 was determined to be 11.56 Å, 12.10 Å and 7.37Å respectively. This suggest that the pore width of MSNPs obtained is 2.312nm, 2.42nm and 1.474nm respectively. This data obtained is consistent with the results observed in TEM and XRD, where there is an increment in pore volume and pore width of MSNPs after the removal of CTAB template followed by a reduction after surface functionalisation with dual responsive ligand.

From the data obtained using XRD and BET, a discrepancy of about 0.67nm in pore width is observed. This difference is due to the different methods in pore width determination between XRD and BET, in which XRD calculates based on the depth reading between the lattice structures of the particle while BET calculated indirectly through the filling of

nitrogen gas molecules into the pore channels of the particle. With this understanding, the difference of 0.67nm is determined to be the thickness of the particles.

With the results obtained above, it confirms that synthesized MSNPs is MCM-41 type of mesoporous material with spherical morphology and diameter of around 160nm, along with pore width to be around 1.99nm.^{78, 91} Isotherm plot point out to be a type IV isotherm plot, which further confirmed that this MSNPs is MCM-41 type.

Figure 15 FTIR spectrum for MSNPs-S1 to MSNPs-S5

Surface functionalisation on MSNPs surface was first examined with FTIR. From the combine spectrum, Si-O-Si absorption peaks at around 1074cm^{-1} and 802 cm^{-1} , along with OH absorption peak at around 1635 cm^{-1} and 3410 cm^{-1} , can be observed in all 5 materials. These characteristic peaks of MSNPs functional group suggest that MSNPs is synthesized^{59, 80}. Next, surface functionalisation of each stage was investigated. To begin, surface functionalisation of MSNPs-S1 with (3-Mercaptopropyl) trimethoxysilane, followed by the removal of CTAB template, is successful as the intensity of C-H alkyl stretch around the region of 2850 cm^{-1} to 2950 cm^{-1} decreases without any changes in the

intensity of Si-O-Si region. At the same time, CTAB C-H peak at 1474cm^{-1} region is absent. These observations suggest the absence of CTAB template and thus indicate the success in template removal.

In MSNPs-S4, a new peak at around 1740 cm^{-1} was observed which belongs to aromatic and carbonyl group, while C-H alkyl peak was observed to be more intense. From this data, it can be determined that surface functionalisation with dual responsive ligand has been successful. After capping with β -CD, peaks around hydroxyl and C-H alkyl regions were intensified, which reflects the success of complexing β -CD with adamantane.

Figure 16 XPS spectrum for MSNPs-S1, MSNPs-S3 and MSNPs-S4

XPS analysis was also carried out to further examine the surface functionalisation of MSNPs. From the combine survey spectrum of MSNPs, peaks corresponding to Silicon, Carbon and Oxygen elements at 103eV, 285eV and 532eV respectively were present in all 4 samples, which correspond to MSNPs and thus it can further be confirmed that the

desired MSNPs have been synthesized. Next, nitrogen peak at around 402eV, which is attributed to the presence of CTAB template, is only reflected in MSNPs-S1 and not reflected in both MSNPs-S3 and MSNPs-S4, indicating the success of CTAB removal from the MSNPs. Aside from that, the appearance of Sulphur peak at around 164eV was observed in MSNPs-S2, MSNPs-S3 and MSNPs-S4, but absent in MSNPs-S1, revealing the success in surface modification of (3-Mercaptopropyl) trimethoxysilane to the surface. In addition, it can be observed that slight shift in $S_{2p}^{3/2}$ and $S_{2p}^{1/2}$ region are present between MSNPs-S2 to MSNPs-S4. According to literature library, this shift reflects the different types of sulfur bonding, where MSNPs-S2 and MSNPs-S3 consist of free SH bond while MSNPs-S4 consist of disulfide bond, which indicates the success in surface functionalisation through disulfide bonding.

Figure 17 Zeta Potential surface charge on MNSPs-S1 to MNSPs-S5

Next, zeta potential analysis was carried out to investigate success of surface functionalisation, through the change in surface charge of MSNPs. From the bar chart, it can be observed that the surface potential for each stage is significantly different, which suggests that the functional groups on the surface of the MSNPs at every stage is different, thus signifying that surface functionalisation was successful.

With the results obtained from FTIR, XPS and Zeta potential, it can be deduced that surface functionalisation with dual responsive ligand had been successful without any disruption or modification of MSNPs morphology.

3.3.2 Drug release under stimulus condition

After the confirmation of dual responsive ligand attachment, drug loading and release capability was investigated. MSNPs was first dispersed in DOX solution (5.00mg/ml) and was placed in shaker (1600rpm, 37°C) under dark for 24 hours. The mixture was then centrifuged and the supernatant was collected. The particles were then dispersed in β -CD solution to initiate complexation with adamantane for another 24 hours, before centrifuging, washing, and collecting the supernatant. The supernatants at each stage were then analyzed using UV instrument ($\lambda_{\text{ex}} = 480\text{nm}$) to determine the concentration of DOX present. The actual amount of DOX loaded within the pores are determined indirectly, from the difference of DOX present at the initial stage with the amount of DOX present in the supernatants. From the calculation, DOX loading efficiency was 5.85%. This relatively low loading could be owing to the presence of ligand which obstruct the pore opening, thereby reduces the ease of DOX diffusion to the channels. This observation can also be supported by the BET analysis, which also indicate a decrease in pore volume of MSNPs after surface modifications, from 0.661cc/g to 0.218cc/g.

Figure 18 Release profile of DOX under the influence of 0mM, 5mM and 10mM of GSH for a period of 35 hours

Next, the efficiency of dual responsive ligand was investigated through the release of DOX into the pores. Redox responsive efficiency was first examined. In this study, DOX loaded MSNPs-S5 (2.00mg) was first dispersed in a centrifuged tube containing pH 7.5 PBS buffer (1.00ml) with GSH (0.00mM, 5.00mM and 10.0mM), followed by shaking for a specific time interval at 37°C and 1600rpm. Thereafter, the mixture was centrifuged and the supernatant was collected, before the addition of fresh buffer with the same concentration of GSH. The amount of DOX present in each batch of supernatant was then determined using fluorescence of $\lambda_{em} = 588\text{nm}$ ($\lambda_{ex} = 480\text{nm}$). From the results, negligible amount of DOX (7.26%) was detected throughout the analysis, indicating that the ligand is very effective in blocking the pores and minimizing premature release under normal physiological condition. On the other hand, significant increase in DOX released was observed upon the addition of GSH. This is due to the cleavage of the disulfide bond that anchored to MSNPs surface, thereby releasing the drug. The release profile of 5.00nM and

10.00nM are 18.9% and 19.1% respectively. This indicates that the sensitivity of disulfide bond in this ligand is very sensitive, suggesting that disulfide bond is not hindered.

Figure 19 Release profile of DOX under the influence of pH 7.5, pH 6.5, pH 5.5 and pH 4.5 for a period of 25 hours

For acid responsive investigation, DOX loaded MSNPs-S5 (2.00mg) was first dispersed in a centrifuged tube containing PBS buffer (1.00ml) of different pH (7.5, 6.5 and 5.5) to mimic physiological and tumour condition, followed by shaking for a specific time interval at 37°C and 1600rpm. Thereafter, the mixture was centrifuged and the supernatant was collected, followed by the addition of fresh buffer of the same pH buffer. The amount of DOX present in every batch of supernatant was then determined using fluorescence of λ_{em} of 588nm ($\lambda_{ex} = 480$ nm). According to the results, it can be observed that the release of DOX from the carrier increases as the pH decreases, from around 7% to about 15%, indicating that the acetal bond can be cleaved under the influence of acidic condition. Not only so, negligible amount of DOX was detected for pH 7.5 and 6.5, 7% and 9% respectively, indicating that the ligand is very effective in blocking the pores and minimizing premature release under normal physiological condition. On the other hand, significant release of DOX was detected with 15% release at pH5.5 which is due to the

cleavage of acetal bond that anchored to MSNPs-S5 surface, thereby releasing the drug. This indicates the effectiveness of this ligand as pH sensitive blocker.

Figure 20 Release profile of DOX under the influence of 0mM, 1mM and 10mM of GSH along with pH of 7.5, pH 6.5, pH 5.5 and pH 4.5 for a period of 33 hours

Following that, the investigation of dual responsive release of the system is performed. DOX loaded MSNPs-S5 (2.00mg) was first dispersed in a centrifuge tube containing PBS buffer (1.00ml) of different pH (7.5, 6.5 and 5.5) along with GSH (0mM, 5.00mM and 10.0mM), followed by shaking for a specific time interval at 37°C and 1600rpm. Thereafter, the mixture was centrifuged and the supernatant was collected, followed by the addition of fresh buffer of the same composition. DOX present in each batch of supernatant was then determined using fluorescence of λ_{em} of 588nm (λ_{ex} = 480nm excitation). From the results obtained, it reflects similar trend obtained when the MSNPs were exposed to either redox condition or pH dependent condition. More importantly, the amount of DOX released was more than two folds when the system is exposed to both acidic and redox environment, suggesting that synergistic release is achieved. With the above results, it

indicates that the ligand is sensitive to redox and acidic conditions along with enhanced drug release effect under both conditions, thereby demonstrating the effectiveness of this dual responsive ligand as a gatekeeper for the system.

3.3.3 In vitro cellular analysis

3.3.3.1 MTT assay

Figure 21 Cell viability assay for MSNPs-S5, Free DOX and DOX loaded MSNPs-S5

Moving on, in vitro cell viability assay was carried out using (3,4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay with HeLa cells. In this study, HeLa cells were seeded to 96 wells with a cell density of 10 thousand cells per well and incubated overnight, followed by the addition of samples and allowed to incubate for another 24 hours. After which, MTT solution was added to each well and incubated for another 4 hours followed by cell viability analysis. In this project, comparison of cell viability for DOX-loaded MSNPs-S5, free DOX and MSNPs-S5 was carried out to examine the toxicity of this drug carrier system. From the results, it can be observed that the cell viability decreases as the concentration of free DOX increases. Besides that, DOX-loaded

MSNPs-S5 also displayed a decrease in cell viability as the concentration increased. However, the decrease is significantly larger as compared to free DOX administration. This indicates that the carrier is internalized by the cells, thereby reflecting the enhancement of drug localization in the cells. Besides that, bare MSNPs-S5 shows minimal toxicity in the cells, with cell viability maintaining at around 66% at a concentration of 400 μ g. This implies that the carrier is highly biocompatible and poses insignificant toxicity to the cells, which also indicates that the toxicity level for DOX-loaded MSNPs-S5 is solely due to DOX. From this MTT assay, it indicates that the system is capable of internalization to cells and provides localized drug delivery within the cancerous cells. On top of that, this result also demonstrates that the carrier is non-toxic to the cells, which is a very promising result for drug delivery carrier.

3.3.3.2 Confocal microscopy

Figure 22 Confocal microscopy images of HeLa cells after incubated with DOX loaded MSNPs-S5. DOX fluorescence and DAPI staining from nucleus can be observed individually as well as in the merged images

Next, confocal laser scanning microscopy analysis was carried out to further investigate internalization of the carrier into the cells. In this study, HeLa cells were seeded to a 6 wells plate with cell density of approximately 250 thousand cells per well and incubated for overnight. Next, DOX-loaded MSNPs-S5 was added to the well at specific time interval (120 minutes, 90 minutes, 60 minutes, 45 minutes, 30 minutes, and 15 minutes) and incubated. After which, the cells were affixed with 4% formaldehyde followed by the staining of cell nucleus with DAPI before analysing through confocal. From the images obtained, minimal DOX fluorescence was observed at the initial stage and the intensity of fluorescence increased as the time interval increased. This suggests that the cleavage of disulphide bond for drug release increases over a span of 120 minutes, indicating that the redox responsive ligand is sensitive within the cellular conditions. Apart from that, it can also be observed that DOX fluorescence is more intense around the nuclear region compared to the other parts of the cells. This indicates that the released DOX migrated and

localized to the nucleus, killing the cells through interactions with DNA⁸¹. From this time-dependent analysis, it demonstrates that the system is capable to internalize into the cells and the disulphide linkage is sensitive to be cleaved in the reducing environment within the cells, thereby releasing DOX. Besides that, movement and localization of DOX from the cytoplasm to the nucleus can also be observed, indicating that the encapsulation of DOX within the system maintains the efficacy of the drug.

3.4 Conclusion

In conclusion, redox responsive mesoporous silica nanoparticles drug delivery system has been successfully developed. From the above characterization analysis, it shows that the MSNPs synthesized belong to MCM-41 mesoporous material with redox responsive ligand functionalized on the surface. The efficiency of redox responsive ligand was demonstrated in solution as well as in the HeLa cells. From the results obtained, it is indicative that the ligand is capable of preventing premature release of DOX in the carrier under normal physiological conditions as well as the ability to release DOX under redox environment. Apart from that, both MTT and confocal analysis also demonstrated the enhancement of cell toxicity through the internalization and localization of the system into the cells as compared to free DOX. In addition, confocal microscopy also demonstrated the different stages of internalization of carrier, as well as the release of DOX, within the cell. Therefore, it can be concluded that this system is promising as a drug delivery carrier for future chemotherapeutic purposes.

Conclusion

In this dissertation, Mesoporous silica Nanoparticles (MSNPs) was chosen as the material for drug delivery along with surface functionalisation of stimuli responsive ligands, with the aim to increase the efficacy of drug delivery directly to cancer cells. MSNPs was first synthesized in both projects, followed by surface functionalisation with redox responsive ligands and dual responsive ligands in chapter 2 and 3 respectively.

Following that, anticancer drug, Doxorubicin, was chosen to be loaded into the system to assess the efficiency as drug delivery system. In these two projects, the morphology of the material was analysed using multiple characterization techniques followed by the investigation of stimuli responsive ligands efficacy as a gatekeeper as well as the sensitivity upon exposure to the respective triggered environment. After that, intracellular analysis such as MTT and confocal were carried out to examine the uptake of the system and the effectiveness as a drug delivery medium as compared to free DOX.

A series of characterisations has been carried out with pore diameters and pore width of 150nm and 3.5nm, 160nm and 2.38nm, for MSNPs synthesised in chapter 2 and 3 respectively. Aside from that, drug loading were 10.1% and 5.83% for system in chapter 2 and 3 respectively. Efficiency of the system were also deemed to be effective through drug release analysis, where Dox is released under specific environment while maintaining negligible Dox release under physiological condition.

From this research, higher toxicity in HeLa cells were observed in DOX loaded MSNPs as compared to free DOX, indicating higher efficiency of DOX loaded MSNPs in anticancer therapy. Aside from that, results obtained in both chapters demonstrated the effectiveness of the system as drug delivery carrier, through negligible release of DOX

under physiological condition and burst release of DOX when the system is exposed to stimulus environment. This demonstrates that DOX loaded MSNPs is an excellent drug delivery system, with enhanced performance in therapeutic effect of DOX as compared to free DOX.

Despite the efficiency of the system using MSNPs, in depth research is still required to further investigate the toxicity and biocompatibility of the material under specific conditions, as well as exploring future possibilities for other applications.

In the future, these two systems can be further enhanced through additional modification. For instance, folic acid can be functionalized onto β -CD where folic acid will serve as a targeting ligand, thereby enabling both active and passive targeting drug delivery. Aside from that, the hydrophilicity and dispersity of the systems can also be further enhanced by functionalizing β -CD with poly ethylene glycol (PEG). These two enhancements can proceed by utilizing free hydroxyl group that is present on β -CD surface, through a series of coupling reactions.

For drug delivery system in Chapter 3, we can further enhance the efficiency by increasing the pore width of the pore channels to allow higher loading and release of Dox. One of the possible way it can be achieved is by changing the surfactant, from C₁₂TAB to C₁₆TAB.

Lastly, further optimisations in the concentration of nanoparticles will be conducted to determine the best concentration of nanoparticles where there is little to no aggregation while maintaining the highest efficiency of the system.

Herein, this dissertation has demonstrated the efficiency of surface functionalized MSNPs with stimuli responsive ligands in delivery of anticancer drug with specific sensitivity to the desired stimulus environment.

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List of Publications

1. Chung Yen Ang, Si Yu Tan, Cathleen Teh, **Wong Mun Fei Eddy**, Qiuyu Qu, Yanli Zhao*, Redox and pH Dual Responsive Polymer Based Nanoparticles for In Vivo Drug Delivery. *Small* **2017**, *13* (7), 1602379-n/a.
2. Chung Yen Ang, Si Yu Tan, Qiuyu Qu, **Wong Mun Fei Eddy**, Yanli Zhao* A dual responsive "turn-on" fluorophore for orthogonal selective sensing of biological thiols and hydrogen peroxide. *Journal of Materials Chemistry C* **2016**, *4* (14), 2761-2774.