Functionalised Mesoporous Silica Nanoparticles for Smart Agrochemical Delivery System

by

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B.E. and M.E.

Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

Deakin University
April, 2015
I am the author of the thesis entitled

**Functionalised Mesoporous Silica nanoparticles for Smart Agrochemical Delivery System**

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Acknowledgements

First of all, I would like to thank my supervisors, Professor Lingxue Kong and Professor David Cahill for their suggestions that have significantly improved my critical thinking, for their help with dealing with the scientific challenges, for their constant encouragement during my studies. I would like to thank Professor Lingxue Kong for his care and support in terms of my development of professional skills and my completion of my thesis. His experience helped me a lot on the scientific writing and doing research comprehensively and systematically. Everything I learnt from him during my study is very crucial for me to build up a system doing scientific research. I am grateful to Professor David Cahill for his support on plant science. He is very patient to explain the technical terms related to plant science and provides me with a lot of knowledge about plants.

I would also like to than Dr. Mary She and Dr. Jim Rookes, for their precious suggestions to improve my writing and critical thinking. A special thank goes to my colleague, Dr. Hashmath I. Hussain from School of Life and Environmental Science, for his constant help with the plant experiments and paper writing. All his work on plant experiments and results obtained from plants are highly appreciated. His efforts have provided me with the plant results to construct the part of my thesis related to plants.

I would like to greatly thank Mr. Dequan Sun from School of Life and Environmental Science for his contributions on the plant experiments, and thank Mr. Tom Cresswell from Australian Nuclear Science and Technology Organisation for providing the results elemental quantification in plants. I am grateful to Dr. Ludovic Dumeè for his support on the synchrotron proposals and experiments and to Dr. Christopher Garvey for his expert suggestions on the modelling and interpretation of synchrotron data.

My deep gratitude goes to thank Ms. Rosey Van Driel and Dr. Andrew Sullivan, for their expert advice on electron microscopy. I would also like to thank Ms. Marion Wright and Mr. Michael Jones for their continuous support on the laboratory work and managing hazards to keep a safe work environment in the lab. I am grateful to all the technicians from Institute of Frontier Materials for their help during my studies.
Special thanks go to Dr. Leonora Velleman for her help with the analysis of electron microscope images and Dr. Jane Allardyce for her support on my thesis and paper writing.

I would also like to thank Deakin University for providing me with the scholarship that constantly supported me to complete my degree. The special acknowledgement goes to the grants M4977, M5826 and M6410 from Australian Synchrotron for providing me with the beamtime to get the data for one chapter of my thesis.

Last, but not least, I would like to thank my parents, my family and my friends for their continuous support to help me complete the doctoral degree. I would also like to thank my wife, Ms. Chunfang Feng, for her support not only in my daily life, but also in the scientific research.

Without the help from my supervisors, my colleagues, my friends and my family, I could not imagine what would be going on during my degree. They are my treasure that support and assist me complete my studies in Institute of Frontier Materials.
Publications

Published


Submitted paper

Localised Functionalization of Mesoporous Silica Nanoparticles with Redox Responsive Gatekeepers for Agrochemical Delivery (submitted to ACS Applied Materials and Interfaces (IF 5.9))

Under preparation

In-situ Small Angle X-ray Scattering Investigation of Formation of Mesoporous Silica Nanoparticles and Swelling-Shrinking Growth Mechanism (Langmuir (IF4.1))
Abstract

Agricultural production benefits from the use of agrochemicals such as fertilizers, herbicides, pesticides, and plant hormones, but the low utilizing efficiency leads to the enormous accumulation of chemicals in the environment, having a great impact on the natural environment. Agrochemical delivery systems using nanomaterials as carriers provide a controllable and eco-friendly application for the efficient utilization of agrochemicals in agriculture. The phytotoxicity of the current nano-deliverers (metal or metal oxide nanoparticles and carbon nanotubes) is the major concern to use nanomaterials in plants. Mesoporous silica nanoparticles (MSNs) as silica based nanomaterials are non-toxic to plants and possess unique properties, including mesoporous structure, controllable particle and pore size, high surface area and easily modified surface. These features make MSNs an ideal candidate as the carrier in agrochemical delivery system. In addition, being different from mammalian cells, the plant cell wall is the natural obstacle that restricts the access of nanoparticles into plant cells. To design and build up an agrochemical delivery system, this thesis addresses five main issues: (1) the fabrication of MSNs with a small particle size to overcome plant cell walls; (2) the exploration of MSN growth mechanisms through time-resolved small angle X-ray scattering (SAXS) at a synchrotron source; (3) the modification of the surface of MSNs with fluorescent dyes to track the translocation of nanoparticles inside plants; (4) the functionalization of redox-responsive gatekeepers at the entrance of the mesopores with decanethiol to control the release of the loaded agrochemicals; and (5) the enlargement of pore size of MSNs with trimethylbenzene and the surface modification with varying surface charges for loading large molecules (like proteins and siRNA) via electrostatic force.

Firstly, the presence of pores within plant cell walls is the pathway for nanoparticles to be internalized but the size of MSNs needs to be smaller than the pore size in the cell walls. The MSNs with small particle size always tend to aggregate and collapse the porous structure. Herein, MSNs with a particle size of approximately 20 nm have been synthesised with cetyltrimethylammonium bromide (CTAB) as a template and tetraethyl orthosilicate as the silica source under neutral and basic conditions. Several experimental parameters were optimized, such as CTAB concentration, the reaction temperature, pH value and ionic strength. Under neutral conditions (pH 7.2), the
CTAB concentration was found to have no effects on the particle size and limited effects on the pore size. The smaller particle size (~20 nm) was obtained at 30 °C and bigger particles (50-60 nm) at 95 °C. Under basic conditions (pH 9, 10 and 12), in the phosphate buffer solution, the pH value has a great impact on the morphology of MSNs. The interaction between particles became strong with increasing pH value, leading to the branched morphology. When the buffer solution is replaced with the ammonia solution under basic condition (pH 10) the ionic strength of the solution decreased by thousand-times. The obtained MSNs in ammonia solution were well dispersed and with the particle size of around 20 nm.

The general explanation of how MSNs grow is the initial hydrolysis and formation of silica monomers, adsorption onto surfactant micelles and condensation of silica coated micelles to form particles. However, the detailed growth mechanism of MSNs still remains unknown especially for the MSNs with small particle size. Herein, time-resolved SAXS with a synchrotron source was employed to investigate the growth of MSNs under different experimental conditions. The fitting models were introduced into the system to interpret the SAXS data. The results suggested that the added TEOS inserted into the hydrophobic core of CTAB micelles, forming an emulsion at the beginning of the growth. The silica monomers were hydrolyzed from the inside of the micelles into the solvent. The CTAB micelles were actually swelled by the addition of TEOS and with the consumption of the TEOS while the pores formed by CTAB was shrunk to form the final mesopores. To the best of our knowledge, this mechanism is studied for the first time, which will contribute to the understanding of MSN growth mechanism.

Detection of the translocation of MSNs inside plants is of importance to understand the interaction between nanoparticles and plant cells. The MSNs were synthesised according to the optimized conditions and functionalised with fluorescein isothiocyanate (FITC) or rhodamine b isothiocyanate (RITC) via the reaction between amine groups in the silane grafted on MSNs and the isothiocyanate groups in the dyes. The successful modification was proved by Fourier transform infrared spectroscopy (FTIR) and fluorescence spectroscopy. The characteristic peak of the successful modification was at 1458 cm⁻¹ (C-N stretching vibration) from FTIR results. The excitation wavelength of FITC obtained from fluorescence spectroscopy changed from 227.96 to 413.93 nm, while the excitation wavelength of RITC maintained the same
The uptake of FITC and RITC labelled MSNs by plants was investigated via confocal laser scanning microscopy. No phytotoxicity was found in plants. The well-dispersed MSNs with ~20 nm particle size were shown to penetrate into the roots of the plant species tested and were transported through the xylem to the stems and leaves.

Mesoporous silica based delivery systems have been widely used to deliver molecules into mammalian cells. However, studies on molecular delivery systems for plants and the controllable release of bio-active molecules has not been widely reported. This study developed a mesoporous silica-based delivery system by using smaller MSNs to overcome the plant cell wall barrier and introduces a redox-responsive function to release agrochemicals only inside plant cells. The plant defence-related hormone salicylic acid (SA) was selected as a model agrochemical to be loaded into mesoporous silica nanoparticles (MSNs). Decanethiol as a short molecule was used as gatekeepers to prevent the premature release of SA from the MSNs before they entered plant cells. The release of SA inside plant cells was mediated by glutathione (GSH) which occurs naturally in cells through cleavage of the disulfide bonds between MSNs and gatekeepers. After capping with decanethiols, the particle size of MSNs did not change. The in vitro release of SA from decanethiol-capped MSNs indicated that the release rate of SA at a GSH concentration of 5 and 10 mM is significantly higher than the release without the presence of GSH. This study demonstrates the potential use of MSNs in plants and the redox-responsive release of agrochemicals for a smart agrochemical delivery system.

The pore size of the MSNs is also expanded to be able to load large molecules (size > 2 nm) such as proteins or siRNA as another potential and exciting development of using MSNs in plants. Based on the expanded CTAB micelle size by trimethylbenzene (TMB), MSNs with large pore size were obtained. The pores can be tuned in size ranging from 10 to 19 nm by changing the ratio between TMB and CTAB. The narrow pore size distribution can be obtained by increasing the amount of TMB. Moreover, the surface of pore expanded MSNs was modified by amine groups to achieve positive charge, which offers an electrostatic force to absorb negatively charged siRNA molecules. The results from the loading of siRNA showed that at least 1 μg of siRNA can completely be adsorbed in 50 μg of positively charged pore expanded MSNs, but there is no siRNA loading detected with the unmodified pore expanded MSNs. This
study established a material platform for genetic and molecular biological application of the MSNs in plants.

In summary, the present work has established an MSN based agrochemical delivery system for the plants and a material platform for the gene transfer applications. The synthesis procedure of MSNs was well manipulated to achieve nanoparticles with good dispersity and small particle size. Time-resolved SAXS was employed to understand the growth mechanism of MSNs in situ. The translocation and accumulation of the synthesised MSNs was detected in the roots, stems and leaves within the intact plants. Redox-responsive gatekeepers were introduced into this agrochemical delivery system to control the release of loaded agrochemicals by the trigger GSH, a natural reductant in cells. The pore expanded MSNs and siRNA loading have provided an insight into molecular delivery in plants. Therefore, the developed agrochemical delivery system can be potentially implemented in real agriculture.
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Chapter 1: Literature Review

1.1 Introduction

Agrochemicals including fertilizers, pesticides and plant growth agents have achieved enormous benefits in agriculture by providing sufficient minerals, controlling pathogenic organisms and enhancing the yield of crops. However, the extensive usage of agrochemicals has long-term side effects on natural ecosystem (Cheng, Salminen & Grewal 2010) as the accumulation of non-degradable compositions will affect the acidity and nutrient balance of soil and threaten both above ground and below ground water systems (Overmyer, Noblet & Armbrust 2005). The abundance of nutrient content in these chemicals may also lead to a large increase in population levels of microorganisms that harm both plants and animals.

The overuse of agrochemicals has been associated with the ineffectiveness of delivery systems currently used in agricultural industries where chemicals are conventionally sprayed to run off when applied to the aerial parts of a crop so that at least some uptake is enabled. Recent developments in nanotechnology and nanomaterials has provided insights into how the efficient and successful delivery of chemicals into plant cells may be afforded by using various nanoparticles. Nanoparticles such as magnetic nanoparticles and metallic nanoparticles (silver, copper, and aluminium) have the potential to be transported through plant cell walls, and carry and release agrochemicals in a controllable fashion, due to their small particle size, chemical and physical stability, high surface area and multi-functionalization with the introduction of versatile polymeric molecules. Even though these nanoparticles are capable of penetrating through cell walls of intact plants, the investigation of their phytotoxicity indicates that the uptake of these nanoparticles by plants have adverse effects on the reproduction of cells and the germination and growth of plants (Dietz & Herth 2011; Nair et al. 2010). Therefore, developing nontoxic vehicles for agrochemical delivery system is necessary to deal with this issue.

Mesoporous silica nanoparticles (MSNs) have recently demonstrated great potential of being used as carriers to deliver chemicals into plant cells. Torney et al (2007) reported that MSNs capped with gold nanoparticles and coated by triethylene glycol
have the ability to penetrate through the cell membrane of protoplasts and deliver DNA and guest molecules (fluorescent dye or β-oestradiol) into plant cells. Other research work has also shown that a biological pesticide loaded into MSNs can be slowly released into the environment (Chen et al. 2010a) due to the good protection offered by the unique MSN structure. More importantly, MSNs as silica based materials are not toxic to plants (Napierska et al. 2010). Therefore, MSNs with proper functionalization can be regarded as an ideal material to deliver agrochemicals.

However, the current MSNs cannot effectively deliver molecules to plants through plant cell walls as the diameter of the MSNs is much larger than the pores on the cell walls. Furthermore, the encapsulation of sufficient agrochemicals into MSNs for real implementation will become difficult particularly when the diameter of the particles is small. Therefore, in this project, I will investigate new mechanisms to effectively encapsulate sufficient amounts of agrochemicals into high porosity MSNs with tailor designed diameters for various plants. The mechanisms will be evaluated on model plants.

Accordingly, understanding the structure of plant cells will be critical to investigate the possible pathway of transporting nanoparticles in plants and to identify the most desirable properties of mesoporous silica nanoparticles for agrochemical delivery.

1.2 Structure and function of plant cell walls

Agrochemicals are comprised of powerful chemicals in order to achieve various goals, such as fertilizers being rich in nutrient elements for plants (nitrogen or minerals), pesticides being useful to kill pathogenic organisms (insects, fungi, bacteria and viruses) and growth regulators to enhance the development of plant tissues (Bhandari 2014). However, plants as living organisms are very sensitive to these chemicals. The over use of agrochemicals and the formulations that they are used in may lead to serious damage to plant cells and tissues as well as the environment (Aktar, Sengupta & Chowdhury 2009; Domene et al. 2008). The accumulation of these chemicals in plant body may gradually affect the reproduction of plant cells, the growth of young plants and even the next generation (Rost et al. 2006). Therefore, efficient and nontoxic delivery methods are sought.
All plant cells are protected by cell walls which in aerial parts is overlain by the waxy and protective plant cuticle. This rigid shield has a unique structure that is different from animal cells and performs as an obstacle between the organelles of plant cells and the environment. Plant cell walls mainly consist of cellulose, hemicellulose and pectin (Sticklen 2008). Cellulose microfibrils and hemicelluloses are linked to form the interpenetrating network of plant cell walls, which are enclosed in a pectin matrix (Figure 1.1). Apart from those main compositions, structural proteins exist in most plant species to complete physical function of plant cell walls, such as the exchange of substances. Even though plant cell walls are tough and rigid, minerals and water can easily transport through cell walls, whereas the transfer of other molecules (protein, fertilizers, pesticides) is determined by the selectivity of structural proteins and the pore size of cell wall ranging from 5 to 20 nm (Nair et al. 2010), and also variations between plant species.

Due to the presence of plant cell walls, the utilization rate of agrochemicals may be decreased to a large extent by 50-90 % (Bhandari 2014). This low utilization rate is mainly because there are no appropriate carriers packaging agrochemicals and transferring them into plant cells (Cifuentes et al. 2010; Colilla, Gonzalez & Vallet-Regi 2013; Sabo-Attwood et al. 2012; Wang et al. 2012). With the help of
nanotechnology, the agrochemicals can be potentially loaded into nanoparticles and delivered into plants, which will significantly increase the efficacy of agrochemicals.

1.3 Assessment on the phytotoxicity of nanoparticles

Nano-materials are advantageous to other materials due to the small-size superiority. As plant cell walls have very small pores to allow for the transportation of chemicals, the agrochemical delivery systems must have morphological advantages with a smaller diameter than that of the pores on plant cell walls. Compared to the polymeric materials, inorganic nanomaterials have the potential to deliver agrochemicals into plants. The advantages of inorganic materials in agrochemical delivery systems include:

- Physical and chemical stability;
- Biocompatibility with plant tissues;
- Excellent adaptivity to the weather;
- High efficiency of agrochemical loadings;
- Effective protection before reaching the specific site;
- Controllable release of agrochemicals.

Many inorganic nanoparticles have been studied in terms of their permeability and phytotoxicity (Dietz & Herth 2011). The first and important requirement as an agrochemical carrier is the low toxicity. The following sections will systematically investigate the existing nano-materials used in plants and their toxic effects on plants.

1.2.1 Carbon nanotubes

Carbon nanotubes (CNTs) possess a long channel with a diameter as small as 1 nm at the centre, which can be a potential carrier to transport drugs to plant cells. The application of carbon nanotubes (single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs)) as nano-carriers has increased at a staggering rate, especially in plants. Due to the broad prospect of their application, the toxicity of CNTs to plants has drawn concern from researchers. Various studies on the
toxicity of CNTs to plants have been carried out with a mixed findings.

Six crop species (cabbage, carrot, cucumber, lettuce, onion, and tomato) are treated by functionalised SWCNTs (fCNTs) and non-functionalised SWCNTs (nCNTs) in order to investigate the effects of carbon nanomaterials on root elongation (Cañas et al. 2008). Results showed that the root length of four crop species (cucumber, onion, lettuce, and tomato) is affected by both fCNTs and nCNTs. The root elongation of cabbage and carrot is not influenced by either fCNTs or nCNTs. Among all the selective crop species, the root length of tomato is reduced most obviously. However, a positive effect on tomatoes was reported by Khodakovskaya et al (2009) that the higher seed germination and growth rate is attributed to the more efficient water uptake related to the addition of MWCNTs in growth medium. Studies also support the positive effects of CNTs in other plants (Nair et al. 2010). Both SWCNTs (in purchased form and purified form) and MWCNTs can enhance germination of rice seeds (Figure 1.2). A recent study reported that the SWCNTs can passively be transported into the chloroplasts and the photosynthetic activity of the SWCNTs group is three times higher than the control groups (Giraldo et al. 2014). The debate on the phytotoxicity of CNTs still goes on. Results are varied from experimental parameters and plant species, which means that CNTs cannot be confirmed to be safe to perform as the agrochemical carriers in plants.

Figure 1.2 Effects of different types of carbon nanotubes in rice seeds (Nair et al. 2010).
1.2.2 Metallic and metal oxide nanoparticles

Toxicity of metallic and metal oxide nanoparticles is also a concern if they are used as vehicles delivering agrochemicals. There are numbers of studies on the influence of the uptake of metal based nanoparticles in plants, including silver (Kim et al. 2009; Panácek et al. 2009; Park et al. 2006), copper (Lee et al. 2008; Shah & Belozerova 2009), titanium dioxide (Hong et al. 2005a; Su et al. 2007), zinc oxide (Lin & Xing 2007, 2008; Stampoulis, Sinha & White 2009) and aluminium (Doshi et al. 2008; Yang & Watts 2005) nanoparticles. Specifically, aluminium and zinc oxide nanoparticles retard the root elongation and germination; titanium dioxide and copper nanoparticles can enhance the germination; other impact on plants is to decrease the production of biomass and transpiration (Nair et al. 2010). Moreover, the effects of nanomaterials in plants vary from different tissues and different plant species. Therefore, the toxicity of metallic atoms and oxides used in agriculture require much further analysis.

1.2.3 Magnetic nanoparticles

Magnetic nanoparticles can be directed and localized by the external magnetic field to release loaded agrochemicals at a specific site, which could be an interesting application of nanotechnology in agriculture. These unique properties make magnetic nanoparticles a major member in metal oxide nanoparticles. A lot of studies have been conducted to evaluate their transportation and effects on plants. Zhu et al. (2008) and Corredor et al. (2009) have reported their work on the translocation and accumulation of magnetic particles in pumpkin plants. They found that the magnetic nanoparticles are mainly accumulated in the vascular system that transports minerals and water from roots to stems and leaves. However, the toxicity of magnetic materials cannot be ignored. Some research in this field illustrates that ferrofluids (colloidal magnetic nanoparticles) can result in chromosomal aberrations in young plants (Pavel & Creanga 2005; Racuciu & Creanga 2007). In addition, the toxic response can be observed with the introduction of magnetic nanoparticles during the germination of maize seeds (Răucuciu & Creangă 2009). The growth of young plants was slightly prohibited and the addition of higher amount of nanoparticles resulted in the blight of leaves. Therefore, magnetic nanoparticles are not safe to plants as an agrichemical
carrier.

1.2.4 Mesoporous silica nanoparticles

Mesoporous silica nanoparticles (MSNs) have attracted increased attention in past decades, due to their high surface area, ordered porous structure, easily functionalised surface and physical and chemical stability. As a drug delivery system for the treatment of cancer in animals, MSNs have been widely used as a vehicle, whereas the application of MSNs in plants is rarely reported. The work from Torney and colleagues (2007) showed that gold capped MSNs with the pore size of 3 nm delivered DNA and its inducers into protoplast (wall-removed plant cell) and intact leaves. Another recent study on MSNs and pesticides (Chen et al. 2010a) reported that raw MSNs loaded with a new biological pesticide showed slow release in an *in vitro* test and prohibited the growth of fungi, which suggests the potential application of MSNs in the treatment of plant pathogens. Popat et al. (Popat et al. 2012) have shown the ability of MSNs to carry and slowly release a biocide to prevent termite infestation. These studies and others have shown that MSNs as a silica based nano-material have no toxicity to plants (Dietz & Herth 2011), which makes MSNs an ideal candidate to be used as an agrochemical carrier. However, the translocation and uptake mechanism of MSNs in plants has not been investigated. A research gap between MSNs and their potential as an agrochemical delivery system still remains.

The phytotoxicity studies on inorganic nanoparticles suggest that the application of CNT and metal based nanoparticles in plants is of concern because of the unexpected phytotoxic effects on plant growth. The recent development on MSNs presents a new delivery system for agrochemicals. As it has been proven that MSNs as silica based materials are nontoxic to plants and have great potential to become a new class of materials for the delivery of agrochemicals. However, the remaining challenges are how MSNs can overcome the plant cell wall barrier and to determine their translocation pathway within plants. Therefore, the morphology and functionalization of MSNs have to be finely controlled to meet the requirements as an agrochemical carrier.
1.4 Fabrication and functionalization of mesoporous silica nanoparticles

As MSNs are ‘friendly’ and do not damage plants following their application compared with other inorganic nanomaterials which may be toxic, they are very suitable candidatures particularly if their size and the properties of their surface can meet the requirement during transportation in plants.

1.4.1 History of MSNs

The first patent referring to MSNs was published in 1970 (Page Madeleine, Beau & Duchene 1970; Vanderpool Clarence, Ritsko Joseph & Chiola 1971; Walsh Robert 1964), but their potential has not fully explored at that time due to the lack of powerful characterization facilities (Carreon 2008). Until the International Union of Pure and Applied Chemistry presented obvious conception for mesoporous materials (pore size ranging from 2 to 50 nm) in 1990s (Rouquerol et al. 1994), mesoporous silica particles attracted researchers’ attention again. Because the length of most chemical molecules and bio-macromolecules ranges from 3-30 nm (Yiu & Wright 2005) and the demand for new drug carriers in drug delivery systems (Charnay et al. 2004; Wang et al. 2011), studies on MSNs became increasingly important. Up till now, MSNs with controllable structure and functionalities have been synthesised to meet the requirements in terms of the solubility of loaded molecules (Cho, Volkov & Sokolov 2010; Qu et al. 2006), the amount of cargo (Charnay et al. 2004; Thomas et al. 2010) and the duration of drug release (Lin et al. 2009; Wu et al. 2007).

1.4.2 Mesoporous structure of MSNs

The mesoporous structure of MSNs is one of the most important properties which are related to the duration of molecule release (Horcajada et al. 2006; Kim, Chung & Lin 2010; Manzano et al. 2008). Table 1.1 illustrates a few porous silica nanoparticles which have been widely studied so far (Wang 2009). According to the parameter of space group, the mesoporous structure can be classified to be hexagonal, cubic, lamella and bicontinuous.
Table 1.1 Pore size and structure of MSNs (Selvam, Bhatia & Sonwane 2001; Wang 2009; Yiu & Wright 2005)

<table>
<thead>
<tr>
<th>Mesoporous silica</th>
<th>Space group</th>
<th>Pore diameter (nm)</th>
<th>Channel structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCM-41</td>
<td>P6mm</td>
<td>2–5</td>
<td>Hexagonal 1D channel</td>
</tr>
<tr>
<td>MCM-48</td>
<td>Ia3d</td>
<td>2–5</td>
<td>Bicontinuous 3D</td>
</tr>
<tr>
<td>SBA-1</td>
<td>Pm3n</td>
<td>2–4</td>
<td>Cubic 3D</td>
</tr>
<tr>
<td>SBA-3</td>
<td>P6mm</td>
<td>2–4</td>
<td>2D hexagonal</td>
</tr>
<tr>
<td>SBA-15</td>
<td>P6mm</td>
<td>5–10</td>
<td>Hexagonal 1D channel</td>
</tr>
<tr>
<td>SBA-16</td>
<td>Im3m</td>
<td>Min 1–6; max 4–9</td>
<td>Body centre arrangement of cages</td>
</tr>
<tr>
<td>FDU-12</td>
<td>Fm3m</td>
<td>Min 4–9; max 10–12</td>
<td>Face centred cubic arrangement of cages</td>
</tr>
<tr>
<td>HMS</td>
<td>P6mm</td>
<td>2–5</td>
<td>Hexagonal</td>
</tr>
</tbody>
</table>

Figure 1.3 TEM image of honeycomb-like MSNs (Left arrow: nanopores in the radial direction; Right arrow: 2-D channel in axial direction) (Vivero-Escoto et al. 2010).
Among all these MSNs, MCM-41, MCM-48 and SBA-15 are the most commonly used mesoporous solids. MCM-41 and SBA-15 have typical two-dimensional hexagonal structures with one-dimensional channel straight forward from the axial direction of a mesostructured cylinder (Figure 1.3 black arrow). Due to the high length-to-diameter ratio, guest molecules to be loaded are difficult to reach the central site of hexagonal channels in MCM-41 and SBA-15, leading to low drug loadings.

![Figure 1.4 SEM (a) and TEM (b) images of mesoporous silica nanoparticles. The insert images are high-resolution electron microscope images of single silica particles (Nandiyanto et al. 2009).](image)

On the contrary, MCM-48 has a cubic 3-dimensional porous structure in which the entrances of each pore are distributed on the surface of spherical particles (Figure 1.4 a and Figure 1.4 b). This structure provides interconnected network between pores, which will provide more opportunity for guest molecules to reach the centre of particles (Kim, Chung & Lin 2010).

### 1.4.3 Fabrication of MSNs

In order to control the morphology of synthesised MSNs, it is needed to understand the mechanism of MSN growth. In this section, the mechanism is systematically investigated. Then, the key factors such as surfactant templates, silica source and catalysts are examined in detail to understand their effects on the formation of MSNs.
1.4.3.1 *Formation mechanism of MSNs*

There are three key components in the synthesis of MSNs, namely surfactant templates, silica precursor and catalysts. Specifically, the surfactant templates form the micelles that evolve mesoporous structure; the silica precursor forms the silica framework outside the micelles; and the catalysts (bases or acids) accelerate the hydrolysis and condensation of silica precursor. The growth mechanism of MSNs has intimate relation to these factors.

The mesostructure is formed after organic surfactants that act as templates are removed (Blin & Imperor-Clerc 2013; Lu & Zhao 2004). The actual process could be more complex, as can be seen from Figure 1.5 which is an example of the synthesis of MCM-41. During the formation of a micelle, some experimental conditions, such as the type and concentration of surfactants, system temperature, reaction time and stirring speed, have an impact on micelle formation (Zhao et al. 1998), controlling the pore and particle sizes (Huo, Margolese & Stucky 1996). Furthermore, an ordered structure of silica shell is established, the organic templates are eliminated and mesoporous particles are obtained. Several general methodologies have been carried out during this process, such as dissolution (Han et al. 2011), dialysis (Liu, Miyoshi & Nakamura 2007) and calcination (Ha et al. 2008). Nevertheless, based on dominating experimental conditions, ideal MSNs can be obtained to fulfil the requirements for drug delivery system.

![Figure 1.5 Possible mechanistic pathways for the formation of MCM-41: (1) liquid crystal phase initiated and (2) silicate anion initiated (Beck et al. 1992).](image)
1.4.3.2 Surfactant templates

In order to prepare regulatory and mesostructured silica nanoparticles, surfactants play an important role in forming the mesopores and directing the mesoporous structure. Currently, the cationic and non-ionic surfactants are used in the synthesis of mesoporous solids (Venkatathri 2007). Most surfactants consist of one non-polar molecular chain with a hydrophobe and another polar part with a hydrophile, which lead to the mutual compatibility to both water and oil. This unique structure makes it possible to act as a template for mesoporous nanoparticles.

![CTAB molecule](image1)

![CTAC molecule](image2)

Figure 1.6 Formulation of CTAB and CTAC.

Cetyltrimethyl ammonium bromide (CTAB) and cetyltrimethyl ammonium chloride (CTAC) are two major cationic surfactants used in the synthesis of MSNs. They contain a cetrimonium group at the hydrophilic terminals (Figure 1.6) and a hydrophobic carbon chain as a tail. The structure of the micelles formed in aqueous solution can be varied and manipulated with the experimental condition.

The formation of surfactants in aqueous solution is of importance during the synthesis process, which will influence the meso-structure. The concentration of CTAB has an impact on the geometric shape of MSNs. The critical micelle concentration (CMC) of CTAB is about $9.0 \times 10^{-4} \, \text{mol/L}$ (Shikata, Hirata & Kotaka 1987). Above CMC, molecules of CTAB can assemble into micelles. When the concentration of CTAB is up to $0.3 \, \text{mol/L}$, spherical micelles with a diameter of $5.2 \, \text{nm}$ are formed in aqueous solution; when the concentration is more than $0.7 \, \text{mol/L}$, micelles in the shape of
hexagonal threadlike tubes are built (Shikata, Hirata & Kotaka 1987). In addition, for cetyltrimonium surfactants, different alkyl chain length will form different mesoporous structure. When the number of carbon atoms increases from 10 to 16, resulting MSNs will have different structures of MCM-41 (C10), medium (C12) and MCM-48 (C14 and C16), respectively (Liu et al. 2012).

However, the pore size is restricted to less than 5 nm, which is the limitation of CTAB and CTAC templates. Thus swelling agents are required for the sake of expanding the pore. Nandiyanto et al. (2009) prepared spherical mesoporous silica particles with a tuneable pore size (4-15 nm) through a CTAB template with an expanding agent (polystyrene nanocluster) (Figure 1.7). Zhang et al. (2011) prepared MSNs with 1,3,5-triisopropylbenzene as a swelling agents to obtain pore size ranging from 3.8 nm to 6.1 nm. Na et al. (Na et al. 2012) prepared MSNs with large pores of 23 nm by using trimethylbenzen as an expanding agent. The results suggested that these MSNs with ultra-large pore size have higher loading of siRNA compared to the MSNs with 2 nm pore size and can deliver siRNA into the cells to achieve the gene transfer.

![Figure 1.7 Expand pore size using PS nanocluster (Nandiyanto et al. 2009).](image)

Apart from CTAB and CTAC mentioned above, surfactants that can form mesoporous micelles, such as some block copolymers can also be regarded as mesoporous templates. Figure 1.8 shows the formulation of several representative types of block
copolymers used in the synthesis of MSNs. Among them, poly(ethylene) oxide–poly(propylene) oxide–poly(ethylene) oxide (PEO-PPO-PEO) as a non-ionic surfactant has been widely used in the synthesis of MSNs (Pei et al. 2005). Sierra et al. (2008) prepared cubic mesoporous silica particles by using triblock copolymer (PEO140PPO39PEO140). Due to the long molecule chain of block copolymer, large pore size (35 nm) of MSNs can also be obtained (Niu et al. 2009). The use of surfactants during the synthesis is not totally independent. They can be mixed together to act as a co-surfactant system. Niu et al. (2014a) introduced polystyrene-b-poly (acrylic acid) as a main surfactant and CTAB as the structure stabilizer and prepared MSNs with ordered meso-structure. Therefore, based on the surfactant templates, the mesoporous morphology of MSNs can be tuned as different pore structures can facilitate the delivery of molecules with different sizes.

Figure 1.8 (a) Block copolymer templates used in mesostructure generation. (b) Typical x-ray diffraction (XRD) and transmission electron microscope (TEM) characterization of a 2D hexagonal SBA-15 material. (c) 2D-XRD and TEM information of an Im3m cubic oriented silica film (Soler-Illia et al. 2003).

1.4.3.3 Silica precursor

The silica precursor provides silica source to form the silica framework that constructs the particles and supports the porous structure. The most commonly used silica source are tetraethyl orthosilicate (TEOS) and tetramethyl orthosilicate (TMOS). The particle size is related to the silica precursor added during the synthesis (Zhao et al. 2012).
Because TEOS is an oil-like liquid that has phase separation with water, the co-solvent, such as ethanol, is normally used to improve the miscibility of TEOS in the aqueous solution. Chen et al. (2010b) prepared mono-dispersed MSNs with different ratio between ethanol and water. The increased content of ethanol increased the particle size of MSNs because the ethanol as a co-solvent decreases. This is because the number of nuclei is decreased by the addition of ethanol, which in turn dominates the growth of MSNs (Chen et al. 2010b). By contrary, TMOS possess high hydrolysis rate and solubility, which leads to the formation of much smaller MSNs. Ma et al. (2012; Ma et al. 2013) fabricated MSNs with ultra-small sub-10 nm particle size by using TMOS as the silica precursor. The TMOS was dissolved in water and hydrolysed without forming a secondary oil phase as TEOS. Even though TMOS has these advantages, it has a higher toxicity than TEOS, which limits its use in the synthesis of MSNs.

1.4.3.4 Catalysts

The hydrolysis rate and condensation rate are affected by the catalysts (bases or acids) added in the aqueous solution (Chiang et al. 2011), which results in different particle morphology. It has been proven that the basic condition leads to a slow hydrolysis and a fast condensation, while the acidic condition is opposite (O¨ye et al. 1997). Due to the difference of basic and acidic conditions, big particles with random particle shapes are obtained under acidic condition while nano-sized particles with uniform shapes can be achieved under basic condition (Chiang et al. 2011; Qiao et al. 2009).

1.4.4 Functionalization of MSNs

Due to the quantum effect of nanosilica, the surface of nanoparticles is covered by tremendous number of active hydroxyl groups. These groups provide potential platform to graft multifunctional polymers onto the surface of external nanoparticles and interior channels (Figure 1.9). The surface modification creates hydrophobic or hydrophilic surfaces and introduces a unique functional group (C=C, –COOH, or –NH₂) onto the surface; the multifunctional grafting focuses on improving biocompatibility and protecting and controlling the release of guest molecules. The surface modification provides MSNs with multifunction that can facilitate the delivery of biomolecules into cells.
Figure 1.9 Possibility to functionalise MSNs to form a delivery system. (A) Nanoparticles as gatekeepers; (B) Guest molecules encapsulated inside channels; (C) Stimuli-responsive linkers; (D) Polymer coating that protects and controls the release of guest molecules; (E) Bioimaging agents; (F) Targeting ligands; (G) DNA; (H) Additional ligands, such as cell-penetration peptides; (I) Incorporation of a diagnostic label; (J) Stimuli-responsive polymers; (K) Attachment of functional groups (Vivero-Escoto et al. 2010).

1.4.4.1 Surface modification

The surface of the MSNs is required to be modified or grafted by organic groups to efficiently encapsulate agrochemicals and enhance their biocompatibility. Silane coupling agents are the most commonly used chemicals in surface modification of silica based materials. Reacting with the hydroxyl groups on the surface, coupling
agents can build a bridge connecting with organic materials.

Silane coupling agents provide a variety of functional groups, such as amine groups, double carbon bonds and epoxy groups (Figure 1.10). There have been large numbers of researchers utilizing this coupling characteristic as a pre-treatment in order to provide the foundation for further modification (Al Othman & Apblett 2009; Manzano et al. 2008; Ramírez et al. 2007; Ritter et al. 2009; Shen et al. 2009). Also, through modified silane coupling, both the capacity of drug loading and the releasing duration are enhanced after silane modification (Manzano et al. 2008). In addition, coupling agents are chemically incorporated on the surface of MSNs, which means that a successful graft group is tolerant of physical treatment such as washing, dissolving and stirring.

![Figure 1.10 The formulation of silane coupling agents.](image)

1.4.4.2 Stimuli-responsive modification

Before MSNs carrying agrochemicals arrive at the targeting tissues or cells, the surrounding condition around MSNs can changes dramatically from one area to another, and agrochemicals without good protection are more likely to leak out during this period. This premature release of the agrochemicals wastes most proportion of loaded cargos, in turn dramatically decreasing the efficiency of delivery. Therefore, an encapsulating strategy is implemented to fulfil two requirements: to maintain a high loading and to control the release via stimuli-response.
Figure 1.11 (A): A schematic indication of assembled gold gate keepers; (B): TEM images of MSNs; and (C) the morphology of gold nanoparticles (Liu et al. 2010).

Various conditions, such as pH value, temperature, magnetism and light, can be regarded as triggers to release chemicals into the environment (Bajpai et al. 2008). A number of stimuli-responsive protectors have been developed to achieve controlled
release. Due to the specific porous structure of MSNs, most stimuli-responsive functionalization happens through gatekeepers at the entrance of the channels (namely mesopores). Gold (Au) nanoparticles are the most commonly used medium for this purpose. Au nanoparticles and MSNs can be connected with pH sensitive polymers (Liu et al. 2010; Torney et al. 2007). In neutral condition, guest molecules are blocked in the channels of MSNs. The results showed that the gold gatekeepers are obvious to be seen at the end of mesopore channels (Figure 1.11 C) under electron microscope after assembly onto the raw MSNs (Figure 1.11 B). When the surrounding pH value tends to be acidic, the hydrolysis of acetal groups allows the gates blocked by gold to be open and leads to the release of entrapped molecules (Figure 1.11 A). For the redox-responsive release, cadmium sulfide nanoparticles (CdS) nanoparticles and organic molecules have been implemented to protect guest molecules in MSNs, based on the theory of conditional stimulation (Du, Song & Liao 2010; Lai et al. 2003). The light mediated release of loaded cargos is always related to the light sensitive materials, such as gold nanorods (Li et al. 2014; Zhang et al. 2012) and rare element particles (Niu et al. 2014b). However, because the main compositions of these gatekeepers are heavy metals that have adverse effects on plant cells and tissues after breaking away from MSNs, the potential use of these types of nano gatekeepers must be carefully considered when used with plants.

1.4.4.3 Biocompatible polymeric coating

After penetrating through plant cell walls, the next obstacle for MSNs to overcome is the cell membrane that essentially consists of proteins and phospholipid bilayers. Biocompatible polymeric coatings are to enhance the permeability and retention. Increased attention is now on how to improve the acceptance of cells in drug delivery systems in animals. PEGylation is a technique that can achieve homogeneous particle dispersion and particularly reinforce the permeability of MSNs across the cell membrane. Several biocompatible materials have been coated on the surface of MSNs, including PEG (poly(ethylene glycol)) (Zhu et al. 2011) and TEG (triethylene glycol) (Torney et al. 2007). After MSNs are PEGylated, it is obvious from the images in confocal microscopy images (Figure 1.12) that the cell uptake of MSNs is improved (Zhu et al. 2011). Therefore, based on the theory of drug delivery, PEGylation is a necessary step for cellular penetration.
Figure 1.12 The distribution of hollow sphere mesoporous silica (HSMS) in Hela cells. (a) HSMS with fluorescent agents; (b) HSMS-PEG with fluorescent agents (Zhu et al. 2011). The cell nuclei are in blue and nanoparticles are in red.

In addition to the enhancement in permeability, surface coating can also manipulate the duration of release to some extent. Different thicknesses of polymeric coating results in different release periods. Niu et al. (2010) prepared core-shell mesoporous silica spheres with controllable shells with a thickness of 5 nm, 25 nm and 60 nm respectively. The shell is also formed by mesoporous silica, which has different pore size. Due to the size gradient of mesopores, chemicals loaded inside can be gradually released according to the thickness of the shell. The results showed that the thicker the shell coatings are, the longer the release takes. This methodology indicates another prospect of the controlled drug delivery systems.

Figure 1.13 Different shells with different release rate of drugs (Niu et al. 2010). The TEM images (left three figures) show the core shell structure of MSNs, and the release kinetics (right graph) show the difference of release duration.
Mesoporous silica nanoparticles, as nanostructured mesoporous materials, possess both properties of nano and porous particles, such as nano-scale particle size and pore size, homogeneous pore size distribution, high surface area and low density, excellent surface chemical activation and stable structural properties. All of the above properties enable MSNs to be an ideal carrier of biomolecules (He & Shi 2011; Wang 2009). In order to load and controllably release biomolecules, the surface functionalization and gatekeeper strategy have to be considered according to the properties of the loaded cargos, such as surface charges and hydrophobicity. In the following section, the loading methods of biomolecules into MSNs will be systematically discussed.

1.4.4.4 Fluorescent labelling and tracking of MSNs in plants

Localizing the nanoparticles in plants is important for a delivery system as it provides useful information on the translocation of nanoparticles within plants (Cho, Volkov & Sokolov 2010; Guo et al. 2011; He et al. 2009b; Kurepa et al. 2010; Liong et al. 2008). Since MSNs with particle size at nano scale are difficult to be observed under normal optical microscope, fluorescent labelling techniques are widely used to track the translocation of nanoparticles in plants.

Commonly used fluorescent dyes in delivery systems include fluorescein isothiocyanate (FITC), green fluorescent protein and quantum dots (Chalfie 2001; Pinto et al. 2008; Yang et al. 2009). FITC can be easily grafted onto the surface of MSNs with covalent bonds that protect FITC from some physical treatment for purifying nanoparticles such as washing, centrifuging and stirring. MSNs bound with FITC show the fluorescence at a certain excitation wavelength. Torney et al. (2007) reported that FITC was incorporated onto the surface of MSNs using a coupling agent as a bridge. The confocal images showed that fluorescence dyed MSNs are localized in tobacco protoplast (Error! Reference source not found.). Therefore, FITC is an deal fluorescent dye for the localization of MSNs in plants.
Figure 1.14 Schematic of MSNs grafted by FITC through coupling agent (A) and Confocal image of MSNs uptake by tobacco mesophyll protoplast (B). Green spots are functionalised MSNs with FITC (white arrow), and the auto-fluorescence of chloroplast is indicated in red (Torney et al. 2007).

1.5 Biomolecule loading method

Due to the porous structure and high surface area, MSNs are able to absorb chemical molecules inside mesopores and on the external surface. Loading agrochemicals inside pores with a diameter of several nanometers is very challenging. In this section, the different loading methods are systematically investigated.
1.5.1 Diffusion method

Utilizing the concentration difference of chemicals in the solution and inside mesopores is one of the main mechanisms to load agrochemicals in MSNs. The chemicals are firstly dissolved in a certain solvent that depends on their solubility, followed by the addition of MSNs (Alsyouri et al. 2011; Zhang et al. 2010). With the concentration gradient, the chemicals move from the higher concentration (solution) into the area with lower concentration (mesopores of MSNs). Once the equal concentration achieves, the diffusion halts. Due to the simple operating procedure, this method is suitable to various types of chemicals as long as they can be dissolved in a certain solvent. However, the low agrochemical loading is the limitation of diffusion method. Because the chemical molecules diffuse into MSNs without external promotion, the loading rate is mainly dependent on the absorbing capacity of MSNs.

1.5.2 Solvent evaporation

Solvent evaporation has been widely used to load drugs in the polymer based drug delivery system (Boateng et al. 2010; Hong et al. 2005b). In this method, the agrochemicals are dissolved in a certain solvent and mixed with the suspension of MSNs. After that, solvent is evaporated at its evaporating temperature and the concentrated agrochemicals are forced to load into mesopores (Charnay et al. 2004). Through this method, high drug loadings can be achieved. But the vapour of organic solvent is toxic to human beings. Rotary evaporation has been developed to collect the vapour of solvent in order to reduce the hazard of this method. More importantly, the drug loading of this method is not affected by the rotary system (Limnell et al. 2011).

1.5.3 Melting method

In melting method, the drugs are heated to the melting point to obtain the fluid drug liquid that easily flows into mesopores of MSNs. Thus, the viscosity of drug fluid at melting temperature is a key parameter. For example, itraconazole and ibuprofen were tested to be incorporated into mesopores of SBA-15 via melting method, and it was found that itraconazole was not properly loaded by this method owing to the high viscosity of melted itraconazole compared to the successful loading of ibuprofen.
(Mellaerts et al. 2008). Moreover, the high temperature has a significant impact on the chemical and physical stability of the drugs, which is a severe limitation for melting method.

1.6 Summary

This literature review systematically and comprehensively summarized the problem of using agrochemicals in agriculture, the assessment of the phytotoxicity of the existing nanomaterials, the advantages of MSNs as the agrochemical carriers, the morphology manipulation of synthesised MSNs and the method of loading and releasing agrochemicals. The previous studies in this area were analytically examined and critically reviewed. This chapter draws the main research question of how to fabricate functionalised MSNs to delivery agrochemicals into plants in a controllable fashion from the current problems in agriculture and raised the efficient and effective solution to the issue. The technical terms and basic theories described in this chapter were anticipated to benefit for the understanding of the following chapters.

The methodology of synthesise and functionalization of MSNs will be incorporated into the following chapter with corresponding results and discussion. This is for the easy understanding of the materials used in each chapter and the fluent idea flow.

Chapter 2 focuses on the morphology control of MSNs. The MSNs with particle size of about 20 nm have been simply and mildly synthesised by controlling experimental conditions such as the temperature, the concentration of CTAB and the ionic concentration of the solution. Temperature was found to be a critical parameter affecting the particle size. The concentration of CTAB had a slight impact on the pore size. The ionic strength greatly affected the dispersity of the final products. The synthesis process of MSNs was systematically evaluated in order to find out the relationship between the experimental conditions and the final morphology of MSNs.

Chapter 3 follows the synthesis of MSNs in Chapter 2 and develops the growth mechanism of MSNs behind the synthesis procedures through time-resolved small angle X-ray scattering (SAXS). Through modelling the SAXS data, the growth stage of the nanoparticles and pores were investigated along with the time. A growth
Chapter 1: Literature Review

mechanism of MSNs was proposed.

Chapter 4 explores the translocation of MSNs within the plants via fluorescent labelling that was chemically bonded onto MSNs. The MSNs were functionalised with FITC or RITC as a fluorescent signal for tracking the movement of MSNs in plants. After plants were treated with fluorescent labelled MSNs, the cross sections from the plant roots, stems and leaves were obtained and the location of MSNs was tracked under confocal laser scanning microscopy. The quantity of MSNs taken up by plants was determined by micro particle induced X-ray emission.

Chapter 5 introduces mesoporous silica based agrochemical delivery systems with stimuli-responsive functionalization. This well-known drug delivery system has been widely used in cancer therapy, however, studies on delivery system for plants and the controllable release are seldom reported. This study was conducted to fill the gap and to build up a mesoporous silica based agrochemical delivery system by using smaller MSNs (particle size ~ 20 nm) to overcome the plant cell wall and introducing redox-responsive functional groups to release agrochemicals only inside plant cells. Glutathione as a natural reductant in the plant cell was used to test the redox-responsive function *in vitro*.

Chapter 6 provides preliminary results for the future perspective in terms of loading and releasing the large molecules into plant cells. The pore size of MSNs was expanded to more than 10 nm with a swelling agent. The morphology of MSNs due to pore expansion turned out to be a flower-like structure. This interesting structure was observed at different angle under transmission electron microscope to understand its 3D architecture. The surface of MSNs was modified to be either positively charged or negative charged. The varying charge on the surface of MSNs and the large pore size favoured the loading of large molecules such as proteins and siRNA via the electrostatic force.

Chapter 7 reviews the conclusions obtained from the research work and suggests future and further work in these areas.

In Appendix A, the materials used in the synthesis and functionalization of MSNs are listed, and in Appendix B, the detailed characterization techniques are described.
Chapter 2: Synthesis of Mesoporous Silica Nanoparticles and the Control of Morphology

Highlights of Contributions:

- Through manipulating experimental conditions such as temperature, concentration of CTAB and ionic strength, the morphology of MSNs can be controlled.
- The influence of these conditions on the morphology of the resulting products is systematically discussed.
- MSNs with small diameters (around 20 nm) were synthesised via a simple and facile procedure in order to facilitate the penetration of MSNs through the plant cell wall.

2.1 Introduction

Silica based nanomaterials have attracted the attention of many researchers in recent decades due to their high surface area, quantum dot effects, controllable structure and low toxicity (Barik, Sahu & Swain 2008). Mesoporous silica nanoparticles (MSNs) with the pore diameter between 2 and 50 nm, as one of the most important candidates in this field, have tunable porous and particulate structure and compelling applications in terms of biosensing, treatment of waste water and controlled drug delivery systems (Angelos et al. 2008; Bonifacio, Lotsch & Ozin 2011; Hoffmann & Fröba 2010; Wang 2009; Wei, Hu & Zhang 2010). In nano-scale, MSNs with the particle size less than 50 nm possess many advantages such as high permeability into tissues with small entries and higher surface area when compared to big particles. In particular in the delivery system to the plants, particle size of MSNs is essential to penetrate through plant cell wall (Nair et al. 2010). MSNs with small particle size (less than 50 nm) is required.

During the synthesis procedure, the resulting morphology of synthesised MSNs is affected by many factors including temperature, pH value, surfactant concentration and structure, and silica precursors. He et al. (2009a) prepared monodispersed MSNs with controllable particle size ranging from 42 to 617 nm at different temperature
under neutral condition through the co-surfactant template of cetyltrimethylammonium bromide (CTAB) and polyoxyethylene (10) cetyl ether. The results showed that the smaller MSNs were obtained at lower temperature and higher surfactant concentration. Chen et al. (2010b) employed cationic germini surfactants synthesised from N,N-dimethylhexadecylamine and (3-bromopropyl) trimethylammonium bromide to synthesise spherical MSNs with tuneable size from 70 to 490 nm under basic condition, and investigated the impacts of temperature and the surfactant concentration on particle size. Through adjusting the experimental conditions, the particle size of MSNs can be tuned in a broad range.

However, the synthesis of MSNs with a particle size less than 50 nm, especially as small as 20 nm, is still very challenging. It is because the porous structure at such a fine particle size tends to collapse (Jervis et al. 2004) and the interaction between particles will intensify the aggregation. Chiang et al. (2011) have prepared MSNs with the particle size of 16.9 nm, but the surface area and pore size of these nanoparticles cannot be characterized. Kobler and Bein (2008) obtained MSNs with the particle size of 15 nm, but the textural porosity measured from nitrogen sorption indicated the aggregation and interconnection between particles. A recent study from Ma and Wiesner (2012) explored the synthesis of MSNs with the particle size less than 10 nm. The size of the smallest MSNs they fabricated was around 6.6 nm. Only half or one pore was formed in one particle because of the sub-10 nm particle size, which impairs the ability of MSNs to be the carriers of biomolecules. Therefore, the most challenging work to prepare MSNs with 20 nm particle size is the maintenance of mesoporous structure.

Herein, we synthesised MSNs with tuneable particle size and undamaged mesoporous structure in a facile and simple way. The CTAB was chosen as the surfactant template to form mesopores, and phosphate buffer solution and ammonia solution were used as catalyst to accelerate the reaction of the silica source. Several experimental conditions including surfactant concentration, temperature, pH value and the ionic strength were investigated. The discrete MSNs with small particle size and uniform dispersity were obtained at low temperature in a reaction solution with very low ionic strength. The relationship between experimental conditions and the final morphology of MSNs was also discussed based on the existing theory about the growth of MSNs.
2.2 Materials, Experimental and Methodology

2.2.1 Materials

All the chemicals used in this work are listed in Appendix A.

2.2.2 Synthesis of MSNs with different experimental parameters

The synthesis process of MSNs was based on the previous studies with modification (He et al. 2009a; Liu et al. 2012; Ma, Sai & Wiesner 2012; Nooney et al. 2002). The aim of controlling the experimental conditions is to achieve small MSNs with uniform dispersity. The detailed experimental procedures are described as follows.

Synthesis of MSNs with different CTAB concentration. A phosphate buffer solution was prepared by mixing 1.74 g NaOH and 10.2 g KH₂PO₄ in 1.5 L Milli-Q water (final pH value is 7.2). Different amount of CTAB was dissolved in 100 mL of this buffer solution. The mixture was heated to 80 °C and kept stirring until the solution of CTAB became homogeneous and then the temperature was kept at 80 °C for 30 min to stabilize CTAB micelles. Subsequently, the aqueous solution of CTAB was cooled down to 30 °C followed by the dropwise addition of 1.86 mL TEOS. The reaction was maintained for 8 h with vigorous stirring. The as-made MSNs were centrifuged and washed with ethanol for three times to remove the free TEOS and CTAB.

Synthesis of MSNs at high temperature. Procedure as above but after stabilizing CTAB micelles at 80 °C for 30 min the temperature of the mixture was heated to 95 °C and the reaction was maintained for 4 h. The as-made MSNs were centrifuged and washed with ethanol for three times to remove the free TEOS and CTAB.

Synthesis of MSNs under different ionic strength. The pH of the previously prepared phosphate buffer solution was adjusted by adding 2 M of NaOH aqueous solution to 9, 10 and 12 (See table 2.1). Then, 2.96 g of CTAB was dissolved in 100 mL of this buffer solution. The following procedure was the same as the previous samples. The reaction was maintained at 30 °C for 4 h. For the synthesis of MSNs under low ionic strength (with ammonium hydroxide as catalyst), 2.96 g of CTAB was
dissolved in 100 mL of Milli-Q water with pH of 10 adjusted by the addition of the diluted ammonium hydroxide. The mixture was heated to 80 °C for 30 min to stabilize CTAB micelles. Then CTAB solution was cooled to 30 °C. TEOS (1.86 mL) was added into CTAB solution dropwise and the reaction was maintained for 24 h, followed by ageing at 80 °C in an oven for 24 h. The as-made MSNs were collected by vacuum filtration.

All the experimental parameters are summarized in Table 2.1.

Table 2.1 Experimental parameters used for MSN synthesis

<table>
<thead>
<tr>
<th>Samples</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>CTAB (mM)</th>
<th>TEOS (mM)</th>
<th>Reaction time (h)</th>
<th>Ageing time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSN-30-60</td>
<td>7.2</td>
<td>30</td>
<td>60</td>
<td>80</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>MSN-30-80</td>
<td>7.2</td>
<td>30</td>
<td>80</td>
<td>80</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>MSN-30-100</td>
<td>7.2</td>
<td>30</td>
<td>100</td>
<td>80</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>MSN-95-60</td>
<td>7.2</td>
<td>95</td>
<td>60</td>
<td>80</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>MSN-95-80</td>
<td>7.2</td>
<td>95</td>
<td>80</td>
<td>80</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>MSN-95-100</td>
<td>7.2</td>
<td>95</td>
<td>100</td>
<td>80</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>MSN-PH9</td>
<td>9a</td>
<td>30</td>
<td>80</td>
<td>80</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>MSN-PH10</td>
<td>10a</td>
<td>30</td>
<td>80</td>
<td>80</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>MSN-PH12</td>
<td>12a</td>
<td>30</td>
<td>80</td>
<td>80</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>MSN-AM</td>
<td>10b</td>
<td>30</td>
<td>80</td>
<td>80</td>
<td>24</td>
<td>24</td>
</tr>
</tbody>
</table>

a The pH value of the reaction solution was adjusted by adding 2 M of NaOH aqueous solution to be 9, 10 and 12.

b The pH value was adjusted by adding ammonium hydroxide to 10.
2.2.3 Removal of CTAB template

To remove the CTAB templates from mesopores, both a solution and a burning method were used in this study. For the solution method, the as-synthesised MSNs were redispersed in 100 mL of ethanol and 1 mL of hydrochloric acid with vigorous stirring for 12 h, followed by centrifugation and washing with ethanol and deionised water for three times. The final MSN powder was collected by drying template-removed MSNs under vacuum. For the burning method, the as-synthesised MSN powders were heated to 550-600 °C for 5 h to burn all the CTAB template. The final products were collected by cooling the samples to room temperature.

2.2.4 Characterization

The TEM and nitrogen isotherms were used to examine the morphology and porous structure of MSNs. The detailed information on various characterization methods will be described in Appendix B. The distribution of micelle size was calculated under room temperature through dynamic light scattering (DLS) (Zetasizer Nano ZS Malven, UK). The CTAB solution with different concentration was prepared by dissolving CTAB powder into the solution. The measurement of small angle X-ray scattering (SAXS) was obtained from Australian Synchrotron under the proposals of M4977 and M6410. The powder samples were sandwiched with the Capton tape and mounted onto a holy aluminium plate.

2.3 Rationale of the synthesis of MSNs

The procedure to form mesoporous structure was divided into two main stages: the formation of micelles (CTAB) and the hydrolysis and condensation of silica source (TEOS), as shown in Figure 2.1. In the aqueous solution, the hydrophobic tails tend to aggregate and the hydrophilic head groups of CTAB face the water phase, which in turn leads to the formation of micelles (step ① in Figure 2.1). After the formation of micelle particles, the second stage was the addition of silica source, TEOS, which was hydrolysed to be negatively charged and condensed on to the positively charged micellar particles (step ② in Figure 2.1). The rate of hydrolysis and condensation can be controlled by adjusting the pH value and the temperature (O’ye et al. 1997). The
morphology of starting CTAB in aqueous solution and the reaction of TEOS have a great impact on the morphology of the particles and pores. Those experimental parameters, such as the concentration of surfactants, the pH value and the temperature, have an impact on the micellar structure and the reaction rate of silica source, which in turn results in different MSN structure (Wu, Mou & Lin 2013). In the following sections, the effects of experimental parameters were investigated in order to find out the relationship between experimental conditions and the final morphology of MSNs, which can be used to control the particle morphology and mesoporous structure.

Figure 2.1 Schematic of MSN synthesis. ① When the concentration of CTAB is over the critical micelle concentration, the micelles are formed in the aqueous solution with the addition of the catalysts. ② Hydrolysed TEOS results in the presence of negatively charged silica monomers that absorb onto the positively charged micelles via electrostatic force. The silica monomers condense to form the silica framework. Once all the silica source is consumed, the existing MSNs re-construct to form spherical particles. After template removal, the mesopores are empty and ready for the loading of guest molecules.


### 2.4 Formation of micelles in aqueous solution

![Image of hydrodynamic diameter distribution of micelles](image)

Figure 2.2 (A): Hydrodynamic diameter distribution of micelles and (B): the interpretation of forming micelles in buffer solution with different concentration of C\textsubscript{16}TAB under pH 7.2 at ambient temperature.

The structure of CTAB as the template in the aqueous solution determines the final porous structure of MSNs. Herein, the CTAB in a phosphate buffer solution was taken as an example to show the behaviour of CTAB with different concentration. The CTAB possesses two critical micelle concentrations (CMC) (Shi, Luo & Li 2011), which have an impact on the micelle size distribution. The first CMC determines the formation of micelles. When the concentration of CTAB (0.5 mM) is lower than the first CMC (0.9 mM (Shikata, Hirata & Kotaka 1987)), micelles have not been formed, and the surfactant molecules are either present individually in aqueous solution (peak at 5 nm) or form a layer at the water/air interface (Figure 2.2 A). With the increased concentration (1~6 mM) over the first CMC but below second CMC (7 mM (González-Pérez et al. 2003; Ma et al. 1998)), CTAB micelles are formed but the size distribution is not uniform. The presence of large aggregates can also be seen at hundred...
nanometers. Further increasing CTAB concentration (10~100 mM) to above the second CMC, micelles with the size of 5-6 nm become the largest population in this system. By measuring the size distribution of CTAB solution, the homogeneity of the formed micelles can also be revealed.

Furthermore, there are two interesting findings regarding the micelle size distribution. Firstly, the abnormal micellar size at 4 mM was observed. This might be because the added salt changes the CMC. To further confirm this assumption, CMC measurement will be required, but as MSNs in this thesis were synthesised at CTAB concentration of over 60 mM where the micelle is stable and micelles size distribution has only one peak (Figure 2.2A). Secondly, the presence of large aggregates in this system is very interesting. The reasons why they present may be because the molecular layer formed at water/air interface, or because the solution was contaminated by dusts. However, these reasons may not explain this phenomenon. On the one hand, DLS should not measure the size distribution at the interface as the abnormal reflection of light affects the results. On the other hand, the dust could be present in all samples if it is the reason, whereas, the DLS results have indicated that when CTAB concentration is over second CMC, the peak for large objects disappeared. To understand this interesting behaviour of CTAB in the buffer solution, future investigation, such as cryo-TEM, will be required.

Another interesting finding is that the size of CTAB micelles decreases with increasing the concentration of CTAB (Figure 2.2A). As the aggregation number of CTAB did not change after 40 mM (Anachkov et al. 2012), the slight drop of the micelle size may be because the high concentration of counter ions (Br⁻) that increases the ionic strength of the solution compressed the micelles. These small changes in fact affect the pore size of the final products, which will be investigated in the following sections.

2.5 Effects of temperature and CTAB concentration

The formation of the CTAB micelles in the aqueous solution provides the template for the silica source to regularly form meso-structured nanoparticles. The morphology of the final products is dependent on the reaction condition. The effects of CTAB concentration and the reaction temperature were investigated.
2.5.1 Morphology of MSNs synthesised at different temperature and with different initial CTAB concentration
Chapter 2: Controllable Synthesis of MSNs

(C) MSN-30-80

(D) MSN-95-80
Figure 2.3 TEM images of MSNs synthesised with different CTAB concentration at 30 °C and 95 °C. The dark phase indicates the silica framework and the white dot indicates the pores on MSNs.
The morphology of the final MSNs is shown in Figure 2.3. The mesoporous structure of all the samples can be seen from the TEM images (white dots). It can be seen from Figure 2.3 A, B and C that at 30 °C, the particle sizes of MSN-30-60, MSN-30-80 and MSN-30-100 are all around 20 nm. The similar particle size can be obtained because the concentration of CTAB used to synthesise these samples is over 60 mM and the micelles formed in the solution are uniform in size (Figure 2.2). The similar results can be seen from the final particle size of MSNs synthesised at 95 °C. The particle size of MSN-95-60, MSN-95-80 and MSN-95-100 were around 50 nm without any significant changes by increasing CTAB concentration. In this phosphate buffer solution system (pH = 7.2), the temperature is the major factor that determines the particle size. In addition, the aggregation can be seen under TEM, thus DLS was employed to investigate the dispersity of these MSNs in water. The results from DLS measurement (Figure 2.4) show that a single size distribution peak present at around 100-200 nm for both MSNs synthesised at 30 °C and 95 °C. Compared to the observation from TEM, the larger particle size detected by DLS suggests the aggregation of MSNs. Further studies are required to examine the aggregation behaviour of MSNs in solution and ensure the dispersity for the good permeability through plant cells.

The particle size of MSNs is determined by two competitive factors, namely the nucleation and the growth of silica (Matsoukas & Gulari 1988). At 95 °C, the fast condensation resulted in the fast growth of MSNs. The silica monomers tended to condense onto the existing nuclei to form particles that provided active spots for the continuous growth of particles. Then nucleation still took place, but the generated nuclei that were much smaller than the already formed particles were dissolved and redeposited on to the particles due to the Ostwald ripening (Botella et al. 2009). The particles tended to grow bigger rather than produce more. In contrast, the nucleation governed the reaction at low temperature (30 °C). Because of the slow growth of particles, the particle cannot grow big enough to swallow other nuclei. More nuclei were generated and present in the solution compared to the high temperature (95 °C), which provided more grown spots for the particles. The small particles were formed at low temperature. Therefore, the temperature in fact affected the domination between the nucleation of silica monomers and the growth of particles, which resulted in the different particle size at high (95 °C) and low (30 °C) temperature.
Figure 2.4 Particle size distribution of MSNs synthesised at different temperature and CTAB concentration. (A) 30 °C, and (B) 95 °C.
2.5.2 Pore size and pore homogeneity affected by the temperature and initial CTAB concentration

Figure 2.5 Scattering data from a synchrotron SAXS. The scattering intensity was plotted against the scattering vector $Q$. The inserts are two dimensional scattering patterns. The scattering rings are pointed out by the yellow arrows.
Chapter 2: Controllable Synthesis of MSNs

The impacts of the initial CTAB concentration on the mesoporous structure and pore size were investigated. A synchrotron SAXS was employed to investigate the scattering from the MSN powders. It can be seen from Figure 2.5 that the SAXS patterns of all the MSN samples possess uniform scattering, which suggested the isotropic mesoporous structure without any pore alignment. The scattering peaks at around 0.1 Å⁻¹ corresponding to 3-4 nm in real space are the diffractions from mesopores. In addition, the adsorption properties of these MSNs were investigated through nitrogen sorption isotherms. The obtained isotherms of MSNs synthesised at 30 ºC and 95 ºC are shown in Figure 2.6 A and B, respectively. All isotherms exhibit typical type IV adsorption curve according to IUPAC classification (Sing et al. 1985). As the absorbed nitrogen at the relative pressure of 0 is less than 100 cm³/g in all the isotherms, the microporous structure occupied very small volume in these MSNs, which means that mesopores are the dominated structure. The hysteresis loops at the relative pressure higher than 0.8 indicate the interconnected porous structure (Hu et al. 2011) (Figure 2.6 A and B).

The temperature and initial CTAB concentration affect not only the structure of CTAB micelles in aqueous solution, but also the interaction between CTAB and TEOS, which in turn leads to the different morphology of MSNs. From the pore size distribution (Figure 2.7), the uniformity and size of mesopores are affected by changing temperature and CTAB concentration. At 30 ºC, with the increase of CTAB concentration, the major distribution of mesopores increases from 2.8 nm for MSN-30-60 to 3.1 nm for MSN-30-100 (Figure 2.7 A). The pore volume occupied by the mesopores reached the highest level with the addition of 80 mM of CTAB (MSN-30-80). The homogeneity of mesopores is improved with the increase of CTAB concentration. At 95 ºC, the uniform pore size distribution can be obtained without observing any impact from the changes in CTAB concentration. The major pore size was 2.5nm, 2.7 and 2.6 nm for MSN-95-60, MSN-95-80 and MSN-95-100 (Figure 2.7 B), respectively and the mesopores accounted for a similar pore volume. The pore homogeneity can also be proved from the SAXS patterns. The isolated scattering ring from MSNs synthesised at 95 ºC also demonstrated the narrow pore size distribution (Figure 2.5).
Figure 2.6 Nitrogen adsorption-desorption isotherms of MSNs synthesised at with varying the concentration of CTAB at 30 °C (A) and 95 °C (B). The isotherms are shifted along with Y axis for clarity (400 for MSN-30-80 and 900 for MSN-30-100 in A; 500 for MSN-95-80 and 900 for MSN-95-100 in B).
The addition of CTAB slightly increases the pore size of the final products. This is because the higher concentration of CTAB leads to the increase of the aggregation.

Figure 2.7 Pore size distribution of MSN synthesised with different CTAB concentration at 30 °C (A) and 95 °C (B).
number of CTAB micelles, which in turn expands the size of the micelles (Israelachvili, Mitchell & Ninham 1976; Rodenas et al. 1994). In addition, the higher temperature makes the pores more uniform but smaller. The framework formed by silanol groups is reconstructed over time especially when the temperature is low (30 °C). The high temperature (95 °C) is helpful to form strong silanol bonding that restricts the reconstruction of the silica framework (Blin & Imperor-Clerc 2013; Wu, Mou & Lin 2013).

Figure 2.8 BET surface area (A) and pore volume (B) of MSNs synthesised with different temperature and CTAB concentration. Note: The pore volume was obtained from the mesopores with the diameter less than 10 nm.
Furthermore, the surface area and total pore volume (calculated when relative pressure is approaching 1 in nitrogen sorption isotherms) are shown in Error! Reference source not found. A and B, respectively. MSNs synthesised at 30 °C possess higher surface area and pore volume than those prepared at 95 °C. The higher surface area is contributed by the smaller particle size (20 nm) while the higher pore volume is because of the larger pore size obtained at 30 °C (Figure 2.7). By changing the concentration of CTAB, different surface area can be obtained at the same temperature. With 80 mM of CTAB, the largest surface area has been obtained at 30 °C. The pore volume at the peak in the pore size distribution is the highest compared to the MSN-30-60 and MSN-30-100, leading to the large inner surface area. At 95 °C, the surface area constantly decreased with the increase of CTAB concentration. This is also because of the decrease of pore volume at the major pore size distribution. Therefore, 80 mM CTAB concentration is ideal to fabricate MSNs with large surface area and pore volume which potentially favours the high loading of biomolecules. In the following sections, the effects of pH and ionic strength on the morphology of MSNs will be investigated with CTAB concentration of 80 mM.

2.6 Effects of the pH and ionic strength

![Image](A) MSN-PH9

50 nm
Figure 2.9 TEM images of MSNs synthesised in the phosphate buffer solution under different pH value. The regions of interest are highlighted by the yellow arrows.

The results of MSNs synthesised at 30 ºC under neutral conditions (pH=7.2) suggested a good spherical shape and an ideal size for the application of MSNs in the plants, but the discrete nanoparticles were not achieved. By setting the temperature of 30 ºC, CTAB concentration of 80 mM and TEOS concentration of 80 mM as constant, the
effects of the pH and ionic strength were further investigated in order to achieve the well-dispersed nanoparticle suspension, because the basic conditions were suggested to be ideal to prepare isolated particles (Qiao et al. 2009; Yamada et al. 2013; Yu et al. 2012).

The morphology of MSNs synthesised under different pH can be seen from Figure 2.9. At pH of 9 and 10, an obvious branched morphology was formed and the nanoparticles were bonded chemically, which deteriorated their dispersity (Figure 2.9 A and B). Mesopores (white dots) can also be seen under TEM. An interesting finding is that the continuous increase of the pH to 12 led to a collapse of the particulate shape and the super-branched silica framework was detected (Figure 2.9 C). These results suggested that a higher pH intensifies the interaction between particles and forms branched silica framework.

Figure 2.10 Nitrogen isotherms of MSNs synthesised at different pH value. The curves are shifted for clarity (1000 for MSN-PH10 and 1700 for MSN-PH12).
Figure 2.11 Pore size distribution of MSNs synthesised at different pH. The inserted graph is the enlarged view for the distribution between 2 and 7 nm.

Figure 2.12 SAXS scattering patterns of MSNs synthesised at different pH value.
The mesoporous structure was still formed even though the interaction between particles became severe. The nitrogen isotherms of MSNs synthesised at different pH also exhibited type IV adsorption branch and type H3 hysteresis loops (Figure 2.10), which indicated the interconnected mesoporous structure, similar to those samples synthesised at pH of 7. The pore size distribution is shown in Figure 2.11. It can be seen that the small pores formed by CTAB template are located at around 2-3 nm and there are large pores formed when the pH was increased to 12 (Figure 2.11). This is not only because the pores formed by CTAB are collapsed, but also because the super-branched silica framework shown in the TEM image (Figure 2.9 C) becomes significant and contributes more pore volume for the adsorption of nitrogen. In addition, the pore size distribution becomes broad, which can be proved by the scattering patterns of these MSNs that there is no obvious diffraction peak at about 0.1 Å⁻¹ as showed in the previous samples (Figure 2.12). Therefore, the increase of pH has a great impact on the mesopore formation especially at a very high level (pH > 10).

![Figure 2.13 BET surface area and pore volume of MSNs synthesised at different pH value. The surface area and pore volume of MSNs synthesised at pH7.2 are derived from Error! Reference source not found. in order to compare the effects of pH value on the morphology of MSNs. (Note: The pore volume for sample synthesised at pH 7.2, 9 and 10 are the effective pore volume obtained from mesopores less than 10 nm. The pore volume at pH 12 is the total pore volume.)](image-url)
The surface area and pore volume can indirectly indicate the adsorption capacity of guest molecules by MSNs. It can be seen from Figure 2.13 that the surface area of MSNs decreases with the increase of pH value because inter-particle connections decrease the surface of each particle. The pore volume of these MSNs is between 0.41 and 0.56 cm$^3$/g without a significant difference at pH 7.2, 9 and 10, but increases to about 1.29 cm$^3$/g when the pH reaches 12. This is because of the presence of large pores within the silica network, which dominates the contribution to the pore volume.

2.7 Effects of ionic strength

Ionic strength of the solution plays an important role in getting isolated particles. Because the ionic strength has an intimate relation to the surface charge of the already grown MSNs. The high ionic strength will screen the charges on the adjacent MSNs from each other, decreasing the repulsion and resulting in particle aggregation (Harker 2011). Thus, the decrease of the ionic strength will help to obtain discrete nanoparticles.

<table>
<thead>
<tr>
<th>Samples</th>
<th>pKa</th>
<th>pH</th>
<th>Ionic strength (mol/L·electron$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSN-30-80</td>
<td>7.20$^a$</td>
<td>7.2</td>
<td>0.10</td>
</tr>
<tr>
<td>MSN-PH9</td>
<td>7.20$^a$</td>
<td>9.0$^d$</td>
<td>0.15</td>
</tr>
<tr>
<td>MSN-PH10</td>
<td>12.38$^b$</td>
<td>10.0$^d$</td>
<td>0.16</td>
</tr>
<tr>
<td>MSN-PH12</td>
<td>12.38$^b$</td>
<td>12.0$^d$</td>
<td>0.20</td>
</tr>
<tr>
<td>MSN-AM</td>
<td>9.25$^c$</td>
<td>10.0$^e$</td>
<td>0.00005</td>
</tr>
</tbody>
</table>

Note: The logarithmic acid dissociation constant (pK$_a$) is obtained from the literature (Conover 1998).

$^a$ The pKa refers to $H_2PO_4^- \rightleftharpoons HPO_4^{2-} + H^+$.

$^b$ The pKa refers to $HPO_4^{2-} \rightleftharpoons PO_4^{3-} + H^+$.

$^c$ The pKa refers to $NH_3 \cdot H_2O \rightleftharpoons NH_4^+ + OH^-$.

$^d$ The pH value was adjusted by adding NaOH (2 M) aqueous solution in the buffer solution.

$^e$ The pH value was adjusted by adding diluted ammonium hydroxide solution.
In the previous section, the phosphate buffer solution was used as the catalyst media to synthesise MSNs. The salt to prepare this buffer solution indirectly increased the ionic strength of the solution and the changing of pH meant that more salt was added to the buffer solution, leading to the further increase of ionic strength of the reaction solution. The ionic strength was calculated and listed in Table 2.2 according to the pH.

Figure 2.14 Morphology of MSN-AM under TEM (A) and SEM (B). The yellow circles in (A) highlight the spherical and isolated particles.
and the dissociation constant of the weak acid/basic salt. The ionic strength increased with increasing the pH value and MSNs with alternative morphologies were obtained. At pH 7.2, the spherical MSNs can be obtained with less branched morphology. When the ionic strength increased to 0.15 and 0.16 mol/L·electron\(^2\), more branched structure can be seen, but because there is no significant difference with the ionic strength at pH 9 and 10, the structures of MSN-PH9 and MSN-PH10 are similar. When the ionic strength goes up to 0.20 mol/L·electron\(^2\) at pH 12, the super-branched silica framework were present. It can be concluded that the low ionic strength is helpful to achieve isolated nanoparticles and high ionic strength increases the interaction between particles.

In this section, MSNs were synthesised under very low ionic strength. The phosphate buffer solution was replaced by the ammonia solution. The ionic strength of the reaction solution was decreased to 0.00005 mol/L·electron\(^2\) (Table 2.2), which is far lower than the ionic strength of the previous buffer solution. Because of the low effects of the solution on the surface charges of silica, the neighbouring particles are repulsed with each other and the growth of nanoparticles is independent. Discrete nanoparticles with uniform particle size can be seen under TEM and SEM (Figure 2.14). The obtained MSNs possess spherical shape and the size is around 20 nm. Mesopores can also be seen on the TEM image (Figure 2.14 A). The targeting application of our MSNs is in agriculture to build up an agrichemical delivery system. Well dispersed MSNs loaded with biomolecules will benefit the penetration into plants and the translocation of MSNs within plants, compared to the aggregated MSNs.
Chapter 2: Controllable Synthesis of MSNs

Figure 2.15 Nitrogen adsorption and desorption isotherms (A) and pore size distribution (B) of MSN-AM. The insert in (B) is the enlarged view for the area between 2 and 6 nm. The scattering patterns (C) of MSN-AM. The scattering ring from the mesopores is pointed by the yellow arrow.

The porous structure of MSN-AM was investigated through the nitrogen sorption isotherms and SAXS. Similar to the previous MSNs, the type IV isotherms of MSN-AM indicate the presence of the mesoporous structure and the hysteresis loops between relative pressure of 0.88 and 0.99 is type H3, suggesting the interconnected porous structure (Figure 2.15 A). The pore size of MSN-AM was calculated from BJH method.
The major pore size distribution is at around 2-3 nm and the textural pore size can also be seen at 37 nm from the pores of inter-particles due to the packing of nanoparticles under dry condition (Figure 2.15 B). The SAXS pattern (Figure 2.15 C) indicates that the mesopores in MSN-AM are isotropic at any directions.

2.8 Theory behind the relationship between experimental conditions and the final morphology of MSNs

Core chemicals i.e., surfactant templates, silica source and the catalysts are the main factors in the synthesis of MSNs, which will be affected by other experimental conditions such as concentration, temperature, pH value and ionic strength. Several representative examples of the synthesised MSNs are shown in Figure 2.16. To explain the relationship between experimental conditions and the morphology of MSNs, several theories are applied.

Firstly, the formation of pores is associated with the behaviour of surfactants in aqueous solution. The final mesoporous structure of MSNs is dependent on the concentration of the surfactant template. The CTAB concentration affects the geometry packing performance of the micelles in the aqueous solution, leading to different meso-phases of MSNs (bicontinuous, hexagonal or cubic) (Wu, Mou & Lin 2013). In this study, the CTAB concentration over 60 mM led to the bicontinuous and interconnected meso-structure, and the increase of CTAB concentration slightly increased the pore size with several angstroms.

Secondly, the formation of silica framework is a sol-gel process in which the hydrolysis and condensation of TEOS play an important role in forming nanoparticles in terms of the shape and size. The hydrolysis and condensation have to be finely controlled to form uniform spherical nanoparticles. In this study, the rate of hydrolysis and condensation was controlled by adjusting the pH value. The super-branched morphology of MSNs can be seen when the pH value is up to 12. This is because the increase of pH leads to the high condensation between silica monomers (Brinker 1988), which in turn accelerates the reaction between particles rather than the growth of single particle.
Last but not least, the ionic strength plays an important role in the stabilization of the silica colloid. As the nanoparticles are silica based, the surface of MSNs is negatively charged, which stabilises MSNs with repulsion between adjacent particles. Whereas, the high ionic strength screened the charges between particles, resulting in aggregation. In this study, the well-dispersed MSNs were obtained in ammonia solution that has an ionic strength as low as 0.0005 mol/L\cdot electron^2 compared to the phosphate buffer solution (>0.1 mol/L\cdot electron^2). Due to the low ionic strength of the solution, the aggregation of particles is minimized and the particles grow independently.

![Diagram](image_url)

Figure 2.16 Summary of the synthesised MSNs under different experimental conditions with CTAB as template and TEOS as silica source.
Chapter 3: Growth Kinetics and Dynamics of MSNs

Highlights of the Contribution:
- Time-resolved SAXS with a synchrotron source was employed to study the growth mechanism of MSNs.
- The growth kinetics of MSNs was investigated through the SAXS invariant. The method was proved to be efficient and powerful to estimate the growth kinetics.
- The growth of MSNs was found to be in TEOS emulsion system.
- A ‘swelling-shrinking’ model is proposed to explain the MSN growth based on the investigation of structural evolution under time-resolved SAXS.

3.1 Introduction

Mesoporous silica nanoparticles (MSNs) have been widely applied as carriers of molecules in targeted and controlled drug delivery systems (Tang, Li & Chen 2012; Vivero-Escoto et al. 2010) and as porous supports of catalyst in high performance catalysis (Meynen, Cool & Vansant 2009). A variety of sol-gel process have been widely used to prepare mesostructured silica (Wu, Mou & Lin 2013). The components to synthesise MSNs contain surfactant templates, silica source and acid or basic catalysts. Through controlling the experimental conditions, MSNs with different porous structure and particle size can be fabricated (Trewyn et al. 2007; Wu, Mou & Lin 2013). The mechanism behind the formation of MSNs is related to the micellar structure formed by surfactant in the aqueous solution, the hydrolysis and condensation of silica source and the interaction between micelles and silica source. Understanding the mechanism of MSN formation is beneficial for reproducing the current materials and also helps to create new structures.

So far, the development of MSN based delivery system has entered new stage, including a focus on the fabrication of MSNs with particle size of less than 30 nm, which allows for improved transportation through cell membrane and rapid degradation within cells (Chiang et al. 2011; Ma, Sai & Wiesner 2012; Urata et al.
Even though these sub-30 nm MSNs have been successfully fabricated, their formation mechanism remains unknown. The existing explanation of MSN growth is mainly on MSNs of large particle size (>100 nm) and ordered porous structure (hexagonal and cubic) (Blin & Imperor-Clerc 2013; Flodström, Wennerström & Alfredsson 2003; Gao et al. 2006; Suteewong et al. 2013). Specifically, hydrolysed silica source either electrostatically assembles on to cationic micelles and is condensed to form particles (Tolbert 2012), or initially forms silica polymer and binds onto non-ionic micelles (Sundblom et al. 2009). Several factors need to be taken into account in order to explore the growing mechanism. When the diameter of MSNs goes below 30 nm, the aggregation of nanoparticles and non-ordered pore structures bring extra difficulties to interpret the mechanism. Because of these challenges, a high resolution and real time investigation of MSN growth is required.

The existing techniques to study the formation mechanism of MSNs include high resolution transmission electron microscopy (Tolbert 2012), nuclear magnetic resonance (Flodström, Wennerström & Alfredsson 2003) and small angle scattering (Zholobenko et al. 2008). When investigating the growth mechanism of small MSNs (diameter less than 30 nm), small angle scattering is more advantageous than others. Because the scattering source (neutrons and electrons) is at atomic level that is much smaller than surfactant micelles and MSNs, it can be more easily used to detect nano features. Furthermore, with the synchrotron source, fast data acquisition can achieve time resolved examination of the growth of MSNs with high resolution, which provides information about the state of the reaction during a short time interval. Recent studies reported the mechanism of MSN growth through time resolved small angle neutron scattering (SANS) (Hollamby et al. 2011; Manet et al. 2011). Even though the unique contrast variation provided by SANS can separate the scattering contribution from silica and surfactant phase, deuterated surfactants did not behave as normal during the synthesis of MSNs (Hollamby et al. 2011). In this case, small angle X-ray scattering (SAXS) as another scattering technique possesses similar online ability and can reveal the synthesis of MSNs under real condition but does not require special treatment of reaction ingredients (Kirby et al. 2013). Therefore, time resolved SAXS brings benefits for precise detection of the formation of MSNs at different growing state.

The existing understanding of MSN growth mechanism stated that the hydrolysed
TEOS forms silica monomer adsorbed on the surface of the surfactant micelles and then the condensation of silica monomer induces the aggregation of micelles to form particles (Blin & Imperor-Clerc 2013; Gao et al. 2006; Suteewong et al. 2013). A few models, like ‘current bun’ model (Hollamby et al. 2011), have also been developed to describe the mechanism. However, the detailed location of TEOS has not been mentioned in these studies, which is the important information to deeply understand the mechanism. Herein, growth mechanism of MSNs with small diameter and non-ordered pore structures is dynamically investigated through time dependent SAXS with a synchrotron source at Australia Synchrotron. The synthesis of MSNs was conducted by using cetyltrimethylammonium bromide as the surfactant template, tetraethyl orthosilicate as the silica precursor and phosphate buffer solution or ammonium hydroxide as the reaction catalyst. The structural changes of micelles before and after the addition of TEOS are discussed in detail to understand the relationship between the hydrodynamic structure of surfactant and the structure of final pores in MSNs. Scattering invariant as a simple but powerful tool and was introduced to describe the growth kinetics of the particles. The effects of experimental conditions on particle growth kinetics were also investigated. The formation mechanism of MSNs was proposed based on the results. This mechanism provides a systematic view of the formation of MSNs, which will benefit the future work on controlling the features of MSNs and designing novel meso-structure.

3.2 Materials, Experimental and Methodology

3.2.1 Materials

The materials used in this work are listed in Appendix A.

3.2.2 Synthesis procedure of mesoporous silica nanoparticles

All the experimental parameters were listed in Table 3.1. The MSNs were synthesised by using CTAB as templates to generate silica mesoporous structure as described in Chapter 2. The aim of selecting the following experimental procedure is to investigate the growth kinetics of MSNs under similar conditions as those described in the
previous chapter viz. different temperature, pH and ionic strength and to find out the mechanism of MSN formation.

### Table 3.1 Experimental parameters for each dynamic setup

<table>
<thead>
<tr>
<th>Samples</th>
<th>CTAB (mM)</th>
<th>TEOS (mM)</th>
<th>Temperature (°C)</th>
<th>pH</th>
<th>Final Completion Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30-7.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>80</td>
<td>80</td>
<td>30</td>
<td>7.2</td>
<td>150</td>
</tr>
<tr>
<td>95-7.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>80</td>
<td>80</td>
<td>95</td>
<td>7.2</td>
<td>57</td>
</tr>
<tr>
<td>30-10-H&lt;sup&gt;b&lt;/sup&gt;</td>
<td>80</td>
<td>80</td>
<td>30</td>
<td>10</td>
<td>150</td>
</tr>
<tr>
<td>30-10-L&lt;sup&gt;c&lt;/sup&gt;</td>
<td>80</td>
<td>80</td>
<td>30</td>
<td>10</td>
<td>150</td>
</tr>
</tbody>
</table>

<sup>a</sup> These two samples are selected to investigate the effect of temperature on MSN growth kinetics.

<sup>b</sup> This sample is synthesised in buffer solution with NaOH to adjust the pH to 10, which is selected to examine the effects of high ionic strength.

<sup>c</sup> This sample is synthesised in ammonium solution with pH of 10. Without adding any salt, the ionic strength is very low, which is selected to be a comparison with the samples synthesised in buffer solution.

### 3.2.3 Setup of time-resolved SAXS by a synchrotron source

SAXS experiments were conducted in a synchrotron source at Australian Synchrotron. A Pilatus 1M detector was used for SAXS data collection. The sample-detector distance was 1.6 m, and the energy was adjusted to 9 keV to cover a Q range of 0.008-0.2 Å<sup>-1</sup>. The Q range calibration was carried out using a silver behenate sample.

The scattering intensity was calibrated into absolute intensity. For each experiment, the empty capillary tubes and the capillary tubes filled with the Milli-Q water were recorded at different exposure time (1 s, 2 s, 5 s and 10 s) before adding CTAB and TEOS. Then the absolute calibration for each samples was done in the software of Scatterbrain provided by SAXS beamline of Australian Synchrotron.

The dynamic investigation of MSN growth was carried out with all the equipment inside SAXS hatch. The schematic of the setup is shown in Figure 3.1. Specifically, MSNs were synthesised in a 200 mL beaker in a water bath that is to control the
temperature of the reaction mixture. During the fabrication of MSNs, the mixture solution was circulated through a peristaltic pump with a flow rate of 0.5 mL/s between the reaction reservoir and capillary tubes. The length of pipes was 2.8 m and the diameter was 2.5 mm. By considering the pipe length, diameter and flow rate, the mixing time is within 30 s. SAXS patterns were collected from the quartz capillary tubes for 2 s every 28 s for 150 min (300 patterns) in order to match the circulating time and to obtain best resolution. Because the reaction of the sample at 95 °C quickly reached the stable state, data collection for this sample was stopped within 1 h (114 patterns). The detailed setup for time resolved experiments was described elsewhere (Hollamby et al. 2011; Michaux et al. 2012; Zholobenko et al. 2008).

![SAXS setup](image.png)

Figure 3.1 SAXS setup to dynamically monitor the growth of MSNs under different experimental conditions. The temperature of the reservoir for fabricating MSNs was controlled by a water bath with magnetic stirrer. The circulated reaction liquid came from the bottom of capillary tubes to the top in order to eliminate any influence of bubbles generated by the influent fluidic flow.

### 3.2.4 Modelling of CTAB micelles

The SAXS data of CTAB micelle in different solution was modelled in the software of “SASview” via least square fit. The total intensity \( I(Q) \) can be interpreted by the
following equation:

\[ I(Q) = P(Q) \otimes S(Q) + \text{background} \]  

where, \( P(Q) \) is the form factor related to the shape of the micelles in the solution and \( S(Q) \) is the structure factor associated with the interaction between micelles. These two factors will be incorporated to model the SAXS data of CTAB micelles before and after TEOS addition.

3.2.3.1 Modelling CTAB micelles before TEOS addition

The CTAB micelles tends to form ellipsoid shape in aqueous solution and the hydrophilic head groups are considered as a shell around the core formed by the hydrophobic tails. Thus ellipsoid core-shell model with fixable shell thickness were used to fit the SAXS data of CTAB micelles in the aqueous solution, which has been proved to be ideal to fit the SAXS data of CTAB under different experimental conditions in this study. The ellipsoid core-shell form factor was given by (Berr 1987; Kotlarchyk & Chen 1983)

\[ P(Q) = \frac{\text{scale}}{V} \int_{0}^{1} |F(Q,r_{\text{min}},r_{\text{max}},\alpha)|^2 d\alpha + \text{background} \]  

where, \( \text{scale} \) is the scale factor, \( V \) is the volume of the ellipse that can be calculated by \( V = \frac{4}{3} \pi r_{\text{maj}}^2 r_{\text{min}} \), \( r_{\text{min}} \) and \( r_{\text{maj}} \) are the minor and major radius of the ellipse respectively and \( \alpha \) is the angle between the major axis and the Q vector.

The interaction between neighbouring CTAB micelles is an electrostatic repulsion because of the charges on the surface of the micelles. This interaction leads to the presence of \( S(Q) \) that can be calculated from a model of rescaled mean spherical approximation (RMSA) reported by Hayter and Penfold (Hansen & Hayter 1982; Hayter & Penfold 1981). This model is based on a screened electrostatics between charged particles in the presence of counterions in the solution, and defined by the following parameters: the volume fraction of the hard sphere, the micellar charges, the radius of particles and the Debye length.

The modelling was conducted through a non-constrained method (Griffiths et al. 2004). The assumption of the structure is required to reach the real physical properties of
micellar structure. Firstly, the core of the micelles is considered to be formed by hydrocarbon tails of CTAB without any water penetration. Thus, the scattering length density (SLD) of the core is constrained for this oil phase. Also, the SLD of water is constrained after considering the effects from added salt. Secondly, the physical properties of the system can be constrained. These parameters include the dielectric constant of water, system temperature and volume fraction of CTAB. All the input parameters are listed in Table 3.2. The rest parameters including the ellipticity of CTAB micelles, shell thickness, charge on the surface of micelles and ionic strength of the solution are calculated during the modelling.

### Table 3.2 Input parameters for the modelling of CTAB micelles before TEOS addition

<table>
<thead>
<tr>
<th>Samples</th>
<th>Dielectric constant(^a)</th>
<th>Temperature (K)</th>
<th>SLD of core ((×10^{-6} \text{ Å}))</th>
<th>SLD of solvent including salt contribution ((×10^{-6} \text{ Å}))</th>
<th>Volume fraction of core(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30-7.2</td>
<td>76.55</td>
<td>303</td>
<td>7.79</td>
<td>9.44</td>
<td>0.0216</td>
</tr>
<tr>
<td>95-7.2</td>
<td>57.01</td>
<td>368</td>
<td>7.79</td>
<td>9.44</td>
<td>0.0216</td>
</tr>
<tr>
<td>30-10-H</td>
<td>76.55</td>
<td>303</td>
<td>7.79</td>
<td>9.44</td>
<td>0.0216</td>
</tr>
<tr>
<td>30-10-L</td>
<td>76.55</td>
<td>303</td>
<td>7.79</td>
<td>9.45</td>
<td>0.0216</td>
</tr>
</tbody>
</table>

\(^{a}\) The dielectric constant of water at different temperature was calculated from the equation reported by Malmberg and Maryott (Malmberg & Maryott 1956).

\(^{b}\) The core contains only the hydrocarbon tails of CTAB.

#### 3.2.3.2 Modelling CTAB micelles after TEOS addition

After the addition of TEOS, a core-shell sphere model was chosen to fit the SAXS data, which is given by (Guinier & Fournet 1955)

\[
P(Q) = \frac{\text{scale}}{V_s} (F_{\text{core}} + F_{\text{shel}})^2 + \text{background} \tag{3}
\]

\[
F_{\text{core}} = 3V_c(\rho_c - \rho_s) \left[\frac{\sin(qr_c) - qr_c \cos(qr_c)}{(qr_c)^3}\right] \tag{4}
\]

\[
F_{\text{shel}} = 3V_s(\rho_s - \rho_{\text{solv}}) \left[\frac{\sin(qr_s) - qr_s \cos(qr_s)}{(qr_s)^3}\right] \tag{5}
\]

where, \(\text{scale}\) is the scale factor, \(V_c\) and \(V_s\) are the volume of the core and shell, \(r_c\) and \(r_s\) are the radius of the core and shell, and \(\rho_c, \rho_s\), and \(\rho_{\text{solv}}\) are the scattering length density...
of the core, shell and the solvent.

The added TEOS forms an oil phase that tends to go inside the micelle core due to the hydrophobicity. Accordingly, the SLD of the core should change and can be calculated from the ratio between CTAB and TEOS. At the initial stage, the silica covered on the surface of the CTAB micelles is not enough to change the structure factor, thus Hayter-Penfold RMSA can still be used as $S(Q)$ to interpret the interaction between micelles. The parameters used in this model are listed in Table 3.3.

Table 3.3 Input parameters for the modelling of CTAB micelles after TEOS addition

<table>
<thead>
<tr>
<th>Samples</th>
<th>Dielectric constant $^a$</th>
<th>Temperature (K)</th>
<th>SLD of core ($\times 10^{-6}$ Å)</th>
<th>SLD of solvent including salt contribution ($\times 10^{-6}$ Å)</th>
<th>Volume fraction of core</th>
</tr>
</thead>
<tbody>
<tr>
<td>30-7.2</td>
<td>76.55</td>
<td>303</td>
<td>7.91</td>
<td>9.44</td>
<td>0.0383</td>
</tr>
<tr>
<td>95-7.2</td>
<td>57.01</td>
<td>368</td>
<td>7.91</td>
<td>9.44</td>
<td>0.0383</td>
</tr>
<tr>
<td>30-10-H</td>
<td>76.55</td>
<td>303</td>
<td>7.91</td>
<td>9.44</td>
<td>0.0383</td>
</tr>
<tr>
<td>30-10-L</td>
<td>76.55</td>
<td>303</td>
<td>7.91</td>
<td>9.45</td>
<td>0.0383</td>
</tr>
</tbody>
</table>

$^a$ The dielectric constant of water at different temperature was calculated from the equation reported by Malmberg and Maryott (Malmberg & Maryott 1956).

$^b$ The core is a mixture consisting of the hydrocarbon tails of CTAB and TEOS.

### 3.2.5 Calculation of invariant over time

Compared to the complicated model fitting, the invariant as a model independent parameter was easy to calculate, thus it was taken to indicate the solid silica growth from the liquid TEOS. The invariant is given by (Kwon et al. 2014; McDermott et al. 2014)

$$\text{Invariant} = \int I(Q)Q^2 dQ = 2\pi^2 \varphi (1 - \varphi)(\Delta \rho)^2$$

where, the integral starts from $Q=0$ and extends to the end of SAXS region, $V$ is the sample volume, $\Delta \rho$ is the SLD difference between two phases and $\varphi_1$ and $\varphi_2$ are the volume fractions of two phases respectively. The MSN growth system was firstly assumed to be a two-phase system containing silica phase and liquid phase (solvent and micelle core). Because the SLD difference ($\Delta \rho$) between silica and solvent and between silica and micelle core are $1.32 \times 10^{-5}$ and $1.35 \times 10^{-5}$ Å$^2$ respectively.
(SLD(silica) = 2.27 × 10^{-5} Å^{-2}, SLD(CTAB) = 9.21 × 10^{-6} Å^{-2}, SLD(solvent) = 9.94 × 10^{-6} Å^{-2}), which does not significantly affect the invariant. Correspondingly, \( \varphi_1 \) and \( \varphi_2 \) are the volume fractions of silica and liquid phase. Secondly, the data at low Q and high Q was extrapolated according to Guinier function and Power law respectively. (Guinier & Fournet 1955) The integrated Q range was chosen between 10^{-5} to 10^{1} Å^{-1}, which was shown to be large enough to get a constant intensity at lower Q’s and a flat background at higher Q’s. Finally, because the SLD of silica is much higher than that of the CTAB micelles and solvent, the interface between micelles and silica and between solvent and silica is assumed as a sharp interface, where the scattering intensity decreases as Q^{-4} and the Porod law can be applied to calculate the invariant.

All the invariants of SAXS data were calculated by the software ‘SASview’ from selected scattering patterns at characteristic time points. The contribution of the invariant from SAXS data is all over 95 % for all the calculated invariants in order to minimize the influence on the invariant from the extrapolated contribution at low and high Q.

### 3.2.6 Further treatment and characterization of MSN samples

The CTAB templates inside pores were removed by soaking all the samples in a solution of 100 mL of ethanol and 1 mL of hydrochloric acid at 60 °C for 12 h. Subsequently, all samples without CTAB were centrifuged and washed with ethanol and dried under vacuum. The morphology of fabricated nanoparticles was characterized under a JEM 2100 transmission electron microscope (TEM) (JEOL, Japan) with the acceleration voltage of 200 kV. All samples were dispersed in ethanol and dropped onto a copper grid with carbon film.

### 3.3 Overview of the SAXS pattern changes over time

The growth of MSNs was monitored in situ by time-resolved SAXS with a synchrotron source. The hydrolysis and condensation rate of TEOS varied from the experimental conditions, thus different growth kinetics can be seen from 3D plots (Figure 3.2). There are several structural changes that can be noted with time in the time-resolved graphs. Firstly, the decrease of the peak contributed by CTAB micelles can be seen at around
0.12 Å⁻¹. At longer time (> 100 min), the peak from CTAB micelles disappeared. Secondly, the presence of bumps at low Q is due to the growth of particles, and as the particles grow bigger, the bumps become clear. Interestingly, the intensity of the shoulder along with the bump from the particles increases, which may be due to the interference between particles caused by aggregation. Thirdly, the increased intensity of diffraction peaks at 0.15 Å⁻¹ corresponding to a real space dimension of 4.1 nm is attributed to the formation of mesopores. Finally, all the scattering patterns reach plateau after a certain time, which means that the fully grown MSNs have been achieved. Similar trends were detected for all four samples. But the time when the particle growth reaches the completion varies from the experimental conditions, which follows the sequence of 95-7.2 (shortest), 30-10-H, 30-7.2 and 30-10-L (longest).

Figure 3.2 3D graphs of dynamic monitoring of MSN growth with time.
3.4 Growth kinetics of MSNs

The growth of MSNs is associated with two reactions, namely hydrolysis of TEOS and condensation of silica monomers. Because the hydrolysis and condensation always take place at the same time (Brinker 1988), it is very difficult to isolate these two processes. A single model could not fit the whole process. Therefore, the size of MSNs and pores were not directly modelled, but the invariant as a model independent parameter was introduced to examine the growth kinetics.

Figure 3.3 The calculated invariant versus time. The invariant was calculated from SAXS patterns obtained from the time-resolved SAXS experiments at different time points.

The invariant changes over time for these samples is displayed in Figure 3.3. The trend of the sharp increase of the invariant followed by a plateaus is similar in all the samples. Because the added amount of TEOS is constant (80 mM), the plateaus has similar altitude. But the sample 30-7.2 is an exception. The invariant keeps going up after 133 min. This is due to the aggregation of the already grown particles. It can be proven by comparing the difference between the SAXS data after 133 min. Figure 3.4 shows the SAXS data of sample 30-7.2 at 145 min and 150 min, and SAXS data of samples 95-
7.2, 30-10-H and 30-10-L at the time point with similar invariant. It can be seen from Figure 3.4 A that the difference is distributed in the whole Q range. The curve shape does not change at 150 min compared to 145 min, and only the intensity increases. At 145 min, the silica particles have been produced and they start to aggregate. The aggregation provide more scattering than the single separate particles, leading to the increase of the intensity without changing scattering patterns. By contrast, the difference from samples 95-7.2, 30-10-H and 30-10-L is much smaller (Figure 3.4 B, C and D). It is important to understand the effects of aggregation on the invariant because the aggregation is not related to the particle or pore growth.

Figure 3.4 Selected time points with similar invariant: (A) 30-7.2, (B) 95-7.2, (C) 30-10-H and (D) 30-10-L. The difference between two curves were calculated by subtract the curve of early time from that of late time.

Based on the understanding of invariant and aggregation effects, the growth kinetics was calculated from the invariant change over time. The structural development of MSNs is associated with the nucleation and particle growth, and the process is related to a phase transformation from liquid (TEOS) to solid (silica), thus Avrami equation can be used to model the growth kinetics of MSNs (Chae, Shi & Huang 2013; Tobler, Shaw & Benning 2009). The relation between the reaction rate and time is expressed
in the following equation,

\[ \alpha = 1 - e^{-(kt)^n} \]  \hspace{1cm} (7)

where, \( \alpha \) is the fraction of invariant (the invariant at time \( t \)/invariant at the end), \( t \) is the reaction time, \( k \) is the reaction constant and \( n \) is a constant related to the growth mechanism and dimensions. Taking a mathematic transformation in Equation (7), it is given

\[ \ln[-\ln(1-\alpha)] = \ln(k) + n \ln t \]  \hspace{1cm} (8)

The reaction related constants \((k \text{ and } n)\) can be calculated from the intercept and slope of the linear fit in a \( \ln[-\ln(1-\alpha)] \) vs \( \ln t \) plot that is shown in Figure 3.5. All the curves have a good coefficient with the linear fitting. The invariants related to the aggregation in sample 30-7.2 were removed to eliminate their effects on the calculated reaction constant.

Figure 3.5 Avrami plot and the corresponding linear fit for each sample. Both axis are taken with the natural logarithm to meet Avrami equation.
Chapter 3: Growth Mechanism of MSNs

The calculated results from linear fitting have been listed in Table 3.4. As can be seen, the reaction constant of the sample 30-7.2 is the lowest at $1.53 \times 10^{-5}$, which is because of the slow hydrolysis and condensation rate of silica source at nearly neutral pH (7.2). The high temperature (95 °C) provides the fastest reaction rate ($k=0.42$), even though the reaction happened at nearly neutral pH. As temperature increases, the molecules move faster and the contacting frequency between them increases, leading to the dramatic rise of reaction rate. In addition, the reaction constants in samples 30-10-H and 30-10-L are at a similar magnitude. This is because the hydrolysis and condensation of TEOS are at the similar pace as the temperature and pH for these two samples are the same. In summary, the MSN growth rate is dependent on the reaction temperature and pH value. Once these parameters are determined, the reaction constant will stay at the same order of magnitude. Higher temperature and pH value leads to fast growth rate. These results appeal a good agreement with the existing work (Blin & Imperor-Clerc 2013; Hollamby et al. 2011), and the invariant method offers a more efficient manner compared to the complicated model fitting.

<table>
<thead>
<tr>
<th>Samples</th>
<th>$n$</th>
<th>$k$</th>
</tr>
</thead>
<tbody>
<tr>
<td>30-7.2</td>
<td>2.47</td>
<td>$1.53 \times 10^{-5}$</td>
</tr>
<tr>
<td>95-7.2</td>
<td>0.73</td>
<td>0.42</td>
</tr>
<tr>
<td>30-10-H</td>
<td>1.02</td>
<td>0.023</td>
</tr>
<tr>
<td>30-10-L</td>
<td>0.73</td>
<td>0.028</td>
</tr>
</tbody>
</table>

Note: The values of $n$ and $k$ are obtained from the slope and intercept respectively in the linear fitting of Avrami plot.

3.5 Mechanism exploration - swelling of CTAB micelles after TEOS addition

To investigate the structural changes of CTAB micelles, the SAXS data was collected before and after TEOS addition. The SAXS data of CTAB micelles was collected after the stabilization of CTAB solution at 80 °C for 30 min. After the addition of TEOS, the first time point in the monitoring of MSN growth was picked up, under which condition the hydrolysis and condensation of TEOS have not greatly affected the scattering. All these data are shown in Figure 3.6.
Before the addition of TEOS, the CTAB structure depends on ionic strength of the reaction solution (the left column of Figure 3.6). In the phosphate buffer solution with similar ionic strength for the samples 30-7.2, 95-7.2 and 30-10-H, the SAXS patterns display a similar peak at around 0.12 Å⁻¹. In contrary, as the ionic strength is very low in ammonia solution, a different scattering pattern is shown, which means that the packing structure of CTAB micelles in non-salt solution is different from that in salt solution. The counterions are not only from bromide, but also from the added salt. Another reason that leads to the different scattering pattern is the electrostatic screen from the added salt, causing a structure factor. In addition, the size, ellipticity and shell thickness of CTAB micelles were obtained from the modelling (Table 3.5). Temperature determines the core radius and ellipticity. It can be seen that even though the scattering patterns are different between the samples with and without salt, at 30 ºC, the major radius of the core and the ellipticity remain the same as 25 Å and 0.55.

High temperature (95 ºC) leads to the decrease of micelle size but increase the curvature of the micelles. Similar to the shape of the scattering pattern, the shell thickness of micelles is 12 Å in the phosphate buffer solution, while it is 10 Å in ammonia solution, which is the evidence of the absorption of counterions from the added salt.

Table 3.5 Summary of CTAB structure parameters calculated from the fitting before and after TEOS addition

<table>
<thead>
<tr>
<th>Samples</th>
<th>Major radius of core (Å)</th>
<th>Axial ratio</th>
<th>Shell thickness (Å)</th>
<th>Polydispersity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before TEOS addition</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30-7.2</td>
<td>25.3</td>
<td>0.55</td>
<td>12.4</td>
<td>0</td>
</tr>
<tr>
<td>95-7.2</td>
<td>23.3</td>
<td>0.62</td>
<td>12.6</td>
<td>0</td>
</tr>
<tr>
<td>30-10-H</td>
<td>25.4</td>
<td>0.55</td>
<td>12.6</td>
<td>0</td>
</tr>
<tr>
<td>30-10-L</td>
<td>25.6</td>
<td>0.57</td>
<td>10.0</td>
<td>0</td>
</tr>
<tr>
<td>After TEOS addition</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30-7.2</td>
<td>27.6</td>
<td>1</td>
<td>8.8</td>
<td>0.1ᵃ</td>
</tr>
<tr>
<td>95-7.2</td>
<td>22.9</td>
<td>1</td>
<td>10.4</td>
<td>0.1ᵃ</td>
</tr>
<tr>
<td>30-10-H</td>
<td>27.4</td>
<td>1</td>
<td>9.1</td>
<td>0.1ᵃ</td>
</tr>
<tr>
<td>30-10-L</td>
<td>23.4</td>
<td>1</td>
<td>12.6</td>
<td>0.1ᵃ</td>
</tr>
</tbody>
</table>

ᵃ Radius polydispersity.
The addition of TEOS has an impact on the structure of CTAB micelles. The peaks for all the samples shifted to a low Q with the increase in intensity (the merged graphs in Figure 3.6). The TEOS inserts into the hydrophobic core of micelles and expands the micelles. Also, a small peak presents at 0.22 Å⁻¹ after TEOS addition, which indicates that the change of the micellar shape from ellipse to sphere according to the model for these two shapes calculated by the software. Based on the above assumption, the SAXS data was fitted by an $F(Q)$ sphere core-shell model with an $S(Q)$ of Hayter-Penfold RMSA. Due to the insertion of the TEOS in the hydrophobic core of micelles, the SLD and the volume fraction of the CTAB core were recalculated as per the amount of added TEOS. The input parameters and the best fit are shown in Table 3.3 and in the middle column of Figure 3.6, respectively. The sphere core-shell model worked
well for all the samples with the addition of TEOS. A polydispersity of the radius was introduced to obtain the best fit, because of the non-homogeneous insertion of TEOS in micelles. This is because the space of CTAB cores are not fully inserted by TEOS, as the molecular volume of added TEOS (1.7 mL) is less than the volume of CTAB cores (2.2 mL calculated from the literature (Tanford 1974)). The fitted parameters from the model are shown in Table 3.5. The radii are all over the critical chain length of hydrocarbon core (21.8 Å (Tanford 1974)). The CTAB micelles were actually swelled by the addition of TEOS.

3.6 Proposed growth mechanism – from the inside of micelles to outside

Figure 3.7 The schematic of the structural changes of CTAB micelles before and after TEOS addition. The red dots represent ethyl groups in TEOS molecules and ethanol in aqueous solution after the hydrolysis of TEOS.

The existing studies on the mechanism of MSN growth illustrate that silica monomers absorb onto micelles and then the condensation of silica monomer induces the aggregation of micelles to form particles (Blin & Imperor-Clerc 2013; Gao et al. 2006; Suteewong et al. 2013). However, an important information about the location of TEOS is always missing in the previous studies. The synthesis of MSNs is always conducted in aqueous solution but the TEOS is an oil-like monomer. According to our experiences, the phase separation was observed in CTAB solution under static
conditions (without stirring), while under vigorous stirring, a turbid uniform system was formed without phase separation. The role of TEOS has not been well investigated. Based on the existing theory of MSN growth, we proposed a new mechanism to describe the growth of MSNs, which is shown in Figure 3.7. The detailed description is as follows:

- **Before the addition of TEOS**, the ellipsoid CTAB micelles are formed in the aqueous solution under the amphiphilic force (Step (1) in Figure 3.7). The micelles are positively charged due to the head groups of quaternary ammonium salt in CTAB molecules. The core of CTAB micelles is formed by hydrocarbon tails that are hydrophobic.

- **After the addition of TEOS**, the oil like TEOS inserts into the hydrophobic core of CTAB micelles, leading to the swelling of micelles (Step (2) in Figure 3.7). The size of the micelles is enlarged by the added TEOS and the shape becomes spherical. Moreover, the difference of TEOS content in each micelle leads to a polydispersity in micelle size.

- **With the hydrolysis of TEOS**, the silica monomers become hydrophilic and are released into the aqueous solution (Step (3) in Figure 3.7). Because the monomers are negatively charged, they absorb onto positively charged CTAB micelles under electrostatic force. With the consumption of TEOS in CTAB core, the micelles shrink and the size becomes smaller. As another product of hydrolysis, ethanol is also present in the solution.

- **Actually**, the hydrolysis and condensation of silica happen at the same time (Brinker 1988). Silica monomers are gradually hydrolysed and released from the core of micelles and form a silica shell around micelles (Step (4) in Figure 3.7). During this process, the micelles continue to shrink until all the TEOS in the core is hydrolysed. The decreased micelle size can be proved by the size at the beginning of TEOS addition (5.4 nm in diameter) and at the final stage (4.1 nm in diameter). The neighbouring micelles with silica shell starts to aggregate, which produces the nuclei for the particle growth.

- **Finally**, with the growth of nuclei and continuous hydrolysis and condensation, silica forms strong framework, and particles and mesopores form.

In summary, the growth of MSN starts from swollen micelles and is followed by the continuous shrinking of micelles, which provides a new insight into the growth mechanism of MSNs.
Chapter 4: Translocation of MSNs inside Plants through Fluorescent Labelling

Highlights of Contributions:

- The FITC and RITC were chemically bonded onto MSNs in order to track the location of MSNs in plants under a fluorescent microscope.
- The RITC labelled MSNs possess an excitation wavelength at 560 nm that is distinguished with the auto-fluorescence from the endogenous agents in plants. The RITC labelled MSNs are an alternative way to avoid the interference of the auto-fluorescence from plants.
- The transport mechanism was proposed which will provide very useful fundamental knowledge for the further study.

4.1 Introduction

Various inorganic nanoparticles have been introduced, such as metallic or metal oxide nanoparticles (silver, copper, aluminium, iron oxide and titanium oxide), carbon based nanomaterials (carbon nanotubes and graphene) and silica nanoparticles (Begum, Ikhtiari & Fugetsu 2011; Chen et al. 2013; Corredor et al. 2009; Doshi et al. 2008; Kunzmann et al. 2011; Panácek et al. 2009; Sim & Wallis 2011; Soenen et al. 2013; Su et al. 2007) to study their translocation of nanoparticles inside plants. The phytotoxicity and the translocation mechanism of these nanoparticles in plants has been increasingly dealt with and require further investigation.

Mesoporous silica nanoparticles (MSNs) can potentially be an ideal carrier to deliver molecules into plants, as it has been proven that they are safe and effective to deliver drugs in animal system (Barkalina et al. 2014; Li et al. 2011; Lu et al. ; Vivero-Escoto, Slowing & Lin 2010; Wang et al. 2013; Wang et al. 2012). MSNs possess such properties as the high surface area, high porosity, low toxicity to cells and stable chemical and physical performance. However, delivering agrochemical into plants with MSNs has been seldom reported (Chang et al. 2013; Torney et al. 2007). Because MSNs are a new material to plant science, the uptake and translocation of MSNs for plants remains unknown.
The existing techniques to track nanoparticles within plants include transmission electron microscope (TEM) and fluorescent labelling. The TEM was widely used to visualize metallic or metal oxide nanoparticles in plants. The iron nanoparticles coated with carbon were synthesised to investigate their penetration through living pumpkin plant (Corredor et al. 2009). The accumulation of carbon coated iron nanoparticles in xylem were observed under TEM, which provided a clear view in the sub-cellular sites. TEM can provide high resolution images of plant cells containing nanoparticles. The drawback of using TEM as the visualizing method is the long and complex preparation process. Plant samples are required to be fixed in the resin and stained by the highly hazardous chemicals (uranyl acetate and lead citrate) (Taylor et al. 2014). In contrast, the detection of fluorescence from nanoparticles is simpler and easier to achieve under fluorescent microscope and confocal laser scanning microscope though the resolution of fluorescent images is much lower than TEM, and the potential auto-fluorescence from plant endogenous biomolecules in plants (Held, Boulafous & Brandizzi 2008; Sun et al. 2014) may disturb the identification of the fluorescence from the nanoparticles. Therefore, in order to investigate the transportation mechanism of MSNs in plants, the combination of multiple detection methods allows for accurate and comprehensive location information of nanoparticles in plants.

In this chapter, two fluorescent dyes with different excitation wavelengths were used to label MSNs to distinguish the auto-fluorescence from plant tissues. Firstly, the dispersity of MSNs will be optimized through controlling the ionic strength of the aqueous solution. The well dispersed MSNs with the particle size of approximately 20 nm were fabricated to ensure the good permeability through plant cell walls. Secondly, the interaction between nanoparticles and plants and the phytotoxicity of MSNs will be investigated, which is to confirm the safety of using MSNs in plants. Thirdly, the uptake and localization of MSNs will be investigated through fluorescent labelling of MSNs with fluorescein isothiocyanate (FITC) and rhodamine b isothiocyanate (RITC). The long distance transport, sub-cellular location and quantification of well-dispersed MSNs will be investigated in the commercial crop species via both apoplastic and symplastic pathways and distributed through plants through the xylem. Results show that well-dispersed MSNs can be taken up through root system, transported through stem and reach the leaves.
4.2 Experimental and Methodology

All the chemicals used in this section are listed in the Appendices.

4.2.1 Synthesis procedure of MSNs

**Synthesis of MSNs in phosphate buffer (pH=7.2).** The synthesis process of MSNs was based on others’ studies with modification as described in Section 2.2.2 and 2.2.3 in Chapter 2.

**Synthesis of MSNs in ammonium hydroxide.** The growth of MSNs was conducted at low temperature to form nanoparticles with small diameter and followed by an ageing process to stabilize the silicon-oxygen bonds in silica framework (Ma, Sai & Wiesner 2012). The detailed procedure has been stated in Section 2.2.2 and 2.2.3 in Chapter 2.

4.2.2 Surface modification and FITC labelling of MSNs

About 40 mg of MSNs without CTAB template were redispersed in 30 mL of absolute ethanol. Under vigorous stirring, 20 μL of APTES was added into this mixture and the mixture was stirred at room temperature for 1 hour. Four milligram of FITC was dissolved in 10 mL of absolute ethanol, which was added dropwise to the mixture of MSNs and APTES. Two hours later, 0.4 mL of deionized water was added into the mixture. The reaction was kept stirring in dark at room temperature for 24 hours. Afterwards, the FITC labelled MSNs (MSN-FITC) was centrifuged and washed with ethanol for 3 times in order to remove the free APTES and FITC. The final yellow particles were dried under vacuum and stored in the dark at room temperature.

For the fluorescent labelling with RITC, the procedure is the same as the above but FITC was replaced by RITC. This sample was denoted as MSN-RITC.
4.2.3 Characterization of MSNs

To investigate the morphology of MSNs, TEM and SEM were employed. The successful labelling of MSNs with FITC and RITC was determined by FTIR spectroscopy. The impact of FITC and RITC grafting on surface area and porosity was investigated from BET-BJH method. The CLSM was used to examine the translocation of MSNs within plants. The detailed characterisation methods were described in Appendix B. The fluorescent properties of samples were obtained from a Cary Eclipse Fluorescence Spectrophotometer (Agilent, US). MSN samples were redispersed in distilled water to make a concentration of 0.5 mg/mL and tested as a water suspension. A Nanosizer ZS (Malvern, UK) was used to measure the zeta potential of the suspension of MSN samples.

4.2.4 Seed germination and root elongation assay

The effect of solid silica nanoparticles (SNPs) and mesoporous silica nanoparticles (MSNs) on seed germination and root elongation of lupin (Naracoorte, South Australia) was examined according to the protocol of U.S. EPA OPPTS 850.4200 (Agency 1996). SNPs of 7 nm purchased from Sigma-Aldrich (Castle Hill, NSW, Australia) and laboratory synthesised MSNs (~20 nm in diameter) at densities of 0.2, 1.0 and 2.0 mg mL\(^{-1}\) in distilled water were used while distilled water was used alone as the control. Firstly, the seeds were tested for their viability by being immersed in deionized water, those that sank to the bottom were deemed viable and were then removed and soaked for 5 min in a surface sterilizing agent solution containing 45 % (v/v) sterile distilled water, 50 % (v/v) ethanol and 1.5 % (v/v) hydrogen peroxide. The seeds were then thoroughly rinsed three times with sterilized deionized water and placed into a 9-cm-diameter glass Petri dish on moistened filter paper. To a Petri dish containing the seeds, 6 mL of 7 nm SNPs and 20 nm MSNs suspensions in distilled water at the desired density or distilled water alone was added. The dishes were sealed with parafilm M\(^\oplus\) (Pecheney Plastics Packaging, Chicago, USA) and covered with aluminum foil, and placed in an incubator at 21 °C. After 5 days, the control and all treatment seeds were calculated by recording germination % and as the root emerged, root length (measured with a fine thread and a ruler). Statistical analyses were performed using a commercial software package (Version 11.5 software, SPSS Inc., Chicago, IL, USA). All data were
collected and analysed by one-way analysis of variance (ANOVA). Significant differences among means at $P < 0.05$ were determined by Duncan’s multiple range tests.

### 4.2.5 The uptake and localization of MSNs

Wheat, maize and lupin seeds were soaked in deionized water after surface sterilization and placed on moistened filter paper in Petri dishes for 1 week and then the germinated seeds were transferred into a soilless plant growth system (SPS) that consisted of two vertical plates that held the germinated seed at the top and allowed roots to grow vertically down between the plates on a sheet of filter paper moistened from a reservoir of nutrient solution (Total Horticultural Concentrate; Excel Distributors, Reservoir, Australia) in the base of a box in which the plates were placed. To treat the roots with MSNs the terminal 5 mm from the root tip of 14-day-old wheat seedlings was immersed in 0.5 mL 20 nm MSNs (as control) or 20 nm MSN-FITC at a concentration of 0.2 mg mL$^{-1}$ within a 1.5 mL centrifuge tubes (Eppendorf) (bound by adhesive tape to the plates). The plates containing the plants and nanoparticles were grown in a hydroponic system as previously described (Martin-Ortigosa et al. 2012). There was a slight change in the experimental setup with lupin because of the large and branched root system that wouldn’t fit the 1.5 mL tubes post three weeks of growth compared to that of wheat that showed slender roots. The principle of this experimental setup is the same as the SPS tested for wheat. Seedlings of lupin and maize were treated similarly except the roots of plants were placed into 50 mL centrifuge tubes containing 3 mL nutrient solution with 0.2 mg mL$^{-1}$ of 20 nm MSNs (as control), MSN- FITC or MSN-RITC. The seedlings were then placed in an environment controlled growth chamber (Thermoline, Coburg North, Australia) with 70% relative humidity at 21°C, and a 14 h light and 10 h dark cycle.

To obtain thin sections of the root, stem and leaves, three samples of each were taken from each plant species and embedded in optimal cutting temperature compound (O.C.T, Tissue-Tek®) and quickly frozen in liquid nitrogen. The embedded tissue samples were cut into 10 μm sections using a cryotome (Zeiss). The sections were placed on a glass slide covered with anti-fading agent 1, 4-Diazobicyclo-(2.2.2) octane (DABCO™, Sigma Aldrich) which prevents photo-bleaching of the dye and observed
using a confocal laser scanning microscopy (Leica) with FITC channel (490–525 nm band width) and RITC (565–610 nm band width).

4.2.6 Quantitative measurement of MSNs in plants by Micro-particle-induced X-ray emission (Micro-PIXE)

Maize plants were exposed to raw MSNs and MSN-RITC for 5 days. Excised leaf tissues were prepared using standard freeze-drying (Kachenko et al. 2008). Root and leaf samples were analysed with a spot size of 10 μm using a beam of 3 MeV and a beam current of 0.5–1 nA. The detection with X-rays used a 100 mm² Ge detector with an angle of 90 msr. To prevent the scattered protons from entering the detector and to reduce the low energy X-ray yield from light elements, a 25-μm Be window and a 80-μm thick aluminium foil with a 0.8-mm diameter pinhole was placed in front of the detector. The data were collected using the Data Acquisition System mpsys4 from Melbourne University together with a Canberra Model 2060 digital signal processor. The quantification of the silicon from the elemental maps was accomplished using GeoPIXE 2 software (Ryan 2001).

4.3 Morphology of MSNs without labelling
Figure 4.1 (A) TEM image of MSNs synthesised in phosphate buffer solution (pH=7.2). (B) Broad view of MSNs under TEM showing the well-dispersed MSNs synthesised in ammonium hydroxide (pH=10) and the inset is the enlarged area showing the silica phase (dark and continuous region) and mesopores (white and scattering dots pointed by the yellow arrows) under TEM. (C) SEM image of well-dispersed MSNs showing the isolated nanoparticles and the spherical shape.
Two methods were used to fabricate MSNs with the diameter of around 20 nm, which is described in detail in Chapter 2. The morphology of synthesised MSNs plays an important role in the uptake by plants. Their morphology can be observed under TEM and SEM. The MSNs synthesised in phosphate buffer solution tend to aggregate (Figure 4.1 A). In contrast, the MSNs fabricated in ammonium hydroxide possess good dispersity (Figure 4.1 B). The enlarged area of MSNs indicate the mesoporous structure that is pointed by the yellow arrow. The spherical nanoparticles with a uniform size can be seen from the SEM images (Figure 4.1 C). The particle size measured from both TEM and SEM is around 20 nm which is not only an ideal size to penetrate through plant cell wall but also possesses sufficient space to encapsulate agrichemicals (Nair et al. 2010).

4.4 Modification of MSN surface with fluorescent dyes

The grafting of FITC onto MSNs was conducted through the chemical reaction between amine groups and isothiocyanate groups. The surface of MSNs was firstly modified with APTES to introduce amine groups that then reacted with isothiocyanate groups in FITC molecules to make FITC chemically bound onto MSNs.

FTIR spectra was employed to investigate the structural change before and after the functionalisation by FITC (Figure 4.2). The successful grafting of FITC onto MSNs is determined through the characteristic absorption peak at C-N stretching vibration of 1458 cm⁻¹ attributed to the reaction between amine groups (-NH₂) on APTES modified MSNs and the isothiocyanate groups (-N=C=S) (Wang et al. 2001). The representative peaks of silica framework indicated on the spectrum of MSNs are at 1087 cm⁻¹ (Si-O-Si asymmetric stretching), 950 cm⁻¹ (Si-O stretching), 840 cm⁻¹ (Si-O-Si symmetric stretching) and 462 cm⁻¹ (Si-O-Si bending vibrations) (Pereira et al. 2011). Because of the coverage of the APTES and FITC molecules on the surface of MSNs, the intensity of these peaks related to silica is lower than that of raw MSNs. The physically absorbed water ascribes a peak at 3425 cm⁻¹ due to –OH stretching vibration and a peak between 1635 cm⁻¹ and 1600 cm⁻¹ attributed to H-O-H bending vibration (Pereira et al. 2009; Pereira et al. 2010). The grafting of RITC was conducted through the same chemical reaction mechanism between amino groups and isothiocyanate groups. The peak of C-
N at 1458 cm$^{-1}$ is also present after grafting RITC onto MSNs.

Mesopores are a unique structure of MSNs and provide the space to load the guest molecules and release them into plants. The mesoporous structure of MSNs before and after grafting fluorescent dyes was investigated through nitrogen adsorption and desorption isotherms. All isotherms exhibit typical type IV adsorption curve according to IUPAC classification (Sing et al. 1985), which indicates the presence of mesoporous structure (Figure 4.3 A). From nitrogen isotherms, it can also be seen that the typical type H2 hysteresis loop presents at the relative pressure over 0.8. The desorption isotherm of MSNs has a very sharp decrease at relative pressure of 0.9. It is because pores are interconnected with others and the absorbed nitrogen molecules have an equal chance to be desorbed from each pore, which indicates the bicontinuous and interconnected mesopores (Wang 2009).
Figure 4.3 (A) Nitrogen isotherms of MSNs, MSN-RITC and MSN-FITC. The inset is the pore size distribution of MSNs. Note: The nitrogen isotherm and pore size distribution of MSNs is the same data set as showed in Figure 2.15 A and B. (B) total absorbed nitrogen gas at relative pressure of 0.99.

Average pore diameter can be calculated through BJH methods. The inset figure in Figure 4.3 A shows the pore size distribution of MSNs before functionalization. The average pore size of MSNs without any functionalization is around 2.58 nm. After the introduction of fluorescent molecules, FITC or RITC, pore size distribution cannot be
detected. The absence of pores can be seen from the decrease in pore volume of the pore diameter less than 10 nm from 0.33 cm$^3$/g for MSNs to 0.11 cm$^3$/g for MSN-FITC or 0.13 cm$^3$/g for MSN-RITC (Table 4.1). This is because the grafted FITC and RITC molecules occupy the pore area inside MSNs. The drop of the total amount of absorbed nitrogen can also prove the pore occupation by the fluorescent molecules (Figure 4.3 B).

Table 4.1 Surface area and pore volume of MSNs before and after functionalization

<table>
<thead>
<tr>
<th>Samples</th>
<th>Surface Area (m$^2$/g)</th>
<th>Pore Volume (Total) (cm$^3$/g)</th>
<th>Pore Volume (&lt; 10 nm) (cm$^3$/g)</th>
<th>Pore Diameter (nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSNs</td>
<td>481.2 ± 1.4</td>
<td>1.71</td>
<td>0.33</td>
<td>2.58</td>
<td>-25.9 ± 4.9</td>
</tr>
<tr>
<td>MSN-FITC</td>
<td>184.8 ± 2.4</td>
<td>0.86</td>
<td>0.11</td>
<td>-</td>
<td>-15.2 ± 3.8</td>
</tr>
<tr>
<td>MSN-RITC</td>
<td>241.2 ± 3.3</td>
<td>0.95</td>
<td>0.13</td>
<td>-</td>
<td>-8.8 ± 3.6</td>
</tr>
</tbody>
</table>

4.5 Fluorescent property of labelled MSNs with different dyes

Table 4.2 Excitation and emission wavelength of fluorescent dye and the labelled MSNs

<table>
<thead>
<tr>
<th>Samples</th>
<th>Excitation Wavelength (nm)</th>
<th>Emission Wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSNs</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>FITC</td>
<td>227.96</td>
<td>516.96</td>
</tr>
<tr>
<td>MSN-FITC</td>
<td>413.93</td>
<td>522.98</td>
</tr>
<tr>
<td>RITC</td>
<td>557.94</td>
<td>580.88</td>
</tr>
<tr>
<td>MSN-RITC</td>
<td>560.00</td>
<td>580.88</td>
</tr>
</tbody>
</table>

All MSNs, FITC labelled MSNs and RITC labelled MSNs can be homogeneously dispersed in aqueous solution (Figure 4.4 A). The FITC labelled MSNs display bright and equal fluorescence from the whole solution under ultraviolet light, whereas MSN-RITC shows no florescence under ultraviolet light (Figure 4.4 B). This is because MSN-FITC and MSN-RITC possess different excitation wavelength that can be detected under fluorescence spectrometer. It can be seen from Error! Reference source not found. that the excitation of MSN-FITC is 413.93 nm and MSN-RITC is
560.00 nm. The radiation of ultraviolet light is not suitable for the excitation of RITC so that no fluorescence is observed.

![Image of fluorescently labelled MSNs under daylight and ultraviolet light](image-url)

Figure 4.4 The dispersion fluorescently labelled MSNs (1), MSN-FITC (2) and MSN-RITC (3) under daylight (A) and ultraviolet light (B). The concentration of particles is 0.5 mg/mL.

Based on the excitation wavelength of MSN-FITC and MSN-RITC obtained from fluorescence spectrometer, the green and red fluorescence can be seen under confocal laser scanning microscopy (Figure 4.5). These fluorescent signals under the
microscope provides the information about the presence of MSNs and more importantly, the different excitation wavelength of MSN-FITC and -RITC. Due to endogenous auto-fluorescence from plants, MSNs modified with different dyes can be used to distinguish with plant tissues in order to precisely and affirmatively determine the uptake of MSNs by plants.

Figure 4.5 Photos of fluorescently labelled nanoparticles on the bottom of centrifuge tubes: (A) MSN-FITC, (E) MSN-RITC. Confocal images of dried powder of nanoparticles on top of glass slides: MSN-FITC under confocal laser scanning microscope, (B) FITC channel, (C) bright field and (D) merged image; MSN-RITC under confocal laser scanning microscope, (F) RITC channel, (G) bright field, (H) merged image. The scale bars in B-D and F-H are 75 μm. The excitation wavelengths of MSN-FITC and MSN-RITC under confocal laser scanning microscopy are 488 nm and 561 nm respectively.

4.6 Phytotoxicity assay of silica based nanoparticles

In addition to the localization of MSNs within plants, the phytotoxicity of MSNs was examined. Lupin, as a common commercial crop, was chosen to investigate the effects of MSNs on the seed germination and root growth. Both solid and mesoporous silica nanoparticles were used to determine the effects of silica based nanoparticles on plant
Figure 4.6 Percentage of lupin seed germination (A) and root length change (B) following incubation with different concentrations of solid silica and mesoporous silica nanoparticles. White bars represent controls, while grey bars represent treatments conducted with SNP and black bars are the treatments with MSN. The values for each data point represents the mean ± SE of three replicates with 12 seeds each. (Derived from the paper published in Journal of Nanoparticle Research (Hussain et al. 2013))
Chapter 4: Plant Uptake of MSNs

The synthesised MSNs were tested for any effects on lupin seed germination (Figure 4.6 A) and radicle length (Figure 4.6 B). The SNPs with the diameter of about 7 nm were used to compare the effects of solid and mesostructured nanoparticles on seed germination. No negative effects were observed on lupin seed germination when the seeds were treated with different concentrations of MSNs and SNPs (Figure 4.6 A). There was no significant difference (p>0.05) at any concentration of nanoparticles tested for seed germination (Figure 4.6 A). To analyse the effect of nanoparticles on root growth, roots were exposed to nanoparticle solutions. It is found that at the highest concentration tested, 2000 mg L\(^{-1}\) SNPs, there is a statistically significant increase in root length compared to the control (p = 0.032; Figure 4.6 B). No significant difference was observed at the lower concentrations. Therefore, there is no toxicity of silica based nanoparticles (SNPs and MSNs) when their concentration is less than 2 mg/mL.

4.7 Cellular and sub-cellular localization of MSNs in roots, stems and leaves and quantification of MSNs in plants

A long-distance transportation of MSNs from the root system to the shoot system was investigated in lupin, which is an important support for MSNs as the agrichemical carriers in the delivery system for plants. From the cross-sections of the lupin root, it can be seen under the confocal laser scanning microscope that the green fluorescence from MSN-FITC can be revealed in the epidermis and associated cells around the casparian band of the wheat root. Nanoparticles were also found in the apoplast and ground tissue of the stem and were transported through the vascular tissue to the palisade and spongy tissue of the lupin leaves (Figure 4.7).

FITC labelled MSNs were found difficult to be detected under confocal laser scanning microscope (Hussain et al. 2013). The auto-fluorescence emitted by endogenous proteins and metabolites in some cells was similar in wavelength to that of FITC. The RITC, similar to FITC but with different excitation wavelength, could be used as an alternative in tracking MSNs. For example, the use of RITC as the fluorophore in the plant root is more useful than in leaves because the red fluorescence from the RITC is indistinguishable from the red auto-fluorescence emitted by chloroplasts. For the uptake of MSNs by maize roots, the control group is the maize root treated by MSNs without any dye. There is no fluorescence from RITC channel MSNs that were labelled
with RITC (Figure 4.8 A-C). The red fluorescence is obvious in the xylem of maize roots (Figure 4.8 D-F), which indicates the accumulation of MSNs is mainly in the transporting system of the roots.

Figure 4.7 Accumulation of MSN-FITC in lupin. Lupin plants were exposed to MSN-FITC solution for 5 days. The lupin plants were 3 weeks old. The translocation of MSN-FITC in the root, stem and leaves of lupin was analysed under confocal laser scanning microscopy. (A-C): cross-sections of lupin root, stem and leaves treated with MSN-FITC under the bright field; (D): aggregates of MSN-FITC in the epidermis and endodermis of the root; (E): MSN-FITC accumulated in the ground tissue of the stem; and (F): accumulation of MSN-FITC in the palisade and surrounding cells. The magnified insets in (D-F) clearly show the accumulation of MSN-FITC. In the images, where ep is epidermis, en is endodermis, pm is palisade mesophyll and sm is spongy mesophyll. Scale bar 25 μm. (Derived from the paper published in Plant Cell Reports (Sun et al. 2014))

The results from confocal microscopy provide strong evidence for the internalization of MSNs in roots, stems and leaves. The determination of the amount of MSNs inside plants is very challenging, because of the uneven distribution of MSNs in plants. Herein, an elemental quantification method at large scale is employed through micro...
particle-induced X-ray emission (Micro-PIXE) in order to understand not only the distribution of MSNs but also the quantity of MSNs inside plants.

Figure 4.8 Distribution of MSN-RITC in maize roots. Three-week-old maize plants were exposed to MSN-RITC for 5 days. A-C: Maize root cross-sections treated with MSNs under bright field and red channel using CLSM. B: As expected, no fluorescence was observed from the roots treated with MSNs. C: Merged image of A and B. D-F: Maize root cross-section treated with MSN-RITC under bright field and red channel using CLSM. E: Bright red fluorescence was observed in the xylem indicating root uptake of MSN-RITC (arrow). F: Merged image of D and E. The excitation wavelength used in RITC channel is 516 nm. Scale bar 25 μm. (Derived from the paper published in Plant Cell Reports (Sun et al. 2014))

The preliminary results from Micro-PIXE elemental detection confirmed the uptake of MSNs in the maize plant (Figure 4.9). Silicon (Si) as the major composition element of MSNs was used as the indicator to determine the presence of MSNs. More silicon in MSN-RITC treated maize plant is detected, compared to the control group that is the maize treated by water. The site with high MSN accumulation is along the vessels of the root (white arrows in Figure 4.9 B), which supports the previous confocal images (Figure 4.8). The Micro-PIXE results of the maize leaves (Figure 4.9 F) indicate the distribution of MSNs mainly in the vascular bundles that are connected to the vessel
to transport the minerals and water to leaves.

Figure 4.9 Elemental maps of cryo-fixed freeze-dried MSN treated roots and leaves of maize. (A): Roots treated with water showed no silica accumulation, (B): MSN-RITC treated root showing heavy accumulation of silica in the maize roots and other essential elements for normal plant growth such as, (C): potassium and (D): calcium were also detected. More interestingly, (E): silica levels were found to be below minimum detection limit in maize leaf when the roots were exposed only with water and (F): leaves showed bright stripes of silica nanoparticle in the vascular bundles of the leaves. Scale bars for (A-D) 300 $\mu$m and (E-F) 500 $\mu$m. (Derived from the paper published in Plant Cell Report (Sun et al. 2014))

The elemental profiles in the roots and leaves of the maize were calculated from the elemental distribution images (Figure 4.9). Maize roots exposed to MSN-RITC showed Si concentrations between 250–750 ppm. The percentage of MSN accumulation in the root was found to range between 25 and 37.5 % of totally added
MSNs. The localised elemental profiles (Figure 4.10) were calculated from the distribution images along the green line in Figure 4.9 E and F. The element of Si in control plants was measured to determine the background contribution of the element and was found to be below the minimum detection level (Figure 4.10 A). Higher concentration of Si was detected at the vascular bundles of the leaves than that at other sites. Apart from silicon, the profiles of other elements such as calcium (Ca) and potassium (K) were also determined. MSNs were found to be co-localized with potassium that is important for normal plant growth (red curve in Figure 4.10 B). The sensitive and reliable information of the accumulation of MSNs in plants can be obtained from Micro-PIXE. This information about MSNs in roots and leaves is very useful to comprehend the transport mechanism of MSNs inside plants.

Figure 4.10 Quantitative elemental profile for MSNs (Si), Potassium (K) and Calcium (Ca) across the freeze-dried maize leaf section. (A) Control group and (B) Treated with MSNs (Derived from the paper published in Plant Cell Report (Sun et al. 2014))
4.8 Uptake of MSNs by plants and their translocation

Figure 4.11 Schematic illustration showing the uptake of MSNs by plant root via symplastic and apoplastic route and the translocation of MSNs throughout the plants.

Plant cell walls have pore sizes between 5 and 30 nm (Adani et al. 2010; Carpita et al. 1979; Fleischer, O’Neill & Ehwald 1999; Rondeau-Mouro et al. 2008; Tepfer & Taylor 1981), which is the main barrier for the nanoparticles entering and transporting in plants. However, the precise measurement of pore size in cell walls is limited to only a few plant species but particles such as copper (50 nm), magnetite (40 nm), gold (10–50 nm), silver, solid silica (14, 50, 200 nm), titanium oxide particles (5 nm) and multi-walled carbon nanotubes (10–150 nm) were found to be accumulated in different plant species (Cifuentes et al. 2010; Kurepa et al. 2010; Larue et al. 2012; Lee, Kwak & An 2012; Nair et al. 2011; Sabo-Attwood et al. 2012; Slomberg & Schoenfisch 2012; Stampoulis, Sinha & White 2009; Wang et al. 2012) in various organelles and cellular compartments indicating that plants may have cell wall pores that vary greatly in size between species. The MSNs in the present study were tuned and fluorescently labelled...
for plant uptake and localization.

The uptake pathway of the engineered nanoparticles is a very controversial topic and the study in this area is still at its infant stage (Nair et al. 2010). In this study, through tracking the accumulation of MSNs in the whole plants, the transportation mechanism can be proposed (Figure 4.11):

- Plants grown in the soilless system absorbed nutrient solution, which provides a driven force for MSNs to penetrate through plant cell walls. The root hair provides large surface for the interaction between MSNs and plant cells.
- Once MSNs entered the plant cell wall, the symplastic and apoplastic routes are two possible ways to move further into the transporting system of the plants. MSNs were detected in the free space between cells and also inside cells (Sun et al. 2014), which indicates that before entering the xylem vessel in the roots, MSNs transport inside roots via both symplastic and apoplastic routes.
- The next step of MSN transportation is through the vessel from the root to the leaf. The accumulation of MSNs happens on the inner surface of the vessel (Figure 4.8). At this step, MSNs accompany the moving water that is the supply for the leaf.
- The transportation of MSNs ends up at the leaves. The main accumulation of MSNs was detected in the vascular vessel of the leaves and some were in the surrounded cells (Figure 4.9).
Chapter 5: Encapsulation of Agrochemicals in MSNs and the Controllable Release Mediated by Glutathione in Plant Cells

Highlights of the Contributions:
- Localized functionalization of the outer surface of mesoporous silica nanoparticles (MSNs) was employed prior to the removal of templates inside mesopores. This not only confers functionality on the surface but provides more space inside the pores.
- Mesopores of MSNs were blocked by short chain molecules to protect the loaded chemicals from being released prematurely, which proves the concept of using small organic molecules as gatekeepers.
- The release of salicylic acid from mesoporous silica nanoparticles was controlled by a glutathione mediated gatekeeper system.

5.1 Introduction

Stimuli-responsive functional groups enable MSNs to transfer cargos without any premature loss and release them in a specific tissue when meeting with trigger factors including pH value (Liu et al. 2010; Muhammad et al. 2011; Yang et al. 2010; Yuan et al. 2011), temperature (Ye et al. 2011), redox mediators (Cui et al. 2012; Kim et al. 2010; Koo et al. 2013), etc. Among them, the behaviour of redox responsive release is advantageous compared to other factors (pH value, temperature, etc) because glutathione (GSH) as a reductant exist naturally in both mammalian and plant cells. The release of loaded cargos is commonly manipulated via cleavage of disulfide bonds between MSNs and gatekeepers (Vivero-Escoto et al. 2010). In the drug delivery system for animals, redox controlled release of drugs has been widely used. Mesopores in MSNs are capped by gatekeepers of surface functionalised nanoparticles such as cadmium sulphide (CdS) (Lai et al. 2003), magnetic nanoparticles (Fe₃O₄) (Giri et al. 2005) and large molecules (thiol terminated polyethylene glycol (PEG-SH)) (Cui et al. 2012). The linkage between MSN and corresponding gatekeepers are disulfide bonds which will be cleaved by GSH inside cells. Victor Lin’s group has provided many
possibilities to enable the functionalization of nanoparticle gatekeepers for redox induced release of drugs from MSNs (Giri et al. 2005; Lai et al. 2003).

However, stimuli-responsive release of agrochemical from MSNs in plants is seldom reported. A report from Victor Lin’s groups indicated that MSNs can be capped by gold nanoparticles and release β-estradiol in tobacco protoplasts (Torney et al. 2007) which are plants cells without plant cell wall. The plant cell wall as a natural obstacle limits many applications of nanomaterials in plant science. To overcome plant cell wall, MSNs with particle size about 20 nm were synthesised and can be taken by intact plants as demonstrated in Chapter 4 (Hussain et al. 2013). In order to transport through plant bodies, the particles has to have finely controlled size (Dietz & Herth 2011) after incorporating functional groups for stimuli-responsive release of agrochemicals. The reported gatekeepers such as CdS, Fe₃O₄ and large molecules are not appropriate as the particle size increases significantly after the functionalization.

Herein, we introduce decanethiol with the chain length slightly larger than the radius of pore size as the gatekeepers to prevent agrochemical molecules loaded in mesopores from being prematurely released before MSN carriers are internalized by plants. Salicylic acid (SA) as a widely used plant hormone is chosen as a model molecule to load into mesopores. Decanethiol is assembled onto thiol modified MSNs through disulfide bonds which are not only to protect loaded SA but also release SAresponsively when exposed to GSH in plant cells.

### 5.2 Experimental and Methodology

All the chemicals used in this section are listed in the Appendices.

#### 5.2.1 Synthesis of mesoporous silica nanoparticles

The MSNs were synthesised by using CTAB as templates to generate silica mesoporous structure as described in Section 2.2.2 in Chapter 2. The ammonia solution was used as the catalyst to induce the hydrolysis and condensation of TEOS.
5.2.2 Grafting of thiol groups on CTAB@MSNs

Thiol groups (-SH) were then grafted onto the surface of the MSNs that contain the template CTAB to allow the functional groups to be present only at the entrance of mesopores.

In detail, 100 mg of CTAB@MSNs were dispersed into 100 mL of absolute ethanol and the mixture was sonicated until a uniform suspension was achieved. Under vigorous stirring, 100 μL of MPTMS was added dropwise into the mixture. After 1 hour, 1 mL of deionized water was added and the reaction was maintained for 24 hours under room temperature with stirring. Then the mixture was heated to 80 °C to stabilize the silanol groups between MSNs and MPTMS. The products were centrifuged and washed with absolute ethanol for 3 times. Then the as-made CTAB@MSNs that were grafted with thiol were redispersed in a solution of 100 mL of absolute ethanol and 1 mL of hydrochloric acid (32%) and the mixture was stirred at 60 °C overnight to remove CTAB inside mesopores. The MSNs with CTAB template removed and modified with thiol were centrifuged in 50 mL centrifuge tubes at 8000 rpm for 7 min, washed with absolute ethanol for 3 times and dried under vacuum at room temperature. Thiol functionalised MSNs were denoted as MSN-SH.

5.2.3 Synthesis of didecyl-disulfide

The disulphide bonds between thiols were formed through a mild oxidation procedure (Kirihara et al. 2008). Specifically, 0.8 mL of decanethiol was dissolved in 12 mL of ethyl acetate under stirring. Six milligrams of sodium iodide and 0.44 mL of hydrogen peroxide (30 wt%) were added to the mixture. After stirring for 30 min at room temperature, the as-synthesised didecyl-disulphide was washed by sodium carbonate aqueous solution (10 wt%) three times to remove abundant iodine and hydrogen peroxide. The final transparent oil-like product was collected by evaporating ethyl acetate in silica gel under room temperature.

5.2.4 Synthesis of thiol capped MSNs

The previously synthesised didecyl disulfide was introduced to form gatekeepers at
the entrance of mesopores. The procedure was as follows: 10 mg of template removed MSN-SH was dispersed in 10 mL of ethyl acetate followed by adding 25 μL of didecyl disulfide. The mixture was sonicated for 1 h and stirred for 24 h at room temperature. The didecyl disulfide capped MSN-SH (MSN-SS-C\textsubscript{10}) was washed and centrifuged with ethyl acetate three times and absolute ethanol three times. Powders were collected by vacuum drying.

**5.2.5 Loading of salicylic acid and assembly of decanethiol onto MSN-SH**

The loading of salicylic acid (SA) was conducted through free diffusion. In detail, 100 mg of SA was dissolved in 5 mL of ethyl acetate. Then, 100 mg of MSN-SH was added into the SA solution and the mixture was sonicated for 30 min. After shaking at room temperature for 24 h, 250 μL of didecyl disulfide was added to the mixture to react with -SH groups on MSN-SH. The reaction was maintained for 24 hours with shaking on a rotating mixer at room temperature. Finally SA loaded and decanethiol capped MSNs were centrifuged at 7500 rpm for 10 min and washed with 40% (v/v) ethanol once to remove most of the ethyl acetate. All the supernatant was collected to measure the loading percentage of SA. MSN samples were quickly frozen in liquid nitrogen to keep the SA in the mesopores and then dried in a freeze dryer for 24 hours. The dried powder was collected for further use and denoted as SA@MSN-SS-C\textsubscript{10}.

The control samples loaded with SA but without any functionalization of the MSNs were also prepared using the same loading procedures and concentration. The loading was maintained for 48 hours. This sample was denoted as SA@MSN.

Loading percentage was calculated from the following equation:

$$\text{Loading percentage} = \frac{\text{Original mass of SA} - \text{Free mass of SA}}{\text{Original mass of SA}} \times 100\%$$

**5.2.6 Characterization**

To investigate the morphology of MSNs before and after assembling decanethiol, SEM and TEM were employed. The surface functional groups were confirmed by Raman
spectroscopy, TGA and SEM-EDS. The success of SA loading and encapsulation was determined through the changes of surface area and porosity obtained from BET-BJH method. The loading capacity and release kinetics of SA from MSNs were examined from UV-Vis spectrometer. The detailed characterization methods were described in Appendix B.

5.2.7 Plant materials and growth conditions

*Arabidopsis thaliana* ecotype Columbia-0 (Col-0) wild-type plants were obtained from the Arabidopsis Stock Centre (Ohio, USA). Seed accessions of Col-0 can be obtained from the Arabidopsis Stock Center (http://arabidopsis.info). A preliminary experiment was conducted to identify the prolonged expression of pathogenesis-related protein 1 (*PR1*) gene to exogenous and MSN loaded SA capped with decanethiol gatekeepers. The plants were grown with slight modification for further experiments (Foster & Chua 1999). The seeds were surface sterilized for 5 min with a solution containing 45 % (v/v) sterile distilled water, 50 % (v/v) ethanol and 1.5 % (v/v) hydrogen peroxide. The sterilization solution was removed and the arabidopsis seeds were rinsed three times with sterile distilled water to remove the residual sterilization solution. The seeds were then transferred to the plates containing the MS medium. The plates were sealed with parafilm and seeds were vernalised at 4°C to improve and synchronise seed germination. The plates were then transferred to a plant growth chamber where seedlings were grown under controlled conditions (Thermoline, Coburg North, Australia) at 21 °C with a 16 h light (light intensity 100 μmol m⁻² s⁻¹) and 8 h dark cycle for two weeks. Small black pots were filled with fresh propagation mix (Debco, Australia) and the pots were placed in a plastic tray containing tap water. The soil on the surface of each pot was moistened with tap water from squeeze bottle and single holes were then made with in the soil of each pot. Arabidopsis seedlings were gently removed from the MS media with forceps and care was taken to avoid any root damage. The seedlings were put into the hole and surrounded by soil. The trays containing the seedlings were covered using a clear transparent lid and placed on top of the pots to minimise wilting during acclimatisation. The seedlings were allowed to grow for one week and were further used for downstream applications.
5.2.8 Vacuum infiltration of SA, MSNs and MSN-SA-C10

Three week old Arabidopsis seedlings were carefully removed from the pot and the roots were washed gently in trays containing tap water with repeated dipping to remove any excessive soil attached to the roots. The vacuum infiltration was performed with slight modifications to the protocol previously reported (Marion et al. 2008; Tian et al. 2014). The vacuum infiltration was done using a PDS-1000 particle delivery system (Bio-Rad, USA). Whole plants were submerged in infiltration mixture containing 3 ml of 5 mM SA solution to a vacuum at ~25 kPa twice, each for 60 s. Treated plants were then planted in pots. Similarly, MSNs and SA@MSN-SS-C10 containing ~5 mM SA were resuspended in 3 ml of water and vacuum infiltrated into the plants and then planted in pots. Leaf samples were collected at 3rd, 5th and 7th day after vacuum infiltration for PRI gene expression analysis and GSH quantification.

5.2.9 Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Semi-quantitative RT-PCR was conducted to test the prolonged up-regulation of PRI gene using RNA samples extracted from plants vacuum infiltrated with SA, MSNs and SA@MSN-SS-C10. For cDNA synthesis, 1 μg of total RNA was isolated by following manufacturer’s instruction (RNAzol, Molecular Research Centre, USA) to reverse-transcribe in a reaction containing Tetro reverse transcriptase, reaction buffer, and 2 μM random hexamers as per the manufacturer’s specifications (Bioline, Alexandria, NSW, Australia). Primers were designed to examine the relative expression of PRI and the housekeeping gene actin that was reported being up-regulated post SA treatment. PCR reactions were carried out with GoTaq green master mix and reagents (Promega, Madison, WI). PCR reactions contained 1μL of cDNA from the RT reaction and 0.5 μM of the appropriate oligonucleotides. PCR cycles were consisted of an initial denaturing step of 3 min at 94 °C, followed by repetitions (18–34 cycles, depending on the primer set) of the following three steps: a 30-s denaturation step at 94 °C, 30-s annealing step ranging between 50 and 52 °C and a 1-min elongation step at 72 °C. Initial reactions were performed to determine the appropriate cycle number for analysis to be conducted within the exponential phase of the PCR reaction (data not shown). PCR products were analysed on 0.5 × TBE-agarose gels and visualised using
ethidium bromide staining. The following primers were used for gene-expression analysis of the plant nanoparticle interaction: PR-1: 5'-AAGAGGCAACTGCAGACTCA-3' and 5'-TCTCGCTAACCCACATGTTC-3' and Actin: 5'-ACGTGGACATCAGGAAGGAC-3' and 5'-GAACCACCAGATCCAGACACT-5.

5.2.10 GSH quantification assay

The GSH was measured using the glutathione reductase assay kit (Sigma). The method relies on the glutathione reductase (GR)-dependent reduction of 5,5-dithiobis-2-nitrobenzoic acid (DTNB), monitored at 412 nm using a 96 well plate reader spectrophotometer (FluostarOptima, BMG labs). The method measures total glutathione i.e. that includes reduced glutathione GSH+GSSG. Fine powders of 50 mg of Arabidopsis leaves treated with SA, MSNs and SA@MSN-SS-C10, respectively were mixed with 1 ml of ice-cold 6% meta-phosphoric acid. The suspensions was then centrifuged at 14000 g for 15 min at 4 °C (Eppendorf centrifuge 5804R). The supernatants were collected and kept on ice in the dark until use. To measure total GSH, triplicate aliquots of 10 μl extract were added to plate wells containing appropriate volumes of assay buffer, nicotinamide adenine dinucleotide phosphate, DTNB and GR from the kit was calculated to reach a total assay volume of 220 μl. Standards were run concurrently in the same plates as triplicate assays of 0 to 50 nmol of GSH in the well. The activity was calculated over 90 s and in all cases was corrected for GSH-independent reduction of DTNB by subtraction of the mean value of triplicate blank assays (0 GSH).

5.3 Schematic description of the modification of MSNs and the encapsulation of SA

For nanoparticles to be used in plants, the size and phytotoxicity of these nanoparticles are critical properties related to the uptake by plants through plant cell walls (Dietz & Herth 2011) and the effects on plant growth. Maintaining high pore surface area and volume after surface modification for a high loading of agrochemicals is quite challenging when the particle size is as small as 20 nm, because silane molecules such
as MPTMS can easily be diffused into deep area of mesopores and react with silanol groups on the inner surface. To avoid the modification in the inner surface of mesopores, localized functionalisation was essential. A process to functionalise the surface of the MSNs with thiol groups was therefore designed before CTAB templates were removed in mesopores of the MSNs, thus the -SH groups were only on the external surface (Steps (1) to (2) in Figure 5.1). After the removal of templates, the pores became vacuous for loading molecules (Step (3) in Figure 5.1).

Figure 5.1 Schematic of functionalization of MSNs with thiol groups and assembly of gatekeepers on to SA loaded MSNs. The localized functionalization of MSNs before removing template inside mesopores allows the thiol groups only to be grafted at the entrance of mesopores.

Gatekeepers are required to prevent the agrochemicals from premature release. The commonly used gatekeepers such as metallic particles (gold nanoparticles (Liu et al. 2010) and CdS nanoparticles (Lai et al. 2003)) and polyethylene glycol (Cui et al. 2012; He et al. 2010; Zhu et al. 2011) will significantly increase particle size which is
dependent on the size of keeper particles and the thickness of polymeric coating and also tend to be coated on the surface rather than around the pores. Decanethiol is a thiol terminated molecule with a short alkyl chain of only 2 nm in length, which can block the pore entrance but have no significant effects on the particle diameter. After that, decanethiol was covalently bonded to thiol modified MSNs through disulfide bonds, to not only protect loaded SA from pre-mature release but also to release SA responsively when the MSNs are exposed to GSH in plant cells (Step (4) in Figure 5.1).

5.4 Synthesis of well-dispersed MSNs

In order to fabricate MSNs with a particle size of about 20 nm, bases were used as catalysts to form silica framework. The slow hydrolysis of TEOS and fast condensation between silanol groups (Chiang et al. 2011; Qiao et al. 2009) normally lead to the formation of spherical particles which possess better morphology than MSNs synthesised under acidic conditions. However, the fast condensation rate in strong basic conditions such as sodium hydroxide may cause the interaction between particles, which results in the aggregation of nanoparticles (Figure 5.2 A). Thus, ammonium hydroxide was used as catalyst to slow down the condensation rate in order to achieve uniformity of MSNs. Figure 5.2 B shows the well dispersed MSNs at low magnification under SEM.

Meso-structure of MSNs plays an important role in loading and releasing agrochemicals. To effectively load and release agrochemicals, interconnected structure is superior to cubic and hexagonal structure, because mesopores are connected to each other throughout the whole particle which in turn provides the molecules with more opportunity to be loaded in MSNs and also to be released from MSNs. In this study, MSNs with interconnected mesopores were fabricated from CTAB templates. From nitrogen isotherms (Figure 2.15 A), it can also be seen that the typical type H2 hysteresis loop presents at the relative pressure over 0.8. The desorption isotherm of MSNs has a very sharp decrease at relative pressure of 0.9. It is because pores are interconnected with others and the absorbed nitrogen molecules have an equal chance to be desorbed from each pore, which indicates the bicontinuous and interconnected mesopores (Wang 2009). The mesoporous structure of MSNs can
be demonstrated from the TEM images of high magnification (Figure 5.2 C). The white dots (arrows) represent the entrance of the mesopores which shows different contrast from the silica framework of the nanoparticles.

The mesoporous structure was investigated through nitrogen sorption isotherms. The MSN isotherms exhibit typical IV adsorption curve (Sing et al. 1985). The average pore size, calculated with Barret-Joyner-Halenda (BJH), reveals a uniform distribution at 2.58 nm as shown in Chapter 4. The mesoporous structure enables the efficient loading of agrochemicals.

(A) TEM image of MSNs synthesised by using sodium hydroxide as catalyst

(B) TEM image of MSNs at low magnification indicating well dispersed nanoparticles.
5.5 Localized functionalization of MSNs with -SH groups

MPTMS was introduced to graft -SH groups onto MSNs. The functionalization of the external surface of MSNs was conducted before CTAB templates were removed. This will ensure that only the silanol groups at the entrance of mesopores will be reacted with MPTMS as mesopores inside MSNs were occupied by CTAB molecules. After the removal of CTAB, mesopores are empty and ready for loading agrochemicals.

The successful grafting of -SH groups was confirmed by Raman spectra (Figure 5.3) where a characteristic peak at around 800 cm\(^{-1}\) that is attributed to the vibration of silanol groups on the surface of silica (Si-OH) and the peak at around 2600 cm\(^{-1}\) represented -SH groups in MPTMS are shown. After silanization of MSNs, both Si-OH and –SH can be seen from the Raman spectrum of MSN-SH, which suggests the successful grafting of -SH groups on the surface of MSNs. Due to the introduction of MPTMS, silanol groups of MSNs were consumed, which in turn led to the decrease of their peak intensity (Figure 5.3).
Chapter 5: Stimuli Response of MSNs

Figure 5.3 Raman spectrums of grafted on the surface of MSNs.

The occurrence of sulphur in MSNs can also be confirmed under SEM-EDS. For a selected region under SEM, elemental mapping was carried out with MSN-SH powder (Figure 5.4 A and B) and a clear peak at 2.26 keV indicates the sulphur (Figure 5.4 C). Because the main elemental composition of MSNs is silicon and oxygen, these two elements are evenly spread throughout the whole region and overlapped with each other. However, due to the modification by -SH groups on the outer surface of MSNs, it can be seen from the merged mapping image that the sulphur is sporadically distributed around the selected area (pink dots in Figure 5.4 B). These results provide qualitative evidence of the position of -SH on MSNs.

Another evidence to prove the presence of -SH groups on the external surface of MSNs but not inner surface is from BET results. It can be seen from Table 5.1 that the surface area of MSN-SH decreases from 363.83 to 346.30 m$^2$/g and the pore volume decreases from 0.33 to 0.20 cm$^3$/g. The slight decrease in surface area is due to the grafting of –SH groups but the mesopore volume does not decrease greatly, which means that the pore area was maintained after functionalization, providing sufficient space for the effective loading of agrochemicals.
Figure 5.4 (A) Morphology of MSN-SH under SEM; (B) Corresponding merged elemental mapping by SEM-EDS of the same area in (A); and (C) Spectrum of EDS mapping for specific elements: Carbon (C), Oxygen (O), Silicon (Si), Sulphur (S).

Table 5.1 BET surface area, average pore size and pore volume of various MSN samples obtained from nitrogen sorption measurement.

<table>
<thead>
<tr>
<th>Samples</th>
<th>BET surface area (m²/g)</th>
<th>Average pore size (nm)</th>
<th>Pore volume (cm³/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSNsᵃ</td>
<td>363.83 ± 2.22</td>
<td>2.59</td>
<td>0.33</td>
</tr>
<tr>
<td>MSN-SH</td>
<td>346.30 ± 1.82</td>
<td>2.22</td>
<td>0.20</td>
</tr>
<tr>
<td>SA@MSN</td>
<td>291.49 ± 2.68</td>
<td>3.10</td>
<td>0.21</td>
</tr>
<tr>
<td>SA@MSN-SS-C10</td>
<td>85.62 ± 1.43</td>
<td>—</td>
<td>0.04</td>
</tr>
</tbody>
</table>

ᵃ The same data set showed in Table 4.1 in Chapter 4.
ᵇ Pore volume is the accumulated pore volume of the mesopores less than 10 nm.

5.6 Assembly of decanethiol onto MSN-SH

The assembly of decanethiol onto MSNs was conducted through thiol-disulfide exchange. In this reaction, -SH groups are provided by MSN-SH and disulfide bonds are from di-decyl disulfide synthesised through a facile oxidation of decanethiol (Kirihara et al. 2008).
Raman spectroscope was employed to detect the formation of disulfide bonds between decanethiol molecules. It can be seen from Figure 5.5 that the absorption peak at around 2600 cm\(^{-1}\) shows the \(-\text{SH}\) groups in decanethiol molecule (Velleman et al. 2011) and a group of strong absorption peaks between 2800 and 3000 cm\(^{-1}\) are the C-H stretching vibration in \(-\text{CH}_2-\) and \(-\text{CH}_3\) from the alkyl chain, which both present in decanethiol and di-decyl disulfide. After oxidation, two decanethiol molecules formed one di-decyl disulfide. The absorption peak between 500 and 550 cm\(^{-1}\) indicates the existence of disulfide bonds (Gosselin et al. 2007; Van Wart & Scheraga 1976). Also the peak at around 2600 cm\(^{-1}\) disappears, which suggests the complete conversion from thiol to disulfide.

Interestingly the \(-\text{SH}\) groups on MSN-SH were not totally exchanged with di-decyl disulfide, after the decanethiols are grafted onto MSNs. Both disulfide bonds and \(-\text{SH}\) groups are present on MSNs. Residual \(-\text{SH}\) groups were detected by Raman spectroscopy. Thiol groups are revealed at around 500 cm\(^{-1}\), which indicates the presence of residual \(-\text{SH}\) groups on nanoparticles (Figure 5.6 A). Disulfide bonds between decanethiol and MSNs lead to the absorption peak at around 2600 cm\(^{-1}\). The obvious enhancement of \(-\text{CH}_2/-\text{CH}_3\) peak is shown at 2800-3000 cm\(^{-1}\) attributed to the introduction of alkyl chain from decanethiol, which can also prove the grafting of decanethiol on MSNs.

![Figure 5.5 Raman spectrums to determine disulfide formation between decanethiols.](image-url)
Figure 5.6 (A) Raman spectrum of MSN-SS-C10 and (B) Thermogravimetric analysis of non-functionalised MSNs and functionalised MSNs (MSN-SH and MSN-SS-C10).
The presence of residual –SH groups on MSNs is important to control the release of loaded agrochemicals. In order to determine the amount of residual –SH groups, TGA was employed. When the functional groups on the surface were removed at high temperature in air atmosphere, the weight loss indicated the amount of molecular decomposition. It can be seen from Figure 5.6 B that MSN-SH and MSN-SS-C10 exhibited one-step and two-step decomposition, respectively. The first weight loss of MSN-SS-C10 started from the same position as MSN-SH did, which means the existence of residual -SH groups on MSNs. The second weight loss starting at around 520 °C illustrated the degradation of disulfide bonds and also alkyl chains from decanethiol. The weight loss caused by -SH groups on MSN-SS-C10 (2.28%) accounted for 41.38% of -SH groups on MSN-SH (5.51%), which means that more than half (58.62%) of the -SH groups on MSN-SH has reacted with didecyl disulfide to form gatekeepers blocking guest molecules.

5.7 Loading and release of salicylic acid

Assembly of decanethiol on to MSN-SH was conducted in ethylacetate because it possesses good miscibility with both SA and didecyl disulfide. Through centrifugation, nanoparticles and free SA can be separated. The supernatants were collected to measure the loading efficacy. The standard calibration curves of SA in water and ethyacetate were obtained with different concentration of SA and linear fitted. The loading percentage calculated from UV-Vis was 6.17% and 9.92% for SA@MSNs and SA@MSN-SS-C10 respectively. The loading percentage of decanethiol capped MSNs is higher than that of MSNs only. Because the particle size was around 20 nm and all the mesopores were interconnected, washing procedure could lead to the loss of most loaded SA. However, when incorporating decanethiol on to MSNs, the loading percentage was increased when the entrance was blocked by gatekeepers.

The main challenge for MSNs as an agrochemical carrier is the particle size which is restricted by the pore size of plant cell wall. The introduction of decanethiol as the gatekeeper did not increase the particle size. It can be seen from Figure 5.7 A and B that the particle size was maintained after SA loading and decanethil capping.
Figure 5.7 SEM images of raw MSNs (A) and SA@MSN-SS-C10 (B).

Mesopores were occupied by SA molecules after loading. The decrease in the total
absorbed volume of nitrogen at relative pressure of 0.99 (Figure 5.8 A) was proved to be attributed to pore filling of MSNs. Nitrogen sorption isotherms of all MSN samples

Figure 5.8 (A) Nitrogen sorption isotherms of various MSN samples. The offset of isothermal curves for SA@MSN, MSN-SH and MSNs are 100, 300 and 500 respectively. (B) Total absorbed volume of nitrogen at relative pressure of 0.99 for different MSN samples. Note: the nitrogen isotherm of MSNs is the same data set as showed in Figure 2.15 A in Chapter 2.
Chapter 5: Stimuli Response of MSNs

exhibited similar IV adsorption curve (Figure 5.8 B), which means the mesoporous structure did not change when SA molecules were incorporated. In addition, the loading of SA and capping with decanethiol greatly decreased the surface area due to the absorption of SA on the surface of MSNs. SA@MSN-SS-C10 had the lowest surface area (85.62 m²/g) and pore volume (0.04 cm³/g) (Table 5.1). This demonstrates the encapsulation of SA with gatekeepers.

In order to investigate the redox-responsive release of MSN-SS-C10, different concentration of GSH was used. The release of SA from MSNs was greatly slowed by decanethiol encapsulation from the release curve of SA@MSN-SS-C10 without GSH (Figure 5.9). According to the results above (Figure 5.5 and Figure 5.6 ), the existence of residual -SH groups provided opportunities for SA to be released from MSN-SS-C10, which can be seen from the release curve of 0 mM GSH after 350 min. In contrast, an increase in concentration of GSH accelerated the release. The release reached the balance at 600 min when 5 mM GSH was added, and at 180 min when 10 mM GSH was added, suggesting that the decanethiol that was covalently bonded on MSNs through disulphide bonds can maintain SA inside mesopores and reduce the release rate.

![Figure 5.9 Cumulative amount of SA released from MSN-SS-C10 under different GSH concentration.](image)

Figure 5.9 Cumulative amount of SA released from MSN-SS-C10 under different GSH concentration.
The release rate of SA was controlled by the concentration of GSH. This special release kinetic provides a dual supply of plant hormones for the plants. Because the gene expression normally needs higher concentration of plant hormones to trigger (Huang et al. 2005), the GSH induced fast release inside plant cells (at 5 or 10 mM GSH) triggers gene expression (Noctor et al. 2012), and the slow release of SA (at 0 mM GSH) when nanoparticles are outside plants provides a sustainable supply of plant hormones.

In order to understand the diffusion mechanism of SA from MSNs under different GSH concentration, Korsmeyer-Peppas model was employed. Equation (1) is the release kinetics developed by Korsmeyer et al. (1983):

$$\frac{M_t}{M_\infty} = K t^n$$

(5.1)

In this equation, $M_t$ is the amount of drug released at time $t$, $M_\infty$ is the amount of drug released at infinite time, $K$ is the release kinetic constant and $n$ is the release exponent indicating the release mechanism. If taking logarithm for both side of Equation (1), the following equation is obtained:

$$\log \frac{M_t}{M_\infty} = \log K + n \log t$$

(5.2)

The released data can be fitted by linear Equation (2). If $n$ is less than 0.45, the diffusion of SA obeys Fickian mechanism; if $n$ is between 0.45 and 0.89, then the diffusion follows non-Fickian mechanism.

The initial release stage of SA was selected before 40 min (Figure 5.10 A), because Korsmeyer-Peppas model is applied to the release percentage less than 60 %. Figure 5.10 B is Korsmeyer-Peppas plot. The release data was fitted by a linear regression and the obtained $n$ value is listed in Table 5.2. The $n$ value of the sample without adding GSH is 0.76 that is between 0.45 and 0.89, which indicates that the movement of SA from MSN-SS-C10 is non-Frickian. In other words, SA cannot be freely diffused from decanethiol capped MSNs. The chemically bonded decanethiol blocked the diffusion of SA from MSNs. In addition, with the addition of GSH at the
concentrations of 5 mM and 10 mM, the $n$ values are 0.32 and 0.39, respectively. Therefore, SA can diffuse from MSNs after cleavage of disulfide bonds by GSH.

![Graph A](image1.png)

**Figure 5.10** Release of SA at initial stage and (B) Logarithmic cumulative amount of SA released versus logarithmic release duration for the sake of fitting with Korsmeyer-Peppas model at the initial stage.
Table 5.2 Output from fitting of released data with different release kinetic model

<table>
<thead>
<tr>
<th>Samples</th>
<th>Korsmeyer-Peppas model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>0 mM GSH</td>
<td>0.86 0.76</td>
</tr>
<tr>
<td>5 mM GSH</td>
<td>0.97 0.32</td>
</tr>
<tr>
<td>10 mM GSH</td>
<td>0.98 0.39</td>
</tr>
</tbody>
</table>

R² - regression coefficient, n – Korsmeyer-Peppas exponent

5.8 Mechanism of SA release from decanethiol capped MSNs

The release of loaded molecules is dependent on the surface properties of pores. In this study, because the inner surface of pores was protected by CTAB molecules during the salinization, the release of salicylic acid is controlled by the functional groups at the entrance of mesopores. The free -SH groups in GSH can cleave disulfide bonds between decanethiol and MSNs. Then GSH will covalently be bonded to MSNs through disulfide bonds. In this case, gatekeepers actually exchanged from decanethiol to GSH. But GSH molecules are not big enough to block the pore entrance, due to the difference of the molecule structure between decanethiol and GSH (Figure 5.11 A). The space between substituted GSH molecules provides SA with a pathway (green dash lines in Figure 5.11 B and C) to diffuse from the inside to outside of the pores. In addition, the concentration of GSH determines the release rate of SA. When exposed to 5 mM GSH, the proportion of cleaved disulfide bonds between decanethiol and MSNs is less than that when exposed to 10 mM GSH. In other words, the release pathway for SA is still blocked by decanethiol with the addition of 5 mM GSH (Figure 5.11 B), and the pathway of adding 10 mM GSH is larger, which possess quick release of SA (Figure 5.9).
Figure 5.11 Release of salicylic acid via cleavage of disulfide bonds between decanethiol and MSNs when exposed to 5 mM (A) and 10 mM (B) of GSH in aqueous solution and the molecular structure of MPTMS, decanethiol, SA and GSH (C).
5.9 Induction of defence genes in Arabidopsis and the corresponding GSH accumulation

Figure 5.12 Housekeeping gene actin and defence gene (PR-1) expression in Arabidopsis with MSN, SA and SA@MSN-SS-C10 treatment on 3rd, 5th and 7th day. M is HyperLadder™ IV (Bioline) and -Ve is the blank channel.

Figure 5.13 Gene expression in the plants without any treatment at day 3, 5 and 7. Only housekeeping gene actin can be detected. No PR-1 gene expression is observed.

The SA can induce the defence gene expression that is related to the resistance to the plant pathogens (Rivas-San Vicente & Plasencia 2011). To investigate the effects of SA loaded MSNs on the gene expression, the defence gene PR-1 and the housekeeping gene actin were measured at third, fifth and seventh day after vacuum infiltration with SA, MSNs and SA@MSN-SS-C10. Plants exposed to MSNs showed a slight increase in PR-1 expression at the third day, but highly expressed when treated with SA at the same day (Figure 5.12). At the fifth and seventh day post-treatment, no signs of PR-1 expression was observed in plants treated with MSNs, but slight expression in the induction of PR-1 was observed on day five with plants treated with SA and on day seven no signs of the defence gene was observed. More interestingly, PR-1 expression
was up-regulated in plants treated with MSN-SA-C10 for all the days tested (Figure 5.12), while for the plants without any treatment, there is no PR-1 expression at these days (Figure 5.13). These results suggested that the SA can induce the *PR-1* gene expression at a very high level, but the gene expression of *PR-1* cannot be maintained due to the consumption of SA by plants, which attribute to the decrease of expression at fifth and seventh day. Because the GSH concentration in plant cells is at nano-mole level, the release of SA is very slow. The slowly released SA maintains the *PR-1* gene expression even at the seventh day after treatment.

![Figure 5.14 GSH accumulation in Arabidopsis leaves post vacuum infiltration with MSN, SA and SA@MSN-SS-C10. The values for each data point represents the mean ± SE of three replicates. FW-Fresh Weight.](image)

The GSH induces the release of SA from MSNs with gatekeepers. It is worthy investigating the level of GSH in plants. All Arabidopsis plants treated with MSN, SA and SA@MSN-SS-C10 were analysed for GSH accumulation in days parallel to that of PR-1 gene analysis. Constitutive accumulation of SA had strongly increased GSH levels with plants three days after SA treatment (Figure 5.14). Five and seven days after the treatment with SA, GSH was decreased, indicating a strong correlation between SA levels and GSH content. Interestingly, the plants treated with SA@MSN-
SS-C10 were still able to maintain the same glutathione redox state as in comparison with plants treated with MSN and SA (Figure 5.14). Deficiency in SA, however, results in lower GSH levels and an inability of the plant to increase the GSH level efficiently. The relationship between SA and GSH is synergetic. According to the release kinetics of SA@MSN-SS-C10 (Figure 5.9), the slowly released SA (0 mM GSH) promotes the accumulation of GSH in plant cells, and the increased GSH level induces more SA to be released from the MSNs with gatekeepers. This cyclic process maintains the supply of SA to plants from MSNs with gatekeepers up to 7 days, leading to the constant expression of PR-1 gene. With the same amount of SA (5 mM) used in this study, the efficiency of the SA encapsulated in MSNs is greatly superior to the bare SA. Therefore, the long term resistance to plant pathogen can be achieved with this constant PR-1 gene expression.
Chapter 6: Pore Size Control of MSNs via Soft Expanding of Surfactant Templates and its Application as siRNA Carriers

Highlights of Contributions:

- The pore size of MSNs can be controlled by adjusting the ratio between TMB and CTAB up to 18 nm in diameter.
- The flower-like mesoporous structure was investigated through TEM at different tilting angles.
- The surface of pore expanded MSNs was modified by amine groups to achieve positive charge, which offers an electrostatic force to absorb negatively charged molecules such as siRNA.
- The pore expanded MSNs with positive charge can absorb siRNA at a high loading capacity.

6.1 Introduction

Small interfering (siRNA) participates in the regulation of gene expression and silencing, thereby influencing protein production (Rost et al. 2006) and is of great importance for plants. To deliver exogenous siRNA into plants, microparticle bombardment is a well-known method. Gold nanoparticles coated with siRNA are bombarded into plant tissues to achieve gene transfer. However, these heavy metal nanoparticles cannot be degraded by plants and have potential side effects on the growth of plants (Dietz & Herth 2011). Furthermore, the harmful agent can easily invade siRNA coated on the surface of gold nanoparticles, leading to the function vanished. Therefore, a system with non-phytotoxicity and protecting shield is required to safely and efficiently deliver siRNA into plants.

In the previous chapters, we have demonstrated the development of MSNs with small particle size and stimuli-responsive gatekeepers to control the release of SA into plants. MSNs are non-toxic to plants, which makes MSNs as ideal candidatures in plant related applications. The pore size of these MSNs is around 2-3 nm that is suitable for
the loading of small molecules like SA. However, large molecules such as siRNA cannot be encapsulated inside mesopores due to the pores being too small. Thus, pore expanding agents, such as trimethylbenzene (Na et al. 2012), polystyrene nanoparticles (Nandiyanto et al. 2009) and co-block polymers (Niu et al. 2014a), are required to enlarge the pores of MSNs. Even though over 10 nm pore size can be achieved, these methods always result in large particles that reduce the permeability through cell membrane. Furthermore, to the best of our knowledge, the application of MSNs with large pore size has not been reported.

In this chapter, the pores of MSNs are enlarged via soft expanding of surfactant templates, and siRNA was used as a model to demonstrate the feasibility of using pore expanded MSNs as carriers for large molecules. Through controlling the ratio between surfactant and expanding agent, the pore size can be controlled in a broad range from 2 to 18 nm. The particle size varies from 25 to 60 nm. Due to the small aspect ratio between pore size and particle size, the flower-like structure can be observed under TEM. The surface of the MSNs with large pores were modified with amine terminated silane to obtain positive charge. Through electrostatic interaction, negative charged siRNA can absorb onto positively charged MSNs and the loading was found to be 100 nM of siRNA in 2 μg of pore expanded MSNs. As a prospective research and to broaden the area of using MSNs in plants, the ability of MSNs to carry siRNA has been demonstrated and the platform for the future work has been established.

### 6.2 Experimental and Methodology

#### 6.2.1 Synthesis of MSNs with large pores

The experimental parameters of MSNs with large pores are based on the synthesis of MSN-AM described in Chapter 2. The 1,3,5-trimethylbenzene (TMB) was introduced into the synthesis process to expand the pore size. The molar ratio between TMB and CTAB is 2, 4 and 8. The specific synthesis of sample with TMB/CTAB ratio of 8 is demonstrated as follows: 2.96 g of CTAB was dissolved in 100 mL of Milli-Q water whose pH had been adjusted to 10 by ammonium hydroxide. The mixture was heated to and kept stirring at 80 °C for 15 min and then 8.91 mL of TMB was added into the CTAB solution stirring for another 15 min at 80 °C. Subsequently, the mixture with
CTAB and TMB was cooled to 30 °C followed by dropwise addition of 1.86 ml TEOS. The reaction was maintained for 24 h under a stirring speed of 550 rpm, and then the mixture was aged at 80 °C for another 24 h. Subsequently, the ageing products were stirred at room temperature for 24 h. After that, the products were filtered through a membrane with the pore size of 100 nm and washed with ethanol for three times to remove the free CTAB and TEOS. In order to remove CTAB templates inside pores, as-synthesised nanoparticles were redispersed in a solution with 100 mL of ethanol and 1 mL of HCl and stirred overnight at 60 °C. The MSNs without CTAB were centrifuge and washed with ethanol for 3 times. Finally, pore expanded MSNs were collected and dried under vacuum for the further use. The samples with different TMB/CTAB ratio were named as TMB-0.5, TMB-2, TMB-4 and TMB-8.

Table 6.1 Experimental parameters for pore expanded MSNs

<table>
<thead>
<tr>
<th>Samples</th>
<th>TMB (mol)</th>
<th>CTAB (mol)</th>
<th>TEOS (mol)</th>
<th>Water (mol)</th>
<th>Stirring time (h)</th>
<th>Ageing time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMB-0.5</td>
<td>0.004</td>
<td>0.008</td>
<td>0.008</td>
<td>5.5</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>TMB-2</td>
<td>0.016</td>
<td>0.008</td>
<td>0.008</td>
<td>5.5</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>TMB-4</td>
<td>0.032</td>
<td>0.008</td>
<td>0.008</td>
<td>5.5</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>TMB-8</td>
<td>0.064</td>
<td>0.008</td>
<td>0.008</td>
<td>5.5</td>
<td>24</td>
<td>24</td>
</tr>
</tbody>
</table>

6.2.2 Modification of surface of pore expanded MSNs

TMB-8 MSNs were selected for the modification with amine groups, because of their uniform pore size distribution. The modification process was conducted post-synthetically or during the synthesis process (co-condensation). The detailed procedure is as follows.
**Post synthesis:** 40 mg TMB-8 with template removed were dispersed into 40 mL of absolute ethanol and the mixture was sonicated until the uniform suspension of TMB-8 was achieved. Under vigorous stirring, different amount (20 μL, 60 μL, 100 μL, 400 μL or 1000 μL) of APTES was added dropwise into the mixture. After 1 hour, 0.5 mL of deionized water was added and the reaction was maintained for 24 hours under room temperature with stirring. The products were centrifuged and washed by absolute ethanol for three times. The APTES modified TMB-8 was collected after a vacuum drying. This sample is denoted as TMB-8-A-post.

**Co-condensation:** Specifically, 2.96 g of CTAB was dissolved in 100 mL of Milli-Q water whose pH had been adjusted to 10 by ammonium hydroxide. The mixture was heated to 80 °C and after 15 min stirring at 80 °C, 8.91 mL of TMB was added into CTAB aqueous solution. The mixture was kept stirring for another 15 min at 80 °C in order to stabilize the structure of CTAB micelles. Subsequently, the aqueous solution of CTAB was cooled down to 30 °C followed by dropwise adding 1.86 ml TEOS. The reaction was maintained for 24 h under a stirring speed of 550 rpm. After that, 0.5 mL of APTES was added to the mixture and stirred at 80 °C for another 24 h. The products were filtered and washed by ethanol for three times to remove free CTAB and TEOS. After filtration, the as-synthesised MSNs were resuspended in 100 mL of ethanol and 1 mL of HCl at 60 °C overnight to remove CTAB micelles in pores. The final products were filtered and washed by ethanol and distilled water for three times and then were dried under vacuum. The dried white powder was kept in a glass bottle for future use. This sample is denoted as TMB-8-A-co.

### 6.2.3 Characterization

The TEM and nitrogen sorption isotherms were used to investigate the morphology of nanoparticles and the mesoporous properties. The detailed characterization methods were described in Appendix B. Zeta potential of nanoparticles dispersed in phosphate buffered saline buffer (PBS) (pH=7.4) was measured using a Zetasizer Nano (Malvern, United Kingdom). The concentration of the nanoparticles in liquid was 0.5 mg/mL.
6.2.4 siRNA loading capacity

The siRNA was purchased from Qiagen (Australia), which is synthetic 22 nucleotide siRNA against GFP expression. The sequence of the siRNA is as follows: sense-gaacuucaggucagcuugccg and antisense-gcaagcuggacccugaaucau. To prepare each siRNA–nanoparticle complex, siRNA (1 μg) was incubated with a predetermined (5, 10, 50 and 100 μg) amount of TMB-8-A-post at 4 °C in phosphate buffer saline solution (PBS). After 1 h of incubation, siRNA loading dye was added to each mixture, and then the mixtures were loaded on the agarose gel (1 %). Gel electrophoresis was carried out at 90 V for 30 min in Tris/borate/EDTA buffer, and the siRNA bands were visualized on a UV trans-illuminator.

6.3 Morphology of pore expanded MSNs

To investigate the effects of TMB on the morphology of nanoparticles, MSNs were synthesised under different TMB/CTAB ratio. The TMB plays an important role in the morphology of final products. The well dispersed nanoparticles can be seen at low magnification under TEM (Figure 6.1), which is because the low ionic strength (only ammonia presence) does not screen the surface charges between adjacent silica particles according to the corresponding discussion in Chapter 2. All the samples are spherical nanoparticles and have a uniform particle size, namely TMB-0.5 (~20 nm), TMB-2 (~30 nm), TMB-4 (~30 nm) and TMB-8 (~50 nm).

Compare to the non-expanded MSNs denoted as MSN-AM in Chapter 2, the obvious expansion of the pores can be seen at high magnification under TEM (insets in Figure 6.1). Lower content of TMB (TMB-0.5) has less impact on the porous structure. When the ratio of TMB/CTAB is 2, the obvious expansion of pores has been observed (Figure 6.1 B), and this is a finding different from the literature (Rathod & Mexico 2007) which stated that the clear pore expansion cannot be seen until the ratio of TMB/CTAB reached 8. The particle size in the previous studies was sub-micro or several hundred nanometers, whereas in this study the particle size is less than 50 nm. This indicates that the TMB has a greater impact on the smaller particles than bigger particles.
(A) TMB-0.5.

(B) TMB-2.
Figure 6.1 TEM images of pore expanded MSNs with different TMB/CTAB ratio. The inset figures are individual particles under high magnification to reveal the porous structure.

Interestingly, a flower-like structure is formed with the expansion of TMB. One
particle was selected and its structure has been shown in Figure 6.2 under high resolution TEM. The pore shape is highly deformed, and with the expansion of pore size, the aspect ratio of pore size and particle size becomes high, but the porous structure is still maintained without collapse.

In order to understand this unique porous structure, TMB-8 was selected to be observed under TEM at different tilting angles from -30° to +30° every 10° due to the obvious large pores. The TEM images of TMB-8 tilted at different angles are shown in Figure 6.3. These images provide a 3-dimensional view of the structure of particles. The particle is spherical and mesopores are distributed throughout the whole particle. But the angles at ± 30° could not provide much more detailed information about the porous structure and further increasing the tilting angles will be the focus in the future work. Despite this, from the view at different angle, the thin silica wall only occupies a small volume of one particle, while the most volume is the mesopore area. This porous structure with open pore area could potentially benefit the loading of siRNA, compared to the spherical nanoparticles with non-porous structure or small pores.

Figure 6.2 One particle of TMB-8 under high resolution TEM. The particle and pore area are highlighted.
Figure 6.3 TEM images of pore expanded MSNs at different angles (from -30° to +30°). The particle pointed by the yellow circle is the focus.

6.4 Porous structure of expanded mesopores

To further investigate the porous structure, the pore size distribution was measured with nitrogen sorption isotherms. It can be seen from the nitrogen sorption isotherms (Figure 6.4 A) that the hysteresis loop of all samples are at the range of 0.8 < P/P₀ < 1, which indicates the pores of larger than 10 nm are presented in MSNs (Zhang et al. 2007).

From the pore size distribution (Figure 6.4 B), the pore size for all samples is still within the range of the meso-scale (2-50 nm). The TMB/CTAB ratio of 0.5 is found to have little impact on the pore size compared to the non-expanded samples described in Chapter 2. The textural pore size can also been seen in TMB-0.5, due to the pores formed by the stacking particles. In TMB-2, the pore size has been expanded to 12.9 nm with another peak at 22.3 nm, which suggests the heterogeneous mesoporous structure. With the increase content of TMB, the uniformity of pore size distribution is improved. In TMB-4 and TMB-8, a narrow distribution at 13.1 and 18.2 nm, respectively can be detected. The size of siRNA is normally 2 nm in diameter and 7 nm in length (Na et al. 2012), thus these samples of TMB-2, TMB-4 and TMB-8 are
suitable for the loading of siRNA.

Figure 6.4 Nitrogen sorption properties of pore expanded MSNs with different TMB/CTAB ratio. For clarity, the curves are shifted with 600 (TMB-2), 1100 (TMB-4), 1600 (TMB-8) in (A), and 0.007 (TMB-2), 0.012 (TMB-4), 0.018 (TMB-8) in (B).
The loading capacity is also related to the surface properties and pore volume. It can be seen from Table 6.2 that all samples have a high surface area up to 537.85 m²/g. The sufficient surface offers area for the siRNA lying on. Pore volume increases to 0.41 cm³/g (TMB-0.5), 1.49 cm³/g (TMB-2), 2.29 cm³/g (TMB-4) and 1.81 cm³/g (TMB-8), compared to the non-expanded MSNs (MSN-AM, 0.31 cm³/g). In TMB-0.5, the pore size was not dramatically enlarged, thus the pore volume is low. When increasing TMB/CTAB ratio the pore size becomes larger. TMB-2 and TMB-4 have a similar pore size distribution, leading to the similar pore volume. The biggest pore size was detected in TMB-8, which possesses the highest pore volume.

<table>
<thead>
<tr>
<th>Samples</th>
<th>BET Surface Area (m²/g)</th>
<th>Pore Volume (cm³/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMB-0.5</td>
<td>537.10</td>
<td>0.41</td>
</tr>
<tr>
<td>TMB-2</td>
<td>451.18</td>
<td>1.49</td>
</tr>
<tr>
<td>TMB-4</td>
<td>535.48</td>
<td>1.50</td>
</tr>
<tr>
<td>TMB-8</td>
<td>537.85</td>
<td>1.81</td>
</tr>
</tbody>
</table>

* a The pore volume was the effective pore volume that is used to load biomolecules rather than the total pore volume including the inter-particle packing volume.

All the results from morphology study and porous properties suggest the potential application of these MSNs as a siRNA carrier. The particle size less than 50 nm can offer high permeability through cell membrane. The large pore size ensure the loading of big molecules and the high surface area and pore volume provide space for molecules to achieve a high loading capacity. Moreover, the molecules can be loaded on the inner surface of pores rather than on the outer surface of non-porous nanoparticles, which can protect these molecules from the harmful agents in the environment.

### 6.5 Charge modification of pore expanded MSNs

Electrostatic force is a simple and powerful means to load siRNA into MSNs. Because the siRNA is negatively charged, the surface of the MSNs needs to be functionalised
with positive charge to facilitate the siRNA encapsulation. In order to introduce positive charge onto pore expanded MSNs, APTES as an amine terminated silane was employed to modify silica surface with amine groups that provide positive charge.

In this study, TMB-8 was selected as the material for surface functionalisation due to its large and uniform pore size. Post-synthesis and one-pot synthesis were conducted to get positive charge. The zeta potential of different samples is shown in Figure 6.5. The raw TMB-8 without APTES modification has a zeta potential of -19 mV. In the post-synthetic process, the particles were already formed and the surfactant in mesopores was removed. When introducing APTES up to 400 μL, a clear trend of increased charge to 0 mV can be detected from the zeta potential measurement, but the charge is still negative and approaching to 0 mV. The positively charged TMB-8 can be obtained with a large amount of APTES (1000 μL). During the one-pot synthesis, APTES was added during the synthesis of TMB-8. The charge of TMB-8 in one-pot is 20.8 mV that is higher than that of TMB-8 with 1000 μL of APTES. The one-pot synthesis was found to be more efficient and only 500 μL of APTES was used during the synthesis, which can achieve a higher surface charge.

![Figure 6.5 Zeta potential of unfunctionalised MSNs and amino-functionalised MSNs with different APTES dosage.](image)

The surface charge is determined by the coverage of amine groups, which can be calculated from the thermogravetric analysis. The TMB-8 with positive charge in post synthesis and one-pot synthesis were compared to understand the relationship between
amination and surface charge. It can be seen from Figure 6.6 that the decomposition temperature of amine groups is at around 300 °C. The weight losses are 6.8 and 11.2 % in samples of post synthesis and one-pot synthesis, respectively and the corresponding coverages are 1.2 and 1.9 mmol/g MSNs for these samples. Thus, the higher amine coverage leads to the higher surface charge on MSNs.

Figure 6.6 Thermogravetric analysis of functionalised MSNs. The weight loss caused by absorbed water was removed and curves are shifted, starting from 100 % weight loss.

The introduction of amine groups onto the surface of TMB-8 has changed the morphology of particles. It can be seen from the high resolution TEM images (Figure 6.7) that the original wall thickness of TMB-8 is 2.9 nm. After grafting amine groups onto the surface, the wall thickness increases to 5.1 and 6.6 nm in the samples of post-synthesis and one-pot synthesis, respectively, which indicates the successful grafting of APTES. The reaction degree of silane is higher in one-pot functionalization than in the post modification. The thicker silica wall means the higher coverage of functional groups on the surface of silica, which can also be proved by the results from TGA (Figure 6.6). More importantly, the porous structure is maintained after grafting, which retains the space for the absorption of siRNA.
(A) non-functionalised TMB-8

(B) amine-functionalised TMB-8 in post synthesis (zeta potential +10.5 mV)
6.6 Loading of siRNA

The TMB-8 with amine groups in post synthesis was used to load siRNA. The porous structure and functionalization of TMB-8 with positive charge benefit the loading of siRNA. On one hand, the opened bowl-like mesopores offer an easy access for siRNA to the inner pore area (Figure 6.8 A). There are fewer than 10 pores in one particle, which means that for one particle, the pores occupy the most space in three dimensional space. The effective use of the spatial area allows for the high loading of siRNA in nanoparticles. On the other hand, the electrostatic force provided by the surface charge retain the absorption of siRNA and carry them into cells.

The siRNA was loaded onto each particle by incubation of the particles (5, 10, 50 and 100 μg) with siRNA (1 μg) in phosphate-buffered saline (PBS, pH 7.4) for 1 h at 4 °C. The use of low temperature is to avoid the malfunction of siRNA. Adsorption and loading capacity of siRNA onto the particles were characterized by agarose gel electrophoresis. The loading capacity was measured to find out the
siRNA/nanoparticle ratio at which the siRNA can be completely absorbed by

Figure 6.8 (A) Cross-section of one TMB-8 functionalised with amine groups and (B) Agarose gel assay showing siRNA loading in standard wells. Lane 1 = Naked siRNA (1 μg), Lanes 2-5 = Ratios of siRNA to MSN18 (1/5, 1/10, 1/50 and 1/100 μg).
nanoparticles. The first channel is the control group which contains 1 μg of siRNA (Figure 6.8 B). After mixing siRNA with 5 and 10 μg of TMB-8-A-post, the siRNA is not completely absorbed by MSNs with a band of unbound siRNA in the electrophoretic gel. When the content of siRNA is over 50 μg, the siRNA band totally disappeared, indicating the complete loading of siRNA. These preliminary results suggested that siRNA could be efficiently loaded inside the TMB-8-A-post at a siRNA/MSN ratio of 1/50-1/100 μg.
Chapter 7: Conclusion and Future Work

7.1 Summary

The development of advanced agrochemical delivery systems is still at its early stage, but when incorporated with nanotechnology, there is potential for this system to provide efficient utilization of agrochemicals. This thesis is to establish an agrochemical delivery system based on functionalised mesoporous silica nanoparticles.

In Chapter 2, a facile and simple process was introduced to successfully synthesise MSNs of small particle size at around 20 nm with non-ordered and interconnected mesoporous structure. The morphology of the MSNs can be controlled through the manipulation of experimental conditions, such as CTAB concentration, temperature, pH value and ionic strength of the reaction solution. The initial CTAB concentration has no significant impact on the pore size with less than 0.5 nm difference when changing the concentration of CTAB from 60, 80 to 100 mM. The low temperature (30 °C) favours the growth of MSNs with small particle size (around 20 nm) and the high temperature (95 °C) benefit the formation of mesopores with uniform size distribution. The high pH and ionic strength result in the interaction between neighbouring nanoparticles, leading to the aggregation. The collapse of particulate structure was detected at the pH of 12 and ionic strength of 0.20 mol/L. The discrete MSNs with spherical shape and uniform particle size were achieved in the ammonia solution that had an ionic strength as low as 0.00005 mol/L. As the aim is to apply MSNs to build up an agrochemical delivery system, the synthesised MSNs in ammonia solution possesses better shape and dispersity than other conditions. These MSNs are ready for the agricultural applications as agrochemical carriers. The following sections will describe the surface modification and the loading and release of a model agrochemical, which will prove the concept of using MSNs in agriculture.

In Chapter 3, Time-resolved SAXS at a synchrotron source was employed to study the growth mechanism of MSNs. The experimental conditions including temperature, pH and ionic strength were also investigated. The growth kinetics of MSNs was investigated through the SAXS invariant. The method was shown to be an efficient and powerful manner to estimate the growth kinetics. The results demonstrated that
under the experimental conditions of high temperature (95 °C) and pH (10), a fast reaction rate was observed compared to the conditions of low temperature (30 °C) and neutral pH (7.2). Through modelling SAXS data before and after TEOS addition, the TEOS was found to be inserted into the hydrocarbon core of CTAB micelles, expanding the micelle size. The growth of MSNs was found to be in TEOS emulsion system. A ‘swelling-shrinking’ model was proposed to explain the MSN growth based on the investigation of structural evolution under time-resolved SAXS, which provides further information about the location of TEOS during the synthesis. This model provides a new insight into the understanding of the MSN growth mechanism and makes a contribution to theoretically interpret the MSN synthesis.

In Chapter 4, the well-dispersed MSNs were grafted with fluorescent dye in order to track the translocation of MSNs inside plants. According to the auto-fluorescence from the endogenous protein in plants, FITC and RITC were selected as indicators to localize MSNs in different plant tissues. Bright and stable fluorescence under confocal microscope can be obtained after dyes were grafted onto the surface of MSNs. The MSNs were found to be non-toxic to plants during seed germination. The concentration of MSNs in the culture medium can be up to 2 mg/mL. The MSNs with fluorescent dyes can be detected in the roots, stems and leaves in the intact plants. The RITC was found to be distinguished with the auto-fluorescence from the plant roots. The quantity of MSNs uptake by plants was determined by the Micro-PIXE. The accumulation of MSNs in the root ranges from 25 to 37.5 % of the totally added MSNs. The above results suggested that both the symplastic and apoplastic pathways can be used by MSNs to get into plants, transport through xylem system and reach the leaves. This information provides the basic understanding of the interaction between MSNs and plants.

In Chapter 5, the concept of small molecules as gatekeepers to protect gust molecules in MSNs was proved. Decanethiol was selected as the gatekeeper bonded onto MSNs through disulfide linkage that can be cleaved when GSH was added. Salicylic acid has been chosen as a model agrochemical to be loaded into MSNs to demonstrate the effectiveness of gatekeepers. Decanethiol gatekeepers can slow down the release rate of SA and be reopened at the reducing condition caused by GSH. The release rate when adding GSH (5 mM and 10 mM) is much higher than the release without GSH. The particle size can be maintained by using small molecules as gatekeepers and with
Chapter 7: Conclusions and Future Work

the help of redox-stimuli disulphide bonds smart release of agrochemicals can be achieved. Through controlling the reaction degree of thiol disulfide exchange between MSN-SH and didecyl-disulfide, the coverage of decanethiol on MSNs can be controlled, which will be a useful method to control the release rate of loaded chemicals without increasing particle size. Therefore, future work will be focusing on optimization of the experimental conditions and phytotoxicity assay in plants. Even though the MSNs can be fully functionalised to be as a carrier to release agrochemicals in a controlled fashion, the synthesis cost of MSN is the another issue that has to be taken into account in order to introduce MSNs into the realistic agricultural industry in the future.

In Chapter 6, the pore size of MSNs was expanded with an expanding agent in order to carry large molecules. The TMB was introduced to enlarge the CTAB micelles before the addition of TEOS to form silica framework. The effects of TMB/CTAB ratio on the particle morphology were investigated. The low TMB content (TMB-0.5) has no significant impact on the pore size, while with the increasing TMB content (TMB-2, TMB-4 and TMB-8), large pores can obviously be observed under TEM. The pore size distribution also suggests the success in pore expansion. The uniformity of pore size is improved with high TMB content (TMB-4 and TMB-8). In order to load siRNA into mesopores, the surface of TMB-8 was modified with amine groups to obtain positive charge, which can adsorb negatively charged siRNA via electrostatic interaction. The loading capacity of amine functionalised TMB-8 was found to be 1 μg of siRNA in 50 μg of MSNs. This preliminary research has provided a material platform for the application of MSNs in the area of gene transfer.

7.2 Future work

7.2.1 Yield of nanoparticles

The yield of MSNs is important for the application of MSNs in agriculture. In our studies, the experimental conditions of MSN synthesis has been optimized in Chapter 2 and well dispersed nanoparticles were obtained in ammonium hydroxide solution at a very low ionic strength. But the yield of this sample was found to be very low compared to the MSNs synthesised in the phosphate buffer solution. Its yield is
improved by increasing TEOS concentration, but this compromises the surface area and pore volume, which deteriorate the loading of agrochemicals. Therefore, further optimization of experimental conditions is required to get an ideal yield of well-dispersed MSNs.

7.2.2 Agrochemical loading and encapsulation

A redox-responsive gatekeeper has been introduced to encapsulate SA in MSNs and release SA triggered by GSH in Chapter 5. The loading of SA in MSNs was found to be less than 10%. The loading capacity is relatively low. The further study will focus on the improvement of agrochemical loading. Another issue is residual thiol groups after assembling gatekeepers on MSNs, which leads to the leaking of SA before opening the gatekeepers. This maybe because the exchange rate between thiol and disulfide is low. The future work will focus on the improvement of thiol-disulfide exchange.

7.2.3 Soil test of SA loaded MSNs

In Chapter 4, the translocation of MSNs in plant has been detected throughout the whole intact plants, through the fluorescent labelling. In Chapter 5, the SA loaded and decanethiol capped MSNs can slowly release SA in plants and induce the expression of pathogen resistance related gene. These plant experiments were conducted only in hydroponic system that is good for the basic research. In agriculture, however, soil system is the main culture medium to grow plant, which is more complicated compared to the soilless system. The future work will shift to the soil system and corresponding experimental design is required.

7.2.4 In-vitro and In-vivo test of gene silencing

The negatively charged siRNA can be loaded into positively charged and pore expanded MSNs at a high loading capacity in Chapter 6. *In-vitro* (protoplast experiment) and *in-vivo* (intact plant experiment) tests are required to investigate the efficiency of gene silencing, as only a loading experiment with siRNA has been done
in Chapter 6. Based on the material platform, the actual application of gene silencing can confirm the usefulness of this system.

7.2.5 Commercial application of MSNs in agriculture

MSNs possess superior properties to other nanoparticles, because MSNs are silica based materials with low toxicity to both plant and mammalian cells and the porous structure is ideal for the loading and encapsulation of biomolecules compared to non-porous nanoparticles. However, the cost is the main aspect that determines if the material can be utilised in a commercial application. In order to extend the application of MSNs to the agricultural industry, the cost on synthesis and functionalization could be decreased by using inexpensive chemicals, such as silicic salt, naturally existed surfactants and simpler gatekeeper system. The efforts on exploring cost-effective method can potentially lead to the development of MSNs with new structures.
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## Appendix A: Materials

<table>
<thead>
<tr>
<th>Chemical names</th>
<th>Abbreviation</th>
<th>Purity</th>
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<tr>
<td>Cyltrimethylammonium bromide</td>
<td>CTAB</td>
<td>≥99%, Sigma-Aldrich</td>
</tr>
<tr>
<td>Tetraethyl orthosilicate</td>
<td>TEOS</td>
<td>98%, Sigma-Aldrich</td>
</tr>
<tr>
<td>Fluorescein isothiocyanate</td>
<td>FITC</td>
<td>≥90%, Sigma-Aldrich</td>
</tr>
<tr>
<td>Rhodamine b isothiocyanate</td>
<td>RITC</td>
<td>≥90%, Sigma-Aldrich</td>
</tr>
<tr>
<td>3-Mercaptopropyltrimethoxysilane</td>
<td>MPTMS</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>(3-Aminopropyl)trimethoxysilane</td>
<td>APTES</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Decanethiol</td>
<td>--</td>
<td>96%, Sigma-Aldrich</td>
</tr>
<tr>
<td>Sodium iodide</td>
<td>--</td>
<td>99.5%, Sigma-Aldrich</td>
</tr>
<tr>
<td>Glutathione reduced</td>
<td>GSH</td>
<td>≥98%, Sigma-Aldrich</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>SA</td>
<td>&gt;99%, Sigma-Aldrich</td>
</tr>
<tr>
<td>1,3,5-Trimethylbenzene</td>
<td>TMB</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>NaOH</td>
<td>AR grade</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>KH$_2$PO$_4$</td>
<td>AR grade</td>
</tr>
<tr>
<td>Sodium carbonate</td>
<td>Na$_2$CO$_3$</td>
<td>AR grade</td>
</tr>
<tr>
<td>Ammonium hydroxide</td>
<td>--</td>
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<tr>
<td>Hydrochloric acid (32%)</td>
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</tr>
<tr>
<td>Ethanol</td>
<td>--</td>
<td>AR grade</td>
</tr>
<tr>
<td>Ethyl acetate</td>
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<td>AR grade</td>
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<tr>
<td>Hydrogen peroxide solution (30%)</td>
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</table>

All chemicals were used without further purification.
Appendix B: Characterization

B.1 Characterization of MSN morphology

B.1.1 Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) images were obtained on a JEM-2100 (JEOL, Japan) electron microscope operating at an accelerating voltage of 200 kV. MSN samples were firstly dispersed in absolute ethanol by ultra-sonication. About 20 μL of MSN suspension was dropped on to a copper gird and dried at room temperature to eliminate the ethanol. The copper grid was placed in the sample holder to visualize the morphology of MSN samples under TEM.

Two types of sample holder can be selected for different purpose. With the help of penta holders, five samples can be inserted into TEM for each time to save the vacuum time. Single tilt holder allows for the rotation of samples along the axis of the holder. MSNs can be visualized at different angles up to ± 30°.

B.1.2 Scanning electron microscopy (SEM)

Scanning electron microscopy images were captured on a Supra 55VP (Zeiss, Germany). MSNs were dispersed in absolute ethanol by ultra-sonication. About 20 μL of MSN suspension was dropped onto an aluminium stub and dried at room temperature. The dried samples were then coated with carbon using a BAL-TEC SCD 050 sputter coater (Leica Microsystems, Australia). Due to the oxidation of carbon coatings in air, the coated samples have to be directly transferred into SEM chamber to minimize the effects of the oxidation.

B.2 Investigation of porous structure - Nitrogen adsorption and desorption isotherms
B.2.1 Theory

Brunauer-Emmet-Teller/Barrett-Joyner-Halenda (BET/BJH) method has been used to measure the pore size and distribution. In this method, nitrogen at 77 K will condense on the solid surface of porous materials. As the nitrogen pressure is lower than saturated vapor pressure, the nitrogen molecules absorbed on the surface will form monolayer that is one molecule thick and then multilayer that will fill the smaller pores first. When nitrogen pressure is approaching to saturated vapor pressure, all pores will totally fill with gas molecules. After that, the decrease of gas pressure will lead to desorption of nitrogen molecules. Finally, the adsorption and desorption of nitrogen is obtained to calculate pore size and distribution.

In BET/BJH method, the calculation of mesopores considers the shape of pores as cylindrical capillaries, and the volume of nitrogen will be measured with the increase of relative pressure \( p^0/p \) in both period of adsorption and desorption. The pore size is calculated from the modified Kelvin equation,

\[
\frac{2\sigma^l g v^l}{RT \ln(p^0/p)}
\]

where \( r_K \) is Kelvin radius, \( \sigma^l g \) is the surface tension of the liquid condensate, \( v^l \) is molar volume, \( p^0/p \) is the relative pressure and \( R \) and \( T \) are constant. If the radius of cylindrical pore is \( r_p \), then

\[
r_p = r_K + t
\]

where \( t \) is the thickness of nitrogen multilayer. In this equation, a certain nitrogen pressure that is expressed in \( p^0/p \) corresponds to a pore size, thus a serial data with the change of gas pressure will be obtained to form a pore size distribution.

B.2.2 Characterization method

Nitrogen adsorption–desorption isotherms were carried out on a Micromeritics Tristar 3000 analyzer (Particle & Surface Science, UK) at 77 K under a continuous adsorption condition. Prior to measurement, all samples were degassed at 40-100 °C for 24 h in a
vacuum oven before measurement. The degassing temperature is dependent on the particle properties. Pure MSNs without any functional groups can be degassed at higher temperature, while the functionalised MSNs should be degassed at lower temperature in order to avoid the decomposition of grafted functional groups. The pore size distribution was calculated from adsorption branches of isotherms by the BJH method. Pore volume and specific surface area were calculated by using BET-BJH methods.

**B.3 Tracking of MSN location in plants - Confocal laser scanning microscopy (CLSM)**

The fluorescence emitted from the dye grafted on the surface of MSNs can be detected under CLSM at a specific excitation wavelength. According to the results from fluorescence spectroscopy in Chapter 4, the excitation wave length of FITC and RITC modified MSNs are 413 and 560 nm. Thus, the channels with the excitation wavelength of 488 nm (490–525 nm band width) and 561 nm (565–610 nm band width) were used and the energy was set to be 15 % to minimize the heat damage of the laser.

**B.4 Determination of agrochemical loading and release - Ultraviolet-visible spectroscopy (UV-Vis)**

**B.4.1 Standard calibration curve**

UV-Vis spectroscopy was used to determine the concentration of SA. The spectra were obtained from USB-2000 or USB-4000 spectrometer (Ocean Optics, United States). Before measuring the concentration of SA in solution, a standard calibration curve was obtained from UV-Vis in different solvent. The absorbance at the wavelength of 306.06 nm in ethyl acetate and 296.59 nm in water were used as the standard in this study. The relationship between the absorbance and SA concentration was plotted and fitted by a linear fitting (Figure B.1).
Figure B.1 Standard calibration curve of SA in ethylacetate and water. The equations are obtained from linear fitting.
B.4.2 Online measurement of SA release

Figure B.2 A UV-Vis cuvette setup for online detection of SA release. The stirring bar is to mix the solution uniformly. The MSNs are trapped inside dialysis tube that is wrapped onto a steel mesh. The steel mesh can help to hold the dialysis tube on the top of the cuvette to keep the bottom clear for data acquisition. Because the measurement normally lasts for 24 h, the evaporation of liquid may lead to the increase of concentration. The lid is to prevent the evaporation.

To investigate the effectiveness of the gatekeepers in vitro, SA loaded MSNs and decanethiol capped and SA loaded MSNs were statistically tested under different concentration of GSH. Ultraviolet visible spectrum was introduced to determine the amount of SA released from MSN samples. Specifically, 3 mg of MSN samples were
placed on the top of a cuvette and the liquid in the cuvette was separated by a piece of dialysis tube in order to prevent the nanoparticles from moving to the bottom of the cuvette. The signal was collected from the bottom of the cuvette every one min. The liquid (3.5 mL) in the cuvette was consisted of water and different concentration of GSH (0 mM, 5 mM and 10 mM). For comparison, the release of SA from non-functionalised MSNs following the same procedures and concentrations was also examined.

B.5 Small angle X-ray scattering (SAXS) in Australian Synchrotron

B.5.1 Brief introduction of SAXS

Figure B.3 Schematic of key features in a SAXS beamline

The SAXS is a powerful characterization method that can obtain a lot of information about the structure of the materials. The key features for a SAXS measurement is shown in Figure B.3. The incident beam is generated from a Cu or Co source and aligned straight to hit on the sample. Because of the different components or different shape structure, the X-ray is scattered and collected by the detector. A 2-dimensional scattering pattern is obtained from the detector and can be integrated into 1-dimensional curve that is for the analysis and modelling. The SAXS data is normally described by the Intensity-Q vector plot in which the Q vector is calculated from the following equation,
where, $\lambda$ is the wavelength of X-ray and $2\theta$ is the angle between the intensity at Q vector and the incident beam. Moreover, the Q range is determined by the sample-to-detector distance ($d$). In order to obtain the information for specific feature, one needs to select a proper $d$ for the test. Detailed information about the relationship between Q range and $d$ can be found in the website of Australian Synchrotron (https://www.synchrotron.org.au/aussyncbeamlines/saxswaxs/saxs-specifications).

Figure B.4 Detection of different structure of MSNs under SAXS beam

In this thesis, SAXS was employed to investigate the structure of MSNs. Because MSNs are porous, there are three different phases that cause different electron density, namely air or solvent phase ($\rho_1$), silica phase ($\rho_2$) and pore area ($\rho_3$). When the samples are interacted with the X-ray beam, different scattering can be obtained from different phase. With the difference of scattering, the specific structural properties can be detected with SAXS, such as the particle size distribution (difference between $\rho_1$ and $\rho_2$) and pore size distribution (difference between $\rho_2$ and $\rho_3$). This is the basis of SAXS measurement on MSNs.
B.5.2 Time-resolved SAXS for investigation of MSN growth

(A) Support of the quartz capillary tube and assembly of rubber pipe connection.

(B) Setup of time-resolved SAXS in Australian Synchrotron.

Figure B.5 Photograph of the design for the time-resolved SAXS.

With the synchrotron source in SAXS, the high energy flux offers many benefits, such
as fast data acquisition, low signal-to-noise ratio and multiple tasks at the same time with other characterization method. In this thesis, the synthesis process of MSNs was examined online under SAXS in Australian Synchrotron. The designed apparatus and SAXS setup are shown in Figure B.5. The purchased quartz capillary tubes was first fixed in the rubber tube (Figure B.5 A). Because the wall thickness of the capillary tubes is 10 μm and it is very fragile, the capillary-rubber tube apparatus was taped onto a cardboard frame as a support. This capillary tube was then connected with the reaction reservoir and a peristaltic pump through the rubber tubes (Figure B.5 B). The reaction solution was circulate with the rubber tube and SAXS data was collected from the capillary tube dynamically. This time-resolved SAXS allows for the investigation of the growth of MSNs online and can also be applied to other particle synthesis system.

B.6 Detection of functional groups

B.6.1 Fourier transform infrared spectroscopy (FTIR)

The FTIR measurements were conducted on a Vertex 70 spectrometer (Bruker, Germany). The MSN samples were mixed with 0.2 g of KBr and grounded into fine powder. The amount of MSN samples used in FTIR is dependent on the sample appearance. The samples with colour are expected to be less amount than white samples. The pellets were made from compressing the powder in a DIE for 4 min. Before the FTIR measurement, the pellets were kept in the oven at 60 °C for 24 hours in order to remove physically absorbed water. The spectra were taken with 32 scans at a resolution of 4 cm⁻¹.

B.6.2 Raman spectroscopy

Raman and FTIR are supplementary techniques with each other to detect the functional groups. The low absorption in FTIR is normally high in Raman. Thus, Raman can be used to detect these groups that are difficult to find in FTIR, such as disulfide bonds in this thesis. Raman spectra were obtained with an inVia Raman microscope (Renishaw, United Kingdom) at the laser wavelength of 514 nm. The powder samples were dropped onto an aluminium foil covered glass slide. The aluminium foil was to
eliminate the background signal from the glass slide. A confocal microscope was used to focus the sample plane.

**B.6.3 Thermogravimetric analysis (TGA)**

The melting point of MSNs as silica based materials is over 1000 °C, while the decomposition temperature of functional groups on the surface of MSNs is normally less than 400 °C. Thus, when heat functionalised MSNs over the decomposition temperature of function groups, these groups should be burnt away and the weight loss can be used to quantify them. The TGA was used to measure the weight loss of functionalised MSN samples at high temperature. The test was conducted on a Netzsch STA 409 (Germany) over a range of temperature from 50 to 700 °C at a rate of 15 °C/min under air atmosphere. Before measurement, all powder samples were maintained in an oven at 60 °C overnight to remove the physically bonded water.

**B.6.4 SEM energy dispersive spectrums (SEM-EDS)**

The SEM-EDS were obtained from an X-Max 20 mm² SDD Energy Dispersive X-ray detector (Oxford, United Kingdom) in an SEM (Supra 55VP). The data acquisition time was 10 min for mapping elements. The MSN samples were dispersed in absolute ethanol with ultra-sonication. About 10 μL of MSN suspension was dropped onto an aluminium stub and dried at room temperature, which was then coated with carbon (5 time flash) in a sputter coater.