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Synthesis and Optimization of Surface Functionalized Mesoporous Silica Nanoparticles for Bioconjugation Platforms

THESIS

submitted in partial satisfaction of the requirements
for the degree of

MASTER OF SCIENCE

in Chemical and Biochemical Engineering

by

Anand Srinath Gopalan

Thesis Committee:
Assistant Professor Jered B. Haun, Chair
Associate Professor Szu-Wen Wang
Assistant Professor Allon I. Hochbaum

2016

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ABSTRACT OF THE THESIS

Synthesis and Optimization of Surface Functionalized Mesoporous Silica Nanoparticles for Bioconjugation Platforms

By

Anand Srinath Gopalan

Master of Science in Chemical and Biochemical Engineering

University of California, Irvine, 2016

Assistant Professor Jered B. Haun, Chair

A large amount of emphasis has been dedicated in recent years to introduce nanoparticles as a viable candidate for targeted therapies. In comparison to other candidates, mesoporous silica nanoparticles have the advantages of being biocompatible, easy to produce, and have the ability to prove to be a theranostic platform. To better study the specific targeting of diseased cells, adhesion studies of drug carrying bioconjugation constructs in fluid environments is required. The goal of this project was to develop a platform based on mesoporous silica nanoparticles that will be used for future multivalent adhesion studies. Specifically, mesoporous silica nanoparticles were synthesized with various sizes and aspect ratios, containing fluorescent dye to enable tracking studies, and with surface treatments that optimized stability and introduced primary-amine functional groups to facilitate attachment with targeted proteins and biomarkers. The effects of various parameters such as solvents, washing methods, secondary modifications and difference in concentrations of the starting materials for the synthesis mixture on the nanoparticles were studied in detail in this thesis. A portion of the study is also dedicated to optimizing a washing procedure to stabilize the particles in an aqueous medium in order to facilitate further modifications. The

results of the work in this project can be utilized to provide a platform for further assays in flow chambers after bioconjugation with targeting proteins through orthogonal chemistries to study the adhesion properties in much greater detail.

CHAPTER 1. INTRODUCTION

1.1 Nanotechnology in Drug Delivery, overview

Over the past few decades, the advent of medical technology has created an onus on finding novel methods to cure and treat serious diseases such as cancer. In the ensuing study of the physicochemical properties of the drugs and the cellular uptake mechanisms, nanoparticles have emerged as a serious contender in targeted therapies (1). Nanotechnology has proven to be useful in both therapeutic as well as diagnostic applications. In diagnostics, they have been shown to have applications in magnetic resonance imaging (MRI) and optical imaging, and therapeutics, and they have been shown to have a lot of potential especially in cancer. Paclitaxel, a cancer drug, has already been released into the market in a loaded nanoparticle form (2).

Despite the numerous advantages offered by nanoparticles in medicine, it is to be noted that there is a corresponding rise in safety concerns over their environmental footprint, toxicity and lack of scientists' knowledge in their biodistribution mechanisms. In order to learn more about the concerns, there has been an increase in the number of in vivo studies involving nanoparticles. In vitro studies do not give the necessary information required to judge the possible inflammatory and toxic responses that can be exhibited by the body in response to the small size of particles (3). Ideal nanoparticles will need to be able to specifically attack the diseased cells alone with no loss of activity or mass. Nanoparticles also are more permeable in the tumor microenvironment with a greater retention time (4). Multifunctional and multiplexed nanoparticles are next on the research radar to produce tailor made drug delivery platforms for diseases like cancer. Nanotechnology enabled systems have also been theorized to provide better collection of patient data. In the near future, the cost effectiveness of personalized medicine using nanoconstructs could see an optimization that would make it affordable in the real world (5).

1.2 Silica nanoparticles and their versatility

Among the various candidates from which to create nanoparticles, silica nanoparticles exhibit a few unique characteristics that could be tuned for various applications. Mesoporous silica exhibits biocompatibility, high encapsulation efficiency, controllable release and ease in production and tuning (1). Mesoporous Silica Nanoparticles (MSN) can be produced in different sizes and shapes and thus provide an opportunity to tune them as theranostic agents which can double as both therapeutic and diagnostic carriers. Unlike Quantum Dots that can be cytotoxic, MSN does not adversely affect cells on contact. Due to their sizes, between 50 to 200 nm, MSNs can be phagocytosed by cells and provide site specific targeting without affecting surrounding healthy tissue. The porous nature of the particles also provides two surfaces; internal and external, which can be selectively functionalized (6). In essence, the high relative surface area, variable surface functionalization and the ability to couple them with traceable modalities make MSNs a highly exploitable resource in nanomedicine (Figure 1) (7).

Another interesting advantage with working with silica is that they can be used to coat other nanoparticles to render them less cytotoxic, such as Quantum Dots and magnetic nanoparticles. A silica shell over the cores gives greater stability and lower cytotoxicity, thus providing better biomarkers (8). In vivo studies in mice to investigate the biocompatability of various sizes have been performed and found no significant side effects (9). MSN functionalized with functional polymers have also been shown to be viable gene delivery vehicles (10).

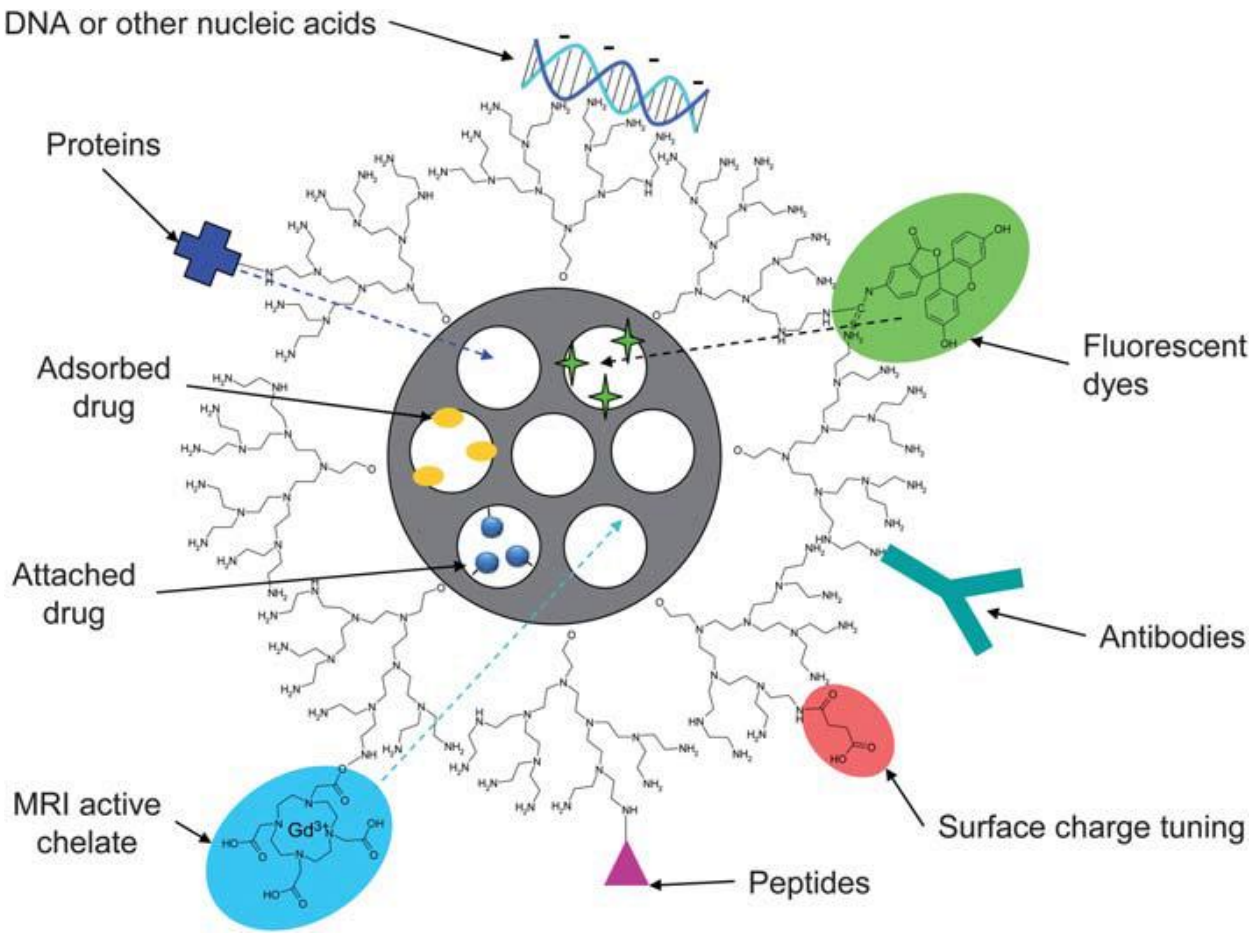

Figure 1 (7): The figure shows the versatility of MSN ranging from encapsulation of drug molecules, attachment to fluorescent dyes and tuning of surface charge.

Mesoporous silica nanoparticles have been known to exist for nearly five decades to scientists. But the idea that they could be used as drug delivery vehicles is relatively new and can be dated back to the late nineties. In vivo studies of silica nanoparticles and their uptake and excretion has been even slower. At this point, the amount of literature to do with the pharmacokinetics and biodistribution is still narrow (11). Werner Stober showed in 1968 that monodisperse silica particles could be produced with suitable precursors and solvents. But there has been a large amount of research in the topic since and nanotechnology has come a long way since. Perhaps the first silica nanoparticles recognized to have uses in medicine were ones in the family of particles discovered by Mobil Corporation in 1992. The pore sizes have been shown to

be tunable based on the micelle forming agent used in conjunction during the synthesis of the particles (12).

1.3 Functionalization of silica nanoparticles

As discussed earlier, one of the major advantages offered by MSN in a biomedical context is the existence of internal and external surfaces of pores for functionalization both by organic and inorganic methods. There has even been some interesting research in using MSN as nanodevices which respond to stimuli and deliver drugs in a controlled manner (13). There are a number of reasons functionalizing the surface of MSN is advantageous. As the particles are generally unstable in aqueous solutions, functionalization can help stabilize them by negating the charge interactions on the surface. Additionally, functionalization can help prepare the surface for secondary coatings and bioconjugation applications. Beyond nanomedicine, functionalizing the particles has been utilized to create catalysts and immobilized enzyme activity substrates (14). Some of the most common functionalizing groups include anionic organoalkoxysilanes, dehydrogenase enzyme molecules and dyes like rhodamine.

With respect to this particular work, (3-aminopropyl) triethoxysilane (APTS) and Trihydroxysilyl propyl methylphosphonate are the reagents most vital (Figure 2). Trihydroxysilyl propyl methylphosphonate has been shown to help reduce aggregation in aqueous medium by Liong et al. when used as a modifier and APTS is used as a silanization agent as well as a grafting agent in many Magnetic Nanoparticle (MNP) applications (15) (16).

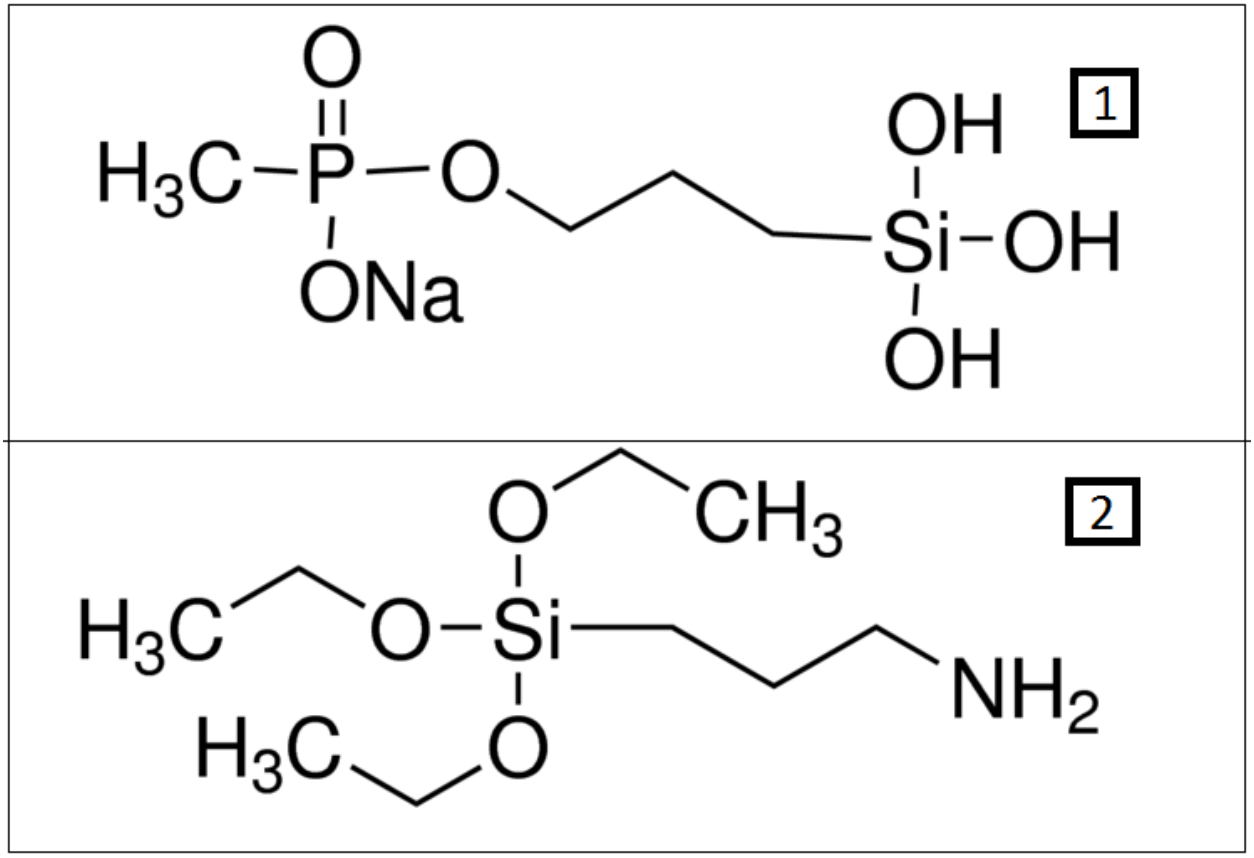

Figure 2 (17): Structures of 1. trihydroxysilyl propyl methylphosphonate and 2. (3-aminopropyl)trimethoxysilane (APTS)

1.4 Project overview

As discussed, MSN has its advantages that prove to make it an interesting choice in many nanoparticle mediated medical applications. Its unique pore structure can be utilized to create functionalized nanoparticles of different shapes and sizes using directing reagents. Most studies have been conducted on spherical particles in the past but MSN with different aspect ratios have received comparatively less attention from researchers. Modeling the adhesion profile and dynamics of non-spherical particles is tough but experimental research has shown that the internalization by cells was by nonspecific cellular intake and didn't cause adverse effects (18). In continuance of this research, we have created MSN of different aspect ratios in the lab and

analyzed the average change in aspect ratio based on the change in the concentration of the directing agent (Figure 3).

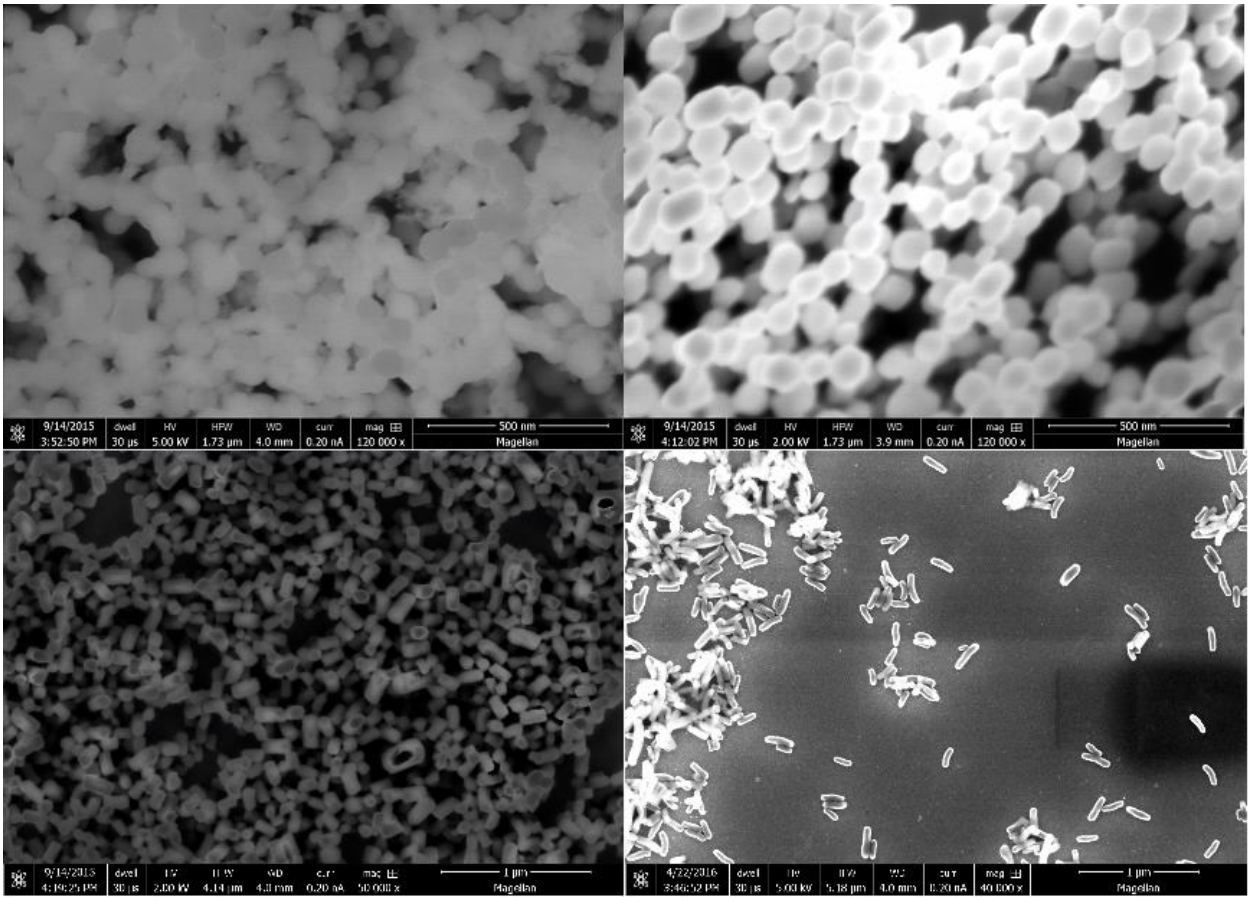

Figure 3: Nanoparticles of different aspect ratios prepared in Haun lab

Among the driving factors behind studying particles of different aspect ratio are that it would be interesting to know how the kinetics is varied in the drug loaded particles when delivered to the body, different cell uptake rates due to the varied geometries, and unique adhesion profiles.

MSN utilizing Cetrimonium bromide as the surfactant and directing agent are notorious for having issues in removing the template during the work up. DLS and SEM usually give different sizes due to the fact that samples that undergo SEM imaging are dried and thus have the template

removed. A section of this work is dedicated to novel ways to work up and wash the synthesis mixture to give a DLS measurement that correlates well with the SEM image sizing. Some of the methods used include different solvents to preferentially remove excess surfactant, treatment with ammonium nitrate and changes in the centrifugation speed and duration during pelleting between washes.

Another important aspect of the study is the incorporation of fluorescence to enable tracking experiments. This required study of fluorescence signal intensity to determine if there are quenching effects were present by correlating the absorbance and the fluorescence values from the spectrofluorometer. The importance of this set of experiments is to prove that the particles can be attached with fluorescent dyes by silanizing them using APTS and infusing them into the particles by co-condensation reactions.

The effects of changing the concentrations of the starting reagents was also studied in detail and reiterates that MSN can be versatile and tailor made to suit the requirements. The starting reagents include a surfactant, solvent, silica providing precursor, base and catalyst. This set of experiments is vital to provide varied templates of consistently produced sizes of MSN for uptake studies.

After a controlled baseline was established in the procedure for synthesizing the particles, the crux of this thesis is based on producing functionalized MSN. The effects of different amounts of the functionalizing reagents and their combinations is studied in detail with quite a few interesting inferences.

In essence, this work establishes a detailed study of the effects of various different factors that influence surface chemistries, size, shape, aggregation, geometries and reactivity of mesoporous nanoparticles in some detail. It lays a platform for future bioconjugation studies which

are envisioned to be conducted using bio-orthogonal coupling reactions like the one between tetrazine and trans-cyclooctene (TCO) (19) which offers a plethora of directions for further studies in selective and simultaneous targeting of biomarkers. Flow chamber assays where the fluorescing nanoparticles flow over a bed of antibodies, proteins or antibody expressing cells with TCO have been envisioned with which it is possible to accurately observe attachment and detachment rates of the particles in great detail.

CHAPTER 2. EXPERIMENTAL APPROACH TO SYNTHESIS OF MESOPOROUS SILICA NANOPARTICLES

2.1 Background

In 1967, Stober et al. discovered a process through which monodisperse silica spheres in the sub-micron range could be produced. Different alcoholic solvents were used including mixtures and the precursors used were also varied. Ammonia was used as a morphological catalyst in this method and depending on the amount of ammonia used, the size of the particles varied. The results were characterized by electron micrograph and became a template to producing silica nanoparticles in the future (20). The findings showed that by varying the ratio of the reagents used in the reaction, the particle morphology too could be changed. The initial range of particle sizes were from 0.05 to 2 microns. Though it was seen that the Stober process produced particles of monodisperse sizes and was extremely easy to replicate, the particle shapes could not be changed and the particles were not mesoporous in nature. For the particles to be useful in the biomedical field, both the above characteristics are desired and sought after.

In 1992, scientists working at the Mobil Corporation first published research with silica nanoparticles created by a sol-gel synthesis with pores. In 1994, Transmission Electron Microscope images of these particles first appeared which showed that they contained hexagonal pore structures (21). The advent of porous silica nanoparticles allowed researchers to attach functional groups to the surface of the particles. Later, it was shown that cationic surfactant head groups could be used to create particles of different shapes and aspect ratios due to their strong interactions with the precursor reagents. Cetyltrimethylammonium Bromide (CTAB) is one such surfactant that is commonly used and is the surfactant used throughout this work (22). It has been

noticed that nanoparticles synthesized with CTAB as the surfactant have a tendency to agglomerate on synthesis.

Recently, research has been conducted with particles of different aspect ratios, synthesized by varying the concentration of CTAB. Cell uptake and cell behavior studies show that larger aspect ratios were interestingly internalized faster and had higher uptake rates. The aspect ratios also played a key role in the different aspects of cell function (23). The synthesis of the particles with varied aspect ratios has been studied in greater detail as will be elucidated below. The methodology for synthesis is inspired by the research work published by Huang et al.

2.2 Experimental reagents and background information

1. Hexadecyltrimethylammonium bromide (CTAB)

CTAB is used as the directing agent and surfactant in our experiments. CTAB is a cationic surfactant and is slightly soluble in water (36.4 g/l at 20˚C). It is often used in production of different types of nanoparticles with various aspect ratios especially in coating gold nanorods (24). CTAB allows fine tuning of the particle size and shape by forming micelles in solution which induce the shape of the formed particles. CTAB has an aggregation number of nearly 95 around room temperature.

The molecule consists of a polar head which is hydrophilic and a hydrocarbon tail which is hydrophobic. The concentration in solvent affects how the micelles behave in solution by changing the interfacial energy and minimizing the exposure of the hydrophobic cores to the surface if the solvent is aqueous. This is what causes the change in shape of the micelle and thus eventually the shape of the particle (Figure 4).

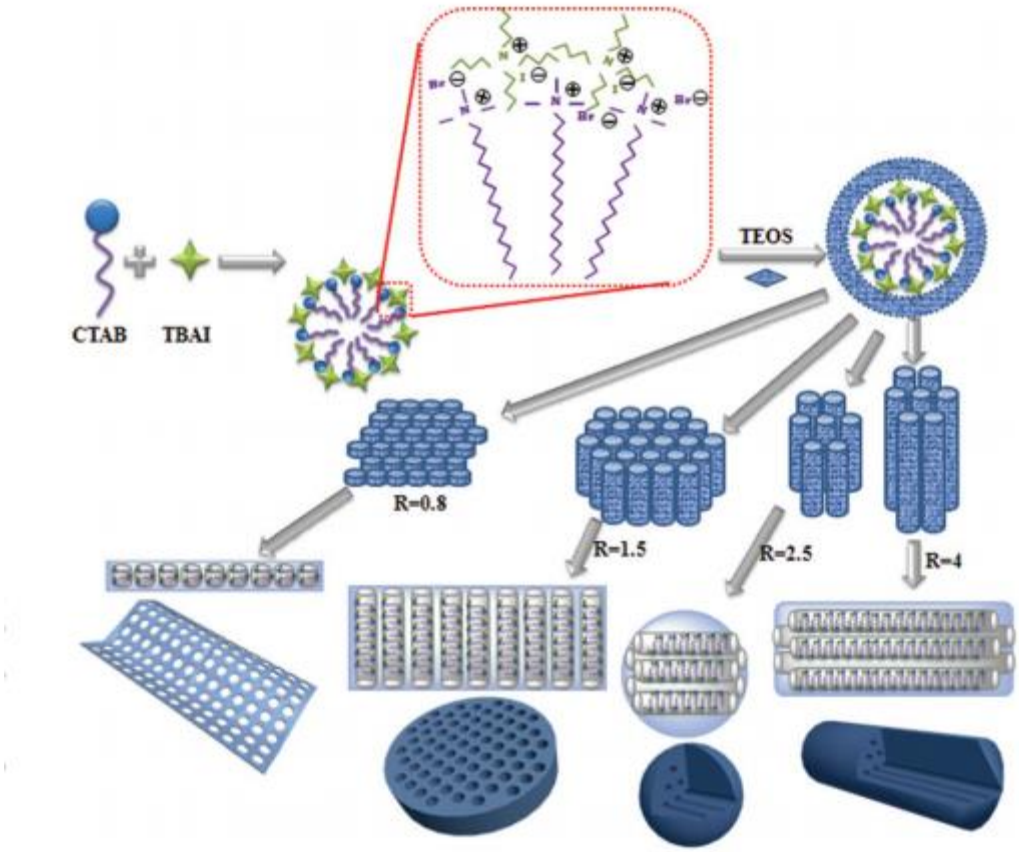

Figure 4 (25): The figure shows how the concentration of CTAB affects the shape of the template due to the difference in micelle structure. TBAI is tetrabutylammonium iodide

2. Tetraethyl orthosilicate (TEOS)

TEOS is used as the precursor for the formation of the silica nanoparticles. The framework of the silica particles is provided by TEOS through the conversion to silicon dioxide when it comes into contact with water.

$$4\ Si(OC_2H_5) + 2\ H_2O \rightarrow SiO_2 + 4\ C_2H_5OH$$

Throughout the project, the formation of silica particles in water is through a sol-gel reaction. This reaction causes TEOS to condense into a solid through the formation of silicon-oxygen linkages (Figure 5).

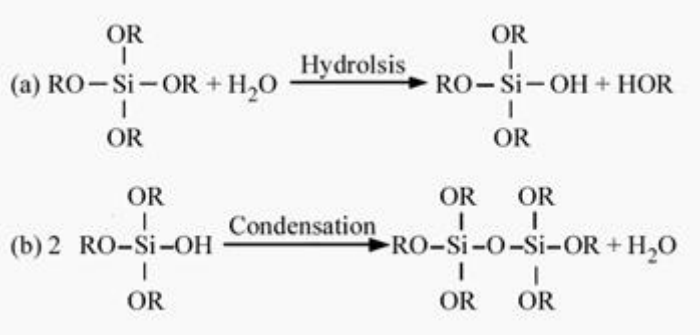

Figure 5 (26): The figure shows the condensation and hydrolysis reaction of TEOS which provides the framework for the synthesis of MSN

3. Ammonium hydroxide

Aqueous ammonia (NH_4OH) is used as a catalyst and size determining agent in this study. Ammonia acts as a morphological catalyst and affects the size of the particles in proportion to its concentration in the synthesis mixture. This is probably due to the interaction between amine groups with the mineral solid formed during the condensation and hydrolysis reactions of TEOS in aqueous medium.

2.3 Nanoparticle synthesis

For the synthesis of control MSN which gave sizes of around 100 nm by SEM, 0.01 g CTAB was added to 10 ml of deionized water at 35˚C on a hot plate. The solution was sonicated and stirred at around 700 rpm with a magnetic stir bar. 200ul of ammonium hydroxide (26° Baume, Fisher Scientific) was added to the solution. The solution was stirred for an hour before adding

90ul TEOS (98%, Sigma-Aldrich) and the synthesis mixture was stirred overnight at 35˚C before work up (washing procedures and centrifugation). When experiments were carried out to study the change in particle morphology with respect to change in concentration of reagents, this was the procedure followed.

Washing procedure: 1 ml of the synthesis mixture was pelleted by centrifuging at 15,000 rpm for 30 min in a 2 ml sample tube. The supernatant was removed from the tube, replaced with the desired solvent, sonicated and centrifuged for 30 min. This process was repeated three times until a stable colloidal solution with no aggregate was observed in the sample tube.

The characterization of the particles was performed by Dynamic Light Scattering (DLS) analysis by dispersing a dilute amount of the washed particles in a suitable solvent. The angle of detection was set at 173˚ and conducted in the backscatter arrangement on the Malvern Zetasizer ZS Nano machine. Scanning Electron Microscope (SEM) images were also taken when necessary but the DLS measurements were the primary indicators used for particle sizing.

2.4 Results and discussion

2.4.1 Effect of CTAB concentration

To synthesize MSN of different shapes, all the initial conditions were kept constant except for the amount of CTAB used. The weight of CTAB was varied from 0.01 g to 0.12 g in a 10 ml reaction. The aspect ratios and average sizes were analyzed using ImageJ. Considering the fact that a major assumption in DLS characterization is that the particle is assumed to be a sphere, DLS was not a viable method of size characterization in this experiment and necessitated the use of SEM. The images and results of the size analysis are denoted below. It was found that if the

concentration of CTAB was increased beyond a certain limit (0.08g), the micellar structure broke down and the particle formation followed no particular order, as can be seen in the case of 6f.

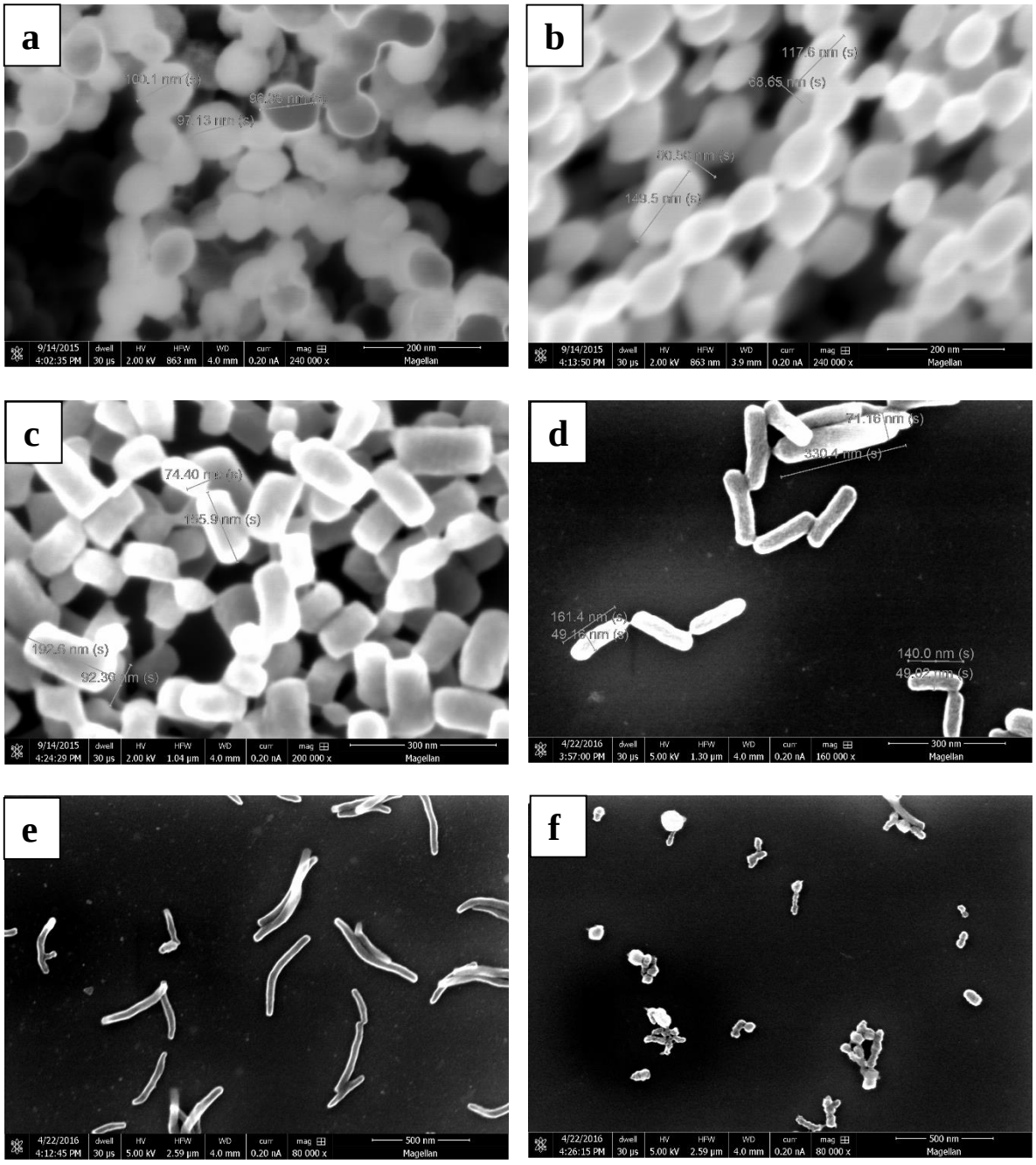

Figure 6: Particle shapes affected by different concentrations of CTAB. a. 0.01 g CTAB, b. 0.02 g CTAB, c. 0.03 g CTAB, d. 0.04 g CTAB, e. 0.08 g CTAB and f. 0.12 g CTAB. All concentrations of CTAB are in g/10 ml

Size analysis:

Sample	Average width (nm)	Average height (nm)	Aspect Ratio (width/height)
0.01 g CTAB	89.00±7.81	87.90±6.22	1.01±0.11
0.02 g CTAB	118.26±7.76	76.45±14.82	1.55±0.20
0.03 g CTAB	159.06±26.33	82.31±9.33	1.93±0.20
0.04 g CTAB	195.45±53.27	53.96±8.86	3.62±0.32
0.08 g CTAB	416.44±127.96	49.65±6.68	8.39±0.34

Table 1: Size analysis of particles with different CTAB concentrations

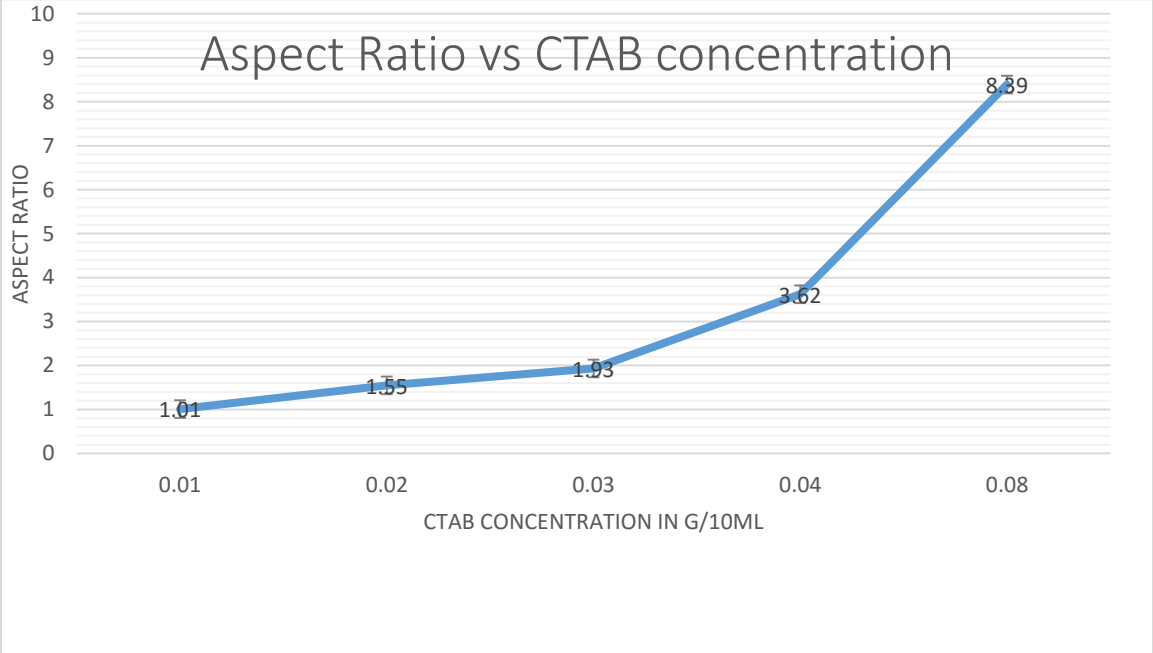

Figure 7: Graph showing change in aspect ratio with respect to concentration of CTAB

The sizes were analyzed using ImageJ, and it is seen that in general, a higher concentration of CTAB results in a higher aspect ratio. As the graph shows, the aspect ratio climbs nearly linearly with increase in concentration of CTAB till a point after which there is no formation of analyzable

particles. Interestingly, it is noted that with increase in CTAB concentration, there is an increase in height but decreases after 0.04 g CTAB. This is because with increase in concentration of CTAB, in order to give a higher surface area to volume ratio, CTAB micelles will pack closer together. The micelles tend to form structures which are energetically more stable.

2.4.2 Effect of washing with different solvents

It has been apparent from past literature that MSN have an issue with aggregation during work up when they form large aggregates that skew DLS readings. This is thought to be due to the charge interactions between the particles in part due to the CTAB (15). Additionally, in order to use the particles for secondary reactions, coatings and adding functional groups to the surface, it is vital to see that the amount of CTAB on the surface is minimized so that the coating is not done on the aggregates. Thus, studying the effect of different procedures of washing the particles took precedent in this work. Calcination could have been a viable option to remove the organic matter from the surface but it provides other problems such as heavy fusing of MSN which necessitates other remedial methods and loss of a large amount of product (15). Liong et al. and others have also published research from which it is apparent that heating the MSN in Ammonium nitrate and washing it in a solvent that preferentially dissolves CTAB like methanol is a useful method to "strip" CTAB from the particle surface (15).

Another method attempted to provide monodisperse DLS readings was to take the measurements in a solution with varied pH. It has been attempted before with promising results for Nickel nanoparticles (27). In this study, the particles were dispersed in 4 millimolar NaOH. The particles prepared in the following studies are identical and the only difference is the method of washing which is mentioned where required.

Ammonium nitrate stripping procedure: 1 ml of the synthesis mixture is centrifuged at 30 min at 15,000 rpm, the supernatant removed and the pellet is dispersed in methanol. The resulting solution is centrifuged again for 30 min and supernatant replaced with methanol. This procedure is repeated twice. The resulting solution is added to 2 ml of ammonium nitrate solution in ethanol (3mg nitrate per ml) and stirred at 60˚C for 30 minutes. The mixture is then pelleted and washed twice in ethanol using the same steps as above. The particles are spherical by design by controlling the amount of CTAB added. The final particles were divided and the DLS was taken in different solvents. The results are tabulated below.

Sample	**N-avg (nm)**	**Z-avg (nm)**	**SD (nm)**	**Polydispersity index**	**Comments**
Control in water	197	649	40	0.8	Washed in water
Control in Ethanol	136	319	110	0.204	Washed in Ethanol
Control in 4mmol NaOH	131	361	129	0.217	Washed in Ethanol
Stripped particles in water	194	326	107	0.248	Highest peak between 120-140
Stripped particles in Ethanol	214	404	149	0.249	
Stripped particles in NaOH	124.6	319	96	0.287	

Table 2: Study of different solvents and stripping method. SD: Standard deviation of N-avg

As can be seen from the results, there is heavy aggregation of particles in an aqueous medium if there is no change in the wash method. However, when using the stripping procedure,

the particles are monodisperse in NaOH. This can be attributed to the fact that a higher pH environment provided by NaOH helps in stabilizing the charge of the particles thereby affecting its ability to form aggregates. It is of note that the intensity average (Z-avg) sizes are not representative of the actual size of the particles due to the fact that a tail of aggregates in the DLS measurement will heavily skew the sizing away from the number average (N-avg) sizes. Thus N-avg sizes are a better representative of the true particle sizes.

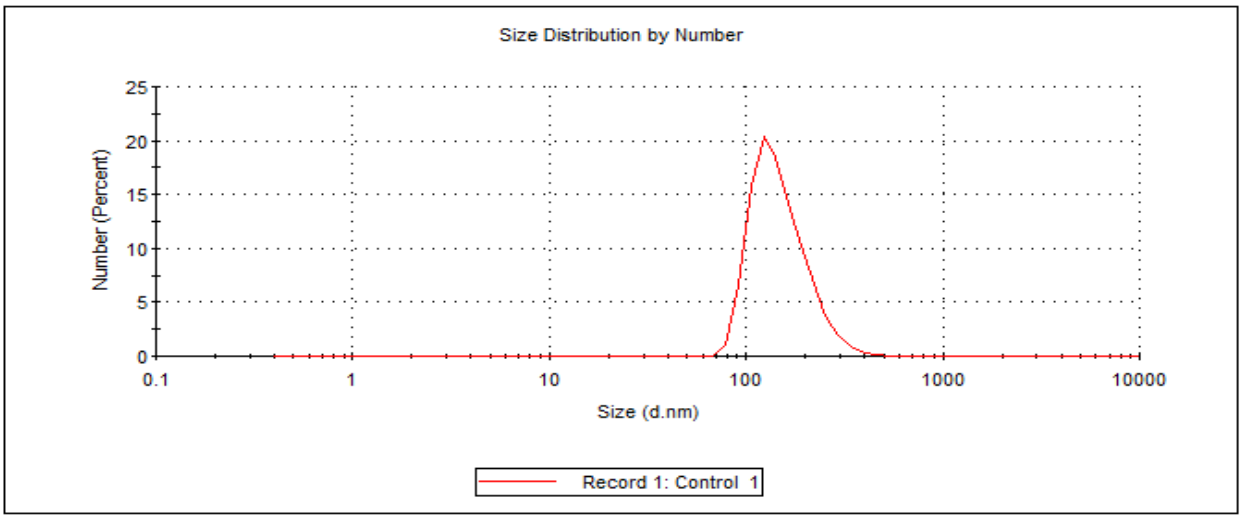

Figure 8: Representative reading of control particles. As can be seen, the peak is extremely close to 100 nm

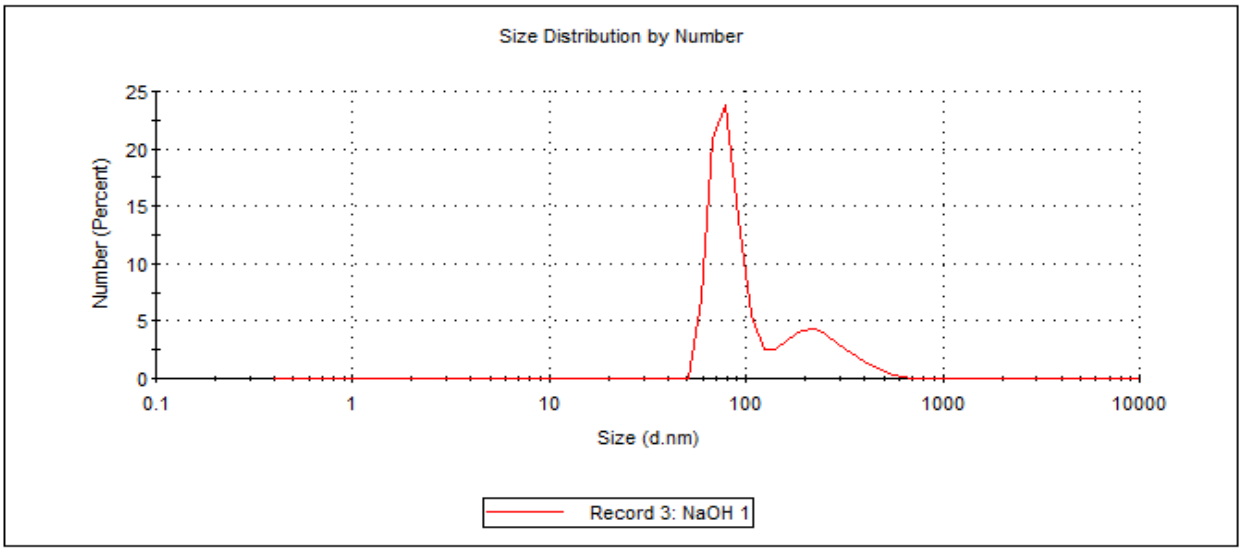

Figure 9: Stripped particles with DLS measured in NaOH show evidence of peak closer to the true size of the particle. However, the tail skews the Z-avg reading as expected

From the experiments conducted, it is evident that the concentration of CTAB affects the particle shape. The micelle is lengthened (25) at higher concentrations causing the particles formed to lengthen in shape. Above a certain concentration, micelle formation breaks down and the particles formed have no correlation and there is no usable data that can be gathered from the images with regard to the aspect ratio of the particles. As discussed, studies on silica particles that have aspect ratios closer to rod than spheres could have interesting insights for the medical industry.

2.4.3 Effect of washing procedures

MSN have a history of being difficult to work with after the synthesis and before secondary modifications especially when working with CTAB as the surfactant and directing agent. One of the main aims of this study was to provide a procedure which would be consistent in its ability to remove CTAB from the surface of the particles. The ammonium nitrate treatment with the addition of heat shows a definite change in the surface morphology of the particle. It could be developed

into a standard for silica nanoparticles prepared in an aqueous medium involving CTAB and a sol-gel reaction.

Besides washing procedures, negating the surface charge on the MSN to prevent agglomeration is another approach taken. The strategy of dissolving the particles in a solvent that is of a pH different than that of deionized water so as to make the particles neutral stable in solution is an approach that researchers have investigated in the past few years. As can be seen from the above results, a 4 millimolar solution of NaOH was found suitable to create stable particles in solution. Other methods to do this involving surface modifications have been discussed in the next chapter.

A point of note from the experimental observations is that when DLS readings are taken, the presence of a Z-avg that is much larger than N-avg is proof that the solution contains large agglomerates of particles which need to be removed from the solution in case of secondary coatings and attachment of functional groups since the potential functional group could coat the agglomerates instead of the individual particles during synthesis.

CHAPTER 3. ADDITIONAL FUNCTIONALIZATION AND MODIFICATIONS OF SILICA NANOPARTICLES

3.1 Background information

Mesoporous silica nanoparticles have provided quite a few interesting possibilities in the realms of nanomedicine that are exciting for the future of the topic. Functionalizing the MSN makes them even more receptive and useful for applications in targeted drug delivery, gene therapy, etc. Silanization is one of the more common methods to create functionalized MSN. Aminopropyltrimethoxysilane (APTS) and Aminopropyltriethoxysilane (APTES) are two of the reagents most commonly used for modification procedures of the particles since they offers many an advantage, creating a more delicate and more functional organic framework with silica nanoparticles. Researchers have already identified functionalized MSN to be DNA delivery vectors for gene therapy (28).

There are generally two common ways that the particles can be modified by using APTS either through grafting after initial synthesis as a secondary coating or during the condensation reaction which instills the APTS during synthesis of the particles. In this project, we have worked on both types of modifications and the results will soon be elucidated in detail. The grafting process is useful in applications where the drug adhesion is studied on a pore-less MSN surface (11). It can act as a protective layer around the original particle. The grafting process takes place in a Stober growth where the medium used for growth is not aqueous but ethanol and does not require CTAB or water for modification. In the co-condensation process, APTS is internalized. This is especially useful when attaching a fluorescent dye for fluorescence studies so that the dye molecules are not completely exposed on the surface and the signal received will be consistent and strong as the dye is protected.

In continuance of the study to create charge stabilized particles in aqueous medium, Trihdroxysilyl propyl methylphosphonate (hereby called phosphonate) modification has shown to help. This compound is usually modified by the grafting procedure in conjunction with APTS.

Another important requirement of functional modification is the ability to label the particle with dyes for diagnostic and bioconjugation study purposes. For this purpose, NHS-Rhodamine has been used for fluorescence studies. To find the optimal amount of dye to use, quenching studies have also been conducted.

3.2 Materials and methods

3.2.1 Co-condensation synthesis experiments

For the co-condensation process of functionalization, most of the steps followed are similar the synthesis steps followed in the previous chapter. For the control particles, 0.01 g of CTAB is dissolved in deionized water and sonicated until dissolution is complete. 200 ul of ammonium hydroxide is added and the mixture left stirring at around 700 rpm and 35˚C for an hour. At this point, 90 ul TEOS is added and after 20 min of further stirring, 1 ul of APTS is added and the reaction left to complete overnight. APTS is obtained from Sigma-Aldrich at 97% purity.

3.2.2 Grafting synthesis experiments

For the grafting modifications, one ml of the synthesis mixture from the previous step is washed as required and placed in ethanol. This solution is left to stir at around 700 rpm at room temperature. If the secondary modification involves TEOS, it is added dropwise and the solution left to stir for 20 min. After this, APTS and/or Trihdroxysilyl propyl methylphosphonate (obtained

from Sigma Aldrich) are coinjected into the synthesis solution. The resulting solution is washed and redispersed thrice in ethanol.

3.2.3 Fluorescence experiments

For the fluorescence experiments, the required dyes are initially silanized by reacting with APTS in the required molar ratio and dissolved in ethanol. The dye used in this case is Alexa Fluor 488 (obtained from ThermoFisher Scientific). The required amount of dye-APTS mixture in ethanol is added to the synthesis mixture like APTS in the co-condensation process in the previous section. The excess ethanol volume is made up by subtracting an equivalent amount of water. For the fluorescence microscopy studies, the particles were modified as required by the grafting synthesis method as mentioned above.

For Rhodamine, the ratio used was 1:20::Alexa Fluor:APTS by moles. Thus, 0.54 mg of Alexa Fluor 488 was sonicated and dissolved in 500 ul of ethanol. 5 ul of APTS was added to the mixture and stirred overnight in absence of light. This gave a resultant mixture of 1ul APTS:100 ul ethanolic solution that could be used in the synthesis reactions. Alexa Fluor 488 has an excitation maxima at 490 nm and emission maxima at 525 nm. The absorbance readings in this set of experiments were taken on the NanoDrop.

3.3 Results and discussions

3.3.1 Co-condensation results

In the co-condesation experiments APTS is added during synthesis of the template MSN, 20 minutes after addition of TEOS. The results are tabulated below. The concentration of ammonia added has also been varied. Two of the samples have undergone the stripping procedure as described in the previous chapter and the others were washed 5 times in ethanol.

Sample	N-avg (nm)	Z-avg (nm)	SD (nm)	Polydispersity index
100 ul NH_3, 1ul APTS	173	577	23	0.293
200 ul NH_3, 1ul APTS	136	276	16	0.24
300 ul NH_3, 1ul APTS	170	273	97	0.2
400 ul NH_3, 1ul APTS	203	320	22	0.165
200 ul NH_3, 2ul APTS	142	301	86	0.36
200 ul NH_3, 5ul APTS	1024	1319	221	0.326
100 ul NH_3, 1ul APTS, stripped	93	333	9.6	0.38
200 ul NH_3, 1ul APTS, stripped	302	366	89	0.27

Table 3: Sizing data on modification by co-condensation process for 10 ml reactions. SD: Standard deviation of N-avg

The results above indicate some useful information about the surface modification procedure. If the concentration of APTS is too high, it gives rise to heavy agglomeration as seen in the 5ul APTS reading. In the other cases, DLS gives a reading that is close to the actual size of the particle. 1 ul of APTS seems to be a good standard to use for co-condensation synthesis based on the results.

3.3.2 Grafting synthesis results

For grafting, the originally synthesized particles were post modified using different ratios of APTS and phosphonate reagents in a Stober growth to ideally give smooth particles with covered pores. These particles will give a better representation during bioconjugation studies as

the attachment of the moieties will be random and unaffected by pores on the surface of MSN. The results are tabulated below. All the modifications were on control synthesis particles.

T: Tetraethyl orthosilicate

P: Trihydroxysilyl propyl methylphosphonate

A: Aminopropyltrimethoxysilane

Sample (reagents in ul)	**N-avg (nm)**	**Z-avg (nm)**	**SD (nm)**	**Polydispersity index**
5T, 4A, 2P	190	291	144	0.22
2T, 2A, 1P	188	282	140	0.225
1T, 2A, 1P	148	341	140	0.451
1T, 1A, 0.5P	163	293	191	0.271
0.5T, 1A, 0.5P	241	303	117	0.217
1T, 1P	190	743	114	0.62
5T, 5P	120	545	80	0.501
15T, 15P	190	314	13.64	0.167

Table 4: Sizing data for grafting synthesized particles

It was expected from the grafting experiments that the size of the particles would increase due to the addition of a second layer. As expected, the sizes do increase. However, a cause for concern would be the standard deviations of N-Avg and Z-avg which give reason to suspect that there is definitely some agglomeration in the synthesis solution even if the highest peak is at an expected size. Phosphonate, which was believed to help in stabilizing the particles does show that it helps negate the aggregation due to surface charges over the experiments.

3.3.3 Fluorescent dye labeling results

For the last set of experiments in this chapter, fluorescent dye labeled particles synthesized by the co condensation method were prepared and analyzed. The mixture of APTS and dye prepared was used in different concentrations. The DLS results of the different concentrations are as tabulated below.

Sample	**N-avg (nm)**	**Z-avg (nm)**	**Polydispersity index**	**SD of N-avg sizes**
Control	197	970	0.8	40
0.5 ul APTS	173	458	0.7	73
1 ul APTS	195	400	0.6	78
2 ul APTS	230	1600	0.3	660

Table 5: DLS results of dye labeled MSN

The dye excitation maxima was at 490 nm and emission maxima at 525 nm. The absorbance results as well as the fluorescence results from the samples are as below.

Sample	**Absorbance at 490 nm**	**Relative absorbance**	**Fluorescence reading**	**(Fluorescence/Relative absorbance)/1000**
Control	0.055	0	0	NA
0.5 ul APTS	0.076	0.021	203	9.67
1 ul APTS	0.095	0.04	447	11.18
2 ul APTS	0.145	0.09	727	8.08

Table 6: Absorbance and fluorescence results

The results from Table 6 show that as expected, the fluorescence readings are in good correlation with the absorbance readings. The last column shows that there is a slight quenching

effect between 1 ul APTS and 2 ul APTS. Since the fluorescence is clearly visible nevertheless, 1 ul APTS is a good template for further dye attachment studies.

For fluorescence microscopy studies, the particles were coated through the grafting process. The three coating combinations were a) Aminopropyltrimethoxysilane + Trihydroxysilyl propyl methylphosphonate+ Tetraethyl orthosilicate, b) Trihydroxysilyl propyl methylphosphonate + Tetraethyl orthosilicate and c) Tetraethyl orthosilicate only. The results are as follows.

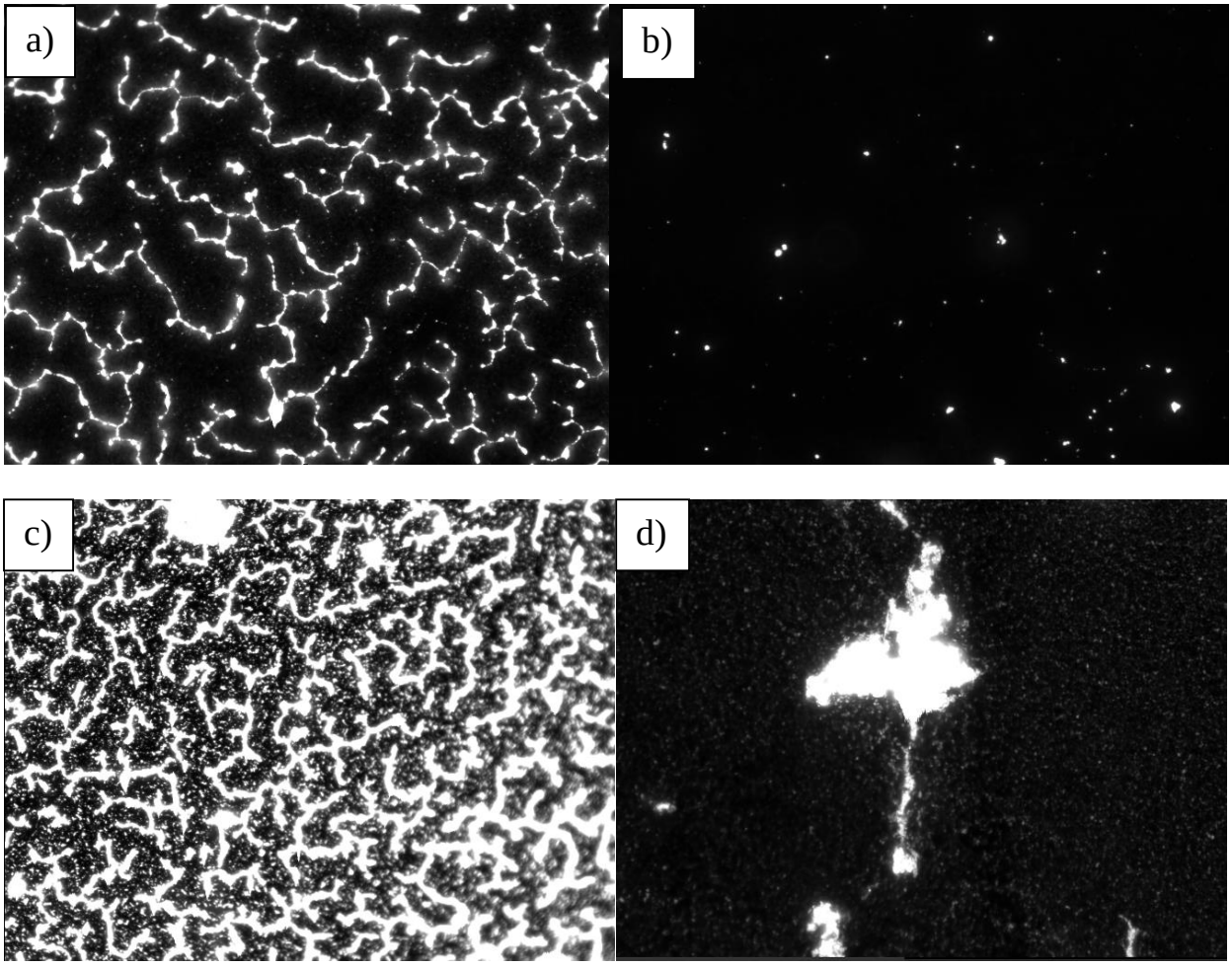

Figure 10: a) A+P+T, b) P+T, c) T only, d) Wash sample showing aggregate

The results show that P+T modification gave the least aggregated results as seen through fluorescence microscopy. The T only modification showed heavy aggregation throughout.

CHAPTER 4. SUMMARY AND CONCLUSIONS

This thesis can broadly be divided into two parts. Chapter 2 primarily dealt with the synthesis itself of Mesoporous Silica Nanoparticles (MSN). Chapter 3 dealt with the functionalization aspects of MSN.

In Chapter 2, it was shown that we can take advantage of the unique properties of each of the reagents used for synthesis of MSN. By changing the concentration of CTAB, it was shown that we could change the aspect ratio of the particles produced. Particles of aspect ratios 1:1 to 8:1 can be produced just by changing the concentration of CTAB and taking advantage of its micelle formation. Apart from the synthesis of particles, we sought to show that it is possible to strip CTAB from the particle surface without having to resort to calcination and risking the fusing of particles. It is possible to use a strict washing procedure and heating with ammonium nitrate solution in ethanol to produce nearly monodisperse particles which are ready for secondary coatings.

It was also seen that the charge interactions between the particles can be reduced or even negated by using solvents of alkaline pH. This particular set of experiments help in producing a situation where the DLS data can be comfortably correlated with SEM data.

The third chapter was dedicated to showing how there are different ways in which MSN can be modified, both by grafting and co-condensation reactions during synthesis. These secondary modifications help in giving functionalities to the nanoparticles which align them further towards biomedical applications especially in targeted drug delivery and gene therapy.

This chapter also focused on showing that it is possible to attach a dye to the MSN by instilling it during the co-condensation reaction. The following fluorescence studies showed that the quenching effects aren’t extreme and it was possible to determine an optimum concentration of APTS to use while modifying the particles.

Through the experiments, this thesis has enabled us to determine the versatility of MSN and created templates which show us how best to utilize the unique advantages afforded by the particles. As discussed, a lot of novel research is being conducted in the field of nanotechnology in biomedicine and to explore the various uses of silica nanoparticles as a theranostic tool.

The future of this work is envisioned to include bioconjugation studies utilizing biorthogonal chemistries to provide a foundation in the topic of targeted delivery systems utilizing functionalized Mesoporous Silica Nanoparticles.

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APPENDIX A

Steps for synthesis of particles:

1. Add 10 ml of deionized water in a 20 ml glass vial.
2. Add required amount of CTAB to vial, sonicate and keep at 35˚C, stirring at 600-700 rpm.
3. Add required amount of ammonium hydroxide and stir for 1 hour.
4. Add required amount of TEOS.
5. Add required amount of functionalizing group after 20 minutes if required.
6. Leave overnight and wash in 2 ml sample collection tubes.

Steps for surface modification:

1. Take 1 ml of washed particles in ethanol in a 10 ml glass vial.
2. If adding TEOS, add required amount and wait for 20 minutes.
3. Add required amount of modifying reagent and stir overnight at 35˚C and 600-700 rpm.
4. Wash in 2 ml sample container in the required solvent.