Design, synthesis and bio-application of functional mesoporous silica nanoparticle

Ma, Xing

2013

Ma, X. (2013). Design, synthesis and bio-application of functional mesoporous silica nanoparticle. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/54992>

<https://doi.org/10.32657/10356/54992>

NANYANG
TECHNOLOGICAL
UNIVERSITY

**DESIGN, SYNTHESIS AND BIO-APPLICATION
OF FUNCTIONAL MESOPOROUS SILICA
NANOPARTICLE**

MA XING

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2013

**DESIGN, SYNTHESIS AND BIO-APPLICATION
OF FUNCTIONAL MESOPOROUS SILICA
NANOPARTICLE**

MA XING

School of Materials Science and Engineering

A thesis submitted to the Nanyang Technological University
in fulfillment of the requirement for the degree of
Doctor of Philosophy

2013

ACKNOWLEDGEMENT

My supervisor, Dr. Zhao Yanli, is the person who opened another window for me when the other one was closed. Dr. Zhao, a wonderful professor who gave me this precious opportunity to continue my PhD education, has helped me a lot during my PhD study period. Under his patient guidance and supervision, I indeed enjoyed my PhD research work and obtained a nice graduate experience during my life.

The success of my PhD study owes many thanks to all the people who support me during the whole period, especially my wife who married me before we started a new life in Singapore. Graduate study is a lonely process in pursuit of scientific truth, but I am the lucky one who has never been alone, as my wife has been accompanying me here for almost three years to fully support my PhD study. With her splendid cooking and most importantly her encouragement which keeps me working hard on research work, I can manage to maintain a smooth progress in my PhD study.

At last, I would like to convey my deep gratitude to all my research group members, lab techniques, administrative staff, as well as those who have ever helped me for my PhD study.

ABSTRACT

The research work concentrated on the design, synthesis and biological application of functional mesoporous silica nanoparticles. Through novel functionalization, several mesoporous silica nanoparticle (MSNP) based systems aiming at different biological applications, including drug/gene co-delivery, photodynamic therapy (PDT) and bio-image, are designed and fabricated. A variety of techniques of both materials and chemical science are used to fully characterize the functional MSNP before application in the biological system. By in vitro and in vivo experiments with cancer cell lines and zebra fish, the effectiveness of these functional MSNP based nano-systems are verified.

TABLE OF CONTENTS

ACKNOWLEDGEMENT	I
ABSTRACT.....	II
TABLE OF CONTENTS.....	III
LIST OF TABLES	V
LIST OF FIGURES	VI
Chapter 1. Research Background and Motivation	1
1.1 Research Background	1
1.1.1 Synthesis, Structure and Property of MSNP	1
1.1.2 Functionalization of MSNP.....	4
1.1.3 Cytotoxicity and biocompatibility of MSNP.....	6
1.1.4 Biological application of MSNP	8
1.2 Research Motivation and Scientific Contribution.....	11
Chapter 2. Drug/Gene Co-delivery by MSNP	14
2.1 Research Background	14
2.2 Redox-responsive Drug/ssDNA co-delivery by functional MSNP.....	15
2.2.1 Research scope and motivation.....	15
2.2.2 Materials and Methods.....	17
2.2.3 Results and Discussion.....	21
2.2.4 Conclusion	33
2.3 Controlled Drug/siRNA Delivery by Ethylenediamine Modified β -cyclodextrin Capped MSNP in Vitro and in Vivo	35
2.3.1 Research scope and motivation.....	35
2.3.2 Materials and Methods.....	37
2.3.3 Results and Discussion.....	44
2.3.4 Conclusion	69
2.4 Target Drug/siRNA Co-delivery by pH-responsive Hollow Mesoporous Silica Nanoparticles.....	70
2.4.1 Research Scope and Motivation.....	70
2.4.2 Materials and Methods.....	72

2.4.3 Results and Discussion.....	78
2.4.4 Conclusion	95
Chapter 3. Monomeric Zinc Phthalocyanine inside Spacer Modified Nano-channels of MSNPs: A Nanohybrid for Target Photodynamic Cancer Therapy	96
3.1 Research Scope and Motivation.....	96
3.2 Materials and Methods.....	98
3.3 Results and Discussion.....	102
3.4 Conclusion	116
Chapter 4. Graphene Oxide Wrapping on Squaraine Entrapped MSNPs for Bio-imaging	117
4.1 Research scope and motivation.....	117
4.2. Materials and Methods.....	119
4.3. Results and Discussion.....	122
4.4. Conclusion	131
Chapter 5. Summary and Outlook.....	132
5.1 Summary of the research work	132
5.2 Outlook and future development.....	136
REFERENCE.....	139
PUBLICATION AND CONFERENCE	155

LIST OF TABLES

Table 3.1: Zeta potential values of functional mesoporous silica nanoparticles.....	47
Table 3.2: DLS size of MSNP-SS-AD-CD-NH ₂ and MSNP-SS-AD+CD-NH ₂ +SiRNA in both distilled water and DMEM cell culture medium.....	49
Table 3.3: Formulations of MSNP-SS-AD+ CD-2NH ₂ +siRNA complex for brain-targeted injections	59
Table 4.1. BET surface area, pore size, and pore volume of HMSNP and MSNP	79

LIST OF FIGURES

Figure 1.1. The mechanism of surfactant directed MSNP synthesis with cetyltrimethylammonium bromide (CTAB) and tetraethylorthosilicate (TEOS) in aqueous solution.....	2
Figure 1.2. Distribution of functional groups depending on the addition time of the organosilane component during synthesis ³⁶	4
Figure 1.3. Schematic representation of nanopistons on mechanized phosphonate-covered silica nanoparticles ¹⁰⁰	8
Figure 1.4. Biological applications of functional mesoporous silica nanoparticle (MSNP)	12
Mechanism 2.1. Illustrative mechanism of MSNP-based drug/gene co-delivery system.....	16
Figure 2.1. (a,b) TEM and (c) HR-TEM images of MSNP-SH	21
Figure 2.2. (a) BET isotherm and BJH pore size distribution of MSNP-SH and (b) powder XRD pattern of MSNP-SH and illustration of a mesoporous structure	22
Figure 2.3. (A) FT-IR spectra of silica nanoparticles under indicated conditions: (a) silica nanoparticles containing surfactants, (b) MSNP without the surfactants, (c) MSNP-SH, and (d) MSNP-SS-NH ₃ ⁺ , and (B) ¹³ C CP MAS NMR spectrum of MSNP-SS-NH ₃ ⁺	23
Figure 2.4. Zeta potential values of silica nanoparticles: (a) MSNP-SH, (b) MSNP-SS-NH ₃ ⁺ , (c) MSNP-SS-NH ₃ ⁺ coated with ssDNA (nanoparticle-to-ssDNA loading weight ratio of 10:1), (d) MSNP-SS-NH ₃ ⁺ loaded with FITC (nanoparticle-to-FITC loading weight ratio of 10:3), and (e) MSNP-SS-NH ₃ ⁺ loaded with FITC, followed by coating ssDNA (nanoparticle-to-FITC-to-ssDNA loading weight ratio of 10:3:1).	24

Figure 2.5. TEM images of (a) MSNP-SS-NH ₃ ⁺ , (b) MSNP-SS-NH ₃ ⁺ coated with 21-mer ssDNA, and (c) MSNP-SS-NH ₃ ⁺ loaded with FITC.....	25
Figure 2.6. Agarose gel electrophoresis of MSNP-SS-NH ₃ ⁺ capped with an increasing ssDNA ratio. Lanes from left to right are free ssDNA, MSNP-SS-NH ₃ ⁺ , nanoparticle-to-ssDNA weight ratios of 5:1, 10:1, 20:1, 30:1, and 30:1 containing 50mM DTT, respectively. Agarose gel electrophoresis was carried out at 100V for 15 min using 1.5wt% agarose gel in TAE buffer	25
Figure 2.7. Fluorescence spectra of FITC-loaded MSNP-SS-NH ₃ ⁺ and equivalent amount of free FITC in PBS buffer.....	26
Figure 2.8. Agarose gel electrophoresis of FITC-loaded MSNP-SS-NH ₃ ⁺ (nanoparticle-to-FITC loading weight ratio of 10:3) capped with an increasing ssDNA ratio. Lanes from left to right are free ssDNA, FITC-loaded MSNP-SS-NH ₃ ⁺ , nanoparticle-to-ssDNA weight ratios of 5:1, 10:1, and 10:1 containing 50mM DTT, respectively. Agarose gel electrophoresis was carried out at 100V for 30 min using 1.5wt% agarose gel in TAE buffer.....	27
Figure 2.9. FITC loading capacity of MSNP-SS-NH ₃ ⁺ under different nanoparticle-to-FITC loading weight ratios.....	28
Figure 2.10. Controlled drug/ssDNA co-release from MSNP-SS-NH ₃ ⁺ in the addition of reduction agent DTT or GSH in PBS buffer (pH = 7.36). (a) Cy5 labeled ssDNA release profile and (b) FITC release profile. Curves ◆ indicate the release profiles of FITC-loaded and ssDNA-coated MSNP-SS-NH ₃ ⁺ upon time without any reduction agents. Curves ■, ▲, and ▼ indicate the release profiles of FITC-loaded and ssDNA-coated MSNP-SS-NH ₃ ⁺ in the addition of 50mM DTT, 50mM GSH, and 10mM GSH at the beginning, respectively. Curves ● indicate the release profiles of FITC-loaded and ssDNA-coated MSNP-SS-NH ₃ ⁺ in the addition of 50mM DTT at 60 min.....	29
Figure 2.11. Fluorescence microscopic images of Hela cells after being treated with 50μg mL ⁻¹ of Dox-loaded and ssDNA/Cy5-coated nanoparticles (MSNP-SS-NH ₃ ⁺ + Dox + ssDNA/Cy5) for 24h. (a) Bright	

field, (b) DAPI (4',6-diamidino-2-phenylindole) stain channel, (c) FITC channel, (d) Dox channel, (e) merged image of b, c, and d, (f) Cy5 channel, and (g) merged image of b, c and e	30
Figure 2.12. Fluorescence microscopic images of Hela cells after being treated with free Dox (0.5 μ g mL ⁻¹). (a) Bright field, (b) DAPI (4',6-diamidino-2-phenylindole) stain channel, (c) FITC channel, (d) Dox channel, (e) merged image of b, c	31
Figure 2.13. Fluorescence microscopic images of Hela cells after being treated with (a) ssDNA/Cy5-coated MSNP-SS-NH ₃ ⁺ (50 μ g mL ⁻¹) or (b) MSNP-SS-NH ₃ ⁺ (50 μ g mL ⁻¹) for 24h.....	32
Figure 2.14. Viabilities of Hela cells after being treated with indicated nanoparticles and equivalent amount of free Dox for 24h. Curves ■, ●, ▲, and ▼ indicate the cell viabilities in the presence of MSNP-SS-NH ₃ ⁺ , ssDNA-coated MSNP-SS-NH ₃ ⁺ (MSNP-SS-NH ₃ ⁺ + ssDNA), Dox-loaded and ssDNA-coated MSNP-SS-NH ₃ ⁺ (MSNP-SS-NH ₃ ⁺ + Dox + ssDNA), and free Dox, respectively, under the concentrations measured	32
Scheme 3.1. Schematic representation of MSNP based siRNA/drug co-delivery system	36
Figure 3.1. a) X-ray diffraction pattern, b) N ₂ adsorption/desorption isotherm, and c) BJH pore size distribution of MSNP-SH prepared by the co-condensation method.....	45
Figure 3.2. a) FESEM and b) TEM images of MSNP-SH, c) FT-IR spectra of MSNP-SH, MSNP-SS-NH ₂ , and MSNP-SS-AD, and d) ¹³ C CP MAS NMR spectrum of MSNP-SS-AD.....	46
Figure 3.3 Photos of MSNP-SS-AD capped with β -CD (left) and MSNP-SS-AD capped with CD-2NH ₂ (right) in aqueous solution (1 mgmL ⁻¹) after a) 5 min sonication and b) stabilized at room temperature for 24 h. Well dispersed sample for MSNP-SS-AD capped with CD-2NH ₂ even after 24 h indicates its good stability in aqueous solution.....	48
Figure 3.4. Controlled drug release profiles in PBS, pH=7.4 for a) RhB release from β -CD capped MSNP-SS-AD, b) RhB release from CD-2NH ₂ capped MSNP-SS-AD, and c) Dox release from CD-2NH ₂ capped MSNP-SS-AD. d) Agarose gel electrophoresis of the siRNA complexation with CD-2NH ₂ capped	

MSNP-SS-AD under different nanoparticle-to-siRNA weight ratio. Lanes from left to right are free siRNA, CD-2NH₂ capped MSNP-SS-AD, and CD-2NH₂ capped MSNP-SS-AD/siRNA complexes under the nanoparticle-to-siRNA weight ratios of 10:1, 30:1, 50:1, 70:1, 90:1, 110:1, and 110:1 containing 50mM DTT, respectively 49

Figure 3.5. Photos of small amount of sample for RhB-loaded MSNP-SS-AD capped with β -CD in PBS buffer (pH 7.4) a) before DTT addition and b) 5 min after DTT (50 mM) addition. Red RhB release was observed 5 min after DTT addition, indicating the redox-triggered RhB release 51

Figure 3.6. CLSM images of HeLa cells treated with a-c) equivalent free Dox, and d-i) FITC-labeled MSNP-SS-AD loaded with Dox and capped with CD-2NH₂ (50 μ g mL⁻¹) for 24 h. a) blue fluorescence from DAPI, b) red fluorescence from Dox, c) overlay of bright field, image a) and b); d) blue fluorescence from DAPI, e) green fluorescence from FITC-labeled MSNP-SS-AD, f) red fluorescence from released Dox, g) overlay of image e) and f), h) overlay of image d) and f), g) overlay of bright field, image d), e, and f) 52

Figure 3.7. CLSM images of a-c) FITC-labeled MSNP-SS-AD, d-e) FITC-labeled MSNP-SS-AD capped with CD-2NH₂. From left to right: blue color from DAPI, green color from FITC labeled MSNP-SS-AD, and overlay images of bright field, blue and green images; g) flowcytometry result (FL1: FITC fluorescence intensity) of HeLa cells treated with 1) control, 2) FITC-labeled MSNP-SS-AD, 3) FITC-labeled MSNP-SS-AD capped with CD-2NH₂; h) flowcytometry result (FL2: Dox fluorescence intensity) of HeLa cells treated with 1) control, 2) equivalent free Dox, 3) FITC-labeled MSNP-SS-AD+Dox+CD-2NH₂+siRNA complex 53

Figure 3.8. Protein concentration collected from HeLa cells after being treated with indicated samples . 54

Figure 3.9. a) Bcl-2 protein knockdown by CD-2NH₂ capped MSNP-SS-AD/siRNA(+) complex (MSNP-SS-AD+CD-2NH₂+siRNA) in HeLa cells (nanoparticle-to-siRNA weight ratio of 110:1). Gene expression was evaluated by the ratio of intensity of the Bcl-2 band to that of the internal standard, β -tubulin. The ratio of each sample was normalized to the value of the sample without any treatment; a-1)

control, a-2) MSNP-SS-AD+CD-2NH₂, a-3) MSNP-SS-AD+CD-2NH₂+siRNA(+), a-4) MSNP-SS-AD+CD-2NH₂+siRNA(-), a-5) free siRNA, a-6) Lipofectamine+siRNA(+). b) Relative cell viability of HeLa cells treated with indicated samples under an equivalent amount of nanoparticle concentration and equivalent amount of Dox; b-1) MSNP-SS-AD+CD-2NH₂+siRNA(-), b-2) free Dox, b-3) MSNP-SS-AD+Dox+CD-2NH₂, b-4) MSNP-SS-AD+Dox+CD-2NH₂+siRNA(+); Nanoparticle-to-siRNA weight ratio is 100:1 55

Figure 3.10. Zebrafish transgenic lines sqKR19 and GW(sq33-e20) are two transgenic lines with fluorescent reporter expression in the choroid plexus, a thin ependymal outgrowth dorsal to the ventricle cavity. GFP expression in this tissue is the readout to address the efficiency of siRNA mediated knockdown in the living animal model. As it is predominantly a single layer of cells at 3-4 dpf, next to the ventricular cavity, it should be the most sensitive readout for interference with GFP expression mediated by EGFP siRNA. (a and b) Dorsal view of 4dpf transgenic zebrafish larvae. (c and d) Common expression domains in the choroid plexus between membrane-localized KillerRed (c) and cytoplasmic GFP (d). (f-g) Immuno-staining of a section of Et(krt4-EGFP) sq33-e20 brain showing how the choroid plexus flank the ventricle cavity. All cells were stained blue with TOPRO3, and the ventricle cavity, demarcated by the absence of cells, is marked by an arrow (f)..... 57

Figure 3.11. *In vivo* uptake of CD-2NH₂ capped MSNP-SS-AD inhibits transcription of GFP in the epithelial cells of choroid plexus at the 4th ventricle of zebrafish larval brain. (a and b) Brain-targeted delivery of FITC labeled and CD-2NH₂ capped MSNP-SS-AD resulted in uptake into some cells largely in peripheral areas of the choroid plexus, highlighted by white boxes. (c-g) Specific weight ratios of CD-2NH₂ capped MSNP-SS-AD/GFP siRNA complexes (C2 and C3) enhance inhibition of GFP transcription *in vivo*. The area (μm²) of GFP expression detected by whole mount *in situ* hybridization in the choroid plexus is quantified by the Image J software. (h) The average size of the choroid plexus is presented in a bar chart as mean ± standard error of mean. Formulations for various weight ratios of nanoparticle/siRNA complexes used for this experiment are as follows: WT (without any treatment), C1 (0.04 μg μL⁻¹ siRNA

only), C2 (0.12 $\mu\text{g } \mu\text{L}^{-1}$ nanoparticle with 0.04 $\mu\text{g } \mu\text{L}^{-1}$ siRNA, 3:1), C3 (0.2 $\mu\text{g } \mu\text{L}^{-1}$ nanoparticle with 0.04 $\mu\text{g } \mu\text{L}^{-1}$ siRNA, 5:1), and C4 (0.4 $\mu\text{g } \mu\text{L}^{-1}$ nanoparticle with 0.04 $\mu\text{g } \mu\text{L}^{-1}$ siRNA, 10:1)..... 58

Figure 3.12. Reduction of EGFP transcript in the choroid plexus of 3dpf GW(sq33-e20) transgenic larvae demonstrates the need to formulate specific weight ratio of CD-2NH₂ capped MSNP-SS-AD complexed with siRNA in order to enhance the efficiency of siRNA mediated GFP knockdown. Area of the EGFP stained choroid plexus was quantified after whole mount in situ hybridization. Formulations for various weight ratios of nanoparticle/siRNA complexes used for this experiment are WT (no treatment); C1 (0.04 $\mu\text{g } \mu\text{L}^{-1}$ siRNA only); C2 (0.12 $\mu\text{g } \mu\text{L}^{-1}$ nanoparticle with 0.04 $\mu\text{g } \mu\text{L}^{-1}$ siRNA; 3:1); C3 (0.2 $\mu\text{g } \mu\text{L}^{-1}$ nanoparticle with 0.04 $\mu\text{g } \mu\text{L}^{-1}$ siRNA; 5:1); C4 (0.4 $\mu\text{g } \mu\text{L}^{-1}$ nanoparticle with 0.04 $\mu\text{g } \mu\text{L}^{-1}$ siRNA; 10:1). In total, 8 samples were analyzed for each experimental set. Area of the choroid plexus outlined in blue is stated in μm^2 60

Figure 3.13. Significant decrease of GFP expression was observed after two reiterative injections of Dox-loaded and CD-2NH₂ capped MSNP-SS-AD complexed with GFP siRNA. (a-e) Same weight ratio of Dox-loaded nanoparticle/GFP siRNA complex injected into the brain ventricle on two consecutive days. The impact on GFP expression in the choroid plexus was assessed two days later. Barely visible GFP expression in the choroid plexus was detected in all transgenic larvae exposed to the highest weight ratio (C4) of Dox-loaded nanoparticle/GFP siRNA complex. (f) The average GFP intensity quantified in the boxed area of the choroid plexus is presented in a bar chart with values stated as mean $\pm$ standard error of mean. Values are compiled from three separate injections. The same acquisition setting was used for image capture using LSM510 META confocal microscope. Image intensity was assessed using the Image J software 62

Figure 3.14. A single brain targeted injection of Dox loaded and CD-2NH₂ capped MSNP-SS-AD/GFP siRNA complex is sufficient to decrease GFP expression in the choroid plexus of GW(Sq33-e20) transgenic zebrafish larvae. (a-e) Representative live images of the choroid plexus after exposure to varying weight ratios of Dox-loaded and CD-2NH₂ capped MSNP-SS-AD/GFP siRNA complexes.

Uptake of Texas Red dextran in embryos injected with free GFP siRNA (b) or different weight ratios of Dox-loaded MSNP-SS-AD/GFP siRNA complexes can be detected in the choroid plexus after two days post injection (c-e). (f) The Average GFP intensity quantified in the boxed area of the choroid plexus are presented in a bar chart with values stated as mean $\pm$ standard error of mean. The same acquisition setting, using a LSM510 META confocal microscope, was employed for *in vivo* imaging. Image intensity was assessed using the Image J software 63

Figure 3.15. Injection of Dox loaded MSNP-SS-AD into the zebrafish circulation elicits less developmental side effects than injection of free doxorubicin when adjusted to the same concentration. 5 out of 6 injected larvae exposed to free doxorubicin elicit developmental defects of reduced growth and edema (a-c; e-f) whereas only 1 out of the 6 larvae exposed to Dox loaded MSNP develop this toxicity associated phenotype (e). Each image is a composite of 3 images that span the anterior-posterior length of the larva. Left: Free Dox 250 μ g/ml; approximate injection volume is 6 nl; 3days post injection; right: 10mg/ml MSNP-SS-AD loaded with Dox and capped with CD-2NH₂; approximate injection volume is 6 nl; 3days post injection 65

Figure 3.16. The liver tumor in the inducible *kras*^{v12} transgenic zebrafish model is reduced after exposure to MSNP-SS-AD loaded with Dox and capped with CD-2NH₂ when compared to untreated control. (a) A view of transgenic zebrafish larvae *Kras*^{v12} experiencing liver hyperplasia at 10 days post fertilization. (b) A view of transgenic zebrafish larvae experiencing liver hyperplasia 6 days post injection of MSNP-SS-AD loaded with Dox and capped with CD-2NH₂, 10 days post fertilization. (c) A magnified view of liver tumor cells exposed to MSNP-SS-AD loaded with Dox and capped with CD-2NH₂. Free released Dox (red) can be seen inside liver tumor cells in green. (d) The average area of liver tumor between non-injected control (n =8) and siblings injected with MSNP loaded Dox (n=10) quantified 6 days post injection is presented as a bar chart with values stated as mean liver area $\pm$ standard error of mean. Liver size from each specimen was quantified using the associated software in LSM510 META..... 66

Figure 3.17. Individual liver tumor area of non-injected control (n =8) in the inducible <i>kras</i> ^{v12} transgenic zebrafish model is bigger when compared to transgenic zebrafish larvae experiencing liver hyperplasia but treated with MSNP-SS-AD loaded with Dox and capped with CD-2NH ₂ (n =10). Each specimen was imaged 6 days post injection. The area of the GFP-positive liver is computed with the Zeiss software in LSM510 META	67
Scheme 4.1. Illustration of target drug/siRNA co-delivery by pH-responsive HMSNP	70
Figure 4.1. TEM images of HMSNP a) at low magnification, b) at high magnification, c) HMSNP+PEI-FA complex, and d) MSNP.....	78
Figure 4.2. N ₂ adsorption/desorption isotherm of a) HMSNP, c) MSNP; and BJH pore size distribution of b)HMSNP, d)MSNP	79
Figure 4.3. Powder X-ray diffraction pattern of HMSNP and MSNP	80
Figure 4.4. FT-IR spectra of PEI, folic acid conjugated PEI (PEI+folic acid), and folic acid.....	80
Figure 4.5. a) UV-vis spectra and b) Zeta potential measurement indicate PEI-FA coating on HMSNP. All the samples are dissolved or suspended in deionized water	81
Figure 4.6. Rhodamine B loading capacity comparison of MSNP & HMSNP at different weight ratios .	82
Figure 4.7. a) Agarose gel electrophoresis of NP(HMSNP or MSNP)+PEI-FA+siRNA(Bcl-2) complex; b) Dox loading capacity of HMSNP and MSNP.....	83
Figure 4.8. Thermal gravimetric analysis (TGA) results of (a) bare HMSNP; (b) as-prepared HMSNP; c) HMSNP+PEI-FA complex; d) HMSNP+DOX+PEI-FA complex	84
Figure 4.9. Agarose gel electrophoresis of HMSNP+Dox+PEI-FA+siRNA(Bcl-2) shows that the loading of Dox inside HMSNPs would not affect the siRNA binding capability of the co-delivery system	84
Figure 4.10. a) pH-controlled Dox release, and b) stimuli-responsive (by changing pH value) Dox release from HMSNP+Dox+PEI-FA+siRNA(Bcl-2) complex in PBS buffer.....	85

Figure 4.11. a) Confocal microscopy images of a) HeLa(FA+) and MCF-7(FA-) cells after treated with 10µg/ml HMSNP(FITC)+Dox+PEI-FA+siRNA(Bcl-2) complex for 24h. From left to right are images under bright field, green channel (FITC: 488/520nm±10nm), red channel (Dox:488/580nm±10nm), and merged channel (Bright field+FITC+Dox+DAPI), respectively; b) merged image of FITC+Dox channels (top), and Bright Field+DAPI+FITC+Dox channels (bottom); c) Flowcytometry results of HeLa(FA+) and MCF-7 (FA-) cells after treated with HMSNP(FITC)+DOX+PEI-FA+SiRNA(Bcl-2) complex for 24h 86

Figure 4.12. Confocal microscopy image of co-cultured HeLa (FA+) and MCF-7 (FA-) cells treated with 10µg mL⁻¹ HMSNP+PEI-FA+siRNA(Bcl-2) complex at HMSNP-to-siRNA weight ratio 20:1 for 24h... 87

Figure 4.13. a) Merged (Bright field+DAPI+FITC+Dox channels) confocal microscopy image of HeLa cells after treated with 10µg/ml HMSNP+DOX+PEI-FA+siRNA(Bcl-2) complex for 24h; b) fluorescence intensity reading of DAPI (blue), FITC (green), and Dox (Red) along the dashed arrow in a)..... 88

Figure 4.14. a) Western blot assay of protein in HeLa and MCF-7 cells; both cells were treated with HMSNP+PEI-FA+siRNA(Bcl-2) complex with HMSNP-to-siRNA weight ratio 10:1, 20:1, and 30:1; b) Relative Bcl-2 protein expression of HeLa and MCF-7 cells. The content of siRNA is 1µg/well; and the content of HMSNP is 10, 20, and 30µg/well respectively; the volume of culture medium in each well is 1ml during the treatment. After 24h treatment, the medium was changed into new medium and the protein was collected after the cells were cultured for another 48h 89

Figure 4.15. Western blot assay of protein in HeLa and MCF-7 cells; lane 1: control (without any treatment); after treated with 2: HMSNP+PEI-FA+siRNA(scrambled), 3: free siRNA(Bcl-2), 4: Lepofectamine+siRNA(Bcl-2), and 5: 10µg/ml HMSNP+PEI-FA+siRNA(Bcl-2) 90

Figure 4.16. a) MTT cytotoxicity assay of MCF-7 and HeLa cells; both cells were treated with HMSNP+Dox+PEI-FA or HMSNP+DOX+PEI-FA+siRNA(Bcl-2) complex with HMSNP-to-siRNA weight ratio 20:1; After 24h treatment, the culture medium was changed into new medium and the MTT

assay was performed after another 48h. b) MTT cytotoxicity assay of MCF-7 and HeLa cells; both cells were treated with HMSNP+PEI-FA+siRNA(scrambled) complex 91

Figure 4.17. Flowcytometry data of cell apoptosis assay by annexin V staining of HeLa cells. Cells were treated with a) 1.5µg/ml free Dox, b) 10µg mL⁻¹ HMSNP+Dox+PEI-FA, and c) 10µg mL⁻¹ HMSNP+DOX+PEI-FA+siRNA(Bcl-2) for 24h; then the culture medium was changed into fresh medium and cultured for another 24h before the flowcytometry assay 92

Figure 4.18. Flowcytometry result of apoptosis assay by annexin V staining of HeLa cells. HeLa cells a) without any treatment, and treated with b) 20µg/ml HMSNP+PEI-FA+siRNA(scrambled), c) 10µg/ml HMSNP+DOX+PEI-FA, d) 10µg/ml HMSNP+DOX+PEI-FA+siRNA(Bcl-2) complex with HMSNP-to-siRNA weight ratio 20:1 for 24h; the medium was replaced with fresh medium after the treatment. For c) and d), extra 5µg/ml HMSNP+DOX+PEI-FA complex was added after 48h incubation. The apoptosis staining and flowcytometry assay was performed after at 72h 93

Scheme 5.1. Schematic illustration for the preparation of hybrid of zinc phthalocyanine (ZnPc) loaded MSNP-Ad and inclusion of CD-2NH₂, **1** for photodynamic singlet oxygen generation. Enlarged area represents a nano-channel modified with adamantane spacer which prevents aggregation of ZnPc..... 97

Scheme S5. Scheme showing preparation of MSNP-Ad. APTES: (3-aminopropyl) triethoxy silane, CTAB: cetyltrimethylammonium bromide, DCC: dicycle hexylcarbodiimide, NHS: *N*-hydroxysuccinimide 100

Figure 5.1. a) X-ray diffraction pattern, b) N₂ adsorption/desorption isotherm, and BJH pore size distribution (inset) of MSNP-Ad..... 102

Figure 5.2. HR-TEM images of a) MSNP-Ad and b) MSNP-Ad loaded with ZnPc and capped with CD-2NH₂, hybrid **1**. Insets are the photographs of corresponding nanoparticles under visible light. c) UV-Vis absorption spectra of ZnPc loaded MANP-Ad complexed with CD-2NH₂ (**1**) in PBS buffer pH 7.2 (red

curve), and ZnPc alone in DMF (black curve, 1×10^{-6} M). d) Emission spectrum of hybrid 1 in PBS buffer at pH 7.2	103
Figure 5.3. a) FE-SEM image of hybrid 1, b) FT-IR spectra of MSNP-NH ₂ (black curve), MSNP-Ad (red curve) and hybrid 1 (blue curve), and c) Values of ζ potential for MSNP-NH ₂ , ZnPc loaded MSNP-Ad, and hybrid 1	104
Figure 5.4. UV-Vis absorption changes (between 340-420 nm) showing photobleaching of the ¹ O ₂ molecular probe, ADMA upon irradiation of hybrid 1 with 675 nm laser light for 40 min. Inset shows enlarged area corresponding to absorption of monomeric ZnPc loaded in 1	105
Figure 5.5. a) Fluorescence microscopy images of HeLa cancer cell lines incubated with 2 and 4, respectively. b) Flow cytometric analysis of HeLa cell lines incubated with and without 2 and 3	106
Figure 5.6. Schematic diagram showing the PDT experimental setup for light irradiation	107
Figure 5.7. MTT viability assay of HeLa cells incubated with 1 for 4 h. Cell viability is expressed as percentage of untreated cells. The concentration refers to the amount of 1 used for the incubation. Green bars indicate control experiments using cells treated with 1 in the dark. Red and blue bars refer to cells treated with 1 under irradiation for 30 and 60 minutes, respectively	108
Figure 5.8. Apoptosis/death cell analysis by flow cytometry. From left to right: HeLa cells without any treatment, HeLa treated with hybrid 1 for 24h and kept in dark, and HeLa cells treated with hybrid 1 and exposed to 674nm irradiation for 20min	109
Figure 5.9. Fluorescence image of HeLa cells after apoptosis/death cells staining; a) control, b) treated with hybrid 1 for 24h and kept in dark, and c) treated with hybrid 1 for 24h and exposed to 674nm irritation for 20min; 1) bright field channel, 2) DAPI channel, 3) Annexin IV channel, 4) PI channel, 5) overlay of 2 and 4, and 6) overlay of 2 and 3	110
Figure 5.10. a-f) Fluorescence microscopic images of HeLa cancer cells treated with 1. Images a-c) before light irradiation, and d-f) after irradiation showing completely damaged cells	111

Figure 5.11. Ultrastructural analysis using TEM. HeLa cells were treated with hybrid 1 for 24 h and exposed to 674nm light irradiation before processed for TEM analysis. (a) A dying cell showed nanoparticles uptake and cellular vacuolization (arrows). (b) Cells experienced PDT displayed the appearance of an apoptotic cell with apoptotic bodies and membrane blebbing. (c) HeLa cells treated with hybrid 1 showed nanoparticles uptake. Some vacuoles evidently contained nanoparticles (arrows). (D, E, F) High magnification images of HeLa cells treated with hybrid 1, ntaining vacuoles within the cytoplasm (arrows). Images were acquired at original magnifications of 5,000 – 40,000.....	111
Figure 5.12. a) UV/vis spectrum of ZnPC loaded MSNP+CD-FA; b) Absorption changes showing singlet oxygen ($^1\text{O}_2$) generation from ZnPc loaded MSNP complex with folic acid attached β -CD	113
Figure 5.13. Fluorescence microscopy image of various cells treated with hybrid 5 (ZnPC loaded MSNP(FITC)-Ad+CD-FA); Channels from left to the right: bright field, DAPI, FITC, ZnPc, overlay of DAPI, FITC and ZnPc	114
Figure 5.14. a) flowcytometry result of cells without any treatment (red) and treated with hybrid 5 for 24h (green peak); b) MTT assay of photocytotoxicity test of cells treated with hybrid 5 and kept in dark or exposed to 674nm light irradiation for 20min	115
Scheme 6.1. Schematic illustration for preparation of GO enwrapped dye loaded MSNPs (GO-MSNP1). Stage 1: loading of 1 in APTES modified MSNP vessel, stage 2: electrostatic wrapping of GO sheets on the surface of the nanoparticles loaded with 1	118
Figure 6.1. a) TEM image of multilayer GO sheets, b) selected area electron diffraction pattern (SAED) of multilayer GO sheets, and c) FT-IR spectrum of GO sheets	122
Figure 6.2. UV/Vis (black curve) and emission (red curve) spectra of 1 in DMF/water (1:1) at pH 6.5	123
Figure 6.3. a) Powder XRD pattern of MSNP-NH ₂ ; b) BET isotherm and BJH pore size distribution (inset) of MSNP-NH ₂	124

Figure 6.4. High resolution transmission electron microscopy (HR-TEM) images of (a) APTES modified MSNPs (MSNPs-NH ₂), (b) GO enwrapped MSNPs without dye loading (GO-MSNPs), (c) GO enwrapped squaraine loaded MSNPs (GO-MSNPs1). The arrows indicate monolayer GO sheets, and (d) the selected area electron diffraction pattern (SAED) of (c), showing ultrathin layer of GO sheets.....	124
Figure 6.5. Typical FE-SEM images (GB mode) of (a) MSNP-NH ₂ and (b) GO-MSNPs1. (c) table showing variation of zeta (ζ) potential values of GO sheets, MSNP-NH ₂ , MSNP1, and GO-MSNPs1 in distilled water. (d) Raman spectra of MSNP-NH ₂ and GO-MSNPs1	126
Figure 6.6. (a) UV/Vis and (b) emission spectra of GO-MSNPs1 (black curve) and GO-MSNPs (red curve) in aqueous solution (1mg mL ⁻¹) at pH 6.5. The excitation wavelength is 580 nm.....	127
Figure 6.7. Changes in the (a) absorption and (b) emission of GO-MSNPs1 and squaraine dye (1) upon addition of 3 mM Cys or GSH in buffer aqueous solution at pH 7 at 24 °C; c) UV/Vis absorption changes of 1 (6×10 ⁻⁶) in DMF/water (1:1) with the addition of Cysteine (3×10 ⁻³ M) during 0-40 min	128
Figure 6.8. MTT viability assay of HeLa cells that were incubated with MSNP and GO-MSNPs1 for 24 h. Cell viability is expressed as percentage of untreated cells. The concentration refers to the amount of MSNP and GO-MSNPs1 used for the incubation	129
Figure 6.9. (a-d) Epifluorescence microscopic images of HeLa cancer cells labelled with GO-MSNPs1. The blue fluorescence is from the nuclear counter stain (DAPI, 1µg mL ⁻¹) and the red fluorescence is from GO-MSNPs1. (a) Image of nuclei, (b) fluorescence of GO-MSNPs1 (dark field), (c) overlay of panels a and b, and d) overlay of Figure c with the phase contrast image	130

Chapter 1. Research Background and Motivation

Mesoporous silica nanoparticle (MSNP) and its related applications have been developed for almost two decades ever since its discovery. But the story is not ending. On the contrary, sharp increasing number of publications about MSNP indicates its importance and great potentials in materials science and engineering. In the first part, a general introduction to MSNP is given, covering basic knowledge of MSNP, the chemical functionalization and related applications focusing on biological area. Then, based on the current research progress of MSNP, specifically its biological applications, several novel designs were inspired, which would be illustrated in the part of the research motivation. And thus the scientific contributions of this work are highlighted.

1.1 Research Background of MSNP

1.1.1 Synthesis, Structure and Property of MSNP

Ever since the invention of ordered mesoporous silica nanoparticle (MSNP) by two research groups independently,^{1, 2} this kind of material has attracted significant attentions. The preparation of new types of ordered mesoporous silica based nanostructure has been significantly advanced and various families of MSNP have been developed, including MCM-n mentioned before, SBA-n (Santa Barbara amorphous silica),^{3, 4} MSU-n (Michigan State University silica),⁵ KIT-1 (Korean Institute of Technology),⁶ IBN (Institute of Bioengineering and Nanotechnology)⁷ and FDU-n (Fudan University).⁸ During the past two decades, the application of these mesoporous silica nanoparticles have been developed and extended into many other fields except for its original intention in the catalyst industry. The unique structure of each type of mesoporous silica is the critical factor that determines the specific application of these novel materials.

Figure 1.1. The mechanism of surfactant directed MSNP synthesis with cetyltrimethylammonium bromide (CTAB) and tetraethylorthosilicate (TEOS) in aqueous solution.⁹

The synthesis of ordered MSNP is usually based on the template directed hydrolysis of silica precursor, tetraethylorthosilicate (TEOS). Cationic surfactant or block polymers that can form micelles are employed as the template for the mesoporous structure formation. Base-catalysed sol-gel process leads to the nanoparticles growth on the micellar template. The formation process should include at least nucleation and self assembly of silica precursor. The morphology of formed particles is determined by the type of catalyst. For example, using NH_4OH will produce rod-like structure, while using NaOH will give smaller size nanospheres. The surfactant can be further removed by refluxing in methanol or ethanol solution containing hydrochloride, in which process the electrostatic interaction between the cationic surfactants and the anionic silicate will be broken and thus the mesopores will be obtained. Obviously, the pore diameter is directly determined by the length of surfactant used in the synthesis process. For example, in MCM-41 type MSNP, cetyltrimethylammonium bromide (CTAB) with 16 carbon containing alkyl chain would yield pore size around 2-3nm,¹⁰ while SBA-15 type MSNP whose formation is directed by block polymer such as pluronic P104¹¹ or P123¹² has much larger pore size. Besides using different surfactant to achieve different pore size, the mesopore diameter can also be varied through addition of pore swelling agent, like trimethylbenzene (TMB). The morphology and particle size of MSNP can be controlled through adjusting the reaction conditions, such as stirring speed, surfactant concentration, pH conditions.¹³

The most characteristic properties of MSNP are the porosity structure which give high specific surface area, large pore volume, and uniform pore size. The porosity of MSNP is normally characterized by N_2 adsorption/desorption. The BET (Brunauer-Emmett-Teller) surface area of MSNP can reach up to as high as $1000\text{m}^2/\text{g}$ for MCM-41 type MSNP or slightly lower surface area for SBA-15 type MSNP. Due to highly porous structure, the pore volume of MSNP is around $1\text{ cm}^3/\text{g}$ which is favorable for cargo molecules loading.¹⁴ In the N_2 adsorption/desorption test, the BJH (Barrett-Joyner-Halenda) method reveals the internal pore size of the mesopores. The MSNP possesses extremely uniform pore diameter. For example, the pore diameter distribution is usually sharply focused on 2-3nm for MCM-41 type and 6-9nm for SBA-15 type MSNP.¹⁵⁻¹⁷ Because of such uniform pore size, the MSNP can orderly pile up in 2D hexagonal structure with p6mm space group, even though they might have different pore size.¹⁸⁻²⁰ The hexagonal structure can be characterized by powder X-ray diffraction and demonstrated by the characteristic (001) peak at extremely low angle.¹⁰ The exact value of 2 theta is determined by the pore size and the relationship based on the classic Bragg's Law. Moreover, the particle size is also a critical factor in the application of MSNP. For MCM-41 type MSNP, the particle size can range from 50nm to 300nm. Through conditions controlling, such as pH values of the reaction system, the particle size distribution can be effectively focused on certain range and uniform nanospheres can be obtained.

As mentioned before, the morphology and mesoporous structure of MSNP are tunable during the synthesis process. Except for the size and shape of MSNP, the surface area, pore size, and pore volume are all tunable in the synthesis of MSNP. It is easily to expect that the surface area and pore volume are closely related with the pore size. Usually, smaller pore size means higher porosity and thus higher surface and pore volume. The mesoporous structure with smaller nanopores is more stable than that of larger pores, which also contributes to such relation. In order to meet different application purposes, the variation of the particle size or pore diameter have been studied by several research groups. They developed several effect methods or synthesis procedures to control the MSNP's morphology,^{14, 17, 21} size²²⁻²⁵, pore size.^{26, 27} In fact, it is great interest to tune the morphology or mesoporous structure of

MSNP. For instance, the enlargement of the pore size allows for the loading of high molecular weight bio-molecules, such as DNA, RNA, protein, and enzyme.²⁸⁻³¹ It is also reported that the particle size of MSNP directly affect the cellular uptake of MSNP in the drug delivery experiment in vitro.³²

1.1.2 Functionalization of MSNP

The specific application of MSNP is always directly related with the functionalization of MSNP. Different surface functional groups can be linked on the MSNP in order to realize different purposes. Therefore, functionalization of MSNP together with its unique structure imposes a variety of functions of MSNP, which is also the major reason for the fast development and numerous reports on MSNP during recent years. For example, the functional groups on MSNP surface can bring hydrophobic or hydrophilic property to the surface of MSNP, which will greatly affect the guest molecules loading property.^{33, 34} Moreover, in catalyst industry the MSNP serves as the scaffold for the catalytic species which are usually functionalized on the mesoporous structure.³⁵

Figure 1.2. Distribution of functional groups depending on the addition time of the organosilane component during synthesis.³⁶ (Copyright 2008 American Chemical Society)

Another reason for the wide application of MSNP is the ease of functionalization on MSNP. Chemically, the presence of Si-OH groups on the surface or inside the internal pores of the MSNP provides the possibility of attaching intended functional groups. Alkoxysilanes with different organic groups are used

to initially modify MSNP with expected groups through interaction with Si-OH groups, which can give great diversity to MSNP, impose distinguished surface property and thus completely different usage of this material.^{37, 38} Typically, there are two major routes for the initial functionalization of MSNP, co-condensation method and post-synthetic grafting method. For the co-condensation method, the functional silane molecules are mixed with silica precursor like TEOS, and MSNP nanoparticles are directly formed through the base-catalyzed nucleation and growth process. As the functional groups are homogeneously mixed with the silica precursor, the formed nanoparticles have uniform distribution of functional groups both on the MSNP surface and inside the mesopores.^{37, 39, 40} However, in the post-synthetic grafting method, the MSNP nanoparticles are prepared first, and the functional groups are linked onto MSNP through refluxing in alkoxy silanes containing solvent. This process can be conducted either before or after removal of the surfactant inside the mesopores, in which way the functional groups can be only attached on the outside surface of MSNP or both outside surface and internal pores.^{41, 42} Hence, post-synthetic grafting is used to selectively functionalize the MSNP, which can even functionalize different groups outside the MSNP surface or inside mesopores.^{36, 43-45} The drawback of post-grafting method is the possibility of non-uniform distribution of functional groups. It is apparent that co-condensation method shows superiority in the aspect of efficiency, as the functionalization procedure is completed together with the MSNP synthesis.

With the initial functionalization, a variety of functional groups, such as primary amine, thiol, phosphate, etc., can be introduced to the MSNP, which allows for the further linkage or modification of required molecules or functional groups. The further linkage or modification on the initial attached functional groups can be performed by various chemical reactions. For example, amidation reaction is one of the most commonly used methods to link additional moiety onto MSNP.⁴⁶⁻⁴⁸ Such reaction is completed in heterogeneous system, and therefore the molecules that will be attached on MSNP are usually added in excessive amount in order to completely convert the initial functional groups on MSNP. So, chemical reactions with high efficiency like “click chemistry” are favorable in the functionalization of MSNP,⁴⁹⁻

⁵¹which can ensure that more than 90% or even 100% of the initial functional groups react with additional moiety.

In addition to linking functional groups or small molecules on MSNP, the MSNP based nano-hybrids have been widely explored for different application purposes. For example, the combination of MSNP with bio-molecules double layer lipid with either positive charge or negatively charge on the hybrid surface has been designed by researchers for the drug and gene delivery purpose.^{52, 53} Besides, cationic^{54, 55} or degradable polymer^{56, 57} coating on MSNP is also developed for drug/gene delivery. Chemically linked ssDNA,^{58, 59} antibody,^{60, 61} or enzyme⁶² on MSNP surface can serve as target or detection probes for the MSNP based nano-hybrid. Moreover, MSNP based nano-hybrid with other smaller nanoparticles decoration were prepared by many research groups, in which a variety of nanoparticles were employed, including to gold nanoparticles,⁶³⁻⁶⁶ iron oxide,⁶⁷⁻⁷⁰ quantum dots,⁷¹⁻⁷³ zinc oxide,⁷⁴⁻⁷⁷ and so forth. Most of these modifications or decorations on the MSNP were either prepared by chemical reactions or electrostatic interaction, which impose different functions to the original material of MSNP.

1.1.3 Cytotoxicity and biocompatibility of MSNP

As this research work mainly concerns on the exploration of the biological application of MSNP, an introduction on the current progress in this specific research area is given in the following.

When applying MSNP into the biological system, the first issue needs to be considered is the safety issues, including toxic effect and biocompatibility of MSNP. The first point considered is the chemical composition of MSNP. Amorphous silica is usually treated as non-toxic and even bio-degradable in living biological system,⁷⁸ and it was widely used in the pharmaceutical industry. Such fact is rather favorable when considering the toxic effect of ordered mesoporous silica nanoparticles. And initial studies also came to similar conclusion that MSNP is generally accepted as low toxicity and regarded safe in biological application when used in low dosage.⁷⁹ For example, early studies compared the cytotoxicity of MSNP

with solid silica spheres, and it was delighted to find that mesoporous structure resulted in much less cytotoxicity than solid silica.⁸⁰ And recently, many more reports about the detailed study on the interaction between mesoporous silica nanoparticles and different cell lines by in vitro experiments, including human colon carcinoma (Caco-2),⁸¹ glioma cells,⁸² and HeLa cells.⁸³⁻⁸⁵ Although MSNP has been widely accepted as non-toxic or low-toxic material, which is favorable for biological application, still concerns on its toxic effect and safety in biological applications have been raised recently. And it has been reported that several factors significant affect its cytotoxicity and biocompatibility.

The most apparent factor affecting MSNP's toxic effect is its dosage administered. As mentioned before, MSNP was found to be low toxic as lower concentration in vitro. However, when increasing the concentration of MSNP used in the biological application, obvious toxic effect was observed. For example, study by Radu group found dosage dependent toxic effect of MSNP and high toxicity was observed at higher concentration⁸⁶.

Then, size-dependent toxic effect is even more interesting. When the size of MSNP is relatively larger and located in the micron range (2.5 μ m), much higher toxicity was found when comparing to the same amount of MSNP with a smaller size (270nm).⁸⁷ However, smaller is not always the better. When the size of MSNP decreased to be smaller than 20nm, they are much more toxic than those with size around 104 and 335nm.⁸⁸ For the intracellular delivery purpose, sub micron size, like 100nm below 500nm, was reported to be easily uptaken by cells without any obvious cytotoxicity effect at relatively high dosage.^{89, 90}

The surface functionalization is expected to directly affect the toxicity of MSNP, as the surface moieties or organic groups directly interact with the living tissues.^{91, 92} Mar á Vallet-Regí's group studied the effect of surface modification of MSNP on its biocompatibility with osteoblastic cell cultures. They found that after coated with osteostatin, a positive effect on the biocompatibility of MSNP was found.⁹³ Besides, surface modification with poly-ethylene glycol (PEG) polymer could enhance the biocompatibility, such as decreasing the non specific serum binding and reducing haemolysis rate.^{94, 95}

In vivo study of the biocompatibility of MSNP has been limited to small rodents animals up to now. Though the biocompatibility of MSNP was demonstrated at low dosage by subcutaneous injections into mice, however deaths were found when treated at high dosage (1.2g kg^{-1} formice) by intrperitoneal injection.⁹⁶ Bio-degradation of MSNP was studied by using simulated biological fluid and textural properties of the mesoporous systems losing after one month incubation was found.⁹⁷ Souris' group studied the hepatobiliary excretion of MSNP with different charge density on MSNP surface in their study.⁹⁸ However, up to now, the biocompatibility of MSNP is still a barrier for its further in vivo application or even clinic test for biological application.

1.1.4 Biological application of MSNP

Due to its unique structure of MSNP, the most widely application in the biological field isto serve as delivery carrier.⁹⁹ The delivered cargos includevarious therapy drugs, DNA or RNA, protein, enzyme and so forth. Because of its tunable pore size, cargos with different size can be easily encapsulated inside the mesopores. The ease of surface modification with organic groups allows for delivery of either hydrophobic or hydrophilic drugs into target tissue or cells. The high surface area and pore volume provide large delivering capability of MSNP.

Figure 1.3. Schematic representation of nanopistons on mechanized phosphonate-covered silica nanoparticles.¹⁰⁰ (Copyright 2010 American Chemical Society)

Among numerous delivery systems designed based on MSNP, on-demand drug release is the major target, as non-specific drug release would cause unwanted side-effect and decrease the drug efficacy. Therefore, stimuli-responsive drug delivery systems based on MSNP have been studied by many research groups during the past decades. The pioneering work was conducted by Tanaka group who used Ultraviolet (UV) sensitive coumarin substitute to graft at the orifice of MCM-41 silica nanoparticles and act as the controllable gate. Photo-irradiation could induce the photocleavage and formation of the dimers, which controlled the access to the internal pore cavity of MSNPs.¹⁰¹ Since then, many stimuli-responsive drug delivery systems based on MSNPs have been developed. The main focuses are (a) the design of controllable “gate keeper” in order to keep the cargos trapped inside before reaching target biological region and (b) stimuli-responsive opening to release the drugs inside. The “gate keeper” selections include various functional groups that are sensitive to pH,¹⁰²⁻¹⁰⁴ redox potential,^{42, 105-108} photo-irradiation,^{41, 109, 110} or thermal change.¹¹¹ The pore opening is dependent on the cleavable covalent bonds, ionic interaction, or competitive binding. Among these on-demand delivery system, one hot topic is mechanized MSNPs. Rotaxanes or pseudorotaxanes were employed to act as the controllable gate-keepers. These mechanically interlocked molecular machines can be functionalized on the MSNP surface, and guest molecules are employed to “close the door”. The bistable characteristic of the rotaxanes or pseudorotaxanes allows reversibly controlled open-close behavior of such drug delivery system.^{112, 113} Dr. Stoddard and Dr. Zink have contributed many excellent works on the mechanized silica nanoparticles for stimuli-responsive drug delivery.^{100, 109} A good review paper on this topic was published by his group recently.^{9, 114}

Besides relatively small organic groups or molecules, researchers have begun to use biomaterials as gate keepers. For example, Dr. Luo used collagen to cap the pores and then to realize cell-specific targeting and redox-responsive controlled drugs.¹¹⁵ Proteins were also utilized to carry out the controlled drug release based on MSNPs. Specific interaction between biotin and avidin was employed to cap the pores by biotin, and such delivery system is protease-responsive.¹¹⁶ Another work by the same group used click

chemistry to covalently link biotin labeled double strand DNA on the pore orifices of MSNPs in order to block the cargo molecules inside by interaction with avidin and achieve controlled release by thermal induced DNA denaturization.¹¹¹ Single strand DNA binding on the MSNP surfaces by charge-charge interaction through functionalized amine groups could also block the pores and the complementary DNA strand acts as the key to open the pores and release internal trapped cargo molecules.¹¹⁷ This new trend has opened a direction for the drug delivery application of MSNPs, which can increase the biocompatibility or target drug delivery with specific biomaterials as “gate keeper” and multi-functional agent. Introduction of biomaterials in the drug delivery system can also promise a good prospect for the future in vivo investigation due to special properties brought by the attached biomaterials. However, at present, these novel designs are not quite feasible in the practical applications. More research work needs to be done in this direction.

Another important biological application of MSNPs is non-viral gene delivery. As a promising candidate for non-viral delivery, MSNPs show obvious superiorities over viral gene delivery systems.¹¹⁸ Various non-viral gene delivery systems have been developed based on MSNP. Cationic polymer is one of the major ways in non-viral gene delivery. The application of silica nanoparticles in gene delivery has been investigated by some researchers. The early work used amine group-modified solid silica nanospheres to form complex with negative charge DNA for gene delivery purpose.¹¹⁹⁻¹²¹ The work by He etc. showed that bioconjugated amino-modified silica nanoparticles with DNA can protect DNA from cleavage by HindIII and proved GFP plasmid DNA expression in COS-7 cells.¹²² Similar work was completed by Pearce etc., who proved that non-viral gene delivery method with amine group-functionalized silicate nanoparticles could increase the transfection efficiency by 230% compared to non-functionalized silicate nanoparticles and also enhance pDNA-PEI complex induced transfection by over 150%.¹²³ Apart from surface modified silica nanoparticles for gene delivery by charge-charge interaction between positive charge silica nanoparticle surface and negative charged DNA chain, some researchers studied on the possibility of adsorption or encapsulation of DNA chains into the mesopores of MSNPs for gene delivery

purposes.¹²⁴⁻¹²⁷ Porous silica nanoparticles with extremely large pores were fabricated to encapsulate DNA or siRNA for gene delivery in vitro and in vivo.^{128, 129}

Apart from drug or gene delivery, MSNP also utilized for many other biological applications. As the MSNP is easily to covalently link fluorescence dyes¹³⁰ or bio-imaging reagent¹³¹ for bio-labeling or bio-image purposes in vitro or in vivo. Besides, MSNP was also employed as the scaffold for bone tissue regeneration, as the surface silanol groups on MSNP impose similar chemical property to bio-classes, so researchers even tried to develop functional MSNP for bone regeneration.^{132, 133} Enzyme immobilization on MSNP aims to achieve high enzyme stability, including both operational stability and storage stability.¹³⁴ The enlargement of pore size is necessary in order to encapsulate biomolecules such as enzymes with larger molecular size. Thus SBA-15^{4, 135} or FDU-12¹³⁶ with larger pore size, ranging from 5nm to 30nm, can effectively encapsulate proteins, as well as facilitating the mass transfer within the porous structure. Positive results were found for enzyme immobilization on MSNP, as higher enzyme activity was observed than free enzyme only.¹³⁷⁻¹³⁹ Through combination with other nanomaterials, such as gold nanoparticles, MSNP based nanohybrid could be used for synergetic cancer treatment by photothermal therapy and chemotherapy therapy simultaneously.¹⁴⁰⁻¹⁴⁴ In addition, MSNPs were also used to immobilize the photosensitizer for photodynamic cancer therapy. Several research groups have successfully prepare photosensitizer modified MSNP through co-condensation method with addition of photosensitizer modified silane.^{145, 146} Post-grafting method was also used to covalently link photosensitizer (protoporphyrin IX) onto the mesoporous structure of MSNP.¹⁴⁷ Effective reactive oxygen species were generated by responsive light irradiation and thus cancer cell killing effect was achieved.

1.2 Research Motivation and Scientific Contribution

In fact, numerous scientific reports based on the MSNP have been published, especially for its biological application, such as serving as drug, gene, or protein delivering carrier, bio-imaging and biochemical

detection. Several excellent review articles summarized the current biological application of MSNP very well.¹⁴⁸⁻¹⁵⁴ On the one side, it seems that this material has been extensively developed. However, on the other side, the fact of exponentially increasing publication numbers about MSNP indicates its great potentials in material science and engineering. Therefore, this research work dedicated to explore new application of MSNP in biological field through novel functionalization. It is expected that this research work would convey a concept of bringing new insight or new life to the traditional materials, which might be taken as the theme of this research work from a philosophical view. Based on the previous research background of MSNP, this research work brought up several proposals that aim at biological problem-solving or improvement on the current systems through novel functionalization on MSNP. Therefore, this work mainly focuses on the design, preparation, and characterization of the novel functional MSNP or MSNP based nano-hybrid. More importantly, the biological applications of these new functional MSNP materials would be well investigated in vitro or even in vivo. Based on the conventional material that was invented about twenty years ago, several novel functional mesoporous silica nanoparticles based nano-delivery systems or nano-hybrids were successfully designed (**Figure 1.4**), fabricated and characterized. The hypothesis of these proposals was successfully certified through various measurements or biological tests in vitro or in vivo.

Figure 1.4. Biological applications of functional mesoporous silica nanoparticle (MSNP).

This research work managed to develop several useful biological functions, such as effective drug/gene co-delivery for enhanced cancer therapy based on MSNP. In addition to novel design of the functional MSNP for the controlled drug/gene delivery, through structure evolution of mesoporous silica, enhanced delivering property was achieved by hollow mesoporous silica nanoparticles. Besides, this research work successfully combined with other scientific disciplines to conduct interdisciplinary research work. In the current research work, MSNP was employed to resolve some critical scientific bottleneck in the biological area through novel functionalization or delicate nano-hybrid design. For example, this research work successfully solved the aggregation problem of the secondary generation photosensitizer zinc phthalocyanine in aqueous solution, and thus extended its effective application in photodynamic therapy for cancer treatment. The success of this research not only provides scientific references for future study in related fields, but also delivering a research concept of developing novelty on conventional materials through structure or morphology evolution.

Chapter 2. Drug/Gene Co-delivery by MSNP

2.1 Research Background

The significance of gene delivery has been widely studied since the discovery of DNA and gene therapy.^{155, 156} DNA, small interfering RNA(SiRNA), microRNA and short hairpin RNA (shRNA) are major types of gene delivered in gene therapy research.¹⁵⁷ The main function of delivered gene is to enhance the expression of therapeutic proteins by exogenous plasmid DNA encoding a specific gene; or on the contrary, to silence certain protein expression to achieve specific biological effects. Due to different working locations inside cells, DNA and siRNA are required to deliver into nucleus or cytoplasm. The sufficient internalization of target gene is the first step for successful gene delivery.

The challenges of gene delivery mainly focus on two issues: efficient delivery/expression and applicability in research and clinical situation. Traditional gene delivery carriers are viral vector-mediated systems, which are the most common and successful methods for gene delivery. However, the limitations of the systems, like toxicity, limited DNA capacity, packaging and recombination issues, as well as high costs, greatly hamper their applications in research work and large scale gene delivery.^{158, 159} Therefore, non-viral delivery systems become increasingly desirable considering their superiorities over the stated limitations in viral gene delivery systems.¹⁶⁰ Various non-viral gene delivery systems have been developed in the past decade. Cationic polymer is one of the major ways in non-viral gene delivery. Biocompatible cationic polymers, like chitosan,¹⁶¹ PEI,¹⁶²⁻¹⁶⁴ as well as their derivatives, were widely studied for gene delivery. However, such systems usually lack effective release mechanism. After internalization, release of target gene mainly depends on the uncontrolled diffusion, which might decrease the expression efficiency due to the strong affinity of gene to its carriers.

Previous research has indicated the superiority of silica nanoparticles in non-viral gene delivery applications as described before. Meanwhile, the idea of drug/gene co-delivery systems sheds light on the treatment of cancers. In addition to delivering anti-cancer drugs for apoptosis, specific genes can be simultaneously delivered in order to promote or silence certain protein expression for gene therapy.^[9a] MSNPs possess apparent advantages in the aspect of drug/gene co-delivery, due to (a) their large pore volume for high drug storage capacity and (b) ease surface functionalization for the gene attachment.

2.2 Redox-responsive Drug/ssDNA co-delivery by functional MSNP

2.2.1 Research scope and motivation

Current research normally utilizes cationic polymer coated MSNPs for binding with genes through electrostatic interactions.¹⁶⁵⁻¹⁶⁸ For example, Nel group successfully delivered siRNA/DNA and Doxorubicin into a cancer cell line and studied the effects.¹⁶⁶ Bhattarai etc. used PEG and PDMAEMA or PDEAEMA modified MSNPs to simultaneously deliver chloroquine and siRNA to increase the transfection and silencing activity.¹⁶⁷ Dendrimer-capped MSNPs showed drug/gene co-delivery capability due to the large amount of amine groups on the dendrimer¹. Another drug/gene co-delivery system fabricated by dendrimer and MSNPs even showed sustained release profile of carried drugs as well as active function of Bcl-2 siRNA.¹⁶⁸ Lin group utilized gold-capped silica nanoparticles to deliver chemicals and plasmid DNA into plant cells.¹⁶⁹ However, these co-delivery systems generally cannot deliver genes and drugs in a controlled release manner. The release mechanism still depends on physical diffusion or dissociation of nanoparticle/ssDNA complex. Since there is such a vacancy of controlled release for the drug/gene co-delivery using the MSNP carriers, we designed and fabricated a MSNP-based drug/ssDNA co-delivery system capable of delivering simultaneously drugs and ssDNA triggered by redox.

The preparation of the novel functionalized MSNPs is illustrated in **Scheme 2.1**. Amino-terminated alkyl chains containing disulfide bonds were functionalized onto the nanoparticle surface by the reaction between *S*-(2-aminoethylthio)-2-thiopyridine hydrochloride (SATH) and thiol-modified nanoparticles (MSN-P-SH). Since the amino groups on the surface can be easily protonated to be ammoniums in aqueous solution at neutral or acidic pH, negatively charged ssDNA chains were introduced onto the surface through electrostatic interactions with the ammoniums. Thus, the ssDNA network at the orifice of mesopores serves as “gatekeepers”, blocking the mesopores in order to avoid the pre-release of the loaded drugs inside the mesopores. The controlled release is based on the redox-triggered disulfide bond cleavage. Addition of the reduction agent such as dithiothreitol (DTT) or glutathione (GSH) can effectively break the disulfide bond and thereby collapse the “gatekeepers” provided by the gene network, leading to a simultaneous drug/ssDNA co-delivery.

Mechanism 1.1. Illustrative mechanism of MSNP-based drug/gene co-delivery system.¹⁷⁰ (Copyright 2012 Wiley-VCH Verlag GmbH & Co.)

2.2.2 Materials and Methods

Materials: Absolute ethanol (EtOH, >99.9%), acetic acid, 2-aminoethythiol hydrochloride, 3-aminopropyltriethoxysilane (APTS), cetyltrimethylammonium bromide (CTAB, 90%), 4',6-diamidino-2-phenylindole (DAPI), diethyl ether, dimethylformamide (DMF, 99%), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,2'-dithiodipyridine, dithiothreitol (DTT), doxorubicin (Dox), dulbecco's modified eagle's medium (DMEM), 5' end/Cy 5 modified 21-mer ssDNA (ssDNA/Cy5), fetal bovine serum (FBS), fluorescein isothiocyanate (FITC), formaldehyde, glutathione (GSH), hydrochloride (HCl, assay 37%), 3-mercaptopyltrimethoxysilane (MPTMS), 21-mer ssDNA, methanol (MeOH, 99.5%), phosphate buffered saline (PBS) buffer, sodium hydroxide (NaOH), tetraethylorthosilicate (TEOS, 99%), and tris-ammonium acetate-EDTA buffer (TAE) were purchased commercially.

Instruments: TEM images were captured by JEOL 2010 TEM at 200kV. X-ray diffraction pattern was collected by low-angle X'Pert XRD. Zeta potential was measured by Mavorn Nanosizer and three independent measurements were conducted for statistic analysis. Surface area and pore size distribution characterization were performed by ASAP 2020 Micromeritics. FT-IR spectra were collected through Fourier transformed infrared spectrometer. Solid state NMR spectra were carried out by 400MHz Jeol ECA400 with CPMAS probe. Release studies were performed by RF-5301 spectrofluorophotometer. Nanodrop 1.0 was used to determine the ssDNA concentration. Agarose gel electrophoresis employs ENDRO GEL XL E0160 electrophoresis system. The fluorescence microscopic images were taken by a confocal fluorescence microscope (Nikon, Eclipse TE2000-E, 60 × oil objective).

Synthesis: Mesoporous silica nanoparticles were prepared by a conventional sol-gel process in base solution. Grafting method was used for the surface functionalization.

MSNP-SH: In a typical synthesis, CTAB (500mg) was dissolved in distilled H₂O (250mL). NaOH aqueous solution (1.75mL, 2M) was added into the above solution. The mixture solution was heated to 80 °C

under vigorous stirring. When the temperature was stabilized, TEOS (2.5mL) was slowly added into the mixture solution and the solution was stirred at 80°C for 2h. For the preparation of the FITC-labeled nanoparticles, fluorescein isothiocyanate (1.3 mg) was dissolved in absolute ethanol (1.5 mL) and mixed with APTES (3μL) for 2 h in the dark before adding into TEOS. Formed nanoparticles were collected by centrifugation at 8000 rpm for 10 min and washed with MeOH and distilled H₂O. The nanoparticles were dried in vacuo at 80°C for 24h. The introduction of thiol units through grafting method was conducted by suspending as-prepared silica nanoparticles (500mg) in MeOH (50mL), followed by the addition of MPTMS (0.5mL). The mixture solution was refluxed at 80°C under N₂ atmosphere for 24h. The thiol group functionalized silica nanoparticles were collected by centrifugation and washed with MeOH and distilled H₂O. Surfactant was removed by suspending the thiol group functionalized silica nanoparticles in MeOH (50mL) containing condensed HCl (3mL, 37%) and refluxing at 80°C for 24h. MSNP-SH was collected by centrifugation and washed thoroughly with MeOH and distilled H₂O.

S-(2-Aminoethylthio)-2-thiopyridine hydrochloride: The preparation of the compound was based on a literature report.¹⁷¹ Typically, thiopyridyldisulfide (4.41g) was dissolved in MeOH (20mL), and acetic acid (0.8mL) was then added. MeOH solution (10mL) containing 2-aminoethylthiolhydrochloride (1.14g) was dropwise added into the above solution within 30 min. After the mixture solution was stirred for 48 h, the solvent was removed in vacuo leading to a yellow oil product. The oil was washed with diethyl ether (50mL) and then dissolved in MeOH (10mL). The product was precipitated by the addition of diethyl ether (200mL), chilled at -20°C for 24h, and collected by filtration in vacuo. The product was reprecipitated in MeOH (10mL) in order to afford a pure compound.

MSNP-SS-NH₃⁺: Disulfide bond formation and terminal ammonium introduction were realized by suspending MSNP-SH (200mg) in MeOH (30mL) followed by the addition of *S*-(2-aminoethylthio)-2-thiopyridine hydrochloride (200mg). The mixture solution was stirred at room temperature for 24h.

Thenanoparticles were collected by centrifugation, washed thoroughly with MeOH and distilled H₂O, and dried in vacuo at 80°C for 24h.

Drug loading efficiency test: Suitable amount of FITC was added into EtOH (1mL), leading to different concentrations of FITC (0.1, 0.3, 0.5, and 0.7 mgmL⁻¹). After the UV-vis absorption measurements, MSNP-SS-NH₃⁺ nanoparticles (1mg) were added to each solution, obtaining various nanoparticle-to-FITC loading weight ratios (10:1, 10:3, 10:5, and 10:7). The mixture solutions were sonicated for 15 min and then stirred at room temperature for 24 h in order to fully disperse nanoparticles in the solutions. The mixture solutions were centrifuged at 13,000 rpm for 5 min and the supernatants were collected for the UV-vis measurements. The FITC loading amounts were evaluated by the UV-vis absorption at 495 nm. Three independent measurements for each loading weight ratio were conducted for statistic analysis. In order to test the loading efficiency of the drug delivery system, doxorubicin-loaded nanoparticles were prepared by mixing MSNP-SS-NH₃⁺ (5mg) with doxorubicin (1mg) in distilled H₂O (1mL, 1mg mL⁻¹) and then stirring the mixture solution overnight. The doxorubicin loading capacity was determined by measuring the UV-vis absorption at 485nm.

Agrose gel electrophoresis: Various nanoparticle-to-ssDNA weight ratios, ranging from 5:1 to 30:1, were prepared by mixing 21-mer ssDNA solution (5μL×100ngμL⁻¹) with proper amount of MSNP-SS-NH₃⁺ solution (2μg μL⁻¹). Free ssDNA solution without silica nanoparticles was also prepared for the control experiment. In order to demonstrate that the drug/ssDNA release is caused by the disulfide bond cleavage, DTT (2.5μL×300mM) was added into an extra lane with the nanoparticle-to-ssDNA weight ratio of 30:1. Proper amount of distilled H₂O was added to each lane in order to keep the concentration of ssDNA constant. The agarose gel electrophoresis was carried out in agarose gel (1.5wt%) within TAE buffer at 100V for 15 min.

DNA capping: MSNP-SS-NH₃⁺ loaded with FITC (nanoparticle-to-FITC loading weight ratio of 10:3) was suspended in distilled H₂O (0.75mL). 21-mer ssDNA or ssDNA/Cy5 (250μL×400 μgmL⁻¹) aqueous solution was added into the suspension (nanoparticle-to-ssDNA weight ratio of 10:1). The mixture solution was mildly stirred at room temperature for 3 h in order to form the nanoparticle/ssDNA complex. The product was collected by centrifugation and washed with distilled H₂O. The ssDNA-capped nanoparticles were suspended in PBS buffer (1mL, pH=7.36) as a stock solution for further tests.

Drug/ssDNA co-delivery test: The drug/ssDNA co-delivery test of the nanoparticles was conducted by adding as-prepared stock solution (300 μL, nanoparticle-to-FITC-to-ssDNA/Cy5 weight ratio of 10:3:1) into PBS buffer (700 μL, pH=7.36) with or without the reduction agent GSH or DTT. The mixture solution was centrifuged at 13,000 rpm for 3 min, and supernatant (10μL) was then collected and diluted by distilled H₂O (3mL) in order to monitor the kinetic release process by fluorescent measurements. Excitation wavelength at 450 nm was used for the FITC detection, and the emission wavelength at 516 nm was observed. The emission wavelength of ssDNA/Cy5 at 660nm was recorded with the excitation wavelength at 620 nm. Released FITC and ssDNA amounts were evaluated from their fluorescence intensities. Three independent measurements were conducted for statistic analysis.

Cell Culture: HeLa cells were cultured in DMEM containing 10% FBS and nonessential amino acids (0.1 mM). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO₂.

MTT cytotoxicity assay: The cytotoxicity of the nanoparticles was evaluated by employing the MTT assay. Equivalent amount of free Dox for each drug-loaded nanoparticle samples was used as control in the cytotoxicity test. HeLa cells were seeded into 96-well plate at a density of 1 × 10⁴ cells/well in DMEM medium. After 24 h exposure, the medium in the wells was replaced with fresh medium (100 μL) containing MSNP-SS-NH₃⁺, ssDNA-coated MSNP-SS-NH₃⁺, or Dox-loaded and ssDNA-coated MSNP-SS-NH₃⁺ with different concentrations. After incubation for another 24 h, the medium was removed and medium (100 μL) containing MTT (0.5mg mL⁻¹) was added. After further incubation for 4 h, the medium

was replaced with DMSO (100 μ L). The plate was gently shaken for 15 min. Then, the absorbance at 560 nm was recorded using a microplate reader (infinite 200 PRO, Tecan). The cell viability related to the control wells that only contain cell culture medium was calculated by $[A]_{\text{test}}/[A]_{\text{control}}$, where $[A]_{\text{test}}$ and $[A]_{\text{control}}$ are the average absorption intensities of the test and control samples, respectively.

Fluorescence microscopic images: HeLa cells were seeded in plastic-bot-tomed μ -dishes (35 mm) and grown in the DMEM medium for 24 h. After Hela cells were exposed to MSNP-SS-NH₃⁺ (50 μ g mL⁻¹), ssDNA-coated MSNP-SS-NH₃⁺ (50 μ g mL⁻¹), Dox-loaded and ssDNA-coated MSNP-SS-NH₃⁺ (50 μ g mL⁻¹), or free Dox (0.5 μ g mL⁻¹) for 24h, the medium was removed and dishes were washed three times with PBS and fixed with 4.0% formaldehyde at room temperature for 15 min. After washing with PBS, the cells were stained with DAPI (1 μ g mL⁻¹) for 15 min.

2.2.3 Results and Discussion

MSNP preparation and characterization

The thiol modified MSNP was prepared by post-synthetic grafting method. The thiol functionalized MSNP (MSNP-SH) were successfully prepared and fully characterized before further application for drug/ssDNA delivery

Figure 2.1. (a,b) TEM and(c) HR-TEM images of MSNP-SH.(Copyright 2012Wiley-VCH Verlag Gmbh& Co.)

Highly ordered mesoporous structure was clearly observed by TEM. For example, the mean nanoparticle size of MSNP-SH (**Figure 2.1a**) is calculated to be 120nm in diameter by counting 30 nanoparticles from the TEM images, the nanoparticle size which is suitable for intracellular drug delivery.^{90, 172, 173} The maximum and minimum diameters of the observed nanoparticles are about 144nm and 95nm respectively, indicating a relatively narrow size distribution. The HR-TEM image shows that the nanoparticles possess hexagonal mesopore arrays (**Figure 2.1c**). Counting 10 independent inter-plane distances by Nano Measure 1.2 reveals that the average inter-plane space (d_{100}) of the mesopores is 3.8nm.

Figure 2.2. (a) BET isotherm and BJH pore size distribution of MSNP-SH and (b) powder XRD pattern of MSNP-SH and illustration of a mesoporous structure. (Copyright 2012 Wiley-VCH Verlag GmbH & Co.)

Surface area of MSNP-SH was characterized by N₂ adsorption/desorption measurement (**Figure 2.2a**). MSNP-SH presents a typical type-IV isotherm, indicating its mesoporous structure. The BET (Brunauer-Emmett-Teller) surface area of MSNP-SH is 738 m²g⁻¹ and its pore size distribution of BJH (Barrett-Joyner-Halenda) desorption sharply focuses on 3.05 nm. Powder XRD further confirms the well-ordered hexagonal structure of MSNP-SH (**Figure 2.2b**). The distance of d_{100} space is 3.92 nm, calculated by Bragg's law from the (100) position at $2\theta = 2.25^\circ$, which is consistent with the value observed from the HR-

TEM image. Thus, the thickness of mesoporous wall can be estimated from the difference ($3.92 - 3.05 = 0.87$ nm) between the internal average pore size given by BJH method and external average pore size calculated from XRD or TEM.

Figure 2.3. (A) FT-IR spectra of silica nanoparticles under indicated conditions: (a) silica nanoparticles containing surfactants, (b) MSNP without the surfactants, (c) MSNP-SH, and (d) MSNP-SS-NH₃⁺, and (B) ¹³C CP MAS NMR spectrum of MSNP-SS-NH₃⁺. (Copyright 2012 Wiley-VCH Verlag GmbH & Co.)

After further functionalization on MSNP, the surface functional groups were characterized by FT-IR and solid state NMR. As seen from FT-IR spectra (**Figure 2.3A**), the peak around 1092 cm^{-1} is attributed to asymmetric stretching of Si-O-Si bond in the silica nanoparticles. The surfactant removal process (from a to b in **Figure 2.3A**) was confirmed by the disappearance of peaks around 2850 and 2924 cm^{-1} , which are attributed to the vibration of the long carbon chains on the surfactant cetyltrimethylammonium bromide (CTAB). Introduction of the thiol groups on MSNP-SH (from b to c in **Figure 2.3A**) were certified by the appearance of the minor peak around 2560 cm^{-1} . After MSNP-SH reacts with SATH (from c to d in **Figure 2.3A**), the peak around 1530 cm^{-1} , characteristic of the amino groups, is present, while the minor peak around 2560 cm^{-1} for the thiol groups disappears,¹⁰⁸ indicating the formation of MSNP-SS-NH₃⁺. The

^{13}C CP MAS NMR spectrum (**Figure 2.3B**) of MSNP-SS- NH_3^+ exhibits strong carbon signals at 11.5, 22.8, 27.9, 34.5, 39.5, and 51.3 ppm, attributing to the aliphatic secondary carbon atoms as assigned in **Figure 3A**. These results indicate the successful introduction of amino-terminated and disulfide bond-bridged alkyl chains on the nanoparticle surface.

Figure 2.4. Zetapotential values of silica nanoparticles: (a) MSNP-SH, (b) MSNP-SS- NH_3^+ , (c) MSNP-SS- NH_3^+ coated with ssDNA (nanoparticle-to-ssDNA loading weight ratio of 10:1), (d) MSNP-SS- NH_3^+ loaded with FITC (nanoparticle-to-FITC loading weight ratio of 10:3), and (e) MSNP-SS- NH_3^+ loaded with FITC, followed by coating ssDNA (nanoparticle-to-FITC-to-ssDNA loading weight ratio of 10:3:1). (Copyright 2012 Wiley-VCH Verlag GmbH & Co.)

21-mer ssDNA was employed as a model gene for complexation with MSNP-SS- NH_3^+ . The chain length of 21-mer ssDNA is suitable for capping the mesopores.¹⁷⁴ Zetapotential results (**Figure 2.4**) indicate the successful transformation of the thiol groups on MSNP-SH to the amino-terminated and disulfide bond-bridged alkyl chains on MSNP-SS- NH_3^+ . MSNP-SH shows a negative zetapotential value of -34.6 mV on account of the Si-O^- groups on the surface. After the formation of MSNP-SS- NH_3^+ , the surface charge turns to a highly positive value of 35.6 mV, because of the introduction of the positively charged NH_3^+ units on the surface. Upon the coverage of the negatively charged ssDNA on the surface, a negative zetapotential value of -35.7 mV was observed. The reversion from a positive value to a negative one proves the ssDNA coating on the nanoparticle surface through the electrostatic interaction between

negatively charged ssDNA and positively charged ammonium group. After the mesopores of MSNP-SS-NH₃⁺ were loaded with a model drug, fluorescein isothiocyanate (FITC), the zeta potential value decreases from 35.6mV to -4.66mV due to the existence of carboxylic group on the FITC molecule. Higher negative value of -37.3mV was measured when MSNP-SS-NH₃⁺ was loaded with FITC followed by capping with ssDNA, indicating that the presence of loaded FITC will not affect the ssDNA binding on the nanoparticle surface.

ssDNA capping on functional MSNP

Figure 2.5. TEM images of (a) MSNP-SS-NH₃⁺, (b) MSNP-SS-NH₃⁺ coated with 21-mer ssDNA, and (c) MSNP-SS-NH₃⁺ loaded with FITC. (Copyright 2012 Wiley-VCH Verlag GmbH & Co.)

By comparing TEM images (**Figure 2.5**) of MSNP-SS-NH₃⁺ before and after capping with ssDNA, the ssDNA coverage on the nanoparticle surface can be estimated. TEM image of MSNP-SS-NH₃⁺ before the ssDNA binding clearly shows hexagonally mesoporous structures. After MSNP-SS-NH₃⁺ binds with ssDNA, the mesoporous structures can be barely observed on account of the surface masking of the ssDNA network. Thus, the successful binding of ssDNA on the nanoparticle surface is fully demonstrated by zeta potential measurements and TEM images. After mesoporous nanoparticles were loaded with FITC, mesoporous structures were also blurred due to the occupation of loaded FITC molecules inside the mesopores (**Figure 2.5c**).

Figure 2.6. Agarose gel electrophoresis of MSNP-SS-NH₃⁺ capped with an increasing ssDNA ratio. Lanes from left to right are free ssDNA, MSNP-SS-NH₃⁺, nanoparticle-to-ssDNA weight ratios of 5:1, 10:1, 20:1, 30:1, and 30:1 containing 50mM DTT, respectively. Agarose gel electrophoresis was carried out at 100V for 15 min using 1.5wt% agarose gel in TAE buffer. (Copyright 2012 Wiley-VCH Verlag GmbH & Co.)

The formation of the nanoparticle/ssDNA complex and the gene delivery capacity of the nanoparticles were estimated by agarose gel electrophoresis (**Figure 2.6**). As expected, free ssDNA clearly shifts in the first lane, while nothing moves out in the second lane for MSNP-SS-NH₃⁺. Upon the increase of the nanoparticle-to-ssDNA weight ratios, the shifted ssDNA amount becomes less and less as indicated by the brightness in the lanes with the nanoparticle-to-ssDNA weight ratios of 5:1 and 10:1. When the weight ratio is over 10:1, the ssDNA shift cannot be observed and ssDNA was completely trapped on the nanoparticle surface. Therefore, the nanoparticle-to-ssDNA weight ratio of 10:1 is suitable for the gene delivery, and was chosen as the experimental condition for the ssDNA delivery in our studies. In the lane 7 (from left to right), when adding 50mM of DTT to the ssDNA-coated MSNP-SS-NH₃⁺, clear ssDNA shift was observed, indicating that ssDNA is released from the nanoparticle surface on account of the disulfide bond cleavage induced by DTT.

Figure 2.7. Fluorescence spectra of FITC-loaded MSNP-SS-NH₃⁺ and equivalent amount of free FITC in PBS buffer. (Copyright 2012Wiley-VCH Verlag Gmbh& Co.)

In order to further prove that the FITC molecules are loaded inside the mesopores, fluorescent intensity of well-suspended FITC-loaded nanoparticles was compared with that of equivalent amount of free FITC in PBS buffer (**Figure 2.7**). Much low fluorescent intensity was observed for the case of the FITC-loaded nanoparticles. The significant fluorescence quenching from the FITC-loaded nanoparticles indicates that the FITC molecules are mainly loaded inside the mesopores of the nanoparticles.

Figure 2.8. Agarose gel electrophoresis of FITC-loaded MSNP-SS-NH₃⁺ (nanoparticle-to-FITC loading weight ratio of 10:3) capped with an increasing ssDNA ratio. Lanes from left to right are free ssDNA, FITC-loaded MSNP-SS-NH₃⁺, nanoparticle-to-ssDNA weight ratios of 5:1, 10:1, and 10:1 containing

50mM DTT, respectively. Agarose gel electrophoresis was carried out at 100V for 30 min using 1.5wt% agarose gel in TAE buffer. (Copyright 2012Wiley-VCH Verlag Gmbh& Co.)

As discussed before, the zeta potential of MSNP-SS-NH₃⁺ loaded with FITC has positive high value and would not affect the ssDNA binding. To further confirm this point, agarose gel electrophoresis was performed with FITC-loaded MSNP-SS-NH₃⁺ (nanoparticle-to-FITC loading weight ratio of 10:3) capped with an increasing ssDNA ratio relatively. ssDNA binding on MSNP was clearly observed (**Figure 2.8**). In addition, DTT can also effectively cleave disulfide bond and release ssDNA from MSNP. During the binding and dissociation processes of ssDNA on FITC-loaded MSNP-SS-NH₃⁺, some of the FITC molecules were observed to be released, which was indicated by the relatively less bright band.

Drug load capacity study

Figure 2.9. FITC loading capacity of MSNP-SS-NH₃⁺ under different nanoparticle-to-FITC loading weight ratios. (Copyright 2012Wiley-VCH Verlag Gmbh& Co.)

In order to measure the drug delivery capacity of MSNP-SS-NH₃⁺, different loading weight ratios of nanoparticle-to-FITC were mixed in absolute EtOH and stirred at room temperature for 24 h. The loading capacity was obtained by comparing the UV-vis intensities of original and supernatant absorptions of FITC at 495 nm. The FITC loading capacity of MSNP-SS-NH₃⁺ was calculated to be around 12~15 μg per 1 milligram of nanoparticles (**Figure 2.9**). The nanoparticle-to-FITC loading weight ratio of 10:3 was

found to be the most suitable loading ratio with the least drug waste during the loading process, since there is no significant loading amount increase observed when increasing the FITC amount.

Controlled drug/ssDNA co-release

Figure 2.10. Controlled drug/ssDNA co-release from MSNP-SS-NH₃⁺ in the addition of reduction agent DTT or GSH in PBS buffer (pH = 7.36). (a) Cy5 labeled ssDNA release profile and (b) FITC release profile. Curves ♦ indicate the release profiles of FITC-loaded and ssDNA-coated MSNP-SS-NH₃⁺ upon time without any reduction agents. Curves ■, ▲, and ▼ indicate the release profiles of FITC-loaded and ssDNA-coated MSNP-SS-NH₃⁺ in the addition of 50mM DTT, 50mM GSH, and 10mM GSH at the beginning, respectively. Curves ● indicate the release profiles of FITC-loaded and ssDNA-coated MSNP-SS-NH₃⁺ in the addition of 50mM DTT at 60 min. (Copyright 2012 Wiley-VCH Verlag GmbH & Co.)

The controlled drug/ssDNA co-delivery of MSNP-SS-NH₃⁺ was investigated by monitoring the fluorescent intensity changes of both model drug FITC and Cy5 labeled ssDNA in PBS buffer (pH = 7.36). Without the addition of any reduction agent, FITC-loaded and ssDNA-coated MSNP-SS-NH₃⁺ does not exhibit obvious cargo leakage (curves ♦ in **Figure 2.10**), indicating that the ssDNA network on the surface can well block FITC inside the mesopores. With the introduction of different amount of reduction agent DTT or GSH, obvious FITC and ssDNA releases were observed. The ssDNA release rate was well controlled by the concentration of the reduction agent, i.e., the released ssDNA amount is proportional to

the concentration of the reduction agent under current experimental conditions. As compared with one GSH molecule containing one thiol group that is responsible for the disulfide bond cleavage, one DTT molecule has two thiol groups. Thus, 50mM of DTT could lead to double amounts of ssDNA release from the nanoparticles than that of 50mM GSH under the same conditions. The addition of 10mM GSH showed an even low ssDNA release amount. The observations further demonstrate that the ssDNA release is controlled by the disulfide bond cleavage. Since each ssDNA chain may interact with multiple ammonium linkers on the nanoparticle surface, the ssDNA chain can be released only when all the disulfide bonds of the ammonium linkers associated with the ssDNA chain are cleaved.

The addition of 50mM DTT to the FITC-loaded and ssDNA-coated MSNP-SS-NH₃⁺ leads to a high FITC release rate as compared with that of 50mM GSH. The addition of 10mM GSH also exhibits a considerable FITC release rate. FITC is initially blocked within the mesopores by the ssDNA network associated with the ammonium linkers. Once a part of the ssDNA network is dissociated from the nanoparticle surface on account of the redox-triggered disulfide bond cleavage, some of the mesopores are open, causing the release of loaded FITC.

In vitro drug/ssDNA co-delivery and cytotoxicity study

Figure 2.11. Fluorescence microscopic images of HeLa cells after being treated with 50μg mL⁻¹ of Dox-loaded and ssDNA/Cy5-coated nanoparticles (MSNP-SS-NH₃⁺ + Dox + ssDNA/Cy5) for 24h. (a) Bright

field, (b) DAPI (4',6-diamidino-2-phenylindole) stain channel, (c) FITC channel, (d) Dox channel, (e) merged image of b, c, and d, (f) Cy5 channel, and (g) merged image of b, c and e. (Copyright 2012Wiley-VCH Verlag GmbH & Co.)

Since a certain amount of GSH is present inside a variety of cancer cells,^{175, 176} drug-loaded and ssDNA-coated MSNP-SS-NH₃⁺ within cells is expected to release both drug and ssDNA triggered by the GSH-induced disulfide bond cleavage. The ssDNA-coated nanoparticles can be effectively endocytosed by HeLa cancer cells and be focused in the cytoplasm as shown from the green fluorescence of FITC-labeled nanoparticles in **Figure 2.11c**. The ssDNA-coated nanoparticles show an appealing property in the Dox/ssDNA co-delivery. Dox fluorescence from both the cytoplasm and nucleus indicates the successful drug release inside the HeLa cells (**Figure 2.11d**). The drug delivery efficacy of the delivery system was demonstrated by comparing to free Dox treatment. Dox delivered by the ssDNA-coated nanoparticles in HeLa cells presents much higher fluorescence intensity (**Figure 2.11d**) than that treated with equivalent amount of free Dox (**Figure 2.12c**).

Figure 2.12. Fluorescence microscopic images of HeLa cells after being treated with free Dox (0.5 μg mL⁻¹). (a) Bright field, (b) DAPI (4',6-diamidino-2-phenylindole) stain channel, (c) FITC channel, (d) Dox channel, (e) merged image of b, c. (Copyright 2012Wiley-VCH Verlag GmbH & Co.)

Red fluorescence intensity of Cy5 on ssDNA excited by 647nm was also observed in the cytoplasm, indicating a successful delivery of ssDNA into HeLa cells (Figure 2.11f). Interestingly, the functional nanoparticles without the ssDNA coating show a low cellular internalization when compared to that of the ssDNA-coated nanoparticles (**Figure 2.13**).

Figure 2.13. Fluorescence microscopic images of HeLa cells after being treated with (a) ssDNA/Cy5-coated MSNP-SS-NH₃⁺ (50 μg mL⁻¹) or (b) MSNP-SS-NH₃⁺ (50 μg mL⁻¹) for 24h. (Copyright 2012 Wiley-VCH Verlag GmbH & Co.)

It is obvious that the ssDNA coating on the nanoparticles can enhance the cellular uptake of the nanoparticles. This enhanced cellular internalization may be due to the biocompatibility of ssDNA. The improved cellular uptake efficiency of the ssDNA-coated nanoparticles is beneficial to the enhanced cytotoxicity of Dox delivered by the nanoparticle-based carrier *in vitro*.

Figure 2.14. Viabilities of HeLa cells after being treated with indicated nanoparticles and equivalent amount of free Dox for 24h. Curves ■, ●, ▲, and ▼ indicate the cell viabilities in the presence of MSNP-

SS-NH₃⁺, ssDNA-coated MSNP-SS-NH₃⁺ (MSNP-SS-NH₃⁺ + ssDNA), Dox-loaded and ssDNA-coated MSNP-SS-NH₃⁺ (MSNP-SS-NH₃⁺ + Dox + ssDNA), and free Dox, respectively, under the concentrations measured. (Copyright 2012Wiley-VCH Verlag Gmbh& Co.)

Anticancer drug Doxorubicin (Dox) was employed in order to investigate the cytotoxicity and co-delivery capacity of the functional nanoparticles. The Dox loading capacity of the nanoparticles was determined to be 9.97 µg per 1 milligram of nanoparticles by measuring the UV-vis absorption intensities of original and supernatant Dox at 485nm. The MSNP-SS-NH₃⁺ nanoparticles have no obvious cytotoxicity towards Hela cells, since the cell viability shows no significant decrease with increasing the nanoparticle concentrations (**Figure 2.14**). The ssDNA coating on the nanoparticles (MSNP-SS-NH₃⁺ + ssDNA) has no obvious effect on the cytotoxicity of the nanoparticles. Relatively high cell viabilities (over 80%) were observed for both samples even at a high nanoparticle concentration of 200µg mL⁻¹, indicating that the nanoparticles as a co-delivery system are biologically benign. The cytotoxicity of the Dox-loaded and ssDNA-coated nanoparticles (MSNP-SS-NH₃⁺ + Dox + ssDNA) displays clearly a dependence on the nanoparticle concentration. The IC₅₀ (drug concentration killing 50% of cells) was determined to be 43µg mL⁻¹ for the drug-loaded nanoparticles, equivalent to a Dox concentration of 0.43µg mL⁻¹. On account of the drug resistance of Hela cells, equivalent amount of Dox cannot actually lead to the efficient apoptosis of cancer cells as compared with that of Dox-loaded nanoparticles, a fact which demonstrates the superiority of the nanoparticle-based co-delivery system.

2.2.4 Conclusion

A functional mesoporous silica nanoparticle carrier for controlled drug/ssDNA co-delivery has been developed. Ammonium units containing cleavable disulfide bond has been introduced onto the nanoparticle surface. Thus, ssDNA has been immobilized onto the nanoparticle surface through electrostatic interactions, blocking the pre-loaded drugs inside the mesopores. The drug/ssDNA co-

delivery can be realized as soon as the nanoparticle system is treated with the reduction agent for the disulfide bond cleavage. The drug/ssDNA release rate is dependent on the concentration of the reduction agent. Relatively abundant presence (up to 10mM) of glutathione in the cells provides an excellent opportunity for the disulfide bond cleavage, while extracellular concentration of glutathione is less than 10 μ M incapable of triggering the disulfide bond cleavage. Therefore, the redox-controlled drug/ssDNA co-delivery system presents promising applications in biological systems, on account of the fact that when the nanoparticle system is internalized inside certain cancer cells, the disulfide bonds can be automatically cleaved by intracellular glutathione for the drug/ssDNA release. The *invitro* investigations have shown that the current nanoparticle system has a very low cytotoxicity with an enhanced cellular internalization capacity. The nanoparticles can successfully deliver both anticancer drug and ssDNA into Hela cells for an improved apoptosis as compared with that of equivalent amount of free drug. The obtained results shed light on further applications of the nanoparticle-based co-delivery system for *in vivo* cancer treatment.

2.3 Controlled Drug/siRNA Delivery by Ethylenediamine Modified β -cyclodextrinCapped MSNP in Vitro and in Vivo

2.3.1 Researchscope and motivation

An effective siRNA/drug co-delivery carrier offers great promise to cancer treatment on account of the synergistic effect provided from cancer-associated genes and anticancer drugs.¹⁷⁷⁻¹⁷⁹ Ever since the discovery of RNA interference (RNAi),¹⁸⁰ gene-specific inhibition has been proposed in cancer treatments to overcome multiple drug resistance (MDR) and to inhibit expression of up-regulated oncogenes that result in un-regulated tumour growth.¹⁸¹⁻¹⁸⁴ In fact, RNAi-based treatments are regarded as a new class of therapeutics for human diseases, including cancer. A hurdle to widespread use of free siRNA-based therapeutics is their inefficient intracellular uptake (1,10).^{185, 186} The use of non-viral siRNA-based carriers is an attractive strategy for RNAi therapeutics. Issues, including how to enhance the efficiency of cellular uptake and how to carry out the controlled release of siRNA from carriers, need to be addressed in the design of such technology. Both criteria are essential for effective targeted gene silencing to be achieved in vivo.¹⁵⁵ Such non-viral-based drug carriers can be used concomitantly to improve the delivery of pharmacological anticancer drugs into cancer cells thereby minimizing undesirable side effects of such drugs.

Although we successfully fabricated a functional MSNP for controlled drug/ssDNA co-delivery in vitro, still no function of the delivered gene was demonstrated. Furthermore, as the MSNP based drug delivery system was used for the cancer treatment, the siRNA based gene therapy is favorable for the application of co-delivery system. Therefore, we aim to design and fabricate a drug/siRNA co-delivery system, aiming to overcome the multiple drug resistance in cancer treatment. In order to study the individual effect of chemotherapy, gene therapy, and synergetic therapy effect respectively, a delivery system

capable of delivering drug/gene individually or delivering both simultaneously is necessary. Thus, we here would like to employ multifunctional MSNPs as carrier for effective co-delivery of siRNA and drug in a controlled-release manner. Such desirable traits will minimize adverse side effects that result from uncontrolled release of both siRNA and drugs. A detailed study of the efficacy of the co-delivery system would be evaluated both in vitro and in vivo.

Scheme 3.1. Schematic representation of MSNP based siRNA/drug co-delivery system.

This study aimed to the efficacy of siRNA/drug co-delivery system based on multifunctional MSNPs, where the mesopores of the nanoparticles are capped by redox-responsive nano-gates, i.e. ethylenediamine modified β -cyclodextrin (CD-2NH₂) rings. The application of CD-2NH₂ nano-gate provides more options in future delivery application, such as individual drug or siRNA delivery, or delivery drug and siRNA simultaneously, which would assist the study on the chemotherapy or gene therapy effect in vitro and in vivo. In this study, the Intracellular efficacy was first evaluated in HeLa cells, where siRNA against Bcl-2 (B-cell lymphoma 2) was employed. Bcl-2 was targeted since this protein promotes tumor formation by preventing cellular apoptosis¹⁸⁷ and contributes to the resistance to

anticancer drugs.¹⁸⁸⁻¹⁹⁰ This co-delivery approach would lead to increased level of apoptosis of HeLa cells treated with CD-2NH₂-capped MSNPs containing Bcl-2 siRNA and doxorubicin (Dox). In vivo siRNA delivery would be further tested in transgenic zebrafish larvae expressing the GFP reporter in the choroid plexus.¹⁹¹ The anti-cancer efficacy was further confirmed in a liver tumor model using transgenic zebrafish that express the oncogenic EGFP-kras^{v12}.¹⁹² This work would use multifunctional MSNP for the drug/siRNA delivery in vitro with HeLa cell line, but also validate the use of transgenic zebrafish larvae as a well-controlled reporter system to develop the formulations of siRNA/drug complexes and to identify those with the highest in vivo potency.

2.3.2 Materials and Methods

Materials: Absolute EtOH (>99.9%), acetic acid, adamantane-1-carboxylic acid (AD-COOH), 2-aminoethylthiol hydrochloride, 3-aminopropyltriethoxysilane (APTS), Bcl-2 primary antibody (Mouse), bovine serum albumin (BSA), cetyltrimethylammonium bromide (CTAB, 90%), 4',6-diamidino-2-phenylindole (DAPI), dicyclohexylcarbodiimide (DCC), diethyl ether, dimethylformamide (DMF, 99%), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,2'-dithiodipyridine, dithiothreitol (DTT), doxorubicin (Dox), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), fluorescein isothiocyanate (FITC), formaldehyde, glutathione (GSH), hydrochloride (HCl, assay 37%), N-hydroxysuccinimide (NHS), Lipofectamine®RNAiMAX reagent (Invitrogen), 3-mercaptopyltrimethoxysilane (MPTMS), methanol (MeOH, 99.5%), phosphate buffered saline (PBS) buffer, siRNA duplex against Bcl-2 protein, sodium hydroxide (NaOH), tetraethylorthosilicate (TEOS, 99%), tris-borate-EDTA buffer (TBE), β -tubulin primary antibody (Rabbit) and secondary antibody (goat anti-mouse and goat anti-rabbit), and universal siRNA negative control were purchased commercially.

Instruments: FESEM and TEM images were captured by FESEM 6340 at 5kV and JEOL 2010 TEM at 200kV, respectively. X-Ray diffraction pattern was collected by low-angle X'Pert XRD from 2 θ =1.5 to 8. Zeta potential value was measured by MavernNanosizer and each sample was measured three times for

statistic analysis. Surface area and pore size distribution of the MSNP-SH nanoparticles were characterized by ASAP-2020 Micromeritics. FT-IR spectra were measured through Fourier transformed infrared spectrometer. ^{13}C Solid state NMR spectrum of MSNP-SS-AD was carried out by 400MHz JEOL ECA400 with CPMAS probe. In vitro release test was monitored by RF-5301 fluorophotometer. Nanodrop 1.0 was used to determine the SiRNA concentration. UV-vis absorption was measured by UV-vis 2501 Spectrometer. Agarose gel electrophoresis was performed by ENDRO GEL XL E0160 electrophoresis system. A microplate reader (infinite 200 PRO, Tecan) was used for the MTT assay. Fluorescence images of transfected HeLa cells were taken by a confocal fluorescence microscope (Nikon, Eclipse TE2000-E, 100 $\times$ oil objective). In vivo images of transgenic zebrafish larvae were acquired with an upright Zeiss Axiovert 200M laser scanning microscope (LSM) Meta 510 (Carl Zeiss), equipped with a $\times 40$ numerical aperture (NA) 0.75 W Achroplan long working distance dipping objective. Bright field zebrafish images were acquired with Olympus AX70 upright compound microscope attached to a ProgRes C10plus digital camera (JENOPTIK Laser, Optik, Systeme GmbH, Germany).

Synthesis of MSNP-SH: MSNP-SH nanoparticles were prepared by a co-condensation method in base solution in order to obtain uniform surface distribution of the functional groups. In a typical synthetic procedure, CTAB (500 mg) was dissolved in distilled H_2O (250 mL). NaOH aqueous solution (1.75 mL, 2 M) was added into the above solution. The mixture solution was heated to 80 $^\circ\text{C}$ under vigorous stirring. When the temperature was stabilized, TEOS (2.5 mL) was slowly added into the mixture solution. MPTMS (0.5 mL) was added dropwise after 15min. The mixture solution was stirred at 80 $^\circ\text{C}$ for another 2 h. As for FITC labeled nanoparticles, FITC (1.3 mg) was dissolved in absolute ethanol (1.5 mL) containing APTES (3 μL), and the mixture solution was gently stirred for 2 h in the dark before adding into the TEOS (2.5 mL). Then, the formed nanoparticles were collected by centrifugation at 8000 rpm for 10 min and washed with MeOH and distilled H_2O . Surfactant was removed by suspending the thiol group functionalized silica nanoparticles in MeOH (50 mL) containing condensed HCl (3 mL, 37%) and

refluxing at 80 °C for 24h. MSNP-SH was collected by centrifugation and washed thoroughly with MeOH and distilled H₂O. The nanoparticles were dried in vacuo at 80 °C for 24h.

S-(2-Aminoethylthio)-2-thiopyridine hydrochloride: The preparation of the compound was according to a previous literature report.¹⁷¹

MSNP-SS-NH₂: Disulfide bond formation on the nanoparticle surface was realized by suspending MSNP-SH (200 mg) in MeOH (30 mL) followed by the addition of *S*-(2-aminoethylthio)-2-thiopyridine hydrochloride (200 mg). The mixture solution was stirred at room temperature for 24h. The MSNP-SS-NH₂ nanoparticles formed were collected by centrifugation, washed thoroughly with MeOH and distilled H₂O, and dried in vacuo at 80 °C for 24 h.

MSNP-SS-AD: Adamantane-1-carboxylic acid (225 mg) was dissolved in DMF (10 mL) containing DCC (645 mg) and NHS (360 mg). After the mixture solution was stirred for 2 h, a DMF (5mL) solution containing fully suspended MSNP-SS-NH₂ (100 mg) was added. After the reaction for 24 h, MSNP-SS-AD nanoparticles were collected by centrifugation and washed with DMF, MeOH, and distilled H₂O extensively.

CD-2NH₂: Ethylenediamine modified β -CD derivative was prepared according to a previous report.¹⁰⁰ Seven ethylenediamine groups were covalently linked at the primary side of the β -CD ring.

Drug Loading and Controlled Release Test: RhB or Dox was loaded into the nanoparticles by stirring aqueous solution (1 mL) containing either RhB or Dox (1 mg) and MSNP-SS-AD (10 mg) at room temperature for 24 h. Then, β -CD or CD-2NH₂ (5mg) was added into the solution in order to cap the mesopores by forming the host-guest complex between AD and β -CD on the nanoparticle surface. After stirring the solution for 5 h, the nanoparticles were collected by centrifugation at 13,000 rpm for 10 min and washed with distilled H₂O for three times. The collected nanoparticles were suspended in PBS buffer (5mL, pH 7.4) as stock solution (2mg mL⁻¹) for future tests. The loading amount of the drugs was calculated by comparing the UV-vis absorption at 553 nm for RhB and 485 nm for Dox. Controlled release experiments were conducted by adding as-prepared stock solution (500 μ L) into PBS buffer (500

μL , pH 7.4) with or without the reducing agent GSH or DTT. The solution was centrifuged at 13,000 rpm for 3 min, and fluorescence intensity ($\lambda_{\text{ex/em}} = 510/572 \text{ nm}$ for RhB, $\lambda_{\text{ex/em}} = 485/553 \text{ nm}$ for Dox) of the supernatant was recorded in order to monitor the kinetic release process of each sample. The final fluorescence intensity after 24 h was regarded as complete release (100%). For each sample, three independent experiments were carried out for statistic analysis.

Agarose Gel Electrophoresis: MSNP-SS-AD (1 mg) was mixed with CD-2NH₂ (1 mg) in aqueous solution, and the mixture was stirred for 5h. CD-2NH₂ capped MSNP-SS-AD (MSNP-SS-AD+CD-2NH₂) was collected by centrifugation and excess CD-2NH₂ was removed by washing the nanoparticles with distilled H₂O for three times. Then, the MSNP-SS-AD+CD-2NH₂ nanoparticles were suspended in distilled H₂O (1mL, 2 mg mL⁻¹). siRNA duplex against Bcl-2 protein, Bcl-2 siRNA, was used in agarose gel electrophoresis. Proper amounts of Bcl-2 siRNA solution (0.2 $\mu\text{g } \mu\text{L}^{-1}$) and MSNP-SS-AD+CD-2NH₂ solution (2 mg mL⁻¹) were mixed to obtain different nanoparticle-to-siRNA(+) weight ratios. Free Bcl-2 siRNA without the nanoparticles was placed in the first lane as the control. In the second line, only MSNP-SS-AD+CD-2NH₂ was added. In order to demonstrate that the siRNA(+) release is induced by the disulfide bond cleavage, DTT (50 mM) was added into the last lane with the highest nanoparticle-to-siRNA(+) weight ratio of 110:1. Proper amount of distilled H₂O was added into each lane in order to keep the concentration of siRNA(+) constant. The MSNP-SS-AD+CD-2NH₂ nanoparticles were incubated with siRNA(+) for 1 h before running the electrophoresis. The agarose gel electrophoresis was carried out in agarose gel (1.5 wt%) within TBE buffer at 100V for 15 min.

Bcl-2 Gene Knockdown and Western Blot Assay: siRNA duplex sequence against Bcl-2 protein, noted as siRNA(+), was presented as follows: sense strand 5'-rGrUrArCrArUrCrCrArUrUrArUrArArGrCrUrGrUrCrGrCdAdG-3', antisense strand 5'-rUrGrCrGrArCrArGrCrUrUrArUrArArUrGrGrArUrGrUrArCrUrU-3'. Non-targeting siRNA duplex, noted as siRNA(-), was used as the negative control. HeLa cells were seeded into 6-well plate at a density of 20×10^4 cells/well in complete DMEM medium and grown for 24 h. Fresh DMEM medium without

any antibiotics and FBS was used for the gene knockdown experiments. MSNP-SS-AD capped with CD-2NH₂ (MSNP-SS-AD+CD-2NH₂, 50 μ L, 2 mg mL⁻¹) was mixed with either siRNA(+) or siRNA(-) (5 μ L, 0.2 μ g μ L⁻¹), and the mixture was incubated for 2 h with gentle stirring every 20 min. The MSNP-SS-AD capped with the CD-2NH₂/siRNA complex (MSNP-SS-AD+CD-2NH₂+siRNA) with an MSNP-SS-AD-to-siRNA weight ratio of 110:1 was stabilized by BSA (1mg mL⁻¹) before adding fresh medium (445 μ L). For siRNA transfection by Lipofectamine as the positive control, same amount of siRNA(+) was used according to the product instruction of Lipofectamine®RNAiMAX reagent. Free siRNA(+) (5 μ L, 0.2 μ g μ L⁻¹) and MSNP-SS-AD+CD-2NH₂ (50 μ L, 2 mg mL⁻¹) were added into fresh medium (495 μ L and 450 μ L), respectively. In total, each sample was prepared in the same volume (500 μ L). The cell culture medium in the 6-well plate was removed and the prepared sample (500 μ L) was added. After 16 h exposure, the medium in each well was replaced by fresh complete DMEM medium (2mL), and HeLa cells were incubated for another 56 h. Then, the cell culture medium was removed and the cells were washed with chilled PBS buffer (pH 7.4) for three times. After adding lysis buffer (500 μ L), the plate was shaken at 4 °C for 30 min in order to break cell membranes. The lysis buffer was collected and centrifuged (13 rpm, 4 °C) for 10min, and the supernatant was then collected. The protein concentration was quantified by Pierce® BCA Protein Assay Kit according to its product instruction. Relative Bcl-2 protein expression level was checked by western blot assay using β -tubulin as internal standard, which was performed according to its standard protocol.

MTT Cytotoxicity Assay: The cytotoxicity was evaluated by employing MTT assay. Equivalent amount of free Dox was used in the cytotoxicity experiments as the control. HeLa cells were seeded into 96-well plate at a density of 1×10^4 cells/well in complete DMEM medium and grown for 24 h. Then, each medium was changed into complete DMEM medium containing free Dox, MSNP-SS-AD capped with CD-2NH₂ (MSNP-SS-AD+CD-2NH₂), Dox-loaded MSNP-SS-AD capped with CD-2NH₂ (MSNP-SS-AD+Dox+CD-2NH₂), Dox-loaded MSNP-SS-AD capped with the CD-2NH₂/siRNA(+) complex (MSNP-SS-AD+Dox+CD-2NH₂+siRNA(+)) with a nanoparticle-to-siRNA weight ratio of 110:1, and Dox-loaded

MSNP-SS-AD capped with the CD-2NH₂/siRNA(-) complex (MSNP-SS-AD+Dox+CD-2NH₂+siRNA(-)) with a nanoparticle-to-SiRNA weight ratio of 110:1, respectively. An equivalent amount of MSNP-SS-AD concentration (50 µg mL⁻¹) was used in each sample. After 16 h exposure, the medium in each well was replaced by fresh complete DMEM medium (100 µL). MTT assay was conducted after 56 h incubation. The medium in each well was changed into medium containing MTT (100 µL, 0.5 mg mL⁻¹). After incubation for 4 h, the medium was removed and DMSO (100 µL) was added into each well. The plate was gently shaken for 15 min and absorbance intensity at 560 nm was recorded using a microplate reader (infinite 200 PRO, Tecan). The relative cell viability related to control wells that were only treated with medium was calculated by $[A]_{\text{test}}/[A]_{\text{control}}$, where $[A]_{\text{test}}$ and $[A]_{\text{control}}$ are the average absorbance of the test and control samples, respectively.

Confocal Laser Scanning Microscopy (CLSM): HeLa cells were seeded in 35 mm plastic-bot-tomed µ-dishes and grown in complete DMEM medium for 24 h. Then, HeLa cells were treated with FITC labeled MSNP-SS-AD, MSNP-SS-AD capped with CD-2NH₂ (MSNP-SS-AD+CD-2NH₂), and Dox-loaded MSNP-SS-AD capped with CD-2NH₂ (MSNP-SS-AD+Dox+CD-2NH₂) under an equivalent amount of MSNP-SS-AD concentration (50 µg mL⁻¹). After 24 h incubation, the medium was removed and the cells was washed three times with PBS buffer (pH 7.4) and fixed with 4.0% formaldehyde at room temperature for 15 min. After removing 4.0% formaldehyde and washing with PBS buffer (pH 7.4) for three times, the cell nucleus was stained with 4',6-diamidino-2-phenylindole (DAPI, 1 µg mL⁻¹) for 15 min and washed with PBS buffer (pH 7.4) for three times before capturing the CLSM images using a confocal microscope (Leica TCS SP5, 60× oil objective).

Flowcytometry Analysis: HeLa cells were seeded in 6-well palte and grown for 24h. Then, the cells were treated with FITC labeled MSNP-SS-AD, MSNP-SS-AD capped with CD-2NH₂ (MSNP-SS-AD+CD-2NH₂), Dox-loaded MSNP-SS-AD capped with CD-2NH₂ (MSNP-SS-AD+Dox+CD-2NH₂) under an equivalent amount of MSNP-SS-AD concentration (50 µg mL⁻¹), and equivalent free Dox. After 24h incubation, the cells were washed by PBS three times and collected by trypsin. The flowcytometry

analysis was performed after washing the collected cells with PBS by centrifugation. The flowcytometry data was collected by BD FACSCalibur Flow Cytometer.

Zebrafish Care and Maintenance: Wild type and transgenic zebrafish lines TG(*fli1:EGFP*), SqKR19, Et(krt4:EGFP)sqet33e20 (or SqET33-e20) and Kras^{v12} were maintained in the IMCB zebrafish facility. The embryos were obtained and experiments were carried on according to the IACUC rules and regulations (the Biopolis IACUC application #090430). To prevent the formation of melanin and ensure optical translucence of transgenic larvae for confocal imaging, 1-phenyl-2-thiourea (PTU) was added to zebrafish embryos at 22 hpf.

Brain Targeted Microinjection of CD-2NH₂ capped MSNP-SS-AD: 36 hours post fertilization or 3 days old transgenic larvae were first immobilized, dorsal side up, in 1% UltraPure™ low melting point agarose (Invitrogen). CD-2NH₂ capped MSNP-SS-AD (6 nL, 0.4 µg µL⁻¹) was then microinjected into the brain ventricle. Microinjected zebrafish larvae and non-injected control were grown in 28.5 °C incubator.

Pericardial Microinjection of Free Dox or Dox loaded in CD-2NH₂ capped MSNP-SS-AD: For microinjection into the zebrafish circulation, 3 days old TG(*fli1:EGFP*) larvae¹⁹³ were mounted ventral side up in 1% molten low melting agarose and 250 µg ml⁻¹ DOX or 10 mg mL⁻¹ Dox loaded in CD-2NH₂ capped MSNP-SS-AD were injected into the sinus venosus of transgenic zebrafish larvae.

Abdominal Microinjection of Dox loaded in CD-2NH₂ capped MSNP-SS-AD: For microinjection into the vicinity of liver tumor¹⁹² 4 days-old transgenic zebrafish experiencing liver hyperplasia were mounted ventral side up in 1% UltraPure™ low melting point agarose (Invitrogen) and 10 mg ml⁻¹ Dox loaded in CD-2NH₂ capped MSNP-SS-AD were injected into the site of the abdomen near the liver without injuring the tissue.

Whole Mount In Situ Hybridization: Embryos at 3dpf were processed for whole-mount in situ hybridization using a technique that was described previously.¹⁹⁴ Dig-labelled (Roche Applied Science, Mannheim, Germany) antisense EGFP RNA probe was used for this analysis. Images were taken using

Olympus AX70 upright compound microscope attached to a ProgRes C10plus digital camera (JENOPTIK Laser, Optik, Systeme GmbH, Germany), and the stained EGFP-positive area was quantified using the Image J software. A quantitative measurement of interference with GFP expression was assessed using WISH to detect choroid plexus expressing EGFP. The stained EGFP-positive area was quantified using the Image J software.

Formulations of CD-2NH₂ Capped MSNP-SS-AD Complexed with siRNA for Brain Targeted Injections: siRNA duplex targeting EGFP (CAA GCU GAC CCU GAA GUU CTT) was purchased from Integrated DNA technologies Inc. Condition 1 (C1): 0.04 µg µL⁻¹ of siRNA against GFP; Condition 2 (C2): 0.12 µg µL⁻¹ CD-2NH₂ capped MSNP-SS-AD with 0.04 µg of siRNA against GFP, 3:1; Condition 3 (C3): 0.2 µg µL⁻¹ CD-2NH₂ capped MSNP-SS-AD with 0.04 µg of siRNA against GFP, 5:1; Condition 4 (C4): 0.4 µg µL⁻¹ CD-2NH₂ capped MSNP-SS-AD with 0.04 µg of siRNA against GFP, 5:1.

In Vivo Imaging of Transgenic Zebrafish Larvae: Transgenic larvae were mounted in 1% UltraPure™ low melting point agarose prior to imaging. Confocal microscopy images of living transgenic larvae were acquired with an upright (Zeiss Axiovert200M) laser scanning microscope (LSM Meta 510, Carl Zeiss) using two laser lines (30 mW Argon and 1 mW HeNe) as the excitation source to visualize GFP and internalized doxorubicin and using the 505/530 nm and 560/615 nm emission band-pass filters, respectively. Images were acquired under the same acquisition settings, for all imaged larvae.

2.3.3 Results and Discussion

MSNP preparation and characterization

In order to develop a viable carrier for controlled siRNA/drug co-delivery, the mesopores of the mesoporous nanoparticles are capped by redox-responsive nano-gates, i.e. CD-2NH₂ rings. As shown in **Scheme 3.1**, the disulfide bond-containing amino groups were initially attached to the MSNP surface. The adamantane (AD) units were then introduced onto the surface through the amidation between the amino groups and adamantane-1-carboxylic acid. Then, AD units on the nanoparticle surface formed stable host-

guest complexes with CD-2NH₂ under the association constant of 10⁴-10⁵ M⁻¹.¹⁹⁵ In addition to capping drug molecules inside the mesopores, the second role of CD-2NH₂ is to bind siRNA through electrostatic interactions. The mechanism of controlled siRNA/drug release within cancer cells can be realized by the cleavage of redox-responsive disulfide bonds triggered by intracellular glutathione (GSH),^{108, 196-198} leading to the uncapping of the β -CD rings followed by the drug release from the mesopores. siRNA can also be dissociated from CD-2NH₂ on account of intracellular acidic pH, thereby achieving high RNA interference at the level required for targeted gene knockdown.

Figure 3.1. a) X-ray diffraction pattern, b) N₂ adsorption/desorption isotherms, and c) BJH pore size distribution of MSNP-SH prepared by the co-condensation method.

Thiol group-containing MSNPs (MSNP-SH) were prepared through the co-condensation reaction of tetraethylorthosilicate (TEOS) and 3-mercaptopyltrimethoxysilane (MPTMS) followed by the removal of structure-directing agent cetyltrimethylammonium (CTAB). The MSNP-SH was characterized by powder X-ray diffraction (XRD) and N₂ adsorption/desorption measurements in order to confirm the mesoporous structure. In the powder XRD pattern (**Figure 3.1a**), the sharp peak at 2θ = 2.12, corresponding to (100) plane, along with two minor peaks at 2θ = 3.96 and 2θ = 4.60 for (110) and (200) planes respectively, clearly prove the hexagonal structure of MCM-41 type MSNPs. In addition, a typical type-IV isotherm was obtained (**Figure 3.1b**), indicating that the MSNP-SH possesses a high BET surface area of 730 m² g⁻¹ and a narrow BJH pore size distribution focusing on 2.7 nm.

Figure 3.2. a) FESEM and b) TEM images of MSNP-SH, c) FT-IR spectra of MSNP-SH, MSNP-SS-NH₂, and MSNP-SS-AD, and d) ¹³C CP MAS NMR spectrum of MSNP-SS-AD.

The surface of MSNP-SH was further treated with S-(2-aminoethylthio)-2-thiopyridine hydrochloride (SATH) for the formation of the disulfide bonds, leading to amino group-terminated nanoparticles (MSNP-SS-NH₂). AD-functionalized MSNPs (MSNP-SS-AD) were finally prepared by the amidation between MSNP-SS-NH₂ and adamantane-1-carboxylic acid (AD-COOH) in the presence of dicyclohexylcarbodiimide in DMF. The morphology of the MSNP-SS-AD nanoparticles was characterized by Field Emission Scanning Electron Microscopy (FESEM) (**Figure 3.2a**) and Transmission Electron Microscopy (TEM) (**Figure 3.2b**), showing uniform nanoparticle size with a mean diameter of 150 nm and well-ordered mesoporous structure with the pore diameter of 2-3 nm. The surface modifications of MSNPs were determined by the FT-IR analysis (**Figure 3.2c**). After converting thiol groups into the disulfide bond-bridged amino groups on the nanoparticle surface, a minor peak at 2560 cm⁻¹ corresponding to the thiol group¹¹⁵ disappeared, while a new peak observed at 1530 cm⁻¹ is assigned to the primary amine. After the amidation for the formation of MSNP-SS-AD, the peak of the amino

group centered at 1530 cm^{-1} disappeared. Signals for the AD units on the nanoparticle surface cannot be well identified, since they overlapped with the broad peak from 1100 to 1300 cm^{-1} . MSNP-SS-AD was further characterized by solid-state ^{13}C cross polarization and magic angle spinning nuclear magnetic resonance (CP MAS NMR) spectroscopy. The ^{13}C CP MAS NMR spectrum (**Figure 3.2d**) of MSNP-SS-AD exhibits strong signals at 11.3, 23, 28, 49.5 and 51.5 ppm, which are attributed to the aliphatic secondary carbon atoms. Signals ranging from 34 to 41 ppm in the spectrum are ascribed to the carbon atoms of the AD units, and the signal at 174 ppm is assigned to the carbonyl carbon of the newly formed amide bond. These observations indicate successful preparation of MSNP-SS-AD.

Table 3.1. Zeta potential values of functional mesoporous silica nanoparticles.

Sample	Zeta Potential in H_2O (AVE $\pm$ STD, mV)
MSNP-SS-NH ₂	32.5 $\pm$ 5.3
MSNP-SS-AD	16.7 $\pm$ 3.7
MSNP-SS-AD +CD	-36.7 $\pm$ 5.1
MSNP-SS-AD +CD-2NH ₂	29.7 $\pm$ 5.5
MSNP-SS-AD +DOX+CD-2NH ₂	32.2 $\pm$ 5.2

Moreover, zeta potential measurements further support the successful surface functionalization of MSNPs. A negative zeta potential of -24.4 mV for MSNP-SH was converted to a positive value of 22.5 mV for MSNP-SS-NH₂, which can be attributed to the positively-charged ammonium species on the surface of MSNP-SS-NH₂. After the introduction of the neutral AD units on the surface, a zeta potential decrease from 22.5 to 12.7 mV was observed in the case of MSNP-SS-AD. The zeta potential measurements also provide clear evidence for the formation of the host-guest complex between AD and CD-2NH₂ on the

nanoparticle surface. When MSNP-SS-AD was capped with β -CD through the complexation, the surface charge of the nanoparticles was reversed from a positive value of 12.7 mV to a negative one of -26.7 mV, on account of the presence of the hydroxyl groups from β -CD. However, when CD-2NH₂ was used to cap the nanoparticles, a highly positive surface charge of 19.7 mV was observed because of the protonated amino groups of CD-2NH₂. A positive value of 22.2 mV was observed when MSNP-SS-AD was loaded with Dox followed by the capping of CD-2NH₂, indicating that the presence of Dox inside the mesopores would not affect the complex formation. Highly positive surface charge provided by CD-2NH₂ can be employed to bind with negatively-charged siRNA through electrostatic interactions, so that the CD-2NH₂ capped MSNP-SS-AD nanoparticles serve as an excellent carrier for the siRNA/drug co-delivery.

Figure 3.3 Photos of MSNP-SS-AD capped with β -CD (left) and MSNP-SS-AD capped with CD-2NH₂ (right) in aqueous solution (1 mgmL⁻¹) after a) 5 min sonication and b) stabilized at room temperature for 24 h. Well dispersed sample for MSNP-SS-AD capped with CD-2NH₂ even after 24 h indicates its good stability in aqueous solution.

In addition, the introduction of CD-2NH₂ onto the MSNP-SS-AD surface enhances the stability of the nanoparticles in both distilled H₂O and cell culture medium DMEM (**Figure 3.3**). After complex with CD-2NH₂, the nanoparticle suspension did not precipitate within 24hour, which might be attributed to the surface change and water solubility imposed by the CD-2NH₂ molecules.

Table 3.2.DLS size of MSNP-SS-AD-CD-NH₂ and MSNP-SS-AD+CD-NH₂+SiRNA in both distilled water and DMEM cell culture medium.

	Size in H ₂ O (nm)	Size in DMEM (nm)
MSNP-SS-AD+CD-NH ₂	1349±22	561±74
MSNP-SS-AD+CD-NH ₂ +SiRNA	1492±40	334±9.0

From the hydrodynamic size measurement, inside the DMEM cell culture medium, the nanoparticles exhibited smaller hydrodynamic size. It could be explained by binding of the small biomolecules inside the DMEM, such as protein, on to the nanoparticle surface, which enhanced the dispersity of nanoparticles further. So, we expect that the surface coverage of CD-2NH₂ on MSNP-SS-AD plays a crucial role in cellular internalization, as it can prevent nanoparticles from aggregation and maintained its sub micron size in the cell culture medium, which is an essential step for intracellular siRNA/drug co-delivery of nanoparticles.

Redox-controlled Drug/siRNA release

Figure 3.4.Controlled drug release profiles in PBS, pH=7.4 for a) RhB release from β -CD capped MSNP-SS-AD, b) RhB release from CD-2NH₂ capped MSNP-SS-AD, and c) Dox release from CD-2NH₂ capped MSNP-SS-AD. d) Agarose gel electrophoresis of the siRNA complexation with CD-2NH₂ capped

MSNP-SS-AD under different nanoparticle-to-siRNA weight ratio. Lanes from left to right are free siRNA, CD-2NH₂ capped MSNP-SS-AD, and CD-2NH₂ capped MSNP-SS-AD/siRNA complexes under the nanoparticle-to-siRNA weight ratios of 10:1, 30:1, 50:1, 70:1, 90:1, 110:1, and 110:1 containing 50mM DTT, respectively.

In order to prove that the drug release was controlled by redox-responsive nano-gates provided by the CD rings, β -CD without any modification was used to complex with MSNP-SS-AD for capping the mesopores. Rhodamine B (RhB) was employed as a model drug, and the loading capacity of MSNP-SS-AD towards RhB was calculated to be 41 μ g of RhB per 1 mg of nanoparticles (4.1 wt%), by comparing UV-vis absorption intensities of RhB solution before and after adding MSNP-SS-AD. As seen from Figure 3.4a and 2.4b, the leakage (black curve) of RhB from β -CD capped MSNP-SS-AD in the absence of reducing agent was negligible, whereas a significant release profile (red curve) of RhB from β -CD capped MSNP-SS-AD was observed upon the addition of reducing agent dithiothreitol (DTT). The observation can be explained by pre-installed disulfide bond cleavage on the nanoparticle surface induced by DTT. This cleavage resulted in the dissociation of the β -CD ring, which in turn led to the release of previously loaded RhB. In comparison, the CD-2NH₂ capped MSNP-SS-AD exhibits slower RhB release after the addition of DTT, which may be attributed to stronger blockage caused by the amino group-containing β -CD ring. Similarly, the RhB release profiles in the presence of GSH (25 mM) can also be observed. In order to understand the release mechanism of real anticancer drugs, similar experiments were carried out using Dox-loaded MSNP-SS-AD capped with CD-2NH₂. The loading capacity of MSNP-SS-AD for Dox was 25 μ g of Dox per 1 mg of nanoparticles (2.5 wt%). Similar redox-controlled Dox release profiles (**Figure 3.4c**) were observed under the same experimental conditions.

Figure 3.5. Photos of small amount of sample for RhB-loaded MSNP-SS-AD capped with β -CD in PBS buffer (pH 7.4) a) before DTT addition and b) 5 min after DTT (50 mM) addition. Red RhB release was observed 5 min after DTT addition, indicating the redox-triggered RhB release.

In fact, redox-controlled release process could even be monitored by eye (**Figure 3.5**). A sample of RhB loaded MSNP-SS-AD capped with β -CD was placed at the bottom of a fluorescence cuvette containing phosphate buffered saline (PBS) buffer (3 mL, pH 7.4). After stabilized for 1 h, 50 mM DTT was added into the solution. Obvious RhB release in red was observed at the bottom of the cuvette after 5 min.

An essential prerequisite in the design of siRNA delivery vehicle is the ability to form stable complexes with free siRNA. In the case of CD-2NH₂ capped MSNP-SS-AD, complex formation is mediated by electrostatic interaction between negatively-charged siRNA and positively-charged diamino groups of CD-2NH₂ on MSNP-SS-AD. The electrostatic interaction was demonstrated by the retarded siRNA band mobility in agarose gel electrophoresis, which was not observed with free siRNA. Bcl-2 siRNA was used in this electrophoresis experiment. As shown in **Figure 3.4d**, free siRNA in the first lane has the highest mobility. Upon increasing the nanoparticle-to-siRNA weight ratios (lanes 3-8, **Figure 3.4d**), a gradual decrease in the band mobility and intensity was observed. siRNA concentration was kept constant in all lanes, except in lane 2, where no siRNA was loaded. siRNA became fully condensed on the nanoparticle surface when the nanoparticle-to-siRNA weight ratio reached 110:1. When DTT (50 mM) was added into a sample with a nanoparticle-to-siRNA weight ratio of 110:1 (last lane, **Figure 3.4d**), the recovery of siRNA band intensity was detected. This result demonstrates that previously attached siRNA can be released from the nanoparticle surface by redox-triggered disulfide bond cleavage. GSH (25 mM) can

also be used to induce the disulfide bond cleavage on the nanoparticle surface, releasing both drug and siRNA for synergistic chemotherapeutic therapy.

In vitro drug/siRNA delivery

Figure 3.6. CLSM images of HeLa cells treated with a-c) equivalent free Dox, and d-i) FITC-labeled MSNP-SS-AD loaded with Dox and capped with CD-2NH₂ (50 μ g mL⁻¹) for 24 h. a) blue fluorescence from DAPI, b) red fluorescence from Dox, c) overlay of bright field, image a) and b); d) blue fluorescence from DAPI, e) green fluorescence from FITC-labeled MSNP-SS-AD, f) red fluorescence from released Dox, g) overlay of image e) and f), h) overlay of image d) and f), g) overlay of bright field, image d), e, and f).

In order to trace the location of the nanoparticles in cells, green fluorescent FITC (fluorescein isothiocyanate) was embedded inside the silica nanoparticles during the fabrication process. Cellular uptake of FITC-labeled MSNP-SS-AD loaded with Dox and capped with CD-2NH₂ was investigated by Confocal Laser Scanning Microscopy (CLSM) using HeLa cells as the cancer cell model. The nuclei in

HeLa cells were stained blue by DAPI (4',6-diamidino-2-phenylindole). Green fluorescence (**Figure 3.6e**) was detected around the cell nucleus, indicating effective nanoparticle internalization by HeLa cells. No green fluorescence was detected outside the cancer cells. In comparison, less green fluorescence was detected in HeLa cells, when cellular uptake of FITC-labeled MSNP-SS-AD was assessed (**Figure 3.7**). Hence, FITC-labeled MSNP-SS-AD capped with CD-2NH₂ presents much higher cellular internalization capacity, confirming its potential for intracellular therapy (**Figure 3.7**). Flow cytometry analysis also supports the cellular uptake enhancement by CD-2NH₂ capping (**Figure 3.7g**).

Figure 3.7.CLSM images of a-c) FITC-labeled MSNP-SS-AD, d-e) FITC-labeled MSNP-SS-AD capped with CD-2NH₂. From left to right: blue color from DAPI, green color from FITC labeled MSNP-SS-AD, and overlay images of bright field, blue and green images; g) flowcytometry result (FL1: FITC fluorescence intensity) of HeLa cells treated with 1) control, 2) FITC-labeled MSNP-SS-AD, 3) FITC-labeled MSNP-SS-AD capped with CD-2NH₂; h) flowcytometry result (FL2: Dox fluorescence intensity)

of HeLa cells treated with 1) control, 2) equivalent free Dox , 3) FITC-labeled MSNP-SS-AD+Dox+CD-2NH₂+siRNA complex.

The CLSM image demonstrated successful release of Dox from MSNP-SS-AD within HeLa cells induced by endogenous GSH (**Figure 3.6d-i**), as shown by the presence of red fluorescent Dox in both cytosol and nucleus. These observations indicate effective redox-triggered intracellular release of Dox from the nanoparticles and successful delivery of Dox into the nucleus, where the anticancer activity of Dox is executed by its interactions with DNA.¹⁹⁹ In addition, the detection of FITC-labeled and CD-2NH₂ capped MSNP-SS-AD containing Bcl-2 siRNA/Dox in perinuclear regions of cytoplasm suggests that the co-delivery carrier could bypass the efflux pump resistance observed in HeLa cells,^{200, 201} thereby enhancing Dox-mediated cytotoxicity. To summarize, Dox delivered by CD-2NH₂ capped MSNP-SS-AD is protected from degradation by extracellular enzymes and exhibits enhanced cellular uptake.²⁰²

Figure 3.8. Protein concentration collected from HeLa cells after being treated with indicated samples.

To assess the efficacy of gene knockdown mediated by Bcl-2 siRNA delivered by CD-2NH₂ capped MSNP-SS-AD, the level of Bcl-2 protein expression in transfected HeLa cells was evaluated by western blot. After disrupting the cell membrane with lysis buffer, the protein concentration of each sample was quantified by Pierce® BCA Protein Assay Kit. Protein concentration of all the six samples was around 1000 µg mL⁻¹ (**Figure 3.8**). No obvious cell death was detected in HeLa cells transfected with CD-2NH₂ capped MSNP-SS-AD alone, CD-2NH₂ capped MSNP-SS-AD/Bcl-2 siRNA complex, or free siRNA.

Commercially available Lipofectamine® RNAiMAX reagent (Invitrogen) was used as a positive siRNA delivery control (siRNA(+)). Non-binding siRNA duplex was used as a negative control (siRNA(-)) to exclude non-specific cytotoxic effect of siRNA. Protein expression results show that Bcl-2 protein was not detected in cells transfected with the Lipofectamine® RNAiMAX reagent, confirming the silencing efficacy of the chosen siRNA(+) sequence against Bcl-2 protein (**Figure 3.8a**, lane F). Transfection of cells with CD-2NH₂ capped MSNP-SS-AD/Bcl-2 siRNA complex resulted in 70% reduction of Bcl-2 protein expression (lane C). Delivery of CD-2NH₂ capped MSNP-SS-AD alone (lane B) did not interfere the expression of endogenous Bcl-2 protein. These observations indicate that no obvious cytotoxicity was associated with the siRNA carrier and that the protein silencing observed in transfected HeLa cells was attributed to siRNA(+) electrostatically associated with the surface of nanoparticles. No Bcl-2 protein knockdown was detected in cells transfected with CD-2NH₂ capped MSNP-SS-AD/siRNA(-) complex (lane D), where siRNA(-) was represented by the non-binding siRNA duplex. Free siRNA alone also failed to decrease the expression of endogenous Bcl-2 protein (lane E). Thus, the transfection studies in HeLa cells demonstrate the efficacy of CD-2NH₂ capped MSNP-SS-AD as a siRNA carrier to mediate intracellular silencing of expression of targeted protein.

Figure 3.9. a) Bcl-2 protein knockdown by CD-2NH₂ capped MSNP-SS-AD/siRNA(+) complex (MSNP-SS-AD+CD-2NH₂+siRNA) in HeLa cells (nanoparticle-to-siRNA weight ratio of 110:1). Gene expression was evaluated by the ratio of intensity of the Bcl-2 band to that of the internal standard, β -tubulin. The ratio of each sample was normalized to the value of the sample without any treatment; a-1)

control, a-2) MSNP-SS-AD+CD-2NH₂, a-3) MSNP-SS-AD+CD-2NH₂+siRNA(+), a-4) MSNP-SS-AD+CD-2NH₂+siRNA(-), a-5) free siRNA, a-6) Lipofectamine+siRNA(+). b) Relative cell viability of HeLa cells treated with indicated samples under an equivalent amount of nanoparticle concentration and equivalent amount of Dox; b-1) MSNP-SS-AD+CD-2NH₂+siRNA(-), b-2) free Dox, b-3) MSNP-SS-AD+Dox+CD-2NH₂, b-4) MSNP-SS-AD+Dox+CD-2NH₂+siRNA(+); Nanoparticle-to-siRNA weight ratio is 100:1.

Synergistic anticancer effect caused by simultaneous delivery of anticancer drug Dox and Bcl-2 siRNA was investigated using the MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cytotoxicity assay. MSNP-SS-AD+CD-2NH₂+siRNA complex alone exhibited little cytotoxic effect, since relative cell viability in transfected cells remains about 80% until extremely high nanoparticle dose (**Figure 3.9b-1**) and the IC₅₀ value was determined to be 600 µg mL⁻¹. A significant increase in cytotoxicity was detected in HeLa cells exposed to Dox-loaded and CD-2NH₂ capped MSNP-SS-AD (**Figure 3.9b-3**), when compared to the exposure with equivalent amount of free Dox (**Figure 3.9b-2**). The results further confirm enhanced cellular uptake and intracellular redox-triggered release of Dox from CD-2NH₂ capped MSNP-SS-AD nanocarrier. The IC₅₀ value of MSNP-SS-AD+Dox+CD-2NH₂ is 37.2 µg mL⁻¹. Simultaneous co-delivery of Dox and Bcl-2 siRNA (siRNA(+)) further enhances the cytotoxic effect, as overall cell viability further decreased and IC₅₀ value was estimated to be 17.5 µg mL⁻¹ (**Figure 3.9b-4**). Thus, CD-2NH₂ capped MSNP-SS-AD presents itself as a multifunctional nanocarrier with enhanced chemotherapeutic efficacy mediated by improved cellular uptake and co-delivery of drug/siRNA complexes.

Figure 3.10. Zebrafish transgenic lines sqKR19 and GW(sq33-e20) are two transgenic lines with fluorescent reporter expression in the choroid plexus, a thin ependymal outgrowth dorsal to the ventricle cavity. GFP expression in this tissue is the readout to address the efficiency of siRNA mediated knockdown in the living animal model. As it is predominantly a single layer of cells at 3-4 dpf, next to the ventricular cavity, it should be the most sensitive readout for interference with GFP expression mediated by EGFP siRNA. (a and b) Dorsal view of 4dpf transgenic zebrafish larvae. (c and d) Common expression domains in the choroid plexus between membrane-localized KillerRed (c) and cytoplasmic GFP (d). (f-g) Immuno-staining of a section of Et(krt4-EGFP) sq33-e20 brain showing how the choroid plexus flank the ventricle cavity. All cells were stained blue with TOPRO3, and the ventricle cavity, demarcated by the absence of cells, is marked by an arrow (f).

In vivo chemotherapeutic efficacy of CD-2NH₂-capped MSNP-SS-AD was further analyzed in transgenic zebrafish larvae. The body plan and most of internal organs of vertebrates are conserved in evolution and many diseases of zebrafish are similar to that in human. The transparent zebrafish larvae with comparatively short development ex-utero represent an attractive and rapid system to validate novel

therapeutic agents.²⁰³ Externally developing zebrafish larvae are amenable to microsurgical manipulations. Bioimaging of tissues expressing a fluorescent protein reporter can be easily achieved with a standard confocal laser scanning microscope (CLSM) due to optical translucence of the relatively small developing larvae, which at 5 days is 4 mm long. We make use of the similarity in high rate of cellular proliferation during embryonic tissue growth to model unregulated proliferations in cancer cells.²⁰⁴ More than 80% of human cancers (skin cancer, lung, stomach and mammary tumors) derive from epithelial tissues.²⁰⁵ The choroid plexus, a tissue of epithelial origin is selected to model primary tumor growth. Two zebrafish transgenic lines with GFP (Et(krt4:EGFP)sqet33e20 or SqET33-e20) and membrane localized KillerRed (SqKR19) expression in the choroid plexus are selected as the *in vivo* epithelial tissue model to assess cellular uptake of drugs delivery by nanoparticles and their impact on choroidal epithelial cell proliferation. Imaging in these optically transparent transgenic larvae provides initial assessment of anti-proliferative therapy mediated by Dox-loaded MSNP-SS-AD *in vivo*.

Figure 3.11. *In vivo* uptake of CD-2NH₂ capped MSNP-SS-AD inhibits transcription of GFP in the epithelial cells of choroid plexus at the 4th ventricle of zebrafish larval brain. (a and b) Brain-targeted delivery of FITC labeled and CD-2NH₂ capped MSNP-SS-AD resulted in uptake into some cells largely in peripheral areas of the choroid plexus, highlighted by white boxes. (c-g) Specific weight ratios of CD-

2NH₂ capped MSNP-SS-AD/GFP siRNA complexes (C2 and C3) enhance inhibition of GFP transcription *in vivo*. The area (μm^2) of GFP expression detected by whole mount *in situ* hybridization in the choroid plexus is quantified by the Image J software. (h) The average size of the choroid plexus is presented in a bar chart as mean $\pm$ standard error of mean. Formulations for various weight ratios of nanoparticle/siRNA complexes used for this experiment are as follows: WT (without any treatment), C1 (0.04 $\mu\text{g } \mu\text{L}^{-1}$ siRNA only), C2 (0.12 $\mu\text{g } \mu\text{L}^{-1}$ nanoparticle with 0.04 $\mu\text{g } \mu\text{L}^{-1}$ siRNA, 3:1), C3 (0.2 $\mu\text{g } \mu\text{L}^{-1}$ nanoparticle with 0.04 $\mu\text{g } \mu\text{L}^{-1}$ siRNA, 5:1), and C4 (0.4 $\mu\text{g } \mu\text{L}^{-1}$ nanoparticle with 0.04 $\mu\text{g } \mu\text{L}^{-1}$ siRNA, 10:1).

We first verified *in vivo* cellular uptake of CD-2NH₂ capped MSNP-SS-AD. Passive targeting was conducted by microinjecting 0.4 $\mu\text{g } \mu\text{L}^{-1}$ FITC loaded and CD-2NH₂ capped MSNP-SS-AD into the brain ventricle of SqKR19 transgenic zebrafish larvae²⁰⁶ at 36 hours post fertilization (hpf). Being located dorsal to the IV ventricle cavity and immediately under the skin the choroid plexus is an easily accessible site. The morphology of choroidal epithelial cells was highlighted by membrane-localized KillerRed in SqKR19 transgenics (**Figure 3.11b**). The fluorescent signal of internalized FITC from MSNP-SS-AD was observed inside choroidal epithelial cells two days after injection. Cells that internalized FITC-loaded nanoparticles were predominantly found in peripheral regions of the choroid plexus defined by white serrated boxes (**Figure 3.11b**).

Table 3.3. Formulations of MSNP-SS-AD+ CD-2NH₂+siRNA complex for brain-targeted injections.

	C1	C2	C3	C4
siRNA (0.2 $\mu\text{g } \mu\text{L}^{-1}$)	1 μL	1 μL	1 μL	1 μL
CD-2NH ₂ capped MSNP-SS-AD	0	1 μL (0.6 $\mu\text{g } \mu\text{L}^{-1}$)	1 μL (1 $\mu\text{g } \mu\text{L}^{-1}$)	2 μL (1 $\mu\text{g } \mu\text{L}^{-1}$)

Figure 3.12. Reduction of EGFPtranscript in the choroid plexus of 3dpf GW(sq33-e20) transgenic larvae demonstrates the need to formulate specific weight ratio of CD-2NH₂ capped MSNP-SS-AD complexed

with siRNA in order to enhance the efficiency of siRNA mediated GFP knockdown. Area of the EGFP stained choroid plexus was quantified after whole mount in situ hybridization. Formulations for various weight ratios of nanoparticle/siRNA complexes used for this experiment are WT (no treatment); C1 (0.04 $\mu\text{g } \mu\text{L}^{-1}$ siRNA only); C2 (0.12 $\mu\text{g } \mu\text{L}^{-1}$ nanoparticle with 0.04 $\mu\text{g } \mu\text{L}^{-1}$ siRNA; 3:1); C3 (0.2 $\mu\text{g } \mu\text{L}^{-1}$ nanoparticle with 0.04 $\mu\text{g } \mu\text{L}^{-1}$ siRNA; 5:1); C4 (0.4 $\mu\text{g } \mu\text{L}^{-1}$ nanoparticle with 0.04 $\mu\text{g } \mu\text{L}^{-1}$ siRNA; 10:1). In total, 8 samples were analyzed for each experimental set. Area of the choroid plexus outlined in blue is stated in μm^2 .

Next, the efficiency of in vivo anti-GFP siRNA interference was assessed using the SqET33-e20 transgenic zebrafish larvae¹⁹¹ expressing cytosolic GFP in the choroid plexus. Briefly, GFP-positive choroidal cells were first detected at 28 hpf in the layer of neuroepithelial cells, and at 36 hpf they formed a cellular sheet representing the roof of the IV ventricle. These cells then converged towards the midline to form the button-like choroid plexus represented by 2-3 layers of cells at 3 days post fertilization (dpf). Microinjection of siRNA-containing nanoparticles into the brain ventricle was conducted at 36 hpf. Injected larvae were grown for two days before being processed for analysis of GFP RNA expression by whole mount in situ hybridization (WISH). siRNA duplex targeting EGFP (CAA GCU GAC CCU GAA GUU CTT; IDT) was used for this experiment. To minimize potential off-target effect of GFP siRNA reported in cell lines,²⁰⁷ a standardized low dose of 0.04 $\mu\text{g}/\mu\text{L}$ GFP siRNA was used in all brain ventricle injections to knockdown GFP transcripts in the choroid plexus. The experiment compared the efficacy of GFP knockdown mediated by free GFP-siRNA and increasing concentration of CD-2NH₂ capped MSNP-SS-AD when complexed to the same amount of GFP-siRNA. Formulations were prepared and incubated for two hours on ice before brain ventricle injection. Free GFP siRNA at 0.04 $\mu\text{g } \mu\text{L}^{-1}$ was inefficient in decreasing GFP transcripts (**Figure 3.11d**). Enhanced GFP knock down was detected when 3:1 or 5:1 weight ratio of MSNP-SS-AD was complexed to 0.04 $\mu\text{g } \mu\text{L}^{-1}$ siRNA (**Figure 3.11e-f**). Increasing the weight ratio to 10:1 reversed this trend (**Figure 3.11g**). CD-2NH₂ capped MSNP-SS-AD/GFP siRNA complexes with weight ratios of 3:1 (C2) and 5:1 (C3) inhibit GFP transcription more efficiently than that

caused by injection of free GFP siRNA alone (C1). Interestingly, the ability to inhibit GFP transcription was lost when the weight ratio of CD-2NH₂ capped MSNP-SS-AD was increased to 10:1 (C4 in **Figure 3.11h**). This observation suggests that an excess of positively charged CD-2NH₂ capped MSNP-SS-AD interferes with intracellular release of GFP siRNA. This in turn impedes GFP silencing mediated by the RNA-induced silencing complex that results in inefficient GFP knockdown.

Figure 3.13. Significant decrease of GFP expression was observed after two reiterative injections of Dox-loaded and CD-2NH₂ capped MSNP-SS-AD complexed with GFP siRNA. (a-e) Same weight ratio of Dox-loaded nanoparticle/GFP siRNA complex injected into the brain ventricle on two consecutive days. The impact on GFP expression in the choroid plexus was assessed two days later. Barely visible GFP expression in the choroid plexus was detected in all transgenic larvae exposed to the highest weight ratio (C4) of Dox-loaded nanoparticle/GFP siRNA complex. (f) The average GFP intensity quantified in the boxed area of the choroid plexus is presented in a bar chart with values stated as mean ± standard error of mean. Values are compiled from three separate injections. The same acquisition setting was used for image capture using LSM510 META confocal microscope. Image intensity was assessed using the Image J software.

In vivo release of internalized Dox mediated by redox-responsive CD-2NH₂ capped MSNP-SS-AD nanocarrier was then investigated. We performed localized delivery of Dox loaded MSNP to sites flanking the choroid plexus to mimic localized delivery of anti-cancer drug into the vicinity of tumor cells. The effect of internalized Dox was assessed based on inhibition of tissue proliferation in choroidal epithelial cells that internalized Dox loaded MSNP. Again, transgenic larvae were injected at 36 hpf and the effect on choroid plexus growth and GFP intensity was assessed. Different weight ratios of injected Dox-loaded nanocarriers complexed with GFP siRNA were evaluated. Six transgenic larvae were imaged two days post injection for each formulation. Untreated transgenic larvae were used as a control of normal growth of choroid plexus and intensity of GFP expression (**Figure 3.13**).

Figure 3.14. A single brain targeted injection of Dox loaded and CD-2NH₂ capped MSNP-SS-AD/GFP siRNA complex is sufficient to decrease GFP expression in the choroid plexus of GW(Sq33-e20)transgenic zebrafish larvae. (a-e) Representative live images of the choroid plexus after exposure to varying weight ratios of Dox-loaded and CD-2NH₂ capped MSNP-SS-AD/GFP siRNA complexes. Uptake of Texas Red dextran in embryos injected with free GFP siRNA (b) or different weight ratios of Dox-loaded MSNP-SS-AD/GFP siRNA complexes can be detected in the choroid plexus after two days

post injection (c-e). (f) The Average GFP intensity quantified in the boxed area of the choroid plexus are presented in a bar chart with values stated as mean $\pm$ standard error of mean. The same acquisition setting, using a LSM510 META confocal microscope, was employed for *in vivo* imaging. Image intensity was assessed using the Image J software.

In larva exposed to C3 (0.2 $\mu\text{g } \mu\text{L}^{-1}$ Dox-loaded nanoparticle with 0.04 $\mu\text{g } \mu\text{L}^{-1}$ siRNA, 5:1) and C4 (0.4 $\mu\text{g } \mu\text{L}^{-1}$ Dox-loaded nanoparticle with 0.04 $\mu\text{g } \mu\text{L}^{-1}$ siRNA, 10:1) formulations, the shape of the choroid plexus has changed and GFP fluorescence was reduced (**Figure 3.14d-e**). These changes were noticed upon single injection of the formulation with concentration above 0.2 $\mu\text{g } \mu\text{L}^{-1}$ of Dox-loaded and CD-2NH₂ capped MSNP-SS-AD nanocarrier. A second set of experiments was conducted in 3-day-old transgenic larvae, where reiterative injections of the same formulation were made into the brain ventricle on two consecutive days. The impact on choroid plexus was then assessed at 7 dpf. When compared to uninjected controls, the injection of free anti-GFP siRNA decreased the mean GFP intensity by 45% (**Figure 3.14b**) without any effect on tissue morphology, i.e. the cellular uptake of GFP siRNA had no effect on maintenance of already formed choroid plexus. Injecting increasing weight ratio of Dox-loaded and CD-2NH₂ capped MSNP-SS-AD/GFP siRNA complex caused defect of the choroid plexus. This phenomenon correlated with the internalization of Dox-loaded nanocarrier and appearance of clones of GFP-negative choroidal epithelial cells (**Figure 3.14c-d**). The most significant effect was detected upon injection by two consecutive doses of C4 formulations (0.4 $\mu\text{g } \mu\text{L}^{-1}$ Dox-loaded and CD-2NH₂ capped MSNP-SS-AD with 0.04 $\mu\text{g } \mu\text{L}^{-1}$ siRNA, 10:1), the highest concentration of Dox used for this set of experiment. Here, the GFP expression was lost not only in epithelial cells of the midline cluster, but also in glial cells of the choroid plexus located in rhombic lips (i.e., adjacent walls of hindbrain, HB; **Figure 3.14e**). Thus, a positive correlation was observed between disturbed morphology of choroid plexus and the amount of injected Dox-loaded and CD-2NH₂ capped MSNP-SS-AD.

Figure 3.15. Injection of Dox loaded MSNP-SS-AD into the zebrafish circulation elicits less developmental side effects than injection of free doxorubicin when adjusted to the same concentration. 5 out of 6 injected larvae exposed to free doxorubicin elicit developmental defects of reduced growth and edema (a-c; e-f) whereas only 1 out of the 6 larvae exposed to Dox loaded MSNP develop this toxicity associated phenotype (e). Each image is a composite of 3 images that span the anterior-posterior length of the larva. Left: Free Dox 250 µg/ml; approximate injection volume is 6 nl; 3days post injection; right: 10mg/ml MSNP-SS-AD loaded with Dox and capped with CD-2NH₂; approximate injection volume is 6 nl; 3days post injection.

Although the drug carrier, CD-2NH₂ capped MSNP-SS-AD, is designed for tumor-targeted delivery we proceed to compare tissue distribution of free Dox (250 µg ml⁻¹) or Dox-loaded MSNP (10 mg ml⁻¹), when introduced into systemic circulation by pericardial injection. The dose for this experiment was adjusted 10 fold higher for whole larvae detection by CLSM. Transgenic zebrafish that express EGFP in all blood vessels Tg(fli1:GFP) is used as the reporter line in this experiment. Larvae toxicity was detected

in the group injected with free Dox. In this group, reduced larval growth and concurrent fluid accumulation in the body (edema) were observed in 5/6 injected larvae 3 days post injection. The developmental defect was only observed in 1/6 larvae injected by Dox-loaded MSNP (**Figure 3.15**). Hence redox triggered release of Dox in CD-2NH₂ capped MSNP-SS-AD causes less side effects than free Dox.

Figure 3.16. The liver tumor in the inducible *kras*^{v12} transgenic zebrafish model is reduced after exposure to MSNP-SS-AD loaded with Dox and capped with CD-2NH₂ when compared to untreated control. (a) A view of transgenic zebrafish larvae *Kras*^{v12} experiencing liver hyperplasia at 10 days post fertilization. (b) A view of transgenic zebrafish larvae experiencing liver hyperplasia 6 days post injection of MSNP-SS-AD loaded with Dox and capped with CD-2NH₂, 10 days post fertilization. (c) A magnified view of liver tumor cells exposed to MSNP-SS-AD loaded with Dox and capped with CD-2NH₂. Free released Dox (red) can be seen inside liver tumor cells in green. (d) The average area of liver tumor between non-injected control (n =8) and siblings injected with MSNP loaded Dox (n=10) quantified 6 days post injection is presented as a bar chart with values stated as mean liver area ± standard error of mean. Liver size from each specimen was quantified using the associated software in LSM510 META.

Area of non-injected liver tumor control (n =8), 10dpf

Area of liver tumor injected with MSNP-SS-AD loaded with Dox (n=10), 10dpf

Figure 3.17. Individual liver tumor area of non-injected control (n =8) in the inducible $kras^{v12}$ transgenic zebrafish model is bigger when compared to transgenic zebrafish larvae experiencing liver hyperplasia but treated with MSNP-SS-AD loaded with Dox and capped with CD-2NH₂ (n =10). Each specimen was imaged 6 days post injection. The area of the GFP-positive liver is computed with the Zeiss software in LSM510 META.

We proceed to verify the anti-cancer property of MSNP-SS-AD loaded with Dox and capped with CD-2NH₂ with a zebrafish mifepristone-inducible liver tumor model that expressed oncogenic EGFP- $kras^{v12}$.

Hyperplastic growth of liver tumors is induced in larvae from 4 days post fertilization. The advantage of this model is a possibility to visualize and quantify changes in liver with CLSM. We injected 5 nl of MSNP-SS-AD loaded with Dox and capped with CD-2NH₂ at a concentration of 10mg/ml into the abdomen of inducible *kras*^{v12} transgenic zebrafish larvae at 4 days post fertilization. The anti-cancer property of Dox loaded in MSNP was assessed 6 days post injection using LSM510 META. Representative images of non-injected transgenic larvae (**Figure 3.16a**) and MSNP loaded Dox-injected transgenic larvae (**Figure 3.16b**) experiencing liver hyperplasia, are presented. All supporting materials that contributed to the bar chart in **Figure 3.16d** are presented in **Figure 3.17**. On average the liver tumor area was 35.5% less in transgenic larvae exposed to MSNP-SS-AD loaded with Dox and capped with CD-2NH₂ (n =10), when compared to non-injected transgenic zebrafish experiencing liver hyperplasia (n=8). Two-tailed T test confirmed that the reduction of liver is statistically significant (P=0.0291). A magnified view of GFP-positive liver tumor cells with internalized Dox (red fluorescent compound; **Figure 3.16c**) further supports the idea that reduction of hyperplastic liver is due to successful internalization and release of Dox from of MSNP-SS-AD+CD-2NH₂.

2.3.4 Conclusion

In conclusion, we have successfully fabricated a redox-responsive drug/siRNA co-delivery system based on mesoporous silica nanoparticles (MSNP-SS-AD). Ethylenediamine-modified β -cyclodextrin (CD-2NH₂) rings have been introduced onto the nanoparticle surface as smart nano-gates to: (1) block drug molecules within the mesopores and (2) provide a platform for the complexation with siRNA through electrostatic interactions. Controlled drug/siRNA release from multifunctional nanoparticles by redox-triggered disulfide bond cleavage has been demonstrated in solution. Successful RNA interference using siRNA to target two different genes has demonstrated the efficacy of CD-2NH₂ capped MSNP-SS-AD as a delivery vehicle for siRNA at a broad range of temperatures. First, the inhibition of Bcl-2 expression has been detected in Bcl-2 siRNA transfected HeLa cell lines maintained at 37°C. Second, the in vivo

experiment carried out in transgenic zebrafish larvae (maintained at 28.5°C) has showed that CD-2NH₂ capped MSNP-SS-AD/GFP siRNA complex injected into the brain ventricle decreases GFP transcription in two different populations of cells of the choroid plexus. This multifunctional nanoparticle could be used as a carrier for simultaneous anticancer drug (Dox) and siRNA delivery. Enhanced cytotoxicity caused by simultaneous delivery of Dox/Bcl-2 siRNA has been demonstrated in HeLa cells. Enhanced cytotoxicity has not been observed upon the injection of Dox-loaded nanoparticle/GFP siRNA complex since GFP mRNA represents physiologically benign target. In contrast, not only Dox-loaded and CD-2NH₂ capped MSNP-SS-AD has been internalized by cells in vivo, but the development of choroid plexus and progression of liver hyperplasia were also inhibited by Dox-loaded nanoparticles in dose-dependent manner. Lack of inhibition of GFP transcripts by high weight ratio formulations of nanoparticle/siRNA complex ($\geq 10:1$) has indicated that the activity of formulations of drug/siRNA needs to be rigorously tested in vivo before optimal effect of synergistic formulation could be achieved.

2.4 Target Drug/siRNA Co-delivery by pH-responsive Hollow Mesoporous Silica Nanoparticles (HMSNP)

Although MSNP is thought to be biocompatible with low cytotoxicity, reports about its dosage dependent toxic effect on biological system still arouse the concern on its safety.^{96, 208, 209} In the introduction part, we also introduced the dosage dependent cytotoxicity effect of MSNP. Thus, it is of great significance to increase the drug and gene delivery capacity of MSNP, which means that required therapy effect can be achieved by much lower amount of silica with negligible toxic effect. And hollow mesoporous silica nanoparticle (HMSNP) stands out as an excellent solution to this problem for its void core can serve as an extra reservoir for drug storage.²¹⁰⁻²¹² Besides, for the same amount of silica, HMSNP can provide larger outside shell surface area for nucleic acid attachment, leading to enhanced gene delivery capacity.

Scheme4.1. Illustration of target drug/siRNA co-delivery by pH-responsive HMSNP.

Another critical issue in cancer treatment is side effect due to non-targeting delivery. Therefore, cancer-target delivery and controlled intracellular drug release are rather essential for successful cancer treatment. The folic acid receptor mediated endocytosis is manipulated by the high affinity between the folic acid molecule and folic acid receptor over-expressed by most cancer cells.²¹³⁻²¹⁵ And folic acid has been widely used as an effect targeting ligand for target cancer therapy and image.²¹⁶⁻²¹⁸ In order to realize enhanced chemotherapy efficacy via target drug/siRNA delivery, we have designed a HMSNP based target drug/siRNA co-delivery vehicle. As showed in **Scheme4.1**, the as-prepared HMSNP was initially functionalized with phosphate groups which has higher affinity of anti-cancer drug Dox and thus can further increase the Dox loading amount.¹⁶⁶ It is also expected that the coating of folic acid conjugated polyethyleneimine (PEI-FA) on HMSNP surface can wrap the particle and block the nano-size channels to prevent stored drugs from pre-releasing. Folic acid molecules conjugated on PEI polymer work as target ligand, resulting in selectively internalization by target cancer cells. HeLa cell lines with significant higher folic acid receptor expression and MCF-7 with low folic acid receptor expression were chosen as the positive and negative models for the investigation of target drug/siRNA delivery.²¹⁹⁻²²² Before internalization into cancer cells, anti-cancer drug Dox can be retained inside the HMSNP with negligible pre-release. Besides, the positive charge given by amine groups of PEI-FA can bind negatively charged siRNA, in which way the nanoparticle serve as siRNA delivery vehicle at the same time. As most cancer cells have acidic intercellular environment,^{223, 224} many researchers focus efforts on the design and fabrication of pH-controlled delivery system.^{100, 225-228} And pH-responsive release property of our co-delivery vehicle ensures the “zero release” before reaching target cancer cells. We suppose that upon the cellular uptake, the acidic intercellular environment leads to the protonization of amine groups on PEI-FA and thus the polymer shell dissociation due to positive charge repelling between polymer chains, leading to release of siRNA and the free Dox diffusion out from the internal core of HMSNP.

2.4.2 Materials and Methods

Materials: Absolute ethanol (EtOH, >99.9%), hydrochloride (HCl, assay 37%), methanol (MeOH, 99.5%), 3-aminopropyltriethoxysilane (APTS), sodium hydroxide (NaOH), tetraethylorthosilicate (TEOS, 99%), 3-(trihydroxysilyl)pro-pylmethylphosphonate monosodium (TPMP), cetyltrimethylammonium bromide (CTAB, 90%), N-hydroxysulfosuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), HMSNP+DOX+PEI-FA +siRNA(Bcl-2) polyethyleneimine (PEI) with molecular weight 1.8kDa, folic acid, 4',6-diamidino-2-phenylindole (DAPI), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), fluorescein isothiocyanate (FITC), doxorubicin (Dox), dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), formaldehyde, phosphate buffered saline (PBS) buffer, siRNA duplex against Bcl-2 protein, β -actin primary antibody (Mouse) and secondary antibody (goat anti-mouse and goat anti-rabbit), and universal siRNA negative control (scrambled), 660nm protein assay kit, western blot assay kit, apoptosis assay reagent annexin V and tris-borate-EDTA (TBE) buffer were purchased commercially.

Instruments: Transmission electron microscopy (TEM) images were captured by JEOL 2010 TEM at 200kV. Specific surface area and pore size distribution of the as-prepared hollow mesoporous silica nanoparticles were measured by ASAP-2020 Micromeritics. X-Ray diffraction pattern was collected by X'Pert powder XRD. Zeta potential value was measured by Mavorn Nanosizer. FT-IR spectra were measured through Fourier transformed infrared spectrometer. Thermogravimetric analysis (TGA) was completed by Thermogravimetric Analyzer Q500 (TGA Q500). Nanodrop 1.0 was used to determine the SiRNA concentration. UV-vis spectra and related absorption intensity was recorded by UV-vis 2501 Spectrometer. Agarose gel electrophoresis was performed by ENDRO GEL XL E0160 electrophoresis system. A microplate reader (infinite 200 PRO, Tecan) was employed for the MTT assay. Western blot assay was completed with Invitrogen's Western Blotting Kits. Confocal microscopy images were taken by

a confocal microscope (Leica TCS SP5, 40 $\times$ oil objective). Flowcytometry data was collected by BD FACSCalibur Flow Cytometer.

MSNP and HMSNP synthesis: Mesoporous silica nanoparticle (MSNP) was prepared by a conventional sol-gel process in base solution and phosphate group was initially functionalized by co-condensation method. In a typical synthesis, CTAB (500mg) was dissolved in deionized H₂O (250mL). NaOH aqueous solution (1.75mL, 2M) was added into the above solution. The mixture solution was heated to 80 °C under vigorous stirring. When the temperature was stabilized, TEOS (2.5mL) was slowly added into the mixture solution. After 15min, 0.5ml TPMP was slowly added to modify the MSNP with phosphate groups and the solution was stirred at 80°C for 2h. Formed nanoparticles were collected by centrifugation at 8000 rpm for 10 min and then washed with MeOH and deionized H₂O thoroughly. Surfactant CTAB was removed by suspending the nanoparticles in MeOH (50mL) containing condensed HCl (3mL, 37%) and refluxing at 80°C for 24h. The nanoparticles were collected by centrifugation and washed thoroughly with MeOH and deionized H₂O. The nanoparticles were dried in vacuo at 80°C for 24h.

HMSNP: Hollow mesoporous silica nanoparticle (HMSNP) was prepared by selective etching method. First, solid silica spheres were prepared by modified Stober method²²⁹. Typically, ethanol (142.8mL), deionized water (20mL) and ammonium aqueous solution (25-28%, 4mL) were mixed and under stirring. After the mixture solution was heated to 30°C, TEOS (6mL) was rapidly added to the mixture and the solution was stirred at 30°C for 2h. The formed solid silica spheres were collected by centrifugation and washed with deionized water and ethanol thoroughly. Then, the solid silica spheres were dried in vacuo at 80°C for 24h. Then, mesoporous silica shell was coated on the solid silica spheres. 100mg of solid silica spheres were homogenously dispersed in 20mL deionized water by ultrasonication for 15min. The prepared solution containing CTAB (150mg), deionized water (30mL), ethanol (30mL), and ammonia solution (0.55mL), was added. The mixture solution was stirred at room temperature for 5min; then TEOS (0.25mL) was added quickly to the mixture. For the preparation of the FITC-labeled HMSP,

fluorescein isothiocyanate (1.3 mg) was dissolved in absolute ethanol (1.5 mL) containing APTES (3 μ L) and mildly stirred for 2 h in the dark before adding into the 0.25mL TEOS. 2h after the addition of TEOS, TPMP (0.05mL) was added into the mixture which was kept stirring for another 4h. The formed mesoporous silica shell coated nanoparticles were collected by centrifugation and washed by deionized water and ethanol before redispersed in 15mL of deionized water. The solid silica sphere was removed by selective etching. With vigorous stirring, 5mL aqueous solution containing Na₂CO₃ (424mg) was slowly added into the well-sonicated water suspension of the above suspension. After stirring at 50°C for 8h, the product of hollow mesoporous silica nanoparticle was collected by centrifugation and extensively washed with deionized water and ethanol. The last step of surfactant CTAB removal was completed by suspending the hollow mesoporous silica nanoparticle in 50mL methanol containing 3mL condensed HCl. The suspension was refluxed under stirring at 80°C for 24h. The as-prepared HMSNP was collected by centrifugation and washed with deionized water and ethanol thoroughly; then dried in vacuo at 80°C for 24h.

PEI-FA conjugate synthesis: 100mg folic acid was dissolved in 5ml DMSO; then 5ml DMSO solution containing 60mg EDC and 180mg NHS was added into the folic acid solution. The mixture was kept stirring for 2h in the dark before added to 15ml aqueous solution containing 700mg PEI (1.8 kDa). Then, the pH value was adjusted to 8 by adding proper amount of 0.2M NaOH. The mixture solution was kept stirring for 3 days and then dialysis against deionized water was performed in a dialysis membrane (1K) for 5 days to remove excess folic acid and byproducts. The PEI-FA conjugate was collected by freeze drying and characterized by FT-IR.

Drug loading efficiency test: Suitable amount of Rhodamine B and 1mg NP(HMSNP or MSNP) at various weight ratios were added into 1 mL deionized water. The mixture solutions were stirred for 24h to allow drug loading into nanoparticles. Then, 4mg PEI-FA was added into the mixture solution and continue the stirring for another 4h. The, RhB loaded HMSNP with PEI-FA coating (HMSNP+RhB+PEI-

FA) was collected by centrifugation and washed with PBS for three times. Then, the HMSNP+RhB+PEI-FA complex was suspended in PBS buffer and the pH was changed to 4.5. After extensively sonication for 30min, the suspension was centrifuged. The released RhB amount was quantified by measuring the UV-vis absorbance intensity of RhB in the supernatant of the suspension. For Dox loading capacity determination, 1mg HMSNP was suspended in 1ml aqueous solution of Dox (1mg/mL) and the mixture was stirred for 24h for the Dox loading. And similar method was employed for the loading capacity determination. For all the experiments, three duplicates were completed for statistic analysis.

Agrose gel electrophoresis: NP(HMSNP or MSNP)+PEI-FA complex was formed by mixing 1mg NP(HMSNP or MSNP) and 4mg PEI-FA in 1ml PBS buffer (pH 7.4), and the mixture was stirred for 4h. The formed complex was collected by centrifugation and washed with PBS (pH 7.4) for three times. Then, the formed NP(HMSNP or MSNP)+PEI-FA complex and siRNA at various NP-to-siRNA weight ratios were mixed and incubated for 2h for the complex formation. Free siRNA and nanoparticles only were also loaded in the first lane and second lane respectively as control. Proper amount of deionized H₂O was added to each lane in order to keep the concentration of siRNA constant. The agarose gel electrophoresis was carried out in agarose gel (1.5wt%) within TBE buffer at 100V for 30 min.

pH-responsive Dox release test: The prepared HMSNP+DOX+PEI-FA+siRNA complex (containing 0.5mg HMNP) was suspended in PBS buffer (1mL, pH=4.5, 6 or 7.4 respectively). For pH=4.5 and 6, appropriate amount of 2M HCl was added to change the pH value of PBS buffer to expected value before using. With certain time interval, the mixture solution was centrifuged and the UV-vis absorbance intensity of Dox (485nm) in the supernatant was recorded to monitor the Dox release process. For pH-stimuli release test, proper amount of 2M HCl was added to the sample of pH=7.4 after the previous experiment. The Dox release was then monitored by similar method. Three independent duplicates were completed for statistic analysis.

Cell Culture: HeLa and MCF-7 were cultured in DMEM containing 10% FBS and nonessential amino acids (0.1 mM). The culture was maintained at 37°C in a humidified atmosphere containing 5% CO₂.

Bcl-2 protein expression silence and western blot assay: siRNA duplex sequence against Bcl-2 protein, noted as siRNA(Bcl-2), was presented as follows: sense strand 5'-rGrUrArCrArUrCrCrArUrUrArUrArArGrCrUrGrUrCrGrCdAdG-3', antisense strand 5'-rUrGrCrGrArCrArGrCrUrUrArUrArArUrGrGrArUrGrUrArCrUrU-3'. Negative control siRNA duplex (Scrambled Negative), noted as siRNA(scrambled), was used as the negative control. Fresh DMEM medium without any antibiotics and FBS was used for the gene knockdown experiments. Both HeLa and MCF-7 cells were seeded into 6-well plate at a density of 20×10^4 cells/well in complete DMEM medium and grown for 24 h respectively. Then, both cells were treated with HMSNP+PEI-FA+siRNA (Bcl-2) complex with various HMSNP-to-siRNA weight ratios, including 10:1, 20:1, and 30:1. The content of siRNA is 1µg/well; and the content of HMSNP is 10, 20, and 30 µg/well respectively. The medium volume of each well is 1mL during the treatment. After 24h treatment, the culture medium was changed into new medium and the cells were cultured for another 48h. Then, the cells were washed by cold PBS and collected by cell scraper. Then, the protein of each sample was collected by lysis buffer and protein concentration was measured by 660nm protein assay kit in order to ensure the protein loading is equal in the western blot assay. Then, western blot assay was performed according to standard procedures with western blot assay kit.

MTT cytotoxicity assay: The cytotoxicity of the nanoparticles was evaluated by employing the MTT assay. HeLa and MCF-7 cells were seeded into 96-well plate at a density of 1×10^4 cells/well in DMEM medium. After 24h incubation, the medium was changed with new medium which containing HMSNP+PEI-FA+siRNA(scrambled), HMSNP+DOX+PEI-FA or HMSNP+DOX+PEI-FA+siRNA(Bcl-2) complex, with HMSNP-to-siRNA weight ratio 20:1. After 24h treatment, the culture medium was changed into new medium and the MTT assay was performed after another 48h. For the MTT assay, typically the old medium was removed and new medium (100 µL) containing MTT (0.5mg/mL) was

added. After 4h incubation, the medium was replaced with DMSO (100 μ L) and the plate was gently shaken for 15 min before measuring the absorbance at 565 nm using a microplate reader (infinite 200 PRO, Tecan). The cell viability related to the control wells that only contain cell culture medium was calculated by $[A]_{\text{test}}/[A]_{\text{control}}$, where $[A]_{\text{test}}$ and $[A]_{\text{control}}$ are the average absorption intensities of the test and control samples (n=8), respectively.

Cell apoptosis assay by flowcytometry: HeLa cells were seeded in 6-well palte and grown for 24h. Then, the cells were treated with 10 μ g/ml HMSNP+PEI-FA, HMSNP+DOX+PEI-FA, and HMSNP+DOX+PEI-FA+siRNA(Bcl-2) complex with HMSNP-to-siRNA weight ratio 20:1 for 24h. After the treatment, the medium was replaced with fresh medium. For the last two samples, 24h after the addition of new medium, an extra 5 μ g/ml HMSNP+DOX+PEI-FA complex was added. Then, flowcytometry assay was performed after at 72h. The cells was collected with trypsin and washed with PBS. Then, the cells were stained by annexin V and flowcytometry assay was performed immediately after the staining. HeLa cells without any treatment were also stained by annexin V as control. In order to show the enhanced chemotherapy effect by the co-delivery system, experiments of HeLa cells only treated with equivalent free Dox (1.5 μ g/ml), 10 μ g/ml HMSNP+DOX+PEI-FA, and HMSNP+DOX+PEI-FA+siRNA(Bcl-2) complex with HMSNP-to-siRNA weight ratio 20:1 were performed. After 24h treatment, the medium was replaced by new medium and the apoptosis assay was performed at 72h.

Confocal microscopic images: HeLa or MCF-7 cells were seeded in plastic-bot-tomed μ -dishes (35 mm) and grown in the DMEM medium for 24 h. Then, the cells were exposed to HMSNP+PEI-FA+siRNA(Bcl-2), and HMSNP+DOX+ PEI-FA+siRNA(Bcl-2) complex (10 μ gmL⁻¹) for 24h. The medium was then removed and dishes were washed three times with PBS. The cells were fixed with 4.0% formaldehyde at room temperature for 15 min. After washing with PBS, the cells were stained with DAPI (30 nM) for 15 min. The cells were further washed with PBS for three times before observation with confocal microscopy. The fluorescence of DAPI was excited by 405nm laser. And the fluorescence of

FITC and Dox were both excited by 488nm laser. The fluorescence of DAPI, FITC, and Dox are collected by $480\pm10\text{nm}$, $520\pm10\text{nm}$, and $580\pm10\text{nm}$ channel respectively.

2.4.3 Results and Discussion

HMSNP preparation and characterization

Selective etching method²³⁰ with modification was used for the preparation of HMSNP. As-prepared HMSNP was initially functionalized with phosphate groups by directly addition of TPMP during the formation of the mesoporous shell structure. To trace the intracellular location of HMSNP, as well as allow the following floccytometry study of the cellular uptake of HMSNP, fluorescein isothiocyanate (FITC) labeled HMSNP was also prepared in the study.

Figure 4.1. TEM images of HMSNP a) at low magnification, b) at high magnification, c) HMSNP+PEI-FA complex, and d) MSNP.

The mesoporous structure of HMSNP was characterized by transmission electron microscope (TEM), individual doughnut-like hollow particles with uniform particle size around 185nm were clearly observed (**Figure 4.1 a**). Relatively higher brightness inside the darker outside circles is due to completely removal of internal solid silica spheres, resulting in hollow cores. The thickness of the outside shell structure is estimated to be 30nm. The mesoporous structure of the shell was formed by CTAB directed condensation of tetraethylorthosilicate (TEOS). Clear perpendicular nano-channels heading the internal core was

indicated by red arrow (**Figure 4.1 b**), which provides the nano-channels for the loading and releasing of drug molecules. In order to compare with normal mesoporous silica nanoparticle (MSNP), phosphate group modified MSNP was also prepared by co-condensation method.²³¹

Figure 4.2. N₂ adsorption/desorption isotherms of a) HMSNP, c) MSNP; and BJH pore size distribution of b)HMSNP, d)MSNP.

Table 4.1. BET surface area, pore size, and pore volume of HMSNP and MSNP.

	Surface area (m ² /g)	Pore volume (cm ³ /g)	Pore size (nm)
MSNP	881.7	0.56	2.3
HMSNP	799.4	0.79	2.6

Similar BET specific surface and BJH pore size (**Table 4.1**) were found for both HMSNP and MSNP, which means that the specific surface area is dominated by the mesoporous structure and pore size is determined by the alkyl chain length of the surfactant CTAB. Type IV isotherm curves of both nanoparticles also proves the successful formation of mesoporous structure. However, bigger hysteresis

loop of the isotherm curve of HMSNP evidences the empty core inside HMSNP, which were also reported by other researchers(**Figure 4.2**).^{230, 232} Moreover, HMSNP has higher pore volume than MSNP, indicating the presence of hollow core.

Figure 4.3. Powder X-ray diffraction pattern of HMSNP and MSNP.

In the XRD pattern, typical (100) peaks at low angle around $2\theta=2.2^\circ$ were found for both MSNP and HMSNP, which is consistent with the hexagonal arrangement of the mesopores. However, due to relatively short-ranged order of the mesoporous shell in HMSNP, the (100) peak of HMSNP is broader than that of MSNP where the ordered region should be larger than HMSNP (**Figure 4.3**).

PEI-FA conjugates preparation and coating on HMSNP

Figure 4.4. FT-IR spectra of PEI, folic acid conjugated PEI (PEI+folic acid), and folic acid.

Folic acid was conjugated on branched cationic polymer PEI (1.8 kDa) through EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide)) chemistry to form amide bond between carboxylic group on folic acid molecule and primary amine group on PEI. The PEI-FA conjugate was characterized by FT-IR spectra (**Figure 4.4**). The presence of a new peak at 1640 cm^{-1} due to C=O amide bond^{233, 234} confirmed the chemical linkage of folic acid onto PEI. PEI-FA conjugate is easily dissolved in deionized water, and the UV-vis spectra of its aqueous solution presents characteristic peaks of folic acid molecules.^{220, 221}

Figure 4.5. a) UV-vis spectra and b) Zeta potential measurement indicate PEI-FA coating on HMSNP.

All the samples are dissolved or suspended in deionized water.

The phosphate groups introduce negative surface charge on HMSNP surface, which allows charge interaction with positively charged PEI-FA. Reversed zeta potential value of HMSNP+PEI-FA complex indicates successfully coating of PEI-FA on HMSNP surface. With the PEI-FA amount increasing, the HMSNP+PEI-FA complex shows increasing positive zeta-potential value (**Figure 4.5 b**), suggesting accumulation of PEI-FA binding on the HMSNP surface. After PEI-FA coating, HMSNP+PEI-FA complex has a broad UV-vis absorbance peak at 280nm indicating presence of folic acid molecule^{220, 221, 233}. Besides, the mesoporous shell structure was blurred after the PEI-FA complex formation (Figure 4.5, c), which further proves the surface coating of PEI-FA. The zeta potential value of Dox loaded HMSNP+PEI-FA complex (HMSNP+Dox+PEI-FA) was determined to be 27.2mV similar to

HMSNP+PEI-FA without Dox loading (28.4mV), which means the presence of Dox inside HMSNP would not affect the PEI-FA coating.

Enhanced Drug and SiRNA delivery capacity

Figure 4.6. Rhodamine B loading capacity comparison of MSNP & HMSNP at different weight ratios.

To demonstrate that the enhanced drug loading capacity of HMSNP is due to the hollow structure, red fluorescence dye rhodamine B (RhB) and HMSNP were mixed at various weight ratios in the same volume of deionized water to perform the loading test. After 24h stirring, RhB was supposed to fully diffuse into the hollow cores. Then, RhB loaded HMSNP was collected by centrifugation and then suspended in deionized water. After 30min ultrasonic treatment to release all the loaded RhB molecules, the supernatant was collected by centrifugation and the UV-vis absorbance intensity of RhB at 553nm was recorded to determine the loading amount of RhB molecules. It is interesting to find that with the portion of RhB increasing, the loading amount of RhB per mg nanoparticle keeps increasing for both HMSNP and MSNP at the beginning (**Figure 4.6**). Then, the loading capacity of MSNP seems to be saturated and no increase was observed with future increase of the RhB amount, but HMSNP remains the increasing trend until the RhB amount was increased by 9 times. The RhB loading capacity of HMSNP was found to be enhanced twice comparing to that of normal MSNP. Then, anti-cancer drug Dox was

loaded in both HMSNP and MSNP at NP-to-Dox weight ratio 1:1. And Dox loading capacity of HMSNP was estimated to be 15.6% which is enhanced by about 100% than MSNP as well (**Figure 4.7 b**).

Figure 4.7. a) Agarose gel electrophoresis of NP(HMSNP or MSNP)+PEI-FA+siRNA(Bcl-2) complex; b) Dox loading capacity of HMSNP and MSNP.

In order to prove the superiority of HMSNP over normal MSNP, the drug loading and siRNA binding capability were demonstrated in figure 4.7. Although both HMSNP and MSNP have similar BET surface area which is mainly contributed by the mesoporous structure, however the outside shell surface of HMSNP should be much larger than that of MSNP because of weight decrease by the removal of the internal solid silica sphere. Thus, for the same amount of both nanoparticles, after coating with the same amount of PEI-FA, the outside shell surface of HMSNP should be larger than that of MSNP, providing more positively charged area to attach siRNA molecules. The agarose gel electrophoresis results certified the hypothesis as well. For HMSNP, when the weight ratio of HMSNP-to-siRNA increases to 12, almost all the siRNA was restrained on nanoparticle's surface, while only the weight ratio of MSNP-to-siRNA was increased to 20 fully binding of siRNA can be achieved (**Figure 4.7 a**). From TGA results, the weight loss of as-prepared HMSNP(**Figure 4.8 b**) that was initially functionalized with phosphate groups increased about 5% when comparing with base HMSNP(**Figure 4.8 a**). Considerable increase of weight loss (about 20 wt%) was observed for HMSNP+PEI-FA complex (**Figure 4.8 c**), comparing to as

prepared HMSNP only (**Figure 4.8 b**), which proves the coating of PEI-FA conjugate on HMSNP. And extra weight loss (6 wt%) in Figure 4.8 d was due to the decomposition of loaded Dox in the HMSNP+Dox+PEI-FA complex.

Figure 4.8. Thermal gravimetric analysis (TGA) results of (a) bare HMSNP; (b) as-prepared HMSNP; c) HMSNP+PEI-FA complex; d) HMSNP+Dox+PEI-FA complex.

Moreover, the loading of anti-cancer drug Dox should not affect the siRNA binding capability. Thus, we performed agarose gel electrophoresis experiment with HMSNP+Dox+PEI-FA+siRNA(Bcl-2) and similar result was observed (**Figure 4.9**). When the HMSNP-to-siRNA weight ratio was higher than 8, all siRNA was effectively binded on the HSMNP and restrained inside the well. Such result allows the drug/siRNA co-delivery simultaneously in the following study.

Figure 4.9. Agarose gel electrophoresis of HMSNP+Dox+PEI-FA+siRNA(Bcl-2) shows that the loading of Dox inside HMSNPs would not affect the siRNA binding capability of the co-delivery system.

Figure 4.10. a) pH-controlled Dox release, and b) stimuli-responsive (by changing pH value) Dox release from HMSNP+Dox+PEI-FA+siRNA(Bcl-2) complex in PBS buffer.

The pH-controlled drug delivery system has attracted considerable attentions recently, because the acidic intercellular environment of most cancer cells^{223, 224} provides natural triggers for drug release inside cancer cells. It is delighted to find that our co-delivery vehicle exhibits pH-responsive drug release property as expected. As showed in Figure 4.10 a, both the release rate and release amount of Dox are pH-dependent and responsive to acidic condition. The Dox release at neutral condition (pH 7.4) is less than 10% and no further release was observed for more than 30 hours, which means the PEI-FA coating on HMSNP can effectively block the nano-channels and prevents Dox from leaking. The pH-responsive release can be explained by the protonation of the amine group on PEI-FA conjugate. At acidic condition, PEI-FA polymer chain would be positively charged due to protonation of amine groups and thus repels each other, which would lead to the swelling and dissociation of the surface PEI-FA polymer layer. Then, the access to the nanochannels and further internal core of HMSNP is available. To certify this hypothesis, another experiment was conducted. After 2 days' release at pH 7.4, appropriate hydrochloride acid was added into the solution to change the pH from 7.4 to 4.5, and then Dox release sharply increased again as showed in figure 4.10 b. Previous study also successfully employed protonation of amine groups to

control the open-close of the mesopores of the mesoporous silica nanoparticles.¹⁰² What's more, the dissociation of PEI-FA coating layer in the acidic intercellular environment would also lead to escape of siRNA molecules that attached on HMSNP surface, delivering siRNA into cytoplasm where RNA interference takes place.²³⁵⁻²³⁷ The pH-responsive release property of the HMSNP based drug/siRNA co-delivery vehicle is rather promising in the following cell study *in vitro*.

Target drug delivery and intracellular drug release

Figure 4.11. a) Confocal microscopy images of a) HeLa(FA+) and MCF-7(FA-) cells after treated with 10 μ g/ml HMSNP(FITC)+Dox+PEI-FA+siRNA(Bcl-2) complex for 24h. From left to right are images under bright field, green channel (FITC: 488/520nm $\pm$ 10nm), red channel (Dox:488/580nm $\pm$ 10nm), and merged channel (Bright field+FITC+Dox+DAPI), respectively; b) merged image of FITC+Dox channels

(top), and Bright Field+DAPI+FITC+Dox channels (bottom); c) Flowcytometry results of HeLa(FA+) and MCF-7 (FA-) cells after treated with HMSNP(FITC)+DOX+PEI-FA+SiRNA(Bcl-2) complex for 24h.

Folic acid receptor has much higher expression level in many cancer cells than that of normal cells²¹³⁻²¹⁵ and specific affinity between folic acid and folic acid receptor can help the cellular uptake of HMSNP through folic acid receptor mediated endocytosis. Thus, PEI-FA coated HMSNP can selectively bind and enter into cancer cells that have high folic acid receptor expression, resulting in target drug/siRNA delivery. The target effect was first tested *in vitro* via observing nanoparticle's uptake by confocal microscopy. To trace the location of HMSNP, green fluorescence dye FITC labeled HMSNP was prepared for the experiment. And HeLa cells with high folic acid receptor expression was chosen as the positive control (FA+), while MCF-7 cells with low folic acid receptor expression was taken as the negative control (FA-). Both cells were treated with the same concentration (10 μ g/ml) of HMSNP+DOX+PEI-FA+siRNA(Bcl-2) complex for 24h. The cell nucleus was stained by DAPI. The cells were washed three times by PBS to remove nanoparticles not taken by the cells before confocal microscopy observation. In figure 4.10 a, it can be seen that much more green fluorescence (FITC) dots indicating the presence of HMSNP are inside the HeLa cells. Meanwhile, only few green fluorescence dots were found in MCF-7 cells, suggesting much less cellular uptake of HMSNP. To further confirm the targeting effect of the drug/siRNA co-delivery system, HeLa cells (FA+) and MCF-7 cells (FA-) were co-cultured and treated with 10 μ g/ml HMSNP(FITC)+PEI-FA+siRNA(Bcl-2) complex. After 24h, cells were washed by PBS three times and observed by confocal microscopy.

Figure 4.12. Confocal microscopy image of co-cultured HeLa (FA+) and MCF-7 (FA-) cells treated with 10 μ g mL⁻¹ HMSNP+PEI-FA+siRNA(Bcl-2) complex at HMSNP-to-siRNA weight ratio 20:1 for 24h.

Selectively cellular uptake is clearly showed, as many more FITC (green) fluorescence dots were observed in HeLa (FA+) cells than MCF-7 (FA-) cells (Figure 4.12). Flowcytometry results confirmed the target delivery effect of the HMSNP statistically (**Figure 4.11, c**), as only 66.31% of MCF-7 cells have HMSNP uptake indicated by FITC fluorescence intensity while almost all the HeLa cells (99.98%) have much higher FITC fluorescence intensity. As expected, its higher FITC fluorescence intensity suggests that folic acid receptor mediated endocytosis indeed happened, resulting in enhanced cellular uptake of HMSNP and thereby target delivery.

Drug release triggered by intracellular acidic pH was quite obvious from the red fluorescence channel indicating the location of anticancer drug Dox (**Figure 4.11, b**). The red fluorescence dots overlapped with green dots suggest residual Dox inside the HMSNP, inside the cytoplasm red color from Dox also observed outside the green dots, which means that considerable amount of Dox released from HMSNP. Besides, purple color from the nucleus due to the overlay of red (Dox) and blue (DAPI) suggests that the released Dox further diffused into the cell nucleus.

Figure 4.13. a) Merged (Bright field+DAPI+FITC+Dox channels) confocal microscopy image of HeLa cells after treated with 10μg/ml HMSNP+DOX+PEI-FA+siRNA(Bcl-2) complex for 24h; b) fluorescence intensity reading of DAPI (blue), FITC (green), and Dox (Red) along the dashed arrow in a).

The intercellular Dox release was further confirmed by fluorescence intensity reading under confocal microscopy (**Figure 4.13**). Green peaks around the nucleus represented by blue curve (DAPI) indicate the

location of HMSNP in the cytoplasm. And high fluorescence intensity of red curve overlapped with blue curve suggests presence of considerable Dox in the cell nucleus. Dox is an anti-cancer drug which mainly interacts with the DNA of cancer cells to induce cell apoptosis and finally cell death¹⁹⁹, so it is favorable to find Dox molecule inside cell nucleus. However, much less red fluorescence was found in MCF-7 cells, which is due to less HMSNP uptake and thus less Dox delivery into the MCF-7 cells. The difference of Dox fluorescence intensities of MCF-7 and HeLa in flowcytometry results also agrees with the confocal microscopy results.

Bcl-2 protein silence and synergetic therapy effect

Figure 4.14. a) Western blot assay of protein in HeLa and MCF-7 cells; both cells were treated with HMSNP+PEI-FA+siRNA(Bcl-2) complex with HMSNP-to-siRNA weight ratio 10:1, 20:1, and 30:1; b) Relative Bcl-2 protein expression of HeLa and MCF-7 cells. The content of siRNA is 1 μ g/well; and the content of HMSNP is 10, 20, and 30 μ g/well respectively; the volume of culture medium in each well is 1ml during the treatment. After 24h treatment, the medium was changed into new medium and the protein was collected after the cells were cultured for another 48h.

The knockdown of the expression of anti-apoptotic protein Bcl-2 is the prerequisite of enhanced chemotherapy. To rule out the possibility of cytotoxicity of the co-delivery vehicle and unequal protein loading, β -actin protein was chosen as the internal standard, and all the results were normalized to the

protein expression of the control group without any treatment. As for HeLa cells, obvious silence of Bcl-2 protein was indicated by the decreasing darkness of the band (**Figure 4.14, a**). In order to clarify the knockdown of Bcl-2 is due to the specific sequence of siRNA against Bcl-2 protein. Universal siRNA duplex (scrambled) which is non-targeting was used as negative control, and no protein silence effect was observed by delivering siRNA(scrambled) (**Figure 4.15**). Besides, equivalent amount of free siRNA (Bcl-2) cannot silence the Bcl-2 protein expression, which certifies the siRNA delivery efficacy of the HMSNP based delivery system (**Figure 4.15**). It is interesting to find that commercialized siRNA tranfection reagent, Lepofectaine cannot effectively perform siRNA transfection in MCF-7 cells. However, with the same amount of siRNA, by increasing the HMSNP's amount to 30 μ g/well, Bcl-2 protein expression was succsfully suppressed in MCF-7 cells (**Figure 4.14 a**), which indicated the superiority of the HMSNP's delivery efficacy.

Figure 4.15. Western blot assay of protein in HeLa and MCF-7 cells; lane 1: control (without any treatment); after treated with 2: HMSNP+PEI-FA+siRNA(scrambled), 3: free siRNA(Bcl-2), 4: Lepofectamine+siRNA(Bcl-2), and 5: 10 μ g/ml HMSNP+PEI-FA+siRNA(Bcl-2).

The targeting effect was also demonstrated in the western blot assay. The knockdown of Bcl-2 protein expression was effective at low amount of HMSNP (10 μ g/well) for HeLa cells, while for MCF-7 cells the knockdown effect can be only found when the amount of HMSNP was increased to 30 μ g/well. This result could be explained by target delivery, which causes higher cellular uptake of HMSNP and siRNA as well

in HeLa cells. Thus, more siRNA can be delivered into HeLa cells comparing to the case of MCF-7 cells, resulting in higher efficacy of protein expression silence.

Figure 4.16. a) MTT cytotoxicity assay of MCF-7 and HeLa cells; both cells were treated with HMSNP+Dox+PEI-FA or HMSNP+Dox+PEI-FA+siRNA(Bcl-2) complex with HMSNP-to-siRNA weight ratio 20:1; After 24h treatment, the culture medium was changed into new medium and the MTT assay was performed after another 48h. b) MTT cytotoxicity assay of MCF-7 and HeLa cells; both cells were treated with HMSNP+PEI-FA+siRNA(scrambled) complex.

We further conducted the MTT cytotoxicity assay and enhanced chemotherapy by co-delivery of Dox and SiRNA(Bcl-2) was found. First, the toxic effect of the co-delivery vehicle was tested. The HMSNP+PEI-FA+siRNA(scrambled) complex shows minor toxic effect even at rather high nanoparticle concentration ($100\mu\text{g mL}^{-1}$). Furthermore, no concentration-dependent relationship was found, which means the toxicity of the carrier and siRNA molecule is negligible (**Figure 4.16, b**). Then, HeLa(FA+) and MCF-7(FA-) cells was treated with HMSNP+Dox+PEI-FA or HMSNP+Dox+PEI-FA +siRNA(Bcl-2) complexes at the same nanoparticle concentration. For MCF-7 cells, both groups have similar cell viability and no enhanced chemotherapy was found (**Figure 4.16, a**), which is reasonable since Bcl-2 protein knockdown does not work at nanoparticle concentration less than $30\mu\text{g/well}$ (**Figure 4.14**). On the other hand, for HeLa cells, cells treated with HMSNP+Dox+PEI-FA +siRNA(Bcl-2) have much lower cell viability than

that treated with HMSNP+DOX+PEI-FA at the same nanoparticle concentration of $10\mu\text{g mL}^{-1}$ or $20\mu\text{g mL}^{-1}$ (Figure 4.16, a). It is clearly that the enhanced chemotherapy is due to combined therapeutic effect of gene therapy by Bcl-2 protein silence and chemotherapy by Dox. Our finding is consistent with previous study which proved the Bcl-2 protein silence induced apoptosis in HeLa cells²³⁸. We suspect that the silence of Bcl-2 protein improved the sensitivity of HeLa cells to conventional anti-cancer drug Dox, which is further proved by the apoptosis assay by flowcytometry. It is worth noting that in the current drug/siRNA co-delivery study, we used relatively much lower amount of mesoporous silica than the previous drug/gene co-delivery system based on mesoporous silica with CD-2NH₂ capping.

Figure 4.17. Flowcytometry data of cell apoptosis assay by annexin V staining of HeLa cells. Cells were treated with a) $1.5\mu\text{g mL}^{-1}$ free Dox, b) $10\mu\text{g mL}^{-1}$ HMSNP+Dox+PEI-FA, and c) $10\mu\text{g mL}^{-1}$ HMSNP+DOX+PEI-FA+siRNA(Bcl-2) for 24h; then the culture medium was changed into fresh medium and cultured for another 24h before the flowcytometry assay.

The enhanced chemotherapy through the co-delivery of Dox and siRNA(Bcl-2) was further confirmed by cell apoptosis assay. The HeLa cells treated with equivalent free Dox and HMSNP+Dox+PEI-FA complex have similar apoptotic cell population indicated by fluorescence intensity of Annexin V, 17.39% and 14.13% respectively. However, for the HeLa cells treated with HMSNP+Dox+PEI-FA+siRNA(Bcl-2) complex, much higher apoptotic cell population(42.19%) was observed, suggesting that silence of Bcl-2 protein would promote the cell apoptosis and improve the efficacy of traditional anti-cancer drugs. Even

though it is widely accepted that the delivery carrier of nanoparticles can enhance the drug uptake and thus obtain higher therapeutic effect, here similar apoptotic population was found for cells treated with equivalent free Dox and HMSNP+Dox+PEI-FA. Such result can be explained by the incomplete release of Dox from HMSNP. We used the same amount of free Dox with loaded Dox in the HMSNP+Dox+PEI-FA complex, but not all the Dox can be released from HMSNP inside cells. As shown in Figure 4.10 a, when the pH value is 6.0, only 50% Dox was released from HMSNP after 24h incubation. HeLa cells without any treatment were also stained with Annexin V as control (3.18). Negligible toxic effect of the co-delivery vehicle and siRNA molecule is also certified by the apoptosis assay, as only 3.88% apoptotic cells were recorded for HeLa cells treated with $20\mu\text{g mL}^{-1}$ HMSNP+PEI-FA+siRNA(scrambled).

Figure 4.18. Flowcytometry result of apoptosis assay by annexin V staining of HeLa cells. HeLa cells a) without any treatment, and treated with b) $20\mu\text{g/ml}$ HMSNP+PEI-FA+siRNA(scrambled), c) $10\mu\text{g/ml}$ HMSNP+DOX+PEI-FA, d) $10\mu\text{g mL}^{-1}$ HMSNP+DOX+PEI-FA+siRNA(Bcl-2) complex with HMSNP-to-siRNA weight ratio 20:1 for 24h; the medium was replaced with fresh medium after the treatment. For c)

and d), extra 5 μ g/ml HMSNP+DOX+PEI-FA complex was added after 48h incubation. The apoptosis staining and flowcytometry assay was performed after at 72h.

In order to better show that the Bcl-2 protein knockdown would overcome multidrug resistance and result in higher sensitivity to anti-cancer drugs, extra amount of 5 μ g mL⁻¹ HMSNP+Dox+PEI-FA was added 24h after the delivery of only Dox or co-delivery of Dox and siRNA(Bcl-2) respectively. It was found that the apoptotic cell population for the co-delivery group is 80.75%, while that of only delivering Dox is 33.87% (Figure 4.18). Therefore, we conclude that our co-delivery system based on HMSNP can effectively deliver Dox and siRNA simultaneously into target cancer cells, resulting in enhanced chemotherapy by synergetic treatment effect of chemotherapy and gene therapy.

2.4.4 Conclusion

In summary, we have successfully fabricated a HMSNP based pH-responsive target drug/siRNA co-delivery vehicle which is capable of simultaneously delivering Dox and siRNA against Bcl-2 protein into target cancer cells. The hollow structure not only improves the Dox loading capability, but also enhances the siRNA binding ability. Selectively target effect by folic acid receptor mediated endocytosis was certified by confocal microscopy and flowcytometry with HeLa (FA+) and MCF-7 (FA-) cells. Effective silence of anti-apoptotic protein Bcl-2 in HeLa cells help to enhance chemotherapeutic efficacy by simultaneously delivery of Dox and siRNA. This work further improved the MSNP based drug/gene co-delivery system from the view of carrier materials' usage and effective targeting capacity, both of which aimed at minimizing the side-effect of the cancer treatment.

Chapter 3. Monomeric Zinc Phthalocyanine inside Spacer Modified Nano-channels of MSNPs: A Nanohybrid for Target Photodynamic Cancer Therapy

3.1 Research Scope and Motivation

Photodynamic therapy (PDT) is a relatively inexpensive and non-invasive method for cancer treatment.²³⁹⁻²⁴² It has been proved to be an ideal approach for the treatment of skin cancer²⁴³ and also found effective for the treatment of early lung cancer,²⁴⁴ bladder cancer,²⁴⁵⁻²⁴⁷ as well as head and neck cancer.²⁴⁸⁻²⁵⁰ In PDT based treatment, tumor cells are destroyed by light irradiation on photosensitizer that induce the production of reactive oxygen species, primarily cytotoxic singlet oxygen ($^1\text{O}_2$), in a localized area. Thus, an ideal photosensitizer should possess high absorption coefficient, high quantum yield of the triplet state, and low dark cytotoxicity in physiological conditions. Although carriers such as polymer conjugates,²⁵¹ liposomes,^{252, 253} and metal nanoparticles²⁵⁴⁻²⁵⁶ have been developed to enhance the solubility and targeted therapy of photosensitizers, the quest for a versatile and safe nanocarrier is still challenging.

As a promising photosensitizer carrier for PDT, the advantages of MSNPs, such as large internal surface area, uniform mesopores, and easy chemical functionalizations, open up new pathways to develop new materials for functional applications. In general, the internalization of nanoparticle-based photosensitizer hybrid demands the optimization of few parameters including the maximum cell targeting, the maximum photosensitizer loading without aggregation, the maximum photodynamic toxicity, and the minimum dark cytotoxicity. Among these parameters, the tumor targeting can be achieved by passive targeting owing to the enhanced permeability and retention (EPR) observed in tumor sites.^{257, 258} Zinc(II) phthalocyanine

(ZnPc) is a second generation photosensitizer, which suffers severe aggregation, thereby losing its singlet oxygen ($^1\text{O}_2$) generation efficiency in aqueous environment. The easy aggregation significantly limits its application potential in physiological environments. Thus, several efforts have been directed forwards developing alternative strategies for using ZnPc effectively as photosensitizer.^{259, 260} For example, recently a report demonstrates the development of a gold nanoparticle/ZnPc conjugate to address this issue by separating ZnPc molecules by alkyl groups on gold nanoparticle surface.²⁶¹

Scheme 5.1. Schematic illustration for the preparation of hybrid of zinc phthalocyanine (ZnPc) loaded MSNP-Ad and inclusion of CD-2NH₂, **1** for photodynamic singlet oxygen generation. Enlarged area represents a nano-channel modified with adamantane spacer which prevents aggregation of ZnPc.

Herein, we describe hitherto unreported method for controlling the self-assembly of ZnPc inside nanochannels of MSNPs (Scheme 5.1). We anticipated that hydrophobic spacers such as adamantane (Ad) inside mesopores of MSNPs could intervene between ZnPc, thus instantly preventing the aggregation of ZnPc. Concurrently, the modified Ad units on the surface of MSNPs complexed with amino substituted β -cyclodextrin (CD-2NH₂) would enhance the dispersion of the hybrid in aqueous conditions. Hence, in

our experiments, we synthesized Ad functionalized triethoxy silane (i.e., *N*-(3-triethoxysilyl)propyl) adamantaneamide) and employed it to prepare Ad-incorporated MSNPs (MSNP-Ad) via co-condensation method. Thus, Ad was coupled onto both the nanoparticle surface and nanochannels. We then loaded hydrophobic ZnPc inside nanochannels of MSNP-Ad in DMF (*N,N'*-dimethyl formamide), followed by the complex formation between Ad on the nanoparticle surface and CD-2NH₂ in aqueous solution, affording the hybrid **1** for further experiments (Scheme 5.1).

3.2 Materials and Methods

Materials: Adamantane-1-carboxylic acid (Ad-acid), 3-aminopropyltriethoxysilane (APTS), cetyltrimethylammonium bromide (CTAB, 90%), dicyclohexylcarbodiimide (DCC), diethyl ether, dimethylformamide (DMF, 99%), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), fluorescein isothiocyanate (FITC), formaldehyde, hydrochloride (HCl, assay 37%), *N*-hydroxysuccinimide (NHS), methanol (MeOH, 99.5%), phosphate buffered saline (PBS, pH 7.2) buffer, sodium hydroxide (NaOH), and tetraethylorthosilicate (TEOS, 99%) were purchased commercially.

Instruments: FESEM and TEM images were captured by FESEM 6340 at 5kV and JEOL 2010 TEM at 200kV, respectively. X-Ray diffraction pattern was collected by Shimazu Powder XRD from $2\theta = 1.6$ to 8° . Zeta potential and hydrodynamic size value were measured by Mavorn Nanosizer and each sample was measured three times for statistic analysis. Surface area and pore size distribution of the nanoparticles were characterized by ASAP-2020 Micromeritics. FT-IR spectra were measured through Fourier transformed infrared spectrometer. UV-vis absorption was measured by UV-vis 2501 Spectrometer. Fluorescence emission spectra were collected by RF 5301 Spectrofluorophotometer. A microplate reader (infinite 200 PRO, Tecan) was used for the MTT assay. Fluorescence images were taken by a confocal fluorescence microscope (Nikon, Eclipse TE2000-E, 60 $\times$ oil objective). Flow cytometry data was collected by BD FACS Calibur Flow Cytometer.

MSNP-NH₂: Amino group functionalized silica nanoparticles (MSNP-NH₂) were prepared by a co-condensation method in base solution in order to obtain uniform distribution of the functional groups both inside the mesopores and on the nanoparticle surface.¹ In a typical synthesis, CTAB (500 mg) was dissolved in distilled H₂O (250 mL). NaOH aqueous solution (1.75 mL, 2 M) was added into the above solution. The mixture solution was heated to 80 °C under vigorous stirring. When the temperature was stabilized, TEOS (2.5 mL) was slowly added into the mixture solution. APTES (0.5 mL) was added dropwise after 15 min. The mixture solution was stirred at 80 °C for another 2 h. As for FITC labeled nanoparticles, FITC (1.3 mg) was dissolved in absolute ethanol (1.5 mL) containing APTES (3 µL), and the mixture solution was gently stirred for 2 h in the dark before adding into the TEOS (2.5 mL). Then, the formed nanoparticles were collected by centrifugation at 8000 rpm for 10 min and washed with MeOH and distilled H₂O. Surfactant was removed by suspending the obtained nanoparticles in MeOH (150 mL) containing condensed HCl (9 mL, 37%) and refluxing at 80 °C for 24 h. MSNP-NH₂ was collected by centrifugation and washed thoroughly with MeOH and distilled H₂O. The nanoparticles were dried in vacuo at 50 °C for 24 h.

MSNP-Ad: Adamantane-1-carboxylic acid (225 mg) was dissolved in DMF (10 mL) containing DCC (645 mg) and NHS (360 mg). After the mixture solution was stirred for 2 h, a DMF (5 mL) solution containing fully suspended MSNP-NH₂ (100 mg) was added. After reacting for 24 h, MSNP-Ad nanoparticles were collected by centrifugation and washed with DMF, MeOH, and distilled H₂O extensively.

CD-2NH₂: Ethylenediamine modified β -cyclodextrin derivative (CD-2NH₂) was prepared according to a previous report.² Seven ethylenediamine groups were covalently linked at the primary side of the β -cyclodextrin ring.

ZnPc Loading and CD-2NH₂ Capping to MSNP-AD (Hybrid I): MSNP-Ad (1 mg) was suspended in DMF solution (1 mL) containing ZnPc (0.1 mg/mL). After stirring for 24 h, ZnPc loaded MSNP-Ad was collected by centrifugation at 13,000 rpm for 3 min. After washing with PBS, CD-2NH₂ (1 mg/mL) was added and then stirred for another 12 h to complete the complex formation, which was then centrifuged at

8000 rpm followed by washing with PBS to obtain hybrid **1**. ZnPc loaded and FITC labeled MSNP-Ad (**2**) and its CD-2NH₂ complex (**3**) were prepared using a similar procedure.

Scheme S5. Scheme showing preparation of MSNP-Ad. APTES: (3-aminopropyl) triethoxy silane, CTAB: cetyltrimethylammonium bromide, DCC: dicyclohexylcarbodiimide, NHS: *N*-hydroxysuccinimide.

Folic acid (FA) conjugation: Folic acid was conjugated on MSNP by EDC/NHS chemistry. ZnPC loaded MSNP with CD-2NH₂ capping was suspended in aqueous solution containing FA, EDC and NHS. After reaction for 24h, the nanoparticles were collected by centrifugation and washed by distilled water.

Cell Culture: HeLa, MCF-7 and MB231 cells were cultured in DMEM containing 10% FBS and nonessential amino acids (0.1 mM). The culture was maintained at 37 °C in a humidified atmosphere containing 5% CO₂.

MTT Cytotoxicity Assay: The photodynamic cytotoxicity effect was evaluated by MTT assay. The cells under study were seeded into 96-well plate at a density of 1×10^4 cells/well in complete DMEM medium and grown for 24 h. Then, ZnPc-loaded MSNP-AD capped with CD-2NH₂ or CD-FA was added into each well at different concentration. After exposure to the nanoparticles for 24 h, the medium in each well was removed, and the cells were washed by PBS twice to remove free nanoparticles in the medium. Fresh complete DMEM medium (100 μ L) was added into each well. After 4h incubation, the cells were exposed to 675 nm laser light for certain period of time. The MTT assay was then conducted after another 48h

incubation by changing the medium in each well into medium containing MTT (100 μ L, 0.5 mg mL⁻¹). After incubation for 4 h, the medium was removed and DMSO (100 μ L) was added into each well. The plate was gently shaken for 15 min and absorbance intensity at 565 nm was recorded using a microplate reader (infinite 200 PRO, Tecan). The relative cell viability related to control wells that were only treated with medium was calculated by $[A]_{\text{test}}/[A]_{\text{control}}$, where $[A]_{\text{test}}$ and $[A]_{\text{control}}$ are the average absorbance of the test and control samples, respectively.

Fluorescence Microscopy Images: The cells were seeded in 35 mm plastic-bottomed μ -dishes and grown in complete DMEM medium for 24 h. Then, HeLa cells were treated with ZnPC-loaded and FITC labeled MSNP-Ad capped with CD-2NH₂ or CD-FA (3, 20 μ g mL⁻¹). After 24 h incubation, the medium was removed, and the cells were washed three times with PBS buffer (pH 7.4) and fixed with 4.0% formaldehyde at room temperature for 15 min. After removing 4.0% formaldehyde, the cells were washed with PBS buffer (pH 7.4) three times before capturing the fluorescence images using a fluorescence microscope.

Flow Cytometry Data Collection: The cells were seeded in 6-well plates and grown in complete DMEM medium for 24 h. The cells were treated with ZnPC-loaded and FITC labeled MSNP-Ad capped with CD-2NH₂ or CD-FA (20 μ g mL⁻¹). After 24 h incubation, the cells were washed by PBS three times and harvested by trypsin treatment. Then, the cells were centrifuged and washed by PBS twice before conducting flow cytometry detection on BD FACS Calibur Flow Cytometer.

Cell TEM: HeLa cells were first treated with hybrid 1 for 24 h and then the medium was removed. The cells were washed three times before adding new medium. After 4 h incubation, the cells were exposed to 674 nm light irradiation for 20 min. Then, the cells were further cultured for 24 h. Afterward, the cells were washed thrice with PBS then fixed with 2.5% glutaraldehyde (SPI, USA) at 4 °C overnight. Hereafter, cells were washed with 0.1% phosphate buffer and post-fixed with 1% osmium tetroxide (SPI, USA) for 1 h at room temperature. Fixed cell pellets were further washed and performed dehydration in the increasing grades of ethanol (25%-100%) and pure acetone. Infiltration in SPI-PonTM-Araldite® (SPI,

USA) resin started at room temperature overnight and followed with pure resin embedding at 60 °C for 72 h. Blocks were sectioned by an ultramicrotome (Leica Ultracut UCT) and ultrathin sections were stained with lead citrate. Grids were viewed under a Philips EM 208 (write your viewed TEM machine name) 100 kV transmission electron microscope.

3.3 Results and Discussion

MSNP preparation and characterization

Amino functionalized MSNPs (MSNP-NH₂) were prepared by co-condensation method in a basic solution in order to endow uniform distribution of the functional groups both inside mesopores and on the nanoparticle surface. Then, the prepared MSNP-NH₂ was further reacted with Ad-COOH to yield MSNP-Ad which was first characterized by powder X-ray diffraction and N₂ adsorption/desorption measurement.

Figure 5.1. a) X-ray diffraction pattern, b) N₂ adsorption/desorption isotherms, and BJH pore size distribution (inset) of MSNP-Ad.

Clear (100) peak at $2\theta = 2.14$ in the Powder X-ray diffraction (PXRD) pattern of MSNP-Ad demonstrates hexagonal packing of MCM-41 type mesoporous structures (**Figure 5.1a**). A typical type-IV isotherm (**Figure 5.1b**), indicative of mesoporous structures, was observed with a BET (Brunauer-Emmett-Teller)

surface area of $852.58\text{ m}^2\text{ g}^{-1}$. The mesoporous size distribution of BJH (Barrett-Joyner-Halenda) desorption sharply focuses at 2.7 nm.

Figure 5.2a shows the HR-TEM image of MSNP-Ad with clear mesoporous structures. Inset is the photograph of MSNP-Ad under visible light. After loading with ZnPc, the nanoparticles exhibit blue color due to the presence of ZnPc molecules inside the mesopores. And in the TEM image of ZnPC loaded MSNP-Ad, the mesoporous structure was completely blurred due to the ZnPc loading inside the mesopores. While preparing ZnPc loaded MSNP-Ad in order to keep ZnPc mostly in its monomeric state inside the mesopores, an optimized solution of ZnPc with 0.1 mg mL^{-1} was used.

Figure 5.2.HR-TEM images of a) MSNP-Ad and b) MSNP-Ad loaded with ZnPc and capped with CD-2NH₂, hybrid 1. Insets are the photographs of corresponding nanoparticles under visible light. c) UV-Vis absorption spectra of ZnPc loaded MANP-Ad complexed with CD-2NH₂ (1) in PBS buffer pH 7.2 (red curve), and ZnPc alone in DMF (black curve, $1 \times 10^{-6}\text{ M}$). d) Emission spectrum of hybrid 1 in PBS buffer at pH 7.2.

The dispersion of ZnPc in MSNP-Ad was monitored through UV-Vis spectrum. ZnPc in DMF has a characteristic absorption maximum at 669 nm (**Figure 5.2c**, black curve). Loading of ZnPc inside the mesopores of MSNP-Ad exhibits a slight bathochromic shift of absorption maximum to 672 nm with a

shoulder at 615 nm that is attributed to dimers (**Figure 5.2c**, green curve). The Ad spacers prevent the aggregation of ZnPc inside the mesopores in PBS buffer to a great extent. The amount of ZnPc loaded in MSNP-Ad was determined spectrophotometrically as 0.6 wt%, by sonicating ZnPc loaded MSNP-Ad in DMF in order to extract out all ZnPc molecules.

Figure 5.3. a) FE-SEM image of hybrid **1**, b) FT-IR spectra of MSNP-NH₂ (black curve), MSNP-Ad (red curve) and hybrid **1** (blue curve), and c) Values of ζ potential for MSNP-NH₂, ZnPc loaded MSNP-Ad, and hybrid **1**.

The hybrid **1** possesses uniform and spherical shape which was observed under FE-SEM and no irregular chunks was found(**Figure 5.3a**), which indicates that the ZnPc molecules were indeed loaded inside the mesopores and no aggregation was formed. The linkage of Ad molecules through amide bond was confirmed by the FT-IR spectra. The peak at 1520 cm⁻¹ due to primary amine disappeared after the reaction with Ad-COOH. The presence of ZnPC was further certified by the FT-IR spectra, since fingerprints of ZnPc molecule at 1388 cm⁻¹ and 667 cm⁻¹ were found in the spectra of ZnPc loaded MSNP-Ad. (**Figure 5.3b**). The hybrid **1** was prepared by making use of the strong inclusion complexation between Ad and β -CD. MSNP-Ad loaded with ZnPc was stirred with CD-2NH₂ in aqueous solution for 12 h to obtain hybrid **1**. Then, hybrid **1** was collected by centrifugation and washed with PBS buffer twice to remove excess CD-2NH₂. Zeta-potential measurement indicates that, after the Ad functionalization, the nanoparticle surface charge was decreased from 24 mV to 6.7 mV owing to the replacement of amino groups with AD units (**Figure 5.3c**). A successful complex formation within **1** between Ad and CD-

2NH₂ was evidenced from enhanced surface charge to 23.3 mV. The HR-TEM of 1 (**Figure 5.2b**) provides evidence for ZnPc loading, as the mesopores occupied by ZnPc cannot be clearly seen when compared to that of MSNP-Ad. Inset of Figure 5.2b shows the photograph of 1 under visible light. As compared with ZnPc loaded MSNP-Ad, hybrid 1 in PBS buffer exhibits a bathochromic shift of the absorption maximum to 675 nm (**Figure 5.2c**, red curve, $\Delta\lambda = 3$ nm), a characteristic monomer signature of ZnPc inside the mesopores. The emission spectrum of 1 (0.5 mg mL⁻¹) was recorded in PBS buffer at pH 7.2. When excited at 610 nm, 1 shows an emission of ZnPc with a maximum at 680 nm (**Figure 5.2d**). The absorption and fluorescence properties of 1 provide clear evidence for the monomeric existence of ZnPc inside the nano-channels with adamantane spacers.

Singlet oxygen (¹O₂) generation

Figure 5.4. UV-Vis absorption changes (between 340-420 nm) showing photobleaching of the ¹O₂ molecular probe, ADMA upon irradiation of hybrid 1 with 675 nm laser light for 40 min. Inset shows enlarged area corresponding to absorption of monomeric ZnPc loaded in 1.

We then investigated the photoinduced ¹O₂ generation capability of 1 through an indirect chemical method using ADMA (9,10-anthracenediyl-bis(methylene)dimalonic acid) as the ¹O₂ trap. ADMA reacts with ¹O₂ to produce corresponding endoperoxide that can be followed by absorption changes of ADMA.²⁶² To hybrid 1 (0.5 mg mL⁻¹) in PBS buffer at pH 7.2, ADMA (6 × 10⁻⁵ M) was added with stirring. A typical absorption spectrum of the mixture shows two signature regions (**Figure 5.4**), i.e., absorption corresponding to ADMA is at 350-415 nm region and ZnPc from 650 to 690 nm. The absorption of

ADMA was decreased continuously; as the mixture was irradiated using 675 nm light for 40 min. Photobleaching of ADMA confirms efficient generation of 1O_2 from ZnPc inside mesopores. The effective 1O_2 generation of 1 in aqueous conditions provides a proof of concept for our strategy of preventing ZnPc from aggregation inside the nanochannels of MSNPs with the adamantane spacers.

Cellular uptake of hybrid 1

Figure 5.5. a) Fluorescence microscopy images of HeLa cancer cell lines incubated with 2 and 4, respectively. b) Flow cytometric analysis of HeLa cell lines incubated with and without 2 and 3.

To prove that the introduction of CD-2NH₂ leads to enhanced cellular internalization of hybrid 1, we carried out parallel in vitro experiments using FITC (fluorescein isocyanate) labeled MSNPs. ZnPc loaded and FITC labeled MSNP-Ad (2) and its CD-2NH₂ complex (3) were prepared accordingly for detailed investigations. Hybrids 2 and 3 containing both ZnPc and FITC dyes with different excitation wavelengths can be easily traced in vitro through green (FITC) and red (ZnPc) excitation channels. We incubated HeLa cells with 2 and 3, respectively. Figure 5.5 shows the images of HeLa cell lines incubated with either 2 (**Figure 5.5a-d**) or 3 (**Figure 5.5e-f**) for 24 h before observation.

Intracellular localization and accumulation experiments conducted using 2 and 3 under the same conditions provide unambiguous evidence for enhanced internalization of 3 that is capped with CD-2NH₂. As seen from row 1 of figure 5.5a, much less fluorescence intensity was observed for both green and red channels, indicating no or low cellular uptake of 2. While under the same conditions, significantly fluorescent signals from both channels (row 2 of **Figure 5.5a**) demonstrate high cellular uptake of hybrid 3. Merged image shows no leakage of ZnPc from the mesopores of hybrid 3, since the green (FITC labeled nanoparticles) fluorescence and red (ZnPc) fluorescence are completely overlapped. In addition, we studied the effect of the CD-2NH₂ complexation in the cell uptake of 2 and 3 using flow cytometry by monitoring the fluorescence intensity of FITC. The flow cytometric analysis in figure 5.5b provides further proof for the enhanced uptake of hybrid 3, since the intracellular fluorescence intensity with 3 is much more significant as compared that with 2. Thus, the enhancement in cellular uptake via the CD-2NH₂ complexation offers high nanoparticle concentration inside the cell lines, capable of leading to apparently high photodynamic toxicity.

Photocytotoxicity study in vitro

Figure 5.6. Schematic diagram showing the PDT experimental setup for light irradiation.

In order to examine the PDT efficiency of hybrid 1, MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazoliumbromide) viability assay on HeLa cancer cell lines incubated with 1 was performed. The in vitro photodynamic toxicity of hybrid 1 was investigated by using a monochromatic light with a power of 2.5 mWcm⁻². HeLa cells were first incubated with hybrid 1 for 24 h, and then washed with PBS

buffer twice to remove residual nanoparticles that were not consumed by cells. Light irradiation experiments were carried out using an in-house developed experimental setup (**Figure 5.6**).

Figure 5.7. MTT viability assay of HeLa cells incubated with 1 for 4 h. Cell viability is expressed as percentage of untreated cells. The concentration refers to the amount of 1 used for the incubation. Green bars indicate control experiments using cells treated with 1 in the dark. Red and blue bars refer to cells treated with 1 under irradiation for 30 and 60 minutes, respectively.

After 48 h incubation followed by irradiation, the cell viability was examined by MTT. Control experiments were also conducted by keeping the samples in the dark. Figure 5.7 shows the results of the MTT viability assay using different amount of 1. Even at the maximum possible concentration of 1 in the dark, relatively low cytotoxicity (green bars) of hybrid 1 was observed. Efficient photodynamic cytotoxicity was observed in the cases of irradiating the samples ($100 \mu\text{g mL}^{-1}$) for 30 and 60 min. Thus, ZnPc photosensitizer loaded hybrid 1 clearly exhibits effective $^1\text{O}_2$ generation, showing obvious cytotoxicity dependence on irradiation time and concentration.

Figure 5.8. Apoptosis/death cell analysis by flowcytometry. From left to right: HeLa cells without any treatment, HeLa treated with hybrid 1 for 24h and kept in dark, and HeLa cells treated with hybrid 1 and exposed to 674nm irradiation for 20min.

To further prove photocytotoxicity induced cell apoptosis and thus cell death, Annexin V/Propidium iodide (PI) dual staining was performed on HeLa cells and statistic analysis by flowcytometry was completed. As shown in figure 5.8, it is reasonable that the HeLa cells without any treatment have low fluorescence intensity of both Annexin V and PI, which means few cells were apoptotic or dead. Then, the cells treated hybrid 1 but kept in dark conditions also mainly concentrated at the same region with low fluorescence intensity of both staining dyes. However, after treated with hybrid 1 and followed light irradiation, there populations of cells present. The normal cells account for 58.67% have lower fluorescence intensity and locate in the same region with the previous two cells. And the population taking 26.43% with high fluorescence intensity of both Annexin V and PI can be attributed to dead cells which were stained with both dyes, while the 12.87% cells only stained with Annexin V was apoptotic cells but still alive.

Figure 5.9. Fluorescence image of HeLa cells after apoptosis/death cells staining; a) control, b) treated with hybrid 1 for 24h and kept in dark, and c) treated with hybrid 1 for 24h and exposed to 674nm irradiation for 20min; 1) bright field channel, 2) DAPI channel, 3) Annexin IV channel, 4) PI channel, 5) overlay of 2 and 4, and 6) overlay of 2 and 3.

The apoptosis/death cells analysis by flowcytometry was confirmed by fluorescence images. The same groups of HeLa cells were stained and observed by fluorescence microscopy. Few green dot and red dots were observed in figure 5.9a-b, suggesting almost no cells were apoptotic or dead. The photocytotoxicity induced cell apoptosis were found in figure 5.9 c, as considerable green dots indicating apoptotic cells and red dots indicating dead cells were observed. By merging image figure 5.9 c-3) and c-4), the individual green dots (**Figure 5.9d-6**) represent the apoptotic cells while yellow dots indicat dead cells which experienced the apoptosis and death process.

Figure 5.10. a-f) Fluorescence microscopic images of HeLa cancer cells treated with 1. Images a-c) before light irradiation, and d-f) after irradiation showing completely damaged cells.

Simultaneously, HeLa cells that were incubated with 1 were imaged with fluorescence microscope. The intracellular localization and accumulation of 1 were visualized by monitoring red fluorescence from loaded ZnPc. Figure 5.10 shows fluorescent microscopic images of HeLa cells ($60\times$ oil objective) treated with 1 for 24 h. Figures 4.8a-c are the images of cell lines kept in the dark. Overlay image (**Figure 5.10c**) indicates the enhanced internalization of hybrid 1.

Figure 5.11. Ultrastructural analysis using TEM. HeLa cells were treated with hybrid 1 for 24 h and exposed to 674nm light irradiation before processed for TEM analysis. (a) A dying cell showed nanoparticles uptake and cellular vacuolization (arrows). (b) Cells experienced PDT displayed the appearance of an apoptotic cell with apoptotic bodies and membrane blebbing. (c) HeLa cells treated with hybrid 1 showed nanoparticles uptake. Some vacuoles evidently contained nanoparticles (arrows). (D, E,

F) High magnification images of HeLa cells treated with hybrid 1, containing vacuoles within the cytoplasm (arrows). Images were acquired at original magnifications of 5,000 – 40,000.

Figures 5.8d-f are the images of cell lines incubated with 1 for 48 h followed by exposure to light at 675 nm for 30 min. After irradiation, the morphology of HeLa cells was significantly changed, which demonstrates that hybrid 1 brings heavy photodynamic damage to cells for eventual apoptosis. When the cells without the treatment of 1 were irradiated by light under the same conditions, no detectable damage was observed. It was confirmed that photodynamic action of hybrid 1 under the defined conditions can be employed for efficient cancer treatment. In-depth study on the photodynamic therapy induced cell damage was performed by cell TEM. HeLa cells treated with hybrid 1 for 24h and then exposed to light irradiation for 30mins. First of all, we clearly found the presence of MSNP inside the cells as indicated by the arrows in figure 5.11c-f, which proves that the hybrid 1 was indeed uptaken by HeLa cells through endocytosis. Furthermore, the clear damage induced by photodynamic therapy was observed (**Figure 5.11a-b**), as apoptotic bodies and membrane blebbing were clearly observed. Thus, we can reach to the conclusion that the designed system based on MSNP can effectively encapsulate ZnPc molecule and prevent it from aggregation, in which way we achieved high efficacy of its singlet oxygen generation property. Then, successfully endocytosis of the hybrid 1 can effectively induce cell apoptosis and cancer cells killing by PDT process which are fully proved by various techniques.

Figure 5.12. a) UV/vis spectrum of ZnPC loaded MSNP+CD-FA; b) Absorption changes showing singlet oxygen ($^1\text{O}_2$) generation from ZnPc loaded MSNP complex with folic acid attached β -CD.

In order to expand the application of the PDT system, we further modified the hybrid 1 with folic acid molecule as a targeting ligand, noted as hybrid 4 (ZnPc loaded MSNP-Ad+CD-FA). Hybrid 1 was reacted with folic acid by EDC/NHS chemistry and washed by PBS three times to remove excessive folic acid molecules. Then, the hybrid 4 was characterized by UV/vis spectrum, extra peak at 280nm due to folic acid molecules was found, while the peak at 674nm due to monometric ZnPc still presented in the spectrum. The singlet oxygen generation of hybrid 4 was confirmed by using ADMA as the $^1\text{O}_2$ probe. And the same efficacy of singlet oxygen generation was found for hybrid 4 (**Figure 5.12b**).

Figure 5.13. Fluorescence microscopy image of various cells treated with hybrid 5 (ZnPC loaded MSNP(FITC)-Ad+CD-FA); Channels from left to the right: bright field, DAPI, FITC, ZnPc, overlay of DAPI, FITC and ZnPc.

To trace the location of MSNP, FITC labeled MSNP was used for the fluorescence image observation. And the ZnPC loaded MSNP(FITC)-Ad+CD-FA complex was noted as hybrid 5. The targeting effect of hybrid 5 was tested with three kinds of cells. HeLa cells and MB231 breast cancer cells have high folic acid receptor expression were used as positive control, while MCF-7 cells with few folic acid receptor expression was used as negative control. Much more green fluorescence dots indicating the presence of hybrid 5 were found in both HeLa and MB231 cells when comparing to MCF-7 cells where few green dots were observed, suggesting effective targeting cancer cells with high folic acid expression. Then, HeLa and MCF-7 were co-cultured and treated with hybrid 5. It is interesting to find that the hybrid 5 selectively bind and enter to HeLa cells, as the MCF-7 colony has few nanoparticle uptake.

Figure 5.14. a) flowcytometry result of cells without any treatment (red) and treated with hybrid 5 for 24h (green peak); b) MTT assay of photocytotoxicity test of cells treated with hybrid 5 and kept in dark or exposed to 674nm light irradiation for 20min.

We continued our study by flowcytometry analysis of various cells treated with hybrid 5, much higher FITC fluorescence intensity was found for both HeLa and MB231 cells, which is indicated by the green peak by comparing to the control cells indicated by read peaks in each cell's histogram (**Figure 5.14a**). However, MCF-7 has lower FITC fluorescence intensity after treated with hybrid 5, which means much less nanoparticles uptake. The current results prove that the hybrid 5 selectively enter cancer cells through folic-acid mediated endocytosis, which certify the targeting effect of hybrid 5. Then, photocytotoxicity test confirmed the selectively endocytosis. From Figure 5.14b, MCF-7 cells still have relatively higher cell viability after the PDT process, while both HeLa and MB231 was effectively killed by PDT process under the same treatment conditions.

3.4 Conclusion

In conclusion, we have developed and demonstrated a novel strategy for preventing aggregation of ZnPc inside the nanochannels of silica nanoparticles. ZnPc existing in its monomeric state inside the pores shows efficient singlet oxygen generation in aqueous environments, leading to significant photodynamic damage. Owing to the complexation of CD-2NH₂, the ZnPc-loaded hybrid exhibits remarkable stability in aqueous solution and low cytotoxicity. Through further linkage of targeting liand on the CD-2NH₂ caps, target PDT process was achieved. This novel hybrid is expected to serve as a platform for developing next generation photodynamic therapeutics for specific cancer treatment.

Chapter 4. Graphene Oxide Wrapping on Squaraine

Entrapped MSNPs for Bio-imaging

4.1 Research scope and motivation

Graphene oxide (GO) is an oxidized derivative of graphene which has a 2D carbon honeycomb lattice.²⁶³ The presence of functional groups and sp^2 -conjugated bonds on the surface of GO provides strong hydrophilic characteristics and feasibility of π - π interactions with aromatic molecules, providing excellent capacities for further functionalizations on the GO surface.^{264, 265} Such chemical property has endowed numerous possibilities of various applications. In particular, owing to the biocompatibility and low cytotoxicity of GO,²⁶⁶ GO-biomolecule conjugate and GO-nanoparticle hybrids were recently used for biological applications such as DNA detection,^{267, 268} cell imaging,^{269, 270} and drug loading/delivery.²⁷¹⁻²⁷⁴ On the other hand, MSNPs have been studied extensively as drug delivery platforms due to their versatility and low cytotoxic features, as we discussed in detail in previous chapters. Advantages of MSNPs, such as large internal surface area and uniform mesopores, have attracted tremendous attention towards their applications for controlled drug delivery, gene delivery, as well as protein or enzyme delivery. Accordingly, significant research effort has centered on developing multifunctional mesoporous nanoparticles for bio-labeling and therapeutic uses, which is also the major target of this research work. Thus, we anticipated that the combination of excellent properties from GO and MSNPs may generate a new research avenue for biological applications.

Squaraine dyes are a class of extensively studied zwitter ionic dyes which exhibit excellent photophysical properties in near-IR (near infrared) region.²⁷⁵⁻²⁷⁷ Squaraines were proved to be suitable for many biological applications, such as metal ion sensors,^{278, 279} NIR fluorescent labels,^{280, 281} two-photon absorbers,²⁸¹ and detection and estimation of thiol containing amino acids.^{282, 283} Recently, these dyes were

proved as second generation of photosensitizers for photodynamic therapy (PDT).²⁸⁴ However, there are two major challenges in using these dyes for biological applications, especially for PDT and imaging applications. One is how to prevent easy formation of dye aggregates in aqueous environments and the other one is how to protect their photophysical characteristics from nucleophilic attack to the squaryl rings. Although several approaches previously reported, such as (i) supramolecular encapsulation using macrocyclic host molecules (e.g. β -cyclodextrin^{285, 286} and serum albumins²⁸⁷) and (ii) the formation of squaraine derived rotaxanes,²⁸⁸⁻²⁹⁰ can enhance their stability and prevent chromophore interactions, the real bottle neck in maintaining the photophysical characteristics of these dyes in aqueous environments remains unresolved. In this work, we aimed to utilize MSNP to accomplish a design of a suitable vessel that can completely prevent squaraine dyes from aggregation and nucleophilic attack.

Scheme 6.1. Schematic illustration for preparation of GO-MSNP1. Stage 1: loading of **1** in APTES modified MSNP vessel, stage 2: electrostatic wrapping of GO sheets on the surface of the nanoparticles loaded with **1**.²⁹¹ (Copyright 2012 American Chemical Society)

Herein, a novel strategy was demonstrated for the fabrication of GO enwrapped MSNPs as protective vessel for squaraine dyes. A model squaraine dye, bis(2,4,6-trihydroxyphenyl)squaraine dye (**1**), was chosen for this work. The present strategy involves loading of **1** into MSNPs followed by the encapsulation of GO on the surface of MSNPs via electrostatic interaction (**Scheme 6.1**). The electrostatic interaction of GO with metal nanoparticles was utilized for the preparation of fluorescent hybrids and anode materials, which procedure was utilized by other report. For example, S. Yang et al. recently reported the fabrication of GO encapsulated metal oxide nanoparticles as high performance anode materials through the co-assembly between GO sheets and positively charged oxide nanoparticles.²⁹² Although it is challenging, it was envisioned that the immobilization of GO sheets onto MSNPs might completely cover the mesopores with GO coating. This approach opens up a novel methodology for the protection of dyes such as squaraines from self-aggregation and nucleophilic attack. We expected that, the resulting hybrid GO-MSNPs has remarkable stability and complete resistance towards nucleophiles such as cysteine (Cys) and glutathione (GSH) in aqueous environments, which could allow the biological imaging application of the chemical sensitive dye.

4.2. Materials and Methods

Materials and instrument: All starting materials were obtained from commercial suppliers and used as received. Electronic absorption spectra were recorded on a Shimadzu UV-3101 PC NIR scanning spectrophotometer and the emission spectra were measured on a Shimadzu RF5301 spectrofluorimeter. TEM images were captured by JEOL 2010 TEM at 200kV. SEM images were captured by Jeol-JSM-7600F. X-ray diffraction pattern was collected by PANalytical X'Pert powder X-ray diffraction. Zeta potential was measured by Mavorn Nanosizer. Raman spectra of the particles on cleaned silicon substrate were measured with a Raman microscope (LabRAM HR, Horiba Yvon). The excitation wavelength of the irradiating light was 633 nm (He-Ne laser, Melles Griot) and signals were collected by using a $\times 100$ objective lens. Surface area and pore size distribution characterization were performed by ASAP 2020

Mircomeritics. FT-IR spectra were collected using Perkin Elmer FT-IR system (Spectrum GX). Fluorescence microscopic images were captured using Nikon D-Eclipse C1 Microscope.

Fluorescence quantum yield was determined in spectroscopic grade methanol and millipore water using optically matching solutions of Cresyl violet ($\Phi_r = 0.52$) as standard. The quantum yield is calculated using equation 1.

$$\Phi_f = \Phi_r (A_r F_s / A_s F_r) (\eta_s^2 / \eta_r^2) \dots (1)$$

where, A_s and A_r are the absorbance of the sample and reference solutions, respectively at the same excitation wavelength, F_s and F_r are the corresponding relative integrated fluorescence intensities and η is the refractive index of the solvent.

Preparation of graphene oxide (GO): GO was prepared by a modified Hammer's method.²⁹³ Graphite (0.5 g) was suspended in concentrated H_2SO_4 (15 mL) under ice bath. $KMnO_4$ (1.5g) was added gradually with stirring. The mixture was stirred at 50 °C for 3h, followed by the addition of distilled water (35mL). After stirring for 15min, distilled water (150 mL) was added to terminate the reaction. Then, H_2O_2 (10mL, 30%) was added and the solution color changed into bright yellow. The mixture was then washed by HCl solution (250mL, 10%) and distilled H_2O . In order to exfoliate the oxidized graphite, the product was treated with an ultrasonic probe at 250W for 1h and centrifuged at 8000rpm for 30min. The supernatant was collected. The process was repeated three times and the supernatant (600mL) was collected as GO stock suspension. GO stock suspension (100mL) was mixed with NaOH (5g) and sodium monochloro acetate (5g), and the mixture was sonicated for 2h to convert the OH groups into COOH groups. The mixture was then washed by distilled water repeatedly until well dispersed GO-COOH suspension was obtained. The solvent of GO-COOH suspension was changed into DMF by centrifugation and then the solution was treated with ultrasonic probe at 250W for 1h in order to break GO sheets into a proper size for the nanoparticle wrapping application.

Synthesis of MSNP-NH₂: Amine containing mesoporous silica nanoparticles (MSNP-NH₂) was prepared by co-condensation method. CTAB (cetyltrimethylammonium bromide, 500mg) was dissolved in distilled H₂O (250mL) followed by the addition of NaOH aqueous solution (1.75mL, 2M). The mixture was heated up to 80 °C under vigorous stirring. When the temperature was stabilized, TEOS (tetraethyl orthosilicate, 2.5mL) was slowly added into the mixture solution. After 15min, APTES (amino propyl triethoxysilane, 0.5ml) was added. The solution was stirred at 80 °C for another 2h. Then, nanoparticles were collected by centrifugation at 8000 rpm for 10 min. The product was washed with MeOH and distilled H₂O thoroughly. Surfactant (CTAB) was removed by suspending the nanoparticles in MeOH (50mL) containing condensed HCl (3mL, 37%). The mixture was refluxed at 80 °C for 24h. The product MSNP-NH₂ was collected by centrifugation at 8000 rpm for 15 min and thoroughly washed by MeOH and distilled H₂O. Collected nanoparticles were dried in vacuo at 80 °C for 24h.

MTT Photocytotoxicity assay: The cytotoxicity test was performed by MTT assay. HeLa cells were seeded into 96-well plate (1×10^4 cells/well) in DMEM (Dulbecco's Modified Eagle's Medium) cell culture medium and grown for 24 h. Then, the medium was removed and DMEM medium (100 μ L/well) containing GO-MSNP1 at different concentrations was added to each well. After 24 h incubation, the medium was changed into DMEM medium (100 μ L/well) containing MTT (0.5mg/mL) followed by incubation for another 4 h. DMSO (100 μ L) was then used to replace the previous cell culture medium. The plate was agitated gently for 15 min, before the absorbance intensity at 560 nm was recorded by a micro plate reader (Infinite 200 PRO, Tecan). Cell viability related to control wells without any nanoparticles was calculated by $[A]_{\text{test}}/[A]_{\text{control}}$, where $[A]_{\text{test}}$ and $[A]_{\text{control}}$ are the average absorbance of the test and control samples (n=8), respectively.

Fluorescence Microscopy Images: HeLa cells were seeded in 35 mm plastic-bottom μ -dishes and grown in complete DMEM medium for 24 h before treated with GO-MSNP1 (50 μ g mL⁻¹). After 24 h incubation, the medium was removed and HeLa cells were washed with PBS (Phosphate buffered saline) buffer

(pH=7.4) for three times to remove any free nanoparticles in the culture dish. Formaldehyde (4%, 1mL) was used to fix the cells at room temperature for 15 min. After removing formaldehyde and washing with PBS buffer (pH=7.4) for three times, the cell nucleus was stained with 4,6-diamidino-2-phenylindole (DAPI, 1 $\mu\text{g/mL}$) for 15 min. Then, the cells were washed with PBS buffer (pH=7.4) for three times before capturing images by using fluorescence microscope (100 $\times$ oil objective).

4.3. Results and Discussion

Materials Preparation and Characterization

GO was synthesized from natural graphite powder by a modified Hammer's method and then the -OH groups were further converted to GO-COOH (hereafter referred as GO sheets) based on previously reported procedures.²⁹⁴

Figure 6.1. a) TEM image of multilayer GO sheets, b) selected area electron diffraction pattern (SAED) of multilayer GO sheets, and c) FT-IR spectrum of GO sheets. (Copyright 2012 American Chemical

Society)

The readily synthesized GO sheets were found dispersible in aqueous and organic solvents such as DMF. The freshly prepared GO sheets were characterized using TEM (**Figure 6.1a**) and FT-IR (**Figure 6.1c**). The TEM image shows multilayer GO sheets and the electron diffraction of a selected area (**Figure 6.1b**) reveals patterns correspond to arranged multilayer GO sheets. Moreover, the presence of -COOH groups on GO sheets is evident from the peaks corresponding to C=O stretching vibration at 1726 cm^{-1} and C-O stretching vibration at 1060 cm^{-1} .

Figure 6.2. UV/Vis (black curve) and emission (red curve) spectra of **1** in DMF/water (1:1) at pH 6.5.

Squaraine dye was prepared by the condensation of 1,2-dihydroxycyclobutenedione or squaric acid with two-fold equivalents of phloroglucinol in acetic acid. The dye was purified by repeated crystallization, obtained in 58% yield and characterized by NMR, FT-IR, and mass spectrometry.²⁹⁵ The UV/Vis spectrum of **1** in DMF/water (1:1 v/v) solution at pH 6.5 shows two bands at 569 ($\epsilon = 1.3 \times 10^5$) and 502 nm, respectively (**Figure 6.2**). The emission spectrum of **1** exhibits a maximum at 596 nm ($\lambda_{\text{ex}} = 580$ nm).

Figure 6.3. a) Powder XRD pattern of MSNP-NH₂; b) BET isotherms and BJH pore size distribution (inset) of MSNP-NH₂.

APTES (aminopropyltriethoxysilane) modified MSNPs (MSNP-NH₂) were prepared via co-condensation method. The formed nanoparticles were first characterized by powder X-ray diffraction (XRD) and N₂ adsorption/desorption. XRD analysis reveals a hexagonal arrangement of the mesopores, and a distance of d_{100} space is calculated to be 4.16 nm by Bragg's law from the (100) position at $2\theta = 2.11^\circ$ (Figure 6.3a). Surface area and porous nature of MSNP-NH₂ were characterized by N₂ adsorption/desorption measurements. A typical type-IV isotherm (Figure 6.3b), indicative of mesoporous structures, was observed with a BET (Brunauer-Emmett-Teller) surface area of 731.4 m²/g. The mesoporous size distribution of BJH (Barrett-Joyner-Halenda) desorption sharply focuses on 2.71 nm. The thickness of mesoporous walls can be estimated as 1.45 nm from the difference between internal average pore size obtained from BJH method and external average pore size calculated from XRD.

Dye loading and graphene oxide wrapping

In the preparation of GO sheet encapsulated MSNPs (GO-MSNP), MSNP-NH₂ (5mg) was suspended in DMF (1 mL) by bath sonication. GO suspension in DMF (4mL) was added to the MSNP-NH₂ solution and the mixture was stirred at room temperature for 24 h in order to complete the electrostatic wrapping process. The product GO-MSNP was then collected by centrifugation at 8000 rpm and thoroughly washed

with DMF in order to remove any suspended GO residues. TEM analysis of GO-MSNPs (Figure 6.4b) reveals a successful wrapping of MSNPs with ultrathin GO sheets.

Figure 6.4. High resolution transmission electron microscopy (HR-TEM) images of (a) APTES modified MSNPs (MSNPs-NH₂), (b) GO wrapped MSNPs without dye loading (GO-MSNPs), (c) GO wrapped squaraine loaded MSNPs (GO-MSNPs1). The arrows indicate monolayer GO sheets, and (d) the selected area electron diffraction pattern (SAED) of (c), showing ultrathin layer of GO sheets.

Inspired from the above mentioned process, we extended the wrapping process with **1** loaded MSNPs. Well dispersed MSNP-NH₂ suspension in DMF (1 mL) was mixed with **1** (3×10^{-4} M) in DMF/water (1:1, 0.5 mL) at pH 6.5 in a glass vial (5 mL). The mixture was stirred at room temperature for 24 h in order to load **1** into mesopores of the nanoparticles. The suspension was then centrifuged twice at 8000 rpm and the supernatant was removed accordingly to obtain **1** loaded MSNPs (MSNPs1) (c. a. 2.5 wt% of **1** with respect to MSNPs). GO suspension (1 mL in DMF) was added to MSNPs1 followed by a gentle bath sonication for 2 min. The mixture was stirred for 24 h to ensure the completion of the wrapping process. The product was then centrifuged at 8000 rpm for 30 min and washed by DMF five times to remove any free dyes and GO residues. After centrifugation, GO-MSNPs1 was well dispersed in water at pH 6.5. HR-

TEM image (**Figure 6.4c**) of GO-MSN1 provides clear evidence for the encapsulation of the dye and the complete wrapping of GO sheets around MSNPs. Apparently, when compared with the TEM image of GO-MSN1, loading of **1** in the mesopores of MSNPs masks the visibility of the mesoporous structures of GO-MSN1. A selected area electron diffraction analysis (SAED) of GO sheets reveals the signature of ultrathin layer of GO sheets coating over the nanoparticles (**Figure 6.4d**). This is evident from the observation that, for ultrathin layer of GO sheets, the intensity of (0-110) spots appears to be more intense relative to that of the (1-210) spots.²⁹⁶⁻²⁹⁸

Figure 6.5. Typical FE-SEM images (GB mode) of (a) MSNP-NH₂ and (b) GO-MSN1. (c) table showing variation of zeta (ζ) potential values of GO sheets, MSNP-NH₂, MSNP1, and GO-MSN1 in distilled water. (d) Raman spectra of MSNP-NH₂ and GO-MSN1. (Copyright 2012 American Chemical Society)

The FE-SEM images recorded in gentle beam mode (GB-high) of MSNP-NH₂ (**Figure 6.5a**) and GO-MSN1 (**Figure 6.5b**) reveal morphologies of GO sheet coated MSNPs loaded with **1**. The surface charges of GO sheets, MSNP-NH₂, MSNP1 and GO-MSN1 were examined using zeta (ζ) potential measurements in distilled water in order to obtain more insights for GO sheet-covered nanoparticles. Summary of ζ -potential measurements is shown in Figure 6.5 c and d. GO sheets show a negative ζ -

potential of -55.3 ± 6.2 mV, indicating a negative charge over the surface of GO sheets. MSNP-NH₂ gives a positive ζ -potential of 28 ± 4.2 mV due to the amine groups on the surface. Thus, strong electrostatic interaction between oppositely charged GO sheets and MSNP-NH₂ nanoparticles leads to the GO-MSNP formation. Dye **1** loaded nanoparticles (MSNP1) exhibit a ζ -potential of 22 ± 4.6 mV, which favours GO wrapping by electrostatic interactions. GO-MSNP1 shows a negative ζ -potential of -39.9 ± 6.5 mV, further proving the surface coverage of MSNPs by negatively charged GO sheets. Figure 6.5 d shows the typical Raman spectra (excitation wavelength 633 nm) of GO-MSNP1 and MSNP-NH₂. MSNP-NH₂ exhibits peaks at 976 cm^{-1} due to silica. GO-MSNP1 shows combination fingerprints of GO,²⁹⁹ including G band at 1593 cm^{-1} and D band at 1332 cm^{-1} respectively and MSNP peaks. So, Raman spectra of GO-MSNP1 also support the wrapping of GO on MSNPs.

Figure 6.6. (a) UV/Vis and (b) emission spectra of GO-MSNP1 (black curve) and GO-MSNP (red curve) in aqueous solution (1mg mL^{-1}) at pH 6.5. The excitation wavelength is 580 nm. (Copyright 2012 American Chemical Society)

The UV/Vis spectrum (**Figure 6.6a**, red curve) of GO-MSNP does not show an obvious absorption under the range of 400-700 nm in aqueous solution at pH 6.5. Interestingly, the UV/Vis spectrum of GO-MSNP1 (**Figure 6.6a**, black curve) presents a characteristic absorption maximum at 595 nm under the same conditions, corresponding to the absorption of loaded dye **1**. When compared to the characteristic absorption maximum of free **1**, ~25 nm of red-shift is observed in case of GO-MSNP1 in aqueous conditions. The observed red-shift in the absorption maximum of GO-MSNP1 can be attributed to the

encapsulation of **1** within the mesopockets of MSNPs, a phenomenon which is similar to the complex formation of the dye with serum albumin.²⁸⁷

The emission spectra of GO-MSN1 and GO-MSN2 were recorded in aqueous solution at pH 6.5 (1mg mL⁻¹). When excited at 580 nm, GO-MSN1 exhibits an emission with the maximum at 607 nm, with a fluorescence quantum yield $\Phi_f = 0.21$ (**Figure 6.6b**, black curve), whereas the nanoparticles without the dye (GO-MSN2) were found to be non-emissive (**Figure 6.6b**, red curve). The photophysical properties of GO-MSN1 provide a proof of concept for the encapsulation of **1** inside the mesopores of MSNPs. The non-interfering absorption and emission bands of GO-MSN1 in aqueous conditions make these novel nanoparticles an excellent candidate for biological applications.

Squaraine dye protection inside MSNP

Figure 6.7. Changes in the (a) absorption and (b) emission of GO-MSN1 and squaraine dye (**1**) upon addition of 3 mM Cys or GSH in buffer aqueous solution at pH 7 at 24 °C; c) UV/Vis absorption changes of **1** (6×10^{-6}) in DMF/water (1:1) with the addition of Cysteine (3×10^{-3} M) during 0-40 min. (Copyright 2012 American Chemical Society)

A detailed investigation on the chemical stability of the GO-MSN1 was carried out by treating the nanoparticles with thiol containing bio-relevant molecules such as Cys and GSH. Separate sets of experiments were conducted on both free **1** (6×10^{-6} M) and freshly prepared GO-MSN1 (1mg mL⁻¹). The corresponding time dependent absorption and emission changes are shown in Figure 6.7a and b, respectively. Extremely high stability of the absorbance intensity and fluorescence emission were observed for GO-MSN1, while free **1** was quickly quenched by Cys and GSH. The reaction of either Cys

or GSH with **1** results in complete bleaching of absorption and emission at 570 and 596 nm respectively, whereas the photophysical characteristics (absorption maximum at 595 nm and emission maximum at 607 nm) of GO-MSNP1 were found unaffected in the presence of either Cys or GSH for a substantial period of time. Furthermore, GO-MSNP1 is very stable and the dyes loaded do not leak out from the mesopores since the nanoparticles can maintain all the photophysical characteristics in aqueous solutions for months. Thus, these observed results stand as a proof of concept for the protection of **1** within the mesopores in the presence of the GO sheet wrapping.

Biocompatibility and living cell image

Figure 6.8. MTT viability assay of HeLa cells that were incubated with MSNP and GO-MSNP1 for 24 h. Cell viability is expressed as percentage of untreated cells. The concentration refers to the amount of MSNP and GO-MSNP1 used for the incubation. (Copyright 2012 American Chemical Society)

In order to examine the potential of GO-MSNP1 for biological applications, the inherent cytotoxicity of GO-MSNP1 was evaluated using the MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) viability assay with HeLa cancer cell lines for an incubation period of 24 h. As shown in Figure 6.8, GO-MSNP1 has no considerable cytotoxicity at low to moderate concentrations.

Figure 6.9.(a-d) Epifluorescence microscopic images of HeLa cancer cells labelled with GO-MSNPs. The blue fluorescence is from the nuclear counter stain (DAPI, $1\mu\text{g mL}^{-1}$) and the red fluorescence is from GO-MSNPs. (a) Image of nuclei, (b) fluorescence of GO-MSNPs (dark field), (c) overlay of panels a and b, and d) overlay of Figure c with the phase contrast image. (Copyright 2012 American Chemical Society)

We next employed GO-MSNPs for both cell surface and subcellular labelling in order to demonstrate their bioimaging ability. In vitro fluorescence imaging studies were performed with HeLa cancer cell lines. Figure 6.9 shows the fluorescent microscopic images of HeLa cells (100 x oil objective) treated with GO-MSNPs for 24 h. The blue fluorescence from the cell nucleus stained by DAPI (4,6-diamidino-2-phenylindole) can be observed in Figure 6.9a. The red fluorescence of GO-MSNPs is distributed inside the cytoplasm as well as on the cell surface. Similarly, the overlay (**Figure 6.9c**) of fluorescence images a and b indicates the accumulation of GO-MSNPs in the cytoplasm of cell lines. The overlay (**Figure 6.9d**) with the phase contrast image reveals both intracellular and cell surface labelling of GO-MSNPs. Thus, the in vitro investigations demonstrate a great potential of the novel hybrid for bioimaging applications.

4.4. Conclusion

In conclusion, we have developed a novel strategy for the protection of squaraine dyes by coating GO sheet onto functional mesoporous silica nanoparticles, leading to a florescent hybrid for bioimaging applications. The ultrathin layer of GO sheets coating over dye loaded nanoparticles prevent the dye from aggregation and nucleophilic attack, successfully reserving the property of the dye. The hybrid exhibits a remarkable stability in aqueous solution and relatively low cytotoxicity. Therefore, it is expected that the novel hybrid can serve as a platform for a variety of biological applications including specific in vitro and in vivo cellular imaging.

Chapter 5. Summary and Outlook

5.1 Summary of the Research Work

As a conventional material which has been invented for about 20 years ever since early 1990, mesoporous silica nanoparticle has been widely developed in a variety of applications, including heterogeneous catalyst development for green chemistry, controlled drug/gene delivery, protein or enzyme delivery, biological imaging and so forth. Among them, the application in biological system has attracted significant attentions due to the lower cytotoxicity of silica to the physiological environment, as well as ease of the chemical modifications. Moreover, the tunable mesoporous structure properties, such as pore size, surface area, and pore volume, also provide lots of possibilities for biological applications. A detailed introduction to mesoporous silica is presented in the first chapter, from which a clear route of the developing of mesoporous silica in biological system can be found. Therefore, even though a traditional mesoporous material which has been extensively studied, mesoporous silica still exhibited great potentials in developing its applications due to its versatile physicochemical properties, as well as material structural variations. As a matter of fact, this special feature of mesoporous silica inspired the author of this thesis to perform research work on the biological application of mesoporous silica nanoparticles, focusing on the MCM-41 type nanoparticles, noted as MSNP in the thesis. Driven by strong curiosity and academic interest, the author explored several possible applications of MSNP in biological system. This research work was successfully accomplished with satisfying experimental results and most of the work has been published on good journals.

In this research work, the author focused on the development of functional mesoporous silica nanoparticle (MSNP) with specific novelty, in order to realize intended biological applications. Comprehensive materials characterizations on the materials structure and chemical modifications were carried out. For example, FESEM and TEM were used to observe the MSNP and determine its real size. BET and BJH

methods were employed to find out the porosity information of the formed materials. The hexagonal packing of mesoporous structure was confirmed by powder X-ray diffraction. FT-IR and NMR were utilized in the tracking of chemical modifications on the MSNP surface. The hydrodynamic size and dispersity of the novel functional MSNP were characterized by DLS, which provided guidance in the preparation of functional MSNP for biological applications. In addition to materials characterizations, extensive biological investigations have been carried as well. Several typical cancer cell lines, like HeLa, MCF-7, and MB231, were chosen as the model for the in vitro study on the drug/gene co-delivery, photodynamic therapy, and bio-imaging. Furthermore, in vivo study was completed with zebra fish which is usually chosen as the primary study model for in vivo drug screening. In-depth biological study has been conducted. For instance, confocal laser scanning microscopy was used to observe the intracellular uptake of MSNP into cancer cells, as well as anti-cancer drug release inside the cells. Western blot assay of Bcl-2 protein was performed to find out the gene delivery efficacy. Apoptosis staining and related flowcytometry study was also completed in this work. Based on all the study, research conclusions of scientific interest and significance were reached. And several complete research projects were accomplished, which was further summarized briefly in the following paragraphs.

In the first work, a functional MSNP covalently conjugated with disulfide bond linked amino groups was design and prepared. The positive surface charge due to protonation of amino groups was utilized to bind negatively charged ssDNA chains at physiological pH. The attached ssDNA chains effectively capped the pores inside which anti-cancer drug Dox was loaded. The formed nano-hybrid material is capable of delivering both anti-cancer drug and ssDNA into cancer cells through endocytosis. It was found that the surface coating of ssDNA could enhance the cellular uptake, which might be attributed to the biocompatibility of ssDNA to the biological system. The controlled release mechanism was based on the redox-responsive disulfide bond. Upon addition of redox reagent, such as DTT or GSH, the disulfide bond could be cleaved, leading to dissociation of the surface coated ssDNA chains, as well as opening of the mesopores from which the delivered drug Dox was released. By utilizing the natural redox potential

given by higher concentration of GSH inside cancer cells, this system could effectively release both drugs and ssDNA once entered into cancer cells. For this point, by using fluorescent dye Cy5 conjugated ssDNA, the internalization of ssDNA was observed by fluorescence microscopy, meanwhile the uptake of nanoparticles, as well as Dox release inside the cancer cells were observed. Enhanced cancer killing effect was presented by the co-delivery system comparing to that of free Dox. Thus, this work clearly indicated the feasibility of controlled drug/gene co-delivery. However, the previous work could not fulfill the requirement of controlled release of drug or gene individually, which function can help to study the chemotherapy and gene therapy deeply. Besides, the ssDNA used in the previous did not exhibit any therapeutic function. Therefore, an improved co-delivery system was developed in order to satisfy such requirement. And siRNA against anti-apoptotic protein Bcl-2 was chosen for the gene therapy purpose. A new co-delivery system based on MSNP was developed. The MSNP was first functionalized with disulfide bond linked adamantane. By forming stable complex between the adamantane and amino group modified β -cyclodextrin, the drug molecules can be capped inside the pores without pre-releasing. The positively charged amino groups modified on the CD rings can effectively bind negatively charged siRNA for the gene delivery purpose. In this work, a synergetic therapeutic concept was employed in the cancer therapy by combining the chemotherapy and gene therapy, in order to overcome the drug resistance in the conventional cancer therapy by anti-cancer drugs. Through western blot assay, it was found that the co-delivery system could effectively deliver siRNA into HeLa cells and resulted in Bcl-2 protein silence which in return led to the increase of sensitivity to the anti-cancer drug Dox. In the co-delivery experiment, enhanced chemotherapy effect was observed in HeLa cells tested by MTT assay. Then, the co-delivery was applied for in vivo study with zebra fish. The siRNA against green fluorescence protein was delivered and obviously decrease of GFP expression was observed. Furthermore, the co-delivery system could also effectively suppress the proliferation of liver tumor planted in zebra fish. Thus, this novel delivery system showed promising future application for cancer treatment by synergetic therapy.

Even though MSNP was reported to be biological friendly, still considerable concerns are raised regarding its safety. Based on the fact of dosage-dependent cytotoxicity effect reported before. A novel structure of MSNP, hollow mesoporous silica nanoparticles, was synthesized. The hollow structure could help enhance the drug and gene delivery capability by about 100%, which were proved in the work. Moreover, as target delivery is another main obstacle in cancer treatment. To address this issue, the co-delivery system was coated with folic acid conjugated PEI polymer. Lower molecular weight PEI (1.8kDa) was used to minimize the possible toxic effect brought by PEI. The strong positive charge given by PEI chains could effectively bind siRNA, the hollow structure could hold much more anti-cancer drug Dox comparing with previous works. Then, the folic acid conjugated on the PEI helped the hybrid nanoparticles to selectively enter into cancer cells through folic acid receptor mediated endocytosis. Target delivery of both drug and siRNA was found by using three kinds of cancer cells, HeLa, MB231 (high folic acid receptor expression) and MCF-7 (lower folic acid receptor expression). Much more effective therapeutic effect was observed by the co-delivery system due to good target delivery and higher delivery capacity, through which much less silica was used when similar therapy effect was achieved. This work made progress in the decreasing of inorganic materials dosage for nanoparticle based delivery system, which is helpful for the biological system as well as human healthy in future clinic application.

In the third chapter, a photodynamic system by using second generation photosensitizer ZnPc was presented. Although ZnPc is proved to be a highly efficiency photosensitizer, the extremely lower solubility in aqueous solution and lose of photodynamic property after aggregation limits its application in biological system for PDT application. This work designed a novel functional MSNP with hydrophobic groups Ad modified both inside and outside the nanochannels. After loading ZnPc inside DMF, it was exciting to find a monodispersed state of ZnPc loaded inside MSNP-Ad in PBS buffer solution from the UV-vis spectra, which suggested the nano-channels could prevent the ZnPc from aggregation. Then, singlet oxygen generation capability was confirmed. By complexing with CD, the hybrid showed enhanced stability and dispersity in aqueous solution, leading to increase in the nanoparticles' uptake in

cancer cells. Effective cancer cells killing by PDT induced apoptosis was confirmed by MTT assay and apoptosis/necrosis staining. From cell TEM, presence of the hybrid inside cell cytoplasm was found, and typical apoptosis evidence was also presented. By further reaction with folic acid on the CD ring, the formed hybrid showed selectivity in cancer treatment, providing target PDT in future application. This work managed to resolve the aggregation problem of the second generation photosensitizer ZnPc and helped to improve its efficiency in aqueous environment, which allows this system for future application in cancer treatment.

At last, a bio-imaging tags based on MSNP and GO nano-hybrid was developed. Squaraine dyes are rather promising in bioimaging due to its emission in NIR region. However, this dye is sensitive to thiol attacks, such as GSH and cysteine which are presented in biological system. In this work, the author creatively combined GO sheet and MSNP to invent a new hybrid. The MSNP was initially functionalized with amino groups and squaraine dye was further loaded inside. Through charge interaction, the GO could closely coat on MSNP surface, which separate the dye from physical contact with external chemicals, in which manner the chemical attacks by thiol containing chemicals were successfully avoid. Extremely stable absorbance and emission were observed comparing to previous strategy used to preserve the dye. MTT results indicated less toxicity of the hybrid material. In vitro imaging was performed in HeLa cell lines and clear NIR fluorescence color was found from inside the HeLa cells where presented high concentration of GSH. Basically, the last two projects managed to use traditional material MSNP to resolve practical problems in biological applications, which enlightened the future development direction of this material.

5.2 Outlook and Future Development

On the basis of the current research work described in the thesis, plus previous studies on the biological application of MSNP, an obvious progress can be concluded. Even though extensive studies on MSNP have been conducted by many research groups, still tremendous possibility can be expected in virtue of the

versatile properties of MSNP, as well as creative minds employed in scientific research. Such concept will continue to guide the future research of the author of this thesis.

As for the biological application of nanoparticles, a new concept, “theranostic” has attracted researchers’ interests, integrating multiple functions into one system. Novel nanoparticles capable of both performing biological detection for disease diagnosis and carrying out drug/gene delivery purpose to complete the therapeutic process, are designed and fabricated for theranostic applications. As an excellent candidate nanoparticle in biological application, MSNP also comes into researchers’ vision for such application. A new route for preparation of theranostic MSNP is to combine MSNP with other functional nanoparticles, such as gold or iron oxide, to take advantage of their special properties for therapeutic or diagnostic purpose. For example, surface plasma resonance (SPR) of gold nanoparticles is utilized in MSNP for detection and synergetic photo-thermal therapy. Magnetic resonance of iron oxide nanoparticles is utilized in the MSNP/Fe₃O₄ hybrid nanoparticles for magnetic resonance image, while accomplish drug delivery by MSNP. And there are some excellent review articles describing such research subject.^{300, 301} But the theranostic application of MSNP is still in the initial stage, there are many research topics that may result in high performance nano-materials in bio-system. So, the combination of MSNP with other novel nano-materials allows for many new applications in biological system, which can provide practical significance in scientific research.

The most concerned issue for application of MSNP into biological system should be its safety. As known to all, non-toxic to healthy tissue and excretion of carrier materials are critical factors in designing new materials for biological application, especially for those with potentials in clinic applications. Using inorganic materials in biological application always faces the issue of degradation. Although very recently there have been reports regarding the degradation of MSNP,³⁰²⁻³⁰⁴ but still no strong evidence showed the complete excretion of MSNP. Therefore, there is a new trend for developing biodegradable mesoporous silica which can be completely “consumed” by the living body. Such requirement presents new research

topics for mesoporous silica nanoparticles in order to extend its biological application into clinic research, which would be a great progress for this research field.

Again, the fundamental reason for the extremely wide application of MSNP should be attributed to its unique mesoporous structure which dominates the versatility of MSNP, verifying the classic relation of “structure” and “application” in materials science. The root of any novelty in MSNP can be traced back to the invention of the brand new structure at early 1990. Therefore, any research in materials science should stick to the rule and innovation in the novel structure might contribute a totally new world, like the case of MSNP in the materials science.

In summary, MSNP is an interesting research subject which produces tremendous publications in the past two decades. However, the story is not ending. Continuous efforts of researchers are being input into this research field and a large number of publications on MSNP are reports every year, suggesting the vitality and great potentials of this traditional material.

REFERENCE

1. C. T. Kresge, M. E. Leonowicz, W. J. Roth, J. C. Vartuli and J. S. Beck, *Nature*, 1992, **359**, 710-712.
2. T. Yanagisawa, T. Shimizu, K. Kuroda and C. Kato, *Bulletin of the Chemical Society of Japan*, 1990, **63**, 988-992.
3. D. Y. Zhao, Q. S. Huo, J. L. Feng, B. F. Chmelka and G. D. Stucky, *Journal of the American Chemical Society*, 1998, **120**, 6024-6036.
4. D. Y. Zhao, J. L. Feng, Q. S. Huo, N. Melosh, G. H. Fredrickson, B. F. Chmelka and G. D. Stucky, *Science*, 1998, **279**, 548-552.
5. S. A. Bagshaw, E. Prouzet and T. J. Pinnavaia, *Science*, 1995, **269**, 1242-1244.
6. R. Ryoo, J. M. Kim, C. H. Ko and C. H. Shin, *Journal of Physical Chemistry*, 1996, **100**, 17718-17721.
7. Y. Han and J. Y. Ying, *Angewandte Chemie-International Edition*, 2005, **44**, 288-292.
8. Q. S. Huo, D. I. Margolese, U. Ciesla, P. Y. Feng, T. E. Gier, P. Sieger, R. Leon, P. M. Petroff, F. Schuth and G. D. Stucky, *Nature*, 1994, **368**, 317-321.
9. K. K. Coti, M. E. Belowich, M. Liong, M. W. Ambrogio, Y. A. Lau, H. A. Khatib, J. I. Zink, N. M. Khashab and J. F. Stoddart, *Nanoscale*, 2009, **1**, 16-39.
10. M. Vallet-Regi, A. Ramila, R. P. del Real and J. Perez-Pariente, *Chemistry of Materials*, 2001, **13**, 308-311.
11. T. W. Kim, Slowing, II, P. W. Chung and V. S. Y. Lin, *Acs Nano*, 2011, **5**, 360-366.
12. X. Ji, K. T. Lee, M. Monjaube and L. F. Nazar, *Chemical Communications*, 2008, **0**, 4288-4290.
13. Q. Cai, Z.-S. Luo, W.-Q. Pang, Y.-W. Fan, X.-H. Chen and F.-Z. Cui, *Chemistry of Materials*, 2001, **13**, 258-263.
14. F. Y. Qu, G. S. Zhu, S. Y. Huang, S. G. Li, J. Y. Sun, D. L. Zhang and S. L. Qiu, *Microporous and Mesoporous Materials*, 2006, **92**, 1-9.
15. A. Nieto, F. Balas, M. Colilla, M. Manzano and M. Vallet-Regi, *Microporous and Mesoporous Materials*, 2008, **116**, 4-13.
16. M. Colilla, F. Balas, M. Manzano and M. Vallet-Regi, *Solid State Sciences*, 2008, **10**, 408-415.
17. M. Manzano, V. Aina, C. O. Arean, F. Balas, V. Cauda, M. Colilla, M. R. Delgado and M. Vallet-Regi, *Chemical Engineering Journal*, 2008, **137**, 30-37.
18. K. Patel, S. Angelos, W. R. Dichtel, A. Coskun, Y. W. Yang, J. I. Zink and J. F. Stoddart, *Journal of the American Chemical Society*, 2008, **130**, 2382-2383.

-
19. S. W. Song, K. Hidajat and S. Kawi, *Chemical Communications*, 2007, 4396-4398.
 20. S. Budi Hartono, S. Qiao, K. Jack, B. P. Ladewig, Z. Hao and G. Lu, *Langmuir*, 2009, **25**, 6413-6424.
 21. L. Zhang, S. Z. Qiao, Y. G. Jin, L. N. Cheng, Z. F. Yan and G. Q. Lu, *Advanced Functional Materials*, 2008, **18**, 3834-3842.
 22. E. B. Cho, D. O. Volkov and I. Sokolov, *Advanced Functional Materials*, 2011, **21**, 3129-3135.
 23. Y. D. Chiang, H. Y. Lian, S. Y. Leo, S. G. Wang, Y. Yamauchi and K. C. W. Wu, *Journal of Physical Chemistry C*, 2011, **115**, 13158-13165.
 24. T. W. Kim, P. W. Chung and V. S. Y. Lin, *Chemistry of Materials*, 2010, **22**, 5093-5104.
 25. J. Gu, J. Liu, Y. Li, W. Zhao and J. Shi, *Langmuir*, 2012, **29**, 403-410.
 26. H. Hata, S. Saeki, T. Kimura, Y. Sugahara and K. Kuroda, *Chemistry of Materials*, 1999, **11**, 1110-1119.
 27. M. Kruk and L. Cao, *Langmuir*, 2007, **23**, 7247-7254.
 28. J. Fan, C. Z. Yu, J. Lei, Q. Zhang, T. C. Li, B. Tu, W. Z. Zhou and D. Y. Zhao, *Journal of the American Chemical Society*, 2005, **127**, 10794-10795.
 29. L. H. Chen, G. S. Zhu, D. L. Zhang, H. Zhao, M. Y. Guo, S. B. Wei and S. L. Qiu, *Journal of Materials Chemistry*, 2009, **19**, 2013-2017.
 30. Q. Gao, Y. Xu, D. Wu, Y. H. Sun and X. A. Li, *Journal of Physical Chemistry C*, 2009, **113**, 12753-12758.
 31. Y. J. Han, G. D. Stucky and A. Butler, *Journal of the American Chemical Society*, 1999, **121**, 9897-9898.
 32. Q. Gan, D. W. Dai, Y. Yuan, J. C. Qian, S. Sha, J. L. Shi and C. S. Liu, *Biomedical Microdevices*, 2012, **14**, 259-270.
 33. F. Balas, M. Manzano, M. Colilla and M. Vallet-Regi, *Acta Biomaterialia*, 2008, **4**, 514-522.
 34. B. Menaa, C. Torres, M. Herrero, V. Rives, A. R. W. Gilbert and D. K. Eggers, *Biophysical Journal*, 2008, **95**, L51-L53.
 35. N. Mizoshita, T. Tani and S. Inagaki, *Chemical Society Reviews*, 2011, **40**, 789-800.
 36. J. Kecht, A. Schlossbauer and T. Bein, *Chemistry of Materials*, 2008, **20**, 7207-7214.
 37. F. Hoffmann, M. Cornelius, J. Morell and M. Froba, *Angewandte Chemie-International Edition*, 2006, **45**, 3216-3251.
 38. F. Hoffmann, M. Cornelius, J. Morell and M. Froba, *Journal of Nanoscience and Nanotechnology*, 2006, **6**, 265-288.
 39. S. L. Burkett, S. D. Sims and S. Mann, *Chemical Communications*, 1996, 1367-1368.

-
40. B. G. Trewyn, Slowing, II, S. Giri, H. T. Chen and V. S. Y. Lin, *Accounts of Chemical Research*, 2007, **40**, 846-853.
 41. T. D. Nguyen, K. C. F. Leung, M. Liong, Y. Liu, J. F. Stoddart and J. I. Zink, *Advanced Functional Materials*, 2007, **17**, 2101-2110.
 42. T. D. Nguyen, Y. Liu, S. Saha, K. C. F. Leung, J. F. Stoddart and J. I. Zink, *Journal of the American Chemical Society*, 2007, **129**, 626-634.
 43. M. S. Kim and J. Y. Chang, *Journal of Materials Chemistry*, 2011, **21**, 8766-8771.
 44. F. de Juan and E. Ruiz-Hitzky, *Advanced Materials*, 2000, **12**, 430-+.
 45. C. M. Yang, H. A. Lin, B. Zibrowius, B. Spliethoff, F. Schueth, S. C. Liou, M. W. Chu and C. H. Chen, *Chemistry of Materials*, 2007, **19**, 3205-3211.
 46. T. Seckin and A. Gultek, *Journal of Applied Polymer Science*, 2003, **90**, 3905-3911.
 47. A. Calvo, M. Joselevich, G. Soler-Illia and F. J. Williams, *Microporous and Mesoporous Materials*, 2009, **121**, 67-72.
 48. E. Ruiz-Hernández, A. Baeza and M. Vallet-Regí *ACS Nano*, 2011, **5**, 1259-1266.
 49. D. I. Fried, A. Schlossbauer and T. Bein, *Microporous and Mesoporous Materials*, 2012, **147**, 5-9.
 50. X. X. He, Y. X. Zhao, D. G. He, K. M. Wang, F. Z. Xu and J. L. Tang, *Langmuir*, 2012, **28**, 12909-12915.
 51. O. De Los Cobos, B. Fousseret, M. Lejeune, F. Rossignol, M. Dutreilh-Colas, C. Carrion, C. Boissiere, F. Ribot, C. Sanchez, X. Cattoen, M. W. C. Man and J. O. Durand, *Chemistry of Materials*, 2012, **24**, 4337-4342.
 52. J. Liu, A. Stace-Naughton, X. Jiang and C. J. Brinker, *Journal of the American Chemical Society*, 2009, **131**, 1354-1355.
 53. J. Liu, A. Stace-Naughton and C. J. Brinker, *Chemical Communications*, 2009, **0**, 5100-5102.
 54. X. J. Wan, G. Y. Zhang and S. Y. Liu, *Macromolecular Rapid Communications*, 2011, **32**, 1082-1089.
 55. S. B. Hartono, W. Gu, F. Kleitz, J. Liu, L. He, A. P. J. Middelberg, C. Yu, G. Q. Lu and S. Z. Qiao, *ACS Nano*, 2012, **6**, 2104-2117.
 56. Y. F. Zhu, W. J. Meng, H. Gao and N. Hanagata, *Journal of Physical Chemistry C*, 2011, **115**, 13630-13636.
 57. X. T. Shi, Y. J. Wang, L. Ren, N. R. Zhao, Y. H. Gong and D. A. Wang, *Acta Biomaterialia*, 2009, **5**, 1697-1707.
 58. D. G. He, X. X. He, K. M. Wang, J. Cao and Y. X. Zhao, *Advanced Functional Materials*, 2012, **22**, 4704-4710.

-
59. D. G. He, X. X. He, K. M. Wang, M. A. Chen, J. Cao and Y. X. Zhao, *Journal of Materials Chemistry*, 2012, **22**, 14715-14721.
60. Slowing, II, P. A. Kapke, S. Goodison and V. S. Y. Lin, *Abstracts of Papers of the American Chemical Society*, 2008, **235**.
61. C. P. Tsai, C. Y. Chen, Y. Hung, F. H. Chang and C. Y. Mou, *Journal of Materials Chemistry*, 2009, **19**, 5737-5743.
62. J. Mendez, A. Monteagudo and K. Griebenow, *Bioconjugate Chemistry*, 2012, **23**, 698-704.
63. L. F. Chen, J. C. Hu and R. Richards, *Journal of the American Chemical Society*, 2009, **131**, 914-+.
64. L. Li, W. J. Ji and C. T. Au, *Progress in Chemistry*, 2009, **21**, 1742-1749.
65. J. J. Zhang and J. J. Zhu, *Science in China Series B-Chemistry*, 2009, **52**, 815-820.
66. L. F. Chen, J. C. Hu, Z. W. Qi, Y. J. Fang and R. Richards, *Industrial & Engineering Chemistry Research*, 2011, **50**, 13642-13649.
67. D. M. Hess, R. R. Naik, C. Rinaldi, M. M. Tomczak and J. J. Watkins, *Chemistry of Materials*, 2009, **21**, 2125-2129.
68. S. Gandhi, S. Venkatesh, U. Sharma, N. R. Jagannathan, S. Sethuraman and U. M. Krishnan, *Journal of Materials Chemistry*, 2011, **21**, 15698-15707.
69. N. Z. Knezevic, Slowing, II and V. S. Y. Lin, *Chempluschem*, 2012, **77**, 48-55.
70. K. Mori, Y. Kondo, S. Morimoto and H. Yamashita, *Journal of Physical Chemistry C*, 2008, **112**, 397-404.
71. P. Wang, Y. H. Zhu, X. L. Yang, C. Z. Li and H. L. Du, *Acta Materialia*, 2008, **56**, 1144-1150.
72. Y. X. Li, Y. H. Zhu, C. Y. Li, X. L. Yang and C. Z. Li, *Materials Letters*, 2009, **63**, 1068-1070.
73. J. Pan, D. Wan and J. L. Gong, *Chemical Communications*, 2011, **47**, 3442-3444.
74. L. I. Burova, D. I. Petukhov, A. A. Eliseev, A. V. Lukashin and Y. D. Tretyakov, *Superlattices and Microstructures*, 2006, **39**, 257-266.
75. J. El Haskouri, L. Dallali, L. Fernandez, N. Garro, S. Jaziri, J. Latorre, C. Guillem, A. Beltran, D. Beltran and P. Amoros, *Physica E-Low-Dimensional Systems & Nanostructures*, 2009, **42**, 25-31.
76. F. Muharnmad, M. Y. Guo, W. X. Qi, F. X. Sun, A. F. Wang, Y. J. Guo and G. S. Zhu, *Journal of the American Chemical Society*, 2011, **133**, 8778-8781.
77. P. B. Lihitkar, S. Violet, M. Shirolkar, J. Singh, O. N. Srivastava, R. H. Naik and S. K. Kulkarni, *Materials Chemistry and Physics*, 2012, **133**, 850-856.
78. K. R. Martin, *Journal of Nutrition Health & Aging*, 2007, **11**, 94-98.

-
79. J. S. Chang, K. L. B. Chang, D. F. Hwang and Z. L. Kong, *Environmental Science & Technology*, 2007, **41**, 2064-2068.
80. A. J. Di Pasqua, K. K. Sharma, Y. L. Shi, B. B. Toms, W. Ouellette, J. C. Dabrowiak and T. Asefa, *Journal of Inorganic Biochemistry*, 2008, **102**, 1416-1423.
81. D.-M. Huang, T.-H. Chung, Y. Hung, F. Lu, S.-H. Wu, C.-Y. Mou, M. Yao and Y.-C. Chen, *Toxicology and Applied Pharmacology*, 2008, **231**, 208-215.
82. J. Yu, H. Zhao, L. Ye, H. Yang, S. Ku, N. Yang and N. Xiao, *Journal of Materials Chemistry*, 2009, **19**, 1265-1270.
83. Y. N. Zhao, B. G. Trewyn, Slowing, II and V. S. Y. Lin, *Journal of the American Chemical Society*, 2009, **131**, 8398-+.
84. J. L. Vivero-Escoto, I. I. Slowing and V. S. Y. Lin, *Biomaterials*, 2010, **31**, 1325-1333.
85. M. Fisichella, H. Dabboue, S. Bhattacharyya, M.-L. Saboungi, J.-P. Salvetat, T. Hevor and M. Guerin, *Toxicology in Vitro*, 2009, **23**, 697-703.
86. D. R. Radu, C.-Y. Lai, K. Jeftinija, E. W. Rowe, S. Jeftinija and V. S. Y. Lin, *Journal of the American Chemical Society*, 2004, **126**, 13216-13217.
87. H. Vallhov, S. Gabrielsson, M. Strømme, A. Scheynius and A. E. Garcia-Bennett, *Nano Letters*, 2007, **7**, 3576-3582.
88. D. Napierska, L. C. J. Thomassen, V. Rabolli, D. Lison, L. Gonzalez, M. Kirsch-Volders, J. A. Martens and P. H. Hoet, *Small*, 2009, **5**, 846-853.
89. Y. S. Lin, C. P. Tsai, H. Y. Huang, C. T. Kuo, Y. Hung, D. M. Huang, Y. C. Chen and C. Y. Mou, *Chemistry of Materials*, 2005, **17**, 4570-4573.
90. Q. He, Z. Zhang, Y. Gao, J. Shi and Y. Li, *Small*, 2009, **5**, 2722-2729.
91. J. L. Vivero-Escoto, Slowing, II and V. S. Y. Lin, *Biomaterials*, 2010, **31**, 1325-1333.
92. V. Lebrete, L. Raehm, J. O. Durand, M. Smahhi, M. H. V. Werts, M. Blanchard-Desce, D. Methy-Gonnod and C. Dubernet, *Journal of Sol-Gel Science and Technology*, 2008, **48**, 32-39.
93. D. Lozano, M. Manzano, J. C. Doadrio, A. J. Salinas, M. Vallet-Regí E. Gómez-Barrena and P. Esbrit, *Acta Biomaterialia*, 2010, **6**, 797-803.
94. Q. He, J. Zhang, J. Shi, Z. Zhu, L. Zhang, W. Bu, L. Guo and Y. Chen, *Biomaterials*, 2010, **31**, 1085-1092.
95. Y. F. Zhu, Y. Fang, L. Borchardt and S. Kaskel, *Microporous and Mesoporous Materials*, 2011, **141**, 199-206.
96. S. P. Hudson, R. F. Padera, R. Langer and D. S. Kohane, *Biomaterials*, 2008, **29**, 4045-4055.

-
97. V. Cauda, A. Schlossbauer and T. Bein, *Microporous and Mesoporous Materials*, 2010, **132**, 60-71.
 98. J. S. Souris, C.-H. Lee, S.-H. Cheng, C.-T. Chen, C.-S. Yang, J.-a. A. Ho, C.-Y. Mou and L.-W. Lo, *Biomaterials*, 2010, **31**, 5564-5574.
 99. Slowing, II, B. G. Trewyn, S. Giri and V. S. Y. Lin, *Advanced Functional Materials*, 2007, **17**, 1225-1236.
 100. Y.-L. Zhao, Z. Li, S. Kabehie, Y. Y. Botros, J. F. Stoddart and J. I. Zink, *Journal of the American Chemical Society*, 2010, **132**, 13016-13025.
 101. N. K. Mal, M. Fujiwara and Y. Tanaka, *Nature*, 2003, **421**, 350-353.
 102. C. Park, K. Oh, S. C. Lee and C. Kim, *Angewandte Chemie International Edition*, 2007, **46**, 1455-1457.
 103. R. Liu, Y. Zhang, X. Zhao, A. Agarwal, L. J. Mueller and P. Feng, *Journal of the American Chemical Society*, 2010, **132**, 1500-1501.
 104. X. Wan, D. Wang and S. Liu, *Langmuir*, 2010, **26**, 15574-15579.
 105. Q. Zhang, Y. Tu, H. Tian, Y.-L. Zhao, J. F. Stoddart and H. Ågren, *The Journal of Physical Chemistry B*, 2010, **114**, 6561-6566.
 106. R. Hernandez, H.-R. Tseng, J. W. Wong, J. F. Stoddart and J. I. Zink, *Journal of the American Chemical Society*, 2004, **126**, 3370-3371.
 107. T. D. Nguyen, H.-R. Tseng, P. C. Celestre, A. H. Flood, Y. Liu, J. F. Stoddart and J. I. Zink, *Proceedings of the National Academy of Sciences of the United States of America*, 2005, **102**, 10029-10034.
 108. H. Kim, S. Kim, C. Park, H. Lee, H. J. Park and C. Kim, *Advanced Materials*, 2010, **22**, 4280-4283.
 109. D. P. Ferris, Y.-L. Zhao, N. M. Khashab, H. A. Khatib, J. F. Stoddart and J. I. Zink, *Journal of the American Chemical Society*, 2009, **131**, 1686-1688.
 110. Q. Lin, Q. Huang, C. Li, C. Bao, Z. Liu, F. Li and L. Zhu, *Journal of the American Chemical Society*, 2010, **132**, 10645-10647.
 111. A. Schlossbauer, S. Warncke, P. M. E. Gramlich, J. Kecht, A. Manetto, T. Carell and T. Bein, *Angewandte Chemie International Edition*, 2010, **49**, 4734-4737.
 112. S. Angelos, Y.-W. Yang, K. Patel, J. F. Stoddart and J. I. Zink, *Angewandte Chemie International Edition*, 2008, **47**, 2222-2226.
 113. S. Angelos, N. M. Khashab, Y.-W. Yang, A. Trabolsi, H. A. Khatib, J. F. Stoddart and J. I. Zink, *Journal of the American Chemical Society*, 2009, **131**, 12912-12914.

-
114. S. Angelos, E. Johansson, J. F. Stoddart and J. I. Zink, *Advanced Functional Materials*, 2007, **17**, 2261-2271.
115. Z. Luo, K. Cai, Y. Hu, L. Zhao, P. Liu, L. Duan and W. Yang, *Angewandte Chemie International Edition*, 2011, **50**, 640-643.
116. A. Schlossbauer, J. Kecht and T. Bein, *Angewandte Chemie International Edition*, 2009, **48**, 3092-3095.
117. E. Climent, R. Martínez-Máñez, F. Sancenón, M. D. Marcos, J. Soto, A. Maquieira and P. Amorós, *Angewandte Chemie International Edition*, 2010, **49**, 7281-7283.
118. K. Kataoka and H. Harashima, *Advanced Drug Delivery Reviews*, 2001, **52**, 151-151.
119. C. Kneuer, M. Sameti, E. G. Haltner, T. Schiestel, H. Schirra, H. Schmidt and C. M. Lehr, *International Journal of Pharmaceutics*, 2000, **196**, 257-261.
120. D. J. Bharali, I. Klejbor, E. K. Stachowiak, P. Dutta, I. Roy, N. Kaur, E. J. Bergey, P. N. Prasad and M. K. Stachowiak, *Proceedings of the National Academy of Sciences of the United States of America*, 2005, **102**, 11539-11544.
121. Z. Csogor, M. Nacken, M. Sameti, C. M. Lehr and H. Schmidt, *Materials Science & Engineering C-Biomimetic and Supramolecular Systems*, 2003, **23**, 93-97.
122. X.-x. He, K. Wang, W. Tan, B. Liu, X. Lin, C. He, D. Li, S. Huang and J. Li, *Journal of the American Chemical Society*, 2003, **125**, 7168-7169.
123. M. E. Pearce, H. Q. Mai, N. Lee, S. C. Larsen and A. K. Salem, *Nanotechnology*, 2008, **19**.
124. M. Fujiwara, F. Yamamoto, K. Okamoto, K. Shiokawa and R. Nomura, *Analytical Chemistry*, 2005, **77**, 8138-8145.
125. X. Li, J. Zhang and H. Gu, *Langmuir*, 2011, **28**, 2827-2834.
126. S. M. Solberg and C. C. Landry, *The Journal of Physical Chemistry B*, 2006, **110**, 15261-15268.
127. F. Gao, P. Botella, A. Corma, J. Blesa and L. Dong, *The Journal of Physical Chemistry B*, 2009, **113**, 1796-1804.
128. M. H. Kim, H. K. Na, Y. K. Kim, S. R. Ryoo, H. S. Cho, K. E. Lee, H. Jeon, R. Ryoo and D. H. Min, *Acs Nano*, 2011, **5**, 3568-3576.
129. H. K. Na, M. H. Kim, K. Park, S. R. Ryoo, K. E. Lee, H. Jeon, R. Ryoo, C. Hyeon and D. H. Min, *Small*, 2012, **8**, 1752-1761.
130. K. Hayashi, M. Nakamura, H. Miki, S. Ozaki, M. Abe, T. Matsumoto and K. Ishimura, *Advanced Functional Materials*, 2012, **22**, 3539-3546.
131. K. M. L. Taylor-Pashow, J. D. Rocca and W. Lin, *Nanomaterials*, 2011, **2**, 1-14.
132. D. Arcos and M. Vallet-Regi, *Acta Biomaterialia*, 2010, **6**, 2874-2888.

-
133. M. Vallet-Regi, I. Izquierdo-Barba and M. Colilla, *Philosophical Transactions of the Royal Society a-Mathematical Physical and Engineering Sciences*, 2012, **370**, 1400-1421.
134. C. Ispas, I. Sokolov and S. Andreescu, *Analytical and Bioanalytical Chemistry*, 2009, **393**, 543-554.
135. H. H. P. Yiu, C. H. Botting, N. P. Botting and P. A. Wright, *Physical Chemistry Chemical Physics*, 2001, **3**, 2983-2985.
136. J. Fan, C. Yu, F. Gao, J. Lei, B. Tian, L. Wang, Q. Luo, B. Tu, W. Zhou and D. Zhao, *Angewandte Chemie International Edition*, 2003, **42**, 3146-3150.
137. A. S. M. Chong and X. S. Zhao, *Catalysis Today*, 2004, **93-95**, 293-299.
138. C. Lei, Y. Shin, J. Liu and E. J. Ackerman, *Journal of the American Chemical Society*, 2002, **124**, 11242-11243.
139. B. Chen, C. Lei, Y. Shin and J. Liu, *Biochemical and Biophysical Research Communications*, 2009, **390**, 1177-1181.
140. M. Ma, H. R. Chen, Y. Chen, X. Wang, F. Chen, X. Z. Cui and J. L. Shi, *Biomaterials*, 2012, **33**, 989-998.
141. L. Y. Zhang, T. T. Wang, L. Yang, C. Liu, C. G. Wang, H. Y. Liu, Y. A. Wang and Z. M. Su, *Chemistry-a European Journal*, 2012, **18**, 12512-12521.
142. P. Botella, I. Ortega, M. Quesada, R. F. Madrigal, C. Muniesa, A. Fimia, E. Fernandez and A. Corma, *Dalton Transactions*, 2012, **41**, 9286-9296.
143. H. Y. Tang, S. Shen, J. Guo, B. S. Chang, X. G. Jiang and W. L. Yang, *Journal of Materials Chemistry*, 2012, **22**, 16095-16103.
144. Z. J. Zhang, L. M. Wang, J. Wang, X. M. Jiang, X. H. Li, Z. J. Hu, Y. H. Ji, X. C. Wu and C. Y. Chen, *Advanced Materials*, 2012, **24**, 1418-1423.
145. D. Brevet, M. Gary-Bobo, L. Raehm, S. Richeter, O. Hocine, K. Amro, B. Looock, P. Couleaud, C. Frochot, A. Morere, P. Maillard, M. Garcia and J.-O. Durand, *Chemical Communications*, 2009, **0**, 1475-1477.
146. T. Wang, L. Zhang, Z. Su, C. Wang, Y. Liao and Q. Fu, *ACS Applied Materials & Interfaces*, 2011, **3**, 2479-2486.
147. H.-L. Tu, Y.-S. Lin, H.-Y. Lin, Y. Hung, L.-W. Lo, Y.-F. Chen and C.-Y. Mou, *Advanced Materials*, 2009, **21**, 172-177.
148. J. M. Rosenholm, C. Sahlgren and M. Linden, *Nanoscale*, 2010, **2**, 1870-1883.
149. F. Q. Tang, L. L. Li and D. Chen, *Advanced Materials*, 2012, **24**, 1504-1534.

-
150. Y. N. Zhao, J. L. Vivero-Escoto, Slowing, II, B. C. Trewyn and V. S. Y. Lin, *Expert Opinion on Drug Delivery*, 2010, **7**, 1013-1029.
 151. L. Pasqua, S. Cundari, C. Ceresa and G. Cavaletti, *Current Medicinal Chemistry*, 2009, **16**, 3054-3063.
 152. P. P. Yang, S. L. Gai and J. Lin, *Chemical Society Reviews*, 2012, **41**, 3679-3698.
 153. M. Manzano and M. Vallet-Regi, *Journal of Materials Chemistry*, 2010, **20**, 5593-5604.
 154. A. Popat, S. B. Hartono, F. Stahr, J. Liu, S. Z. Qiao and G. Q. Lu, *Nanoscale*, 2011, **3**, 2801-2818.
 155. D. Luo and W. M. Saltzman, *Nature Biotechnology*, 2000, **18**, 33-37.
 156. Y. W. Won, K. S. Lim and Y. H. Kim, *Journal of Controlled Release*, 2011, **152**, 99-109.
 157. D. J. Gary, N. Puri and Y.-Y. Won, *Journal of Controlled Release*, 2007, **121**, 64-73.
 158. R. G. Crystal, *Science*, 1995, **270**, 404-410.
 159. S. K. Tripathy, H. B. Black, E. Goldwasser and J. M. Leiden, *Nature Medicine*, 1996, **2**, 545-550.
 160. K. Kataoka and H. Harashima, *Advanced Drug Delivery Reviews*, 2001, **52**, 151.
 161. N. Saranya, A. Moorthi, S. Saravanan, M. P. Devi and N. Selvamurugan, *International Journal of Biological Macromolecules*, 2011, **48**, 234-238.
 162. A. Baker, M. Saltik, H. Lehrmann, I. Killisch, V. Mautner, G. Lamm, G. Christofori and M. Cotten, *Gene Therapy*, 1997, **4**, 773-782.
 163. S. M. Zou, P. Erbacher, J. S. Remy and J. P. Behr, *Journal of Gene Medicine*, 2000, **2**, 128-134.
 164. T. Blessing, M. Kurs, R. Holzhauser, R. Kircheis and E. Wagner, *Bioconjugate Chemistry*, 2001, **12**, 529-537.
 165. T. Xia, M. Kovichich, M. Liong, H. Meng, S. Kabehie, S. George, J. I. Zink and A. E. Nel, *ACS Nano*, 2009, **3**, 3273-3286.
 166. H. Meng, M. Liong, T. Xia, Z. Li, Z. Ji, J. I. Zink and A. E. Nel, *ACS Nano*, 2010, **4**, 4539-4550.
 167. S. Bhattacharai, E. Muthuswamy, A. Wani, M. Brichacek, A. Castañeda, S. Brock and D. Oupický, *Pharmaceutical Research*, 2010, **27**, 2556-2568.
 168. A. M. Chen, M. Zhang, D. Wei, D. Stueber, O. Taratula, T. Minko and H. He, *Small*, 2009, **5**, 2673-2677.
 169. F. Torney, B. G. Trewyn, V. S. Y. Lin and K. Wang, *Nature Nanotechnology*, 2007, **2**, 295-300.
 170. X. Ma, K. T. Nguyen, P. Borah, C. Y. Ang and Y. Zhao, *Advanced Healthcare Materials*, 2012, **1**, 690-697.
 171. Y. W. Ebright, Y. Chen, Y. Kim and R. H. Ebright, *Bioconjugate chemistry*, 1996, **7**, 380-384.
 172. W. Jiang, B. Y. S. Kim, J. T. Rutka and W. C. W. Chan, *Nature Nanotechnology*, 2008, **3**, 145-150.

-
173. F. Lu, S.-H. Wu, Y. Hung and C.-Y. Mou, *Small*, 2009, **5**, 1408-1413.
174. E. Climent, R. Martínez-Máñez, F. Sancenón, M. D. Marcos, J. Soto, A. Maquieira and P. Amorós, *Angewandte Chemie*, 2010, **122**, 7439-7441.
175. G. K. Balendiran, R. Dabur and D. Fraser, *Cell Biochemistry and Function*, 2004, **22**, 343-352.
176. A. Russo, J. Carmichael, N. Friedman, W. Degraff, Z. Tochner, E. Glatstein and J. B. Mitchell, *International Journal of Radiation Oncology Biology Physics*, 1986, **12**, 1347-1354.
177. M. M. Janat-Amsbury, J. W. Yockman, M. Lee, S. Kern, D. Y. Furgeson, M. Bikram and S. W. Kim, *Molecular Therapy*, 2004, **9**, 829-836.
178. T. Kishida, H. Asada, Y. Itokawa, K. Yasutomi, M. Shin-ya, S. Gojo, F. Cui, Y. Ueda, H. Yamagishi, J. Imanishi and O. Mazda, *Molecular Therapy*, 2003, **8**, 738-745.
179. A. A. Seyhan, *Human Genetics*, 2011, **130**, 583-605.
180. A. J. Hamilton and D. C. Baulcombe, *Science*, 1999, **286**, 950-952.
181. S. M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber and T. Tuschl, *Nature*, 2001, **411**, 494-498.
182. G. D. Eytan, *Biomedicine & Pharmacotherapy*, 2005, **59**, 90-97.
183. L. S. Jabr-Milane, L. E. van Vlerken, S. Yadav and M. M. Amiji, *Cancer treatment reviews*, 2008, **34**, 592-602.
184. M. Saad, O. B. Garbuzenko and T. Minko, *Nanomedicine*, 2008, **3**, 761-776.
185. H. M. Aliabadi, B. Landry, C. Sun, T. Tang and H. Uludag, *Biomaterials*, 2012, **33**, 2546-2569.
186. A. de Fougerolles, H. P. Vornlocher, J. Maraganore and J. Lieberman, *Nature Reviews Drug Discovery*, 2007, **6**, 443-453.
187. S. Cory and J. M. Adams, *Nature Reviews Cancer*, 2002, **2**, 647-656.
188. M. L. Cleary, S. D. Smith and J. Sklar, *Cell*, 1986, **47**, 19-28.
189. R. Kim, M. Emi, K. Tanabe and T. Toge, *Cancer*, 2004, **101**, 2491-2502.
190. P. T. Straten and M. H. Andersen, *Oncotarget*, 2010, **1**, 239-245.
191. M. Garcia-Lecea, I. Kondrychyn, S. H. Fong, Z. R. Ye and V. Korzh, *Plos One*, 2008, **3**.
192. A. T. Nguyen, A. Emelyanov, C. H. V. Koh, J. M. Spitsbergen, S. Parinov and Z. Y. Gong, *Disease Models & Mechanisms*, 2012, **5**, 63-72.
193. N. D. Lawson and B. M. Weinstein, *Developmental Biology*, 2002, **248**, 307-318.
194. L. M. McCaffrey and I. G. Macara, *Trends in Cell Biology*, 2011, **21**, 727-735.
195. W. C. Cromwell, K. Bystrom and M. R. Eftink, *Journal of Physical Chemistry*, 1985, **89**, 326-332.

-
196. S. Giri, B. G. Trewyn, M. P. Stellmaker and V. S. Y. Lin, *Angewandte Chemie International Edition*, 2005, **44**, 5038-5044.
 197. A. Meister and M. E. Anderson, *Annual Review of Biochemistry*, 1983, **52**, 711-760.
 198. A. Verma, J. M. Simard, J. W. E. Worrall and V. M. Rotello, *Journal of the American Chemical Society*, 2004, **126**, 13987-13991.
 199. E. J. Gabbay, D. Grier, R. E. Fingerle, R. Reimer, R. Levy, S. W. Pearce and W. D. Wilson, *Biochemistry*, 1976, **15**, 2062-2070.
 200. D. Goren, A. T. Horowitz, D. Tzemach, M. Tarshish, S. Zalipsky and A. Gabizon, *Clinical Cancer Research*, 2000, **6**, 1949-1957.
 201. H. L. Wong, R. Bendayan, A. M. Rauth, H. Y. Xue, K. Babakhanian and X. Y. Wu, *Journal of Pharmacology and Experimental Therapeutics*, 2006, **317**, 1372-1381.
 202. T. Minko, P. Kopeckova and J. Kopecek, *Pharmaceutical Research*, 1999, **16**, 986-996.
 203. M. Mimeault and S. K. Batra, *Drug Discovery Today*, 2013, **18**, 128-140.
 204. Y. L. Ma, P. Zhang, F. Wang, J. J. Yang, Z. Yang and H. L. Qin, *Journal of Cellular and Molecular Medicine*, 2010, **14**, 2697-2701.
 205. Y. Fujita, *Cancer Science*, 2011, **102**, 1749-1755.
 206. V. Korzh, C. Teh, I. Kondrychyn, D. M. Chudakov and S. Lukyanov, *Zebrafish*, 2011, **8**, 23-29.
 207. C. Tschuch, A. Schulz, A. Pscherer, W. Werft, A. Benner, A. Hotz-Wagenblatt, L. S. Barrionuevo, P. Lichter and D. Mertens, *Bmc Molecular Biology*, 2008, **9**.
 208. A. J. Di Pasqua, K. K. Sharma, Y. L. Shi, B. B. Toms, W. Ouellette, J. C. Dabrowiak and T. Asefa, *J Inorg Biochem*, 2008, **102**, 1416-1423.
 209. Z. Tao, B. B. Toms, J. Goodisman and T. Asefa, *Chemical Research in Toxicology*, 2009, **22**, 1869-1880.
 210. X. W. Lou, L. A. Archer and Z. Yang, *Advanced Materials*, 2008, **20**, 3987-4019.
 211. Y. Jiao, J. Guo, S. Shen, B. Chang, Y. Zhang, X. Jiang and W. Yang, *Journal of Materials Chemistry*, 2012, **22**, 17636-17643.
 212. Q. Zhang, T. Zhang, J. Ge and Y. Yin, *Nano Letters*, 2008, **8**, 2867-2871.
 213. S. D. Weitman, R. H. Lark, L. R. Coney, D. W. Fort, V. Frasca, V. R. Zurawski and B. A. Kamen, *Cancer Research*, 1992, **52**, 3396-3401.
 214. A. C. Antony, *Annu Rev Nutr*, 1996, **16**, 501-521.
 215. M. M. Doucette and V. L. Stevens, *The Journal of Nutrition*, 2001, **131**, 2819-2825.
 216. A. R. Hilgenbrink and P. S. Low, *Journal of Pharmaceutical Sciences*, 2005, **94**, 2135-2146.

-
217. P. S. Low, W. A. Henne and D. D. Doorneweerd, *Accounts of Chemical Research*, 2007, **41**, 120-129.
218. J. Sudimack and R. J. Lee, *Advanced Drug Delivery Reviews*, 2000, **41**, 147-162.
219. H. Chen, R. Ahn, J. Van den Bossche, D. H. Thompson and T. V. O'Halloran, *Molecular Cancer Therapeutics*, 2009, **8**, 1955-1963.
220. Y. Song, W. Shi, W. Chen, X. Li and H. Ma, *Journal of Materials Chemistry*, 2012, **22**, 12568-12573.
221. G. A. Mansoori, K. S. Brandenburg and A. Shakeri-Zadeh, *Cancers*, 2010, **2**, 1911-1928.
222. S.-M. Lee, H. Chen, T. V. O'Halloran and S. T. Nguyen, *Journal of the American Chemical Society*, 2009, **131**, 9311-9320.
223. M. Stubbs, P. M. J. McSheehy, J. R. Griffiths and C. L. Bashford, *Molecular Medicine Today*, 2000, **6**, 15-19.
224. I. F. Tannock and D. Rotin, *Cancer Research*, 1989, **49**, 4373-4384.
225. H. Meng, M. Xue, T. Xia, Y.-L. Zhao, F. Tamanoi, J. F. Stoddart, J. I. Zink and A. E. Nel, *Journal of the American Chemical Society*, 2010, **132**, 12690-12697.
226. H. P. Rim, K. H. Min, H. J. Lee, S. Y. Jeong and S. C. Lee, *Angewandte Chemie International Edition*, 2011, **50**, 8853-8857.
227. F. Muhammad, M. Guo, W. Qi, F. Sun, A. Wang, Y. Guo and G. Zhu, *Journal of the American Chemical Society*, 2011, **133**, 8778-8781.
228. C.-H. Lee, S.-H. Cheng, I. P. Huang, J. S. Souris, C.-S. Yang, C.-Y. Mou and L.-W. Lo, *Angewandte Chemie International Edition*, 2010, **49**, 8214-8219.
229. W. Stöber, A. Fink and E. Bohn, *Journal of Colloid and Interface Science*, 1968, **26**, 62-69.
230. X. Fang, C. Chen, Z. Liu, P. Liu and N. Zheng, *Nanoscale*, 2011, **3**, 1632-1639.
231. R. Richer, *Chemical Communications*, 1998, **0**, 1775-1777.
232. Y. Chen, H. Chen, M. Ma, F. Chen, L. Guo, L. Zhang and J. Shi, *Journal of Materials Chemistry*, 2011, **21**, 5290-5298.
233. L. Zhang, J. Xia, Q. Zhao, L. Liu and Z. Zhang, *Small*, 2010, **6**, 537-544.
234. J. Coates, in *Encyclopedia of Analytical Chemistry*, John Wiley & Sons, Ltd, 2006.
235. M. A. Valencia-Sanchez, J. Liu, G. J. Hannon and R. Parker, *Genes & Development*, 2006, **20**, 515-524.
236. M. Monaghan and A. Pandit, *Advanced Drug Delivery Reviews*, 2011, **63**, 197-208.
237. Y.-L. Chiu, A. Ali, C.-y. Chu, H. Cao and T. M. Rana, *Chemistry & Biology*, 2004, **11**, 1165-1175.

-
238. T. Futami, M. Miyagishi, M. Seki and K. Taira, *Nucleic Acids Symposium Series*, 2002, **2**, 251-252.
239. D. Bechet, P. Couleaud, C. Frochot, M. L. Viriot, F. Guillemin and M. Barberi-Heyob, *Trends in Biotechnology*, 2008, **26**, 612-621.
240. T. J. Dougherty, C. J. Gomer, B. W. Henderson, G. Jori, D. Kessel, M. Korbelik, J. Moan and Q. Peng, *Journal of the National Cancer Institute*, 1998, **90**, 889-905.
241. S. B. Brown, E. A. Brown and I. Walker, *Lancet Oncology*, 2004, **5**, 497-508.
242. J. P. Celli, B. Q. Spring, I. Rizvi, C. L. Evans, K. S. Samkoe, S. Verma, B. W. Pogue and T. Hasan, *Chemical Reviews*, 2010, **110**, 2795-2838.
243. L. E. Rhodes, M. de Rie, Y. Enstrom, R. Groves, T. Morken, V. Goulden, G. A. E. Wong, J. J. Grob, S. Varma and P. Wolf, *Archives of Dermatology*, 2004, **140**, 17-23.
244. H. Kato, *Journal of Photochemistry and Photobiology B: Biology*, 1998, **42**, 96-99.
245. M. Kriegmair, R. Baumgartner, W. Lumper, R. Waidelich and A. Hofstetter, *British Journal of Urology*, 1996, **77**, 667-671.
246. U. O. Nseyo, J. DeHaven, T. J. Dougherty, W. R. Potter, D. L. Merrill, S. L. Lundahl and D. L. Lamm, *Journal of Clinical Laser Medicine & Surgery*, 1998, **16**, 61-68.
247. P. Jichlinski and H. J. Leisinger, *Urological Research*, 2001, **29**, 396-405.
248. D. E. Schuller, J. S. McCaughan and R. P. Rock, *Archives of Otolaryngology-Head & Neck Surgery*, 1985, **111**, 351-355.
249. P. J. Lou, H. R. Jager, L. Jones, T. Theodossy, S. G. Bown and C. Hopper, *British Journal of Cancer*, 2004, **91**, 441-446.
250. M. Biel, *Lasers in Surgery and Medicine*, 2006, **38**, 349-355.
251. S. J. Lee, H. Koo, D. E. Lee, S. Min, S. Lee, X. Y. Chen, Y. Choi, J. F. Leary, K. Park, S. Y. Jeong, I. C. Kwon, K. Kim and K. Choi, *Biomaterials*, 2011, **32**, 4021-4029.
252. E. Reddi, C. Zhou, R. Biolo, E. Menegaldo and G. Jori, *British Journal of Cancer*, 1990, **61**, 407-411.
253. A. S. L. Derycke and P. A. M. de Witte, *Advanced Drug Delivery Reviews*, 2004, **56**, 17-30.
254. B. Z. Zhao, J. J. Yin, P. J. Bilski, C. F. Chignell, J. E. Roberts and Y. Y. He, *Toxicology and Applied Pharmacology*, 2009, **241**, 163-172.
255. H. L. Tu, Y. S. Lin, H. Y. Lin, Y. Hung, L. W. Lo, Y. F. Chen and C. Y. Mou, *Advanced Materials*, 2009, **21**, 172-+.
256. I. Roy, T. Y. Ohulchanskyy, H. E. Pudavar, E. J. Bergey, A. R. Oseroff, J. Morgan, T. J. Dougherty and P. N. Prasad, *Journal of the American Chemical Society*, 2003, **125**, 7860-7865.

-
257. L. Brannon-Peppas and J. O. Blanchette, *Advanced Drug Delivery Reviews*, 2004, **56**, 1649-1659.
258. K. J. Cho, X. Wang, S. M. Nie, Z. Chen and D. M. Shin, *Clinical Cancer Research*, 2008, **14**, 1310-1316.
259. N. Nombona, K. Maduray, E. Antunes, A. Karsten and T. Nyokong, *Journal of Photochemistry and Photobiology B-Biology*, 2012, **107**, 35-44.
260. M. Fadel, K. Kassab and D. A. Fadeel, *Lasers in Medical Science*, 2010, **25**, 283-292.
261. G. Obaid, I. Chambrier, M. J. Cook and D. A. Russell, *Angewandte Chemie International Edition*, 2012, **51**, 6158-6162.
262. F. F. Wang, X. J. Yang, L. Ma, B. R. Huang, N. Na, C. E. Ying, D. C. He and J. Ouyang, *Journal of Materials Chemistry*, 2012, **22**, 24597-24604.
263. A. K. Geim and K. S. Novoselov, *Nature Materials*, 2007, **6**, 183-191.
264. D. R. Dreyer, S. Park, C. W. Bielawski and R. S. Ruoff, *Chemical Society Reviews*, 2010, **39**, 228-240.
265. Y. W. Zhu, S. Murali, W. W. Cai, X. S. Li, J. W. Suk, J. R. Potts and R. S. Ruoff, *Advanced Materials*, 2010, **22**, 3906-3924.
266. K. Wang, J. Ruan, H. Song, J. L. Zhang, Y. Wo, S. W. Guo and D. X. Cui, *Nanoscale Research Letters*, 2011, **6**.
267. S. Bi, T. T. Zhao and B. Y. Luo, *Chemical Communications*, 2012, **48**, 106-108.
268. M. Luo, X. Chen, G. H. Zhou, X. Xiang, L. Chen, X. H. Ji and Z. K. He, *Chemical Communications*, 2012, **48**, 1126-1128.
269. J. L. Li, H. C. Bao, X. L. Hou, L. Sun, X. G. Wang and M. Gu, *Angewandte Chemie-International Edition*, 2012, **51**, 1830-1834.
270. T. N. Narayanan, B. K. Gupta, S. A. Vithayathil, R. R. Aburto, S. A. Mani, J. Taha-Tijerina, B. Xie, B. A. Kaiparettu, S. V. Torti and P. M. Ajayan, *Advanced Materials*, 2012, **24**, 2992-2998.
271. X. M. Sun, Z. Liu, K. Welsher, J. T. Robinson, A. Goodwin, S. Zaric and H. J. Dai, *Nano Research*, 2008, **1**, 203-212.
272. L. M. Zhang, J. G. Xia, Q. H. Zhao, L. W. Liu and Z. J. Zhang, *Small*, 2010, **6**, 537-544.
273. H. Y. Wen, C. Y. Dong, H. Q. Dong, A. J. Shen, W. J. Xia, X. J. Cai, Y. Y. Song, X. Q. Li, Y. Y. Li and D. L. Shi, *Small*, 2012, **8**, 760-769.
274. H. Q. Bao, Y. Z. Pan, Y. Ping, N. G. Sahoo, T. F. Wu, L. Li, J. Li and L. H. Gan, *Small*, 2011, **7**, 1569-1578.
275. A. Ajayaghosh, *Chemical Society Reviews*, 2003, **32**, 181-191.
276. A. Ajayaghosh, *Accounts of Chemical Research*, 2005, **38**, 449-459.

-
277. S. Sreejith, P. Carol, P. Chithra and A. Ajayaghosh, *Journal of Materials Chemistry*, 2008, **18**, 264-274.
278. E. Arunkumar, P. Chithra and A. Ajayaghosh, *Journal of the American Chemical Society*, 2004, **126**, 6590-6598.
279. A. Ajayaghosh, E. Arunkumar and J. Daub, *Angewandte Chemie-International Edition*, 2002, **41**, 1766-+.
280. E. Terpetschnig, H. Szmecinski, A. Ozinskas and J. R. Lakowicz, *Analytical Biochemistry*, 1994, **217**, 197-204.
281. B. L. Renard, Y. Aubert and U. Asseline, *Tetrahedron Letters*, 2009, **50**, 1897-1901.
282. J. V. Ros-Lis, B. Garcia, D. Jimenez, R. Martinez-Manez, F. Sancenon, J. Soto, F. Gonzalvo and M. C. Valdecabres, *Journal of the American Chemical Society*, 2004, **126**, 4064-4065.
283. S. Sreejith, K. P. Divya and A. Ajayaghosh, *Angewandte Chemie-International Edition*, 2008, **47**, 7883-7887.
284. R. R. Avirah, D. T. Jayaram, N. Adarsh and D. Ramaiah, *Organic & Biomolecular Chemistry*, 2012, **10**, 911-920.
285. K. T. Arun, B. Epe and D. Ramaiah, *Journal of Physical Chemistry B*, 2002, **106**, 11622-11627.
286. K. T. Arun, D. T. Jayaram, R. R. Avirah and D. Ramaiah, *Journal of Physical Chemistry B*, 2011, **115**, 7122-7128.
287. V. S. Jisha, K. T. Arun, M. Hariharan and D. Ramaiah, *Journal of the American Chemical Society*, 2006, **128**, 6024-6025.
288. J. J. Gassensmith, J. M. Baumes and B. D. Smith, *Chemical Communications*, 2009, 6329-6338.
289. E. Arunkumar, C. C. Forbes, B. C. Noll and B. D. Smith, *Journal of the American Chemical Society*, 2005, **127**, 3288-3289.
290. J. R. Johnson, N. Fu, E. Arunkumar, W. M. Leevy, S. T. Gammon, D. Piwnica-Worms and B. D. Smith, *Angewandte Chemie-International Edition*, 2007, **46**, 5528-5531.
291. S. Sreejith, X. Ma and Y. Zhao, *Journal of the American Chemical Society*, 2012, **134**, 17346-17349.
292. S. B. Yang, X. L. Feng, S. Ivanovici and K. Mullen, *Angewandte Chemie-International Edition*, 2010, **49**, 8408-8411.
293. W. S. Hummers and R. E. Offeman, *Journal of the American Chemical Society*, 1958, **80**, 1339-1339.
294. N. I. Kovtyukhova, P. J. Ollivier, B. R. Martin, T. E. Mallouk, S. A. Chizhik, E. V. Buzaneva and A. D. Gorchinskiy, *Chemistry of Materials*, 1999, **11**, 771-778.

-
295. A. Treibs and K. Jacob, *Angewandte Chemie-International Edition*, 1965, **4**, 694-&.
296. J. C. Meyer, A. K. Geim, M. I. Katsnelson, K. S. Novoselov, T. J. Booth and S. Roth, *Nature*, 2007, **446**, 60-63.
297. Y. Hernandez, V. Nicolosi, M. Lotya, F. M. Blighe, Z. Y. Sun, S. De, I. T. McGovern, B. Holland, M. Byrne, Y. K. Gun'ko, J. J. Boland, P. Niraj, G. Duesberg, S. Krishnamurthy, R. Goodhue, J. Hutchison, V. Scardaci, A. C. Ferrari and J. N. Coleman, *Nature Nanotechnology*, 2008, **3**, 563-568.
298. W. Zhang, J. Cui, C.-a. Tao, Y. Wu, Z. Li, L. Ma, Y. Wen and G. Li, *Angewandte Chemie International Edition*, 2009, **48**, 5864-5868.
299. K. N. Kudin, B. Ozbas, H. C. Schniepp, R. K. Prud'homme, I. A. Aksay and R. Car, *Nano Letters*, 2008, **8**, 36-41.
300. W. X. Mai and H. Meng, *Integrative Biology*, 2013, **5**, 19-28.
301. N.-T. Chen, S.-H. Cheng, J. S. Souris, C.-T. Chen, C.-Y. Mou and L.-W. Lo, *Journal of Materials Chemistry B*, 2013, **1**, 3128-3135.
302. W. Zhai, C. He, L. Wu, Y. Zhou, H. Chen, J. Chang and H. Zhang, *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, 2012, **100B**, 1397-1403.
303. K. Chen, J. Zhang and H. Gu, *Journal of Materials Chemistry*, 2012, **22**, 22005-22012.
304. V. Cauda, A. Schlossbauer and T. Bein, *Microporous and Mesoporous Materials*, 2010, **132**, 60-71.

PUB LICATION AND CONFERENCE

Publication

1. **Xing Ma**, Kim Truc Nguyen, Parijat Borah, Chung Yen Ang, Yanli Zhao,* “Functional silica nanoparticles for redox-triggered drug/ssDNA co-delivery” *Adv. Healthcare Mater.***2012**, *1*, 690-697. (This work has been highlighted as Frontispiece.)
2. Sivaramapanicker Sreejith‡, **Xing Ma**‡, Yanli Zhao,* “Graphene oxide wrapping on squaraine-loaded mesoporous silica nanoparticles for bioimaging” *J. Am. Chem. Soc.***2012**, *134*, 17346-17349.(‡: equal contribution)
3. **Xing Ma**, Cathleen Teh, Quan Zhang, Parijat Borah, Cleo Choong, Vladimir Korzh, and Yanli Zhao*. “Redox-Responsive Mesoporous Silica Nanoparticles: A Physiologically Sensitive Co-delivery Vehicle for siRNA and Doxorubicin”. *Antioxidants & Redox Signaling*. **2013**. accepted
4. **Xing Ma**‡, Sivaramapanicker Sreejith‡ and Yanli Zhao*. “Monomeric Zinc Phthalocyanine inside Spacer Modified Nano-channels of Mesoporous Silica Nanoparticles: A Nanohybrid for Photodynamic Cancer Therapy”. (under revision).
5. **Xing Ma**, Yun Zhao, Kee Woei Ng and Yanli Zhao*. “Integrated hollow mesoporous silica nanoparticles for target drug/siRNA co-delivery”. *Chem. Eur. J.* **2013**, accepted.
6. **Xing Ma**, Ong Onn Shaun and Yanli Zhao*. “Dual-responsive Drug Release from Functional Mesoporous Silica Nanoparticles with Oligonucleotide Capping”. *Biomaterials Science*.**2013**, *1*, 912-917.
7. Truong Thi Hong Anh, **Ma Xing**, Duc Huynh Tien Le, Ayae Sugawara-Narutaki, Eileen Fong. “Elastin-based silver-binding proteins with antibacterial capabilities”. *Nanomedicine*. 2013, *8* (4): 567-575

-
8. Parijat Borah, **Xing Ma**, Kim Truc Nguyen, and Yanli Zhao*. "A vanadyl complex grafted to periodic mesoporous organosilica: A green catalyst for selective hydroxylation of benzene to phenol" *Angew. Chem. Int. Ed.***2012**, 51, 7756–7761
 9. Huacheng Zhang, **Xing Ma**, Kim Truc Nguyen, Yanli Zhao*. "Biocompatible pillararene-assembly-based carriers for dual bioimaging" *ACS Nano***2013**, accepted.
 10. Kim Truc Nguyen, Dehui Li, Parijat Borah, **Xing Ma**, Zhaona Liu, Liangliang Zhu, George Grüner, Qihua Xiong, Yanli Zhao,* "Photoinduced charge transfer within polyaniline-encapsulated quantum dots decorated on graphene" *ACS Appl. Mater. Interfaces***2013**, accepted.
 11. Jinhong Guo, Wen Lei, **Xing Ma**, Peng Xue, Yu Chen, Yuejun Kang*. "Design of A Fluidic Circuit-based Microcytometer for Circulating Tumor Cell Detection and Enumeration", **2013**. accepted
 12. Hong Yan, Cathleen Teh, Sivaramapanicker Sreejith, Liangliang Zhu, Anna Kwok, Weiqin Fang, **Xing Ma**, Kim Truc Nguyen, Vladimir Korzh, Yanli Zhao*. "Functional mesoporous silica nanoparticles for photothermal-controlled drug delivery in vivo" *Angew. Chem. Int. Ed.***2012**, 51, 8373–8377
 13. Quan Zhang, Fang Liu, Kim Truc Nguyen, **Xing Ma**, Xiaojun Wang, Bengang Xing, Yanli Zhao*. "Multifunctional mesoporous silica nanoparticles for cancer-targeted and controlled drug delivery" *Adv. Funct. Mater.***2012**, 22, 5144-5156
 14. Huacheng Zhang, **Xing Ma**, Junfei Guo, Kim Truc Nguyen, Quan Zhang, Xiao-Jun Wang, Hong Yan, Liangliang Zhu, Yanli Zhao*. "Thermo-responsive fluorescent vesicles assembled by fluorescein-functionalized pillar[5]arene" *RSC Adv.***2013**, 3, 368-371.
 15. Huacheng Zhang, Kim Truc Nguyen, **Xing Ma**, Hong Yan, Junfei Guo, Liangliang Zhu, Yanli Zhao*. "Host–guest complexation driven dynamic supramolecular self-assembly" *Org. Biomol. Chem.***2013**, 11, 2070-2074..

-
16. **Ma X**; Klosterman L; Hu Y Y; Liu X P; Schmidt-Rohr K; Mallapragada S; Akinc M *. “Aqueous Route Synthesis of Mesoporous ZrO₂ by Agarose Templatation”. *Journal of the American Ceramic Society*. **2012**, 95:3455-3462.
 17. Hu Y Y; Liu X P; **Ma X**; Rawal A; Prozorov T; Akinc M; Mallapragada S K; Schmidt-Rohr K . “Biomimetic Self-Assembling Copolymer-Hydroxyapatite Nanocomposites with the Nanocrystal Size Controlled by Citrate”. *Chemistry of Materials*. **2011**, 23:2481-2490.
 18. J.C. Yan, Z.W. Xu, L. Shi, **X. Ma**, S.Q Yang. “Ultrasonic assisted fabrication of particle reinforced bonds joining aluminum metal matrix composites”. *Materials and Design*. **2011**, 32: 343–347.

Conference

1. **Functional Silica Nanoparticles for Redox-Triggered Cargo/ssDNA Co-Delivery**. The 5th MRS-S Conference on Advanced Materials (Poseter: P00520 – 22, March 2012). Singapore
2. **Redox responsive nanoparticles for drug/gene Co-delivery, as an ORAL presentation**. International Conference of Young Researchers on Advanced Materials (ICYRAM) (Oral presentation, July 1-6, 2012). Singapore
3. **Functional Mesoporous Silica Nanoparticle Based Photodynamic Therapy** The 7th International Conference on Materials for Advanced Technologies (ICMAT) 2013. (Poster July 1-6, 2013). Singapore