Mesoporous silica nanoparticle-based protein delivery systems for biomedical applications

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Chapter I

General Introduction

1.1 Mesoporous silica nanoparticle for protein delivery

The interest in proteins as promising therapeutic agents has increased remarkably in almost every field of medicine, like cancer, inflammatory diseases, vaccines, and also in diagnostics.[1, 2, 3] Human insulin was the first recombinant biopharmaceutical approved by FDA in 1982 and ever since, more than 100 proteins have been approved for clinical use[4] as proteins typically show a high specificity with less interference with normal biological processes.[5, 6] However, proteins used as a therapeutic are usually characterized by their short plasma half-life, high elimination rate, limited ability to cross cell membranes, and poor bioavailability through intestinal administration.[2, 7] Once proteins are present in blood, these biomacromolecules are prone to degradation and cannot be targeted to the desired site of action.[7] In order to improve the therapeutic efficacy of proteins, various nanocarriers are developed to overcome these barriers.

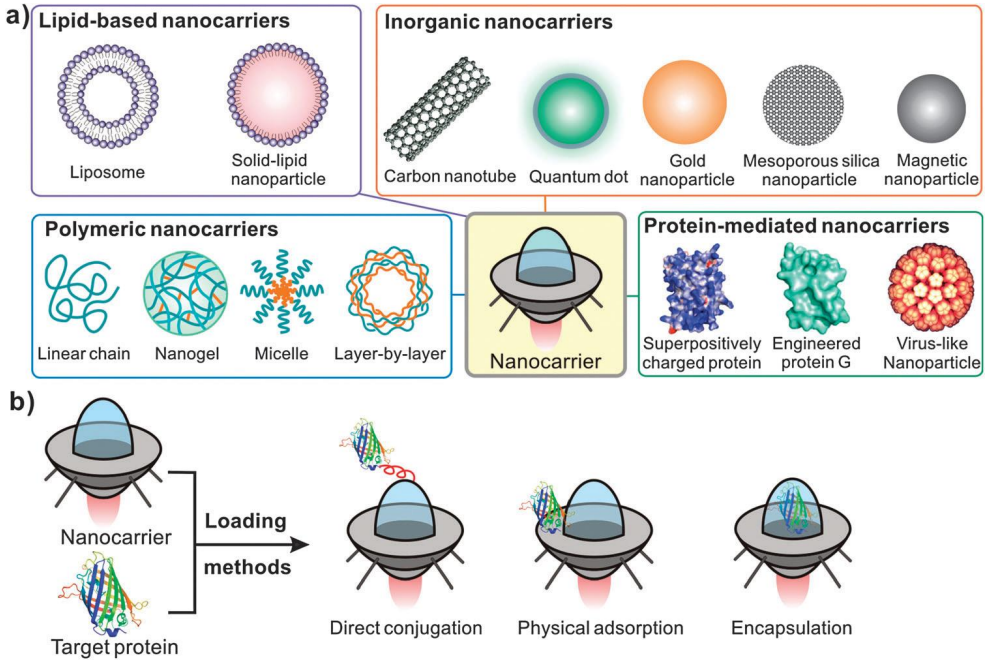

Figure 1.1 *(a) Various types of nanocarriers used for intracellular protein delivery. (b) The three commonly used methods for preparing protein/nanocarrier composites.[3] Figure taken from reference 3.*

Delivering therapeutic proteins into cells remains an important challenge[8] and therefore nanocarriers enabling efficient protein delivery are in high demand.[3, 5] Over the last decades,

various nanocarriers (Figure 1.1)[3] such as lipid based assemblies,[4, 9] and polymeric nanoparticles[10, 11] and inorganic nanoparticles have been developed. Loading multiple cargos, for example short peptides and large proteins, within a particulate delivery systems can overcome drawbacks exhibited by conventional free proteins (*e. g.* rapid clearing),[12] retaining pharmacological and enzymatic activity by protecting the cargo against harsh environments.[7, 13] Compared to carbon-based nanomaterials and assemblies, inorganic materials show the unique feature of high thermal/chemical stability and exhibit typically good biocompatibility with relatively low rates of degradation.[14]

Since the M41S family of ordered Mesoporous silica nanoparticles (MSNs) was first reported in the early 1990s,[15] the number of publications on MSNs have increased dramatically.[2, 16 17-20] MSNs have attracted considerable attention for their application in biomedicine because of their fascinating properties.[2, 17, 21, 22] The unique properties of MSNs include the large surface area and pore volume, tunable pore diameter and structure, good chemical and thermal stability and a large variety in chemistry for surface functionalization.[16, 17, 23-26] MSNs contain channels which are able to encapsulate and release therapeutic molecules, including low molecular weight drugs, small interfering RNA,[27] and proteins.[28] Furthermore, MSNs are biocompatible and accepted as "general recognized as safe (GRAS)" by the U. S. Food and Drug Administration (FDA).[18, 29] As a nanomedicine, they possess several requirements to be considered as an ideal biocompatible particle, *i. e.* they are safe to use,[30] are efficiently internalized into cells by phagocytosis or the receptor-mediated endocytosis (Figure 1.2).[22, 31-33] Most importantly, these MSNs can be loaded with drugs, and the drug release can be (externally) triggered and/or controlled.[34]

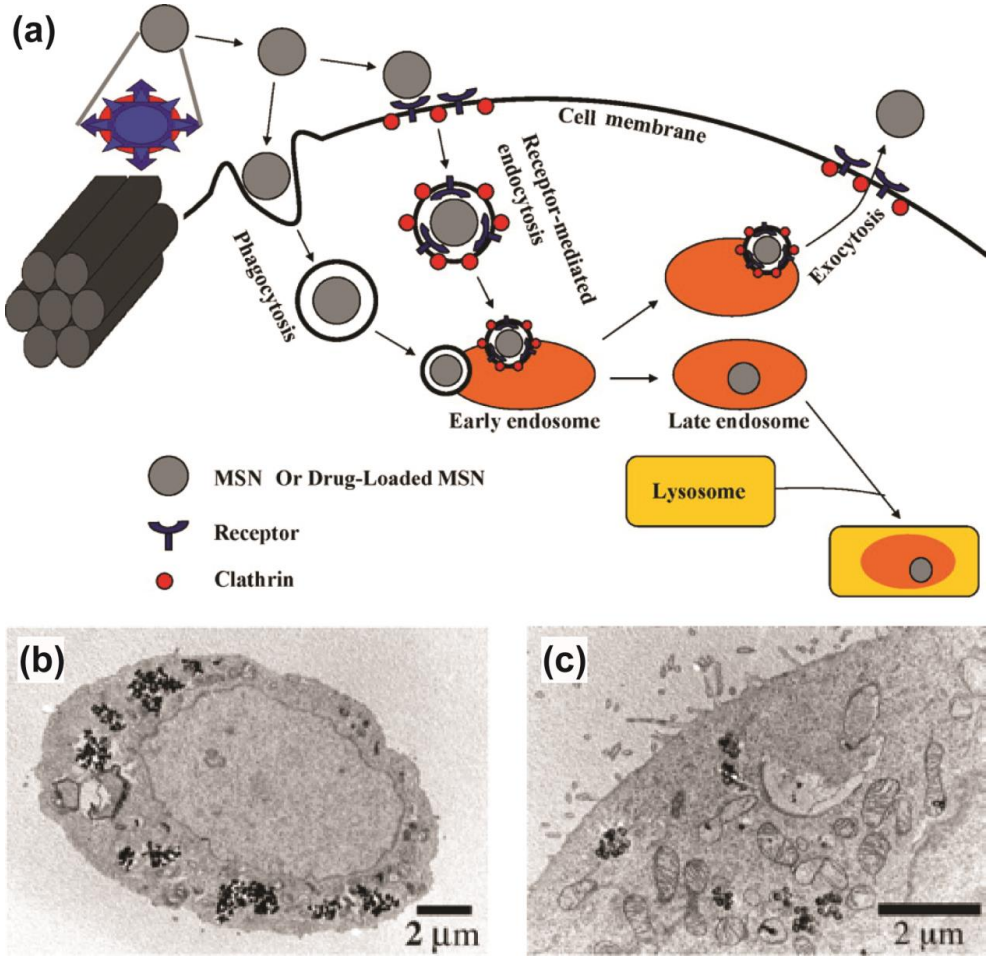

Figure 1.2 *(a) Endocytotic pathway of MSNs in mammalian cells, taken from reference 22. TEM micrographs of PAMAM-MSN (black dots) endocytosed by (b) Chinese hamster ovarian (CHO), taken from reference 32 and (c) human cervical cancer (HeLa) cells, taken from reference 33.*

More recently, MSNs with an open-pore structure have emerged as a potential carrier for protein delivery. Due to their structure, MSNs protect proteins from premature degradation in body fluids, thereby increasing the efficiency of protein delivery *in vivo*, thus reducing renal filtration.[3] Furthermore, the silanol-containing surface of MSNs can be easily functionalized,[21, 35] enabling the adsorption of various proteins with different surface properties,[36] or modification with targeting molecules such as folate to enhance cellular uptake.[21, 37] Indeed, several reports have shown that MSNs can facilitate the transport of

protein into the cytosol via an endocytosis pathway and subsequent endosomal escape.[2, 3, 20, 33, 38, 39]

Numerous synthetic protocols for the preparation of MSNs have been developed.[40-45] Encapsulating large biomacromolecules (*e.g.* proteins) in MSNs is still challenging however, because the most commonly used type of MSN have a rather small mesopore diameter (< 3 nm)[44] preventing the effective encapsulation of a broad range of proteins.[46, 47] Therefore MSNs with a large pore size have been synthesized, however in most cases, these particles have a large particle size resulting in decreased cellular uptake efficiency.[44, 48, 49] This is in line with previous studies showing that a particle size between 50 and 200 nm is preferred for endocytic uptake.[33, 48, 50] Therefore, there is still a need for monodisperse MSNs with a particle size in the 50-200 nm range, controllable surface chemistry, and a large pore size (> 5 nm) for applications in the field of drug delivery.[46, 51-53]

1.2 Design and synthesis large pore and small diameter of MSNs for protein delivery

In general, MSNs are synthesized using a templating agent,[2] typically a surfactant in aqueous solution which is either neutral or charged.[20] The pH of aqueous solutions used in MSN synthesis controls the hydrolysis and condensation reaction (Figure 1.3).[13, 54] The size, morphology, mesostructures of MSNs can be rationally designed by controlling the reaction parameters (*e.g.* surfactant concentration, pH, temperature, silica source*)*.[17, 18, 43] With the abundant availability of various types of surfactants,[18] numerous synthetic protocols for the preparation of MSNs have been developed.[43, 52]

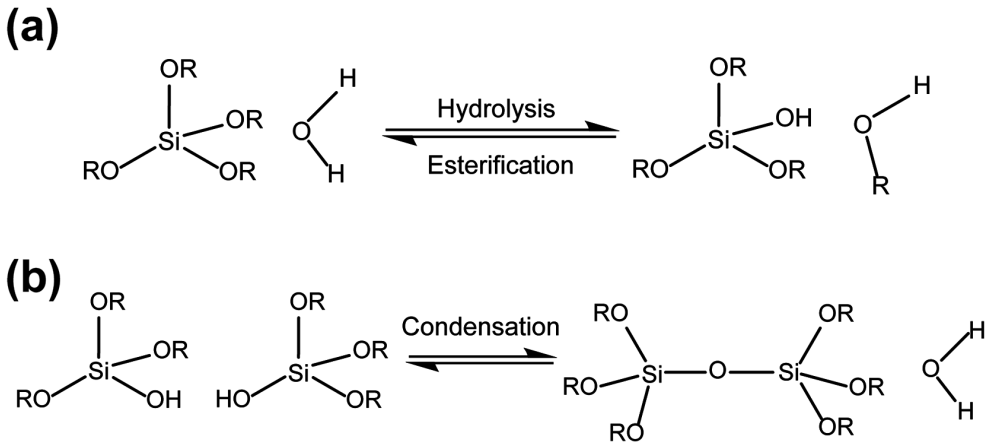

Figure 1.3 *Mechanism of (a) hydrolysis and (b) condensation reactions of alkoxysilanes and organylakoxysilanes, resulting in a molecular formula of* $Si(OR)_{4-n}(OH)_n$.

The two classical examples of MSNs are MCM-41[55] (Mobil Composition of Matter no. 41) and SBA-15[15, 56] (Santa Barbara Amorphous no. 15) which use cetyltrimethylammonium bromide (CTAB) and amphiphilic block copolymers (Pluronics P123, $PEO_{20}PPO_{70}PEO_{20}$) as a template respectively. MCM-41-type nanoparticles typically have a diameter ranging from 20-500 nm while pore sizes ranges from 2-6 nm (Figure 1.4).[39, 57-59] Therefore, these materials can be used for the encapsulation and release of low to medium molecular weight biomolecules like small proteins (*e. g.* cytochrome, Mw 12384 Da, geometric size 2.6 × 3.2 × 3.3 nm).[8, 58]

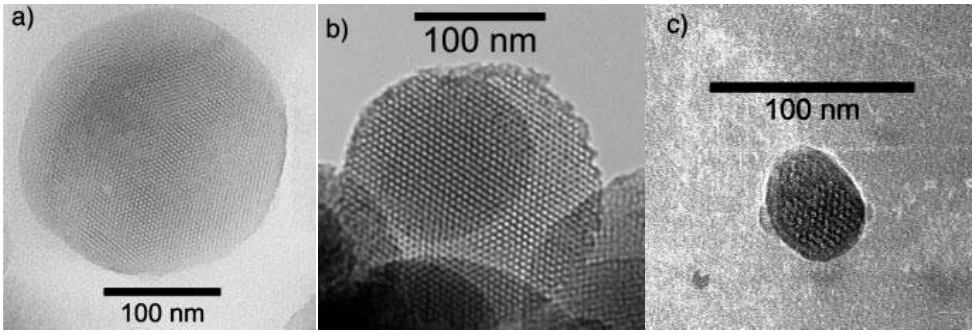

Figure 1.4 *TEM images of three spherical MSNs with different particle and pore diameter: a) particle size ca. 250 nm, pore diameter ca. 2.3 nm.[57] B) particle size ca. 200 nm; pore diameter ca. 6.0 nm.[58] C) particle size ca. 50 nm; pore ca. 2.7 nm.[39] Figure taken from reference 39.*

Due to their size, large proteins are predominantly adsorbed onto the external surface of MSNs and do not make use of the protective environment inside MSNs, nor do they utilize the large internal surface area presented by these pores.[16, 60-62] SBA-15 does have large pores (5-30 nm) but the average particle size of these materials is in the range of sub-micrometer to several micrometers rendering these particles less suited for biomedical applications (Figure 1.5).[56, 63]

Therefore several groups have developed methods to control the outer particle diameter and pore-size of MSNs in the nanometer range.[8, 41, 42, 45, 50, 51, 64, 65] Based on geometric considerations, it is expected that MSNs with a large pore-size (> 5 nm) and small diameter (< 200 nm) should facilitate the efficient loading and delivery of biomacromolecules with large molecular sizes.[66] Covalent immobilization of protein into MSNs is one approach to retain their functional characteristics,[47, 67-69] however, irreversible covalent attachment can result in a loss of activity compared to physical adsorption.[70] To avoid complicated chemical reactions, encapsulation of proteins inside MSNs driven by electrostatic interactions (physisorption) will be discussed in this thesis.

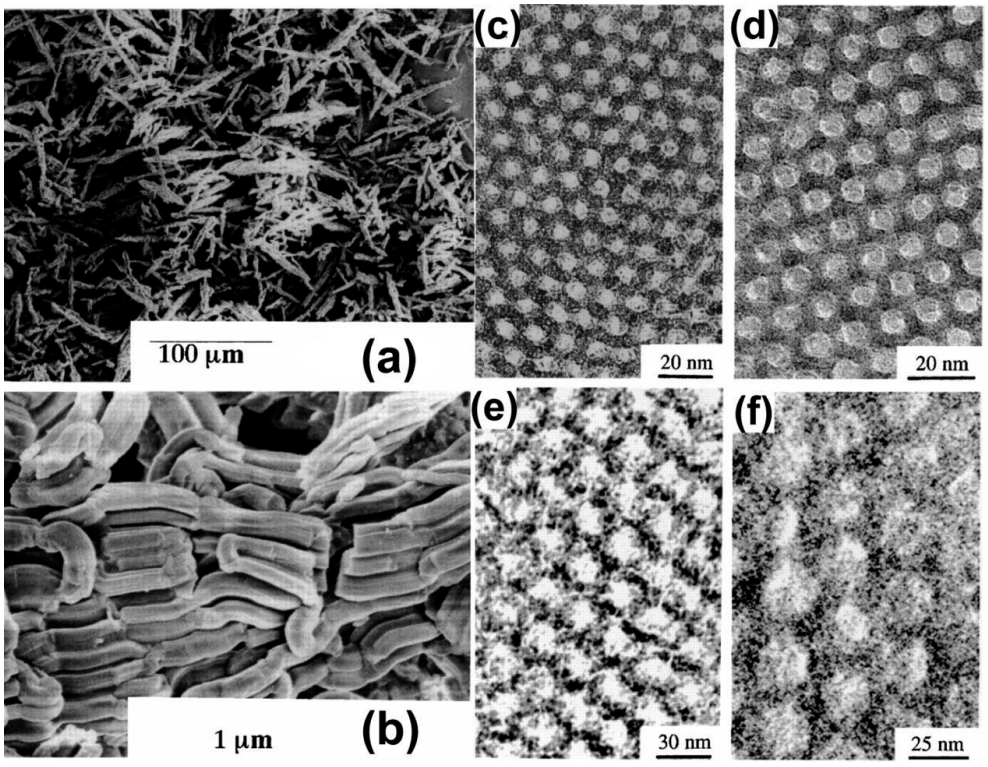

Figure 1.5 *(a) and (b) Scanning electron microscopy and (c-f) transmission electron microscopy images of hexagonal mesoporous silica SBA-15. SBA-15 can be synthesized over a range of reaction mixture compositions and conditions.*[56, 63] *TEM images show well-ordered hexagonal arrays of mesopores. Figure taken from reference 56.*

In this section, a few examples of MSNs with a large pore size and a small particle diameter that previously were used for the intracellular delivery of functional proteins using MSNs are presented.

MCM-41-type MSNs with larger pores (5.4 nm) could be obtained by using mesitylene (TMB) as a pore-expanding agent.[58, 71] Lin and coworkers reported the successful intracellular delivery of the membrane-impermeable protein cytochrome c (molecular weight 12.4 kDa, isoelectric point 11.35) using these MSNs as the nanocarrier and it was shown that the released proteins were still functional and highly active in catalyzing the oxidation of 2,2'-aznio-bis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) by hydrogen peroxide.[58]

Yu and coworkers reported a facile and oil-free approach to synthesize dendritic MSNs with a center-radial pore structure, tunable particle size (79-160 nm) and large pores (22

nm).[64] These MSNs were synthesized in an aqueous phase using sodium trifluoroacetate as an additive to control the growth of MSNs. Antimicrobial lysozyme (molecular weight 14.3 kDa, isoelectric point 10-10.5) was chosen as cargo to study the delivery efficiency of these MSNs.[64, 72] It was found that MSNs with a large pore size and small particle size (MSNs-3) show significantly better antibacterial activity as compared to the particles with small pore sizes (MSNs-1 and MSNs-2, Figure 1. 6).[64]

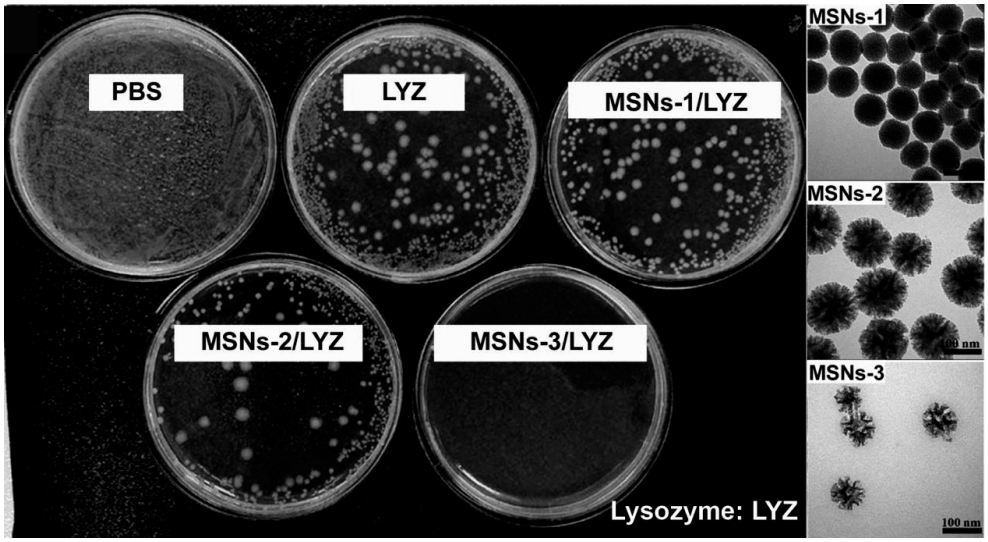

Figure 1.6 *Lysozyme loaded MSNs (500 µg/mL) for Escherichia coli (E. coli) inhibition after 5 days incubation. Figure taken from reference 64.*

While some progresses has been made with MSNs mediated protein delivery using cytochrome c or lysozyme as model proteins,[44, 73-76] reports on intracellular delivery of large functional proteins (more than 100 kDa) are rare.[8] Yu et al. designed a new type of monodispersed MSNs with a core-cone structure (MSN-CC) with an average pore size of 45 nm (Figure 1.7). These particles were synthesized in a chlorobenzene-water system, using cetyltrimethylammonium chloride (CTAC) as a structure-directing agent and tetraethyl orthosilicate (TEOS) as the silica source. The loading capacity for large molecular weight proteins (*i. e.* IgG, 150 kDa; β-galactosidase, 119 kDa) is 560 mg/g MSNs and 190 mg/g MSNs, respectively. The efficient delivery of active β-galactosidase (119 kDa) into N2a cells (mouse neuroblastoma cells) was quantified by monitoring the enzymatic activity of β-Galactosidase, showing that this core-cone structured MSN is a potential nanocarrier for protein delivery.[8]

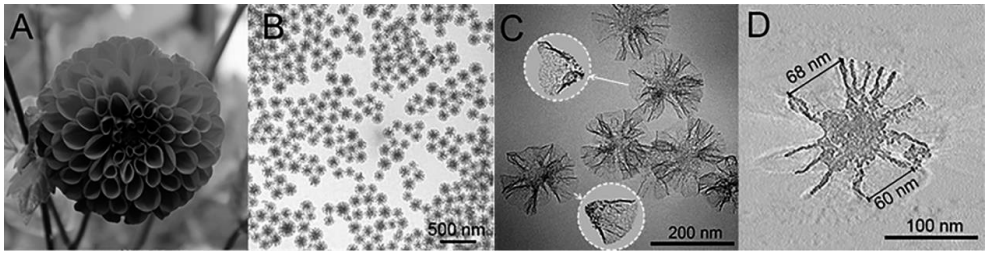

Figure 1.7 *(A) A picture of a dahlia photographed by C. Xu at Tasmania.(B) TEM images at low magnification, (C) high magnification, MSN-CC with uniform diameters of 180 nm, and (D) an electron tomography (ET) slice of MSN-CC. Figure taken from reference 8.*

For future *in vivo* applications of these protein-loaded MSNs, there are still some limitations that to be resolved, such as premature protein release and the colloidal instability of MSNs in salt-containing solutions. More specifically, after protein encapsulation the surface properties of MSNs change (*e.g.* zeta-potential becomes neutral), resulting in aggregation and colloidal instability in physiologic environments.[24] As a result, these aggregated particles are recognized and removed by the mononuclear phagocytical cells in the reticuloendothelial system.[49] As mentioned earlier in this chapter, the efficiency of cellular uptake of MSNs is size-dependent[23] and aggregated particles are therefore less suitable for *in vivo* delivery applications.

1.3 A lipid bilayer coated mesoporous silica nanoparticles

Following the pioneering work on supported phospholipid membranes in the 1980s,[77, 78] these reconstituted planar membranes on solid supports (or supported lipid bilayers, SLBs) have become popular as a model system mimicking the membrane of cells, with potential bio-technological applications. For example, as a model of biological membranes to study the transport of ions and molecules (Figure 1.8).[78, 79] Considerable work has been done to investigate the process of bilayer the formation by exposing small lipid vesicles to a hydrophilic support (*e.g.*, silica).[77-81] It was shown that phospholipid membranes are able to assemble into different structures upon contact with a hydrophilic surface,[82] involving vesicle adsorption, rupture and spreading into planar membranes.[79] Cryo-electron microscopy imaging convincingly demonstrated that the lipid bilayers faithfully follow the topology of the silica substrate.[83]

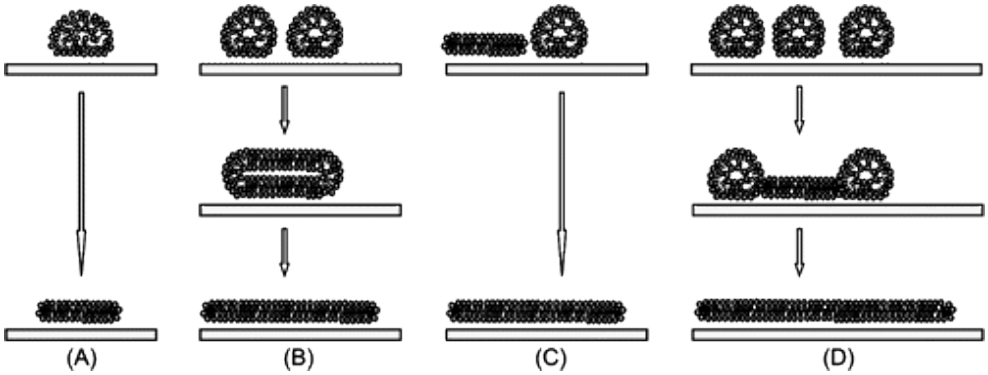

Figure 1.8 *Mechanisms of vesicle rupture: (A) an isolated adsorbed vesicle ruptures spontaneously, driven by its support-induced deformation; (B) neighboring adsorbed vesicles fuse and eventually rupture; (C) the active edge of a supported bilayer patch induces the rupture of a neighboring vesicle; (D) the cooperative action of several neighboring vesicles leads to the rupture of a first vesicle (at the critical vesicular coverage). The active edge thereby exposed triggers the rupture of adjacent vesicles.* [79] *Figure taken from reference 79.*

The long-term colloidal stability of MSNs remains a challenge, especially in *in vivo* experiments. Since the repulsive force between MSNs decreases after protein encapsulation, aggregation readily occurs. Therefore, application of a lipid bilayer on the exterior surface of MSNs could be useful to enhance the colloidal stability. Furthermore, it will also affect the rate of protein release by covering the pores of MSNs.[84, 85] In addition, the composition of supported lipid bilayer, can be modified for specific biological applications, for example by

conjugation of specific molecules in order to achieve cell-specific targeting and controlled intracellular delivery. [27, 86]

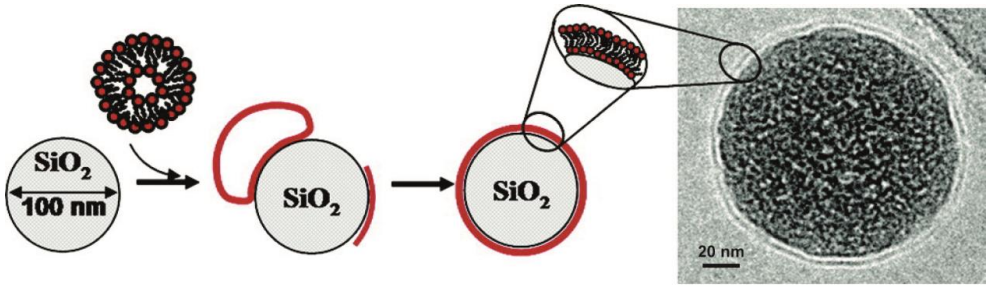

Figure 1.9 *Schematic representation of the elaboration process of silica nanoparticles covered with lipids.[87] Relative cryo-transmission electron microscopy image, the red lines represent lipid patch or a lipid bilayer.[83] Figure taken from reference 83.*

The use of supported lipid bilayer coated MSN, sometimes called "protocells" as a new drug delivery tool to ensure efficient cell uptake, was pioneered by Brinker and others.[84, 85 12, 84, 88-91] These protocells can be considered as a mimic of the cellular envelopes and cytoskeleton, combining the favorable properties of MSNs and liposomes.[23] While MSNs possess a high surface area and large accessible pore volumes enabling loading with guest molecules, the lipid bilayer acts as a physical barrier keeping the cargo inside the MSN core and provides colloidal stability.[85, 92, 93] The cargo is therefore protected from the environment and can be transported and released into the cell.[88] MSNs can be coated non-covalent with a physically adsorbed bilayer[23, 83, 86, 87, 92] or modified through covalent bonds with the phospholipids [85, 94] Efforts have been made to develop biocompatible and multifunctional protocells, such as using peptide-modified lipid bilayer coated MSN in order to target a specific tissue or cell type.[12, 27, 86, 95] Furthermore, magnetic field controlled[93] or light-controlled release of cargo,[96] PEGylation lipid composition have been applied to realize effective targeting or extending the circulation time.[25, 88, 94] However, most studies to date with these nanocarriers using lipid bilayer coated MSN mediate the delivery of low molecular weight dyes and drugs only.[84, 85, 90, 97, 98] So far, only a few reports on the delivery of small interfering RNA, [27] DNA[23] and proteins (*i.e.* ricin toxin A-chain, 32 kDa) have been reported.[12, 86]

1.4 Aim and outline of this thesis

In this thesis, we designed a facile synthetic route of a new type of MSNs that are able to effectively encapsulate and release proteins. The delivery of three commonly used proteins (ovalbumin, cytochrome c and hemoglobin) with different surface charges and molecular weight was studied, to investigate the potential biomedical application of large pore MSNs as a vaccine (ovalbumin), in protein therapy (hemoglobin) and in cancer therapy (cytochrome c).

In **Chapter 2**, we present a new type of elongated cuboidal MSNs with average dimensions of 90 × 43 nm that process disk-shaped cavities, stacked on top of each other, oriented parallel to the short axis of the particle. The MSN surface was modified with amines using 3-aminopropyltriethoxysilane or 3-[2-(2-aminoethylamino)ethylamino]propyltrimethoxysilane (AP-MSNs and AEP-MSNs), in order to obtain a positive surface charge at physiological pH. This unique open mesostructure makes it an ideal scaffold for protein encapsulation and delivery. Seven model proteins (α-lactalbumin, ovalbumin, bovine serum albumin, catalase, hemoglobin, lysozyme and cytochrome c) were encapsulated and the release was studied. It was found that these large-pore MSNs are suitable protein nanocarriers with favorable physical properties such as rapid encapsulation and with a high loading capacity for a wide range of model proteins. The protein release from these large-pore MSNs was examined showing that the structure of the released proteins remained unaltered. This study proved that these new cuboidal MSNs can be used as an effective platform for therapeutic protein delivery.

To study the scope of potential biomedical applications, a new intradermal delivery system is described in **Chapter 3**, which synergistically integrates the advantages of nanoparticles with microneedle delivery. In this study, ovalbumin-loaded, lipid bilayer-covered MSNs (LB-MSNs) were adsorbed onto pH-sensitive microneedles. The large pores (10 nm) facilitated the rapid encapsulation of ovalbumin (OVA) with a high loading capacity. The introduction of a lipid bilayer significantly improved the colloidal stability of OVA loaded AEP-MSNs and concomitantly reduced the premature release of OVA. In addition, it enables the coating of LB-MSNs on the surface of pH-sensitive microneedle arrays based on electrostatic interactions. Application of LB-MSNs coated microneedle arrays into human skin (*ex vivo*) resulted in the successful delivery of the OVA-loaded nanoparticles into the skin in a pH dependent manner. This microneedle-mediated intradermal delivery system for MSNs can be a promising tool to deliver a wide range of compounds into the skin.

Protein Delivery into the cytoplasm of cells is still a challenging topic in the field of nanomedicine as inefficient cellular uptake and endosomal escape limits potential clinical applications. In **Chapter 4** a complementary pair of coiled-coil lipopeptides (CP_4E_4 and CP_4K_4) was introduced as a way to trigger and control the targeted delivery of lipid bilayer coated MSNs into cells. The positively charged membrane-impermeable protein cytochrome c was used as a model protein in this study. MSNs with large pores rapidly encapsulated cytochrome c and were coated by a phospholipid bilayer containing CP_4E_4. These nanoparticles were added to CP_4K_4 pre-treated HeLa resulting in efficient cell uptake. By applying the fusogenic coiled coil system, cytochrome c was efficiently transported into the cytoplasm of cells within 30 minutes.

The preparation and (bio)physical properties of hemoglobin-based oxygen carrier nanoparticles (Hb-NPs) is described in **Chapter 5**. MSNs with large pores were used to encapsulate bovine hemoglobin (MSNs/Hb) and coated with a lipid bilayer. These large pore MSN act as rigid core to store and provide a protective environment for the encapsulated Hb. The lipid bilayer enhanced the colloidal stability of MSNs/Hb, as well as prevented the premature release of Hb. The *in vivo* circulation was studied in zebrafish embryos, demonstrating the potential for future pharmaceutical applications.

As nanoparticles (NPs) are attractive for pharmaceutical applications due to their unique features, such as its large surface to mass ratio resulting in the ability to encapsulate a wide variety of compounds[99] (*e.g.* low-molecular weight drugs, proteins, DNA/RNA). For a better characterization of these nanoparticles, it would be helpful to know the mass of a single nanoparticle, and therefore be able to calculate the number of molecules encapsulated per MSN. Nanoparticle Tracking Analysis (NTA) can determine the size distribution, and high sensitively in terms of particle-number concentration.[100] Based on this technique, we developed a simple and low-cost method to estimate the mass of single MSNs (**Chapter 6**).

Finally in **Chapter 7,** the findings and conclusions of this thesis are summarized and further research for potential applications of MSNs are presented.

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