Expert Opinion

Capped mesoporous silica nanoparticles as stimuli-responsive controlled release systems for intracellular drug/gene delivery

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Importance of the field: The incorporation of stimuli-responsive properties into nanostructured systems has recently attracted significant attention in the research of intracellular drug/gene delivery. In particular, numerous surface-functionalized, end-capped mesoporous silica nanoparticle (MSN) materials have been designed as efficient stimuli-responsive controlled release systems with the advantageous 'zero premature release' property.

Areas covered in this review: Herein, the most recent research progress on the design of biocompatible, capped MSN materials for stimuli-responsive intracellular controlled release of therapeutics and genes is reviewed. A series of hard and soft caps for drug encapsulation and a variety of internal and external stimuli for controlled release of different cargoes are summarized. Recent investigations on the biocompatibility of MSN both *in vitro* and *in vivo* are also discussed.

What the reader will gain: The reader will gain an understanding of the challenges for the future exploration of biocompatible stimuli-responsive MSN devices.

Take home message: With a better understanding of the unique features of capped MSN and its behaviors in biological environment, these multifunctional materials will find a wide variety of applications in the field of drug/gene delivery.

Keywords: biocompatibility, capped mesoporous silica nanoparticles, intracellular drug/gene delivery, nanoparticle endocytosis, stimuli-responsive controlled release systems

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1. Introduction

The parallel developments in the design of pharmaceutical drugs and in the controlled manipulation of materials at the nanometer scale have recently begun to merge in order to produce new generations of diagnostic and therapeutic agents. Many agents used for pharmacotherapy, such as antitumor drugs, show side effects and limited effectiveness that restrict their clinical application. To maximize therapeutic efficacy and minimize side effects, numerous efforts have been made in the design of target-specific drug delivery systems that can securely transport the medications to targeted cells and tissues, without degradation or untimely release.

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Article highlights.

- Stimuli-responsive end-capped MSN materials are promising drug carriers that securely deliver a large payload of drug molecules without degradation or premature release.
- MSN can be readily internalized by animal cells at low concentrations without posing any cytotoxicity issue *in vitro* or any apparent negative health effects *in vivo*. Detailed characterizations of cellular activities with MSN and long-term biocompatibility of MSN *in vivo* remain poorly understood.
- The selective surface functionalization of MSN and the capping of the mesopores with hard or soft caps bring controlled release capabilities and multifunctionalities to these materials. The use of biomolecules as a new type of capping agent should be continuously investigated to develop next-generation capped MSN systems.
- Among a variety of internal and external stimuli that have been applied to capped MSN systems, biomolecules and light have attracted growing interest. Further efforts on the development of new stimuli and on the combination of two or more stimuli in a single capped MSN system are needed to achieve precise spatial and temporal delivery of the medications to targeted site.

This box summarizes key points contained in the article.

Of the various drug nanocarriers explored, stimuli-responsive end-capped mesoporous silica nanoparticle (MSN) materials have been shown to be excellent candidates to fulfil the above-mentioned requirements owing to their advantageous 'zero premature release' property. This property is particularly useful when the drug to be delivered is toxic or its therapeutic dosage requires precise control. Conventional polymer-based drug delivery systems suffer from inherent problems, including limited capacity of drug loading and poor stability in blood after injection and the difficulty in temporally controlling the release of matrix-encapsulated compounds, as it usually takes place immediately on dispersion of these materials. In contrast to traditional polymer-based 'soft' nanomaterials, these highly stable inorganic MSN drug carriers are able to deliver a large payload of drug molecules with much lower degradation kinetics. The use of capping agents controls the pore opening and closing so that the encapsulated cargoes can be released with precise temporal control. In addition, the unique internal and external surfaces of these materials make them ideally suited to the design of sophisticated drug delivery systems by incorporating one or more different diagnostic and therapeutic capabilities into a single vehicle with precise release control in response to one or more stimuli, either reversibly or non-reversibly. As illustrated in **Figure 1**, the internal mesopores of these materials can serve as a safe microenvironment where molecules can be loaded and protected from degradation or deactivation before entering target cells. On drug loading, the openings of the mesoporous channels can be blocked with a series of hard caps such as iron oxide (Fe_3O_4), cadmium sulfide (CdS) and gold nanoparticles (Au NPs), or soft caps including organic molecules, biomolecules and supramolecular assemblies, to prevent the drugs from leaching before reaching the targeted site. The external surface can be functionalized further with ligands capable of cell targeting and diagnosing disease [1]. In addition, the fact that these materials are readily taken up by animal and plant cells at low concentrations without posing any *in vitro* cytotoxicity [2-4], or any apparent negative health effects *in vivo* [5-8], makes these multifunctional nanoparticles promising candidates for target-specific stimuli-controlled delivery of therapeutics.

This review focuses on the latest developments of biocompatible, capped MSN with special attention given to sophisticated stimuli-responsive systems with new capping agents and controlled release mechanisms designed for intracellular drug/gene delivery.

2. Intracellular delivery and biocompatibility of MSN

The first hexagonally ordered mesoporous silica material (designated as MCM-41) reported by researchers at the Mobil Oil Company consisted of micrometer-sized particles with variable morphology [9]. Whereas mesoporous silica microparticles are potentially useful for many non-biological applications such as adsorption, catalysis and chemical separations, they are not ideally suited for biotechnological and biomedical devices owing to the large particle size and irregular morphology. For example, for these materials to serve as intracellular carriers for drug/gene delivery, they have to be efficiently internalized by mammalian cells, which require the particle size of the materials to be on the submicrometer scale [10,11]. In addition, microparticles are within the size window of many pathogens and could potentially trigger acute immune responses when introduced *in vivo*. In the pursuit of biocompatible materials for controlled release and drug delivery applications, extensive research effort has been devoted to achieving control over particle size and morphology.

Mesoporous silica nanoparticles are prepared by a simple and rapid synthetic approach characterized by uniform particle size (80 – 500 nm), adjustable particle morphology, high surface areas (900 – 1100 m^2/g), large accessible pore volumes (0.5 – 1.5 cm^3/g), tunable pore size (2 – 10 nm), and a wide variety of surface functional groups that can be attached on the internal and external surfaces of MSNs to manipulate surface properties for drug loading and release [12]. The MSN shown in **Figure 1B**, for example, has a particle size ~ 150 nm with a hexagonal mesoporous structure, and pores ~ 2 nm in diameter; this material has a surface area ~ 900 m^2/g, and pore volume ~ 0.9 cm^3/g. Some reviews on the synthesis, size and morphology control and surface functionalization of MSN have been published recently [13,14].

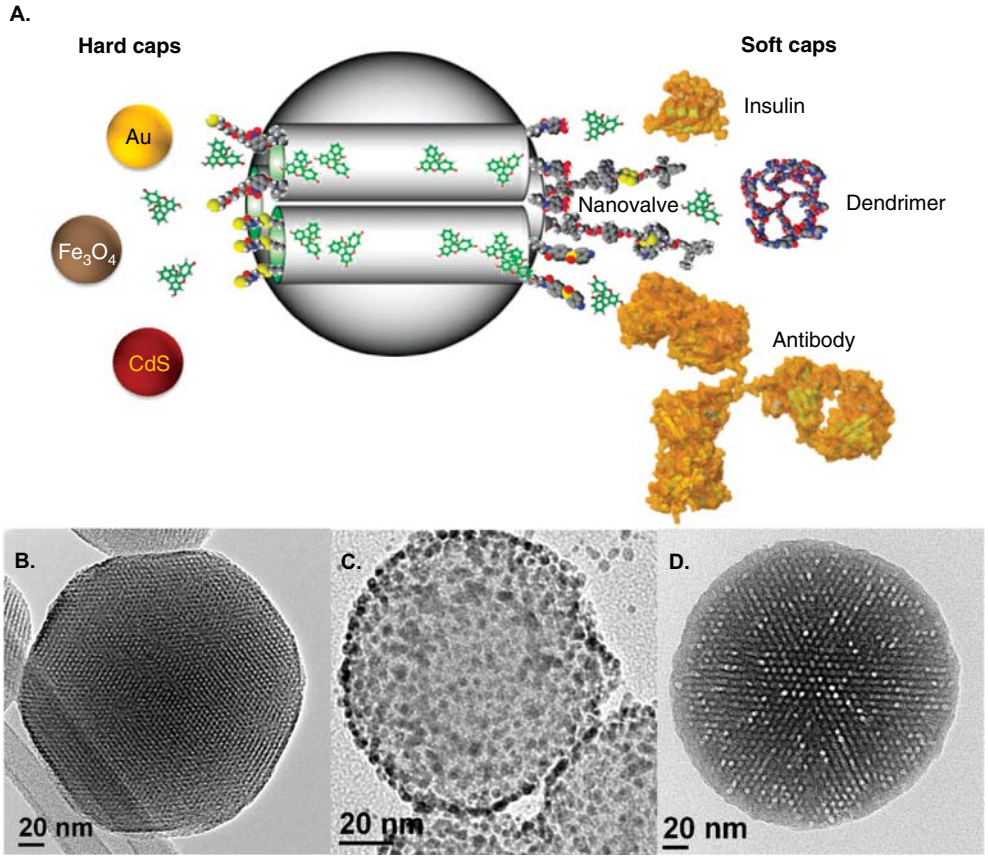

Figure 1. A. Schematic representation of a MSN loaded with drugs and capped with hard caps and soft caps highlighted in this review. Transmission electron microscopy images of (**B**) a MSN along the axis of the mesopores, (**C**) capped with hard (Au NP) and (**D**) with soft (polymer) caps.
MSN: Mesoporous silica nanoparticle.

2.1 Intracellular uptake of MSN and *in vitro* biocompatibility

2.1.1 Intracellular uptake of MSN

After the first discovery by Lin and co-workers that MSNs are readily internalized by eukaryotic cells without inducing cytotoxicity and are capable of delivering DNA [2], intensive efforts have been directed to understanding the mechanism of cellular uptake and various biological applications of MSN *in vitro*. Many different research groups have demonstrated that MSN can be rapidly and efficiently endocytosed by a variety of mammalian cells, including cancer cells (HeLa, CHO, lung, PANC-1, breast cancer MCF-7, pancreatic RIN-5F), non-cancer cells (neural glia, liver, endothelial, skin fibroblast), macrophage, stem cells (3TL3, mesenchymal), and others [2,3,15-23].

To determine the efficiency and mechanism of the cellular uptake of MSN, several spectroscopic techniques have been used, including flow cytometry, confocal fluorescent microscopy, transmission electron microscopy (TEM) and differential interference contrast microscopy (DIC) [2,3,15-17,22,24]. The uptake efficiency (EC_{50}) of MSN by mammalian cells ranges from 1 to 50 μg/ml, dependent on the surface properties of the particles. The uptake occurs in a relatively short time frame, usually within 30 min of introduction of MSN into the culture medium [3,25]. By using confocal fluorescence microscopy, MSNs can be observed inside the cells and are capable of escaping from endosomes to reach the cytosol (**Figure 2D–F**) [3]. TEM micrographs also provide direct evidence on the internalization and subcellular localization of the particles (**Figure 2A–C**) [2]. Recently, Lin, Yeung and co-workers followed the uptake of individual MSNs into single cells by DIC, in real time [24].

As outlined below, various factors have been demonstrated to influence the efficiency, kinetics and mechanism of the intracellular uptake of MSN materials.

(1) *Surface property of MSN.* Lin and co-workers showed that the functionalization of the external surface of MSNs affects not only the efficiency of their internalization, but also the uptake mechanism and their ability to escape from the endosomal entrapment [3]. In general, positively charged MSNs have a higher endocytosis

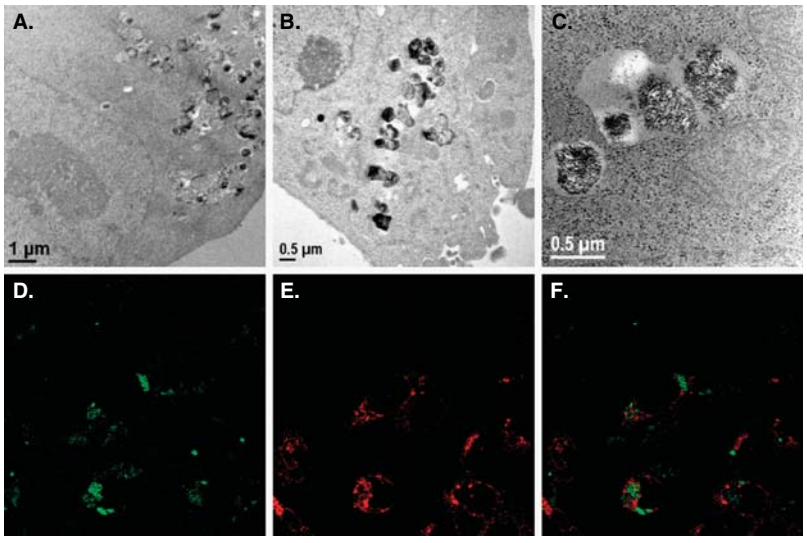

Figure 2. A – C. Transmission electron microscopy images of CHO cell with endocytosed MSN. **D – F.** Confocal fluorescence images of fluorescein-labeled MSN (green, **D**) endocytosed by HeLa cells co-stained with an endosome marker (FM 4 – 64, red, **E**). The merged images (**F**) show little coincidence of green and red spots (giving yellow), indicating that MSN (green) have already escaped from the (red) endosomes.
MSN: Mesoporous silica nanoparticle.

efficiency compared with negatively charged materials owing to the higher affinity to the negatively charged cell surface. The uptake of MSN has been found to take place mainly through a clathrin-mediated endocytosis, whereas some surface-functionalized MSNs, such as amine- and guanidinium-functionalized MSNs, enter the cells through a clathrin- and caveolae-independent mechanism. In addition, a notable increase in the endocytosis efficiency by cancer cells was observed for folic acid grafted MSN by means of folic acid receptor-mediated endocytosis. It has also been observed that MSNs with a highly negatively charged surface can easily escape from endosomal entrapment, as depicted in **Figure 2D–F**, probably attributable to the 'proton sponge effect'. Similar results were reported later by Mou and co-workers and Linden and co-workers [16,26].

(2) *Particle size and aggregation ability.* The particle shape, size and agglomeration effect on the endocytosis of MSN has been investigated by the Lin research group [21]. The smaller particles with higher dispersibility in aqueous solution were shown to be endocytosed with a higher efficiency and faster kinetics than the larger particles. Later, Mou and co-workers also studied the effect of particle size on cellular uptake of MSNs, showing that the maximum uptake by HeLa cells occurs at a particle size of 50 nm [23].

(3) *Particle morphology.* Lin and co-workers also reported that the cellular uptake of MSNs is morphology and cell line dependent [21]. A cancer cell line showed a higher endocytosis efficiency and rate for both spherical and tubular particles compared with a normal cell line. Interestingly, tubular MSNs achieved a more efficient uptake by cancerous and non-cancerous cells than the spherical ones. This was later confirmed by Tang and co-workers [27].

All these factors lead to the conclusion that intracellular uptake of MSN can be regulated by choosing an appropriate nanoparticle formulation, which opens the possibilities of achieving high specificity and efficacy of the intracellular controlled delivery of therapeutic agents.

2.1.2 *In vitro biocompatibility*

The biocompatibility of MSN with cellular systems has been tested by different methods. Studies on the viability and proliferation of various mammalian cells indicate that these properties are not affected by MSN at dosages < 100 µg/ml even after 6 days of incubation [2,3]. Cell morphology and membrane integrity are conserved after the internalization of MSN as determined by microscopic analysis and selective DNA staining followed by flow cytometry [3]. Colorimetric assay with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) shows that mitochondrial activity remains at normal levels after uptake of MSN [16]. However, the effects of MSN on diverse aspects of cellular metabolism still need to be evaluated more carefully and deeply. For example, although several studies have indicated that MSN internalization does not affect human mesenchymal stem cell (hMSC) morphology, viability, proliferation and differentiation capacities [25,28], Chen and co-workers have recently observed that the internalization of MSN induces a significant but transient protein response (actin polymerization, small GTP-bound protein RhoA activation) and the generation of

osteogenic signals in hMSCs [29]. Meanwhile, Asefa and co-workers also reported on the physical property-, concentration- and time-dependent effects of MSNs on cellular bioenergetics (cellular respiration and ATP content) [30]. These findings suggest that more research efforts should be directed to the detailed characterization of cellular activities with MSN internalization to provide comprehensive baseline information for the use of MSN as therapeutic drug carriers.

2.2 *In vivo* biocompatibility

Any clinical application of MSN is contingent on good biocompatibility. The small particle size of MSNs allows for their use as intravenous drug delivery systems. However, one prerequisite for intravenous administration of MSN drug carriers is their biocompatibility with red blood cells. Lin and co-workers have recently reported that in contrast to the pronounced hemolytic activity of amorphous silica, MSNs show high biocompatibility towards red blood cells at concentrations up to 100 μg/ml, as shown in **Figure 3** [4]. By comparing the hemolytic activities of different surface-functionalized MSNs and amorphous silica with the same surface functional groups, it was demonstrated that the enhanced biocompatibility of MSN with red blood cells is related to its unique honeycomb-like structure with arrays of mesopores where most silanol groups are hidden in the interior of the particles, resulting in a low surface density of silanols accessible to the cell membranes of red blood cells.

Also, recent investigations on the biodistribution and circulation properties of MSN in mice and rats demonstrated that the intravenous administration of these nanoparticles did not cause observable toxicity at doses < 200 mg/kg [5-8,31]. However, when the dosage is increased to > 200 mg/kg, toxic effects start to appear, as reported by Kohane and co-workers [31]. It should be noted that the dosage (1200 mg/kg) used in Kohane's study is two orders of magnitude higher than the one that would be necessary for drug delivery applications, especially considering the high drug loading capacity of MSN. Nevertheless, the biocompatibility of MSN could be further enhanced by surface functionalization. For example, PEGylated mesoporous silicates have been shown to be non-toxic in peripheral lung tissue [32]. Such surface coating strategy may also mitigate any systemic toxicity.

Hyeon and co-workers studied the biodistribution of the NPs (< 200 nm) in murine models of cancer, observing the accumulation of the NPs in tumors 24 h after injection. The authors attributed the localization to the enhanced permeability and retention (EPR) effect. They also observed NP accumulation in the rest of the organs, including liver and kidney, with no apparent toxicity [6]. In another study reported recently by Mou and co-workers [8], the intravenous injection of MSN (50 – 100 nm, surface modified with positively charged groups) led to accumulation of the NPs mainly in the liver (35.3%), followed by the kidney (9.0%), lung (8.3%), spleen (8.0%) and heart (4.5%). In a long-term biodistribution study, they also observed the accumulation of MSNs in the liver for up to 3 months without any apparent toxicity, suggesting that MSNs are resistant to decomposition and biocompatible *in vivo* at low concentrations [5]. These interesting findings are promising steps towards the *in vivo* biomedical application of these multifunctional nanoparticles.

In a few words, MSN materials have been demonstrated to be biocompatible drug carriers at dosage < 100 μg/ml for up to 1 week *in vitro* and < 200 mg/kg *in vivo* for up to 3 months. The final fate and long-term toxicity of MSNs should be investigated continuously before biomedical applications. Surface modifications should also be explored to reduce toxicity for applications in drug delivery and other biomedical fields.

3. Capped MSN-based stimuli-responsive controlled release systems

Since Vallet-Regí and co-workers proposed using mesoporous silica materials for drug loading and release [33], many mesoporous silica-based drug delivery systems have been studied and research efforts directed to attaining controlled release. The first capped mesoporous silica material for controlled release was reported by Tanaka and co-workers [34]. A reversible photo-triggered controlled release was developed by taking advantage of a photodimerization reaction of coumarin to control the opening and closing of the mesopores. However, these early applications were based on MCM-41 materials without defined shapes or monodispersed sizes. Shortly after Tanaka's report, Lin's research group prepared a CdS NP capped MSN for drug release, the uncapping of which was triggered by disulfide reducing agents [35]. The same research group later developed the first examples of using biocompatible MSNs as drug carriers and nanoparticles as capping agents for stimuli-responsive controlled release, the caps including chemically or physically removable nanoparticles such as Fe_3O_4 [17] and Au NPs [18,36]. In related works, Tseng, Nguyen and co-workers, designed a variety of stimuli-responsive nanovalves for the controlled release of dyes [37-39]. Although many of their early, established capping and release systems operated exclusively in non-aqueous solvents, they presented challenges in terms of the operational applicability under physiological conditions. Cyclodextrin (CD) and cucurbit[n]uril (CB[n]) were later used to increase the biocompatibility of the nanovalve systems [40,41]. However, intracellular drug delivery behaviors of these systems are still under investigation. Nevertheless, these systems greatly improved the diversity of capped mesoporous silica materials for stimuli-responsive controlled release with their 'on–off' reversible control. Many excellent reviews of nanovalve-based mesoporous silica materials for controlled release have been written by Liong and co-workers [42-44].

The cellular uptake and intracellular drug/gene delivery property of a capped MSN system was demonstrated for the first time by Lin and co-workers using G2-PAMAM-coated

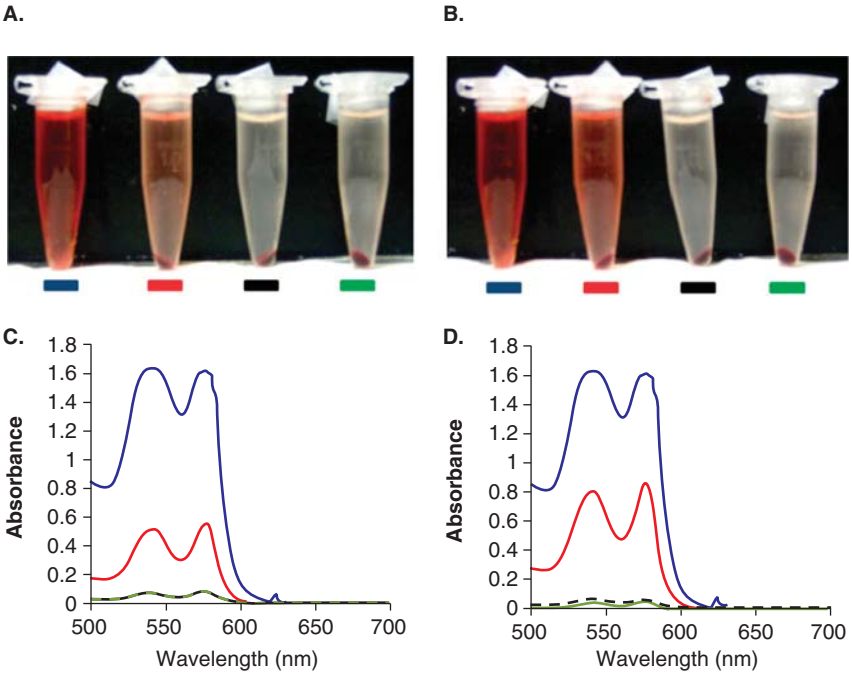

Figure 3. Hemolysis assay for amorphous silica (red lines) and MSN (green lines), compared with water as a positive control (blue lines) and PBS as a negative control (dashed black lines). The presence of hemoglobin (red) in the supernatant was detected visually (**A, B**) and by absorption at 541 nm (**C, D**) after centrifugation of the cells. The materials were suspended at (**A, C**) 60 and (**B, D**) 100 mg/ml.

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MSN: Mesoporous silica nanoparticle.

MSN as a vehicle to deliver plasmid DNA into astroglia, human and hamster cancer cells [2]. This system proved to be able to protect plasmid DNA against enzymatic digestion and induce the expression of enhanced green fluorescent protein (EGFP) in cells more effectively than commercial transfection reagents. This work opened the door to the design of many stimuli-responsive polymer-based soft caps and the investigation of drug delivery behavior of capped MSN systems inside cells. So far, only a few capped MSN systems have been applied to cellular systems for controlled drug/gene delivery and biocompatibility investigations [2,17-20,36], which is fundamental for future cell- and organ-specific delivery of therapeutics and other *in vivo* applications.

In this review, the capping agents that were introduced onto mesoporous silica materials are classified as hard caps such as CdS, Fe_3O_4 and Au NPs and soft caps including organic molecules, biomolecules and supramolecular assemblies. This review is focused on those capped MSNs that have been designed and/or applied to the intracellular controlled release of drugs and genes (**Table 1** and **Figure 1A**). Interestingly, among the various caps that have been exploited, biomolecules have emerged only recently as a new type of biocompatible capping agent for highly specialized tasks, including insulin for diabetes treatment, antibody for target-specific controlled release, and biotin–avidin for cell-targeted drug delivery, as will be described later.

In general, stimuli-responsive capped MSN systems have the following three features that distinguish them from other drug delivery materials.

1) Capped MSN can encapsulate a large payload of unmodified therapeutic compounds to achieve high intracellular concentrations, reducing undesired side effects resulting from leaching of the cargoes, and protecting the therapeutics from degradation by the environment. The mesoporous structure of MSN with tunable pore size offers the possibility of loading a large quantity of biogenic molecules, including antitumor drugs, imaging dyes, DNA, proteins and other chemicals of pharmaceutical interest, and in particular those that are cell membrane-impermeable or incompatible with biological fluids. In a typical cargo loading process, MSNs functionalized with organic groups (linkers) are incubated with concentrated drug solution to facilitate the diffusion of drug molecules into the mesopores of MSN. Capping agents are then added to the solution to block the pore entrance by forming covalent bonds or through electrostatic interaction with the

Table 1. Stimuli and triggers applied in capped MSNs for intracellular drug/gene delivery.

Stimuli	Trigger	Cap	Responsive moiety of linker or stalk	Ref.
Internal stimuli				
pH	Acid	PDDA	Carboxylic acid	[48]
	Acid	CD	PEI	[49]
	Acid	CB[6]	Trisammonium	[50]
	Acid	CB[6]	Dialkyl-4,4′-bipyridinium (viologen)	[51]
	Acid	Borate	Saccharide	[69]
Temperature	Temperature increase	PNIPAM	Thermoresponsive polymer	[53,54]
Redox	DTT, ME	CdS NP	Disulfide	[35,46]
	DTT, DHLA	Fe_3O_4 NP	Disulfide	[17]*
	DTT	Au NP	Disulfide	[36]*
	DTT, TCEP	PAMAM dendrimer	Disulfide	[2,46]*
	DTT	Crosslinked PNAS	Disulfide	[70]
	DTT	Polyelectrolyte multilayers PEM-aptamer	Disulfide	[20]*
Biomolecule				
Enzyme	PLE	α-CD	Ester-linked adamantyl stopper	[41]
	β-D-Galactosidase	Lactose	Glycosidic bond	[59]
	Protease (trypsin)	Biotin–avidin	Avidin	[58]
Blood sugar	Glucose	G-Insulin	Phenylboronic acid	[19]*
Antigen	STZ	Antibody	Hapten	[60]
External stimuli				
Light	UV light	Au NP	*o*-Nitrobenzyl ester	[18]*
	UV light	β-CD	*o*-Nitrobenzyl ester	[65]
	UV light	Py-β-CD	Azobenzene stalks	[63]
Ultrasound	Ultrasound	Ferrocene	Amide	[67]
Electric field	Voltage	β-CD	Ferrocene	[68]
Double/multiple stimuli	Visible light, pH	Carboxylate-terminated G1.5 PAMAM	Spiropyran	[64]
	UV, pH	Au NP	Phenylboronic acid	[45]
	UV, pH	CB[6], Azobenzene nanoimpeller	Trisammonium	[61]
	UV, DTT, α-CD	Crosslinked β-CD-bearing PNAS	Diazo, Disulfide	[62]
	α-Amylase, lipase, UV	β-CD	CD, ester, *o*-nitrobenzyl ester	[66]

*Intracellular drug/gene release and *in vitro* biocompatibility have been tested with mammalian/plant cells.
CB[6]: Cucurbit[6]uril; CD: Cyclodextrin; DHLA: Dihydrolipoic acid; DTT: Dithiothreitol; ME: Mercaptoethanol; MSN: Mesoporous silica nanoparticle; PAMAM: Polyamidoamine; PDDA: Poly(dimethydiallylammonium chloride); PEI: Polyethyleneimine; PLE: Porcine liver esterase; PNAS: Poly(*N*-acryloxysuccinimide); PNIPAM: Poly(*N*-isopropylacrylamide); STZ: Sulfathiazole; TCEP: Tris(2-carboxyethyl)phosphine.

linkers on the surface of MSN, so that loaded cargoes are prevented from leaching out of the mesopores. Physisorbed drug molecules could be washed off and the loading could be calculated by subtracting the amount of cargo remaining in the solution and that has been washed off from the initial concentrated drug solution. Typically, the amount of cargo that can be loaded in the mesoporous channels of MSN varies from 0.2 to 50 μmol/g. In contrast to conventional drug carriers that require covalent attachment of therapeutic compounds to their matrices, a capped MSN system does not require any modification of the drug molecules but physically traps them inside the mesopores. Capping prevents the loaded species from leaching out and allows drug release only in the presence of specific stimuli that trigger the removal of the caps. In this aspect, the structural integrity and consequently the pharmacological properties of encapsulated drugs can be retained. These encapsulation methods make possible the simultaneous delivery of one or more therapeutic agents to achieve synergistic therapeutic outcomes. Furthermore, the pharmacokinetic properties and biodistribution of the payloads can be controlled by manipulating the surface properties of the capped MSN carrier.

2) The capping agent can provide extra functionality to the MSN drug carrier, such as cell targeting, facilitating endosomal escape, loading more therapeutics and serving as diagnostic agents. For example, by using superparamagnetic iron oxide nanoparticles as

removable caps for MSN [17], Lin and co-workers were able to manipulate cells that had internalized Fe_3O_4-MSN by applying an external magnetic field. The combination of this magnetic motor effect with the stimuli-responsive controlled release property, also demonstrated for this capped MSN, showed that it was possible to direct therapeutic agents to cells or tissues of interest by loading them inside the material. Gold nanoparticles have also been well established as hard caps for MSN [18,36]. Besides their role as biocompatible caps, Au NPs can also increase the density of individual MSNs to enable their use with a gene gun system. In that way, Lin and co-workers were able to demonstrate for the first time the ability of Au NP capped MSN to act as a co-delivery agent of a gene and its promoter into plant cells (**Figure 4**) [36]. The laser-induced plasmonic heating property of Au NPs was utilized for triggering drug release in addition to other stimuli-cleavable chemistries. This has been demonstrated recently by Martínez-Máñez and co-workers in a pH and near infrared (NIR) laser-controlled delivery system [45]. The local plasmonic heating induced by a NIR laser resulted in the cleavage of the boronic ester linkage between the Au NPs and the MSN, allowing the release of entrapped Safranine O molecules. The cap itself could also play a role in therapeutic treatment. For example, insulin is known to regulate blood glucose level in the treatment of diabetes. In a glucose-responsive double delivery system recently published by the Lin group, gluconic acid-modified insulin (G-insulin) was used both as a cap to control the delivery of cyclic AMP to pancreas beta-cells and as a therapeutic agent to regulate directly blood glucose levels (**Figure 5**) [19].

3) The versatile and selective surface functionalization of MSN allows pore uncapping and drug release with a high degree of control. The introduction of one or more types of stimuli-responsive functional group to the capped MSN system enables them to perform a series of special tasks on command, as described in the next section. The diffusion of encapsulated molecules can be controlled by selectively decorating the interior surface and by choosing appropriate caps; in other words, the release kinetics of drugs can be tuned to match the needs of the biological system of interest. For example, by using real-time imaging Yeung, Lin and co-workers demonstrated that the kinetics and amounts of ATP encapsulated in MSN could be tuned by using different types of cap. The study revealed that hard nanoparticle caps such as CdS are more suitable for the fast release of relatively small amounts of payload, whereas flexible soft caps such as PAMAM dendrimers are more convenient for slow and sustained release of larger amounts of cargo [46].

Above all, the ability to functionalize independently each section of capped MSN (interior surface, exterior surface and caps), along with the good biocompatibility and tunable endocytosis efficiency, makes these capped MSN materials with multiple, orthogonal and controllable functions for biomedical applications.

4. Stimuli-responsive controlled release mechanisms

To achieve precise spatial and temporal delivery of therapeutic agents to target sites, a variety of stimuli-responsive groups have been introduced to MSN, including groups that respond to stimuli found in the interior of biological systems (pH, temperature, redox potential and biomolecules) and stimuli that can be applied externally from biological systems (light, ultrasound and electrical field). Various responses to stimuli are feasible, including bond cleavage, competitive binding and conformational changes. Capped MSN systems are designed to take advantage of these responses and to trigger the release of encapsulated molecules. An overview of stimuli and triggers that have been applied to capped mesoporous silica materials for controlled release and intracellular drug/gene delivery is given in **Table 1**. In this section, the most recent stimuli-responsive controlled release systems found in the literature are analyzed, with a particular focus on biomolecule, light and double-responsive controlled release systems.

4.1 Internal stimuli-responsive controlled release

Stimuli-responsive controlled release systems are especially advantageous when the triggering stimuli are unique to the targeted pathology. This allows the drug carriers to respond specifically to the desired species and release drugs in a self-regulated fashion. Examples of internal stimuli that have been exploited for intracellular drug and gene delivery include pH, temperature, redox state and some specific biomolecules such as enzymes, carbohydrates and antigens.

4.1.1 pH

The acidic pH found in tumor and inflammatory tissues (pH ~ 6.8) as well as in the endosomal and lysosomal compartments of cells (pH ~ 5 – 6) provides a potential internal trigger for the release of drugs from a pH-responsive drug carrier [47]. To exploit this condition the carrier must be stable at physiological pH (~ 7.4) but release its encapsulated payload in acidic environments. A series of pH-responsive caps including polyelectrolyte, pseudorotaxanes and organic molecules have been used for controlling the release of drug molecules, as summarized in **Table 1** [48-51]. Although exciting, none of these systems has been tested with cells or animals for intracellular pH-responsive controlled release, possibly owing to the weak response of these systems at mildly acidic conditions.

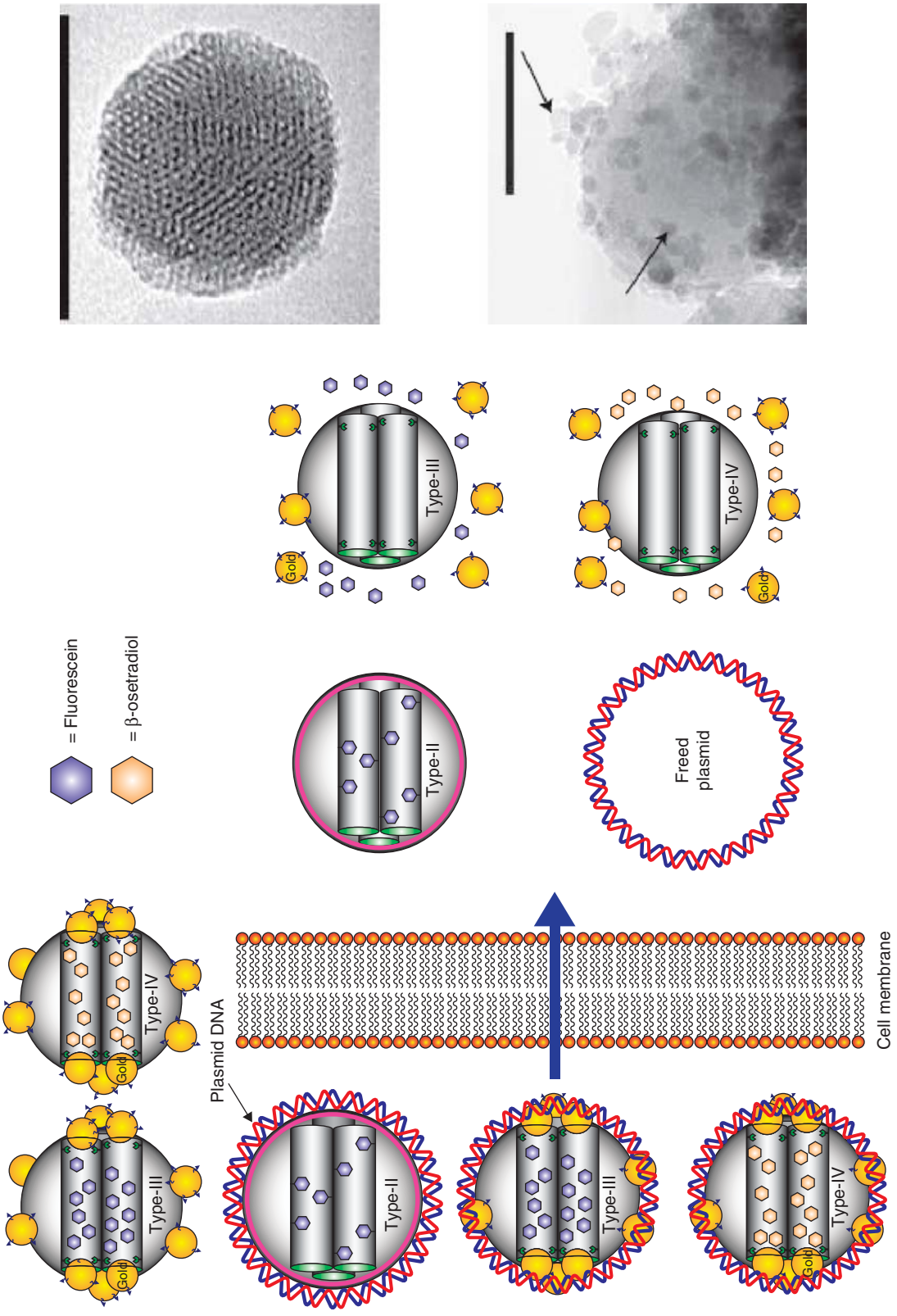

Figure 4. Schematic representation of a series of surface-functionalized MSNs for intracellular controlled release of genes and chemicals into plant cells triggered by addition of reducing agent, DTT.

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DTT: Dithiothreitol; MSN: Mesoporous silica nanoparticle.

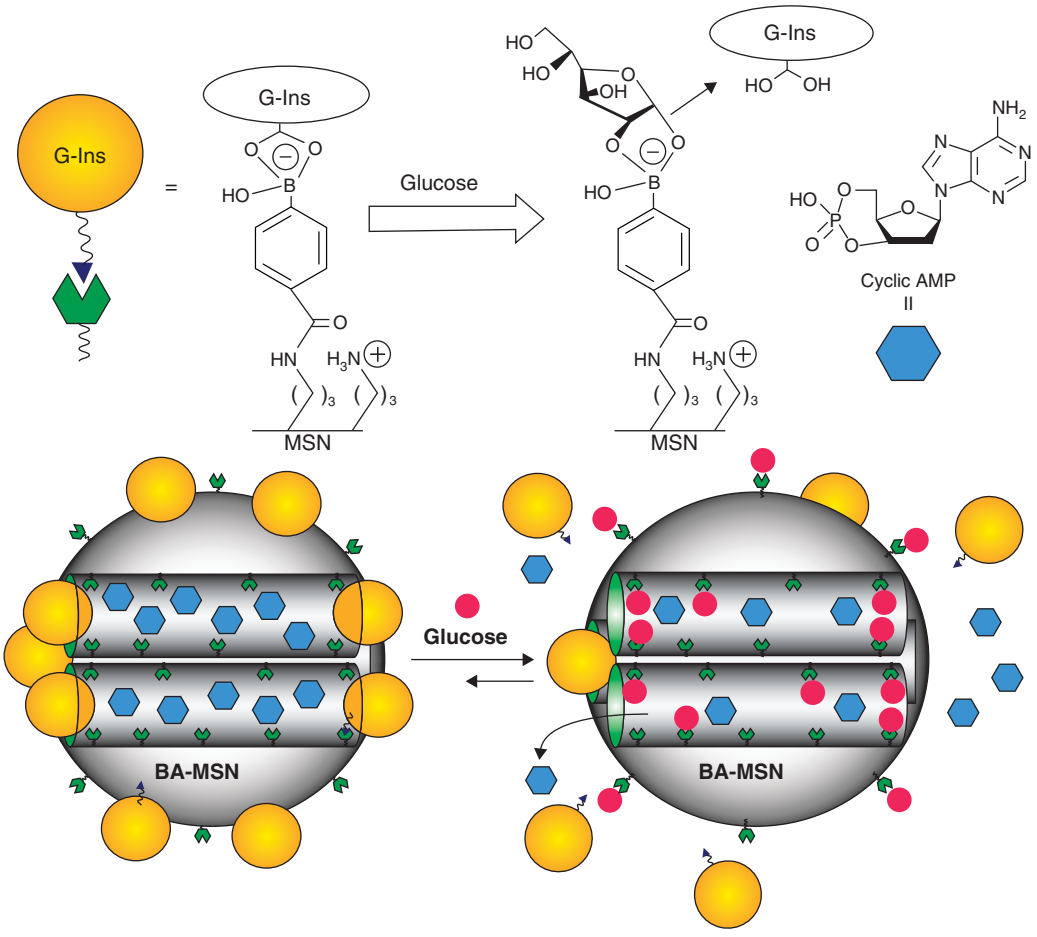

Figure 5. Schematic representation of the glucose-responsive MSN-based double delivery system for controlled release of bioactive G-Ins and cyclic AMP. The controlled release mechanism was achieved by means of the displacement reaction between blood glucose and G-Ins based on reversible boronic acid-diol complexation. High glucose concentration triggers the G-Ins uncapping and the release of cyclic AMP sequentially to diminish the higher than normal level of blood glucose.
Reproduced with permission from [19].
G-Ins: G-insulin; MSN: Mesoporous silica nanoparticle.

4.1.2 *Temperature*

Temperature is another internal stimulus that can be exploited for triggering drug/gene release at specific sites. For example, it has been shown that the local temperature in many tumors is slightly higher than normal body temperature. Therefore, a temperature-responsive drug carrier that releases drugs only at temperatures > 37°C but keeps the drugs encapsulated while in circulation is highly desirable. Poly-(*N*-isopropylacrylamide) (PNiPAM) is a well-studied thermoresponsive polymer for controlled release studies. By growing PNiPAM on the external surface of MSN, Lin and co-workers were able to control thermally its partition between water and toluene, which could lead to applications in temperature-dependent phase transfer and thermoresponsive controlled release in different solution environments [52]. López and co-workers prepared PNiPAM-coated mesoporous silica microparticles and demonstrated that the coated particles were able to absorb fluorescein from an aqueous solution at high temperature and release their cargo to a fresh solution on temperature increase [53,54]. However, it should be noted that depending on the pore size of the particle, the drug loading and release behavior could be different [55,56].

4.1.3 *Redox potential*

It is known that intracellular glutathione (GSH) levels in most tumor cells are 100 – 1000-fold higher than the extracellular levels, therefore the naturally occurring redox potentials between the mildly oxidizing extracellular space and the reducing intracellular space can be utilized as a stimulus to trigger the release of encapsulated molecules from drug carriers [57]. The potential of disulfide bonds to reduce into free thiols as a response to this reductive condition has attracted much attention for the design of redox-responsive controlled release systems. This has been well established by the use

of disulfide linked nanoparticles (CdS, Fe_3O_4 and Au NP) [17,18,35,36] and PAMAM dendrimers [2,46] as capping agents. Remarkably, these redox-responsive capped MSN systems have been utilized as intracellular delivery devices for dyes, drugs and genes into mammalian and plant cells [17,20,36].

In an effort to extend the applications of MSN as an intracellular delivery agent for plant cells, Lin and co-workers were able to deliver DNA and chemicals into plants by the use of Au NP-capped redox-responsive MSNs, as depicted in **Figure 4** [36]. The MSN materials were first loaded with β-estradiol, which is a promoter for the activation of a green fluorescent protein (GFP) encoding plasmid DNA (GFP pDNA) to be delivered into the plant cells, and the pore entrances were then capped with Au NPs by means of a disulfide bond. The Au NP-capped MSN was then coated with the GFP pDNA and introduced into the plant cells by a gene gun. Interestingly, the plant cells could express the delivered gene only when the reducing agent was added by perfusion to induce the release of the entrapped β-estradiol for DNA activation. This demonstrated for the first time that MSN has the ability to co-deliver different chemicals into plant cells with a precise control of location, time and the sequence of release.

Later, the strategy of introducing disulfide bonds for redox-responsive controlled release was also used by Yang, Wang and co-workers, when they used it for the reversible crosslinking of a polyelectrolyte multilayer-coated MSN (MSN-PEM) [20]. On addition of disulfide reducing agent dithiothreitol (DTT), they were able to observe the release of loaded fluorescein. Interestingly, a cancer-specific DNA aptamer was also attached to the MSN-PEM for targeted drug delivery. By comparing the endocytosis efficiency of aptamer-bound MSN-PEM with the aptamer-free system, as well as the viability of cancer and the non-cancerous cells when incubated with these two materials loaded with anticancer drugs, they demonstrated cell-targeted redox-responsive controlled release ability of the aptamer-bound MSN-PEM.

4.1.4 *Biomolecules*

Biomolecules have recently emerged as a new type of internal stimulus that has attracted a growing interest owing to their biocompatibility and interesting biological activities. So far, the types of biomolecule that have been introduced to capped mesoporous silica materials include enzymes, blood sugars and antigens.

In a first proof-of-concept, Patel *et al.* developed an enzyme-responsive capped mesoporous silica material [41]. The material was loaded with luminescent rhodamine B (RhB) and capped with [2]-rotaxane by threading β-cyclodextrin onto a polyethylene glycol stalk and held with an ester-linked adamantyl stopper. The release of RhB was observed only on addition of porcine liver esterase (PLE), resulting in the hydrolysis of the adamantyl ester, which led to the dethreading of the [2]-rotaxane. In another example, Bein and co-workers exploited biotin–avidin as a protease-responsive cap to encapsulate fluorescein molecules [58]. The controlled release was achieved by the addition of the protease trypsin, leading to the hydrolysis of the attached protein avidin and the release of the entrapped fluorescein dyes. Recently, Bernardos *et al.* developed a lactose-capped mesoporous silica support with RhB dye encapsulated and capped by a network of lactose linked by hydrogen bonding interactions [59]. The presence of β-D-galactosidase caused the hydrolysis of glycosidic bond in the anchored lactose, leaving only a glucose derivative on the surface. This decrease in the size of the capping agent induced the release of the entrapped dye. In addition, β-amylase and lipase have also been used as triggers in a multiresponsive controlled release system, as will be described later.

Blood sugars have been shown to be excellent biomolecular triggers by the Lin group in the development of a glucose-responsive double delivery system for sequential delivery of insulin and cyclic adenosine monophosphate (cAMP), as illustrated in **Figure 5** [19]. As mentioned above, G-insulin was exploited to encapsulate cyclic AMP inside mesopores and also served as a therapeutic agent to regulate blood glucose level. Phenylboronic acid linkers on the external surface of MSN could sense the glucose level and regulate the pore opening and closing. A competitive binding between G-insulin and saccharides with phenylboronic acid resulted in the G-insulin uncapping once a higher than normal blood glucose level was encountered. Surface zeta-potential change on the G-insulin uncapping enhanced the cellular uptake of the material for efficient intracellular cAMP delivery. The fast insulin release (within 30 min) is especially important for diabetic patients requiring high dosage of insulin after meals, and the sustained intracellular release of cAMP can induce insulin production from pancreas beta cells in between meals for a long-term effect. This co-delivery system with control over the sequence of release is particularly attractive for biomedical applications.

Also, antigens were exploited as biomolecule-based stimuli for triggering drug release from an antibody-capped mesoporous silica nanocarrier [60]. Martínez-Máñez and co-workers attached a hapten linker to the external surface of a Ru $(bipy)_3^{2+}$ dye-loaded mesoporous silica and capped the pores with a polyclonal antibody. A selective uncapping of the pores with consequent release of the dye was observed by addition of sulfathiazole (STZ) antigen by means of a displacement reaction. The use of bio-controlled drug delivery systems is highly appealing for a wide variety of biomedical applications.

4.2 External stimuli-responsive controlled release

4.2.1 *Light*

Light is very attractive as a remote control for the site-specific delivery of drugs. In principle, the release of entrapped molecules can be rapidly induced on exposure to light at a specific time and location without any change in the chemical environmental. Suitable chromophores, such as azobenzene [61-63], spiropyran [64] and a photocleavable linker *o*-nitrobenzyl

ester [18,65,66] have been incorporated into capped MSN systems to render them susceptible to light for photoresponsive controlled release.

Recently, Kim and co-workers reported a photoresponsive cyclodextrin-capped MSN by the introduction of a photocleavable *o*-nitrobenzyl ester linker. On irradiation at 350 nm, this system was able to release preloaded calcein [65]. At the same time, a photoresponsive Au NP-capped MSN was reported by Lin *et al.,* as shown in **Figure 6** [18]. The gold nanoparticles were functionalized with the photocleavable linker thioundecyl-tetraethyleneglycoestero-nitrobenzylethyldimethyl ammonium bromide (TUNA), and were incorporated onto the MSN surface by means of electrostatic interaction. On irradiation with UV light, TUNA is converted to the negatively charged thioundecyltetraethyleneglycolcarboxylate (TUEC), leading to the dissociation of the Au NPs from the MSN surface owing to charge repulsion, with the consequent release of the cargo from the mesopores. This system was then loaded with the anticancer drug paclitaxel and administered to fibroblast and liver cells. The drug-loaded MSNs were readily endocytosed by the cells without inducing any cytotoxicity, indicative of 'zero premature release'. On irradiation under biocompatible conditions, the preloaded drugs were released, leading to significant cell death. An alternative approach by Ferris *et al.* took advantage of the difference between the binding affinity of β-CD with the *cis* and *trans* isomers of azobenzene to control the release of cargo molecules from MSN [63]. Irradiation of azobenzene stalks with 351 nm light induced the isomerization from the *trans* to the *cis* isomer, resulting in the β-CD rings dethreading from the stalks and releasing a previously loaded cargo.

4.2.2 *Other external stimuli*

Ultrasound can also serve as a stimulus to trigger the release of drugs and to achieve the targeted delivery by local sonication. An ultrasound-responsive MSN system was developed recently by Kwon and Lee by means of amide bond coupling of a carboxy-substituted ferrocene complex and an aminopropyl functionalized MSN [67]. They demonstrated that the complex could be cleaved on ultrasound irradiation, consequently opening the pores of MSN.

Redox-responsive drug carriers may also find applications in the externally controlled release of drugs by applying electric current. For example, Khashab *et al.* used the inclusion complex between β-CD and ferrocene to encapsulate rhodamine B [68]. By applying a voltage (+1 V) to the solution, ferrocene threads were oxidized to the positively charged ferrocenium ions, resulting in the dethreading of the β-CD macrocycles and release of their cargo.

4.3 Double stimuli-responsive controlled release

Dual-controlled or multiresponsive controlled release systems are able to use two or more stimuli either in an independent or in a synergistic fashion, which opens the possibility of developing more complex controlled release behaviors.

The first dual-controlled system was reported by Martínez-Máñez and co-workers based on the use of a spiropyran photochrome. The compound they used can be reversibly transformed from a neutral spiropyran structure into a positively charged merocyanine by irradiation with UV light. The merocyanine form is stable when kept in the dark but converts into the spiropyran on irradiation with visible light or by heating [64]. The researchers anchored the positive merocyanine moieties to the pore entrance of mesoporous silica and added carboxylate-terminated G1.5 PAMAM to block the pores by means of electrostatic interaction. Two responses were established for this system: photoresponsiveness, achieved through merocyanine transformation to the neutral spiropyran form by irradiation with visible light; and pH-responsiveness by decreasing the pH to neutralize the carboxylate groups of the dendrimer. Both stimuli proved to be capable of disrupting the electrostatic interaction between the negatively charged dendrimer and the positively charged merocyanine-functionalized surface, leading to the release of preloaded dye molecules. The same authors demonstrated recently a pH and photoresponsive Au NP-capped mesoporous silica material [45]. The interaction between boronic acid functionalized Au NP and saccharide-derivatized mesoporous silica surface could be reversibly regulated by pH owing to the formation of boronic esters. The laser-induced plasmonic heating properties of Au NP account for the photoresponsiveness of this system.

Another pH and photoresponsive controlled release system based on the combination of pH-responsive nanovalves and light-responsive nanoimpellers was reported recently by Angelos *et al.* [61]. They demonstrated that the release of the encapsulated molecules requires activation of both stimuli, acting as an AND logic gate. Feng and co-workers published a multiresponsive supramolecular capped mesoporous silica system by grafting β-CD-bearing polymer on the surface of mesoporous silica and crosslinking by the addition of disulfide groups to form a polymeric network that blocked the pores [62]. They demonstrated the release of preloaded calcein dye on UV irradiation, on addition of α-CD and on the introduction of disulfide reducing agents such as DTT, causing the isomeric transformation of azobenzene groups, displacement of β-CD by α-CD, and cleavage of disulfide bond between β-CD and polymer main chains, respectively. In addition, an enzyme- and photoresponsive cyclodextrin-capped MSN was established by Kim and co-workers [66]. The CD was anchored on the MSN surface through an *o*-nitrobenzyl ester-containing stalk, which could be ruptured by UV irradiation or hydrolyzed by lipase. Besides, the CD caps could also be degraded by α-amylase, allowing enzyme- and photoresponsive controlled release of guest molecules.

5. Conclusion

The development of several capped MSN systems containing stimuli-responsive linkers has shown promising properties for the intracellular delivery of drugs and nucleic acids. Capped

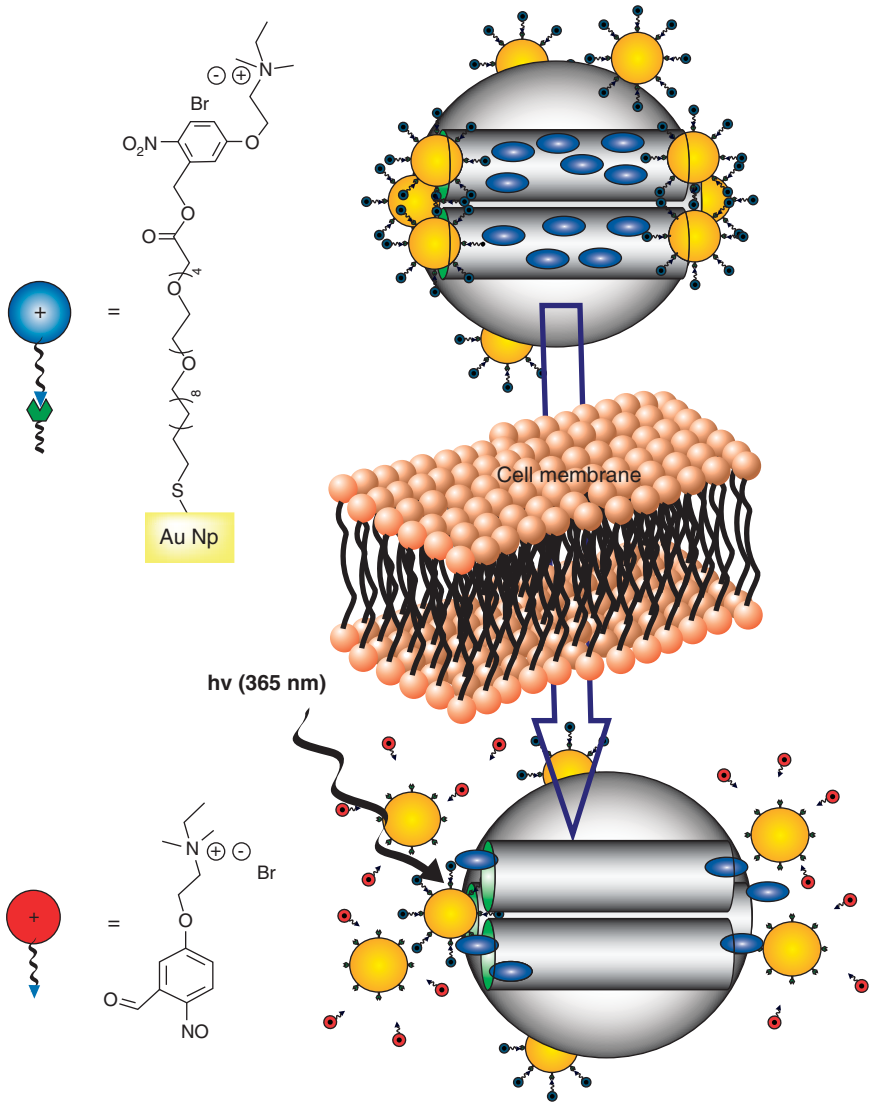

Figure 6. Schematic representation of a photoresponsive gold nanoparticle-capped MSN. On UV irradiation, the photolabile linker on the Au NPs was cleaved, changing the surface charge property (zeta-potential) of these gold nanoparticles from positive to negative. The charge repulsion between the Au NPs and MSN would then uncap the mesopores and allow the release of guest molecules.

MSN: Mesoporous silica nanoparticle; NPs: Nanoparticles.

MSN-based controlled release systems are continuously evolving, giving rise to newer, more sophisticated multifunctional devices that are gradually approaching a state at which their biomedical application is imminent. It is particularly encouraging to witness the fast expansion of capping agents and controlled release mechanisms introduced to MSN drug carriers, which now includes the use of bioactive molecules (insulin, antibody and biotin–avidin) as capping agents and biomolecules of pharmaceutical interest (blood sugar, antigens and enzymes) as triggers. It is also remarkable to observe that one or more types of stimulus, drug or other functionality have been integrated into single MSN carriers to achieve highly specialized delivery tasks. The recent studies on the biocompatibility of these materials both *in vitro* and *in vivo* also lead us to believe that these stimuli-responsive capped MSN materials will find a wide variety of applications in the field of cell-targeted and organ-specific drug and nucleic acids delivery.

6. Expert opinion

Major efforts have been put forth to create increasingly sophisticated stimuli-responsive MSN materials that release one or more therapeutics with ever more control. Significant

challenges remain, however, as the controlled release properties of many of the existing stimuli-responsive capped MSN systems are yet to be tested with living cells or tissues, and most of them have not been tested in organisms. Furthermore, some systems have minimal potential to succeed as vehicles for therapeutics unless significant improvements are made to increase their response to real biological conditions. For example, some of the reported pH-responsive capped MSNs require relatively strong acidic environments (pH < 5) for pore opening and drug release, which is not compatible for *in vitro* or *in vivo* drug delivery. Although there is now a large number of release mechanisms available, the field remains open for the discovery of even more internal and external stimuli to expand the diversity of triggers for release by capped MSN. This progress will certainly provide even higher degrees of specificity and control in drug delivery by capped MSN. It is worthwhile highlighting that biomolecules with advantageous biocompatibility and selectivity properties have been actively investigated both as triggers and as capping agents in recent years. Still, reports on biomolecular caps are rare throughout the literature. More work is necessary to enlarge this promising area of the field.

The introduction of switchable properties to these stimuli-responsive capped MSNs is another subject for future development. Many of the established systems release drugs on uncapping irreversibly, which limits their applications. Besides using capping agents that can be reversibly controlled, switches could also be created by using guest molecules that interact with the capping agents or other sensing moieties once released, providing a closed feedback loop capable of self-regulating the amount and rate of release. Introducing such a reversible control to these materials will lead to active smart drug delivery devices.

It is also of particular interest to develop new biocompatible, capped MSN systems for intracellular delivery of bioactive molecules to reduce or enhance certain cellular activities. Intracellular delivery of cyclic AMP to stimulate insulin production [19] and the release of gene expression promoter β-estradiol inside cells for DNA activation [36] are examples of such a goal. Although a great number of anticancer drugs and imaging dyes have been loaded and released, demonstrations of controlled release of biogenic molecules, such as genes, enzymes and proteins, and other molecules of pharmaceutical interest are still scarce. In the future, it will be highly desirable to design a drug carrier that could be efficiently internalized by specific cells and participate in a variety of biochemical or catalytic reactions inside cells. Much work still lies ahead in developing such smart and biocompatible, capped MSN devices.

Despite recent encouraging progress in improving the biocompatibility of these materials, most of today's materials are still investigated outside biological systems. In other words, what sets today's synthetic materials apart from *in vivo* biomedical applications of tomorrow are the lack of proof on biocompatibility, cellular uptake and intracellular controlled release properties of these capped MSN materials. Further work is required to fully understand how these systems function both *in vitro* and *in vivo*. As more biocompatibility and drug delivery data both *in vitro* and *in vivo* become available, it is envisaged that these multifunctional stimuli-responsive capped MSN systems will play a key role in clinical and other biomedical and biotechnological applications.

Declaration of interest

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