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Targeted Drug Delivery

Enzyme-Responsive Controlled Release of Covalently Bound Prodrug from Functional Mesoporous Silica Nanospheres**

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To address the limitations of conventional drug delivery systems, many novel nanomaterials such as polymers,[1] liposomes,[2] dendrimers,[3] and inorganic nanoparticles[4] have been proposed as smart drug-delivery systems capable of site-specific delivery. Amongst these, mesoporous silica nanoparticles (MSNs) have attracted a lot of attention as a robust inorganic alternative to the polymeric nanoparticles owing to their high porosity, ease of modification, biocompatibility,[5] and physicochemical stability. MSNs have been utilized effectively as drug and bioactive molecule carriers,[6] magnetic resonance imaging (MRI) contrast agents,[7] and for the immobilization of enzymes.[8] The use of MSNs for on-command delivery of drugs or bioactive molecules has, however, only been developed in recent years. Several sophisticated systems based on MSNs have been described in which the efficient release of cargo from MSNs into targeted sites is regulated either by external stimuli, such as thermal,[9] electrostatic,[10] time,[11] light,[12] or by internal stimuli such as pH[13] or enzymes.[14] This stimuli-responsive approach is typically achieved by the functionalization of the external surface of the particles or the mesopore walls, yielding nanogates which can be opened selectively under stimulus conditions (e.g. pH, light, enzymes, or temperature) to release the cargo from the mesopores.[15] Nevertheless, despite numerous studies of stimuli-responsive structures, the cargo is rarely covalently bound to the MSNs; instead it is physically adsorbed into the pores,[14a] which may cause undesired leaching of the cargo before it reaches the target site. Also, in most of the studies on enzyme-responsive delivery using MSNs, the enzyme-responsive nanogating mechanism is not caused by the drug itself or bioactive molecule.

Furthermore, selective delivery of therapeutics to the colon by the oral route is critical for treating Crohn's disease, inflammatory bowel disease (IBD), and colon cancer. This selectivity has traditionally been achieved by delivery systems that are pH-, time-, or enzyme-dependent (prodrugs).[16] Among these approaches, targeting with prodrugs is favored because of their higher selectivity and site specificity.[17] Although vast efforts were devoted to design site-specific colon delivery systems, enzyme-responsive systems based on both passive targeting (using nanoparticles) and selectivity (towards azo-reductase) are seldom discussed.

To address the above issues, we have designed herein a novel bacterial azo-reductase-responsive delivery system for IBD (Scheme 1). For this, sulfasalazine (SZ, a prodrug first-line therapy for IBD) is covalently attached to the surface of MCM-48 mesoporous silica nanospheres acting as the enzyme-responsive carrier; the anchored SZ molecules can then be reduced into 5-aminosalicylic acid (5-ASA) and

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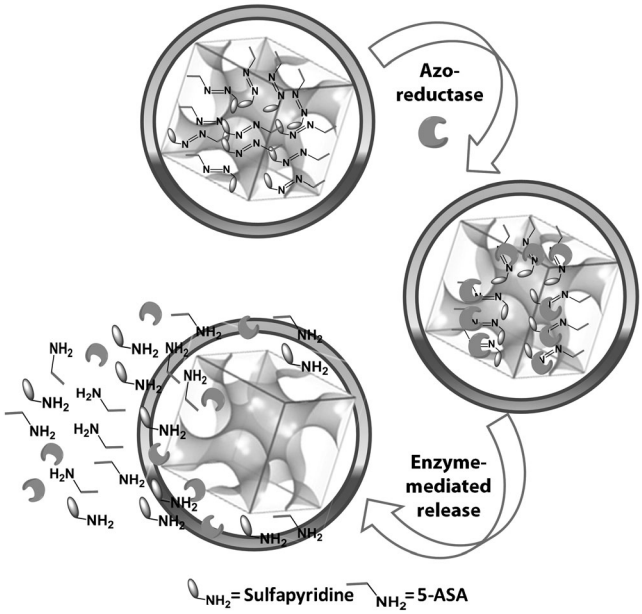


Scheme 1. Representation of the process of enzymatic release of 5-ASA and sulfapyridine from the 3D nanoreactors in the presence of azo-reductase.

sulfapyridine (SP) only inside the colon through interaction with azo-reductase. In our system, the functional MSNs will serve the dual purpose of targeted delivery and being a biocompatible carrier for this type of molecule. MCM-48 mesoporous silica nanospheres were chosen as the inorganic nanoscaffold owing to their unique 3D cubic pore structure, large enough pore size to accommodate biomolecules (3.2 nm),[8c] small particle size (< 200 nm), high surface area (approximately 1285 $m^2 g^{-1}$), and easily tunable surface chemistry. Additionally, the choice of MSNs was also motivated by their higher biocompatibility and lower cytotoxicity than solid silica spheres.[8a] Moreover, our recent studies demonstrated the superiority of the MCM-48 pore network over its 2D hexagonal counterpart (MCM-41) in terms of adsorption capacity and mass transport.[7,18]

Typically, the MCM-48 nanospheres were synthesized using a combination of cetyltrimethylamonium bromide (CTAB) and Pluronic F127 as structure-directing species at room temperature under basic conditions according to the procedure reported by Kim, et al.[19] Following this, the synthesis of enzyme-responsive nanocarriers was carried out in a two-step procedure. As shown in Scheme 2, MSNs were treated first with (3-iodopropyl)trimethoxysilane (IPTMS) to yield 3-iodopropyl-functionalized silica (**M-IP**). The 3-iodopropyl groups then undergo nucleophilic substitution with the drug SZ under alkaline conditions to covalently bind SZ forming **M-IP-SZ** (see Supporting Information for experimental details, Reaction S1). The **M-IP-SZ** particles were then washed several times with methanol to remove unreacted adsorbed species and dried under vacuum.[20]

Transmission electron microscopy (TEM) investigations show that the native MCM-48 nanoparticles are almost monodisperse nanospheres (about 200 nm in diameter) with a 3D cubic internal pore structure (Scheme 2 and Supporting Information, Figure S1). Further, the TEM analyses also indicate that the surface modification procedure was successful without altering the overall morphology and the mesostructure after IP and SZ attachment (Figure 1 a,b and Figure S1). Interestingly, sulfasalazine-attached particles (**M-IP-SZ**) are clearly coated with organic moieties (Figure 1 b). Also, partial disappearance of the mesopores is believed to be because of the attachment of SZ onto the MCM-48 nanospheres, forming a core–shell structure. These observations are also confirmed by low-angle powder X-ray diffraction (Figure S2). In terms of porosity, the native particles show type IV nitrogen adsorption–desorption isotherms with a sharp capillary condensation step at intermediate P/P_o, which is characteristic of the presence of uniform mesopores (Figure S3). A second capillary condensation step is also observed above $P/P_o = 0.95$, which can be attributed to the interparticle voids of the materials with small particle size (Figure S3). After functionalization, **M-IP** and **M-IP-SZ** exhibit typical isotherms of materials with mesopores filled with organics, and a significant reduction of pore size and surface area was observed (Figure S3, Table S1).[14a] Elemental analysis (EA) of the materials revealed a high percentage of carbon and the presence of nitrogen and sulfur in **M-IP-SZ** confirmed the attachment of SZ to the MSNs (Table S2). Thermogravimetric analysis (TGA) of the organic functional materials shows a total weight loss of about 21 % for **M-IP** and 24 % for **M-IP-SZ** (Figure S4). The very small difference in weight loss between **M-IP-SZ** and **M-IP** is thought to be due to the substitution of iodine, a heavy atom, with SZ. According to the TGA curves, the thermal decomposition of the 3-iodopropyl group and the propyl-linked SZ occurs in the interval of temperatures between 150 and 650 °C.

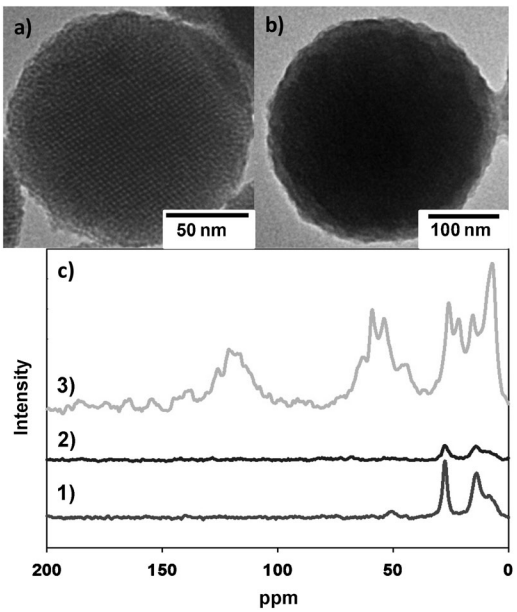
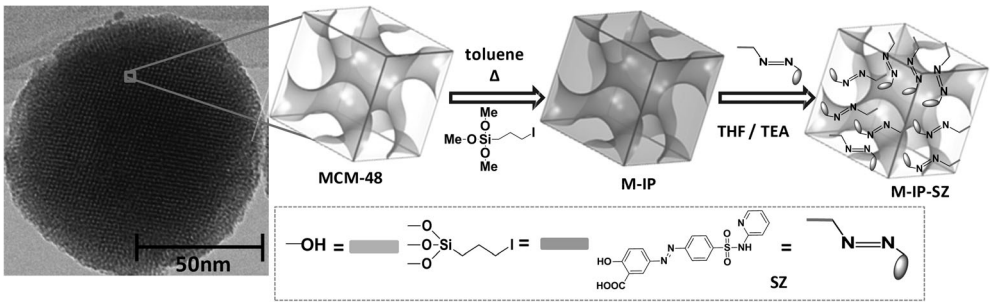


Figure 1. TEM images of a) IPTMS functionalized MCM-48 (**M-IP**), and b) sulfasalazine-attached MCM-48 (**M-IP-SZ**), c) ^{13}C CP NMR of 1) **M-IP**, 2) **M-IP-SZ** without TEA, 3) **M-IP-SZ** with TEA.

The dynamic light scattering (DLS) mean hydrodynamic diameter of the MCM-48 MSNs increases at each stage of functionalization, from 201 nm for the native MCM-48 particles up to 285 nm for **M-IP-SZ** (Table S1). This substantial increase in particle size (observed in suspension) could be attributed to attachment of bulky SZ molecules and partly also owing to aggregation. Furthermore, zeta-potential measurements were per-



Scheme 2. Synthesis and design of the enzyme-responsive drug delivery system based on mesoporous silica nanoparticles with a 3D pore structure. THF = tetrahydrofuran, TEA = triethylamine.

formed to monitor the evolution of the nanoparticle surface charges. MCM-48 MSNs show a zeta potential of −30 mV owing to anionic silanol groups. **M-IP** and **M-IP-SZ** exhibited similar zeta potential values of −40 mV and −20 mV, respectively, which is due to the residual silanol groups, with some modulation of the zeta potential caused by functionalization with organic groups. The highly negative zeta potential means that the particles are stable in suspension (Table S1). These changes in zeta potential and the particle size increase for **M-IP** (230 nm) and **M-IP-SZ** (285 nm) are both in line with the results of the EA and TGA analyses. Note that with a particle size of about 285 nm, **M-IP-SZ** remains small enough to be used for targeted delivery applications.[5a] Additionally, Fourier-transformed infrared (FTIR) spectra of the native and functionalized hybrid silica nanoparticles substantiated the proper surface functionalization (Figure S5). The absorbance bands related to SZ and the iodopropyl group were found in **M-IP-SZ** further validating successful chemical binding.

Solid-state ^{13}C CP NMR and ^{29}Si MAS NMR were performed to further confirm functionalization and to investigate the nature of the bonding between the propyl group and SZ (Figure 1c and Figure S6, respectively). The 3-iodopropyl functionalized MCM-48 particles (**M-IP**) show three distinct signals in the alkane region with chemical shifts at 8.5 ppm ($-\mathbf{C}H_2-I$), 13.9 ppm ($CH_2-\mathbf{C}H_2-CH_2$), and 27.6 ppm ($-\mathbf{C}H_2-Si$) corresponding to the propyl carbon atoms, and a fourth weak resonance is observed at 50.9 ppm indicating a small amount of residual methoxysilane groups ($Si-O-\mathbf{C}H_3$).[20a,21] In addition to the propyl signals in the 5–30 ppm region, **M-IP-SZ** displays resonances in the regions: 50–65 ppm owing to the C3 carbon atom of the propyl group, which forms a covalent bond with the N or O atoms of SZ; signals around 110–165 ppm can be ascribed to aromatic ring carbon atoms; and greater than 165 ppm is due to the carbonyl group. The number of resonances in the upfield region of the spectrum (less than 65 ppm) clearly indicates that a mixture of compounds is present, owing to the nucleophilic N and O atoms of SZ reacting with the 3-iodopropyl groups. These data confirm that SZ is covalently bound to the MSNs by way of C–N and C–O linkages. Notably, particles prepared in the absence of triethylamine (TEA) show no evidence of SZ attachment by ^{13}C CP NMR (Figure 1c) and elemental analysis (Table S2), indicating that the reaction occurs only in the presence of base.[20] The ^{29}Si MAS NMR data of native MCM-48, **M-IP**, and **M-IP-SZ** samples are depicted in Figure S6. The non-functionalized MCM-48 nanospheres show three signals in the Q region attributed to different types of silanol groups present on the surface of the silica. Signals at −93 ppm, −101 ppm, and −110 ppm correspond to Q^2, Q^3, and Q^4 species, respectively.[22] The spectra of the functionalized **M-IP** and **M-IP-SZ** reveal three signals in the Q region and two additional signals in the T region confirming the presence of Si–C bonds originating from the organosilane grafting, that is, yielding T^2 and T^3 signals.[13c]

The driving force for the triggered release of drug from **M-IP-SZ** is reductive cleavage of the aromatic azo bond of SZ, which is a substrate of azo-reductase, an extracellular enzyme produced by colonic anaerobic microflora, leading to formation of primary amines.[23,16] Hence, we hypothesized that both 5-ASA and SP will be released from the silica nanocarriers in the presence of the enzyme, co-delivering both molecules at the colonic site (Scheme 1 and Supporting Information, Reaction S2). To predict the viability of the enzyme-responsive system, in vitro drug release experiments were carried out in a simulated intestinal fluid with anaerobic bacteria that release azoreductase (for experimental details, see the Supporting Information). The release of 5-ASA and SP was monitored both qualitatively and quantitatively using UV/Vis spectroscopy and high performance liquid chromatography (HPLC), respectively (Figures S7 and S8).[24] Figure 2 shows the percentage release of 5-ASA and SP from **M-IP-SZ** at various pH values, with and without the presence of azo-reductase, confirming the selective process of reduction of enzyme-treated SZ into SP and 5-ASA. As expected, both molecules were released (5-ASA 35% and SP 55% release), which further validates our hypothesis of an attachment from two ends of the SZ molecule through a C–N or C–O bond. These results are corroborated by color changes (insert in Figure 2) wherein the SZ solution changes from yellow to colorless after bacterial conversion. Similarly, SZ-immobilized silica particles turn from yellow to white after bioconversion of the covalently attached SZ in **M-IP-SZ**, with no color change observed in samples without enzyme treatment, thus further verifying our hypothesis. Therefore, our study establishes that SZ remains covalently bound to the MSNs avoiding premature release in the absence of a trigger.

The reduction of pure SZ at pH 1.2 (simulated gastric fluid), pH 7.4 (PBS), and in PBS with bacteria was also tested to study the effect of pH on the release/degradation of SZ (Figure S9a). At pH 1.2 and at pH 7.4, no degradation of SZ was observed in the absence of bacteria. Conversely, more than 90% of SZ, in the form of pure SZ, was reduced in the presence of the bacteria. Moreover, no significant leaching of SZ was observed at pH 1.2 or pH 7.4 from **M-IP-SZ**, indicating that the bonding is strong enough to ensure that the functionalized MSNs can transit through the stomach

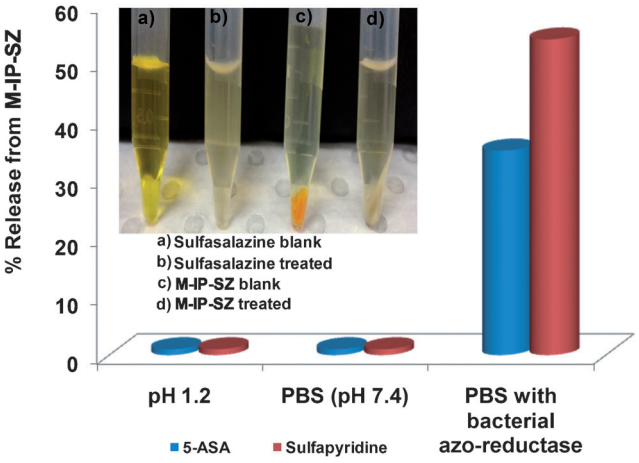


Figure 2. Percentage release of 5-ASA and SP from **M-IP-SZ** at different pH values and in the presence and absence of bacterial azo-reductase. Insert: color change owing to bioconversion of SZ into 5-ASA and sulfapyridine upon treatment with azo-reductase.

(pH 1.2) and intestine (pH 7.4) without undesirable SZ release, which proves its effectiveness as an enzyme-responsive carrier (Figure S9 b).

In conclusion, we have described a proof-of-concept design permitting the attachment of prodrugs on MSNs for controlled release of cargo in the presence of bacterial enzymes. We have used 3-iodopropyl functionalized MSNs as carriers, on which biological molecules can be attached to achieve stimuli-responsive release. In the presence of the azo-reductase, the system efficiently releases 32 mg of 5-ASA per gram of silica, which is ten times higher than previously reported methods.[25a] However, this value is still low in view of dosing necessities and needs to be improved for its clinical use. Nevertheless, when used orally, silica nanoparticles have shown high accumulation in inflamed tissues and release of drug at the site of inflammation, significantly reducing the effective dose.[25b] Further improvement can be achieved by specific C–N attachment of SZ to the MSNs, which avoids the release of SP and increases the release of 5-ASA. These matters will be addressed in our future studies, but overall **M-IP-SZ** shows promise as a treatment for IBD. Future work will include cell-adhesion and cytotoxicity studies of these nanoparticles. Additionally, other prodrug models will be investigated to further probe the versatility of this approach for colon cancer therapy. Finally, combination of the system with magnetic or optical probes integrated in the pores is possible to allow for coupled targeting/diagnostic applications.

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