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Stimulus-responsive Controlled Release System by Covalent Immobilization of an Enzyme into Mesoporous Silica Nanoparticles

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Abstract

Mesoporous silica nanoparticles (MSN) have emerged as an attractive class of drug delivery carriers for therapeutic agents. Herein, we explored the covalent immobilization of proteins into MSN to generate a stimulus-responsive controlled release system. First, MSN were functionalized with thiol groups using (mercaptopropyl)-trimethoxysilane (MPTMS). Functionalization was verified by X-ray photoelectron spectroscopy (XP), Fourier-transform infrared (FTIR) spectroscopy, and dynamic light scattering. The model enzyme carbonic anhydrase (CA) was coupled to sulfosuccinimidyl 6-[3'(2-pyridyldithio)-propionamido]hexanoate (Sulfo-LC-SPDP) at a low ratio of 1:1 to prevent enzyme inactivation and subsequently covalently immobilized into MSN via thiol-disulfide interchange. The enzyme could be released from MSN with 10 mM glutathione which represents intra-cellular redox conditions while it remained bound to the MSN at extra-cellular redox conditions represented by 1 μ M glutathione. The activity of the released enzyme was >80% demonstrating that the enzyme was still largely functional and active after immobilization and release. Human cervical cancer (HeLa) cells were incubated with the MSN-CA bioconjugates at various concentrations for 24 h and the data show good biocompatibility. In summary, we demonstrate the potential of MSN as potential drug delivery systems for proteins.

INTRODUCTION

There has been a growing interest in utilizing nanotechnology in the development of drug delivery systems.¹ Nanomaterials, such as polymers, mesoporous silica nanoparticles and nanotubes have emerged as potent drug delivery devices.^{2–4} Likewise, the employment of proteins including enzymes as therapeutic agents has increased due to their selectivity and catalytic efficiency.⁵ Due to recent advances in nanotechnology, the integration of nanosized materials (e.g., nanoparticles) and therapeutic agents (e.g., enzymes) is now conceivable.

Drug delivery systems provide important tools for enhancing the efficacy of therapeutic agents thus reducing unwanted side effects. For example, locally higher doses of chemotherapeutic agents can be delivered using nanoparticles with 100–300 nm in diameter because they accumulate preferentially in tumors.^{6,7} However, challenges remain in the design of nanosized delivery systems. For example, it is highly desirable that drug delivery systems show zero-premature release until the target is reached and that the release of the

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SUPPORTING INFORMATION Scanning electron microscopy micrographs of (A) MSN, (B) MSN-SH, and (C) MSN-CA. Residual activity of CA-SPDP bioconjugates at different levels of modification. This information is available free of charge via the Internet at <http://pubs.acs.org/>.

drug is controlled by a stimulus-responsive design.^{8,9} Stimuli can include pH,² electric fields,¹⁰ and photoirradiation⁵ and have been utilized for drug release.^{2, 9, 11} In general, stimulus-responsive release systems allow a smart release of drugs by responding to endogenous or exogenous activation.^{4,12–17} Recently, specific chemical reactions, such as disulfide reduction, have emerged as alternative mechanisms for drug release.¹⁶ The main driving force of the drug release in the cell is by reduced glutathione for such systems.¹⁸

Herein we selected silica as the material for the development of a nanosized delivery system. Once injected, MSN degrade into biocompatible monomeric silicic acid ($\text{Si}(\text{OH})_4$). Si is an essential human nutrient and been implicated in protecting against the toxic effects of aluminum and in promoting calcification.³ In recent years, the use of silica nanoparticles has been extended to biomedical and biotechnological fields, such as biosensors, DNA deliver, drug delivery, and enzyme immobilization.¹⁹ It has been demonstrated that MSN can be endocytosed by cells and thus can serve as carriers for the controlled intracellular release of drugs.^{20–23} In addition, MSN are capable to escape the endosomal entrapment^{6, 21, 24–26} and can potentially protect the delivered protein drug from exposure to proteases. In addition, the large pore volume that MSN possess allow for loading of large protein amounts into the nanoparticles.^{21, 24–26} Slowing *et al.* encapsulated cytochrome c (Cyt-c) into MSN and realized studies related to the uptake and release of Cyt-c by mammalian cells.²¹ They demonstrated that the Cyt-c remained active after its release from MSN. However, as far as we know, there are no reports on conjugating enzymes into MSN via stimulus-responsive bonds, which would allow for the smart release under physiological conditions solely in the presence of a targeted stimulus. This was the goal of our work.

Herein, we report and demonstrate the use of chemically functionalized MSN to form conjugates with an enzyme via formation of stimulus-responsive covalent bonds (Figure 1). Carbonic anhydrase (CA) was selected as the model biological molecule since it is a well-characterized enzyme with easily measurable bioactivity, which makes it an ideal candidate for the proof-of-concept of our system.^{27,28} CA (EC **4.2.1.1**) in this study was from bovine erythrocytes and thus belongs to the group of α -CAs, Zn^{2+} containing metalloenzymes. CAs catalyze the conversion of CO_2 and H_2O to H_2CO_3 , essential to various biological processes including maintaining the acid-base balance in blood and tissues, enabling efficient transport of carbon dioxide out of tissues, and increasing the CO_2 concentration in chloroplasts.²⁹ We demonstrate that CA can be released from the silica nanoparticles under conditions emulating intra- but not extra-cellular redox conditions with more than 80% of residual activity. The system developed is the first step in the direction of a smart protein delivery system which could be used to treat various diseases.

EXPERIMENTAL PROCEDURES

Mesoporous silica nanoparticles (product number 643637), (3-mercaptopropyl)trimethoxysilane (MPTMS), 4-nitrophenyl acetate (*p*-NPA), L-glutathione, dithiothreitol (DTT), toluene (99.9%) and acetone (99.9%) were from Sigma-Aldrich (St. Louis, MO). Carbonic anhydrase (CA, EC **4.2.1.1**) from bovine erythrocytes was from Worthington Biochemical Corporation (Lakewood, NJ). Sulfosuccinimidyl 6-(3'-[2-pyridyldithio]-propionamido)hexanoate (Sulfo-LC-SPDP) was from Proteochem (Denver, CO). The CellTiter 96® AQueous non-radioactive cell proliferation assay was from Promega (Madison, WI). HeLa cells were purchased from ATCC (Manassas, VA). Dialysis membranes were obtained from Spectrum Laboratories (Rancho Dominguez, CA). All reagents were used as supplied without further purification.

Thiol-functionalized Mesoporous Silica Nanoparticles (MSN-SH)

MSN were activated by functionalization with thiol groups. The activation was done by the addition of 3.75 ml of (mercaptopropyl)-trimethoxysilane (MPTMS, 95%) to 3.75 ml of a stirred suspension of 125 mg of MSN in refluxing toluene at 110°C for 1 h. After slow cooling of the mixture, the solid phase was recovered by filtration and washed with 200 ml of toluene and 500 ml of acetone. The product was dried overnight under vacuum.

X-ray Photoelectron Spectroscopy (XPS)

XPS was performed using a PHI 5600ci spectrometer with an Al K α monochromatic source (350 W, 15 kV) and at a takeoff angle of 45°. Survey spectra were obtained at 187.85 eV.

FTIR Spectroscopy

FTIR studies were conducted with a ThermoNicolet Nexus 470 FTIR spectrometer. Silica nanoparticles and the conjugates were measured as KBr pellets (0.5 mg sample per 200 mg of KBr). Each sample was measured at least five times.

Dynamic Light Scattering (DLS)

The size of particles was determined by dynamic light scattering (DLS) using a DynaPro Titan.

Carbonic Anhydrase Modification with Sulfo-LC-SPDP

The protein was dissolved in 50 mM phosphate buffered saline (PBS) with 0.15 M NaCl and 10 mM EDTA at pH 7.2 to a final concentration of 2 mg/ml. Sulfosuccinimidyl 6-[3'(2-pyridyldithio)-propionamido] hexanoate (Sulfo-LC-SPDP, 8.5 mg) was added directly to the reaction flask. The mixture was reacted for 30 min at room temperature under gently stirring and dialyzed thrice against nanopure water at 4°C using cellulose ester membranes with a 10 kDa cut-off. A volume ratio of 1:100 (sample-tonanopure water) was used during dialysis. Modified carbonic anhydrase (CA-SPDP) was subsequently lyophilized for 48 h using a 6-L lyophilizer (model 77530, Labconco, Kansas City, MO) at a condenser temperature of -45°C and a pressure of <60 m Hg. The lyophilized powder was stored until use at -20°C.

Degree of Protein Modification

The extent of enzyme modification (i.e., the number of amine groups coupled to the cross-linker) was determined by measurement of the release of pyridine-2-thione and the 2,4,6-trinitrobenzene sulfonic acid (TNBSA) assay. For the pyridine-2-thione release assay modified carbonic anhydrase was dissolved in 50 mM PBS with 0.15 M NaCl and 10 mM EDTA at pH 7.2 to achieve a protein concentration of 0.5 mg/ml. The absorbance of the samples was measured and recorded at 343 nm. Then, 10 μ l of 15 mg/ml dithiothreitol (DTT) solution was added to each sample. After 15 min, the absorbance of the samples was measured and recorded at 343 nm against a blank treated as above. The molar ratio of SPDP to protein was calculated. For the TNBSA assay modified carbonic anhydrase was dissolved in 0.1 M sodium bicarbonate buffer, pH 8.5 at a concentration of 0.05–0.2 mg/ml. Then 0.25 ml of 0.01% (w/v) of a TNBSA solution was added to 0.5 ml of sample solution. The solutions were incubated at 37°C for 2 h. After incubation 0.25 ml of 10% SDS solution and 0.125 ml of 1 N HCl were added to each sample. The absorbance of each sample was measured at 335 nm against a blank treated as above. The amount of SPDP bound to the protein was obtained from a calibration curve. For both assays the samples were prepared in triplicate.

Circular Dichroism (CD) Spectroscopy

CD spectra were acquired with an OLIS DSM-10 UV-Vis CD spectrophotometer at 20°C in the near-UV region (250–320 nm) at 0.5 nm spectral resolution using a 10 mm quartz cell with a protein concentration of 0.6 mg/ml in 15 mM Trissulfate buffer at pH 7.6 and 25°C. Each spectrum was obtained by averaging six scans. Spectra of buffer blanks were measured prior to the samples and were digitally subtracted from the sample CD spectra.

Carbonic Anhydrase Activity

Carbonic anhydrase (1.2 μ M) activity was determined at 25°C by monitoring the hydrolysis of *p*-nitrophenol acetate (*p*-NPA) in 15 mM tris-sulfate buffer, pH 7.6. The hydrolysis was monitored at 400 nm using a Shimadzu 2450 UV/Vis spectrophotometer. The assay was performed in 1 ml cuvette. The reaction was started by mixing 700 μ l of 15 mM tris-sulfate buffer, pH 7.6, 100 μ l of 16.56 mM *p*-NPA dissolved in acetonitrile, and 200 μ l of enzyme solution. The residual activity was calculated with respect to the specific activity of native carbonic anhydrase.^{30,31}

Covalent Immobilization of CA into MSN

2.0 mg of MSN-SH was sonicated for 3 min in 50 mM PBS, 0.15 M NaCl, and 10 mM EDTA, pH 7.2 in Eppendorf vials to create a suspension. Different volumes of a carbonic anhydrase stock solution were added to each MSN-SH suspension. The mixtures were gently stirred overnight at 4°C. Then the samples were centrifuged at 14,000 rpm for 20 min. To remove the unreacted enzyme three cycles of washing/centrifugation were performed. The washing steps were performed with 50 mM PBS containing 0.15 M NaCl and 10 mM EDTA at pH 7.2. The amount of immobilized enzyme was determined by depletion by measuring the protein concentration in the supernatants obtained during washing and compare it to the total protein amount employed. Protein concentration in the supernatant was determined from the absorbance at 280 nm using a calibration curve generated with native carbonic anhydrase.

Cytotoxicity Assay

Experiments were performed using a human cervical cancer cells line (HeLa). The cells were cultured as recommended by ATCC in *minimum essential medium* (MEM) with L-glutamine supplemented with 10% fetal bovine serum (FBS) and 1% penicillin. HeLa cells were incubated at 37°C under 5% CO₂ and used before 25 passages. Mitochondrial function was assessed using the CellTiter 96 aqueous non-radioactive cell proliferation assay (Promega, Madison, WI). About 40,000 cells/well were seeded into 96-well microtiter plates in 100 μ L of penicillin free culture medium with 10% FBS. After 24 h, culture medium was replaced with culture medium containing serial dilutions of the MSN-CA bioconjugate suspensions. The cells were incubated with the suspended bioconjugate for 24 h. The MSN suspension settled down over time and consequently the cells were in direct contact with the nanoparticles during incubation. Then, 20 μ L of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) was added to each well. After 2 h, the optical intensity of each well was measured spectrophotometrically at 490 nm in a microplate reader (Thermo Electron Corporation, Multiskan Ascent).^{32,33} The spectrophotometer baseline was obtained using culture medium without cells. Experiments were performed 8 times.

Controls: HeLa cells treated with 2 μ M staurosporin for 6 h were used as positive control and cells without any treatment were used as negative control.³⁴ The statistical analysis for the cell viability results indicated that under the tested conditions the cytotoxicity obtained for a CA-SPDP concentration ≥ 30.87 μ g/mL is highly significant from the obtained for the

negative control ($p < 0.001$). Consequently the concentration range used for the experiments was 0.3 – 30.87 $\mu\text{g/mL}$.

Residual Activity of Released CA from MSN

The MSN-CA bioconjugates were suspended in 1 ml of 50 mM PBS containing 10 mM DTT. The samples were incubated overnight at 37°C under constant shaking at 125 rpm. Then, the samples were centrifuged at 14,000 g for 20 min to precipitate the MSN. DTT was removed from the supernatant using Centricon devices with an exclusion size of 10 kDa and the released enzyme obtained in the supernatant. The specific activity of modified carbonic anhydrase was obtained using these supernatants and the residual activity determined.

Release of Carbonic Anhydrase from MSN in the Presence of Glutathione

The release of CA from MSN was measured by preparing a suspension of 5 mg of MSN-CA conjugates in 1 ml of 50 mM PBS with 1 mM EDTA at pH 7.4 and glutathione (GHS) concentrations of 0, 0.001, and 10 mM. The chemical stability of thiols is affected by parameters such as temperature, pH, and the presence of metal ions. Addition of a chelating agent, i.e., EDTA, greatly improves the chemical stability of thiols.³⁵ Incubation was performed for 24 h at 37°C and the MSN were pelleted by centrifugation at 14,000 rpm for 20 min. The supernatant was removed and used to determine the concentration of released CA and the pelleted MSN were resuspended in fresh GHS-PBS buffer. The solubility of CA Carbonic is >10 mg/ml according to the manufacturer. The MSN-CA conjugate used in the release experiments contained a total of 1.1 mg of immobilized CA. Therefore, all the release experiments were performed under sink conditions.

Statistical Data Treatment

All data were obtained at least in triplicate, the data averaged and standard deviations calculated. Statistical significance for comparisons of multiple groups was established using one-way multiple Tukey comparison post-test ANOVA. All statistical analysis were performed with InStat 3.06 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS AND DISCUSSION

MSN Activation and Characterization

The goal of our investigation was to immobilize CA in MSN by introducing a redox-sensitive covalent SS-bond to accomplish release under intracellular redox conditions. To accomplish this it was necessary to first chemically decorate MSN with thiol groups. This was accomplished using (3-mercaptopropyl)trimethoxysilane (MPTMS). MPTMS reacts with the hydroxyl groups of silica forming a covalent bond. Thiol groups are introduced in this way into the material and presented terminal of the propyl groups. XPS was used to investigate the MSN prior to and after modification to confirm the introduction of the thiol groups (Figure 2). The introduction of SH groups into the MSN was evident from photoemission peaks of the 2s and 2p orbitals of S in the MSN-SH spectrum, which were absent in the spectrum of MSN. FTIR spectroscopy was also employed to characterize the product. A broad band centered at 3442 cm^{-1} was observed in the MSN-SH spectrum corresponding to hydroxyl stretching vibrations (Figure 3). Peaks at lower frequencies are due to various vibrational modes of the silicate (Si-OH and Si-O-Si). Bands at 2922 and 2861 cm^{-1} are due to partial hydrolyzation of the material used to synthesize the silica nanoparticles.³⁶ However, importantly the spectrum of MSN-SH shows a band at 2571 cm^{-1} consistent with SH-stretching vibrations which is missing in the spectrum of MSN.

Dynamic light scattering (DLS) was employed to determine if the chemical modification process had an effect on the particle size or would cause aggregation of the suspended silica

nanoparticles (Table 1). In principle introduction of SH-groups could cause formation of interparticle disulfide bonds and thus covalent aggregation. DLS data revealed two different hydrodynamic particle size distributions for both samples. Furthermore, somewhat in contrast to our expectations, the data revealed a reduction in the hydrodynamic radius upon chemical modification of the particles (Table 1). SEM micrographs were obtained to evaluate the effect of the modification process on the morphology of the MSN. No substantial morphology changes were detected in the micrographs (**for details see Supporting Information**). The characteristics of the particles after modification were still in line with the end application in mind, namely passive targeted delivery. It should be noted, however, that for clinical applications nanoparticles with a diameter of over 400 nm should be removed from the preparation because these particles will be effectively cleared by the mononuclear phagocytic system (MPS).

CA Activation and CA-SPDP Characterization

We selected carbonic anhydrase (EC 4.2.1.1, CA) as the model enzyme in our studies because it is a well-studied, accessible, and relatively inexpensive enzyme.²⁷ First we introduced a SH-group at the surface of the enzyme in order to immobilize it into the thiol functionalized MSN. This was accomplished by utilizing the reagent sulfo-LC-SPDP which reacts with amines through the succinimidyl group. The SPDP molecule also has a disulfide-containing linkage that can be cleaved with reducing agents such as DTT and glutathione.³⁷ After performing the reaction with the enzyme, the extent of enzyme modification was assessed using two different assays. In the first the release of pyridine-2-thione was determined, which is the product of the reaction of Sulfo-LC-SPDP with a thiol containing reagent.³⁶ The results were corroborated by using the 2,4,6-trinitrobenzene sulfonic acid (TNBSA) assay, which determines the amount of amino groups on the protein surface (largely the ϵ -amino groups of lysines).³⁸ Under the conditions employed in this work, both assays demonstrated that the protein was modified with the cross-linker at a linker-to-carbonic anhydrase molar ratio of 1.0 ± 0.5 , which represents a ca. 5% modification of the available enzyme surface amino groups. The enzyme activity was reduced to $80.0 \pm 0.7\%$ by the chemical modification. [It should be noted that higher levels of modification caused larger enzyme inactivation (**for details see Supporting Information**).] Near UV-circular dichroism (CD) spectroscopy was employed to investigate if the enzyme tertiary structure was affected by the modification. The spectra revealed that no significant changes in tertiary structure of SPDP-CA occurred after attaching the cross-linker (Figure 4).

MSN-CA Bioconjugate and its Characterization

SPDP-CA was covalently immobilized into the thiol-functionalized MSN. The strategy was that the 2-pyridyl disulphide group of the immobilized cross linker on the enzyme surface would react with the thiol groups introduced into MSN by thiol-disulfide interchange to form aliphatic disulphides.³⁷ To establish the amount of SPDP-CA that could be immobilized into the MSN-SH we reacted different amounts of SPDP-CA with 2 mg of MSN-SH. The amount of immobilized enzyme increased proportionally to the amount of enzyme added into the MSN-SH suspension (Figure 5). The maximum immobilization was determined to be 577 ± 65 mg of carbonic anhydrase per 1.0 g of MSN-SH under our conditions.

The bioconjugate was characterized by XPS and FTIR to confirm protein immobilization. The XPS spectrum of MSN-CA presents a new N (1s) signal confirming enzyme immobilization (Figure 2). The FTIR spectrum of the MSN-CA bio-conjugate shows the appearance of two strong bands at 1645 cm^{-1} and 1542 cm^{-1} due to the protein amide I and II vibrational modes. Thus, these data unequivocally demonstrate immobilization of the

enzyme into the functionalized MSN. However, these data do not distinguish adsorption from covalent immobilization.

However, proof was obtained by being able to remove the CA from the MSN using 10 mM DTT but not with buffer. The activity of the released enzyme was measured and was compared with that obtained for CA-SPDP (see Materials and Methods section for details). The enzyme retained $79 \pm 11\%$ of activity after immobilization and release. This demonstrates some detrimental impact of the procedure on enzyme integrity. While some inactivation of the enzyme is probably inevitable using such strategies, future experiments are being designed to minimize this by increasing protein thermodynamic stability and minimizing protein-surface interactions. To identify potential changes in the morphology of the MSN due to enzyme immobilization we employed SEM and dynamic light scattering. The SEM micrographs show that no major morphology changes (size and porosity) occurred due to enzyme immobilization (**for details see** Supporting Information). Dynamic light scattering showed that the size of the MSN-CA bioconjugates was similar to that of non-modified MSN (Table 1).

Controlled Release of CA from Nanoparticles

In vitro release studies were conducted to assess the release of immobilized CA from the nanoparticles under somewhat physiological conditions in PBS at pH 7.4 and 37°C. Intracellular glutathione concentrations (1–10 mM) are sufficient to cleave disulphide bonds and thus should afford release of the immobilized enzyme while the concentration of 1 μ M glutathione representing extracellular plasma conditions should have no effect.¹⁶ However, release could also be promoted by other cell-produced antioxidants, such as NADH and dihydrolipoic acid.³⁹ It is in consequence possible that the intra-cellular release *in vivo* could be faster than in our *in vitro* assay. Fresh glutathione had to be added daily to each sample during release due to limited glutathione stability in the *in vitro* release assays. We found that only a small amount of 1% and 3% of CA was released using no or 1 μ M concentrations of glutathione (Figure 6). In contrast, when exposing the CA-MSN to 10 mM glutathione, CA was released from the nanoparticles (Figure 6). The fact that carbonic anhydrase release only occurred in the presence of glutathione proves that the enzyme was indeed covalently immobilized and not absorbed at the surface in agreement with our spectroscopic evidence.

The protein was completely released from the MSN under reducing conditions in 480 h. In contrast, in systems using adsorption for protein immobilized typically only 45–55% of the immobilized protein was released.²¹ Figure 6 also shows that the CA release profile at intracellular glutathione concentrations is sigmoidal which is characteristic of an energy-dependent release process. This type of system is characterized by slower release at initial stages followed by increased release at later stage.⁴⁰

In summary, our results demonstrate the ability of our system to release the enzyme under intracellular conditions but not at extracellular conditions.

Cell Viability Studies

Cell viability is essential when creating a new drug delivery system to avoid non-selective cytotoxic events and was thus studied herein. Several publications have already established the non-cytotoxicity of MSN.^{19,23,41} However, the cytotoxicity of MSN-CA bioconjugates has not been investigated. Cell viability was studied via the measurement of cell metabolic activity. The mitochondrial function was measured using the MTS assay after incubating HeLa cells with different concentrations of MSN-CA bioconjugate for 24 h. Viable cells convert tetrazolium salts (MTS) to formazan dyes that are measured spectrophotometrically.³³ The assay was performed varying the concentrations of the silica

and the enzyme. Figure 7a shows the obtained results of the assay performed in terms of varying silica concentration. In general, the cytotoxic effect of the MSN-CA conjugate is lower than that of the MSN-SH alone (except for the 17.15 $\mu\text{g/mL}$ concentration). Cell viability decreased from 97% to 63% at increasing CA concentration compared with 100% to 80% at increasing MSN-CA conjugate concentration, respectively (Figure 7b). However, statistical analysis by ANOVA demonstrated that there was no significant difference between the two groups ($p > 0.05$).

CONCLUSION

In summary, we have explored the covalent immobilization of a model protein into MSN to generate a stimulus-response controlled release. We demonstrate that the release of the immobilized enzyme occurs under intra-cellular but not extra-cellular redox conditions. The released enzyme was still functional and active but some activity decrease was encountered. Cell viability studies establish good biocompatibility of the generated bioconjugate. Future experiments include studies related to endocytosis and endosomal escape of the bioconjugate. We envision that this bioconjugate could serve as a platform for the creation of a drug delivery system utilizing therapeutic proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Representation of the immobilization of modified carbonic anhydrase into activated mesoporous silica nanoparticles to generate MSN CA conjugates and the smart release of CA in the presence of the redox stimulus. The crystal structure of carbonic anhydrase at 1.95 Å atomic resolution was acquired from the protein data bank (access code 1V9E).

Figure 2.
Wide-scan XPS spectra for MSN, MSN-SH and MSN-CA conjugates.

Figure 3.

FTIR spectra of MSN, MSN-SH and MSN-CA conjugates. The inset shows the area corresponding to the SH stretching vibrational mode (2800-2300 nm) expanded (same experiments as main spectra).

Figure 4. Near UV CD spectra of non-modified CA and SPDP-CA. The samples were dissolved in 15 mM Tris-sulfate, pH 7.6 at 20°C.

Figure 5. Amount of immobilized CA at various CA concentrations employed during synthesis. The reaction employed 2 mg of MSN-SH.

Figure 6. In vitro release profile of carbonic anhydrase from MSN at different glutathione concentrations.

Figure 7. HeLa cell viability in the presence of different concentrations of MSN-SH and MSN-CA conjugates (A) and of SPDP-CA and MSN-CA conjugates (B) after incubation for 24 h.

Table 1

Particle size of MSN and MSN-SH, and MSN-CH determined by dynamic light scattering.

Sample	Diameter (nm) ^a	% Intensity
MSN in water	317.2	90.8
	626.0	9.2
MSN-SH in water	184.3	70.6
	687.5	29.4
MSN-CA in water	312.5	100
MSN-CA in 0.9% NaCl	311.5	100

^a Polydispersity index for all the samples was less than 20%