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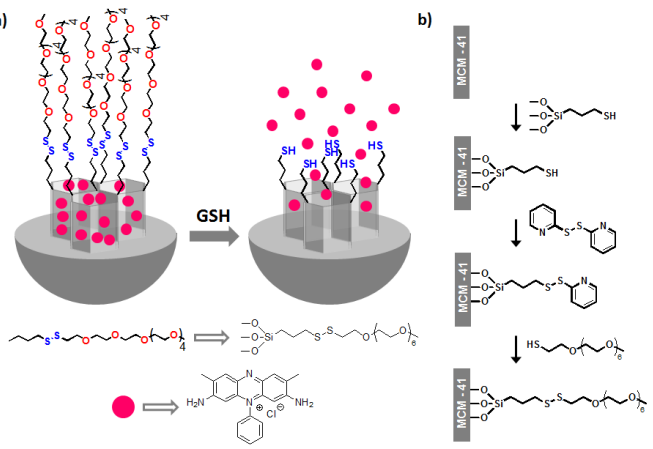
Mesoporous silica nanoparticles loaded with safranin O and capped with disulfide-containing oligo(ethylene glycol) chains were used for the selective and sensitive fluorimetric detection of glutathione

Biothiols such as cysteine (Cys), homocysteine (Hcy), and glutathione (GSH) are of vital importance in cellular processes, especially in preventing the damage to cellular components caused by reactive oxygen species.[1] Biothiols are controlled by the equilibrium between thiols and disulfides and their relative levels in cells are closely related to aging and many diseases, such as cancer,[2] AIDS,[3] cystic fibrosis,[4] and neurodegenerative pathologies.[5] In particular, GSH is a fundamental small peptide having many biological functions such as maintenance of intra-cellular redox activity, xenobiotic metabolism, intra-cellular signal transduction and gene regulation.[6] Besides, several diseases have been associated with a decrease in plasma of GSH concentrations. For instance, low levels of GSH are involved in leucocyte loss,[7] psoriasis,[8] liver damage,[9] cancer[2] and AIDS.[3]

In this context, different analytical methods are currently available for GSH determination including HPLC,[10] capillary electrophoresis,[11] mass spectrometry,[12] etc. Moreover recently, the preparation of chromo-fluorogenic probes for the detection of thiol-cotaining bio-molecules has increased in interest. In most cases, these probes are designed following the chemodosimeter approach, which makes use of the high nucleophilic reactivity of the thiol functional group.[13] However, many of these probes only display sensing features in organic or mixed water-organic solvents and it is not unusual to observe a similar response for closely related species such as Cys, Hcy and GSH.[14] Besides, very recently, simple molecular probes for the selective recognition of GSH in aqueous environments have also been described.[15] As an alternative to molecular probes for GSH detection, the development of nano sensing systems has emerged in the last years. In particular, gold nanoparticles,[16] gold nanoclusters[17] and quantum dots[18] have been used for the optical sensing of GSH.



Scheme 1. (a) Schematic representation of the sensing mechanism of solid **S1** in the presence of GSH. (b) Sequence of reactions used to anchor the molecular gate onto the outer surface of the loaded inorganic support.

From a different point of view there is an increasing interest in the design of responsive nanoscopic hybrid gated materials containing caps and showing the ability to release entrapped guests upon application of external stimuli.[19] These devices

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usually contain a silica mesoporous support, in which the cargo is stored, and molecular or supramolecular entities attached on the external surface that act as "gates", allowing the controlled release of entrapped molecules at will. Both components, i.e. gate and cargo, have been carefully selected to achieve a wide range of control functions in most cases for drug delivery applications. Moreover, we have recently suggested that the use of gated materials can also be applied to the development of optical probes.[20] The underlying idea here is that the coordination or reaction of the target analyte with the binding sites could modulate dye delivery from pores to the solution, resulting in a chromo-fluorogenic signal. One advantage of this approach is the potential existence of amplification features; in particular, the presence of few analyte molecules may induce the release of a relatively high amount of entrapped dye molecules. For these reasons, very recently, the application of gated materials in sensing protocols has deserved great attention.[21]

Given our interest in the application of capped mesoporous materials in sensing and recognition protocols,[22] we report herein the design of a simple capped system for the selective optical detection of GSH. Our proposed paradigm is briefly represented in Scheme 1. MCM-41 mesoporous silica nanoparticles (MSN) of ca. 100 nm were selected as support. MSN were loaded with a suitable dye (safranine O), and then the external surface was functionalized with disulfide-containing oligo(ethylene glycol) groups which act as molecular gates (solid **S1**). The signalling paradigm relies on the selective reduction of the disulfide bond by GSH which was expected to result in pore opening and dye release. In this context it should be noted that although disulfide-containing gated materials have been used for drug delivery in cells[23] there are not, as far as we know, specifically designed capped systems for GSH detection. However, taking into account the possible use of different thiol-selective gate-like systems and choice of a wide range of indicator dyes, this tailor-made strategy displays enormous potential yet still has not been explored.

MCM-41 mesoporous nanoparticles were synthesized according to reported procedures.[24] Then, the pores of calcined MCM-41 were loaded with safranine O by stirring overnight at room temperature a suspension of the nanoparticles in an acetonitrile solution of the dye. Afterward, an excess of (3-mercaptopropyl) trimethoxysilane was added and the thiol-functionalised solid was further reacted with 2,2'-dipyridyl disulfide. Finally the preparation of **S1** was achieved by reaction of with *O*-(2-mercaptoethyl)-*O*'-methyl-hexa(ethylene glycol) (0.14 mmol). The mixture was stirred for 12 h and the final support **S1** was isolated by centrifugation, washed with abundant water and dried (see Supporting Information for details).

The MCM-41 scaffold and the final sensing mesoporous solid **S1** were characterized using standard techniques. The main structural properties obtained from these studies such as particle diameter, BET specific surface area, pore volumes and pore sizes are listed in Table 1 (see Supporting Information for additional information). Powder X-ray diffraction (PXRD) and transmission electron microscopy (TEM) carried out on the MCM-41 nanoparticles showed clearly the presence of a mesoporous structure that remained in the final solid **S1** despite the loading process with the dye and further functionalization with oligo(ethylene glycol) chains (see Figure 1 for TEM images). Moreover, contents of safranin O and the disulfide-containing oligo(ethylene glycol) groups in **S1** were determined by elemental and thermogravimetric analyses, gaving values of 0.30 mmol g^{-1} and 0.86 mmol g^{-1}, respectively.

Table 1. Main structural properties calculated from TEM, PXRD and N_2 adsorption analysis.

Sample	Diameter particle[a] (nm)	S_{BET} (m^2 g^{-1})	Pore Volume[b] (cm^3 g^{-1})	Pore size[a] (nm)
MCM-41	94.0 ± 5.0	1045.7	0.90	2.76
S1	92.0 ± 8.0	491.1	0.28	-

[a] Measured by TEM. [b] BJH model.

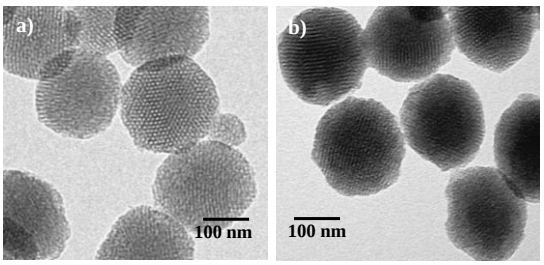

Figure 1. TEM images of (a) calcined MCM-41 and (b) **S1** solid.

In a first step the response of **S1** in the presence of the bio-relevant thiol GSH was studied. In a typical experiment, 1 mg of solid **S1** was suspended in 2 ml of water at pH 7.0 in the presence of GSH (1 mM). At certain times fractions were collected, centrifuged to eliminate the solid and the emission in the solution of safranin O at 575 nm (λ_{ex} = 520 nm) measured. Moreover cargo delivery from **S1** in the absence of GSH was also studied. Safranin O release kinetics are depicted in Figure 2. As seen, in the absence of GSH, **S1** showed nearly a "zero release" indicating tight pore closure. In contrast, the presence of GSH induced the opening of the pores and the subsequent release of the dye. Delivery was quite fast and for instance after 10 min ca. 90% (referred to the total amount of dye released after 4 h) of the cargo was released.

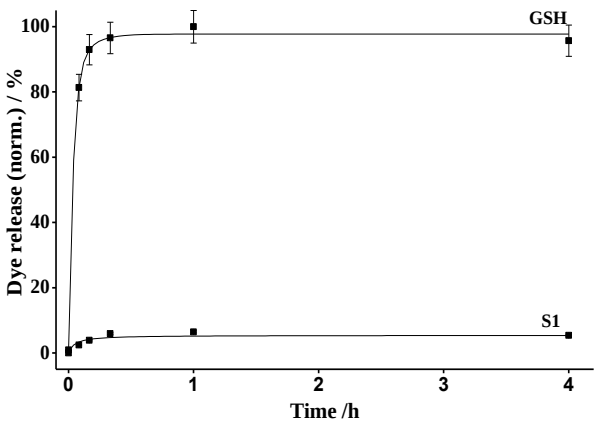

Figure 2. Kinetics of the release of safranin O from solid **S1** in the absence and in the presence GSH (1 mM).

Following a similar approach dye delivery from **S1** was studied as a function of the amount of GSH (see Figure 3). A correlation between the concentration of GSH and dye delivered was observed in agreement with an uncapping protocol involving reduction of the disulfide bond in the capping molecules in **S1**. A saturation of the delivery was observed for concentrations of GSH of about 10 μM. Moreover the limit of detection (LOD) of GSH following this procedure was determined to a concentration as low as 0.1 μM. As stated above one main characteristic of analyte-induced uncapping in gated mesoporous supports is the inherent feature of signal amplification in which only few analyte molecules (i.e. GSH) reacting at the pore openings are necessary to release a significant number of signalling units (i.e. safranine) entrapped in the nanoparticles. In the present case, for an intermediate point in Figure 3, the presence of one GSH molecule results in the delivery of ca. 100 safranine molecules.

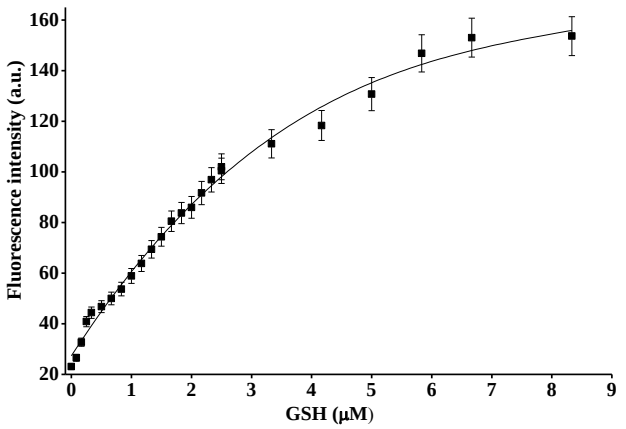

Figure 3. Release of safranin O from solid **S1** in the presence of different amounts of GSH in water at pH 7.0 after 1h upon addition.

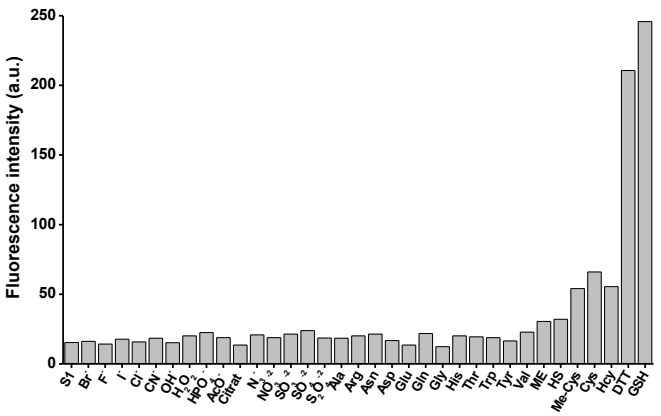

Figure 4. Emission intensity of safranin O at 575 nm (excitation at 520 nm) released from solid **S1** (water at pH 7.0) in the presence of selected anions, amino acids and reducing agents (10 mM) after 30 min upon addition.

In order to verify the selectivity of the method, similar experiments with solid **S1** were performed in the presence of 10 mM of selected anions (HS^-, F^-, Br-, Cl^-, I^-, CN^-, OH^-, HPO_4^-, AcO^-, Citrate, N_3^-, NO_3^{-2}, SO_3^{-2}, SO_4^{-2} and $S_2O_4^{-2}$), oxidants (H_2O_2), amino acids (Hcy, Cys, Me-Cys, Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Thr, Trp, Tyr and Val) and GSH. Dye delivery from **S1** after 30 min upon guest addition is shown in Figure 4. The results show that solid **S1** is highly selective to the presence of GSH, whereas other relevant bio-thiols (i.e. Hcy, Cys and Me-Cys) and HS^- induced a rather poor cargo delivery. This opens the way to the possible use of **S1** material for GSH detection in highly competitive media.

The uncapping mechanism was tentatively attributed to a selective reduction by GSH of the disulphide bond in **S1**. In order to further asses the mechanism, release experiments in the presence of 2-mercaptoethanol (ME) and dithiothreitol (DTT), which are reagents typically used to reduce disulfides to thiols, were carried out. The redox potentials for ME and DTT are -0.253 V and -0.33 V, respectively, whereas that for GSH is -0.262 V. As a result it was expected that **S1** would be opened in the presence of DTT (with is a stronger reducing agent than GSH) whereas ME (with a slightly lower reduction potential than GSH) should uncap the pores in a much lower extent. This hypothesis was confirmed experimentally and the results are shown in Figure 4, pointing to a reduction of the disulfide bond in the oligo(ethylene glycol) chain as the uncapping mechanism.

Table 2. Determination of total GSH in spiked human serum samples using solid **S1**.

Sample	GSH spiked (μM)	GSH determined (μM)	Recovery (%)
1	5.99 + 2.5	8.12 ± 0.54	95
2	5.99 + 4.0	10.75 ± 0.84	107
3	5.99 + 6.0	10.98 ± 1.03	92
4	5.99 + 10.0	15.53 ± 1.86	97

Encouraged by these findings we attempted to detect GSH in a more complex system and selected human serum as a more realistic environment. Typical amounts of GSH in serum are in the 3-8 μM range, whereas other aminothiols such as Cys and Hcy are at concentrations of ca. 140-300 μM and 8-30 μM, respectively.[25] The LOD of **S1** for GSH is 0.1 μM, hence the probe is expected to sense GSH in typical concentrations in serum samples. Moreover **S1** displays a poor response to Cys and Hcy (see Figure 3) and therefore probe **S1** was expected to be selective for GSH. A human serum containing a concentration of GSH of 5.99 μM was used for the studies. The serum was spiked with additional known amounts of GSH (2.5, 4, 6, and 10 μM) and the total GSH concentration was determined using **S1** following the method of standard addition. Results are shown in Table 2. As seen in the table, **S1** was satisfactorily applied to the detection of GSH in a highly competitive environment such as human serum with rather high recovery ratios ranging from 92 to 107 %.

In summary, we have reported herein the design of mesoporous silica nanoparticles capped with a disulfide-

containing derivative and loaded with safranin O as a suitable system for the selective chromo-fluorogenic detection to GSH in pure water. The observed emission enhancement was ascribed to the GSH-induced selective uncapping of **S1** via the reduction of the disulfide bond that allowed the release of the dye entrapped in the porous network of the inorganic scaffold. The observed response was highly selective toward GSH whereas similar sulfur-containing derivatives, such as Cys, Hcy and HS^-, were unable to uncap the gated material to a significant extent. **S1** can detect GSH down to concentrations of 0.1 μM. Besides, **S1** was used to detect GSH in human serum. Despite there are some reports on the use of disulfide-containing gated materials for drug delivery applications, this is, as far as we know, the first report in which capped nanoparticles have been used for the selective and sensitive detection of biothiols (in particular GSH). Moreover, the possibility of selecting different disulfide derivatives as caps, porous supports, cargos (such as fluorophores, dyes or redox active species for electrochemical sensing) and the inherent signal amplification features observed in capped materials make this approach attractive in the design of new probes for bio-thiols.

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