

Enzyme-Mediated Controlled Release Systems by Anchoring Peptide Sequences on Mesoporous Silica Supports**

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The design of delivery systems that contain gatelike scaffolds and that are able to release entrapped guests in the presence of well-defined target-dependent triggers has recently attracted great attention.^[1] In this field, and as an alternative to polymer materials, the incorporation of gated mechanisms on mesoporous silica nanoparticles (MSNs) has been recently employed for advanced on-command delivery applications by using diverse physical and chemical stimuli.^[2,3] However, there are very few MSN-based systems designed to trigger cargo release by using biomolecules. In particular, the design of enzyme-responsive biogated MSNs is a barely studied area.^[4] Furthermore this is a promising field of research, taking into account that the use of tailor-made peptide sequences and specific proteases is envisioned to have a large potential that may provide exquisite selectivity in the design of advanced gate-opening devices. Despite these appealing features, there is only one recent report that deals with the capping of porous silica with a peptide shell, in which the

capped solid is employed for the release of entrapped dextran in the presence of certain proteases.^[5] In this example, the authors describe the use of a simple tripeptide containing an 9-fluorenylmethoxycarbonyl (Fmoc) protecting group that allows the gate to be closed by π - π interactions between adjacent Fmoc moieties. However, novel closing properties would be attained based on the large number of possibilities that could offer complex unprotected peptide sequences upon demonstration that these sequences could be anchored on silica supports to design materials with zero release. We were initially attracted by the design of such materials where long modular protease-sensitive peptides regulate the gate-opening properties.

In our proof-of-concept study, we selected mesoporous MCM-41 silica nanoparticles (ca. 100 nm) as inorganic scaffolds that show distinctive properties such as a high homogeneous porosity, inertness, high load capacity, and easy surface functionalization. The MCM-41 support was loaded with a fluorescent dye [Ru(bipy)₃]Cl₂·6H₂O, and the external surface was chemically modified with 3-(azidopropyl)triethoxysilane (solid **S1**). This azide group serves as a handle to attach the capping groups, that is, in the case presented here, a peptide sequence (see below, Figure 1). **S1** was characterized by using standard procedures. The X-ray pattern of **S1** shows the mesoporous characteristic (100)

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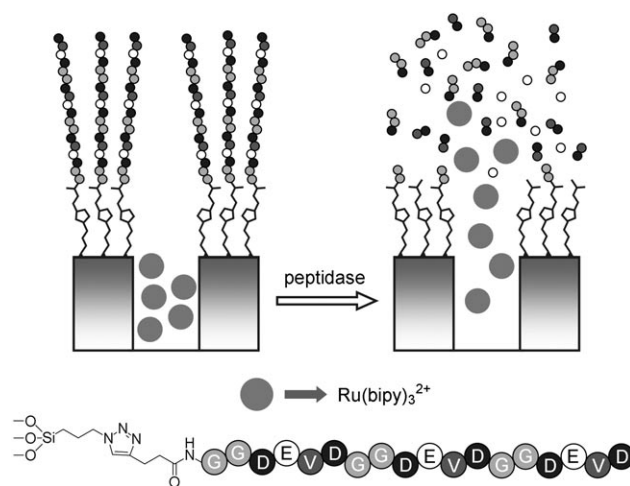


Figure 1. Representation of the gated material **S1** functionalized with 3-(azidopropyl)triethoxysilane and capped with the peptide sequence **P1**. The release of the loaded [Ru(bipy)₃]²⁺ dye was achieved through enzymatic cleavage at the C-terminus amide bonds of the negatively charged amino acids contained in **P1** (D and E). The sequence of the **P1** peptide is shown.

peak diffraction, thus indicating that the dye-loading process and the subsequent functionalization with 3-(azidopropyl)-triethoxysilane have not damaged the mesoporous scaffolding.

The presence of the mesoporous structure in the final functionalized solid **S1** is also confirmed by TEM analysis, in which the typical channels of the MCM-41 matrix are visualized (see Figure 2 and the Supporting Information).

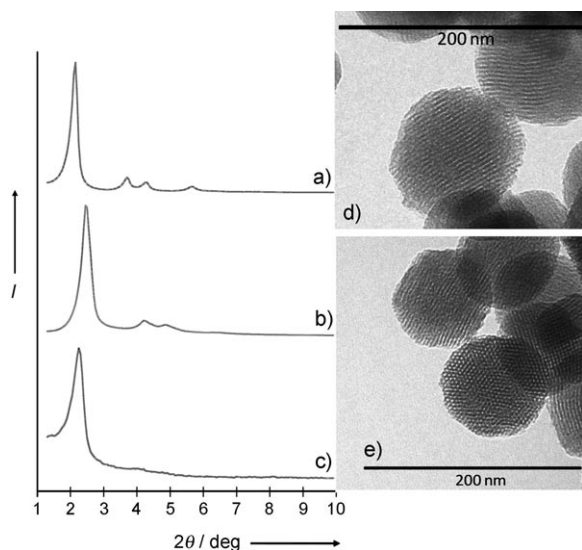


Figure 2. Powder X-ray patterns of: a) as-synthesized MCM-41, b) calcined MCM-41, and c) solid **S1** containing the $[\text{Ru}(\text{bipy})_3]^{2+}$ dye and 3-azidopropyltriethoxysilane. TEM images of: d) calcined MCM-41 sample and e) solid **S1-P1**, showing the typical hexagonal porosity of the MCM-41 mesoporous matrix.

Figure 2 also shows that the prepared material is obtained as spherical particles with diameters of approximately 100 nm. The nanoparticulate MCM-41 calcined starting material showed a typical adsorption–desorption isotherm (see the Supporting Information). The application of the Brunauer–Emmet–Teller (BET) model resulted in a value for the total specific surface of $1145 \text{ m}^2 \text{ g}^{-1}$ and a pore volume of $0.89 \text{ cm}^3 \text{ g}^{-1}$. From the XRD, porosimetry, and TEM studies, the a_0 cell parameter (4.16 nm), the pore diameter (2.47 nm), and a value for the wall thickness (1.69 nm) were calculated. In contrast, the N_2 adsorption–desorption isotherm of **S1** is typical of mesoporous systems with filled mesopores (see the Supporting Information), and a significant decrease in the N_2 volume adsorbed and the surface area ($356 \text{ m}^2 \text{ g}^{-1}$) is observed.

The modular peptide H-GGDEVDGGDEVDGG-DEVD-OH (**P1**) was designed to act as substrate of the proteolytic enzymes obtained from *Streptomyces griseus* (PESG) that cleave at the C terminus of the amide bond from negatively charged aspartic (D) and glutamic (E) amino acids. The peptide sequence was prepared by Fmoc-based solid-phase synthesis employing a peptide synthesizer for the preparation of the fragment H-DGGDEVDGGDEVD-OH; the rest of the amino acids were introduced manually. Once the complete sequence was obtained, 4-pentynoic acid was

attached at the N-terminal side by an amide bond. Then the peptide was cleaved from the resin and purified by preparative reversed phase (RP) HPLC. The identity and purity of the corresponding N-terminally alkyne-modified peptide **P1** was confirmed by HPLC and MALDI-TOF mass spectrometry.

P1 was attached to the solid **S1** by the copper(I)-catalyzed Huisgen azide/alkyne 1,3-dipolar cycloaddition “click” reaction,^[6] which induces the formation of a 1,2,3-triazole heterocycle upon reaction of the azide group (on the solid) and the triple bond (on **P1**). The peptide-functionalized solid (**S1-P1**) was isolated by centrifugation and washed to eliminate both residual dye and free peptide. TEM analysis confirmed that **S1-P1** retains the mesoporous structure (see Figure 2). The content of $[\text{Ru}(\text{bipy})_3]^{2+}$, azide groups, and **P1** in the solid **S1-P1** was quantified through thermogravimetry and elemental analysis and amounts to 0.179, 0.053, and $0.012 \text{ mmol g}^{-1} \text{ SiO}_2$ respectively.

To investigate the enzyme-responsive gating properties of **S1-P1**, 500 μg of the solid were added to an aqueous solution (4.5 mL) that contained PESG ($c_{\text{enzyme}} = 0.12 \text{ mg mL}^{-1}$, pH 8) and the suspension was stirred. Uncapping and subsequent delivery of the dye was determined through the measurement of the luminescence emission at 610 nm ($\lambda_{\text{exc}} = 453 \text{ nm}$) of the free $[\text{Ru}(\text{bipy})_3]^{2+}$ complex in solution. As a control experiment, the dye release was also determined by using suspensions of **S1-P1** under similar conditions but in absence of PESG. The difference in dye delivery in both experiments is shown in Figure 3; a remarkable enzyme-induced release is observed, whereas “zero release” is found in the absence of PESG at pH 8. “Zero delivery” was also detected when similar experiments were carried out with **S1-P1** in the absence of PESG at pH 5, thus indicating that possible changes of pH do not induce cargo release.

Anchoring of the **P1** peptide on the **S1** nanoparticles by the “click” reaction turned out to be an efficient procedure to place the peptide in a suitable arrangement on the MSN surface in order to obtain the **S1-P1** “zero release” material containing peptide sequences as gatekeepers. It is worth noting that the effective closing of the pores has a clear relation with the peptide-anchoring procedure. Thus, in

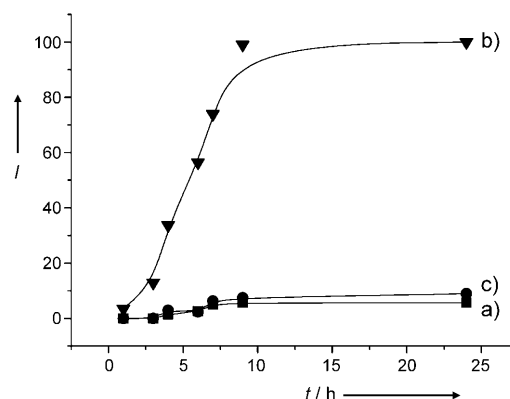


Figure 3. Kinetics of the release of the $[\text{Ru}(\text{bipy})_3]^{2+}$ dye from solid **S1-P1** a) in the absence and b) in the presence of protease enzyme at pH 8; c) kinetics of the release of the $[\text{Ru}(\text{bipy})_3]^{2+}$ dye from solid **S1-P1** at pH 5 in the absence of protease enzyme.

addition to the delivery studies with **S1–P1**, further control experiments were carried out using the solid **S2–P2**. **S2** is a mesoporous solid similar to **S1** but contains 3-(triethoxysilyl)propyl isocyanate groups on the external surface and **P2** is a control peptide with several lysine residues as anchoring-reactive amino acid. Reaction of **S2** with **P2** gives **S2–P2**, where the peptide is anchored on the surface in a “disordered” manner caused by the random reaction of the thiocyanate with the different primary amino groups from the peptide to give thiourea moieties. Interestingly, whereas the ordered anchoring in solid **S1–P1** results in zero release, the material **S2–P2** shows significant delivery, thus indicating a poor pore closing (see the Supporting Information).

In addition to the delivery studies with **S1–P1**, experiments were carried out using the materials **S1–P3** and **S1–P4**. These are similar solids to **S1–P1** but contain peptides of different length: **P3** (H-GDEVVDG-OH) and **P4** (H-DEVVDGGDEVVDG-OH). When the release kinetics of the ruthenium dye from **S1–P1**, **S1–P3**, and **S1–P4** are compared, it was observed that the delivery is proportional to peptide length. Thus, it was found that whereas the peptide **P1** allows zero release (see Figure 3), neither **P3** nor **P4** are able to completely block the delivery of the dye (kinetic details of these solids are included in the Supporting Information).

In summary we have demonstrated, as a proof-of-concept, that it is possible to anchor complex peptide sequences on silica supports to develop nanodevices with zero release that are specifically opened in the presence of targeted proteolytic enzymes. In particular the **P1** peptide attached to the azide-functionalized MSN was able to hamper the release of the dye trapped in the pores, whereas delivery is triggered in the presence of a protease. The work also demonstrates the efficiency of the click chemistry reaction in anchoring the peptide sequence on the mesoporous support. Despite proving the concept, we are aware that many interesting proteases reside in the cytoplasm and it is well-known that most of the nanoparticles enter the cell by endocytosis. Therefore it would be of interest to selectively direct the nanoparticles to specific cellular compartments in order to target selective enzymes. To this end, we plan in the near future to exploit the potential of peptides to control the cell-entry mechanism that would mediate the final intracellular setting and protease exposure of the device. The fact that synthetic peptides can be designed containing both amino acid sequences recognized by the target enzyme and amino acid sequences in charge of cell-entry control has also to be considered as a suitable approach when using these new peptide-based nanodevices. Based on the observation that the field of peptide–protease pairs is well-known and offers high reliability for on-command-delivery protocols, we believe that the design of enzyme-responsive materials is a promising field of research that can further have biomedical relevance in the design of custom-made materials for advanced drug delivery and applications in regenerative medicine.

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