Dual Enzyme-Triggered Controlled Release on Capped Nanometric Silica Mesoporous Supports

Alessandro Agostini,[a, b] Laura Mondragón,[a, b, c] Carmen Ramón Martínez-Máñez,[a, b, c] Félix Sancenón,[a, b, c] Juan Coll,[a, b, c] Elena Aznar,[a, c] M. Dolores Marcos,[a, b, c] Elena Pérez-Paya,[d] and Pedro Amorós[e]

The development of nanoscopic hybrid materials equipped with “molecular gates” showing the ability of releasing target entrapped guests upon the application of an external trigger has attracted great attention and has been extensively explored during recent years.[1] These nanodevices are composed of two subunits, namely, a suitable support and certain capping entities grafted on the surface of the scaffolding.[2] The support is used as a suitable reservoir in which certain chemicals can be stored whereas the molecules grafted in the outer surface act as a “gate” and can control the release of the entrapped molecules at will. Both components are carefully selected and arranged in order to achieve a wide range of required functionalities.

As support, mesoporous silica nanoparticles (MSN) have been widely used due to their unique properties, such as large load capacity, biocompatibility, high surface area and well-known functionalization procedures.[3, 4] Moreover, gated MSN have recently been used for the development of on-command delivery nanodevices by using several physical and chemical triggers. For instance, MSN displaying controlled-release features with the use of light,[5] redox reactions,[6] and pH changes[7] have been described. In contrast, gated nanomaterials able to deliver the cargo triggered by biomolecules are scarce although some illustrative examples that use antigen–antibody interactions,[7] hybridisation of single stranded oligonucleotides,[8] and enzymes[9-14] have been reported. In particular, the use of enzymes is especially appealing taking into account the possibility to synthesise tailor-made enzyme-specific sequences as molecular caps. However, in spite of these interesting features there are few examples that use enzymes in opening protocols. The first enzyme-responsive gate in a mesoporous support was described by Stoddart and co-workers. In that work, a [2]rotaxane ended with a bulky adamantyl ester stopper that acted as molecular gate was removed by porcine liver esterase treatment.[9] Further hybrid systems involving avidin–biotin,[10] lactose,[11] starch,[12] β-cyclodextrins[13] and peptide sequences[14] as capping groups have been reported. These examples offer a chemically simple approach that can benefit from the vast knowledge on enzyme–substrate pairs for the design of versatile systems for controlled release.

A further step in the field should take into account that the flow of defined biological processes rely on biochemical networks with the participation of multiple enzyme-dependent stages. It would be then useful to define future applications with the development of dual or multiple enzyme-triggered systems by using capped mesoporous supports. This would require the design of capping threads containing different enzyme-specific hydrolysable linkers located at defined positions on the external surface of MSN. The whole design will provide highly versatile and specific-release nanodevices the delivery profiles of which could be controlled and fine-tuned by defined combinations of enzymes. As a first-of-its-kind proof-of-concept, we have prepared an MSN support capped with the molecular entity 1 that contains amide and urea linkages, and we have evaluated it as a multi-enzyme-tuned delivery system (Scheme 1).

As inorganic carrier vehicle we selected mesoporous MCM-41 silica nanoparticles of about 100 nm in diameter, which were prepared following well-known procedures using TEOS as hydrolytic inorganic precursor and hexadecyltrimethylammonium bromide (CTABr) as porogen species.[15] The structure of the nanoparticulated calcined MCM-41 starting material was confirmed by X-ray diffraction (Figure 1), TEM and SEM microscopy. The N2 adsorption–desorption isotherms showed a typical type IV curve with a specific surface of 999.6 m2 g-1, and a pore volume of 0.79 cm3 g-1. From the XRD, porosimetry and TEM studies, the a0 cell parameter (4.46 nm), the pore diameter (2.46 nm) and a value for the wall thickness (1.99 nm) were calculated. For the preparation of S1, the calcinated MSN was first loaded with [Ru(bipy)3]Cl2, which was used as dye for monitoring the enzyme-triggered protocol, and then treated with the capping molecule 1. The derivative 1 was synthesised following a two-step procedure from diethylentriamine. In a
The presence of amide and urea moieties in the capping molecule 1 allowed the analysis of a multi-enzyme-dependent release of the [Ru(bipy)$_3$]$_{2}^{2+}$ dye from S1 by using amidase and urease enzymes. In a typical experiment, solid S1 (5 mg) was suspended in water (12.5 mL) at pH 7.5. Then amidase or/and urease (1 μL of the purchased solution) were added and the final suspension was stirred. As the control experiment dye release was determined by using suspensions of S1 under similar conditions but in the absence of enzyme. Uncapping and subsequent delivery of the dye to the aqueous solution was easily detected by monitoring the metal-to-ligand charge transfer transition band of the [Ru(bipy)$_3$]$_{2}^{2+}$ dye at 451 nm or through the emission band at 619 nm ($\lambda_{ex} = 451$ nm).$^{[17]}$ The different delivery profiles for both experiments are shown in Figure 2. In

![Scheme 1. Schematic representation of solid S1 and the enzymatic uncapping mechanism.](image)

The presence of amidase a relatively quick delivery was found (ca. 20% of the cargo was released in 2 h); however, only a moderate delivery was observed for long periods of time (only 40% of the dye was delivered after 15 h). In contrast the urease-stimulated release was slower (for instance, no delivery was observed after 2.5 h) yet at longer time periods urease was able to deliver a significantly larger amount of the cargo from S1 (ca. 80% of the dye was delivered after 15 h) than amidase. We also observed that there is a very low payload release in the absence of urease or amidase (Figure 2). The different enzyme-dependent delivery rates obtained on solid S1 were related to the design of gate 1, in particular to the relative position of the hydrolysable groups on the molecule. Solid S1 treatment with amidase induced the hydrolysis of the amide bonds located far away from the surface with the subsequent release of two bulky trimethylacetate moieties. In spite of this reduction in the steric crowding around the pore outlets, the organic residual that remained anchored was large enough to hamper, to some extent, the release of the dye. As a consequence, the degree of cargo release is low but fast. On changing from amidase to urease a completely different response was obtained. Addition of urease induced the hydrolysis of the urea bond located deeper inside the structure of 1 and led to a drastic reduction of the thread size. As a consequence, the degree of cargo release is high but quite slow. Release experiments with S1 in the presence of both enzymes were also carried out. In this case, a synergic effect was
observed; the action of the amidase induced a rapid cargo release, whereas at longer time periods the combined action of the urease allows a nearly complete cargo delivery (see the Supporting Information). In order to further demonstrate that the enzyme treatment is entirely responsible for the cargo release, two additional experiments were carried out. Solid S1 was incubated in the presence of non-related enzymes, such as esterase and pronase. In a different experiment, prior to incubation with solid S1, the amidase and urease were heat denatured at 60 °C for 60 min. In both experiments no release of the dye was observed.

Once we demonstrated the in vitro aperture mechanism of S1, our next objective was to test the feasibility of using this gated nanodevice in cells. For this purpose, the HeLa cell line was chosen and treated with S1 at different doses for 24 h. Cell viability and cellular uptake of nanoparticles was assessed by using the WST-1 assay and confocal microscopy (Figure 3).12 The same experiments were also performed by using the MCF-7 cell line (see the Supporting Information for further details). Confocal images demonstrated that the intracellular vesicular localization of S1 nanoparticles (red) is probably associated to lysosomes. Also, S1 solid was biocompatible at the concentrations tested as no significant reduction in cell viability was observed.

With the aim to demonstrate a possible therapeutic application of these nanodevices as drug carriers, a new S1 solid was synthesised containing the chemotherapeutic agent camptothecin (CPT). CPT is a cytotoxic quinoline alkaloid that inhibits DNA polymerase I and disrupts DNA replication to induce cell death. HeLa cells were treated as described before in the presence of this new material, S1-CPT.

In order to obtain a more detailed analysis of the cell death processes related to the in-cell release of CPT from the nanoparticles, the dye propidium iodide (PI) and the early stage cell death marker Annexin V (Ann V) were employed (see the Supporting Information for further details). Figure 4 shows the results obtained by confocal microscopy and flow cytometry. A significant reduction in cell viability was observed 24 h after the addition of S1-CPT by confocal microscopy studies (cells detached from the plate, plasma membrane blebbing, and presence of cellular debris, among other features). These results were confirmed by flow cytometry experiments. Just 24 h after the addition of S1-CPT (200 µg mL⁻¹) 50 % of the cells were dead and 25 % had initiated cell death processes. By contrast, no significant reduction in cell viability was observed when cells were treated with S1-E, an S1 solid with no cargo molecule (see the Supporting Information for further details).

In summary, we have reported here the synthesis of new nanoscopic silica mesoporous supports capped with enzyme hydrolysable groups for the design of nanodevices for zero release that are specifically opened in the presence of defined enzymes. In particular we have designed gated materials capped with bulky organic moieties containing amide and urea linkages that could be selectively hydrolysed in the pres-
ence of amidase and urease, respectively. A remarkably distinct delivery profile was observed depending on the enzyme used. Amidase induced the hydrolysis of two amidase bonds located far away from the inorganic support; this allowed immediate, yet incomplete, release of the dye. In contrast, urease hydrolysed the urea bond located deeper inside the capturing molecule and closer to the surface of the silica nanoparticle; this allowed a near total cargo release but was delayed in time. Simultaneous treatment with both enzymes displayed a synergistic effect and a delivery profile showing fast and complete payload release was observed. These results demonstrate that it is possible to use relatively simple molecules containing enzyme-hydrolysable groups for the design of versatile capped materials that can be opened at will.

The possibility of including, in the capturing molecule, different enzyme-hydrolysable groups located in predefined positions allows the control of the delivery profiles. Based on the fact that enzyme–substrate pairs offer a vast range of combinations, proof-of-concept of the possible application of this nanodevice as drug carrier was performed and proved the ability of the S1-CPT solid to be internalized by cells and release its cargo. We believe that the design of multi-enzyme-responsive capped materials can be important in the design of custom-made systems for delivery applications with the aim of controlling the flow of key biological processes in nano- and regenerative medicine.

Acknowledgements

We thank the Spanish Government (project MAT2009-14564-C04 and CTQ2007-64735-AR07) the Generalitat Valenciana for their Santiago Grisolia Fellowship and PROMETEO/2009/016) for support. A.A. and L.M. thank the Generalitat Valencia (project MAT2009-14564-C04 and CTQ2007-64735-AR07) the Generalitat Valencia (project MAT2009-14564-C04 and CTQ2007-64735-AR07) the Generalitat Valencia (project MAT2009-14564-C04 and CTQ2007-64735-AR07) the Generalitat Valencia (project MAT2009-14564-C04 and CTQ2007-64735-AR07). We thank the Spanish Government (project MAT2009-14564-C04 and CTQ2007-64735-AR07) the Generalitat Valencia (project MAT2009-14564-C04 and CTQ2007-64735-AR07). We thank the Spanish Government (project MAT2009-14564-C04 and CTQ2007-64735-AR07). We thank the Spanish Government (project MAT2009-14564-C04 and CTQ2007-64735-AR07). We thank the Spanish Government (project MAT2009-14564-C04 and CTQ2007-64735-AR07).

Keywords: controlled release · drug delivery · mesoporous materials · nanoparticles · urease

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Received: October 28, 2011
Published online on February 17, 2012

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