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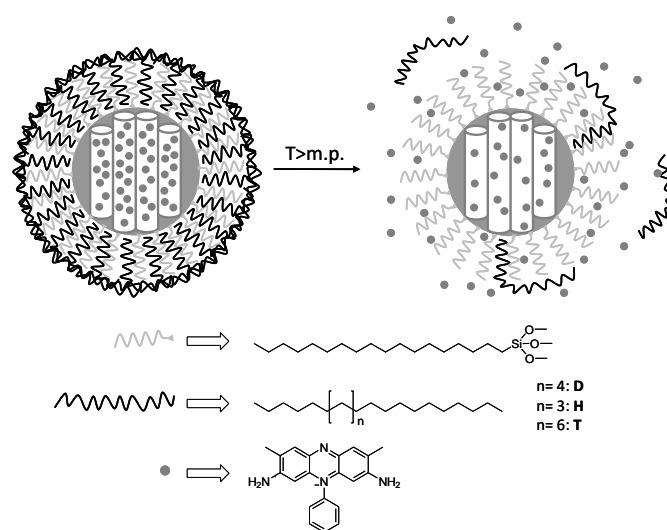
Finely tuned temperature-controlled cargo release using paraffin-capped mesoporous silica nanoparticles

Elena Aznar, Laura Mondragón, José V. Ros-Lis, Félix Sancenón, M. Dolores Marcos, Ramón Martínez-Mañez,* Juan Soto, Enrique Pérez-Payá, Pedro Amorós

Stimuli-responsive capped mesoporous materials have demonstrated to be fascinating vehicles for the storage and controlled release of entrapped guests.^[1] Chemical triggers as the presence of anions,^[2] small redox molecules,^[3] antigens,^[4] oligonucleotides^[5] and enzymes^[6] have been used to develop gated materials with controlled release features, but also physical stimuli as light^[7] and temperature^[8] have attracted the attention thanks to the possibility of remote activation. To date, temperature-controlled release of molecules from pore voids of mesoporous materials has mainly been achieved using the thermosensitive poly(N-isopropyl acrilamide) (PNIPAAm) derivative.^[8a-e] More recently Bein et al. described a molecular valve that releases the entrapped fluorescein upon heating up to the specific melting temperature of double stranded DNA sequences attached to the pore openings of mesoporous nanoparticles.^[8h] Despite these examples, temperature-controlled release of molecules from mesoporous materials is still in

its infancy and for instance there are not reported examples where the temperature release could be easily customized depending on target requirements.

Taking into account these concepts and being aware of the promising features of silica mesoporous supports (SMPS) as containers,^[9] we focused our attention on the development of new tailor-made temperature-responsive gated mesoporous materials.



Scheme 1. Representation of the gated material **S1** functionalized with octadecyltrimethoxysilane and capped with paraffin. The delivery of the entrapped guest (Safranin O) is triggered when temperature rises above paraffin melting point.

The expected performance of the new gated material, depicted in Scheme 1, is based on the use of paraffins as capping molecules that can be melted at a fixed temperature. In this work silica mesoporous nanoparticles (ca. 100-150 nm) have been selected as inorganic scaffold. The MCM-41 support was loaded with a suitable guest (Safranin O) and the external surface was functionalized with octadecyltrimethoxysilane (solid **S1**). These alkyl chains are not able to close the pores but are suitable to interact with paraffins (vide infra) via London forces setting up a hydrophobic layer that could block the pores inhibiting guest release. An increase of the temperature above paraffin melting point would result in the paraffin melting and the subsequent uncapping of the pores.

S1 was characterized using standard procedures (see Supporting Information). X-ray diffraction pattern of **S1** (Figure 1) shows the mesoporous characteristic (100) diffraction peak indicating that the loading process with the dye and the further functionalization with octadecyltrimethoxysilane have not modified the structure of the mesoporous scaffolding. Moreover, 0.44 mmol g⁻¹ SiO₂ of dye and 0.76 mmol g⁻¹ SiO₂ of octadecyl on solid **S1** were determined by elemental analysis and thermogravimetric studies.

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For the preparation of gated material **S1-D**, the paraffin docosane **D** was selected (melting point 42 °C). 150 mg of **S1** were dispersed in 120 mL of hexane sonicating during 30 minutes. Then, 1.5 g of **D** were added and the suspension (see Supporting Information for details). The final **S1-D** solid was isolated by centrifugation and dried under vacuum. This synthetic procedure assures that the final hybrid solid contains Safranin O inside of mesopores and the corresponding paraffin as coating surrounding the nanoparticle. The contents in solid **S1-D** of Safranin O and paraffin **D** (determined via thermogravimetric analysis) were 0.42 and 0.15 mmol g⁻¹ SiO₂, respectively. Figure 1 shows TEM images of calcined MCM-41 and **S1-D**. For the latter the paraffin coating can be clearly observed.

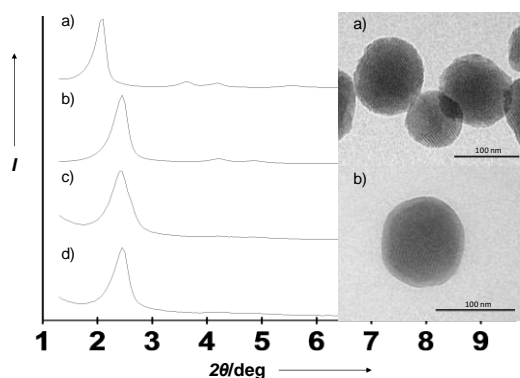


Figure 1. Left: powder X-ray diffraction patterns of (a) MCM-41 as synthesized (b) calcined MCM-41 (c) **S1** and (d) **S1-D**. Right: TEM images of (a) calcined MCM-41 and (b) **S1-D** showing the typical porosity of the MCM-41 mesoporous matrix and the paraffin coating.

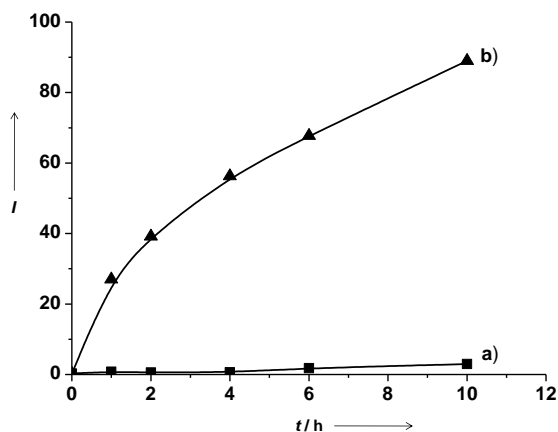


Figure 2. Release of Safranin O from solid **S1-D** at 40 °C (curve a) and 44 °C (curve b).

In order to investigate the gating properties of the solid, **S1-D** was suspended in a thermostated vase containing deionized water (see Supporting Information). The suspension was stirred at 40 °C. The same procedure was repeated with **S1-D** adjusting the temperature at 44 °C. The fluorescence emission at 585 nm ($\lambda_{ex} = 520$ nm) of the released Safranin O at the two temperatures as a function of time is displayed in Figure 2. At 40 °C solid **S1-D** was tightly capped and showed a negligible release of the dye (curve a). In contrast, when temperature was adjusted at 44 °C, the entrapped Safranin O probe escaped from the pore voids due to a temperature-dependent

uncapping event (curve b). Quantitatively, the release at 44 °C was 30-fold higher than at 40 °C in 1 hour and the difference exceeded 100-fold in 4 hours. Further experiments showed that a 36% of total entrapped dye was released after 10 hours at 44 °C.

In a second step delivery from **S1** was also studied as a function of the melting point of the temperature-responsive layer. For these experiments the paraffins heneicosane **H** (melting point 39 °C) and tetracosane **T** (melting point 49 °C) were selected. The corresponding solids **S1-H** and **S1-T** were prepared following the same procedure as that used for **S1-D**. The new solids showed similar Safranin O release profiles to **S1-D** (see Figures SI-1 and SI-3). Furthermore, the temperature-dependent performance of the hybrid materials was evaluated by suspending the corresponding solid in deionized water at a given temperature for 2 hours (Figure 3). After this time, suspensions were filtered and the release of Safranin O determined.

The results, depicted in Figure 3, confirmed how an appropriate paraffin selection allows a fine tune triggering event customization. In fact, the temperature needed to release 50% (TR50) of the entrapped probe were 39 °C, 42 °C and 50 °C for solids **S1-H**, **S1-D** and **S1-T**, respectively. It is remarkable that solid **S1-T** showed a negligible release below 49 °C although at such high temperatures diffusion processes could have some relevance.

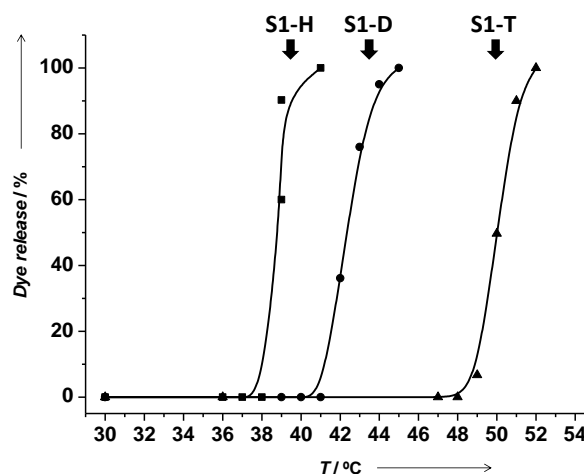


Figure 3. Release profile of solids **S1-H**, **S1-D** and **S1-T** at different temperatures. Release data were normalized to the maximum delivery obtained for each solid.

These hybrid nanoparticles can have biological applications as nanodevices for intracellular temperature-controlled release of molecules. To study this possibility, solid **S1-H** (TR50, 39 °C) was initially evaluated in HeLa cells (see Supporting Information). Briefly, cells were incubated in duplicate in the presence of **S1-H** for one hour; excess of solid **S1-H** was removed and cells were placed in fresh medium for three additional hours to allow a complete internalization of the nanoparticles. Subsequently, one cellular sample set was incubated for three hours at 42 °C in order to induce melting of the solid **S1-H** coverage, while the second was kept at 37 °C. Twelve hours later, the release of Safranin O was analysed by confocal microscopy. Figure 4 shows representative images of HeLa cells treated with solid **S1-H** at 37 °C (4a) and at 42 °C (4b). The Safranin O probe (in red colour) was found spread in the cell cytoplasm in cells incubated at 42 °C, but not in those cells

incubated at 37 °C due to a temperature-dependent uncapping of the internalized solid **S1-H**.

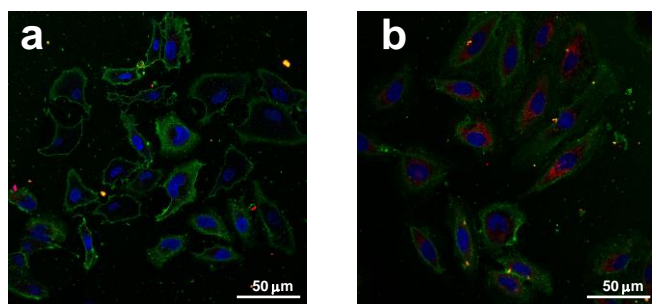


Figure 4. Confocal microscopy images corresponding to HeLa cells treated with solid **S1-H** (200 μg/mL) and incubated at 37 °C (a) and 42 °C (b). The cellular internalization of **S1-H** was followed by Safranine O associated fluorescence (red) in the presence of DNA marker Hoechst 33342 (blue) and the plasma membrane marker WGA Alexa Fluor 647 (green)

Moving a step forward in our research, our next purpose was to demonstrate the possible application of these nanoparticles as temperature-dependent drug-carriers for on-command delivery inside cells. In this case, **S1-H** was loaded with the chemotherapeutic agent doxorubicin (DOX). The release profile and performance of **S1-H-DOX** were similar to solids loaded with Safranine O (see Figure SI-4). **S1-H-DOX** induced cell death that can be traced at the early events by an exposure of phosphatidylserine to the external face of the cellular membrane (Annexin V – Ann V - is an appropriate marker – see Supporting Information). Late events of cell death are characterized by membrane cell permeabilization that allows the incorporation to the cell of nuclear markers such as DAPI. HeLa cells were treated as described before in the presence of this new nanoparticle **S1-H-DOX**. At 37 °C both, control and **S1-H-DOX** treated cells showed characteristics of healthy cells as determined by flow cytometry (negative for both Ann V and DAPI – Figure 5). However, confocal images of cells treated with **S1-H-DOX** and incubated at 42 °C, showed morphological features such as cell detachment, reduction of cellular volume and cellular shrinkage. Moreover, at 42 °C, 60% of **S1-H-DOX**-treated, but not control, cells showed characteristics of DOX-induced cell death and the analysis suggested that close to 40% of cells were developing the cell death program (Ann V positive, DAPI negative) while close to 20% of the cells (DAPI positive) were at the late stages of such program. These results confirmed the temperature-dependent release of DOX in cells.

In short, we reported herein a new family of temperature-responsive hybrid nanoscopic solids that are uncapped at a given temperature. The materials were prepared using MCM-41 supports functionalized with octadecyltrimethoxysilane and the further addition of paraffins that are able to form a hydrophobic layer that inhibited cargo release. An increase of the temperature above paraffin melting point results in the release of the entrapped guest. The finely tuned tailor-made temperature triggered delivery achieved in these capped systems and the possibility to select a wide range of paraffins with specific melting points in a wide range of temperatures make these solids suitable for applications that demand zero release before stimuli implementation and render them important in delivery applications triggered by selected global or local temperature changes.

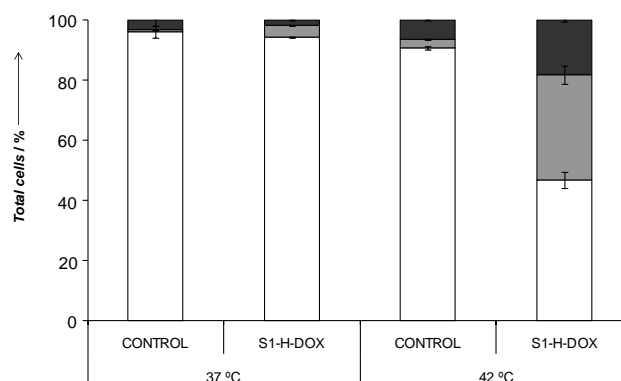


Figure 5. Cell viability studies. HeLa cells were treated or not with **S1-H-DOX** as described for **S1-H** and incubated at 37 °C or 42 °C. Then, DAPI and Ann V stainings were performed to determine cell viability and cell death induction, respectively. Percentage of dead cells (black), cells undergoing cell death (grey) and healthy cells (white) are depicted after 18 hours of treatment. Two independent experiments were performed. Data are reported as (mean ± SE).

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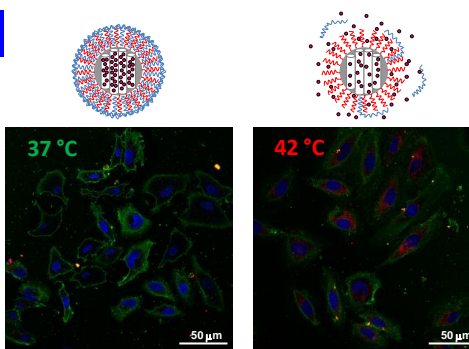
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Molecular gates

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Intracellular-temperature controlled release via paraffin-capped mesoporous silica nanoparticles



We reported herein a new family of finely tuned tailor-made temperature-responsive hybrid nanoscopic mesoporous materials that are uncapped at a given temperature and their potential application as vehicles for intracellular on-command delivery of chemotherapeutics.