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Caspase 3 targeted cargo delivery in apoptotic cells using capped mesoporous silica nanoparticles

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Abstract: Excessive cell death through apoptosis is at the origin of several pathologies, such as degenerative disorders, stroke or ischemia-reperfusion damage. In this context strategies to improve control inhibition of apoptosis and cell death are of interest and may represent a pharmacological opportunity for the treatment cell death-related disorders. In this scenario new peptide-containing delivery systems (solids **S_1-P_1** and **S_1-P_2**) are described based on mesoporous silica nanoparticles (MSNs) loaded with a dye and capped with the KKGDEVDKKARDEVDK (**P_1**) peptide that contains two repeats of the DEVD target sequence which is selectively hydrolysed by the caspase-3 (**C3**) enzyme that plays a central role in the execution-phase of cell apoptosis. HeLa cells electroporated with **S_1-P_1** are able to deliver the cargo in the presence of staurosporin (STS) which induces apoptosis with the consequent activation of the cytoplasmic **C3** enzyme. Moreover the nanoparticles **S_1-P_2**, containing both a cell-penetrating TAT peptide and **P_1** also entered in HeLa cells and delivered the cargo preferentially in cells treated with the apoptosis inducer cisplatin.

Recently the combination of new advances in nanotechnology and biomolecular chemistry has turned out in the design of new nanodevices able to develop innovative biologically related functions in areas such as bioengineering, biosensing and bionanotechnology.[1] In this context, mesoporous silica nanoparticles (MSNs) have proved to be excellent containers for storage and delivery due to their unique mesoporous structure and large loading capacity.[2] Moreover mesoporous silica can be easily functionalized, and this has allowed the design MSNs equipped with switchable molecular/supramolecular pore-capping ensembles able to be selectively opened with a number of different chemical, physical and biochemical stimuli.[3] This results in the design of stimuli-responsive hybrid gated materials showing zero release yet able to deliver the cargo at-will.[4]

In this scenario, the use of biomolecules has been widely used in the design of gated MSNs.[5] Among them, the combination of peptides as caps and enzymes as triggers offers great potential. In fact, although not as popular as other stimuli, the use of enzymes in gated materials is highly appealing as the use of tailor-made peptides and specific enzymes may induce remarkable selectivity to advanced gated devices for on-command delivery in realistic biological environments. Although certain examples have been reported using esterases,[6] glycosidases,[7] peptidases,[8] reductases,[9] ureases[10] and DNases[11] this subfield in the area of gated nanochemistry has not been fully explored. Moreover the participation of enzymes in multiple biological steps makes this approach unique for the design of advanced delivery devices and there is still plenty of room in this field of research. In particular, and as far as we know, there are not MSNs specifically designed to be selectively opened in the presence of caspase enzymes.

A number of gated materials have been designed for delivery of cytotoxics, with the main objective of reducing the secondary effects associated to classical chemotherapy due to the unspecific biodistribution of the drugs.[12] In contrast to this approach we have put our attention on the design of gated MSNs with the objective of decreasing unwanted cell death. Excessive cell death is at the origin of several pathologies, such as degenerative disorders, stroke or ischemia-reperfusion damage.[13] Therefore, controlled inhibition of cell death represents a pharmacological opportunity for the treatment of these disorders. Among the different types of cell death in this study we have focused on apoptosis,[14] a biological process in which the cell undergoes a series of biochemical and morphological changes that induce the destruction of the cell in a silent and ordered way. This process is initiated by a series of ligands that activate a family of cell death receptors located in the plasma membrane (extrinsic apoptosis) or after the induction of mitochondrial outer membrane permeabilization (MOMP) by internal cell death stimuli (intrinsic apoptosis).[15] No matter the origin, both pathways converge in the activation of a family of cysteine aspartyl proteases known as caspases. Once activated, these enzymes act as the executioner machinery of apoptosis

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cleaving essential substrates of the cell, causing the dismantling of the cells.[16] In this scenario we have focused on caspase-3 (**C3**). The **C3** cleavage site on protein substrates is highly conserved and consists of a four amino acid target site (i.e. DEVD; Asp-Glu-Val-Asp) which is cleaved after the Asp residue.[17] This specificity allows **C3** to be highly selective.

A first approach to target apoptotic cells would be to develop selective-delivery carriers that are able to release their cargo in these particular cells. In this context, and taking into account our previous experience in the development of gated nanoparticles containing peptides as molecular caps,[18] we report herein the preparation of gated MSNs capable of selectively deliver their cargo in the presence of activated **C3** enzyme in cells (Figure 1A), once apoptosis has been induced. We selected mesoporous MCM-41 nanoparticles (ca. 100 nm) as inorganic scaffold which was synthesized according to reported procedures.[19] The nanoparticles were loaded with safranin O dye and the external silica surface was functionalized with 3-(azidopropyl)triethoxysilane (solid **S_1**). The initial MCM-41 and **S_1** support were subsequently characterized using standard techniques. The mesoporous structure of MCM-41 and **S_1** were confirmed by TEM and XRD studies (Figure 1B and 1C). The starting MCM-41 material and **S_1** showed a spherical shape of ca. 110 nm (see Supporting Information). Moreover the MCM-41 calcined starting material showed a typical adsorption-desorption isotherm of MSNs. The application of the BET model resulted in a value for the total specific surface of 1001.10 m^2g^{-1} and a pore volume of 0.79 cm^3g^{-1}. Besides a pore diameter of 2.52 nm was calculated. In contrast, the N_2 adsorption-desorption isotherm of **S_1** was typical of mesoporous systems with partially filled mesopores and a significant decrease in the N_2 volume adsorbed and in the surface area was found. In particular **S_1** showed a specific surface of 437.57 m^2g^{-1} and a pore volume of 0.31 cm^3g^{-1} (see Supporting Information for further details).

Finally **S_1** was capped with the peptidic sequence NH_2-KKGDEVDKKARDEVDK- alkyne, **P_1**, (containing two repeats of the DEVD target sequence). **P_1** was attached to **S_1** by the copper(I)-catalyzed Huisgen azide/alkyne 1,3-dipolar cycloaddition "click" reaction,[20] 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 **P_1**). A scheme of the final material is depicted in Figure 1A. The **P_1**-functionalized solid (**S_1-P_1**) was isolated by centrifugation and washed to eliminate both residual dye and free peptide. TEM analysis confirmed that **S_1-P_1** retained the mesoporous structure (see Figure 1B). The safranine O and peptide contents in **S_1-P_1** were determined by elemental analysis and thermogravimetric studies, and amounted to 0.130 and 0.034 mmol g^{-1} SiO_2, respectively (see Supporting Information).

To investigate the enzyme-responsive gating properties of **S_1-P_1** in the presence of **C3** 0.5 mg of **S_1-P_1** were suspended in PBS (10% glycerol, 1 mM DTT) and the suspension stirred at 37ºC in the absence and in the presence of **C3** enzyme. Uncapping and subsequent delivery of the dye was determined through the measurement of the fluorescence emission at 585 nm (λ_{ex} = 520 nm) of the safranine O dye released. The delivery profile is displayed in Figure 1D. It is show values of Safranin release at each time normalized against total release. In the absence of caspase-3, solid **S_1-P_1** showed a negligible dye release, indicating tight pore closure, whereas in the presence of **C3** enzyme a clear cargo release was observed due to the hydrolysis of the peptide sequence **P_1**. After 400 minutes, ca. 80% of the total delivery was observed for **S_1-P_1**.

Proved the specific cargo release *in vitro*, the proper aperture of the gated **S_1-P_1** material was studied *ex vitro* using HeLa cells. It is well know that when nanoparticles of ca 100 nm are in contact with cells these are usually internalized by endocytosis reaching lysosomes. However in this case due to the cytoplasmic localization of **C3** endocytosis of **S_1-P_1** must be avoided. For that purpose, **S_1-P_1** was introduced in HeLa cells by electroporation.

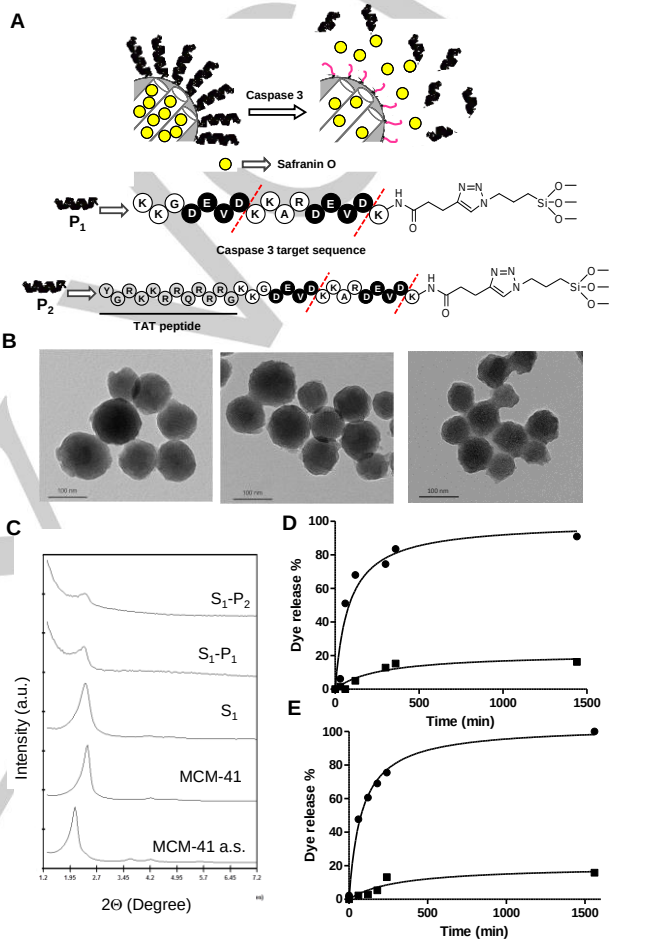

Figure 1. A) Schematic representation of the gated materials **S_1-P_1** and **S_1-P_2** capped with peptides **P_1** and **P_2**, red lines indicate the locations of caspase hydrolysis. B) From left to right, TEM images of calcined MCM-41, **S_1-P_1** and **S_1-P_2** showing the typical porosity associated to MCM-41 mesoporous matrix. C) Powder X-ray patterns of MCM-41 as synthesized, calcined MCM-41, **S_1**, **S_1-P_1** and **S_1-P_2**. D, E) *In vitro* release profiles of safranin O in the absence (■) and in the presence (●) of recombinant caspase-3 (**C3**) at 37°C and pH 7.2 for **S_1-P_1** and **S_1-P_2**, respectively.

Staurosporin, a nonspecific protein kinase inhibitor that induces apoptosis with the consequent activation of **C3,** was used for the activation of the caspase enzymes in HeLa cells.[21] Cleavage and aperture of the molecular gate of **S_1-P_1** by **C3** was assessed by confocal microscopy by means of safranin O associated fluorescence. For that purpose, cells were also stained with the DNA marker Hoechst 33342 and the cellular membrane marker Wheat Germ Agglutinin Alexa Fluor 647 (WGA). Results are shown in Figure 2A. HeLa cells electroporated in the presence of **S_1-P_1** in the absence of STS showed a poor cytoplasmic fluorescence associated to safranin

O, whereas when similar experiments were carried out with cells treated with STS a clear safranin O fluorescence was observed. This difference was attributed to **C3** activation and the subsequent hydrolysis of the $\mathbf{P_1}$ peptide containing the DEVD target sequence that resulted in cargo delivery. Moreover, in order to further prove the specific **C3**-mediated aperture of $\mathbf{S_1}$-$\mathbf{P_1}$, HeLa cells were electroporated with $\mathbf{S_1}$-$\mathbf{P_1}$ and treated with the **C3** inhibitor Q-VD-OPh[22] for 24 hours previous the addition of STS. The objective was to block **C3** activation. Under these conditions confocal microscopy images showed significant reduction of safranin O cytoplasmic signal, proving the major role of **C3** in the aperture of the molecular gate (Figure 2A). Quantification of the cytoplasmic fluorescence associated to safranin O was determined for the different treatments and it is shown in Figure 2B. In agreement with confocal studies a significantly larger fluorescence was observed in cells electroporated with $\mathbf{S_1}$-$\mathbf{P_1}$ in the presence of STS ($\mathbf{S_1}$-$\mathbf{P_1}$+STS) when compared with $\mathbf{S_1}$-$\mathbf{P_1}$ and $\mathbf{S_1}$-$\mathbf{P_1}$+STS+Q-VD-OPh treatments. Besides to discard toxicity associated with $\mathbf{S_1}$-$\mathbf{P_1}$, cell viability in the absence of apoptotic stimulus at different nanoparticle concentrations was evaluated by WST-1 (Figure 2C). No toxicity was found for $\mathbf{S_1}$-$\mathbf{P_1}$ up to concentrations of 100 $\mu g\ mL^{-1}$.

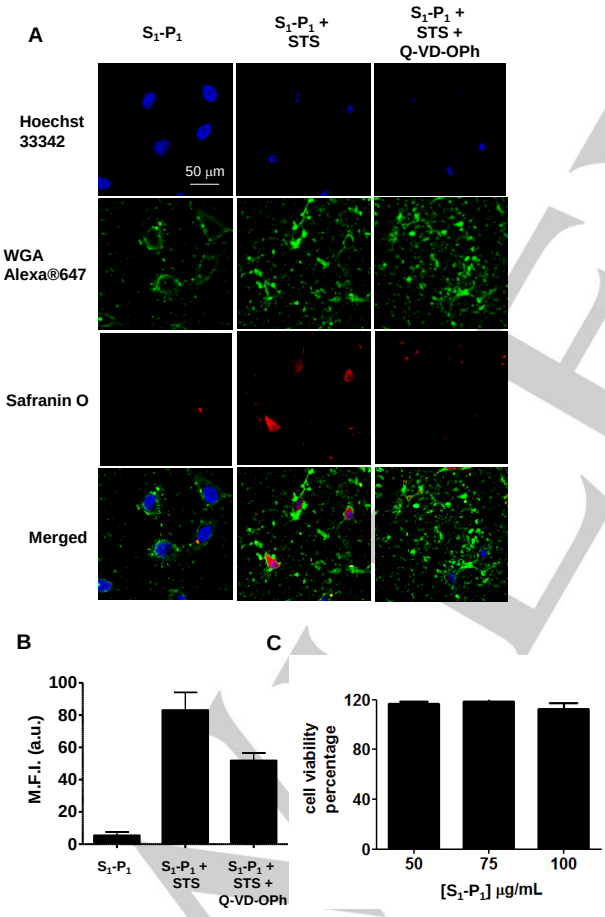

Figure 2. A) HeLa cells were electroporated in the presence of 100 μg/mL $\mathbf{S_1}$-$\mathbf{P_1}$ and incubated for 24 hours, previous the addition of STS 1.5 μM when indicated for 4 hours. Inhibition of **C3** by Q-VD-OPh 20 μM was also performed 24 hours prior the addition of STS. The cellular uptake of $\mathbf{S_1}$-$\mathbf{P_1}$ was followed by safranin O associated fluorescence (red) in the presence of DNA marker Hoechst 33342 (blue) and plasma membrane marker WGA Alexa Fluor®647 (green). B) Quantification of the safranin O associated fluorescence intensity for the different treatments. Three independent experiments were developed obtaining similar results. Data are expressed as mean ± s. Statistically significant differences were found among the different treatments when paired t Student test were applied, C) Cell viability analyzed by WST-1 at different nanoparticle concentrations.

The experiments above demonstrated that MSNs were able to display cargo delivery in the presence of **C3** enzyme via hydrolysis of the $\mathbf{P_1}$ capping peptide. However *ex vivo* experiments were performed by electroporation in order to increase the permeability of the cell membrane allowing $\mathbf{S_1}$-$\mathbf{P_1}$ MSNs to be introduced in the cytosol where **C3** enzyme exists. In this context we also considered the fact that synthetic peptides can be designed containing both, amino acid sequences recognized by target-enzyme and amino acid sequences in charge of cell entry-control. In this scenario a new peptidic sequence based on the original $\mathbf{P_1}$ was synthesized but this time adding to the N-terminal the sequence corresponding to the cell penetrating peptide TAT (peptide $\mathbf{P_2}$, see Figure 1A).[23] The TAT peptide is known to facilitate cellular uptake of various molecular cargo from nanosize particles to small chemical molecules and large fragments of DNA.

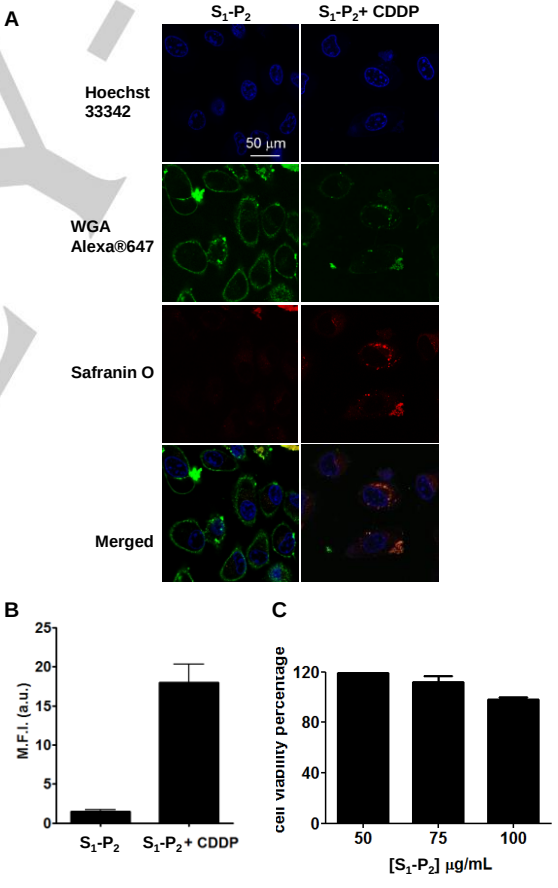

Figure 3. A) HeLa cells were treated with 100 μg/mL $\mathbf{S_1}$-$\mathbf{P_2}$ and incubated for 4 hours previous the addition of CDDP 50 μM when indicated. After 24 hours of incubations confocal microscopy studies were performed. The cellular uptake of $\mathbf{S_1}$-$\mathbf{P_2}$ was followed by safranin O associated fluorescence (red) in the presence of DNA marker Hoechst 33342 (blue) and plasma membrane marker WGA Alexa Fluor®647 (green). Cytoplasmic safanin O associated fluorescence was observed in samples treated with $\mathbf{S_1}$-$\mathbf{P_2}$ and CDDP, but not in case of control samples treated only $\mathbf{S_1}$-$\mathbf{P_2}$. B) Quantification of the safranin O associated fluorescence intensity for the different treatments. Three

independent experiments were developed obtaining similar results. Data are expressed as mean ± s. Statistically significant differences were found among the different treatments when paired t Student test were applied, C) Cell viability analyzed by WST-1 at different nanoparticle concentrations.

Solid **S_1-P_2** was synthesized following a similar procedure to that used for the preparation of **S_1-P_1** (see Figure 1A). The mesoporous structure in **S_1-P_2** was confirmed by TEM and XRD studies (see Figure 1B and 1C). The safranin O and peptide contents in **S_1-P_2** were determined by elemental analysis and thermogravimetric studies, and amounted to 0.205 and 0.016 mmol g^{-1} SiO_2, respectively (see Supporting Information). **S_1-P_2** nanoparticles were suspended in PBS (10% glycerol, 1 mM DTT) at 37ºC in the absence and in the presence of **C3** enzyme. The delivery kinetics profile is displayed in Figure 1E. **S_1-P_2** showed a poor cargo delivery in the absence of recombinant **C3** whereas a clear cargo release was observed in the presence of this enzyme. Moreover studies in HeLa cells were performed following similar procedures than those used for **S_1-P_1** but without the use of electroporation. Besides in this case cisplatin (CDDP), which causes DNA crosslinks, was employed as apoptosis inducer to activate **C3**.[24] In a typical experiment HeLa cells were seeded, and 24 hours later, were treated with **S_1-P_2** for 6 hours and then CDDP was added when indicated. 24 hours later, cells were stained with the DNA marker Hoechst 33342 and the cellular membrane marker WGA and cells were studied by confocal microscopy. Appearance of cytoplasmic associated safranin O fluorescence can be observed in case of the cells treated with **S_1-P_2** in the presence of CDDP (Figure 3A). By contrast, a very poor safranin O signal was detected in **S_1-P_2** treated cells in the absence of the apoptosis inducer. Subsequent quantification of safranin O cytoplasmic associated fluorescence showed a significant emission increase in cells treated with CDDP when compared to control cells (Figure 3B). Besides to discard toxicity associated with **S_1-P_2** cell viability in the absence of apoptotic stimulus at different nanoparticle concentrations was evaluated by WST-1 (Figure 3C). No important toxicity was found for **S_1-P_2** nanoparticles up to concentrations of 100 μg mL^{-1}.

In summary, we have shown here that it is possible to use capping peptide sequences on mesoporous silica supports in order to develop nanodevices showing nearly “zero” release yet specifically opened in the presence of the targeted proteolytic enzyme **C3** in cells. HeLa cells electroporated with **S_1-P_1** showed a poor cytoplasmic fluorescence associated to safranin O while a clear cargo release was observed in the presence of the apoptosis inducer STS. Moreover the nanoparticles **S_1-P_2**, containing both a cell-penetrating TAT peptide and **P_1**, entered in HeLa cells and delivered the cargo preferentially in cells treated with the apoptosis inducer cisplatin. These can be considered preliminary results towards adopting new therapeutic strategies to inhibit cell death due to apoptosis. Based in the fact that the field of peptide-protease pair is well-known and offers advanced opportunities for on command delivery protocols, we believe that the synthetic strategy followed herein, which combines the use of mesoporous nanoparticles with highly specific targeting peptides, can have biomedical relevance to further design improved custom-made delivery systems for other particular diseases.

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Keywords: caspase 3 • controlled release • peptides • gated mesoporous materials • nanoparticles

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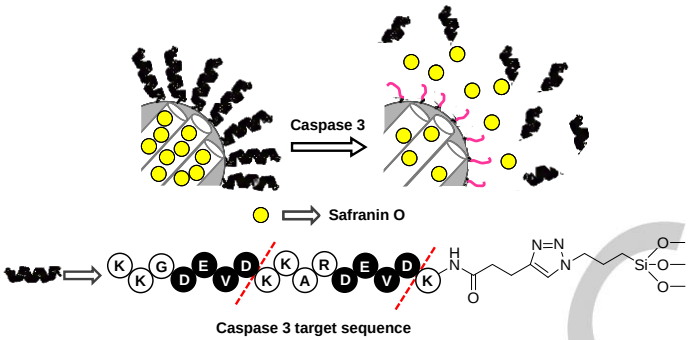
COMMUNICATION

S_1-P_1 and **S_1-P_2** nanoparticles capped with a peptide sequence recognized and hydrolyzed by caspase 3 enzyme were synthesized and characterized. *In vitro* and *ex vitro* studies of both materials proved the caspase 3 induced release of the entrapped dye.



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Caspase 3 targeted cargo delivery in apoptotic cells using capped mesoporous silica nanoparticles