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Enzyme-Responsive Intracellular Controlled Release Using Nanometric Silica Mesoporous Supports Capped with 'Saccharides'.

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TITLE RUNNING HEAD Nanoscopic hybrid systems containing gate-like scaffoldings.

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ABSTRACT. The synthesis of new capped silica mesoporous nanoparticles for on-command delivery applications is described. The gate-like functional hybrid systems consisted of nanoscopic MCM-41-based materials functionalized on the pore outlets with different 'saccharide' derivatives and a dye contained in the mesopores. A series of hydrolyzed starch products as 'saccharides' were selected. The mesoporous silica nanoparticles **S1**, **S2** and **S3** containing the grafted starch derivatives Glucidex® 47, Gludicex® 39 and Glucidex® 29, were synthesized. Additionally, for comparative purposes solid **S4**, containing lactose was prepared. Delivery studies in pure water in the presence of pancreatin or β-D-galactosidase were carried out for **S1**-**S3** and **S4**, respectively. **S1**, **S2** and especially **S3** showed very low release in the absence of enzyme, but displayed cargo delivery in the presence of the corresponding enzyme. Moreover, nanoparticles of **S1** were used to study the controlled release of the dye in intracellular media. Cell viability assays using HeLa and LLC-PK1 cells indicated that **S1** nanoparticles were devoid of unspecific cell toxicity. The Endocytosis process for **S1** nanoparticle internalization in HeLa cells was confirmed and the anchored starch was degraded by the lysosomal enzymes. Furthermore, a new mesoporous silica nanoparticle functionalized with Glucidex® 47 and loaded with a cytotoxic, **S1-DOX** was developed. The cell viability with **S1-DOX** decreased due to the internalization

of the nanoparticle, enzyme-dependent opening of the "saccharide" molecular gate and the consequent release of the cytotoxic agent. As far as the authors know, this is the first example of enzyme-induced in cell delivery using capped silica mesoporous nanoparticles.

KEYWORDS. Carbohydrates, enzyme, intracellular controlled release, mesoporous, and gate.

BRIEFS. Mesoporous silica nanoparticles functionalized with certain 'saccharides' were prepared, fully characterized and used for an enzyme-triggered controlled release of an entrapped guest in *in vitro* and in *ex vivo* assays.

Controlled release of therapeutic drugs, peptides or nucleic acid fragments in a specific cell is a new and promising research field in biomedical sciences. Traditional delivery systems are in most cases based on polymers that usually release their cargo *via* diffusion-controlled processes or through degradation of the polymer container.[1-7] In recent years, as an alternative to polymeric materials, silica mesoporous supports (SMPS) have been used as inorganic scaffolds for the storage and release of drugs and organic molecules.[8-10] Silica mesoporous supports provide unique features such as stability, biocompatibility, large load capacity, and the possibility to include gate-like scaffoldings on the external surface for the design of nanodevices for on-command delivery applications.[11-16] Most of the gated stimuli-responsive systems are based on bio-channels and bio-gates that utilize movable mechanisms triggered by specific stimuli. SMPS-based systems with control release properties have been reported. Such systems, containing different gate-like scaffoldings, are sensitive to changes in the media pH or redox conditions as well as to light as triggers for uncapping the pores.[17-19]

The first gated SMPS was developed by Fujiwara *et al.*[20,21] *via* reversible photodimerization of coumarin attached to the pore outlets that were sensitive to light. Since then, other photochemical gated systems based on *cis-trans* isomerization of azobenzene moieties,[22-25] the association and light-operated dissociation of a α-cyclodextrin with azobenzene groups[26-28] and the spiropyran-merocynine photo

switchable transformation[29] have been reported. pH is also a widely used stimulus for the control of mass transport in mesoporous materials. The authors recently reported the first gated hybrid system, using SMPS functionalized with polyamines on the external surface, operating in an aqueous solution under pH and anion control.[30-32] Additional systems involving carboxylates[33] pseudorotaxanes,[34-37] inclusion complexes with cucurbit[6]uril[38-40] cucurbit[7]uril[41] and α-cyclodextrins[42] have also been prepared. Furthermore dual pH- and photo-switched release of guests has been achieved in SMPS capped with boronic acid-functionalized gold nanoparticles.[43] Changes in the media redox conditions are another popular approach to control delivery in capped mesoporous materials. Lin *et al*. developed capped materials using mesoporous scaffoldings capped with CdS, gold or magnetic nanoparticles attached to the SMPS through disulfide linkages that were broken up upon addition of certain reducing compounds.[44-54] Also, the rupture of disulfide linkages in molecular based systems[55] and polymeric networks have been used for on-command delivery.[56,57] Zink and Stoddart prepared several gated materials triggered by redox inputs that were equipped with nanovalves based on rotaxanes and pseudorotaxanes containing redox active moieties.[58-62] Apart from the examples described above, which use redox-, light- and pH-triggered systems, some additional exploratory studies that use alternative external stimuli such as temperature[63] and the presence of certain anions[64] have also been reported. Another use of these hybrid gated nanomaterials deals with the development of new signaling protocols. The release of an entrapped dye triggered by a specific guest is used in this paradigm which leads to the development of novel hybrid sensing materials showing enhanced signaling features.[65-67]

Although some efforts have been made to prepare SMPS containing different gate-like scaffoldings, the development of real systems for controlled release is still in its incipient stage. For instance, some of the reported systems display gating features in non-aqueous solvents, employ gate-like scaffoldings that require large synthetic efforts or use external stimuli that are difficult to apply to certain delivery applications. In addition, there is an almost complete lack of SMPS-based systems showing a selective delivery induced by target bio-molecules. One of the few examples involves the use of antibody-capped

mesoporous nanocontainers that are specifically uncapped in the presence of the corresponding antigen[68]. The enzyme-substrate system can also offer opportunities for the design of sensitive and specific SMPS-based nanodevices. In a first proof-of-the-concept Zink *et al*. functionalized the external surface of SMPS with a [2]rotaxane capped with an ester-linked adamantyl stopper.[69,70] The functionalized SMPS device showed "zero release" until the addition of porcine liver esterase (PLE) which induced dethreading of the [2]rotaxane due to hydrolysis of the adamantyl ester. More recently, Bein *et al.* have attached avidin caps on biotinylated SMPS. [71] The avidin-biotin complex formation resulted in a tight closure of the pores. Addition of the protease trypsin resulted in the hydrolysis of the attached protein avidin and the release of the entrapped guest. The authors have contributed to the field reporting mesoporous silica supports capped with lactose which were selectively uncapped in the presence of enzyme β-D-galactosidase.[72] Also, Kim *et al.* reported the preparation of silica nanoparticles functionalized through a click chemistry reaction, with β-cyclodextrin as gatekeepers.[73] Addition of α-amylase induced the release of calcein due to hydrolysis of the cyclodextrin core.

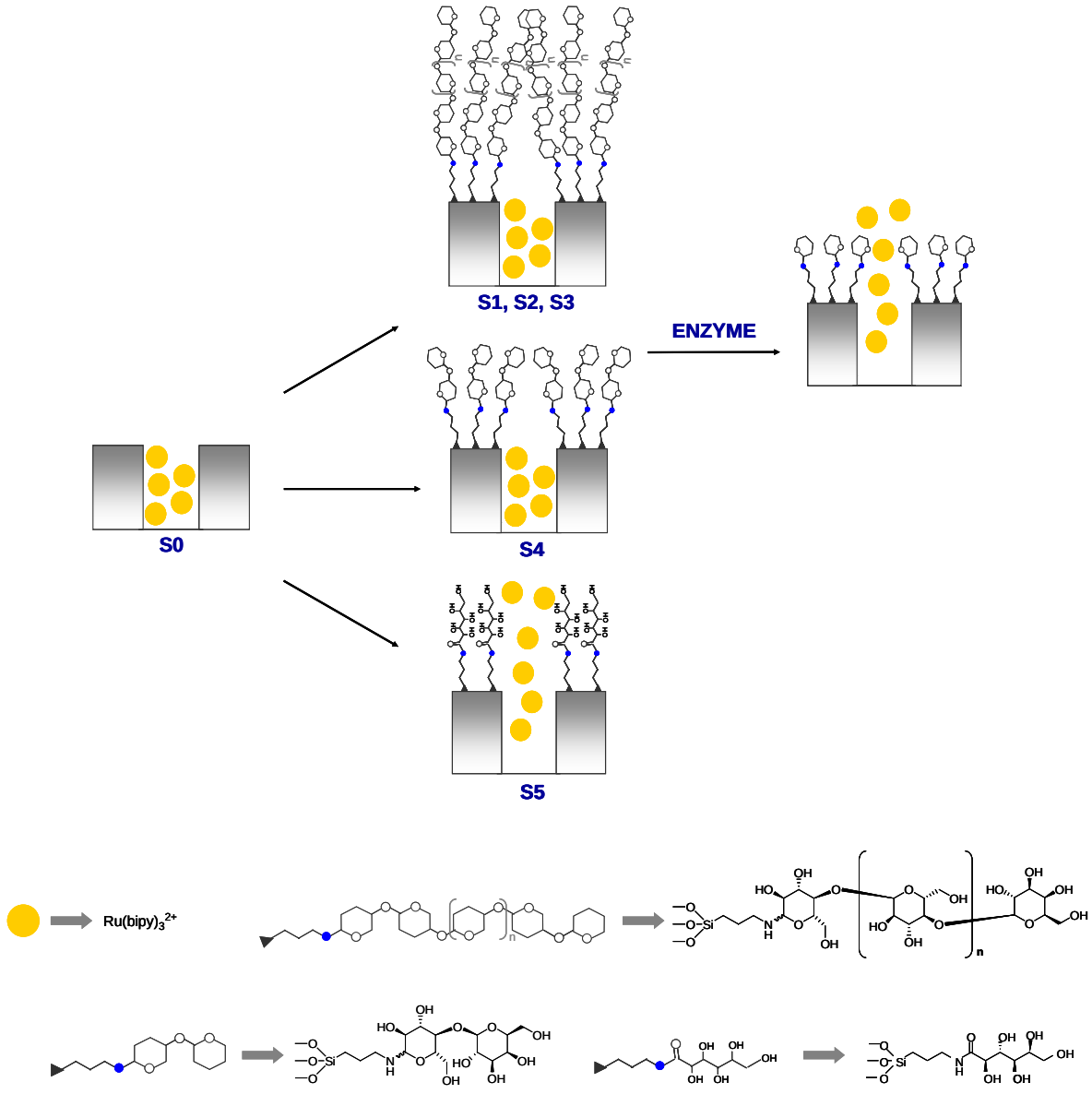
The groundwork done suggests that enzymatic degradation of capped scaffoldings in SMPS is a suitable protocol for the on-command delivery of entrapped substances. In fact, the possibility of using enzymes for selective release applications opens a wide range of new perspectives in the development of bio-compatible delivery systems using silica mesoporous supports. However, as far as the authors know, examples of enzyme-induced delivery in living cells using capped silica mesoporous nanoparticles have not yet been reported. As a continuation of the authors' previous work in this field, the synthesis, characterization and controlled release experiments using nanometric SMPS functionalized with 'saccharides' are reported herein. Delivery processes in the presence of enzymes are studied and the internalization of 'saccharide'-capped nanoparticles in cells is also demonstrated.

RESULTS AND DISCUSSION.

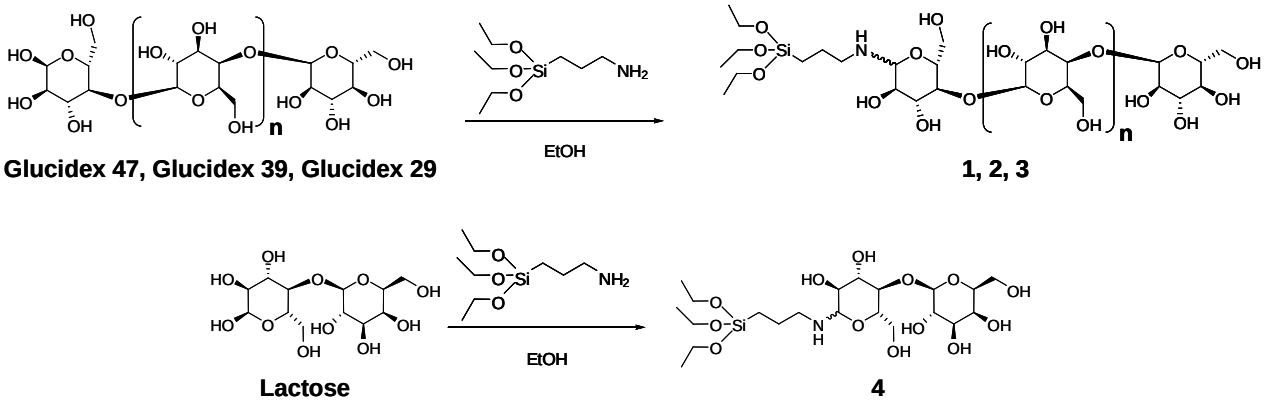
The gated materials. The incorporation of gate-like ensembles on mesoporous scaffoldings has proved to be a suitable approach for the development of nanoscopic solids for mass transport and controlled release applications. As stated in the introduction section, most of the gated materials developed make use of molecular/supramolecular interactions for the fine control of cargo delivery. Very few examples use bio-molecules for capping or uncapping protocols. Following the authors' interest in the application of biological interactions, as triggered control for delivery, attention here was centered on the use of enzymes as "biological-keys".

Scheme 1 shows the proposed paradigm for the preparation of the gated material. In this approach, MCM-41 was used as an inorganic scaffold in the form of nanoparticles (*vide infra*). The MCM-41 support contains mesopores in the 2-3 nm range that allow the encapsulation of certain guests. The aim was to design bio-compatible, easy-to-prepare and low-cost capping systems in order to synthesize simple gated-scaffoldings to be used efficiently in delivery applications. Among different possibilities, attention was focused on the use of 'saccharides'. With this goal in mind, and at the same time seeking to avoid complex synthetic routes to saccharide derivatives, the use of commercially-available hydrolyzed starch products was opted for.[74] Hydrolyzed starch is obtained by the moderate hydrolysis of starch. The degree of starch hydrolysis is indicated by the dextrose equivalent (DE)[75] which denotes the percentage fraction of reducing sugars in the sample (DE 1 is equivalent to non-hydrolyzed starch whereas DE 100 is equivalent to glucose). For this work the following dextrose equivalents (DE) were used; Glucidex® 47 (5% glucose, 50% maltose, 45% oligosaccharides and polysaccharides), Glucidex® 39 (3% glucose, 37% maltose, 60% oligosaccharides and polysaccharides) and Glucidex® 29 (10% glucose, 9% maltose, 81% oligosaccharides and polysaccharides). Additionally, for comparative purposes, lactose[76] was also used as a capping molecule. Carbohydrate polymers and mixtures of carbohydrate polymers are readily available simple saccharides that have been widely used as food additives and biodegradable systems and matrices for the stabilization of certain species.[77]

The hydrolyzed starch derivatives (Glucidex® 47, 39 and 29) were reacted with 3-aminopropyltriethoxysilane in ethanol to yield the corresponding alkoxysilane derivatives (**1**, **2**, **3**) (see Scheme 2), whereas the capping molecule **4** was prepared *via* reaction of 3-aminopropyltriethoxysilane with lactose (see Scheme 2).[78] The further anchoring of these silane derivatives **1**, **2**, **3** and **4** on the external surface of the SMPS resulted in the preparation of solids **S1**, **S2**, **S3** and **S4**. The anchoring of these saccharide derivatives were expected to inhibit cargo delivery due to the formation, around the pore outlets, of a dense hydrogen bonding, interaction-based, 'saccharide' network. The working hypothesis predicts that in the presence of pancreatin (containing amylases able to hydrolyze the 1→4 glycosidic bond between β-D-glucoses present in the starch) or β-D-galactosidase (able to hydrolyze 1→4 glycosidic bond between β-D-galactose and β-D-glucose in lactose) the hydrolysis of the 'saccharide' network results in an uncapping of the pores in **S1**-**S3** and **S4**, respectively, allowing delivery of the entrapped molecule (*vide infra*). $[Ru(bipy)_3]^{2+}$ was selected as a guest for delivery. The delivery of the ruthenium complex from the pore voids to the aqueous solution (the uncapping protocol) can be easy followed *via* monitoring of the absorption band at 453 nm in the aqueous phase.



Scheme 1. Schematic representation of the synthesis of hybrid mesoporous nanoparticles **S1**-**S4** capped with trialkoxysilane carbohydrate derivatives and **S5** functionalized with *N*-(3-triethoxysilylpropyl)gluconamide. Addition of enzymes to **S1**-**S4** solids would uncap the mesopores *via* the selective hydrolysis of the 'saccharides' anchored on the mesoporous external surface.



Scheme 2. Synthesis of trialkoxysilane hydrolyzed starch derivatives **1, 2** and **3**, and synthesis of trialkoxysilane lactose derivative **4.**

The final solids (**S1**, **S2**, **S3** and **S4**) must ideally contain the oligosaccharide derivative anchored to the external surface, whereas the dye must be contained in the mesoporous channels. In order to prepare the final hybrid materials with these requirements a previously reported two-step synthetic procedure was used. In the first step, the mesoporous nanoparticles were added to a solution containing a high concentration of $[Ru(bipy)_3]^{2+}$ dye in order to achieve an efficient loading of the pores. Then, in the same mixture (second step of the material preparation) the hydrolyzed starch derivatized with trialkoxysilane moieties **1** (from Glucidex® 47), **2** (from Glucidex® 39), **3** (from Glucidex® 29) or the lactose derivative **4** was added to the suspensions. This would lead to hybrid materials in which the 'saccharides' are basically placed on the external surface of the mesoporous support bearing in mind that the anchoring of the corresponding 'saccharide' is carried out when the mesopores are filled with the dye. Finally the yellow/orange solids (**S1, S2, S3** and **S4**) were filtered, washed with water and dried at 40 ºC for 12 hours. As appropriate controls, solids **S0**, **S5** and **S6** were prepared. Solid **S0** is an SMPS with $[Ru(bipy)_3]^{2+}$ dye filling the pores but lacking any further functionalization on the surface. Solid **S5** contains the $[Ru(bipy)_3]^{2+}$ dye in the pores and was additionally functionalized on the surface with the commercially available *N*-(3-triethoxysilylpropyl)gluconamide derivative (see Scheme 1). This solid, functionalized with a monosaccharide (a glucose derivative), assesses the degree of effect, if any, that the hydrolyzed starch (**1**, **2** and **3**) and the disaccharide lactose (**4**) grafted on the pore outlets have on

the dye delivery from solids **S1**-**S4** in the presence of suitable enzymes. Solid **S6** is an SMPS without any dye in the interior of the pores and functionalized with the hydrolyzed starch derivative **1** (from Glucidex® 47). This solid was prepared exactly according to the surface functionalization procedures used.

Characterization of the solids. The solids prepared were characterized using standard techniques. Figure 1 shows powder X-ray patterns of the solids MCM-41 as-synthesized, MCM-41 calcined and **S1**, **S2**, **S3** and **S6**. The XRD of siliceous MCM-41 as-synthesized (curve a) shows the typical four low-angle reflections of a hexagonal ordered array indexed as (100), (110), (200), and (210) Bragg peaks. From the XRD data an a_0 cell parameter of 46.43 Å (d_{100} spacing of 40.21 Å) was calculated. In curve b, corresponding to the MCM-41 calcined sample, a significant shift of the (100) reflexion in the XRD is clearly observed. This displacement, together with the broadening of the (110) and (200) reflexions, is consistent with an approximate cell contraction of ca. 6-8 Å and attributed to the condensation of silanols during the calcination step. Figure 1 also shows the XRD patterns for solids **S1, S2, S3** and **S6** (curves c, d, e and f, respectively). For these materials, the reflections (110) and (200) are lost, most likely due to a reduction of contrast as consequence of the functionalization process. Nevertheless, the presence of the d_{100} peak in the XRD patterns in all cases indicated that the process of pore loading with the $[Ru(bipy)_3]^{2+}$ complex, and the additional functionalization with the corresponding saccharides, did not to a large extent modify the mesoporous MCM-41 scaffolding. **S4** and **S5** show XRD profiles similar to those from **S1**-**S3** (not shown). The presence in the final functionalized solids of the mesoporous structure was also confirmed from the TEM analysis, in which the typical channels of the MCM-41 matrix are visualized as alternate black and white stripes (see Figure 2 for MCM-41 calcined (a) and solid **S3** (b)). The figure also shows that the prepared MCM-41-based materials are obtained as spherical particles with diameters ranging from 100 to 200 nm.

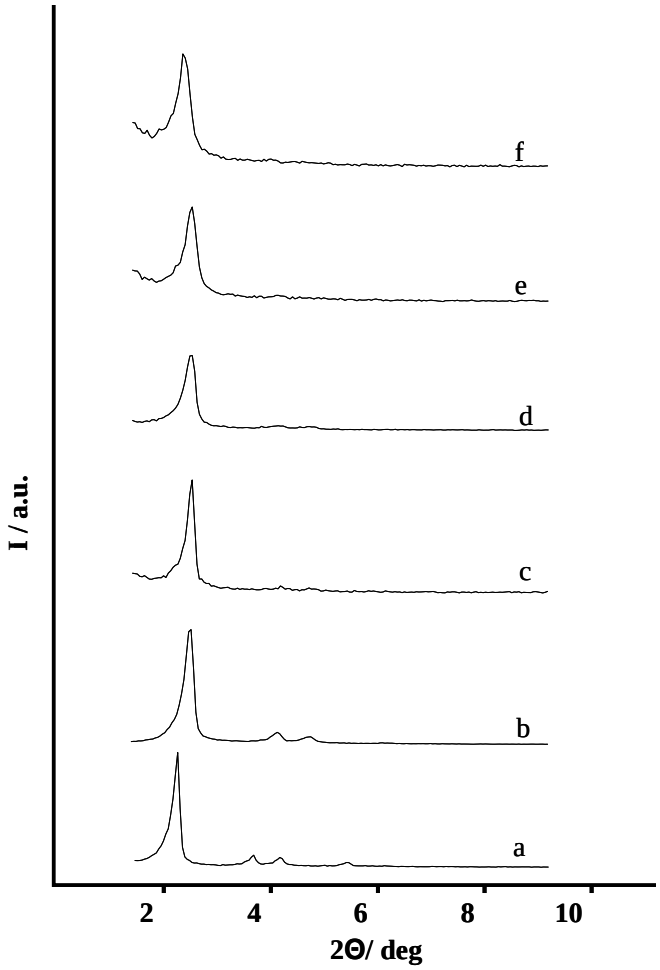


Figure 1. X-ray pattern of (a) MCM-41 as-synthesized, (b) MCM-41 calcined, (c) **S1** (d) **S2** (e) **S3** and (f) **S6**

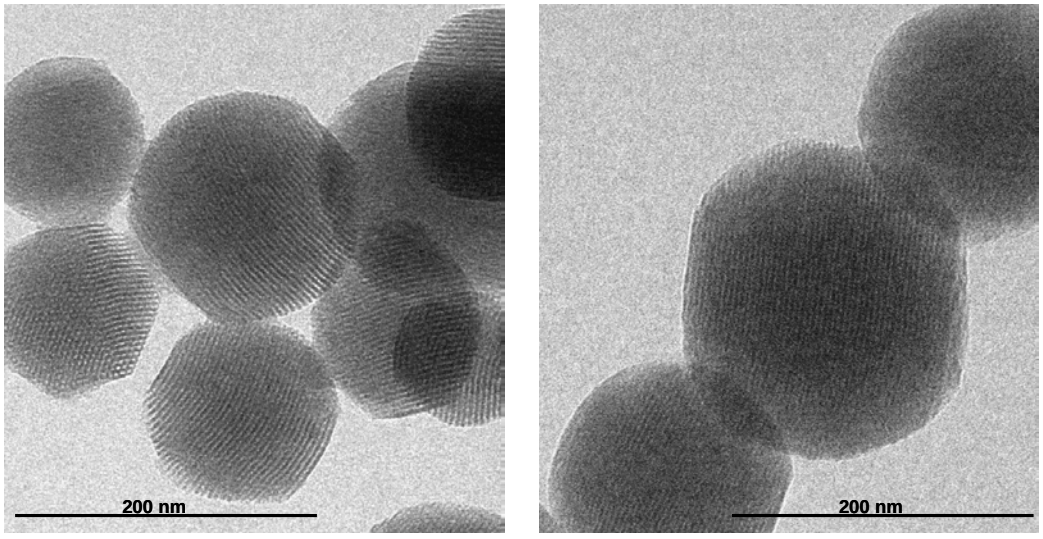


Figure 2. TEM image of MCM-41 calcined (a) and solid **S3** (b) showing the typical porosity of the MCM-41 matrix.

The N_2 adsorption-desorption isotherms of the nanoparticulated MCM-41 calcined material is shown in Figure 3. A typical curve for these mesoporous solids consisting of an adsorption step at intermediate P/P_0 value (0.1-0.3) can be observed. This curve corresponds to a type IV isotherm, in which the observed step deals with nitrogen condensation inside the mesopores. The absence of a hysteresis loop in this interval and the narrow BJH pore distribution suggest the existence of uniform cylindrical mesopores (pore diameter of 2.29 nm and pore volume of 0.62 $cm^3\ g^{-1}$calculated by using the BJH model on the adsorption branch of the isotherm). The application of the BET model resulted in a value of 975 m^2/g for the total specific surface. From the XRD, porosimetry and TEM studies, the a_0 cell parameter (3.98 nm), the pore diameter (2.29 nm) and a value for the wall thickness of 1.69 nm were calculated. In addition to this adsorption step associated to the micelle generated mesopores, a second feature appears in the isotherm at a high relative pressure ($P/P_0 > 0.8$). This adsorption corresponds to the filling of the large voids among the particles (0.48 $cm^3\ g^{-1}$calculated by using the BJH model) and therefore must be considered as a textural-like porosity. In this case, the curves show a characteristic H1 hysteresis loop and a wide pore size distribution.

The N_2 adsorption-desorption isotherm of **S1** is typical of mesoporous systems with practically filled mesopores (see Figure 3 c). Consequently, relatively low N_2 adsorbed volume (BJH mesopore volume = 0.13 $cm^3\ g^{-1}$) and surface area (231 m^2/g) values were calculated. In fact, this solid shows flat curves when compared (at the same scale) to those of the MCM-41 parent material and the **S6** solid without dye, this indicates significant pore blocking and the subsequent absence of appreciable mesoporosity. Despite the significant pore volume decrease, some features can still be observed in the BJH mesopore size distribution such as a maximum at 1.8 nm (on the border between meso and micropores) and several shoulders in the 2-3 nm range. Additionally, the textural porosity is preserved and only a certain decrease (0.31 $cm^3\ g^{-1}$) when compared to the parent silica is observed. **S2**, **S3**, **S4** and **S5** samples show similar N_2 adsorption-desorption isotherms to that shown by **S1**. This is an expected result bearing in mind that all the solids have dye molecules sited at the pores and assuming a certain collapse of the

saccharide groups anchored to the pore entrances, while taking into account the dry conditions achieved during the sample evacuation prior to the analysis (*vide infra*). Similar results have been observed by the authors in related systems.

An intermediate behavior between those of nanoparticulated MCM-41 and functionalized and dye charged samples (**S1**, **S2**, **S3**, **S4** and **S5**) is observed for **S6**. Although the N_2 adsorption-desorption isotherm of **S6** (see Figure 3 b) is qualitatively similar to the isotherm of MCM-41 (the two well defined adsorption steps ascribed to the mesopores and the textural pores are preserved) an appreciable decrease both in the N_2 volume adsorbed (BJH mesopore volume = 0.28 cm^3 g^{-1}) and surface area (567 m^2/g) was measured as expected. The area of solid **S6** was reduced ca. 40%, when compared to that presented by MCM-41, due to the external grafting of derivative **1**. On the other hand, the difference between **S1** and **S6** isotherms is clearly related to the filled or empty (with the ruthenium dye complex) nature of the mesopores, respectively. Therefore, for a similar functionalization degree with sacharides, the higher surface area and pore volume for **S6** must be due to the absence of the dye in the interior of the mesopores. Application of the BJH model leads to an average pore diameter of 2.16 nm. As observed for **S1**, the BJH pore size distribution shows a maximum at 1.85 nm (on the border between meso and micropores) and several shoulders. The presence or absence of dye inside the mesopores does not affect the textural-like porosity. In fact, the second adsorption step at high relative pressure (P/P_0 > 0.8) which appears in the isotherm of **S6** and similar BJH textural pore volumes can be estimated for **S1** (0.31 cm^3 g^{-1}) and **S6** (0.30 cm^3 g^{-1}). This data supports the fact that the dye molecules are specifically incorporated inside the ordered mesopores. BET specific surface values, pore volumes, and pore sizes calculated from the N_2 adsorption-desorption isotherms for MCM-41, **S1**, **S2**, **S3**, **S4**, **S5** and **S6** are listed in Table 1.

Considered as a whole, the N_2 adsorption-desorption data illustrate the effective pore blocking that occurs when the saccharide functional groups outside the pore and the dyes inside the mesopores are present in a cooperative way. Moreover, as previously described for related gate-like ensembles, the dry

conditions required for sample preparation previous to the adsorption experiments, must favor a certain saccharide dehydration with the subsequent collapse on the external surface. This collapse would block the mesopores to a large extent. In the absence of dye molecules inside the mesopores, a slightly higher porosity seems to be accessible for nitrogen. This last behavior is a direct consequence of the evolution of large complexes occupying the mesoporous voids. This loss of the cooperative blocking effect favors a certain increase of the internal empty volume. In all materials containing dye molecules and functional groups, similar pore sizes and volume values were measured. The BJH pore size distributions of these last materials (**S1**, **S2**, **S3**, **S4** and **S5**) show maximum peaks and/or appreciable shoulders at pore sizes around 1.8-2.0 nm. These values (lower than those calculated for the pure silica parent material), seem to be more realistic than those estimated by the application of the BJH model. The decrease in mesopore size to the range of supermicropores could be viewed as a non-completely efficient collapse of the functional arms on the mesopore entrances and even to a certain inhomogeneity in the density of functionalized saccharides.

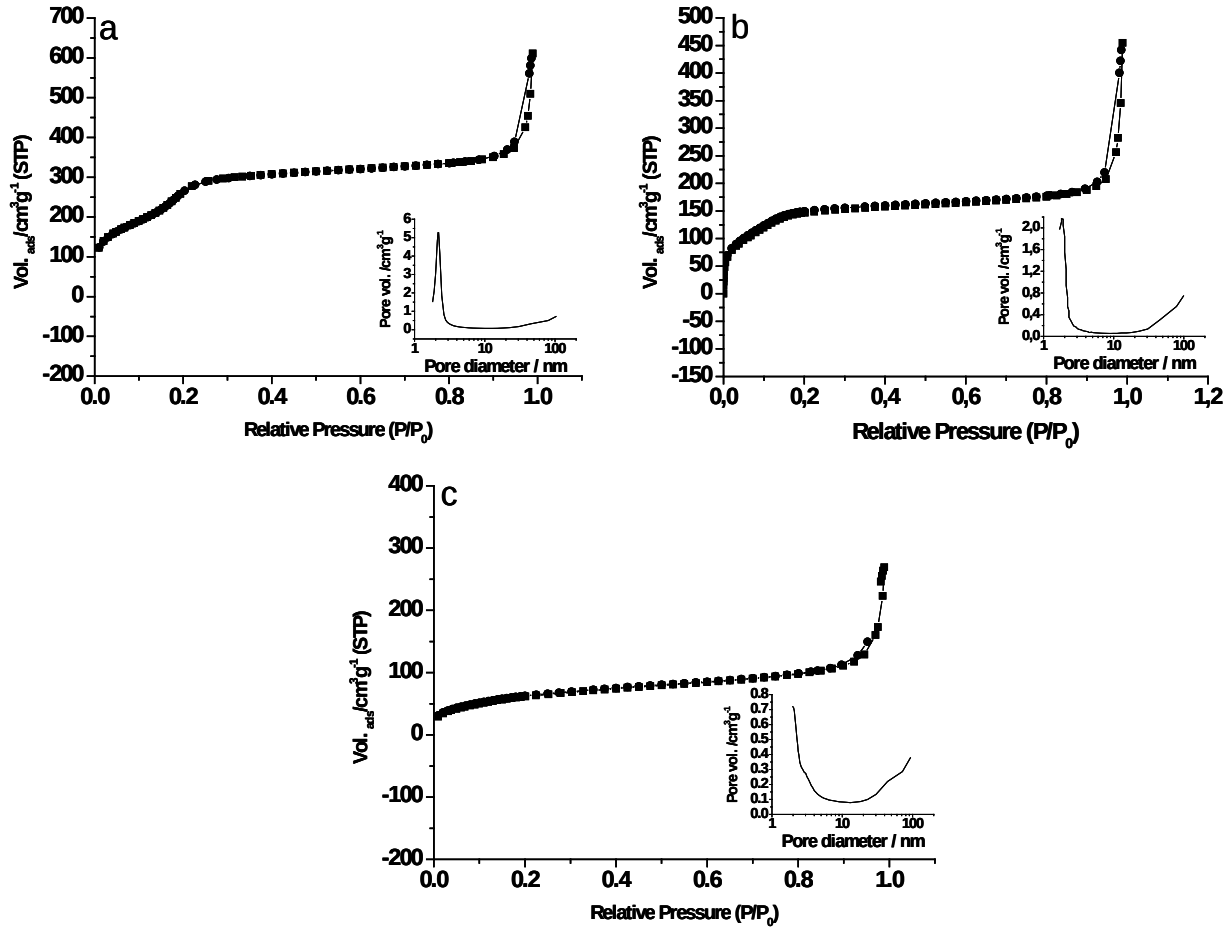


Figure 3. Nitrogen adsorption-desorption isotherms for (a) MCM-41 mesoporous material (b) **S6** and (c) **S1** materials. Insets: Pore size distribution of each material.

The contents of 'saccharides' and dye in solids **S0**, **S1**, **S2**, **S3**, **S4**, **S5** and **S6** were determined by elemental and thermogravimetric analysis and are shown in Table 2. Solids **S1**, **S2** and **S3** functionalized on the outer surface with hydrolyzed starch showed very similar contents that range from 0.055 to 0.073 g/g for the polysaccharide/SiO_2 ratio and from 0.085 to 0.099 g/g for the dye/SiO_2 ratio.

Table 1. BET specific surface values, pore volumes and pore sizes calculated from the N_2 adsorption-desorption isotherms for selected materials.

	S_{BET}	Pore Volume [a]	Pore size [a,b]
	(m^2 g^{-1})	(cm^3 g^{-1})	(nm)
MCM-41 (nanoparticle)	975	0.62	2.29
S1	231	0.13	2.46
S2	804	0.49	2.44
S3	193	0.09	2.29
S4	45	0.04	2.30
S5	10	0.01	2.20
S6	567	0.28	2.16

[a]Pore volumes and pore sizes are only associated with intraparticle mesopores. [b]Pore size estimated by using the BJH model applied on the adsorption branch of the isotherm.

Table 2. Content (α) in grams of 'saccharide' and dye per gram of SiO_2 for solids **S0**, **S1**, **S2**, **S3**, **S4**, **S5** and **S6**.

Solid	$\alpha_{'saccharide'}$ (g/g SiO_2)	α_{dye} (g/g SiO_2)
S0	____	0.112
S1	0.067	0.099
S2	0.073	0.085
S3	0.055	0.090
S4	0.114	0.064
S5	0.068	0.257
S6	0.124	____

Functional enzyme-driven controlled release. As stated above the aim was to develop enzyme-triggered delivery systems using 'saccharide'-capped mesoporous nanoscopic scaffolds. In this section, several experiments were carried out in order to study the enzyme-responsive controlled-release

protocol, using the capped materials, in detail. The behavior of **S1, S2** and **S3** in the presence of pancreatin and of solid **S4** in the presence of β-D-galactosidase was initially analyzed in water at pH 7.5, as these have been described as optimal conditions for enzyme-based degradation of saccharides. Pancreatin (a mixture of amylase, lipase and proteases) would uncap the pores through selective hydrolysis of the 1→4 glycosidic bond in the starch chains, whereas β-D-galactosidase, able to hydrolyse 1→4 glycosidic bond between β-D-galactose and β-D-glucose, would induce the pores to open in **S4**.

In a typical experiment, **S1**, **S2**, **S3** and **S4** were suspended in water at pH 7.5 in the presence of and in the absence of the corresponding enzyme. The suspension was then stirred and the dye delivery was monitored through the absorption band of the $[Ru(bipy)_3]^{2+}$ dye at 453 nm (Figure 4 shows the delivery profiles). As a significant feature, the "zero release" obtained in the absence of enzyme should be noted. This is especially relevant when analyzing solid **S3** (*vide infra*). In clear contrast, the solids displayed an enzyme-dependent cargo delivery. In more detail, solids **S1**, **S2** and **S3** released less than 2 % of the entrapped dye after five hours in water whereas solid **S4** released 7 % of its cargo. In the presence of the corresponding enzyme (pancreatin for **S1**, **S2** and **S3** and β-D-galactosidase for **S4**) **S1**, **S2** and **S3** delivered 63, 48 and 31 % of the cargo respectively, whereas solid **S4** released 85 % of the dye (see Figure 4). It is clear that a simple choice of the hydrolysis degree of the starch has a dramatic influence in the delivery profile. Thus, at a certain time, **S1** is able to deliver more cargo than **S2,** and **S2** is able to deliver more cargo than **S3** which is directly related to the different DE values of the hydrolyzed starch acting as capping scaffoldings; *i.e.* the lesser the hydrolysis the lower the delivery rate. Although the rate of hydrolysis using pancreatin (acting on **S1**, **S2** and **S3**) and β-D-galactosidase (for **S4**) may not be the same, it is apparent from Figure 5 that in the presence of the corresponding enzyme the use of a disaccharide such as lactose as a capping system results in a higher delivery rate when compared with the use of hydrolyzed starch.

Another important feature of the prepared capped materials is the ability to release their cargo in a progressive fashion for prolonged periods of time. This is especially the case for **S1**, **S2** and **S3**. These systems are able to deliver the cargo over a period of at least 60 hours (not shown), a property that could be of special interest to avoid unwanted drug delivery peaks. Low delivery rates have been observed particularly for **S3** nanoparticles that release ca. 68 % of the cargo after 60 hours and would be active for longer periods of time. This progressive release could be related to a lowered degree of hydrolysis of the appended starch in **S3** when compared to **S1** or **S2.**

In order to confirm the proposed enzyme-mediated gating mechanism solid **S5** was prepared, which is similar to **S1**-**S4** but contains the commercially available monosaccharide derivative *N*-(3-triethoxysilylpropyl)gluconamide on the surface. This is a suitable model bearing in mind that the enzymatic hydrolysis of the chain capping structures in **S1**-**S4** will ideally result in a unique glucose unit appended on the external surface of the solid. As can be observed in Figure 4, the 'saccharide'-functionalized **S1**-**S4** showed a very low cargo release in the absence of the corresponding enzyme, whereas the monosaccharide-containing **S5** displays a significant $[Ru(bipy)_3]^{2+}$ delivery under similar experimental conditions. These results stress the conclusion that the delivery induced in the presence of pancreatin or β-D-galactosidase is due to the enzymatic-mediated rupture of the glycosidic bond and reduction in length of the saccharide chain at the surface. To further demonstrate that both, pancreatin and β-D-galactosidase are responsible of the release of $[Ru(bipy)_3]^{2+}$ dye, additional experiments were carried out. First, the enzymes were denaturated by heating the solutions containing the enzymes (pH 7.5) at 60ºC for 60 minutes before the addition of **S1**-**S4**. In a second experiment, solids **S1**, **S2**, **S3** and **S4** were incubated in the presence of the digestive protease pepsin. In both experimental settings no dye release was observed, strongly suggesting the selective activity of pancreatin or β-D-galactosidase enzymatic hydrolysis as the mechanism responsible of the opening of the mesopores.

In order to corroborate that the gating mechanism arises from the grafting of polysaccharides in the pore outlets of the MCM-41 scaffold, the hybrid material **S0** that contains only the $[Ru(bipy)_3]^{2+}$ dye in

the pores but lacks the hydrolyzed starch derivatives **1, 2, 3** or the disaccharide derivative **4** was prepared. Aqueous suspensions at pH 7.5 of solid **S0** alone showed a very fast dye release, however dye delivery was strongly inhibited when **S0** was in the presence of β-D-galactosidase or pancreatin, most likely due to an unselective adsorption of the enzyme on the external mesoporous surface through interactions with the silanol groups. This indicated that the grafting of the polysaccharides on the surface of mesoporous materials does not only act as capping systems that can be selectively uncapped but at the same time the functionalized surface with the saccharides prevent the unspecific adsorption of the enzymes.

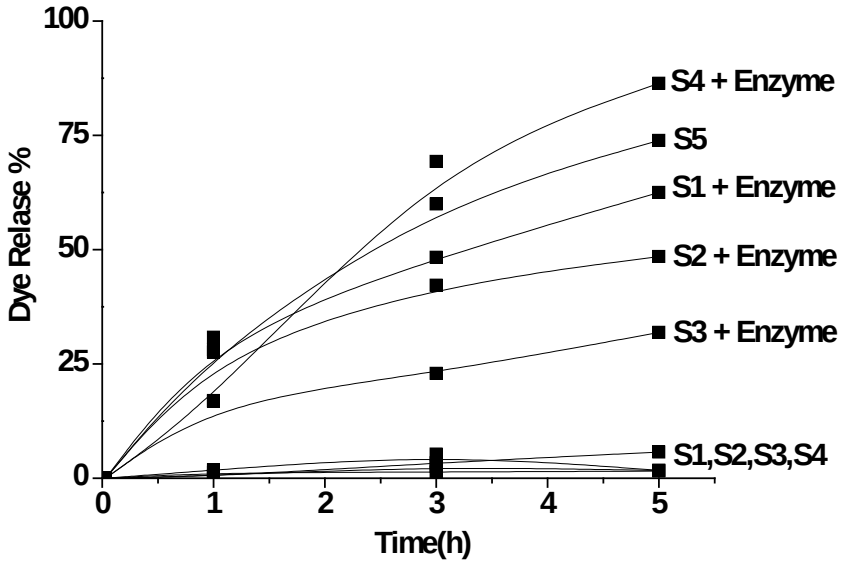


Figure 4. Kinetic release of $Ru(bipy)_3^{2+}$ dye from water suspensions of gated solids **S1**, **S2**, **S3**, **S4** in the absence and in the presence of enzyme at pH 7.5, and water suspensions at pH 7.5 of solid **S5**, for five hours.

To complete the study of the effect that the enzyme activity has on the saccharide, the solid **S1-1** (obtained from solid **S1** after the uncapping process using pancreatin and delivery of the entrapped dye) was prepared and ^{13}C-NMR studies were carried out. The ^{13}C MAS NMR spectrum of samples **S1** and **S1-1** are shown in Figure 5. The ^{13}C-NMR spectrum of **S1** displays signals in the 11-50 ppm range

assigned to the 3-aminopropyltriethoxysilane linker, resonances between 70-90 ppm corresponding to the HO-C- units of the 'saccharide', and signals between 115-260 ppm attributed to the $[Ru(bipy)_3]^{2+}$ dye. Additionally, the characteristic ^{13}C signal of the (-O-CH-O-) glycosidic bond is clearly found in the **S1** solid at 100 ppm (see Figure 5). The ^{13}C-NMR spectrum of solid **S1-1** still shows some signals at the above mentioned shift ranges indicating the presence of certain 'saccharide' still anchored to the solid and the presence of some undelivered dye that may remain stoutly adsorbed on the walls of the mesopores. However, the most remarkable feature in **S1-1** is the pronounced relative decrease of the ^{13}C signal intensity at 100 ppm, strongly indicating that the enzymatic hydrolysis of the glycosidic bonds was highly effective. In fact, a certain (low) proportion of non-hydrolysed sacharides is reasonably expected in the **S1-1** due to some steric hindrance for the complete enzymatic attack. This could be the origin of the complex NMR spectra in the 11-50 ppm chemical shift range associated with mixtures of slightly different aminopropyl arms.

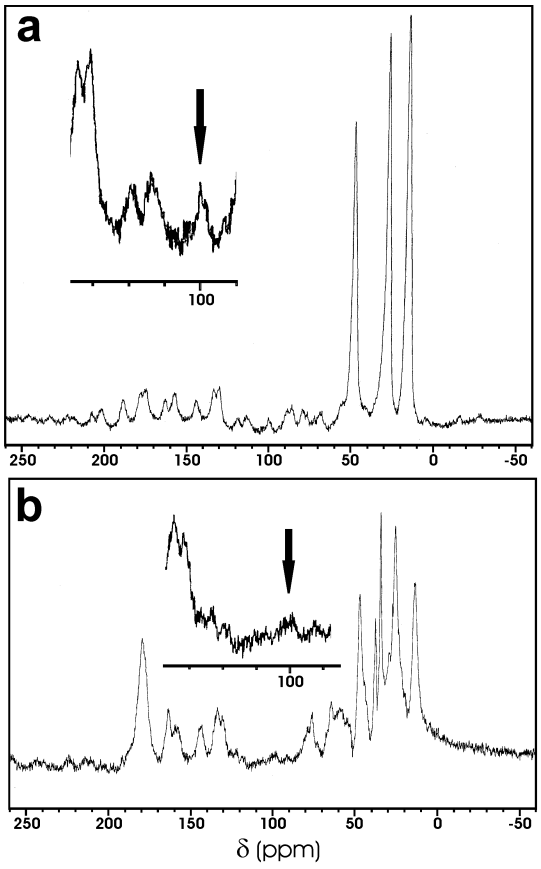


Figure 5. ^{13}C MAS NMR spectrum of (a) **S1** and (b) **S1-1.**

Delivery of gated materials in intracellular media. Cell internalization of mesoporous silica nanoparticles bearing gated stimuli-responsive scaffoldings is an appealing new interdisciplinary research field on the frontier of nanoscience.[79-89] As stated above one goal in this study was to demonstrate that easy-to-prepare 'saccharide' capped silica mesoporous nanoparticles could be used for in cell delivery applications. Thus, after the *in vitro* characterization of the different 'saccharide'-capped mesoporous scaffolds (*vide ante*), **S1** nanoparticles were selected for further *ex vivo* assays. Previous reports suggested that saccharides can be used as targeting molecules in modified nanoparticles in order to induce efficient uptake by target cells *via* endocytosis.[90,91] Therefore, saccharide-capped SMPS could be good candidates for the design of intracellular cargo-releasing specialized nanodevices.

Initially, an evaluation of the ability of cells to internalize **S1** and the suitability of the nanoparticles in terms of cellular toxicity was performed (Figure 6). Confocal microscopy analysis was used in order to evaluate whether or not **S1** was internalized in both the tumoral HeLa and non-tumoral LLC-PK1 cell lines (see Materials and Methods section for further information) tracking the $[Ru(bipy)_3]^{+2}$ fluorescence. In these experiments cell nuclei were stained with Hoechst 33342 and cellular membrane with the fluorescent marker WGA Alexa Fluor® 647 as cellular markers. A dotted pattern of a $[Ru(bipy)_3]^{+2}$ fluorescent signal associated to intracellular vesicles was observed (Figures 6a and 6b for HeLa and LLC-PK1 cells, respectively) suggesting **S1** internalization in both cell lines. A WST-1 cell viability assay was employed to determine any unspecific toxicity of **S1**. The output of the assay suggested that **S1** was well tolerated by the cells in the experimental conditions (Figure 6c).

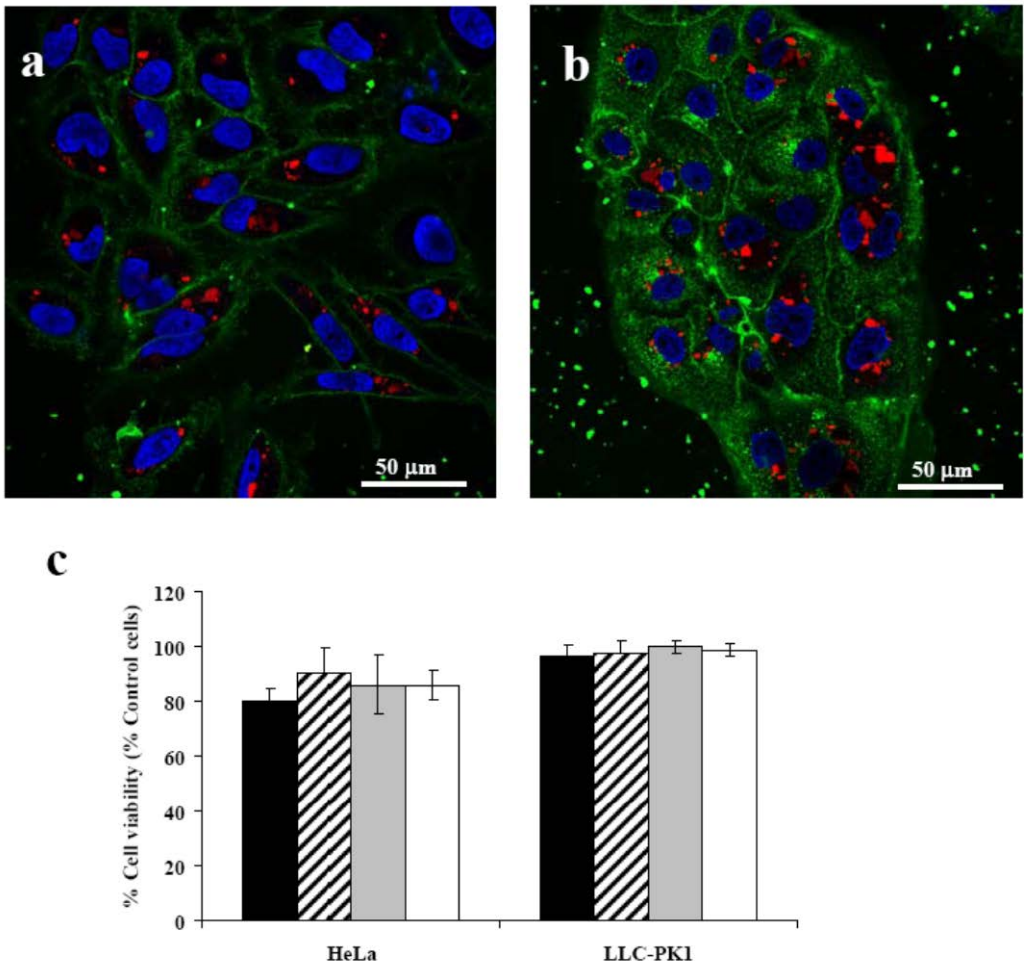


Figure 6. Cellular uptake of **S1**. a) HeLa and b) LLC-PK1 cells were incubated for 20 minutes in the presence of **S1** 50 µg/mL. Next, the medium was removed and fresh medium was added. After 15 hours, confocal microcopy studies of **S1** cellular uptake were performed by means of $[Ru(bipy)_3]^{+2}$-**S1** associated fluorescence (red) in the presence of DNA marker Hoechst 33342 (blue) and the plasma membrane marker WGA Alexa Fluor® 647 (green). For cell viability studies, (c) HeLa and LLC-PK1 cells were treated with **S1** in the same conditions at concentrations of 50, 25, 10 and 5 µg/mL (black, grey, stripped and white bars, respectively) for 24 h. Then, cell viability was quantified by means of a WST-1 assay. Three independent experiments were performed and data are reported as (mean ± s).

The presence of **S1**-containing intracellular vesicles suggested an endosomal (or endosomic)-mediated cellular internalization. Such a mechanism is generally used in cellular systems to transport different

substrates to the autolysosomes for bulk degradation in an energy-dependent process.[92-94] Therefore it is conceivable to postulate that cargo-containing **S1** is transported to the autolysosomes where the activity of lysosome enzymes (including amylases) induces a saccharide hydrolysis-dependent release of the entrapped guest. To characterize the way **S1** nanoparticles are internalized, HeLa and LLC-PK1 cells were incubated in the presence of **S1** at 37 ºC and 4 ºC and the $[Ru(bipy)_3]^{+2}$ associated fluorescence was measured (Figure 7a and 7b). A statistically significant reduction of **S1** cellular uptake in both cell lines was observed upon incubating cells at 4 ºC when compared to the uptake obtained at 37 ºC. This fact would indicate an energy dependent internalization mechanism. Additionally, and interestingly, an increased uptake at all temperatures analyzed for tumoral HeLa cells when compared to non-tumoral LLC-PK1 cells was observed. To further characterize the internalization process, HeLa cells were transfected with the autolysosome marker microtubule-associated protein 1 light chain 3 (LC3-I) fusionated to the green fluorescent protein (GFP, LC3-eGFP).[95] The LC3-I protein presents a cytoplasmic localization under normal conditions. In the presence of inductors of autolysosome formation, it is processed and recruited to the autolysosomes, where LC3 II is generated by site specific proteolysis and lipidation near to the C-terminus. This fact induces the appearance of a dotted pattern related to the membrane associated LC3-II new subcellular localization. Once transfected, cells were treated with **S1** and the fluorescence associated to the $[Ru(bipy)_3]^{+2}$ dye was determined. As a result, a certain number of cells showed a dotted $[Ru(bipy)_3]^{+2}$ pattern surrounded by LC3-eGFP-associated vesicles suggesting that the actual location of cell internalized **S1** is in the autolysosomes (Figure 7c).

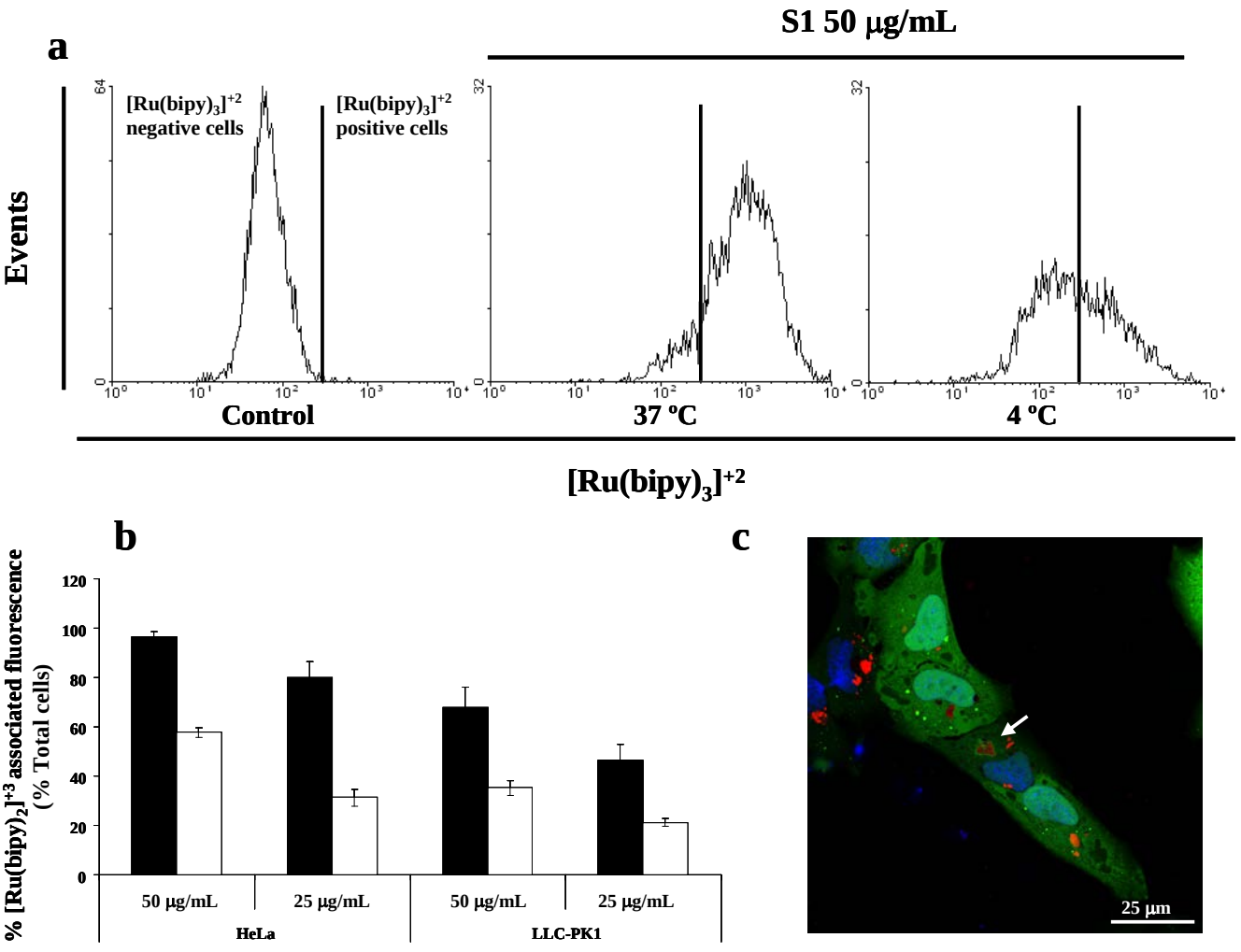


Figure 7. Implication of endosomes and lysosomes in the cellular degradation of **S1**. HeLa and LLC-PK1 cells were treated **S1** 50 and 25 μg/mL at 37 ºC and 4 ºC. After 20 minutes, nanoparticles were removed and cells were further incubated at 37 ºC for 24 h. Then, $[Ru(bipy)_3]^{+2}$ associated fluorescence was studied by means of flow cytometry. In figure b, columns indicate the percentage of cells which stained positive for $[Ru(bipy)_3]^{+2}$ in HeLa and LLC-PK1 cells at 37 ºC (black bars) and 4ºC (white bars), respectively. Three independent experiments were performed employing duplicates. Results are expressed as (mean ± s). Statistically significant data (Student's t test, $P < 0.05$) were obtained in all cases when comparing the two different incubation temperatures. To determine the autolysosomal localization of **S1** nanoparticles, HeLa cells were transfected with LC3-eGFP and 24 h later treated with **S1** 50 and 25 μg/mL for 20 minutes. After the treatment, the medium was changed and further incubated

for 24 h prior to determination through confocal microscopy of the localization of $[Ru(bipy)_3]^{+2}$ associated fluorescence (red) and LC3-eGFP (green). As a result, autolysosomal localization of **S1** was determined (white arrow).

In order to prove the lysosomal amylase-mediated degradation of the capping "saccharides" of **S1**, new capped mesoporous nanoparticles were synthesized and loaded with the chemotherapeutic agent doxorubicin (dox) as a guest molecule **(S1-DOX)**.[96] **S1-DOX** shows a similar N_2 adsorption-desorption isotherm, XRD profile and TEM images to that observed for **S1** (data not shown). The content of dox in solid **S1-DOX** was determined by elemental and thermogravimetric analysis and amounted to 0.278 g of dox/ g SiO_2. Kinetic release of dox from water suspensions of the gated **S1-DOX** solid in the presence and absence of enzyme at pH 7.5 were carried out, and a similar delivery behavior to that found for **S1** was observed; *i.e.* zero delivery in the absence of amylases and delivery in the presence of the enzymes. Based on these observations *in vitro*, further *ex vivo* assays were carried out. Dox is broadly employed in the treatment of cancer. However, the non-specific cell internalization of this drug causes significant secondary effects in treated patients (*i.e.* cardiotoxic and nephrotoxic effects). Confining dox in nanoparticles for more-specific cellular release has been reported to be an interesting strategy to decrease the unwanted secondary effects, thereby increasing the efficiency of this drug.[97] **S1-DOX** nanoparticles were added to the cells at different concentrations and cellular localization and cell toxicity was examined by confocal microscopy and flow cytometry after 24 h (Figure 8). As experimental controls, MCM-41 material and the supernatant of the **S1-DOX** suspension employed to treat the cells were also added in the same conditions as **S1-DOX.** MCM-41 was used in order to discard any toxic effect associated with the mesoporous material, while a **S1-DOX** suspension was employed to discard any unspecific aperture of the molecular gate outside the cells. A significant percentage of **S1-DOX** treated cells detached from the plate, due to the activation of dox-induced cell death, were observed by confocal microscopy studies. By contrast, no cell death was observed in MCM-41-treated cells (Figures 8 a-d) or in the case of the supernatant of the **S1-DOX** suspension (data not

shown). These results strongly suggest that dox delivery is only observed upon **S1-DOX** internalization and enzyme-mediated aperture of the “saccharide” gate. The quantification of the percentage of cell death was performed by flow cytometry (measured by DNA content staining with DAPI). Cellular population in normal conditions typically distributes in a two peak graphic depending on their DNA content; *i.e.* cells in G1- or G2-phase, whereas a cell undergoing cell death usually presents a sub-G1 DNA content due to cellular fragmentation and subsequent DNA loss. A typical cell cycle distribution was observed in cells treated with MCM41 (Figure 8 e). In the case of cells treated with **S1-DOX**, the typical cell cycle distribution was lost and a significant percentage of cells presented a sub-G1 DNA content corresponding to dead cells (Figure 8 e-f). These results indicate that nanoscopic SMPS functionalized with ‘saccharide’ derivatives are suitable supports for the delivery of entrapped molecules into cells for biological applications.

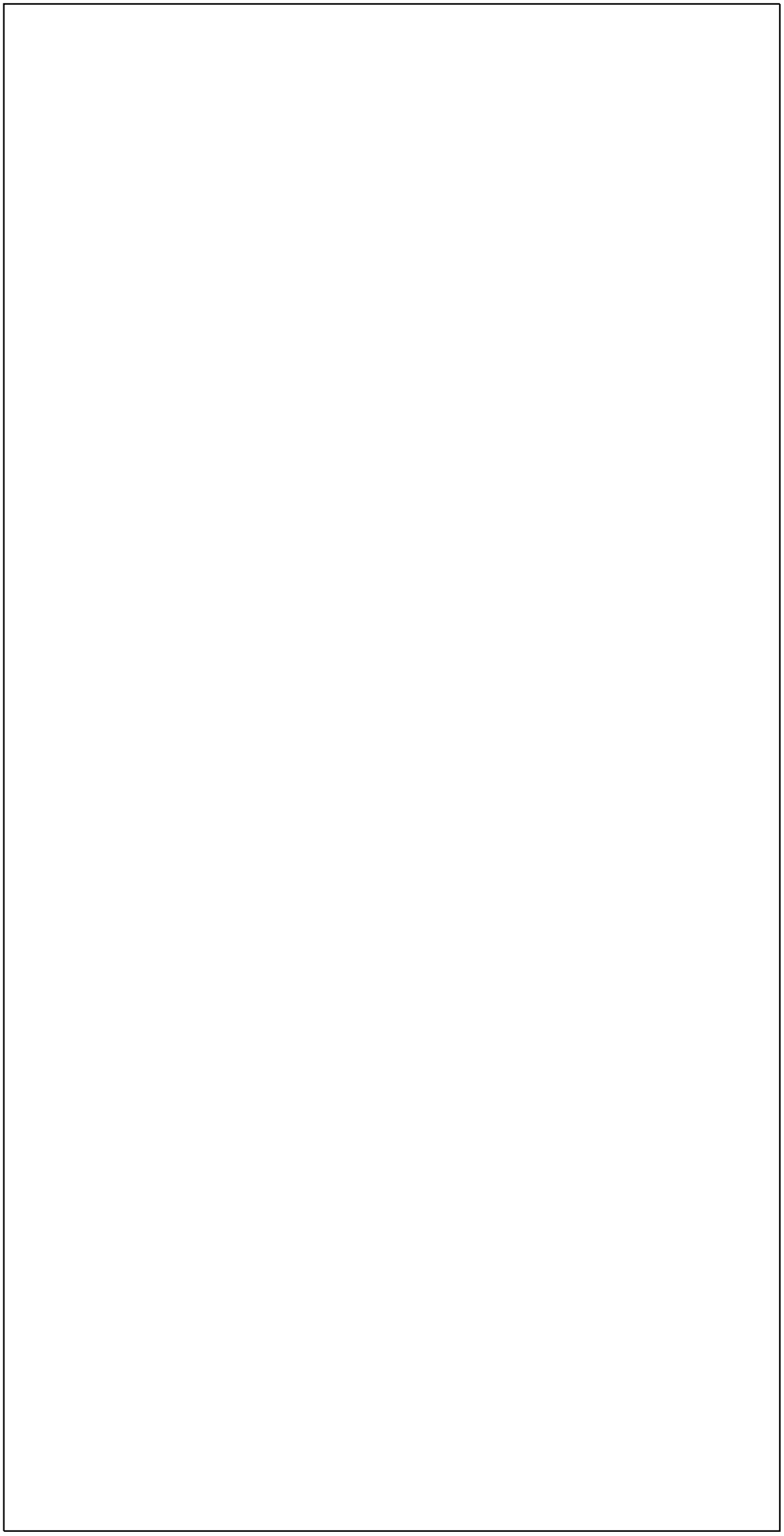


Figure 8. S1-DOX cell internalization induces cell death. HeLa cells were treated with control MCM-41 (a, b) or **S1-DOX** (c, d) at 50, 25 μg/mL and after 20 minutes, the medium was removed and cells were further incubated for 24 h. MCM-41-treated cells remained attached to the plate (a) and showed standard phenotype in confocal microscopy analysis (b) when nuclei were stained with Hoechst 33342 (blue) and plasma membrane with WGA Alexa Fluor® 647 (green). In contrast, **S1-DOX**-treated cells were detached from the plate (c) and an 'in cell' diffuse pattern of dox-associated fluorescence (red) together with cellular corpses were observed (d) suggesting dox-induced cell death. Quantification of cell death was performed by flow citometry. DNA content was studied by fixing the cells in ethanol and staining them with DAPI. MCM-41 treated cells presented a standard cell cycle distribution, while a significant percentage of **S1-DOX** treated cells presented a sub-G1 DNA content proving the existence of cell death processes (Figure 8e). A quantification of the percentage of cells in sub-G1 phase is shown in Figure 8f. Two independent experiments were performed employing duplicates in both cases. Results are expressed as (mean ± s). An asterisk indicates statistically significant data (Student's t test, $P < 0.05$).

CONCLUSIONS.

In conclusion, it has been demonstrated that the attachment of a hydrolyzed starch derivative as a gatekeeper on the surface of MSNs supports provides a suitable method for the design of mesoporous systems able to deliver the entrapped guest in the presence of suitable enzymes. Specifically, the mesoporous silica nanoparticles **S1**, **S2** and **S3** containing different grafted starch derivatives (*i.e.* Glucidex® 47, Gludicex® 39 and Glucidex® 29) showing a different degree of hydrolysis were prepared. Additionally, for comparative purposes, solid **S4** containing a lactose derivative as a capping molecule was also prepared. Whereas the capped **S1**-**S4** solids showed "zero release", the same solids in water in the presence of pancreatin (for **S1**-**S3**) and galactosidase (for **S4**) released the cargo in a controlled fashion due to the enzyme-induced hydrolysis of the glycosidic bonds in the anchored 'saccharides'. A

clear control of the delivery rate was additionally found depending on the used hydrolyzed starch derivative. The delivery results indicate that it is possible to design different delivery profiles *via* a simple selection of the degree of hydrolyzation of the starch (*i.e.* related to the lengths of different 'saccharide' components and their relative proportions). It was shown that the 'saccharide'-functionalized nanoparticles **S1** are efficiently taken up by both tumoral (HeLa) and non-tumoral (LLC-PK1) cells although a more efficient internalization in HeLa cells was observed. The cellular uptake of the nanoparticles occurs *via* endocytosis targeting them to the autolysosomes, where the capping polysaccharides are degraded by lysosomal enzymes and the cargo is delivered. Finally, the possible application of 'saccharide'-functionalized nanoparticles as suitable delivery systems in cells of chemotherapeutic agents such as doxorubicin was demonstrated, and a substantial reduction of cell viability was observed in cells treated with solid **S1-DOX**. These results suggest that it might be possible to use bio-scaffoldings (for instance 'saccharide' as demonstrated here) as capping systems for the preparation of biocompatible delivery nanodevices based on silica mesoporous supports. The possibility of using these "bio-gates" which could be selectively opened by bio-molecules (for instance enzymes) opens a wide range of possibilities in the design of advanced nanodevices for controlled delivery applications and a number of new advances in this area are anticipated.

EXPERIMENTAL.

Synthesis. General methods. XRD, TGA, elemental analysis, TEM, N_2 adsorption-desorption, NMR and UV-visible spectroscopy techniques were employed to characterize the synthesized materials. Powder X-ray measurements were performed on a Philips D8 Advance diffractometer using Cu K_α radiation. Thermo-gravimetric analyses were carried out on a TGA/SDTA 851e Mettler Toledo balance, using an oxidant atmosphere (air, 80 mL/min) with a heating program consisting of a heating ramp of 10 ^oC per minute from 393 to 1273 K and an isothermal heating step at this temperature for 30 minutes. TEM images were obtained with a 100 kV Philips CM10 microscope. N_2 adsorption-desorption

isotherms were recorded with a Micromeritics ASAP2010 automated sorption analyzer. The samples were degassed at 120 ºC in vacuum overnight. The specific surface areas were calculated from the adsorption data in the low pressure range using the BET model. Pore size was determined following the BJH method. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were acquired with a Varian 300 spectrometer (Sunnyvale, CA, USA). ^{13}C MAS NMR spectrum was recorded on a Varian Unity 300 spectrometer operating at 128.3 MHz using a magic angle spinning speed of 4.0 kHz. UV-visible spectroscopy was carried out with a Lambda 35 UV/vis spectrometer (Perkin-Elmer Instruments). Live cellular internalization studies were performed with a Cytomics FC 500 (Beckman Coulter Inc.) and a confocal Leica microscope handled with a TCS SP2 system, equipped with an acoustic optical beam splitter (AOBS). Cell viability measurements were carried out with a Wallac 1420 workstation.

Chemicals. The chemicals tetraethylorthosilicate (TEOS), n-cetyltrimethylammonium bromide (CTABr), sodium hydroxide (NaOH), 3-aminopropyltriethoxysilane, tris (2, 2'bipyridyl) ruthenium (II) chloride hexahydrate($[Ru(bipy)_3]Cl_2 \cdot 6H_2O$), Pancreatin from porcine pancreas, β-D-galactosidase from *Kluyveromyces lactis*, D-(+)-Lactose monohydrate and tissue culture grade dimethylsulfoxide (DMSO) were provided by Aldrich. N-(3-triethoxysilylpropyl) gluconamide was provided by ABCR. The hydrolyzed starch Glucidex® 47 (5% glucose, 50% maltose, 45% oligosaccharides and polysaccharides), Glucidex® 39 (3% glucose, 37% maltose, 60% oligosaccharides and polysaccharides) and Glucidex® 29 (10% glucose, 9% maltose, 81% oligosaccharides and polysaccharides) were provided by Roquette. D-MEM with L-glutamine, fetal calf serum (FCS), trypan blue solution (0.4 %) cell culture grade, trypsin, wheat germ agglutinin Alexa Fluor® 647 and Hoechst 33342 were provided by Gibco-Invitrogen. The cell proliferation reagent WST-1 was obtained from Roche Applied Science. Doxorubicin hydrochloride was provided by Sequoia Research Products Ltd. All the products were used as received.

Synthesis of the hydrolyzed starch derivative ***1, 2, 3*** (Scheme 2). A solution of 3-aminopropyltriethoxysilane (5.85 mL, 25 mmol) in ethanol was added to a suspension of hydrolyzed starch (Glucidex® 47, Glucidex® 39 and Glucidex® 29) in ethanol (total volume 250 mL). The reaction

mixture was stirred for 24 h at room temperature and then heated at 60ºC for 30 min. The solvent was evaporated under reduced pressure to give a white solid (**1**, 7.32 g, yield 84 %; **2**, 7.15 g, yield 82 %; **3**, 7.55 g, yield 87 %. ^{1}H NMR (300 MHz, D_2O): δ 0.42 (t, 2 H, -CH_2-Si-), 1.02 (t, 9 H, CH_3-CH_2-O-Si-), 1.53 (m, 2 H, -CH_2-CH_2-Si-), 2.74 (t, 2 H, -NH-CH_2-CH_2-CH_2-Si-), 3.20-3.77 (m, n H, starch hydrolyzed, CH_3-CH_2-O-Si-), 5.13 (d, 1 H, -O-CH-O-) ppm. ^{13}C{1H } NMR (75MHz, D_2O): δ 9.62 (-CH_2-Si-), 16.65 (CH_3-CH_2-O-Si-), 21.74 (-CH_2-CH_2-Si-), 41.96 (-NH-CH_2-CH_2-CH_2-Si-), 57.25 (CH_3-CH_2-O-Si-), 61.84 (HO-CH_2-CH-), 72.67-78.11 (HO-CH-), 89.57 (-O-CH-CH), 94.84 (-O-CH-NH-), 100.25 (-O-CH-O-) ppm.

*Synthesis of the lactose derivative **4*** (Scheme 2). A solution of 3-aminopropyltriethoxysilane (5.85 mL, 25 mmol) in ethanol was added to a suspension of lactose monohydrate (5.4 g, 15 mmol), in ethanol (total volume 250 mL). The reaction mixture was stirred for 24 h at room temperature and then heated at 60ºC for 30 min. The solvent was evaporated under reduced pressure to give a white solid (**4**, 7.22 g, 13.24 mmol, yield 89 %). ^{1}H NMR (300 MHz, D_2O): δ 0.46 (t, 2 H, -CH_2-Si-), 1.03 (t, 9 H, CH_3-CH_2-O-Si-), 1.56 (m, 2 H, -CH_2-CH_2-Si-), 2.77 (t, 2 H, -NH-CH_2-CH_2-CH_2-Si-), 3.34-3.93 (m, 19 H, Lactose, CH_3-CH_2-O-Si-), 4.29 (d, 1 H, -O-CH-O-) ppm. ^{13}C{1H } NMR (75MHz, D_2O): δ 10.89 (-CH_2-Si-), 17.52 (CH_3-CH_2-O-Si-), 23.32 (-CH_2-CH_2-Si-), 43.18 (-NH-CH_2-CH_2-CH_2-Si-), 58.17 (CH_3-CH_2-O-Si-), 61.77 (HO-CH_2-CH-), 69.31 (HO-CH-),71.72 (HO-CH-), 73.27 (HO-CH-), 76.11 (-O-CH-CH), 79.19 (-O-CH-NH-), 103.68 (-O-CH-O-) ppm. Mass spectra (M = $C_{21}H_{43}NO_{13}Si$): 545 (M+1), 461(M−(CH_3-CH_2-)$_3$), 369 (M−(CH_3-CH_2-O-)$_3$-Si-CH_2-), 326 (M−(CH_3-CH_2-O-) 3-Si-CH_2-CH_2-CH_2-NH-), 221(M−Lactose), 135 (M−Lactose-NH-CH_2-CH_2-CH_2-Si-).

Synthesis of mesoporous MCM-41 nanoparticles. The MCM-41 mesoporous nanoparticles were synthesized using the following procedure: n-cetyltrimethylammoniumbromide (CTABr, 2.00 g, 5.48 mmol) was first dissolved in 960 mL of deionized water. NaOH (aq) (2.00 M, 7.00 mL) was added to the CTABr solution, followed by adjusting the solution temperature to 95ºC. TEOS (10.00 mL, $5.14.10^{-2}$ mol) was then added dropwise to the surfactant solution. The mixture was allowed to stir for 3 h to

give a white precipitate. The solid product was centrifuged, and washed with deionized water and ethanol. Finally the solid was dried at 60ºC (MCM-41 as-synthesized). To prepare the final porous material (MCM-41), the as-synthesized solid was calcined at 550 ºC using an oxidant atmosphere for 5 h in order to remove the template phase.

Synthesis of **S1, S2, S3**. (Scheme 1). In a typical synthesis, 1.00 g of templated-free MCM-41 and the dye tris(2,2'-bipyridyl) ruthenium (II) chloride (0.6 g, 0.8 mmol) were suspended in 40 mL of water in a round-bottomed flask under inert atmosphere. The mixture was stirred for 24 h at room temperature with the aim of achieving the maximum loading in the pores of the MCM-41 scaffolding. Then, an excess of the corresponding alkoxysilane derivative **1**, **2** and **3** (1g) in 20 mL of water, was added and the final mixture was stirred for 5.5 h at room temperature. Finally, the solid (**S1**, **S2**, **S3**) was filtered off, washed with 40 mL of water, and dried at 40ºC for 12 hours.

Synthesis of **S1-DOX.** In a typical synthesis, 1.00 g of templated-free MCM-41 and doxorubicin hydrochloride (0.5 g, 0.91 mmol) were suspended in 40 mL of water in a round-bottomed flask under inert atmosphere. The mixture was stirred for 24 h at room temperature with the aim of achieving the maximum loading in the pores of the MCM-41 scaffolding. Then, an excess of the alkoxysilane derivative **1** (1g) in 20 mL of water was added, and the final mixture was stirred for 5.5 h at room temperature. Finally, the solid (**S1-DOX**) was filtered off, washed with 40 mL of water, and dried at 40ºC for 12 hours.

Synthesis of **S4**. (Scheme 1). In a typical synthesis, 1.00 g of templated-free MCM-41 and the dye tris(2,2'-bipyridyl) ruthenium (II) chloride (0.6 g, 0.8 mmol) were suspended in 40 mL of water in a round-bottomed flask under inert atmosphere. The mixture was stirred for 24 h at room temperature with the aim of achieving the maximum loading in the pores of the MCM-41 scaffolding. Then, an excess of the alkoxysilane derivative **4** (1g, 1.8 mmol) in 20 mL of water was added, and the final mixture was stirred for 5.5 h at room temperature. Finally, the solid (**S4**) was filtered off, washed with 40 mL of water, and dried at 40ºC for 12 hours.

Synthesis of **S5**. (Scheme 1). 1.0 g of templated-free MCM-41 and the dye tris(2,2'-bipyridyl)ruthenium(II) chloride (0.6 g, 0.8 mmol) were suspended in 40 ml of anhydrous acetonitrile and heated at 120º C in a Dean-Stark in order to remove the adsorbed water by azeotropic distillation under inert atmosphere. The suspension was stirred for 24 hours at room temperature with the aim of loading the pores of the MCM-41 scaffolding. After this, an excess of the commercially available N-(3-triethoxysilylpropyl)gluconamide (50% in ethanol, 1.05 mL, 1.31 mmol) was added and the suspension was stirred for 5.5 hours. The final orange solid (**S5**) was filtered, washed with acetonitrile and dried at 40 ºC for 12 hours.

Synthesis of **S6**. In a typical synthesis, 1.00 g of templated-free MCM-41 was suspended in 40 mL of water in a round-bottomed flask under inert atmosphere, an excess of the alkoxysilane derivative **1** (1g) was added, and the mixture was stirred for 5.5 h at room temperature. Finally, the solid **S6** was filtered off, washed with 40 mL of water, and dried at 40ºC for 12 hours.

Synthesis of **S0**. For the sake of comparison, and as a control solid, hybrid material containing only the $[Ru(bipy)_3]^{2+}$ dye (**S0**) was synthesized in order to assess the effect of the saccharide grafted in the outer of the MCM-41 pores. The procedure was the same as described for **S1**, **S2**, **S3** and **S4** but without grafting the saccharide derivative. The final orange solid (**S0**) was filtered, washed with water and dried at 40 ºC for 12 hours.

Dye release studies. In a typical experiment, 10 mg of **S1**, **S2**, **S3** and **S4** were suspended in 18.75 mL of water at pH 7.5 and then 6.25 mL of enzyme solution (0.4 g of enzyme in 100 mL of water at pH 7.5) was added. Pancreatin was used for solids **S1**, **S2**, **S3** and β-D-galactosidase for solid **S4**. The suspensions were used for the evaluation of the gate-like effect by studying dye release from the pore voids of the functionalized material *via* the rupture of a glycosidic bond. For release studies with **S0** and **S5** 10 mg of the solid were placed in 25 mL of water at pH 7.5 and at a certain time an aliquot was separated and filtered. The delivery of the dye from the pore voids to the aqueous solution was monitorized *via* the absorbance of the dye at 453 nm.

Cell culture conditions. The HeLa human cervix adenocarcinoma and the LLC-PK1 pig kidney cells were purchased from the German Resource Centre for Biological Materials (DSMZ) and were grown in D-MEM and Medium 199 supplemented with 10 % and 6% FCS, respectively. Cells were maintained at 37 ºC in an atmosphere of 5 % carbon dioxide and 95 % air and underwent passage twice a week. Cells were transfected with eGFP-C1 plasmid containing the rat LC3 gene (LC3-eGFP) (kindly provided by G. Kroemer) by means of lipofectamine transfection reagent (Invitrogen), according to the manufacturer's recommendations.

WST-1 cell viability assay. Cells were cultured in sterile 96-well microtiter plates at a seeding density of $2.5 \cdot 10^3$ and $8 \cdot 10^3$ cells/well for HeLa and LLC-PK1 , respectively, and they were allowed to settle for 24 h. **S1** in DMSO was added to cells at a final concentration of 50, 25, 10 and 5 μg/mL. After 23 h, WST-1[98] (10 μL of a 5 mg/mL solution) was added to each well. Cells were further incubated for 1 h (a total of 24 h of incubation was therefore studied) and absorbance was measured at 595 nm.

*Live confocal microscopy **S1** cellular internalization.* HeLa and LLC-PK1 cells were seeded in 24 mm Φ glass cover-slips in six-well plates at a seeding density of 10^5 cells / well. 24 h later, cells were treated when indicated with **S1** or **S1-DOX** at a final concentration of 50 μg/ml. 20 minutes later, the medium was removed to eliminate compounds. After 15 h of incubation, cells were stained with 10 ng/mL of Hoechst 33342 and 5 mg/mL wheat germ agglutinin (WGA) Alexa Fluor® 647 for 30 min in PBS containing 10 % FCS or keeping the medium in case of **S1-DOX** treatments. Slides were visualized under a confocal microscope.

*Cytofluorometry studies employing **S1***. To develop the cytofluorometry studies, HeLa and LLC-PK1 cells were seeded at 50×10^3 cells/well in a twelve-well plate. After 24 h, cells were treated with **S1-DOX** 50 μg/mL for 20 minutes before removing the culture medium. Cells were incubated for 15 h before fixing them with ethanol 80%, keeping them at – 20 ºC for 24 h. Finally, cells were stained with DAPI CONCENTRATION and cell cycle studies were performed to determine the cell viability of the samples.

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