Smart Nanoparticles for Controlled Release Applications

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Abstract

Nowadays, nanotechnology is getting a lot of attention due to its potential in several applications. One of these applications is controlled drug delivery systems. To have a good drug delivery system, it is necessary that it only delivers once it reaches the desired location and at a controlled rate. Among several inorganic-based materials, silica nanoparticles are being studied due to their unique characteristics such as tunable pore size, high pore volume and surface area, biocompatibility and versatility achieved by surface modification. The drug release systems are often triggered by diverse stimuli such as pH, temperature, enzymes or light but there is still a need for systems to keep the cargo longer inside the platform.

The aim of this work was to develop a novel release system based on the hydrophobic-hydrophilic and cationic-anionic interactions between the functionalization compound and the cargo molecule. Two types of nanoparticles were produced by two different methods: MCM-41 nanoparticles by sol-gel and OMSM nanoparticles by emulsion-based method to obtain different particle and pore sizes. SEM analysis showed monodisperse nanoparticles, with $51 \pm 9nm$ nm for OMSN and $61 \pm 9nm$ for MCM-41. And nitrogen adsorption showed higher pore volume and surface area for MCM-41 nanoparticles. The template was extracted with solvent. Both nanoparticles were functionalized either with trimethoxy(propyl)silane (PTES) or N-trimethoxysilylpropyl- N, N, N - trimethylammonium chloride (CAT) successfully.

The proof of concept for these functionalized MSNs was made through the release study of two fluorescent molecules, sulforhodamine B (SRB), fluorescent dye and doxorubicin (DOX), chemotherapeutic drug. Two pair of interactions were here studied, SRB with CAT (cationic) and DOX with PTES (hydrophobic). The first pair proved to be efficient in controlling the release. The percentage of cargo released after 10 h, was 50 times higher in non-functionalized nanoparticles than in functionalized ones. The nanoparticle type did not show influence either in the cargo incorporation or release of SRB. The incorporation of DOX was done successfully, with better results in nanoparticles functionalized with PTES, followed by nanoparticles without functionalization. The tests with DOX revealed a much slower kinetics of cargo release. Even though there was incorporation in NPs functionalized with PTES, there wasn't release after 9 days due to strong interactions between DOX and the hydrophobic functional groups.

Keywords: mesoporous, silica, nanoparticles, controlled release, pore functionalization

1. Introduction

Nanotechnology is an emerging science that has created a lot of interest in the last decades, mainly due to the wide range of applications, from electronics and communications to chemistry, energy and biology [1].

Due to their diverse applications several nanostructures, element combination and synthesis methods are being developed and optimized, such as polymer/silica nanocomposite for catalysis [2], gold nanorods for cancer imaging and photothermal therapy [3,4] or quantum dots for sensing applications [5].

Nanoparticles (NPs) can be divided according with their composition: they can be organic (polymeric or lipid nanocarriers), inorganic (silica NPs, gold NPs and quantum dots) or hybrid (combining two or more components) [6].

Among the materials mentioned, mesoporous silica nanoparticles (MSNs) stand out due to their unique properties such as their ordered and tunable pore size (2-30 nm), high pore volume ($>1\ cm^3$/g) and high surface area ($>700\ nm^2$). In addition, two surfaces can be modified to contain different functional groups and the particles are biocompatibility [7-9].

Porous materials are classified by IUPAC, according to their pore size. Materials with pores smaller than 2 nm are defined as microporous, between 2 and 50 nm they are mesoporous and above 50 nm

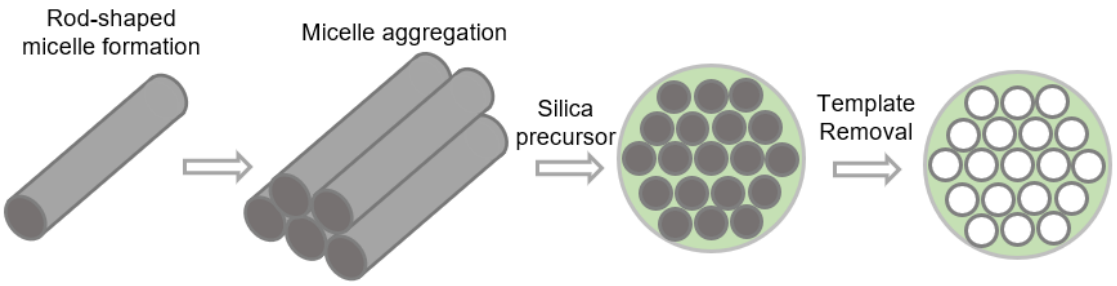

Figure 1: Example of mesoporous silica nanoparticles synthesis.

are macroporous [10]. The first ordered mesoporous silica family, named M41S, was developed in 1992 at the Mobil Research and Development Corporation. It includes three structures; hexagonal, cubic and lamellar. The hexagonal phase, MCM-41, is the most well-known and studied [11,12].

To synthetize MSNs with a high surface area, tunable pore size and large pore volumes it is required the use of an organic template that confers the pores structure to the material after silica precursor condensation and template removal (Figure 1).

The sol-gel process has been widely used to produce silica nanoparticles. A sol is a dispersion of colloidal particles in a liquid, with sizes between 1 and 100 nm, and a gel consists in an interconnected rigid network with sub-micron pores and polymeric chains [13]. In the case of sol-gel synthesis, a colloidal suspension (sol) forms a network through gelation, thus forming a continuous liquid phase (gel) [14].

Stöber *et al.* first reported in 1967, a technique to produce uniform and monodisperse silica particles, ranging from 50 nm to 2 μm. This method is initiated with metal alkoxide precursors, such as tetramethylorthosilicate (TMOS) or tetraethoxysilane (TEOS), followed by hydrolysation and polymerisation through a condensation mechanism in basic media [14,15].

The process starts with the silica source hydrolysis (such as TEOS) in the presence of water, ammonia and ethanol. The condensation reaction occurs between two hydroxyl groups (-OH) to form a network of Si-O-Si. Once a significant number of Si-O-Si bonds are formed, these oligomers condensate to form colloidal particles or a sol. Over time, these particles bind to form a three-dimensional network [14,15].

Another approach to produce mesoporous silica nanoparticles is by emulsion templating [16-19]. Emulsions are a two-phase system containing water droplets dispersed in an oil phase (water-in-oil - W/O) or vice-versa (oil-in-water - O/W). They are thermodynamically unstable systems but can be kinetically stabilized using surfactants [20].

A W/O emulsion can be emulsified into another aqueous phase, this is known as double emulsion. When the continuous phase and the innermost droplets are water the system is referred as W/O/W [17,18,20]. In these systems there are two different interfaces, one between the small water droplet and the oil around, and another between the oil phase and the adjacent water. The interfaces are commonly stabilized by two different amphiphiles, one more hydrophobic in the first interface and another more hydrophilic for the second. This process also requires mechanical energy, normally in the form of vigorous stirring to produce monodisperse nanoparticles [18].

Gustafsson *et al.* [19] suggested a method to produce MSNs that consists in an water-in-oil-water (W/O/W) emulsion, an O/W emulsion is usually a water-in-oil microemulsion dispersed in an oil-in-water emulsion. In this procedure, it is used an oil phase that contains TEOS and octane, an emulsifier and ethanolamine as catalyst to TEOS hydrolysis. TEOS is solubilized in the oil droplets, with the hydrolysis starting at the interface between the water and oil [19,20]. Surfactants solubilize water into oil forming a W/O microemulsion (reverse micelle). So, the oil droplet contains small water droplets besides oil. The water droplets inside the oil droplet start to coalesce and form a silica network [18,19].

The final step in the synthesis process is the template removal. It can be removed either by calcination or solvent extraction [8,21].

MSNs have two functional surfaces, the outer surface of the particles and the pore channels. This feature allows both surfaces to be selectively functionalized. There are two different approaches, either by co-condensation or by postsynthetic methods [11,22-24].

The most common process of nanoparticles functionalization is by postsynthesis modification (also known as grafting). This method can be performed either before or after the template removal [8,10].

The silica condensation around the micelles is incomplete, meaning there are silanol groups available at the surfaces, with a density of 2-3 -Si-OH

per nm^2 [21]. Therefore, the functionalization can be done by reacting an organosilane, usually $(R'O)_3SiR$, with those free silanol groups. By selecting the R group it is possible incorporate different organic groups (for example NH_2, methacrylate or ionic) [10].

This method has the disadvantage that it can be difficult to achieve a homogeneous distribution of the organic group due to the fact the functionalization occurs in the most accessible sites. This drawback can be overcome using a mesoporous silica with larger pore width [22-24].

The co-condensation method consists in adding an organosilane precursor, $(R'O)_3SiR$, with a terminal functional group along with silica precursor and templating agent, into the reaction. They co-condense and the organic groups will end up covalently anchored on the pore wall [10].

Unlike the previous method, pore blocking is not a problem, since the organic functionalities are introduced during the synthesis stage. Also, it is possible to obtain a more homogeneous distribution of the functional groups as they are components of the silica network [21]. However, there is one major problem related with the final density of functional groups. By increasing the silica precursor concentration in the mixture, the degree of mesoscopic order decrease, since it has a disrupting effect on the structural integrity of the template agent, leading to disordered materials [10-21].

MSNs have large pore volumes which allows the loading of a large cargo amount. However, to avoid the premature release of that cargo is necessary to control the release and the delivery site. By blocking the pore entrance with a stimuli-responsive system the cargo will be entrapped, and the premature release will be avoided. It can be achieved either by coating the outer nanoparticle surface or by blocking the pores entrance or increasing the interaction of the cargo with the internal surface of the material. These systems can be triggered by pH, temperature, redox potential and enzymatic or by applying light or magnetic field [15,25-29].

The aim of this work was to develop a novel controlled release system based on the interactions between functional groups present in the silica internal surface and the cargo molecule.

Two types of nanoparticles were produced by two different methods: MCM-41 nanoparticles by sol-gel and OMSM nanoparticles by emulsion-based method. The template removal method chosen was solvent extraction since this method preserves the silanol at the MSNs surface and reduces pore shrinkage. The NPs were functionalized by post-synthetic functionalisation with two different compounds: trimethoxy(propyl)silane, further mentioned as PTES and N-trimethoxysilylpropyl- N, N, N - trimethylammonium chloride, further mentioned as CAT. The main interest in choosing these compounds was to test two different interactions between the functionalization and the chosen cargo. PTES, known to be as a hydrophobic substance, was chosen to test a hydrophobic-hydrophilic interaction with doxorubicin hydrochloride (DOX). CAT, a cationic substance, was chosen to test cationic-anionic interaction with sulforhodamine B (SRB).

2. Experimental Part

2.1. Materials

Tetraethoxysilane (TEOS, 99%, Aldrich), cetyltrimethylammonium bromide (CTAB, 99%, Sigma), n-octane (+99%, ACROS Organics) ethanolamine (99%, Sigma), sodium hydroxide (NaOH, pure, EKA), ethanol absolute (EtOH, >99%, Fisher Chemical) and hydrochloric acid (HCl fuming, 37% ACS reagent, Sigma-Aldrich). The deionized water was produced from a Millipore system Milli-Q 18 MΩcm (with a Millipak membrane filter 0.22 μm).

Regarding nanoparticles functionalization, they were surface modified with trimethoxy(propyl)silane (97%, Sigma Aldrich) and N-trimethoxysilylpropyl- N, N, N - trimethylammonium chloride (50% ethanol, Gelest) in dried toluene (toluene distilled over calcium hydride before use).

For NMR analysis samples was used 1,3,5-trioxane (≥99.0%, Fluka), deuterium oxide (D_2O, 99.9% atom, CIL) and dimethyl sulfoxide (DMSO, 99,9%, CIL). For the loading process, was prepared phosphate buffer solution (PBS, pH 7.5) with disodium hydrogen phosphate (Na_2HPO_4, 99%, Riedel-de-Han) and sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$, 98%, Panreac). It was also used dimethyl sulfoxide (DMSO, >99.9%, Sigma Aldrich).

The cargo molecules used in the release studies were doxorubicin hydrochloride (DOX, >95%, TCI) and sulforhodamine B (SRB, Molecular Probes). It was also used a polypropylene dialysis device with a cellulose membrane (Slide-A-Lyzer Mini Dialysis Devices, 10K MWCO, 0.5mL).

2.2. Equipment

The nanoparticles were characterized by SEM and nitrogen adsorption. The SEM apparatus was JEOL scanning electron microscope (model JSM7001F, JEOL, Tokyo, Japan), with an accelerating voltage of 15 kV. The samples were coated with gold/palladium using a turbo-pumped sputter coater from Quorum Technology (model Q150T ES, Quorum Technology, Ashford, UK) for 1 minute. The N_2 adsorptiondesorption isotherms were ob-

tained at 77 K of the degassed samples, using a Micromeritics ASAP 2010. ^{1}H-NMR quantification was obtained on a Bruker Avance III 400 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) operating at 400 MHz. The cargo release was studied by fluorescence spectroscopy, Horiba-JobinYvon Fluorolog-3 spectrofluorimeter, in Right Angle mode and measures were carried out in PMMA cuvettes from VWR (with dimensions of 1 cm × 1 cm) at 25 °C. For the absorbance spectra was used a V-660 UV-Vis spectrophotometer from Jasco International (Tokyo, Japan) with a double monochromator and photomultiplier tube detector for high resolution. The measurements were carried out in quartz cells (with dimensions of 1 cm × 1 cm) at 25 °C. The NPs centrifugations were carried out on a Sigma 2K15, with a 12141 rotor, at 12000 rpm. And during release studies, the centrifugations were carried out on a Hitachi Himac CT 15RE at 15000 rpm.

2.3. Methods

2.3.1. MSNs Synthesis

The OMSN synthesis was carried out as described by Gustafsson *et al.* [19]. In a polypropylene flask was stirred 0.200 g of CTAB with 62 g of Mili-Q water, 19.9 g (28.4 ml) of n-octane and 18.6 μL of ethanolamine at 70 °C for 1 h. Then, 2.0 g of TEOS was added dropwise and was kept stirring also at 70 °C for 20 h. After cooling, the dispersion was decanted and then centrifuged (12000 rpm, 20 minutes) and washed 3 times with absolute ethanol. In the last cycle, the supernatant was removed, and the nanoparticles were left drying in a ventilated oven at 50 °C.

MCM-41 were synthesised as described by Rodrigues, A. S. [30]. In a polypropylene flask was added 0.5 g of CTAB with 240 g of Mili-Q water and then it was stirred at 80 °C. Afterwards, 1.75 mL of NaOH (1.4 M) was added and finally 2.5 mL of TEOS, dropwise. It was left stirring for 2 h at 80 °C. After cooling, the mixture was filtered and washed with 300 mL of absolute ethanol. Then it was left drying in a ventilated oven at 50 °C.

The template was removed by solvent extraction. The MSNs, already washed and dried, were placed in a polypropylene flask with an acidified ethanolic solution (0.5 M HCl, 20 mL for each 500 mg of NPs), and stirred for 2 h at 40 °C. Then, they were centrifuged (12000 rpm, 20 minutes) and washed 3 times with absolute ethanol and left drying overnight at 50 °C in a ventilated oven.

2.3.2. NPs Functionalization

For surface functionalization was used two different alkoxysilane: Trimethoxy(propyl)silane and N-trimethoxysilylpropyl-N, N, N-trimethylammonium chloride further mentioned as PTES and CAT respectively. For the functionalization, 0.2 g of MCM-41 or OMSN nanoparticles were dispersed in 10 mL of dry toluene. Then, was added to the mixture the quantity indicated in Table 1 of PTES or CAT and it was maintained at 125 °C under reflux with argon atmosphere for 24 h. The nanoparticles were recovered by centrifugation and washed three times with ethanol (12000 rpm, 20 minutes), after the last centrifugation the supernatant was removed, and the particles were left drying at 50 °C.

Table 1: Volumes of functionalization compounds used in different NPs

Compound	NP type	Volume (mL)
PTES	OMSN	0.9
	MCM-41	0.7
CAT	OMSN	1.3
	MCM-41	1.1

2.3.3. SRB Loading and Release

MCM-CAT-SRB and OMSN-CAT-SRB: For the nanoparticles functionalized with CAT the cargo molecule was sulforhodamine B (SRB). First, a phosphate buffer solution (pH 7.5) was prepared. In a 250 mL volumetric flask was added 0.324 g of sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$) and 1.441 g of disodium hydrogen phosphate (Na_2HPO_4). Then, milli-Q water was added up to 250 mL and the solution was shaken until dissolution. Afterwards it was transferred to a polypropylene flask and was kept in the fridge. With the buffer solution previously prepared, two SRB solutions in PBS were prepared (1×10^{-4} M and 1×10^{-2} M). For the loading, 3 mg of MCM-CAT or OMSN-CAT nanoparticles were dispersed in 3 mL of SRB in PBS solution and it was kept at 25 °C overnight. Subsequently, 1 mL of that dispersion was centrifuged (15000 rpm, 5 min at 25 °C) to remove the SRB that was not loaded, and the supernatant was removed and saved. Then, the loaded NPs were redispersed in 1 mL of phosphate buffer (pH 7.5) and centrifuged. The second supernatant was also removed and saved. Afterwards all supernatants were weighted. Finally, 200 μm of PBS was added to the nanoparticles and immediately before starting the release experiment, the mixture was transferred to the dialysis device and inserted on top of the fluorescent measurement cuvette (that was already filled with 3.5 mL of PBS). The release studies were carried out by measuring the fluorescence intensity of the SRB molecules in the bottom cuvette for 10 h at constant temperature at 25 °C, under stirring.

MCM-SRB and OMSN-SRB: To study the difference between functionalized and non-

functionalized nanoparticles, the same procedure was performed for non-functionalized nanoparticles.

Free SRB: For comparison, the release experiment was performed for free SRB (without nanoparticles), using the average number of moles that are released from the nanoparticles functionalized and non-functionalized, calculated from the supernatants absorbance.

2.3.4. DOX Loading and Release

MCM-PTES-DOX and OMSN-PTES-DOX: For the nanoparticles functionalized with trimethoxy(propyl) the cargo molecule was doxorubicin hydrochloride (DOX). A solution of DOX in DMSO (1×10^{-4} M) was prepared. Then 2 mg of MCM-PTES or OMSN-PTES nanoparticles were dispersed in 2 mL of the prepared solution and kept at 25 °C overnight. It was carried out as mentioned in section 2.3.3, with the exception that the first addition was DMSO instead of PBS. In this case, the kinetic study was carried out over 9 days. The fluorescence intensity of the sample was measured every day and after the measurements the cuvette with the dialysis membrane was left stirring at constant temperature at 25 °C until the next day. To track changes in the release measurements along the experiment (timeframe of days), a control sample of the initial solution of DOX in DMSO (1×10^{-4} M) was used. Complementary, the release of DOX in nanoparticles functionalized with N-trimethoxysilylpropyl-N, N, N-trimethylammonium chloride was studied following the same procedure.

MCM-DOX and OMSN-DOX: To study the difference between functionalized and non-functionalized nanoparticles, the same procedure was made for non-functionalized nanoparticles.

3. Results and Discussion

A novel control release system, consisting in a silica mesoporous silica NP functionalized with CAT or PTES was synthesized. These compounds interact with the cargo molecules controlling the delivery rate.

3.1. MSNs Caracterization

The nanoparticles diameters, before template removal, were obtained by SEM. The estimated average diameters were 61 ± 9 nm for the MCM-41 type nanoparticles (Figure 2 - A) and 51 ± 9 nm for OMSN type (Figure 2 - B).

Then, the template was removed with solvent and nitrogen adsorption-desorption was performed for both nanoparticles. From nitrogen analysis, it was obtained the nanoparticles surface area and pore diameter and volume, as presented in Table 2.

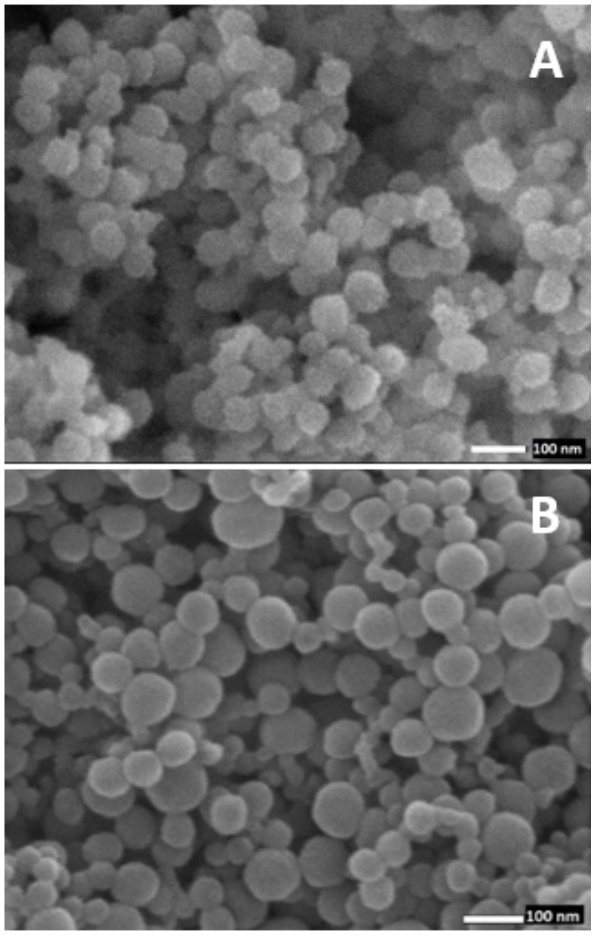

Figure 2: SEM images of A) MCM-41 and B) OMSN

Table 2: Results from nitrogen adsorption

	MCM-41	**OMSN**
Surface area (m^2/g)	960	470
Pore diameter (nm)	2.8	7.7
Pore volume (mL/g)	0.8	0.52

From the nanoparticles caracterization is possible to conclude that the NPs were syntesised sucessffuly with the desired caracteristics: different pore sizes and diameters.

3.2. Functionalization

After MSN synthesis and template removal, both types of nanoparticles were functionalized with two different compounds PTES and CAT. The quantification was carried out via ^{1}H NMR as described by Crucho *et al.* [31]. This method destroys the nanoparticles and requires an internal standard, such as trioxane, to be used in the quantification. The results obtained are presented in Table 3.

The values for the concentration of functionalization compound is different in MCM and OMSN

Table 3: Functional group quantification

	[PTES or CAT) (mmol/g)	Surface Coverage (molecule/nm^2)
MCM-PTES	3.31	2.08
OMSN-PTES	1.62	2.08
MCM-CAT	1.90	1.19
OMSN-CAT	1.79	2.29

nanoparticles, which goes according to the expected since they are two different types of nanoparticles with different surface areas and pore volumes. MCM nanoparticles have higher concentrations of CAT or PTES, due to its larger surface area and pore volume that, consequently, can accommodate more functional groups in their surface

When taken into account the surface area, the number of molecules per nm^2 is about the same for MCM and OMSN nanoparticles, which means that the functionalization is very effective regardless the type of nanoparticle and compound. Although, the surface coverage values are similar for both type of nanoparticles functionalized, the value is lower for MCM-CAT. This value diverges probably due to an experimental error.

3.3. Loading and Release Studies

The proof of concept for these functionalized MSNs as controlled release platforms was made through the release study of two fluorescent molecules, SRB which is a fluorescent dye and DOX a chemotherapy drug. The molecules were chosen due to their fluorescence properties and their behaviour with different functionalisations. The hydrophobic, PTES, was chosen to test a hydrophobic-hydrophilic interaction with DOX. CAT, a cationic substance, was chosen to test cationic-anionic interaction with SRB.

3.3.1. SRB loading

The loading process starts by placing the dry nanoparticles in a solution of SRB as mentioned in 2.3.3. The supernatants removed by centrifugation are used for the molar quantification, by the absorbance obtained from the UV-Vis spectroscopy. With the maximum value of the absorbance and with a calibration curve it is obtained the concentration of the supernatant and then, the moles number, presented in Table 4.

From Table 4 is possible to see that the number of moles of SRB incorporated is similar for all nanoparticles systems, which is about 50% of the amount used in loading (Table 4). This means that the NP type (OMSN and MCM) do not influenced the amount incorporated and the pore size and surface area don't influence directly the cargo loading.

Also, the incorporation efficiency remains the same in spite of the concentration of the loading solution. Therefore, it is possible to control the amount of cargo inside the nanoparticles by changing the loading solution concentration.

It is possible to confirm that the internal functionalization of the nanoparticles does not affect the cargo capacity.

3.3.2. SRB controlled release

After the loading process and centrifugations, the NPs were transferred to the dialysis device and inserted on top of the fluorescence measurement cuvette.

The release studies were carried out by measuring the fluorescence intensity of the SRB molecules in the bottom cuvette for 10 h. After 10 h, an emission spectrum of the cuvette is performed in order to quantify the number of moles released.

Then, the maximum value of the spectrum is used in the emission calibration curve and the number of moles of SRB released are obtained (Table 4).

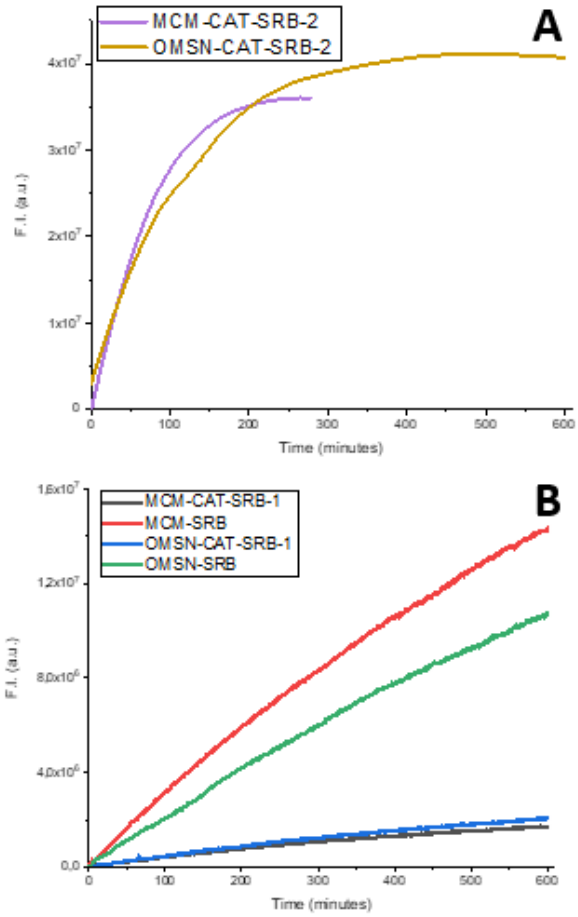

Figure 3: Release studies of A) MCM-CAT-SRB-2 and OMSN-CAT-SRB-2; B) MCM-CAT-SRB-1, MCM-SRB, OMSN-CAT-SRB-1 and OMSN-1

The percentage of released SRB after 10 hours is similar for all cationic functionalized NPs.

Table 4: Data for the all experiments: SRB incorporated

Sample	$\mathbf{n}_{SRBadd}$ (mol)[a]	$\mathbf{n}_{supernatants}$ (mol)[b]	$\mathbf{n}_{MSNs}$ (mol)[c]	Incorp. (%)[d]	$\mathbf{n}_{rel}$ (mol)[e]	Released (%)[f]
OMSN-CAT-SRB-1	1.023×10^{-7}	4.698×10^{-8}	5.533×10^{-8}	54.1	8.068×10^{-11}	0.15
MCM-CAT-SRB-1	1.023×10^{-7}	5.189×10^{-8}	5.042×10^{-8}	49.3	7.026×10^{-11}	0.14
OMSN-CAT-SRB-2	1.169×10^{-5}	5.390×10^{-6}	6.298×10^{-6}	53.9	1.737×10^{-8}	0.28
MCM-CAT-SRB-2	1.169×10^{-5}	5.003×10^{-6}	6.685×10^{-6}	57.2	1.388×10^{-8}	0.21
OMSN-SRB	1.023×10^{-7}	5.993×10^{-8}	4.237×10^{-8}	41.4	4.404×10^{-9}	10.39
MCM-SRB	1.023×10^{-7}	4.388×10^{-8}	5.843×10^{-8}	57.1	6.607×10^{-9}	11.31
Free SRB	1.023×10^{-7}	-	-	-	2.835×10^{-9}	95.77[g]

[a] number of SRB moles in the loading solution for the release study (in 1 mL, except for free SRB that is in 200 μL); [b] number of SRB moles in the supernatants; [c] number of SRB moles inside the MSNs; [d] number of SRB moles inside the MSNs per gram of nanoparticles; [e] percentage of moles incorporated: $\frac{n_{MSNs}}{n_{SRBadd}} \times 100\%$; [f] total number of moles released after 10 hours; [g] percentage of moles released after 10 h: $\frac{n_{rel}}{n_{MSNs}} \times 100\%$; [g] for this value instead of using n_{MSNs}, it was used the value of n_{SRBadd}

For non-functionalized nanoparticles the percentage released was about 50 times higher than functionalized nanoparticles, which indicates that the system can indeed control the release rate.

Then, to confirm that the dialysis membrane was not a barrier in the diffusion process, a release experiment was performed using free SRB in solution with, approximately the same number of moles that are released in other experiments. It was confirmed that the membrane is not a barrier in the diffusion since 95% of the SRB molecules diffuse across the membrane.

As shown in Figure 3-B, the nanoparticles without functionalization release their cargo faster than functionalized nanoparticles which means that by adding functional groups with some affinity with the cargo molecules it is achieved a slower release rate.

The percentage released from MCM-SRB and OMSN-SRB is about 10% after 10 h, which was expected to be higher. Despite the plateau was not yet reached, two effects may be happening to reduce the fluorescence intensity analysed. First some photobleaching (SRB degradation), that can be taking place, can be balanced by the moles release (fluorescence intensity). Another hypothesis is the interaction between SRB and the nanoparticle. Initially, a large amount of SRB is released and an equilibrium can be achieved with the osmotic pressure due to the difference in SRB concentration inside and outside the nanoparticle. If the system only depended on the osmotic pressure, the cargo would be released continuously to balance the pressure. But that is not the case, there is also, the interaction between the SRB and the pore wall. Eventually, the osmotic pressure for the drug to be release will be balanced by the drug-pore adsorption energy. In this closed system, the release reaches an equilibrium.

Comparing all functionalized nanoparticles, the OMSN-CAT-SRB-1 and MCM-CAT-SRB-1 release more since they incorporated more SRB.

Both OMSN-CAT-SRB-2 and MCM-CAT-SRB-2 have a plateau. That is explained by the same reasons as mentioned above for MCM-SRB and OMSN-SRB, that is, the osmotic pressure for the SRB to be release will be balanced by the drug-pore adsorption energy.

The OMSN-CAT-SRB-2 curve ((Figure 3A) slightly decreases at the end due to some SRB photobleaching that can be happening after the long exposure to the excitation light. Also, the MCM-CAT-SRB-2 curve ((Figure 3-A) was acquired for a shorter time because of a stability problem of the equipment.

With these results it is possible to conclude that the pore size does not influence the release at least after 10 hours and with SRB as cargo. Probably because SRB is a small molecule for which even smaller pores are not barriers to the diffusion, so that larger pores do not increase the release. This means that in our case, the functionalization is the most important parameter, since the amount of SRB released is 50 times lower in the cationic-functionalized nanoparticles.

3.3.3. DOX loading

Initially, the DOX experiments were to be carried out as the ones with SRB. The loading process of DOX was similar to SRB as well as the release experiment. The first experiment was performed in OMSN nanoparticles functionalized with PTES. It was expected that the fluorescence intensity increased with time, however that was not verified. The kinetics curve only registered noise, with no fluorescence from DOX.

To overcome this problem, one possible solution was to change the release media. Since PTES-functionalized nanoparticles are hydrophobic, the PBS/PTES interaction could be affecting the release of the cargo. Therefore, PBS was substituted by DMSO.

Table 5: Data for the all experiments: DOX incorporated

Sample	$\mathbf{n}_{DOXadd}$**(mol)**[a]	$\mathbf{n}_{supernatants}$ **(mol)**[b]	$\mathbf{n}_{MSNs}$ **(mol)**[c]	**Incorporated (%)**[d]
OMSN-CAT	1.330×10^{-7}	1.374×10^{-7}	-4.370×10^{-9}	≈ 0
MCM-CAT	1.303×10^{-7}	1.371×10^{-7}	-4.000×10^{-9}	≈ 0
OMSN-PTES	1.330×10^{-7}	1.136×10^{-7}	1.929×10^{-8}	14.5
MCM-PTES	1.330×10^{-7}	1.068×10^{-7}	2.624×10^{-8}	19.7
OMSN	1.330×10^{-7}	1.244×10^{-7}	8.650×10^{-9}	6.5
MCM	1.330×10^{-7}	1.263×10^{-7}	6.750×10^{-9}	5.1

[a] number of DOX moles in the loading solution for the release study (in 1 mL); [b] number of DOX moles in the supernatants; [c] number of DOX moles inside the MSNs; [d] percentage of moles incorporated: $\frac{n_{MSNs}}{n_{DOXadd}} \times 100\%$

Despite this change, the result remained the same, the cargo was still not released. This can be related with the strong van der Waals interactions between DOX and the hydrophobic PTES. Considering that, it was performed a release experiment using nanoparticles without functionalization but DOX was still not released. Since DOX is a quite large molecule it has a lot of interactions (van der Walls) with the silica surface, that might be preventing the release.

The possibility of the nanoparticles not being loaded with DOX was discarded because it was clearly visible that the nanoparticles became orange after the centrifugations. Therefore, the duration of the experiment was increased to 9 days. Instead of analysing the cuvette continuously, an emission spectrum was measured at days 1, 2, 3, 8 and 9.

The amount of DOX incorporated was quantified using a calibration curve and the maximum absorbance of the supernatants (Table 6).

For nanoparticles functionalized with CAT, the value for the number of moles incorporated was close to zero. This can be explained by the residual amount of DOX incorporated that can result in a measurement in the range of the equipment threshold. Also, both DOX and CAT are cationic since DOX hydrochloride is protonated in this solution (the amine group pKa is 8.4 which is higher than the PBS pH of 7.5).

The amount of DOX incorporated by non-functionalized nanoparticles is smaller than the nanoparticles functionalized with PTES, since the functionalization increases the amount of cargo inside, by interacting with the drug (hydrophobic and van der Waals interactions between silanols in the surface and the drug), keeping it in the nanoparticles even after the washing. Overall, the incorporation achieved was good.

3.3.4. DOX controlled release

The drug release was analysed by the emission spectra and results are presented in Figures 4 and 5 for all samples during the 9 days.

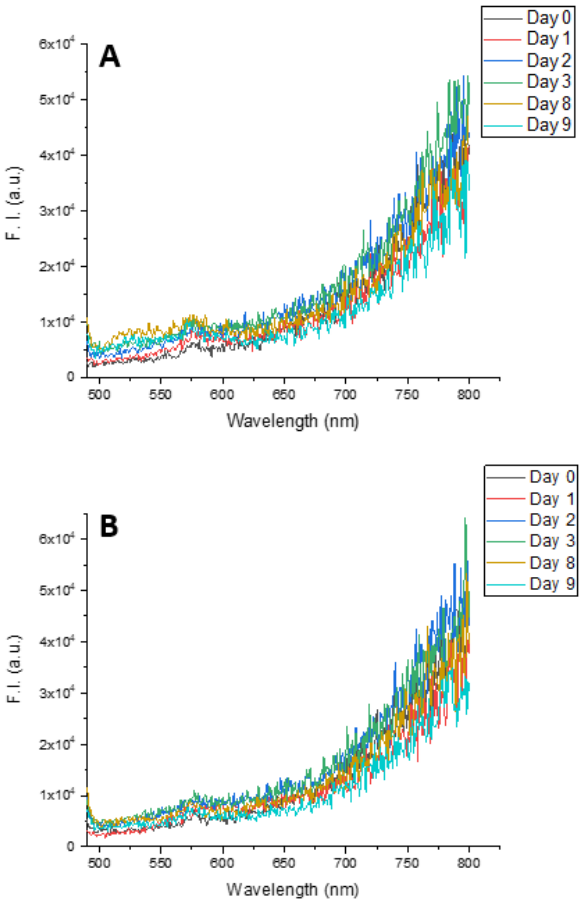

Figure 4: Release studies of A) OMSN-CAT and B) MCM-CAT

The main difference between the results is a clear peak that appears in the spectra of the non-functionalized nanoparticles (Figure 4 - C and D).

An increase in fluorescence intensity is observed between day 0 and day 1, and it is almost constant until day 9, which confirms some drug release in the absence of functionalization.

For the CAT-functionalized nanoparticles no release was expected since those nanoparticles did not incorporate any considerable amount of DOX (Figure 4 - A and B).

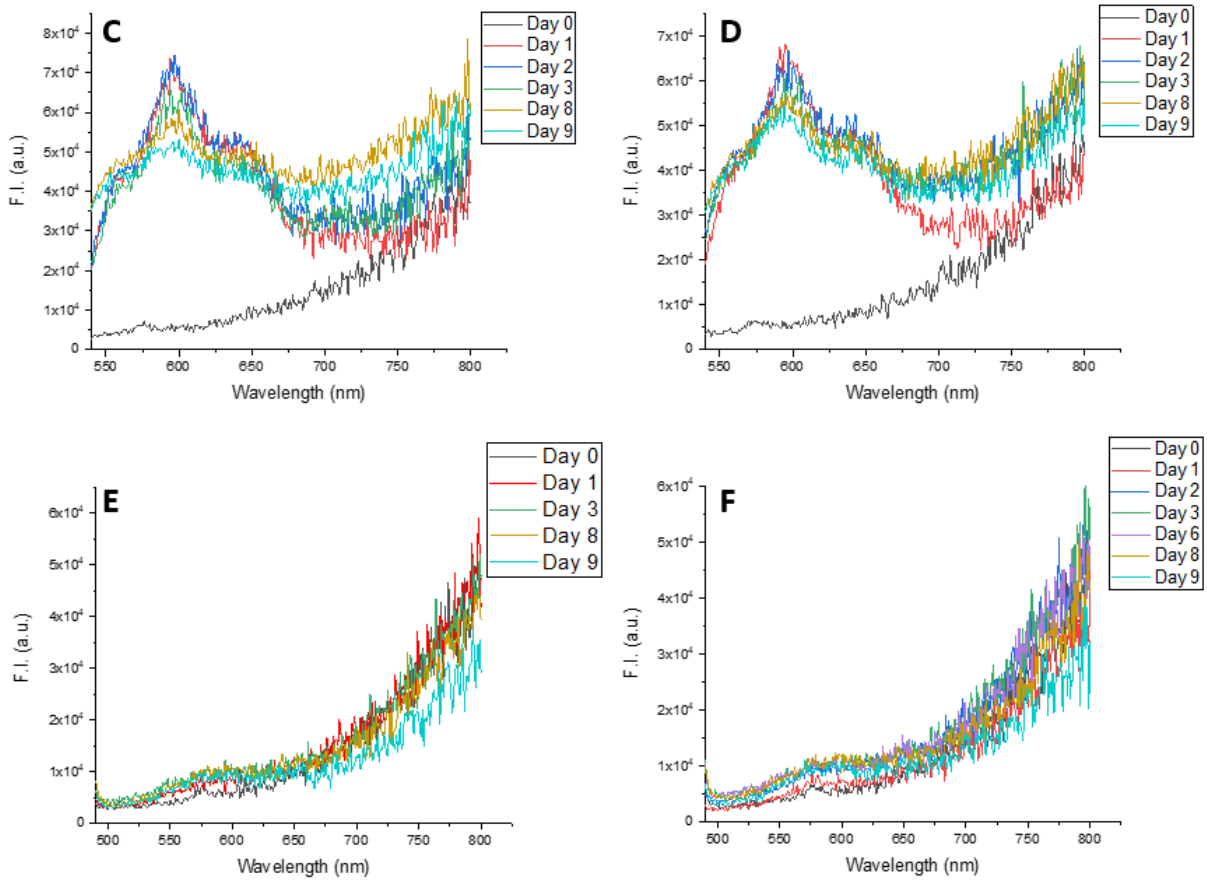

Figure 5: Release studies of C) OMSN, D) MCM, E) OMSN-PTES and F) MCM-PTES

For nanoparticles functionalized with PTES, also no release was detected during the 9 days (Figure 4 - E and F). PTES is a hydrophobic compound, so it probably has strong London interactions with DOX. These interactions strongly stabilize the DOX in the nanoparticles pores, so that there is incorporation of DOX in the NPs, but no release is observed in aqueous media in 9 days. Although the release rate is extremely low in this case, it could be possibly detected in longer experiments.

4. Conclusions

In this work a novel controlled release system based on the interactions between the functionalized MSN pore walls and the cargo molecule was prepared. Nanoparticles with different sizes, pore volumes and width, were successfully synthesized by sol gel method, MCM, and by emulsion-based method, OMSN.

After template removal, silica NPs were surface modified with two functional groups: trimethoxy(propyl)silane (PTES) and N-trimethoxysilylpropyl- N, N, N - trimethylammonium chloride (CAT). It was achieved a surface coverage higher than 2 molecule/nm^2 for almost all nanoparticles, which means that the functionalization was very effective.

The proof of concept for these functionalized MSNs as controlled release platforms was made through the release study of two fluorescent molecules, SRB and DOX.

The main interest in choosing these compounds was to test different interactions between the functionalization and the chosen cargo. CAT, a cationic substance, was chosen to test cationic-anionic interaction with sulforhodamine B (SRB). The hydrophobic, PTES, was chosen to test a hydrophobic-hydrophilic interaction with doxorubicin hydrochloride (DOX).

In the studies with SRB, the cationic functionalization proved to be very efficient in controlling the release. The percentage of cargo released after 10 h, was 50 times higher in non-functionalized nanoparticles (about 10%) than in functionalized ones (between 0.14 to 0.28%).

The concentration of the loading solution was confirmed to be important. A more concentrated solution leaded to a greater amount of SRB incorporated, with those NPs also releasing more, even though in a slower rate compared to non-functionalized nanoparticles (10% compared to 0.2%).

It was also proven that the type of nanoparticle, with larger pore size/volume, did not considerably influence either the incorporation or release of SRB.

The tests with DOX revealed a much slower kinetics of cargo release. The incorporation was done successfully for nanoparticles functionalized with PTES and also for nanoparticles without functionalization (with lower efficiency). Nanoparticles functionalized with the cationic compound CAT did not incorporate relevant amounts of DOX, since both are cationic.

Although there was incorporation of DOX in NPs functionalized with PTES, no release was observed in aqueous media during the 9 days of the experiment, possibly due to the strong interactions between the functional groups and the drug. Nanoparticles without functionalization released some DOX; but not in a large amount when compared with the results obtained with SRB.

Overall, this novel platform proved that by tuning the pore functionalization of mesoporous silica nanoparticles according to the cargo that will be incorporated, it is possible to create a system where the cargo is delivered in a controlled rate.

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