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Incorporation of methylene blue into mesoporous silica nanoparticles for singlet oxygen generation[†]

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Methylene blue is a known photosensitizer with an ability to generate singlet oxygen. The main active species is the monomeric form. However, this dye has a tendency to self-aggregate to form dimers and higher aggregates, which are less active for ${}^{1}O_{2}$ generation. In this study we propose different strategies to incorporate methylene blue into porous silica nanoparticles. The design of the synthesis is a key parameter to avoid both leaching of the dye and its self-aggregation. The negatively charged silica matrix allows the incorporation of the dye, which is cationic, with no leaching, whereas the presence of phenyl functions in the matrix favors the monomeric form of methylene blue (MB). We observe that both dimeric and monomeric forms of MB can generate ${}^{1}O_{2}$ species when confined inside a silica matrix, improving the activity compared to the bare dye in solution.

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Introduction

Photodynamic therapy (PDT) is a promising technology compared to conventional chemotherapy and radiation therapy for cancer treatment. Traditional chemotherapy is often associated with systemic side effects and a high recurrence rate, while radiation therapy is restricted by the cumulative radiation dose. Obviously, PDT has its own advantages due to its minimal invasiveness, repeatability without cumulative toxicity, excellent functional and cosmetic results, reduced long-term morbidity, and improved quality of life of the patients.¹

PDT is achieved by the excitation of a photosensitizer (PS) with visible light at certain wavelengths, and then the excited PS may either drive electron-transfer reactions to/from biological molecules (type I mechanism) or, alternatively, transfer its energy to molecular oxygen, thus generating singlet oxygen ($^{1}O_{2}$, type II mechanism) that plays a dominant role in *in vivo* PDT.^{2,3} In the first path, one-electron reduced PS may in turn react with oxygen to produce a superoxide anion and other reactive oxygen species (ROS). Among all the photosensitizers, methylene blue (MB) is a desirable and water-soluble PS that can undergo both types of PDT mechanisms and, through the

Type II route, it is able to generate a high ratio of singlet oxygen $(\Phi \Delta \sim 0.5)$, with strong electrophilic activation of the oxidizing electron-rich double bonds in biological molecules and macromolecules.⁴ It is also an FDA approved medicine and has been extensively used as a diagnostic agent or histological dye in clinical applications.⁵⁻⁷ However, it can easily selfaggregate, which leads to the formation of dimers, trimers and H-aggregates.⁸ All forms of aggregates often result in less-effective ¹O₂ generation which becomes a problem to be tackled.9,10 Regarding this issue, encapsulation of MB in nanoparticles is a practical method to ameliorate its efficiency¹⁰⁻¹² and control the self-aggregation at the same time.^{10,13,14} Among all the nanoparticles, porous silica emerges as a versatile support because of its tuneable size, variable porous morphologies and high pore volume. In addition, it is easy to functionalize silica with certain groups by silane chemistry.^{15–18} Most importantly, silica nanoparticles also display excellent biocompatibility and they are frequently used as MB-drug carriers.^{16,19–21}

In most cases, the incorporation of MB into mesoporous silica results in the MB dimer and higher aggregates being the dominant adsorbed species, mainly when one-pot approaches are used, which is a disadvantage for singlet oxygen generation.^{13,14,22} However, some highly effective strategies have been designed for controlling MB aggregation in silica-MB composites. In particular, Wang *et al.*¹⁸ loaded MB into silica, and the obtained silica-MB material was subsequently incubated in an acetate (Ac) buffer solution and finally treated with an assembly of coordination complexes of tannic acid (TA) and Fe(m) ions to get a core-shell silica-MB-Ac@TA system. The generation of singlet oxygen, and photodynamic efficiency and stability against *in vitro* reduction of monomeric MB

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released from silica-MB-Ac@TA were considerably higher than those for silica-MB-Ac, due to the effective control of the aggregation state of MB molecules during outward diffusion through the complexes of TA and Fe(III) ions. Kohle et al. investigated two different particle designs deviating from ultrasmall poly(ethyleneglycol)-coated (PEGylated) fluorescent coreshell silica nanoparticles (SNPs) by replacing the fluorescent dye with a photosensitizer (PS).²¹ In the first approach, the MB derivative is encapsulated within the silica network, while in the second one it is grafted onto the SNP surface between the PEG corona chains. In both cases, the PS molecules are covalently bound to the SNPs via a thiol-Michael addition click reaction between maleimide-functionalized MB and (3mercaptopropyl)trimethoxysilane (MPTMS) co-condensed into the silica matrix. While in the first system the photosensitizer is protected from photobleaching, a higher rate of photobleaching is observed for the second one when compared to the free PS. Encapsulation significantly improves PS photostability, while surface conjugation diminishes it. However, the second strategy shows relatively less MB dimerization, consequently generating more ¹O₂, compared to the free dye. Saita et al.¹³ showed that the incorporation of MB into porous silica nanoaggregates of 10-20 nm nanoparticles (P-SiOx NAs/MB) avoided MB aggregation compared to a one-pot system. This nanocomposite showed 1.36 times higher ¹O₂ production under light irradiation compared to free MB. The authors performed MB release tests to study the retention ability of P-SiO_x NAs. For free MB as a comparison, almost 93% of it was released from the dialysis membrane after 30 min, as expected, while the release of MB by P-SiO_x NAs/MB, as measured outside the membrane, was controlled to approximately 27% on account of the interaction between MB and P-SiO_x NAs.

In summary, in the above studies it was shown that the silica matrix can protect the photosensitizer and tune ${}^{1}O_{2}$ production. However, the synthesis strategy to incorporate a photosensitizer such as MB into the matrix is a key parameter to control both its aggregation and leaching, which are essential issues for singlet oxygen generation. In this study we propose the use of mesoporous silica nanoparticles as a matrix, which have radial porosity to optimize diffusion.²³ In order to minimize the aggregation of methylene blue and also prevent its leaching, silica nanoparticles will be decorated with phenyl functions. Different strategies to incorporate both phenyl groups and methylene blue are described and the obtained materials are characterized using a panel of physicochemical techniques. The results of this study show that the MB monomer versus dimer ratio can be largely increased due to the presence of phenyl groups. However, the synthesis strategy to incorporate the PS is the key parameter to control leaching and fine-tune ¹O₂ generation.

Results

Synthesis of the materials

The objective of this work was the incorporation of methylene blue, a photosensitizer (PS) broadly used to generate singlet oxygen (¹O₂), into an inorganic matrix to optimize its optical properties by avoiding dimer and higher aggregates formation, thus providing a good environment to stabilize both the PS and the generated singlet oxygen species. Porous silica nanoparticles are known to be suitable systems for applications in medicine since they can combine several functions and their shape and size can be easily controlled.^{24,25} Indeed, this type of matrix has been extensively used to develop nanoparticles for PDT applications.^{21,26} In addition, we wanted to minimize the leaching of the PS in solvents such as water and alcohols. Indeed, in most cases, an important leaching is observed when the supported PS is suspended in a solvent.¹³ This issue is particularly crucial for certain applications, since the properties of the PS may differ inside and outside of the matrix. It also limits the recyclability of the PS system.

Our strategy was to use mesoporous silica nanoparticles functionalized with phenyl groups in order to minimize both methylene blue (MB) aggregation and leaching in polar environments. We expected the phenyl rings to (1) intercalate between the molecular units of MB to avoid self-aggregation and (2) exert an improved retention of the PS through π -stacking interactions. We chose mesoporous silica nanoparticles with radial porosity as a matrix, which are easy to synthesize with good control of the particle and pore size.²³ These so-called stellate nanoparticles are synthesized starting from tetraethoxysilane (TEOS) as a silica source and using a cationic surfactant, cetyltrimethylammonium p-toluenesulfonate (CTATos), which plays the role of a template to generate the porosity of the nanoparticles. The size of the particles is controlled by the presence of triethanolamine (TEAH₃) in the reaction mixture and by tuning the synthesis parameters (time and temperature). We used the procedure already reported,²³ with the following initial molar composition: 1.0 TEOS: 0.06 CTATos: 0.03 TEAH₃: 88 H₂O, obtaining stellate silica nanoparticles (ST¹) of 57 \pm 7 nm after two hours at 80 °C. We also prepared nanoparticles (ST) with identical molar composition but added TEOS in two sequential half-loads with a 15 minutes gap between them, because this two-step addition will be a strategy applied later for the incorporation of methylene blue (vide infra). In the latter case we obtained larger nanoparticles of 70 \pm 7 nm size, as shown in the SEM image (Fig. 1 and Table 1).

In order to incorporate phenyl functions into the silica matrix, phenylsilane ($C_6H_5SiH_3$) was used to covalently graft phenyl (Ph) groups onto the silica. Two main strategies were then developed (Scheme 1): (a) simultaneous incorporation of phenyl functions and MB molecules during the synthesis of the silica nanoparticles (one-pot incorporation, strategy 1), and (b) initial synthesis of stellate silica nanoparticles, in some cases incorporating phenyl groups in a first one-pot step, followed by the addition of the MB molecules (post-synthesis incorporation, strategy 2). The nomenclature used for the different materials prepared is the following: **o** and **p** refer respectively to one-pot or post-synthesis procedures, and the components are denoted as **MB** for methylene blue, **Ph** for the phenyl group and **ST** for stellate silica nanoparticles. Additionally, a number indicates the methylene blue/phenyl molar ratio used in the



Fig. 1 SEM images of ST silica nanoparticles: one step (a) vs. two step TEOS addition (b).

Table 1 Mean particle sizes of the samples obtained from SEM in	nages
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	Sample	Particle size (nm)
Bare NPs	ST ¹ -H (TEOS added in one step) ST-H (TEOS added in two steps) ST ^c	57 ± 7 70 ± 7 48 ± 6
One-pot strategy	o-Ph@ST o-MB@ST o-[1MB/Ph]@ST-H o-[0.1MB/Ph]@ST-H o-[0.02MB/Ph]@ST-H o-[0.01MB/Ph]@ST-H o-[0.001MB/Ph]@ST-H ^a	$\begin{array}{c} 61 \pm 7 \\ 51 \pm 5 \\ 55 \pm 6 \\ 34 \pm 3 \\ 40 \pm 5 \\ 74 \pm 6 \\ 86 \pm 6 \end{array}$
Post-synthesis strategy	p,o-[0.01MB/Ph]@ST-H p-MB@ST ^c	$\begin{array}{c} 67\pm7\\ 56\pm5\end{array}$

^{*a*} Synthesized in 1 L aqueous solution while the other samples were synthesized in 100 mL; the particle size value was deduced from 50–100 particles analysed using ImageJ software in SEM pictures.

synthesis. For example, **o**-[**0.01MB**/**Ph**]**③ST** stands for one-pot incorporation of both methylene blue (MB) and phenyl functions (Ph) using a molar MB/Ph ratio of 0.01 in stellate silica nanoparticles (ST) and **p,o**-[**0.01MB**/**Ph**]**④ST** for postsynthesis incorporation of methylene blue in one-pot phenylfunctionalized ST silica nanoparticles, with a MB/Ph molar ratio of 0.01.

As mentioned above, in order to minimize MB leaching, the original synthesis of the ST nanoparticles was modified, using a TEOS addition in two steps with a time gap of 15 minutes between the two additions. The aim was to attain nanoparticles in which both the Ph and MB functions will be located mostly in the core of the nanoparticles. As stated earlier (Fig. 1 and Table 1), the control synthesis involving the addition of TEOS in two steps in the absence of Ph yields particles with increased size, from 57 \pm 7 nm (TEOS addition in one step) to 70 \pm 7 nm (TEOS addition in two steps). This size difference can be explained as follows: the lower TEOS concentration at the beginning of the synthesis in the two-step procedure leads to the formation of a smaller number of silica nuclei and the second addition of TEOS induces the growth of these already existing nuclei rather than the formation of new ones. The same protocol-with TEOS addition in two steps-was tested in



Fig. 2 SEM images of (a) ST-H, (b) o-[0.001MB/Ph]@ST-H and (c) ST^c ; insets: size distribution of the nanoparticles, using 100 particles.

the presence of around 1% molar composition of phenylsilane $(C_6H_5SiH_3)$ with regard to SiO₂, and the size of the particles diminished from 70 \pm 7 to 61 \pm 7 nm compared to the synthesis carried out in a single step without phenyl functions, as observed previously in similar mesoporous silica nanoparticles upon pyridine-silane addition.²⁷ A similar reduction effect is observed in the presence of methylene blue in the reaction medium, yielding nanoparticles of 51 \pm 5 nm size. In conclusion, the gradual addition of TEOS increased the size of the final particles, while the presence of phenyl moieties or MB diminished it (Table 1).

The first strategy to incorporate MB was the one-pot incorporation of both Ph and MB together with the first TEOS loading during the growth of silica ST nanoparticles (Scheme 1, strategy 1). This is a fast (2 h at 80 °C) and ecofriendly method, as it uses only water as solvent. The initial Ph/TEOS molar ratio was

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maintained constant and equal to 0.008 in all syntheses. Different amounts of methylene blue were incorporated, with MB/Ph ratios of 1, 0.1, 0.02, 0.01 and 0.001, in the latest case using a tenfold higher volume of solvent to increase the precision of the weighted amount of MB. For example, for the o-[0.01MB/Ph]@ST sample, the molar composition of the initial mixture was 1.0 SiO₂:0.06 CTATos:0.03 TEAH₃:88.0 H₂O:0.008 Ph-silane:0.00008 MB. Sample o-MB@ST was also prepared for comparison purposes by following the same one-pot two-step procedure but without the addition of Ph.

The second strategy was to incorporate MB in a postsynthesis treatment into previously prepared NPs, either with or without phenyl functions (Strategies 2A and 2B, respectively, see Scheme 1). In the case of phenyl-decorated nanoparticles (Strategy 2A), the Ph functions were introduced along with the first TEOS loading, in a two-step TEOS addition. In this case only a MB/Ph ratio of 0.01 was studied and the same molar ratio of reactants as that of strategy 1 was used. Finally, in the postsynthesis strategy 2B, MB was incorporated into either calcined (ST^c) or acid extracted (ST-H) nanoparticles.

Surfactant extraction

We performed a thorough optimization of the procedures to remove the surfactant with the aim of making the pores accessible without damaging the organic molecules (Ph, MB) present in the NPs. We tested two different techniques: on one hand, NH₄OAc treatment in an EtOH/H₂O (1/1) solution at 45 °C and, on the other hand, HCl treatment in ethanol (96%) at the same temperature (see the Experimental section). In both cases, the number of washing reactant equivalents compared to the surfactant present in the sample was optimized. The best procedure was the use of 2.5 equivalents of HCl *vs.* the amount of surfactant calculated from TGA and this methodology was then used to treat the whole series of samples (Fig. S2–S4, ESI†). An "–H" suffix was added to the name of the acid-extracted samples. For example, o-Ph@ST-H stands for a one-pot synthesis of ST nanoparticles using phenylsilane, which were subsequently treated with HCl in an EtOH–water mixture to extract the surfactant.

Evaluation of MB release

Leaching tests were performed in water for all the samples by monitoring the presence of MB in the washing solutions, after several centrifugation/washing cycles, using UV-visible spectroscopy. Surprisingly, for almost all the one-pot samples continuous MB leaching was observed upon acid treatment in a EtOH/ H_2O mixture to remove the surfactant and also during water washes (Fig. S5, ESI†). However, we observed that the presence of Ph functions seems to help the MB to remain inside the silica matrix. Thus, in one-pot materials, the lower the MB/Ph ratio the lower the MB release, with negligible leaching observed for the sample containing the lowest amount of MB, *i.e.*, o-[0.001MB/Ph]@ST. To our delight for the samples prepared using post-synthesis (Scheme 1, Strategy 2), such as p-MB@ST^c and p,o-[0.01MB/Ph]@ST-H, no leaching was observed at all, the washing solutions obtained from the first washes were colourless. No MB leaching was observed even in the absence of Ph functions, indicating that the synthesis strategy determines the location of the MB functions inside the nanoparticles. The MB molecules, which are cationic, are likely retained in the silica nanoparticles by ionic interactions with the silanolate functions present in the matrix. In the case of one-pot synthesis, the minimal retention of MB molecules could be explained by a wrong location of both Ph and MB during the synthesis. Indeed, we have used a reactant, PhSiH₃, that is not very reactive and it may partially stay inside the surfactant micelles, therefore precluding its quantitative copolymerisation with TEOS to be incorporated in the matrix (*vide infra*). MB could have been partially driven to the micelles by π -stacking interactions with phenyl, thus being removed upon washing.

Chemical quantification analyses of sulphur and carbon were performed through ICP for selected samples (o-Ph@ST-H, p,o-[0.01MB/Ph]ST-H, p-MB@ST^c and o-[0.01MB/Ph]@ST-H) to determine the MB/silica content. The results indicate that the amount of sulphur was under the quantification limit (0.10%) in all cases, thus precluding the straightforward estimation of the MB content. Conversely, the percent of carbon could be evaluated (see Table S1 in the ESI[†]). The data are pretty consistent with the %C estimated from TGA analyses (Fig. S6 and Table S1, ESI⁺), where weight losses attributable to MB⁺ and Ph-(Si) units take place around 200 and 600 °C respectively. Surprisingly, TGA curves evidence minimal incorporation of phenyl functions in the one-pot o-[0.01MB/Ph]@ST-H sample, since no significant mass losses were found at 600 °C. This result is consistent with IR spectroscopy (Fig. 3(A)): typical features of the phenylsilane are observed for o-Ph@ST-H and p,o-[0.01MB/Ph]ST-H whereas in o-[0.01MB/Ph]@ST-H these signals are not present. However, the presence of phenylsilane in the last sample was indeed detected before surfactant extraction (Fig. 3(B)) indicating that, as mentioned above, most of the phenlylsilane did not react, being probably located inside of the surfactant micelles, and was mostly removed during surfactant extraction. Nevertheless, a little amount is still present as shown in the UV-Vis spectrum (vide infra, Fig. 5), where transitions corresponding to phenyl functions are found at wavelengths close to 215 and 260 nm.

Study of the morphology and the porosity of the samples

The different nanoparticles studied here are spherical particles with a narrow size distribution as shown in SEM images (Fig. 1, 2 and Fig. S7, ESI[†]). The diameters of the particles are presented in Table 1. It is interesting to highlight again that the presence of phenylsilane diminishes the size of the nanoparticles, and the calcination step contracts them, from 70 nm (ST) to around 50 nm (ST^c). TEM images were collected for the NPs containing only phenyl functions (o-Ph@ST) and also for a series of samples containing methylene blue: o-[0.01MB/Ph]@ST-H, p,o-[0.01MB/Ph]@ST-H and (d) p-MB@ST^c (Fig. 4 and Fig. S8–S11, ESI[†]). The porosity of the particles is radial in all cases. The polycondensation with phenylsilane renders the particles smaller and less homogeneous in shape (Fig. 4(a) and



Fig. 3 IR spectra of selected samples: (A) IR spectra of (a) ST-H, (b) o-Ph@ST-H, (c) o-[0.01MB/Ph]@ST-H, (d) p,o-[0.01MB/Ph]@ST-H, (e) p-MB@ST^c and (f) phenylsilane (Ph-SiH₃), where the vertical dashed line at 697 cm⁻¹ indicates a typical band of phenylsilane; (B) spectra of (a) "one-pot" sample before extraction of the surfactant (o-[0.01MB/Ph]@ST), (b) o-[0.01MB/Ph]@ST-H extracted using HCl/EtOH-H₂O, (c) CTATos and (d) phenylsilane. Vertical lines also indicate bands corresponding either to the surfactant or to phenylsilane.

(c)). For the calcined sample p-MB@ST^c the particles seem to be denser, in agreement with the particle size decrease after thermal treatment. In addition, some aggregates were observed in these samples. Indeed, covalent links between the particles can be formed during the calcination process (Fig. S11, ESI†). Finally, the one-pot sample (o-[0.01MB/Ph]@ST-H) presents a morphology and size similar to bare ST nanoparticles,²³ which confirms that most of the Ph functions were not incorporated in the structure of the solid.

Nitrogen sorption isotherms at 77 K were performed for some of the samples (Fig. S12, ESI[†]). The adsorption profile is typical of stellate nanoparticles, with a progressive increase of N_2 adsorption, in agreement with radial conical pores.²³ At high



Fig. 4 TEM images of (a) o-Ph@ST-H, (b) o-[0.01MB/Ph]@ST-H, (c) p,o-[0.01MB/Ph]@ST-H and (d) p-MB@ST^c.

values of p/p° drastic N₂ adsorptions were observed, which correspond to inter-particle porosity. Therefore, the porous volume was measured at $p/p^{\circ} = 0.9$ in order to eliminate all the volume included between the particles (Table S2, ESI⁺), obtaining internal porous volumes of around 1.2 mL g^{-1} for calcined nanoparticles and around 2 mL g^{-1} for samples synthesized in the presence of phenylsilane, with particles smaller and less defined in the latter case. Another slight difference between the isotherms for samples synthesized from extracted (ST-H) and calcined (ST^c) stellate nanoparticles was observed. Indeed, for ST^c we can identify the primary mesopores at a very small scale generated by single surfactant micelles in the N_2 sorption isotherm (slight N_2 increase at p/ $p^{\circ} \sim 0.4$), because all the surfactant was totally removed, in agreement with TGA (Fig. S6, ESI⁺). However, for the acidwashed samples some surfactants may remain at the inner cones of the radial pores and, therefore, the access to the deepest mesopores was hindered (TGA curves evidence that some of the surfactant was remaining even after 5 washes, Fig. S4, ESI[†]).

DLS measurements were performed for some of the samples in order to analyse the trend of the primary nanoparticles to form aggregates (Fig. S13–S16, ESI[†]). For samples p-MB@ST^c and o-[0.01MB/Ph]@ST-H the average size in methanol was 115–120 nm, indicating the formation of small aggregates, though higher aggregates were also detected. For the other samples, it was difficult to stabilize the individual particles, since big aggregates formed. The size of the aggregates depended on the sample preparation (ultrasound treatment or not) and it could evolve with time (higher aggregates for longer times).

Control of methylene blue (MB) aggregation

UV-visible spectroscopy was used to monitor the MB aggregation state in each studied sample. The spectrum of MB in water at a low concentration is characterized by a typical cyanine-like quasi solitonic absorption band in the far-red, with a characteristic vibronic shoulder of lower intensity at a lower wavelength (Fig. 5 and Fig. S17, ESI⁺). In methanol, a slight blue-shift is observed, while in the solid state a broadening of the absorption band witnesses the formation of small molecular aggregates (Fig. S17, ESI[†]). The signature of MB in water and methanol diluted solutions indicates that MB is present in the form of individual, well solvated molecules in this aqueous environment. A similar pattern is also seen in the case of p,o-[0.01MB/Ph]@ST-H (Fig. 5), indicating that the molecule is almost exclusively present in its monomeric form within the particle. A different trend is seen for both p-MB@ST^c and p-MB@ST-H: in both cases, a similar narrow and almost structureless absorption band is obtained devoid of the vibronic structure generally associated with cyanine transition. According to the existing literature,²⁸ this band shape points towards the quasi-exclusive presence of low-dimension aggregates, such as MB dimers within the mesoporous network. By analogy, the spectrum of o-[0.01MB/Ph]@ST-H suggests that monomers and dimers of MB coexist within the particle in the latter case. To



Fig. 5 Solid DR-UV-visible spectra of the samples selected for the singlet oxygen generation (Kubelka–Munk, arbitrary units), and UV-visible spectrum of a 1×10^{-5} M solution of methylene blue in water (black line, Abs in arbitrary units).

rule out the hypothesis of an involvement of demethylated MB forms (such as Azure A, Azure B or Thionine), which would also produce an hypsochromic shift of the maximum wavelength,²⁹ electrospray ionization mass spectrometry (ESI-MS) experiments were performed for selected samples in methanol (Fig. S18, ESI[†]). A low amount of demethylated products were detected for the post-synthesis of p,o-[0.01MB/Ph]@ST-H and p-MB@ST^c nanoparticles, though most of the MB remained in its original form, thus discarding a relevant role of these demethylated species in the spectroscopic features of the samples. We can thus conclude that, consistent with our initial hypothesis, phenyl groups are able to completely suppress MB dimerization in the post-synthesis samples, whereas aggregation is only partially reduced in the one-pot o-[0.01MB/Ph]@ST-H sample.

In parallel to the family of stellate silica nanoparticles described until now, another set of samples based on the MCM-41 type of silica called LUS was prepared for comparison (experimental procedures are given in the ESI,† and the corresponding solid UV-vis spectra are depicted in Fig. S1, ESI†).^{30,31}

The DR-UV-visible spectrum of one of these samples, p_{Wa},p_{Cy} -[0.01MB/Ph]@LUS, is included in Fig. 5. In striking contrast with the rest of the samples, the absorption spectrum of p_{Wa},p_{Cy} -[0.01MB/Ph]@LUS shows a significantly blueshifted, broadened and ill-defined band structure, generally associated with H-aggregation and/or demethylated forms of the dye, presumably originated in part by the more basic character of this material.³²

In summary, these spectroscopic investigations highlighted the high tenability of the aggregation state of MB that could be achieved by subtle modifications of the silica support.

Singlet oxygen generation

Five samples containing the photosensitizer methylene blue (MB) were selected to study ${}^{1}O_{2}$ generation and compare their behaviour to free MB: o-[0.01MB/Ph]@ST-H, p,o-[0.01MB/

Ph]@ST-H, p-MB @ST^c, p-MB@ST-H and also the LUS-based p_{Wa}, p_{Cy} -[0.01MB/Ph]@LUS sample. To do so, we monitored the kinetics of the photobleaching of anthracene dipropionic acid (ADPA) in aqueous solution for this series of samples. The concentration of the latter was adjusted to obtain the isoabsorbance of MB species at the excitation wavelength (see the experimental part for details). By monitoring the evolution of the luminescence intensity of ADPA *versus* irradiation times (Fig. S19 and S20, ESI[†]), we obtained in each case an evolution that could satisfactorily be fitted to a first order model (Fig. 6). Taking MB in water as a reference (to which we attribute the arbitrary photosensitizing ability of 1), the studied systems divide into two categories.

On the one hand, p_{Wa} , p_{Cy} -[0.01MB/Ph]@LUS and o-[0.01MB/ Ph]@ST-H experienced a marked decrease of their photocatalytic properties. The sample p_{Wa} , p_{Cy} -[0.01MB/Ph]@LUS, where MB is exclusively present in the form of large H-aggregates, presents much decreased photosensitizing ability (0.25), in good agreement with what has previously been reported for such aggregates^{33,34} in nanosuspensions. More unexpectedly, o-[0.01MB/Ph]@ST-H, where a mixture of MB in its monomeric and dimeric form is present, is also characterized by complete inactivation of its photosensitizing capability. We suspect that the one-pot procedure used in the synthesis of the material might result in the closure of the mesopores that in turn restricts the in and out diffusion of molecular species and thereby precludes the interaction between the photosensitizer and molecular oxygen.

On the other hand p,o-[0.01MB/Ph]@ST-H, p-MB@ST^c and p-MB@ST-H experience an increase of their photocatalytic activity. Thus, in spite of the similarity of their spectral signature p,o-[0.01MB/Ph]@ST-H behaves as a significantly more efficient photosensitizer (1.5) when compared to molecular MB in aqueous solution. Such singlet oxygen generation enhancement following immobilization of a photosensitizer



Fig. 6 First order plot of the evolution of the luminescence intensity of ADPA vs. irradiation time: (a) ADPA/p-MB@ST^c, (b) ADPA/p,o-[0.01MB/Ph]@ST-H, (c) ADPA/p-MB@ST-H, (d) ADPA/MB, (e) ADPA/p_{Wa},p_{Cy}-[0.01MB/Ph]@LUS, and (f) ADPA/o-[0.01MB/Ph]@ST-H. The irradiation wavelength is 629 nm. ADPA luminescence is recorded at 409 nm, using 358 nm excitation.

into porous mineral support has already been reported in the past, and correlated with an enhancement of the triplet excited state lifetime stemming from confinement effects within the support.^{35,36} More surprisingly a similar efficiency is found for p-MB@ST-H in spite of the dimeric nature of the MB assembly within the silica matrix. This seemingly contradicts existing studies involving MB dimers, which concluded that the energy transfer pathways – including singlet oxygen generation – were suppressed in favour of electron transfer ones (superoxide formation) and therefore type I phototherapeutic mechanisms.^{13,37} Even more impressively, p-MB@ST^c, which presents the same spectral signature as p-MB@ST-H, displays the highest photosensitization efficiency among the series, leading to an increase in the kinetics of ADPA oxidation, which is more than double with regard to MB in aqueous solution (2.1).

Discussion

From the above results, we can deduce that the synthesis strategy is a key parameter to achieving the incorporation of the dye into a matrix. In our case, the presence of phenyl moieties in the structure favours the occurrence of the monomeric species of the dye, here methylene blue, as shown for sample p,o-[0.01MB/Ph]@ST-H, where MB was incorporated using post-synthesis treatment and only its monomeric form was present in the final solid. However, when a "one-pot" approach was used to incorporate at the same time the phenyl functions and the dye during the growth of the silica nanoparticles a very low amount of phenyl was incorporated. This could be due to the use of a low reactive precursor (PhSiH₃) and also to π - π interactions between the phenyl functions and MB that may interfere in the polycondensation of phenylsilane and TEOS. Indeed, part of the phenylsilane may be located inside the surfactant micelles, avoiding polycondensation with TEOS, and is therefore likely removed during the surfactant extraction. In this case, a mixture of dimeric and monomeric forms was found and, in addition, MB was not retained in the matrix, since continuous leaching was observed for these "one-pot" samples (Strategy 1, Scheme 1). In this case, part of the dye (which is positively charged) may not reach the silanolate functions present in the silica, and is therefore released.

The incorporation of the dye was optimized using a postsynthesis strategy both on calcined or extracted silica nanoparticles (Strategy 2B), and also on extracted silica nanoparticles with Ph functions (Strategy 2A). The dye is likely retained in these solids by electrostatic interactions and no leaching at all was observed. After the incorporation of the dye in these three solids, the samples p-MB@ST^c, p-MB@ST-H and p,o-[0.01MB/Ph]@ST-H presented the best results concerning singlet oxygen generation, displaying higher activity than that for the bare dye in solution. The best material was p-MB@ST^c, which contains exclusively dimeric MB, unlike p,o-[0.01MB/ Ph]@ST-H, where only the monomer is present. This unexpected result could tentatively be explained by higher stabilization of the generated ${}^{1}O_{2}$ species by the matrix. Indeed, the framework of the calcined silica nanoparticles (ST^c) is more condensed, with a lower number of Si–OH functions and higher hydrophobicity. Such an environment is expected to decrease non-radiative deactivation of ${}^{1}O_{2}$ through a vibronic interaction with O–H vibrators thus enhancing the ${}^{1}O_{2}$ species lifetime in the confined space of the silica matrix.

To mitigate these positive conclusions, the hybrid nanoparticles studied here tend to form aggregates, since the external surface was not functionalized to optimise their colloidal stability. For applications in nanomedicine such as photodynamic therapy this drawback could be in the future addressed by surface modification of the colloidal particles with water solubilising entities such as PEG chains or water solubilising polymers.^{38–40} However, this trend to aggregate may become an asset in the field of heterogeneous catalysis, as it would enable easy separation of the oxidation photocatalyst from the reaction mixture.

Conclusions

Different synthetic methodologies were designed for the incorporation of methylene blue into mesoporous silica particles with the aim to minimize MB aggregation and thus improve its efficiency towards singlet oxygen photogeneration, while avoiding the leaching of the dye. Two different strategies (one-pot and post-synthesis) were employed to incorporate phenyl functions into the NPs with the goal of intercalating it between MB molecules and thus favour the monomeric form. When using one-pot procedures, only negligible amounts of Ph were anchored and, as inferred from UV-vis spectroscopy, mixtures of monomeric and dimeric MB forms were present in the corresponding o-[xMB/Ph]@ST-H samples. In contrast, the incorporation of MB through a post-synthesis strategy into phenyl-functionalized ST nanoparticles leads to the purely monomeric form in the p,o-[0.01MB/Ph]@ST-H material. In parallel, calcined ST^c nanoparticles have also been functionalized with MB in the post-synthesis procedure and, in this case, the p-MB@ST^c nanoparticles contain exclusively MB dimers. It is remarkable that although a certain degree of MB leaching is found for one-pot samples, the materials obtained through post-synthesis methods perfectly retain MB upon washing, with no leaching at all.

The carbon content of the selected samples was determined by ICP and is consistent with the results provided by TGA. On the other hand, the characterization of the functionalized ST materials evidences that the nanoparticles display a spherical shape with a narrow size distribution and a smaller mean size for the samples containing Ph, with radial porosity in all cases.

Finally, the ability to generate singlet oxygen upon irradiation in the MeOH suspension was evaluated for selected samples, with the ones prepared by post-synthesis procedures (p-MB@ST^c, p,o-[0.01MB/Ph]@ST-H and p-MB@ST-H) showing the best results and improving the performance of molecular MB in solution while, in contrast, the one-pot o-[0.01MB/ Ph]@ST-H is not active at all. The unexpectedly high capability of the calcined p-MB \otimes ST^c nanoparticles to generate ${}^{1}O_{2}$ is outstanding despite MB being in its dimeric form. The results obtained make the post-synthesis NP materials promising candidates to be tested in either PDT or photocatalysis.

Experimental

Materials and methods

Methylene blue trihydrate (MB, 98%), sodium hydroxide (pearl, NaOH, 97%), LUDOX HS-40 colloidal silica (40 wt% suspension in H_2O), tetramethylammonium bromide (98%) and tetraethyl orthosilicate (TEOS, 99%) were purchased from Alfa Aesar. Hexadecyltrimethylammonium *p*-toluenesulfonate (CTATos, 98%) and triethanolamine (TEAH₃, 99%) were purchased from Sigma-Aldrich. Anhydrous ethanol (99.9%), methanol (99.9%), acetone (99.8%), and cyclohexane (99.8%), as well as HCl (37%), were purchased from Carlo Erba. Phenylsilane (Ph, 96%) was purchased from TCI Europe. All the chemicals and solvents were used as received.

Synthesis of the materials

Stellate silica nanoparticles (ST). Stellate silica nanoparticles were prepared according to the work of Zhang *et al.* with little modifications.²³ CTATos (1.66 g, 3.64 mmol) and triethanolamine (TEAH₃, 0.30 g, 2.01 mmol) were added into 100 mL of water and the solution was stirred and heated at 80 °C. After 1 h, 7 mL of TEOS were added to the solution, and then another 7 mL (overall 63.16 mmol) of TEOS were added after 15 minutes. The mixture was heated for 1 h 45 min, and the solution was filtered and washed with water. The sample was dried at 80 °C overnight.

Calcination: ST nanoparticles were heated at 600 °C for 4 hours under air with a ramping rate of 1.5 °C minute⁻¹. The calcined nanoparticles are named ST^e.

One-pot synthesis of o-Ph@ST. The synthesis was similar to the procedure of synthesizing ST nanoparticles but 0.060 mL (0.49 mmol) of phenylsilane ($C_6H_5SiH_3$) were added together with the first dose of 7 mL of TEOS. The obtained solid was dried at 80 °C overnight.

One-pot synthesis of o-MB@ST. The synthesis was analogous to the procedure of synthesizing ST nanoparticles but 1.85 mg (0.0049 mmol) of methylene blue trihydrate (MB) were added together with the first dose of 7 mL of TEOS. The sample was dried at 80 °C overnight.

One-pot synthesis of o-[XMB/Ph]@ST. The synthesis was similar to the procedure described to prepare ST nanoparticles but 0.060 mL (0.049 mmol) of phenylsilane (Ph-SiH₃) and a certain amount of methylene blue trihydrate were added simultaneously to the first dose of 7 mL of TEOS. For different samples, 'X' indicates the methylene blue/phenylsilane ($n_{\rm ME}/n_{\rm Ph}$) initial molar ratio used, with X = 1, 0.1, 0.02, 0.01 and 0.001. In the last sample, the volume of water used was 1 L instead of 100 mL. The molar amount of phenylsilane relative to the silica source (TEOS), $n_{\rm Ph}/n_{\rm TEOS}$, was constant and equal to 0.008 for all the samples.

Extraction of the surfactant using NH₄OAc as washing reagent. 1 g of ST was dissolved in 150 mL of an EtOH/H₂O solution ($V_{\text{ethanol}}/V_{\text{water}} = 1$) and a certain amount of NH₄OAc was added (tests were performed with 1, 5, 12.5, 20 or 30 equivalents compared to the surfactant present in the solid, to optimize the procedure). The mixture was stirred at 45 °C for 1 h, filtered and washed three times with 100 mL of an EtOH/H₂O mixture (v/v = 1:1). This process was repeated twice and the final solid was dried at 80 °C overnight. The samples were named ST-XNH₄OAc (X: $n_{\text{NH}_4\text{OAc}}/n_{\text{CTA+}}$, see ESI,† Fig. S2).

Extraction of the surfactant using HCl as washing reagent. 1 g of ST was dissolved in 40 mL of ethanol (96%) and then certain amounts of HCl (1.5, 2.5 or 15 equivalents compared to the surfactant present in the solid) were added. The mixture was stirred at 45 °C for 30 min and then it was filtered and washed with 40 mL of ethanol and 40 mL of acetone. This process was repeated twice, and the solid was dried at 80 °C overnight. The samples were named ST-XHCl (X = $n_{\text{HCl}}/n_{\text{CTA+}}$). After the optimisation of the surfactant removal, a final protocol using 2.5 equivalents of HCl was then applied to remove the surfactant from all the series of samples (ST, o-Ph@ST, o-MB@ST and o-[XMB/Ph]@ST). The washed samples were named ST-H, o-Ph@ST-H, o-MB@ST-H and o-[XMB/Ph]@ST-H.

Post-synthesis of p-MB@ST^c, p-MB@ST-H and p,o-[0.01MB/ Ph]@ST-H. 0.425 g of calcined stellate nanoparticles (ST^c), 0.446 g of HCl-washed stellate nanoparticles (ST-H) or 0.465 g of phenyl-functionalized washed nanoparticles (o-Ph@ST-H) were added into three different Erlenmeyer flasks each containing 25 mL of methylene blue solution (2 mg of methylene blue trihydrate dissolved in 25 mL of water). After 1 h of stirring, the solutions were filtered, washed with 25 mL of water 3 times and dried at 80 °C overnight. The resulting samples were named p-MB@ST^c, p-MB@ST-H and p,o-[0.01MB/Ph]@ST-H, respectively.

Characterization of the materials. Infrared spectra were recorded either on KBr pellets in the 4000-400 cm⁻¹ range using an ATI Mattson Genesis Series FT-IR spectrometer or using an ATR PerkinElmer Spectrum 65 FT-IR spectrometer in the 4000-550 cm⁻¹ range. Thermogravimetric analyses (TGAs) were performed with a Netzsch STA 409 PC Luxx device under aerobic conditions with a 10 °C min⁻¹ temperature increase. Nitrogen sorption isotherms at 77 K were obtained with a Belsorp Max volume device on solids that were dried under vacuum overnight at 80 °C. Solid state UV-visible spectra were recorded for samples diluted with calcined silica using a Jasco V-770 spectrometer. UV-visible spectra in solution were recorded using a JASCO V-730 spectrometer. Chemical analyses (C, H and N) were performed at the CNRS Institute of Analytical Sciences in Lyon, France. Carbon quantification: the sample, sealed in a silver capsule, was dropped into a flow of oxygen, the unit combustion was held at 1050 °C and followed by postcombustion in a furnace at 850 °C containing copper oxide. The flash combustion allowed for the complete transformation of carbon into carbon dioxide, which was quantified using a nondispersive infrared detector. Scanning electron microscopy (SEM) images were recorded using a Zeiss Supra 55 VP microscope on samples that were first redispersed in ethanol and

then deposited on a copper foil tape. Transmission electron microscopy (TEM) images were performed at 100 kV (JEOL 1200 EXII). Samples for TEM measurements were deposited from suspensions on copper grids and allowed to dry before observation. Dynamic light scattering (DLS) was performed using a Cordouan VASCO particle size analyser. The Advanced Padé Laplace algorithm and SBL theory were used to treat the data. Padé-Laplace assumes that there is a discrete number of different sizes and SBL (Sparse Baysian Learning) looks for multi-modal and multi-dispersity size distribution. ESI-MS: analyses were performed on a Navigator LC/MS chromatograph from Thermo Quest Finnigan, using methanol as the mobile phase.

Luminescence studies and singlet oxygen generation. Luminescence spectra were recorded using a Horiba-Jobin-Yvon Fluorolog-3[®] spectrofluorimeter, equipped with a three-slit double-grating excitation and emission monochromator with dispersions of 2.1 nm mm⁻¹ (1200 grooves mm⁻¹). An R928 detector was used for emission intensity measurements in the visible range [400-845 nm]. Spectra were reference-corrected for the emission spectral response (detector and grating). In order to study the ¹O₂ generation efficiency using 3,3'-(anthracene-9,10-diyl)dipropanoic acid (ADPA) as a scavenger, 2 mL of freshly prepared methanolic solutions of ADPA and the sample to be studied were placed in a three mL open quartz cuvette $(1 \times 1 \times 3 \text{ cm})$, and stirred at 100 rpm for the whole irradiation period. The initial optical density of ADPA in the reaction mixture was 0.1 at the irradiation wavelength, which was centered at 358 nm.

All the samples were adjusted in concentration so that the initial absorbance of the MB photosensitizer at 629 nm was similar in all cases (OD ca 0.15). Samples were then irradiated over given periods of time using a spectrofluorometer xenon arc lamp as an irradiation source. The irradiation wavelength was centred at 629 nm, and entrance slits were set at 10 nm opening. This setup ensured constant irradiation power throughout all the irradiation experiments.

Conversion of the ADPA scavenger upon reaction with photogenerated oxygen was followed by regular measurements of the luminescence signal of the latter (considering it to be linearly proportional to its concentration, which is valid in the investigated range of absorbance, with OD < 0.1), consecutive to the excitation performed at 358 nm. The plot of the ADPA concentration decay was performed using the luminescence intensity recorded at 409 nm.

Abbreviations

ADPA	3,3'-(Anthracene-9,10-diyl)dipropanoic acid
DLS	Dynamic light scattering
DR UV	Diffuse reflectance ultra-violet
MB	Methylene blue
OD	Optical density
PEG	Polyethylene glycol
PS	Photosensitizer

SEM	Scanning electron microscopy
ST	Stellate
TEM	Transmission electron microscopy
TEOS	Tetraethoxysilane
TGA	Thermogravimetric analysis

Author contributions

J. X. conducted the synthesis and characterization of the materials. L. B. provided suggestions for the experiments. T. P. and Y. G. helped to characterize the nanoparticles using TEM. C. M. and J. X. carried out the study of the luminescent properties and generation of singlet oxygen. B. A. and M. R. P. coordinated the research project, supervised the experiments and organized the preparation of the manuscript. All the authors participated in the preparation of the manuscript.

Conflicts of interest

There are no conflicts to declare.

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