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ORIGINAL PAPER

Synthesis and Characterization of Oxazine-doped Silica Nanoparticles for Their Potential Use as Stable Fluorescent Reagents

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Abstract The synthesis process to obtain silica nanoparticles (NPs) doped with two oxazine dyes, nile blue and cresyl violet, has been investigated using a modification of the reverse micelle microemulsion method and a procedure based on the Stöber method. A micellar medium provided by the non-ionic surfactant Triton X-100 in a hexanol:water mixture and an ethanol:water mixture, have been used to provide the synthesis medium in each case. Tetraethoxysilane has been used as the initiator of the polymerization and condensation reactions after its hydrolysis in basic medium using ammonium hydroxide. Dye-silane precursor NPs have been also synthesized in order to compare their potential advantages against the NPs obtained by the direct encapsulation of the oxazine dyes. Size distribution and fluorescence of the synthesized NPs, which were monitored using Transmision Electron Microscopy (TEM) and a microplate reader, respectively, depend on the molar ratio and total concentration of the reagents involved in the synthesis. NPs obtained using the developed synthesis procedures had sizes below 400 nm in most instances and the best luminescent properties were observed for NPs with sizes ranging from 100 to 300 nm. Lower sizes result in a decrease in the fluorescence intensities of these nanomaterials. Parameters related with the luminescence features of these NPs were calculated in order to compare the feasibility of both synthesis approaches. The repeatability of the reverse-micelle microemulsion procedure performed in different days gave a relative standard deviation of 10% for the fluorescence intensity values.

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Introduction

The use of nanomaterials is having a huge impact in the bioanalysis field [1–9], being their composition and physicochemical properties the main factors to choose the detection system best suited for each assay. The use of silica NPs as labels is interesting in bioassays with optical detection because of the transparency of silica to visible light. Other desirable properties are: 1) silica polymerization chemistry is well known, 2) silica material is not microbiologically degraded, and 3) porosity or swelling changes of silica NPs do not happen at moderate pH variations. However, the porosity of these materials can originate drawbacks in their performance as labels due to losses of the encapsulated substances as it will be discussed below.

Different silica precursors, such as tetramethoxysilane (TMOS) and tetraethoxysilane (TEOS) are commonly used to synthesize silica NPs. These precursors undergo hydrolysis and polycondensation reactions, which result in the formation of monodisperse spherical silica particles [10]. There are two general routes to synthesize silica NPs, namely reverse-micelle microemulsion and Stöber methods. The reverse-micelle microemulsion method relies on the formation of a water-in-oil microemulsion formed by a small amount of water, an organic solvent and a surfactant. Water nanodroplets inside the reverse micelles act as reactors in which the growth of silica NPs takes place. The Stöber method involves the hydrolysis of the silica precursor in an alcohol/water mixture and the silicic acid formed nucleates and condenses to give rise to spherical

monodisperse NPs. The choice of every synthesis approach depends on the physicochemical properties of the species to be encapsulated [2, 4, 6]. In general, hydrophobic and hydrophilic dyes can be encapsulated by using the Stöber method whereas the reverse-micelle microemulsion method has been mainly used for the synthesis of metal-chelate doped silica NPs [2, 4]. An advantage of the reverse-micelle microemulsion method is that usually provides NPs with narrower size distributions than those obtained by the Stöber method [2].

A common issue to both synthesis approaches is the probability of dye leakage, especially for organic dyes. This phenomenon can be minimized or avoided by coupling the organic fluorophor to hydrophilic species with high molecular weight, such as dextrans [11] or proteins [12], for two reasons: 1) the resulting derivative is hydrophilic as the silica matrix, and 2) the large size of the macromolecule can prevent the diffusion through silica matrix pores. Another option is the synthesis of precursors by covalent linking of the dye or a derivative, usually containing carboxyl [13], sulfonyl chloride [14] and isothiocyanate [15] groups, to a silane precursor, previously to the synthesis of silica NPs.

Metal chelates and organic dyes have been traditionally used as labels in bioassays with luminescent detection [7]. Conventional fluorophores, such as fluorescein isothiocyanate, fluorescamine or umbelliferone derivatives, provide the achievement of relatively low detection limits, but these compounds suffer from photobleaching processes due to the relatively high energy of their incident excitation wavelengths. However, this drawback can be minimized by embedding these dyes into a silica matrix, which protects them from environmental factors that would affect their fluorescence [2, 6]. Another limitation of conventional fluorophores is that their emission can be overlapped by static background fluorescence signals from sample matrix, which usually happens at short wavelengths. An alternative is the use of long-wavelength fluorophores, such as organic dyes (cyanines, oxazines, alexa dyes) and lanthanide and ruthenium chelates [16–18].

The work presented here encompasses different approaches to synthesize long-wavelength emitting silica NPs doped with cresyl violet acetate and nile blue chloride for the first time. Two different sol-gel methods, based on the modification of the reverse-micelle microemulsion and Stöber approaches and on the use of the silane precursor TEOS and ammonium hydroxide, have been developed for the direct encapsulation of the fluorophore. The fluorescence of the NPs obtained by the first method has been improved in the absence of cyclohexane, which is used in the conventional procedure. The reverse-micelle microemulsion method has been also applied to the encapsulation of a newly synthesized silane precursor from nile blue chloride using glutaraldehyde and 3-aminopropyl triethoxysilane (APS). The influence of the different reagents on the features of the NPs formed in both synthesis procedures has been studied and optimized by measuring the fluorescence intensity using a microplate reader and the NP size by Transmission Electron Microscopy (TEM). The luminescent features of the NPs synthesized by both methods have been compared in order to select the synthesis procedure more suitable to obtain nanomaterials useful as stable fluorescent reagents for analytical purposes, such as labels in fluoroimmunoassays. Some properties of these long-wavelength emitting NPs, such as photodegradability, chemical stability and dye leakage are also discussed.

Experimental

Instrumentation

A 1420 Multilabel counter Victor ^{3}V microplate reader (Perkin Elmer and Analytical Sciences, Wallac Oy, Turku, Finland) was used to perform fluorescence measurements. Different filters (nominal wavelength/passband) were used to select the excitation (531/25 nm and 590/7 nm for cresyl violet, 620/8 nm for nile blue) and emission (620/8 nm for cresyl violet, 680/10 nm for nile blue) wavelengths of the doped NPs. An SLM-Aminco (Urbana, IL, USA), Model 8100 photon-counting spectrofluorimeter, equipped with a 450 W xenon arc source and a R928 photomultiplier tube, was used to perform fluorescence excitation and emission scans of pure dyes and synthesized NPs, as well as photobleaching experiments. Size characterization was performed by Transmision Electron Microscopy (TEM), using a CM10 Philips Microscope. Copper grids (200C-FC) coated with a Formvar® carbon film 200 mesh supplied by Aname (Madrid, Spain) were used as support in TEM experiments.

Reagents

All reagents were of analytical grade. 3-Aminopropyl triethoxysilane (APS) was obtained from Aldrich (Aldrich, Milwaukee, USA), and tetraethoxysilane (TEOS) and Triton X-100 from Fluka (Buch, Switzerland). Cresyl violet acetate, nile blue chloride and 25% glutaraldehyde aqueous solution (Grade II) were supplied by Sigma (St Louis, MO, USA) and absolute ethanol, acetone and ammonium hydroxide (25% as NH_3) by Panreac (Castellar del Vallès, Spain). Cyclohexane was supplied by Probus (Badalona, España) and 1-hexanol by Merck (Schuchardt, Germany). Cresyl violet and nile blue solutions (1×10^{-3} M) were prepared by dissolving the appropriate amount of each dye in the minimum amount of methanol and then, raised up with distilled water until a final concentration of methanol of 10%, and stirring the mixture for 24 h.

Procedures

Synthesis of cresyl violet and nile blue-doped silica NPs by a modified reverse micelle microemulsion method

The synthesis was performed according to the following procedure: an amount of Triton X-100 (510–530 mg or 0.79–0.82 mmol) was dissolved in 9.6 ml (0.53 mol) of distilled water by stirring vigorously this mixture for 5 min. Then, a volume of 100 μl (0.44 mmol) of TEOS was added and the solution was stirred for 5 min. A volume (1.8 ml) of 10^{-3} M (1.8 μmol) cresyl violet or nile blue was added and the mixture stirred again for 5 min. Afterwards, 3 ml (0.024 mol) of hexanol were added and the microemulsion formed was stirred for 15 min. Concentrated ammonium hydroxide (70 μl, 0.9 mmol) was then added and the mixture stirred for 5 min to start the TEOS hydrolysis and condensation reactions. The mixture was then placed in a thermostated tank at 35 °C for 7.5 h in the dark.

The reaction mixture of each fluorophore-doped NPs, which was composed by two phases clearly differenciated, was centrifuged for 5 min at 2,000 rpm to complete the separation of two phases. The upper phase, which was strongly colored, was extracted and 5–7 ml of acetone were added to break the microemulsion formed, and the precipitate was separated by centrifugation for 20 min at 3,000 rpm. Then, NPs were washed with ethanol using ultrasound sonication for 30 s to desorb the fluorophore from the NP surface and then centrifuged for 5 min at 10,000 rpm to get rid of unreacted precursors and the excess of surfactant and fluorophore. The washing process was repeated several times with ethanol and distilled water until the fluorescence signal of the supernatant was the same as the blank signal. NPs were finally re-dispersed in 1 ml of distilled water.

Synthesis of cresyl-violet doped NPs using a Stöber method-based procedure

To a volume of 25 ml of ethanol (0.43 mol) were added 500 μl of 10^{-3} M (0.5 μmol) cresyl violet. Then, 1 ml (4.4 mmol) of TEOS and 1.5 ml (19.5 mmol) of concentrated ammonium hydroxide solution were added. The mixture was stirred for 1 h at room temperature. After this time, it was centrifuged at 3,000 rpm for 10 min. The NPs synthesized were purified by washing them with ethanol and water for several times as mentioned above for the reverse micelle microemulsion method.

Synthesis of NPs using nile blue-APS precursor as fluorophore

A volume (12 ml) of 8.9×10^{-4} M (10 μmol) nile blue solution was mixed with 100 μl of 1% glutaraldehyde (10 μmol) aqueous solution and, immediately after, 20 μl (85 μmol) of APS were added and the mixture stirred for 5 min before its use in the reverse-micelle microemulsion method above described. A volume (1.2 ml) of the reaction mixture, without any additional purification step, was added to the mixture instead of the unchanged fluorophore.

Characterization of cresyl violet- and nile blue-doped silica NPs

Synthesized NPs were characterized by TEM and fluorescence intensity measurements. TEM experiments were carried out by spotting 10-μl drops of NP suspensions in ethanol onto the copper grids, which were placed above a filter paper and let to dry at room temperature for several minutes. Fluorescence measurements were obtained by dispensing aliquots of 200 μl of NP suspensions onto microwells in triplicate and using the filters above described to choose the adequate excitation and emission wavelengths.

Fluorescence stability of oxazine-doped silica NPs

Silica NPs were divided in five 1-ml aliquots in Eppendorf tubes. All of them were washed several times with ethanol and water until the supernatants presented a fluorescence intensity corresponding to the blank signal and, then, NPs were stored at 4 °C. At the time of the performance of the fluorescence measurements, each aliquot was sonicated for 30 s and then centrifuged at 10,000 rpm for 5 min to remove the supernatant. Then, they were again reconstituted and re-dispersed in water. A volume (200 μl) of the NP suspension was added to a microwell plate and the fluorescence intensity was measured in triplicate.

Photobleaching study of oxazine-doped silica NPs

Free organic dye solution or dye-doped NP dispersion was placed in a quartz cuvette and the fluorescence intensity was measured at the corresponding maximum excitation and emission wavelengths by continuosly irradiating the cuvette with light from the 450 W xenon arc lamp of the photon counting spectrofluorimeter. Fluorescence intensity measurements were monitored at room temperature for 1 h, with an integration time of 1.0 min.

Results and discussion

Synthesis of cresyl-violet- and nile blue-doped silica NPs

Both synthesis approaches were optimized by using mainly the univariate method although, in some instances, the influence of variable ratios of the reagents was also studied.

Optimization of reverse micelle microemulsion method

As indicated above, the formation of microemulsions involves the use of a surfactant, an aqueous solution and an organic solvent. Depending on the relative amounts of the components, water-in-oil microemulsions (rich in organic solvent) or oil-in-water microemulsions (rich in water) can be formed. In the first case, a co-surfactant, generally a medium or long hydrocarbon chain alcohol, is often incorporated in the micelles. Cyclohexane, n-hexanol, Triton X-100 and water were chosen to develop the reverse-micelle microemulsion method, according to the procedures previously reported [2, 4, 6, 12–14]. These approaches involve continuous mechanical stirring to originate stable water-in-oil microemulsions. However, the use of the experimental conditions previously described did not give rise to satisfactory results for the synthesis of the oxazine-doped silica NPs. The study of the stirring conditions showed that, after mixing the reagents in the addition order described in the procedure and then leaving the mixture to stand, two phases appeared: an upper phase (rich in organic solvent) and a lower phase (rich in water). The upper phase, which was strongly coloured, was a stable water-in-oil microemulsion containing the brightest NPs.

Studies carried out about the microemulsion composition revealed that NPs can be synthesized in the absence of cyclohexane, yielding NPs with similar features (number, size) but with slightly higher fluorescence intensity than that obtained using cyclohexane. Figure 1 shows TEM images of the NPs obtained in the presence of different volumes of cyclohexane. The water volume was also modified to keep constant the total volume. As can be seen, the amount and sizes of NPs synthesized in the absence of cyclohexane (Fig. 1a) were comparable to those achieved using 7.5 ml of cyclohexane (Fig. 1c). However, the use of intermediate volumes of cyclohexane (Fig. 1b) provided larger NPs with wider size distributions. According to these results, the use of cyclohexane was precluded and only water, Triton X-100 and 1-hexanol were the main components of the microemulsion.

The phases obtained were separated by centrifugation of the mixture at 2,000 rpm for 5 min. A precipitate appeared in the botton of the lower phase at reagent concentrations higher than the optimum values, which indicated the

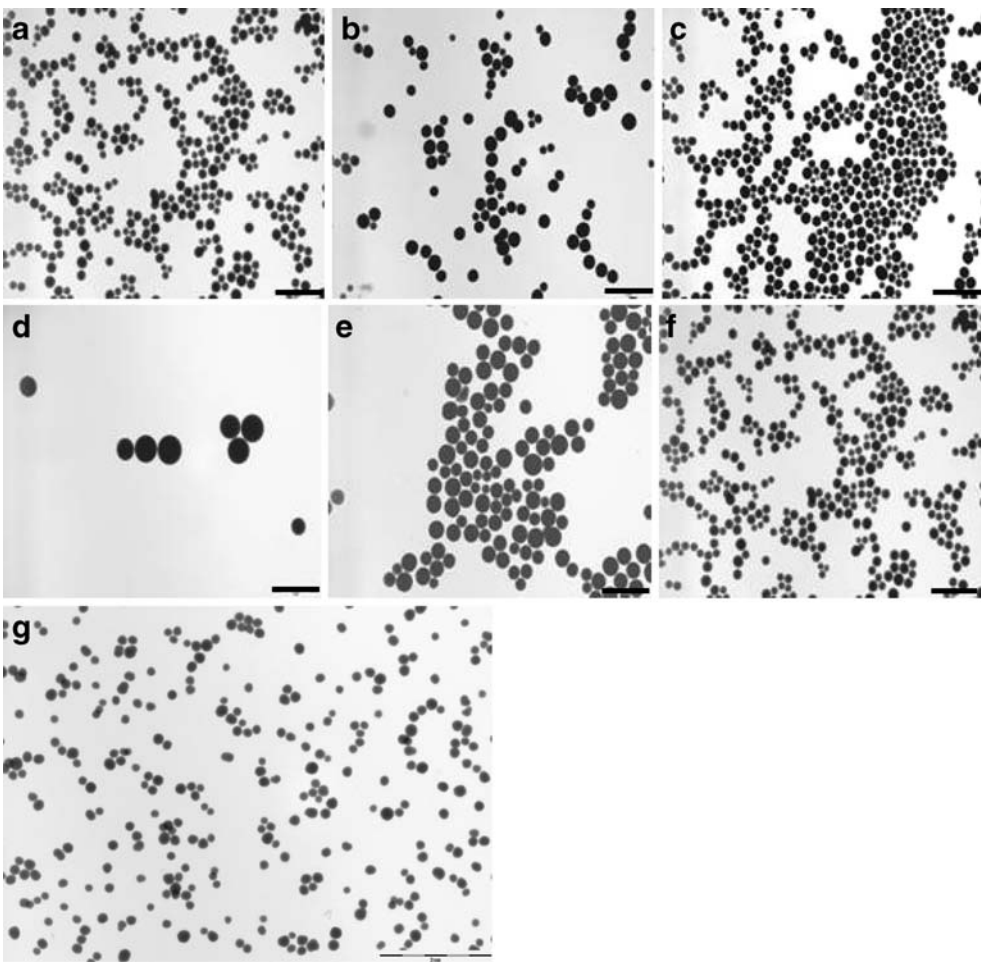

Fig. 1 TEM images of nile blue-doped NPs synthesized using different cyclohexane volumes (**a**, **b** and **c**), different Triton X-100 concentrations (**d**, **e**, **f**) and those for nile-blue-APS doped silica NPs (**g**). Experimental conditions: 100 μl (0.44 mmol) TEOS, 0.2 ml of 1×10^{-3} M (0.2 μmol) nile blue, 3 ml (0.024 mol) hexanol, 70 μl (0.9 mmol) NH_4OH. In (Fig. 1a–c): 510–520 mg (0.79–0.82 mmol) Triton X-100. In Fig. 1a 11.3 ml (0.63 mol) water, 0 ml cyclohexane, in Fig. 1b, 7.5 ml (0.42 mol) water, 3.75 ml (0.034 mol) cyclohexane, in Fig. 1c 3.8 ml (0.21 mol) water, 7.5 ml (0.068 mol) cyclohexane. In Fig. 1d–f 11.3 ml (0.63 mol) of water, In Fig. 1d 0 mg, in Fig. 1e 310.9 mg (0.48 mmol), in Fig. 1f 510.9 (0.79 mmol) of Triton X-100. Scale bar: 1 μm. In Fig. 1g Experimental conditions: 510–520 mg (0.79–0.81 mmol) of Triton X-100, 10.3 ml (0.57 mol) of water, 100 μl (0.44 mmol) TEOS, 1.2 ml precursor reaction mixture, 3 ml (0.024 mol) hexanol, 70 μl (0.9 mmol) of NH_4OH. Scale bar: 2 μm

saturation of the upper phase. The analysis by TEM of the re-suspended solid revealed that it was mainly constituted by large size particles.

The distribution of the reagents in both phases is related with the total volume of solution, the volume of hexanol and the amount of surfactant used. The reduction of the water volume from 11.5 ml to 5 ml, keeping constant the amount of the other components, resulted in the no appearance of NPs after breaking the microemulsion, which would be ascribed to the change of the effective reagent concentrations.

The influence of water amount was studied by keeping constant the total mixture volume, and hence, the final concentrations of the synthesis components. Water volume was decreased from 12.5 ml to 7.3 ml, increasing hexanol volumes from 1.8 ml to 7 ml. The number of NPs decreased and their sizes were higher than 200 nm when the hexanol volume was higher than 3 ml, choosing this value as optimum.

The influence of the amount of Triton X-100 (Fig. 2) showed that the number of nile blue-doped NPs increases and their size decreases as Triton X-100 amount increases. The best results were obtained using 510–520 mg of Triton X-100 (Fig. 1f), because lower amounts gave NPs of sizes higher than 200 nm (Fig. 1d and e). Very small and partially aggregated nanoparticles were obtained when the Triton X-100 amount was close to 1.0 g. It was also checked that the Triton X-100/hexanol ratio is a critical variable, as Fig. 2 shows. As it can be seen, a ratio of 170 mg Triton X-100/ml hexanol gave the maximum fluorescence intensity of the

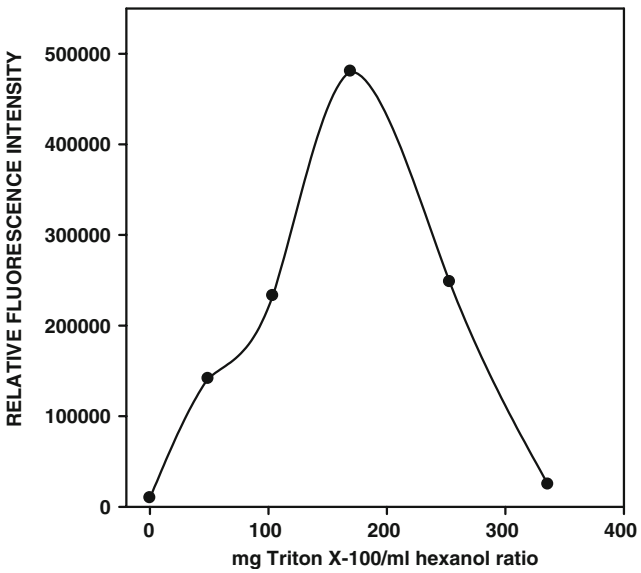

Fig. 2 Influence of Triton X-100/hexanol ratio on the fluorescence intensity of nile blue-doped NPs. Experimental conditions: 11.3 ml (0.63 mol) of water, 100 μl (0.44 mmol) TEOS, 0.2 ml of 1×10^{-3} M (0.2 μmol) nile blue, 3 ml (0.024 mol) hexanol, 70 μl (0.9 mmol) NH_4OH

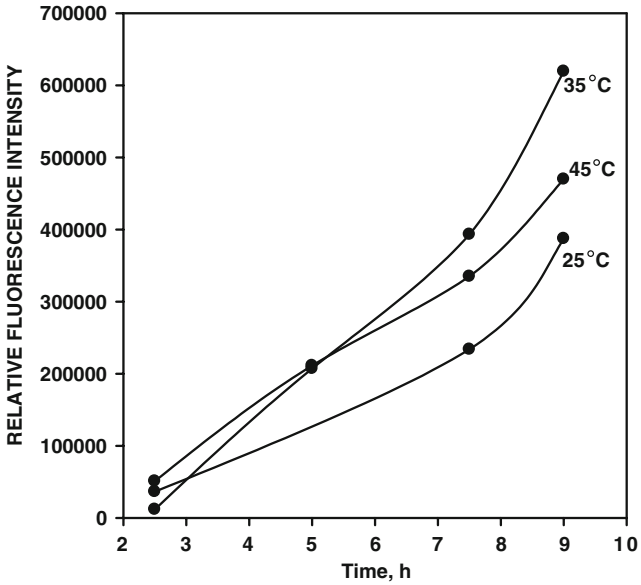

Fig. 3 Influence of reaction time and temperature on the fluorescence intensity of nile blue-doped NPs. Experimental conditions: 510–520 mg (0.79–0.81 mmol) of Triton X-100, 11.3 ml (0.63 mol) of water, 100 μl (0.44 mmol) TEOS, 0.2 ml of 1×10^{-3} M (0.2 μmol) nile blue, 3 ml (0.024 mol) hexanol, 70 μl (0.9 mmol) NH_4OH

synthesized material. The amount of the surfactant used is relatively high, but is in agreement with the fact that the critical microemulsion concentration of a non-ionic surfactant in water-in-oil microemulsions is higher [10] than the critical micelle concentration of the same surfactant in water.

The fluorescence intensity and the size of the NPs were evaluated modifying the temperature and the reaction time. Figure 3 shows the influence of these variables in the fluorescence intensity of nile blue-doped NPs, finding that the fluorescence increased from 25 to 35 °C, but it decreased at 45 °C. Although the fluorescence intensity was higher after 9 h of reaction, the size of the NPs obtained also increased so, a time of 7.5 h was chosen as optimum. The NP sizes slightly decreased with increasing temperatures in the range of 25–45 °C. This study was also performed for cresyl violet doped-NPs finding similar results.

The features of nile blue-doped NPs obtained after a reaction time of 7.5 h and at 35 °C are compared in Table 1 with the features of similar NPs obtained after 24 h at 25 °C. Some parameters were calculated in a similar way as described elsewhere [19] using 200 μl of 0.1 mg/ml NP dispersions in each well. The number of NPs was obtained by dividing the amount of NPs into the weight of one particle, which was calculated from the volume of one particle (assuming that it is a perfect sphere and expressing the volume in mm^3) and multiplying this volume by the specific gravity of silica (2.3). The intensity/particle ratio is defined as the fluorescence intensity divided by particle amount, and the specific intensity is the intensity/particle

Table 1 Comparison of the properties of nile blue-doped silica nanoparticles obtained at two reaction time and temperature values by the modified reverse-micelle microemulsion method

Conditions	24 h, 25 °C	7.5 h, 35 °C
Concentration	0.1 mg/ml	0.1 mg/ml
Amount (mg)	0.02	0.02
Mean diameter, nm	323	170
Particle volume, mm^3	1.76×10^{-11}	2.57×10^{-12}
Particle number	4.92×10^{8}	3.38×10^{9}
Intensity[a]	180927	111708
Intensity/particle ratio	3.67×10^{-4}	3.3×10^{-5}
Specific intensity	2.09×10^{7}	1.29×10^{7}

[a] Measurements performed in microplates (200 μl) using excitation and emission filters of 620/8 and 680/10 nm, respectively

ratio divided by the volume of one particle. An increase in the synthesis time yields larger NPs, which are more luminescent, since the specific intensity, which is independent on the particle volume, is about 1.6 times higher than that of NPs obtained at 7.5 h and 35 °C.

The influence of ammonium hydroxide was studied in the range of 35–140 μl of a commercial concentrated solution (0.45–1.8 mmol). The number of nile blue-NPs formed was scarce at the limits of the assayed interval, being also the fluorescence intensity obtained quite low (Fig. 4a). The size of the NPs obtained increased in the range of 70–610 nm of diameter as NH_4OH was increased. The decrease in the fluorescence intensity at high NH_4OH values would be ascribed to the partial degradation of the fluorofore in the alkaline medium, as it has been reported elsewhere for a cyanine dye [20]. The volume chosen as optimum was 70 μl (0.9 mmol).

The influence of TEOS was evaluated for nile blue NPs in the range of 50–800 μl (0.22–3.52 mmol). Increasing TEOS volumes, the size of NPs increased from 140 to 300 nm, obtaining sizes larger than 200 nm above 100 μl. These results were obtained under the optimal experimental conditions, excepting for the fluorophore amount, which was ten times lower than the optimum value. As it was found that the fluorescence intensity also increased with the TEOS amount, a compromised solution was adopted and 100 μl (0.44 mmol) of TEOS was chosen as the optimum value to obtain intermediate sizes and intensities.

The amount of fluorophore used is a critical variable. The volume of 1×10^{-3} M aqueous nile blue solution was varied from 100 μl to 7.2 ml by adjusting the total volume of the aqueous phase to 11.5 ml with distilled water, in order to keep constant the initial concentrations of the other reagents. The influence of this variable on the fluorescence intensity is shown in Fig. 4b, in which can be seen that the maximum value was obtained using 1.8 μmol of nile blue. The decrease in the fluorescence intensity at higher values is ascribed to the appearance of a coloured precipitate in the tube bottom after the centrifugation process, which was mainly formed by large size particles. The formation of fluorophore aggregates can also contribute to this behaviour as they are less fluorescent than their monomers [6]. The fluorophore concentration also affects the size of the NPs, which decreases until the amount of nile blue is 0.43 μmol, from which their diameter remains constant, with a mean value of about 170 nm. A similar behaviour was observed for cresyl violet-doped NPs.

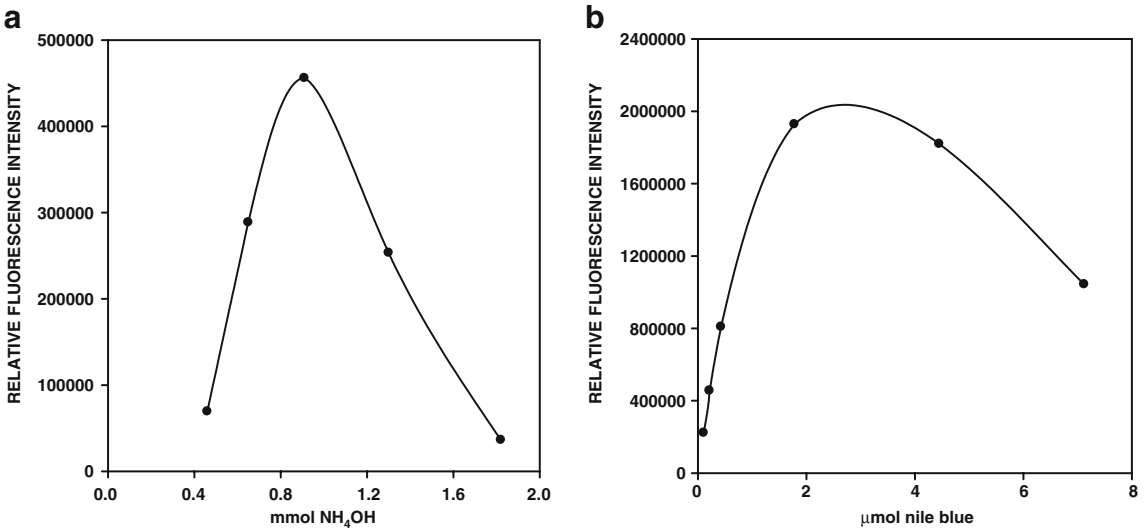

Fig. 4 Influence of the amount of ammonium hydroxide (**a**) and nile blue (**b**) added on the fluorescence intensity of nile blue-doped NPs. Experimental conditions: 510–520 mg (0.79–0.81 mmol) of Triton X-100, 11.3 ml (0.63 mol) of water, 100 μl (0.44 mmol) TEOS, 3 ml (0.024 mol) hexanol. In Fig 4a 0.2 ml of 1×10^{-3} M (0.2 μmol) nile blue, in Fig. 4b 70 μl (0.9 mmol) of NH_4OH

The synthesis of precursors involving the covalent linking of the primary amino groups of the fluorophore and the silane derivative APS was assayed to obtain NPs less susceptible to dye leakage. It was found that the use of equimolar ratios of nile blue and glutaraldehyde, and APS in an 8.5-fold molar excess, gave the best results in terms of fluorescence intensity of the NPs obtained. The time of reaction of the mixture was studied in the interval of 0–10 min, and 5 min were found to be enough, since larger irregular particles were obtained at longer reaction times. This mixture was used for the NP synthesis without any further purification step. The influence in the fluorescence intensity of the precursor volume added to the synthesis procedure was studied in the range of 0.6–2.4 ml, providing 1.2 ml of precursor the highest value. The mean size of the NPs synthesized by using 1.2 ml of precursor mixture was 150 nm, decreasing the size at higher volumes. Figure 1.g shows the aspect of the NPs obtained using 1.2 ml of precursor, which are not completely spherical, although they have a uniform size with a relative standard deviation around 10%.

Optimization of the synthesis by the Stöber method

The approach used was similar to previously described methods for the encapsulation of a fluorescein-silane precursor and a ruthenium chelate [19, 21], which encompassed the use of an ethanol:water mixture as the medium for the hydrolysis and condensation of silane reagent. The application of this method has been carried out using cresyl violet as fluorophore, with the aim of comparing the features of the NPs obtained with those of the NPs obtained with the above described method.

Ethanol volume was assayed in the range of 10–25 ml (0.17–0.43 mol), obtaining the best fluorescence intensity values with the highest ethanol amount. This would be ascribed to the increase in the effective fluorophore concentration at low volumes of ethanol, which gives rise to the formation of the non fluorescent fluorophore dimers above mentioned.

The influence of water was studied in the range of 0.5–25 ml, yielding sizes below 180 nm, which increased (300–350 nm) when the water volume was 5–10 ml. This behaviour could be explained bearing in mind that the TEOS hydrolysis, which would be the limiting step at low water volumes, should be favoured by an increase in water volumes leading to faster NP synthesis and larger NP sizes. The use of 25 ml of water provided a lower amount of NPs, owing to the dilution of the reagents involved in the synthesis.

The optimization of the fluorophore amount, performed from 0.5 to 10 ml of 1×10^{-3} M (0.5–10 μmol) cresyl violet at a fixed water volume of 0.5 ml, showed that the best results were obtained for 0.5 μmol of fluorophore. The influence of TEOS was assayed in the range of 0.1–10 ml finding that NPs obtained at low TEOS volumes (0.1–2 ml) were practically monodisperse whereas those synthesized at higher TEOS volumes (5–10 ml) were quite irregular in shape and in size distribution.

Table 2 shows the comparison of the cresyl violet-doped silica NPs obtained by the modified reverse-micelle microemulsion and the Stöber methods. The excitation filter used for cresyl violet was 531/25 nm, which is an excitation wavelength shorter than the maximum, in order to minimize scattering phenomena. The specific intensity of the NPs obtained by the modified microemulsion method was about 12 times higher than that obtained by the Stöber procedure. This would be ascribed to the fact that cresyl violet is less degraded by the alkaline conditions owing to the protection that micelles can confer.

Fluorescence spectra of cresyl violet- and nile blue- silica NPs

The spectral features of the synthesized NPs were compared to those showed by pure dyes and by bare silica NPs. Figure 5 shows the emission spectra obtained at the maximum excitation wavelength of each fluorophore, in which can be seen that there is not appreciable fluorescent signal from bare silica NPs. The emission bands of the oxazine-doped silica NPs show a slight shift of approximately 9 nm towards shorter wavelengths. This behaviour is in accordance to the results obtained after encapsulating other fluorophores, such as fluorescein isothiocyanate or

Table 2 Comparison of the properties of cresyl violet-doped NPs obtained by the proposed reverse-micelle microemulsion and Stöber methods

	Reverse micelle microemulsion method	Stöber method
Concentration, mg/ml	0.1	0.1
Particle amount, mg	0.02	0.02
Diameter, nm	178	133
Particle number	2.94×10^{9}	7.06×10^{9}
Intensity[a]	125563	10138
Intensity/particle ratio	4.27×10^{-5}	1.44×10^{-6}
Specific intensity	1.45×10^{7}	1.17×10^{6}

[a] Measurements were performed using microplates (200 μl) and excitation and emission filters of 531/25 and 620/8 nm, respectively

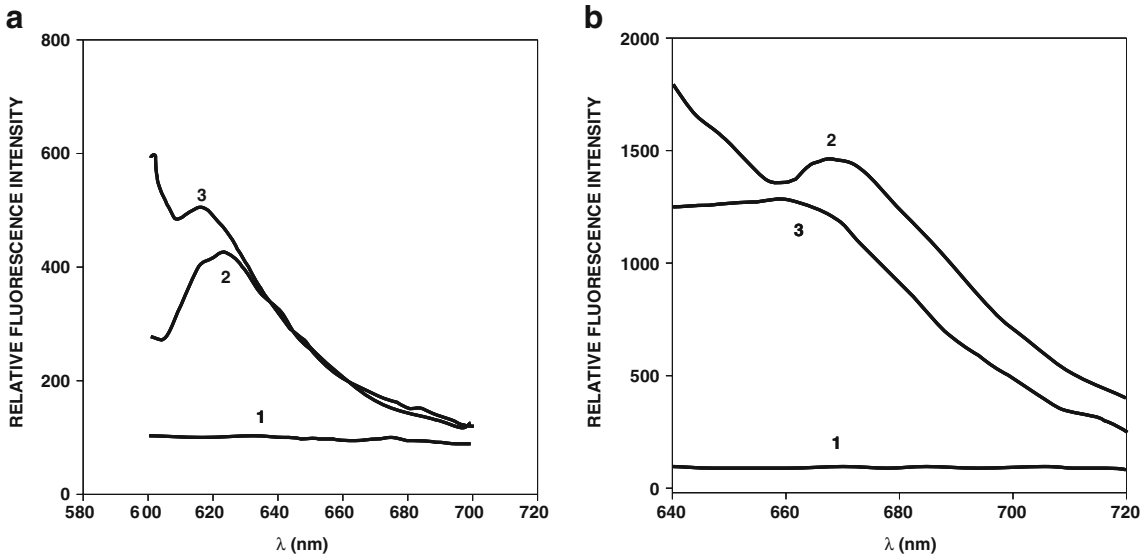

Fig. 5 Emission spectra in distilled water of: bare silica NPs (**a**1, **b**1), 0.07 μM pure cresyl violet (**a**2) and 0.5 μM nile blue (**b**2) dye solutions, and cresyl violet- (**a**3) and nile blue-doped (**b**3) NPs, respectively

ruthenium dyes [6], which experienced shifts towards shorter and longer wavelengths, respectively. This fact could be ascribed to the interaction of the dyes, which are cationic, with the negatively charged silanol groups from the silica matrix. Figure 5 also shows that the emission spectrum for nile blue-APS-doped NPs is practically the same as that obtained for nile blue-doped silica NPs.

Fluorescence stability of NPs

The suspensions of cresyl violet and nile blue NPs were divided in five aliquots of 1 ml each, which were stored at 4 °C. At the assay time, NPs were washed, centrifuged and re-dispersed in water. The fluorescence intensity was measured the same day that NPs were synthesized and 1, 2, 5 and 9 days later, using in all instances the same number of washes to each aliquot (three times with 1 ml of ethanol and four times with distilled water), until supernatant fluorescence close to blank signal. Under these conditions, the fluorescence intensity remained almost constant for at least 9 days without appreciable changes in the fluorescence intensity caused by dye leakage. The fluorescence intensity from nile blue- and nile blue-APS-doped NPs decreased after the seven washes, being the decrease of the

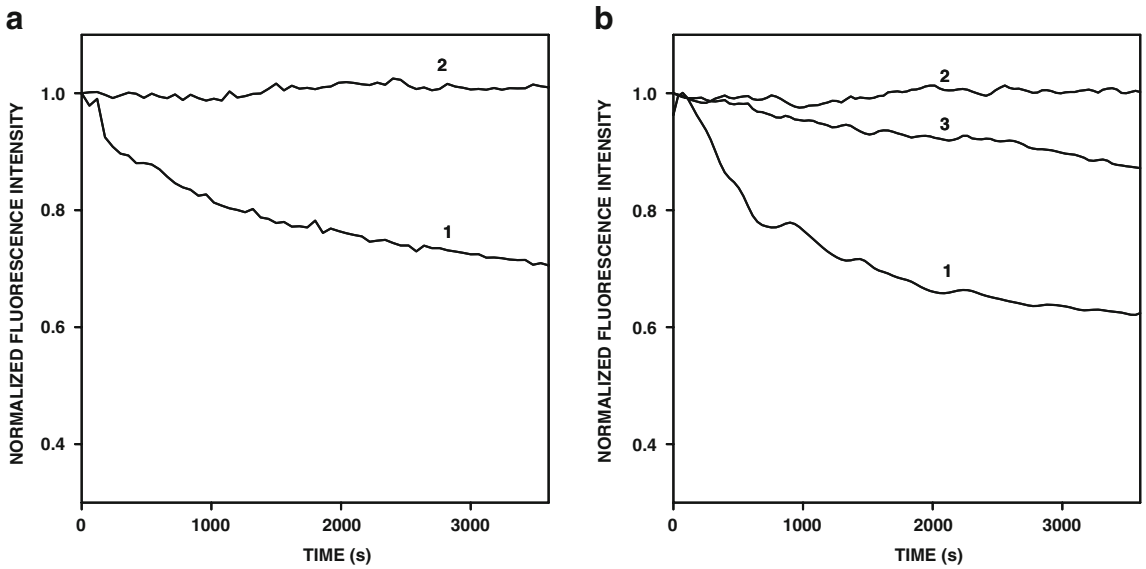

Fig. 6 Photobleaching experiments for pure cresyl violet (**a**1) and nile blue (**b**1) solutions, respectively; cresyl violet (**a**2) and nile blue-doped (**b**2) NPs, respectively; and nile blue-APS-doped NPs (**b**3)

first NPs two fold that of the second ones, which would be ascribed to the covalent binding of the fluorophore to the silane reagent, which minimizes dye leakage.

Once the NPs were totally purified (supernatant fluorescence close to blank signal), they were subjected to additional subsequent washes with 1 ml of distilled water to study the potential dye leakage from the core of NPs, finding that the fluorescence of both nile blue- and nile blue-APS-doped NPs remained almost constant. The stability of nile blue-doped NPs could be ascribed to the abovementioned fact that nile blue is a cationic dye and would interact with the negative silanol groups from silica matrix. In this way, dye leakage would be less favoured than for anionic dyes, such as Cy5, which are more prone to repulsion forces with the silanol groups from silica matrix [12].

Photostability experiments

To investigate the potential photobleaching of the synthesized NPs in aqueous solution, they were irradiated for 1 h and the results were compared to those obtained for pure dye solutions (Fig. 6). The intensity of pure cresyl violet (Fig. 6a, curve 1) and nile blue (Fig. 6b, curve 1) dropped until the 72% and the 65%, respectively, of the initial intensity. However, the fluorescence intensity of both cresyl violet NPs (Fig. 6a, curve 2) and nile blue-doped NPs (Fig. 6b curve 2) remained almost constant and the NPs prepared by using the nile blue-APS precursor (Fig. 6b, curve 3) experienced a decrease by a 10% of the initial intensity. Similar results have been reported in recently synthesized NPs containing Cy5 [12] and fluorescein [19], which confirm the increased photostability of encapsulated dyes due to the protection that silica matrix confers them.

Conclusions

The work presented here reports the synthesis of cresyl violet- and nile blue-doped silica NPs for the first time. The emission at long wavelengths of these NPs is a useful option to avoid the potential interferences of static background signals from sample matrix. The systematic study of the experimental variables involved in the synthesis process has given rise to the achievement of NPs with homogeneous size and high and stable fluorescence intensity. The modified reverse microemulsion method proposed precludes the use of cyclohexane as organic solvent, which is replaced by 1-hexanol. The use of the oxazine dye and the APS precursor to obtain the NPs has shown that the second one reduces dye leakage in the purification process. The comparison of the luminescent properties of cresyl violet-doped NPs synthesized by microemulsion and Stöber methods has shown the usefulness of the first one to obtain more luminescent NPs.

The increased photostability of the synthesized NPs with respect to the pure dye proves the succesful dye encapsulation and the protection that silica matrix confers them. The features of the synthesized NPs seem to make them suitable as analytical reagents in fluoroimmunoassays after their functionalization, which is currently under research.

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References

1. Gómez-Hens A, Fernández-Romero JM, Aguilar-Caballos MP (2008) Nanostructures as analytical tools in bioassays. Trends Anal Chem 27(5):394–406
2. Yan J, Estévez MC, Smith JE, Wang K, Ho X, Wang L, Tan W (2007) Dye-doped nanoparticles for bioanalysis. Nanotoday 2 (3):44–50
3. Seydack M (2005) Nanoparticles labels in immunosensing using optical detection methods. Biosens Bioelectron 20(12):2454–2469
4. Tansil NC, Gao Z (2006) Nanoparticles in biomolecular detection. Nanotoday 1(1):28–37
5. Liu G, Lin Y (2007) Nanomaterial labels in electrochemical immunosensors and immunoassays. Talanta 74(3):308–317
6. Yao G, Wang L, Wu G, Smith J, Xu J, Zhao W, Lee E, Tan W (2006) FloDots: luminescent nanoparticles. Anal Bioanal Chem 385(3):518–524
7. Wang F, Tan WB, Zhang Y, Fan X, Wang M (2006) Luminescent nanomaterials for biological labelling. Nanotechnology 17(1):R1–R13
8. Wiwanitkit V, Seereemaspun A, Rojanathanes RJ (2007) Increasing the agglutination reaction in slide test for weak B blood group by gold nanoparticle solution: the first world report. J Immunol Methods 328(1–2):201–203
9. Du B, Li Z, Cheng Y (2008) Homogeneous immunoassay based on aggregation of antibody-functionalized gold nanoparticles coupled with light scattering detection. Talanta 75(4):959–964
10. Arriagada FJ, Osseo-Asare K (1999) Synthesis of nanosize silica in a nonionic water-in-oil microemulsion: effects on the water/surfactant molar ratio and ammonia concentration. J Colloid Interface Sci 211(2):210–220
11. Zhao X, Bagwa RP, Tan W (2004) Development of organic-dye-doped silica nanoparticles in a reverse microemulsion. Adv Mater 16(2):173–176
12. He X, Chen J, Wang K, Qin D, Tan W (2007) Preparation of luminescent Cy5 doped core-shell SFNPs and its application as a near-infrared fluorescent marker. Talanta 72(4):1519–1526
13. Chen XL, Zou JL, Zhao TT, Li ZB (2007) Preparation and fluoroimmunoassay application of new red-region fluorescent silica nanoparticles. J Fluoresc 17(2):235–241
14. Yang W, Zhang CG, Qu HY, Yang HH, Xu JG (2004) Novel fluorescent silica nanoparticle probe for ultrasensitive immunoassays. Anal Chim Acta 503(2):163–169
15. Hu SR, Liu JM, Yang TL, Lin HZ, Huang JL, Lin QW, Zhu GH, Huang XM (2005) Determination of human alpha-fetoprotein (AFP) by solid substrate room temperature phosphorescence

enzyme-linked immune response using luminescent nanoparticles. Microchim Acta 152(1–2):53–59
16. Gómez-Hens A, Aguilar-Caballos MP (2004) Long-wavelength fluorophores: new trends in their analytical use. Trends Anal Chem 23(2):127–136
17. Hemmilä I, Laitala V (2005) Progress in lanthanides as luminescent probes. J Fluoresc 15(4):529–542
18. Gómez-Hens A, Aguilar-Caballos MP (2002) Terbium-sensitized luminescence: a selective and versatile analytical approach. Trends Anal Chem 21(2):131–141
19. Nakamura N, Shono M, Ishimura K (2007) Synthesis, characterization, and biological applications of multifluorescent silica nanoparticles. Anal Chem 79(17):6507–6514
20. Bringley JF, Penner TL, Wang R, Harder JF, Harrison WJ, Buonemani L (2008) Silica nanoparticles encapsulating near-infrared emissive cyanine dyes. J Colloid Interf Sci 320(1):132–139
21. Rossi LM, Shi L, Quina FH, Rosenzweig Z (2005) Stöber synthesis of monodispersed luminescent silica nanoparticles for bioanalytical assays. Langmuir 21(10):4277–4280