Fluorescent labelled SiO_2 nanoparticles as tracers in natural waters. Dependence of detection limits with environmental conditions

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The identification of engineered nanomaterials (ENM) in the environment is of uttermost importance, in view of the possibility of harmful effects in living organisms. While it is clear that monitoring their presence and accumulation is essential in any risk assessment scenario, this is a challenging task: On the one hand, ENM are present at trace concentration levels, requiring extremely sensitive sampling and analysis methods. On the other, specific identification of ENM is further complicated by the simultaneous presence of other nanomaterials (often analogous in terms of size and properties) already present in the environment. Therefore, the development of labels that allow unequivocal, highly sensitive identification of specific nanomaterials is desirable. Here we report on the development of stable fluorescent labels for silica (SiO_2) nanoparticles. The markers developed allow monitoring their presence in environmentally relevant media at low detection levels. Identification of labelling signals has been performed using both online and offline techniques in a variety of conditions.

Introduction

Engineered materials manufactured at the nanoscale have the potential to improve quality of life, providing benefits to the environment and enabling societal advances [1]. Some of the engineered nanomaterials (ENM) that are currently attracting attention, in terms of their wide technological applicability include nanosilver, carbon nanotubes, cerium dioxide, silica, titanium dioxide and zinc oxide [2]. These are still produced and marketed in smaller quantities than the traditional materials, but their use is increasing extremely fast. The global market of nanotechnology products was estimated at $731 billion ($7.31·10^9) in 2012, and projected to grow over $4 trillion ($4·10^{12}) by 2018 [3].

It is widely expected that nanomaterials will be a key tool in preserving the natural environment through applications aimed to destroy or remove hazardous chemicals and by enabling cleaner production technologies. Indeed, remediation technologies stand to benefit strongly from nanotechnology [4-7]. However, it is still not yet clear what are the potential impacts of nanosized matter in the air, water and soils. The question is still whether ENM will leave an environmental heritage and in which way this potential legacy should be tackled [8,9]. Although the cumulative amount of released ENM into the environment is very small compared to conventional chemicals, their release rate will noticeably grow as the production and applications of nanomaterials increase [10,11].

It is a matter of fact that nanosized matter is ubiquitous in the air we breathe, both in research and industrial locations [12,13] and also in natural environments [14,15]. Environmental nanoparticles, such as silica or carbon-based nanoparticles may be generated through natural processes (such as attrition, volcano eruptions or wild fires [16]). These nanoparticles may also have an anthropogenic origin, and are being released in the context of common daily life activities, such as transport or combustion processes. Metal nanoparticles may also be generated incidentally, through the use of metal utensils, soldering operations or the use of electric engines [17-19]. Of special concern are emerging ENM, new compositions or structures to which there has been scarce or null previous human exposure. Indeed, many toxicity studies are underway using these materials under controlled laboratory conditions [20-22]. However, the relevance for practical purposes of many of the nanotoxicity studies published has been seriously questioned [23].

While the discussion on how to best conduct studies of the toxicity or ENMs will take time to settle, there is a clear need to assess the levels of exposure by monitoring their presence in the environment with which we are in contact (air, water or solid surfaces for instance) and, whenever possible, their evolution and fate [24]. Indeed, a variety of instrumental techniques have been used for this challenging task [25]. However, detection and characterization have been limited, as no single technique or method is suitable to identify and quantify ENMs in environmental samples [26]. Thus for instance, a high-resolution transmission electron microscope

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would be perfectly suited to characterize the size, shape and composition of even a single nanoparticle in a sample of environmental particulate matter. However, for this analysis to be effective, the nanoparticle to be identified has to be located on the path of the electron beam. Since usually the existing proportion of ENMs in relation to background nanosized matter is extremely low, the probability of finding the desired nanoparticle within the observation window would be close to zero. It is obvious that the use of these techniques would involve impossibly long observation times, and therefore can be excluded for practical exposure assessment.

In view of these difficulties, it would be highly desirable to include identification features in the design of ENMs, in such a way that they can be detected at low concentrations and discriminated from environmental ENMs. A strong interest has emerged on the developing of labelling techniques using specific tags that are not likely to be present in the sampling media. For instance, fluorescent dyes [27], radioactive tracers [28-30], stable isotopes [31, 32], lanthanide elements [33] or even the intrinsic catalytic activity [34] have been proposed for the labelling of nanoparticles in environmental health and safety applications.

A very important aspect that is often overlooked in labelling refers to the use of marked nanoparticles for specific purposes (*e.g.* cell trafficking or monitoring of nanoparticle aerosols). In this case it should be taken into account that the modification of the substrates by different markers may lead to different behaviour of the nanomaterials (see for instance [31]) and the label selected might interfere with the aspect to be studied. Thus for instance, surface modifications such as the grafting of fluorescent molecules on the outside surface of nanoparticles would be unsuitable for monitoring of nanoparticle aerosols, since aspects such as surface charge and aggregation behaviour would be directly affected. Furthermore, there are other labelling methods, such as radioactive tracing that might raise concerns about their release in both indoor settings and in the environment.

In this work we attempt to develop highly sensitive labelled silica nanoparticles as tracers for the release of ENMs in aquatic environments at very low concentrations. The objective is to identify the potential impact of these ENMs at long exposure times, which requires very stable fluorescent nanomaterials in different environments. To this end, we have designed an adequate procedure to incorporate and stabilizeinnocuous [35] fluorescent molecules in70-nmSiO_2 nanoparticles. We have determined the detection limits of the fluorescent-labelled nanoparticles in different water environments, and studied their stability under different conditions of acidity, O_2 concentration and temperature.

Experimental Section

Materials synthesis and characterization

Chemicals. Tetraethyl orthosilicate (TEOS, 98%, Aldrich) and ethanol (EtOH, 99%, Sigma) were used as received. Milli-Q grade water (Millipore, Billerica MA) and ammonium hydroxide (NH_4OH, 25-28% solution in water, Aldrich) were used as reagents for the hydrolysis of silicate precursors. The fluorescent labelwasTris(1,10-phenanthroline) ruthenium (II) chloride hydrate ($Ru(phen)_3Cl_2{\cdot}H_2O$, 98%, Aldrich), which was used without previous purification.

Synthesis procedure. The synthesis methods for fluorescent silica nanoparticles have been adequately adapted from the literature dealing with materials for bioimaging. These were attractive because avoiding interference with biological systems often means that the concentration of fluorescent groups on the surface has to be minimized. Briefly, $Ru(phen)_3$:SiO_2 nanoparticles were synthesized using a sol-gel process reported elsewhere [36,37]. In a 50-mL flask, 23 mL of absolute ethanol, 1.5 mL of NH_4OH solution and 0.5 mL of an aqueous solution (10 mg/mL) of $Ru(Phen)_3Cl_2$ were stirred in darkness. After 10 min, 5 mL of a 2:3 TEOS:EtOH mixture was added and kept under stirring for 1h. The obtained suspension was subjected at ultrasonic stirring for 10 min, followed by several cycles of centrifugation (10000 rpm, 10 min), washing in EtOH and sonication (80 W, 2 min). The $Ru(phen)_3$:SiO_2 nanoparticles and supernatant phases were stored in dark.

Characterization. The morphology of the synthesized nanoparticles was assessed by means of electron microscopy techniques. Transmission electron microscopy (TEM) images were taken in a Tecnai T20 (FEI Co, Hillsboro OR) electron microscope at a 200 kV. Particle size distributions were obtained from statistical analysis of TEM images using the ImageJ processing software with a number of measured particles (N) more than 75 in every image. Scanning Electron Microscopy (SEM) images were obtain in a FEI Inspect Field Emission Gun microscope. Dynamic light scattering (DLS) measurements were performed using a Brookhaven 90Plus instrument to determine the hydrodynamic diameter and ζ-potential of the fluorescent-labelled nanoparticles in water suspension. XPS was used to confirm the presence of ruthenium in the $Ru(phen)_3$:SiO_2 nanoparticles.

Fluorescent labelling and quantification in aqueous media

Spectrophotometry. The fluorescence emission of $Ru(phen)_3$:SiO_2 nanoparticles was analysed in a Perkin-Elmer spectrophotometer. The absorption and emission wavelengths were 448 and 595 nm for $Ru(phen)_3$:SiO_2 at 25ºC. The scan speed was set at 100 nm/min with a 7.5 nm grid monochromator. Samples were dispersed in different aquatic media to test the influence of the environment in the fluorescent emission and the limit of detection. The selected media were Milli-Q water (Millipore, Billerica MA), equivalent to Grade 1 water (ISO 3696:1987), tap water from Zaragoza municipal water grid, Ebro river water (extracted from Station 0507 of the Canal Imperial at Zaragoza, Aragon, Spain) and Atlantic Ocean seawater (extracted from Zarautz seashore, Basque Country, Spain). To avoid biological evolution, these latter media were immediately frozen upon extraction and thawed just before the addition of labelled nanoparticles. Finally, to test the influence of silicate particles in the water environment, a 10-ppm suspension of Stöber SiO_2 particles without fluorescent labels (DLS particle size 100 ± 5 nm) in Milli-

Q water was also used as medium of dispersing labelled nanoparticles. The physicochemical characteristics of the tested media are shown in Table 1.

Limits of detection. Fluorescent-labelled nanoparticles were dispersed in the different media at a starting concentration of 10 ppm, which was sequentially diluted down to 5 ppm, 1 ppm, 500 ppb, 100 ppb, 50 ppb, 10 ppb, 5 ppb and 1 ppb. Blank spectra were recorded using Milli-Q water in the same conditions as described for labelled nanoparticles in the dispersion media. Spectra of the suspended samples were sequentially recorded in the fluorescence spectrometer at the above-cited excitation and emission wavelength for every labelled material.Fluorescence spectra of the suspensions of $Ru(phen)_3$:SiO_2 nanoparticles in every aqueous medium were taken at 25ºC. Samples were dispersed in three similar flasks at the same concentration and every sample was measured three times in the fluorescence spectrometer. This measurement scheme provided nine values of fluorescence intensity per sample and nanoparticle concentration and per dispersion media. The intensity of the emission peak at every tested wavelength was plotted against the concentration in every dispersion media and subjected to linear least squares regression. Fitting parameters were calculated with a 95% of confidence level ($\alpha = 0.05$).

Results and discussion

Labelling and analysis of nanomaterial stability

The fluorescent labelled $Ru(phen)_3$:SiO_2nanoparticles showed a round shape with mean size 68.7 ± 11 nm (see TEM pictures in Figure 1 and also SEM pictures in ESI). The SiO_2 based materials showed fluorescent emission spectra at 595 nm (Figure 2), which translated into reddish coloured nanoparticles.

Table 1. Characterization of the environmentally relevant media for fluorescent-labelled nanoparticles used in this study (data ± σ)

	Milli-Q	SiO_2	Tap	Canal	Sea
Turbidity[(a)] (NTU)	0.01 ± 0.01	4.5± 0.2	0.2± 0.1	153 ± 2	2.3± 0.1
[O_2] (ppm)	4.9± 0.2	4.8± 0.2	4.9± 0.3	5.1 ± 0.3	5.0 ± 0.2
O_2Saturation (%)	68± 3	67± 2	67± 2	68± 3	67± 2
Conductivity (20ºC; µS/cm)	19.1 ± 0.9	21 ± 1	572± 29	748± 37	(29±2)·10^3
pH (20ºC)[b]	6.8± 0.1	6.7± 0.1	8.3± 0.1	8.3± 0.1	8.2± 0.1
Dry residue (mg/mL)	0.026 ± 0.003	0.053 ± 0.005	0.52± 0.01	0.84 ± 0.02	33.5± 0.1

(a)Determined by nephelometry at 20ºC and expressed in nephelometric turbidity units (NTU); (b) The measurement of pH in Milli-Q water was carried out adding 1 mL of a 10-2 M NaCl solution in 20 mL of final suspension volume.

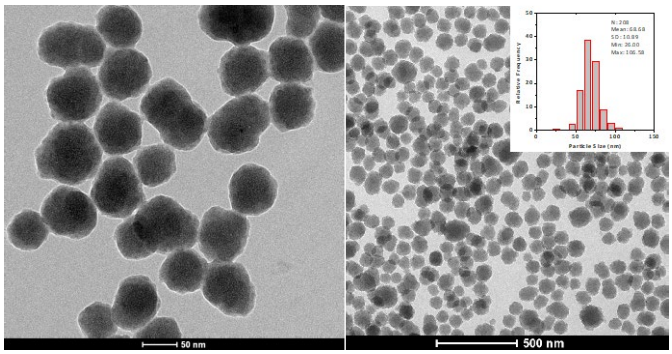

Figure 1. TEM images of $Ru(phen)_2$-labelled SiO_2 nanoparticles. Inset shows the particle size distribution obtained from TEM images with N = 208 particles

The fluorescent emission of the $Ru(phen)_3$-labelled nanoparticles showed a noticeable dependence on the environment temperature (Figure 3a). The emission decay was estimated as the ratio of the emission intensity of the nanoparticle suspension after every elapsed time of storage, $I(t_e)$, to the emission intensity upon stabilization of the nanoparticle suspension, I_0.Results showed anintensity loss of about 50% during the initial 24 h of storage at all tested temperatures. Intensity loss was constant afterwards up to 240 h of storage. The storage at low temperatures (4ºC) induced lower emission decay than those observed at 25º and 40ºC, where a loss ofabout 60% was detected. This decay could be attributed to the increased diffusion of fluorescent labels trapped into the SiO_2 bulk phase when increasing the medium temperature.

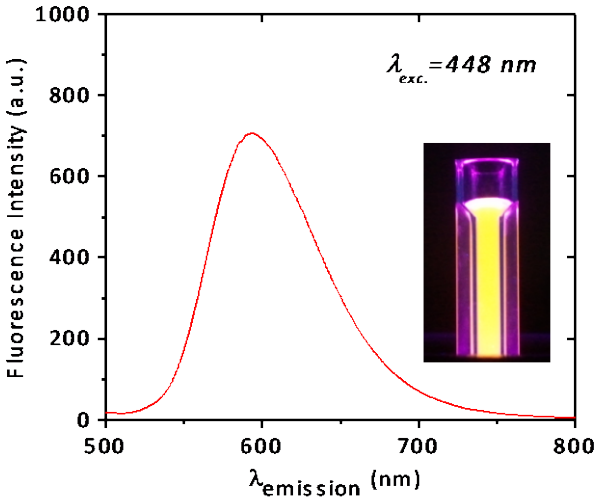

Figure 2. Typical fluorescence emission spectra of $Ru(phen)_2$-labelled SiO_2 nanoparticles in aqueous dispersion at 10 ppm. Emission was excited by laser irradiation at 448 nm. Inset shows an image of the 10-ppm suspension of $Ru(phen)_2$:SiO_2 nanoparticles under spectrometer irradiation.

Similar effect has been observed for the dependence on the suspension acidity (Figure 3b). The fluorescent emission presented a constant decay in slightly acidic environments (pH 5) during the initial 24 h of storage, showing a fluorescent emission loss of about 30% of the initial intensity. During the

initial 24 h of storage, the increase in alkalinity exerted more noticeable intensity loss, losing about 65% of the initial intensity when the suspension was buffered at pH 8.

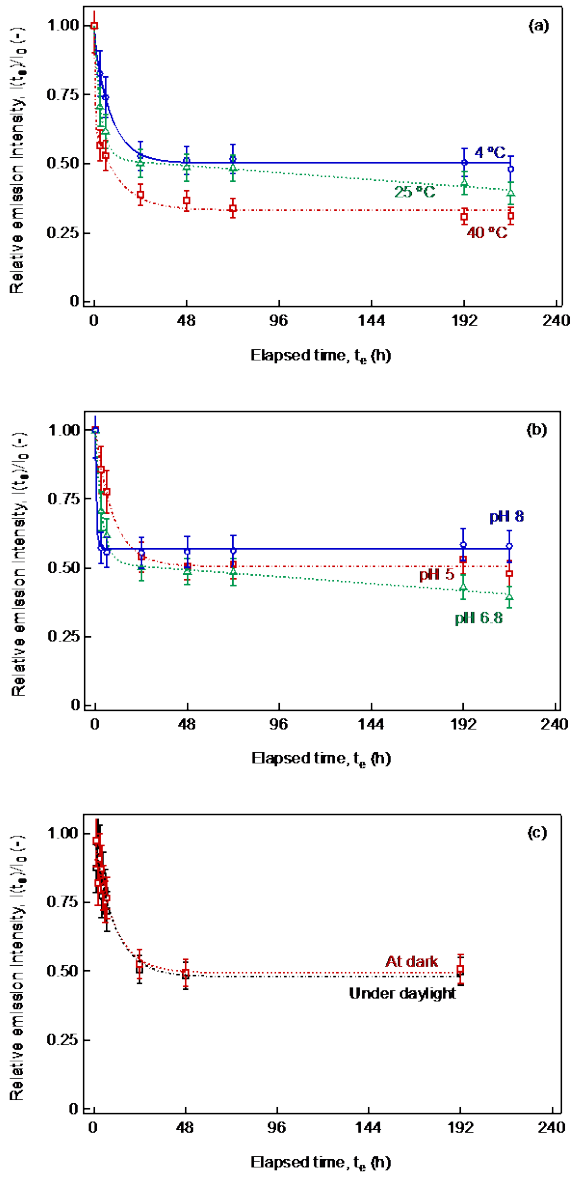

Figure 3.Dependence of the fluorescence emission intensity of a typical10-ppm aqueous suspension of $Ru(phen)_3$:SiO_2 nanoparticles as function of the elapsed time of storage at different temperatures (a) and pH conditions (b), as well as under different illumination environmentsusing a daylight lamp(c). Values were expressed as the intensity ratio of the fluorescent emission of suspensions at the elapsed time of storage, $I(t_e)$,to the emission intensity measured upon nanoparticle stabilization in Milli-Q® water, I_0.Dotted lines were given as visual guide of data and did not imply any decay model.

This feature was attributed to the favoured solubility of the SiO_2 shell in alkaline environments, which might support the release of fluorescent labels out of the labelled nanoparticles. This release reached a maximum around 48 h of immersion, which resulted in a constant emission for particles after that period. The stability under sunlight exposure of both labelled-nanoparticles was studied by monitoring the evolution of the fluorescent signal of particle a 10-ppm suspension in Milli-Q grade water in darkness and under sunlight at 25ºC and pH 6.5 (Figure 3c). The signal intensity showed a maximum loss of about 20% regardless the light conditions during storage. Interestingly, the labelled nanoparticles exhibited a rapid loss in fluorescence during the initial hours of exposure and then remained stable for the rest of the tested period (up to a week of exposure). It was also worth noticing that the intensity loss of a 10-ppm solution of $Ru(phen)_3Cl_2$ revealed a different behaviour at the same conditions. In this latter case, the fluorescent emission loss under darkness was noticeably smaller than under sunlight. This suggested the protective effect of the SiO_2 shell on the $Ru(phen)_3^{2+}$complex cations in the fluorescent nanoparticles and therefore their potential utility for identification purposes.

The fluorescent emission of labelled nanoparticles was stable in diverse environments, which would enable their application as tracers in different conditions. The surface layer of the fluorescent nanoparticles showed a negative electrical charge in almost all the pH range (Figure 4), as it was commonly reported for Stöber SiO_2 with a point of zero charge in the pH range from 1.5 to 4 [38]. At pH between 5 to 7, the labelled SiO_2 nanoparticles showed ζ-potential values under -30 mV, which implies high colloidal stability in environmentally relevant conditions.

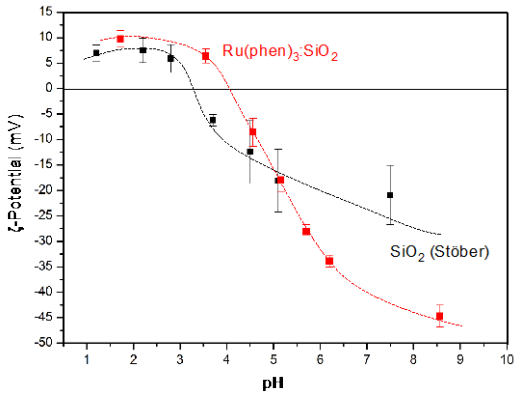

Figure 4. ζ-Potential vs. pH plot of a typical 10-ppm aqueous suspension of $Ru(phen)_3$:SiO_2 nanoparticles showing the negative surface charge beyond pH 4 (point of zero charge, PZC). For comparison purposes, similar plot of a 10-ppm aqueous suspension of unlabelled Stöber-like SiO_2 nanoparticles was included. Negative surface charge was stronger for fluorescent nanoparticles in almost all pH range.

Limits of detection

The limit of detection (c_D) was defined as the analyte concentration with sufficiently high detection probability leading to a correct positive measurement decision of their presence in the environment [39-42].In practical terms, the values of c_D were determined using the calibration data of the intensity of the fluorescence signal at the emission wavelength and the standard error of the blank measurements (the complete derivation of the expression for the calculation of c_D can be found in the ESI file).

$$c_D = \frac{(3.719)s_B}{A}$$

In this expression, s_B was the standard error of the intensity of the blank signal and A was the slope of the calibration curve for the labelled nanoparticles obtained in every aquatic media (data shown in ESI). A confidence level of 95% (α = 0.05) was assumed. It is worth to mention that the values of c_D were only dependent on the analytical technique and independent of the measuring procedure and scale [42]. The obtained values could be consequently used asstandards for the determination of labelled nanoparticles in water media using fluorescence spectrometry.

The labelled nanoparticles could be clearly detected in the aquatic environments considered in this study at enough low concentrations over the blank signal (Figure 5). An increase in fluorescent intensity was noticed as the concentration of the labelled nanoparticles was increased up to 10 ppm in all tested media. The lowest value of analytical sensitivity for $Ru(phen)_3$:SiO_2 (Table 2) was found in Milli-Q water (at 25.9ppb), since this was the clearest environment in which nanoparticles were dispersed. It was interesting to notice here that values of c_D of $Ru(phen)_3$-labelled nanoparticles in natural water media (river and seawater) where low enough to detect their presence at concentrations as low as 287 ppb for theCanal water and 76.5 ppb for seawater, which pointed out the effectiveness of the proposed labelling and identification procedure in natural environments.

The immersion of labelled nanoparticles stored in the dispersion environments for long periods affected to the fluorescent emission in all tested media. An increase in the values of c_D was observed in low-interfering environments (Figure 5and Table 2). When labelled nanoparticles were kept in the river water (Canal) for more than two days, the measured fluorescent emission of suspensions under 10 ppm was nearly constant. This fact affected the linear range of fluorescence used to estimate the c_D in those conditions (see ESI). The overall effect was an effective reduction in the analytical sensitivity of fluorescence for Canal water. Therefore, the values of c_D increased upon immersion for longer periods in testing environments. The reduction in sensitivity of $Ru(phen)_3$:SiO_2 nanoparticles in river waters was attributed to the growth of biological matter in the medium after long immersion periods. This increase in turbiditymasked part of the fluorescent emission of labelled nanoparticles at low concentrations and therefore increased the effective value of c_D in these conditions.

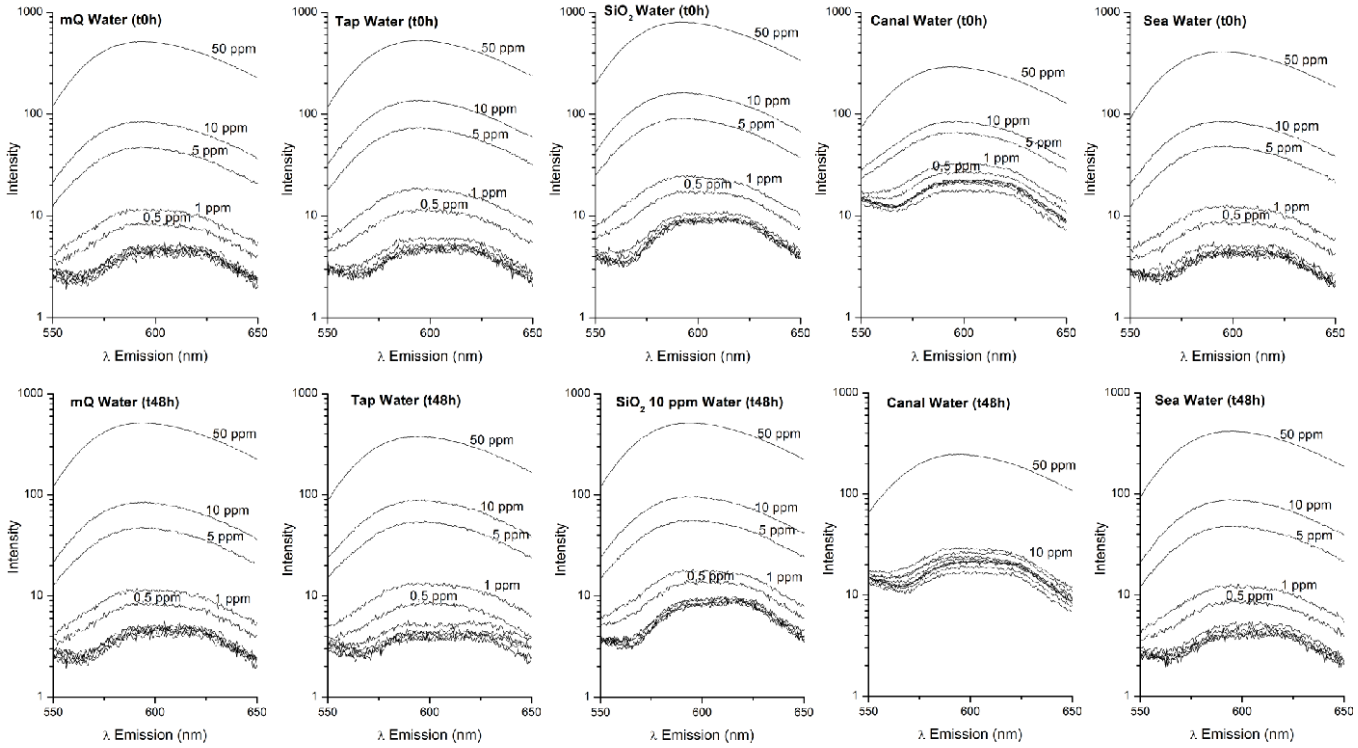

Figure 5. Fluorescence emission spectra in the range of 550 to 650 nm for suspensions of$Ru(phen)_3$:SiO_2 nanoparticles at concentrations from 50 ppm down to 0.01 ppm in the different aquatic environments considered in Table 1. Spectra weretaken at 25ºC, showingthat the fluorescent emission of $Ru(phen)_3$:SiO_2 upon dispersion (t 0h) and after soaking for two days in the media (t 48h) was stable. Onlya small decrease in fluorescencewas observed in all environments except for river water (Canal), where the presence of suspendedbiological matter affectedthestability and fluorescence during the measurement.

Table 2. Limits of detection (c_D) in ppb calculated for fluorescent-labelled $Ru(phen)_3$:SiO_2 nanoparticles in different environment upon dispersion (0h) and after several periods of suspension. Data were obtained from ordinary least-squares linear regression (y = B + Ax) within a confidence level of 95% ($\alpha = 0.05$)

	Immersion time				
	0 h	24 h	48 h	192 h	5040 h
Milli-Q	25.9	50.9	68.1	87.9	56.4
Tap water	50.9	61.9	87.3	97.1	40.6
SiO_2 (10 ppm)	37.6	48.2	80.9	91.8	140
Canal water [a]	286	372	155	327	982
Sea water	76.5	75.2	74.4	72.7	76.1

[a] Values of c_D for Canal water calculated after immersion formore than 48 h were affected by the changes in the linear range of the calibration of fluorescence emission (see ESI for details).

The observed values of c_D for labelled nanoparticles stored in the media for more than 48 h remained stable in Milli-Q, tap water and seawater. In the case of samples dispersed in the river water and in presence of SiO_2 nanoparticles, the linear regression of the calibration was worse than for labelled nanoparticles in the other environments, with lower values of R^2 as well as higher values of standard error of the blank measurements, s_B(see ESI). This rendered higher values of c_D for $Ru(phen)_3$:SiO_2 nanoparticles in those media (Table 2).

In general, the silica structure protected the fluorescent labels inside the structure, thus allowing a long-time stability of the fluorescence. Since the synthesis procedure allowed the preparation of dense nanoparticles, the penetration of water through the surface to the fluorescent labels in the nanoparticle bulk was reduced. This feature, along with the stability of silica,could favourtheir use as efficient markers of the release of nanomaterials during their life cycle.

Conclusions

Fluorescent molecules could be incorporated into Stöber-like SiO_2 nanoparticles, which led to the synthesis of labelled SiO_2 with structures and properties similar to those of pristine nanomaterials. High degree of analytical sensitivity and low detection limits were achieved for fluorescent-labelled SiO_2 nanoparticles in different aquatic environments. The acidic conditions of the dispersion media affected the detection limits of labelled nanoparticles, as well as the presence of already-suspended matter. This labelling procedure could be therefore applied to assess the presence of labelled ENMs in the aquatic media in both natural environments and to reduce potential exposure risks. In addition, the sensitivity of the method opens up possibilities of application for monitoring the release of nanosized matter in different environmental compartments during all stages of their life cycle.

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