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ORIGINAL PAPER

Radio (^{14}C)- and fluorescent-doubly labeled silica nanoparticles for biological and environmental toxicity assessment

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Abstract A new and efficient synthetic route to fluorescent and ^{14}C-double-labeled silica-based nanoparticles (NPs) is described. The synthesis has been carried out using the "oil-in-water" micro-emulsion technique. Fluorescent and radioactive labeling have been achieved entrapping labeled poly(ethylene glycol) (PEG) molecules in the NPs. The produced particles have been analyzed by means of scanning electron microscopy, photon correlation spectroscopy, confocal microscopy, scintillation counting and oxidation/combustion experiments. Fluorescence quenching experiments confirm that the label is entrapped in the particles. The results presented suggest that the silica matrix does not block the β-radiations emitted from the labeled PEG molecules entrapped in the NPs.

Keywords Silica nanoparticles · Fluorescence · Radioactivity · Micro emulsion

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Introduction

Among the nanotechnology-based systems developed and because of their outstanding properties, nanoparticles (NPs) have been found to be highly valuable for a broad range of applications such as food, cosmetics, drugs, paintings, electronic devices, to name but a few (Rotello 2004). Because of the almost unlimited potential of nanosystems, one may expect that the next few years will see a wide range of new nanoparticle-based products rushing up the market. Indeed, the production of engineered nanomaterials was estimated to be approximately 2,000 tons in 2004 and is expected to rise up to 58,000 tons in 2011–2020 (Maynard et al. 2006).

However, owing to the relative novelty of these materials, very little is known about their potential to cause adverse biological effects and the use of NPs is gathering an increasing attention and concerns in the public opinion and in the media. Since several years and because of their potential commercial applications in biomedicine for drug transport and targeting (De Villiers et al. 2008), numbers of research programs for the study of nanoparticle effects on human health have been initiated and carried out (Brannon-Peppas and Blanchette 2004) while the risks of environmental contamination by NPs remain largely unknown (Hasselloev et al. 2008). Because of the lack of suitable analytical methods, knowledge about the behavior of NPs in complex matrices is very limited (Nel et al. 2006).

Among the wide range of nanoparticulate systems developed, silica NPs represent an important class because of the non-toxicity of the bulk material and the possibility to produce them in a highly controllable manner with relatively low production costs. In addition, their chemistry is fairly well known and their chemical

modification may, therefore, be achieved without major problems. At the laboratory scale, silica NPs are generally produced using one of the two following chemical approaches: reverse micro emulsion (Arriagada and Osseo-Asare 1999) or sol–gel synthesis (Bogush et al. 1988); while flame-spray pyrolysis (Kammler et al. 2001) is more appropriate for large-scale production. The fluorescent labeling of silica NPs with various dyes such as organic and metal-organic systems have been described (Burns et al. 2006). Nevertheless, to our knowledge, no results on radiolabeling of silica NPs have been published to date.

In this paper, we report on the synthesis of silica NPs, using the micro-emulsion approach, doubly labeled with both fluorescence and radioactivity. Because of the ease of ^{14}C-signal detection, these systems are expected to be highly efficient tools for assessing in a sensitive and quantitative manner the behavior of these particles in terms of distribution, accumulation, aggregation and toxicity in complex matrices such as living organisms or environmental models. The addition of the fluorescent properties will enable a rapid microscopic observations and straightforward localization of the particles in these systems to support toxicity assessment.

Experimental

General

Chemicals and solvents were purchased from Fluka (Switzerland) at analytical grade and used without further purification. Fluorescein-PEG was purchased form Nanocs (USA) and ^{14}C-PEG from Hartmann Analytic (Germany) with a specific radioactivity of 27.75 MBq/g.

Synthesis

A solution of triton X-100 (21.2 mL), 1-hexanol (21.6 mL) and cyclohexane (90 mL) was submitted to a vigorous magnetic stirring during 15 min. A 7 mL of this solution was transferred into a 15 mL glass vial and stirred. After 15 min, pure water (132 μL, resistivity $\geq$18 MΩ cm) containing from 0.0286 to 286 g/L poly(ethylene glycol) MW 4000 was added. The solution was kept under stirring for 15 min before adding 80 μL of tetra-ortho-silicate and additional 15 min before adding 42.2 μL of ammonium hydroxide (33%). The reaction mixture was then kept under stirring at room temperature for additional 24 h. After being transferred in a 50 mL conical flask, the micro emulsion was destabilized by adding 30 mL of ethanol and an ultrasonic treatment of 3 min. The resulting suspension was subsequently centrifuged (4,000*g*, 4°C, 30 min), the supernatant eliminated and the white solid pellet obtained was re-suspended in 1.5 mL of ethanol. This suspension was subsequently transferred in a 2 mL vial and centrifuged (31,000*g*, 4°C, 10 min). The supernatant was eliminated and this step was repeated twice with ethanol and three times with pure water. The produced particles were finally suspended in 1 mL of water and kept at 4°C.

After 1, 2, 13, 16, 17 and 20 days, the sample was centrifuged (31,000*g*, 4°C, 10 min) and the radioactivity of both the pellet and the supernatant measured as described below.

Photon correlation spectroscopy

Particle size measurements were carried out by the mean of photon correlation spectroscopy (PCS) using a Nanosizer (Malvern, UK) system, on nanoparticle suspensions in nanopure water. The measurements were done in triplicate to ensure the reproducibility of the results.

Scanning electron microscopy (SEM)

Samples for SEM studies were prepared spreading 3 μL of the respective nanoparticle suspensions on freshly cleaved mica surfaces and dried at room temperature. After Au–Pd sputter coating, imaging was carried using a Supra 40 V system (Carl Zeiss, Switzerland) at an accelerating voltage of 20 kV using an in-lens detector.

Radioactivity measurements

Radioactivity measurements on NP suspensions were carried out using a Perkin Elmer 2800TR Tri Carb Liquid scintillation analyzer. Samples were prepared adding a known volume of freshly suspended and sonicated particles in 18 mL of a scintillation cocktail Lumasafe (Lumac-LSC, Groningen, The Netherlands). Nanoparticle concentrations were evaluated by measuring the optical density of the respective suspensions at 600 nm wavelength and compared with a linear standard curve obtained with non-labeled particles. At this wavelength, the influence of the FITC can be neglected.

Oxidation/combustion experiments were performed using a 307 PerkinElmer Sample Oxidizer. Aliquots of nanoparticle suspensions were diluted on micro crystalline cellulose contained in the combusto-cone and then combusted for 2 min. During the combustion cycle, ^{14}C is oxidized to gaseous carbon dioxide which is trapped by the carbon dioxide absorbent (Carbo-Sorb® E) and later mixed with the scintillator cocktail (Permafluor E+), ratio absorbent/scintillator cocktail equal to 5 mL/13 mL.

Results and discussion

Silica-based NPs have been synthesized following a standard water-in-oil micro-emulsion procedure, using cyclohexane and 1-hexanol as the organic phase, tetraethyl orthosilicate as a silica source and Triton X-100 as a surfactant (Abarkan et al. 2006). When using the micro-emulsion method, the hydrophobicity of the common organic dyes make them inappropriate to be encapsulated in the NPs. Nevertheless, the use of for instance dextran-dye conjugates have been shown to be a successful approach to incorporate the organic dye in the silica matrix (Selvin 2000). In a similar approach, we decided to introduce the radioactivity and/or the fluorescence properties using a low-molecular weight (4,000) poly(ethylene glycol) ^{14}C or fluorescently labeled (cf. Fig. 1). The choice of PEG was driven by the following parameters: (1) high-water solubility which is expected to improve the encapsulation of the molecule during the micro-emulsion synthesis, (2) low toxicity, (3) relative high-molecular weight molecule with respect to typical labels in order to minimize the release from the produced particles.

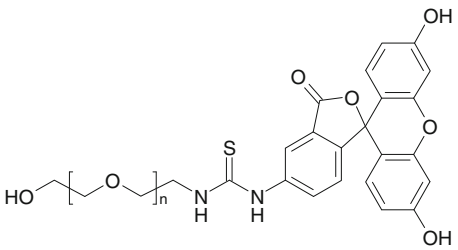

Fig. 1 Molecular formula of FITC-PEG used for the fluorescent labeling. FITC: fluorescein isothiocyanate

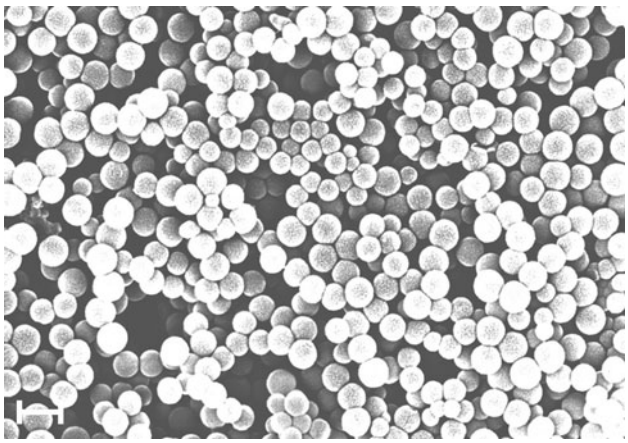

Fig. 3 SEM image of nanoparticles (magnification 100 K)—with a concentration of PEG applied in the microemulsion of 2.86 g/L, *scale bar* 200 nm. SEM: scanning electron microscopy

It is expected that during the formation of the silica matrix, the PEG present in the aqueous phase will be entrapped in the produced particles, as represented in Fig. 2.

The effect of increasing amounts of PEG (non-labeled) ranging from 0.0286 to 286 g/L in the aqueous phase on the size and morphology of the produced particles was studied by means of scanning electron microscopy (SEM); a characteristic image obtained for a PEG concentration of 2.86 g/L is presented in Fig. 3.

From the SEM image, it could be clearly seen that the silica NPs have a spherical shape with a mean diameter of 105 ± 20 nm, with a reasonably narrow size distribution. PCS experiments are in good agreement with the result as they reveal a hydrodynamic diameter of 100 nm and a polydispersity index of 0.15. The presence of the PEG in

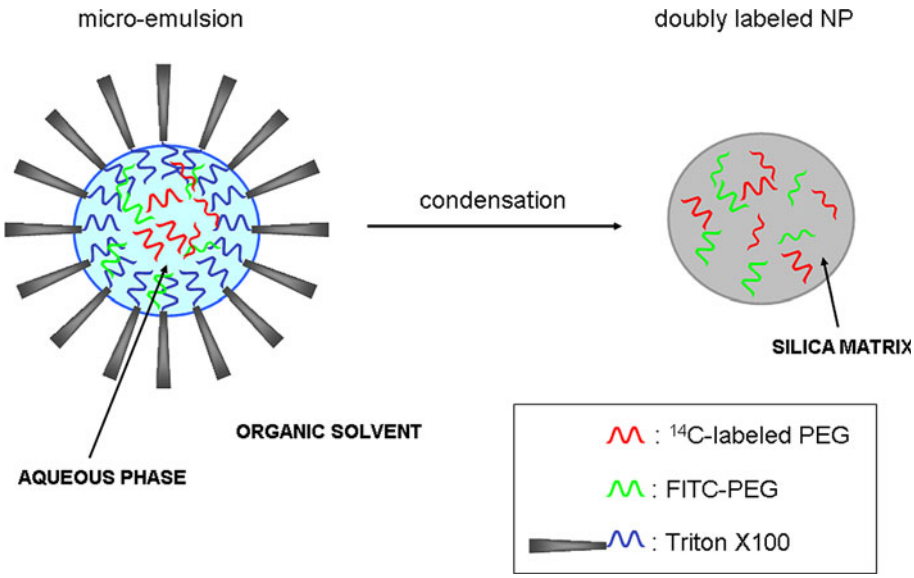

Fig. 2 Schematic representation of the strategy used to label the NPs during the synthesis. SEM: scanning electron microscopy

the micro emulsion did not cause any relevant change in the morphology or the size of the produced particles for concentrations up to 32.1 g/L. For the highest concentration studied, 286 g/L, the SEM analysis (cf. ESI) revealed the presence of a much broader distribution with the presence of non-spherical solids. From this result, it may be assumed that for concentrations up to 32.1 g/L PEG may be introduced in the micro emulsion without the risk of major disturbance of the synthetic process.

The same experiments were performed using a fluorescently labeled PEG, fluorescein–PEG (FITC-PEG, MW 3400), and the results analyzed by confocal microscopy; one representative image obtained for a FITC-PEG concentration of 2.86 g/L in the aqueous phase is presented in Fig. 4.

Even if the resolution is not high enough to observe single particles, it could be clearly seen that the sample shows a strong fluorescence, easily detectable at 520 nm (when excited at 458 nm). Nevertheless, additional experiments are needed to assess the localization of the FITC-PEG within the particles. It is expected that if the fluorescent dye is entrapped inside the particle, the silica matrix acts as protecting barrier against photo-bleaching. Using the confocal fluorescence microscope, the fluorescence decay of the fluorescently labeled NPs and the free dye were studied, the results are presented in Fig. 5.

From Fig. 5, it could be seen that the decrease in the relative fluorescence intensity is much slower in the case of the fluorescently labeled NPs compared with the free dye.

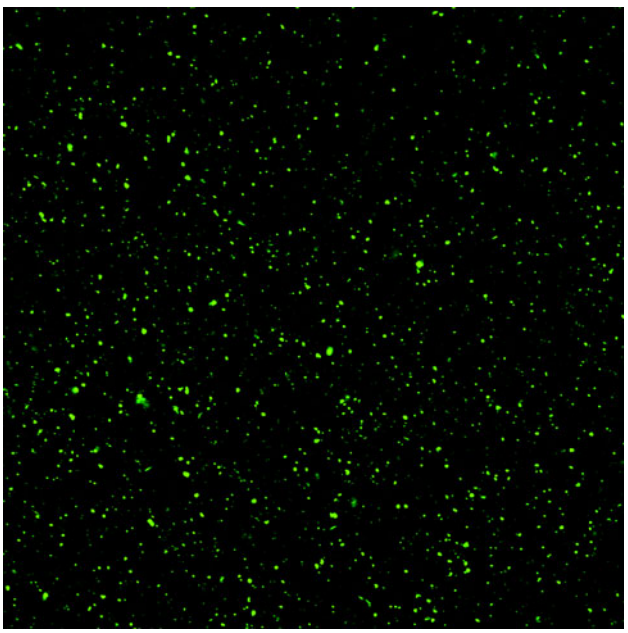

Fig. 4 Confocal microscope image of FITC-labeled nanoparticles; image dimension 250 × 250 μm, using a maximum excitation wavelength of 458 nm and a maximum emission at 520 nm

This is in good agreement with the results published by Zhao et al. (2004) regarding the development of a synthetic procedure to encapsulate a tetramethylrhodamine-dextran conjugate in silica core-shell NPs where it was demonstrated that the encapsulation of the hydrosoluble conjugate leads to a photo-protecting effect on the fluorescent dye and results in a lower photobleaching rate. This results was attributed to the complete encapsulation of the dye within the silica matrix. In the present work, it can be assumed that the PEG molecules are not adsorbed at the surface of the particles but encapsulated.

In order to optimize the incorporation of the ^{14}C-PEG in the silica NPs, increasing concentrations in the aqueous phase ranging from 23.68 mg/L to 32.21 g/L were used and the radioactivity of the produced particles tested; the results are presented in Table 1.

The results presented in Table 1 show that for all ^{14}C-PEG concentrations tested, a part of the radio-labeled PEG is successfully entrapped within the NPs. The radioactivity incorporation yield drops from 32.6, 28.7, 5.9 to 3.5% increasing the quantity of ^{14}C-PEG from 23.6 mg/L to 32.2 g/L, equivalent to 87–118.10^3 Bq, respectively. Nevertheless, increasing the amount of ^{14}C-PEG the specific radioactivity of the corresponding NPs increases up to 326 Bq/mg. As mentioned above, when increasing the PEG concentration to higher values the micro-emulsion process is disturbed and does not yield spherical NPs. This implies that in order to increase the specific radioactivity of the produced particles, the most obvious way would be to increase the specific radioactivity of the ^{14}C-PEG. Interestingly, the radioactivity measurements of the NPs in suspension and after oxidation (with a destruction of the silica matrix) are very close. This suggests that the silica matrix does not block the β-radiations emitted from the labeled PEG molecules entrapped in the NPs.

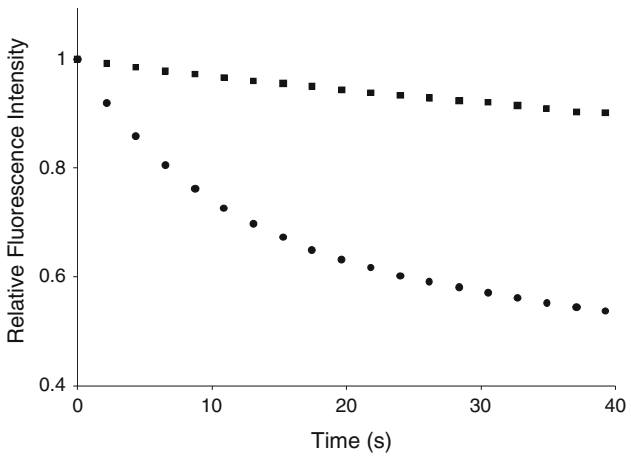

Fig. 5 Photostability test of FITC-PEG (*filled circle*) and FITC-PEG NPs (*filled square*)

Table 1 Radioactive recovery in the NPs for varying concentrations of PEG

RA applied Bq[a]	RA recovery in NPs suspensions[b]	RA recovery from oxidized NPs Bq[c]	RA incorporation yield (%)	Specific RA Bq /mg
87	29	27	32.6	1.90
885	254	251	28.7	17.9
9390	562	550	5.9	35.9
$118 \cdot 10^3$	4,136	4,236	3.5	327

[a] Radioactivity (RA) applied in the synthesis

[b] RA total recovery in the NPs (direct measurement of the final product with scintillation counting)

[c] RA total recovery from oxidized NPs (direct measurement of the final product oxidized)

[d] Specific radioactivity of the final product per mass

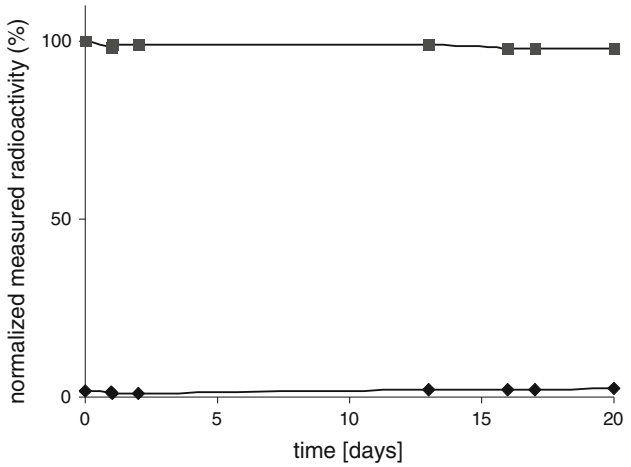

Fig. 6 Normalized radioactivity measured over time for ^{14}C-labeled nanoparticles prepared with 3.21 g/L of ^{14}C-labeled PEG measured in the pellet (*filled square*) or in the supernatant (*filled diamond*) after centrifugation

An additional proof of the stability of the encapsulation was obtained from the study of the possible release of the ^{14}C-labeled PEG over time (20 days) keeping the particles in suspension in water; the results are given in Fig. 6.

From these results, it can be seen that all the radioactivity remains in the particles (i.e. the pellet after centrifugation) and no release of the ^{14}C PEG is measured after 20 days confirming the encapsulation of the label inside the silica NPs and the lack of release over a period of 20 days.

Conclusion

In this paper, the synthesis of doubly labeled (^{14}C and fluorescent) silica NPs is described, these systems are expected to be of value to assess the behavior of this model system in biological systems and in the environment.

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