Biodegradable dye-doped silica fluorescent coating: multifunctional nanoparticles for cell assays and toxicity reduction

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In vivo imaging and therapy represent one of the most promising areas in nanomedicine. Particularly, the identification and localization of nanomaterials within cells and tissues is a key issue to understand their interaction with biological components, namely their cell internalization route, intracellular destination, therapeutic activity and possible cytotoxicity. Here, we show the development of multifunctional nanoparticles (NPs) by providing luminescent functionality to zinc (ZnO) and iron oxide (Fe 3 O 4 ) NPs, which have already demonstrated cellular toxicity and application in nanotherapy for imaging and cancer treatment, respectively. Specifically, we describe simple synthesis methods based on modified Stöber procedures to incorporate fluorescent molecules on the surface of oxide NPs. These procedures involve successful coating of NPs with a size-controlled shell of amorphous silica (SiO 2 ) incorporating standard chromophores like fluorescein, rhodamine B or rhodamine B isothiocyanate. Our coating procedures allow identification and location of ZnO and Fe 3 O 4 NPs inside the cellular cytoplasm using confocal microscopy imaging. Besides, the SiO 2 shell provides a platform for further functionalization that enables the design of targeted therapeutic strategies. Finally, we studied the degradation of the shell in different physiological environments.

Introduction

Nanotechnology is a multidisciplinary area which usually combines input from different fields like chemistry, physics, cellular biology or materials science. In particular, the fabrication of devices at the nano-scale is an area of increasing research, where synthesis, and manipulation of nanomaterials have become of paramount importance. Since a couple of decades, the use of nanomaterials oriented to the application in medicine and biology has procured the emergence of a new field, nano-bio-medicine. It focuses on the study of nanomaterials interaction with cells, tissues and bacteria, and their potential application in drug delivery, biomarkers detection and quantification and cancer therapy, among others. A great variety of nanomaterials has been proposed in nanomedicine. Among the most relevant, gold, silver and iron oxides constitute the paradigms. Besides, silica- or carbon-derived materials like mesoporous silica nanoparticles (NPs), single/multi-wall carbon nanotubes, nanodiamonds or graphene and graphene oxide are highly attractive in the search for functional biocompatible nanomaterials. 1–6 Nanomaterials to be used in medicine must present low or null cytotoxicity, greatly restricting the potential applications to in vivo bio-labelling. Importantly, cytotoxic effects of nanomaterials are not only related to their chemical composition, but also to structural features, such as particle size, morphology or even their crystallinity degree.

Metal and metal oxide NPs, particularly magnetic NPs, hold a great potential to be used in a variety of biomedical applications. However, so far, only iron oxide NPs have been approved by the US Food and Drug Administration (FDA) for their use in humans, as a contrast agents in magnetic resonance imaging (MRI) 7 and also as iron replacement therapies to treat anemia related to chronic kidney disease. 8 Additionally, these magnetic NPs can be used in hyperthermia, i.e. they become heated up when exposed to an external AC magnetic field, a phenomenon that can be applied in therapy for localized killing of temperature sensitive malignant cells and tissues. 9 However, as-synthesized NPs are frequently organic-soluble, thus requiring post-synthetic surface modifications to exploit their properties in biomedicine.

In addition, a large number of current trends include the use of non-magnetic oxide NPs. For instance, zinc oxide (ZnO) is present in numerous industrial products such as paints, coating and cosmetics as whitener and UV-filter, increasing human and environmental exposure. 10–12 However, ZnO NPs have shown to cause acute cytotoxicity when they are dissolved intracellularly. 13,14 Thus, since they can interfere with cell proliferation and survival, conveniently modified ZnO NPs are also interesting candidates as potential nanotherapy in different diseases treatments. 15–18 A major objective in nanomedicine is the development of high-quality chemically-stable and biocompatible core-shell nanomaterials with tailored physical properties. 19,20 Among various types of coatings, silica (SiO 2 ) shells represent a fine
strategy. These show optical transparency, high biocompatibility, strong chemical and colloidal stability and tunable thickness and porosity.\textsuperscript{21,22} This type of surface modification improves the nanoparticle dispersion in aqueous saline media, reducing aggregation that often limits the use and therapeutic effect of many materials in biomedicine offering an excellent platform to attract a variety of ligands like proteins, nucleic acids, drugs, or even other nanomaterials,\textsuperscript{23,24} allowing the assembly of multifunctional nanomaterials. In addition, SiO\textsubscript{2} shells can isolate the nanomaterial and prevent cytotoxic effects.\textsuperscript{25,26}

An important challenge regarding the development of multifunctional NPs with magnetic and optical functionalities is to avoid the quenching of the chromophore luminescence when closely/chemically attached to a magnetic core. In the case of magnetic iron oxide nanoparticles, this problem has been previously addressed by developing multi-step reactions in which the magnetite (Fe\textsubscript{3}O\textsubscript{4}) NPs are firstly coated with a SiO\textsubscript{2} shell, and chromophores are subsequently and covalently attached to amine groups of a polyelectrolyte.\textsuperscript{27,28} Here we demonstrate the possibility to use a simple procedure for the simultaneous functionalization of metal oxide NPs with both amorphous silica and fluorescent dyes. This procedure is similar to the co-condensation method described by Lin et al. to simultaneously incorporate organic dyes in a mesoporous SiO\textsubscript{2} coating of magnetite NPs.\textsuperscript{29}

In this paper, we functionalize Fe\textsubscript{3}O\textsubscript{4} and ZnO NPs with a fluorescent amorphous silica shell that incorporates different dyes. Specifically, Fe\textsubscript{3}O\textsubscript{4} nanoparticles prepared by a hydrothermal approach and commercial BASF ZnO nanoparticles were coated with SiO\textsubscript{2} incorporating rhodamine B isothiocyanate (RBITC), fluorescein (F) or rhodamine B (RhB). Structural and magnetic characterization of the core-shell nanoparticles is presented. In addition, in vitro studies in Hela cells were performed to optically characterize the cellular uptake and to provide insight in the SiO\textsubscript{2} influence on toxicity. Firstly, coating with a non-toxic shell gives protection and shielding of the potentially toxic oxide core, as previously mentioned. Secondly, a luminescent shell is extremely useful in biomedicine as it allows monitoring the distribution of NPs within tissues or cells. This is relevant to determine the uptake route of nanomaterials and the cytoplasmic distribution/targeting of these NPs in in vivo assays. This feature is particularly relevant in emerging targeted nanotherapeutics, where the fluorescent molecules in the SiO\textsubscript{2} shell allows verifying that NPs accumulate only in the target tissue (i.e. magnetic hyperthermia). This fluorescent coating-based strategy will open new possibilities for nanomaterials to be applied in many different biotechniques, including precision cellular recognition by fluorescence microscopy or flow cytometry, complementary diagnostic in biopsies, or their use as optical sensors for determining intracellular temperature or pH. Finally, the stability and degradation of the amorphous silica shell in different physiological media is studied and compared to previous mesoporous silica shell results.

Experimental

Nanoparticle synthesis and coating

Chemicals

FeCl\textsubscript{3}·6H\textsubscript{2}O (99%), FeCl\textsubscript{2}·4H\textsubscript{2}O (99%), sodium oleate (82%), Rhodamine B (RhB, ≥90%), Rhodamine B isothiocyanate (RBITC, 70% labelling efficiency), fluorescein sodium salt (F, ≥ 90%) (3-amino-propyl)trimethoxysilane (APMS, 97%), tetraethoxysilicate (TEOS, 98%), and ammonium hydroxide solution (NH\textsubscript{4}OH, 28-30%) were obtained from Sigma-Aldrich. Cyclohexane (99.5%) and isopropyl alcohol were supplied by Panreac, and ethanol (96%) from Scharlab.

Synthesis

Hydrophobic Fe\textsubscript{3}O\textsubscript{4} NPs were prepared by a hydrothermal approach described elsewhere.\textsuperscript{30} For the fluorescent-SiO\textsubscript{2} coating, an adapted reverse microemulsion method was followed. Briefly, in this procedure the fluorophore RBITC was covalently bound to the silica matrix by a previous conjugation with APMS by dissolving 0.02 g of APMS in 5 mL of ethanol containing 0.01 g of RBITC, and stirring for 12 h in dark. The reverse microemulsion system was prepared by mixing 8.73 mL of cyclohexane, 0.27 mL of ferrofluid (7 mg/ml), 0.07 mL of NH\textsubscript{4}OH (25%), 0.1 mL of RBITC-APMS conjugate and 0.15 mL of TEOS, which were sequentially added with ultrasonic homogenization between each reagent addition. The resulting solution was sealed and kept in dark at room temperature (RT) for 15 h. Then, 6 mL of ethanol were added to disrupt the microemulsion and precipitate the NPs, followed by a first separation by centrifugation at 3000 rpm for 5 min. The excess of reagents was then removed by repeated centrifugation/redispersion cycles with ethanol (12000 rpm, 15 min) and NPs were finally redispersed in ethanol for storage.

Commercial ZnO Z-Cote® (BASF Chemical Company) NPs without any further treatment of ca. 130 nm average size were coated with amorphous SiO\textsubscript{2} and functionalized with different dyes like F and RhB, by using a novel one-step procedure based on Stöber method.\textsuperscript{31} Briefly, 160 mg of ZnO nanopowder sample were dispersed in isopropyl alcohol by sonication for 30 minutes. Then, 4.5 mL of a solution of F or RhB in deionized water (0.1 mg/mL) and 6 mL of hydrous ammonia were added dropwise, and the solution was kept under stirring at 50ºC for 10 minutes. After that, 200 µL of TEOS were added, and the mixture reacted under stirring in dark conditions at 50ºC for 1 hour. Finally, the reaction mixture was centrifuged at 6000 rpm for 10 minutes, and the resulting NPs were washed with deionized water several times to remove the dye in solution and dried at 90ºC. Thus, the ZnO NPs were coated with a SiO\textsubscript{2} shell and the fluorophore was embedded into the SiO\textsubscript{2} shell in the same reaction.

Nanoparticle characterization

X-ray diffraction (XRD) patterns were recorded using the Cu-K\textalpha radiation (λ = 1.5418 Å) in a Bruker D8 Advance x-ray diffractometer. Typically, the diffraction diagrams were obtained as a function of 2θ in the 10º-135º range with a
European Molecular Biology Laboratory Cell Bank, passage 10) were cultured under standard conditions in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics (Gibco, Thermo Fisher Scientific). Cells were incubated for 24 hours with the particles that were previously dispersed by mild sonication in standard culture medium containing 10% serum. Cells were fixed in 4% paraformaldehyde, and were stained with Hoechst (Bisbenzimide, Sigma-Aldrich) Confocal microscopy images were obtained with a Nikon A1R confocal microscope and processed with NIS-Elements Advanced Research software. All confocal cell images are pseudo-colored.

Determination of cell viability
HeLa cells were seeded into a 6-well plate and were exposed to Fe$_3$O$_4$@SiO$_2$-RBITC (6 and 15 μg/ml) or ZnO@SiO$_2$-FITC (15 and 30 μg/ml of ZnO) for 24, 48, 72 and 96 h. The harvested cell suspension was mixed with an equal volume of 0.4% trypan blue solution (Sigma) and was incubated at RT for 5 min. The viable cells were counted using a TC20™ automated cell counter (Bio-Rad). Values of viability of treated cells were expressed as a percentage of that from corresponding untreated control cells. All cellular assays were repeated at least three times.

Analysis of SiO$_2$ stability and degradation
As-prepared Fe$_3$O$_4$@SiO$_2$-RBITC and ZnO@SiO$_2$-F were incubated at a NPs concentration of 100 μg/ml in three different physiological media: (a) phosphate buffer saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$, pH 7.4); (b) DMEM culture medium supplemented with 10% FBS; (c) Synthetic lysosomal medium (25 mM MES-NaOH pH 4.5 containing 0.5 mM CaCl$_2$, 1 mM MgCl$_2$, and 200 mM KCl)$^{15}$. Samples were incubated at 37 °C with mild rotation, and 200 μl of each sample were taken every 24 h for a total period of a week. Aliquots were centrifuged for 20 min at 10,000 g and washed several times with distilled water. Finally, samples were resuspended and mounted on TEM grids for analysis.

Results and discussion

Structural characterization
All prepared samples were analyzed by X-ray diffraction (XRD). Figure 1 shows the diffraction patterns of the as-grown and silica-coated Fe$_3$O$_4$ nanocrystals. The Rietveld refinement is consistent with the expected cubic magnetite structure, space group Fd-3m (standard Bragg residual: $R_B = 3.24\%$). The refined lattice parameter, $a = 8.3492(7)$ Å, is 0.5% smaller than the value obtained from the bulk. This reduction has been...
Previously reported in this material, no traces of maghemite or other impurity phases have been detected in the XRD patterns on both samples. The silica-coated NPs show an additional broad diffraction feature at around 2θ = 239° (d = 3.87 Å), typical of an amorphous arrangement. The diffraction peaks show a significant broadening in comparison with those from the bulk samples, due to the small particle size. A rough estimation on the average crystallite size from the diffraction pattern analysis can be obtained from the Williamson-Hall (W-H) model.  

The instrumental broadening effect is independently accounted for by using a standard LaB$_6$ sample. A more complex yet accurate method is the Double Voigt (DV) approach, which provides size and strain related parameters based on a convolution approach for the whole powder pattern analysis, as implemented in TOPAS software. The size and strain contributions are both represented by Voigt functions, whereas the instrumental contribution can be derived either from a standard sample analysis or from the Fundamental Parameters approach (FPA). The obtained average crystallite size of the bare Fe$_3$O$_4$ NPs was 13.2(3) nm from the W-H method (assuming k = 0.89 for spherical NPs) and 8.9(3) nm from the DV approach (FPA and standard methods providing consistent results). In addition, silica-coated particle sizes were 17.6(6) nm to 9.1(5) nm (W-H and DV methods, respectively), in agreement with transmission electron microscopy (TEM) results, pointing out single-domain magnetite NPs (see Fig. 2).  

A similar analysis was performed on commercial ZnO NPs. The recorded XRD pattern shows only reflections corresponding to the hexagonal zincite structure (space group P6$_3$mc), and no secondary phases were detected within experimental uncertainty. Refined lattice parameters, a = 3.25187(3) Å, c = 5.20987(6) Å, atomic coordinates and isotropic displacement parameters (R$_B$ = 2.20 %) were in good agreement with values reported elsewhere. An average grain size of L = 96(2) nm was obtained from XRD by the W-H method assuming single domain, whereas L = 88(2) nm was obtained according to the DV method.

**TEM characterization**

Fe$_3$O$_4$ and ZnO particle morphology and SiO$_2$ coating thickness were evaluated through TEM images. Figure 2 shows images of silica-coated Fe$_3$O$_4$ and ZnO NPs. TEM images confirm the spherical particle shape of the magnetite NPs. The estimated average diameter of Fe$_3$O$_4$ core for the silica-coated particles was 9.9 nm (log-normal distribution with σ = 2.5 nm, see Fig. 2A, B), in very good agreement with the average crystallite size estimated from XRD analysis through the DV approach. The amorphous SiO$_2$ coating shell of Fe$_3$O$_4$ particles (responsible for the peak at 2θ = 239° in Fig. 1B) had an estimated thickness of 15 nm from image evaluation. By contrast, ZnO NPs presented an irregular shape, with an elongated trend. In this case, silica coating was ca. 24 nm thick (Fig. 2C).

**Spectroscopic measurements**

The surface modification of functional NPs has enabled to develop hybrid nanoparticle probes with distinct properties. The optical properties of the fluorescent dye doped silica core-shell Fe$_3$O$_4$ and ZnO NPs are shown in Figure 3. It compares the excitation and emission spectra of free dyes RBITC, RhB and F dyes, and the same dyes incorporated in the SiO$_2$ shell of the coated NPs as resuspended in aqueous media. In all cases, the wavelength of the excitation and emission maxima is very similar to the values obtained for the dyes in solution (502 and 530 for F, 549 and 575 nm for RhB and RBITC, respectively). Only slight shifts could be noted that can be attributed to the different environment of the fluorophore molecules inside the silica matrix and the different dye concentration in solution. The large number of dye molecules inside the silica shell makes these hybrid NPs suitable for biolabeling and provides the final particles with improved hydrophilicity and biocompatibility.

**Magnetic characterization of Fe$_3$O$_4$**

Hysteresis loops M (H, T = 300 K) of Fe$_3$O$_4$ and Fe$_3$O$_4$@SiO$_2$ ensembles of NPs taken at RT are shown in Fig. 4A, while Fig. 4B compares the zero-field-cooled (ZFC) and field-cooled (FC) thermal dependence of the DC-magnetization M$_{DC}$ (T) of both ensembles. The sample was first cooled down from RT to 5 K without magnetic field (ZFC). Just after cooling, a DC magnetic

Fig. 2 TEM images of (A) Fe$_3$O$_4$ after SiO$_2$ coating, displaying an average total size of 25(3) nm (shell thickness of ca. 15 nm). (B) The magnetite core size diameter. Fig. 3 Normalized excitation and emission fluorescence spectra of (A) Fe$_3$O$_4$@SiO$_2$-RBITC, (B) ZnO@SiO$_2$-RhB, (C) ZnO@SiO$_2$-F NPs (solid line) in comparison, with free fluorophores in solution (dotted lines). For A and B spectra, λ$_{ex}$ = 550 nm, λ$_{em}$ = 585 nm; for C, λ$_{ex}$ = 440 nm, λ$_{em}$ = 550 nm.
field $H = 50$ Oe was applied and the temperature increased to 300 K while the magnetization was measured as a function of temperature. Then, the sample was further cooled down to 4 K with the applied magnetic field (FC), and the $M_{DC}(T)$ was collected while increasing the temperature again.

The magnetization reduction of Fe$_3$O$_4$ compared with Fe$_3$O$_4$ in Fig. 4 confirms the formation of the silica shell. The saturation magnetization ($M_s$) values are 65 emu/g for the bare magnetite NPs and 12 emu/g for the corresponding SiO$_2$-coated NPs, the same NPs shown in Fig. 2B. The saturation magnetization of bulk magnetite is around 91 emu/g but there is a significant reduction when the particle size decreases down to the nanoscale due to the enhanced surface-to-volume ratio. From the above-mentioned values, the $M_s$ should correspond to a nanoparticle size around 10 nm, which confirms the values obtained via XRD and TEM. Assuming that the magnetic core is 10 nm for all particles, we can obtain the mass percentage of Fe$_3$O$_4$, which is 19.4%, whereas 80.6% corresponds to SiO$_2$. This allows us to estimate a volume of $V = 3.423 \times 10^{-14}$ cm$^3$. If particles are perfectly spherical, then the number of magnetic cores in the NPs is $6.54 \times 10^{14}$. If now we assume that the shell is homogeneous and of the same thickness for all particle assemble, the $V(SiO_2) = 5.208 \times 10^{-18}$ cm$^3$, which gives a thickness for the concentric coating shell of 10.9 nm. This value appears to be reasonable compared to what it is visible in TEM images (Fig. 2). The hysteresis loops are connected to the values of coercivity ($H_c$) at RT, which are below 30 Oe, for both samples, indicating that most of the NPs are superparamagnetic.

The ZFC-FC $M_{DC}(T)$ curves show a common behavior for the two samples, but with subtle distinct details as well. For both bare and coated magnetite NPs, there is irreversibility indicating the existence of magnetic disorder. In bare magnetite, the FC branch is soon flat ($M_{DC}(T) = 3.4$ emu/g for $T < 250$ K) when going down in temperature, whereas the ZFC branch presents a maximum at blocking temperature $T_B = 252.9(5)$ K, and then an $M_{DC}(T)$ decrease towards low temperature. The high temperature maximum at $T_B$ is connected to the blocking of the magnetic NPs, that is, the point where the magnetic moments in the particles begin to flip freely (for $T > T_B$). In addition, the extremely large width of the maximum is indicating that some NPs are becoming blocked/unblocked in a coherent manner due to the existence of dipolar interactions. A rough estimation of the magnetic size derived by inserting the value of $K = 1.3 \times 10^4 J/m$ for magnetite in $25kT_B = kV_{MAG}$ gives $D_{MAG} = (\frac{4V_{MAG}}{\pi})^{1/3}$, $D_{MAG} = 23$ nm. This simple formula stems from the Néel-Brown expression of the relaxation process for fine magnetic nanoparticles. This $D_{MAG}$ is slightly higher than the size obtained from x-ray diffraction. This means that the particles suffer dipolar interactions among them.

A deeper interpretation of the ZFC-FC $M_{DC}(T)$ is possible for Fe$_3$O$_4$@SiO$_2$. There are two clear differences respect to the bare Fe$_3$O$_4$: i) there is a well-defined maximum at $T_B(H = 50$ Oe) = 128.3(5) K in the ZFC branch. This is due to the increase of

![Image of magnetic properties](https://example.com/image.png)

**Fig. 4** Magnetic properties of uncoated and SiO$_2$-coated Fe$_3$O$_4$. (A) Field dependent isothermal magnetization at 300 K show that $M_s$ decreases upon coating with silica while $H_c$ remains nearly constant. (B) ZFC and FC magnetization curves show a decrease in blocking temperature in coated NPs.
interparticle distance among NPs, thus reducing the dipolar interactions, and ii) the FC $M_{DC}(T)$ still increases when $T < T_B$ in ZFC, and for $T < 50$ K finally becomes somewhat flat reaching $M_{DC}(T = 5 \text{ K}) = 3.9 \text{ emu/g}$. The i) observation point indicates that there exists a very clear blocking/unblocking process at $T_B$, which is directly connected with the existence magnetic NPs. Yet, it is higher than we expect and hence there are still some dipolar interactions, which increase $T_B$. Regarding point ii), the FC branch those dipolar interactions impedes such an increase and hence the observed low-temperature saturation.

It has been recently established that MNPs should be defined with care for biomedical applications and for that, two standardized definitions have been raised: single core NPs are formed by a single magnetic core surrounding by a coating. This coating may be functionalized and prepared for a biological agent. Secondly, there could be magnetic NPs (multicore nanoparticles) formed by several cores embedded in a surrounding coating, which in addition can include another layer (shell of a functionalizing materials). In consequence, we propose that Fe$_3$O$_4$@SiO$_2$ samples are classified as single core (~10 nm) magnetic NPs.

**Nanoparticles as fluorescent bioprobes**

The coating with a fluorescent silica shell permits: i) the localization of the particles with techniques like confocal fluorescence microscopy and, ii) incorporate on the surface other functional molecules. Identifying the location of NPs after administration in cells or in animal models can be challenging, especially in non-fluorescent nanomaterials used in therapy, such as ZnO or Fe$_3$O$_4$. Herein we show how incorporating an organic fluorophore in the SiO$_2$ shell represents a useful strategy to overcome this difficulty, maintaining the ability of these particles to be functionalized following different strategies.

To test the cellular uptake sequence of the silica-coated nanomaterials we chose HeLa cells as a reference system. This human epithelial cell line, obtained from a cervical carcinoma, has been widely employed as a reference cellular model to test numerous compounds and nanomaterials because its cellular and molecular features are known in great detail. Different concentrations of the fluorescent silica-coated NPs (ca. 10 μg/mL for Fe$_3$O$_4$ and 15 μg/mL for ZnO), were added to the culture medium of exponentially growing HeLa cells that were incubated with Fe$_3$O$_4$@SiO$_2$-RBITC for 5 h (caption from Video S1). In all cases, the NPs were internalized by the cells accumulating in the cytoplasmic perinuclear region, reaching its maximum uptake 24 h after exposure to the NPs in the media (Fig. 5). As for conventional silica NPs, these multifunctional nanomaterials, upon incubation in cell culture medium containing serum proteins, acquire a protein biocorona that drives them to promiscuously express cellular receptors triggering receptor-mediated endocytosis.

The fluorescence of the NPs displayed long standing stability in the extracellular and intracellular environments due to the entrapment and shielding of fluorophores in the silica matrix, allowing their tracking through the exposure time (ESI, Video S1). There are no signs of free dye fluorescence within the cells (Fig. 5). The fact that different fluorophores with distinct chemical structure and fluorescence emission wavelengths can be successfully incorporated into the silica shell enables a tailored design of the NPs depending on the particular needs of each experiment, avoiding fluorescence spectra overlapping if further functionalization with another emitting molecule or antibody is required. This procedure can serve to endow NPs with radically new properties such as integrating nano-sensors to detect local temperature or pH within the different cell compartments.

**Biocompatibility: Cell viability of bare Vs SiO$_2$-coated nanoparticles**

Biosafety assessment of nanomaterials is a matter of concern. Thus, an in-depth analysis of toxicity is timely in all cases, since it is clear that each engineered nanoparticle needs a tailor-designed evaluation. For this purpose, HeLa cells were incubated with doses of NPs ranging from 6 to 30 μg/mL (depending on the nanomaterial) of fluorescent silica-coated Fe$_3$O$_4$ and ZnO and bare ZnO (Fig. 6) as a positive control. Cell...
survival was quantified using the trypan blue exclusion assay at different time points as in previous studies.\textsuperscript{13}

Firstly, there are several factors involved in Fe\textsubscript{3}O\textsubscript{4} toxicity including particle size, charge and surface chemistry, dosage and coating.\textsuperscript{56–59} In our results, the cytotoxic effect of the coated Fe\textsubscript{3}O\textsubscript{4}@SiO\textsubscript{2}-RBITC NPs in HeLa cells was negligible at 6 and 15 μg/ml or the exposure time (Fig. 6A) during the first 96 h. In addition, the SiO\textsubscript{2} shell provided hydrophilicity to the synthesized Fe\textsubscript{3}O\textsubscript{4} NPs that were initially only organic-soluble and could not be suitable for in vivo diagnosis or therapeutic approaches. Also, this shell protected the magnetic core from cell exposure, hindering the degradation and release of Fe ions.\textsuperscript{60} Iron oxides have been traditionally considered as innocuous because iron ions are naturally found in cells. However, over a certain level, they can generate reactive oxygen species (ROS) and other toxic effects.\textsuperscript{59,61}

Secondly, ZnO NPs toxic effects have been widely demonstrated in different cell types in literature,\textsuperscript{52–66} were prevented when coating bare ZnO with a SiO\textsubscript{2} shell. Previous studies reported how incubation of HeLa cells with the identical ZnO NPs underwent necrosis, significantly reducing cell viability in a time and concentration dependent manner.\textsuperscript{13} For the cytotoxicity study, we employed equivalent dosages of uncoated and coated particles (15 and 30 μg/mL of ZnO) and compared the effects in cultured HeLa cells. Fig. 6B shows how doses of 15 μg/ml of uncoated NPs in the culture medium triggered a subtle cell death effect (less than 5% in all incubation times) compared to that triggered by 30 μg/ml dosages, where the viability did not exceed a 31% in any case. By contrast, comparable dosages of SiO\textsubscript{2}-coated ZnO particles revealed a remarkable protective effect, where the cell viability was maintained above 75% for all the incubation times. Similar to the case of Fe\textsubscript{3}O\textsubscript{4} above described, SiO\textsubscript{2} shell prevents the ZnO dissolution after cellular uptake thus preventing cytotoxicity.\textsuperscript{13,66,67} These results highlight the importance of surface engineering to control the degradation of the core, avoiding acute oxidative stress and cytotoxicity processes in cells.

**In vitro SiO\textsubscript{2} stability under physiological conditions**

It is known that silica is unstable in aqueous media and it can hydrolyze into water-soluble silicic acid (Si(OH)\textsubscript{4}) by a sequential process of hydration, hydrolysis and ion exchange. The degradation rate depends on the media where particles are incubated, as well as on the saturation of the system, that is, the silica concentration. When silicic acid reaches a certain level, the degradation reaction is no longer favored. Moreover, the physicochemical properties of the NPs, such as size, shape, porosity and chemical surface, are also crucial.\textsuperscript{68,69} On this basis, with the great variability on the experimental conditions among all the published studies of silica degradation, robust conclusions cannot be extrapolated. However, it is well established that, contrary to mesoporous SiO\textsubscript{2}, amorphous silica is more stable and can survive in physiological conditions for longer periods of time. Previous works have reported that the mesoporous particles has a first and really fast degradation stage and can dissolve almost completely within few hours. By contrast, non-porous particles do not dissolve before a week incubation or do not even reach a complete degradation, always depending on the dissolution medium and the particle concentration.\textsuperscript{70–72}

In this context, as-synthesized Fe\textsubscript{3}O\textsubscript{4}@SiO\textsubscript{2}-RBITC and ZnO@SiO\textsubscript{2}-F NPs were incubated in different simulated physiological media to study the stability of the coating. Phosphate saline buffer (PBS, pH 7.4) was chosen as the simplest buffer with physiological conditions. DMEM culture medium supplemented with serum was chosen to simulate more accurately cellular conditions. Finally, synthetic lysosomal

![Fig. 6](image-url) **Fig. 6** Time-dependent quantification of cell viability after incubation of HeLa cells with the silica-coated nanomaterials. (A) Fe\textsubscript{3}O\textsubscript{4}@SiO\textsubscript{2} NPs were added at 6 and 15 μg/ml without important cytotoxic effects in neither case. (B) Comparison of the cellular survival upon exposure to doses of 15 and 30 μg/mL ZnO NPs with and without SiO\textsubscript{2} coating. Quantification of the cell survival revealed how nanotoxicity was substantially reduced when NPs were coated with a silica shell (extended data of uncoated ZnO can be found in ref.\textsuperscript{11}). Viability of nanoparticle-treated cells is expressed relative to non-treated control cells, which represents a 100% viability. Data are reported as mean ±SEM from three independent experiments.
media (pH 4.5) was used to mimic the acidic environment of endolysosomal vesicles inside cells. Dispersions of 100 µg/ml NPs \(^71\) in each medium were incubated at 37 °C with mild agitation, and aliquots were taken every 24 h for a total period of one week. Samples were washed several times and finally analyzed by TEM (Fig. 7).

Incubation in PBS promoted subtle surface irregularities after 24 h, that became more significant over time. After a week, the SiO\(_2\) shell was nearly imperceptible in ZnO NPs and the core began to be attacked and dissolved. Some Fe\(_3\)O\(_4\) NPs still show SiO\(_2\) around the core but with degraded appearance. Similar results were obtained with DMEM incubation, though the degradation rate was even higher. This can be attributed to the presence of Mg\(^{2+}\) and Ca\(^{2+}\) ions that are present in DMEM but not in PBS, which are known to accelerate the dissolution rate. Likewise, Na\(^+\) ions contribute to the deprotonation of silica.\(^71\)

The degradation process in synthetic lysosomal media was radically different. Even after one week, the shell surface of Fe\(_3\)O\(_4\)@SiO\(_2\) NPs remained unaltered. Interestingly,
degradation occurred from the inside of the shell, producing a hole around the Fe$_3$O$_4$ core. These results can be interpreted as due to the strong dependence on pH for SiO$_2$ dissolution in aqueous media. In alkaline conditions, the levels of deprotonation of silanol groups and hydrolysis of Si-O-Si bonds by nucleophilic attack by OH$^-$ ions increase. By contrast, the dissolution in acidic conditions is much lower, as seen in our results with lysosomal medium incubation. The presence of minimal porous channels in the silica shell could explain the inner degradation observed in the lysosomal incubation medium, where the acidic medium reaches the interphase between the NP core and the SiO$_2$ shell. This hypothesis could also explain the difficulty to collect a pellet after centrifuging the ZnO aliquots in lysosomal media for further washing and analysis (TEM images could not be obtained). If acidic medium is able to reach the core of ZnO@SiO$_2$, it will be dissolved, as previously reported.

With all these results, we can conclude that SiO$_2$ coating has enough stability to allow NPs to reach their target in cells or tissues with its original nanostructure. Moreover, the shell can be slowly degraded over time in silicic acid, which is a non-toxic product, so NPs could be finally eliminated from the organism. As seen in the viability assays (Fig. 6), even at longer incubation times where partial degradation has taken place with core exposure, cell death is minimal, highlighting the protective effect of SiO$_2$ shell from acute cytotoxic effects. Also, the acid stability can provide some advantages, e.g., if NPs are administrated orally and therefore exposed to the acidic gastrointestinal environment.

Conclusions

Here we have shown how different oxide NPs such as Fe$_3$O$_4$ or ZnO can be effectively coated with fluorescent silica following either a reverse microemulsion route and a modification of Stöber method, respectively, depending on the nature and surface of the nanoparticle, thus simplifying and reducing the steps required in previously described procedures. We have reported a comprehensive analysis of the physicochemical properties of the synthetized NPs, which is fundamental to fully understand the behavior of nanomaterials in biological environments and to evaluate their suitability in biomedical applications. In cellulo studies have shown that silica shell confers high stability in aqueous media and biocompatibility, protecting the core of the nanoparticle from environmental and thus, from dissolution which can trigger exposure undesired acute cytotoxic effects. The stable fluorescent labelling of the silica shell allows the tracking and analysis of the interactions of the nanoparticles with biological systems for times long enough to perform standard measurements such as epifluorescence or confocal microscopy. Moreover, the progressive dissolution of the silica shell in biological conditions contributes to the final degradation and clearance of the NPs, crucial prerequisites for a potential translation in clinics. Hence, this coating procedure opens up the possibility of developing novel multifunctional nanoparticle systems with different and complementary properties, which are of particular relevance in biomedicine and related fields.

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