Bio-identity and fate of albumin-coated SPIONs evaluated in cells and by the *C. elegans* model

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Keywords: iron oxide nanoparticles, protein corona, cytotoxicity, *C. elegans*, biodistribution,

Nanoparticles which surfaces adsorb proteins in an uncontrolled and non-reproducible manner will have limited uses as nanomedicinal products. A promising approach to avoid nanoparticles non-specific interactions with proteins is to design bio-hybrids by purposely pre-forming a protein corona around the inorganic cores. Here, we investigate, *in vitro* and *in vivo*, the newly acquired bio-identity of superparamagnetic iron oxide nanoparticles (SPIONs) upon their functionalization with a pre-formed and well-defined bovine serum albumin (BSA) corona. Cellular uptake, intracellular particle distribution and cytotoxicity were studied in two cell lines: adherent and non-adherent cells. BSA decreases nanoparticles internalization in both cell lines and protects the iron core once they have been internalized. The physiological response to the nanoparticles is then *in vivo* evaluated by oral administration to *Caenorhabditis elegans*, which was selected as a model of a functional intestinal barrier. Nanoparticles biodistribution, at

single particle resolution, is studied by transmission electron microscopy. The analysis reveals that the acidic intestinal environment partially digests uncoated SPIONs but does not affect BSA-coated ones. It also discloses that some particles could enter the nematode's enterocytes, likely by endocytosis which is a different pathway than the one described for the worm nutrients.

1. Introduction

The biological identity of a synthetic nanoparticle (NP) is modified by the proteins and other biomolecules (i.e., DNA, lipids and sugars) adsorbed on its surface when placed in biological milieus. NP synthetic identity includes the size, shape, and surface chemistry, while biological identity includes the structure and composition of the protein corona, along with the size and aggregation state of the nanoparticles in a physiological environment. Biological response includes the triggering of signalling pathways, NP fate, and NP toxicity. Protein adsorption onto NPs in biological media is a determining factor that influences both their uptake and cytotoxicity.[1-6] An increasing number of publications have studied the impact on cytotoxicity and cellular uptake of aggregates of NPs in protein-rich media.[7-10] In addition, when those aggregates flocculate, the consequence is a heterogeneous distribution of the NPs in cell media that adds more variability in the uptake at the single-cell level and hinders the understanding of the population-wide biological response[11] Nevertheless, particle aggregation in cell media can also be advantageous.[12] Aggregates of iron oxide NPs increase cell uptake and enhance cell contrast in magnetic resonance imaging (MRI) without compromising cell viability.[13] Protein–nanoparticle aggregates were proposed as biocompatible drug carriers[14] and their use was suggested to increase the cell viability of some nanomaterials.[15, 16] However, adsorption of proteins by NPs in an uncontrolled and non-reproducible manner will have limited applications in

nanomedicine. A recurring and successful approach to avoid the out-of-control formation of a protein corona is grafting a protein-resistant polymer on the surface, such as polyethylene glycol (PEG), which increases their stealth character and blood circulation time.[17-20] Although this strategy is effective at lowering the nonspecific interactions of nanomaterials with proteins and cells, it suffers several drawbacks: PEG can be oxidized in a physiological environment, either spontaneously or by enzyme action[21] and even high PEG densities can't prevent protein adsorption and macrophage uptake.[22, 23] Moreover, PEGylated nanomaterials can induce the production of antibodies against them.[6] Some studies have monitored, with radiolabels, the fate of the grafted polymer after cell uptake both *in vitro* and *in vivo*. [24, 25] For a particular case, authors reported the loss of polymer integrity,[24] making difficult the correlation of the potential toxic effects of the polymer fragments or the NPs themselves. Other studies also suggest preclinical evidence that PEGylation can lead to storage diseases interfering with kidney, spleen or liver function in the long term and high dosed therapies.[26, 27]

A different approach to safeguard the stability of engineered nanoparticles in biofluids is to pre-form a protein corona around the inorganic core in a controlled and reproducible mode, commonly labelled as hard-corona in contraposition of weaker bound biomolecules, soft-corona.[28] Such systems demonstrate enormous potential, not only because they show longer blood circulation and superior stealth than when bare nanoparticles are used but also because the protein can be exploited as a theranostic vehicle, where a drug,[29-31] an imaging probe,[32] or a non-masked targeting agent can be anchored. We have followed this latter approach by performing a well-defined bovine serum albumin (BSA) hard corona on the surface of superparamagnetic iron oxide nanoparticles (SPIONs) and, subsequently exposing the BSA-SPIONs to other

biomolecules, cells, and living organisms to investigate their newly acquired bio-identity. Albumin is a non-immunogenic and nontoxic endogenous protein, while engineered SPIONs are extensively used in preclinical studies as contrast agents in magnetic resonance imaging (MRI), magnetic hyperthermia, and as drug-delivery vehicles.[33, 34] In addition, the use of SPIONs for cell labelling to guide and image their engraftment in target tissues has also been reported.[35] Besides applications in nanomedicine, SPIONs offer other advantages that help to address standing questions related to their biological identity, since precise quantification of NP uptake in cells and small organisms can be done by using magnetometry techniques, while Prussian blue staining can easily be used to confirm the homogeneity of their uptake. Quantification of NP uptake is not straightforward for other inorganic nanomaterials.

In previous work, we showed that SPIONs synthesized in a microwave oven can be readily stabilized in water without tedious ligand exchanges mandatory in colloidal organic routes,[36] while a second step allowed us to compactly coat the NPs with a monolayer of BSA.[37] In addition, *C. elegans* were used to monitor the SPIONs *in vivo*. [38] This animal model encompasses simplicity, transparency, short lifecycle, sequenced genome, small body size (1 mm long), and ease of cultivation, which makes it a very convenient host in the materials science laboratory for initial NP biological evaluation.[39] It has been long used as an animal model in development, neurobiology and functional genomics, and is currently emerging as a promising alternative model organism in toxicology.[40, 41] In this work, we take advantage of its anatomical complexity and in particular, of the functional and structural similarities between the *C. elegans* alimentary system and the human [42,43] to evaluate the nano/bio interaction of orally administered NPs and study the NP *in vivo* behaviour with or without protein pre-coating. This simple animal allows materials scientists to perform

information-rich experiments before using superior models that require higher infrastructure and dedicated laboratories.

Here, we thorough investigate the synthetic and the biological identity, and the physiological responses of albumin precoated iron oxide nanoparticles, BSA-SPIONs, by using *in vitro* and *in vivo* assays and comparing the results with SPIONs stabilized with citrate, protein uncoated particles, C-SPIONs. For the *in vitro* studies we use electron microscopy and magnetometry to image and quantify the effect of the pre-formed protein corona on the nanoparticles, their subcellular localization, and uptake in two different cell lines. We also study the digestion kinetics of the NPs in a solution that mimics the lysosomal environment. The physiological response of BSA-SPIONs is evaluated by their oral administration to *C. elegans*; a model for the mammalian intestinal barrier. By analyzing thin cross-sections of the worms by transmission electron microscopy (TEM) we visualize the NPs and track their biodistribution with subcellular precision. We focus on two different regions of the body of the *C. elegans*: an anterior region of the intestine (pH = 6) and a region of the posterior intestinal segment (pH = 3.6). Most particles are visualized inside the intestinal lumen although we identify nanoparticles that crossed the lumen and they were endocytosed by the enterocytes. BSA-SPIONS were taken-up by the digestive cells in a similar magnitude regarding endosome size and number as the C-SPIONs.

2. Experimental Section, Materials and Methods

SPIONs: Synthesis and characterization

Chemicals: Iron (III) acetylacetonate ($\geq 97.0\%$) and trisodium citrate dihydrate (Na_3Cit) were purchased from Sigma-Aldrich. Benzyl alcohol was obtained from Scharlau. Acetone was bought from Panreac. Fetal bovine serum (FBS), cell-culture medium developed in the Roswell Park Memorial Institute (RPMI) with Glutamax,

phosphate-buffered saline (PBS), Alamar Blue, and MTT were obtained from Invitrogen. All materials were used as received if not stated otherwise.

Synthesis of C-SPIONs and BSA-SPIONs

C-SPIONs were synthesized by using microwave-assisted thermal decomposition [36, 37] BSA-SPIONs were fabricated by performing a BSA adsorption protocol.[37] The pH of the C-SPIONs dispersion was adjusted to 11 with 0.01 M NaOH, then 100 μ L BSA (18 mg/mL) solution were added dropwise under mild vortex. BSA and C-SPIONs were incubated for 1 h at room temperature. Finally, the pH of the solution was adjusted to 7.4 by adding 0.05 mM HNO₃. The final concentration of BSA-SPIONs dispersion was maintained at 1 mg/mL (in terms of Fe₂O₃). Note that BSA and HSA (human serum albumin) are 76.36% identical (based on the alignment of their sequences using UNIPROT tool). No significant differences between the two proteins should be expected on the nano/bio assays presented here. In addition, *C. elegans* does not have albumin and lack albumin-receptors; hence no species-dependent reaction could occur.

Dynamic light scattering (DLS) and zeta-potential analysis: DLS measurements were performed in a Zetasizer Nano ZS (Malvern) equipped with a He/Ne 633 nm laser at 25°C. For each sample, three independent measurements were performed with 15 scans for each measurement.

Superconductive quantum interference device (SQUID) analysis: Magnetometry was performed with a Quantum Design system (MPMS5XL) to determine the magnetization of SPIONs. 150 μ L SPIONs of known concentration were dried in a polycarbonate capsule in a 60 °C oven overnight. Magnetization curves at 5K were recorded as a function of applied magnetic field up, to 6 Tesla.

Negative staining TEM: BSA-SPIONs were visualized by performing negative-staining TEM. One drop of the purified BSA-SPIONs was placed on a carbon-coated grid and

blotted with filter paper. Subsequently, 5 μ L 2 % uranyl acetate was placed on the grid for 1 min before draining off. The grid was then placed in a 2011 JEOL electron microscope at the working voltage of 100 kV.

Flame absorption spectroscopy: To determine the iron concentration of C-SPIONs, samples were sonicated for 10 min in an ultrasound bath. An aliquot of the sample was diluted with HCl (1 %) and the iron content of the resulting solution was determined by flame absorption spectroscopy (air–acetylene) with a Perkin-Elmer 2100 spectrometer in a triplicate assay.

SPIONs: Degradation study

Color intensity determination: 20 mM citrate buffer (CB; pH 4.6) was prepared by mixing equal volumes of 10 mM citric acid and 10 mM sodium citrate monobasic solution. CB with 10 % FBS (CB-FBS) was also prepared by adding the appropriate amount of FBS. C-SPIONs and BSA-SPIONs were dispersed in CB or CB-FBS to a final SPION concentration of 0.5 mg/mL. During the whole degradation process, digital images of the dispersions were taken every day, at the same time and with identical conditions, using a digital camera. To quantify the color intensity of each image, ImageJ software was used. Images in grayscale were analyzed and the mean color intensity of each image was determined by averaging the intensity of at least three different regions. For an accurate determination of the changes during the degradation process, the “gray” intensity of Fe^{3+} ions from a FeCl_3 solution in the same buffer was used as the reference for a fully digested system. The gray intensity of C-SPIONs or BSA-SPIONs dispersion at day 0 was labeled as I_0 , those measured at different days during the degradation process were I_d , and that of FeCl_3 was labeled as I_{FeCl_3} . Intensity of gray in each image, and thus percentage of digestion, was calculated as:

$$\text{Gray intensity (\%)} = \frac{I_0 - I_d}{I_0 - I_{\text{FeCl}_3}} \times 100\%$$

Size-evolution determination by using transmission electron microscopy

During the degradation process, 100 μL sample solutions were added to the copper grid for TEM measurements. SPIONs were imaged in a JEOL TEM-1210 electron microscope at an operating voltage of 120 KV. Size-distribution histograms were computed from 200–300 SPIONs and fitted to a Gaussian curve to obtain the mean nanoparticle size. Selected-area electron diffraction (SAED) was used to identify the crystallinity of SPIONs.

***In vitro* cell experiments**

Cell viability: MDA-MB231 cells were cultured at a concentration of approximately 1×10^6 cells/mL in a 96-well plate (0.2 mL per well), in complete medium (RPMI supplemented with 10 % FBS and 6 mM GlutaMax) and allowed to grow for 24 h. HL 60 cells were cultured in complete medium (RPMI medium supplemented with 10 % FBS, 6 mM GlutaMax and 0.5 mM sodium pyruvate) at a concentration of 2×10^6 cells/mL in a 96-well plate (0.1 mL per well). Both cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA), and grown at 37 °C in 5 % CO_2 until reaching 70 % confluence.

For MDA-MB231 cell lines, after 24 h growth with a confluence of 70 %, the medium was replaced with fresh complete medium containing C-SPIONs or BSA-SPIONs, with a final SPION concentration of 40 $\mu\text{g/mL}$, 75 $\mu\text{g/mL}$, 100 $\mu\text{g/mL}$, and 150 $\mu\text{g/mL}$. Cells and SPIONs were co-incubated for another 24 h.

For HL60 cell lines, after 24 h growing with a confluence of 70 %, another 0.1 mL fresh complete medium containing C-SPIONs or BSA-SPIONs, with concentrations double those for MDA MB 231 cell lines, was added to each well to reach a final volume of

200 μL . The final SPIONs concentration in each well was also maintained at 40 $\mu\text{g/mL}$, 75 $\mu\text{g/mL}$, 100 $\mu\text{g/mL}$, and 150 $\mu\text{g/mL}$. Cells were then allowed to co-incubate with SPIONs for another 24 h.

Cell viability after SPION exposure was evaluated by using MTT (Biomedica) assays. The appropriate amount of reactive MTT (10 % of the sample volume in each well, 20 μL) was added to each well, plates were incubated at 37 °C for 2–5 h and read in a micro-plate reader (Victor3, Perkin Elmer) at 450 nm, and at 620 nm as reference. Viability test was performed in a total of 20 samples for those cultures incubated with C-SPIONs for MDA MB 231 and HL60 cells, and in 14 and 10 samples incubated with BSA-SPIONs for MDA MB 231 and HL60 cells respectively. Experiments were compared with the control using one-way ANOVA and Tukey test post-hoc analysis, (*) $p < 0.05$; (***) $p < 0.005$.

Cellular uptake: Briefly, after 24 h incubation of cells with C-SPIONs and BSA-SPIONs, cell cultures were thoroughly washed three times with PBS, and counted. Cells were centrifuged and pellets obtained were dried overnight in polycarbonate capsules at 60 °C and 25–35 mmHg vacuum. Magnetization curve of samples was recorded on a superconductive quantum interference device (SQUID) measurement magnetometer (Quantum Design MPMS5XL). Magnetization curve of SPIONs at 5K was recorded as a function of applied magnetic field under 6 Tesla. At temperatures lower than the blocking temperature, superparamagnetic particles are blocked in a ferromagnetic state and display remnant magnetization at zero applied magnetic field. The uptake of the SPIONs can be evaluated by measuring the remnant magnetization value of treated cells ($M_{r \text{ cells}}$) after they were magnetized up to 6 Tesla. $M_{r \text{ cells}}$ was obtained by dividing the measured M_r value of the cell sample by the number of counted cells. To know the amount of iron oxide per cell (g $\text{Fe}_2\text{O}_3/\text{cell}$), the remnant magnetization value of the

SPIONs ($M_{r \text{ SPIONs}}$) (emu/g Fe_2O_3) was divided by the $M_{r \text{ cells}}$; the calculation is given below.

$$\text{Fe}_2\text{O}_3 \text{ uptake (pg/cell)} = \frac{M_{r \text{ cells}} \text{ (emu/cell)}}{M_{r \text{ SPIONs}} \text{ (emu/g}_{\text{Fe}_2\text{O}_3})}$$

Intracellular localization: MDA MB 231 cells were seeded in a 96-well petri dish, grown, and treated with 75 and 150 $\mu\text{g}\cdot\text{ml}^{-1}$ BSA-SPIONs. After 24 h of incubation, cells were trypsinized, washed twice with PBS, and collected by centrifugation (1400 rpm, 5 min). Supernatants were discarded and 2 mL of 2 % glutaraldehyde (served as fixation solution) in cacodylate buffer was added to the pellet. Cells were incubated in the fixation solution at 4 °C and further stained by using 1 % OsO_4 . They were then dehydrated in an alcohol series and embedded in Epon resin. Ultrathin sections (70 μm) were transferred onto copper grids and analyzed by TEM, using a Jeol-JEM 1400 microscope operating at 200 kV.

In vivo C. elegans experiments

Growth and treatment of C. elegans: *C. elegans* were grown on NGM agar seeded with *E. coli* OP50 following standard practices.[35] A 48 h synchronous culture was either fed SPIONs at 100 $\mu\text{g}/\text{mL}$ in MilliQ water (treated worms) or incubated in MilliQ water (control worms) for 24 h at 20 °C.

Sample preparation: Control and treated worms were washed twice with 20 % BSA in M9 buffer and then fixed by high-pressure freezing, freeze-substituted, flat-embedded in Epon resin and sectioned by following a targeted ultra-microtomy protocol, as described previously.[36]

Visualization and analysis of C. elegans sections: 60-nm cross-sections were examined by transmission electron microscopy (TEM), high-angle annular dark-field scanning transmission electron microscopy (HAADF STEM), electron energy loss spectroscopy (EELS) and energy-dispersive X-ray spectroscopy (EDX). TEM was performed using a

PHILIPs CM 120 microscope. HAADF STEM, EDX, and EELS were performed using a FEI Tecnai G2 F20 S-TWIN HR(S)TEM equipped with an EDX system and an EELS system.

3. Results and Discussion

Citrate-coated SPIONs (C-SPIONs) were synthesized by microwave-assisted thermal decomposition of iron acetylacetonate to yield γ -Fe₂O₃ nanoparticles with a mean diameter of 6 ± 1 nm. Particles were readily stabilized in water at pH=7 with a zeta potential, $\zeta = -44$ mV. Cryo-TEM confirmed the individual stability of the SPIONs. The anionic aqueous dispersion was stable for more than six months without any noticeable changes by DLS.[36, 37] The system is superparamagnetic above 45 K and displays a high saturation magnetization value of 85 emu/g Fe₂O₃ at 5 K (Figure S1 a–d). Further coating of the C-SPIONs by using a BSA-adsorption protocol yields the BSA-SPIONs system in which each nanocrystal is coated with approximately ten proteins, electrostatically bonded to the citrate through the positively charged moieties of the protein (**Figure 1a**).[44] BSA forms a monolayer of a hard protein corona as seen in the negative staining TEM image of **Figure 1b**. An increase of 7 nm in the hydrodynamic diameter (D_h) by DLS supports the side-on binding of the protein (**Figure 1c**). BSA-SPIONs have an anionic character with a surface charge of $\zeta = -22$ mV (**Figure 1d**).

Figure 1. a) Representation of a pre-formed protein corona coated nanoparticle BSA-SPION resulting from molecular dynamic modelling analysis, each protein is displayed with a different colour,[44] b) uranyl-stained TEM images; the inorganic SPIONs (dark) and the BSA hard protein corona (white) are marked with arrows, c) hydrodynamic diameter of the C-SPIONs (15 nm) and of the BSA-SPIONs (22 nm), c) zeta potential values for BSA, C-SPIONs, and BSA-SPIONs.

C-SPIONs and BSA-SPIONs were investigated *in vitro*. For this purpose, we chose two cell lines: adherent cells, MDA-MB231 (MDA), and suspension cells, HL60. MDA cells are highly invasive human mammary epithelial tumour cells and show low nonspecific binding to BSA.[45] It is expected that suspension cells are the first cell type encountered by the NPs after intravenous administration of NPs and that suspension cell lines have different nanoparticles internalization behaviour.[46] Thus, human HL60 promyelocytic leukaemia cells were also selected. **Figure 2** displays the viability tests for the two cell lines upon 24 h exposure to C-SPIONs and BSA-SPIONs at different concentrations. In all cases, cell viability is higher than 80 %, even for concentrations as high as 150 µg/mL. As expected, cytotoxic profiles are rather similar

for the two systems since the experiments were carried out in a protein-rich media and therefore a protein corona also forms on the C-SPIONs. Even though, some differences between the two systems were identified; an increase in C-SPION concentration leads to a decrease in cell viability for MDA and HL 60 cells ($p<0.05$ and $p<0.005$, respectively, when SPION concentration is higher than 75 $\mu\text{g/mL}$); in the case of BSA-SPION exposure, a significant decrease in cell viability is observed for MDA ($p<0.005$ at BSA-SPION concentration higher than 75 $\mu\text{g/mL}$), while no significant changes in viability were observed for HL 60 cell line in all concentrations studied. In short, the presence of BSA coating did not modify the viability of the cells for the adherent MDA cell line, while it moderately improved non-adherent HL 60 cells viability.

Figure 2. Viability analysis (MTT assay) for two cell lines after 24 h incubation with increasing SPION concentration. Experiments were compared with the control, (*) $p<0.05$; (***) $p<0.005$.

SPION uptake after 24 h incubation for the two concentrations was quantified ($\text{g Fe}_2\text{O}_3/\text{cell}$) by determining the remanent magnetization at 5 K of a known number of cells (emu/cell) and the remanent magnetization of a given mass of nanoparticles ($\text{emu/g Fe}_2\text{O}_3$). Unsurprisingly a concentration-dependent process was found; higher concentrations of SPIONs resulted in increased cellular uptake in all cases. Also, adherent cells internalized SPIONs (C-SPIONs and BSA-SPIONs) more efficiently than suspension cells. The iron oxide content of the labeled MDA-MB231 cells was almost

three fold higher than in the HL60 cells under the same incubation conditions (**Figure 3**).

Figure 3. Iron content versus two iron oxide concentration for two cell lines: MDA-MB231 (solid color) and HL60 (stripped pattern) and for the two systems: C-SPIONs (gray) and BSA-SPIONs (cyan). Statistical analysis by Anova: $p < 0.05$ (*).

Cellular uptake of SPIONs was greatly affected by the presence of the BSA coating in both cell lines; a considerable lower uptake of BSA-SPIONs was observed than of the C-SPIONs (more than double). This finding clearly shows that the BSA coating modifies the interaction of SPIONs with cells by substantially decreasing unspecific cell internalization. Moreover, the BSA surface would enable the anchoring of cell-targeting specific moieties. Decrease in the cellular uptake of NPs with the BSA coating was previously reported.[47, 48] Charge-mediated adhesion of nanoparticles to the plasma membrane leads to more efficient internalization. In our case, although both systems are negatively charged, BSA-SPIONs attach less to the plasma membrane, similarly to the neutrally charged PEG-nanoparticles (**Figure 4**).[23] After 24 h the colloidal stability

and hydrodynamic sizes for the two systems are highly comparable as seen in Figure S2. From Figure 4, it appears that citrate-coated and protein-coated nanoparticles are taken up by endocytosis. A characteristic feature of endosomal compartments for the BSA-SPIONs is the lower packing fraction, which we attribute again to the lesser affinity of those NPs to the cellular membrane compared to the C-SPIONs. Thus, we argue that differences in cellular uptake must be related to the nanoparticle surface and their level of “stickiness” to the cellular membrane rather than to particle aggregation or to different endosomal pathways.

Figure 4. TEM representative images of MDA-M231 cells after being incubated 24 h with C-SPIONs (upper panels) and BSA-SPIONs (lower panels). Left panels show an endosome containing nanoparticles (marked with white arrow heads). The right panels show a fraction of the cell membrane; nanoparticle aggregates interacting with the plasma membrane are visible for the C-SPIONs (white arrows heads).

To complete the *in vitro* investigations we followed the digestion process of the two NP systems in a solution that mimics a cell lysosome, for up to 30 days. We used a

citrate buffer (CB) solution at a pH=4.5. We also performed the same experiments in a buffer solution at a pH=4.5 complemented with 10 % FBS (CB-FBS) to better mirror the real lysosome environment. To monitor particle digestion, we analyzed the images of the solutions at fixed times; the results are shown in **Figure 5**. For C-SPIONs incubated with CB, nanoparticles were digested fast during the first 4 days, and a complete degradation was observed at day 7. The addition of FBS in the buffer lowered the degradation rate; still, a complete dissolution was found by day 15. We thus assume that the FBS weakly adsorbs onto the C-SPIONs surface and hence slightly protects the SPIONs from degradation.[18] In contrast, BSA-SPIONs displayed completely different digestion profile. In CB, BSA-SPIONs are only slightly digested during the first 7 days and 50 % of the BSA-SPIONs remained after 30 days incubation. Remarkably, in the presence of FBS, there was only negligible degradation for BSA-SPIONs, even at day 30. Thus both the hard corona of the pre-formed BSA monolayer and the additional adsorbed soft FBS protein corona synergistically protected the SPIONs from being complexed and chelated by the free citrate ions present in the buffer. We further monitored the hydrodynamic diameter (D_h) by DLS and inorganic nanocrystal size by TEM (Figures S4 and S5). Consistent with previous results, D_h for C-SPIONs incubated in CB displayed a gradual decrease upon time, from the initial 17 nm to less than 3 nm at day 6. After 6 days, particle size was too small to be measured by DLS. Size reduction was also evident in the TEM images, where the loss of crystallinity is established from the fewer and less intense diffraction rings visible at a greater number of days. However for BSA-SPIONs incubated in CB we could not detect differences in D_h over time, even after 30 days incubation. D_h is maintained at around 25 nm; even though 50 % of the iron from BSA-SPIONs is in ionic form, the rest maintained their initial structure intact. This aspect will be studied in more detail in the future.

Figure 5. Images of vials containing the same initial concentration of iron oxide as C-SPIONs or BSA-SPIONs in a citrate buffer (CB) at pH=4.5 (two upper panels) and in a citrate buffer supplemented with 10% FBS (CB+10% FBS). Left figures represent the color intensity, directly linked to Fe³⁺ ions as a function of time for the two buffers. C-SPIONs are depicted in gray and BSA-SPIONs in blue.

It is important to understand how the passage of nanoparticles through biological barriers and between compartments and organs is influenced by the protein corona.[49] The *in vivo* fate of orally administered SPIONs was evaluated in the model organism *Caenorhabditis elegans*. *C. elegans* has some key features that may allow it to become an alternative animal model for initial screening and evaluation of the oral delivery of nanomaterials. Because of its resemblances to the mammalian intestinal barrier, the *C. elegans* intestine is proposed as a model for studying intestinal functions (**Figure 6a**).[50-52] In particular, the polarity of the intestinal cells is conserved, showing an apical membrane with microvilli that project to the lumen, and a basolateral membrane,

both separated by apical junctions that also connect together adjacent cells. Such apical junctions are reminiscent of the apical junction complex found in mammals.[53] The mechanisms of biomolecule transport across the gastrointestinal barrier are also highly conserved. In addition, the pH value[54] and the variety of digestive enzymes[55] in the intestinal microenvironment of the worm allow us to investigate the nanoparticles' biodegradability after oral administration.

We studied the two iron oxide systems in *C. elegans*, where multiple parameters (i.e., ionic strength, pH value, diameter of the anatomical structures, etc.) could modify the NPs' original properties. We attempted to correlate the different bio-identity of C-SPIONs and BSA-SPIONs regarding cell internalization mechanisms. Previously, we reported on the similar uptake of C-SPIONs and BSA-SPIONs in *C. elegans* due to the rhythmic pharyngeal pumping that is responsible for nanoparticle entrance to *C. elegans* body during oral feeding. However, we observed coating-dependent toxicological effects in the worm, in which the BSA-SPIONs are more biocompatible than the uncoated particles, in particular at high dosage (500 µg/mL).[38] Here, we evaluate the fate of SPIONs by TEM in ultrathin cross-sections of two different regions of the body of the *C. elegans*: an anterior region of the intestine (pH = 6), very close to the pharynx ending; and a more posterior region of the intestine (pH = 3.6; **Figures 6b and 6c**).[54] We have been able to observe that most particles are located inside the intestinal lumen and only a very small number of individual particles could be detected in the area surrounding the lumen; the glycocalix, which appears to act as a buffer zone.[56] All along the intestine, SPIONs remained separated from the microvilli surface, and very few individual NPs were observed closer to the microvilli (Figure S7). Remarkably, we detected endosomes loaded with particles in the enterocytes of worms treated with both SPION species (**Figure 6d** and S8c), contrasting with our findings for 10-nm gold

nanoparticles where all the NPs visualized were restrained to the intestinal lumen.[57] BSA-SPION treated worms contained slightly larger endosomes filled with NPs (600 ± 300 nm, $n=19$) than C-SPION treated ones (470 ± 200 nm, $n=17$). Control worms occasionally had endosomes that contained features resembling nanoparticle-like structures but both EDX and EELS confirmed the presence of iron in the endosomes of treated worms and not in the controls (**Figure 6e** and S8d). The same cross-sections were further analysed by HAADF STEM, where the intensity of the materials is approximately proportional to the square of the atomic number, Z^2 . In Figure 6e and S8b–c, the high intensity of the SPIONs both inside the lumen and inside the endosomes can be attributed to Fe ($Z=26$) whereas C ($Z=6$) has lower intensity, thus SPIONs are seen as bright spots, not observed in the control worms. Both C-SPIONs and BSA-SPIONs remained monodisperse, both in the lumen and inside the endosomal/lysosomal compartment. A histogram with the nanoparticle size distribution showed that C-SPIONs in the lumen underwent a 10 % size decrease after 24 h treatment in the anterior part of the intestine, while BSA-SPIONs remained unchanged (Figure S9). In posterior regions, smaller clumps (< 200 nm) of individual SPIONs were observed. In this area, C-SPIONs had a slightly smaller size than in the anterior, corresponding to a 15 % size decrease compared to the as-synthesized SPIONs, while the size of the BSA-SPIONs again remained unchanged (Figure S9). These observations support the hypothesis that C-SPIONs undergo a partial digestion under the intestinal conditions of *C. elegans*. [38] Note that the microvilli structure was not affected in the worm studies and that no apoptotic digestive cells were observed. The absence of food during NP treatment caused cessation of defecation; TEM observations showed that no NPs were present in the lumen at the level of the excretory system (Figure S10).

Figure 6. a) Schematic drawing of the *C. elegans* anatomy and cross-section. b–d) TEM images of control *C. elegans* (first column), *C. elegans* treated with C-SPIONs (central column) and *C. elegans* treated with BSA-SPIONs (right column). *C. elegans* were cut at two body locations: b) anterior and c) posterior. d) Endosomes containing NP or NP-like content in treated and control worms, respectively. e) HAADF STEM images of endosomes. EDX analysis (insets) confirmed the absence or presence of iron in control and treated worms, respectively.

Overall, the observations on the fate of C-SPIONs and BSA-SPIONs in *C. elegans* suggest that the BSA coating protects the NP inorganic core from acidic degradation, in accordance with results in Figure 5. Moreover, both NPs remained monodisperse in the worm intestine. However, NPs can be also endocytosed during their confinement in the intestine, and no remarkable differences have been observed regarding the number of endosomes, their size, or their loading in the two sections of the intestine studied. These findings indicate that nanoparticles are internalized by the cells through a different pathway than the nutrients feeding the worm. This point deserves further studies.

3. Conclusion

Unravelling meaningful relationships between the physiological impact of engineered nanoparticles and their synthetic and biological identity is rather complex but of vital importance when considering nanoparticles biomedical uses and when establishing their nanotoxicological profile. In this work, we focused on the acquired bio-identity, *in vitro* and *in vivo*, of anionic citrate-SPIONs (C-SPIONs) outfitted with a well-defined albumin corona (BSA-SPIONs). Cytotoxicity, intracellular particle distribution, and uptake were studied in two cell lines: adherent and suspension cells. The intracellular content of iron oxide in adherent cells is three-fold higher than in non-adherent whilst the uptake of the BSA-SPIONs was greatly reduced in both cell lines compared to the same particles without the pre-formed protein corona. Thus, the pre-formed corona substantially decreases unspecific cell internalization. Moreover, it protects the particles from acidic digestion. The fate of orally administered nanoparticles was then evaluated *in vivo* in the model organism *Caenorhabditis elegans*. Analysis of ultrathin cross-sections of the worm by transmission electron microscopy allowed us to screen the biodistribution of the particles showing that both nanoparticles systems remained monodispersed in the lumen microenvironment while we identify a small fraction that

crossed the intestinal barrier by endocytosis of the intestinal cells. No remarkable differences were observed in the number of endosomes, their size or their loading, in either SPION coating. Observations on the NP fate in *C. elegans* suggest that the BSA coating protects the inorganic core from acidic degradation in the worm intestine and prevents the direct contact of the inorganic core with the worm's body which may result in a higher biocompatibility of the BSA-SPIONs.

The information gathered from the *in vitro* and the *in vivo* experiments on exactly the same NPs system with different surface coating is extremely relevant and complementary but the comprehensive analysis of results in an ensemble mode is challenging reflecting the complexity of this enterprise. Even though, we can conclude that the BSA coating did not significantly affect the cytotoxicity of NPs in the two cell lines studied but it showed a marked influence on the *C. elegans* survival rates at high doses underpinning the need of NPs evaluation in higher complex organisms.[38] Secondly, cell uptake was considerably reduced for BSA-SPIONs but the protein coating did not affect the NPs transit across the *C. elegans* intestinal barrier. Importantly both *in vitro* and *in vivo* studies identified endocytosis as the likely nanoparticle–cell invagination mechanism. Moreover, although the *in vitro* results show that uncoated SPIONs form aggregates in protein-rich media and stay monodispersed when precoated with BSA, we observed that both C-SPIONs and BSA-SPIONs retained their individuality in the biomolecule-rich intestinal microenvironment of the *C. elegans*. Finally, we observed that BSA-SPIONs are efficiently protected from acidic media, both in the lysosome environment and in the *C. elegans* lumen, in contrast to the more easily digested uncoated SPIONs.

Acknowledgments This research was partially funded by the Spanish Ministry of Economy (MAT2015-64442-R, Ramon y Cajal program (AL, RyC-2010-06082), FPU program (LGM, FPU12/05549), Severo Ochoa Program (SEV-2015-0496) co-funded by European Social Funds), the Generalitat de Catalunya (2014SGR213), People Program of the European Commission (grant agreement no. 303630, co-funded by the European Social Fund), a Chinese Scholarship Council fellowship (SMY, 201206150053), the Christian Boulton fellowship 2015 from EMBL (LGM), the COST Actions HINT (Action No. MP1202), and GENIE (Action No. BM1408-A). We thank Lorenzo Albertazzi (IBEC, Spain) for the critical reading of the manuscript.

References

- [1] Lynch I, Dawson KA. Protein-nanoparticle interactions. *Nano Today* 2008;3:40-7.
- [2] Roecker C, Poetzel M, Zhang F, Parak WJ, Nienhaus GU. A quantitative fluorescence study of protein monolayer formation on colloidal nanoparticles. *Nat Nanotech* 2009;4:577-80.
- [3] Ritz S, Schoettler S, Kotman N, Baier G, Musyanovych A, Kuharev J, Landfester K, Schild H, Jahn O, Tenzer S, Mailänder V. Protein Corona of Nanoparticles: Distinct Proteins Regulate the Cellular Uptake. *Biomacromolecules* 2015;16:1311-21.
- [4] Assaad L, Monyoncho E, Pitzschel K, Allagui A, Petit M, Hanbuecken M, Baranova EA, Santinacci L. 3D-nanoarchitected Pd/Ni catalysts prepared by atomic layer deposition for the electrooxidation of formic acid. *Beilstein J Nanotechnol* 2014;5:162-72.
- [5] Lundqvist M, Stigler J, Elia G, Lynch I, Cedervall T, Dawson KA. Nanoparticle size and surface properties determine the protein corona with possible implications for biological impacts. *PNAS* 2008;105:14265-70.
- [6] Walkey CD, Chan WCW. Understanding and controlling the interaction of nanomaterials with proteins in a physiological environment. *Chem Soc Rev* 2012;41:2780-99.
- [7] Albanese A, Chan WCW. Effect of Gold Nanoparticle Aggregation on Cell Uptake and Toxicity. *ACS nano* 2011;5:5478-89.
- [8] Safi M, Courtois J, Seigneuret M, Conjeaud H, Berret JF. The effects of aggregation and protein corona on the cellular internalization of iron oxide nanoparticles. *Biomaterials* 2011;32:9353-63.
- [9] Liu X, Chen Y, Li H, Huang N, Jin Q, Ren K, Ji J. Enhanced Retention and Cellular Uptake of Nanoparticles in Tumors by Controlling Their Aggregation Behavior. *ACS nano* 2013;7:6244-57.
- [10] Gliga AR, Skoglund S, Wallinder IO, Fadeel B, Karlsson HL. Size-dependent cytotoxicity of silver nanoparticles in human lung cells: the role of cellular uptake, agglomeration and Ag release. *Part Fibre Toxicol* 2014;11.
- [11] Ware MJ, Godin B, Singh N, Majithia R, Shamsudeen S, Serda RE, Meissner KE, Rees P, Summers HD. Analysis of the Influence of Cell Heterogeneity on Nanoparticle Dose Response. *ACS nano* 2014;8:6693-700.
- [12] Hristov DR, Rocks L, Kelly PM, Thomas SS, Pitek AS, Verderio P, Mahon E, Dawson KA. Tuning of nanoparticle biological functionality through controlled surface

- chemistry and characterisation at the bioconjugated nanoparticle surface. *Sci Rep* 2015;5.
- [13] Carenza E, Barcelo V, Morancho A, Montaner J, Rosell A, Roig A. Rapid synthesis of water-dispersible superparamagnetic iron oxide nanoparticles by a microwave-assisted route for safe labeling of endothelial progenitor cells. *Acta Biomaterialia* 2014;10:3775-85.
- [14] Sanpui P, Paul A, Chattopadhyay A. Theranostic potential of gold nanoparticle-protein agglomerates. *Nanoscale* 2015;7:18411-23.
- [15] Landgraf L, Christner C, Storck W, Schick I, Krumbein I, Daehring H, Haedicke K, Heinz-Herrmann K, Teichgraeber U, Reichenbach JR, Tremel W, Tenzer S, Hilger I. A plasma protein corona enhances the biocompatibility of Au@Fe₃O₄ Janus particles. *Biomaterials* 2015;68:77-88.
- [16] Kah JCY, Chen J, Zubietta A, Hamad-Schifferli K. Exploiting the Protein Corona around Gold Nanorods for Loading and Triggered Release. *ACS nano* 2012;6:6730-40.
- [17] Schoettler S, Becker G, Winzen S, Steinbach T, Mohr K, Landfester K, Mailaender V, Wurm FR. Protein adsorption is required for stealth effect of poly(ethylene glycol)- and poly(phosphoester)-coated nanocarriers. *Nat Nanotech* 2016;11:372-7.
- [18] Gref R, Luck M, Quellec P, Marchand M, Dellacherie E, Harnisch S, Blunk T, Muller RH. 'Stealth' corona-core nanoparticles surface modified by polyethylene glycol (PEG): influences of the corona (PEG chain length and surface density) and of the core composition on phagocytic uptake and plasma protein adsorption. *Colloids Surf, B* 2000;18:301-13.
- [19] He Q, Zhang J, Shi J, Zhu Z, Zhang L, Bu W, Guo L, chen Y. The effect of PEGylation of mesoporous silica nanoparticles on nonspecific binding of serum proteins and cellular responses. *Biomaterials* 2010;31:1085-92.
- [20] Dai Q, Walkey C, Chan WCW. Polyethylene Glycol Backfilling Mitigates the Negative Impact of the Protein Corona on Nanoparticle Cell Targeting. *Angew Chem Int Ed* 2014;53:5093-6.
- [21] Kawai F, Kimura T, Fukaya M, Tani Y, Ogata K, Ueno T, Fukami H. Bacterial Oxidations of Polyethylene--Glycol. *Appl Environ Microbiol* 1978;35:679-84.
- [22] Stolnik S, Dunn SE, Garnett MC, Davies MC, Coombes AGA, Taylor DC, Irving MP, Purkiss SC, Tadros TF, Davis SS, Illum L. Surface Modification of Poly(Lactide-co-Glycolide) Nanospheres by Biodegradable Poly(Lactide)-Poly(Ethylene Glycol) Copolymers. *Pharm Res* 1994;11:1800-8.
- [23] Pelaz B, del Pino P, Maffre P, Hartmann R, Gallego M, Rivera-Fernandez S, de la Fuente JM, Nienhaus GU, Parak WJ. Surface Functionalization of Nanoparticles with Polyethylene Glycol: Effects on Protein Adsorption and Cellular Uptake. *ACS nano* 2015;9:6996-7008.
- [24] Bargheer D, Nielsen J, Gebel G, Heine M, Salmen SC, Stauber R, Weller H, Heeren J, Nielsen P. The fate of a designed protein corona on nanoparticles in vitro and in vivo. *Beilstein J Nanotechnol* 2015;6:36-46.
- [25] Kreyling WG, Abdelmonem AM, Ali Z, Alves F, Geiser M, Haberl N, Hartmann R, Hirn S, Jimenez de Aberasturi D, Kantner K, Khadem-Saba G, Montenegro J-M, Rejman J, Rojo T, Ruiz de Larramendi I, Ufartes R, Wenk A, Parak WJ. In vivo integrity of polymer-coated gold nanoparticles. *Nat Nanotech* 2015;10:619-+.
- [26] Klinker K, Barz M. Polypept(o)ides: Hybrid Systems Based on Polypeptides and Polypeptoids. *Macromol Rapid Commun* 2015;36:1943-57.
- [27] Young MA, Malavalli A, Winslow N, Vandegriff KD, Winslow RM. Toxicity and hemodynamic effects after single dose administration of MalPEG-hemoglobin (MP4) in rhesus monkeys. *Transl Res* 2007;149:333-42.

- [28] Kelly PM, Åberg C, Polo E, O'Connell A, Cookman J, Fallon J, KrpetićŽeljka, Dawson KA. Mapping protein binding sites on the biomolecular corona of nanoparticles. *Nat Nano* 2015;10:472-9.
- [29] Peng Q, Zhang S, Yang Q, Zhang T, Wei X-Q, Jiang L, Zhang C-L, Chen Q-M, Zhang Z-R, Lin Y-F. Preformed albumin corona, a protective coating for nanoparticles based drug delivery system. *Biomaterials* 2013;34:8521-30.
- [30] Aires A, Ocampo SM, Cabrera D, de la Cueva L, Salas G, Teran FJ, Cortajarena AL. BSA-coated magnetic nanoparticles for improved therapeutic properties. *J Mater Chem B* 2015;3:6239-47.
- [31] Mariam J, Sivakami S, Dongre PM. Albumin corona on nanoparticles - a strategic approach in drug delivery. *Drug delivery* 2015;1-9.
- [32] Wang X, Xing X, Zhang B, Liu F, Cheng Y, Shi D. Surface engineered antifouling optomagnetic SPIONs for bimodal targeted imaging of pancreatic cancer cells. *Int J Nanomedicine* 2014;9:1601-15.
- [33] Ling D, Lee N, Hyeon T. Chemical Synthesis and Assembly of Uniformly Sized Iron Oxide Nanoparticles for Medical Applications. *Acc Chem Res* 2015;48:1276-85.
- [34] Colombo M, Carregal-Romero S, Casula MF, Gutierrez L, Morales MP, Boehm IB, Heverhagen JT, Prosperi D, Parak WJ. Biological applications of magnetic nanoparticles. *Chem Soc Rev* 2012;41:4306-34.
- [35] Carenza E, Barcelo V, Morancho A, Levander L, Boada C, Laromaine A, Roig A, Montaner J, Rosell A. In vitro angiogenic performance and in vivo brain targeting of magnetized endothelial progenitor cells for neurorepair therapies. *Nanomedicine* 2014;10:225-34.
- [36] Pascu O, Carenza E, Gich M, Estrade S, Peiro F, Herranz G, Roig A. Surface Reactivity of Iron Oxide Nanoparticles by Microwave-Assisted Synthesis; Comparison with the Thermal Decomposition Route. *J Phys Chem C* 2012;116:15108-16.
- [37] Yu S-M, Laromaine A, Roig A. Enhanced stability of superparamagnetic iron oxide nanoparticles in biological media using a pH adjusted-BSA adsorption protocol. *Journal of Nanoparticle Research* 2014;16.
- [38] Gonzalez-Moragas L, Yu S-M, Carenza E, Laromaine A, Roig A. Protective Effects of Bovine Serum Albumin on Superparamagnetic Iron Oxide Nanoparticles Evaluated in the Nematode *Caenorhabditis elegans*. *ACS Biomater Sci Eng* 2015;1:1129-38.
- [39] Gonzalez-Moragas L, Roig A, Laromaine A. *C. elegans* as a tool for in vivo nanoparticle assessment. *Adv Colloid Interface Sci* 2015;219:10-26.
- [40] Leung MCK, Williams PL, Benedetto A, Au C, Helmcke KJ, Aschner M, Meyer JN. *Caenorhabditis elegans*: An Emerging Model in Biomedical and Environmental Toxicology. *Toxicol Sci* 2008;106:5-28.
- [41] Tejeda-Benitez L, Olivero-Verbel J. *Caenorhabditis elegans*, a Biological Model for Research in Toxicology. In: de Voogt WP, editor. *Rev Environ Contam T*. Cham: Springer International Publishing; 2016. p. 1-35.
- [42] Hall DH, Altun ZF. *C. Elegans Atlas*: Cold Spring Harbor Laboratory Press; 2008.
- [43] McGhee JD. The *C. elegans* intestine. *WormBook : the online review of C elegans biology* 2007:1-36.
- [44] Yu S, Peralvarez-Marin A, Minelli C, Faraudo J, Roig A, Laromaine A. Albumin-coated SPIONs: an experimental and theoretical evaluation of protein conformation, binding affinity and competition with serum proteins. *Nanoscale* 2016.
- [45] Taherian A, Li X, Liu Y, Haas TA. Differences in integrin expression and signaling within human breast cancer cells. *Bmc Cancer* 2011;11.

- [46] Lin J, Yu Y, Li B, Huang H, Lin S, Li C, Su Y, Feng S, Chen G, Li Y, Huang Z, Zeng H, Chen R. Electrical pulse - mediated enhanced delivery of silver nanoparticles into living suspension cells for surface enhanced Raman spectroscopy. *Laser Phys Lett* 2012;9:240-6.
- [47] Baier G, Costa C, Zeller A, Baumann D, Sayer C, Araujo PHH, Mailaender V, Musyanovych A, Landfester K. BSA Adsorption on Differently Charged Polystyrene Nanoparticles using Isothermal Titration Calorimetry and the Influence on Cellular Uptake. *Macromol Biosci* 2011;11:628-38.
- [48] Ruge CA, Kirch J, Canadas O, Schneider M, Perez-Gil J, Schaefer UF, Casals C, Lehr C-M. Uptake of nanoparticles by alveolar macrophages is triggered by surfactant protein A. *Nanomedicine* 2011;7:690-3.
- [49] Monopoli MP, Aberg C, Salvati A, Dawson KA. Biomolecular coronas provide the biological identity of nanosized materials. *Nat Nanotech* 2012;7:779-86.
- [50] Gillard G, Shafaq-Zadah M, Nicolle O, Damaj R, Pecreaux J, Michaux G. Control of E-cadherin apical localisation and morphogenesis by a SOAP-1/AP-1/clathrin pathway in C-elegans epidermal cells. *Development* 2015;142:1684-94.
- [51] Shafaq-Zadah M, Brocard L, Solari F, Michaux G. AP-1 is required for the maintenance of apico-basal polarity in the C. elegans intestine. *Development* 2012;139:2061-70.
- [52] Martorell P, Llopis S, Gonzalez N, Monton F, Ortiz P, Genoves S, Ramon D. *Caenorhabditis elegans* as a model to study the effectiveness and metabolic targets of dietary supplements used for obesity treatment: the specific case of a conjugated linoleic acid mixture (Tonalin). *J Agric Food Chem* 2012;60:11071-9.
- [53] Michaux G, Legouis R, Labouesse M. Epithelial biology: lessons from *Caenorhabditis elegans*. *Gene* 2001;277:83-100.
- [54] Chauhan VM, Orsi G, Brown A, Pritchard DI, Aylott JW. Mapping the pharyngeal and intestinal pH of *Caenorhabditis elegans* and real-time luminal pH oscillations using extended dynamic range pH-sensitive nanosensors. *ACS nano* 2013;7:5577-87.
- [55] McGhee JD. The *Caenorhabditis elegans* intestine. *Wiley Interdisciplinary Reviews-Developmental Biology* 2013;2:347-67.
- [56] Gobel V, Barrett PL, Hall DH, Fleming JT. Lumen morphogenesis in C-elegans requires the membrane-cytoskeleton linker erm-1. *Dev Cell* 2004;6:865-73.
- [57] Gonzalez-Moragas L, Berto P, Vilches C, Quidant R, Kolovou A, Santarella-Mellwig R, Schwab Y, Roig A, Laromaine A. In vivo testing of gold nanoparticles using the *Caenorhabditis elegans* model organism. Submitted 2016.

GRAPHICAL ABSTRACT

Bio-identity and fate of albumin-coated SPIONs evaluated in cells and by the *C. elegans* model

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Supporting Information

Bio-identity and fate of albumin coated SPIONs evaluated in cells and by the *C. elegans* model

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Figure S1. a) TEM image of the synthesized $\gamma\text{-Fe}_2\text{O}_3$ nanoparticles, insets contains their response to an external magnet, the selected-area electron diffraction (SAED) indexed to diffraction bands corresponding to maghemite, and the size-distribution histogram yielding a mean particle size = $6 \pm 1\text{ nm}$, b) cryo-TEM image of the aqueous colloidal dispersion of citrate-coated SPION. C-SPIONs and a schematic representation of such, c) hydrodynamic diameter of the water-stable C-SPIONs as-synthesized and after six months and d) M(H) hysteresis curve at 5 K, the inset contains field-cooled and zero-field-cooled curves of M(T) with a 50 Oe applied field. A blocking temperature below 50 K is apparent.

Figure S2. Changes in the hydrodynamic diameter (D_h) of the C-SPIONs and BSA-SPIONs in the MDA-MB231 cell media at different incubation times; 0, 1, and 24 h.

Figure S3. Hydrodynamic size distribution and D_h (inset) in citrate buffer at several measurement time-points of the C-SPIONs (a,c) and BSA-SPIONs (b,d).

Figure S4. Upper panel: TEM images of C-SPIONs in citrate buffer at day 1, 2, 3, and 7; middle panel: SAED of these systems. At day 7 only the most intense band is visible; lower panel: particle-size histograms.

Figure S5. Upper panel: TEM images of BSA-SPIONs in citrate buffer at day 1, 7, 15, and 25 days; middle panel: SAED of these systems. At day 25 only the most intense diffraction bands are seen, lower panel: includes particle-size histograms.

Figure S6. Size evolution vs. days of incubation in a citrate-buffer solution of C-SPIONs (left) and BSA-SPIONs (right), monitored by TEM.

Figure S7. TEM image showing individual SPIONs (indicated with arrowheads) in the glycocalix of the *C. elegans* intestine. The dotted line marks the limit of the glycocalix.

Figure S8. TEM and HAADF STEM images of *C. elegans* cross-sections showing the intestinal lumen (panel A,B) and endosomes of the intestinal cells (panel C). The high intensity of the SPIONs both inside the lumen and the endosomes of treated worms can be attributed to Fe ($Z=26$), whereas C ($Z=6$) has lower intensity. Such bright spots are not observed in the control worms. EELS (panel D) further confirmed the presence of iron in the endosomes of SPION-treated worms, but not in control ones.

Figure S9. Size distribution of as-synthesized SPIONs and SPIONs in *C. elegans*. The plots correspond to the Gaussian fitting of the size- distribution histograms obtained after measuring more than 150 nanoparticles from TEM images. The mean size value decreases in the case of the C-SPIONs, indicated as percentage of the initial size of the SPIONs both in the anterior and posterior part of the *C. elegans* intestine when compared with the as-synthesized mean size. Mean particle size remains unchanged for BSA-SPIONs.

Figure S10. TEM image of the lumen of the anus of SPION-treated *C. elegans*.