# *In vitro* biocompatibility study of sub-5nm silica-coated magnetic iron oxide fluorescent nanoparticles for potential biomedical application

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# Supplementary Methods

### Nanoparticles synthesis

Magnetite nanoparticles were synthesized by procedure reported in Vayssières *et al.*, [1] using simpler co-precipitation of ions $Fe^{2+}$ and $Fe^{3+}$ in a solution containing a mixture of $Fe(NO)_3$ and $FeCl_2$ and $NaNO_3$, in a 3M $NaNO_3$ solution. The pH of the medium was kept constant in a range from 10 to 12, by addition of the corresponding base (NaOH). The reactions were performed at room temperature, controlled atmosphere, with continuous bubbling of nitrogen. $Fe_3O_4$ nanopowder was collected after centrifugation (11000 rpm, 10 min) and dried under dynamic vacuum.

1. Vayssieres, L., Chaneac, C., Tronc, E. & Jolivet, J. P. Size Tailoring of Magnetite Particles Formed by Aqueous Precipitation: An Example of Thermodynamic Stability of Nanometric Oxide Particles. *J. Colloid Interface Sci.* **205**, 205-212 (1998).

### X-ray diffraction analysis (XRD)

XRD measurements were performed using a powder diffractometer (Philips X'Pert Pro) operating in the reflection mode with CuKa radiation and equipped with a graphite back monochromator.

### Magnetization measurements

The magnetic property of the nanoparticles was analyzed by vibrating sample magnetometry (VSM) and superconducting quantum interference device (SQUID) at a magnetic field of 100 Oe for both field-cooling (FC) and zero-field-cooling (ZFC).

### Phase contrast and Fluorescence Microscopy

The CaCo-2 cells ($2x10^4$ cells/$cm^2$) were seeded on cover glasses in a 24-well plate and allowed to adhere for 24h. Then sub-5 SIO-Fl nanoparticles, treated ultrasonically for 5 min to break up aggregation, were dispersed in cell culture medium and added to the CaCo-2 cells at a concentration of 10 µg/ml, 50µg/ml and 100 µg/ml. 48h after exposure the cells were repeatedly washed with PBS and fixed in paraformaldehyde 4% at 4°C for 10 min, then washed twice in $Ca^{2+}$/$Mg^{2+}$-free PBS and stained for nuclei localization with Hoechst 33342 (trihydrochloride trihydrate). Phase contrast and fluorescence measurements were performed by using an inverted microscope (Olympus IX51, RT Slider SPOT - Diagnostic instruments, Sterling Heights, MI, USA) equipped

with a 20X, 40X and 60X objective and with a cooled CCD camera (Spot RT Slider, Diagnostic Instruments).

**Fluorescence microscopy analysis of Rhodamine phalloidin-labelled F-actin**

The CaCo-2 cells ($2x10^4$ cells/$cm^2$) were seeded on cover glasses in a 24-well plate and after 24 h of culture incubated with sub-5 SIO-Fl nanoparticles at three different concentrations (10µg/ml, 50µg/ml, and 100 µg/ml). 7 days after exposure the cells were repeatedly washed with PBS and fixed in paraformaldehyde 4% at 4°C for 10 min, washed twice in $Ca^{2+}$/$Mg^{2+}$-free PBS and permeabilized at room temperature for 15 min (0.1% Triton X-100, 1% BSA; Sigma-Aldrich). The cells were also incubated with phalloidin tetramethylrhodamine isothiocyanate conjugated (1:100), an anti-actin toxin (Sigma), in a blocking buffer for 1 h. Cells were washed three times with PBS, counterstained for nuclei localization with Hoechst 33342 (trihydrochloride–trihydrate), and examined. Fluorescence measurements were using an inverted microscope (Olympus IX51, RT Slider SPOT - Diagnostic instruments, Sterling Heights, MI, USA) equipped with a 20X, 40X and 60X objective and with a cooled CCD camera (Spot RT Slider, Diagnostic Instruments).

**Real-time quantitative reverse transcriptase polymerase chain reaction analysis (qPCR)**

Amount of target was calculated using the $2^{-\Delta\Delta Ct}$ equation.

$^{\Delta}Ct$ = (average target Ct – average GAPDH Ct)

$^{\Delta\Delta}Ct$ = (average $^{\Delta}Ct$ treated sample – average $^{\Delta}Ct$ untreated sample)

Before using $^{\Delta\Delta}Ct$ method for quantification, we performed a validation experiment to demonstrate that efficiency of target genes and reference GAPDH were equal. Real-time PCR was performed with Sybr Green I Mastermix (Applied Biosystems) using an ABI PRISMTM 7000 Sequence Detection System. Each reaction was run in triplicate and contained 0,5 µl of cDNA template along with 250 nM primers in a final reaction volume of 25 µl. The genes investigated were ALP1 and VIL1. The specific primers used are the following:

ALP1: 5’-ccaggacatcgccactcag-3’; 5’-tcagtgcggttccacacata-3’

VIL1: 5’-gctatctatggtgtgggaag-3’; 5'-cctgtagtctcttggtgttg-3’

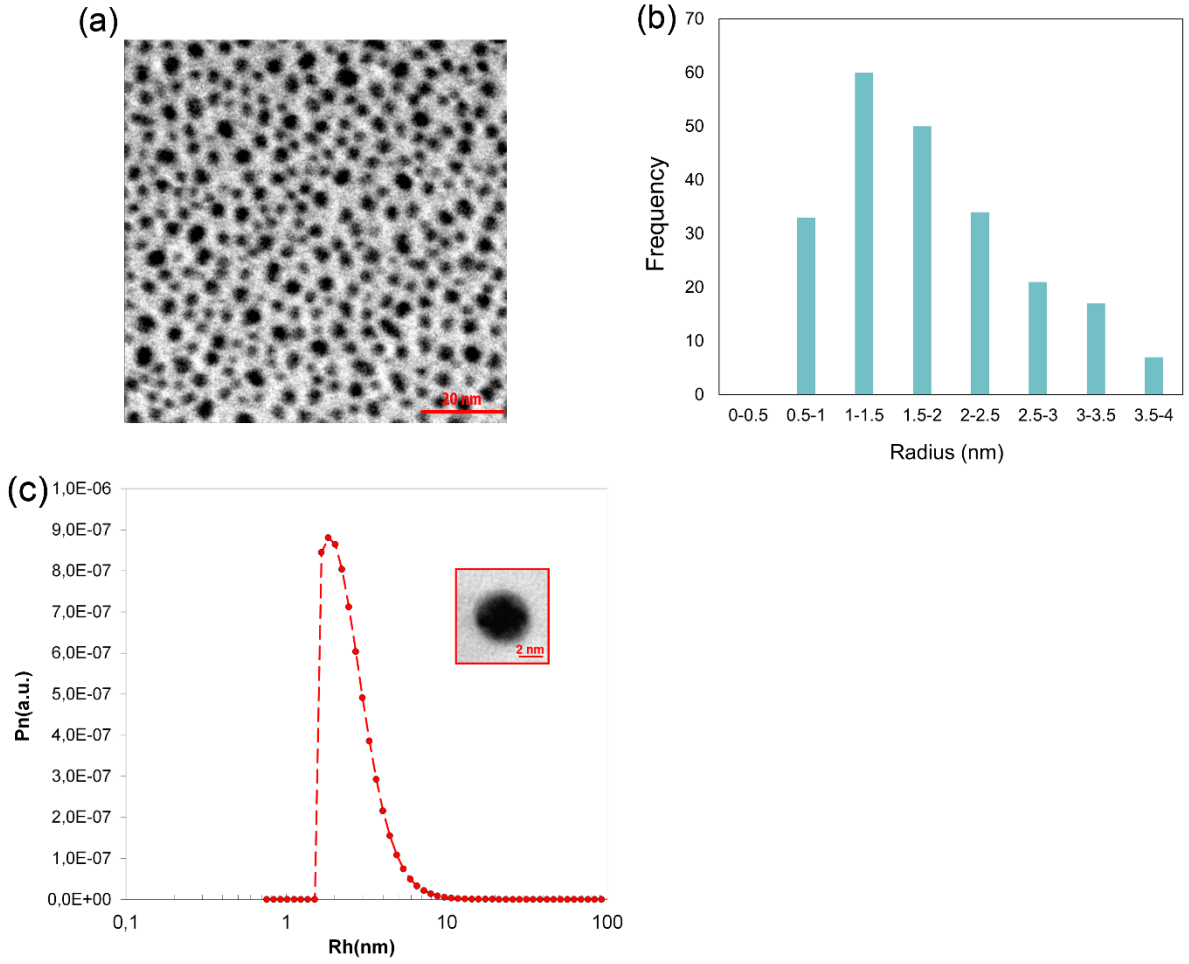
GAPDH: 5’-catcatctctgccccctct-3’; 5’-caaagttgtcatggatgacct-3’

Cycling parameters were 50°C for 2 min., then 95°C for 10 min. in order to activate DNA polymerase, then 40 cycles of 95°C for 15 seconds and finally 60°C for 1 min. The Melting curves were performed using Dissociation Curves software (Applied Biosystems) to ensure that only a single product was amplified. As negative controls, we used tubes where RNA or reverse transcriptase was omitted during the RT reaction.

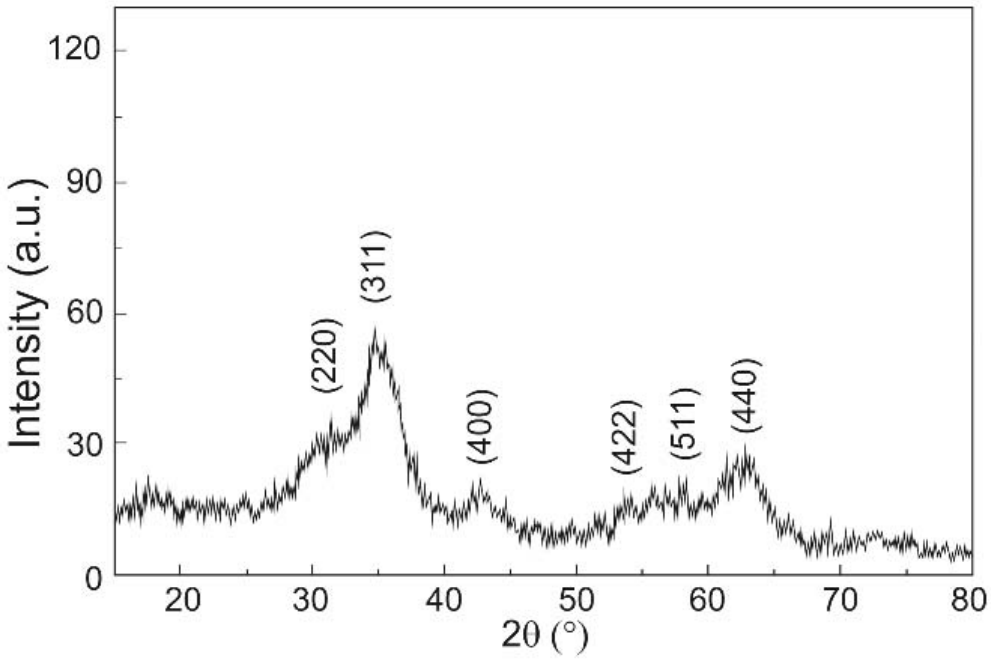
**TEM analysis**

The SIO nanoparticles, treated ultrasonically for 5 min to break up aggregation, were dispersed in cell culture medium and added to the CaCo-2 cells at a concentration of 50µg/ml. After 1h exposure the cells were repeatedly washed with PBS and fixed using glutaraldehyde (2.5%) for 1 h. The cells were washed several times with 0.15M cacodylate buffer and post-fixed with 1.5% osmium tetroxide for 1 h. After further washing with cacodylate buffer, the samples were dehydrated through an ethanol gradient from 70% to 100%, embedded and cut into ultrathin sections and examined as previously described.

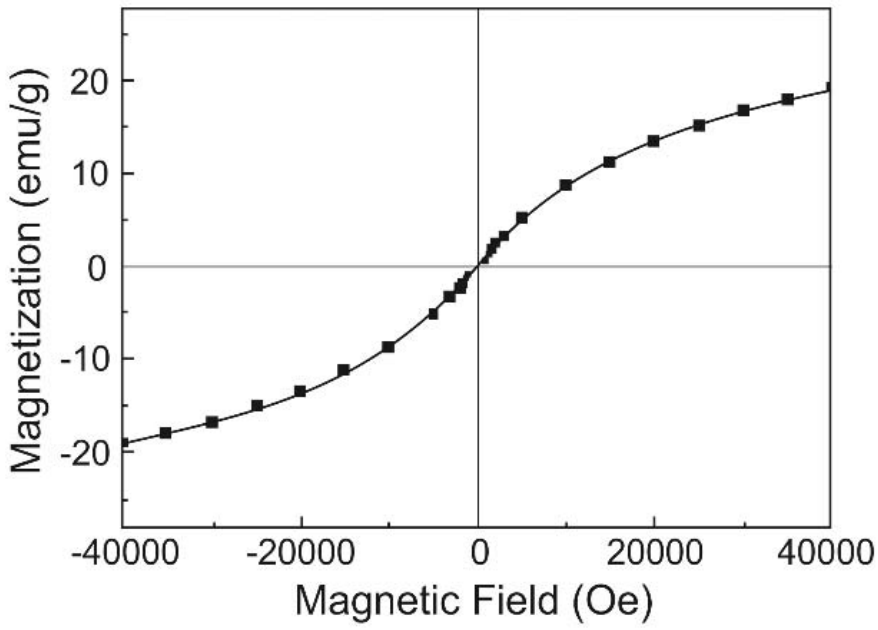
# Supplementary Figures



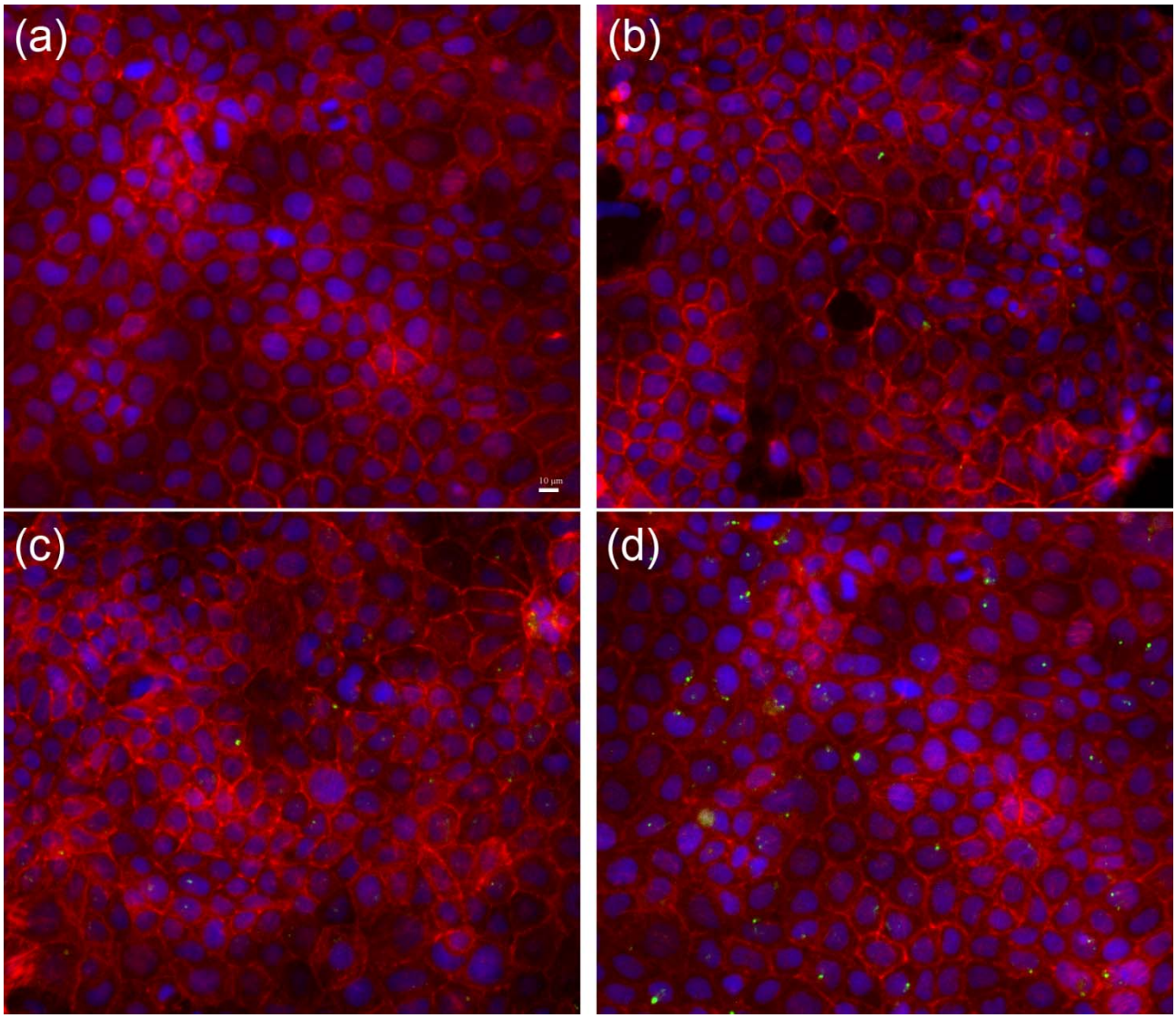

**Supplementary Figure S1. Characterization of 3 nm ultrafine superparamagnetic nanoparticles.** Representative TEM image of spherical 3 nm ultrafine superparamagnetic nanoparticles (a). 3 nm ultrafine nanoparticles size distribution obtained from the TEM images (sample size=220 nanoparticles) (b) and from DLS (c). A representative high-resolution image of a single 3 nm ultrafine nanoparticles is reported in the (c) inset.



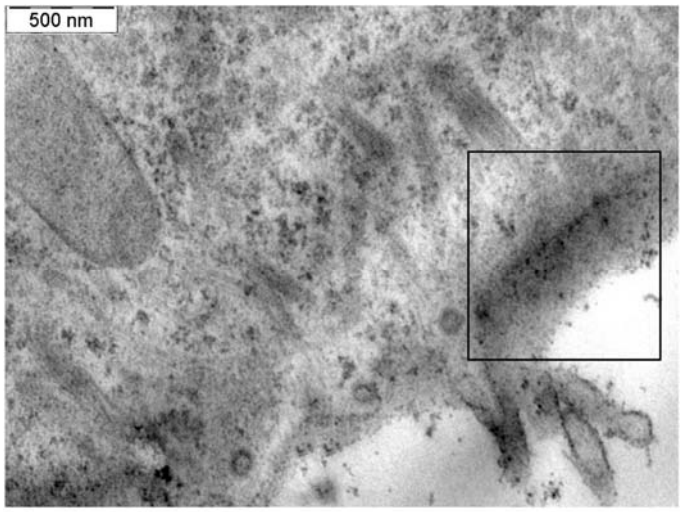

**Supplementary Figure S2. X-ray diffraction pattern of 3 nm ultrafine nanoparticles.**




**Supplementary Figure S3. Magnetization curve of 3 nm ultrafine nanoparticles obtained by VSM at room temperature.**




**Supplementary Figure S4. Fluorescence microscopy analysis of rhodamine phalloidin-labelled F-actin (red) of CaCo-2 cells.** Untreated cells (a) and cells treated with 10µg/ml (b), 50 µg/ml (c) and 100 µg/ml (d) of sub-5nm silica-coated magnetic iron oxide fluorescent nanoparticles (green) after 7 days of exposure. The sub-5 SIO-Fl nanoparticles inside the cells are shown and do not interfere with the cytoskeletal organization. Nuclei are counterstained with Hoechst (blue). Photographs were taken at a magnification of 20X.




**Supplementary Figure S5. TEM image of Caco-2 cells after 1h of exposure with SIO nanoparticles (50µg/ml).** The box highlights the iron oxide nanoparticles within Caco-2 cells.

# Supplementary Table

| Time (h) | 1 | 12 | 24 | 36 | 48 | 60 |
|---|---|---|---|---|---|---|
| $D_H$ (nm) | 8.5±2.2 | 9.4±2.8 | 9.5±2.8 | 9.3±2.7 | 9.5±3.1 | 9.4±2.9 |
| $\zeta$ (mV) | -15.5±3.4 | -9.2±3.1 | -9.2±2.8 | -9.1±3.0 | -9.1±3.1 | -9.2±3.4 |

**Supplementary Table S1. Dynamic Light Scattering and Zeta potential measurements of SIO nanoparticles after exposure to complete cell culture medium.**