1. Introduction

Engineered nanoparticles (NPs) have attracted much attention during the last decade because of their size-related properties, large surface-to-volume ratios and high surface reactivity. Inorganic NPs are already applied in biotechnology, nanomedicine, and materials science. Safety issues have developed however concerning their potential cyto- and genotoxicity. For in vivo and in vitro experimentations, recent developments have heightened the need for simple and facile methods to measure the amount of nanoparticles taken up by cells or tissues. In this work, a rapid and highly sensitive method for quantifying the uptake of iron oxide nanoparticles in mammalian cells is reported. The approach exploits the digestion of incubated cells with concentrated hydrochloric acid reactant and a colorimetric-based UV–visible absorption technique. The technique allows the detection of iron in cells over 4 decades in masses from 0.03 to 300 picograms per cell. Applied on particles of different surface chemistry and sizes, the protocol demonstrates that the coating is the key parameter in the nanoparticle/cell interactions. The data are corroborated by scanning and transmission electron microscopy, and the results stress the importance of resiliently adsorbed nanoparticles at the plasma membrane.
quantitative flow cytometry was also shown to be a straightforward method,[11] from which the proportions of adsorbed versus internalized NPs can be determined,[12]

In the present communication, we report on a facile and cost-effective method to quantify the total iron content of mammalian cells labeled with maghemite (γ-Fe$_2$O$_3$) NPs. For iron oxide-based materials, several analytical techniques were designed during the last years, including magnetophoresis,[13,14] superconducting quantum interference device,[15] magnetic resonance imaging,[16,17] relaxometry,[16–18] and UV–visible spectrometry.[18–20] Concerning this later technique, reports from the literature exploit colorimetric assays but mention different chemical reactants for the detection of iron. Combined with the acid digestion of cells, ferrozine, a chelating agent of ferrous ions (Fe$^{2+}$) is probably the most commonly used biomarkers. The UV–visible absorbance of Fe–ferrozine complexes exhibits a characteristic peak at 562 nm and its amplitude scales linearly with concentration. This analytical technique provides a highly sensitive detection method,[19] but is not directly applicable to maghemite, which contains iron atoms in their Fe(III) oxidation state. Hydrochloric acid has also been examined as a biomarker for iron, alone,[18,20] or in conjunction with other acids and reducing agents (i.e., potassium ferrocyanide as in the Prussian blue staining protocol).[21] Concerning the sensitivity of the techniques mentioned previously, 1 pg/cell appears as a typical limit.[13–15,17,18]

In this work, we developed a new protocol, dubbed MILC for mass of metal internalized/adsorbed by living cells, which appears as a simple, rapid, and highly sensitive method for quantifying the uptake of iron oxide NPs in mammalian cells. Our approach exploits the digestion of incubated cells with concentrated hydrochloric acid reactant and a colorimetric-based UV–vis absorption technique, as described above. In contrast to previous reports, MILC uses the entire absorption spectrum between 200 and 1000 nm and the fitting against calibrated references to derive the iron content. As a result, this new technique allows the detection of iron in cells over 4 decades in masses, typically between 0.03 to 300 pg/cell. Applied on maghemite NPs of different coating and sizes, MILC demonstrates that the coating is the key parameter in the NP/cell interactions. The data are corroborated by scanning and transmission electron microscopy, and stress the importance of resilient adsorbed NPs at the cell membrane.

2. Results and Discussion

2.1. The MILC Protocol

The amount of iron taken up by the cells was measured following the protocol MILC. MILC makes use of UV–vis spectrometry to determine the iron concentration from pelleted cells dissolved in concentrated hydrochloric acid. The results are the mass of iron, $M_{\text{Fe}}$, expressed in picograms per cell. We provide here a description of the data analysis, and refer the reader to the Experimental Section for the description of the methodology. MILC proceeds in three steps. The live cells are first cultured and incubated with the particles for times between $t_{\text{inc}} = 5$ min to 24 h. The supernatant is removed and the cells are thoroughly washed with phosphate buffered saline (PBS). The cells are then tryspinized, numbered using a Mallassez chamber, and centrifuged to get a pellet at the bottom of the Falcon tube. The pellets are dissolved in hydrochloric acid (35%), and later investigated by UV–vis spectrophotometry. The cell pellets dissolved in HCl display the yellow color characteristic of tetrachloroferrate ions FeCl$_4^{-}$ (Figure 1a). The absorbance of the dissolved pellets is compared to those of iron oxide and of fibroblasts determined separately. Figure 1b shows the absorbance curves at 1 pg/µL for γ-Fe$_2$O$_3$ and at 1 cell/µL for NIH/3T3. Over broad ranges of wavelengths, both exhibit characteristic dependences such as exponential decays of the form $\text{Abs}(\lambda) = -\exp(-\lambda/\lambda_0)$. Exponential decreases indicated by straight lines in the figure were obtained for $\lambda_0 = 21$ and 358 nm, respectively. For fibroblasts treated with NPs, the absorbance expresses as a linear combination of the two above contributions. The number densities of cells retrieved from the absorbance were checked with direct Malassez counting for further validation. Figure 1c displays the UV–vis absorbance of a series of cell samples prepared with increasing iron oxide concentrations, [Fe] = 0.1, 1, and 10 mM. The continuous lines through the data points...
are linear combinations of the two reference curves shown in Figure 1b, from which the masses of iron per cell are derived. One gets here $M_{Fe} = 1, 4, \text{ and } 12 \text{ pg/cell}$ respectively, with an uncertainty of 10%. Assuming for the absorbance an absolute uncertainty of 0.03, the minimum amount of iron detectable by this technique is 0.03 pg/cell, i.e., 30 fg/cell. With MILC, the high sensitivity arises from the fact that the whole UV–vis spectrum is taken into account in the adjustment.\cite{18–20}

### 2.2. Effect of Coating, Particle Size, and Cell Line

Figure 2 displays the masses of internalized and adsorbed iron obtained by varying different physico-chemical and biological parameters: the coating of the particles (Figure 2a), the particle size (Figure 2b), and the cell type (Figure 2c). The straight line in Figure 2 depicts the maximum amount of iron $M_{Fe}^{\text{max}}$ that can be taken up by a cell. It is calculated by dividing the mass of iron present in the supernatant by the number of cells in the assay ($3 \times 10^6$ fibroblasts exposed to a concentration of 1 mM correspond to $M_{Fe}^{\text{max}} = 37 \text{ pg/cell}$). Note that this limit is independent on the particle size or aggregation state, and thus appears similarly in the three diagrams. For uncoated and citrate-coated NPs, $M_{Fe}$ increases linearly with [Fe] and levels off above 10 mM. Here, the masses of internalized/adsorbed iron are high and above 100 pg/cell. In the linear parts, i.e., below 3 mM, it represents about 30% of the maximum value $M_{Fe}^{\text{max}}$. For the neutral PAA$_{3K}$-b-PAM$_{30K}$ and charged PAA$_{3K}$ coating, the [Fe] variations are similar, but the saturation plateaus are found at much lower levels (0.2 and 10 pg/cell as compared to 100 pg/cell for citrate); PAA and PAM represent poly(acrylic acid) and poly(acrylamide), respectively. As shown in Figure 2a, MILC allows the detection of iron in cells over 4 decades in masses, typically between 0.03 to 300 pg/cell. Figure 2b explores the role of the NP size on the internalized amount, all NPs being now coated with PAA$_{3K}$. The mass of iron per NIH/3T3 cell is displayed for magnetic cores varying between 6.7 and 10.7 nm (see the Supporting Information Section 1, SI-1). The data of the four specimens agree well with each other, suggesting that for this specific coating, there is no major effect of the NP size on the internalization amounts. Figure 2c compares the $M_{Fe}$ values for cells adhering on a substrate (NIH/3T3 fibroblast) and in suspensions (2139 lymphoblasts, THP1 monocytes). PAA$_{3K}$-γ-Fe$_2$O$_3$ NPs with core size 8.3 nm were used in this assay. Lymphoblastoid and monocytes are in the 0.1–1 pg/cell range that is slightly lower than for fibroblasts. From these experiments, it is found that the coating is the most important parameter affecting the internalized/adsorbed amounts of iron.\cite{22,23} with amplitudes of variation larger than 1000 between the saturation levels (0.2 pg/cell for PAA$_{3K}$-b-PAM$_{30K}$ against 300 pg/cell for uncoated NPs). Cell type plays a minor role, whereas NP size has no noticeable impact. This later result could be explained by the fact that the sizes of the PAA$_{3K}$-coated particles cover a narrow range in the present work. The diameters of the magnetic cores range for instance from 6.7 to 10.7 nm (SI-1). Previous studies have reported size-dependent uptake, but for NPs spanning over a broader size range, typically up to 1 μm.\cite{23–26} Although these studies did not discuss the stability of the NPs in biological fluids, it is found as a general trend that the numbers on NPs internalized decrease with the size.

### 2.3. Localization of Particles Inside the Cells

Fibroblasts seeded with iron oxide NPs were further investigated by transmission electron microscopy (TEM). Figure 3a AND d provides representative images of NIH/3T3 cells
incubated with Cit–γ-Fe₂O₃ and PAA₂K–γ-Fe₂O₃, respectively. The experimental conditions were an incubation time of 24 h and an iron concentration of 10 mM. The corresponding $M_{Fe}$ levels were 100 ± 20 and 7 ± 2 pg/cell respectively (Figure 2a).

A careful analysis of the TEM images shows that the NPs were primarily located in membrane-bound compartments or endosomes.[13,14,27–30] Close-up views of the selected areas (rectangles) clearly identify the lipidic membrane separating the cytosol from the NPs (Figure 3c-e). In this work, NPs were found neither in the cytosol nor in the nucleus. A statistical analysis of the endosome sizes was performed and revealed weak variations as a function of concentration and coating. For citrate-coated NPs at 1 and 10 mM, the endosomal distribution was peaked at 500 and 800 nm; respectively (Figure 3c). Compartments larger than 1 μm were also detected. For the PAA₂K–γ-Fe₂O₃ at 10 mM, the average size was 500 nm, i.e., in relative accordance with those of the cell control (data not shown).

The similarities of the iron oxide loaded compartments in Figure 3a,d suggest similar mechanisms of entry into the cells, i.e., endocytosis.[13,31] The major difference between the two coating specimen lies in the spatial distribution of the particles inside endosomes; with citrate, the NPs appear as aggregated under the form of clusters, whereas with polymers they are randomly spread and unassociated. With the PAA₂K-coated NPs, the endosomes were also more homogeneously filled. In conclusion to this part, and in good agreement with the literature,[12,13,27–30,32] we have found that the carboxylate-coated NPs are internalized by the NIH/3T3 fibroblasts and located in endosomal membrane-bound compartments. The coating has here a moderate impact on the endosome size distributions. These results contrast with those obtained by the MILC technique that highlights large differences in the internalized/adsorbed iron masses. They also suggest that large quantities of NPs should be extracellular and located on the plasma membrane.

2.4. Adsorption and Internalization Kinetics

Figure 4 displays the variations of the mass of internalized and/or adsorbed iron with the incubation time $t_{inc}$ upon addition of uncoated, citrate, and PAA₂K-coated particles. The investigated concentrations were [Fe] = 4 mM for the bare particles, and 1 and 10 mM for the coated specimens. As noted earlier, the evolution of the recorded masses at 37 °C imparts two interaction mechanisms: the adsorption of the NPs on the plasma membrane and their internalization into endocytic vesicles.[12,13,28,29] For the present case studies, the mass of internalized/adsorbed iron increases following a similar pattern: within the first 5 min of contact with NPs, $M_{Fe}(t_{inc})$ jumps to an initial value noted $M_{Fe}^0$ which depends on both concentration and coating. This initial jump is followed by a progressive leveling-off towards a saturation value noted $M_{Fe}^\infty$. In these assays, the $M_{Fe}^\infty$ values were consistent with those recorded at 24 h (Figure 2a). The continuous lines between the data points are exponential growth functions of the form:

$$M_{Fe}(t_{inc}) = M_{Fe}^0 + \Delta M_{Fe} \left(1 - e^{-\frac{t_{inc}}{\tau}}\right)$$  \hspace{1cm} (1)$$

where $\Delta M_{Fe} = M_{Fe}^\infty - M_{Fe}^0$ denotes the difference between the final and initial states and $\tau$ the characteristic time of the kinetics. For the time profiles in Figure 4, the $\tau$ values are of the order of 300–500 min for all specimens tested.
Numerous protrusions identified as microvilli appeared as entangled by the clusters, as identified in Figure 5c or acquired a more spherical shape (size 200 nm). Fibroblasts incubated with citrate-coated particles appeared very differently. Figure 5d provides an image of a cell that is merely covered with particles and particle clusters. There, the deposited materials form a thick and porous layer of aggregates, the largest being of a few micrometers (arrowhead). On this image the plasma membrane cannot be seen. Citrate-coated particles also induced a dramatic transition in the morphology of the microvilli. They are now elongated threads with an average length of 1.2 μm, going up to 3 μm. In a few examples, the microvilli were terminated with a sphere covered with an iron oxide cap (inset). Other SEM images obtained with citrate showed similar features, but with less adsorbed material at the membrane. Phase-contrast optical microscopy provided further evidence that NP clusters were still present at the cell surface after an incubation time of 24 h (SI-3), confirming the strong resilience of the adsorbed layer. In conclusion, the SEM studies demonstrate the importance of using visualization techniques to determine the distribution of particles on a cellular and subcellular level. This technique enables us to infer that the large MFe values found by MILC for Cit–γ-Fe2O3 can be attributed to extracellular iron oxide adsorbed at the plasma membrane in the form of highly heterogeneous clusters.

**2.5. Particles at the Plasma Membrane**

Scanning electron microscopy (SEM) experiments were performed to visualize the plasma membrane of cells treated with Cit–γ-Fe2O3 and PAA2K–γ-Fe2O3 NPs. The experimental conditions investigated were an incubation time of 2 h and an iron concentration of 10 mM. The corresponding MFe levels found by MILC were 24 ± 5 and 1.1 ± 0.2 pg/cell, respectively, i.e., ~20 times larger for citrate than for PAA2K (Figure 4).

Figure 5a shows a SEM image of a control untreated cell. The cylindrical body of the NIH/3T3 exhibits its surface numerous protrusions identified as microvilli and indicated by arrows. Formed as cell extensions starting from the membrane, microvilli are involved in a wide variety of functions, including internalization, cellular adhesion, and mechanotransduction. For NIH/3T3 fibroblasts, their average length, diameter, and density are 600 nm, 160 nm, and 3 μm⁻², respectively, in good agreement with literature data (see Supporting information SI-2).

Figure 5b and c display representative images of the plasma membrane for a cell incubated with PAA2K–γ-Fe2O3 NPs. The cell body and the microvilli covering the cell surface maintained their morphologies after a 2 h exposure. The average length and density of the protrusions remained also unchanged at 690 nm and 3 μm⁻² respectively (SI-2). Scattered on the cell surface, the NPs were difficult to localize. On a few locations, SEM showed particles attached to the cell membrane as single entities or as clusters. In some other cases, the microvilli appeared as entangled by the clusters, as identified in Figure 5c or acquired a more spherical shape (size 200 nm). Fibroblasts incubated with citrate-coated particles appeared very differently. Figure 5d provides an image of a cell that is merely covered with particles and particle clusters. There, the deposited materials form a thick and porous layer of aggregates, the largest being of a few micrometers (arrowhead). On this image the plasma membrane cannot be seen. Citrate-coated particles also induced a dramatic transition in the morphology of the microvilli. They are now elongated threads with an average length of 1.2 μm, going up to 3 μm. In a few examples, the microvilli were terminated with a sphere covered with an iron oxide cap (inset). Other SEM images obtained with citrate show similar features, but with less adsorbed material at the membrane. Phase-contrast optical microscopy provided further evidence that NP clusters were still present at the cell surface after an incubation time of 24 h (SI-3), confirming the strong resilience of the adsorbed layer. In conclusion, the SEM studies demonstrate the importance of using visualization techniques to determine the distribution of particles on a cellular and subcellular level. This technique enables us to infer that the large MFe values found by MILC for Cit–γ-Fe2O3 can be attributed to extracellular iron oxide adsorbed at the plasma membrane in the form of highly heterogeneous clusters.

**Table 1.** List of the fitting parameters obtained from the adjustment of the adsorption and internalization kinetics data (Equation 1). Here, MFe is the mass of iron extrapolated at t0 = 0 and ΔMFe the difference MFe – MFe, where MFe is the mass of iron extrapolated at t0 = ∞. τ denotes the relaxation time of the process of the exponential rise.

<table>
<thead>
<tr>
<th>γFe2O3</th>
<th>[Fe] [μm]</th>
<th>MFe [μg/cell]</th>
<th>ΔMFe [μg/cell]</th>
<th>τ [min]</th>
</tr>
</thead>
<tbody>
<tr>
<td>uncoated</td>
<td>4</td>
<td>4</td>
<td>104</td>
<td>350 ± 50</td>
</tr>
<tr>
<td>citrate-coated</td>
<td>1</td>
<td>0.4</td>
<td>22</td>
<td>330 ± 100</td>
</tr>
<tr>
<td>10</td>
<td>3.2</td>
<td>70</td>
<td>320 ± 40</td>
<td></td>
</tr>
<tr>
<td>PAA2K-coated</td>
<td>1</td>
<td>0.36</td>
<td>2.4</td>
<td>530 ± 100</td>
</tr>
<tr>
<td>10</td>
<td>0.44</td>
<td>4.0</td>
<td>490 ± 140</td>
<td></td>
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</table>

(Charge 1). Similar exponential profiles together with a leveling-off of the uptake were found in the recent literature. Interestingly, these behaviors did not depend on the cell type. Note that for PAA2K-coated NPs at 10 mM the measured mass at 5 min is much lower than that of the citrate or uncoated particles (0.44 versus 3.2 pg/cell). This later result suggests that in case of an initial MFe jump, a large quantity of nanoparticles should be adsorbed at the surface of the cells. Some studies have also reported similar trends.
2.6. Adsorption at the Cell Membranes and Colloidal Instability

The bare and the citrate-coated NPs exhibit the highest masses of adsorbed/internalized iron. In culture media such as RPMI (Roswell Park Memorial Institute) and DMEM (Dulbecco’s modified eagle medium), the particles aggregate and form highly polydisperse and micrometer-sized clusters.\(^{[39,40]}\) For Cit–\(\gamma\text{Fe}_2\text{O}_3\), this destabilization was studied recently and was attributed to the displacement of the citrates from the particle surfaces towards the bulk, as they are preferentially complexed by calcium and magnesium counterions of the culture medium. For noncoated particles, the precipitation occurred because of the change of pH between that of the synthesis medium (pH 2) and that of the culture medium (pH 7.4). To study the kinetics of aggregation, light scattering experiment was performed using the same protocol as for the incubation in vitro. In these assays, a few microliters of a concentrated iron oxide dispersion was poured rapidly in a test tube containing the cell culture medium, the hydrodynamic diameter \(D_{\text{H}}\) being thereafter monitored as a function of the time (SI-4). For Cit–\(\gamma\text{Fe}_2\text{O}_3\), the hydrodynamic diameter was shown to increase within the first seconds of contact from 23 to 180 nm and remained at this level for one hour. For uncoated NPs, the initial increase was even more important \((D_{\text{H}} > 1 \mu\text{m})\). At longer times, the aggregates settled down at the bottom of the tube, resulting in a decrease of the scattering intensity and hydrodynamic diameter. Similar observations were made in Petri dishes when the particles were incubated with cells. At this point, it is unclear whether some NPs precipitate directly on the cell membranes, or if there is first the formation of aggregates and then a deposition of the cells. However, the similarity of behaviors for \(M_{\text{Fe}}\) (Figure 4) and \(D_{\text{H}}\) (SI-4) at short times supports the first scenario. It is also plausible that the cells act as sites of nucleation for the destabilization process. On a longer time scale, the sedimentation of the NP clusters down to the adherent cell layer enters into play and further increases the adsorption of the NPs, in agreement with several earlier reports.\(^{[14,43]}\) From the above data, we conclude that the strong NP adsorption seen by SEM and optical microscopy is heightened by the destabilization of the NPs in the cell culture media.

3. Conclusion

In the present paper we propose a simple and facile assay to measure the iron content of living mammalian cells incubated with iron oxide NPs. This assay is based on UV–vis spectrometry and on the digestion of seeded cells with hydrochloric acid. With a 10% accuracy and a minimum sensitivity as low as 30 femtograms per cell, the technique allows the dosage of iron in cells over 4 decades in masses, measuring the iron content and cell number at once. The present study was also designed to examine the effect of physico-chemical parameters on the NP/cell interactions.

One of the more significant findings to emerge from this work is the correlation between the phase behavior of the NPs in biological fluids and their interactions with adherent mammalian cells. Iron oxide NPs coated with citrate ligands are found to be destabilized in cell culture media and to adsorb massively onto NIH/3T3 fibroblast cells. Particles made from the same magnetic core, but coated with a 3 nm polymer adlayer exhibit much weaker interactions with cells. These findings confirm those obtained with human lymphoblasts in suspension and treated with the same particles.\(^{[12]}\) The most striking result revealed here came from SEM, which disclosed huge amounts of resiliently adsorbed NPs at the cell membrane. This technique permits one to attribute the large iron contents found for Cit–\(\gamma\text{Fe}_2\text{O}_3\) (typically 100 pg/cell at high [Fe]) to extracellular iron oxides adsorbed at the plasma membrane in the form of highly heterogeneous clusters. In parallel, the low amount of PAA\(_{39}\)–\(\gamma\text{Fe}_2\text{O}_3\) NPs detected by SEM at the cell membrane suggests that the MILC data for this compound (around 10 pg/cell at saturation) corresponds mainly to the iron content internalized by the cells. Such data are in good agreement with those of the literature.\(^{[10,15,20,23]}\)

Given the wide use of citrates for coating-engineered NPs (as in the cases of gold\(^{[42–44]}\) and rare-earth oxide\(^{[45]}\)), the present results, together with those of the recent literature\(^{[46–48]}\) confirm the poor coating performances of such low-molecular-weight ligands. Taken together, these results suggest two types of NP/cell behavior: NPs are either adsorbed on the cell membranes, or internalized into membrane-bound endocytic compartments. In the case of adsorption, as shown by SEM, the layer of materials deposited on the membranes can grow up to 500 nm and is still observed after 24 h of incubation. More importantly, this method of measuring internalized/adsorbed iron in living environments can be easily extended to other living environments such as bacteria, tissues, and organs.

4. Experimental Section

4.1. Chemicals, Synthesis, and Characterization

Nanoparticles Synthesis: the iron oxide nanoparticles (bulk mass density \(\rho = 5100 \text{ kg m}^{-3}\)) were synthesized according to the Massart method\(^{[49]}\) by alkaline co-precipitation of iron(II) and iron(III) salts and oxidation of the magnetite \((\gamma\text{Fe}_3\text{O}_4)\) into maghemite \((\gamma\text{Fe}_2\text{O}_3)\). The nanoparticles were then size-sorted by subsequent phase separations.\(^{[50]}\) At pH 1.8, the particles are positively charged, with nitrate counterions adsorbed on their surfaces. The resulting interparticle interactions are repulsive, and impart an excellent colloidal stability to the dispersion. For the present study, three batches of \(\gamma\text{Fe}_2\text{O}_3\) NPs of median diameter 6.7, 8.3, and 10.7 nm were synthesized. The size and size distribution were retrieved from vibrating sample magnetometry (VSM) and from TEM, and were found in good agreement. Table 2 lists the results obtained for the median diameters noted \(D_{\text{VSM}}\) and \(D_{\text{TEM}}\) and for the polydispersities. The polydispersity was defined as the ratio between standard deviation and average diameter. VSM was also used to determine the volumetric magnetization \(m_{\text{v}}\) of the NPs, which was found to be \(2.9 \times 10^5 \text{ A m}^{-1}\), i.e., slightly lower than the volumetric magnetization of bulk maghemite \((m_{\text{v}} = 3.9 \times 10^5 \text{ A m}^{-1})\).
Table 2. Characteristics of the iron oxide particles used in this work. The diameter of the bare particles determined by VSM and by TEM. Similarly, s^VSM and s^TEM are the values of the polydispersity for the size distributions. D_0 is the hydrodynamic diameter of particles dispersed in water and determined by dynamic light scattering. The D_0 values for the bare and citrate-coated particles are identical. Values of the hydrodynamic diameters in the cell culture medium are given in SI-4. [19]

<table>
<thead>
<tr>
<th>Coating</th>
<th>Hydrodynamic Diameter D_0 [nm]</th>
</tr>
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<tbody>
<tr>
<td>citrate</td>
<td>12.7</td>
</tr>
<tr>
<td>PAA_{2K}</td>
<td>17.7</td>
</tr>
</tbody>
</table>

This process resulted in the adsorption of a highly resilient polymer adlayer surrounding the particles. The hydrodynamic diameters of the bare, citrate- and PAA_{3K}-coated particles in deionized (DI) water were determined by dynamical light scattering (Table 2). From these values, the layer thickness was estimated at 3 ± 1 nm. Hydrodynamic sizes of the particles dispersed in DMEM after one day and after one week are provided in SI-4. [19] The density of chargeable carboxylic groups was evaluated by acid titration at 25 ± 3 nm^-2. For the particles of diameter 8.3 nm, it corresponded to 5400 structural anionic charges in average. As a final step, the dispersions were all dialyzed against DI water which pH was first adjusted to 8 (Spectra Por 2 dialysis membrane with molecular weight cut-off, MWCO, 12 kDa). At this pH, 90% of the carboxylate groups of the citrate and PAA_{3K} coatings were ionized.

The synthesis of the copolymer poly(acrylic acid)-b-poly(acrylamide) was based on the Madix technology which uses the xanthate as chain-transfer agent in the controlled radical polymerization. [22] The molecular weights targeted by the synthesis were 5000 and 30 000 g mol^-1 for the charged and neutral blocks respectively. The molecular weight of the chain as determined from static light scattering was slightly higher, at M_w = 43 500 ± 1000 g mol^-1. Dynamic light scattering revealed a hydrodynamic diameter of D_h = 11 nm, and size exclusion chromatography revealed a polydispersity index of M_w/M_n = 1.6. The adsorption of the PAA_{2K}-b-PAM_{3K} layer was realized via an entrap-driven ligand exchange process between citrates ions and copolymers. The hydrodynamic diameter of the resulting core-shell colloid was 50 nm.

Cell Culture and Cellular Growth: In this work, one type of adherent cells (NIH/3T3 fibroblast) and two types of cells in suspensions (human lymphoblasts 2139 and the THP1 monocytes) were studied. NIH/3T3 fibroblast cells from mice were grown in T25-flasks as a monolayer in DMEM with high glucose (4.5 g L^-1) and stable glutamine (PAA Laboratories GmbH, Austria). This medium was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (PAA Laboratories GmbH, Austria). Exponentially growing cultures were maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C, and in these conditions the plating efficiency was 70–90%, and the cell duplication time was 12–14 h. Cell cultures were passaged twice weekly using trypsin–ethylenediaminetetraacetic acid (EDTA) (PAA Laboratories GmbH, Austria) to detach the cells from their culture flasks and wells. The cells were pelleted by centrifugation at 1200 rpm for 5 min. Supernatants were removed and cell pellets were re-suspended in assay medium and counted using a Malassez counting chamber.

The lymphoblastoid cell line 2139 is a normal line provided to us by Dr. Janet Hall from the Institut Curie (Orsay, France). Lymphoblasts are one of the different stages of physiological differentiation inside the lymphoid line leading to lymphocytes. This cell line was immortalized by the virus Epstein-Barr (EBV) and was obtained by Dr. Gilbert Lenoir from the Institut Gustave Roussy (Villejuif, France). [53, 54] The human acute monocytic leukemia cell line (THP1) was provided to us by the Karlsruhe Institute of Technology.
full papers

(Karlsruhe, Germany). Both lymphoblasts and monocytes were grown in suspension in T25-flasks in RPMI with high glucose (2.0 g L⁻¹) and stable glutamine. RPMI was supplemented with 10% FBS and 1% penicillin/streptomycin. The culture and counting protocols for the cells in suspension were similar to those of the NIH/3T3 fibroblasts. Note finally that the lymphoblasts and monocytes have a duplication time of 20 h.

4.2. Experimental Methods

**MILC Protocol:** Cells were seeded onto 3.6 cm Petri dishes, incubated until reaching 60% confluence and then incubated with NPs at different concentrations for times between 5 min and 24 h. The concentrations in the supernatants were varied from [Fe] = 0.03 to 30 mM. Note that the concentrations investigated are representative of those reported in the literature for in vivo and in vitro assays.⁴ Previos cytotoxicity studies have shown that γ-Fe₂O₃ NPs were nontoxic, as the cell viability remained around 100%⁵ over periods from 1 to 4 days (SI-6). After the incubation period, the supernatant was removed and the layer of cells washed thoroughly with PBS. The cells were then trypsinized and mixed again with culture medium without serum. Aliquots of 20 μL were taken up for counting using a Malassez counting chamber. The cells were finally centrifuged and pellets were dissolved in 35 vol.% HCl. The cells dissolved in HCl were poured in a UV–vis microcell, studied with a Variant spectrophotometer (Cary 50 Scan) and calibrated with the help of a reference.¹⁷ For the absorbance curve of tetrachloroferrate ions displayed in Figure 1b, the concentration was determined by flame atomic absorption spectroscopy using a Perkin-Elmer A100 spectrometer. MILC protocol was performed in triplicate for different types of nanoparticles and coating (for data treatment, see Section 2.2 and the Supporting Information, SI-7).

**Transmission Electron Microscopy:** TEM on nanomaterials was carried out on a Jeol-100 CX microscope at the SIARE facility of Université Pierre et Marie Curie (Paris 6). It was utilized to characterize the sizes of the γ-Fe₂O₃ NPs (SI-1). For the TEM studies of cells, the following protocol was applied. NIH/3T3 fibroblast cells were seeded onto the 6-well plate. After the 24 h incubation with NPs, excess medium was removed, and the cells were washed in 0.2 mM phosphate buffer (PBS), pH 7.4. They were fixed in 2% glutaraldehyde-phosphate buffer (0.1 M) for 1 h at room temperature. Fixed cells were washed in 0.2 M PBS. Then, they were postfixed in 1% osmium-phosphate buffer (0.1 M) for 45 min at room temperature in dark conditions. After 0.1 M PBS washes, the samples were dehydrated in increasing concentrations of ethanol. Samples were then infiltrated in 1:1 ethanol:epon resin for 1 h and finally in 100% epon resin for 48 h at 60 °C for polymerization. Sections of 90 nm thickness were cut with an ultramicrotome (LEICA, Ultracut UCT) and picked up on copper-rhodium grids. They were then stained for 7 min in 2% uranyl acetate and for 7 min in 0.2% lead citrate. Grids were analyzed with a transmission electron microscope (JEOL, EM 912 OMEGA) equipped with a LaB₆ filament, at 80 kV and images were captured with a digital camera (SS-CCD, Proscan 1024 × 1024), and the iTEM software.

**Scanning Electron Microscopy:** Cells were primarily fixed 1 h at room temperature and overnight at 4 °C by immersion in a fixative solution (2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.5), washed 3 times with cacodylate buffer 0.2 M, and post fixed during 1 h at room temperature in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.5) and washed again 3 times in 0.1 M cacodylate buffer. Dehydration until 100% ethanol was completed through graded ethanol-water mixtures at room temperature. Cells were then dried according to the CO₂ critical point drying method (using a Bal-Tec CPD030). After mounting onto scanning stubs samples were coated with a conductive layer (10 nm) of carbon using a thermal carbon evaporator (Cressington C208). Scanning electron microscopy was performed either on a Zeiss ULTRA 40 or a Hitachi SU-70 field emission scanning electron microscopes. Microanalysis and mapping was performed either with Edax CDU or Oxford X-Max EDX detectors installed on the microscopes columns.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author. Section SI-1 provides a complete characterization of the iron oxide NPs in terms of structural and magnetic properties. The diameter and length distributions of microvilli at the cell membrane in various seeding conditions are shown in SI-2, whereas SI-3 displays transmission optical microscopy images of cells incubated with γ-Fe₂O₃ NPs. The stability of the coated and bare particles in cellular media is studied in SI-4. The release amounts and release rates of ferric ion Fe³⁺ at neutral and acidic pH for the Massart dispersions are estimated in SI-5. In SI-6, MIT toxicity assays insure that the NPs used in this study do not present acute short-time toxicity, and in SI-7 the UV–vis absorption versus wavelength is shown for comparison.

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