Preventing Corona Effects: Multiphosphonic Acid Poly(ethylene glycol) Copolymers for Stable Stealth Iron Oxide Nanoparticles

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Supporting Information

ABSTRACT: When dispersed in biological fluids, engineered nanoparticles are selectively coated with proteins, resulting in the formation of a protein corona. It is suggested that the protein corona is critical in regulating the conditions of entry into the cytoplasm of living cells. Recent reports describe this phenomenon as ubiquitous and independent of the nature of the particle. For nanomedicine applications, however, there is a need to design advanced and cost-effective coatings that are resistant to protein adsorption and that increase the biodistribution in vivo. In this study, phosphonic acid poly(ethylene glycol) copolymers were synthesized and used to coat iron oxide particles. The copolymer composition was optimized to provide simple and scalable protocols as well as long-term stability in culture media. It is shown that polymers with multiple phosphonic acid functionalities and PEG chains outperform other types of coating, including ligands, polyelectrolytes, and carboxylic acid functionalized PEG. PEGylated particles exhibit moreover exceptional low cellular uptake, of the order of 100 femtograms of iron per cell. The present approach demonstrates that the surface chemistry of engineered particles is a key parameter in the interactions with cells. It also opens up new avenues for the efficient functionalization of inorganic surfaces.

I. INTRODUCTION

The concept of “stealth particles” was introduced some years ago to describe therapeutic drug nanocarriers showing an increased blood circulation in vivo. The expression was first associated with a series of self-assembled organic particles including liposomes, lipid-based complexes, and biodegradable polymeric micelles. As for the micelles, poly(lactic acid)-b-poly(ethylene glycol) or poly(caprolactone)-b-poly(ethylene glycol) were among the most studied copolymers because their core−shell structure was found to be resistant to plasma protein adsorption. This remarkable property was attributed to the presence of a repulsive poly(ethylene glycol) (PEG) brush playing the role of protective layer and was further investigated in various drug delivery contexts. Poly(ethylene glycol) offers many advantages, among which to be hydrophilic and soluble at body temperature, inexpensive, and approved by regulatory health and control agencies.

In parallel, engineered particles with dimensions from 1 to 100 nm made from carbon (nanotubes, graphene) or from metallic atoms (gold, silver, oxide, semiconductor) were also the subject of intense research during these last years. When dispersed in biological fluids, however, the particles were found to be selectively coated with serum proteins, which eventually leads to their agglomeration and precipitation. In a broad survey on nanoparticle dispersions prior to in vitro exposure, Murdock et al. demonstrated that many metal (silver, copper) and metal oxide (alumina, silica, anatase) nanomaterials were agglomerating in solutions and that, depending on the system, on the presence of serum or on sonication, agglomeration was either agitated or mitigated. The agglomeration of engineered particles is a phenomenon of critical importance since it results in the loss of the nanometer character of the probes, in changes of their hydrodynamic properties and of their interactions with cells. This behavior was found to be quite general, and it has led researchers to propose the paradigm of the “protein corona”. Recent reports described this phenomenon as ubiquitous and independent of the nature of particles. The conclusions relative to the protein corona contradict the results found on PEGylated endosomes and micelles, which exhibit a clear and significant resistance against protein adsorption and stealthiness in vivo. In this work, we address the question of the coating of engineered particles and show that, thanks to an appropriate choice of polymer functionalities, stealth iron oxide particles can be obtained.

For applications in nanomedicine, particles need to be functionalized by chemical and physical-chemical modifications of their surfaces. Besides covalent grafting and efficient way to stabilize particles makes use of the layer-by-layer assembly

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technique.19 The technique is founded on simple mixing protocols in which charged polymers adsorbed spontaneously on the oppositely charged surfaces by multiple point attachment. Allowing the deposition of single or multilayers, the technique was applied to various particle types20–24 and, notably, to iron oxide nanocrystals.25–29 In the search of PEGylated coating with better anchoring properties, new polymer architectures have also been proposed. Na et al. designed multidentate catechol and PEG derivatized oligomers that provided greatly enhanced stability of iron oxide nanocrystals over a broad range of pH and of electrolytes.30 To replace the carboxylic groups used as linkage to cationic surfaces, single31–33 or multiple34–38 phosphonic acid based polymers were synthesized and tested. Indeed, phosphonic acid has been shown to exhibit a higher binding affinity toward metallic atoms as compared to carboxylic acid, especially in acidic conditions.36,37,39 Recently, Sandiford et al. exploited PEG polymer conjugates containing a terminal bifunctional phosphonic acid group for binding to magnetic nanoparticles. This strategy provided high densities of tethered PEG chains (about 1 PEG per nm2) as well as stable dispersions.55 Last, but not least, phosphonic acid based polymers are recognized for their excellent biocompatibility and for their usefulness in nanomedicine.37

In the present study, statistical copolymers containing phosphonic acid and PEG functional groups (Specific Polymers, France, http://www.specipolymers.fr/) were synthesized by free radical polymerization. The copolymer composition was optimized to provide a simple and scalable protocol for the preparation of large quantities of products, and to an excellent stability of the coating in biological conditions. The efficacy of the phosphonic acid PEG copolymers as a coat was evaluated using iron oxide nanoparticles. Iron oxide is already present in numerous biomedical applications, such as magnetic resonance imaging and hyperthermia, and there is a strong demand for advanced and high-performance coating in these fields. In 2008, our group proposed a simple protocol for the coating of cationic nanoparticles with monofunctionalized phosphonic acid terminated oligomers.32,33 The same strategy is used here with copolymers that have multiple functional phosphonic acid end groups. It is demonstrated that the coated particles are stable in biological fluids for months and that the PEG layer strongly reduces the uptake by living cells, including macrophages.

II. EXPERIMENTAL SECTION

Polymer Synthesis. All phosphonic and carboxylic acid PEG copolymers (Patent FR14/00899960) were synthesized by Specific Polymers, (France, http://www.specipolymers.fr/).

Methacrylic acid (MAA; CAS: 79−81−5) was supplied by Acros Organics and used as received. PEG-methacrylate (PEGMA; SP-43,302, CAS: 26915−72−0) and dimethyl(methacryloyloxy)methylphosphonate (MAPC1, SP-41,003, CAS: 86242−61−7) monomers were produced by Specific Polymers. 2,2′-Azobis(isobutyronitrile) (AIBN; CAS: 78−67−1) was supplied by Sigma-Aldrich and used after recrystallization in methanol.

Poly(poly(ethylene glycol) methacrylate-co-methacrylic acid), or poly(PEGMA-co-MAA) was synthesized by free radical polymerization involving PEGMA and MAA monomers, AIBN as radical initiator, and tetrahydrofuran (THF) as solvent. A typical polymerization procedure is described here: MAA (0.21 g, 2.4 mmol), PEGMA (5 g, 2.4 mmol), and AIBN (0.02 g, 0.12 mmol) were added along with 40 mL of THF in a Schlenk flask. The mixture was degassed by three freeze−evacuate−thaw cycles and then heated at 70 °C under argon in a thermostated oil bath for 24 h, leading to 100% conversion. The copolymers were precipitated in cold ether after synthesis to remove the low molecular weight chains. Poly(PEGMA-co-MAA) statistical copolymer was finally recovered after evaporation of the solvents under reduced pressure (Supporting Information, S1). 1H NMR (CDCl3, 300 MHz) δ (ppm): 4.0−3.4 (CH2−CH2−O), 2.2−1.7 (CH2(OCH2)2), 0.9−1.5 (CH2(CH3)2). Poly(poly(ethylene glycol) methacrylate-co-dimethyl(methacryloyloxy)methyl phosphonic acid) or poly(PEGMA-co-MAPC1) was synthesized by free radical polymerization involving PEGMA and MAPC1 monomers, AIBN as radical initiator, and methacryloyloxyethylketone (MEK) as solvent.40 A typical polymerization procedure is described here: MAPC1 (0.50 g, 2.4 mmol), PEGMA (5 g, 2.4 mmol), and AIBN (0.02 g, 0.12 mmol) were added along with 20 mL of MEK in a Schlenk flask. The mixture was degassed by three freeze−evacuate−thaw cycles and then heated at 70 °C under argon in a thermostated oil bath for 24 h, leading to 100% conversion. The PEGMA and MAPC1 monomer conversion rates were measured by 1H NMR spectroscopy and were found to have similar time dependences, indicating that the chains have a similar composition throughout the synthesis (Supporting Information, S2). The copolymers were precipitated in cold ether after synthesis to remove the low molecular weight chains. Poly(PEGMA-co-MAPC1) statistical copolymer was recovered after evaporation of the solvents under reduced pressure. 1H NMR (CDCl3, 300 MHz) δ (ppm): 4.4−3.5 (CH2−CH2−O, O−CH2−P, O−P(OCH2)3), 2.1−1.7 (CH2(CH3)2), 0.9−1.3 (CH2(CH3)2). 31P NMR (CDCl3, 300 MHz) δ (ppm): 21.8. The hydrolysis of the phosphonated ester into phosphonic acid was performed using bromotrimethylsilane and then methanol at room temperature, as already reported in the literature.41,42 Solvents were finally evaporated under reduced pressure leading to the poly(PEGMA-co-MAPC1add) final product. The scheme of the synthesis of phosphonic acid poly(ethylene glycol) copolymers is shown in Figure 1.30 1H NMR (CDCl3, 300 MHz) δ (ppm): 4.5−3.5 (CH2−CH2−O, O−CH2−P, 2.1−1.7 (CH2(CH3)2), 0.9−1.3 (CH2−CH2)). 31P NMR (CDCl3, 300 MHz) δ (ppm): 18.0.

![Figure 1. Two-step synthesis of poly(poly(ethylene glycol) methacrylate-co-dimethyl(methacryloyloxy)methyl phosphonic acid), abbreviated as poly(PEGMA-co-MAPC1add) and described as phosphonic acid PEG copolymer in this work.](image-url)

Polymer Characterization. 1H and 31P NMR spectra were recorded using a Bruker Avance 300 (300 MHz) with CDCl3, or CD3OD as solvent. For 1H NMR, chemical shifts were referenced to the peak of residual nondeuterated solvents at 7.26 ppm for CDCl3. The weight-averaged molecular weight of the polymers was determined by static light scattering measurements using a NanoZS Zetasizer from Malvern Instrument. Quartz cuvettes compatible with water solutions and toluene for calibration were used. The polymer solutions were prepared with 18.2 MΩ Milli-Q water, filtered with 0.2 μm cellulose filters, and their pH was adjusted to 8 by addition of ammonium hydroxide. The Rayleigh ratio R(c) was measured as a function of the scattering vector c.
The refractive index increment was determined by refractometry (Corduane Technologies; Supporting Information, S4). For phosphonic and carboxylic acid PEG copolymers, we obtained \( dn/dc = 0.148 \pm 0.06 \text{ cm}^3 \text{ g}^{-1} \) and \( dn/dc = 0.137 \pm 0.07 \text{ cm}^3 \text{ g}^{-1} \), respectively. These values are close to that of PEG, \( dn/dc = 0.135 \text{ cm}^3 \text{ g}^{-1} \), indicating that the contrast is dominated by that of the PEG segments. The refractive index increment \( dn/dc \), the scattering contrast \( K \), and molecular weight of the two polymers are listed in Table 1. The \( M_w \) values were 12950 \( \pm \) 500 and 15500 \( \pm \) 2000 g mol\(^{-1}\) for the polymers with phosphonic and carboxylic acid anchoring groups, in good agreement with those targeted by the synthesis (11000 g mol\(^{-1}\)).

The molar-mass dispersity for poly(PEGMA-co-MAPC1) and poly(PEGMA-co-MAA) were determined by size exclusion chromatography using polystyrene columns and found at 1.81 and 1.78, respectively (see Supporting Information, S2, for details). From the UV absorbance at 254 nm, the number-averaged molecular weight \( \langle M_n \rangle \) and standard deviation \( \sigma \) and average diameter \( D_{\text{TEM}} \) were determined by size exclusion chromatography using polystyrene columns and found at 1.81 and 1.78, respectively.

Table 2 lists the results from vibrating sample magnetometry (VSM) and from transmission electron microscopy (TEM). Similarly, \( D_{\text{VSM}} \) and \( D_{\text{TEM}} \) are the values of the size dispersity. \( D_0 \) is the hydrodynamic diameter of the bare particles in water. The weight-averaged molecular weight \( M_w \) was determined from static light scattering.

The particles were demonstrated by the appearance of five diffraction rings which wave vectors matched precisely those of the maghemite structure (Supporting Information, S6 and S7). In this work, the nanoparticle concentrations are defined by the percentage by weight of \( \gamma\text{-Fe}_2\text{O}_3 \) in the dispersion or by the iron molar concentration \([\text{Fe}]\). With these units, \( c(\gamma\text{-Fe}_2\text{O}_3) = 8 \times 10^4 \text{ wt}\% \) or 80 \( \mu\text{g/mL} \) corresponds to \([\text{Fe}] = 1 \text{ mM}\).

**Coating.** Citric acid is a weak triacid of molecular weight \( M_w = 192 \text{ g mol}^{-1} \) with three acidity constants (\( \text{pK_a} = 3.1, \text{pK}_{a2} = 4.8 \) and \( \text{pK}_{a3} = 6.4 \)). Complexation of the surface charges with citric acid was performed during the synthesis through simple mixing. At pH 8, citrate-coated particles are stabilized by electrostatics. As a ligand, citrate ions were characterized by adsorption isotherms and the adsorbed species were in equilibrium with free citrates in the bulk. The concentration of free citrates was kept at the value of 8 mM, both in DI water and in culture medium. The hydrodynamic diameter of the citrate-coated particles was identical to that of bare particles, indicating a layer thickness under 1 nm (Table 3). Poly(sodium acrylate), the salt form of poly(acrylic acid) (PAA), with a weight-average molecular weight \( M_w = 2100 \text{ and } 5100 \text{ g mol}^{-1} \) and a polydispersity of 1.7, was purchased from Sigma-Aldrich and used without purification. To adsorb polyelectrolytes on the particles, the precipitation-redispersion protocol was applied. The precipitation of the iron oxide dispersion by PAA was performed in acidic conditions (pH 2). The dispersion was further purified by ultracentrifugation (100 000 g, 48 h).

Table 3. Hydrodynamic Diameter \( D_h \) of Coated Particles, as Determined by Dynamic Light Scattering

<table>
<thead>
<tr>
<th>coating</th>
<th>6.8 nm ( \gamma\text{-Fe}_2\text{O}_3 ) ( \text{nanoparticles} )</th>
<th>13.2 nm ( \gamma\text{-Fe}_2\text{O}_3 ) ( \text{nanoparticles} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>citrate</td>
<td>13 ( \pm ) 1</td>
<td>27 ( \pm ) 4</td>
</tr>
<tr>
<td>poly(acrylic acid) 2K</td>
<td>18 ( \pm ) 2</td>
<td>32 ( \pm ) 2</td>
</tr>
<tr>
<td>poly(acrylic acid) 5K</td>
<td>23 ( \pm ) 2</td>
<td>37 ( \pm ) 3</td>
</tr>
<tr>
<td>carboxylic acid-PEG</td>
<td>26 ( \pm ) 2</td>
<td>50 ( \pm ) 4</td>
</tr>
<tr>
<td>phosphonic acid-PEG</td>
<td>25 ( \pm ) 2</td>
<td>37 ( \pm ) 2</td>
</tr>
</tbody>
</table>

The first column denotes the molar equivalent of acid groups per gram (milli equiv g\(^{-1}\)) of polymer as determined by \( ^1\)H NMR. From the refractive index increment \( dn/dc \), the scattering contrast \( K \) is calculated. The weight-averaged molecular weights were obtained by static light scattering. The numbers of the functional groups are determined from the molecular weight and from the molar equivalent of acid groups. Note that the refractive index increments are close to that of poly(ethylene glycol), 0.135 cm\(^3\) g\(^{-1}\).
precipitate was then separated by magnetic sedimentation and its pH was increased by addition of ammonium hydroxide. The precipitate dispersed spontaneously at pH 8. The hydrodynamic sizes of PAA_{Ac}/Fe_{3}O_{4} coated γ-Fe_{2}O_{3} are listed in Table 3. These values were 5 and 10 nm larger than the hydrodynamic diameter of the uncoated particles, indicating a corona thickness 2.5 and 5 nm, respectively. Values of the electrophoretic mobilities and zeta-potentials are provided in the Supporting Information, S8.

**Cell Culture.** Adherent cells from mice including NIH/3T3 fibroblasts and RAW264.7 macrophages were studied. Fibroblasts are the most common cells of connective tissues in animals, in particular, in the skin, whereas macrophages are professional phagocytes with a role that is to engulf and eliminate cellular debris and circulating pathogens. NIH/3T3 fibroblast cells were grown in T25 flasks as a monolayer in Dulbecco’s Modified Eagle’s Medium (DMEM) with high glucose (4.5 g L^{-1}) and stable glutamine (PAA Laboratories GmbH, Austria; Supporting Information, S9). The 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-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 resuspended in assay medium and counted using a Malassez counting chamber.

The RAW264.7 were grown in suspension in T25-flasks in Roswell Park Memorial Institute (RPMI) with high glucose (2.0 g L^{-1}) and stable glutamine (PAA Laboratories GmbH, Austria). RPMI was supplemented with HEPES 10 mM, 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 macrophages have a duplication time similar to that of fibroblasts.

**Static and Dynamic Light Scattering.** Static light scattering is a noninvasive technique used to characterize polymers and particles in solution. In this experiment, the dispersion prepared at a concentration c was illuminated by a laser source. The scattered light intensity I_s(c, θ) was recorded at a given angle θ over a period of 5 min and averaged. The molecular weight of the scatterers was determined by measuring the Rayleigh ratio R(c, θ):

\[ R(c, θ) = \frac{I_s(c, θ) n_r^2}{I_r n_r^2} R_c \]

where I_s(c, θ) and I_r are the scattering intensities of the sample and of toluene, respectively, n_r and n_c are their refractive indexes and R_c is the Rayleigh ratio of toluene. In the present study, we had n_r = 1.333, n_c = 1.497, and R_c = 1.352 × 10^{-5} cm^{-1} and λ = 633 nm. For the analysis of the scattering data, the Zimm representation was used. This Zimm representation consists in plotting the ratio K_c/R(c, θ) versus c. For diluted solutions, it varies as

\[ \frac{K_c}{R(c, θ)} = \frac{1}{M_w} + 2A_2 c + P(θ) \]

where P(θ) is the form factor of the scattering particles, A_2 the second virial coefficient, and K = \frac{24n_r^2 n_c^2}{N_A} (N_A being the Avogadro number and λ is the wavelength of light). In the K_c/R(c, θ) versus c representation, the inverse molecular weight is the ordinate at origin and slope is twice the virial coefficient. For particles and/or polymers in the nanometer range as it is the case here, P(θ) = 1.68.

With dynamical light scattering, the collective diffusion coefficient D(c) was determined from the second-order autocorrelation function of the scattered light. The autocorrelation functions were analyzed using the cumulants and the CONTIN algorithm fitting procedure provided by the instrument software, and it was checked that both gave comparable results. The hydrodynamic sizes determined by light scattering D_h were found to be systematically larger than those obtained by electron microscopy (D_{EM}) or by magnetometry (D_{M}). The reason for this difference is related to the size dispersity of the particles, light scattering being sensitive to the largest objects of the distribution.

**Mass of Iron Internalized/Adsorbed by Living Cells.** The live cells were cultured and incubated with the particles for 24 h. The supernatant was removed and the cells were thoroughly washed with phosphate buffer saline (PBS). The cells were trypsinized, numbered using a Malassez chamber and centrifuged. The pellets were dissolved in hydrochloric acid (35%), and later investigated by UV-vis spectrophotometry. The cell pellets dissolved in HCl displayed the yellow color characteristic of tetrachloroferrate ions FeCl_4^-.

The absorbance of the dissolved pellets was compared to those of iron oxide and of cells determined separately. For the sake of accuracy, MILC was calibrated against regular titration techniques such as flame atomic absorption spectroscopy. Assuming for the absorbance an absolute uncertainty of 0.03, the minimum amount of iron detectable by this technique is 0.3 picogram (i.e., 30 femtogram) per cell. With MILC, the high sensitivity arises from the fact that the whole UV-vis spectrum was taken into account in the adjustment. For the 13.2 nm particles used in this study, a mass of iron of m_{Fe} = 1 pg/cell corresponds to 5 × 10^4 particles.

**Toxicity Assays.** The method measured the mitochondrial activity of cells. Subconfluent cell cultures (90% confluency at treatment time) on 96-well plates were treated with 100 μL/well of nanoparticles at different concentrations for 24 h (or at different time points according to the final end point), culture medium was removed, cells were rinsed with culture media without phenol red and incubated with 100 μL/well of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium (WST-1, Roche Diagnostics), diluted 1/10 (or more according to cell lines) in culture medium without phenol red for 1 to 4 h. The assay principle is based upon the reduction of the tetrazolium salt WST-1 to formazan by cellular dehydrogenases. The generation of the dark yellow colored formazan was measured at 450 nm in a multiwell-plate reader against a blank containing culture media and WST-1 and it was corrected from the absorbance at 630 nm. The optical density of the supernatant is directly correlated to cell number.

**III. RESULTS AND DISCUSSION**

**III.1. Phase Behavior and PEG Coating.** Poly(poly-(ethylene glycol) methacrylate-co-dimethyl(methacryloyloxy)-methyl phosphonic acid), abbreviated in the following poly(PEGMA-co-MAPC1_{acid}) and depicted as phosphonic acid PEG copolymer (Figure 1) was synthesized and characterized according to techniques provided in the Materials and Methods and in Supporting Information, Sections S1–S4. This polymer was compared to a poly(poly(ethylene glycol) methacrylate-co-methacrylic acid), in short poly(PEGMA-co-MAA), which presented the same composition as poly(PEGMA-co-MAPC1_{acid}), but where carboxylic groups replace the phosphonic acid moieties. Solutions of iron oxide nanoparticles and polymers at the same concentration (c = 0.1 wt %) and same pH (pH 2) were mixed at different volume ratios X. This method ensures that the total concentration remains constant and that no aggregation of particles occurs during mixing because of pH or salinity gap. The pH of the mixed solution was then raised to 8 by addition of ammonium hydroxide. Figure 2a,b displays the stability diagram of mixed dispersions at physiological conditions for 6.8 and 13.2 nm iron oxides mixed with phosphonic acid PEG copolymers. On the left-hand side, the hydrodynamic diameter D_h is displayed as a function of X. On the right-hand side, images of the vials containing the dispersions are presented. The color change of the dispersions is due to the changes in iron oxide
concentration, this concentration varying as $c_{\text{part}} = cX/(1 + X)$. Likewise, the polymer concentration decreased with $X$ as $c_{\text{pol}} = c/(1 + X)$. The hydrodynamic diameter of single polymer chains was 16 nm, as indicated by the square at $X = 10^{-5}$. Upon addition of particles, $D_h$ exhibits a plateau over 2 to 3 decades in $X$ and then increases sharply with the formation of large micron-size aggregates. The precipitated ranges are identified by colored backgrounds for each assay. In this range, the dispersions are turbid and sediment with time. Supplementary investigations were carried out with the carboxylic acid PEG copolymers with 6.8 nm particles. The hydrodynamic diameters were measured from samples displayed on the right-hand side. Above a critical ratio $X_C$, dispersions are turbid and the particles agglomerate. For micron-sized aggregates, $D_h$ is set at 1 $\mu$m for the sake of simplicity.

The mixing was performed slightly below $X_C$, as compared to their initial values. Iron oxides coated with phosphonic or carboxylic acid PEG copolymers were formulated following the method set out in the section II.1. Subsequent measurements of the intensity and diameter were carried out at 1 day, 1 week, and 1 month. For particles aggregating over time, both $X_S$ and $D_h$ are expected to increase as compared to their initial values. Iron oxides coated with phosphonic or carboxylic acid PEG copolymers were formulated following the method set out in the section II.1. The mixing was performed slightly below $X_C$, that is, where the dispersions are stable whatever the pH. The dispersions were dialyzed against deionized water using a 50 kDa cutoff membrane to remove the excess polymer, and further concentrated by ultrafiltration. Iron oxide with the functionalizing carboxylic acid PEG copolymers.

Figure 2. Stability diagram of mixed $\gamma$-Fe$_2$O$_3$/polymers dispersions at pH 8 as a function of the mixing ratio $X$. $X$ is defined as the ratio between the volumes of nanoparticle and polymer dispersions: (a) phosphonic acid PEG copolymers with 6.8 nm particles, (b) phosphonic acid PEG copolymers with 13.2 nm particles, and (c) carboxylic acid PEG copolymers with 6.8 nm particles. The hydrodynamic diameters were measured from samples displayed on the right-hand side. Above a critical ratio $X_C$, dispersions are turbid and the particles agglomerate. For micron-sized aggregates, $D_h$ is set at 1 $\mu$m for the sake of simplicity.

$$n_{\text{ads}} = \frac{1}{X_C} \frac{M^\text{part}_{\text{ads}}}{M^\text{pol}}$$

where $M^\text{part}$ and $M^\text{pol}$ are the molecular weights of the particle and polymer, respectively. For the 6.8 nm iron oxide particles (Figure 2a), $X_C = 1.3$ and $n_{\text{ads}} = 97$. For the 13.2 nm particles (Figure 2b), $X_C = 5$ and $n_{\text{ads}} = 207$. These $n_{\text{ads}}$ values correspond to a polymer density of $0.50 \pm 0.15$ nm$^{-2}$. A density of adsorbed chains independent of the diameter implies a constant density of structural charges at the iron oxide surface, a result that is expected for this type of particles. A polymer density of $0.50$ nm$^{-2}$ corresponds to a coverage of 1.5 PEG chain per nm$^2$, one of the highest densities reported for core–shell assembled structures. In the plateau regime, the hydrodynamic sizes of the two coated particles were found at 25 and 37 nm respectively, that is, 12 and 10 nm larger than those of the bare particles (Tables 3). This result indicates the existence of a 5–6 nm thick PEG layer around the iron oxide nanocrystals, consistent with partially stretched PEG chains. With zeta potentials of $-6$ mV for both particles, electrokinetic measurements confirmed that the PEGylated particles were globally neutral (Supporting Information, S8). A schematic representation of the coating is illustrated in Figure 3.

III.2. Colloidal Stability in Cell Culture Media. In the present study, particles were said to be stable if their hydrodynamic size remained constant as a function of the time after a change of medium, and equal to that found in DI water at physiological pH. The stability criterion defined here takes into account the possible scenarios observed in cell culture media, that is, the adsorption of proteins at the outer coating layer, aggregation, or both. To assess the colloidal stability of coated iron oxides, the following protocol was outlined. A few microliters of a concentrated dispersion were poured and homogenized rapidly in 1 mL of the solvent to be studied, and simultaneously the scattered intensity $I_h$ and diameter $D_h$ were measured by light scattering. The targeted weight concentration was 0.1 wt %. After mixing, the measurements were monitored over a period of 2 h. Subsequent measurements of the intensity and diameter were carried out at 1 day, 1 week, and 1 month. For particles aggregating over time, both $I_h$ and $D_h$ are expected to increase as compared to their initial values. Iron oxides coated with phosphonic or carboxylic acid PEG copolymers were formulated following the method set out in the section II.1. The mixing was performed slightly below $X_C$, that is, where the dispersions are stable whatever the pH. The dispersions were dialyzed against deionized water using a 50 kDa cutoff membrane to remove the excess polymer, and further concentrated by ultrafiltration. Iron oxide with the functionalizing carboxylic acid PEG copolymers.
PEG polymers was first studied in buffer (PBS) and in NH₄Cl salted solutions and displayed excellent stability over several weeks (Supporting Information, S10). In a second study, cell culture media including Dulbecco’s Modified Eagle’s Medium (DMEM) and Roswell Park Memorial Institute medium (RPMI), with and without fetal bovine serum were investigated. Figure 4a displays the hydrodynamic diameter $D_h$ of 13.2 nm iron oxide coated with various polymers. As the concentrated dispersion is added to the cell medium, $D_h$ exhibits a jump from 18 nm to 40–50 nm. The initial value of 18 nm before mixing corresponds to the average size of the proteins and biological macromolecules present in the medium. For 2 h $D_h(t)$ remains unchanged for particles coated with phosphonic acid PEG copolymers, whereas it increases continuously for particles coated with carboxylic acid PEG copolymers. This later evolution is indicative of a slow destabilization of the dispersion, which is due to the detachment of the coating55) or to the slow association with binding macromolecules in the medium. The results on PEGylated particles are compared with those prepared with a poly(acrylic acid) layer. For the 13.2 nm particles, destabilization also occurs, as evidenced by the slow increase in $D_h$ after the initial jump is indicative of the destabilization of the dispersion. Only particles covered with phosphonic acid PEG copolymers are stable and devoid of plasma proteins. (b) One-week stability diagram for particles dispersed in water and cell culture media DMEM and RPMI, with and without fetal calf serum (FCS). (c) Comparison of the colloidal stability of bare and coated 13.2 nm $\gamma$-Fe₂O₃ nanoparticles in various suspending media. Green squares indicate that the particles are stable, and red squares indicate the particles are unstable.

Recently for iron oxide nanocrystals by Faure et al.56 and it was found to be 3 nm for 13.2 nm particles. Hence, for particles with a layer thinner than 3 nm, van der Waals forces dominate the particle–particle interactions and induce agglomeration. This effect is not present for 6 nm thick PEG coating which is insensitive to the ionic strength.

After 1 week, the stability diagram of Figure 4b exhibited similar features: the 6.8 and 13.2 nm particles covered with phosphonic acid PEG chains were still disperse, whereas partial aggregation was observed with the carboxylic acid PEG polymers. Note that, for the carboxylic acid modified polymer, the particles are slightly more stable in the presence of serum, indicating the role played by the proteins in the interactions.5,55,58 However, over a longer period, the carboxylic acid PEG coating leads to a macroscopic aggregation and sedimentation. Figure 4c summarizes the long-term stability behavior of 13.2 nm iron oxide particles in various solvents, including PBS, NH₄Cl 1 M solution, and cell culture media. Comparison is extended to bare particles and to particles covered with citrate ligands.55,59 From these data, it can be seen that polymers with multiple phosphonate functionalities and PEG chains outperform all other types of coating examined.

**III.3. In Vitro Assays and Toxicity.** In in vitro assays, the amount of particles taken up by the cells is of particular interest for it is a reliable and quantitative evaluation of the interactions with cells. With magnetite or maghemite, this amount is expressed in terms of mass iron atom per cell, noted $m_{g Fe}$ and expressed in pg/cell.47,60,61 Here, we exploit the MILC protocol (Mass of metal Internalized/adsorbed by Living Cells) to measure $m_{g Fe}$ in NIH/3T3 mouse fibroblasts and in RAW 264.7 macrophages.47 Both cell lines are representative of in vitro assays currently performed. Based on the digestion of incubated cells (Figure 5a,b) with concentrated hydrochloric acid reactant
(Figure 5c) and complemented by a colorimetric assay (Figure 5d), the technique detects particles that are adsorbed at the plasma membrane or internalized by the cells. In this assay, the 13.2 nm γ-Fe$_2$O$_3$ particles were selected and coated with three different types of ligands or polymers: citrate, poly(acrylic acid), and the phosphonic acid PEG copolymer. MILC was performed in six-well plates cultivated with 3 million cells per Petri dish in average (Figure 5). Incubation time was 24 h. The concentrations investigated were [Fe] = 0.03, 0.1, 0.3, 1, 3, and 10 mM, and covered the range of those reported in the literature for in vivo and in vitro assays. Data are displayed in Figure 6 for the NIH/3T3 and RAW264.7.

The straight thick lines in Figure 6 depict the maximum amount of iron that can be taken up by a single cell at the treatment conditions. For citrate-coated particles, $m_{Fe}$ increases linearly with [Fe] and leveled off above 10 mM (Figure 6a,e). Here, the masses of internalized/adsorbed iron are high and in the range 50−70 pg/cell, independent of the cell lines. In the linear parts it represents about 10−20% of the maximum value discussed previously. For particles coated with PAA$_{2K/5K}$, the variations are similar, but the saturation plateaus are at a lower level, between 5 and 20 pg/cell for NIH/3T3 and RAW264.7 (Figure 6b,c,f,g). The data with fibroblasts are in good agreement with those determined recently with smaller particles. Note also that the carboxylic acid PEG coated particles exhibit the same behavior as those coated with PAA$_{2K/5K}$, the particles being slowly destabilized over time. Conversely, with phosphonic acid PEG copolymers, the uptake and adsorption levels are of the order of 0.1 pg/cell, that is, 50−700x below those found with citrate and poly(acrylic acid) (Figure 6d,h). The monitoring of three types of behaviors in biofluids (from rapid precipitation to excellent dispersibility) allows establishing a correlation between the stability of particles in cell culture media and the amount of adsorbed and internalized particles. Precipitating particles are prone to adsorb at the cellular membranes and to enter the cells, a process that is accelerated by sedimentation. For particles coated with polymers in contrast, sedimentation is less important. Significant differences are however observed between the charged PAA$_{2K/5K}$ and the neutral PEG coating. These differences were explained by the behavior of the polyelectrolyte brush in physiological solvent, which has the tendency to shrink at high ionic strength and to be less protective against aggregation.

Figure 7a,b displays the viability of NIH/3T3 and RAW264.7 cells treated with phosphonic acid PEG copolymers and with the 13.2 nm PEGylated particles. The toxicity assay was based on the WST1 protocol that assessed the mitochondrial activity. The experimental conditions were an incubation time of 24 h and an iron concentration between 0.03 and 10 mM. Viability experiments with phosphonic acid PEG copolymers (full symbols in Figure 7) were performed at concentrations that were equivalent to those calculated from the amounts adsorbed on the particles. The results on the PEGylated copolymers and on the polymer coated particles showed no sign of toxicity, even at high doses, confirming the excellent biocompatibility of phosphonic acid PEG coating. The viability of particles coated with citrate and PAA$_{2K/5K}$ was also measured and it was in agreement with previous reports.
IV. CONCLUSION

In this study, phosphonic acid poly(ethylene glycol) copolymers are synthesized and used as a coat for iron oxide particles. The copolymers put under scrutiny are consistent with 3–4 PEG chains of molecular weight 2000 g mol\(^{-1}\) and 3–4 anchoring moieties grafted on the same methacrylate backbone. The interplay between the number of PEGylated chains, the nature of the anchoring groups and total molecular weight of the copolymers is optimized to allow a facile formulation of coated particles. With a polymer density of 0.5 nm\(^{-2}\) at the iron oxide surface, 10 g of polymers (as obtained from a single synthesis) are sufficient to coat 40 g of iron oxide with diameter 13.2 nm. This later value may be compared to the mass of particles required in standard in vitro toxicity or in vivo studies, which is of the order of milligrams. Time-resolved light scattering provides clear evidence that the coated particles are stable in biologically relevant conditions for months and are protective against proteins adsorption. The PEG coating efficacy is tested against different types of coating agents, including a copolymer with carboxylic acid groups in lieu of the phosphonic acid moieties. Carboxylic acid PEG copolymers show mitigated stability and protection against protein adsorption. The difference between the two polymers is interpreted in terms of binding affinity toward the iron oxide surface, which is assumed to be higher for phosphonic acid. Further work is being performed to clarify this issue. The present study confirms moreover previous findings: when proteins adsorbed at the particle surfaces and built a protein corona, they induce the agglomeration of the particles and in fine a destabilization of the dispersion. In this respect, the present study poses the question of the ubiquity of the protein corona and of its relevance in the context of applications related to nanomedicine. As the composition of the phosphonic acid PEG copolymers can be further implemented using stimuli responsive or fluorescent comonomers, the strategy proposed here opens up new avenues for functionalizing inorganic surfaces.

ASSOCIATED CONTENT

Supporting Information
Sections on the synthesis of poly(poly(ethylene glycol) methacrylate-co-methacrylic acid) (S1), on the reactivity of the monomers during synthesis (S2), on the characterization of the polymers by light scattering (S3) and by refractometry (S4), on the characterization of iron oxide nanoparticles by transmission electron microscopy (S5), by electron beam microdiffraction (S6), by vibrating sample magnetometry (S7), and by zeta potential (S8). Section S9 displays the composition of the cell culture medium used, and S10 shows the stability of the coated particles as a function of the salt concentration. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

REFERENCES
