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# Chitosan-Coated Superparamagnetic Fe<sub>3</sub>O<sub>4</sub> Nanoparticles for Magnetic Resonance Imaging, Magnetic Hyperthermia, and Drug Delivery

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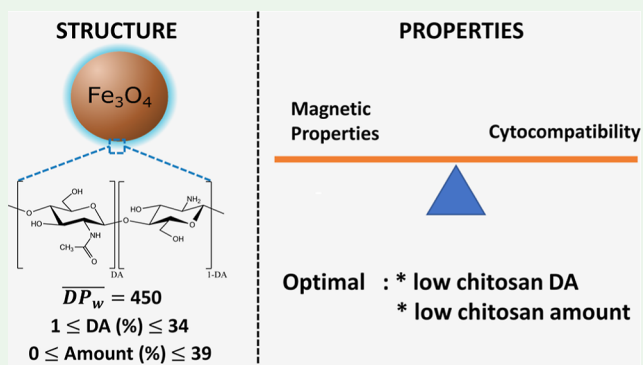


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Supporting Information

**ABSTRACT:** Superparamagnetic iron oxide nanoparticles (SPIONs) have great potential for biomedical applications as multipurpose magnetic resonance imaging (MRI) contrast agents, and some systems have already been approved by The United States Food and Drug Administration for clinical use. Superparamagnetic behavior, high magnetic saturation, and fast synthesis are the major advantages of these nanomaterials. Polymer coatings are often used to prevent cluster formation and to improve biocompatibility of nanoparticles. In this work, we propose to use chitosan (CS) coatings as a means to tune the biocompatibility and magnetic properties of the SPIONs. For this purpose, magnetite (Fe<sub>3</sub>O<sub>4</sub>) SPIONs with various CS coatings were synthesized. CS with identical degree of polymerization (DP<sub>w</sub> = 450) but different



degrees of acetylation (DA of 1, 14, and 34%) were employed to tune the hydrophilic properties of the SPIONs' coatings. A highly crystalline magnetite phase with a superparamagnetic behavior was evidenced for all the studied nanoparticles, whose average sizes varied between 5 and 10 nm. Adjusting the preparation process enabled us to control precisely the amount of coating on the SPIONs. Such coatings significantly impacted their magnetic properties, which were found to decrease with the quantity of CS and also with its DA. Interestingly, cytocompatibility was enhanced by the presence of the CS coating so that all CS-coated SPIONs studied were found to be nontoxic, independently of the coating amount. This trade-off approach suggests that optimal SPIONs systems would consist of a magnetite core, coated with a low amount of low-DA CS. Finally, the multimodal features of the SPIONs were evidenced by performing magnetic hyperthermia and MRI measurements. Despite significant differences, for all the CS-coated SPIONs, the magnetic properties remained strong enough to envision their use in various biomedical applications such as magnetic hyperthermia, MRI contrast agents, magnetic field-assisted drug delivery, and as platforms for further biological functionalization.

**KEYWORDS:** magnetic nanoparticles, chitosan, hyperthermia, MRI, cytotoxicity, nanomedicine

## 1. INTRODUCTION

Superparamagnetic iron oxide nanoparticles (SPIONs) can be used for a variety of biomedical applications including carrier for drug delivery, magnetic hyperthermia for cancer treatment or as magnetic resonance imaging (MRI) contrast agents.<sup>1–3</sup> Among SPIONs, magnetite (Fe<sub>3</sub>O<sub>4</sub>) and maghemite (γ-Fe<sub>2</sub>O<sub>3</sub>) have been widely studied due to their good magnetic properties and low toxicity.<sup>4</sup> Magnetite has a cubic inverse spinel structure (space group *Fd3m*), with oxygen ions in a cubic packed arrangement that contains both divalent Fe(II) and trivalent Fe(III) ions, with an ideal ratio of Fe(III)/Fe(II) = 2. The Fe(II) ions occupy half of the octahedral sites, while the Fe(III) ions are evenly split between the other half of the octahedral and tetrahedral sites.<sup>5</sup> The magnetic property of iron oxide nanoparticles depends on their crystalline phase, but

it is well-known that superparamagnetism is observed for SPIONs with sizes below ~20 nm. Thus, at ambient temperature and in the absence of magnetic field, the magnetic potential energy that governs the direction of the spontaneous magnetization is lower than the thermal bath energy,<sup>6</sup> resulting in zero net magnetic moment. The size and shape of magnetite nanoparticles are key properties for biomedical applications as they directly impact their magnetic and biological properties.<sup>7,8</sup>

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53 The superparamagnetic behavior of  $\text{Fe}_3\text{O}_4$  SPIONs is thus  
54 characterized by almost zero coercivity and no hysteresis.  
55 When injected in the body, this type of SPIONs present a  
56 magnetic moment only under the presence of an external  
57 magnetic field and reach saturation magnetization ( $M_s$ ) orders  
58 of magnitude above that of biological components. The  
59 SPIONs can therefore be detected and manipulated in the  
60 body, making superparamagnetism property the basis for  
61 biomedical applications.<sup>9</sup>

62 Among the methods to synthesize  $\text{Fe}_3\text{O}_4$  SPIONs, the  
63 coprecipitation is the most employed route as it is quick,  
64 simple, and effective.<sup>10</sup> This method consists in mixing of iron  
65 ions in an acidic solution in the absence of oxygen (under an  
66 argon inert atmosphere) to prevent their oxidation and the  
67 formation of hematite ( $\alpha\text{-Fe}_2\text{O}_3$ ), which has no (or very weak)  
68 magnetic response. Then, the pH is increased by the addition  
69 of a basic solution, leading to the formation of ferrous and  
70 ferric hydroxides and then to the precipitation of magnetite  
71  $\text{Fe}_3\text{O}_4$  SPIONs.<sup>11</sup>

72 However, bare magnetite SPIONs may have cytotoxic  
73 effects.<sup>12,13</sup> Thus, in order to address a variety of biomedical  
74 applications, SPIONs are usually coated to improve their  
75 colloidal stability and their biocompatibility.<sup>14</sup> Such coated  
76 particles are usually referred to as “core–shell” SPIONs.<sup>15</sup>  
77 Many types of shells have been reported. Among them, metals  
78 such as silver and gold have been widely studied,<sup>16,17</sup> as well as  
79 silica, titanium, or zinc-based oxides.<sup>18–20</sup> Also, synthetic  
80 polymers like poly(ethylene glycol) (PEG) or natural  
81 polysaccharides such as alginate and dextran have been  
82 extensively used.<sup>21–23</sup> Overall, coating addition was found to  
83 drastically improve the biological properties compared to those  
84 of bare SPIONs, so that a coating is systematically present on  
85 all  $\text{Fe}_3\text{O}_4$  SPION-based medical devices approved world-  
86 wide.<sup>24,25</sup> However, despite the improvement of the biological  
87 properties, the coatings have also been reported to dramatically  
88 decrease the magnetic properties of SPIONs, regardless of the  
89 coating type.<sup>26–29</sup> Therefore, a biocompatibility/magnetic  
90 property trade-off needs to be found, certainly depending on  
91 the desired applications.

92 Among the various possibilities of polymer coatings for the  
93 SPIONs, chitosan (CS) and CS derivatives were investigated  
94 in several previous works.<sup>30–32</sup> Indeed, CS has many  
95 interesting properties, such as to be biocompatible, biodegrad-  
96 able, antibacterial, and nontoxic.<sup>33</sup> Also, CS has reactive amine  
97 and hydroxyl sites, which allow its complexation with metals.  
98 However, these studies very often adopt a focused scope,  
99 evaluating one given type of CS-coated SPION designed for a  
100 specific application and particular evaluation conditions.<sup>34,35</sup>  
101 Thus, the impact of CS coating parameters such as degree of  
102 acetylation (DA) and weight percentage is rarely addressed.  
103 Finally, CS polymers are not always fully characterized (e.g., in  
104 terms of molar mass distributions).

105 In a previous work of our group,<sup>36</sup> we synthesized  $\text{Fe}_3\text{O}_4$   
106 SPIONs coated with CS oligosaccharides (COS) with  
107 controlled DA and degree of polymerization. We evidenced a  
108 narrow size distribution, high magnetic saturation ( $M_s$ ), and  
109 low cytotoxicity of these COS-coated SPIONs. However, COS  
110 may be difficult to prepare and characterize. Therefore, we  
111 choose here to use longer CS chains as coating materials. In  
112 this new work, we addressed the problematic competition  
113 between the biological and magnetic properties of core–shell  
114 SPIONs, using  $\text{Fe}_3\text{O}_4$  as core and tuned amounts of fully  
115 characterized and comparable CS polymers as shells, with such

SPIONs being synthesized and coated in aqueous conditions  
116 without use of cross-linkers. In a systematic study of SPIONs’  
117 physicochemical, magnetic, and biological properties, we  
118 deeply investigated the influence of CS coatings’ characteristics  
119 on SPIONs’ efficacy and relevancy for MRI and magnetic  
120 hyperthermia (MH) applications. Besides, a wide range of  
121 experimental configurations were explored, with MRI measure-  
122 ments on five devices with field strengths ranging from 0.5 to  
123 11.7 T, and MH experiments at three different field amplitudes  
124 (100–370 G) and frequencies (168–491 kHz). This in-depth  
125 study generated a wide range of converging data, which clearly  
126 indicated the existence of an optimal DA and weight  
127 percentage for CS coating in order to guarantee excellent  
128 magnetic and biological SPION properties. Additionally, this  
129 work highlighted the multifunctional features of these CS-  
130 coated SPIONs, allowing to envision their use for MRI, MH,  
131 and drug delivery applications, as well as platforms for  
132 theragnostics and biofunctionalization tools. 133

## 2. EXPERIMENTAL SECTION

**2.1. Materials.** Commercial CS from shrimp shells (batch type  
134 244/020208; DA < 1%;  $M_w$  = 186 kg/mol;  $M_n$  = 97 kg/mol;  $D$  = 1.9),  
135 was bought from Mahtani Chitosan Pvt. Ltd (Veraval, India) and  
136 characterized by proton nuclear magnetic resonance ( $^1\text{H}$  NMR)<sup>37</sup> and  
137 Size-exclusion chromatography–multi angle laser-light scattering  
138 (SEC-MALLS).<sup>38</sup> Iron(III) chloride hexahydrate ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ),  
139 iron(II) chloride tetrahydrate ( $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ ), hydrochloric acid  
140 (37%), acetic acid, aqueous ammonia (28%), propylene glycol,  
141 ammonium acetate, and sodium nitrite were purchased from Sigma-  
142 Aldrich. Extra-pure pork gelatin (platinum, 240 bloom) was  
143 purchased from Roth. 144

**2.2. CSs and  $\text{Fe}_3\text{O}_4$  SPION Syntheses.** **2.2.1. Depolymerization**  
**of CS.** A controlled depolymerization of the initial 244 CS was  
145 performed. For that, a CS solution at a concentration of 0.5% (w/v)  
146 was prepared using 20 g of CS 244 and 4 L of a 0.2 M acetic acid/0.15  
147 M ammonium acetate buffer (pH = 4.5). The mix was mechanically  
148 stirred for 24 h to ensure the complete solubilization of CS. A  
149 filtration under pressure was performed to remove insolubles. The  
150 solution was filtered twice, using consecutive filters (Millipore) of 3  
151 and 1.2  $\mu\text{m}$  pore size. Once purified, the CS solution was placed  
152 under mechanical stirring, and the depolymerization process was  
153 initiated by adding 77 mL of a sodium nitrite solution at 1 mg/mL in  
154 order to reach a stoichiometric ratio  $n_{\text{nitrite}}/n_{\text{glucosamine}}$  of 0.1.  
155 According to pre-established depolymerization curves,<sup>39</sup> the reaction  
156 time was set to 55 min. The reaction was stopped by the addition of  
157 ammonium hydroxide until reaching a basic pH ( $\sim 10$ – $11$ ) to  
158 precipitate CS. The obtained CS was washed and centrifuged several  
159 times until reaching neutral pH ( $\sim 6.3$ ). Finally, CS was freeze-dried.  
160

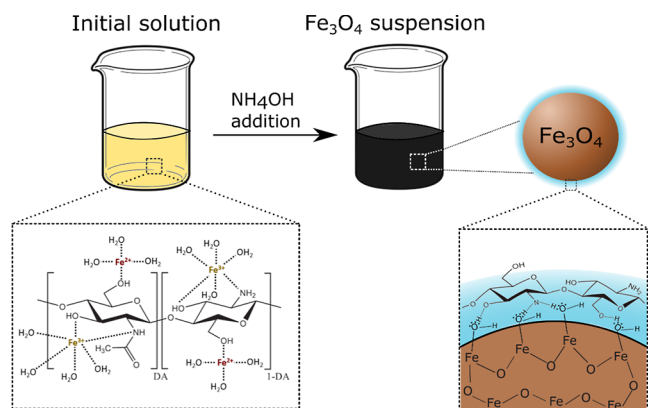
**2.2.2. Reacetylation of CS.** A homogeneous series of CSs with  
161 different DAs was prepared.<sup>40</sup> To this end, two reacetylations yielding  
162 DAs of 14 and 34% were performed starting from the previously  
163 described depolymerized CS ( $\text{CS}_1$ ). These reacetylations were  
164 performed following a method described elsewhere.<sup>41</sup> Briefly, 6 g of  
165  $\text{CS}_1$  was diluted for each case in 480 mL of a water/propylene glycol  
166 mixture (50/50 v/v) with acetic acid at a concentration of 3.3 g/L.  
167 Then, given amounts of acetic anhydride diluted in propylene glycol  
168 were added dropwise to reach DAs of 14 and 34%, and solutions were  
169 stirred overnight. At last, CSs were precipitated by addition of  
170 ammonium hydroxide until pH ranged 10–11. CSs were washed by  
171 successive centrifugations (10 min, 10,000 rpm) and dilutions with  
172 deionized water several times to remove all propylene glycol and  
173 reach a neutral pH. Finally, CSs were then freeze-dried.  
174

**2.2.3. Synthesis of  $\text{Fe}_3\text{O}_4$  SPIONs.** The synthesis of  $\text{Fe}_3\text{O}_4$  SPIONs  
175 was achieved by chemical coprecipitation method. The advantage of  
176 this technique is its simplicity and high production yield, but other  
177 processing methods such as flame spray pyrolysis<sup>42</sup> and electro-  
178 chemical method are also scalable. Thus, iron(III) chloride  
179 hexahydrate ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ) and iron(II) chloride tetrahydrate 181

182 (FeCl<sub>2</sub>·4H<sub>2</sub>O) were dissolved at a stoichiometric ratio of 2:1  
 183 deionized water at 60 °C under an inert atmosphere (Ar). HCl was  
 184 added to the mixture to reach the concentration of 0.1 M and stabilize  
 185 the iron ions. Precipitation of iron ions to form magnetite was  
 186 achieved by dropwise addition of NH<sub>4</sub>OH to reach a pH in the range  
 187 ~10–11. The formation of magnetite by chemical precipitation could  
 188 be clearly evidenced by the immediate change of color of the solution  
 189 from yellow to black upon addition of NH<sub>4</sub>OH. Then, the SPIONs  
 190 were washed several times with deionized water and collected using  
 191 magnetic decantation until reaching neutral pH. They were finally  
 192 freeze-dried and stored at room temperature.

193 **2.2.4. Synthesis of Fe<sub>3</sub>O<sub>4</sub> SPIONs Coated with CS.** Synthesis of  
 194 Fe<sub>3</sub>O<sub>4</sub> SPIONs coated with CS of various DA (Fe<sub>3</sub>O<sub>4</sub>/CS<sub>DA</sub>) was  
 195 carried out through the same chemical coprecipitation method in the  
 196 presence of solubilized CS. First, 50 mL of CS solution at a  
 197 concentration of 2.5 g/L was prepared in an aqueous acidic medium  
 198 (HCl). For CS of DA 1%, solutions were prepared at different  
 199 concentrations depending on the desired amount of CS on SPIONs  
 200 (see Table S1). 0.905 g of FeCl<sub>3</sub>·6H<sub>2</sub>O and 0.333 g of FeCl<sub>2</sub>·4H<sub>2</sub>O  
 201 were dissolved in 20 mL of water at 60 °C under an inert atmosphere  
 202 and mechanical stirring, followed by the addition of HCl for ion  
 203 stabilization. Then, CS solution was added slowly to the iron salts  
 204 solution. After 1 h, stirring was increased, and NH<sub>4</sub>OH was added  
 205 dropwise to the solution to increase the pH (~10–11) and therefore  
 206 to precipitate the CS-coated Fe<sub>3</sub>O<sub>4</sub> SPIONs. The obtained CS-coated  
 207 SPIONs were washed in deionized water and separated by magnetic  
 208 decantation until the pH became neutral. Finally, SPIONs were  
 209 freeze-dried and stored at room temperature. The same synthesis  
 210 route was repeated to obtain CS-coated Fe<sub>3</sub>O<sub>4</sub> SPIONs with the  
 211 reacylated CSs analyzed in this work, and its principle is illustrated  
 212 in Scheme 1 below.

**Scheme 1. Synthesis Principle of Fe<sub>3</sub>O<sub>4</sub>/CS<sub>DA</sub> SPIONs from a CS Acetate Solution Including Fe(II) and Fe(III) Ions<sup>a</sup>**



<sup>a</sup>Precipitation under inert gas is induced by pH increase in the presence of ammonia and results in CS-coated nanoparticles.

213 **2.3. Materials' Characterization.** **2.3.1. Determination of the**  
 214 **DA of CSs.** Depolymerized (low DA) and reacylated CSs were  
 215 characterized using <sup>1</sup>H NMR in order to determine more precisely  
 216 their respective DAs.<sup>37</sup> Samples were prepared by dissolving 10 mg of  
 217 CS in 1 mL of D<sub>2</sub>O with 5 μL of HCl (12 N). The solutions were  
 218 then transferred to NMR tubes. Analyses were carried out at room  
 219 temperature using a 400 MHz spectrometer (Bruker). Trimethylsilyl-  
 220 3-propionic-2,2,3,3-*d*<sub>4</sub> acid sodium salt was used as an internal  
 221 reference.

222 **2.3.2. Analysis of CS Average Molar Mass.** SEC was performed to  
 223 analyze both depolymerized and reacylated CSs. First, CS was  
 224 dissolved overnight at 1 mg/mL in 0.2 M AcOH/AcONH<sub>4</sub> 0.15 M  
 225 buffer. The solution was then filtered through cellulose methyl ester  
 226 membranes with 0.22 μm pore size and put in an SEC vial. The  
 227 analyses were done with a system comprising a 1260 infinity pump  
 228 (Agilent technology) connected to two columns, TSKgel G2500PW

and G6000PW (Tosoh Bioscience). The MALLS detector used was a 229  
 Dawn Heleos II (Wyatt) operating at 690 nm and coupled to an  
 230 Optilab T-Rex refractometer (Wyatt). The refraction index incre-  
 231 ments were selected according to the values of DA.<sup>38</sup> 232

233 **2.3.3. FTIR Analysis of CS-Coated SPIONs.** Samples were mixed  
 234 with KBr and pressed to form pellets. Infrared spectra were recorded  
 235 with a Nicolet iS-10 FTIR spectrometer (Thermo Scientific). The  
 236 range of analysis was 4000–400 cm<sup>-1</sup>. For each measurement, 200  
 237 scans were performed with a resolution of 4 cm<sup>-1</sup>.

238 **2.3.4. Determination of Organic Coating Amount on SPIONs.**  
 239 Analyses were carried out with a TGA Q-500 device (TA  
 240 Instruments). Thermograms were recorded from 30 to 700 °C with  
 241 a heating rate of 10 °C/min under dry air. Sample mass loss above  
 242 200 °C was used to determine the amount of CS on the SPIONs.

243 **2.3.5. Size Distributions of SPIONs.** SPIONs were observed by  
 244 transmission electron microscopy (TEM) (at the Centre Technolo-  
 245 gique des Microstructures CTμ, Villeurbanne, France). Suspensions  
 246 were prepared in deionized water for both "pristine" Fe<sub>3</sub>O<sub>4</sub> SPIONs  
 247 and for CS-coated SPIONs and were sonicated for 15 min. Then, one  
 248 droplet of each suspension was put on separated ultrafine carbon  
 249 TEM grids, and the solvent was evaporated at room temperature.  
 250 Images of nanoparticles were obtained using a 1400 flash (JEOL)  
 251 microscope equipped with a camera Rio 16 (Gatan). For each sample,  
 252 size distribution was obtained by measuring the diameter of 250  
 253 SPIONs using ImageJ program and by fitting the data with a log-  
 254 normal distribution function.

255 **2.3.6. Analysis of the Crystalline Structure of SPIONs.** Wide-angle  
 256 X-ray scattering (WAXS) experiments were performed at European  
 257 Synchrotron Radiation Facility (ESRF) (Grenoble, France), on  
 258 D2AM beamline. SPIONs were placed on a homemade sample  
 259 holder and entrapped between two Kapton tapes. The incident  
 260 photon energy was set to 15.7 keV, with a beam diameter of 45 μm. A  
 261 WAXS Open for SAXS (WOS) 2D-detector was used, from  
 262 IMXPAD.<sup>43</sup> The distance between the sample and the detector was  
 263 set to ~10 cm. The scattering vector *q* calibration was performed  
 264 using a chromium oxide (Cr<sub>2</sub>O<sub>3</sub>) powder reference. All scattered  
 265 images were normalized by the transmitted intensity, and azimuthal  
 266 averages were calculated around the image center (mean center of  
 267 incident beam). The signal corresponding to an empty cell with only  
 268 2 Kapton tapes was recorded and subtracted of each sample in order  
 269 to obtain the scattering diagram of the samples. The estimation of  
 270 crystallite size was done using the isotropic Williamson–Hall model,<sup>44</sup>  
 271 corresponding to the following equation

$$\beta_{hkl} \cos(\theta_{hkl}) = \frac{k\lambda}{D} + 4\epsilon \sin(\theta_{hkl}) \quad (1)$$

272 where  $\beta_{hkl}$  is the full width at half-maximum (fwhm) of the diffraction  
 273 peak corresponding to the (*hkl*) family of reticular planes.  $\beta_{hkl}$  was  
 274 determined using a fitting of the scattering profile with Voigt function  
 275 (combination of Gauss and Lorentz fittings) for (220), (311), (400),  
 276 (511), and (440) diffraction peaks.  $\theta_{hkl}$  is the Bragg angle (in radians)  
 277 corresponding to the (*hkl*) diffraction peak, *k* is a constant (*k* ~ 0.9  
 278 for isometric crystals),  $\lambda$  is the wavelength of the incident radiation ( $\lambda$   
 279 = 0.7907 Å), *D* is the average crystallite size of the sample, and  $\epsilon$  is the  
 280 lattice strain. A linear fit was performed to extract the crystallite size of  
 281 each sample. 282

283 **2.3.7. Analysis of SPIONs Suspensions.** Small-angle X-ray  
 284 scattering (SAXS) experiments were also performed at ESRF on  
 285 D2AM beamline using the same beam in the conditions of  
 286 simultaneous SAXS/WAXS experiments. SPION suspensions were  
 287 prepared at 1 mg/mL in ultrapure water for Fe<sub>3</sub>O<sub>4</sub> and in ultrapure  
 288 water + 1 μL/mL of pure acetic acid for CS-coated Fe<sub>3</sub>O<sub>4</sub> SPIONs in  
 289 order to facilitate their redispersion from powders. The suspensions  
 290 were sonicated during 40 min and then transferred in glass tubes  
 291 (Deutero GmbH, ref 600020-200, external diameter: 3 mm, length:  
 292 60 mm, width: 0.2 mm). The sample to detector distance was set to  
 293 1.89 m. D5 solid state detector (IMXPAD) was used for the 2D image  
 294 collection. Again, the *q*-range calibration was performed with a  
 295 standard (silver behenate), the scattering diagrams were normalized

296 by transmission, and the signal of the empty cell (solvent + tube) was  
297 subtracted in order to deduce the SPIONs' signal at small angles.

298 **2.3.8. Magnetization Measurements.** The magnetic curves were  
299 determined at room temperature, under an applied magnetic field  
300 ranging from  $-10$  to  $10$  kOe, using a vibrating sample magnetometer  
301 (VSM). The magnetic measurements as a function of temperature  
302 have been carried out in a Cryomagnetics ModelC—Mag Vari-9T  
303 Research System. Temperature-dependent magnetization  $M$  was  
304 measured in the zero-field-cooling (ZFC) and field-cooled (FC)  
305 regimes. In ZFC protocol, the sample was cooled down to the lowest  
306 temperature ( $4$  K) in the absence of any external magnetic field ( $H$ ).  
307 At  $T = 4$  K, the measurement field ( $100$  Oe) was applied, and  $M \times T$   
308 curve was recorded during warming. After recording  $M$  in the ZFC  
309 mode, the sample was subsequently cooled down until  $T = 4$  K at the  
310 same field and temperature dependence. The temperature sweep rate  
311 during the measurements of  $M$  was  $1$  K/min.

312 **2.3.9. MH Measurements.** For each measurement,  $1$  mL of  
313 suspension at a concentration of  $5$  mg/mL was prepared. Samples  
314 were then sonicated for  $15$  min. D5 series thermomagnetometer  
315 (Nanoscale Biomagnetics) equipped with a G2 Driver and a cal 2 coil  
316 (Nanoscale Biomagnetics) was used for the measurements. We  
317 investigated three different frequencies ( $491$ ,  $306$ , and  $168$  kHz) and  
318 magnetic field amplitudes ( $370$ ,  $200$ , and  $100$  G) for each sample.  
319 Temperature was initially stabilized at  $\sim 25$  °C, followed by  $10$  min of  
320 acquisition at fixed field amplitude and frequency. The obtained  
321 temperature vs time curves were used to determine the specific  
322 absorption rate (SAR) value of the SPIONs, according to

$$\text{SAR} = C_{p\text{-solvent}} \frac{\Delta T / \Delta t}{C_{\text{Fe}}} \quad (2)$$

323 where  $C_{p\text{-solvent}}$  is the specific heat capacity of the solvent ( $4.186$  J K $^{-1}$   
324 g $^{-1}$  for water),  $\Delta T / \Delta t$  is the initial temperature rate in °C/s, and  $C_{\text{Fe}}$   
325 is the concentration of iron oxide in g/mL. In this work,  $\Delta T / \Delta t$  was  
326 calculated considering a time range between  $0$  and  $60$  s.

327 **2.3.10. MRI Analyses.** SPIONs were first embedded in gelatin  
328 hydrogels to prevent any movement or aggregation during the  
329 experiment. Briefly, gelatin was dissolved in water ( $3\%$  m/v) under  
330 mechanical stirring at  $80$  °C. Then,  $4$  mL of the gelatin solution and  
331  $0.5$  mL of a SPIONs suspension at a given concentration were  
332 introduced in  $5$  mL glass tubes ( $180 \times 7.5 \times 0.6$  mm). The  
333 concentration of the SPION suspension was adjusted depending on  
334 the final concentration needed in the hydrogel. The tubes containing  
335 gelatin + SPIONs suspension were manually stirred to obtain a  
336 homogeneous mixture and then placed in the fridge overnight for  
337 gelation. MRI experiments were conducted at CREATIS Laboratory  
338 (Villeurbanne, France) for  $11.7$  and  $7$  T using Bruker Biospec MR  
339 scanners and at  $0.5$  T using a Pure Devices benchtop MRI.  $3$  T  
340 acquisitions were carried out at Léon Bérard center (Lyon, France) on  
341 a Siemens Syngo MRI scanner and at Hospital Lyon Sud (Pierre-  
342 Bénite, France) for  $1.5$  T using an Elekta Unity MRI scanner. For  $T_1$   
343 acquisitions, saturation-recovery sequences were used at  $0.5$ ,  $1.5$ ,  $7$ ,  
344 and  $11.7$  T, while modified look-locker imaging (MOLLI) sequence  
345 was employed at  $3$  T. For  $T_2$  acquisitions, multiecho spin-echo  
346 sequences were used for all analyses.

347 **2.3.11. Cytotoxicity Analysis.** Canine dermal fibroblasts were  
348 isolated from a dead neonatal dog obtained from VetAgro Sup—  
349 Campus Vétérinaire de Lyon (Marcy l'Etoile, France) in accordance  
350 with the VetAgro Sup animal ethics committee. Skin specimens were  
351 collected from the abdominal surface of the dog, cut into small  
352 fragments, and digested with  $0.25\%$  trypsin/ethylenediaminetetra-  
353 acetic acid (EDTA) (Eurobio, France) for  $15$  min at  $37$  °C. After  
354 digestion, the skin fragments were washed with phosphate-buffered  
355 saline (PBS). Dermal fibroblast cultures were established by explant  
356 culture in  $25$  cm $^2$  flasks (Falcon, VWR international, France)  
357 containing Dulbecco's modified Eagle's medium (Eurobio, France)  
358 supplemented with  $20\%$  fetal calf serum,  $2$  mM L-glutamine, and  $2\%$   
359 penicillin/streptomycin/amphotericin B (Eurobio, France) at  $37$  °C  
360 in a humidified atmosphere with  $5\%$  CO $_2$ . After  $2$  days, the fibroblasts  
361 started to grow out, and fragments were removed. After  $5$  days, the

cells reached confluence and were subcultured after  $0.25\%$  trypsin/  
EDTA treatment in  $75$  cm $^2$  flasks (Falcon, VWR international,  
France). The culture medium was changed two times a week. Cells  
are used between three and six passages.

Cell viability was investigated in accordance with the international  
standard operation procedure "Tests for in vitro cytotoxicity" ISO  
10993-5. Cell cytotoxicity was assessed by the CCK-8 assay (Sigma-  
Aldrich, France) according to the manufacturer's instructions. This  
assay is based on reduction of soluble yellow tetrazolium into  
insoluble purple formazan crystals by mitochondrial succinate  
dehydrogenase of viable cell. Therefore, the rate of formazan crystal  
formation is directly proportional to number of viable cells, which is  
measured in terms of absorbance. For this, canine fibroblasts were  
seeded at a density of  $1 \times 10^4$  cells/well in  $96$ -well plate at  $37$  °C in  
humidified atmosphere with  $5\%$  CO $_2$ . After  $2$  days of culture, the  
medium was replaced with fresh medium containing Fe $_3$ O $_4$   
nanoparticle formulations with concentrations of  $0$  [control],  $0.060$ ,  
 $0.125$ ,  $0.250$ ,  $0.500$ , and  $1$  mg/mL. Three exposure times ( $24$ ,  $48$ , and  
 $72$  h) were tested in all experiments. After incubation,  $10$   $\mu$ L CCK8  
was added into each well and incubated at  $37$  °C for  $2$  h. The  
absorbance was measured at  $450$  nm using a microplate reader  
(MultiSkan, Thermo Fisher). Cell viability was determined as the  
ratio of the optical density (OD) of exposed cells to the OD of the  
untreated cells  $\times 100$ . For each concentration of nanoparticles, mean  
values of the mean absorbance rates from eight wells were calculated,  
and results were expressed as mean  $\pm$  standard deviation. All  
experiments were performed in duplicate. Graphs and statistical  
analyses (Tukey test) were performed using Origin software.  $P$ -values  
 $\leq 0.05$  were considered significant.

## 3. RESULTS AND DISCUSSION

**3.1. Coating Analysis of SPIONs.** **3.1.1. In-Depth CS**  
**Average Molar Weight Characterization.** Table S2 gathers  
the macromolecular characterizations of different CSs that  
were prepared and used in this work. As expected, a decrease  
of  $M_w$  was observed from initial CS to CS $_1$ , which underwent a  
depolymerization process and was further used for the  
preparation of CS $_{14}$  and CS $_{34}$ . The depolymerization by  
nitrous deamination also contributed to narrow the molar  
weight distributions, with a significant decrease of dispersity  $\mathcal{D}$ .  
The average DA was determined by  $^1\text{H}$  NMR, with DAs for  
CS $_{14}$  and CS $_{34}$  close to the targeted values. This reacetylation  
induced an increase in the average molar weights, but the value  
of the mean degree of polymerization was quite constant.  
Thus, CS $_1$ , CS $_{14}$ , and CS $_{34}$  constitute a homologous series of  
statistical CSs. Details about calculations can be found in the  
Supporting Information and chromatograms in SI2.

**3.1.2. CS–Magnetite Nanoparticle Interactions Studied**  
**by Vibrational Spectroscopy (FTIR).** Figure S3a shows the  
Fourier transform infrared (FTIR) spectra of CSs with  
different DAs (CS $_{\text{DA}}$ ). Several characteristic bands of CS  
were identified.<sup>45</sup> The bands in the range of  $3600$ – $3000$  cm $^{-1}$   
were attributed to axial stretching of O–H and N–H bonds.  
Absorption bands at  $2930$  and  $2875$  cm $^{-1}$  indicated C–H axial  
stretching of CH $_2$  and CH $_3$  groups, respectively. For CS $_1$ , the  
absorption band at  $1600$  cm $^{-1}$  was attributed to the angular  
deformation of N–H in amine groups ( $-\text{NH}_2$ ). Some  
differences were observed for CS $_{14}$  and CS $_{34}$  due to different  
DA, with bands at  $1660$  and  $1550$  cm $^{-1}$  indicating, respectively,  
C=O axial stretch and angular deformation of N–H bonds for  
amino groups. As expected, the relative intensity of the C=O  
band increases with DA, evidencing the effective reacetylation  
of CS. For all CS $_{\text{DA}}$ , coupling of C–H angular deformation and  
C–N axial stretching were found at  $1380$  and  $1420$  cm $^{-1}$ .  
Finally, the vibration bands for the polysaccharide skeleton  
evidencing C–O and C–O–C stretching were found in the

range [1150–900  $\text{cm}^{-1}$ ].<sup>46</sup> Figure S3b shows IR spectra for noncoated and coated  $\text{Fe}_3\text{O}_4$  SPIONs. The absorption bands of  $\text{Fe}_3\text{O}_4$  SPIONs appeared at 590 and 440  $\text{cm}^{-1}$ . These bands are shifted in comparison to those of bulk magnetite, with absorption bands at 570 and 375  $\text{cm}^{-1}$ , respectively.<sup>47</sup> This change may be attributed to nano-size effects of the SPIONs. The Fe–O bonds close to the surface are bended and distorted, therefore they vibrate at higher wavenumbers than that in bulk magnetite, where the bonds are not physically constrained. In comparison to noncoated  $\text{Fe}_3\text{O}_4$  SPIONs, the FTIR spectra of  $\text{Fe}_3\text{O}_4/\text{CS}_{\text{DA}}$  SPIONs show the characteristic absorption bands of magnetite at 590 and 440  $\text{cm}^{-1}$ , but the typical bands of CS were also present and appeared slightly shifted. For  $\text{Fe}_3\text{O}_4/\text{CS}_1$ , C=O axial stretching appeared at 1595  $\text{cm}^{-1}$ . For  $\text{Fe}_3\text{O}_4/\text{CS}_{14}$  and  $\text{Fe}_3\text{O}_4/\text{CS}_{34}$ , C=O axial stretching and angular deformation of NH bonds shifted, respectively, to 1635 and 1540  $\text{cm}^{-1}$ . For all types of  $\text{CS}_{\text{DA}}$  SPIONs exhibited coupling of C–H angular deformation and C–N axial stretching at 1380 and 1420  $\text{cm}^{-1}$ , the polysaccharide skeleton at 1065  $\text{cm}^{-1}$ , and other bands for glycosidic bonds in the range of 1153–894  $\text{cm}^{-1}$ . As a result, we conclude that significant interactions are formed between magnetite nanoparticles and CS chains, possibly through amine groups and acetyl groups for  $\text{CS}_{14}$  and  $\text{CS}_{34}$ , respectively.

**3.1.3. Thermal Degradation of CS: Dosage and Interactions with Magnetite.** The obtained thermogravimetric analysis (TGA) results are listed in Table 1. Figure S4a shows

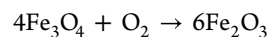
**Table 1. Structural Characteristics of the Synthesized Magnetite SPIONs<sup>a</sup>**

samples	coating wt %	$d_{\text{TEM}}$ (nm)	$D_{\text{WAXS}}$ (nm)	$\epsilon_{\text{WAXS}}$ ( $\times 10^{-4}$ )	$d_{\text{e,SAXS}}$ (nm)	$D_{\text{m}}$ (nm)
$\text{Fe}_3\text{O}_4$	0	9.6	9.7	0.3	13.1	9.5
$\text{Fe}_3\text{O}_4/\text{CS}_1$	38.0	5.4	5.9	−10.0	6.7	8.1
$\text{Fe}_3\text{O}_4/\text{CS}_{14}$	38.8	5.7	5.2	−9.0	6.7	7.5
$\text{Fe}_3\text{O}_4/\text{CS}_{34}$	36.8	6.2	5.6	−6.4	7.5	8.2

<sup>a</sup>Values  $d_{\text{TEM}}$ ,  $D_{\text{WAXS}}$ ,  $d_{\text{e,SAXS}}$ , and  $D_{\text{m}}$  correspond to size of SPIONs measured using TEM, WAXS, SAXS, and VSM techniques, respectively.

the thermograms of CSs with different DAs ( $\text{CS}_{\text{DA}}$ ). Mass losses below 200 °C are attributed to water desorption.<sup>48</sup> The degradation of CS started at 250 °C and was complete at 625 °C. No major differences were observed in the thermograms with the evolution of DA. Figure S4b shows the thermograms of uncoated and CS-coated SPIONs. Polymer degradations start at lower temperature in  $\text{CS}_{\text{DA}}$ /SPIONs in comparison to that in their pure CS references. Again, these changes are indicative of interactions between CS and the SPIONs' surface. On the noncoated  $\text{Fe}_3\text{O}_4$  SPION curve, a slight mass increase of 0.5% was observed between 100 and 180 °C. This mass gain has been reported in several studies and is characteristic of magnetite.<sup>49,50</sup> It is due to the oxidation process of magnetite  $\text{Fe}_3\text{O}_4$  to maghemite  $\gamma\text{-Fe}_2\text{O}_3$ . However, the theoretical mass gain for such conversion is 3.3%.<sup>51</sup> In our work, as in previously mentioned studies, it was found to be less than 1%. This is due to the overlap of two different processes, where the mass gain due to the oxidation of magnetite is superimposed with the elimination of OH groups and  $\text{H}_2\text{O}$  molecules present at the SPIONs surface.

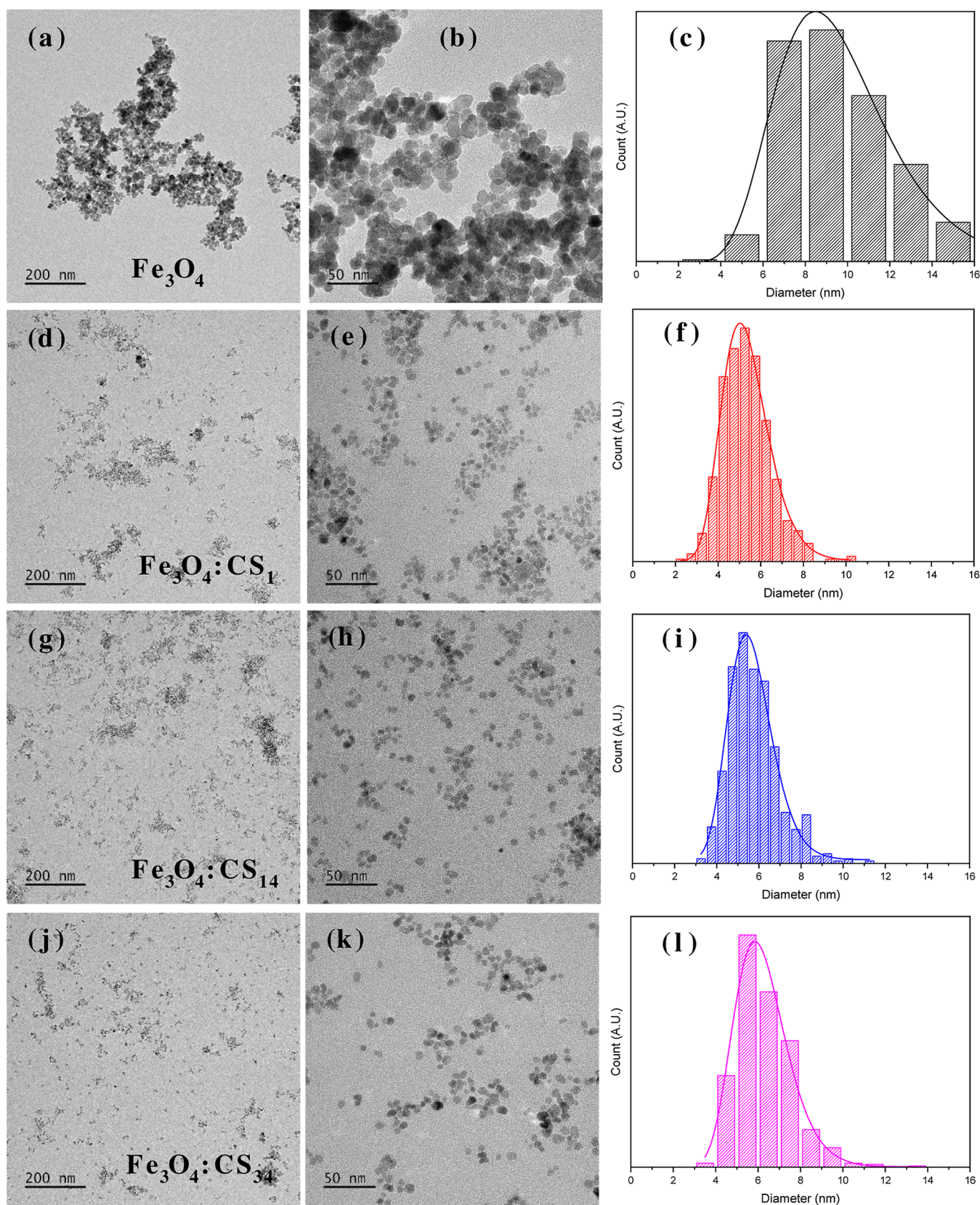
The amount of coating on SPIONs was estimated by two different methods. In contact with air and above 250 °C, magnetite  $\text{Fe}_3\text{O}_4$  tends to be oxidized in hematite  $\alpha\text{-Fe}_2\text{O}_3$  following the reaction



In this study, thermograms were recorded under dry air flow from 30 to 700 °C with a temperature rate of 10 °C/min. As the formation of hematite occurs at high temperature, it can be assumed that a complete conversion of  $\text{Fe}_3\text{O}_4$  to  $\alpha\text{-Fe}_2\text{O}_3$  was achieved at the end of thermal analyses at 700 °C. The first method relies on the hypothesis that the amount of iron remains constant through the entire TGA analysis and that the conversion of pure magnetite to hematite is characterized by a mass increase of 3.3%. Also, full degradation of the coatings was supposed at 700 °C, given the previous thermograms for CSs alone. Thus, a second method consists in measuring the mass loss associated with the complete degradation of CS, accounting again for the mass gain associated with magnetite to hematite conversion. Full calculations are available in S15. Finally, both methods lead to comparable results, with a weight fraction of CS close to 38%. Logically, we obtained here higher weight percentages (wt %) of CS coatings than in our previous study with COS<sup>37</sup> (~35–40% for CS coating in comparison with ~10–15% for COS) even though the same protocol was applied and the same amount of iron salts and CS or COS were used. This is likely due to the higher molar mass of CS, contributing to a thicker coating in comparison to short COS.

In a second step, the amount of coating was varied, and two new batches of  $\text{CS}_1$ -coated SPIONs were synthesized.  $\text{CS}_1$  was chosen for these tests as  $\text{Fe}_3\text{O}_4/\text{CS}_1$  SPIONs showed the best results in MH experiments (see Section 3.3.2). For molar ratios  $n_{\text{CS}_1}/n_{\text{Fe}}$  of 0.3, 0.2, and 0.1 (see Section 2.2.4), results indicated coating mass fractions of 38, 29.6, and 19.3% w/w (of dry material), respectively, showing the possibility of tuning the coating amount on the SPIONs. This allows to find an optimal equilibrium between magnetic and biological properties (see Section 1). Therefore, with such CS coating, these two properties can be easily and precisely adjusted depending on the desired application. Figure S4c displays the thermograms for these experiments.

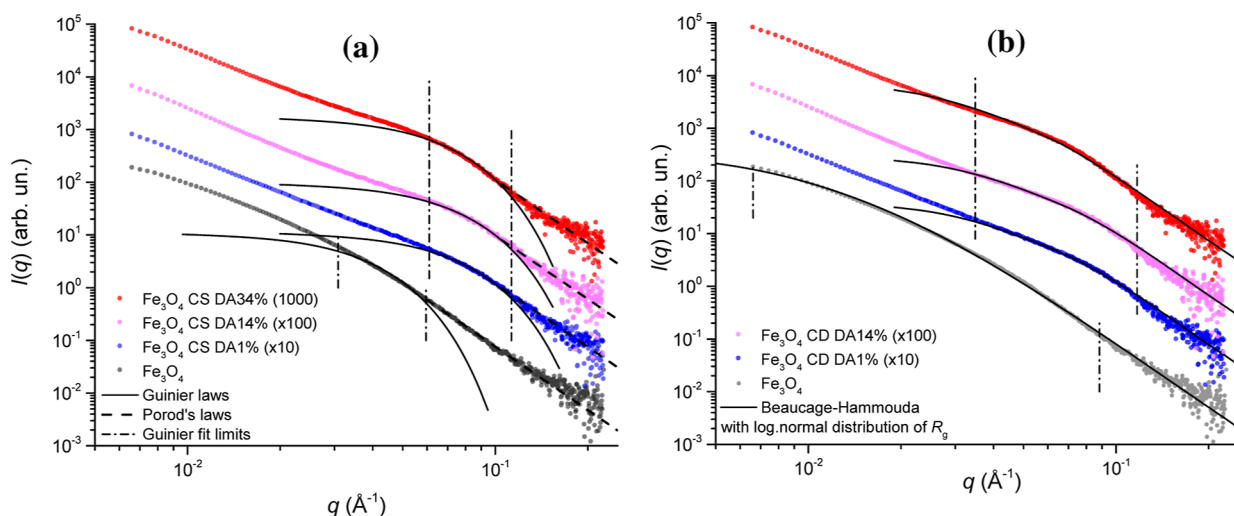
**3.2. Structural Characterizations of  $\text{Fe}_3\text{O}_4$  and  $\text{Fe}_3\text{O}_4/\text{CS}_{\text{DA}}$  SPIONs.** **3.2.1. Microscopic Analyses of SPION Dried Suspensions.** TEM experiments were performed to determine the size and the morphology of the SPIONs. Figure 1 shows the TEM images of SPIONs and the particle size histograms adjusted with a log–normal distribution.<sup>52</sup> All particles had a nearly spherical shape, with sizes ranging from 5 to 10 nm. The smallest sizes were obtained for CS-coated SPIONs, with an average size of 5.8 nm. Similar results were found in our previous study with  $\text{Fe}_3\text{O}_4$  SPIONs coated with COSs.<sup>36</sup> This can be explained by the in situ method of synthesis of SPIONs, where CS is added to the solution of ferrous and ferric ions before the coprecipitation. CS may complex the iron ions at amino and hydroxyl sites.<sup>53</sup> Thus, such a complexation may promote dispersion and nucleation of SPIONs, reducing their size and narrowing their dispersity. The slight increase of SPIONs' size with DA of CS may be due to the less numerous amine groups as more amino complexation sites are substituted by acetyl groups when the DA increases. These results are in agreement with those we previously obtained with COS,<sup>36</sup> showing that the molar weight of CS should play a minor role



**Figure 1.** TEM pictures and histograms of size distribution profiles for (a–c)  $\text{Fe}_3\text{O}_4$ , the size distribution fitted with a log–normal distribution giving an average diameter  $d_{\text{TEM}} = 9.6 \pm 2.9$  nm, (d–f)  $\text{Fe}_3\text{O}_4/\text{CS}_1$ , with  $d_{\text{TEM}} = 5.4 \pm 1.1$  nm, (g–i)  $\text{Fe}_3\text{O}_4/\text{CS}_{14}$ , with  $d_{\text{TEM}} = 5.7 \pm 1.1$  nm, (j–l)  $\text{Fe}_3\text{O}_4/\text{CS}_{34}$ , with  $d_{\text{TEM}} = 6.2 \pm 1.3$  nm.

535 on the complexation and nucleation of magnetite nano-  
536 particles.

TEM pictures also show aggregates of the SPIONs. As 537  
mentioned previously, this may be partly due to the large 538



**Figure 2.** (a) SAXS diagrams showing experimental data and their modeling using the Guinier law for  $\text{Fe}_3\text{O}_4$ ,  $\text{Fe}_3\text{O}_4/\text{CS}$ ,  $\text{Fe}_3\text{O}_4/\text{CS}_{14}$ , and  $\text{Fe}_3\text{O}_4/\text{CS}_{34}$  samples. The shoulder present in the coated systems is well reproduced, but the scattering of uncoated  $\text{Fe}_3\text{O}_4$  nanoparticle suspension is poorly characterized as a result of a broader distribution of elementary particles. (b) Modeling of scattering data with a distribution of gyration radii evidencing the distribution of gyration radii in the noncoated  $\text{Fe}_3\text{O}_4$  system.

539 surface/volume ratio of SPIONs, which leads to the formation  
540 of clusters to reduce the surface energy, but aggregation  
541 necessarily occurs after the drying of the SPION suspension,  
542 which was performed for TEM observations. Therefore, the  
543 aggregation state characterized by TEM pictures may not be  
544 representative of the aggregates present in suspension. CS-  
545 coated SPIONs appeared less aggregated than COS-coated  
546 ones,<sup>36</sup> possibly thanks to the steric hindrance effect enhanced  
547 by longer CS chains. It was not possible to clearly see the  
548 coatings on SPIONs, probably because of an insufficient  
549 contrast for CS compared to that for magnetite  $\text{Fe}_3\text{O}_4$ .  
550 However, given the single-crystal morphology of SPIONs, CS  
551 should not be inserted within the SPIONs' core but rather  
552 surround them and form a shell around the magnetite cores.

553 **3.2.2. Microstructural Study of SPIONs by SAXS.** The  
554 SAXS patterns of the  $\text{Fe}_3\text{O}_4$  SPIONs are shown in Figure 2.  
555 For coated particles, we can divide the pattern in three  
556 domains. (i) In the low vector range ( $q < 3 \times 10^{-2} \text{ \AA}^{-1}$ ),  
557 scattering is dominated by the presence of aggregates of  
558 primary particles. Their analysis with the generalized Porod's  
559 law ( $I(q) = Bq^{-\alpha}$ ) yields fractal exponents  $\alpha \approx 2.3$  for all  
560 coated nanoparticles, typical of a rather compact aggregation of  
561 ultrafine particles.<sup>54</sup> Interestingly, this scattering behavior with  
562 an intensity upturn in the low  $q$  range is not clearly found in  
563 the noncoated  $\text{Fe}_3\text{O}_4$  nanoparticle system. This indicates that  
564 after freeze-drying, the coating may favor the formation of  
565 aggregates by chain interdiffusion and entangling, with such  
566 interparticle interactions remaining after redispersion with  
567 sonication in aqueous media and further dilution to 0.1 g/mL.  
568 (ii) In the intermediate scattering range, from  $q = 5 \times 10^{-2}$  to  
569  $0.1 \text{ \AA}^{-1}$ , a shoulder is present on the scattering diagrams of the  
570 coated systems, which can be modeled with a Guinier law

$$I(q) = I_0 \exp\left(-\frac{R_g^2}{3} q^2\right) \quad (3)$$

571

572 (iii) In the  $q$ -range from 0.1 to  $0.15 \text{ \AA}^{-1}$ , the Porod's regime  
573 is present as

$$I(q) = \frac{C}{q^4} \quad (4)$$

The results obtained for the gyration radii are displayed in  
575 SI6 and Table 1 as  $d_{e,\text{SAXS}} = 2\sqrt{\frac{5}{3}} R_g$ . Interestingly, the  
576 modeling of the uncoated  $\text{Fe}_3\text{O}_4$  suspension may not reflect  
577 the actual distribution of gyration radii as the overlap between  
578 the Guinier model and the experimental data is limited to a  
579 rather restricted  $q$ -range, and no shoulder is clearly present on  
580 the scattering diagram. A more complex analysis was thus  
581 applied to the noncoated  $\text{Fe}_3\text{O}_4$  nanoparticles to better  
582 account for a broader gyration radius distribution, as suggested  
583 by TEM observations. The scattering patterns were modeled  
584 accounting for a log-normal distribution of gyration radius of  
585 the particles, i.e., invoking a sum of Guinier and Porod laws,  
586 weighted by the distribution function  $g(R_g)$ . Hence, the  
587 scattered intensity can be theoretically written as  
588

$$I(q, \mu, \sigma) = \int_0^{+\infty} k \cdot g(R_g) \cdot I_p(q) \cdot dR_g \quad (5)$$

where  $I_p(q)$  can be written to combine the Guinier and Porod's  
590 laws<sup>55</sup>  
591

$$I_p(q) = k R_g^6 \cdot \exp\left(-\frac{R_g^2 \cdot q^2}{3}\right) + \frac{C}{q^d} \left[ \text{erf}\left(\frac{q R_g}{\sqrt{6}}\right) \right]^{3d} \quad (6)$$

where  $C = k R_g^{6-d} \left[ \frac{6d^2}{(2+d)(2+2 \times d)} \right]^{d/2} \Gamma\left(\frac{d}{2}\right)$   
593

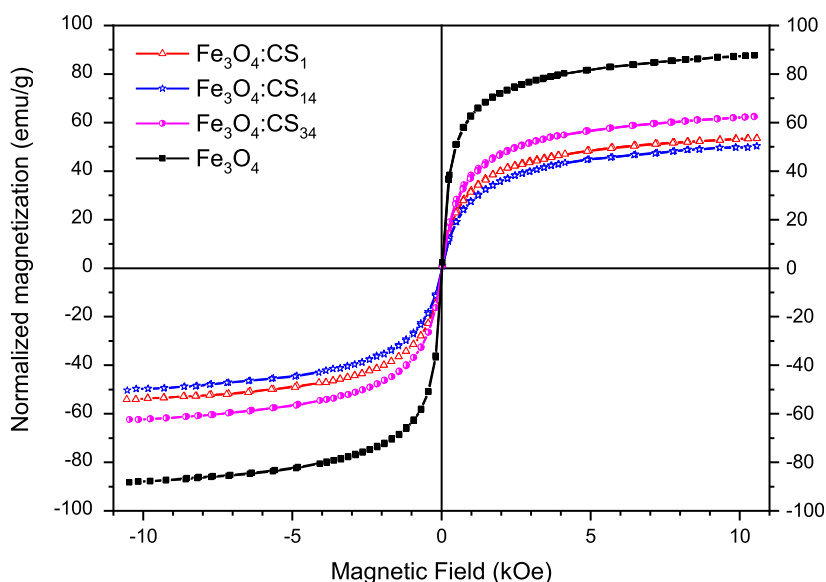
Hypothesizing a net interface of the  $\text{Fe}_3\text{O}_4$  cores,  $d = 4$   
594 further results in  $C = 10.24 \text{ \AA}^2$ .  $k$  is a scaling factor,  $q =$   
595  $4\pi\lambda^{-1} \sin(\theta)$ ,  $2\theta$  is the Bragg angle, and  $g(R_g)$  is the log-  
596 normal number distribution function of gyration radii  $R_g$   
597

$$g(R_g) = \frac{1}{R_g \cdot \sigma \cdot \sqrt{2\pi}} \exp\left(-\frac{(\ln(R_g) - \mu)^2}{2\sigma^2}\right) \quad (7)$$

where  $R_g$  is the mean radius of gyration ( $\text{\AA}$ ).  
599

The calculation of  $I(q, \mu, \sigma)$  integral was performed by  
600 Lobatto's recursive adaptive quadrature, using the `quadl()`  
601





**Figure 3.** Normalized  $M \times H$  curves (emu/g of  $\text{Fe}_3\text{O}_4$ ) for  $\text{Fe}_3\text{O}_4$  and  $\text{Fe}_3\text{O}_4/\text{CS}_{\text{DA}}$ . CS coating decreases  $M_{\text{S}}$  but does not impact coercivity.

602 function within Matlab or Octave environments, adapting the  
 603 sampling of  $R_{\text{g}}$  values in the relevant decades of the  
 604 investigated  $q$ -range. Then, the determination of parameters  
 605  $\mu$  and  $\sigma$  was performed using the nonlinear least-squares solver  
 606 *lsqcurvefit*(). The numerical results arising from the determi-  
 607 nation of the  $g(R_{\text{g}})$  distribution function shape parameters are  
 608 shown in the bottom part of SI6. Such evaluation offers a way  
 609 to deduce not only the number-average radius of gyration  
 610  $M_1 = \langle R_{\text{g}} \rangle = e^{\mu + \sigma^2/2}$  but also all moments of the  $g(R_{\text{g}})$   
 611 distribution  $M_k = \langle R_{\text{g}}^k \rangle = e^{k\mu + k^2\sigma^2/2}$  and their ratios. As  
 612 shown in Figure 2 and SI6, the scattering curve of noncoated  
 613 systems is well reproduced in the entire  $q$ -range with a rather  
 614 broad distribution of particle gyration radii, with coefficient of  
 615 variation  $\text{Cv} = \sqrt{e^{\sigma^2} - 1} \approx 0.52$ .

616 However, when the same distributed Beaucage–Hammouda  
 617 analysis is applied on coated systems, a satisfactory modeling  
 618 can only be obtained restricting the analyzed data to the  $q$ -  
 619 range from  $\sim 3.5 \times 10^{-2}$  to  $0.12 \text{ \AA}^{-1}$ , thus excluding the large  
 620 aggregate contribution, and choosing a generalized Porod  
 621 exponent  $d \sim 4.5$ , indicative of the presence of a gradual  
 622 decrease of electron density from the surface of the  
 623 nanoparticles. In agreement with the TEM observations, the  
 624 CS coatings induce a significant decrease of the mean gyration  
 625 radius. A strong decrease of the breadth of the gyration radius  
 626 distribution is reflected by very small values of parameter  $\sigma$  and  
 627 Cv. In fact, such negligible breadth parameters values may  
 628 partly result from the restricted  $q$ -range used for the fits of the  
 629 coated systems: it may not be possible to separate  
 630 unambiguously the contribution of small aggregates of  
 631 elementary particles and that of larger elementary particles.

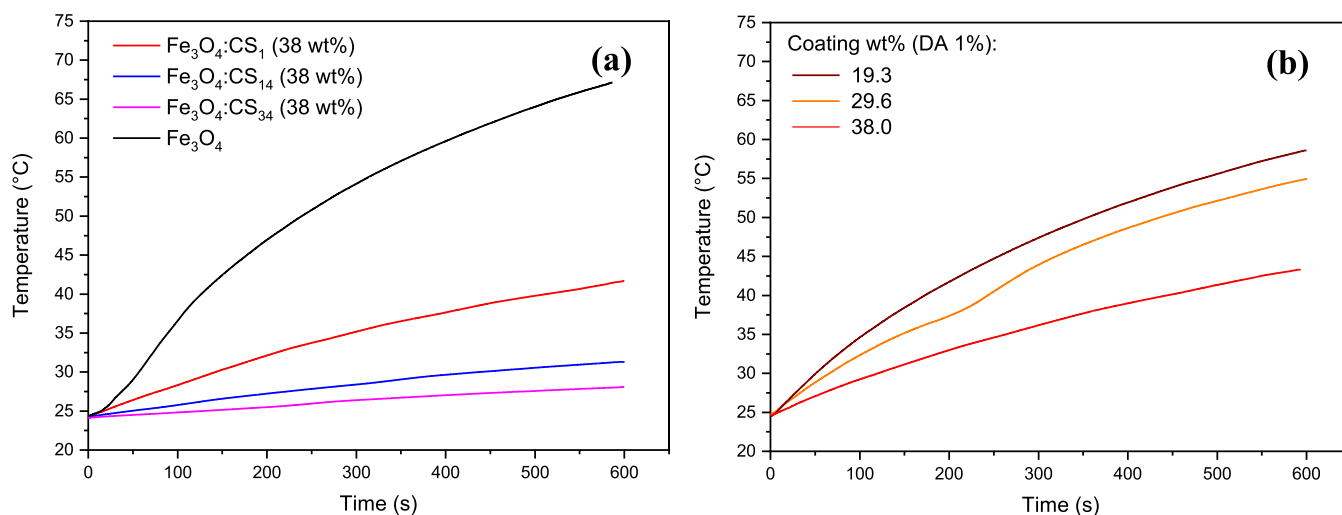
632 As a general trend, comparing coated and noncoated  
 633 SPIONs, CS coatings lead to a narrow distribution of particle  
 634 sizes during the precipitation of magnetite nanoparticles,  
 635 resulting in a net shoulder in the scattering patterns. However,  
 636 it can also favor the formation of aggregates during freeze-  
 637 drying of the suspension, the contribution of which was not  
 638 taken into account in the TEM analysis of elementary particles.  
 639 **3.2.3. Crystalline Structure of Magnetite Cores Invest-**  
 640 **igated by WAXS.** Figure S5 shows the diffraction diagrams (in

transmission mode) obtained on SPION powders. The signal 641  
 is largely dominated by the SPION  $\text{Fe}_3\text{O}_4$  cores. The 642  
 crystalline structure of the SPIONs can clearly be identified 643  
 with several peaks corresponding to (111), (220), (311), 644  
 (222), (400), (422), (511), (440) family of planes of 645  
 magnetite. For  $\text{CS}_{\text{DA}}$ -coated SPIONs, a slight diffraction peak 646  
 ( $q \sim 1.42 \text{ \AA}^{-1}$ ) can be identified on each of the three spectra, 647  
 showing the effective presence of CS on the surface of 648  
 magnetite SPIONs. Larger diffraction peaks were observed for 649  
 $\text{CS}_{\text{DA}}$ -coated SPIONs. Such broadening can be induced both 650  
 by size and strain.<sup>56</sup> In many works, the apparent crystallite 651  
 sizes ( $D_{\text{WAXS}}$ ) are calculated using Scherrer's equation, by 652  
 considering the fwhm of the most intense (311) diffraction 653  
 peak. However, this approach must be applied carefully as 654  
 measuring crystallite size with a single diffraction peak only 655  
 gives an information about the apparent dimension in one 656  
 direction. Moreover, the Scherrer's equation does not consider 657  
 the potential strain-induced peak broadening, and this may 658  
 therefore lead to an underestimation or overestimation of 659  
 crystallite size. Here, we chose to use the Williamson–Hall 660  
 (WH) isotropic strain model for the analysis of peak 661  
 broadening.<sup>44</sup> In this model, the lattice strain and the crystallite 662  
 size are independent parameters, and the total peak broadening 663  
 is calculated by adding the two contributions (see Section 664  
 2.3.6). The results are reported in Table 1. The WH plots as 665  
 well as the results obtained using Scherrer's equation are 666  
 shown in SI7. Interestingly, the obtained values with the WH 667  
 model are very close to those measured by TEM, which 668  
 indicates the monocrystalline structure of the SPIONs. Also, 669  
 the tendency of CS to reduce the SPION size was also 670  
 evidenced with these WAXS experiments. In addition, 671  
 calculations of the apparent strain  $\epsilon$  from the WH model 672  
 showed that the presence of coating is inducing a compression 673  
 strain onto the cores (see Table 1, with negative strain values 674  
 for  $\text{CS}_{\text{DA}}$ ), unlike naked  $\text{Fe}_3\text{O}_4$  SPIONs for which the strain is 675  
 minimal. This compressive strain on cores of CS-coated 676  
 SPIONs may be attributed to a complexation mechanism of 677  
 surface  $\text{Fe}^{3+}$  ions, partial oxidation of the surface of particles, 678  
 and surface effects at the nanoscale. The strain values obtained 679  
 here using the WH model can be found to be relatively small in 680  
 comparison with those reported in other works in literature.<sup>57</sup> 681

**Table 2. Thermo-Magnetic Characteristics of Synthesized Magnetite SPIONs<sup>a</sup>**

samples	$M_s$ (emu/g)	SAR (W/g)	$r_1$ (mM <sup>-1</sup> s <sup>-1</sup> )	$r_2$ (mM <sup>-1</sup> s <sup>-1</sup> )	$r_2/r_1$
Fe <sub>3</sub> O <sub>4</sub>	87.7	85.1	0.8	154	185
Fe <sub>3</sub> O <sub>4</sub> /CS <sub>1</sub> (19.3 wt %)		83.0	0.7	127	177
Fe <sub>3</sub> O <sub>4</sub> /CS <sub>1</sub> (29.6 wt %)		49.6	1.0	137	141
Fe <sub>3</sub> O <sub>4</sub> /CS <sub>1</sub> (38.0 wt %)	53.4	40.5	1.2	124	104
Fe <sub>3</sub> O <sub>4</sub> /CS <sub>14</sub> (38.0 wt %)	50.3	14.0	1.5	137	92
Fe <sub>3</sub> O <sub>4</sub> /CS <sub>34</sub> (38.0 wt %)	62.4	7.0	1.9	123	65

<sup>a</sup>Values of  $r_1$ ,  $r_2$ , and  $r_2/r_1$  are given at  $B_0 = 3$  T. Values measured at other field strengths are given in SI12.



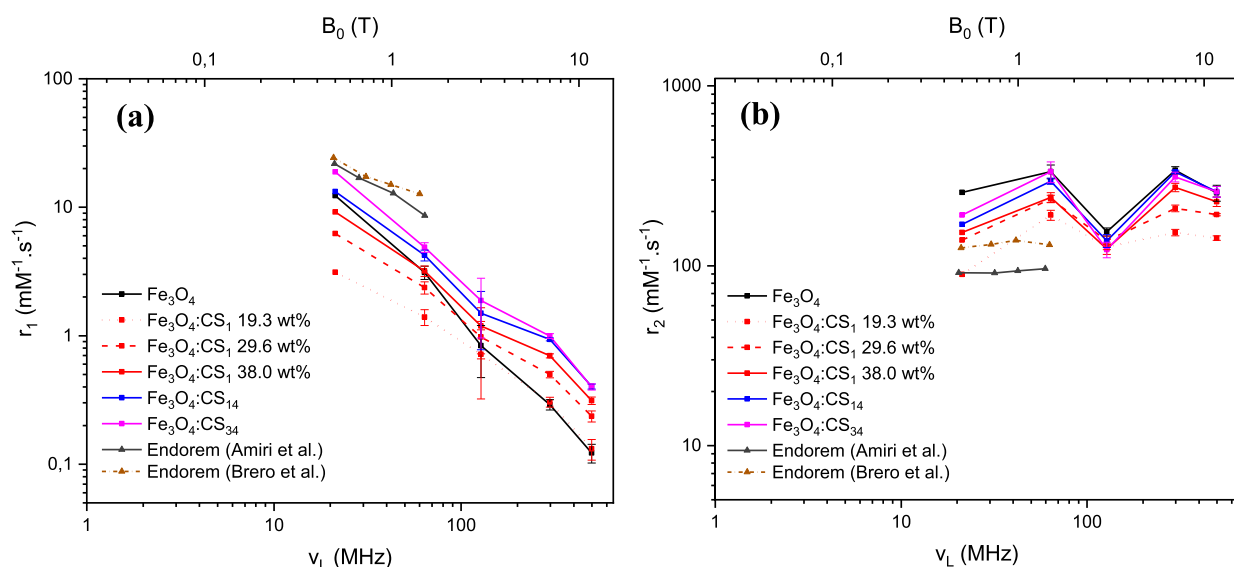
**Figure 4.** (a) Heating curves of Fe<sub>3</sub>O<sub>4</sub>, Fe<sub>3</sub>O<sub>4</sub>/CS<sub>1</sub>, Fe<sub>3</sub>O<sub>4</sub>/CS<sub>14</sub>, and Fe<sub>3</sub>O<sub>4</sub>/CS<sub>34</sub> SPIONs at a concentration of 5 mg/mL of Fe<sub>3</sub>O<sub>4</sub>, and a magnetic field amplitude and frequency of, respectively, 370 G and 491.45 kHz. (b) Heating curves of Fe<sub>3</sub>O<sub>4</sub>/CS<sub>1</sub> SPIONs with different amounts of coating, in the same experimental conditions.

682 However, it is known that the synthesis method is a key  
683 element contributing to residual microstrains in the samples.<sup>57</sup>  
684 Higher strains are more favored by mechanical and thermal  
685 processes than milder chemical syntheses. Our results stay in  
686 agreement with those of the studies in which the same  
687 synthesis protocols were used.<sup>58,59</sup>

688 **3.3. Magnetic Properties.** 3.3.1. *Measurement of*  
689 *Magnetic Properties.* Figure 3 shows the  $M$  (magnetization)  
690 vs  $H$  (magnetic field) curves recorded at room temperature,  
691 normalized by the exact percentage of magnetite. The apparent  
692 magnetization at saturation ( $M_s$ ) for noncoated Fe<sub>3</sub>O<sub>4</sub>  
693 SPIONs was  $\sim 87$  emu/g of nanoparticles, which is similar to  
694 values reported in the literature for bulk magnetite.<sup>60</sup> This  
695 result was not expected, since  $M_s$  is usually lower for SPIONs  
696 than for the bulk material because of finite size effects (i.e.,  
697 canting of the surface spins) and surface defects contributing to  
698 breaking of the crystal symmetry.<sup>61</sup> In this work, the decrease  
699 in  $M_s$  also depended on the coating amount: the highest  $M_s$   
700 was reached for noncoated Fe<sub>3</sub>O<sub>4</sub> SPIONs. It was lower for  
701 coated systems and measured to lie between  $\sim 50$  and  $\sim 63$   
702 emu/g (coating representing 36.8–38.8 wt %) (see Table 2).  
703 In order to exploit further the VSM curves, we used a  
704 modeling to separate the different components in the  
705 magnetization of the SPION nanoparticles. The magnetization  
706 for a single superparamagnetic nanoparticle can be classically  
707 expressed with the Langevin function  $L(x)$ <sup>62</sup>

$$708 \quad M(H, T) = M_s \cdot L(x) + \chi H \quad (8)$$

where  $L(x) = \coth(x) - \frac{1}{x}$ ,  $x = \frac{\mu_p H}{k_B T}$ ,  $\mu_p$  is the mean magnetic  
709 moment of the nanoparticle,  $k_B$  the Boltzmann constant,  $\chi$  the  
710 magnetic susceptibility, and  $H$  the magnetic field. This  
711 modeling for  $M(H, T)$  consists in two different contributions.  
712 The nonlinear Langevin term expresses the magnetization for  
713 an ideal superparamagnetic system, while the linear term  
714 characterizes the deviation to the Langevin behavior due to  
715 interparticle interactions, anisotropy, and inhomogeneities  
716 such as volume and moment distribution. Equation 8 was  
717 then used to fit the experimental VSM data. The results are  
718 shown in SI8. A good agreement was observed between the  
719 theoretical model and the experimental values. The two  
720 distinct Langevin and linear contributions were clearly  
721 evidenced. Interestingly, despite the size reduction of CS-  
722 coated SPIONs (see previous TEM, WAXS, and SAXS  
723 results), the linear contribution was found comparable to  
724 that of noncoated Fe<sub>3</sub>O<sub>4</sub> SPIONs. It was also possible to  
725 extract the mean SPION diameters from the model using  
726 structural considerations to link the particle diameter to the  
727 mean magnetic moment  $\mu_p$ . The values of magnetic sizes are  
728 shown in Table 1. Briefly, magnetite crystallizes following an  
729 inverse spinel cubic structure with a unit cell volume  $V_{UC} =$   
730  $592.07 \text{ \AA}^3$  containing  $N = 8$  formula units. The mean magnetic  
731 moment  $\mu_p$ , which was one of our fit parameters, can therefore  
732 be expressed by the equation  $\mu_p = N \times mbfu \times P$ , where  $mbfu$   
733 is the number of Bohr magneton per formula unit (which is  
734  $4.07 \mu_B$  for bulk magnetite<sup>61</sup>) and  $P$  the average number of unit  
735 cells in a single domain. The mean particle volume  $V$  and so its  
736



**Figure 5.** Evolution of  $r_1$  (a) and  $r_2$  (b) at different magnetic fields  $B_0$  and Larmor frequencies  $\nu_L$ . Different systems synthesized in this work were analyzed at 0.02 and 0.05 g/L to deduce the relaxivities, and the results are compared with Endorem.<sup>68,69</sup>

737 diameter are therefore assessable by the relation  $V = V_{UC} \times \mu_p /$   
738  $(N \times mbfu)$ .

739 Finally, in the magnetic measurements as a function of  
740 temperature in the ZFC and FC regimes (see S19), the samples  
741 showed a distinct irreversibility in a temperature known as the  
742 “blocking temperature”  $T_B$ . The  $\text{Fe}_3\text{O}_4$  SPIONs presented a  $T_B$   
743  $\sim 180$  K, and the CS-coated SPIONs show a  $T_B \sim 125$  K. This  
744 corroborates the results presented previously and suggests a  
745 decrease in the magnetic interaction between the nanoparticles  
746 due to CS-coating. Therefore, we can conclude that at room  
747 temperature, the samples are in a superparamagnetic state.

### 748 3.3.2. Characterization of Thermo-Magnetic Properties.

749 To evaluate the potential of the SPIONs for cancer treatment  
750 by MH, their magnetic heating (MH) abilities were studied at  
751 different field amplitudes and frequencies. The complete set of  
752 heating curves are available in S110, and the corresponding  
753 temperature increases  $\Delta T$ , as shown in S111. Figure 4a shows  
754 the heating curves of the different SPIONs in suspension at a  
755  $\text{Fe}_3\text{O}_4$  concentration of 5 mg/mL, for a field amplitude and  
756 frequency of, respectively, 370 G and 491,45 kHz. As expected,  
757  $\Delta T$  was higher for high magnetic fields and high frequencies.  
758 No resonance phenomena were observed. These results are in  
759 agreement with those of other studies conducted on different  
760 types of SPIONs.<sup>63,64</sup> Comparing our different systems, the

761 characteristics of SPIONs impacted the MH efficacy. The  
762 highest  $\Delta T$  values were obtained for noncoated  $\text{Fe}_3\text{O}_4$   
763 SPIONs, then a decrease was observed for  $\text{Fe}_3\text{O}_4/\text{CS}_{\text{DA}}$   
764 SPIONs. Finally, we investigated the evolution of MH  
765 properties with the amount of coating on SPIONs. Three  
766 batches of  $\text{Fe}_3\text{O}_4/\text{CS}_1$  with 19, 29, and 38 wt % of  $\text{CS}_1$  were  
767 compared, and the results are shown in Figure 4b.  $\Delta T$   
768 significantly increased for SPIONs with 19 and 29 wt % of  $\text{CS}_1$   
769 in comparison with SPIONs with 38 wt % of  $\text{CS}_1$ . These  
770 results showed that it is possible to tune MH properties to  
771 reach an optimal equilibrium between the need for a coating  
772 and the strength of the MH effect. We also observed that  $\Delta T$   
773 decreased with the DA of CS used as coating. This was more  
774 surprising and could not be explained by a difference of coating  
775 wt % as TGA showed that they were very similar (see Table 1).  
776 To the best of our knowledge, we could not find papers in

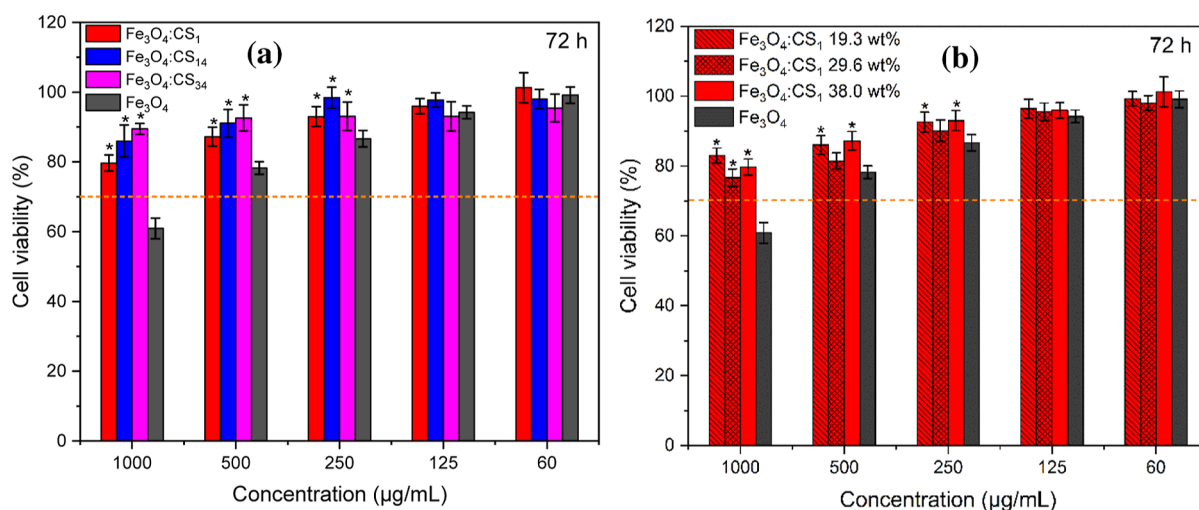
literature treating this question. The explanation may reside in  
777 the physicochemical and structural properties of CS that  
778 change with the DA.  
779

Even though  $\Delta T$  obtained with MH testing could sharply  
780 vary with the type of SPIONs considered, it is worthy to note  
781 that many configurations investigated here could be suitable  
782 for MH. Indeed, MH requires the temperature of the zone of  
783 the treatment to be between 40 and 43 °C,<sup>65,66</sup> which means a  
784 temperature increase between 3 and 6 °C from the body  
785 temperature. Therefore, many of the tests performed in this  
786 work would satisfy this criterion (see S111). To complete the  
787 analyses, we also calculated the SAR for each type of SPIONs.  
788 The SAR can be defined as the power absorbed per mass unit  
789 of the SPIONs. S111 shows the SAR values for all MH  
790 experiments conducted. As for  $\Delta T$ , SAR increases with the  
791 amplitude and the frequency of the magnetic field. For  
792 noncoated  $\text{Fe}_3\text{O}_4$  SPIONs, the SAR value measured at 491  
793 kHz is multiplied by 12.2 within the amplitude range of 100–  
794 370 G. In the same way, the SAR value measured at 370 G is  
795 multiplied by 8.7 within the frequency range of 168–491 kHz.  
796 Regarding the different types of SPIONs, the highest SAR  
797 values are obtained for noncoated  $\text{Fe}_3\text{O}_4$  SPIONs. This is in  
798 agreement with the evolution of  $\Delta T$  and with other  
799 experiments conducted in this work, for which an increase of  
800 coating amount tends to decrease the magnetic properties as  
801 CS coating reduces the magnetic interaction between the  
802 SPIONs.  
803

### 804 3.3.3. Investigation of SPIONs as Contrast Agents for MRI.

$T_1$  and  $T_2$  values at various static magnetic field  $B_0$  (ranging  
805 from 0.5 to 11.7 T) were measured for all systems at two  
806  $\text{Fe}_3\text{O}_4$  concentrations (0.02 and 0.05 g/L, respectively,  
807 corresponding to iron concentrations  $C = 0.259$  and 0.648  
808 mM), by combining measurements on several MR scanners.  
809 The data can be rationalized and compared with those from  
810 previous studies by the calculation of the relaxivities according  
811 to ref 67  
812

$$r_i = \frac{\frac{1}{T_i(C)} - \frac{1}{T_i(C=0)}}{C} \quad i = 1; 2 \quad (9) \quad 813$$



**Figure 6.** Cell viability results of canine fibroblasts after 72 h of interaction time against (a) Fe<sub>3</sub>O<sub>4</sub> and Fe<sub>3</sub>O<sub>4</sub>/CS<sub>DA</sub> SPIONs at different concentrations with same amount of CS<sub>DA</sub> coating 38%, (b) Fe<sub>3</sub>O<sub>4</sub> and Fe<sub>3</sub>O<sub>4</sub>/CS<sub>1</sub> at different concentrations and different amounts of CS<sub>1</sub> coating. \* indicated statically different results of Fe<sub>3</sub>O<sub>4</sub>/CS<sub>DA</sub> from Fe<sub>3</sub>O<sub>4</sub> SPIONs, using Tukey test and *p* value < 0.05. Orange dashed line indicates threshold for cell viability, according to international standard procedure ISO 10993-5.

Figure 5 captures the evolution of  $r_1$  and  $r_2$  in comparison with published data for Endorem reference sample.<sup>68,69</sup> Interestingly, our  $r_1$  values are lower, while we found higher  $r_2$  values, evidencing the strongest efficacy of our systems for use as  $T_2$  contrast agents. This strong  $T_2$  high-field magnetic imaging performance is expressed by the ratio  $r_2/r_1$ , with values ranging from 65 to 185 at  $B_0 = 3$  T (see Table 2). The complete set of data at different  $B_0$  strengths is available in SI12. Regardless of the SPION type considered, the high-field magnetic imaging performance evaluated by the ratio  $r_2/r_1$  (see Table 2) increases with the field strength. This is coherent with already reported results for other SPION types.<sup>70</sup> Interestingly,  $r_2/r_1$  values were found to decrease with the coating amount for all SPION types. This tendency was similar for MH results (see Figure 4b), thus corroborating the idea that CS coating quantity directly lowers magnetic properties while keeping them satisfying. We also identified that  $r_2/r_1$  is impacted by the DA of CS. Surprisingly, smaller DAs yield better imaging performance despite the strongest CS-Fe ions interactions through amine groups than with acetylated CS.

**3.4. Cytotoxicity Assay.** First, the SPIONs were observed with optical microscope after 24 h of contact with canine fibroblasts (see Figure S10). Different behaviors were found, depending on the formulation. As a general tendency, SPIONs were found aggregated in the culture medium. Aggregation was found stronger for the highest SPION concentration, and the dispersions were better for the low concentrations, with a better dispersion in the culture wells on both cellular layers and spaces without cells. Micrographs clearly showed that the presence of CS coating and the DA of CS had an influence on SPION aggregates, with larger aggregates forming at higher DAs. Also, an increase of coating mass fraction for DA = 1% appeared to favor the formation of aggregates. Overall, the spatial distribution of SPIONs in wells was heterogeneous. From these results, CS coating seemed to act as a sticking agent rather than a steric stabilizer (see SAXS results in Section 3.2.2). Moreover, SPIONs distributions seemed colocalized with cells. Indeed, no particles were observed in spaces without cells, while cellular layers were covered by SPIONs interacting with the cell surface or being internalized within fibroblasts.

SPIONs' cytotoxicity was assessed with CCK8 assays on canine fibroblasts after 24, 48, and 72 h of interaction. The data for 24 and 48 h are reported in SI13. As shown in Figure 6, cell viability increased with dilution of SPIONs. For concentrations of 125 µg/mL and below, viability was shown to be higher than 80% for all the formulations investigated and for the three interaction times. In the case of bare magnetite SPIONs, a toxicity was observed particularly at 24 and 72 h with a viability around 60% at a concentration of 1 mg/mL. The addition of a CS coating apparently decreased this toxicity (viability >80%), regardless of the DA and of the amount of CS coating. Therefore, we suggest that the thinnest CS coating layers onto the magnetite SPIONs are enough to preserve the biocompatibility of such systems while maintaining strong magnetic properties and minimizing their aggregation (see Figure S10). However, such results have to be interpreted carefully as SPION aggregation in culture medium causes a heterogeneous spatial distribution of the nanoparticles, which may affect cell viability in different ways: first, aggregates may be more difficult to internalize, possibly introducing a bias in the evaluation of their intrinsic cytocompatibility; second, in the form of aggregates, fibroblasts may not be in contact at all with primary SPIONs. Therefore, we consider such results as encouraging, and further efforts will be made to obtain well dispersed systems even for highest coating amounts, in order to study the impact of SPION aggregation.

Altogether, these results tend to show that the CS coating improved biocompatibility of magnetite-based SPIONs. Fe<sub>3</sub>O<sub>4</sub>/CS<sub>DA</sub> systems are thus fully compatible with the standard international procedure "Tests for in vitro cytotoxicity" ISO 10993-5 for all tested concentrations and interaction times, while bare Fe<sub>3</sub>O<sub>4</sub> systems exhibit some toxicity at high concentrations (above 500 µg/mL).

Finally, most of the results obtained in this work were in qualitative agreement with those obtained previously with CS oligomers, which are more difficult to synthesize. Therefore, both options may be suitable for designing biocompatible SPIONs. Further improvements may focus on the development of an efficient method to prevent or control the aggregation of CS-coated SPIONs.

## 4. CONCLUSIONS

894 We successfully synthesized and fully characterized Fe<sub>3</sub>O<sub>4</sub>  
895 magnetite SPIONs coated with well characterized and  
896 comparable CSs, using a simple coprecipitation method  
897 without the use of any organic solvent within CS solutions.  
898 The SPIONs obtained were spherical and had a size between 5  
899 and 10 nm, and the presence of highly crystalline magnetite  
900 phase was clearly evidenced by WAXS analyses. TGA analyses  
901 showed that CS coating amounts onto magnetite cores could  
902 be easily and precisely adjusted, playing on the initial  
903 concentration of CS in the precipitation medium. The  
904 presence of a coating impacted the magnetic properties ( $M_s$ ,  
905 SAR,  $r_2$ ), which decreased with coating addition, while the  
906 biological properties were improved by the presence of CS  
907 coating but remained independent of its quantity and DA in  
908 the investigated mass fraction range. Also, the impact of CS  
909 DAs on both property types was investigated. Higher DAs  
910 tended to decrease MRI and hyperthermia efficacy, while  
911 SAXS analyses and optical microscopy observations revealed  
912 that they promoted aggregation. Therefore, we suggest that  
913 optimal CS-coated SPIONs would result from the addition of a  
914 thin shell (20%) of low DA (1%) CS in order to strengthen  
915 biocompatibility while maintaining excellent magnetic proper-  
916 ties. We believe that such SPIONs will find applications in the  
917 fields of MRI, MH, and magnetic field-assisted drug delivery,  
918 among others.

## ■ ASSOCIATED CONTENT

### Supporting Information

921 The Supporting Information is available free of charge at  
922 <https://pubs.acs.org/doi/10.1021/acsnm.3c06118>.

923 CSs characterization by SEC and <sup>1</sup>H NMR, FTIR  
924 spectra, TGA graphs, TGA and SAXS calculation details,  
925 WAXS diffraction profiles, WH plots, magnetization  
926 curves, FC-ZFC curves, MH curves, SAR values, MRI  
927 data, and cytotoxicity assay results at 24, 48, and 72 h  
928 (PDF)

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## Notes

The authors declare no competing financial interest.

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