In vitro studies on ultrasmall superparamagnetic iron oxide nanoparticles coated with gummic acid for T2 MRI contrast agent

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Ultrasmall superparamagnetic iron oxide nanoparticles coated with gummic acid have been investigated as possible constituents of aqueous ferrofluids for biomedical applications and especially for MRI contrast agent. The structural characteristics and the size of the nanoparticles have been analyzed as well as the magnetic properties. In order to evaluate any possible capabilities as a contrast agent, the relaxation time, T2, of hydrogen protons in the colloidal solutions of nanoparticles have been measured in order to gain information on the relaxation behavior compared to other MRI contrast agents. The *in vitro* cytotoxicity of the obtained magnetic nanoparticles of iron oxide coated with gummic acid was investigated by two separate methods (MTT and FACS analysis) and by using three different normal and transformed cell lines. Our results showed that the synthesized nanoparticles had no toxic effect on any of the cell lines used. © *2007 American Institute of Physics.* [DOI: 10.1063/1.2821757]

I. INTRODUCTION

Magnetic fluids or ferrofluids are stable colloidal suspensions of ultrafine particles of a ferrite in any ordinary liquid (e.g., water, liquid hydrocarbon, etc.).[1] The dimensions of the ultrafine particles lie in the nanometer scale (hence the term magnetic nanoparticles is also encountered)

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and for magnetite—the most popular ferrite material to prepare magnetic fluids, it is found that for stable colloids, which do not coagulate and aggregate, the diameters of the particles' core must be less than around 15 nm.[1] During the preparation of such suspensions or ferrofluids, biocompatible coatings on the particles are necessary to avoid particle agglomeration due to steric or electrical repulsions and to achieve compatibility with the human body.[2]

The prepared suspensions of coated magnetic nanoparticles have recently been the focus of intensive research not only for the study of the physics of magnetism in the nanometer scale,[3] but also for the use of ferrofluids in many technological and biomedical applications such as technical heating processes, media contrast agents in magnetic resonance imaging (MRI), and as therapeutic agents for RF-magnetic hyperthermia.[4–13]

Specifically, MRI contrast agents are chemical substances introduced to the anatomical or functional region being imaged, in order to increase the differences between different tissues or between normal and abnormal tissue, by altering the relaxation times. MRI contrast agents are classified according to the different changes in relaxation times after their injection.

To date, gadolinium or manganese salts as well as superparamagnetic iron oxide-based particles are by far the most commonly used materials as MRI contrast agents.[14] Superparamagnetic contrast agents have the advantage of producing an enhanced proton relaxation in MRI better than those produced by paramagnetic ions. Consequently, lower doses are needed to reduce to a great extent the secondary effects in the human body.

Colloidal T2-agents are often called USPIO for ultrasmall superparamagnetic iron oxide. They consist of iron oxide cores, whose composition and physicochemical properties vary continuously from magnetite to maghemite. They are generally synthesized in a one-step process by alkaline coprecipitation of iron (II) and iron (III) precursors in aqueous solutions of hydrophilic macromolecules that serve (i) to limit the magnetic core growth during the synthesis, (ii) to stabilize via sterical repulsions the nanoparticle dispersion in water (and later in physiological medium), and (iii) to reduce *in vivo* the opsonization process. These colloidal contrast agents would be more realistically described as several magnetic cores, more or less aggregated, embedded in the hydrophilic macromolecules, which are sometimes cross-linked in a second step for enhancing the mechanical entrapment. Such a step-by-step synthesis allows control of the magnetic core size and size distribution and the overall hydrodynamic diameter, thanks to accurate and reproducible experimental conditions, e.g., colloidal stability control, molecular weight, etc.

USPIOs, also called monocrystalline iron oxide nanoparticles (MIONs) do satisfy a set of requirements needed of a superparamagnetic contrast agent, given that they are intravenously administered into the body of a patient—a small size to avoid lung ischemia exhibit an overall hydrodynamic diameter lower than 40 nm.[8] Thanks to their small size and their gummic acid corona, they act as stealth particles not easily recognizable by the mononuclear phagocyte system. Their plasma half-life could increase therefore they remain in the blood long enough to act as blood-pool agents for magnetic resonance angiography (MRA).[10] In comparison with other MRI contrast agents, USPIOs exhibit lower relaxivities which lead to a higher contrast on T2-weighted images.

In this paper, magnetic nanoparticles of iron oxide coated with gummic acid obtained by coprecipitation method with a mean core size of 9 nm and a hydrodynamic size of 20–25 nm have been successfully dispersed, in aqueous medium, giving rise to stable and biocompatible colloidal suspensions that could be used as T2 contrast agents in MRI. The nature of the nanoparticles, as well as the effect of the particle size on the magnetic and relaxometric behavior of the ferrofluid has been studied. Techniques of particle sizing such as transmission electron microscopy, dynamic light scattering, x-ray diffraction, x-ray photoelectron and resonance micro-Raman spectroscopy and magnetic measurements have been used to characterize powder and ferrofluids form and the absolute size values were analyzed and compared.

In order to evaluate any possible capabilities as a contrast agent, the relaxation time, T2, of hydrogen protons in the colloidal solutions of nanoparticles have been measured and the corresponding relaxivities were correlated with the structural characteristics of the powder and the magnetic properties of the suspensions. The *in vitro* cytotoxicity of the obtained magnetic nano-

particles of iron oxide coated with gummic acid was investigated by two separate methods (MTT and FACS analysis) and by using three different normal and transformed cell lines. Our results showed that the synthesized nanoparticles had no toxic effect on any of the cell lines used.

II. MATERIALS AND METHODS

A. Particle preparation

A dispersion of USPIO nanoparticles coated with gummic acid was synthesized by the reaction of ferric chloride and ferrous chloride in the presence of ammonia, using coprecipitation method.[15,16] Gum arabic is generally accepted to be a mixture of salts of calcium, magnesium, and potassium formed by the union of these elements with *gummic acid*. The gum is chiefly composed of calcium arabate. *Gummic acid* forms salts containing an excess of acid. It is considered identical with the *metapectic acid* of Frémy, and is obtained from a solution of the gum, acidulated with chlorhydric acid, by precipitation with alcohol. Before drying *gummic acid* is soluble in water, but after drying it becomes *metagummic acid*, and refuses to dissolve in either hot or cold water unless alkalinized. The nominal molecular weight of the gummic acid used was 250,000. Aqueous solutions of 0.1 M Fe(III) (30 ml) and 0.1 M Fe(II) (15 ml) were mixed, and 3 ml of 5 M ammonia solution was added dropwise over 1 min with stirring on a magnetic stir plate. The stirring continued for 20 min under a nitrogen-gas atmosphere. The particles obtained were washed 3 times using ultracentrifugation (30000 rpm for 20 min at 10 °C) with nitrogen purged water. The iron oxide nanoparticle yield, determined by weighing of the lyophilized sample of the preparation, was 400 mg. Formulations of iron oxide nanoparticles were developed, first by optimizing the amount of gummic acid coating required to coat iron oxide nanoparticles completely, and then by optimizing the amount of coating required to form an aqueous dispersion of polysaccharide coated nanoparticles. The formulations were heated to 60 °C while being stirred for 30 min and then cooled to room temperature. The black precipitate thus obtained was washed twice with 15 ml of water. The precipitate was lyophilized for 2 days at −60 °C. Finally, the colloidal suspension was filtered through a 0.22 μm pore size filter yielding a ferrofluid that was stable for more than one year.

B. Characterization techniques

Dry powders and aqueous suspensions of the coated magnetic nanoparticles were prepared and subsequently characterized by a number of techniques including transmission electron microscopy, dynamic light scattering, x-ray diffraction, x-ray photoelectron and resonance micro-Raman spectroscopy, vibrating sample magnetometry, and nuclear magnetic resonance imaging.

Crystallographic analysis of the samples was carried out from the x-ray diffraction patterns, recorded between 20° to 100° (2θ) at 0.5° min^{-1} in a Siemens D500 diffractometer with Cu $K\alpha$ radiation. X-ray tube voltage and current were set at 40 kV and 35 mA, respectively. The crystallite size was obtained from the full width half maximum of the (311) reflection typical for iron oxide using the Debye-Scherrer Eq.[17]

For the Transmission Electron Microscopy analysis, a 200 keV Phillips CM20 microscope was used to observe the morphology, mean size, and distribution of the nanoparticles in the powder, and the mean aggregate size in the colloidal suspensions. This determination was made on a large number, by measuring at least 200 particles, from different micrographs. The specimen for TEM imaging was prepared from the USPIO suspension in deionized water by using sonication for 3 min. The colloidal suspensions were highly diluted and nebulized onto the microscope grid in order to avoid particle agglomeration during the preparation of the samples. After sonication, 1 ml of the USPIO suspension was centrifuged for 5 min at 14000 min^{-1}. A drop of well dispersed supernatant was placed on a carbon-coated 200 mesh copper grid, followed by drying the sample at ambient conditions before it was attached to the sample holder on the microscope. The mean size measured by electron microscopy corresponds to the magnetic core of the aggregates.

The magnetization of the powder samples as a function of the applied magnetic field was obtained by means of a vibrating sample magnetometer, Model DANFYSIK System 8000. The powder was packed tightly in the sample holder avoiding any fiber space inside.

The saturation magnetization (Ms) was evaluated by extrapolating M versus $1/H$ at $1/H=0$ in the high field region. The initial susceptibility of the powders was measured in the field range of ±20 kOe.

Finally, the effect of USPIO nanoparticles coated with gummic acid on proton relaxation time was studied by measuring the relaxivities. MR imaging was performed with a Siemens MRI spectrometer operating at 1.5 T. The dependence of the spin-spin (T2) relaxation times on kind of nanoparticle and nanoparticle concentration was determined by applying MRI on phantoms containing various ferrofluids. The initial concentration for both Resovist and our gummic coated ultrasmall iron oxide nanoparticles was 20–30 mg of iron oxide per ml. T2-weighted images were measured at 25 °C at various concentrations of the ferrofluids examined, diluted by 1%, 0.1%, 0.05%, 0.01%, 0.005%, and 0.001% volume concentration.

T2-weighted images were measured with the use of a single-slice multiecho pulse train sequence with parameters TR: 2500 ms, TE: 12 ms in 31 symmetrically repeatable echoes. The choice of the above parameters was based on phantom studies for T2 values ranging from 10 up to 80 ms. For this sequence, the field of view was 42 cm and the image reconstruction matrix was 224×160. The longer anatomical axis (horizontal) was chosen as the frequency encoding axis and the shorter as the phase encoding axis.

C. Cell lines and culture conditions

The human skin fibroblast strain AG01523c was obtained from the Coriell Institute for Medical Research (Camden, NJ, USA). Another fibroblast strain (DSF22) developed in our laboratory from a healthy 22-year-old donor as previously described[18] was also used to confirm the results. Cells were routinely cultured in Dulbecco's Minimal Essential Medium (DMEM) supplemented with antibiotics and 10% Fetal Bovine Serum (FBS) in an environment of 5% CO_2, 85% humidity and 37 °C, and they were subcultured using trypsin-citrate (0.25%–0.3%, respectively) solution at a 1:2 split ratio. Cells were tested and found to be mycoplasma-free. All cell culture media were from Gibco-BRL (*Paisley, U.K.*).

HEK293 cells, a human embryonic kidney adenovirus immortalized cell line (ATCC, Cat. No. CRL-1573), were continuously grown in DMEM culture medium containing 25 mM glucose, 10% FBS, 4 mM glutamine, and antibiotics in 5% CO_2 at 37 °C. Cells are released from their tissue culture flasks for passaging by treatment with 0.05% trypsin/0.03% EDTA.

D. Cytotoxicity assays

1. MTT assay

Cytotoxicity was estimated by a modification of the MTT assay.[19,20] Cells were plated in 96-well flat-bottomed microplates at a density of approximately of 7,000 cells/well. 18 h after plating, the culture medium was replaced with serial dilutions of the suspensions under study in fresh medium. The maximum suspension concentration used was less than 5% v/v to avoid further culture medium dilution and thus adverse effects on cell physiology. After a 72-h-incubation, the cells were washed with phosphate buffered saline (PBS) to remove any residual traces of the suspension and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] *(Sigma, St. Louis, MO, USA)* dissolved at a final concentration of 1 mg/ml in serum-free, phenol-red-free DMEM (*Biochrom KG*) was added for a further 4-h-incubation. Then, the MTT-formazan was solubilized in isopropanol and the optical density was measured at a wavelength of 550 nm and a reference wavelength of 690 nm.

2. Quantitative PI-FACS analysis of HEK293 cell survival

The potentially induced cytotroxicity was also examined by flow cytometry using propidium iodide staining of fixed cells. Cells were plated in 6-well microplates at a density of approximately

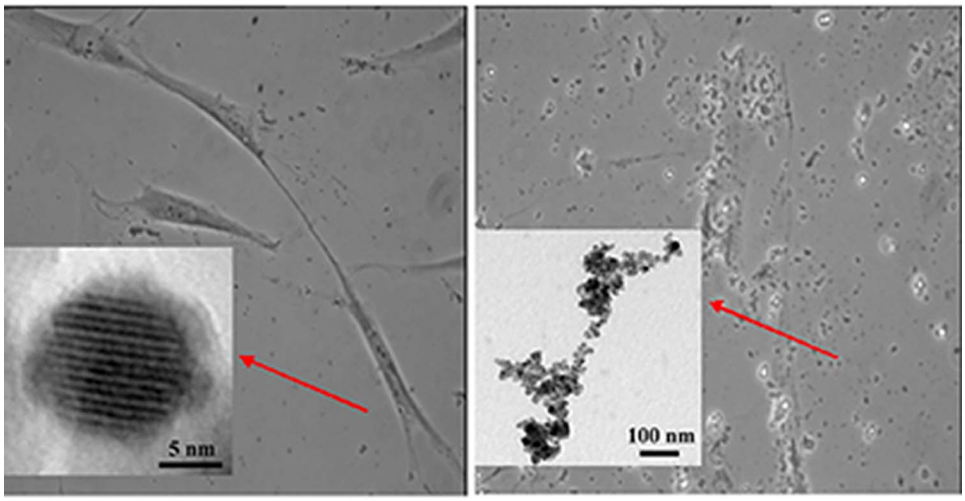

FIG. 1. Standard microscopy (main frames) and transmission electron microscopy [small inside frames (inset)] images (i) coated USPIO nanoparticles (ii) uncoated USPIO nanoparticles on normal human skin fibroblasts.

3×10^4 cells/well. 18 h after the plating, the culture medium was replaced with serial dilutions of the suspensions under study in fresh medium (0.02% v/v, 0.05% v/v, and 0.5% v/v) and the cells were subsequently cultured for another 48 h.

Following incubation, HEK293 cells were trypsinized and 2×10^6 cells were collected by centrifugation at $300 \times g$ for 5 min. After being washed in phosphate-buffered saline (PBS), cells were fixed in 70% ethanol for at least 30 min at 4 °C. Cells were subsequently centrifuged as before, and resuspended in 1.5 ml PBS at room temperature for 10 min, followed by another centrifugation. Finally, cells were resuspended in DNA staining solution (20 μg/ml propidium iodide, 200 μg/ml RNAse A in PBS) and incubated for 30 min in the dark, at room temperature. Cells were analyzed by flow cytometer (FACScan, Beckton-Dickinson), using CellQuest software (Becton Dickinson, Franklin Lakes, NJ).

E. Statistical analysis

Results were presented as means ± SD based on data derived from three independent experiments. Statistically significant differences between values were evaluated by one-way ANOVA or Student's t test as appropriate. A p value below 0.05 was considered statistically significant.

III. RESULTS AND DISCUSSION

A. TEM, XRD, and XPS techniques

Agglomeration of the USPIO particles is initiated from collisions between the particles during nucleus formation creating clusters in the liquid carrier. Growth of the agglomerate takes place by attraction forces and capillary forces between magnetite clusters and single particles. The cluster size increases until the forces balance between the clusters and equilibrium is reached. USPIO particles have large surface area and small curvature. This large surface energy accelerates the agglomeration process in order to decrease the free energy of the system. To avoid precipitation of USPIO, stabilization is introduced against aggregation using polysaccharide molecules such as gummic acid.

A combination of standard microscope and transmission electron microscopy images of the gummic acid coated USPIO colloidal suspension and the uncoated USPIO samples on normal human skin fibroblasts is shown in Fig. 1. From the analysis of the TEM images of the coated sample [small frame (inset) (i) scale bar 5 nm], the core size of the nanoparticles is ranging from 7 to 15 nm with a mean core size around 9 nm. In Fig. 2, XRD powder patterns of iron oxide

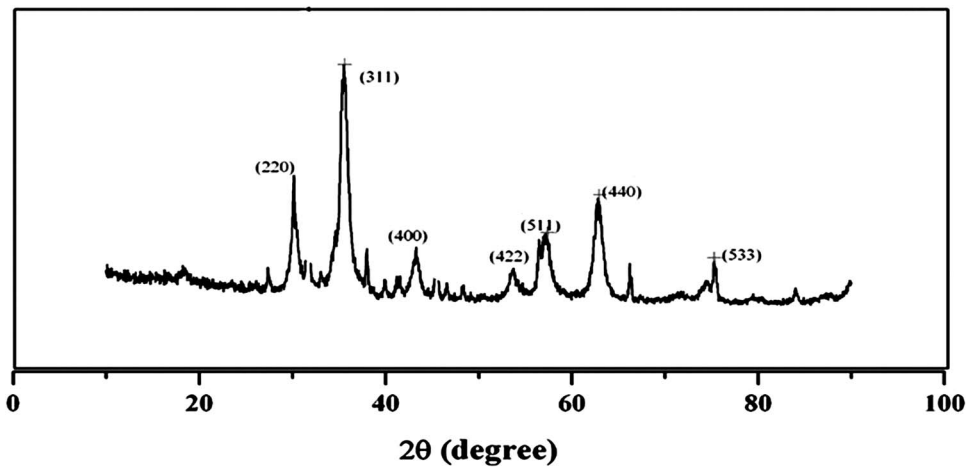

FIG. 2. XRD patterns of the coated USPIO nanoparticles of maghemite (γ-Fe_2O_3). Size was calculated by Scherrer formula to be 9 nm.

phases, allows the identification of a major cubic spinel phase. This indicates the presence of maghemite γ-Fe_2O_3 with a average size of 7–8 nm (space group $P4_132$). Figure 3 shows the XPS spectra of the γ-Fe_2O_3 in the Fe $2p$ core-level region. The background has been subtracted using the Shirley method, the electron take-off angle has been adjusted to 45° with respect to the surface normal. Two weak but well defined peaks at approximately 710.8 eV and 724.2 eV, are observed, in agreement with the typical values of the Fe $2p_{3/2}$ and $2p_{1/2}$ core-level XPS peaks for ferric oxides.[21,22] In addition, the main Fe $2p_{3/2}$ and $2p_{1/2}$ peaks are accompanied by weak satellite structures on their high binding energy side, about 8 eV above the respective Fe $2p$ line. Such a spectrum is typical of Fe_2O_3 oxides (α- and γ-Fe_2O_3 polymorphs).

The hydrodynamic size from dynamic light scattering data, Fig. 4, was 25 nm with a very narrow distribution. Due to the small size no aggregates could be observed in the micron-size standard microscopy image on skin fibroblast. On the contrary in Fig. 1(ii) on both nano– and micron-sized images we observe large agglomerates due to interparticle interaction of the uncoated USPIO because their size is in the range of 200 nm–900 nm they can be observed even with a standard microscope.

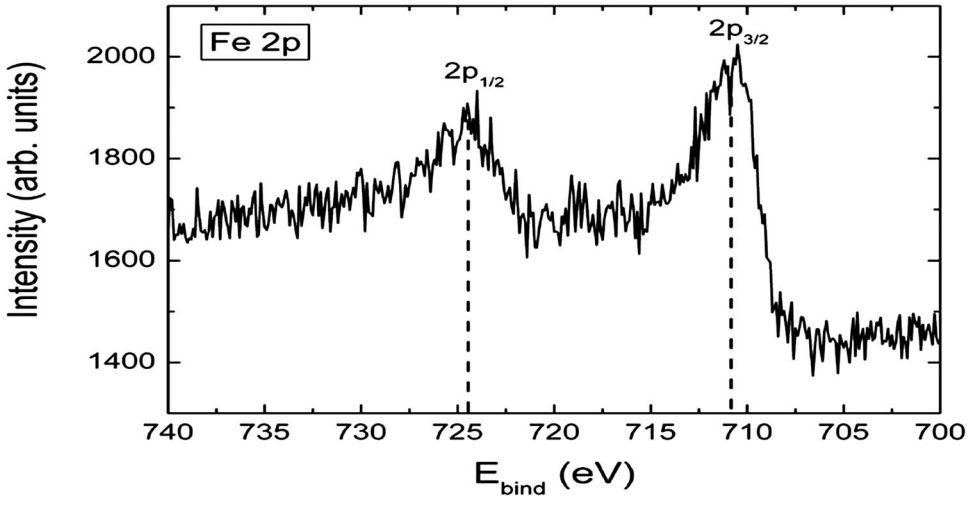

FIG. 3. XPS Fe $2p$ core-level spectra of the coated USPIO nanoparticles of maghemite (γ-Fe_2O_3).

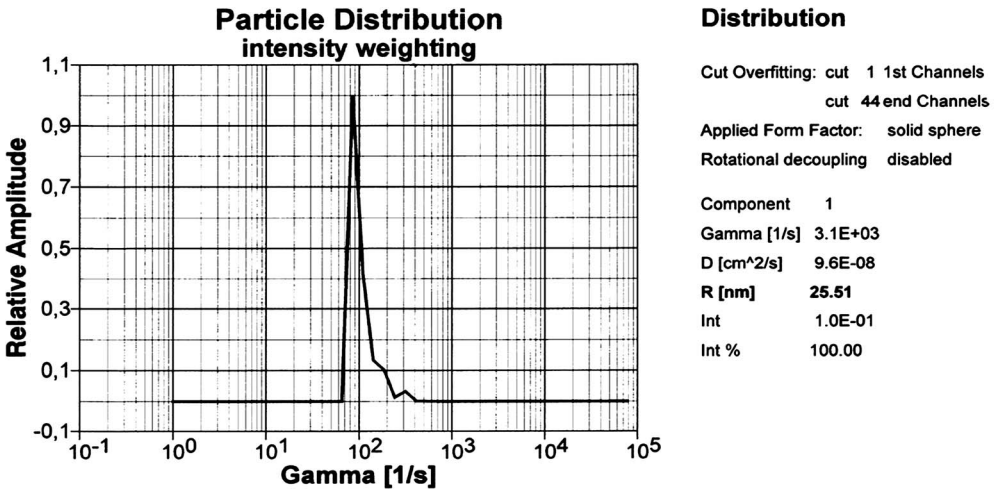

FIG. 4. Coated USPIO nanoparticles of maghemite (γ-Fe_2O_3) lognormal particle distribution by DLS.

B. Magnetization measurements

The macroscopic magnetic moment of the coated magnetic nanoparticles was measured at room temperature as a function of the magnetic field using a vibrating sample magnetometer with the applied field varying between ±20 kOe. The magnetization data for the gummic acid coated nanoparticles are shown in Fig. 5.

Assuming that the sample consists of noninteracting single-domain particles, in the superparamagnetic state, the magnetization is described by the Langevin function:[23]

$$L\left(\frac{\mu H}{k_B T}\right) = \coth\left(\frac{\mu H}{k_B T}\right) - \frac{\mu H}{k_B T}, \tag{1}$$

where μ is the magnetic moment of a particle, H is the applied field and k_B is the Boltzmann constant. Since inevitably there will be a distribution of the sizes of the magnetic particles, it is a common practice[24] to introduce a distribution function of the magnetic moments $f(\mu)$ and the magnetization $M(H,T)$ is then described by:

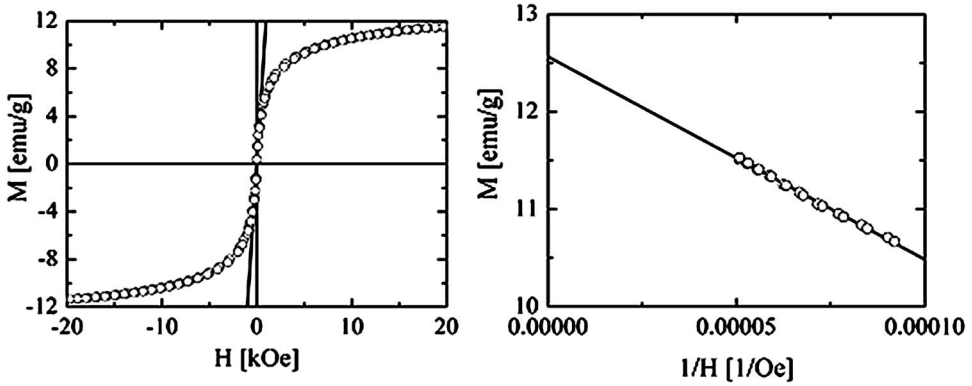

FIG. 5. (i) Magnetization for dry powder sample of coated nanoparticles. The line is a linear fit to the low-field data near the origin, (ii) Magnetization data near saturation, as a function of $1/H$.

$$M(H,T) = \int_0^\infty \mu L\left(\frac{\mu H}{k_B T}\right) f(\mu) d\mu. \quad (2)$$

The magnetization data plotted M as a function of $1/H$ are shown in Fig. 5. The saturation magnetization $M_s = N\langle\mu\rangle$ thus obtained was found to be $M_s = 12.6$ emu/gr much lower than the corresponding one for the bulk magnetite (50 emu/gr), a reduction frequently encountered in the literature.[3] The USPIO are ferri- or ferromagnetic single domain particles that have significantly small thermal energy with the same order of magnitude as the anisotropy energy barrier. Reduced magnetization in small ferrite particles is well documented in the literature, but the nature of the spin structure in such particles has not been well understood. Proposed mechanisms for moment reduction include shell-core structures, spin canting, spin-glass behavior, etc.[3]

Using the initial slope of the magnetization data in Fig. 5, the saturation magnetization and the moments of the lognormal distribution function, the following statistical parameters for the magnetization are obtained: $\mu_0 = 1239\ \mu_B$, $\langle\mu\rangle = 2707\ \mu_B$, $\sigma = 1.25$, and $N = 5.02 \times 10^{17}$/g.

The mean magnetic moment $\langle\mu\rangle = 2707\ \mu_B$ corresponds to a mean particle diameter $\langle d\rangle_{magn} = 5$ nm, if $I_s = 500$ emu/cm^3 is assumed for the saturation magnetization of bulk magnetite.[3] This magnetically derived mean particle diameter $\langle d\rangle_{magn} = 5$ nm, is somewhat smaller than the one derived from TEM images (Fig. 1). This difference is frequently encountered in the literature and may be due to the presence of a magnetic dead layer around the particle, resulting in a magnetic particle volume derived from magnetization data smaller than the crystalline core volume.[4] Also, some of the iron oxide species exist as a nonmagnetic amorphous phase, which does not contribute to the magnetic behavior, or as an iron phase that will have a smaller effect on magnetization than a magnetite only phase.

C. Magnetic resonance imaging

MRI contrast agents are classified by the different changes in relaxation times after their injection. *Positive contrast agents* cause a reduction in the T1 spin lattice relaxation time (increased signal intensity on T1 weighted images). They (bright on MRI) are typically small molecular weight compounds containing as their active element Gadolinium, Manganese, or Iron. *Negative contrast agents* (dark on MRI) are ultrasmall superparamagnetic iron oxides (USPIO) usually consist of a crystalline iron oxide core containing thousands of iron atoms and a shell of polymer. These agents produce predominantly spin spin relaxation effects (local field inhomogeneities), which results in shorter T2 relaxation times. The MRI results imply that for MR imaging with gummic acid-coated USPIO, both the image intensity and contrast might be severely amended by the existence of USPIO. T2-weighted images represent signal intensity changes corresponding to decreasing T2 values indicating a concentration gradient of gummic acid-coated USPIO. In Fig. 6(i) water, fat, a T1 contrast agent (coated gadolinium oxide) and a T2 contrast agent (Resovist) are compared with gummic acid USPIO. As one can observe gadolinium oxide appears bright as water in $TE = 26.8$ ms because this is a positive T1 contrast agent with relaxation time in the region of 500 ms. It was used in the figure only to show the difference of T1 and T2 agents. In the case of Resovist and gummic acid USPIO samples, even if they were diluted from the same initial concentration of 20–30 mg iron oxide per ml, one could see that 1% of volume concentration of Resovist has the same negative (dark) contrast as 0.01% of gummic acid coated USPIO. Focus on that observation in Fig. 6(ii) the same area of water, 1% Resovist, 0.05% and 0.01% of gummic acid coated USPIO were measured (~3.55 qcm) with mean brightness of 735, 192.1, 39.5, and 201, respectively. The same effective brightness of Resovist can be achieved using a hundred times more dilute sample of gummic acid-coated nanoparticles. This could be explained by the different rate of aggregation time between different coated nanoparticles such as Resovist and ours coated by carboxydextran and gummic acid, respectively, even though the amount of iron oxide and the core size are similar. One could see that in our case the hydrodymanic size of the coated nanoparticles is very small and with very narrow distribution, Fig. 4, sign of low aggregation.

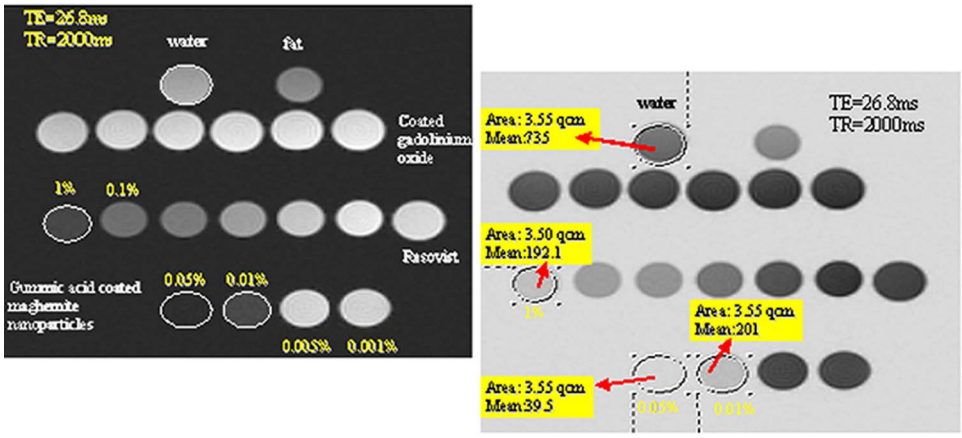

FIG. 6. (i) T2-weighted images at various concentrations of the ferrofluid examined, diluted by 1%, 0.1%, 0.05%, 0.01%, 0.005%, and 0.001% volume concentration in comparison with other T1 (gadolinium oxide) and T2 (Resovist) contrast agents (ii) measurements of surface area/mean brightness of water, 1% v/v of resovist and 0.01% of gummic coated USPIO.

D. Cytotoxicity

1. MTT assay

The USPIO colloidal suspensions we have prepared were tested for possible cytotoxic activity on normal human skin fibroblasts. As shown in Fig. 7, there was no reduction of the cells' viability at any concentration tested, the highest being approximately 4.5% v/v of the suspension in culture medium, approximately corresponding to the highest concentrations to be administered *in vivo*. The slight increase in viability at the highest concentration tested is due to absorbance of residual colloidal, which adhered very strongly to the cells and could not be removed even through

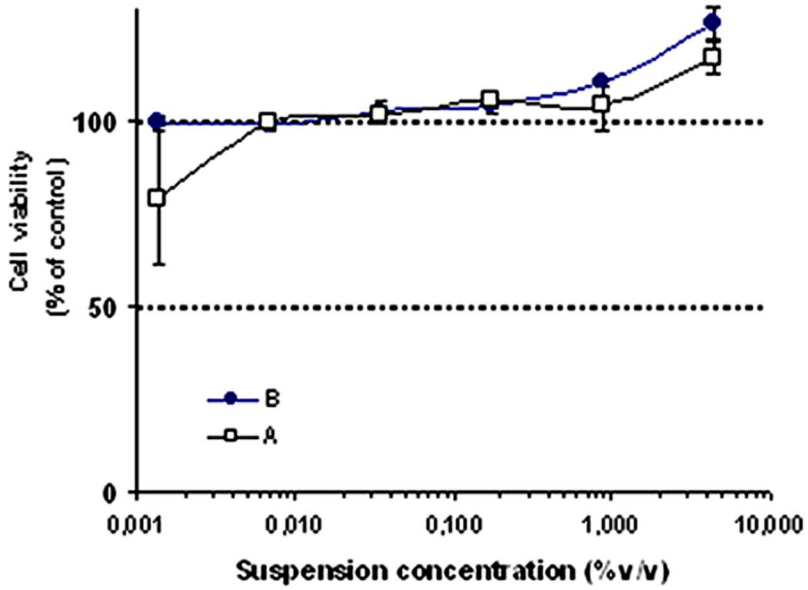

FIG. 7. Viability of human skin fibroblasts exposed to USPIO coated with gummic acid suspension before (a) and after (b) filtration through a 0.2 μm-membrane. The MTT-method was applied as described in Sec. II. A representative experiment out of three similar ones is depicted; each point is the average of six replicates (error-bars indicate standard deviation).

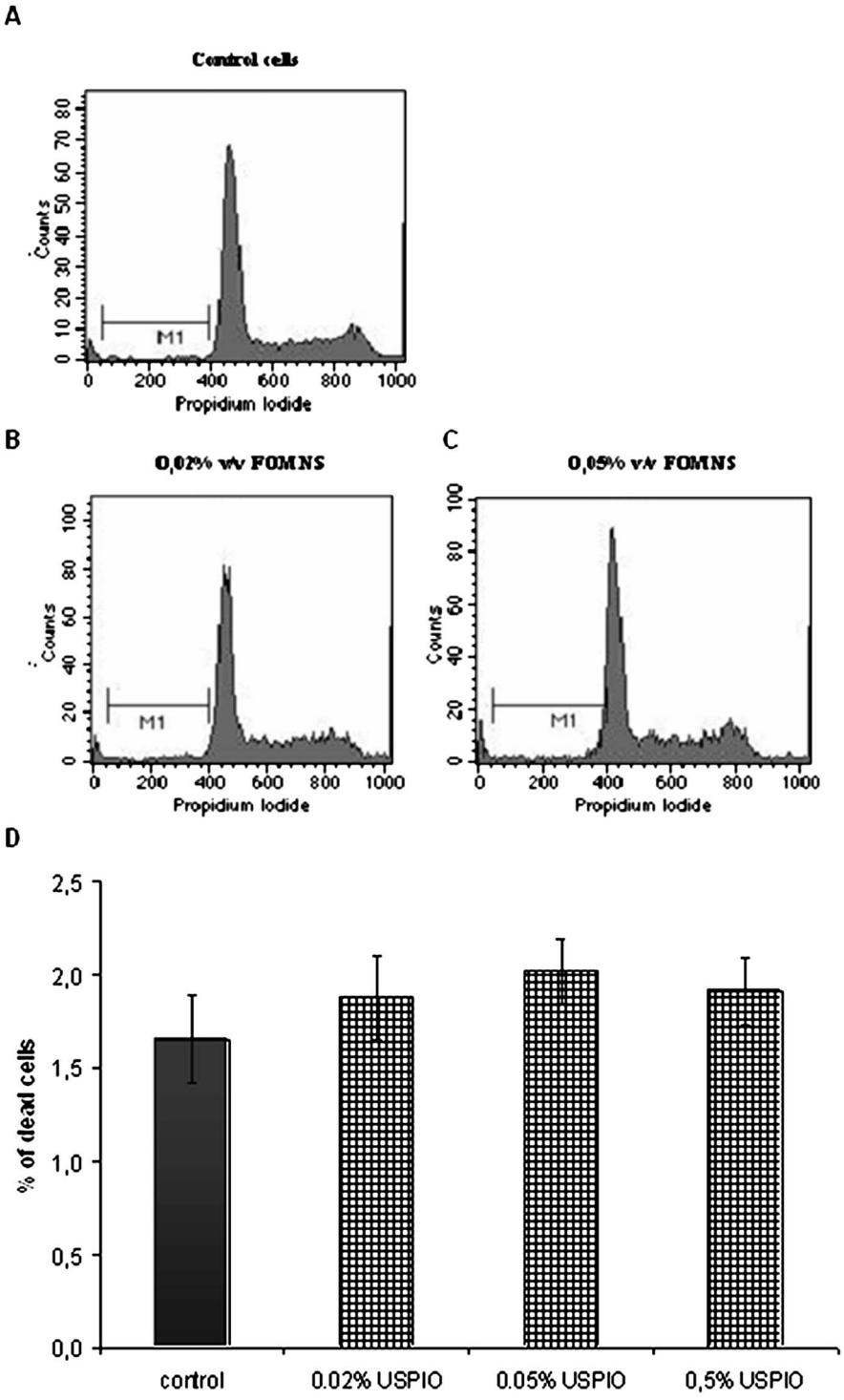

FIG. 8. Effect of USPIO coated with gummic acid suspension on HEK293 cell survival. HEK293 cells continuously growing in DMEM were exposed for 48 h to 0.02% or 0.05% v/v of nanoparticle in the presence serum. The population of dead cells was analyzed by flow cytometry analysis as described in Sec. II E (a) Flow cytometric analysis of control HEK293 cells. Six thousand events per sample were counted. The M1 gate demarcates the population of dead cells. (b) Cells treated with 0.02% v/v and (c) 0.05% of FOMNS for 48 h. (d) Quantification of the percentage of cells undergoing cell death (PI-positive cells gated in M1 gate). Results are shown as means ± SD from three independent experiments. $^{*}P<0.05$ relative to control cells.

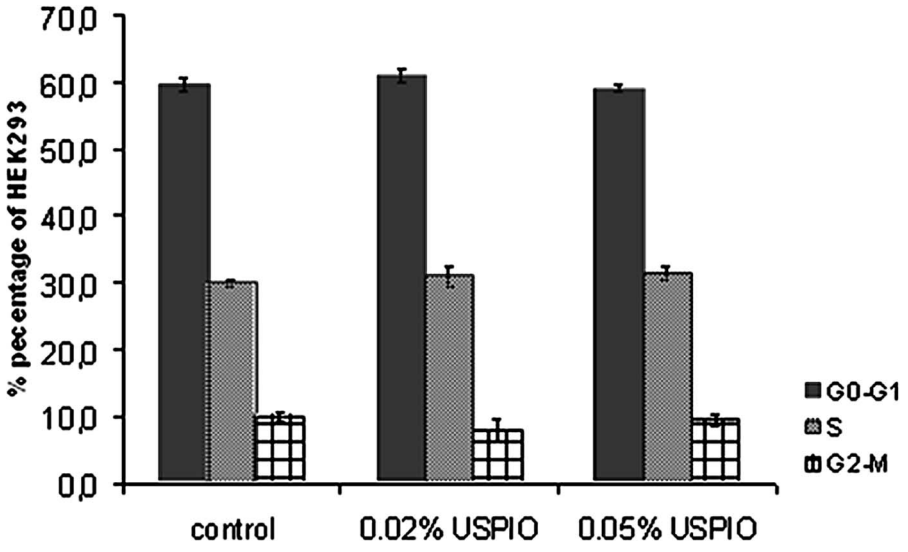

FIG. 9. Effect of USPIO coated with gummic acid suspension on HEK293 cell proliferation. Quantification of the percentage of cells in different cell cycle phases. Results are shown as means ± SD from three independent experiments. $P < 0.05$ relative to control cells.

extensive PBS-washes. Filtration of the suspension through a 0.2 μm filter reduces this effect marginally (Fig. 7, series B), most probably by eliminating aggregates which may have been produced during the preparation of the colloidal suspension.

2. Effects of the USPIO colloidal suspensions on HEK293 cell survival and cell proliferation

We investigated whether the synthesized USPIO colloidal suspensions could be associated with any impairment on HEK293 cell survival. Following incubation of cells in the presence or absence of 0.5% v/v (data not shown) or 0.05% v/v or 0.02% v/v of the suspension for 48 h, cell death was evaluated by flow cytometry after propidium iodide staining of fixed cells. As shown in Figs. 8(a)–8(d), cell death, estimated by the percentage of sub-G1 events, after 48 h incubation in the presence of 0.05% v/v or 0.02% v/v of the suspension was not increased significantly, when compared with control cells. Serum deprivation treatment did not affect HEK293 cell survival in the control/basal state (cells grown in the absence of the suspension) (data not shown).

Additionally, the USPIO colloidal suspensions prepared were also tested for possible effects on HEK293 cell proliferation, by meaning of cell cycle didstribution. As shown in Fig. 9 there were no effects on cell cycle regulation under any concentration tested.

IV. CONCLUSIONS

The magnetic properties of USPIO nanoparticles coated with gummic acid were investigated in order to access their effectiveness of the associated ferrofluids in biomedical applications. The nature of the nanoparticles, as well as the effect of the particle size on the magnetic and relaxometric behavior of the ferrofluid has been studied. The TEM, XRD, XPS, DLS, and magnetic measurements showed that the particles exhibited a superparamagnetic behavior as expected for nanometer particles with an average magnetic core diameter of 9 nm and a hydrodynamic core of 20–25 nm.

In order to evaluate any possible capabilities as a T2 contrast agent, the relaxation time, T2, of hydrogen protons in the colloidal solutions of nanoparticles have been measured and the corresponding relaxivities were correlated with the structural characteristics of the powder and the magnetic properties of the suspensions. Comparing a T2 contrast agent Resovist, already in the market, and gummic acid USPIO samples both with the same initial concentration of iron oxide

showed that 1% of volume concentration of Resovist has the same negative (dark) contrast as 0.01% of gummic acid coated USPIO. The same effective brightness of Resovist can be achieved with a hundred times more dilute sample of gummic acid coated nanoparticles. This could be explained by the different rate of aggregation time between different coated nanoparticles such as Resovist and ours coated by carboxydextran and gummic acid respectively. The *in vitro* cytotoxicity of the obtained magnetic nanoparticles of iron oxide coated with gummic acid was investigated by two separate methods using three different cell lines, the human skin fibroblast strain AG01523c, a fibroblast strain (DSF22) developed from a healthy 22-year-old donor and a human embryonic kidney cell line (HEK293). Our results showed that the synthesized nanoparticles had no toxic effect on any of the cell lines used.

It appears that the USPIO coated with gummic acid colloidal suspensions can be administered at different concentrations to normal human cells without affecting their viability. If this initial indication is supplemented by optimal results in systemic toxicity and inflammation assays, then these materials are very promising candidates for *in vivo* use. This agent demonstrates great promise for improving the quality of MR angiography, and in quantificating capillary permeability and myocardial perfusion. The design objectives for the next generation of MR contrast agents will likely focus on prolonging intravascular retention, improving tissue targeting, and accessing new contrast mechanisms.

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