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Journal of Magnetism and Magnetic Materials 293 (2005) 127–134
Journal of magnetism and magnetic materials
www.elsevier.com/locate/jmmm

A method for synthesis and functionalization of ultrasmall superparamagnetic covalent carriers based on maghemite and dextran

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Available online 3 March 2005

Abstract

A new generation of susceptibility contrast agents for MRI and based on maghemite cores covalently bonded to dextran stabilizing macromolecules was investigated. The multistep preparation of these versatile ultrasmall superparamagnetic iron oxides (VUSPIO) consisted of colloidal maghemite synthesis, surface modification by aminopropylsilane groups, and coupling of partially oxidized dextran via Schiff's bases and secondary amine bonds. The dextran corona might be easily derivatized, e.g. by PEGylation.

Keywords: Ferrofluid; Infrared spectroscopy; MRI; Contrast agent; PEGylation; Silanation; Transmission electron microscopy; Zeta potential; Photon correlation spectroscopy; USPIO; VUSPIO; 3-Aminopropyltrimethoxysilane; α-Amino poly(ethylene glycol); α,ω-Diamino telechelic poly(ethylene glycol); Dextran; Maghemite

1. Introduction

Magnetic iron oxide nanoparticles have attracted attention in particular because of their usefulness as contrast agents for magnetic resonance imaging (MRI) [1,2]. The superparamagnetic behaviour of these subdomain magnetic cores (3–10 nm) is similar to that of paramagnetic substances, in that they lose their magnetization when the magnetic field is removed, but differs by the value of the magnetic moment which is markedly higher. Therefore their relaxivities are much higher than those of the widely-used Gd-chelates. In most situations, they are used for their significant capacity to produce predominantly T_2 relaxation effects, which result in signal reduction on T_2-weighted images. They are also called susceptibility agents or (U)SPIO for (Ultrasmall) SuperParamagnetic Iron Oxide.

For intravenous administration, they are generally synthesized in a one-step process by alkaline coprecipitation of iron (II) and iron (III)

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0304-8853/$ - see front matter © 2005 Published by Elsevier B.V.
doi:10.1016/j.jmmm.2005.01.053

precursors in aqueous solutions of hydrophilic macromolecules, such as dextran [3], followed by chromatographic separation for narrowing the size polydispersity. The dextran macromolecules serve to limit the magnetic core growth during the synthesis and to stabilize via sterical repulsions the nanoparticle dispersion in water (and later in physiological medium). These colloidal contrast agents would be more realistically described as several magnetic cores, more or less aggregated, embedded in the dextran corona, which are sometimes cross-linked in a second step for enhancing the mechanical entrapment of the inorganic cores [4].

Two different classes of iron oxides are currently clinically approved or in phase-III trials. Because of their large overall hydrodynamic volume (over 40 nm in diameter), SPIO agents are efficiently accumulated in the organs of the Mononuclear Phagocyte System (MPS): ca. 80–90% of the injected dose in liver and 5–8% in the spleen with plasma half-life less than 10 min. Therefore, SPIO decrease liver and spleen signals and allow diagnosing malignant tumours or metastases in these organs. Thanks to their smaller size and the hydrophilicity of their dextran corona, USPIO act as stealth particles. Their plasma half-life is more than 2 h [5] and therefore they remain in the blood long enough to act as blood-pool agents (MR angiography) [6] or to be accumulated in the lymph nodes (MR lymphography) [7]. This size-dependent distribution in tissues (passive targeting) is a current limitation for the diagnosis of pathologies in any other organ. For instance, imaging specific tissues would need contrast agents as stealth as USPIO with an extra requirement, as their surface labelling with ligands that specifically bind to surface epitopes or receptors on the target sites (active targeting).

Only very few attempts for conjugating biomolecules to the dextran coating were reported using electrostatic or chelating interactions [8] or reduction of Schiff's bases formed from dextran activated by oxidation [9,10]. Indeed, the interactions between magnetic cores and dextran macromolecules, e.g. Van der Waals and hydrogen interactions [11], are too weak and generally prevent any efficient derivatization of dextran corona without macromolecule depletion [12]. Recently, for a similar purpose, attempts were made for preparing USPIO from dextran whose terminal sugar had been previously reduced [13].

The aim of this work is to investigate a new generation of USPIO, based on maghemite cores covalently bonded to their dextran corona through the use of silane coupling agents. Because of their potential ability to be tailor-derivatized, they are called VUSPIO for Versatile USPIO [14]. Their multistep synthesis and their characterization by IR spectroscopy, transmission electron microscopy (TEM), zeta potential measurement and photon correlation spectroscopy (PCS) are described and discussed in this article.

2. Experimental section

2.1. Materials and purification methods

Iron (III) chloride hexahydrate (98% +), iron (II) chloride tetrahydrate (99% +), iron (III) nitrate nonahydrate (99% +), sodium borohydride (99%) sodium metaperiodate (99%) and 3-aminopropyltrimethoxysilane APS (97%) were purchased from Aldrich. Dextran 70 kD (from leuconostoc mesenteroides B512) was obtained from Sigma. Monoamino- and diaminotelechelic poly(ethylene glycol), $M_W \sim 2000$ g/mol, were purchased from Huntsman, Salt Lake City. All other reagents were of analytical grade. In all experiments, water was previously deionized ($R < 10\,M\Omega$) by reverse osmosis (PM600, USF, France). Tangential ultrafiltration system consists of a perilstatic pump (Millipore N80EL005), silicon tubing and a poly(ethersulfone) membrane (Prep/Scale^{TM}, cut-off 100,000 g/mol). Dialysis tubing (cellulose, cut off 12,400 g/mol) was obtained from Sigma.

2.2. Maghemite ferrofluid preparation

Maghemite-based cationic ferrofluids were prepared according to a method previously described [15]. Briefly, the cores were synthesized by alkaline coprecipitation of iron (II) and iron (III) precursors in aqueous solution with an excess of

concentrated ammonia hydroxide. Then, the magnetite nanoparticles were oxidized to maghemite with iron (III) nitrate at 80 °C. The cationic maghemite ferrofluid was obtained by peptization of nanoparticles flocculates using nitric acid. The final iron concentration was adjusted to 0.35 mol/L (28 g/L of maghemite) and pH to 2.5.

2.3. Surface silanation of maghemite nanoparticles

Two hundred millilitres of methanol and 17.6 mL (0.1 mol) of APS were added to 200 mL of the maghemite ferrofluid. After stirring at room temperature for 12 h, 200 mL of glycerol were added. Then, methanol and water were roughly removed, respectively, at 40 and 80 °C by using rotary evaporator and the dispersion was dehydrated in vacuum at 100–110 °C for 2 h. Flocculated APS-modified nanoparticles were washed three times with 400 mL of water/acetone 30:70. Following the addition of 400 mL of water, peptization was performed by slowly decreasing pH from 9 to 3 with nitric acid under vigorous stirring.

2.4. Dextran conjugation

Ten grams dextran macromolecules were partially oxidized in 200 mL of water by 10 mL of a 2.06 mol/L $NaIO_4$ solution for 12 h at room temperature and purified by dialysis against 5 L of water. The dialysis procedure was repeated every 2 h for a total of 5 times. One gram of activated dextran was then mixed with 20 mL of the dispersion of modified maghemite nanoparticles ($[Fe] = 0.08$ mol/L) at pH 3 during 24 h and then at pH 9 for 4 h. The so-prepared magnetic particles (VUSPIO) were then washed through tangential ultrafiltration against 3 L of water. The generated Schiff's bases were stabilized by reductive amination using 10 mL of 0.206 mol/L $NaBH_4$ solution at pH 9 for 4 h. Borate salts were removed by dialysis.

2.5. VUSPIO PEGylation

Before reductive amination of the previous Schiff's bases, 20 mL of a VUSPIO dispersion (adjusted to $[Fe] = 0.08$ mol/L) were let to react with an excess (3.7 g) of monoamino-PEG or (20.5 g) of diamino-PEG in 100 mL of water at pH 9 for 4 h.

2.6. Analytical and characterization methods

Total iron concentrations in the suspensions were determined by dissolution of maghemite nanoparticles in concentrated HCl, complete reduction of iron (III) by a $SnCl_2$ solution, oxidation of excess $SnCl_2$ by $HgCl_2$ and titration of iron (II) by $K_2Cr_2O_7$. For diluted maghemite suspensions, iron concentrations were also determined by spectrophotometric titration at 480 nm (molar extinction coefficient = 420 L/mol/cm). Transmission electron micrographs were obtained using a JEOL-2000 FX electron microscope operating at 200 kV. Samples were prepared by air-drying drops of diluted dispersions of the preparations on carbon films supported by copper grids. For negative staining, a droplet of 2 wt% of uranyle acetate (99.9%, Electron Microscopy Sciences, Washington, USA) was deposed after drying of the sample and let to adsorb for 30 s. The excess was then removed by sucking-up with filter paper. Magnetic measurements were achieved using a SQUID 5MPMS in the temperature range 15–290 K. Specific surface area of the nanoparticles was measured by nitrogen adsorption (BET method) using a Micromeritics ASAP. Thermogravimetry (Setaram MTB 10-8) of 20–60 mg of dried nanoparticles was performed in static air at a ramp rate of 120°/h to 650 °C. Photon correlation spectroscopy and zeta potential measurements were performed using the Zetasizer 3000 HS (Malvern Instruments) equipped with a laser He–Ne (50 mW, 532 nm). Diffuse reflectance infrared Fourier transform (DRIFT) and infrared transmission spectra were recorded using a Bruker IFS Equinox 55 FTIR spectrometer (signal averaging 30 scans at a resolution of 4 cm^{-1}). For DRIFT experiments, the spectrometer was equipped with a selector Graseby Specac diffuse reflection cell (Eurolabo, France). Samples were prepared by spreading crushed powders (3.3 wt%) in anhydrous KBr on a conical support.

3. Results and discussion

In order to fulfill their function of targeting specific tissues or organs, VUSPIO have to first circulate in the blood compartment for a long time by minimizing or delaying the nanoparticle uptake by the MPS [16]. It is now well established that such a requirement depends on (i) the hydrodynamic diameter of the nanoparticles, which has to be as low as possible, and (ii) their surface characteristics, as high hydrophilicity and electrical neutrality. For USPIO, dextran corona plays simultaneously the role of steric stabilizer of the nanoparticles (slowing down of aggregation or ripening processes) and of surface repellant towards plasma opsonins (first forces of the MPS clearance mechanism). In the case of the VUSPIO synthetic strategy, dextran corona is anchored to the magnetic cores in the very last step (Fig. 1). Therefore, the colloidal stability of the magnetic dispersion during the first steps has to be ensured by electrostatic repulsions as for conventional aqueous ferrofluids. The difficult stage of this new process consists of the successful transition of the colloidal stability control from electrostatic repulsions to steric ones, in order to avoid the irreversible aggregation of the magnetic cores.

The original maghemite ferrofluid was prepared in large scale and reproducible manner according to the well-known Massart's method [15]. It consists of alkaline coprecipitation of iron (II) and iron (III) precursors, complete oxidation of the nanoparticles into maghemite and peptization of nanoparticles flocculates for leading to a cationic ferrofluid. In such conditions, the mean diameter of the maghemite nanoparticles was 8.5 nm as determined by PCS (Table 1) and confirmed by TEM (7.5 nm). The superparamagnetic behaviour at room temperature of the nanoparticles was checked by magnetization measurement. The magnetization curve vs. applied magnetic field (not displayed here) followed the well-known Langevin function. It may be noticed that in conventional (U)SPIO the composition and the physicochemical properties of the iron oxide cores vary continuously from magnetite Fe_3O_4 to maghemite γ-Fe_2O_3. Thanks to our synthetic route, the obtained magnetic cores are essentially maghemite and are therefore more stable towards any oxidation reaction.

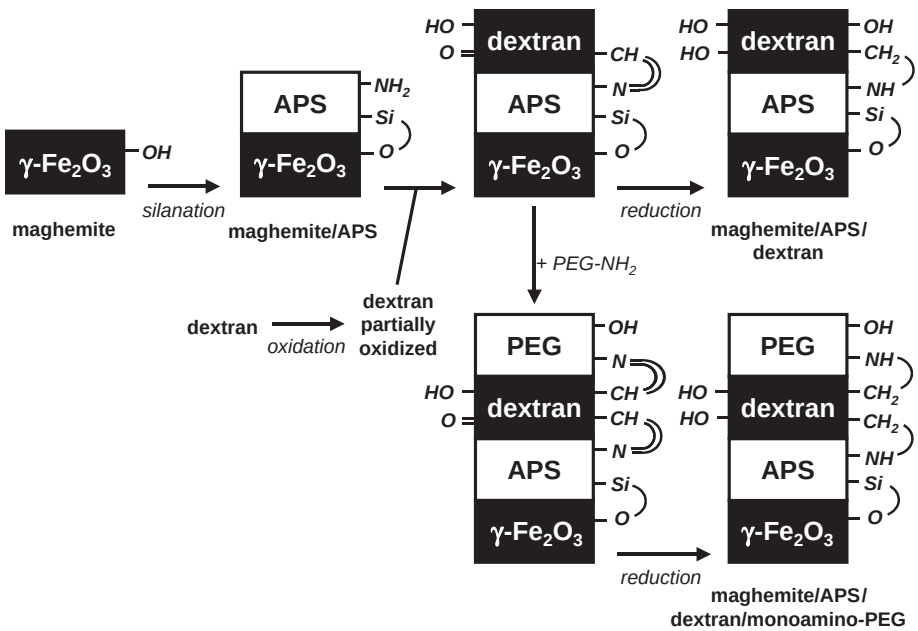

Fig. 1. Multistep synthesis route investigated for the building of VUSPIO.

Table 1
Evolution as determined by PCS of the hydrodynamic particle size (Z) of nanoparticles as far as surface modification occurs

Nanoparticles	Z average mean (nm)
Maghemite	8.5
Maghemite/APS	15
Maghemite/APS/dextran	60
Maghemite/APS/dextran/monoamino-PEG	80
Maghemite/APS/dextran/diamino-PEG	135

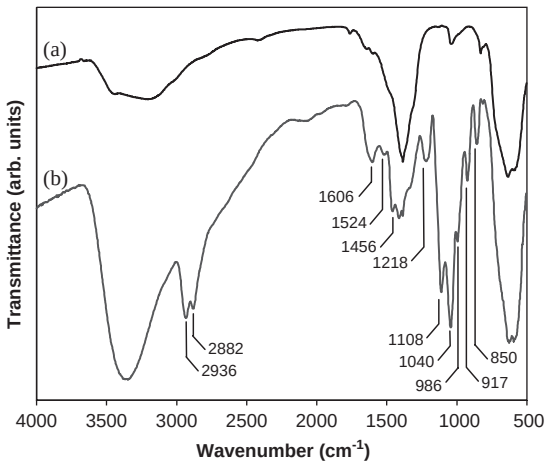

Fig. 2. DRIFT spectra of (a) maghemite and (b) maghemite/APS.

For the covalent conjugation of dextran to the maghemite surface, the use of a coupling agent is necessary. APS was chosen because of the reactivity of alkoxysilane groups towards oxide surface [17] and the presence of the amino group which is hydrophilic, protonable in acidic conditions to ensure the electrostatic colloidal stability, condensable with aldehyde group into Schiff's bases and may act as catalyst towards the alkoxysilane surface condensation.

DRIFT spectrum of maghemite/APS is compared to that of pristine maghemite in Fig. 2. For pristine nanoparticles, the spectrum shows two wide vibrational Fe–O bands at 636 and 586 cm^{-1} and is typical of oxide nanoparticle surface whose the spinel structure is disordered [18]. The band in the 820–830 cm^{-1} range is characteristic of surface hydroxyl groups and corresponds to the stretching mode ν_s(FeOH). The weak band around 850 cm^{-1} corresponds to O–N–O deformation mode of physisorbed nitrate counter-ions while the wider at 1382 cm^{-1} is assigned to their stretching mode. This last band has disappeared on the maghemite/APS spectrum. Moreover, new bands have appeared at 1040, 1108, 1218, 1456, 1524, 1606, 2882 and 2936 cm^{-1}. The last six bands may also be observed in the transmission infrared spectrum of pure APS (Table 2). They are assigned to Si–CH_2, methylene group and protonated or deprotonated amino group and confirm the presence of aminopropylsilane. The 917 cm^{-1} band could be assigned to vibration modes involving C–O stretch and could be due to non-hydrolyzed methoxy groups or physisorbed residual glycerol molecules. If some bands have not been identified (e.g. 986 cm^{-1}), there is no evidence for assigning them to Fe–O–Si bond, because the literature data are contradictory on this subject. On the other hand, the strong bands at 1040 and 1108 cm^{-1} are assigned to siloxane Si–O–Si bonds [19]. Therefore, it could be derived from these infrared spectra, that, in addition to hypothetical Fe–O–Si bonds, the anchoring of aminopropylsilane groups is mainly due to a highly cross-linked polysiloxane film entrapping each maghemite nanoparticle. Moreover, a great number of amino groups are free and arranged on the external side of the polysiloxane film, because it was observed the isoelectric point

Table 2
IR absorption bands of pure APS molecule

ν (cm^{-1})	Intensity	Assignments
3380	m	ν_{as}(NH)
3289	w	ν_s(NH)
2937	S	ν_{as}(CH_2)
2843	S	ν_s(O–CH_3)
2755	w	ν_s(CH)
1584	m	δ(NH_2)
1462	m	δ(CH_2)
1299	m	ω(CH_2)
1191	S	ρ(O–CH_3)
1083	VS	ν_{as}(Si–O–C)
812	S	ν_s(Si–O–CH_3)

Abbreviations: w, weak; m, medium; S, strong; VS, very strong; ν_s, symmetric stretching; ν_{as}, asymmetric stretching; δ, shearing; ω, bending; ρ, rocking.

(IEP) has increased from pH 7 in maghemite sample to pH 10.5 after silanation (Fig. 3). Such a value is consistent with primary amines as majority charge carriers. BET measurement and thermogravimetric analysis gave a specific surface area of $130\,m^2/g$ and APS surface density of $7.5\,\mu mol/m^2$. This last value is consistent with those already reported in the literature for alkylsilane-modified oxide nanoparticles [20]. It may be noticed that this APS surface density as determined by TGA corresponds to 1/20 of the APS molecules added in the reactor. Therefore, it may be reasonably considered that in such conditions the surface is saturated by aminopropylsilane groups.

As shown in Fig. 3, maghemite/APS nanoparticles may be peptized at a pH value lower than 8. Therefore, any further surface chemical reaction in this pH range was performed on isolated nanoparticles, that is to say not on flocculates. For anchoring the dextran corona around the maghemite/APS cores, the reaction was performed at pH 3 in order to let the dextran macromolecules interact with aminopropyl and/or silanol groups essentially through weak interactions such hydrogen bonding. In this way, the colloidal stability of the dispersion goes over from electrostatic repulsive forces to steric ones. In a second time, pH is increased to 9 in order to allow the Schiff's base formation. These imine bonds were stabilized through reductive amination. In Fig. 3, the zeta potential value is very weak (−3 mV at pH 7.4) and is nearly insensitive to pH value. It is consistent with the fact that the majority of free amino groups has been used during the dextran corona anchoring. In Fig. 4, spectra *a* and *b* display dextran infrared absorption bands before and after partial oxidation, respectively. Only the appearance of a new band at $1729\,cm^{-1}$ attests that aldehyde groups have been formed. From the comparison of spectra *b* and *c*, it may be observed that the band at $1729\,cm^{-1}$ is always present after reaction with maghemite/APS, but it has disappeared after reductive amination. Moreover, imine bonds were identified on spectrum *c* ($\nu_{C=N}$ at $1634\,cm^{-1}$) and replaced by secondary amine bonds on spectrum *d* (ν_{NH} at $1586\,cm^{-1}$). TGA gave a weight loss of 60% that is to say a dextran-to-iron ratio of 2.10. The average hydrodynamic size of maghemite/APS/dextran was found to be 60 nm (Table 1). This value is higher than the

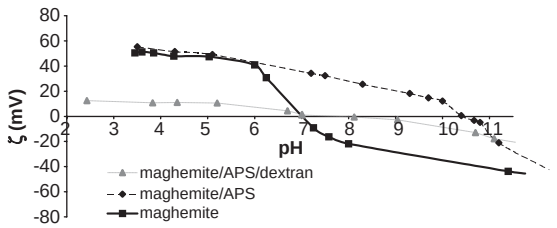

Fig. 3. Evolution of zeta potential curves vs. pH as far as the surface modification of maghemite nanoparticles occurs.

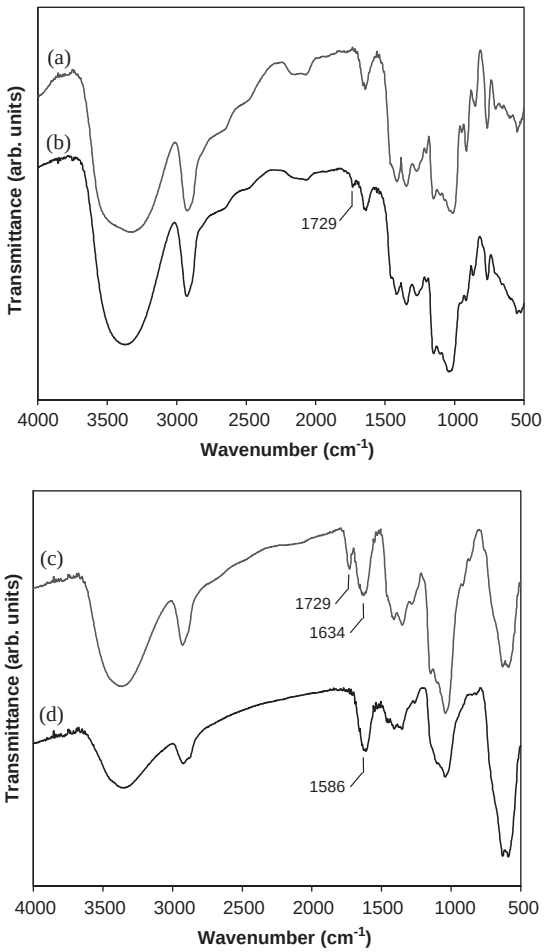

Fig. 4. IR spectra of dextran before (a) and after (b) oxidation by $NaIO_4$ and DRIFT spectra of maghemite/APS/dextran before (c) and after (d) reductive amination by $NaBH_4$.

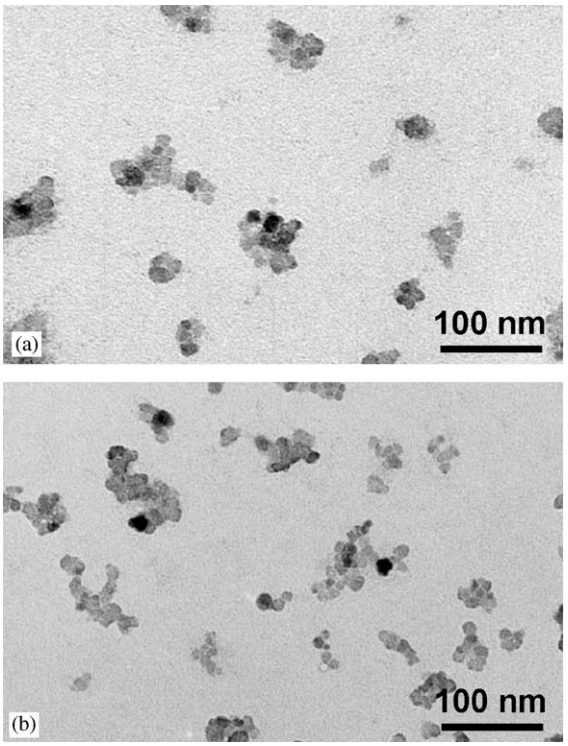

Fig. 5. TEM images of negatively stained VUSPIO (a) maghemite/APS/dextran and (b) maghemite/APS/dextran/diamino-PEG.

magnetic core dimension. Indeed, TEM image is more consistent with a VUSPIO morphology made of small aggregates of a few number of magnetic cores embedded in the dextran corona (Fig. 5a).

In order to check the ability of VUSPIO to be surface derivatized, PEGylation was performed with maghemite/APS/dextran before reductive amination. The hydrodynamic size increases from 60 to 80 nm with monoamino-PEG and up to 135 nm with diamino-PEG (Table 1). In this last case, a further cross-linking reaction probably occurred because of the presence of two amino-end groups on each PEG macromolecules (Fig. 5b).

4. Conclusion

A new multistep synthesis route was investigated for designing more stable and versatile USPIO. Their preparation consists first of colloidal maghemite synthesis, its surface modification through the grafting of aminoalkylsilane groups and the coupling of partially oxidized dextran via formation of Schiff's bases. Such a step-by-step synthesis permits one to control the magnetic core size and the overall hydrodynamic diameter thanks to accurate and reproducible experimental conditions, e.g. colloidal stability control. This synthetic route needs neither ultrasonication for keeping the dispersion stable nor fractionation for narrowing the size polydispersity. These stable MR contrast agents fulfill the main requirements for long circulation in blood compartment. Relaxivity measurements are currently in progress. Moreover, they may be derivatized through the residual aldehyde groups for surface labelling, e.g. PEGylation, but also ligand and/or drug coupling. Moreover, because the magnetic cores are prepared in a preliminary step, they are not restricted to co-precipitated iron oxides. For all these reasons, it is suggested to call these agents "VUSPIO" for Versatile USPIO and several in vivo applications are already expected and investigated, e.g. molecular MR imaging, cell tracking, targeted intracellular hyperthermia, heat-triggered drug delivery platform.

Acknowledgements

The experiments were performed at the Bordeaux Institute of Condensed Matter Chemistry which is supported by the Centre National de la Recherche Scientifique and the University of Sciences and Technology of Bordeaux. Stéphane Mornet was supported by a grant from the Conseil Régional d'Aquitaine.

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