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Development and characterization of solid lipid nanoparticles loaded with magnetite

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Abstract

This paper describes the preparation of colloidal lipid particles containing magnetite from warm emulsions. A two step method was used to obtain the nanoparticles: (i) formulation of a transparent phase by heating a O/W emulsion (aqueous surfactant solution melted with a lipid phase, containing the ethyl oleate and soybean lecithin) in which modified lipophilic magnetite is incorporated, and (ii) preparation of the nanoparticles by dispersing the warm transparent phase in cold water (7 °C) under mechanical stirring. The latter method gives spherical nanoparticles of a mean size of 62 nm measured by Photon Correlation Spectroscopy and Transmission Electronic Microscopy. The magnetite entrapment efficiency was determined by use of a magnetophoretic sedimentation method.

Keywords: Emulsion; Nanoparticles; Magnetite; Magnetophoresis

1. Introduction

Interest in lipid nanoparticles as colloidal drug carriers has grown during the last years (Müller et al., 2000). Several authors have demonstrated that lipophilic drugs are easily included in the internal phase of these systems (Sjöström et al., 1993; de Chasteigner et al., 1996; Zur Mühlen and Mehnert, 1998). Similarly, in the case of hydrophilic drugs, it was shown that they can be also incorporated provided they became rather lipophilic by formation of complexes (Cavalli et al., 1993; Morel et al., 1996). These delivery systems are considered able to target drugs to certain organs or tissues and can be used in various administration routes including oral, ocular and parenteral (Alyautdin et al., 1998; Yang et al., 1998).

Nanoparticle systems have been prepared from different polymers or lipids, such as glycerides and

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fatty acids, using several procedures: high pressure homogenization, warm microemulsion dispersion, polymerisation in an acidic medium and nanoprecipitation (Ueda and Kreuter, 1997; Müller et al., 1997; Dunn et al., 1997). Among them, the process based on the dispersion of a warm microemulsion in cold water is a simple method that has been widely employed (Aquilano et al., 1993; Daubresse et al., 1996; Cavalli et al. 1997; Heiati et al., 1998). Microemulsions are clear, thermodynamically stable, optically isotropic systems obtained spontaneously by mixing water and oil stabilized by surfactant and co-surfactant (Bourrel and Schechter, 1988; Gallarete et al., 1988). Besides this, the microemulsions obtained are often stable in a narrow temperature range. The nanoparticles obtained by dispersion of the microemulsion in cold water allow the encapsulation of the drug previously dispersed into the microemulsion. However, the relatively high temperatures required for their preparation when using lipids with high melting points (i.e. stearic acid, mp: 70 °C; glycerol tristearate, mp: 69–71 °C; glycerol behenate, mp: 70–73 °C) can severely limit the applicability of this technique when the exposition time of the encapsulated drug at high temperatures cannot be minimized.

Therefore, the purpose of the present work was the preparation and characterisation of colloidal lipid like particles from warm transparent emulsions containing ethyl oleate as a lipid phase. These systems are stable in quite a large range of temperatures ranging from 34 to 47 °C. In this study, magnetite was encapsulated to constitute markers after being modified in order to increase its lipophilicity and consequently their incorporation into lipid phase. Further studies are presently in progress to examine the biodistribution of these lipid nanoparticles by Magnetic Resonance Imaging (IRM).

2. Materials and methods

2.1. Materials

Ethyl oleate was supplied by Croda SA (Trappes Cedex, France), soybean lecithin (Lipoid® S75 3) was obtained from Lipoid GmbH (Ludwigshafen, Germany), Butanol, and Polysorbate 80 (Tween® 80) were from Prolabo (Paris, France). All reagents were used as received.

2.2. Synthesis of magnetite

Magnetite particles with a diameter of approximately 15 nm were prepared by coprecipitation of $FeCl_2$ and $FeCl_3$ in HCl solution (10%) in the presence of an excess of ammonia and subsequently coated by oleic acid and washed with acetone to obtain a stable dispersion (Khalafalla, 1975; de Cuyper and Joniau, 1988; Pouliquen et al., 1991). A 4.5 ± 0.5 mg/ml magnetite concentration was evaluated by atomic emission spectroscopy. Therefore, the tendency of the particles to agglomerate is inhibited, the dispersion is stabilized and the lipophilicity of the magnetite is increased.

2.3. Preparation of nanoparticles

The preparation of lipid nanoparticles was performed according to a two step method described in Fig. 1: preparation of a transparent phase, followed by its dispersion in cold water in order to obtain nanoparticles.

2.3.1. Transparent phase formulation

A mixture of water and 5% w/w Tween® 80 is warmed to the same temperature (60 °C) as the lipid phase containing a 5% w/w ethyl oleate and 1.5% w/w lecithin. Under mild mixing the aqueous phase is added to the melted lipophilic suspension, successively butanol (7.5% w/w) was added to the emulsion obtaining a clear system under continuous shaking (at 34–47 °C). Then the magnetite suspension (5% w/w) was added to the system without modification of its visual aspect.

In order to characterize this transparent system, measure of conductivity as a function of temperature was carried out using a Conductivity cell WTW LF325-B (Weilheim, Germany). This technique is used to characterize the evolution of a classical emulsion with the temperature. More particularly this method allows the determination of the Phase Inversion Zone where a o/w emul-

sion systems continuously reverse to form a w/o one (Förster et al., 1992; Sing, 1997). The samples were assessed visually to determine the regions of transparency.

2.3.2. Nanoparticles formulation

Finally, an aqueous suspension of colloidal particles was obtained by dispersing the warm transparent phase in cold water (7 °C) at a 1:5 ratio (microemulsion:water, v/v). This particle suspension was washed with distilled water by ultrafiltration using a Minitan® System (Millipore, Saint-Quentin-Yvelines, France) equipped with polysulfone membranes of 300 000 Da molecular weight cut-off (Minitan® PTMK filter sheets, Millipore®), in order to eliminate the large proportion of surfactants used to obtain the transparent phase. The suspension was then freeze-dried for 48 h (Lyophilisateur RP2V®, Serail, Argenteuil, France) with Trehalose as cryoprotectant (5% w/w).

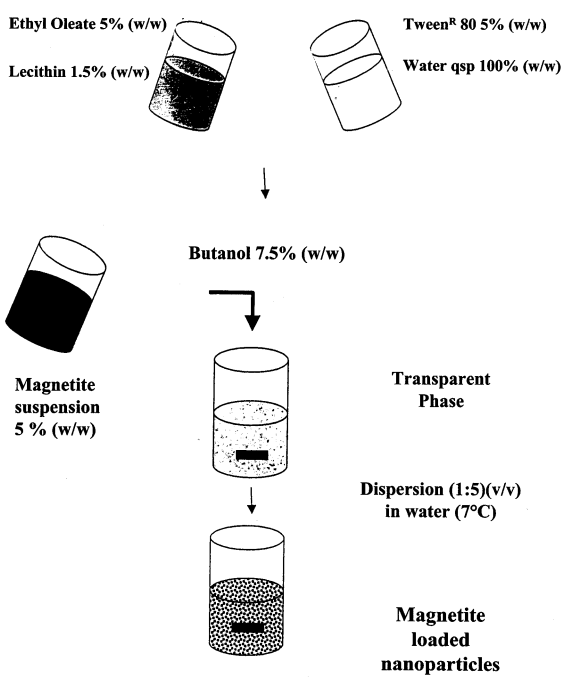

Fig. 1. Nanoparticle formulation

2.4. Characterization methods

2.4.1. Particle size analysis

The average particle size and polydispersity index were determined by Photon Correlation Spectroscopy (PCS) using a Malvern Autosizer® 4700 (Malvern Instruments Ltd., Malvern UK). Measurements were done in triplicate and the photodetector was perpendicular to the laser beam.

2.4.2. Transmission Electron Microscopy (TEM)

Observations were realised using an Electronic Transmission Microscope at 80 kV (JEOL 100 CX, Japan). For the preparation of the samples, some copper shutters recovered by a collodion layer are put over the dialysed dispersion of nanoparticles which consequently adhere spontaneously to this film. In order to obtain sufficient contrast and before observation the film recovered with NP is exposed to osmium tetroxide (4% in aqueous solution) for 3 h.

2.4.3. Atomic Force Microscopy (AFM)

Experiments were carried out in a non-contact mode using an Autoprobe CP (Park Scientific Instruments, Geneva, Switzerland). We have used a 2 μm cantilever and monocrystalline silicium tip (Ultralever UL020, Park Scientific Instruments). The linear scanning rate was 1.5 Hz.

2.4.4. Zeta potential analysis

The electrophoretic mobility and zeta potential measurements were determined using a Malvern Zetasizer 3000 (Malvern Instruments Ltd., Malvern UK). All the samples were diluted in 0.1 M NaCl before analysis.

2.4.5. Magnetite entrapment

The amount of magnetite incorporated into the nanoparticles was determined after separation of free magnetite from loaded nanoparticles by magnetophoresis (de Cuyper and Joniau, 1990). The magnetic field was generated with a water-cooled Bruker electromagnet (Type BE10) (Karlsruhe, Germany) equipped with two pole pieces 2 cm from each other. For this purpose, the magnetite was previously radio labelled during the synthesis

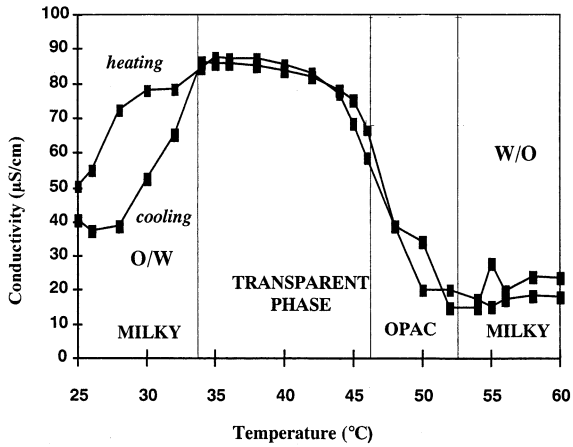

Fig. 2. Conductivity of the mixing versus temperature.

process with the gamma-emitting isotope Fe^{59}. Aliquots of the samples were analyzed by gamma counting before and after magnetophoresis using a Minaxi γ Auto-Gamma® 5000 Series (Packard Instruments Company, Meriden, USA).

3. Results and discussion

3.1. Transparent phase characterisation

In order to study the phase behaviour of the emulsion prepared, conductivity measurements were carried out firstly when cooling down (from 60 to 25 °C) the initial water in oil emulsion under magnetic stirring and a second time when heating the preparation from 25 to 60 °C.

As shown in Fig. 2, one can see (first zone) an increase of the conductivity (40 to 80 μS/cm) with temperature which can be related to the stability increase of the o/w emulsion with more and more defined and small drops dispersion. When the temperature increases (in this particular zone), the oily droplets size decreases along with increasing emulsion stability (strongly related to a lower rate of coalescence of the more and more fine oily droplets) until we reach the beginning of the ZIP where no dispersed and continuous phases can be observed (microemulsion state). This temperature effect is often used in order to stabilise classical emulsions. In this zone one can observe a pronounced hysteresis between cooling and heating consequently delaying the transformation processes which is more important when the temperature decreases.

Between 34 and 46 °C (second zone) the system has a transparent aspect with high conductivity (about 80 mS/cm) characterising a bicontinuous phase corresponding to a microemulsion single phase system. This transparent system is stable in a broad temperature range (34–47 °C) and can be considered as a system of preformed bicontinuous structures that will allow the formulation of lipid particles in the nanometer size range. In this range, the phase behaviour of the initial o/w emulsion changes to a single-phase and transparent system. The transparent phase was identified as the area where a clear and transparent formulation was obtained based on visual inspection of the samples.

The third zone is related to the inversion of an oil-in-water emulsion to a water-in-oil emulsion and does not occur at a fixed temperature but over a range of temperatures (43–53 °C) which represent the Phase Inversion Zone (Sing, 1997) and where the system presents an opaque aspect.

Finally (last zone), at temperatures above 57 °C a conductivity of about 20 μS/cm can be related to an oil continuous phase of a water-in-oil emulsion (milky aspect).

As we have previously described, the technique used in this study is based on the rapid dilution of the transparent phase (at 40 °C) which provokes the formation of a suspension of colloidal lipid particles (Cavalli et al., 1996).

3.2. Nanoparticles characterization

3.2.1. Photon correlation spectroscopy

In the case of blank nanoparticles after ultrafiltration, results obtained by Photon Correlation Spectroscopy examination demonstrated the formation of one population at 49 nm (quite laplacian shape distribution) with a narrow size distribution (polydispersity index = 0.102) (Fig. 3a). One can note a good correlation between the three properties (intensity, volume and number)

indicating one single distribution without contaminating small objects.

In the same way, loaded nanoparticles after ultrafiltration (Fig. 3b), show one population at 62 nm with a satisfying size distribution (polydispersity index = 0.121). Here also the three analysis modes results are well correlated to a single population.

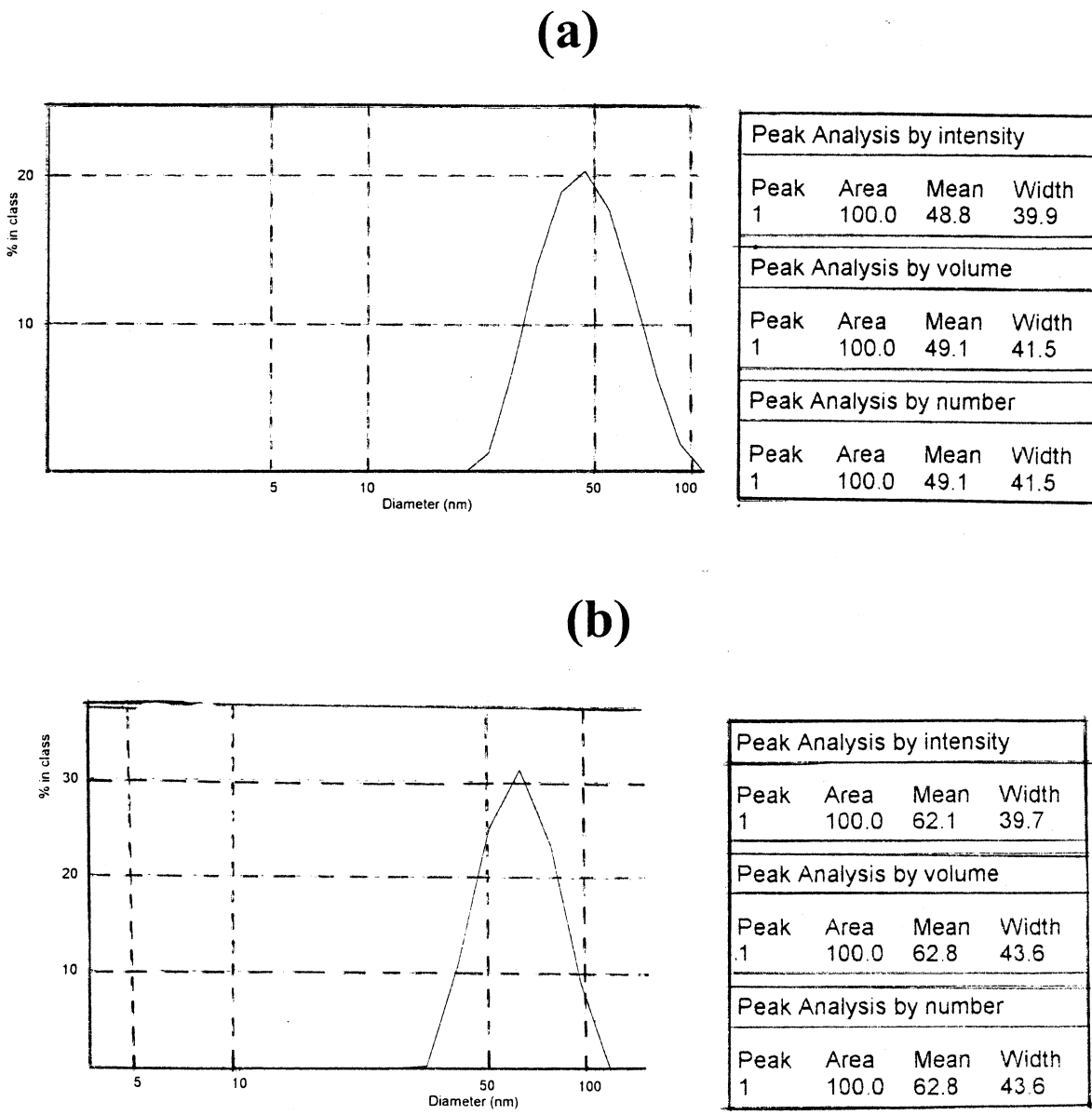

Fig. 3. Size granulometry of unloaded nanoparticles (a) and magnetite loaded nanoparticles (b), determined by Photon Correlation Spectroscopy.

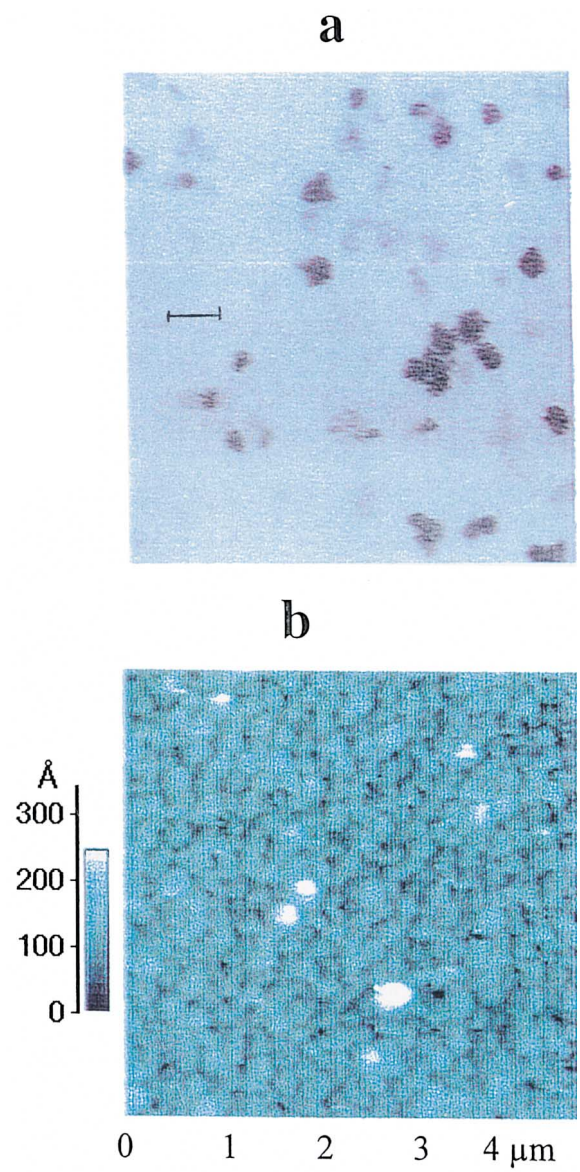

Fig. 4. Transmission Electronic Microscopy photomicrographs of unloaded nanoparticles (a). Atomic Force Microscopy image of magnetite loaded nanoparticles (b).

3.2.2. Transmission Electronic Microscopy

The photomicrographs obtained by TEM showed the regular aspect and fairly spherical shape of the particles with a mean diameter of 50 nm (Fig. 4a). The scale bar in the figure represents 100nm.

Osmium recovery allows their singularisation because of its affinity with the double bounds of the unsaturated alkyl chains of the lecithin but also with the ethyl oleate double bond. In this way, the core but also the surface of the particles can be visualised.

3.2.3. Atomic Force Microscopy

AFM was performed to image the shape and surface appearance of nanoparticles. A drop of diluted aqueous suspension (without preliminary ultrafiltration) was placed on a mica slide and dried out at room temperature for 24 h. We present in Fig. 4b the image related to magnetite loaded nanoparticles after water evaporation. One can remark a shape and size homogeneity. The mean vertical particle resolution is about 20 nm which is good agreement with the 60 nm size obtained by PCS and TEM. But unfortunately we obtain a lateral resolution for one particle of about 200 nm. This result is not surprising considering the well known ability of particles to fuse during the water evaporation consequently to the molecular diffusion at the surface of the particles (Juhué and Lang, 1994). Moreover the presence of free surfactant in the suspension should increase this characteristic size. One week after the spreading on the mica plate, no particle can be distinguished on AFM images which present a homogeneous and flat aspect.

3.3. Magnetite encapsulation efficiency

Since free magnetite was strongly attracted by the magnetic field, the entrapment efficiency was calculated as the percentage of the activity recovered in the supernatant after magnetophoresis.

To increase the lipophilicity and impart stability to magnetite particles, they should be coated with a dispersing agent (sometimes called peptizing or protective colloid) (de Cuyper and Joniau, 1990; Cavalli et al., 1996). The molecules of the dispersing agent must possess the dual property of being strongly adsorbed on the surface of the dispersed phase in addition to being soluble in the dispersion medium. Magnetite was coated with oleic acid in order to obtain a poorly water-soluble particles, so this coating procedure would allow us to incorporate it into the lipid nanoparti-

cles. The coated magnetite was added to the system once the transparent phase was obtained. The incorporation of magnetite slightly increased the average size of the resulting particles (60 nm) compared to unloaded nanoparticles (see Fig. 3b).

3.3.1. Visual aspect of the sample

A further experiment was performed by addition of a similar quantity of magnetite to a suspension of blank particles. The colour of the nanoparticle suspensions obtained may be take as the first evidence of the incorporation of magnetite into the particles. The sample containing the nanoparticles with encapsulated magnetite was pale-brown while the colour of the sample obtained by adding the magnetite after the preparation of nanoparticles was the same of blank particles. Hence, a phase separation was observed when the magnetite was added to pre-formed particles.

3.3.2. Zeta potential measurements

The Zeta potential of the particles containing magnetite (-9.6 ± 2.5 mV) was similar to that of blank particles (-13.0 ± 3.1 mV). However, the value for the oleic acid coated magnetite was -33.0 ± 1.4 mV. Therefore, this results show that no free magnetite can be detected by zeta potentiometry.

3.3.3. Magnetophoresis

As we have previously outlined, after nanoparticle preparation, magnetophoresis was used to separate non encapsulated magnetite from loaded nanoparticles (de Cuyper and Joniau, 1988). In an initial stage, radio-labelled magnetite (see Section 2) was added to a suspension of blank nanoparticles and was subjected to magnetophoresis under different magnetic field intensities. In order to optimise the efficiency of the magnetic sedimentation, a magnetic field of 1.8 T was applied for 5 min. In these conditions we measured a supernatant radioactivity which represents 0.08% of the initial value (before the magnetophoresis). In that way 99.92% of magnetite precipitate when this magnetic field is applied.

Therefore, based on these results the same procedure was applied to the magnetite loaded nanoparticle suspension. After a 1.8 T magnetic field exposition (5 min) we have measured a supernatant radioactivity which represents 92% of the initial value (before the magnetophoresis).

From these results three important points can be deducted. Firstly sedimentation of encapsulated or covered magnetite nanoparticles strongly decreases by using a 1.8 T magnetic field. In this way the consequent lipid layer (4*magnetite size) around the magnetic particle could decrease their flocculation ability. If one can consider that the ferro-magnetic properties (orientation of dipolar magnetic moments) of the coated magnetite are still unchanged when they are coated by the surfactant shell, the magnetic attraction forces are not enough important for this magnetic field to induce flocculation. As a direct consequence, the sedimentation is affected.

Secondly the precipitate is mainly composed by free magnetite particles but probably also by lipidic nanoparticles with free magnetite located at their surface. Finally and consequently to the previous points, 92% (or more) of magnetite particles are encapsulated.

4. Conclusion

In this paper a method is developed for the preparation of lipidic nanoparticles (< 60 nm) according to a microemulsion preformulation method. Conductivity measurements versus temperature allow the choice of a convenient mixing temperature zone for which the finest microemulsions can be obtained. This microemulsion initiates the formation of blank lipidic nanoparticles after an adequate dispersion in cold water.

In the same way this experimental protocol is well adapted when we want to incorporate hydrophobed magnetite nanoparticles in the previous lipidic ones. Visual aspect of the samples, zeta potential measurements but also magnetophoretic experiments give strong evidences of the presence of the magnetic particles in the core of the lipidic nanoparticles. We have shown that the consequent lipidic layers around the magnetic nanoparticles are sufficient to modify electrokinetical effects in an electrical field but also aggregation and sedimentation phenomena in a magnetic field.

On the basis of the reported preliminary data, the presently described lipid nanoparticles might constitute a promising formulation for magnetite targeting. Their small size, high entrapment efficiency and partially lipidic nature suggest that they could be an interesting attempt for site specific magnetite delivery. One can hope that their soft surfactant and lipidic shell will represent an ideal compromise between stabilisation and protection during the blood transport and a satisfying magnetite release across an injured Brain–Blood Barrier. To check this latter point in more detail in vivo distribution of these nanoparticles will subsequently be tested.

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