Available online at www.sciencedirect.com
ScienceDirect
International Journal of Pharmaceutics 347 (2008) 156–162
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

# Preparation and characterization of extruded magnetoliposomes

Raimon Sabaté [a], Ramon Barnadas-Rodríguez [a], José Callejas-Fernández [b], Roque Hidalgo-Álvarez [b], Joan Estelrich [a,*]

[a] *Departament de Fisicoquímica, Facultat de Farmàcia, Universitat de Barcelona, Avda. Joan XXIII, s/n, 08028-Barcelona, Catalonia, Spain*
[b] *Departamento de Física Aplicada, Facultad de Ciencias, Universidad de Granada, Campus de Fuente Nueva, 18071-Granada, Spain*

Received 20 March 2006; received in revised form 21 June 2007; accepted 27 June 2007
Available online 4 July 2007

**Abstract**

Phospholipid vesicles encapsulating magnetic nanoparticles (here after called magnetoliposomes) have been prepared for targeting a drug to a specific organ using a magnetic force, as well as for local hyperthermia therapy. Magnetoliposomes are also an ideal platform for use as contrast agents. We describe the preparation and characterization of liposomes containing magnetite, a ferrimagnetic material. These liposomes were obtained by extrusion. To prevent the aggregation of particles, the magnetite was treated—prior to encapsulation—with a surfactant, resulting in a stable ferrofluid suspension. Once the ferrofluid had been obtained, it was used to hydrate the phospholipid layers. Magnetoliposomes had a diameter of around 200 nm, the same pore size as the membranes used for the extrusion. The encapsulation efficiency was dependent on the initial amount of ferrofluid present at the encapsulation stage, and in the worst case was 19%. This value corresponded to 82.06 mmol of magnetite per mole of phospholipid. Although we have used a determined membrane pore to obtain the magnetoliposomes, the method described here allows to prepare magnetoliposomes of different sizes as well as of different magnetite content.


*Keywords:* Liposome; Magnetism; Microencapsulation; Phospholipid

## 1. Introduction

Since they were produced some 40 years ago (Bangham, 1993), liposomes have proved to be unique model systems for studying both structural and dynamic aspects of natural membranes. Although, given their biocompatibility, biodegradability, low toxicity and immunogenicity, there are many possible applications for liposomes, in fields as varied as mathematics and biophysics (for a review, see Lasic, 1993), one of their most successful applications is as drug delivery systems (Barenholz, 2001).

In chemotherapy, the use of magnetic force for targeting a drug to a specific organ or tissue was first proposed by Widder et al. (1979), on the assumption that magnetic fields are harmless to biological systems. For this purpose, they used albumin microspheres containing magnetite ($Fe_3O_4$). Magnetite is a ferrimagnetic material with an inverse spinel structure. There are two magnetic sublattices with antiparallel spin orientations. However, the opposing magnetic moments of the two sublattices are not equal in magnitude, which results in a net magnetic moment.

Magnetic nanospheres and ferrofluids have also been used in numerous biological fields including diagnostics, drug targeting, molecular biology and cell purification. The first magnetoliposomes were prepared by Kiwada et al. (1986). They dispersed the magnetite into the liposome-forming lipids, and achieved a final mean liposome diameter of 1.54 μm. De Cuyper and Joniau (1988) observed that different types of phospholipids adsorbed onto magnetizable solid particles, and they used nanometre-sized $Fe_3O_4$ particles stabilized by a lauric acid envelope as a solid support to obtain magnetoliposomes. They state that the lipids adopt a bilayer configuration around the iron oxide core with one of the monolayers adsorbed onto the magnetite surface (De Cuyper and Joniau, 1991). The resulting structures denoted by these authors as "magnetoliposomes", mimic biological membranes to the same extent as classical liposomes. Moreover, it has been demonstrated that, whereas the outer shell of the bilayer can be extracted by organic solvents, the

* Corresponding author. Tel.: +34 93 4024559; fax: +34 93 4035987.
*E-mail address:* joanestelrich@ub.edu (J. Estelrich).

0378-5173/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2007.06.047

inner leaflet remains firmly attached (De Cuyper and Noppe, 1996). Müller-Schulte et al. (1997) proposed the use of a kind of magnetoliposome for local hyperthermia therapy, after intravenous injection of the particles and subsequent placement of the malignant tissue in an alternative magnetic field. Each cycle of a hysteresis cycle of any magnetic material involves an energy loss proportional to the area of the cycle. Hence, if magnetoliposomes are remotely positioned at a given site in the body, perhaps the site of malignancy, then the application of an alternating magnetic field can be used to selectively warm a given area. It has been proposed that this simple physical effect could be used both to destroy cells directly and to induce a modest increase in temperature so as to increase the efficiency of either chemotherapy or radiotherapy. In this regard, Viroonchatapan et al., 1995) prepared thermosensitive liposomes using dextran magnetite, that is, a magnetic iron oxide core surrounded by dextran chains. Since dextran magnetite is highly soluble in water, these liposomes had high magnetite content. More recently, De Cuyper et al. (2003) and Hodenius et al. (2002) used polyethylene glycol (PEG) derivatized phospholipids to coat the iron oxide core. These PEGylated phospholipids circumvent the quick removal of liposomes from the bloodstream by liver cells (Allen, 1996). Moreover, they are wholly biocompatible (Lacava et al., 2004). This approach has also been used by Martina et al. (2005).

Magnetoliposomes are an ideal platform as contrast agents due to their strong effect on $T_2$ relaxation (Bulte and De Cuyper, 2003). In this regard, Martina et al. (2005) have shown that the magnetic properties of liposomes encapsulating maghemite rank them among the most efficient $T_2$ magnetic resonance contrast agents.

In this paper we describe a method for obtaining magnetoliposomes, in which the magnetic material is incorporated into the aqueous space of the vesicles during their preparation (as used, for example, for the encapsulation of water-soluble drugs). This method has a number of advantages over above mentioned: it affords relatively high encapsulation efficiency and enables vesicles of different sizes to be prepared.

## 2. Materials and methods

### 2.1. Materials.

A strong neodymium-iron-boron ($Nd_2Fe_{12}B$) magnet was from Halde GAC (Barcelona, Spain); tetramethylammonium hydroxide (TMAH) was from Aldrich (Steinheim, Germany); soybean phosphatydylcholine (Lipoid S-100) (PC) was a gift from Lipoid (Ludwigshafen, Germany). All other reagents were of analytical grade.

### 2.2. Preparation of magnetite and ferrofluid

Magnetite and the corresponding ferrofluid were prepared as described by Berger et al. (1999). The method is based on the stoichiometric mixture of $Fe^{2+}$ and $Fe^{3+}$ in aqueous media, the coprecipitation of the corresponding hydroxides [$Fe(OH)_2$ and $Fe(OH)_3$] upon the addition of a strong alkali, and the relatively fast aging of those hydroxides under vigorous stirring to form magnetite:

$$2FeCl_3 + FeCl_2 + 4H_2O + 8NH_3 \rightarrow Fe_3O_4 + 8NH_4^+ + 8Cl^- \quad (1)$$

A detailed protocol for the preparation of magnetite and ferrofluid can be read in Berger et al. (1999).

### 2.3. Preparation of magnetoliposomes

PC was dissolved in a mixture (2:1, volume ratio) of chloroform and methanol in a round-bottom flask and dried in a rotary evaporator under reduced pressure at 40 °C to form a thin film on the flask. The film was hydrated for 24 h with the appropriate amount of the mixture of water and ferrofluid to give a lipid concentration of 20 mmol $L^{-1}$. Multilamellar liposomes (MLV) were formed by gentle sonication in a Transonic Digitals bath sonifier (Elma, Germany) for 10 min. MLV were downsized to form oligolamellar vesicles by extrusion at room temperature in a Liposofast device (Avestin, Canada) through two polycarbonate membrane filters of 0.2-μm pore size a minimum of 21 times (MacDonald et al., 1991).

### 2.4. Characterization of the iron oxide cores

Prior to characterization of the iron oxide cores by X-ray diffractometry, the magnetite was pre-dried at reduced pressure, and then heated to 80 °C for 2 h. The X-ray patterns were taken in a Bragg-Brentano $\theta/2\theta$ Siemens D-500 diffractometer (radius = 215.5 mm) with Cu $K_\alpha$ radiation, selected by means of a secondary graphite monochromator. The divergence slit was 1° and the receiving slit was 0.15°. The starting and final $2\theta$ angles were 10° and 100°, respectively. The step size was 0.05° $2\theta$ and the measuring time was 3 s per step. The average particle diameter for the crystalline precipitate was determined by Scherrer's formula using the half-widths of the most intense X-ray diffraction peaks (Nuffield, 1966),

$$t = \frac{0.9\lambda}{B\cos\theta_B},$$

where $t$ is the particle diameter in nm, $\lambda$ is the wavelength of the X-ray radiation in nm, $\theta_B$ is the Bragg angle of the peak, and $B$ is the peak broadening, which is a measure of the size of the nanoparticles with respect to reference particles. As a reference, we used a commercial ferritine (Aldrich, Milwaukee, WI) with a crystal thickness greater than ~200 nm. The average size of magnetite particles was determined using the peak at $2\theta = 35.6°$ (Miller indices (3 1 1)).

### 2.5. Size determination

In ferrofluid and magnetoliposomes, the mean and distribution of the particle size were determined by dynamic light scattering at 25 °C with an Zetasizer Nano (Malvern, UK). To measure particle size distribution of the dispersion, a polydispersity index (PI), ranging from 0.0 for an entirely monodisperse sample to 1.0 for a polydisperse sample, was used.

### 2.6. Electrokinetic characterization

Electrophoretic mobility was measured with a Zetasizer 4 (Malvern, UK). Magnetoliposomes were diluted with KBr 1 mmol $L^{-1}$ until a concentration of approximately 1 mg $mL^{-1}$ of magnetite. To avoid that magnetoliposomes can settle due to the high density of the magnetite, the measurement was carried out with three different samples.

### 2.7. Purification of magnetoliposomes

Non-entrapped ferrofluid particles were removed by size exclusion chromatography (SEC). 250 μL of magnetoliposomes was applied to a D-Salt Excellulose column (Pierce, Rockford, IL) saturated with lipids before sample elution. The eluent was bidistilled water.

### 2.8. Transmission electron microscopy

Ferrofluid and magnetoliposomes were observed by transmission electron microscopy (TEM) using a Jeol 1010 microscope (Jeol, Japan), operating at 80,000 kV. Samples were prepared by placing a drop of magnetoliposomes onto a 400-mesh copper grid coated with carbon film, and after staining with uranyl acetate they were allowed to dry in the air before introduction into the microscope. Images were recorded with a Megaview III camera. Acquisition was accomplished with the Soft-Imaging software (SIS, Germany).

### 2.9. Assay of magnetite

The amount of encapsulated magnetite was determined based on ferrous ion by using *o*-phenanthroline as follows (Kiwada et al., 1986): A liposomal or ferrofluid sample (0.1 mL) was mixed with 0.1 mL of Triton X-100 solution (5%, v/v), before ionizing the magnetite by adding 0.5 mL of concentrated HCl; 0.5 mL of hydroxylamine hydrochloride solution (1.44 mol $L^{-1}$) was then added to reduce ferric ion. After 15 min, 1 mL of *o*-phenanthroline solution (12.6 mmol $L^{-1}$) was added, the mixture was neutralized with 0.25 mL of 12 mol $L^{-1}$ NaOH, and the pH was adjusted to about 4.0 by adding the necessary volume of 50 mmol $L^{-1}$ citrate buffer. Finally, the absorbance was read at 509 nm in a Shimadzu UV-2401 PC UV–vis spectrophotometer (Shimadzu, Japan). The calibration curve was performed with several amounts of a solution of $Fe_3O_4$ in 12 mol $L^{-1}$ HCl (Aldrich, Milwaukee, WI).

### 2.10. Assay of phospholipids

Phospholipid content in magnetoliposomes was determined by the method of Steward-Marshall (1980). It was confirmed in advance that the presence of ferritine had no influence on colour development. An aliquot of 0.150 mL of magnetoliposomes was mixed with 1.5 mL of $CHCl_3$, before adding 1.5 mL of the reagent (0.1 mol $L^{-1}$ ammonium ferrothiocyanate). After shaking energetically for 45 s, the sample was centrifuged for 10 min at 2000 rpm. The absorbance of the aqueous phase was read at 490 nm. The calibration curve was performed with several amounts of a 40 mmol $L^{-1}$ solution of phosphatydylcholine in $CHCl_3$.

### 2.11. Encapsulation efficiency determination

In order to establish the best magnetite/phospholipid ratio, liposomes containing 1.25–125 mg of magnetite per mol of phospholipids were prepared. After SEC purification, the amount of magnetite and the phospholipid content were determined, the magnetite/phospholipid ratio was calculated, and this value was compared to the initial pre-SEC values to obtain the percentage of encapsulation.

## 3. Results

### 3.1. Characterization of the iron oxide cores

We determined the half-widths of the five strongest reflections in the diffraction pattern (Fig. 1), in order to calculate the peak broadening of each individual reflection. The mean crystal size was determined from Scherrer's formula, which gave a value of 12.5 nm. The lattice spacing was calculated from the position of the 311-peak using Bragg's law. This yielded a value of the cubic unit cell, $a = 0.837$ nm. The density of magnetite, determined by picnometry, was 5240 kg $m^{-3}$. Magnetite presented a mass magnetic susceptibility, $\chi_m$, of 0.638/g of magnetite (Magnetic Susceptibility Meter SM20, GF Instruments, Czech Republic). Expressed in g of iron this value is 0.881.

### 3.2. Characterization of ferrofluid

Ferrofluid, as a dark brown suspension, presented a size of $58 \pm 12$ nm (PI = $0.21 \pm 0.01$) determined by dynamic light scattering and a mass magnetic susceptibility of 0.895/g of iron. This value is totally in accordance with that obtained for magnetite. Ferrofluid exhibited positive magnetic behaviour in

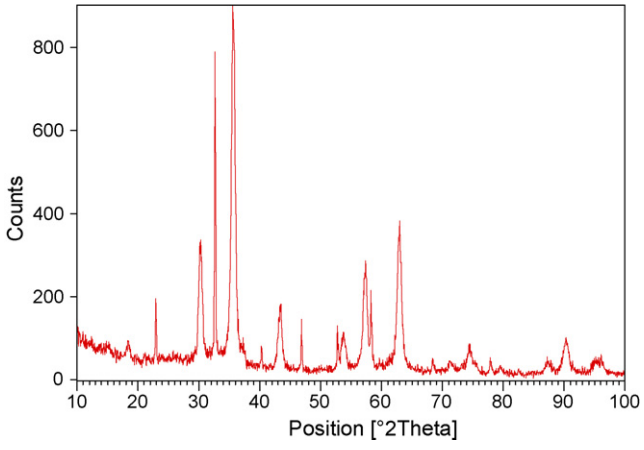


Fig. 1. X-ray powder diffraction power of the magnetite synthesized by the technique outlined in the text. The peak used to determine the average particle size was at $2\theta = 35.65°$ (Miller indices (3 1 1)).

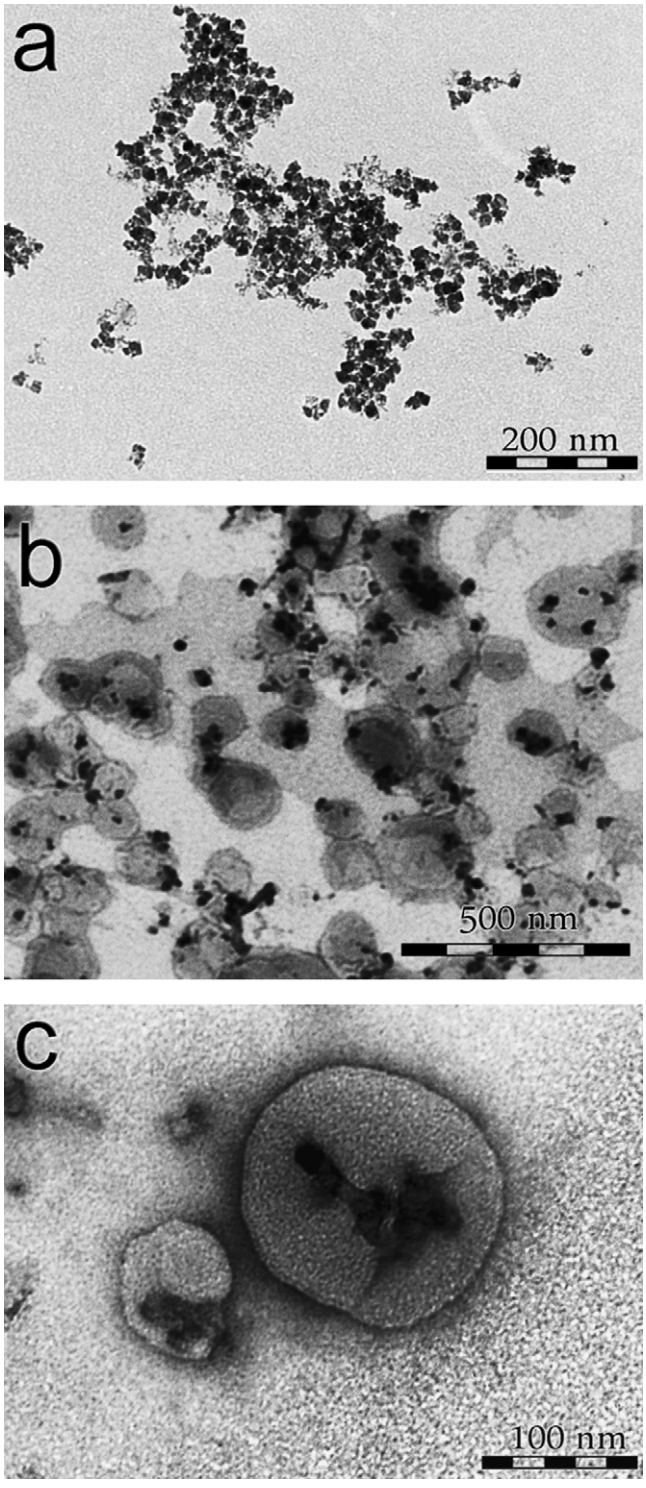


Fig. 2. TEM micrograph of (a) ferrofluid and (b and c) magnetoliposomes loaded with a concentration of ferrofluid equivalent to 72.89 g of magnetite per mole of phospholipid. Ferrofluid was observed directly and only the magnetic cores are visible, whereas magnetoliposomes were stained with uranyl acetate.

the presence of a permanent magnet. No sedimentation was observed when the sample was centrifuged at 12,000 rpm for 5 min at room temperature. The particles of ferrofluid are shown in Fig. 2a. This micrograph was obtained without staining the sample.

### 3.3. Characterization of magnetoliposomes

Magnetoliposomes were of brownish appearance. When liposomes encapsulated the ferrofluid their size, determined by dynamic light scattering and expressed as *z*-average, was $140 \pm 1$ nm (PI = $0.25 \pm 0.02$) before purification by SEC, and $197 \pm 9$ nm (PI = $0.21 \pm 0.03$) after it. The electrophoretic mobility of purified magnetoliposomes was—$0.42 \times 10^{-8}$ $m^2\ V^{-1}\ s^{-1}$. Fig. 2(b and c) shows two micrographs at two magnifications of magnetoliposomes. Principally closed liposomes with different size were seen, although diameters near below 200 nm predominate. Ferrofluid particles were found in a higher extent inside of the liposomes although their distribution was rather irregular. Entrapped particles did not present any specific interaction with lipid bilayer.

### 3.4. Purification and encapsulation efficiency determination

After SEC purification, liposomes were recovered completely free of contaminating external ferrofluid in eluates corresponding to the void volume of the column. However, most liposomes contained ferrofluid, since only a few ferrofluid-free liposomes were seen in the TEM pictures. The encapsulation efficiency was determined in liposomes with varying initial weight ratios of magnetite to phospholipids, ranging from 1.25 g $Fe_3O_4$/mol PC to 125 g $Fe_3O_4$/mol PC. Magnetite and phospholipid concentrations were determined before and after the purification of liposomes by SEC. Table 1 shows the results obtained for encapsulation efficiency. Before purification, the amount of magnetite per mole of lipid increased when increasing the initial amount of magnetite. This finding confirmed that ferrofluid was stable and that it was not retained by the extrusion membrane. However, after purification the concentration of magnetite was lower, especially at initial magnetite concentrations higher than 12.50 g $Fe_3O_4$/mol PC. This could be due to the interaction between ferrofluid and phospholipids. Having in mind the presence of positive charge of ferrofluid, it is possible an electrostatic interaction with the phospholipids phosphate group. This fact would justify the high encapsulation levels at low initial magnetite/phospholipids ratios. At higher ratios, the ferrofluid is also entrapped into the aqueous space. The concentration of encapsulated magnetite increased gradually as a function of initial concentration. At the highest initial concentration, an encapsulation of 22.19 g (or 82.06 mmoles) $Fe_3O_4$ per mole of phospholipid was achieved. This figure is equivalent to 0.10 moles of magnetite per mole of phospholipids. In contrast, the percentage of encapsulation, which was very high at the lowest initial concentrations (from 1.25 g $Fe_3O_4$/mol PC to 12.5 g $Fe_3O_4$/mol PC), decreased and finally remained almost constant.

Concerning the vesicle stability, the size and size distribution of magnetoliposomes stored at room temperature and in perikinetic conditions did not undergo significant changes for almost three weeks. After this time, a dark precipitate appeared, although magnetoliposomes did not coagulate, since after shaking by inversion and left to rest for 15 min, values of size

Table 1
Encapsulation efficiency of ferrofluid-loaded liposomes (FEF)

| Initial concentration used for liposome preparation (g $Fe_3O_4$/mol PC) | Concentration in liposomes before purification (g $Fe_3O_4$/mol PC) | Concentration in liposomes after purification (g $Fe_3O_4$/mol PC) | Percentage of encapsulation (%) |
|---|---|---|---|
| 1.25 | 1.22 ± 0.06 | 1.18 ± 0.10 | 96.6 |
| 5.00 | 4.96 ± 0.07 | 4.90 ± 0.07 | 98.8 |
| 12.50 | 11.26 ± 1.21 | 10.21 ± 1.17 | 90.7 |
| 25.00 | 17.94 ± 3.44 | 11.49 ± 3.08 | 64.1 |
| 37.50 | 31.52 ± 2.33 | 11.61 ± 3.65 | 31.7 |
| 50.00 | 43.17 ± 3.21 | 12.28 ± 3.87 | 28.3 |
| 62.50 | 55.24 ± 4.09 | 13.72 ± 3.63 | 24.8 |
| 75.00 | 72.89 ± 2.84 | 13.77 ± 4.24 | 18.9 |
| 100.00 | 98.15 ± 0.93 | 18.13 ± 5.41 | 18.5 |
| 125.00 | 119.95 ± 2.75 | 22.19 ± 4.50 | 18.5 |

Values (±S.D.) are the mean of three determinations. Percentage of encapsulation was calculated by (concentration after purification/concentration before purification) ×100.

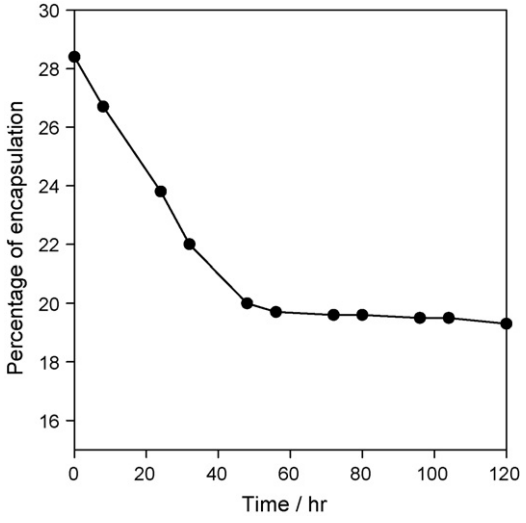


Fig. 3. Time-course of changes in the encapsulation efficiency of 3 weeks-old magnetoliposomes stored at room temperature (≅25 °C). During the forward 5 days, at the indicated times, an aliquot of the dispersion was withdrawn and purified by size exclusion chromatography, and the contents in iron and in phospholipids were determined. The initial concentration of magnetoliposomes was 62.50 g $Fe_3O_4$/mol phospholipid.

and distribution size returned to the original values at least for two weeks more. However, after the first three weeks and during five days, the encapsulation efficiency showed a significant reduction in comparison with the obtained previous values (Fig. 3).

## 4. Discussion

Magnetic particles capable of forming superparamagnetic dispersions in a carrier fluid are pure metals and metal oxides, mainly formed by iron. Pure metals include Ni, Co, and Fe; they present the highest magnetic susceptibility, but are highly toxic and extremely sensitive to oxidation. Therefore, iron oxides, despite their lower magnetization, offer great potential as oxidatively stable magnetic particles. Magnetite ($Fe_3O_4$), maghemite ($\gamma$-$Fe_2O_3$), and hematite ($\alpha$-$Fe_2O_3$) are the iron oxides, which present ferromagnetic properties, the maximal saturation magnetization being found in magnetite. These materials have been used with grain sizes down to the nanoscale for longer than any other type of material. This is because of a fundamental change in the magnetic structure of ferrimagnetic materials when grain sizes are reduced. Below a critical particle size (<15 nm for the common materials), there is a single magnetic domain, i.e. a particle that is in a state of uniform magnetization in any field. Magnetic nanoparticles lie between soft materials and normal permanent magnet materials (Berry and Curtis, 2003). As with any kind of nanoparticle, liposomes that possess magnetic properties offer exciting new opportunities in biomedicine and biological research, and have applications both *in vivo*—drug delivery, hyperthermia, magnetic resonance imaging (MRI) contrast enhancement, etc. (Pankhurst et al., 2003)—and *in vitro*, providing new alternatives to conventional methods of separation and selection. The so-called magnetic solid-phase extraction uses magnetic and magnetizable adsorbents to separate and preconcentrate a substance (Tartaj et al., 2003).

We obtained magnetite by means of a co-precipitation of Fe(II) and Fe(III) in the presence of ammonium, with a size of 12.5 nm. Magnetite is comprised of Fe(II) and Fe(III) ions in a 1:2 molar ratio, where half of the Fe(III) ions are tetrahedrally coordinated and the other half are octahedrally coordinated and all of the Fe(II) are octahedrally coordinated (Berger et al., 1999). In order for magnetite to remain in suspension, its diameter must be in the order of 10 nm. In this case, at room temperature, the thermal energy associated with these particles is the same order of magnitude as the gravitational and magnetic attraction, $\sim 4 \times 10^{-21}$ J, and therefore the particles remain suspended (Berger et al., 1999). To obtain a stable dispersion in water as a carrier medium, magnetite was coated with a surfactant. This afforded a hydrophilic interface formed by a layer of hydroxide anions and tetramethylammonium cations creating a system of magnetic particles stabilised electrostatically. The coating brought about an increase in size up to 60 nm. If we compare this value with the average size of a magnetite particle (12.5 nm), it is evident that a ferrofluid particle contains more than one particle of magnetite. The small size and large surface area of magnetite particles provokes the particle aggregation in order to reduce their surface energy. The clustering of magnetic nanoparticles has been also observed in magnetite

stabilized with polymers (Ditsch et al., 2005). A common situation is when attractive Van der Waals interactions initially overcome electrostatic repulsion forces to allow cluster formation but where the electrostatic energy barrier to coagulation increases as clusters grow so that beyond a finite cluster size no further growth occurs. Shen et al. (2001) have reported that approximately 50 particles participate in these clusters, and these aggregates do not grow further once this size. Upon dilution with water, these clusters grow to form chainlike structures as the dispersion is destabilized because of the desorptive loss of the surfactant. In Fig. 2a we can see some of these clusters. However, the clusters observed in TEM measurements may simply be artefacts of the preparation method since the particles must be deposited on a grid and may be aggregated differently than in solution. For this reason, dynamic light scattering was used to ascertain the sizes of the clusters in suspensions. Some of the clusters of the micrograph agree well with dynamic light scattering measurements. On the other hand, the ferrofluid is colloidally stable since no sedimentation was observed when it was centrifuged at 12,000 rpm for 5 min at room temperature.

To avoid the charge effects due to the surfactant, we then encapsulated the ferrofluid in neutral liposomes, using the method of extrusion. This method has been used by Martina et al. (2005). However, they encapsulated maghemite stabilized by citrate. We encapsulated the ferrofluid in the same way as any aqueous phase is encapsulated. According to images obtained by TEM (Fig. 2b and c) it can be inferred that ferrofluid is mainly located in the aqueous compartment of liposomes. Our liposomes present a size of around 200 nm, corresponding to the diameter of the pore of the membranes used. The value of 150 nm obtained prior to the purification is due to the existence of two different kinds of scatters, ferrofluid and magnetoliposomes, and the resulting value is the consequence of averaging the corresponding diameters. The obtained magnetoliposomes have relatively high magnetite content; they encapsulate, from 1.18 g to 22.19 g of magnetite per mole of phospholipid. These values agree with those obtained by other groups (Kiwada et al., 1986; Martina et al., 2005).

As can be seen in Table 1, the percentage of encapsulation varied as a function of the initial amount of magnetite used in the preparation of liposomes, and in those liposomes, which encaged the maximal amount of magnetite, the percentage of encapsulation was around 19%.

## 5. Conclusions

Magnetoliposomes were prepared from 12.5-nm iron oxide particles. Weakly interacting particles were prepared by covering the oxide particles with tetramethylammonium hydroxide, resulting a ferrofluid that could be easily dispersed in an aqueous solvent. The ferrofluid was the aqueous phase used to hydrate the phospholipid films. Once hydrated, the lipid dispersion was extruded, obtaining the magnetoliposomes. The described procedure is very simple and it permits to prepare 200-nm unilamellar magnetoliposomes encapsulating different amounts of magnetite as a function of the iron concentration present in the hydration solvent.

## Acknowledgements

The authors are grateful for the financial support of the "Ministerio de Educación y Ciencia, Plan Nacional de Investigación, Desarrollo e Innovación Tecnológica (I + D + i)" to the projects MAT2006-12918-C05-01 and -05.

## References

Allen, M.T., 1996. Liposomal drug delivery. Curr. Opin. Colloid Interface Sci. 1, 645–651.
Bangham, A.D., 1993. Liposomes: The Babraham connection. Chem. Phys. Lipids 64, 275–285.
Barenholz, Y., 2001. Liposome application: problems and prospects. Curr. Op. Coll Interface Sci. 6, 66–77.
Berger, P., Adelman, N.B., Beckman, K.J., Campbell, D.J., Ellis, A.B., Lisensky, G.C., 1999. Preparation and properties of an aqueous ferrofluid. J. Chem. Ed. 76, 943–948.
Berry, C.C., Curtis, A.S.G., 2003. Functionalisation of magnetic nanoparticles for applications in biomedicine. J. Phys. D. 36, R198–R206.
Bulte, J.W.M., De Cuyper, M., 2003. Magnetoliposomes as contrast agents. Methods Enzymol. 373, 175–198.
De Cuyper, M., Joniau, M., 1988. Magnetoliposomes. Formation and structural characterization. Eur. Biophys. J. 15, 311–319.
De Cuyper, M., Joniau, M., 1991. Mechanistic aspects of the adsorption of phospholipids onto lauric acid stabilized $Fe_3O_4$ nanocolloids. Langmuir 7, 647–652.
De Cuyper, M., Noppe, W., 1996. Extractability of the phospholipid envelope of magnetoliposomes by organic solvents. J. Colloid Interf. Sci. 182, 478–482.
De Cuyper, M., Müller, P., Lueken, H., Hodenius, M., 2003. Synthesis of magnetic $Fe_3O_4$ particles covered with a modifiable phospholipids coat. J. Phys.: Condens. Matter 15, S1425–S1436.
Ditsch, A., Laibinis, P.E., Wang, D.I.C., Hatton, T.A., 2005. Controlled clustering and enhanced stability of polymer-coated magnetic nanoparticles. Langmuir 21, 6006–6018.
Hodenius, M., De Cuyper, M., Desender, L., Müller-Schulte, D., Steigel, A., Lueken, H., 2002. Biotinylated Stealth magnetoliposomes. Chem. Phys. Lipids 120, 75–85.
Kiwada, H., Sato, J., Yamada, S., Kato, Y., 1986. Feasibility of magnetic liposomes as a targeting device for drugs. Chem. Pharm. Bull. 34, 4253–4258.
Lacava, Z.G.M., García, V.A.P., Lacava, L.M., Azevedo, R.B., Silva, O., Pelegrini, F., De Cuyper, M., Morais, P.C., 2004. Biodistribution and biocompatibility investigation in magnetoliposomes treated mice. Spectroscopy 18, 597–603.
Lasic, D., 1993. Liposomes: From Physics to Applications. Elsevier, Amsterdam, p. 177.
MacDonald, R.C., MacDonald, R.I., Menco, B.P., Takeshita, K., Subbarao, N.K., Hu, L.R., 1991. Small-volume extrusion apparatus for preparation of large, unilamellar vesicles. Biochim. Biophys. Acta 1061, 297–303.
Martina, M.S., Fortin, J.P., Ménager, C., Clément, O., Barratt, G., Grabielle-Madelmont, C., Gazeau, F., Cabuil, V., Lesieur, S., 2005. Generation of superparamagnetic liposomes revealed as highly efficient MRI contrast agents in vivo imaging. J. Am. Chem. Soc. 127, 10676–10685.
Müller-Schulte, D., Füssl, F., Lueken, H., De Cuyper, M., 1997. In: Häfeli, U., Schütt, W., Teller, J., Zborowki, M. (Eds.), Scientific and Clinical Applications of Magnetic Carriers. Plenum, Plenum, New York, pp. 517–526.
Nuffield, E.W., 1966. X-ray Diffraction Methods. Wiley, New York.
Pankhurst, Q.A., Connolly, J., Jones, S.K., Dobson, J., 2003. Applications of magnetic nanoparticles in biomedicine. J. Phys. D. 36, R167–R181.
Steward-Marshall, J.C., 1980. Colorimetric determination of phospholipids with ammonium ferrothiocyanate. Anal. Biochem. 104, 10–14.

Shen, L., Stachowiak, A., Fateen, S.-E.K., Laibinis, P.E., Hatton, T.A., 2001. Structure of alkanoic acid stabilized magnetic fluids. A small-angle neutron and light scattering analysis. Langmuir 17, 288–299.

Tartaj, P., Morales, M.P., Veintemillas-Verdaguer, S., González-Carreño, T., Serna, C.J., 2003. The preparation of magnetic nanoparticles for applications in biomedicine. J. Phys. D. 36, R182–R197.

Viroonchatapan, E., Ueno, M., Sato, H., Adachi, I., Nagae, H., Tazawa, K., Horikoshi, I., 1995. Preparation and characterization of dextran magnetite-incorporated thermosensitive liposomes: an on-line flow system for quantifying magnetic responsiveness. Pharm. Res. 12, 1176–1183.

Widder, K., Flouret, G., Senyei, A., 1979. Magnetic microspheres: synthesis of a novel parenteral drug carrier. J. Pharm. Sci. 68, 79–82.