Journal of Colloid and Interface Science 425 (2014) 118–127

Contents lists available at ScienceDirect

Journal of Colloid and Interface Science

www.elsevier.com/locate/jcis

Characterization and cytotoxicity studies on liposome–hydrophobic magnetite hybrid colloids

Alice Floris [a], Chiara Sinico [a,*], Anna Maria Fadda [a], Francesco Lai [a], Francesca Marongiu [a], Alessandra Scano [b], Martina Pilloni [b], Fabrizio Angius [c], Carlos Vázquez-Vázquez [d], Guido Ennas [b,*]

[a] Dipartimento di Scienze della Vita e dell'Ambiente, Sezione Scienze del Farmaco, Università di Cagliari, Via Ospedale 72, 09124 Cagliari (CA), Italy
[b] Dipartimento di Scienze Chimiche e Geologiche, Cagliari Unità di Ricerca del Consorzio Nazionale di Scienze e Tecnologie dei Materiali (INSTM), Cittadella Universitaria di Monserrato, SS 554 bivio Sestu , 09042 Monserrato (CA), Italy
[c] Dipartimento di Scienze Biomediche, Sezione Patologia, Università di Cagliari, Via Porcell 4, 09124 Cagliari (CA), Italy
[d] Departamento de Química Física, Facultad de Química, Universidad de Santiago de Compostela, Santiago de Compostela, 15782 Galicia, Spain

ARTICLE INFO

Article history:
Received 18 December 2013
Accepted 15 March 2014
Available online 25 March 2014

Keywords:
Magnetoliposomes
Nanoparticles synthesis
SPION
Magnetite
Stealth liposomes

ABSTRACT

The aim of this study was to highlight the main features of magnetoliposomes prepared by TLE, using hydrophobic magnetite, and stabilized with oleic acid, instead of using the usual hydrophilic magnetite surrounded by sodium citrate. These biocompatible magnetoliposomes (MLs) were prepared with the purpose of producing a magnetic carrier capable of loading either hydrophilic or lipophilic drugs. The effect of different liposome/magnetite weight ratios on the stability of magnetoliposomes was evaluated by monitoring the mean diameter of the particles, their polydispersity index, and zeta potential over time. The prepared magnetoliposomes showed a high liposome–magnetite association, with magnetoliposomes containing PEG (polyethylene glycol) showing the best magnetite loading values. To verify the position of magnetite nanoparticles in the vesicular structures, the morphological characteristics of the structures were studied using transmission electron microscopy (TEM). TEM studies showed a strong affinity between hydrophobic magnetite nanoparticles, the surrounding oleic acid molecules, and phospholipids. Furthermore, the concentration above which one would expect to find a cytotoxic effect on cells as well as morphological cell–nanoparticle interactions was studied in situ by using the trypan blue dye exclusion assay, and the Prussian Blue modified staining method.

1. Introduction

The high biocompatibility and versatile nature of liposomes have made these particles unique nanometric systems, with a wide spectrum of biomedical applications. In particular, due to their size and surface tailoring, they have proved to be efficient drug-delivery systems. Their amphiphilic properties permit different types of drugs, both hydrophilic (contained within their internal aqueous compartments) or hydrophobic (contained inside their lipid bilayer shell) molecules to be incorporating within their structures. Liposomes that have been sterically stabilized by grafting hydrophilic polyethylene glycol (PEG) chains onto their lipid bilayer, also called Stealth® liposomes, allow for a prolonged circulation time and a reduced uptake by the mononuclear phagocyte system (MPS) [1–4]. To limit systemic toxic effects related to the non-specific distribution of a drug within the body, and to deliver drugs to a precise lesion or region, different types of targeting mechanisms have been developed. Such mechanisms include active targeting, such as coupling to the PEG chain coated surface special ligands in order to increase liposome–cell association [5], and physical targeting, such as inclusion of magnetic nanoparticles in order to permit guidance by an external magnetic field [6–8].

Physical targeting can be exploited by including magnetite or other magnetizable materials (e.g. maghemite or gadolinium ions) in the vesicular structure of the liposome. Different magnetic behaviors can be observed depending on the size of the magnetic grains. At sizes <30 nm, Fe_3O_4 nanoparticles are superparamagnetic (SPM), and do not retain magnetism after the removal of a magnetic field [9]. To prevent particle aggregation, the nanoparticles can be surrounded by a coating consisting of either a polymer

* Corresponding authors. Fax: +39 070 6754388.
E-mail addresses: alicefl@tiscali.it (A. Floris), sinico@unica.it (C. Sinico), mfadda@unica.it (A.M. Fadda), frlai@unica.it (F. Lai), fmarongiu@unica.it (F. Marongiu), alescano80@tiscali.it (A. Scano), martina.pilloni@gmail.com (M. Pilloni), fangius@unica.it (F. Angius), carlos.vazquez.vazquez@usc.es (C. Vázquez-Vázquez), ennas@unica.it (G. Ennas).

http://dx.doi.org/10.1016/j.jcis.2014.03.046
0021-9797/

(synthetic or natural e.g., dextran [10–12] or proteins [13]) or amphiphilic molecules (e.g. fatty acids [14] or phospholipids [15]).

The advantageous characteristics of both magnetic nanoparticles and liposomes are combined in the so-called magnetoliposomes (MLs).There are two main types of magnetoliposomes. Classical MLs, as prepared by De Cuyper and Joniau [15], consist of an iron oxide core immediately surrounded by a phospholipid bilayer. The absence of an aqueous core has a disadvantage in that a hydrophilic drug cannot be loaded into the liposomal structure. Kiwada et al. [16] prepared a second type of magnetoliposomes by using the thin layer evaporation (TLE) technique. TLE and reverse phase evaporation (RPE), usually followed by an extrusion process, are used to form extruded magnetoliposomes [17], and are the methods most often used for preparation of this second type of magnetoliposome. Magnetite nanoparticles are usually hydrophilic, stabilized by sodium citrate [18,19], and dispersed in water [20–22]. Magnetoliposomes prepared with sodium citrate-magnetite generally have the magnetic nanoparticles located in the aqueous core, as demonstrated by Martina et al. [18].

Although there are several publications concerning magnetoliposomes [12,16–18], some aspects of their structural and chemical/physical properties are still not understood and only in the very recent years, publications concerning magnetoliposomes prepared with magnetite stabilized by oleic acid, have been reported in the literature [23]. Moreover, a few of them studied the oleic acid interaction with the phospholipid bilayer, or how the oleic acid/magnetite ratio affects the positioning of magnetite and the percentage of liposome–magnetite association. The aim of this study was to highlight the main features of magnetoliposomes prepared by TLE, using hydrophobic magnetite, and stabilized with oleic acid, instead of using the usual hydrophilic magnetite surrounded by sodium citrate. In a previous paper [24] we reported the results of a comparative study of magnetoliposomes prepared with either hydrophobic (surrounded by a high percentage of oleic acid) or hydrophilic magnetite nanoparticles, where the influence of the different nature of the nanoparticles on the assembly features was evident. The present study focuses on both characterizing the liposome–magnetite assembly structure by using TEM visualization and DLS (Dynamic Light Scattering) measurements, and the influence of the magnetite concentration on the liposome–magnetite association percentage. Finally, cytotoxicity and cellular uptake studies were conducted to verify the biocompatibility and association with cells of both the hydrophobic magnetite nanoparticles, and the respective magnetic hybrid colloid (magnetoliposome).

2. Materials and methods

2.1. Materials

A sample of magnetite nanoparticles, surrounded by oleic acid and dispersed in chloroform, containing around 10 wt% of magnetite (brand name: NGAP FeO-03#01; hereafter OA–Fe_3O_4) was kindly provided from NANOGAP SUB-NM-PARTICLES (Santiago de Compostela, Spain). Soya phosphatidylcholine (PhospholiponP90 G) was purchased from Phospholipid GmbH (Nattermannallee, Köln Germany). Chloroform solutions of egg-yolk L-R-phosphatidylcholine (EPC, molar mass 760.08) and 1.2-diacyl-SN-glycero-3-phosphoethanolamine-N-[methoxy(poly(ethyleneglycol))-2000] (DSPEPEG2000, molar mass 2805.54) were purchased from Avanti Polar Lipids (Alabaster, AL). Phosphate buffer at pH 7 with an ionic strength of 163 mM, was purchased from Carlo Erba Reagenti. Nuclear Fast Red, RPMI 1640 medium, potassium ferric ferrocyanide-Prussian Blue, aluminum sulfate, potassium hexacyanoferrate, hydroxylamine hydrochloride, 1,10-phenanthroline monohydrate, sodium hydroxide, hydrochloric acid, citric acid, sodium citrate tribasic dehydrate, trehalose, and ethanol were purchased from Sigma–Aldrich (Germany). Fetal calf serum (FCS), streptomycin, penicillin, and L-glutamine, were purchased from Life Technologies, Monza, Italy. Deionized water was used for aqueous solutions and washing.

Magnetite characterization. Average particles size and size distributions of the as received magnetite were determined by TEM and DLS analysis.

The amount of oleic acid in the capped magnetic iron oxide nanoparticles was determined by thermogravimetric analysis (TGA) with a Perkin–Elmer TGA7 in N_2 atmosphere. Sample was previously dried at room temperature for several hours and then heated from 25 to 600 °C at a heating rate of 10 °C/min, including two isotherms steps: at 120 °C for 60 min and at 600 °C for 180 min.

X-ray diffraction patterns were collected with a Seifert diffractometer operating at 40 kV and 30 mA using Cu Kα radiation (λ = 0.154186 nm) and equipped with a graphite monochromator on the diffracted beam. Several drops of OA–Fe_3O_4 suspension were deposited and evaporated on silicon single crystal "zero background" specimen holder. Rietveld refinement was carried out using the Maud program [25]. Usual recommended fitting procedures were adopted [26]. The instrumental profile broadening was derived from the fitting of XRD data obtained from standard samples.

The infrared spectrum of a dried sample (diluted in KBr) was recorded between 4000 and 400 cm^{-1} in a Bruker IFS-66V FTIR.

Magnetization of the samples was recorded in a Quantum Design PPMS Model 6000 magnetometer.

Magnetoliposome preparation. Magnetoliposomes (MLP) were prepared from pure soya phosphatidylcholine or a mixture of EPC:DSPE-PEG2000 (95:5 mol%), according to the thin film hydration method (TLE). An appropriate amount of magnetite chloroform dispersion, at magnetite concentration of 2.72 mg/ml, was added to the chloroform used to dissolve the phospholipids. Then, the organic phase was evaporated at 40 °C using a rotary evaporator (Rotavapor R110, Büchi) under reduced pressure. The obtained thin lipid film was placed under vacuum for 24 h, and hydrated with an appropriate amount of phosphate buffer to give a final lipid concentration of 20 mmol L^{-1}. All MLP formulations were sonicated by 10 cycles of 30 s (and subsequently referred to MLP*) in a MSE Sanyo Soniprep 150 sonicator (England). In order to evaluate the influence of the magnetite concentration on dimension, morphology and stability of the resulting magnetic hybrid colloids, formulations with an increasing magnetite concentration, from 0.17 to 4.32 mM, were studied. Denomination of each formulation according to preparation parameters is reported in Table 1. Some

Table 1
Denomination of each formulation according to preparation parameters as used lipids and magnetite concentration. Sonicated formulation is indicated as *.

Formulation	Lipid	Magnetite concentration (mM)
LP	P90G	0
LP*	P90G	0
LPA	PC:DSPE-PEG 95:5 mol%	0
LP*A	PC:DSPE-PEG 95:5 mol%	0
MLP_1	P90G	0.17
MLP*_1	P90G	0.17
MLP_2	P90G	0.43
MLP*_2	P90G	0.43
MLP_3	P90G	0.86
MLP*_3	P90G	0.86
MLP_4	P90G	1.51
MLP*_4	P90G	1.51
MLP_5	P90G	2.80
MLP*_5	P90G	2.80
MLP_6	P90G	4.32
MLP*_6	P90G	4.32
MLP_6A	PC:DSPE-PEG 95:5 mol%	4.32
MLP*_6A	PC:DSPE-PEG 95:5 mol%	4.32