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The *in vitro* kinetics of the interactions between PEG-ylated magnetic-fluid-loaded liposomes and macrophages

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Abstract

Binding and uptake kinetics of magnetic-fluid-loaded liposomes (MFL) by endocytotic cells were investigated *in vitro* on the model cell-line J774. MFL consisted of unilamellar phosphatidylcholine vesicles (mean hydrodynamic diameter close to 200 nm) encapsulating 8-nm nanocrystals of maghemite (γ-Fe_2O_3) and sterically stabilized by introducing 5 mol% of distearylphosphatidylcholine poly(ethylene glycol)$_{2000}$ (DSPE-PEG$_{2000}$) in the vesicle bilayer. The association processes with living macrophages were followed at two levels. On one hand, the lipid vesicles were imaged by confocal fluorescence microscopy. For this purpose 1 mol% of rhodamine-marked phosphatidylethanolamine was added to the liposome composition. On the other hand, the iron oxide particles associated with cells were independently quantified by magnetophoresis. All the experiments were similarly performed with PEG-ylated or conventional MFL to point out the role of polymer coating. The results showed cell association with both types of liposomes resulting from binding followed by endocytosis. Steric stabilization by PEG chains reduced binding efficiency limiting the amount of MFL internalized by the macrophages. In contrast, PEG coating did not change the kinetics of endocytosis which exhibited the same first-order rate constant for both conventional and PEG-ylated liposomes. Moreover, lipids and iron oxide particle uptakes were perfectly correlated, indicating that MFL vesicle structure and encapsulation rate were preserved upon cell penetration.
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1. Introduction

Advances in nanotechnology have stimulated the development of innovative systems for the delivery of drugs or diagnosis agents. Among the different systems available, liposomes continue to arouse great interest, mainly due to their biocompatibility and vesicular structure which can include a wide range of substances, either hydrophilic within the internal aqueous compartments or hydrophobic inside the lipid bilayer shell. Over the last 10 years, hybrid systems formed by encapsulating iron oxide nanoparticles within liposomes, known as magnetoliposomes, have been investigated as new tools for magnetic cell sorting [1], magnetic drug targeting [2] or delivery [3,4], hyperthermia [5–7] as well as contrast agents for magnetic resonance imaging (MRI) [8,9]. In this context, we recently developed nanosized superparamagnetic liposomes referred to as magnetic-fluid-loaded liposomes (MFL), composed mainly of a natural phospholipid, egg phosphatidylcholine (EPC), and loaded with calibrated nanocrystals of maghemite (γ-Fe_2O_3). These magnetoliposomes first served as a model membrane to study the pore formation induced by solubilizing surfactants [10]. A second potential application is *in vivo* imaging [11,12].

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An important limitation to the use of conventional liposomes in biological application is their rapid removal from blood by the mononuclear phagocytic system (MPS) [13–16]; consequently, the widely applied strategy of grafting poly(ethylene glycol) (PEG) chains onto the surface to create a steric barrier hindering interactions with opsonins or cells and, thus, increasing their circulation half-life [17–21], has been applied to MFL. Our previous studies have demonstrated the physical stability of such MFL in biological media, rendering them appropriate for intravenous administration. They have proved to be effective MRI contrast agents that can be magnetically guided *in vivo* towards solid tumors implanted in mice, and their long-circulating behaviour *in vivo* has also been clearly demonstrated [11,12,22]. However, after intravenous administration, although a part of the injected MFL remain in the blood for more than 24 h, a proportion is taken up by the cells of the reticuloendothelial system and recovered in the liver and the spleen of the animals [22]. This could be a result of the liposome diameter close to 200 nm, which is in the upper range of size allowing long-circulating properties [23,24]. Nevertheless, it should be considered that MFL contain nanoparticles of high density with a loading varying from 1.4 to 2.5 mol of iron per mole of lipids [12]. It is possible that such a peculiar hybrid structure may influence the mechanism of uptake by the macrophages. To date, this question has not been studied. Furthermore, MFL were first proposed as MRI contrast agents for *in vivo* diagnosis and in this application high resolution of the images closely depends on their ability to associate with the cells of the target tissue while preserving their initial vesicle structure and maghemite loading. This is a second reason for investigating the mechanism of MFL uptake by living cells.

As a preliminary approach to these two concerns, we decided to study the interactions of MFL with macrophages *in vitro* by using the murine cell line J774. Macrophages are the cells responsible for removing liposomes from the circulation *in vivo* [13,14,25]. This particular cell line has already been validated as an *in vitro* model to predict the kinetics of blood clearance of colloid drug delivery systems [13,16,26]. Experiments were performed on unfixed living cells in the presence of 10% of foetal calf serum (FCS), in order to approach physiological conditions and avoid artefacts due to fixing procedures [27]. The lipid bilayers were labelled with 1 mol% of the fluorescent phospholipid *N*-[lissamine rhodamine B sulfonyl] phosphatidyl ethanolamine. MFL association with macrophages was followed and analyzed by confocal fluorescence microscopy, and in addition the internalized iron oxide particles were quantified by cell magnetophoresis according to a procedure described elsewhere [28]. The kinetics and mechanism of association with cells were investigated, as a function of both lipid concentration and temperature in order to describe the intracellular fate of MFL in detail. PEG-ylated and conventional (without PEG coating) MFL were compared to highlight the influence of surface PEG on the uptake process.

2. Materials and methods

2.1. Materials

Chloroform solutions of L-α-phosphatidylcholine (EPC) extracted from egg yolk, 1,2-diacyl-*SN*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (distearylphosphatidylcholine poly(ethylene glycol)$_{2000}$ (DSPE-PEG$_{2000}$)) and *N*-(lissamine rhodamine B sulfonyl) phosphatidylethanolamine (Rho-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). Sodium chloride, sodium citrate, *N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) and cytochalasin B were provided by Sigma (St. Louis, MO). Antibody Rat-anti-mouse CD11b was ordered at Molecular Probes (Oregon, USA). The buffer used for magnetoliposome preparation was 108 mM NaCl, 20 mM sodium citrate, 10 mM HEPES, pH 7.4 and 285 mosmol (measured with a cryoscopic micro-osmometer, Bioblock Scientific, France). Phosphate-buffered saline (PBS, pH 7.4, 290 mosmol) was supplied by Invitrogen-Lifetechnologies (Cergy Pontoise, Paris).

2.2. Synthesis of maghemite nanoparticles

Nanocrystals of maghemite (γ-Fe_2O_3) were synthesized according to a procedure already described [10,12,29]. Final adjustment of both aqueous medium and maghemite concentration was performed by ultrafiltration on MACROSEP filters (cutoff 50 kD; Fisher Scientific Labosi, France) followed by addition of buffer in order to obtain the desired Fe(III) concentration of 5.4 M (checked by flame spectrometry).

2.3. Liposome preparation

Fluorescent rhodamine-labelled magnetic-fluid-loaded liposomes (Rho-MFL) were prepared as previously described [10,12]. The fluorescent phospholipid was added to the mixture used to form a film. Briefly, thin lipid film hydration was followed by sequential extrusion under nitrogen pressure ($<$10 bars) at 25 °C through polycarbonate filters with decreasing pore diameters of 0.8 μm/0.4 μm/0.2 μm (PORETICS, Osmotics, Livermore, USA). The lipid content of the films, either (EPC:Rho-PE; 99:1 mol%) for conventional Rho-MFL or (EPC:DSPE-PEG$_{2000}$:Rho-PE; 94:5:1 mol%) for PEG-ylated Rho-MFL, was determined by weight (precision, 5×10^{-5} g). Non-entrapped maghemite particles were removed by gel exclusion chromatography (GEC) performed with a 0.4×5.8 cm Sephacryl S1000 superfine (Pharmacia) microcolumn (TERUMO 1 mL-syringe) saturated with lipids before sample loading. The eluent was the buffer used for liposome preparation. Iron loading was determined after GEC purification by flame spectrometry.

2.4. Quasi-elastic light scattering (QELS)

The hydrodynamic diameters of the liposomes were determined with a Nanosizer (N4 MD, Coultronics), at 25 °C, 90° scattering angle and using size distribution processor (SDP) analysis (total lipid concentration: 0.15 mM). Mean hydrodynamic diameters d_h were calculated from the mean translational diffusion coefficient D of the particles according to the Stokes–Einstein law for spherical and non-interacting particles: $d_h = k_BT/3\pi\eta D$ (k_B the Boltzmann constant; η the viscosity of the aqueous medium).

2.5. Cell culture and preparation

The J774 A1 murine macrophage cell line (ECACC catalogue no. 91051511) was maintained as an adherent culture and was grown as a monolayer in a humidified incubator (95% air; 5% CO_2) at 37 °C in 75-cm^2 flasks (Nunc) containing RPMI 1640 Glutamax-I medium (GIBCO)

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