Biomaterials 29 (2008) 4137–4145

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

The effect of magnetic targeting on the uptake of magnetic-fluid-loaded liposomes by human prostatic adenocarcinoma cells

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ARTICLE INFO

Article history:
Received 25 April 2008
Accepted 10 July 2008
Available online 29 July 2008

Keywords:
Magnetoliposome
Iron oxide nanoparticles
Confocal fluorescence microscopy
Magnetic cell labeling
Magnetophoresis
Uptake kinetics

ABSTRACT

Interactions of magnetic-fluid-loaded liposomes (MFL) with human adenocarcinoma prostatic cell line PC3 were investigated *in vitro*. MFL consisted of unilamellar phosphatidylcholine vesicles (mean hydrodynamic diameter close to 180 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 cells, including binding and effective internalization, were followed versus time at two levels. On one hand, the lipid vesicles labeled by 1 mol.% of rhodamine-marked phosphatidylethanolamine were imaged by confocal fluorescence microscopy. On the other hand, the iron oxide particles associated with cells were independently quantified by magnetophoresis. This allowed modeling of MFL uptake kinetics as a two-step process involving first binding adsorption onto the outer cell membrane followed by subsequent internalization. Capture efficiency was significantly improved by guiding MFL in the near vicinity of the cells by means of a 0.29-T external magnet developing a magnetic field gradient close to 30 mT/mm. Double detection of lipids by fluorescence tracking and of iron oxide by magnetophoresis showed excellent correlation. This demonstrated that MFL associate with tumor cells as intact vesicle structures which conserve their internal content.

1. Introduction

Cancer is induced by uncontrolled growth and spreading of abnormal cells. This life-threatening disease still causes more than 6 million deaths every year worldwide [1]. Effective treatments include a combination of surgery, radiotherapy, chemotherapy, even hormone therapy and/or immunotherapy. Chemotherapeutic agents are directed at killing or controlling the cancer cells; but the most effective drugs tend to be the most toxic as well [2]. The dosage of a chemotherapeutic agent is an important determinant for its success. It is necessary to expose cancer cells to a sufficiently high concentration of the drug for a long enough duration while avoiding normal cells. This is the motivation for targeted delivery of anticancer drugs.

In this respect, long-circulating liposomes have shown increased deposition in solid tumors through a hyperpermeable microvasculature [3,4]. Liposomal formulations of doxorubicin (Doxil®) and daunorubicin (Daunoxome®) are already commercialized for Kaposi sarcoma [5,6]. Innovation tends to active targeting of liposomes either by anchoring a specific ligand, namely immunoliposomes, or by using a physical pathway: pH-, temperature-sensitive or magnetic liposomes [1,2,6–9].

Magnetic targeting is a promising non-invasive method to locally concentrate particulate carriers in a tissue of interest [10–12]. It has already been evaluated using nanovectors such as magnetic nanoparticles [13–16] or magnetoliposomes [17–19]. The last systems have shown they could vehicle and deliver drugs and/or diagnosis agents, enclosed inside the vesicle structure or incorporated in the lipid bilayer, next to the magnetic site without any side-effect on the organism and at efficient levels for treatment efficacy.

For several years, our strategy has been to design new magnetoliposomes called magnetic-fluid-loaded liposomes (MFL) which consist of superparamagnetic iron oxide (maghemite, γ-Fe_2O_3) nanoparticles encapsulated within unilamellar phospholipid vesicles close to 200 nm in diameter and sterically stabilized by poly(ethylene glycol) (PEG) coating [20,21]. They have proved to be efficient as contrast agents for magnetic resonance imaging (MRI). On the basis of *in vivo* experiments, PEGylated MFL showed stealth behavior when injected intravenously and could be magnetically guided into human prostatic adenocarcinoma (PC3) tissue. The experiments were performed on tumors subcutaneously implanted in mice. We showed that the vesicle structure and iron loading were preserved upon magnetic driving that occurred via the vasculature

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0142-9612/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2008.07.011

[21–25]. Even if successful accumulation was confirmed in the targeted tissue by histological analysis, the question that remained open was whether cellular uptake effectively took place or not and to what extent the magnetic field gradient could improve it.

For this purpose, the present study focuses on the influence of a magnetic field gradient on PEGylated MFL interactions *in vitro* with PC3. The experiments were performed on living cells using two complementary and independent techniques successfully used in the study of MFL endocytosis by macrophages [26]. The new challenge here is to compare both mechanism and kinetics of liposome binding to and uptake by cancer cells in the absence and in the presence of a magnetic field. On one hand, the uptake of the vesicle structures was followed by confocal fluorescence microscopy. In this respect, a fluorescent lipid probe, N-(lissamine rhodamine B sulfonyl) phosphatidylethanolamine (Rho-PE) was incorporated within the liposome bilayer. On the other hand, the cell-internalization of the magnetic material contained by the liposomes was analyzed by magnetophoresis according to a methodology recently developed for the internalization of magnetic nanoparticles [27,28].

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] (DSPE-PEG2000) and N-(lissamine rhodamine B sulfonyl) phosphatidylethanolamine (Rho-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). Sodium chloride, sodium citrate, HEPES buffer and fluorescein isothiocyanate FITC-PKH 67 were provided by Sigma-Aldrich (St Louis, MO). The buffer used was 108 mM NaCl, 20 mM sodium citrate, 10 mM HEPES, pH 7.4 and 285 mOsm (measured with a cryoscopic micro-osmometer, Bioblock Scientific, France). PBS and all cell culture media were supplied by Gibco®, Invitrogen-Lifetechnologies (Cergy Pontoise, Paris).

2.2. Synthesis of maghemite nanoparticles

Nanocrystals of maghemite (γ-Fe_2O_3) were synthesized according to procedures already described [20,21,29]. The nanoparticles presented a superparamagnetic behavior. The magnetic and hydrodynamic diameters were 7.7 nm (S.D. 0.37) and 18 ± 2 nm, from magnetization curve and quasi-elastic light scattering, respectively. Final aqueous medium and maghemite concentration (5.4 M Fe(III) checked by flame spectrometry) were adjusted by ultrafiltration (MACROSEP filter, cutoff 50 kD, Fisher Scientific Labosi, France) followed by addition of the same buffer as that used for liposome preparation.

2.3. Liposome preparation

Fluorescent magnetic-fluid-loaded liposomes (Rho-MFL) were prepared according to the methodology already developed [20,21]. Briefly, the technique coupled thin film hydration method with 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 final lipid composition (EPC: DSPE-PEG2000: Rho-PE; 94:5:1 mol.%) 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 elution. The eluent was the buffer used for liposome preparation. The final vesicle preparation contained 20 mM total lipids (checked by fluorescence spectroscopy [24]) and 40 mM iron oxide (from flame spectrometry).

2.4. Quasi-elastic light scattering (QELS)

Hydrodynamic diameters were determined with a nanosizer apparatus (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: $d_h = k_B T/3\pi\eta D$ (k_B, Boltzmann constant; η viscosity of the aqueous medium).

2.5. Cell culture and preparation

Human prostatic adenocarcinoma cells (PC3) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with Glutamax, 10% fetal calf serum (FCS) and 100 IU/mL penicillin/streptomycin. They were maintained as an adherent culture and were grown as a monolayer in a humidified incubator (5% CO_2) at 37 °C in 75-cm^2-flasks. When confluence was reached, cells were trypsinized and counted in a hemocytometer.

2.6. Liposome uptake experiments

PC3 cells were plated onto a sterile cover-slip placed in a 3.5 cm-diameter well (COSTAR, France) and left to adhere and recover over 24 h at 37 °C in a humidified atmosphere of 5% CO_2. Each test was carried out when cell density reached 10^6 cells per well (1 mL total volume). A cylindrical magnet (14 mm diameter, 5 mm high, 0.29 T at the magnet surface, 30 mT/mm 2 mm apart from the surface, along the magnet axis) was attached under the cover-slip for the magnetic targeting experiments. Prior to confocal fluorescence microscopy experiments, liposomes were diluted by putting 20 μL of the initial liposome preparation (20 mM total lipids in buffer) in 1 mL DMEM buffer supplemented with 10% FCS to get final concentrations of 0.4 mM phospholipids or equivalently of 0.8 mM iron oxide in total sample volume. An aliquot of 1 mL of the diluted dispersion was added to each test well and cells were incubated for 1 or 4 h at 37 °C while rocking on a plate rocker at approximately 10 cycles/min in order to avoid any liposome sedimentation. After incubation, cells were washed three times with cold PBS to remove non-associated liposomes. Then, the cover-slip was immediately recovered by fresh PBS and placed in a special chamber for confocal microscopy analysis. Living cells were maintained at 37 °C during imaging. Each experiment was repeated three times.

For magnetophoresis experiments, the same incubation times and washing protocols were followed and the same magnet was used for the magnetic targeting experiments. Liposomes were diluted in DMEM buffer supplemented with 10% FCS at different concentrations of iron oxide from 0.4 mM to 160 mM. For the highest concentrations, the initial liposome preparation was concentrated by magnetic sorting to a final iron oxide concentration of 0.4 M, checked by flame spectroscopy, according to a procedure previously described [23]. Before incubation with cells, the concentrated liposomes were diluted in DMEM supplemented with 10% FCS to get the liposomes dispersions at 40 mM, 80 mM and 160 mM equivalent concentrations of iron.

2.7. Confocal fluorescence microscopy

Before incubation with liposomes, the PC3 cells were labeled by the green FITC-PKH67 cell linker dye (Sigma, St Louis, MO) [30] which incorporates into cell membrane with no modification of biological activity (Fig. 1). A mixture of 0.5 μL of the 10^{-3} M PKH67 solution and 100 μL of PBS was added to 10^6 cells per well for 1 min, at room temperature in the dark. The association of liposomes with cells was examined immediately after different periods of incubation by confocal laser scanning microscope CLSM 510 (Zeiss, Germany), coupled to LSM 5 Image Browser (Zeiss, Germany). All images were acquired with an air-cooled ion laser at 488 nm (FITC) and 543 nm (rhodamine), performed using either a Plan Neofluar 10×/0.3 or a Plan Apochromat 63×/1.4NO oil objective lens. Fluorescence was collected by using a 505–550 nm band pass filter for FITC and a long pass filter beginning at

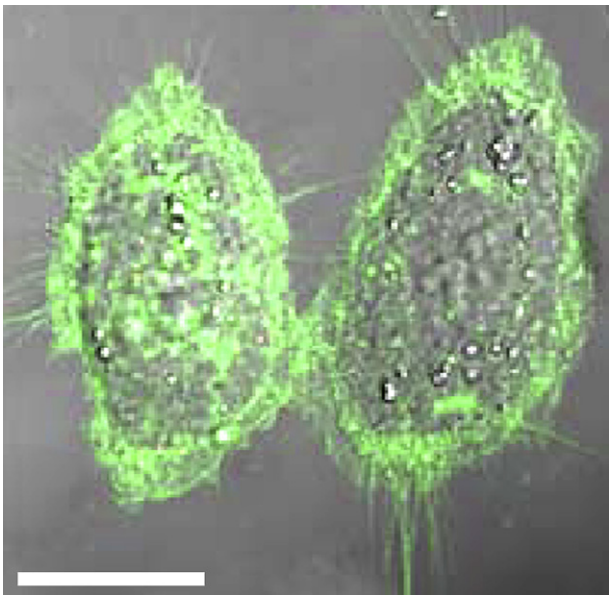

Fig. 1. Confocal fluorescence microscopy of PC3 cells after labeling of the cytoplasmic membrane (FITC-PKH67). Here is represented a single image viewed in the mid-point of the cell thickness ($73 \times 73 \times 0.41$ μm). White bar represents 22 μm.