MAGMA (2005) 18: 186–192
DOI 10.1007/s10334-005-0111-y

RESEARCH ARTICLE

Relaxivity of liposomal paramagnetic MRI contrast agents

G. J. Strijkers
W. J. M. Mulder
R. B. van Heeswijk
P. M. Frederik
P. Bomans
P. C. M. M. Magusin
K. Nicolay

Received: 6 April 2005
Revised: 13 June 2005
Accepted: 27 June 2005
Published online: 9 September 2005

G. J. Strijkers(✉) · W. J. M. Mulder
R. B. van Heeswijk · K. Nicolay
Biomedical NMR,
Department of Biomedical Engineering,
Eindhoven University of Technology,
P.O. Box 513, 5600 MB,
Eindhoven, The Netherlands
E-mail: g.j. strijkers@tue. nl
Tel: +31 40 2473727
Fax: +31 84 7154343

P. M. Frederik · P. Bomans
Department of Pathology,
EM Unit, University of Maastricht,
Maastricht, The Netherlands

P. C. M. M. Magusin
Department of Chemical Engineering
and Chemistry,
Eindhoven University of Technology,
P.O. Box 513, 5600 MB,
Eindhoven, The Netherlands

Abstract Paramagnetic liposomes, spherical particles formed by a lipid bilayer, are able to accommodate a high payload of Gd-containing lipid and therefore can serve as a highly potent magnetic resonance imaging contrast agent. In this paper the relaxation properties of paramagnetic liposomes were studied as a function of composition, temperature and magnetic field strength. The pegylated liposomes with a diameter of approximately 100 nm were designed for favorable pharmacokinetic properties in vivo. The proton relaxivity, i.e. the T_1 relaxation rate per mmol of Gd(III) ions, of liposomes with unsaturated DOPC phospholipids was higher than those with saturated DSPC lipids. Addition of cholesterol was essential to obtain monodisperse liposomes and led to a further, although smaller, increase of the relaxivity. Nuclear magnetic relaxation dispersion measurements showed that the relaxivity was limited by water exchange. These results show that these paramagnetic liposomes are very effective contrast agents, making them excellent candidates for many applications in magnetic resonance imaging.

Keywords Liposome · Contrast agent · Molecular imaging · Nuclear magnetic relaxation dispersion

Introduction

Liposomes and polymeric nanospheres have been studied extensively during the last decade for their potential as a carrier for drugs, such as chemotherapeutic agents [1, 2]. They can be designed to ensure efficient delivery to the diseased area, resulting in a high target-to-background ratio, and protect the drug against metabolism and inactivation in the blood plasma [1–3]. Recently, there has been renewed interest in such nanoparticles for applications in molecular imaging, which aims to visualize molecular processes in vivo using a combination of imaging and active targeting of a contrast agent [4]. To this end the colloidal particle has to be conjugated with a ligand, e.g. a peptide or an antibody, as well as with a contrast agent, such as a radioactive tracer or a magnetic resonance imaging (MRI) label allowing for in vivo tracing of the particle [5]. Furthermore, a combination of this targeting, drug delivery, and imaging offers the unique possibility to follow the delivery and effects of local targeted drug treatment in vivo.

For MRI the contrast agents used are generally based on either iron-oxide nanoparticles, providing negative contrast in $T_2^{(*)}$-weighted images, or Gd complexes, providing positive contrast in T_1-weighted images. There are several Gd-based contrast agents currently on the

Table 1 Formulations of the liposomes, differing in the fatty-acid chain composition (saturated DSPC versus unsaturated DOPC), the presence of cholesterol and the addition of Gd-containing lipid (Gd-DTPA-BSA or Gd-DTPA-BOA)

Formulation	PEG_{2000}-DSPE	Gd-containing lipid	Phospholipid	Cholesterol
A	0.15	0.75 Gd-DTPA-BSA	1.10 DSPC	1
B	0.15	0.75 Gd-DTPA-BSA	1.10 DSPC	–
C	0.15	–	1.85 DSPC	1
D	0.15	0.75 Gd-DTPA-BOA	1.10 DOPC	1
E	0.15	0.75 Gd-DTPA-BOA	1.10 DOPC	–
F	0.15	–	1.85 DOPC	1

Lipids and cholesterol were mixed in a ratio of 2:1, except for formulation B and E, which contain no cholesterol

market, among which the best known are Gd-DTPA (Magnevist, Schering, Germany), Gd-DOTA (Dotarem, Guerbet, France), Gd-DTPA-BMA (Omniscan, Amersham, UK), Gd-HP-DO3A (ProHance, Bracco, Italy), Gd-DTPA-BMEA (Optimark, Mallinkrodt, USA), and Gd-DO3A-Butrol (Gadovist, Schering, Germany). For these contrast agents a tissue concentration of the order of 10^{-7} mol/g is typically needed to obtain sufficient contrast in the resulting MR image. Such a number is too high to be able to image sparse molecular epitopes expressed in the living body, since typical receptors are normally present in low concentrations of approximately 10^{-9}–10^{-13} mol/g [6].

Because of this sensitivity problem an effective amplification strategy is needed for Gd-based contrast agents. Liposomes and also other colloidal particles offer a solution here because a high payload of Gd-containing amphiphilic lipid can be incorporated in the lipid (bi)layer, which increases the effective relaxivity r_1 per particle spectacularly [5, 7–11]. Nevertheless, the addition of large amounts of Gd alone does not guarantee a low detection limit, since negative effects on the sensitivity may result from a disproportionate increase of the transverse relaxivity r_2 with respect to r_1. Moreover, the relaxivity that is obtained is dependent on many parameters, including the water accessibility, and rotation, translation and diffusion correlation times, and therefore strongly depends on the liposome composition, stability, and size. Furthermore, an optimal liposome formulation in terms of the relaxivity does not necessarily concur with a composition desired for optimal biodistribution and pharmacokinetics [1, 2].

In this paper we report on the relaxation properties of Gd-containing liposomal contrast agents as a function of composition, temperature, and magnetic field strength. As a starting point, the size and basic formulation of the liposomes, which includes pegylated phospholipids (PEG_{2000}-DSPE) to prolong the in vivo circulation time [12] as well as cholesterol for stability, were chosen on the basis of criteria and experimental observations described in literature concerning particles with a long circulation time [1, 2, 5]. Additionally, the liposomes have incorporated a high payload of Gd-containing lipid in the bilayer, resulting in a high relaxivity r_1 per liposome. Liposomal formulations were based on either phospholipids with saturated or unsaturated fatty-acid chains, to determine the influence of membrane fluidity on the MRI relaxation properties.

Materials and methods

1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1, 2-dioleoyl-*sn*-Glycero-3-phosphocholine (DOPC), 1,2-distearoyl-*sn*-Glycero-3-phosphoethanolamine-*N*-[methoxy (polyethylene glycol) -2000] (PEG_{2000}-DSPE), and cholesterol (Chol), were obtained from Avanti Polar Lipids (Alabaster, AL). Gd-DTPA-bis(sterylamide) (Gd-DTPA-BSA), and Gd-DTPA-bis(oleylamide) (Gd-DTPA-BOA), were purchased from Gateway Chemical Technology (St. Louis, MO). HEPES was obtained from Merck (Darmstadt, Germany). All other chemicals were of analytic grade or the best grade available. Polycarbonate filters for liposome extrusion were from Costar (Cambridge, MA). Magnevist Gd-DTPA (Gadopentetate Dimeglumine) was obtained from Schering (Germany).

Liposomes were prepared by lipid-film hydration. A mixture of the appropriate amounts of lipids (typically: 120 μmol of total lipid) was dissolved in chloroform/methanol 1:1 (v/v) and evaporated to dryness by rotary evaporation at 313 K. The lipid film was subsequently hydrated in 3 ml of HEPES buffered saline (HBS) (20 mM HEPES, 135 mM NaCl, pH 7.4). The resulting lipid dispersion was extruded sequentially six times through polycarbonate membrane filters with a pore diameter of 200 nm and subsequently ten times through filters with a pore diameter of 100 nm using a Lipofast Extruder (Avestin, Canada). The temperature during extrusion was 333 K for the DSPC and room temperature for the DOPC-based liposomes.

The liposomes were made in six formulations, differing in the fatty-acid chain composition (saturated versus unsaturated), the presence or absence of cholesterol and the Gd-containing lipid (Gd-DTPA-BSA or Gd-DTPA-BOA). Lipids and cholesterol were mixed in a ratio of 2:1. Details about the compositions of the six formulations are given in Table 1. For clarity, in the remainder of this paper the six liposome formulations will be referred to as formulations A to F. Formulations A to C are based on saturated phospholipid DSPC, of which A and B are paramagnetic liposomes containing Gd-DTPA-BSA and C is diamagnetic. Formulation B contains no cholesterol. Formula-

tions D to F are based on unsaturated DOPC, of which D and E are paramagnetic liposomes containing Gd-DTPA-BOA and F is diamagnetic. Formulation E contains no cholesterol.

Gd(III) ion concentrations were determined using two techniques. The first method used was inductively coupled plasma atomic emission spectrometry (ICP-AES, Leeman Labs Echelle Unicam 701 emission spectrometer, TNO, Eindhoven, the Netherlands). The second method was to measure the relaxivity r_1 of a solution of the liposomes after destruction for 3 h in perchloric acid. The Gd concentration was subsequently determined by comparison with a calibration line of r_1 versus Gd concentration of a stock solution of $GdCl_3$ destructed with perchloric acid.

Cryo-transmission electron microscopy (cryo-TEM) was used to assess the morphology of the liposomes. Suspensions of the liposomes were applied to bare grids (700-mesh, hexagonal pattern) within an environmental chamber (relative humidity 100%, 297 K), after which excess liquid was blotted away using filter paper. The grid was subsequently dropped into melting ethane. The vitrified specimens were stored under liquid nitrogen and observed at 103 K (Gatan 626 cryoholder) in a Philips CM12 microscope, operating at 120 kV [13].

The temperature dependence of the relaxivities was measured with a Bruker MSL200 (Bruker, Rheinstetten, Germany) NMR spectrometer at a magnetic field strength of 4.7 T, using standard 10-mm liquid NMR probes. Temperatures were calibrated from the peak separation in the ^{1}H NMR spectrum of ethylene glycol. The proton spin–lattice relaxation time, T_1 was determined using an inversion recovery sequence with variable inversion times between 10 and 3000 ms. T_2 was measured using a standard spin-echo sequence with variable echo time ranging between 100 μs and 300 ms, in an exponential fashion. The longitudinal and transverse relaxivities r_1 and r_2 were determined from linear fits of $1/T_1$ and $1/T_2$ as a function of Gd(III) ion concentration for total lipid concentrations of 0.3, 0.9, and 1.2 mM. The temperature was varied between 298 and 358 K.

^{1}H nuclear magnetic relaxation dispersion (NMRD) measurements were performed on a Stelar Spinmaster FFC-2000 (Stelar, Mede, Italy). T_1 was determined at 25 points exponentially distributed in the frequency range 0.01–35 MHz. Below 15 MHz a prepolarization field of 0.7 T was applied with a duration of at least four times the estimated T_1. The T_1 at each relaxation field was determined by an exponential fit of the signal intensity as a function of 16 relaxation field durations, measured at a readout field of 0.31 T. Measurements were performed at 298, 310, and 333 K, i.e. at room temperature, at physiological temperature, and at a temperature above the phase-transition temperature of the DSPC-based liposomes. The paramagnetic relaxivities r_1 were calculated taking into account diamagnetic contributions, as measured from the liposomes without Gd-containing lipid. The NMRD data were analyzed according to the theory of nuclear spin relaxation in paramagnetic systems by Bertini et al. [14] and Kruk et al. [15].

Results

Cryo-TEM images for liposomal formulations with (A, D) and without cholesterol (B, E) are shown in Fig. 1. The addition of cholesterol leads to spherical bilayer particles, both for the lipids with saturated (A) as well as unsaturated (D) fatty acid chains. Triangular-shaped vesicles are formed for formulation B, containing no cholesterol and lipids with saturated fatty-acid chains. The same triangular-shaped vesicles were observed for formulation C, without Gd-containing lipid (data not shown). Formulation E (Fig. 1) and F (data not shown), containing no cholesterol and lipids with unsaturated fatty-acid chains, result in a mixture of circular, elongated, and cigar-shaped vesicles.

The temperature dependence of the ^{1}H relaxivities r_1 and r_2, measured at 200 MHz, are presented in Fig. 2. The relaxivities for all liposomal formulations are higher than for the traditional Gd-DTPA. Note that the relaxivities are reported in terms of the Gd(III) ion concentrations. Therefore the relaxivity per liposome particle is much higher as a liposome contains approximately 40000 Gd(III) ions, which was calculated using a liposome size of 100 nm and a lipid surface area of 0.6 nm^2 [16]. The liposomes containing cholesterol have a higher r_1 than those without cholesterol. The DSPC-based liposomes (A, B) display a maximum in r_1 around 335 K. A similar temperature dependence is found for r_2. For the DOPC-based liposomes (D, E) the r_1 shows remarkably little temperature dependence at this field strength. In contrast, a large increase in r_2 with increasing temperature is observed. At physiological temperature (310 K) and a resonance frequency of 200 MHz the r_2/r_1 ratios are 6.2, 9.3, 5.4, and 6.3 for formulations A, B, D, and E, respectively.

Relaxivity dispersion curves for aqueous solutions of liposomal formulations A and D, and for Gd-DTPA are displayed in Fig. 3. The ^{1}H NMRD profiles of the liposomes are similar to those reported for Gd complexes bound to a macromolecule, with a peak at around 25 MHz caused by an increased motional correlation time [17]. For both formulations the relaxivity increases with temperature. For cholesterol-containing DOPC liposomes at physiological temperature (310 K), a local relaxivity maximum of 11.3 $mM^{-1}s^{-1}$ occurs at 25 MHz. As a reference the relaxivity dispersion curves of an aqueous solution of Gd-DTPA are presented in the right-hand panel of Fig. 3. The relaxivity of Gd-DTPA decreases with increasing temperature and drops off from an essentially constant value at low field strengths when going to higher frequencies, in agreement with the literature [17].

The solid lines in Fig. 3 are best fits of the NMRD curves of the liposomal solutions according to the theory of nuclear spin relaxation in paramagnetic systems [14, 15]. For formulation A the fit of the NMRD data at a temperature of 298 K did not converge and is therefore not presented. Fitting parameters included: Δ_t = the magnitude of the transient zero-field-splitting (ZFS), τ_v = the correlation time for electronic relaxation, τ_r = the rotational correlation time, τ_m = the water exchange correlation time, and D_{ZFS} = the static ZFS. The following parameters

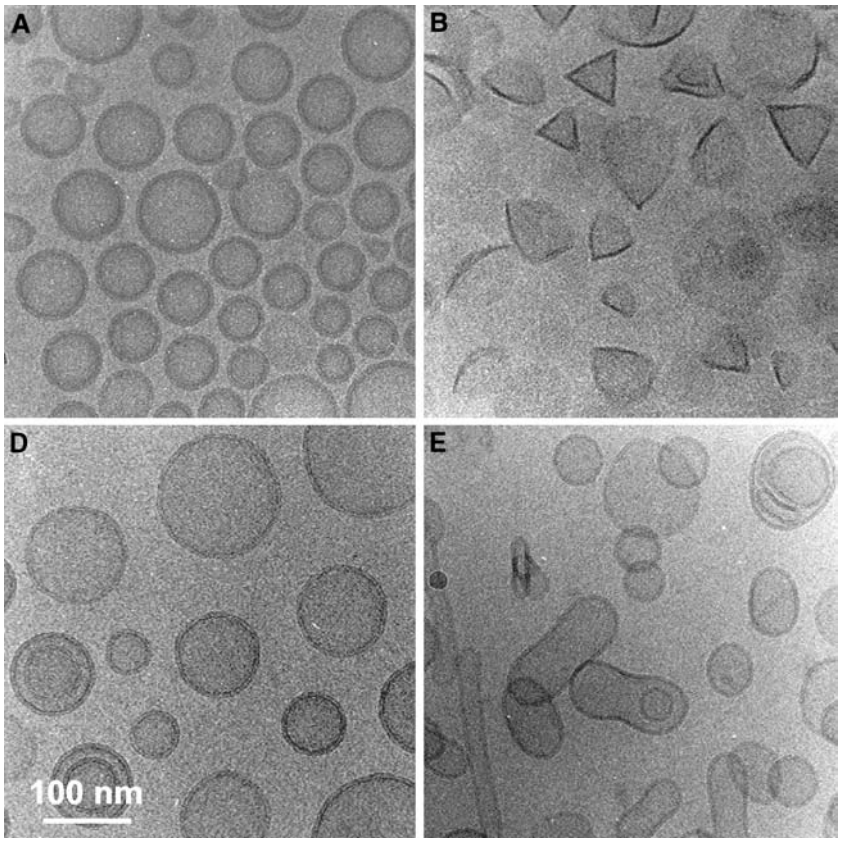

Fig. 1 Cryo-transmission electron microscopy images of DSPC-based liposomes (formulations A and B), and DOPC-based liposomes (formulations D and E). Formulations A and D contain cholesterol, while formulations B and E contain no cholesterol. For liposome compositions, see Table 1

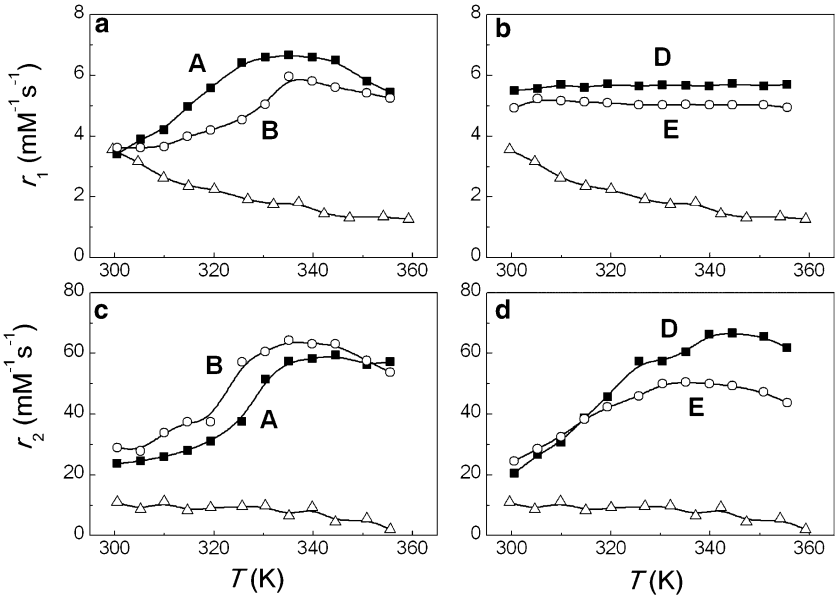

Fig. 2 Temperature dependence of the ^{1}H longitudinal and transverse relaxivities r_1 (a, b) and r_2 (c, d) at 200 MHz of aqueous solutions of liposomes with different formulations A (*filled squares*), B (*open circles*), D (*filled squares*), E (*open circles*), and Gd-DTPA (*open circles*). The *solid lines* are guides to the eye only

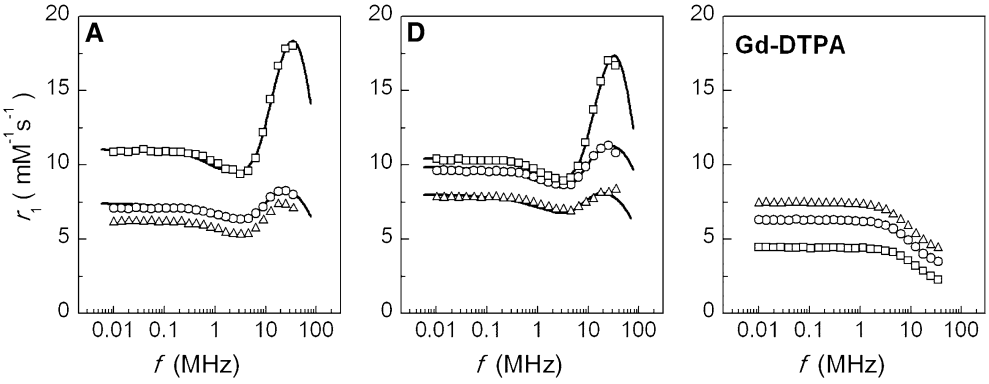

Fig. 3 ^{1}H nuclear magnetic relaxation dispersion curves of aqueous solutions of liposomes (formulations A and D, left and middle panel) and Gd-DTPA (right panel) at temperatures of 298 (*open triangles*), 310 (*open circles*), and 333 K (*open squares*). The solid lines are fits according to the parameters given in Table 2 and the text

were fixed: the hydration number q = 1, the distance between water proton and unpaired electron spin r = 3.1 Å, the distance of closest approach d = 3.6 Å, and the water diffusion constants $D_{\text{diff}} = 2.2 \times 10^{-3}$, 3.2×10^{-3}, and 5.5×10^{-3} mm^2/s for T = 298, 310, and 333 K, respectively [17]. The fitting parameters obtained are listed in Table 2. The water exchange seems most affected by a change in temperature, resulting in a dramatic decrease of the exchange correlation time with increasing temperature.

Discussion

Liposomes containing a high payload of Gd seem well suited as sensitive T_1-lowering MRI contrast agents. In a number of previous studies their in vitro and in vivo applicability has already been demonstrated [5, 10, 18–21]. Liposomes have been studied for more than 20 years as carriers for the improved delivery of drugs, including chemotherapeutic agents, antigens, and genetic material. From these studies there exists extensive knowledge about optimal formulations, depending on the desired application [1, 2].

For application as a MRI contrast agent a formulation that ensures long circulation time is often required. This is particularly true when the goal is to use the liposomes as a blood-pool agent or to achieve accumulation at a specific site in the body, e.g. a tumor. Active targeting can be achieved by conjugating a targeting ligand to the liposome, which can be attached to the distal ends of the PEG chains [5]. The most popular way to obtain long circulation times is to attach polyethylene glycol (PEG) covalently to the outer surface of the liposomes. The PEG coating is highly hydrated and this layer protects against interactions with molecular and biological components in the blood stream. In contrast, conventional liposomes without PEG have short circulation times due to rapid uptake by phagocytic cells of the reticulo-endothelial system (RES), primarily in the liver and the spleen. A supplementary way to ensure long circulation times is adding cholesterol. Cholesterol acts as a spacer, because of its inflexible structure, preventing demixing of the lipids and reducing the PEG chain–chain interactions, resulting in improved steric stabilization of the liposome. Most optimal formulations for long circulation times contain more than 30% cholesterol and low concentrations of PEG, viz. typically less than 7%. The general trend is that increasing the size of the liposomes results in more rapid uptake by the RES.

Taking into account the considerations above, the liposomes in this study were designed for long circulation time, and thus included cholesterol and PEG. The size of the liposomes was chosen to be approximately 100 nm, which does not compromise the long circulation times, while still allowing a large amount of Gd-containing lipid to be incorporated. The TEM images in Fig. 1 indeed confirm that the addition of cholesterol is essential to obtain monodisperse unilamellar vesicles, for the liposomes with saturated as well as unsaturated fatty-acid chains. The addition of considerable amounts of Gd-containing lipid apparently does not negatively influence the stabilization of the cholesterol-containing liposomes. The membrane permeability of the liposomes with and without Gd-containing lipid was the same as measured with a calcein leakage assay [5].

The relaxivities per mmol of Gd(III) ions, at 200 MHz and variable temperature, are higher for all liposomal formulations compared to traditional Gd-DTPA. Gløgård et al. [10] have found the highest r_1 relaxivity for liposomes containing no cholesterol and low content of Gd-chelate. In contrast, we observe a higher r_1 relaxivity for the vesicles with cholesterol. This may be caused by an increase of the motional correlation times under influence of the steric hindrance of these specific Gd lipids by cholesterol. However, considering the large differences in shape and size of the vesicles with and without cholesterol, as observed by cryo-TEM shown in Fig. 1, it is probably not viable to make a direct comparison between these two formulations here.

The peak observed in the temperature dependence of r_1 for the DSPC-based liposomes (Fig. 2a) coincides with the well-known gel to liquid-crystalline phase transition

Table 2 Best-fit parameters of the ^{1}H NMRD data for formulations A and D at different temperatures

Temperature (K)	Δ_t (cm^{-1})	τ_v (ps)	τ_r (ns)	τ_m (μs)	D_{ZFS} (cm^{-1})
Formulation A					
310	0.028	57	3.7	1.2	0.04
333	0.016	18	1.8	0.1	0.05
Formulation D					
298	0.023	54	4.4	1.2	0.04
310	0.019	46	1.5	0.5	0.05
333	0.017	20	2.5	0.2	0.06

The fit curves are presented in Fig. 3 as the solid lines. The fitting parameters are: Δ_t = the magnitude of the transient ZFS, τ_v = the correlation time for electronic relaxation, τ_r = the rotational correlation time, τ_m = the water exchange correlation time, and D_{ZFS} = the static ZFS

[16]. This phase transition was confirmed for these liposomes independently with differential scanning calorimetry (data not shown). For the DOPC-based liposomes this phase transition lies below 273 K and hence such a peak is not observed. At physiological temperature and a resonance frequency of 200 MHz the r_2/r_1 ratios are between 5 and 9 for the different formulations. This ratio is quite high, which is in general not favorable for a T_1-lowering contrast agent, although negative effects due to high r_2 can be effectively limited by using pulse sequences with short TE. The r_1 per mM liposomes at physiological temperature and 4.7 T is an estimated $40000 \times 5 = 0.2 \times 10^6\,mM^{-1}s^{-1}$, which brings these liposomes very close to the sensitivity of FeO particles with an estimated r_2 per mM particles of $1 \times 10^6\,mM^{-1}s^{-1}$ (assuming 5000 Fe ions per particle and r_2 per ion of $200\,mM^{-1}s^{-1}$). Certainly at higher magnetic fields, the r_2/r_1 ratio of the paramagnetic liposomes will increase and therefore T_2-lowering contrast agents, such as FeO nanoparticles, may have more favorable properties in terms of sensitivity. Nevertheless, for a given in vivo application the choice between paramagnetic liposomes and FeO particles will depend on whether the liposome pros (positive contrast, flexible particle that is easily conjugated with antibodies/peptides, possibility to encapsulate drugs) outweigh the cons (not as sensitive as FeO, T_2 effects at higher fields). The r_2/r_1 ratio usually decreases with decreasing resonance frequency and therefore we expect that this ratio will be somewhat more favorable at the lower, clinically applicable field strengths, although we did not confirm this.

NMRD curves were only measured for formulations A and D, since only these lead to useful, stable monodisperse liposomes. At these resonance frequencies, for both formulations the relaxivity r_1 increases with temperature, indicating that the T_1 relaxation is limited by water exchange, in contrast to earlier observations by Tilcock et al. [11]. We infer that the Gd at the inner leaflet of the membrane particles becomes more accessible to water at higher temperatures, thus contributing more to the relaxivity. This is confirmed by the fact that the DOPC-based liposomes have a higher relaxivity, since they are in the liquid-crystalline phase for all measured temperatures and thus have a less rigid membrane that is more permeable to water.

The theory of nuclear spin relaxation of paramagnetic systems includes many parameters, describing in much detail the complex interactions between water and the paramagnetic Gd relaxation entity. Therefore a fit of an NMRD curve usually results in quite unreliable estimates of the relaxation parameters, unless several variables can be determined using independent methods, such as ^{17}O NMR. Nonetheless, we have fitted the NMRD curves, keeping some of the variables fixed to literature values [17], restricting the number of fitting parameters to five (Table 2). Since we cannot separately measure the relaxivity of the inner and outer leaflet of the lipid bilayer, the resulting parameters must be considered to contain contributions from both compartments.

For both the DSPC- and DOPC-based liposomes we observe similar trends with temperature. The most obvious is the rapid decrease of the exchange correlation time τ_m with temperature, consistent with a faster exchange of water in a more permeable membrane. We think that a fit of the NMRD curve of formulation A at 273 K is not possible because the inner leaflet of the membrane is not, or is only slightly, accessible for bulk water. Since r_1 is calculated from a division through the total Gd concentration, including Gd in the inner leaflet, this leads to an underestimation of the actual r_1 of Gd in the outer leaflet. As expected, the rotational correlation times tend to decrease with increasing temperature as a consequence of the faster rotational motion of the Gd lipids at higher temperatures.

The parameters governing the electronic relaxation processes are D_{ZFS}, Δ_t, and τ_v. The static ZFS is a weak measure for the averaged distortion of the cubic symmetry around the Gd(III) ion, which slightly increases with increasing temperature. The transient ZFS amplitude decreases with increasing temperature, while the corresponding correlation time τ_v decreases. It is not obvious why the transient ZFS amplitude, which is a measure for the de-

formability of the complex by collisions with the water molecules, should decrease with increasing temperature. However, it seems that this could well be due to some covariance in the fit, because effectively the combination of Δ_t and τ_v still results in increased relaxation at higher temperatures.

Conclusions

In conclusion, we have characterized paramagnetic liposomes with saturated DSPC and unsaturated DOPC phospholipids, and with and without cholesterol. Addition of cholesterol is necessary to obtain monodisperse unilamellar liposomes. The incorporation of large amounts of Gd-containing lipids in the sterically stabilized pegylated DSPC- and DOPC-based liposomes results in spherical nanoparticles with a higher relaxivity r_1 per Gd(III) ion compared to traditional Gd-DTPA, although the improvement is modest. The relaxivity of liposomes with unsaturated phospholipids is higher compared to those with saturated lipids, because of the higher accessibility of water. Cholesterol leads to a further, although smaller, increase of the relaxivity that is probably caused by an increase in the motional correlation times. The highest relaxivity per Gd(III) ion of $11.3\,mM^{-1}s^{-1}$ at physiological temperature (310 K) was obtained for DOPC-based liposomes containing cholesterol at 25 MHz. These results show that these paramagnetic liposomes are highly potent contrast agents, making them excellent candidates for many applications in molecular MR imaging.

Acknowledgements The authors would like to thank G. Parigi from the Magnetic Resonance Center (CERM) of the University of Florence for kindly providing the NMRD measurement time and the fitting program, as well as for useful discussions. This study was financially supported by the BSIK program entitled Molecular Imaging of Ischemic Heart Disease (project number BSIK03033).

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