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# Liposomes as carriers of amphiphilic gadolinium chelates: the effect of membrane composition on incorporation efficacy and in vitro relaxivity

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**Abstract**

The effects of membrane composition (phospholipid type and amount of cholesterol), liposome size, drug/lipid ratio (loading) and nature of the amphiphilic gadolinium (Gd) chelate on the incorporation efficacy and magnetic resonance (MR) contrast efficacy (longitudinal ($T_1$) relaxivity) were investigated using a fractional factorial design. A highly lipophilic Gd-chelate was required to ensure complete liposome incorporation. High $T_1$-relaxivity was obtained by using liposomes composed of cholesterol and phospholipids with short acyl chain lengths (dimyristoyl phosphatidyl choline (DMPC) and dimyristoyl phosphatidyl glycerol (DMPG). Two key factors, the loading of Gd-chelate and the amount of cholesterol in small-sized DMPC/DMPG liposomes, were studied further in a central composite optimising design. A robust high relaxivity region was identified, comprising high loading of cholesterol and Gd-chelate. However, the highest $T_1$-relaxivity (52 $mM^{-1}$ $s^{-1}$) was found in an area containing no cholesterol and low content of Gd-chelate. Nuclear magnetic resonance dispersion (NMRD) profiles were obtained for five of the liposome compositions from the optimising design, and high relaxivity peaks in the 20 MHz region confirmed the presence of Gd-chelates with a long $\tau_R$. A liposome formulation was selected for surface modification with polyethylene glycol (PEG), without having any effect on the $T_1$-relaxivity. 

*Keywords:* Liposomes; Amphiphilic gadolinium chelates; Magnetic resonance; Relaxivity; Membrane composition; Incorporation efficacy; Nuclear magnetic resonance dispersion profile; Experimental design

## 1. Introduction

Magnetic resonance imaging (MRI) is a diagnostic modality based on the principles of NMR. Although MRI is a non-invasive technique,

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around 25% of the present MR-examinations are including a contrast agent (CA). CAs can improve the diagnostic value by increasing the sensitivity, but also by enhancing the differentiation between tissues, including normal and pathological tissues. Present paramagnetic MRI CAs used in the clinic are mostly low molecular weight compounds with extra cellular distribution. For the future, the development of new CAs is important to find new applications for MRI, especially in the fields of organ specific and responsive agents.

To enhance the MR-efficacy and/or to improve the site-specific delivery of gadolinium (Gd) based MR-contrast agents, various systems have been suggested as carriers (Okuhata, 1999). Among these are liposomes, micelles (André et al., 1999) and nanoparticles (Morel et al., 1998), as well as macromolecular systems, such as polylysine (Aime et al., 1999).

Liposomes are bilayer vesicles built up by amphiphiles, and are formed spontaneously when amphiphiles are dispensed in aqueous media. The amphiphiles are usually phospholipids and among the most common components are phosphatidyl choline (PC) and phosphatidyl glycerol (PG). Other materials, such as cholesterol are frequently included in the formulation. Hydrophilic compounds can be trapped within the liposome interior, while lipophilic or amphiphilic compounds normally are incorporated into the liposome membrane.

Liposomes have been extensively investigated as a possible carrier of Gd-chelates to enhance the contrast efficacy and to change the pharmacokinetic properties of the CA. The bio-distribution of liposomes is highly dependent on liposomal physicochemical properties such as size, surface charge or membrane composition. However, conventional liposomes are to a large extent taken-up by the reticuloendothelial system (RES). Liver-targeted Gd-labelled liposomes have been investigated extensively (Unger et al., 1989; Kabalka et al., 1991; Schwendener, 1994; Fossheim et al., 1999). By prolonging the circulation time, liposomes with potential application for MR-angiography and tumor imaging have been obtained. One approach included the use of Gd-labelled polymerised liposomes prepared by using a polymerisable amphiphilic Gd–diethylenetriaminepentaacetic acid (Gd–DTPA) derivative (Storrs et al., 1995). This system showed an improved physical stability, originating from the increased membrane rigidity and was able to avoid, to some extent, uptake by the RES. Recently; another class of Gd-loaded liposomes has been suggested as a blood pool agent. This liposome system containing a polychelating amphiphilic polymer, was surface modified with polyethylene glycol (PEG), which increased the in vivo circulation time due to reduced RES uptake (Weissig et al., 2000).

To be able to design 'optimal' liposomal MR contrast agents, it is necessary to have an understanding of the relaxation theory. A review fully covering the relaxation theory of Gd-chelates for medical diagnostics is written by Caravan et al. (1999).

In contrast to other imaging modalities (X-ray, scintigrapy and ultrasound), the effect of a MR-contrast agent is not seen directly on the image, but rather the effect it exerts on proton relaxation, normally water protons. The relaxation enhancement of water protons in aqueous solution of Gd-chelates can be described with a model considering four contributions: inner sphere ($R_1^{is}$), outer sphere ($R_1^{os}$) and second sphere ($R_1^{2s}$), in addition to the diamagnetic contribution from the matrix ($R_1^{m}$):

$$R_1^{obs} = R_1^{is} + R_1^{os} + R_1^{2s} + R_1^{m} \quad (1)$$

where $R_1^{obs}$ is the experimentally measured $T_1$-relaxation rate ($s^{-1}$). When one or more water molecule is coordinated to the gadolinium ion, such as in Gd–HDD–DO3A and Gd–HHD–DO3A, the most important contribution to the relaxation rate is coming from the inner sphere term. This contribution is given by:

$$R_1^{is} = \frac{q \cdot C}{55.6} \cdot \frac{1}{T_{1M} + \tau_M} \quad (2)$$

where $q$ is the number of inner sphere coordinated water molecules (hydration number), $C$ is the molar Gd-ion concentration, $T_{1M}$ is the longitudinal relaxation time of the protons located at the inner-sphere water molecules, and $\tau_M$ is the residence time of the protons bound to the water

molecules in the inner coordination sphere. Eq. (2) gives that the water molecule residence time must be fairly short in order for the bulk water to experience any relaxation rate ($1/T_{1M}$) enhancement from the water molecule present in the inner sphere; that is the water exchange rate must be so fast that $\tau_M \ll T_{1M}$. The relaxation rate of the inner sphere water molecule can be calculated using the Solomon–Bloembergen–Morgan approach:

$$\frac{1}{T_{1M}} \propto \left[ \frac{3\tau_{C1}}{1+\omega_H^2\tau_{C1}^2} + \frac{7\tau_{C2}}{1+\omega_S^2\tau_{C2}^2} \right] \quad (3)$$

where $\omega_H$ and $\omega_S$ are the proton and electron Larmor frequencies, respectively; $\tau_{Ci}$ ($i = 1, 2$) are the correlation times of the modulation of the dipolar electron-proton coupling. $\tau_{Ci}$ is made up of three additive contributions:

$$\frac{1}{\tau_{Ci}} = \frac{1}{\tau_R} + \frac{1}{\tau_M} + \frac{1}{\tau_{Si}} \quad (4)$$

where $\tau_R$ is the rotational correlation time of the Gd-chelate and $\tau_S$ is the electronic relaxation time of the Gd-ion. By incorporating the Gd-chelate into the liposomal membrane a lengthened $\tau_R$ can be achieved due to the reduced molecular tumbling. This effect can in turn give rise to an enhanced relaxation rate, as long as fast-exchange ($\tau_M \ll T_{1M}$) conditions are present.

Lately, a novel series of amphiphilic Gd–1,4,7-tris(carboxymethyl) - 1,4,7,10 - tetraazacyclododecane (DO3A) derivatives have been synthesised for the purpose of incorporation into the liposomal bilayer (Gløgård et al., 2000). In the present study, two of these compounds (Gd–(2-hydroxydodecyl)–DO3A (Gd–HDD–DO3A) and Gd–(2-hydroxyhexadecyl)–DO3A (Gd–HHD–DO3A)) were incorporated into the liposome membrane (Fig. 1). A fractional factorial design was used to evaluate effects of membrane composition (phospholipid type and amount of cholesterol), liposome size, drug/lipid ratio (loading) and nature of amphiphilic Gd-chelate. Incorporation efficacy of the Gd-chelate into the liposome lamella and the contrast efficacy, in term of the $T_1$-relaxivity, were used as responses. With starting point in the results from the screening, optimisation of the liposome formulation was performed with respect to high $T_1$-relaxivity and incorporation efficacy of the Gd-chelate. The optimised liposome formulation was surface modified with PEG in order to monitor any effect on the contrast efficacy.

## 2. Materials and methods

### 2.1. Materials

Dimyristoylphosphatidylcholine (DMPC), dipalmitoylphospatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), dimyristoylphosphatidylglycerol (DMPG) dipalmitoylphosphatidylglycerol (DPPG) and distearoylphosphatidylglycerol (DSPG) were kindly provided by Nattermann Phospholipids, Cologne, Germany. PEG 5000-L-α-dimyristoylphosphatidylethanolamine and cholesterol were obtained from Avanti Polar Lipids Inc. (Alabaster, AL) and Fluka Chemical Co. (St. Louis, MO), respectively. Gadolinium 1,4,7-tris(carboxymethyl)-10-(2-hydroxydodecyl)-1,4,7,10-tetraazacyclododecane(Gd–HDD–DO3A) and gadolinium 1,4,7-tris(carboxymethyl) - 10 - (2 - hydroxyhexadecyl) - 1,4,7,10-tetraazacyclododecane (Gd–HHD–DO3A) were synthesised according to procedure published elsewhere (Gløgård et al., 2000). All other chemicals were of analytical grade.

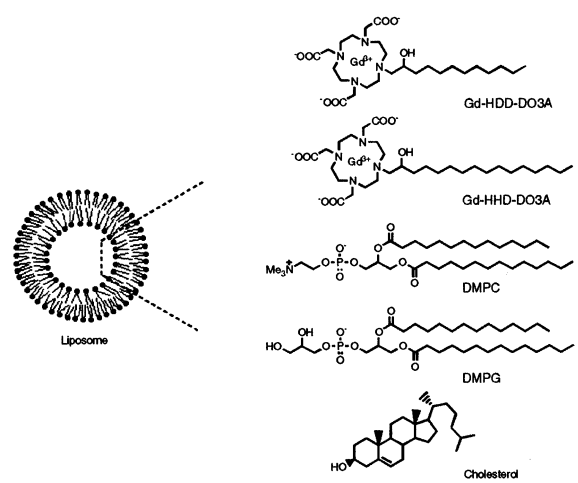


Fig. 1. A schematic presentation of the liposome system, exemplified with DMPC/DMPG and cholesterol.

### 2.2. Methods

#### 2.2.1. Liposome preparation and characterisation

Liposomes were prepared by the thin film-method as follows; the phospholipids, Gd-chelate and cholesterol were co-dissolved in chloroform/methanol mixture (10:1 v/v) and evaporated to dryness under reduced pressure. The thin film obtained was hydrated with an aqueous glucose solution (50 mg $ml^{-1}$) pH 7.4. The resulting liposomes were gently shaken above the phase transition temperature ($T_c$) for 2 h. The liposomes (5 μmol $ml^{-1}$) were extruded (Lipex extruder, Bio-membranes Inc., Vancouver, Canada) through either 400, 200 or 50 nm polycarbonate membranes (Nucleopore®, Costar Corp., Cambridge, MA) in the last ten extrusion steps.

Extra liposomal Gd-chelate was removed by dialysis (Slide-A-Lyzer® dialysis cassette ($M_w$ cut off 10 000), Pierce, Rockford, IL) against glucose (50 mg $ml^{-1}$) pH 7.4 overnight.

The phospholipid concentration was determined by phosphorus analysis according to Rouser et al. (1970). The Gd-chelate incorporation efficacy (%) was calculated by comparing the amount of Gd present in the sample before and after dialysis as determined by inductively coupled plasma atomic emission spectrophotometry (ICP-AES) analyses.

The mean diameter of the liposomes was measured at 90° angle (25 °C) by photon correlation spectroscopy (PCS) using a Coulter DELSA 440 (Beckman Coulter Inc., Fullerton, CA).

### 2.3. In vitro relaxometry

The relaxation measurements were performed at 0.47 T (Minispec PC-120b, Bruker GmbH, Rheinstetten, Germany) and the $T_1$-relaxation rates ($R_1^{obs}$) were obtained by the inversion recovery method at 39 °C. The $T_1$-relaxivitiy ($r_1$) for the liposomal Gd-chelate in each formulation was obtained either by the relationship:

$$r_1 = \frac{R_1^{obs} - R_1^{m}}{C} \quad (5)$$

where $R_1^{obs}$ and $R_1^{m}$ are the $T_1$-relaxation rates ($s^{-1}$) of the sample and the matrix (glucose 50 mg $ml^{-1}$), respectively, and $C$ is the Gd-concentration (mM), or from a linear least squares regression analysis of the relaxation rate ($R_1^{obs}$) versus $C$.

### 2.4. NMRD measurements

The $1/T_1$ nuclear magnetic relaxation dispersion (NMRD) profiles were obtained on a Spinmaster FFC (fast field cycling) NMR relaxometer (Stelar s.r.l., Mede (PV), Italy), which covered a continuum of magnetic fields from 0.03 to 12 MHz. In addition $T_1$-relaxation rates were recorded at 20 MHz (variable field (8–60 MHz) Stelar Spinmaster, Stelar s.r.l., Mede (PV), Italy), 90 MHz (Jeol EX-90, Jeol Ltd., Tokyo, Japan) and 300 MHz (Bruker Spectrospin Avance DPX 300, Bruker GmbH, Karlsruhe, Germany).

The relaxation parameters obtained from the experimental NMRD data were calculated using a program written by E. Terreno (University of Turin, Italy) and the computer software Origin version 4.0 (Microcal™ Origin®, Northampton, MA).

### 2.5. Experimental design

The designs of the statistical experiments were performed using Unscrambler software (Camo, Trondheim, Norway), whereas the evaluations of the results were performed with Unscrambler and Modde 4.0 (Umetri AB, Umeå, Sweden) for the screening and optimisation, respectively.

#### 2.5.1. Screening

The effects of membrane composition (phospholipid chain lenght and amount of cholesterol), liposome size, drug/lipid ratio (loading) and nature of amphiphilic gadolinium (Gd) chelate on the incorporation efficacy and $T_1$-relaxivity were studied in a $2^{5-1}$ fractional factorial design, comprising 16 runs. In order to estimate the experimental error and check the linearity, triplicates were added at two centre points (one for each Gd-chelate) giving a total of 22 runs. The design resolution was V+, i.e. main effects and two-factor interactions are unconfounded. The levels of the factors are shown in Table 1. The liposome batches were produced in a random order.

Table 1
The levels of the factors studied in the $2^{5-1}$ fractional factorial design

| Factor | Level | | |
|---|---|---|---|
| | −1 | 0 | +1 |
| Phospholipid type | DMPC | DPPC | DSPC |
| Chelate loading (mol%) | 2.5 | 3.75 | 5 |
| Liposome size (nm) | 50 | 200 | 400 |
| Gd-chelate type | HDD–DO3A | | HHD–DO3A |
| Cholesterol content (mol%) | 0 | 16.67 | 33.33 |

−1, 0 and +1 represent low level, middle point and high level, respectively.

#### 2.5.2. Optimisation

Based on the results from the fractional factorial design, a central composite design with two variables was applied to find the optimum condition and to analyse how sensitive the responses were to variations in the settings of the experimental variables. This design is useful for estimating the coefficients in a higher-order polynomial. A total of 11 experiments were performed including triplicates of the centre point. The centre points improve the assessment of the response surface curvature and simplify estimation of the model error. The Gd-chelate loading and cholesterol content were varied and the incorporation efficacy and $T_1$-relaxivity were assessed. The liposome batches were produced in random order.

#### 2.5.3. Evaluation

A partial least square analysis (PLS1) was performed on the data sets from the fractional factorial design (V+) using Unscrambler software. Multiple linear regression (MLR) was used to evaluate the data from the optimisation design (CCD) using Modde. The significance of the estimated effects was tested by analysis of variance. The accuracy of the statistical model used is described by the parameter $R^2$. $R^2$ is the fraction of the data explained by the model and values close to 1 indicate a good model.

## 3. Results

### 3.1. Screening

The liposomes were sized down, according to the experimental plan, by extruding the liposomes through 50 nm, 200 or 400 nm polycarbonate filters. The actual sizes obtained where 63–84, 139–149 and 150–190 nm, respectively. All batches produced showed a relatively narrow size distribution.

#### 3.1.1. Incorporation efficacy

Liposomes were prepared according to the fractional factorial plan. Fig. 2 shows the main effects of increasing the factors from low to high level. The results show that use of the most lipophilic chelate gives the highest incorporation efficacy. Gd–HHD–DO3A was completely incorporated into the liposome lamella, whereas an incorporation efficacy between 12 and 23% was obtained for Gd–HDD–DO3A. Higher extent of incorporation was also observed when using DSPC-membranes compared to DMPC-ones. Larger liposomes showed only a minor positive effect on the incorporation efficacy, while the cholesterol content and loading extent of Gd-chelate had no effect. The $R^2$ for the model was 0.970.

#### 3.1.2. Relaxivity

The main effects of increasing the factors from low to high level in the fractional factorial design

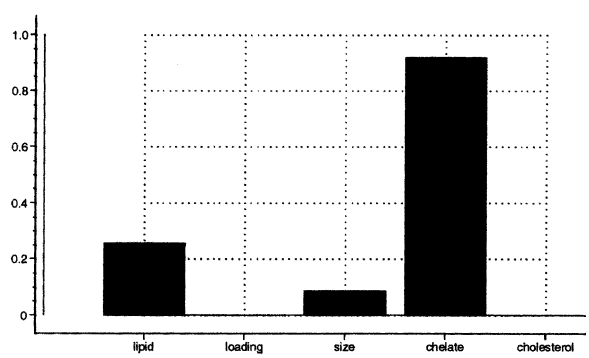


Fig. 2. The effect of increasing the factors from a low (−1) to a high level (+1) on the incorporation efficacy in the $2^{5-1}$ fractional factorial design.

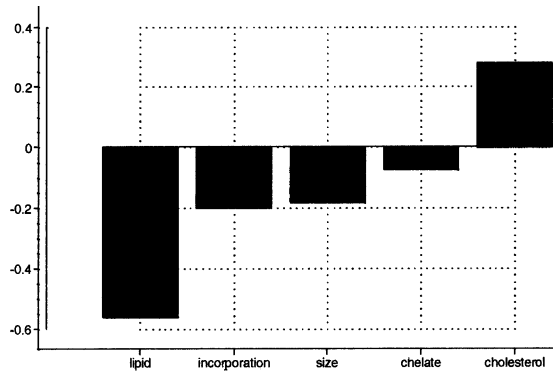


Fig. 3. The effect of increasing the factors from a low ($-1$) to a high level ($+1$) on the $T_1$-relaxivity in the $2^{5-1}$ fractional factorial design.

are shown in Fig. 3. A higher $T_1$-relaxivity was obtained for the DMPC-based liposomes compared to the DSPC-ones. The highest relaxivities among the investigated DMPC- and DSPC-liposome series were 42 and 24 $mM^{-1}\ s^{-1}$, while the average values were 28 and 20 $mM^{-1}\ s^{-1}$, respectively. The second most important factor with regard to the relaxivity was the liposomal cholesterol content where a positive effect was seen with increased content. High loading of Gd-chelate, the use of large liposomes or employing Gd–HHD–DO3A as the chelate, all had a small negative impact on the $T_1$-relaxivity. The $R^2$ for the model were 0.703. An improved model was obtained by including interactions. As the main factors remained unchanged, the simpler model was preferred.

### 3.2. Optimisation

The $T_1$-relaxivity response surface constructed from the results obtained in the optimisation design is shown in Fig. 4. Two factors, amount of cholesterol and type of Gd-chelate, were varied between 0–40 and 1–10 mol%, respectively. Gd–HHD–DO3A was chosen due to the favourable effect on the incorporation efficacy, while small DMPC-based liposomes (extruded through 50-nm polycarbonate filters) were chosen in order to ensure a high contrast efficacy. The sizes of the liposome batches produced in the optimisation design were between 65 and 81 nm. The highest $T_1$-relaxivity (52 $mM^{-1}\ s^{-1}$) was obtained in the region with both low content of both Gd-chelate and cholesterol. By increasing the loading of Gd-chelate without increasing the cholesterol content, the $T_1$-relaxivity decreased to below 40 $mM^{-1}\ s^{-1}$. The negative effect on the relaxivity was much less prominent when allowing the cholesterol content to increase simultaneously. This made it possible to establish a robust high relaxivity region with a high Gd-chelate loading. The $R^2$ for the model were 0.781.

### 3.3. NMRD profiles

The NMRD profiles at 27 and 39 °C for liposomes (65 nm) composed of DMPC/DMPG containing 5.5% Gd–HHD–DO3A and 20% cholesterol (corresponds with the centre in the optimisation plane) are shown in Fig. 5. The NMRD data were fitted and the parameters obtained are given in Table 2. In addition, NMRD-profiles were recorded with liposomal compositions corresponding to the four corners of

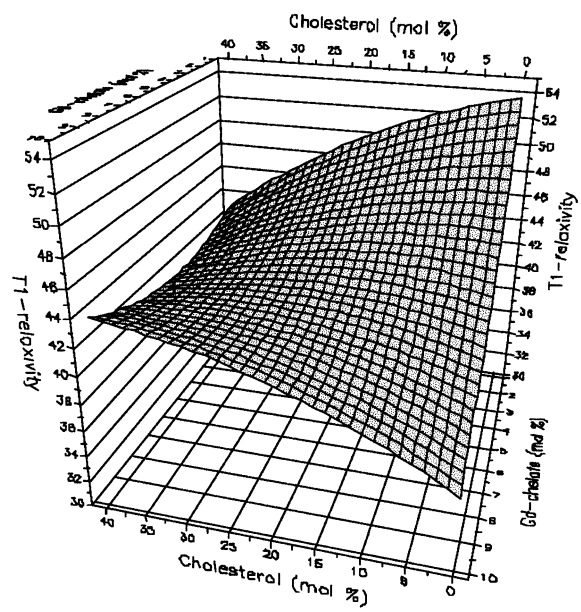


Fig. 4. The response surface obtained from the central composite design showing the $T_1$-relaxivity ($mM^{-1}\ s^{-1}$) as a function of the cholesterol (0–40 mol%) and the Gd-chelate Gd–HHD–DO3A loading (1–10 mol%).

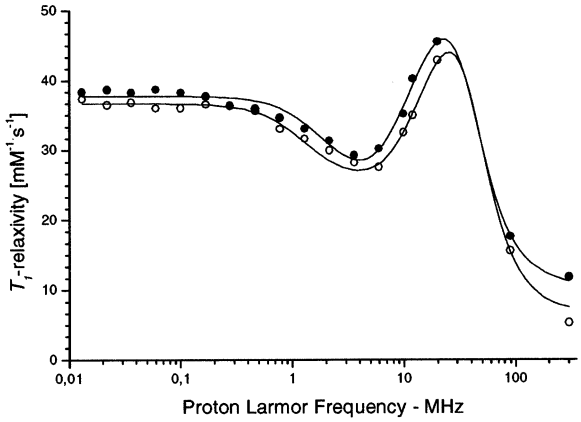


Fig. 5. NMRD-profiles of 65 nm DMPC/DMPG liposomes containing 5.5% Gd–HHD–DO3A and 20% cholesterol at 27 °C (●) and 39 °C (○); the lines are represent the best fitting procedure.

the optimising plane. All profiles showed a relaxivity peak around 20 MHz, as expected for Gd-chelates having an increased $\tau_R$. The relaxivity obtained at 20 MHz in the five NMRD profiles were all in accordance with the values predicted from the surface plot obtained in the optimisation experiments.

### 3.4. PEG modification

The influence of surface-grafted PEG on the $T_1$-relaxivity was investigated for the DMPC/DMPG liposome formulation containing 5.5% Gd–HDD–DO3A and 20% cholesterol. The incorporation of 5% DMPE–$PEG_{5000}$ did not affect the relaxivity.

Table 2
Relaxation parameters at 27 and 39 °C obtained from the least-squares analysis of the NMRD data of DMPC/DMPG liposomes (65 nm) containing 5.5% Gd–HHD–DO3A and 20% cholesterol

| Parameter | 27 °C | 39 °C |
|---|---|---|
| $\Delta^2$ ($s^{-2}/10^{19}$) | $1.6 \pm 0.2$ | $1.5 \pm 0.1$ |
| $\tau_V$ (ps) | $35 \pm 2$ | $27 \pm 2$ |
| $\tau_V^{is}$ (ns) | $44 \pm 4$ | $24 \pm 7$ |
| $\tau_M$ (ps) | $599 \pm 52$ | $544 \pm 35$ |
| $q_{is}$ | 1.5 | 1.5 |
| $R_{is}$ (Å) | 3 | 3 |
| $\tau_R^{2s}$ (ps) | $137 \pm 38$ | $68 \pm 11$ |
| $q_{2s}$ | $10 \pm 2$ | $11 \pm 1$ |
| $R_{2s}$ (Å) | 4 | 4 |

The values underlined were fixed.

## 4. Discussion

### 4.1. Screening

#### 4.1.1. Incorporation efficacy

The nature of Gd-chelate proved to be the most important factor in order to obtain a high degree of incorporation. This seems to be correlated to the lipophilic moiety of the chelates. Gd–HHD–DO3A, with the highest partition (P) coefficient between 1-octanol and water, showed a higher membrane incorporation compared to the less lipophilic Gd–HDD–DO3A. The log $P$ values of the two compounds are 3.7 and 1.9, respectively (Gløgård et al., 2000).

The higher incorporation of the Gd-chelates into the DSPC-liposomes compared to the DMPC-liposomes might be explained by the higher rigidity of the former, improving the interaction between the lipophilic tail of the Gd-chelate and the liposome membrane.

#### 4.1.2. $T_1$-relaxivity

A higher $T_1$-relaxivity was obtained for the DMPC-based liposomes compared to the DSPC-ones. At 39 °C the DMPC-membrane ($T_c$ 24 °C) is in the liquid-crystalline state whereas the DSPC-membrane ($T_c$ 55 °C) is in the solid gel state. In the liquid-crystalline state the water exchange rate between the liposome interior and exterior is high, thereby allowing bulk water to experience magnetic interaction with the Gd-chelates located on the inner surface. In the gel state the water exchange rate is lower, making the inner surface chelates 'less accessible' for the bulk water and thereby decreasing their contribution to the overall relaxivity. On the other hand, the lateral motion on the liposome surface is about twice as high in a liquid-crystalline membrane compared to a gel state membrane (Tilcock, 1999). This should give rise to a longer $\tau_R$ for the Gd-chelates incorporated into a gel state DSPC-membrane, thereby increasing the relaxivity. No

such increase in the efficacy is observed in the screening-experiments; despite a higher expected $\tau_R$. This implies that the relaxivity is exchange limited.

A positive effect was seen when cholesterol was included in the formulations. The influence of cholesterol on the liposome membrane is associated to the phase transition temperature of the latter. With the incorporation of cholesterol into a solid, DSPC-liposome fluidises the membrane, leading to increased transmembrane water permeability and hence an increased relaxivity of the Gd-chelates present inside the liposomes. In the liquid-crystalline DMPC-liposome the positive effect observed upon cholesterol incorporation is most likely related to an increase in the membrane rigidity. The rotational correlation time ($\tau_R$) of the Gd-chelate is prolonged, without affecting adversely the conditions of fast water exchange.

Negative effects were seen on the relaxivity both when increasing the incorporation of the Gd-chelates, and when increasing the carbon chain length of the Gd-chelates. A possible explanation is the negative influence of the Gd-chelate on the membrane packing in the sense of creating disorder. A likely impact of this is an increase in the lateral surface motion on the liposome surface, leading to a shortening of $\tau_R$ for the Gd-chelates and thereby a decrease in the relaxivity. The negative effect of increasing the lipophilic chain length might be due to an indirect effect. As seen in the incorporation efficacy study, increasing the lipophilic chain gives a higher degree of incorporation, and hence an increased surface motion of the Gd-chelates on the liposome, which in turn decreases the relaxivity.

The reduction in relaxivity with the use of large liposomes might be attributed to the reduced surface area-to-volume ratio and the presence of multilamellar bilayers, which slow down the water exchange between the liposome interior and exterior.

### 4.2. Optimisation

The decrease seen in the response surface when increasing the loading of Gd–HHD–DO3A is already explained in terms of increased lateral motion of the Gd-chelates on the liposomal surface. The phase organisation amphiphilic molecule prefers, alone or in mixtures with other amphiphiles can be explained in terms of the packing parameter ($P$) (Israelachvili, et al., 1980). In order to create a particle with a bilayer structure, the amphiphile needs to have a $P$-value close to 1, which corresponds to a rectangular shape. Phospholipids, e.g. PC and PG, have $P$-value near 1. A single chained amphiphile, such as Gd–HHD–DO3A or a lysophospholipid, has a $P$-value less than 1 (conical shape) and prefers to form micellar structures. When combining one compound that prefers bilayer structure with one that prefers micelle structure, the result might be a membrane with little compliance and a high lateral surface motion. The improvement in relaxivity efficacy observed when incorporating cholesterol at a high loading of Gd–HHD–DO3A might also be explained in terms of the packing parameter. Cholesterol has a shape opposite of Gd–HHD–DO3A ($P > 1$). When combining these two compounds they should, at a particular ratio, create units with a packing parameter equal to 1. It is therefore reasonable to argue that the increase in relaxivity is due to a lengthening of the $\tau_R$ caused by improved membrane packing. However, with a low loading of Gd–HHD–DO3A, cholesterol has a negative influence on the relaxivity. Cholesterol is known to both rigidify a fluid-crystalline membrane, and thereby increase $\tau_R$, and to decrease the water exchange across the membrane. The relaxivity decrease observed in this case is most likely caused by a decreased water exchange between the liposome interior and exterior, limiting the relaxivity contribution of the inner surface associated Gd-chelates.

### 4.3. NMRD profiles

Measuring the relaxivity at one magnetic field is rarely adequate to fully characterise a MR-contrast agent. Determining the relaxivity as a function of field strength (e.g. NMRD-measurements) allows the evaluation of $\tau_R$ and other relaxation parameters, providing valuable insight into the factors contributing to the relaxivity. The parameters obtained from the analysis of the NMRD

data of DMPC/DMPG liposomes (65 nm) containing 5.5% Gd–HHD–DO3A and 20% cholesterol at 27 and 39 °C are listed in Table 2.

The NMRD-profiles of liposomal systems show a broad peak in the 20–35 MHz region, indicating that the $\tau_R$ of the Gd-chelates is prolonged due to liposome incorporation. Upon fitting of the NMRD-data, $\tau_R$ values of 24 and 44 ns were obtained at 39 and 27 °C, respectively. A similar peak, but with a lower peak relaxivity has been obtained for a comparable liposomal system investigated by Tilcock et al. (1992). The Gd-chelates used in that study were bisamide and bisester derivatives of DTPA, and the $\tau_R$ value reported was 10 ns (40 °C). DTPA and especially the bisamide derivatives are known to have a slow water exchange time ($\tau_m$) compared to (2-hydroxyalkyl)–DO3A derivatives, which mainly explains the difference in relaxivity between the two systems.

A hydration number ($q$) of 1.5 was used to obtain good correlation between the model and the experimental data. This is a slight increase compared to the parent gadolinium (2-hydroxypropyl)–DO3A with a reported $q$ value of 1.3, indicating a reduced coordination of the hydroxyl moiety towards the Gd in the liposomal system (Zhang et al., 1992). A possible explanation is that the hydroxyl group in Gd–HHD–DO3A, when present in the liposome, is interacting with polar groups on the surface.

In addition, a relatively high number of second sphere water molecules had to be included to obtain a good model. A second sphere water molecule can be defined as a water molecule held in the second coordination shell of the Gd-chelate by hydrogen bonding to the polar groups present in the ligand (Botta, 2000). Normally, the number of molecules present in the second coordination sphere is restricted due to few coordination sites on the ligand and the short distance from the Gd-ion this effect is observed. However, in a liposomal system a large number of phospholipid head groups, capable of forming strong hydrogen bonds with water (Cevc, 1990), are surrounding the Gd-chelates, thereby increasing the number of water molecules with a longer $\tau_R$ than the bulk water in the proximity of the Gd-centre. Another possibility is that this number ($q = 1.5$) does not reflect the inner sphere water molecules directly, but rather the high density of protons, together with other positively charged ions, present in the Stern plane that is surrounding the negatively charged liposome (Tocanne and Teissié, 1990).

The $\tau_R$-values obtained from the NMRD-data could not explain the variation in relaxivity seen in the surface plot (Fig. 5). The reason might be a relatively high uncertainty in the values obtained from the NMRD-data. On dealing with multi-parameter equations it would be an advantage to fix several of the parameters by using independent methods. Unfortunately, the low concentration of Gd in the liposome formulations prevented the use of such methods, e.g. the $^{17}O$-method in order to determine $\tau_m$.

### 4.4. PEG-coating

Surface modification with PEG is known to prolong the circulation time due to reduced extent of opsonisation of the liposomes (Klibanov et al., 1990; Patel and Moghimi, 1990). It has also been reported that PEG-coating has a positive effect on the relaxometric properties of liposome incorporated Gd-chelates. Experiments performed on PEG-modified Gd labelled liposomes have shown $T_1$-relaxivities about twice as high as liposomes without surface modification (Trubetskoy et al., 1995). However, in the present study no increase in relaxivity was observed.

## 5. Conclusion

The incorporation of amphiphilic Gd-chelates into the liposomal membrane markedly enhanced the $T_1$-relaxivity. The statistical experimental plans were useful to identify and optimise the most important factors affecting the incorporation efficacy and the relaxivity. High incorporation was achieved by using highly lipophilic chelates, while using liposomes composed of DMPC/DMPG and cholesterol ensured the highest MR-contrast efficacy. Coating of liposomes with PEG showed no effect on the $T_1$-relaxivity.

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