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Novel high relaxivity colloidal particles based on the specific phase organisation of amphiphilic gadolinium chelates with cholesterol

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Abstract

To obtain high T_1-relaxivity colloidal particles with a simultaneously high loading of amphiphilic Gd-chelates, a novel drug dosage form based on the phase organisation of amphiphilic gadolinium chelates with cholesterol was developed.

In order to find a formulation, which exhibit both high T_1-relaxivity and gives small particles a D-optimal mixture design (experimental design) was applied. Gadolinium 1,4,7-tris(carboxymethyl)-10-(2-hydroxyhexadecyl)-1,4,7,10-tetraazacyclododecane (Gd-HHD-DO3A) and cholesterol at approximately equimolar ratio proved to form thermodynamic stable disc-like colloidal particles as seen by cryo-electron micrographs. T_1-relaxivity of these particles was typically around 20 $mM^{-1}\,s^{-1}$ and the size below 100 nm (photon correlation spectroscopy (PCS)). The particles do most probably not interact with blood components as no change in T_1-relaxivity was observed when the particles were mixed with whole blood. The particles were stable at room temperature for at least 6 months.

Keywords: Particles; Lipid disc; Gadolinium chelates; MR contrast agent

1. Introduction

Particles have been extensively investigated as carriers of paramagnetic MR-contrast agents due to site-specific delivery and/or enhanced contrast efficacy. Most attention has been focused around lipid-based systems such as liposomes, but also solid lipid nanoparticles (SLN) (Morel et al., 1998), micelles (André et al., 1999) and particles of polysaccharides (Fossheim et al., 1999) have been widely used. Initially, liposomes were considered attractive due to the accumulation in macrophage rich tissue, i.e. liver and spleen (Kabalka et al., 1991). Later, the interest turned towards long circulating vehicles and imaging of the vascular system (Storrs et al., 1995).

In order to ensure high contrast efficacy (T_1-relaxivity), two parameters have proved to be important for particulate paramagnetic MR-contrast agents, high rigidity and access to bulk water. To obtain high rigidity, the particle needs tight packing with a restricted motion of the contrast agent. Combined with a high molecular weight of the particle this will normally increase the contrast efficacy by prolonging the rotational correlation time (τ_R) of the Gd-chelate, i.e. the rotation of the contrast agent in solution will be slowed down. Simultaneously, fast exchange between

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the water molecule co-ordinated to the Gd-ion and the bulk water (τ_M) needs to be maintained. Restricted availability of bulk water to the gadolinium will have a negative impact on the T_1-relaxivity. In order to ensure high access to bulk water, the Gd-chelate should be present on the particle surface or in the case of liposomes by using a fluid membrane, which allows high water transport across the membrane. However, low loading of the contrast agent and a complicated production are major drawbacks for liposomes as carrier for paramagnetic MR-contrast agents. Other colloidal systems might, therefore, be favourable. For an extensive theoretical treatment on design of paramagnetic MR contrast agents see Caravan et al. (1999).

The phase organisation of the amphiphilic Gd-chelates with cholesterol to form layers at approximately equimolar ratio was suggested in an earlier paper (Gløgård et al., 2002). According to the theory of phase organisation, the structure a composition of amphiphiles prefers is determined by the packing parameters (P) of the individual molecules (Israelachvili et al., 1980). A single chained amphiphile with a large hydrophilic head group, such as gadolinium 1,4,7-tris(carboxymethyl)-10-(2-hydroxyhexadecyl)-1,4,7,10-tetraazacyclododecane (Gd-HHD-DO3A), has a P-value less than 1 (conical shape) and prefers to form micellar structures. In order to form bilayer structures, the amphiphile needs to have a P-value close to 1, which corresponds to a rectangular shape. However, when Gd-HHD-DO3A is combined with a molecule, such as cholesterol, which has an opposite shape ($P > 1$) they should at a particular ratio be able to create bilayers. Similar studies on lipid-based formulations of the antifungal drug amphotericine B have showed how the particle structure can change with its composition (Hillery, 1997). One interesting structure, other than the liposome, is the lipid discs made up of amphotericine B and cholesteryl sulfate in molar ratio 1:1 (Guo et al., 1991).

In order to find a system that meets the criteria of high contrast efficacy (T_1-relaxivity), small particle size, high loading, good stability and easy preparation, the specific phase organisation of amphiphilic Gd-chelates with cholesterol was investigated. Palmitic acid (PA) was used to create negatively charged particles. A D-optimal mixture design was used to evaluate the effects of Gd-HHD-DO3A, cholesterol and PA and find the optimum level of the components. The structures of Gd-HHD-DO3A, PA and cholesterol are shown in Fig. 1.

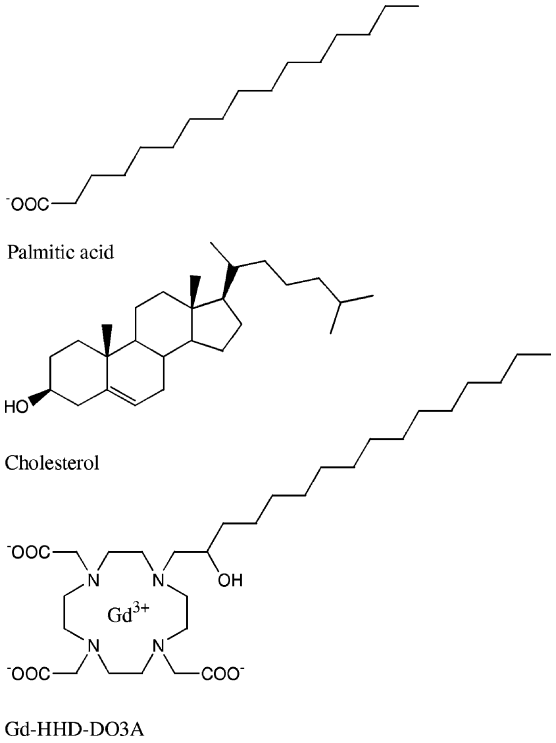

Fig. 1. Molecular structures of Gd-HHD-DO3A, PA and cholesterol.

2. Materials and methods

2.1. Materials

Palmitic acid, cholesterol and glucose were obtained from Fluka Chemical Co. (St. Louis, MO). All purchased chemicals were used as received and had a purity above 99%. Gd-HHD-DO3A was prepared according to published procedure (Gløgård et al., 2000).

2.2. Methods

2.2.1. Formulation and characterization of particles

Gd-HHD-DO3A, cholesterol and PA were co-dissolved in a mixture of chloroform and methanol (4:1, v/v) and evaporated under reduced pressure. The Gd-chelate was added from stock solution. The thin

film obtained was hydrated using aqueous isotonic glucose (50 mg ml^{-1}, pH 7.4). The total amount of particles was approximately 20 mg ml^{-1}(0.25–3.0 mM Gd). The resulting dispersion was heated at 70 °C for 2 h. The particles were irradiated using an ultrasound probe for 10 min and dialysed against glucose (50 mg ml^{-1}, pH 7.4) overnight using Slize-A-Lyzer® dialysis cassette (M_w cut-off 10,000) (Pirce, Rockford, IL). The T_1-relaxation time (s) was measured before and after dialysis.

The mean particle size was measured at 90° angle (25 °C) by photon correlation spectroscopy (PCS) after dilution with isoton glucose using a Coulter DELSA plus (Beckmann Coulter Inc., Fullerton, CA). The cryo-micrographs were recorded by G. Karlsson, Department of Physical Chemistry, University of Uppsala, Sweden (Almgren et al., 2000).

2.2.2. In vitro relaxometry

The relaxation measurements were performed at 20 MHz (Minispec mq 20 NMR Analyzer, Bruker GmbH, Rheinstetten, Germany) and the T_1-relaxation rates (R_1^{obs}) were obtained by the inversion recovery method at 37 °C. For the temperature profile curve, the temperature was varied between 5 and 70 °C.

The T_1-relaxivitiy (r_1) for each formulation was obtained either by the relationship:

$$r_1 = \frac{R_1^{obs} - R_1^{m}}{C},$$

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.

2.2.3. Stability during storage

All the samples were stored at room temperature for 6 months. The size and T_1-relaxivity was measured during storage.

2.2.4. Stability in whole blood

Human whole blood (EDTA as anticoagulant) was mixed with the particles to give final Gd-concentrations of 0.5, 1.0 and 5.0 mM. Samples were incubated at 37 °C for up to 3 h. The T_1-relaxivity was monitored during this period.

2.2.5. Experimental design

The design and evaluation of the statistical experiments were performed using Unscrambler software (Camo, Trondheim, Norway). In order to find a region with small particles and high T_1-relaxivity a D-optimal mixture design was applied with a total of 17 experiments. Eight different formulations were made, where replicates were included in order to estimate the experimental error. The different formulations are shown in Table 1. The batches were produced in a random order.

A principal component analysis (PCA) was first performed on the data set from the D-optimal mixture design (Esbensen, 2000). The data was autoscaled before any statistical operations were performed. Full cross validation was used. A partial least square analysis (PLS 1) with full cross validation was further used to fit each response (size and T_1-relaxivity) to the variables (cholesterol, Gd-HHD-DO3A and PA) (Esbensen, 2000). The response surfaces for particle size and T_1-relaxivity are based on these models.

3. Results and discussion

3.1. D-optimal mixture design

The particles were prepared according to the experimental plan. The different compositions, the size and the T_1-relaxivity of the particles are shown in Table 1. T_1-relaxation rate (R_1^{obs}) and particle size were measured before and after dialysis. No change was observed, indicating that the Gd-HHD-DO3A was completely incorporated into the particles.

3.1.1. Effects of the size and the T_1-relaxivity of the formed particles

Fig. 2 shows a PCA bi-plot (loading and scores) with the three factors (Gd-HHD-DO3A, cholesterol, PA), the two responses (T_1-relaxivity, particle size) and all the samples (1–17). Variables close to each other and along the same straight line through the origin covary. They are positively correlated if they are situated on the same side of origin and negatively correlated if they lie on the opposite side. Variables of little importance lie near the origin. The PCA explained 86% of the variance in the original matrix by the first two components.

Table 1
Different composition of the particles and the corresponding size and T_1-relaxivity

Run order	Formulation no.	Gd-HHD-DO3A (mol%)	Cholesterol (mol%)	Palmitic acid (mol%)	Size (nm)	T_1-relaxivity ($mM^{-1}\,s^{-1}$)
1	III	27.5	62.5	10.0	117	12.3
2	I	60.0	40.0	0	83.7	23.3
3	II	5.00	95.0	0	1850	17.5
4	IV	5.00	75.0	20.0	785	14.1
5	III	27.5	62.5	10.0	190	11.5
6	II	5.00	95.0	0	1290	17.1
7	III	27.5	62.5	10.0	177	11.5
8	VII	5.00	85.0	10.0	896	13.9
9	IV	5.00	75.0	20.0	402	13.6
10	II	5.00	95.0	0	1630	17.3
11	VIII	50.0	40.0	10.0	80.2	21.7
12	VI	32.5	67.5	0	261	11.6
13	I	60.0	40.0	0	89.9	23.8
14	I	60.0	40.0	0	128	22.4
15	V	40.0	40.0	20.0	102	19.7
16	V	40.0	40.0	20.0	157	20.9
17	VI	32.5	67.5	0	236	11.9

Three groups of scores may be distinguished in the plot. In the lower left corner a group (formulation no. II) is situated in direction of the particle size and hence shows large particles (>1280 nm). The group shows a medium high relaxivity ($\sim$17.3 $mM^{-1}\,s^{-1}$). These particles contain high amounts of cholesterol and corresponding less Gd-HHD-DO3A. In the upper left corner a group (formulation no. III, IV, VI

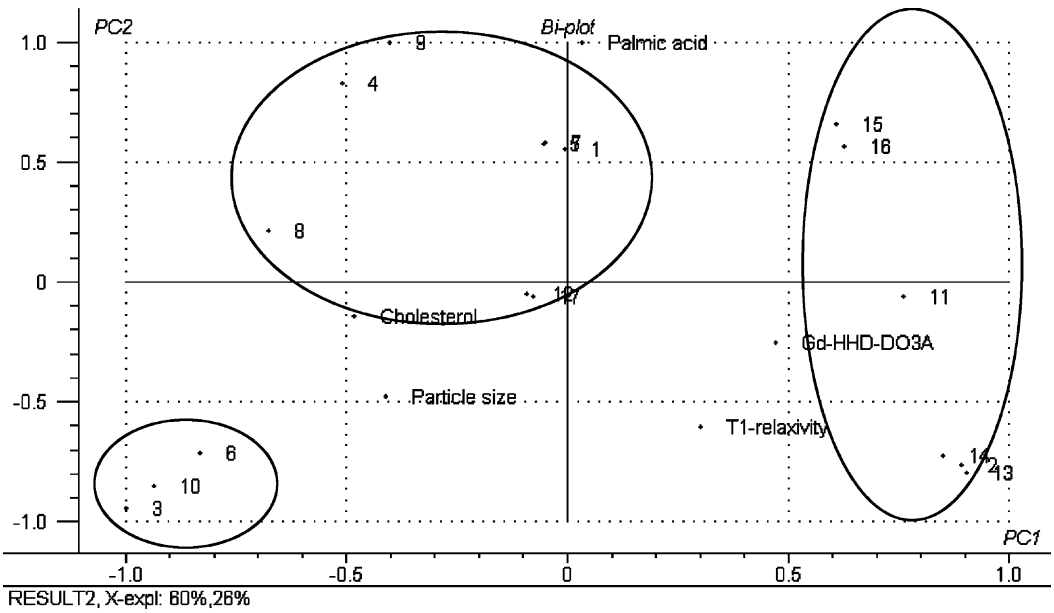

Fig. 2. PCA bi-plot. Loadings (factors and responses) are shown together with the scores (samples). Three groups of scores are distinguished in the plot.

and VII) with particles of medium size (117–896 nm) and low relaxivity (11.5–14.1 $mM^{-1}\,s^{-1}$) is located. These particles contain medium level cholesterol. To the right in the plot, a group is situated in the direction of the response T_1-relaxivity (formulation no. I, V and VIII) and hence shows high T_1-relaxivity (19.7–23.8 $mM^{-1}\,s^{-1}$). All these particles have small sizes (83.7–157 nm). These particles contain high amounts of Gd-HHD-DO3A and corresponding less cholesterol. The group is stretched out due to the negative influence of PA on the T_1-relaxivity.

The correlation between size of the particles and the T_1-relaxivity is further shown in Fig. 3. The plot clearly shows that two different effects are influencing the T_1-relaxivity. This implies that at least two different structures of the particles are formed depending upon the composition. First, a strong correlation is shown between the size of the particles and the T_1-relaxivity. The formulations with these properties are the two groups to the left in Fig. 1. By increasing the size, a higher T_1-relaxivity is obtained. These formulations contain medium to high levels of cholesterol, where an increased level of cholesterol results in a higher T_1-relaxivity. A higher T_1-relaxivity with increased cholesterol content and corresponding increased particle size might be due to an increased rigidity of the particle, which results in decreased lateral motion and thereby a prolonged τ_R for the Gd-chelates. The overall low T_1-relaxivity of this group implies that the structures formed not fully allow bulk water to experience magnetic interaction with the Gd-chelates. Some Gd-chelates might be located in the interior of the structures where the water exchange rate is low. Another explanation might be that the lateral motion on the surface is higher which results in a shorter τ_R for the Gd-chelates and thereby decreasing the relaxivity. Second, the group with the highest T_1-relaxivity contains the particles with the smallest size. This group is the same as the formulations to the right in Fig. 1 (formulation no. I, V and VIII). No effect of size on the T_1-relaxivity is shown within this group. However, the negative effect of PA on the T_1-relaxivity is shown. The higher T_1-relaxivity implies particle-structures, which allow bulk water to experience magnetic interaction with the Gd-chelates, incorporated in the particles.

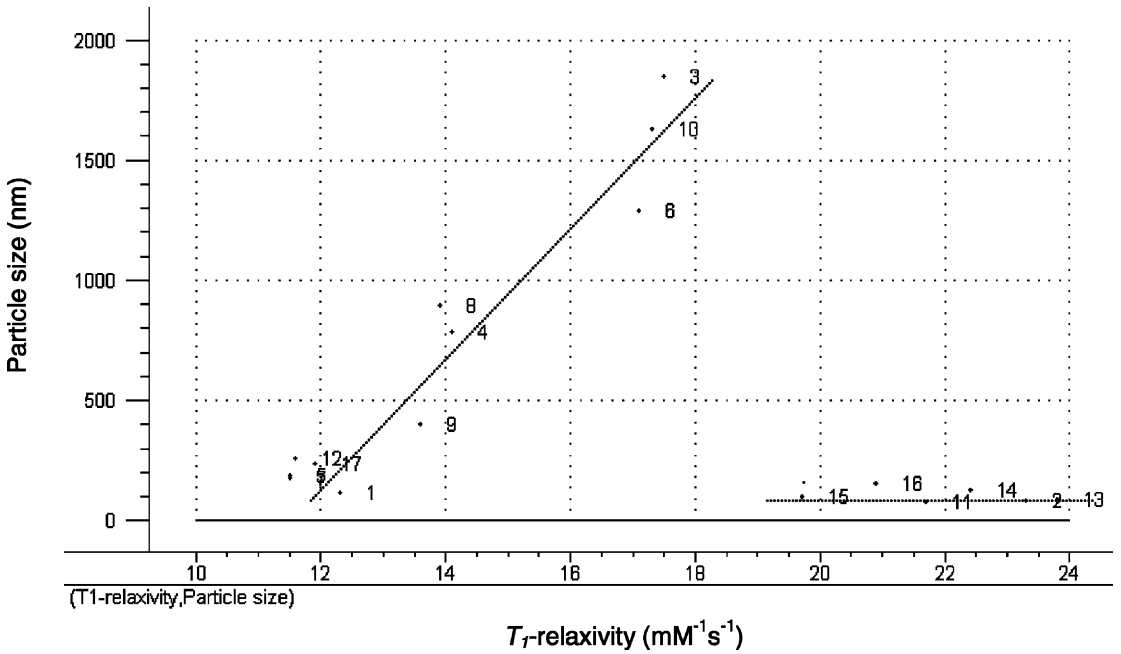

Fig. 3. Correlation between particle size (nm) and T_1-relaxivity ($mM^{-1}\,s^{-1}$).

3.1.2. Optimisation of the size and T_1-relaxivity of the formed particles

The response surfaces constructed from the results obtained in the D-optimal matrix design with size and T_1-relaxivity of the formed particles as responses are shown in Figs. 4 and 5. In addition to the main effects, the square effect of cholesterol and Gd-HHD-DO3A was included in the model for size whereas the interaction between cholesterol and Gd-HHD-DO3A was included in the model for T_1-relaxivity. The *X*-explained variance was 92% and the *Y*-explained variance was 76% with two principal components (PC1 and PC2) in the PLS 1 model for size. The corresponding values for the PLS 1 model for the T_1-relaxivity was 99 and 93%, respectively.

From the response surface (Fig. 4), smaller particles can be obtained in a region of medium to low level of cholesterol and hence medium to high level of Gd-HHD-DO3A. The smallest size of the particles (below 100 nm) in this design was achieved by keeping the ratio between cholesterol and Gd-HHD-DO3A between 40:40 and 40:60 (mol:mol) (formulation no. I, V and VIII). This can most probably be explained by the phase organisation of amphiphilic molecules prefers, alone or in mixtures with other amphiphiles (Israelachvili et al., 1980). Cholesterol ($P > 1$) has a shape opposite of Gd-HHD-DO3A ($P < 1$) and when combining these two compounds they should create units with a packing parameter equal to 1, which corresponds to a rectangular shape. Only when the ratio is approximately 1:1 the formation of the small structures seems to be favourable. An excess of Gd-HHD-DO3A might aim in the formation of these structures, but as Gd-HHD-DO3A forms micelles this might not be preferable.

As shown in the response surface in Fig. 5, the highest T_1-relaxivity is found in the same region of the design as the particles with the smallest size (Fig. 4). A ratio between cholesterol and Gd-HHD-DO3A between 40:40 and 40:60 (mol:mol) (formulation no. I, V and VIII) results in the highest T_1-relaxivity (up to 23.8 $mM^{-1}\,s^{-1}$), which most probably is due to a preferable structure of the particles resulting in high water availability for the Gd-chelates. On the

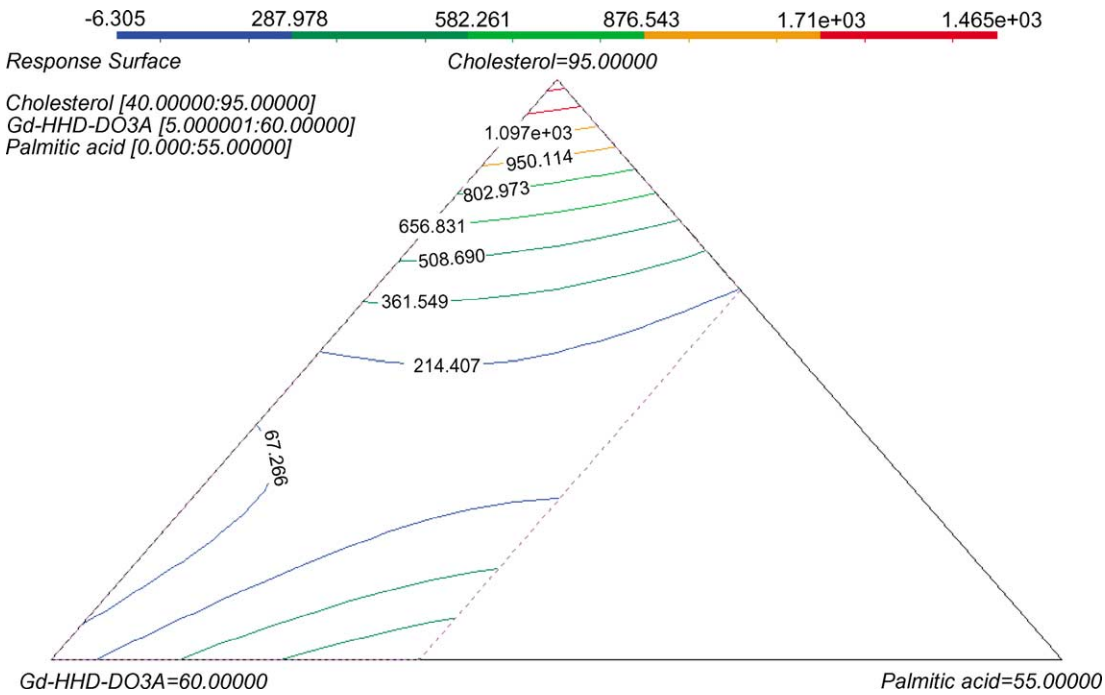

Fig. 4. Response surface obtained from the D-optimal mixture design showing the particle size (nm) as a function of Gd-HHD-DO3A (5–60 mol%), cholesterol (40–95%) and PA (0–20%).

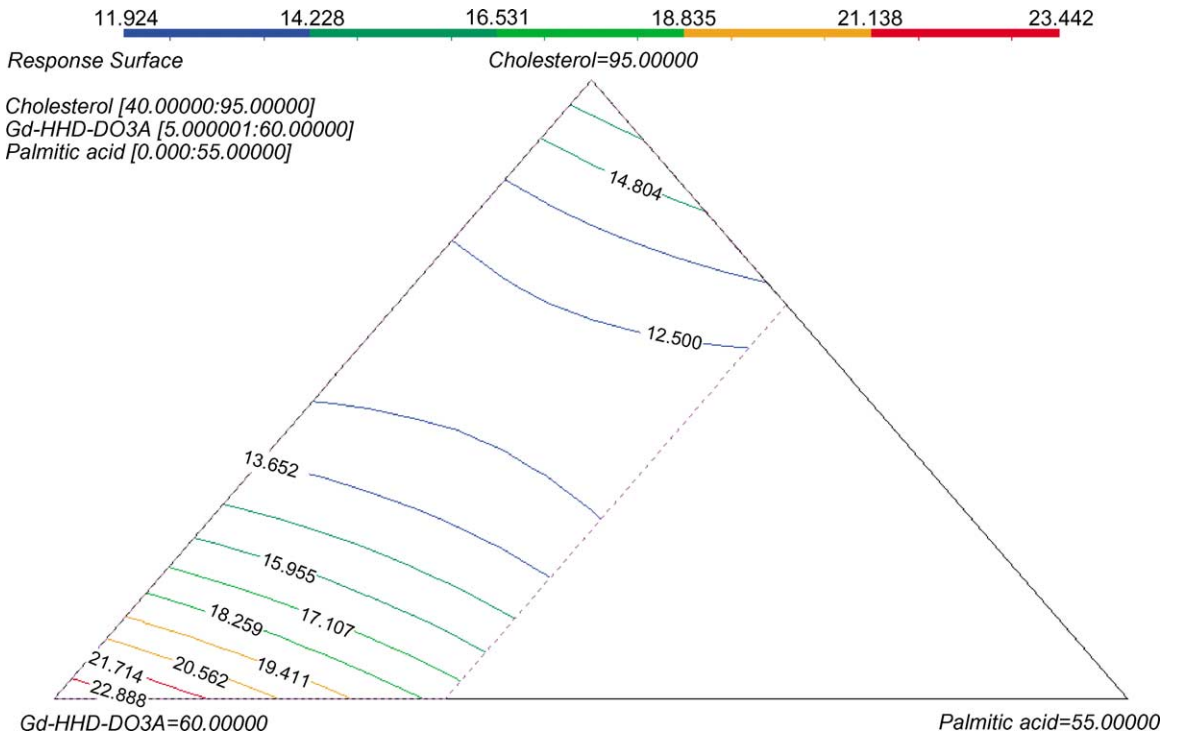

Fig. 5. Response surface obtained from the D-optimal mixture design showing the T_1-relaxivity ($mM^{-1}\,s^{-1}$) as a function of Gd-HHD-DO3A (5–60 mol%), cholesterol (40–95%) and PA (0–20%).

other end of the plot, medium high relaxivity is obtained due to the corresponding larger size of the particles.

The ability of amphiphilic Gd-chelates and cholesterol to form bilayers at approximately equimolar ratio was suggested in an earlier paper (Gløgård et al., 2002). In this paper amphiphilic Gd-chelates were incorporated into the liposomal lamella. The present drug dosage form allows higher incorporation of amphiphilic Gd-chelates compared to liposomes. Concentrations of the amphiphilic Gd-chelate, Gd-HHD-DO3A, was successfully included in liposomal formulation up to 10 mol%, compared to 40–60 mol% in the present study. Higher loading of Gd-HHD-DO3A in liposomes results in instability of the colloidal structure and has also shown to reduce the T_1-relaxivity (Gløgård et al., 2002). Another advantage is the simpler preparation method for these particles and the possibility to prepare higher concentrations compared to liposomes. Unfortunately, the T_1-relaxivity is lower for the novel drug system compare to the liposomes where values as high as $52\,mM^{-1}\,s^{-1}$ was obtained. This implies that the structures formed not fully allow bulk water to experience magnetic interaction with the Gd-chelates or that the lateral motion on the surface is higher which results in a shorter τ_R for the Gd-chelates.

3.2. Stability of the formed particles

3.2.1. Stability on storage

The size and the T_1-relaxivity of the formed particles during 6 months storage is shown in Table 2. Of the selected formulations (I, V and VIII), only formulation no. I and VIII proved to be stable during this period. As excess Gd-HHD-DO3A is not preferable and a negative charge might be valuable for long-term stability, formulation no. VIII was selected as lead formulation for further work.

For the other formulations, an increase in particle size most often resulted in a decrease in T_1-relaxivity due to formation of particle-structures, which do not

Table 2
Size and T_1-relaxivity of the formed particles during 6 months storage at room temperature

Run order	Formulation no.	Size (nm)				T_1-relaxivity ($mM^{-1} s^{-1}$)	
		T_0	3 weeks	3 months	6 months	T_0	6 months
1	III	117	118	122	121	12.3	13.8
2	I	83.7	84	83	85	23.3	23.3
3	II	1850	1800	1645	1500	17.5	12.0
4	IV	785	801	836	110	14.1	12.9
5	III	190	185	187	189	11.5	11.3
6	II	1290	1130	1600	2430	17.1	15.8
7	III	177	184	171	165	11.5	12.5
8	VII	896	903	1030	1800	13.9	15.3
9	IV	402	396	408	441	13.6	13.2
10	II	1630	1500	1650	1700	17.3	11.7
11	VIII	80.2	90	83	79	21.7	20.6
12	VI	261	256	243	159	11.6	22.8
13	I	89.9	88	102	nd	23.8	nd
14	I	128	132	129	nd	22.4	nd
15	V	102	155	306	495	19.7	13.1
16	V	157	180	1175	695	20.9	13.4
17	VI	236	233	230	229	11.9	12.1

nd, not determined due to microbial growth in the sample.

allow water to experience magnetic interaction with the Gd-chelates incorporated in the particles.

3.2.2. Stability in blood

No change in the T_1-relaxivity was observed when formulation no. VIII was mixed with whole blood and incubated (37 °C) up to 3 h. This shows that the particles, most probably, do not interact with the blood components. The data also suggest that the Gd-HHD-DO3A form thermodynamically stable colloidal particles with cholesterol as no dilution effect was observed. Also the size-measurements point in this direction as no change was observed during measurements of the diluted samples. This is an advantage compare to micellar systems where the thermodynamic equilibrium depends largely on the critical micellar concentration and rapid dilution leads to instant instability.

3.3. Structure and size of the colloidal particles

A cryo-electron micrograph of formulations no. VIII is shown in Fig. 6. The sample contains mainly disc-like structures of different sizes. The discs appear with a face-on orientation or edge-on orientation in the cryo-TEM image. The sample contains in addition a few liposomes/veshicles and in the background of the micrograph some small spots that might be globular micelles can be seen.

The mean size of the particles (formulation no. VIII) measured by PCS was 80 nm, which corresponds well with the size of the discs showing the face-on orientation in the image. Estimating the edge thickness of the particles from the cryo-TEM image indicates that they are less than 5 nm thick.

Similar disc-shaped particles are characteristics for Amphocil® containing amphotericin B and sodium cholesteryl sulfate in a 1:1 molar ratio (Guo et al., 1991; Hillery, 1997) and other cholesterol rich formulations with amphiphiles (Edwards et al., 1997). Guo and co-workers suggested that the interaction with amphotericin B and sodium cholesteroyl sulfate results in rigid and tightly packed lipid layers that are unable to bend sufficiently to form closed vesicular structures. Amphotericin B probably forms a shield at the disc edges, orientated to expose the seven hydroxyl groups to the polar aqueous environment. These disc-shaped particles do not have any entrapped aqueous volume.

A possible structure in this case is that Gd-HHD-DO3A and cholesterol are arranged in a 1:1 interdigitated complex, in which the polar head group of the Gd-chelates and the hydroxyl group of cholesterol

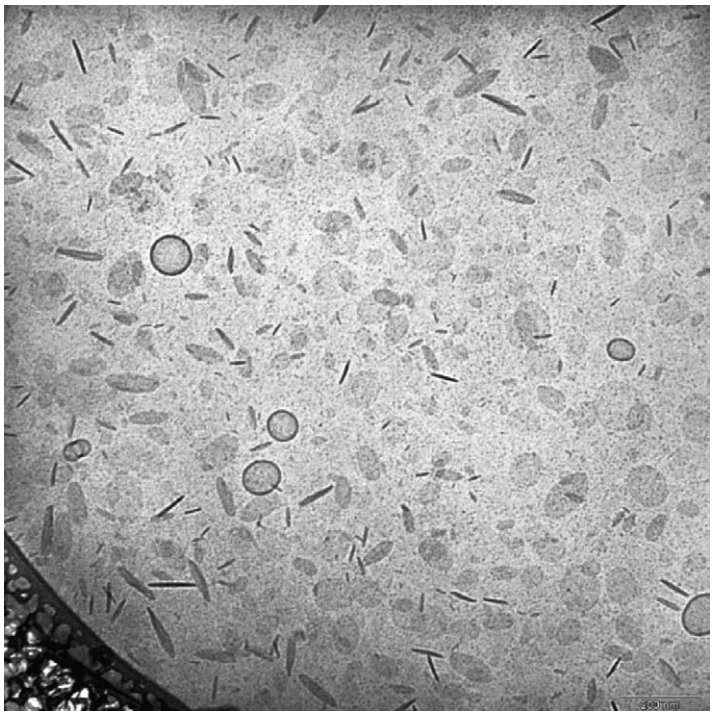

Fig. 6. Cryo-electron micrographs of Gd-HHD-DO3A, cholesterol, PA in the molar ratio 50:40:10.

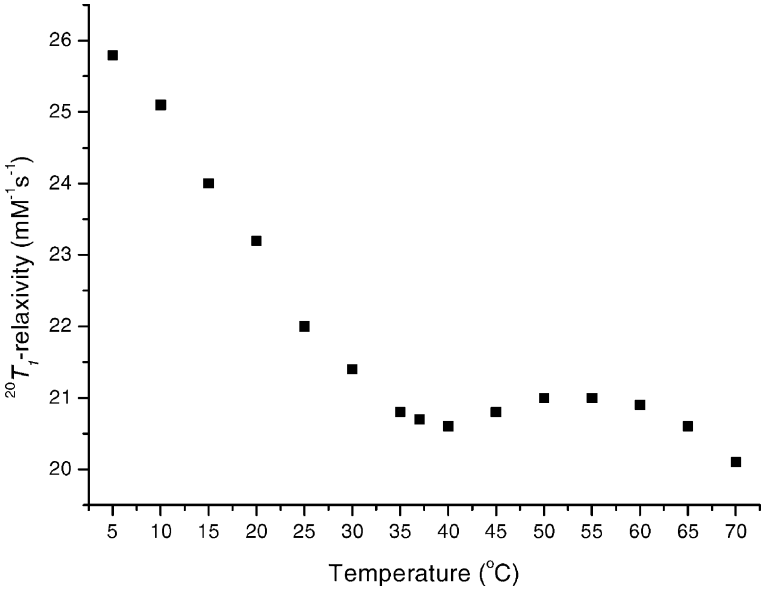

Fig. 7. The temperature (°C) vs. T_1-relaxivity (mM^{-1} s^{-1}).

shield the lipidic chain of the Gd-chelate and the cholesterol backbone from the aqueous environment.

3.4. Effect of temperature

The temperature profile of formulation no. VIII is shown in Fig. 7. The observed decrease in T_1-relaxivity with increase in temperature is probably due to a shortening of τ_R. The relaxivity peak around 55 °C might be explained by increased hydration of the particles. Alternatively, the increase in relaxivity might be accounted for by the small amounts of liposomes present in the sample. The water exchange across the liposome membrane increases at higher temperatures allowing Gd-chelates present on the inner side of the bilayer to contribute more to the overall relaxivity.

4. Conclusions

Novel high relaxivity particles based on the phase organisation of amphiphilic gadolinium chelates with cholesterol are developed. At an approximately equimolar ratio, thermodynamically stable discs of colloidal sizes are formed. This novel drug dosage form allows high loading of amphiphilic Gd-chelates and proved to be stable both in whole blood and upon long-term storage.

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