Liposomes with conjugates of a calix[4]arene and a Gd-DOTA derivative on the outside surface; an efficient potential contrast agent for MRI†

Daniel T. Schüle,a Patrick van Rijn,b Sophie Laurent,c Luce Vander Elst,c Robert N. Muller,c Marc C. A. Stuart,d Jürgen Schatze and Joop A. Peters*a

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Paramagnetic liposomes used as contrast agents in magnetic resonance imaging (MRI) often suffer from low efficacies because of slow water diffusion through the membrane. We present an approach to overcome this limitation by incorporation of a calix[4]arene based agent that expresses the chelates towards the bulk water.

Paramagnetic Gd(mn) complexes are widely used contrast agents (CAs) in magnetic resonance imaging (MRI). They function by a shortening of the longitudinal relaxation time ($T_{1,\text{obs}}$) of surrounding water protons, which is determined by the local concentration of the CA ([Gd]) and its relaxivity ($r_1$) (eqn (1)).

$$\frac{1}{T_{1,\text{obs}}} = \frac{1}{T_{1,\text{tissue}}} + r_1 [\text{Gd}]$$

Whereas $r_1$ can be enhanced by, for instance, an increase of the size and therefore of the rotational correlation time ($\tau_R$) of the CA, a higher [Gd] can be achieved by the introduction of targeting functions to the agent and/or the attachment of several Gd-chelators to a molecular platform leading to a higher Gd-payload per molecule CA.$^{1,2}$ Self-assembled CAs are very promising since they do not only allow the delivery of a huge amount of chelates to the site of interest, they also result in relatively large systems with favorably long $\tau_R$. $^3$ Moreover, micelles and liposomes of proper sizes are able to accumulate selectively in tumor tissue by enhanced permeability and retention.

Liposomes with MRI CAs included in their membranes offer the additional possibility to entrap therapeutics or diagnostics for other molecular imaging techniques (e.g. PET and SPECT) in their cavities. $^4$ A disadvantage of liposome systems with MRI CAs incorporated in the membrane is that in the systems reported to date, half of the Gd-chelate molecules are oriented inside. These Gd-chelates have poor relaxivities caused by the low water permeability ($P_w$) of the hydrophobic membranes.$^5$ This is limiting significantly the overall relaxivity.$^6$ Higher $r_1$ can be reached by the design of systems with higher $P_w$, but these may have decreased serum stability. Here, we report the preparation and characterization of a system with very high relaxivity by inclusion of calix[4]arene I (Fig. 1) into the outer layer of the membrane of DSPC/DSPE-PEG2000-OMe/cholesterol liposomes.

Calix[4]arene I was synthesized by alkylation of 5,11,17,23-tetra-tert-butyl-25,27-dihydroxy-26,28-dipropoxy-calix[4]-arene with octadecylbromide. Isopinotration and subsequent reduction yielded a tetra-amino-calix[4]arene that was coupled with tert-butyl-DO3A-monoacid. After deprotection of the tert-butyl functions, the ligand was complexed with Gd(mn).

Since the presence of 1 during the lipid film preparation leads to disruption of the vesicles, it was impossible to prepare the liposomes by mixing all components prior to the lipid film preparation. Therefore, first a mixture of DSPC/DSPE-PEG2000-OMe/cholesterol was dissolved in CHCl$_3$ and the solvents were removed to yield a lipid film. After resuspension in HEPES buffer, 8.8 mol% of 1 was added and the mixture was extruded at 85 °C subsequently through filters with diameters of 200 and 100 nm. Dynamic Light Scattering (DLS) showed that the liposomes obtained had a narrow size distribution (PDI < 0.1) and an average hydrodynamic radius of 100 nm (Fig. S2 in ESI†).

This was confirmed by transmission electron microscopy (cryo-TEM) and size exclusion chromatography (SEC). The
shift in the phase transition temperature, \( T_m \) as determined by
differential scanning calorimetry, from 51.6 °C for a blank
sample to 48.4 °C for the sample containing 1 is an indication
that 1 is included in the liposomal bilayer. The stability of
these liposomes was investigated by monitoring the size and
polydispersity of samples stored at 4 °C in time. Even after
8 weeks, no change was observed suggesting that the particles
are highly stable, which may be ascribed to the presence of
cholesterol (33 mol%) and to the steric protection by
PEGylation.

Nuclear Magnetic Relaxation Dispersion (NMRD) is one of
the most important techniques used to characterize MRI
CAs.\(^7\) The field dependence of \( r_1 \) gives important information
on the parameters governing the \( r_1 \) of a given system. The
NMRD profiles of the presently studied liposomes show local
maxima at about 20 MHz, which is typical for systems with a
long \( t_B \).\(^1,2\) For previously reported liposomal agents having
equal amounts of Gd-DTPA-BSA pointing outward and
inward from the membrane, NMRD profiles typically show a
steep increase of \( r_1 \) with temperature around \( T_m \).\(^8,9\) This is
ascribed to the increase of \( P_w \) with temperature leading to an
increased contribution to the overall \( r_1 \) of the inward pointing
Gd-chelates. Above \( T_m \), \( P_w \) is normally high enough to ensure
sufficient water exchange between in- and outside the lipo-
somes so that the permeability is no longer limiting the
relaxivity. By contrast, liposomes containing 1 only show a
gradual relatively small increase in \( r_1 \) with temperature, even
when \( T_m \) of the membranes is exceeded (Fig. 2).

This and the overall very high relaxivities compared to
both those of the previously studied monomeric tetratropil
analogue of 1\(^10\) and Gd-DTPA-BSA/DSPC/DSPE-PEG2000-
OMe/cholesterol suggest that either \( P_w \) is extremely high and
thus, in the presently studied system, there is no quenching
effect due to the membrane and/or that a significant amount of
Gd is oriented towards the bulk water. Therefore, relaxometric
\( P_w \) measurements were performed on the diamagnetic Y(III)
analog of the liposomes according to the procedure described
by Terreno.\(^11\) The \( P_w \) of this sample (17.3 × 10^{-5} \text{ cm s}^{-1})
is too low for efficient water transport. This in combination with
the overall high relaxivity suggests that the majority of the
Gd-chelates are pointing outward in the presently studied
system.

Further support for the predominant location of the
Gd-chelates on the outer layer of the membrane was obtained
by simulations of the relaxivity at 0.01 MHz as a function of
the temperature. These were performed by means of a two-step
model that considers the system as an aequous solution of
Gd-chelates in the interior of the liposome with the included
water molecules being in exchange with the Gd-chelates and
the bulk water outside of it. With the use of parameters
estimated from those for the previously studied calix[4]arene
systems\(^10\) and assuming that 50% of the chelates are located
inside the vesicles (\( f_{in} = 0.5 \)), a temperature jump in relaxivity
of 5 s^{-1} mM^{-1} is expected if the residence time of water in the
cavity of the liposomes decreases from 9.1 (the value corre-
sponding to \( P_w \) to 0.1 ms. By contrast, the relaxivity remains
almost constant when \( f_{in} \) is zero (see ES1†). From these
simulations, it may be concluded that at least 80% of the
Gd-chelates are oriented towards the outside of the liposomes.
The slight increase of \( r_1 \) with temperature is a consequence
of an accelerated exchange rate of water directly bound to
the metal center (\( k_{ex} \)). Finally, a good simultaneous fit of
the NMRD profiles was obtained with the Solomon-
Bloembergen-Morgan theory adapted with the Lipari-Szabo
approach to take into account the effects of local mobility
assuming that all Gd-chelates are located in the outer
membrane layer (see ES1†). The best-fit parameters (see Fig. 2
(lines) and ES1†) are in the range to be expected based on
values obtained for the previously studied low-molecular
weight calix[4]arene system.\(^10\)

Attempts to prepare liposomes with about equal amounts of
chelate on the inner and outer membrane layer with the use of
the “classical” preparation protocol\(^6,12\) failed; cryo-TEM
images showed that complex mixtures of aggregates with a
high content of disk-like micelles (bicelles) along with vesicles
that were not completely closed or multilamellar (see ES1†)
were always obtained.

In conclusion, we present a novel approach towards stable
liposomal MRI contrast agents with high relaxivity. The
calix[4]arene derivative is mainly incorporated at the outer
surface of the vesicles. The Gd(III) ion is chelated with DOTA,
which guarantees a high kinetic stability, which is an
advantage in view of the toxicity of free Gd(III). Apparently,
the steric demands of I favor its inclusion in the outer layer
of the membrane. The bulkiness of 1 practically excludes a
flip-flop of the Gd-chelates from the outer to the inner layer
of the membrane.\(^13\) The cavity of these liposomes is available
for therapeutics and other diagnostics and, therefore, these
systems may have potential as dual modality probes for
molecular imaging and as theranostics.

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Notes and references