Magnetoliposomes with high USPIO entrapment efficiency, stability and magnetic properties

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Abstract

The DRV technique (followed by extrusion) was used for construction of hydrophilic-USPIO encapsulating liposomes. Magnetoliposomes (ML) were characterized for size, surface charge, entrapment, physical stability and magnetic properties (relaxivity). Results show that nanosized extruded-DRV MLs encapsulate higher amounts of USPIOs in comparison with sonicated vesicles. Fe (III) encapsulation efficiency (EE) is 12%, the highest reported to date for nanosized MLs. EE of MLs is influenced by ML membrane composition and polyethyleneglycol (PEG) coating. PEG-coating increases ML EE and stability; however, r2-to-r1 ratios decrease (in comparison with non-PEGylated MLs). Most ML-types are efficient T2 contrast agents (because r2-to-r1 ratios are higher than that of free USPIOs). Targeted MLs were formed by successfully immobilizing OX-26 monoclonal antibody on ML surface (biotin-streptavidin ligation), without significant loss of USPIOs. Targeted MLs retained their nanosize and integrity during storage for 1 month at 4°C and up to 2 weeks at 37°C.

From the Clinical Editor: Skouras and colleagues present a method for high encapsulation of hydrophilic USPIO-s in magnetoliposomes using the DRV extrusion technique. The goal is to optimize the production of MRI detectable contrast agents with functionalized homing capability based on immobilizing specific antibodies in the surface of magnetoliposomes.

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Key words: Magnetoliposomes; USPIO; Nanoparticle; Magnetic; Targeting; Monoclonal antibody

Magnetic fluids in the form of colloidal suspensions or nanoparticles (NPs) have widespread applications as magnetic resonance imaging (MRI) contrast agents.1,2 In this context, they can be used in combination with nanosized drug carriers to serve as theranostic systems (i.e., nanoscale delivery systems with combinatory therapeutic diagnostic imaging modalities).3-5 One type of such NPs is the ultra-small (with diameters \(<50\) nm) super paramagnetic iron oxide cores (USPIOs), which are efficient T2 contrast agents; however, USPIO stability is usually a problem for their successful in vivo use. To increase USPIO stability (decrease their tendency to aggregate) several types of coatings have been considered; as dextrans, proteins, polymers, fatty acids or phospholipids.6-8 Although stabilization is achieved by coating USPIOs with phospholipids, and the phospholipid coating can be used as an anchor for decoration of their surface with targeting ligands, when such magnetoliposomes (MLs) are used as diagnostics, one targeted vesicle transfers only one paramagnetic particle. Furthermore, co-loading with drugs (for theranostic applications) is not efficient. USPIO-entrapping nanosized liposomes may be considered as alternative ML formulations, with better magnetic properties (due to the possibility of entrapping many USPIOs in one vesicle) and ability to co-load large amounts of drugs. In recent years, such systems have been formulated by conventional liposome-formation techniques as sonication,9 film hydration,10 extrusion through polycarbonate membranes,11,12 or reverse

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inclusion. In these previous studies, the entrapment efficiency of magnetic NPs in the MLs was either not measured or not optimized. Recently doxorubicin (DOX) and magnetic NPs were co-encapsulated in folate-decorated phase evaporation/extrusion. In these previous studies, the measured or not optimized. Recently doxorubicin (DOX) and magnetic NPs were co-encapsulated in folate-decorated liposomes; and although the DOX encapsulation was high, it was claimed that “there is a need to optimize magnetic particle incorporation.” When targeting applications are designed, any optimization of magnetic particle incorporation in MLs should not be followed by an increase of ML size. Indeed, MLs need to have high magnetic content for optimized magnetic responsiveness, as well as nano-dimensions for exploitation of the enhanced permeability and retention (EPR) effect in the case of tumor targeting, and/or ability to circumvent biological barriers (depending on the application). The former two requirements are difficult to reconcile.

Herein, for the first time the dried-rehydrated vesicle (DRV) technique was used to prepare USPIO-P00904 (Guerbet, Roissy, France) entrapping MLs. The specific USPIOs used were stabilized by a hydrophilic coating. Several lipid compositions and preparative aspects were initially investigated to optimize the ML encapsulation efficiency, size distribution, zeta potential, stability, integrity and magnetic properties (relaxivity values). Finally, the potential of the proposed technique for preparation of targeted MLs was evaluated by preparation of OX-26-monoclonal antibody (MAb)-decorated MLs.

Methods

Phosphatidyl choline (PC), 1,2-distearoyl-sn-glycerol-3-phosphatidylcholine (DSPC), Phosphatidyl glycerol (PG), 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG-lipid) and 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N-[biotinyl (polyethylene glycol)-2000] (Biotin-lipid) were purchased from Avanti Polar Lipids (Alabaster, Alabama). Cholesterol (Chol), streptavidin from S. avidinii (STREP), 10 nm gold nanoparticles (AuNPs) labeled with rabbit anti-mouse antibodies (immune-nanogold) and Sepharose CL-4B were purchased from Sigma-Aldrich (Chemilab SA, Athens, Greece). Spectrapore Dialysis tubing (MW cutoff 10,000) was from Serva (Heidelberg, Germany). Biotinylated OX-26 anti-transferin MAb was obtained from Guerbet and used as an isotonic solution in mannitol (28.7 mg Fe/mL).

ML preparation

Different types of MLs were prepared. Multilamellar vesicles (MLVs), small unilamellar vesicles (SUVs), dried-rehydrated vesicles (DRVs) as well as extruded-DRV MLs with the following lipid compositions (molar ratios reported): PC/Chol (2:1), PC/Chol (4:1), PC/Pg/Chol (9:1:5), DSPC and DSPC/Chol (2:1). In some cases, PEG-lipid was added at 1 mol%, 4 mol % or 8 mol% (of total phospholipid [without Chol]).

For MLVs, the thin-film hydration method was used. In brief, lipids were solvated in a chloroform/methanol (2:1 v/v) mixture and placed in a round flask. Lipid solution was dried by rotary evaporation until development of a thin lipid film. Residual organic solvents were removed by nitrogen and lipids were hydrated with appropriate amounts of the USPIO dispersion (usually 20 – 200 μl) and/or PBS buffer (10 mM, pH 7.40), at a temperature above the transition temperature of the lipid used. Next: (i) For MLVs, the liposome dispersion was sonicated for 15 minutes in a bath-type sonicator (Branson, Danbury, Connecticut); (ii) For SUVs, the liposome dispersion was sonicated by a probe microtip (Sonic and Materials, Suffolk, United Kingdom) for 2 10-minute cycles; (iii) For DRVs the method described earlier was applied. In brief, empty SUVs were prepared (as described above); then 1 mL SUV dispersion was mixed with USPIO dispersion (20 – 200 μl), the mixture was freeze-dried and rehydrated. Finally, all types of MLs prepared were incubated for 1 hour at a temperature above the lipid Tm (for annealing any structural defects).

The DRV-ML size was decreased by extrusion (20 times) through 2 stacked polycarbonate membranes (pore size 100 nm) in a Lipo-so-fast extruder system (Avestin, Mannheim, Germany) and the MLs produced by this method are referred to as extruded-DRVs.

The separation of liposome-encapsulated and free USPIOs was accomplished by gel exclusion chromatography (GEC) on a Sepharose–4B CL column, eluted with PBS buffer, pH 7.40. Lipid-containing fractions were pooled and concentrated (if needed) by ultrafiltration. To exclude the possibility that USPIOs aggregate and co-elute with liposomes during GEC, free USPIO dispersions were treated by the identical procedure used for ML formation (without addition of lipids) and assessed by GEC.

Targeted-ML preparation

Targeted-MLs were prepared by adding Biotin-lipid at 0.05 mol% with respect to total lipid in the lipid-phase during ML preparation. The biotin-streptavidin-biotin ligation was used. For antibody conjugation, biotinylated MLs were incubated first with 10-fold molar excess STREP (in comparison with biotin) for 1 hour at 25°C and then overnight at 4°C. Then free STREP was removed by GEC and finally OX-26 MAb decoration of the biotinylated- STREP-labeled MLs was performed by incubation with excess of biotin-MAb (attachment yield >90%).

ML characterization and stability

Vesicle entrapment efficiency

The Fe-to-lipid (mol/mol) ratio was calculated in all liposome types. For this, the Fe concentration was measured in liposomes, after vesicle disruption by a colorimetric method. In brief, 100 μl of HCl (37 %) and 10 μl of H2O2, were mixed (vortex) with 100 μl MLs and 1 mL of KSCN (2% w/v) was added. Sample was mixed (vortex) and optical density was measured (480 nm). The accuracy of this method for MLs was realized after construction of a calibration curve of USPIOs in the absence or in the presence of lipids and surfactant. The Fe
content of ML dispersions was also measured by inductively coupled plasma ICP-MS. For this purpose, 4500 µL of nitric acid 65% was added to 500 µL of the ML sample. The mixture was heated at 80°C during 8 hours, allowing a complete dissolution of the ML. After the mineralization step, the sample was dissolved in MilliQ water to obtain a final concentration of nitric acid at 32.5%. Total Fe concentration was quantified by ICP-MS (Optima 3300 RL, Perkin-Elmer, Shelton, Connecticut) using a set of calibration standards ranking from 0.2 mg/L to 10 mg/L of iron with yttrium as internal standard prepared in nitric acid 32.5% (RF power at 1500 W, Fe = 238,204 nm, Y = 324,227 nm).

The lipid concentration of ML dispersions was measured by the Stewart colorimetric assay.23 Phospholipids form a colored complex with ammonium ferrothiocyanate (OD-485 nm), which is extracted with chloroform. Lipid concentration is calculated based on a standard curve prepared with known phospholipid amounts. The lipid concentration of MLs was adjusted, as needed.

**Encapsulation Efficiency and physicochemical characteristics of MLs**

Concerning the effect of ML preparation technique on USPIO EE (Table 1), MLs prepared by DRV method have more than 2 times higher EE in comparison with those prepared by thin-film hydration (MLVs); also extruded-DRV MLs with mean diameters around 100 nm, have approximately 10 times higher EE in comparison with SUVs of similar size. The near 70-times decrease of mean diameter of the DRV MLs results in only a about 5-times decrease of Fe (III) EE. Thereby, it was decided to use the DRV-extrusion method for further optimization of USPIO-loading.

As presented in Table 2, higher EEs are achieved for both DRVs and extruded-DRVs, when diluted PBS (10% v/v) is used in the first step of DRV formation, (in comparison with full-strength [10 Mm] buffer) in accordance with previous results obtained for other types of encapsulated molecules.17 ML iron loading increases as initial Fe(III)-to-lipid ratio increases, in the specific Fe(III)–to-lipid ratio range evaluated. Indeed, when the initial Fe(III)-to-lipid ratio is increased by 10 times (from 0.25 to 2.5), a 7.7-times increase of the EE occurs for DRVVs and a corresponding 5-times increase for extruded DRVVs. When 4 mol % PEG-lipid is added in ML membranes, USPIO loading increases significantly (Figure 1). This change occurs when high (>4.5) initial Fe(III)-to-lipid ratios are used but is less pronounced (and statistically insignificant) at initial ratios below 2.56 (µmole Fe(III)/mg lipid). However the EE of MLs

### Table 1

<table>
<thead>
<tr>
<th>Type of liposome</th>
<th>Size (nm)</th>
<th>EE (mmole Fe / mg lipid)</th>
<th>Fe/lipid (mole/mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUV</td>
<td>125.3</td>
<td>0.01354</td>
<td>0.0107</td>
</tr>
<tr>
<td>MLV</td>
<td>5412</td>
<td>0.2792</td>
<td>0.221</td>
</tr>
<tr>
<td>DRV</td>
<td>8156</td>
<td>0.60564</td>
<td>0.478</td>
</tr>
<tr>
<td>DRV(extruded)</td>
<td>117.8</td>
<td>0.12417</td>
<td>0.098</td>
</tr>
</tbody>
</table>

MHz (1.42 Tesla). For this, the ML dispersions were diluted to obtain 5 points between 0.05 and 1.5 mM of Fe. The relaxivity was finally calculated by the slope of the linear regression performed on the 5 data points.

**Results**

### ML preparation

A complete separation of MLs from nonencapsulated USPIOs was achieved under the conditions applying for GEC (see Supplementary Figure S1, available online at http://www.nanomed-journal.com). It was demonstrated that plain USPIOs do not aggregate; thus, the MLs eluted from the GEC column are not “infected” by aggregated USPIOs (which may have co-eluted with the MLs). Additionally, the technique used for detection of Fe (III) in MLs was found to be reliable for the determination of the USPIO content of MLs (see Supplementary Figure S2).

### TEM studies

MLs (1-2 mg/ml) were deposited for 2 minutes on formvar-coated carbon-reinforced 300-mesh copper grids (Agar Scientific, Ltd., Essex, United Kingdom) and negatively stained with 5% ammonium molybdate (Sigma) for 2 minutes, washed with H2O (x 2), drained and observed at 100.000 eV with JEOL (JEM-2100) TEM. For demonstration of OX-26 MAb presence on the targeted-ML surface, control MLs (with no MAb) and sample MLs (with MAb) were allowed to react “on grid” with 10 nm immuno-nanogold for 30 minutes. The excess of immuno-nanogold was washed with PBS, and then MLs were stained as above. For comparison, empty liposomes were also visualized, after being processed using the same protocol.

### Magnetic properties of MLs

Relaxivity measurements were performed on various types of MLs with a Minispec Relaxometer (Bruker, Billerica, Massachusetts) at 37 ± 1°C, operating at 20 MHz (0.47 Tesla) and 60
does not increase any further when higher PEG-lipid contents are used (8 mol%), even at the highest initial ratios evaluated.

MLs with DSPC + 8 mol% PEG-lipid composition were used for attachment of OX-26 MAb on their surface (by the method described above) using a 5.1 (μmoles/mg) initial Fe-to-lipid ratio, and the EE of the targeted MLs was 0.436 ± 0.022 μmoles Fe/mg lipid. In fact EE was practically the same before and after the ML decoration procedure.

Stability of MLs

The stability of MLs with respect to mean diameter and PI was initially evaluated during incubation of extruded-DRV MLs with various lipid compositions at 4°C for periods up to 35 days. It is evident from Figure 2, A., that MLs that are not coated with PEG tend to aggregate after 25 days. The increase of ML surface charge (by addition of a charged lipid [PG] in the liposome membrane) does not provide higher liposome stability. In contrast, PEG-lipid coated MLs retained their initial size (approximately 100 nm) after 35 days of incubation (and even up to 2 months [not shown]), at 4°C. Furthermore, the PIs of PEGylated MLs were stable for the full incubation period (Figure 2, B). Figure 2 shows also that the targeted MLs are also very stable (size and PI) under the applying conditions. Because non-PEGylated MLs were seen to be unstable at 4°C, no further stability investigations were conducted for this ML type.

PEGylated MLs were demonstrated to retain their initial mean diameters and PIs also after 35 days of incubation at 37°C (Figure 3, A and B), with the exception of PC/Chol 4.1 + 4 mol % PEG-lipid MLs, which became aggregated after 7 days of incubation (not measured on day 10, due to large size). For the targeted MLs (appearing as red dots on the graphs), mean diameter and PI values were practically unchanged during 10 days at 37°C; however, significant aggregation was noticed on day 20. Nevertheless, the stability demonstrated is more than adequate for in vivo applications.

The integrity (retention of encapsulated quantities of USPIOs) of PEGylated ML types, as well as MAb-decorated MLs were also evaluated, during incubation at 4°C and 37°C for a period of 3 weeks. As demonstrated by the results (Figure 4) the ML types that demonstrated high stability in

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**Table 2**

<table>
<thead>
<tr>
<th>Initial USPIO volume (μl)</th>
<th>Initial ratio (Fe to lipid)</th>
<th>Encapsulation efficiency (μmoles Fe/mg lipid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>–hydration buffer</td>
<td>μmoles Fe/mg lipid</td>
<td>particles/mg lipid</td>
</tr>
<tr>
<td>10 -10%PBS</td>
<td>0.257</td>
<td>1.93 E+13</td>
</tr>
<tr>
<td>10 -PBS</td>
<td>1.285</td>
<td>5.79 E+14</td>
</tr>
<tr>
<td>50 -10%PBS</td>
<td>2.569</td>
<td>1.93 E+14</td>
</tr>
<tr>
<td>50 -PBS</td>
<td>1.285</td>
<td>5.79 E+14</td>
</tr>
<tr>
<td>100-10%PBS</td>
<td>2.569</td>
<td>1.93 E+14</td>
</tr>
<tr>
<td>100-PBS</td>
<td>1.285</td>
<td>5.79 E+14</td>
</tr>
</tbody>
</table>

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Figure 1. Encapsulation efficiencies (EE) of various types of DRV and extruded-DRV MLs in respect to initial Fe-to-lipid ratio. Each EE value (reported as μmole Fe(III)/mg lipid) is the mean value of at least 3 different preparations. Standard deviations (SDs) are presented as bars. Symbol key is included in the figure insert.

Figure 2. Size stability of various ML types during storage at 4°C for up to 35 days (A) mean diameter, and (B) PI. Each value is the mean of at least 3 different preparations. SDs are presented as bars. Red dots are for OX-26 MAb decorated MLs. Sample key is in figure insert.
terms of size characteristics can also retain the encapsulated USPIO loads under similar incubation conditions. In the same way, the OX-26 MAb-decorated targeted MLs retain their contents for at least 20 days at 4°C and 14 days at 37°C, which is in good correlation with the corresponding size stability (Figures 2 and 3).

Magnetic properties of MLs

Relaxometric properties of plain USPIOs and various ML types were measured (Table 3) and in most cases, the relation of inversed relaxation times $T_1^{-1}$ and $T_2^{-1}$ versus Fe concentration was linear ($R^2$ values between 0.99 – 1), with a few exceptions for some non-PEGylated MLs for which the relations were not very linear ($R^2$ between 0.97 – 0.98); $r_1$ and $r_2$ relaxivities were calculated from the slope of each line. For both, plain USPIOs and MLs the traversal relaxivity $r_2$ was found to be significantly higher in comparison with longitudinal $r_1$, confirming that they are suitable as T2 contrast agents. With the exception of MLs consisted of PC/Chol (4:1) + 4 mol% PEG-lipid, the $r_1$ relaxivities of MLs are lower than that of plain USPIOs. This indicates a “quenching” of the USPIO efficacy on the $T_1$ relaxation time, due to accessibility of water to the interior of liposomes. As seen in Table 3, when PEG-lipids are added in the membranes of specific ML types, the corresponding $r_1$ values increase (in comparison with those of non-PEGylated MLs).

The ratio of $r_2$ to $r_1$ is a measure of ML potential as a contrast agent. This ratio decreases when DSPC-MLs are PEGylated with 4 mol% PEG-lipid (Figure 5), mostly due to increase of $r_1$. However, in the case of PC/Chol (2:1) MLs (Figure 5), PEGylation does not have such effect, suggesting that other

![Figure 3](image1.png)  
**Figure 3.** Mean diameter (A graph) and PI (B graph) of various MLs during incubation at 37°C. Each value is the mean of at least 3 different preparations. SDs are presented as bars. Red dots are for OX-26 MAb decorated MLs. Sample key is in the figure insert.

![Figure 4](image2.png)  
**Figure 4.** Retention of encapsulated USPIOs in PEGylated MLs and MAb-decorated MLs, during incubation at 4°C and 37°C, for 3 weeks. Each value is the mean of at least 3 different preparations; SDs are presented as bars. Sample key is included in the figure insert; solid symbols are for 4°C and the corresponding open symbols are for 37°C.

<table>
<thead>
<tr>
<th>ML Lipid Composition</th>
<th>Fe/lipid (mol/mol)</th>
<th>$r_1$ (mM⁻¹.s⁻¹)</th>
<th>$r_2$ (mM⁻¹.s⁻¹)</th>
<th>$r_1$ (mM⁻¹.s⁻¹)</th>
<th>$r_2$ (mM⁻¹.s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC/Chol 2:1</td>
<td>0.142</td>
<td>7.4</td>
<td>126.6</td>
<td>5</td>
<td>115.3</td>
</tr>
<tr>
<td>PC/Chol 2:1</td>
<td>0.156</td>
<td>7.3</td>
<td>114.3</td>
<td>4.9</td>
<td>120</td>
</tr>
<tr>
<td>PC/Chol 4:1</td>
<td>0.125</td>
<td>9.1</td>
<td>129.9</td>
<td>5.7</td>
<td>148.8</td>
</tr>
<tr>
<td>PC/Chol 4:1</td>
<td>0.158</td>
<td>8.6</td>
<td>139.4</td>
<td>5.4</td>
<td>128.7</td>
</tr>
<tr>
<td>DSPC</td>
<td>0.087</td>
<td>2.6</td>
<td>95.4</td>
<td>1.2</td>
<td>95.8</td>
</tr>
<tr>
<td>DSPC/Chol 4:1</td>
<td>0.053</td>
<td>2.4</td>
<td>68.5</td>
<td>1.8</td>
<td>81.3</td>
</tr>
<tr>
<td>+ PEG-lipid (1 mol% of total lipid)</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>PC/Chol 4:1</td>
<td>0.133</td>
<td>7.1</td>
<td>153.8</td>
<td>4.2</td>
<td>144.3</td>
</tr>
<tr>
<td>+ PEG-lipid (4 mol% of total lipid)</td>
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<td>PC/Chol 2:1</td>
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<td>PC/Chol 4:1</td>
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<td>33.1</td>
<td>95.6</td>
<td>13</td>
<td>94.2</td>
</tr>
<tr>
<td>DSPC/Chol 4:1</td>
<td>0.066</td>
<td>6.9</td>
<td>43.2</td>
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<td>46</td>
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<tr>
<td>DSPC</td>
<td>0.367</td>
<td>11.7</td>
<td>144.4</td>
<td>5.9</td>
<td>154.4</td>
</tr>
<tr>
<td>DSPC/Chol 9:1:5</td>
<td>0.286</td>
<td>11.4</td>
<td>120</td>
<td>6</td>
<td>125</td>
</tr>
<tr>
<td>+ PEG-lipid (8 mol% of total lipid)</td>
<td></td>
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<td></td>
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<tr>
<td>PC/Chol 2:1</td>
<td>0.335</td>
<td>11.9</td>
<td>79.7</td>
<td>7.3</td>
<td>86.9</td>
</tr>
<tr>
<td>USPIO-00904</td>
<td>2.21 (mM)</td>
<td>33.0</td>
<td>80.7</td>
<td>14.0</td>
<td>76.4</td>
</tr>
</tbody>
</table>
parameters of ML composition (as inclusion of Chol in the lipid membrane) may also be important for magnetic properties. When the PEG-lipid content of MLs is increased to 8 mol%, r1 values are lower (in comparison with those of the 4 mol% PEG-lipid MLs) and the r2-to-r1 ratios are reduced even more (in comparison with the MLs with 4 mol% PEG), mainly due to reduction of the r2 values, which are very close to r2 of plain USPIOs (Table 3), indicating a black-hole effect (loss of magnetic efficacy because relaxation is faster than water exchange across the lipid membrane). In general, it is very interesting that most MLs evaluated herein have better efficiency in comparison with plain USPIOs, in accordance with their significantly higher r2-to-r1 ratio (Figure 5).

**Discussion**

The main requirements for use of MLs as carriers for theranostic applications (carriers that can be loaded with both diagnostic and therapeutic agents) are nanosized dimensions, efficient magnetic properties (which are correlated with USPIO loading), potential to co-load high quantities of drugs, stability and targeting potential.4-14,24-28 The formation of liposomes that encapsulate USPIOs has been reported; however, in most cases the liposome-preparation techniques utilized were conventional methods, such as thin-film hydration, sonication or extrusion. Furthermore, the effect of the lipid-membrane composition on the stability and magnetic efficiency of MLs has not been studied to date (to the best of our knowledge). Herein, it is proven that the DRV technique followed by extrusion is efficient for the formation of nanosized MLs with high USPIO loading (Table 1), in comparison with values achieved by others for MLs of similar size.12 A 24% EE reported for larger MLs (∼360 nm mean diameter) may not be accurate because cryo-TEM revealed that most magnetic NPs were in aggregated form and outside of MLs.11 Herein, P00904-USPIOs retain their size and are

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**Figure 5. Ratios of transversal (r2) to longitudinal (r1) relaxivity at 37°C, at 20 MHz (upper graph) or 60 MHz (lower graph), for free P00904-USPIOs (presented as line parallel to x-axis) and MLs (as bars). The corresponding EE for each ML measured is in Table 3. In the axis legend PEGylated ML types are labeled as +P1 (means 1 mol% PEG-lipid) and +P4 (means 4 mol% PEG-lipid).**

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**TEM morphological studies (control and MAb-decorated MLs)**

Morphological evaluation of MLs with TEM revealed that no free USPIOs or aggregates were present in the purified ML dispersions; only monomeric USPIO encapsulated in vesicles were observed (Figure 6, A and B). OX-26 MAb is attached on MLs, as proven by immuno-colloidal gold staining (Figure 6, D-F), but not on the control liposomes (Figure 6, A and B). In the micrographs which have colloidal gold on and USPIOs in MLs, due to the higher contrast of gold particles in comparison with encapsulated USPIOs, it is difficult to distinguish encapsulated USPIOs. A micrograph of control empty OX-26 MAb-decorated liposomes is included (Figure 6, C) for comparison.
entrapped in the vesicles at high amounts, without any aggregated states present in ML samples (see Supplementary Material) as confirmed by TEM studies (Figure 6).

It is realized that the stability and magnetic properties of the DRV-extruded MLs are influenced differently by various composition-related factors. Experimental data confirm that by increasing the rigidity of the lipid membrane (Chol content) and especially by increasing the PEG-lipid content (surface PEG density) of MLs, it is possible to achieve highly stable MLs, as well as MAb-decorated MLs (Figures 3 and 4). The well documented effect of PEGylation on liposome integrity is explained by the highly hydrated PEG headgroups that extend out and surround the liposome in a 4–10 nm corona, providing a protective steric barrier which prevents liposome aggregation and fusion. Concerning the magnetic properties of extruded-DRV MLs and the corresponding influence of PEGylation, it is seen that $r_1$ values of PEGylated MLs (with 4 mol% PEG-lipid) are generally higher in comparison with those of the non-PEGylated ones (Table 3). This could be explained by hypothesizing that PEGylation increases water exchange between interior and exterior of liposomes; however, such effects of PEGylation have not been reported before (to the best of our knowledge). It is known that the extension of the PEG corona from the liposome surface and its configuration (“mushroom” or “brush”) is dependent on the concentration of PEG-lipids in liposomes (and on PEG moiety length). When 8 mol% PEG-lipid is added in the MLs, the magnetic properties are again modulated (in comparison with 4 mol% containing MLs) and $r_2$ values are reduced (almost 2 times). One hypothesis could be that membrane of 8 mol% PEG-lipid MLs (brush) is “magnetically” more permeable than the one of 4 mol% PEG-lipid ones (mushroom), and the $r_2$ magnetic efficacy is thereby lost because of a “black hole” effect.

Therefore, it is important to consider all the abovementioned factors when designing DRV-extruded MLs to have the best equilibrium between required characteristics for theranostic applications. It is known that classical MRI sequences (gradient echo, RARE, etc.) are T2-weighted, which means that one needs to have the highest possible $r_2$-to-$r_1$ ratio because $r_1$ relaxation contributes to signal degradation. However, today, new MRI sequences are available, even if not yet in clinical use, that allow the abolishment of $r_1$ relaxation contribution to signal, which means that magnetic efficiency is best as $r_2$ values increase (irrespective of corresponding $r_1$ values). In this context, the optimal ML compositions in terms of magnetic efficiency and stability are DSPC or PC/Chol (2:1) MLs with 4 mol% PEG-lipids.

In conclusion, the current results demonstrate that the extruded-DRV technique can ensure high USPIO loading in nanosized PEGylated MLs with high stability, although the targeting potential of such MLs can be increased by surface decoration (without affecting their loading and size). Lipid-membrane composition and surface-coating parameters influence the stability and magnetic efficiency of MLs and the proposed ML formulations (with sufficient stability and efficient T2 contrast) are the ones consisting of PC/Chol (2:1) MLs with 4 mol% PEG-lipids.

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Appendix A. Supplementary data

Supplementary materials related to this article can be found online at doi:10.1016/j.endend.2010.04.036.

References