Passive targeting of lipid-based nanoparticles to mouse cardiac ischemia–reperfusion injury

Tessa Geelen, Leonie E. Paulis, Bram F. Coolen, Klaas Nicolay and Gustav J. Strijkers*

Reperfusion therapy is commonly applied after a myocardial infarction. Reperfusion, however, causes secondary damage. An emerging approach for treatment of ischemia–reperfusion (IR) injury involves the delivery of therapeutic nanoparticles to the myocardium to promote cell survival and constructively influence scar formation and myocardial remodeling. The aim of this study was to provide detailed understanding of the *in vivo* accumulation and distribution kinetics of lipid-based nanoparticles (micelles and liposomes) in a mouse model of acute and chronic IR injury. Both micelles and liposomes contained paramagnetic and fluorescent lipids and could therefore be visualized with magnetic resonance imaging (MRI) and confocal laser scanning microscopy (CLSM). In acute IR injury both types of nanoparticles accumulated massively and specifically in the infarcted myocardium as revealed by MRI and CLSM. Micelles displayed faster accumulation kinetics, probably owing to their smaller size. Liposomes occasionally co-localized with vessels and inflammatory cells. In chronic IR injury only minor accumulation of micelles was observed with MRI. Nevertheless, CLSM revealed specific accumulation of both micelles and liposomes in the infarct area 3 h after administration. Owing to their specific accumulation in the infarcted myocardium, lipid-based micelles and liposomes are promising vehicles for (visualization of) drug delivery in myocardial infarction. Copyright © 2012 John Wiley & Sons, Ltd.

Supporting information may be found in the online version of this paper

Keywords: liposomes; micelles; myocardial infarction; drug delivery; MRI

1. INTRODUCTION

The best treatment option for acute myocardial infarction is currently early reperfusion of the affected myocardium. The aim of restoring blood flow is to limit the extent of myocardial necrosis and scarring, which are important factors in the development of systolic heart failure and determine prognosis. The downside of reperfusion treatment is that it causes adverse secondary ischemia–reperfusion (IR) injury by the generation of cytotoxic reactive oxygen species, resulting in additional apoptosis (1). After the initial IR event a dynamic cascade of events is initiated to promote myocardial infarct healing. In the acute phase, early after the ischemic event, infarct healing is characterized by cell death and inflammation. At later time points, in the chronic phase, the affected myocardium remodels into scar tissue (2–5).

An emerging approach for further treatment of IR injury involves the delivery of therapeutic compounds to the myocardium to promote cell survival and constructively influence scar formation and myocardial remodeling (6,7). The effectiveness of intravenously injected therapeutics may be hampered by fast clearance from the blood circulation and by lack of retention in the infarcted myocardium. To improve drug delivery to the infarct, nanoparticles offer a suitable vehicle as they can be designed to display long blood circulation half-lives and are able to encapsulate a large amount of drug molecules. Furthermore, owing to their size, nanoparticles demonstrate prolonged retention in the infarct area, which is enabled by the presence of leaky vasculature (8,9). Previously, liposomal nanoparticles were exploited for such purposes. Injection of ATP-, coenzyme Q10-, PGE₁- or adenosine-loaded liposomes resulted in a reduction of the extent of irreversibly damaged myocardium

within the area at risk (10–15). Furthermore, accumulation of pegylated micelles in the infarcted myocardium has been demonstrated in a rabbit model of myocardial infarction (16). Therefore, micelles were proposed for delivery of lipophilic drugs (16,17). The above studies have in common that information on accumulation kinetics in the heart was lacking and the exact location of the lipid-based nanoparticles at cellular scale was not visualized. Obviously, these need to be determined for optimization of drug delivery. Furthermore, only acute IR injury (up to 3 h reperfusion time) was considered, which is clinically less relevant, and therefore later time points after IR injury should be studied as well.

Therefore, the aim of this study was to provide detailed understanding of the *in vivo* accumulation and distribution kinetics of long-circulating lipid-based nanoparticles in a mouse model of acute (1 day old) as well as chronic (up to 2 weeks) myocardial IR injury. We studied micelles (diameter approximately 15 nm) and liposomes (diameter approximately 100 nm) to investigate influence of nanoparticle size. Both micelles and liposomes were equipped with paramagnetic Gd-containing lipids and with fluorescent lipids. Importantly, this enabled us to study the distribution of the nanoparticles in the infarcted myocardium *in vivo* using MRI, as well as to localize the nanoparticles at the

* Correspondence to: G. Strijkers, Biomedical NMR, Department of Biomedical Engineering, Eindhoven University of Technology, PO Box 513, 5600 MB, Eindhoven, The Netherlands. E-mail: g.j.strijkers@tue.nl

T. Geelen, L.E. Paulis, B.F. Coolen, K. Nicolay, G.J. Strijkers
Biomedical NMR, Department of Biomedical Engineering, Eindhoven University of Technology, PO Box 513, 5600 MB, Eindhoven, The Netherlands

cellular level *ex vivo* with confocal laser scanning microscopy (CLSM). As a control late gadolinium enhancement (LGE) MRI was performed after administration of Gd-DTPA. Furthermore, contrast-enhanced *in vivo* MRI was complemented with cine MRI to determine cardiac function.

2. RESULTS AND DISCUSSION

2.1. Nanoparticle Characterization

Micelles and liposomes were first characterized with respect to hydrodynamic size and MRI relaxivity properties at 1.41 and 9.4 T (Table 1). Gd-DTPA was included as a reference. Micelle diameter was approximately 15 nm and the liposome diameter of 100 nm was significantly larger. At 1.41 T, the micelle and liposome longitudinal relaxivity (r_1) values were higher than those for Gd-DTPA. Thus, the nanoparticles are powerful MRI contrast agents, which is further amplified by the high payload of Gd-DOTA-carrying lipids incorporated in the lipid membranes. As expected, at 9.4 T, r_1 values were considerably lower compared with 1.41 T (18). Nevertheless, the r_1 of micelles was still higher than the r_1 of Gd-DTPA. The ratio of transversal and longitudinal relaxivities (r_2/r_1) of both micelles and liposomes was relatively high at 9.4 T, indicating that the nanoparticles will display a pronounced T_2 shortening effect at high field strengths. From the relaxivity data we concluded that the nanoparticles possessed ample sensitivity for *in vivo* MR imaging of nanoparticle accumulation in cardiac IR injury.

2.2. Blood Circulation Half-lives and Biodistribution

To investigate the blood circulation half-lives and biodistribution, mice were injected with Gd-DTPA, micelles or liposomes and

blood samples were obtained up to 48 h after administration. Blood ΔR_1 values ($= 1/T_{1,\text{post}} - 1/T_{1,\text{pre}}$) determined at 9.4 T served as a measure of the concentration of nanoparticles in the circulation (Fig. 1a) (19). From mono-exponential fits the blood circulation half-lives were calculated (Fig. 1a). As expected, Gd-DTPA had a short blood circulation half-life of 0.30 ± 0.05 h owing to fast renal clearance enabled by its small size. Micelles and liposomes displayed relatively long blood circulation half-lives of 3.90 ± 0.44 and 2.31 ± 0.40 h, respectively. Previously, van Bochove *et al.* observed longer blood circulation half-lives in mice for micelles (22.5 ± 2.8 h) and liposomes (7.0 ± 1.0 h) containing neutral Gd-DTPA-carrying lipids (20). The Gd-DOTA-conjugated lipids used in this study have a charge of -1 , which could induce faster blood clearance via ingestion by phagocytic cells (21).

To determine the *in vivo* fate of micelles and liposomes, animals were killed 48 h after administration of the nanoparticles. Organs were excised and the biodistribution was studied with CLSM (Fig. 1b). Micelles accumulated mainly in the kidney, suggesting a clearance pathway that partly involved renal elimination. Liposomes were mainly detected in the spleen and in smaller amounts in the liver, lungs and kidneys. The large size of liposomes prohibits renal clearance and therefore it is likely that they are removed from the blood by the reticuloendothelial system.

2.3. In Vivo MRI

Mice underwent transient occlusion (30 min) of the left anterior descending (LAD) coronary artery to induce cardiac IR injury. Contrast-enhanced *in vivo* MRI was performed at day 1 (acute), or at week 1 or week 2 (chronic) after IR injury to visualize the distribution and extravasation kinetics of Gd-DTPA, micelles and liposomes. The MRI protocol is depicted schematically in Fig. 2.

Table 1. Characterization of Gd-DTPA, micelles and liposomes

	Hydro-dynamic diameter (nm)	r_1 ($\text{mM}^{-1} \text{s}^{-1}$) at 1.41 T, 37 °C	r_2 ($\text{mM}^{-1} \text{s}^{-1}$) at 1.41 T, 37 °C	r_2/r_1 at 1.41 T, 37 °C	r_1 ($\text{mM}^{-1} \text{s}^{-1}$) at 9.4 T, 20 °C	r_2 ($\text{mM}^{-1} \text{s}^{-1}$) at 9.4 T, 20 °C	r_2/r_1 at 9.4 T, 20 °C
Gd-DTPA	ND	3.3 ± 0.2	3.7 ± 0.3	1.14 ± 0.01	3.9^a	4.2^a	1.1^a
Micelles	15.6 ± 0.2	29.7 ± 1.4	45.9 ± 1.0	1.55 ± 0.05	6.3 ± 0.3	51.5 ± 2.2	8.1 ± 0.1
Liposomes	$100.1 \pm 3.6^*$	$14.1 \pm 0.7^*$	$22.2 \pm 1.0^*$	1.58 ± 0.01	$3.2 \pm 0.1^*$	56.7 ± 3.0	$17.9 \pm 0.3^*$

Mean $\pm$ standard error of the mean ($n=3$), except: ^a $n=1$. * $p < 0.05$ vs micelles.

Figure 1. Blood circulation half-lives and biodistribution. (a) ΔR_1 ($= 1/T_{1,\text{post}} - 1/T_{1,\text{pre}}$) of all blood samples, as measured at 9.4 T, plotted vs time after injection. Blood circulation half-lives were determined by fitting with a mono-exponential decay function (solid lines), leading to the blood circulation half-lives ($t_{1/2}$) as shown in the table (Mean $\pm$ SD). (b) Biodistribution of nanoparticles in several organs. The red color originates from the near-infrared (NIR) signal of micelles and liposomes. As a non-fluorescent negative control, organs from mice injected with Gd-DTPA are shown. Scale bar = 100 μm .

Figure 2. *In vivo* MRI protocol. T_1 -weighted scans were acquired in all sessions to investigate accumulation of Gd-DTPA, micelles or liposomes in myocardial tissue up to 48 h after injection. During the last imaging session of a series cine MRI scans were recorded as well, to evaluate cardiac function. Arrows indicate the time point of injection of Gd-DTPA, micelles or liposomes. In the chronic ischemia-reperfusion (IR) injury groups, one mouse was killed after 3 h of circulation of micelles or liposomes (day 6/13) for histology, while the other mice were followed up to 48 h after injection. IR, Ischemia-reperfusion surgery; &, cine MRI; and †, sacrifice and tissue harvesting for histological validation.

In addition, cine MRI was performed to determine cardiac function. Myocardial IR injury resulted in a similar reduction in cardiac function at all investigated time points (Supporting Information, Fig. S1). Ejection fractions (EF) were 52 ± 2 , 54 ± 3 and $59 \pm 4\%$ at day 1, week 1 and week 2, respectively, which are lower than EF values reported for healthy mice (70–80%), confirming the presence of myocardial infarction (22,23). Relatively high standard deviations in EF suggested heterogeneous infarct sizes within groups. Cardiac output (CO) and the left ventricular mass were significantly higher at week 1 and week 2 as compared with day 1, which can be explained by left ventricular remodeling after IR injury.

2.4. Acute IR Injury – Day 1

Gd-DTPA, micelles or liposomes were injected immediately or at day 1 after induction of IR injury. For both groups the accumulation of Gd-DTPA and paramagnetic nanoparticles was visualized by T_1 -weighted short-axis multi-slice MRI at day 1 after the IR injury. Injection of Gd-DTPA at day 1 resulted in immediate hyperenhancement of the infarcted myocardium (Fig. 3a). The hyperenhancement slowly disappeared within the next 30 min, in agreement with the extensively described LGE effect used to measure infarct size (24). The contrast-to-noise ratio (CNR) of

infarct versus remote tissue at 0.1 h after administration (Fig. 3b) was significantly enhanced compared with CNRs pre injection and at later time points (46.9 ± 3.7 , $p < 0.05$ vs all time points). No residual contrast enhancement was observed at day 1 when Gd-DTPA was injected directly after IR injury and circulated for 24 h. As a negative control for the fluorescence microscopy analysis of hearts from nanoparticle-injected mice, CLSM of mouse hearts injected with Gd-DTPA was performed. As expected, no near-infrared (NIR) autofluorescence was observed (Fig. 4).

In contrast to the observations for Gd-DTPA, administration of nanoparticles at day 1 resulted in hyperenhancement of remote cardiac tissue at 0.1 h after injection, while the infarct area remained isointense and, therefore, appeared as a dark rim (Fig. 3a). Consequently, the CNR of infarct versus remote tissue at 0.1 h was negative, amounting to -11.7 ± 1.4 and -9.3 ± 3.1 for micelles and liposomes, respectively (Fig. 3b). At this early time point after administration micelles and liposomes were still circulating in the blood, while accumulation into infarcted myocardium might not be present yet. We propose that the absence of signal intensity changes in the infarct reflected the impaired perfusion of the infarct. Often the dark rim of infarct tissue was present in the subendocardium. Indeed, the subendocardium is most prone to IR injury as it is located most distal from the coronary arteries (25,26). These observations suggest that the lipid-based

Figure 3. *In vivo* MRI of acute IR injury. (a) Short-axis T_1 -weighted MR images obtained before and after injection of Gd-DTPA (row 1), micelles (row 2) or liposomes (row 3). In columns 1–3, the agents were injected on day 1 after IR injury, while in column 4 the agents were administered at the start of reperfusion and circulated for 24 h. Arrows indicate the areas of contrast enhancement. (b) Group-averaged CNRs between infarct and remote tissue at different time points after injection. * $p < 0.05$ vs all time points; ** $p < 0.05$ vs pre; † $p < 0.05$ vs 0.1 h after injection; ‡ $p < 0.05$ vs 1.4 h after injection; and § $p < 0.05$ vs Gd-DTPA at 24 h after injection.

Figure 4. Confocal laser scanning microscopy (CLSM) of infarcted myocardium and border zones at day 1 after IR injury. Images were acquired after short (3 h) or after long (24 h) circulation of the nanoparticles. In all images the fluorescence of NIR lipids incorporated in micelles and liposomes is shown in red. As a fluorescence-negative control, mice injected with Gd–DTPA are shown. Scale bar = 100 μ m. Row 1: green corresponds to autofluorescence of the heart. Autofluorescence is less intense in the infarcted myocardium. Laser intensities for NIR visualization are given as percentages of the maximal laser intensity. For NIR visualization after staining, this laser intensity was always set to 50%. Row 2: leukocytes (CD18⁺) in blue; row 3: macrophages (CD68⁺) in green; row 4: vessels (CD31⁺) in blue; and row 5: laminin in green.

nanoparticles may offer a surrogate diagnostic readout, as a blood-pool agent, of the viable and perfused myocardium. Nevertheless, for the determination of myocardial infarct size traditional LGE MRI with Gd–DTPA is still the method of choice.

After 1.4 h, micelle accumulation in the infarct area became apparent resulting in disappearance of the dark rim in the infarct area (Fig. 3). The CNR increased significantly to a positive value of 6.9 ± 5.4 ($p < 0.05$ vs 0.1 h). For the larger liposomes, the T_1 -weighted signal increase proceeded more slowly. The subendocardial infarct remained hypoenhanced at this time point and this resulted in a persistent negative CNR of -4.0 ± 2.4 . CLSM was used to detect the NIR lipid present in the micelles and liposomes to determine nanoparticle localization at the cellular level. For this purpose, short-axis histological sections of multiple mice at multiple longitudinal positions and multiple locations in the infarct, in the border zone and in the remote myocardium were studied. CLSM of hearts at 3 h after injection confirmed extensive accumulation of micelles in the infarcted myocardium (Fig. 4, Table 2). Accumulation was high in the infarct border zones and in the core of the infarcted myocardium. NIR signal of micelles did not co-localize with inflammatory cells or vessels. Staining of laminin (extracellular matrix) revealed that micelles were primarily associated with infarcted cardiomyocytes.

Table 2. Visual scoring of confocal laser scanning microscopy images for intensity of near-infrared fluorescence signal intensities after acute ischemia–reperfusion injury

Time after administration	nanoparticle	Remote	Border zone	Infarct
3 h	Gd–DTPA	--	--	--
	Micelles	$\pm$	+	++
	Liposomes	–	+	–
24 h	Gd–DTPA	--	--	--
	Micelles	--	$\pm$	++
	Liposomes	--	+	++

-- = No; – = low; $\pm$ = moderate; + = high; and ++ = very high near-infrared fluorescence signal intensity.

Liposomes, on the other hand, were present in distinct spots in the infarct border zone area and occasionally co-localized with macrophages, indicating phagocytosis.

After 24 h of circulation (Fig. 3), both micelles and liposomes had accumulated in the infarcted myocardium and caused

hyperenhancement compared with the remote myocardium with significantly enhanced CNR values of 24.0 ± 5.2 and 19.9 ± 2.9 for micelles and liposomes, respectively ($p < 0.05$ vs pre and vs Gd-DTPA at 24 h). CLSM at 24 h revealed that micelles had accumulated diffusively and very specifically in the infarcted myocardium, which is in agreement with MRI (Fig. 4, Table 2). Liposomes had extravasated after 24 h of circulation and were massively associated with the infarcted myocardium, although the accumulation pattern appeared more scattered compared with micelles. Immunohistochemistry demonstrated that part of the liposomes was still present in vessels, probably owing to their long blood circulation half-lives. Some NIR signal of liposomes co-localized with leukocytes, while macrophages were almost absent. Staining of laminin revealed that most liposomes were present inside cells, most probably cardiomyocytes.

2.5. Chronic IR Injury – Weeks 1 and 2

LGE MRI with Gd-DTPA resulted in hyperenhancement of chronic infarcts at 0.1 h after injection (Fig. 5a, c). At this time point after administration CNR values were 24.3 ± 1.1 and 25.4 ± 5.8 at weeks 1 and 2, respectively ($p < 0.05$ vs all time points, Fig. 5b, d). Owing to fast washout, Gd-DTPA enhancement was absent at later time points.

Injection of micelles at week 1 resulted in immediate hyperenhancement of the remote tissue on T_1 -weighted MRI, again with an isointense subendocardial infarct, consequently appearing as a dark rim. CNR of the infarct with remote tissue was -6.3 ± 1.9 (Fig. 5a, b). Thereafter, micelles accumulated in the infarct resulting in CNR values of 5.3 ± 1.9 , 5.6 ± 3.4 and 3.6 ± 2.2 for measurements at 1.4, 24 and 48 h after administration ($p < 0.05$ vs 0.1 h). After short circulation of the nanoparticles (3 h), CLSM revealed diffuse accumulation of micelles in the infarcted myocardium, which sometimes co-localized with inflammatory cells (Figs 6 and 7). Some remaining NIR fluorescence was observed in scattered spots 48 h after administration. However, these fluorescent spots were also present in mice injected with Gd-DTPA and are related to autofluorescence of inflammatory cells. Liposome injection at week 1 resulted in a (small) hypoenhancement of the infarct at 0.1 h and slight hyperenhancement at the later time points. However, group-averaged CNR values for liposome-injected animals were not significantly different from pre-injection values (Fig. 5a, b). Remarkably, CLSM showed accumulation of liposomes in the infarcted myocardium 3 h after administration, which sometimes co-localized with macrophages (Figs 6 and 7). At 48 h after administration fewer spots remained.

Administration of micelles or liposomes at week 2 after IR injury did not result in visible hypo- or hyperenhancement on MR images

Figure 5. *In vivo* MRI of chronic IR injury. (a) Short-axis T_1 -weighted MR images obtained before and after injection of Gd-DTPA (row 1), micelles (row 2) and liposomes (row 3) at week 1 after IR injury. (b) Group-averaged CNRs between infarct and remote tissue at different time points after injection. * $p < 0.05$ vs all time points; † $p < 0.05$ vs 1.4 h after injection; and †† $p < 0.05$ vs all contrast agents at 0.1 h after injection. (c) MRI at week 2 after IR injury before and after injection of Gd-DTPA (row 1), micelles (row 2) and liposomes (row 3). (d) Group-averaged contrast-to-noise ratios between infarct and remote tissue at different time points after injection. * $p < 0.05$ vs all time points and †† $p < 0.05$ vs all contrast agents at 0.1 h after injection.

Figure 6. CLSM of infarcted myocardium and border zones at week 1 and 2 after IR injury. Images were acquired after short (3 h) or after long (48 h) circulation of the nanoparticles. NIR fluorescence of micelles and liposomes is shown in red. Green corresponds to autofluorescence of the heart. Autofluorescence is less intense in the infarcted myocardium. Laser intensities are given as percentages of the maximal laser intensity. As a negative control, mice injected with Gd-DTPA are shown. Scale bar = 100 μ m.

Figure 7. CLSM of infarcted myocardium at week 1 and 2 after IR injury. All images were obtained after 3 h circulation of the nanoparticles. Fluorescence of NIR-lipids incorporated in micelles and liposomes is shown in red and 50% laser intensity was used. Scale bar = 100 μ m. Row 1: leukocytes (CD18⁺) in blue; row 2: macrophages (CD68⁺) in green; row 3: vessels (CD31⁺) in blue; and row 4: laminin in green.

or in changes in quantified CNR values ($p > 0.05$ vs all). Two weeks after IR injury the infarcted myocardium is mainly replaced by fibrotic tissue, in which only few vessels are present. This will hamper extravasation and accumulation of the large nanoparticles in the infarct. With CLSM diffuse accumulation of micelles and scattered spots of liposomes NIR fluorescence were observed in the infarcted myocardium at the early time point

after injection (3 h), whereas some of the fluorescence remained at 48 h for both nanoparticles (Fig. 6).

2.6. Implications

Micelles and particularly liposomes have extensively been studied and applied as drug carriers, mainly for oncological

purposes (27,28). Cardiotoxicity, a well-known side effect of several cytotoxic anti-tumor drugs, for example, doxorubicin, can be diminished by encapsulation of the therapeutics (29,30). When the diseased myocardium is the target, micelles and liposomes offer a suitable platform for drug delivery as well. As indicated before, encapsulation of therapeutic compounds in liposomes resulted in a reduction of infarct area. Furthermore, encapsulation of the therapeutics had the advantage of preventing drug degradation (10,11), diminishing or even preventing adverse side effects (13,14) and overcoming poor drug solubility (15).

In this study we showed that micelles and liposomes, over a time period of 24 h, accumulated very specifically in the acute IR injured myocardium. Importantly, in our study little to no accumulation of micelles and liposomes was observed in the healthy remote myocardium, indicating specific delivery of the nanoparticles to infarcted myocardium. Whereas micelles associated with the cardiomyocytes, liposomes were found more scattered in the tissue and were associated with vessels, inflammatory cells or cardiomyocytes, rendering both nanoparticles suitable for targeting different aspects of ischemic injury in the acute phase. Also in the chronic phase, when the infarct is characterized by extensive fibrosis, micelles and liposomes were accumulating, although to a lesser extent, in the infarcted myocardium 3 h after administration. Therefore, these nanoparticles are suitable for drug delivery purposes in chronic infarction as well, for example, to prevent further adverse remodeling of the scar and reduce the risk of heart failure.

In this study semi-quantitative T_1 -weighted MRI was used to study the accumulation of paramagnetic nanoparticles in the infarcted myocardium. Future studies may focus on the *in vivo* estimation of local nanoparticle concentration by the application of quantitative T_1 mapping (31,32). In future drug delivery studies this could result in the quantification of delivery of the carrier to the infarcted myocardium, as a measure of local drug delivery, which can be related to the observed therapeutic effect.

Although we have shown that passive targeting of micelles and liposomes to the infarct is very specific, there may be an additional benefit of an active targeting approach. Verma *et al.* showed that non-targeted ATP-containing liposomes diminished IR injury. However, targeting of the ATP-containing liposomes to myosin resulted in almost complete recovery of cardiac function (33). Therapy with VEGF-loaded liposomes targeted against myosin resulted in improved cardiac function, while this was not observed for non-targeted VEGF-liposomes (34). Incorporation of phosphatidylserine in the lipid membrane of liposomes for macrophage targeting resulted in diminished left ventricular remodeling in a permanent ligation model of myocardial infarction (35). The micelles and liposomes in the current study can be made target specific straightforwardly by incorporation of different lipids in the lipid membrane or by conjugation of ligands to the distal ends of polyethylene glycol (PEG) chains. Previously, such nanoparticles were used for MR imaging of angiogenesis and apoptosis (36–38).

3. CONCLUSIONS

In this study the extravasation and accumulation properties of paramagnetic micelles and liposomes in mouse myocardial IR injury were studied with MRI and CLSM. Micelles and liposomes accumulated massively and specifically in the acute infarcted myocardium. Micelles displayed faster accumulation kinetics

compared with liposomes, presumably owing to their smaller size. In 1- and 2-week-old chronic infarcts, micelle and liposome accumulation was less but still detectable 3 h after administration. Because the nanoparticles generated significant contrast in MRI they are suitable for combined imaging and therapy. Altogether, we conclude that the presented lipid-based nanoparticles are promising vehicles for drug targeting purposes in myocardial infarction.

4. EXPERIMENTAL

4.1. Nanoparticle Preparation and Characterization

Micelles were prepared by lipid film hydration of a lipid mixture containing 50 μmol of total lipid. The lipid mixture consisted of gadolinium-DOTA-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (Gd-DOTA-DSPE, SyMO-Chem BV, Eindhoven, the Netherlands), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy [poly(ethylene glycol)]-2000] (PEG2000-DSPE, Lipoid, Ludwigshafen, Germany) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[maleimide[poly(ethylene glycol)]-2000] (Mal-PEG2000-DSPE, Avanti Polar Lipids, Alabaster, AL, USA) at a molar ratio of 0.5:0.4:0.1 and was dissolved in chloroform:methanol 4:1 (v/v). Next, 1 mol% of near-infrared664-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (NIR664-DSPE, SyMO-Chem BV) was added. Solvents were removed by rotary evaporation at 30 °C and the resulting lipid film was dried overnight under a constant nitrogen flow. The lipid film was dissolved in HEPES buffered saline (HBS, 10 mM HEPES, 135 mM NaCl, pH 6.7) and stirred at 65 °C for 1 h. Finally, the micelles were concentrated using a Vivaspin concentrator (20 ml, MWCO 100 000 Da, 3000 rpm) and resuspended in HBS (pH 7.4) at a concentration of approximately 35 mM lipid.

Liposomes were prepared as described earlier by lipid film hydration of a lipid mixture (21). Briefly, the lipid mixture (50 μmol of total lipid) consisting of Gd-DOTA-DSPE, 2-distearoyl-*sn*-glycero-3-phosphocholine (Lipoid), cholesterol (Avanti Polar Lipids), PEG2000-DSPE and Mal-PEG2000-DSPE was dissolved at a molar ratio of 0.75:1.1:1:0.075:0.075 in chloroform-methanol, 8:1 (v/v). Additionally, 0.1 mol% NIR664-DSPE was incorporated. After rotary evaporation at 30 °C and overnight drying under a nitrogen flow, the lipid film was hydrated in HBS (pH 6.7). The resulting particles were sized by extrusion through 400 nm filters (twice), 200 nm filters (four times) and 100 nm filters (eight times). Finally, the liposomes were concentrated using ultracentrifugation (45 min, 55 000 rpm, 4 °C) and resuspended in HBS (pH 7.4) at a concentration of approximately 70 mM lipid.

The final lipid concentrations of micelles and liposomes were measured by a phosphate determination according to Rouser (39). Number-weighted hydrodynamic size and size distribution were assessed with dynamic light scattering (ZetaSizer NanoS, Malvern Instruments Ltd, Malvern, UK) at 23 °C.

Longitudinal and transversal relaxivities r_1 and r_2 of micelles and liposomes were measured in HBS solution (pH 7.4). Values for r_1 and r_2 are reported in $\text{mM}^{-1}\text{s}^{-1}$. High-field relaxivities were measured at 9.4 T and room temperature with a small animal MR scanner (Bruker BioSpin GmbH, Ettlingen, Germany). For T_1 measurements an inversion recovery fast low angle shot (FLASH) sequence was used, with the following parameters: overall repetition time (TR) 15 s, TR 4 ms, echo time (TE) 2 ms, flip angle (α) 15°, number of excitations (NEX) 4, field of view (FOV) $3 \times 3 \text{ cm}^2$, matrix 128×128 , 1 mm slice thickness, 32

segments and 60 inversion times ranging from 72.5 to 4792.5 ms. T_2 relaxation times were determined using a multi-slice multi-echo sequence with the following parameters: TR 2000 ms, 32 echo times ranging from 9 to 288 ms, α 180°, NEX 4, FOV $3 \times 3 \text{ cm}^2$, matrix 128×128 and 1 mm slice thickness. Low-field relaxivities were determined at 1.41 T and 37°C with a Bruker Minispec (Bruker BioSpin GmbH). T_1 values were measured using an inversion recovery sequence, with the following parameters: recycle delay 20 s, 10 inversion times ranging from 5 ms to 10 s and four averages. T_2 values were determined with a CPMG sequence, with: recycle delay 20 s, inter-echo time 0.4 ms, 10 000 echoes and 16 averages.

4.2. Blood Circulation Half-lives and Biodistribution

All animal experiments were performed in accordance with the declaration of Helsinki and were approved by the local ethical committee for animal experiments of University of Maastricht. To determine blood kinetics, healthy Swiss mice ($n = 3/\text{group}$, $35.6 \pm 1.1 \text{ g}$, Charles River Laboratories, Sulzfeld, Germany) were injected intravenously with Gd-DTPA ($0.3 \text{ mmol Gd kg}^{-1}$ body weight (b.w.)), Bayer HealthCare Pharmaceuticals, Mijdrecht, the Netherlands), micelles ($0.05 \text{ mmol Gd kg}^{-1}$ b.w.) or liposomes ($0.05 \text{ mmol Gd kg}^{-1}$ b.w.). Before and at different time points after injection (2, 15, 30, 45 and 60 min and 4, 8, 24 and 48 h), blood samples of $20 \mu\text{l}$ were taken from the *vena saphena* and diluted in $20 \mu\text{l}$ heparinized saline to prevent coagulation. T_1 relaxation times were measured at 9.4 T using the same inversion recovery FLASH sequence as described above. For further study of the biodistribution of the nanoparticles, mice were killed by cervical dislocation after the last blood sample (48 h) and organs were excised, frozen in melting isopentane and cryosections of $5 \mu\text{m}$ were prepared.

4.3. In Vivo Cardiac MRI

Cardiac IR injury was induced in $n = 49$ male Swiss mice ($35.3 \pm 0.4 \text{ g}$, Charles River Laboratories). First, mice were anesthetized with 3% isoflurane in 0.4 ml min^{-1} medical air and anesthesia was maintained using 1.5–2.5% isoflurane. Next, mice underwent transient (30 min) ligation of the LAD coronary artery. Ligation of the LAD coronary artery was confirmed by blanching of the myocardium. After waking up, mice were kept overnight in a recovery room at 30°C.

Micelles or liposomes were injected intravenously via a tail vein catheter at a dose of $0.05 \text{ mmol Gd kg}^{-1}$ b.w. As a control, mice were injected with Gd-DTPA ($0.3 \text{ mmol Gd kg}^{-1}$ b.w.). Administration of the agents was performed either at the start of reperfusion, or at day 1, week 1 or week 2 after the start of reperfusion ($n = 3\text{--}5/\text{group}$, for details see Supporting Information, Table S1, and Fig. 2). Animals were randomly assigned to one of these groups before IR injury. Cardiac MRI was performed before and up to 48 h after administration of the contrast agents. To prevent the influence of differences in infarct composition during the experimental protocol, the day 1 groups were followed up to 24 h after injection only. For *in vivo* MRI, anesthesia was induced with 3% isoflurane in 0.4 L min^{-1} medical air and maintained with 1.5–2.5% isoflurane. Mice were positioned supine in a custom-built animal-holder with a heating pad and the ECG, breathing rate and temperature were monitored. The cardiac rate was kept at 400–600 beats per min, the breathing rate at 60–100 breaths per min and the temperature at 36–37°C.

MR imaging was performed with a 9.4 T small animal MR scanner (Bruker BioSpin GmbH) equipped with a 35 mm-diameter quadrature birdcage RF coil (Rapid Biomedical, Rimpf, Germany). Images were acquired using ECG triggering and respiratory gating (Small Animal Instruments Inc., Stony Brook, NY, USA). To visualize nanoparticle accumulation and washout, T_1 -weighted multi-slice FLASH measurements were performed, with the following parameters: TR 63 ms, TE 1.8 ms, α 60°, NEX 6, FOV $3 \times 3 \text{ cm}^2$, matrix 192×192 and a slice stack of nine short-axis slices of 1 mm thickness, which covered the left ventricle from apex to base. For measurement of cardiac function, single-slice cine FLASH measurements were performed in which the following parameters were adjusted: TR 7 ms, α 15°, 1 slice of 1 mm thickness and typically 13–15 cardiac frames. Nine short-axis slices from apex to base and two long-axis slices were obtained. After MRI, mice were killed by cervical dislocation and organs were excised, snap-frozen in isopentane and cryosections of $5 \mu\text{m}$ were prepared to study the cellular distribution of the nanoparticles.

4.4. Confocal Laser Scanning Microscopy

Localization of NIR664-DSPE present in micelles and liposomes was visualized by CLSM using a Zeiss LSM META system (Carl Zeiss BV, Sliedrecht, the Netherlands). Sections were thawed and covered with PBS and a cover glass. Excitation of NIR664 was performed with a 633 nm HeNe laser (5.0 mW) and emission was filtered with a 680/60 nm band-pass filter. Autofluorescence of heart tissue was excited with a 488 nm Ar laser and the emission was filtered with a 525/50 nm band-pass filter. Cryosections of animals injected with Gd-DTPA were used as negative controls.

To investigate co-localization of the nanoparticles, consecutive sections were dual-stained for macrophages and leukocytes or for vessels and extracellular matrix. Macrophages were labeled with rat-anti-mouse CD68-FITC ($4 \mu\text{g ml}^{-1}$, AbD Serotec, Düsseldorf, Germany) and leukocytes with rat-anti-mouse CD18-PE ($2 \mu\text{g ml}^{-1}$, BioLegend, Uithoorn, the Netherlands). Vessels were stained with rat-anti-mouse CD31-biotin ($2 \mu\text{g ml}^{-1}$, BioLegend) coupled to streptavidin-Cy3 ($1 \mu\text{g ml}^{-1}$, BioLegend) and the extracellular matrix was labeled with rabbit-anti-mouse laminin ($9 \mu\text{g ml}^{-1}$, Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands) conjugated to goat-anti-rabbit Alexa488 ($10 \mu\text{g ml}^{-1}$, Molecular Probes, Bleiswijk, the Netherlands). Coverglasses were mounted with Mowiol. CLSM of stained macrophages (FITC) and laminin (Alexa488) was performed with settings as described above for autofluorescence detection. Labeled leukocytes (PE) and vessels (Cy3) were excited with a 543 nm HeNe laser and emission was filtered with a 580/75 nm band-pass filter. All images were acquired with a $20\times$ objective at an in-plane resolution of $0.31 \times 0.31 \mu\text{m}^2$, using the same settings for each fluorophore. CLSM images were visually scored for the localization of the nanoparticles and for co-localization of NIR signal and stainings.

4.5. Data Analysis and Statistics

T_1 and T_2 relaxation times for relaxivity measurements and blood samples were determined with a custom-built fitting program in Mathematica 6 (Wolfram Research Europe, Oxfordshire, UK). For the blood samples $\Delta R_1 = R_{1,\text{post}} - R_{1,\text{pre}}$ vs time after injection was fitted with a mono-exponential decay function in OriginPro 7.5 (OriginLab Corporation, Northampton, MA, USA) to determine the circulation half-lives of the nanoparticles and Gd-DTPA.

Global cardiac functional parameters were determined from the single-slice cine FLASH measurements by semi-automatic analysis with MRV FARM 2.0 software (Pie Medical Imaging, Maastricht, the Netherlands). Cardiac functional parameters were not different between the groups of mice injected with different contrast agents and therefore parameters were pooled per time point. This software was also used to determine the regional wall thickening in 20 segments per slice. *In vivo* T_1 -weighted FLASH images were analyzed by manually drawing regions-of-interest (ROIs) in infarct and remote ventricular tissue and in a noise-only region. For all measurements, the infarct ROI was positioned in a region of reduced wall thickening in the anterolateral left ventricular wall. The remote ROI was placed in the septum. For mice measured at week 1 or 2 after IR injury, infarct ROIs were carefully positioned in the mid-myocardium, because histology confirmed this as the location of the infarct. Mean signal intensity (μ) and standard deviation (σ) were determined for all ROIs. Subsequently, the contrast-to-noise ratio (CNR) between infarct and remote ROIs was calculated as: $CNR = (\mu_{\text{infarct}} - \mu_{\text{remote}}) / \sigma_{\text{noise}}$. Finally, a running average over three consecutive scans was applied.

All data are presented as mean $\pm$ standard error of the mean. To test differences between nanoparticle characteristics Student's *t*-tests for independent samples were performed. One-way analysis of variance with Bonferroni correction for multiple comparisons was applied to test whether: (a) the CNR values for a specific contrast agent changed significantly in time and (b) the CNR values of contrast agents were significantly different at a specific time point after injection. Values of $p < 0.05$ were considered significant.

5. SUPPORTING INFORMATION

Supporting information can be found in the online version of this article.

Acknowledgments

This work was sponsored by Dutch Technology Foundation STW, applied science division of the Netherlands Organisation for Scientific Research (NWO) and the Technology Program of the Ministry of Economic Affairs; grant number: 07952. Peter Leenders and Leonie Niesen are acknowledged for biotechnical assistance.

REFERENCES

- Michael LH, Ballantyne CM, Zachariah JP, Gould KE, Pocius JS, Taffet GE, Hartley CJ, Pham TT, Daniel SL, Funk E, Entman ML. Myocardial infarction and remodeling in mice: effect of reperfusion. *Am J Physiol Heart Circul Physiol* 1999; 277(2 Pt 2): H660–H668.
- Frangogiannis NG. The mechanistic basis of infarct healing. *Antioxid Redox Signal* 2006; 8(11–12): 1907–1939.
- Frangogiannis NG. The immune system and cardiac repair. *Pharmacol Res* 2008; 58(2): 88–111.
- Blankesteijn WM, Creemers E, Lutgens E, Cleutjens JPM, Daemen MJAP, Smits JFM. Dynamics of cardiac wound healing following myocardial infarction: observations in genetically altered mice. *Acta Physiol Scand* 2001; 173(1): 75–82.
- Cleutjens JPM, Blankesteijn WM, Daemen MJAP, Smits JFM. The infarcted myocardium: simply dead tissue, or a lively target for therapeutic interventions. *Cardiovasc Res* 1999; 44(2): 232–241.
- St John Sutton MG, Sharpe N. Left ventricular remodeling after myocardial infarction: pathophysiology and therapy. *Circulation* 2000; 101: 2981–2988.
- Sanz-Rosa D, García-Prieto J, Ibanez B. The future: therapy of myocardial protection. *Ann NY Acad Sci* 2012; 1254: 90–98.
- Dauber IM, VanBenthuyzen KM, McMurtry IF, Wheeler GS, Lesnfsky EJ, Horwitz LD, Weil JV. Functional coronary microvascular injury evident as increased permeability due to brief ischemia and reperfusion. *Circul Res* 1990; 66(4): 986–998.
- Horwitz LD, Kaufman D, Keller MW, Kong Y. Time course of coronary endothelial healing after injury due to ischemia and reperfusion. *Circulation* 1994; 90(5): 2439–2447.
- Verma DD, Hartner WC, Levchenko TS, Bernstein EA, Torchilin VP. ATP-loaded liposomes effectively protect the myocardium in rabbits with an acute experimental myocardial infarction. *Pharm Res* 2005; 22(12): 2115–2120.
- Verma DD, Levchenko TS, Bernstein EA, Torchilin VP. ATP-loaded liposomes effectively protect mechanical functions of the myocardium from global ischemia in an isolated rat heart model. *J Control Release* 2005; 108(2–3): 460–471.
- Verma DD, Hartner WC, Thakkar V, Levchenko TS, Torchilin VP. Protective effect of coenzyme Q10-loaded liposomes on the myocardium in rabbits with an acute experimental myocardial infarction. *Pharm Res* 2007; 24(11): 2131–2137.
- Feld S, Li G, Wu A, Felli P, Amirian J, Vaughn WK, Gornet T, Swenson C, Smalling RW. Reduction of canine infarct size by blous intravenous administration of liposomal prostaglandin E_1 : comparison with control, placebo liposomes, and continuous intravenous infusion of prostaglandin E_1 . *Am Heart J* 1996; 132(4): 747–757.
- Smalling RW, Feld S, Ramanna N, Amirian J, Felli P, Vaughn WK, Swenson C, Janoff A. Infarct salvage with liposomal prostaglandin E_1 administered by intravenous bolus immediately before reperfusion in a canine infarction–reperfusion model. *Circulation* 1995; 92(4): 935–943.
- Takahama H, Minamino T, Asanuma H, Fujita M, Asai T, Wakeno M, Sasaki H, Kikuchi H, Hashimoto K, Oku N, Asakura M, Kim J, Takashima S, Komamura K, Sugimachi M, Mochizuki N, Kitakaze M. Prolonged targeting of ischemic/reperfused myocardium by liposomal adenosine augments cardioprotection in rats. *J Am Coll Cardiol* 2009; 53(8): 709–717.
- Lukyanov AN, Hartner WC, Torchilin VP. Increased accumulation of PEG-PE micelles in the area of experimental myocardial infarction in rabbits. *J Control Release* 2004; 94(1): 187–193.
- Torchilin VP. Micellar nanocarriers: pharmaceutical perspectives. *Pharm Res* 2007; 24(1): 1–16.
- Strijkers GJ, Mulder WJM, van Heeswijk RB, Frederik PM, Bomans P, Magusin PCMM, Nicolay K. Relaxivity of liposomal paramagnetic MRI contrast agents. *Magn Reson Mater Phy* 2005; 18(4): 186–192.
- van Tilborg GAF, Strijkers GJ, Pouget EM, Reutelingsperger CPM, Sommerdijk NAJM, Nicolay K, Mulder WJM. Kinetics of avidin-induced clearance of biotinylated bimodal liposomes for improved MR molecular imaging. *Magn Reson Med* 2008; 60(6): 1444–1456.
- van Bochove GS, Paulis LEM, Segers D, Mulder WJM, Krams R, Nicolay K, Strijkers GJ. Contrast enhancement by differently sized paramagnetic MRI contrast agents in mice with two phenotypes of atherosclerotic plaque. *Contrast Media Mol Imag* 2011; 6(1): 35–45.
- Hak S, Sanders HMHF, Agrawal P, Langereis S, Grüll H, Keizer HM, Arena F, Terreno E, Strijkers GJ, Nicolay K. A high relaxivity Gd(III) DOTA-DSPE-based liposomal contrast agent for magnetic resonance imaging. *Eur J Pharm Biopharm* 2009; 72(2): 397–404.
- Kober F, Iltis I, Cozzone PJ, Bernard M. Cine-MRI assessment of cardiac function in mice anesthetized with ketamine/xylazine and isoflurane. *Magn Reson Mater Phy* 2004; 17(3–6): 157–161.
- Ojha N, Roy S, Radtke J, Simonetti O, Gnyawali S, Zweier JL, Kuppusamy P, Sen CK. Characterization of the structural and functional changes in the myocardium following focal ischemia–reperfusion injury. *Am J Physiol Heart Circul Physiol* 2008; 294(6): H2435–H2443.
- Yang Z, Berr SS, Gilson WD, Toufektsian M-C, French BA. Simultaneous evaluation of infarct size and cardiac function in intact mice by contrast-enhanced cardiac magnetic resonance imaging reveals contractile dysfunction in noninfarcted regions early after myocardial infarction. *Circulation* 2004; 109(9): 1161–1167.
- Kumar D, Hacker TA, Buck J, Whitesell LF, Kaji EH, Douglas PS, Kamp TJ. Distinct mouse coronary anatomy and myocardial infarction consequent to ligation. *Coronary Artery Dis* 2005; 16(1): 41–44.
- Michael LH, Entman ML, Hartley CJ, Youker KA, Zhu J, Hall SR, Hawkins HK, Berens K, Ballantyne CM. Myocardial ischemia and reperfusion: a murine model. *Am J Physiol* 1995; 269(6 Pt 2): H2147–H2154.
- Strijkers GJ, Kluzza E, Van Tilborg GAF, Van der Schaft DWJ, Griffioen AW, Mulder WJM, Nicolay K. Paramagnetic and fluorescent liposomes for target-specific imaging and therapy of tumor angiogenesis. *Angiogenesis* 2010; 13(2): 161–173.

28. Torchilin VP. Targeted pharmaceutical nanocarriers for cancer therapy and imaging. *AAPS J* 2007; 9(2): E128–E147.
29. Gabizon A, Shmeeda H, Barenholz Y. Pharmacokinetics of pegylated liposomal doxorubicin: review of animal and human studies. *Clin Pharmacokinet* 2003; 42(5): 419–436.
30. Abraham SA, Waterhouse DN, Mayer LD, Cullis PR, Madden TD, Bally MB. The liposomal formulation of doxorubicin. *Meth Enzymol* 2005; 391: 71–97.
31. Coolen BF, Geelen T, Paulis LEM, Nauerth A, Nicolay K, Strijkers GJ. Three-dimensional T₁ mapping of the mouse heart using variable flip angle steady-state MR imaging. *NMR Biomed* 2011; 24(2): 154–162.
32. Coolen BF, Geelen T, Paulis LEM, Nicolay K, Strijkers GJ. Regional contrast agent quantification in a mouse model of myocardial infarction using 3D cardiac T₁ mapping. *J Cardiovasc Magn Reson* 2011; 13: 56.
33. Verma DD, Levchenko TS, Bernstein EA, Mongayt D, Torchilin VP. ATP-loaded immunoliposomes specific for cardiac myosin provide improved protection of the mechanical functions of myocardium from global ischemia in an isolated rat heart model. *J Drug Target* 2006; 14(5): 273–280.
34. Scott RC, Rosano JM, Ivanov Z, Wang B, Chong PL-G, Issekutz AC, Crabbe DL, Kiani MF. Targeting VEGF-encapsulated immunoliposomes to MI heart improves vascularity and cardiac function. *FASEB J* 2009; 23(10): 3361–3367.
35. Harel-Adar T, Ben Mordechai T, Amsalem Y, Feinberg MS, Leor J, Cohen S. Modulation of cardiac macrophages by phosphatidylserine-presenting liposomes improves infarct repair. *Proc Natl Acad Sci USA* 2011; 108(5): 1827–1832.
36. Mulder WJM, Strijkers GJ, Griffioen AW, van Bloois L, Molema G, Storm G, Koning GA, Nicolay K. A liposomal system for contrast-enhanced magnetic resonance imaging of molecular targets. *Bioconjug Chem* 2004; 15(4): 799–806.
37. Mulder WJM, Strijkers GJ, Habets JW, Bleeker EJW, van der Schaft DWJ, Storm G, Koning GA, Griffioen AW, Nicolay K. MR molecular imaging and fluorescence microscopy for identification of activated tumor endothelium using a bimodal lipidic nanoparticle. *FASEB J* 2005; 19(14): 2008–2010.
38. van Tilborg GAF, Mulder WJM, Deckers N, Storm G, Reutelingsperger CPM, Strijkers GJ, Nicolay K. Annexin A5-functionalized bimodal lipid-based contrast agents for the detection of apoptosis. *Bioconjug Chem* 2006; 17(3): 741–749.
39. Rouser G, Fleischer S, Yamamoto A. Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids* 1970; 5(5): 494–496.