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The Effect of Nanoparticle Polyethylene Glycol Surface Density on Ligand-directed Tumor Targeting Studied *in vivo* by Dual Modality Imaging

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Abstract

The development and application of nanoparticles as *in vivo* delivery vehicles for therapeutic and/or diagnostic agents has seen a drastic growth over the last decades. Novel imaging techniques allow real-time *in vivo* study of nanoparticle accumulation kinetics at the level of the cell and targeted tissue. Successful intravenous application of such nanocarriers requires a hydrophilic particle surface coating, of which polyethylene glycol (PEG) has become the most widely studied and applied. In the current study, the effect of nanoparticle PEG surface density on the targeting efficiency of ligand-functionalized nanoemulsions was investigated. We synthesized 100 nm nanoemulsions with a PEG surface density varying from 5 to 50 mol%. Fluorescent and paramagnetic lipids were included to allow their multimodal detection, while RGD peptides were conjugated to the PEG coating to obtain specificity for the $\alpha_v\beta_3$-integrin. The development of a unique experimental imaging setup allowed us to study, in real time, nanoparticle accumulation kinetics at (sub)-cellular resolution in tumors that were grown in a window chamber model with confocal microscopy imaging, and at the macroscopic tumor level in subcutaneously grown xenografts with magnetic resonance imaging. Accumulation in the tumor occurred more rapidly for the targeted nanoemulsions than for the non-targeted versions, and the PEG surface density had a strong effect on nanoparticle targeting efficiency. Counter intuitively, yet consistent with the PEG density conformation models, the highest specificity and targeting efficiency was observed at a low PEG surface density.

Keywords

Nanoemulsion; targeting-ligand; polyethylene glycol; surface density; intravital microscopy; dorsal window chamber; DCE-MRI

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Supporting Information

Supporting figures and calculations are provided in the supporting information. This material is available free of charge *via* the Internet at http://pubs.acs.org.

The use of nanoparticles for *in vivo* diagnostics and drug delivery has increased tremendously over the last two decades.[1, 2] The exciting possibility of incorporating multiple functionalities within the same nanoparticle allows for *in vivo* monitoring of biodistribution and cargo delivery with a variety of imaging modalities.[3–5] Functionalization of the nanoparticles with targeting ligands, such as peptides [6] or aptamers, [7] has provided more control over *in vivo* nanoparticle distribution, and has enabled their use as molecular imaging agents.[3, 8]

The pharmacokinetics and biodistribution of nanoparticles are to a large extent governed by their surface properties. Hence, a prerequisite for successful intravenous administration of nanoparticles is a suitable hydrophilic and biocompatible particle surface or surface coating. Such surface coatings can consist of polysaccharides, [9] poly-amino acids,[10] or synthetic polymers.[11] Within the latter class polyethylene glycol (PEG) was identified in the early nineties to be highly suitable [12–14] and has become the most widely used nanoparticle surface coating.[13–15] PEG is highly hydrophilic, has the lowest level of protein or cellular adsorption of any known polymer, is non-toxic, and many PEGylated therapeutics have been FDA-approved since its introduction.[1, 14, 15] Although the mechanism by which PEG coatings increase circulation times and improve biodistribution profiles is not fully understood, the most widely accepted explanation is that PEG provides a steric barrier, which prevents nanoparticle opsonization, thereby delaying removal from the circulation by the mononuclear phagocyte system (MPS).[14, 15] A critical factor is the PEG density on the nanoparticle surface which has been found to modulate nanoparticle circulation times [16, 17] and nonspecific cellular uptake.[18]

Nanoparticle targeting using cell surface receptor specific ligands, can enhance the cellular uptake of nanoparticles.[19] However, a topic that remains largely uninvestigated is the effect PEG surface density has on the targeting potential of ligand-functionalized nanoparticles. Studies have shown that at low PEG density, the PEG units on a surface are organized in a so-called mushroom configuration, which transforms to a brush configuration at higher PEG density.[20] In the mushroom regime, no lateral interaction between neighboring polymers occurs, implying that the nanoparticle surface is not completely covered with PEG. In the brush regime the polymers overlap, fully covering the surface and providing optimal surface protection against opsonization. However, in the brush regime the lateral interactions between the polymers induce chain stretching outwards from the nanoparticle surface, increasing the thickness of the PEG layer with increasing PEG density. A hypothesis is that when ligands are conjugated to the distal ends of the PEG chains in the brush confirmation, this interaction with neighboring PEG chains may reduce the ability of interaction with their molecular or cellular targets.

To investigate the above hypothesis, we developed a unique multimodal *in vivo* imaging setup which allowed us to study the effect of PEG surface density on target-specific nanoparticle accumulation in tumor tissue using both high resolution intravital microscopy and magnetic resonance imaging (MRI) on mice. The nanoparticle platform used is based on a recently introduced multimodal nanoemulsion,[21] of which the surface PEG-density can be judiciously varied. The $\alpha_v\beta_3$-integrin specific Arginine-Glycine-Aspartic acid peptide RGD was used as a targeting ligand. $\alpha_v\beta_3$-integrin is highly expressed on angiogenically activated endothelial cells and is an extensively studied marker for tumor angiogenesis.[22] *In vitro*, $\alpha_v\beta_3$-integrin expressing human endothelial cells were used to study nanoemulsion targeting efficiency and cellular handling as a function of their PEG surface density. For *in vivo* experiments, we employed a dorsal window chamber tumor mouse model[23, 24] and confocal laser scanning microscopy (CLSM) to evaluate nanoparticle targeting and accumulation in tumor tissue at a (sub)-cellular resolution in real time. Different fluorophores were used to distinguish targeted and non-targeted nanoparticles within the same tumor tissue, making this set up highly suitable to study the interactions between nanoparticles and the living tumor. Finally, to corroborate the CLSM observations on the whole tumor level with a clinically relevant imaging modality, dynamic contrast enhanced MRI (DCE-MRI) was explored to study the nanoemulsion tumor targeting dynamics.

Materials and Methods

Nanoparticle synthesis

Stock solutions of all the components in chloroform were prepared. Typically a total of 20 µmoles of the amphiphillic lipids (1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), cholesterol, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000 (PEG2000-DSPE), maleimide-PEG2000-DSPE, purchased from Avanti Polar Lipids) were mixed at molar ratios as indicated in Figure 1B. The amount of soybean oil was defined as mg per µmole of the amphiphillic lipid mixture. For fluorescence imaging, 0.1 mol% of NIR664-PEG2000-DSPE (SyMO-Chem) and/or 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-lissamine rhodamine B sulfonyl ammonium salt (rhodamine-PE, Avanti Polar Lipids) was added, and for MRI 25 mol% gadolinium-diethylenetriaminepentacetate-bis(stearylamide) (Gd-DTPA-DSA, Avanti Polar Lipids) was added at the expense of DSPC. Subsequently the chloroform was evaporated with rotation evaporation (60 min at maximum vacuum and room temperature) and the resulting lipid film was hydrated with hepes buffered saline (HBS, 2.38 g/L Hepes and 8 g/L NaCl, pH 6.7). The obtained crude emulsion was sonicated for 20 min (Heat Systems-Ultrasonics, W-225R, duty cycle 35%, 30 Watt and 20 kHz) in a water bath keeping the emulsion at ambient temperature. Half of the final nanoemulsions were conjugated with c(RGDf(-S-acetylthioacetyl) K) (Ansynth) (RGD, 13.5 µg RGD per µmole lipid). Before adding the peptide to the maleimide functionalized nanoemulsion, the thiol group on the RGD peptide was deacetylated at pH 7 for 1 h. The activated peptide was added to the nanoemulsions and the resulting mixture was left to react overnight at 4 °C. The nanoemulsions were concentrated when necessary with vivaspin concentrators (100 kDa MWCO) and finally dialysed (Spectra/Por Float-A-Lyzer G2, 100 kDa MWCO, Spectrum Laboratories) against HBS of pH 7.4 to remove unconjugated RGD and to obtain physiological pH. After preparation, the nanoemulsions were stored at 4 °C for a minimum of 5 days before using them in experiments to assure hydrolysis of unreacted maleimide groups.

Dynamic light scattering and zeta potential measurement

The hydrodynamic size and size distribution, and the zeta potential were measured using dynamic light scattering techniques (Malvern, Zetasizer Nano PS). For size measurements 8 µl of nanoemulsion suspension was dispersed in 800 µl HBS in an ordinary cuvette. Reported values were the average of approximately 50 measurements. The reported values are the means and PDIs after combining the 50 measurements with the Malvern zetasizer series software. For zeta potential measurements 20 µl of nanoemulsion suspension was dispersed in 1.5 ml dH_2O in a capillary cell (Malvern, DTS1061). Reported values were the average of 300 measurement runs.

Cell culturing and incubations

HUVEC (Lonza Bioscience) were cultured in gelatin coated cell culture flasks using Dulbecco's modified eagle medium (DMEM, Gibco/Invitrogen) supplemented with endothelial cell growth medium (EGM) BulletKit (1% FBS, Lonza Bioscience). The cells were used for experiments at passage 4 to 7. In all experiments, the cells were incubated in medium containing either no (blank), RGD or CTRL (non-targeted) nanoemulsions at a 1 mM lipid concentration for 3 h, followed by 3 times washing with PBS. For live cell imaging with CLSM, HUVEC were grown in gelatin coated 8-well µ-slides (Ibidi). After the incubation and washing, the cells were maintained in fresh medium. For flow cytometry experiments, HUVEC were grown in gelatin coated 12-well plates. After nanoemulsion incubation, the cells were detached from the wells with 0.25% trypsin-EDTA solution (Sigma-Aldrich), spun down at 1500 rpm for 5 min, resuspended in ice cold PBS and placed on ice. In the endocytosis inhibition assay, the cells were pre-incubated with either chlorpromazine (10 μg/ml) or genistein (70 mg/ml) for 30 min before incubating with medium containing both the endocytic inhibitor and the nanoemulsions.

Flow cytometry

Cellular uptake of nanoemulsions was measured by flow cytometry (Gallios, Beckman Coulter). The 633 nm laser line was used to excite the NIR664 fluorochrome and the fluorescence was detected using a 650–670 bandpass filter. The flow cytometry data was analyzed using the software Kaluza (Beckman Coulter). Cellular fragments and debris were excluded from the analysis by gating the fluorescence on side scatter vs forward angle light scatter signal. Within one experiment all the samples were analyzed using the same gate. The cellular uptake was measured both as the percentage of cells with higher fluorescence intensity than cellular autofluorescence as well as the amount of internalized nanoemulsion, which was estimated as the median fluorescence intensity divided by the median of the autofluorescence and normalized to the observed maximum cellular uptake. The targeting effect was defined as the ratio between the normalized fluorescence intensity of cells incubated with RGD or CTRL nanoparticles.

Live cell CLSM

Live HUVEC were imaged by CLSM (Zeiss LSM 510 META) using an apochromate 40×/1.2 water immersion objective and a frame size of 1024×1024 pixels. The 8-well μ-slide was placed in an incubation chamber (PECON, CTI-controller 3700 and temp control 37-2) mounted on the CLSM object stage, and the cells were imaged at 37° C and 5% CO_2. NIR664-PEG-DSPE was excited at 633 nm and the fluorescence detected using a meta-detector at 676–719 nm, and rhodamine-PE was excitated at 543 nm and detected using a meta-detector 569–612 nm.

To assess particle integrity, the cells were incubated with P5, P10, and P20 CTRL and RGD nanoemulsions containing both NIR664-PEG-DSPE and rhodamine-PE and imaged up to 3 h post incubation.

Intracellular localization of the P5 RGD and CTRL nanoemulsions were compared by co-incubating the cells with CTRL nanoemulsions labeled with rhodamine-PE and RGD nanoemulsions labeled with NIR664-PEG2000-DSPE.

To assess lysosome colocalization of the nanoemulsion, the cells were incubated for 45 min with medium containing 50 nM lysotracker green (Invitrogen) and washed before the incubations with nanoemulsions. Lysotracker green was excited at 543 nm and detected at 565–615 nm

Intravital CLSM

For intravital CLSM, tumors grown in dorsal window chambers in mice were used. The window chambers (made of polyoxymethylene, build in house) were implanted as previously described [45] in male athymic Balb/c Nu/nu mice of 20 to 25 gram. The mice were anesthetized with a subcutaneous injection of 12 mg/kg midazolam/fentanyl/haloperidol /water (3/3/2/4). 24 h after implanting the chambers, $2–3\cdot10^6$ HeLa cells (from the cell line of human cervical carcinoma cells) were injected in the center of each chamber. The surgical procedures were performed under sterile conditions. The animals were kept under pathogen-free conditions at a temperature of 19 to 22 °C, 50 to 60% humidity, and 65 air changes per h, and animals were allowed food and water *ad libitum*. The drinking water contained 2% sucrose and 67.5 mg/L Baytril (enrofloxacin). After 12–16 days when the tumors were 0.2 to 0.7 cm thick and filled 30 to 100% of the window area, the mice were used for experiments. The mice were anesthetized by subcutaneous injections of 12 mg/kg midazolam/fentanyl/Haldol/water (3/3/2/4) and either P5 or P50 nanoemulsions were intravenously (i.v.) injected at a dose of 80 μmole lipid/kg bodyweight. Six mice received each PEG formulation and from these 2 mice received RGD (NIR664-PEG-DSPE labeled), 2 mice CTRL (NIR664-PEG-DSPE labeled), and 2 mice both RGD (rhodamine-PE labeled) and CTRL (NIR664-PEG-DSPE labeled) nanoemulsions. This allowed us to study the distribution of each agent in 4 mice. For visualization of the vasculature, 100 μl of 20 mg/ml 2MDa FITC labeled Dextran (Sigma-Aldrich) was injected i.v. The anesthetized mice were placed on a heating pad at 37°C which was fixed to the microscope stage in a specially designed mouse holder. The tumors were imaged using a C-Apoplan 40×/0.8 water objective with long working distance. NIR664 and rhodamine were excited and detected as for imaging of cells. FITC-dextran was excited at 488 nm and detected with a bandpass filter at 500–550 nm. The tumors were imaged from the coverslip and approximately 100 μm into the tissue with a frame size of 512×512 pixels. 3D visualizations of intravitally acquired z-stacks were obtained using Amira (Visage Imaging)

MRI

All the MRI experiments were performed in a Bruker Biospec 7.05 Tesla horizontal bore magnet. The T_1 relaxivity of the Gd in the nanoemulsions was obtained using a dilution series in HBS (pH 7.4) of 6 different Gd concentrations ranging from 0.05 to 1 mM in 2 ml eppendorf tubes. The tubes were placed in an in house built sample holder which was imaged with a volume resonator. The T_1 relaxation time was measured using a spin-echo protocol: echo time (T_E) of 8.4 ms; repetition times (T_R) of 15, 25, 50, 75, 100, 200, 300, 400, 600, 800, 1200, 1600, 3200, 6400, 12800, 18000 ms; field of view (FOV) of 60×40 mm; matrix (MTX) of 128×128; slice thickness of 2mm; 2 averages.

For P5 nanoemulsion detection *in vitro* in cell pellets, HUVEC were incubated with nanoemulsions for 3 h (n=3 in each group: blank, RGD nanoemulsion, and CTRL nanoemulsion), washed and detached with trypsin. The cell suspension was centrifuged and the pellet fixed with 150 μl 4% paraformaldehyde and transferred to a 200 μl eppendorf tube. In the eppendorf tube a cell pellet was formed by gravity. The cell pellets were imaged using a quadrature surface coil with a T_1-weighted spin echo sequence: T_E of 7.7; T_R of 1000 ms; FOV of 20×16 mm, MTX of 100×80, slice thickness of 0.5 mm; 20 averages. Subsequently a T_1-map was obtained with a spin echo protocol: T_E of 8.4 ms; T_R of 42, 80, 160, 320, 640, 1280, 2000, 3500, 5000, 8000, 12000, 16000 ms; FOV of 16×16 mm; MTX of 80×80; slice thickness of 0.6 mm; 4 averages.

For DCE-MRI, the xenografts of the ovarian cancer cell line TOV21G in 12 week old female athymic Balb/c Nu/nu mice were used. Tumors were imaged around 4 weeks after subcutaneous inoculation of $1.5 \cdot 10^6$ tumor cells in the flank. The animals were kept under pathogen-free conditions at a temperature of 19 to 22 °C, 50 to 60% humidity, and 65 air changes per h, and animals were allowed food and water *ad libitum*. The mice were anesthetized with isoflurane (2% in 67% N2 / 33% O2) and the tail vein was canulated. Respiration rate and body temperature were monitored using pressure-sensitive and rectal temperature probes (SA Instruments, New York, NY, USA) and hot air flow and isoflurane flow were adjusted accordingly. The tumors were imaged with a quadrature surface coil using a dynamic imaging sequence with a temporal resolution of 21.6 s and using 60 repetitions it lasted 21.6 min (RARE pulse sequence with T_E of 7 ms; T_R of 300 ms, RARE factor 2, zero filling acceleration of 1.34, FOV25.2×14.4 mm, MTX of 56 × 32, slice thickness of 1 mm, 1 slice, 6 averages). The nanoemulsions were injected i.v. at the start of the 11th repetition as a bolus lasting approximately 20 s. Either P5 RGD nanoemulsions (n=3) or P5 CTRL nanoemulsions (n=3) were injected at a dose of 80 μmole of lipid/kg bodyweight, resulting in 20 μmole Gd per kg bodyweight. In the frames with the highest relative signal enhancement in the DCE-MRI, pixels in the tumor with at least 6 % relative signal enhancement were color coded using Matlab (The MathWorks, Natick, MA, USA). Those color coded relative signal enhancement maps were merged with high resolution T1-weighted post-injection images to visualize the distribution of signal enhancement throughout the tumor. The high resolution T_1-weighted images were obtained 25 minutes post nanoemulsion injection (FLASH pulse sequence, T_E of 5.4 ms, T_R of 350 ms, FOV 25.2×14.4 mm, MTX of 56 × 32, slice thickness of 1 mm, 1 slice, 4 averages). Those images were also used as a reference when defining ROIs in the dynamic sequence. The analysis of the T_1-maps and the DCE-MRI curve plotting was performed using Matlab.

Statistical analysis

Where appropriate, statistical testing was performed using 2 sample, upper tailed student t-tests with Minitab software (Minitab Inc., State College, PA, USA). The significance criterion was $p \leq 0.05$.

Results and discussion

Nanoparticle synthesis and characterization

Nanoemulsions with six different PEG2000-DSPE contents (5, 10, 20, 30, 40, and 50 mol%, referred to as P5, P10, P20, P30, P40, and P50 respectively) were synthesized and characterized (Figure 1A), and all the components were incorporated at molar ratios as shown in Figure 1B. Increasing PEG2000-DSPE content resulted in a substantial decrease in nanoemulsion droplet size (data not shown). Plausibly this is due to PEG2000-DSPE having a very low critical packing parameter (≈0.05), which reflects the dimensional proportion between the hydrophobic and hydrophillic part of an amphiphile.[25] Hence, in PEG2000-DSPE the hydrophilic polymer is very large relative to the hydrophobic fatty acid tails. This large hydrophilic polymer tends to increase the curvature of the membrane in which the lipid incorporates, decreasing the diameter of the particle. As nanoparticle size may have significant effect on cellular interaction and uptake,[26] the nanoparticle size was kept constant by increasing the amount of soybean oil with increasing PEG2000-DSPE content in the nanoemulsions (Figure 1B–C). The hydrodynamic diameters of the six nanoemulsions with different PEG content were approximately 100 nm with polydispersity indices (PDI) well below 0.2 (Figure 1C–D). The zeta potential of the nanoemulsions was below −25 mV, providing good colloidal stability caused by electrostatic repulsive forces between individual particles. The amino moiety in PEG-DSPE is negatively charged at neutral pH,[27] which was reflected by a more negative zeta potential for increasing PEG2000-DSPE content (Figure 1E). RGD conjugation increased the zeta potential slightly, which might be explained by shielding of the negative surface charge by the RGD peptides.[28] More detailed information about dynamic light scattering (DLS) results can be found in Table S1 in supporting information.

Cellular uptake and targeting as function of PEG content

To study the effect of PEG density on targeting efficiency, we used the $\alpha_v\beta_3$-integrin specific RGD peptide as a model targeting ligand. This integrin plays an important role in vascular angiogenesis for solid tumor growth and is significantly upregulated on angiogenically activated endothelial cells. Human umbilical vein endothelial cells (HUVEC) grown in culture are reported to express high levels of this integrin as well,[29] providing an *in vitro* model to study the targeting of the RGD conjugated nanoemulsions. HUVEC were incubated with medium containing either no emulsion (blank), or the RGD or non-targeted control (CTRL) version of the six formulations described above, and the cellular uptake was quantified using flow cytometry. For P5 and P10, RGD functionalization resulted in increased cellular uptake compared to uptake of CTRL nanoemulsions (Figure 2A–B). For P20, P30, P40 and P50 no difference between cellular uptake of targeted or non-targeted nanoemulsions was observed (Figure 2A–B). Furthermore, in case of P5, P10 and P20 both targeted and non-targeted nanoemulsions internalized in 100% of the HUVEC, whereas for P30, P40 and P50 only 60 to 90% of the cells internalized the nanoemulsion, indicating decreased non-specific cellular interaction at higher PEGylation densities (Figure S1 in Supporting Information). The uptake ratio between RGD and CTRL nanoemulsions demonstrated that the targeting efficiency was highest at low PEG densities (P5 and P10) and virtually no targeting effect was observed at 20 mol% and higher PEG2000-DSPE densities (Figure 2C). Using the model published by Gennes *et al.*,[30] we calculated (Section S1 in Supporting Information) that on P5, the PEG was predominantly present in a mushroom configuration whereas in the formulations with higher PEG densities, the polymer would be mostly present in the brush configuration, extending outwards from the nanoemulsion surface. Taken together, this confirmed our hypothesis that high PEG surface densities inducing the brush configuration decrease the nanoparticle targeting efficiency.

Klibanov *et al.* observed that increasing the PEG5000-DSPE content up to 7 mol% in biotinylated liposomes progressively hampered their binding to avidin.[12] They conjugated biotin directly to the phosphate headgroups where they will readily be covered by surface bound PEG. Although Klibanov *et al.* did demonstrate that nanoparticle PEGylation can affect ligand-directed nanoparticle targeting, it is likely that in such a configuration the PEG5000 interferes in the avidin-biotin binding in a similar fashion as in delaying opsonization. To circumvent this issue, Kirpotin *et al.* conjugated anti-HER2 Fab′ fragments to the distal ends of PEG chains in liposomes.[31] They observed no effect on the targeting efficiency of varying PEG2000 densities up to 5.7 mol%. This PEG density is what is maximally achievable for liposomes without affecting their morphology (obtaining micellular aggregates instead of liposomes), [32] and at this surface density no PEG brush conformation is induced. The unique features of our nanoparticle platform allow increasing the PEG surface density up to 50% and thus study the influence of PEG on ligand targeting in both a wider range of densities and as well as at densities high enough to induce the brush regime. Another possible explanation for why Kirpotin *et al.* did not detect effects of PEG density on targeting is that due to the large size of the Fab fragment (~55000 g/mol) compared to both RGD (720 g/mol) and the PEG polymer used (2000 g/mol), the PEG coating may have less effect on ligand-target interactions.

Cellular uptake mechanism and intracellular trafficking

The cellular uptake and intracellular trafficking of the RGD and CTRL nanoemulsions were studied by live cell CLSM as a function of PEG surface density. To study particle integrity after internalization, the P5, P10 and P20 nanoemulsions were double labeled with two spectrally different fluorochromes conjugated to two different lipids; rhodamine-PE and NIR664-PEG2000-DSPE. In case of rhodamine-PE, rhodamine is directly conjugated to the PE headgroup, whereas NIR664 is conjugated to the distal end of PEG in NIR664-PEG2000-DSPE. Importantly, as those two lipids differ significantly in their molecular structure, they could be expected to be trafficked differently when the nanoemulsions disintegrate intracellularly. For P5 it was found that in the first two hours after incubation with the nanoemulsion, the fluorescence from the two different lipids generally colocalized in case of RGD nanoemulsion, but was more separated in case of CTRL nanoemulsions (Figure 3A–B). This demonstrated that the RGD functionalization had a significant effect on the intracellular fate of the nanoemulsions. The RGD nanoemulsions were internalized intact and stayed mostly intact up to 2 h after incubation, whereas the CTRL nanoemulsions were mostly disintegrated immediately upon internalization. In case of P10 and P20, no differences in integrity of the RGD and CTRL nanoemulsions could be detected, both the RGD and CTRL nanoemulsions seemed partially disintegrated during the first 2 h after internalization (Figure S1 in Supporting Information). At these higher PEG surface densities, the RGD nanoemulsions behaved similar to non-specific CTRL nanoemulsions, an observation which was also reflected by the poor targeting at higher surface PEG density found by flow cytometry measurements (Figure 2C). Therefore the following *in vitro* experiments were done with P5 nanoemulsions.

To study whether the difference in integrity for P5 RGD and CTRL nanoemulsion could be related to different intracellular localization, HUVEC were co-incubated with RGD and CTL nanoemulsions labeled with respectively NIR664-PEG-DSPE and rhodamine-PE. Minimal colocalization between the RGD and CTRL nanoemulsions was observed in the first 2 h after incubation, confirming different intracellular localization and potentially different internalization mechanisms (Figure 3C). Endocytic pathways were studied by utilizing inhibitors of clathrin- and caveolae-mediated endocytosis. HUVEC were incubated with nanoemulsions in the presence of the inhibitors and cellular uptake was subsequently quantified by flow cytometry (Figure 3D). For P5 RGD nanoemulsions a slight, but not statistical significant reduction in cellular uptake was observed when clathrin-mediated endocytosis was inhibited (approximately 25% reduction, p=0.131). Inhibition of caveolae-dependent pathways resulted in approximately 80% reduction in cellular uptake (p=0.004), indicating that the RGD nanoemulsions were mainly internalized through a caveolae-dependent pathway. This is consistent with the reports that $\alpha_v\beta_3$-integrin can be internalized via caveolae-mediated endocytosis.[33]

Similar results were obtained for CTRL nanoemulsion, however, the differences were less pronounced. The CTRL nanoemulsion was hardly internalized via clathrin-dependent pathways, but mainly through the caveolae-dependent pathway (clathrin-mediated endocytosis inhibition: approximately 10% reduction in uptake with p=0.305, caveolae-dependent endocytosis inhibition: approximately 50% reduction in uptake with p=0.0001). Hence, as the inhibition of caveolae-dependent pathways decreased cellular uptake by only 50%, other caveolae- and clathrin-independent pathways are probably involved in cellular entry of the CTRL nanoemulsions as well.

Various intracellular destinations such as endoplasmatic reticulum, Golgi apparatus, endosomes and lysosomes have been observed for material internalized via a caveolae-dependent pathway.[34] Thus, we labeled lysosomes with lysotracker, which stains acidic compartments such as late endosomes and lysosomes, to study the colocalization between lysosomes and nanoemulsions. A clear difference in association between lysosomes and the RGD and CTRL nanoemulsions was observed (Figure 3E–F). The P5 CTRL nanoemulsions mostly colocalized with lysosomes shortly after incubation, whereas the RGD nanoemulsions showed little association with the lysosomes in the first 2 h after internalization. These results are in agreement with the findings of Oba *et al.* studying the internalization mechanism and lysosomal colocalization of RGD-conjugated and non-conjugated PEG-polylysine-DNA polyplexes[35, 36]. They also found that the RGD-conjugated nanoparticles internalized in a caveolae-dependent manner, colocalized with lysosomes to a lower extend than non-targeted nanoparticles, and predominantly localized to perinuclear regions. Thus, the RGD-peptide caused a different intracellular route for the targeted nanoemulsions than for non-targeted nanoemulsions. CTRL nanoemulsions seemed to follow the classical route from early endosomes to late endosomes and lysosomes, whereas the RGD nanoemulsions were shown to escape this route. It is known that $\alpha_v\beta_3$-integrin is localized in caveolae and that after internalization it is recycled back to the plasma membrane via various routes[37]. These integrin recycling routes bypass lysosomes, which may explain that the transport to late endosomes and lysosomes of RGD nanoemulsions was avoided. Moreover, certain viruses[38] and bacteria[39] have been found to enter cells in a receptor-mediated caveolae-dependent manner and avoid lysosomal degradation as well. As lysosomes constitute the intracellular digestion compartment, the observed colocalization between lysosomes and CTRL nanoemulsions explain the disintegration of the CTRL nanoemulsions upon cellular entry as opposed to the RGD nanoemulsions, which stayed intact after internalization.

Intravital CLSM

To study *in vivo* vascular targeting efficiencies, we employed a tumor model growing in dorsal window chambers in mice. This model allows intravital CLSM, providing real time imaging of nanoemulsion dynamics and localization at a subcellular resolution. Employing spectrally different fluorescent labels for simultaneously administered CTRL and RGD functionalized nanoemulsion, this model offers the exciting possibility to study the tissue distribution of both nanoemulsions in the same tumor tissue, excluding individual differences affecting the observations. *In vitro,* we found that 5 mol% PEGylation resulted in optimal targeting efficiency. However, a recent study has shown that RGD conjugated nanoemulsions containing 50 mol% PEG2000-DSPE exhibited specificity for the tumor vasculature.[40] Thus, vascular targeting of both P5 and P50 nanoemulsions were studied *in vivo*.

P5 RGD nanoemulsions were confined to the vessel wall, and essentially no extravasation was observed approximately 6 h post injection (Figure 4A–B), demonstrating high affinity for the tumor vasculature. At the same time point, the P5 CTRL nanoemulsion hardly accumulated in the vessel wall, but extravasated from the tumor vasculature (Figure 4A–B). Simultaneous injection of the differently fluorescently labeled CTRL and RGD nanoemulsions, demonstrated that at spots where the CTRL nanoemulsions displayed extensive extravasation (Figure 4B), being indicative for high vascular permeability, the RGD nanoemulsions were restricted to the vessel wall (Figure 4B). This confirmed that the P5 RGD nanoemulsions selectively target the angiogenic tumor vasculature. Previous studies have also demonstrated that RGD multivalency directs nanoparticles to angiogenic endothelial cells and restricts extravasation.[41, 42] 3D reconstructions of z-stacks obtained intravitally showed that throughout the imaged volumes, RGD nanoemulsions remained relatively homogeneously confined to the vessel wall, whereas CTRL nanoemulsions extravasated from the vasculature in a more heterogeneous manner (Figure 4C–D and Figure S2 in Supporting Information).

For P50, only a minor targeting effect was observed approximately 6 h post injection. In mice injected with spectrally different RGD and CTRL P50 nanoemulsion, the agents were distributed very similarly throughout the tumor tissue as demonstrated by the high degree of colocalization of the two agents (Figure 4E). Although this indicated non-specific association of the CTRL nanoemulsions with the vessel wall and extravasation of RGD nanoemulsion, the RGD nanoemulsions colocalized with the vessel wall to a slightly higher extent, as illustrated in the magnified insets in Figure 4E. Although this demonstrated some specificity of P50 for the tumor vasculature, our main conclusion is that these *in vivo* results confirmed the *in vitro* observation that targeting of the nanoemulsions is most efficient at low surface PEG density and that at high PEG surface density the RGD nanoemulsions behave very similar to CTRL nanoemulsions.

Studying the tissue accumulation kinetics of the RGD and the CTRL agents, it was found that the P5 RGD nanoemulsions started to accumulate in the vessel wall within 10 min post injection, as shown by a speckeled pattern colocalized with the vessel wall, which persisted to 30 min post injection (Figure 5A). In addition to the speckled pattern, the nanoemulsion was clearly observable inside the vasculature at these early timepoints. At 2 h post injection, less circulating nanoparticles were observed, and the vessel wall had become highly fluorescent resulting in a distinct delineation of the tumor vasculature, which remained clearly visible up to 24 h post injection (Figure 5A). Hence, the accumulation of the P5 RGD nanoemulsions started upon injection and the particles accumulated and remained in the vessel wall throughout the imaging period. In case of the P5 CTRL nanoemulsion, only few of the inspected tumor regions contained extravasated nanoemulsions within the first 30 min post injection, visible as high fluorescence intensity foci, and probably only in regions with high vascular permeability (Figure 5B). Two to 4 h after injection some regions showed clear extravasation, however, other regions remained without any detectable accumulation of the agent. It was not before approximately 8 h post injection that the majority of the tumor contained significant quantities of extravasated nanoemulsion, which persisted up to 24 h post injection (Figure 5B). Taken together, the P5 RGD nanoemulsions accumulated both faster and in a more homogenous manner in the tumor tissue than the P5 CTRL nanoemulsions.

The P50 nanoemulsions displayed similar dynamics as the P5 nanoemulsions (Figure S3 in Supporting Information); faster accumulation of the RGD nanoemulsions than of the CTRL nanoemulsions. However, significant extravasation of both P50 agents was observed at later time points and as pointed out earlier, the specificity for the vessel wall of the P50 RGD nanoemulsions was substantially lower than that of P5 RGD nanoemulsions.

DCE-MRI

With the subcellular resolution of the intravital CLSM it was possible to demonstrate pronounced differences for particle distribution and accumulation dynamics of the P5 RGD and CTRL nanoemulsions. However, it did not provide information on the particle behavior on the whole tumor level. For this purpose we used MRI. First we investigated whether it was possible to detect the cellular uptake of the nanoemulsions with MRI *in vitro*. HUVEC were incubated with medium containing either no (blank) or RGD or CTRL P5 nanoemulsions labeled with gadolinium (Gd-DTPA-DSA). After thorough washing, cell pellets were formed and imaged with a T_1-weighted protocol including quantification of T_1 relaxation times (Figure 6A–B). A pronounced decrease in T_1 relaxation times of cell pellets incubated with the paramagnetic nanoemulsions was found. However, the decrease in T_1 relaxation times was most pronounced in the cell pellets incubated with the RGD version. The Gd concentration in cells was calculated, based on an ionic relaxivity of 3.2 $mM^{-1}s^{-1}$, as we measured at 7 Tesla and room temperature, and the uptake ratio between RGD and CTRL nanoemulsions was found to be very similar to the uptake ratio determined by flow cytometry. This demonstrated that our P5 nanoemulsions are observable with MRI and that relative quantification of the nanoparticle uptake with MRI is feasible *in vitro*.

MRI and other clinical imaging modalities lack the spatial resolution to readily discriminate between contrast agent binding to the tumor endothelium and contrast agent extravasation to the tumor interstitium in static images. As the intravital CLSM not only demonstrated differences in the nanoemulsion distribution, but also showed a distinct difference between the dynamics of the CTRL and the RGD nanoemulsions, we explored DCE-MRI for its ability to study the dynamics of the nanoemulsions on the whole tumor level and thereby corroborate the CLSM findings.

The thin tumors in the dorsal window chamber do not allow MRI at sufficient signal to noise ratio. Therefore we used ovarian cancer xenografts growing subcutaneously on the flank of mice for the DCE-MRI. The P5 nanoemulsions were injected i.v. and the tumor was imaged at a temporal resolution of 21.6 s over the time course of 20 min. Signal enhancement in the tumor during the DCE-MRI predominantly occurred in the tumor rim, whereas the core region of the tumor displayed limited signal increase (inset in Figure 6C). It has also been shown that in solid tumors, $\alpha_v\beta_3$-integrin expression by vascular endothelial cells is predominantly occurring at the tumor rim.[41] Therefore, the signal intensity *versus* time curve from the DCE-MRI was obtained from ROIs containing only the tumor rim. These curves showed a clear difference between the dynamics of the RGD and the CTRL nanoemulsions. For both nanoemulsions an increase in signal intensity was observed, however for the RGD nanoemulsions the increase was larger (Figure 6C). Additionally, the signal intensity increased faster for the RGD nanoemulsion, reflecting the more rapid accumulation of the RGD nanoemulsions as was observed in the intravital CLSM. This not only confirmed that the different dynamics of the RGD and the CTRL nanoemulsions as was observed in the intravital CLSM experiments occurred throughout the angiogenic tumor rim, but also demonstrated that DCE-MRI can become a potent tool to study the targeting efficacy of targeted MRI contrast agents *in vivo*. Actually, recently, the first studies employing dynamic MRI techniques to study the dynamics of nanoparticles targeted to the tumor vasculature have appeared.[43, 44] Oostendorp *et al.* used a paramagnetic nanoparticle and also observed their targeted agent to accumulate both faster and in larger amounts in the tumor rim in the first 20 min post injection.[43]

As the DCE-MRI curves mainly reflect the dynamics of two processes, namely blood clearance of the agent, which causes a reduction in signal intensity, and increasing contrast agent concentration in the tumor, which increases the signal intensity, absolute quantification of $\alpha_v\beta_3$-integrin expression levels is not straightforward. Furthermore, the accumulation of the RGD nanoemulsions is not necessarily solely occurring via integrin targeting, but might partially occur also through non-specific processes. Moreover, the nanoemulsions are likely to change their relaxivity upon accumulation in the tissue, further complicating quantification. However, the present approach can be used to qualitatively assess $\alpha_v\beta_3$-integrin expression, which would be a useful tool in monitoring response to anti-angiogenic therapy in both preclinical and clinical settings.

Conclusion

We developed a multimodal nanoparticle system in which the PEG surface density can be judiciously varied. Employing this nanoparticle platform and a unique combination of real time intravital microscopy and DCE-MRI allowed us to systematically investigate the effect of PEG surface density on *in vivo* nanoparticle behavior. Specifically, in this study we investigated the effect of PEG surface density on the targeting potential of ligand-functionalized nanoparticles. Intravital microscopy provided real-time information on nanoparticle targeting to the angiogenic tumor vasculature at the cellular level, while DCE-MRI could be used to study targeting kinetics at the macroscopic tumor level. Tumor accumulation occurred much faster for the targeted nanoemulsions than for the non-targeted versions. Counter intuitively, yet consistent with the PEG density conformation models, both the *in vitro* and *in vivo* studies demonstrated that a low PEG surface density, at which the PEG chains are present in a mushroom configuration, was optimal for efficient and specific nanoparticle targeting. Therefore, we conclude that the optimal PEG surface density for peptide conjugated nanoparticle systems around 100 nm in size should be set below 10 %. Conveniently, typical nanoparticle PEG surface densities used to improve nanoparticle pharmacokinetics and biodistribution are within this range. This is crucial knowledge in the quest for the optimal design of ligand-functionalized nanoparticles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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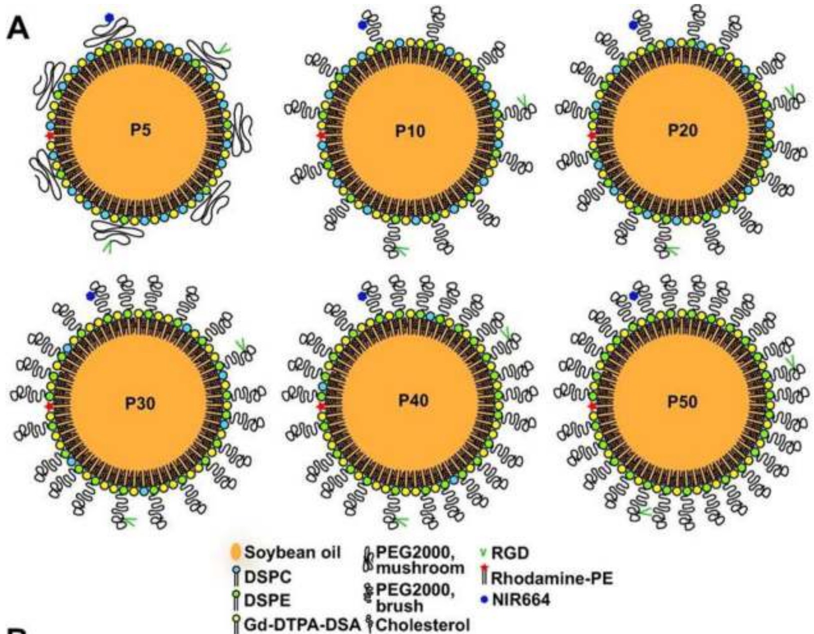


B

	P5	P10	P20	P30	P40	P50
DSPC (mol%)	62	57	47	37	27	17
Cholesterol (mol%)	33	33	33	33	33	33
PEG2000-DSPE (mol%)	2.5	7.5	17.5	27.5	37.5	47.5
Mal-PEG2000-DSPE (mol%)	2.5	2.5	2.5	2.5	2.5	2.5
Soybean oil (mg/μmol lipid)	3	3.5	3.7	4.4	5.3	5.8

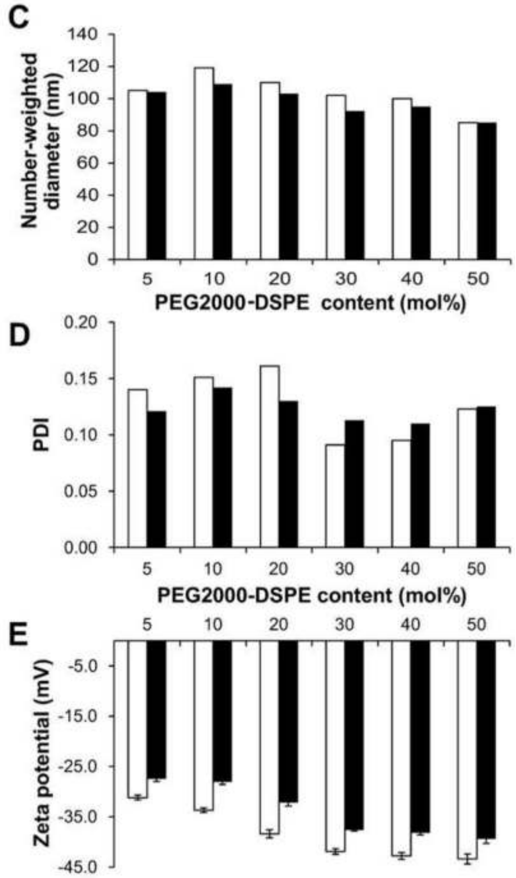


Figure 1.

Nanoemulsion schematics and characteristics. A: Cartoons of the nanoemulsions with the different PEG2000-DSPE content and mushroom/brush configuration indicated. B: Lipid and soybean oil content of the different nanoemulsions. C: Hydrodynamic diameters of the nanoemulsions as a function of the PEG2000-DSPE content as measured with dynamic light scattering. D: Polydispersity indexes (PDI) as a function of the PEG2000-DSPE content of the measured diameters as reported in figure C. E: Zeta potentials as a function of the PEG2000-DSPE content. For C–E: white bars: CTRL nanoemulsion, black bars: RGD nanoemulsion.

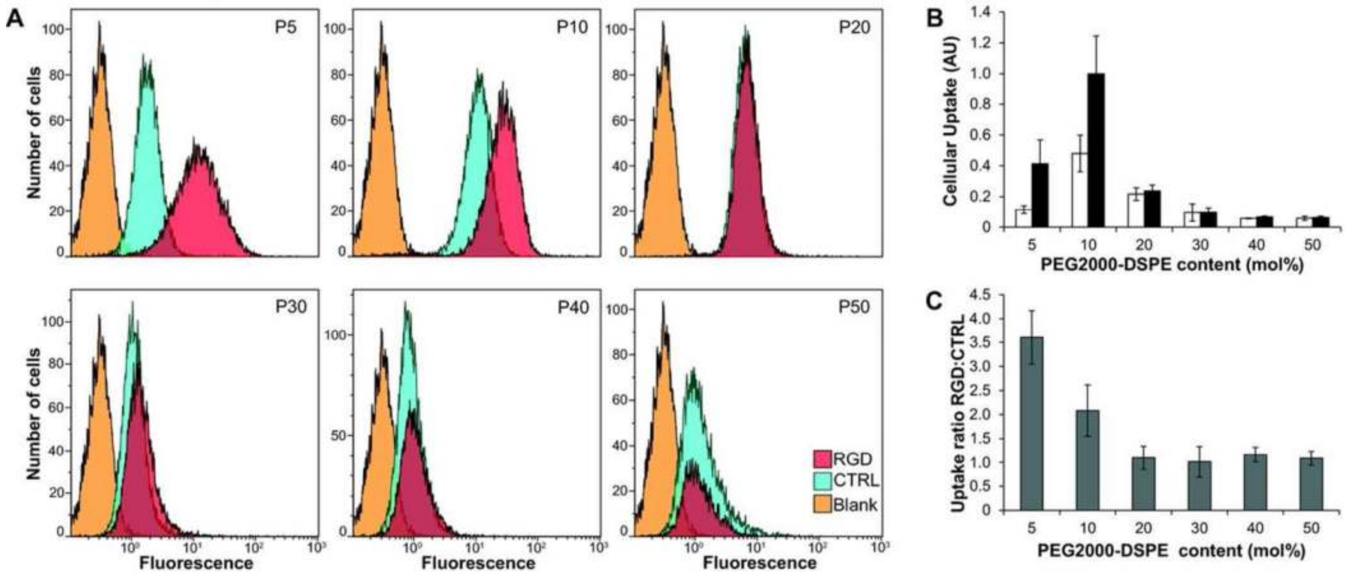


Figure 2.

Cellular uptake as a function of PEG2000-DSPE content. A: Logarithmic flow cytometry histograms for the nanoemulsions with the different PEG2000-DSPE content. B: Normalized cellular uptake as the median fluorescence intensity of the positive cells divided by the median of the cellular autofluorescence for CTRL (white bars) and RGD (black bars) nanoemulsion. The error bars represent the standard deviation (n=6). C: Cellular uptake ratio between RGD and CTRL nanoemulsions. The error bars represent the standard deviation (n=6).

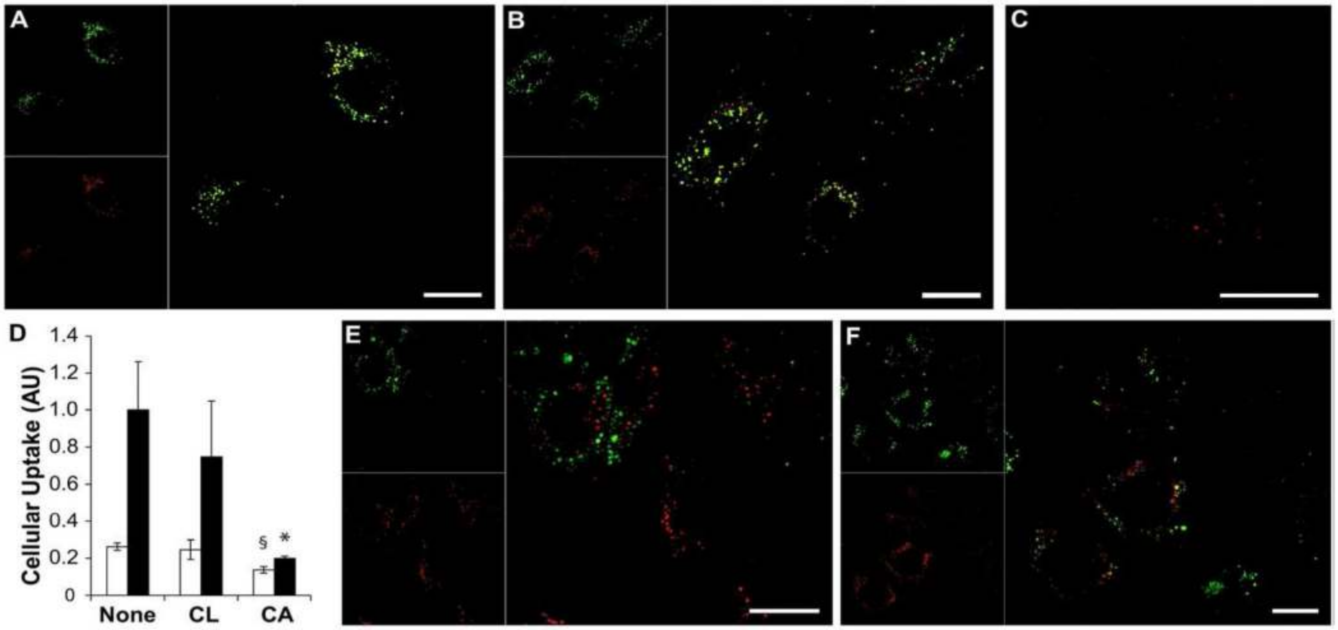


Figure 3.

HUVEC incubated with P5 nanoemulsion for 3 h. White bars represent 20 μm. A–B: RGD nanoemulsions (A) and CTRL nanoemulsions (B) which were double labelled with rhodamine-PE (green) and NIR664-PEG2000-DSPE (red). 2 h post incubation RGD emulsions remained intact (yellow in overlay) whereas the CTRL emulsions partially disintegrated. C: HUVEC simultaneously incubated with RGD nanoemulsion (red) and CTRL nanoemulsion (green) demonstrating different intracellular localization. D: Cellular uptake for CTRL (white bars) and RGD (black bars) nanoemulsions when no endocytosis blocking was performed (None), when clathrin-dependent pathways were blocked (CL) and when caveolae-dependent pathways were blocked (CA). (§: CTRL None vs CA $p=0.0001$ and *: RGD None vs CA $p=0.004$). The error bars represent the standard deviation ($n=4$). E–F: HUVEC with lysosomes labelled in green incubated with RGD nanoemulsions (red in E) and CTRL nanoemulsions (red in F).

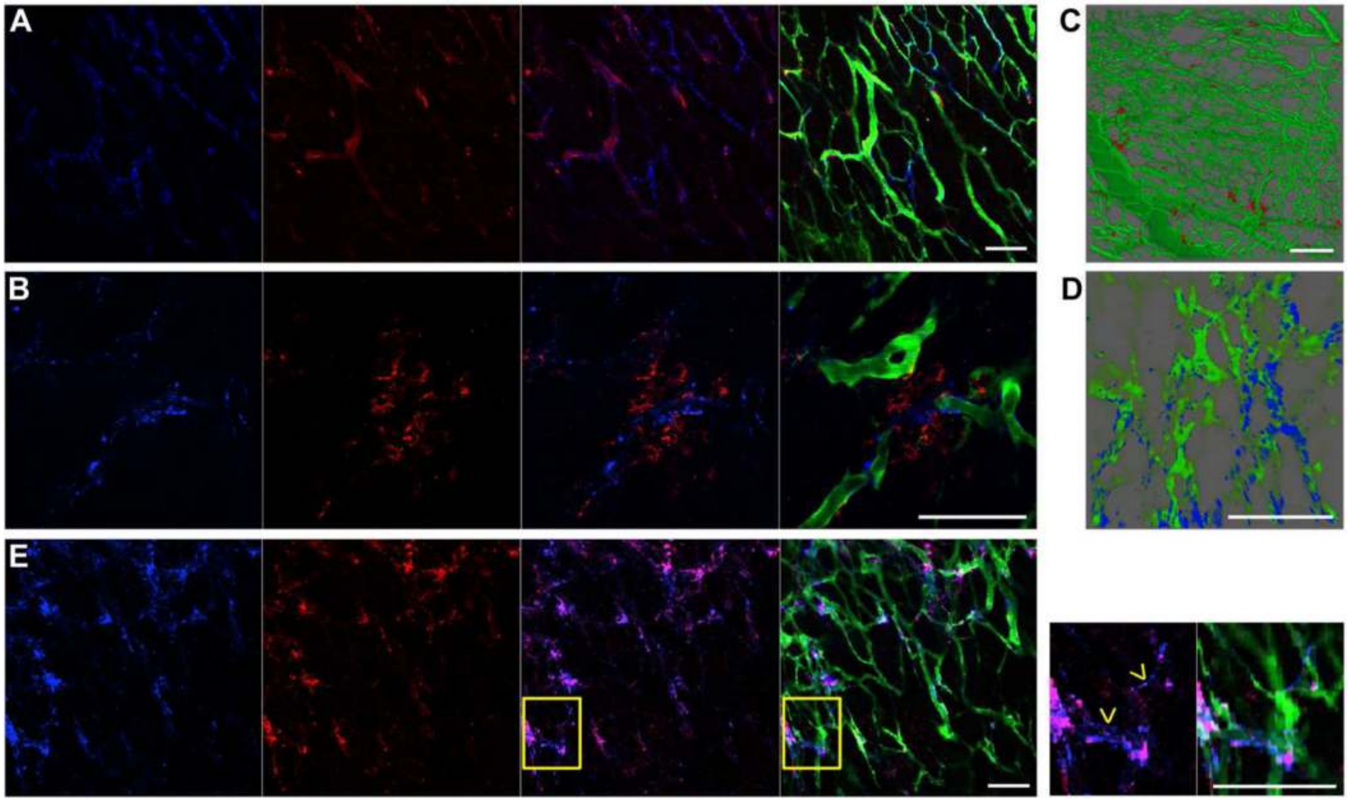


Figure 4.

Nanoemulsion distribution imaged by intravital microscopy. Images obtained after injection of P5 nanoemulsions (A–D) and P50 nanoemulsions (E). RGD nanoemulsion in blue, CTRL in red and the vasculature in green. Scale bars represent 100 μm. A: At 6 h post injection, the P5 RGD nanoemulsion was confined to the vessel wall, whereas the CTRL nanoemulsion extravasated. B: A region with extensive extravasation of the P5 CTRL nanoemulsion, where the P5 RGD nanoemulsion was confined to the vessel wall 6 h post injection. C–D: 3D reconstructions from z-stacks obtained 4 h post injection of P5 RGD nanoemulsion (C) and 8 h post injection of P5 CTRL nanoemulsion (D). E: At 6 h post injection of P50 nanoemulsions the distribution of the RGD and the CTRL nanoemulsions was very similar as evident from the pink color in the overlay. In the magnified insets the arrows indicate RGD nanoemulsion localizing to the vessel wall.

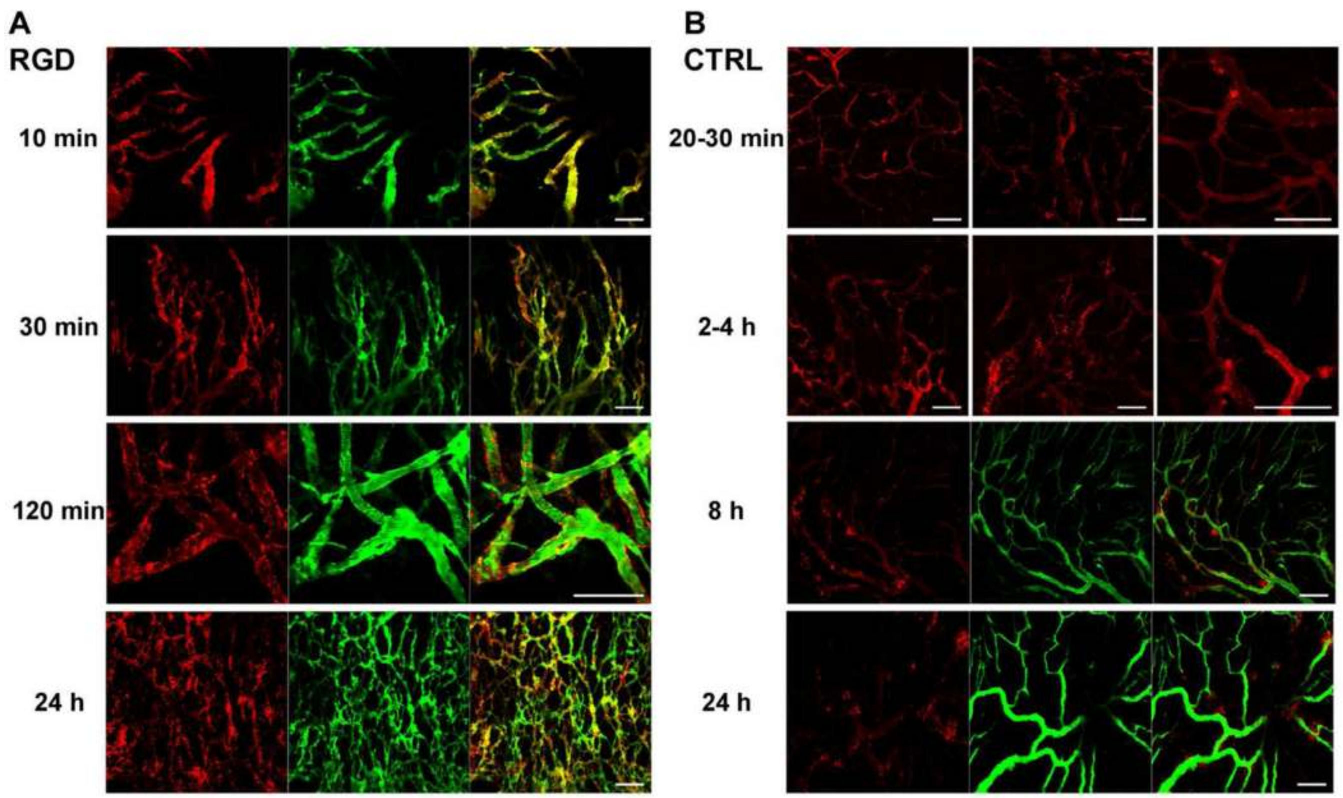
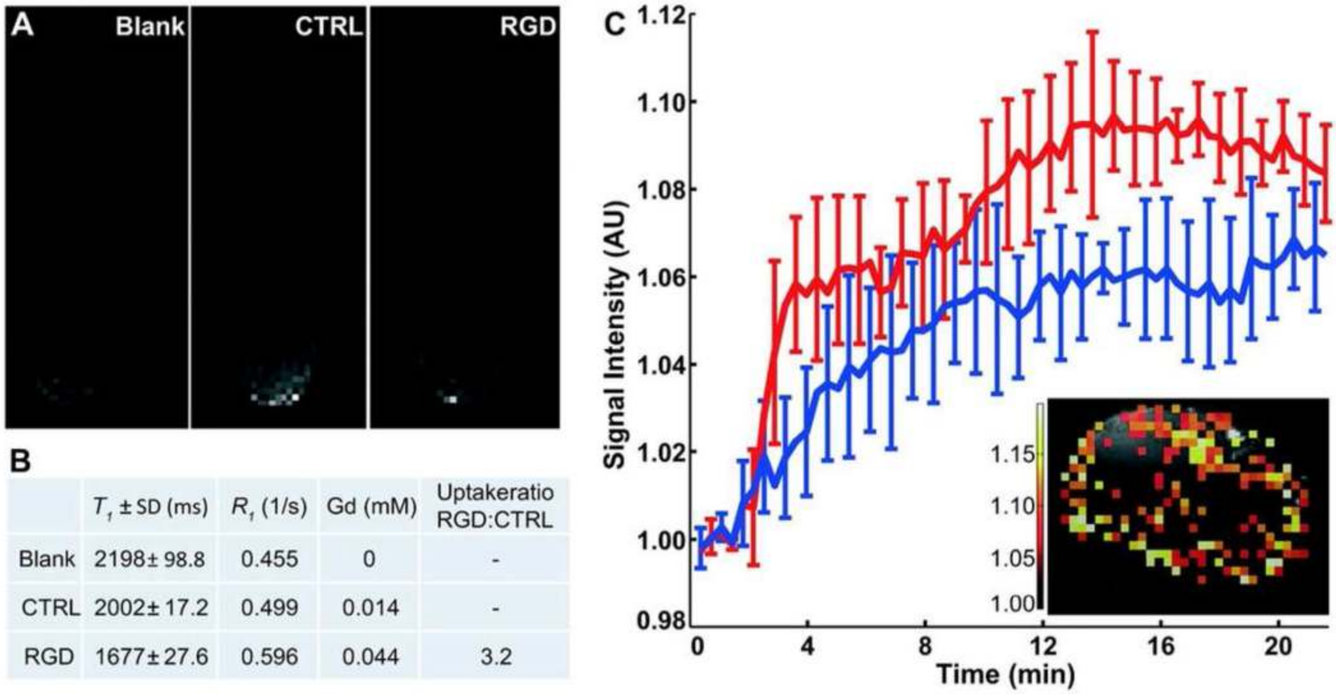


Figure 5.
P5 nanoemulsion accumulation kinetics imaged by intravital microscopy. Nanoemulsions in red and the vasculature in green. White bars represent 100 μm. A: Already at 10 and 30 min post injection of RGD nanoemulsions, a speckled accumulation pattern was observed and 2 h post injection and onwards a clear binding to the vasculature wall occurred. B: Up to 4 h post injection, the extravasation of CTRL nanoemulsion was very heterogeneous as shown in the multiple images at those time points. Regions with significant accumulations and extravasation, visible as high fluorescence intensity foci, and also regions with hardly any fluorescence were observed. At 8 h post injection and onwards the CTRL nanoemulsion had extravasated throughout the tumor.



	T_1 ± SD (ms)	R_1 (1/s)	Gd (mM)	Uptakeratio RGD:CTRL
Blank	2198± 98.8	0.455	0	-
CTRL	2002± 17.2	0.499	0.014	-
RGD	1677± 27.6	0.596	0.044	3.2

Figure 6.

MRI results obtained with P5 nanoemulsion containing Gd-DTPA-DSA. A: T1-weighted images of pellets of HUVEC incubated with either no (blank), or RGD or CTRL nanoemulsion. B: T1 and R1 (1/T1) values in the cell pellets (n=3) and the calculated cellular Gd concentration. The uptake ratio was determined by dividing the Gd concentration in RGD incubated pellets by the Gd concentration in CTRL incubated pellets. C: DCE-MRI curves in the tumor rim in mice injected with CTRL nanoemulsion (blue, n=3) or RGD nanoemulsion (red, n=3). The error bars represent the standard deviations.¬ The inset shows a high resolution T1-weighted image of a tumor on a mouse flank obtained 25 min post RGD nanoemulsion injection. The color coded overlay shows the relative signal intensity in the DCE-MRI in the same units as the curves.