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Iron oxide core oil-in-water emulsions as a multifunctional nanoparticle platform for tumor targeting and imaging

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Abstract

Nanoemulsions are increasingly investigated for the delivery of hydrophobic drugs to improve their bioavailability or make their administration possible. In the current study, oil-in-water emulsions with three different mean diameters (30, 60, and 95 nm) were developed as a new multimodality nanoparticle platform for tumor targeting and imaging. To that aim, hydrophobically coated iron oxide particles were included in the soybean oil core of the nanoemulsions to enable their detection with magnetic resonance imaging (MRI), while the conjugation of a near infrared fluorophore allowed optical imaging. The accumulation of this novel nanocomposite in subcutaneous human tumors in nude mice was demonstrated with MRI and fluorescence imaging *in vivo*, and with Perl's staining of histological tumor sections *ex vivo*.

1. Introduction

Research on the development and application of nanoparticles for biomedical purposes, popularly referred to as nanomedicine, has had unprecedented growth in recent years [1–3]. Specifically, iron oxide [4], quantum dots [5] and gold nanoparticles [6] were recognized to have extraordinary features for diagnostics as well as therapeutics [7,8]. The past decade has also witnessed tremendous advances in the field of nanoparticle facilitated molecular imaging, while non-invasive imaging has shown to be especially valuable to investigate aspects such as target specificity, the pharmacokinetic profile as well as the therapeutic potential of novel materials [3,9,10]. For example polymeric nanoparticles have shown utility in the field of targeted drug delivery as well as for diagnostic purposes, including magnetic resonance imaging (MRI) [11,12,13].

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The design of effective nanocomposites for molecular imaging and/or therapy involves careful consideration of the properties required for the application in question. Once the required properties have been established, candidate nanoparticle platforms may be identified. The particle synthesis can then be optimized to generate an assembly with the appropriate contrast/therapeutic agents included, optimized surface coating, targeting properties, defined size and a high degree of biocompatibility [3].

It is highly desirable to have a strategy to create all-in-one nanocomposites that are easy to synthesize and functionalize, are flexible, multifunctional, biocompatible, allow the inclusion of both nanocrystals and (macro)molecules, and of which the size can be judiciously varied [10,14]. Oil-in-water emulsions are an example of a nanoparticle platform that is capable of carrying both high payload of hydrophobic materials in their core and an amphiphilic payload in their surfactant corona [15,16].

In the current study we developed such an all-in-one nanoparticle platform that is based on oil-in-water emulsions. They are composed of a hydrophobic oil core component, which also includes nanocrystals (iron oxides). These oil droplets are subsequently stabilized by a lipid mixture that favors the formation of small particles in a size-range of 30 nm to 100 nm. The latter is a prerequisite to exploit the Enhanced Permeability and Retention (EPR) effect, i.e. the gathering of long-circulating particles in tumors [17–19]. To increase the longevity of the particles in the circulation, PEGylated lipids were included in the synthesis [20,21]. The iron oxide particles provide contrast for MRI, whereas lipids labeled with a near infrared (NIR) fluorophore (Cy5.5) were incorporated into the nanoemulsion corona for optical imaging. The concept is schematically depicted in Figure 1A.

The particles were extensively characterized and applied to mice bearing subcutaneous human xenograft tumors of EW7 (Ewing's) sarcoma, where we were able to visualize the accumulation of the particles in the tumors by MRI and fluorescence imaging *in vivo* as well as with histological techniques *ex vivo*.

2. Materials and methods

2.1. Materials

Distearoyl-sn-glycero-3-phosphocholine (DSPC), distearoyl-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] ammonium salt (PEG-DSPE) and distearoyl-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] were all purchased from Avanti Polar Lipids and used as received. The colloidal suspension of oleic acid coated magnetite (Fe_3O_4) particles of 3–15 nm in toluene was obtained from the “Polytehnica” of Timisoara (Romania). The Cy5.5 NHS ester was purchased from Amersham Biosciences (Piscataway, NJ). Cell culture supplies were purchased from Invitrogen (Carlsbad, CA).

2.2. Synthesis of the Cy5.5-PEG-DSPE lipid

73.5 mg NH_2 -PEG-DSPE was dissolved in a 500 μl 9:1 chloroform: methanol mixture and 10 mg Cy5.5 NHS ester in 500 μl DMSO. 25 ml of a 0.1 M sodium bicarbonate solution (pH~8.5) was prepared, heated to 70 °C and the NH_2 -PEG-DSPE solution added dropwise under vigorous stirring to form a micelle preparation. After cooling down to room temperature, the DMSO solution containing the Cy5.5 NHS ester was added to the micelle solution and stirred overnight in a beaker under N_2 . The preparation was washed extensively with millipore water by using a Vivaspin MW 10000 column concentrator. After 6 wash cycles the preparation was redispersed in 1 ml millipore water and freeze dried for 3 days. The residue was solubilized in a 2:1 chloroform: methanol mixture to a final concentration of 2 mg/ml Cy5.5 and stored at –80 °C.

2.3. Synthesis of the nanoemulsions

For the synthesis of the nanoemulsions, separate stock solutions of all the lipophilic components were prepared in chloroform. The concentrations were as follows: DSPC stock solution: 25 mg/ml, PEG-DSPE: 100 mg/ml, soybean oil: 0.5 g/ml, iron oxide particles: 100 mg/ml, Cy5.5 PEG-DSPE: 2 mg/ml. The composition of the formulation with the highest total amount of soybean oil was: 2.8 mg of DSPC, 10 mg of PEG-DSPE, 38 mg of soybean oil, 9 mg of iron oxide particles and 989 μ g of Cy5.5 PEG-DSPE lipids. The formulation with the medium total amount of oil consisted of 2.8 mg DSPC, 10 mg PEG-DSPE, 20 mg soybean oil, 4.74 mg iron oxide particles and 760 μ g Cy5.5 PEG-DSPE. The nanoemulsion with the lowest amount of oil contained 2.8 mg DSPC, 10 mg PEG, 10 mg soybean oil, 2.36 mg iron oxide particles and 502 μ g Cy5.5 PEG-DSPE. The components were mixed together and added dropwise under stirring to 8 ml of boiling millipore water. Subsequently, the crude emulsion was homogenized by sonication using a thin sonicator tip (BioLogics, Inc., 3.9 mm). The preparation was sonicated for 20 min (level 20 %, pulse 70 %, device: Biologics, Inc., ultrasonic homogenizer model 150 V/T) while cooled with room temperature water. Finally, the preparation was concentrated to a final volume of 2.8 ml. The preparations were measured with a dynamic light scattering device before and after filtration through a 0.2 μ l filter. The filtered nanoemulsions were stored in the dark at 4 °C.

2.4. Dynamic light scattering and zeta potential measurements

The hydrodynamic sizes of the particles were measured using a dynamic light scattering (DLS) device from Malvern Instruments (model HPPS). The sizes of the nanoemulsions were determined directly after they were synthesized. The formulations were diluted before the measurement as follows: 500 μ l millipore water was mixed with 50 μ l of the particles with the smallest amount of oil or 20 μ l for the other two nanoemulsions. The mean sizes were determined with independent measurements from three drawn samples of the preparation with 5 runs per sample, 15 determinations in total. The Zeta potential was determined by using a device from Brookhaven Instruments Corporation (Zeta PALS, Zeta Potential Analyzer). Due to the interference of Cy5.5 fluorescence with the measurement, three nanoemulsions containing all the aforementioned components except the Cy5.5 lipids were used. In order to achieve measureable samples, 2000 μ l of millipore water were mixed with 20 μ l of the formulation with the highest content of oil, whereas 60 μ l of the medium one and 150 μ l of the smallest one were used. Three different samples of one nanoemulsion were measured.

2.5. Transmission electron microscopy

A Hitachi H7650 linked to a SIA (Scientific Instruments and Applications) digital camera run by Maxim CCD software was used. TEM was performed on the nanoemulsions diluted 1:10 and 1:20 in an ammonium acetate buffer using a 2 % sodium phosphotungstate (pH=7) negative stain as described by Forte and Nordhausen [34].

2.6. Relaxometry

In order to determine the longitudinal (r_1) and transverse relaxivities (r_2) of the particles, T_1 and T_2 measurements of solutions were performed on a 60 MHz Bruker Minispec (Bruker Medical BmbH, Ettingen) operating at 40 °C. Dilutions of 1:10, 1:20 and 1:40 of the nanoemulsions in millipore water were prepared and 200 μ l were used for the measurement. R_1 and r_2 of the samples was then calculated from the slope of the graph of $1/T_1$ and $1/T_2$ plotted against the iron concentration.

2.7. Cell culture and tumor model

Human EW7 (Ewing's sarcoma) cancer cells were maintained in RPMI 1640 medium supplemented with 10 % FCS. The cells were grown in a 5 % CO₂, water saturated atmosphere

at 37 °C and subculturing was performed twice a week by 1:10 dilution after trypsinization. Six-week-old male swiss nude mice were purchased from Taconic (Albany, NY) and all animal handling approved by the Mount Sinai School of Medicine Institutional Animal Care and Use Committee. To establish subcutaneous tumors in nude mice 2 million cells were injected. The tumors used in this study were in a volume range of 65–125 mm³.

2.8. Fluorescence imaging

Ex vivo and *in vivo* fluorescence imaging experiments were performed using an IVIS Imaging System 200 (Xenogen, Alameda, CA). To enable detection of the Cy5.5 fluorescence a 615–665 nm excitation filter and a 695–770 nm emission filter were used.

For the determination of the pharmacokinetics and the half-life of the three nanoemulsions, the compounds were intravenously administered and blood was drawn from the saphenous vein of the mouse leg after 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h and 24 h. The blood was collected in pre-weighed 1.5 ml cups containing 40 µl heparin (10 U/ml), centrifuged for 7 min at 3000 rpm with a table centrifuge and 20 µl of the supernatant pipetted into 0.2 ml cups. The photon count in the different samples was determined with the IVIS 200 using the following parameters: focus height of 0.3 cm, field of view 6.5 × 6.5 cm, and an excitation time of 0.5 s.

For the *in vivo* fluorescence imaging experiments, the highly fluorescent food of the nude mice was withdrawn at least 6 hours prior to the experiment. Moreover, remaining food powder traces in the fur were removed by wiping the mice with wet gauze. The mice were pre-scanned, injected with the nanoemulsions, and scanned at several time points post injection (5 min, 15 min, 30 min, 1 h, 2 h, 4 h and 24 h). A field of view of 12.2 × 12.2 cm was chosen and the focus height was set to 1.5 cm, while the excitation times varied from 1 s, up to 3 s and 5 s, depending upon the fluorescence intensity. After the imaging experiments the mice were sacrificed by saline perfusion under isoflurane anesthesia and the tumor, liver, lung, spleen and kidneys were excised and imaged together with the corresponding organs of a control mouse that was not injected. The parameters for the image acquisition were set as follows: focus height of 0.5 cm, field of view of 12.2 × 12.2 cm and excitation times of 0.5 s, 1 s and 3 s.

2.9. MR imaging

Nude mice under isoflurane anesthesia bearing subcutaneous EW7 tumors were scanned using a 9.4 T MRI system (Bruker Instruments). MR imaging was performed by using a spin-multiple echo sequence with a repetition time of 2500 ms and ten echos, i.e. 6.7 ms, 13.3 ms, 20 ms, 26.7 ms, 33.3 ms, 40 ms, 46.7 ms, 53.3 ms, 60 ms and 66.7 ms. The field of view was 2.6 × 2.6 cm, the matrix size 128 × 128, the no. of slices ten with a slice thickness of 1 mm and the no. of averages 4 which amounted to a total scan time of 16 min. After the pre-scans, mice were intravenously injected with one of the three nanoemulsions (equivalent of 16.7 mg/kg iron oxide) and scanned again 24 h afterwards according to the protocol above.

3. Results and discussion

3.1. Synthesis and physical characterization of the nanoemulsions

To synthesize the nanoemulsions, an elegantly simple procedure was developed. All necessary components, i.e. ordinary phospholipids, PEGylated lipids, soybean oil and, when appropriate, oleic acid coated iron oxide particles were added to chloroform. The resulting mixture was added dropwise and under vigorous stirring to boiling millipore water. This procedure led to an instantaneous evaporation of the chloroform and to the immediate formation of a crude oil-in-water emulsion. Subsequently, the preparation was homogenized using a probe sonicator to form a uniform nanoemulsion. An extended description of the synthesis method can be found in the Methods section.

After concentrating the formulation, the hydrodynamic mean sizes of the different preparations were measured by dynamic light scattering (DLS) before and after filtration through a 0.2 μm pore sized filter. To generate stable oil-in-water emulsions with different mean particle sizes, we first focused on nanoemulsions without the inclusion of iron oxide nanocrystals. By increasing the amount of soybean oil while maintaining a constant lipid content, we were able to achieve this goal. Many of the oil-in-water emulsions available today and reported in literature are well above 150 nm in diameter [22]. In the current study we applied lipid mixtures that favor the formation of small particles. Consequently we were able to reproducibly manufacture stable particles with mean sizes of 30, 60, or 95 nm (Supplementary Figure S1).

To enable their detection with MRI we included oleic acid capped iron oxide nanoparticles into the preparations by replacing equivalent volumes of soybean oil to yield particles whose mean sizes were again 30, 60, or 95 nm. In the photographs depicted in Figure 1B an apparent increase of turbidity can be observed for the preparations with increasing size. Inductively coupled plasma mass spectrometry (ICP-MS) analysis measurements revealed a nearly 100 % encapsulation efficacy of magnetite. Assuming magnetite to be composed of iron oxide nanoparticles with a mean size of 10 nm (Figure S2, Supplementary Information), one can calculate that on average there are 36 iron oxide particles per 95 nm, 9 per 60 nm and 1 per 30 nm nanoemulsion particle. To allow optical imaging of the nanoemulsions, we first included a lipophilic near-infrared dye in the oil core of the particle along with the iron oxides. We observed, however, very low emission intensity in this case. This effect is consistent with a report in the literature, where the simultaneous inclusion of magnetic particles and a quantum dot fluorophore in a microemulsion led to greatly reduced fluorescence [23]. Therefore, the NIR fluorophore Cy5.5 was attached to the outside lipid layer to provide a strong fluorescence signal. Cy5.5 was linked distally to NH₂-PEG-DSPE lipids in a NHS ester synthesis (described in the Methods section) and incorporated into the “30 nm”, “60 nm”, and “95 nm” formulations at respectively 1.8 mol%, 2.7 mol % and 3.5 mol%, at the expense of regular PEG-lipids. The increasing percentage of Cy5.5 was incorporated to compensate for the expected increasing fluorescence quenching effects for the larger particles (due to higher iron oxide content). We will refer to the nanoemulsions containing iron oxide in the core and Cy5.5 attached as “95 nm”, “60 nm”, and “30 nm”, according to their mean particle size.

Negative stain transmission electron microscopy (TEM) was performed on the three different sized nanocomposites (Figure 1C) and corroborated our particle size findings obtained with DLS (Figure 1B). To establish the potential of the iron oxide loaded nanoemulsions to serve as MRI contrast agents, the longitudinal relaxivity (r_1) and transverse relaxivity (r_2) were measured at 1.41 T. Both r_1 and r_2 are expressed in $\text{mM}^{-1}\text{s}^{-1}$ and the higher the r_2/r_1 ratio the more the agent becomes favorable for so-called T_2^* -weighted MRI over T_1 -weighted MRI [24]. Interestingly, we observed r_1 and r_2 to be size dependent, with r_1 increasing slightly from 2.5 ± 0.4 , to 2.7 ± 0.3 , to $2.9 \pm 0.3 \text{ mM}^{-1}\text{s}^{-1}$ for 30, 60, and 95 nm emulsions, respectively. A more pronounced increase was found for the r_2 values, ranging from $76 \pm 1.6 \text{ mM}^{-1}\text{s}^{-1}$ for 30 nm, to $136 \pm 9.7 \text{ mM}^{-1}\text{s}^{-1}$ for 60 nm, and up to $184 \pm 7.1 \text{ mM}^{-1}\text{s}^{-1}$ for 95 nm. Long-term stability and shelf life was investigated by monitoring the sizes of the nanoemulsions with repeated DLS measurements over a period of 3 months. In Figure S3A and B it can be appreciated that the particle mean size as well as the polydispersity index did not significantly alter in this period. No indication of aggregation or phase separation was observed. Zeta potential determinations, which provide a reliable characterization of the stability of a colloid with respect to coagulation or flocculation, revealed values corresponding to a good stability of below -30 mV for all three formulations, namely $-31.7 \pm 3.1 \text{ mV}$ for 30 nm, $-39.5 \pm 1.5 \text{ mV}$ for 60 nm, and $-52.8 \pm 6.7 \text{ mV}$ for 95 nm. Importantly, we also determined the stability of the nanoparticles in a setting where we mimicked physiological conditions by incubating them in PBS containing 10 % fetal bovine serum at 37 °C for 20 hours. We did not observe significant changes in the particle diameter as determined by DLS.

3.2. *In vitro* results

The biocompatibility of the 60 nm nanoemulsions (both with and without iron oxide nanocrystals) was investigated using a viability assay on three different cell lines at 143 $\mu\text{g Fe/ml}$ for 6 hours. The investigated cells, EW7 tumor cells, J774A1 macrophages and human umbilical vein endothelial cells (HUVECs), were selected since they are an integral part of tumors. No decrease in cell viability was observed for EW7 cells, while the viability of macrophages and the HUVECs marginally decreased to 90% for the nanoemulsions without iron oxide and to 83% and 74% for the formulations with iron oxide, for macrophages and HUVECs, respectively (see Supplementary Figure S4). The viability of the cells at the applied high nanoparticle dose is comparable to other commercially available particles or newly developed nanoparticulate contrast agents described in the literature [13].

The uptake of the three nanoemulsions by EW7 cells (incubated at a Fe concentration of 40 $\mu\text{g/ml}$ for 9 hours) was assessed with T_2 -weighted MR imaging of loosely packed cell pellets. The particles with the highest mean size of 95 nm generated the highest signal loss, which was gradually followed by the 60 and 30 nm formulations (Supplementary Figure S5). This variation can be explained by the higher ionic r_2 values for the larger particles.

3.3. *In vivo* fluorescence imaging

The NIR label included in the established oil-in-water nanocomposites enabled us to perform *in vivo* fluorescence imaging experiments to determine the pharmacokinetic profile and organ distribution. The agents were injected into nude mice via the tail vein and blood samples were collected up to 24 h post administration. The analysis of the corresponding fluorescence images of the serum samples over time revealed that there is a small, but significant size dependent decrease in half-life with an increase in particle size (Figure 2A and Supplementary Figure S6). Moreover, we acquired fluorescence images of nude mice ($n=2$ for the three different sizes) intravenously injected with the nanoemulsions in order to follow the distribution of the particles and to monitor their accumulation in the tumors. At a dose of 16.6 mg/kg magnetite we appreciated an increase of fluorescence in the tumors for the “30 nm” and the “60 nm” formulations, whereas no tumor signal was observed for the “95 nm” particles (Figure 2B, 2C). Nonetheless, after sacrificing the animals 24 h post injection and excision of the tumors and the organs, Cy5.5 fluorescence accumulation could be observed for all three particles (Supplementary Figures S7, S8). To ensure the absence of contrast agent in the circulation, the mice were sacrificed by perfusion with saline under isoflurane anesthesia. In these *ex vivo* measurements it was appreciated that besides accumulating in the tumor, the different agents were primarily present in the organs of the reticuloendothelial system (RES), i.e. liver and spleen. In the *in vivo* scans liver accumulation could only be observed in the anterior scans (Supplementary Figure S9), but not in the posterior scans presented in Figure 2. The reason detectable Cy5.5 fluorescence can be appreciated in the tumor in the *ex vivo* situation for the 95 nm nanoemulsion compared to the *in vivo* situation is likely due to the absorbance of light by the mouse's skin. As shown in the normalized charts of Figure S8 of the Supplementary Information, there is an increase in the percentage of fluorescence per ROI of the tumors injected with the 30 nm and 60 nm formulations compared to the ones injected with the 95 nm nanoemulsion.

3.4. *In vivo* magnetic resonance imaging

The tumor targeting and imaging potential of the iron oxide containing nanoemulsions was also investigated using T_2 -weighted MRI. This type of MRI depicts iron oxide accumulations as hypointense regions in the acquired image. To ensure the observed effect was due to iron oxide, control nanoemulsions were manufactured that included all earlier mentioned components except the magnetite nanocrystals. Nude mice bearing subcutaneous EW7 tumors were pre-scanned using a Bruker 9.4 T small animal dedicated MRI system under constant

isoflurane anesthesia. A spin echo MRI sequence (details in Methods section) was used to generate images for analysis. After the pre-scans the three different sized particles and the control particles were intravenously injected, and the mice were subsequently scanned at 24 h post injection. Hypointense regions in the tumors can be clearly appreciated for all three agents (Figure 3), while no significant signal attenuations were found for the tumors of animals that were injected with the control formulations (Figure S10 of Supplementary Information). The analysis of the different tumors (n=4 mice per agent) revealed that the mean normalized signal intensity over the entire tumor volumes decreased by 7 % for 30 nm, 8 % for 60 nm, and 22 % for 95 nm at 24 hours after administration. Although the trend is obvious, the latter data were not significant because of the inherent heterogeneity of the tumor tissue, which causes large variations in the tumor intensity as observed by MRI. Nevertheless, apparent and significant signal loss was found in localized regions within the tumors that varied from 18 % to 35 % for 30 nm, 19 % to 37 % for 60 nm, and 28 % to 49 % for 95 nm.

3.5. *Ex vivo* histology

Perl's staining of histological sections of the tumors was used to provide further evidence for the accumulation of iron oxide. The tumors were excised after the 24 h post MRI scan and fixed overnight in a 4% paraformaldehyde solution containing 5% sucrose to avoid dehydration and shrinking of the cells in the tissue. Following cutting with a cryomicrotome, the stained sections showed massive uptake of iron oxide (Supplementary Figure S11). Interestingly, we were also able to match the hypointense regions of the MRI image with a histological composite brightfield image obtained of a corresponding tumor cross-section (Figure 4).

In the current study we were able to manufacture stable nanoemulsions with accurately controlled mean sizes of 30, 60, and 95 nm that carry contrast agents to enable their multimodality visualization. The particles were extensively characterized, applied *in vivo*, and their capability for the delivery of nanocrystals to subcutaneous tumors in a nude mouse model was demonstrated.

The unique blend of using synthetic materials, such as nanocrystals, and natural molecules makes it a very attractive candidate for *in vivo* applications [25,26]. In addition, the preparation method is original, but relatively easy, which allows for facile production of large quantities that are necessary for *in vivo* experiments. Importantly, the encapsulation efficiency for hydrophobic compounds is close to 100% (determined via ICP-MS), because the surfactant molecules encapsulate the entire hydrophobic phase, along with its components. Therefore, further purification steps to remove untrapped materials are not required. Another important advantage is that the lipid-mixture that is applied as a surfactant to stabilize this oil-in-water platform is chosen such that it has the tendency to form micelles instead of bilayered vesicles [27]. This allows the formation of emulsions in a size ranging from 30 nm to 100 nm, by simply varying the ratios of the different components without the necessity to use a co-surfactant. This is ideal for establishing an optimized combination of the pharmacokinetic profile, penetration into diseased tissue and uptake by cells. In comparison to microemulsion systems applied for diagnostics [22,28] our platform is much smaller and the preparation strategy avoids contamination with large vesicular structures.

The possibility of encapsulating hydrophobic drugs or other materials and the ability of monitoring their delivery to tumors or other pathological sites makes this platform especially versatile. Instead of (or in addition to) imaging methods like MRI and optical imaging used in the current study, this platform can be extended for computed tomography (CT) or positron emission tomography (PET) imaging [29,30]. This can be accomplished by using hydrophobically coated gold particles [6] in the core and/or radiolabeled lipids [31] as part of the corona.

Many newly developed drugs belong to the BCS (Biopharmaceutics Classification System) class IV, characterized by low permeability, low solubility and low oral bioavailability [32], which strongly limits their administration to patients. By solubilization of the compounds in the oil phase of the presented nanoemulsion platform their diminished applicability can be overcome [33]. Long known and highly cytotoxic drugs like the very hydrophobic compound Camptothecin could potentially become suitable for chemotherapy.

Moreover, the delivery of the iron oxide particle loaded nanoemulsions together with poorly water soluble drugs could be used in combination with magnetic thermal therapy [8]. The latter technique utilizes an alternating magnetic field together with the presence of magnetic particles to generate heat that triggers the release of the drug from the oil droplets. In addition to the controlled release of the nanoemulsion's content, the local hyperthermia could also have a synergistic effect and thereby increase the therapeutic efficacy.

4. Conclusion

A multifunctional and biodegradable nanocarrier system based on water-in-oil nanoemulsions was developed that can be employed for the delivery of hydrophobic nanocrystals and other hydrophobic agents. Moreover, we have demonstrated tunability of the overall particle diameter by changing the mean droplet sizes in a range of 30 nm to 100 nm using two different lipids only, without the need for co-surfactants. In the current study tumor accumulation of the particles was accomplished by exploiting the EPR effect. The multimodality features of the system, the ease of fabrication, the flexibility and the long shelf live of several months generates significant advantages over other nanoparticle systems. The high payload potential of the nanoemulsions allowed us to load high quantities of iron oxide nanocrystals, causing an remarkably high transverse relaxivity (r_2), which is desirable for T_2^* -weighted MRI. This nanoparticle system also allows for the inclusion of other nanocrystals and therefore can be easily extended to other imaging techniques, but also has potential to be used as a drug delivery vehicle.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix

Additional description of the methods used for Perl's staining of histological sections as well as the 3D rendering of the MR images; long-term DLS measurements; fluorescence images for *ex vivo* pharmacokinetics and biodistribution; evaluation of the biodistribution data; results of MR imaging of subcutaneous tumors after administration of control particles; additional histological images (Perl's staining); TEM image of the oleic acid capped iron oxide particles.

Figure 1. Concept, size determinations and relaxometry

(A) Schematic depiction of the three different sized nanoemulsions used in this study. (B) Dynamic light scattering (DLS) size measurements and photographs of the nanoemulsion preparations. (C) Negative stain transmission electron microscopy (TEM) images of the corresponding formulations (smallest size left). (D) Corresponding values for the longitudinal (r_1) and the transverse (r_2) relaxivities ($n=3$, mean $\pm$ standard deviation). (E) Zeta potential values.

Figure 2. Pharmacokinetics

(A) Chart showing normalized pharmacokinetic curves of the three nanoemulsions after intravenous injection into nude mice. (B) *In vivo* NIR fluorescence imaging series showing the accumulation of the 30 nm nanoemulsion in a subcutaneous tumor after intravenous injection. (C) Region of interest (ROI) fluorescence analysis to determine the tumor to skin ratio, i.e. the quotient of the fluorescence photon count from the tumor and the skin determined in a ROI of the same size, for the three different nanoemulsions after intravenous administration.

Figure 3. MR images of nude mice bearing subcutaneous EW7 tumors, injected with the three different nanoemulsions

(A) T₂-weighted MR images of the pre-scans (TR 2500 ms, TE 53.3 ms), (B) corresponding T₂-weighted MR images 24 h after intravenous injection. The insets show the enlarged MR images of the tumors, respectively. White arrows indicate local hypointense regions within the tumor caused by iron oxide accumulations. (C) 3D images that show the proton density MR image (grey), the whole tumor in 3D (transparent green) and the areas of iron oxide deposition in red. The inset images show an oblique view of the tumor with contrast agent in order to illustrate its 3D structure.

Figure 4. Correlation between MRI and histology

(A) T₂-weighted MR image (TR 2500 ms, TE 53.3 ms) of a subcutaneous EW7 tumor in a nude mouse. (B) Corresponding T₂-weighted MR image 24 h after injection with the 30 nm nanoemulsion. The hypointense signal within the yellow outline indicates a local area of high iron content. (C) Image of a Perl's stained histological section of the same tumor showing the iron deposits as blue stained areas. The inset depicts a higher magnification. The area of signal loss seen in the MR image from (B) matches the location of the iron staining (yellow outline) in the histology image from (C).