Published in final edited form as:

Bioconjug Chem. 2012 May 16; 23(5): 941–950. doi:10.1021/bc200543k.

Nanoclusters of iron oxide: effect of core composition on structure, biocompatibility and cell labeling efficacy

Geralda A.F. van Tilborg^{1,*}, David P. Cormode², Peter A. Jarzyna², Annette van der Toorn¹, Susanne M.A. van der Pol³, Louis van Bloois⁴, Zahi A. Fayad², Gert Storm⁴, Willem J.M. Mulder², Helga E. de Vries³, and Rick M. Dijkhuizen¹

¹Biomedical MR Imaging and Spectroscopy Group, Image Sciences Institute University Medical Center Utrecht, Utrecht, The Netherlands ²Translational and Molecular Imaging Institute, Mount Sinai School of Medicine, New York, NY, USA ³Department of Molecular Cell Biology and Immunology, VU University Medical Center, Amsterdam, The Netherlands ⁴Department of Pharmaceutics, Institute for Pharmaceutical Sciences, Utrecht, The Netherlands

Abstract

Inorganic nanocrystals have a variety of applications in medicine. They may serve as contrast agents, therapeutics and for in vitro diagnostics. Frequently, the synthesis route yields hydrophobically capped nanocrystals, which necessitates their subsequent coating to render a water-soluble and biocompatible probe. Biocompatibility is crucial for cellular imaging applications, which require large quantities of diagnostically active nanoparticles to be loaded into cells. We have previously reported the design and synthesis of a fluorescent and magnetic resonance imaging-detectable core-shell nanoparticle that encapsulates hydrophobically coated iron oxide nanocrystals. The core of soybean oil and iron oxide is covered by a shell mixture of phospholipids, some of which contained polyethylene glycol. Despite the biocompatibility of these components, we hypothesize that we can improve this formulation with respect to *in vitro* toxicity. To this aim, we measured the effect of six different core compositions on nanoparticle structure, cell labeling efficacy and cell viability, as well as cell tracking potential. We methodically investigated the causes of toxicity and conclude that, even when combining biocompatible materials, the resulting formulation is not guaranteed to be biocompatible.

Keywords

MRI; fluorescence; nanoparticle; cytotoxicity

Introduction

Inorganic nanocrystals such as quantum dots, gold nanoparticles and iron oxides have a wide variety of applications in the field of biomedicine. Semiconductor nanocrystals, also known as quantum dots, are useful in a variety of fluorescence related applications ¹, such as fluorescence imaging ², and probing biological systems using Förster resonance energy transfer (FRET) ³. Gold nanostructures can be used as computed tomography contrast agents ^{4, 5}, as tracers for optical ^{6, 7} and photoacoustic imaging ⁸, for photothermal

*Dr. Geralda A.F. van Tilborg, Yalelaan 2, 3584 CM Utrecht, The Netherlands, gtilborg@gmail.com, Phone: +31 30 2535515.

Supporting Information Available

Additional materials and methods, figures and tables are available in the supporting information, as described in the text. This information is available free of charge via the Internet at <http://pubs.acs.org/>.

ablation⁹, and for in vitro diagnostics¹⁰. Iron oxide nanoparticles can be used in magneto-hyperthermia therapies¹¹, for in vitro diagnostics¹², and as magnetic resonance imaging (MRI) contrast agents¹³. One of the most important uses of iron oxide based contrast agents is to label cells to allow them to be tracked non-invasively by MRI. In vivo cell tracking may provide an important tool in (pre)clinical diagnostics and therapeutics, for instance, by enabling monitoring of leukocyte infiltration¹⁴ or stem cell migration¹⁵.

Inorganic nanocrystals may be ideally synthesized under hydrophobic conditions, which produce nanocrystals of highly defined size and high stability. For instance, synthesis of iron oxides in octadecene and other hydrophobic solvents result in very monodisperse cores of controllable diameter, which can be coated with oleic acid and dispersed in hydrophobic solvents^{16, 17}. In contrast, aqueous phase synthesis of iron oxides via the Molday method results in amorphous iron oxide cores¹⁸. However, despite their advantageous properties, such hydrophobically coated nanocrystals are not miscible with biological media. Therefore, the development of methods to render hydrophobically coated nanocrystals biocompatible is of great importance and is pivotal for biomedical applications. This is particularly relevant for cell tracking studies, which requires cell loading with large quantities of contrast agent, while preserving biological function.

There have been several methods proposed for making hydrophobic nanocrystals biocompatible. For example, Dubertret et al. used phospholipids to suspend quantum dots in aqueous media¹⁹. Polymers such as poly-maleic anhydride-alt-1-octadecene derivatives have been used by the group of Colvin and others, to solubilize quantum dots and iron oxides^{20–23}. A variety of nanocrystals have been encapsulated in lipoprotein-like coatings^{24–26}. We have previously reported a method to create biocompatible, core-shell nanoparticles of controllable diameter via the use of a carefully chosen phospholipid mixture for the shell and soybean oil as the core material²⁷. These nanoemulsions were utilized to suspend oleic acid coated iron oxides in aqueous media, creating iron oxide nanoclusters and allowing their use as an MRI contrast agent, as demonstrated in tumor-bearing mice. Fluorophores were included in the lipid coating to provide contrast for fluorescence imaging. The formulation was well-tolerated by mice. Furthermore, we have lately used this platform for delivery of hydrophobic glucocorticoid drugs, where we observed significant reductions in tumor growth compared to controls²⁸. Nevertheless, at moderate to high incubation doses we also observed in vitro toxicity of the formulation *without drug*, indicating an adverse reaction to this soybean oil-iron oxide platform even though composed of biocompatible building blocks.

In the current study we developed this core-shell platform for simultaneous fluorescent and magnetic labeling of cells and tested the effect of a variety of core materials on nanoparticle formation and biocompatibility. To be specific, we compared nanoparticles with nanoclusters of iron oxide, and either soybean oil, medium chain triglycerides, olive oil, cottonseed oil, corn oil or no oil as core materials, with the aim of developing an improved formulation for cell tracking applications. Of the two formulations with highest biocompatibility, we extensively characterized the nanoparticles as well as their cellular uptake by means of MRI, confocal microscopy, transmission electron microscopy, and other methods, in comparison with the original formulation. Furthermore, we methodically assessed to what extent nanoparticle composition, storage conditions and storage time affect cell viability and activity.

Experimental Procedures

Information on the materials, nanoparticle characterization, intracellular iron content determination and thiobarbituric acid reactive substances assay can be found in the supplementary information.

Nanoparticle formation

Nanoparticles were prepared according to a solvent evaporation method, as described by Jarzyna et al.²⁷ First, oleic acid-coated iron oxide nanocrystals were retrieved from the toluene carrier fluid by evaporation under nitrogen flow. Next, lipids, oil and dry iron oxide nanocrystals were individually dissolved in chloroform to obtain the following stock solutions: 25 mg/ml DSPC, 100 mg/ml PEG2000-DSPE, 1 mg/ml Rhodamine-PE, 0.5 g/ml oil and 100 mg/ml iron oxide. Stock solutions were used to obtain fresh lipid/oil/iron oxide mixtures, containing 2.8 mg DSPC, 10 mg PEG2000-DSPE and 8.4 mg iron oxide nanocrystals. In case of oil inclusion, 29.5 mg soybean oil ($d = 0.919$ g/ml), 30.5 mg MCT ($d = 0.945$ g/ml), 28.9 mg corn oil ($d = 0.9$ g/ml), 29.5 mg cottonseed oil ($d = 0.918$ g/ml) or 29.2 mg olive oil ($d = 0.91$ g/ml) was also added to the mixture. Fluorescent nanoparticles were obtained by inclusion of 1 mol% (8.1 μ g) Rhodamine-PE in the lipid mixture. Oil-containing nanoparticles without iron were prepared by inclusion of 11% extra oil in the lipid/oil mixture, thereby correcting for the volume of the iron oxide nanocrystals. Directly following its preparation, the lipid/oil/iron oxide/chloroform mixture, with a maximum volume of 447 μ l, was added dropwise under stirring to 8 ml of boiling reverse osmosis water. In order to evaporate all chloroform, the resulting nanoparticle suspension was continuously heated and concentrated to 3 ml. Next, the suspension was placed under nitrogen or air and homogenized by 45 min sonication in an ultrasonic water bath (UR=198, Retsch GmbH, Haan, Germany). Following sonication, the nanoparticle suspension was cooled to room temperature, filtered through a 0.2 μ m filter (Minisart, Sartorius Stedim Biotech GmbH, Goettingen, Germany) and concentrated to approximately 1 ml using a 300 kDa MWCO Vivaspin concentrator (Sartorius Stedim Biotech GmbH, Goettingen, Germany) for 10 min at 3500 rpm. Iron concentrations were measured, and all nanoparticle preparations were diluted in reverse osmosis water to a concentration of 3.5 mg Fe/ml. Nanoparticles were stored in the dark at 4 °C under nitrogen or air, similar to the conditions used during sonication.

Cell culture and incubations

The murine macrophage cell line RAW 264.7 was grown in RPMI 1640 medium (Gibco, Invitrogen, Breda), supplemented with 10% FCS and 1% PSG. Cells were cultured at 37 °C in a humidified atmosphere and 5% CO₂.

For each experiment, cells were plated at a concentration of $0.5 \cdot 10^6$ cells/ml RPMI 1640 at 24 hours before incubation. At the onset of the experiment, medium was changed and nanoparticles were added to a final concentration of 200 μ g Fe/ml. Microscopy, MRI and labeling efficiency experiments, were performed with freshly prepared nanoparticles, whereas nanoparticles at 1, 7, 14 and 24 days post preparation were used for viability assays. Cells were incubated for three hours, followed by extensive washing in PBS and harvesting for subsequent analyses, unless stated otherwise.

Flow cytometry of fluorescently labeled cells

Cells were incubated with fluorescent nanoparticles in 24 wells plates, washed and harvested by incubation with 1 mM EGTA in PBS for 5 min at 37 °C. Cell suspensions were centrifuged for 5 min at 1500 rpm and resulting pellets were resuspended in 100 μ l PBS/0.1% BSA. Cellular fluorescence, excited with a 488 nm argon-ion laser, was measured in

fluorescent channel 2 (FL-2: 575/25 nm) of a FACSCalibur flow cytometer (BD Biosciences, Breda, The Netherlands), counting 20,000 cells per sample.

Fluorescence and light microscopy of labeled cells

Cells were grown on coverslips in 24 wells plates, incubated with fluorescent nanoparticles, washed and fixed in 4% formalin for 20 min. Subsequently, cells were either stained for iron with Prussian Blue or prepared for fluorescence microscopy. For Prussian Blue staining, cells were incubated for 30 min with a fresh mixture of 20 mg/ml Potassium Ferricyanide ($K_3[Fe(CN)_6]$) and 2 N HCl. Nuclei were counterstained for 10 min with nuclear fast red (NFR), dehydrated and mounted with Entellan. For fluorescence microscopy, nuclei were counterstained with Hoechst (5 min; 1:1000 dilution), washed in PBS and mounted in fluorescence mounting medium. Fluorescence microscopy images were obtained on a Leica DM6000 microscope (Leica, Rijswijk, The Netherlands), whereas Prussian Blue labeled cells were visualized on a Nikon light microscope (Nikon, Amstelveen, The Netherlands).

Transmission electron microscopy of labeled cells

Cells were incubated with non-fluorescent nanoparticles in 6 wells plates, washed and harvested by scraping in RPMI 1640 medium. Subsequently, cells were pelleted (5 min, 1500 rpm) and resuspended in 2.5% glutaraldehyde in PBS for fixation. The cells were embedded and stained using standard TEM pathology tissue processing techniques²⁹. The grids were imaged using a Hitachi H7650 instrument (Hitachi High Technologies, Pleasanton, CA). A minimum of fifteen cells per sample was imaged at a range of magnifications (2–50k).

MRI of labeled cells

Cells were incubated with non-fluorescent nanoparticles in T25 flasks, washed, harvested in RPMI 1640 by scraping and counted using a hemocytometer. Next, cells were pelleted by centrifugation (5 min, 1500 rpm) and resuspended to a concentration of $2 \cdot 10^3$ cells/ μ l PBS. Part of the cell sample was used to determine the intracellular iron content, while the other part was further diluted in PBS to obtain 4 samples within a range of $0.25 \cdot 10^3$ – $2 \cdot 10^3$ cells/ μ l. The obtained samples were carefully mixed with 0.8% warm agarose, at a 1:1 volume ratio in 250 μ l PCR tubes, and rapidly cooled on ice. MRI experiments were performed in a 9.4 T horizontal bore MR system (Varian Inc., Palo Alto, CA, USA). Cell-containing PCR tubes were placed in a water container, and T_2 as well as T_1 values of homogeneously distributed cells in agarose were obtained using similar sequences and parameters as described for nanoparticle characterization (see supplementary information). T_2^* -weighted MR images were acquired using a gradient echo sequence (TR/TE = 100/15 ms, $\alpha = 35^\circ$, 16 averages and $100 \times 100 \times 100 \mu\text{m}^3$ voxel resolution).

T_2^* -weighted images were analyzed in Matlab. First, a relatively large region-of-interest (ROI) was manually drawn on the image of the water reservoir surrounding the cell samples. Next, the mean signal intensity (SI_{ref}) and standard deviation (SD_{ref}) within this reference ROI were calculated, and used to define the threshold for detection of contrast enhancement: $\text{Threshold} = SI_{\text{ref}} - (5 \cdot SD_{\text{ref}})$. Pixels in the cell-containing tubes were considered contrast-enhanced in case their signal intensity (SI_{pixel}) dropped below the defined threshold: $SI_{\text{pixel}} < \text{Threshold}$.

Threshold. ROIs were manually drawn within the images of the individual sample containers, and the fraction of contrast-enhanced pixels was determined for each ROI.

Cell viability

Cells were incubated with non-fluorescent nanoparticles in 24 and 96 wells plates at 1, 7, 14 and 28 days after nanoparticle preparation. Following incubation, cells were directly washed

in PBS and subjected to viability assays (3/0h). Alternatively, cells were washed in RPMI 1640 and cultured up to 24 hours post incubation, followed by washing in PBS and viability assays (3/24h).

The viability of labeled cells was assessed with fluorescent 7-amino-actinomycin D (7-AAD) and a colorimetric MTT assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide). For 7-AAD staining, cells from the 24 wells plates were harvested by incubation with 1 mM EGTA in PBS (5 min at 37 °C), pelleted by centrifugation (5 min at 1500 rpm) and resuspended in 50 μ L PBS/0.1% BSA. A 1:500 dilution of 7-AAD staining solution (BD Pharmingen, BD Biosciences, Breda, The Netherlands) was added to each cell sample in a 1:1 volume ratio and fluorescence, excited with a 488 nm argon-ion laser, was directly measured in fluorescent channel 3 (FL-3) of a FACSCalibur flow cytometer (BD Biosciences, Breda, The Netherlands), counting 20,000 cells per sample. Untreated control cells were also stained with 7-AAD to determine baseline levels of cell death within the population, which is typically less than 15%. For the MTT assay, cells were directly incubated with MTT in the 96 wells plate (100 μ L/well, 1:10 diluted in RPMI 1640) for 1.5 hours at 37 °C and 5% CO₂. Next, MTT was removed and the formed water-insoluble formazan was dissolved in a mixture of dimethylsulfoxide and glycine (6:1 volume ratio; 175 μ L/well). The absorption of the resulting purple solution was measured at 540 nm. Untreated control cells were used as a reference with 100% metabolic activity.

Statistics

Statistical analysis was performed in SPSS (16.0). A one-way ANOVA with a post hoc Bonferroni multiple-comparison procedure was applied to test for significant differences in cellular iron content, mean fluorescent intensity per cell (MFI), metabolic activity, thiobarbituric acid reactive substances (TBARS) and nanoparticle properties compared to IO-Soy-NP and, if applicable, nanoparticles without iron and/or untreated control cells. A two-way ANOVA with Bonferroni pairwise comparisons was applied to test for significant effects of storage time and storage conditions on cell viability for each of the nanoparticles. $P < 0.05$ was considered statistically significant.

Results

Initial screening

In order to obtain a more biocompatible platform than the previously described soybean-iron oxide formulations^{27, 28}, we first synthesized five alternatives to the original soybean oil-iron oxide formulation, with medium chain triglycerides (MCT), olive oil, cottonseed oil, corn oil or no oil as core materials (Figure 1A). The described solvent-evaporation technique resulted in stable suspensions of lipid-coated nanoparticles with nanoclusters of iron oxide. Our initial screening step was characterization of the nanoparticles with TEM, to ensure that the nanoparticles had correctly formed. As can be seen in Figure 1B, inclusion of corn, cottonseed or olive oil allowed homogeneous dispersion of the oleic-acid coated iron oxide nanocrystals throughout the nanoparticle core, similar to that of soybean oil (included for comparison). MCT forced the encapsulated iron oxides outwards, to the interface of the MCT core and lipid coating (IO-MCT-NP). In the absence of the oil phase (IO-NP), iron oxide nanocrystals were densely packed inside the lipid monolayer (Figure 1B), and resulted in more irregularly shaped nanoparticles compared with the spherically shaped oil-containing nanoparticles. As the oleic acid coating of the iron oxide nanoparticle and all the oils are hydrophobic, similar iron oxide incorporation patterns were expected. The differing behavior of the iron oxide in the MCT compared to the other oils likely reflects a favorable interaction between the oleic acid (C18, unsaturated) coating of the iron oxide with the long chain unsaturated fatty acids of soybean, corn, cottonseed and olive oils, whereas such an

interaction with the saturated, short fatty acids of MCT is disfavored. Nevertheless, every formulation allowed good incorporation of the iron cores and oil within lipid coatings, and were thus included in the next screening step.

The second screening step was the *in vitro* MTT cell viability assay, which measures metabolic activity. We used murine macrophage RAW 264.7 cells to study the nanoparticles as these cells are known to efficiently internalize contrast agents. We tested the metabolic activity of these cells incubated with 200 $\mu\text{g Fe/ml}$ of media, with formulations synthesized 14 days earlier (*infra vide*). Incubation with corn, cottonseed or olive oil-based formulations resulted in considerable loss of metabolic activity to less than twenty percent of control values, which was comparable with the soybean oil-based formulation (Figure 1C). On the other hand, incubation with formulations using MCT or no oil resulted in significantly higher metabolic activity. The latter formulations were therefore selected for in depth characterization, along with the soybean oil formulation; for cell labeling purposes; and for further assessment of effects on cell viability.

Nanoparticle characteristics

Dynamic light scattering (DLS) measurements showed that IO-NP were significantly smaller than IO-Soy-NP, while IO-MCT-NP and IO-Soy-NP were of comparable size (Table 1). Nanoparticle size was highly reproducible between different batches and demonstrated a polydispersity index (PDI) < 0.2 for each preparation. Zeta potentials varied marginally from -51.3 ± 2.1 mV for IO-Soy-NP to -46.0 ± 0.3 mV for IO-NP (Table 1). Lipid-coated oil-containing nanoparticle formulations without entrapped iron oxide crystals showed equivalent size and zeta potentials compared to their corresponding iron-containing nanoparticle (Table S1), indicating that the zeta potential was predominantly determined by the lipid coating.

Relaxivities r_1 and r_2 , measures of the efficiency of the contrast agent to reduce T_1 and T_2 , respectively, and thereby generate contrast in T_1 - and T_2 -weighted MR images, were determined for each nanoparticle at 9.4 T. IO-Soy-NP and IO-MCT-NP showed similar r_2 values, whereas the r_2 of IO-NP was significantly higher (Table 1). In all cases the r_2/r_1 ratio was high (> 400), which indicates that the nanoparticles are extremely potent $T_2^{(*)}$ contrast agents that can induce strong hypointensities on $T_2^{(*)}$ -weighted MR images. The distinctive distribution of iron oxide within the nanoparticle core apparently induces differences in r_1 and r_2 relaxivities (Table 1). Most importantly, elimination of the oil phase caused a significant increase in relaxivity r_2 , which agrees with studies demonstrating that r_2 increases with iron oxide core size or aggregation of iron oxide particles in suspension³⁰. Longitudinal relaxivities (r_1) were rather low ($< 1 \text{ mM}^{-1}\text{s}^{-1}$), which is expected at high field strength, but may also be ascribed to restricted exchange of water protons with iron oxide particles in the hydrophobic core, especially when iron oxide is homogeneously distributed throughout the entire oil phase as is the case for soybean oil. The relaxivity values for IO-Soy-NP differ from those we reported previously³¹, however, this is expected as the previous measurements were acquired at a different field strength, *i.e.* 1.5 T.

Inclusion of Rhodamine-PE within the lipid monolayer provided multimodal nanoparticles that additionally allowed their detection with optical techniques. Nevertheless, it is important to note that nanoparticle fluorescence was largely quenched in the presence of iron oxide (Figure S1).

Cell labeling efficacy

RAW 264.7 cells, *i.e.* murine macrophages, were incubated with IO-Soy-NP, IO-MCT-NP and IO-NP to assess and compare their labeling efficiency. Following 3 hours of incubation

at 200 $\mu\text{g Fe/ml}$, cells labeled with IO-Soy-NP contained 1.0 ± 0.1 pg iron per cell, which was significantly less than the iron content of RAWs labeled with IO-MCT-NP (2.6 ± 0.4 pg iron) or IO-NP (5.3 ± 0.7 pg iron) (Figure 2A). Fluorescence measurements also showed significantly increased uptake of IO-MCT-NP and IO-NP compared to IO-Soy-NP (Figure 2B). However, in contrast to the iron uptake, fluorescence was higher for IO-MCT-NP than for IO-NP labeled cells. Possibly, this may be attributed to quenching of fluorescence in the presence of high iron concentrations.

Additional fluorescence measurements of cells labeled with oil-containing nanoparticles without incorporated iron oxide revealed significantly reduced uptake compared to their iron-containing counterparts (Figure S2). Considering fluorescence is quenched by the presence of iron oxide within the nanoparticles (Figure S1), this suggests that actual differences in uptake between nanoparticles with and without iron oxide are larger than observed by the mean fluorescent intensity per cell.

Microscopy of labeled cells

Following internalization, nanoparticles were easily detected by fluorescence microscopy and light microscopy (Figure 3). In the latter case cells were stained for iron by Prussian Blue. Compartmentalized intense areas of fluorescence and iron were observed throughout the cytoplasm for all nanoparticles. Transmission electron microscopy (TEM) images of labeled cells revealed nanoparticle accumulation in endolysosomal compartments (Figure 4), which corresponded with the localized areas of fluorescence and iron observed with fluorescence and light microscopy (Figure 3). In case of IO-Soy-NP, internalized iron oxide was predominantly restricted to the endosomal membrane (Figure 4C,D), while cells labeled with IO-MCT-NP showed a characteristic disperse, and circular shaped, distribution of iron oxide throughout the entire endosome, similar to that observed in TEM images of the IO-MCT-NP alone (Figure 4E,F). Labeling of cells with IO-NP resulted in completely filled endosomes with high amounts of densely packed iron oxide (Figure 4G,H).

MRI of labeled cells

Labeling of cells with iron oxide encapsulating nanoparticles (Figure 3F,I,L) induced a visibly detectable reduction in signal intensity on T_2^* -weighted MR images compared to images of unlabeled control cells (Figure 3C). This signal decrease was most pronounced for IO-NP, followed by IO-MCT-NP and IO-Soy-NP, and corresponded with reductions in T_1 and T_2 values in the same cell samples (Figure S3). Decreasing the density of labeled cells from 1000 cells/ μl to 125 cells/ μl agarose allowed us to study the potential of these lipid-coated clusters of iron oxide for MR-based detection of small cell clusters or even individual cells (Figure 5). Contrast-enhanced pixels were not observed in samples containing unlabeled cells, while cells labeled with IO-NP and IO-MCT-NP showed contrast-enhanced pixels even at cell concentrations as low as 125 cells/ μl , *i.e.* 0.125 cell/voxel (Figure 5A). At such a low cell density, the measured fraction of contrast-enhanced pixels underestimated the actual cell density for each of the nanoparticles (Figure 5B). This data suggests that cells were either clustered, or some cells were insufficiently labeled for single cell detection. Furthermore, we have to take into account that the 'blooming' T_2^* effect of extensively labeled cells also affects neighboring voxels, which further complicates accurate cell density measurements based on the extent of contrast enhancement. Nevertheless, the MRI data convincingly demonstrate that IO-NP and IO-MCT-NP allowed the detection of small numbers of cells.

Nanoparticle-related cytotoxicity

Nanoparticle-induced cytotoxicity was assessed by flow cytometric analysis of fluorescently stained late-apoptotic and/or necrotic cells with 7-AAD, while a second measure of cell

viability, *i.e.* metabolic activity, was assessed with the MTT assay. Directly following three hours of incubation with freshly prepared nanoparticles, only cells treated with IO-MCT-NP showed a small, yet significant, particle-related increase in 7-AAD staining compared to untreated control cells (Figure 6A). Metabolic activity was also reduced and significant differences between the three nanoparticles were observed. IO-NP showed most preserved metabolic activity, followed by IO-MCT-NP and IO-Soy-NP (Figure 6B). Metabolic activity was partially restored at 24 hours after labeling with IO-Soy-NP and IO-MCT-NP, and activity in cells labeled with IO-NP returned to reference levels. Only cells labeled with IO-Soy-NP showed a small but significantly increased percentage of 7-AAD positive cells within the culture after 24 hours.

IO-Soy-NP-induced cytotoxicity was also found to progress with nanoparticle storage time, as the fraction of 7-AAD positive cells after 3 hours of incubation consistently and significantly increased with time after nanoparticle preparation (Figure 7A). These results were corroborated by the metabolic activity measurements, which showed significant loss of metabolic activity as a function of nanoparticle storage time (Figure 7D). In both assays it was demonstrated that this is a partially oxygen-dependent process, as storage under nitrogen significantly delayed the storage time-dependent increase in nanoparticle-induced cytotoxicity (Figure 7A,D). IO-MCT-NP and IO-NP nanoparticles did not demonstrate such a storage time effect, and significant differences with nitrogen storage were not observed (Figure 7B,C,E,F).

The storage time-dependent cytotoxic effects of IO-Soy-NP could not be attributed to nanoparticle degradation during storage, as DLS measurements demonstrated that particle size and PDI remained stable over a time period of at least 18 weeks after production (own observations, data not shown). The fact that oxygen deprivation during nanoparticle storage significantly delayed this time-dependency strongly suggests that cytotoxicity may be partially ascribed to the oxidative formation of fatty acid hydroperoxides and secondary degradation products from polyunsaturated fatty acids in the soybean oil. We hypothesize that this effect is enhanced by dispersion of the iron oxide crystals throughout the oil phase, as iron acts as a catalyst in the oxidation of unsaturated fatty acids³². This was confirmed by a thiobarbituric acid reactive substances (TBARS) assay, which demonstrated significantly increased production of fatty acid hydroperoxide degradation products in IO-Soy-NP compared to Soy-NP, IO-MCT-NP, MCT-NP and IO-NP (Figure 8).

Discussion

Recent developments in nanoparticulate agents for multimodal imaging have opened a window of opportunities for advanced biomedical research³³. In the current study, a relatively simple solvent evaporation procedure was applied to prepare six different fluorescently labeled lipid-coated nanoparticles, with a diameter < 100 nm and characteristic distributions of the iron oxide crystals throughout the nanoparticle core, depending on the oil phase (Figure 1). We characterized structural properties of formulations composed of combinations of biocompatible materials, and measured the effect of such combinations on nanoparticle characteristics, cell labeling potential and cell viability. We found that combinations of individually biocompatible materials do not necessarily result in biocompatible nanoparticles, with the catalytic or oxidizing properties of iron oxides potentially degrading the oil core, depending on its composition.

The increasing interest in nanomaterials for *in vivo* cell imaging studies requires development of particles that allow efficient cell labeling with minimal effects on cell viability and function. In general, nanoparticle internalization is affected by numerous factors, including cell type and cell density^{34,35}, as well as nanoparticle size, charge, shape

and concentration^{36–39}. We found that incubations of RAW cells with the negatively charged lipid-coated nanoparticle preparations resulted in an intracellular iron content ranging from 1.0 ± 0.1 pg iron/cell for IO-Soy-NP to 5.3 ± 0.7 pg iron/cell for IO-NP (Figure 2A). Labeling of human monocytes with negatively charged carboxydextran-coated SPIO (65 nm) was found to be higher³⁶. Based on size, charge and cell type (Table 1), one may also expect higher labeling efficiency of RAW cells with IO-Soy-NP, IO-MCT-NP and IO-NP. However, internalization of these nanoparticles is most probably hampered by the high amount of PEG polymers (50 mol%) in the lipid coating, which sterically stabilizes these nanoparticles and decreases their interaction with macrophages due to reduced absorption of serum proteins on the nanoparticle surface⁴⁰. This is corroborated by other studies in macrophages, demonstrating reduced uptake of PEGylated iron oxide particles compared to carboxy-dextran coated SPIO⁴¹ and dextran-coated nanoparticles⁴². We chose the RAW macrophage cell line as a test bed, as these cells are known to efficiently internalize contrast agents. Cells that extensively internalize nanoparticles were a prerequisite for efficient evaluation of cytotoxicity and detectability. The investigation of nanoparticle internalization by a variety of different cell types would be an interesting future extension of this work.

Our study demonstrates that coating and size, which were comparable between the nanoparticles, are not the only factors affecting nanoparticle uptake, as substantial differences in labeling efficiency were observed between IO-Soy-NP, IO-MCT-NP and IO-NP (Figure 2), as well as their non-iron containing counterparts (Figure S2). These differences could also not be explained by dissimilarities in charge (Table 1 and S1), but may originate from differences in nanoparticle weight, caused by differences in core density due to inclusion/exclusion of iron oxide nanocrystals, shape and/or cytotoxicity.

Cells labeled with the selected nanoparticles generated visibly detectable hypointensities on T_2^* -weighted images with an isotropic resolution of 100 μm , which reflects the potential to detect small clusters of cells (Figure 5). We expect that the actual detection threshold in our experimental setting could be further optimized, as single cell MR imaging studies have already been conducted at lower magnetic field strength (1.5 T) and lower intracellular iron content (0.2 pg iron/cell)⁴³.

The inclusion of fluorescent entities additionally allowed the detection of labeled cells with optical techniques (Figure 2–3), which extends the application of these nanoparticles for biodistribution studies using optical in vivo imaging systems²⁷, phenotyping of intravenously labeled cells with flow cytometry⁴⁴ and validation of MRI data at the subcellular level with (confocal) fluorescence microscopy. Unfortunately, fluorescence measurements did not permit quantitative analysis of labeling efficiency due to fluorescent quenching in the presence of iron, especially at high concentrations (Figure S1). Most probably, this also explains the discrepancy between measurements of nanoparticle load based on iron content and mean fluorescent intensity for IO-NP-labeled cells (Figure 2), in which nanoparticles are densely packed within endolysosomal compartments (Figure 4H). Such effects may be minimized by coupling the fluorescent label to the distal end of the PEG chain, instead of directly to the phospholipid.

Despite the regular assumption that iron oxide particles are non-toxic at concentrations typically applied for cellular MR imaging, recent studies revealed that excessive intracellular labeling with iron oxide particles may seriously affect cell proliferation, migration and differentiation⁴⁵. In the current study we evaluated the cytotoxicity of the different nanoparticle preparations and found that cellular metabolic activity as assessed with an MTT assay was initially lowered, but recovered after 24 hours, while the degree of cell death observed from flow cytometric analysis of 7-AAD stained cells remained

relatively stable or increased somewhat (Figure 6). The measured increase in metabolic activity at 24 hours post cell labeling may be associated with increased reactive oxygen species (ROS) production, which is in agreement with earlier studies demonstrating transiently increased ROS production within 3–24 hours of labeling with magnetoliposomes ⁴⁶, VSOP ⁴⁷ and dextran-coated nanoparticles ⁴⁸.

Cytotoxicity after nanoparticle loading is dependent on a number of factors, including intracellular iron concentration and nanoparticle coating ⁴⁵. However, in our study the differences in cytotoxicity between formulations were likely not caused by these two factors as all nanoparticles had the same coating, and the formulations with highest iron content had lowest toxicity. Potentially, these differences may be ascribed to lipotoxicity, since the uptake of triglycerides, irrespective of their degradation in free fatty acids, has been associated with oxidative stress and consequent cell death in J774 macrophages ^{49, 50}. Especially polyunsaturated fatty acids (PUFA), which are abundantly present in soybean oil (59 %), are known to be highly susceptible to oxidation into hydroperoxides, followed by rapid degradation into more stable and toxic secondary products such as reactive aldehydes ⁵¹. In contrast to PUFA, monounsaturated fatty acids (MUFA), such as the oleic acid coating of the iron oxide crystals, and saturated fatty acids (SFA), as present in MCT and the DSPC/PEG2000 coating, are more resistant to oxidation. Accordingly, replacement of the soybean oil core by alternative vegetable oils consisting of long chain triglycerides with varying fractions of MUFA and polyunsaturated PUFA, *i.e.* corn, cottonseed or olive oil, was expected to result in nanoparticles with reduced toxicity after cell labeling. Highest cell viability at 14 days after nanoparticle preparation was achieved with olive oil preparations (Figure 1C), which may be attributed to the relatively low PUFA content of olive oil (7 %) compared to corn (59 %) and cottonseed (48 %) oil. Nevertheless, each nanoparticle reduced the cell viability to 10–20%, suggesting that MUFA peroxidation in the oil phase may also play a role in oxidative deterioration, perhaps by propagation of a chain reaction initiated in PUFA.

The nanoparticle platform presented in the current study is considered highly flexible and allows a wide range of variations in its coating as well as materials encapsulated within the core. Present formulations contained high amounts of PEGylated phospholipids and may therefore be suitable for future applications requiring a long blood half-life, such as in vivo labeling of circulating monocytes. However, in case of in vitro labeling, the labeling efficiency of phagocytes as well as non-phagocytic cells may be increased by decreasing the PEG content and/or by inclusion of different phospholipids, such as cationic lipids. Alternatively, functionalized PEG lipids may be incorporated and covalently conjugated to peptides, proteins or antibodies that induce nanoparticle internalization. Last, nanocrystals such as gold or quantum dots may also be loaded as cargoes for alternative applications such as CT or fluorescence imaging.

Conclusions

Lipid-coated nanoparticles containing nanoclusters of iron oxide, either in the presence or absence of an oil phase, allowed the detection of single cells with MRI, fluorescence microscopy and flow cytometry. Nanoparticles without incorporated oil exhibited superior properties in terms of MR relaxivities, cell labeling efficiency, biocompatibility and shelf-life, whereas medium-chain triglycerides (MCT) and long-chain triglycerides (soybean, corn, cottonseed and olive oil) were associated with lipotoxicity. The inclusion of vegetable oils caused a further increase in cytotoxicity as a function of storage time, which we ascribe to iron-catalyzed peroxidation of (poly)unsaturated fatty acids in the oil phase. We therefore conclude that lipid-coated iron oxide nanoparticles without an oil phase are most suitable for future cellular imaging studies, and represent an attractive, potent and flexible platform that

can be easily adjusted for alternative multimodal imaging set-ups or therapeutic purposes²⁸. Additionally, this study emphasizes that nanoparticles for in vivo applications, even when composed of biocompatible compounds such as phospholipids, iron oxide and vegetable oils, should be carefully designed and evaluated in terms of cell homeostasis, as the combination of biologically safe building blocks may still lead to cytotoxic products.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Marina Talelli (Department of Pharmaceutics, Institute for Pharmaceutical Sciences, Utrecht, The Netherlands) for the assistance with the iron quantification. This research was supported by the Netherlands Organization for Scientific Research (NWO-Vidi 917.76.351; RMD), the European Union's Seventh Framework Programme (FP7/2007-2013) under grant agreements n° 201024 and n° 202213 (European Stroke Network), and NIH grant K99 EB012165 (DPC).

References

1. Medintz IL, Uyeda HT, Goldman ER, Mattoussi H. Quantum dot bioconjugates for imaging, labelling and sensing. *Nat Mater.* 2005; 4:435–446. [PubMed: 15928695]
2. Choi HS, Liu W, Misra P, Tanaka E, Zimmer JP, Ipe BI, Bawendi MG, Frangioni JV. Renal clearance of quantum dots. *Nat Biotech.* 2007; 25:1165–1170.
3. Medintz IL, Clapp AR, Mattoussi H, Goldman ER, Fisher B, Mauro JM. Self-assembled nanoscale biosensors based on quantum dot FRET donors. *Nature Mater.* 2003; 2:630–638. [PubMed: 12942071]
4. Hainfeld JF, Slatkin DN, Focella TM, Smilowitz HM. Gold nanoparticles: a new X-ray contrast agent. *Brit J Radiol.* 2006; 79:248–253. [PubMed: 16498039]
5. Cormode DP, Roessl E, Thrane A, Skajaa T, Gordon RE, Schlomka JP, Fuster V, Fisher EA, Mulder WJM, Proksa R, AFZ. Atherosclerotic plaque composition: analysis with multicolor CT and targeted gold nanoparticles. *Radiology.* 2010; 256:774–782. [PubMed: 20668118]
6. Wang HF, Huff TB, Zweifel DA, He W, Low PS, Wei A, Cheng JX. In vitro and in vivo two-photon luminescence imaging of single gold nanorods. *Proc Natl Acad Sci USA.* 2005; 102:15752–15756. [PubMed: 16239346]
7. Qian X, Peng XH, Ansari DO, Yin-Goen Q, Chen GZ, Shin DM, Yang L, Young AN, Wang MD, Nie S. In vivo tumor targeting and spectroscopic detection with surface-enhanced Raman nanoparticle tags. *Nat Biotech.* 2008; 26:83–90.
8. Song KH, Kim C, Cobley CM, Xia Y, Wang LV. Near-infrared gold nanocages as a new class of tracers for photoacoustic sentinel lymph node mapping on a rat model. *Nano Lett.* 2009; 9:183–188. [PubMed: 19072058]
9. von Maltzahn G, Park JH, Agrawal A, Bandaru NK, Das SK, Sailor MJ, Bhatia SN. Computationally guided photothermal tumor therapy using long-circulating gold nanorod antennas. *Cancer Res.* 2009; 69:3892–3900. [PubMed: 19366797]
10. Rosi NL, Mirkin CA. Nanostructures in Biodiagnostics. *Chem Rev.* 2005; 105:1547–1562. [PubMed: 15826019]
11. Jordan A, Scholz R, Wust P, Fähling H, Felix R. Magnetic fluid hyperthermia (MFH): Cancer treatment with AC magnetic field induced excitation of biocompatible superparamagnetic nanoparticles. *J Mag Mag Mater.* 1999; 201:413–419.
12. Haun JB, Castro CM, Wang R, Peterson VM, Marinelli BS, Lee H, Weissleder R. Micro-NMR for rapid molecular analysis of human tumor samples. *Science Trans Med.* 2011; 3:71ra16.
13. Bulte JWM, Kraitchman DL. Iron oxide MR contrast agents for molecular and cellular imaging. *NMR Biomed.* 2004; 17:484–499. [PubMed: 15526347]
14. Stoll G, Bendzus M. Imaging of inflammation in the peripheral and central nervous system by magnetic resonance imaging. *Neuroscience.* 2009; 158:1151–60. [PubMed: 18651996]

15. de Vries IJM, Lesterhuis WJ, Barentsz JO, Verdijk P, van Krieken JH, Boerman OC, Oyen WJG, Bonenkamp JJ, Boezeman JB, Adema GJ, Bulte JWM, Scheenen TWJ, Punt CJA, Heerschap A, Figdor CG. Magnetic resonance tracking of dendritic cells in melanoma patients for monitoring of cellular therapy. *Nat Biotech.* 2005; 23:1407–1413.
16. Kwon SG, Piao Y, Park J, Angappane S, Jo Y, Hwang NM, Park JG, Hyeon T. Kinetics of monodisperse iron oxide nanocrystal formation by “heating-up” process. *J Am Chem Soc.* 2007; 129:12571–12584. [PubMed: 17887758]
17. Hyeon T, Lee SS, Park J, Chung Y, Na HB. Synthesis of highly crystalline and monodisperse maghemite nanocrystallites without a size-selection process. *J Am Chem Soc.* 2001; 123:12798–12801. [PubMed: 11749537]
18. Molday RS, Mackenzie D. Immunospecific ferromagnetic iron-dextran reagents for the labeling and magnetic separation of cells. *J Immun Methods.* 1982; 52:353–367.
19. Dubertret B, Skourides P, Norris DJ, Noireaux V, Brivanlou AH, Libchaber A. In vivo imaging of quantum dots encapsulated in phospholipid micelles. *Science.* 2002; 298:1759–1762. [PubMed: 12459582]
20. Qi LF, Gao XH. Quantum dot-amphipol nanocomplex for intracellular delivery and real-time imaging of siRNA. *ACS Nano.* 2008; 2:1403–1410. [PubMed: 19206308]
21. Cormode DP, Skajaa GO, Delshad A, Parker N, Jarzyna PA, Calcagno C, Galper MW, Skajaa T, Briley-Saebo KC, Bell HM, Gordon RE, Fayad ZA, Woo SL, Mulder WJ. A versatile and tunable coating strategy allows control of nanocrystal delivery to cell types in the liver. *Bioconjugate chemistry.* 2011; 22:353–61. [PubMed: 21361312]
22. Yu WW, Chang E, Falkner JC, Zhang J, Al-Somali AM, Sayes CM, Johns J, Drezek R, Colvin VL. Forming biocompatible and nonaggregated nanocrystals in water using amphiphilic polymers. *J Am Chem Soc.* 2007; 129:2871–2879. [PubMed: 17309256]
23. Muir BW, Moffat BA, Harbour P, Greg Coia G, Zhen G, Waddington L, Scoble J, Krah D, Thang SH, Chong YK, Mulvaney P, Hartley P. Combinatorial discovery of novel amphiphilic polymers for the phase transfer of magnetic nanoparticles. *J Phys Chem C.* 2009; 113:16615–16624.
24. Bruns OT, Ittrich H, Peldschus K, Kaul MG, Tromsdorf UI, Lauterwasser J, Nikolic MS, Mollwitz B, Merkel M, Bigall NC, Sapra S, Reimer R, Hohenberg H, Weller H, Eychmuller A, Adam G, Beisiegel U, Heeren J. Real-time magnetic resonance imaging and quantification of lipoprotein metabolism in vivo using nanocrystals. *Nat Nano.* 2009; 4:193–201.
25. Cormode DP, Skajaa T, van Schooneveld MM, Koole R, Jarzyna P, Lobatto ME, Calcagno C, Barazza A, Gordon RE, Zanzonico P, Fisher EA, Fayad ZA, Mulder WJM. Nanocrystal core high-density lipoproteins: A multimodal molecular imaging contrast agent platform. *Nano Lett.* 2008; 8:3715–3723. [PubMed: 18939808]
26. McMahon KM, Mutharasan RK, Tripathy S, Veliceasa D, Bobeica M, Shumaker DK, Luthi AJ, Helfand BT, Ardehali H, Mirkin CA, Volpert O, Thaxton CS. Biomimetic high density lipoprotein nanoparticles for nucleic acid delivery. *Nano Lett.* 2011; 11:1208–14. [PubMed: 21319839]
27. Jarzyna PA, Skajaa T, Gianella A, Cormode DP, Samber DD, Dickson SD, Chen W, Griffioen AW, Fayad ZA, Mulder WJ. Iron oxide core oil-in-water emulsions as a multifunctional nanoparticle platform for tumor targeting and imaging. *Biomaterials.* 2009; 30:6947–54. [PubMed: 19783295]
28. Gianella A, Jarzyna PA, Mani V, Ramachandran S, Calcagno C, Tang J, Kann B, Dijk WJR, Thijssen VL, Griffioen AW, Storm G, Fayad ZA, Mulder WJ. A multifunctional nanoemulsion platform for imaging guided therapy evaluated in experimental cancer. *ACS Nano.* 2011 in press.
29. Horak D, Babic M, Jendelova P, Herynek V, Trchova M, Pientka Z, Pollert E, Hajek M, Sykova E. D-mannose-modified iron oxide nanoparticles for stem cell labeling. *Bioconjug Chem.* 2007; 18:635–44. [PubMed: 17370996]
30. Perez JM, Josephson L, Weissleder R. Use of magnetic nanoparticles as nanosensors to probe for molecular interactions. *Chembiochem.* 2004; 5:261–4. [PubMed: 14997516]
31. Jarzyna PA, Skajaa T, Cormode DP, Samber DD, Dickson SD, Chen W, Griffioen AW, Fayad ZA, Mulder WJM. Iron oxide core oil-in-water emulsions as a multifunctional nanoparticle platform for tumor targeting and imaging. *Biomaterials.* 2009; 30:6947–6954. [PubMed: 19783295]

32. Minotti G, Aust SD. The role of iron in oxygen radical mediated lipid peroxidation. *Chem Biol Interact.* 1989; 71:1–19. [PubMed: 2550151]
33. Mulder WJ, Strijkers GJ, van Tilborg GA, Cormode DP, Fayad ZA, Nicolay K. Nanoparticulate assemblies of amphiphiles and diagnostically active materials for multimodality imaging. *Acc Chem Res.* 2009; 42:904–14. [PubMed: 19435319]
34. Boutry S, Brunin S, Mahieu I, Laurent S, Vander Elst L, Muller RN. Magnetic labeling of non-phagocytic adherent cells with iron oxide nanoparticles: a comprehensive study. *Contrast Media Mol Imaging.* 2008; 3:223–32. [PubMed: 19072771]
35. Arbab AS, Bashaw LA, Miller BR, Jordan EK, Bulte JW, Frank JA. Intracytoplasmic tagging of cells with ferumoxides and transfection agent for cellular magnetic resonance imaging after cell transplantation: methods and techniques. *Transplantation.* 2003; 76:1123–30. [PubMed: 14557764]
36. Metz S, Bonaterra G, Rudelius M, Settles M, Rummeny EJ, Daldrup-Link HE. Capacity of human monocytes to phagocytose approved iron oxide MR contrast agents in vitro. *Eur Radiol.* 2004; 14:1851–8. [PubMed: 15249981]
37. Matuszewski L, Persigehl T, Wall A, Schwindt W, Tombach B, Fobker M, Poremba C, Ebert W, Heindel W, Bremer C. Cell tagging with clinically approved iron oxides: feasibility and effect of lipofection, particle size, and surface coating on labeling efficiency. *Radiology.* 2005; 235:155–61. [PubMed: 15749976]
38. Roser M, Fischer D, Kissel T. Surface-modified biodegradable albumin nano- and microspheres. II: effect of surface charges on in vitro phagocytosis and biodistribution in rats. *Eur J Pharm Biopharm.* 1998; 46:255–63. [PubMed: 9885296]
39. Sharma G, Valenta DT, Altman Y, Harvey S, Xie H, Mitragotri S, Smith JW. Polymer particle shape independently influences binding and internalization by macrophages. *J Control Release.* 2010; 147:408–12. [PubMed: 20691741]
40. Verma A, Stellacci F. Effect of surface properties on nanoparticle-cell interactions. *Small.* 6:12–21. [PubMed: 19844908]
41. Tromsdorf UI, Bruns OT, Salmen SC, Beisiegel U, Weller H. A highly effective, nontoxic T1 MR contrast agent based on ultrasmall PEGylated iron oxide nanoparticles. *Nano Lett.* 2009; 9:4434–40. [PubMed: 19799448]
42. Xie J, Xu C, Kohler N, Hou Y, Sun S. Controlled PEGylation of monodisperse Fe₃O₄ nanoparticles for reduced non-specific uptake by macrophage cells. *Adv Mater.* 2007; 19:3163.
43. Smirnov P, Poirier-Quinot M, Wilhelm C, Lavergne E, Ginefri JC, Combadiere B, Clement O, Darrasse L, Gazeau F. In vivo single cell detection of tumor-infiltrating lymphocytes with a clinical 1.5 Tesla MRI system. *Magn Reson Med.* 2008; 60:1292–7. [PubMed: 19030162]
44. Fogel U, Ding Z, Hardung H, Jander S, Reichmann G, Jacoby C, Schubert R, Schrader J. In vivo monitoring of inflammation after cardiac and cerebral ischemia by fluorine magnetic resonance imaging. *Circulation.* 2008; 118:140–8. [PubMed: 18574049]
45. Soenen SJ, Himmelreich U, Nuytten N, De Cuyper M. Cytotoxic effects of iron oxide nanoparticles and implications for safety in cell labelling. *Biomaterials.* 32:195–205. [PubMed: 20863560]
46. Soenen SJ, Brisson AR, De Cuyper M. Addressing the problem of cationic lipid-mediated toxicity: the magnetoliposome model. *Biomaterials.* 2009; 30:3691–701. [PubMed: 19371948]
47. Stroh A, Zimmer C, Gutzeit C, Jakstadt M, Marschinke F, Jung T, Pilgrim H, Grune T. Iron oxide particles for molecular magnetic resonance imaging cause transient oxidative stress in rat macrophages. *Free Radic Biol Med.* 2004; 36:976–84. [PubMed: 15059638]
48. Bulte JW, Arbab AS, Douglas T, Frank JA. Preparation of magnetically labeled cells for cell tracking by magnetic resonance imaging. *Methods Enzymol.* 2004; 386:275–99. [PubMed: 15120257]
49. Aronis A, Madar Z, Tirosh O. Mechanism underlying oxidative stress-mediated lipotoxicity: exposure of J774.2 macrophages to triacylglycerols facilitates mitochondrial reactive oxygen species production and cellular necrosis. *Free Radic Biol Med.* 2005; 38:1221–30. [PubMed: 15808420]

50. Aronis A, Madar Z, Tirosh O. Lipotoxic effects of triacylglycerols in J774.2 macrophages. *Nutrition*. 2008; 24:167–76. [PubMed: 18165129]
51. Gueraud F, Atalay M, Bresgen N, Cipak A, Eckl PM, Huc L, Jouanin I, Siems W, Uchida K. Chemistry and biochemistry of lipid peroxidation products. *Free Radic Res*. 44:1098–124. [PubMed: 20836659]

Figure 1. Schematic overview of the different nanoparticles either with or without incorporated oil (A). Negative stain TEM of the different nanoparticles (B) and metabolic activity of RAW cells after 3 hours of incubation at 200 $\mu\text{g Fe/ml}$ with the six different nanoparticle formulations (C). Cell incubations were performed at 14 days post nanoparticle preparation. Bars represent mean $\pm$ standard deviation ($n=6$ for IO-Soy-NP, IO-MCT-NP and IO-NP; $n=4$ for IO-Corn-NP, IO-Cot-NP and IO-Olive-NP). * $p < 0.05$ compared to IO-Soy-NP.

Figure 2.

Cellular iron content (A) and flow cytometry-based mean fluorescent intensity (MFI) in fluorescent channel 2 (FL-2: 575/25 nm) (B) after 3 hours of incubation with 200 μ g Fe/ml RPMI 1640. Bars represent mean $\pm$ standard deviation (n=3). * $p < 0.05$ compared to IO-Soy-NP incubation. # $p < 0.05$ compared to untreated control cells.

Figure 3.

Fluorescence microscopy, Prussian Blue staining and MRI of unlabeled control cells (A–C) and cells labeled with IO-Soy-NP (D–F), IO-MCT-NP (G–I) or IO-NP (J–L) nanoparticles. Fluorescence: fluorescent nanoparticles, containing Rhodamine lipids, are shown in red, while nuclei, stained with Hoechst, are shown in blue. Prussian Blue: Iron, stained by Prussian Blue, is shown in blue, while nuclei, stained by Nuclear Fast Red, are shown in red. MRI: T_2^* -weighted gradient echo images of homogeneously dispersed labeled cells in 0.4% agarose at a concentration of 1000 cells/ μ l (TR/TE = 100/15 ms, 100 μ m isotropic resolution, 9.4 T).

Figure 4. TEM images of unlabeled cells (A,B) and cells labeled with IO-Soy-NP (C,D), IO-MCT-NP (E,F) or IO-NP (G,H) nanoparticles. Single cells are shown in the left column (A,C,E,G) and magnifications of the corresponding images in the right column (B,D,F,H).

Figure 5.

(A) T_2^* -weighted MR images (100 μm isotropic resolution) of homogeneously distributed cells in 0.4% agarose, at concentrations varying from 125–1000 cells per μl (*i.e.* 0.125–1 cells/voxel), overlaid by color-coded significantly contrast-enhanced (CE) pixels. (B) Percentage of CE pixels measured within each cell sample.

Figure 6.

Effect of cell labeling on cell death, as determined from 7-AAD staining (A), and metabolic activity, as determined with an MTT assay (B). 3/0h = 3 hours incubation, harvesting directly after incubation, and 3/24h = 3 hours incubation, harvesting 24 hours after incubation. Bars represent mean $\pm$ standard deviation (n=3). Degree of 7-AAD staining was defined as the percentage of 7-AAD positive cells within a population of 20,000 cells, whereas normal metabolic activity was set at 100%, based on the levels in untreated control cells. * $p < 0.05$ compared to IO-Soy-NP incubation at the corresponding time point. # $p < 0.05$ compared to untreated control cells at the corresponding time point.

Figure 7.

Cell viability as a function of time after nanoparticle preparation, as assessed by 7-AAD staining (A–C) and an MTT assay (D–F). Nanoparticles were prepared on day 0, followed by storage under normal conditions (Air) or nitrogen (N₂). Cells were incubated with nanoparticles or remained untreated (control) for 3 hours at 200 µg Fe/ml. Bars represent mean ± standard deviation (n=4 for 7-AAD, n=6 for Metabolic activity). * p < 0.05 compared to its corresponding storage condition on Day 1 after preparation. # p < 0.05 compared to its corresponding nanoparticle treatment under Air storage at the same time point after nanoparticle preparation.

Figure 8.

Thiobarbituric acid reactive substances (TBARS) in nanoparticle suspension at day 1 after nanoparticle preparation under air storage. Bars represent mean $\pm$ standard deviation (n=3).

* $p < 0.05$ compared to IO-Soy-NP. # $p < 0.05$ compared to its iron-containing counterpart.

Table 1

Properties of the different nanoparticles in reverse osmosis water. Diameters were obtained with dynamic light scattering, as given by the number-weighted average of three different nanoparticle preparations (n=9). Zeta potentials were determined on a single nanoparticle sample (n=3). Relaxivities, expressed in $\text{mM}^{-1}\text{s}^{-1}$ iron, were measured at 9.4 T on two different nanoparticle preparations (n=2). Values represent mean $\pm$ standard deviation.

Nanoparticle	Diameter (nm)	Zeta potential (mV)	r_1 ($\text{mM}^{-1}\text{s}^{-1}$)	r_2 ($\text{mM}^{-1}\text{s}^{-1}$)
IO-Soy-NP	76.2 $\pm$ 8.6	-51.3 $\pm$ 2.1	0.29 $\pm$ 0.06	268.89 $\pm$ 17.80
IO-MCT-NP	78.9 $\pm$ 5.7	-50.4 $\pm$ 0.4	0.55 $\pm$ 0.08	267.19 $\pm$ 19.25
IO-NP	62.4 $\pm$ 4.1 *	-46.0 $\pm$ 0.3 *	0.62 $\pm$ 0.12	402.48 $\pm$ 8.82 *

*
p < 0.05 compared to IO-Soy-NP.