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Iron oxide - loaded liposomes for MR imaging

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1. ABSTRACT

In this study a liposome cell labeling system was developed for non - target - specific labeling of glioma cells with superparamagnetic iron oxide nanoparticles for magnetic resonance imaging (MRI). A high non - target - specific uptake is ideal for *in vitro* labeling of cells and subsequently for cell tracking and visualization of phagocytic cells *in vivo*. The preparation of iron oxide - loaded liposomes was optimized and the biological properties of the liposomes were investigated. Cytotoxicity and cell viability were examined and showed limited cytotoxic effects. Non - target - specific labeling of glioma cells *in vitro* for subsequent specific labeling of molecules for MR imaging was tested by T2* - weighted MRI at 3T. The glioma cells showed a strong initial uptake of the iron oxide liposomes and the uptake was not saturable within 24 h exposure. The uptake of liposomes was superior to non - coated magnetite nanoparticles. Using PEG - ylated liposomes, the non - specific uptake could be decreased fundamentally (86 % lower) in comparison to conventional liposomes. Furthermore, the ability of liposomes as contrast agents for MR imaging was investigated. Cells labeled with iron oxide nanoparticles by treatment with liposomes showed a negative contrast in MRI and consequently successful cellular labeling. Thus, iron oxide - loaded liposomes are well suited for non - target - specific cell labeling for MR imaging.

2. INTRODUCTION

Complexes, nanoparticles and liposomes filled with paramagnetic substances are potential contrast agents for MRI. Liposomes have been studied intensively for drug delivery and diagnostic purposes (1,2). Liposomes are microscopic vesicles consisting of one or more membrane - like phospholipid bilayers surrounding an aqueous medium. They can be formulated into small structures (between 80 - 110 nm) that encapsulate either hydrophilic drugs in the interior or hydrophobic drugs within the bilayer. Their ability to extravasate at sites with increased vascular permeability (for example in solid tumors) enables selective targeting to these sites (enhanced permeability and retention (EPR) effect) (3). But conventional liposomes are rapidly cleared from the circulation by the reticoluendothelial system (RES) (4). Steric stabilization of liposomes with hydrophilic polymers such as polyethyleneglycol (PEG) results in a reduced uptake by cells leading to prolonged circulation times, a decrease in the elimination rate of liposomes from the blood stream and thereby to an increased localization in the tumor (5,6).

The purpose of this study was to develop a liposome cell labeling system, which would be amenable to labeling glioma cells with iron oxide nanoparticles to show if liposomes could facilitate unspecific labeling of tumor cells with iron oxide nanoparticles. For this, we first investigated the biological properties of liposomes filled

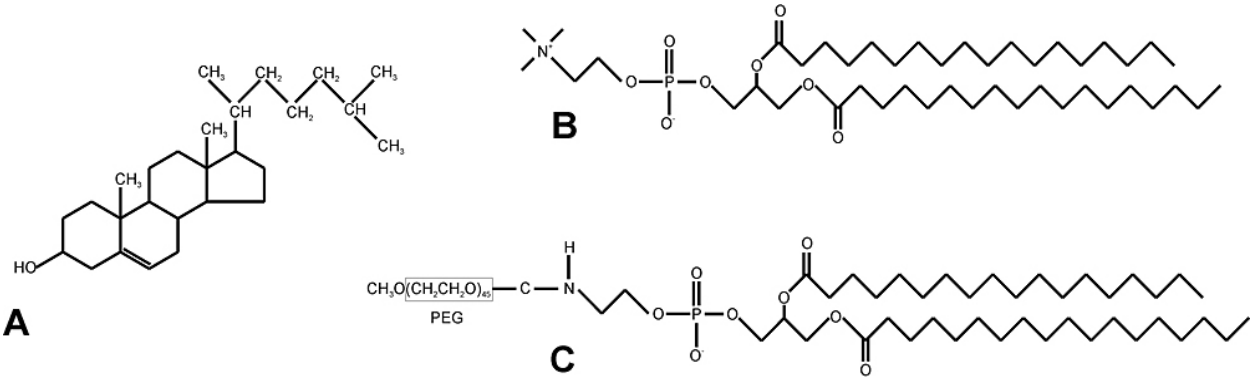


Figure 1. The mean components of the liposomes prepared. A. Cholesterol (Mw = 386 g/mol); B. DSPC (1,2 - Diasteroyl - sn - glycero - 3 - phosphocholine, Mw = 790 g/mol); C. DSPE - PEG(2000)(1,2 - Diasteroyl - sn - glycero - 3 - phosphoethanolamine - N - [methoxy - poly(ethylenglycol)2000], Mw = 2805 g/mol)

with superparamagnetic iron oxide nanoparticles. Cytotoxicity, cell viability and their ability for non - target - specific labeling of cells *in vitro* for subsequent labeling of molecules in MR imaging were examined. Unlabeled cells always served as controls.

3. MATERIALS AND METHODS

3.1. Materials

1,2 - Diasteroyl-sn-glycero-3-phosphocholine (DSPC, M = 790.16 g/mol), 1,2-diasteroyl-sn-glycero-3-phosphoethanol- amin-N-[methoxy-poly(ethyleneglycol) - 2000] (Ammonium salt) (DSPE - PEG (2000), M = 2805.54 g/mol) were donated by Lipoid (Ludwigshafen, Germany). Cholesterol (Chol, M = 386.66 g/mol) was purchased from Sigma-Aldrich (Deisenhofen, Germany) (Figure 1). Fluid colloidal iron oxide nanoparticles (aqueous dispersion of non - coated magnetite Fe_3O_4; c_{Fe} = 27 mg/ml; particle size: 10 nm) were obtained from Dr. Norbert Buske (Berlin, Germany). Resovist (maghemite / magnetite with a carboxyl-dextran cover; c_{Fe} = 28 mg/ml; particle size: 60 nm) was received from Schering (Berlin, Germany).

3.2. Cell culture and media

The human U343 glioma cell line (WHO IV grade), which is characterized by a high phagocytic potential, was obtained from the "Deutsches Krebsforschungs - Institut" (Heidelberg, Germany) and cultivated in glutamine - supplemented Dulbecco´s modified Eagle´s medium (DMEM) plus 10 % (v/v) fetal calf serum (FCS, Gibco, Germany). The cells were only used between the fifth and 15th subculture. The cell line was cultured as a monolayer in a humidified atmosphere of 5 % CO_2 at 37°C in 75 cm^2 - flasks (Nunc, Wiesbaden; Germany) and transferred every 3 days.

3.3. Preparation of liposomes

Conventional liposomes were composed of DSPC : Chol (2.3 : 1 molar ratio) and PEG - ylated liposomes of DSPC : Chol : DSPE - PEG(2000) (13 : 6 : 1 molar ratio). For preparation based on the film hydration method, the lipids were mixed at the indicated molar ratios. A thin lipid film was formed by dissolving the lipid - mixtures in 0.4 ml $CHCl_3$: MeOH (1: 1) and subsequent removal of the solvent by evaporation under a nitrogen stream at 25°C to complete dryness. The lipid film was hydrated with 10 ml phosphate buffered saline (PBS (136 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4 /1l aqua bidest); pH 7.4) including the suspended iron oxide nanoparticles (Fe_3O_4; 0.1 ml; c = 27 mg/ml) to be loaded. The newly formed multilamellar vesicles (MLV) were warmed to a temperature slightly above the phase transition temperature (54.7°C) of the dominant lipid of preparation (DSPC) in a water bath at 60°C under stirring until the lipid film was completely resuspended. The resulting MLV were frozen (liquid nitrogen, -196°C) and thawed (water bath, 60°C) five times.

Non - incorporated iron oxide nanoparticles were separated from the Fe_3O_4 - liposomes by repeated centrifugation at 2000 x g for one minute. The liposome dispersion was sized by repeated discontinuous extrusion (LiposoFast Extruder, Avestin Inc.; Mannheim, Germany) through polycarbonate membranes with decreasing pore sizes of 200 and 100 nm for 21 times each.

The final liposome preparations were stored in 10 ml vials at 4°C and used within one week after preparation. The final concentration was 0.012 mmol lipid/ml. After preparation, iron loading was measured quantitatively by a photometrical iron test (described in 3.8) and iron content was varied from 60 up to 95 micrograms Fe(III)/ml liposome dispersion.

3.4. Liposome size and stability determinations

The mean particle size of the prepared unilamellar liposome dispersions was evaluated by photon correlation spectroscopy (PCS) using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). PCS is a non - invasive well - established technique for measuring particle size and size distribution in liquids over the size range from a few nanometers to a few microns. The particles in liquid are illuminated with a laser and the intensity of the scattered light fluctuates at a rate that is dependant upon the size of the particles. Analysis of these fluctuations yields the velocity of the Brownian motion and hence the particle size using the Stokes-Einstein relationship.

To specify the mean diameter, the liposome dispersion was diluted with PBS (1 : 33) and the average of three measurements was taken at 25°C. The average vesicle size distribution was determined by number - based Gaussian distribution. For determining the liposomal stability, the diameter of three preparations was measured in an interval of two days over a period of two weeks.

3.5. Unspecific uptake and labeling of cells by liposomes

To determine the time - dependant uptake of maghemite - loaded liposomes, 10^6 cells were incubated with liposomes in DMEM - 10 % FCS for different incubation times (15 min, 45 min, 90 min, 3 h, 6 h, 12 h and 24 h; iron concentration 0.04 mg/ml). Resovist (a clinically approved contrast agent in the European Union) and colloidal Fe_3O_4 without or with addition of protamine sulfate (0.01 mg/ml) applied at the same iron concentration (0.04 mg/ml) were used for comparison. Iron content of the liposomes / nanoparticles as well as iron uptake in the cells was determined spectrophotometrically as described in paragraph 3.8. Internalization of the iron oxide nanoparticles was visualized by Prussian Blue Staining.

3.6. Cytotoxicity studies

In order to show whether liposomes show any (cytotoxic -) effects *in vitro*, cell proliferation with and without internalized liposomes was determined with CyQuant Cell Proliferation Assay (Molecular Probes, Eugene, Oregon, USA). This assay is a fast, sensitive and convenient method for counting cells in a population and for measuring their proliferative activity by determining the total amount of cell DNA. Substances with a negative influence on the proliferation rate, like dimethylsulfoxide (DMSO), cause a decrease in cell number and therefore a decrease of total cell DNA. Cells were seeded at 1 x 10^5 cells/Petri dish (Sarstedt, Nuembrecht, Germany; effective growth area: 35 x 10 mm^2) and were allowed a sufficient amount of time (24 h) to adhere to plate. Subsequently, the adherent cells were treated with liposomes incubated with iron oxide for 24 h (iron concentration in the culture medium: 0.04 mg/ml and 0.08 mg/ml). After incubation, the cells were washed with PBS two times to remove unbound liposomes and cellular debris. The adherent cells were frozen at - 20°C for at least 24 h. Then they were thawed and lysed by adding a buffer containing the CyQuant GR dye; afterwards fluorescence was measured directly by a fluorescence spectrophotometer (F-2000, Hitachi; wavelength: Ex = 480 nm / Em = 520 nm) and compared with the fluorescence of untreated cells. For comparison, cells were also incubated with Resovist, colloidal Fe_3O_4 (iron concentration 0.04 mg/ml) and 4 % and 8 % DMSO.

3.7. Internalization studies by MRT

For MRI measurements the cells were seeded on cell culture inserts incorporating polyethylene terephthalate (PET) track etched membranes (BD Falcon, catalog# [35]3180, BD Biosciences, San Jose, Ca, USA). The cells (cell count 10^5) grew as a monolayer on the PET - membranes (effective growth area 4.2 cm^2) which are coated with D - lysine and which show no or little contrast in T2* - weighted MRI (7). Cells were allowed to adhere to the membranes overnight. Then, Fe_3O_4 - labeled liposomes were added for one hour (iron concentration 0.04 mg/ml). The cells were washed with PBS to remove unbound liposomes and fixed with paraformaldehyde (4 % (w/v), dissolved in PBS) for 20 min. Subsequently, cells were washed again with PBS and stored at 4°C. The membranes were checked for the presence of cells by light microscopy. Cells labeled with Resovist (0.04 mg/ml) served as a positive control, unlabeled cells as a negative control. MRI - measurements at 3T (Achieva, Philips, Eindhoven, Netherlands) were performed in a water bath at room temperature. A T2*- weighted pulse sequence with TE / TR = 35 / 1000, a flip angle of 50°, a spatial resolution of 0.15 mm in plane, a slice thickness of 1.5 mm and four repetitions were used.

3.8. Quantitative measurement of iron oxide content

Cells were seeded on Petri dishes (Sarstedt; effective growth area: 35 x 10 mm^2) and were allowed to adhere for two days (cell count 1 x 10^6). The cells were incubated with liposomes or other iron oxide nanoparticles (iron concentration at incubation: 0.04 g/ml) for a specific time (from 15 min up to 24 h), washed with PBS to remove unbound liposomes or non - internalized particles and dry frozen at - 20°C. After 24 h the cells were refrozen and lysed by adding 1 ml concentrated HCl (36 %). After scraping off, the cells were heated at 70°C for 10 minutes. After cooling down to room temperature, 0.1 ml of the cell suspension was diluted with 0.9 ml of citrate phosphate buffer (0.2 M; pH 2.9). 0.1 ml of this dilution was incubated in a 96 well plate after adding 0.1 ml citrate phosphate buffer and 0.015 ml of the Spectroquant test kit solution (Fe-AN, Merck, Darmstadt, Germany; measuring range 0.05 - 5.00 mg/l Fe). Fe^{3+} was reduced to Fe^{2+} by thioglycolacid and complexed by Ferrospectral (chromogenic reagent, Na_2[3-(2-pyridyl)-5,6- bis(4-phenylsulfonic acid)-1,2,4-triazine]). The bathochrome shift was measured photometrically after 30 min by an Elisa reader (λ = 550 nm). Iron standards were used for calibration, and by linear regression the iron contents of the cells could be evaluated.

3.9. Internalization studies by Prussian Blue Staining

1 x 10^5 cells were seeded on sterile cover slips (24 x 50 mm^2) and were allowed to adhere overnight. The cells were incubated with iron oxide - labeled liposomes for one hour (iron concentration 0.04 mg/ml), washed briefly with PBS and fixed by acetone (- 20°C) for 10 min. Subsequently, a filtered aqueous potassium ferrocyanide - hydrochloric acid solution (2 % (w/v), diluted with 1 M HCl (aqueous solution) in a ratio of 1 : 2) was added, and the cells were incubated for 10 min at 37°C. Fe-ions are captured and replace cations of potassium ferrocyanide, forming insoluble ferric ferrocyanide, which precipitates and results in the formation of a bright blue pigment called Prussian blue. After rewashing with PBS, the cells were counterstained with Nuclear Fast Red (0.1 % (w / v); aqueous solution; Chroma, Stuttgart) in 5 % aluminum sulfate in distilled water for 1 min, which stained the nuclei and cytoplasm of cells red. Finally the cells were washed in distilled water and mounted in Aquatex (aqueous mounting

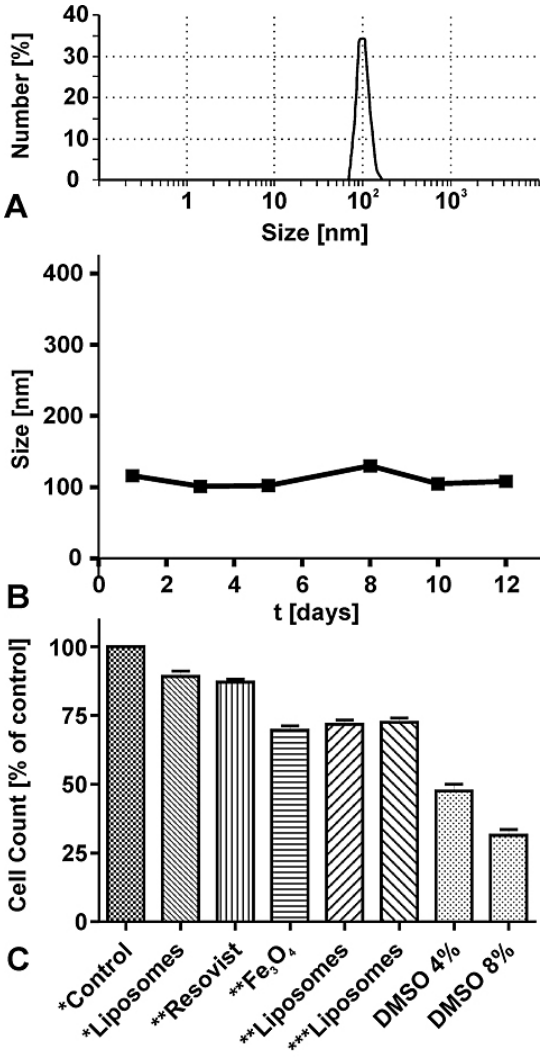


Figure 2. Size, stability and cytotoxicity of liposomes. A. Size distribution of the liposomes. The mean particle size (nm) of prepared iron oxide - loaded liposomes was determined after each preparation by photon correlation spectroscopy (PCS) at 25°C (Zetasizer Nano ZS, Malvern Instruments) ($n \geq 3$). The mean diameter averaged over 3 measurements was 101 nm. Data evaluation was done by number - based Gaussian analysis. B. Size stability. For determining the stability of the liposomes the size was measured by PCS in an interval of two days over a period of 12 days. The mean diameter was calculated at 110 +/- 11 nm (expressed as means +/- sd; n = 3). The maximum of the standard deviation is 15 nm. The mean diameter was from three runs per measurement at 25°C. C Influence of nanoparticles and liposomes on cell proliferation. Proliferation of U 343 glioma cells in standard culture medium (with 10 % FCS) was determined by DNA quantification after 24 h; control = 100 % without additions, * without iron, ** 40 micrograms Fe/ml, *** 80 micrograms Fe/ml.

agent; Merck, Darmstadt) on microscope slides (76 x 26 mm^2, super frost; Roth, Karlsruhe, Germany).

4. RESULTS

4.1. Size and stability of liposomes

The size of liposomes is an important factor for their biological properties and their stability. Therefore, after every preparation of Fe_3O_4 - liposomes their hydrodynamic diameter was repeatedly determined by PCS. In all preparations, photon correlation spectroscopy yielded a single sharp peak at 100 +/- 11 nm for liposomal size (Figure 2A), indicating a homogeneous liposomal dispersion. For determining the liposomal stability, their sizes were measured in an interval of 2 days over a period of two weeks. The mean diameter was specified by three runs per measurement at 25°C and did not deviate much over time (Figure 2B). This indicates that there is no aggregation of the liposomes, and the preparations were physically stable at 4°C for at least two weeks.

4.2. Uptake internalization of conventional Fe_3O_4 - loaded liposomes as compared with other particles

Using corresponding iron concentrations, the cellular uptake of iron oxide - loaded liposomes was compared to the uptake of iron oxide nanoparticles with or without the addition of protamine sulfate (Figure 3A). The U 343 cells showed a strong initial uptake of Fe_3O_4 - liposomes with no saturation within 24 h. After 15 min incubation time, the initial uptake averaged by 13.3 % of the final liposomal Fe uptake after 24 h and the iron content in the cells continued to increase up to 46.5 % the next 45 min. Finally, after 24 hours the iron oxide content constituted 25 +/- 1 micrograms Fe/10^6 cells (=100 %). The initial uptake of Resovist was found to be 0.8 % of the liposomal Fe uptake after an incubation time of 15 min and increased to an average of 2.4 % after an incubation time of 45 min. After incubation with Resovist for 24 hours, a final iron content of 22.2 % of liposomal Fe uptake was measured. After incubation of U 343 cells with coll. Fe_3O_4 for 15 min, the iron content was found to be 14.5 % of liposomal Fe uptake. With the addition of protamine sulfate, this value could be increased to 25 % of liposomal uptake. An increase of iron uptake continued up to 30 % (with addition of protamine sulfate up to 50 % of liposomal Fe uptake) the next 45 min. The final iron oxide content constituted 68 % (with addition of protamine sulfate 66 %) after 24 hours. In comparison to liposomes neither colloidal Fe_3O_4 (with and without the addition of protamine sulfate) nor Resovist reached comparable values after 24 h incubation time.

4.3. Effect of mPEG - DSPE on the uptake of iron oxide - loaded liposomes by U 343 glioma cells

PEG - coated liposomes are referred to as sterically stabilized liposomes. Since they are less well recognized by macrophages, they are also known as stealth - liposomes. The PEG stabilizing effect results from local surface concentration of highly hydrated groups that sterically inhibit both hydrophobic and electrostatic interactions at the liposome surface. In this study we selected DSPE - PEG containing PEG with an MW of 2000.

The high uptake of conventional liposomes by U 343 after 45 min incubation (11.5 +/- 0.5 micrograms Fe/10^6

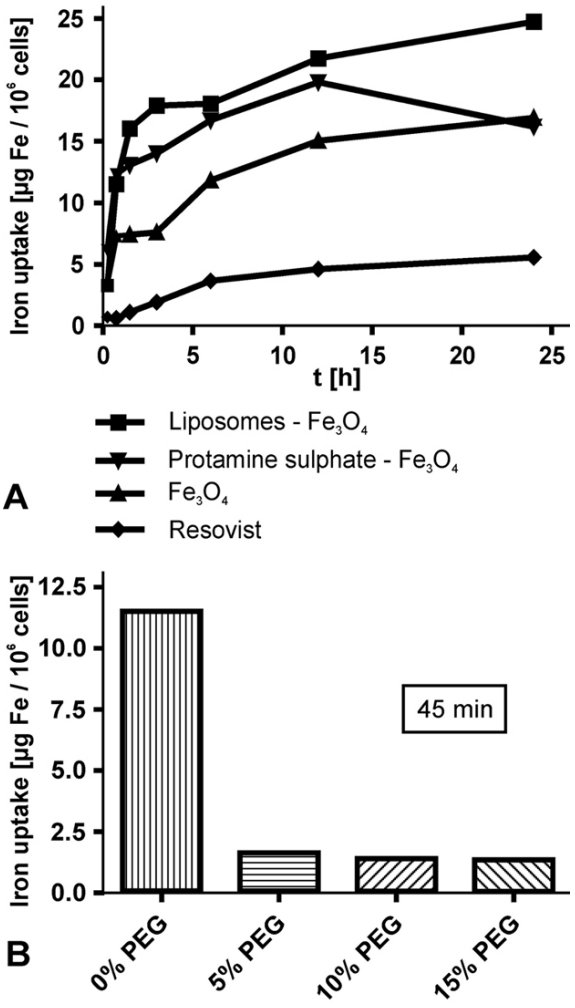


Figure 3. Cellular uptake of iron oxide nanoparticles and iron oxide - filled liposomes. A. Time - dependent uptake of iron oxide - loaded liposomes, colloidal Fe_3O_4 (with and without addition of protamine sulfate) and Resovist (positive control) over a period of 24 h (iron concentration: 40 micrograms/ml). The content of iron oxide in 10^6 U 343 cells was measured colorimetrically (expressed as means +/- sd, n = 3). B. Uptake of iron oxide - filled liposomes. 10^6 U 343 cells were incubated with Fe_3O_4 - loaded liposomes with increasing PEG - content over a period of 45 min. Iron content (micrograms Fe/ml) in the cells was measured colorimetrically (n = 3). The values of the standard deviations are given in table 1 (Iron uptake dependent on PEG - content).

cells) was significantly ($p < 0.001$, unpaired t - test) reduced by the addition of 5 % DSPE - PEG (2000) (1.64 micrograms Fe/10^6 cells) (Figure 3B). This means a decrease of iron content about 86 % with PEG. Raising the PEG - concentration from 5 % up to 15 % showed no further decrease of non - specific uptake (Table 1).

4.4. Cytotoxicity study

Any effective cell labeling vehicle must be able to transfer the label without interfering cytotoxic effects while maintaining an adequate level of cell viability. To investigate if the used lipids for liposomal preparation affect the cell viability or proliferation of cells, the glioma cells were incubated with liposomes without loading up with nanoparticles. By the use of proliferation assays, it could be proved that the lipids showed no cytotoxic effects on cells (Figure 2C). All iron oxide liposomes as well as colloidal Fe_3O_4 - particles and Resovist showed limited cytotoxicity over a period of 24 h (iron concentration 0.04 mg/ml). Even after an increase of iron concentration up to 0.08 mg/ml the liposomes showed the same low toxic effects. In contrast, DMSO (4 % and 8 %) strongly affected the cell viability. After 24 h of incubation, the percentage of surviving cells incubated with DMSO (4 %) was lower (decreased by 53 %) in comparison to the untreated cells. Increasing DMSO to 8 % reduced the percentage of surviving cells by 69 %.

Therefore, the used liposomes did not influence cell viability or cell proliferation and consequently the iron oxide liposomes could be used for effective cell labeling.

4.5. Internalization studies by Prussian Blue Staining

In order to study the cellular distribution of internalized iron, U 343 glioma cells were incubated with Fe_3O_4 - liposomes for one hour and iron was visualized by Prussian blue staining. As seen in Figure 4A-D (different images of one preparation), the blue pigment was found inside the cells and not on the cell surface. This confirms an internalization of the iron - loaded liposomes and excludes an adsorption.

4.6. Internalization studies by MRT

Cells labeled with the iron oxide liposomes were analyzed by MRI to determine if they induce a visible contrast. MR images of U 343 treated with liposomes show a negative contrast and consequently successful cellular labeling. There is a clear decrease in signal intensity (darkening of the image) originating from the cells treated with liposomes compared to the control with untreated U 343 cells, which appears bright (Figure 4E).

5. DISCUSSION

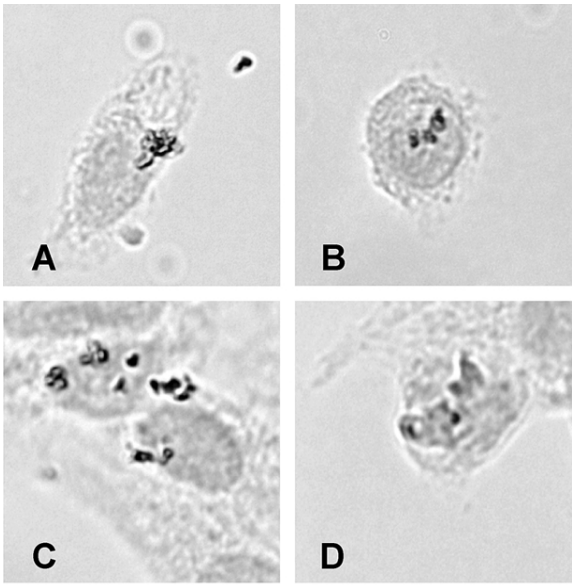
This study describes the development of iron oxide liposomes as a contrast agent for MRI designed to enter and label cells. The optimized liposome formulation and labeling conditions were ascertained by examining the effect of variations in lipid formulations, liposome - cell incubation time and liposome dose on uptake of the iron oxide probe into the cells.

The lipid hydration as a method for preparing liposomes appears to be a suitable method because of its good compromise between iron load, which should be as high as possible to improve the contrast in T2* - weighted MRI by decreasing the local signal amplitude, and vesicle diameters, which should be small enough to allow systemic administration. Tumor vessels are permeable, presumably as the result of large pores in the vessel endothelium (7). Gabizon declares that the largest endothelial fenestrations are approx. 100 - 140 nm in size (8). Thus, the optimum

Table 1. Iron uptake dependent on PEG - content

PEG - content [%]	Iron - oxide content [micrograms Fe/10^6 cells]
0	11.5 +/- 0.5
5	1.6 +/- 0.3
10	1.4 +/- 0.2
15	1.3 +/- 0.3

Iron oxide content of 10^6 U 343 glioma cells after incubation for 45 min with Fe_3O_4 - loaded liposomes with different PEG fraction (n = 3, means +/- S.D.)



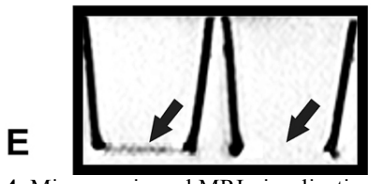


Figure 4. Microscopic and MRI visualization of iron oxide - filled liposomes. A-D. Microscopic visualization of internalized iron oxide - filled liposomes by U 343 cells after incubation with Fe_3O_4 - loaded liposomes for 45 min by Prussian Blue Staining and counterstaining with Nuclear Fast Red (iron concentration 0.04 mg/ml). A-D = different images of one preparation. E. Visualization of cells (10^5) which were labeled with iron oxide - loaded liposomes for 1 h (iron concentration 0.04 mg/ml) (left membrane) by MRI at 3T as compared to untreated cells (10^5) (right membrane).

diameter of liposomal delivery to tumor tissues has been shown to be around 100 nm (9-11). After each preparation of the liposomes their size was determined by PCS and adjusted to 100 nm. Over a period of 12 days the liposomes remained stable in size. So there is no aggregation of the liposomes, and the preparations were physically stable for at least two weeks.

Using DNA measurements (DNA amounts), the cytotoxicity and cell viability induced by liposomes was also assessed and found to be minimal. The Fe_3O_4 - liposomes did not result in any difference in cell proliferation and survival compared to a control.

In comparison to the uptake and internalization of other iron oxide nanoparticles into the cells, the highest uptake was obtained by using liposomes. Neither coll. Fe_3O_4 (with and without addition of protamine sulfate) nor Resovist could reach similar values. It is evident that increasing the liposome - cell incubation time results in a substantial increase in the total liposome content (and subsequently Fe_3O_4 label) with no saturation within 24 h. Our results are in line with the results of O. Morag *et al.* (12). Therefore, using liposomes is an effective way for labeling cells with iron oxide nanoparticles and is superior to Resovist or other iron oxide nanoparticles.

The use of PEG - ylated liposomes (also known as stealth liposomes) provides drug carriers with steric stabilization that show a reduced uptake by the RES and prolonged circulation in the body (10). Pharmacokinetics analysis and therapeutic studies have shown that PEG - liposomes have considerable potential as drug carriers for tumor therapy (13). These liposomes can exploit the "enhanced permeability and retention (EPR)" effect for preferential extravasation from tumor vessels which results in increased liposome accumulation in tumor tissues. By determining the uptake of liposomes *in vitro* by U 343, the uptake of sterically stabilized PEG - liposomes was reduced significantly (86 % lower) compared to conventional liposomes as expected. Raising PEG -contents from 5 % to 15 % showed no further significant decrease of the non - specific uptake.

Liposomes are effective contrast agents for MRI (14,15). Cells treated with Fe_3O_4 - liposomes showed a clear decrease in intensity in T2* - weighted MR images (darkening of the image) compared to a control which appeared bright.

In conclusion, this study describes an optimized and simple procedure to prepare unilamellar liposomes containing superparamagnetic nanoparticles. The liposomes are highly stable, non-toxic and suitable as imaging contrast agents for MR imaging *in vitro*. However, achieving a more effective targeting of tumor cells requires the development of novel liposomes exhibiting both a longer circulation, for example by PEGylation, and active targeting by a ligand.

6. ACKNOWLEDGEMENTS

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