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Lysozyme Transport to the Brain by Liposomes

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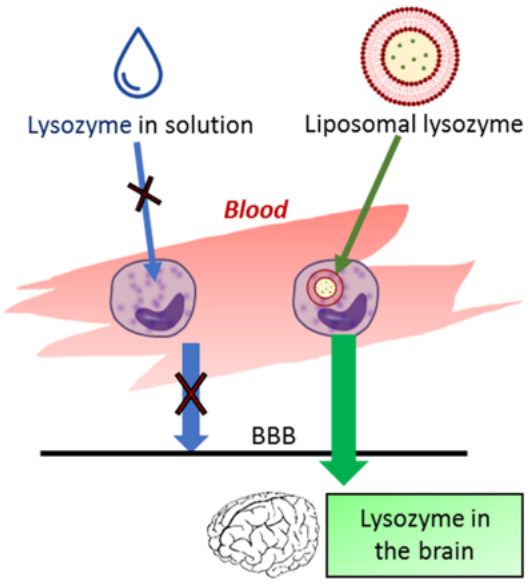
Graphical Abstract



Abstract

Delivery of drugs into the brain is limited due to poor penetrability of many drugs via the blood-brain barrier. Previous studies have shown that the brain is kept under close surveillance by the immune system, implying that circulating phagocytic cells, such as neutrophils and monocytes, are crossing the blood-brain barrier. We hypothesized that charged liposomes could be transported to the brain following their phagocytosis by circulating monocytes. In this work, we investigated the capacity of circulating monocytes to be exploited as a drug delivery system following IV administration of nano-sized, positively fluorescently labeled liposomes containing the protein lysozyme. Negatively charged fluorescently labeled liposomes were used for comparison. By using a modified thin-film hydration technique, the desired properties of the liposomal formulations were achieved including: size, polydispersity index, high drug concentration and stability. In vitro results showed a significant time-dependent uptake of positively charged liposomes by RAW264.7 cells. In vivo results revealed that circulating white blood cells (mainly monocytes) contained high levels of fluorescently labeled liposomes. Screening of brain sections using confocal microscopy uncovered that a substantial amount of fluorescently labeled liposomes, in contrast to the fluorescent markers in solution, was transported into the brain. In addition, anti-CD68 immunofluorescent staining of brain sections, demonstrated co-localization of positively charged liposomes and macrophages in different brain sections. Furthermore, significantly higher levels of lysozyme were detected in brain lysates from rats treated with positively charged liposomes compared to rats treated with lysozyme solution. Taken together this confirms our hypothesis that the designed liposomes were transported to the brain following their phagocytosis by circulating monocytes.

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Keywords

Blood-brain Barrier, Monocytes, Liposomes; Lysozyme, Brain delivery, Drug delivery system

Abbreviations

- WBC white blood cells
- MPS mononuclear phagocytic system
- HP 8-Hydroxypyrene-1,3,6-trisulfonic acid trisodium salt
- DSPC 1,2-distearoyl-sn-glycero-3-phospocholine
- DOTAP 1,2-dioleoyl-3-trimethylammonium-propane
- DPPE-Rhod 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine Rhodamine B sulfonyl); DPPE-Rhodamine
- DSPG 1,2-distearoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol)
- FBS fetal bovine serum
- Lip$^{(+)}$LYS Liposomal lysozyme (positively charged)

Rationale and Purpose

The blood-brain barrier (BBB) is a formidable permeability barrier, which excludes most drugs from entering the brain. Hence, developing drug delivery systems for brain disorders, through intact BBB, is of importance. The mononuclear phagocytic system (MPS), phagocytize particulate matter in the circulation. In addition, the brain is under immuno-surveillance. Consequently, we hypothesized that monocytes could be utilized to transport particulate delivery systems to the brain. We describe here the extent and mechanism of liposomal lysozyme (a 14 kDa protein) transport to the brain in rats.

Introduction

One of the major limiting factors in the development of new drugs for brain disease is the presence of an intact BBB. Only certain small and positively charged lipophilic molecules can passively diffuse across an undisrupted BBB, while certain nutrients and specific molecules can only enter the brain via transporters [1]. Understandably, developing an efficient and non-invasive drug delivery system for brain therapy, without affecting the BBB, is of high merit [2].

The MPS is a part of the immune system that consists of phagocytic cells, primarily monocytes and macrophages. Both circulating monocytes, and neutrophils [3] phagocyte foreign particles in the blood, including drugs delivered as nanoparticles (NP). Since the brain is under immunological surveillance, allowing monocytes and neutrophils to cross the BBB [4-7], these cells can be exploited to deliver particulate drugs across the BBB and into the brain. The propensity of monocytes for rapid recognition of particulate matter, the major clearance mechanism of IV administered particulate delivery systems [8, 9], has provided a rational approach to formulate specific "non-stealth" liposomes for increased uptake by circulating monocytes.

We have shown that circulating monocytes could be exploited as transporters of non-PEGylated, negatively charged liposomes to the brain [10]. Brain uptake after IV administration of negatively charged serotonin liposomes in healthy rats was 2 times higher than the uptake after free drug administration. However, markedly higher brain uptake seems to be required to serve as a viable solution for brain drug delivery. It is also known that positively charged particles are more avidly internalized by the MPS in comparison to negatively charged particles [11, 12]. Accordingly, we hypothesized that positively charged liposomes could be exploited to effectively deliver high-molecular weight drugs to the brain following their phagocytosis by circulating monocytes.

The objective of this work was to develop and characterize positively charged and fluorescently labeled liposomal formulations containing the lysozyme protein (MW = 14 kDa). In addition, we examined the biodistribution of positively charged liposomes in comparison to negatively charged liposomes and to free drugs in solution, and further elucidated the mechanism of transport into the brain of intact rats.

Experimental design

A nanoparticulated delivery system of positively charged liposomes encapsulating lysozyme was developed. The liposomes contained fluorescent markers, the hydrophilic 8-Hydroxypyrene-1,3,6-trisulfonic acid tri-sodium salt (HP) in the aqueous core or the hydrophobic DPPE-Rhodamine (DPPE-Rhod) in the liposome membrane for monitoring uptake and biodistribution. Formulations studied were, (i) lysozyme encapsulated in positively charged liposomes, labelled with DPPE-Rhod; (ii) negatively charged liposomes containing HP (since lysozyme was not encapsulated efficiently in this liposomal formulation) for comparison purposes; and serving as controls, (iii) empty liposomes and iv) drugs in solution (lysozyme, DPPE-Rhod, and HP). First, we characterized the liposomal delivery systems, and evaluated the uptake and cytotoxicity in cell cultures. Next, we examined the biodistribution and brain transport in intact rats (animal care and procedures conformed to the standards for care and use of laboratory animals of the Hebrew University of Jerusalem, Israel and the National Institutes of Health, USA). Following treatment (IV or IP) with various formulations, phagocytosis by the MPS, biodistribution, toxicity, and brain uptake were determined. The co-localization of the liposomes with monocytes in the brain was assessed for validating the proposed mechanism of brain transport.

Materials and Methods

Liposomes preparation

Positively charged liposomes were prepared using a modified film thin hydration technique. Liposomes were composed of 1, 2-distearoyl-sn-glycero-3-phospocholine (DSPC, Lipoid, Ludwigshafen, Germany), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP, Lipoid) and cholesterol (Sigma-Aldrich), at a molar ratio of 3:1:2, respectively. The lipophilic fluorescent marker, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine Rhodamine B sulfonyl), (DPPE-Rhod, Avanti Polar Lipids) was added to the film at a molar ratio of 0.05. The lipids were dissolved in tert-butanol (Arcos Organics) and lyophilized overnight. The obtained film was hydrated with 10 mg/mL of chicken egg-white lysozyme (Sigma-Aldrich) in phosphate-buffered saline, and rotated in a 60°C bath for 40 minutes at 90 rpm ($Lip^{(+)}LYS$). The obtained liposomes were then homogenized using a thermo barrel extruder (Lipex Biomembranes). Non-encapsulated drug was removed by means of dialysis (300 kDa MW, Spectrum Laboratories, Inc.) in PBS overnight. Empty liposomes (empty-$Lip^{(+)}$) were prepared by the same procedure without lysozyme in the hydration solution.

Despite numerous efforts, only minor encapsulation of lysozyme in negatively charged liposomes was obtained (most probably due the electrostatic interaction). Therefore, HP was encapsulated in negatively charged liposomes to serve as a comparison for the positively charged liposomes. Negatively charged liposomes were prepared by replacing DOTAP with the negatively charged lipid, 1,2-distearoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (DSPG, Lipoid) at a molar ratio of 3:1:2. The lyophilized film was then hydrated with 100 mM of the hydrophilic fluorescent marker 8-Hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HP, Sigma-Aldrich) in PBS ($Lip^{(-)}HP$). Un-encapsulated HP was removed by passing the liposomal suspension through a Sephadex G-50 column and eluted using PBS.

Characterization of liposomal formulations

Size and zeta potential

Size, polydispersity index (PDI) and surface charge of drug-loaded and empty liposomes were determined at room temperature, following a 1:100 dilution with PBS, by means of a Zetasizer (Malvern Instruments, Malvern).

Cryogenic transmission electron microscopy (Cryo-TEM)

A drop (3 µl) of the liposomal suspension was applied to a glow discharged TEM grid (300-mesh Cu grid) coated with a holey carbon film (Lacey substrate, Ted Pella, Inc.). The excess liquid was blotted, and the specimen was vitrified by rapid plunging it into liquid ethane, pre-cooled with liquid nitrogen using Vitrobot Mark IV (FEI). The vitrified samples were examined at −177°C using a FEI Tecnai G2 12 TWIN TEM equipped with a Gatan 626 cold stage, and the images were recorded (4K × 4K FEI Eagle CCD camera) at 120 kV in a low-dose mode.

Determination of lipids concentration

Total phospholipid concentration was determined by means of HPLC (Alliance e2695, Waters) equipped with an ELS detector (Alltech 3300, Grace). Liposomes were dissolved with an IPA:chloroform solution (1:1), and were injected to a YMC-Pack PVA-Sil-NP column (250 × 4.6 mm, 5 µm, YMC America, Inc.), heated to 35°C. The mobile phases were composed of IPA:chloroform (1:1) (phase A), and IPA:chloroform:water (8:5:1) (phase B), both containing 0.02% v/v TEA and 0.005% v/v TFA, using a gradient starting at 80/20 to 100% with a flow rate of 1 mL/min.

Adsorption to serum proteins

Liposomes were diluted in fetal bovine serum (FBS, Biological Industries, Israel) at a ratio of 1:40 and incubated at 37°C for 24 and 48 hours. The dilution factor was chosen based on the maximal expected intravenous (IV) injection volume (0.5 mL) in rats, 0.3 kg body weight (BW) and blood volume of 20 mL. The affinity of serum proteins to positively and negatively charged liposomes was evaluated as a function of the liposomes size distribution pre- and post-incubation period, measured by means of a Zetasizer.

Drug loading and encapsulation yield

The concentration of encapsulated lysozyme was determined using a gradient reverse phase HPLC method. Drug containing liposomes were dissolved in 200 mM Octyl β-D-glucopyranoside (OGP, Sigma-Aldrich) and injected to an Xbridge BEH300 C18 column (100 × 4.6 mm, 3.5 µm, Waters). The mobile phase consisted of A, 0.1% TFA in water and B, 0.1% TFA in acetonitrile. Chromatographic separation was performed using a gradient of 95/5 to 5/95, at a flow rate of 1 mL/min, detected at 280 nm (PDA 2998 detector, Waters). Concentration of non-encapsulated lysozyme was determined using the filtrate from liposomes centrifuged with a 100 kDa centrifugal filter unit (Vivaspin, GE Healthcare). The concentration inside the vesicles was determined by subtracting the external concentration from total lysozyme concentration in the suspension.

Lysozyme bioactivity

The bioactivity of lysozyme was assessed using lyophilized *Micrococcus lysodeikticus* cells (Sigma-Aldrich). A *Micrococcus lysodeikticus* cell suspension was prepared using 66 mM potassium phosphate buffer (Merck), pH 6.2 where after 2.5 mL was pipetted into polystyrene cuvettes (Sarstedt). A 100 µl of a liposome suspension treated with or without OGP (*test*), and potassium phosphate buffer (*blank*) were added into the cuvette, mixed by inversion, and the decrease in absorption was recorded by means of a spectrophotometer (UV/VIS-Ultraspec 2100 pro) at λ=450 for 7 minutes. The slope of ΔA_{450} per minute was evaluated and lysozyme concentration was calculated using the following equation:

$$\text{Units/ml lysozyme} = (\Delta A450/\min\ Test - \Delta A450/\min Blank)\frac{df}{(0.001)(0.1)}$$

Where *df* is the dilution factor, *0.001* is the ΔA_{450} per unit of lysozyme, and *0.1* is the volume (mL) of liposomes or buffer added to the cell suspension.

In vitro evaluation

Quantitative uptake evaluation in vitro (FACS)

RAW264.7 cells (murine monocyte/ macrophage) were seeded in 12-well plates, 1 x 10^5 cells per well, containing Dulbecco's modified eagle medium (DMEM, BI) enriched with 10% FBS, 2 mM L-glutamine and 100 Units/mL penicillin and 100 mg/mL streptomycin. The next day, cells were treated with 0.05, 0.25, 0.5, and 1.2 mg/mL Lip$^{(+)}$LYS diluted in PBS, for 1, 4, and 24 hours. After treatment the cells were washed, trypsinized, and collected by centrifugation. Cells were thereafter re-suspended with PBS and analyzed by means of iCyt eclipse flow cytometer (Sony Biotechnology, Inc.). A total of 50,000 cells were counted for each measurement in a rate of 20 µl/min. Untreated cells served as a control group, and fluorescence of the gated cells was measured using the FCS Express software (De Novo Software).

Qualitative uptake evaluation (confocal microscopy)

RAW264.7 cells were seeded on coverslips and treated as described above. After treatment the cells were washed and fixed for 10 minutes using a 4% paraformaldehyde solution (J.T. Baker chemicals). Cell's nucleus was thereafter stained with 10 µg/mL Hoechst solution (Sigma-Aldrich) for 10 minutes. Each coverslip was mounted on a microscope slide with mounting medium (Sigma-Aldrich), and the slides were examined by means of an Olympus FV10i confocal laser scanning microscope 1 x 60 (Olympus America, Inc.).

Cell viability assay (MTT assay)

The RAW264.7 cells were seeded in 24-well plates (40,000 cells/well) containing complete growth medium. The cells were treated on the following day with 0.5 and 1.2 mg/mL $Lip^{(+)}LYS$, or lysozyme solution (20 and 40 µg/mL). Each experiment was performed in duplicate and 10% v/v dimethyl sulfoxide (DMSO, Sigma-Aldrich) served as a positive control. After 24 hours or 48 hours incubation, 100 µl of 5 mg/mL thiazolyl blue tetrazolium bromide (MTT, Sigma-Aldrich) was added to each well, containing 1 mL of growth medium, and incubated for 60 minutes. The unreacted dye was thereafter removed, and the purple formazan product was dissolved in 100 µl/well DMSO for 30 minutes at 37°C. Cell viability was determined by means of a plate reader (Cytation 3, BioTrek) at λ=540 nm and the number of viable cells were normalized to untreated cells.

In vivo evaluation

Animal care and procedures conformed to the standards for care and use of laboratory animals of the Hebrew University of Jerusalem, Israel, and the National Institutes of Health (NIH, USA). Animals were fed with standard laboratory chow and tap water ad libitum. A total of 34 naïve male Sabra rats (BW 250–320 g BW; Harlan, Jerusalem, Israel) were used in this study. In all experiments, the animals were randomly divided into subgroups. In the treatment groups, rats were injected IV (jugular vein) with $Lip^{(+)}LYS$, $Lip^{(-)}HP$, or IP with the free fluorescent marker in solution (DPPE-Rhod dissolved in corn oil or HP in PBS). Untreated rats served as control.

Toxicity

$Lip^{(+)}LYS$ (4 mg/kg lysozyme, 1.2 mg/kg DPPE-Rhod) and DPPE-Rhod (1.2 mg/kg) were injected IV and IP, respectively, (n=5 each group). Untreated rats served as control. Heparinized blood was drawn 24 h after treatment by cardiac puncture under general anesthesia. Blood specimens were centrifuged (4,000 rpm, 10 min, 4°C), and enzyme levels of aspartate transaminase (AST) and alanine transaminase (ALT), enzymes typically used as biomarkers for liver toxicity assessment, were analyzed according to the routine protocol of the Department of Clinical Biochemistry, Hadassah Hospital.

Biodistribution

Uptake evaluation

Animals (n=22) were randomly divided into 5 groups and injected IV with $Lip^{(+)}LYS$ (2 mg/kg lysozyme, 1.2 mg/kg DPPE-Rhod), $Lip^{(-)}HP$ (6 mg/kg HP), or IP with the free fluorescent marker in solution (1.2 mg/kg DPPE-Rhod or 6 mg/kg HP). Intact rats served as control. Four and 24 hours after treatment, the rats were anesthetized by isoflurane inhalation and subjected to intracardiac perfusion with PBS via the left ventricle. Following perfusion, liver, spleen, kidneys, and the brain were harvested and washed with PBS. Organs were scanned by means of a typhoon scanner at λ_{ex} 560 nm; λ_{em} 580 nm ($Lip^{(+)}LYS$) or λ_{ex} 488 nm; λ_{em} 519 nm ($Lip^{(-)}HP$) followed by ImageJ analysis. The relative mean fluorescence intensity of the tested organ was obtained by subtracting the measured mean fluorescence intensity from corresponding untreated organ. Brains were incubated in 30% sucrose solution (Sigma-Aldrich) overnight, embedded in optimal cutting temperature compound and thereafter snap frozen. The frozen tissue was sectioned (10–30 µm thick sections) with a cryostat (Sakura Finetek). Two or 3 sections were mounted on each slide and stained with DAPI (DAPI Fluoromount-G; SouthernBiotech) after fixation with 4% PFA and scanned by means of a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss Microscopy) followed by ImageJ analysis.

Quantification of uptake by WBC

Heparinized blood was drawn 4 and 24 h after treatment by cardiac puncture under anesthesia. Red blood cells were lysed (Erythrolyse, 1:20

dilution, AbD, Serotec), and the pellet was washed twice with FACS buffer (1% BSA in PBS). Data were acquired on a BD FACScan (BD Bioscience) and analyzed with FCS Express software (De Novo Software). The population of white blood cells (WBC) was gated according to forward and side scattering, monocyte and granulocyte populations were gated according to their typical forward and side scattering, and the fluorescence of the gated cells was measured.

Macrophages co-localization with liposomes in the brain

Slides containing brain sections were fixed with 4% PFA in PBS for 15 minutes, followed by washing with 0.1% polyoxyethylene 20 sorbitanmonolaurate (J.T.Baker) in PBS (PBS-T). Sections were blocked by 3% Fraction V BSA in PBS for one hour and then incubated over night with a primary CD68 antibody (Bio-Rad), diluted 1:25 with CAS block (Life Technologies). One day after, sections were washed with PBS-T and incubated with secondary Cy2 conjugated antibody (Jackson ImmunoResearch) diluted 1:50 in CAS block. Each slide was mounted, stained with DAPI, and scanned by laser scanning confocal microscope (Olympus).

Quantification of lysozyme in the brain

Protein extraction from brain tissue

Animals were divided into 3 groups (n=7 each) and injected IV with Lip$^{(+)}$LYS (3 mg/kg lysozyme) or lysozyme solution (3 mg/kg). Intact rats served as control. Four hours after treatment, the rats were anesthetized by isoflurane inhalation and subjected to intracardiac perfusion with PBS via the left ventricle. Following perfusion brains were harvested and washed with ice-cold PBS and then weighed and minced in a petri dish, followed by the addition of a protease inhibitor cocktail (Sigma-Aldrich), diluted 1:100 in PBS. Brains were then homogenized in a plastic tube on ice for 10 seconds, followed by sonication for 10 minutes using a tip-sonicator (Qsonica, LLC). Homogenates were then centrifuged for 10 minutes at 14,000× g, and the supernatant was removed and stored at −20°C pending analysis.

Quantification of lysozyme (Western blot)

Semi-quantitative analysis of lysozyme levels in the brains was conducted by means of Western blot. Total protein concentration was measured using the Bradford protein assay, and protein lysates were diluted to achieve similar amounts of loaded protein in each well. SDS-PAGE was performed under reducing conditions on a 12% polyacrylamide gel for an hour, followed by protein transfer to a nitrocellulose membrane overnight. The membrane was probed with primary lysozyme antibody (anti-lysozyme; hen egg white, rabbit antibody), diluted 1:1000 with 5% BSA in TBS-T for 2 h and then with goat anti-rabbit HRP-conjugated secondary antibody, diluted 1:10,000 in TBS-T for 30 minutes. Finally, the membrane was shaken in peroxide-enhanced chemiluminescent (ECL) mix for one minute and signal intensity was measured with a ChemiDoc Imager and analyzed using the Image Lab software (Bio-Rad).

Statistical analysis

Data are expressed as the mean ± standard error of mean (SEM). Statistical analysis of liposomes uptake *in vitro*, cytotoxicity *in vitro*, uptake by WBC and WB quantification was conducted using one or two-way analysis of variance (ANOVA). For statistical analysis of biodistribution and brain uptake *in vivo*, student's t-test for independent means was used. Differences were termed statistically significant at $p<0.05$.

Results

Liposome physicochemical properties

Liposomal formulations obtained by the modified thin-film hydration technique are described in Table 1. The mean vesicle size was ~175 nm, with a positive (+19 mV) or negative (−38 mV) zeta potential, a low PDI (<0.1), and an encapsulation yield (EY%) of 19% and 4%, for lysozyme and HP, respectively.

Table 1. *Composition and physicochemical properties of the tested liposomal formulations.*

Name	Formulation	Composition			Physicochemical properties			
		Molar ratio	Lipids (mg/mL)	Drug* (mg/mL)	Size (nm)	PDI	ζ potential (mV)	EY (%)
Lip$^{(+)}$LYS	DSPC:Chol:DOTAP	3:2:1	49 ± 4	Lysozyme 2 ± 0.4	179±7	0.09 ± 0.01	+19±0.5	19 ±4
Lip$^{(-)}$HP	DSPC:Chol:DSPG	3:2:1	46 ± 2	HP 2 ± 0.2	169±0.8	0.08 ± 0.02	-38 ± 0.2	4±0.3

Mean ± SEM. *After removal of free drug. EY: Encapsulation yield

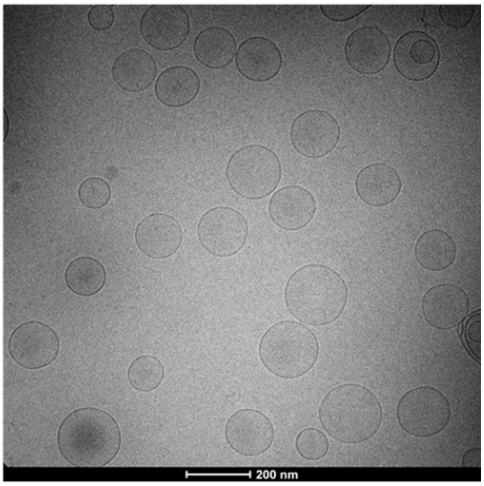

Figure 1. Representative cryo-TEM micrograph of Lip$^{(+)}$LYS, scale bar = 200 nm.

In order to evaluate the stability of Lip$^{(+)}$LYS over time, the formulation was stored in PBS for one year at 4°C, and examined periodically for size, PDI, and zeta potential changes. After a period of one, six and 12 months the observed changes in liposomes size, PDI, and zeta potential were found insignificant (less than: 10 nm, 0.005 and 3 mV, respectively; Fig. 2A-C).

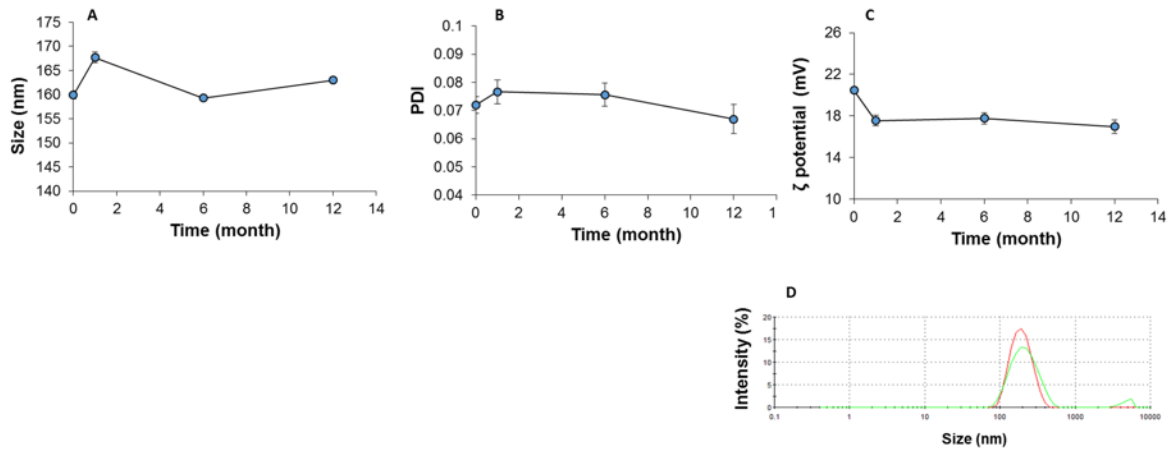

Fig. 2. The effect of storage time and incubation in serum on liposome stability. Lip$^{(+)}$LYS stability over time at 4°C was verified by size (A), PDI (B) and zeta potential (C) (mean ± SEM). Adsorption of serum proteins to Lip$^{(+)}$LYS was measured before and 24 hours after incubation in FBS, red and green lines, respectively, (D).

Adsorption to serum proteins could change the biodistribution and stability of the liposomes, therefore we examined size changes following incubation in serum (Fig. 2D and Fig S1). A significant change in size and PDI of Lip$^{(+)}$LYS following 24 hours of incubation with serum proteins was noted. The liposomes exhibited a higher PDI (0.276) with an increase of 25% from their initial mean size (Fig. 2D). It should be noted that no leakage of lysozyme was observed.

Bioactivity of lysozyme

Lysozyme bioactivity was determined by calculating the rate of decreased optical density (of lysozyme) following reaction with the substrate, *Micrococcus lysodeikticus* normalized to lysozyme solution (Fig. S2B). Intact Lip$^{(+)}$LYS demonstrated partial enzymatic bioactivity of 45%, whereas when broken, following addition of OGP (clear solution), the enzymatic bioactivity was found to be 90%. Empty liposomes (empty-Lip$^{(+)}$) served as a negative control as they did not show any bioactivity (Fig. 3).

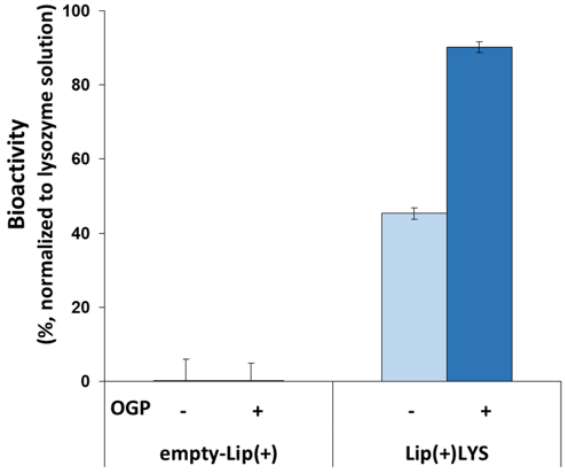

Fig. 3. Bioactivity of lysozyme encapsulated in liposomes. Data normalized to lysozyme solution (200 Units/mL). Empty liposomes served as negative control (mean ± SEM).

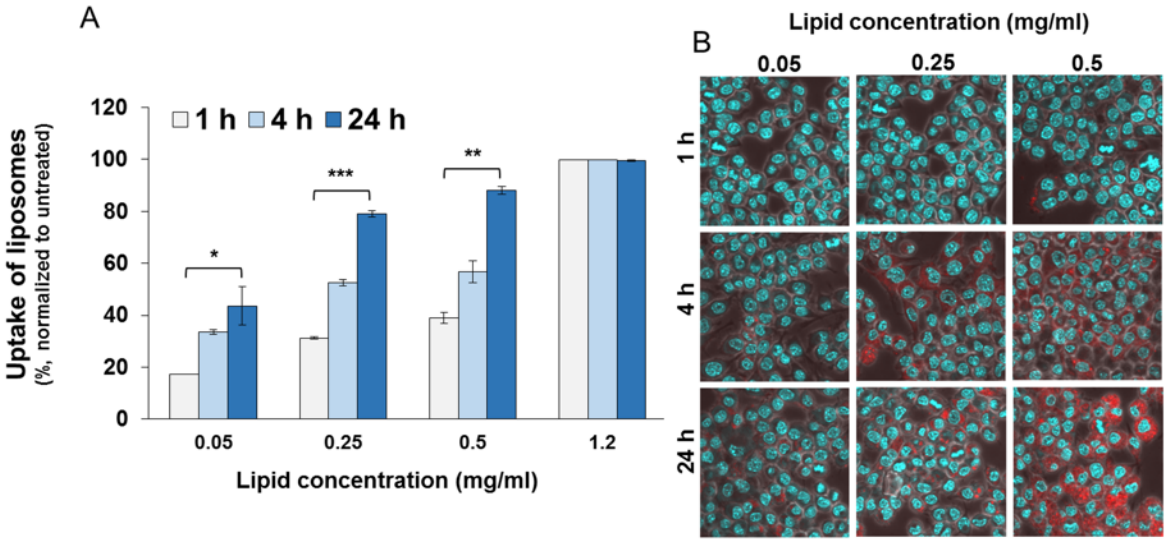

*Fig. 4. Dose- and time-dependent uptake of Lip$^{(+)}$LYS into RAW264.7 cells. Uptake was measured by FACS (A) and confocal microscopy (B) after treatment with 0.05, 0.25, 0.5, and 1.2 mg/mL Lip$^{(+)}$LYS. Untreated cells served as a negative control (*p<0.05; **p<0.005; ***p<0.001, mean ± SEM). Cell nuclei is shown in blue (Hoechst); fluorescent membrane marker of the liposomes (DPPE-Rhod) is shown in red. The fluorescent intensity is normalized to untreated cells (magnification ×60 nm).*

Quantification of Lip(+)LYS cellular uptake

Cellular uptake of Lip(+)LYS was evaluated in RAW264.7 cells using flow cytometry (FACS) analysis. Uptake was observed within one hour and the number of liposomes increased in both a dose- and a time-dependent manner ($p<0.001$ and $p<0.05$, respectively; Fig. 4A).

Evaluation of Lip(+)LYS toxicity

The possible toxic effect of Lip(+)LYS formulation was examined *in vitro* and *in vivo* (Fig. 5A and 5B, respectively). RAW264.7 cells, treated with 0.5 mg/mL Lip(+)LYS or equivalent concentration of lysozyme solution, showed no cytotoxic effect (viability > 80%), 24 and 48 hours after treatment. In contrast, treatment with 1.2 mg/mL Lip(+)LYS exhibited slight (77%) but significant decrease in cell viability, 48 h after treatment ($p<0.05$; Fig. 5A). *In vivo* hepatic toxicity of Lip(+)LYS formulation was evaluated by measuring levels of liver enzymes associated with hepatotoxicity- AST and ALT. Results showed that AST and ALT levels were unaffected 24 hours after IV injection of Lip(+)LYS or IP injection of a DPPE-Rhod solution containing an equivalent concentration (Fig. 5B).

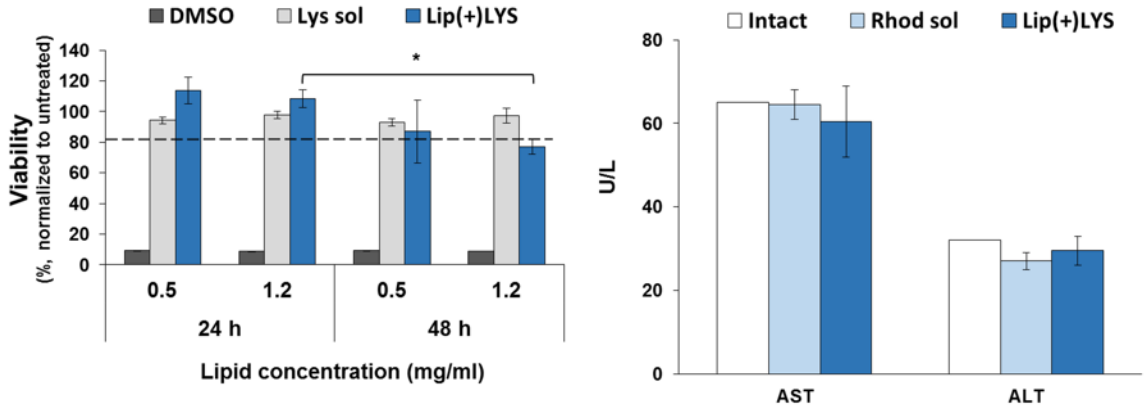

*Fig. 5. Toxicity evaluation of Lip(+)LYS. In vitro cytotoxicity determined by the MTT assay (A). RAW264.7 cells were treated for 24 hours and 48 hours with 0.5 and 1.2 mg/mL Lip(+)LYS (lysozyme conc. 20 and 40 µg/mL) or lysozyme solution (20 and 40 µg/ml). DMSO (10% v/v) served as a positive control. Cells viability is expressed as a percentage of non-treated cells (mean ± SEM; *p<0.05). In vivo hepatotoxicity was determined by measuring AST and ALT levels (B) after treatment with Lip(+)LYS (4 mg/kg lysozyme, 1.2 mg/kg DPPE-Rhod, n=2), in comparison to levels after Rhodamine solution treatment (1.2 mg/kg DPPE-Rhod, n=2), and in an intact rat (n=1; mean ± SEM).*

In vivo biodistribution

Liposomes uptake by circulating WBC

Uptake of fluorescently labeled liposomes (positively and negatively charged) and the corresponding fluorophores solutions by circulating WBC were measured 4 hours and 24 hours after treatment by FACS (Fig. 6).

In the free fluorescent marker treated rats (DPPE-Rhod sol. and HP sol.), only negligible fluorescence was detected in cells 4 hours or 24 hours after treatment. In contrast, Lip(+)LYS exhibited a significant uptake by monocytes and granulocytes in comparison to DPPE-Rhod solution (after 4 hours and 24 hours; $p<0.05$). Negatively charged liposomes (Lip(-)HP) had significantly increased uptake by lymphocytes ($p<0.01$), granulocytes ($p<0.05$), monocytes ($p<0.005$), and total WBC ($p<0.01$; data not shown) compared to HP in solution. Liposomes uptake by the monocyte gated cells was significantly higher than lymphocytes uptake in both liposomal formulations. Uptake by blood lymphocytes, granulocytes, and monocytes was similar in the positively- and negatively charged formulations (Fig. 6).

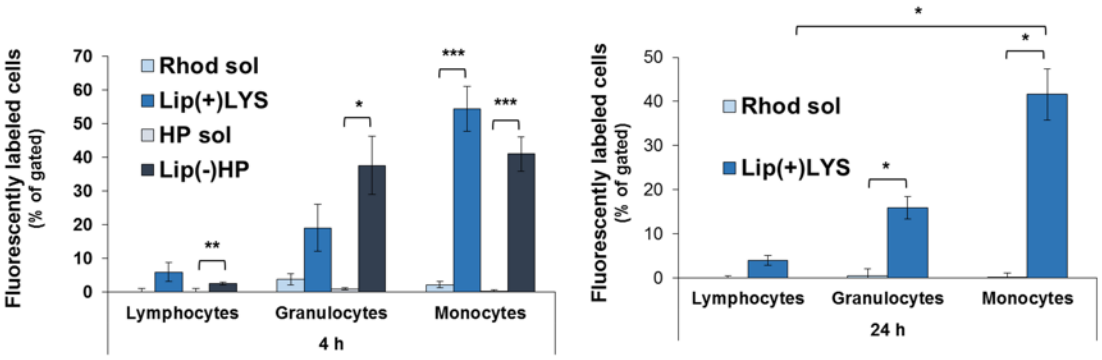

*Fig. 6. In vivo biodistribution. Liposome uptake by circulating WBC, 4 hours and 24 hours after injection. Blood was analyzed by means of FACS. Cells were gated based on their granularity and size scattering and the percentage of positive fluorescent cells recorded. Lip(+)LYS, 2 mg/kg lysozyme, 1.2 mg/kg DPPE-Rhod (n=2), Rhod solution, 1.2 mg/kg DPPE-Rhod, (n=2), Lip(-)HP, 6 mg/kg HP (n=3), HP sol, 6 mg/kg HP, (n=3). *p<0.05, **p<0.01, ***p<0.005; mean± SEM.*

Liposomes transport into the brain

In addition to typhoon imaging, the brain tissue was cryo-sectioned and confocal microscopy was used to qualitatively examine biodistribution of the different liposomal formulations in different brain regions, 4 hours and 24 hours after treatment (Fig. 7A-B).

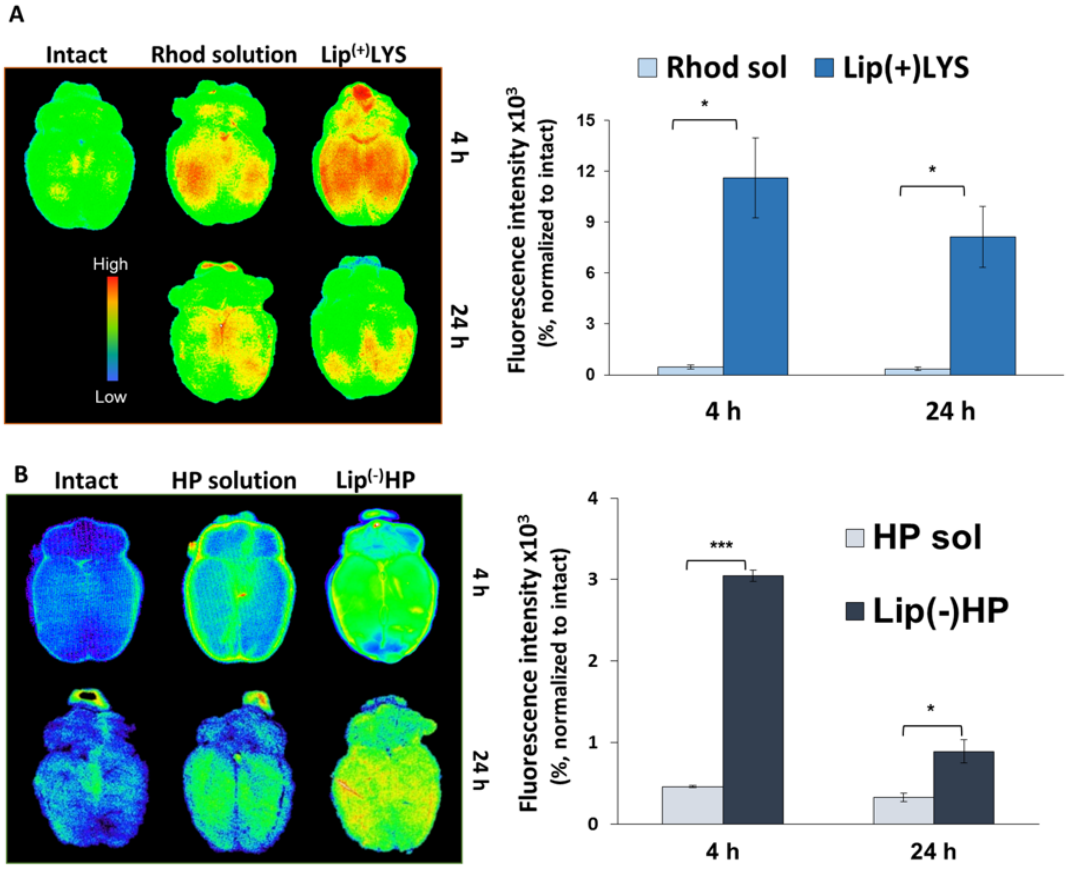

*Fig. 7. Brain transport of liposomes in comparison to free drugs in solution. Typhoon images and ImageJ analyses of brain sections (confocal imaging) demonstrating transport after treatment of rats with positively charged liposomes containing lysozyme (Lip(+)LYS) in comparison to the liposome fluorescent marker, DPPE-Rhodamine (Rhod) in solution (A), and the brain transport of negatively charged liposomes containing hydroxypyrene (Lyp(-)HP) in comparison to HP in solution (B). Fluorescence intensity was calculated in different cryo-sections of the brain. Lip(+)LYS (2 mg/kg lysozyme, 1.2 mg/kg DPPE-Rhod, n=2), Rhod sol. (1.2 mg/kg DPPE-Rhod, n=2), Lip(-)HP (6 mg/kg HP, n=3), and HP sol. (6 mg/kg HP, n=3). *p<0.05, **p<0.01, ***p<0.005; (mean ± SEM).*

Fluorescence intensity of the liposomal formulations was compared to rats treated with the corresponding fluorescent markers solution (DPPE-Rhod sol and HP sol) and normalized to untreated rats' background. Lip$^{(+)}$LYS accumulated in the brain 26 times more than its free fluorescent marker. At 24 hours, fluorescence intensity was decreased insignificantly and was still significantly higher (×16) in comparison to DPPE-Rhod solution. Lip$^{(-)}$HP also accumulated significantly more than its free fluorescent marker (×7). At 24 hours, Lip$^{(-)}$HP concentration was decreased significantly compared to 4 hours but was still significantly higher than HP solution. A significantly higher accumulation in the brain of Lip$^{(+)}$LYS was observed in comparison to Lip$^{(-)}$HP, after 4 hours and 24 hours, 4 times and 9 times, respectively.

Co-localization of liposomes with macrophages in the brain

The mechanism of brain transport was elucidated by examining brain cryo-sections stained with the monocyte/macrophage marker ED1 (CD68). Co-localization of monocytes/macrophages with Lip$^{(+)}$LYS was observed in some regions of the brain, 24 hours after treatment (Fig. 8).

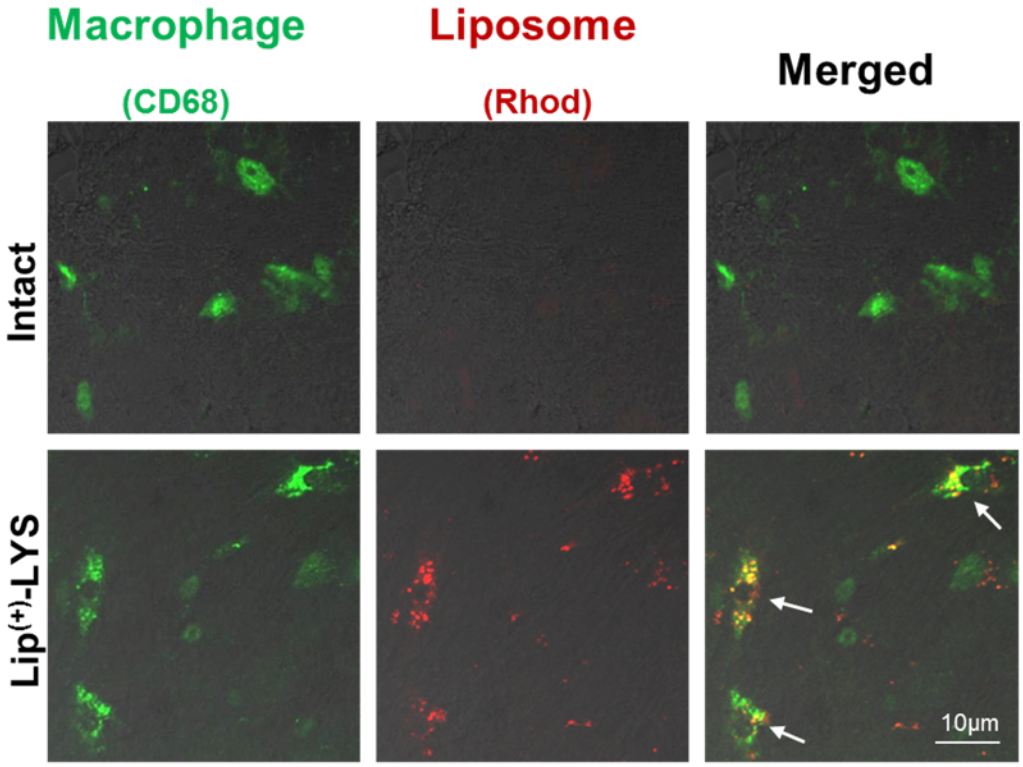

Fig. 8. Co-localization of liposomes with macrophages in the brain. Brain confocal microscopy images 24 hours after treatment with Lip(+)LYS in comparison to intact rats. CD68 is shown in green (macrophage); Rhodamine is shown in red (Lip(+)LYS), and co-localization (marked by arrows) is shown in yellow/orange (magnification ×60 nm; Scale bar = 10 μm).

In contrast, negligible amounts of Rhodamine were found in the brain after treatment with DPPE-Rhod solution, and no co-localization of monocytes and DPPE-Rhod was detected.

Quantification of lysozyme in the brain

Western blot analysis was used to semi-quantify lysozyme levels in brain lysates after treatment with Lip$^{(+)}$LYS. Lysozyme brain levels 4 hours after treatment with Lip$^{(+)}$LYS were found to be significantly higher in comparison to levels after lysozyme solution treatment (Fig. 9, top).

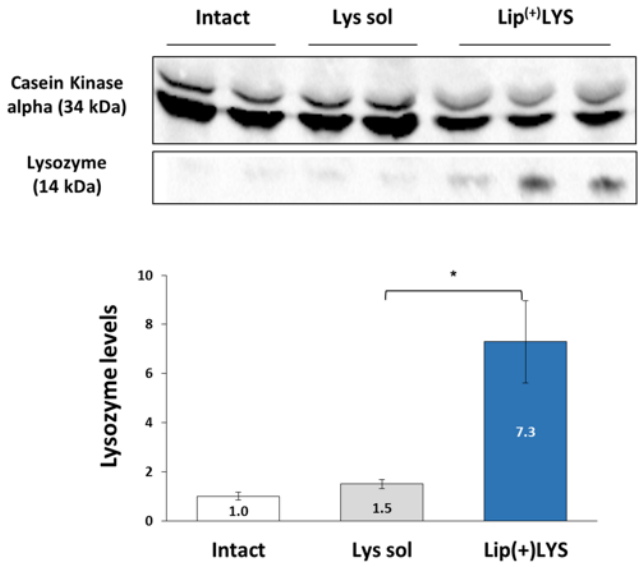

*Fig. 9. Brain lysozyme levels following Lip(+)LYS administration. Western blot analyses of lysozyme in the brain 24 h after treatment with Lip(+)LYS and lysozyme in solution (the house keeping gene bands, casein kinase alpha, served as control). Quantification of bands intensity by ImageJ analysis is shown in the bar graph. Intensity of intact bands was determined as 1. Treatments, Lip(+)LYS (3 mg/kg lysozyme, n=3), Lys sol (3 mg/kg lysozyme, n=2), and intact rat (n=2). *p<0.05 Lip(+)LYS vs. Lys sol.*

Lysozyme protein levels in the brain following Lip$^{(+)}$LYS treatment were 4.8 times higher than those obtained in animals treated with lysozyme solution (Fig. 9, bottom). Similar brain lysozyme levels were found in lysozyme solution treated rats and in intact rats, indicating that lysozyme as a free drug was not transported to the brain.

Discussion

In this study, we validated our hypothesis, and demonstrated that a positively charged particulate delivery system accumulates in the brain via circulating monocytes, bypassing the BBB. Moreover, intact lysozyme, a 14 kDa protein, was detected in the brain following liposome administration.

Positively charged liposomes containing lysozyme were successfully formulated. Attempts to encapsulate lysozyme in negatively charged liposomes failed, probably due to electrostatic interaction between lysozyme and the negatively charged lipid. Therefore, in order to compare brain transport of positively versus negatively charged vesicles, the fluorescent dye, HP, was encapsulated in negatively charged liposomes.

NP size is a significant determinant of the formulation safety and efficacy. Large particles (> 0.5 μm) are known to cause adverse effects after injection by accumulating in the lungs and causing thrombosis [13], whereas vesicles smaller than ~80 nm are capable of penetrating various tissues and escaping the MPS [8, 9, 13, 14]. In addition, it is known that positively charged particles are more avidly internalized by the MPS in comparison to negatively charged particles [11, 12]. It was expected that liposomes, which do not have a neutral membrane, and are neither ultra-small in size nor hydrophilic (PEGylated i.e., non 'stealth' liposomes) [8, 15-17], would efficiently be taken up by circulating phagocytotic cells (monocytes and neutrophils). Since our target cells are circulating monocytes, a liposome size of 100 to 250 nm seems preferable and suitable for filter-sterilization. Indeed, the formulated liposomes obtained (Table 1) are characterized by a spherical shape of unilamellar vesicles, mean vesicle size of ~175 nm, with a positive (+19 mV) or negative (-38 mV) zeta potential, and a low PDI (<0.1).

The stability and 3-dimensional structure of proteins are prone to degradation by various factors [18, 19]. The enzymatic activity of lysozyme extracted from dissolved (OGP) liposomes remained >90%, indicating high stability (Fig. 3). Intact liposomes exhibited a 45% bioactivity implying that a portion of the enzyme was either bound to the outside or not

encapsulated inside the liposomes. Nevertheless, following centrifugal filtration (Vivaspin), discarding possible free lysozyme residing outside the liposomes, lysozyme bioactivity was unchanged implying there was no free lysozyme outside the liposomes. This finding is in accord with the HPLC analyses, where only a negligible amount of lysozyme was found outside the liposomes. The above observations suggest that a certain amount of the enzyme is integrated or adsorbed to the membrane, which is bioactive. More importantly, intact lysozyme, a 14 kDa protein, was successfully encapsulated in the liposomal formulation.

Serum proteins act as opsonins, by binding to the liposomal surface, making them more susceptible to ingestion by phagocytic cells through adsorptive endocytosis [8, 9, 17, 20]. After 24 hours incubation in 10% FBS the Lip$^{(+)}$LYS formulation, exhibited a significant increase in mean size and PDI (Fig. 2). In contrast, negatively charged empty liposomes (empty-Lip$^{(-)}$) did not show any changes in size or PDI (Fig. S1). Empty liposomes that were positively charged (empty-Lip$^{(+)}$) also showed a significant change in mean size and PDI (Fig. S1) indicating that the positive charge of the membrane rather than the encapsulated drug, lysozyme, is responsible for the electrostatic interaction with serum proteins. Adsorption of serum proteins can be of advantage since opsonization has been shown to result in better particles recognition by the MPS mediating efficient internalization by the immune system [21-23].

Uptake of Lip$^{(+)}$LYS by the murine RAW264.7 macrophage cell line revealed a rapid dose- and time-dependent uptake, reaching saturation at lipid concentration of 1.2 mg/mL (Fig. 4). Treatment of intact rats with positively and negatively charged liposomes showed a significantly higher uptake by monocytes as compared to lymphocytes. No significant differences of uptake between the positively- and negatively charged formulations was observed by blood lymphocytes, granulocytes or monocytes (Fig. 6). It is concluded that circulating monocytes efficiently engulf the charged liposomes.

Biodistribution of the liposomal formulations was evaluated in rats by means of typhoon imaging (Fig. 7A-B and Fig. S4) and by confocal microscopy of brain sections (Fig. 7A-B). As expected, the highest fluorescence intensity was observed in the liver for both Lip$^{(+)}$LYS and Lip$^{(-)}$HP formulations, 4 hours after injection. While liver and spleen are monocyte-rich organs, especially in an inflammatory state [24], higher levels of fluorescence were found in those organs after liposome treatment in comparison to the free fluorescent markers in solution (Fig. 7A-B). Fluorescence levels in Lip$^{(+)}$LYS treated rats were significantly decreased in all organs, except the kidneys, 24 h after injection. Excretion from the kidneys commenced 4 hours after treatment, and exaggerated excretion occurred 24 hours after treatment. This could be due to clearance of liposomes from the brain, liver and spleen between 4 and 24 hours.

Lip$^{(+)}$LYS was found to be practically non-toxic (77% cell viability after 48 hours; Fig. 5) in the RAW264.7 cell line at the same high concentration of saturable uptake. In primary SMC no reduction in cell viability was found even at high concentrations or prolonged exposure time (Fig. S3). Positively charged NP, as opposed to negatively charged ones, have been shown to cause cytotoxicity due to reduction in mitochondrial metabolic activity and production of intracellular ROS [25-27]. When evaluating the formulation *in vivo* using liver enzymes, AST and ALT, as markers for liver damage and hepatotoxicity, Lip$^{(+)}$LYS did not show any toxicity (Fig. 5). Thus, it seems that the liposomal formulations are non-cytotoxic. Nevertheless, the dose of Lip$^{(+)}$LYS in the *in vivo* biodistribution studies was similar to the completely non-cytotoxic concentration found in RAW264.7 cell culture studies.

Both DPPE-Rhod and HP solutions treatments resulted in significantly lower brain levels in comparison to liposome treatments (Fig. 7B). DPPE-Rhod is a phospholipid, labeled on the head group with the red-fluorescent Rhodamine B, having a MW of 1249.6, and HP is water-soluble with a MW of 524.39. There was no significant difference in the relatively small uptake of the fluorescent markers following administration in solutions. It could be suggested that the lipophilic nature of DPPE-Rhod compensated for its higher MW transporting both dyes similarly via passive transmembrane diffusion [28, 29]. Significantly higher accumulation in the brain was found

following treatments with both Lip$^{(+)}$LYS and Lip$^{(-)}$HP than the corresponding fluorescent markers injected as a solution (×26 and ×7, respectively). Moreover, the fluorescence resulting from DPPE-Rhod solution treatment was not detected deep in the brain tissue (Fig. S5) as opposed to Lip$^{(+)}$LYS treatment.

The fluorescent levels in the brain, after 24 hours, following Lip$^{(+)}$LYS treatment were insignificantly decreased (Fig. 7). In contrast, the fluorescent levels in the brain following Lip$^{(-)}$HP treatment were significantly decreased after 24 hours (but were still significantly higher than those obtained following HP in solution treatment). This could be due to the faster elimination of the water-soluble molecule from the brain tissue.

Quantification of lysozyme in brain lysates by means of Western blot confirmed the effective transport of lysozyme to the brain. Lysozyme protein levels in the brain following Lip$^{(+)}$LYS treatment were ~5 times higher than those obtained in animals treated with lysozyme solution (Fig. 9). The levels of Lip$^{(+)}$LYS in the brain determined by fluorescent intensity (x26 times than those obtained following DPPE-Rhod administered in solution) are apparently higher than those obtained by determining actual lysozyme levels in the brain by the Western blot analysis (~×5 vs. lysozyme administered in solution). There are several plausible explanations for this apparent discrepancy including the small number of animals in the Western blot study, the fluorescence in brain tissue is over estimated, and that only part of lysozyme was released from the monocytes in the brain. Nevertheless, taken together the results clearly imply that lysozyme in solution is not able to penetrate the BBB, and relatively high levels of the drug are transported to the brain when encapsulated in liposomes.

In our previous studies, IV administration of the negatively charged serotonin liposomes in intact rats exhibited 2 times higher uptake than the free drug [10]. In this study, both the negatively- and positively charged liposomes exhibited better transport to the brain. The better transport to the brain of the negatively charged liposomes containing HP in the current study (×7 vs. ×2) could be explained by the different formulation used in the current work: Same lipids were used but in a different molar ratio (DSPC:DSPG:Cholesterol, 1:1:1 and 3:2:1, respectively). It is well known that any change in the liposome physicochemical properties, including membrane composition, charge and vesicles size could directly affect the uptake by the MPS.

Finally, we confirmed the mechanism of liposomal brain transport validating our hypothesis. Although CD68 stains also microglia, it is suggested that the liposomes were co-localized in the brain with blood-borne monocytes/macrophages. This is because (i) Liposomes size and charge impedes passive diffusion; (ii) A substantial number of circulating monocytes engulfed liposomes (~40%) and rapidly cleared from the blood; and (iii) Co-localization of macrophages with liposomes was found only following Lip$^{(+)}$LYS but not DPPE-Rhod solution treatment (Fig. 8). This is corroborated by the negligible number of blood monocytes engulfing DPPE-Rhod. This clearly suggests that the carrier of the liposomal formulation to the brain are circulating monocytes.

Conclusions

In conclusion, a new positively charged formulation encapsulating a high-molecular weight molecule, the protein lysozyme, was successfully developed with an intact bioactive protein. The liposomal formulation is characterized by desirable properties suitable for uptake by circulating monocytes. Treatment of intact rats with positively and negatively charged liposomes showed a significantly higher uptake by monocytes as compared to lymphocytes. Delivery to the brain by circulating monocytes was confirmed by co-localization of positively charged liposomes and CD68, and by demonstrating significantly higher levels of lysozyme in brain lysates from Lip$^{(+)}$LYS treated rats as compared to lysozyme solution treated rats. Taken together, the positively charged liposomes are more efficient than negatively charged liposomes in transporting drugs to the brain, bypassing the BBB. The developed liposomal NP delivery system could be utilized to deliver various drugs into the brain, which could be useful in inflammatory associated disorders characterized by increased recruitment of phagocytic cells into the brain.

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Conflict of Interest

The authors declare no conflicts of interest. For signed statements, please contact the journal office: editor@precisionnanomedicine.com

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Bibliography

[1] W.M. Pardridge, The blood-brain barrier: bottleneck in brain drug development, NeuroRx, 2 (2005) 3-14.

[2] W.M. Pardridge, Drug targeting to the brain, Pharm Res, 24 (2007) 1733-1744.

[3] M.T. Silva, M. Correia-Neves, Neutrophils and macrophages: the main partners of phagocyte cell systems, Front Immunol, 3 (2012) 174.

[4] I. Bechmann, J. Priller, A. Kovac, M. Bontert, T. Wehner, F.F. Klett, J. Bohsung, M. Stuschke, U. Dirnagl, R. Nitsch, Immune surveillance of mouse brain perivascular spaces by blood-borne macrophages, Eur J Neurosci, 14 (2001) 1651-1658.

[5] W.F. Hickey, Leukocyte traffic in the central nervous system: the participants and their roles, Semin Immunol, 11 (1999) 125-137.

[6] W.F. Hickey, Basic principles of immunological surveillance of the normal central nervous system, Glia, 36 (2001) 118-124.

[7] H.S. Nottet, Y. Persidsky, V.G. Sasseville, A.N. Nukuna, P. Bock, Q.H. Zhai, L.R. Sharer, R.D. McComb, S. Swindells, C. Soderland, H.E. Gendelman, Mechanisms for the transendothelial migration of HIV-1-infected monocytes into brain, J. Immunol., 156 (1996) 1284-1295.

[8] S.M. Moghimi, A.C. Hunter, Capture of stealth nanoparticles by the body's defences, Crit Rev Ther Drug Carrier Syst, 18 (2001) 527-550.

[9] S.M. Moghimi, A.C. Hunter, J.C. Murray, Nanomedicine: current status and future prospects, FASEB J, 19 (2005) 311-330.

[10] E. Afergan, H. Epstein, R. Dahan, N. Koroukhov, K. Rohekar, H.D. Danenberg, G. Golomb, Delivery of serotonin to the brain by monocytes following phagocytosis of liposomes, J Control Release, 132 (2008) 84-90.

[11] A.S. Zahr, C.A. Davis, M.V. Pishko, Macrophage uptake of core-shell nanoparticles surface modified with poly(ethylene glycol), Langmuir, 22 (2006) 8178-8185.

[12] H. Epstein-Barash, D. Gutman, E. Markovsky, G. Mishan-Eisenberg, N. Koroukhov, J. Szebeni, G. Golomb, Physicochemical parameters affecting liposomal bisphosphonates bioactivity for restenosis therapy: internalization, cell inhibition, activation of cytokines and complement, and mechanism of cell death, J Control Release, 146 (2010) 182-195.

[13] F. Alexis, E. Pridgen, L.K. Molnar, O.C. Farokhzad, Factors affecting the clearance and biodistribution of polymeric nanoparticles, Mol Pharm, 5 (2008) 505-515.

[14] M.A. Dobrovolskaia, P. Aggarwal, J.B. Hall, S.E. McNeil, Preclinical studies to understand nanoparticle interaction with the immune system and its potential effects on nanoparticle biodistribution, Mol Pharm, 5 (2008) 487-495.

[15] H.M. Patel, Serum opsonins and liposomes: their interaction and opsonophagocytosis, Crit Rev Ther Drug Carrier Syst, 9 (1992) 39-90.

[16] Y. Sato, H. Kiwada, Y. Kato, Effects of dose and vesicle size on the pharmacokinetics of liposomes, Chem Pharm Bull (Tokyo), 34 (1986) 4244-4252.

[17] A.J. Schroit, J. Madsen, R. Nayar, Liposome-cell interactions: in vitro discrimination of uptake mechanism and in vivo targeting strategies to mononuclear phagocytes, Chem Phys Lipids, 40 (1986) 373-393.

[18] E.Y. Chi, S. Krishnan, T.W. Randolph, J.F. Carpenter, Physical stability of proteins in aqueous solution: mechanism and driving forces in nonnative protein aggregation, Pharm Res, 20 (2003) 1325-1336.

[19] M.C. Manning, D.K. Chou, B.M. Murphy, R.W. Payne, D.S. Katayama, Stability of protein pharmaceuticals: an update, Pharm Res, 27 (2010) 544-575.

[20] K.D. Lee, K. Hong, D. Papahadjopoulos, Recognition of liposomes by cells: in vitro binding and endocytosis mediated by specific lipid headgroups and surface charge density, Biochim Biophys Acta, 1103 (1992) 185-197.

[21] C. He, Y. Hu, L. Yin, C. Tang, C. Yin, Effects of particle size and surface charge on cellular uptake and biodistribution of polymeric nanoparticles, Biomaterials, 31 (2010) 3657-3666.

[22] E. Cohen-Sela, M. Chorny, D. Gutman, S. Komemi, N. Koroukhov, G. Golomb, Characterization of monocytes-targeted nanocarriers biodistribution in leukocytes in ex-vivo and in-vivo models, Nano Biomed Eng, 2 (2010) 91-99.

[23] H. Epstein, D. Gutman, E. Cohen-Sela, E. Haber, O. Elmalak, N. Koroukhov, H.D. Danenberg, G. Golomb, Preparation of alendronate liposomes for enhanced stability and bioactivity: in vitro and in vivo characterization, AAPS J, 10 (2008) 505-515.

[24] S. Epelman, K.J. Lavine, G.J. Randolph, Origin and functions of tissue macrophages, Immunity, 41 (2014) 21-35.

[25] S. Bhattacharjee, L.H. de Haan, N.M. Evers, X. Jiang, A.T. Marcelis, H. Zuilhof, I.M. Rietjens, G.M. Alink, Role of surface charge and oxidative stress in cytotoxicity of organic monolayer-coated silicon nanoparticles towards macrophage NR8383 cells, Part Fibre Toxicol, 7 (2010) 25.

[26] N.M. Schaeublin, L.K. Braydich-Stolle, A.M. Schrand, J.M. Miller, J. Hutchison, J.J. Schlager, S.M. Hussain, Surface charge of gold nanoparticles mediates mechanism of toxicity, Nanoscale, 3 (2011) 410-420.

[27] X. Luo, M. Feng, S. Pan, Y. Wen, W. Zhang, C. Wu, Charge shielding effects on gene delivery of polyethylenimine/DNA complexes: PEGylation and phospholipid coating, J Mater Sci Mater Med, 23 (2012) 1685-1695.

[28] W.A. Banks, Characteristics of compounds that cross the blood-brain barrier, BMC Neurol, 9 Suppl 1 (2009) S3.

[29] Q. Wang, H. Yang, D.W. Miller, W.F. Elmquist, Effect of the p-glycoprotein inhibitor, cyclosporin A, on the distribution of Rhodamine-123 to the brain: an in vivo microdialysis study in freely moving rats, Biochem Biophys Res Commun, 211 (1995) 719-726.

Supporting information

Adsorption to serum proteins

Adsorption of serum proteins to negatively empty liposomes (empty-Lip$^{(-)}$) and positively empty liposomes (empty-Lip$^{(+)}$) was examined by means of size changes following incubation in serum. There was a significant change in size (20%) and PDI (0.233) of the empty-Lip$^{(+)}$ formulation following 24 hours of incubation with FBS. In contrast, empty-Lip$^{(-)}$ did not show any changes in size or PDI (Fig. S1).

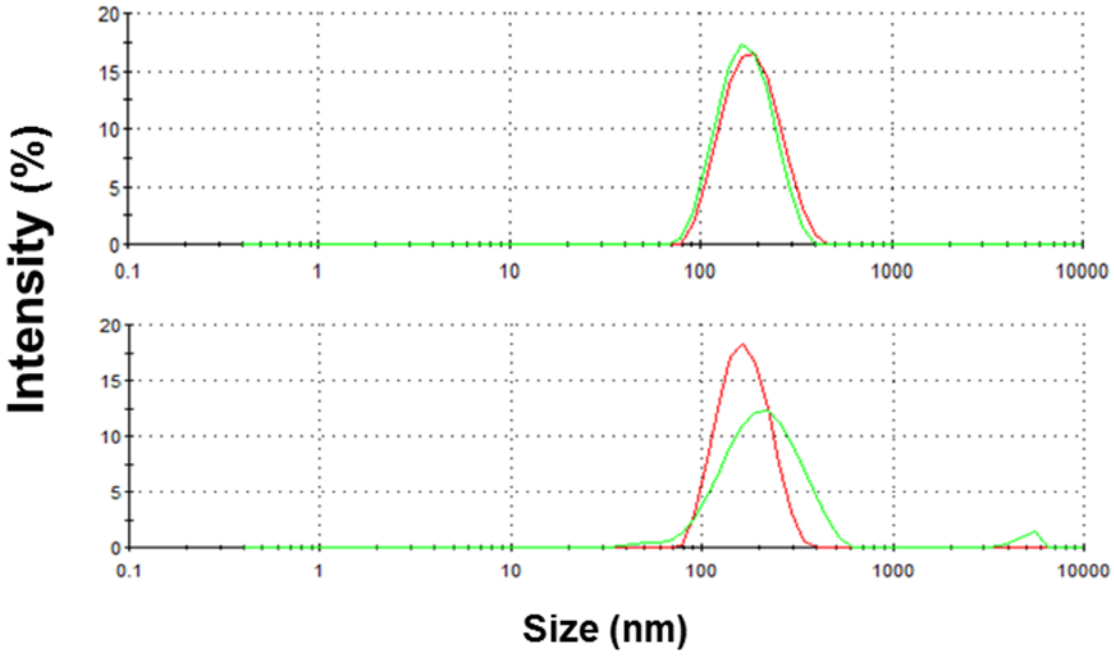

Fig. S1. Adsorption of serum proteins to negatively charged empty liposomes (empty-Lip$^{(-)}$) before incubation in FBS (red line) and after 24 hours of incubation (green line) (top) in comparison to positively charged empty liposomes (empty-Lip$^{(+)}$), before incubation (red line) and after 24 hours of incubation (green line) (bottom). Changes in sample size and PDI distribution was evaluated after incubation in FBS (dilution factor 1:100 v/v) at 37°C.

Lysozyme assay and bioactivity following encapsulation

Concentration of encapsulated lysozyme was measured by means of HPLC at a wavelength of 280 nm. The obtained retention time for lysozyme was 6.4 minutes (Fig. S2). Minimum detection limit of lysozyme concentration was 1 µg/mL. Lysozyme concentrations outside and inside the liposomes was 9 µg/mL and 2.71 mg/mL, respectively, resulting in an encapsulation yield of 19% (Table 1). Lysozyme bioactivity was determined by calculating the rate of decreased OD (of lysozyme) following reaction with the substrate, *Micrococcus lysodeikticus* normalized to lysozyme solution (Fig. S2).

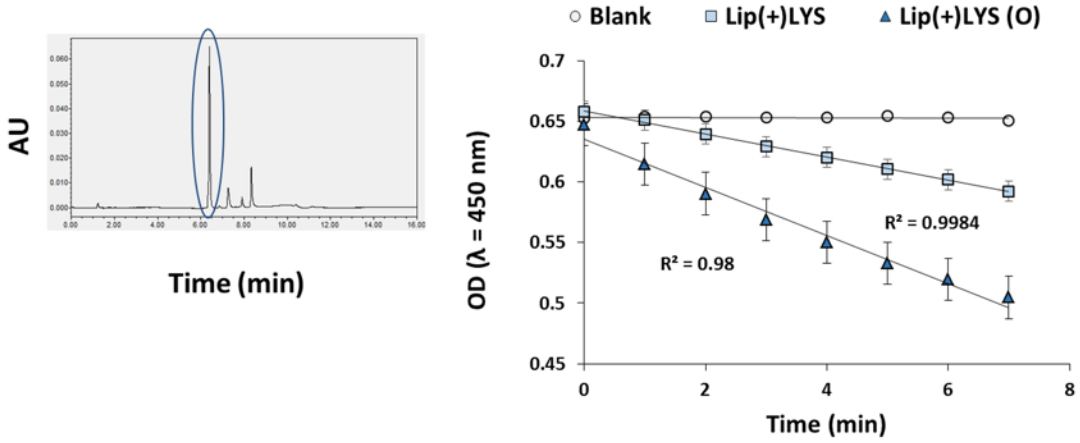

Fig. S2. Identification of lysozyme by HPLC analysis. Representative lysozyme peak is shown on the left obtained in Lip(+)LYS formulation analysis after disruption in OGP solution (6.4 minutes retention time). Representative decrease in OD after adding Lip(+)LYS into the substrate suspension, with or without the nonionic surfactant OGP (O), compared to blank, is shown on the right. Slopes were calculated from the plots demonstrating a linear rate of decreased OD (of lysozyme) after reacting with the substrate (ΔA450/min).

In vitro cytotoxicity evaluation

Primary smooth muscle cells treated with Lip(+)LYS showed no cytotoxic effect at concentrations of 1.2 and 2.4 mg/mL, and after 24 and 48 hours; viability >80%. Cells treated with lysozyme solution also did not show any decrease in viability for all concentrations and time points (Fig. S3).

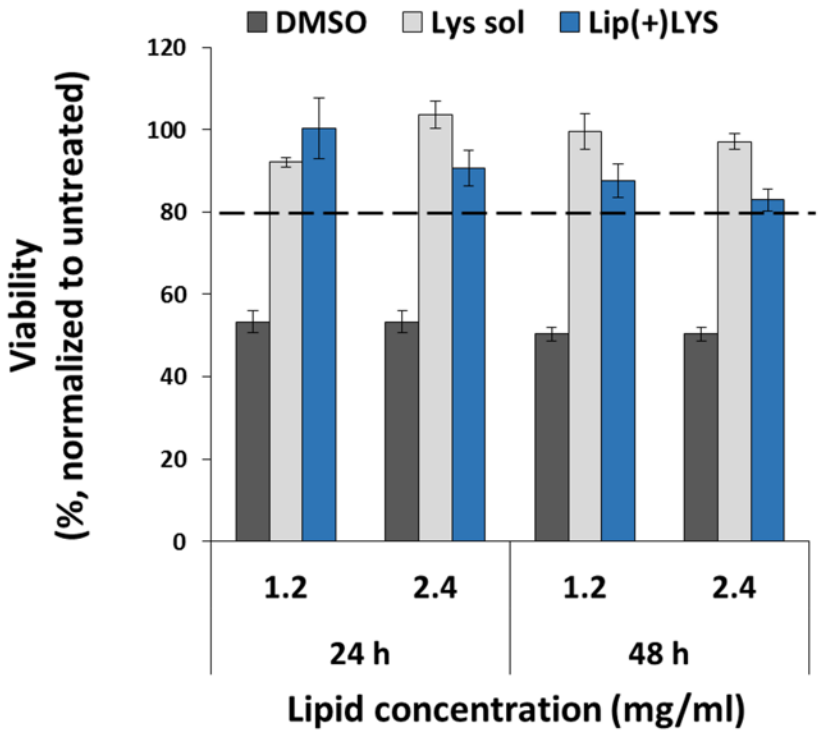

Fig. S3. Primary smooth cells were treated for 24 hours and 48 hours with 2.5% and 5% v/v of Lip(+)LYS (lipid conc. 1.2 mg/mL and 2.4 mg/mL, lysozyme conc. 40 and 80 µg/mL) or lysozyme solution (40 and 80 µg/mL). DMSO (10% v/v) served as positive control. Cells viability is expressed as % of non-treated cells (mean ± SEM).

In vivo biodistribution

Biodistribution of positive and negative liposomes in vivo was evaluated in rats by means of typhoon imaging of kidney, spleen and liver (Fig. S4). The highest fluorescence intensity was observed in the liver for both positive and negative liposomes, 4 hours after injection. Fluorescence levels decreased significantly 24 hours after treatment in all organs except the kidneys in Lip$^{(+)}$LYS treated rats (Fig. S4A).

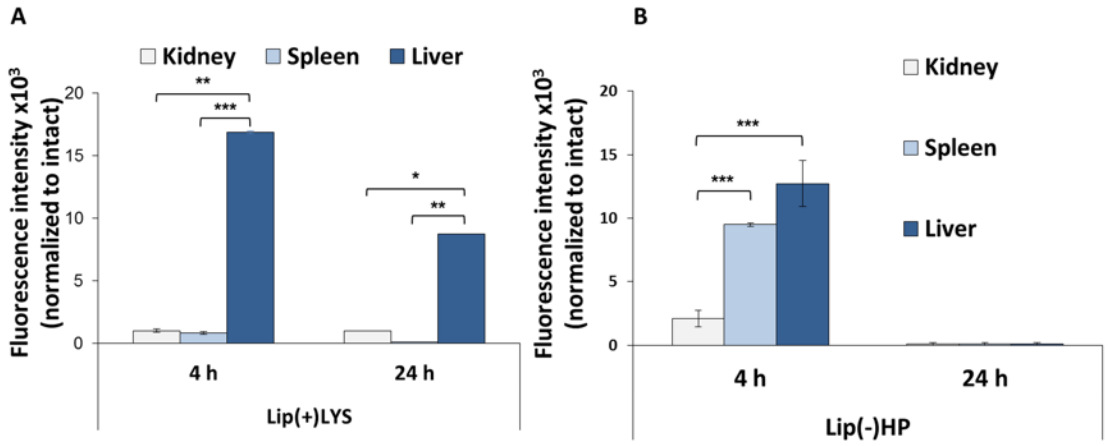

*Fig. S4. Quantitative uptake of positively charged liposomes (A) and negatively charged liposomes (B) in kidneys, spleen and liver, 4 and 24 hours after injection. Lip$^{(+)}$LYS (4 mg/kg lysozyme, 1.2 mg/kg DPPE-Rhod, n=2, 4 h; n=2, 24 h), Lip$^{(-)}$HP (6 mg/kg n=3, 4 h; n=4, 24 h). Mean ± SEM; *p<0.01, **p<0.001, ***p<0.0001.*

Qualitative uptake evaluation

For assessment of Lip$^{(+)}$LYS biodistribution in different brain regions, brains were cryo-sectioned and scanned by confocal microscopy. Uptake was compared between rats treated for 24 hours with Lip$^{(+)}$LYS, DPPE-Rhod solution or intact (Fig. S5). For quantitative assessment of biodistribution, brains were scanned in the "tiles" mode using confocal microscopy followed by ImageJ analysis. Brain levels of Lip$^{(+)}$LYS were significantly higher than those observed in DPPE-Rhod solution treated rats of the tissue.

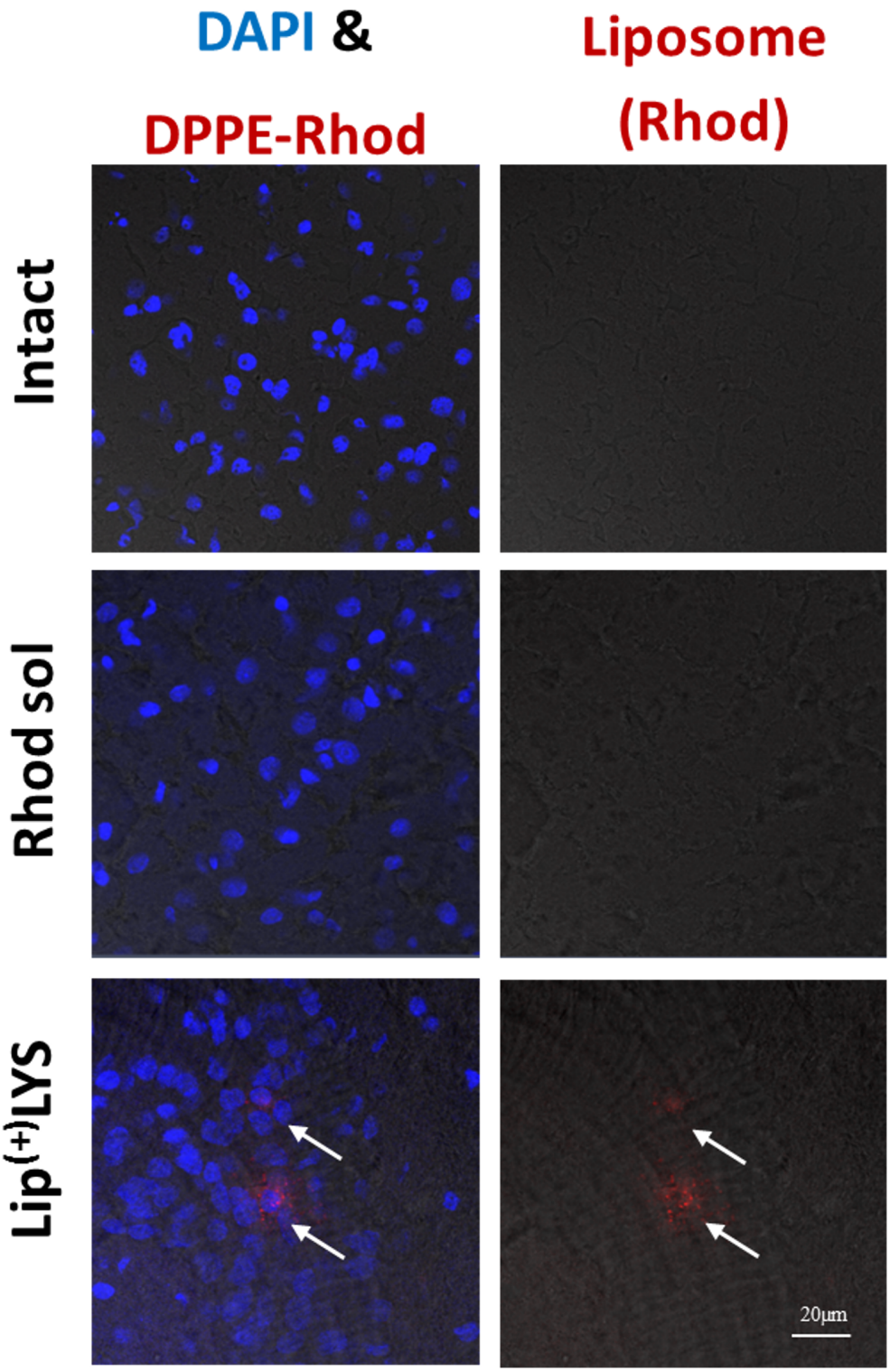

Fig. S5. Representative brain confocal images comparing the transport of DPPE-Rhodamine into the brains 24 h after treatment with Lip$^{(+)}$LYS, Rhodamine solution or intact rat (NT). Nuclei are shown in blue (DAPI); DPPE-Rhodamine is shown in red; liposomes transported into the brain are indicated by arrows. Images were obtained by means of confocal microscopy and analyzed with ZEN 2012 software; magnification ×40 nm.