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In vivo toxicity of cationic micelles and liposomes

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Abstract

This study investigated toxicity of nanocarriers comprised of cationic polymer and lipid components often used in gene and drug delivery, formulated as cationic micelles and liposomes. Rats were injected intravenously with 10, 25 or 100 mg/kg and sacrificed after 24 or 48 h, or 24 h after the last of three intravenous injections of 100 mg/kg every other day. Histological evaluation of liver, lung and spleen, clinical chemistry parameters, and hematology indicated little effect of treatment. DNA strand breaks were increased in the lung and spleen. Further, in the dose response study we found unaltered expression levels of genes in the antioxidant response (\textit{HMOX1}) and repair of oxidized nucleobases (\textit{OGG1}), whereas expression levels of cytokines (\textit{IL6}, \textit{CXCL2} and \textit{CCL2}) were elevated in lung, spleen or liver. The results indicate that assessment of genotoxicity and gene expression add information on toxicity of nanocarriers, which is not obtained by histology and hematology.

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Key words: Nanocarriers; Liposomes; Micelles; In vivo toxicology; Nanomedicine

Background

Nanotechnology has expanded widely over the last decades not only to involve application areas for solar cells and electronics, but also pharmaceuticals, bringing a whole new perspective into health and medicine.\textsuperscript{1,2} There is a great interest in using the unique physico-chemical properties of nanoparticles (NPs) in drug delivery systems for transport and targeting of specific compartments, normally inaccessible to some pharmacutes.\textsuperscript{3,4} Some of the targeting areas currently being investigated are various cancer types, as well as the central nervous system (CNS) in relation to drug and gene delivery.\textsuperscript{5-10} For gene delivery a cationic surface can facilitate a higher degree of interaction between the compounds and the cells and thereby enhance the entrance to the CNS by adsorptive endocytosis.\textsuperscript{11}

Abbreviations: 8-oxodG, 8-oxo-7,8-dihydroguanine; 2’-deoxyguanosine; ATP, Adenosine triphosphate; BW, Body weight; CCL2, Chemokine C-C motif ligand 2; cDNA, Complementary DNA; CNS, Central nervous system; CXCL2, Chemokine C-X-C motif ligand 2; DLS, Dynamic light scattering; ELISA, Enzyme-linked immunosorbent assay; FBS, Fetal bovine serum; FPG, Formamidopyrimidineglycosylase; FPGs, FPG sensitive sites; HCS, High content screening; H&E, Haematoxylin and Eosin; HMOX1, Heme oxygenase (decycling) 1; IL-6, Interleukin-6; IV, Intravenous; LDH, Lactate dehydrogenase; NPs, Nanoparticles; OECD, Organization for economic Co-operation and Development; OGG1, 8-Oxoguanine glycosylase; PBS, Phosphate-buffered saline; PCR, Polymerase Chain Reaction; PEG, Polyethylene glycol; REACH, Registration, evaluation, authorization and restriction of chemicals; RES, Reticuloendothelial system; ROS, Reactive oxygen species; SB, strand breaks; SD, standard deviation; TNF-alpha, Tumor necrosis factor alpha.

Conflicts of interest: No interest of conflicts.

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Liposomes show great possibilities as drug carriers and are already used as drug delivery systems in treatment of cancer, but they are occasionally known to induce complement activation which can lead to hypersensitivity reactions. Liposomes consist of lamellar phospholipid bilayers surrounding aqueous compartments, where the composition can be altered, in order to control the targeting, distribution, drug delivery and toxicity.

Another great potential for drug delivery is polymeric micelles, with a hydrophilic shell and a hydrophobic core. The micelles can be constructed in small sizes of 15–80 nm, and are suggested as promising carriers of poorly water soluble drugs due to their hydrophilic shell. For gene transfection, the use of polymers has shown to be successful in vitro; however cellular uptake, gene transfection and cytotoxicity are dependent on the properties of the specific polymers. These properties include size, structure and surface charge, which all have to be considered when constructing new polymers.

Adverse effects of pharmaceuticals can be attributed to their lack of targeting specificity. Improving this parameter by implementing a nanocarrier could decrease the risk of unwanted adverse effects as found by Pereverzeva et al, where an intravenous injection of the cancer drug doxorubicin in rats showed a reduced cardio- and testicular toxicity when being encapsulated by polysorbate 80 coated NPs. However, there is a potential risk when using nanomaterials, a consideration supported by findings of toxicological effects of e.g. metal oxides, carbon nanotubes or combustion particles that may promote inflammation, cytotoxicity and genotoxicity in lungs after airway exposure and to promote allergic sensitization. Due to limited information available in terms of potential toxicity, including genotoxicity and inflammation, of NPs for drug delivery, we conducted a dose–response study of systemic NP administration by intravenous injection to mimic the delivery route of a drug carrier. In the present study we investigated the short term toxicity of a well-characterized cationic polymeric micelle system and a cationic standard DOTAP/CHOL liposome used in gene delivery.

We assessed the toxicological endpoints by histopathology, hematology and clinical chemistry parameters. We further investigated genotoxicity and gene expression of inflammatory markers (CCL2, CXCL2, IL6), oxidative stress response (HMOX1) and repair of oxidatively-generated DNA nucleobases such as 8-oxo-7,8-dihydroguanine (8-oxoGua) and ring-opened formamidopyrimidine DNA glycosylase (FPG) sensitive sites. Findings from this study were used to further evaluate the NPs in a dose response study, where time and doses were evaluated after a single administration of NPs and examined after 24 or 48 h. The particles were tested as empty vesicles without ligands attached and without any associated drugs, to establish the immediate toxicity of the pristine drug carrier.

**Methods**

**Liposomes**

Lipids were purchased from Avanti Polar Lipids, Alabaster, AL, USA. Cationic liposomes were prepared from N-[1-(2,3-dioleyl)propyl]-N,N,N-trimethylammonium-chloride (DOTAP, synthetic) and cholesterol (plant source) at a concentration of 10 mg/ml in sterile phosphate-buffered saline (PBS) as previously described. Briefly, equimolar amounts of DOTAP and cholesterol were dissolved in chloroform and mixed in a glass vial. The solvent was evaporated under an argon gas stream and the lipid film was dried by high-vacuum overnight. Hydration was performed in PBS at 50 °C for 2 h followed by sonication for 30 min using a Branson water bath (MT-1510, 42 kHz, 80 W, setting “sonics”, Branson Ultrasonics, Danbury, CT, USA) as previously described.

For characterization of the liposomes, 20 µl of cationic liposomes were diluted in 1.7 ml buffer (glucose (5%), Na-HEPES (10 mM, pH 7.4) in disposable cuvettes and the size distribution of the NPs was measured by dynamic light scattering (DLS) using a ZetaPALS Zeta Potential Analyzer (Brookhaven Instruments, NY, US) at standard settings, typically 10 sub-runs of 30 seconds. Data were fitted using built-in software to estimate size and polydispersity index (PDI). Subsequently, zeta potential was measured in same sample using a conditioned electrode, typically by 10 runs observing the relative residual from model fitting, P < 0.04. The preparation had an average size of 92 ±1 nm (average ± standard error), polydispersity index 0.14 and a zeta potential of +53 ± 2 mV.

**Micelles**

The amphiphilic triblock-copolymer poly(methylenecrylate)-b-poly(amineoethyl methacrylate)-b-poly(ethylene glycol) (PMMA-b-PAEMA-b-PEG) (500 mg, 0.04 mmol) synthesized by our previously described method was dissolved in 14 ml of DMF (Sigma Aldrich, Dorset, UK). To this clear polymer solution, 4 ml of MilliQ water was added during constant stirring within a time interval of 30 min followed by drop wise addition of 24 ml MilliQ water. The cloudy micelle solution was then transferred into a dialysis tube (MWCO = 12 kDa) and dialysis was performed against MilliQ water for two days (Dh = 50 ± 1 nm, PDI 0.10, zeta potential of 32 ± 3 mV, measured in glucose (5%), Na-HEPES (10 mM, pH 7.4) and then against PBS buffer (pH 7.4) for another three days. This procedure was also described previously. The final micelle concentration was 9.9 mg/ml.

The stability of the particle suspension was tested after storage for 1 month without any significant change in size or zeta potential.
have been published previously. The rats were sacrificed by sacrificing day 8 or 24 h after the last dose. Some toxicological BW) or PBS (n = 6 per group) IV on day 1, 4 and 7 and study 18 rats were administered cationic particles (100 mg/kg (10, 25 or 100 mg/kg BW) or PBS by IV injection (n = 8 per single-dose study 72 rats were administered cationic particles during the dosing period and changes were recorded. Body-weights (BW) were recorded three times (every 2nd day) in the consumption was recorded per cage on a weekly basis. Body-weights (BW) or PBS groups 24 and 48 h are pooled. **Three administrations every other day over seven days.

| Layout of the study groups. | Single dose study | Repeated dose study |
|----------------------------|-------------------|---------------------|
| PBS (24 and 48 h)          | n = 8*            | n = 6               |
| PBS (7 days)**             |                   |                     |
| Micelle 10 mg/kg (24 h)    | n = 8             |                     |
| Micelle 25 mg/kg (24 h)    | n = 8             |                     |
| Micelle 100 mg/kg (24 h)   | n = 8             |                     |
| Micelle 100 mg/kg (48 h)   | n = 8             |                     |
| Micelles 100 mg/kg (seven days)** | n = 6             |                     |
| Liposome 10 mg/kg (24 h)   | n = 8             |                     |
| Liposome 25 mg/kg (24 h)   | n = 8             |                     |
| Liposome 100 mg/kg (24 h)  | n = 8             |                     |
| Liposome 100 mg/kg (48 h)  | n = 8             |                     |
| Liposome 100 mg/kg (7 days)** | n = 6             |                     |
| Total n                    | 72                | 18                  |

* PBS groups 24 and 48 h are pooled. **Three administrations every other day over seven days.

Animals

Ninety male Han Wistar rats, (Crl:WI (Han)) were obtained from Charles River Wiga GmbH, Germany. Only male rats were used in the present study because a pilot study did not reveal any sex differences on weight gain, hematology and clinical chemistry (data not shown). The rats were four weeks old at arrival and were housed in humidity and temperature-controlled ventilated cupboards (Scantainers, Scanbur Technology, Karlslunde, Denmark) with two or three rats per cage after randomization. They were acclimatized for 1–2 weeks prior to examination and a 12 h day/night cycle was maintained. A standard rodent diet (Altromin 1324, Brogaarden, Denmark) and water were provided ad libitum. Food consumption was recorded per cage on a weekly basis. Body-weights (BW) were recorded three times (every 2nd day) in the week before treatment and on a daily basis during the study. Cage-side clinical observations were conducted several times daily during the dosing period and changes were recorded.

The design of the two study parts is shown in Table 1. In the single-dose study 72 rats were administered cationic particles (10, 25 or 100 mg/kg BW) or PBS by IV injection (n = 8 per group) once and sacrificed after 24 or 48 h. In the repeated dose study 18 rats were administered cationic particles (100 mg/kg BW) or PBS (n = 6 per group) IV on day 1, 4 and 7 and sacrificed day 8 or 24 h after the last dose. Some toxicological endpoints from the repeated dose study not included here have been published previously.32 The rats were sacrificed by exsanguination while anaesthetized by Avertin (Ampliqon, Odense, Denmark) 10 ml/kg BW intraperitoneally and after collection of intracardial blood for analyses. Organs (liver, lungs, spleen, kidneys, adrenal glands, urinary bladder, jejunum, ileum, colon, caecum, duodenum, rectum, lymph nodes (mesenteric and mandibular), thymus and brain) were isolated and examined macroscopically. The weights of brain, liver, spleen, kidneys, adrenal glands, lungs and thymus were recorded and individual organ to BW ratios were calculated. All gross lesions were retained and examined. Tissue samples from brain, kidneys, liver, lungs and spleen were snap frozen in liquid nitrogen for genotoxicity tests.

All experiments were conducted under the Danish Federal guidelines for use and care of laboratory animals and were approved by the Danish Animal Inspectorate.

Sample collection for hematology and clinical chemistry

Intracardial blood samples were drawn for hematology (suspended in 1.6 mg EDTA/ml; Sarstedt, Nümbrecht, Germany) including red- and white blood cell count, hemoglobin, hematocrit and platelet count, by a Advia 120 (Siemens Healthcare Diagnostics, Tarrytown, NY, USA). Further, blood was used for clinical chemistry (suspended in 35 international units Heparin/ml; Sarstedt, Nümbrecht, Germany) with centrifugation at 2200 × g for 15 min at 6 °C for separation of plasma stored at –20 °C until analysis. Clinical chemistry parameters, alanine amino transferase, alkaline phosphatase, aspartate transaminase, albumin, cholesterol, creatinin, glucose, urea, calcium, chloride, potassium, sodium, phosphate and bilirubin, were analyzed on a Pentra 400 (Horiba ABX, Montpellier, France).

Histology

Tissue samples from the collected organs and gross lesions were fixed in 4% neutral buffered formaldehyde solution for at least 48 h. Samples from liver, lung, spleen, kidney, brain and gross lesions were trimmed, dehydrated on a Leica ASP300S (Leica Microsystems, Wetzlar, Germany) and embedded in paraffin. Sections were cut at 5 μm on a Shandon Finesse Microtome (Axlab, Vedbæk, Denmark). Sections for light microscopic examinations were stained with Haematoxylin and Eosin (H&E-staining) in a Leica ST4040 Linear Staining System (Leica Microsystems, Wetzlar, Germany). The sections were examined by light microscopy (Leica Microsystems, Wetzlar, Germany).

Genotoxicity

Tissues were homogenized into 1.5 ml ice-cold Merchants buffer followed by filtration through a 80 μm pore filter (Millipore) as described previously.32,35 The filtrate was resuspended in 37 °C agarose and transferred to a Gelbond® Film (Lonza, Basel, Switzerland). Cells were lysed overnight and subsequently incubated with FPG (45 min, 37 °C) or buffer. After the enzyme treatment, the samples were submerged in alkaline electrophoresis buffer at pH 13 for 40 min before they were electrophoresed in the same solution (25 V, 200 mA, 20 min, 4 °C). Afterwards, they were washed in neutral buffer (0.4 M Trizma base, pH 7.5, 15 min, 25 °C) and finally fixed with 96% ethanol (2 h, 25 °C). The DNA was stained with YOYO-1 (Life Technologies, Nærum, Denmark) and damage with 96% ethanol (2 h, 25 °C). The DNA was stained with YOYO-1 (Life Technologies, Nierum, Denmark) and damage was scored in a fluorescence microscope (Olympus CX40) by visual classification according to a 5-class scoring system and transformed to lesions/106 basepairs by a calibration curve as described previously.32 The samples were scored in duplicate and repeated on three different days, and the level expressed as mean lesions/106 basepairs. The results are expressed as SB and FPG sensitive sites (FPGss). The latter represents the net level of lesions that have been detected by the FPG enzyme (difference in DNA damage between samples that have been treated with FPG enzyme or buffer). The FPGss represent...
Table 2
Clinical chemistry and hematology from single dose study for all groups reported as mean ± SD (n = 8) for animals exposed to control (PBS) or micelles.

| Parameters                      | Micelle Groups ± SD                                      | Control | 10 mg/kg | 25 mg/kg | 100 mg/kg | 100 mg/kg (48 h) |
|--------------------------------|---------------------------------------------------------|---------|----------|----------|-----------|-----------------|
| Body weight (g)                | 141 ± 18.9                                              | 125 ± 20.7 | 133 ± 25.4 | 134 ± 18.1 | 147 ± 14.9       |
| Erythrocytes (10¹²/L)          | 5.72 ± 5.31                                             | 5.9 ± 4.30 | 5.92 ± 5.17 | 5.87 ± 2.61 | 6.18 ± 4.8       |
| Hemoglobin (mmol/L)            | 7.00 ± 0.42                                             | 7.08 ± 0.59 | 6.90 ± 0.57 | 6.96 ± 0.39 | 7.16 ± 0.42       |
| Hematocrit (L/L)               | 0.35 ± 0.03                                             | 0.36 ± 0.03 | 0.36 ± 0.03 | 0.36 ± 0.02 | 0.37 ± 0.03       |
| Thrombocytes (10¹¹/L)          | 9.12 ± 5.73                                             | 8.80 ± 1.01 | 8.62 ± 1.35 | 9.26 ± 8.56 | 9.04 ± 1.36       |
| Leucocytes (10¹²/L)            | 4.25 ± 1.07                                             | 3.92 ± 1.22 | 3.55 ± 1.19 | 4.11 ± 1.07 | 4.45 ± 1.44       |
| Reticulocytes (10¹¹/L)         | 4.71 ± 6.81                                             | 4.66 ± 6.42 | 4.45 ± 6.41 | 4.60 ± 9.03 | 4.23 ± 4.26       |
| Chloride (mmol/L)              | 99.9 ± 1.25                                             | 100 ± 1.49 | 101 ± 1.41 | 101 ± 0.74 | 100 ± 0.99       |
| Potassium (mmol/L)             | 4.41 ± 0.28                                             | 4.24 ± 0.47 | 4.09 ± 0.44 | 4.28 ± 0.36 | 4.53 ± 0.58       |
| Sodium (mmol/L)                | 133 ± 1.73                                              | 133 ± 1.46 | 133 ± 2.27 | 134 ± 1.85 | 134 ± 1.77       |
| Bilirubin (µmol/L)             | 1.04 ± 0.54                                             | 0.96 ± 0.25 | 1.15 ± 0.34 | 0.88 ± 0.47 | 0.81 ± 0.44       |
| Alkaline Phosphatase (µkat/L)  | 4.90 ± 0.81                                             | 5.63 ± 1.13 | 5.03 ± 1.14 | 4.83 ± 0.69 | 4.39 ± 1.16       |
| Phosphorus (mmol/L)            | 2.31 ± 0.55                                             | 2.11 ± 0.30 | 2.01 ± 0.48 | 2.38 ± 0.46 | 2.36 ± 0.27       |
| Albumin (g/L)                  | 26.7 ± 5.31                                             | 27.06 ± 1.46 | 27.2 ± 1.44 | 28.0 ± 2.61 | 6.18 ± 4.8       |
| Creatinine (µmol/L)            | 7.43 ± 1.20                                             | 5.96 ± 0.56* | 6.30 ± 0.97* | 6.36 ± 1.17* | 7.51 ± 0.77       |
| Calcium (mmol/L)               | 3.26 ± 0.22                                             | 3.11 ± 0.12 | 3.03 ± 0.06* | 3.16 ± 0.12 | 3.30 ± 0.30       |
| Triglycerides (mmol/L)         | 1.24 ± 0.33                                             | 1.10 ± 0.20 | 1.06 ± 0.41 | 1.12 ± 0.58 | 1.30 ± 0.42       |
| Alanine aminotransferase (µkat/L) | 1.59 ± 0.28                                       | 1.61 ± 0.36 | 1.55 ± 0.20 | 1.50 ± 0.19 | 1.48 ± 0.35       |
| Glucose (mmol/L)               | 7.00 ± 0.42                                             | 7.08 ± 0.59 | 6.90 ± 0.57 | 6.96 ± 0.39 | 7.16 ± 0.42       |
| Leucocytes (10⁹/L)             | 4.25 ± 1.07                                             | 3.92 ± 1.22 | 3.55 ± 1.19 | 4.11 ± 1.07 | 4.45 ± 1.44       |
| Aspartate aminotransferase (µkat/L) | 4.71 ± 6.81                                    | 4.66 ± 6.42 | 4.45 ± 6.41 | 4.60 ± 9.03 | 4.23 ± 4.26       |
| Urea (mmol/L)                  | 7.43 ± 1.20                                             | 5.96 ± 0.56* | 6.30 ± 0.97* | 6.36 ± 1.17* | 7.51 ± 0.77       |
| Creatinine (µmol/L)            | 22.1 ± 3.87                                             | 21.0 ± 2.72 | 20.4 ± 3.79 | 23.5 ± 5.93 | 27.9 ± 5.57*      |

* indicates significant change compared to control groups.

oxidatively-generated DNA nucleobase lesions including 8-oxoGua and ring-opened formamidopyrimidine lesions.

Gene expression by RT-PCR

The mRNA was extracted from tissue samples using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Frozen tissue from each sample was added to TRIzol and homogenized. The samples were incubated at room temperature for 5 min before adding 0.2 ml chloroform and centrifuging at 12,000 g for 15 min. The supernatant was transferred to a new tube and the mRNA precipitated with 0.5 ml isopropanol. The samples were incubated at room temperature for 10 min before centrifuging at 12,000 g for 10 min. The pellet was washed in ice-cold 75% ethanol followed by centrifugation at 7,600 g for 5 min. The pellet was allowed to air dry. The pellet was resuspended in 30 µl RNase free water by incubation at 55 °C for 10 min.

The extracted mRNA samples were DNase treated using RQ1 RNase-Free DNase kit (Promega, Madison, WI, USA).

The total mRNA was converted into cDNA by reverse transcriptase PCR using the High Capacity cDNA Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). A negative control with no Reverse Transcriptase was included in each run.

The quantification of gene expression was determined by real-time PCR using the Taqman® gene expression assay. The probe and primers used were: CCL2 (Rn00580555_m1), CXCL2 (Rn00586403_m1), IL6 (Rn01410330_m1), OGG1 (Rn00578409_m1), HMOX1 (Rn00561387_m1) and as endogenous control: eukaryotic 18S rRNA (X03205.1), (Applied Biosystems). All samples were determined as triplicates. The level of mRNA expression normalized to the level of 18S rRNA was calculated as 2-ΔΔCt. The relative mRNA levels in the exposure groups were standardized in order to obtain fold increases in mRNA level relative to the level in the control groups.

Cytokine levels in plasma

IL6 and TNF-alpha were determined by ELISA (R&D Systems, MN, USA), according to the manufacturer’s instructions.

Statistics

The statistical analysis for the single dose study was carried out as a one-factor ANOVA on stratified datasets on micelles or liposomes. The control group consisted of 8 animals (a pool of the control animals sacrificed after 24 and 48 h respectively). The same control group was used for both datasets (micelles and liposomes). For the dataset on repeated exposures we used one-factor ANOVA with exposure (control, micelles or liposomes) as categorical variables. Homogeneity of variance between groups was assessed by Levene’s test at 5% level. Log-transformation was necessary for some variables to achieve homogeneity of variance. The results on HMOX1 in the liver in the repeated exposure study did not have homogeneity of variance between groups and these results were thus analysed by non-parametric Kruskal-Wallis test. Statistically significant results were accepted when $P < 0.05$ in the overall ANOVA. The P-values in the text correspond to the post-hoc least significant difference tests. The statistical analysis was carried out in Statistica 5.5 from StatSoft, Inc. (Tulsa, OK, USA). All data are...
presented as mean and standard error of the mean (SEM) values in the figures and standard deviation (SD) in the tables.

Results

The study was conducted in two setups, where the first study was a seven day repeated dose toxicity study, with the aim to observe potential toxicological effects. The results from the first study were subsequently used for an in-depth investigation of the response in a single dose exposure setup with three dose levels and two time points.

Histopathology, bodyweight and clinical signs

There was no effect on BW in animals given cationic particles as compared to controls (data not shown). No clinical signs of reactions to treatment and no macroscopic changes were noted. Furthermore, there were no significant differences in absolute or relative organ weights between the treated and control groups; neither did the histopathological examination reveal any findings associated with the administration of the cationic particles.

Hematology and clinical chemistry

The repeated dose study revealed no significant changes in hematology or clinical chemistry parameters as described elsewhere.32

For the single dose study the hematology parameters were in general within the normal range and none showed any statistically significant changes in animals exposed to micelles or liposomes when compared to the controls. Regarding clinical chemistry, both exposure to micelles and liposomes resulted in a significant decrease in the concentration of circulating urea and calcium – the effect appeared to be stabilized after 48 h. The level of creatinine was increased significantly for the high dose at 100 mg/kg BW for both micelles and liposomes at 24 h and 48 h, respectively (Tables 2 and 3). The micelles induced a significant increase in the level of albumin, but had no effect on triglycerides, whereas the liposomes resulted in a significant decrease in albumin as well as triglycerides (Tables 2 and 3). All the statistically significant changes in hematology and clinical chemistry were small. All other parameters in hematology or clinical chemistry showed no statistically significant changes.

Genotoxicity and gene expression

Exposure to both micelles and liposomes three times during one week in the repeated dose study increased levels of SB in lung tissue (Figure 1, A), but not in the liver (Figure 1, B). Neither exposure increased the level of FPGss of any sampled tissues. An increase was observed for expression of the pro-inflammatory cytokines CCL2 and IL6 as well as for the oxidative stress response gene HMOX1 and the DNA repair enzyme OGG1 in the liver (Figure 2). The increase was statistically significant for the groups exposed to micelles. For the groups exposed to liposomes the same trend appeared, but the effect was only significant for CCL2 and CCL3. In the lung tissue, no significant effect on gene expression levels was seen for any of the exposures (Figure 3).

In the single dose study the highest dose of micelles caused a significant increase in SB in the lung tissue, which was further increased after 48 h, whereas the rats administered liposomes showed a significant increase at 25 and 100 mg/kg BW after 24 h exposure which was diminished after 48 h.
Discussion

This study aimed at investigating the toxicological effect of cationic lipid-based NP carriers with a standard set of in vivo toxicology tests supplemented with assessment of genotoxicity and gene expression patterns in potential target organs. Biomarkers often investigated within particle toxicology were assessed.26,36,37 SB levels were increased in both lung and spleen after administration of liposomes with less response after micelle treatment, which however, caused more inflammatory gene expression in these target organs.

Using the general toxicology parameters investigating clinical signs, histopathology, hematology and clinical chemistry, we found low to no effect on any of the groups tested as the values were all within the normal range and levels. When using the comet assay to further investigate the genotoxic effects, an adverse effect of treatment was observed in all groups given the liposomes and for the highest dose of the micelles, indicating that the comet assay might be a sensitive method that would be of interest to include in the test battery when assessing NP carriers. These results are in agreement with another study showing that cationic liposomes in vitro showed genotoxic effects even at non-cytotoxic concentrations, where also an increase in surface charge was correlated with an increase in genotoxicity.26

We used a non-PEGylated type of liposomes in the present study. These are recognized relatively fast by reticuloendothelial cells in the liver or spleen due to binding to proteins such as immunoglobulins, complement proteins and apolipoproteins. Moreover, it has been shown that non-PEGylated liposomal doxorubicin had half-life in plasma of 12.5 and 31.7 h after IV administration of 6 and 10 mg/kg BW, respectively.38 Thus the difference in dose alone could influence the distribution of liposomes to the spleen and other organs, where the cationic properties of the liposomes, which are not protected by PEG, could interact with the negatively charged surface of the cell membranes through electrostatic interaction and absorption to the cell surface resulting in for instance crossing of the cellular membrane and potentially causing toxicity to the cell.39-41 Cationic liposomes have also been associated with dose-dependent toxicity and pulmonary inflammation in mice after i.t. instillation. Dokka et al found that cationic liposomes induced generation of reactive oxygen species in lung cells causing inflammation and toxicity due to the cationic head group and not the liposome itself.42 The DNA damage observed in the present study could largely be a result of their cationic surface charge, which enables the particles to cross the cell membrane, thereby interfering with the intracellular space, which implies that the properties of the chosen liposome could be of interest in regard to lowering the toxicity for future applications. The surface of the NPs can cause oxidative stress resulting in increased intracellular calcium and gene activation or the intracellular distribution of NPs may affect mitochondria thereby generating oxidative stress. These pathways could both lead to inflammation in which DNA damage could be a possible consequence.25,43,44 The micelles could have a longer circulation time due to the PEGylation that could increase the toxicity to the cell.16,45

The gene expression of HMOX1 is considered to be marker of oxidative stress, which we previously have found increased in lung tissue after exposure to NPs.35 The unaltered levels in the single dose study of HMOX1 in liver, lung and spleen tissue indicates that the exposure to liposomes and micelles was not associated with oxidative stress. This is supported by
the unaltered levels of OGG1 and FPGs in the same tissues.

We have previously observed that repeated exposure to diesel exhaust particles was associated with unaltered levels of oxidatively damaged DNA and increased expression of OGG1 in lung tissue. It indicates that the increased levels of SB by exposure to liposomes and micelles in the present study represent lesions that could be generated by non-oxidative reactions to DNA.

The gene expression of the inflammatory cytokine IL6 was most consistently increased as observed after exposure to 100 mg/kg BW micelles in lung and spleen in the single dose study, although there also were increased levels of CCL2 in the lung and CXCL2 in the spleen. In the repeated dose study the level of IL6 was increased in the liver after exposure to both liposomes and micelles. Nevertheless, IL6 in plasma was below the detection levels of the ELISA methods. This could be due to a too low sensitivity of the assay or that cytokine production in these organs contributes little to the systemic levels found in plasma. A study showed that inhalation of negatively charged lipid NPs was not associated with pulmonary inflammation in terms of elevated IL6 in mice after inhaled doses of up to 200 μg. It indicates that the cationic charge on our particles could be a key parameter in the induction of an inflammatory response.

The two studies described here, had different setups, which also can have different impact on the animals. After repeated dosing the animal’s tolerance to the effects of NPs could be developed. The total dose in the seven day repeated dose study

Figure 2. mRNA level observed in the 7 day repeated dose study in the liver tissue. Rats exposed to either PBS as control vehicle (dark bars), micelles (light grey) or liposomes (dark grey). The level of the following genes is reported: CCL2 (A); CXCL2 (B); HMOX1 (C); IL6 (D) and OGG1 (E). Data are reported as mean level of 8 animals + SEM. * indicate that p < 0.05 compared to the control group.
was higher and the exposure time longer. The reason for this approach was that this study was designed as a sighting study to establish where effects were seen, and not to measure how large the effects were. In the single dose study, the experiment was performed within a realistic range with regards to both the duration and dose of the exposure.

The doses of NPs in our study can be compared to Doxil, which is a PEGylated liposomal doxorubicin that is approved for treatment of e.g. recurrent ovarian cancer. The liposomal vesicle in Doxil consists of a single lamellar envelope with a mean size of 80–90 nm. The total lipid content of Doxil is approximately 16 mg/ml and the doxorubicin is around 2 mg/ml, indicating a 8:1 ratio. Doxil is administered by IV injection once a month in a dose of 50 mg/m² (Doxil.com), corresponding to 92 mg for an adult. A pharmacokinetic study used a dose range of 10–80 mg/m² for IV administration, which corresponds to between 18.5-147 mg of Doxil. This would mean that patients treated with Doxil will receive between 148 mg and 1176 mg of the lipid vehicle, resulting in between 30 mg/L and 235 mg/L of lipids in blood. In our treatment regime, the rats were given 10, 25 and 100 mg/kg BW corresponding to (rats BW: ~135 grams) 1.35, 3.38 and 13.5 mg per rat, which equals 135, 337.5 and 1350 mg/L in the blood. We included two concentrations close to the clinical use as well as a higher concentration for possible higher dosing situations.

Overall conclusions from this study are that both NP carriers showed limited toxicological effects at low doses, whereas higher doses produced some effects that probably were related to the cationic surface charge. Where the standard general toxicity parameters did not reveal any systemic changes by the NPs, the genotoxicity and gene expression responses in potential target organs showed effects. The liposomes appeared to induce more toxicity than the micelles, since there were increased DNA

Figure 3. mRNA level observed in the 7 day repeated dose study in the lung tissue. Rats exposed to either PBS as control vehicle (dark bars), micelles (light grey) or liposomes (dark grey). The level of the following genes is reported: CCL2 (A); CXCL2 (B); HMOX1 (C); IL6 (D) and OGG1 (E). Data are reported as mean level of 8 animals + SEM. * indicate that p < 0.05 compared to the control group.
damage in the lung and spleen at all doses tested. The micelles only caused genotoxicity after exposure to the highest dose but, as opposed to that of the liposomes, the effect did not appear to decline over the time span tested (24–48 h).

For future testing of the toxicity of NPs it could therefore be important to include multiple end points rather than only standard parameters. We would suggest including genotoxicity and gene expression patterns in potential target organs with the standard general toxicity test battery in order to determine the full toxic potential of a NP. Furthermore, it would be of interest to investigate the effect of PEGylation in more detail.

The use of genotoxicity assessment shows that this is a sensitive screening method that can be used to assess the toxicity of compounds with low systemic toxicity, which is already acknowledged, since the comet assay was introduced as a draft for OECD guideline for testing of chemicals in 2012.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nano.2014.08.004.

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Table 4
Level of mRNA in the single dose study, data are from lung, liver and spleen and reported as mean ± SD.

| mRNA  | 24 h after micelles | Control 10 mg/kg | 25 mg/kg | 100 mg/kg | 100 mg/kg (48 h) |
|-------|---------------------|-----------------|----------|-----------|-----------------|
| Lung  |                     |                 |          |           |                 |
| CCL2  | 1.76 ± 1.07         | 1.16 ± 0.91     | 0.48 ± 0.32 | 2.24 ± 2.01* | 1.91 ± 2.16 |
| CXCL2 | 0.32 ± 0.33         | 0.18 ± 0.13     | 0.14 ± 0.09 | 0.22 ± 0.18 | 0.56 ± 1.04 |
| IL6   | 0.10 ± 0.11         | 0.16 ± 0.15     | 0.65 ± 1.55 | 8.39 ± 6.85* | 0.17 ± 0.16 |
| HMOX1 | 10.5 ± 16.7         | 3.50 ± 2.39     | 5.48 ± 2.82 | 8.21 ± 12.7 | 4.17 ± 4.11 |
| OGG1  | 4.83 ± 4.62         | 1.90 ± 0.97     | 2.35 ± 0.89 | 6.40 ± 7.16 | 16.3 ± 37.8 |
| Liver |                     |                 |          |           |                 |
| CCL2  | 0.36 ± 0.32         | 0.02 ± 0.01     | 0.10 ± 0.05 | 0.06 ± 0.05 | 0.08 ± 0.08 |
| CXCL2 | 8.88 ± 7.61         | 2.92 ± 1.72     | 7.59 ± 5.07 | 12.0 ± 14.0 | 7.48 ± 6.48 |
| HMOX1 | 4.30 ± 1.43         | 1.46 ± 0.59     | 2.50 ± 1.28 | 2.12 ± 1.29 | 2.33 ± 1.69 |
| OGG1  | 1.53 ± 0.49         | 0.33 ± 0.25     | 0.52 ± 0.38 | 2.44 ± 2.20 | 0.79 ± 0.42 |
| Spleen|                     |                 |          |           |                 |
| CCL2  | 1.37 ± 0.85         | 0.71 ± 0.40     | 1.23 ± 1.12 | 3.94 ± 2.73 | 1.27 ± 1.31 |
| CXCL2 | 0.02 ± 0.01         | 0.01 ± 0.01     | 0.04 ± 0.04 | 0.16 ± 0.19* | 0.02 ± 0.02 |
| IL6   | 1.29 ± 1.01         | 0.83 ± 0.46     | 0.51 ± 0.31 | 7.88 ± 10.2* | 3.75 ± 4.98 |
| HMOX1 | 116 ± 81.1          | 84.6 ± 33.9     | 70.6 ± 39.0 | 185 ± 128  | 50.4 ± 35.8 |
| OGG1  | 12.0 ± 7.08         | 12.5 ± 13.8     | 14.8 ± 10.2 | 38.7 ± 67.6 | 3.89 ± 1.94 |

24 h after Liposomes

| mRNA  | 24 h after Liposomes | Control 10 mg/kg | 25 mg/kg | 100 mg/kg | 100 mg/kg (48 h) |
|-------|---------------------|-----------------|----------|-----------|-----------------|
| Lung  |                     |                 |          |           |                 |
| CCL2  | 1.76 ± 1.07         | 4.21 ± 7.73     | 1.49 ± 2.04 | 3.00 ± 3.24 | 2.60 ± 2.76 |
| CXCL2 | 0.32 ± 0.33         | 0.62 ± 1.09     | 0.21 ± 0.27 | 0.24 ± 0.26 | 0.42 ± 0.48 |
| IL6   | 0.10 ± 0.11         | 0.05 ± 0.04     | 0.08 ± 0.08 | 0.13 ± 0.12 | 0.12 ± 0.13 |
| HMOX1 | 10.5 ± 16.7         | 31.8 ± 69.9     | 9.64 ± 16.3 | 7.09 ± 4.60 | 8.78 ± 5.65 |
| OGG1  | 4.83 ± 4.62         | 2.92 ± 2.33     | 4.38 ± 5.84 | 3.56 ± 1.84 | 4.22 ± 3.74 |
| Liver |                     |                 |          |           |                 |
| CCL2  | 0.36 ± 0.32         | 0.17 ± 0.10     | 0.16 ± 0.13 | 0.81 ± 0.36* | 0.11 ± 0.08 |
| CXCL2 | 8.88 ± 7.61         | 8.54 ± 4.27     | 9.23 ± 5.99 | 13.7 ± 5.73 | 5.44 ± 1.36 |
| HMOX1 | 4.30 ± 1.43         | 3.70 ± 1.57     | 3.24 ± 0.80 | 5.41 ± 3.16 | 3.63 ± 1.94 |
| OGG1  | 1.53 ± 0.49         | 2.01 ± 1.03     | 1.93 ± 0.05 | 2.85 ± 1.63 | 1.05 ± 0.37 |
| Spleen|                     |                 |          |           |                 |
| CCL2  | 1.37 ± 0.85         | 0.47 ± 0.27     | 1.16 ± 0.72 | 2.71 ± 2.22 | 1.30 ± 0.75 |
| CXCL2 | 0.02 ± 0.01         | 0.01 ± 0.00     | 0.01 ± 0.01 | 0.03 ± 0.03 | 0.02 ± 0.02 |
| IL6   | 1.29 ± 1.01         | 0.98 ± 0.93     | 1.94 ± 1.18 | 1.54 ± 0.96 | 2.64 ± 1.73 |
| HMOX1 | 116 ± 81.1          | 39.2 ± 23.9     | 71.2 ± 38.5 | 82.1 ± 56.2 | 119 ± 84.1 |
| OGG1  | 12.0 ± 7.08         | 3.75 ± 1.88     | 7.88 ± 6.09 | 6.42 ± 3.95 | 4.35 ± 4.47 |

* indicates significant increase compared to control group.
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