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RESEARCH PAPER

Magnetized Aerosols Comprising Superparamagnetic Iron Oxide Nanoparticles Improve Targeted Drug and Gene Delivery to the Lung

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ABSTRACT

Purpose Targeted delivery of aerosols could not only improve efficacy of inhaled drugs but also reduce side effects resulting from their accumulation in healthy tissue. Here we investigated the impact of magnetized aerosols on model drug accumulation and transgene expression in magnetically targeted lung regions of unanesthetized mice.

Methods Solutions containing superparamagnetic iron oxide nanoparticles (SPIONs) and model drugs (fluorescein or complexed plasmid DNA) were nebulized to unanesthetized mice under the influence of an external magnetic gradient directed to the lungs. Drug accumulation and transgene expression was subsequently measured at different time points.

Results We could demonstrate 2–3 fold higher accumulation of the model drug fluorescein and specific transgene expression in lung regions of mice which had been exposed to an external magnetic gradient during nebulization compared to the control mice without any exposure to magnetic gradient.

Conclusions Magnetized aerosols present themselves as an efficient approach for targeted pulmonary delivery of drugs and gene therapeutic agents in order to treat localized diseases of the deeper airways.

KEY WORDS aerosol · cancer · gene delivery · lung · magnetic drug targeting

INTRODUCTION

Drug delivery to the lungs through aerosol inhalation is widely used for the treatment of severe lung diseases such as asthma, COPD or cystic fibrosis and could become a promising alternative for systemically applied chemotherapeutic drugs which are currently used for the treatment of lung cancer but are usually associated with severe side effects when administered systemically (1).

Inhalation of chemotherapeutic drugs is likely to combine desireable feature of considerable pulmonary deposition of the desired drug together with minimization of its severe systemic side effects due to high drug concentrations in the blood circulation. In our recent study, therapeutic effects in a murine lung tumor model could be demonstrated after aerosol administration of plasmid DNA (pDNA) coding for diphtheria toxin (2). As several lung diseases do not affect

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the entire lung, not even in advanced stages, scientists and clinicians seek for more precise alternatives to direct aerosols into distinct regions of the lungs. Targeted aerosol delivery could be beneficial for the following reasons: (i) a high drug concentration could be achieved in diseased tissue including tumors, and (ii) healthy tissue, required for tissue regeneration, could be prevented from harmful side effects of the applied drugs. Previous studies have been performed to address these key issues either by coupling targeting ligands to nanoparticles (3–5) or by loco-regional lung targeting using magnetized aerosols – so called nanomagnetosols – consisting of droplets comprising a soluble drug together with superparamagnetic iron oxide nanoparticles (SPIONs) guided by an external magnetic gradient field (6).

Although magnetic drug delivery of nanomagnetosols has recently been demonstrated in the lungs of anesthetized, mechanically ventilated mice, we were further interested in investigating if (i) magnetic drug targeting to the lung could also be feasible in unanesthetized mice under spontaneous breathing, and (ii) this could lead to targeted expression of exogenously delivered pDNA in the lungs of mice. We were particularly interested in the former because particle deposition has been previously demonstrated to largely depend on breathing patterns (7) which could be relevant in awake patients under physiological breathing conditions. MacIntyre *et al.* have shown that aerosol delivery in mechanically ventilated patients is significantly reduced compared to nonintubated subjects. Among other factors, this reduced delivery was attributed to a combination of suboptimal breathing pattern and endotracheal tube presenting itself as a site for aerosol deposition (8). One major limitation for aerosol delivery in mice as animal models is the low observed deposition rate. Our recent studies in mice demonstrated that less than 0.05% of the total nebulized pDNA reaches the lung tissue. Even with these low amounts, substantial transgene expression indicating high gene transfer efficiency, could be observed when pDNA was complexed to branched polyethylenimine (PEI) (9). Against this background one may assume that even a low increase of the deposited dose would be capable of leading to a considerable enhancement of its biological effect.

In the present study, we therefore applied nanomagnetosols to unanesthetized mice by using two different inhalation systems under exposure to an external magnetic gradient field during the nebulization procedure. These experiments were conducted either with nanomagnetosols comprising fluorescein sodium together with SPIONs or nanomagnetosols comprising PEI-pDNA complexes and SPIONs. At the end of the experiment, mice were euthanized and lungs were isolated for quantification of fluorescein sodium and SPIONs. Furthermore, magnetic field targeted gene expression was demonstrated using *in vivo* bioluminescence imaging and further quantified *ex-vivo* in the isolated lungs.

MATERIALS AND METHODS

Chemicals and Plasmid DNA

Fluorescein sodium, branched PEI (average molecular weight 25 kDa), heparan sulfate and Drying-Pearls orange were obtained from Sigma Aldrich (Deisenhofen, Germany). D-luciferin was purchased from Synchem OHG (Flensburg/Altenburg, Germany). Superparamagnetic spindle shaped iron oxide nanoparticles *fluidMAG/SP-D* (average hydrodynamic diameter of about 200 nm) were kindly provided by Chemicell GmbH (Berlin, Germany).

The plasmid pCMV-luc containing the *Photinus pyralis* luciferase gene under the control of the cytomegalovirus immediate early promotor (CMV) was kindly provided by Prof. E. Wagner (Department of Pharmacy, Ludwig-Maximilians-University Munich, Germany). pCpG-luc was constructed by Manfred Ogris (Department of Pharmacy, Ludwig-Maximilians-University Munich, Germany). Both plasmids were propagated in *Escherichia coli* and provided in a highly purified form (LPS content ≤0.1 E.U./μg DNA) by PlasmidFactory GmbH (Bielefeld, Germany). The amount of supercoiled pDNA was ≥90% ccc (covalently closed circular) for pCMV-luc and greater than 98% ccc grade for pCpG-luc.

Assembly of Inhalation Systems

Animal experiments were conducted using either a nose-only inhalation system (NOIS) or a whole-body inhalation chamber (WBIC). The NOIS was purchased from TSE-Systems (Bad Homburg, Germany) and was provided with an integrated ultrasound nebulizer, generating aerosol droplets with a Mass Median Aerodynamic Diameter (MMAD) between 0.5 and 1 μm. The WBIC was driven with a PARI Turboboy® compression nebulizer (kindly provided by PARI Starnberg, Germany), generating aerosol droplets with an MMAD between 1 and 6 μm. As mice are obligate nasal breathers, aerosol droplets larger than 1 μm are usually captured in the nose of the animals, therefore reducing the total deposition of the applied aerosol in the lungs. To bypass this obstacle, the generated aerosol was dried through a spacer, which was interconnected between the nebulizer and the inhalation chamber and filled with 100 g of Drying Pearls Orange (10).

The NOIS consisted of eight single animal inhalation chambers with two adapters: one connected to a high-pressure port, which was needed for aerosol supply, and another to a vacuum pump to withdraw excessive aerosol from the chamber. Furthermore, the system was driven with a flow rate of 8 l/min. The WBIS, however, consisted of one inhalation chamber which housed 8 animals and was assembled as described previously (10). In order to avoid

adduction of the animals (due to magnets placed on the animals' chests), single separated chambers were constructed by using a fine wire fence (11). All aerosol applications were conducted using air enriched with 5% CO_2 as described previously (12).

Assembly of Permanent Magnets

To ensure a strong magnetic gradient field in the vertical direction, four identical magnets were arranged into a magnetic quadrupole. The magnetic field strength was measured with a Hall probe at nine distinct points lying in the same plane. The spatial variation of the magnetic field strength, denoted by its gradient was determined by taking measurements at five different planes with a vertical separation of $\Delta z=1$ mm. The gradient in Z-direction was then calculated according to $(Bxz1 - Bxz2)/(\Delta z)$, where Bxz1 and Bxz2 are the magnetic field strengths (in Tesla) at points x lying in planes z1 and z2, respectively.

In Vivo Application of Nanomagnetosols

Female 6–8 weeks old BALB/c mice were obtained from Elevage Janvier (Le Genest St Isle, France) and maintained under specific pathogen-free conditions. Mice were acclimatized to the environment of the animal facility for at least 7 days prior to the start of the experiments. All animal procedures were approved and controlled by the local ethics committee and carried out according to the guidelines of the German law of protection of animal life. Prior to nebulization, mice were anesthetized for a short duration using Isoflurane (Abbott, Germany). The duration was sufficient to shave their skin and fix the quadrupole magnet on the right chest of the animals. Subsequently, mice were placed in the chambers of the two different inhalation systems. Nanomagnetosol application was started as soon as the animals regained consciousness.

Preparation of Nanomagnetosol Suspensions

Fluorescein/nanomagnetosol suspension was prepared by mixing 4 ml fluorescein sodium (5 mg/ml) with 4 ml fluidMAG/SP-D (250 mg/ml). PEI/pDNA-solution was prepared by mixing 1 mg pCpG-luc with 1.305 mg branched PEI (each diluted in 2 ml of water for injection, B. Braun Melsungen AG, Germany) resulting in a final concentrations of 250 μg/ml pCpG-luc and 0.362 mg/ml branched PEI, respectively (corresponding to an N/P ratio of 10). Furthermore, the PEI/pDNA-solution was mixed by pipetting up and down 8 times and incubated for 20 min at room temperature. Prior to the nebulization, 4 ml fluidMAG/SP-D (250 mg/ml) was added to the PEI/pDNA-solution and mixed gently.

In Vivo Luciferase Assay

Twenty-four hours after aerosol application, animals were anesthetized by intraperitoneal injection of medetomidine (11.5 μg/kg BW), midazolame (115 μg/kg BW) and fentanyl (1.15 μg/kg BW). Fifty microliters of D-luciferin substrate (1.5 mg/50 μl PBS) was applied to the lungs via sniffing (13). Ten minutes later bioluminescence was measured using an IVIS-100 imaging system (Xenogen, Alameda, USA) with the camera settings field of view 10, f1-stop, high resolution binning, and exposure time of 10 min.

Ex Vivo Luciferase Assay

To quantify pulmonary gene expression per mg tissue weight, mice were euthanized by cervical dislocation post *In Vivo* Imaging. After opening the peritonea by midline incisions, lungs were dissected out and washed in PBS. Lungs were snap-frozen in liquid nitrogen and homogenized using a mortar and pestle in the frozen state. After addition of 400 μl of lysis buffer containing 25 mM Tris pH 7.4, 0.1% Triton X-100 and Complete Protease Inhibitor (Roche Diagnostics GmbH, Penzberg, Germany), samples were incubated for 20 min on ice. The protein lysates were subsequently centrifuged at 10,000 g, 5 min and luciferase activity in the supernatant was measured using a Lumat LB9507 tube luminometer (EG&G Berthold, Munich, Germany). Recombinant luciferase (Roche Diagnostics GmbH, Penzberg, Germany) was used as a standard to quantify luciferase expression per mg lung tissue.

Ex Vivo Fluorescein Quantification in Lung Tissue

Mice were euthanized immediately after the inhalation procedure by intraperitoneal injection of pentobarbital. Subsequently, the lungs were dissected out, separated into left and right lobes, weighed and stored on ice until further processing. For fluorescein quantification, 600 μl of 0.2 M phosphate buffer (pH=8.0) was added to each tissue sample followed by homogenization using a Polytron dispersing and mixing device (Kinematica AG, Littau/Luzern, Switzerland). Subsequently, samples were centrifuged for 5 min at 10.000 g and the fluorescence intensity was measured in 100 μl of the supernatant using a Walac Victor^2 1420 Multilabel Counter (Perkin Elmer, Boston, USA).

DNA Retardation Assay

SPION/PEI/pDNA suspension and PEI/pDNA solution were prepared as described above. SPIONs were separated from one half of the SPION/PEI/pDNA suspension using MACS separation columns (Miltenyi Biotec, Germany) while the other half of the suspension was left untreated.

Subsequently, 5 µl of each particle suspension was mixed either with 2 µl of double-distilled water or with 2 µl of a heparan sulfate solution (5 mg/ml). After 45 min of incubation at room temperature, samples were mixed with 1 µl of loading buffer (0.25% bromphenol blue, 0.25% xylene cyanol FF, 30% glycerol in water), loaded into individual wells of a 0.8% agarose gel and separated by agarose gel electrophoresis at 125 V for 1 h. Staining of the gel was performed with ethidium-bromide and DNA bands were visualized under UV light.

Magnetorelaxometry

The right and left lungs were measured using a single-channel superconducting quantum interference device (SQUID) gradiometer in a magnetically shielded room as described previously (6).

Determination of Non-heme Iron

To determine non-heme iron in lungs, 40–200 mg tissue was incubated overnight at 65°C with 250–500 µl of acid mixture containing 3 M HCl and 0.6 M trichloracetic acid. Supernatant was used for non-heme iron determination by a colorimetric method using 1,10-phenanthroline as described (14). Briefly, 50 µl of the supernatant was mixed with 20 µl 10% hydroxylamine hydrochloride solution, 100 µl ammonium acetate buffer (25 g ammonium acetate, 70 ml glacial acetic acid adjusted with water to a final volume of 100 ml) and 50 µl 0.1% 1,10-phenanthroline solution. The mixture was incubated for 20 min at room temperature and optical density at 510 nm was measured.

Histology

Post nebulization, mice were euthanized and lungs dissected as described above. The isolated lungs were fixed in 4% PFA for 36 h, paraffin embedded and sectioned into 5 µm slices. Subsequently, slices were stained with the Perls Prussian blue for non-heme iron by a standard protocol and examined with an Axiovert 135 microscope (Carl Zeiss AG, Germany).

Statistical Analysis

Results are reported as mean values +/- standard deviation. Statistical significance was calculated using Mann-Whitney-*U*-Test and $p<0.05$ was considered significant.

RESULTS

Quadrupole Assembly of Cylindrical Magnets Generates an Effective Magnetic Gradient

Our previous results, both computer-aided simulations and experiments in mice, have demonstrated that a magnetic flux gradient $\nabla B > 100\ Tm^{-1}$ in close proximity to the magnet tip of a stationary electromagnet is sufficient to target nanomagentosols site-specifically to regions of the lungs subjected to magnetic field (6). Based on these results, we designed a portable magnetic device, which met the following requirements: a) generate a high enough magnetic field gradient and b) allow to be fixed on the chest of non-restrained mice. We found that a magnetic quadrupole assembled from four cylindrical neodymium-ironboron (Nd-Fe-B) permanent magnets in a reciprocal assembly fulfilled these requirements. This conformation resulted in a magnetic field, which when applied, was highly focused on the desired regions of the animal.

The amplitude of the magnetic field was calculated at different distances from the quadrupole (0 to 5 mm). The calculated values were subsequently verified using a Hall prope. The magnetic field was strongest at the center of a single magnet with a value of 0.68 Tesla at a distance of 0 mm. The magnetic field strength decreased with increasing distance from the magnet. At a distance of 5 mm its value was 0.03 Tesla. Furthermore, in order to get an insight into the rate of change of the magnetic field strength, a magnetic gradient field was calculated. Close to the surface of the magnet, the gradient was 300 T/m which revealed that there is a strong change in the magnetic field intensity. The gradient decreased 12-fold within a distance of 5 mm, having a value of 18 T/m at 5 mm. The distance between the skin of the animals' chest, where the magnet was attached, and the lung tissue was estimated to be a minimum of 2 mm during inspiration, consequently resulting in a magnetic field of 0.2 Tesla and a magnetic gradient of 140 T/m (Fig. 1).

Deposition of Fluorescein in the Lungs of Mice upon Nanomagnetosol Administration

Fluorescein-nanomagnetosol was nebulized and delivered to mice using either a whole body inhalation chamber (WBIC) or a nose only inhalation systems (NOIS) with or without exposure to a magnetic gradient field generated by applying the quadrupole magnet to the right side of the chest during nebulization. Immediately after nebulization, fluorescein content was measured in the homogenized left and right lung lobes. Fluorescein deposition was significantly 3.8- and 3.6-fold higher in the left and right lungs of mice exposed to a magnetic gradient field after WBIC nebulization

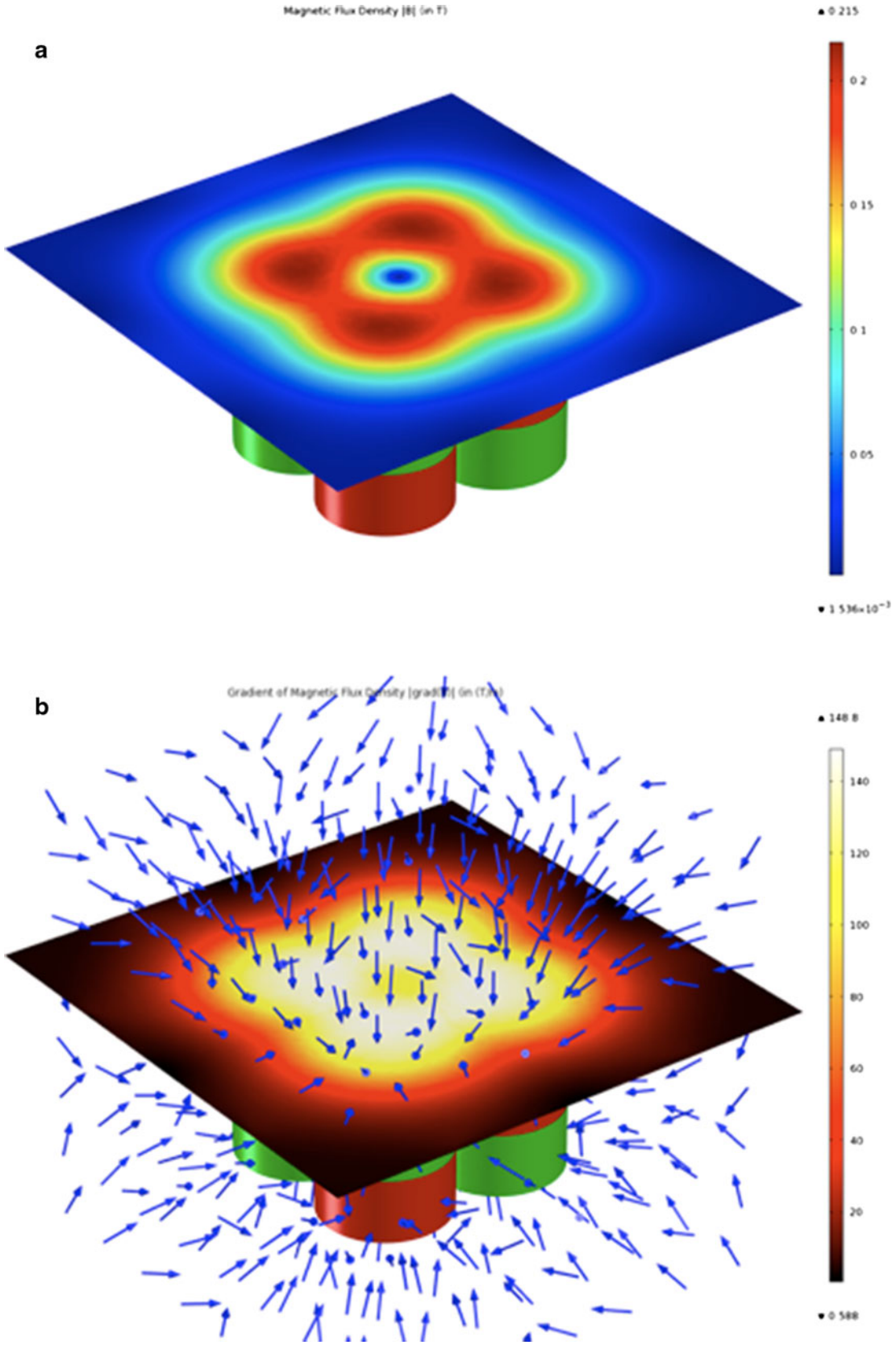

Fig. 1 Magnetic Flux Density (in Tesla) at a distance of 2 mm from the surface of the magnets. (**a**) The highest values are located directly above the centres of the individual magnets. (**b**) Gradient of the magnetic flux density in Tesla per meter at a distance of 2 mm from the surface of the magnets. *Arrows* indicate the direction of the force acting on the magnetosols (proportional to gradient and magnetic properties).

compared to control animals (Fig. 2a). There was no significant difference in fluorescein deposition between the left and right lung lobes for both magnetized and control mice. Total lung deposition increased from 0.01% of the aerosolized dose for control mice to 0.028% for mice exposed to the magnetic field. Similar observation were made after nanomagnetosol delivery to murine lungs using NOIS nebulization but the increase of deposition in magnetized lungs compared to control mice was only 3.7- and 2.6-fold for the left and right lungs, respectively (Fig. 2b). Furthermore, total fluorescein deposition in the lungs was lower for both control and magnetized lungs compared to nebulization using WBIC. However, considerable difference between the duration of nebulization process between WBIC (80 min) and NOIS (20 min) needs to be taken into account when comparing total pulmonary deposition. In summary, these observations demonstrate that a low molecular weight model drug molecule such as fluorescein could be magnetically enriched in the lungs of unanesthetized mice using nanomagnetosols. Moreover, for both nebulization devices, comparable levels of fluorescein deposition was observed in both right and left lung lobes.

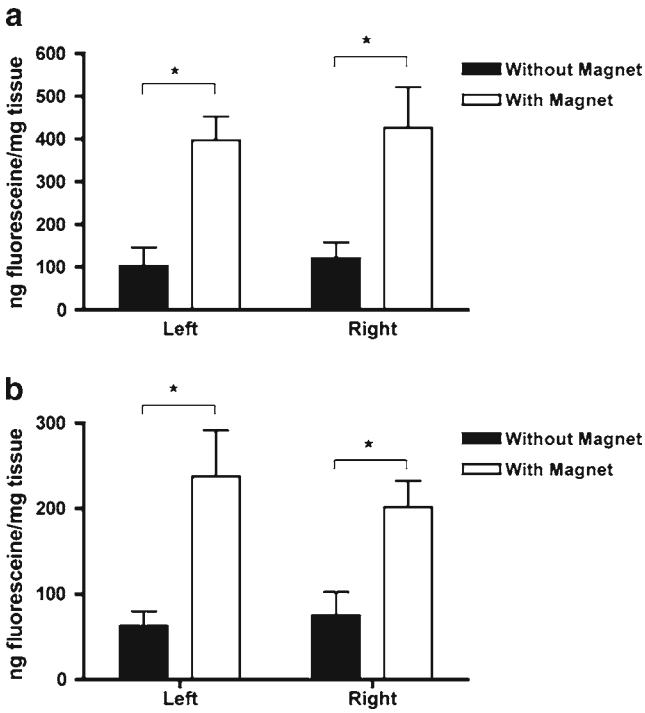

Fig. 2 Fluorescein deposition in the mice lungs after nebulization of a nanomagnetosol with/without magnetic field application to the right side of the chest. A nanomagnetosol comprising fluorescein sodium and SPIONs was applied to the lungs of Balb/c-mice using (**a**) WBIC or (**b**) NOIS nebulization. One group of mice was exposed to an external magnetic field gradient on the right chest during nebulization. Subsequently, fluorescein deposition was measured in the lung tissue. Asterisks (*) stand for statistically significant ($p=0.008$) difference in fluorescein deposition between groups of animals subjected to magnetic field and those without magnetic field during nebulization.

Deposition of SPIONs in the Lungs upon Nanomagnetosol Administration

The results from fluorescein deposition studies demonstrated that although an overall increase in pulmonary deposition could be achieved, no targeted delivery to only the right lung lobe could be attained when quadrupole magnet assembly was centered above the right chest during nebulization. We wondered whether this unexpected finding was associated with imprecise magnet positioning above the right lung resulting in additional exposure of the left lung to magnetic field, or whether fluorescein sodium was rapidly biodistributed from the right to the left lung after deposition via a diffusion processes. To address this issue, we additionally measued the SPION content in the lungs of mice as rapid diffusion of SPIONS in the tissue is restricted by their size. The amount of iron in the lungs was measured by using two different analytical methods. Magnetrelaxometry has been previously used for this purpose in a recent study from our group and has been shown to be highly sensitive in detection of SPIONs in the lung tissue (6). In order to corroborate these measurements, we additionally conducted an alternative method to quantify non-heme iron in lung tissue (14). Similar results were obtained using these two different methods. The deposition rate of SPIONs increased 2.1-fold in the right lung exposed to the magnetic field compared with the control left lung independent of the nebulization device, whereas no difference in SPION deposition between the left and right lungs was observed in control mice which were not exposed to a magnetic gradient field (Fig. 3). These observations establish the proof of principle for targeted magnetic aerosol delivery to the lungs in unanesthetized mice.

Analysis of SPION Deposition in the Lungs of Mice by Histology

Deposition of SPIONs within the lung tissue was further analyzed by histology to assess their distribution in the lung tissue and any histopathological alterations which could have been caused by the iron particles or/and the magnetic gradient field. Prussian blue staining of lung tissue samples prepared immediately after nebulization of the nanomagnetosol, showed iron particles in the alveoli of the right lung of mice, which where exposed to a magnetic field during inhalation. Hardly any deposition could be observed in lungs of control mice (Fig. 4a and d). At higher magnification, iron particles could be located predominantly in alveolar macrophages of magnetized lungs. No other tissue alteration, such as emphysema, which could have been caused by the magnetic field and its effect on SPION distribution could be observed.

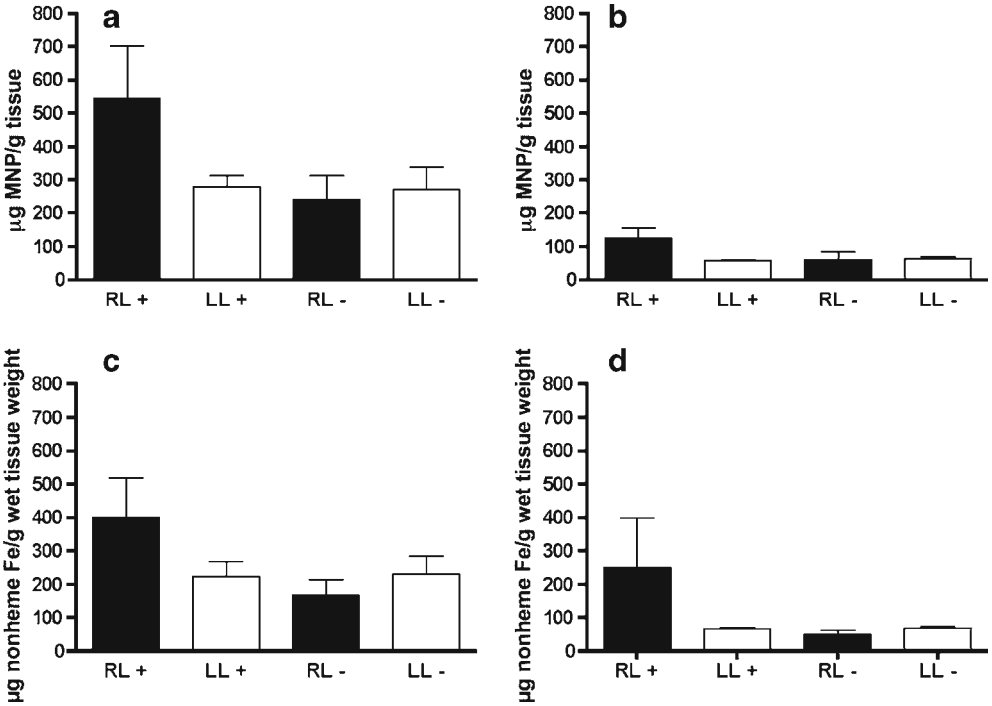

Fig. 3 SPION concentration in mice lungs measured after nanomagnetosol application with the WBIC (**a**, **c**) or NOIS (**b**, **d**) device using either magnetorelaxometry (**a**, **b**) or non-heme iron quantification (**c**, **d**).

Gene Expression in the Lungs upon Nanomagnetosol Application

In a next step, we analyzed transgene expression in the lungs upon nanomagnetosol application in mice. A suspension comprising plasmid DNA (coding for the reporter gene *luciferase*) complexed with brPEI (25 kDa) and SPIONs was generated and nebulized to mice using the WBIC procedure as described above. Transgene expression was measured 24 hours later using *in vivo* bioluminescence imaging. We observed considerable luciferase activity in the right lungs of mice which had been exposed to a magnetic field during nebulization (Fig. 5a), whereas no gene expression was observed in control mice (Fig. 5b).

To quantify the amount of luciferase in the lung tissue, an *ex vivo* luciferase assay was conducted. Similar to results obtained with *in vivo* bioluminescence imaging, no luciferase activity could be detected in lungs from control mice (n=3) whereas lungs from mice exposed to a magnetic field during nebulization (n=3) contained 7.2 pg luciferase per gram tissue (Fig. 5c).

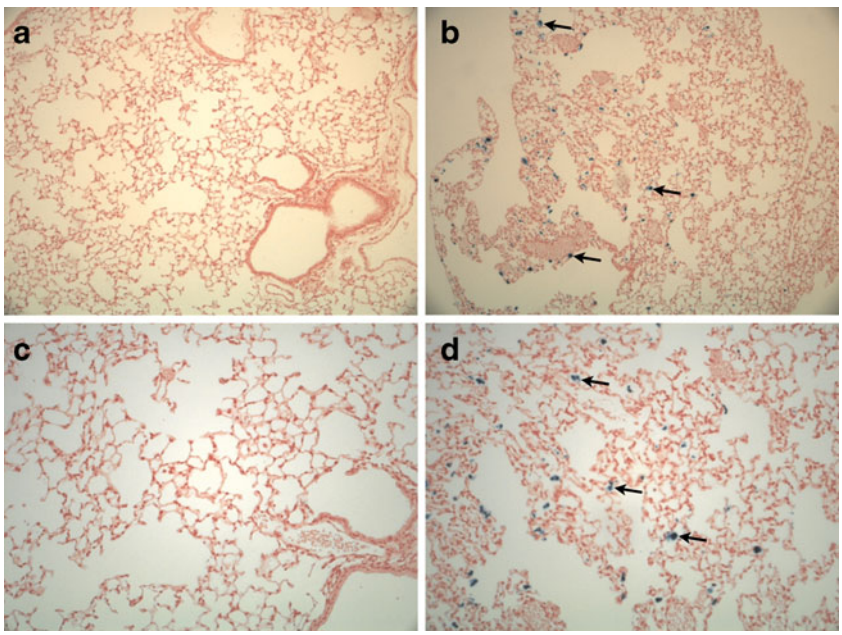

Fig. 4 Prussian blue stained sections from the right (**b**, **d**) and left (**a**, **c**) lungs at low (100x, **a**, **b**) and high (200x, **c**, **d**) magnification. SPIONs were cleared from the lungs by alveolar macrophages, which stained blue for iron (**d**).

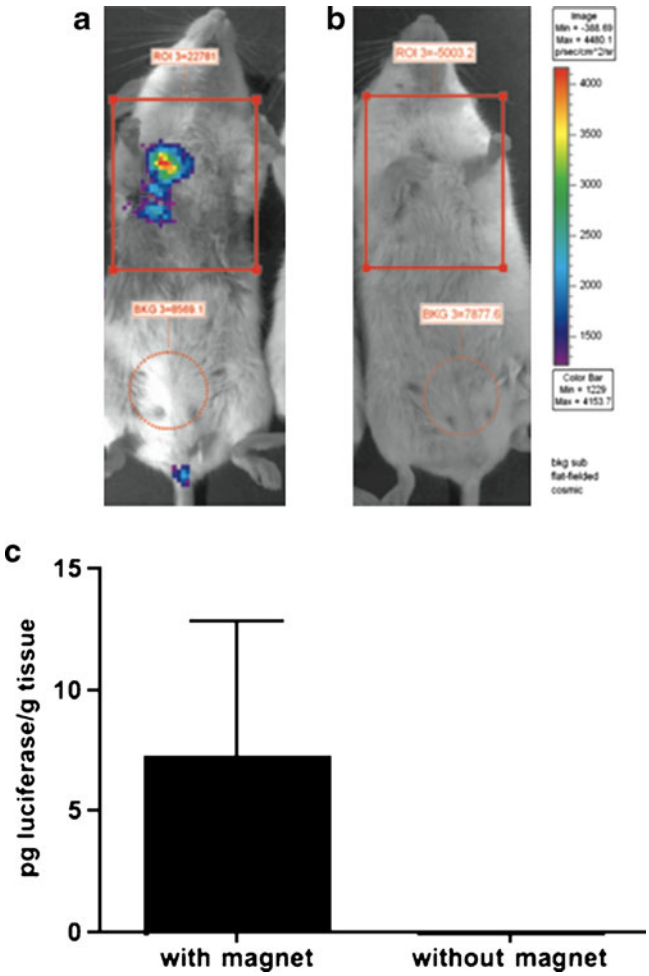

Fig. 5 A nanomagnetosol solution comprising PEI-pDNA gene vectors coding for the luciferase gene and SPIONs was nebulized to Balb/c-mice (n=3) either with (**a**) or without (**b**) an external magnetic gradient field applied to the right chest. Twenty-four hours after nebulization luciferase activity in the lungs was measured using *in vivo* bioluminescence imaging in mice. The lungs were removed subsequently to conduct an *ex vivo* luciferase assay, revealing a mean luciferase expression of 7.2 pg luciferase per gram tissue in mice that had been exposed to a magnetic gradient and no luciferase in mice without a magnetic gradient during the nebulization procedure (**c**).

Analysis of Interaction of PEI-pDNA Gene Vectors and fluidMAG SPIONs

In order to analyze the interaction of PEI-pDNA complexes and SPIONs in more detail, a DNA retardation assay was performed. In the first step, the nanomagnetosol was processed on a magnetic separation column (Miltenyi, Germany) to remove any magnetic material and associated non-magnetic material from the suspension. In a second step, the eluted fraction was analyzed by agarose gel electrophoresis with and without the addition of heparin sulfate to detect any eluted and released pDNA. As shown in Fig. 6, pDNA released from the PEI-pDNA complexes was detectable on the agarose gel electrophoresis in the eluted fraction of the nanomagnetosol after passing the column positioned at the magnet. Semiquantitative analyses of band intensity revealed similar amount of released pDNA as observed for the untreated control sample. These observations demonstrate that the PEI-pDNA gene vectors and SPIONs are predominantly present in the nanomagnetosol solution as individual particle populations which are not tightly associated to each other.

DISCUSSION

Localized pulmonary delivery of chemotherapeutic drugs or nucleic acids could largely advance treatments for patients suffering from severe lung diseases such as lung cancer or tuberculosis. Even though therapeutic benefits for the patients are of utmost importance, potential drug induced side effects such as necrosis, inflammation or hemoptysis, should be considered before substances are applied to the airways. Quite a few diseases are restricted to particular areas of the lung. Therefore, it would be advantageous to attain high concentrations of the desired drugs in the diseased parts of the lung, while leaving the healthy tissue unaffected. Targeted aerosol application of drugs and SPIONs under a magnetic gradient field could be one option to achieve this goal. The dose required for a therapeutic effect could be reduced and this reduction consequently is expected to attenuate drug-related side effects. Proof-of-principle for this approach has previously been demonstrated by Dames *et al.* who proved the feasibility of magnetic aerosol delivery of nebulized SPIONs in anesthetized mechanically ventilated mice (6). However, disadvantageous in this set up was the fact that mice had to be anesthetized and ventilated through a tracheal catheter during the inhalation of nanomagnetosols. Given that nanomagnetosol technology could principally be transferred to the clinic, it would be inevitable that patients should be conscious during the inhalation procedure.

For this reason, in the present study nanomagnetosol application was conducted in unanesthetized mice under physiological respiration conditions. Furthermore, fluorescein sodium was used as a model drug molecule, representative for potential drugs, which could be applied by this method. Our results demonstrated a 3-fold higher accumulation of fluorescein sodium in the entire lungs when a magnetic field was used during inhalation. Surprisingly, the fluorescein deposition increased not only in the magnetized lung lobe but also the control, non-magnetized lung lobe. This observation can most likely be attributed to diffusional transport of fluorescein from the right to the left lung. We speculate that the influence of the magnetic gradient was strong enough to increase deposition of aerosol droplets in the right lungs exposed to the magnetic field but too weak to avoid rapid clearance of the SPIONs by

		A	B	C	D	E	F	G		
	1 kB	pDNA	pDNA/ PEI	pDNA/ PEI	pDNA/ PEI	pDNA/ PEI	pDNA/ PEI	pDNA/ PEI	1 kB	
SPION	-	-	-	-	+	+	+	+	-	SPION
MAG	-	-	-	-	-	-	+	+	-	MAG
HS	-	-	-	+	-	+	-	+	-	HS

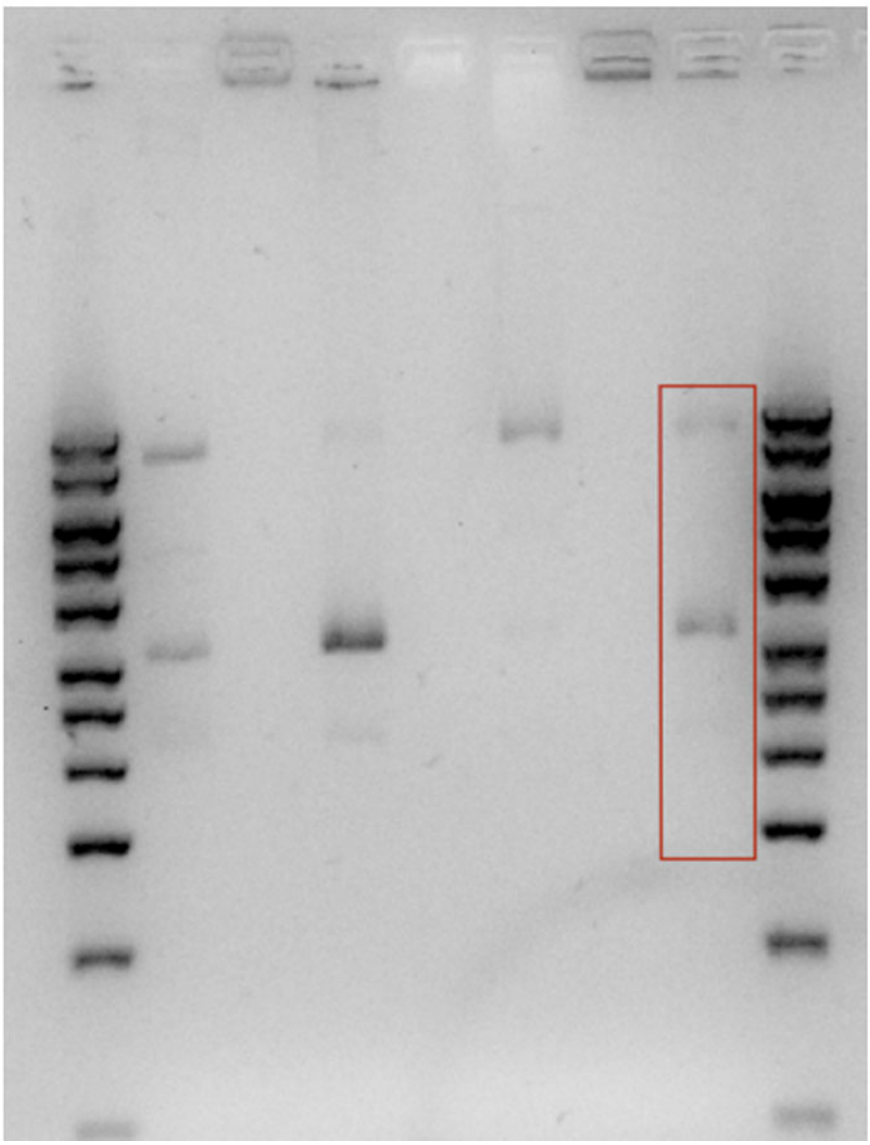

Fig. 6 Nanomagnetosol samples were withdrawn during the nebulization procedure in order to perform a DNA-retardation-assay. Gel electrophoresis revealed characteristic patterns for the positive control - pDNA alone (A) and PEI/pDNA complexes after treatment with heparansulfate (HS) (C). No characteristic pattern was observed for the negative control - PEI/pDNA-complexes without HS treatment (B). Weak pDNA-retardation was observed for samples consisting of PEI/pDNA-complexes and SPIONs when they were treated with HS (E) but not when treatment with HS was omitted (D). Comparable patterns to the positive controls (A, C) were obtained when PEI/pDNA-complexes were separated from the SPIONs with a magnetic column and HS treatment (G), but not when HS treatment was omitted (F).

mucociliar clearance and/or phagocytosis. This hypothesis was further supported by the fact that fluorescein was not bound to the SPIONs but free in solution which allowed its rapid tissue biodistribution after reaching the lung surface. Moreover, these observations are in agreement with a previously published report by Wittgen et (15) who demonstrated that some cytotoxic drugs such as cisplatin were systemically resorbed even though aerosol delivery was used for drug application. Encapsulation of the candidate drug into liposomes (16) or targeted EGF-modified gelatin nanoparticles (17) could be one possibility to achieve delayed and sustained release of drugs in the lungs.

In addition to pulmonary deposition of fluorescein as model drug molecule, we were also interested in the fate of the SPIONs. The deposition of SPIONs in the lungs was quantified by two different methods: magnetrelaxometry

and determination non-heme iron. Both methods yielded similar results with respect to relative differences in SPION deposition in the lungs. Higher amounts of SPIONs were accumulated in the right lungs exposed to the gradient magnetic field compared to the left lungs. These observations were confirmed by lung histology which provided evidence for SPION deposition in the alveolar region but also revealed pronounced phagocytosis by alveolar macrophages. Similar deposition and tissue distribution was observed in our previous study (6). Our results are in agreement with previous report by Lehmann *et al.* who demonstrated that iron nanoparticles are taken up predominantly by macrophages and dendritic cells rather than by epithelial cells (18). Even though the authors discovered a particle dose dependent increase of TNFα in immune cells, no histopathological alterations could be detected in the lungs of mice after exposure to nanomagnetosols in our study.

The efficacy of nanomagnetosols predominately depends on the magnetic gradient field. In the present work a magnetic quadrupole was used which was fixed on the chests of the mice. Using this arrangement a strong magnetic gradient field was generated and applied to the right lungs of the mice. Our results demonstrate that such magnetic device could be used to successfully realize magnetic drug targeting to the lungs in unanesthetized mice. Pulmonary deposition of fluorescein and SPIONs post nebulisation was about doubled by magnetization.

Furthermore, we investigated the capability of nanomagnetosols to be used for targeted gene delivery to the lungs. In our previous study, using magnetic gradient field, 2-fold higher pDNA amounts were detected in the magnetized right lung lobe compared to the unmagnetized left lobe (6). Our current fluorescein and SPION quantification data is in strong agreement with pDNA quantifications made by Dames *et al.* (6). Even though the difference in pDNA deposition between magnetized and unmagnetized lobes was only 2-fold, strong differences were observed with respect to luciferase expression where almost no expression could be detected in the lungs from control mice. It needs to be mentioned that absolute quantification of pDNA cannot be directly compared to transgene expression, in this case luciferase, resulting after pDNA delivery. As each plasmid molecule is subjected to multiple rounds of transcription, many copies of transgene mRNA would be produced in the cells from a single copy of pDNA.

To gain mechanistic insights into the interaction of SPIONs with PEI-pDNA gene vectors, investigations were made to check if the SPIONs and PEI-pDNA gene vectors were physically bound to each other or were present as two independent populations in solution. The findings of the DNA retardation assay revealed that intact pDNA was only detected in the eluted fraction after magnetic separation on the column but not together with the separated SPIONs. This suggests that the SPIONs simply act as an aerosol magnetizer for PEI-pDNA complexes but do not bind to them or DNA.

The results demonstrate the feasibility of targeted gene delivery to the lungs of mice using magnetic aerosols and furthermore raise the possibility of the applied magnetic field altering the cell conditions to enhance transgene expression. This aspect merits immediate and detailed investigations aimed at understanding the effect of magnetic field on the cellular uptake of nanomagnetosols, their intracellular trafficking, structure of nuclear pore complex, pDNA nuclear transport etc. Such mechanistic studies are likely to bring nanomagnetosols a step closer to the realisation of their clinical potential. However, scaling up the magnetic field gradient to address the size of the human lung, still remains a tedious hurdle conderning clinical feasibility of nanomagnetosols. On the other hand, biocompatibility of SPIONs (19), their interaction with lungs and airways (20–22) and availability of high field gradient electromagnets for use in pigs (23), make us believe that magnetic targeting of nanomagnetosols has great potential for targeted inhalation therapy in the future.

CONCLUSIONS

To conclude, the results of this study document the potential of magnetic drug targeting to the lungs for the treatment of a variety of pulmonary diseases. Nanomagnetosol application could improve aerosol therapy by decreasing harmful side effects and increasing treatment efficacy of the applied drugs.

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