THE INFLUENCE OF NATURAL PULMONARY SURFACTANT ON THE EFFICACY OF SIRNA LOADED DEXTRAN NANOGELS

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ABSTRACT

Aims: Topical administration of small interfering RNA (siRNA) nanocarriers is a promising approach in the treatment of pulmonary disorders. Pulmonary surfactant, covering the entire alveolar surface of mammalian lungs, will be one of the first interfaces that siRNA nanocarriers encounter upon inhalation therapy. Therefore, it is of outstanding importance to evaluate the impact of pulmonary surfactant on the performance of siRNA nanocarriers. **Materials & Methods:** The effect of natural lung-derived surfactants on the siRNA delivery capacity of dextran nanogels (DEX-NGs) is evaluated *in vitro* using flow cytometry and confocal microscopy. **Results:** Although the interaction with pulmonary surfactant decreases the cellular internalization of siDEX-NGs significantly, the gene silencing potential of siDEX-NGs was maintained. On the other hand, cationic lipid-based siRNA nanocarriers (Lipofectamine RNAiMAX^{TM}) are incompatible with pulmonary surfactants. **Conclusions:** Our data suggest that pulmonary surfactant can enhance the intracellular siRNA delivery by DEX-NGs, thereby possibly providing new therapeutic opportunities.

KEYWORDS

Polymeric nanoparticles

Dextran nanogels

siRNA delivery

Pulmonary surfactant

Lipoplex

Curosurf®

Infasurf®

INTRODUCTION

RNA interference (RNAi) represents a powerful gene silencing mechanism wherein ~21 nt RNA duplexes, termed small interfering RNA (siRNA), function as the effector molecules for sequence-specific mRNA cleavage. Since synthetic siRNA can be designed to target nearly any human gene, RNAi has become the method of choice to suppress gene expression for therapeutic purposes. Over the last decade, several excellent reviews have been published on the concept of RNAi [1-3] and its clinical potential [4, 5]. Different target tissues and modes of administration have already been evaluated for siRNA, but most successes to date have emerged from local delivery. Given the prevalence of various pulmonary diseases, such as asthma, COPD and lung cancer, the lung is an important target organ in RNAi therapy. Direct siRNA delivery to the lung by local administration has become an established method and offers several advantages over systemic administration in the treatment of respiratory diseases. Inhalation therapy ensures reduction of undesirable systemic side effects, allows direct contact of the siRNA with lung target cells and is noninvasive. Moreover, the required therapeutic dose of siRNA can be significantly decreased [6, 7].

To be therapeutically active, siRNA needs to be guided to the diseased area and efficiently delivered in the cytoplasm of the target cells. Therefore, appropriate nanocarrier formulations are imperative to improve pharmacokinetics and pulmonary distribution, to enhance specific cell targeting and to potentiate intracellular delivery [6]. However, pulmonary administration exposes the nanocarriers to lung-specific physical and chemical barriers that may hamper efficient siRNA delivery. Beside the mucociliary clearance and complex lung anatomy, the airway surface liquid (containing pulmonary surfactant) covering the lower airway epithelial cells is an

important barrier for nanocarriers [7-10]. Pulmonary surfactant contains approximately 90% lipids and 10% surfactant proteins (SP), which are synthesized and secreted by alveolar type II pneumocytes [11]. The lipid fraction consists mainly of phospholipids (about 80-85 wt%) and a minor portion of neutral lipids (~10-20 wt%). SP-A and SP-D are high molecular weight hydrophilic proteins, that play a role in the innate immune response [12]. In contrast, SP-B and SP-C are small hydrophobic proteins that facilitate formation and stabilization of the surfactant film [13].

As pulmonary surfactant is one of the first endogenous interfaces that nucleic acid nanocarriers encounter upon pulmonary delivery, it is of key importance to evaluate the consequences of this interaction. Hence, the impact of pulmonary surfactant on the stability and transfection efficiency of specific nucleic acid nanocarriers has been evaluated in the past [14-16]. A general finding of these studies is the incompatibility of cationic lipid based nanocarriers with pulmonary surfactants. However, nanocarriers consisting of cationic polymers seem to be less prone to inactivation following surfactant interaction [15, 16].

Little information is available in the literature on the effect of pulmonary surfactant on novel siRNA delivery vectors such as matrix nanoparticles. Recent work from our research group demonstrated that cationic biodegradable dextran nanogels (DEX-NGs) are promising carriers for siRNA, due to their high siRNA loading capacity, efficient cellular uptake *in vitro* and their ability to safely deliver intact siRNA to the cell cytoplasm [17, 18]. The main objective of this study was therefore to provide a detailed evaluation on the interaction of pulmonary surfactant with siRNA loaded DEX-NGs (siDEX-NGs) *in vitro*. Curosurf® and Infasurf® were considered as a model to mimic human lung surfactant. Both are modified natural lung-derived surfactants,

clinically approved for the treatment of Respiratory Distress Syndrome in premature infants. This work describes the influence of the different surfactant preparations on the physicochemical characteristics, cellular internalization and gene silencing potential of the siDEX-NGs in a lung epithelial and alveolar macrophage cell line. This information may contribute to a better understanding of the impact of pulmonary surfactant on the efficacy of polymeric nanocarriers.

MATERIALS & METHODS

SiRNA duplexes

Twenty-one nucleotide siRNA duplexes targeting the enhanced green fluorescent protein (siEGFP) and negative control duplexes (siCTRL) were purchased from Eurogentec (Seraing, Belgium). siEGFP: sense strand = 5'-CAAGCUGACCCUGAAGUUC**tt**-3'; antisense strand= 5'-GAACUUCAGGGUCAGCUUG**tt**-3'. siCTRL: sense strand = 5'-UGCGCUACGAUCGACGAUG**tt**-3'; antisense strand = 5'-CAUCGUCGAUCGUAGCGCA**tt**-3' (lower case bold letters represent 2'-deoxyribonucleotides, capital letters are ribonucleotides). For fluorescence experiments, siCTRL duplex was labeled with a Cy®5 dye at the 5' end of the sense strand (Eurogentec). siGENOME SMARTpool targeting the protein tyrosine phosphatase receptor type C (PTPRC, CD45), as well as non-targeting siGENOME, were purchased from Dharmacon (Thermo Fisher Scientific, Tournai, Belgium).

Preparation of cationic dextran nanogels and loading with siRNA

For the preparation of DEX-NGs an inverse mini-emulsion photopolymerization method was applied as reported previously [17]. Briefly, dextran hydroxyethyl methacrylate (dex-HEMA, DS 5.2 [19]) (65 mg) was dissolved in a solution containing 40 µL irgacure 2959 1% (w/v), 80 µL lipopolysaccharide (LPS) free water and 40 µL of the cationic methacrylate monomer, [2-(methacryloyloxy)-ethyl] trimethylammonium chloride (TMAEMA, 80 wt% solution in water). The obtained dex-HEMA solution was emulsified in 5 mL of mineral oil, supplemented with the

surfactant ABIL EM 90 (10% (v/v)), through ultrasonication (50 s, Branson Tip Sonifier, amplitude 15%). The formed emulsion nanodroplets were immediately crosslinked by UV irradiation (900 s, Bluepoint 2.1 UV source, Hönle UV technology) under cooling (4°C). The resulting dex-HEMA-*co*-TMAEMA nanogels were collected by precipitation in acetone, followed by centrifugation, and washed three times with acetone:hexane (1:1). Traces of organic solvent were removed by vacuum evaporation and the pellet was redispersed in 5 mL LPS free water. To assure long-term stability, the DEX-NGs were lyophilized and stored desiccated.

To load DEX-NGs with siRNA, first a DEX-NG stock (2 mg/mL) was prepared by dispersing a weighed amount of lyophilized particles in ice-cooled N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (pH 7.4, 20 mM), followed by short sonication (amplitude 10%). Subsequently, equal volumes of DEX-NG dilutions and siRNA were mixed and incubated at 4°C for ≥ 15 min to allow complexation.

Natural lung-derived surfactant and anionic synthetic liposomes

Curosurf® (Poractant Alfa) is a product of Chiesi Pharmaceuticals (Parma, Italy). Infasurf® (Calfactant) was a kind gift of ONY, Inc. (Amherst, New York). Both surfactants differ in their method of extraction, which can change their overall proportion of fatty acids and surfactant proteins. Infasurf® is lipid extracted from calf lung lavage and Curosurf® is extracted from minced bovine lung, which leads to a lower concentration of hydrophilic surfactant proteins (SP-B and SP-C) and a depletion of cholesterol during production. A detailed comparison of lipid and protein compositions can be found in the literature [11, 20, 21].

Depending on the experimental needs, the surfactants were diluted in HEPES buffer or in Opti-MEM® (Invitrogen, Merelbeke, Belgium). Prior to use, Curosurf® and Infasurf® dispersions were homogenized by CapMix™ (3M ESPE, Seefeld, Germany) for 60 s.

1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and L-α-phosphatidylglycerol (egg, chicken) (egg:PG) were purchased from Avanti Polar Lipids, Inc (Alabaster, AL, USA). Anionic synthetic liposomes (ASLs) were prepared as follows. The lipids were dissolved in chloroform and mixed in a round bottomed flask. A lipid film was formed by evaporation of the chloroform under vacuum at 60 °C. The dried lipid film was hydrated in HEPES buffer resulting in a final lipid concentration of 10 mg/mL. Finally the formed liposomes were extruded 11 times through a polycarbonate membrane filter with 100 nm pores (Whatman, Brentfort, UK) at room temperature.

Dynamic light scattering (DLS) and zeta potential

Particle size and surface charge were determined by DLS and zeta potential measurements respectively, using a Zetasizer Nano ZS (Malvern, Worcestershire, UK), equipped with Dispersion Technology Software (DTS). The average values and the corresponding standard deviation (SD) were calculated from three measurements performed in HEPES buffer (20mM, pH 7.4).

To evaluate the size and surface charge of DEX-NGs as a function of the siRNA loading, DEX-NGs (20µg/mL) were incubated with sequential siRNA dilutions during 10 min at 4°C. Following, the different samples were measured at 25°C. To assess the influence of Curosurf® on the size and surface charge of siDEX-NGs, siDEX-NGs (2 pmol siRNA per µg DEX-NGs) were incubated with

different amounts of Curosurf® for 10 min at 4°C. This samples were subsequently measured at 37°C.

Polyacrylamide gel electrophoresis (PAGE)

The maximal loading capacity of the preformed DEX-NGs for siRNA was assessed by gel electrophoresis on a 20% non-denaturing polyacrylamide gel. Increasing concentrations of DEX-NGs were mixed with a fixed amount of siRNA (10 pmol). After 15 min incubation at 4°C, gel loading solution (Ambion, Lennik, Belgium) was added to each sample and this mixture was loaded onto a 20% non-denaturing polyacrylamide gel prepared in TBE buffer (10.8 g/L Tris base, 5.5 g/L boric acid, 0.74 g/L $Na_4EDTA.2H_2O$). Electrophoresis was performed at 100 V during 1.5 h. Afterwards the siRNA was visualized by incubation of the gel in $1/10^4$ SYBR Green II RNA staining dilution (Molecular Probes, Invitrogen) followed by UV transillumination and gel photography.

To evaluate the influence of natural lung-derived surfactants on siRNA complexation, siDEX-NGs (10 pmol siRNA/µg DEX-NGs) were incubated with the surfactants in HEPES buffer. After 10 min incubation at room temperature, gel loading solution was added. The samples were analyzed as described above.

Fluorescence fluctuation spectroscopy

Fluorescence fluctuation spectroscopy (FFS) monitors fluorescence intensity fluctuations in the excitation volume of a confocal microscope. The fluorescence fluctuations originate from the movement of fluorescent molecules in and out a fixed excitation volume. It can be used to evaluate the complexation of fluorescently labeled molecules [22]. The influence of natural

pulmonary surfactant on the complexation of siRNA with DEX-NGs (versus Lipofectamine RNAiMAX™) was examined. Equal volumes of Cy®5 labeled siRNA and DEX-NGs (or Lipofectamine RNAiMAX™) were gently mixed. After complexation, Curosurf® and Infasurf® dilutions were added. All samples were further diluted in HEPES buffer or Opti-MEM® after 10 min incubation and transferred to a glass-bottomed 96-well plate (Greiner Bio-one, certified DNase/RNase free). Subsequently, the focal volume of the microscope was positioned in the sample and the fluorescence fluctuations were recorded during a 30 s time interval. All samples were prepared in triplicate. The average fluorescence intensity of freely diffusing and complexed siRNA in the fluorescence fluctuation profile was determined as described previously [23]. FFS measurements were performed with a motorized Nikon TE2000-E inverted microscope equipped with a water immersion objective lens (Plan Apo 60X, NA 1.2, collar rim correction, Nikon), using a 637 nm laser line for the excitation of fluorescent siRNA. It was combined with the detection channels of the fluorescence correlation spectrometer MicroTime 200 (Picoquant GmbH, Berlin, Germany), equipped with SymPhoTime software.

Cell lines and culture conditions

Cell culture experiments were performed using human alveolar epithelial cells (H1299), that stably express the enhanced green fluorescent protein (H1299_EGFP), and murine alveolar macrophages (MH-S). H1299_EGFP cells are grown in RPMI 1640, supplemented with 2 mM glutamine, 10% heat-inactivated fetal bovine serum (FBS, Hyclone) and 100 U/mL penicillin/streptomycine at 37 °C in a humidified atmosphere containing 5% CO_2. The MH-S cell

culture medium is obtained by further addition of 0.05 mM 2-mercaptoethanol, 0.01 M HEPES and 1 mM sodium pyruvate .

Quantification of siRNA cellular internalization by flow cytometry

H1299_EGFP cells were plated in 24-well plates (1.75 x 10^4 cells per cm^2) and MH-S macrophages were seeded in 12-well plates (4 x 10^4 cells per cm^2) and allowed to attach overnight. According to the experimental needs, natural surfactants or ASLs were diluted in HEPES buffer and added to the DEX-NGs complexing red fluorescent siRNA (3.33 pmol siRNA per µg DEX-NGs; 0.75 mol% Cy®5 labeled). After 5-fold dilution in Opti-MEM®, the samples were brought onto the cells. Following 4 h incubation (37°C, 5% CO_2), the cells were washed with PBS and incubated during 5 min with dextran sulfate sodium salt (0.1 mg/mL) in PBS to remove surface bound fluorescence. Next, the cells were trypsinized (trypsin / EDTA 0.25 %) and diluted with 1 mL complete cell culture medium. Following centrifugation (7 min, 1500 rpm), the cell pellet was resuspended in 250 µL flow buffer (PBS supplemented with 1% BSA and 0.1% sodium azide) and placed on ice until flow cytometric analysis. A minimum of 10^4 cells was analyzed in each measurement, using a BD Biosciences FACSCalibur™ flow cytometer. The fluorescence detector for Cy®5 labeled siRNA detects wavelengths of 661nm (±16nm). Data analysis was performed by using the BD CellQuest Pro analysis software.

Additionally, the cellular uptake of siRNA mediated by the lipid based siRNA transfection reagent Lipofectamine RNAiMAX™ (Invitrogen) was analyzed. H1299_EGFP cells were plated in 24-well plates (1.75 x 10^4 cells per cm^2). Cy®5 labeled siRNA was diluted in Opti-MEM® and gently mixed with equal volumes Lipofectamine RNAiMAX™ (6 pmol siRNA per µL

Lipofectamine RNAiMAX™). After 15 min incubation at room temperature, natural surfactants diluted in Opti-MEM® were added and incubated for 10 min at room temperature. Cells were transfected and analyzed by flow cytometry as described above.

Visualization of siRNA cellular internalization by confocal microscopy

H1299_EGFP cells and MH-S cells were seeded in sterile, glass-bottomed 8-well Lab-Tek Chamber Slides™ (Thermo Fischer Scientific, Ghent, Belgium) at a density of 1.25 x 10^4 and 1.88 x 10^4 cells per cm^2 respectively and allowed to attach overnight. DEX-NGs loaded with Cy®5 labeled siRNA (3.33 pmol siRNA per µg siDEX-NGs) and incubated with natural surfactants (20 mg per mg DEX-NGs) were diluted in Opti-MEM® and incubated with the cells for 4 h (37°C, 5% CO_2). Subsequently, cells were incubated with 0.1 mg/mL dextran sulfate sodium salt in PBS (5 min) and washed with PBS. Next, cells were fixed with a 4% paraformaldehyde solution in PBS for 20 min at room temperature. After fixation, the cells were washed twice with PBS and mounted with VectaShield® Mounting Medium containing DAPI (Vector Laboratories, Brussels, Belgium). The cellular internalization of fluorescent labeled siRNA was visualized with a Nikon C1si confocal scanning module installed on a motorized Nikon TE2000-E inverted microscope (Nikon Benelux, Brussels, Belgium), equipped with a 60x oil objective lens (Plan Apo 60x, NA 1.2, collar rim correction, Nikon), using a 408 nm and 637 nm laser line for the excitation of DAPI and Cy®5 labeled siRNA, respectively.

Gene silencing

H1299_EGFP cells were plated in 24-well plates (1.75 x 10^4 cells per cm^2) and transfected 24 h later with DEX-NGs complexing 3.33 pmol siRNA per µg DEX-NGs, prepared as described above.

Alternatively, siRNA was complexed with Lipofectamine RNAiMAX™ according to manufacturer's instructions (6 pmol siRNA per µL Lipofectamine RNAiMAX™). The sequence of the active siRNA was designed to selectively reduce EGFP expression. siRNA induced EGFP suppression was normalized to the EGFP gene silencing due to an negative control siRNA sequence (siCTRL). After 4 h incubation (37°C, 5% CO_2), the cells were washed with PBS and incubated in 500 µL cell culture medium for 48 h. At this time point, maximal gene silencing was obtained. Further cell sample preparation and flow cytometric analysis was performed as described above. The fluorescence detector for EGFP detects wavelengths of 530nm ±30nm.

MH-S cells were plated in 12-well plates (4 x 10^4 cells per cm^2). After incubation overnight, the cells were treated during 4 h (37°C, 5% CO_2) with DEX-NGs loaded with 3.33 pmol siRNA per µg DEX-NGs targeting the CD45 gene, washed with PBS and incubated in cell culture medium (1 mL) for 48 h. For flow cytometric analysis, the cells were detached with a non-enzymatic cell dissociation buffer (10 min incubation at 37°C, 5% CO_2). After centrifugation (7 min, 1500 rpm), the cell pellet was diluted in 1 mL ice-cold staining buffer (PBS supplemented with 3% FBS and 0.1% sodium azide) and placed on ice for 30 min to block aspecific binding sites for the CD45 antibody. Subsequently, the cells were incubated with PerCP-Cy™ 5.5 Rat Anti-Mouse CD45 (BD Biosciences, Erembodegem, Belgium) diluted in staining buffer (0.008 µg/µL) during 45 min on ice. Following three washing steps with 1 mL ice-cold staining buffer, the cell pellet was re-suspended in 250 µL ice-cold staining buffer and analyzed with flow cytometry as described above.

RESULTS

Physicochemical characterization of siRNA loaded dextran nanogels (siDEX-NGs)

To successfully deliver siRNA across the plasma membrane into the cytosol, a good complexation of the negatively charged siRNA to DEX-NGs is required. To evaluate this, siRNA was incubated with sequential DEX-NG dilutions and loaded on a polyacrylamide gel. Figure 1A demonstrates a complete complexation of siRNA between 50 and 33.3 pmol siRNA per µg lyophilized DEX-NGs, which corresponds to a loading capacity of ≥ 50 wt%. Next, the changes in size and zeta potential of the DEX-NGs upon the addition of siRNA were evaluated (Figure 1B). DEX-NGs are characterized by a hydrodynamic diameter (d_H) of 184.4 ± 0.8 nm and a surface charge of 25.1 ± 0.9 mV. The addition of increasing amounts of siRNA gradually decreases the surface charge of DEX-NGs. Aggregation, as a result of neutralization of the DEX-NGs, occurred at an siRNA loading of 30 pmol siRNA per µg DEX-NGs. Note that further addition of siRNA results in negatively charged particles that have the same d_H as the original unloaded DEX-NGs. These data are in agreement with the observations in previous work by Raemdonck et al [17]. All together, to ensure complete loading of the siRNA and to obtain stable cationic nanoparticles after siRNA complexation, a siRNA loading < 20 pmol per µg lyophilized DEX-NGs was used in further experiments.

Influence of natural lung-derived surfactants on physicochemical characteristics of siDEX-NGs

To examine the interaction of pulmonary surfactant with siDEX-NGs, two commercially available surfactants were used, i.e. Curosurf® and Infasurf®. Prior to their use, the surfactant dispersions

were homogenized at high speed (CapMix™) leading to the formation of surfactant vesicles with a d_H ~ 120 nm and a zeta potential ~ -30 mV. The effect of both surfactants on the physicochemical characteristics of the nanogels was examined. The addition of Curosurf® reverses the net surface charge of siDEX-NGs from positive to negative (Figure 2A). Neutralization of the surface charge (~2 mg Curosurf® per mg DEX-NGs) leads to colloidal instability as observed by the emergence of micrometer aggregates. Starting from a 5-fold weight excess of surfactant to DEX-NGs, stable nanosized particles are detected with a marked increase in hydrodynamic diameter (d_H = ~400 nm). Comparable results were obtained when incubating siDEX-NGs with Infasurf® (data not shown).

The surface charge reversal of siDEX-NGs indicates a direct (electrostatic) interaction between the surfactant vesicles and the nanogel surface. To assure that this interaction does not cause a release of siRNA from siDEX-NGs, gelelectrophoresis and FFS were performed on the nanogels after addition of Curosurf® or Infasurf®. Figure 2B presents that even a high excess of Curosurf® does not interfere with the siRNA complexation as measured by PAGE. Figure 2C shows that these results can be confirmed by FFS. The same result was observed for Infasurf® (evaluated up to 15 mg per mg DEX-NGs, data not shown).

Assessing the influence of natural surfactants on cellular uptake of siDEX-NGs

The impact of Curosurf® and Infasurf® on the cellular uptake of siDEX-NGs in lung epithelial cells (H1299_EGFP) and alveolar macrophages (MH-S) was evaluated by flow cytometry and confocal laser scanning microscopy. For this purpose, DEX-NGs were loaded with red fluorescent siRNA (Cy®5 labeled) and incubated with the cells. Fluorescent particles bound to the surface of the

cells were removed by an incubation step with dextran sulfate (0.1 mg/mL in PBS) [24]. Flow cytometric quantification of the intracellular fluorescent signal is shown in Figure 3A. Incubating the siDEX-NGs with Curosurf® significantly lowers the cellular internalization (~65% drop in mean fluorescence intensity) which is even more pronounced in the case Infasurf® is used (~92.5%). Figure 3B shows the corresponding fluorescence micrographs of internalized siDEX-NGs in both cell lines. These confocal images support the data obtained by flow cytometry and affirm that both Curosurf® and Infasurf® strongly diminish the cellular uptake of siDEX-NGs. It was verified that addition of surfactant did not interfere with the fluorescence signal as such (data not shown).

RNAi effect of siDEX-NGs before and after addition of natural surfactants

The gene silencing potential of siDEX-NGs has been demonstrated in a human hepatoma cell line (Huh-7) in earlier work [17]. Here we aimed to confirm the RNAi activity of siDEX-NGs in the H1299_EGFP cells. The sequence of the active siRNA was designed to selectively reduce EGFP expression. A negative control siRNA sequence was used in the experiment to calculate percentage EGFP expression (Figure 4). It is demonstrated that the maximal gene silencing is obtained at a DEX-NG concentration of ≥25 μg/mL. In MH-S cells the same DEX-NG concentration leads to a maximal gene silencing when formulated with siRNA targeting the CD45 gene (data not shown). Hence, to ensure an optimal RNAi effect all subsequent gene silencing experiments are conducted at a DEX-NG concentration ≥ 25 μg/mL.

Next, the gene silencing of the siDEX-NGs after interaction with Curosurf® or Infasurf® was quantified. H1299_EGFP and MH-S cells were transfected with DEX-NGs loaded with siRNA

targeting the EGFP and CD45 gene respectively. Figure 5A clearly shows that the gene suppression in both cell lines is maintained after addition of the surfactants. In the H1299 epithelial cell line even a significant increase in gene silencing is observed after interaction with Curosurf®. It was verified that the addition of Curosurf® did not cause unspecific inhibition of EGFP expression (data not shown). Silencing of the pan-leucocytic marker CD45 in the alveolar macrophage cell line was less pronounced, a result that may be linked to macrophages being notoriously difficult to transfect [25]. Nonetheless, the interaction of siDEX-NGs with either pulmonary surfactants again did not impede the RNAi effect. The influence of Curosurf® on EGFP gene silencing was also evaluated in a siRNA dose-response experiment (Figure 5B). Even at a suboptimal amount of siRNA, Curosurf® does not reduce EGFP knockdown efficiency resulting in a comparable IC_{50} value ~ 3 pmol/mL under the experimental conditions. It is important to note that the incubation of the H1299_EGFP cells with a mixture of uncomplexed siRNA and Curosurf® failed to elicit any EGFP gene silencing as opposed to the high gene suppression obtained with siDEX-NGs and Curosurf® (data not shown). This indicates that the complexation of siRNA into cationic nanoparticles is required for gene silencing.

Interaction of natural surfactant with anionic siDEX-NGs

It was shown in Figure 1B that the addition of increasing siRNA concentrations reverses the net surface charge of DEX-NGs. Therefore, we also assessed the influence of the siDEX-NG surface charge on the cellular uptake and gene silencing potential in the presence of lung surfactant. H1299_EGFP cells were incubated with DEX-NGs loaded with 4 pmol siRNA or 40 pmol siRNA per µg NG resulting in stable cationic and anionic nanoparticles respectively

(Figure 1B). As expected, negatively charged nanogels are less efficiently internalized (Figure 6A), leading to

reduced EGFP knockdown (Figure 6B). Interestingly, although siRNA internalization was even further decreased, pre-incubation of anionic siDEX-NGs with natural lung-derived surfactant could almost completely recover the gene silencing potential.

Effect of synthetic ternary phospholipid mixture on biological activity of siDEX-NGs

In an effort to gain additional insight in the activity of the pulmonary surfactant, we examined the impact of a simple protein-free model system, i.e. ASLs, consisting of DPPC, POPC and egg:PG (50:35:15 wt%). The influence of these ASLs on the efficacy of siDEX-NGs was compared with the effect of Curosurf®. Figure 7 depicts the flow cytometric data of the cellular internalization (A-B) and gene silencing (C-D) experiments in both lung epithelial and alveolar macrophage cells. The data illustrate that Curosurf® and ASLs both markedly reduce the cellular uptake of the Cy®5 labeled siDEX-NGs. ASLs also reverse the surface charge of cationic siDEX-NGs (data not shown), thereby likely hampering the interaction with the cell membrane. More importantly, the decreased cellular uptake, following ASLs interaction results in a strong inhibitory effect on the gene silencing potential of siDEX-NGs, in contrast to pulmonary surfactant.

Effect of natural surfactants on cellular uptake and gene silencing of siRNA lipoplexes

Besides polymeric nanoparticles, many siRNA nanoformulations under (pre-)clinical evaluation are based on cationic liposomes [26]. To test the effect of pulmonary surfactant on the cellular

uptake and efficacy of siRNA lipoplexes (siLPXs) we formulated respectively Cy®5 labeled and anti-EGFP siRNA in Lipofectamine™ RNAiMAX LPXs and incubated the particles with increasing amounts of Curosurf®. The data depicted in Figure 8 (A-B) show that both Curosurf® and Infasurf® completely block the cellular internalization of siRNA by the lipoplexes in H1299_EGFP cells. In stark contrast with the result s obtained for siDEX-NGs, the gene silencing capacity of siLPXs was lost already at low concentrations of pulmonary surfactant. These data corroborate earlier reports in the literature stating significantly hindered lipid-mediated gene transfer in the presence of pulmonary surfactant [15]. It is critical to relate the influence of the surfactants on the siRNA complexation with DEX-NGs to their influence on the siRNA complexation with cationic liposomes (Lipofectamine RNAiMAX™). As can be seen in Figure 8C, adding increasing surfactant concentrations to siLPXs results in a complete release of the encapsulated siRNA. This is in stark contrast with the observations for siDEX-NGs, where no siRNA dissociation could be observed following incubation with pulmonary surfactant.

DISCUSSION

Topical administration of siRNA nanocarriers is a promising approach in the treatment of pulmonary disorders. However, an efficient siRNA delivery vehicle should still overcome the various extra- and intracellular barriers encountered following inhalation therapy [27]. Pulmonary surfactant will be one of the first interfaces that drug loaded nanoparticles encounter after topical administration. Therefore, it is of great importance to assess the impact of pulmonary surfactant on the performance of nucleic acid nanocarriers.

In this study, the effect of pulmonary surfactant on the siRNA delivery capacity of DEX-NGs to lung epithelial cells and alveolar macrophages was evaluated. These cell types form the primary entry point for respiratory pathogens, are affected in several lung diseases and contribute to the underlying inflammation in e.g. cystic fibrosis, asthma and chronic obstructive pulmonary disease, which makes them attractive targets for RNAi therapy [7, 27].

Consistent with our earlier reports, DEX-NGs display a high loading capacity for siRNA (Figure 1) and are able to induce a marked RNAi effect in both pulmonary cell lines (Figure 4-5). However, the interaction with lung surfactant decreases the cellular internalization of siDEX-NGs significantly (Figure 3). This effect can be attributed to the influence of pulmonary surfactants on the net surface charge of siDEX-NGs. Pulmonary surfactants have an overall negative surface charge, due to the presence of anionic phospholipids, including phosphatidylglycerol and phosphatidylinositol (8-15 wt% of total phospholipids) [11]. In this way, the addition of lung surfactant reverses the net surface charge of siDEX-NGs from positive to negative (Figure 2). This will lower the interaction of the nanocarriers with the negatively charged cell surface and thereby decrease the cellular deposition of siRNA. Also, pre-incubation of pulmonary surfactant

increases the d_H of siDEX-NGs (Figure 2), which can have a considerable influence on the mechanism and kinetics of endocytic uptake.[28, 29].

Remarkably, the RNAi effect of siDEX-NGs after interaction with Curosurf® or Infasurf® in both cell lines was maintained. Moreover, incubation of siDEX-NGs with Curosurf® induced a stronger knockdown in alveolar epithelial cells (Figure 5) and was able to recover the gene silencing potential of anionic siDEX-NGs, although cellular uptake remained compromised (Figure 6). These data support the hypothesis that the surfactants may influence the intracellular trafficking of siDEX-NGs, thereby possibly facilitating the cytosolic siRNA delivery [30]. Kissel and co-workers also recently reported on the interaction of a natural lung-derived surfactant (Alveofact™) with polymeric nucleic acid nanoparticles in a lung epithelial cell line [31, 32]. In contrast to our data, they observed an enhanced cellular uptake of pDNA [31] or siRNA [32] after addition of the surfactant. This effect was attributed to hydrophobic interactions or a membrane fusion process stimulated by the presence of phospholipids on the nanoparticle surface [32]. It is important to note that these authors did not report a surface charge reversal of their nanocarrier following surfactant interaction. The influence of Curosurf® and Infasurf® on the intracellular fate of polymeric nanoparticles however still remains an open question and requires further investigation.

ASLs that consist of the most abundant phospholipids in pulmonary surfactant [11] but lack surfactant proteins, also significantly blocked the cellular internalization of siDEX-NGs. In contrast to Curosurf®, the interaction of siDEX-NGs with ASLs additionally inhibited their gene silencing activity (Figure 7). Likely the complex composition of pulmonary surfactant is of significant importance to the observed effects.

Curosurf® and Infasurf® both contain predominantly phospholipids, whose primary component is 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), with a minor fraction of hydrophobic surfactant proteins. The hydrophilic surfactant proteins present in native surfactant, SP-A and SP-D, are removed due to their role in the innate immune response [12]. It is important to consider that SP-A, which is the major protein component in native lung surfactant, can stimulate cellular binding and uptake in alveolar macrophages [33]. Furthermore, it plays an important role in the surfactant recycling by type II alveolar epithelial cells. The binding of pulmonary surfactant to type II cells is mediated by a SP-A specific cell surface receptor (P63) [34]. To elucidate the potential role of the hydrophilic surfactant proteins in the efficacy of siRNA loaded nanocarries, further experiments are required.

It is believed that pulmonary surfactant poses a critical barrier for lipid based nucleic acid nanocarriers, making them unlikely candidates for lung delivery. Also in our hands, cationic lipoplexes (siLPXs) were unable to transfect lung epithelial cells after incubation with pulmonary surfactant. The components of pulmonary surfactant are thought to mix with the lipid constituents of the lipoplexes, resulting in a complex with a suboptimal composition that is unable to transfect the target cells efficiently [14]. An alternative explanation is that the surfactants compete with the lipoplexes for cell-surface binding [9, 14, 15]. However, our experimental results clearly indicate that lipid mixing of siLPXs with pulmonary surfactant entails a quantitative release of the encapsulated siRNA (Figure 8C) and induces the formation of large aggregates (data not shown), which was not yet demonstrated previously.

Altogether, we have experimental evidence that pulmonary surfactant may potentiate polymeric siRNA delivery, thereby possibly providing new therapeutic opportunities for co-

administration of polymeric siRNA nanocarriers and pulmonary surfactant for the treatment of pulmonary diseases with underlying surfactant dysfunction. In addition, endogenous surfactant function is often impaired in the inflamed airways [35, 36], suggesting that instillation of exogenous surfactant may have additional therapeutic benefits. Exogenous surfactant replacement therapy has already evolved to a standard therapeutic intervention for neonatal respiratory distress syndrome [37] and positive effects in the treatment of acute respiratory distress syndrome in adults have been described [38]. Further research must be pursued in order to understand how surfactant replacement could benefit a wider range of lung diseases.

CONCLUSIONS

In the context of developing an efficient nanocarrier for pulmonary delivery, the compatibility of siDEX-NGs with natural pulmonary surfactant was investigated. The results presented in this paper indicate that the interaction with natural pulmonary surfactant does not impede the gene silencing potential of siDEX-NGs in H1299 or MH-S cells, in contrast to a lipid based siRNA nanocarrier. The observations detailed above could therefore have important implications for therapeutic pulmonary administration of siRNA nanocarriers.

EXECUTIVE SUMMARY

- Cationic biodegradable dextran nanogels (DEX-NGs) are promising carriers for siRNA delivery since they can be loaded efficiently with siRNA, they are taken up by cells *in vitro*, and they are able to induce a marked RNA interference (RNAi) effect.
- The interaction of siRNA loaded DEX-NGs (siDEX-NGs) with natural pulmonary surfactant, Curosurf® or Infasurf®, decreases the cellular internalization in a lung epithelial (H1299_EGFP) and alveolar macrophage (MH-S) cell line significantly.
- Remarkably, the RNAi effect of siDEX-NGs after interaction with Curosurf® or Infasurf® in both H1299_EGFP and MH-S cell lines was maintained.
- The cellular internalization of siRNA is strongly decreased for anionic siDEX-NGs, likely due to the negative surface charge, leading to a substantial inhibition of gene suppression. Remarkably, after the addition of Curosurf®, the gene silencing potential was partially recovered.
- Anionic synthetic liposomes (ASLs), that consist of the most abundant phospholipids in pulmonary surfactant, have a comparable effect as Curosurf® on the cellular internalization of siDEX-NGs. But in contrast to Curosurf®, ASLs almost completely abrogate the gene silencing activity of siDEX-NGs.
- Interestingly, lipid based siRNA nanocarriers (Lipofectamine RNAiMAX™) seem to be incompatible with pulmonary surfactants.

FUTURE PERSPECTIVES

The interaction of a promising siRNA carrier with an important extracellular barrier in the lung is described here. Experimental evidence that pulmonary surfactant potentiates siRNA delivery by dextran nanogels is shown. We anticipate that our approach may provide new therapeutic opportunities for co-administration of polymeric siRNA nanocarriers and pulmonary surfactant for the treatment of pulmonary diseases with underlying surfactant dysfunction. The evaluation of the *in vivo* performance of this formulation in validated animal models is scheduled for the near future.

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(*) **Davidson BL, Mccray PB, Jr.: Current prospects for RNA interference-based therapies. Nature reviews. Genetics 12(5), 329-340 (2011)**

A comprehensive review on RNA interference and its possibilities in the clinic.

(**) **Merkel OM, Kissel T: Nonviral Pulmonary Delivery of siRNA. Acc Chem Res, 45, 961-70 (2012).**

A recent review on pulmonary siRNA delivery. It describes the different biological barriers that various siRNA formulations encounter upon topical administration.

(*) **Ernst N, Ulrichskotter S, Schmalix WA et al.: Interaction of liposomal and polycationic transfection complexes with pulmonary surfactant. Journal of Gene Medicine 1(5), 331-340 (1999).**

A report highlighting the different influence of exogenous pulmonary surfactant on polyfection and lipofection.

(**) **Raemdonck K, Naeye B, Buyens K et al.: Biodegradable Dextran Nanogels for RNA Interference: Focusing on Endosomal Escape and Intracellular siRNA Delivery. Adv Funct Mater 19(9), 1406-1415 (2009).**

Describes the preparation of biodegradable cationic dextran based nanogels, that are promising carriers for siRNA.

(**) **Zuo YY, Veldhuizen RA, Neumann AW, Petersen NO, Possmayer F: Current perspectives in pulmonary surfactant--inhibition, enhancement and evaluation. Biochim Biophys Acta 1778(10), 1947-1977 (2008).**

An important review on the biophysical properties of pulmonary surfactant.

(*) **Benfer M, Kissel T: Cellular uptake mechanism and knockdown activity of siRNA-loaded biodegradable DEAPA-PVA-g-PLGA nanoparticles. Eur J Pharm Biopharm, (2011).**

Demonstrates the improved gene silencing of siRNA loaded polymeric nanoparticles (DEAPA-PVA-g-PLGA) after interaction with exogenous pulmonary surfactant (Alveofact®).

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FIGURES

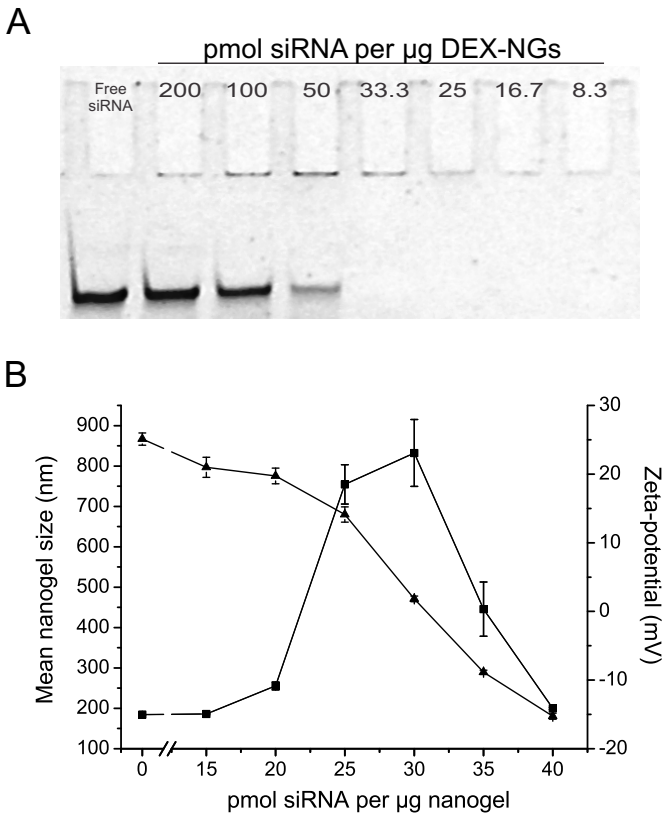

Figure 1: Evaluation of the siRNA loading in dextran nanogels (DEX-NGs). (**A**) Polyacrylamide gel electrophoresis on siRNA loaded DEX-NGs. (**B**) Size (—■—) and zeta potential (—▲—) of DEX-NGs (20 µg/mL) as a function of siRNA loading. The measurements were performed at 25°C in HEPES buffer 20 mM, pH 7.4. Data points represent average values ± SD.

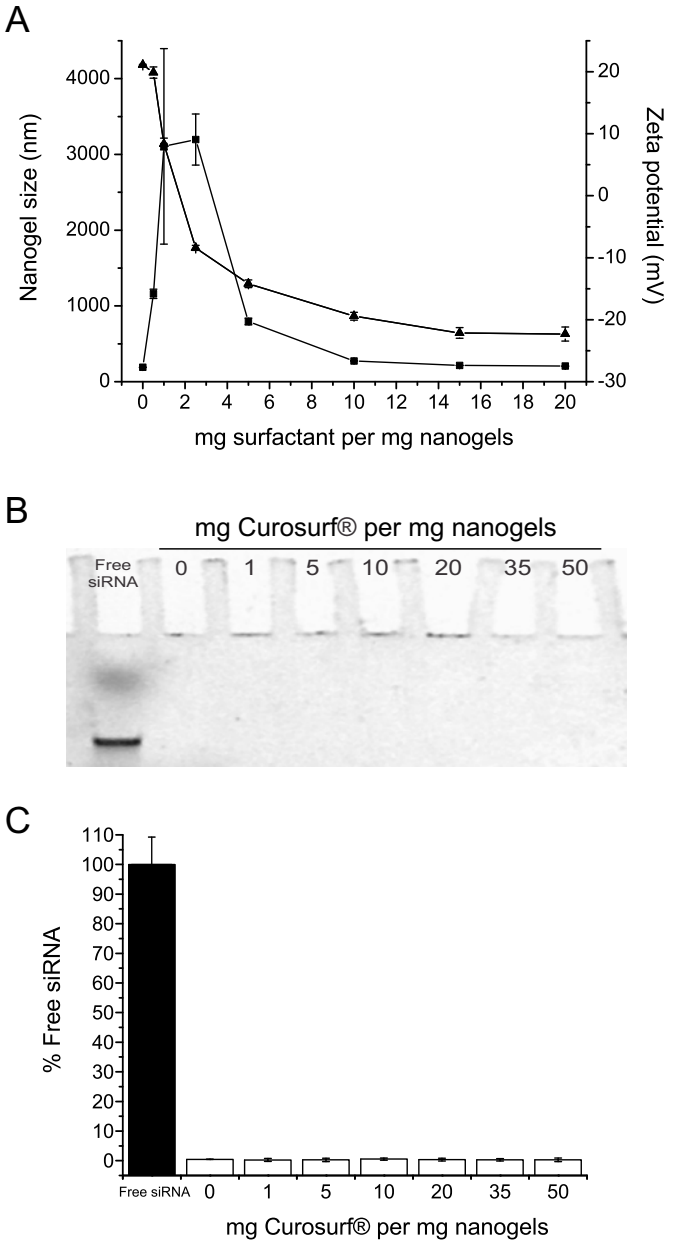

Figure 2: Incubation of siRNA loaded dextran nanogel (siDEX-NG) dispersion with natural lung-derived surfactant. (**A**) Size (—■—) and zeta potential (—▲—) of siDEX-NGs (2 pmol siRNA per µg DEX-NGs) after the addition of Curosurf® was measured by dynamic light scattering at 37°C in HEPES buffer 20 mM, pH 7.4. Data points represent average values ± SD. (B) Polyacrylamide gel electrophoresis on siDEX-NGs (10 pmol siRNA per µg DEX-NGs) following incubation with increasing amounts of Curosurf®. (**C**) Percentage of free siRNA (Cy®5 labeled), dissociated from siDEX-NGs (10 pmol siRNA per µg DEX-NGs) as a function of the Curosurf® concentration; measured by fluorescence fluctuation spectroscopy (FFS) in HEPES buffer. The concentration of fluorescent siRNA equaled 25 pmol/mL in all samples. Data points represent mean ± SD (n=3).

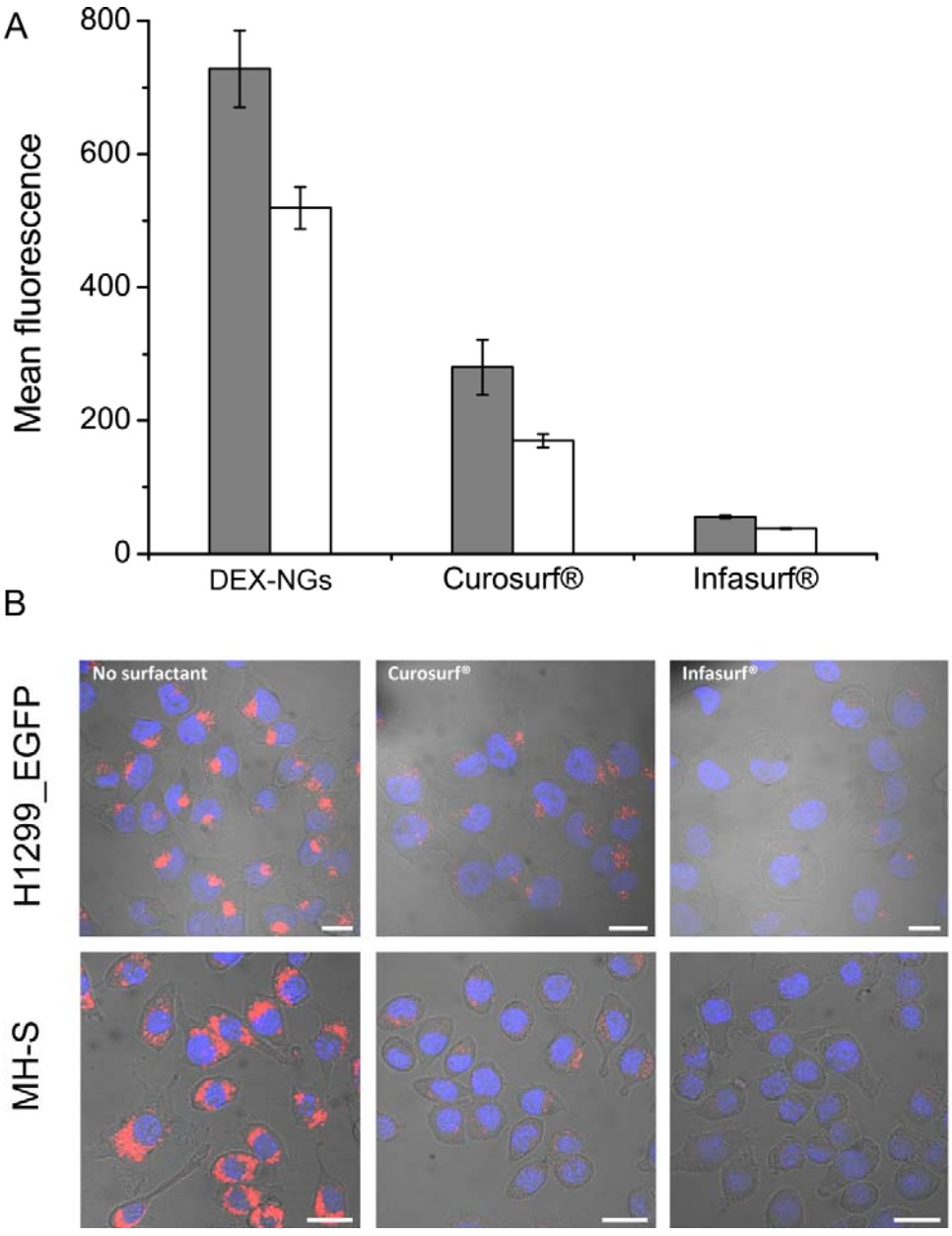

Figure 3: (**A**) Evaluation of the intracellular uptake of dextran nanogels (DEX-NGs) loaded with fluorescent siRNA by flow cytometry in H1299_EGFP cells (gray bars) and in MH-S cells (white bars) after incubation with Curosurf® and Infasurf®. (**B**) Confocal images of DEX-NGs in H1299_EGFP and MH-S cells. The DEX-NGs are red fluorescent as they are loaded with Cy®5 labeled siRNA. Cell nuclei appear blue by staining with DAPI. Scale bar corresponds with 20 µm.

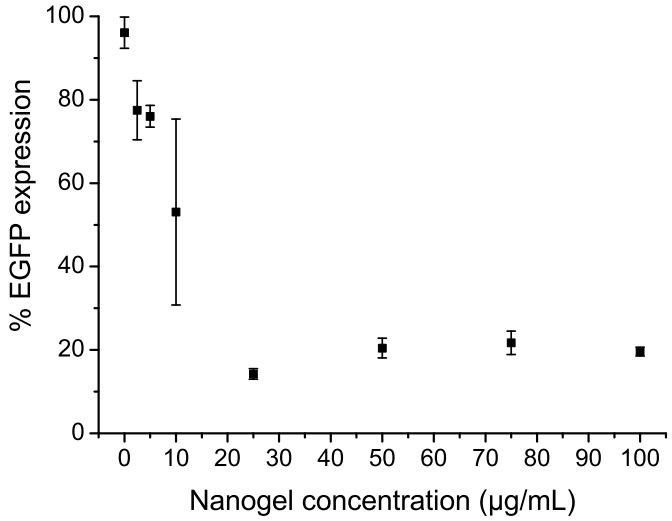

Figure 4: EGFP gene silencing with increasing concentrations of dextran nanogels (DEX-NGs) loaded with 30 pmol siCTRL or siEGFP (24-well plate). 100% indicates the EGFP expression of cells treated with DEX-NGs loaded with an negative control siRNA sequence (siCTRL). Data points represent average values ± SD.

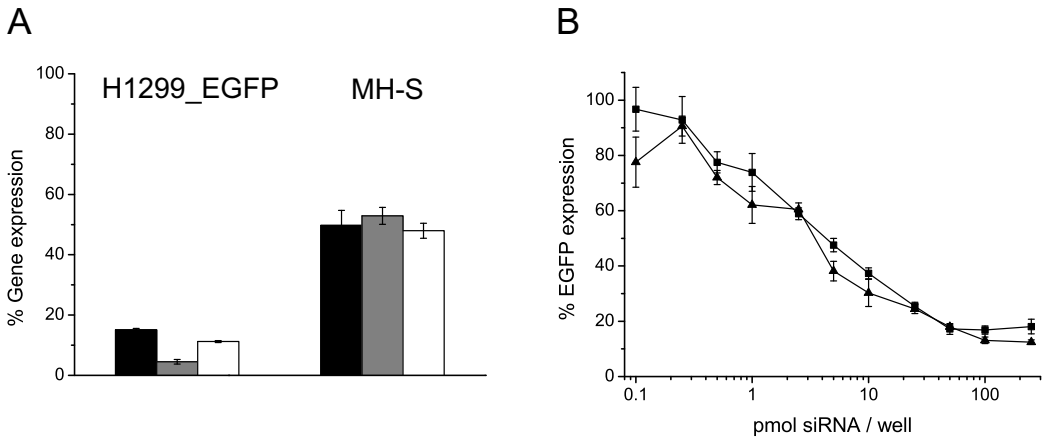

Figure 5: Analysis of the gene silencing potential of siRNA loaded dextran nanogels (siDEX-NGs) with flow cytometry. (**A**) EGFP and CD45 gene silencing in the H1299_EGFP cells and MH-S cells respectively. Curosurf® (gray bars) and Infasurf® (white bars) were incubated with the siDEX-NGs in a ratio of 15 mg surfactant per mg DEX-NGs. The gene expression for siDEX-NGs without the addition of surfactants is illustrated with the black bars. Every well contained 30 pmol (24-well plate) and 125 pmol (12-well plate) siRNA for the H1299_EGFP cells and MH-S cells respectively. (**B**) EGFP gene silencing by siDEX-NGs as a function of siRNA amount with (—▲—) and without (—■—) the addition of Curosurf® (20 mg per mg DEX-NGs).

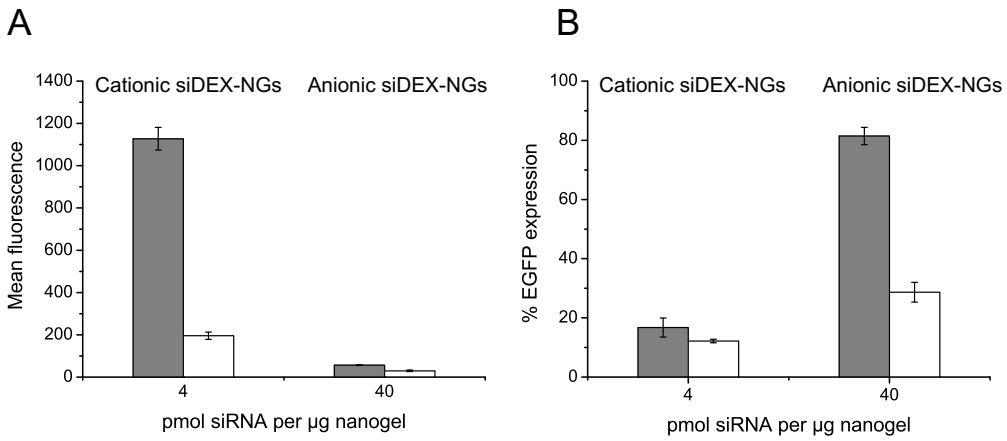

Figure 6: (**A**) Cellular uptake and (**B**) EGFP silencing of cationic siRNA loaded dextran nanogels (siDEX-NGs) (loaded with 4 pmol siRNA per µg DEX-NGs) and anionic siDEX-NGs (loaded with 40 pmol siRNA per µg DEX-NGs) in the absence (gray bars) and presence of Curosurf® (white bars). Transfection experiments were performed with a fixed concentration of DEX-NGs (25 µg/mL). Data points represent average values ± SD.

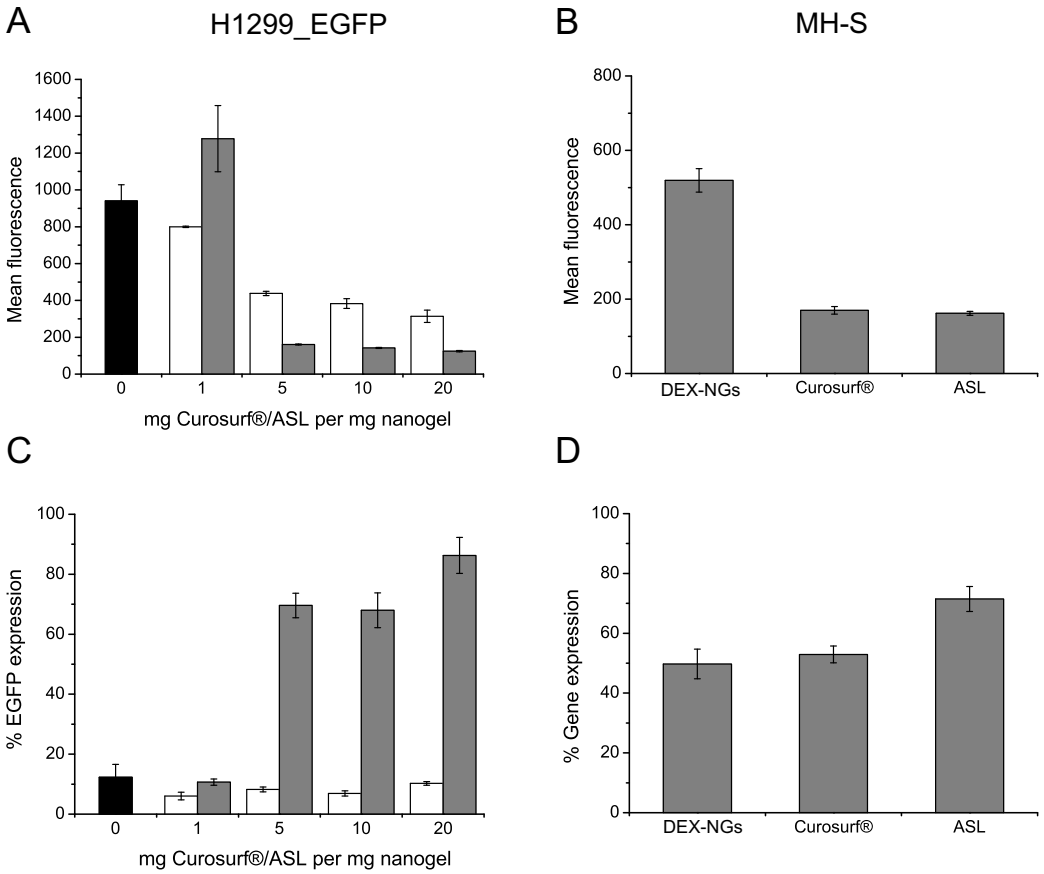

Figure 7: (**A**) Cellular internalization and (**C**) gene silencing potential of the siRNA loaded dextran nanogels (siDEX-NGs) as function of the added Curosurf® (white bars) and anionic synthetic liposomes (ASLs) (gray bars) concentration in H1299_EGFP cells. (**B**) Cellular internalization and (**D**) gene silencing potential in MH-S cells that were incubated with siDEX-NGs, after interaction with 15 mg per mg Curosurf® or ASLs. Every well contained 30 pmol siRNA (H1299_EGFP; 24-well plate) or 125 pmol siRNA (MH-S; 12-well plate). Data points represent mean ± SD.

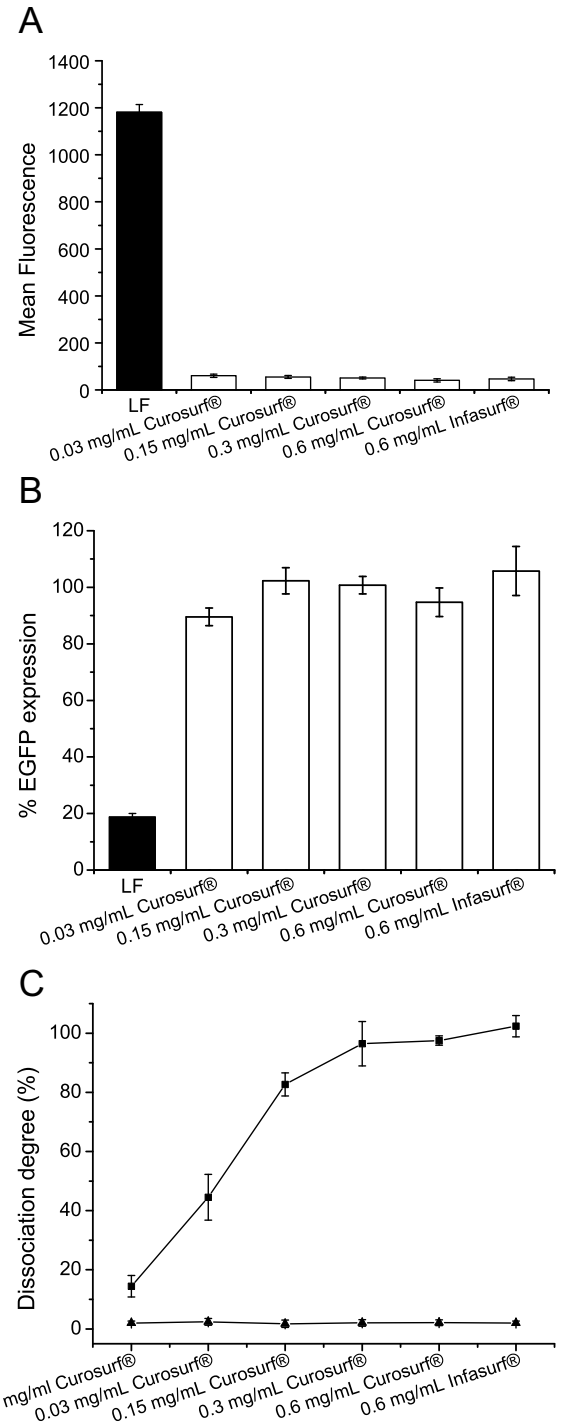

Figure 8: (**A**) Cellular internalization and (**B**) gene silencing potential of siRNA complexed with Lipofectamine™ RNAiMAX after interaction with Curosurf® or Infasurf® in H1299_EGFP. Every well contained 15 pmol siRNA. Data points represent mean ± SD. (**C**) Comparison of the dissociation degree of fluorescent siRNA (Cy®5 labeled) complexed with Lipofectamine™ RNAiMAX (—■—) or DEX-NGs (—▲—) in Opti-MEM® as determined by fluorescence

fluctuation spectroscopy (FFS). The concentration of fluorescent siRNA equaled 25 pmol/mL in all samples. Data points represent mean ± SD (n=3).