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Research paper

Solid lipid nanoparticles can effectively bind DNA, streptavidin and biotinylated ligands

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Abstract

Cationic solid lipid nanoparticles (SLN) have recently been suggested for non-viral gene delivery, as these particles consist of well tolerated substances, can bind DNA directly via electrostatic interactions and mediate gene transfer in vitro. We here report the development of SLN complexes, which can be targeted to specific surface receptors. A formulation of SLN was prepared by the microemulsion technique comprising of stearylamine and the matrix lipid Compritol ATO 888 with a size of approximately 100 nm and a zeta-potential of +15. These SLN are able to condense DNA in complexes, which are very stable under physiological conditions, and they display low cytotoxicity in cell culture. In addition to binding of DNA, the SLN can simultaneously bind substantial amounts of streptavidin directly via electrostatic interactions. The SLN:DNA:streptavidin complexes are stable and are capable of binding biotinylated ligands, which can interact with surface receptors. This method allows for development of a fast and simple method of preparing a targeted non-viral gene therapy vector.

Keywords: Solid lipid nanoparticles; SLN; Cancer gene therapy; Targeted delivery; Streptavidin

1. Introduction

Several types of approaches have been attempted for development of drug delivery systems for genes including both viral and non-viral approaches. Apart from the many undesirable effects of most of these gene therapy vectors, such as immunogenic effects, toxicity, rapid clearance, trapping in specific organs etc., one of the major disadvantages of most formulations is that they confer no cellular specificity and have to be applied locally. For disseminated cancer diseases, such as many cancer forms, treatment must be administered systemically and therefore must be targeted to the cancer cells. Many efforts have been made for preparing targeted non-viral gene therapy vectors based on liposomes including cationic lipids (reviewed in [1]) or polyplexes, such as polyethyleneimine (PEI) (reviewed in [2]).

In the recent years, solid lipid nanoparticles (SLN) have been developed as potential carriers for a number of drugs (reviewed in [3,4]). SLN usually consists of physiologically well-tolerated ingredients already approved for pharmaceutical application in humans, can readily be produced in large scale, have good storage capabilities including freeze-drying, can be sterilised and show low cytotoxicity, when injected intravenously [3,5–7]. In addition, the advantage of SLN is that the charge of the particles can be modulated via the composition, thus allowing binding of oppositely charged molecules via electrostatic interactions. SLN can be produced in nano-scale size (100–200 nm), wherefore the particles are sufficiently small to traverse the microvascular system and prevent macrophage uptake and are therefore particularly suitable for systemic delivery.

There are only a few reports of the use of SLN for delivery of genes [8–11]. Olbrich et al. [8] prepared several SLN compositions by the high-pressure homogenisation method with positive ζ-potentials, but found only one composition, which could bind DNA and mediate expression of a reporter gene in cell culture. However, the transfection efficiency was extremely low compared to conventional polyplex transfection

Abbreviations BSA, bovine serum albumin; EGF, epidermal growth factor; EGFP, enhanced green fluorescent protein; PBS, phosphate buffered saline; PEI, polyethyleneimine; SLN, solid lipid nanoparticles.

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with poly-*L*-Lysine (P-*LL*) or PEI. This and other formulations were recently further tested by Tabatt et al. [9,11] and several formulations were demonstrated to have low cellular toxicity and bind significant amounts of DNA, but the transfection efficiencies remained very low compared to conventional agents. The transfection efficiency was significantly improved in vitro by pre-complexing the DNA with a dimer of the HIV-1 TAT-peptide, which contains a cell penetrating domain for improvement of cellular uptake and a nuclear localisation sequence for translocation of the DNA into the nucleus [10]. Although this SLN complex could not mediate substantial expression in vivo application, it may prove a potential method for administration of a therapeutic gene in a local environment, such as the pulmonary tract or for solid tumours.

The aim of the present study was to develop a SLN vector, which was able to complex with DNA, confer low or no transfection efficiency per se, but be able to be coupled to a ligand for targeted uptake via internalising receptors. We here report the production of an SLN formulation prepared by the microemulsion technique, which in addition to binding of significant amounts of DNA also could bind streptavidin in a stable complex. This SLN complex could bind directly to biotinylated ligands.

2. Materials and methods

2.1. Materials

Stearylamine, human recombinant EGF, bovine BSA, chloroquine and streptavidin were purchased from Sigma-Aldrich (Denmark), glyceryl dibehenate (Compritol® ATO 888) from Gattefossé (France), polysorbate 80 (Tween 80) from Bioworld (Germany), BODIPY 492/515 and LysoTracker Red® DND99 from Molecular Probes (Denmark). Cell culture reagents were purchased form Invitrogen (Denmark). The reporter plasmid pEGFP-N1 encoding enhanced green fluorescent protein was from Clontech (Denmark).

2.2. SLN production

The SLN were produced from a warm oil-in-water microemulsion as described in [12]. Briefly, the SLN formulation (final total lipid concentration 3.75 μg/μl) consist of the cationic lipid stearylamine (0.075% w/w), the uncharged lipid Compritol (0.3% w/w) and butanol (0.7% w/w) melted in an oil bath with gentle stirring at 75–80 °C. After completely melting of the lipids, 1 ml of pre-heated MilliQ water was added. The surfactant Tween 80 was added drop-wise until a clear microemulsion was obtained and additional 2 ml pre-warmed water is added. The warm microemulsion was suctioned into a 75 °C pre-warmed syringe and under constant pressure instantly sprayed into 40 ml of ice cold water with stirring for 30 min. To remove surfactant, aliquots of the SLN were dialysed against PBS (137.9 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2 (Invitrogen, Denmark) or water at 4 °C for 24–48 h using dialysis tubes of cellulose ester (Spectrum, Denmark) with a 300 KDa cut off and stored up to 1 month at 4 °C. The SLN were tested for toxicity on cell culture for each new batch (see below). For SLN labelled with the fluorescent dye BODIPY, this was added to the lipid phase during preparation of the microemulsion.

2.3. ζ-potential measurements

The ζ-potential measurements were performed using a Zeta Master (Malvern Instrument). Field strength in the measurement cell was 30 V/cm. All measurements were carried out in a degassed NaCl solution with an ionic strength of 0.001 M pre-incubated 10 min prior to ζ-potential measurement.

2.4. Size measurements

Size measurements were carried out using dynamic light scattering technique using a DynaPro (protein solution). The measurements were performed in MilliQ water. The laser sensitivity was set to 100%, baseline tolerance was 10% and the channels from 4 to 120 were included in the measurements. According to these settings, at least 10 fitting measurements were performed in order to get a representative result. The final result was calculated from the results obtained from the regularisation histogram.

2.5. Binding of DNA and agarose gel electrophoresis

Plasmid DNA was added to the dialysed SLN in water or PBS in the ratios indicated in the various experiments and incubated for 30 min at room temperature. For analysis of DNA binding by gel electrophoresis the samples were diluted in water, PBS or serum free medium, OPTIMEM (Invitrogen, Denmark). Analysis by agarose gel electrophoresis was performed at 2.5 V/cm in 0.8% agarose gels in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA) containing μg/μl ethidium bromide. The total amount of DNA added per lane was 0.5 μg for all samples.

2.6. Biotinylation of ligands

EGF and BSA were labelled with biotin using the EZ-Link™-NHS-Biotin biotinylation kit from Pierce (Denmark) according to manufacturers instructions in phosphate buffer pH 6. BSA was biotinylated using 10 M excess of biotin and EGF using 5 and 10 molar excess of biotin. Biotinylation degree was determined by the HABA-Avidin-assay from Pierce. The proteins were labelled with three biotin molecules per molecule BSA and by one biotin per molecule EGF.

2.7. Labelling of DNA

Plasmid DNA was fluorescently labelled with TRITC (rhodamine) using the Mirus Label IT® kit (Mirus Corporation, Denmark) according to manufacturers instructions with the ethanol precipitation method for purification.

2.8. Binding of streptavidin, biotinylated BSA and biotinylated EGF to SLN

After and between all additions of streptavidin, biotinylated BSA or biotinylated EGF the SLN were dialysed 24–48 h against water using dialysis tubes with a 300 kDa cut off. Streptavidin was incubated with SLN in ratios of 5:1–20:1 (SLN/streptavidin, w/w). Biotinylated BSA was added in four times and biotinylated EGF in five times molar excess to estimated SLN bound streptavidin. Prior to SDS-PAGE, the samples were concentrated by drying under vacuum. For analysis of SLN:streptavidin+/− biotinylated EGF the samples were precipitated by acetone to remove the lipid moiety of the complex. The samples were analysed by SDS-PAGE using a the NuPage system with 4–20% bis–tris gels (Invitrogen, Denmark) and Coomassie Brilliant Blue staining or silver staining using the SilverXpress® kit from Invitrogen.

2.9. Cell lines and tissue culture

The human small cell lung cancer cell line CPH 54A [13] was propagated in Eagles Minimum Essential Medium (EMEM) and a mouse fibroblast cell line transfected to express the human EGF receptor, NR6-WA [14] was propagated in Dulbecco's Modified Eagle's Medium. All media were supplemented with 10% fetal calf serum and antibiotics. Cells were plated 4×10^4 cells in 0.5 ml medium in 24 well cell culture plates (NUNC, Denmark) coated with fibronectin (1 μg/cm^2) (Sigma) one day prior to addition of SLN or SLN complexes. 18.8 μg SLN (with or without complexion with DNA, streptavidin or biotinylated EGF) was added to 200 μl serum free medium (OPTIMEM) containing 0.1 mM chloroquine and added to the cells after the growth medium was removed. After 2 h incubation the medium was replaced with 1 ml growth medium and cells evaluated after 1–72 h as indicated. The toxicity (using various amounts of SLN) was evaluated by phase microscopy or by live staining in 0.1% Trypan Blue (Invitrogen, Denmark) in PBS. Evaluation of gene expression was assessed visually by fluorescence microscopy. Staining with Lysotracker was for 10 min at a dilution of 1:10.000, after which the cells were washed once in PBS and fixated in 3.7% formaldehyde for 10 min. Microscopy of stained SLN uptake was performed using a Zeiss confocal laser scanning microscope (CLSM) with a C-Apochromat 63× objective and a laser wavelength of 488 or 560 nm. For determination of EGF receptor activation, the NR6-WA cells were starved for 24 h in DMEM with 0.5% FCS prior to addition of EGF or SLN complexes. After 15 min incubation with EGF, biotinylated EGF or SLN:DNA:streptavidin:biotinylated EGF complexes, the cells were washed once in PBS and lysed directly in SDS-PAGE sample buffer.

2.10. Determination of EGF receptor phosphorylation

Cell lysates from equal amounts of cells incubated with EGF, biotinylated EGF or SLN:DNA:streptavidin:biotinylated EGF complexes were separated by SDS-PAGE using the NuPage system with 3–8% tris–acetate gels (Invitrogen, Denmark), transferred to nitrocellulose membranes and incubated with rabbit polyclonal anti-phospho Tyr1068 EGFR (cell signalling) according to manufacturers specifications. Visualisation of phosphorylated EGF receptor was performed using horseradish peroxidase coupled secondary antibody (DAKO) and developed using the ECL (enhanced chemiluminescence) Western blotting Detection system™ (Amersham) and exposure to Hyperfilm (Amersham).

3. Results

3.1. Characterization of DNA binding capacities of SLN

We have previously prepared and characterised SLN prepared by the microemulsion technique using different formulations and purification methods, where we characterised several formulations with an overall positive charge (ζ-potentials from +10 to +20 mV), with similar size distributions (100–200 nm) and low cellular toxicity after removal of the surfactant by dialysis [12]. These SLN formulations were tested for their ability to bind DNA via electrostatic interactions. The stability and capacity of the binding was measured by the ability of the SLN to retain DNA in an electric field by gel shift analysis using agarose gel electrophoresis. Only one formulation consisting of Compritol ATO 888 and the cationic lipid stearylamine could retain binding of DNA in an electric field. This formulation was characterised further. The SLN:DNA were mixed in various ratios and estimated to retain significant amounts of DNA in the ratio 20:1 and 10:1 (SLN:DNA, w/w) (cationic lipid:DNA 4:1, 2:1 w/w). For the ratio 20:1 and 10:1 (equivalent of 0.05 and 0.1 μg DNA/μg SLN) the SLN:DNA particles retained their positive charge and did not release DNA during electrophoresis. The particles migrated towards the cathode, both when the SLN had been dialysed against PBS or water (Fig. 1(A)). At higher concentrations of DNA (ratio 5:1), the SLN dialysed against water had a slight less binding capacity revealed by some release of DNA during electrophoresis (Fig. 1(A)). The binding of DNA was confirmed by measurements of the ζ-potentials, which showed a positive ζ-potential of +13.5 for the SLN alone, +0.2 for SLN:DNA ratio of 20:1, but a negative ζ-potential of −5.1 at ratio 10:1. The sizes of the particles was not influenced significantly by binding of DNA, as the average diameter of the SLN alone was 115 nm, of SLN:DNA 20:1 was 96 nm and for 10:1 was 130 nm, thus demonstrating that the DNA does not cause aggregation of the SLN particles and the SLN confer a significant condensation effect on the DNA. Incubation of SLN and DNA for different periods (30 min–72 h) and at different temperatures (4, 22 and 37 °C) revealed no difference in binding capacity. Linearising the plasmid prior to binding decreased the binding to SLN (data not shown).

As transfections of cells in culture generally are performed in serum free medium (e.g. OPTIMEM), it was essential to assure that the SLN:DNA complexes were stable in this

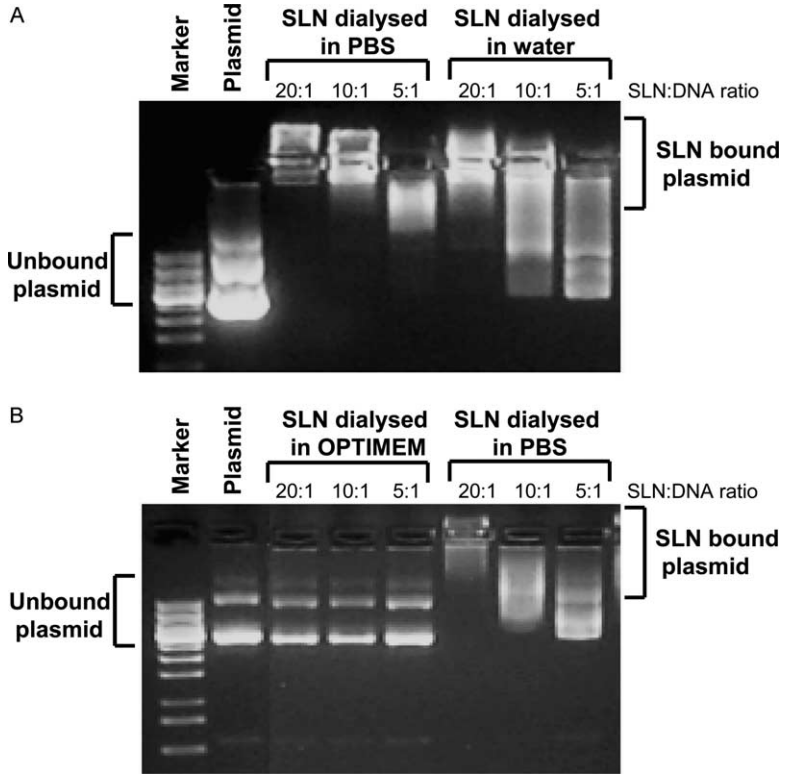

Fig. 1. DNA binding capacities of SLN:DNA complexes. (A) SLN dialysed against water or PBS at 4 °C for 24 h and incubated with DNA in the ratios: SLN:DNA (w/w) 20:1, 10:1 and 5:1.The samples were diluted in water, incubated at room temperature for 2 h and analysed by agarose gel electrophoresis. (B) SLN dialysed against OPTIMEM or PBS at 4 °C for 24 h and incubated with DNA in the ratios: SLN:DNA (w/w) 20:1, 10:1 and 5:1.The samples were diluted (10–20×) in OPTIMEM, incubated at room temperature for 2 h and analysed by agarose gel electrophoresis. The samples were loaded to contain equal amounts of DNA.

medium. When the SLN were dialysed against OPTIMEM, no binding of DNA was observed (Fig. 1(B), lanes 3–5). However, if the SLN were dialysed in water or PBS, added DNA and subsequently diluted into OPTIMEM, only a slight decrease in binding capacity was observed (Fig. 1(B) lanes 6–8).

3.2. Cellular tolerance and uptake of SLN with and without DNA

The toxicity of the SLN was measured by diluting the SLN in serum free medium and incubation with the CPH 54A and NR6-WA cell lines for 2 h, after which the cells were changed to growth medium. Cell survival was evaluated after 24 h by visual evaluation and by staining with Trypan Blue. No toxicity was observed at concentrations up to 70 μg SLN/ml. Complexing DNA to SLN slightly increased the tolerance level of SLN to 100 μg SLN/ml.

To determine if the SLN particles are taken up by cells per se, the SLN were labelled with a fluorescent lipophilic stain and incubated with cells in tissue culture. Uptake of labelled SLN was observed in a few cells (less than 1%) and was mainly distributed in small vesicles (Fig. 2(A)). Co-staining with the acidotropic stain Lysotracker (Fig. 2(B)) revealed co-localisation with endosomes and/or lysosomes. The lipophilic fluorescent dye is not covalently attached to the SLN and therefore could be released from the SLN and integrate in cellular membranes (including endosomal/lysosomal membranes). Addition of dye without SLN showed that the dye was capable of staining extra- and intracellular membranes (data not shown). However, after refeeding and incubation in growth medium for 24 h, the labelled SLN still remained in vesicles (Fig. 2(C)) co-localising with the endosomal/lysosomal marker (Fig. 2(D)), showing that there is no release to the cytoplasm or degradation of these SLN. No staining was observed after addition of the dye alone after 24 h. The same results were observed for the NR6-WA cell line (data not shown). Staining with the live-stain Trypan Blue after 24 h revealed that cells with high uptake of SLN were dead (data not shown). Therefore an alternative staining method-using SLN bound to TRITC-labelled DNA was performed (Fig. 2(E) and (F)). There was only observed uptake in a few cells (less than 1%), displaying vesicular localisation and no labelled DNA in the nucleus. The presence of labelled DNA in the cells confirms that the SLN:DNA complexes are stable in OPTIMEM and during cellular uptake, as there was no cellular staining with excess amounts of TRITC-labelled DNA added alone. No gene expression was observed (visual evaluation using EGFP as reporter gene) for SLN complexed with DNA even after 72 h incubation in growth medium. Addition of the endosomolytic agent chloroquine, previously shown to increase gene expression both of liposomal [15] and SLN transfections [8,9] did not increase uptake nor result in gene expression.

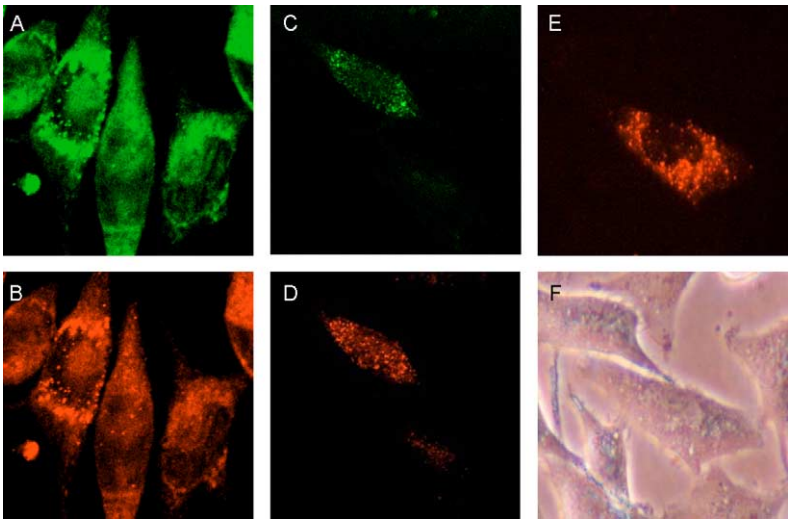

Fig. 2. Localisation of labelled SLN and SLN bound to DNA. CPH 54 A cells incubated 2 h with SLN labelled with a fluorescent lipophilic stain (Bodipy) and staining for 10 min with an endosomal/lysosomal marker (Lysotracker Red). (A) Bodipy staining. (B) Lysotracker staining. CPH 54 A cells incubated 2 h with SLN labelled with a fluorescent lipophilic stain (Bodipy) followed by incubation in growth medium for 24 h and staining for 10 min with an endosomal/lysosomal marker (Lysotracker Red). (C) Bodipy staining. (D) Lysotracker staining. CPH 54 A cells incubated with SLN:TRITC labelled DNA in a ratio of 20:1 for 2 h followed by incubation in growth medium for 72 h. (E) DNA staining. (F) phase microscopy (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

3.3. Binding of streptavidin and DNA to SLN

Unspecific uptake of SLN:DNA did not mediate gene expression, which could be due to insufficient uptake and/or lack of release from the endosomes. For cell specific delivery—e.g. of a therapeutic gene—a ligand mediated specific internalisation could be expected to greatly increase uptake. Many ligands such as proteins and peptides can readily be biotinylated and the binding of biotin to streptavidin has exceptionally high affinity [16]. It has previously been shown that the negatively charged streptavidin ($pI = 5$) can bind directly to PEI via electrostatic interactions and is capable of binding a biotinylated ligand [17]. To test if the same approach could be applied to SLN, streptavidin was added directly to the SLN using a 20:1 w/w ratio of SLN:streptavidin. The SLN-streptavidin complexes were analysed by SDS-PAGE and stained. Streptavidin remains bound to the SLN after extensive dialysis (Fig. 3 lane 3). Streptavidin alone in equivalent amounts without SLN was totally removed by dialysis (not shown). It was estimated from the staining that the binding capacity of SLN was approximately 0.02 μg streptavidin/μg SLN (equivalent to 0.08 μg streptavidin/μg cationic lipid). Dialysis up to 1 week did not reduce the amount of streptavidin bound, indicating that the binding is strong. The size of the SLN:streptavidin particles increased from 146 to 288 nm. SLN particles pre-complexed with DNA (20:1 w/w) could only bind about a third amount of streptavidin, thus confirming that some of the positive charges are occupied by DNA (Fig. 3 lane 4). However, this demonstrates that SLN can bind significant amounts of DNA and streptavidin simultaneously. Binding of DNA to SLN incubated with saturating amounts of streptavidin was totally inhibited, demonstrating that all the accessible positive charges were saturated with streptavidin (data not shown). Addition of streptavidin to SLN did not influence cellular toxicity.

3.4. Binding of ligands to SLN:streptavdin complexes

To ensure that the SLN bound streptavidin was functional, the SLN:streptavidin complexes were incubated with biotinylated BSA. After extensive dialysis the samples were analysed by SDS-PAGE, which showed that biotinylated BSA was bound to SLN:streptavidin (Fig. 4 lanes 2–4). As not all biotinylated BSA was removed by dialysis presumably due to the ability of BSA to form aggregates [18] (Fig. 4 lanes 5–7), the binding efficiency could not be directly evaluated. However, the results demonstrate that the SLN bound streptavidin is capable of binding a biotinylated ligand.

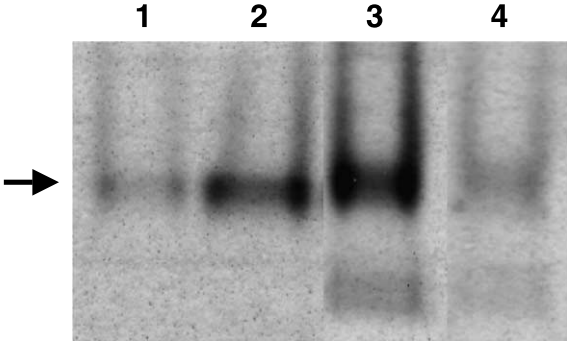

Fig. 3. Binding of streptavidin to SLN. Lane 1, 0.5 μg streptavidin. Lane 2, 2 μg streptavidin. Lane 3, SLN:streptavidin complex after dialysis (280 μg SLN mixed with 50 μg streptavidin). Lane 4, SLN:DNA:streptavidin complex after dialysis (280 μg SLN bound with DNA in a ratio 20:1 mixed with 50 μg streptavidin). Samples were concentrated and analysed by SDS-PAGE (non-reducing) and Coomassie staining. Arrow indicates the 60 kD tetramer streptavidin.

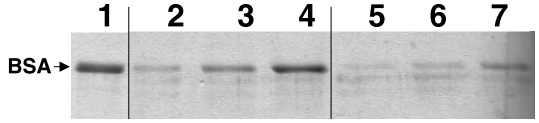

Fig. 4. Binding of SLN:streptavidin complexes to biotinylated BSA. Lane 1, BSA (0.5 μg). Lanes 2–4, 40, 80 and 250 μg lipid of SLN:streptavidin: biotinylated BSA complexes after dialysis (50 μg BSA added to 750 μg SLN: streptavidin). Lanes 5–7, Biotinylated BSA after dialysis (same amounts as added to samples in lanes 2–4, respectively). Samples were analysed by SDS-PAGE (reducing) and Coomassie staining.

An attractive ligand for targeted gene therapy is EGF, as this ligand is readily internalised and many cancers over express the EGF receptor on the cell surface [19]. Therefore biotinylated EGF was added to SLN:streptavidin complexes in a similar manner as for biotinylated BSA. However, as EGF also is an acidic protein and negatively charged at physiological pH (pI=4.6), EGF by itself binds the SLN in the absence (Fig. 5 lane 3) and presence (Fig. 5 lane 4) of streptavidin. Therefore it could not be determined how large a fraction of the bound EGF was bound via streptavidin and how much was directly bound to the SLN. However, as EGF is a very small protein (6 kD) it would be unlikely that SLN embedded EGF has biological activity. To determine if the bound EGF was biologically active, an assay utilising EGF activation of the EGF receptor was employed. EGF, biotinylated EGF and SLN:DNA:streptavidin:biotinylated EGF complexes were added to a cell line (NR6-WA) with high expression of the EGF receptor. Receptor activation by EGF was analysed by phosphorylation of the receptor by western blotting of cell lysates probed with a phospho-EGF receptor antibody. There was a basal low level of phosphorylation without addition of EGF (Fig. 6 lane 1), but a substantial activation of the receptor leading to higher phosphorylation was observed in a dose dependent manner with EGF (Fig. 6 lanes 2–4). Biotinylated EGF was approximately 15 times less efficient than native EGF (Fig. 6 lanes 5–7), demonstrating that the addition of biotin to this small ligand to some extent impairs the biological activity. There was no difference in the activities of EGF biotinylated with five or 10-fold molar excess of biotin. Importantly, there was a substantial activation after addition of the SLN:streptavidin:biotinylated EGF complexes (Fig. 6 lanes 7–8), showing that the SLN bound EGF indeed is biologically active.

To test if the EGF associated SLN complexes could mediate gene expression, the complexes were tested on the EGF receptor expressing cell line (NR6-WA). However, after a 2 h

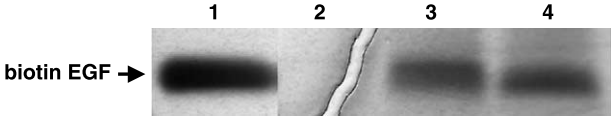

Fig. 5. Binding of SLN:+/−streptavidin to biotinylated EGF. Lane 1, Biotinylated EGF (0.2 μg). Lane 2, SLN after dialysis (94 μg lipid). Lane 3, SLN mixed with biotinylated EGF after dialysis (94 μg lipid, 1 μg EGF)). Lane 4, SLN:streptavidine mixed with biotinylated EGF after dialysis (94 μg lipid, 1.9 μg DNA (50:1),1.7 μg streptavidin incubated with 1 μg biotinylated EGF). Samples were precipitated with acetone, analysed by SDS-PAGE (reducing) and silver staining.

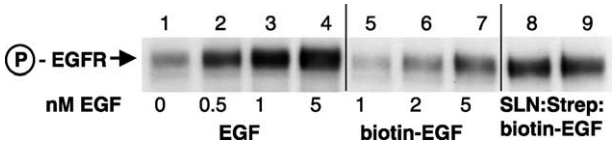

Fig. 6. Activation of the EGF receptor by EGF, biotinylated EGF and SLN: DNA:streptavidin:biotinylated EGF complexes. Western blot of lysates from cells expressing the EGF receptor (NR6-WA) after 15 min incubation with EGF, biotinylated EGF or SLN complexes. The blot is probed with an antibody to the activated EGF receptor (anti-phospho-EGF receptor Tyr 1068) and visualised by ECL. Equal amounts of cell lysates were loaded per lane. Lane 1, control. Lanes 2–4, Cells incubated with EGF (0.5, 1 and 5 mM). Lanes 2–4, Cells incubated with biotinylated EGF (1,2 and 5 mM). Lanes 7–8, Cells incubated with dialysed SLN:streptavidin:biotinylated EGF complexes (94 μg lipid, 1.9 μg DNA (50:1), 1.7 μg streptavidin incubated with 1 μg biotinylated EGF). Lanes 8 and 9 are complexes using EGF biotinylated with 1:5 and 1: 10 M excess of biotin, respectively.

incubation with the SLN complex in the presence of chloroquine and 48 h of incubation in growth medium no reporter gene expression was observed. SLN complexes with DNA, streptavidin, biotinylated BSA or EGF did not display higher toxicity than SLN alone.

4. Discussion

The aim of this study was to develop a formulation of SLN prepared by the microemulsion method for targeted gene transfer with parenteral administration. It was therefore necessary to develop a formulation with low toxicity and an appropriate surface charge to bind various components. Non-toxic compositions and a simple purification method of positively charged SLN by the microemulsion method have been demonstrated previously [12]. We found that one of these formulations could efficiently bind and condense DNA and did not cause aggregation of the particles. The SLN:DNA complexes were stable during extensive dialysis and DNA remained bound in an electric field during electrophoresis. SLN dialysed against OPTIMEM could not bind DNA, whereas SLN dialysed against water or PBS were able to retain the DNA after dilution in OPTIMEM, as judged by both gel electrophoresis and uptake of TRITC labelled DNA:SLN complexes in cell culture. Therefore it cannot be the high ionic strength during dialysis, but rather other components such as magnesium, calcium or amino acids, which inhibit the binding of DNA in OPTIMEM, but are not able to displace already bound DNA. Therefore, the SLN:DNA complexes were stable at physiological ionic strength, pH and temperature, thus allowing testing in cell culture. As cells in culture can tolerate up to 100 μg SLN/ml, DNA bound to SLN can be added to cell culture at a concentration up to 2.5 μg/ml, which, if delivered and released efficiently, should be sufficient for high gene expression. We could only detect unspecific uptake in very few cells and we did not observe gene expression of the SLN:DNA particles per se, even in the presence of the endosomolytic agent chloroquine.

In order to be able to target the particles for cell specific uptake via highly internalising receptors, attachment of ligands to the particles was performed. Chemical covalent coupling of

a ligand to the SLN could impair the biological activity of the ligand and thereby the binding capacity to a receptor. Direct biotinylation of SLN and coupling of ligands via streptavidin could cause crosslinking of the SLN, thus making the particles too large for systemic delivery. We therefore chose to attach streptavidin directly to the SLN, which would allow binding of biotinylated ligands directly to the SLN. The SLN were indeed capable of binding of significant amounts of streptavidin via electrostatic interactions and the complex was stable even after extensive dialysis. The SLN bound streptavidin was functional, as it could bind biotinylated BSA and EGF. Importantly, the SLN particles were capable of binding both DNA and streptavidin, thereby generating a potential vector for targeted gene therapy.

The complex was tested using the biotinylated ligand EGF, as the receptor for this ligand is overexpressed in many cancer forms [19] and has previously been demonstrated to mediate receptor targeted gene transfer coupled to poly-*L*-lysine [20,21] and PEI [17,22,23]. To determine if the EGF bound to the SLN was biologically active, we tested activation of the EGF receptor after incubation a cell line expressing the receptor with SLN:DNA:streptavidin:biotinylated EGF. We found activation of the receptor, showing that some of the SLN bound EGF was biologically active. The activity was 50 times less than calculated from the amounts of EGF, which theoretically could be bound to the SLN:DNA:streptavidin complexes. This is probably due to the lower biological activity of biotinylated EGF and that not all streptavidin-binding sites can be expected to be accessible in the SLN complex. These results show that biologically active biotinylated EGF can be coupled to the SLN:DNA:streptavidin complexes.

We obtained no gene expression after incubating a cell line with high expression of the EGF receptor with the complex. The lack of gene expression could be due to insufficient amounts of functional EGF bound or that the complex binds, but is not internalised. Another possibility is that the SLN complexes are trapped in the endosomes and the DNA therefore is not released to the cytoplasm. PEI in itself has intrinsic endosomolytic activity, but this is probably not the case for SLN. Release of DNA from endosomes has been demonstrated to increase transfection efficiency by inclusion of endosomolytic peptides in the gene transfer vector, such as the fusogenic hemagglutinin HA-2 N-terminal peptide [24]. These peptides can readily be biotinylated and therefore bound directly to the SLN bound streptavidin in addition to the ligand. Alternatively, the ligand can be produced as a chimeric protein with the pseudomonas exotoxin A translocation domain [25,26]. Other highly internalised ligands, such as transferrin could be tested, as malignant cells often over express the transferrin receptor and has been shown to mediate efficient gene delivery using polyplexes both in vitro and in vivo [23,27–29].

The observation that it is possible to stably bind streptavidin, DNA and biotinylated ligands simultaneously to SLN particles without increasing toxicity nor increasing the size of the particles significantly is therefore the first step for development of an SLN based targeted gene therapy vector for treatment of disseminated cancer.

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