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Quantifying the average number of nucleic acid therapeutics per nanocarrier by Single Particle Tracking microscopy

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ABSTRACT Nucleic acid biopharmaceuticals are being investigated as potential therapeutics. They need to be incorporated into a bio-compatible carrier so as to overcome several biological barriers. Rational development of suitable nanocarriers requires high quality characterization techniques. While size, concentration and stability can be very well measured these days, even in complex biological fluids, a method to accurately quantify the number of nucleic acid therapeutics encapsulated in nanocarriers is still missing. Here we present a method, based on concentration measurements with Single Particle Tracking microscopy, with which it is possible to directly measure the number of plasmid DNA molecules per nanoparticle, referred to as the plasmid/NP ratio. Using DOTAP/DOPE liposomes as model carrier, we demonstrate the usefulness of the method by investigating the influence of various experimental factors on the plasmid/NP ratio. We find that the plasmid/NP ratio is inversely proportional with the size

of the pDNA and that the plasmid/NP decreases when lipoplexes are prepared at lower concentrations of pDNA and nanocarrier, with values ranging from 6.5 to 3 plasmid/NP. Furthermore, the effect of pre- and post-PEGylation of lipoplexes was examined, finding that pre-PEGylation results in a decreased plasmid/NP ratio, while post-PEGylation did not alter the plasmid/NP ratio. These proof-of-concept experiments show that Single Particle Tracking offers an extension of the nanoparticle characterization toolbox, and is expected to aid in the efficient development of nanoformulations for nucleic acids based therapies.

KEYWORDS SPT, nanocarrier, liposomal gene delivery, nucleic acid delivery

ABBREVIATIONS

CCM, Cell Culture Medium; Ceramide-PEG$_{2000}$, N-octanoyl-sphingosine-1-[succinyl[methoxy(polyethylene glycol)2000]]; DOPE, 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine; DOTAP, (2,3-Dioleoyloxy-propyl)-trimethylammonium-chloride; DSPE-PEG$_{2000}$ 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-(methoxy (polyethyleneglycol)-2000); LPX, Lipoplexes; NA, Nucleic acid; pDNA, Plasmid DNA; PEG, Polyethylene glycol; Plasmid/NP ratio, the average number of complexed plasmids for every nanocomplex; SPT, Single Particle Tracking.

INTRODUCTION

Nucleic acid biopharmaceuticals present a tremendous potential for treatment of life-threatening and incurable diseases. DNA vaccines, siRNA and gene–based treatments are attracting an ever-growing interest, although delivering these nucleic acid molecules to the desired cells is not an easy task. The human body has evolved to resist against organisms whose main purpose is to insert nucleic acids into the cells of our body. To reach the desired target, the therapeutic nucleic acids need to overcome numerous *in vivo* extracellular and intracellular

barriers. Just to name a few, they have to cross multiple tissues (mucus and extracellular matrices), evade the immune system, remain intact in a nuclease rich environment, reach the target cell and enter the cytoplasm or nucleus[1]. To be able to survive such a long and perilous journey, therapeutic nucleic acids require a safe, biocompatible carrier that protects and delivers them to the target cells. As a consequence, over time studies focusing on engineering gene delivery systems have increased exponentially. Liposomes, cationic polymers, nanogels, inorganic and magnetic particles, as well as cell penetrating peptides have been studied at length[1-7]. During development they are thoroughly characterized in terms of size, surface charge, stability and tendency to adsorb proteins.

A typical route to incorporate nucleic acid (NA) molecules into nanocarriers is by electrostatic complexation. Cationic carriers are mixed with the negatively charged nucleic acids at a certain charge ratio to form stable complexes. While the charge ratio between both is easily calculated, typically it is not known how many nucleic acid copies are present per nanocarrier. Yet, even at a fixed charge ratio, it is quite plausible that differences in the complexation conditions, such as the volume in which the complexes are prepared, may introduce differences on the loading degree per nanocarrier, which in turn may influence the final effectivity of the nanoformulation. Being able to know and consequently manipulate the number of therapeutic molecules per carrier may prove beneficial for rational optimization of the preparation method to ensure maximal effectivity. However, determining the number of NA copies in nanoparticles is not easily accomplished. One strategy based on single molecule photobleaching has been proposed[8, 9], but it requires a very low background noise and single fluorophore detection sensitivity. Moreover, care must be taken to ensure that every nucleic acid molecule is labeled with one and only one fluorophore, next to the fact that this method is only suited for a very small number of cargo molecules per nanoparticle as bleach steps of

individual fluorophores have to be resolved. Indeed, for larger numbers of molecules these small changes in fluorescence intensity will drown in the overall shot noise.

To address this need, here we propose a new approach to determine the number of DNA copies in nanoparticles. It is based on single particle tracking (SPT) microscopy with which nanoparticle number concentrations can be accurately determined[10-14]. In brief, the Brownian motion of individual nanoparticles is imaged, and the frequency of entering and leaving the focal plane is used as a fundamental metric from which the absolute nanoparticle number concentration, that is the number of nanoparticles in a given volume, can be determined. In this paper, we demonstrate that by making use of SPT concentration measurements it is possible to determine the number of DNA copies per nanoparticle as follows. As a first step, the pDNA number concentration of a pDNA solution is measured, which can be done either by SPT or by classic absorbance measurements. The pDNA is subsequently mixed with the carrier material to form nanocomplexes. Next, the number concentration of the formed DNA-carrier complexes is measured with SPT. Upon full complexation of the pDNA, the ratio of the two quantities gives the average number of plasmids for every nanoparticle, referred to in this paper as the plasmid/NP ratio.

To demonstrate the proof-of principle, we focus on cationic DOTAP:DOPE liposomes as carriers for pDNA complexation, which have been frequently evaluated for gene therapy[15-17]. The interested reader is referred to excellent reviews for more information on this carrier system for gene therapy [1, 18, 19]. We use the described SPT methodology to investigate how pDNA size, lipoplex (LPX) charge ratio and concentration at which LPX are prepared influence the number of pDNA copies per lipoplex. Also, on the carrier side, we investigate the influence of pre- or post-PEGylation of the LPXs on the number of pDNA copies per nanoparticle. Taken together we demonstrate that SPT concentration measurements present a unique method to precisely determine the average number of plasmids per nanoparticle for a given nanoformulation. This

new type of information is expected to contribute to the rational optimization of gene complexes for improved gene delivery.

EXPERIMENTAL SECTION

Preparation of liposomes

(2,3-Dioleoyloxy-propyl)-trimethylammonium-chloride (DOTAP) and 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were purchased from Corden Pharma LLC (Liestal, Switzerland).

1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-(methoxy(polyethyleneglycol)-2000) (DSPE-PEG$_{2000}$) and N-octanoyl-sphingosine-1-{succinyl[methoxy(polyethylene glycol)2000]} (Ceramide-PEG$_{2000}$) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). The appropriate amount of the lipids (28 μl DOTAP and 29.8 μl DOPE) was mixed at a 1:1 ratio in a round-bottom flask and dissolved in 1000 μl of chloroform (Sigma Aldrich, Bornem, BELGIUM). PEGylated liposomes were prepared by adding the desired amount of DSPE-PEG$_{2000}$ or Ceramide-PEG$_{2000}$, corresponding to 3 mol% or 10 mol% of the total lipids.

The flask was then placed into the rotavapor (IKA, Staufen, Germany) and put in contact with a water bath pre-heated at 40°C. The pressure was reduced to 0.1 bar and the flask was rotated at 180 rpm for 15-20 min. The dried lipid film was then rehydrated in 0.2 ml distilled water and detached from the glass walls using glass pearls. The obtained liposome suspension had a concentration of 5 mM DOTAP and 5 mM DOPE. Since, at neutral pH, 1 mole of DOTAP has 1 mole of positive charges, the suspension had 5 mM of positive charges. The liposome suspension was then placed into a 0.5 ml vial and pulse-sonicated (10 s on, 15 s off) for 6 cycles using a probe sonicator (Branson Ultrasonics Digital Sonifier, Danbury, USA). The size and zeta-potential of the liposomes were checked using the Zetasizer Nano-ZS (Malvern, Worcestershire, UK). Depending on the batch, liposomes had a size between 65 nm and 75 nm

in diameter with a PDI that was always smaller than 0.25 and a zeta-potential of around +45 mV for the cationic liposomes and around +7 mV for the PEGylated liposomes.

Preparation of pDNA

Two types of plasmid DNA were used, gWiz™ pDNA (≈ 5800 bp) and pBlueScript KS+ pDNA (≈ 3000 bp). The plasmids are multiplied in *E.coli* bacteria (Genlantis, San Diego, CA, USA) in a sterile LB-medium (NaCl; Sigma-Aldrich, St Louis, Missouri, USA / Yeast extract; Lab M, Heywood, UK / Tryptone; Lab M, Heywood, UK) to which the antibiotic Kanamycin (Sigma-Aldrich, St Louis, Missouri, USA) is added and the bacteria were incubated overnight at 37°C, shaken at 300 rpm. We used the QIAGEN plasmid purification protocols (QIAGEN, Hilden, Germany) to obtain a purified pDNA solution. The purity and concentration of the solution was assessed using Nanodrop 2000C (ThermoFisher Scientific, Waltham, USA). The pDNA was labelled with Cy™5 (*Label* IT® Nucleic Acid Labeling Kits, Mirus Bio LLC, Madison, USA) by using 1 µl of Label IT Reagent for every 2 µg of pDNA. pDNA was purified from non-conjugated Cy™5 using the provided G50 Microspin Purification columns (Mirus, Madison, USA), as described by the manufacturer.

Lipoplex formation and plasmid/NP experiment

LPXs were formed by mixing, in equal volumes, a fixed amount of 1 µg of labeled pDNA with RNase free water and a solution with the appropriate amount of liposomes to reach the desired charge ratio of N/P 6, 8, 10 and 12. The lipoplex solution, which had a total volume of 20 µl, was then vortexed and placed in the dark at room temperature for 30 minutes, to allow complex formation. The pDNA concentration of the solution was 50 ng/µl. Afterwards, the solution was diluted to a pDNA concentration of 0.3 ng/µl and the LPX concentration was measured with SPT. Variations in this protocol were introduced to monitor the influence on the resulting plasmid/NP ratio. In one set of experiments, the charge ratio was adapted by changing the amount of pDNA in the solution, while keeping the amount of lipids fixed at 1.2 mM

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positive charges. In another set of experiments, both the pDNA and the liposome solutions were diluted ten and fifty times, respectively, before complex formation, to study the role of the concentration in LPX formation. In other words, the volumes of the pDNA and liposome solutions that were mixed together were increased 10 and 50 times, while keeping the charge ratio constant (at N/P 8). The undiluted, 10 and 50 times diluted stock solutions used for complex formation are referred to as 50 ng/µl, 5 ng/µl and 1 ng/µl respectively, based on the pDNA concentration.

Also PEGylated lipoplexes were included in this study, formed by adding the desired amount of PEG (linker chain: DSPE or Ceramide) to the DOTAP and DOPE in the round-bottom flask (pre-insertion technique) or to the LPX solution after complexation (post-insertion technique). In the latter case, the solution was vortexed and maintained 1 h at 37°C under continuous stirring at 200 rpm.

Gel Electrophoresis

Complexation of pDNA with liposomes was verified using gel electrophoresis. 400 ng labeled pDNA/eppendorf was complexed with the appropriate amount of liposomes to reach the desired charge ratio (4, 6, 8, 10 or 12) and water was added to a total volume of 15 µl. Then 5 µl TBE buffer (Tris; Merck Millipore, Billerica, Massachusetts, USA / Borate; VWR, Haasrode, Belgium / EDTA; Merck KGaA, Darmstadt, Germany) and 5 µl Loading buffer (ThermoFisher Scientific, Waltham, USA) were added to each solution. The samples were loaded on a 0.7% agarose gel (ThermoFisher Scientific, Waltham, USA) and left to run at 100 V for 50 min. Results are shown in Fig. 1 SI and 2 SI.

Measuring Nanoparticle Number Concentrations by Single Particle Tracking

Single Particle Tracking is a well-known advanced microscopy technique that allows to analyze the mobility of nanoparticles. Briefly, SPT captures the motion of individual particles in time-lapse videos, which are then analyzed to reconstruct the trajectories of each of these

nanoparticles. From the analysis of the trajectories it is possible to calculate for instance the diffusion coefficient of each nanoparticle. Recently we developed a dedicated algorithm that allows to extract the absolute number concentration from SPT videos of nanoparticles diffusing freely in a liquid[10, 20]. The number concentration is the average number of particles identified per frame divided by the detection volume V. As SPT is a microscopy based method, the lateral dimensions of V are simply the field of view of the microscope, which is easily calibrated with a graded ruler. The difficulty, however, is to precisely determine the effective axial dimension, which is unknown and impossible to calibrate a priori since it depends on several parameters, including the image processing settings used to detect the particles. It can, however, be derived from the time particles spend inside V, which can be measured directly from the SPT movies. Small and fast moving particles will spend less time within V as compared to larger and slower ones. Thus, thanks to the fact that the diffusion coefficient can be directly measured from SPT data and by making use of the statistical model reported by Roding et al.[10], the axial dimension of V can be readily determined for each SPT movie and used to calculate the absolute number concentration of the particles in the dispersion.

The plasmid/NP ratio, which is of interest in this study, is then simply calculated by dividing the concentration of pDNA in the solution before complexation by the concentration of LPX after complexation, with the assumption that all the plasmid DNA is complexed.

For every data point, a minimum of 20 videos is recorded, which all together provides a minimum of 1000 trajectories per data point. Every batch is tested 4 times, while every experiment is repeated 3 times, to ensure the validity of the results.

RESULTS

Validation

First we evaluated if SPT is able to correctly measure pDNA and LPX concentration. A solution of gWiz™ pDNA was prepared at a concentration of 166 ± 3 ng/µl, corresponding to

$2.84 \pm 0.05*10^{13}$ pDNA strands per ml. While SPT can be operated in light scattering mode, the best sensitivity is obtained in combination with fluorescence. Therefore, the experiments reported here were all done in fluorescence mode, with pDNA labelled covalently with a far-red fluorophore, Cy5, for optimal visualization with SPT. A dilution series was prepared from the stock solution at sufficiently low concentrations (0.006%, 0.013%, 0.019%, 0.025%, 0.032%) so that the diffusion of individual pDNA copies can be imaged with SPT. The pDNA number concentrations as measured with SPT are presented in Fig. 1A. A linear trend line through the data extrapolated to 100% predicts a pDNA stock concentration of $2.66 \pm 0.23*10^{13}$ plasmid/ml, with an R^2 of 0.989, in excellent agreement with the actual concentration reported above. This shows that SPT is well suited to measure the number concentration of Cy5-labelled pDNA.

Next, we evaluated whether SPT allows to determine the number concentration of DOTAP:DOPE LPX containing Cy5-labelled pDNA. A dilution series was prepared from a LPX formulation (charge ratio of 8) and measured with SPT, as shown in Fig. 1B. Charge ratio 8 was chosen to ensure complete complexation of the cargo while keeping the amount of carrier to a minimum to reduce cytotoxicity as much as possible in case the lipoplexes would be used for cell transfections. As expected, the LPX number concentration follows a linear trend, with an R^2 of 0.985. From the linear fit the LPX stock concentration is extrapolated to be $1.9 \pm 0.1*10^{11}$ particle/ml.
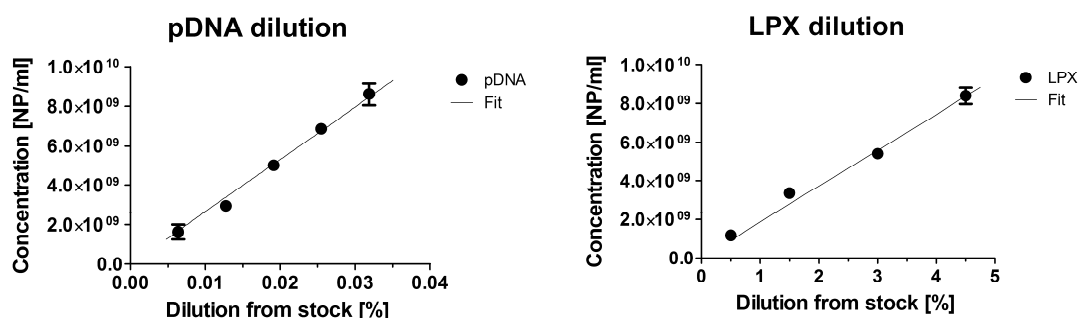
Fig. 1. SPT number concentration measurements of a dilution series of (A) Cy5-labelled gWiz pDNA and (B) DOTAP:DOPE LPX containing Cy5-pDNA at a charge ratio of 8.

Knowing the number concentration of plasmids and LPX, the average number of plasmids per LPX now readily follows from the ratio C_{pDNA}/C_{LPX}. The pDNA solution, when complexed with DOTAP:DOPE liposomes, was diluted 20 times. Thus, for the above mentioned LPX of charge ratio 8 it yields a plausible number of 7.0 ± 0.7 plasmids per LPX. This result is based on the assumption that all pDNA is complexed (i.e. no remaining free pDNA). Based on gel electrophoresis this is the case here, as no uncomplexed pDNA could be detected (see SI). Further on we will discuss how the method can be extended in the future to also deal with partially uncomplexed pDNA.

Influence of plasmid size

As a first application of the method we investigated if the number of plasmid/NP is influenced by the size of the plasmid, keeping the charge ratio constant. We prepared LPX at charge ratio 8 with gWiz^{TM} pDNA ($\approx$ 5800 bp) and pBlueScript KS+ pDNA of about half the size ($\approx$ 3000 bp). 50 ng/µl pDNA was added to DOTAP:DOPE liposomes corresponding to 1.2 mM positive charges. As can be seen in Fig. 2A we find that 2.2 ± 0.2 times as many plasmids per LPX are found for the smaller pDNA, which corresponds well with the difference in size of both plasmids. In any case it shows that the plasmid size can influence the number of plasmid/NP.

Influence of the concentration of pDNA and liposomes

Next, we explicitly investigate the influence of both pDNA and liposomes concentration on the number of plasmid copies per LPX. LPXs were prepared at a constant charge ratio 8 for different concentrations of pDNA and liposomes. Fig. 2B confirms that the plasmid/NP ratio is highly dependent on the concentration of the initial stock solutions that are mixed when the LPXs are prepared. At a pDNA concentration of 1 ng/µl the LPXs contain only 3 copies of

pDNA, while it is about 6.5 at a concentration of 50 ng/µl. This again shows that the plasmid/NP ratio has the tendency to increase at higher concentrations of the interaction partners. Surprisingly, the LPX size showed the opposite trend, indicating that size alone is not sufficient to infer if LPXs have more or less copies of pDNA per particle.

Influence of charge ratio

To investigate how the number of plasmid/NP changes for various LPX charge ratios, we first start by keeping the concentration of lipids constant at 1.2 mM and adjust the amount of gWiz™ pDNA to obtain charge ratios of 6, 8, 10 and 12 (see 'Fixed Lipid Content' in Table 1). All solutions are prepared in a constant total volume of 20 µl. The results in Fig. 2C (light gray bars) show a clearly decreasing trend for the plasmid/NP ratio at increasing charge ratio, with values ranging from 10.5 ± 0.6 plasmid/NP (charge ratio 6) to 5.7 ± 0.3 plasmid/NP (charge ratio 12). To test if the reported values are statistically different, an unpaired t-test was conducted to compare the values of charge ratio 6 to the values of charge ratio 8, 10 and 12. In the last two cases the values are significantly different ($p < 0.05$), while it is not the case between charge ratio 6 and 8 ($p = 0.07$).

The decreasing trend could be explained by the fact that the same amount of liposomes are available at higher charge ratios to complex less pDNA, so that it seems reasonable that complexes will be formed with less pDNA per particle. Although not very pronounced, this is to some extent also reflected in the LPX size as measured by SPT shown in Fig. 2C (light gray line), with about 20% decrease in size between charge ratio 6 and 12.

Alternatively, these data can be interpreted from the pDNA point of view, demonstrating that more pDNA per particle is captured when a higher pDNA starting concentration is used (for the lowest charge ratio).

The same charge ratio series can also be obtained by keeping the pDNA concentration constant at 50 ng/µl and adjusting the amount of lipids instead. In this case we find a slightly

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increasing trend for the plasmid/NP ratio as the charge ratio increases (Fig. 2C, dark gray bars), with values ranging from 7.7 ± 0.2 plasmid/NP (charge ratio 6) to 9.4 ± 0.5 plasmid/NP (charge ratio 12). At the same time a moderate size increase is observed (Fig. 2C, dark gray line).

An unpaired t-test comparing the values of charge ratio 6 to the values of charge ratio 8, 10 and 12 shows that the values are significantly different in all three cases ($p < 0.05$). An important difference with the previous charge ratio series is that the number concentration of pDNA and lipids is different, as seen in Table 1.

PDNA AND LIPOSOMES CONCENTRATION

CHARGE RATIO	Fixed Lipid Content		Fixed pDNA Content	
	Concentration pDNA [ng/μl]	Concentration lipids [mM]	Concentration pDNA [ng/μl]	Concentration lipids [mM]
6	67	1.2	50	0.9
8	50	1.2	50	1.2
10	40	1.2	50	1.5
12	33	1.2	50	1.8

Table 1. Concentration of plasmid DNA and lipids, expressed in ng/μl and nM of positive charges respectively, in the LPX dispersions. The volume was always kept constant at 20 μl. "Fixed lipid content" corresponds to 1.2 mM of lipids, and "fixed pDNA content" corresponds to 50 ng/μl pDNA.

For instance for the charge ratio of 12 we now have 50% more lipids and pDNA as compared to the previous series. This means that the complexes are now formed at a higher concentration, which apparently leads to larger complexes containing more pDNA per particle. On the contrary, for a charge ratio of 6 we now have 25% less lipids and pDNA as in the previous series, which leads to smaller complexes with less pDNA per particle. Therefore, it is clear that the plasmid/NP ratio is not only determined by the charge ratio, but also by the concentration at which the complexes are prepared, as was observed in Fig. 2B.
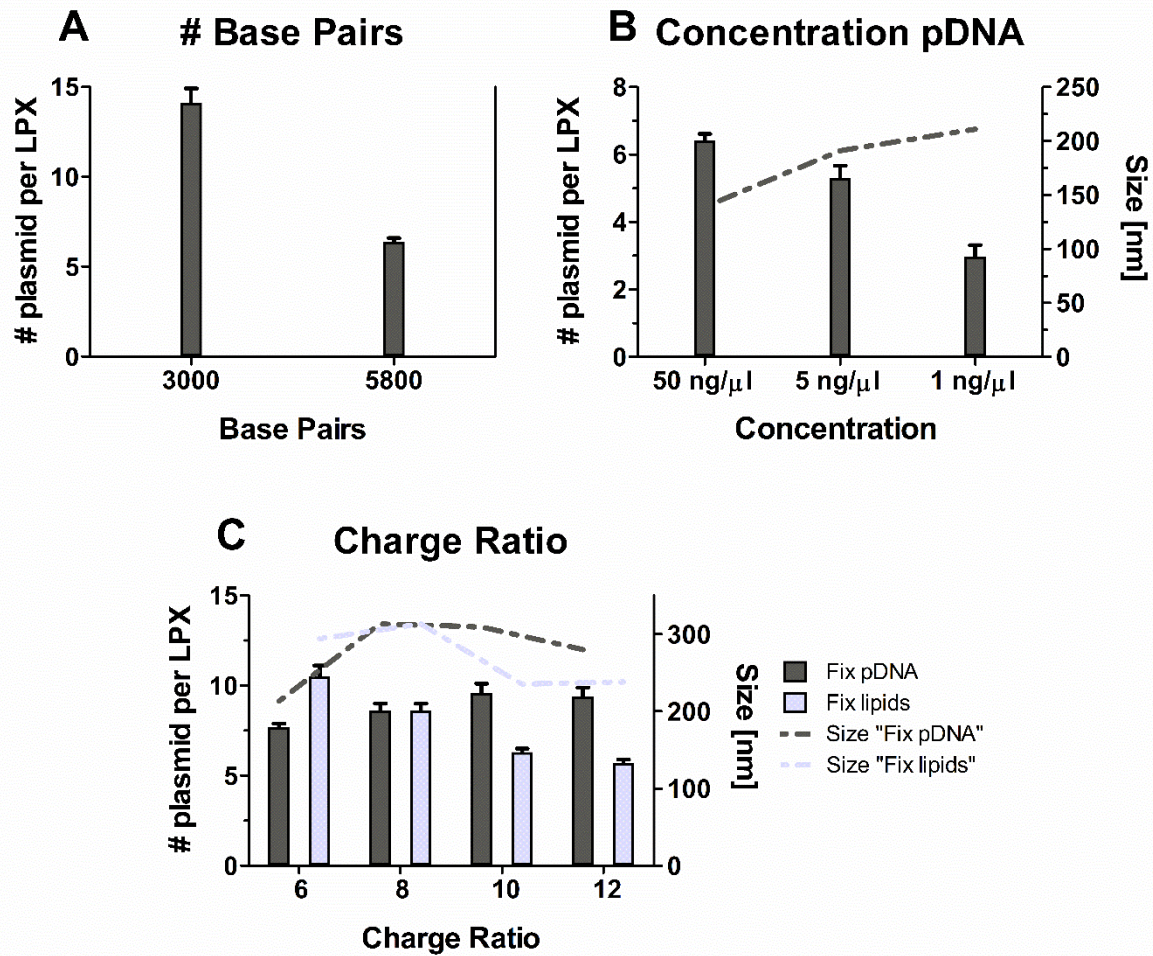
Fig. 2. The effect of pDNA size, LPX charge ratio and pDNA concentration on the number of plasmids per LPX. (A) Effect of plasmid size on plasmid/NP ratio. Halving the number of base pairs in the plasmid doubles the plasmid/NP ratio, that goes from 6.4 ± 0.2 plasmids/NP to 14.1 ± 0.8 plasmids/NP. (B) Effect of concentration on the plasmid/NP ratio. Both the pDNA and the liposome solutions were diluted ten and fifty times (pDNA concentrations from 50 ng/μl to 5 ng/μl and 1 ng/μl) to form LPXs at a fixed charge ratio of 8. The decrease in plasmid/NP ratio confirms that the complexation is concentration-dependent. (C) Plasmid/NP ratio in function of charge ratio. LPXs were complexed in a fixed volume (20 μl) by keeping the amount of plasmids constant at 50 ng/μl (dark gray) or by keeping the amount of lipids constant at 1.2 mM (light gray).

PEGylated lipoplexes

PEGylation of nanoparticles is a common strategy to improve their transport and stability in biological tissues. For instance, it can prevent mucoadhesion[21] and increases the nanoparticle half-life and colloidal stability in biofluids[22, 23]. However, PEG chains may also reduce the interaction strength between pDNA and liposomes, which in turn could affect the plasmid/NP ratio. Here, we investigate this for two common PEGylation strategies. In the first PEGylation approach, referred to as PRE-PEGylation, PEGylated lipids are added to the lipid mixture of the liposomes in the round-bottom flask, before water is added to form liposomes, thus before complexation with pDNA[21, 24, 25]. In the second strategy the PEG chains are added to the LPX dispersion after formation of the complexes, referred to as POST-PEGylation[21, 25, 26]. We used DSPE-PEG$_{2000}$ and tested the effect of PRE- and POST-PEGylation, as well as the influence of the amount of PEG on the plasmid/NP ratio of LPXs (at fixed charge ratio 8). The graph shown in Fig. 3 indicates a substantial drop in the plasmid/NP ratio of PRE-PEGylated LPXs as the amount of PEG increases (from 6.4 ± 0.2 to 3.5 ± 0.1 to 1.8 ± 0.2 plasmid/NP). An unpaired t-test comparing the values of non-PEGylated complexes to the values of PRE-PEGylated complexes shows that the means are significantly different in all cases ($p < 0.05$). The substantial drop in plasmid/NP ratio can be explained by the steric hindrance of the PEG chains and the shielding of cationic charges due to which the interaction with pDNA is reduced. At the same time the LPX size increased, again showing that LPX size alone cannot be used to infer the plasmid/NP ratio. POST-PEGylation, on the other hand, did not have such a strong impact on the plasmid/NP ratio with only a very small reduction in the number of plasmid/NP, that from 6.4 ± 0.2 plasmid/NP is reduced to 5.0 ± 0.1 and 5.6 ± 0.2 plasmid/NP. This is likely due to the fact that the LPX are already formed at the time that the DSPE-PEG is added so that (most of) the pDNA remains stably incorporated. An unpaired t-test comparing the values of

non-PEGylated complexes to the values of POST-PEGylated complexes shows that the means are significantly different in all cases in which DSPE is used as a linker chain ($p < 0.05$), while.

Finally, we tested if the anchor lipid that is used to post-functionalize LPX with PEG may have an influence. Therefore, we evaluated POST-PEGylation with 10 mol% of Ceramide-PEG_{2000} instead of DSPE-PEG. Ceramide-PEG is sometimes preferred to enhance the interaction with the anionic cellular membrane as it is less stably incorporated into LPX as compared to DSPE-PEG[23, 27]. Post-PEGylation with Ceramide-PEG again had no influence on the plasmid/NP ratio compared to the non-pegylated LPX ($p = 0.36$), showing that it influences LPX composition even less than DSPE-PEG.
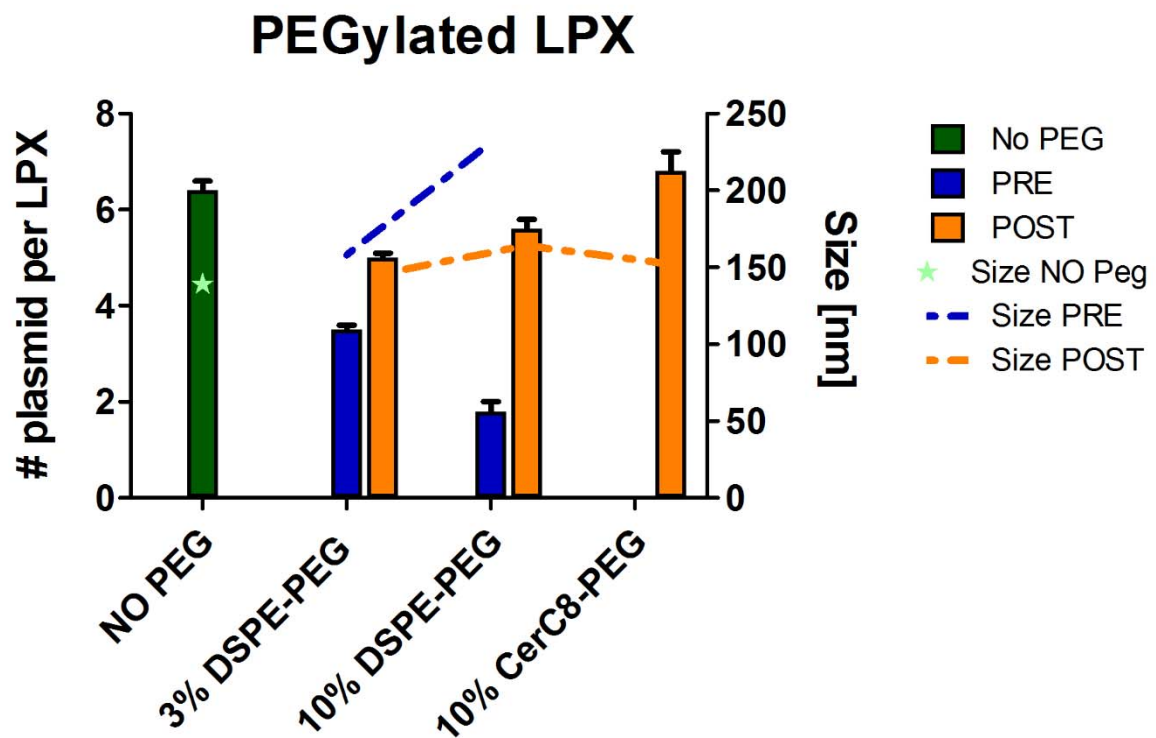

Fig. 3. Influence of PRE- and POST-PEGylation on the number of plasmid copies per LPX. Liposomes were PRE-PEGylated by incorporation of the indicated percentage of PEGylated DSPE lipids. POST-PEGylation of LPX was performed with both DSPE-PEG and Ceramide C8-PEG. All LPX were prepared at a charge ratio of 8.

DISCUSSION

   To obtain a safe and efficient delivery of nucleic acid-based therapeutics, a complete and thorough characterization of the carrier-cargo complex is required. The efficiency of the carrier in encapsulating or complexing the therapeutic cargo molecules is an important parameter that may influence the effectiveness of the carrier. To our knowledge, we present here the first reported method for directly measuring the plasmid/NP ratio in a flexible manner. Another reported method makes use of photobleaching to count the number of therapeutic molecules each labelled with a single fluorophore. When continuously excited, the fluorophores will photobleach one after the other, resulting in a step-wise decrease in intensity. By counting the number of steps it is possible to calculate the amount of cargo molecules in the complex. Importantly, this method requires each NA to be labelled with exactly one fluorophore. While there are methods to do that, such as the *in vitro* incorporation of modified nucleotides in the polynucleotide backbone, or the introduction of specific sequences in the plasmid DNA to allow sequence-specific fluorophore attachment[28], these are rather complex procedures and need to be considered carefully. Another limitation of the method is that it is inherently limited to a low number of molecules, because else the step-wise decrease in fluorescence is buried in the overall shot-noise. Instead, our approach based on SPT nanoparticle concentration measurements, does not pose any particular limit on the number of plasmids per nanoparticle. While in principle it does not require the nucleic acids to be labelled when operated in light scattering mode, we did label the plasmids in our study as it improves the detection sensitivity. In any case, the labelling requirements are not as stringent as in the photobleaching method since the number of fluorophores per plasmid does not need to be controlled. Indeed, the only requirement is that the plasmids and complexes are visible in the SPT microscope. The

downside, however, is that only the average number of plasmid/NP is obtained, while the photobleaching methods yield information on a single particle basis.

As a case study, we focused on DOTAP:DOPE liposomes as a model carrier and studied its plasmid/NP ratio for a variety of conditions. The influence of pDNA size, charge ratio of the LPXs and concentration of pDNA vs. liposomes were thoroughly investigated. Remarkably, each of those factors had an influence on the number of plasmids per carrier. First it was noted that LPXs formed with pDNA of half the size had double the amount of plasmids per LPX when prepared at the same charge ratio. It must be noted that by halving the size of the pDNA while keeping the weight concentration (in ng/µl) constant, the actual number of pDNA molecules doubled. This is exactly as one would expect and provides a validation of the proposed method. Then a slight decrease in plasmid/NP ratio was noticed when the charge ratio was increased by keeping constant the amount of lipids and varying the pDNA concentration, while the opposite trend was observed when the charge ratio was modified by keeping the concentration of pDNA constant and adjusting the amount of liposomes. Finally, we observed that preparing lipoplexes of fixed charge ratio at lower concentrations of pDNA and liposomes reduces the plasmid/NP ratio.

In general, these observations indicate that the charge ratio itself is not the main factor that influences the number of plasmid per carrier, but it seems that this number also depends on the potential collisions between the plasmids and the liposomes. Indeed, the plasmid/NP ratio per nanoparticle substantially increases when the concentration of the nanocarriers constituents increases and/or when the pDNA size is decreased, leading to a higher amount of pDNA molecules for the same weight concentration.

It should be noted that to calculate the number of pDNA per nanoparticle, the empty liposomes that did not bind fluorescent pDNA remain invisible for the SPT instrument. Also, we cannot determine how many liposomes come together to form a lipoplex with the pDNA.

All observations above, however, point out that it is mainly the concentration of the pDNA solution that dictates the plasmid/NP ratio, with more plasmid/NP when a higher pDNA starting concentration is used. This also points to the fact that complexation is a very fast process, as the amount of pDNA in the microenvironment seems to determine the amount of plasmid/NP, and not the overall ratio of pDNA and liposomes for which one could expect that similar ratios should be obtained when a large enough equilibration time is allowed. Based on our direct experience and the works of Pires et al.[29] and Balbino et al.[30], we expect complexation to happen in the first few seconds to minutes. Since we allowed 30 min of complexation before measuring the LPX concentration, the equilibrium has already been reached. Therefore we conclude that when the number of plasmid/NP decreases, this indicates that the complexation conditions helped the spreading of pDNA among the otherways uncomplexed liposomes, or vice versa, when the plasmid/NP increases, there are more uncomplexed liposomes in the suspension (or more liposomes fused together to form the complexes).

Another factor that we investigated was the influence of PEGylation on the plasmid/NP ratio. Functionalization of nanoparticles with PEG chains to improve their colloidal stability in buffers and biofluids, and to prolong their circulation time *in vivo* has been widely studied[21, 22, 24, 31]. Here we evaluated the effect of PEGylation on the number of plasmids per LPX and this revealed that the PEGylation method does have a significant impact. With PRE-PEGylated liposomes a decrease in the plasmid/NP ratio was observed with increasing PEG content. Instead, POST-PEGylation of LPX had virtually no effect on the incorporated number of plasmids per nanoparticle. This is most likely due to the fact that PRE-PEGylated liposomes have better colloidal stability, so that they have less tendency to agglomerate when the pDNA-LPX complexes are formed. As a result those LPX will contain less liposomes per NP, also leading to the complexation of less pDNA per LPX. Also, the PEG-chains shield the surface

charge of the liposomes and provide steric hindrance, most likely lowering the affinity of pDNA for the liposomes.

Optimization of nanoparticles for nucleic-based therapy should not focus only on the optimization of the carrier, but also of the carried nucleic acid molecules. As shown in Fig. 2A, the lower the number of base pairs, the higher the number of plasmids per particle. A smaller size is not only beneficial in the complexation process, but also during transfection. It has been reported that plasmid DNA bigger than 6000 bp have a substantially decreased expression compared to smaller constructs[32]. It is believed that this size-dependent reduced efficiency is due to the lower mobility big plasmid DNA experience in the cytoplasm. Minicircle DNAs are the perfect solution for an optimized transfection efficiency. Minicircle DNAs are non-viral closed circular gene expressing vectors to which all the sequences not directly linked to gene transfection are removed. They are smaller and safer than the commonly used plasmids, since the sequence coding for antibiotic resistance is removed, and present longer gene expression due to the removal of their bacterial origin of replication[32, 33].

On the technological side, while we used a custom-built program to analyze SPT data in this study, it is of note that SPT instruments are also commercially available, like those from Malvern (NanoSight) and ParticleMetrix. They are gaining in popularity for nanoparticle characterization to complement DLS measurements. As such the reported method should find easy entrance in the nanomedicine community. For optimal accuracy we do advise though to make use of our reported concentration algorithms which were extensively validated to give correct absolute concentration values in a calibration-free manner[10-12, 14].

A current limitation of the method as reported here is that it requires complete complexation of the cargo and its carrier. Indeed, in case there is still free pDNA in the LPX dispersion, the visible particles in the SPT images cannot be unambiguously assigned to LPX so that its concentration cannot be reliably determined. A workaround could be to label the carrier with a

different fluorophore so that LPX can be identified as particles having both colors while uncomplexed pDNA would only have one color. An alternative approach is to quantify and remove uncomplexed DNA by adding nucleases to the LPX sample after measuring its apparent concentration. The protocol would then require to measure the particle concentration two times, before and after the addition of DNase I. The unbound DNA can thus be quantified as the difference between the particle concentration in the LPX solution (C_{NC}), measured before the addition of DNase I, and the LPX concentration C_{LPX}, measured after degradation of pDNA by the DNase. Thus the number of plasmids per particle in this case can be calculated from

$$\frac{pDNA}{NC}ratio = \frac{C_{complexed\ pDNA}}{C_{LPX}} = \frac{C_{pDNA} - (C_{NC} - C_{LPX})}{C_{LPX}}$$

where C_{pDNA} is the concentration of pDNA in the solution, complexed and unbound, and must be measured or calculated before complexation with the carriers.

Finally, it is important to note that the presented SPT method is not limited to plasmids, but could be used for other and smaller nucleic acids as well. The only requirements are that (1) the concentration of nucleic acids is known or can be measured precisely beforehand, (2) that 100% complexation is obtained, and (3) that the final complexes can be visualized by SPT (scattering or fluorescence). Furthermore, in case the (liposomal) carrier would be labeled, the same SPT method could be used to determine the carrier number concentration, which would allow to get even more detailed insight into the formation and composition of pDNA-carrier complexes.

CONCLUSION

Prior to fulfilling their therapeutic effect, nucleic acid/carrier complexes must overcome numerous barriers that vary according to the administration route. To exploit their full potential, such nanomedicines must be extensively characterized. However, differences in the

complexation conditions, such as pDNA size, concentration of the components and type of protocol used to PEGylate the nanoparticles, introduce differences on the loading degree per nanoparticle. In future research it is of interest to investigate for lipoplexes but also for other carriers how that may influence the biological effectivity of the formulation. We have proposed to take advantage of SPT nanoparticle concentration measurements and apply it to determine the number of plasmids per nanoparticle, a type of characterization that was not possible before. Given the fact that SPT instruments are commercially available and are increasingly being used for nanoparticle characterization, we believe that the demonstrated method can find rapid acceptance in the field and contribute to the development of safer and more efficient nanoformulations for nucleic acids based-therapies.

FIGURE CAPTION

Fig. 1. SPT number concentration measurements of a dilution series of (A) Cy5-labelled gWiz pDNA and (B) DOTAP:DOPE LPX containing Cy5-pDNA at a charge ratio of 8.

Fig. 2. The effect of pDNA size, LPX charge ratio and pDNA concentration on the number of plasmids per LPX. (A) Effect of plasmid size on plasmid/NP ratio. Halving the number of base pairs in the plasmid doubles the plasmid/NP ratio, that goes from 6.4 ± 0.2 plasmids/NP to 14.1 ± 0.8 plasmids/NP. (B) Effect of concentration on the plasmid/NP ratio. Both the pDNA and

the liposome solutions were diluted ten and fifty times (pDNA concentrations from 50 ng/µl to 5 ng/µl and 1 ng/µl) to form LPXs at a fixed charge ratio of 8. The decrease in plasmid/NP ratio confirms that the complexation is concentration-dependent. (C) Plasmid/NP ratio in function of charge ratio. LPXs were complexed in a fixed volume (20 µl) by keeping the amount of plasmids constant at 50 ng/µl (dark gray) or by keeping the amount of lipids constant at 1.2 mM (light gray).

Fig. 3. Influence of PRE- and POST-PEGylation on the number of plasmid copies per LPX. Liposomes were PRE-PEGylated by incorporation of the indicated percentage of PEGylated DSPE lipids. POST-PEGylation of LPX was performed with both DSPE-PEG and Ceramide C8-PEG. All LPX were prepared at a charge ratio of 8.

ASSOCIATED CONTENT

Supporting Information.

Gel electrophoresis pictures and their description (Word, docx).

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The manuscript was written through contributions of all authors. All authors have given

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