Mechanistic insights into the superior DNA delivery efficiency of multicomponent lipid nanoparticles: an *in vitro* and *in vivo* study

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KEYWORDS. Lipid nanoparticles; gene delivery; transfection efficiency; membrane disintegration; vaccination.

ABSTRACT Lipid nanoparticles (LNPs) are currently having an increasing impact in nanomedicine as delivery agents, among others, of RNA molecules (e.g., short interfering RNA for the treatment of hereditary diseases, or messenger RNA for the development of COVID-19 vaccines). Despite this, the delivery of plasmid DNA (pDNA) by LNPs in preclinical studies is still unsatisfactory, mainly due to the lack of systematic structural and functional studies on DNA-loaded LNPs. To tackle this issue, we developed, characterized, and tested a library of 16 multicomponent DNA-loaded LNPs, which were prepared by microfluidics and differed in lipid composition, surface functionalization, and manufacturing factors. 8 out of 16 formulations exhibited proper size and zeta potential and passed to the validation step, i.e., the simultaneous quantification of transfection efficiency (TE) and cell viability in human embryonic kidney cells (HEK-293). The most efficient formulation (LNP15) was then successfully validated both in vitro, in an immortalized adult keratinocyte cell line (HaCaT) and in an epidermoid cervical cancer cell line (CaSki), and in vivo, as nanocarrier to deliver a cancer vaccine against the benchmark target tyrosine-kinase receptor HER2 in C57BL/6 mice. Finally, by a combination of confocal microscopy, transmission electron microscopy (TEM), and synchrotron small angle X-ray scattering (SAXS) we were able to show that the superior efficiency of LNP15 can be linked to its disordered nanostructure consisting of small size unoriented layers of pDNA sandwiched between closely apposed lipid membranes that undergoes massive destabilization upon interaction with cellular lipids. Our results provide new insights into the structure-activity relationship of pDNA-loaded LNPs and pave the way to the clinical translation of this gene delivery technology

Introduction

Considering the increasing impact of gene therapy and gene vaccines on the contemporary medical and epidemiological scenario, new strategies to tackle the limitations linked to the use of naked nucleic acids (NAs) in vivo are highly desirable. In this regard, it is well accepted that the employment of gene-delivery systems (GSDs) increases the cellular uptake of NAs and protects them from nuclease-mediated degradation and phagocyte sequestration [1, 2]. In the field, non-viral GDSs represent an attractive platform due to their higher safety profile. Among them, lipid-based nanoparticles (LPNs) proved to be chemically modifiable and poorly toxic or immunogenic GDS [3]. Most of the available LNPs are composed of a mixture of amino lipids generally comprising cationic or ionizable lipids (for 50%). Cationic lipids are naturally able to encapsulate nucleic acids (NA) which are negatively charged [4]. Similarly, ionizable lipids are cationic at acid pH and neutral at physiological pH. This feature allows the interaction with the NA cargo during the synthesis procedure, performed using acid buffer, and later into the endosomal compartment promoting fusion with cellular organelle membrane and leading to massive NA release into the cytosol [5]. The conventional methods to obtain lipid-based delivery systems (e.g., bulk mixing) are multistep procedures suffering from poor reproducibility and scalability. Innovations in manufacturing technology led to the shift to the most reproducible methods, including the microfluidic mixing [6]. This technique is based on the rapid mixing of two solutions, one constituted by the lipid dissolved in organic solvent and the second by NA in an aqueous buffer. These are forced to pass through thin channels in which lipids and NAs assemble under controlled conditions generating small and monodisperse LNPs suspension[7]. This single-step bottom-up approach ensures high reproducibility and lowers variability with respect to other synthesis procedures [8]. Furthermore, by changing manufacturing conditions such as lipid formulation, microfluidics parameters (total

flow rate (TFR) and flow rate ratio (FRR)) and the lipid/NA ratio it is possible to tune the chemical-physical properties of LNPs, aiming to obtain safe and effective GDSs. Building on these technological improvements, lipid-based systems for siRNA and mRNA delivery are currently successfully employed both in pharmaceutics and vaccinology. For the first time in 2018, the FDA approved the clinical use of an LNP-formulated siRNA, Onpattro®, which is used for the treatment of polyneuropathies caused by hereditary transthyretin-mediated amyloidosis (hATTR)[9]. The latest clinical success achieved by LNPs as a delivery system is the mRNA vaccines developed to face the SARS-Cov2 emergency (Moderna, BioNThec, and CureVac)[8]. Despite the clinical successes obtained by lipid-based GDSs for mRNA and siRNA delivery, the same objective has not been achieved by LNPs encapsulating pDNA.

DNA vaccines are genetically engineered DNA plasmids that express an antigen. They reserve several advantages with respect to RNA-based, including a lower cost of production, a higher thermostability (they do not require a strict cold chain for distribution), an easier and more rapid manufacturing process. Thus, they represent a valuable alternative to mRNA vaccines. However, an efficient pDNA delivery system is still lacking. mRNA vaccines need to cross only one membrane to reach the cytoplasm, where they can be quickly translated into antigenic proteins, whereas DNA vaccines need to cross the cytoplasm and the nuclear membrane, to be first transcribed in the nucleus and then translated in the cytoplasm. These differences make more difficult to develop LNPs for efficient DNA delivery with respect to RNA. To overcome this issue, physical methods of DNA delivery, such as electroporation, are usually used. They enhance cell transfection and consequently antigen expression and immune stimulation. Recently, two DNA vaccines developed against SARS-CoV-2, ZyCoV-D and INO-4800, demonstrated that DNA vaccines are safe and able to induce a protective immune response[10-12]. Vaccination with ZyCoV-

D, administered intradermally via a needle-free injection system, was found to be efficacious and resulted in generation of both humoral and cellular response in a phase 3 clinical trial. INO-4800, when administered via intradermal injection followed by electroporation, displayed excellent safety, tolerability and immunogenicity. However, widespread administration of DNA vaccines might be limited by production of these physical devices (i.e. electroporators or injectors). Considering that the development of effective DNA delivery system is essential to achieve therapeutic goals, in order to optimize LNP specifically conceived for pDNA delivery, in this work we synthesized a library of multicomponent LNP formulations for DNA encapsulation which differed in lipid composition, surface functionalization, and manufacturing factors and we explored their application in the delivery of an anticancer DNA vaccine. To assess the efficiency of the systems, we applied a multistep experimental strategy which starts from the intra-structural characterization of the LNPs to the *in vitro* and *in vivo* biological validation.

We believe that our findings provide insights into the structure-activity relationship of DNA-loaded LNPs and contribute to filling the knowledge gap that still limits the clinical translation of this gene delivery technology.

Results

Chemical-physical characterization of LNPs

A library of 16 LNPs with systematic changes in lipid composition was obtained through microfluidic mixing with plasmid DNA (pDNA) coding for the firefly luciferase reporter gene (Table S1 in the Supporting Information). LNPs were characterized for their size and zeta potential through DLS and micro-electrophoretic measurements. The results are summarized in Table S2 in the Supporting Information. As shown in Fig. 1a, the size of LNPs ranged from about 100 nm to

1000 nm, whereas the polydispersity index (PdI) values were comprised within the range between 0.1 and 0.5.

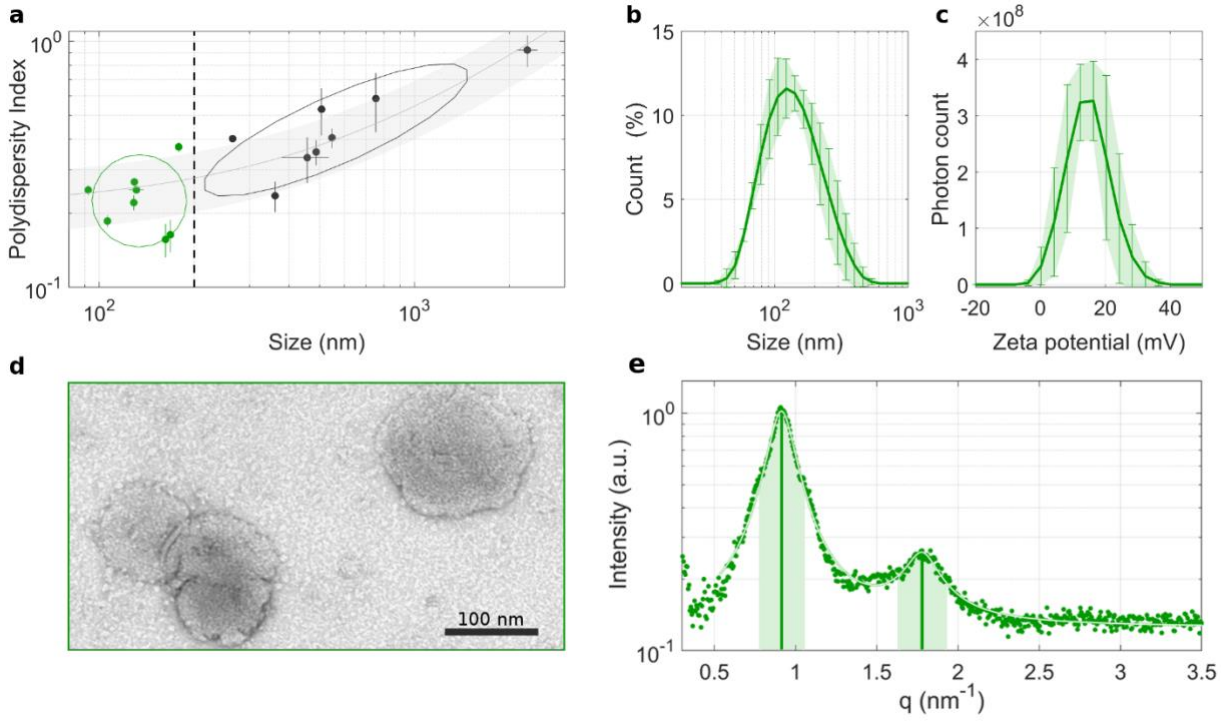


Figure 1. Synthetic identity of LNPs. (a) Scatterplot of polydispersity index vs size for all the employed LNPs (green and black dots ± standard deviation). The vertical dashed line indicates the size threshold for further in vitro validations. The solid line indicates the best linear fit to the data. (b) Size distribution, (c) zeta potential distribution. (d) Representative transmission electron microscopy (TEM) image for LNP15. (e) Synchrotron small-angle X-ray (SAXS) pattern of LNP15.

A positive correlation between size and PdI was detected, i.e., the lower the particle size, the lower the PdI. This trend is depicted in Fig. 1a as a solid line in a shaded area, which represents a linear correlation within the experimental errors. Thus, depending on the employed lipid mixture and mixing parameters, the microfluidic manufacturing procedure yielded monodisperse solutions

of small LNPs (green dots) or less-homogeneous dispersions of large LNPs (black dots). According to the literature[13], we set a threshold value of 200 nm as a cutoff size (indicated as a vertical dashed line in Fig. 1a), beyond which LNPs were excluded from further analyses. Out of 16 formulations, 8 LNPs were smaller than the threshold, with an average size of about 130 nm and an average PdI of 0.235. Representative size distribution of a representative sub-threshold LNP formulation (i.e., LNP15) is reported in Fig. 1b, along with the corresponding zeta potential distribution (Fig. 1c). Structural analysis was performed through TEM and synchrotron SAXS. TEM experiments (Fig. 1d) confirmed the DLS findings, whereas synchrotron SAXS experiments were aimed at exploring the inner particle structure. A representative synchrotron SAXS pattern is shown in Fig. 1e (green dots), along with the corresponding multi-Lorentzian fitting curve (solid line), according to Eq. 1. All the measured fitting parameters are listed in Table S3. The SAXS curve exhibited two broad Bragg peaks located at q100= 0.91 nm-1 and q200 = 1.79 nm-1 (i.e., $q200 \approx 2q100$), indicating a spatial periodicity along the normal direction to the lipid bilayer, with a d-spacing = $2\pi/q100$ = 6.83 nm. This is the typical pattern of a lamellar lipid/DNA phase[14] whose repeat unit is made of a monolayer of hydrated DNA molecules (~ 2,5 nm) sandwiched between opposing lipid membranes ~ 4 nm thick. Interestingly, despite the amplitude decrease of the second peak with respect to the first one (i.e., B < A in Eq. 1), no significant peak broadening was detected (i.e., $b \approx a$). The occurrence of Bragg peaks of equal width whose amplitudes decrease exponentially with the diffraction order is due to the thermal disorder of lamellar lattices [15]. This kind of disorder usually referred to as “first order disorder”, is generated by small fluctuations around well-defined positions of equal separation with the result that the structure factor decreases exponentially with the Debye-Waller temperature factor. Taking into account the full width at half maximum (FWHM = Δq = 0.22) of the Bragg peak, the average domain size of DNA-lipid layers

can be measured by using the Debye-Sherrer relation: $Lm = 2\pi/\Delta q \sim 28$ nm. This value corresponds to an average domain size that is compatible with stacks made on average of N=4 repeating units. SAXS experiments performed on the other LNP formulations provided similar results. The main difference was found for the average domain size, i.e., in the number of repeating units. This aspect could play a key role in TE and shall be deeply discussed in the following sections.

In vitro validation: transfection efficiency and cell toxicity of LNPs

After completing the first step of the screening procedure, the 8 selected LNPs were screened for their ability to induce firefly luciferase expression in human embryonic kidney (HEK-293) cell line. Transfection reagent Lipofectamine™3000 was used as a positive control. As our goal was to achieve LNPs with high TE and low toxicity, cell viability expressed as a percentage with respect to non-treated cells was assessed for each of the investigated systems. In Fig. 2a, negative controls are reported as blue histograms having 100% cell viability and no luciferase expression (TE= 0), while positive controls are represented as red histograms and exhibited average TE= 4 and about 50% cell viability. Results for all the tested formulations are depicted as green histograms. The best candidate among the LNPs included in the library can be identified by coupling TE and cell viability values in a scatter plot (Fig. 2b). Each dot corresponds to a specific LNP, and its location is determined by the measured values of cell viability (x-axis) and TE (y-axis). Highly transfecting LNPs are generally cytotoxic, leading to cell viability values of about 50% (e.g., LNP12 and LNP14), whereas the most biocompatible system was the less performant as a transfecting agent (i.e., LNP13). LNP16, LNP8, and LNP15 exhibited comparable TE values, but increasing cell viability, which read 40%, 75%, and 86%, respectively. Based on the results of Fig. 2, LNP15 was identified as the most promising formulation for further validation, as it

exhibited the best compromise between high TE and good biocompatibility. Further considerations about the role of PEGylation and the lipid/DNA ratio on the TE of LNPs are reported in the Supporting Information.

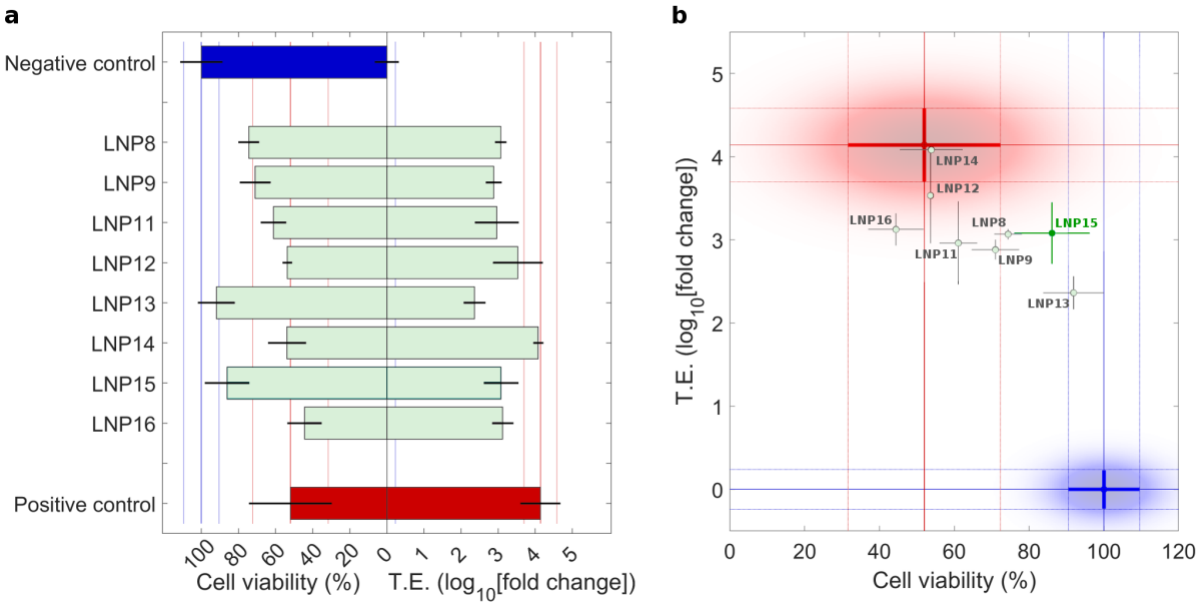


Figure 2. Transfection efficiency and cell viability of LNPs. (a) Histogram chart and (b) scatter plot of HEK-293 cell viability (with respect to non-treated cells) and transfection efficiency (expressed as fold-change with respect to non-treated cells) for LNPs. relationship.

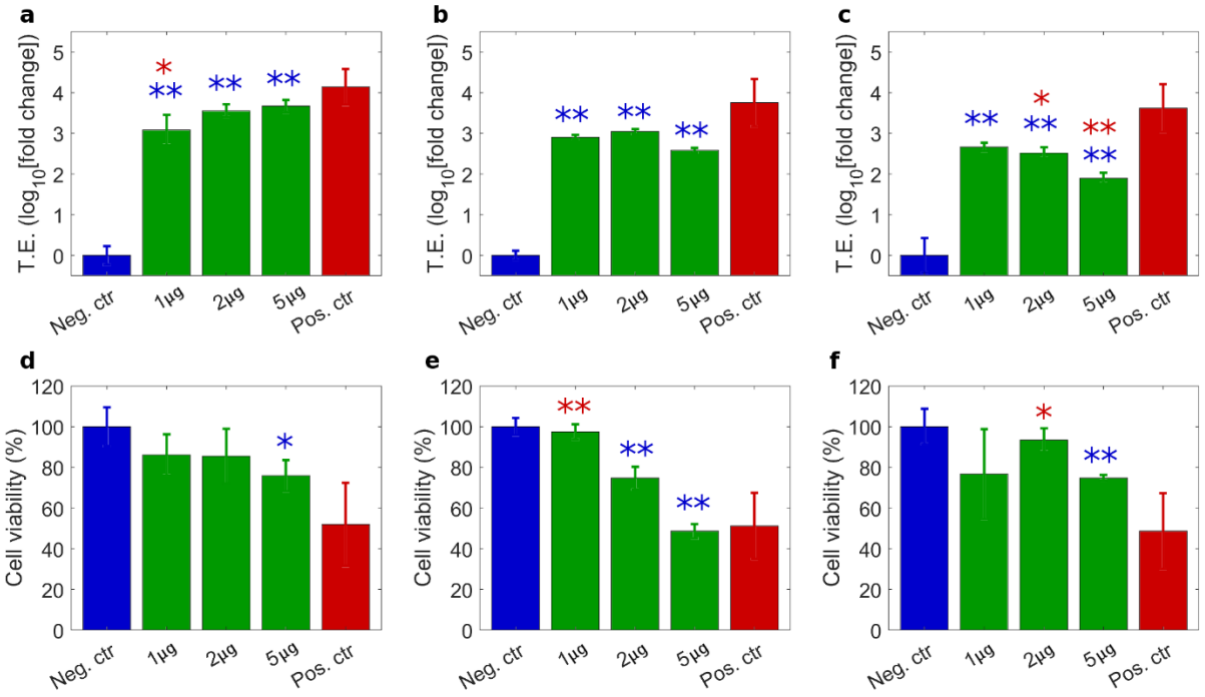


Figure 3. Effect of DNA dose on transfection efficiency (TE) and cell viability of LNP15. TE and cell viability of LNP15 on (a, d) HEK-293, (b, e) HaCaT, and (c, f) CaSki cell lines at different pDNA amounts, as indicated in the x-tick labels. Statistical significance was evaluated by p-value from Student's t-test, results with respect to negative and positive controls are expressed with blue and red asterisks, respectively. ** $p<0.01$, * $p<0.05$, no asterisks $p>0.05$.

Previous investigations[16] identified the DNA dose as a key factor regulating the transfection performances of lipid systems, thus we further validated LNP15 at three different doses (i.e., 1, 2, and 5µg DNA/well) in HEK-293 cells. As the skin is a usual vaccination site, due to its ability to induce both humoral and cellular immunity, we also transfected two epidermal cell lines, i.e., immortalized adult keratinocytes (HaCaT) and epidermoid cervical cancer cells (CaSki) cells. TE and cell viability are shown in Fig. 3 a-c and Fig. 3 d-f respectively. As Fig. 3 a-c clearly shows, the trend of TE as a function of the DNA dose was strictly dependent on the cell line.

Administration of LNP15 to HEK-293 and CasKi resulted in TE boost and diminution respectively. On the other hand, when HaCaT cells were treated with LNP15, TE showed poor dependence on the DNA dose. Cell viability decreased with the DNA dose in HEK-293 and HaCaT cells, while a minor effect was detected in CaSki cells.

As a final validation step of our study, we evaluated the ability of LNPs in delivering anti-HER2 DNA vaccine pVAX-hECTM both *in vitro* and *in vivo*. This cancer vaccine has been developed against the tyrosine-kinase receptor HER2, which is considered an ideal target in cancer immunotherapy due to its crucial role in the epithelial transformation and its selective overexpression in cancer tissues, such as breast cancer [17-19]. Since HER2 is exposed on the cell membrane, it can be targeted both by antibodies and cell-mediated immunity. It was previously reported that pVAX-hECTM is able to induce an antitumor immune response in breast cancer preclinical models when administered by electroporation [20-22]. Thus, we encapsulated the DNA vaccine into LNP15, while the less effective formulation (i.e., LNP13) was used as a negative control. Immunofluorescence experiments reported in Fig. 4 and in Fig. S1 in the Supporting Information were aimed at demonstrating that transiently transfected cells can express the oncoantigen HER2 encoded by the DNA vaccine and display it on the cell surface.

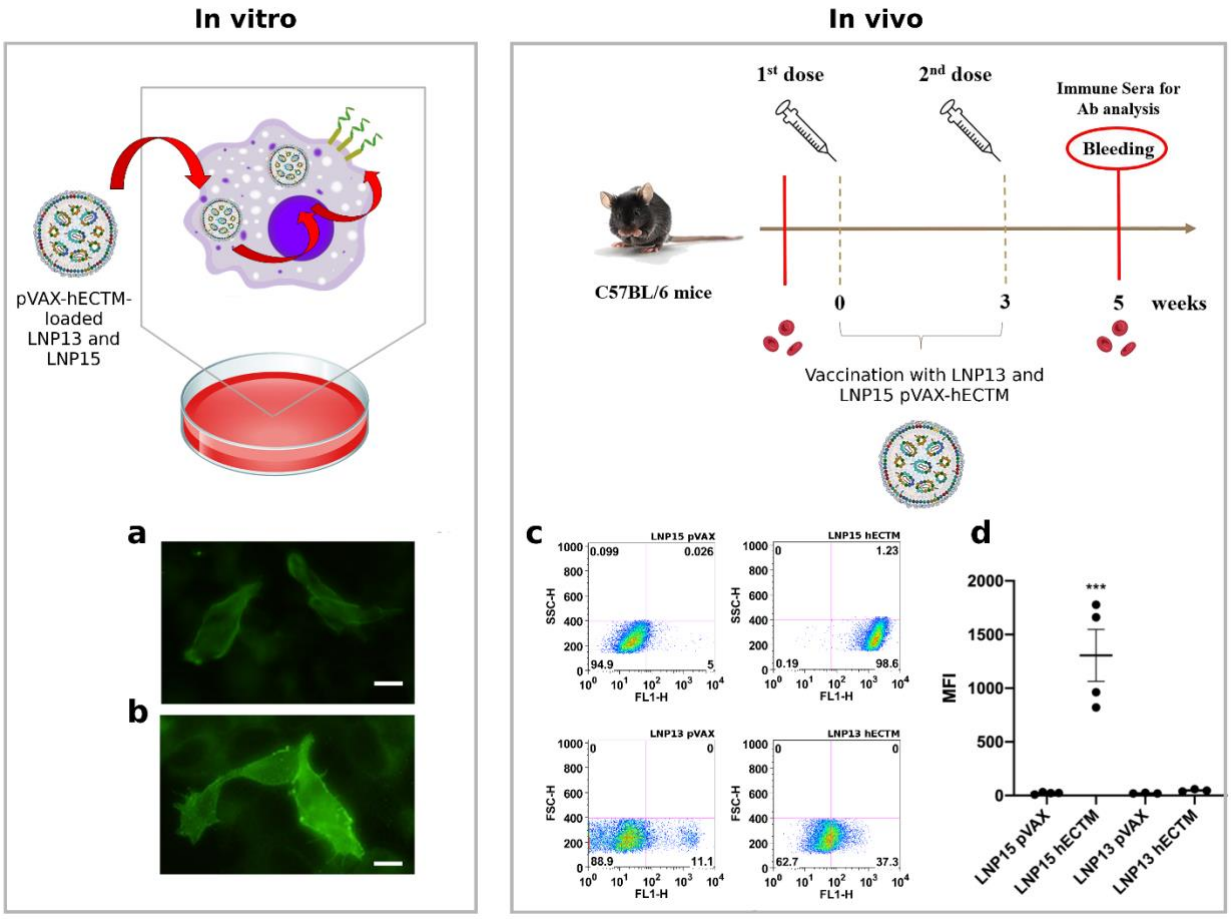


Figure 4. In vitro and in vivo validation of vaccine-loaded LNPs. HEK-293 cells were transiently transfected with the pVAX-hECTM-loaded LNP13 (a) and LNP15 (b) and analyzed under a fluorescence microscope 48 hours after transfection. To detect the expression of the HER2 antigen in the membrane, trastuzumab was used as the primary antibody, and cells were stained using a secondary fluorescent antibody (Alexa-fluor 488). Magnification 40x (scalebar = 1 μm). (c) FACS analysis of the anti-HER2 antibody response induced in C57BL/6 mice (n=4/experimental group) by the pVAX-hECTM vaccine in comparison with pVAX1 empty vector (control), both encapsulated in LNP15 or LNP13, administered by intramuscular (i.m.) injections into the tibial muscle two times at 3 weeks interval. Sera from immunized mice were harvested two weeks after

the last booster and tested on SK-BR-3 target cells. (d) Mean Fluorescence intensity (MFI). Data are shown as MFI ± SEM; t-test **** P <0.0001.

As shown in Fig. 4 a-b, HEK-293 cells were successfully transfected by LNP15 and, to a less extent, by LNP13. HER2 was exposed on the cell membrane, as required for inducing an effective humoral response. Then, the immunogenicity of pVAX-hECTM encapsulated in LNP15 or LNP13 was assessed by vaccinating C57BL/6 mice and evaluating the elicited anti-HER2 antibody response by FACS analysis. Of note, mice immunization with LNP15-pVAX-hECTM triggered a significantly higher antibody titer not only with respect to encapsulated empty plasmids (LNP15-pVAX, LNP13-pVAX), which were used as negative controls but also in comparison to LNP13-pVAX-hECTM, that was not able to induce a detectable antibody production (Fig. 4 c-d).

Motivated by in vivo results we explored the mechanisms of action of LNP15. An accepted paradigm in gene delivery states that efficient GDSs must be able to overcome transfection barriers and release the gene payload into the intracellular environment. Although the unbinding of DNA from lipid vesicles is not completely understood, several reports demonstrated that it depends on charge neutralization by cellular anionic lipids[23]. To estimate the role of this process, an established approach consists in mixing GDSs with negatively charged anionic liposomes, intended as a model system of cellular membranes, and then measuring DNA release[24]. Indeed, this methodology proved to be a robust and versatile tool for predicting the TE of lipoplexes [25]. Thus, we incubated LNP15 with DOPG liposomes and quantified DNA release by fluorescence-based measurements. LNP13 was used as a negative control. As shown in Fig. 5a, the amount of DNA released from LNPs monotonously increased as the DOPG/LNP mass ratio increased.

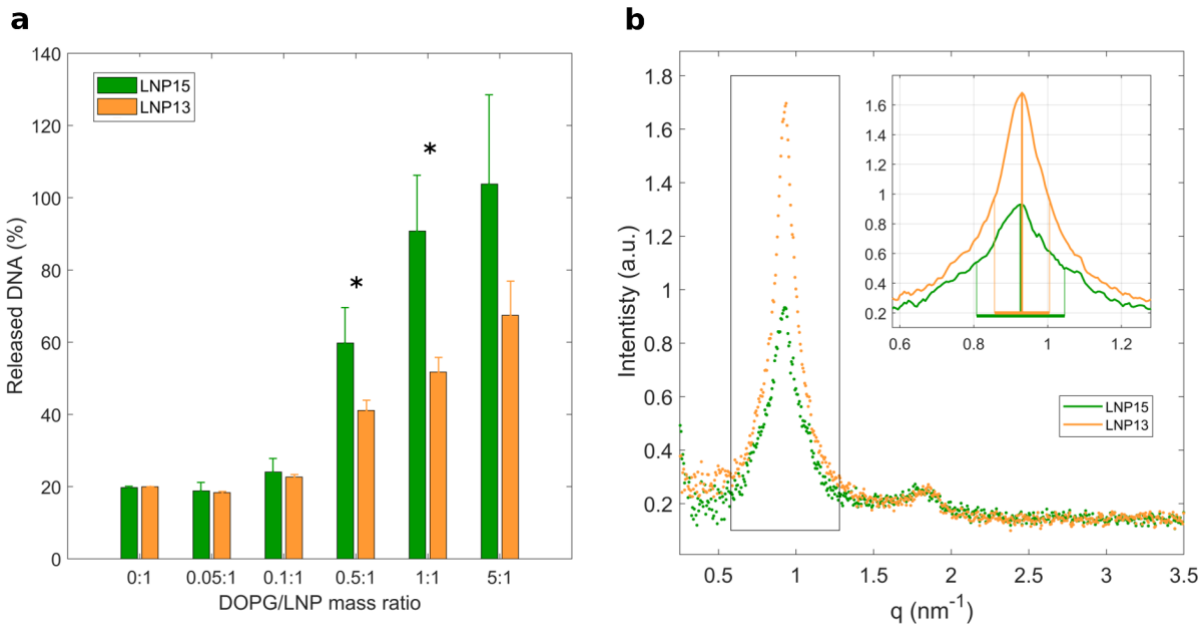


Figure 5. (a) Amount of pDNA released from LNP15 and LNP13 upon interaction with anionic liposome DOPG as a function of the DOPG/LNP mass ratio. (b) Synchrotron SAXS patterns of LNP15 and LNP13. The inset shows the first-order Bragg peaks.

However, starting from DOPG/LNP mass ratio = 0.5, LNP15 released significantly more DNA than LNP13. As a next step, we investigated the mechanism underlying such a different ability in releasing the gene payload. The research performed over the last two decades clarified that the structure of lipidic GDSs changes radically upon interaction with cellular lipids and that these changes are critical for endosomal escape[25, 26]. It was also clarified that lipid mixing that leads to the fusion and formation of transient structures is the main factor controlling the release of the gene payload from lipid GDSs[27]. Thus, we employed synchrotron SAXS to verify whether the different abilities of LNP15 and LNP13 to release DNA may be related to their inner structure. As Fig. 5b shows, the SAXS patterns of LNP15 and LNP13 exhibited a set of two equidistant Bragg peaks (q001 ~ 0,9 nm-1 and q002 ~ 1,8 nm-1). On the other side, the first-order Bragg peaks of

LNP15 and LNP13 (Fig. 5b, inset) had different heights and widths. In light of the above-reported considerations, we conclude that both formulations are made of locally ordered domains that are oriented in different directions. The main difference between the two structures resides in the spatial extension of the lamellar domains that is larger for LNP13 (compatible with N > 6 repeated units) than for LNP15 (compatible with N = 4 repeated units). Multilayered lipid systems have been long used in pharmacology to improve the structural stability against solubilization and fusion[28]. Moreover, it is also well known that the membrane stability of multilayered systems directly correlates with the number of layers. Therefore, we are tempted to conclude that the superior ability of LNP15 in releasing DNA may correlate with the low number of layers it is made of and, in turn, with a natural tendency to be destabilized by cellular membranes. In a series of previous investigations[29, 30], we used fluorescence confocal microscopy in live cells to explore the mechanisms of action of lipid systems at the cellular level. From previous investigations[31], we learned that lipoplexes and nanoparticle systems enter the cells through a combination of energy-dependent (ED) and energy-independent (EI) mechanisms. ED internalization routes include some well-characterized endocytic pathways, such as phagocytosis, macropinocytosis, caveolar endocytosis, and clathrin-mediated endocytosis. On the other hand, EI internalization mechanisms include fusion, embedment, and direct translocation. These mechanisms are still poorly understood due to experimental limitations and are currently under investigation by computational methods[32]. It has been shown that EI processes may contribute to efficient cell internalization for some NP types. Thus, we first measure TE at 37 °C where all the internalization mechanisms are active, and at 4°C where endocytosis is blocked and only EI uptake can take place. Results reported in Fig. 6a show that at 4°C the TE of LNP15 is one order of magnitude lower than at 37 °C suggesting a minor involvement of EI mechanisms in particle internalization. Among intracellular barriers to

efficient gene delivery, lysosomal accumulation is probably the most rate-limiting one[33]. Upon entrapment within the lysosomal compartment, LNPs undergo metabolic degradation and transfection fails. We, therefore, quantified the colocalization of LNP15 with lysosomes in HEK-293 cells at both 37°C and 4°C. Representative confocal images are reported in Fig. 6 b-e, where the green signal corresponds to fluorescently labeled lipids, the red signal identifies lysosomes, and two-channel colocalization is depicted as yellow pixels. At 37C° we observed that some intact fluorescently labeled LNPs colocalize with lysosomes (Fig. 6b). The presence of these spot-like structures is compelling evidence of lysosomal accumulation. On the other hand, however, we noticed that large regions of the cell cytosol were uniformly stained in green (zoomed in Fig. 6d). This peculiar fluorescence distribution does not originate from intact lipid vesicles and has been previously identified as a fingerprint of fusion-like mechanisms [34]. No diffuse fluorescence was observed when cells were treated with poorly efficient LNP13 (Fig. S2 in the Supporting Information). In that case, the intracellular green fluorescence was only visible in the form of spot-like structures, presumably originating from intact vesicles. When experiments were replicated at 4°C such diffuse green fluorescence was not observed in cells, but only localized at the cell membrane (Fig. 6c and magnification in Fig. 6e). Our TE and confocal microscopy results at 4°C agree with recent findings showing that siRNA loaded LNPs bind to cell membranes at low temperature (0-4°C), but are not effectively taken up by the cells with the result that their silencing activity is completely inhibited[35]. Indeed, LNP-lysosome colocalization was remarkably lower at 4°C as compared to 37°C, as calculated in Fig. 6f by the Mander's coefficient that quantifies the portion of green pixels (the lipids) which colocalizes with the red ones (the lysosomes).

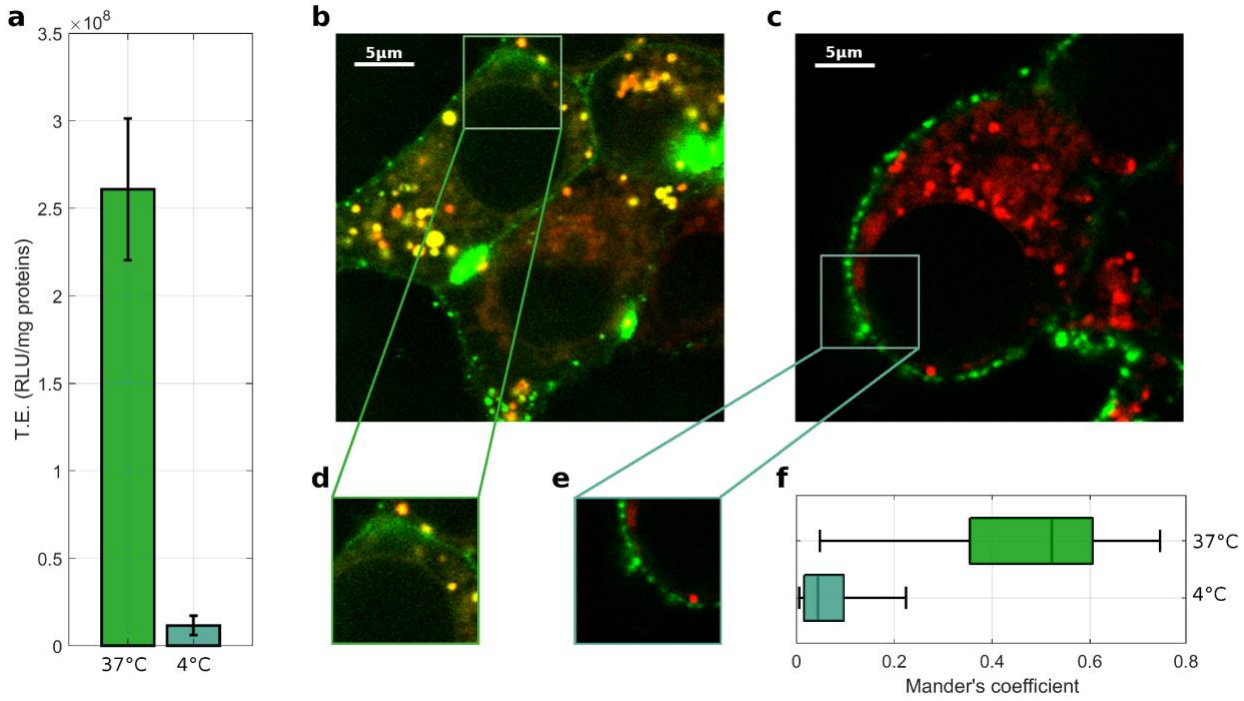


Figure 6. Mechanistic investigation of the intracellular behavior of LNP15. (a) Transfection efficiency of LNP15 at 37 °C and 4°C. Confocal images of LNP15 (green channel) and lysosomes (red channel) after administration to HEK-293 cells at 37°C (b) and 4°C (c). Magnifications are displayed in panels (d) and (e). (f) Quantification of colocalization in terms of the green-to-red Mander's coefficient. Boxplots depict the distributions of measured values over data sets made of n=10 images per class.

Collectively, we conclude that robust intracellular disintegration by anionic cellular lipids can be the main factor accounting for the high TE of LNP15. This mechanism likely involves fusion with cellular membranes and is differentially activated depending on the lipid composition of the LNP formulation.

Discussion

Most efforts have been devoted to encapsulating RNA in LNPs so far while loading DNA has been only marginally investigated. Detailed knowledge of the structure and biological activity of pDNA-LNPs may extend the use of this emerging delivery technology to several biomedical applications that require the delivery of DNA instead of RNA. To fulfill this gap this study started with a screening multistep procedure to optimize LNPs for DNA delivery and thus achieve highly efficient and not cytotoxic GDSs. As a first step, we designed a library of LNPs with systematic changes in influential factors (i.e., lipid composition, PEGylation, microfluidic parameters) that are known to affect the physicochemical properties of GDSs [36-38]. To this end, 16 multicomponent LNPs were prepared through the microfluidic mixing of lipid mixture dissolved in absolute ethanol and pDNA in acetate buffer. A threshold size was set to ~ 200 nm according to the cutoff originally set by Torchilin and co-workers for GDSs in vivo [13]. 2- and 3-component plain formulations were found to be larger in size than PEGylated LNPs. The increase in size correlated with the increase of their polydispersity and is likely due to the presence of aggregates and different-sized populations in the suspension. This finding confirms the relevance of PEGylated lipids, which are commonly used to avoid particle-particle interactions by creating a steric hindrance on the particle surface, as a critical ingredient of DNA-loaded LNPs [39]. We also observe that 3 out of 4 unPEGylated 4-component LNPs had the proper size for in vitro validation. This result indicates that microfluidics determines the formation of LNPs whose size depends on the lipid species involved and their relative molar ratio. As most of the knowledge on the microfluidic LNPs preparation is due to siRNA [40-42] or mRNA [43, 44] encapsulating formulations while little is known on the of LNPs encapsulating pDNA [45], we also explored the effect of the lipid/DNA weight ratio, Rw. LNP10, LNP11, and LNP12 were prepared for this purpose using the same lipid composition

but encapsulating double and half the DNA dose (i.e., Rw= 5 and Rw= 20 respectively). This parameter had a great impact on the resulting LNPs, as the size of the systems increased along with increasing DNA dose, while the zeta-potential followed an opposite trend, i.e., the much lower the DNA concentration more positive the particle charge. Finally, we changed the total flow rate (TFR) from TFR= 2 ml/min to TFR= 8 ml/min in LNP15 and LNP16 respectively. Even with the same lipid composition and amount of encapsulated DNA, these LNPs were different in size and zeta potential, as the increase in the flow rate caused an increase in size and PdI, along with an increase in positive charge. Globally, the physical-chemical characterization of the LNP library allowed us to demonstrate that PEGylation is fundamental to obtaining small-sized LNPs when the number of lipid components is less than 3. Increasing the number of lipid components (n> 4) is a valid strategy for keeping the size of the LNPs below a cutoff of 200 nm. Moreover, lipid/DNA ratio and microfluidic parameters should be carefully evaluated. In our dataset, we found that high TE is usually accompanied by a reduction in cell viability as shown in Fig. 3 both LNP12 and LNP14, even if as effective as the positive control (TE= 3.5 and 4 respectively), exerted non-negligible toxicity on treated HEK-293 cells. With that in mind, we considered LNP15 the best compromise between high TE and biocompatibility, as this system was slightly less effective than the positive control, but considerably less toxic (85% vs. 50% cell viability). Furthermore, to find the best concentration for LNP15 in relation to the toxicity, three different concentrations were tested in three different cell lines, namely HEK-293, HaCaT, and CasKi. The first cell line is a common cell line used for research purposes, while HaCaT and CasKi are epidermal cell lines that were selected given the potential use of LNPs for DNA vaccination. The TE of a lipofection system is not only dependent on the vector but also the cell-to-cell differences. Depending on the cell type and their intracellular trafficking and uptake mechanisms peculiarity, different cell lines may be

easy- or hard-to-transfect [46]. The general trend observed for LNP15 indicated that increasing the particle dose administered to cells resulted in reduced cell viability, but this effect is not accompanied by a clear effect on TE. Finally, we studied LNP15-cell interaction at a cellular and subcellular level. The sketch reported in Fig. 7 summarizes our mechanistic understanding of LNP15-cell interaction. Electrostatic attractions let LNPs approach the anionic surface of the cell, and attachment is followed by endocytosis. Fluorescence confocal microscopy revealed that a portion of administered LNP15 underwent lysosomal degradation, while another one produced a diffuse intracellular fluorescence from labeled lipids (Fig. 6). This is an unusual behavior for lipid systems (e.g. Lipofectamine™3000), as lipoplexes are usually observed as single spots in the cytosol[47]. On the other hand, the behavior of LNP15 resembles that of enveloped viruses that fuse with cellular membranes to infect cells [48]. With respect to viral particles, LNP15 exploits electrostatic attractions with the plasma membrane to lower the barrier due to the repulsive "hydration force" between hydrated lipid bilayers [49]. Combined TEM and synchrotron SAXS analyses explain that LNP15 nanoparticles are small and made of unoriented layers of pDNA sandwiched between closely apposed lipid membranes. The reduced number of layers (N=4) makes this formulation unstable against disintegration by cellular lipids. This event is likely to occur through interactions with anionic lipids that form ionic pairs with cationic lipids [50] and may induce the flattening and opening of LNPs. According to previous literature[51], we suppose that the destabilization rate could depend upon the surface charge density and the lipid composition. This aspect deserves further consideration and will be addressed in future investigations. The instability observed for LNP15 is reminiscent of that reported for multicomponent lipoplexes which undergo entropy-driven mixing with cellular lipids leading to structure destabilization, endosomal escape, and massive DNA release in the cytoplasm [52-55].

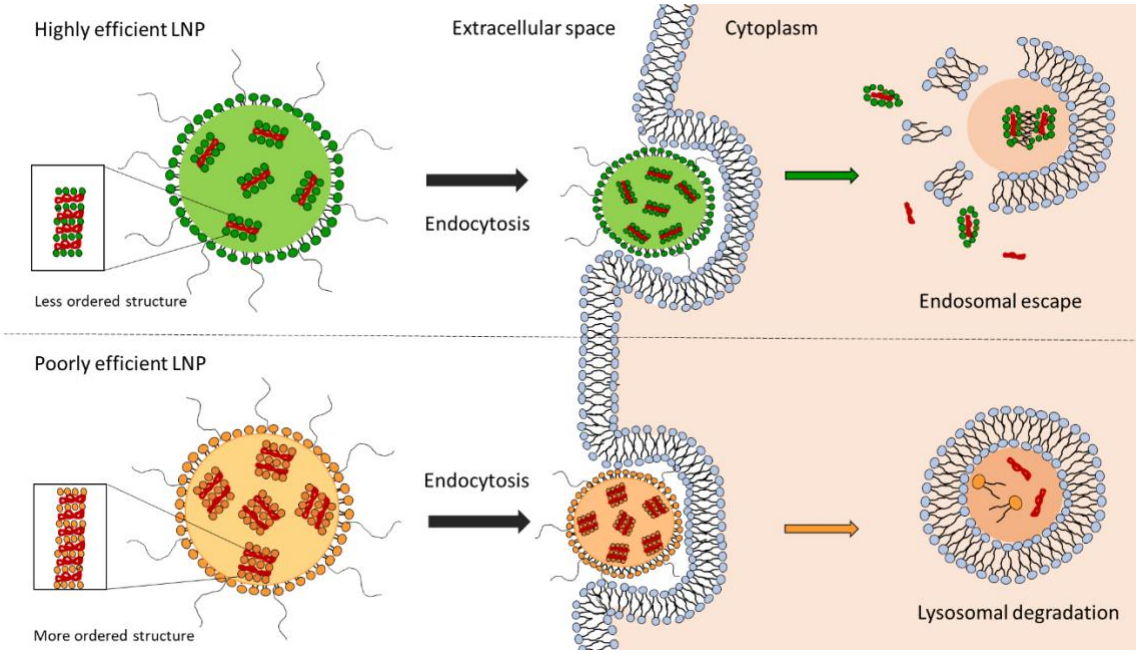


Figure 7. DNA-loaded LNPs exploit electrostatic attractions with the plasma membrane to drop off the repulsion barrier due to the "hydration force" between hydrated lipid bilayers. Particle cellular association is followed by endocytosis. Intracellular disintegration of nanoparticle structure contributes to the discharge of DNA from highly efficient LNPs thus creating optimized conditions for successful transfection.

Materials and methods

Microfluidic Preparation of LNP-pDNA complexes

Cationic lipids 1,2-Dioleoyl-3-trimethyl-ammonium-propane (DOTAP) and (3-[N- (N0,N0-dimethyl-aminoethane)-carbamoyl])-cholesterol (DC-Chol, zwitterionic lipids: dioleoyl phosphatidylethanolamine (DOPE), 1,2-Dioleoyl-sn-glycerol-3-phosphocholine (DOPC), Cholesterol and PEG-lipids: 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DOPE-PEG) and 1,2-distearoyl-sn-glycero-3-

phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). The plasmid used for LNP synthesis, pGL3 (Firefly luciferase encoding plasmid) was purchased from Promega (Fitchburg, WI, USA). Lipid nanoparticles (LNPs) were obtained through a microfluidic mixing device (NanoAssemblr® Benchtop from Precision NanoSystems Inc., Vancouver, BC, Canada) with a Y-shape staggered herringbone micromixer (SHM). All the formulations were prepared by dissolving lipids in different combinations and molar ratios, in absolute ethanol to reach a final concentration of 12.5 mM. PmirGLO (Promega, Italy) was dissolved in 25 mM sodium acetate buffer (pH = 4), to 0.2 mg/mL. The organic solvent where lipids are dissolved and pDNA aqueous solutions are pumped by syringes into the microfluidic device and at the Y-junction they interact forming LNP complexes. Two different total flow rates (TFR; 2 mL/min and 8 mL/min), were explored to get LNP complexes. The formulations were produced at different DNA/lipid weight ratios (Rw = 5, 10, and 20) corresponding to nitrogen to phosphate charge ratio (N/P; nitrogen from the cationic lipid and phosphate from the nucleic acid) of 1.5, 3, and 6. After the micromixing process, the ethanol concentration decreases until 25% of the final LNP suspension volume. LNPs complexes were subsequently loaded on Dialysis cassettes (0.5–3 mL, MWCO 3 kDa, Thermo Scientific, Rockford, MI, USA) and dialyzed for 19 h against 500 mL of phosphate-buffered saline (PBS) at pH 7.4 to remove the residual ethanol.

Size and zeta-potential measurements

Particle size and zeta potential were measured by dynamic light scattering (DLS) and micro-electrophoresis at 25°C using a Zetasizer Nano ZS90 (Malvern, UK). LNP suspensions were

diluted 1:100 with distilled water for the measurements. Results are reported as mean ± standard deviation.

Transmission Electron Microscopy

Transmission Electron Microscopy (TEM) was performed using a TEM Morgagni 268D (Philips, Netherlands). Briefly, samples (total volume = 8 μL) were dropped on formvar–carbon-coated copper grids (EMS, PA, USA) and allowed to adsorb for 5 min, then stained with a 2% uranyl acetate solution for 1 min at room temperature. Before the imaging, the excess staining solution was removed with filter paper.

Synchrotron Small Angle X-ray Scattering

Synchrotron Small Angle X-ray Scattering (SAXS) measurements were carried out at the Austrian SAXS station of the synchrotron light source ELETTRA (Trieste, Italy). A Pilatus3 1 M (Dectris, Baden, Switzerland) detector was employed for data acquisition and calibrated by using silver behenate powder (d-spacing = 58.376). q-range was set within 0.05 and 5 nm-1, with an exposure time of 10 s, which did not yield radiation damage). Correction for background, primary beam intensity, and detector efficiency were included in the analysis of SAXS patterns. SAXS patterns were finally fitted by a multi-Lorentzian function, according to the following equation:

$$I(q) = \sum_{i=1}^{M} \frac{A}{1 + i^2 \left(\frac{q - q_a}{a} \right)^2} + \sum_{i=1}^{M} \frac{B}{1 + i^2 \left(\frac{q - q_b}{b} \right)^2} + k \qquad (1)$$

Where the first and the second sum of Lorentzian functions describe the first and the second Bragg's peak, located at qa and qb, and with width proportional to a and b, and amplitude equal to A and B, respectively. The fitting procedure converged for M=2 with a goodness of R2=0.996.

Transfection Efficiency Assay

In vitro validation of the LNP library was achieved by transfection experiments on human embryonic kidney 293 (HEK-293) cells (ATCC, Rockville, MD, USA), human immortalized keratinocytes (HaCaT) cells, and human cervical cancer (CaSki) cells) (ATCC, Rockville, MD, USA) cell line. HEK-239 and HaCaT cell lines were grown in DMEM while the CaSki cell line in RPMI-1640 and supplemented with 10% FBS and maintained at 37°C with 5% CO2. In transfection experiments, cells were seeded on 24-well plates (40,000 cells per well). Each experiment has been performed in triplicates. Cells were treated with 1 μg formulated pDNA (PmirGLO expressing firefly luciferase, Promega, Italy. Three different DNA amounts 1 μg, 2 μg, and 5 μg per well were explored for LNP15. Lipofectamine™3000 was used as a positive control at 1 μg DNA per well following the standardized protocol (Life Technologies, Carlsbad, CA, USA). Cell lines were treated in Optimem medium (Life Technologies, Carlsbad, CA, USA), with selected LNPs (LNP8, LNP9, LNP11, LNP12, LNP13, LNP14, LNP15, LNP16) or Lipofectamine™3000 and then incubated for 3 h. After treatment, an appropriate medium supplemented by 20% FBS was added to each well to reach a final FBS concentration of 10%, then cells were incubated at 37°C. Luciferase expression was measured after 48 h, through Luciferase Assay System (Promega, Madison, WI, USA). Briefly, cells were washed in a phosphate saline buffer and 60 μL of lysis buffer 1X (Promega) were added to each well. Then, 30 μL of the cell lysate was placed in 3 wells of white Corning® 96Well Solid Polystyrene

Microplate (Sigma-Aldrich, Milan, Italy). The 10 µL present in each well were diluted with 100 µL per well of luciferase substrate (Promega) while the remaining 30 µL were divided into 3 wells (10 µL per well) and used for BCA assay. The transfection efficiency (TE) is expressed as Relative Light Units (RLU) per mg of cell proteins, while the protein amount was determined by Pierce BCA Assay Protein Kit (Thermo Fisher Scientific, Waltham, MA, USA). TE for the generic i-th sample is expressed as the logarithmic fold change with respect to the TE of the negative control, i.e.,

$$TE_i = \log_{10}\left(\frac{I_i/m_i}{I_0/m_0}\right) \quad (2)$$

Where Ii and I0 indicate the detected luminescence for the sample and the negative control, respectively, while mi and m0 represent the measured protein amount by BCA assay for the sample and the negative control, respectively.

Cell Viability Assay

Cell viability of HEK-293, HaCaT, and CaSki cells was evaluated by 2, 3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT assay, cell proliferation Kit II, Roche). Cells were seeded on 96-well plates (10,000 cells/well) for 24 h and incubated at 37°C and 5% CO_2. Cells were treated with LNP complexes or Lipofectamine™ 3000 in Optimem medium for 3 h. After the treatment, DMEM 20% was added to HEK-293 and HaCaT cells and RPMI 20% to CaSki cells, and the cells were incubated for 48 h at 37°C. Then, the XTT solution, prepared as indicated in the kit protocol, was added 50 µL per well and cells were incubated at 37°C for 3 h. After that, the absorbance (450-500 nm) was measured with the Glomax Discover System (Promega, Madison, WI, USA).

Confocal microscopy

Live-cell imaging experiments were performed with a Zeiss LSM 800 confocal microscope equipped with a 63X, 1.4 N.A. oil immersion objective, and GaAsP detectors. Approximately $2x10^5$ HEK-293 cells were seeded in a WillCo glass bottom dish (22 mm) 24 h before the experiment. On the day of confocal acquisitions cells were incubated with LNP13- and LNP15 - TexasRed 1X for 3 hours at 37 C° (or 4 C° to inhibit endocytosis). 30 minutes before confocal acquisition cells were stained with LysoTracker DeepRed (ThermoFisher) for lysosome staining. A series of confocal acquisitions (512x512 pixels, 50 nm pixel size) were taken exciting TexasRed at 561 nm (HeNe laser) and the emission was collected in the 570–630 nm range. LysoTracker DeepRed was excited at 633 nm and emission was collected in the 650-750 nm range. To evaluate the colocalization level between LNPs and lysosomes Manders' and Pearson correlation coefficients were calculated using the JaCoP plugin for ImageJ software.

Immunofluorescence

HEK-293 cells were plated in a 24-well plate (1x105 cells/well). One day after plating, 70–90% of confluent cells were transiently transfected with 1 μg pVAX-hECTM encapsulated in LNP15. Forty-eight hours after transfection, cells were fixed for 10 minutes with phosphate-buffered saline (PBS)–4% paraformaldehyde (Sigma, St. Louis, MO). After incubation in blocking buffer (PBS–10% bovine serum albumin (BSA; Sigma)) for 20 minutes, cells were incubated for 1 hour at 37 °C with the primary antibody trastuzumab (anti-human HER2 antibody, 1:50). After washing, cells were incubated with Alexa Fluor 488-conjugated anti-human IgG secondary antibody (Invitrogen Molecular Probes, Eugene, OR) at a dilution of 1:200 for 1 hour at 37°C. Finally, cells were

examined under Fluorescence Microscope (Carl Zeiss GmbH, Germany), to assess membrane expression of the oncoantigen HER2.

Vaccine preparation

pVAX1 Vector (Life Technologies) and the DNA vaccine pVAX-hECTM (encoding the human extracellular and transmembrane domains of the human HER2 receptor; about 5000 bp) were transformed into *E. coli* strain DH5-alpha and grown in Luria-Bertani medium supplemented with kanamycin. DNA plasmids were purified using a Maxiprep kit (Qiagen) for in vitro transfections and an EndoFree Plasmid-Giga kit (Qiagen, Chatsworth, CA, USA) for in vivo immunization, and their concentration was determined spectrophotometrically at 260 nm. pVAX1 and pVAX-hECTM were encapsulated in LNP15 and LNP13 nanoparticles by microfluidics.

Mice experiments

C57BL/6 mice were housed under controlled temperature (20 °C) and circadian cycle (12 hours light / 12 hours dark). The animals were fed on a chow diet (Mucedola) and tap water ad libitum. Mice were treated in accordance with the U.K. Animals (Scientific Procedures) Act, 1986, and associated guidelines, EU Directive 2010/63/EU for animal experiments, and with the 3Rs principles. All animal experiments were authorized by the Italian Ministry of Health (#708/2021-PR) and by the Animal Research Committee (OPBA) of the National Institute of Health and Science on Ageing (INRCA – Istituto Nazionale di Riposo e Cura per Anziani), Ancona (Italy). C57BL/6 male mice (8-10-week-old) were immunized by intramuscular (i.m.) injections into the tibial muscle two times at 3 weeks intervals with 100 μg of hECTM DNA vaccine or pVAX empty control vector encapsulated in LNP15 (4 mice/ experimental groups). Two weeks after the last

vaccination, blood was collected from the retro-orbital plexus under anesthesia. To collect serum, whole blood samples were left to clot at room temperature for 30 minutes. Serum separation was accomplished by centrifugations at 6.000 rpm at 4°C. Sera from immunized mice were analyzed by flow cytometry (BD FACSCalibur), using human HER-2 overexpressing SK-BR-3 cells as target cells. SK-BR-3 cells were obtained from American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's Modified Essential Medium (DMEM, Gibco, Life Technologies) supplemented with 10 % fetal bovine serum (FBS, Gibco, Life Technologies) and 1 % penicillin-streptomycin (P/S) (Gibco, Life Technologies). Cells were maintained at 37 °C in an atmosphere of 5 % CO2. Briefly, subconfluent cells were detached and dispensed at a density of 10^6 cells per Falcon® 5 mL Round Bottom Polystyrene Test Tube. After a 3 minutes centrifugation at 1.000 rpm at 4°C, the obtained cell pellet was resuspended in staining buffer (0.05% NaN3, 2% FBS in 1x PBS) and incubated with sera of vaccinated mice (1:40 dilution in staining buffer) for 1 hour at 4°C. After incubation, cells were washed three times and incubated with Alexa Fluor® 488 goat anti-mouse IgG secondary antibody (1:200 dilution in staining buffer) for 1 hour, at 4°C. Samples were washed three times with staining buffer, and resuspended in 1x PBS, ready for analysis with BD FACSCalibur. Cell Quest Pro (version 6.0.2) and FlowJo (version 8.7) were used as acquisition and analysis software, respectively.

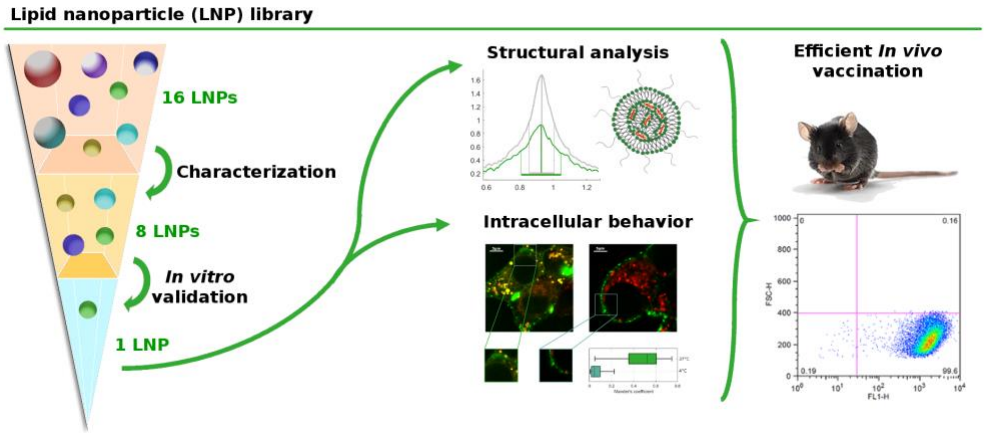
Statistical information

The results of each experiment are reported as mean ± standard deviation of at least three replicates. Experimental errors are represented as error bars in scatterplots and histograms. Where indicated, the p-value from the two-tail Student's t-test was computed to evaluate the statistical significance of the detected differences.

Conclusion

In this work, we investigated the structure-activity relationship of a library of 16 DNA-loaded LNPs with systematic changes in lipid composition and microfluidic mixing parameters. We individuated LNP15 as the most promising formulation by a step-by-step screening procedure ranging from physical-chemical characterization to in vitro validation and animal vaccination in vivo. This superior efficiency correlated with the particle nanostructure that was prone to be disintegrated by cellular lipids. This peculiar nanoscale arrangement induces DNA release and limits lysosomal degradation. Our results provide new insights into the correlation between the structure and functioning of DNA-loaded LNPs and pave the way to the clinical translation of this gene delivery technology.

Table of Content



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Author Contributions

[#]E.Q. and J.W. contributed equally. Conceptualization was done by A.A., D.P., C.M., and G.C.; methodology was done by E.Q., J.W., S.R., L.C., G.F., L.P., V.D.L., G.M., H.A., L.M., R.B., D.P., F.C., A.A., C.M., and G.C.; investigation was done by E.Q., J.W., S.R., L.C., G.F., L.P., V.D.L., G.M., H.A., L.M., R.B.; writing of the original draft preparation was done by E.Q., S.R., L.D., D.P., F.C., and G.C.; writing with reviewing and editing was done by A.A., D.P., F.C, C.M., and G.C.; supervision was done by H.A., A.A., D.P., F.C, C.M., and G.C., and funding acquisition was done by F.C., D.P, and G.C. All authors have read and agreed to the published version of the manuscript.

Notes

Any additional relevant notes should be placed here.

Supporting Information

Table S1. Lipid composition and mixing parameters for the preparation of the LNPs.

Table S2. Characterization of LNPs.

Table S3. Fitting parameters of synchrotron SAXS data.

Figure S1. *In vitro* validation of vaccine loaded LNPs.

Figure S2. Mechanistic investigation of the intracellular behavior of LNP13

ACKNOWLEDGMENT

The research leading to the results reviewed here has received funding from the Sapienza University of Rome (grant number RM12117A87BA3B80 to G.C.) and from the Italian Minister for University and Research (MUR) for the research project "TITAN"(Nanotecnologie per l'immunoterapia dei tumori) - Programma PON «R&I» 2014-2020 (ARS01_00906 to G.C.) This work was supported in part, by the European Research Council (ERC) under the Horizon 2020 Programme (grant agreement No 866127 to F.C., project "CAPTUR3D"). JW was supported by Fondazione Umberto Veronesi.

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