

The Impact of Using Different Cationic Polymers on the Formation of Efficient Lipopolyplexes for pDNA Delivery

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Purpose: Lipopolyplexes (LPP), i.e. hybrid ternary complexes of cationic polymers, nucleic acids and liposomes, represent a second-generation non-viral vector aiming to overcome the limitations of the first-generation polyplexes and lipopolyplexes like in vivo toxicity and ineffective transfection efficiency. Although their potential has already been proven in vitro and in vivo, lipopolyplexes are still poorly explored as gene delivery systems. Here, we provide evidence of the effect of lipopolyplexes composition on their physico-chemical features, cytotoxicity, and biological activity (i.e. cell uptake, endosomal escape, and transfection efficiency).

Methods: Lipopolyplexes were prepared by either bulk mixing or a two-step microfluidic process consisting of i) the formation of polyplexes by complexing a plasmid DNA encoding the green fluorescence protein with a panel of cationic polymers (either chitosan, poly-L-lysine (PLL) or polyethyleneimine (PEI)) followed by ii) the formation of the ternary complex by mixing polyplexes with neutral liposomes. The optimal polymer/DNA/lipid Nitrogen/Phosphate ratios and microfluidic operating parameters (volume ratio and total flow rate (TFR)) were preliminarily defined to obtain lipopolyplexes with desired properties.

Results: The optimized conditions led to obtain lipopolyplexes with a mean diameter of ~180 nm, a PDI < 0.2 and a slightly positive or neutral z-potential. FRET, SAXS and Cryo-EM analyses demonstrated the formation of a ternary complex in which the type of polymer dictated particles' structure. Lipopolyplexes displayed negligible toxicity in vitro, while promoting higher protein expression compared to the corresponding polyplexes and control 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) lipopolyplexes. Moreover, despite the three lipopolyplexes displaying similar uptake kinetics, those made of PEI showed the highest endosomolytic activity and promoted the most effective DNA transfection.

Conclusion: Overall, this study demonstrates that lipopolyplexes are a valid platform for pDNA delivery, with PEI lipopolyplexes being the best performing formulation, and that the type of cationic polymer plays a major role in the nanoparticles intercellular trafficking.

Keywords: lipopolyplexes, chitosan, poly-L-lysine, polyethyleneimine, microfluidics, DNA delivery

Introduction

Gene therapy has recently emerged as a valid approach to treat pathologies through the modification of a genetic sequence underlying a human disease; compared to conventional protein-targeted drugs, gene therapy modulates gene expression, therefore promoting site-specific, durable or possibly curative therapeutic outcomes.¹ However, efficient in vivo delivery of nucleic acids is often limited by i) degradation processes of the genetic material in biological fluids and ii) poor cell penetration of the payload due to its high molecular weight, high hydrophilicity and electrostatic repulsion with the cell membrane, ultimately resulting in insufficient accumulation in target tissues upon administration.^{2,3} Thus, the loading of nucleic acids in appropriate delivery systems is essential to provide adequate stability, improve biodistribution and allow effective intracellular delivery. To date significant advances in RNA delivery have been made as demonstrated by the increasing number of licensed products, including the transthyretin amyloidosis

inhibitor Patisiran, SARS-CoV-2 vaccines mRNA-BNT162b2 and mRNA-1273⁴ and lastly RSV Vaccine mRESVIA(R).⁵ The use of DNA therapeutics instead remains challenging due to the limited nuclear delivery, particularly in non-dividing cells, and ineffective transcription process. Therefore, developing adequate and highly controlled carriers for DNA-delivery is of great interest.

Viral vectors are currently used to deliver DNA into cells⁶, however, their utility is often hampered by anti-vector immunity, production limitations, restricted packaging capacity of the payload and safety concerns. Conversely, synthetic non-viral delivery systems are less immunogenic, easy to manipulate and produced by scalable and cost-effective manufacturing processes.⁷ A range of nanoparticle-based systems have been developed for DNA delivery, with those based on cationic lipids (lipoplexes) and cationic polymers (polyplexes) being the most studied. These binary complexes demonstrated to be effective transfecting agents *in vitro*; in particular, lipoplexes have been the most widely used for gene delivery applications.⁸ However, the strong ability of cationic lipids to interact with cell membranes leads to a dose-dependent, apoptosis mediated cell death and tissue damage, which arise safety concerns and limit their clinical applications.⁸ Indeed, to date lipoplexes based transfection reagents such as Lipofectamine are approved only for research use. On the other hand, polyplexes have poor cell uptake ability, inevitably resulting in ineffective transfection.⁹

To combine the advantages of lipid based nanosystems (ie high stability and efficient cellular uptake) and those of polymeric particles (ie full nucleic acid condensation and facilitated endosomal escape), ternary complexes composed of nucleic acid, cationic polymer and liposomes (lipopolyplexes – LPP) have emerged as second-generation nonviral vectors.¹⁰ Lipopolyplexes are versatile platforms as a wide combination of polymers and lipids can be tailored to meet several medical needs. Recently, they have been proposed as vaccine,¹¹ for the treatment of neurodegenerative disorders¹² and in cancer therapy.¹³

The choice of polycation is crucial as it strongly dictates lipopolyplexes' efficiency.^{12,14} Synthetic (ie polyethylenimine (PEI), poly-L-lysine (PLL)) as well as natural (ie chitosan, spermidine, spermine, protamine sulfate and collagen) polymers are frequently reported.^{15,16} PEI is considered the gold standard transfecting agent due to its high endosomolytic activity and nucleic acid packing capacity.^{17,18} Specifically, linear PEI of 22 kDa and branched PEI of 25 kDa are the most potent transfectants both *in vitro* and *in vivo*.¹⁷ However, increasing PEI molecular weight resulted in enhanced cytotoxicity and reduced biocompatibility.^{19,20}

PLL is conventionally reported as an alternative to PEI for gene therapy due to its poor immunogenicity; however, its transfection capacity is generally limited as PLL-based particles trapped after endocytosis.^{21,22} To this end, lipidation of PLL polyplexes is considered a valid strategy to improve polymer's efficacy.²³ Natural polymers are also attractive since they are usually non-toxic at a wide concentration range, biocompatible and biodegradable.²⁴ Among them, chitosan is considered advantageous as chitosan-based vectors can load large nucleic acids and provide effective protection of the payload. Moreover, chitosan can act as pH responsive polymer since the pKa of its amine groups is below physiological pH, thus promoting endosomal escape of the genetic material.^{25,26}

Herein, we tested a panel of lipopolyplexes containing either the cationic polymer chitosan, PLL or PEI for the delivery of a plasmid DNA encoding the green fluorescence protein as model (pDNA-EGFP) to study the effect of the polymer's choice on the cytotoxicity and DNA transfection efficiency. To the best of our knowledge, a systematic comparison of the polymer nature on nucleic acid complexation, ternary complex formation and transfection efficiency has not been conducted yet.

To formulate lipopolyplexes, a two-step process consisting of first the preparation of the polymer-pDNA-EGFP binary complexes (polyplexes), followed by its mixing with 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) based liposomes, was applied. We hypothesized that the association between cationic polymers (to promote the condensation of the genetic material) and neutral liposomes (to reduce the overall positive charge of the final formulation) might improve lipopolyplexes' performances *in vitro*. Microfluidic manufacturing approach was applied in spite of conventional bulk mixing to improve scalability and reproducibility of lipopolyplexes. Formulations were prepared at different polymer/DNA/lipid molar ratios and screened according to their physicochemical attributes as well as their ability to condense nucleic acid payload. Three independent techniques, ie fluorescence resonance energy transfer (FRET), small-angle x-ray scattering (SAXS) and cryogenic-electron microscopy (Cryo-EM), were combined to precisely correlate the structure of polyplexes and lipopolyplexes with their biological activity. Lastly, the efficiency of polyplexes and lipopolyplexes in

promoting endosomal disruption and protein expression in vitro was compared to that of a DOTAP lipoplex as benchmark for nucleic acid in vitro delivery.

Materials and Methods

Materials

Cholesterol, α -poly-L-lysine 150–300 kDa, branched polyethylenimine 25 kDa, Dimethyl sulfoxide (DMSO), Fluorescein-5-isothiocyanate, Tris(hydroxymethyl)aminomethane, GelRed, disodium phosphate (Sigma Aldrich); Chitosan hydrochloride 150–600 kDa (NovaMatrix); Acetonitrile, ethanol (Riedel-de-Haen); Hydrochloric acid, Potassium dihydrogen phosphate (VWR); Chloroform (Honeywell) Sodium chloride (VWR); 96-well black microtiter plates (Greiner); 96-well microtiter plates transparent, 24-well polystyrene microtiter plates, L-Glutamine 100X (200 mM), Dulbecco's Phosphate Buffer Saline w/o Calcium and Magnesium, Trypsin-EDTA with Phenol Red, Dulbecco's Modified Eagle's Medium high glucose, Fetal Bovine Serum (Euroclone). DNA Gel Loading Dye, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), Calcein, (4',6-diamidino-2-phenylindole (DAPI) (ThermoFisher); XT MOPS Running Buffer, 1% precast agarose gels (Biorad). 1 kb Molecular Ruler DNA standard, 1–15 kb (Biorad). 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (18:1 Liss Rhod PE), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) (Avanti polar), (HeLa) (obtained from American Type Culture Collection, ATCC). Plasmid DNA (pDNA) encoding for green fluorescent protein (GFP) was extracted from competent *Escherichia coli* and purified using Qiagen Plasmid Maxi Kit (Qiagen GmbH). About 1.2 mg of pDNA was recovered from 500 mL of culture.

Preparation of the Formulation by Conventional Methods

Liposomes

Liposomes were prepared by thin lipid film hydration method. Lipid mixtures composed of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and cholesterol (Chol) (55:35:10 mol/mol) were prepared in chloroform at 12 mM and put into a round bottom flask. The organic solvent was evaporated under reduced pressure (80 mbar) at 50°C and 100 rpm for 1 h using a rotatory evaporator (RII, Buchi). The lipid film was rehydrated with phosphate buffer 40 mM, NaCl 70 mM pH 7.4 for 1 h at 50°C, leading to the formation of multilamellar vesicles. Subsequently, liposomes were extruded 5 times through 0.2 μ m polycarbonate membranes and then 10 times through 0.1 μ m polycarbonate membranes (Nucleopore Track-Etch Membrane, Whatman®, Maidstone, UK) to obtain unilamellar vesicles.

Polyplexes

Polyplexes were prepared by bulk mixing. Chitosan and PEI were dissolved in phosphate buffer 50 mM pH 4.5 at a concentration of 2 mg/mL, while for PLL a commercially available solution of 0.1% in water was used, and the pH was adjusted to 4.5. To prepare polyplexes, a pDNA solution in phosphate buffer 50 mM pH 4.5 was added dropwise under vigorous stirring to a 0.5 μ g/mL polymer solution to have Nitrogen of polymer to Phosphate of pDNA (N/P) ratio of 6, 10 or 14 w/w (which correspond to 9, 15 or 22 mol/mol for chitosan, 13, 23 or 32 for PLL and 15, 26 or 36 mol/mol for PEI, calculated based on the average Mw). The mixture was incubated for 20 minutes at room temperature to allow complexation.

Lipopolyplexes

To prepare lipopolyplexes, 1.8 or 0.72 mM liposomes (corresponding to 0.5 or 0.2 mM final concentration) were added in a 1:5 v/v ratio to selected polyplex dispersions and incubated for additional 30 minutes at RT under vigorous stirring.

Preparation of the Formulations by Microfluidics

An in-house microfluidic system consisting of a commercially available micromixer equipped with a staggered herringbone geometry and a 3D-printed injection system was employed as a cost-effective alternative. The system consisted of a commercially available staggered herringbone micromixer (ChipShop) connected to two glass syringes by two PTFE tubes. Syringes are allocated into two coordinated syringe pumps (Formlabs Form 3B+, Manufat, Italy) and their

movement is regulated via the Pronterface Software. The device was programmed to push the plunger of each syringe at different speeds depending on the desired total flow rate, lipid/aqueous phase ratio and total volume.

Liposomes

For the preparation of anionic liposomes used in lipopolyplexes formulations, lipid mixtures composed of DPPC:DOPE:Chol (55:35:10 mol/mol) were prepared in ethanol at 12mM; phosphate buffer 40 mM pH 7.4 was used as aqueous phase. The aqueous:organic volume ratio and the total flow rate (TFR) were set at 3:1 and 12mL/min, respectively. To remove the organic solvent, 1 mL of formulations was dialyzed against 200 mL phosphate buffer 40 mM pH 7.4 supplemented with 70 mM to provide isotonicity.

Polyplexes

For polyplexes preparation, microfluidics parameters were first optimized using PLL. Briefly, PLL at 0.5, 0.7 and 1mg/mL in phosphate buffer 50 mM pH 4.5 was injected in the chip with a solution of pDNA-EGFP at N/P 23 mol/mol. The polymer:pDNA volume ratio was tested at 30 and 40%, while the TFR varied from 1 to 6 mL/min. Selected parameters were applied to prepare remaining polyplexes. Specifically, chitosan and PEI solutions at 0.7 mg/mL in phosphate buffer 50 mM pH 4.5 were injected in the chip with a solution of pDNA-EGFP at N/P of 15 mol/mol (corresponding to 10 and 6 w/w for chitosan and PEI, respectively), for both Chitosan and PEI. The polymer:pDNA volume ratio and the TFR were set as 40% and 3 mL/min, respectively.

Lipopolyplexes

To prepare lipopolyplexes, polyplexes and 1.4 mM anionic liposomes were further injected into the microfluidic cartridge at lipid/polyplex N/P of 2, 2.8 and 3.4 mol/mol for Chitosan PLL and PEI, respectively. Polyplex/liposome volume ratio of 30% and TFR of 3mL/min were used.

Lipoplexes

To prepare lipoplexes, DOTAP:DOPE:Chol (55:35:10 mol/mol) in ethanol at 7 mM and Tris buffer 50 mM pH 7.4 were used as organic and aqueous phase, respectively; process parameters were set as follows: TFR 6mL/min, volume ratio 1:1. Subsequently, both phases were injected simultaneously in the micromixer, and particles were collected in a 1.5 mL Eppendorf. Afterwards, the prepared liposomes were mixed with the pDNA in 100mM Tris buffer 40 mM NaCl pH 7.4 at N/P 4 (w/w), based on a previous formulation study (data not shown). Process parameters were set as follows: TFR 6mL/min, volume ratio 1:1. Subsequently, upon simultaneous injection of both phases in the micromixer, lipoplexes were collected and dialyzed for 1 hour against TRIS buffer 100 mM supplemented with 40mM NaCl to provide isotonicity.

Physicochemical Characterization of Formulations

All formulations were characterized in terms of hydrodynamic diameter, polydispersity index (PDI) by dynamic light scattering (DLS) and zeta potential (ZP) by electrophoretic light scattering (ELS) in a Zetasizer Nano ZS (Malvern Panalytical, Malvern, UK) by diluting formulations 1:10 in filtered ultrapure water at 25°C. Particles stability was assessed both in 10% fetal bovine serum at 37°C or in saline solution at 4°C by incubating samples in each medium and measuring changes in particles size, PDI and ZP up to 48 hours (sampling every 4 hours) or 14 days, respectively. Particles counts and particles distribution profiles by number were obtained by Nano Tracking Particle Analysis (NTA) performed in a NanoSight NS300 device (Malvern Panalytical, Malvern, UK). In brief, samples were diluted in filtered ultrapure water to get accurate acquisition. Camera settings were fixed and maintained for all samples (gain: 14; camera level: 13–14, detection threshold 6–7). For each sample, five videos of 60 seconds were recorded and analyzed with NanoSight NTA software 3.3 (Malvern Analytical).

Gel Retardation Assay

pDNA condensation by both polyplexes and lipopolyplexes was assessed by performing a gel retardation assay. Samples containing 1 µg/well pDNA were mixed with DNA Gel Loading Dye at 5:1 vol/vol and loaded on 1% precast agarose

gels under non-denaturing conditions. The gel was run at 90 V for 60 min in 3-(N-morpholino)propanesulfonic acid (MOPS) running buffer. 1 kb DNA ladder was used as molecular weight standard. The gel was further stained with GelRed following manufacturers' instruction and visualized using GelDoc Go Gel Imaging System (Bio-Rad).

Quantification of DNA Loading Efficiency

DNA encapsulation efficiency (EE) was measured using the Quant-iT PicoGreen dsDNA Assay Kit (ThermoFisher) following the manufacturer's instructions in 96-well black microtiter plates. Briefly, 100 μ L of the diluted fluorescent dye was added to 100 μ L of each formulation and incubated in absence of light for 5 min. This allowed the dye to quantitatively bind free nucleic acid. The concentration of non-loaded pDNA was determined by measuring fluorescence ($\lambda_{em} = 480$ nm, $\lambda_{ex} = 520$ nm) using Tecan Spark Microplate reader (Tecan). The actual loading was obtained by subtracting the unloaded pDNA to the initial nucleic acid concentration.

Fluorescence Resonance Energy Transfer Analysis

To perform Fluorescence Resonance Energy Transfer (FRET) analysis polymers and liposomes were fluorescently labelled with Fluorescein Isothiocyanate (FITC) and Rhodamine (Rhod), respectively.

Labeling of Cationic Polymers

Chitosan was dissolved in phosphate buffer 50 mM pH 4.5, while PLL and PEI were dissolved in 100mM Na₂CO₃ pH 9 and 11, respectively. Each polymer was mixed with a solution of FITC in DMSO at 1:4 mol/mol dropwise and incubated for 3 hours under stirring at room temperature in the dark. This allows the isothiocyanate group of FITC to spontaneously react with unprotonated primary amines of each polymer, thus resulting in the formation of a stable thiourea bond. Unconjugated FITC was removed via 24h-dialysis (cut off = 14,000 Da) and conjugation was confirmed by reverse phase HPLC (Agilent HP 1100 series) by detecting unconjugated FITC at 490 nm. In brief, separation was achieved using a reversed-phase analytical column (InertClone 5 μ m ODS (3) 100 Å 150x4,6mm column), with a linear gradient between eluent A (phosphate buffer 20mM pH 7.4) and eluent B (acetonitrile). After 1 min at 10%, eluent B was increased to 40% in 15 min, then taken back to the initial conditions in 2 min and held for an additional 3 min. Process parameters were set as follows: flow rate 1 mL/min, temperature of analysis 25°C and injection volume 10 μ L. Rhod-labelled liposomes were prepared incorporating 18:1 Liss Rhod PE (1 mol %) within the lipid mixture before micro-fluidic injection.

FITC-labelled polymers and Rhod-labelled liposomes were subsequently used in the formation of polyplexes and lipopolyplexes as described above. FITC-to Rhod molar ratios were 0.4, 0.5 and 2 for Chitosan, PLL and PEI LPP, respectively. This molar range is usually reported to ensure an efficient energy transfer between the fluorescence donor and the acceptor.^{27–29} For FRET experiments, FITC fluorescence was normalized by diluting FITC-polymer conjugates (as controls), polyplexes and lipopolyplexes to FITC concentration of 2.3 μ g/mL, while control Rhod-liposomes concentration was normalized by that within lipopolyplexes. Subsequently, 200 μ L of each formulation was placed in 96-well black microtiter plates and samples fluorescence was measured at λ_{ex} of 480 nm and λ_{em} from 518 to 800 nm (bandwidth 20 nm) using Tecan Spark Microplate reader (Tecan). Data analysis was performed as described previously³⁰ with some modifications. The decrease in fluorescence emission intensity at 522 nm as a result of fluorescence quenching was expressed as percentage of quenching (Q%) according to

$$\text{percentage of quenching} = \left(1 - \frac{I(PP)}{I(FITC - polymer)} \right) \times 100 \quad (1)$$

Where I(PP) and I(FITC-polymer) are the fluorescence emission intensities of FITC within polyplexes and FITC conjugated with different polymers in absence of DNA. FRET efficiency (E%) was calculated from FITC emission at 522 nm within polyplexes and lipopolyplexes and expressed as percentage according to

$$\text{FRET efficiency} = E = \left(1 - \frac{I(LPP)}{I(PP)} \right) \times 100 \quad (2)$$

Subsequently the average distance (R) between FITC and Rhod in each lipopolyplexes was determined according to

$$R = R_0 \sqrt[6]{\frac{1}{E} - 1} \quad (3)$$

where R_0 is the Forster radius of FITC-Rhod dye pair which is defined as the distance at which energy transfer of the donor-acceptor pair is 50% of the maximum. R_0 was set as 5.5 nm as previously reported.³¹

Small Angle X-Ray Scattering Analysis

Small Angle X-ray Scattering (SAXS) measurements were performed at the beamline ID02 of the European Synchrotron Radiation Facility (ESRF, Grenoble, France), experiment IN-949. Solutions were loaded in a flow-through cell with 2 mm internal diameter. This set-up allowed for measurements of samples and buffers in the same experimental configuration, necessary to obtain a good subtraction of the background contribution to the scattered intensity. Furthermore, during the acquisition, the samples were gently pushed to flow in the cell, to avoid any radiation damage. Ten short frames (0.1 s) were acquired and averaged. The scattered intensities lay in the momentum transfer (q) range $10^{-2} \leq q \leq 7 \text{ nm}^{-1}$ being $q = (4\pi/\lambda)\sin(\theta/2)$, where θ is the scattering angle and λ is the X-ray wavelength ($\lambda = 0.1 \text{ nm}$). All measurements were performed at room temperature. Data were fitted using SaSView program (www.sasview.org).

Cryo-Electron Microscopy

Sample vitrification was carried out with a Mark IV Vitrobot (Thermo Fisher Scientific). Three μL of the samples at 0.5 mg/mL of both polyplexes and lipopolyplexes in aforementioned buffers were applied to a Quantifoil R1.2/1.3 Cu 300-mesh grid previously glow-discharged at 30 mA for 30" in a GloQube (Quorum Technologies). After sample application to the grids, we incubated for 20 s, then blotted the grids in a chamber at 4°C and 100% humidity, and then plunge-frozen into liquid ethane. Vitrified grids were transferred to a Talos Arctica (Thermo Fisher Scientific) operated at 200 kV and equipped with a Ceta 16M detector (Thermo Fisher Scientific). Images were acquired at a nominal magnification of 45'000x, corresponding to a pixel size of 0.229 nm/pixel with a defocus of $-3.0 \mu\text{m}$.

Cell Culture

Human cervical adenocarcinoma cells (HELA, ATCC, CCL-2) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 50 $\mu\text{g/mL}$ streptomycin, and 100 U/mL penicillin (complete medium) at 37°C and 5% (5% CO₂ and 95% air).

Human umbilical vein endothelial cells (HUVEC, ATCC, CRL-1730) were cultured in endothelial cell growth medium-2 (Lonza). HEK-293 (American Type Culture Collection, ATCC®, Manassas, VA, USA) were routinely grown at 37°C (5% CO₂ and 95% air), in DMEM (Euroclone) supplemented with 10% FBS (ThermoFischer), 2mM glutamine, 100 IU/mL penicillin/streptomycin (all Sigma-Aldrich).

Mycoplasma contamination was evaluated every two weeks using MycoAlert® Mycoplasma Detection Kit (Lonza, Italy).

Cell Toxicity Assay in Human Cervical Adenocarcinoma Cells

For the experiments, cells were seeded in 96-well polystyrene microtiter plates at a density of 3×10^4 cells in 200 μL of complete medium and incubated for at least 12 hours to allow cell adhesion. Then, medium was removed, and cells were incubated with serial dilutions of polyplexes or lipopolyplexes in DMEM 2% FBS,^{32,33} starting from a polymer concentration of 60 $\mu\text{g/mL}$. Cells incubated with DMEM 2% FBS or a DOTAP based lipoplex diluted in DMEM 2% FBS at aforementioned concentrations were used as negative and positive controls, respectively. After 48 hours, supernatant was replaced with 180 μL of fresh medium, and 20 μL of MTT solution at 5mg/mL were added in each well. Cells were further incubated for 2 hours at 37°; upon medium removal, 200 μL /well of DMSO were added to solubilize formazan crystals, and the absorbance of released formazan at 570 nm was measured using Tecan Spark Microplate reader (Tecan). The percentage of viable cells was calculated with respect to untreated cells.

In vitro Transfection Efficiency

On the day of the experiment, HeLa, HEK-293 and HUVEC cells (5×10^4 cells/well) were seeded in 24-well polystyrene microtiter plates and incubated in complete corresponding medium for 12 hours to allow cell adhesion. Cells were then incubated for 48 hours with polyplexes and lipopolyplexes at pDNA concentration of 1 $\mu\text{g}/\text{well}$ (corresponding to 20 $\mu\text{g}/\text{mL}$ of chitosan and PLL and 12 $\mu\text{g}/\text{mL}$ of PEI) in DMEM 2% FBS (for HeLa and HEK-293) or endothelial cell growth medium-2 (for HUVECs). DOTAP lipoplex was used as a positive control, as mentioned above. For flow cytometry experiment, cells were subsequently harvested from the well-plates, washed, and resuspended in 200 μL of DPBS for the analysis. The percentage of EGFP+ cells with respect to untreated control was measured by Novocyte 3000-Agilent cytometer. Cell populations of interest were identified based on forward (FSC-A) and side (SSC-A) scatter and fluorescence intensity thresholds were set using appropriate negative control. Data were then analysed by using NovoExpress Software (Agilent). For fluorescence microscopy experiment, medium was removed, and cells were washed with DPBS subsequently fixed with 4% paraformaldehyde for 5 min at room temperature then washed with DPBS and further stained with 0.1 $\mu\text{g}/\text{mL}$ 4',6 diamidino-2-phenylindole (DAPI) in DPBS for 15 min at room temperature. After extensive washing with DPBS, cells were imaged using a fluorescent microscope (Nikon ECLIPSE Ts2R; excitation filter for GFP detection 470 nm, excitation filter for DAPI 385 nm, magnification 20X) and analysed using the ImageJ software.

In vitro Endosomal Escape Induced by Polyplexes and Lipopolyplexes

Circular glass coverslips (20 mm diameter) were treated with 1M HCl for 1 hour, then washed with DPBS and sterilized by immersing in absolute ethanol overnight. Then, they were transferred to a 12-well plate where HeLa cells (1×10^5 cells/well) were subsequently seeded and incubated for 12 hours in complete medium to allow cell adhesion. Medium was then replaced with DMEM 2% FBS-containing calcein (0.1 mg/mL) and polyplexes or lipopolyplexes at 1 $\mu\text{g}/\text{well}$ of DNA and incubated for 4 hours at 37°C. Cells containing calcein only or calcein and DOTAP lipopolyplexes at 1 $\mu\text{g}/\text{well}$ of DNA were used as negative and positive control, respectively. Calcein is a cell membrane impermeable dye which gets entrapped into endocytic vesicles and results in punctuate fluorescence within endosomes. Upon endosomal disruption, calcein is released into the cytoplasm leading to a diffused fluorescence signal. Cells were then washed with PBS, fixed with 4% paraformaldehyde, stained with DAPI as described above, imaged using a fluorescent microscope (Nikon ECLIPSE Ts2R; excitation filter for GFP detection 470 nm, excitation filter for DAPI 385 nm, magnification 40X) and pseudocolored using ImageJ. To quantitate the endosomal escape, nuclei of cells displaying a diffused fluorescence signal were counted and normalized over the total number of DAPI+ nuclei in each image. A total of 3 different images of at least 20 cells were analyzed per sample.

In vitro Uptake of Lipopolyplexes

HeLa cells (5×10^4 cells/well) were seeded in 24-well polystyrene microtiter plates and incubated in complete medium for 12 hours to allow cell adhesion. Rhod-FITC double labelled lipopolyplexes were prepared as described above in paragraph 2.7. Cells were then incubated for 1, 4 and 24 hours with double labelled lipopolyplexes at Rhod-labelled liposomes concentration of 42 μM in DMEM 2% FBS. Cells incubated with medium only were used as negative control. For flow cytometry experiment, cells were subsequently harvested from the well-plates, washed, and resuspended in 200 μL of DPBS for the analysis. Cells were acquired using a FACSymphony™ cytometer (Becton Dickinson, USA) with 5 lasers (UV, Violet, Blue, Yellow-Green, and Red). The percentage of EGFP+ and Rhod+ cells with respect to untreated control were analyzed using FlowJo software (version 10.9.1, BD Biosciences, USA). Cell populations of interest were identified based on forward (FSC-A) and side (SSC-A) scatter and fluorescence intensity thresholds were set using appropriate negative control.

Statistical Analysis

Unless stated otherwise, the results were calculated as mean $\pm$ standard deviation (SD). One-way analysis of variance (ANOVA) followed by Tukey's post hoc analysis was performed for comparison, and significance was acknowledged

for p values <0.05 . Analysis and interpretation were made using GraphPad Prism 9 (GraphPad Prism software, San Diego, CA, USA).

Results

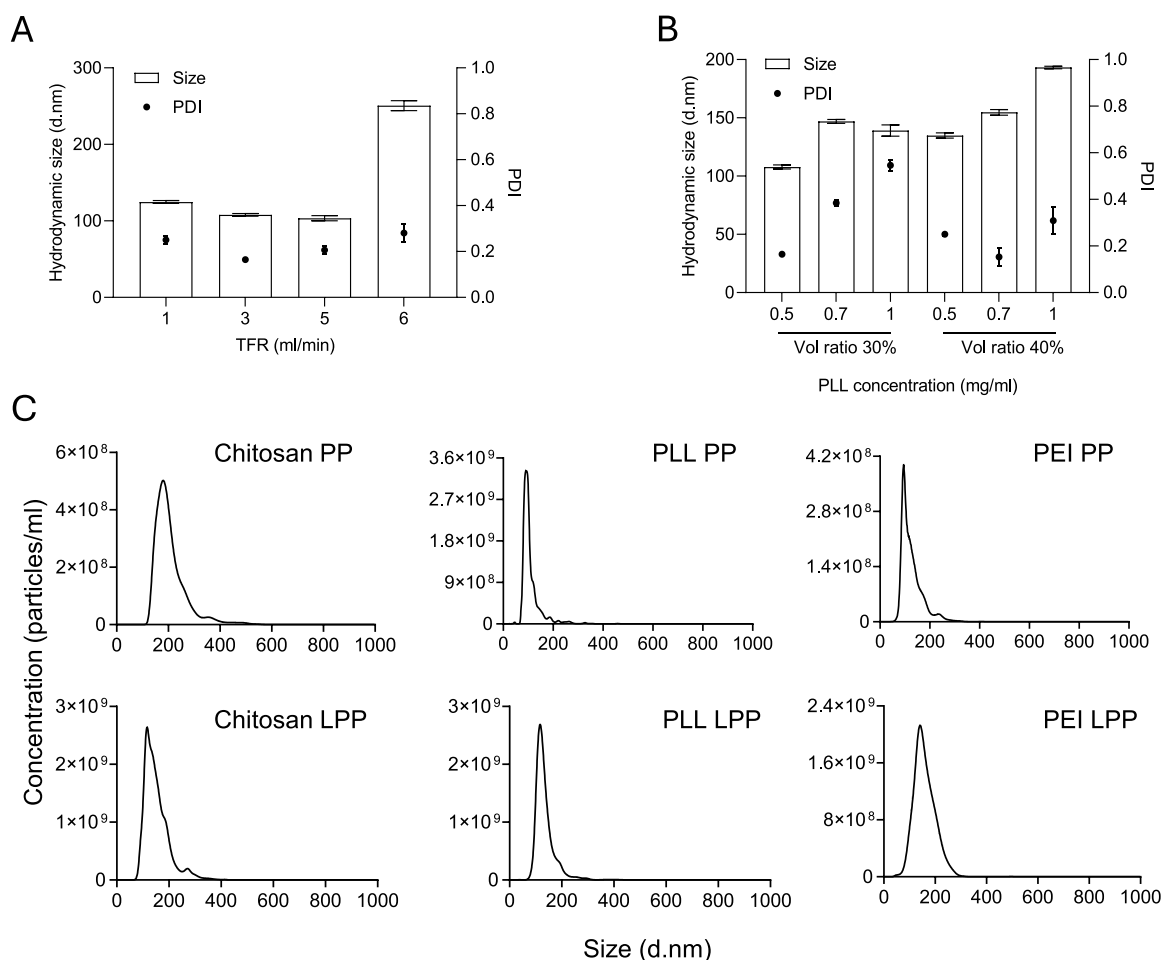
Polyplexes and Lipopolyplexes Preparation and Physico-Chemical Characterization

Nitrogen-to-phosphate ratio (N/P) is a key element which influences particles' physicochemical properties such as net surface charge, size and stability. In the attempt to find a suitable N/P for each polymer, polyplexes were prepared by bulk mixing and the polymer/DNA mass ratios varied between 6, 10, 14 (which correspond to 9, 15 or 22 mol/mol for chitosan, 13, 23 or 32 for PLL and 15, 26 or 36 mol/mol for PEI). Data showed that the optimal mass ratios were 10 for chitosan and PLL and 14 for PEI polyplexes (which correspond to 15, 23 and 36 mol/mol for chitosan, PLL and PEI respectively). These particles had a mean diameter of around 270 nm, PDI between 0.27 and 0.34 and a neat positive surface charge ranging from +32 to +40 mV irrespective of the polymer used (Figure S1A). Upon complexation of liposomes at 0.5 mM with selected chitosan and PEI polyplexes or at 0.2 mM with selected PLL polyplexes (corresponding to a polyplex/lipid N/P of 1.8, 3 and 2.7, respectively), the resulting ternary complexes showed better physicochemical attributes (mean diameter of 165 nm and PDI < 0.2 , Figure S1B). Of note, while chitosan and PLL lipopolyplexes were slightly positively charged (+13.8 and +8.4 mV respectively), PEI lipopolyplexes were neutral (data not shown). Notably, when preliminarily tested for in vitro toxicity, both PEI polyplexes and lipopolyplexes induced cell death at relevant doses for DNA transfection (data not shown). Therefore, for subsequent preparation of binary and ternary complexes via microfluidics, the N/P weight ratio of PEI polyplexes was reduced to 6 (corresponding to 15 mol/mol) to minimize the quantity of polymer to incubate with cells, while N/P weight ratio of 10 were maintained for chitosan and PLL polyplexes (which correspond to 15 and 23 mol/mol respectively).

To set up microfluidics process parameters, the effect of TFR on particle size distribution was first evaluated on PLL polyplexes (N/P 10 w/w; 23 mol/mol). Polymer concentration and DNA/polymer volume ratio were set at 0.5 mg/mL and 30%, respectively. Decreasing the injection speed from 6 mL/min to 5 mL/min resulted in a reduction of both particles size (from about 250 nm to 103 nm) and PDI (from 0.3 to 0.2, Figure 1A). TFR decrease up to 1 mL/min further improved the monodispersity of the dispersion, with 3 mL/min being the optimal injection speed which allowed to formulate PLL polyplexes with the lowest polydispersity index (PDI 0.16 ± 0.01). Increasing polymer content from 0.5 to 0.7 mg/mL did not significantly change particles' dimensions (Figure 1B). Conversely, polyplexes prepared using PLL concentration of 1 mg/mL were highly heterogeneous (PDI > 0.5 ; Figure 1B). The DNA/polymer volume ratio was increased to 40% to increase the DNA loading within the polyplexes formulation (Figure 1B). Results showed that particles maintained an average diameter of around 120 nm and were monodispersed at PLL concentrations below 0.7 mg/mL. As expected by data determined at 30% volume ratio, the increase of PLL content to 1 mg/mL induced formulation's aggregation and consequent precipitation. Consequently, TFR 3 mL/min, 40% DNA/polymer volume ratio and polymer concentration of 0.7 mg/mL were selected to prepare Chitosan and PEI polyplexes. Resulting polyplexes had a size of 252 ± 4 nm and 213 ± 6 nm, respectively, with PDI < 0.23 (Table 1). All polyplexes had a neat positive surface charge ranging from +32 to +35 mV, irrespective of the polymer nature (Table 1).

To prepare lipopolyplexes, the injection speed was maintained at 3 mL/min while the liposomes/polyplex volume ratio was set at 30%, aiming to minimize DNA dilution. Upon complexation of selected polyplexes with DPPC liposomes, the final optimal polyplex/liposome molar ratio was found to be 2, 2.8 and 3.4 for chitosan, PLL and PEI lipopolyplexes, respectively (Table 1). These conditions allowed achieving monodispersed (average PDI < 0.25 ; Table 1) lipopolyplexes with mean diameter of 170 nm.

Overall, LPP showed a smaller diameter and less positive surface charge (from around +35 mV to between +14 and +22 mV) compared to the corresponding PP ($p < 0.001$) and this might be considered as an indirect proof of the formation of a ternary structure in which the polyplexes are condensed with the lipids (Table 1). Nanoparticle tracking analysis results confirmed formulation homogeneity, in line with DLS analysis (Figure 1C and Table 1). It is worth noting that Chitosan and PLL PP showed around 10-folds lower particles concentration than corresponding LPP ($p < 0.01$), conversely the particles count of PEI PP and LPP was comparable (17×10^{10} particles/mL on average, Table 1). This



Formulation	Hydrodynamic diameter (d.nm)	Polydispersity index (PDI)	Zeta potential (mV)	Concentration (particles/ml*10 ¹⁰)	DNA Encapsulation efficiency (%)
Chitosan PP	252±4	0.22±0.02	+35±0.14	4.51±0.07	89.2± 3.6
Chitosan LPP	189± 3	0.18±0.04	+22.7±0.49	20.5±0.54	93.4 ±3.8
PLL PP	154±2	0.15±0.03	+32.8±0.63	1.83 ±1.29	98.7 ±1.0
PLL LPP	178±5	0.22±0.03	+21.3±1.18	22.3 ±6.10	96.5± 1.1
PEI PP	213±6	0.15±0.03	+34±0.17	9.52 ±5.27	95.8± 2.2
PEI LPP	143±1	0.07±0.03	+14.3±0.74	25.5 ±14.90	98.1 ± 0.2

Figure 1 Effect of microfluidic process parameters on polyplexes (PP) and lipopolyplexes (LPP) physicochemical properties. **(A)** effect of TFR increase on PLL PP particles size (bar) and PDI (dots), **(B)** effect of the polymer/DNA volume ratio and polymer concentration on PLL PP particles size (bar) and PDI (dots), **(C)** representative NTA graphs of optimized Chitosan, PLL and PEI based PP and LPP prepared by microfluidics. Results showed in each graph are represented as mean ± SD of three independent experiments.

suggests that differences in the interactions among the three polymers with DNA and lipids might occur, with PEI generating more regular and homogeneous structures.

Microfluidics was also employed to prepare DOTAP lipopolyplexes at N/P 4 w/w, to be used as a positive control for subsequent *in vitro* studies; these particles exhibited a size of approximately 150 nm and a PDI < 0.2 (data not shown). The interaction between the cationic polymers and the payload was deepened by gel electrophoresis; gel images showed that, while unformulated pDNA migrated into the gel, that associated with polyplexes or lipopolyplexes was retained in the wells (Figure S2). This demonstrates that the selected N/P ratios were sufficient to totally condense DNA, as no free

Table 1 Physicochemical Characterization of Polyplexes (PP) and Lipopolyplexes (LPP). Particles Attributes of Chitosan, PLL and PEI Based PP and LPP Prepared by Microfluidics. Results are Represented as Mean $\pm$ SD of Three Independent Experiments

Formulation	Hydrodynamic Diameter (nm)	Polydispersity Index (PDI)	Zeta Potential (mV)	Concentration (Particles/mL*10 ¹⁰)	DNA Encapsulation Efficiency (%)
Chitosan PP	252 $\pm$ 4	0.22 $\pm$ 0.02	+35 $\pm$ 0.14	4.51 $\pm$ 0.07	89.2 $\pm$ 3.6
Chitosan LPP	189 $\pm$ 3	0.18 $\pm$ 0.04	+22.7 $\pm$ 0.49	20.5 $\pm$ 0.54	93.4 $\pm$ 3.8
PLL PP	154 $\pm$ 2	0.15 $\pm$ 0.03	+32.8 $\pm$ 0.63	1.83 $\pm$ 1.29	98.7 $\pm$ 1.0
PLL LPP	178 $\pm$ 5	0.22 $\pm$ 0.03	+21.3 $\pm$ 1.18	22.3 $\pm$ 6.10	96.5 $\pm$ 1.1
PEI PP	213 $\pm$ 6	0.15 $\pm$ 0.03	+34 $\pm$ 0.17	9.52 $\pm$ 5.27	95.8 $\pm$ 2.2
PEI LPP	143 $\pm$ 1	0.07 $\pm$ 0.03	+14.3 $\pm$ 0.74	25.5 $\pm$ 14.90	98.1 $\pm$ 0.2

nucleic acid was detected. Picogreen showed that DNA was fully encapsulated within polyplexes and lipopolyplexes, with a loading efficiency ranging between 89% and 98% for all the formulations prepared, further confirming the suitability of selected N/P ratios for polyplexes and lipopolyplexes preparation (Table 1).

Analysis of the Ternary Structure of Lipopolyplexes

In an effort to demonstrate the formation of ternary complexes, FRET analysis was applied. To assess that, polymers and liposomes were fluorescently labelled with Fluorescein Isothiocyanate (FITC) and Rhodamine (Rhod), respectively. It was determined that the conjugation efficiency for Chitosan and PLL was 73% while for PEI was about 83%.

Overall, it was observed a decrease in FITC emission at 522 nm for FITC-polymers compared to corresponding polyplexes for all three samples, although the percentage of quenching differed among polymers (Figure 2A); specifically, Q% of PEI was significantly higher than that of Chitosan and PLL (36.12 $\pm$ 4.44%, 23.26 $\pm$ 3.74% and 17.19 $\pm$ 2.71%, respectively, $p < 0.05$), indicating more effective nucleic acid complexation by PEI (Figure 2). The addition of Rhod-liposomes to FITC-polyplexes further decreased FITC emission of resulting double labeled lipopolyplexes, with PLL and PEI lipopolyplexes showing the highest reduction as quantified by FRET efficiency values (E%) (11.60 $\pm$ 1.6%, 46.69 $\pm$ 1.98% and 39.56 $\pm$ 2.83% for chitosan, PLL and PEI, respectively, $p < 0.001$, Figure 2). Besides, an increase of Rhod emission of lipopolyplexes compared to Rhod-liposomes was also observed; this suggests that FITC-polyplexes and Rhod-liposomes were closed enough to modify each other's surrounding environment likely by electrostatic interaction, thus confirming the formation of ternary complexes. Of note, differences in E% were not due to variabilities in the ratio of conjugated probes; indeed, despite the absolute quantity of FITC and Rhod in each LPP varied due to differences in the N/P, the relative FITC-to-Rhod molar ratios were comparable among lipopolyplexes.

Measurements of FRET efficiency can be used to estimate the distance (R) between the donor and the acceptor in double-labeled lipopolyplexes, aiming to deepen the structure of these complexes generated with different polymers. The calculated average distances of FITC-Rhod within ternary complexes followed a similar trend observed for FRET efficiency, with PLL and PEI lipopolyplexes showing significantly lower R than Chitosan counterparts (5.62 $\pm$ 0.07%, 5.90 $\pm$ 0.11% and 7.73 $\pm$ 0.20%, respectively, $p < 0.001$, Figure 2).

To provide insight into the nanoscale structure of polyplexes and lipopolyplexes, small-angle x-ray scattering (SAXS) experiment was conducted.³⁴ To verify the formation of lipopolyplexes by mixing the components by microfluidics, it is necessary to compare the scattered intensity data obtained for lipopolyplexes with those measured for liposomes and polyplexes. The scattered intensity curves for liposomes, polyplexes and lipopolyplexes are reported in Figure 2B, normalized by their concentration within LPPs final formulation. Generally, differences in absolute intensity values and the features of the intensity profiles were visible between polyplexes and lipopolyplexes, as well as between systems with

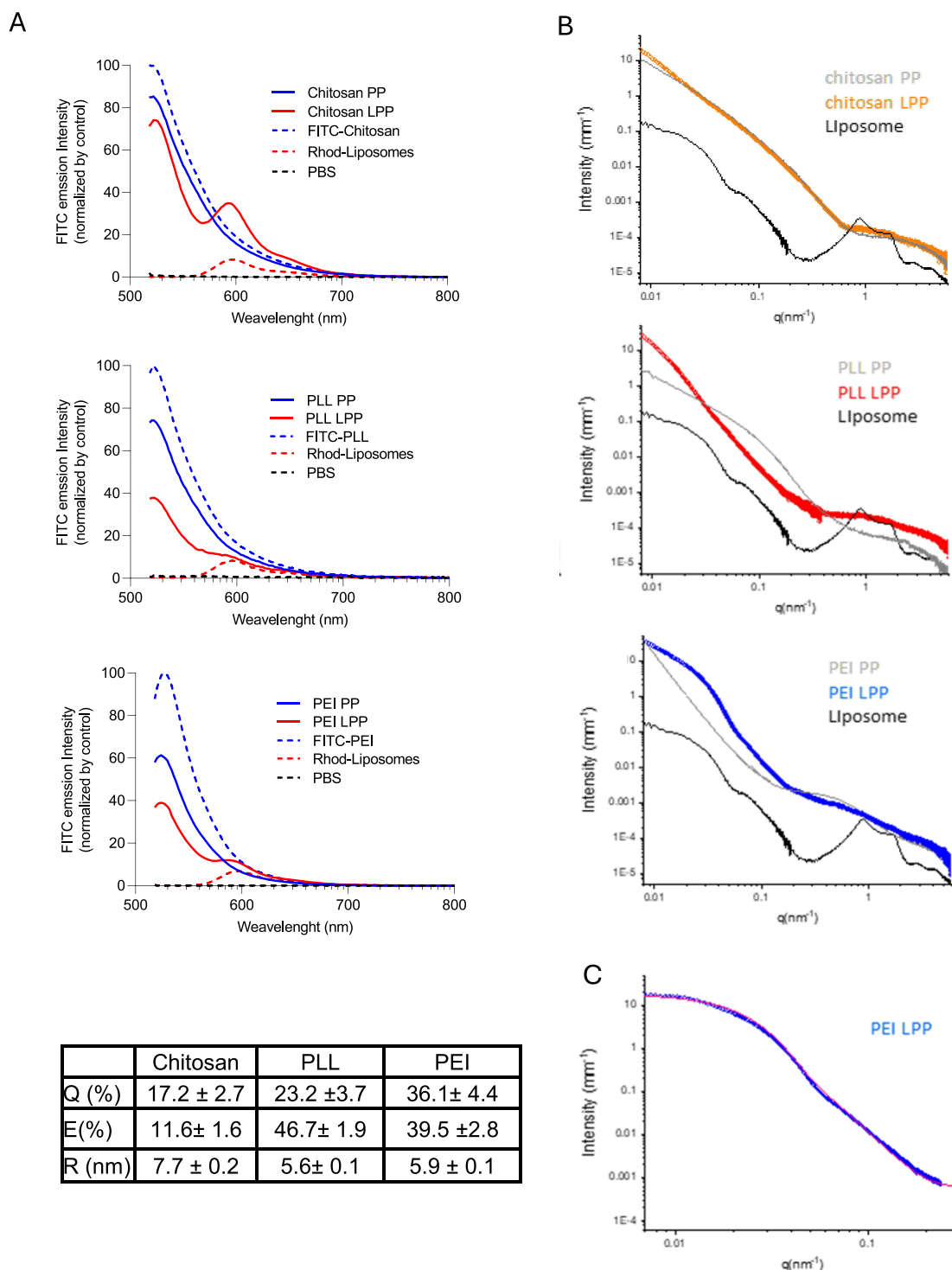


Figure 2 Structural analysis of polyplexes and lipopolyplexes by FRET and SAXS. **(A)** Representative normalized fluorescence emission spectra of Chitosan (top), PLL (center) and PEI (bottom) formulations. For each polymer, the spectra of free FITC-polymer (blue dotted line), FITC-labeled polyplexes (PP, blue solid line), Rhod-liposomes (red dotted line), FITC/Rhod-labeled lipopolyplexes (LPP, red solid line) and buffer (black dotted line) were acquired. The table in the bottom left shows the quantification of FRET. The interaction between FITC-polymers and pDNA was evaluated by quantifying the percentage of quenching (Q%) using equation (1), while the interaction between FITC-PP and Rhod-liposomes was quantified by calculating the FRET efficiency (E%) using equation (2). From E%, the distance between FITC and Rhod within ternary complexes was estimated using equation (3). Results are represented as mean ± SD of two or three independent experiments. **(B)** SAXS intensity profiles of Chitosan (top), PLL (center) and PEI (bottom) formulations: liposome (black line) $c = 0.3$ mg/mL, PPs (grey lines) $c = 0.3$ mg/mL, and LPPs (coloured symbols) $c = 0.6$ mg/mL. **(C)** Intensity profile of PEI LPP after subtracting the contribution to the scattered intensity of a fraction of non-interacting components. The magenta line is the reconstruction by a spherical core-shell model. Two independent formulations batches were analyzed and representative graphs of one batch were reported.

different cationic polymers. Specifically, the features of the scattering curves of polyplexes indicated a multiple-level structure of these particles, with a local arrangement of polymer and pDNA chains and a fractal cluster arrangement on the longer length scale (tens and hundreds of nms). The fits of the experimental data are reported in [Figure S3](#). The reconstruction of the intensity profiles has been obtained by modeling the local structure with an entangled network of polymer chains, with a mass fractal arrangement (fractal dimension 3), with a very small characteristic size for all polyplexes (around 6, 10 and 32 nm for PEI Chitosan and PLL polyplexes respectively). These substructures aggregated in larger clusters with a loose packing for chitosan polyplexes (mass fractal dimension 2) and PLL polyplexes (mass fractal dimension 1.8) and a close packing for PEI polyplexes (fractal dimension 3.8). Overall, these results indicate the formation of clusters with a close-packed substructure in PEI polyplexes and a loose-packed substructure in the other two binary complexes.

The form factor of liposomes has been reconstructed by a closed bilayer model, with a size of about 135 nm, in agreement with DLS results (data not shown). The bilayer profile (reported in [Figure S4](#)) displayed the characteristic central lipid core and the two external slightly asymmetric layers, with a total thickness of about 5 nm. In addition, the experimental intensity spectrum ([Figure 3B](#)) showed two peaks, the first centered at $q = 0.89 \text{ nm}^{-1}$, superimposed on the form factor. These peaks are characteristic of a fraction of multilamellar liposomes with interlamellar distance $d = 7 \text{ nm}$.

If no interactions occurred between liposomes and polyplexes and the final formulations were constituted by the simple coexistence of the two components, the intensity spectrum of lipopolyplexes should be the simple sum of those of liposomes and polyplexes. Instead, the intensity spectra had different characteristics throughout the explored q range, revealing the effective formation of lipopolyplexes. In [Figure S5](#), a comparison between experimental data and those of the hypothetical non-interacting systems is reported.

Generally, the intensity profiles of lipopolyplexes varied for the different polymers, though some common features could be observed. In all the formulations, the high multilamellar intensity peak ($q = 0.89 \text{ nm}^{-1}$) of liposomes reduced to a residue, indicating that only a low fraction of simple liposomes (less than 10%) is still present in the formulations, while the majority has complexed with polyplexes. In the low- q region, corresponding to the internal arrangement of complexes on long distances, the lipopolyplexes spectra showed the features of globular particles, particularly for PEI and PLL lipopolyplexes, although quite polydisperse.

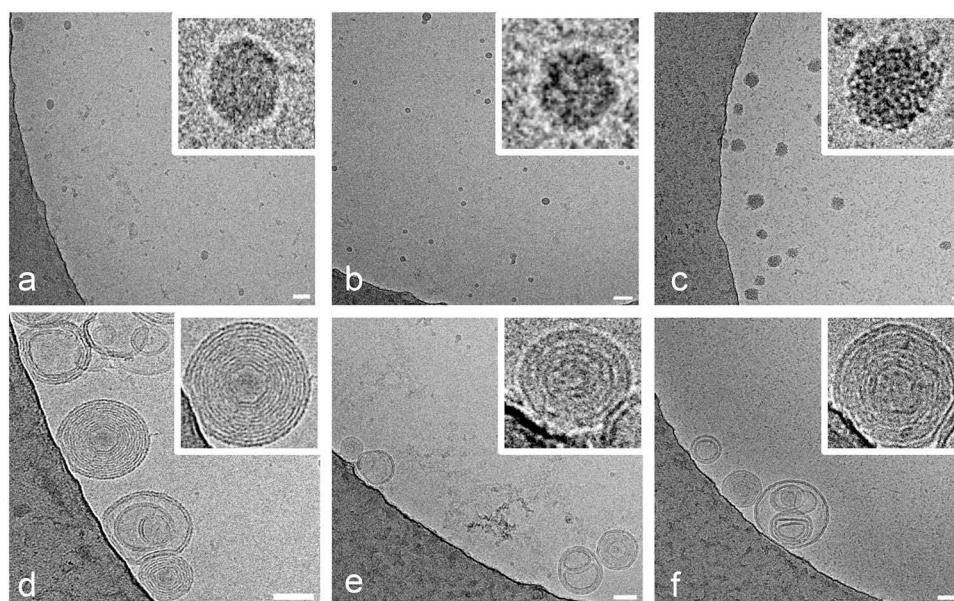


Figure 3 Structural analysis of polyplexes and lipopolyplexes by Cryo-EM. Cryo-EM images of Chitosan polyplexes (a), PLL polyplexes (c), PEI polyplexes (e) and corresponding lipopolyplexes (b–d and f). Scale bars represent 50 nm. Two independent formulations batches were analyzed and 20 images per sample were acquired. One representative image per sample was shown.

The reconstruction of the intensity profiles was not possible by modelling the particles with a single form factor. This was due to the coexistence of particles with diversified internal structure. As for chitosan lipopolyplexes, their spectrum was quite similar to that of chitosan polyplexes (Figure 2B), except for the low- q and the high- q regions, suggesting that a large fraction of complexes kept the internal mass fractal arrangement of chitosan polyplexes, possibly stabilized by a lipid shell. Conversely, PLL lipopolyplexes showed an intensity profile that was markedly different from that of the corresponding polyplexes (Figure 2B), indicating a rearrangement of the majority of components in a complex with a closer packing (fractal dimension 3.6). Moreover, the formulation of PEI lipopolyplexes displayed a more defined intensity profile (Figure 2B). We then subtracted the intensity contributions of a fraction of liposomes (10% of the experimental liposome signal) and a fraction of polyplexes (40%), corresponding to a fraction of polyplexes in the final formulation or particles with the same internal arrangement. The subtracted intensity profile is reported in Figure 2C along with the reconstruction, obtained by modelling the form factor of the lipopolyplexes with a spherical particle with a core-shell internal structure. The core had an electron density higher than that of the solvent, confirming the formation of a spherical complex, surrounded by a lipid bilayer. Of note, the internal arrangement of these lipopolyplexes was different from that of the polyplex, suggesting a true interaction between the polymers and the lipids that rearrange with a tighter packing.

Cryo-EM further confirmed that polyplexes were dense structures of consistent and homogeneous morphology with a tendency to assume round shape, irrespective of the cationic polymer used (Figure 3A–C). Lipopolyplexes were still spherical shaped, though their structure strongly differed; indeed, all ternary complexes predominantly assumed a multilamellar structure upon complexation and internalization of polyplexes (Figure 3D–F).

In vitro Assays

Cytotoxicity of Formulations

The cytotoxic effect of all formulations on HeLa cells was evaluated after 48 hours by an MTT assay. Formulations were diluted to reach a polymer concentration ranging between 60 and 0.7 $\mu\text{g/mL}$ and DOTAP lipopolyplexes were used as positive control. Generally, as shown in Figure 4A, cytotoxicity was driven mainly by polymer nature and secondly by the type of delivery system. At polymer concentrations above 7.5 $\mu\text{g/mL}$, the viability of cells incubated with all formulations remained above 80% (which is the standard cutoff for considering cells as viable) with no significant differences across the concentrations. Of note, chitosan turned out to be the most biocompatible polymer; indeed, the percentage of viable cells incubated with both polyplexes and lipopolyplexes was above 80% irrespective of the polymer dose tested. As for the other polymers, PLL was significantly more cytotoxic than PEI and as toxic as DOTAP lipopolyplexes, particularly at the highest doses. For instance, at 60 $\mu\text{g/mL}$ of polymer, the percentage of viable cells were 12.5 ± 5.8 , 26.3 ± 4.4 and 20.8 ± 10.6 for PLL polyplexes, PEI polyplexes and DOTAP lipopolyplexes, respectively (Figure 4A). Corresponding lipopolyplexes behaved similarly, with those made of PEI being significantly less toxic ($p < 0.05$) though the addition of liposomes augmented cell viability for both polyplexes ($32.8 \pm 12.6\%$ and $50.1 \pm 4.3\%$). Moreover, the subtoxic polymer dose of PLL and PEI lipopolyplexes was 1.5-fold higher than that of corresponding polyplexes (30 vs 20 $\mu\text{g/mL}$), overall indicating that ternary complexes were more biocompatible than binary counterparts (Figure 4A).

In vitro Cellular Uptake of Formulations

In vitro cellular uptake of Rhod-FITC double labelled lipopolyplexes both at 4°C and 37°C was performed (representative dot plots are reported in Figure S6). At 4°C, the temperature at which endocytosis is inhibited, the percentage of Rhod+/FITC+ cells (ie lipopolyplexes +) was below 4% after 1 h and did not increase above 10% over time, irrespective of the polymer choice (Figure S7). In contrast, at 37°C, lipopolyplexes rapidly interacted with HeLa, particularly those made of PEI, with approximately 22% Rhod+/FITC+ cells after 1 h incubation, whereas only 5.5% of cells incubated with Chitosan or PLL lipopolyplexes were Rhod+/FITC+ (Figure 4B). However, increasing the incubation times, the difference among LPPs became non-significant, with approximately 40% and 70% Rhod+/FITC+ cells after 4 h and 24 h incubation for all the formulations (Figure 4B), suggesting that uptake kinetics of lipopolyplexes is independent from the polymer choice.

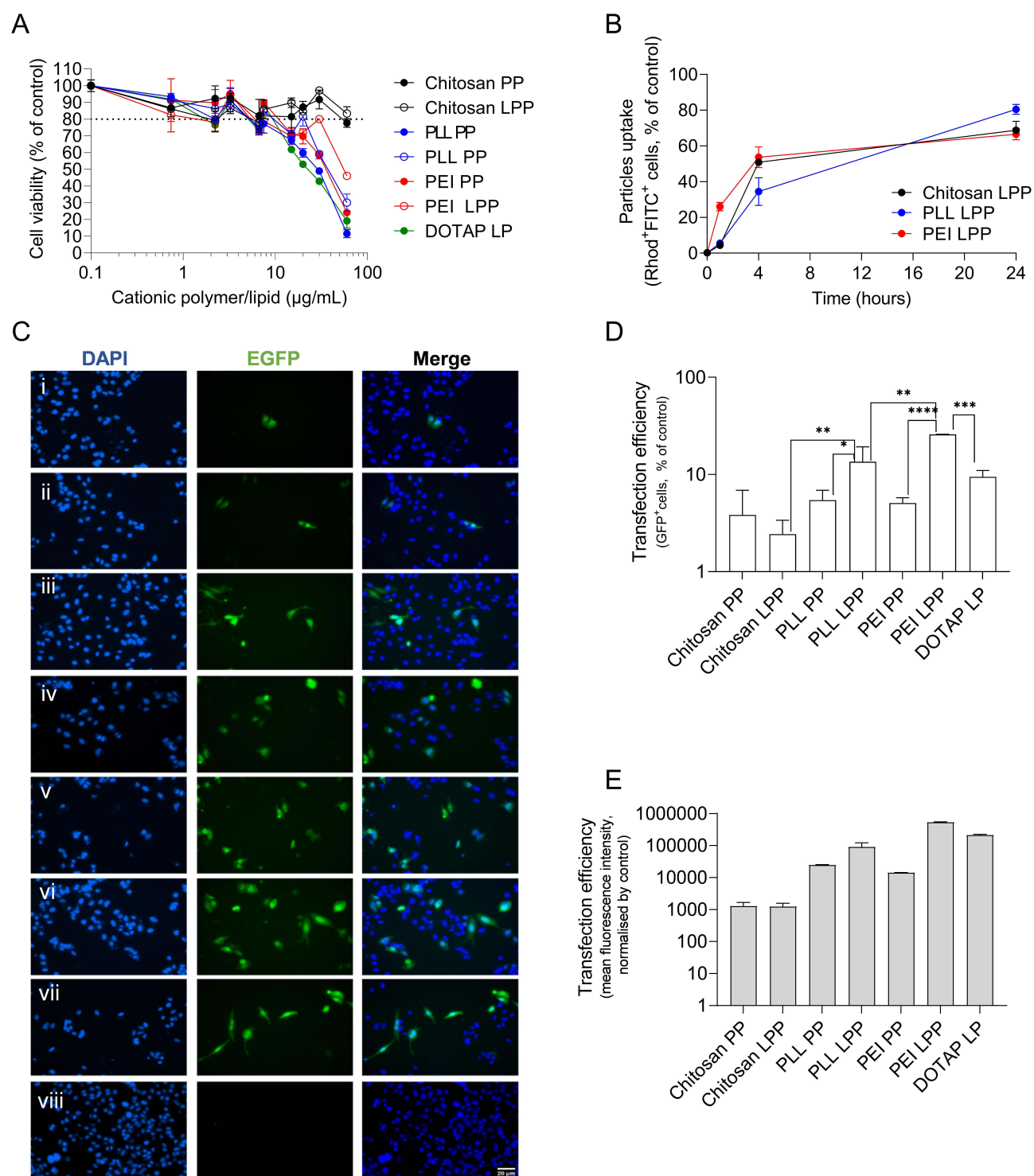


Figure 4 In vitro cytotoxicity, cell uptake and transfection efficiency of polyplexes and lipopolyplexes in HeLa cells. **(A)** Cell viability of Chitosan, PLL and PEI polyplexes (PP) and corresponding lipopolyplexes (LPP) on HeLa cells after 48h incubation. DOTAP lipopolyplexes (LP) were used as positive control. The dotted line represents the threshold for cytotoxicity level of 80% of cells viability compared to untreated cells. The results of two or three independent experiments each performed in triplicate were shown (mean $\pm$ SD). **(B)** Cellular uptake of Chitosan, PLL and PEI LPP in HeLa cells represented in terms of percentage of Rhod⁺/FITC⁺ cells. The means $\pm$ SD of one experiment performed in duplicate were reported. Transfection efficiency of PP and LPP in HeLa cells after 48h incubation (blue DAPI, Green GFP). **(C)** Representative images of transfected HeLa cells by i) Chitosan PP, iii) PLL PP and v) PEI PP and corresponding LPP (ii, iv and vi); DOTAP LP (vii) and cells incubated with media only (viii) were used as positive and negative controls respectively. Scalebar represents 20 μm . The experiment was performed in duplicate. Quantitative analysis of formulations' transfection efficiency expressed as **(D)** percentage of GFP⁺ cells and **(E)** mean fluorescence intensity. The results of one or two experiments, performed in duplicate, are shown (mean $\pm$ SD). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Transfection Efficiency of Formulations

Based on cytotoxicity assay, chitosan and PLL concentration of 20 $\mu\text{g/mL}$, and PEI concentrations of 12 $\mu\text{g/mL}$ (corresponding all to 1 $\mu\text{g/well}$ DNA) were selected to assess formulations' in vitro efficiency on HeLa cells. As reported in [Figure 4C–E](#), although all candidates were effective in promoting cell transfection after 48 hours in complete medium, the efficiency of the transfection varied. As qualitatively shown by fluorescent microscopy images, PEI lipopolyplexes were the most effective in promoting gene expression ([Figure 4C](#)). Quantification of GFP signal by flow cytometry suggested that the addition of liposomal component increased PLL and PEI formulations' performance ([Figure 4D](#)), while no difference was observed between chitosan polyplexes and lipopolyplexes (approximately 3%) in terms of percentage of transfected cells. Specifically, PLL lipopolyplexes transfected cells were almost three-times higher compared to those transfected by polyplexes counterpart (around 5% vs 13%); similarly, PEI lipopolyplexes transfection efficiency was 5-fold higher than that of corresponding polyplexes (25% vs 5% respectively). Overall, among the three lipopolyplexes, the percentage of GFP+ cells were ranked in the order PEI > PLL > Chitosan lipopolyplexes. Specifically, PLL lipopolyplexes and PEI lipopolyplexes were as potent as and significantly more potent, respectively, than DOTAP lipopolyplexes which is considered the gold standard for in vitro transfection ([Figure 4D](#)). Similarly, the mean fluorescence intensity (MFI) of cells incubated with PEI lipopolyplexes was the highest among the formulation tested ([Figure 4E](#)), indicating that PEI lipopolyplexes transfected a higher number of cells expressing higher protein content. Interestingly, PEI lipopolyplexes were effective even when tested in non-tumor cells (HEK-293 embryonic kidney cell line and primary HUVECs endothelial cells). Again, PEI lipopolyplexes were the best performing formulation which transfected both cell lines with comparable efficacy (29.2 ± 2.8 and $36.2 \pm 0.7\%$ of GFP+ cells on HEK-293 –[Figure S8A](#) – and HUVECs – [Figure S8B](#) – respectively), similarly to HeLa cells, and they were also more potent than the gold standard DOTAP lipopolyplexes ([Figure S8](#)). Finally, this formulation was highly stable both in saline solution over 14 days at 4°C and in 10% FBS at 37°C for 48 hours as the size did not markedly vary (size around 125 nm, PDI around 0.25 in PBS, [Figure S9A](#) and around 100 nm in serum, [Figure S9B](#)).

Endosomal Escape Promoted by Formulations

To further investigate the mechanism of cell entry and gene transfection of polyplexes and lipopolyplexes, the endosomal escape ability of binary and ternary complexes was evaluated using a calcein based assay (schematic representation of the calcein assay reported in [Figure 5B](#)). Of note, our preliminary expression kinetics results reported no GFP signal at this timepoint (data not shown), ensuring that only the real signal from calcein is detected. The percentage of cells that showed calcein release after 4 h is displayed in [Figure 5](#) as a function of the different delivery systems. Cells incubated with calcein alone or in combination with DOTAP lipopolyplexes were used as negative and positive controls, respectively. As expected, when cells were exposed to calcein alone, the probe got entrapped into endosomes during the vesicle formation resulting in punctate fluorescence in cell cytoplasm with no endosomal release ([Figure 5A](#) panel viii); conversely, the co-incubation of calcein with DOTAP lipopolyplexes resulted in diffused cytoplasmic fluorescence showed by the majority of cells ([Figure 5A](#) panel vii). Regarding polyplexes and lipopolyplexes, particles' endosomolytic effect was driven mainly by the polymer nature, in line with EGFP expression; specifically, the co-incubation of calcein with chitosan based particles ([Figure 5A](#) panels i and ii) poorly promotes the release of the probe from endosomes, as only few cells displayed diffused fluorescence in the cytoplasm indicating calcein-loaded endosomal disruption (3.46 and 5% for Chitosan PP and LPP, respectively, [Figure 5C](#)). Conversely, both PLL and PEI containing particles were effective in inducing endosomal escape, as many cells displayed diffused fluorescence ([Figure 5A](#) panels iii–vi), though with different potencies. Indeed, considering ternary complexes, PEI lipopolyplexes provoked calcein endosomal release in almost 33% of cells, and this value was more than 3-fold higher than that of PLL lipopolyplexes incubated cells ([Figure 5C](#)). Of note, PEI lipopolyplexes endosomal escape efficiency was also significantly ($p < 0.01$) higher than that of DOTAP lipopolyplexes (18.67% - [Figure 5C](#)).

Moreover, the presence of a lipid moiety did not strongly vary the mode of endosomal disruption induced by lipopolyplexes compared to polyplexes as only a tendency of ternary complexes to promote greater escape from endosomes than binary complexes was observed, and this difference became significant only in the case of PEI particles.

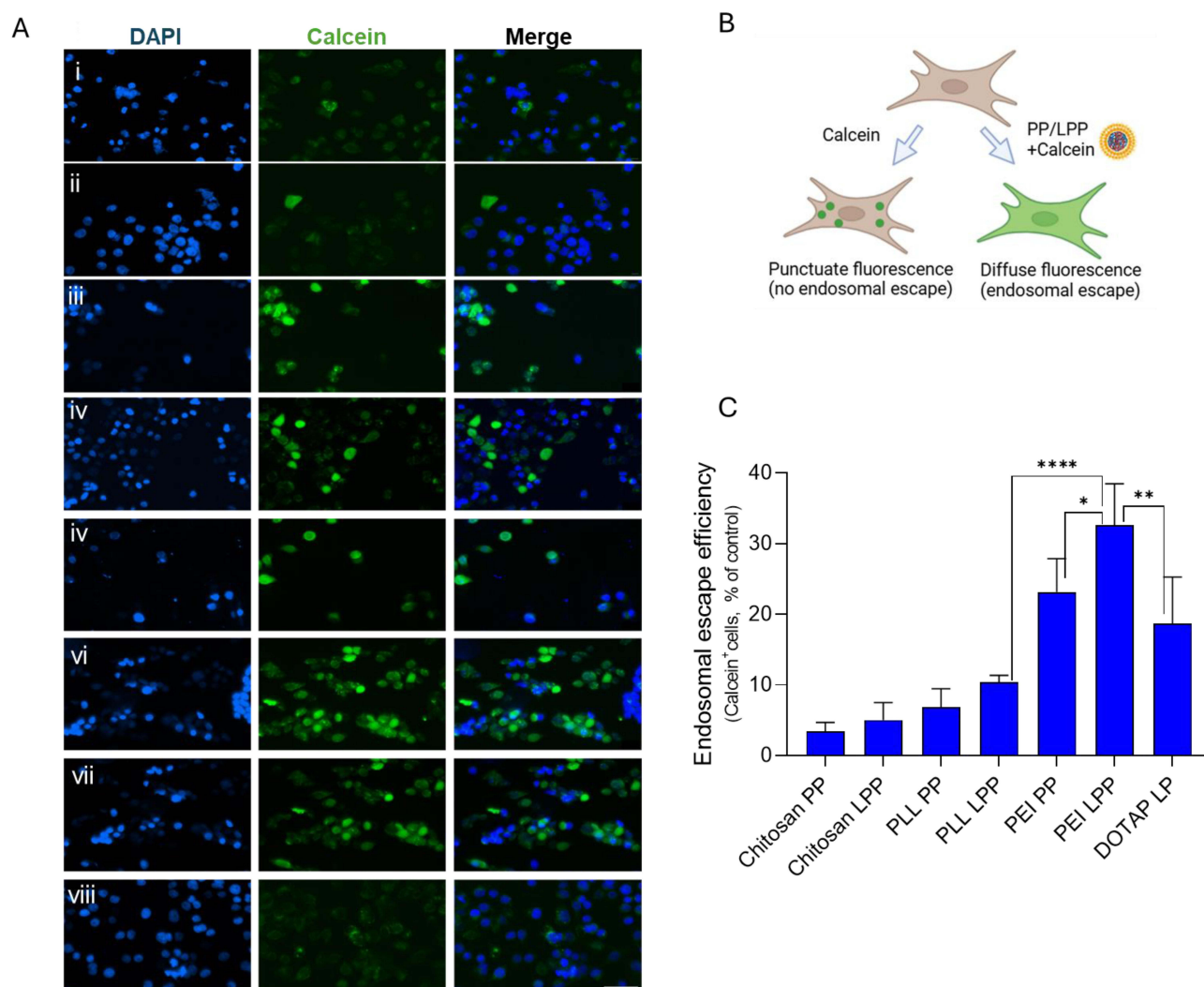


Figure 5 Endosomal escape capacity of polyplexes (PP) and lipopolyplexes (LPP). **(A)** Representative images of HeLa cells incubated with calcein only (viii) and i) Chitosan PP, iii) PLL PP and v) PEI PP or corresponding LPP (ii, iv and vi) or DOTAP lipopolyplexes (LP;vii). Blue DAPI, green Calcein. Scalebar represents 20 μ m. **(B)** Schematic representation of the calcein assay used for endosomal escape quantification. **(C)** Quantitative analysis of the percentage of diffused Calcein⁺ cells among the total DAPI + cells. The results of one experiment, performed in triplicate, are shown (mean $\pm$ SD). * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

Specifically, the endosomal escape efficiencies of chitosan, PLL and PEI lipopolyplexes were around 1.5-fold higher than those of corresponding polyplexes (5, 10.35 and 33% vs 3.46 and 6.89 and 23%, respectively, Figure 5C).

Discussion

Lipopolyplexes represent second-generation non-viral vectors for gene therapy aiming to enhance nucleic acid delivery by combining the favorable properties of both components. However, manufacturing is still the main limiting factor which strongly restricts the preclinical and clinical applications of these nanovectors. To date, bulk mixing is still the widest used method for lipopolyplexes preparation and, to the best of our knowledge, only one work applied a microfluidic system for lipopolyplexes production,³⁵ notably, an additional sonication step and a premixing step of polymer and lipids were required to obtain narrow-sized particles³⁵ in the aforementioned work. Conversely, the microfluidic system we developed provides a unique tool to formulate polymer-lipid ternary complexes with consistent size and size distribution compared to conventional bulk mixing without the need of any downsizing techniques. Our data demonstrated that, by adjusting process operating parameters such as flow rate ratio, total flow rate and polymer concentration, lipopolyplexes can be prepared within a limited size range. Moreover, as opposed to the cited work in

which a hydrodynamic flow focusing mixing was employed, here we applied a staggered herringbone micromixer which is known to improve mixing efficiency, facilitate scalability and limit sample dilution.^{36–39} Both binary and ternary complexes prepared via microfluidics possessed adequate physicochemical attributes (Z-average around 150 nm and PDI < 0.2) and high DNA condensation and loading (above 89%) though the high molecular weight of Chitosan and PLL used herein (200–400KDa on average). Microfluidics appear to address several formulation challenges associated with the use of these high molecular weight polymers,⁴⁰ which are less frequently discussed in the literature compared to their medium and low molecular weight counterparts. In particular, the use of high molecular weight chitosan (>400 KDa) for lipopolyplexes preparation to the best of our knowledge has never been reported. Herein, we demonstrated the feasibility to obtain nanosized complexes using also this polymer. Indeed, the increase of the molecular weight of polymers might provide significant advantages for cell internalization and enhance the overall effectiveness of the delivery systems.^{41,42}

Of note, the addition of a lipid envelope on polyplexes core resulted in lipopolyplexes with equivalent or reduced particles size. This might indicate that lipopolyplexes tended to adopt a more compact and ordered structure than corresponding polyplexes, possibly due to additional charge-to-charge interactions between the negatively charged liposome and the positively charged polymer. These findings are consistent with those of Perche and colleagues who showed that entrapping PEI-mRNA PP within neutral liposomes did not significantly change the size of binary complexes.¹¹ However, an increase in particle size of PP upon liposome complexation has also been described.^{43,44} These contradictory results might be due to i) the change in polymers and lipids composition within LPP which possess different physicochemical properties, ie molecular weight, charge density and transition temperature and ii) the application of different techniques for particles size determination (AFM vs DLS vs PCS vs TEM) which might not be always comparable.

Regarding particles surface, the combination of cationic polyplexes and neutral liposomes used herein resulted in hybrid nanosystems with slightly positive surface charge (around +14mV) compared to binary counterparts (around +30 mv) irrespective of the polymer nature. The use of neutral/slightly anionic liposomes in place of cationic counterparts was proved to reduce cytotoxicity and improve biocompatibility and transfection efficiency of LPP.^{12,45} Differences in particles count and the reduction in surface charge of cationic polyplexes upon neutral liposomes addition are ways to indicate encapsulation of polyplexes in lipid bilayers, though insufficient to precisely define the structure of binary and ternary complexes. Here, by combining three independent techniques – FRET, SAXS and Cryo-EM – a more comprehensive structural analysis of polyplexes and lipopolyplexes was provided.

SAXS analysis indicated that the type of polymer used to produced polyplexes by microfluidics affected the overall structure of binary complexes. Specifically, PEI polyplexes organized in clusters with a close-packed substructure, whereas those made of chitosan and PLL formed loose-packed substructure. This correlated with FRET analysis which showed greater nucleic acid complexation ability (higher Q%) of PEI polyplexes over chitosan and PLL. Both techniques confirmed the formation of a ternary complex upon liposomes mixing with polyplexes, and that the structure of lipopolyplexes is influenced by the polymer nature. As suggested by SAXS, chitosan lipopolyplexes tended to assume a globular core-shell structure of an external lipid layer and a dense core, likely composed of chitosan polyplexes. Conversely, spectra of PLL and PEI lipopolyplexes showed a rearrangement among polymer, DNA and liposomes and a true interaction between the polymer and the lipids to form a packed complex, which strongly differed from the original polyplex. These findings were also supported by FRET data, where lipopolyplexes of PEI and PLL induced higher FRET efficiency and shorter Forster radius compared to Chitosan counterpart.

The presence of these ternary structures was also confirmed by cryo-EM images. Overall, our data indicated that reducing polymer molecular weight (MW) and increasing its pI, promote its interaction with liposomes and DNA. It might be worth noting that the MW range of chitosan and PLL used herein is broad (150–600 and 150–300 KDa, respectively) and this might impair a precise correlation between MW and condensation ability. Besides, though of lower MW (around 25KDa), PEI is a branched polymer, and this increases the number of nitrogen groups per monomer thus augmenting the charge density, balancing the lower MW on condensation ability.

Polymers' pI is a key factor for DNA complexation, which dictates the degree of protonation and affects polymer's charge and consequent interaction with the payload. The pI of PLL and PEI tested herein is around 9 and 11, respectively, while the pKa of the primary amine of chitosan is usually 6.5, depending on the degree of N-deacetylation and degree of

polymerization.⁴⁶ Therefore, a higher degree of ionization at the pH at which complexation occurred is expected for basic polymers. It is reported that, at acidic pH, PEI showed two modes of DNA binding - both to DNA grooves and to the DNA phosphate backbone^{47,48} - and this increases its tendency to form stable complexes. The high buffer capacity of PEI compared to the other polymers tested is expected to increase the protonation ratio of amine groups at low pH, thus leading to stable DNA complexes.

In vitro, polyplexes and lipopolyplexes performances were mainly driven by the polymer nature and secondly by the type of delivery system. Chitosan was the most biocompatible polymer, however, this did not translate into enhanced GFP expression despite the use of high molecular weight chitosan in lipopolyplex formulations has been associated with increased transfection efficiency.⁴²

Besides, the addition of liposomes improved the viability of polyplexes, particularly in the case of PEI particles which resulted to be significantly less toxic even when compared to a DOTAP lipopolyplexes. These data were in line with previous findings which reported the improved toxicity profile of lipopolyplexes over polyplexes,^{10,49} additionally, the use of microfluidics allowed to reduce the N/P of PEI lipopolyplexes without affecting the stability (up to 14 days at 4°C in saline solution), ultimately minimising both unbound polymer in PEI-based formulations and the total polymer content for in vitro administration, which would possibly trigger a necrotic response caused by excessive cationic charges and membrane destabilization.⁵⁰ Further, PEI ternary complexes were significantly more potent than the control DOTAP lipopolyplexes and PLL lipopolyplexes in promoting protein expression in three different cell lines (ie HeLa, HEK-293 and HUVECs), thus being a versatile platform for effective DNA delivery potentially suitable for a range of therapeutic applications.

Our data demonstrated that the higher transfection efficiency of PEI lipopolyplexes correlated with augmented endosomal disruption capacity. The efficiency of a delivery system to trigger a successful transfection process depends on several factors including the ability to prevent the cargo degradation, to interact with cell membranes and being internalized, to favor endolysosomal escape and DNA nuclear entry. PEI have been shown to facilitate endosomal escape via osmotic rupture - *proton sponge effect* - as the amine groups can enhance the protons concentrations within the endosomes increasing the influx of chloride ions and water, thus leading to differential pressure levels on both sides of the endosomal membrane and consequent vesicles disruption.^{51,52} Other studies proposed that a direct interaction between PEI and the endosomal membranes would be an additional mechanism to proton sponge effect which triggers membrane destabilization.⁵³ Additionally, endocytosed PEI was found to undergo nuclear localization, thus inevitably favoring DNA entry into the nucleoplasm.⁵⁴ The poor endosomal escape promoted by chitosan might be related to the lower buffering capacity of the cationic polymer, also reported in literature,^{55,56} thus suggesting that the endolysosomal disruption mechanism of chitosan might not be proton sponge based.

Herein we further demonstrated that the superior capacity of PEI to trigger endosomal escape was not related to an enhanced internalization of PEI lipopolyplexes over other ternary complexes, as the uptake kinetics was comparable among the three ternary complexes. Notably, similar percentage of positive cells at 4 and 37°C for Chitosan and PLL LPP suggest that, after 1 h, these lipopolyplexes could be surface-associated rather than internalized. However, the contribution of surface-associated particles observed at 4°C could be considered negligible at later timepoints thus indicating an actual cellular uptake rather than a combination of surface-association and internalization of lipopolyplexes. An active internalization could facilitate endosomal escape of particles, as they usually enter cells via endocytosis.^{57,58} These findings align with previous studies which indicate an ATP mediated endocytic pathway for PEI lipopolyplexes, further reporting a caveolar and clathrin-dependent mechanism of internalization.⁵⁹ Overall, these data indicate that the presence of a lipid envelope rather than the polymer nature drove lipopolyplexes internalization process, while the type of cationic polymer played the major role in the intercellular trafficking of these particles.

Conclusions

The present study demonstrates the successful preparation of pDNA- lipopolyplexes of different cationic polymers via microfluidics possessing adequate physico-chemical attributes and providing full DNA condensation. All lipopolyplexes were ternary complexes in which interactions among lipopolyplexes components were confirmed by FRET, SAXS and Cryo-TEM analyses. Structurally, while chitosan lipopolyplexes appeared as globular core-shell particles of an external

lipid multilamellar layer and a dense core of polyplexes, those of PLL and PEI were packed complexes in which a rearrangement among polymer, DNA and liposomes and a true interaction between the polymer and the lipids occurred. The choice of cationic polymer also strongly dictated in vitro performances of these synthetic vectors. In essence, though uptake kinetics of the three lipopolyplexes were comparable, PEI lipopolyplexes promoted the highest transfection efficiency in both tumor and non-tumor/primary cells, and this correlated with a greater endosomolytic activity. Overall, this study demonstrates that lipopolyplexes are a versatile and valid platform for pDNA delivery with PEI lipopolyplexes being the most potent formulation; moreover, the type of cationic polymer played the major role in the intercellular trafficking of these nanovectors. This work poses the basis for future studies in which the composition and surface properties of these ternary complexes can be modified to optimize their performance based on the selected target and pathology. Further research is also needed to optimize the microfluidic method for preparing lipopolyplexes in a single injection step.

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Disclosure

The authors report no conflicts of interest in this work.

References

- Kulkarni JA, Witzigmann D, Thomson SB, et al. The current landscape of nucleic acid therapeutics. *Nature Nanotechnol.* **2021**;16(6):630–643. doi:10.1038/s41565-021-00898-0
- Jones CH, Chen CK, Ravikrishnan A, Rane S, Pfeifer BA. Overcoming nonviral gene delivery barriers: perspective and future. *Mol Pharmaceut.* **2013**;10(11):4082–4098. doi:10.1021/mp400467x
- Mendes BB, Connot J, Avital A, et al. Nanodelivery of nucleic acids. *Nat Rev Method Primers.* **2022**;2(1). doi:10.1038/s43586-022-00104-y
- Chemaitelly H, Ayoub H, Coyle P, et al. BNT162b2 versus mRNA-1273 vaccines: comparative analysis of long-term Protection against SARS-CoV-2 infection and severe COVID-19 in Qatar. *Influenza Other Respir Viruses.* **2024**;18(10). doi:10.1111/irv.13357
- Wilson E, Goswami J, Baqui AH, et al. Efficacy and safety of an mRNA-based RSV pref vaccine in older adults. *N Engl J Med.* **2023**;389(24):2233–2244. doi:10.1056/NEJMoa2307079
- Taghdiri M, Mussolino C. Viral and non-viral systems to deliver gene therapeutics to clinical targets. *Int J Mol Sci.* **2024**;25(13):7333. doi:10.3390/ijms25137333
- Geall AJ, Verma A, Otten GR, et al. Nonviral delivery of self-amplifying RNA vaccines. *Proc Natl Acad Sci U S A.* **2012**;109(36):14604–14609. doi:10.1073/pnas.1209367109
- Tros de Ilarduya C, Sun Y, Düzgüneş N. Gene delivery by lipoplexes and polyplexes. *Eur J Pharm Sci.* **2010**;40(3):159–170. doi:10.1016/j.ejps.2010.03.019
- Loeche A, Bruyns-Haylett M, Ballester PJ, Borros S, Oliva N. A machine learning approach to predict cellular uptake of pBAE polyplexes. *Biomater Sci.* **2023**;11(17):5797–5808. doi:10.1039/D3BM00741C
- Rezaee M, Oskuee RK, Nassirli H, Malaekheh-Nikouei B. Progress in the development of lipopolyplexes as efficient non-viral gene delivery systems. *J Control Release.* **2016**;236:1–14. doi:10.1016/j.jconrel.2016.06.023
- Perche F, Clemençon R, Schulze K, et al. Neutral lipopolyplexes for in vivo delivery of conventional and replicative RNA vaccine. *Mol Ther Nucleic Acids.* **2019**;17:767–775. doi:10.1016/j.omtn.2019.07.014
- Chen W, Li H, Liu Z, Yuan W. Lipopolyplex for therapeutic gene delivery and its application for the treatment of Parkinson’s disease. *Front Aging Neurosci.* **2016**;8. doi:10.3389/fnagi.2016.00068
- Le Moignic A, Malard V, Benvegnu T, et al. Preclinical evaluation of mRNA trimannosylated lipopolyplexes as therapeutic cancer vaccines targeting dendritic cells. *J Control Release.* **2018**;278:110–121. doi:10.1016/j.jconrel.2018.03.035
- Welser K, Campbell F, Kudsiya L, et al. Gene delivery using ternary lipopolyplexes incorporating branched cationic peptides: the role of peptide sequence and branching. *Mol Pharm.* **2013**;10(1):127–141. doi:10.1021/mp300187t
- Dai J, Zou S, Pei Y, et al. Polyethylenimine-grafted copolymer of poly(l-lysine) and poly(ethylene glycol) for gene delivery. *Biomaterials.* **2011**;32(6):1694–1705. doi:10.1016/j.biomaterials.2010.10.044
- Kö Ping-Hö Ggård M, Tubulekas I, Guan H, et al. Chitosan as a nonviral gene delivery system. structure-property relationships and characteristics compared with polyethylenimine in vitro and after lung administration in vivo. *Genet Ther.* **2001**;8.
- Casper J, Schenk SH, Parhizkar E, et al. Polyethylenimine (PEI) in gene therapy: current status and clinical applications. *J Control Release.* **2023**;362:667–691. doi:10.1016/j.jconrel.2023.09.001
- Nouri F, Sadeghpour H, Heidari R, Dehshahri A. Preparation, characterization, and transfection efficiency of low molecular weight polyethylenimine-based nanoparticles for delivery of the plasmid encoding cd200 gene. *Int J Nanomed.* **2017**;12:5557–5569. doi:10.2147/IJN.S140734

19. Hua P, Yang D, Chen R, Qiu P, Chen M. ROS responsive polyethylenimine-based fluorinated polymers for enhanced transfection efficiency and lower cytotoxicity. *Bosn J Basic Med Sci.* **2022**;22(4):593–607. doi:10.17305/bjbms.2021.6704
20. Taheri Z, Kazemi M, Khalvati B, et al. Dihydroxyphenylalanine-conjugated high molecular weight polyethylenimine for targeted delivery of plasmid. *Sci Rep.* **2024**;14(1). doi:10.1038/s41598-024-71798-1
21. Tian H, Lin L, Jiao Z, et al. Polylysine-modified polyethylenimine inducing tumor apoptosis as an efficient gene carrier. *J Control Release.* **2013**;172(2):410–418. doi:10.1016/j.jconrel.2013.06.026
22. Ahmad A, Khan JM, Paray BA, Rashid K, Parvez A. Endolysosomal trapping of therapeutics and endosomal escape strategies. *Drug Discovery Today.* **2024**;29(8):104070. doi:10.1016/j.drudis.2024.104070
23. Heyes J, Palmer L, Chan K, et al. Lipid encapsulation enables the effective systemic delivery of polyplex plasmid DNA. *Mol Ther.* **2007**;15(4):713–720. doi:10.1038/sj.mt.6300101
24. Ponnusamy PG, Mani S. Material and environmental properties of natural polymers and their composites for packaging applications—a review. *Polymers.* **2022**;14(19):4033. doi:10.3390/polym14194033
25. Harugade A, Sherje AP, Pethe A. Chitosan: a review on properties, biological activities and recent progress in biomedical applications. *React Funct Polym.* **2023**;191:105634. doi:10.1016/j.reactfunctpolym.2023.105634
26. Dong L, Li Y, Cong H, Yu B, Shen Y. A review of chitosan in gene therapy: developments and challenges. *Carbohydr Polym.* **2024**;324:121562. doi:10.1016/j.carbpol.2023.121562
27. Widmer S, Reber MJ, Müller P, et al. Incorporation of a FRET dye pair into mesoporous materials: a comparison of fluorescence spectra, FRET activity and dye accessibility. *Analyst.* **2015**;140(15):5324–5334. doi:10.1039/C5AN00850F
28. Das S, Manam J. Fluorescein isothiocyanate and rhodamine B dye encapsulated mesoporous SiO₂ for applications of blue LED excited white LED. *Opt Mater.* **2018**;79:259–263. doi:10.1016/j.optmat.2018.03.052
29. Stanzel M, Brilmayer R, Langhans M, Meckel T, Andrieu-Brunsen A. Systematic study on FRET-pair functionalization of mesoporous thin films for correlation of pH-sensing and ionic mesopore accessibility. *Microporous Mesoporous Mater.* **2019**;282:29–37. doi:10.1016/j.micromeso.2019.03.009
30. Ko YT, Bickel U, Huang J. Polyethylenimine/oligonucleotide polyplexes investigated by fluorescence resonance energy transfer and fluorescence anisotropy. *Oligonucleotides.* **2011**;21(2):109–114. doi:10.1089/oli.2010.0271
31. Vogel SS, Van der Meer BW, Blank PS. Estimating the distance separating fluorescent protein FRET pairs. *Methods.* **2014**;66(2):131–138. doi:10.1016/j.ymeth.2013.06.021
32. Sekar RP, Lawson JL, Wright ARE, et al. Poly(l-glutamic acid) augments the transfection performance of lipophilic polycations by overcoming tradeoffs among cytotoxicity, pDNA delivery efficiency, and serum stability. *RSC Applied Polymers.* **2024**;2(4):701–718. doi:10.1039/D4LP00085D
33. Jordan M, Wurm F. Transfection of adherent and suspended cells by calcium phosphate. *Methods.* **2004**;33(2):136–143. doi:10.1016/j.ymeth.2003.11.011
34. Di Cola E, Cantu' L, Brocca P, et al. Novel O/W nanoemulsions for nasal administration: structural hints in the selection of performing vehicles with enhanced mucopenetration. *Colloids Surf B Biointerfaces.* **2019**;183:110439. doi:10.1016/j.colsurfb.2019.110439
35. Koh CG, Zhang X, Liu S, et al. Delivery of antisense oligodeoxynucleotide lipopolyplex nanoparticles assembled by microfluidic hydrodynamic focusing. *J Control Release.* **2010**;141(1):62–69. doi:10.1016/j.jconrel.2009.08.019
36. Joshi S, Hussain MT, Roces CB, et al. Microfluidics based manufacture of liposomes simultaneously entrapping hydrophilic and lipophilic drugs. *Int J Pharm.* **2016**;514(1):160–168. doi:10.1016/j.ijpharm.2016.09.027
37. Webb C, Forbes N, Roces CB, et al. Using microfluidics for scalable manufacturing of nanomedicines from bench to GMP: a case study using protein-loaded liposomes. *Int J Pharm.* **2020**;582:119266. doi:10.1016/j.ijpharm.2020.119266
38. Lopes C, Cristóvão J, Silvério V, Lino PR, Fonte P. Microfluidic production of mRNA-loaded lipid nanoparticles for vaccine applications. *Expert Opin Drug Delivery.* **2022**;19(10):1381–1395. doi:10.1080/17425247.2022.2135502
39. Jürgens DC, DeBloch L, Porras-Gonzalez D, et al. Lab-scale siRNA and mRNA LNP manufacturing by various microfluidic mixing techniques – an evaluation of particle properties and efficiency. *OpenNano.* **2023**;12:100161. doi:10.1016/j.onano.2023.100161
40. Schmid F. Understanding and modeling polymers: the challenge of multiple scales. *ACS Polymers Au.* **2023**;3(1):28–58. doi:10.1021/acspolymersau.2c00049
41. Zheng M, Pan M, Zhang W, et al. Poly(α -L-lysine)-based nanomaterials for versatile biomedical applications: current advances and perspectives. *Bioact Mater.* **2021**;6(7):1878–1909. doi:10.1016/j.bioactmat.2020.12.001
42. Huang M, Fong CW, Khor E, Lim LY. Transfection efficiency of chitosan vectors: effect of polymer molecular weight and degree of deacetylation. *J Control Release.* **2005**;106(3):391–406. doi:10.1016/j.jconrel.2005.05.004
43. Pinnapireddy SR, Duse L, Strehlow B, Schäfer J, Bakowsky U. Composite liposome-PEI/nucleic acid lipopolyplexes for safe and efficient gene delivery and gene knockdown. *Colloids Surf B Biointerfaces.* **2017**;158:93–101. doi:10.1016/j.colsurfb.2017.06.022
44. Schäfer J, Höbel S, Bakowsky U, Aigner A. Liposome-polyethylenimine complexes for enhanced DNA and siRNA delivery. *Biomaterials.* **2010**;31(26):6892–6900. doi:10.1016/j.biomaterials.2010.05.043
45. Ewe A, Schaper A, Barnert S, et al. Storage stability of optimal liposome-polyethylenimine complexes (lipopolyplexes) for DNA or siRNA delivery. *Acta Biomater.* **2014**;10(6):2663–2673. doi:10.1016/j.actbio.2014.02.037
46. Bakshi PS, Selvakumar D, Kadirvelu K, Kumar NS. Chitosan as an environment friendly biomaterial – a review on recent modifications and applications. *Int J Biol Macromol.* **2020**;150:1072–1083. doi:10.1016/j.ijbiomac.2019.10.113
47. Sun C, Tang T, Uludağ H, Cuervo JE. Molecular dynamics simulations of DNA/PEI complexes: effect of PEI branching and protonation state. *Biophys J.* **2011**;100(11):2754–2763. doi:10.1016/j.bpj.2011.04.045
48. Utsuno K, Uludağ H. Thermodynamics of polyethylenimine-DNA binding and DNA condensation. *Biophys J.* **2010**;99(1):201–207. doi:10.1016/j.bpj.2010.04.016
49. Costa B, Boueri B, Oliveira C, Silveira I, Ribeiro AJ. Lipoplexes and polyplexes as nucleic acids delivery nanosystems: the current state and future considerations. *Expert Opin Drug Deliv.* **2022**;19(5):577–594. doi:10.1080/17425247.2022.2075846
50. Monnery BD, Wright M, Cavill R, et al. Cytotoxicity of polycations: relationship of molecular weight and the hydrolytic theory of the mechanism of toxicity. *Int J Pharm.* **2017**;521(1–2):249–258. doi:10.1016/j.ijpharm.2017.02.048

51. Benjaminsen RV, Mattheijer MA, Henriksen JR, Moghimi SM, Andresen TL. The possible “proton sponge” effect of polyethylenimine (PEI) does not include change in lysosomal pH. *Mol Ther*. 2013;21(1):149–157. doi:10.1038/mt.2012.185
52. Vermeulen LMP, De Smedt SC, Remaut K, Braeckmans K. The proton sponge hypothesis: fable or fact? *Eur J Pharm Biopharm*. 2018;129:184–190. doi:10.1016/j.ejpb.2018.05.034
53. Yue Y, Jin F, Deng R, et al. Revisit complexation between DNA and polyethylenimine - effect of uncomplexed chains free in the solution mixture on gene transfection. *J Control Release*. 2011;155(1):67–76. doi:10.1016/j.jconrel.2010.10.028
54. Jerzykiewicz J, Czogalla A. Polyethyleneimine-based lipopolyplexes as carriers in anticancer gene therapies. *Materials*. 2022;15. doi:10.3390/ma15010179
55. Riva R, Ragelle H, Des Rieux A, et al. Chitosan and chitosan derivatives in drug delivery and tissue engineering. *Adv Polym Sci*. 2011;244:19–44. doi:10.1007/12_2011_137
56. Rezaei A, Hashemi E. A pseudohomogeneous nanocarrier based on carbon quantum dots decorated with arginine as an efficient gene delivery vehicle. *Sci Rep*. 2021;11(1). doi:10.1038/s41598-021-93153-4
57. Qiu C, Xia F, Zhang J, et al. Advanced strategies for overcoming endosomal/lysosomal barrier in nanodrug delivery. *Research*. 2023;6. doi:10.34133/research.0148
58. Cullis PR, Hope MJ. Lipid nanoparticle systems for enabling gene therapies. *Mol Ther*. 2017;25(7):1467–1475. doi:10.1016/j.ymthe.2017.03.013
59. Xiang S, Tong H, Shi Q, et al. Uptake mechanisms of non-viral gene delivery. *J Control Release*. 2012;158(3):371–378. doi:10.1016/j.jconrel.2011.09.093

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