



Tailoring Zein-based lipid–polymer hybrid vectors for efficient pulmonary siRNA delivery by nebulization

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ABSTRACT

For the local treatment of lung diseases, siRNA inhalation could represent a promising approach, but it requires suitable formulations for efficient RNAi therapy. In this work, we developed a cationic and amphiphilic copolymer, obtained from the grafting on α , β -poly(N-2 hydroxyethyl)-DL-aspartamide (PHEA) of 1,2-Bis(3-aminopropylamino)ethane (bAPAE) and zein, with a derivatization degree (DD%), respectively, equal to 35 ± 0.3 mol% and 2.0 ± 0.2 mol%. The obtained graft PHEA-bAPAE-g-zein copolymer self-assembled in water and efficiently complexed siRNA. From this, nanoparticles (NPs) were produced by complexation with siRNA at N/P ratios of 10 and 20; by surface coating with phospholipid shell, polymer-lipid hybrid systems (LPHNPs) were also produced. These carriers showed nanometric size which was confirmed by TEM analysis. Nebulization with two commercial VMNs preserved the size and stability of most formulations, producing droplets suitable for inhalation ($3.5\text{--}4.5\text{ }\mu\text{m}$). Specifically, the Aerogen Solo device achieved higher product recovery than the Beurer IH 55. All samples, except for LPHNPs 10, demonstrated a high cellular uptake and efficient eGFP knockdown pre and post nebulization and mediated a sustained endosomal escape, validated through Pearson's correlation coefficient analysis. NPs 20 and LPHNPs 20 samples showed the highest eGFP knockdown compared to the scrambled control sequence. Finally, the muco-diffusive properties, evaluated using two air–liquid interface (ALI) cellular models with different muco-secretory activity, showed that the LPHNPs 20 sample was the most internalized vector, as its diffusion and cellular uptake were less influenced by the amount of mucus on the cells.

1. Introduction

Inflammation is a defense mechanism involving complex cellular and molecular interactions, which, if unresolved, can become chronic and cause tissue damage [1]. In chronic respiratory diseases such as asthma, cystic fibrosis (CF), and chronic obstructive pulmonary disease (COPD), persistent inflammation worsens disease progression [2]. Direct pulmonary drug delivery is a promising approach for the treatment of lung diseases because it offers high bioavailability and significantly low systemic side effects [3]. In patients with cognitive, neuromuscular, or respiratory limitations, advanced devices such as vibrating-mesh nebulizers (VMNs), offer clear advantages over other inhalation devices [4], but the therapy success also depends on the use of suitable formulations

[5,6]. However, despite the progress in pharmaceutical devices and formulation development, optimal anti-inflammatory therapies remain a challenge, due to the complexity of inflammation and the insufficient selectivity of current treatments in modulating key molecular pathways without causing adverse effects [2].

Small interfering RNAs (siRNAs) represent a promising strategy for treating inflammatory lung diseases by selectively modulating pathogenic gene expression [7]. Through sequence-specific degradation of complementary mRNA, siRNAs silence target genes, reducing the expression of key pro-inflammatory mediators involved in pulmonary inflammation [8]. In contrast, conventional anti-inflammatory drugs, acting on broader signalling pathways, increase the likelihood of off-target effects. However, while six siRNA-based therapies are in clinical

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use, their application to chronic lung diseases unfortunately remains a great challenge due to the presence of several barriers which hamper efficient delivery and cellular uptake [5]. Therefore, literature describes siRNA delivery systems developed to overcome pulmonary barriers, with polymeric carriers representing one of the most widely investigated strategies [9]. These materials are suitable for siRNA delivery because they can be properly designed and synthesized with dedicated structural and functional properties to carry siRNA across mucus and into target cells [10]. Amphiphilic polymers, such as those based on poly(amino acids), represent highly versatile vectors for nucleic acid delivery owing to their easily modifiable structures. By combining electrostatic condensation with hydrophobic self-assembly, these systems effectively overcome critical delivery barriers by stabilizing nanoparticles at lower N/P ratios, lowering cationic toxicity, and enabling multi-cargo transport. The functional utility of these vectors has been demonstrated across diverse therapeutic applications. For instance, modifying hyper-branched poly(L-lysine) (PLL) with adamantane and acetylated β -cyclodextrin yields a supramolecular host-guest platform capable of pH-triggered aggregation, which enhances anti-TNF- α siRNA delivery within immune cells for rheumatoid arthritis therapy [11]. In cancer treatment, amphiphilic PEG-PLL backbones conjugated with hydrophobic oleic acid and carrying gemcitabine via glutathione-cleavable disulfide bonds enabled the co-delivery of chemotherapy and SERPINB9 siRNA, successfully remodelling the tumor microenvironment to overcome chemo-immunoresistance [12]. In another approach, grafting PLL dendrons onto zein via click chemistry (ZDP) resolved the native protein's poor nucleic acid binding capacity, producing amphiphilic micelles that facilitate the safe, pH-dependent endosomal release of doxorubicin and siRNA [13]. Importantly, amphiphilic modifications consistently improve vector performance across polymer classes; for example, introducing hydrophobic moieties into pullulan significantly enhanced polyplex-mediated silencing efficiency compared to its non-amphiphilic counterpart, while imparting critical structural stability against shear stress during nebulization [14]. Together, these strategies highlight how the architectural flexibility of amphiphilic and polypeptide-containing copolymers provides a rational route toward robust, stimuli-responsive RNAi delivery systems.

In this study, a novel cationic and amphiphilic graft copolymer was synthesized starting from the α,β -poly(N-2-hydroxyethyl)-DL-aspartamide (PHEA) [15,16] to obtain a suitable carrier for the efficient delivery of siRNA to the lung. To stabilize siRNA through electrostatic interactions into stable nanoparticles [17], PHEA was functionalized with the proper amount of 1,2-bis(3-aminopropylamino)ethane (bAPAE) as hydrophilic-cationic portion [18], and zein to confer amphiphilic behavior, obtaining the graft copolymer PHEA-bAPAE-g-zein. By the chosen proper ratio between copolymer and siRNA, expressed as N/P ratio, polyplex nanoparticles (NPs) were obtained, and by coating the nanoparticle surface with phospholipids, lipid-polymer hybrid nanoparticles (LPHNPs) were obtained. To better understand how the surface composition of both NPs and LPHNPs samples impact on their properties such as size and muco-diffusion, we evaluated these properties before and after lyophilization and nebulization with devices such as the Beurer IH55 (home use) and the Aerogen Solo (clinical use). Moreover, performance of both systems on cellular uptake, cell colocalization and endosomal escape, gene silencing (before and after nebulization) was carried out in a submerged cell culture model. To simulate the complexity of lung barriers and the pulmonary environment, we additionally carried out biological investigations of NPs and LPHNPs performance by employing air-liquid interface (ALI) cellular models.

2. Materials and methods

2.1. Materials

Zein (product code Z3625), carbonyldiimidazole (CDI), diethylamine (DEA), 1,2-bis(3-aminopropylamino)ethane (bAPAE), Anhydrous

dimethyl sulfoxide (a-DMSO), dichloromethane, acetone, ethanol, diethyl ether, agarose, ethidium bromide 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) were purchased from Sigma-Aldrich (Milan, Italy). HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), RPMI 1640 medium, EMEM medium, Triton X-100, heparin sodium salt from porcine intestinal mucosa, heat-inactivated fetal bovine serum (FBS), geneticin (G418), Dulbecco's phosphate-buffered saline (PBS), were obtained from Sigma-Aldrich (Darmstadt, Germany). PneumaCult™ ALI medium was obtained from STEMCELL Technologies, Vancouver, Canada. Lipofectamine 2000, OPTI-MEM serum reduced medium, 0.05% trypsin-EDTA, Alexa Fluor 647 NHS ester (AF 647), Alexa Fluor 488 NHS ester (AF 488), Hoechst 33,342 powder, AlexaFluor488-conjugated Wheat-Germ-Agglutinin (AF488-WGA) and a SYBR Gold Nucleic Acid Gel Stain 10.000X concentrate in DMSO and Lysotracker™ green solution were purchased from Thermo Fisher Scientific (Schwerte, Germany). Transwell® inserts (6.5 mm diameter, 0.4 μ m pore size) were purchased from Corning (NY, USA). Chambered coverslip (μ -Slide 8 Well) were bought from Ibidi (Gräfelfing, Germany).

α,β -Poly(N-2-hydroxyethyl)-DL-aspartamide (PHEA) and PHEA-bAPAE were synthesized and characterized according to already reported procedures [15,18].

Dicer substrate double-stranded siRNA targeting enhanced green fluorescent protein (eGFP) (siGFP, 25/27mer), and scrambled siRNA (siNC, 25/27mer) were purchased from IDT (Integrated Technologies, Inc., Leuven, Belgium). Sequences are given in Table 1.

2.2. Synthesis and characterization of PHEA-bAPAE-g-zein graft copolymer

2.2.1. Synthesis

The PHEA-bAPAE-g-zein graft copolymer was prepared through a multistep synthesis. Initially, by activation of PHEA hydroxyl groups by using Bis(4-nitrophenyl) carbonate (BNPC), PHEA-bAPAE was obtained by reacting with an excess amount of bAPAE, as described in a prior study [18].

Subsequently, the PHEA-bAPAE was further functionalized with zein. For this purpose, zein (1369.86 mg; 0.068 mmol) was dissolved in anhydrous DMSO (14 mL), and subsequently CDI (14 mg; 0.086 mmol), was added. The solution was stirred at 40 °C for 4 h. After this time, the dispersion of PHEA-bAPAE [300 mg; 1.37 mmol of repeating units (RU)] in anhydrous DMSO, was added dropwise to the activated zein dispersion. The amounts of each reagent were properly determined according to mmol ratio (R_1) = (mmol of zein/ PHEA-bAPAE mmol of RU) = 0.05, R_2 = (mmol of CDI/ mmol of zein) = 1.2. After leaving the resulting reaction mixture at 40 $\pm$ 0.1 °C for 48 h, it was added dropwise into an appropriate amount of ethyl ether/dichloromethane mixture (ratio 50:50 v/v), and the precipitate separated by centrifugation at 10,000 rpm, at 4 °C for 10 min, washed several times with an ethanol/water mixture (ratio 90:10 v/v). Then, the obtained product was dried under vacuum. The solid residue was dissolved in DMSO and then the solution was purified by dialysis (RC Tubing, at MWCO 50 kDa) first against DMSO, and then against bidistilled water; subsequently, the dispersion was freeze-dried and stored as powder or further characterization. PHEA-bAPAE-g-zein graft copolymer was obtained with a yield of 42 wt %. The derivatization degree of the graft copolymer was evaluated by ^1H NMR and by UV spectroscopic analyses [19].

PHEA-bAPAE-g-zein ^1H NMR (300 MHz, DMSO- d_6 , 25 °C, TMS): δ 0.7–0.9 (6H_{Leu} of zein (CH₃)₂CHCH₂-CH(NH₂)CO-); δ 1.4–1.8 (m, 4H_{bAPAE}, -NHCH₂CH₂CH₂NHCH₂CH₂NHCH₂CH₂CH₂NH-); δ 2.6 (m, 2H_{PHEA}, -COCHCH₂CONH-); δ 2.8 (m, 8H_{bAPAE}, -NHCH₂CH₂CH₂NHCH₂CH₂NHCH₂CH₂CH₂NH₂); δ 3.12 (t, 2H_{PHEA}, -NHCH₂CH₂O-); δ 3.3 (m, 4H_{bAPAE}, -CONHCH₂CH₂-CH₂CH₂CH₂NH₂); δ 3.4 (t, 2H_{PHEA}, -NHCH₂CH₂OH); 4.0 (m, 2H_{PHEA}, -NHCH₂CH₂OCO-); δ 4.65 (m, 1H_{PHEA}, -NHCH(CO)CH₂-); δ 7.0–7.3 (5H_{phenylalanine} of zein, H-Ar); δ 6.5–6.8 (4H_{tyrosine} of zein, H-Ar).

Table 1

Sequences of siRNAs used in the study.

siRNA sequence name	Sense strand (5'-3')	antisense strand (3'-5')	Length (nt)	
			sense	antisense
siGFP	pACCCUGAAGUUAUCUGCACCACcg	ACUGGGACUUAAGUAGACGUGGUGGC	25	27
siNC	pCGUUAUUCGCGUAUAUACGCGUat	CAGCAAUUAAGCGCAUUAUUGCGCAUAp	25	27

The UV spectra of zein, at different concentrations, and of the copolymer PHEA-bAPAE-g-zein (1 mg/ml in DMSO) were measured by UV spectrophotometer (UV-2600, Shimadzu, Japan). The scanning range was 200–340 nm. The measured copolymer absorbance was interpolated with a previously obtained calibration line, plotting the absorbance values as a function of the concentration of zein dispersions at known concentrations between 0.0625 mg/ml and 1 mg/ml in DMSO.

To produce a fluorescent labelled derivative via amine-reactive NHS-ester chemistry, PHEA-bAPAE-g-zein (50 mg; 0.08 mmol of PHEA-bAPAE RU) was dissolved in anhydrous DMSO (2 mL), and then DEA (1.06 mg, 0.0145 mmol) was added to the dispersion. After complete solubilization, AF488- NHS ester (0.25 mg; 0.00038 mmol) was added dropwise to the dispersion. The resulting reaction mixture was left under stirring at 25 ± 0.1 °C overnight. The solution was purified by dialysis (RC Tubing, at MWCO 50 kDa), first against DMSO and then against bidistilled water, subsequently, the solution was freeze-dried and stored for further characterization. The amounts of each reagent were properly determined according to mmol ratio R_3 (mmol of AF 488 /PHEA-bAPAE mmol of RU) = 0.0047, R_4 = (mmol of DEA/PHEA-bAPAE-g-zein mmol of RU) = 0.18. The derivatization degree in AF488 was evaluated by UV spectroscopic analysis (UV-2600, Shimadzu, Japan) at the scanning range of 400–600 nm. The absorbance of a copolymer dispersion (2 mg/ml in DMSO) was interpolated with a calibration line obtained by plotting the absorbance of AF488 solutions in DMSO (ranging between 0.005–0.02 mg/ml) as a function of these concentrations.

2.2.2. Size Exclusion Chromatography (SEC)

The weight-average molecular weight ($\bar{M}_w$) and the polydispersity ($\bar{M}_w/\bar{M}_n$) of PHEA-bAPAE-g-zein copolymer was determined by SEC analysis using Phenomenex Phenogel 5u•10⁴ column (California, USA) connected to an Agilent 1260 Infinity Multi-Detector GPC/SEC system (Santa Clara, United States), equipped with a refractive index (RI) and multi-angle light scattering (MALS) detectors. The analyses were performed by relative method, using DMF + LiBr 0.1% as mobile phase, with a flow rate of 0.8 ml/min and polystyrene (2, 20 and 150 kDa) as calibration standard, a column temperature of 50 °C and a polymer concentration of 18 mg/ml. SEC analysis of PHEA-bAPAE was performed as previously described [18].

2.2.3. Critical aggregation concentration (CAC)

The CAC of PHEA-bAPAE-g-zein copolymer has been determined by pyrene assay [20]. Briefly, a stock solution of pyrene (6.0•10⁻² M) was prepared in acetone and stored at 5 °C. The pyrene solution was diluted with double-distilled water to a final concentration of 12•10⁻⁷ M. It was then kept at 60 °C under vacuum for 1 h to remove acetone. The pyrene solution was then mixed 1:1 v/v with PHEA-bAPAE-g-zein aqueous dispersion at concentrations ranging from 1•10⁻⁵ to 7.5 mg/mL. The final concentration of pyrene in each sample was 6•10⁻⁷ M. Fluorescence spectra were recorded using a JASCO spectrofluorometer. The Critical Aggregation Concentration (CAC) was determined by monitoring the excitation intensity ratio ($I_{336\text{ nm}}/I_{333\text{ nm}}$, $\lambda_{\text{em}} = 390\text{ nm}$) of pyrene as a function of copolymer concentration.

2.2.4. Complexation study

The formation of polyplexes between siRNA and the PHEA-bAPAE-g-zein copolymer was evaluated by electrophoretic mobility shift assay (EMSA) by agarose gel electrophoresis [18]. In particular, equal volumes of a siRNA dispersion in Hepes nuclease-free at pH 7.4 (at a fixed

concentration of 20 mM) and of the copolymer PHEA-bAPAE-g-zein in DMSO (at different concentrations) were mixed, to obtain a N/P ratio (R) ranging from 2.5 to 10; then the mixtures were vortexed and incubated in an orbital shaker at 37 °C for 30 min. Then, each mixture was analyzed by gel electrophoresis. Of each sample 20 µl was loaded onto a 1.5 w/v% agarose gel containing 100 µL of EtBr and run at 100 V in triacetate/EDTA buffer (TAE) at pH 8 for 30 min. The bands were visualized by UV transilluminator and recorded.

2.3. Production and characterization of siRNA-loaded nanocarriers

2.3.1. Production

Nanoparticles (NPs) from PHEA-bAPAE-g-zein and siRNA were obtained by the modified antisolvent precipitation/solvent evaporation. Specifically, the copolymer and siRNA were dispersed in an acetone/water mixture (70:30 v/v), to obtain a polymer/siRNA weight ratio of 20 and 40, corresponding to a N/P ratios of 10 and 20, respectively. The resulting dispersions slowly added dropwise to a fivefold volume of 10 mM Hepes buffer under moderate agitation and then placed under reduced pressure at 30 °C to obtain each nanoparticle dispersion, respectively named NPs 10 and NPs 20. Polymer-lipid hybrid systems (LPHNPs) were obtained by dispersing the copolymer and siRNA in an acetone/water mixture (70:30 v/v), to obtain a polymer/siRNA weight ratio of 20 and 40. The resulting dispersions were first dripped into an ethanolic phospholipid dispersion, containing 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC) (1:1 w/w), with a polymer-to-phospholipid weight ratio of 1:1; then, the resulting dispersion was diluted with the same volume of Hepes 10 mM and then placed under reduced pressure at 30 °C. The dispersion was then transferred to a Vivaspin 500 centrifugal concentrator (100 kDa molecular weight cut-off, Sartorius, Germany) and centrifuged at 12,500 rpm for 30 min at 4 °C. The resulting concentrate was then resuspended in Hepes 10 mM to obtain each nanoparticle dispersion, respectively named LPHNPs 10 and LPHNPs 20. The formulations were characterized after preparation, post freeze-drying and after the nebulization process.

2.3.2. Mean size and ζ potential determination

The hydrodynamic diameter, polydispersity index (PDI), and ζ -potential of NPs and LPHNPs samples, freshly prepared, post freeze-drying and after nebulization, were determined using a Zetasizer Advance Ultra (Malvern Instruments, Malvern, UK). Dynamic light scattering (DLS) was performed at 25 °C in 173° backscatter mode to measure size and PDI, while ζ -potential was assessed by phase analysis light scattering (PALS) using folded capillary zeta cells (DTS1070, Malvern Instruments). All measurements were conducted in triplicate ($n = 3$) and results expressed as mean size (nm) $\pm$ standard deviation (SD).

2.3.3. Encapsulation efficiency

The siRNA encapsulation efficiency was quantitatively evaluated by using a modified SYBR Gold assay [21]. Briefly, siRNA loaded into each sample was quantified by using a particle disruption approach (direct method); to do this, 20 µL of each sample dispersion was transferred into a Fluotrac 384 well plate (Greiner Bio-One, Frickenhausen, Germany) and incubated at room temperature (RT) for 1 h under agitation with 10 µL of a 2% Triton X detergent solution and 2 µL of a 2000 U/mL heparin solution. Then, 6 µL of 4 × SYBR Gold Nucleic Acid Gel Stain was added and incubated for 5 min in the dark. Free siRNA was also evaluated with

the same assay (indirect method); to do this, 20 μL of each sample dispersion was incubated for 5 min with $4 \times \text{SYBR Gold Nucleic Acid Gel Stain}$ in the dark. After incubation, fluorescence was measured using a microplate reader (TECAN Spark, TECAN, Männedorf, Switzerland) with an excitation wavelength of 492/20 nm and an emission wavelength of 537/20 nm.

2.3.4. Phospholipids amount

The lipid content of LPHNPs, post freeze-drying and after nebulization, was determined by using the ammonium ferrioxalate colorimetric assay [22]. In detail, each sample was dispersed in chloroform and emulsified with an aqueous solution of ammonium ferrioxalate. After 15 min, the organic phase was separated, and its absorbance was measured at 488 nm using an JASCO spectrofluorometer (Shimadzu, Italy). All experiments were conducted in triplicate. Lipid quantification was performed using a calibration curve generated from DPPC/DLPC mixtures (1:1 w/w) at concentrations ranging from 0.1 to 0.00625 mg/mL ($y = 11,266x$, $R^2 = 0.999$).

2.3.5. TEM analysis

The morphology of the NPs and LPHNPs were assessed using transmission electron microscopy (TEM). Onto 200 mesh copper grids coated with a carbon support film, 5 μL of each sample dispersion at 1 mg/mL was deposited and left to dry. Afterward, a drop (5 μL) of a phosphotungstic acid solution (1% w/v) was added to the same grid, as a contrast agent. After allowing the samples to air dry, grids were analyzed using a FEI Tecnai G2 s-Twin transmission electron microscope operated at 200 kV and equipped with a 4 K digital camera (Thermo Fisher Scientific) [18].

2.3.6. Polyanionic exchange

The stability of NPs and LPHNPs samples to polyanionic exchange was determined after incubation with mucin dispersion. In detail, dispersions of NPs and LPHNPs in Hepes 10 mM pH 7.4, with N/P ratios of 10 and 20, were mixed with a mucin dispersion at different concentrations: 0.05% w/v (M1), 0.08% w/v (M2), 0.1% w/v (M3) and 0.2% w/v (M4) and incubated at room temperature for 2 h. After this time, the resulting dispersion was analyzed by a gel electrophoresis assay, and by using a modified SYBR Gold assay [21]. To account for mucin autofluorescence, free siRNA samples were prepared in the presence of the corresponding mucin concentration, and the fluorescence of free siRNA was set as 100% siRNA release. All measurements were conducted in triplicate, and results are presented as mean values $\pm$ SD.

2.3.7. Turbidimetric analysis

The interactions of NPs and LPHNPs samples with mucin were evaluated through turbidimetric analysis following a modified protocol from the literature [6]. Type II mucin was dispersed in Hepes 10 mM to achieve a concentration of 0.08% w/v, under stirring overnight for complete hydration. Each sample dispersion was prepared at concentration of 0.8 mg/mL, mixed with an equal volume of mucin dispersion, and the resulting mixture was incubated at 37 °C on an orbital shaker for 1, 2, 4 and 6 h. At these time points, scattered light of each resulting mixture was measured at λ 500 nm by a UV spectrophotometer (UV-2600, Shimadzu, Kyoto, Japan). All experiments were conducted in triplicate, and the results are expressed as Transmittance % mean values $\pm$ SD as a function of incubation time.

2.3.8. Nebulization

Two commercially available nebulizers were utilized: The Beurer IH 55 nebulizer (Beurer, Ulm, Germany), and the Aerogen Pro (Aerogen, Ratingen, Germany). All siRNA-loaded NPs and LPHNPs samples were dispersed in Hepes 10 mM at pH 7.4, and 1000 μL of the resulting dispersions were loaded in the nebulizer reservoirs. During nebulization, the produced aerosols were collected in cooled Falcon tubes for analysis.

2.3.9. Aerosol characterization

Aerosols were analyzed by laser diffraction, by nebulization into a laser diffractor (HELOS, Sympatec, Clausthal-Zellerfeld, Germany) equipped with an R2 lens and INHALER module. Each aerosol was delivered through a perforated silicone mouthpiece using an Aerogen Pro T-piece, positioned approximately 50–100 mm upstream of the laser beam. Experiments were conducted at 40% relative humidity and at room temperature. Aerosol extraction was performed at 13.9 L/min to prevent re-circulation into the laser path. Each measurement was done by three consecutive runs of 5 s with a signal integration time of 200 ms. Triplicate measurements ($n = 3$) were performed. Results are reported as Q3 volume median diameter (Q3-VMD) $\pm$ SD of nebulized droplets, based on Mie theory applied to aqueous buffer with corresponding complex refractive indices.

2.4. Biological characterization

2.4.1. Cell culture

2.4.1.1. Submerged cultures. Cell lines of human non-small cell lung carcinoma line H1299 and H1299-eGFP stably expressing enhanced green fluorescent protein (eGFP) as a reporter were used in submerged culture systems. The H1299-eGFP cells were maintained in 75 cm² flasks using RPMI-1640 medium supplemented with 10% fetal bovine serum, and 0.4% G418 disulfate salt. The H1299 cells were cultured under identical conditions but without G418 selection.

2.4.1.2. Air-liquid interface (ALI) cultures. The Calu-3 and 16HBE14o-cells line were cultured in EMEM medium supplemented with 10% FBS. To establish an air-liquid interface (ALI) cultures of Calu-3 cells, 2.5×10^5 cells in 100 μL of medium were seeded onto uncoated Transwell® inserts. The basolateral compartment received 700 μL of EMEM. After three days of submerged growth, ALI conditions were established by removing the apical medium. The inserts were then transferred to new wells containing 200 μL of PneumaCult™ ALI medium which was refreshed every 48 h. ALI of 16HBE14o-cells was realized by seeded at 1×10^5 cells in 100 μL of medium onto uncoated Transwell®, and then they were treated in the same way as Calu-3 cells. Transepithelial electrical resistance (TEER) was monitored using an EVOM volt/ohm meter (World Precision Instruments, Sarasota, USA), and NPs and LPHNPs-mediated transfection experiments were initiated one-week post-airlift, when TEER values above 300 $\Omega \cdot \text{cm}^2$ were reached. All cell cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO₂. Subculturing and reseeded were carried out at approximately 80% confluence.

2.4.2. Cell viability assay

The cell viability of H1299 cells, incubated in the presence of PHEA-bAPAE-g-zein, NPs and LPHNPs, was assessed using an MTT assay. Cells were seeded in 96-well plates at a density of 15,000 cells per well and incubated at 37 °C with 5% CO₂ for 24 h. A DMSO dispersion of the polymer was diluted in RPMI-1640 complete medium to obtain a concentration range of 0.67 to 0.04 mg/mL and incubated with H1299 cells for 24 and 48 h. After incubation, the supernatant was removed, and each plate was washed with sterile PBS. The cells were then incubated at 37 °C for 3 h with 100 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), prepared in sterile PBS (1 mL) and diluted on the day of analysis with serum-free RPMI-1640 medium (0.5 mg/mL). The MTT solution was then replaced with DMSO and incubated at room temperature for 30 min. Absorbance was measured at 570 nm using a microplate reader (TECAN, Männedorf, Switzerland). The formulations were prepared as described above NPs and LPHNPs, 10 and 20, with a content range of 200 pmol to 10 pmol siNC (corresponding to a polymer concentration range of 0.34 to 0.017 mg/mL for the formulations obtained at N/P ratio of 20, and of 0.17 to 0.008 mg/mL for the

for the formulations obtained at N/P ratio of 10), were added to each well and incubated with the cells for 24 h. The plates were then treated and analyzed following the same procedure described above.

2.4.3. Cellular uptake

Cellular uptake was quantified by flow cytometry [17]. H1299 cells were plated in 24-well plates at a density of 25,000 cells per well and incubated for 24 h at 37 °C with 5% CO₂, before transfection. The NPs and LPHNPs, 10 and 20 were prepared as described above by using siRNA, of which 20% was labeled with Alexa Fluor 647. As controls, siRNA (negative control) and siRNA complexed with Lipofectamine 2000 (positive control) were used. Formulations containing 50 pmol of siRNA were added to each well and incubated with the cells for 24 h. The medium with siRNA formulation was then discarded, and the cells were rinsed with PBS and treated with 0.05% trypsin–EDTA (100 µL/well) for 4 min to detach the cells. The trypsin was deactivated by addition of fresh medium (500 µL/well). The cell suspensions were subsequently transferred into microcentrifuge tubes and washed with PBS two times. Fluorescence intensity was measured using a flow cytometer (Attune NxT Acoustic Focusing Cytometer, Thermo Fisher Scientific, USA).

2.4.4. Gene silencing

Gene silencing of NPs and LPHNPs, 10 and 20, pre- and post-nucleation, was evaluated in H1299-eGFP cells grown in submerged culture. Cells were seeded in 24-well plates at a density of 25,000 cells per well. At 24 h after the seeding, NPs and LPHNPs containing 100 pmol of either non-coding control siRNA (siNC) or GFP-targeting siRNA (siGFP) were added to each well, and cells were transfected for 48 h under standard culture conditions. For comparison, cells treated with siGFP or siNC complexed to Lipofectamine 2000 were used as positive control, while free siGFP and siNC served as negative control. Following the incubation period, the medium was removed, and the cells were gently rinsed with phosphate-buffered saline (PBS), then detached using 0.05% trypsin–EDTA. The collected cells were washed twice with PBS and subsequently resuspended in buffer for analysis. Flow cytometric analysis was carried out using an Attune NxT Acoustic Focusing Cytometer (Thermo Fisher Scientific, USA). Excitation was performed with a 488 nm laser, and emission was detected using a 530/30 nm bandpass filter (BL-1H channel). The mean fluorescence intensity (MFI) was used as a quantitative measure of gene silencing efficiency. Results were first normalized to untreated blank cells and then expressed as relative eGFP expression (%) by dividing the values of siGFP formulations by those of the corresponding non-targeting control (siNC) formulations. Data are reported with the corresponding standard deviation (SD).

2.4.5. Endosomal escape

The intracellular trafficking and endosomal release of NPs and LPHNPs, 10 and 20, were investigated in H1299 cells grown in submerged cultures by using confocal laser scanning microscopy (CLSM). Cells were seeded at a density of 10,000 cells per well on a chambered coverslip and incubated for 24 h under standard conditions. The formulations containing 50 pmol of siRNA (20% of which was Alexa Fluor 647-labeled) were added to each well and incubated with the cells for an additional 3 h. Following incubation, the medium was removed and replaced with complete RPMI-1640 medium containing LysoTracker™ green solution (75 nM) for 1 h at 37 °C to label acidic compartments. After PBS washes, nuclear staining was performed using 1 µg/mL Hoechst 33,342 for 20 min. Excess stains were removed by two final PBS washes. Fluorescence imaging was performed using a Leica TCS SP8 inverted confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a 63X objective. The confocal images were processed using the software ImageJ with the plugin JACoP to assess the Pearson's correlation coefficient.

2.4.6. Intracellular co-localization

Colocalization was evaluated in H1299 cells grown in submerged

cultures by using confocal laser scanning microscopy (CLSM). Cells were seeded under the same conditions described before. Cells were incubated with samples, obtained between PHEA-bAPAE-g-zein_{AF488} (DD_{AF488} 0.43%) and 50 pmol of siRNA_{AF647} (20% labeled), and then washed twice with PBS, nuclear staining was performed using 1 µg/mL Hoechst 33,342 for 20 min. Excess staining was removed by two final PBS washes. Fluorescence images were captured with Leica TCS SP8 inverted confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany).

2.4.7. Cellular uptake in different ALI models

Calu-3 and 16HBE14o- cells cultured at the ALI were obtained as previously described. For transfection, the NPs and LPHNPs samples were applied on top of Calu-3 or 16HBE14o- monolayers and incubated for 24 h (100 pmol of siRNA, 20% Alexa Fluor 647-labeled). Free siRNA served as a negative control and lipofectamine 2000 complex served as a positive control. For imaging, cell nuclei were stained from the basolateral side with 300 µL of Hoechst 33,342 solution (10 mg/mL) diluted 1:200, while mucus was labeled from the apical side with 100 µL of AlexaFluor488-conjugated Wheat-Germ-Agglutinin (AF488-WGA, 1 mg/mL) diluted 1:100 and incubated for 20 min. Confocal microscopy images were acquired after washing the cells three times with PBS on both the apical and basolateral sides, cutting out the Transwell membrane, and mounting it on a glass slide with a drop of FluorSave™ Reagent (Merck KGaA, Darmstadt, Germany). Imaging was performed using a Leica TCS SP8 inverted confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a 40X objective. Image processing was carried out with ImageJ software.

Cellular uptake on these models was quantified by flow cytometry. In particular, the cells were transfected with the same concentrations of NPs and LPHNPs reported for the imaging studies. After 24 h the cells were washed twice with PBS and collected in an Eppendorf tube by gently scraping them off the membrane using a pipette tip. Prior to the readout via flow cytometry (Attune NxT instrument, Thermo Fisher Scientific, Darmstadt, Germany), the cells were centrifuged for 5 min at 400 x g and washed twice with PBS. The cell pellet was resuspended in PBS containing 2 mmol EDTA (Sigma Aldrich, Taufkirchen, Germany). To evaluate the uptake of the labeled siRNA, the MFI was recorded using an excitation at 647 nm and a 660/20 nm bandpass emission filter.

2.5. Statistical analysis

Data are presented as mean ± SD. Statistical differences between the groups were analyzed using Student's *t*-test. Statistical significance was set at **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

3. Results and discussion

3.1. Synthesis and characterization of PHEA-bAPAE-g-zein graft copolymer

To realize polymeric vectors for efficient pulmonary RNAi therapy to the bronchial epithelium, a multifunctional graft copolymer was synthesized starting from the α,β-poly(N-2 hydroxyethyl)-DL-aspartamide (PHEA) [15,20], which was first functionalized with the proper amount of 1,2-bis(3-aminopropylamino)ethane (bAPAE), as already described in literature [18]. The efficacy in complexing siRNA and inducing gene silencing of the obtained cationic PHEA-bAPAE derivative has already been demonstrated in the literature [18,23]. Then, the PHEA-bAPAE was conjugated with zein, to obtain the cationic and amphiphilic graft copolymer PHEA-bAPAE-g-zein.

Zein is a prolamin extracted from corn, considered "GRAS" (Generally Recognized as Safe) by the FDA, easily functionalizable [24], and whose degradation products have potential anti-inflammatory properties [25,26]. It is currently being used to design inhalable delivery systems [27]. The grafting of hydrophobic chains on the hydrophilic PHEA

backbone was performed based on previously reported evidence demonstrating that polymeric siRNA delivery vectors incorporating hydrophobic moieties exhibit significant advantages over their hydrophilic counterparts, increasing complex stability and endosomal escape, which ultimately translate into enhanced gene silencing efficacy [17,21,28]. The PHEA-bAPAE copolymer functionalization with zein is carried out through a coupling reaction via carbonyldiimidazole (CDI), as shown in Fig. 1. Since it was not possible to determine exactly which amino acid (and therefore which carboxyl group) zein is bound to, the reaction scheme was depicted as shown below considering that experimental conditions are set in a such way to avoid multipoint reaction and that SEC analysis confirmed that no species at high molecular weight were obtained. The theoretical amount of zein to be conjugated was chosen by considering an appropriate hydrophilic/lipophilic balance to the resulting amphiphilic copolymer.

By ^1H NMR analysis, the degree of derivation of PHEA-bAPAE with zein ($\text{DD}_{\text{zein}}\%$) was obtained by relating the sum of the zein integrals at δ 7.3–7.0 ppm corresponding to five aromatic protons of phenylalanine, and those at δ 6.8–6.5 ppm corresponding to four aromatic protons of tyrosine [29], to the signal at δ 3.1 ppm attributed to two protons of the PHEA backbone [15]. In Fig. S1, the ^1H NMR spectrum of PHEA-bAPAE-g-zein in $\text{DMSO}-d_6$ is shown. Based on this evaluation, $\text{DD}_{\text{zein}}\%$ of the copolymer was found to be 2.0 ± 0.2 mol%. This $\text{DD}_{\text{zein}}\%$ value was confirmed by UV spectroscopic analysis carried out as reported in the experimental section [19], resulting at 1.9 ± 0.3 mol%.

The PHEA-bAPAE-g-zein graft copolymer was further characterized via SEC analysis in organic solvent to determine the weight-average molecular weight ($\bar{M}_w$) and polydispersity ($\bar{M}_w/\bar{M}_n$). The SEC chromatograms of the PHEA-bAPAE and PHEA-bAPAE-g-zein copolymers, expressed as LS 90° signal intensity as a function of retention time, are provided in Fig. S2A and S2B, respectively. The monomodal SEC profile of PHEA-bAPAE-g-zein indicates a low incidence of a potential cross-linking reaction due to the presence of multiple carboxyl groups on the zein chain. All the data obtained from the characterization of the PHEA-bAPAE-g-zein graft copolymer were summarized in Table 2.

To evaluate the self-assembling behavior of the copolymer, the Critical Aggregation Concentration (CAC) study was performed by measuring changes in the pyrene excitation spectra as a function of copolymer concentration [20]; in such way the self-aggregating properties of PHEA-bAPAE-g-zein copolymer were determined and obtained data are shown in Fig. 2A. In detail, the I_{336}/I_{333} intensity ratios of peaks measured in the excitation spectra of pyrene are plotted as a function of

Table 2

Weight-average molecular weight ($\bar{M}_w$), polydispersity index ($\bar{M}_w/\bar{M}_n$), and DD in bAPAE and zein of the PHEA-bAPAE-g-zein graft copolymer.

Copolymer	Molecular Weight		Derivatization Degree (mol%)		
	$\bar{M}_w$ (g/mol)	$\bar{M}_w/\bar{M}_n$	DD _{bAPAE}	DD _{zein} ^1H NMR	UV
PHEA-bAPAE-g- Zein	88,100*	1.30	35 ± 0.3	2.0 ± 0.2	1.9 ± 0.3
PHEA-bAPAE	20,900**	1.41	35 ± 0.3	—	—
Zein	24,100*	1.90	—	—	—

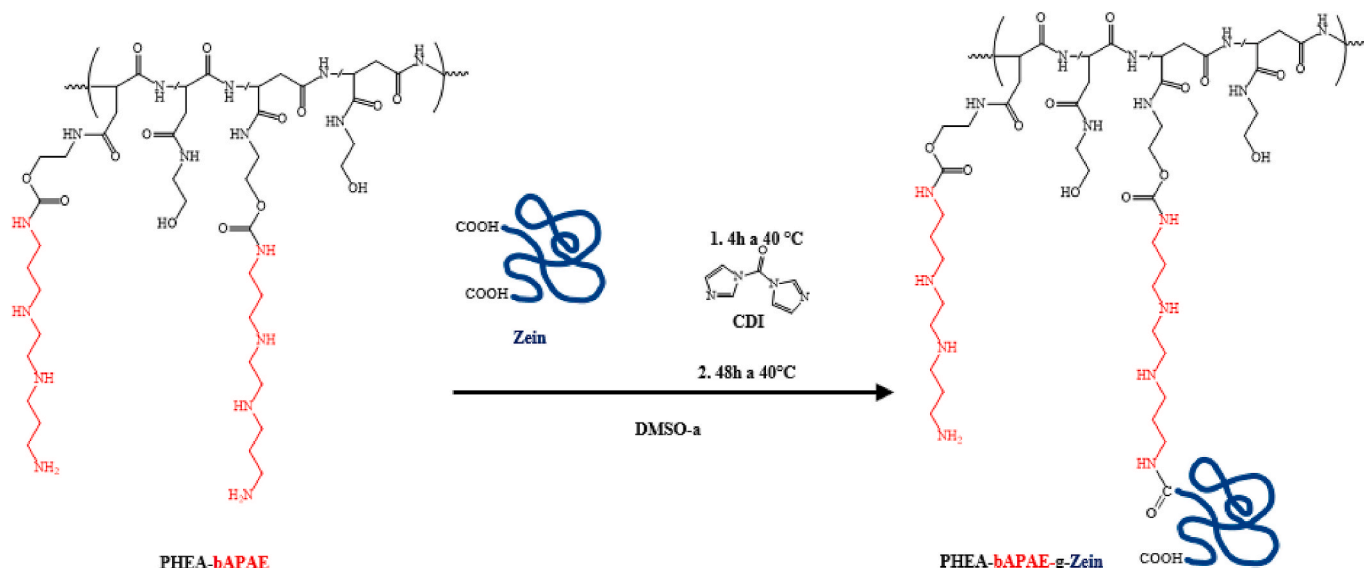
*SEC performed in DMF + LiBr.

** SEC performed in buffer citrate/phosphate 0.15 M + NaCl 0.1 M pH 5.

the logarithm of the aqueous copolymer concentrations. From the obtained sigmoidal curve, the CAC value was graphically calculated from the point of intersection of the tangent to the rising portion of the curve with the tangent to the flat portion of the curve itself. The obtained CAC value of PHEA-bAPAE-g-zein was 0.025 mg/mL, corresponding to $2.83 \cdot 10^{-7}$ M. This CAC value is similar to that reported for Zein-poly(L-lysine) dendron (ZDP) conjugates (0.0180 mg/mL, corresponding to $2.17 \cdot 10^{-7}$ M) [13], indicating that the strong inter- and intramolecular hydrophobic interactions of zein effectively lower the concentration required to trigger copolymer self-assembly.

Preliminary biological studies on the copolymer were performed to explore the concentration range in which the copolymer maintained good biocompatibility. The viability of treated H1299 cells was high at copolymer concentration until 0.17 mg/mL, after 24 and 48 h of incubation under the chosen experimental conditions (Fig. 2B). Overall, compared to cytocompatibility of already reported amphiphilic cationic copolymers on the same cell line, these results highlight a favorable cytocompatibility profile of PHEA-bAPAE-g-zein copolymer, which show low intrinsic toxicity up to higher concentration [17,30], probably due to a well-balanced hydrophobic-cationic design.

To assess the ability of the obtained copolymer to stably complex siRNA due to cationic behavior, a complexation study was carried out by incubating equal volumes of siRNA and copolymer dispersions for 30 min, obtaining different N/P ratios, and by evaluating the complex formation and stability by agarose gel electrophoretic analysis; results are shown in Fig. 2C. The minimum N/P ratio that allowed the formation of stable complexes was 10, a value sufficiently low to suggest sufficient tolerability in cell culture.

**Fig. 1.** Synthetic scheme of the PHEA-bAPAE-g-zein copolymer.

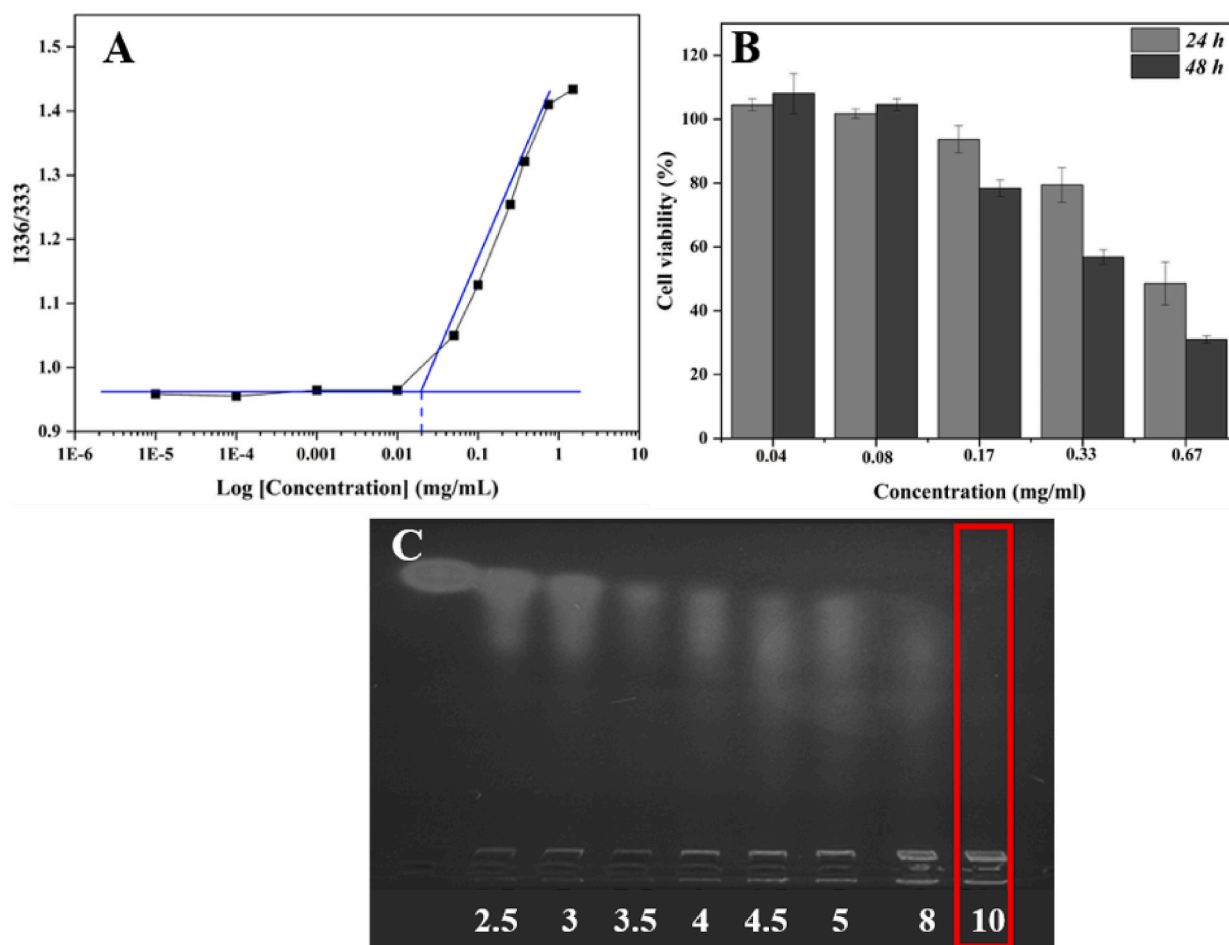


Fig. 2. (A) Trend in the I_{336}/I_{333} intensity ratios of pyrene, measured in the excitation spectra, as a function of the aqueous concentration of the PHEA-bAPAE-g-zein copolymer, expressed as $\log[P]$; (B) Cell viability % of H1299 cells after 24 and 48 h incubation in the presence of PHEA-bAPAE-g-zein copolymer at concentrations ranging between 0.04–0.67 mg/ml. Results are shown as mean $\pm$ SD ($n = 3$); (C) Agarose gel electrophoresis of PHEA-bAPAE-g-zein/siRNA polyplexes obtained at phosphorus/nitrogen (N/P) ratios between 2.5 and 10.

4. Production and characterization of siRNA/PHEA-bAPAE-g-zein nanoparticles (NPs) and lipid/polymer hybrid nanoparticles (LPHNPs)

In this section, the production of both nanoparticles and hybrid systems was carried out through controlled modulation of key formulation parameters. This optimization enables the development of adaptable formulations whose physicochemical and functional characteristics were rationally tuned to address specific application requirements, including the intended purpose and pulmonary delivery. Exploiting the complexing behavior of PHEA-bAPAE-g-zein graft copolymer, nanoparticles (NPs) were produced by using the nanoprecipitation and solvent evaporation technique; then, by adding a proper lipid mixture in the organic solvent, the lipid-polymer hybrid nanoparticles (LPHNPs) were also obtained. 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and the 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC) were chosen as lipid components because they mimic pulmonary surfactants [18,31], and the obtained shell enhanced biocompatibility, cellular uptake efficiency, and transport across mucosal barriers of obtained systems [18].

Furthermore, the combination of phospholipids with different acyl chain lengths modulated the mechanical properties of the coating shell, resulting in a reduction in the size of the resulting hybrid systems [32]. Both NPs and LPHNPs were produced using a N/P ratio of 10 and 20, thus obtaining samples named as NPs 10, NPs 20, LPHNPs 10, and LPHNPs 20, respectively.

The phospholipid content of LPHNPs, was determined using a colorimetric analysis [18] and expressed as a weight percentage between the phospholipids amount and the sample total weight, resulting in 37.5 wt% and 47.3 wt% for LPHNPs 10 and 20, respectively. siRNA quantification in each sample was performed by SYBR gold analysis in the presence of both Triton X-100 and heparin. Triton X-100 was used as a surfactant capable of breaking the hydrophobic interactions that allow the copolymer to self-assemble into nanoparticulate structures, while heparin, being a polyanion, competes with siRNA to form electrostatic interactions with the cationic portions of the copolymer; therefore, the presence of both destabilizes the nanoparticulate structure and allows its release from the system, making the siRNA accessible for detection by SYBR gold [21]. The analysis showed 100% loading, suggesting that the polyplex stability is supported by both hydrophobic and electrostatic interactions.

Fresh NPs and LPHNPs samples were characterized by determining their average size, the polydispersity index (PDI) and ζ potential values. Fig. 3A shows sizes below 200 nm, which are suitable for diffusion through the mucus network after pulmonary inhalation [33,34].

Moreover, all samples showed a positive ζ potential close to +25 mV (Fig. 3A), except LPHNPs 10 (+15 mV). In all cases, these positive values could potentially improve the cellular internalization of formulations because they promote interaction with the negatively charged cell membrane [35]. Since the freeze-drying (FD) process may affect polyplexes and nanoparticles stability, particle size, and ζ potential, these parameters were evaluated post-lyophilization. The results showed that

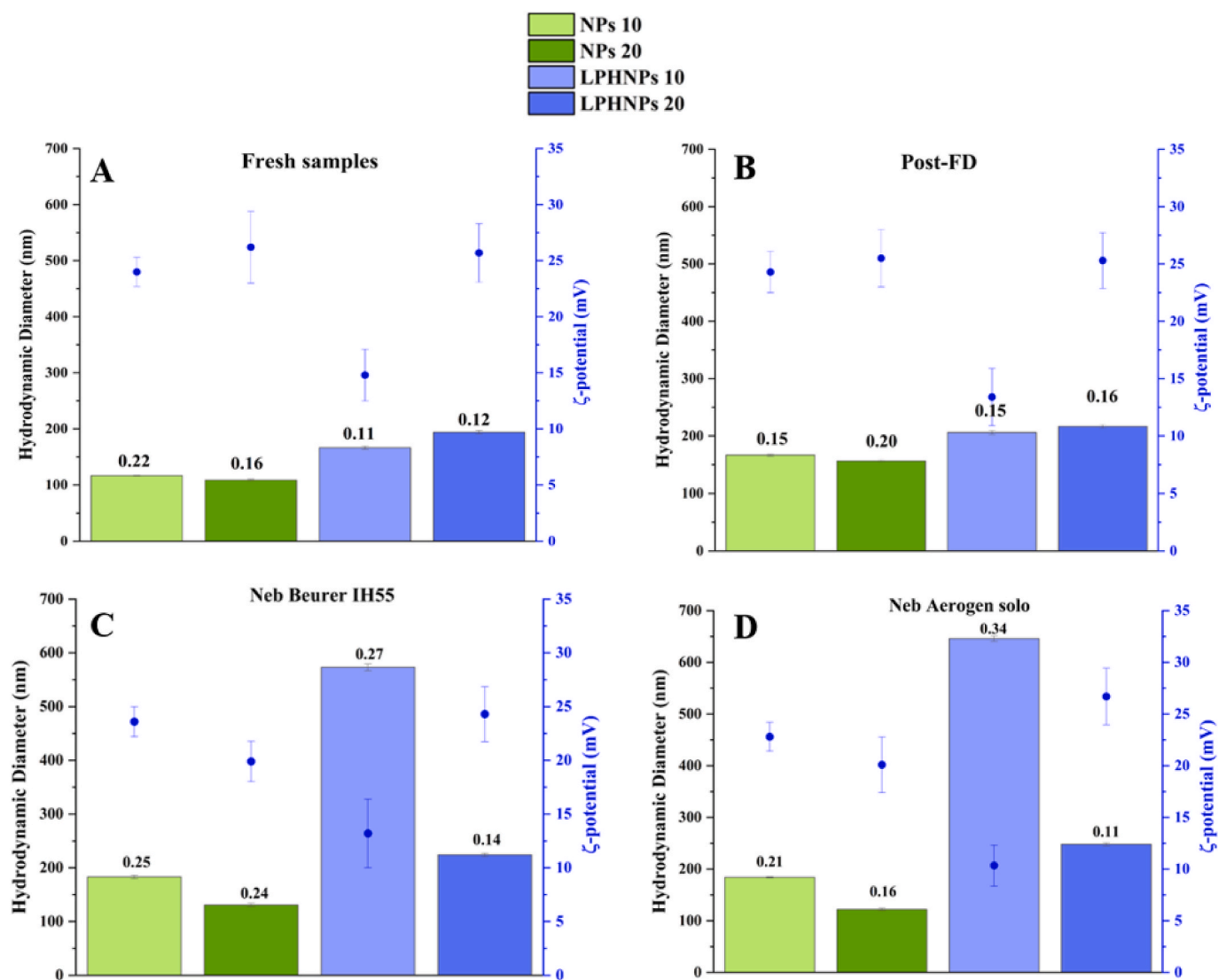


Fig. 3. Hydrodynamic diameter, polydispersity index (PDI, indicated on each histogram) and ζ -potential (blue dots) of NPs and LPHNPs samples at N/P ratio of 10 and 20, freshly prepared (A), after freeze-drying (B), and after nebulization with the Beurer IH55 (C) or with the Aereogen Solo devices (D). (FD = freeze-drying).

the freeze-drying (FD) process didn't significantly affect the samples characteristics, because, after redispersion in water, the size values and ζ potential values (Fig. 3B) were comparable to those of fresh samples.

The nanometric size was confirmed by Transmission Electron Microscopy (TEM), which also showed spherical shape. Moreover, an additional signal was detected on the surface of LPHNPs 20 that was

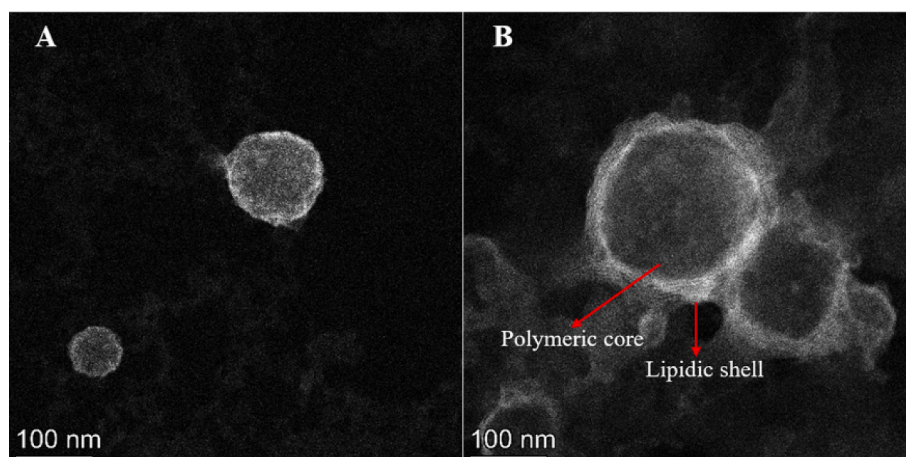


Fig. 4. TEM images of: NPs 20 (A) and LPHNPs 20 (B). The scale bars represent 100 nm (magnification 170,000x).

absent on the NPs 20 surface; this could be attributed to the presence of the lipid shell on the hybrid nanoparticles (reported as examples in Fig. 4A and B, respectively).

Designed for inhalation, the sample stability was evaluated after nebulization by using two types of vibrating-mesh nebulizers (VMN), the Beurer IH55 and the Aerogen Solo. Both rely on the same vibrating-mesh technology, but differing in mesh materials, which may influence how the formulations interact with each device [36]. As shown in Fig. 3C and D, all samples (except LPHNPs 10) show no significant changes in size and PDI than fresh samples, indicating that the particles are stable when subjected to the stresses induced by nebulization. In contrast, LPHNPs 10 showed a significant increase in hydrodynamic diameter, probably due to the nebulization process which promotes aggregation phenomena if sufficiently high surface charges are not protected against it. To learn more about the effects of nebulization on samples, physical stability of nebulized samples, in terms of component loss, was evaluated by determining the content in phospholipids [18], polymer [37], and siRNA [21]. Data, reported in Table 3, showed that there was a loss of all detected materials after nebulization, higher if using the Beurer IH55. This result suggests that mechanical and thermal stresses induced by nebulization may cause sample physical instability and retention in the nebulizer, resulting in some components no longer being detectable in the nebulized product. These effects were significantly less pronounced on the LPHNPs 20 formulation nebulized with the Aerogen Solo, which showed phospholipids, polymer and siRNA recovery of 85.7, 85.1 and 98.6 wt%, respectively. Moreover, the aerosol analysis by laser diffraction, conducted under controlled humidity conditions (~40% RH) [38], showed that the median volume diameters (Q3-VMD) were maintained in range between 3 and 5 μm , resulting potentially optimal droplet sizes for deposition in the deep region of the lungs [21].

Overall, these results confirm that both VMNs tested were suitable to produce aerosols for pulmonary administration, but the Aerogen Solo device seemed less destructive on samples, especially on hybrid systems.

The mucus present in the conducting airways, mainly composed of negatively charged glycoproteins dispersion forming a viscous network [39], could act as a physical barrier for these vectors and compete with the complexed siRNA, destabilizing our samples. Therefore, stability studies were carried out on sample dispersions in the presence of increasing amount of mucin (ranging between 0.05–0.2% w/v) to mimic different pulmonary conditions. After 2 h of incubation at 37 °C, dispersions were analyzed by gel electrophoresis. Results showed that LPHNPs 20 were the most stable complexes at all tested mucin concentrations except that at 0.2 w/v% (M4) (Fig. 5B), giving also the lowest siRNA release (Fig. 5C) and the lowest interaction with mucin at concentration of 0.08% w/v (Fig. 5D). On the contrary, the other samples showed instability at all the tested mucin concentrations (Fig. 5A and 5B), faster siRNA release (Fig. 5C) and higher interactions with mucin (Fig. 5D) than the LPHNPs 20 sample. Therefore, the presence of the phospholipid shell improved the stability of the systems in the presence of mucin, slowing down the premature release of siRNA.

4.1. Biological characterization

Cell viability assays, carried out on human non-small cell lung carcinoma cell line (H1299 cells) after 24 h of incubation (Fig. 6A), demonstrated that samples were cytocompatible up to 100 pmol of siRNA per well (corresponding to a polymer concentration of 0.08 mg/mL for N/P 10 and 0.17 mg/mL for N/P 20), which was set as the maximum siRNA concentration used for subsequent biological experiments. By comparing these results with cell viability in the presence of the copolymer alone, both NPs and LPHNPs were tolerated better after charge-neutralization due to complexation with siRNA.

Before investigating the siRNA knockdown effect when delivered by these nano vectors, we performed a test to evaluate their internalization, by using siRNA_{AF647} and the PHEA-bAPAE-g-zein copolymer labeled with 0.42 ± 0.2 mol% of AF488, as described in the experimental part. Indeed, we found that all the systems were internalized as intact particles, with fluorescence emitted by both the PHEA-bAPAE-g-zein_{AF488} copolymer and siRNA_{AF647} within the cells (Fig. 6C). The quantification of siRNA_{AF647} fluorescence showed that almost all vectors allowed siRNA internalization in significantly higher quantities than the free ones (Fig. 6B). The least effective formulation in terms of siRNA internalization was represented by the LPHNPs 10 sample, probably due to the lower zeta potential value.

In the next step, we performed an eGFP gene silencing assay on H1299-eGFP cells, which showed that most of the samples, except for LPHNPs 10, reduced eGFP expression compared to control (Fig. 7A). The LPHNPs 20 formulation showed the highest eGFP knockdown (~77%), calculated from a siGFP/siNC expression ratio of ~ 23%, but not significantly higher than NPs 20 (eGFP knockdown ~ 76%). The transfection and gene silencing capabilities of amphiphilic polymeric systems appear to be dictated by the presence, nature, and functionalization degree of the hydrophobic domain, which likely modulate cellular internalization and endosomal escape. For instance, incorporating highly lipophilic unsaturated chains (e.g., oleylamine) promotes endosomal escape, achieving > 90% eGFP knockdown at low charge ratios (N/P 5), though strictly requiring an optimal grafting percentage (~50%) [17]. Conversely, systems embedding bulkier or more rigid hydrophobic cores exhibit intermediate efficiencies: Zein-based cationic nanomicelles (ZDP) achieved a 37.3% transfection efficiency yielding ~ 60% mRNA knockdown [13], while PHEA-bAPAE-PLGA lipid-polymer hybrids engineered for pulmonary delivery reached ~ 50% target protein inhibition [18]. This experimental data aligns with these comparisons, demonstrating that the silencing capacity of these systems is primarily governed by the polymeric core architecture, as no significant differences in gene knockdown were observed between our polymeric nanoparticles and their corresponding lipid-hybrid counterparts. Instead, the phospholipid shell enhance formulation stability and overcome physiological barriers during regional administration, such as pulmonary delivery. Silencing efficiency of samples evaluated after nebulization of sample dispersions with both Beurer IH55 and Aerogen

Table 3

Percentage content of siRNA, polymer (Pol), and phospholipids (PL), in each sample after nebulization with Beurer IH55 and Aerogen Solo, after dispersion in Hepes 10 mM pH 7.4; nebulized droplet size, expressed as $X(Q3 = 50\%)$ (Data indicate mean $\pm$ SD, with $n = 3$).

Sample	BEURER IH55 Sample composition			Droplet size X ($Q3 = 50\%$) (μm)	AEROGEN Solo Sample composition			Droplet size X ($Q3 = 50\%$) (μm)
	siRNA recovery (%)	Pol recovery (%)	PL recovery (%)		siRNA recovery (%)	Pol recovery (%)	PL recovery (%)	
Dispersing medium	—	—	—	4.6 ± 0.0	—	—	—	4.5 ± 0.1
NPs 10	89.6 ± 0.5	57.4 ± 1.0	—	4.5 ± 0.1	97.6 ± 0.7	71.7 ± 0.9	—	4.4 ± 0.1
NPs 20	95.5 ± 0.7	54.7 ± 0.9	—	3.8 ± 0.4	102.1 ± 0.5	70.6 ± 1.0	—	4.4 ± 0.1
LPHNPs 10	70.2 ± 0.6	55.6 ± 1.1	59.9 ± 1.1	3.5 ± 0.1	98.5 ± 0.7	75.2 ± 0.8	74.0 ± 0.9	4.5 ± 0.0
LPHNPs 20	92.9 ± 0.4	46.3 ± 1.0	66.1 ± 1.0	3.9 ± 0.2	98.6 ± 0.6	85.1 ± 0.9	85.7 ± 0.7	4.6 ± 0.1

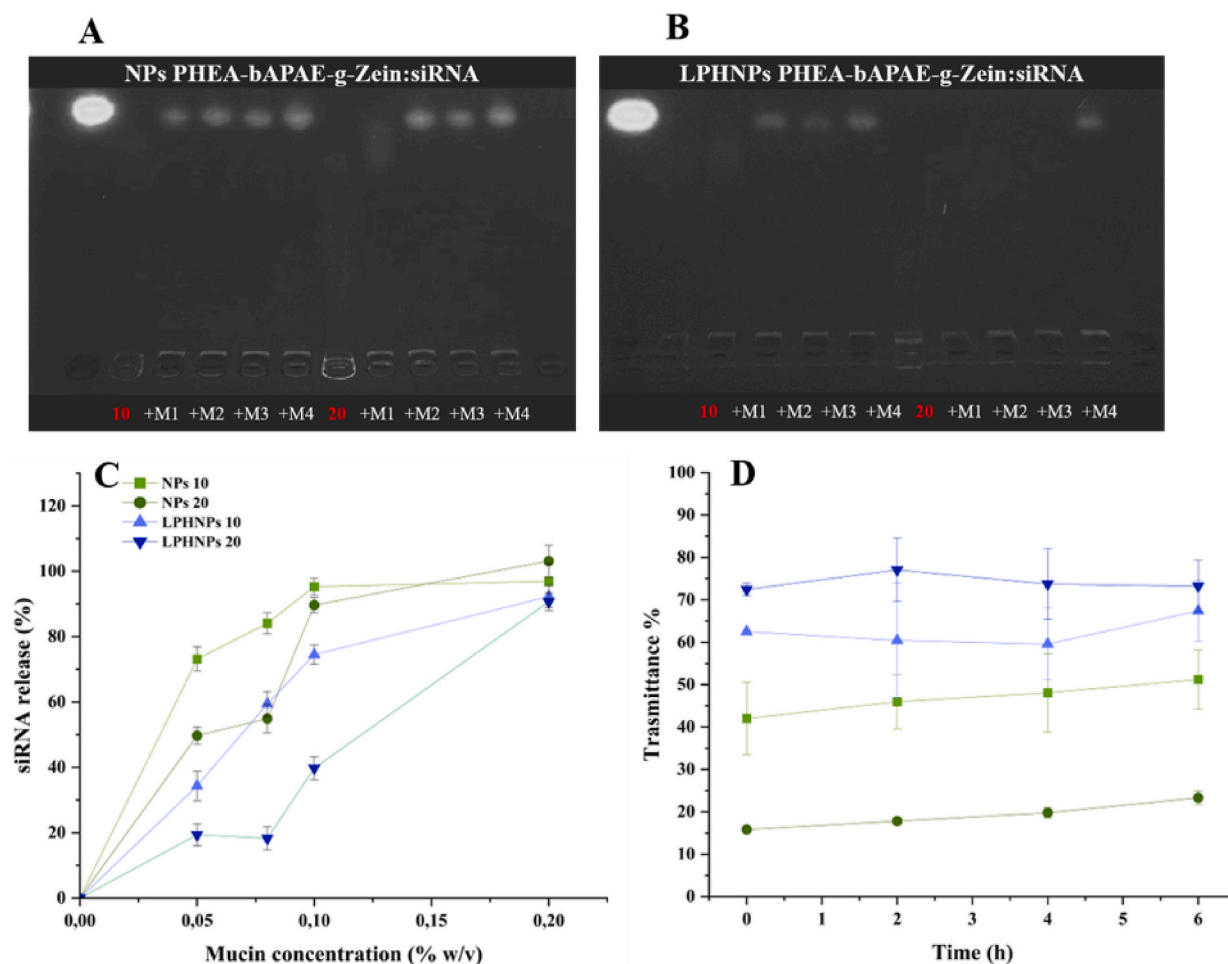


Fig. 5. Stability studies in the presence of mucin: agarose gel electrophoresis of samples (A) NPs and (B) LPHNPs, and (C) siRNA release after incubation for 2 h with mucin (concentrations: M1 0.05% w/v, M2 0.08% w/v, M3 0.1% w/v, and M4 0.2% w/v). (D) Turbidimetric analysis of mucin dispersion (0.08% w/v) in the presence of NPs and LPHNPs samples.

Solo indicated that nebulization did not compromise the biological activity of siRNA loaded into the carriers. Effectively, although both devices caused a partial loss, nebulized samples (except LPHNPs10) mediated a significant gene silencing compared to the control, slightly lower than those obtained with fresh samples (Fig. 7B and C, respectively), highlighting their potential suitability for pulmonary delivery.

The LPHNPs 10 formulation, which did not show biological effects in terms of gene silencing, was internalized in significantly higher quantities than free siRNA; therefore, the poor silencing efficiency (~20%) might have been due to limited endosomal escape [40]. Therefore, we evaluated the endosomal localization of the samples on H1299 cells after 3 h of incubation by Confocal Laser Scanning Microscopy (CLSM) analysis; results showed a high concentration of siRNA at the endosomal level when cells were incubated with sample LPHNPs 10 (Fig. 8A), consistent with poor eGFP gene silencing efficiency. In particular, the yellow signal given by the overlap of green (lysosomes labeled with green LysoTracker) and red fluorescence (siRNA_{AF647}), indicated the presence of siRNA in endosomes, without efficient release of the latter into the cytosol. In contrast, the other samples showed diffuse intracellular distribution with relatively low siRNA localization in lysosomes, consistent with efficient endosomal escape translated into efficient eGFP knockdown activity.

The co-localization was also quantified using Pearson's correlation coefficient (PCC), which statistically measured the degree of overlap between the two fluorescence channels. A PCC value close to 1 indicates no release of siRNA from the endosomes (maximum co-localization),

whereas a significant decrease in the PCC value reflects successful endosomal escape and cytoplasmic dispersion [40,41]. The PCC also confirmed the endosomal localization of siRNA, as it was significantly higher for the LPHNPs 10 formulation than for most other samples (Fig. 8B).

Since the nebulization allowed the deposition of these systems on the mucus layer, the intracellular uptake of these nanocarriers in cellular models with different mucus secretory capacities was evaluated. Specifically, Calu-3 and 16-HBE cells cultured in Air-Liquid Interface (ALI) models were chosen, as they were able to form a pseudostratified epithelium with tight junctions, mucus secretion (higher in Calu-3), and cilia-like structures that closely mimic the *in vivo* respiratory tract [41,42].

After incubation, we used flow cytometry analysis to quantify siRNA_{AF647} (red) carried by the NPs and LPHNPs into the ALI cultures, which showed that it could diffuse through the mucus, reaching the underlying cell monolayer. The uptake was higher in 16HBE14o- ALI cells (Fig. 9A) than in Calu-3 ALI cells (Fig. 9B). These findings were consistent with the varying mucus secretion levels between the two cell lines, with higher secretion in Calu-3 limiting siRNA uptake. It was underlined that the diffusion and cellular internalization of sample LPHNPs 20 appeared to be little influenced by the different secretory cell capacity, probably due to the presence of the surface shell provided by the phospholipids. Fig. 9C shows CLSM images of ALI cells after incubation with the LPHNPs 20 formulation, where the presence of a greater quantity of mucus (green color) in Calu-3 and the presence of siRNA (red

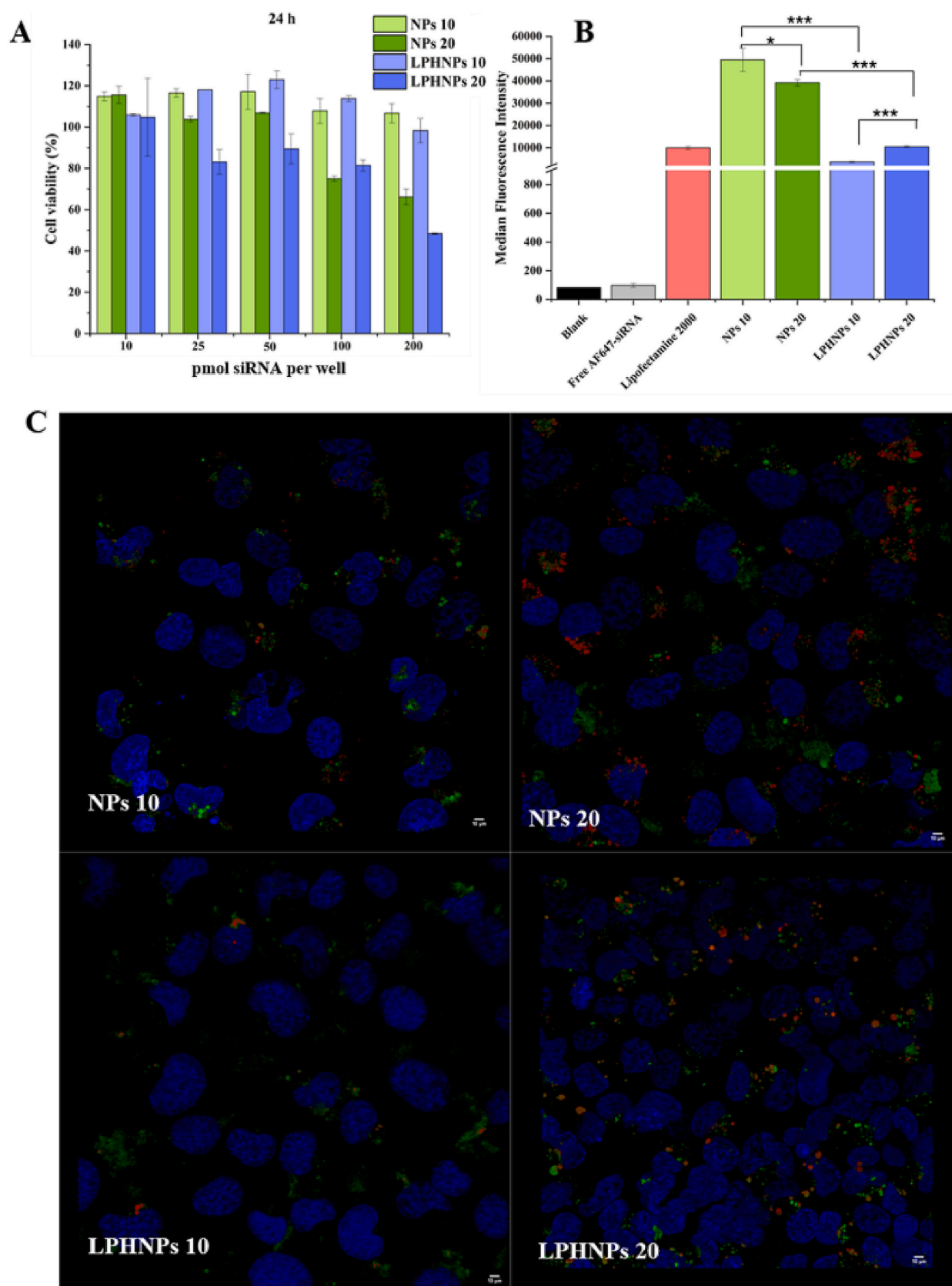


Fig. 6. (A) Cell viability of H1299 cells, in the presence of NPs and LPHNPs 10 and 20, after 24 h of incubation expressed in terms of siRNA incubated per well. Data are expressed as mean $\pm$ SD ($n = 3$). (B) siRNA_{AF647} internalization in H1299, expressed as Mean Fluorescence Intensity (MFI), after incubation for 24 h with NPs and LPHNPs 10 and 20 (50 pmol siRNA/well). Negative control: cells treated with free siRNA_{AF647}. Positive control: cells treated with Lipofectamine 2000. (Data points indicate mean $\pm$ SD, $n = 3$). * $p < 0.05$, *** $p < 0.001$. (C) Three-dimensional (3D) view of CLSM Z-stack images of H1299 cells incubated with NPs or LPHNPs 10 and 20 after 24 h of incubation. The nuclei were stained with DAPI (blue). Green spots indicate the AF488 labeled copolymer, while the red spots siRNA_{AF647}.

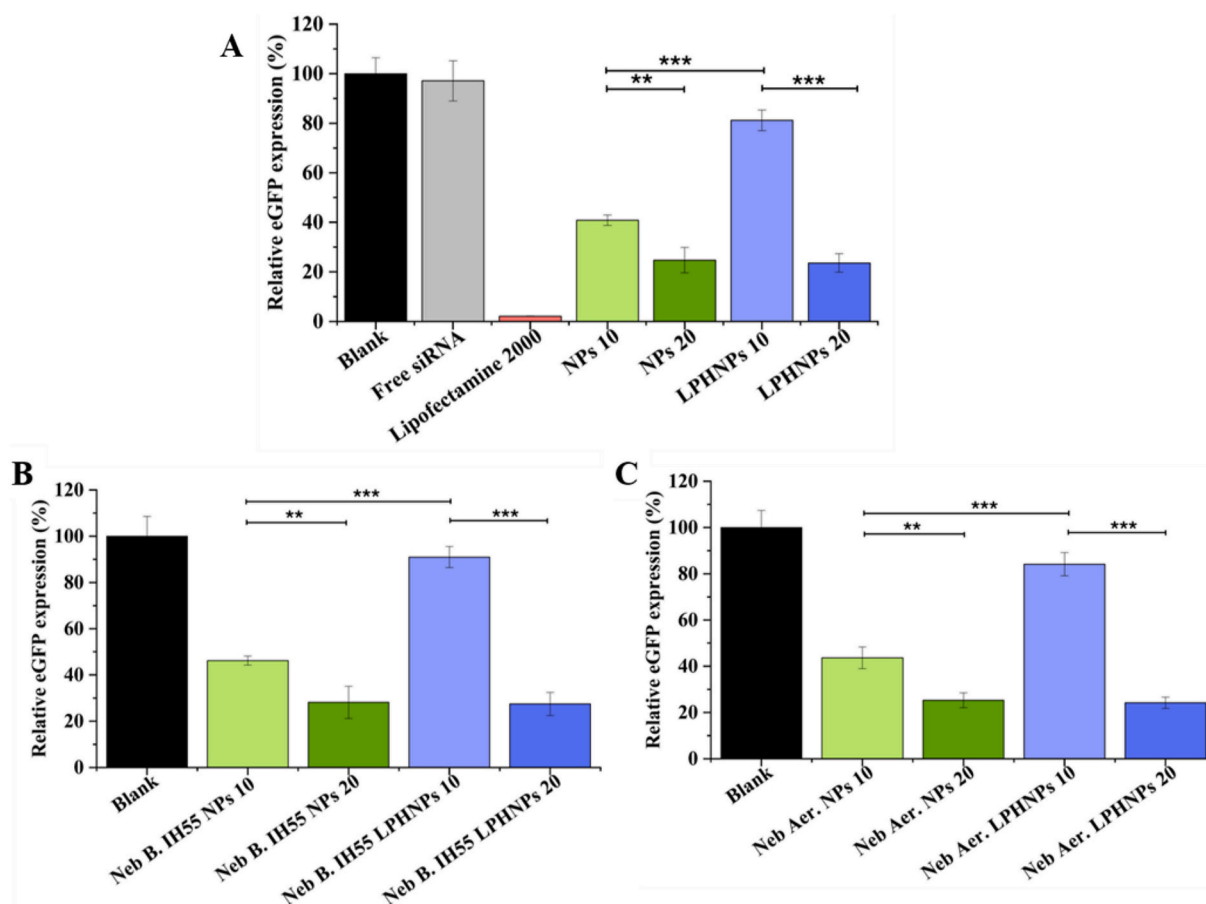


Fig. 7. Gene silencing is reported as relative eGFP expression (%) in H1299-eGFP cells after 48 h of transfection with naked siRNA (siGFP, siNC), or siRNA complexed in (A) fresh NPs and LPHNPs (N/P 10 and 20), or samples nebulized with (B) the Beurer IH55 or (C) the Aerogen Solo device. Lipofectamine 2000 is used as positive control. All samples were tested using 100 pmol siRNA/well. Controls include untreated cells (black bar). ** $p < 0.01$, *** $p < 0.001$.

spots) in both the cell layers (nuclei colored blue) underlying the mucus are evidenced.

5. Conclusions

In this study, the amphiphilic copolymer PHEA-bAPAE-g-zein was designed and synthesized to achieve a well-defined balance between its hydrophilic and hydrophobic domains, a key structural feature that promoted efficient self-assembly in aqueous environments at low concentration values. This property allows the formation of stable nanostructured carriers with siRNA starting from an N/P ratio of 10. Moreover, this work investigated how formulation parameters, specifically variations in the N/P ratio and the presence of a lipid shell, affect the physicochemical and biological performance of the systems in view of pulmonary administration, a route that imposes stringent requirements in terms of stability, mucus interaction, and aerosolization behavior. Accordingly, both polymeric nanoparticles (NPs) and lipid-polymer hybrid nanoparticles (LPHNPs), developed at N/P ratios of 10 and 20, exhibited acceptable cytocompatibility and efficient intracellular siRNA release, promoting endosomal escape and enabling effective gene silencing. Notably, modulation of the N/P ratio in the hybrid systems significantly influenced transfection efficiency, likely due to the formation of distinct molecular assemblies as a function of polymer content, which in turn affected endosomal escape mechanisms.

For inhalation delivery, surface composition emerged as a critical determinant of formulation performance. In particular, the presence of a phospholipid shell in LPHNPs 20 markedly improved formulation behavior, resulting in limited interaction with mucin at different concentrations, reduced premature siRNA release, and enhanced cellular

uptake in two air-liquid interface (ALI) cell culture models. These findings highlight the importance of surface composition in overcoming mucus-related barriers to pulmonary siRNA delivery.

Furthermore, nebulization of both NP and LPHNP formulations, using two different vibrating mesh nebulizer (VMN) devices, showed no negative effects on their physicochemical properties or gene silencing efficacy and the generation of droplets within a size range suitable for inhalation, supporting the translational relevance of the proposed delivery systems.

Overall, the use of PHEA-bAPAE-g-zein-based lipid-polymer hybrid nanoparticles was shown to overcome key limitations of polymer-only systems for pulmonary delivery, owing to the presence of the phospholipid shell, which enhances mucus stability and facilitates mucus penetration. Collectively, these results confirm the promise of combining amphiphilic copolymers with phospholipid coatings, at optimized N/P ratios, as a versatile formulation strategy for pulmonary siRNA delivery, highlighting the potential of these hybrid systems as prospective vectors for inhalable gene therapy.

CRedit authorship contribution statement

Sofia Bonsignore: Writing – original draft, Formal analysis, Conceptualization. **Min Jiang:** Resources, Methodology. **Salvatore Emanuele Drago:** Validation, Data curation, Conceptualization. **Cinzia Scialabba:** Resources, Data curation. **Leonie DeBloch:** Resources. **Sebastian Sturm:** Investigation, Formal analysis. **Emanuela Fabiola Craparo:** Writing – review & editing, Supervision, Conceptualization. **Knut Müller-Caspar:** Resources. **Olivia M. Merkel:** Writing – review & editing, Supervision, Funding acquisition. **Gennara Cavallaro:**

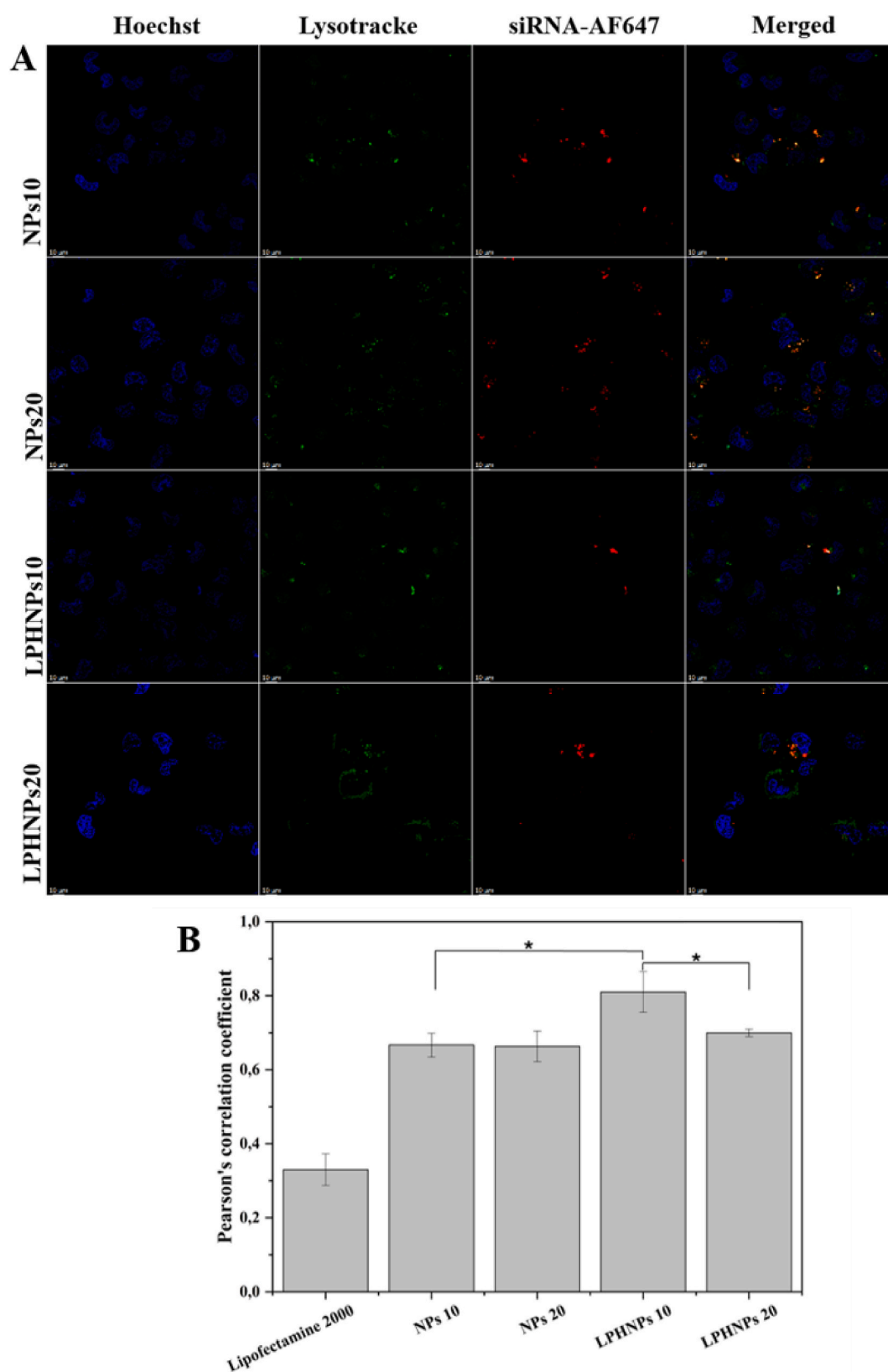


Fig. 8. Endosomal localization in H1299 cells after 3 h of incubation with NPs and LPHNPs 10 and 20 (50 pmol siRNA/well); positive control: cells treated with Lipofectamine 2000. (A) Images acquired by CLSM, where Hoechst 33342-stained nuclei are shown in blue, Lysotracker green-labeled endosomes are shown in green, and siRNA_{AF647} is shown in red. (B) Pearson's correlation coefficient ($n = 6$, values are expressed as mean $\pm$ SEM, unpaired t -test, * $p < 0.05$).

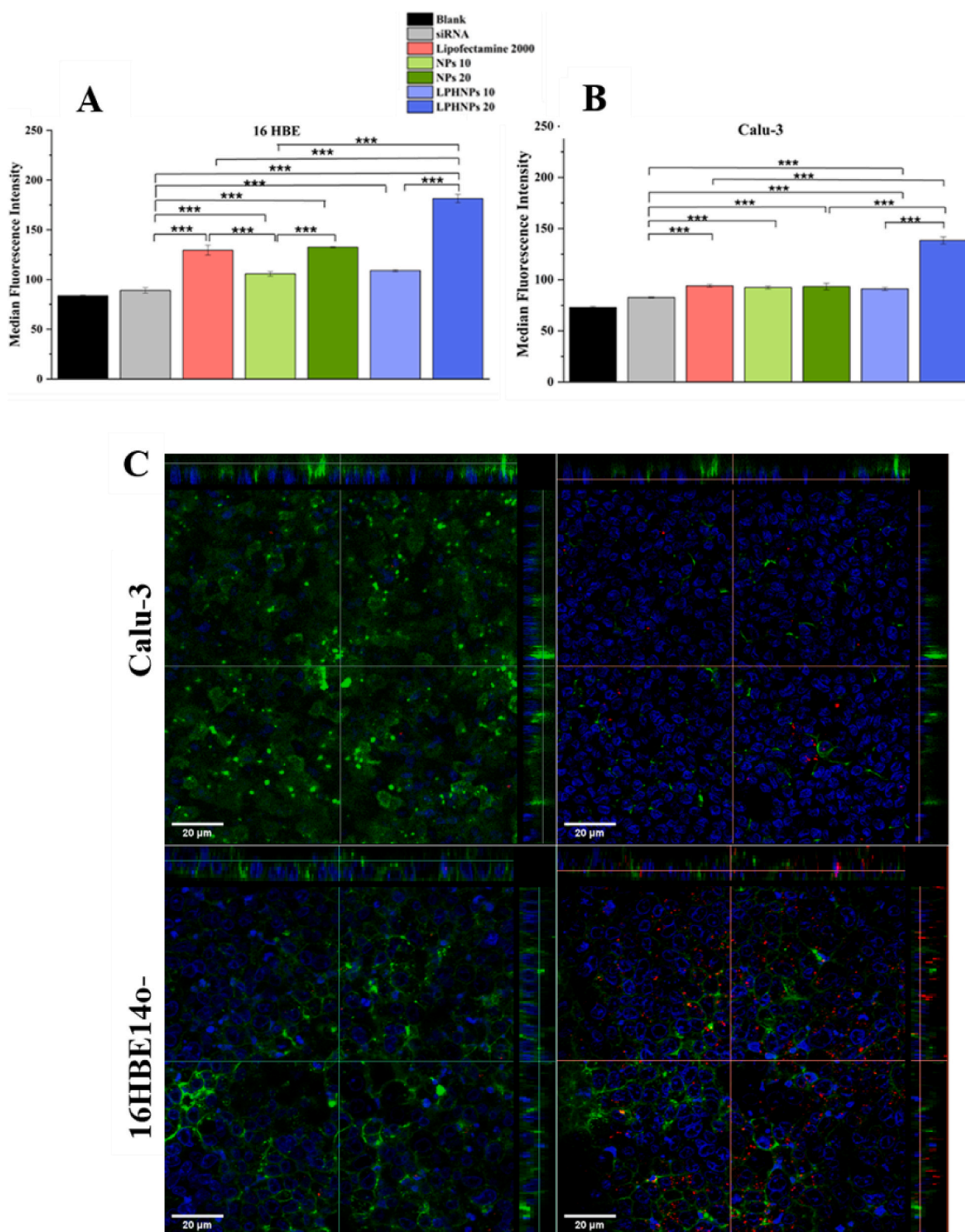


Fig. 9. AF647-labeled siRNA ($\text{siRNA}_{\text{AF647}}$) cellular uptake, expressed as MFI %, in Calu-3 cells (A) and 16-HBE cells (B) cultured at ALI after 24 h of incubation with NPs and LPHNPs 10 and 20 (100 pmol $\text{siRNA}_{\text{AF647}}$ /well containing 20% $\text{siRNA}_{\text{AF647}}$). Untreated Calu-3 cells represent the blank sample. Lipofectamine encapsulating 100 pmol $\text{siRNA}_{\text{AF647}}$ is positive control. *** $p < 0.001$. (C) Confocal microscopy of ALI of Calu-3 and 16-HBE, incubated with LPHNPs 20, 100 pmol of $\text{siRNA}_{\text{AF647}}$. Images represent individual XY planes from a Z-stack, showing the upper mucosal layer (right micrographs) and the lower basal layer toward the nuclei (left micrographs). Nuclei were stained with Hoechst 33,342 (blue) and the mucosal layer with Wheat-Germ-Agglutinin-AlexaFluor488 (green).

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Declaration of competing interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.eurpolymj.2026.115040>.

Data availability

Data will be made available on request.

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