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Highly branched poly β-amino ester/CpG-depleted *CFTR* plasmid nanoparticles for non-viral gene therapy in lung cystic fibrosis disease

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Lung cystic fibrosis (CF) is a lethal inherited disease caused by mutations in the CF transmembrane conductance regulator (*CFTR*) gene, leading to a dysfunctional CFTR protein. Gene therapy offers promise for the treatment of lung CF. However, the development and clinical application of CF gene therapy have long been hampered by the absence of safe and highly efficient delivery vectors. In this work, a novel polymer-based gene replacement treatment approach was developed. A series of poly (β-amino esters) (PAEs) with various topological structures and chemical compositions were screened to create non-viral therapeutic systems for CFTR restoration in lung CF disease. A nanoparticle, formed by the selected highly branched PAE (HPAE) with a CpG-depleted CFTR plasmid, demonstrated *CFTR* gene expression and biocompatibility in lung epithelial cells, outperforming leading commercial gene transfection reagents such as Lipofectamine 3000 and Xfect. The newly developed gene therapy system successfully restored functional CFTR protein production in lung CF epithelial monolayers. This therapeutic approach holds great potential for use as an efficient and safe non-viral treatment for CF patients.

INTRODUCTION

Cystic fibrosis (CF) is a lethal inherited disease caused by mutations in the CF transmembrane conductance regulator (*CFTR*) gene, which affects over 100,000 people worldwide.[1] Although it is a multi-organ systemic disease, its leading cause of morbidity and mortality is the progressive infection and damage to the lungs, making the lung a priority target organ for therapeutic intervention.[2] More than 2,000 mutations of this gene have been identified.[3] These mutations lead to the production of a dysfunctional CFTR protein that decreases or loses the ability to regulate the chloride ion (Cl^-) transport in lung epithelial cells.[4,5] Consequently, this dysfunctional state gives rise to dehydration and pH alterations of the airway mucus, predisposing the individual to recurrent lung infections and inflammations, and ultimately leading to pulmonary failure.[6,7]

Although several medications, including bronchodilators, mucus thinners, and antibiotics, can alleviate patients' symptoms, they cannot solve the problem from the origin for this disease.[8] More recently, CFTR modulators, including correctors and potentiators (like ivacaftor), have been applied to patients to correct the malfunction of the CFTR protein.[9] These modulators demonstrated highly effective treatments in CF patients.[10] However, patients with certain genetic profiles may be ineligible for CFTR modulator therapies, given that the majority of these modulators are specifically tailored to certain mutation types.[11] As a result, approximately 10% of CF patients are left requiring new treatments to address this disease.[10] Gene replacement is a promising strategy, as it is independent of the mutation type and can produce functional CFTR protein in the lung to regulate the chloride ion transport, holding promise for addressing over 2,000 different mutation types associated with CF disease.

The progress in the development of gene therapy for lung CF has been hindered by the lack of safe and highly efficient delivery vectors.[12] Conventional viral vectors, such as adenovirus (Ad), can induce immune responses within the therapeutic framework.[13] For example, Zuckerman et al. in 1999 used Ad5 for *CFTR* gene delivery, resulting in flu-like symptoms in all volunteers with CF.[14] Adeno-associated virus (AAV) is an alternative type of viral vector used for lung CF gene therapy due to its relatively low immunogenicity compared with Ad. However, AAV has not demonstrated efficacy in CF patients, as its packaging capacity is limited, leading to a restricted range of selectable expression elements.[13] Moreover, AAV-induced antiviral immune response still hampered its widespread application in patients.[15] The therapeutic efficacy of virus-mediated treatments in

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lung CF is relatively limited. On the contrary, non-viral vectors have been employed as vectors that present solutions to the issues pertaining to immunogenicity, thereby rendering them as more promising prospects for the development of safe and efficient therapeutic systems.[12,16,17] Non-viral vectors primarily encompass cationic lipid and polymer vectors. The development of non-viral therapeutic systems for CF has predominantly focused on lipid-based vectors, given their advantages including a broad range of payloads, excellent biocompatibility, and encapsulation efficiency. However, the lipid-based delivery approaches failed to meet the clinical requirement for CF gene therapy owing to their limited delivery efficiency, thereby reaching a bottleneck in their development.[12,16] In contrast, polymer vectors have been rarely explored in the development of CF therapeutic systems.[17] With the significant strides forward made in polymer synthesis technology in recent years, this offers a credible route for the development of a novel non-viral therapeutic system targeting lung CF, for example using poly(lactic-co-glycolic) acid (PLGA) and PEI in pDNA delivery in treating lung CF disease.[18] The development of both polymers, however, faces challenges due to their inherent limitations. PLGA has low efficiency to encapsulate the nucleic acid,[19] while PEI exhibits higher cytotoxicity than other cationic polymers *in vitro* and *in vivo*.[20–22]

Given the requirements of efficacy and safety in CF therapy, poly (β-amino esters) (PAEs) have emerged as a promising and highly versatile vector for efficient gene delivery. Since the first reported PAEs as gene delivery vectors, thousands of PAE vectors have been developed and employed in various genetic disease studies.[23] The use of PAEs has proven to achieve exceptional transfection efficiency, even in cell types that are notoriously challenging to transfect.[24] Moreover, PAEs have been used for lung delivery *in vivo*, demonstrating their potent efficiency. These studies have highlighted the advantages offered by PAEs, including great biocompatibility, substantial genetic payload capacity, and cost-effective production, rendering them outstanding candidates for CF gene therapy.[25] It has been reported that the topology and chemical composition of PAEs, especially the nature of terminal groups, significantly influence their gene transfection efficiency.[23,24] Moreover, the optimal PAE structures might differ among various cell lines.[26] Therefore, the design and selection of PAE structures are necessary to develop an efficient non-viral therapy tailored to lung CF.

In gene replacement therapy, the DNA plasmid also plays a vital role in determining overall safety and efficacy. The recent investigations have explicitly shown that the construction of the plasmid by optimizing the CFTR cDNA sequence, eliminating the CpG sequence, incorporating S/MAR domains, and employing a safer human promoter such as human elongation factor 1-α (hEF1α) can notably enhance the duration of CFTR expression without posing any safety concerns.[27–30] Based on these studies, a CpG-depleted CFTR plasmid was employed for CFTR expression in this study.

Here, we have pioneered the development of a polymer-based therapeutic approach for the treatment of lung CF. A series of PAE vectors with diverse chemical compositions and topological structures (linear, branched, and cyclic) were selected from our lab. Their transfection efficiency was assessed in lung CF epithelial cells and the most efficient and safe vector was selected for the rest of the study. The highly branched PAE number 3 (HPAE3) resulted in the selected polymer to deliver the CpG-depleted *CFTR* plasmid for the CFTR protein restoration as a potential therapeutic treatment for lung CF.

RESULTS

In vitro PAEs screening

The PAEs, involving a branched (HPAE1), linear (LPAE1), and cyclic types (CPAE1) of structures were evaluated (Figures 1A and S1; Table S1).[31,32] These three PAEs employed BDA and S5 as functional monomers according to Langer previous research,[33] along with 103 as branched monomer and endcap group (Figure 1A, step 1; Table S1; Figure S1). By delivering gWiz-GFP plasmid in CFBE41o-cells, the transfection results demonstrated that the HPAE1 had higher transfection efficiency when compared to other structural polymers LPAE1($p = 0.0003$) and CPAE1 ($p = 0.0002$) (Figures 1B, S16, and S18). To further improve the HPAE's structure and transfection efficiency, another HPAE2 based on pentaerythritol tetraacrylate (PTTA) branched monomer was further evaluated (Figure 1A, step 2; Table S1; Figure S1). The HPAE2 demonstrated higher transfection efficiency than HPAE1 ($p = 0.0003$) (Figures 1C, S17, and S19). Subsequently, based on the optimized HPAE2 backbone, three different endcap groups E7, 122, or LDA were introduced into the HPAE terminus to synthesize HPAE3-5 (Figures 1A and S1; Table S1). Notably, HPAE3 with E7 endcap had higher transfection efficiency than HPAE4 and HPAE5, showcasing a remarkable 1.18-fold increase in transfection efficiency compared with the HPAE2 in CFBE14o-cells ($p < 0.0001$) (Figures 1D, S17, and S19), indicating that HPAE3 can work as a promising vector in lung CF gene therapy.

gWiz-GFP polyplexes characterization and transfection efficiency evaluation of HPAE3 and commercial reagents

Considering that gene vectors need to overcome several barriers associated with DNA delivery to achieve successful gene replacement in lung CF, the transfection behavior of HPAE3 was investigated, taking Lipofectamine 3000 and Xfect as controls. These included the analysis of the vector-DNA encapsulation efficiency, surface charge of nanoparticles, cellular uptake, and the target gene expression. PicoGreen assay (Figure 2A) was conducted to evaluate the DNA binding capacity, preliminary analysis could indicate a higher DNA binding capacity of HPAE3 than Xfect and lower than Lipofectamine 3000. However, this observed difference was not supported by statistical analysis (multiple comparisons via Kruskal-Wallis test). Afterward, agarose gel electrophoresis was conducted to evaluate the DNA condensation ability of these three transfection reagents (Figure S20). No shifting of DNA bands was observed when complexed with HPAE3. Furthermore, HPAE3 effectively condensed DNA into nanoparticles with sizes around 200 nm and exhibited a zeta potential of around 30 mV (Figures 2B and 2C). Moreover, it was observed that HPAE3-based polyplexes exhibited higher uptake in CFBE41o-cells than Lipofectamine 3000, while lower uptake compared with Xfect,

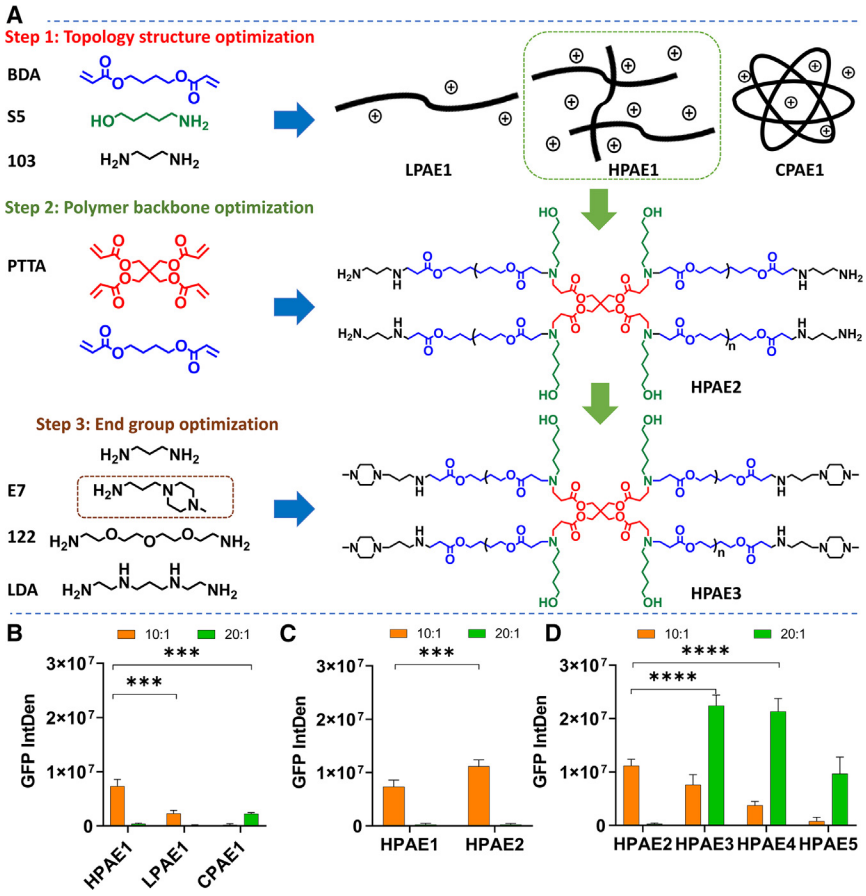

Figure 1. PAEs optimization for gene transfection in CFBE41o- cells

(A) Iterative optimization of the topological structure, polymer backbone, and end group tailors the leading HPAE3 with high transfection efficiency and biocompatibility in CFBE41o- cells. (B) GFP fluorescence intensity (GFP IntDen) from CFBE41o- cells treated by HPAE1, LPAE1, CPAE1/gWiz-GFP complexes at w/w of 10 and 20, after 48-h transfection. (C) GFP IntDen comparison from CFBE41o- cells treated by HPAE1 and HPAE2/gWiz-GFP complexes at w/w of 10 and 20, after 48-h transfection. (D) GFP IntDen from CFBE41o- cells treated by HPAE2, compared with HPAE3, HPAE4, and HPAE5/gWiz-GFP complexes at w/w of 10 and 20, after 48 h transfection. All data were collected from at least three independent experiments ($n \geq 3$). All bar graph data are depicted as means ± SDs. $^{***}p < 0.001$, as compared with HPAE1; $^{****}p < 0.0001$, as compared with HPAE2. Fluorescence microscopy images are representative of three independent experiments.

but statistical analysis revealed no differences among the three transfection reagents tested (Figures 2D and S21). The gene transfection efficiency of the HPAE3 was further compared with these two commercial transfection reagents. The statistical results showed that the GFP expression of cells following the transfection with HPAE3 exhibited a comparable level to Xfect and was 12-fold higher than that of Lipofectamine 3000 ($p = 0.0225$), while performing similar cell viability as Xfect and higher cell viability than Lipofectamine 3000 ($p = 0.0146$) (Figures 2F, 2E, S22, and S23).

HPAE3/CpG-depleted CFTR plasmid complex characterization

Plasmid construct and CFTR DNA sequence also play an important role in treating this disease. Therefore, the CpG-depleted CFTR plasmid was designed by subcloning the optimized and CpG-depleted CFTR fragment into the CpG-free plasmid backbone with S/MAR sequence flanking alongside the CFTR sequence (Figure 3A). The CpG-depleted CFTR plasmid further recruited the hEF1α promoter (Figures 3A and S15). Given the size of CpG-depleted CFTR plasmid (~7.7 kbp), achieving efficient delivery poses a significant challenge. Therefore, the transfection behavior of HPAE3/CpG-depleted CFTR plasmid system was further evaluated. Characterization of the HPAE3/CpG-depleted CFTR plasmid demonstrated that this polyplex system has similar characteristics compared with HPAE3/gWiz-GFP plasmid in terms of DNA binding, condensation, polyplex size, zeta potential, and uptake (Figures 2B, 2C, and S24–S27).

CFTR transcription and protein expression in CFBE41o-cells

The effect of HPAE3/CpG-depleted CFTR plasmid complexes was further evaluated for transcription and expression levels, along with cell viability, after 48 h of transfection in CFBE41o-cells. As expected, the CFTR mRNA levels of plasmid complexes were found to be 14-fold of 16HBE41o- (HBE) CFTR mRNA levels (Figure 3D). Further, HPAE3/CpG-depleted CFTR plasmid complexes exhibited a 26-fold CFTR protein expression of HBE CFTR expression (Figures 3E and 3F). In terms of cell viability, this developed polyplex achieved levels exceeding 75% of the untreated cells (Figure 3G). When normalizing the CFTR expression capacity by combining the cell viability with CFTR mRNA and CFTR protein level, the normalized CFTR mRNA level of HPAE3 was around 2-fold Xfect ($p = 0.0004$) and similar to Lipofectamine3000 (Figure 3H). Notably, the normalized CFTR protein level of HPAE3/CpG-depleted CFTR plasmid was around 2-fold of Xfect ($p = 0.0392$) and 2-fold of Lipofectamine 3000 ($p = 0.006$) (Figure 3I). Subsequently, immunocytochemistry and flow cytometry confirmed the CFTR protein restored 48 h post-transfection of HPAE3/CpG-depleted CFTR plasmid complexes was localized in the cellular membrane (Figures 4A and S28), being around 10% of the cells positive for CFTR protein restoration on the cell membrane (Figure S28).

CFTR function restoration evaluation in CFBE41o-cells

In addition to assessing the CFTR protein expression at the molecular biological level, it is also crucial to assess its functional recovery to

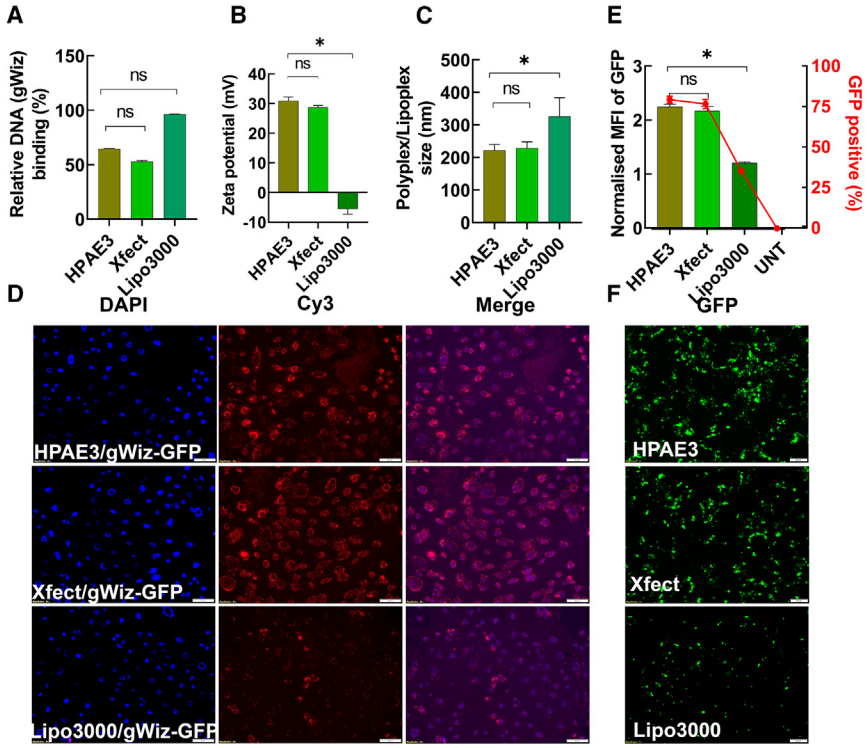

Figure 2. HPAE3/gWiz-GFP polyplexes characterization and transfection efficiency evaluation

(A) Relative DNA binding of HPAE3 at w/w of 20:1 (polymer:DNA). Lipofectamine 3000 and Xfect were used as controls. (B) HPAE3/gWiz-GFP polyplexes size. (C) HPAE3/gWiz-GFP polyplexes zeta potentials. (D) Cellular uptake in CFBE41o- after 4-h transfection with HPAE3/gWiz-GFP polyplexes. The nucleus was stained with DAPI (blue), and DNA was labeled with Cy3 (red) using a 20× fluorescence microscopy objective. The area with the Cy3 regions without DAPI staining is owing to the labeled DNA nanoparticles attached to the bottom of the cell culture plates. (E) Normalized MFI and percentage of GFP-positive CFBE41o- cells with flow cytometry. (F) GFP expression after 48-h transfection of HPAE3/gWiz-GFP polyplexes. All data were collected from at least three independent experiments ($n \geq 3$). All bar graph data are depicted as means ± SDs. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$; ns: not significant, as compared with HPAE3. Fluorescence microscopy images are representative of three independent experiments.

evaluate the clinical potential of lung CF gene replacement therapy. Using the Ussing Chamber Assay, the transepithelial chloride short-circuit current (*I*sc), was measured in response to the CFTR stimulator and inhibitor. After forskolin stimulation, an increase in *I*sc (Δ*I*sc) of 4.6 ± 1.018 μA/cm^2 was observed with HPAE3/CpG-depleted CFTR plasmid complexes, until CFTR channel inhibition was induced (Figures 4B and 4C). After adding CFTRinh_172 inhibition, 4.19 ± 1.683 μA/cm^2 Δ*I*sc (from 131 ms to 166 ms) was detected on the apical monolayer induced by transfection with HPAE3/CpG-depleted CFTR plasmid complexes (Figures 4B and 4D).

DISCUSSION

CF is a monogenetic disease with over 2,000 different mutation types, rendering it one of the earliest diseases that is suited for the gene replacement approach. Over the past 30 years, this approach has been one of the most promising technologies for treating lung CF. However, it is more complex than anticipated to develop a therapeutic gene replacement system in this disease, as the physical barriers to achieve the target cells require safe and highly efficient delivery vectors.[12,13,16]

Considering the imperative quest for highly efficient vectors, an HPAE-based therapeutic system targeting lung CF gene delivery was developed. The CF bronchial epithelial cells, which are primarily affected by the disease,[2] were identified as ideal target cells. Therefore, its immortalized cell line, CFBE41o-, was used in this study. By comparing the transfection efficiency of different PAEs in CFBE41o- cells, the highly branched PAE number 3 (HPAE3) emerged as the optimal gene delivery vector for treating lung CF disease. Furthermore, the developed HPAE3/DNAs system was evaluated encompassing key parameters such as DNA binding of HPAE3, polyplex size, zeta potential, cell uptake, cell viability, CFTR mRNA, and protein expression, and CFTR function restoration. The developed HPAE3/DNAs system exhibited a favorable profile for CF gene therapy, demonstrating remarkable restoration of CFTR protein and excellent cell viability.

In the screening process for PAEs, a comparison of topological structures revealed that HPAEs exhibited superior transfection efficiency in CFBE cells. This observation aligns with existing studies highlighting the enhanced DNA binding efficiency and delivery potential of HPAEs, attributed to their distinctive three-dimensional (3D) architecture. The 3D structure provides an increased number of acrylate termini on the base polymer, facilitating the attachment of end-capping groups and thereby enhancing protonation ability.[34,35] Subsequently, the HPAE structures were further optimized by "A2+B4+C2" Michael addition strategy, showcasing heightened transfection performance coupled with increased biocompatibility. Furthermore, by optimizing the end-capping for the HPAEs, tailored HPAE3 with E7 end-capping for gene delivery in CFBE41o- cells.[26,32]

Regarding polyplex transfection behavior, HPAE3 demonstrated an impressive nearly 100% cellular uptake efficiency, while the GFP-positive percentage reached approximately 76%. This discrepancy may be elucidated by HPAE3's proficient cellular uptake capability, coupled with a relatively lower escape ability of the polyplex within CFBE41o- cells, resulting in a final transfection

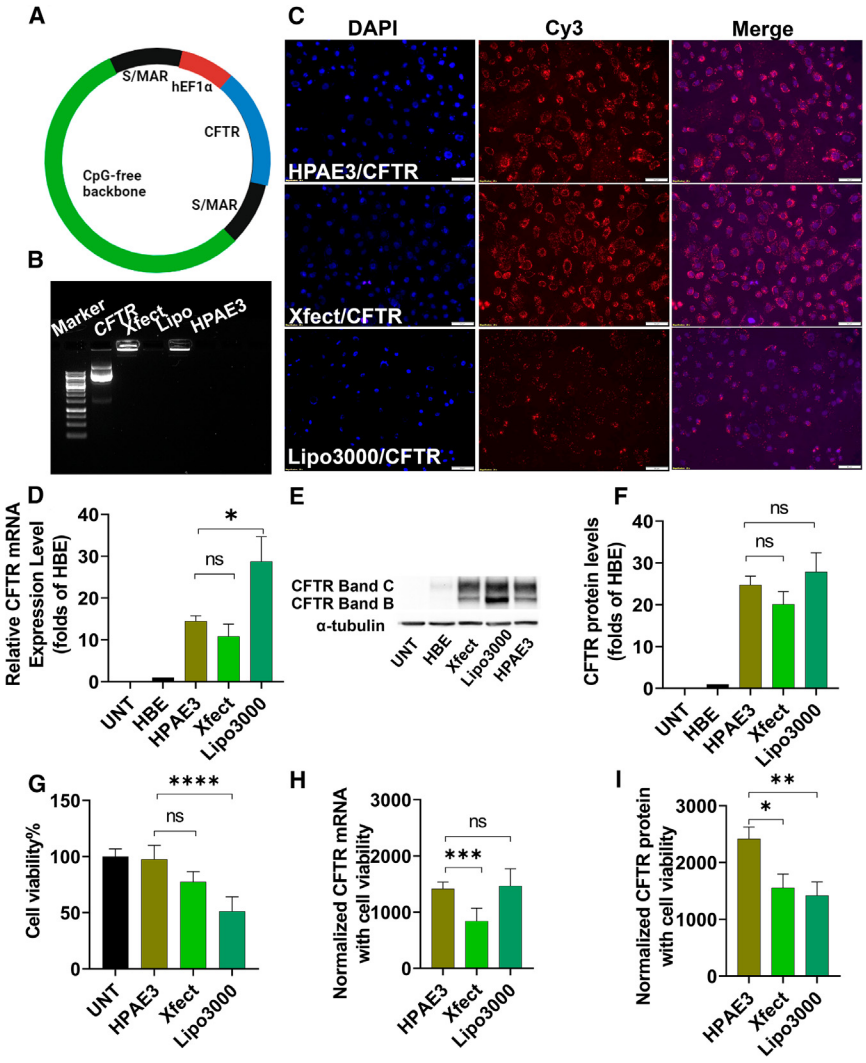

Figure 3. Polyplex characterization and CFTR expression of HPAE3/CpG-depleted CFTR plasmid complexes in CFBE41o- cells

(A) The map of CpG-depleted CFTR plasmid. (B) Agarose gel results of HPAE3/CpG-depleted CFTR plasmid complexes at w/w of 20. Comparison with Lipofectamine3000 and Xfect. (C) Cellular uptake of HPAE3/CpG-depleted CFTR plasmid complexes in CFBE41o-after 4-h transfection. (D) The CFTR mRNA quantification by RT-qPCR normalized by GAPDH. (E) CFTR protein expression by western blot. (F) Semi-quantified CFTR protein expression by western blot normalized by α-Tubulin. (G) Cell viability of transfected CFBE41o-cells. (H) CFTR mRNA and (I) CFTR protein expression normalized with cell viability of transfected CFBE41o-cells. The normalized data were presented by combining CFTR mRNA or CFTR protein expression to cell viability. All data were collected from at least three independent experiments ($n \geq 3$). All bar graph data are depicted as means ± SDs. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, as compared with HPAE3. ns: not significant, as compared with HPAE3. Western blot and immunocytochemistry images are representative of three independent experiments ($n = 3$).

efficiency of around 76%. To enhance the polyplex escape ability in the context of lung CF gene delivery, future investigations could focus on improving the endosomal escape mechanisms associated with the HPAE3-based polyplex.[23,24,26] Gel electrophoresis analysis revealed no discernible DNA band migration, although the PicoGreen assay indicated a binding rate of approximately 65%. This inconsistency might be attributed to the partial encapsulation of DNA by the polymers, leaving a portion accessible for PicoGreen binding. This observation is further supported by the higher cellular uptake exhibited by HPAE3, although there existed no significant difference after Kruskal-Wallis testing. Additionally, HPAE3 also demonstrated stable DNA binding capacity across different plasmid sizes, indicating its robust stability and high potential for gene therapy in CF disease.

To achieve therapeutic CFTR expression levels in CF lung epithelial cells, which should be at least 6%–10% of the CFTR expression of healthy bronchial epithelial cells,[36] a CpG-depleted CFTR plasmid was employed to enhance CFTR expression. This plasmid possesses distinct features, including a CpG-free backbone,[27,28] the incorporation of S/MARs domains,[30] a codon-optimized and CpG-free CFTR gene sequence, and the human promoter hEF1α. The inclusion of the hEF1α promoter is particularly significant, as it can prolong CFTR expression, thereby aiding in the sustained recovery of CFTR levels. Furthermore, the presence of CpG sequences can stimulate immune responses when introduced into the lungs.[27] Therefore, the CpG-depleted CFTR plasmid would be safe when applied to CF patients, as it exhibited no safety concerns during the clinical trial.[16]

Upon complexation of HPAE3 with the CpG-depleted CFTR plasmid, the polyplex system exhibited remarkable restoration of CFTR protein levels in diseased cells at 48 h post-transfection. Notably, there was a 25-fold increase in CFTR levels compared with healthy human bronchial epithelial cells, a significant achievement considering the therapeutic CFTR expression levels are typically targeted to reach 6%–10% of the CFTR expression in healthy bronchial epithelial cells.[36] Given the pivotal role of CFTR protein as a chloride ion channel and its dysfunction leading to lung disorders,[5] further evaluation of chloride ion secretion function recovery is imperative to assess the potential clinical applications of the developed HPAE3/CpG-depleted CFTR plasmid polyplex system. Subsequent evaluations revealed a partial restoration of chloride secretion function in epithelial monolayers after treatment with

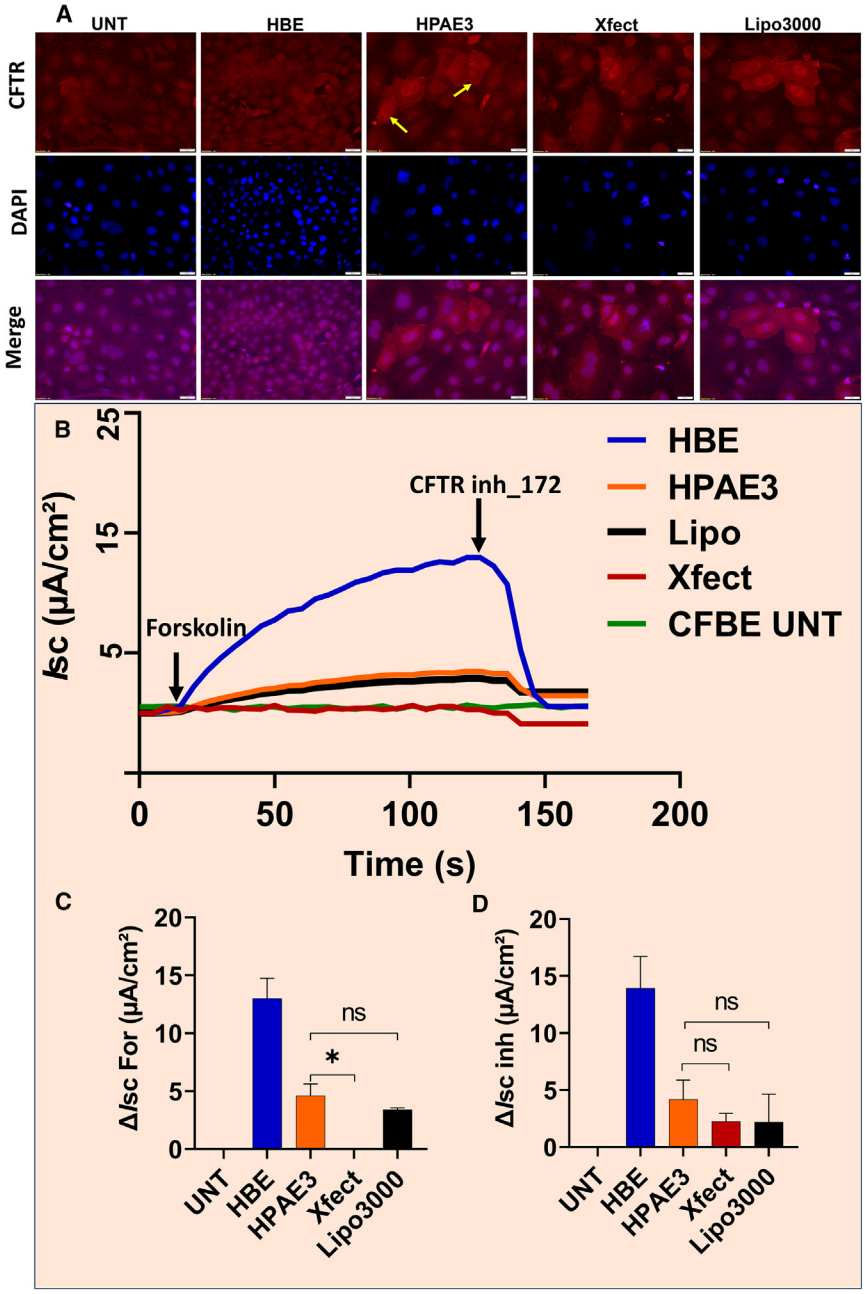

Figure 4. CFTR expression and function restoration of HPAE3/CpG-depleted CFTR plasmid complexes in CFBE41o- cells and monolayer

(A) Immunocytochemistry images of CFBE41o- cells after 48-h transfection with HPAE3/CpG-depleted CFTR plasmid complexes. Lipofectamine 3000 and Xfect were used as controls. CFTR detected in red, and nucleus stained by DAPI in blue, using a 40× fluorescence microscopy objective. (B) The chloride short-circuit current (*I*sc) measurement for CFTR function testing in CFBE41o- monolayer after 48-h transfection with HPAE3/CpG-depleted CFTR plasmid complexes. (C) The quantified Δ*I*sc after adding forskolin (For) for CFTR function testing in CFBE41o- monolayer after 48-h transfection with HPAE3/CpG-depleted CFTR plasmid complexes. (D) The quantified Δ*I*sc after adding CFTRinh_172 (inh) for CFTR function testing in CFBE41o- monolayer after 48-h transfection with HPAE3/CpG-depleted CFTR plasmid complexes. All data were collected from at least three independent experiments ($n \geq 3$). All bar graph data are depicted as means ± SDs. $^{*}p < 0.05$ as compared with HPAE3. ns: not significant, as compared with HPAE3. Immunocytochemistry images and *I*sc tests are representative of three independent experiments ($n = 3$).

this system. Although the transepithelial resistance of CFBE41o- cells was found to be lower than that of 16HBE14o cells, there is no paracellular access for ions or reagents in CFBE41o- cells. This observation can be explained by the lack of change of Δ*I*sc observed in untreated CFBE41o- cells even after exposure to the different buffer conditions like using Cl-decreased Krebs-bicarbonate Ringer (KBR) buffer, adding forskolin and CFTRinh_172 inhibition reagents. In addition, by employing forskolin and an inhibitor, the HPAE3/CpG-depleted CFTR plasmid polyplex system demonstrated approximately 35.28% and 30.08% recovery of CFTR function in CFBE monolayer cells compared with healthy bronchial epithelial cells. The results demonstrated the capacity of developed polyplex system in restoring CFTR protein function *in vitro*. The transepithelial resistance of CFBE41o-cells was observed to be lower than that of 16HBE14o cells, potentially resulting in paracellular ion leakage and inaccurate measurements of chloride secretion. Therefore, in the future, the transepithelial resistance of CFBE41o- cells should be improved by using air-liquid interface culture before testing the current values. Additionally, it is important to note that the feasibility of the HPAE3/CpG-depleted CFTR plasmid polyplex system has been primarily established in CFBE41o- cells *in vitro* in this study. Future work should focus on evaluating the efficacy of the developed system in primary cells, *ex vivo*, and *in vivo* CF models to ascertain its potential clinical relevance and therapeutic impact.

The majority of non-viral gene therapy clinical trials have utilized lipid-based vectors, while Xfect is the gold standard among cationic polymers.[12,26] Therefore, a critical step in assessing the potential clinical application of HPAE3 is to compare it with established vectors like Lipofectamine 3000 and Xfect.[26,27] The results demonstrated that HPAE3 achieved a 12-fold transfection efficiency and around 4-fold cell viability compared with Lipofectamine 3000. In

addition, HPAE3 demonstrated competitive transfection performance compared with Xfect in these two parameters. Regarding CFTR expression, the HPAE3-based system exhibited superior restoration of CFTR mRNA and protein levels compared with Xfect and comparable levels to Lipofectamine 3000. On the other hand, the results of HPAE3 demonstrated similarity in CFTR function restoration compared with Lipofectamine 3000. However, the introduction of forskolin did not yield any discernible alterations in the transfection process mediated by Xfect. This lack of observable change can be attributed to the lower transfection efficiency of Xfect when employed in monolayer cultures formed by non-dividing cells.[37]

The superior efficacy of the HPAE3/CpG-depleted CFTR plasmid polyplex system, compared with lipid-based vectors and cationic polymers, highlights its potential for clinical applications. However, it is crucial to note that this evaluation is solely based on *in vitro* studies, which represented the initial step toward developing a non-viral vector-based gene replacement strategy. Thereby, other factors such as large-scale manufacturing of HPAE-based polyplexes, clinical practicability, and *in vivo* efficacy should be considered for future clinical application.[38,39] One promising direction to address one of these challenges is using microfluidic mixing for preparing polyplexes, offering advantages such as precise control, cost-effectiveness, manufacture at scale, and flexibility for modifications.[40,41] In addition, given that lung cystic fibrosis is a chronic disease, exploring nebulization administration methods is pivotal for the clinical application of the HPAE3/CpG-depleted CFTR plasmid polyplex system. The production of nebulized polyplexes relies on their shear resistance, which influences both their transfection efficacy and stability pre- and post-nebulization.[22]

On the other hand, the primary limitation for *in vivo* development of inhaled nanoparticles lies in the preparation of a concentrated gene therapy system to attain optimal therapeutic efficacy, due to the constrained rate of lung deposition.[42] However, preparing the concentrated formulations are more complexed than anticipated, given that the polyplexes at higher concentration tend to precipitate owing to their limited colloidal stability, thereby diminishing their efficacy.[43] Therefore, in the future, several methods can be employed for concentrating HPAE-based systems, such as lyophilization and reconstitution, ultrafiltration, and tangential flow filtration.[43,44] Additionally, to better evaluate its clinical potential, further *in vivo* study of the developed HPAE3/CpG-depleted CFTR plasmid polyplex system is important, as the mouse model is closer to the human physiological environment compared with *in vitro* cell lines. The efficacy of this system would be further determined by the CFTR expression level in the lung and toxicity evaluation.[27]

MATERIALS AND METHODS

Synthesis of PAEs

For linear PAE 1 (LPAE1) synthesis, monomers 1,4-butanediol diacrylate (BDA), and 5-amino-1-pentanol (S5) were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 30% w/v. The reaction occurred at 90°C under argon protection. Once the polymer reached the desired molecular weight, the end-capping amine, 1,3-diaminopropane (103), was added to stop the reaction, and the reaction system was diluted to 10% w/v. In the end, the polymer was precipitated into diethyl ether for purification and was dried under vacuum. The polymer was stored at −20°C for the following studies.

The cycled PAE 1 (CPAE1) was synthesized using a reaction stage-dependent dilution strategy reported in a recent work.[31] Monomers BDA, S5, and 103 were dissolved in DMSO at the concentration of 30% w/v. The reaction occurred at 90°C under argon protection. Once the polymer reached the desired molecular weight, the end-capping amine 103 was added to stop the reaction, and the reaction system was diluted to 10% w/v. In the end, the polymer was precipitated into diethyl ether for purification and was dried under vacuum. The polymer was stored at −20°C for the following studies.

The highly branched PAEs from 1 to 5 (HPAE1, HPAE2, HPAE3, HPAE4, and HPAE5) were synthesized using an "A2+B4+C2" strategy reported in previous studies.[32] Monomers (Table S2) were dissolved in DMSO at the concentration of 30% w/v. The reaction was conducted at 90°C under argon protection. Once the polymer reached the desired molecular weight, an end-capping amine, 103, 1-(3-aminopropyl)-4-methylpiperazine (E7), 1,11-Diamino-3,6,9-trioxaundecane (122), or Lithium diisopropylamide (LDA) was added to stop the reaction, and the reaction system was diluted to 10% w/v. In the end, the polymers were precipitated into diethyl ether for purification and were dried under vacuum. The polymers were stored at −20°C for the following studies.

Gel permeation chromatography was used to monitor the reaction, and nuclear magnetic resonance was used to confirm the chemical structures of PAEs. All the reagents mentioned above were purchased from Merck (Rahway, NJ, USA).

Plasmid information and construction

The CpG-depleted *CFTR* plasmid (7.7 kbp) consists of a human cytomegalovirus (hCMV) enhancer-plus hEFIα promoter (hCEFIα)-SI126 Intron-*CFTR*-BGH polyA-R6K origin-Zeocin-resistant sequence (Figure S15). The hCMV enhancer (GenBank: acc# JQ439994) and BGH polyA (GenBank: acc# JQ439997) are CpG-free sequences.[45] The hEFIα promoter and SI126 Intron sequences are CpG-free and designed based on the pcpgf-mcs plasmid (InvivoGen, San Diego, CA, USA). The *CFTR* sequence has the characteristics of codon optimization and CpG-free sequence, which is based on the so*CFTR*2 sequence[27] with 6 bp changes. These hCMV enhancer-hEFIα promoter-SI126 Intron-*CFTR*-BGH polyA components were commercially synthesized by Eurofins (Munich, Germany) and ligated into the pcpgf-mcs plasmid at EcoRI sites. In addition, R6K origin and Zeocin-resistant sequences are from pcpgf-mcs plasmid and serve as the plasmid backbone in CpG-depleted *CFTR* plasmid.

Cell culture

CFBE41o-human CF bronchial epithelial and 16HBE14o-human bronchial epithelial cell lines, derived from a CF patient with Phe508-del CFTR mutation and a healthy donor, respectively, and immortalized with the origin-of-replication defective SV40 plasmid (pSVori-), were purchased form Merck. Cells were grown in flasks or plates coated with a fibronectin/collagen/bovine serum albumin (BSA) extracellular matrix cocktail (Merck) in minimum essential medium (MEM) cell culture medium (Merck) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA), 1X MEM non-essential amino acid (only for CFBE41o- cell culture) (Merck), 2 mM glutamine (Thermo Fisher Scientific), 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate (Thermo Fisher Scientific), incubated at 5% CO_2 at 37°C.

Cell transfection and fluorescence signal analysis

CFBE41o- cells were seeded (~20,000/cm^2) 24 h before transfection, and when the cell confluence arrived at 50%–70%, transfections were performed. The cells were transfected with a series of PAE polymers that were designed and synthesized by our group (Figure 1; Table S1), and the commercial transfection reagents Lipofectamine 3000 (Thermo Fisher Scientific) and Xfect (Takara, Kusatsu, Japan) were taken as controls. CFBE41o- cells were transfected in the culture media with the plasmids at a final concentration of 5 ng/μL, previously complexed to the different synthesized polymers. PAEs and gWiz-GFP were diluted in 25 mM NaOAc at w/w of 10:1 and 20:1 (polymer:DNA) and then mixed at a 1:1 volume/volume ratio (v/v). HPAE3 and CpG-depleted CFTR plasmids were diluted in 25 mM NaOAc at w/w of 20:1 (polymer:DNA) and then mixed at v/v of 1:1. Lipofectamine 3000 and Xfect were used following the manufacturer's instructions. Expression of the reporter gene from gWiz-GFP plasmid (Aldevron, South Fargo, ND, USA) was visualized 48 h post-transfection by using an Olympus IX81 fluorescence microscope (Olympus, Tokyo, Japan). The intensity of GFP fluorescence was analyzed and semi-quantified using the ImageJ software (NIH, Bethesda, MD, USA), as performed in previous studies.[32,46]

Cell viability

The alamarBlue Kit (Thermo Fisher Scientific) was used to assess cell viability, following the method previously described.[32,46] In brief, cell viability was tested 48 h post-transfection by replacing the previous medium with fresh medium containing 10% (v/v) alamarBlue reagent. A SpectraMax M3 multi-plate reader (Molecular Devices, San Jose, CA, USA) was used for excitation/emission values determination, which were recorded at 570 nm and 590 nm after incubation for 1 h protected from light at 37°C. Untreated cells were used to normalize fluorescence values and set to 100% cell viability.

Cellular uptake of polyplexes and fluorescence signal analysis

The Cy3 DNA labeling kit (Mirus, Marietta, GA, USA) was used to label DNA, according to the manufacturer's instructions. Using 5 ng/μL of DNA, CFBE14o-cells were transfected with HPAE3/DNA polyplexes (w/w = 20:1), Lipofectamine 3000/DNA lipoplexes, and Xfect/DNA polyplexes for 4 h and then incubated with NucBlue Live Ready Probes Reagent (Thermo Fisher Scientific) at room temperature for 30 min. Fluorescent images were taken with an Olympus IX81 fluorescence microscope. The intensity of Cy3 fluorescence was analyzed and semi-quantified using the ImageJ software, as mentioned above.

DNA binding assay

Quant-iT PicoGreen dsDNA Assay Kits (Thermo Fisher Scientific) were used to quantify the DNA binding capacity of each polymer. This experiment was performed as previously described.[32] Polyplexes were prepared with the same formulation used for cell transfection and added in an equal volume (100 μL) to the PicoGreen working solution. The mixture was incubated for 5 min, protected from light. Fluorescence was measured using a SpectraMax M3 plate reader. DNA binding efficiency (BE) was calculated using the equation $BE = ((F_{DNA} - F_{Sample})/(F_{DNA} - F_{Blank})) \times 100\%$, where F_{DNA} is the florescence measurement of free DNA without polymer, F_{Sample} is the florescence of a polyplex or lipoplex at a given w/w between polymer or lipid to DNA, and F_{Blank} is the florescence from PicoGreen working solution only, with the buffer used for polyplex or lipoplex formulation.

Agarose gel electrophoresis

Agarose gel electrophoresis was performed as previously described.[32] The agarose gel electrophoresis was performed with the same formulations used for cell transfection. Polyplexes, prepared as described above, were loaded into 1% agarose (Merck) gel wells containing 1X SYBR Safe DNA Gel Stain (Thermo Fisher Scientific), and electrophoresis was conducted at 110 V for 40 min.

Polyplex size and zeta potential

The size and zeta potential of polyplexes or lipoplexes formulated by the polymer or lipid and DNA (gWiz-GFP or CpG-depleted CFTR plasmids), were measured using a Zetasizer Pro (Malvern Panalytical, Malvern, UK). Polyplexes or lipoplexes were prepared as described above and diluted to 1 mL using their corresponding transfection buffer for measurement. The temperature of the samples was controlled at 25°C.

Western blot analysis

After 48 h post-transfection, cells were washed with DPBS (Thermo Fisher Scientific). Then radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific) with protease inhibitor (Thermo Fisher Scientific) was added to each sample to lyse the cells and solubilize the proteins. To quantify protein concentration, the Pierce BCA Protein Assay was performed using the kit from Thermo Fisher Scientific. Samples were loaded onto 7% NuPAGE Tris-Acetate protein gels (Thermo Fisher Scientific) and later transferred to a nitrocellulose membrane (Thermo Fisher Scientific). For the detection of the CFTR protein, the primary antibody, mouse anti-human CFTR UNC-596 (University of North Carolina, NC, USA), was diluted at 1:500, and the secondary antibody, an anti-mouse immunoglobulin (Ig)G horseradish peroxidase (HRP)-linked antibody (Cell Signaling, Danvers, MA, USA), was diluted at 1:2,000. Human α-tubulin (Cell

Signaling) was used to detect the loading control proteins at the dilutions of 1:1,000. The secondary anti-rabbit IgG, HRP-linked antibody (Cell Signaling) was diluted at 1:4,000. The protein bands were developed and captured using Pierce ECL western blotting substrate (Thermo Fisher Scientific) and the iBright CL750 imaging system (Thermo Fisher Scientific). Moreover, the semi-quantitative analysis of CFTR was determined using ImageJ Fiji software (NIH), as described in previous studies.[46]

RT-qPCR analysis

RT-qPCR was performed to quantify the CFTR mRNA expression, following similar protocol described in our previous study.[46] After 48 h post-transfection, cells in a 24-well plate were washed with DPBS. The RNeasy Mini Kit (QIAGEN, Hilden, Germany) was used for total RNA extraction from cells. cDNA was generated using a SuperScript III First-Strand Synthesis SuperMix kit (Thermo Fisher Scientific) and qPCR was performed by using TaqMan PCR mix (Thermo Fisher Scientific) in a QuantStudio 7 Flex System, (Thermo Fisher). *CFTR* TaqMan primer (Hs00357011_m1) or customized OP-*CFTR* TaqMan primer (ART2DCW) (Thermo Fisher Scientific) was used for CFTR mRNA expression detection, and *GAPDH* TaqMan primer (Hs99999905_m1) (Thermo Fisher Scientific) was used as the endogenous control. The delta-delta Ct ($2-\Delta\Delta Ct$) method was used for the CFTR mRNA quantification.

Immunocytochemistry analysis

After three washes in DPBS, the treated or untreated cells were fixed in 4% paraformaldehyde (Thermo Fisher Scientific) and permeabilized in 0.5% Triton X-100 (Merck). Afterward, the cells were blocked in 3% BSA (Merck). For the detection of CFTR protein, the primary mouse anti-human CFTR UNC-570 antibody (University of North Carolina, NC, USA) was diluted to 1:200. The AlexaFluor 568-labeled secondary goat anti-mouse IgG (H + L) antibody (Thermo Fisher Scientific) was diluted to 1:800. Cell nuclei were stained with DAPI (Abcam) and imaged using an Olympus IX83 fluorescence microscope.

Flow cytometry analysis

Living cells were detached through trypsinization. The mean fluorescence intensity (MFI) and GFP-positive cells were quantified by flow cytometry (Beckman Coulter Life Sciences, Indianapolis, IN, USA). GFP fluorescence was detected at 525 nm using a 525/40-nm band-pass filter. For the CFTR protein detection, the live cells were incubated for 30 min with mouse anti-human CFTR antibody (CFTR/1643) (Alexa Fluor 647) at a 1:150 dilution (Novus, St. Louis, MI, USA). CFTR fluorescence was detected at 660 nm using a 660/10 nm band-pass filter. For each run, 1×10^4 cells were counted. Afterward, cells were analyzed by flow cytometry using CytoFLEX Flow Cytometer.

Short-circuit current (Isc) measurement and analysis

Cells were cultured forming monolayers on Millicell inserts (Merck). After 48 h post-transfection, they were ready to be mounted in the Ussing chamber (Physiologic Instruments, Reno, NV, USA) in KBR buffer (135 mM NaCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 2.4 mM K_2HPO_4, 0.6 mM $KHPO_4$, 10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid [HEPES], and 10 mM dextrose, pH 7.4).[47] By using chloride-depleted KBR buffer to replace the normal KBR buffer on the apical side, the monolayer was exposed to a decreasing basolateral-to-apical chloride gradient environment.[47] After adjusting for voltage offsets, the transepithelial voltage was clamped at 0 mV, and measurements of the current and resistance were recorded using the Acquire and Analyze software package (Physiologic Instruments). The transepithelial resistance of CFBE41o- cells and 16HBE14o cells were $169.3 \pm 6.37\ \Omega \times cm$ and $497.9 \pm 7.24\ \Omega \times cm^2$. Short-circuit current (*Isc*) measurements were performed in the presence of amiloride (100 μM) (Merck), forskolin (10 μM) (Merck) and CFTRinh-172 (20 μM) (Merck) to the apical part sequentially. The process involved the elimination of current produced by the epithelial sodium channels (ENaC) through amiloride, followed by stimulation with forskolin to induce chloride efflux and subsequent inhibition of CFTR channel opening with a CFTR inhibitor. These steps led to current flowing and changing across the CF apical monolayer.

Statistical analysis

The mean ± standard deviation (±SD) was reported for continuous variables. Significance was determined using Kruskal-Wallis test for multiple comparison and following Dunn's test to determine exactly which groups were different. $p < 0.05$ was considered significant ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$). GraphPad Prism 8.0 (San Diego, CA, USA) was used for statistical analysis.

Conclusions

In this work, a PAE-based vector is applied for the first time in the development of gene therapy in lung CF. Through experimental comparisons and optimizations, a series of PAE vectors with different structures were designed and synthesized. The optimal HPAE3/CpG-depleted CFTR plasmid system exhibited not only good CFTR expression but also exceptional biocompatibility. Moreover, it demonstrated partial CFTR function recovering in CF lung epithelial monolayer. These results offer a promising potential for non-viral gene therapy in the treatment of lung cystic fibrosis and thereby might open new possibilities for the treatment of lung CF disease.

DATA AND CODE AVAILABILITY

The authors confirm that the data supporting the findings of this study are available within the article and its supplemental information. Additional data are available upon request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2024.101292.

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AUTHOR CONTRIBUTIONS

Conceptualization, methodology, investigation, writing – original draft, and funding acquisition, B.Q.; writing – review & editing, Y.L., X.W.; investigation, D.M., Z.L., S.T., Z.H., J.L.; supervision, writing – review & editing, and funding acquisition, W.W.; conceptualization, supervision, writing – review & editing, and funding acquisition, I.L.-S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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