

Statistical, Block, and Gradient Copolymers Reveal Microstructure-Dependent Binding and Intracellular Delivery of Genome Editor Proteins

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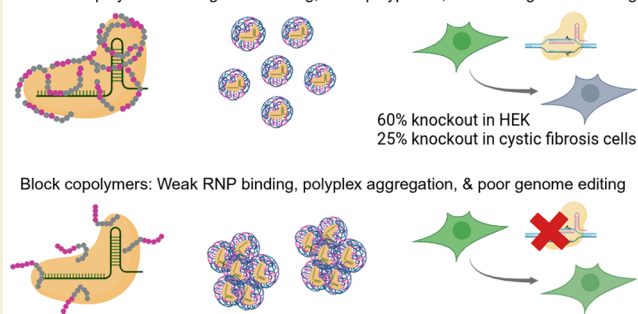
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ABSTRACT: Ribonucleoproteins (RNPs) are powerful and precise genome editor proteins that promise to cure diseases with an underlying genetic basis. However, nanocarriers such as polymers or lipids still struggle to bind, complex, and deliver fragile RNP payloads. Thus, far, polymer chemists have focused on the chemical identity rather than the spatial arrangement of repeat units to identify design rules for polymer-mediated RNP delivery. To elucidate microstructure effects in polymer-mediated RNP delivery, we synthesized compositionally equivalent, length-matched statistical, block, and gradient copolymers. Then, we compared their polymer–RNP binding affinities, polyplex sizes, and editing performance. Statistical copolymers, which are polymer ensembles with rich sequence diversity, bound RNPs more tightly and formed smaller ($R_h \sim 50$ nm) polyplexes than structurally well-defined block copolymers, which surprisingly promoted polyplex aggregation. Further, microstructure-dependent trends (statistical > gradient > block) in RNP binding strength propagated across cellular uptake and editing efficiency. For instance, in human embryonic kidney cells, statistical copolymers matched state-of-the-art commercial reagents in nonhomologous end joining (NHEJ) editing efficiency (60% at near-stoichiometric N/P ratios), whereas block copolymers performed no better than free RNPs. In human bronchial epithelial cells bearing G542X cystic fibrosis mutations, statistical and gradient copolymers exhibited robust NHEJ editing (25% for statistical copolymers S) with canonical spCas9 RNPs, suggesting that these polymers are promising for future investigations with base editor delivery. Our work reveals that RNP binding and delivery demand chemical heterogeneity along polymer backbones supplied by a statistical distribution of hydrophobic, electrostatic, and hydrogen-bonding motifs. Microstructure is a powerful yet overlooked design handle that complements traditional compositional screening to tailor bespoke polymers for diverse genome editor payloads and cellular targets.

KEYWORDS: Ribonucleoproteins, CRISPR/Cas9, polymer microstructure, gradient copolymers, block copolymers, statistical copolymers, cystic fibrosis

Statistical copolymers: Strong RNP binding, small polyplexes, & efficient genome editing



Block copolymers: Weak RNP binding, polyplex aggregation, & poor genome editing

INTRODUCTION

Genome editor proteins are powerful tools in basic biological research and in treating diseases with an underlying genetic basis (e.g., sickle cell anemia, cystic fibrosis, cancer, hypercholesterolemia).¹ Genome editor proteins usually take the form of ribonucleoproteins (RNPs), which comprise DNA-modifying editor proteins complexed with single guide RNA (sgRNA) directing editor protein activity toward target DNA sequences.² These large fragile proteins rely on synthetic (e.g., polymers and lipid nanoparticles) or biological (e.g., virus-like particles and bacteriophages) nanocarriers to enter nuclei and initiate therapeutic edits.^{3–6} Although RNP-mediated genome editing is safer and more efficient than pDNA and mRNA editors,^{7,8} RNPs face steeper intracellular delivery challenges than typical nucleic acid payloads because their biomolecular

properties impose unique constraints on nanocarriers.⁹ For instance, RNPs are highly susceptible to misfolding, imperiling the safety and site-specificity of genome editing.^{10–13} Hence, nanocarriers must maintain these fragile payloads in their catalytically competent conformations. Furthermore, both components of RNPs, editor proteins (e.g., editors such as spCas9 and next-gen editors such as base editor proteins) and sgRNA, are quite expensive to express and purify.²

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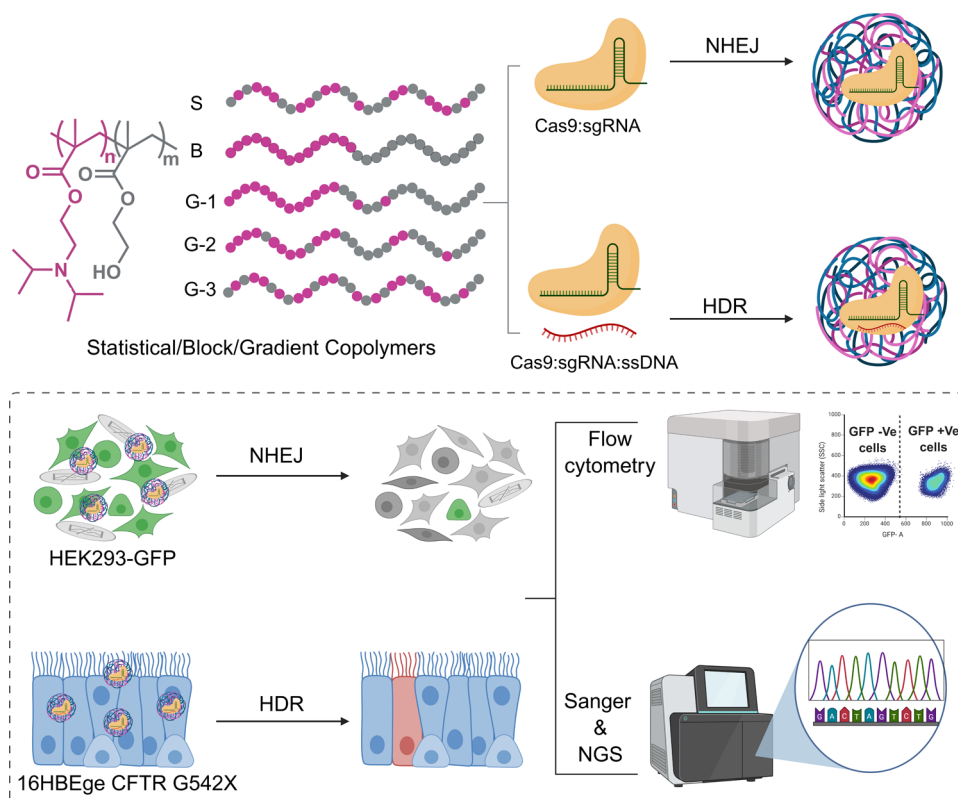


Figure 1. Compositionally equivalent and length-matched statistical, block, and gradient copolymers with varied gradient steepness were complexed with CRISPR/Cas9 ribonucleoproteins (RNPs). We asked how polymer microstructure governs RNP binding affinity, polyplex size distributions, and resistance to thermally triggered protein unfolding. We also compared editing outcomes across polymer microstructures by assessing nonhomologous end joining (NHEJ) and homology-directed repair (HDR). Created with Biorender.com.

Consequently, nanocarriers such as polymers that load RNPs efficiently¹⁴ are highly promising. To tailor polymers for diverse genome editor protein variants and cellular targets, we must first define structure–function relationships governing polymer–RNP complexation and intracellular delivery. In this work, we augment the RNP delivery capabilities of polymers by mapping how microstructure, or the spatial arrangement of cationic and neutral repeat units along polymer backbones, governs polymer–RNP complexation, RNP stabilization, and intracellular delivery.

While other delivery platforms struggle with binding and protecting these fragile RNP payloads,¹⁵ polymers excel in loading RNPs. For instance, electroporation consumes thrice as much RNP reagent as polymeric carriers.¹⁴ Biological nanocarriers such as engineered bacteriophages and virus-like particles, while promising, cannot be manufactured as easily and inexpensively as polymers.³ LNPs also encounter debilitating challenges during RNP encapsulation because they struggle to protect fragile RNPs from the denaturing effects of ethanol, an essential solvent for lipid precursors. To counter this, researchers tether RNPs to empty preformed LNPs, employing what are known as “post-loading” formulation protocols, rather than encapsulating RNPs concomitantly during LNP formation.¹⁶ Unlike mRNA payloads, which readily penetrate LNP interiors even with postloading protocols, RNP payloads tend to occupy LNP exteriors rather than cores, where they are poorly protected from rapid enzymatic degradation and aggregation.^{5,16} Recently, Doudna, Murthy, and co-workers engineered and delivered thermostable Cas9 variants that survive encapsula-

tion within LNPs without resorting to postloading.¹⁷ In contrast, polymers do not require specially engineered thermostable genome editor proteins that potentially suffer from limited editing scope and functionality. Additionally, RNPs are far more susceptible to loss of therapeutic activity than canonical nucleic acid payloads such as pDNA. Therefore, polymers must conserve delicate networks of hydrogen bonding between sgRNA and DNA-modifying proteins that maintain RNPs in correctly folded and catalytically active conformations. Misfolded RNPs may fail to discriminate between off-target and target genome sequences, compromising the site-specificity and safety of genome editing.¹¹ To avoid the deleterious consequences of RNP misfolding, polymers must preserve catalytically active protein–sgRNA conformations.

We recently demonstrated how polymer microstructure modulates pDNA binding affinity, pDNA loading per polyplex, and pDNA delivery efficiency.¹⁸ Gradient copolymers combined the cytocompatibility and colloidal stability of block copolymers and the pDNA delivery efficiency of statistical copolymers.¹⁸ However, microstructure-dependent trends in colloidal stability, payload loading, toxicity, and delivery efficiency identified from pDNA payloads may not apply in structurally dissimilar RNP payloads. Unlike double-stranded nucleic acids with high anionic charge densities, RNPs are proteins with cationic, anionic, and polar neutral amino acid residues distributed heterogeneously on their mosaic-like surfaces. Therefore, cationic polymers that condense pDNA to form polyelectrolyte complexes (polyplexes) may bind RNPs via entirely different pathways beyond

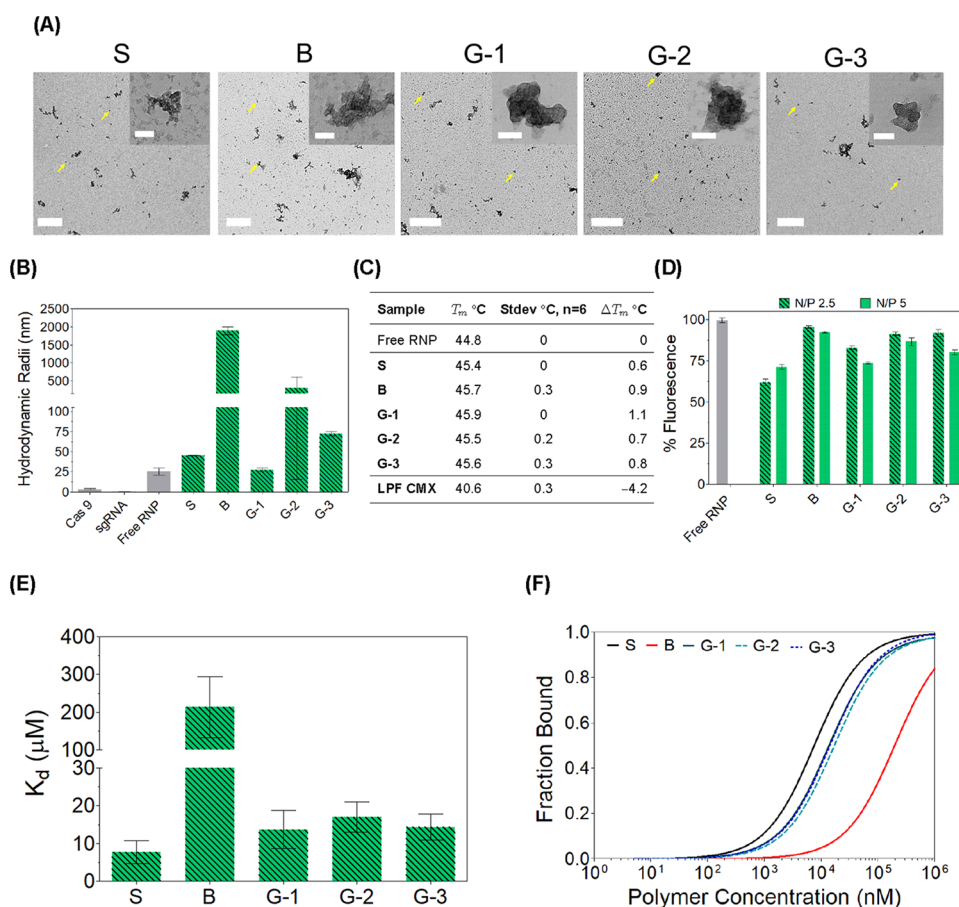


Figure 2. Polymer microstructure modulates polyplex physical properties. (A) **B** formed aggregation-prone aspherical polyplexes whereas **S** resulted in small well-defined spherical polyplexes. **G-1**, **G-2**, and **G-3** formed polyplexes with broader size distributions. Scale bars are 1 μ m and 200 nm for the images and insets. (B) The hydrodynamic radii of polyplexes formed by **B** are larger than those of polyplexes formed by **S**. (C) The melting temperature T_m from differential scanning fluorimetry was used to evaluate RNP conformational integrity across polyplexes. Unlike polymers, commercial reagents such as LPF CMX compromised the capacity of RNPs to remain correctly folded in catalytically active conformations under heating. Differences in melting point, T_m , between polyplexes and free RNPs are tabulated. (D) Dye exclusion measurements estimated polymer–RNP association. (E) Microscale thermophoresis estimates polymer–RNP binding affinities in the form of apparent dissociation constants or K_d . **S** exhibited K_d of less than 10 μ M whereas **B** bound RNPs at least 20 times more weakly (K_d of 200 μ M). (F) Binding curve generated by monitoring the thermophoretic response of fluorescently labeled RNPs as a function of polymer concentration.

electrostatic interactions. Indeed, polymer chemists encoded nonelectrostatic binding motifs such as hydrophobic¹⁹ and hydrogen bonding (phenylboronic acid^{20–23} and guanine^{24–28}) functionalities to enhance RNP binding. Chemists have identified and incorporated amino acid-mimetic repeat units that exploit weak noncovalent interactions such as hydrophobic, hydrogen bonding, or electrostatic interactions to bind RNPs.^{29,30} Additionally, polymer chemists seek to mirror the heterogeneous charge distributions on RNP surfaces by engineering heterogeneity along polymer backbones and incorporating a mix of cationic and anionic charge residues.³¹ Engineering sequence distributions, that is, modulating the spatial distribution of anionic, cationic, and hydrophobic repeat units, will realize polymer segments complementing the surface chemistry of their RNP binding partners. Thus, far, polymer chemists seeking to engineer RNP delivery platforms have focused on exploring the compositional design space rather than microstructure, a knowledge gap we address in this paper. The well-defined structures of block copolymers are said to impart colloidal stability.³² However, we posit that chemical and electrostatic heterogeneity, hallmarks of statistical

copolymers, are essential for polymers to bind RNPs in a manner reminiscent of protein–protein recognition.

Anticipating that the spatial distribution of repeat units will modulate polymers' capacity to bind, stabilize, and deliver RNPs, we asked how compositionally equivalent and length-matched statistical, block, and gradient copolymers bind and bind RNPs, maintain RNP conformational integrity under thermal stress, and resist aggregation. Our working hypothesis is that microstructural differences will specify polymer–RNP binding affinities and editing outcomes. We address three outstanding questions in polymer-mediated RNP delivery: How do polymer–RNP binding affinity (K_d) and polyplex size compare across statistical, block, and gradient copolymers? How do we engineer polymer microstructure to augment editing efficiency while minimizing toxicity? How does the cellular internalization of RNPs vary with microstructure? To the best of our knowledge, our work is the first to demonstrate that polymer microstructure is a powerful design handle to navigate trade-offs between RNP binding affinity, delivery efficiency, and toxicity.

RESULTS AND DISCUSSION

We varied the spatial arrangement of lipophilic cations (2-(diisopropylamino)ethyl methacrylate) and hydroxylated (2-hydroxyethyl methacrylate) neutral repeat units along polymer backbones and then asked how these microstructural differences impact polymer–RNP binding affinity, resistance to aggregation and protein unfolding, and intracellular delivery (Figure 1). We synthesized compositionally equivalent, length-matched statistical (S), block (B), and gradient (G-1, G-2, and G-3) copolymers (Figure S1). First, we measured the polymer–RNP dissociation constants K_d , the hydrodynamic sizes, and the RNP melting temperatures T_m across polyplexes from five microstructurally distinct variants. Then, we compared polymers' capacity to mediate functional gene editing outcomes via nonhomologous end joining (NHEJ) and homology-directed repair (HDR) assays in human embryonic kidney (HEK) and cystic fibrosis (CF) cells, respectively. We reveal that the strength of polymer–RNP binding is strongly microstructure-dependent, with statistical copolymers exhibiting the highest binding affinity (or lowest K_d), block copolymers binding RNPs the weakest, and gradient copolymers exhibiting intermediate RNP binding behavior. Microstructural variants that bound RNPs tightly, complexed RNPs efficiently, and formed sub-100 nm polyplexes mediated genome editing most efficiently. Our work reveals the importance of tailoring the spatial distribution of cationic repeat units while designing polymers for RNP delivery.

Polymer Microstructure Governs Polyplex Aggregation Propensity and RNP Binding Strength

We first investigated whether polyplexes formed from statistical, block, and gradient copolymers diverged in their size distributions, RNP binding affinities, and capacity to maintain RNP conformational integrity under thermal stress (Figure 2). Recognizing that polyplex size dictates cellular uptake, toxicity, and immune response,³³ we assessed the hydrodynamic radii of polyplexes (Figure 2B) via dynamic light scattering (DLS). We expected B to form highly structured and well-defined core–shell polyplexes, with a complex core comprising cationic blocks and RNPs and a neutral hydrophilic corona shielding polyplexes from aggregation. Conversely, we anticipated that S polyplexes would form poorly defined aggregates due to the dispersed distribution of cationic repeat units along S polymer backbones. Surprisingly, we observed the opposite results with S forming well-defined polyplexes sized less than 50 nm in radius whereas B polyplexes aggregated into 1–2 μm structures. G-1, G-2, and G-3 polyplexes straddled these extremes, spanning a broad range of sizes (70–500 nm). While polyplexes formed from gradient copolymers were larger than S polyplexes, they did not aggregate as severely as B. To confirm these microstructure-dependent trends in polyplex size (Figure 2A and Figure S3), we performed transmission electron microscopy (TEM). S formed spherical monodisperse polyplexes consistent with DLS observations. Gradient copolymers (G-1, G-2, and G-3) exhibited a bimodal distribution of sizes and shapes, forming both small spherical polyplexes sized around 50 nm as well as nonspherical irregular aggregates, explaining the wide variation observed in DLS for gradient copolymers. We note that DLS assumes spherical morphologies whereas TEM images show that B formed polyplexes with high aspect ratios. Overall, we see a gradual transition from small well-defined spherical polyplexes to aggregated polyplexes with

nonspherical morphologies as we go from statistical to gradient to block copolymers. S, G-1, G-2, and G-3 have a higher pK_a (6.6) than B (pK_a of 6.2). The latter has a lower degree of protonation in PBS (Figure S1), where we formed polyplexes, and is therefore prone to aggregation at a pH of 7.4 (Figure S2) due to the hydrophobic collapse of deprotonated segments. Further, B also has the highest octanol–water partition coefficient (which parametrizes hydrophobicity), among the polymers studied, due to the hydrophilic-to-hydrophobic transition of deprotonated polymer segments (Figure S1). If B polymers formed large aggregates due to polycation deprotonation and hydrophobic collapse,¹⁹ it could explain these differences in polyplex size and morphology. If RNPs associate with aggregated polymers (as is the case with B) rather than single chains (e.g., S), then resulting polyplexes would also be severely aggregated.

Next, we measured RNP complexation by performing dye exclusion assays (Figure 2D). Because OliGreen was developed for single-stranded oligonucleotides, this assay may not completely capture binding interactions between RNPs and polymers. OliGreen measurements for polymers and commercial reagents alike (Figure S5) must therefore be interpreted with caution. Although we cannot obtain absolute values of RNP encapsulation efficiency, this assay is still a useful high-throughput tool to reveal broad trends in polymer–RNP binding for compositionally diverse, combinatorially designed polymer libraries.³⁴ Because our five microstructural variants have comparable composition, we did not expect to observe striking differences in polymer–RNP association via OliGreen. Yet, S polyplexes recorded a 38% decrease in fluorescence compared to free RNPs whereas B exhibited the least OliGreen displacement (5% decrease).

Given the limitations of OliGreen, we measured polymer–RNP binding affinity via microscale thermophoresis (MST), a binding assay that approaches isothermal titration calorimetry in its precision and sensitivity.³⁵ Microscale thermophoresis calculates dissociation constants or K_d , with lower K_d values signifying stronger polymer–RNP binding affinities. While S exhibited an average K_d of less than 10 μM , B bound RNPs much more weakly, with a K_d of 200 μM (Figure 2E). G-1, G-2, and G-3 had intermediate binding affinities, with dissociation constants of 10–20 μM . From the binding curves (Figure 2F), it is evident that gradient copolymers occupy binding states intermediate between statistical and block copolymers. Overall, MST measurements establish that microstructure governs polymer–RNP binding strength.

Statistical copolymers, polymer ensembles comprising diverse sequence distributions, mimic the chemical heterogeneity of genome editor proteins, which display anionic, cationic, and polar amino acid residues.³⁶ Previous work has indicated that matching the spatial distribution of charged, hydrophilic, and hydrophobic segments in polymers to the amino acid residues of proteins may promote polymer–protein binding.^{37,38} We speculate that S polymers may contain a higher proportion of segments that mirror RNP surfaces purely due to sequence diversity in their ensemble, augmenting their RNP binding affinity. B, in contrast, consists of uniform polymer populations with well-separated blocks of cationic and hydrophilic repeat units. Not only is sequence diversity entirely absent in B, but B's cationic and hydrophilic segments are larger than complementary patches on proteins, resulting in poor RNP binding.³⁹

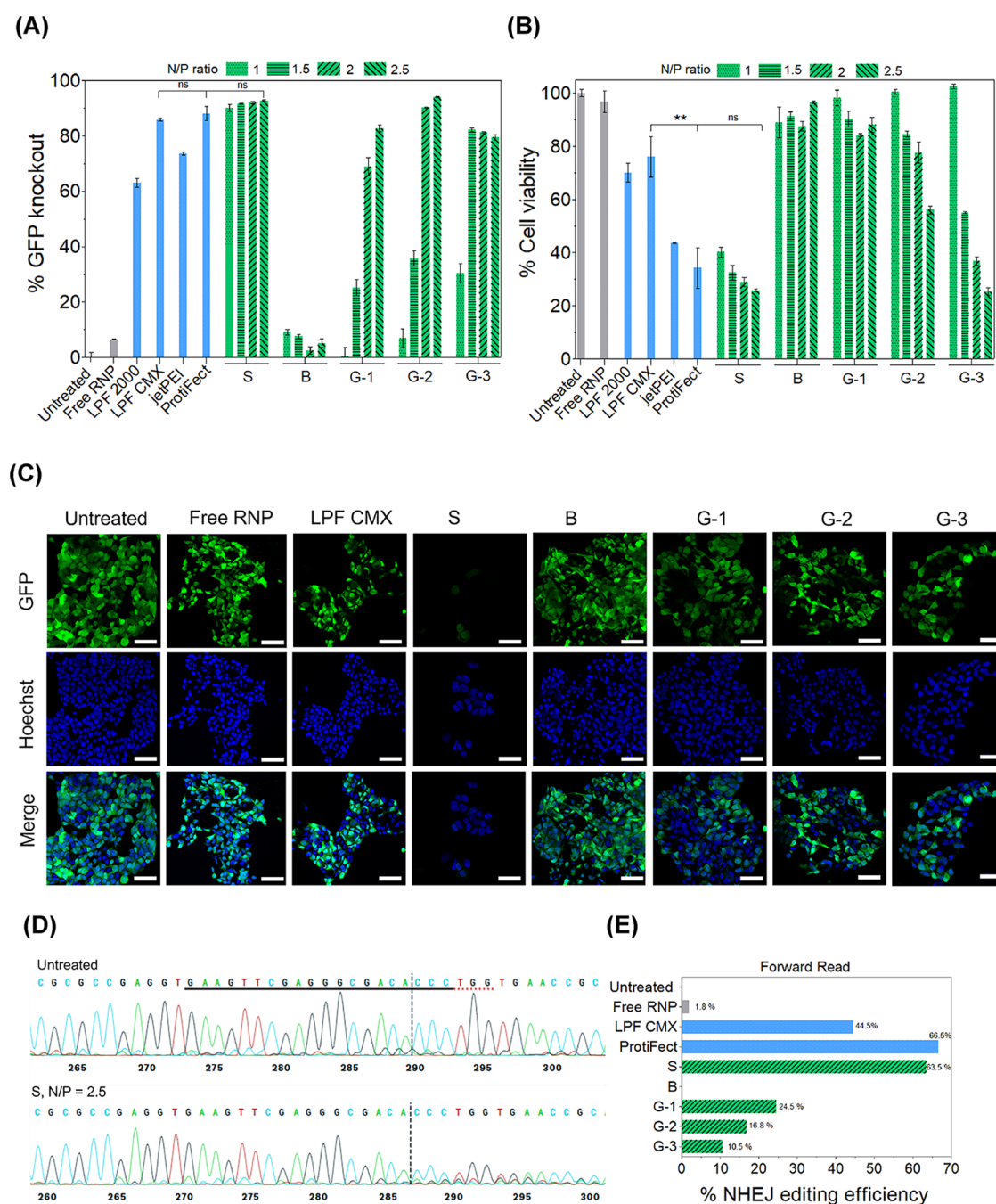


Figure 3. Polymer microstructure governs editing efficiency. (A) To quantify editing efficiency, we measured GFP knockout in HEK293-GFP cells via flow cytometry. Statistical copolymers **S** and some gradient copolymers, **G-1** and **G-2**, achieved editing efficiencies comparable to those of commercial protein delivery reagents (LPF CMX and ProtiFect). In contrast, editing performance of RNPs delivered by block copolymers **B** was indistinguishable from free RNPs (ns denotes $p \geq 0.05$). (B) In contrast to **S**, gradient copolymers realized high editing efficiencies while still maintaining cell viability (* * denotes $p \leq 0.01$). (C) Fluorescent micrographs further confirm GFP knockout trends. Scale bar is 50 μm . (D) Representative chromatograms from Sanger sequencing depict insertions and deletions stemming from nonhomologous end joining (NHEJ). (E) NHEJ editing efficiency estimates from Sanger sequencing mirrored trends from GFP knockout.

Finally, we performed differential scanning fluorimetry (DSF) to determine whether complexation with polymers modified the melting temperature of RNPs. We were interested in seeing whether polymers like **S** with high RNP binding affinities stabilized RNPs under thermal stress more effectively than weaker-binding polymers like **B**. During DSF, polyplexes (or free RNPs) are gradually heated in the presence of a hydrophobic dye (Figure S4). As RNPs unfold under heating, dye molecules bind to exposed hydrophobic amino

acid residues and become fluorescent. The inflection point of the fluorescence curve represents the melting temperature (T_m), which has been previously used to compare the thermostability of engineered Cas9 variants.¹³ In our work, we apply T_m measurements to assess the capacity of microstructural variants to delay the onset of RNP unfolding under thermal stress (Figure 2C). Free RNPs had a melting point of 44.8 °C whereas RNPs encapsulated within commercial lipid-based reagents exhibited a lower T_m of 40.6

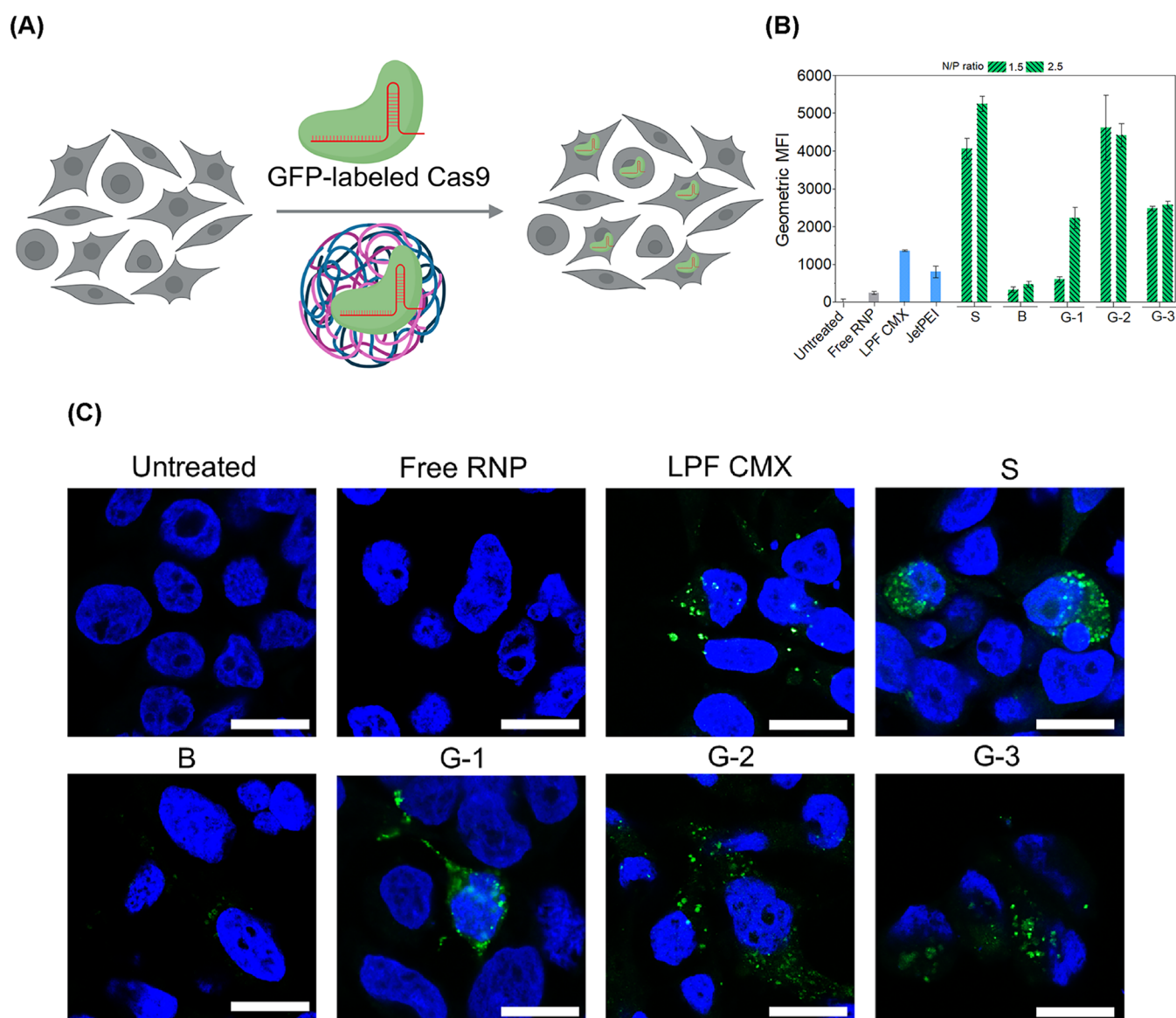


Figure 4. Differences in cellular uptake of RNPs underlie microstructure-dependent editing efficiency disparities. (A) We employed fusion proteins of spCas9 and green fluorescent protein (GFP) to detect and quantify intracellular RNPs. Polyplexes were formed from GFP-labeled RNPs and delivered to HEK293T cells. The geometric mean fluorescence intensity (MFI) of GFP was measured via flow cytometry to quantify cellular internalization of RNPs. (B) Free RNPs (no delivery vehicle) recorded the lowest levels of internalization whereas S and G-2 achieved the highest MFI values. B exhibited internalization levels comparable to free RNPs. (C) RNPs were largely absent within cells in the case of free RNPs and B whereas RNPs appeared in perinuclear regions for S, G-1, G-2, G-3, and LPF CMX. Nuclei appeared blue (Hoechst) and RNPs appeared as punctate green spots. Scale bar is 10 μ m. Created with Biorender.com.

$^{\circ}\text{C}$, a 4 $^{\circ}\text{C}$ drop. This indicates that Lipofectamine CRISP-RMAX (LPF CMX) accelerates the onset of thermally triggered unfolding. In contrast, S, B, G-1, G-2, and G-3 increased the RNP T_m , with ΔT_m values ranging from 0.6 to 1.1 $^{\circ}\text{C}$. Unlike with LPF CMX, RNPs complexed with all five polymers maintained their conformational integrity, irrespective of the contrasting RNP binding affinities of these five polymers. Having established that polymer microstructure dictates RNP binding affinity and the size and morphology of polyplexes, we next asked whether RNP delivery efficiency is microstructure-dependent.

Statistical Copolymers Deliver RNPs Efficiently; Block Copolymers Fail

In the first section, we revealed that microstructural tuning modulated polymer–RNP binding interactions and engineered

desirable polyplex size distributions. Next, we asked whether RNP delivery outcomes are sensitive to microstructural differences and applied these results to formulate polymer design rules for genome editor proteins (Figure 3). Polymer–RNP complexes or polyplexes were delivered to HEK cells engineered with destabilized GFP genes (HEK293-GFP). We designed sgRNA sequences (Figure S6) for GFP knockout and estimated NHEJ editing efficiency by measuring the decrease in GFP expression via flow cytometry. Untreated cells and free RNPs served as negative controls. Polymers were benchmarked against commercial RNP delivery reagents such as LPF CMX and ProtiFect and commercial pDNA delivery reagents Lipofectamine 2000 (LPF 2000) and jetPEI. Additionally, we performed Sanger sequencing to analyze the frequency of

insertions and deletions (indels) to verify flow cytometry estimates of NHEJ editing efficiency.

Even at nitrogen-to-phosphorus (N/P) ratios as low as 1 and 1.5 (Figure 3A), **S** polyplexes achieved editing efficiency (90%) comparable to those of commercial RNP delivery reagents, LPF CMX and ProtiFect (80–90%). LPF 2000 and jetPEI performed modestly (60–70%) because these reagents are optimized for pDNA rather than RNP payloads. Unlike previous reports on RNP delivery with HEK293-GFP,^{27,29,40–42} we achieved 90% editing efficiency at nearly stoichiometric N/P ratios. In contrast to **S**, **B** resulted in drastically lower editing efficiencies (below 10%) irrespective of the N/P ratio used. Unlike **S**, **G-1**, **G-2**, and **G-3** exhibited N/P-dependent editing efficiencies; N/P ratios of 1 and 1.5 resulted in subpar editing efficiencies whereas at higher N/P ratios of 2 and 2.5, these three gradient copolymers approached the editing performance of **S**. Because gradient polymers have lower sequence heterogeneity than statistical copolymers, we needed an excess of free polymers⁴³ and therefore higher N/P ratios to bind and deliver RNPs. To better visualize trends observed from flow cytometry, we performed fluorescence imaging (Figure 3C and Figure S7). HEK293-GFP cells retained high GFP expression in the case of free RNPs and **B** but expressed far less GFP for **S**, **G-1**, **G-2**, **G-3**, LPF CMX, and ProtiFect, consistent with flow cytometry measurements.

S polyplexes triggered higher toxicity than **B** and gradient (**G-1**, **G-2**, and **G-3**) polyplexes (Figure 3B). This observation is consistent with our earlier work in pDNA delivery¹⁸ where we noted that the spatial distribution of cationic groups along polymer backbones dictates the frequency of polyplex interactions with cell membranes. A more dispersed distribution of cationic groups, as is the case with **S**, results in more frequent polyplex–cell contacts and greater toxicity. jetPEI and ProtiFect were somewhat toxic (30–45% viability), but LPF 2000 and LPF CMX were tolerated much better (70–80% viability). **G-1** and **G-2** balanced high editing efficiency with good viability, affirming that gradient copolymers can combine the excellent cytocompatibility of block copolymers and the exceptional editing performance of statistical copolymers.

We performed Sanger sequencing to analyze the distribution of indels (Figure 3D and Figures S13–S14). A Pearson correlation coefficient of 0.83 was observed between GFP knockout and Sanger estimates of editing efficiencies (Figure S15), indicating that flow cytometry measurements captured trends in editing efficiency. As seen in Figure 3E and Figure S12, **S** and ProtiFect showed the highest editing efficiency (64–66%) followed by LPF CMX (45%) and **G-1**, **G-2**, and **G-3** (11–25%). Together, flow cytometric measurements of GFP knockout and Sanger analysis of indel frequencies establish that careful microstructural tuning is a powerful design principle to navigate trade-offs between editing performance and cell viability. The sequence heterogeneity resulting from a statistical distribution of repeat units favors strong RNP binding and also maximizes RNP delivery efficiency.

Cellular Uptake of RNPs Explains Microstructure-Dependent Editing Efficiency Trends

Having established that polymer–RNP binding affinities and editing efficiency are governed by microstructure, we then tested whether cellular uptake, particularly polymer–cell membrane interactions that precede nonspecific endocytosis,

is also microstructure-dependent (Figure 4). Although **S**, **B**, **G-1**, **G-2**, and **G-3** all have comparable compositions and lengths, **B** exhibited substantially lower editing efficiencies than statistical and gradient analogs. Whether **B** polyplexes exhibited poor RNP delivery due to their inability to penetrate cell membranes or whether intracellular trafficking barriers hindered functional RNP delivery remained unclear. Unless polymers help RNPs overcome cellular internalization barriers, they cannot mediate functional genome editing. At the same time, RNP internalization is a necessary but insufficient condition for genome editing. Hence, we quantified the cellular uptake by forming polyplexes with GFP-tagged Cas9 (Figure 4A). After extensive washing of HEK293T cells to remove extracellularly bound RNPs and polyplexes, we measured GFP intensity associated with fluorescent polyplexes by computing the geometric mean fluorescence intensity (MFI).

The cellular internalization of RNPs, as measured by GFP MFIs, was highest for **S** (Figure 4B). Importantly, the RNP uptake mediated by **B** was extremely low, approaching the near-zero internalization levels of free RNPs. Interestingly, commercial reagents LPF CMX and jetPEI exhibited lower internalization than **S** although LPF CMX mediated efficient NHEJ editing. **G-2** was comparable to **S** whereas **G-1** and **G-3** exhibited up to 50% lower uptake. RNP uptake trends mirrored editing efficiency trends for all five microstructural variants but not for LPF CMX. This suggests that maximizing the amount of internalized RNPs alone does not boost editing efficiency; rather, downstream intracellular processing determines the fate of internalized RNPs. A larger fraction of internalized RNPs access the cytosol and then enter nuclei for LPF CMX than for **S**. Further, if **S** loses more RNP payloads to endolysosomal processing than LPF CMX, **S** will require higher numbers of internalized RNPs to achieve the same level of genome editing as LPF CMX.

When we visualized the intracellular localization of GFP-labeled polyplexes, **S**, **G-1**, **G-2**, and **G-3** partitioned between the cytosol and the nuclei whereas **B** polyplexes were scarce and could not be found within the cell volume (Figure 4C and Figure S8). Flow cytometric measurements of RNP uptake and fluorescence imaging together suggest that **B** polyplexes were indistinguishable from free RNPs. This observation that **B** fails to bind RNPs and mediate association with cell membranes is consistent with the poor RNP binding affinities observed for **B** in Figure 2E.

Among the five microstructural variants tested, internalization was highly predictive of editing efficiency, suggesting that downstream intracellular delivery barriers such as endosomal escape were less influential. Hence, we did not probe microstructure-dependent differences in endosomal entrapment via colocalization measurements. However, in future work, we will assess whether a statistical distribution of cationic repeat units is essential to perforate endosomal membranes and ensure that RNPs are released into the cytosol. We speculate that polymer design features shaping RNP binding—dispersed statistical-like distribution of charged repeat units—may also promote cellular internalization. We will investigate these questions in a later paper.

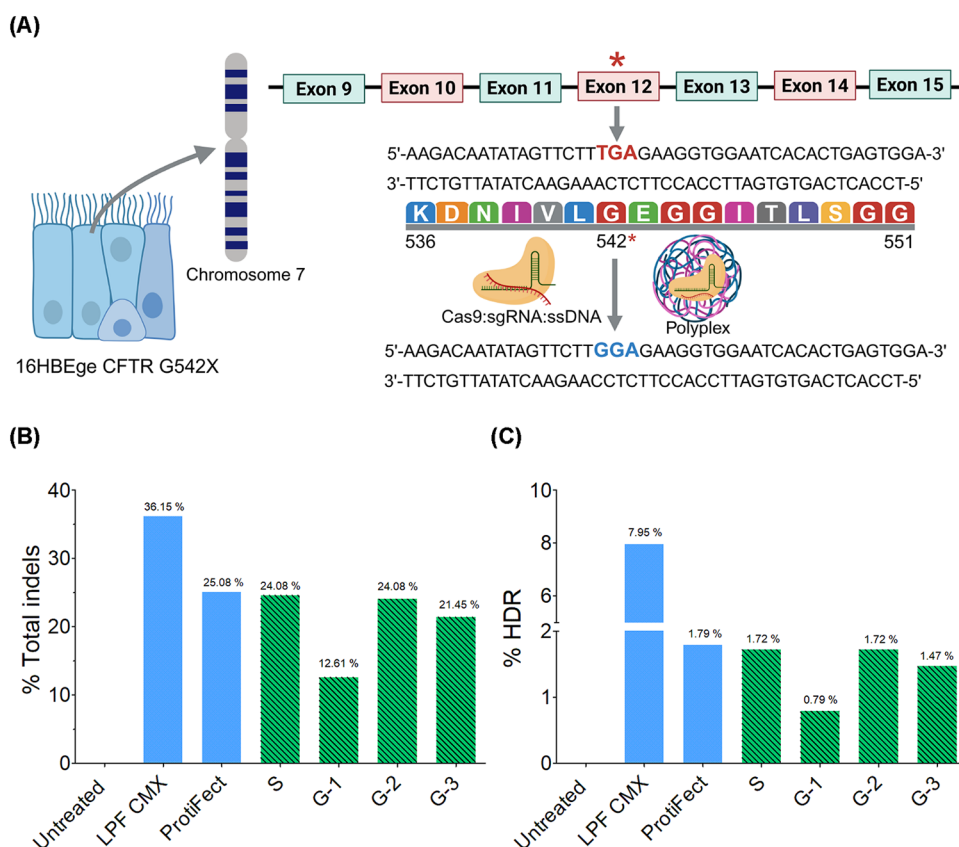


Figure 5. Statistical and gradient copolymers delivered RNPs to mediate genome editing in human bronchial epithelial cells with cystic fibrosis mutations. (A) By codelivering single-stranded DNA templates (ssDNA) along with spCas9 RNPs, we corrected cystic fibrosis mutations in exon 12 in bronchial epithelial cells (16HBEge CFTR G542X). Sequences in the donor ssDNA that are intended to block recutting by spCas9 are not shown in the schematic for simplicity. Comparison of (B) NHEJ and (C) HDR editing efficiencies evaluated via next-gen sequencing. Created with Biorender.com.

Statistical and Gradient Copolymers Mediate Robust NHEJ Editing But Suboptimal HDR in Cystic Fibrosis Bronchial Epithelial Cells

Earlier, we learned that statistical and gradient copolymers deliver RNPs efficiently to HEK293-GFP cell lines. We then asked whether these polymers can mediate functional genome editing in a more challenging disease-relevant cellular target: human bronchial epithelial (HBE) cells bearing G542X cystic fibrosis mutations (16HBEge CFTR G542X).^{44–46} Although 90% of CF patients benefit from existing CFTR modulator therapies such as Trikafta,⁴⁷ patients with rare mutations, which typically induce more severe CF phenotypes, do not benefit from these CFTR modulators. In particular, patients bearing G542X mutations (4.6% prevalence) fail to produce CFTR proteins altogether, requiring either HDR-based genome editors or adenine base editors (ABEs) to restore CFTR expression.⁴⁸ To correct G542X mutations, HDR or ABE payloads must edit TGA stop codons (Figure 5A) to prevent nonsense-mediated mRNA degradation and the truncation of CFTR proteins.^{49–51} ABEs and HDR are not the only therapeutically relevant editing modalities for CF treatment; a rarer subset of CF variants (<2%) with deep intronic mutations benefited from NHEJ-based correction.⁵² In this study, we evaluated NHEJ and HDR efficiencies since both editing pathways hold therapeutic relevance in CF (Figure 5).

NHEJ and HDR frequencies (Table S4) were measured via next-gen sequencing (NGS). While LPF CMX realized the

highest NHEJ efficiency (36%), ProtiFect (25%) and S (25%) were not far behind. Gradient copolymers exhibited a broad range of editing efficiencies (12–24%) but were all lower than S. We did not test B in 16HBEge CFTR G542X cells because B recorded near-zero editing efficiencies in HEK293-GFP cells, and HBE cells are even more recalcitrant to RNP delivery. Overall, we observed promising spCas9 RNP delivery efficiencies in HBE cells with statistical and gradient copolymers that can be translated to ABE RNPs in the future.

For HDR editing, LPF CMX mediated 8% HDR editing (Figure 5C), comparable to what was obtained by Doudna and co-workers.¹⁷ The HDR frequencies of statistical (1.7%) and gradient (0.8–1.7%) polyplexes were comparable to ProtiFect (1.8%). Even extremely low frequencies of CFTR gene knock-in (HDR as low as 2%) can realize therapeutically relevant CFTR protein expression levels and mediate phenotypic rescue of G542X mutations.⁵¹ In contrast to NHEJ, where polymers need to bind and deliver RNPs alone, efficient HDR editing requires polymers to cocomplex and codeliver RNPs with ssDNA templates. Suspecting that poor ssDNA complexation was responsible for low HDR editing by polymers, we performed OliGreen assays (Figure S5B) and observed lower complexation of HDR payloads for polymers compared to LPF CMX. However, dye exclusion assays cannot determine whether competitive displacement of RNP payloads by ssDNA played a role in undermining HDR editing. To initiate HDR pathways, polyplexes must realize sufficiently high nuclear concentrations of both RNP and ssDNA and co-

localize both payloads at the site of DNA cleavage. We suspect that these conditions cannot be realized by highly dynamic nanoassemblies such as polyplexes where small nucleic acids such as ssDNA readily hop from one polyplex to another. In contrast, enveloped nanocarriers such as LPF CMX are more likely to co-complex and co-deliver ssDNA with RNPs and present both payloads together at cleavage sites. To improve HDR rates, we must covalently tether RNPs to ssDNA, synchronize RNP delivery with the cell cycle to promote HDR during the synthesis and second growth phases of the cell cycle, or use HDR-promoting drugs.^{53,54} Further, we neither optimized the total nucleic acid dose nor the molar ratio of ssDNA to RNP so further improvements may be observed when we increase the total dose beyond 1 μ g and depart from a 1:1 ssDNA to RNP ratio.^{50,55,56}

Although **S** realized modest HDR efficiencies ($\sim 1.7\%$) that are slightly below knock-in rates required for therapeutically beneficial CFTR expression (2%), we observed high ($\sim 25\%$) indel rates via NHEJ (Figure 5B). Clearly, cellular uptake and endosomal escape do not hinder HDR payload delivery; otherwise NHEJ efficiencies would also be depressed. Instead, ssDNA donors may not be shuttled to the nuclei either due to poor polymer–ssDNA binding and subsequent DNA degradation. Given that **S** delivers CRISPR/Cas9 RNPs to CF epithelial cells to mediate NHEJ for roughly 1 in 4 cells, these polymers should be tested for adenine base editor RNPs to restore function in G542X variants.

LIMITATIONS

The gradient copolymers synthesized in this study, **G-1**, **G-2**, and **G-3**, were prepared via semibatch polymerization with a linear monomer addition schedule, limiting gradient steepness variations.¹⁸ Future work will leverage kinetic models to design nonlinear monomer addition schedules and continuous flow polymerization to obtain fine-tuned control over gradient steepness.^{57–60} Given that statistical copolymers and gradient copolymers are polymer ensembles, it is difficult to reproduce precisely the same population distribution from one synthesis batch to the next. Even polymer batches with comparable NMR compositions, pK_a values, and molecular weights may exhibit varying biological performance due to their ensemble character. Hence, we used the same batch of prepared polymers for the entirety of the study. We anticipate that continuous flow polymerization methods may improve reproducibility from batch to batch due to superior heat and mass transfer. Cellular uptake trends explained why block copolymers exhibited poor editing efficiency. However, to establish whether endosomal escape governs differences in editing performance between statistical and gradient copolymers, we need to perform endosomal escape assays such as split-luciferase endosomal escape quantification (SLEEQ).⁶¹ Typically, intracellular pathways are identified via fluorescence microscopy assays that map organelle distribution or via endocytosis inhibition assays that use small molecule drugs. For protein payloads, neither approach is sufficiently sensitive. Unlike these qualitative indirect assays, SLEEQ measurements would have quantified endosomal escape efficiency across all five copolymers. Intracellular delivery of ABE RNPs to bronchial epithelial cells (16HBEge CFTR G542X) and base editing efficiency measurements were not performed due to the high costs of ABE proteins. Finally, we lacked the capability to verify disease rescue and CFTR functioning after polymer-

mediated genome editing via electrophysiological measurements such as transepithelial electrical resistance.⁵¹

CONCLUSIONS

Ribonucleoproteins (RNPs) require bespoke polymeric delivery platforms that are attuned to their fragility, chemical heterogeneity, and unique binding parameters. While other researchers have identified composition-based design rules for polymeric RNP carriers, microstructure has long been marginalized as a design principle. In this work, we report a systematic head-to-head comparison of how compositionally equivalent, length-matched statistical, block, and gradient copolymers bind, stabilize, and deliver spCas9 RNPs to potentiate NHEJ and HDR editing.

Statistical copolymers had the highest binding affinity (K_d lower than 10 μ M) for RNPs while also forming spherical well-defined sub-100 nm polyplexes. In contrast, block copolymers bound RNPs weakly (K_d around 200 μ M) and formed micron-sized aggregates. Gradient copolymers had intermediate binding strengths (K_d of 10–20 μ M) and sizes (70–500 nm). All five microstructural variants conserved RNP conformational integrity under thermal stress, as indicated by higher melting temperatures for polyplexes than free RNPs. Statistical copolymers mediated NHEJ editing most efficiently, with GFP knockout comparable to what was achieved with commercial protein delivery reagents. Block copolymers had near-zero editing efficiencies whereas gradient copolymers achieved gentle yet efficient RNP delivery. Statistical copolymers, which have a more dispersed distribution of lipophilic cations along their polymer backbones, maximized RNP complexation as well as polyplex–cell contacts, culminating in efficient RNP internalization by cells. Block copolymers had the lowest levels of RNP internalization. Internalization trends were similar to microstructure-dependent trends in RNP delivery efficiency, explaining why statistical and gradient copolymers outperformed block copolymers. Finally, in human bronchial epithelial cells with G542X cystic fibrosis mutations, statistical copolymers achieved HDR rates slightly below therapeutically beneficial levels but mediated robust NHEJ editing. We expect that statistical and gradient copolymers will mediate efficient delivery of adenine base editors such as ABE8e RNPs for correcting G542X mutations in cystic fibrosis. We are currently exploring how modulating sequence distributions within statistical copolymers can augment RNP delivery. Our work establishes polymer microstructure as a critical design variable for binding, stabilizing, and delivering RNPs, particularly for therapeutic applications such as cystic fibrosis.

METHODS

Materials

2-hydroxyethyl methacrylate ($\geq 99\%$); 2-(diisopropylamino)ethyl methacrylate (97%); and V501-initiator 4,4'-azobis(4-cyanovaleric acid) ($>98\%$) were obtained from Sigma-Aldrich (St. Louis, MO). Chain transfer agent 4-cyano-4-((ethylthio)carbonothioyl)thio-pentanoic acid (95%) was obtained from AmBeed (Arlington Heights, IL). *N,N*-Dimethylformamide was purchased from Spectrum Chemicals (New Brunswick, NJ). Monomers were purified through basic alumina columns (Sigma-Aldrich). spCas9 was obtained from Aldevron (Fargo, ND). sgRNA GFP (S1 and S2) and sgRNA G542X (Table S1) were procured from Synthego, CA. Both spCas9 and sgRNA stock solutions were further diluted in PBS (1 $\times$) for polyplex formation. Lipofectamine CRISPRMAX Cas9 delivery reagent,

Lipofectamine 2000, Opti-MEM (reduced serum media), heat-inactivated fetal bovine serum (FBS), Dulbecco's modified Eagle medium (DMEM), minimal essential medium (MEM), Ultrapure DNase/RNase-Free Distilled Water, premium FBS, 0.1 mM MEM nonessential amino acids, penicillin-streptomycin (100 $\times$), and antibiotic-antimycotic were purchased from Thermo Fisher. ProtiFect STAR protein delivery reagent was purchased from Novus Biologicals, CO. jetPEI, HTS DNA (catalog #89129–916) was acquired from Polyplus. Calcein Deep Red AM ester was purchased from AAT Bioquest (Pleasanton, CA). Bovine collagen solution type 1 was purchased from Advanced Bio Matrix, Carlsbad, CA. LHC-8 Basal medium, bovine serum albumin 7.5%, Fibronectin Human plasma 1 mg/mL, Phosphate-Buffered Saline (PBS, 1 $\times$), FluoroBrite DMEM, TrypLE express, and Hoechst 33342 Solution were procured from Thermo Fisher. Pierce 16% Formaldehyde (w/v), ProLong Glass Antifade Mountant, Triton X-100 (1%), and RNaseZap RNase Decontamination Solution were purchased from Invitrogen, Thermo Fisher. QuickExtract DNA extraction solution was purchased from Lucigen (WI). AccuPrime Taq DNA Polymerase kit was ordered from Thermo Fisher and primers (Table S2) from Integrated DNA Technologies (IDT, Skokie, IL). Monarch DNA gel extraction kit was obtained from New England Biolabs, MA. Cell counting kit (CCK-8) was from Dojindo Molecular Technologies. Cas9-GFP Protein (Catalog no. CAS9GFPPRO-250UG) was purchased from Millipore Sigma.

Polymer Synthesis and Characterization

Statistical (S), block (B), and gradient copolymers (G-1, G-2, and G-3) were synthesized via reversible addition–fragmentation chain transfer (RAFT). Briefly, all the polymerization reactions were carried out using the Carousel 12 parallel synthesizer (Heidolph, Wood Dale, IL). All the polymerization reaction mixtures were degassed, heated at 80 $^{\circ}\text{C}$, and stirred at 200 rpm in inert conditions (nitrogen) for the duration of the reactions. The detailed procedure of each reaction condition was reported in our previous work.¹⁸ The synthesized copolymers were purified via a dialysis membrane with an MWCO of 3.5 kDa (Repligen, Waltham, MA) at pH 5.5. Dialysis media was exchanged twice daily for three to 5 days. Then copolymers were lyophilized and stored at 4 $^{\circ}\text{C}$. All the synthesized copolymers were characterized by NMR spectroscopy (JEOL ECA 500, JEOL; Peabody, MA) and size exclusion chromatography (SEC-MALS, Wyatt; Santa Barbara, CA) to characterize differences in microstructure, composition, and molecular weight distribution. The pK_a values of synthesized polymers were measured by Orion Star T910 pH titrator (Thermo Fisher Scientific; Waltham, MA). We caution the reader that statistical and gradient copolymers are nearly impossible to reproduce perfectly from batch to batch because they are ensembles. It is advisable to synthesize large quantities of statistical or gradient copolymers for a study like this. For this paper, we synthesized S with DMSO as the solvent instead of DMF (which was used as solvent in Lawson et al.¹⁸) When we switched solvents, we obtained a slightly higher M_n than targeted: 18.5 kDa instead of the targeted 15.4 kDa. We obtained a $\bar{D}$ of 1.13, which is well below 1.2. Satisfyingly, we obtained the targeted polymer composition of 50% cationic incorporation. We used the same set of polymers from the same synthesis batch throughout for all studies in this paper.

Polyplex Characterization

RNP Complexation. spCas9 and sgRNA were diluted with PBS (1 $\times$) and mixed at equimolar ratios and incubated for 15 min at 23 $^{\circ}\text{C}$. Polymer stock solutions were prepared at 1 mg/mL and diluted using PBS (1 $\times$) to achieve the desired N/P ratios (1, 1.5, 2, 2.5) and sterile-filtered. Immediately, all the copolymer solutions were complexed with RNPs (spCas9:sgRNA) and incubated for 45 min at 23 $^{\circ}\text{C}$. The concentration of spCas9 and sgRNA was varied according to the experimental assay performed. For intracellular RNP delivery, sgRNA and spCas9 were prepared at concentrations of 0.039 and 0.19 mg/mL, respectively. Higher concentrations were employed for polyplex physical characterization. OliGreen assays were performed to estimate RNP complexation using protocols described

elsewhere but with higher sgRNA concentrations (1 $\mu\text{g/mL}$) and polyplex volumes (200 μL).³⁴

Microscale Thermophoresis to Calculate Polymer–RNP K_d Values. Microscale thermophoresis (MST) experiments were run on the Monolith NT.115 Pico. Cas9-GFP protein was combined with a stoichiometric equivalent amount of sgRNA and diluted in 1 $\times$ PBS to 100 nM with 0.1% Tween-20 added to prevent protein sticking to the capillaries. S, B, G-1, G-2, and G-3 were diluted in 1 $\times$ PBS to 1 mM. The polymer solutions were serially diluted over 16 vials by a factor of 2 with 1 $\times$ PBS. Then, 10 μL of the RNP solution was added to each of the 10 μL polymer dilutions, resulting in a final RNP concentration of 50 nM and polymer concentrations ranging from 0.5 mM to 15 nM. All 16 concentrations were loaded into standard monolith label-free capillaries. Using the software MO.AffinityAnalysisv2.3, a binding affinity measurement was conducted at 40% MST power and 40% excitation power of the blue laser. Binding curves were generated by dividing the hot fluorescence reading (interval from 1.5 to 2.5 s) by the cold fluorescence reading (interval from -1 to -0.5 s), then plotting these values as a function of polymer concentration. The selection of an early time frame for the hot fluorescence reading prevented aggregation (seen at higher times) from altering the calculation of K_d . PALMIST software was utilized to globally fit a binding curve to triplicates from each polymer group, yielding an estimated K_d with a ± 1 μM uncertainty.⁶²

Differential Scanning Fluorimetry. Different protocols were optimized for polymers and positive controls (LPF CMX). DSF buffer was prepared using Tris base (20 mM), KCl (150 mM), MgCl_2 (5 mM), and DTT (1 mM) at pH 7.5.

spCas9 and sgRNA were dissolved separately in DSF buffer to achieve a 5 μM concentration. Next, sgRNA was slowly added to the spCas9 solution and incubated at 23 $^{\circ}\text{C}$ for 10 min. Polymer dilutions were prepared at desired concentrations in DSF buffer and mixed with equal volumes of RNP solutions (2.5 μM) to achieve an N/P ratio of 2.5. After 45 min, Invitrogen SYPRO Protein Gel Stain was diluted to 100 $\times$ in DSF buffer and then added to the polyplexes at a final concentration of 20 $\times$. 25 μL was transferred to each well (96-well Alkali Scientific ProCycle Clear Roche 480 Light Cyclers qPCR plate with a full edge). Using a Roche 480 Light Cyclers, the temperature was increased from 25 to 95 $^{\circ}\text{C}$ at a ramp rate of 0.02 $^{\circ}\text{C/s}$ in continuous acquisition mode, with 30 acquisitions per $^{\circ}\text{C}$.

6 μL of LPF CMX reagent was added to 22 μL of Opti-MEM reduced serum media (instead of 94 μL of Opti-MEM as described in the protocol). spCas9 and sgRNA were separately prepared into 5.5 μM solutions in Opti-MEM to form RNPs. The sgRNA solution was added to the spCas9 solution, and the resulting RNPs were incubated at 23 $^{\circ}\text{C}$ for 10 min. LPF CMX Cas9 Plus reagent was added to the RNPs to form a final concentration of 9% Cas9 Plus Reagent in the RNP by volume. Then, that solution was added to the previously diluted LPF CMX solution and allowed to incubate for 5 min at 23 $^{\circ}\text{C}$. Again, 100 $\times$ Invitrogen SYPRO Protein Gel Stain was added to achieve a final concentration of 20 $\times$, and 25 μL of the stained complexes were added to each well before performing the same melting protocol stated above. The same protocol was repeated using DSF buffer to dilute the RNPs and LPF CMX reagent but a more interpretable melting curve was obtained with Opti-MEM instead of DSF buffer for LPF CMX.

Dynamic Light Scattering. Polymer stock solutions (1 mg/mL) were prepared using sterile, syringe-filtered Ultrapure water. Similarly, spCas9 and sgRNA solutions were diluted from stock solutions. Briefly, 19 μL of spCas9 stock was diluted to 1 mL of PBS (1 $\times$), and 12.35 μL of sgRNA stock was diluted to 1 mL of PBS (1 $\times$). Equal volumes of diluted spCas9 and sgRNA were pipet-mixed to achieve a 1:1 molar ratio. The mixture was incubated for 10 min to form RNPs. Simultaneously, polymer stock solutions were diluted in PBS (1 $\times$) to achieve an N/P ratio of 2.5. Finally, equal volumes of diluted copolymer solutions were mixed with RNPs and incubated for 45 min at 23 $^{\circ}\text{C}$ to form polyplexes. After 45 min incubation, samples were transferred into 96-well clear-bottom glass plates. All DLS measurements were performed using a DynaPro Plate Reader III (Wyatt Instruments, CA).

Transmission Electron Microscopy. Polyplexes were formulated in PBS at a concentration of 10 ng/ μ L. After 45 min of incubation, 2 μ L of polyplexes were deposited on copper-coated Formvar grids (200 mesh) and incubated for 2 min. The excess polyplexes were removed by washing with ultrapure water for 30 s. Following that, TEM grids were stained with 2% uranyl acetate for 30 s. Finally, TEM grids were washed (3–4 $\times$) with ultrapure water to remove the excess uranyl acetate stain. All the TEM grids were dried in a vacuum desiccator overnight. The morphology of polyplexes was imaged using a FEI Talos F200X TEM (Thermo Fisher Scientific, Waltham, MA) operated at 200 kV and equipped with a field emission gun (FEG).

Biological Studies

HEK293-GFP cells with destabilized GFP were purchased from Gentarget, San Diego, CA. Cystic fibrosis cells (16HBEge CFTR G542X) were provided by the Cystic Fibrosis Foundation (CFF) through an institutional materials transfer agreement. HEK293T cells were obtained from American Type Culture Collection (ATCC). All the cell lines were revived according to provided instructions and grown in uncoated T-75 flasks using either DMEM or DMEM with 0.1 mM MEM. 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin or antibiotic-antimycotic solution were added. The cells were maintained at 37 $^{\circ}$ C/5% CO₂ in a humidified atmosphere. At periodic intervals (48 or 72 h), the cell culture media was replaced with fresh media. Cells were passaged once they attained 80–90% confluency.

HEK293-GFP Cell Culture. HEK293 cells engineered with destabilized GFP (HEK293-GFP) were revived and maintained according to instructions from Gentarget. Briefly, cells were grown in complete media containing DMEM (high glucose), 2 mM glutamine, 10% FBS, 0.1 mM MEM nonessential amino acids, and 1% penicillin-streptomycin using T-75 tissue culture flasks. Every 2 days, the media was removed, the cells were washed with PBS (1 $\times$), and the media replaced with fresh complete media. Once cells attained confluency (80%), cells were passaged in a 1:10 dilution.

Cystic Fibrosis Cell Culture. The 16HBEge CFTR G542X cells were obtained from the Cystic Fibrosis Foundation. We prepared the complete cell culture media using MEM (89%), FBS (10%), and penicillin-streptomycin (1%). T-75 tissue culture flasks were coated with 2 mL coating solution (LHC-8 basal medium, bovine serum albumin 7.5%, type 1 bovine collagen solution, and fibronectin from human plasma). Coated flasks were transferred into an incubator (37 $^{\circ}$ C/5% CO₂) for 2–3 h. After incubation, the coating solution was carefully removed from the flasks and stored at 4 $^{\circ}$ C. 16HBEge CFTR G542X cells were revived, and cells were added to the coated flasks and incubated at 37 $^{\circ}$ C/5% CO₂ overnight. The next day the media was removed, and fresh complete media was added and incubated. At regular intervals (3 $\times$ weekly), media was changed, and cell growth and morphology were monitored.

Flow Cytometric Measurement of GFP Knockout in HEK293-GFP Cells. HEK293 cells engineered with destabilized GFP (HEK293-GFP) were cultured until they reached around 80–90% confluency. Cells were detached via 0.25% trypsin and centrifuged. Pelleted cells were diluted and seeded in 24-well plates at a density of 30,000 cells per well. The cells were then incubated at 37 $^{\circ}$ C/5% CO₂ for 24 h. Polyplexes were formed at N/P ratios from 1 to 2.5 in sterile conditions and incubated for 45 min. Positive controls, Lipofectamine CRISPRMAX Cas9 delivery reagent, Lipofectamine 2000, jetPEI, and ProFect STAR protein delivery reagent, were prepared according to manufacturers' instructions. Polyplexes and positive controls were diluted in Opti-MEM (reduced serum media), added to cells, and incubated at 37 $^{\circ}$ C/5% CO₂ for 4 h. For all treatment conditions, the spCas9:sgRNA mole ratio was maintained at 1:1 and the RNP dose remained constant at 1 μ g/well. DMEM (1 mL) was added at the end of 4 h. After 24 h incubation, the media was replaced with fresh DMEM media. Three days after RNP delivery, cells were trypsinized and centrifuged for 5 min and cell pellets were collected. The cell pellets were washed with PBS and stained with 400 nM of Calcein Red viability dye in PBS at 23 $^{\circ}$ C for 30 min. The GFP knockout of CR-viable cells was quantified using

flow cytometry (Cytek Northern Lights, CA). Flow cytometry gating strategies are provided in the [Supporting Information section \(Figure S9\)](#). The NHEJ editing efficiency in all cell lines was calculated by determining the proportion of GFP-negative cells. The percentage GFP knockout was calculated by using the following formula. Here, GM_{Polyplex} is the geometric mean of GFP in polyplex-treated HEK293-GFP cells, GM_{Untreated} is the geometric mean of GFP in untreated HEK293-GFP cells, and GM_{Negative} is the background autofluorescence from regular HEK293T cells lacking the GFP gene.

$$\% \text{GFP knockout} = \left[1 - \frac{\left(\text{GM}_{\text{Polyplex}} - \text{GM}_{\text{Negative}} \right)}{\left(\text{GM}_{\text{Untreated}} - \text{GM}_{\text{Negative}} \right)} \right] \times 100 \quad (1)$$

Viability Measurements. Cytotoxicity was assessed using the CCK-8 assay. CCK-8 stock solution was diluted according to manufacturers' instructions using FluoroBrite DMEM with FBS. Three days after RNP delivery, cell culture supernatant media was removed from each well and replaced with diluted CCK-8 solutions. Cells were incubated for 3–4 h at 37 $^{\circ}$ C/5% CO₂. After incubation, 100 μ L of supernatant media was transferred into 96-well plates. Immediately, the absorbance was measured at 460 and 720 nm using a microplate reader (Synergy H1, BioTek, Winooski, VT). The background absorbance from CCK-8 was subtracted, and corrected absorbance values were normalized to that of untreated cells.

Flow Cytometry for Polyplex Uptake. The cellular uptake of polyplexes was determined using HEK293T cells. HEK293T cells were cultured using T-75 tissue culture flasks and 50,000 cells per well were seeded in 24-well plates then incubated at 37 $^{\circ}$ C/5% CO₂ for 24 h. GFP tagged Cas9 stock was suspended using Tris-buffer pH 7.4 and diluted according to manufacturers' instructions. GFP tagged Cas9 was complexed with sgRNA at 1:1 mol ratio to form stable RNPs. S, B, G-1, G-2, and G-3 polymers were mixed with RNPs to achieve desired N/P ratios (1.5 and 2.5). The polyplexes were incubated for 4 h at 37 $^{\circ}$ C/5% CO₂. Positive controls jetPEI and Lipofectamine CRISPRMAX were prepared according to manufacturers' protocols. After 4 h of incubation, 1 mL of DMEM media was added to polyplexes and cells were returned to the incubator. On the next day (24 h after RNP delivery), cells were detached, centrifuged, and pelleted. The cells were washed with 1 $\times$ PBS and incubated with Cell Lotion (Genlantis, San Diego, CA) for 20 min to remove the surface bound free GFP Cas9. After incubation, cells were centrifuged and washed with 1 $\times$ PBS. Then cells were suspended in 200 μ L of 1 $\times$ PBS with 2% FBS. The geometric mean fluorescence intensity (MFI) of GFP was calculated using flow cytometry. Gating strategies are furnished in the [Supporting Information \(Figure S10\)](#).

Fluorescence Imaging of GFP Knockout. Polyplex-mediated GFP knockout and cell morphology were visualized using laser confocal imaging. The day before cell seeding, the coverslips were placed in 24-well polystyrene dishes. The gelatin (1 mg/mL) solution was coated on the coverslips and UV sterilized overnight. HEK293-GFP cells (50,000 per well) were seeded on sterilized precoated coverslips and incubated at 37 $^{\circ}$ C/5% CO₂ for 24 h. Polyplexes were delivered as reported in previous sections. On day 5 (72 h after RNP delivery), cells were washed with PBS (1 $\times$) and their nuclei were stained with Hoechst 33342 and incubated for 15 min. After incubation, cells were washed with ice-cold PBS (1 $\times$) twice. Then cells were fixed with formaldehyde (4% v/v) for 30 min at 23 $^{\circ}$ C. Once the fixation process was completed, the cells were again washed with ice-cold 1 $\times$ PBS thrice. Then, coverslips were carefully removed from the well plates and mounted on glass slide using ProLong Glass Antifade Mountant (Thermo Fisher, MA). Excess mounting media was removed using blotting paper and the coverslips' edges were fixed with nail polish and dried at 23 $^{\circ}$ C for 2 h. The GFP expression was observed using Leica STELLARIS 5 Confocal Microscope with LIA chroic laser supply unit. Images were captured using a 40 $\times$ water immersion objective, with 405 and 488 nm solid-state lasers being used for excitation, and emission was collected with HyD spectral detectors.

The cellular uptake of polyplexes was also imaged using laser confocal imaging. The day before cell seeding, the glass coverslips were placed in 24-well polystyrene dishes. The gelatin (1 mg/mL) solution was coated on the coverslips and UV sterilized overnight. HEK293T cells (50,000 per well) were seeded on sterilized precoated coverslips and incubated at 37 °C/5% CO₂ for 24 h. Polyplexes were formed and delivered as reported in previous sections. At 24 h incubation, cells were washed with 1× PBS and their nuclei were stained with Hoechst 33342 and incubated for 15 min. After incubation, cells were washed with ice-cold 1× PBS thrice. Then cells were fixed with 4% v/v formaldehyde for 30 min at 23 °C. Once the fixation process was completed, the cells were again washed with ice-cold 1× PBS thrice. Then coverslips were carefully removed from the well plates and mounted on a glass slide using ProLong Glass Antifade Mountant (Thermo Fisher, MA). Excess mounting media was removed using blotting paper and the coverslips' edges were fixed with nail polish and dried at 23 °C for 2 h. The GFP expression was observed using Leica STELLARIS 5 Confocal Microscope with LIA chroic laser supply unit. Bidirectional imaging was enabled, and the z-step size was set to 200 nm. Images were taken using a 63× oil immersion objective. 405, 488, and 638 nm solid-state lasers were used for excitation, and emission was collected with HyD spectral detectors.

Sanger Sequencing to Analyze Editing Outcomes in HEK293-GFP Cells. After 48 h, cell culture media was aspirated and edited cells were washed with PBS. 200 μL of QuickExtract solution was added to each well and incubated for 10 min. Cell suspension was transferred into microcentrifuge tubes and vortexed for 15–30 s. After heating at 65 °C for 15 min, samples were removed from the water bath and again vortexed for 15–30 s. Samples were transferred to a preheated 98 °C bath for 4 min. Extracted DNA templates were checked for purity on a Nanodrop. Finally, the DNA templates were aliquoted and stored at –20 °C.

DNA templates were amplified by setting up PCR reactions using the AccuPrime Taq DNA Polymerase kit (Thermo Fisher) according to the manufacturers' instructions. PCR conditions were optimized by setting up a gradient PCR. The reaction mixture comprised diluted extracted DNA (200 ng to 500 ng), forward primer (FP), reverse primer (RP), nuclease-free water and Taq DNA polymerase. The following thermo-cycler program was performed: 95 °C for 2 min (melting), 55 °C (annealing) for 30 s and 68 °C (extension) for 3 min. The PCR products were checked in the agarose gel electrophoresis by casting a 1.5% agarose gel (Figure S11). SYBR Safe DNA Gel Stain was used to visualize the DNA bands. Gel electrophoresis was performed at 100 V for 60–90 min and imaged using a UV transilluminator. Bands were excised and purified using a Monarch DNA gel extraction kit (New England Biolabs, MA). Finally, purified DNA templates were eluted in Milli-Q water and submitted for Sanger sequencing (Azenta Life Sciences, MA).

Homology-Directed Repair in Cystic Fibrosis Cells. 16HBEge CFTR G542X cells were grown in cell culture flasks (T-75) and maintained until they reached 70–90% confluency. Media was removed and the cells were washed with D-PBS. Then TrypLE express was added and incubated for 5 to 10 min at 37 °C until complete cell detachment was observed. Cells were centrifuged for 3 to 5 min. Pelleted cells were diluted with complete media, counted, and 25,000 cells per well were seeded in 24-well tissue culture plates. The plates were transferred into an incubator (37 °C/5% CO₂) for 24 h for cell attachment. The next day, once the cells were attached to the culture substrates, polyplex samples were prepared for HDR editing as a three-step process. First, the RNP complexes (spCas9 and sgRNA G542X) were prepared at a 1:1 mol ratio and incubated for 10 min. Second, RNP was complexed with ssDNA at a 1:1 mol ratio and incubated for 10 min at 23 °C. Polymer solutions (1 mg/mL) were diluted according to the target N/P ratio of 2.5 using PBS and sterile filtered. Third, equal volumes of polymer solutions were carefully pipetted, mixed with equal volumes of ssDNA-RNP complexes, and incubated for 45 min. The ratio of Cas9/sgRNA/ssDNA was maintained as 1:1:1 for all the wells. Positive controls, such as ProtiFect STAR protein delivery reagent and Lipofectamine CRISP-

RMAX Cas9 delivery reagent, were prepared according to the manufacturers' instructions. All polyplexes and positive controls were diluted with reduced serum media (Opti-MEM) and added to the cell monolayer of 16HBEge CFTR G542X cells. After 4 h in the incubator, 1 mL of MEM was added to all the wells. Media changes were performed every 2 days and cells were grown and passed for 15 days.

Next-Gen Sequencing. After 15 days, edited cells were trypsinized, and DNA was extracted using QuickExtract solution. Briefly, media was aspirated from each well/plate and cells were washed with 1× PBS. After complete removal of PBS in each well, QuickExtract solution (500 μL) was added, and plates were incubated for 10–20 min. Then, plates were tapped gently to lift the cells, and a cell scraper was used to collect the undetached cells. Then cells were transferred into centrifuge tubes (2 mL). The cell suspension was vortexed for 30 s. Sample tubes were incubated at 65 °C for 20 min and vortexed for 30 s. The sample tubes were transferred to a preheated 95 °C water bath for 20 min. The purity of DNA templates was analyzed using the BioTek Take3 microvolume plate. Extracted DNA was amplified by setting up PCR reactions using corresponding primers (amplicon size: 263 bp). We used the Thermo Fisher AccuPrime Taq DNA Polymerase kit for all PCR reactions, and protocols were followed as per the manufacturers' instructions. The PCR products were purified using gel electrophoresis and ExoSAP-IT PCR product cleanup reagent (Thermo Fisher). Gel sections (Figure S16) were purified as before. The purified DNA templates were diluted and submitted for NGS analysis (Azenta Life Sciences). DNA templates were ligated with Illumina partial adapters (Forward: 5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3'; Reverse: 5'-GACTGGAGTTCAGACG-TGTGCTCTTCCGATCT-3') before being subjected to the NGS Illumina MiSeq platform, at 2 × 300 bp sequencing with 50,000 reads per sample.

Bioinformatics Analysis. CRISPR editing was evaluated at the CFTR locus (ENSG0000001626; chr7:117,465,784–117,715,971; GRCh38/hg38; transcript CFTR-001, ENST0000003084). A 263-bp amplicon was amplified using primers (forward: AGAAGGAA-GATGTGCCTTTCAAATTCAG; reverse: CAGAATATAAAGCAATAGAGAAATGTCTG) and annotated in Geneious Prime to define the CDS (positions 42–268 bp), sgRNA protospacer (GGTGAAT-CACACTGAGTGG), and the target stop-loss codon TGA (position 145, corrected to GGA) along with an HDR donor template. A CRISPR analysis window was set at positions 75–240 bp, spanning the spCas9 cut site.

Raw paired-end FASTQ files were assessed using FastQC (Table S3) within Geneious Prime. Samples with fewer than 10,000 reads or mean read length below 200 bp were excluded. R1 and R2 reads were merged to generate consensus fragments, resolving base-level errors at the cut site and removing improperly overlapping pairs. Merged reads were aligned to the annotated CFTR wild-type amplicon using the Geneious mapper (medium sensitivity, no pretrimming). Duplicate reads were removed, and only successfully aligned, nonduplicate reads were retained for downstream variant analysis.

Variants were identified within the CRISPR target window (positions 75–240 bp) at a minimum frequency of 0.1%, with translation frames assigned by CDS annotation. Six editing metrics were quantified using the "Analyze CRISPR Editing Results" function: % total indels (NHEJ), % gene knockout (frameshift/stop-gain), % knock-in (HDR-templated corrections), % effective knock-in, overall CRISPR editing efficiency (NHEJ + HDR), and % TGA → GGA conversion.

Statistical Analysis

Significance testing was performed by one-way ANOVA (Tukey multiple comparison) using GraphPad Prism. Measurements are reported as average ± standard deviation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acspolymersau.6c00169>.

Polymer characterization data, supplemental TEM data, supplemental DLS data, supplemental DSF data, notes on single guide RNA design and optimization, list of primers and single guide RNA sequences, gel electrophoresis snapshots, supplemental Sanger sequencing, next-gen sequencing, flow cytometry data, and supplemental fluorescence micrographs (PDF)

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Notes

The authors declare no competing financial interest.

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