Quinine copolymer reporters promote efficient intracellular DNA delivery and illuminate a protein-induced unpackaging mechanism

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Materials.

Synthesis. The monomers 2-hydroxyethyl acrylate (HEA), N-hydroxyethyl acrylamide (HEAm), N,N-dimethylacrylamide (DMAm), N-isopropylacrylamide (NIPAm), and 2-(dimethylaminoethyl) methacrylate (DMAEMA); the chain transfer agent 4-cyano-4-[(dodecylsulfanylthiocarbonyl)sulfanyl]pentanoic acid (CDP); the initiators azobisisobutyronitrile (AIBN) and 4,4'-azobis(cyanovaleric acid) (V-501); and the endocytosis inhibitors amantadine hydrochloride, filipin III from *Streptomyces filipinensis*, and 5-(N,N-dimethyl)amiloride hydrochloride (DMA) were purchased from Sigma-Aldrich (St. Louis, MO). Quinine (anhydrous, 99% total base with ≤ 5% dihydroquinine) was purchased from Alfa Aesar (Tewksbury, MA). All chemicals were used as received unless mentioned otherwise. All solvents were ACS grade. Dialysis tubing (\(M_w\) cut-off = 1 kDa) was purchased from Spectra/Por, treated with 0.1 wt % ethylenediaminetetraacetic acid (EDTA) solution, stored in ~0.05 wt % sodium azide solution, and triple rinsed with Milli-Q water before use.

Polyplex/Transfection Reagents. The pZsGreen (4.7 and 10 kb), gWiz-luc, and pCMV-lacZ plasmids were purchased from Aldevron (Fargo, ND). CCK-8 cell counting kit was purchased from Dojindo Molecular Technologies (Rockville, MD). Lipofectamine 2000, propidium iodide (1.0 mg/mL solution in water), PicoGreen (Quant-iT PicoGreen, dsDNA reagent), UltraPure ethidium bromide (10 mg/mL), methanol-free formaldehyde (16% w/v), and Pierce BCA Protein Assay Kit was purchased from ThermoFisher Scientific (Waltham, MA). Cy-5 labelled pZsGreen plasmid was used as prepared in a previous study by Tan et al.¹ Luciferase Assay System with lysis buffer was purchased from Promega (Madison, WI).

Cell Culture Reagents. Dulbecco’s Modified Eagle Medium (DMEM; high glucose, pyruvate, and Glutamax supplemented), Fluorobrite DMEM (phenol red-free media), Iscove’s Modified Dulbecco’s Medium (IMDM), Reduced Serum Medium (Opti-MEM), Defined Keratinocyte Serum-Free Media (K-SFM) with Growth Supplement, Trypsin-EDTA (0.05%) with and without phenol red, Phosphate Buffered Saline (PBS) pH = 7.4, UltraPure DNase/RNase-Free distilled water (DI H₂O) Antibiotic-Antimycotic (100×), Gentamicin/Amphotericin (500×), Heat Inactivated Fetal Bovine Serum (HI FBS), and were purchased from Life Technologies-ThermoFisher Scientific (Carlsbad, CA).

Cell Lines. Human cervical carcinoma cells (HeLa, ATCC CCL-2) were purchased from ATCC (Manassas, VA). Human embryonic kidney cells (HEK 293T) were received as a gift from the laboratory of Mark Osborne at the University of Minnesota. Chronic myelogenous leukemia (CML) lymphoblasts (K562) were received as a gift from the laboratory of Jennifer Adair at the Fred Hutchinson Cancer Research Center. Immortalized keratinocytes (N/TERT) were received as a gift from Ellen van den Bogaard at the Dermatology Department at Radboudumc, Netherlands.

Instrument Details.

Synthesis. ¹H NMR spectra used to characterize polymers were obtained on a Bruker (Billerica, MA) Avance III AX-400 MHz NMR Spectrometer with a BBO SmartProbe with 64 scans per
spectra and a relaxation delay of 10 seconds. $^1$H NMR spectra gathered to monitor polymerization kinetics were obtained on a temperature-controlled Bruker Avance III AV-500 MHz NMR spectrometer with a TBO triple resonance PFG probe with 4 scans per spectra and a relaxation delay of 1 second. Molar mass ($M_n$ and $M_w$) and dispersity ($D$) of polymers were characterized by aqueous size exclusion chromatography (SEC) with an Agilent Technologies (Santa Clara, CA) 1260 Infinity system with an aqueous mobile phase containing 0.10 M Na$_2$SO$_4$ and 1.0 wt % acetic acid and a flow rate of 0.4 mL/min, Eprogen (Downers Grove, IL) columns [CATSEC1000 (7 µm, 50 × 4.6), CATSEC100 (5 µm, 250 × 4.6), CATSEC300 (5 µm, 250 × 4.6), and CATSEC1000 (7 µm, 250 × 4.6)], and variable wavelength UV-detector. A Wyatt HELEOS II light scattering detector ($\lambda = 662$ nm) and Optilab rEX refractometer ($\lambda = 658$ nm; Wyatt technologies; Santa Barbara, CA) were used as in-line light scattering and differential refractive index detectors for SEC analysis. Astra VII software (Wyatt Technologies; Santa Barbara, CA) was used for the determination of $M_n$, $M_w$, $D$, and $dn/dc$ of polymers.

**Polyplex/Biological.** Zeta potential and dynamic light scattering (DLS) measurements (not contributing to kinetic plot) were made with a Zetasizer Nano ZS (Malvern; Worcestershire, UK) with a 4.0 mW He-Ne laser ($\lambda = 633$ nm). DLS measurements contributing to the kinetic plot were made with a DynoPro Plate Reader (Wyatt Technologies; Santa Barbara, CA). Gels in the electrophoretic mobility shift assay (EMSA) were illuminated using a Bi-O-Vision UV transilluminator ($\lambda_{ex} = 366$ nm) (Spectrolin; Westbury, NY) and photographed with a 16 MP digital camera with 28 mm lens (LG G4; Seoul, South Korea). Cell suspensions were counted with a Countess II automated cell counter (ThermoFisher Scientific; Waltham, MA) with dead cell discrimination by dilution (1:1) with trypan blue (0.4%). Percent ZsGreen expression of transfected cells were measured with a BD FACSVerse flow cytometer (BD Biosciences; San Jose, CA) with dual lasers ($\lambda = 488$ nm and 640 m), seven detectors, and analyzed using FlowJo software (Ashland, OR). Widefield fluorescence microscopy was carried out using an EVOS Digital Microscope (AMG Life Technologies; Grand Island, NY). Widefield fluorescence microscopy with deconvolution was carried out using a Zeiss TIRF Scope (Oberkochen, Germany). Fluorescence and absorbance measurements were acquired using a Synergy H1 multimode plate reader (BioTek; Winooski, VT). pH measurements were made with AB15 digital pH meter (Accumet Basic, Fisher Scientific, Pittsburgh, PA).

**Polymer Synthesis.**

*Poly(Quinine-co-HEA).* Prior to reacting, HEA was passed through activated basic alumina prior to reacting it to remove stabilizer and then passed through a PTFE filter (0.22 µm). Quinine (1.56 g, 4.80 mmol), HEA (0.836 g, 7.20 mmol), and 2,2’-azobis(2-methylpropionitrile) (AIBN) (19.7 mg, 0.120 mmol) were dissolved in 200 proof ethanol (5 mL) and mixed with a magnetic stir bar in a sealed vial. The reaction mixture was sparged with N$_2$ for 30 min, heated to 70 °C, and left to stir for 24 hours. The reaction mixture was dialyzed in 75/25 (v/v) methanol/THF (solvent replaced 4× over 48 hours) and then dialyzed in deionized water (solvent replaced 4× over 48 hours). The resulting off-white precipitate and suspension were lyophilized yielding an off-white powder.
(0.978 g, 56% yield, 13.6% quinine incorporation). (Note: yield based on molar incorporation of comonomers in copolymer). Product was characterized using $^1$H NMR (Fig. S3, Table 1) and aqueous SEC (Fig. S9, Table 1).

*Poly*(**Quinine-co-HEAm**). Prior to reacting, (HEAm) was passed through activated basic alumina to remove stabilizer, dissolved in ethanol (1 mL), and then passed through a PTFE filter (0.22 μm). Quinine (0.973 g, 3.00 mmol), HEAm (0.230 g, 2.00 mmol), and AIBN (8.2 mg, 0.050 mmol) were dissolved in 200 proof ethanol (5 mL) and mixed with a magnetic stir bar in a sealed vial. The reaction mixture was sparged with N$_2$ for 30 min, heated to 70 °C, and left to stir for 24 hours. The reaction mixture was precipitated into acetone (3×), filtered, and dried under vacuum. The resulting off-white flakes (0.226 g, 32% yield, 13.3% quinine incorporation) (Note: yield based on molar incorporation of comonomers in copolymer). Product was characterized using $^1$H NMR (Fig. S4, Table 1) and aqueous SEC (Table 1).

*Poly*(**Quinine-co-Am**). Quinine (0.973 g, 3.00 mmol), acrylamide (0.213 g, 3.00 mmol), and AIBN (9.9 mg, 0.06 mmol) were dissolved in 200 proof ethanol (5 mL) and mixed with a magnetic stir bar in a sealed vial. The reaction mixture was sparged with N$_2$ for 30 min, heated to 70 °C, and left to stir for 24 hours. The off-white suspension formed during the course of the reaction was centrifuged and washed with ethanol (3×) and then dried under vacuum. The resulting off-white flakes (0.214 g, 38% yield, 8.9% quinine incorporation) (Note: yield based on molar incorporation of comonomers in copolymer). Product was characterized using $^1$H NMR (Fig. S5, Table 1) and aqueous SEC (Table 1).

*Poly*(**Quinine-co-DMAm**). Prior to reacting, DMAm was passed through activated basic alumina to remove stabilizer. Quinine (1.95 g, 6.00 mmol), DMAm (0.595 g, 6.00 mmol), and AIBN (197 mg) were dissolved in 200 proof ethanol (10 mL) and mixed with a magnetic stir bar in a sealed vial. The reaction mixture was sparged with N$_2$ for 30 min, heated to 70 °C, and left to stir for 24 hours. After dialyzing the reaction mixture in ethanol, the dialysis solvent was gradually transitioned to ethanol/deionized water mixtures with increasing water content until all ethanol was removed. The purified product was lyophilized yielding an off-white solid (0.438 g, 32% yield, 6.5% quinine incorporation). (Note: yield based on molar incorporation of comonomers in copolymer). Product was characterized using $^1$H NMR (Fig. S5, Table 1) and aqueous SEC (Table 1).

*Poly*(**Quinine-co-NIPAM**). Quinine (0.487 g, 1.50 mmol), NIPAm (0.170 g, 1.50 mmol), and AIBN (49.7 mg, 0.300 mmol) was dissolved in DMF (6 mL) and mixed with a magnetic stir bar in a sealed vial. The reaction mixture was sparged with N$_2$ for 30 min, heated to 70 °C, and left to stir for 24 hours. One method of work-up involved dialyzing the reaction mixture in methanol and then transitioning the dialysis solvent to methanol/deionized water (1:1 v/v), and then to pure deionized water. Lyophilization of the purified product yielded a white powder (38 mg, 10% yield, 7.7% quinine incorporation). Alternatively, the reaction mixture was worked up by precipitation
in 3:1 (v/v) ether/hexanes (3×) to yield an off-white solid (50 mg, 11% yield, 21% quinine incorporation). Products were characterized by $^1$H NMR (Figure S6) and aqueous SEC (Table S1).

**Poly(DMAEMA-co-HEA).** Prior to reacting, DMAEMA was passed through activated basic alumina to remove stabilizer. DMAEMA (0.110 g, 0.700 mM), HEA (0.499 g, 4.30 mM), and AIBN (8.2 mg, 0.050 mmol) were dissolved in DMSO (4.4 mL) and mixed with a magnetic stir bar in a sealed vial. The reaction mixture was dialyzed in deionized water (solvent replaced 6× over 3 days) and lyophilized to yield an amorphous solid (0.447 g, 73.6% yield, 13.0% DMAEMA incorporation). Product was characterized by $^1$H NMR and aqueous SEC (Table 1).

**Poly(HEA).** Prior to reacting, HEA) was passed through activated basic alumina to remove stabilizer and then passed through a PTFE filter (0.22 µm). HEA (1.39 g, 12.0 mmol), and 2,2’-azobis(2-methylpropionitrile) (AIBN) (19.7 mg, 0.120 mmol) were dissolved in 200 proof ethanol (5 mL) and mixed with a magnetic stir bar in a sealed vial. The reaction mixture was sparged with N$_2$ for 30 min, heated to 70 ºC, and left to stir for 24 hours. The reaction mixture was dialyzed in 75/25 (v/v) methanol/THF (solvent replaced 4× over 48 hours) and then dialyzed in deionized water (solvent replaced 4× over 48 hours). Lyophilization of the purified product yielded a clear amorphous solid (1.24 g, 89% yield). Product was characterized by $^1$H NMR and aqueous SEC (Table 1).

**Poly(DMAEMA).** The RAFT polymerization of DMAEMA was performed according to the protocol described in a previous study.² Prior to reacting, DMAEMA was passed through activated neutral alumina to remove stabilizer. DMAEMA (5.00 g, 31.8 mmol), CPD (0.183 g, 0.454 mmol), and V-501 (12.7 mg, 0.0454 mmol) were dissolved in dimethylformamide (DMF) with a final monomer concentration of ~30 wt %. The reaction mixture was degassed via freeze-pump-thaw cycles, pressurized with argon, and polymerized at 80 ºC overnight. The polymerization was quenched by cooling the reaction mixture in an icebath. The polymer was purified by precipitation in cold hexane (3×), dissolved in benzene, and freeze dried, yielding a light-yellow powder (4.50 g, 90% yield). Products were characterized by $^1$H NMR and aqueous SEC (Table 1).
Fig. S1. Compatibility of comonomers in copolymerization with quinine. (a) The reaction scheme shows the conditions used for the free radical copolymerization of HEA and quinine with a 50/50 monomer feed ratio. (b) Other acrylate- and acrylamide-based monomers were copolymerized with quinine at 50/50 feed ratios and showed a range of quinine (Q) incorporation (%) in the final isolated polymer as determined by $^1$H NMR.
Fig. S2. Synthesis of DMAEMA and DMAEAm control polymers. Free radical copolymerization of HEA was performed with (a) DMAEMA and (b) DMAEAm. (c) Homopolymerization of DMAEMA was performed via RAFT. The methacrylate (DMAEMA) and acrylamide (DMAEAm) tertiary amine-containing monomers were used instead of the acrylate-based analogue (DAEA) because DAEA showed significant degradation due to self-catalyzed hydrolysis of the ester by back-biting of the amine.\textsuperscript{3}
Fig. S3. $^1$H NMR spectra and peak assignments of poly(quinine-co-HEA). The copolymer was dissolved in D$_6$-DMSO, and the comonomer ratio in the copolymer was obtained using the integrations from peaks corresponding to each comonomer. Ratio of HEA/quinine = $((v,j)-3.00)/2 = 6.38$
**Fig. S4.** $^1$H NMR spectra and peak assignments of poly(quinine-\textit{co}-HEAm). The copolymer was dissolved in D$_6$-DMSO, and the comonomer ratio in the copolymer was obtained using the integrations from peaks corresponding to each comonomer. Ratio of HEAm/quinine = (\textit{b,c,d,e})-\textit{f} = 4.00 = 6.57
**Fig. S5.** $^1$H NMR spectra and peak assignments of poly(quinine-$co$-Am). The copolymer was dissolved in D$_6$-DMSO, and the comonomer ratio in the copolymer was obtained using the integrations from peaks corresponding to each comonomer. Ratio of Am/quinine $= ((c,d,e)-3.00)/2 = 10.28$
Fig. S6. $^1$H NMR spectra and peak assignments of poly(quinine-cO-DMAm). The copolymer was dissolved in MeOD, and the comonomer ratio in the copolymer was obtained using the integrations from peaks corresponding to each comonomer. Ratio of DMAm/quinine = $((m,u)-5.00)/6 = 14.05$
Fig. S7. $^1$H NMR spectra and peak assignments of poly(quinine-co-NIPAm). The copolymer was dissolved in MeOD, and the comonomer ratio in the copolymer was obtained using the integrations from peaks corresponding to each comonomer. Ratio of NIPAm/quinine = ($j,v,k$)-4.00 = 11.97
**Fig. S8.** Aqueous size exclusion chromatography (SEC) trace of poly(quinine-co-HEA). Light scattering (LS) trace (red) and refractive index (RI) trace (blue) were used to determine Mn, Mw, and Đ for quinine copolymers. The UV absorption peak (λ = 280 nm) overlaps with LS/RI peaks, indicating that quinine is present in copolymer and not as a separate homopolymer or monomeric contaminant.

The following equation was used for determining the $dn/dc$ of quinine copolymers using measured $dn/dc$ of quinine and homopolymers.\(^4\)

$$
(dn/dc)_{ab} = x_a (dn/dc)_a + (1 - x_a)(dn/dc)_b
$$  \hspace{1cm} (S1)
Table S1. Structural properties of polymers used in biological studies

| Polymer            | % Cationic Repeat Units in Polymer (Molarity)a | % Cationic Repeat Units in Polymer (Mass) | % Cationic Monomer in Feed (Molarity) | M_n b [kg/mol] | D  |
|--------------------|-----------------------------------------------|------------------------------------------|--------------------------------------|----------------|----|
| poly(HEA-co-quinine) | 13.6                                          | 30.5                                     | 40                                   | 22             | 1.76 |
| poly(HEAm-co-quinine) | 13.3                                          | 30.2                                     | 60                                   | 9.2            | 1.39 |
| poly(Am-co-quinine)  | 8.9                                           | 30.8                                     | 50                                   | 9.3            | 1.39 |
| Poly(NIPAm-co-quinine) | 7.7                                           | 19.3                                     | 50                                   | 9.1 c          | 1.03 |
| Poly(DMAm-co-quinine) | 6.5                                           | 18.5                                     | 50                                   | 6.3 d          | 1.53 |
| poly(HEA-co-DMAEAMA) | 13.0                                          | 16.8                                     | 14                                   | 19 e           | 2.17 |
| poly(HEA-co-DMAEAm)  | 14.3                                          | 17.0                                     | 12                                   | 38 e           | 2.05 |
| poly(HEA)           | 0                                             | 0                                        | 0                                    | 25             | 2.47 |
| poly(DMAEAM)        | 100                                           | 100                                      | 100                                  | 11 e           | 1.02 |

a Determined via 1H NMR as shown in Figure S3-7.
b Unless otherwise noted, M_n and D was determined via aqueous SEC. The dn/dc of the copolymers was calculated using eqn S1 with measured dn/dc values of quinine (0.266) and corresponding homopolymers; pHEA (0.128), pHEAm (0.186), and pAm (0.169). The values are p(quinine-co-HEA) dn/dc = 0.170, p(quinine-co-HEAm) dn/dc = 0.186, p(quinine-co-Am) dn/dc = 0.199.
c The dn/dc value for the homopolymer p(NIPAm) in aqueous buffer (0.167) was determined from literature and used to calculate dn/dc of copolymer (0.1861) using eqn S1.
d M_n and D determined via THF SEC with an estimated dn/dc = 0.180 (polystyrene)
e The dn/dc value for the homopolymer pDMAEMA (0.174) was measured in the aqueous SEC buffer. This value was used to calculate the dn/dc of poly(DMAEAM-co-HEA) (0.135) using eqn S1, which was used as the dn/dc for DMAEAm copolymer.

Table S2. Characterization of poly(quinine-co-HEA) copolymers with varying degrees of quinine incorporation

| % Quinine in Polymer (molarity)a | % Quinine in Monomer Feed (molarity) | M_n (kDa)b | D  |
|----------------------------------|--------------------------------------|------------|----|
| 17                               | 50                                   | 15         | 1.44 |
| 14                               | 40                                   | 22         | 1.76 |
| 10                               | 33                                   | 23         | 2.71 |
| 6                                | 15                                   | 108        | 4.05 |
| 3                                | 10                                   | 42         | 4.47 |
| 0                                | 0                                    | 25         | 2.47 |

a Determined via 1H NMR as shown in Figure S3.
b Unless otherwise noted, M_n and D was determined via aqueous SEC. The dn/dc of copolymers was calculated using eqn S1 with measured dn/dc values of quinine (0.266) and corresponding homopolymer pHEA (0.128).
Reactivity Ratios.
The reactivity ratios between quinine and several acrylate/acrylamide comonomers (including HEA, HEAm, and Am) were measured by running free radical copolymerizations (1 M total comonomer concentration) with AIBN (0.01 M) in an NMR tube (0.5 mL total volume), which was sealed by a rubber septa de-gassed with N₂. The reaction was run at 70 °C in D₆-DMSO while monitoring via ¹H NMR (1 min between each measurement). The conversion of each comonomer was calculated by integrating the corresponding alkene peak and normalizing to an internal standard (aromatic proton of quinine). Copolymerizations were run at eleven different comonomer feed molar ratios (f₁) per comonomer (ranging between 0.95-0.10). The conversion of each comonomer was determined at the time of 5% total monomer conversion (Fig. S9a). It was assumed that the ratio of the comonomers consumed at 5% total monomer conversion was equivalent to the ratio of comonomers incorporated into the copolymer (F₁) at that time. For each comonomer, F₁ was plotted against f₁ and fitted with the Mayo-Lewis equation (Fig. S9b) to give the reactivity ratios (r₁ and r₂) with quinine.

Calculation of the reactivity ratios between quinine and HEA/HEAm/Am gives insight into the microarchitecture of the polymer chains. In these three copolymerizations, HEA, HEAm, and Am are defined as M₁ and quinine is defined as M₂. The reactivity ratios r₁ and r₂ are defined as a ratio of rate constants:

\[ r₁ = \frac{k₁₁}{k₁₂} \]  \hspace{1cm} (S2)

\[ r₂ = \frac{k₂₂}{k₂₁} \]  \hspace{1cm} (S3)

Here, k₁₁ is the rate constant for the addition of M₁ to a propagating chain ending with M₁ (homo-propagation) and k₁₂ is the rate constant for the addition of M₂ to the same chain end (hetero-propagation). The ratio r₂ describes the homo- and hetero-propagation rate constants for propagating chains ends consisting of M₂. The reactivity ratios can be determined by plotting f₁ against F₁ and fitting the points with the Mayo-Lewis equation:

\[ F₁ = \frac{r₁f₁^2 + r₂f₂^2}{r₁f₁^2 + 2r₁f₂f₂ + r₂f₂^2} \]  \hspace{1cm} (S4)

Here, the value F₁ is the mole fraction of M₁ in copolymer. The value f₁ is the mole fraction of M₁ in the comonomer feed while f₂ corresponds to mole fraction of M₂ in the comonomer feed.

The reactivity ratios r₁ and r₂ for all three comonomers were greater than 10 and less than 0.12, respectively, which indicate that the propagating radical chain-ends, consisting of either M₁ or M₂, prefer addition of the activated comonomer M₁ (acrylate or acrylamide) over M₂ (quinine) at all points in the polymerization. This tendency means that quinine is incorporated as isolated units statistically throughout the polymer chain (Figure S9c) and is unlikely to form quinine-rich blocks, even upon consumption of the activated monomer M₁.
Fig. S9. Determination of reactivity ratios in the free radical copolymerization of quinine with acrylate HEA and acrylamides HEAm and Am. (a) Variable-temp $^1$H NMR was used to monitor alkene peak integrations, which correlates to comonomer conversion, during radical polymerization. The conversion of comonomers was plotted over time. (b) Copolymerizations were run at eleven different comonomer feed ratios ($f_1$). The resulting comonomer ratio in the copolymer ($F_1$) was measured via $^1$H NMR and plotted above. The points for each comonomer were fitted with the Mayo-Lewis equation (see above) to give the reactivity ratios for each comonomer with quinine. (c) Representation of the resulting copolymers of quinine with HEA, Am, and HEAm.
**Potentiometric Titration.**

Poly(quinine-co-HEA) (7.50 mg) and quinine (2.30 mg) were dissolved separately in aqueous HCl (7.5 mL, 13.3 mM HCl) to afford solutions containing equal molar concentrations of quinine (0.947 mM) at pH = 2.0. While measuring the pH of the solution while stirring with a pH-probe at 23 °C, known increments of NaOH (0.01 M) were added to the solution. The pKα’s of the polymer and monomer were calculated from the derivative values of the titration curve, which corresponds to the inflection point.

![Figure S10. Potentiometric titration of poly(quinine-co-HEA) and monomeric quinine. Quinine contains a basic amine on the quinuclidine head (pKα = 8.5) as well as on the quinoline ring (pKα = 4.1). In this potentiometric titration, the pKα for the quinuclidine amine is easily observed for both the monomer (pKα = 8.5) and copolymer (pKα = 6.8), which is the only relevant protonatable amine at physiological pH. This decrease in the pKα of the cationic moiety upon incorporation polymerization is commonly observed for polycations. Although it is not easily measured via standard aqueous titration, the pKα quinoline amine, which is likely suppressed further (< 4.1), may aid in buffering of lysosomes to promote escape into the cytoplasm.](image-url)
**Polyplex Formation.**
All solutions were prepared by diluting components in DNAse/RNAse-free water, unless otherwise specified. Plasmid DNA solution in water (1.0 mg/mL) was diluted to achieve a concentration of 0.02 μg/μL. A polymer stock solution (11.0 mg/mL) was obtained by dissolving dried polymer powder into aqueous acetic acid (0.507 M). This polymer stock solution is diluted to the appropriate concentration necessary so that upon mixing with the diluted plasmid solution at equal volumes, the appropriate N/P ratio is achieved. In the case of poly(quinine-co-HEA), 0.291 μL of the polymer stock is necessary for every 1 μg of plasmid at an N/P = 1. For example, to make a polyplex sample containing 3.3 μg of plasmid at N/P = 6 for a transfection, 6.34 μL of polymer stock was diluted in 175.16 μL of water to yield an excess (1.1×) of dilute polymer solution. The dilute polymer solution (165 μL) was added slowly to the dilute plasmid solution (165 μL) and left to incubate for 30 min at room temperature prior to use.

**Electrophoretic Mobility Shift Assay.**
Polyplexes with N/P ratios ranging between 0-10 were formed with pZsGreen in water. In order to formulate the polyplexes prior to gel loading, a pZsGreen stock (50 ng/μL) was aliquoted in batches of 10 μL. Polymer solutions (>10 μL) were formulated in accordance with the protocol outlined above. Equal volume aliquots of the polymer solutions were added to the DNA solutions to form polyplex solutions (total volume = 20 μL each), which were then incubated at room temperature for 30 min. Each polyplex solution was then spiked with 2 μL of 30% glycerol solution in water prior to loading 10 μL of solution onto an agarose gel (0.6% in TAE buffer) containing ethidium bromide (0.3 μg/mL) and run at 80 V for 45 minutes. The plasmid and quinine-containing polymer within gel was imaged upon illumination by a UV transilluminator with UV-blocking cover (λ<sub>ex</sub> = 366 nm).
**Fig. S11.** Electrophoretic mobility shift assay (EMSA) of quinine copolymers with ZsGreen-encoding plasmid (4.7 kb, 0.5 µg) with increasing N/P ratios of (a) poly(quinine-co-HEA), (b) poly(quinine-co-HEAm), (c) poly(quinine-co-Am), and (d) poly(quinine-co-DMAm) as shown in Table S1. In (e), poly(quinine-co-NIPAm) contained 21% quinine by molar incorporation (derived via precipitation as discussed in Materials and Methods). For the controls in (a) and (f), the N/P ratio = 10 or contained the molar equivalence of subunits to poly(quinine-co-HEA) at N/P =10. The plasmid was visualized with ethidium bromide (orange) excited at medium-wavelength UV (312 nm). Under UV excitation, quinine and quinine-copolymers fluoresce blue (see Fig. S13) and are shown migrating towards anode.
Dynamic Light Scattering.

The hydrodynamic diameter ($d_h$) of polyplexes in water and DMEM was determined via DLS using a Malvern Zetasizer Nano ZS ($\lambda = 633$ nm) with a scattering angle of 173° at a temperature of 25 °C. Polyplexes were formed with pZsGreen in accordance with the protocol outlined above for a range of N/P ratios. The concentration of plasmid in the DLS samples, for both polyplexes in water and polyplexes diluted in DMEM (serum-free), was equivalent to those used in the PicoGreen dye exclusion studies and transfection studies. The interval of time between addition of media and sample measurement was kept constant for all samples. The mean $d_h$ was the mean Z-average diameter (calculated by the instrument) for three independent replicates.

The kinetic plot showing the change in $d_h$ of poly(quinine-co-HEA) polyplexes upon dilution in DMEM (serum-free) (Fig. 3e) was obtained using a Wyatt DynaPro Plate Reader at 25 °C. The $d_h$ of poly(quinine-co-HEA) polyplexes (N/P = 8) at time = 0 min corresponds to the mean $d_h$ of the polyplexes prior to diluting in DMEM. Upon diluting the polyplex solution in DMEM, the $d_h$ was measured repeatedly over the course of 1 hour. The measurement at each timepoint was determined by 10 acquisitions (5 sec each) with a 1 min waiting period between measurements leading to approximately 120 second intervals between measurements. The mean $d_h$ at each timepoint was determined by averaging three independent replicates.

DLS of polyplexes after addition of DMEM (w/ 10% FBS) (Fig. S12b) show far less aggregation compared to serum-free DMEM (Fig. S12a) showing inhibition of aggregation by binding of serum to polyplexes. This supports that protein directly binds to polymer (as discussed in Main Manuscript). The nearly identical aggregation of behavior of poly(quinine-co-HEA) only (without plasmid) (Fig. S12e) shows that the rapid aggregation upon addition buffered media is primarily due to the hydrophobic collapse of the polymer upon reduction protonation due to neutralization of solution pH.
Fig. S12. Dynamic light scattering (DLS) of polyplexes. (a) Polyplexes were formed from mixing poly(quinine-co-HEA) with plasmid under aqueous conditions with increasing N/P ratios. Sufficient signal was not observed for plasmid only, polymer only (N/P = 6), quinine only (N/P = 6), poly(HEA) + plasmid (mass equiv. to N/P = 6), and poly(HEA) + quinine + plasmid (mass equiv. to N/P = 6). (b) Poly(quinine-co-HEA) polyplexes were diluted (3×) in DMEM (+FBS, 10% v/v) with increasing N/P ratios. (c) Control polymers were mixed with plasmid (N/P = 6) in water and diluted (3×) in serum-free DMEM. (d) Comparison of poly(quinine-co-HEA) vs poly(quinine-co-HEA) in water and after dilution (3×) in serum-free DMEM (after ~60 minutes of incubation). (e) Poly(quinine-co-HEA) polyplexes and polymer-only after incubation in buffer containing NaHCO₃ buffer (44 mM) and dextrose (25 mM) as used in zeta potential measurements (Fig. S13). Data represented as the mean ± SD (n = 3).
**Zeta Potential.**

Zeta potential measurements of polyplexes were obtained with a Malvern Zetasizer Nano ZS at 25 °C in folded capillary cell cuvettes. Polyplexes were formed with pZsGreen in water in accordance with the protocol outlined above at equivalent concentrations. After measuring the zeta potential of the polyplexes in water, the polyplex solution was diluted (3×) in a NaHCO₃ buffer (44 mM) containing dextrose (25 mM) that was pre-incubated overnight in a 5% CO₂ environment. This NaCl-free buffer was chosen to mimic DMEM as closely as possible while maintaining the conductivity of the solution <5 mS/cm. The reported zeta potential was the mean of independent replicates (n ≥ 3).

**Fig. S13.** Zeta potential of poly(quinine-co-HEA) polyplexes in water and buffer (composition as described above). The zeta potential of the polyplexes are compared to aggregates formed from polymer only. Data represented as the mean ± SD (n ≥ 3).
**PicoGreen Dye Exclusion Assay.**

The dye exclusion assay protocol was adapted from a previous study by McLendon et al. All solutions were prepared by diluting components in DNase/RNase-free water, unless otherwise specified. Aqueous PicoGreen solution was formed by diluting the PicoGreen stock in water (1:200). Two plasmid stocks (0.02 μg/μL) were made by diluting pZsGreen with either the aqueous PicoGreen solution or water alone. Polyplexes were formed as described above for N/P ratios of 0, 1-10, 15, and 20 with both the plasmid stock containing PicoGreen well as with the plasmid stock containing no dye (to serve as a blank) and left to incubate for 30 min at room temperature. The polyplex solutions (50 μL) were dispensed into a black flat-bottom 96-well microplate using a multichannel pipettor in triplicate, and the fluorescence endpoint was measured using a microplate reader equipped with fluorescence filter cube (λ<sub>ex</sub> = 485/20 nm, λ<sub>em</sub> = 528/20 nm). The relative fluorescence of each sample was calculated by subtracting the blank (dye-free sample of the corresponding N/P ratio) and then normalizing to the polymer-free sample (N/P = 0). The polyplex solutions in the plate were then diluted 3× with phenol red-free DMEM (serum-free), and the fluorescence endpoints were taken at 5, 15, and 30 min after dilution. Subsequently, a 10% (v/v) addition of FBS (15 μL) was added to the polyplex solutions diluted with DMEM, and fluorescence endpoints were taken at 5, 15, and 30 min after dilution.
Fig. S14. Monitoring plasmid binding by quinine copolymers and controls at various N/P ratios via PicoGreen Dye Exclusion Assay. The exclusion of PicoGreen from intercalating into the plasmid is due binding of the polymer to DNA and is compaction in the polyplex. Increases in relative PicoGreen fluorescence ($\lambda_{ex} = 480$ nm, $\lambda_{em} = 520$ nm) correlate to decreased polymer-induced plasmid compaction. Serum-free cell media (DMEM) was added to polyplexes in water at concentrations identical to those performed in transfection. Fetal bovine serum (FBS, 10% v/v) was then added to the diluted polyplexes and the relative change in fluorescence was monitored. Polyplexes were at N/P = 0-10, 15, and 20 for polymers (a) poly(quinine-co-HEA), (b) poly(quinine-co-HEAm), (c) p(DMAEMA), and (d) poly(DMAEMA-co-HEA). Data represented as the mean ± SD (n = 3).
**Fig. S15.** Comparison of poly(quinine-co-HEA) and poly(quinine-co-HEAm) at an N/P = 6 showing change in plasmid decompaction through sequential dilution with DMEM and addition FBS (+10%). Data represented as the mean ± SD (n = 3).
Cell Culture.
The HeLa and HEK 293T cells were cultured in DMEM containing FBS (10%) and Antibiotic/Antimycotic (1×). N/TERT cells were cultured in supplemented K-SFM with Gentamicin/Amphotericin (1×). Cell media was supplemented with and cells were cultured in 75 cm² flasks at 37 °C under 5% CO₂ atmosphere. The cell cultures were monitored for confluency (maintained below 70%) and passaged every 2-3 days. The K562 cells were cultured in IMDM containing FBS (10%) and Antibiotic/Antimycotic (1×). Cells were cultured in 75 cm² flasks at 37 °C under 5% CO₂ atmosphere. The cell culture was kept below (1×10⁶ cells/mL) and passaged every 2-3 days.

Transfection Assay with ZsGreen Reporter Plasmid.
Transfection protocol for adherent cells. The following protocol was adapted from previous reports (ref). Twenty-four hours prior to transfection, cells were seeded in 24-well microplates with 1 mL/well of cell suspension (5.0 × 10⁴ cells/mL) in FBS-supplemented DMEM (HEK 293T and HeLa) or supplemented K-SFN (N/TERT). Just prior to the transfection, polyplexes were prepared in the manner described above. For each sample in triplicate (3 wells total), 330 μL of polyplex solution (containing 3.3 μg of plasmid at a concentration of 0.01 μg/μL) was prepared, which was left to incubate at room temperature for 30 minutes. Just prior to the addition of the polyplexes to the cells, strongly adherent cells (HeLa and N/TERTs) were washed with PBS to remove residual protein-containing media. For HEK 293T cells (which can more easily be removed via washing), the cell media was gently aspirated (without washing) prior to addition of polyplexes. After removal of the cell media/washing, the polyplex solution was diluted 3x with serum-free DMEM (all adherent cell types). Unless noted otherwise, the polyplex solution was added immediately to the freshly washed cells (300 μL/well for a total dose of 1 μg of plasmid/well) after dilution with DMEM. Lipofectamine 2000 was administered at 0.5 µg/well in accordance with the commercial protocol. 24 hours post-transfection, the media was replaced with fresh media (1ml, DMEM with 10% FBS) and the cells were placed back into the incubator. 48 hours post-transfection, adherent cells were washed with PBS, lifted from the well surface with phenol red-free trypsin (200 μL), and diluted in 300 μL of Fluorobrite DMEM (with 10% FBS). The cell suspension was
homogenized (no visible cell clumps) and 50 μL of each suspension was added to a clear flat-bottom 96-well microplate for cell viability analysis (vide infra). The remaining cell suspension was placed in Falcon tubes in order to prepare them for flow cytometry analysis (vide infra).

Transfection protocol for suspension cells. Just prior to transfection, cells were seeded in 24-well microplates with 100 μL/well of cell suspension (4.0 × 10⁶ cells/mL) in PBS. Polyplexes were prepared in the manner described above except that the dose and volume was scaled up (4×) while maintaining constant concentration. For each sample in triplicate (3 wells total), 1.320 mL of polyplex solution (containing 13.2 μg of plasmid at a concentration of 0.01 μg/μL) was prepared, which was left to incubate at room temperature for 30 min. The polyplex solution was diluted 3× with IMDM (serum-free) and then added directly to the cells (1.200 mL/well for a total dose of 4 μg of plasmid/well). The dose delivered with Lipofectamine 2000 was also scaled by 4× (2 μg plasmid total) and administered according to the commercial protocol. The cells were placed in the incubator and left to incubate with the polyplexes for 4 hours. Following this incubation, 800 μL of the polyplex solution was carefully aspirated from the top of the solution so as to not disturb the cells at the bottom of the wells. After this, 1 mL of IMDM (with 10% FBS) was gently added to the well and the cells were placed back into the incubator. After 48 hours following transfection, the cells were transferred into Falcon tubes for cell viability analysis. These cells were pelleted via centrifuge and the remaining supernatant was aspirated. The cells were resuspended in PBS, pelleted via centrifuge, and resuspended in 500 μL of Fluorobrite DMEM (with 10% FBS). A homogeneous portion of this cell suspension (50 μL) was added to a clear flat-bottom 96-well microplate for cell viability analysis (vide infra). The cell suspension remaining in the Falcon tubes was carried forward in processing for flow cytometry analysis.

Flow cytometry analysis. The cells were pelleted via centrifuge (set to 4 ºC) and the remaining supernatant was aspirated. The cells were resuspended in ice-cold PBS, pelleted via centrifuge (4 ºC), and resuspended in ice-cold PBS containing 1% FBS and propidium iodide (PI, 10 μg/mL). The samples were analyzed via flow cytometry by collecting 10,000 events per replicate. The gates for ZsGreen+ live cells (PI negative, ZsGreen positive) were set with the untreated control.

Transfection Assay with Luciferase Reporter Plasmid.
The polyplex formulation with the gWiz-luc plasmid and the transfection procedure with adherent cells (HeLa and PFBs) are identical to the protocol described above with the ZsGreen reporter plasmid. After 48 hours following the transfection, the cells were washed with PBS followed by addition of lysis buffer (100 μL) to each well. After incubating at room temperature for 10 min, an aliquot of cell lysate (5 μL) was pipetted both into an opaque white flat-bottom 96-well microplate for luciferase quantitation. The plate was placed in a plate reader equipped with an automatic injection system that added luciferase substrate (100 μL) to the lysate and measured the chemiluminescence for each well. The protein concentration for each sample was quantitated by following the manufacturer's protocol using a Pierce BCA Protein Assay Kit and standard curve made with bovine serum albumin (BSA). The mean relative light units (RLUs) for each sample,
determined from the luciferase assay, was normalized to the mean mass of protein (mg) measured for each sample.

**Fig. S16.** Transfection screens in HeLa cells comparing transfection efficiency of quinine copolymers with various hydrophilic comonomers. (a) Luciferase transfection assays showing ineffective transfection with poly(quinine-co-NIPAm), poly(quinine-co-DMAm), and poly(quinine-co-Am) (see Table S1 for polymer characteristics). (b) ZsGreen transfection assay comparing poly(quinine-co-HEA) and poly(quinine-co-HEAm) shows clear superiority of the acrylate copolymer in promoting transfection. Data represented as the mean ± SD (n = 3).

**Fig. S17.** Luciferase reporter transfection assay in HeLa cells with poly(quinine-co-HEA) with varying levels of quinine incorporation. Polypelexes were formed with the polymers outlined in Table S2 at N/P = 10. Data represented as the mean ± SD (n = 3).
**Fig. S18.** Fluorescence of quinine copolymers and its quenching by chloride. (a) The fluorescence spectra ($\lambda_{ex} = 350$ nm) of quinine was compared to those obtained with poly(quinine-co-HEA) and poly(quinine-co-HEAm) at equimolar concentrations of quinine (equivalent to the concentration of quinine moieties used in transfection). This reduction in fluorescence intensity of the copolymers is likely due to self-quenching by quinine repeat units.\(^\text{11}\) (b) A difference spectrum of the compounds before vs after addition of plasmid (N/P = 6) shows a significant decrease in the fluorescence intensity upon introduction of DNA. This reduction in fluorescence is indicative of intercalation of quinine into DNA.\(^\text{12}\) (c) Quenching of quinine fluorescence by chloride ions, under physiologically relevant concentrations, is dose-dependent for both quinine monomer and copolymers.\(^\text{13}\) The dramatic decrease in fluorescence intensity of polyplexes outside the cell vs inside the cell can be explained, in part, by the concentration gradient that exists between the extracellular environment (cell media $[\text{Cl}^-] = 110$ mM) vs the intracellular space ($[\text{Cl}^-] \sim 4$ mM).
Widefield Fluorescence Microscopy, Colocalization.

Widefield epifluorescence microscopy of HEK 293T cells, transfected with poly(quinine-co-HEA) and Cy5-labelled ZsGreen plasmid, was performed using a Zeiss TIRF scope. Three separate fluorescence channels were collected on each sample, corresponding to three fluorophores present: Cy5 ($\lambda_{ex} = 640/30$ nm, $\lambda_{em} = 690/50$ nm), ZsGreen ($\lambda_{ex} = 470/40$ nm, $\lambda_{em} = 525/50$ nm), and poly(quinine-co-HEA) ($\lambda_{ex} = 365$ nm, $\lambda_{em} = 445/50$ nm). Images were taken with dimensions of $81.92 \mu m \times 81.92 \mu m$ with z-slices of 240 nm, with an objective of 100×. Slice number in samples ranged from a minimum of 16 to a maximum of 202. Slices and image dimensions were refined when processed to exclusively reflect cellular volume. Raw images were collected using ZEN Black 2.3 sp1 (Zeiss, Stockholm, Sweden) at the University Imaging Center at the University of Minnesota. One limitation in the area of widefield microscopy is convolution from out of focus z-stacks. Widefield images contain residual noise from other image stacks, whereby the degree and location of the out of focus convolution is proportional to its point spread function. Deconvolution has been an iterative mathematical tool shown to alleviate this problem, and allow for point based image analysis. Huygens deconvolution software version:17.10.0p5 (Scientific Volume Imaging, The Netherlands) was used to perform batch deconvolution on all widefield images using the CMLE algorithm with SNR:40, Q threshold of 0.01, with a maximum iterations of 50. The Minnesota Supercomputing Institute was used to execute the necessary calculations.

Colocalization of Cy5 and poly(quinine-co-HEA) was computed using an ImageJ colocalization plugin JACoP with normalized threshold values for like channels. Colocalization was quantified as an average across all z-stacks per deconvolved sample image array (n= 4; mean $\pm$ SD) (Fig. S19). We find the Mander’s coefficient for colocalization of Cy5 to poly(quinine-co-HEA) to be $0.68 \pm 0.09$ (4 hrs) and $0.53 \pm 0.04$ (24 hrs). These Mander’s coefficients indicate significant colocalization, but it was lower than expected visually. This discrepancy can be attributed to the slight translocation caused by the switching fluorescence channels, as well as the slight time delay, as the cells analyzed were alive. Despite this, the data shows that the poly(quinine-co-HEA) successfully delivers plasmid into the cell and that the internalized polyplexes contain plasmid.
Fig. S19. Internalization of Cy5-labelled DNA and its colocalization with poly(quinine-co-HEA). (a) Three-dimensional image of live HEK 293T cells (100× magnification) 24 hrs post-transfection with poly(quinine-co-HEA) (blue) and Cy5-labelled plasmid (red) exhibiting ZsGreen expression (green). Dimensions: 81.92 µm × 81.92 µm × 3.84 µm. (b,c) Slices of z-stacked images produced with widefield fluorescence microscopy with deconvolution of live HEK 293T cells transfected at (a) 4 hrs and (b) 24 hrs prior to analysis. Component images include (i) poly(quinine-co-HEA), (ii) Cy5-labelled plasmid, and (iii) overlay image. Scale bar = 25 µm.
**Widefield Fluorescence Microscopy, Particle Size.**

*Sample preparation and image acquisition.* Twenty-four hours prior to transfection, HeLa cells were seeded (1 × 10^5 cells/well) on sterilized glass coverslips in 12-well microplates. The cells were transfected with poly(quinine-co-HEA) and pZsGreen (N/P = 8) in accordance with the aforementioned protocol (with all components scaled 2× in order to accommodate 12-well microplates). At time points of 6 and 48 hours post transfection, samples were washed with PBS and fixed by incubation with 4% formaldehyde in PBS for 10 min. The fixed samples were washed with PBS (3×) and mounted with 50/50 PBS/glycerol. Images were taken with an EVOS Digital Microscope at 4, 10, and 40× magnification under transmitted light and fluorescence with filters for DAPI (λ_{ex} = 357/44 nm, λ_{em} = 447/60 nm) and GFP (λ_{ex} = 470/22 nm, λ_{em} = 525/50 nm).

*Image-Derived Particle Sizing.* Widefield fluorescence and transmission microscopy images were taken of HeLa cells transfected with poly(quinine-co-HEA) with varying incubation periods as described above. Images were analyzed using ImageJ software, where the brightness/contrast of the images were adjusted using a linear function. Fluorescence images were then background subtracted using a rolling ball radius between 50 and 15 pixels, depending on signal to noise ratio. To determine particle cell count, the transmission images (depicting specific cell dimensions) were used in conjunction with the fluorescence images to manually tally the particles per cell. Discrimination of extracellular vs intracellular particles was aided by the increased fluorescence of intracellular particles compared to extracellular particles (see Fig. S18 for details). In addition, intracellular and extracellular particle size was analyzed. Particles outside of the cell were analyzed by thresholding background subtracted transmission images and using the “analyze particle” function built within ImageJ. Intracellular particles were analyzed by applying a color threshold to fluorescence images and using the “analyze particle” function. In all cases boundaries for particle analysis included a particle circularity between 0.50 and 0.10 with minimum particle size of 0.04 μm^2. Furthermore, a boundary of maximum particle size between 1.54 and 3.50 μm^2 was used, depending on image type, to discriminate against clusters of individual particles that could not be resolved individually.

As shown in Figure 4d-e and Figure S20-21, particle size and the number of internalized particles positively correlated to ZsGreen expression efficiency. For example, the increased mean particle diameter (780 to 1190 nm) between samples [0,5] to [30,5] led to increased transgene expression (0.1 to 5.3% ZsGreen+). In addition, increased uptake of these large particles over longer exposure times (1190 nm) from samples [30,5] and [30,30] led to nearly a ten-fold increase in transgene expression (5.3 to 52.3% ZsGreen+). Upon reaching a threshold of internalized particles at 30 minutes cell incubation (approximately 27 particles/cell), however, there is increased cellular toxicity (Fig. 4e). These data shows that particle aggregation and increased particle uptake leads to an increased fraction of transfected cells, and we quantified this physiochemical effect on the biological properties. This data corroborates similar trends see for polyethyleneimine (PEI).
Indeed, a fine balance between transfection performance and cytotoxicity in this system exists and can be tailored by controlling the incubation periods that affects both polyplex size and amounts of particles internalized by cells. The ability to tightly control particle size and number allows for differential transgene delivery to target cell populations without introducing variability into cargo load.

**Fig. S20.** Widefield fluorescent images of HeLa transfected with poly(quinine-co-HEA) with various incubation parameters. The HeLa cells were fixed 6 hrs post-transfection with poly(quinine-co-HEA) at various formulation and cell incubation times. The sample names [i,ii] are derived from (i) formulation time (Fig. 3d) and (ii) cell incubation time (Fig. 4a). Left image in each pair is an overlay of transmission and polymer (blue), while the right image is of polymer only. Scale bar = 10 um.
**Fig. S21.** Imaging analysis of poly(quinine-co-HEA) polyplexes. (a) Mean particle diameter of poly(quinine-co-HEA) polyplexes *in vitro* both in, and outside of cells at various incubation periods. (b) Dependence of incubation period on polyplex particle count determined via optical microscopy. Data represented as the mean ± SD (n = 3).

Figure S21a shows a steady increase in mean particle diameter from 0 to 30 minutes of pre-incubation. This trend is in line with DLS data and fits the hypothesis that the particles aggregate during the pre-incubation period. Furthermore, for some cases (like 0,30 and 15.5) we find that the polyplex size inside cells is smaller than the polyplex size outside of cells. This suggests, especially early on in the cell-incubation period, that there is a selective uptake mechanism that favors smaller polyplexes over larger aggregates. In addition, as shown in Figure S21b, there is a significant difference in the number of polyplexes between cell incubation periods of 5 and 30 minutes. Increasing the time that polyplexes incubate with the cells allows for a corresponding increase in the number of endocytosed particles. This finding suggests that modulating the cell-incubation period influences cellular plasmid intake, which in turn provides control over balancing cell viability and transfection efficiency.
CCK-8 Viability Assay.
After procuring all cell suspensions (50 μL each) in a clear flat-bottom 96-well microplate as described in the Transfection Method, the samples were subjected to a CCK-8 viability assay protocol adapted from the manufacturer’s instructions. Each well was spiked with 50 μL of a 5× dilution of CCK-8 reagent in phenol red-free DMEM (with 10% FBS). The samples were placed in an incubator and left to incubate for 1-3 hours depending on cell type and density. Due to settling and potential clumping of the cells, the cell suspension was homogenized by gentle pipetting (with care taken not to introduce any bubbles) prior to analysis in the plate reader. The absorption of the cell suspensions and a blank (containing all components minus cells) was taken at 450 and 650 nm. The absorption values correlating to the blank and the OD at 650 nm (the contribution to the absorption due to the light scattered by cells) was subtracted from the samples’ absorption at 450 nm. This value was then normalized to the untreated sample and multiplied by 100 to give a relative % cell viability at the 48 hours time point after transfection.

Endocytosis Inhibition Assay.
The transfection of HeLa cells in the presence of endocytosis inhibitors was adapted from the protocol above. The concentrations and cell incubation times needed for amantadine (clathrin), filipin III (caveolae), and DMA (macropinocytosis) was optimized in previous studies.2,20-23 The cells were transfected with poly(quinine-co-HEA) (N/P = 8) and pZsGreen with pre-incubation and cell incubation times (as defined in Main Manuscript) of 30 min and 15 min, respectively. Prior to adding the polyplexes to the cells, the cells were incubated in DMEM (with 10% FBS) containing either amantadine (1 mM), filipin III (1 μg/mL), and DMA (100 μM) for 1 hour, 1 hour, and 5 min, respectively. In addition, the polyplexes were diluted (2×) in serum-free DMEM containing the respective endocytosis inhibitor at concentrations equivalent to those above. After the cell incubation period, the polyplex-containing media was aspirated, the cells were washed with PBS, and 1 mL of fresh DMEM (with 10% FBS) was added to the cells. The toxicity and transgene expression of the cells were quantified 48 hours later as described above.
**Fig. S22.** ZsGreen reporter transfection screen HEK 293T with all controls. (a) Transgene expression as measured with flow cytometry after 48 hours. (b) Cell viability was measured with CCK-8 assay after 48 hours. Data represented as the mean ± SD (n = 3).
Fig. S23. ZsGreen reporter transfection screen in K562 cell line. (a) Transgene expression as measured with flow cytometry after 48 hours. (b) Cell viability was measured with CCK-8 assay after 48 hours. Data represented as the mean ± SD (n = 3).

Fig. S24. Effect of protein on transfection efficacy. HEK 293T cells were transfected with pZsGreen and poly(quinine-co-HEA) (N/P = 8). After diluting the polyplexes with serum-free DMEM, FBS was added to the mixture prior to its incubation with cells. Increasing concentrations of FBS in the transfection media led to reductions in transfection efficiency in a dose-dependent manner. Data represented as the mean ± SD (n = 3).
Examination of Hydrolytic Stability of the Hydroxyethyl Pendant Groups.

Poly(HEA) was dissolved (10 mg/mL) in several D$_2$O solutions with a range of pH values. These include ~0.1% v/v TFA (pH 2.3), unbuffered D$_2$O (pH 5.3), and PBS-buffered D$_2$O (pH 7.4). D$_2$O with NaOH (~1% w/v) (pH 13.5) served as a positive control for hydrolysis. These solutions were analyzed via $^1$H NMR immediately after dissolving (1 hr) and 72hrs later after incubation at 37°C. By monitoring the integration reduction of the methylene peak closest to the ester (4.1 ppm, 2H) in relation to the backbone protons (1.4-2.6 ppm, 3H), we could quantify the percent of pendant group hydrolysis.

Fig. S25. The dependence of poly(HEA) hydrolysis in terms of pH as determined with $^1$H NMR. a) A representative $^1$H NMR spectra of poly(HEA) in D$_2$O is shown. The integration of methylene protons closest to the ester group was monitored in relation to the protons on the hydrolysis-resistant backbone of the polymer. b) The percent of hydroxyethyl pendant group hydrolysis was quantified at variety of pH values soon prior and post incubation at 37°C. As expected, our positive control in basic conditions showed rapid hydrolysis within minutes, but no other solution showed significant levels of hydrolysis (>5%) after 72 hrs. These results showed that the acid-catalyzed hydrolysis of the pendant groups would be negligible in the mildly acidic conditions encountered in the cell.
Raman spectroscopy DNA Binding studies.

To determine the binding mechanism of poly(quinine-co-HEA) polymers with DNA, the Raman spectrum of poly(quinine-co-HEA) was measured in the presence of calf-thymus DNA at N/P ratio of 5 (Fig. S26a). Concentrated stock solutions of DNA and poly(quinine-co-HEA) were prepared in water under acidic conditions (1.2 % v/v HCl) to keep the polymer soluble and to mimic the conditions under which the polynplexes were performed for dye exclusion, DLS, and transfection experiments. The stock solutions were prepared with 2% (v/v) acetonitrile, which was used as an internal standard. To prepare polynplexes, the stock solutions were mixed together in a 1:1 ratio (v/v). Upon mixing, a noticeable increase in the viscosity and the formation of aggregates was observed. The viscosity change was similar to what we previously observed with monomeric quinine and indicates that the poly(quinine-co-HEA) polymer chains are binding DNA to form polynplex aggregates.¹²

The Raman spectra of the polymer, DNA, and polynplex samples were measured using a home-built setup that used a 785 nm diode laser purchased from Innovative Photonic Solutions. The beam was directed through a 30/70 beamsplitter and focused onto the sample with an infinity-corrected Olympus Ach 10×/0.25 NA objective. An excitation power of 40 mW was used at the sample. The Raman scattered light was collected using a 180° backscattering geometry and redirected through the beamsplitter into an Acton SpectraPro 2500i spectrometer. The light was dispersed in the spectrometer using a 600 gr/mm grating and imaged using a PIXIS CCD camera purchased from Princeton Instruments. The spectra shown here were measured by averaging 30 spectra collected with 60 s acquisition times.

The quinoline ring symmetric mode of quinine was used as a spectroscopic probe to monitor poly(quinine-co-HEA) binding to DNA due to its sensitivity to the local chemical environment. We previously demonstrated that the frequency of this vibration characteristically shifts in a manner that depends on whether the quinoline ring is engaging in electrostatic, hydrogen bonding, or π-stacking interactions. We also showed that the frequency of this mode in monomeric quinine in aqueous solution is at 1370 cm⁻¹ when the quinoline ring is deprotonated, but upshifts to 1390 cm⁻¹ upon protonation. These frequency shifts indicate that the quinoline ring mode can be used to diagnose the specific interaction mechanisms of quinine with its local chemical environment, as well as local pH changes, in cells.¹²

Fig. S26a shows the Raman spectrum of the poly(quinine-co-HEA) polynplexes. The spectrum is complex, showing spectral features that derive from both DNA and polymer vibrational modes. The most intense spectral feature is located at 1388 cm⁻¹ and can be assigned to the protonated quinoline ring symmetric stretching mode of quinine in poly(quinine-co-HEA) polymers. Most of the other spectral features are significantly weaker than the quinoline ring mode and can be mainly assigned to vibrational modes localized to the nucleobases of DNA. To understand the spectral changes that occur due to poly(quinine-co-HEA) binding to DNA, the polynplex spectrum was compared to the spectra of DNA and aggregated poly(quinine-co-HEA) polymers.
In order to highlight the polymer spectral changes due to DNA binding, a difference spectrum (Fig. S26d) was calculated by subtracting the spectral contributions of aggregated poly(quinine-co-HEA) polymer and DNA. The difference spectrum shows Raman bands that derive from the DNA in the form of two broad negative features at 1241 cm\(^{-1}\) (assigned to deoxythymine, deoxyadenosine, and deoxycytidine modes) and 1415 cm\(^{-1}\) (assigned to a deoxyadenosine mode), as well as a positive feature at 1486 cm\(^{-1}\) (assigned to deoxyadenosine and deoxyguanosine modes). The difference spectrum also shows a strong positive feature at 1386 cm\(^{-1}\), which derives from the quinoline ring mode of poly(quinine-co-HEA) polymers bound to DNA. The presence of the two negative features in the difference spectrum indicates that the Raman cross sections of the 1241 cm\(^{-1}\) and 1415 cm\(^{-1}\) modes is lower in the bound DNA of polyplexes (Fig. S26a) relative to the unbound DNA (Fig. S26b). The reason for the lower cross sections stems from a well-known hypochromism that occurs in some Raman bands due to changes in the stacking of base pairs in the DNA duplex.\(^{24,25}\)

**Fig. S26.** Raman spectra of poly(quinine-co-HEA) and DNA in solution. The traces include Raman spectra of (a) poly(quinine-co-HEA) polyplexes, (b) DNA, and (c) poly(quinine-co-HEA) polymer aggregates. The difference spectrum shown in (d) was calculated by subtracting the spectra shown in (b) and (c) from that shown in (a).
As summarized in Table S3, the frequency of quinoline ring mode was also analyzed in detail to identify spectroscopic signatures of polyplex unpackaging. Compared to the difference spectrum, the quinoline ring mode of poly(quinine-co-HEA) polymers dissolved in aqueous solution is upshifted 3 cm\(^{-1}\) (Fig. 2f(i) in Main Manuscript). For monomeric quinine, the loss of \(\pi\)-stacking interactions between quinine and DNA nucleobases that occurs upon deintercalation results in a characteristic frequency upshift in the quinoline ring mode. We therefore interpret the 3 cm\(^{-1}\) frequency upshift in the quinoline ring mode as the spectroscopic signature of polyplex unpackaging due to the deintercalation of poly(quinine-co-HEA) quinine moieties from DNA.

**Table S3.** Comparison of quinoline ring mode frequencies and their interpretation for monomeric quinine and poly(quinine-co-HEA) polymer in various states.

| Quinine Type          | State     | \(\tilde{\nu}\) (cm\(^{-1}\))\(^a\) | \(\Delta\tilde{\nu}\) (cm\(^{-1}\))\(^b\) | Interpretation                                                                 |
|-----------------------|-----------|----------------------------------------|------------------------------------------|-------------------------------------------------------------------------------|
| Monomeric Quinine     | Dissolved | 1390                                   | -1                                       | Quinoline ring-water interactions in monomeric quinine                        |
| poly(Quinine-co-HEA)  | Dissolved | 1389                                   | -                                        | Quinoline ring-water interactions in poly(quinine-co-HEA)                     |
| poly(Quinine-co-HEA)  | Aggregated| 1388                                   | 1                                        | Possibly either weak \(\pi\)-stacking interactions between quinoline ring moieties in poly(quinine-co-HEA) or quinoline ring-water interactions |
| poly(Quinine-co-HEA)  | Aggregated| 1386                                   | 3                                        | \(\pi\)-stacking interactions between quinoline rings of poly(quinine-co-HEA) and DNA nucleobases |

\(^a\)frequency of the protonated quinoline ring symmetric stretching mode  
\(^b\)frequency difference with respect to poly(quinine-co-HEA) polymer in solution

**Raman Imaging Studies on Transfected Cells.**  
HeLa cells were grown on glass coverslips in 6-well plates and subsequently transfected with poly(quinine-co-HEA) polyplexes. After this, the media was removed, and the cells were rinsed with PBS. The cells were then fixed by incubating them in a 4\% (v/v) solution of formaldehyde in PBS at room temperature for 15 minutes. The formaldehyde solution was subsequently removed, and the cells were then rinsed twice with PBS. After removing residual PBS, 10 \(\mu\)L of mounting solution containing glycerol was applied to the fixed cells to maintain their morphologies.

Raman images of the transfected cells were measured along with control samples that contained polyplexes treated with the 4\% (v/v) formaldehyde solution to assess the potential influence of fixation on the Raman spectrum of poly(quinine-co-HEA) polymers (vide infra). The images were measured using a commercial confocal microscope system (alpha 300R) purchased from WITec (Ulm, Germany) equipped with a UTS300 spectrometer, a DV401 CCD detector, and a piezo-
driven feedback-controlled scanning stage. Samples were excited at 532 nm using a frequency doubled Nd:YAG laser. The laser light was focused onto the sample using a 100× Nikon air objective with a numeric aperture of 0.90. We typically used 10 mW of laser light power at the sample and an accumulation time of 1 s/pixel. The back-scattered Raman light was directed to the spectrometer via a fiber optic connection. The light was dispersed in the spectrometer using an 1800 gr/mm grating. The images of the cells were collected in the form of a hyperspectral datacube over a 50 μm × 50 μm area in spatial increments of 250 nm.

Each pixel in the Raman images contains a Raman spectrum that roughly spans the region from 900 - 1800 cm\(^{-1}\). Prior to visualizing and further analyzing the images, the spectra were pre-processed using custom-written MATLAB scripts and functions that automatically removed cosmic ray spikes and baselined each spectrum. Most of the prominent Raman bands in the spectra derive from glycerol (1050, 1110, and 1465 cm\(^{-1}\)), which was used in the cell mounting solution. However, as discussed in the Main Manuscript, HeLa cells could be visualized by integrating the intensity of the protein-derived Amide I band (1660 cm\(^{-1}\)), while polyplexes could be visualized by integrating the intensity of the unprotonated quinoline ring symmetric stretching mode of quinine (1369 cm\(^{-1}\)).

**Principal Component Analysis (PCA) of Hyperspectral Raman Images.**

PCA is a multivariate analysis method that was used to extract information from the Raman images in the 1300 – 1500 cm\(^{-1}\) region based on recurring spectral variations in the data. PCA decomposes the spectral data contained in the images into an orthogonal set of principal components (PCs) in the form of loading vectors and their corresponding scores. The advantage of PCA is that it enables the identification of spectral patterns that are not easily discernible by eye.
Fig. S27. Representative results from PCA of Raman hyperspectral images. The score maps (a-j) and corresponding loading vectors (k-t) for the first ten PCs are shown for HeLa cells that were imaged after fixation 48 h post-transfection.

To perform PCA, we used the `pca` function in MATLAB. Fig. S27 shows representative loading vectors and score maps obtained from our analysis of the Raman hyperspectral images. In the case of Fig. S27, the PC loading vectors and score maps to HeLa cells that were fixed 48 h after transfection (Fig. 6c in Main Manuscript). In our analysis, we initially considered the first ten PCs for each hyperspectral image and generally found that the first three or four PCs exhibited loading vectors that contained physically meaningful Raman spectral features. In Fig. S27, for example, the first four PCs show spectral features that can be readily assigned to distinct chemical species. The loading vectors corresponding to PCs 1, 2, and 4 show features that derive CH$_2$ deformation modes. In the case of PCs 1 and 2, these features appear at 1465 cm$^{-1}$ and can be readily assigned to glycerol, which was used in the mounting solutions for imaging the fixed cells. In contrast, the CH$_2$ deformation mode for PC 4 appears at 1447 cm$^{-1}$ and can be assigned to lipids.

The loading vectors corresponding to PCs 1 and 3 contain spectral features that can be assigned to packaged and unpackaged poly(quinine-co-HEA) polyplexes, respectively. In the case of PC 1, the quinoline ring mode appears at 1369 cm$^{-1}$ and can be assigned to intercalated quinine moieties in
poly(quinine-co-HEA) polyplexes that are engaged in π-stacking interactions with DNA nucleobases. In PC 3, the quinoline ring mode is upshifted 3 cm\(^{-1}\) to 1372 cm\(^{-1}\), which matches the frequency shift that occurs due to the deintercalation of quinine from DNA (c.f. Table S3).

It is also interesting to note that the loading vector corresponding to PC 3 additionally contains two broad features that occur between roughly 1300 - 1350 cm\(^{-1}\) and 1400 - 1460 cm\(^{-1}\) and negative peaks that occur at roughly 1320 cm\(^{-1}\) and 1420 cm\(^{-1}\). There are generally few clearly-defined features that derive from the cells in these spectral regions (partly the region between 1300 - 1350 cm\(^{-1}\)), aside from Raman bands corresponding to nucleic acids such as DNA.\(^{27}\) The negative peak that occurs at 1320 cm\(^{-1}\), for example, can potentially be assigned to a deoxyguanosine mode, while the negative peak at 1420 cm\(^{-1}\) can be attributed to a deoxyadenosine mode.\(^{27}\) If these spectral features derive from DNA bands, we speculate that they appear as negative peaks because the local concentration of DNA is decreased due to poly(quinine-co-HEA) polyplexes unpackaging and releasing their cargo.

We additionally performed PCA on the Raman hyperspectral images of polyplex samples (no HeLa cells present) to rule out the possibility that formaldehyde fixation gave rise to the spectral features observed in the PC 3 loading vectors. As shown in Fig. S28, PC loading vector corresponding to polyplexes that were fixed with 4% (v/v) formaldehyde are essentially identical to that corresponding to the unfixed control sample. The frequency of the quinoline ring mode is located at 1369 cm\(^{-1}\) for both fixed and unfixed polyplexes, matching the frequency of the PC 1 loading vector observed in Fig. S27. This result indicates that fixation does not significantly perturb the structure of polyplexes or give rise to spectral artifacts that would confound our analysis of the PC loading vectors shown in Fig. S27.
Fig. S28. Raman images and PC loading vectors of poly(quinine-co-HEA) polyplexes. Polyplexes were (a) unfixed and (b) fixed by treatment with 4% (v/v) formaldehyde. The PC loading vectors for both samples show that the frequency of the quinoline ring mode is 1369 cm\(^{-1}\) for both samples, indicating that formaldehyde fixation does not perturb the structure of polyplexes. Scale bars = 2 μm.
Calculating the Deintercalation Image Map.

The PC 1 and 3 score maps in Fig. S27 were used to determine the deintercalation map shown in Fig. 7f in the Main Manuscript. The workflow for processing the PC score maps and calculating the deintercalation map is shown in Fig. S29. To calculate the deintercalation map, the uneven backgrounds present in the PC score maps were first corrected by using a “rolling ball” algorithm (Fig. S29b). A raw deintercalation map (Fig. S29c) was calculated by determining the percent deintercalation for every pixel using the following equation:

\[ P_i = \frac{S_{PC1,i}}{S_{PC1,i} + S_{PC3,i}} \times 100\% \quad (S6) \]

where \( P_i \), \( S_{PC1,i} \), and \( S_{PC3,i} \) are the percent deintercalation, PC 1 score map intensity, and PC 3 score map intensity at the \( i^{th} \) pixel, respectively. Due to the imperfect background correction in the PC score maps, the resulting raw deintercalation map (Fig. S29c) contains noise features that obscures the polyplex particles. To better highlight the polyplexes, a more refined deintercalation map (Fig. S29d) was obtained by using an intensity threshold to select all particles and to reject as much of the noise background as possible. The intensities shown in Fig. S29c represent deintercalation percentages that are scaled from 0 to 100%. We found that all of the particles could be selected and most of the noise could be rejected by uniformly setting the intensity thresholds between 1 and 99%. As a result of this thresholding method, the polyplex particles are clearly visible in the refined deintercalation map (Fig. 29d), although some residual noise features can still be observed in the upper half of the image. To remove this residual noise, we therefore applied a binary mask to filter the refined deintercalation map and produce the image shown in Fig. S29f.
**Fig. S29.** Workflow for determining the percent deintercalation map of polyplexes in HeLa cells 48 hrs post-transfection. The PC score maps shown in (a) were first converted into grayscale images and (b) background corrected using a rolling ball algorithm. (c) A raw deintercalation map was determined from the background corrected PC score maps by calculating the percent deintercalation for every pixel using eq. S6. The raw deintercalation map contains noise that obscures the polyplex particles. As a result, a (d) refined deintercalation map was calculated by selecting polyplexes using a uniform intensity threshold. The refined map still contains residual noise and was subsequently filtered by applying a (e) binary mask to the image. The filtered deintercalation map is shown in panel (f). Scale bars = 5 μm.

**Correlation Between Protein Concentration and Polyplex Unpackaging.**
As described in the Main Manuscript, the deintercalation map shown in Fig. S29f (Fig. 7f in the Main Manuscript) was further analyzed to understand the structural role that intracellular proteins play in polyplex unpackaging. To do this, radially-averaged cross sections of individual particles located inside the cells were calculated to quantify the distribution of polymer, protein, and deintercalated quinine moieties in the polyplexes as a function of distance (in microns), $R$ (Fig. S30). The relative concentration of polymer and protein was determined by normalizing the intensities of the quinoline ring mode and the Amide I mode, respectively, to their corresponding maximum Raman band intensities. In order to compare the different polyplexes, the cross sections were normalized with respect to the apparent radius, $r$, of each individual particle (assuming a sphere). Fig. S30 shows the median and standard deviation of all the radially-averaged polymer, percent deintercalation, and protein cross sections for the polyplex particles analyzed.
Fig. S30. Radially-averaged cross sections quantifying the distribution of polymer, percent deintercalated quinine moieties, and protein for polyplex particles in HeLa cells after 48 hrs post-transfection. (a) Deintercalation map show the percent deintercalated quinine moieties of poly(quinine-co-HEA) polymers for polyplex particles. Panels (i) and (ii) show the radially-averaged cross sections of two different polyplexes with respect to the percent deintercalation. The radially-averaged cross sections quantify the relative concentration of (b) polymer, (c) deintercalated quinine moities, and (d) protein for all polyplex particles colocalized with cells. Scale bars = 5 μm.
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