Catch and Release: Photocleavable Cationic Diblock Copolymers as a Potential Platform for Nucleic Acid Delivery

Matthew D. Green[≠], Abbygail A. Foster[≠], Chad T. Greco, Raghunath Roy, Rachel M. Lehr, Thomas H. Epps, III*, and Millicent O. Sullivan*

Department of Chemical and Biomolecular Engineering, University of Delaware, Newark, DE 19716, USA. E-mail: thepps@udel.edu; msulliva@udel.edu; Fax: +1 302 831 1048; Tel: +1 302 831 8072

[≠]These authors contributed equally to this work.

Supplementary Information

Materials. Di-*tert*-butyl dicarbonate (Boc_2O), 5-Hydroxy-2-nitrobenzaldehyde (HNBA), bromopropylamine hydrobromide, 18-crown-6, sodium borohydride ($NaBH_4$), triethylamine (Et_3N), methacryloyl chloride, concentrated hydrochloric acid (HCl), anisole, copper bromide (Cu(I) Br), *N,N,N',N',N''*-pentamethyldiethylenetriamine (PMDETA), methoxy PEG (mPEG), *α*-bromoisobutyryl bromide, calcium hydride (CaH_2), sodium dodecyl sulfate (SDS), and anhydrous 4.0 N HCl in dioxane were purchased from Sigma Aldrich and used as received. Potassium carbonate (K_2CO_3) was purchased from Sigma Aldrich and dried at 120 ˚C for at least 18 h before use. Tetrahydrofuran (THF), sodium bicarbonate ($NaHCO_3$), diethyl ether, sodium sulfate (Na_2SO_4), methanol, acetone, ethyl acetate, and hexanes were purchased from Fisher Scientific and used as received. Dichloromethane (DCM) was purchased from Fisher

Scientific and distilled from CaH_2 prior to use. Deionized water (DI water) used during monomer and polymer synthesis and for dialysis was obtained from an in-house source. Water used in characterization was obtained from a Milli-Q water purification system (resistivity = 18.2 MΩ•cm). 1× Dulbecco's phosphate buffered saline (DPBS) (150 mM NaCl, with calcium and magnesium) was purchased from Fisher, Opti-MEM® I Reduced Serum Media (buffered with *4*-(*2*-hydroxyethyl)-*1*-piperazineethanesulfonic acid (HEPES) and sodium bicarbonate, and supplemented with hypoxanthine, thymidine, sodium pyruvate, L-glutamine, trace elements, and growth factors) was purchased from Life Technologies. The sodium salt of HEPES was purchased from Fisher and dissolved at 20 mM in ultrapure water, and then the pH was adjusted to ≈6 using 1.0 M HCl or 1.0 M NaOH.

^{1}H NMR Spectroscopy. Solutions of Boc-APNBMA, mPEG-*b*-P(Boc-APNBMA)$_n$, and mPEG-*b*-(APNBMA•HCl)$_n$ were prepared in $CDCl_3$ or DMSO-d_6. ^{1}H NMR spectra were collected at 600 MHz using a Bruker AVIII 600 MHz spectrometer.

Synthesis of *tert*-butyl(3-bromopropyl)carbamate. Bromopropylamine hydrobromide salt (10.03 g, 45.8 mmol) was dissolved in 300 mL of THF in a 500 mL round-bottomed flask. A solution of $NaHCO_3$ (7.77 g, 92.4 mmol) in DI water (180 mL) was added, and the solution became cloudy. Boc_2O (10.04 g, 46.0 mmol) was weighed out in a scintillation vial, dissolved in 10 mL THF, and added to the round-bottomed flask. The solution was stirred at 23 ˚C for 18 h. Afterward, the reaction was quenched with 300 mL of DI water to form two clear, colorless layers. This mixture was extracted with

diethyl ether (100 mL, 3×), and the organic phase was washed with brine (100 mL, 3×). Then, the organic phase was dried over Na_2SO_4 and concentrated on a rotary evaporator to yield a clear, slightly yellow liquid. The product was recrystallized from hexanes 3× at -20 ˚C, yielding white crystals. Then, the product was dried under reduced pressure, and characterized using ^{1}H NMR spectroscopy. ^{1}H NMR (600 MHz, $CDCl_3$) δ 4.66 (s, 1H), 3.44 (t, J = 6.5 Hz, 2H), 3.27 (q, J = 6.3 Hz, 2H), 2.05 (p, J = 6.4 Hz, 2H), 1.44 (s, 9H).

Synthesis of 3-(hydroxymethyl)-4-nitrophenol (HMNP). HNBA (10.01 g, 59.8 mmol) was dissolved in 300 mL of methanol and cooled to 0 ˚C. $NaBH_4$ (4.64 g, 122.6 mmol) was added slowly over 20 min. **The reaction bubbles violently upon $NaBH_4$ addition, take caution!** The reaction proceeded for 2 h after $NaBH_4$ addition. Afterward, the reaction was quenched with 70 mL of 5 vol% HCl (aq), and the solvent was removed using a rotary evaporator to yield a white solid. The product was characterized with ^{1}H NMR spectroscopy. ^{1}H NMR (600 MHz, DMSO) δ 10.92 (s, 1H), 8.05 (d, J = 9.0 Hz, 1H), 7.26 (d, J = 2.7 Hz, 1H), 6.79 (dd, J = 9.0, 2.8 Hz, 1H), 5.52 (d, J = 4.8 Hz, 1H), 4.82 (d, J = 3.8 Hz, 2H).

Synthesis of *tert*-butyl(*3*-(*3*-(hydroxymethyl)-4-nitrophenoxy)propyl)carbamate (HNPC). HMNP (3.52 g, 20.8 mmol) and dried K_2CO_3 (7.21 g, 52.1 mmol) were dissolved in 100 mL of acetone and heated for 15 min at 60 ˚C in a two-neck round-bottomed flask equipped with a reflux condenser and a Teflon-coated septum. Then, a solution of *tert*-butyl(*3*-bromopropyl)carbamate (7.44 g, 31.2 mmol) in minimal acetone was added using a syringe, and the solution was stirred for 10 min. Subsequently, a

solution of 18-crown-6 (0.55g, 2.0 mmol) in minimal acetone was added via syringe, and the solution was heated under reflux for 18 h. Following the reaction, the solution was concentrated using a rotary evaporator to yield a brown liquid. The product was dissolved in 50 mL of DCM and washed with DI water (50 mL, 3×). Then, the organic phase was dried over Na_2SO_4 and removed using a rotary evaporator, yielding a brown oil. The oil was added to hexanes resulting in two phases: a brown oil and cloudy supernatant. The mixture was heated until the supernatant became clear (40-50 ˚C), after which the supernatant was discarded. The oil was washed twice more to yield the purified product, which was dried under vacuum to yield a brown solid. The product was characterized using ^{1}H NMR spectroscopy. ^{1}H NMR (600 MHz, DMSO) δ 8.13 (d, J = 9.1 Hz, 1H), 7.33 (d, J = 2.6 Hz, 1H), 7.01 (dd, J = 9.1, 2.8 Hz, 1H), 6.95 (t, J = 5.3 Hz, 1H), 5.60 (t, J = 5.5 Hz, 1H), 4.85 (d, J = 5.5 Hz, 2H), 4.11 (t, J = 6.1 Hz, 2H), 3.08 (q, J = 6.6 Hz, 2H), 1.86 (p, J = 6.5 Hz, 2H), 1.36 (s, 12H).

Synthesis of *5*-(*3*-(Boc-amino)propoxy)-*2*-nitrobenzyl methacrylate (Boc-APNBMA). Et_3N (2.51 g, 24.8 mmol) and HNPC (4.20 g, 12.8 mmol) were dissolved in 400 mL of DCM in a 500 mL round-bottomed flask and cooled to 0 ˚C. Methacryloyl chloride (1.50 g, 14.4 mmol) was added slowly, and the solution was stirred for 23 h. After the reaction, the solution was washed with 5 vol% HCl (aq) (75 mL, 3×), saturated $NaHCO_3$ (aq) (100 mL, 3×), and DI water (70 mL, 3×). The organic layer was dried over Na_2SO_4 and concentrated on a rotary evaporator to yield a brown oil. The product was further purified using silica gel column chromatography (1/1 ethyl acetate/hexanes mobile phase) to yield a white solid, which was dried under vacuum and characterized using ^{1}H NMR

spectroscopy (final yield 2.55 g). ^{1}H NMR (600 MHz, DMSO) δ 8.21 – 8.16 (d, 1H), 7.15 – 7.09 (m, 2H), 6.94 (t, *J* = 5.5 Hz, 1H), 6.11 (s, 1H), 5.79 – 5.74 (m, 1H), 5.51 (s, 2H), 4.12 (t, *J* = 6.1 Hz, 2H), 3.08 (q, *J* = 6.5 Hz, 2H), 1.93 (s, 3H), 1.85 (p, *J* = 6.3 Hz, 2H), 1.36 (s, 11H).

Synthesis of methoxy poly(ethylene glycol) isobutyryl bromide (mPEG-Br). mPEG (5.0 g, 1.0 mmol) and Et_3N (0.38 g, 3.7 mmol) were dissolved in 25 mL of dry DCM in a 50 mL round-bottomed flask equipped with a magnetic stir bar and cooled to 0 ˚C. To this solution, α-bromoisobutyryl bromide (0.4 mL, 3.2 mmol) was added dropwise, and the solution was allowed to warm to 23 ˚C and stirred for 18 h. After the reaction, the solution was quenched with 1 mL of water and washed with a saturated $NaHCO_3$ (aq) solution (25 mL, 3×). The organic layer was dried over Na_2SO_4 and concentrated on a rotary evaporator. Then, mPEG-Br was dissolved in methanol, precipitated in cold diethyl ether, and pelleted via centrifugation at 4000 rpm at 4 ˚C for 15 min. The precipitation and centrifugation steps were repeated twice to yield a white powder. ^{1}H NMR (600 MHz, $CDCl_3$) δ 4.35 - 4.29 (m, 2H), 3.64 (s, 459H), 3.37 (s, 3H), 1.93 (s, 6H).

Synthesis of mPEG-*b*-P(Boc-APNBMA)$_n$. As an example, for the synthesis of mPEG-*b*-P(Boc-APNBMA)$_{7.9}$, Boc-APNBMA (0.60 g, 1.5 mmol) and mPEG-Br (0.54 g, 0.10 mmol) were weighed out and transferred into a glove box. Inside a glove box, a solution of Cu(I)Br (15 mg, 0.10 mmol) and PMDETA (21 mg, 0.12 mmol) in 4 mL of anisole was prepared in a 25 mL round-bottomed flask equipped with a magnetic stir bar. Subsequently, mPEG-Br and Boc-APNBMA were dissolved in 4 mL of anisole and

transferred into the round-bottomed flask, which then was sealed with a rubber septum. The flask was transferred out of the glove box and heated to 70 ˚C. After 24 h, the diblock copolymer was precipitated in cold diethyl ether and pelleted *via* centrifugation at 4000 rpm at 4 ˚C for 15 min. The pellet was dissolved in minimal methanol, and the precipitation and centrifugation were repeated twice to yield a white powder. Then, the polymer was added to DI water, yielding a cloudy solution. mPEG-*b*-P(Boc-APNBMA)$_n$ was pelleted via centrifugation at 4000 rpm at 4 ˚C for 25 min, which removed residual mPEG-Br macroinitiator.

Synthesis of mPEG-*b*-P(APNBMA•HCl)$_n$. As an example, for the synthesis of mPEG-*b*-P(APNBMA•HCl)$_{7.9}$, mPEG-*b*-P(Boc-APNBMA)$_{7.9}$ (0.72 g, 0.09 mmol) was placed in a 25 mL round-bottomed flask equipped with a magnetic stir bar and sealed with a rubber septum. The flask was purged with Ar (gas) for 10 min, and then cooled to 0 ˚C. An anhydrous 4 N HCl solution in *1,4*-dioxane (15 mL) was added, and the solution was stirred for 2 h. After the reaction, the polymer was precipitated in cold diethyl ether, redissolved in DI water and dialyzed (3500 MWCO) against DI water for 24 h with three solvent exchanges.

Polyplex formulation. Polyplexes were formed using mixtures of the gWiz-GFP plasmid (pDNA) and mPEG-*b*-P(APNBMA•HCl)$_{7.9}$. pDNA solutions were prepared at 40 μg/mL in 20 mM HEPES (pH 6.0). Polyplexes were formed by dropwise addition of polymer solution to an equal volume of pDNA while vortexing. Solutions contained polymer over the range of concentrations appropriate to form polyplexes at the desired

ammonium to phosphate (N/P) ratio. Polyplexes were incubated at 23 ˚C for 10 min prior to further analyses.

pDNA condensation using the ethidium bromide exclusion assay. Polyplex formation was analyzed using agarose gel electrophoresis. Polyplexes were prepared at N/P ratios from 0 to 7 by mixing 0.5 μg pDNA with the appropriate amount of polymer, and the resulting mixture was added to 5 μL of gel loading dye (2.5 mg/mL bromophenol blue in 3/7 (v/v) glycerol/water). Then, the polyplex solution was added to the wells of a 1.0% agarose gel containing 0.2 μg/mL of ethidium bromide. Gels were run at 100 V for 2 h and subsequently imaged using a Biorad Gel Doc XR.

SDS-mediated pDNA release. Polyplexes were prepared at N/P = 5 and incubated for 2 h at 37 ˚C in the presence of SDS at a sulfate/phosphate (S/P) ratio of 20. The resulting mixture was irradiated with 365 nm UV light at 200 W/m^2 (Omnicure S2000, Lumen Dynamics, Mississauga, Ontario, Canada) for 60 min and collected for agarose gel electrophoresis (performed as described above).

Polymer/Polyplex Cleavage. Polymer and polyplex solutions were prepared such that the final polymer concentration was 0.1 mg/mL for analysis by agarose gel electrophoresis and UV-Vis spectroscopy and 2.0 mg/mL for analysis by ^{1}H NMR spectroscopy. Solutions were loaded into a chamber prepared by sealing two glass slides with a rubber gasket. The chamber was irradiated with 365 nm light at 200 W/m^2 for 0 min, 10 min, 20 min, 40 min, or 60 min. Samples were removed from the chamber and

collected for absorbance measurements (Thermo Scientific, NanoDrop 1000 Spectrophotometer), agarose gel electrophoresis, and ^{1}H NMR spectroscopy.

Preparation of polyplexes for dynamic light scattering (DLS). Polyplexes (1.5 μg pDNA, 75 μL of solution) were mixed with 200 μL HEPES buffer, H_2O, PBS, or Opti-MEM, and subsequently incubated at 23 ˚C for 60 min prior to particle size determination. DLS experiments were performed using a CNI Optoelectronics Co., Ltd. 532 nm, 427.6 mW laser, coupled with a Brookhaven Instruments Corporation BI-200SM goniometer that had an inline 532 nm filter from Intor, Inc. The intensity auto-correlation function was recorded at 90˚ and analyzed using a quadratic cumulant fit. All light scattering experiments were performed at 25 ˚C.

YOYO-1 Fluorescence Quenching Assay. gWiz-GFP plasmid was mixed with the bis-intercalating dye YOYO-1 iodide at a base pair/dye ratio of 50 and incubated at room temperature for 1 h. Polyplexes were formed at N/P ratios of 0, 1, 2, 3, 4, 5, 6, or 7 by combining 1 μg YOYO-1 labeled pDNA and the appropriate amount of PEG-*b*-P(APNBMA·HCl)$_{7.9}$ as described previously. Subsequently, 50 μL of polyplex solution was added to a 96-well plate, and the fluorescence was measured using a GloMax Multi Detection System reader.

Cell Culture. Mouse embryonic fibroblast (NIH/3T3) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured under conditions suggested by ATCC: 37 ˚C, 95% relative humidity, and 5 vol% CO_2 in

Dulbecco's modified Eagle medium (DMEM) supplemented with 10 vol% fetal bovine serum (FBS) and 1 vol% penicillin-streptomycin.

Alamar Blue Assay for Cytotoxicity. Polymer toxicity and cell viability under UV irradiation were evaluated in NIH/3T3 cells using the Alamar Blue (AB) assay according to the manufacturer's protocols. Polymer solutions were prepared in Opti-MEM at the specified concentrations. To test the polymer cytotoxicity, the cells were rinsed once with PBS and incubated in polymer solutions of varying concentration for 3 h at 37 ˚C with 5 vol% CO_2. To test the cytotoxicity of the UV irradiation conditions and the photocleavage reaction products, the cells were incubated at 37 ˚C for 20 min under UV irradiation in either Opti-MEM (phenol red free) or a polyplex solution (0.1 mg/mL mPEG-*b*-P(APNBMA•HCl)$_{7.9}$, N/P = 5) prepared in Opti-MEM (phenol red free). After UV irradiation, the cells were rinsed with PBS, complete growth medium was added, and the cells were incubated at 37 ˚C with 5 vol% CO_2 for 48 h. After the incubation, AB was added directly into the culture medium at a final concentration of 10% by volume for viability measurements, and then the cells were incubated for 6 h at 37 ˚C with 5 vol% CO_2. AB fluorescence was measured using a GloMax-multi detection system plate reader (Promega, Madison, WI). To determine the baseline fluorescence, a solution to which AB was added to media without cells was analyzed.

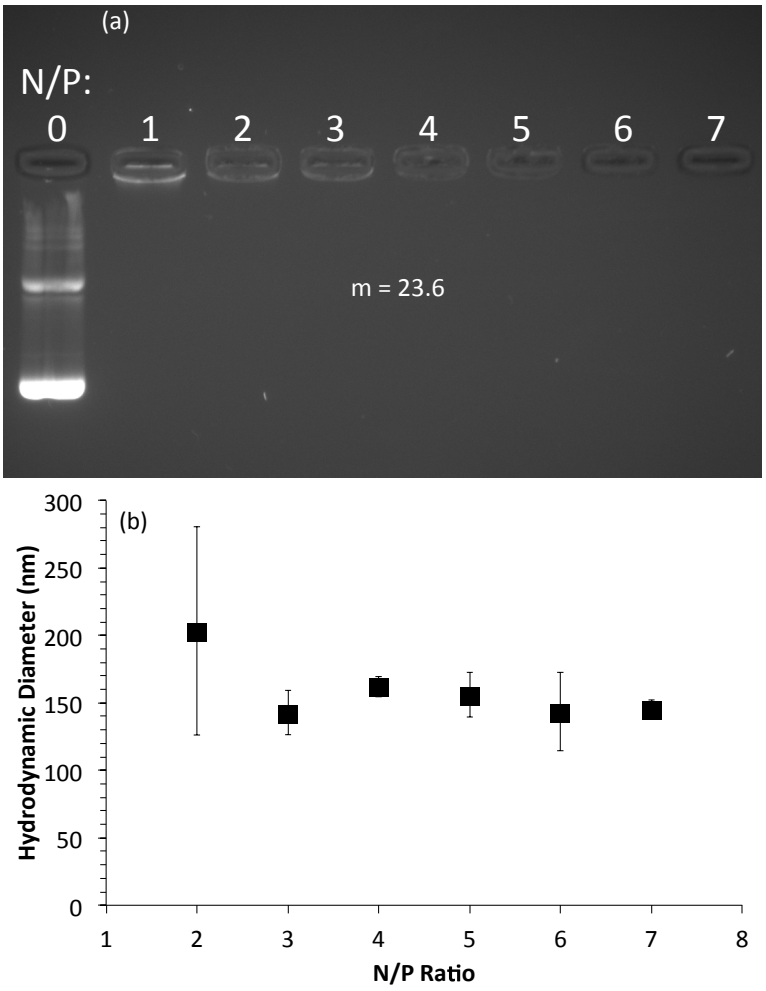

Fig. S1 Characterization of mPEG-*b*-P(APNBMA•HCl)$_{23.6}$/pDNA polyplexes using (a) gel electrophoresis and (b) DLS. Error bars in DLS represent the standard deviation from the mean of three independent measurements of polyplex hydrodynamic diameter.

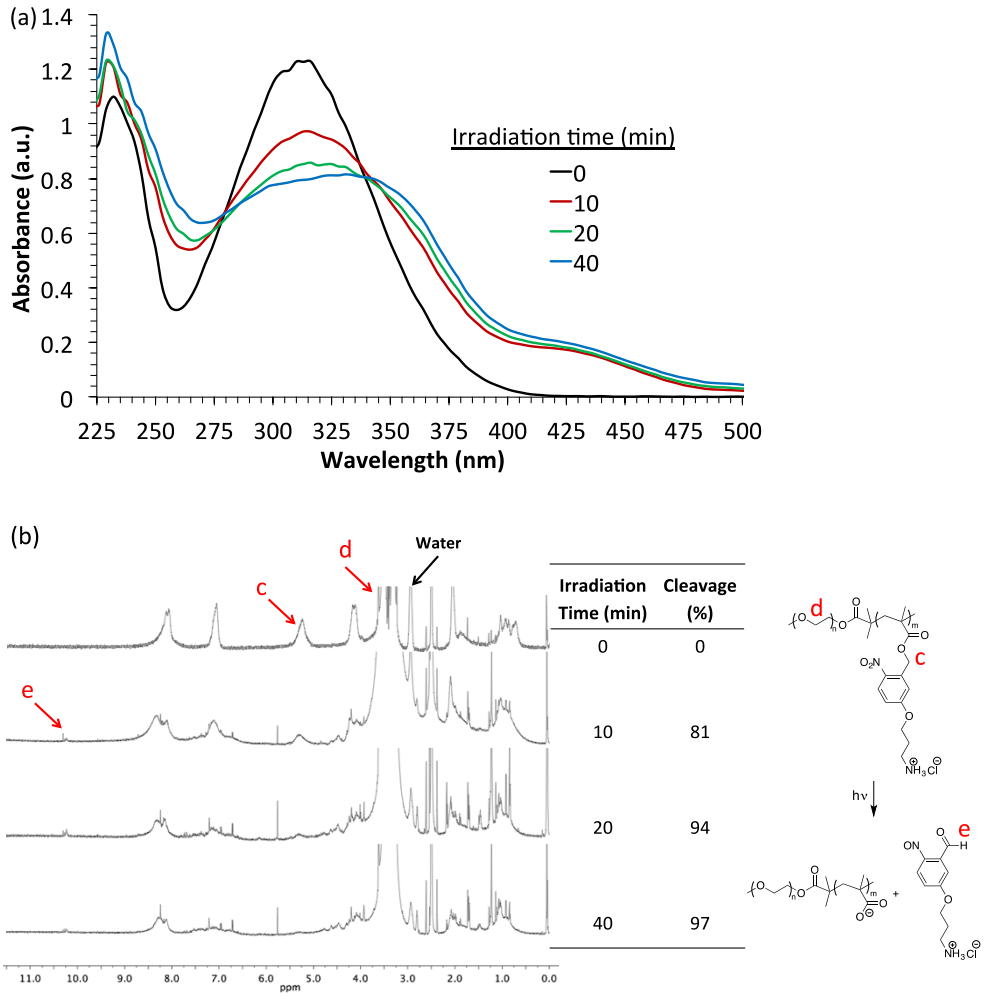

Fig. S2 Characterization of polymer structural changes during the photolysis reaction. (a) UV-Vis spectra of mPEG-*b*-P(APNBMA•HCl)$_{7.9}$ as a function of irradiation time, and (b) ^{1}H NMR spectra of the irradiated polymer. The extent of the reaction (cleavage (%)) was calculated by integrating the resonances for the benzylic –CH_2– ("c") relative to the resonances for the PEG methylenes ("d"). Additionally, the resonance at ~10.2 ppm ("e") from the benzaldehyde proton was analyzed to confirm the formation of the nitrosobenzaldehyde salt.

Determination of Exponential Decay Constant. We fit the relative absorbance data with an exponential decay according to a literature precedent.[1] The decay follows the relationship:

$$I = exp^{f(}\frac{-t}{\tau},$$

in which I is the relative absorbance, t is time (min), and τ is the decay constant (min). Increasing the molecular weight of the cationic block led to a decrease in the decay constant (Fig. S3a: 5.7 min for n = 7.9 and Fig. S3b: 3.7 min for n = 23.6), which

suggested an accelerated photocleavage. The use of mPEG-*b*-P(APNBMA•HCl)$_{7.9}$ to encapsulate pDNA into polyplexes decreased the decay constant to 2.7 min (Fig. S3c), which suggested further acceleration of the photocleavage reaction upon complexation into polyplexes. We are investigating potential photocleavage mechanisms to develop relationships between polymer block length or polyplex formation and the photocleavage kinetics.

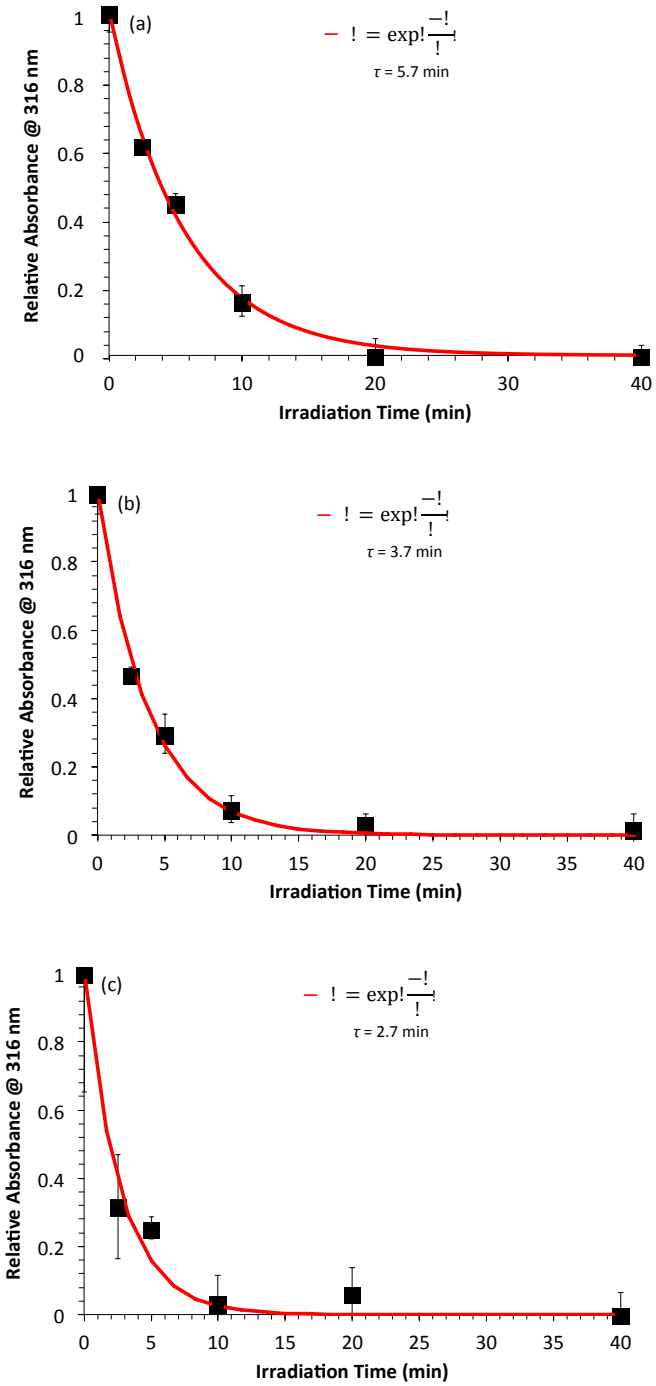

Fig. S3 Normalized absorbance (filled squares) at 316 nm for (a) mPEG-*b*-P(APNBMA•HCl)$_{7.9}$, (b) mPEG-*b*-P(APNBMA•HCl)$_{23.6}$ and (c) mPEG-*b*-P(APNBMA•HCl)$_{7.9}$/pDNA polyplexes as a function of UV irradiation time. The log of the normalized intensity was fit using an exponential decay to determine τ [$I = \exp(-t/\tau)$, in which I is normalized absorbance, t is time, and τ is the exponential decay constant].

References

1. Kloxin, A. M.; Kasko, A. M.; Salinas, C. N.; Anseth, K. S. *Science* **2009,** 324, (5923), 59-63.