

# MAG vs. PEG: Incorporating a poly(MAG) Layer to Promote Colloidal Stability of Nucleic Acid/"Click Cluster" Complexes

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## **Experimental Procedures**

**Chemical and Biological Reagents:** Pentaethylenehexamine, iodine, and *N*-hydroxy succinimide were purchased from Sigma Aldrich (St. Louis, MO) and used as received unless otherwise noted. Pentaethylenehexamine was purified through double distillation prior to use. *N,N'*-Dicyclohexylcarbodiimide (DCC), adamantane methylamine, ethyl trifluoroacetate, triphenylphosphine, sodium azide,  $\beta$ -cyclodextrin, and acetic anhydride were purchased from Acros Organics (Thermo Fisher Scientific, New Jersey, NJ) and used without purification.  $\beta$ -cyclodextrin was dried by dissolving in dry DMF, followed by evaporation of the solvent, and further drying under vacuum. Propiolic acid was purchased from Alfa Aesar (Ward Hill, MA) and used without further purification. The NHS activated polyethylene glycol (PEG-SC) was

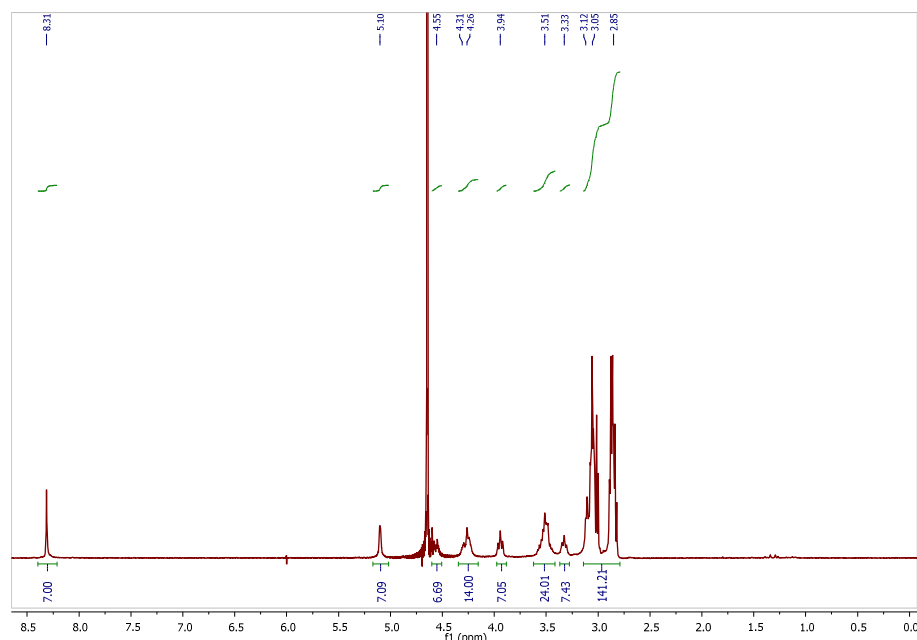
purchased from NOF America Corporation (White Plains, NY) and used without purification. The heptakis{6-[4-(2-pentaethylenepentaminoethylcarbamoyl)-1*H*-1,2,3-triazol-1-yl]}cycloheptaamylose (click clusters) were synthesized and purified using our previously reported methods.<sup>1</sup> All the dilutions of the plasmid DNA and N/P dilutions of the click clusters were completed using UltraPure™ DNase/RNase-Free Distilled Water (Gibco, Carlsbad, CA). All of the cell culture reagents such as Dulbecco's Modified Eagles Medium containing GlutaMAX™ (DMEM), reduced serum media OptiMEM™, phosphate buffered saline at pH 7.4 (PBS), trypsin, heat-inactivated fetal bovine serum (FBS), antibiotic and antimycotic (AB/AM), DNase RNase Free Ultrapure Water were purchased from GIBCO, Invitrogen (Carlsbad, CA). The cell culture supplies from Corning, Inc. (Lowell, MA), such as cell culture plates and 75 cm<sup>2</sup> flasks, were purchased from Fisher Scientific. All of the cell lines used in this study (human adenocervical carcinoma cell line (HeLa), and human glioblastoma cell lines U251 and U-87 MG) were purchased from American Type Cell Culture Collection (Manassas, VA). The positive control, jetPEI™, was purchased from Polyplus-Transfection, Inc. (New York, NY). The polymer Glycofect™ was generously donated by Techulon, Inc. (Blacksburg, VA). The nucleic acid gWiz™ High-Expression Luciferase plasmid DNA (6732 bp) was purchased from Aldevron, Inc. (Fargo, ND). This same plasmid DNA labeled with Cy5™ dye was purchased from Mirus Bio (Madison, WI). The [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) powder was purchased from Invitrogen, Inc. (Carlsbad, CA).

**Synthesis of the Click Clusters.** Experimental detail for preparation of click clusters can be found in previously reported publication by Srinivasachari et al.<sup>1</sup> A brief description of the click cluster synthesis is provided below.

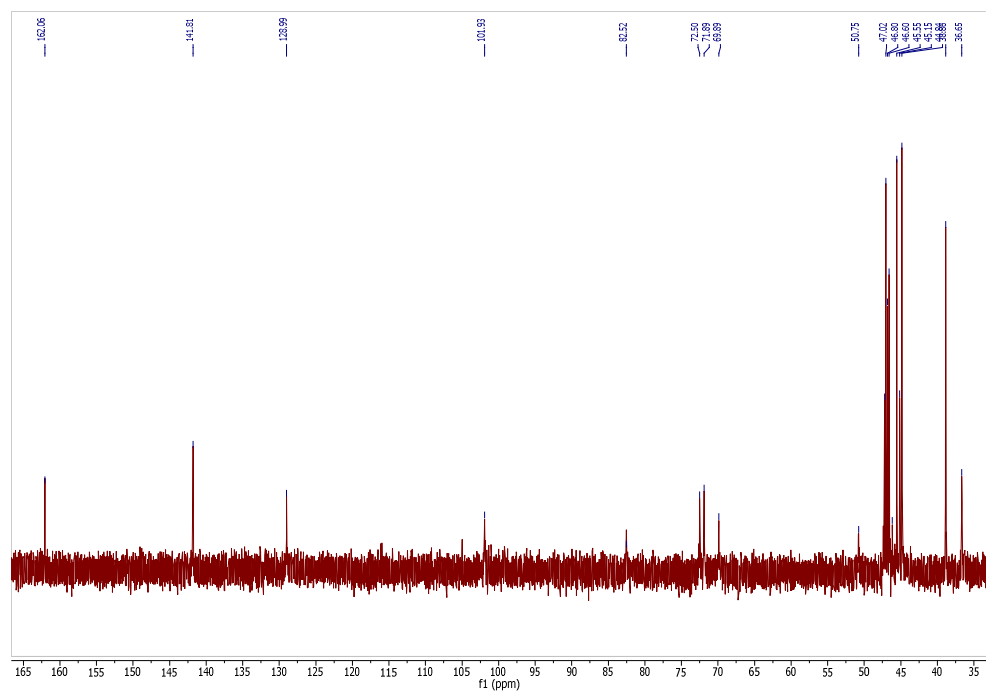
Pure, dried  $\beta$ -cyclodextrin was reacted with triphenyl phosphine and iodine to afford the fully 6-substituted per-iodo  $\beta$ -cyclodextrin. A nucleophilic substitution using sodium azide was then used followed by protection of the hydroxyl groups using acetic anhydride to afford the acetylated per-azido- $\beta$ -cyclodextrin core. Next, the oligoethyleneamine arms were synthesized by first reacting the double distilled pentaethylenhexamine with one equivalent of ethyl trifluoroacetate to protect one of the primary amines. The resulting free internal secondary amines and primary amine were protected *in situ* with (Boc)<sub>2</sub>O to afford the fully protected pentaethylenhexamine. Deprotection of the primary amine was performed by cleavage of the trifluoroacetyl group in basic aqueous methanol and the desired Boc derivative was obtained via column chromatography. The Boc protected monoamines were then conjugated to propiolic acid using DCC coupling. The pure oligoethyleneamine alkyne arm was obtained after silica gel column chromatography followed by recrystallization 29% overall yield.

The final click cluster was synthesized by utilizing the azide-alkyne 1,3-dipolar cycloaddition reaction using copper sulfate/sodium ascorbate in *t*BuOH/H<sub>2</sub>O at a ratio of 1:1. The fully protected click cluster was precipitated and washed with a 10% ammonium hydroxide solution multiple times to remove the copper, then rinsed with pure H<sub>2</sub>O. Without any further purification the Boc and acetyl protecting groups were removed followed by extensive dialysis against pure H<sub>2</sub>O followed by ion exchange chromatography with an increasing gradient of NH<sub>4</sub>HCO<sub>3</sub> aqueous solutions to elute the pure click clusters.<sup>1</sup> This yielded a white, fluffy product after lyophilization. The click clusters were analyzed by MALDI-TOF mass spectrometry using an Applied Biosystems 4800 MALDI ToF/ToF operated in reflective positive mode. The matrix for click cluster analysis was comprised of 2,5-dihydroxybenzoic acid in acetone with 5 mg/ml of NaI. The click cluster structure was also confirmed by <sup>1</sup>H, <sup>13</sup>C NMR with peak assignments

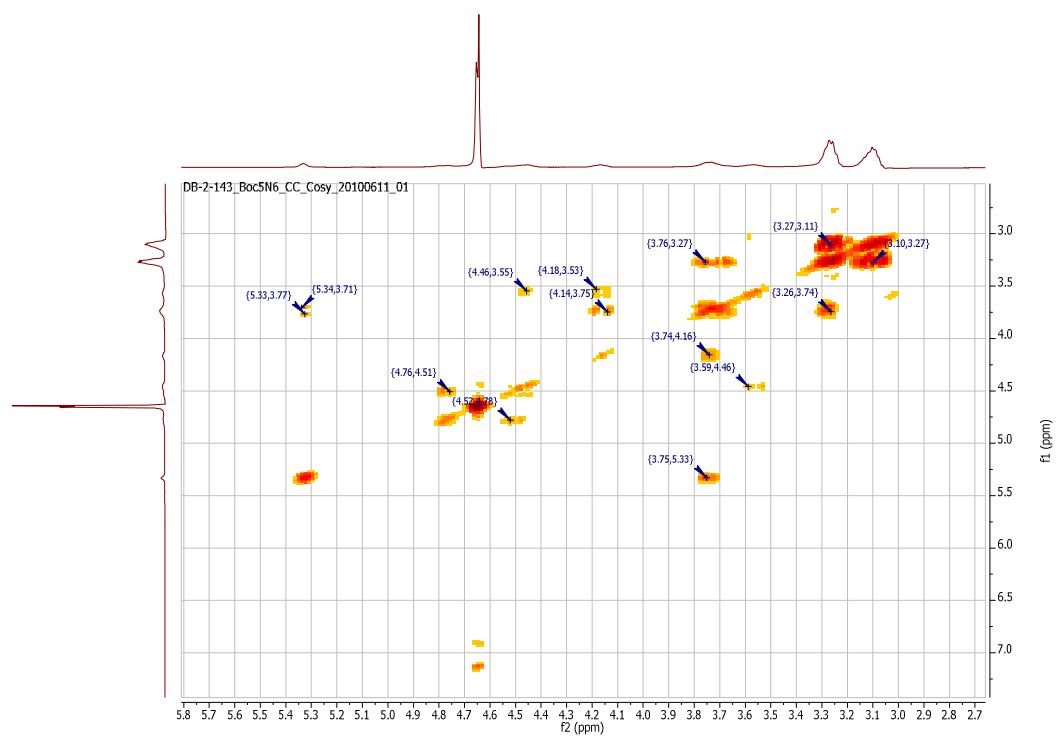
determined with 2D COSY and HSQC NMR experiments.  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ ):  $\delta = 2.85\text{-}3.21$  (m, 140H,  $\text{CH}_2$ ), 3.33 (t, 7H, H4), 3.51 (t, 7H, H2), 3.94 (t, 7H, H3), 4.20-4.30 (b, 14H, H5&H6), 4.55 (d, 7H, H6), 5.10 (s, 7H, H1), 8.31 (s, 7H).  $^{13}\text{C}$  NMR ( $\text{D}_2\text{O}$ ):  $\delta = 36.65, 38.86, 44.84, 45.15, 45.55, 46.60, 46.80, 47.02, 47.23, 50.75, 69.89, 71.89, 72.50, 82.52, 101.93, 128.99, 141.81, 162.06$ . MALDI-TOF: Calc.  $\text{M}^+ \text{C}_{133}\text{H}_{259}\text{N}_{63}\text{O}_{35}$ : 3299.04 Found: 3299.89 g/mol



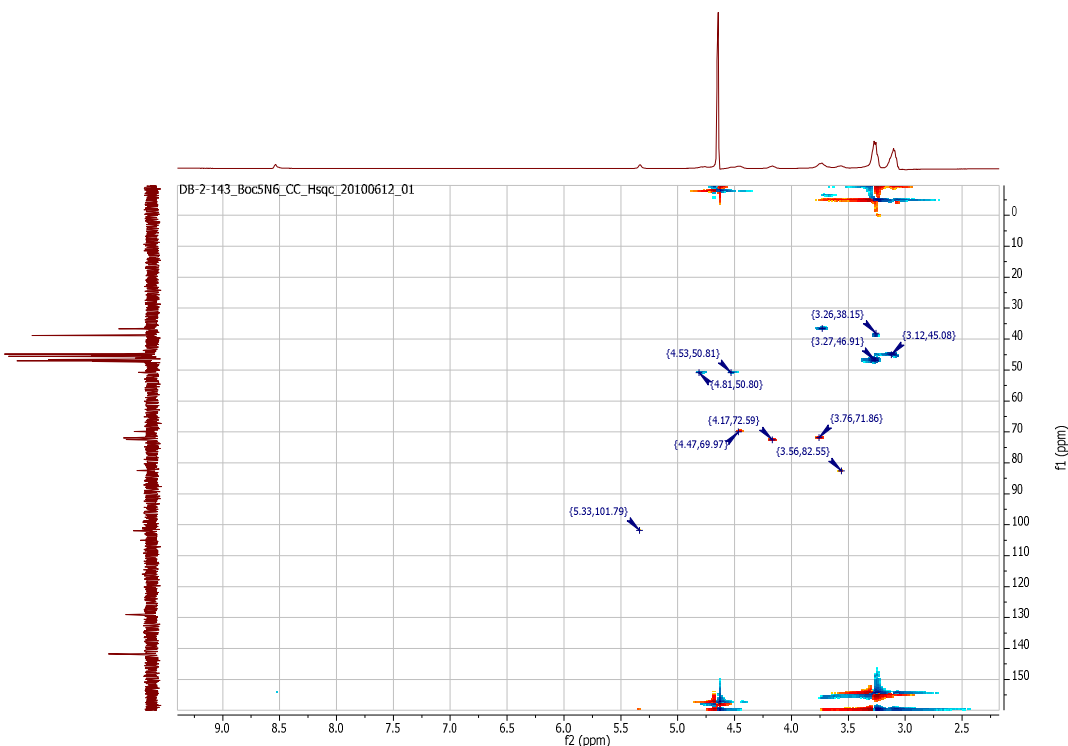
**SI Figure 1a.** Proton NMR of pure click cluster in  $\text{D}_2\text{O}$  with a small amount of dioxane impurity overlapping H2 of click cluster.



SI Figure 1b. Carbon 13 NMR of pure click cluster in D<sub>2</sub>O.



SI Figure 1c. 2D COSY NMR of pure click cluster in D<sub>2</sub>O.



**SI Figure 1d.** 2D HSQC NMR of pure click cluster in D<sub>2</sub>O.

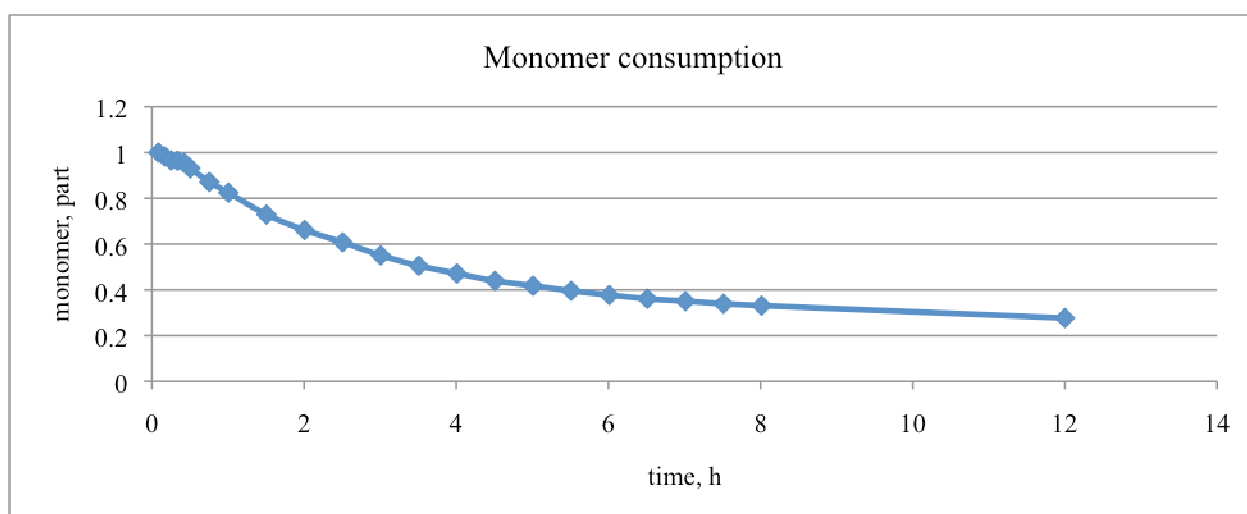
**Synthesis of Ad-PEG<sub>5000</sub>** Preparation of the AD-PEG molecules were completed using a method similar to one by Pun et al.<sup>2</sup> In brief, PEG<sub>5000</sub>-SC, in a vial, was dissolved in CHCl<sub>3</sub> after which 5 equivalents of adamantane methylamine were added and stirred at room temperature for 24 h. All CHCl<sub>3</sub> was then removed under vacuum and ultra-pure H<sub>2</sub>O was added to dissolve this residue. The aqueous solution was centrifuged at 4000 rpm for 30 min to separate the excess adamantane methylamine. The supernatant was removed and dialyzed against ultra-pure H<sub>2</sub>O, then finally freeze-dried to yield white product with near quantitative yield. The PEG derivative was analyzed by MALDI-TOF in a matrix consisting of 4 mg/mL alpha-cyano-4-hydroxy-cinnamic acid in 50:50 water:acetonitrile supplemented with 0.2% trifluoroacetic acid and 20 mM ammonium citrate. Product purity was analyzed by HPLC using an Agilent 1100 series

pump (ACN: H<sub>2</sub>O gradient) equipped with C18 column and Alltech ELSD detector. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ = 1.61 (s, 6H), 1.70-1.90 (dd, 6H), 2.09 (s, 3H), 2.94 (s, 2H), 3.52 (s, 3H), 3.84 (PEG peak), 4.34 (t, 2H).

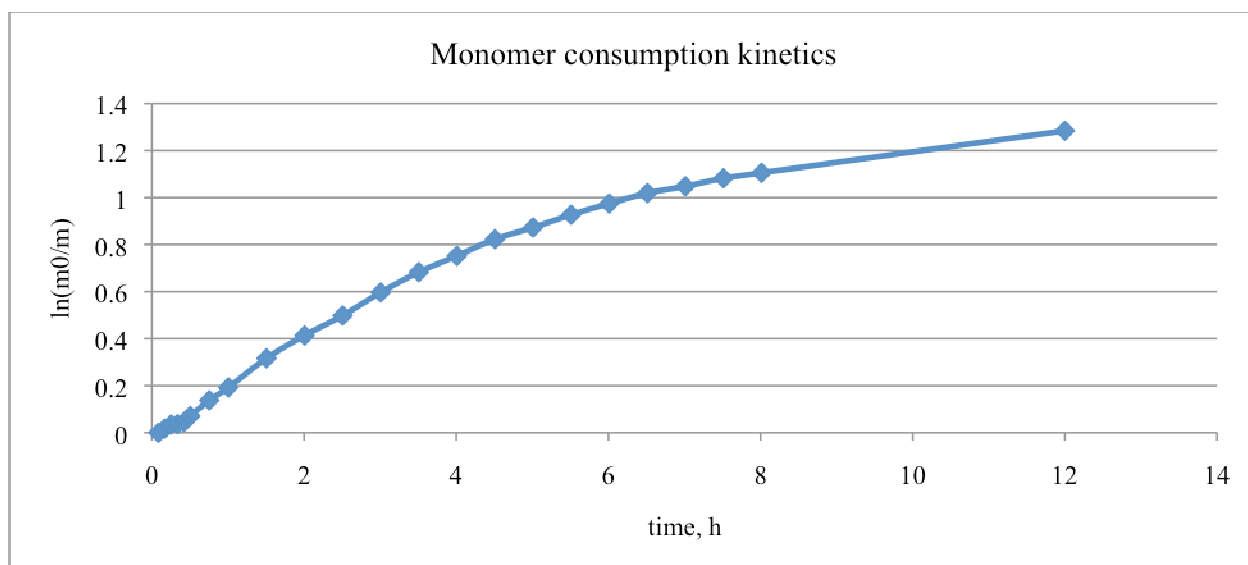
**Synthesis of Ad-CTA.** 4-cyano-4-(propylsulfanylthiocarbonyl) sulfanylpentanoic acid (CPP) CTA (199.5 mg, 8.77x10<sup>-4</sup> mol, 1.1 eq) and HOBT (O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate) (332.7 mg, 8.77x10<sup>-4</sup> mol, 1.1 eq) were dissolved in 3 mL of DMSO. After stirring at room temperature for 10 min, 131.7 mg (7.97x10<sup>-4</sup> mol, 1.0 eq) of adamantane methylamine, dissolved in 1 mL of DMSO, was added to the reaction and stirred at room temperature for 3 h. The reaction mixture was then poured into 40 mL of pH 4 water, extracted with DCM, and organic extracts were dried over Na<sub>2</sub>SO<sub>4</sub>. The product was isolated by silica gel column purification with DCM:ethyl acetate (9.5:0.5) eluent. The isolated Ad-CTA was purified by recrystallization in CCl<sub>4</sub>. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ = 1.02 (t, 3H), 1.48 (d, 6H), 1.60-1.71 (dd, 6H), 1.75 (m, 2H), 1.90 (s, 3H), 2.04 (s, 3H), 2.35-2.58 (m, 4H), 2.97 (d, 2H), 3.32 (t, 2H). LC-MS: Calcd. for (M+H)<sup>+</sup> 425.71 g/mol, found 425.20 g/mol.

**Synthesis of Ad-p(MAG)<sub>52</sub>.** The monomer, 2-deoxy-2-methacrylamide-glucopyranose (MAG), was synthesized according to previously published procedures.<sup>3</sup> Monomer consumption rate was determined by NMR analysis using the following reaction conditions: 210 mg (0.849 mmol, 1.04 eq) of MAG and 0.238 mg (8.49x10<sup>-4</sup> mol, 0.104 eq) of V-501 were dissolved in 1.521 mL of D<sub>2</sub>O and 0.234 mL of 0.1 M acetate buffer. 3.48 mg (.819 mmol, 1.00 eq) of Ad-CTA was dissolved in 1.755 mL of *d*<sub>6</sub>-DMSO. Flasks containing these solutions and empty NMR tubes were capped with septa connected with cannula and deoxygenated in line by bubbling N<sub>2</sub> through the solutions for 45 min at room temperature. Solution of MAG and initiator was cooled down in ice-bath and DMSO solution of Ad-CTA was pushed in with

nitrogen pressure. This resulted in formation of a milky suspension that was further purged with nitrogen into NMR tube. The NMR tube was placed in a NMR spectrometer preheated to 70 °C. The progress of monomer consumption was assessed by monitoring the disappearance of the vinyl peak at 5.60 ppm.



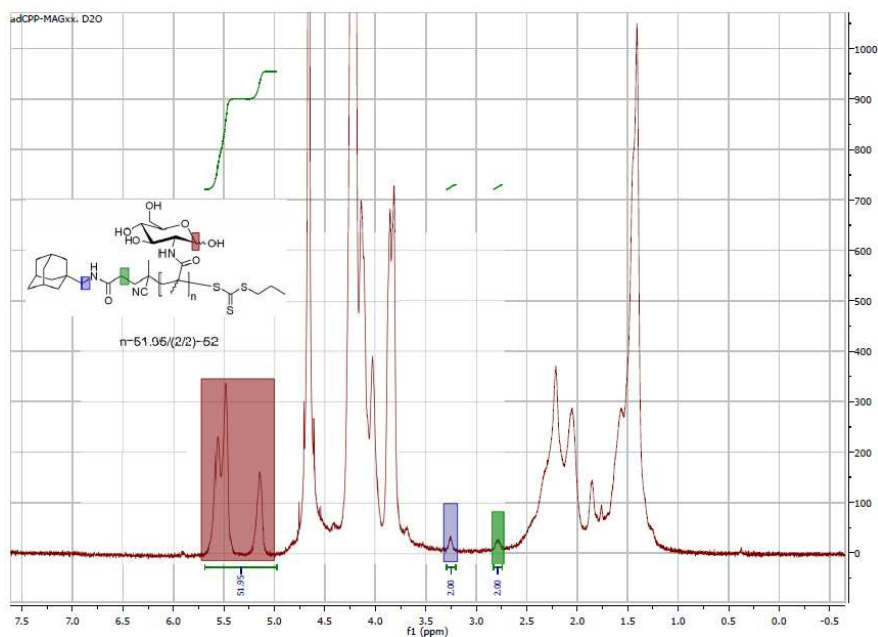
**SI Figure 2.** Part monomer was calculated by normalizing integration value for 5.51-5.68 ppm (corresponds to one of the vinyl protons), against integration value for 4.42-5.10 ppm (corresponds to anomeric protons of both  $\alpha$  and  $\beta$  forms combined).



**SI Figure 3.** Monomer consumption kinetics.  $\ln(m_0/m)$  was calculated as natural logarithm of the ratio between normalized integration values 5.51-5.68 ppm (corresponds to one of the vinyl protons) at time  $t=0$  and time  $t$ .

Ad-conjugated MAG polymer was synthesized following the established kinetics as follows: 1.653 g (6.685 mmol, 104 eq) of MAG and 1.87 mg ( $6.67 \times 10^{-3}$  mmol, 0.104 eq) of V-501 were dissolved in 11.976 mL of H<sub>2</sub>O and 1.842 mL of 0.1M acetate buffer. 27.4 mg ( $6.45 \times 10^{-2}$  mmol, 1.00 eq) of Ad-CTA were dissolved in 13.818 mL of DMSO. Flasks containing these solutions were capped with septa, connected with cannula, and deoxygenated in line by bubbling N<sub>2</sub> through the solutions for 45 min at room temperature. The solution of MAG and initiator was cooled down in ice-bath and DMSO solution of Ad-CTA was pushed in with nitrogen pressure. This resulted in formation of a milky suspension. The reaction flask was disconnected from the nitrogen line and placed in an oil bath pre-heated to 70 °C. The solution became clear after ~1 minute of stirring. Reaction was allowed to proceed for 3.5H and was stopped by removing the septum and cooling the reaction mixture in ice/water bath. The reaction mixture was diluted with 25 mL of water and dialyzed against 4 L x 9 of ultra-pure water for 72

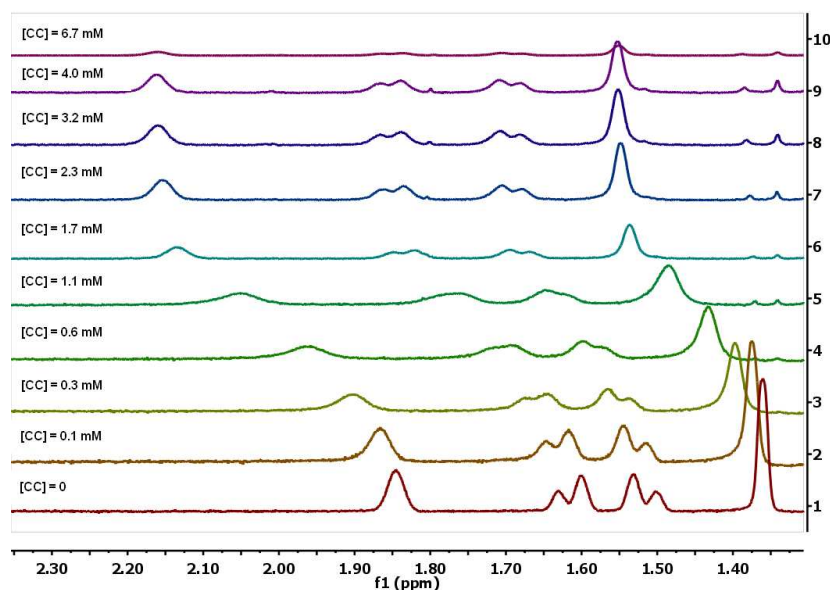
h using a dialysis membrane with 3500 Da molecular weight cut-off. Dialyzed polymer solution was freeze-dried to yield 0.584 g of fluffy solid. The DP=52 was calculated from NMR analysis as the integral ratio between the signals of terminal adamantane-CH<sub>2</sub>-NHR and anomeric hydrogen protons on the MAG structure as shown below.



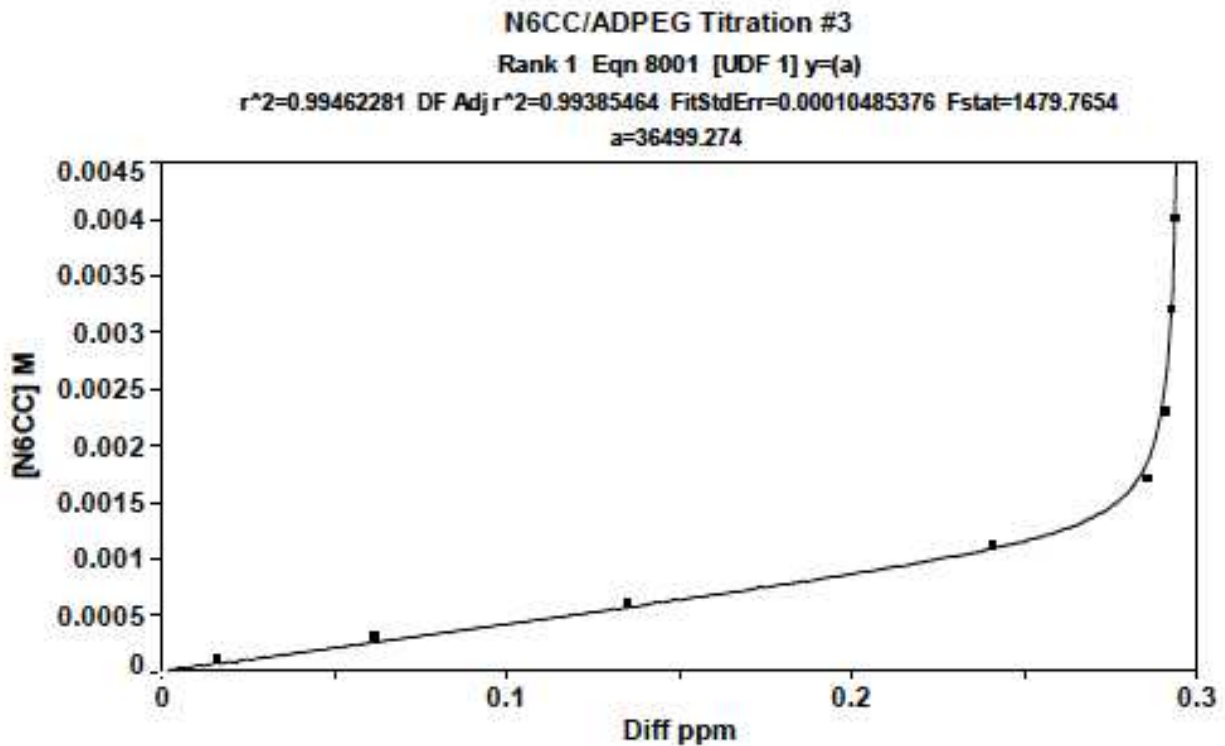
**SI Figure 4.** NMR peaks used to calculate the degree of polymerization through NMR integration.

**Determination of Relative Association Constants via NMR Titration.** The relative association constants ( $K_a$ ) were determined by titration of the click clusters into an Ad-PEG stock solution. The data was analyzed using Conner's Method<sup>4</sup> and TableCurve 2D<sup>®</sup> data analysis software (Systat Software Inc., San Jose, CA). The titration was carried out by first preparing a stock solution of the guest molecule (Ad-PEG, 1.2 mM) in UltraPure™ DNase/RNase-Free Distilled Water. A stock solution of click cluster (guest) was prepared using the previously prepared Ad-PEG stock solution at a concentration of 6.7 mM to keep the

adamantane polymer concentration constant. This click cluster stock solution, containing 1.2 mM Ad-PEG, was subsequently used to prepare 10 separate concentrations of click cluster ranging from 0 to 6.7 mM by diluting with the original Ad-PEG stock solution. Each sample was prepared in a clean 5mm NMR tube with a final volume of 700  $\mu$ L each. After all the samples were prepared, they were vortexed and left to incubate at RT for 24 h. A Varian 400MR NMR was used to record the  $^1\text{H}$  spectra of each of the ten samples under identical conditions. The chemical shift of the tertiary proton on the adamantane head group was identified and the difference in chemical shift of this proton between the uncomplexed and complexed Ad-PEG was recorded. The data was then fit with the Conner's equation using non-linear regression with the Table Curve data analysis software. A  $K_a$  of around  $36,000 \text{ M}^{-1}$  was observed for the Ad-PEG<sub>5000</sub> guest inclusion.



**SI Figure 5.** Stacked  $^1\text{H}$  NMR of Ad-PEG/click cluster NMR titration experiment that was used to determine the association constant between the Ad-X macromolecules and the click clusters.

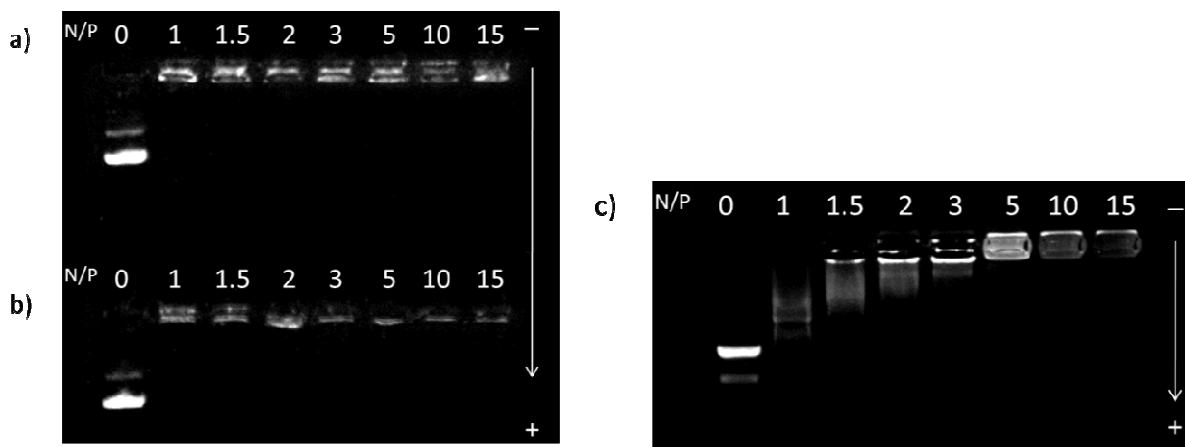


**SI Figure 6.** Table Curve data fitting using Conner’s equation showing calculation of the Ad-PEG/click cluster binding constant.

**Polyplex Formulation.** Stock solutions of pure click clusters as well as Ad-X/click cluster complexes were prepared at a stock solution concentration N/P (N = number of protonatable amines on the click cluster, P = phosphate groups on the pDNA) ratio of 250, based on a potential 0.02 mg/mL pDNA concentration, and then the polymers were subsequently diluted to the desired N/P for complexation with pDNA using UltraPure™ DNase/RNase-Free distilled water. Pure click cluster, 0.5 mg, was dissolved in 350  $\mu$ L UltraPure™ DNase/RNase-Free distilled water to give the N/P 250 stock solution. The complexed Ad-X/click cluster stock solutions were prepared in the same manner except that a 1 molar equivalent of either Ad-PEG

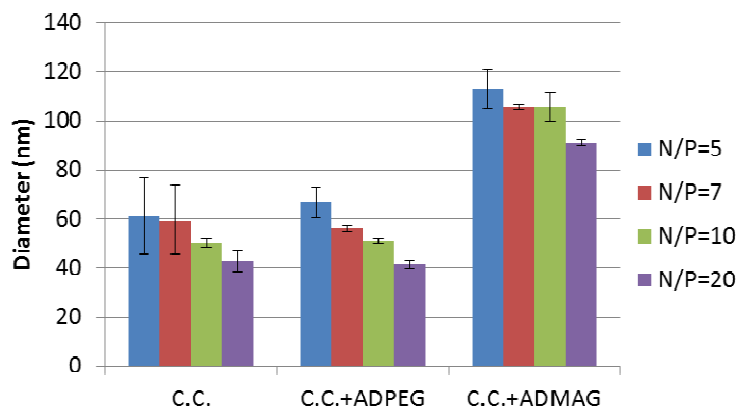
or Ad-MAG<sub>52</sub> was included in the solution. These complexed stock solutions were vortexed and left to incubate for 24 h before use to complex with pDNA. Click cluster-pDNA polyplexes were prepared at various N/P ratios by adding click cluster solution, 150  $\mu$ L, of appropriate concentration to an equal volume of pDNA solution for both the complexed and uncomplexed solutions. Polyplexes were allowed to incubate for 1h at RT before any further manipulations. Final pDNA concentration in polyplex formulation solutions was 0.1 mg/mL for gel electrophoresis experiment and 0.02 mg/ml for all other experiments. JetPEI<sup>TM</sup> polyplexes were formulated at an N/P of 5 and Glycofect<sup>TM</sup> polyplexes at N/P of 20 following the manufacturer protocols

**Gel Binding Assay.** Gel electrophoresis (60V) was carried out on Agarose gel (0.6%, w/v) containing ethidium bromide (0.6  $\mu$ g/mL) made with 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA). 2  $\mu$ L of BlueJuice<sup>TM</sup> loading buffer was added to each polyplex solution, and 15  $\mu$ L of resulting mixture were loaded onto the gel for analysis.

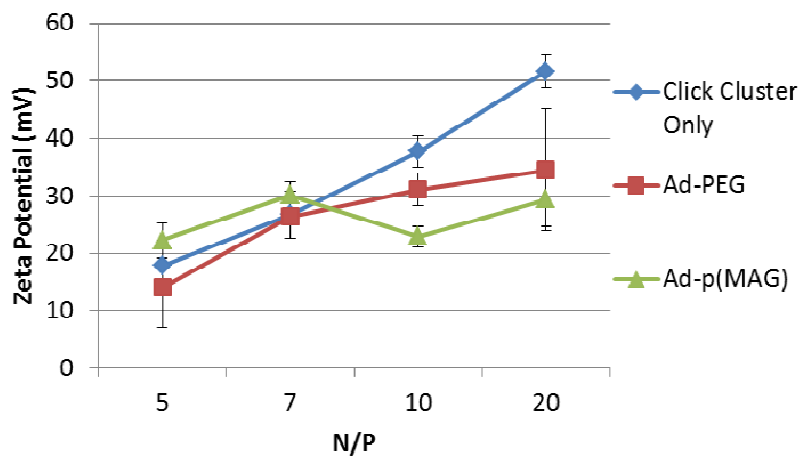


**SI Figure 7.** Gel binding assay results for a) click cluster/pDNA only, b) Ad-PEG, and c) Ad-p(MAG)<sub>52</sub> polyplex formulations.

**Polyplex Size and Zeta Potential in Water.** Polyplexes, formulated at N/P of 5, 7, 10, and 20 using the N/P 250 stock solutions described in detail above in the Polyplex Formulation section, were diluted with 700  $\mu$ L of pure water and transferred in cuvettes. Hydrodynamic diameters were measured at 25  $^{\circ}$ C using a Zetasizer Nano ZS DLS (Malvern Instruments, Malvern, UK) at a detection angle of 173 $^{\circ}$ . Polyplex solutions were transferred in zeta potential cuvettes and zeta potential measurements were carried using the same instrument at 25  $^{\circ}$ C.

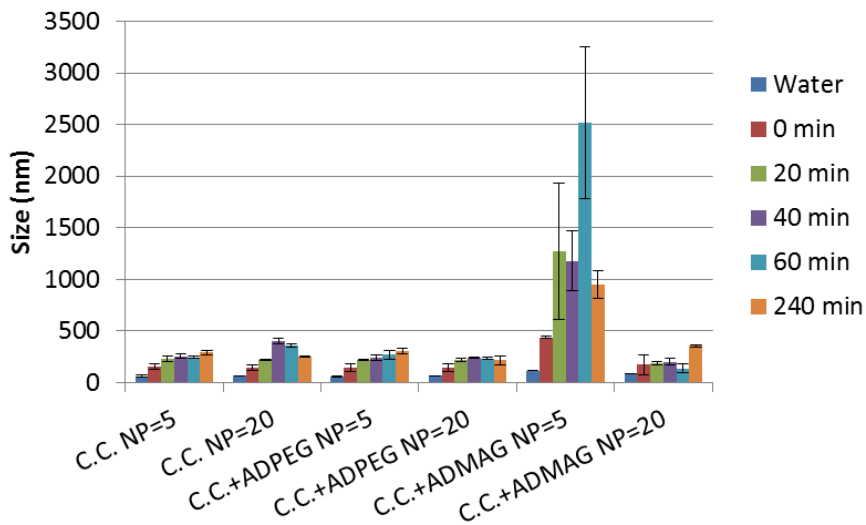


**SI Figure 8.** Size of the click cluster-pDNA polyplex formulations in water at the indicated N/P ratios.

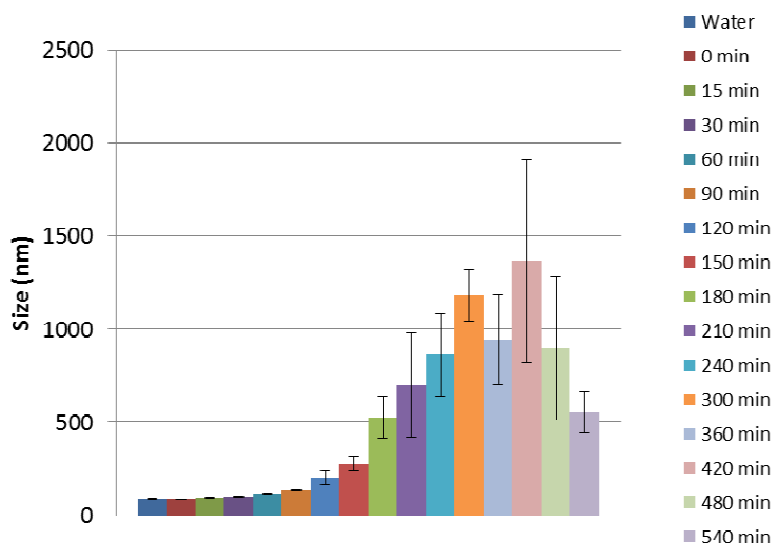


**SI Figure 9.** Zeta potential of the click cluster-pDNA polyplex formulations in water at the indicated N/P ratios.

**Polyplex Stability in Salt and Serum Conditions.** To investigate the stability of these polyplexes in more physiologically relevant conditions, the polyplexes were diluted with 700  $\mu$ L of either Opti-MEM<sup>TM</sup> or DMEM containing 10% FBS. Hydrodynamic diameters were measured at different time points between 0 to 4 hours. All measurements were done at 25 °C with a detection angle of 173°. In order to get a better picture of the very slow aggregation taking place with Ad-p(MAG)<sub>52</sub> at N/P of 20 in salt conditions, the size measurements were done every 15 min up to 9 h.



**SI Figure 10.** Hydrodynamic diameter of the polyplex formulations as a function of time after dilution with 700  $\mu$ L of DMEM containing 10% serum.



**SI Figure 11.** Hydrodynamic diameter of Ad-p(MAG)<sub>52</sub> polyplexes, at N/P 20, after dilution with Opti-MEM™.

**Cell Culture.** The cells were cultured in 75 cm<sup>2</sup> flasks in DMEM containing 10% FBS and 5% AB/AM (hereafter referred as 10% FBS DMEM) in incubator at 37 °C and 5% CO<sub>2</sub>. The cells were cultured in 24-well plates for (1) MTT cell viability assay and (2) DC Protein Assay (Bio-Rad Laboratories, Hercules, CA) and luciferase expression assay ‘Luciferase Assay System’ (Promega, Madison, WI). The cells were cultured in 6-well plates for the uptake experiment.

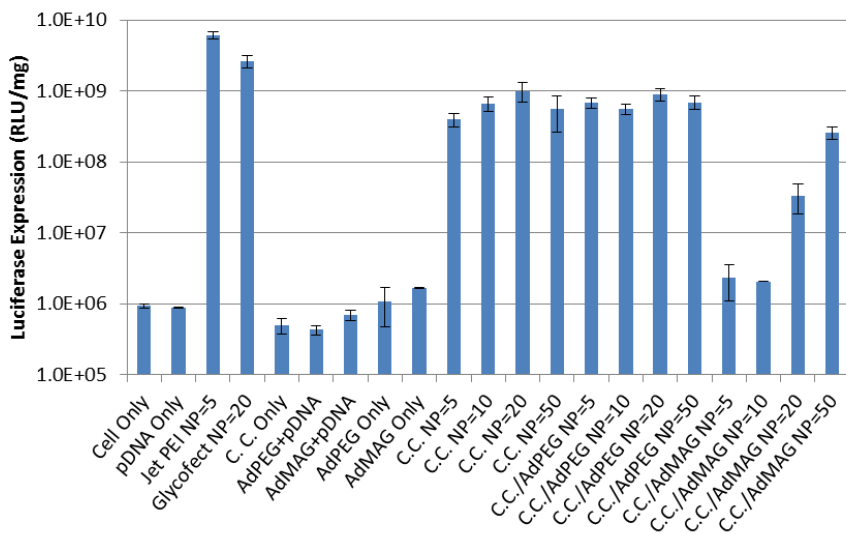
The cells were seeded at a cell density of 50,000 cells/well in 24 well plates at 24 h prior to transfection in 10 % FBS DMEM for both (1) MTT assay and (2) protein and luciferase expression assay. In the case of the uptake experiment, the cells were seeded in 6-well plates at a 250,000 cells/well density 24 h prior to transfection.

**Transfection Assays.** The solution of click cluster, click cluster+Ad-PEG, or click cluster+Ad-(MAG)<sub>52</sub> (50  $\mu$ L/well) was prepared at pre-determined N/P ratios in UltraPure™ DNase/RNase-Free Distilled Water using the previously prepared N/P 250 stock solutions described above in the Polyplex Formulation section. The pDNA solutions (50  $\mu$ L/well) were prepared at a concentration of 0.02  $\mu$ g/ $\mu$ L in UltraPure™ DNase/RNase-Free Distilled Water. The resulting mixture of solution was allowed to incubate at room temperature for 1 h. The polyplexes were then diluted in 200  $\mu$ L/well OptiMEM™ immediately before adding to the wells during transfection. Thus a final solution of 300  $\mu$ L/well of diluted polyplex solution was added to the wells. After transfection, the cells were returned to the incubator at 37 °C and 5% CO<sub>2</sub> for 4 h. After which 1 mL/well of 10% FBS DMEM was added to each well and again incubated for another 20 h. Furthermore, after 24 h post transfection, the media in each of the wells was aspirated off and replaced with 1 mL/well fresh 10% FBS DMEM and then incubated for another 24 h. After 48 h post-transfection, the cells were assayed.

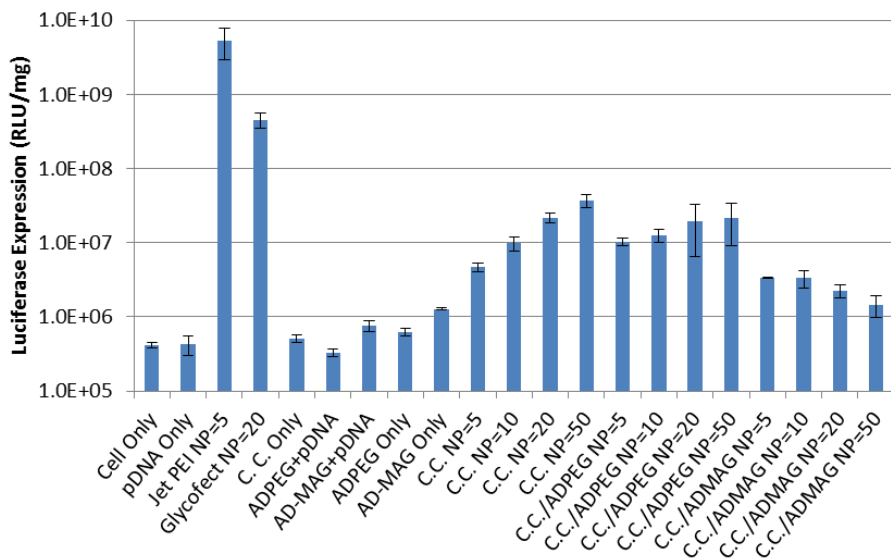
For the uptake experiment, Cy5™ labeled gWiz™-luciferase plasmid DNA was used. The polyplexes were prepared by adding 250  $\mu$ L of click cluster solution at predetermined N/P ratio, using the N/P 250 stock solutions described in detail in the Polyplex Formulation section, to 250  $\mu$ L Cy5™ labeled-pDNA solution at 0.02  $\mu$ g/ $\mu$ L into an eppendorf tube and allowed to incubate in the dark for 1 h. The polyplexes were diluted with 1000  $\mu$ L/well OptiMEM™ immediately before adding to the transfection solution to the wells. Thus, the final solution of diluted polyplex that was added to each well was 1500  $\mu$ L/well. The cells were then incubated at 37 °C and 5% CO<sub>2</sub> for 4 h. After, the cells were washed with 500  $\mu$ L/well PBS and again washed with 500  $\mu$ L/well CellScrub™ Buffer (Gelantis, Gene Therapy Systems, Inc., San Diego, CA) to remove polyplexes that may be adhering to outside surface of the plasma membrane of the cells

(to avoid false positive signals for polyplex internalization). Later, the cells were centrifuged and washed three times with PBS before analyzing on a BD FACS Canto™ II (BD Bioscience, San Jose, CA). The data were analyzed using BD FACS Diva™ software. The Cy5™ fluorescence was gated using the cells only control.

In the case of protein and luciferase assay the cells were lysed with 100 µL/well of 1x dilution of Luciferase Cell Culture Lysis 5X Reagent (Promega, Madison, WI). The protein assay was performed using 5 µL of cell lysate to clear 96 well plate and absorbance at 750 nm was measured using a TECAN Genios Pro plate reader (TECAN US, Inc., Durham, NC) at room temperature using Magellan™ software. The luciferase assay was performed on the same plate reader. The experiment consisted of adding 5 µL/well of cell lysate to an opaque (white) 96-well plate and then 95 µL/well of luciferase substrate (Promega, Madison, WI) was added using the auto injector on the plate reader. The luminescence was measured using XFluor4™ software with an integration time of 10000 ms.

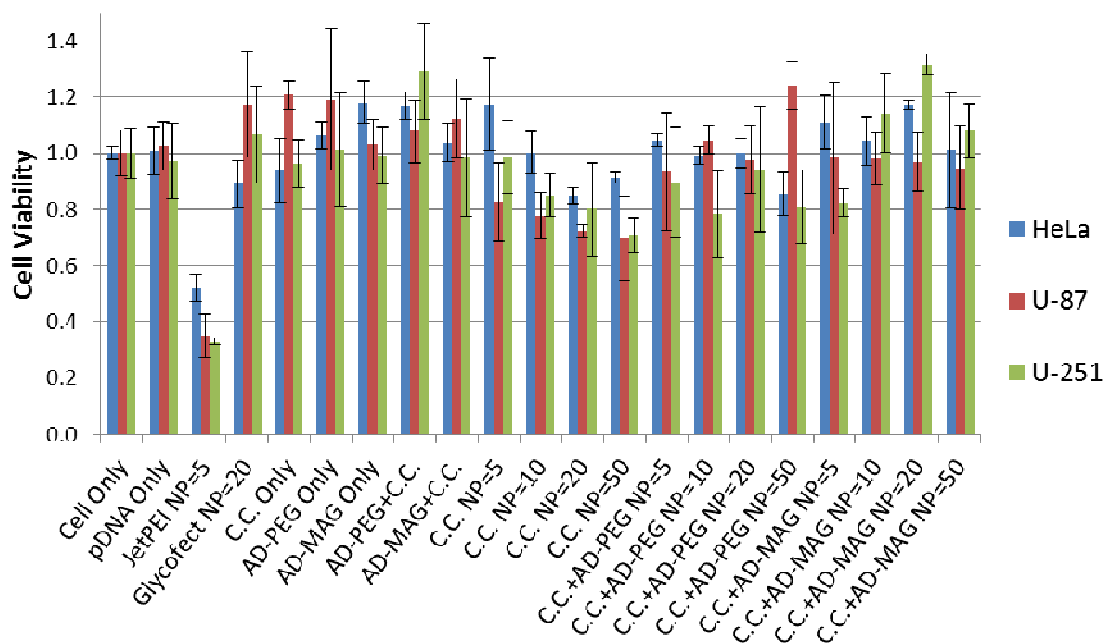


**SI Figure 12.** Transfection efficiency of the three polyplex formulations using gWiz<sup>TM</sup>-luciferase plasmid DNA in U-251 cells for N/P 5, 10, 20, and 50.



**SI Figure 13.** Transfection efficiency of the three polyplex formulations using gWiz<sup>TM</sup>-luciferase plasmid DNA in HeLa cells for N/P 5, 10, 20, and 50.

In case of the MTT assay, 1 mL/well of 0.5 mg/mL MTT containing Opti-MEM<sup>TM</sup> was added to each well and the cells were incubated for 1 hour at 37 °C and 5% CO<sub>2</sub>. After 1 h the cells were washed with 500 μL/well PBS and lysed with 600 μL/well dimethyl sulfoxide (DMSO). Then 200 μL/well of the violet-colored cell lysis solution was then added to a clear 96-well plate and absorbance was measured at 570 nm using plate reader TECAN Genios Pro (TECAN US, Inc., Durham, NC) at room temperature.



**SI Figure 14.** MTT toxicity assay of the three polyplex formulations [click cluster only, click cluster+Ad-PEG, or click cluster+Ad-(MAG)<sub>52</sub>] and the controls (cells only, pDNA only, Jet-PEI, and Glycofect) using gWiz<sup>TM</sup>-luciferase plasmid DNA in all three cell lines (HeLa, U-87, and U-251) from N/P 5, 10, 20, and 50.

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