Nanoplex delivery of siRNA and Prodrug Enzyme for Multimodality Image-Guided Molecular Pathway Targeted Cancer Therapy

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Supporting Information

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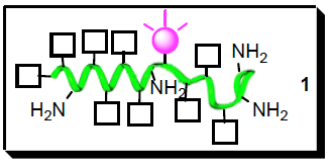
1. Materials and General Experimental Methods

All organic chemicals and solvents were of analytical grade from Aldrich (St. Louis, MO) and Alfa Aesar (Ward Hill, MA) unless otherwise specified. Poly-L-lysine hydrobromide (PLL, 6.0 kDa), branched polyethyleneimine (bPEI, 25 kDa), phenylmethanesulfonyl fluoride (PMSF), cytosine, uracil, 5-fluorocytosine, 5-fluorouracil, DNaseI and lysozyme were from Sigma (Milwaukee, WI). *N*-[ε-maleimidocaproyloxy]sulfosuccinimide (Sulfo-EMCS), *N*-succinimidyl S-acetylthiopropionate (SATP), succinimidyl 4-hydrazinonicotinate acetone hydrazone (SANH), succinimidyl 4-formylbenzoate (SFB) and Ellman's reagent were from Pierce (Rockford, IL). Rhodamine Red-X succinimidyl ester was from Molecular Probes (Eugene, OR). Cy5.5-NHS was from GE Healthcare (Piscataway, NJ). Fetal bovine serum (FBS), penicillin, streptomycin, isopropyl b-$_D$-thiogalactopyranoside (IPTG) and dithiothreitol (DTT) were form Invitrogen (Carlsbad, CA). siRNA-chk was purchased from Dharmacon Research (Lafayette, CO) with the following sequences for sense: 5'-CAUGCUGUUCCAGUGCUCCUU-3' and antisense: 5'-GGAGCACUGGAACAGCAUGUU-3'. FITC was modified in the 3' end of the sense strand. Scrambled siRNA (ON-TARGET*plus* Non-targeting siRNA #1) with minimal targeting of known genes in human, mouse and rat cells was confirmed by the producer. The MTT cell proliferation kit was from ATCC (Manassas, VA). Amicon ultra-15 centrifugal filter tubes (5,000 and 10,000 MW cutoff) were from Millipore (Bedford, MA). The invasive human mammary breast cancer cell line MDA-MB-231 was obtained from American Type Culture Collection (Rockville, MD). The molecular weight of conjugate was measured from Waters Ultrahydrogel^{TM} 500 (7.8 mm × 300 mm) gel permeable column, which was operated by a High Performance Liquid Chromatography (HPLC) system equipped with a Waters 1525 binary pump and Waters 2487 dual wavelength absorbance detector (Waters Inc. Milford, MA). ^{1}H NMR spectra were recorded at 400 MHz on a Varian Mercury400 (Varian Inc. Palo Alto, CA), and chemical shifts were reported in ppm relative to tetramethylsilane. The hydrodynamic radius,

molecular size distribution and zeta potentials of the conjugates were measured on a Malvern Zetasizer (Malvern Instruments Inc., Southborough, MA) dynamic light scattering instrument equipped with 50 mW 532 nm laser. Gadolinium ion concentrations were determined with a Perkin Elmer/Sciex Elan DRC II (Concord, ON, Canada) ICP-MS system in the Department of Geography and Environmental Engineering, Johns Hopkins University. UV-VIS spectra were recorded on a Beckman Coulter DU® 530 UV–VIS spectrophotometer (Fullerton, CA). Fluorescence spectra were obtained on a Jobin Yvon-Spex® FluoroMax-3 spectrofluorometer (HORIBA Jobin Yvon Inc., Edison, NJ). Wavelength scanning was carried out in increments of 1.0 nm with 0.1 second integration time, and the slit widths were set to 4.0 nm/4.0 nm (excitation/emission). Confocal fluorescent images were generated from a Zeiss LSM 510 META confocal laser scanning microscope (Carl Zeiss, Inc.) using a Plan-Apochromat 63×1.4 oil immersion lens (Zeiss). *In vivo* optical images were obtained with an IVIS 200 small animal imaging system (Caliper Life Sci., Hopkinton, MA). *In vitro* T_1 values and *in vivo* MRI, MRSI and ^{19}F MRS were acquired on a 9.4 T Omega spectrometer (Bruker Instruments, Fremont, CA) with 15 cm diameter shielded gradients.

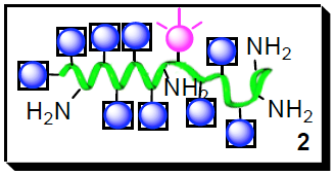
2. Synthesis of bCD-111/siRNA-chk nanoplex 11

Preparation of Cy5.5 and DOTA chelate labeled PLL (**1**). Cy5.5-NHS ester (5 mg, 4.4×10^{-6} mol, 1.2 equiv.) dissolved in 100 μL anhydrous DMSO was added dropwise to PLL (20 mg, 3.6×10^{-6} mol) in 1.0 mL 0.1 M HEPES pH 8.5 solution. After stirring at 25 °C for one hour, the mixture was loaded to a filtration tube (MW 5000 cut off) and centrifuged at a speed of 4000 rpm to remove the hydrolytic by-products. The purified Cy5.5 labeled PLL (3.5×10^{-6} mol) was stored in 2.0 mL 0.5 M HEPES pH 8.5 solution and home-made DOTA-NHS ester [1] (264 mg, 5.3×10^{-4} mol, 3.0 equiv. of the primary amines in the PLL) as solid powder was added slowly over 30 min. During the reaction procedure, the pH of the solution was

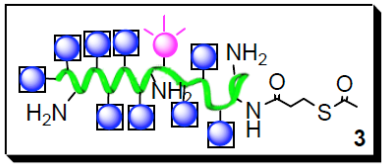


monitored and maintained at around 8.5 by adding 5.0 M NaOH solution. The mixture was further stirred at room temperature for 6 h and purified by centrifuging in a filtration tube (MW 5000 cut off). The resulting Cy5.5 and DOTA labeled PLL (**1**) as a deep blue color clear solution was concentrated in 1.0 mL 0.1 M HEPES. The degree of the Cy5.5 labeling was determined by measuring the absorbance of PLL (ε_{213} = 46,800 $M^{-1}cm^{-1}$) and Cy5.5 (ε_{680} = 250,000 $M^{-1}cm^{-1}$). On average, 0.7 Cy5.5 fluorophore per PLL molecule was obtained and the yield of compound **1** was 90%.

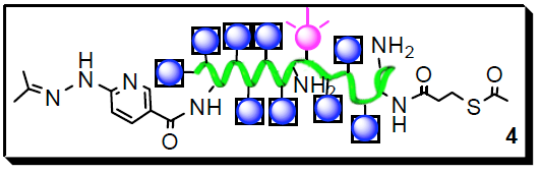
Preparation of gadolinium complex of imaging reporter labeled PLL (**2**). $Gd_2(CO)_3$ as a solid powder (75 mg, 1.5×10^{-4} mol, 50 equiv. of PLL moiety) was added to the solution of ccompound **1** (3.0×10^{-6} mol based on PLL) in 5.0 mL 0.1 M HEPES. The mixture was stirred at 50 °C overnight. At the end of the reaction, the excess $Gd_2(CO)_3$ was removed by centrifuging with a speed of 2000 rpm. The clear solution of compound **2** was loaded to a filtration tube (MW 5000 cut off) and concentrated to a final volume of 1.0 mL in 0.1 M HEPES pH 8.5. The yield of compound **2** as a blue color solution was 91% as measured by the absorbance of Cy5.5.



Preparation of S-acetylthioacetate functionalized PLL (**3**). *N*-succinimidyl S-acetylthiopropionate (SATP) (4.4 mg, 6.0 equiv., 1.8×10^{-5} mol) dissolved in 100 µL anhydrous DMSO was added to the solution of compound **2** (3.0×10^{-6} mol) in 1.0 mL 0.1 M HEPES pH 8.4. The mixture was stirred at room temperature for 1 h, then purified and concentrated into 1 mL 0.1 M HEPES pH 8.4 by centrifugal filtration (MW 5,000 cut off filter tube) as described above. The compound **3** with a yield of 92% (based on the PLL) was collected as a blue color solution.

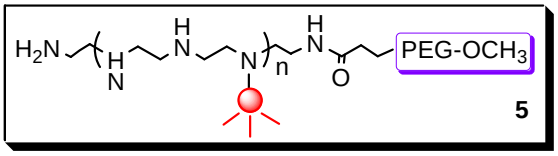


Preparation of S-acetylthioacetate and hydrazine functionalized PLL (**4**). *N*-succinimidyl 4-



hydrazinonicotinate acetone hydrazone (SANH) (3.5 mg, 6.0 equiv., 1.2×10^{-5} mol) dissolved in 100 µL anhydrous DMSO was added to the solution of compound **3** (2.0×10^{-6} mol) in 1 mL 0.1 M HEPES pH 8.5. The mixture was stirred at room temperature for 1 h, then purified and concentrated into 1 mL 0.1 M HEPES pH 8.4 by centrifugal filtration (MW 5,000 cut off filter tube) as described above. The dual functional group labeled PLL (based on the PLL) was isolated as a blue color solution with a yield of 95%.

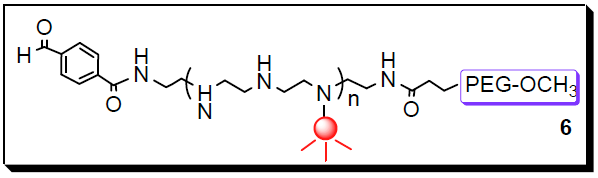
Preparation of rhodamine and PEG (2 kDa) labeled branched polyethyleneimine (bPEI, 25 kDa) (**5**).



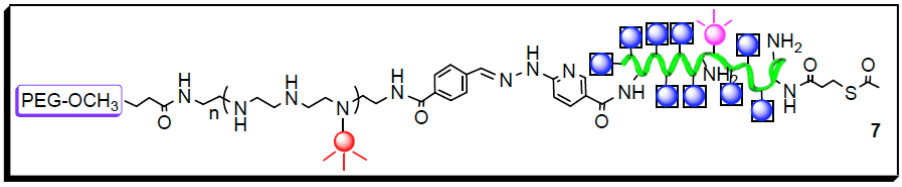
Rhodamine-NHS ester (1.9 mg, 3.0 equiv., 3.6×10^{-6} mol) dissolved in 100 µL anhydrous DMSO was added to the solution of branched polyethyleneimine (bPEI, 25 kDa) (30 mg, 1.2×10^{-6} mol) in 1.0 mL 0.1 M HEPES pH 8.5. After stirring at room temperature for 1 h, the rhodamine labeled PEI was purified and concentrated into 1 mL 0.1 M HEPES pH 8.4 by centrifugal filtration (MW 10,000 cut off filter tube). To prepare PEG-bPEI copolymer, PEG-NHS ester (24 mg, 10 equiv., 1.2×10^{-5} mol) with a molecular weight of 2 kDa in 200 µL DMSO was added to the solution of Rho-bPEI. After stirring for 1 h, the resulting rhodamine and PEG modified PEI copolymer was purified into 1 mL 0.1 M HEPES pH 8.4 as a deep red color solution by centrifugal filtration (MW 10,000 cut off filter tube). The labeling degree of the rhodamine on PEI was determined by measuring the absorbance of rhodamine (ε_{572} = 120,000 $M^{-1}cm^{-1}$) in the solution of compound **5**. On average, 1.5 rhodamine moieties per PEI molecule were obtained. To quantify the molar ratio between PEG and PEI polymer, compound **5**

was washed in MW 10,000 cut off filter tube with pure water for three times to remove the buffer salts. The resulting solution was frozen in liquid nitrogen and lyophilized overnight. The red cotton-like solid was weighted and re-dissolved in deuterium water and measured by ^{1}H NMR. The ratio between PEG and PEI in compound **5** was determined as 8.5 by quantifying the integrated proton numbers of CH_2-O (~ 3.7 ppm) in PEG chain and CH_2-N (2.4~3.0 ppm) in bPEI. The overall yield of compound **5** was measured as 86%.

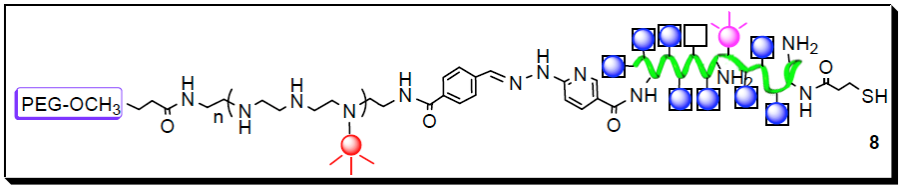
Preparation of benzaldehyde functionalized PEI (**6**). *N*-succinimidyl 4-formylbenzoate (SFB) (1.5 mg, 6 equiv., 6.0×10^{-6} mol) in 100 µL DMSO was added to a solution of compound **5** (1.0×10^{-6} mol) in 1 mL 0.1 M HEPES pH 8.4. After stirring at room temperature for 1 h, the benzaldehyde labeled PEI was purified with a yield of 92% and concentrated into 1 mL 0.1 M HEPES pH 8.4 by centrifugal filtration (MW 10,000 cut off filter tube).



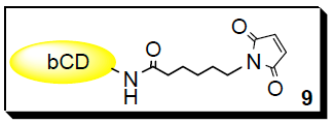
Preparation of PEI-PLL conjugate (**7**). The PEI-PLL **7** conjugate was prepared by mixing compound **4** (2.4×10^{-7} mol, 1.2 equiv.) and **6** (2.0×10^{-7} mol) in the 0.1 M MES buffered solution with a pH value of 4.7. The mixture was stirred overnight at room temperature. At the end of the reaction, the resulting PEI-PLL conjugate was purified in a MW 10,000 cut off filter tube by centrifugation and concentrated in 1 mL 1X PBS at pH 7.4. The yield of compound 7 as a purple clear solution was 92% (based on PEI) and the molar ratio between PEI/PLL was calculated as 1/1.1 by measuring the absorbance of rhodamine labeled in PEI and the Cy5.5 labeled in PLL.



Preparation of sulfhydryl group modified PEI-PLL conjugate (**8**). The purified PEI-PLL conjugate **7** (2.0 × 10^{-7} mol) was washed by 1X PBS pH 7.4 in a MW 10,000 cut off filter tube and concentrated to 1 mL. 100 µL deacetylation solution (0.5 M hydroxylamine, 25 mM EDTA in PBS, pH 7.4) was added to compound **7**. After incubation for 2 h at room temperature, the mixture was purified into PBS pH 7.0 supplemented with 10 mM EDTA by centrifugal filtration (MW 10,000 cut off). The yield of compound **8** was calculated as 96% by measuring the absorbance of rhodamine labeled in PEI. The determination of the degree of sulfhydrylation labeling on the PLL moiety was further measured using Ellman's reagent. Briefly, 250 µL of compound **8** with a concentration less than 0.1 mM (PLL moiety) was added to the mixture of 2.5 mL reaction buffer (PBS, pH 8.0 supplemented with 1 mM EDTA) and 50 µL Ellman's reagent solution (4 mg/mL). Meanwhile, cysteine standards at concentrations of 1.5, 1.25, 1.0, 0.75, 0.5, 0.25 and 0 mM were also incubated with 2.5 mL reaction buffer. After incubating for 15 min, the absorbance of the compound **8** at 412 nm was read and fitted to the calibration curve that was prepared by plotting the A_{412} of the cysteine standards with the cysteine concentrations. The concentration of sulfhydryls can be calculated from the absorbance of the product TNB (ε_{412} = 714,150 $M^{-1}cm^{-1}$). On average, 2.5 sulfhydryl groups were functionalized on each PPL polymer.

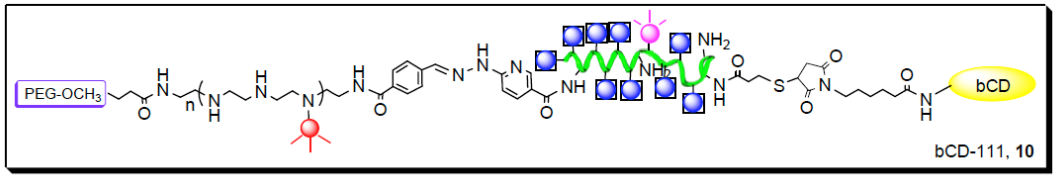


Preparation of maleimide group modified bacterial cytosine deaminase (bCD) protein (**9**). Free bCD protein was isolated with a good yield consistent with our previous work [1].



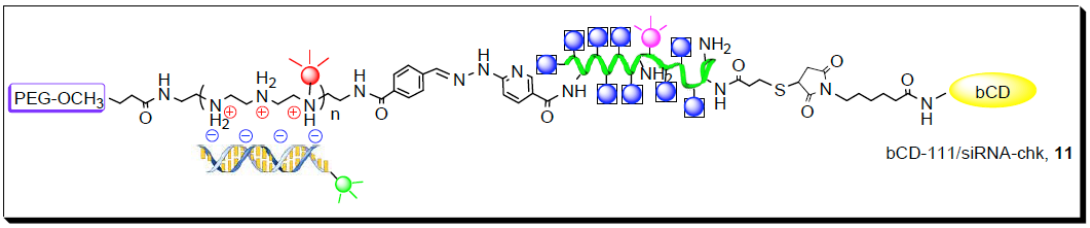
N-[ε-maleimidocaproyloxy]-*N*-succinimide (EMCS) (0.5 mg, 1.2×10^{-6} mol, 6.0 equiv.) dissolved in 50 µL anhydrous DMSO was added to the solution of bCD in 2.0 mL PBS pH = 7.4 (60 mg, 2.0×10^{-7} mol). The mixture was rocked gently for 1 h, then purified and concentrated into 1 mL PBS pH 7.4 by centrifugal filtration (MW 10,000 cut off) as described above. The yield of compound **9** was calculated as 90% by measuring the absorbance of bCD protein (A_{279} = 76 $mM^{-1}cm^{-1}$).

Preparation of bCD-111 conjugate (**10**). 1.1 equivalent of sulfhydryl functionalized PEI-PLL copolymer **8**



(2.2×10^{-7} mol) in 1 mL PBS 7.0 was mixed with the sulfhydryl group modified bCD **9** (2.0×10^{-7} mol) in 1 mL PBS pH 7.0. After incubation for 1 h at room temperature, the mixture was purified into PBS pH 7.4 by centrifugal filtration (MW 100,000 cut off). The yield of bCD-111 conjugate was about 86% (based on bCD) by measuring the absorbance of bCD at 279 nm. The molar ratio between bCD hexamer/PEI/PLL was determined as 1/1.05/1.2 by measuring the absorbance of bCD (ε_{279} = 76 $mM^{-1}cm^{-1}$), rhodamine (ε_{572} = 120,000 $M^{-1}cm^{-1}$) and Cy5.5 (ε_{680} = 250,000 $M^{-1}cm^{-1}$) respectively. The final bCD-PEI-PLL conjugate: bCD-111 **10** was stored in a mixture of 1X PBS pH 7.4 and glycerol with a volume ratio of 1/1 under -20°C.

Preparation of bCD-111/siRNA-chk nanoplex (**11**). **10** in 250 µL 1X PBS 7.4 with a concentration of 2×10^{-4} M (5×10^{-8} mol) was mixed with the siRNA-chk in 30 µL pure water with a concentration of 4.8×10^{-4} M.



The bCD-111/siRNA-chk nanoplex **11** as a purple color solution was formulated by keeping the mixture at room temperature for 30 min. The N/P ratio of the resulting nanoplex should be 50. Nanoplex **11** could be used directly without further purification before the experiments.

3. Characterization of Nanoplex 11

The molar equivalent ratios of Cy5.5 and Gd^{3+}-DOTA to PLL in **4** and labeled rhodamine to PEI in **5** were determined by measuring the absorbance of Cy5.5 (ε_{680} = 250,000 $M^{-1}cm^{-1}$), rhodamine (ε_{520} = 94,500 $M^{-1}cm^{-1}$), PLL (ε_{213} = 46,800 $M^{-1}cm^{-1}$), and the Gd^{3+} ion concentration was determined by inductively coupled plasma mass spectrometry (ICP-MS). The molar ratio between grafted PEG and PEI in co-polymer **5** was measured by quantifying the integrated proton numbers of C*H*$_2$-O (≈ 3.7 ppm) in PEG chain and C*H*$_2$-N (2.4~3.0 ppm) in PEI by ^{1}H NMR spectroscopy. In compound **10**, the molar ratio of bCD hexamer/PEI/PLL was determined by measuring the absorbance of bCD (ε_{279} = 76 $mM^{-1}cm^{-1}$), rhodamine and Cy5.5. The integrity of nanoplex **11** was verified by the overlapping of the eluting peaks monitored at 280 nm (bCD), 520 nm (rhodamine) and 490 nm (FITC) in separated gel permeation chromatographic (GPC) studies. The molecular weight of nanoplex **11** was determined by fitting to a calibration curve obtained by running protein standards with a gel permeable chromatography (GPC) column. The mean hydrodynamic diameter and zeta potential of nanoplex **11** was measured by dynamic light scattering (DLS). The average longitudinal relaxivities of **3**, **7** and **10** was measured by 9.4 T MRI at 25 °C.

3.1. Determination of the Molecular Weight of Nanoplex 11

All size exclusion chromatography (SEC) studies were typically carried out as outlined below unless otherwise stated. Mobile phase: 1X PBS, pH 7.4; isocratic flow rate: 0.7 mL/min; operating temperature: 25 °C; injected volume: 20 µL. The effluent peaks of bCD-111/siRNA-chk nanoplex **11** were monitored at 280, 520 and 488 nm that correspond to the absorbance of the bCD protein, rhodamine labeled in the PEI vector, and FITC labeled in the siRNA-chk in separate experiments (**Figure S1A**). Overlapping of the three peaks verifies the entity of nanoplex **11**.

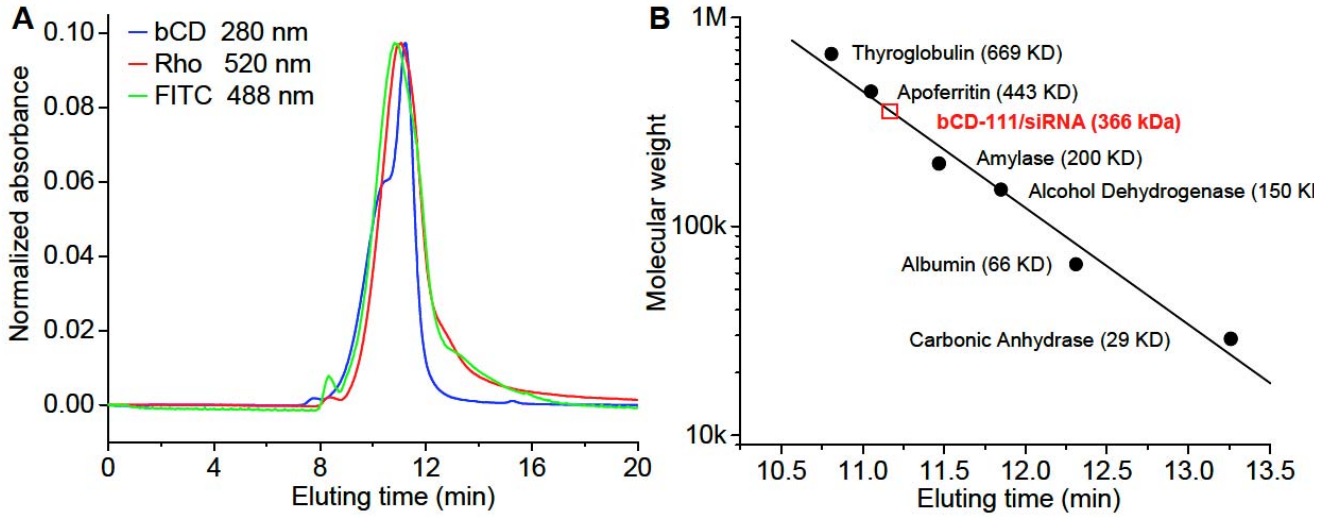


Figure S1. Determination of the molecular weight of nanoplex **11** by size exclusion chromatography (SEC). (**A**) The effluent peaks of nanoplex **11** (N/P = 50) were monitored at 280 nm (the absorbance of bCD protein), 520 nm (the absorbance of rhodamine labeled in PEI vector) and 488 nm (the absorbance of FITC labeled in siRNA-chk duplex). Overlapping of the three peaks verified the entity of the bCD-111/siRNA-chk nanoplex. (**B**) The calibration curve of the SEC column that was fitted by running a series of protein ladders (molecular weight ranged from 29–669 kDa) by using the same HPLC method. The molecular weight of nanoplex **11** was labeled in this calibration curve with red.

The gel-permeable column was calibrated by the Gel Filtration Molecular Weight Kit (Aldrich, St. Louis, MO), which comprises of a set of protein markers in the range of 29 kDa to 669 kDa. The eluting times of the different protein standards were measured with the same HPLC method. The molecular weight calibration curve was obtained by plotting molecular weight *vs*. eluting time of each protein standard

(**Figure S1B**). The average molecular weights of nanoplex **11** were obtained as 366 kD by fitting its peak eluting time against the calibration curve (**Figure S1B**).

3.2. Determination of Size Distribution and Zeta Potential of Nanoplex 11

Hydrodynamic radius and size distribution of free bCD, conjugate **10** and bCD-111/siRNA-chk nanoplex **11** were determined by dynamic light scattering (DLS).

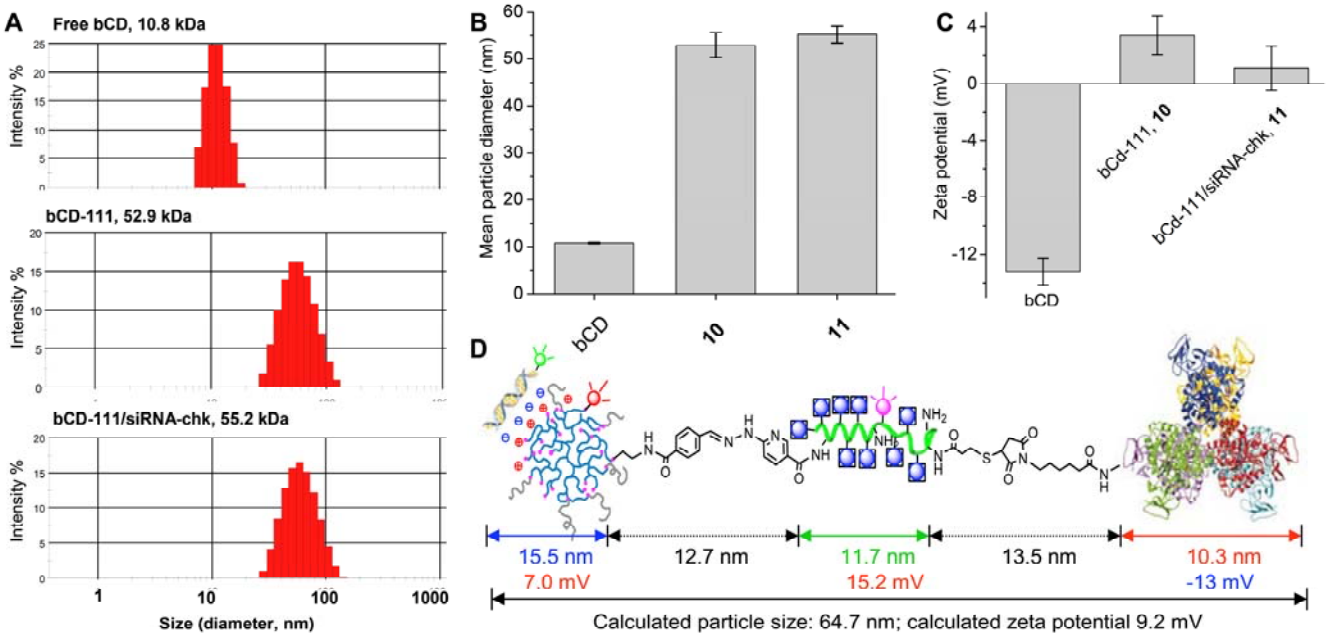


Figure S2. Determination of the particle size distribution (**A**), average particle sizes (**B**) and average zeta potential (C) of bCD-111 conjugate **10** and bCD-111/siRNA-chk nanoplex **11** by dynamic light scattering (DSL) (n = 6). The particle size distribution and average particle diameter were measured in 1X PBS (pH 7.4) with a bCD hexamer concentration of 3–6 μM. The diluted free bCD, conjugate bCD-111 **10**, nanoplex **11** in PBS were filtered through a 0.22 μm PVDF filter. (**D**) The average size and surface charge of the three individual components including bCD hexamer (10.3 nm, -13 mV), PEI-siRNA-chk complex (15.5 nm, 7.0 mV) and PLL (11.7 nm, 15.2 mV) in the bCD-111 **10** conjugate were measured by DLS. The length of the two linkers (12.7 and 13.5 nm) were estimated from the vendor's protocol (Pierce). The particle size and zeta potential of conjugate **10** were calculated as 64.7 nm and 9.2 mV by addition of the corresponding values of the individual components.

To prepare the sample for DLS studies, the bCD derivatives were diluted to PBS pH 7.4 at a concentration of 1 mg/mL and filtered through a 0.22 μm filter to remove any bulky dust. The instrument was calibrated by the standard polystyrene beads with a diameter of 60 nm in PBS before the measurements. The zeta

potential of nanoplex **11** in 0.1 M NaCl was measured in a clear zeta Cell cuvette with a concentration of about 2 mg/mL. The instrument was calibrated with 300 nm polystyrene latex in a pH 9.2 buffer with a zeta potential of -50 mV. The average zeta potential was estimated from 20 acquisitions.

3.3. Determination of the Relaxivity of the Conjugate bCD-111 10

The molar ratio of bCD hexamer/PLL/PEI in conjugate bCD-111 **10** was determined by measuring the absorbance of bCD subunits (ε_{279} = 76,000 $M^{-1}cm^{-1}$), rhodamine (ε_{520} = 94,500 $M^{-1}cm^{-1}$) and Cy5.5 (ε_{680} = 250,000 $M^{-1}cm^{-1}$) moieties in **10** (**Figure S3A**) according to the Beer-Lambert law: A = εbc, where **A** is absorbance, **ε** is the molar extinction co-efficacy with units of L mol^{-1} cm^{-1}, **b** is the path length of the sample with unit of cm, **c** is the concentration of the compound in solution, expressed in mol L^{-1}. To measure the relaxivity of Gd^{3+} ion in **10**, the number of gadolinium chelates on **10** was firstly measured by ICP-MS. The standard solutions with the Gd^{3+} concentration of 1, 5, 10, 20, 50, 100, 200 ppm in 3% nitric acid were prepared, and a calibration curve was made by plotting the corresponding intensity counts *vs.* the Gd^{3+} concentrations (**Figure S3B**). Meanwhile, **10** was diluted to 3% nitric acid with a final concentration of 1.0 μM. The Gd^{3+} concentrations of different samples were obtained by fitting the detected Gd^{3+} peak intensities against the calibration curve (**Figure S3B**). The experimental result showed that approximately 11 Gd^{3+}-DOTA chelates were labeled in conjugate **10**. The longitudinal relaxivities of conjugate **10** and its precursors including compound **3**, **7** and commercial available MR contrast agent Gd^{3+}-DOTA as a control molecule were determined according to the equation of r_{1p} = $(1/T_{sample}-1/T_{PBS})/[Gd]$.

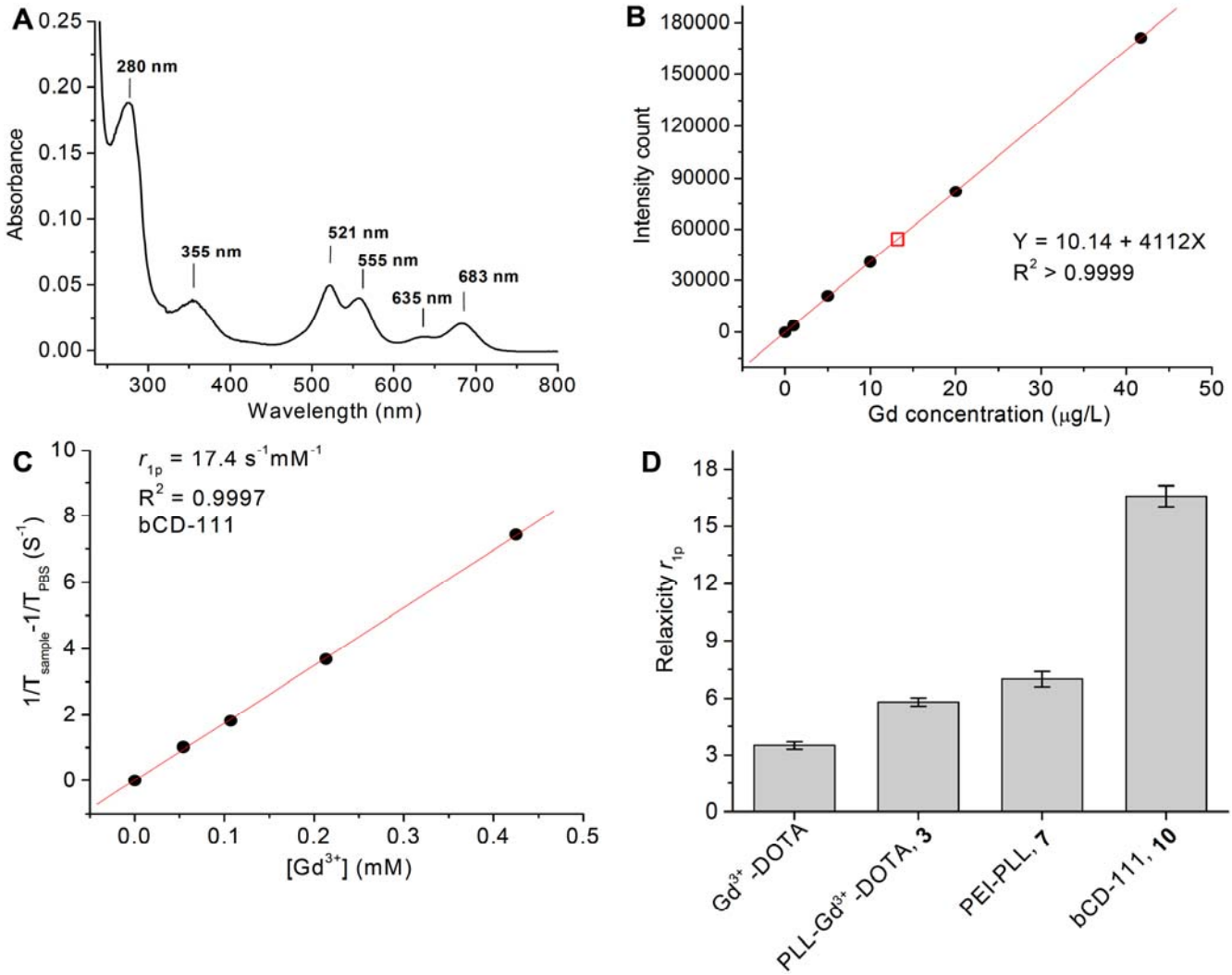


Figure S3. Determination of the absorbance and longitudinal relaxivity of conjugate bCD-111 **10**. (**A**) Absorbance of **10** (1 μM) in 1X PBS (pH 7.4) at 25 °C. The main absorption peaks can be attributed to bCD protein (279 nm), hydrazone (355 nm), rhodamine (521 and 555 nm) and Cy5.5 (635 and 683 nm). (**B**) The calibration curve of gadolinium ion concentration that was fitted by measuring the Gd standard solutions with concentration of 1, 5, 10, 20 40 μg/L in ICP-MS. The number of the labeled Gd^{3+}-DOTA chelates in conjugate **10** was calculated as 11 by fitting the measured Gd^{3+} concentration with the calibration curve. (**C**) The longitudinal relaxivity of **10** was determined according to the equation of r_{1p} = $(1/T_{sample}-1/T_{PBS})$/Conc[Gd]. To measure the r_{1p} value, the T_1 values of four bCD-111 solutions with different concentrations in PBS pH 7.4 were measured at 9.4 T at 25 ° C. Gd^{3+} concentration of each samples can be calculated from **10** concentration. (**D**) The measured relaxivities of compounds **3**, **7** and **10** at 9.4 T at 25 °C.

The T_1 values of PBS and selected compounds with four different concentrations in PBS pH 7.4 were measured with a 9.4 T MR spectrometer at 25 °C. Plotting the $(1/T_{sample}-1/T_{PBS})$ values of **10** with corresponding Gd^{3+} concentrations measured by ICP-MS provided the relaxivity of **10** (**Figure S3C**). The longitudinal relaxivities of Gd^{3+}-DOTA, **10** and its derivatives are demonstrated in **Figure S3D**.

3.4. Kinetic Studies of conjugate 10 to Substrates Cytosine and 5-FC

Enzyme assays with cytosine as the substrate were performed according to a previously reported method [1]. Kinetic values of conjugate **10** relative to bCD were obtained by measuring the time-dependent absorbance of cytosine at 286 nm. Reaction solutions with the cytosine concentrations of 0.05, 0.1, 0.2, 0.4, 0.5, 0.6, 0.8, 1.0 mM were prepared in 50 mM Tris-HCl pH 7.5. After adding the enzyme, the absorbance at 286 nm was recorded immediately at every 15 s for a total of 5 min at 25 °C. The initial velocity of enzyme to catalyze cytosine at corresponding concentration can be calculated from the absorbance decrease at 286 nm ($\Delta\varepsilon_{286}$ = –0.68 $mM^{-1}cm^{-1}$). These measurements were repeated 3–4 times by using the same amount of enzyme for each cytosine concentration. Woolf plots were used to determine Michealis-Menton constant K_m and turn over number, k_{cat}, ($k_{cat} = V_{max}/[E]$, where V_{max} is the maximal catalytic velocity; [*E*] is the total enzyme concentration) of bCD-111 **10** or bCD (**Table S1**).

Table S1: Kinetic values of bCD and conjugate bCD-111 **10**[a]

	Cytosine (5-Fluorocytosine)				
	K_m	k_{cat}[c]	k_{cat}/K_m	specific activity	R.S.[f]
	(*mM*)	(s^{-1})	($mM^{-1}\ s^{-1}$)	($\mu mol \cdot min^{-1} mg^{-1}$)	
bCD[b]	0.19 (3.9)	185 (71)	973 (18)	144[d] (58)[e]	0.018
bCD-111[b]	0.18 (3.7)	142 (69)	788 (19)	119[d] (61)[e]	0.024

[a]Determined in 50 mM Tris buffer, pH 7.5 at 25 °C; [b]Protein concentrations were measured from the absorbance of bCD monomer (ε_{279} = 76 $mM^{-1}cm^{-1}$). [c]k_{cat} values were calculated using the equation $k_{cat} = V_{max}/[E]$. [d]Determined in 0.5 mM cytosine. [e]Determined in 9.6 mM 5-FC. [f]Relative specificities (R.S.) were calculated using the equation: $[k_{cat}/K_m(5\text{-FC})]/[k_{cat}/K_m(\text{cytosine}) + k_{cat}/K_m(5\text{-FC})]$.

Similar enzyme assays with 5-FC as the substrate were performed [2]. Reaction solutions (2 mL) containing 0.4, 0.8, 1.6, 3.2, 4.8, 6.4, or 9.6 mM 5-FC in 50 mM Tris-HCl pH 7.5 were incubated with **10** or bCD at 25 °C separately. Aliquots (50 uL) of the reaction solution were taken every 90 s over a 15 min period, and immediately quenched in 1.95 mL 0.1 M HCl. Initial catalytic velocity corresponding concentration can be calculated from the absorbance decrease at 297 nm ($\Delta\varepsilon_{297}$ = –0.41 $mM^{-1}cm^{-1}$).

Assays were repeated 3–4 times by using the same amount of enzyme for each 5FC concentrations. The kinetic parameters as K_m and k_{cat} were determined in the same way as for cytosine (**Table S1**).

4. Determination of Cytotoxicity of Conjugate 10 *in vitro*

The concentration dependent cytotoxicities of free bPEI, bPEI-PEG copolymer **5**, and conjugate bCD-111 **10** in highly invasive/malignant MDA-MB-231 human breast cancer cells were measured using an MTT assay. As demonstrated in **Figure S4A**, free bPEI showed high cytotoxicity and the IC_{50} was about 0.21 μM after 4 days incubation.

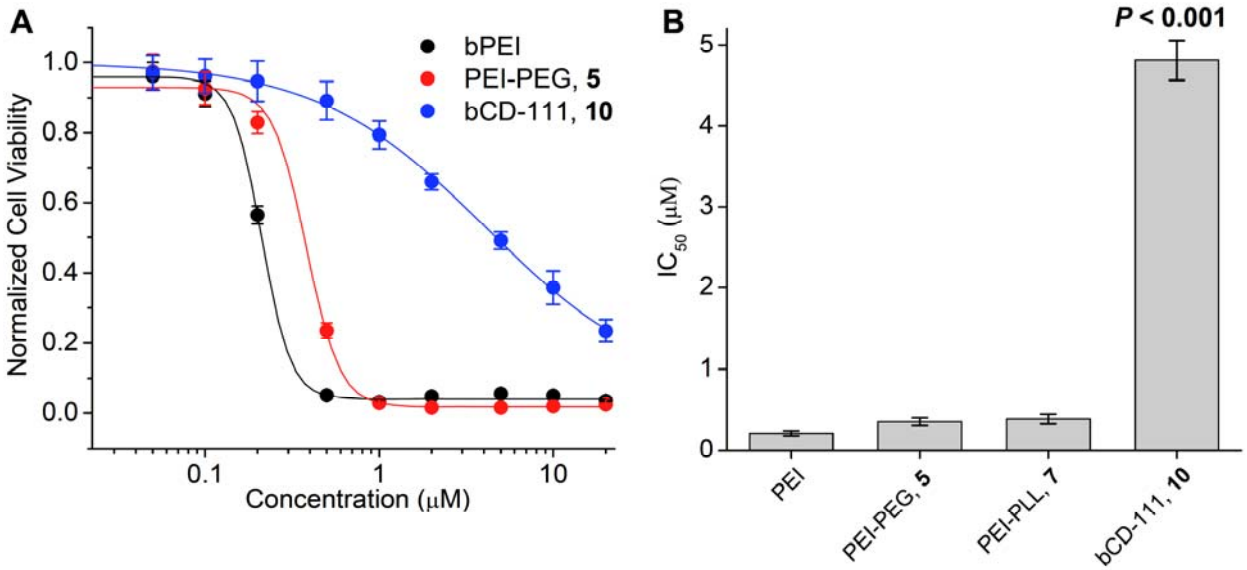


Figure S4. bCD-111 conjugate demonstrated much lower cytotoxicity than PEI and its derivatives in human breast MDA-MB-231 cancer cells. (**A**) Concentration (based on PEI moiety) dependent cytotoxicities of PEI, PEI-PLL **5** and bCD-111 **10** measured by MTT assay (n = 4). (**B**) IC_{50} of PEI and its derivatives in MDA-MB-231 cultured cells according to the results of panel A. In a typical MTT assay, cultured cells were treated with the selected compound at various concentrations (based on PEI moiety) for 4 days prior to MTT assay.

The PEGylation on bPEI substantially decreased the cyotoxicity of bPEI polymer, and the IC_{50} of compound **5** increased 67% to 0.35 μM. The bPEI-PLL copolymer **7** demonstrated very similar IC_{50} value (0.38 μM) compared to that of compound **5**, which indicates the grafted PLL moiety does not obviously affect the cytotoxicity. The cytotoxicity of **10** decreased significantly and the IC_{50} value increased 23

times to 4.81 μM in comparison with free bPEI (**Figure S4B**). Although the mechanism of the bCD protein induced reduction of cytotoxicity is not clear yet, it is possible that the bulky bCD hexamer may substantially shield the highly positive surface charge of bPEI polymer, which is a major cause of the high toxicity of the PEI polymer [3].

5. Determination of Enzymatic Stability of Internalized bCD-111 10 *in vitro*

The enzymatic stabilities of free bCD and conjugate **10** were tested in cultured MDA-MB-231 cells (**Figure S5**).

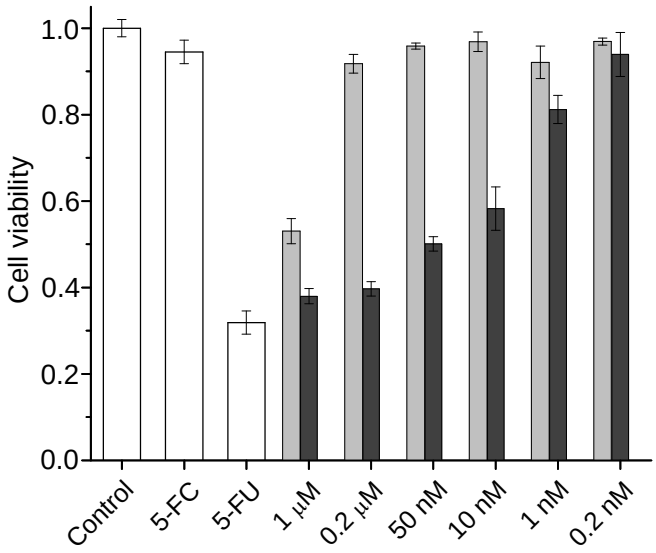


Figure S5. Conjugate **10** demonstrated higher enzymatic stability than free bCD in MDA-MB-231 human breast cancer cells. Cultured cells were treated with free bCD or **10** with various concentrations (based on bCD hexamer) for 4 h, washed and incubated for further 24 h. 5-FC (final concentration: 1.5 mM) was added and further incubated for 4 days prior to MTT assay. Light grey bar: free bCD; grey bar: **10**. Cells treated with media alone, 5-FC alone (1.5 mM) and 5-FU alone (1.5 mM) were used as the negative and positive controls.

The cells were treated with free bCD or **10** at various concentrations (0.2 nM–1.0 μM based on bCD hexamer) for 4 h. At the end of incubation, the cells were washed three times with 1X PBS and continually incubated for a further 24 h. 5-FC at a final concentration of 1.5 mM was added and the cells

were further incubated for 4 days prior to MTT assay. In the control experiments, the cells were treated with media only, prodrug 5-FC alone (1.5 mM) or active drug 5-FU alone (1.5 mM) for 5 days before MTT assay. As demonstrated in **Figure S5**, conjugate **10** showed much higher intracellular enzymatic activity than free bCD, and converted sufficient non-toxic 5-FC to toxic 5-FU that efficiently inhibited cell growth. Significantly, comparable cell viabilities were achieved between the treatment of 5-FU directly and the bCD-111/5-FC strategy. Moreover, significant cell growth inhibition was demonstrated even with a **10** concentration as low as 10 nM. In comparison, free bCD protein showed much lower stability in the intracellular surroundings than **10**, and no obvious therapeutic effect was observed when its concentration was below 1 μM. It is possible that the highly positive charges of bPEI and/or PLL polymers may prevent the interaction of the bCD moiety and the endogenous proteases that degrade the bCD protein in the cytoplasm.

6. N/P Value Dependent Stability of Nanoplex 11

The N/P ratio that represents the molar ratio of nitrogen (N) atoms in the delivery vector and phosphorus (P) atoms in the nucleotide is crucial for determining the stability and delivery efficacy of the polymer/siRNA nanoplex. Electrophoretic gel mobility shift assay (EMSA) was used to investigate the N/P value dependent condensation between conjugate **10** and siRNA-chk. As demonstrated in **Figure S6**, **10** and siRNA-chk were mixed in PBS pH 7.4 with N/P values of 0, 1, 5, 10, 20 and 30 with identical siRNA concentration of 10 μM. After 30 min incubation at room temperature, the resulting nanoplexes were loaded on a 1% agarose gel (1.0 μg siRNA-chk per well). After electrophoresis, the nucleotide was stained by ethidium bromide and UV light images were obtained (**Figure S6A**). Fluorescence images of the same agarose gel were also captured after exciting the rhodamine label in bPEI (**Figure S6B**), and FITC in siRNA-chk (**Figure S6C**) respectively, using an optical imaging system.

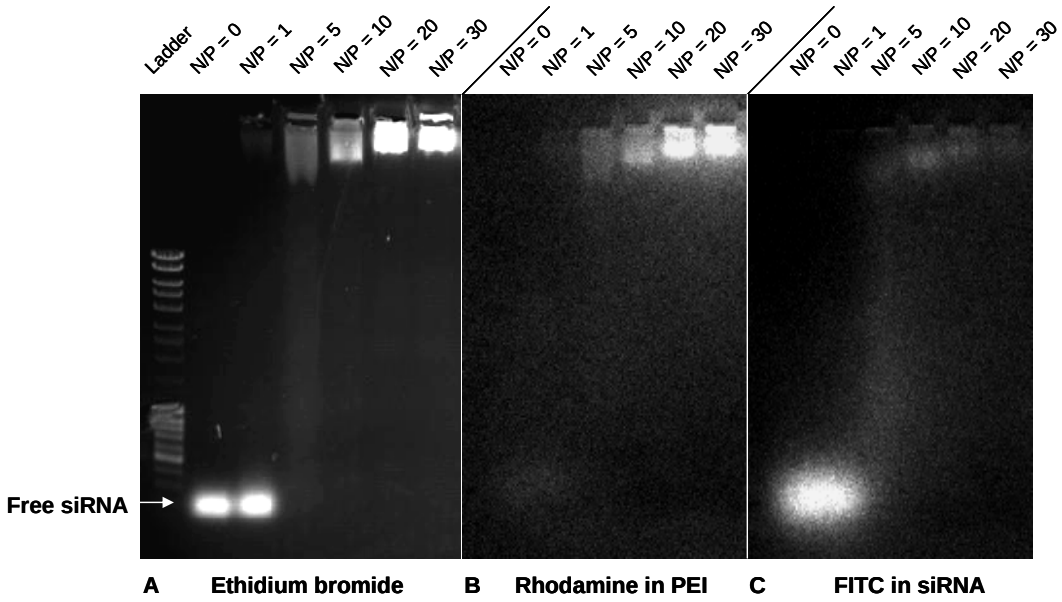


Figure S6. Electrophoretic gel mobility shift assay demonstrated that the stability of **10**/siRNA-chk nanoplex **11** is proportional to the N/P ratio. Stable **10**/siRNA-chk nanoplex was formed when the N/P value was above 20. Panel A shows the UV nucleotide image that was pre-stained by ethidium bromide. Rhodamine and FITC fluorescence images of the same gel are listed in panel B and C respectively.

EMSA studies demonstrated that **10** and siRNA-chk formed a tight nanoplex if the N/P ratio was greater than 20. A free siRNA-chk band was detected under UV and FITC fluorescence images when the N/P ratio was below 20.

7. The Stability of Encapsulated siRNA-chk in Fresh Mouse Serum

A nuclease stability assay was conducted to determine the stability of siRNA-chk encapsulated in nanoplex **11**. Typically, nanoplex **11** was formulated in PBS, pH 7.4 with a N/P ratio of 50 for 30 min (siRNA-chk concentration: 10 μM). The nanoplex was incubated for 0, 1, 4, 8 h in 70% fresh mouse serum at 37 °C. In a control experiment, naked siRNA-chk was incubated for identical periods under the same experimental conditions. At the end of incubation, all encapsulated siRNA was displaced from the nanoplex by adding 1% SDS and quickly loaded to the wells (0.67 μg/well siRNA) of 1% agarose gel.

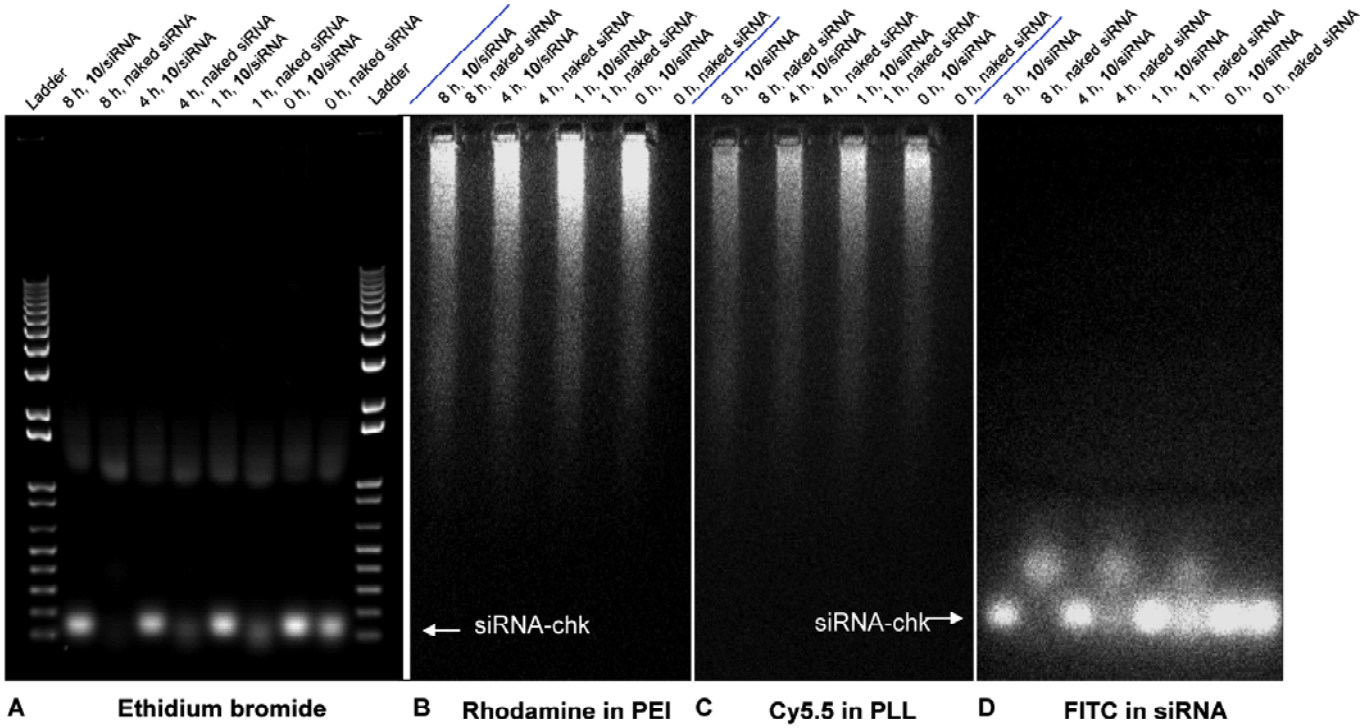


Figure S7. siRNA-chk encapsulated within the bCD-111/siRNA-chk nanoplex **11** showed high stability in fresh mouse serum. (**A**) Agarose gel electrophoresis of the nanoplex **11** (N/P = 50) and naked siRNA-chk after incubation in 75% fresh mouse serum for 0, 1, 4 and 8 h at 37 °C (nucleotide was stained by ethidium bromide). Rhodamine (**B**), Cy5.5 (**C**) and FITC (**D**) fluorescence images of the same agrose gel were demonstrated in panel **B**, **C** and **D** respectively. The fluorescence images in panel **B**–**D** were captured by the DsRed, Cy5.5 and GFP excitation and emission filters respectively in the optical imaging system.

As shown in the ethidium bromide stained UV image (**Figure S7A**) and FITC excited fluorescence image (**Figure S7D**) of the agarose gel, while naked siRNA-chk fully degraded in less than an hour, siRNA-chk encapsulated in nanoplex **11** was well protected from degradation even after 8 h incubation in 70% fresh mouse serum. Notably, similar siRNA delivery vector migration patterns were demonstrated in the rhodamine excited fluorescence images (**Figure S7B**) and Cy5.5 excited fluorescence images (**Figure S7C**) of the same agrose gel, which further verifies the covalent conjugation between rhodamine labeled PEI and Cy5.5 labeled PLL in the conjugate **10**.

8. The N/P Ratio Dependent Transfection Efficiency of Nanoplex 11 in vitro

Since we identified the important role of the N/P ratio in determining the stability of bCD-111/siRNA-chk

nanoplex, we further investigated the relationship between the N/P ratio and the siRNA transfection efficacy of the nanoplex in MDA-MB-231 cell culture. As demonstrated in **Figure S8**, the chk down-regulation efficiency of the nanoplex in MDA-MB-231 cells increased with the N/P ratio in the range of 10–50 with the final siRNA-chk concentration kept constant at 100 nM. This result correlated with the electrophoretic gel mobility shift studies (**Figure S6**), in which the stability of bCD-111/siRNA-chk nanoplex increased with the N/P ratio.

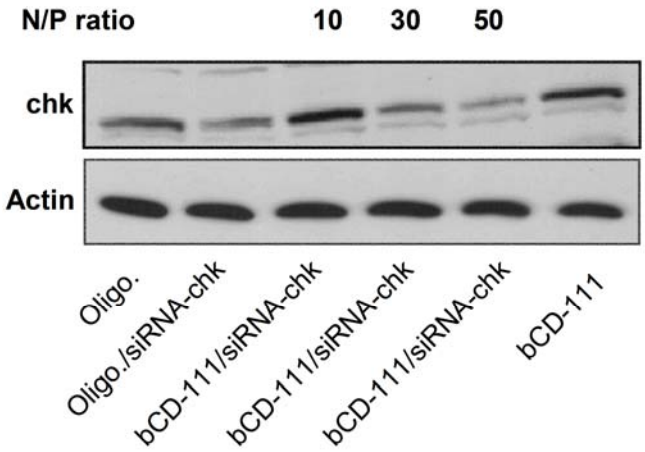


Figure S8. Transfection efficiency of bCD-111/siRNA-chk nanoplex **11** is proportional to the N/P ratio in the range of 10–50. Choline kinase western blots of MDA-MB-231 cells treated with oligofectamine alone (negative control), oligofectamine/siRNA (positive control), bCD-111 **10** vector alone (negative control), and nanoplex **11** with N/P ratio of 10, 30 and 50. Actin levels were probed as loading controls.

9. References

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(2) Hayden, M. S.; Linsley, P. S.; Wallace, A. R.; Marquardt, H.; Kerr, D. E. *Protein Expr Purif* **1998**, *12*, 173-84.
(3) Lee, C. C.; Liu, Y.; Reineke, T. M. *Bioconjug Chem* **2008**, *19*, 428-40.