Supporting Information

Synthesis of Polydixylitol based gene transporter (PdXYP):

A highly hyperosmotic gene delivery vector, PdXYP, was synthesized in three steps. Dixylitol (dXY) containing eight hydroxyl groups was synthesized by xylitol dimerization for which xylitol and acetone were condensed into crystalline xylitol diacetone (Xy-dAc). The terminal hydroxyl groups of Xy-dAc were then reacted with trifluoromethyl suphonyl chloride to make trifluoromethylsulphonyl xylitol diacetone (TMSXD). TMSXD was then reacted with an equal molar ratio of Xy-dAc in the presence of dry THF to form the xylitol diacetone dimer (dXy-dAc). dXy-dAc was finally converted into dXY by opening the rings in HCl–MeOH solution.

(a) Synthesis of dixylitol: Xylitol was first converted into crystalline diacetone xylitol by the method used by Raymond and Hudson for the condensation of xylitol and acetone[1]. The terminal hydroxyl group of xylitol diacetone was reacted with trifluoromethane sulphonyl chloride to make TMSXD. TMSDX was reacted with Xy-Ac (1.2 eq) in presence of dry THF to form dXy-Ac in dry THF [2] and the product was finally converted in to dXY by acidic ring opening as described in a previous reported method[3].

(i) XYdAc: Mixture of 10.0 g. of crystalline Xylitol, 200ml of acetone, 20 g of anhydrous copper sulfate and 200ul of concentrated sulfuric acid was stirred for 48 hours at 35^{o}C. Copper sulfate was separated by filtration and the filtrate was stirred with 10 g. of powdery calcium hydroxide for one hour to neutralize the acid; the solids were removed by filtration and the solvent by distillation in vacuum. The syrupy residue, weighing 7g was obtained. Crystallized by dissolving in hexane and cooling at -70^{o}C.

(ii) TMSXD: 2ml Trifluoromethanesulfonic anhydride (dissolved in 3ml DCM) was added drop wise to a solution of Xy-dAc (3g) and pyridine (2ml) in DCM (10 mL) at -30 °C. The reaction was stirred at -30 to -10 °C for 3 h after which time TLC (ethyl acetate/DCM, 1:1) showed the complete consumption of the starting material and the formation of one major product. The reaction was diluted with DCM (30 mL) and washed with HCl (2M, 20 mL), and the aqueous layer was extracted with DCM (3 x 20 mL). The combined organic layers washed with brine (30 mL), dried (magnesium sulfate) and concentrated under reduced pressure**,** compound is used without further purification for next step.

(iii) dXYdAc: Xy-dAc (2.0 g) and sodium hydride (65% suspension in oil, 0.6 g) were dissolved in dry tetrahydrofuran (THF, 50 mL) at room temperature and then a solution of TMSXD (1.2 eq) in dry THF (25 mL) was added slowly. The mixture was stirred for 12 h at room temperature. The solution was diluted with EtOAc and washed with brine. After evaporating the solvent, the crude compound was purified through column chromatography (SiO2; CH2Cl2/EtOAc, 4:1) to yield a transparent liquid.

(iv) dXY: dXYdAc was suspended in 2 M HCl (50 mL) and then MeOH was added to form a homogenous solution. The mixture was stirred at 90 oC for 6 h and then the solvent was evaporated under vacuum to yield the product.

(b) Synthesis of dXYdA: Dixylitol diacrylate (dXYA) monomer was synthesized by esterification of dixylitol with 2 equivalents of acryloyl chloride. An emulsion was prepared by dissolving dixylitol (1 g) in DMF (20 ml) and pyridine (10 ml) followed by dropwise addition of acryloyl chloride solution (1.2 ml dissolved in 5 ml DMF) at 4 ^{o}C with constant stirring [4] . After reaction completion, HCl–pyridine salts were filtered and the filtrate was dropped into diethyl ether. The product was precipitated in as a syrupy liquid and dried under vacuum.

(c) Synthesis of PdXYP. PdXYP was prepared by Michael addition reaction between LMW bPEI (1.2 kDa) and dXYA. Briefly, the synthesized dXYdA (0.38 g in 5ml DMSO) was added dropwise to 1 equivalent of bPEI (1.2 kDa, dissolved in 10 ml DMSO) and reacted at 60 ^{o}C with constant stirring for 24 h. After reaction completion, mixture was dialyzed using a Spectra/Por membrane (MWCO: 3500 Da; Spectrum Medical Industries, Inc., Los Angeles, CA, USA) for 36 h at 4 ^{o}C against distilled water. Finally, the synthesized polymer was lyophilized and stored at -70 ^{o}C.

Characterization of PdXYP and PdXYP/DNA polyplexes. ^{1}H NMR spectra dXY and PdXYP in D_2O were recorded using Advanced 600 spectrometer (Bruker, Germany). The mass spectra of the polymer was measured

by Matrix-Assisted Laser Desorption Ionization Mass Spectrometer Voyager-DETM STR Biospectrometry Workstation, MALDI TOF-TOF 5800 System. The elemental composition (C, H, N and O) of the monomer and polymer was measured by Thermo Flash EA1112 Elemental Analyser. The absolute molecular weight of PdXYP polymer was measured by gel permeation chromatography coupled with multiangle laser light scattering (GPC-MALLS) using a Sodex OHpack SB-803 HQ (Phenomenex, Torrells, CA, USA) column (column temperature 25°C; flow rate 0.5 ml/min). PdXYP polymer was then complexed with DNA (0.1µg) at various N/P ratios (0.1, 0.5, 1, 2, 3, and 5) for 30 min at RT and resolved on a 0.8% agarose gel (with 0.1 µg/ml EtBr) casted in 1X TAE buffer at 100 V for 40 min in 0.5X TAE running buffer. Images were captured under ultraviolet illumination. For DNase protection assay, PdXYP/DNA (N/P 20) polyplexes and free DNA were incubated with DNase I (1 µl, 50 units) in DNase/Mg^{2+} digestion buffer at 37°C. After 30 min, DNase was inactivated by adding 5 µl EDTA (100 mM) at 70°C for 10 min and incubated for another 30 min at RT. Finally, the protected DNA was released from the complexes with the addition of 5 µl 1% sodium dodecyl sulfate (SDS) for 2 h and resolved on a 0.8% agarose gel (with 0.1 µg/ml EtBr) in 0.5X TAE running buffer at 100 V for 40 min.

PdXYP/DNA polyplexes were characterized using a transmission electron microscope (EF-TEM) (LIBRA 120, Carl Zeiss, Germany) and a dynamic light scattering spectrophotometer (DLS 8000, Otsuka Electronics, Osaka, Japan). The specimens for TEM were prepared by drop-coating the PdXYP/DNA (N/P 20) and PEI25k/DNA (N/P 10) polyplex dispersion onto a carbon grid and then dried for 2 h, after which it was stained with 1% uranyl acetate (10 s) and observed for its morphology. DLS samples were prepared at various N/P ratios (5, 10, 15, 20, 25, and 30) of PdXYP/DNA polyplexes with 40 µg/ml DNA and then measured for their hydrodynamic size and zeta potential with 90° and 20° scattering angles at 25°C.

Osmolarity measurement. The osmolarity of sorbitol, mannitol, dixylitol and PdXYP/DNA polyplexes at different concentrations (2%, 3%, 5%, 10%) were measured as mOsm using a cryoscopic osmometer 030 (GANAte, USA) and calculated as depression in freezing point of solutions.

Cell culture and animal studies. Low passage human hepatocellular liver carcinoma (HepG2) cells, human cervix epithelial carcinoma (HeLa) cells and adenocarcinoma human alveolar basal epithelial (A549) cells were cultured in T-75 culture flasks (Nunc, Thermo Scientific) in 15 ml complete medium containing low glucose Dulbecco's Modified Eagle's culture medium (DMEM) (Sigma, USA) for HepG2 and HeLa cells and Roswell Park Memorial Institute (RPMI)-1640 culture medium for A549 supplemented with 10% heat-inactivated FBS (Hyclone Laboratories, USA) and 1% antibiotic cocktail of streptomycin and penicillin. Primary rat astrocytes were maintained in DMEM F-12 medium supplemented with fetal bovine plasma derived serum (10%), heparin (1850 U/ml), bFGF (1.5 ng/ml), insulin (5 µg/ml)-transferrin (5 µg/ml)-sodium selenite(5 ng/ml), hydrocortisone (500 nM) and gentamicin (50 µg/ml). Cells were maintained under standard culture conditions of 37°C and 5% CO_2 for 4 or 5 days, with the culture duration selected to maintain cells under exponential growth and reach ~80% confluency. Cells were then trypsinized, centrifuged at 1200 RPM for 5 min to pellet cells and counted using hemocytometer to seed the cells for experimental assays. For animal study four weeks old nude Balb/c mice were obtained from Orient Bio Inc. (Republic of Korea) and kept in a laboratory animal facility maintained at 23 ± 2°C and 50 ± 20% relative humidity under a 12 h light/dark cycle. All experimental protocols were reviewed and approved by the Animal Care and Use Committee at Seoul National University (SNU-120409-3).

In vitro transfection and cytotoxicity of PdXYP/DNA polyplexes.

Cytotoxicity of PdXYP/DNA and PEI25k/DNA polyplexes at various N/P ratios (5, 10, 15, 20, and 30) were measured in three cancer cell lines (A549, HeLa, and HepG2) and primary rat astrocytes by the reduction of a tetrazolium component (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, or MTT) (Sigma, St. Louis, Mo, USA) into insoluble purple colored formazan crystals by the mitochondria of the viable cells. Polyplex transfected cells that had been incubated in a 24-well plate (10 x 10^4 initial cell density/well) for 36 h were then incubated with MTT reagent (0.5 mg/ml in 1X PBS) for 3 h, followed by the addition of DMSO (500 µl) to solubilize the colored crystals, and absorbance was measured at 540 nm using a Sunrise™ TECAN ELISA reader (Grödig, Austria).

A549, HeLa, HepG2 cells and primary rat astrocytes at 80% confluency (10 x 10^4 initial cell density/well) in a 24-well plate were transfected with PdXYP/pGL3 (1 µg) and PEI25k/pGL3 polyplexes at various N/P ratios (5,

10, 15, 20 and 30) in serum-free medium, which 3 h later was exchanged with 10% serum containing medium . After 24 h, luciferase assay was performed according to the manufacturer's protocol. A chemiluminometer (Autolumat, LB953; EG&G Berthold, Germany) was used to measure relative light units (RLUs) and normalized with protein concentration in the cell extract estimated using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Transfection activity was measured in triplicate as RLUs per mg protein. Transfection of PdXYP/DNA (N/P 20) was also compared with PEI1.2k (N/P 10) and PEI1.2k + dixylitol (42 mol%). The percentage transfection efficiency of PdXYP/tGFP (1 µg) (N/P 20) was measured and compared with PEI25k/tGFP (N/P 10) in A549 cells 36 h post-transfection using flow cytometry (BD Biosciences, San Jose, CA, USA). Cell expressing GFP acquired from a total of 10000 cells were scored through a FACS calibrator.

Degradation study. TRITC (Molecular Probes, Invitrogen, Oregon, USA) (25 µl, 1 mg/100 µl in DMF) was added to PdXYP (1 ml, 10 mg/ml in H_2O) to block ~1% of its total amines, and the mixture was then stirred overnight ($PdXYP^T$). Unreacted TRITC was removed by washing with ethyl acetate (3 x 2 ml), which was then lyophilized and resuspended in water. A549 cells (3 x 10^5 initial cell density/well) after 24 h of incubation in a cover glass bottom dish (SPL Lifesciences, Korea) were transfected with $PdXYP^T$/DNA polyplexes and further incubated for 3 h, 2 d, 3 d, 5 d, and 7 d to study the degradation profile of $PdXYP^T$ polyplexes. The transfected cells were washed 3 times with 1X PBS and then fixed with 4% paraformaldehyde for 10 min at 4°C. DAPI was used to counter-stain the nuclear DNA of fixed cells, and images were procured using a Carl Zeiss LSM 710 inverted laser scanning confocal microscope with ZEN software to monitor fluorescently labeled $PdXYP^T$/DNA polyplexes inside the treated A549 cells. Cell viability was also observed after 3 h, 2 d, 5 d, and 7 d of transfection by MTT assay.

BBB transmigration assay. Transmigration experiments were conducted on day 5 after the complete activation of BBB kit (Pharmacocell, Japan) when TEER values in each well were > 150 Ωxcm^2. DNA was fluorescently labeled ($tGFP^F$) with bisBenzimide H33258 (Sigma, USA). 1.2 ml media was added to the lower chamber and 0.3 ml media containing free $tGFP^F$ or PdXYP/$tGFP^F$ or PEI25k/$tGFP^F$ polyplexes were added to the upper chamber of the in vitro BBB kit and incubated at 37 °C for 3 h. 1 ml media was aspirated from the lower chamber and analyzed spectrofluorometrically to determine polyplex BBB-permeability assay. Inserts without cells on the transwell membrane were used as blanks. In other experiments upper chamber of BBB were added with free tGFP and PdXYP/tGFP polyplexes for 3 h. After 48 h the primary rat astrocytes in the lower chamber were analyzed for transfection efficiency using FACS and their lysates for western blot.

Western blot analysis. After transfection, cells were harvested and lysed with 1X RIPA lysis buffer (Millipore, MA, USA). A BCA protein assay kit (Thermo scientific, MA, USA) was used to measure the protein concentrations. Equal amounts of the protein (25 µg) from each sample were separated by a Novex NuPAGE 4-12% SDS-PAGE gel (Life technologies, CA, USA), transferred to nitrocellulose membrane using iBlot (Invitrogen, USA) and then non-specific binding sites were pre-blocked with 5% skim milk for 1 h at RT. The membrane was washed and probed with anti-caveolin1 (Abcam, ab17052), anti-GFP (Santa Cruz, CA, USA) and anti-β-actin (Abfrontier, Seoul, Korea) antibodies (1:500 dilution) overnight at 4°C. Then, the membrane was incubated with secondary antibody (1:1000 dilution) conjugated with HRP (Invitrogen, CA, USA). Bands were captured using a ChemiDocTM XRS+ (Biorad, CA, USA) imaging system. The band intensities were analyzed quantitatively using ImageJ software (NIH, USA) and plotted as the mean pixel value.

Tumor implantation, in vivo bioimaging and biodistribution. Five weeks old nude Balb/c mice (male, 4 mice/group) were subcutaneously injected with 100 µl of a single cell suspension containing 3 x 10^6 A549 cells. When the tumor size reached 800-1000 mm^3, 100 µl of PdXYP/pGL3 (30 µg) complexes (N/P 20) in normal saline was locally injected into the tumor. PEI25k/pGL3 (N/P 10) complexes prepared under identical conditions were used as vector control, while naked pGL3 was used as a negative control and the mice were bioimaged after 7 days. The IVIS Imaging system 100 (Xenogen) with Living Image software was used for in vivo bioimaging to analyze the luciferase expression. The mice were anaesthetized by intraperitoneal (IP) injection of a Zoletil (40 mg/kg): Rompun (10 mg/kg) (4:1) mixture diluted 8 times in sterile 1X PBS. 200 µl of D-luciferin (15 mg/ml stock solution in DPBS) for a 20 g mouse (3 mg/mouse) was injected intraperitoneally and was quickly distributed throughout the body. Luciferase expressed in cells reacts with luciferin to emit luminescence, which was captured by the IVIS system to show images with intensity proportional to luciferase expression. Images were captured in the plateau phase which usually occurs after 15 min and lasts for 15-20 min.

For in vivo biodistribution, 6 week old nude Balb/c mice (male, 4 mice/group) were intravenously injected with

100 µl of PdXYP (N/P 20) and PEI25k (N/P 10) polyplexes in normal saline. Naked pGL3 (30 µg) was used as control. After 5 d, luciferase expression was monitored in various organs of mice using IVIS Imaging system. For luciferase quantification different organs of mice were dissected out, weighed, homogenized, suspended to 25% w/v homogenate in 2.5X lysis buffer (Promega, USA) and protein concentration in cell lysates were estimated using chemiluminometer.

Lipid raft co-localization study. DNA (1 µg) was labeled with YOYO-1 iodide (2 µl, 1 mM in DMSO) by stirring for 2 h at 25°C in dark and stored at -20°C (DNA^F). A549 cells (3 x 10^5 initial cell density/well) after 24 h of incubation in a cover glass bottom dish (SPL Lifesciences, Korea) were transfected with PdXYP/DNA^F and PEI25k/DNA^F polyplexes and further incubated for 15, 30 min. Cells were rinsed with 1X PBS, fixed with 4% paraformaldehyde at 37°C for 10 min, washed twice with ice cold PBS and then permeabilized with ice-cold 0.2% Tween 20 (in PBS) for 10 min. Non-specific binding was blocked using 10% BSA in 1X PBS for 5 min at RT and then at 4°C for 1 h because cooling prevents endocytosis of antibodies. Lipid rafts of the fixed cells were then labeled using the orange-fluorescent Alexa Fluor 555 lipid raft labeling kit (Invitrogen) according to the recommended protocol. Nuclei were stained with DAPI (0.1 µg/ml) for 10 min and mounted with Aqua poly/mount (Polysciences, PA, USA). The images were procured from confocal microscopy at 555/565 Abs/Em.

Endocytosis inhibition study. A549 cells were incubated with caveolae endocytosis inhibitor, methyl β-cyclodextrin (0, 2.5, 6.5, 10 mg/ml) for 1 h at 37°C and then transfected with PdXYP/DNA polyplexes. Similarly, A549 cells were incubated with endosome proton pump inhibitor, bafilomycin A1 (200 nM) for 10 min and transfected with PdXYP/DNA and PEI25k/DNA polyplexes. Luciferase activities were measured as RLUs per mg protein.

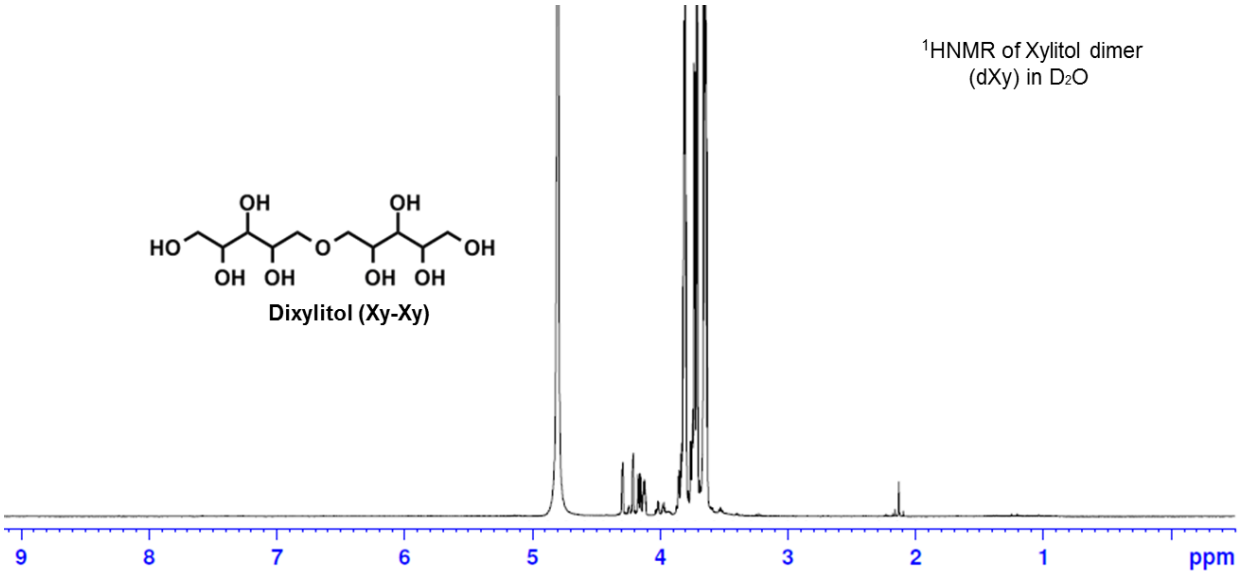

Fig. S1 ^{1}HNMR of Xylitol dimer (dXy) in D_2O

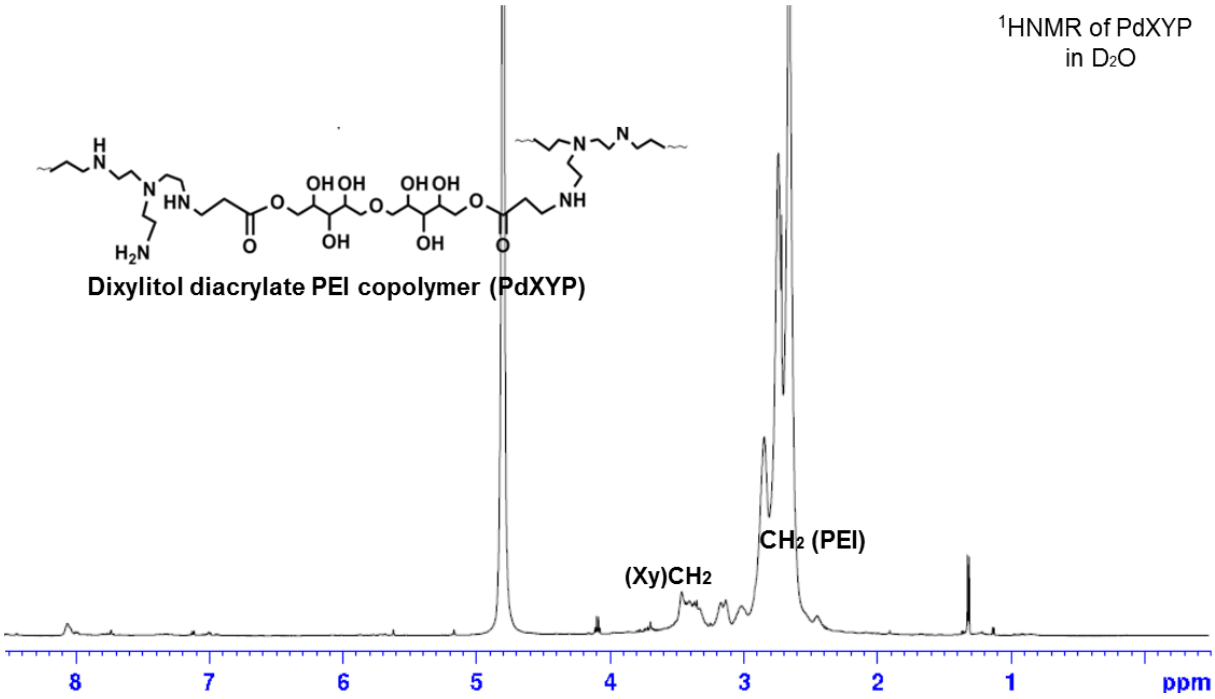

Fig. S2 ^{1}HNMR of PdXYP in D_2O

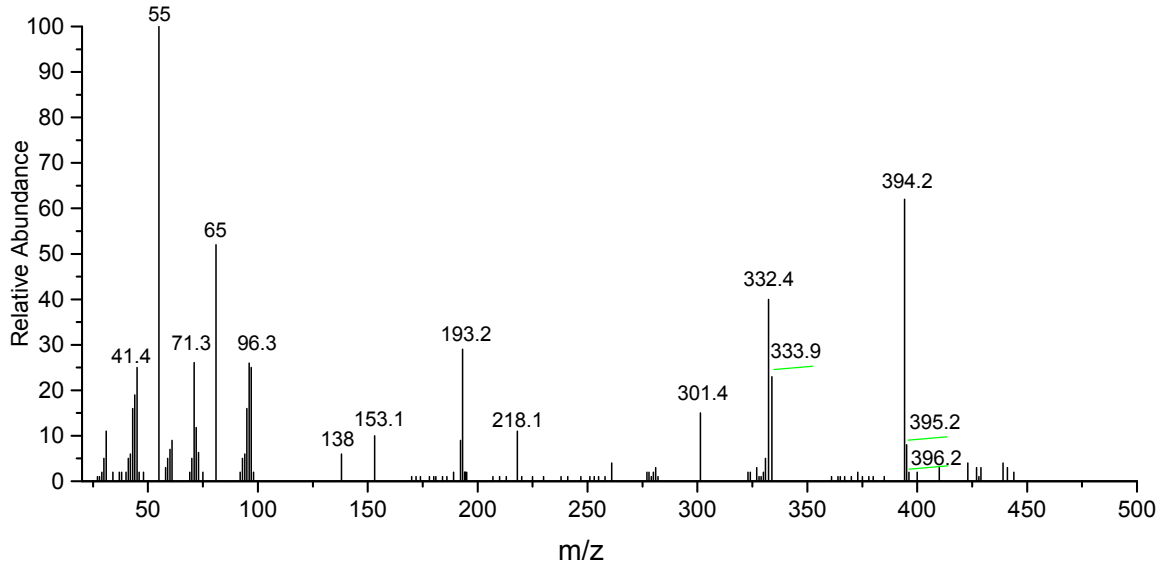

Fig. S3 MS spectra of dXYdA

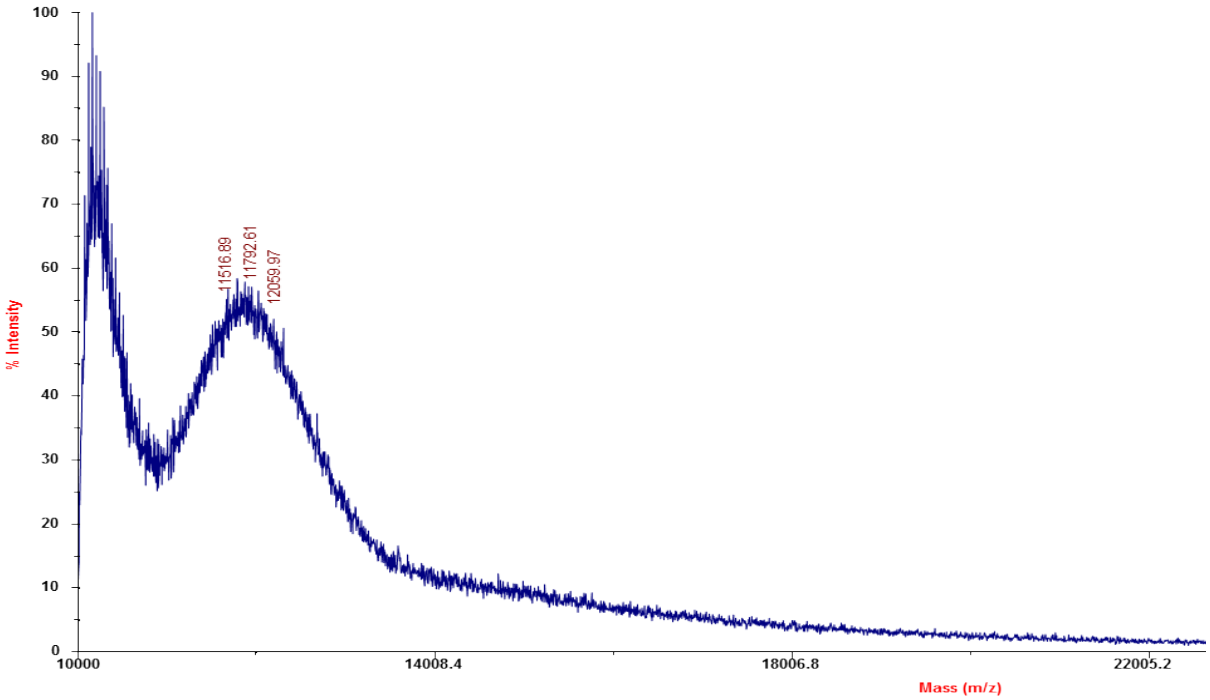

Fig. S4 MALDI-TOF-MS of PdXYP

Elemental analysis PdXYP and dXYdA. Elemental analysis of PdXYP found: C, 53.43; H, 11.03; N, 26.88; O, 8.66. Calcd. for C90H214N38O11: C, 53.92; H, 10.76; N, 26.55, O, 8.78 %. For dXYdA Elemental analysis found: C, 48.66; H, 6.81; O, 44.49. Calcd. for C16H26O11: C, 48.73; H, 6.65; O, 44.63%

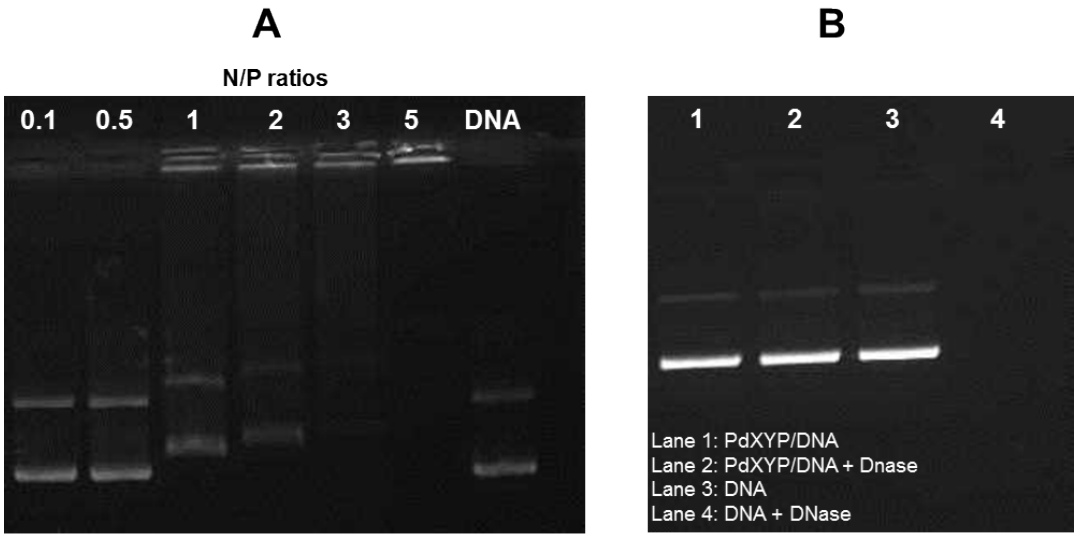

Fig. S5 Electrophoretic mobility shift assay (A) Gel electrophoresis of PdXYP/DNA (0.1ug) complexes at various N/P ratios shows complete retardation at an N/P ratio of 3. (B) DNase protection and release assay. Complexed DNA with PdXYP (N/P 20) was released using 1% SDS. Lane 2 demonstrates protection of the complexed DNA, while Lane 4 show its complete degradation in the absence of PdXYP.

Z-Average (d.nm):	77.94		Size (d.nm):	% by Intensity	% by Volume
PdI:	0.157	Peak 1:	84.43	98.7	99.2
Intercept:	0.814	Peak 2:	4966	1.3	0.8
CPF:	0.00	Peak 3:	0.000	0.0	0.0
Result quality :	Good				

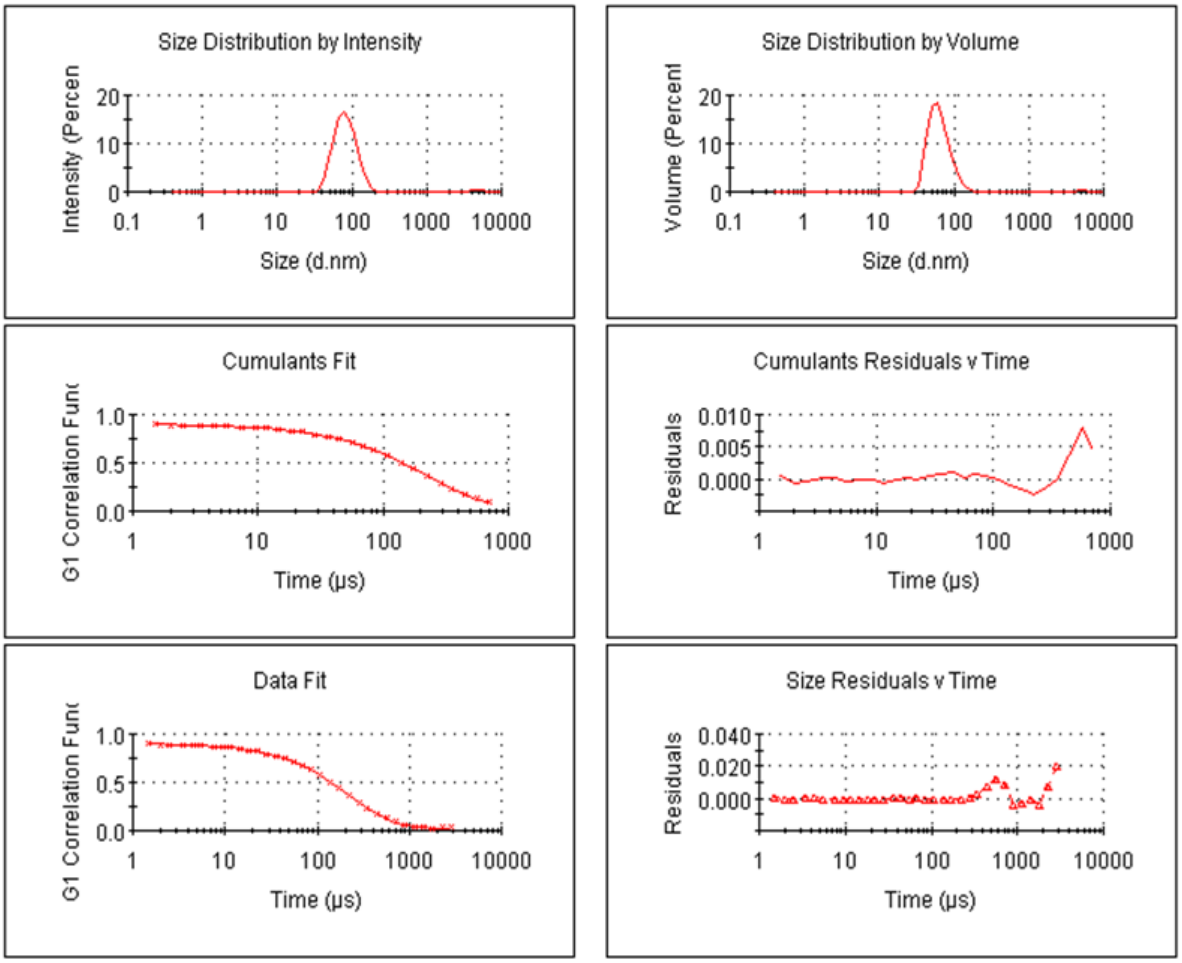

Fig. S6 Particle size and distribution measurement by Dynamic Light Scatttering (DLS)

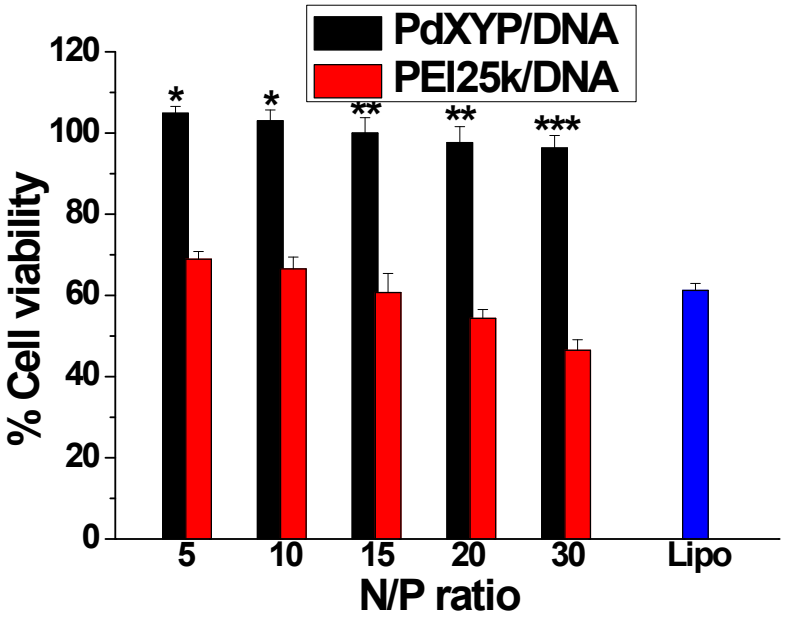

Fig. S7 Cytotoxicity of PdXYP/DNA complexes compared to PEI25k/DNA complexes at various N/P ratios in A549 cells (n=3, error bar represents SD) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, one-way ANOVA)

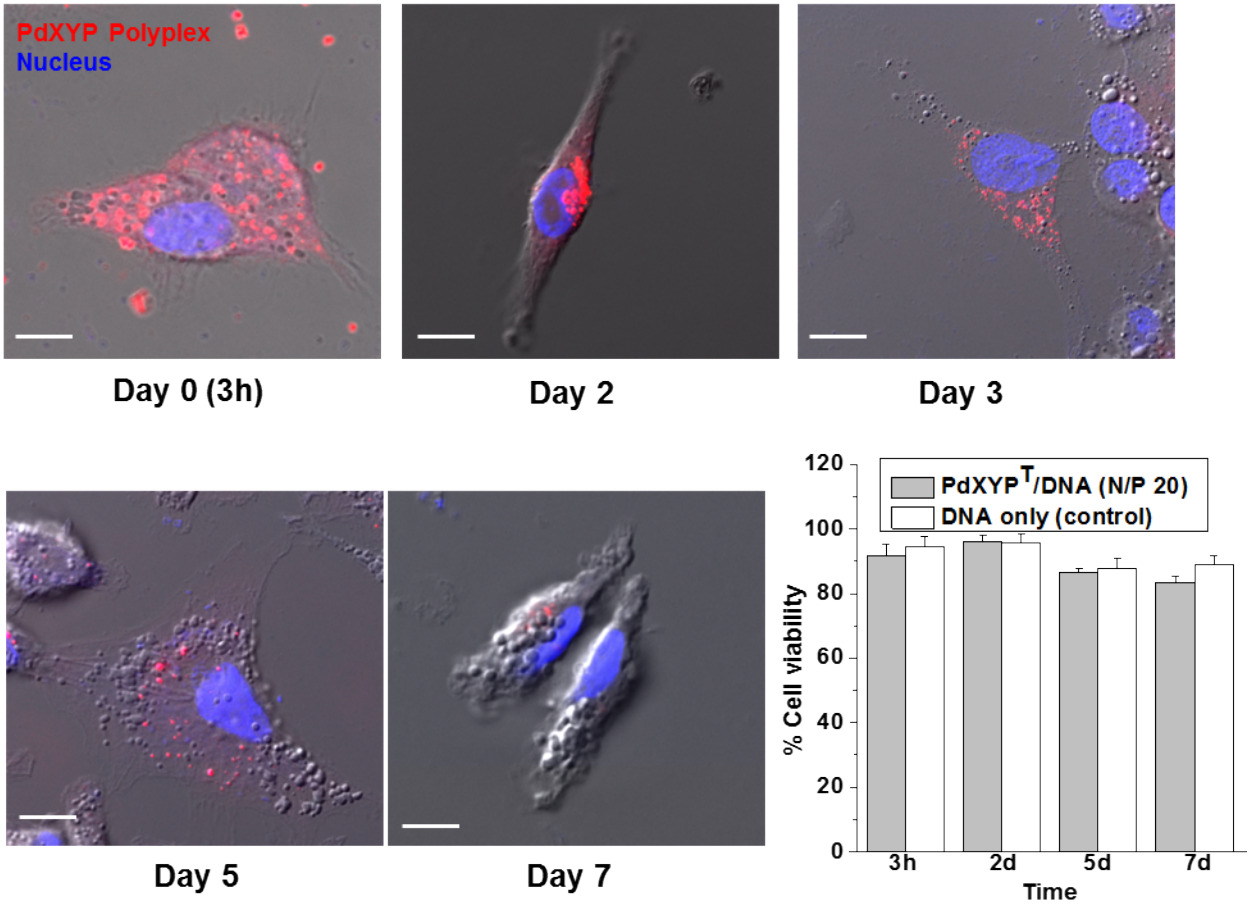

Fig. S8 Uptake and degradation of PdXYP^T/DNA complexes in A549 cells. Confocal microscopic images of A549 cells with DAPI nuclear staining (blue), observed up to 7 days following transfection with TRITC-labeled PdXYP^T (red). PdXYP^T after cellular uptake (3h) (scale bar: 10 μm) is gradually degraded up to day 7, and the occurrence of vesicular structure represents the increased exocytosis of fragmented PdXYP^T. Cytotoxicity measurements of PdXYP^T/DNA (N/P 20) complexes by MTT assay after 3h, 2d, 5d and 7d of transfection in A549 cells show no cytotoxic effects. Statistical significance was determined using one-way ANOVA (n=3, error bar represents SD).

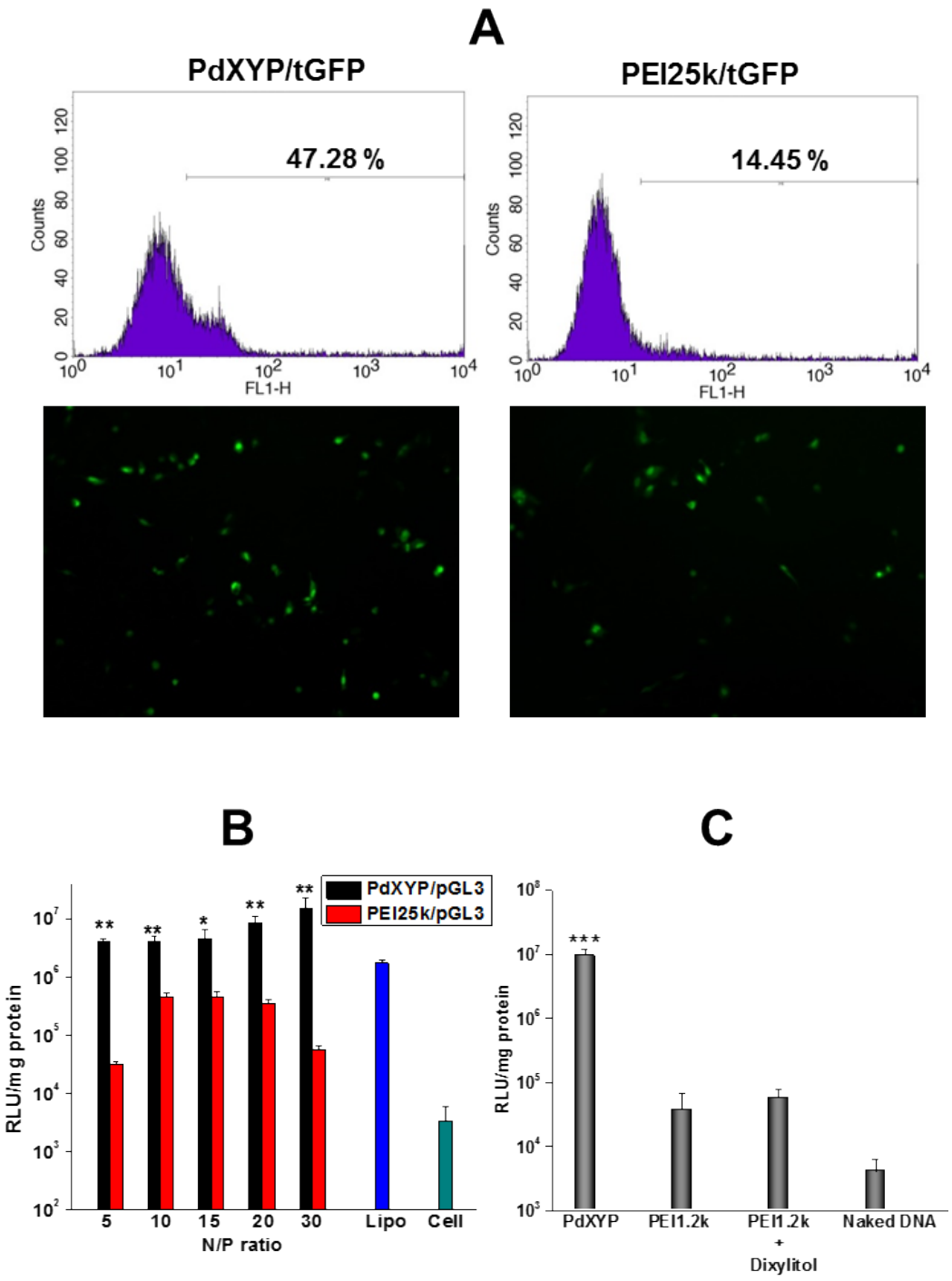

Fig. S9 Transfection efficiency of PdXYP/DNA in A549 cells. (A) FACS studies showing 47% transfection efficiency of PdXYP/tGFP (N/P 20) over 14% of PEI25k/tGFP (N/P 10) complexes with corresponding transfection images. (B) Luciferase activity of PdXYP/pGL3 complexes at various N/P ratios (C) Luciferase activity of PdXYP/pGL3, PEI1.2k/pGL3 and PEI1.2k/pGL3 + dixylitol in rat astrocytes shows no effect of free dixylitol on transfection efficiency of PEI1.2k/pGL3 polyplexes . (n=3, error bar represents SD) (**$p < 0.01$, one-way ANOVA).

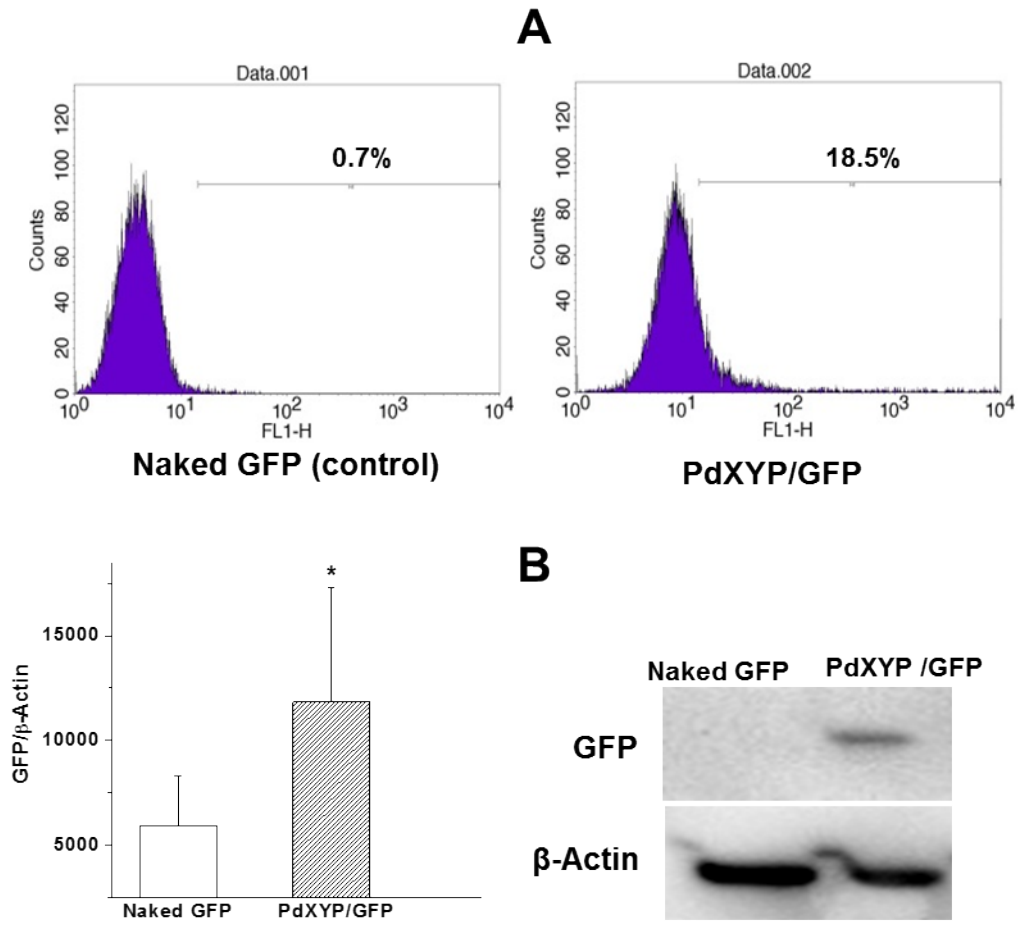

Fig. S10 Transfection efficiency of PdXYP/tGFP in astrocytes after crossing the BBB. (A) FACS analysis (B) Western blot analysis of GFP protein from the lysate of the transfected astrocytes after 48 h, showing a significant increase in GFP protein expression in cells treated with PdXYP/GFP complexes in contrast to the control. Data are shown as the mean ± SD of three independent experiments (*$p < 0.05$, one-way ANOVA).

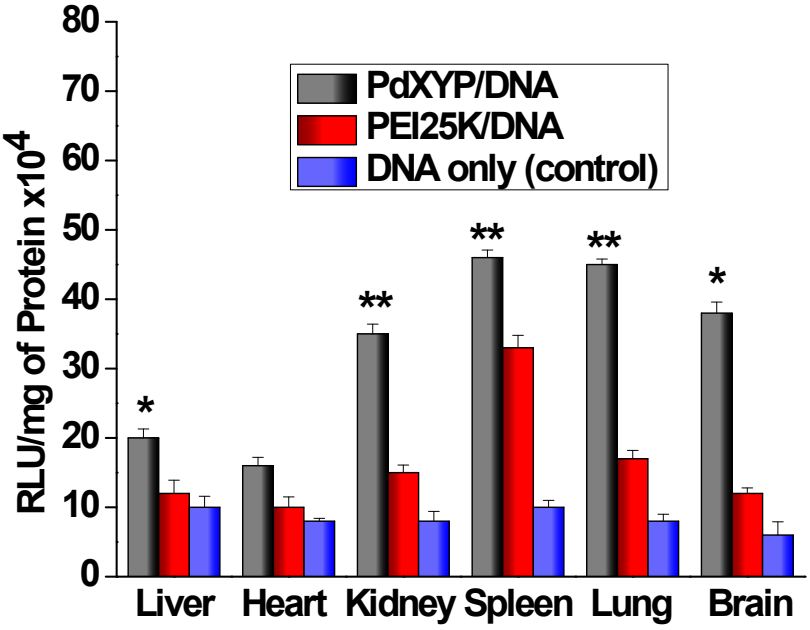

Fig. S11 In vivo biodistribution of PdXYP/DNA polyplexes in Balb/c mice (n=4) after 5 days of intravenous injection was analyzed as luciferase expression in various organs (error bar represents SD) (*$p < 0.05$, **$p < 0.01$, one-way ANOVA).

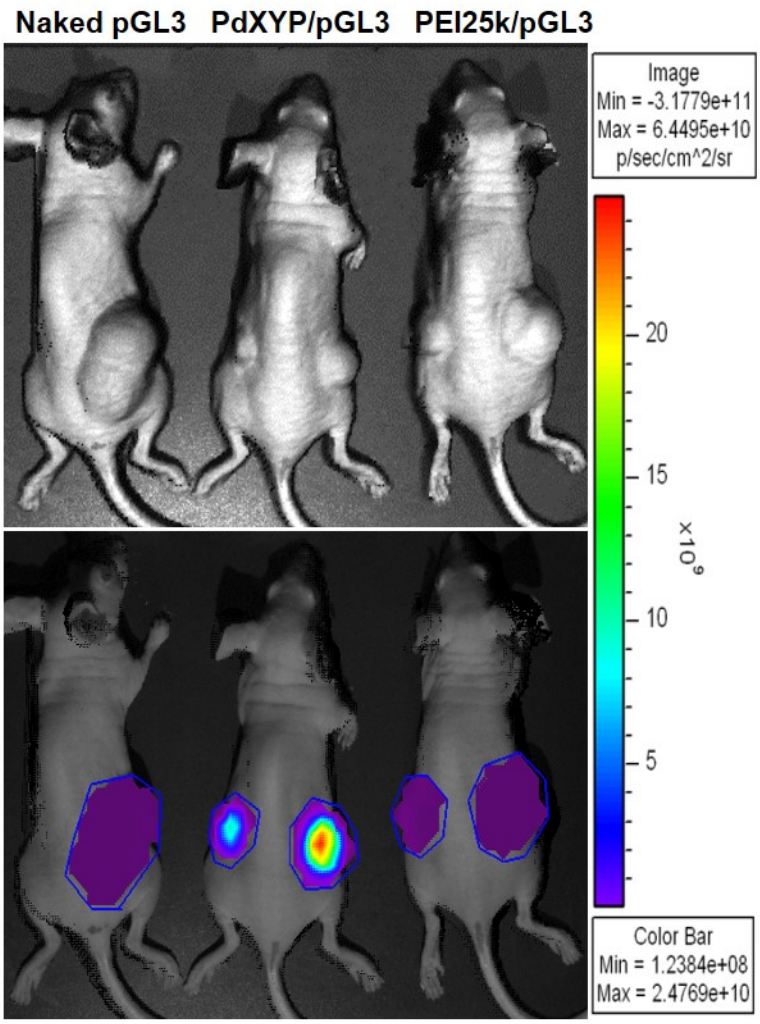

Fig. S12. *In vivo* bioimaging after 7 days of local injection of PdXYP/pGL3, PEI25k/pGL3 and naked pGL3 in xenograft mice showing luciferase expression of PdXYP in deeper tumor locations.

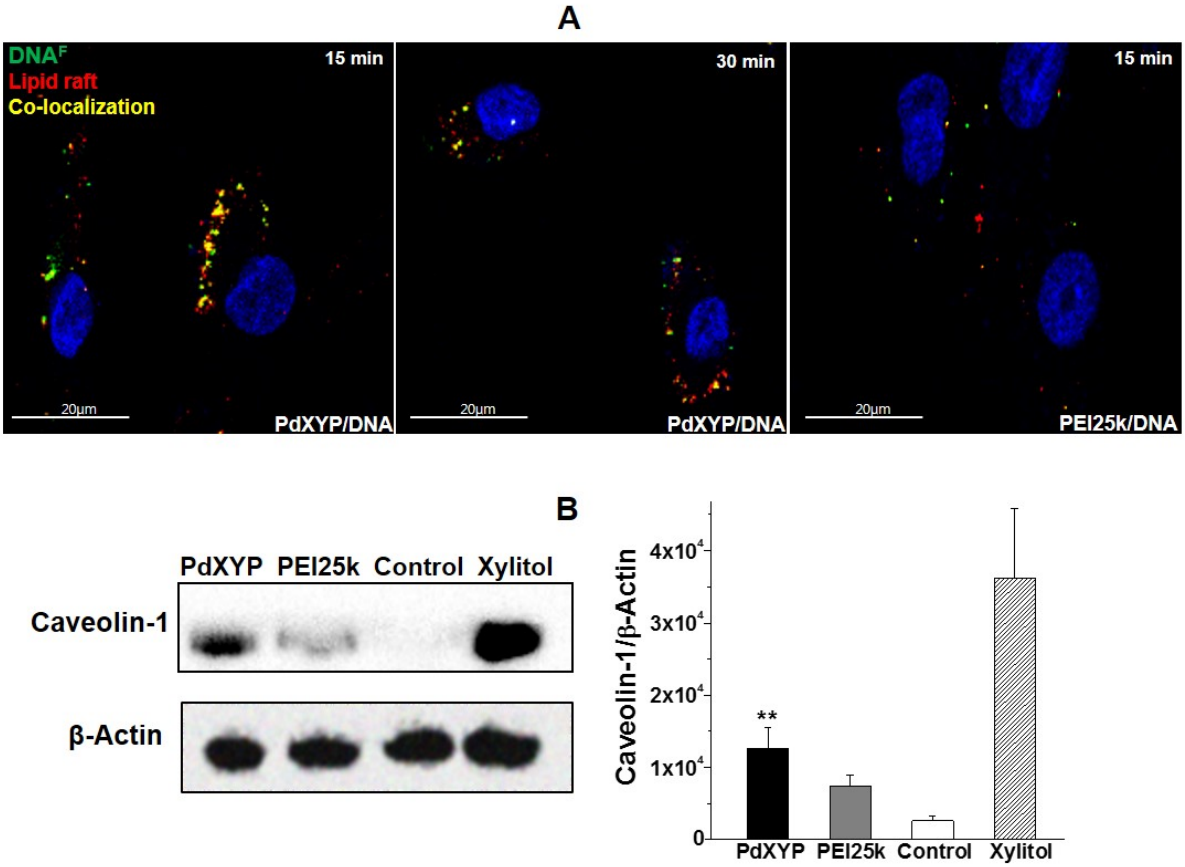

Fig. S13. Mechanistic investigations for caveolae mediated endocytosis of PdXYP/DNA polyplexes in A549 cells. (A) Co-localization (yellow) of PdXYP/DNA polyplexes (green) with caveolin rich lipid rafts (orange) after 15 and 30 min of transfection suggests its caveolae-mediated endocytosis (scale bar: 20μm). (B) Western blot analysis of caveolin-1 protein from the lysate of transfected A549 cells after 30 min shows a significant increase in caveolin-1 protein expression in cells treated with PdXYP/DNA polyplexes in contrast to PEI25k/DNA. Data are shown as the mean±SD (**P < 0.01, one-way ANOVA).

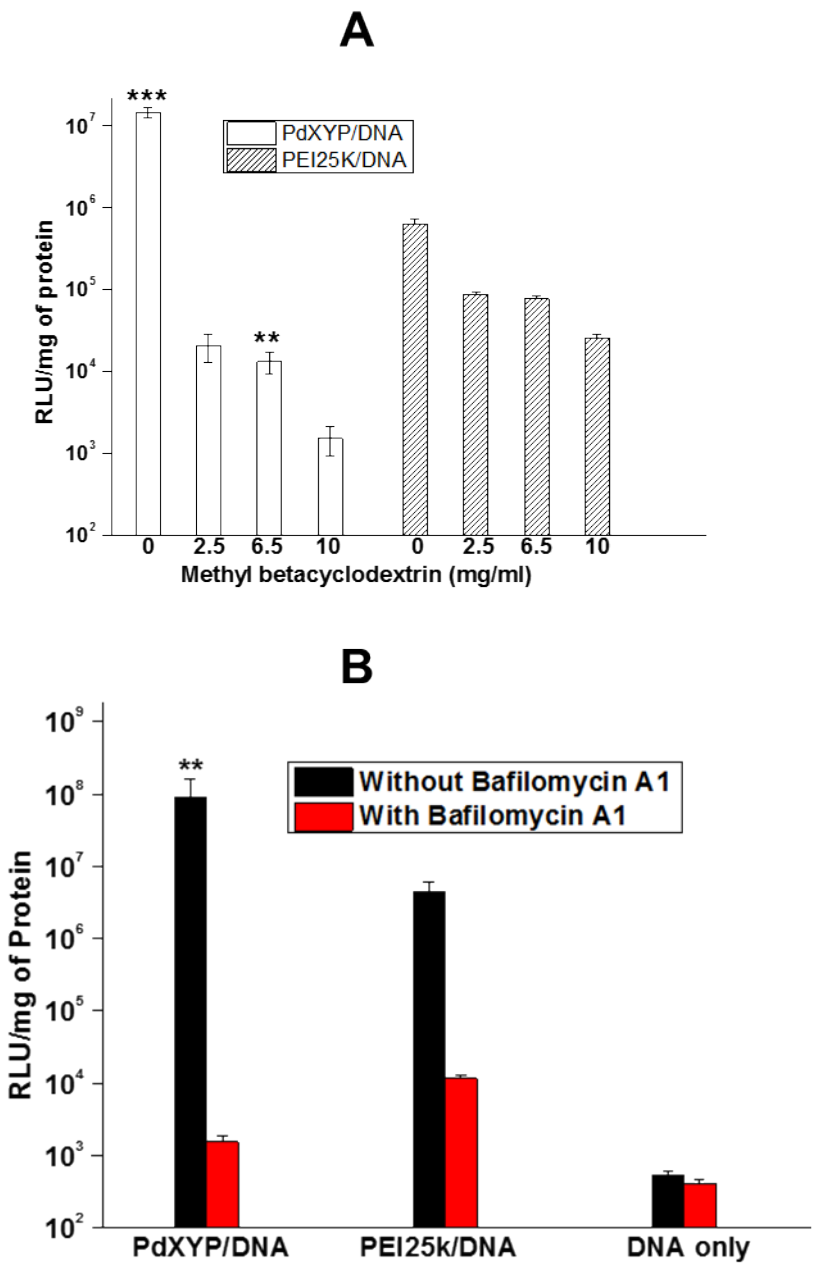

Fig. S14 Endocytosis inhibition study. (A) Methyl betacyclodextrin inhibition at various concentrations shows decreased luciferase expression suggesting caveolae mediated endocytosis of PdXYP/DNA polyplexes. (B) Vacuolar type H^+ ATPase inhibition by Bafilomycin A1 (200nM in DMSO) shows decreased transfection suggesting endosomal escape of PdXYP/DNA polyplexes, (error bar represents SD) (**$p < 0.01$, ***$p < 0.001$, one-way ANOVA).

References

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