Supplementary Information

Zwitterionic polymer with minimal reactivity against PEG antibodies to enhance therapeutic effects of cytokine-targeting DNA aptamer

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1. Materials and Methods

General Information

Reagents were purchased from commercial suppliers and used without further purification, unless otherwise described. All DNA samples were synthesized by and purchased from GeneDesign, Inc. (Osaka, Japan) or Fasmac (Kanagawa, Japan). Refolding of DNA aptamer was performed with a thermal cycler (95 °C for 5 min, and then cooled at –0.1 °C/sec to 25 °C). Dulbecco's phosphate buffer saline (DPBS–, pH 7.4) was used as PBS in all experiments. Recombinant mouse IFN-gamma protein (#50709-MNAH) was purchased from Sino Biological (Beijing, China). Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL ECS400 (^{1}H 400 MHz) spectrometer. Male Sprague-Dawley (SD) rats were obtained from Japan SLC, Inc. (Shizuoka, Japan) and Male ICR mice were obtained from The Jackson Laboratory Japan, Inc. (Kanagawa, Japan).

Polymer synthesis and purification

Methanol (5 mL) was purged with nitrogen over 30 minutes. Tri(ethylene glycol) methacrylate (TEGMA, #729841, Sigma-Aldrich) was passed through a neutral aluminium oxide (#101077, Merck Millipore) column to remove polymerization inhibitors. Methacryloyloxyethyl phosphorylcholine (MPC, #730114, Sigma-Aldrich) or TEGMA was mixed with catalysts Cu(I)Br and 2,2'-bipyridyl (#040-04242, Fujifilm Wako Pure Chemical Corporation), and the purged methanol was added to the mixture under nitrogen. Polymerization was initiated by the addition of 2-((*tert*-butoxycarbonyl)amino)ethyl 2-bromo-2-methylpropanoate to the reaction mixture under nitrogen. The reaction solution was stirred at room temperature and, after 22 hours, was exposed to air to stop the reaction. After removing the solvent by evaporation, 2 mL of trifluoroacetic acid (TFA) was added, and the solution was stirred at room temperature for 2 hours to remove the terminal *tert*-butoxycarbonyl protecting group. After removing TFA under reduced pressure, the product was precipitated by adding 6 mL of diethyl ether, which was re-dissolved by 2 mL of water and added with 0.5 M EDTA to chelate the copper. The solution was then dialyzed in water for 2 days using Spectra/Por 3 Dialysis Tubing (3.5 kDa MWCO, Spectrum Labs), passed through 0.45 μm filter (#06542-14, Nacalai Tesque), and lyophilized.

Gel Permeation Chromatography

Number-averaged molecular weight (M_n) and weight-averaged molecular weight (M_w) of the polymers were determined by gel permeation chromatography. The polymers were dissolved in the mobile phase (2 mg/mL, 0.2 M sodium nitrate for PMPC and PEG; MeOH/50 mM sodium nitrate = 1:1 for PTEGMA) and passed through 0.45 μm filter (#06542-14, Nacalai Tesque). The analysis was conducted using TSKgel G4000 PW_{XL}

column (Tosoh) on HPLC system equipped with refractive index detector (Shimadzu). After equilibration of the column, 50 μL of the sample solution was injected, and separation was performed with a mobile phase at a flow rate of 1 mL/min (column temperature: room temperature). PEO/PEG standards (#PSS-PEOKITR1, Agilent) with peak molecular weight (M_p) from 599 to 478,000 Da were used for calibration.

Dynamic Light Scattering

Hydrodynamic diameter (D_h) of the polymers was determined by dynamic light scattering (Malvern Panalytical zetasizer, ZSU3200). The polymers were dissolved in PBS (10 mg/mL), passed through 0.22 μm filter (#SLGVX13NL, Merck Millipore), and analyzed in transparent polystyrene cuvette (#67.754, Sarstedt) at 25 °C.

DBCO-polymer synthesis and purification

DBCO-NHS ester (3 μmol) (#D5999, TCI chemicals) was dissolved in dimethylsulfoxide (to be 100 mg/mL) and mixed with amine-functionalized polymers (1 μmol) dissolved in methanol (to be 200 mg/mL). After the addition of *N*,*N*-diisopropylethylamine (3 μmol), the mixture was incubated at room temperature for 12 hours. To remove excess DBCO-NHS ester, the mixture was washed with 200 μL diethyl ether for three times, filtered (0.45 μm), and lyophilized.

Synthesis of aptamer-polymer conjugates and purification

Aptamer-polymer conjugates were synthesized by strain-promoted alkyne-azide click (SPAAC) reaction using gradual freeze-thawing method described in the previous report.[1] Azide-functionalized aptamer (100 μM) and DBCO-polymer (300 μM) were mixed in PBS and frozen overnight at –30 °C. After the mixture was thawed at 4 °C for over an hour, aptamer-polymer conjugates were purified by anion exchange chromatography.

For the purification of PEG- or PMPC-TXB0063 conjugates, the reaction mixture was diluted by binding buffer (20 mM Tris-HCl, pH 8.0) and loaded onto an anion exchange column (HiTrap Q HP, Cytiva) equilibrated with the binding buffer. Aptamer-polymer conjugates were separated from unreacted DBCO-polymer and azide-functionalized aptamer by gradient elution (20 mM Tris-HCl + 1 M NaCl). PTEGMA-TXB0063 conjugate was purified as described in the previous report,[2] with minor modifications as follows. The reaction mixture was diluted by binding buffer (20 mM ammonium carbonate (pH 8.2), loaded onto the anion exchange column (HiTrap Q HP) equilibrated with the binding buffer, and eluted by gradient elution (20 mM ammonium carbonate + 1 M NaCl).

The purified polymer-aptamer conjugates were concentrated and desalted by Amicon Ultra Centrifugal Filter (10 kDa MWCO, Merck Millipore).

Anti-PEG ELISA

Amine-terminal polymers were coated onto polystyrene plate (#DY990, R&D Systems) by incubating 20 μg/mL of polymer solution (100 μL/well) in PBS at 4 °C for 2 hours. After the plate was washed and blocked with PBS with 0.5% Bovine Serum Albumin (BSA) (#9048-46-8, Fujifilm), 50 μL Horseradish peroxidase (HRP)-conjugated anti-PEG antibody (#1D9-6, Life Diagnostics, 1/2000 diluted in PBS with 0.5% BSA) was added to each well and incubated at 25 °C and incubated for 1 hour, shaken at 300 rpm. The plate was then washed with PBS with 0.5% BSA, and ELISA-POD substrate 3,3′,5,5′-tetramethylbenzidine (TMB) solution (100 μL/well, #05299-54, Nacalai Tesque) was dispensed to each well. The plate was incubated at 25 °C for 5 minutes, and the reaction was stopped by adding 100 μL of 1M phosphoric acid. The binding of HRP-conjugated antibody was quantified by measuring the absorbance at 450 nm (570 nm as reference).

Competitive ELISA

Competitive ELISA was conducted using PEG ELISA (#PEG, Life Diagnostics) according to the manufacture's protocol. Aptamer or aptamer-polymer conjugates (1 μM in water) were diluted 10-fold with HRP PEG diluent (#PEGD50-1), and 50 μL aliquot was added to each well of PEG-BSA coated plate. After the addition of HRP-conjugated anti-PEG antibody (#1D9-6, Life Diagnostics, 1/4000 diluted in HRP PEG diluent), the mixture was incubated at 25 °C for 1 hour, shaken at 300 rpm. The plate was washed with HRP PEG Wash buffer (#PEGW50-20), and TMB reagent (#TMB11-1) was dispensed to each well. The plate was incubated at 25 °C for 10 minutes, shaken at 300 rpm, and the reaction was stopped by adding 100 μL of Stop solution (#SS11-1). The binding of HRP-conjugated antibody was quantified by measuring the absorbance at 450 nm (570 nm as reference).

Plasma retention

Male Sprague-Dawley rats were obtained from Japan SLC, Inc. (Shizuoka, Japan). Fifteen rats (6 weeks old, weight range of 184-201 g) were randomly assigned to six groups (n = 3 per group). TXB0063, TXB0063-PEG_{40kDa}, TXB0063-$PMPC_{53}$, TXB0063-$PMPC_{93}$, or TXB0063-$PMPC_{206}$ (40 nmol/kg) were administered into the lateral tail vein. Two hundred μL of blood were collected from the cervical vein at 5, 15, and 30 minutes, as well as at 1, 3, 6, 12, 24, and 24 hours post-injection. Each blood sample was mixed with EDTA-2K (final concentration of 1 mg/mL), centrifuged at 12,000 × g and 4 °C for 5 minutes to prepare plasma samples. The

concentration of aptamer in the plasma samples was measured by quantitative PCR using KOD SYBR qPCR mix (#QKD-201, TOYOBO, Osaka, Japan). The plasma samples were diluted 200-fold with nuclease-free water. Samples for standard curve were prepared by serial dilution of aptamer or polymer-aptamer conjugates with EASY Dilution (#9160, Takara Bio, Shiga, Japan), spiked in plasma obtained from unadministered rats. To prepare 20 μL PCR reaction solution, 2 μL of diluted sample were mixed with 0.4 μM forward primer (5′-GGCCGGTACCCGAACCACAGTTTATAGTTGTACTA-3′) and 0.4 μM reverse primer (5′-CGCTTCGCGG GCCAGACCCTGC-3′). Quantitative PCR was performed by a CFX connect instrument (Bio-Rat, CA, US) as follows: 95 °C for 2 minutes, followed by 40 cycles of 95 °C for 10 seconds, 52 °C for 10 seconds and 68 °C for 30 seconds. Pharmacokinetic parameters were determined using the moment analysis program available on Microsoft Excel, which was programmed by the Graduate School of Pharmaceutical Sciences at Kyoto University.

Circular dichroism (CD)

CD spectra of TXB0063, TXB0063-$PMPC_{206}$, TXB0063-PEG_{40kDa}, or TXB0063-$PTEGMA_{200}$ (4 μM) in 20 mM Tris-HCl (pH 7.5) containing 5 mM KCl were measured at 25 °C with a CD spectrometer (JASCO, J-1500) using 1 mm path length quartz cell. Data pitch was set to 0.1 nm. Spectra were averaged from 8 scans. Spectral baseline was recorded using 20 mM Tris-HCl (pH 7.5) containing 5 mM KCl, and all data points were baseline subtracted.

Nuclease stability

TXB0063, TXB0063-$PMPC_{206}$, or control 50 nt oligonucleotide (0.5 μM) were incubated in the presence of 50 % normal mouse serum (#146-06551, Fujifilm Wako) in PBS for 0, 2, 4, 6 or 8 h at 37 °C. After the incubation, the reaction was stopped by freezing the mixture in liquid nitrogen and the samples were stored at –30 °C until they were analyzed.

For the Quantitative PCR analysis of TXB0063 and TXB0063-$PMPC_{206}$, the serum-incubated mixtures were diluted 100-fold with 0.1 % tween-20 (#P2287, Sigma-Aldrich). To prepare 10 μL PCR reaction solution, 2 μL of the diluted samples were mixed with 0.4 μM forward primer (5′-GGCCGGTACCCGAACCACAGTTTATT GTTG-3′), 0.4 μM reverse primer (5′-GCGGGCCAGACCCTGCAAAAC-3′), and PowerUP SYBR Green Master Mix (#A25742, Applied biosystems). Quantitative PCR was performed by Applied Biosystems StepOne Real-Time PCR system (#StepOne-01, Thermo).

For the denaturing polyacrylamide gel electrophoresis (PAGE) analysis of TXB0063 and control 50 nt oligonucleotide, the serum-incubated mixtures were incubated at 70 °C for 7 minutes in the presence of 8 M

urea and loaded onto 8 % polyacrylamide gel containing 8 M urea. After the electrophoresis in TBE buffer, the gel was stained by GelRed (#41002, Biotium, Inc.).

Cell stimulation and flow cytometry analysis

Cell stimulation with IFN-gamma and flow cytometry analysis were conducted following the protocol in the previous report[3] with modifications as follows. L929 murine fibroblast cell line (#RCB1422 from RIKEN Bioresource Research Center Cell Bank, Tsukuba, Japan) was maintained in Minimum Essential Medium (MEM) with Non-Essential Amino Acid (#21443, Nacalai Tesque) supplemented with 10 % heat-inactivated FBS (Origin South America, #CCP-FBS-BR-500, Biowest) and 1 % antibiotic-antimycotic mixed stock solution (#09366-44, Nacalai Tesque). In the IFN-gamma neutralization test, TXB0063 or TXB0063-polymer conjugates serially diluted with PBS (10 μL, final concentration of 0.3–300 nM) were added to L929 cells (4×10^5 cells in 180 μL MEM) incubated at 37 °C. Cells were then stimulated with 2 ng/mL murine IFN-gamma by addition of 10 μL of 40 ng/mL IFN-gamma in MEM for 15 minutes, centrifuged and washed with ice-cold PBS to stop the reaction. For the flow cytometry analysis, cells were resuspended with 200 μL of 4 % paraformaldehyde (#24-0630-5, Sigma-Aldrich) in 0.1 M phosphate buffer (pH 8.0) for fixation, incubated at room temperature for 20 minutes, centrifuged, resuspended with 20 μL ice-cold PBS, added with ice-cold methanol 200 μL, and incubated on ice for 30 minutes for permeabilization. Then the cells were centrifuged, washed twice with staining buffer (PBS with 0.5 % BSA), and stained by incubation in 40 μL of staining buffer containing 40-fold diluted Alexa Fluor 488-conjugated mouse anti-STAT1 (pY701) antibody for 30 minutes on ice in the dark. The cells were added with 200 μL of staining buffer, centrifuged, washed with 200 μL of staining buffer, and analyzed using flow cytometer (Guava Easycyte TM, Merk Millipore).

Therapeutic efficacy

Male ICR mice (7 weeks old) were obtained from The Jackson Laboratory Japan, Inc. (Kanagawa, Japan). Thirty mice (8 weeks old, weight range of 31-40 g) were assigned into three groups (n = 10 per group): PBS + LPS, TXB0063 (10 mg/kg) + LPS, and TXB0063-$PMPC_{199}$ (10 mg/kg) + LPS groups, respectively. To induce endotoxic shock model, mice received 12.5 mg/kg of LPS (E. coli, O111:B4, Sigma) into the peritoneal cavity. TXB0063 or TXB0063-$PMPC_{199}$ intravenous administration was performed 4, 24, and 48 h after LPS injection, and mortality was monitored over the next 7 days. The statistical analysis of mortality was performed using EZR (Easy R)[4]. The difference in survival rate was analyzed with a Kaplan-Meier nonparametric model, and the curves were compared using the log-rank test. P values less than 0.05 were considered significantly different.

Ethical approval

All animal experiments were performed with the approval of the department animal care and use committee of the University of Tokyo.

2. Figures S1–S5

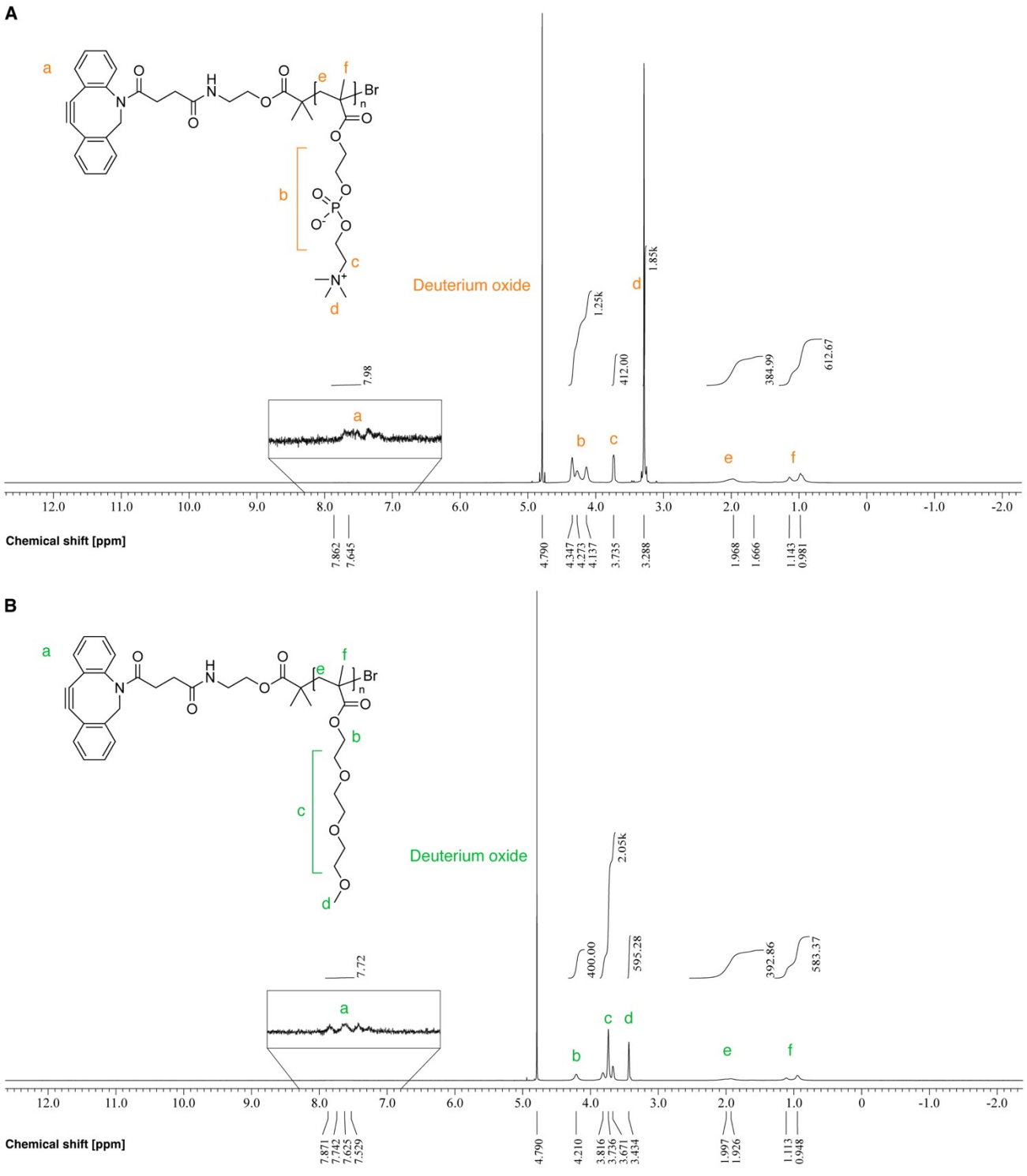


Figure S1. **^{1}H NMR spectra of DBCO-polymers in D_2O**. (A) $PMPC_{206}$-DBCO (B) $PTEGMA_{200}$-DBCO

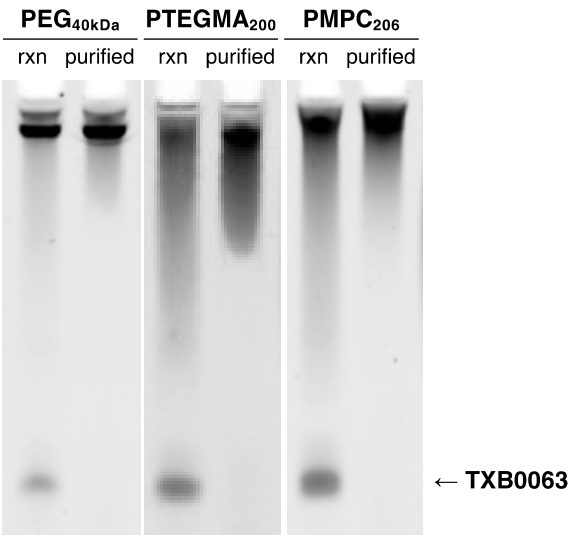
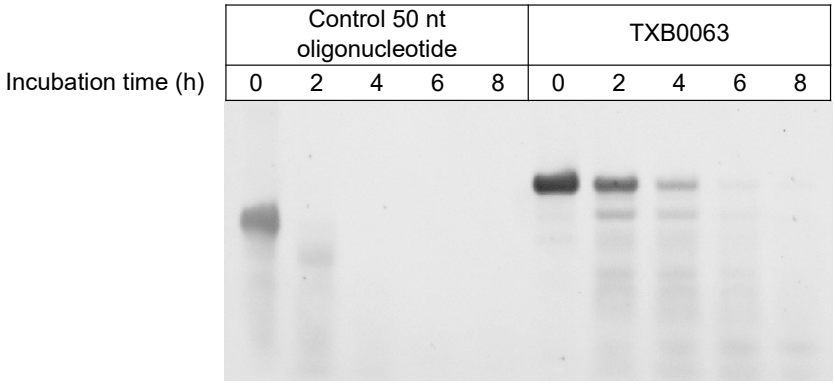


Figure S2. **PAGE analysis of TXB0063-polymer conjugates after anion exchange chromatography.** Left lane: Click reaction solution (containing both TXB0063 and TXB0063-polymer conjugates); Right lane: purified TXB0063-polymer conjugates.

A



control 50 nt oligonucleotide

5′-TCAATCCATCATTTACTCATGCTAACACCAACTATTTGCCTTCTACTAAC-3′

No secondary structure was predicted by VectorBuilder.

B

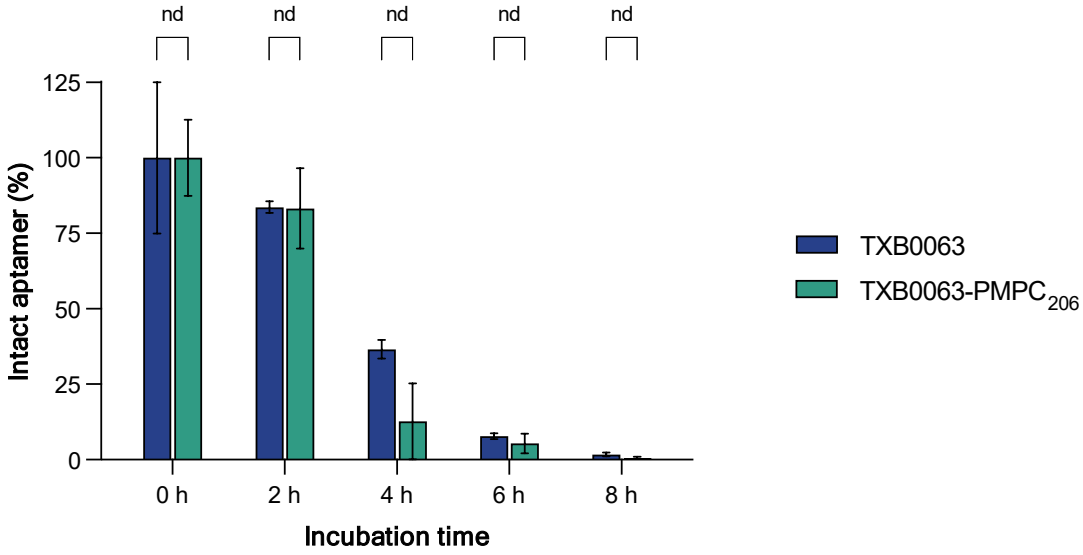


Figure S3. **Evaluation of nuclease resistance of TXB0063 and TXB0063-PMPC$_{206}$.** Oligonucleotides (0.5 μM each in 50 % v/v of mouse serum/PBS) were incubated at 37 °C, frozen in liquid nitrogen to stop the reaction at the designated time points, and analyzed by (A) denaturing PAGE or (B) qPCR. Statistical significance was examined by multiple t-test and *p*-values were corrected by False Discovery Rate method of Benjamini and Hochberg. nd (not a discovery, $p > 0.05$).

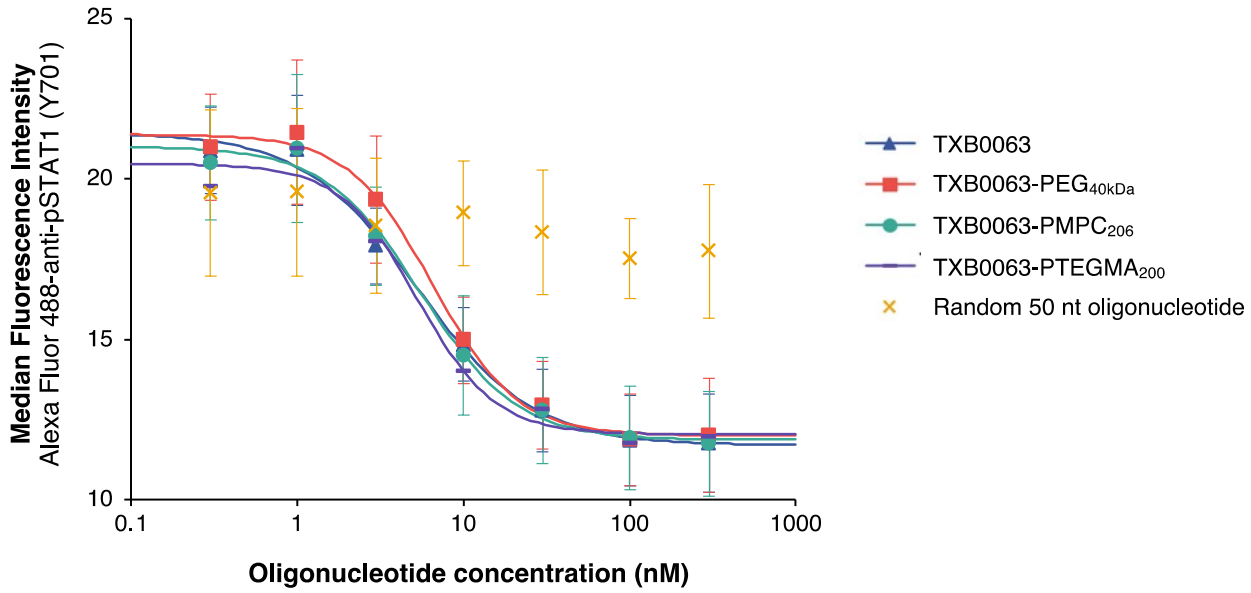


Figure S4. **Inhibition of IFN-gamma activity by TXB0063 or TXB0063-polymer conjugates.** L929 murine fibroblast cells were stimulated by 2 ng/mL IFN-gamma in the presence of TXB0063, TXB0063-polymer conjugates, or control random 50 nt oligonucleotide at 37 °C for 15 min. Phosphorylation of STAT1, activated by IFN-gamma, was analyzed using flow cytometry by staining cells with Alexa Fluor 488-conjugated anti-pSTAT1 (Y701) antibody.

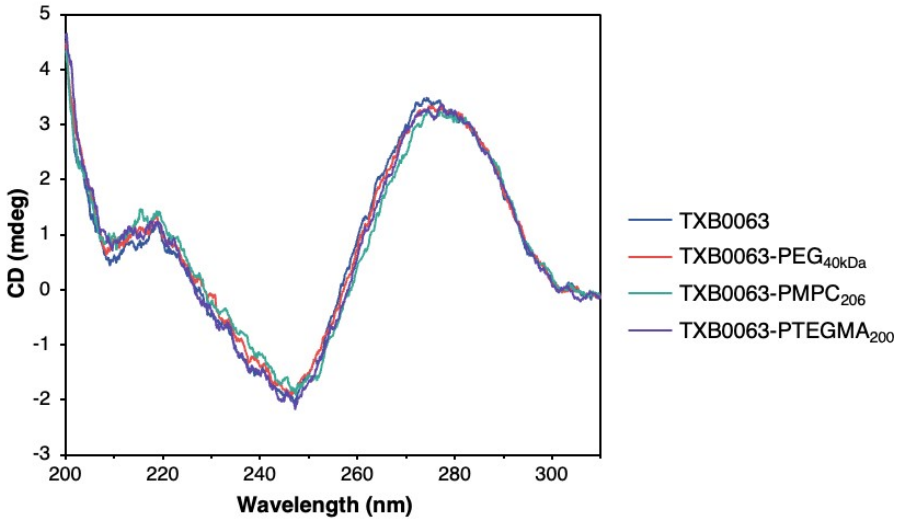


Figure S5. **CD spectra of TXB0063 and TXB0063-polymer conjugates.** CD spectra of TXB0063 and TXB0063-polymer conjugates (4 μM) in 20 mM Tris-HCl buffer (pH 7.5) containing 5 mM KCl were acquired at 25 °C.

3. References

(1) Takemoto, H.; Miyata, K.; Ishii, T.; Hattori, S.; Osawa, S.; Nishiyama, N.; Kataoka, K. Accelerated Polymer-Polymer Click Conjugation by Freeze-Thaw Treatment. *Bioconjugae Chem.* 2012, **23**, 1503–1506.

(2) Ozer, I.; Pitoc, G.; Layzer, J.; Moreno, A.; Olson, L., Layzer, K.; Hucknall, A.; Sullenger, B.; Chilkoti, A. PEG-Like Brush Polymer Conjugate of RNA Aptamer That Shows Reversible Anticoagulant Activity and Minimal Immune Response. *Adv. Mater.* 2022, **34**, 2107852.

(3) Akiyama, Y.; Harada, K.; Miyakawa, J.; Kreder, K. J.; O'Donnell, M. A.; Daichi, M.; Katoh, H.; Hori, M.; Owari, K.; Futami, K.; Ishikawa, S.; Ushiku, T.; Kume, H.; Homma, Y.; Luo, Y. Th1/17 Polarization and Potential Treatment by an Anti-Interferon-γ DNA Aptamer in Hunner-Type Interstitial Cystitis. *iScience* 2023, **26**, 108262.

(4) Kanda, Y. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. *Bone Marrow Transplant.* 2013, **48**, 452–458.