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

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Overcoming poor *in vivo* pharmacokinetics is a critical challenge in developing therapeutic aptamers, and conjugation to poly(ethylene glycol) (PEG) is a well-established technique for aptamers to prolong blood circulation. However, the existence of antibodies that specifically recognize PEG and their adverse effects on *in vivo* behaviors have been increasingly reported, highlighting the necessity of alternative modification strategies for aptamers. To address this issue, we focused on a zwitterionic polymer, particularly poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC), as a PEG alternative to modify DNA aptamers. We conjugated PMPC to a DNA aptamer targeting IFN- $\gamma$  and investigated the properties of the PMPC-conjugated DNA aptamer as a therapeutic agent. PMPC modification did not affect the neutralizing activity of the aptamer. PMPC demonstrated lower reactivity against anti-PEG antibodies than PEG-like aptamer modifiers previously reported to exhibit low reactivity against PEG antibodies. In addition, PMPC extended the blood circulation time of the aptamer as long as or longer than PEG with a similar molecular size. In the LPS-induced inflammation animal model, the survival rate after treatment with the PMPC-aptamer conjugate was significantly superior to that with unmodified aptamer. These results indicate that PMPC has potential as an aptamer or other nucleic acid drug modifier to replace or be compatible with PEG.

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## Introduction

Aptamers, also called chemical antibodies, are highly structured, short, single-stranded oligonucleotides that bind to their targets with high affinity and selectivity.<sup>1–3</sup> Because they can be chemically synthesized rather than produced biologically and are resistant to heat denaturation unlike antibodies, researchers have been attempting to develop aptamers for use in targeted therapy, *in vitro* diagnosis, and *in vivo* imaging.<sup>2</sup>

Proper modifications are often introduced for nucleic acid-based therapeutics, including aptamers, to tailor their pharmacokinetic properties. Without any modification, aptamers

are rapidly eliminated from the *in vivo* circulation. This is because aptamers are mostly smaller (6–30 kDa) than the glomerular effective size cutoff (30–50 kDa) and are subject to renal filtration.<sup>2</sup> Therefore, while several aptamers have been or were in clinical trials so far, most of them were conjugated to polymers to lengthen their circulation times, thereby decreasing the dosing frequency and enhancing their efficacy.<sup>3</sup>

Poly(ethylene glycol) (PEG) has long been considered to be biocompatible and non-immunogenic, making PEG modification (PEGylation) a primary option for drug delivery.<sup>4</sup> However, there is an increasing number of reports that repeated injection of PEG-modified materials can evoke the production of anti-PEG antibodies.<sup>5</sup> These anti-PEG antibodies could specifically bind to PEGylated drugs and trigger the immune response.<sup>6</sup> In the phase 2b clinical trial of the PEGylated RNA aptamer *Pegnivacogin*, 3 of 640 patients experienced severe allergic reactions after the first dose, to which a high level of pre-existing anti-PEG antibodies is considered to majorly contribute, resulting in early termination of the trial.<sup>7</sup> Due to exposure to various consumer products containing PEG, anti-PEG antibodies were revealed to be prevalent even in patients naive to PEGylated drugs.<sup>6</sup> Furthermore, administration of PEGylated pharmaceuticals, including mRNA vac-

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## Results and discussion

### Synthesis of polymeric aptamer modifiers and evaluation of their reactivity against anti-PEG antibodies

PMPC and PTEGMA used in this study were synthesized by atom transfer radical polymerization using 2-((*tert*-butoxycarbonyl)amino)ethyl 2-bromo-2-methylpropanoate as a starting compound (Fig. 1B). After the polymerization reaction, the terminal *tert*-butoxycarbonyl protecting group was deprotected by trifluoroacetic acid (TFA) treatment to afford PMPC and PTEGMA with terminal amino groups. The degree of polymerization and molecular weight profile were determined by  $^1\text{H}$  NMR and gel permeation chromatography (GPC), respectively.

We first investigated the anti-PEG reactivity of the polymers with approximately the same number of repeating units per molecule, PMPC<sub>206</sub> and PTEGMA<sub>200</sub> (Fig. 1C). Mouse anti-PEG antibodies (1D9-6), selective to the PEG backbone, were used for the assay. PTEGMA did exhibit less binding to anti-PEG antibodies than PEG, as reported. However, it was also reported that anti-PEG antibodies recognizing the ethylene glycol (EG) backbone bind to PEG-like brush polymers when there are more than two EG moieties in repeated units, while poly(diethylene glycol methacrylate) bearing only two EG repeats is not appropriate for *in vivo* use due to its lower critical solution temperature, above which it becomes hydrophobic and loses its antifouling effect.<sup>10,19–21</sup> Consistent with this, PTEGMA, which has 3 EG moieties in each repeating unit, could not completely prevent anti-PEG antibodies from binding, as shown in Fig. 1C. On the other hand, PMPC was less recognized by anti-PEG antibodies than PTEGMA. These results suggest that PMPC, which has the potential to successfully evade the immune system, could be a new polymeric modifier for DNA aptamers.

### Synthesis of polymer-aptamer conjugates and evaluation of their reactivity against anti-PEG antibodies

In the present study, an aptamer, TXB0063, that binds to murine IFN- $\gamma$  was used (Fig. 2A). TXB0063 is a 62-nucleotide DNA aptamer (19.7 kDa without polymer modification) with the expanded genetic alphabet 7-(2-thienyl)imidazo[4,5-*b*]pyridine (Ds) for enhanced affinity and a mini-hairpin structure at the 3'-terminus for improved serum stability. TXB0063 binds to IFN- $\gamma$  with a  $K_D$  value of 2.47 nM and inhibits its activity *in cellulo*,<sup>22</sup> but does not function *in vivo* effectively because of its rapid clearance and requires appropriate modification.

Aptamer-polymer conjugates were synthesized by copper-free strain-promoted alkyne-azide cycloaddition (SPAAC, Fig. 2B). PMPC<sub>53, 93, 206</sub>, PTEGMA<sub>200</sub>, and PEG<sub>40kDa</sub> with a terminal amino group, shown in Fig. 1B, were the polymer modifiers used. The dibenzocyclooctyne (DBCO) group was introduced into the polymer terminus (DBCO-polymer, Fig. S1† for typical  $^1\text{H}$  NMR spectra). TXB0063 was modified with an azide group (TXB0063-azide) through the reaction between an azido-PEG<sub>4</sub>-*N*-hydroxysuccinimide ester and an amine group on the thymine residue, shown in red in Fig. 2A.

TXB0063-polymer conjugates were prepared using the SPAAC click chemistry between the DBCO-polymer and TXB0063-azide through a gradual freeze-thawing method<sup>23</sup> and purified by ion-exchange chromatography (Fig. 2B and S2†).

To characterize the synthesized conjugates, the anti-PEG reactivity of TXB0063-polymer conjugates was examined by the competitive enzyme-linked immunosorbent assay (ELISA) (Fig. 2C). TXB0063-PMPC<sub>206</sub> showed a significantly lower reactivity than TXB0063-PTEGMA<sub>200</sub> and TXB0063-PEG<sub>40kDa</sub>. Since TXB0063 without polymer modification did not show non-specific binding to the anti-PEG antibody, the antibody is considered to recognize PTEGMA and PEG, as was the case for the polymer alone (Fig. 1C).

### Evaluation of the effect of PMPC modification on the molecular size and *in vivo* circulation time of aptamers

The conjugates were also analyzed by size exclusion chromatography (SEC) (Fig. 2D). It suggests that modification with PMPC increased the size of the aptamer in a molecular weight-dependent way, with TXB0063-PMPC<sub>206</sub> having a comparable size to TXB0063-PEG<sub>40kDa</sub>.

After confirming that TXB0063 with a 3'-mini hairpin structure<sup>24</sup> is more stable in serum than single-stranded DNA with no predictable secondary structure and that PMPC modification does not affect the stability of TXB0063 in serum (Fig. S3†), we evaluated the *in vivo* pharmacokinetic properties. TXB0063 conjugated to PEG<sub>40kDa</sub> and PMPC<sub>53, 93, 206</sub> was intravenously administered (40 nmol kg<sup>-1</sup>) into rats, and the concentration of aptamers in the blood circulation over time was quantified by qPCR (Fig. 2E). All aptamer-polymer conjugates showed extended half-lives during the distribution phase ( $\alpha$  phase  $t_{1/2}$ ) from 0.09 h for non-modified TXB0063 to 0.18–0.54 h. TXB0063-PMPC conjugates exhibited size-dependent improvement in plasma retention half-lives. Notably, PMPC<sub>206</sub> surpassed PEG<sub>40kDa</sub>, a conventional choice for aptamer modification, improving both the half-life and AUC of TXB0063 better than PEG<sub>40kDa</sub>.

Considering that PMPC<sub>206</sub>, determined by GPC and DLS (Fig. 1B), was close in size to PEG<sub>40kDa</sub> and TXB0063-PMPC<sub>206</sub> was shown to be with a similar size to TXB0063-PEG<sub>40kDa</sub> in SEC (Fig. 2D), this result suggests that PMPC with a comparable molecular size can work correspondingly and be used as a substitute for PEG to prolong the survival of DNA aptamers in blood circulation.

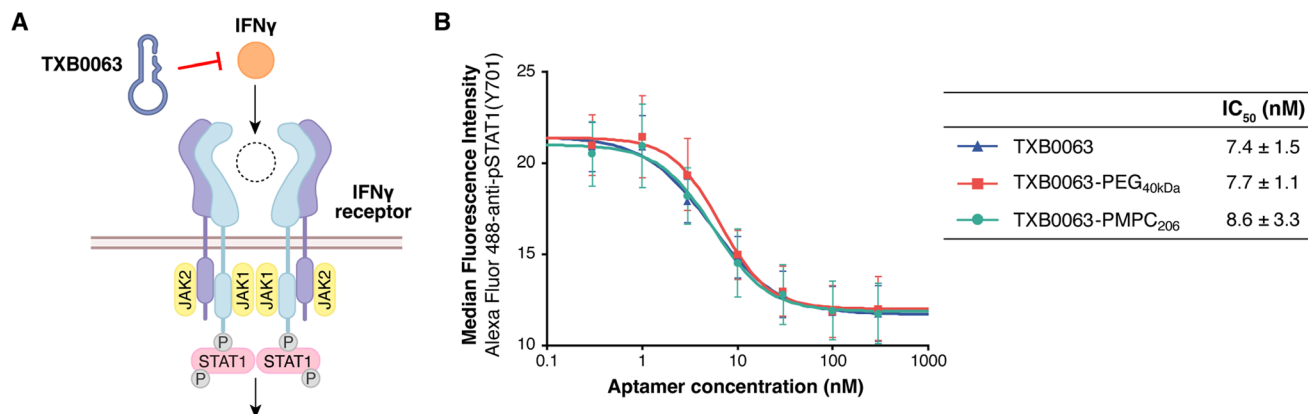
Therefore, we focused on PMPC<sub>206</sub> as a modifier capable of enhancing aptamer circulation and reducing the reactivity to anti-PEG antibodies, and proceeded to more detailed *in vitro* and *in vivo* evaluation studies of TXB0063-PMPC<sub>206</sub>.

### Evaluation of *in cellulo* and *in vivo* function of the PMPC-modified aptamer

We evaluated TXB0063-PMPC<sub>206</sub> for its function as an inhibitor for IFN- $\gamma$  activity. Initially, we investigated the IFN- $\gamma$  inhibitory activity of TXB0063-PMPC<sub>206</sub> in cultured cells. IFN- $\gamma$  induces the phosphorylation of signal transducer and activator of transcription (STAT1), and TXB0063 can







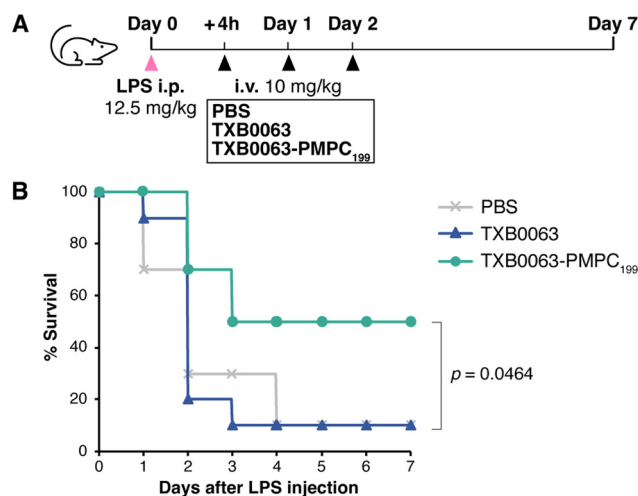
**Fig. 3** (A) Inhibitory mechanism of TXB0063 in IFN-gamma signaling. (B) Inhibition of STAT1 phosphorylation by TXB0063 and TXB0063-polymer conjugates. L929 cells were treated with IFN-gamma ( $2 \text{ ng mL}^{-1}$ ) at  $37^\circ\text{C}$  for 15 minutes in the presence of TXB0063 or TXB0063-polymer conjugates. After being washed with ice-cold PBS, the cells were fixed, permeabilized, and immunostained to measure the STAT1(Tyr701) phosphorylation level by flow cytometry. Plotted curves and the median fluorescence intensities are representative of three independent experiments performed in triplicate. IC<sub>50</sub> values are expressed in average  $\pm$  standard deviation of the values obtained from each experiment. Inhibition experiments using TXB0063-PTEGMA<sub>200</sub> and random oligonucleotide control are shown in Fig. S4.†

spectrum of TXB0063-PMPC<sub>206</sub> shows no notable difference from TXB0063 with no polymer modification (Fig. S5†). These results suggest that PMPC modification does not alter the conformation and inhibitory efficacy of TXB0063.

Finally, we investigated if PMPC modification can realize the therapeutic efficacy of TXB0063 *in vivo*, by systemic administration. In the previous study, TXB0063 was shown to mitigate bladder inflammation in Hunner-type interstitial cystitis-like model mice, when applied locally to the bladder *via* intravesical instillation.<sup>22</sup> In addition, previous observations on animal models and humans have suggested that neutralization of IFN-gamma could be an effective therapeutic approach to hyperinflammation.<sup>25</sup> We, therefore, tested the therapeutic potential of TXB0063 using a lipopolysaccharide (LPS)-induced endotoxic shock model.

IFN-gamma-deficient mice are resistant to endotoxic shock,<sup>26,27</sup> and mice treated with anti-IFN-gamma antibodies can better survive LPS-mediated septic shock.<sup>28,29</sup> Since TXB0063 is an IFN-gamma binding aptamer, if it functions as intended *in vivo* with blood circulation extended by PMPC modification, it is expected to neutralize IFN-gamma, alleviate acute inflammatory responses, and improve the survival rate of the LPS-injected mice.

We monitored the survival rate of mice after intravenous administration of PBS, TXB0063, and TXB0063-PMPC<sub>199</sub> at a dose of  $10 \text{ mg kg}^{-1}$  (aptamer equivalent) injection at 4, 24, and 48 hours after LPS injection ( $12.5 \text{ mg kg}^{-1}$ ) (Fig. 4A). During the one-week observation period, only 10% of mice that received PBS control survived longer than 3 days. When treated with TXB0063, the survival rate of mice was no better than the PBS-treated group, with only 10% survival by the 4th day. In contrast, TXB0063-PMPC<sub>199</sub> significantly improved survival rates, reaching 50% by the end of the observation week (Fig. 4B). This result clearly shows that PMPC modification significantly enhances the function of cytokine-inhibitory aptamers to achieve therapeutic efficacy *in vivo*.



**Fig. 4** Efficacy of TXB0063-PMPC in a mouse model of LPS-induced endotoxic shock. (A) Scheme for the study of therapeutic efficacy. The dosage of TXB0063 and TXB0063-PMPC<sub>199</sub> is expressed in terms of the weight of the aptamer. (B) Survival rates of the endotoxic shock model mice repeatedly injected with PBS, TXB0063, and TXB0063-PMPC<sub>199</sub> ( $N = 10$ ). Statistical significance was examined using a log-rank test. Data were considered to be statistically significant when  $p < 0.05$ .

## Conclusions

In the present study, we report a strategy to improve the pharmacokinetic properties of DNA aptamers by modifying them with the zwitterionic polymer PMPC.

DNA aptamers often require polymeric modifications for clinical applications, as their molecular weights are usually below the molecular cut-off for glomerular filtration. PEG has been considered non-immunogenic and commonly used to modify aptamers and various injectable drugs. However, it has been revealed that anti-PEG antibodies are generated and can



trigger immune reactions. With the increased sensitivity of assays and shared awareness about the immunogenicity of PEG, it has been increasingly reported that many healthy individuals, even without known exposure to PEGylated drugs, possess anti-PEG antibodies. This is due to daily exposure to PEG-containing products such as food or cosmetics, increasing the probability of possible immune reactions that occur when these antibodies bind to PEGylated aptamers.

PTEGMA, a polymer with three ethylene glycol moieties grafted along its methyl methacrylate backbone, was previously proposed as a candidate to modify aptamers with reduced reactivity against anti-PEG antibodies. However, our investigation showed that PTEGMA cannot fully evade recognition by anti-PEG antibodies due to its PEG-like chemical structure. On the other hand, PMPC, which includes no ethylene glycol backbone structure, was found not to bind to anti-PEG antibodies.

Aside from minimizing the potential for immunological side effects against PEG antibodies, it is notable that PMPC fulfilled its role as a macromolecular modifier to improve the *in vivo* behavior of DNA aptamers. First, PMPC<sub>206</sub> modification did not affect the structure of TXB0063, a DNA aptamer targeting murine IFN-gamma, and retained its neutralizing activity. Second, PMPC<sub>206</sub> modification prolonged the half-life of TXB0063, to a level comparable to or slightly superior to PEG<sub>40kDa</sub>. Finally, prolonged circulation of the PMPC-modified aptamer contributed to its enhanced therapeutic efficacy. While no difference in the survival rate was observed between TXB0063 and PBS control in the LPS-induced endotoxic shock model, the PMPC-modified aptamer significantly improved the survival rate from 10% to 50%. Further investigation on the optimization of the dose and frequency of administration may enhance the efficiency of the therapeutic potential of this aptamer.

These results comprehensively support the potential of PMPC as an aptamer modifier that can replace or be used alongside PEG. It is also expected that our approach of using PMPC as an aptamer modifier may be extended to other aptamer therapeutics, including those previously studied with PEG modifications as well as newly discovered aptamers.

## Author contributions

S.S. conceived the project. S.C., M.H., R.U., K.F. and S.S. designed the experiments. S.C., M.H., R.U., Y.S., Y.N., H.I., K. O., H.P., and K.F. carried out experiments and analyzed data, with support from A.T. and T.K. R.U. and S.S. provided resources and supervision. S.C. and S.S. wrote the manuscript, and all authors commented on and approved the manuscript.

## Ethical approval

All animal experiments were performed with the approval of the Animal Care and Use Committee of the University of

Tokyo, in accordance with the guidelines for the care and use of laboratory animals as stated by this institution.

## Data availability

The data supporting this article have been included as part of the ESI.†

## Conflicts of interest

The authors declare the following competing financial interests: the authors (S.C., M.H., R.U., Y.S., K.O., H.P., K.F., and S. S.) have filed a patent application (PCT/JP2022/022601). All other authors declare that they have no competing interests.

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