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## Influence of hydrophilic polymers on the accelerated blood clearance of mRNA lipid nanoparticles upon repeated administration

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mRNA lipid nanoparticles (LNPs) have emerged as a leading delivery system for mRNA-based vaccines and therapeutics. However, a significant limitation of this system is the presence of poly(ethylene glycol) (PEG). It is widely known that repeated doses of PEG-based therapeutics can induce an anti-PEG antibody response, leading to the accelerated blood clearance (ABC) of LNP therapeutics requiring frequent dosing, as anti-PEG antibodies have been found present in a large proportion of the population. To address this issue, we developed a mouse model for LNP clearance after a repeated dose. We then synthesised LNPs with the PEG component replaced by a library of hydrophilic polymers: poly(oligo(ethylene glycol) methyl ether methacrylate) (POEGMA), POEGMA-methacrylic acid (POEGMA (-)), POEGMA-2-(dimethyl-amino)ethyl methacrylate (POEGMA (+)), poly(*N,N*-dimethyl-acrylamide) (PDMA), and poly(*N*-(2-hydroxypropyl) methacrylamide) (PHPMA). Our results demonstrated that all three POEGMA LNPs, especially POEGMA (+) LNPs, exhibited minimal ABC effect after two weekly doses; in contrast, PDMA LNPs demonstrated significantly lower clearance in the presence of anti-PEG antibodies. This study highlights the potential of PEG-free polymer-LNPs as promising mRNA carriers that avoid rapid clearance with repeated administration.

### New concepts

Advancement of mRNA-lipid nanoparticle (mRNA-LNP) therapeutics beyond vaccines is stymied by increasing prevalence of antibodies generated against polyethylene glycol (PEG), a key component in mRNA-LNP formulations, as applications of this polymer ranging from medications to common household products have resulted in widespread exposure. Anti-PEG immune responses can mediate accelerated blood clearance (ABC) of mRNA-LNPs, necessitating repeat dosing to achieve a desired therapeutic effect. Replacement of PEG with polymers able to stabilise mRNA-LNPs while avoiding premature clearance by anti-PEG antibodies is of keen interest for therapeutics that require frequent, multiple dosing, but products have yet to progress beyond the bench. We generated a library of novel PEG-free mRNA-LNP formulations by replacing the PEG component with a range of hydrophilic polymers. We then developed a mouse model expressing anti-PEG antibodies after repeated administration of mRNA-LNPs, resulting in their rapid clearance from circulation, and investigated clearance of PEG-free mRNA-LNPs under the same conditions. Here we present a proof-of-concept exploration of clearance profiles and anti-PEG cross-reactivity of PEG-free mRNA-LNPs after repeated administration. This short communication not only provides valuable insight into PEG-free mRNA-LNPs as promising therapeutic candidates, but additionally presents a robust model for researchers to assess clearance profiles of their own mRNA-LNP formulations.

## Introduction

Lipid nanoparticles (LNPs) are a rapidly expanding system in the nanomedicine field, with mRNA vaccines and therapeutics emerging as promising new platforms, highlighted by the success of the Moderna and BioNTech-Pfizer vaccines against the COVID-19 (SARS-CoV-2 virus) pandemic in 2020.<sup>1–3</sup> The LNP system typically consists of a mixture of lipids such as the ionisable cationic lipids, cholesterol, phospholipids, and lipid-conjugated poly(ethylene glycol) (PEGylated lipid), as well as the nucleic acids (*e.g.*, mRNA or pDNA) that encode the protein of interest.<sup>4,5</sup> Among these components, the PEGylated lipid plays a crucial role in the surface modification of the LNP. It is known for its anti-fouling properties which prevent particle aggregation and enhance particle colloidal stability, as well as

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its ability to prevent plasma protein adsorption to the LNP surface, thereby reducing the LNP uptake by cells of the mononuclear phagocyte system (MPS) in the liver and spleen.<sup>6–8</sup> This ‘stealth’ property allows LNP therapeutics to maintain longer blood circulation time and reduce off-target effects.<sup>9</sup>

A major limitation exists with the PEGylated nanoparticle system: its propensity to trigger rapid clearance of PEGylated therapeutics after repeated administration following an initial exposure to PEG.<sup>10,11</sup> This phenomenon, known as accelerated blood clearance (ABC), can significantly reduce the effectiveness of PEGylated therapeutics and increase undesirable adverse effects through altering their biological distribution.<sup>11,12</sup> Since most treatment approaches for mRNA therapeutics necessitate multiple doses due to their short half-lives,<sup>13</sup> a PEGylated therapeutic may lose its extended circulation properties and efficacy if frequent dosing is required.<sup>10,11,14,15</sup> This could be problematic for LNP-based therapeutic applications, such as gene therapy and cancer immunotherapy, which depend on repeated dosing over an extended period without compromising their therapeutic efficacy.<sup>16</sup> Recent studies have discovered that mRNA–LNP formulations can induce or boost anti-PEG antibodies in humans and have advanced our understanding of the structure–function relationships and immunological consequences of PEGylated LNPs, underscoring the critical need to overcome PEG immunogenicity.<sup>17–21</sup> Addressing this immune response is essential for improving the safety and efficacy of PEGylated therapeutic platforms, as well as expanding the utility of PEGylated LNPs in the long term.

To overcome the PEG immunogenicity, and therefore reduce the incidence of ABC of PEGylated nanoparticles, various strategies have been developed and adopted. These include altering the physicochemical properties of the nanoparticles by introducing cleavable PEG, adjusting its molecular weight, or substituting PEG entirely with other hydrophilic polymers.<sup>22–24</sup> Liposomes modified with hydrophilic polymers such as poly-(hydroxyethyl L-asparagine) (PHEA), poly(*N*-(2-hydroxypropyl)methacrylamide) (PHPMA), poly-(vinylpyrrolidone) (PVP), poly(2-methyl-2-oxazoline) (PMOX), poly(*N*-acryloyl morpholine) (PACM), and poly(*N,N*-dimethylacrylamide) (PDMA) have not been shown to trigger the ABC phenomenon.<sup>24–26</sup> However, to the best of our knowledge, the ABC effect of these polymers has not yet been studied in the context of LNPs. Investigating this is crucial, as the LNP platform is rapidly advancing in both vaccine development and therapeutic applications. As the LNP structure is inherently more complex than that of a liposome – due to differences in lipid composition, which can affect polymer density and conformation on the particle surface, as well as its more fragile nucleic acid cargo – the results gathered from previous liposomal studies may not be directly applicable to LNPs, even when similar experimental conditions are used. Concordantly, as researchers seek to expand mRNA–LNP technologies beyond vaccine applications, in particular those requiring more frequent dosing *via* intravenous (i.v.) routes, it is imperative that *in vivo* screening of formulations to identify candidates susceptible to ABC after repeat dosing is undertaken prior to advancing to in-depth pre-clinical studies.

Herein, we replaced the PEG component in mRNA–LNPs with poly(oligo(ethylene glycol) methyl ether methacrylate) (POEGMA), POEGMA-methacrylic acid (POEGMA (–)), POEGMA-2-(dimethylamino)ethyl methacrylate (POEGMA (+)), PDMA, and PHPMA (Fig. S1), and evaluated their ABC phenomenon in mice using a mouse model that we developed in our lab. The side chains of POEGMA contain a similar ethylene glycol repeating unit as the main backbone of PEG, though the POEGMA side chains have far fewer ethylene glycol units (9 units compared to 44–45).<sup>27</sup> Our mouse model, utilising a facile fluorescence-based approach to track blood clearance of mRNA–LNPs after both an initial and repeated weekly dose, provided a robust and efficient method for screening formulations for ABC. We also investigated the cross-reactivity between anti-PEG IgM and each polymer–LNP of our library. To keep comparisons relevant, we utilised polymer–lipids with 18-carbon lipid chains (C18) with a comparative C18 PEG control (PEG-DSG) accordingly. Longer acyl chains are known to result in slower shedding of PEGylated lipids from the LNP surface, resulting in extended circulation time and allowing the PEGylated drug to remain in the body longer. Additionally, this leads to increased production of anti-PEG IgM, which can induce the ABC phenomenon.<sup>28,29</sup> Intravenously administered mRNA–LNPs containing C18 PEG have also been reported to be particularly effective for targeting liver and splenic immune cells.<sup>30,31</sup> Therefore, this approach will provide a more effective comparison of polymers that may induce the ABC effect, aiding in the identification of polymers suitable for longer-acting LNP treatments requiring multiple doses within the ABC effect window.

## Experimental

### Materials

mRNA encoding muGFP–NLuc was synthesised in our laboratory as previously described.<sup>32</sup> Cholesterol was purchased from Sigma-Aldrich. 1,2-Distearoyl-*rac*-glycerol-3-methoxypolyethylene glycol (DSG-PEG 2000) and 1,2-distearoyl-*sn*-glycero-3-phosphorylcholine (DSPC) were sourced from Avanti Polar Lipids. ((4-Hydroxybutyl)azanediyl)bis(hexane-6,1-diyl)bis(2-hexyldecanoate) (ALC-0315) was obtained from Sinopeg Biotech. 1,1''-Dioctadecyl-3,3, 3'',3''-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (DiD solid; DiC18(5)) was purchased from Thermo Fisher Scientific.

### LNP preparation

LNPs were prepared as previously reported.<sup>27,33,34</sup> Lipid components – ALC-0315, DSPC, cholesterol, PEG/polymer–lipid and DiD (if present) – were dissolved in ethanol at molar ratio of 46.3 : 9.4 : 42.7 : 0.5 (polymer–lipid)/1.6 (PEG) : 0.25. mRNA concentrations were measured using a NanoDrop (Nd 3300 Fluorospectrometer, Thermo Fisher Scientific) and dissolved in 25 mM sodium acetate buffer (pH 4). The solutions were mixed *via* a microfluidic chip with a staggered herringbone mixer (SHM) in the NanoAssemblr Benchtop (Cytiva) at an N/P charge



ratio of 6, a total flow rate of 8 mL min<sup>-1</sup> and a 3:1 flow rate ratio. The mixture was dialysed against PBS (pH 7.4) for 18 hours. Prior to *in vivo* studies, LNPs were filtered and then either concentrated (Amicon Ultra-0.5 mL Centrifugal Filters 50 K, Merck) or diluted with sterile PBS (pH ~7.4) as needed. All LNPs were stored at 4 °C until use.

### LNP characterisation

LNPs were characterised using dynamic light scattering (DLS) on a Zetasizer Nano ZS (Malvern Instruments) to determine average particle size and polydispersity index with a ZEN0040 disposable cuvette. Zeta-potential measurements were conducted using a DTS107 disposable folded capillary cell after diluting LNPs 10-fold with nuclease-free water. All measurements were done in triplicate and reported as mean ± standard deviation, with a single population observed unless stated otherwise.

RNA entrapment efficiency was measured using the Quant-iT RiboGreen RNA assay kit (Thermo Fisher Scientific). LNP samples were diluted 50-fold in TE buffer and plated in duplicate in a black 96-well microplate. To lyse the LNPs, 0.1% Triton X-100 was added to one well per sample, followed by 10 minutes incubation at room temperature (RT) and centrifugation at 300 rpm. Quant-iT Ribogreen RNA reagent was later added to all wells and the plate was further incubated at RT for 5 minutes. Fluorescence was measured using an EnVision 2103 Multilabel Reader (PerkinElmer) at excitation/emission wavelengths of 500/535 nm. Encapsulation efficiency (%) was calculated using the formula: (lysed LNP – non-lysed LNP)/lysed LNP × 100.

### Animal work

All experiments were approved by Monash Institute of Pharmaceutical Sciences (MIPS) and University of Melbourne (UoM) animal ethics committee and conducted according to Australian and New Zealand Council for the Care of Animals in Research and Teaching guidelines; Approval code: 2023-39320-98700, 19 September 2023 (MIPS) and 2023-26558-44218-4, 11 September 2023 (UoM). Male BALB/c mice (9–11 weeks old) with standard diet and free access to water were housed on a 12-hour light/dark cycle at ambient temperatures (21–22 °C). In all experiments, a minimum of four mice were randomly selected and used per group to confirm data reproducibility.

### Mouse model of LNP clearance

Mice were administered with 100 μL PBS or non-labelled LNPs (0.5 μg mRNA) *via* the i.v. route. Seven days later, a second i.v. dose of 100 μL DiD-labelled LNPs (24 μg mRNA) was given to the mice. At 0–4 h post-injection of the second dose, blood was collected and centrifuged twice at 3000 g (5 min per run) to collect the serum. The fluorescence intensity of DiD-labelled LNPs was measured using an EnVision 2103 Multilabel Reader (PerkinElmer) at excitation/emission wavelengths of 620/665 nm.

### Anti-PEG IgM detection

The ELISA was performed using a slightly modified previously published method.<sup>17</sup> Maxisorp plates (Thermo Fisher Scientific) were coated overnight at 4 °C with 200 μg mL<sup>-1</sup> 8-arm PEG-NH<sub>2</sub>

(40 kDa) (JenKem Technology). The plates were washed four times with PBS then blocked with 5% (w/v) skim milk powder in PBS for 2 h. Serum samples were diluted 1:10 in 5% skim milk and serially diluted 2-fold on a separate microplate. The diluted samples were transferred to the PEG-coated plates and incubated for 1 h. Plates were washed twice with 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (Sigma-Aldrich) in PBS and four times with PBS before being incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgM (Thermo Fisher Scientific) at 1:2000 dilution for 1 hour at RT. Plates were washed again as above, and developed using 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich) for 5 minutes. The reaction was stopped with 0.16 M H<sub>2</sub>SO<sub>4</sub> and absorbance was read at 450 nm on an EnVision 2103 Multilabel Reader (PerkinElmer).

### Statistical analysis

Graphs and statistics were prepared in GraphPad Prism (version 9.3.1 for Windows). Statistical differences were analysed using two-way ANOVA (followed by Dunnett multiple comparisons test), with  $\alpha = 0.05$  used to indicate significance. All samples were reported as mean ± standard error of the mean.

## Results and discussion

### Polymer-LNP characterisation

All PEG-alternative polymer-lipids used in this study were synthesised *via* reversible addition-fragmentation chain transfer (RAFT) polymerisation. The detailed synthesis and comprehensive characterisation data for polymers used in this work are fully described in a recent publication from our lab.<sup>27</sup> In this study, we first synthesised six mRNA-LNPs, each incorporating one of the respective polymer-lipids (Fig. S1 and Table 1). After successful synthesis, the LNPs were characterised to determine their size, polydispersity index (PDI), zeta potential and encapsulation efficiency of the mRNA cargo (Table S1). All particles were smaller than 80 nm, with PDIs below 0.2, indicating a narrow particle size distribution. The zeta potential measurements showed net negative charges for all LNPs, and the encapsulation efficiencies of the mRNA cargo were all greater than 65%. Overall, these results are consistent with previous data obtained from our lab,<sup>27</sup> confirming the reproducibility and suitability of these LNPs for *in vivo* experiments.

### ABC model

Next, we evaluated the effect of polymer-LNPs on the induction of ABC effect in mice by first establishing a mouse model of

Table 1 RAFT polymer characteristics

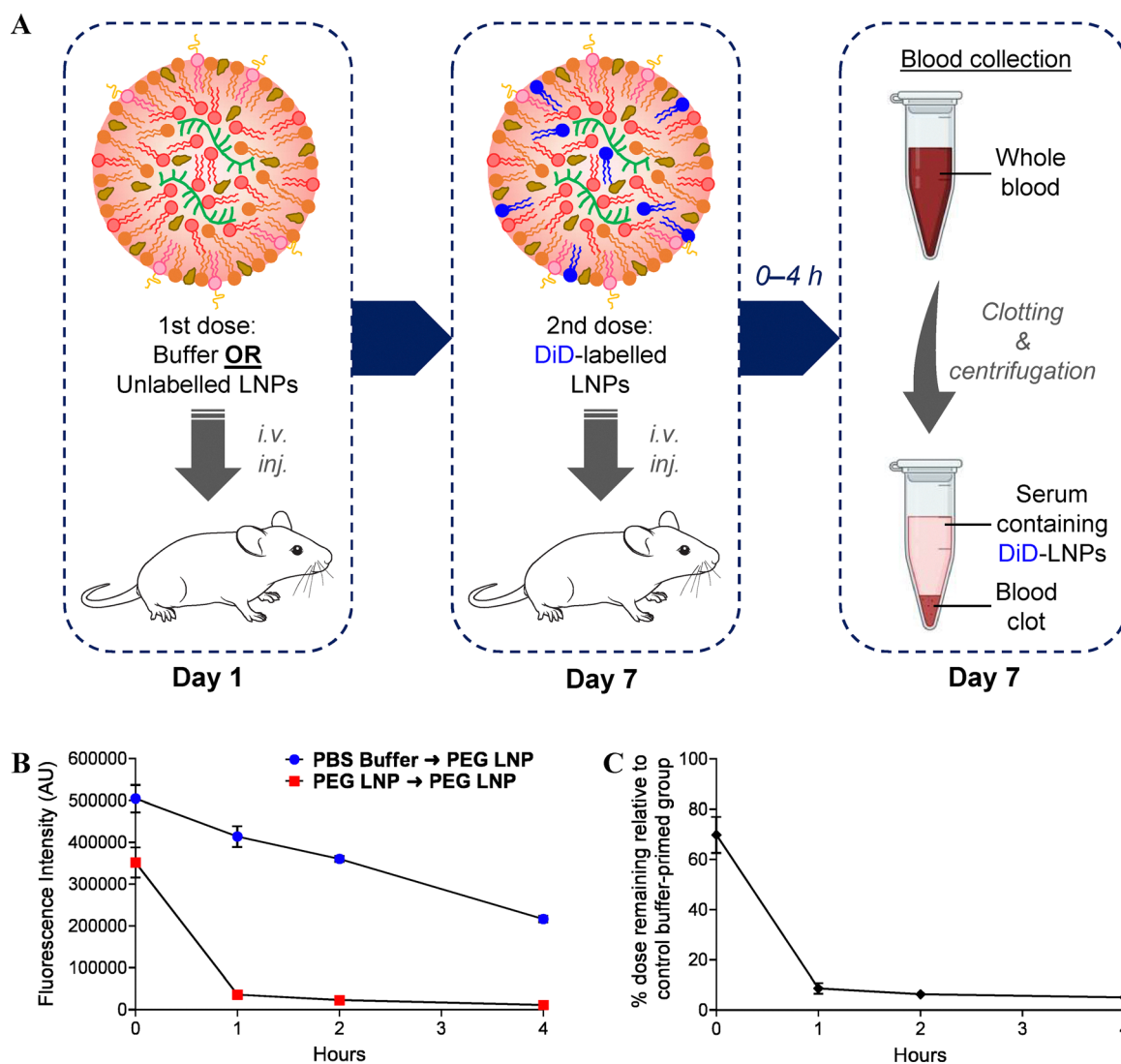
Polymer	Structure	Charge
POEGMA	Branched	Neutral
POEGMA (-)	Branched	Negative
POEGMA (+)	Branched	Positive
PDMA	Linear	Neutral
PHPMA	Linear	Neutral



PEGylated LNP clearance (Fig. 1A). Dosing parameters were optimised in-house and selected based on those established with other PEGylated nanodrugs: typically, more anti-PEG antibodies are generated when a smaller initial dose is administered followed by a larger second dose of PEGylated materials 6–8 days later.<sup>11,35</sup> Larger initial doses of PEGylated nanoparticles are avoided because they have been shown to induce immune tolerance (B cell inactivity) to PEG in mice, leading to a decrease in the production of anti-PEG IgM.<sup>36</sup> Given the short half-life of mRNA–LNP therapeutics,<sup>13</sup> studying the ABC effect within the first few hours after the second dose is critical, as this is when the effect is most pronounced.<sup>24,37–39</sup> As such, mice were divided into 8 groups and injected according to the dosing regimen presented in Table S1. Mice were first primed with LNPs at a dose of 0.5  $\mu\text{g}$  mRNA per mouse *via* the i.v. route. Seven days later, mice were boosted i.v. with LNPs

labelled with the lipophilic dye DiD at a dose of 24  $\mu\text{g}$  mRNA per mouse. Blood samples were collected from the mice between 0–4 h post-second injection to measure DiD fluorescence and determine the level of DiD-labelled LNP clearance.

As shown in Fig. 1B and C, LNP clearance in our anti-PEG mouse model was most pronounced within the first hour post-second dose. The ‘PEG LNP  $\rightarrow$  PEG LNP’ treatment group, in which mice previously exposed to PEG LNPs received another PEG LNP injection, showed a significant loss of circulating LNPs during this initial hour. The control ‘PBS Buffer  $\rightarrow$  PEG LNP’ group exhibited a longer blood circulation time in the absence of prior PEG exposure, with DiD fluorescence showing a 50% reduction only at 4 hours. Overall, these LNP clearance dynamics align with findings from previous ABC studies on PEGylated liposomes, showing that the ABC phenomenon is typically most pronounced within the first four hours after a



**Fig. 1** Development of a rapid clearance model in BALB/c mice for LNPs after one repeated dose. (A) Schematic illustration of mouse i.v. injection and blood collection schedule at days 1 and 7. (B) Serum clearance of DiD-labelled LNPs in absence (PBS Buffer  $\rightarrow$  PEG LNP) and presence (PEG LNP  $\rightarrow$  PEG LNP) of an initial LNP dose. (C) Serum clearance of DiD-labelled LNPs in mice pre-exposed to LNPs relative to the control group at 0–4 h after the second injection ( $n = 5–7$ ).



second i.v. injection in pre-exposed mice,<sup>24,37–39</sup> and often occurs most rapidly within the first 15 minutes.<sup>10,11</sup> This clearance of PEG LNPs may be attributed to the production of anti-PEG IgM, which is known to lead to increased liver uptake and a reduced blood half-life of PEGylated nanoparticles upon subsequent dosing.<sup>40–42</sup>

### ABC of polymer-LNPs

Having established a mouse model primed with PEG LNPs, which results in rapid LNP clearance within the first four hours following a second dose, we proceeded to evaluate the ABC effect of the other PEG-alternative polymer-LNPs in mice at 4 h. Mice were divided into 17 groups and injected according to the dosing regimen outlined in Table 2. In this experiment, we also assessed the cross-reactivity of each polymer to PEG by administering PEG LNPs to the mice in the first dose and our polymer-LNPs in the second dose. To ensure consistency, the fluorescence intensity (reported as relative fluorescence arbitrary units (AU)) of all DiD-labelled LNPs was established prior to injection. As shown in Fig. S2, no significant differences in fluorescence intensity were observed between all DiD-labelled LNPs prior to administration.

Following the second injection of DiD-labelled LNPs, the 'PEG → PEG' group exhibited the highest blood clearance among all LNP groups (Fig. 2A and B), consistent with the findings in Fig. 1, demonstrating that our mouse model is both robust and reproducible. POEGMA LNPs, whose polymer shares a similar but much shorter ethylene glycol repeating unit with the main backbone of PEG, exhibited either similar or reduced blood clearance compared to the 'PEG → PEG' group when administered to mice previously dosed with PEG LNPs. It is hypothesised that anti-PEG IgM may bind to a similar epitope with less specificity for PEG,<sup>41</sup> indicating some degree of cross-reactivity between anti-PEG IgM and POEGMA. Additionally, the branching of POEGMA, which offers more comprehensive protection to the LNPs against opsonisation,<sup>43</sup> may further reduce premature clearance mediated by a pre-existing anti-PEG response. Notably, POEGMA (+) LNPs demonstrated the

lowest blood clearance compared to POEGMA and POEGMA (–) LNPs when PEG LNPs were administered in the first dose, suggesting that the positive charge may play a role in prolonging circulation half-life under the tested conditions. In contrast, all three branched polymer-LNP groups with the same prime-boost schedule – 'POEGMA → POEGMA', 'POEGMA (–) → POEGMA (–)', and 'POEGMA (+) → POEGMA (+)' – showed relatively low blood clearance. This suggests that these three polymer-LNPs do not trigger the same high level of ABC effect and induce minimal production of anti-polymer antibodies after the first dose, compared to the linear PEG LNPs (Fig. 2A and B). This finding aligns with Liu *et al.*'s study, which demonstrated that branched PEG on liposomes produced noticeably lower levels of anti-PEG IgM compared to linear PEG-modified nanocarriers and did not induce the ABC phenomenon after repeated injections.<sup>44</sup>

Interestingly, the two linear polymer-LNP groups when administered in the same prime-boost schedule – 'PDMA → PDMA' and 'PHPMA → PHPMA' – exhibited relatively high blood clearance (Fig. 2A and B), suggesting that they may have triggered the ABC effect and production of anti-polymer antibodies. However, their clearance was still significantly lower than that of the control 'PEG → PEG' group. In contrast, PDMA and PHPMA LNPs demonstrated different clearance patterns in mice pre-exposed to PEG LNPs: the 'PEG → PDMA' group showed very low blood clearance, whereas the 'PEG → PHPMA' group displayed relatively high blood clearance. This observation warrants further investigation beyond the scope of this study, as differences in polymer molecular weight, flexibility, and conformation could also influence their shedding and/or opsonisation propensities, and consequently, the circulation half-life of the LNPs.<sup>24</sup>

### Anti-PEG IgM detection

Finally, we evaluated the cross-reactivity of anti-PEG IgM against all five polymer-LNPs using an enzyme-linked immunosorbent assay (ELISA) to determine whether differences in anti-PEG IgM cross-reactivity influenced the varying clearance

Table 2 Injection protocols for LNPs formulated with different polymer-lipids

Dosing regimen (1st injection → 2nd injection)	First injection (unlabelled polymer-LNPs)	Second injection (DiD-labelled polymer-LNPs)
PBS → PEG	PBS buffer control (no LNPs)	PEG
PBS → POEGMA	PBS buffer control (no LNPs)	POEGMA
PBS → POEGMA (–)	PBS buffer control (no LNPs)	POEGMA (–)
PBS → POEGMA (+)	PBS buffer control (no LNPs)	POEGMA (+)
PBS → PDMA	PBS buffer control (no LNPs)	PDMA
PBS → PHPMA	PBS buffer control (no LNPs)	PHPMA
PEG → PEG	PEG	PEG
PEG → POEGMA	PEG	POEGMA
PEG → POEGMA (–)	PEG	POEGMA (–)
PEG → POEGMA (+)	PEG	POEGMA (+)
PEG → PDMA	PEG	PDMA
PEG → PHPMA	PEG	PHPMA
POEGMA → POEGMA	POEGMA	POEGMA
POEGMA (–) → POEGMA (–)	POEGMA (–)	POEGMA (–)
POEGMA (+) → POEGMA (+)	POEGMA (+)	POEGMA (+)
PDMA → PDMA	PDMA	PDMA
PHPMA → PHPMA	PHPMA	PHPMA



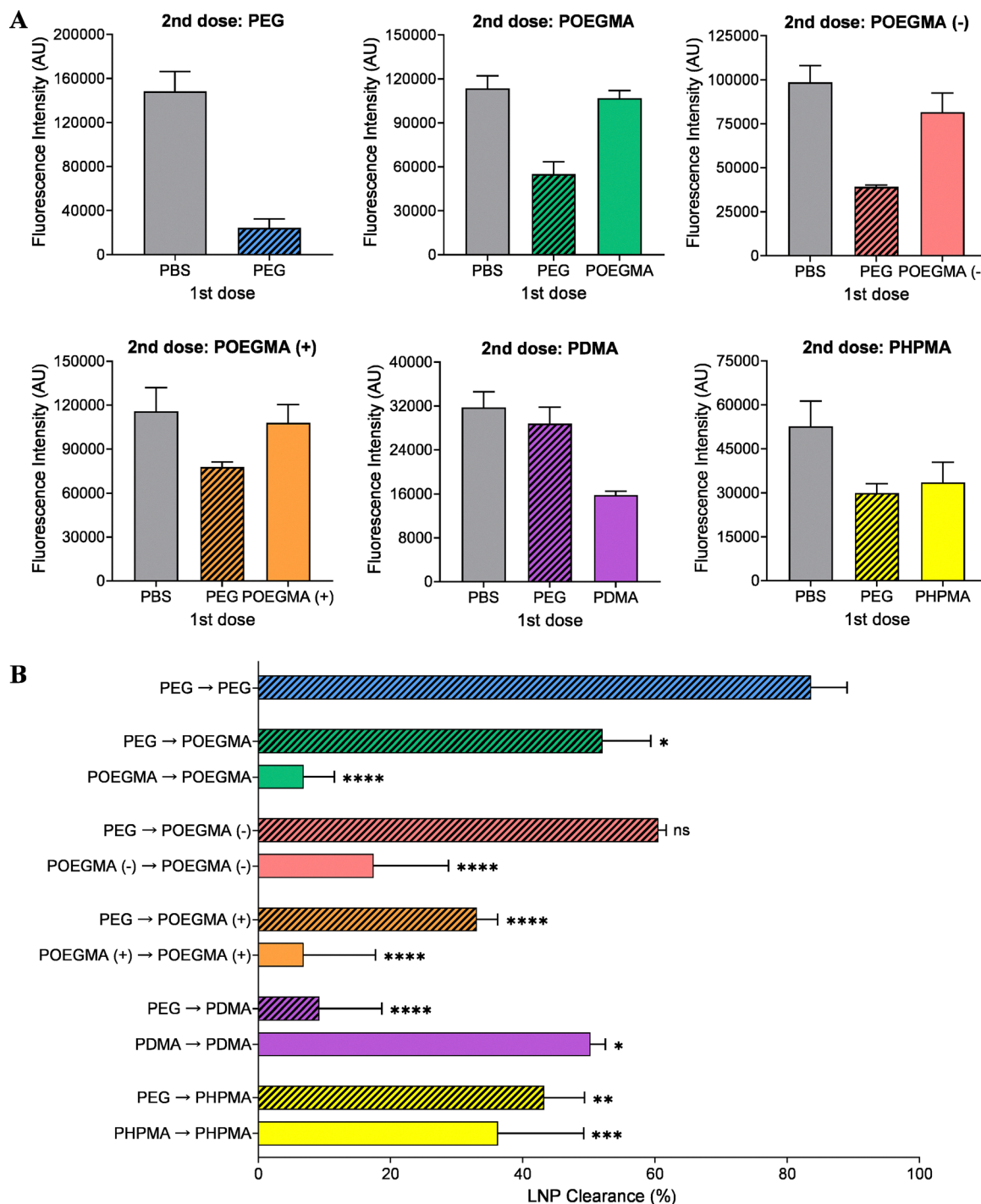


Fig. 2 (A) Serum concentrations and (B) blood clearance (%) of DiD in mice after two polymer-LNP i.v. injections (one week apart) at 4 h. ( $n = 4-9$ ; significant differences shown are compared to the control 'PEG → PEG' group: \* $p < 0.0202$ , \*\* $p = 0.0017$ , \*\*\* $p = 0.0006$ , \*\*\*\* $p < 0.0001$ ).

patterns of polymer-LNPs at the time of injection for the second dose (day 7) in mice pre-exposed to PEG. The ABC phenomenon has been linked to the cross-linking of PEGylated materials with PEG-specific splenic B-cell receptors triggering anti-PEG IgM production.<sup>45</sup> Our results demonstrated that all five polymer-LNPs induced detectable but significantly lower IgM titres compared to PEG LNPs (Fig. 3), indicating antibodies generated against these polymers overarchingly exhibited low

cross-reactivity to anti-PEG IgM. Although not statistically significant, the average serum antibody titres of all three POEGMA LNPs, and PHPMA LNPs, appeared notably higher compared to that of PDMA LNPs. As mentioned previously, in the case of POEGMA LNPs, cross-reactivity with anti-PEG antibodies may be attributed to the similar ethylene glycol repeating unit shared with the PEG backbone. Overall, it is interesting that polymers with differing moieties from PEG (*i.e.* PDMA and



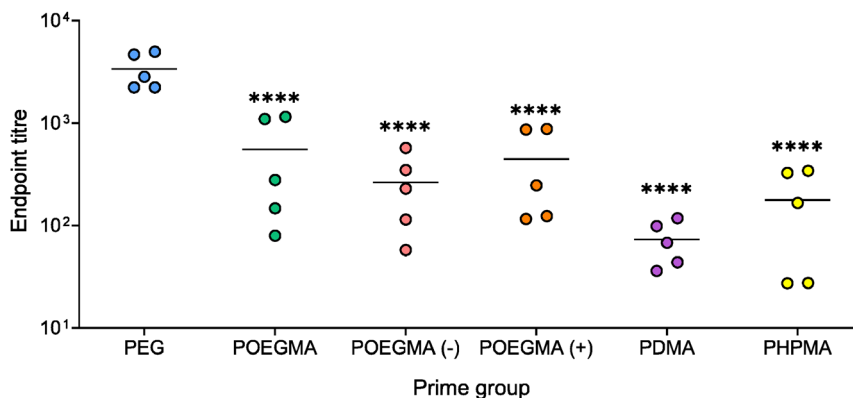


Fig. 3 Evaluation of anti-PEG IgM in sera on day 7 following a single injection of each LNP formulation. ( $n = 5$ ; significant differences shown are compared to the control PEG group: \*\*\*\* $p < 0.0001$ ).

PHPMA) still demonstrated some cross-reactivity with anti-PEG IgM, warranting further investigation into the mechanisms underlying this response.

## Conclusion

In this study, we investigated the ABC effect and PEG cross-reactivity of a panel of hydrophilic polymer-lipids on mRNA-LNPs through the development of a fluorescence-based mouse model of ABC. Our findings demonstrate that the polymer branching properties and addition of a charge, which are not possible with PEG, allowed POEGMA (+) LNPs to exhibit minimal ABC effect after two weekly doses. This could be valuable in developing mRNA-LNP therapeutics requiring repeated administration, particularly when there is minimal pre-exposure to PEG or anti-PEG antibodies. Additionally, we showed that PDMA LNPs displayed significantly low clearance in the presence of anti-PEG antibodies, highlighting their potential for use in mRNA-LNP therapeutics where anti-PEG antibodies are present in the body. These results underscore the potential of alternative polymers to PEG in the development and application of LNP therapeutics, especially when mRNA-LNPs have short half-lives which necessitate repeated dosing. Evidently, there is no 'one-size-fits-all' solution to the ongoing ABC phenomenon, and LNP formulations will ultimately need to be tailored to each patient's individual profile for optimal outcomes. Further research is necessary to gain a deeper understanding of the clearance mechanisms of these polymer-LNPs, their ABC effect over time, and their correlation with the induction of anti-polymer antibodies. As such, our mouse model provides a simple and effective 'first-pass' screening assay to assess clearance profiles of mRNA-LNP formulations after an initial repeated dose and identify candidates to advance to further studies. In line with this, future studies could expand the pre-clinical evaluation by incorporating additional animal models such as rats or non-human primates, to better assess the translatability and generalisability of the results. Ultimately, these polymers present a promising alternative to PEG in mRNA-LNPs for minimising the ABC effect, and will aid in the future design and polymer applications of PEG-free LNP systems including, but not

limited to, gene therapy, protein replacement therapy, and cancer immunotherapy.

## Author contributions

Dayangku Nordiyana B. P. Hassanel: conceptualization, methodology, validation, formal analysis, investigation, data curation, writing – original draft, writing – review & editing, visualisation, project administration. Yi Ju: methodology, writing – review & editing. Asuka Takanashi: investigation (mice injections). Azizah Algarni: investigation (mice injections). Chee Leng Lee: investigation (mice injections). Stephen J. Kent: resources, writing – review & editing, supervision, funding acquisition. Colin W. Pouton: resources, writing – review & editing, supervision, funding acquisition. Emily H. Pilkington: conceptualization, writing – review & editing, supervision.

## Conflicts of interest

All the contributing author(s) report(s) no conflict of interests in this work.

## Data availability

The data supporting this article have been included as part of the supplementary information (SI). See DOI: <https://doi.org/10.1039/d5nh00230c>.

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