

GENE THERAPY

# Genetic engineering of transfusable platelets with mRNA-lipid nanoparticles is compatible with blood banking practices

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KEY POINTS

- Donor platelets can be directly genetically modified in plasma and platelet additive solution using mRNA-lipid nanoparticles.
- mRNA-LNP transfection is scalable to physiological and supraphysiological platelet concentrations, and engineered platelets can be stored.

**Platelets contribute to a variety of physiological processes, including inflammation, sepsis, and cancer. However, because of their primary role in hemostasis, platelet transfusions are largely restricted to managing thrombocytopenia and bleeding. One way to expand the utility of platelet transfusions would be to genetically engineer donor platelets with new or enhanced functions. We have previously shown that lipid nanoparticles containing mRNA (mRNA-LNP) can be used to genetically modify authentic platelets in a nonclinical crystalloid solution. Currently, platelets collected for transfusion are stored in plasma or in plasma supplemented with platelet additive solution (PAS) at supraphysiological concentrations at room temperature, or at 4°C if intended for use in acute hemorrhage. Here, we describe a new plasma-optimized mRNA-LNP for transfecting platelets directly in plasma and plasma supplemented with PAS that is scalable to physiological and supra-physiological platelet concentrations. Transfecting platelets in clinical solutions with mRNA-LNP does not affect aspects of in vitro physiology, and transfected platelets are storable. The compatibility of this transfection system with current clinical practices could**

**enable future mRNA-LNP-based platelet products and cell therapies.**

## Introduction

Platelet transfusions are an important cell therapy used to prevent or stop bleeding and manage thrombocytopenia.<sup>1</sup> Beyond hemostasis, platelets are also key immune cells and have been implicated in inflammatory diseases among other physiological processes.<sup>2</sup> However, despite their multifunctional roles, platelets have not been expanded as a cell therapy in other indications. One of the major hurdles in developing new, platelet-based cell therapies is the limited options available for genetically modifying them with new or enhanced functions. Indirect methods for genetically engineering platelets exist and rely on the modification of platelet precursor cells using lentiviral vectors, resulting in the production of modified platelets.<sup>3,4</sup> However, this process can be costly, and there are immunologic challenges.<sup>5</sup> Previously, our laboratory demonstrated that donor platelets collected for transfusion can be engineered in nonclinical, crystalloid solution using lipid

nanoparticles (LNPs) containing mRNA to produce exogenous protein.<sup>6</sup> Engineering new or enhanced function directly in donated platelets with this technology and improving its clinical compatibility could widely expand the current uses of platelet cell therapy.<sup>7</sup>

Transfecting platelets with mRNA rather than DNA is necessary because platelets are anucleate and unresponsive to DNA delivery.<sup>6</sup> Furthermore, platelets are translationally active, synthesizing new proteins from endogenous mRNA, which is a natural property leveraged to achieve the production of exogenous protein.<sup>8</sup> We have previously shown that mRNA delivery requires LNPs, as other delivery agents do not work for transfecting platelets.<sup>6</sup> LNPs are clinically validated, highlighted by the US Food and Drug Administration–approved drug, Onpatro, which is a silencing RNA (siRNA) therapeutic for hereditary transthyretin amyloidosis, and the mRNA-based COVID-19 vaccines Spikevax and Comirnaty.<sup>9-11</sup> Another major advantage

of LNPs for platelet engineering is their modularity, whereby lipid components and mRNA can be modified to change cell tropism and improve transfection potency. For example, LNP composition can be specifically designed to maximize expression in cultured cells<sup>12</sup> or for targeting extrahepatic tissues.<sup>13</sup>

In our previous work, platelets were washed and resuspended in crystalloid buffer before transfection with mRNA-LNP. Although they retained hemostatic function, this approach is limited. It requires excessive platelet handling in transferring platelets from their normal plasma-based storage solution into pure crystalloid solution, which can cause unwanted activation.<sup>14,15</sup> Platelets are easily activated by stressors, which results in the release of intracellular  $\alpha$ -granules rich in membrane bound P-selectin (CD62P), the externalization of anionic phospholipid, and the formation of pseudopodia.<sup>2</sup>

Currently, platelets are stored in donor plasma alone or in plasma supplemented with platelet additive solution (PAS), which replaces 60% to 70% of the plasma with a crystalloid nutrient solution.<sup>16</sup> Storing platelets in plasma supplemented with PAS improves platelet quality during storage and reduces the risk of transfusion-associated allergic responses.<sup>17,18</sup> Platelet units are typically stored at room temperature,<sup>19</sup> but they can alternatively be refrigerated if destined for transfusion into patients with acute hemorrhage, as cold-stored platelets have improved hemostatic properties.<sup>20,21</sup> To improve clinical compatibility of the mRNA-LNP platform, achieving transfection directly in platelet storage solutions is needed. However, there has been limited investigation into directly transfecting cells ex vivo suspended in plasma using LNPs, which would be required.

To enhance the clinical compatibility of mRNA-LNP platelet transfection, we systematically optimized an LNP formulation to enable transfection in plasma alone and plasma supplemented with PAS. To determine if this second-generation mRNA-LNP would be scalable and easily integrated into current blood center practices, transfection at supraphysiological concentrations, platelet function, and storage stability were also investigated.

## Materials and methods

### Platelet collection and storage

Platelets used in this study were produced and sampled as previously described.<sup>6,22</sup> Two types of pooled platelet concentrates were used in this study: (1) platelet concentrates composed of platelets pooled from 4 ABO-matched donors resuspended in 100% plasma (plasma), which is a standard product provided by the Canadian Blood Services<sup>23</sup>; and (2) platelet concentrates composed of platelets pooled from 7 ABO-matched donors and resuspended in Macopharma platelet additive solution (SSP+; PAS-E) at a ratio of approximately 60:40 or 70:30 PAS:plasma by volume (PAS<sub>70:30</sub>), produced according to an adapted protocol for producing pooled platelet psoralen-treated products for transfusion that are not pathogen inactivated.<sup>24</sup> Platelets were transfected immediately after unit production, which was 1 day after whole blood collection. Protocols used in this study were approved by the University of British Columbia Ethics Committees (H21-01516 and H16-00773) and the Canadian Blood Services Research

Ethics Board (2021-007). Further details on platelet preparation and transfection can be found in the supplemental Data (available on the *Blood* website).

### Preparing and characterizing mRNA-LNP

LNPs were formulated and mRNA was synthesized as previously described using unmodified nucleotides.<sup>6,25</sup> All mRNA sequences were encoded for the reporter protein NanoLuc Luciferase (NanoLuc) or firefly luciferase (fLuc). For some studies, 0.5 mol% of the lipophilic tracer dye, DiD oil; DiIC18(5) oil (1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate) (Invitrogen), was included and the cholesterol mol % was adjusted accordingly. Polydispersity index, zeta potential, and particle size were measured using the Malvern Zetasizer Nano (Malvern Panalytical, Malvern, England). Representative size distributions for mRNA-LNP composed of NTX-001-DSPC-1.5% DMG-PEG<sub>2000</sub>, NTX-001-POPC-1.5% DMG-PEG<sub>2000</sub>, and NTX-001-POPC-0.5% DMG-PEG<sub>2000</sub> (PO-mRNA-LNP) are included in supplemental Figure 1. Further details can be found in the supplemental Data.

### Flow cytometry

Platelets were stained to evaluate levels of CD62P, CD42b, mRNA-LNP uptake, anionic phospholipid exposure, mitochondrial content, activated glycoprotein (GP) IIb/IIIa, and micro-particle enumeration. Further details about the staining procedures, antibodies, sources, dilutions, buffer compositions, and gating strategy can be found in the supplemental Data.

### Platelet characterization

Platelets were functionally characterized using rotational thromboelastometry (ROTEM) and light transmission aggregometry, whereas the morphology was assessed using transmission electron microscopy. Further details can be found in the supplemental Data.

### Statistical analysis and software

All experiments included a minimum of 3 biological replicates (3 platelet bags each containing platelets from several donors), with data presented as mean  $\pm$  standard error of the mean, and assumptions of normality and equal variance made for these analyses. Statistical comparisons were conducted and visualized as previously described.<sup>6</sup> Flow cytometry data were analyzed using FlowJo version 10.9.0, and artwork and schematics were created using Adobe Illustrator version 28.

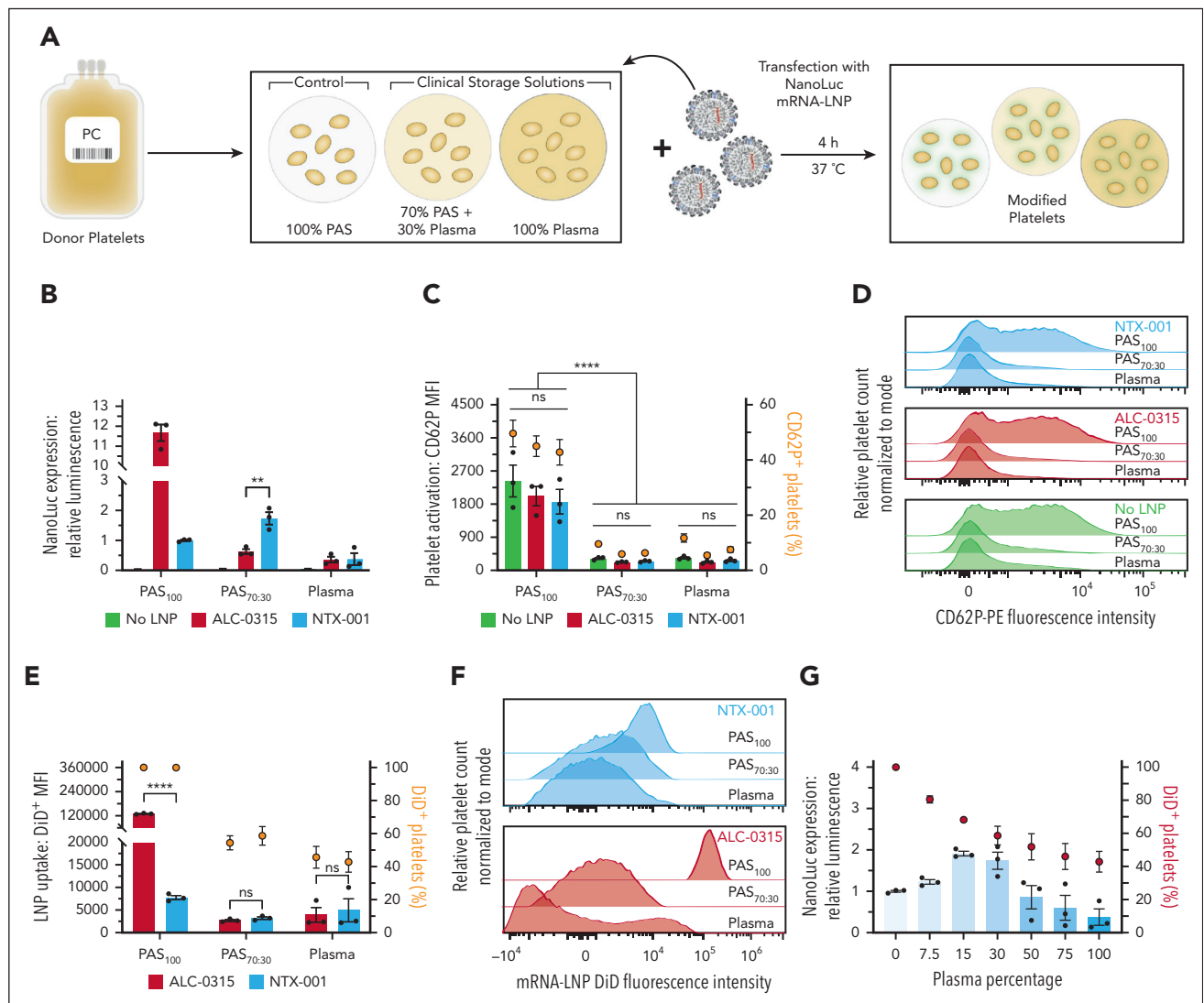
## Results

### Platelets can be transfected with mRNA-LNP in clinical storage solutions

LNPs generally consist of 4 major lipid components, including an ionizable lipid, structural phospholipid, PEGylated lipid, and cholesterol, all of which can be optimized to modulate cellular tropism and transfection potency.<sup>26</sup> To identify an mRNA-LNP that enables exogenous protein expression in platelets stored in clinical solutions, we first optimized the ionizable lipid by evaluating a small library of ionizable cationic lipids with predicted tropism for extrahepatic tissue in vivo.<sup>27</sup> Each ionizable lipid was screened in an LNP formulation incorporating 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol, and 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-

2000 (DMG-PEG<sub>2000</sub>) at the molar ratios used in Onpattro.<sup>28,29</sup> Ionizable lipid NTX-001 was deemed most suitable for transfecting platelets in plasma (supplemental Figure 3). We next determined if LNPs formulated with NTX-001 are superior for transfecting platelets in clinical storage solutions of 100% plasma (plasma) or 70% PAS supplemented with 30% plasma (PAS<sub>70:30</sub>) compared with the previously identified platelet-optimized ionizable lipid ALC-0315.<sup>6</sup> Platelets were also transfected in 100% PAS (PAS<sub>100</sub>) as a nonclinical crystalloid control (Figure 1A). Platelets transfected with mRNA-LNP formulated with NTX-001 had 2.5-fold higher NanoLuc expression in PAS<sub>70:30</sub> compared with ALC-0315, which had limited potency in plasma solutions but was highly potent in PAS<sub>100</sub> (Figure 1B).

The ionizable lipid used in the mRNA-LNP can dramatically affect platelet activation, making some formulations undesirable.<sup>6</sup> Platelets suspended in PAS<sub>100</sub> were significantly more activated than those suspended in PAS<sub>70:30</sub> or plasma (Figure 1C-D; supplemental Table 1). There was no difference in activation between mRNA-LNP containing ALC-0315 or NTX-001, and no differences compared with platelets without LNP in any of the 3 storage solutions. Furthermore, levels of platelet GPIb (CD42b), a surface receptor that binds to exposed sub-endothelial matrix, were similar between mRNA-LNP and no LNP platelets in all solutions (supplemental Figure 4). To determine if differences in NanoLuc expression were related to differences in mRNA-LNP uptake, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine (DiD), a fluorescent lipophilic



**Figure 1. Donor platelets can be transfected with mRNA-LNP while in plasma and PAS.** (A) Schematic describing platelet transfection in PAS<sub>100</sub>, or in clinical storage solutions of PAS<sub>70:30</sub> or plasma. (B) Relative NanoLuc expression, measured as the normalized luminescence per platelet donor unit, using mRNA-LNP containing the ionizable lipid ALC-0315 or NTX-001 in PAS<sub>100</sub>, PAS<sub>70:30</sub>, and in plasma. (C and D) Quantification of median fluorescence intensity (MFI) (bars, left y-axis) and percentage of platelets (yellow circles, right y-axis) positive for the platelet activation marker CD62P (C) and representative flow cytometry plots (D). (E and F) Quantification of DiD MFI of platelets positive for DiD-labeled mRNA-LNP with statistical significance (bars, left y-axis) and percentage of platelets (red circles, right y-axis) positive for DiD (E) and representative flow cytometry plots (F). (G) NanoLuc expression in platelets transfected in PAS with increasing percentages of plasma present (v/v) (bars, left y-axis) and percentage of platelets (red circles, right y-axis) positive for DiD-labeled mRNA-LNP. Platelets were transfected at a concentration of  $50 \times 10^6 \text{ mL}^{-1}$ . P values were determined by 2-way analysis of variance. Values reported as mean  $\pm$  standard error of the mean. **\*\*** $P < .01$ ; **\*\*\*\*** $P < .0001$ .  $n = 3$ . Ns, not significant.

dye that incorporates into the mRNA-LNP during formulation, was used.<sup>30</sup> The percentage of platelets positive for mRNA-LNP was similar, and the median fluorescence intensity (MFI) of DiD-positive platelets was not significantly different in platelets transfected with ALC-0315 mRNA-LNP compared with NTX-001 in PAS<sub>70:30</sub> and plasma (Figure 1E–F). However, transfection with ALC-0135 mRNA-LNP resulted in significantly higher uptake in PAS<sub>100</sub>. We then wanted to determine if the ratio of plasma/PAS affects exogenous protein expression. NanoLuc expression peaked when platelets were transfected with NTX-001 mRNA-LNP at 15% to 30% plasma, whereas both uptake and platelet activation decreased as the amount of plasma increased, with CD42b remaining stable (Figure 1G; supplemental Figure 5).

### The composition of the mRNA-LNP affects platelet transfection potency in PAS<sub>70:30</sub> and plasma

To further improve the mRNA-LNP formulation for transfecting platelets in PAS<sub>70:30</sub> or plasma, we systematically optimized other lipid components, including the structural phospholipid and PEGylated lipid, which also influences particle potency (Figure 2A).<sup>31</sup> A variety of structural phospholipids with different head and tail groups can be used to generate LNPs. Twelve different structural phospholipids were screened, combining lipid head groups phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), or phosphatidylserine (PS), with distearoyl (DS), 1,2-dioleoyl (DO), or 1-palmitoyl-2-oleoyl (PO) tails. Each structural phospholipid was formulated with ionizable lipid NTX-001, DMG-PEG<sub>2000</sub>, cholesterol, and DiD.

In both clinical storage solutions, mRNA-LNP containing PG or PS lipids yielded low levels of NanoLuc expression regardless of the lipid tail. In contrast, mRNA-LNP formulated with PC- or PE-containing structural phospholipids achieved the highest NanoLuc expression in platelets (Figure 2B–C). PC and PE phospholipids with PO tail groups (1 degree of unsaturation) achieved the highest levels of NanoLuc expression, followed by DO (2 degrees of unsaturation) and DS (fully saturated). Overall, PO combined with PC (POPC), resulted in a 3.3- and 4.1-fold increase in NanoLuc expression compared with DSPC in platelets suspended in PAS<sub>70:30</sub> or plasma at 4 hours, respectively (Figure 2D–E). Likewise to NanoLuc expression, mRNA-LNP uptake also varied with the structural phospholipid used. There was slightly higher mRNA-LNP uptake into platelets when particles were formulated with PC and PE structural phospholipids compared with PG and PS in PAS<sub>70:30</sub> (supplemental Figure 6A–B).

To optimize the PEGylated lipid for platelet transfection in PAS<sub>70:30</sub> and plasma, we tested DMG-PEG<sub>2000</sub>, 2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide (ALC-0159; used in Comirnaty<sup>32</sup>) and 1-(monomethoxy polyethylene glycol 2000)-2,3-distearylglycerol (DSG-PEG<sub>2000</sub>) at different molar ratios with NTX-001 and POPC. Across PEGylated lipid species tested, NanoLuc expression increased in PAS<sub>70:30</sub> and plasma as the molar percentage decreased to 0.5% in PAS<sub>70:30</sub> and plasma, with less PEGylated lipid resulting in increased mRNA-LNP particle size (Figure 2F–H). Despite this trend, PEGylated lipid is a necessary component of the mRNA-LNP, as LNP formulated without PEGylated lipid failed to encapsulate mRNA (supplemental Table 2). Again similar to NanoLuc expression,

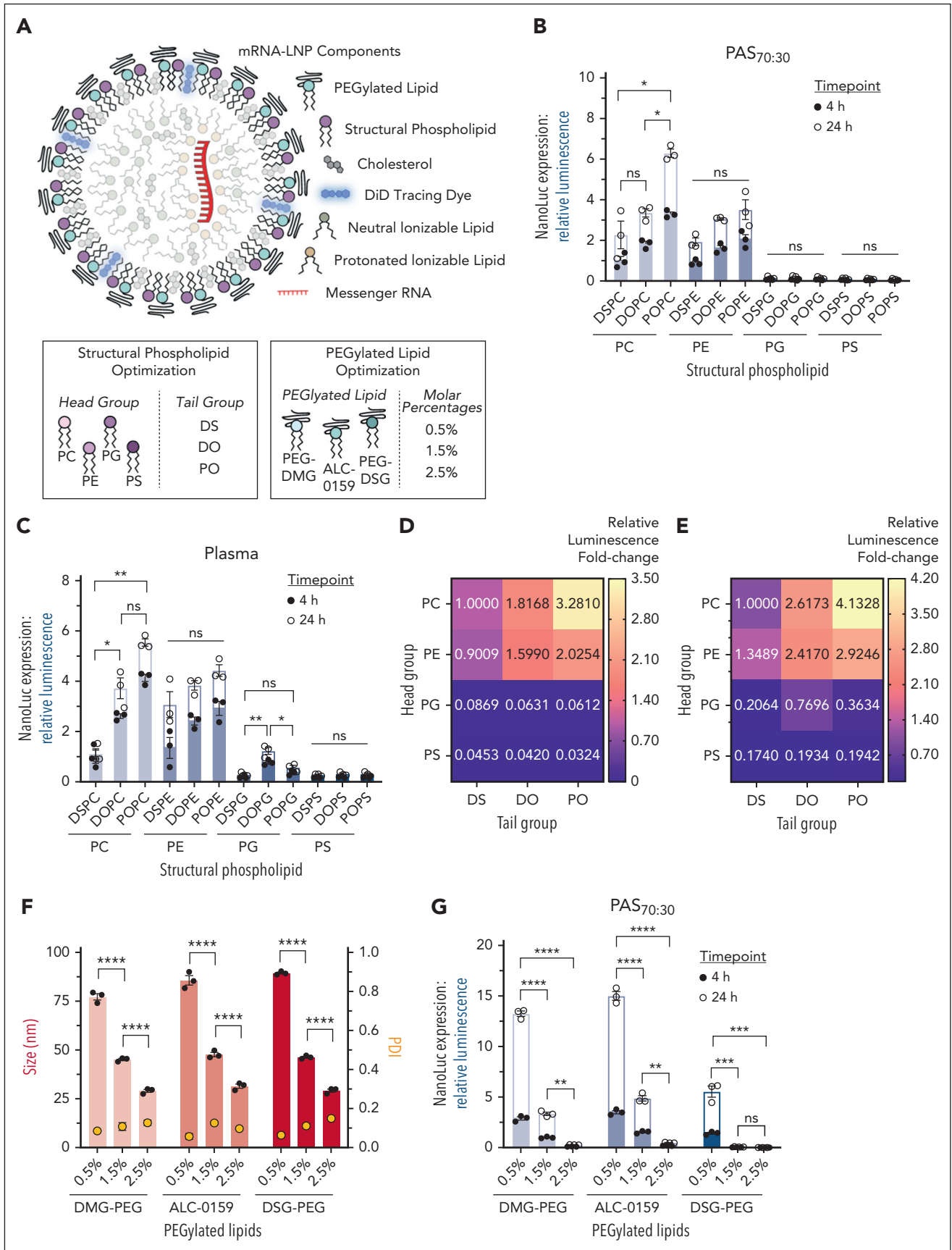
the MFI of DiD-positive platelets decreased in most transfections as molar percentage of PEGylated lipid increased in PAS<sub>70:30</sub> (supplemental Figure 6C–D). NanoLuc expression in PAS<sub>70:30</sub> and plasma and particle size correlated to mRNA-LNP uptake (supplemental Figure 7). Progressive optimization resulted in a plasma-optimized mRNA-LNP (PO-mRNA-LNP) composed of NTX-001, POPC, cholesterol, and 0.5% DMG-PEG<sub>2000</sub>, which yielded a five- and fourfold increase in NanoLuc expression compared with original mRNA-LNP (NTX-001, DSPC, cholesterol, and 1.5% DMG-PEG<sub>2000</sub>) in PAS<sub>70:30</sub> and plasma after 4 hours, respectively (Figure 2I). The PO-mRNA-LNP also enabled the expression of firefly luciferase in transfected platelets, which did not affect activation (supplemental Figure 8).

### mRNA-LNPs with various structural and PEGylated lipids do not affect platelet activation in PAS<sub>70:30</sub> and plasma

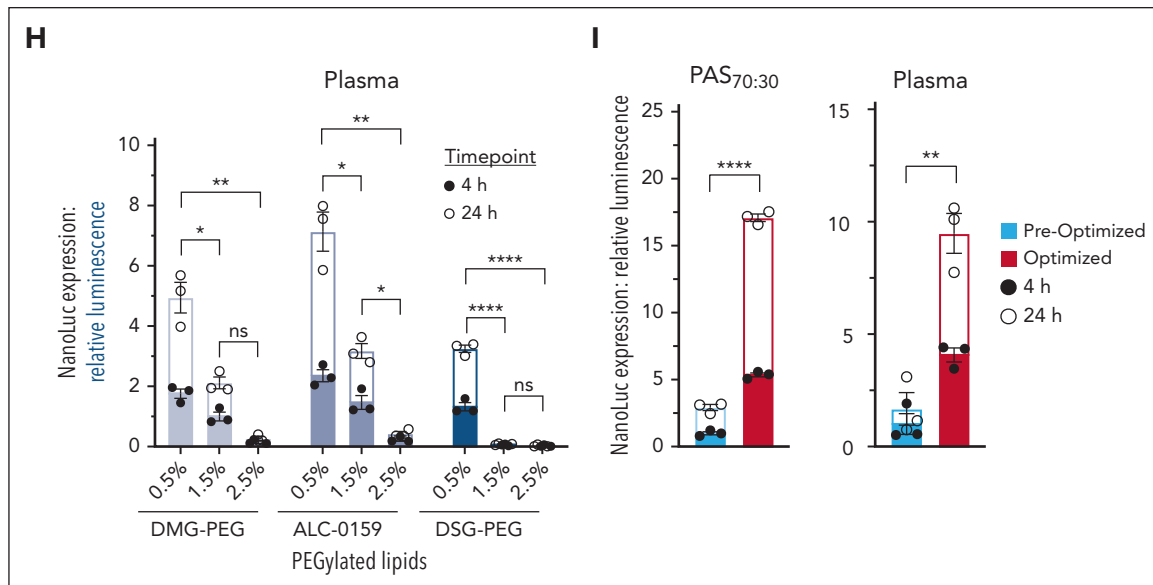
We next evaluated whether the different structural phospholipids and PEGylated lipids used in the mRNA-LNP impact platelet activation in PAS<sub>70:30</sub> and plasma for each unique formulation. In both PAS<sub>70:30</sub> and plasma, none of the structural phospholipids caused significant platelet activation compared with no LNP platelets, and platelets stimulated with synthetic cross-linked collagen-related peptide (CRP-XL)<sup>33,34</sup> activated as expected (Figure 3A–B; supplemental Table 3). Platelet CD42b surface levels were also comparable between mRNA-LNP and no LNP platelets in PAS<sub>70:30</sub> and plasma regardless of the structural phospholipid (supplemental Figure 9A–B). To determine if the structural phospholipids used in the mRNA-LNP affect platelet PS exposure, another hallmark of platelet activation, transfected and no LNP platelets were stained with annexin V, which binds to surface anionic phospholipids and is detectable by flow cytometry.<sup>35,36</sup> Annexin V MFI was not significantly different irrespective of the structural lipid tested in PAS<sub>70:30</sub> and plasma and was comparable to no LNP platelets, whereas platelets activated with calcium ionophore (CaI) as a positive control displayed high levels of PS (Figure 3C–D). Similar to the structural phospholipid screen, platelet activation and CD42b were not affected by any of the PEGylated lipids tested or their molar ratio in both PAS<sub>70:30</sub> and plasma (Figure 3E–F; supplemental Figure 9C–D; supplemental Table 4). Finally, the forward and side scatter distribution of the platelet population by flow cytometry was comparable between no LNP and PO-mRNA-LNP-transfected platelets in PAS<sub>70:30</sub> and plasma (supplemental Figure 10).

### Transfection with PO-mRNA-LNP is scalable to physiological and supraphysiological platelet concentrations

The average physiological platelet concentration ranges from  $150 \times 10^6$  to  $450 \times 10^6$  mL<sup>-1</sup>.<sup>37</sup> In units collected for transfusion, platelets are stored at supraphysiological concentrations of  $\approx 900 \times 10^6$  to  $1400 \times 10^6$  mL<sup>-1</sup>.<sup>23</sup> To determine if mRNA-LNP transfection is scalable, we evaluated NanoLuc expression in platelets at concentrations of  $250 \times 10^6$  mL<sup>-1</sup> (physiological) and  $800 \times 10^6$  mL<sup>-1</sup> (supraphysiological) after transfection with PO-mRNA-LNP. NanoLuc expression increased with mRNA-LNP dosage in both PAS<sub>70:30</sub> and plasma and at both platelet concentrations (Figure 4A–B). At physiological and supraphysiological concentrations, the optimal dose was 24 and



**Figure 2. Transfection in PAS<sub>70:30</sub> and plasma is best with mRNA-LNP formulated with PC helper lipids and low molar percentage of PEGylated lipid.** (A) Schematic of an mRNA-LNP and the various optimized components. (B-C) Relative NanoLuc expression in platelets transfected with mRNA-LNP formulated with a library of structural



**Figure 2 (continued)** phospholipids at 4 hours (shaded bars) and 24 hours (open bars) in PAS<sub>70:30</sub> (B) or plasma (C). (D and E) Heat map showing the relative NanoLuc expression fold change between structural phospholipids used in the mRNA-LNP for platelets transfected in PAS<sub>70:30</sub> (D) and plasma (E). (F) mRNA-LNP size (nm) (bars, left y-axis) and polydispersity index (PDI) (yellow circles, right y-axis) after formulation with various PEGylated lipids and at various molar percentages. (G and H) Relative NanoLuc expression in platelets transfected with NTX-001-POPC mRNA-LNP formulated with various molar percentages of PEGylated lipids after 4 hours (shaded bars) and 24 hours (open bars) in PAS<sub>70:30</sub> (G) or plasma (H). (I) NanoLuc expression in platelets transfected with preoptimized and optimized mRNA-LNP. Platelets were transfected at a concentration of  $250 \times 10^6 \text{ mL}^{-1}$ . P values were determined by the 2-way analysis of variance and applied to 24-hour data. Values are reported as mean  $\pm$  standard error of the mean. \* $P < .05$ ; \*\* $P < .01$ ; \*\*\* $P < .001$ ; \*\*\*\* $P < .0001$ .  $n = 3$ . Ns, not significant.

$48 \mu\text{g mL}^{-1}$  for platelets transfected in PAS<sub>70:30</sub> and plasma, respectively.

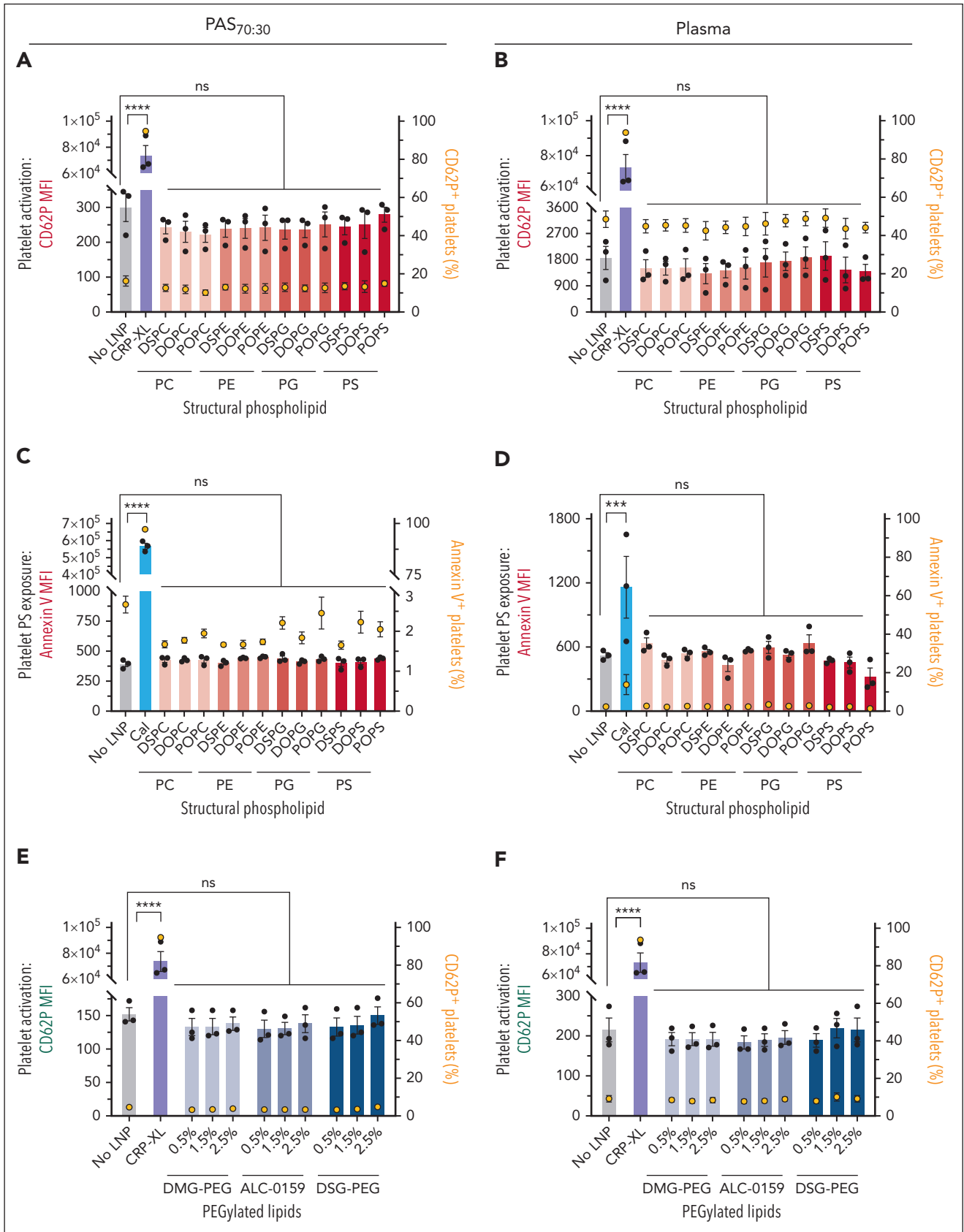
Platelet activation at the different concentrations and at the various PO-mRNA-LNP doses did not increase compared with no LNP platelets in PAS<sub>70:30</sub> or plasma (Figure 4C–D; supplemental Table 5). There was an increase in platelet activation at supraphysiological concentrations compared with physiological concentrations, which was not influenced by mRNA-LNP exposure. PO-mRNA-LNP uptake increased as the dosage increased in both solutions regardless of the platelet concentration (Figure 4E–F). To determine if PO-mRNA-LNP exposure at different doses affects platelet reactivity, platelets suspended in PAS<sub>70:30</sub> were stimulated with adenosine diphosphate (ADP).<sup>37</sup> Regardless of the PO-mRNA-LNP dose, transfected platelets retained equivalent responsiveness to ADP as no LNP platelets, although platelets at supraphysiological concentration were less responsive than those at physiological concentration (Figure 4G). CD42b levels were similar across PO-mRNA-LNP doses in PAS<sub>70:30</sub> and plasma (supplemental Figure 11).

### Platelets transfected with PO-mRNA-LNP maintain their responsiveness to agonists, are coagulable in vitro, and do not display major morphologic changes

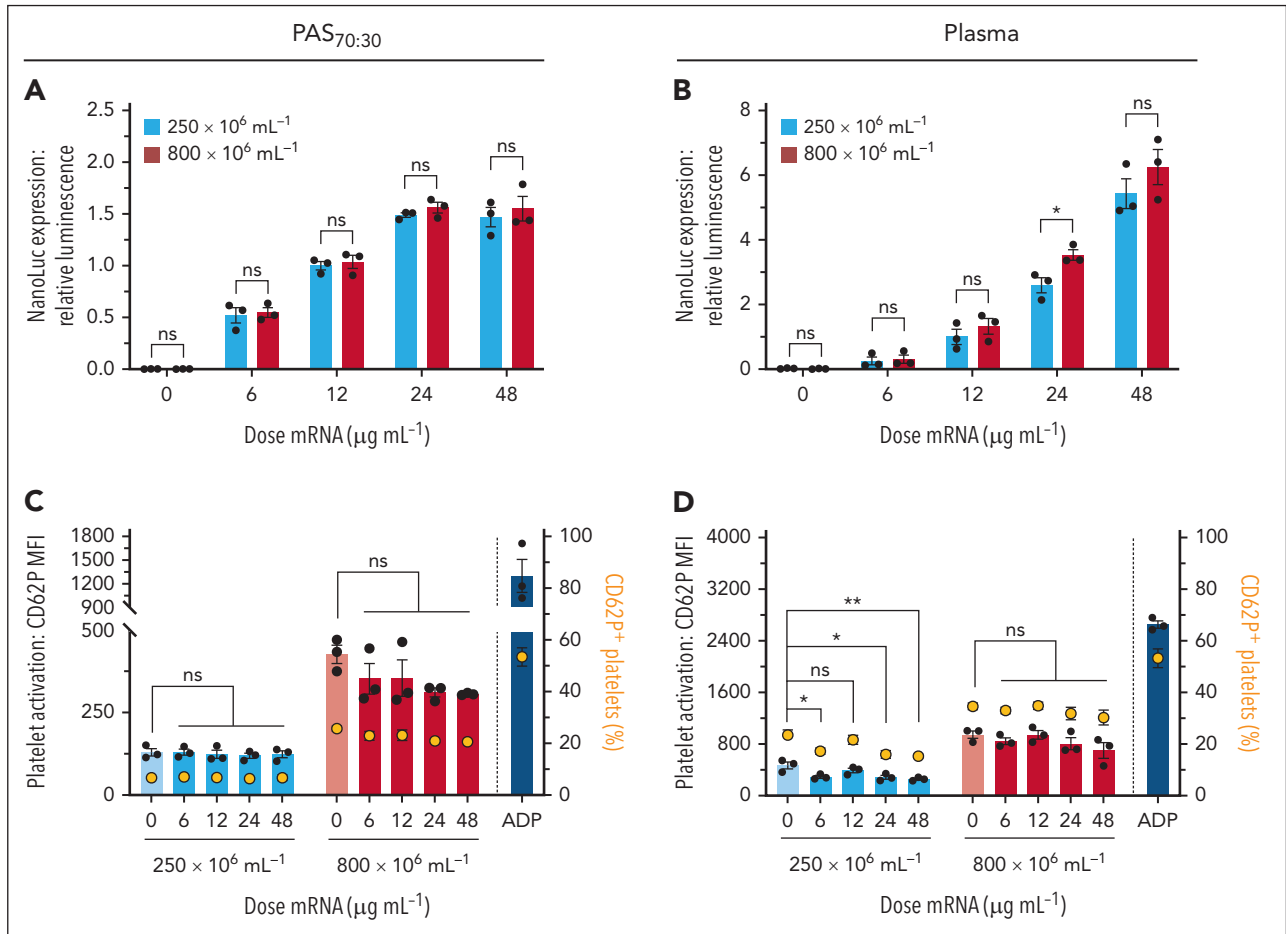
To determine if PO-mRNA-LNP transfection impacts platelet reactivity to agonists, no LNP and transfected platelets were activated with ADP, thrombin, Cal, or CRP-XL. Platelets transfected at supraphysiological concentration with PO-mRNA-LNP at  $24 \mu\text{g mL}^{-1}$  in PAS<sub>70:30</sub> did not have significantly impaired

reactivity to all agonists tested (Figure 5A; supplemental Table 6). Depending on the agonist, CD42b binding did decrease, consistent with previous reports,<sup>38</sup> but the MFI was similar for each agonist between transfected and no LNP platelets (supplemental Figure 12). No significant difference in annexin V binding was observed between no LNP platelets and PO-mRNA-LNP-transfected platelets, whereas a clear increase in annexin V binding was observed when platelets were stimulated with Cal (Figure 5B). Because platelet subpopulations form after activation,<sup>38,39</sup> we next evaluated activated GPIIb/IIIa expression by PAC-1 binding. Activated GPIIb/IIIa was not significantly different between no LNP platelets compared with PO-mRNA-LNP-transfected platelets (Figure 5C). Furthermore, PO-mRNA-LNP did not impact CD62P/PAC-1 platelet subpopulations before or after activation with CRP-XL (supplemental Figure 13).

To determine if platelets transfected with PO-mRNA-LNP aggregate, are coagulable, and contribute to the strength of forming clots, light transmission aggregometry and ROTEM were used. PO-mRNA-LNP-transfected platelets aggregated similarly to no LNP platelets (supplemental Figure 14). The coagulability of PO-mRNA-LNP and no LNP platelets suspended in PAS<sub>70:30</sub> was tested by ROTEM at a concentration of  $800 \times 10^6 \text{ mL}^{-1}$  after clotting was initiated via the extrinsic pathway using thromboplastin. Platelets transfected with PO-mRNA-LNP clotted similarly to no LNP platelets as there were no significant differences in the clot time and maximum clot firmness (Figure 5D–F; supplemental Figure 15). Transfection with PO-mRNA-LNP did not induce notable morphologic changes, and stimulation with CRP-XL resulted in increased pseudopod formation and degranulation (Figure 5G; supplemental Figure 16).<sup>34</sup>



**Figure 3. The structural phospholipid and PEGylated lipid used in the mRNA-LNP does not affect platelet activation.** (A-B) Quantification of MFI (bars, left y-axis) and percentage of platelets (yellow circles, right y-axis) positive for the platelet activation marker CD62P for platelets treated with mRNA-LNP consisting of different structural phospholipids or activated with CRP-XL at 4 hours in PAS<sub>70:30</sub> (A) and plasma (B). (C-D) Quantification of MFI (bars, left y-axis) and percentage (circles, right y-axis) of PS exposure measured using annexin V staining in platelets treated with mRNA-LNP consisting of different structural phospholipids or activated with calcium ionophore (Cal) in



**Figure 4. Transfection of platelets with PO-mRNA-LNP is scalable to physiological and supraphysiological concentrations in PAS<sub>70:30</sub> and plasma.** (A-B) Relative NanoLuc expression per unit platelet transfected with mRNA-LNP at various doses suspended at physiological ( $250 \times 10^6 \text{ mL}^{-1}$ ) and supraphysiological concentration ( $800 \times 10^6 \text{ mL}^{-1}$ ) in PAS<sub>70:30</sub> (A) and plasma (B) at 4 hours. (C-D) Quantification of MFI (bars, left y-axis) and percentage of platelets (yellow circles, right y-axis) positive for the platelet activation marker CD62P in PAS<sub>70:30</sub> (C) and plasma (D). (E-F) Quantification of MFI of platelets positive for DiD-labeled mRNA-LNP (bars, left y-axis) and percentage of platelets (yellow circles, right y-axis) positive for DiD in PAS<sub>70:30</sub> (E) and plasma (F). (G) Percentage of platelets positive for the platelet activation marker CD62P when activated with ADP ( $10 \mu\text{M}$ ) or unactivated. *P* values were determined by the unpaired Student *t*-test (2-tailed) or the 1-way analysis of variance. Values are reported as mean  $\pm$  standard error of the mean. \**P* < .05; \*\**P* < .01; \*\*\**P* < .001; \*\*\*\**P* < .0001. *n* = 3. Ns, not significant.

### Platelets transfected in PAS<sub>70:30</sub> with PO-mRNA-LNP can be stored

Platelet units are stored at room temperature or refrigerated depending on their intended use.<sup>41</sup> We next determined if mRNA-LNP-transfected platelets could be stored by first scaling transfection with preoptimized mRNA-LNP to physiological platelet concentration ( $250 \times 10^6 \text{ mL}^{-1}$ ) in a small-volume, pilot study and found transfected platelets stored similarly to no LNP platelets (supplemental Figure 17-18). To better model clinical practices, no LNP and PO-mRNA-LNP-transfected platelets were stored at pediatric platelet unit volumes<sup>42</sup> in single-donor clinical-grade platelet bags at supraphysiological platelet concentration ( $800 \times 10^6 \text{ mL}^{-1}$ ) at either room temperature (RTP) with constant agitation or cold-stored (CSP) at  $4^\circ\text{C}$  with no agitation. Stored platelet count,

blood gas, baseline activation, responsiveness to ADP, PS exposure, and coagulability were measured over storage. Because NanoLuc expression is temperature dependent (supplemental Figure 19),  $50 \times 10^6$  platelets were removed from the storage bag before each sampling time and incubated at  $37^\circ\text{C}$  for 4 hours to determine if extended storage affects inducible NanoLuc expression (Figure 6A).

Both baseline and induced NanoLuc expression profiles were different between transfected RTP and CSP. In transfected RTP, baseline NanoLuc expression increased over the first week of storage up to 1.8-fold higher than the inducible expression observed after 4 hours on day 1 (Figure 6B). Inducible NanoLuc expression peaked in CSP on day 1 and rapidly decreased over the storage duration (Figure 6C). Interestingly, inducible

**Figure 3 (continued)** PAS<sub>70:30</sub> (C) and plasma (D). (E-F) Quantification of MFI (bars, left y-axis) and percentage of platelets (yellow circles, right y-axis) positive for the platelet activation marker CD62P for platelets treated with mRNA-LNP consisting of different PEGylated lipids or activated with CRP-XL at 4 hours in PAS<sub>70:30</sub> (E) and plasma (F). In some cases, activation by CRP-XL or Cal was measured in separate bags of platelets. Platelets were transfected at a concentration of  $250 \times 10^6 \text{ mL}^{-1}$ . *P* values were determined by the 1-way analysis of variance. Values are reported as mean  $\pm$  standard error of the mean. \*\*\**P* < .001; \*\*\*\**P* < .0001. *n* = 3. Ns, not significant.

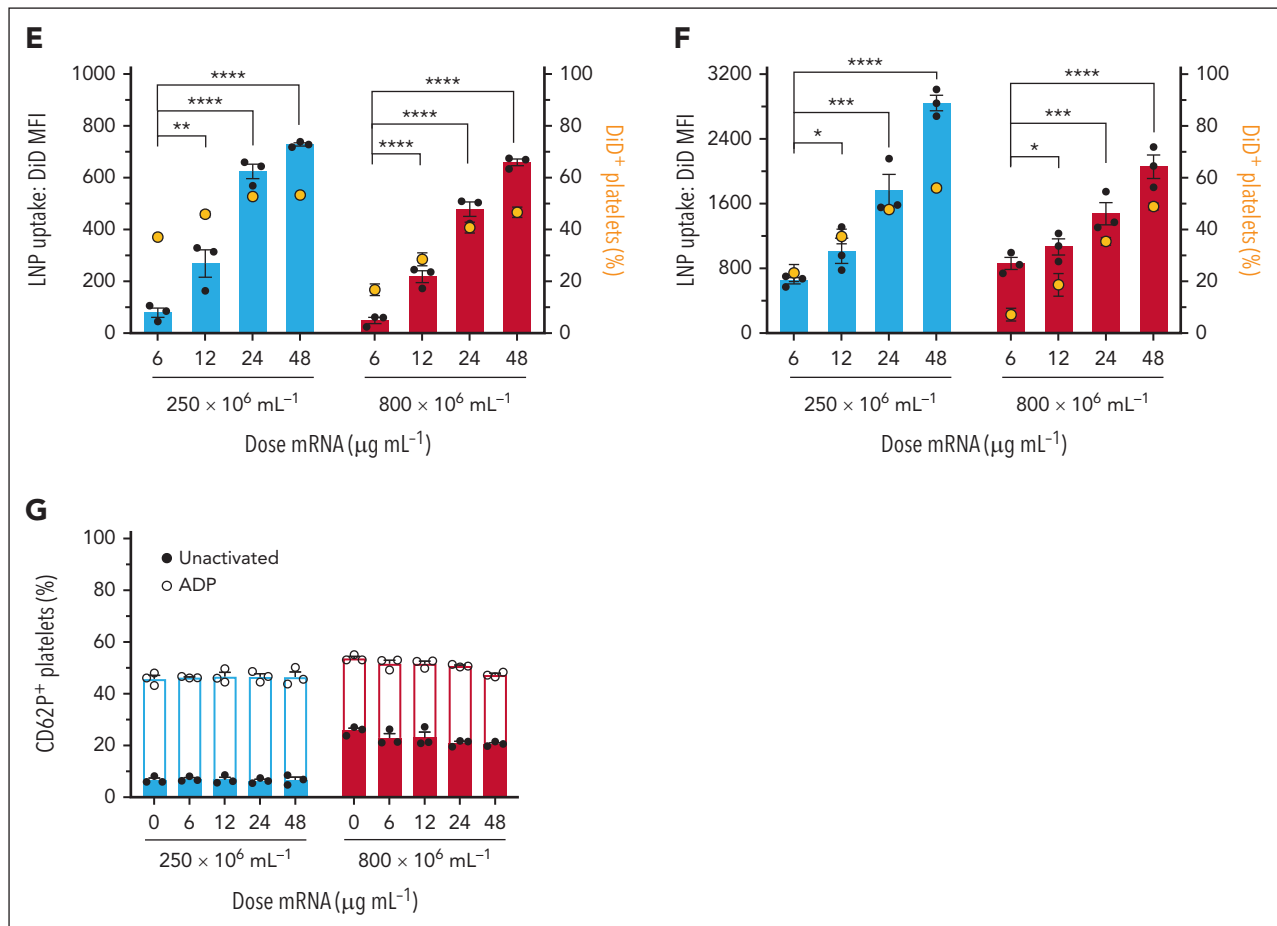


Figure 4 (continued)

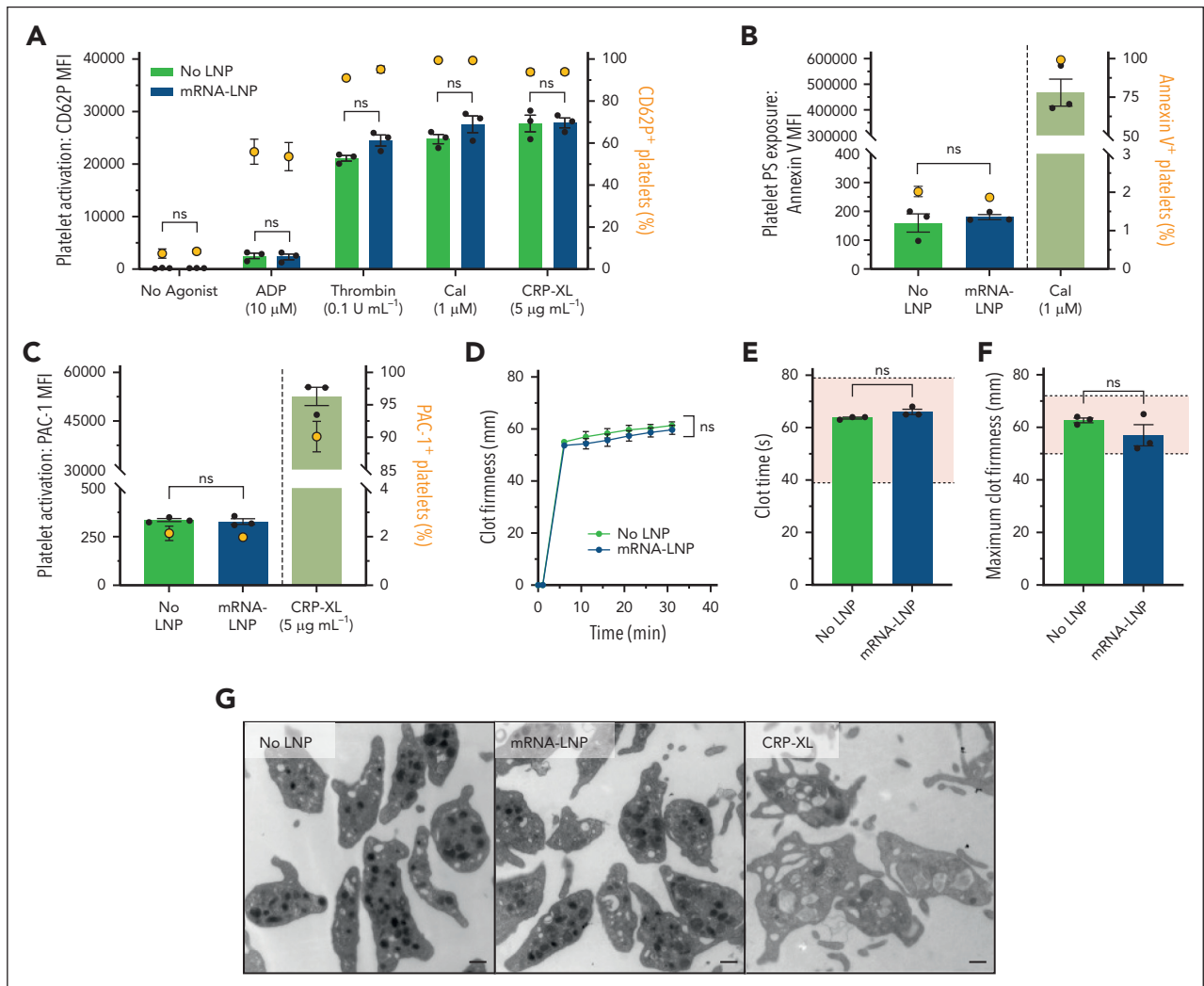
NanoLuc expression was only observed up to day 2 for RTP but was preserved in CSP for up to 14 days, albeit at substantially lower levels than the first day. Baseline CD62P activation in RTP increased over the 14 days of storage for both RTP and CSP; however, activation of RTP treated with PO-mRNA-LNP was significantly lower than no LNP platelets from day 4 onwards (Figure 6D-E; supplemental Table 7). ADP responsiveness was equivalent between no LNP and PO-mRNA-LNP-transfected platelets across all time points for CSP and up until day 4 for RTP (supplemental Figure 20; supplemental Table 8). Although PO-mRNA-LNP RTP were less responsive than no LNP RTP on days 4, 7, and 14, because their baseline activation was lower than no LNP RTP, their overall magnitude of ADP responsiveness was not significantly different. PS exposure increased over storage but was not influenced by mRNA-LNP transfection (Figure 6F-G). The maximum clot firmness of transfected RTP and CSP was unaffected compared to no LNP platelets over the entire storage duration (Figure 6H-I). CSP count was unaffected by mRNA-LNP transfection over 2 weeks of storage, and PO-mRNA-LNP transfection did not affect RTP counts until days 7 and 14, at which point it was significantly higher (supplemental Figure 21). PO-mRNA-LNP transfection did not influence blood chemistry, including pH, glucose, and lactate, nor CD42b expression over 14 days of storage for both RTP and CSP (supplemental Figures 22-23). The morphology of stored platelets changed between days 2 and 7, hallmarked by increased degranulation, but importantly, morphology was

comparable between no LNP and PO-mRNA-LNP-transfected platelets on each day (supplemental Figure 24). Finally, we examined the effects PO-mRNA-LNP treatment on the age-related parameters of mitochondrial and microparticle content. Mitochondrial content was not significantly different between PO-mRNA-LNP and no LNP platelets over 7 days of storage and was also not different between platelets positive or negative for mRNA-LNP uptake in most cases (supplemental Figure 25). Total CD41a-positive microparticle content as well as the annexin V-positive subfraction were similar between no LNP and PO-mRNA-LNP-transfected RTP and CSP on days 1 and 7 of storage (supplemental Figure 26).

To determine if platelets were the most amenable to transfection early during storage, RTP and CSP were stored over 7 days in PAS<sub>70:30</sub> and transfected on days 1, 2, 4, and 7 with fresh mRNA-LNP. Inducible NanoLuc expression was highest at day 1 and decreased at each subsequent day of transfection during storage (supplemental Figure 27).

## Discussion

Platelet units require minimal handling before transfusion into patients with thrombocytopenia or active bleeding. Newer techniques to improve platelet transfusions, including pathogen inactivation and refrigeration,<sup>43</sup> only address storage limitations but were developed to be easily integrated into clinical

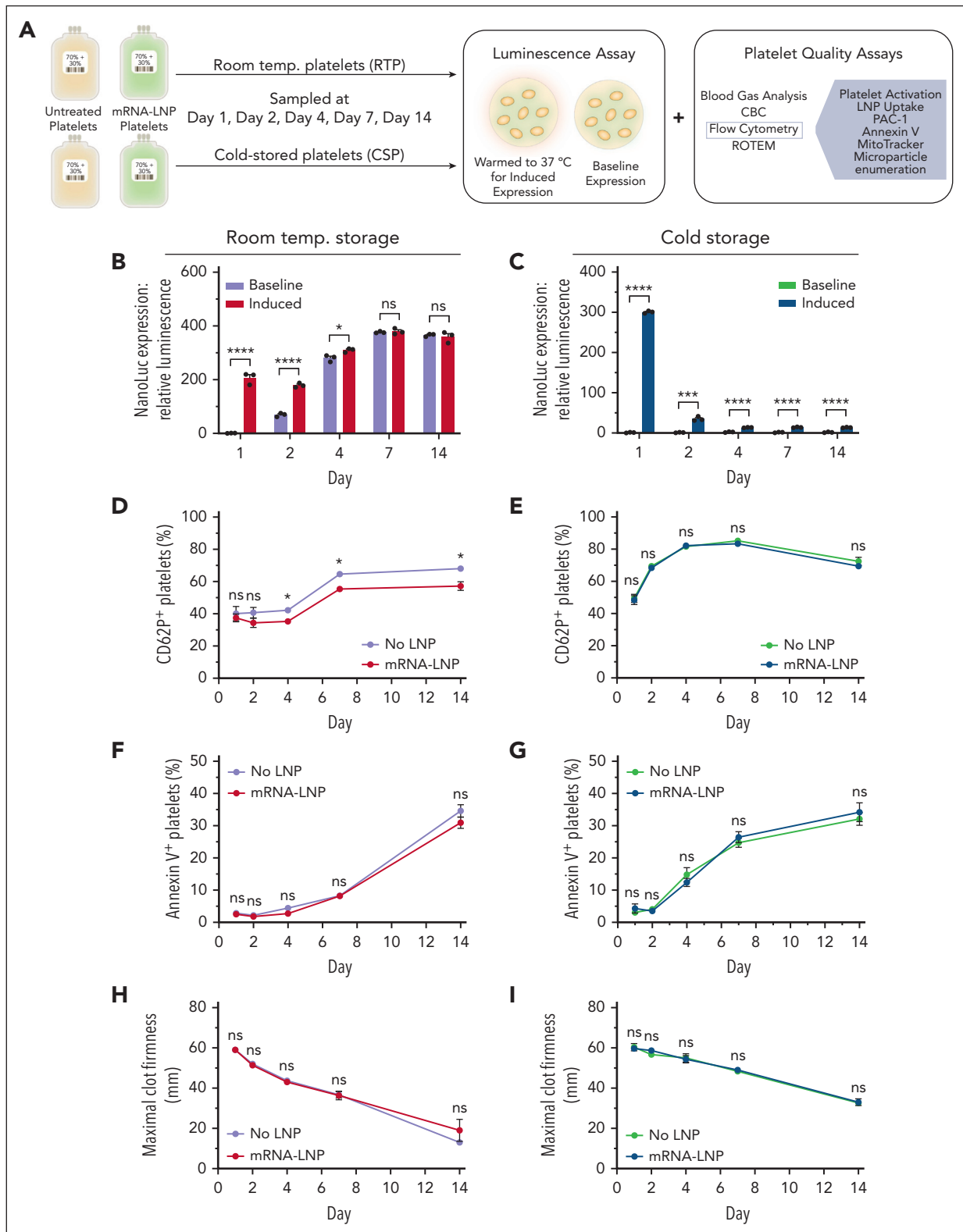


**Figure 5. PO-mRNA-LNP transfected platelets respond appropriately to agonists, are coagulable, and have normal morphology.** (A) Platelet activation without agonist or after stimulation by ADP, thrombin, Cal, or CRP-XL measured by the MFI (bars, left y-axis) and percentage platelet activation (yellow circles, right y-axis) of surface CD62P. (B) Quantification of MFI (bars, left y-axis) and the percentage of platelets (yellow circles, right y-axis) positive for annexin V. (C) Quantification of MFI (bars, left y-axis) and percentage of platelets (yellow circles, right y-axis) positive for PAC-1. (D) Representative ROTEM curves, with clotting initiated by thromboplastin. (E and F) Quantification of ROTEM clot time (E) and maximum clot firmness (F). The red shaded region is the clinically accepted reference ranges for each parameter.<sup>40</sup> Platelets were transfected at a supraphysiological concentration of  $800 \times 10^6 \text{ mL}^{-1}$ . (G) Transmission electron micrographs of platelets without LNP (No LNP), treated with PO-mRNA-LNP, or activated with  $5 \mu\text{g mL}^{-1}$  CRP-XL. The scale bar indicates 500 nm. Platelets were transfected at a concentration of  $250 \times 10^6 \text{ mL}^{-1}$ . *P* values were determined by the 2-way analysis of variance (ANOVA), the 1-way ANOVA, or the unpaired Student *t*-test (2-tailed). Values are reported as mean  $\pm$  standard error of the mean. *n* = 3. Ns, not significant.

practice. We previously found that washed platelets can be genetically modified with mRNA-LNP<sup>6</sup>; however, washed platelet products are used infrequently and are restricted to specific indications.<sup>44</sup> To improve the clinical compatibility of mRNA-LNP for platelet engineering, we developed PO-mRNA-LNP capable of transfecting transfusion-ready platelets directly in clinical storage solutions that is scalable and does not affect aspects of platelet physiology.

Properties of the ionizable lipid, structural phospholipid, and PEGylated lipid contribute to final mRNA-LNP characteristics and potency.<sup>31</sup> In plasma, LNPs form a unique protein corona driven by plasma protein affinities for the LNP surface. The profile of adsorbed proteins largely depends on the innate properties of the lipids, which strongly influence uptake mechanisms and cell tropism.<sup>45,46</sup> We found that mRNA-LNP containing NTX-001 are more potent in plasma compared

with mRNA-LNP containing ALC-0315, likely because of the formation of a favorable corona configuration. Similarly, structural phospholipid headgroups PC and PE, which are present on the LNP surface,<sup>47</sup> likely contribute to a corona that increases uptake into platelets compared with LNP with PG or PS lipids. However, uptake does not always mean increased potency<sup>31</sup> as platelets transfected with PE or PC containing mRNA-LNP had similar uptake but different NanoLuc expression. The saturation of structural phospholipid tails can affect mRNA endosomal escape. In tandem with ionizable lipids,<sup>48</sup> unsaturated lipids increase intracellular delivery of nucleic acids by improving disruption of endosomal membranes,<sup>31,49</sup> which may explain why structural phospholipids with unsaturated tails were more potent in platelets. Finally, formulating at a molar ratio of  $>0.5\%$  PEGylated lipid reduced particle potency and size, which may be due to increased steric hindrance from elevated surface PEGylated lipid and reduced cell



**Figure 6. PO-mRNA-LNP transfection is compatible with platelet storage in that it does not adversely affect platelet quality.** (A) Schematic describing platelet transfection, storage, and platelet quality assays performed. (B-C) Baseline and induced NanoLuc expression in room temperature stored platelets (RTP) (B) and cold-stored platelets (CSP) (C) transfected with PO-mRNA-LNP on day 1 (D1) and stored at  $800 \times 10^6$  platelets  $\text{mL}^{-1}$  in PAS<sub>70:30</sub>. (D-E) Percentage of CD62P-positive RTP (D) and CSP (E). (F-G) Percentage of annexin V-positive RTP (F) and CSP (G). (H-I) Quantification of ROTEM maximum clot firmness with clotting initiated by thromboplastin in RTP (H) and CSP (I). *P* values were determined by the unpaired Student *t*-test (2-tailed). Values are reported as mean  $\pm$  standard error of the mean. \**P* < .05; \*\*\**P* < .001; \*\*\*\**P* < .0001. *n* = 3. *Ns*, not significant.

interactions.<sup>50</sup> Reduced transfection efficacy may also be due to reduced PEGylated lipid desorption that can then impede the protein corona from forming,<sup>26,51</sup> or reduced mRNA delivery as smaller particles contain less mRNA.<sup>30</sup>

mRNA-LNP-transfected platelets were comparably activated to control platelets in PAS<sub>100</sub>, confirming that platelet handling and not mRNA-LNP exposure is what causes unwanted activation. Proteins present in PAS<sub>70:30</sub> and plasma, such as albumin, stabilize platelets and reduce activation.<sup>52</sup> In the presence of plasma, mRNA-LNP-transfected and control platelets were not activated and, importantly, displayed no differences in activation induced by mRNA-LNPs of different compositions. This was surprising given that we previously noted that some mRNA-LNPs induce platelet activation more than others,<sup>6</sup> and some cationic mRNA-LNP formulations even cause platelet aggregation and clotting when administered to the lung.<sup>53</sup> By design, platelets can activate each other,<sup>54</sup> resulting in an activation feedback loop. It is likely that the stabilizing effects of plasma during transfection and predicted neutral charge of the PO-mRNA-LNP are preventing mRNA-LNP-induced activation that would otherwise occur if transfected in crystalloid solution alone. At supraphysiological concentration, PO-mRNA-LNP-transfected platelets had comparable activation, aggregation, morphology, and coagulation profiles to control and standard banked platelets.<sup>19,55</sup> We anticipate that platelets modified with PO-mRNA-LNP will not lead to unwanted platelet aggregation or clearance in vivo based on these in vitro results.

Differences in kinetics of NanoLuc expression in RTP vs CSP during storage may be exploited for specific applications. NanoLuc expression accumulated in RTP over the first week of storage and inducible expression occurred up to day 2. This suggests that platelets have a limited translational capacity but synthesize new proteins during storage at room temperature, consistent with previous reports.<sup>56</sup> The plateau in NanoLuc expression in RTP may be attributed to reduced mitochondrial activity with a shift to the less efficient glycolysis pathway for adenosine triphosphate synthesis, in addition to reduced ribosomal recycling, leading to reduced protein synthesis.<sup>57-59</sup> Although significantly lower NanoLuc expression was detected in CSP over storage, platelets could still be induced to express low levels of NanoLuc even after 2 weeks in the cold. Refrigeration reduces platelet metabolic activity and extends unit storability.<sup>41</sup> Because transfected CSP produced bursts of NanoLuc expression after warming, it is likely that cold storage is preserving some translational capacity for transfected mRNA, albeit at substantially reduced levels. In an analysis of platelet subpopulations<sup>39</sup> and microparticles after PO-mRNA-LNP treatment, microparticle release and mitochondrial content were comparable to no LNP platelets, suggesting that transfection does not negatively impact aging during storage.<sup>60</sup> Overall, stored PO-mRNA-LNP-treated platelets, either at room temperature or 4°C, were not different from controls across quality measures except that interestingly, transfected platelets were less activated later into room temperature storage. Although the quality of all platelet product generally diminishes during long periods of storage,<sup>19,55,57,61,62</sup> there were no major differences detected between control and transfected platelets.

Because platelet transfusions are commonly administered after trauma and during acute bleeding, engineering platelets to

express prohemostatic agents, such as antifibrinolytic factors, is a major goal. Expressing proteins that are used by other emerging platelet-based cell therapies, such as coagulation factor VIII<sup>3</sup> or thrombolytic enzymes,<sup>63</sup> can be explored with mRNA-LNP, although the kinetics of expression would have to be considered. This platform could also be used to express anti-cancer agents in platelets given that platelets are commonly transfused to oncology patients,<sup>64</sup> and naturally exchange cargo to tumor cells.<sup>65</sup>

Further understanding the role of the protein corona, uptake mechanisms, and mRNA escape kinetics provide even more opportunities to rationally design mRNA-LNP with increased potency for platelet engineering. Finally, given that platelet warming is important for inducing high levels of protein expression, and that fresh platelets are preferred for transfusion, integrating this technology into the clinic will require early mRNA-LNP modification by blood collection centers or hospital blood banks. As product use fluctuates, and platelet shortages can occur, determining how many units are modified and for what purposes will need to be optimized. We expect that the clinical compatibility of the optimized mRNA-LNP described here will further advance this technology and enable new platelet cell therapies.

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## Authorship

Contribution: C.S., J.L., and E.K. designed and performed experiments, analyzed and interpreted data, made the figures, wrote the initial draft of the manuscript, and edited the final manuscript; K.E.B., N.P., E.M.R., A.W., B.M., and Z.N. helped perform the experiments; D.A. and M.A.C. synthesized the ionizable lipid library; M.R. provided intellectual input on experimental design and edited the manuscript; D.V.D., E.J., and P.R.C. helped design the experiments and edited the manuscript; C.J.K. designed experiments, interpreted data, and wrote the manuscript; and all authors approved submission of the manuscript.

Conflict-of-interest disclosure: D.A. is an employee of, and M.A.C., E.J., P.R.C., and C.J.K. are cofounders and hold equity in NanoVation Therapeutics Inc. K.E.B., P.R.C., and C.J.K. are cofounders and hold equity in SeraGene Therapeutics Inc. P.R.C. is cofounder and holds equity in Acuitas Therapeutics. Most authors have submitted intellectual property on lipid nanoparticles containing RNA. J.L., C.S.,

K.E.B., M.R., D.V.D., E.J., P.R.C., and C.J.K. have filed 1 patent application (US Patent and Trademark Office number 63/344,247) and 3 provisional patent applications on lipid nanoparticle transfection of platelets. These interests have been fully disclosed to each institution, and plans are in place for managing any potential conflicts arising from licensing these patents. The remaining authors declare no competing financial interests.

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## Footnotes

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All data needed to evaluate the conclusions in the article are present in the article and/or the supplemental Data. There are no major restrictions in the availability of materials, but some lipids may require material transfer agreements. Original data are available on request from the corresponding author, Christian J. Kastrup ([ckastrup@versiti.org](mailto:ckastrup@versiti.org)).

The online version of this article contains a data supplement.

There is a *Blood Commentary* on this article in this issue.

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## REFERENCES

1. Stroncek DF, Rebullia P. Platelet transfusions. *Lancet*. 2007;370(9585):427-438.
2. van der Meijden PEJ, Heemskerk JWM. Platelet biology and functions: new concepts and clinical perspectives. *Nat Rev Cardiol*. 2019;16(3):166-179.
3. Shi Q, Kuether EL, Chen Y, Schroeder JA, Fahs SA, Montgomery RR. Platelet gene therapy corrects the hemophilic phenotype in immunocompromised hemophilia A mice transplanted with genetically manipulated human cord blood stem cells. *Blood*. 2014; 123(3):395-403.
4. Du LM, Nurden P, Nurden AT, et al. Platelet-targeted gene therapy with human factor VIII establishes haemostasis in dogs with haemophilia A. *Nat Commun*. 2013;4:2773.
5. Charlesworth CT, Hsu I, Wilkinson AC, Nakauchi H. Immunological barriers to haematopoietic stem cell gene therapy. *Nat Rev Immunol*. 2022;22(12):719-733.
6. Leung J, Strong C, Badior KE, et al. Genetically engineered transfusable platelets using mRNA lipid nanoparticles. *Sci Adv*. 2023;9(48):ead0508.
7. Leung J, Cau MF, Kastrup CJ. Emerging gene therapies for enhancing the hemostatic potential of platelets. *Transfusion*. 2021; 61(suppl 1):S275-S285.
8. Weyrich AS, Schwertz H, Kraiss LW, Zimmerman GA. Protein synthesis by platelets: historical and new perspectives. *J Thromb Haemost*. 2009;7(2):241-246.
9. Akinc A, Maier MA, Manoharan M, et al. The Onpatro story and the clinical translation of nanomedicines containing nucleic acid-based drugs. *Nat Nanotechnol*. 2019;14(12): 1084-1087.
10. Polack FP, Thomas SJ, Kitchin N, et al. Safety and efficacy of the BNT162b2 mRNA Covid-19 vaccine. *N Engl J Med*. 2020;383(27): 2603-2615.
11. Baden LR, El Sahly HM, Essink B, et al. Efficacy and safety of the mRNA-1273 SARS-CoV-2 vaccine. *N Engl J Med*. 2021; 384(5):403-416.
12. Sato Y, Hashiba K, Sasaki K, Maeki M, Tokeshi M, Harashima H. Understanding structure-activity relationships of pH-sensitive cationic lipids facilitates the rational identification of promising lipid nanoparticles for delivering siRNAs in vivo. *J Control Release*. 2019;295:140-152.
13. Chander N, Basha G, Yan Cheng MH, Witzigmann D, Cullis PR. Lipid nanoparticle mRNA systems containing high levels of sphingomyelin engender higher protein expression in hepatic and extra-hepatic tissues. *Mol Ther Methods Clin Dev*. 2023;30: 235-245.
14. Walkowiak B, Kralisz U, Michalec L, et al. Comparison of platelet aggregability and P-selectin surface expression on platelets isolated by different methods. *Thromb Res*. 2000;99(5):495-502.
15. Siewiera K, Labieniec-Watala M, Wolska N, Kassassir H, Watala C. Sample preparation as a critical aspect of blood platelet mitochondrial respiration measurements—the impact of platelet activation on mitochondrial respiration. *Int J Mol Sci*. 2021;22(17):9332.
16. Spelmink SE, Jager ST, van de Watering L, et al. Efficacy and safety of platelet additive solution-E stored platelet concentrates. *Transfusion*. 2023;63(12):2273-2280.
17. van der Meer PF, de Korte D. Platelet additive solutions: a review of the latest developments and their clinical implications. *Transfus Med Hemother*. 2018;45(2):98-102.
18. Tobian AA, Fuller AK, Uglik K, et al. The impact of platelet additive solution apheresis platelets on allergic transfusion reactions and corrected count increment (CME). *Transfusion*. 2014;54(6):1523-1529. quiz 1522.
19. Devine DV, Serrano K. The platelet storage lesion. *Clin Lab Med*. 2010;30(2):475-487.
20. Strandenes G, Sivertsen J, Bjerkvig CK, et al. A pilot trial of platelets stored cold versus at room temperature for complex cardiothoracic surgery. *Anesthesiology*. 2020;133(6): 1173-1183.
21. Center for Biologics Evaluation and Research. Alternative Procedures for the Manufacture of Cold-Stored Platelets Intended for the Treatment of Active Bleeding when Conventional Platelets Are Not Available or Their Use Is Not Practical. U.S. Department of Health and Human Services Food and Drug Administration; 2023.
22. Levin E, Culibrk B, Gyongyossy-Issa MI, et al. Implementation of buffy coat platelet component production: comparison to platelet-rich plasma platelet production. *Transfusion*. 2008;48(11):2331-2337.
23. Circular of Information For the Use of Human Blood Components. *Canadian Blood Services*. 2022.
24. Blais-Normandin I, Tordon B, Anani W, Ning S. Pathogen-reduced platelets. In: Khandelwal A, Abe T, eds. *Clinical Guide to Transfusion*. Ottawa: Canadian Blood Services; 2022:chap 19.
25. Kulkarni JA, Witzigmann D, Leung J, et al. Fusion-dependent formation of lipid nanoparticles containing macromolecular payloads. *Nanoscale*. 2019;11(18):9023-9031.
26. Kulkarni JA, Cullis PR, van der Meel R. Lipid nanoparticles enabling gene therapies: from concepts to clinical utility. *Nucleic Acid Ther*. 2018;28(3):146-157.
27. World Intellectual Property Organization. Mc3-type lipids and use thereof in the preparation of lipid nanoparticles. In: Ciufolini MA, Kulkarni J, Kurek D, Saadati F, Tam AC, Witzigmann, eds. *Patent application WO 2022/246571 A1*. 2022.
28. Adams D, Gonzalez-Duarte A, O'Riordan WD, et al. Patisiran, an RNAi therapeutic, for hereditary transthyretin amyloidosis. *N Engl J Med*. 2018;379(1):11-21.
29. Jayaraman M, Ansell SM, Mui BL, et al. Maximizing the potency of siRNA lipid nanoparticles for hepatic gene silencing

- in vivo. *Angew Chem Int Ed Engl.* 2012; 51(34):8529-8533.
30. Cheng MHY, Leung J, Zhang Y, et al. Induction of bleb structures in lipid nanoparticle formulations of mRNA leads to improved transfection potency. *Adv Mater.* 2023;35(31):e2303370.
  31. Hald Albersen C, Kulkarni JA, Witzigmann D, Lind M, Petersson K, Simonsen JB. The role of lipid components in lipid nanoparticles for vaccines and gene therapy. *Adv Drug Deliv Rev.* 2022;188:114416.
  32. Schoenmaker L, Witzigmann D, Kulkarni JA, et al. mRNA-lipid nanoparticle COVID-19 vaccines: structure and stability. *Int J Pharm.* 2021;601:120586.
  33. Morton LF, Hargreaves PG, Farndale RW, Young RD, Barnes MJ. Integrin alpha 2 beta 1-independent activation of platelets by simple collagen-like peptides: collagen tertiary (triple-helical) and quaternary (polymeric) structures are sufficient alone for alpha 2 beta 1-independent platelet reactivity. *Biochem J.* 1995;306(Pt 2): 337-344.
  34. De Simone I, Baaten C, Gibbins JM, et al. Repeated platelet activation and the potential of previously activated platelets to contribute to thrombus formation. *J Thromb Haemost.* 2023;21(5):1289-1306.
  35. Dachary-Prigent J, Freyssinet JM, Pasquet JM, Carron JC, Nurden AT. Annexin V as a probe of aminophospholipid exposure and platelet membrane vesiculation: a flow cytometry study showing a role for free sulfhydryl groups. *Blood.* 1993;81(10): 2554-2565.
  36. Sodergren AL, Ramstrom S. Platelet subpopulations remain despite strong dual agonist stimulation and can be characterised using a novel six-colour flow cytometry protocol. *Sci Rep.* 2018;8(1):1441.
  37. Tyagi T, Jain K, Gu SX, et al. A guide to molecular and functional investigations of platelets to bridge basic and clinical sciences. *Nat Cardiovasc Res.* 2022;1(3):223-237.
  38. Vadgama A, Boot J, Dark N, et al. Multiparameter phenotyping of platelets and characterization of the effects of agonists using machine learning. *Res Pract Thromb Haemost.* 2024;8(5):102523.
  39. Spurgeon BEJ, Frelinger AL 3rd. Comprehensive phenotyping of human platelets by single-cell cytometry. *Cytometry A.* 2022;101(4):290-297.
  40. Görlinger K, Dirkmann D, Hanke AA. Rotational thromboelastometry (ROTEM®). In: Gonzalez E, Moore HB, Moore EE, eds. *Trauma Induced Coagulopathy.* Springer International Publishing; 2016:267-298.
  41. Zhao H, Devine DV. The missing pieces to the cold-stored platelet puzzle. *Int J Mol Sci.* 2022;23(3):1100.
  42. New HV, Berryman J, Bolton-Maggs PH, et al. Guidelines on transfusion for fetuses, neonates and older children. *Br J Haematol.* 2016;175(5):784-828.
  43. Devine DV. Novel platelet products including cold-stored platelets. *Hematology Am Soc Hematol Educ Program.* 2022;2022(1):421-423.
  44. Tobian AA, Savage WJ, Tisch DJ, Thoman S, King KE, Ness PM. Prevention of allergic transfusion reactions to platelets and red blood cells through plasma reduction. *Transfusion.* 2011;51(8):1676-1683.
  45. Francia V, Schifferers RM, Cullis PR, Witzigmann D. The biomolecular corona of lipid nanoparticles for gene therapy. *Bioconjug Chem.* 2020;31(9):2046-2059.
  46. Dilliard SA, Cheng Q, Siegwart DJ. On the mechanism of tissue-specific mRNA delivery by selective organ targeting nanoparticles. *Proc Natl Acad Sci U S A.* 2021;118(52): e2109256118.
  47. Zhang R, El-Mayta R, Murdoch TJ, et al. Helper lipid structure influences protein adsorption and delivery of lipid nanoparticles to spleen and liver. *Biomater Sci.* 2021;9(4): 1449-1463.
  48. Hafez IM, Maurer N, Cullis PR. On the mechanism whereby cationic lipids promote intracellular delivery of polynucleic acids. *Gene Ther.* 2001;8(15):1188-1196.
  49. Kulkarni JA, Myhre JL, Chen S, et al. Design of lipid nanoparticles for in vitro and in vivo delivery of plasmid DNA. *Nanomedicine.* 2017;13(4):1377-1387.
  50. Seynhaeve ALB, Dicheva BM, Hoving S, Koning GA, Ten Hagen TLM. Intact Doxil is taken up intracellularly and released doxorubicin sequesters in the lysosome: evaluated by in vitro/in vivo live cell imaging. *J Control Release.* 2013;172(1):330-340.
  51. Mui BL, Tam YK, Jayaraman M, et al. Influence of polyethylene glycol lipid desorption rates on pharmacokinetics and pharmacodynamics of siRNA lipid nanoparticles. *Mol Ther Nucleic Acids.* 2013; 2(12):e139.
  52. Hayashi Y, Takenaka S, Kohmura C, Ikeda H. Preparation of discoid washed platelets by differential centrifugation. *Clin Chim Acta.* 1998;275(1):99-105.
  53. Omo-Lamai S, Zamora ME, Patel MN, et al. Nanoparticles to the lungs induces clotting: mechanisms and solutions. *Adv Mater.* 2024; 36(26):e2312026.
  54. Golebiewska EM, Poole AW. Platelet secretion: from haemostasis to wound healing and beyond. *Blood Rev.* 2015;29(3):153-162.
  55. de Wit YES, Vlaar R, Gouwerok E, et al. Platelet concentrates in platelet additive solutions generate less complement activation products during storage than platelets stored in plasma. *Blood Transfus.* 2023;21(2):157-167.
  56. Thon JN, Devine DV. Translation of glycoprotein IIIa in stored blood platelets. *Transfusion.* 2007;47(12):2260-2270.
  57. Ng MSY, Tung JP, Fraser JF. Platelet storage lesions: what more do we know now? *Transfus Med Rev.* 2018;32(3):144-154.
  58. Mills EW, Green R, Ingolia NT. Slowed decay of mRNAs enhances platelet specific translation. *Blood.* 2017;129(17):e38-e48.
  59. Mills EW, Wangen J, Green R, Ingolia NT. Dynamic regulation of a ribosome rescue pathway in erythroid cells and platelets. *Cell Rep.* 2016;17(1):1-10.
  60. Pienimaeki-Roemer A, Kuhlmann K, Bottcher A, et al. Lipidomic and proteomic characterization of platelet extracellular vesicle subfractions from senescent platelets. *Transfusion.* 2015;55(3):507-521.
  61. Green SM, Padula MP, Dodgen TM, Batarseh A, Marks DC, Johnson L. Lipidomic changes occurring in platelets during extended cold storage. *Transfus Med.* 2024; 34(3):189-199.
  62. Cardigan R, Williamson LM. The quality of platelets after storage for 7 days. *Transfus Med.* 2003;13(4):173-187.
  63. Kufrin D, Eslin DE, Bdeir K, et al. Antithrombotic thrombocytes: ectopic expression of urokinase-type plasminogen activator in platelets. *Blood.* 2003;102(3): 926-933.
  64. Schiffer CA, Bohlke K, Delaney M, et al. Platelet transfusion for patients with cancer: American Society of Clinical Oncology clinical practice guideline update. *J Clin Oncol.* 2018;36(3):283-299.
  65. Haemmerle M, Stone RL, Menter DG, Afshar-Kharghan V, Sood AK. The platelet lifeline to cancer: challenges and opportunities. *Cancer Cell.* 2018;33(6):965-983.

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