

Title: PEG-associated efficacy loss in lipid nanoparticles persists across formulation modifications for mRNA delivery

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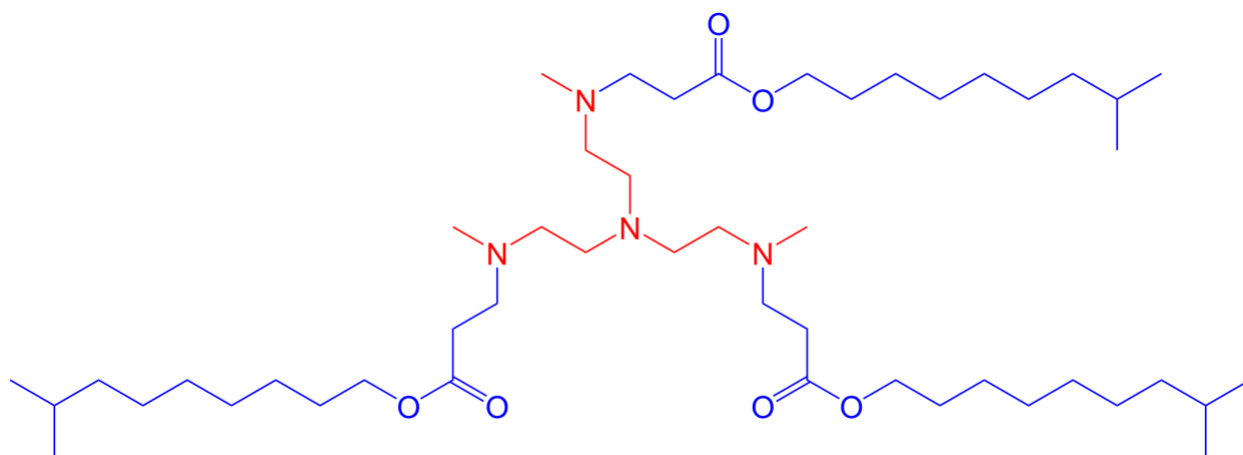
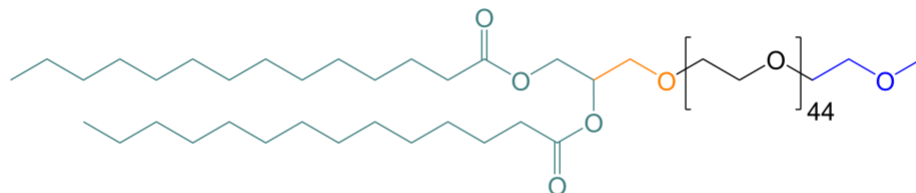
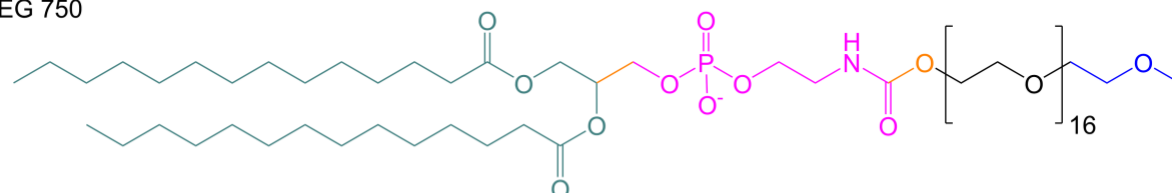


Fig S1 The ionizable lipidoid 304O_{i10} comprises an amine headgroup and an acrylate tail. The previously identified head group (304, red) and tail group (O_{i10}, blue) were reacted for 3 days at 90°C, with purification by flash chromatography.

DMG-PEG 2000



PE-PEG 750



PE-PEG 2000

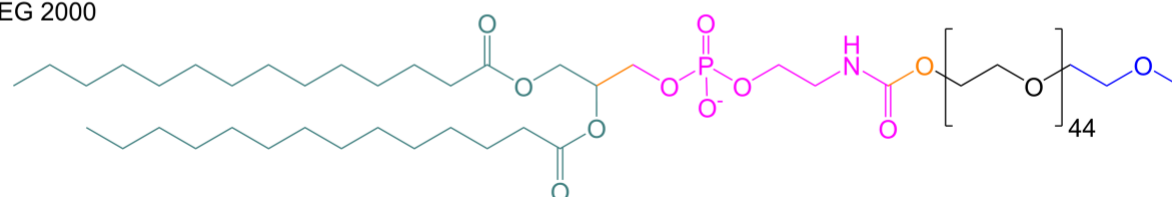


Fig S2 The three PEG-lipids used in this study share a common fatty acid tail structure but differ in their linker chemistry. The PEG-lipids used in this study each contain two fatty acid tails of 14 carbons (myristic acid, green). DMG-PEG2000 contains a glycerol linker between the fatty acid tails and the PEG chain, while PE-PEG750 and PE-PEG2000 contain a phosphoethanolamine linker. Each PEG-lipid ends with a methoxy group.

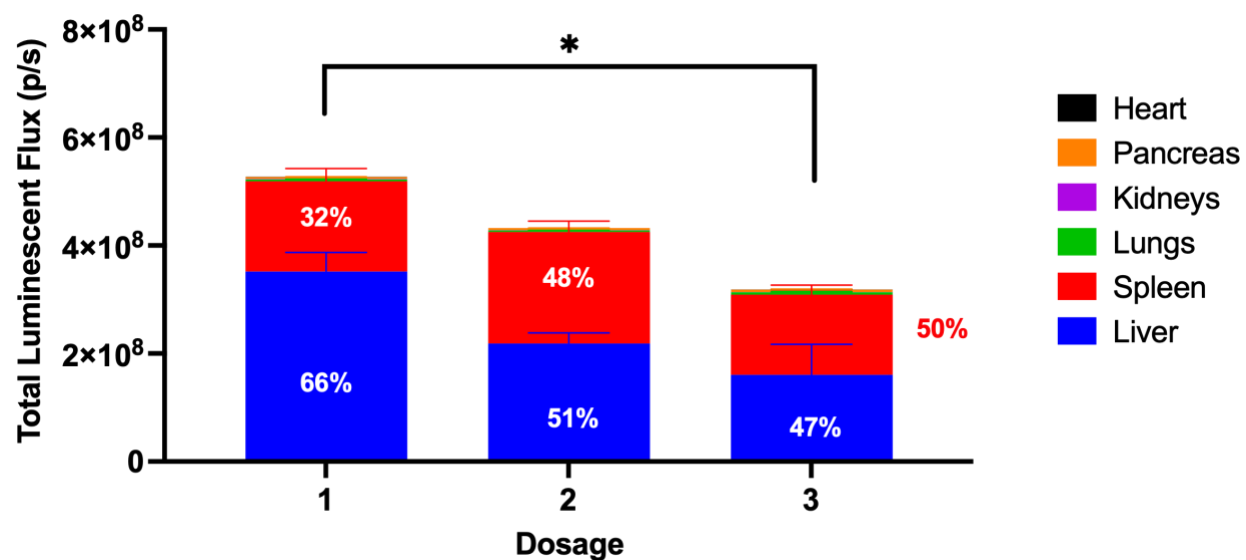


Fig S3 LNP 304O₁₁₀ exhibits liver and spleen tropism and loses efficacy upon repeated administration. LNPs were formulated with luciferase mRNA (mLuc) at 0.5 mg/kg and delivered IV. Over three sequential dosages, a notable shift is observed from the liver to the spleen. (N=3-4); error bars represent SEM, with * indicating P < 0.05, by one-way ANOVA.

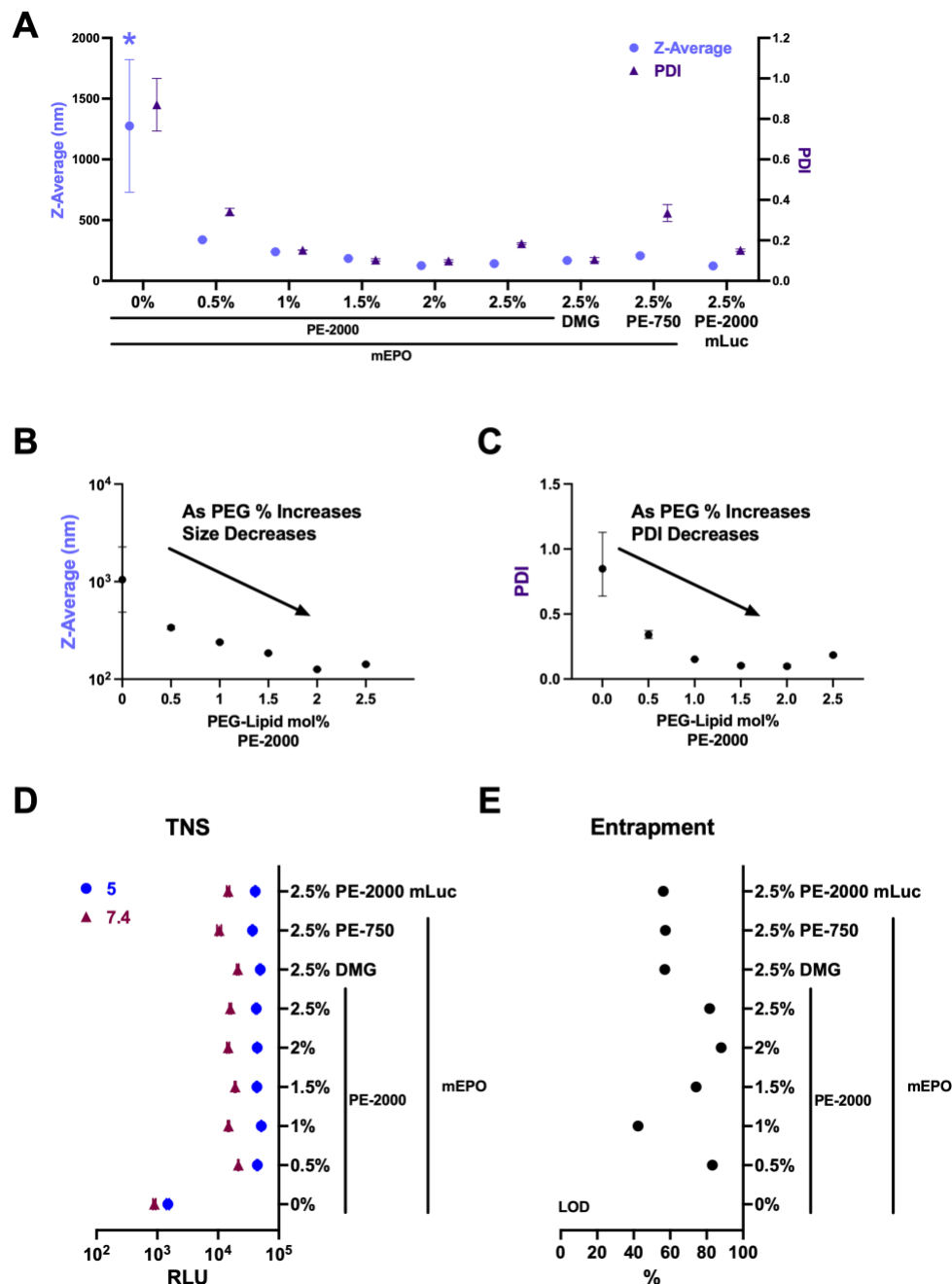


Fig S4 Characterization of 3040i10 LNP size and entrapment reflects previous studies on the effect of PEG percentage. Lipid nanoparticles were formulated with varying mRNA cargo (mEPO or mLuc), PEG-lipid percentage, or type: PE-PEG2000 (PE-2000), PE-PEG750 (PE-750), and DMG-PEG2000 (DMG). A) In the absence of PEG (0%), LNP size exceeded 1000 nm, and the polydispersity index (PDI) was over 0.8. B-C) As the PEG percentage increased, the size and PDI decreased, similar to previous study findings. D) TNS fluorescence was higher at pH 5 (endosomal) across all formulations than at pH 7.4 (physiological), consistent with expected ionizable lipid behavior. E) RNA entrapment values were highest for 2 and 2.5% PE-PEG2000 mEPO formulations, and the 0% PEG formulation had no measurable entrapment, with values below the limit of detection (LOD). (N=3-4); error bars represent SEM, with * indicating $P < 0.05$, by one-way ANOVA.

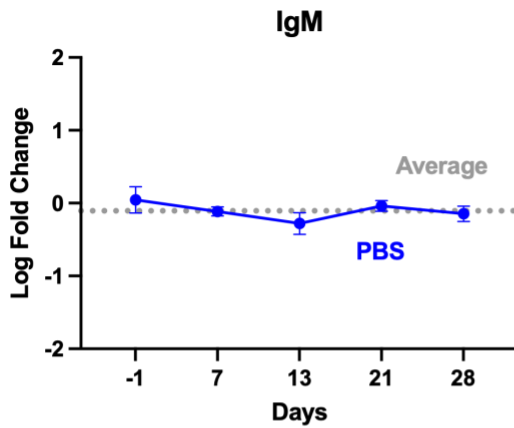
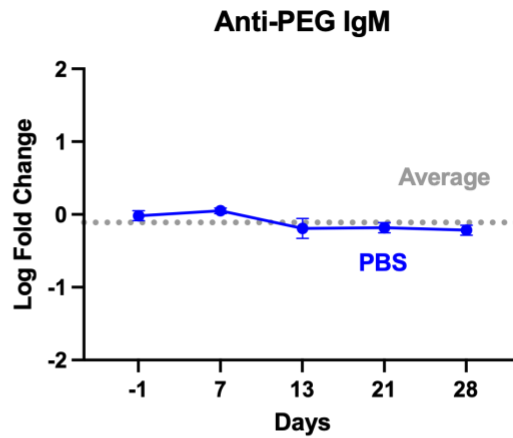
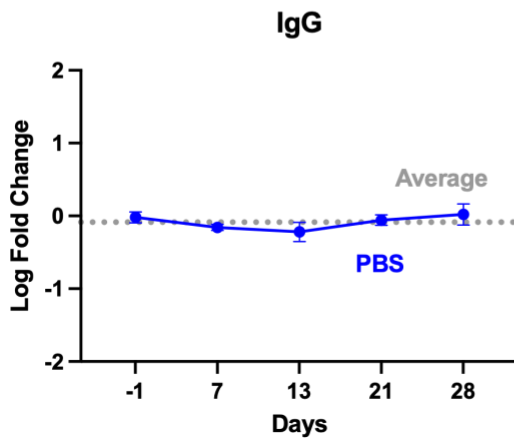
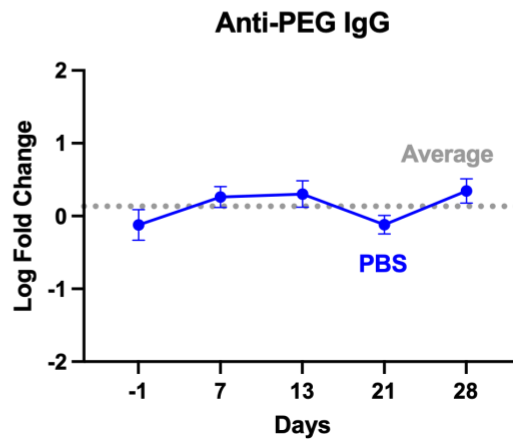
A**B****C****D**

Fig S5 In the PBS control, levels of IgM, Anti-PEG IgM, IgG, and Anti-PEG IgG remained consistent across the 28-day study. Serum antibody levels were quantified over time by ELISA. All antibody levels remained consistent for A) IgM, B) Anti-PEG IgM, C) IgG, D) Anti-PEG IgG. The gray dashed line indicates the average serum level. N=3-4, error bars represent SEM.

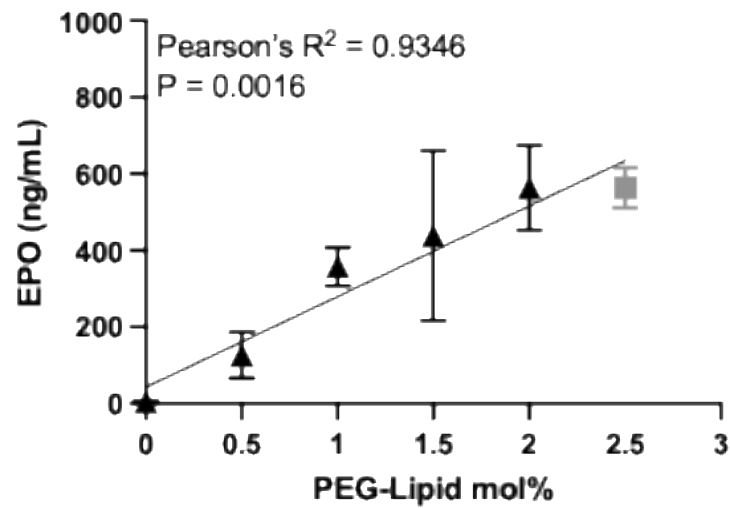


Fig S6 EPO expression correlated positively with PEG-lipid molar percentage. EPO concentrations were measured from blood collected 6 hours after the first dose across PEG-lipid concentrations from 0 to 2.5 mol%. N=3-4, error bars represent SEM. Pearson's Correlation, $R^2 = 0.9346$, $p < 0.05$.

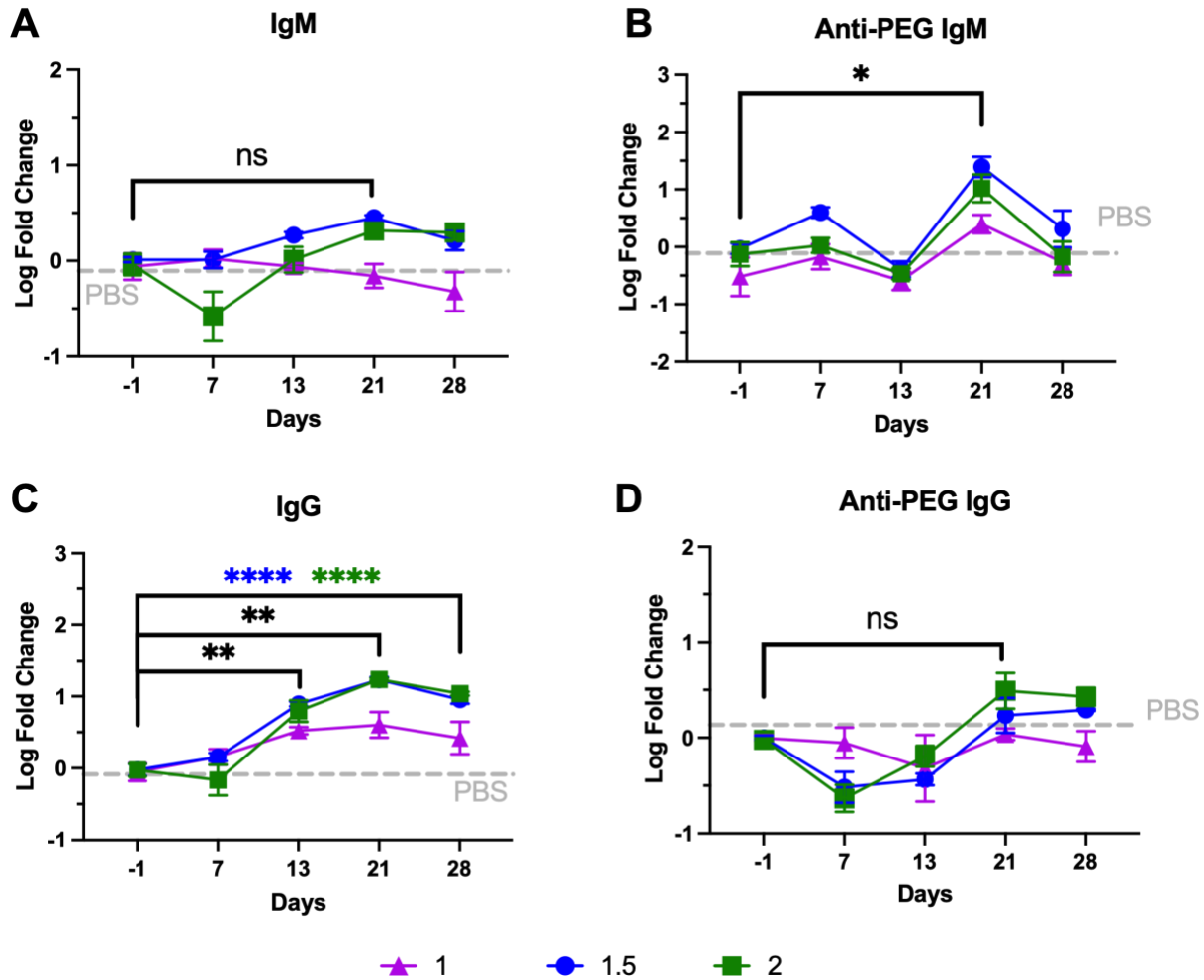


Fig S7 Anti-PEG IgM and total IgG increased significantly for PEG-lipid concentrations of 1-2 mol%. Lipid nanoparticles formulated with lipidoid 304O_{i10} and PE-PEG2000 were delivered intravenously to mice on Day 0 at 0.5 mg/kg with EPO mRNA for PEG molar concentrations of 1% (purple triangles), 1.5% (blue circles), and 2% (green squares). A) IgM, B) anti-PEG IgM, C) IgG, and D) anti-PEG IgG serum levels were quantified by ELISA. Anti-PEG IgM increased significantly for 1.5% and 2% PEG. Total IgG increased significantly for all groups, but anti-PEG IgG did not increase significantly. The gray dashed line indicates the average serum PBS control value. N=3-4, error bars represent SEM, with * and **, **** indicating P < 0.05, P < 0.01, and P < 0.0001, respectively, by two-way ANOVA. Black asterisks indicate that all groups within a panel differed significantly from their respective later time point, while color-matched asterisks indicate that only the corresponding treatment group reached significance.

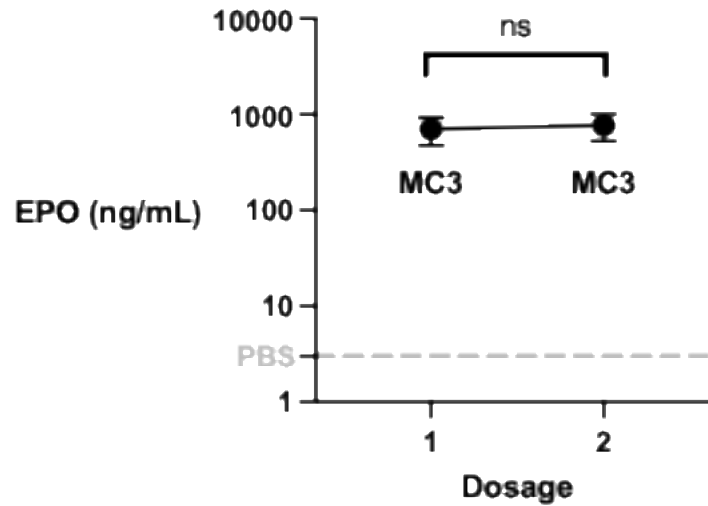


Fig S8 LNP_{MC3} maintains efficacy upon repeat administration. Lipid nanoparticles formulated with MC3 were delivered intravenously to mice at 0.5 mg/kg with EPO mRNA. Serum EPO concentrations were quantified by ELISA 6 hours after each dose. The gray dashed line indicates the PBS control serum level. N=3-4, error bars represent SEM, ns = not significant by Welch's t-test.