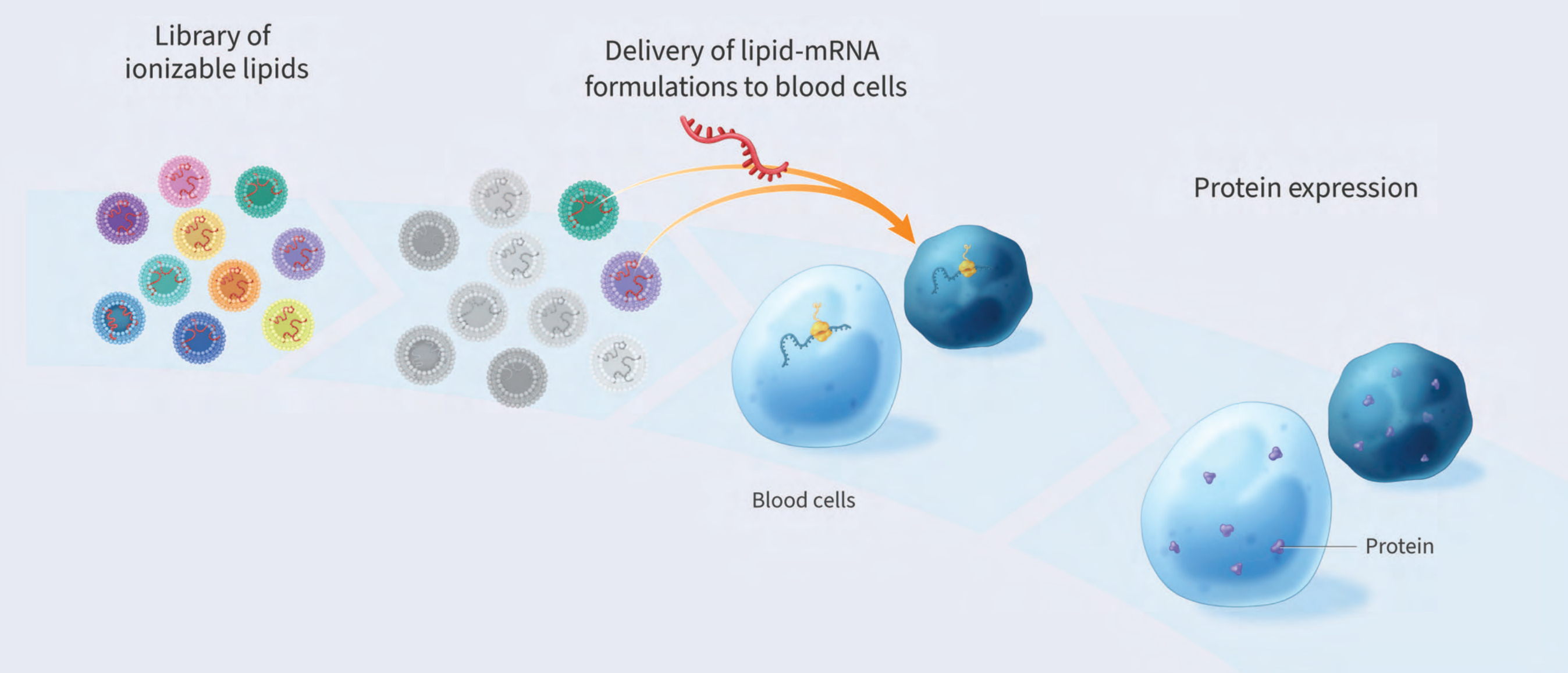


Introduction

In recent years, lipid-based mRNA delivery has become the gold standard method for inducing exogenous protein production *in vivo* as evidenced by the success of the COVID-19 mRNA vaccines. While intramuscular injections are ideal for vaccine applications, intravenous injections are generally more suitable for achieving broad internal distribution of therapeutic payloads. Following systemic administration, blood cells are the first cells encountered by lipid nanoparticles (LNPs) and therefore serve as high interest targets for *in situ* protein production. With the goal of identifying a lipid formulation capable of efficiently transfecting blood cells, we synthesized and screened a library of over 20 multivalent ionizable lipids with variations in headgroup and lipid tail. Lipids were characterized as lipoplexes and as solid LNPs, using mRNA encoding green fluorescent protein (GFP) as representative nucleic acid cargo. Lipid formulations were analyzed for transfection efficiency and cytotoxicity using both THP-1 monocytes and Jurkat T cells to select the most effective candidate, FB3-54. The ability to efficiently target blood cells using lipid delivery systems opens the door to a variety of applications, including potent *in vivo* mRNA delivery and cell reprogramming.

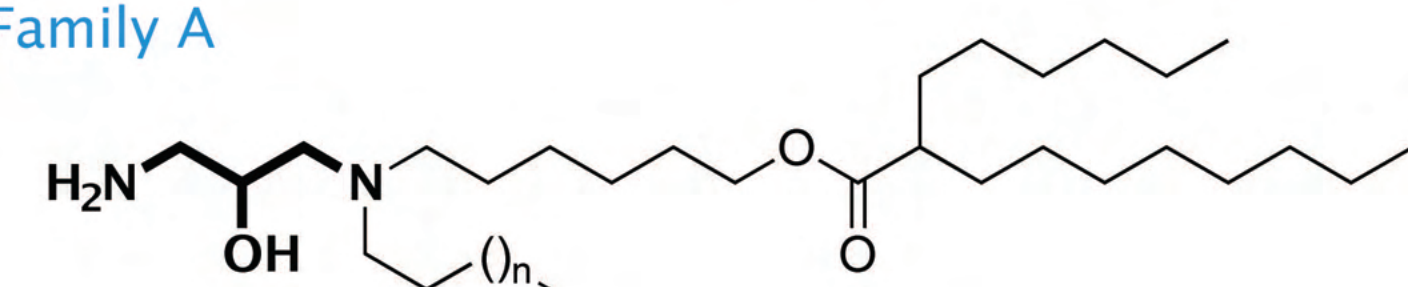


Conclusions

- We have identified a new lipid composition, FB3-54, which is potent and able to transfect blood cells in both lipoplex and LNP form.
- Our lipid design strategy has identified a family of novel lipids with similar characteristics able to effectively transfect blood cells.
- In designing our library, we observed that varying lipid head group, tail, and spacer link greatly affects lipid transfection ability.
- Efficiently targeting blood cells with lipid/RNA systems may allow for applications such as potent *in vivo* mRNA delivery and cell reprogramming.

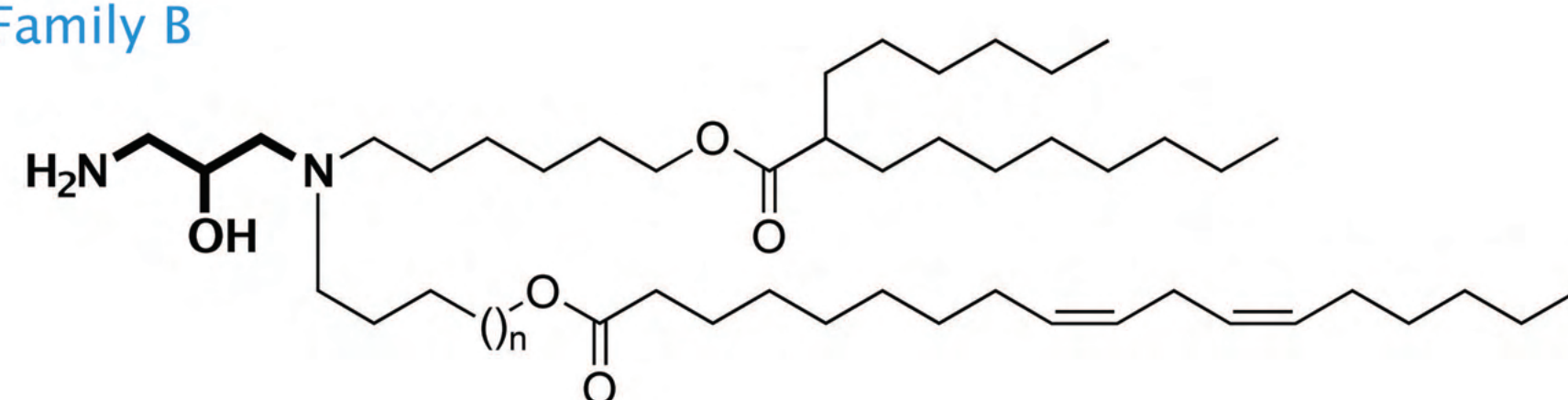
1 Lipid Library Design

Family A



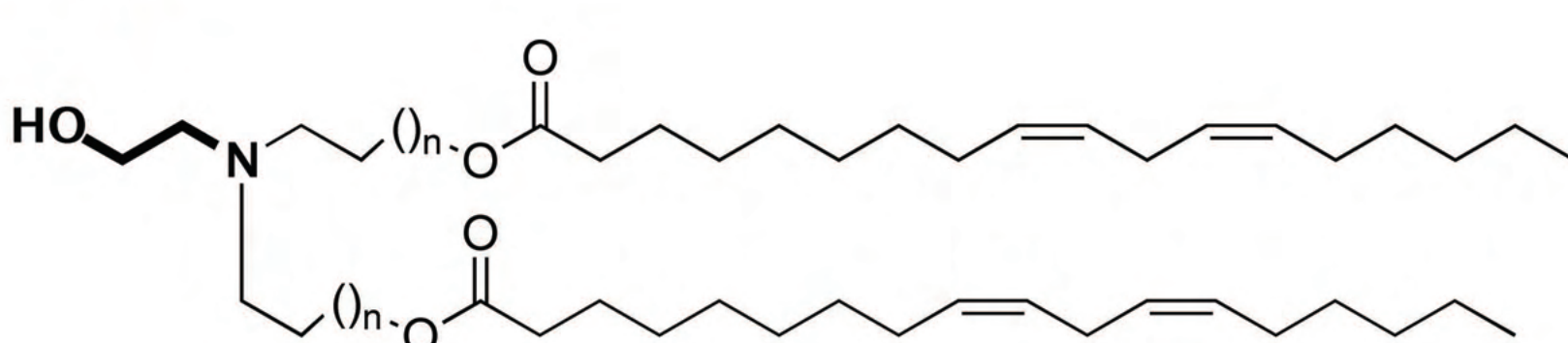
- Headgroup: 2-hydroxypropylamine (half of the headgroup in our ToRNAado™ lipid)
- Ester tail facilitates breakdown of lipids into less toxic carboxylic acids and alcohols

Family B



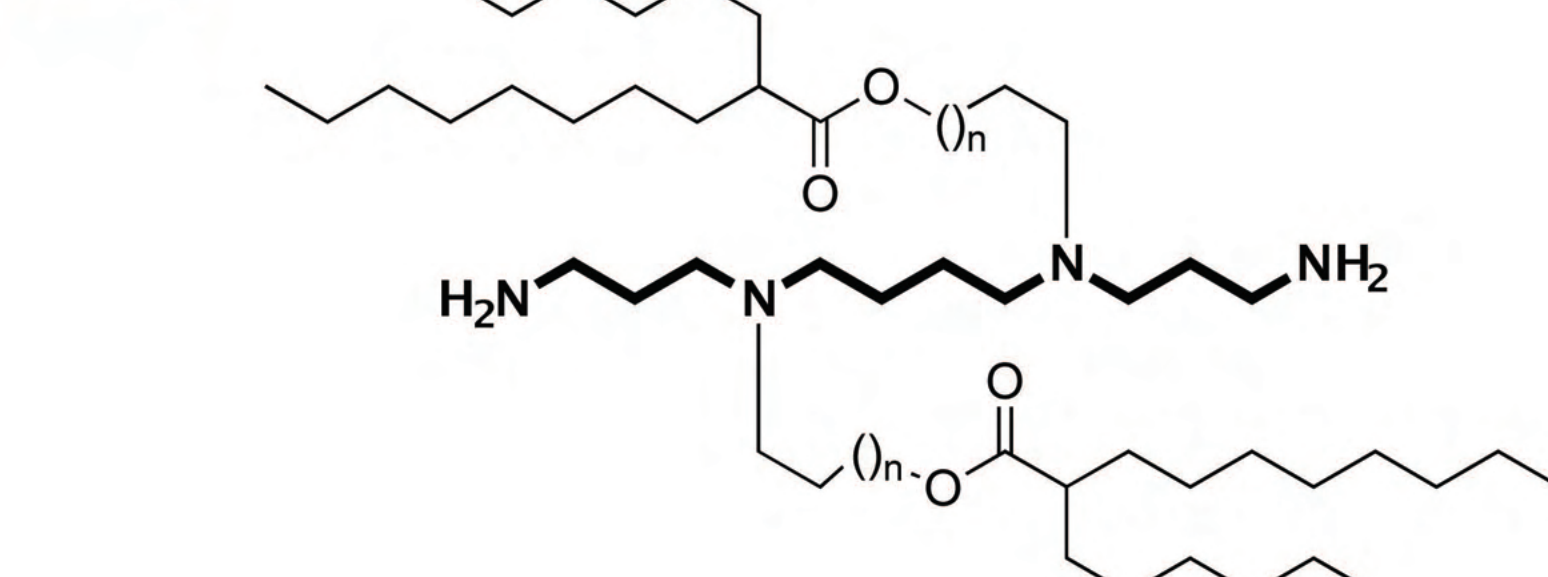
- Headgroup: 2-hydroxypropylamine
- Ester tails
- Lower tail is a derivative of ToRNAado™ tails with ester addition

Family C



- Headgroup: ethanolamine (the headgroup of SM-102, the ionizable lipid component of Spikevax)
- ToRNAado™ tails with esters

Family D



- Headgroup: spermine (naturally occurring, known to effectively bind RNA in ToRNAado™)
- Ester tails

Figure 1. Composition of lipid library. The library is comprised of 4 unique multivalent ionizable lipid families. Lipid tails varied in head-to-tail spacer length, n , ranging from 1 to 15.

3 Transfection Efficacy

Methods: To evaluate transfection efficiency and cytotoxicity, THP-1 monocytes and Jurkat T cells were transfected with lipoplexes and lipid nanoparticles.

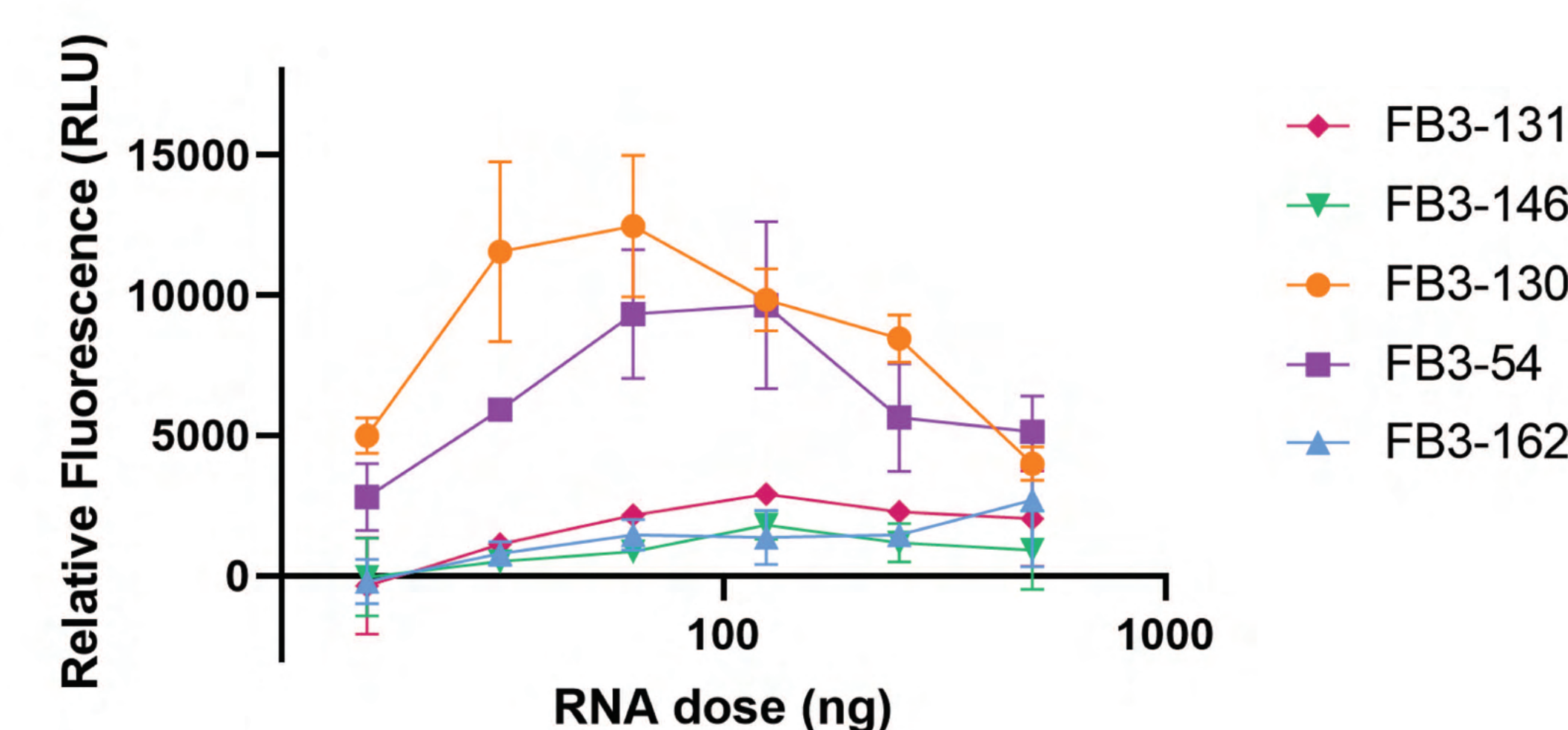


Figure 6. Lipoplex screen in THP-1 monocytes. These 5 lipids are all in the Family D with a spermine headgroup and bis hexyl 2-hexyldecanoate tails. Lipids listed in order of increasing carbon spacer link length. Lipoplexes were created using a lipid:mRNA weight ratio of 2:1 for each formulation. FB3-130 and FB3-54 had the greatest transfection efficiency, varying in structure by only one carbon.

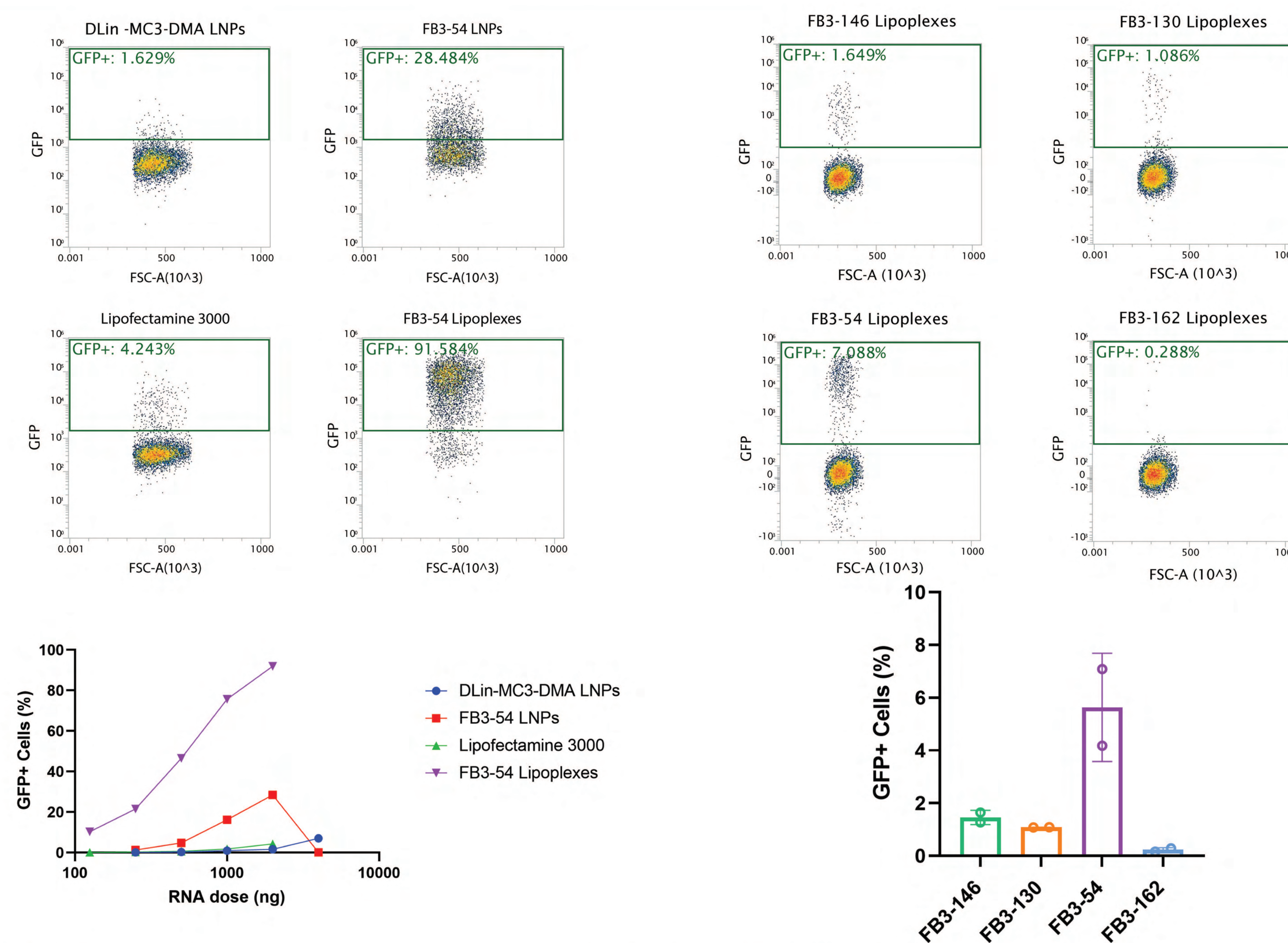


Figure 5. THP-1 monocyte transfection. Dose response curve of LNP and lipoplex transfections analyzed using flow cytometry. Density plots of GFP+ transfected cells from doses of 2000 ng RNA per 120,000 cells for each formulation. FB3-54 LNPs and lipoplexes exhibited greater *in vitro* transfection efficiency of THP-1 monocytes than commercially-available controls.

2 Formulation and Physical Characterization of Nanoparticles

Methods: LNPs were formulated through bulk mixing using a molar ratio of 50:38.5:10:1.5 (ionizable lipid/cholesterol/DSPC/DMG-PEG2000) and a 0.067 weight ratio of mRNA to ionizable lipid. Lipoplexes were formulated at various weight ratios of lipid:mRNA, and mRNA-loading efficiency was determined via gel electrophoresis. The lowest lipid:mRNA weight ratio that displayed complete complexation for each lipid was used in subsequent *in vitro* analyses.

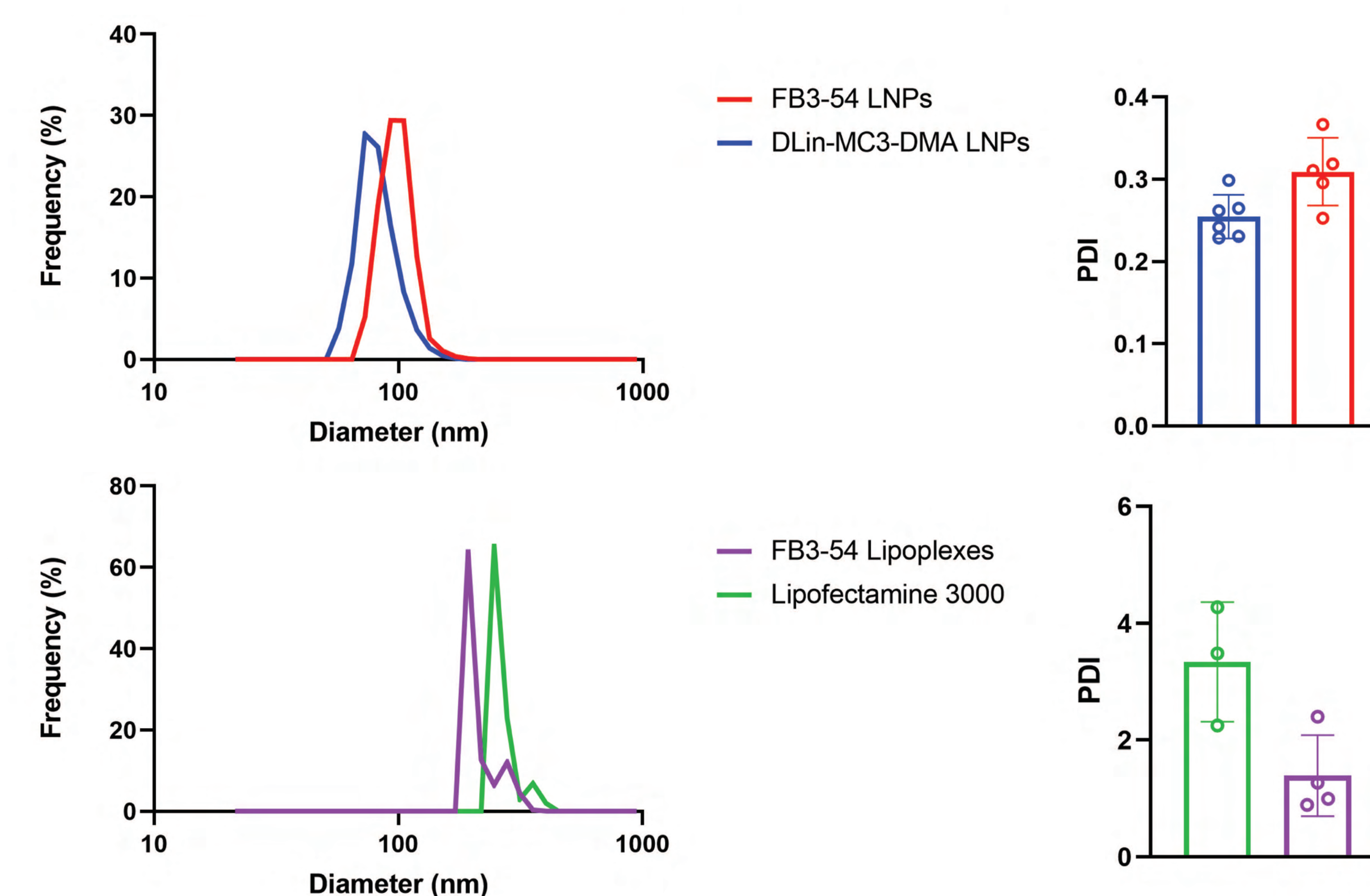


Figure 2. Dynamic light scattering (DLS) was used to determine hydrodynamic diameter of the nanoparticles, graphed as number particle size distribution. Polydispersity index (PDI) was also measured.

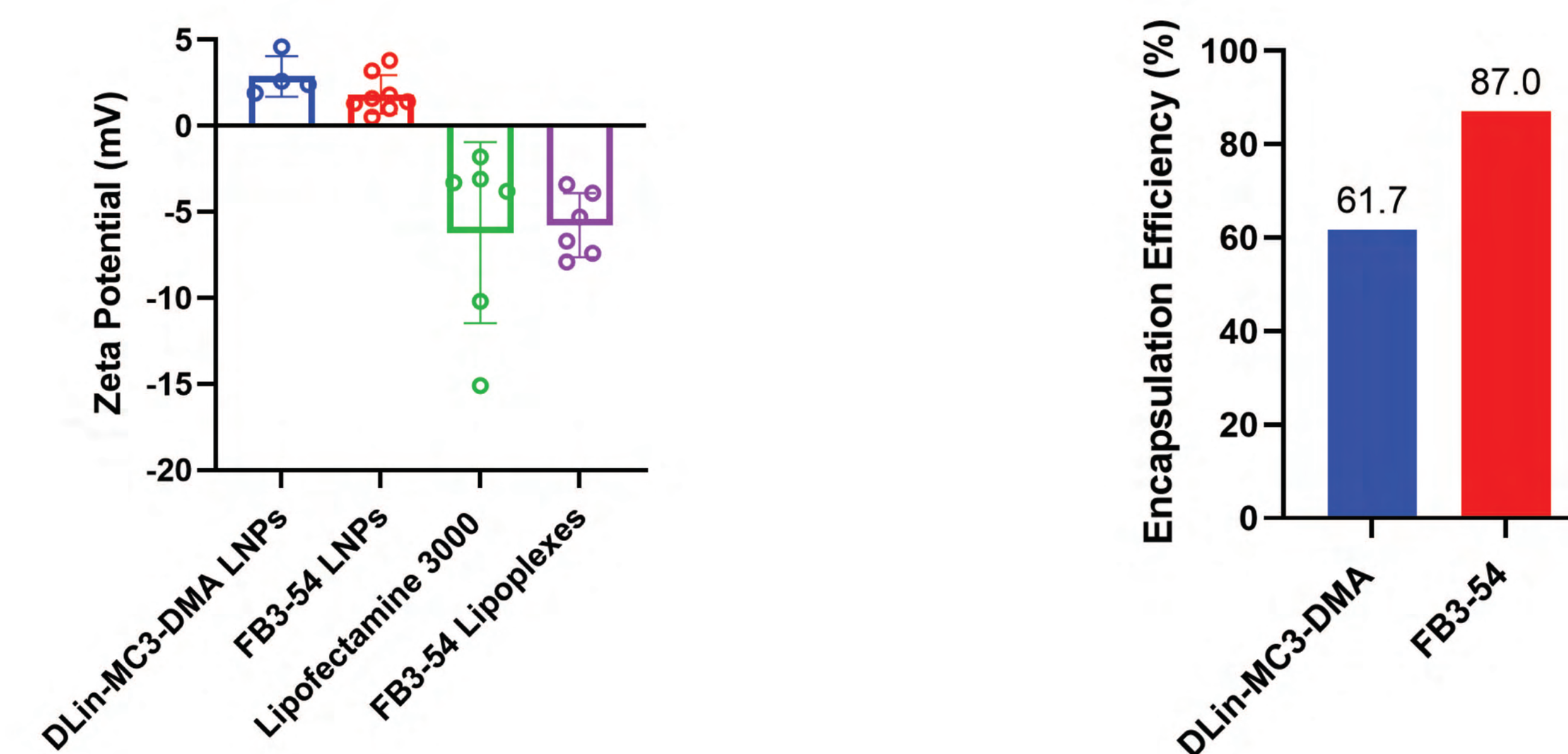


Figure 3. Zeta potential of each nanoparticle formulation was measured by diluting samples in 10 mM NaOH solution at a pH of 5.5.

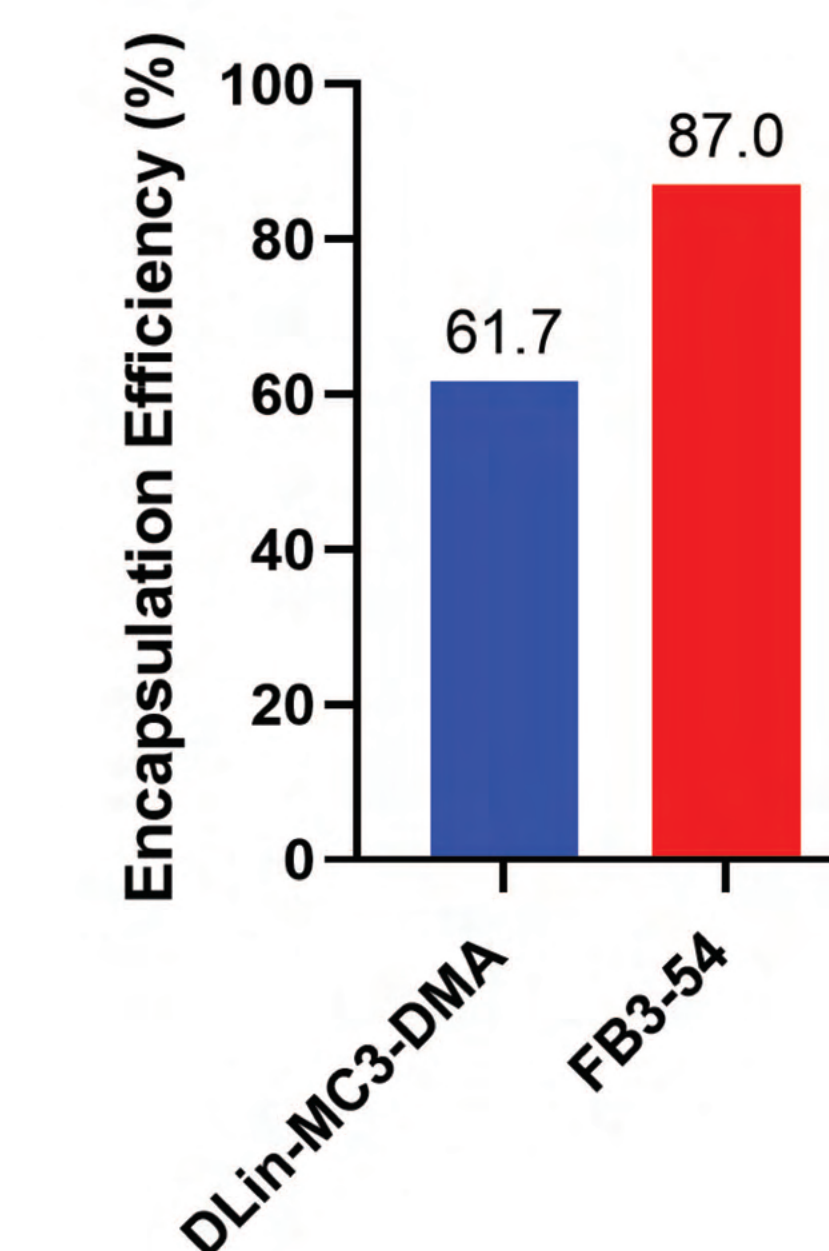


Figure 4. Encapsulation efficiency of LNPs was measured using a RiboGreen assay. FB3-54 LNPs demonstrated greater encapsulation of mRNA.

4 Relative Cytotoxicity

Figure 8. Relative viability of THP-1 monocytes after transfection. Determined using Cell Titer Glo viability assay and calculating viability relative to untransfected cells. FB3-130 which had the greatest transfection in Fig 7 also demonstrates greatest toxicity.

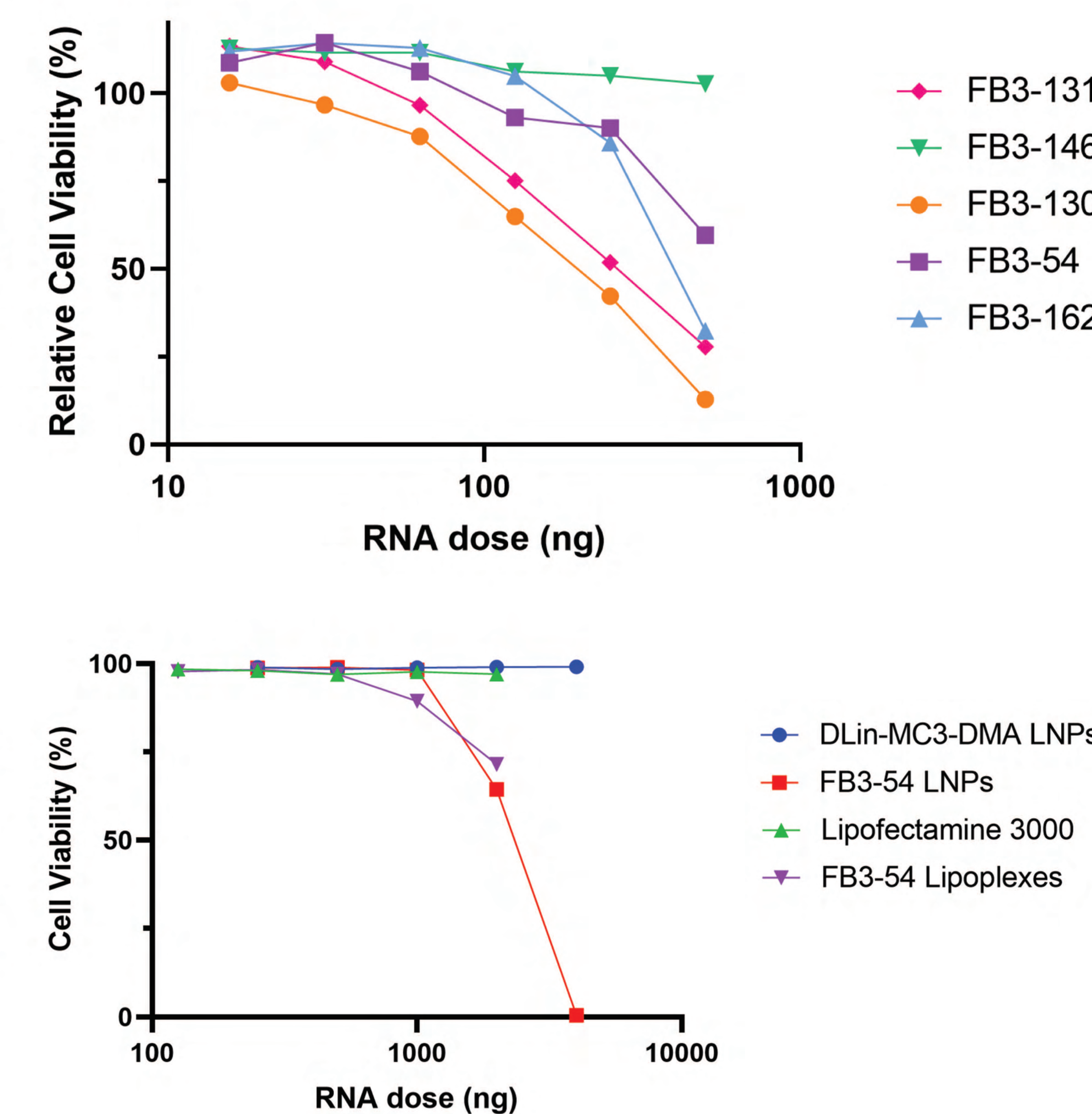


Figure 9. Viability of THP-1 monocytes after transfection. DLin-MC3-DMA LNPs and Lipofectamine had very little toxicity, but also had low transfection as seen in Fig 5. FB3-54 LNPs had significantly greater toxicity than respective FB3-54 lipoplexes, likely due to the greater lipid:mRNA ratio in the formulation.