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Protocol

Preparation of placenta-tropic mRNA lipid nanoparticles for pregnancy disorders

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Abstract

Lipid nanoparticles (LNPs) have garnered tremendous enthusiasm in preclinical and clinical settings for the delivery of nucleic acids such as mRNA. With applications in protein replacement therapies, vaccines and gene editing, mRNA LNPs have only recently been explored in the context of pregnancy disorders. There is a significant need for the design of novel therapeutic technologies such as mRNA LNPs to treat obstetric disorders like pre-eclampsia that are associated with placental pathology and detrimental effects on maternal and fetal health. Here, we present a step-by-step procedure for the preparation and evaluation of placenta-tropic mRNA LNPs for researchers from varied disciplines to explore their application in treating pregnancy disorders. In this Protocol, we describe steps for synthesizing and purifying the key ionizable lipid excipient of the placenta-tropic LNP formulation (4 d) before preparing mRNA LNPs using microfluidic mixing (1 d). Then, we detail in vitro mechanistic evaluations of the effect of protein adsorption on LNP-mediated mRNA transfection to placental trophoblasts (3 d). Finally, we outline methods for isolating reproductive tissues from time-dated pregnant mice to assess in vivo LNP biodistribution and mRNA transfection to the murine placenta (16 d). Compared to alternative LNP formulation procedures, this Protocol focuses on delivering mRNA LNPs to the placenta with a workflow that can be applied for a range of obstetric disorders. This Protocol seeks to increase interdisciplinary work at the interface of nanomedicine, gene modulation and reproductive health.

Key points

- The procedure covers synthesis and purification of the key ionizable lipid excipient, preparation of mRNA LNPs by microfluidic mixing, assessment of the effect of protein adsorption on LNP transfection to placental trophoblast cells and evaluation of LNP biodistribution and mRNA expression in the placentas of time-dated pregnant mice.
- The mRNA LNP formulation presented here has demonstrated applications in treating pre-eclampsia in murine models of the condition and has the potential to be expanded for the treatment of other pregnancy disorders.

Key references

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Introduction

Nucleic acid therapeutics including mRNA have emerged as a new generation of pharmaceuticals, following decades of clinical success for small-molecule drugs and biologics such as proteins, peptides and antibody therapies[1]. The widespread application of nucleic acids in protein-replacement therapies, vaccines and gene editing[2–5] is due in part to the development of the lipid nanoparticle (LNP) technology that facilitates safe and effective mRNA delivery in the body. Although the first LNP formulations have been approved by the US Food and Drug Administration (FDA) for siRNA therapy since 2018[6] and explored preclinically for delivery to tissues such as the liver[7,8], lungs[9,10], spleen[9], bone[11] and pancreas[12], as well as immune[13–15] and hematopoietic stem cells[16,17], they have only recently garnered attention in the context of women's health.

Research in the field of women's health has historically been and continues to be underfunded. In 2020, only 1% of research and development funding worldwide was dedicated to non-cancer-related women's health[18–20]. Within the broad field of women's health, pregnant patients and the conditions that affect them are perhaps the most underserved; >99% of drug trials in the United States fail to enroll pregnant patients[21]. This has a devastating impact on patients in the clinic: there are few to no treatment options for pregnancy disorders such as pre-eclampsia, preterm birth, placenta accreta spectrum disorders and gestational trophoblastic disease. There is a significant need for the design, evaluation and implementation of novel therapeutic technologies to treat pregnancy disorders, many of which are associated with well-characterized pathologies in the placenta.

Development of the Protocol

Although the COVID-19 mRNA LNP vaccines have been shown to be safe and effective for use in pregnant mothers[22–24], at the time of their emergency use authorization by the FDA, they had yet to be tested in a controlled, randomized trial in pregnant patients[25]. In addition, there was little to no literature reporting the pharmacokinetics or biodistribution of mRNA LNPs in animal models of pregnancy after intramuscular or intravenous administration. This inspired our group[26–30] and others[31–33] to explore mRNA LNP delivery during pregnancy.

Using a small library of novel LNP formulations (<20), we demonstrated mRNA delivery to the placenta—a novel tissue target for LNP delivery—upon intravenous administration in pregnant mice[26]. Through this work, we showed that small alterations in the ionizable lipid structure resulted in differential delivery to the placenta, inspiring further work toward identifying a placenta-tropic LNP formulation for potent mRNA delivery to the placenta[29]. Specifically, we used a high-throughput in vivo screening approach to evaluate a large library of 98 LNP formulations with unique ionizable lipid structures and excipient compositions. As a result of this high-throughput screen, we identified the placenta-tropic LNP formulation[29] described here, which enables more than an order of magnitude greater mRNA delivery to the placenta than the industry standard C12-200[7] and clinical standard DLin-MC3-DMA[34] LNP formulations.

We further sought to explore the targeting mechanism by which this LNP enables mRNA delivery to the placenta (Fig. 1). To this end, we turned to the growing body of literature that describes and assesses protein corona formation on the surface of nanoparticles such as LNPs after intravenous administration[35–37]. As has been robustly shown for LNPs that facilitate nucleic acid delivery to the liver, serum proteins immediately adsorb on the LNP surface in the blood stream, forming a corona that facilitates subsequent cellular interactions (Fig. 1a). Using a combination of in vitro and in vivo assays, we proposed a mechanism based on the adsorption of the serum protein β_2-glycoprotein I (β_2-GPI) by which the LNP formulation described in this Protocol enables mRNA delivery to the placenta[29] (Fig. 1b).

Finally, we have used this placenta-tropic LNP formulation for the delivery of therapeutic mRNA for the treatment of pre-eclampsia; pre-eclampsia is a hypertensive disorder associated with impaired vascularization in the placenta. Thus, we evaluated the potential for vascular endothelial growth factor (VEGF) mRNA delivery to remodel placental vasculature,

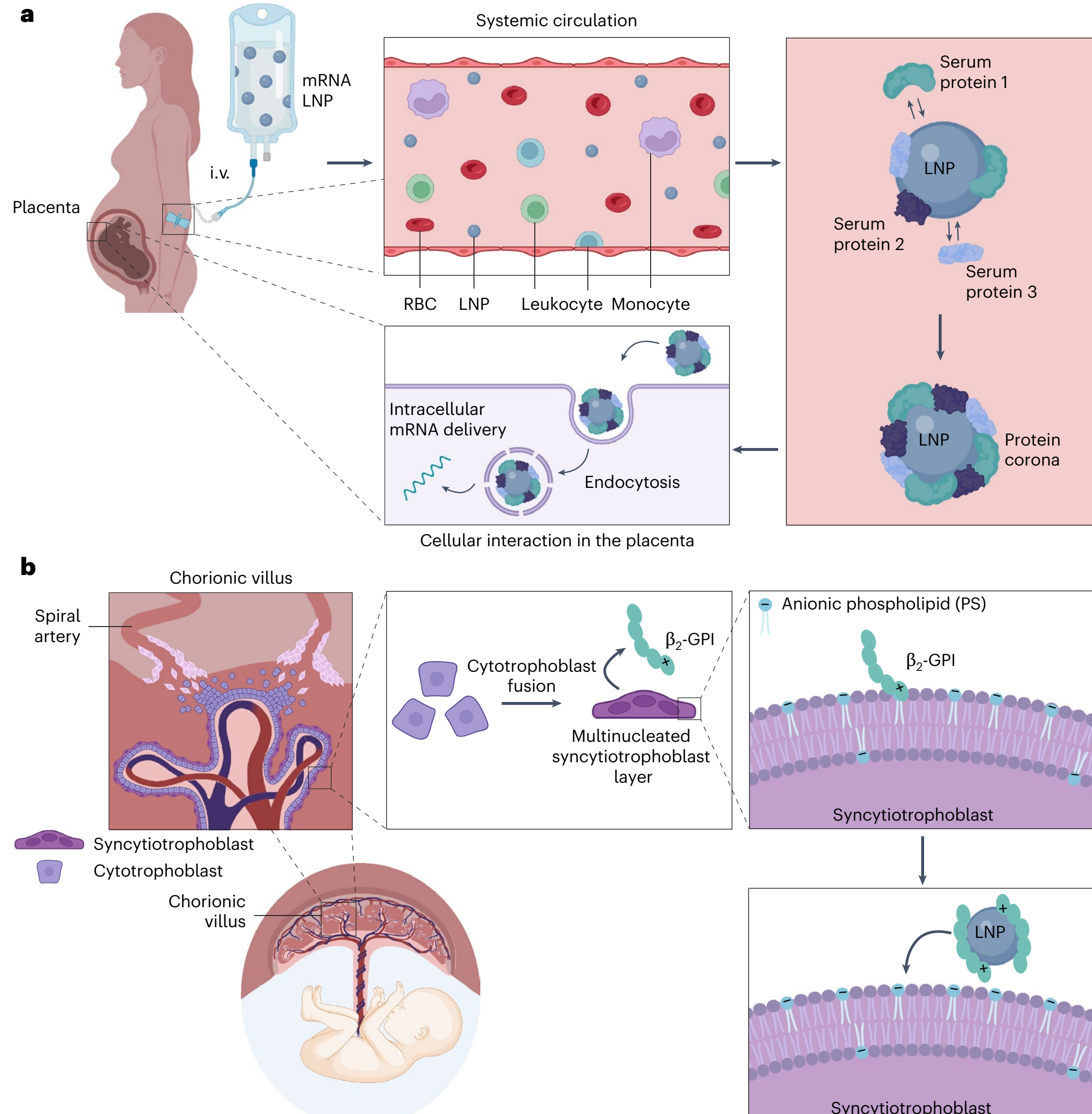


Fig. 1 | Delivering mRNA LNPs to the placenta upon systemic administration. **a**, Immediately after intravenous (i.v.) administration, LNPs interact with cells and proteins present in circulation; serum proteins with high affinity for the lipids in the LNP formulation adsorb on the LNP surface, forming a protein corona[35]. LNP-cell interactions in a particular tissue such as the placenta are facilitated by the proteins in the corona, which then enable intracellular uptake via endocytosis and subsequent mRNA delivery to the cell cytosol. RBC, red blood cell. **b**, Unique aspects of placental physiology may have a role in promoting tissue-specific mRNA LNP delivery upon systemic administration. Specifically, syncytialization occurs at the chorionic villi—the site of blood exchange between maternal and fetal circulation—whereby cytotrophoblasts fuse into a multinucleated syncytiotrophoblast layer. The syncytiotrophoblast secretes the five-domain protein β_2-glycoprotein I (β_2-GPI), responsible for regulating coagulation and complement activation[64]. During syncytialization, the anionic phospholipid phosphatidylserine (PS) is externalized from the inner leaflet of the cell membrane to the outer leaflet, enabling β_2-GPI binding to the syncytiotrophoblast surface via a highly cationic amino acid sequence[65]. In this Protocol, we assess the effect of β_2-GPI adsorption on the surface of placenta-tropic LNPs on mRNA transfection to placental trophoblasts. Panel **a** is adapted with permission from ref. 35, ACS. Panel **b** is adapted with permission from ref. 72, Wiley. The illustrations were created in BioRender. Swingle, K. (2026) https://BioRender.com/e2p5mz9.

induce vasodilation and restore a high–blood flow environment in pre-eclampsia. In both inflammation- and hypoxia-induced mouse models of pre-eclampsia, this placenta-tropic mRNA LNP technology ameliorated hypertension, improved fetal health and remodeled placental vasculature[29].

Overview of the Protocol

Here, we present a workflow for the preparation and evaluation of LNPs for mRNA delivery to the placenta (Fig. 2). Before formulating placenta-tropic LNPs, we first synthesize the crude C14-494 ionizable lipid by combining the epoxide-terminated 14-carbon alkyl tail (1,2-epoxytetradecane) with the 494 piperazine-based polyamine core[29] (Steps 1–13). This S_N2 reaction is performed under gentle stirring at 80 °C for 2 d in ethanol. The crude product is purified to isolate the C14-494 lipid using flash chromatography; product is identified in chromatography fractions via liquid chromatography–mass spectrometry (LC–MS). Rotary evaporation is used to remove organic solvents and isolate the C14-494 ionizable lipid as a clear-to-yellow viscous oil.

Second, we prepare placenta-tropic mRNA LNPs by combining a lipid mixture in ethanol with an mRNA mixture in citrate buffer via microfluidic mixing (Steps 14–28). The lipid mixture is made by combining ionizable lipid (C14-494), phospholipid (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, DOPE), cholesterol and lipid-poly(ethylene glycol) (1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000, C14-PEG_{2000}) at a molar ratio of 35:24:46.5:2.5 (Fig. 3b). LNPs are formulated using a microfluidic device containing channels with staggered herringbone micromixers (Fig. 3c) and dialyzed against 1× PBS for 2 h. Finally, we use the Quant-it RiboGreen reagent and RNA assay kit to measure the concentration of encapsulated mRNA in the LNP formulation by comparison to a standard curve. Obtaining this encapsulated mRNA concentration is essential for the remaining in vitro and in vivo assays described in this Protocol.

Third, we evaluate the effect of protein adsorption on in vitro luciferase mRNA delivery to placental trophoblasts (BeWo b30 cells) (Steps 29–40). To this end, BeWo b30 cells are seeded in a 96-well plate and allowed to adhere overnight. On the following day, we incubate LNPs in increasing amounts of protein—either β_2-GPI or apolipoprotein E (ApoE)—for 15 min at 37 °C to mimic the physiologic conditions in circulation after intravenous administration. Before treating the cells, culture medium containing FBS is removed from the well plate and replaced with Opti-MEM reduced serum medium. The cells are treated with (i) 1× PBS, (ii) uncoated LNPs, (iii) LNPs coated in increasing amounts of β_2-GPI or (iv) LNPs coated in increasing amounts of ApoE and left to incubate for 24 h. After incubation, the cells are lysed, and luciferin substrate from the Promega luciferase assay system is added to the well plate, enabling luminescence quantification via a plate reader.

Finally, we assess biodistribution and mRNA transfection in gestational day 16 pregnant mice for placenta-tropic luciferase mRNA LNPs that have been fluorescently labeled with the lipophilic dye DiD (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate) (Steps 41–58). This section of the Protocol begins by setting up matings by housing one sexually mature male mouse with 1–4 female mice ≥10 weeks of age overnight. On the following day (gestational day 0), the male and female mice are separated, and the vaginal cavity of the female mice is checked for the presence of a vaginal plug, indicating that mating has occurred. Starting on or around gestational day 11, pregnancy is confirmed visually in females with a rounded abdomen. On the basis of the number of pregnant mice, placenta-tropic mRNA LNPs are prepared, adjusted to an mRNA concentration of 60 ng μl^{-1} and labeled with the fluorescent dye DiD. LNPs are administered intravenously to the pregnant mice; 6–12 h after administration, D-luciferin substrate is injected intraperitoneally to measure luminescence signal from mRNA transfection using an in vivo imaging system (IVIS). The mice are then euthanized, and tissues, including placentas and fetuses, are dissected and imaged. DiD fluorescence signal (i.e., a measure of LNP biodistribution) and luminescence signal (i.e., a measure of mRNA transfection) are quantified in each tissue by using the ROI tool in the Living Image software.

Applications of the Protocol

We have previously demonstrated the application of the placenta-tropic mRNA LNPs described in this Protocol for the treatment of pre-eclampsia[29], an obstetric condition with a well-characterized vascular pathology in the placenta of human patients[38]. We envision that this Protocol can be readily adapted to investigate the capacity for mRNA LNPs to treat a much broader range of pregnancy disorders including fetal growth restriction, preterm

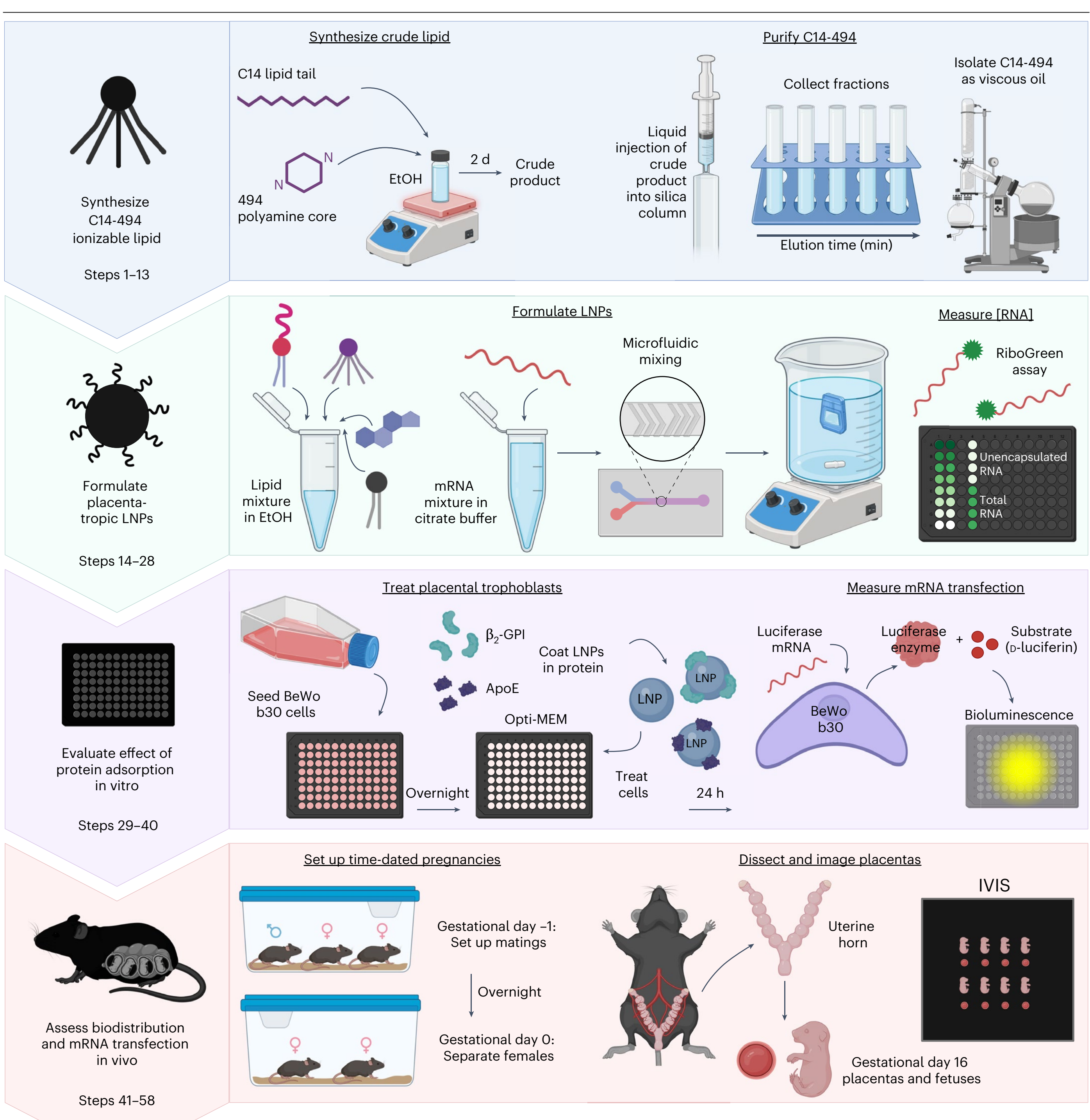
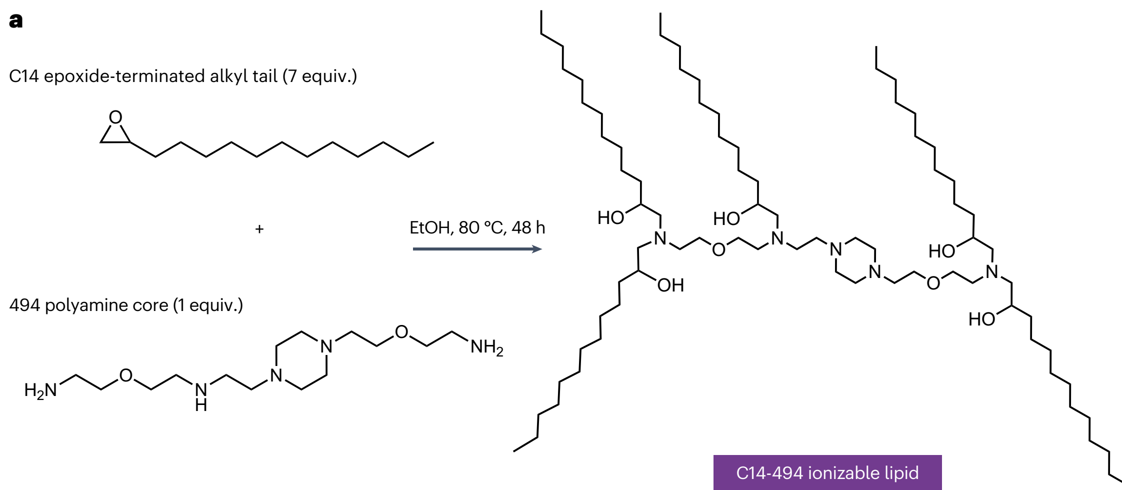


Fig. 2 | Overview of the Protocol for formulating and evaluating placenta-tropic mRNA LNPs. Before formulating placenta-tropic LNPs, the C14-494 ionizable lipid was synthesized via S_N2 reaction chemistry and purified via flash chromatography (Steps 1–13). The purified lipid was then used to formulate placenta-tropic LNPs by combining a lipid mixture in ethanol (EtOH) and an mRNA mixture in citrate buffer via chaotic mixing in microfluidic channels with staggered herringbone features. After dialysis, the concentration of encapsulated mRNA was measured using a RiboGreen assay (Steps 14–28). Placenta-tropic LNPs encapsulating luciferase mRNA were used to assess the effect of protein (i.e., β_2-GPI and apolipoprotein E) adsorption on mRNA transfection to BeWo b30 placental trophoblasts (Steps 29–40). Finally, in vivo LNP biodistribution and luciferase mRNA transfection was assessed in time-dated pregnant mice using an in vivo imaging system (IVIS) (Steps 41–58). The illustrations were created in BioRender. Swingle, K. (2026) https://BioRender.com/nan25of.

a



b

LNP formulation			
Lipid	Structure and function	Molar ratio	Molar percent
C14-494	Multi-tailed ionizable lipid that adopts a positive charge in the acidic compartment of the endosome, enabling endosomal escape and intracellular nucleic acid delivery	35	32.41
DOPE	Zwitterionic phospholipid with unsaturated lipid tails that promotes fusion with the endosomal membrane by forming the inverted hexagonal phase (H_{II})	24	22.22
Cholesterol	Steroid excipient with a tetracyclic structure that facilitates LNP stability and rigidity	46.5	43.06
C14-PEG_{2000}	Hydrophilic poly(ethylene glycol) polymer conjugated to a lipid tail for limiting serum protein adsorption and immune system–mediated opsonization for a prolonged circulation time	2.5	2.31

c

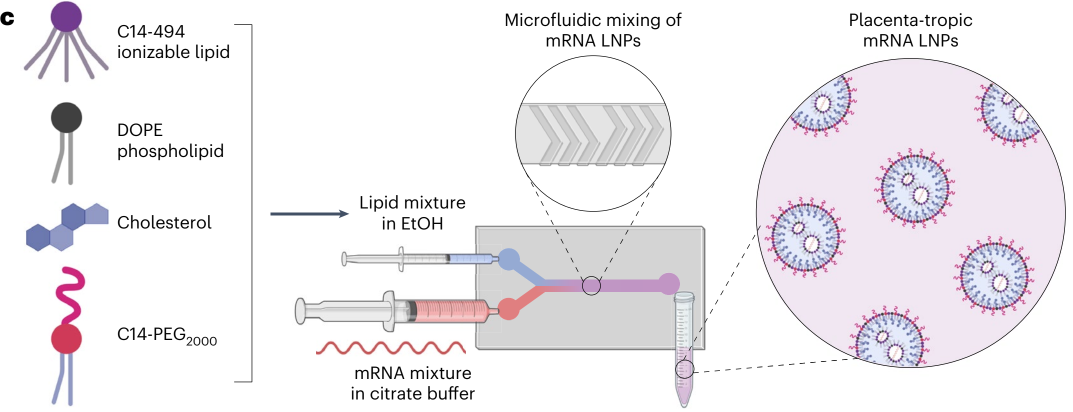


Fig. 3 | Preparing placenta-tropic mRNA LNPs with the C14-494 ionizable lipid. a, C14-494 is a multi-tailed ionizable lipid with a piperazine-based polyamine core that contains multiple ether linkages. It is synthesized via simple and fast S_N2 reaction chemistry, whereby seven molar equivalents (equiv.) of the C14 epoxide-terminated alkyl tail are combined with one molar equivalent of the 494 polyamine core in EtOH for 2 d. **b**, The purified C14-494 ionizable lipid is then combined with the remaining LNP excipients including the phospholipid DOPE, cholesterol and lipid-PEG at molar ratios of 35:24:46.5:2.5. Each of these lipids plays a unique role in the structure and function of the placenta-tropic mRNA LNPs. **c**, To formulate mRNA LNPs, chaotic mixing between the lipid and mRNA mixtures is induced in a microfluidic device fabricated with staggered herringbone features. The illustrations in **c** were created in BioRender. Swingle, K. (2026) https://BioRender.com/se6he1l.

birth, placenta accreta spectrum disorders and gestational trophoblastic disease. Because of the modular nature of LNPs, the nucleic acid payload—here, luciferase mRNA—can be readily switched to other therapeutic cargoes including VEGF mRNA[29], placental growth factor mRNA[31], interleukin-4 mRNA[33] or soluble Fms-like tyrosine kinase-1 siRNA[39], all of which have been explored in the context of therapeutic drug delivery to the placenta.

In this Protocol, we describe the preparation of protein-coated LNPs to evaluate the effect of β_2-GPI or ApoE adsorption on in vitro luciferase mRNA delivery to BeWo b30 placental trophoblasts. We envision that this section of the Protocol will have broad applications in the field of LNP-mediated nucleic acid delivery, particularly with the growing interest in exploring LNP structure-function relationships with respect to protein corona formation[29,36,40,41]. For example, depending on the intended administration route for the LNP therapeutic, this section can be readily adapted to explore the effect of various proteins from biological fluids such as serum (intravenous administration), peritoneal fluid (intraperitoneal administration), interstitial fluid (intramuscular or subcutaneous administration) or lung surfactants and mucus (inhalation). Similarly, our group and others[29,36] have used versions of this Protocol to assess the effect of protein adsorption on mRNA transfection in a range of cell types beyond placental trophoblasts, including liver hepatocytes, splenic macrophages, lung epithelial cells and circulating immune cells such as T and B cells.

Finally, we outline here the steps by which researchers can work with and isolate reproductive tissues from time-dated pregnant mice in their laboratory, aiming to expand preclinical investigations in pregnant rodents across the fields of science, engineering and medicine. For novel drug and therapeutic technologies, only a small proportion of literature reports biodistribution or pharmacokinetic data in pregnant rodents before advancing the technology along the preclinical-to-clinical pipeline toward larger animals and humans. To address this gap in research, we describe key logistical and experimental details for working with time-dated pregnant mice in an effort to increase the feasibility and accessibility for working with pregnant animals in the context of novel drug, biomaterial or gene modulation technologies.

Comparison with other methods

Here, we describe the preparation of placenta-tropic LNPs formulated with C14-494, a novel, multi-tailed ionizable lipid with several ether and hydroxyl moieties (Fig. 3a). This LNP was identified via high-throughput in vivo screening from a library of 98 formulations and has demonstrated potent mRNA delivery to the placenta with applications in treating pre-eclampsia[29]. Note that besides the synthesis and evaluation of novel ionizable lipid structures, other LNP optimization approaches have also been used for achieving LNP delivery to the placenta.

First, several studies report promising results using design-of-experiments approaches to alter the relative amounts of the traditional four components (i.e., ionizable lipid, phospholipid, cholesterol and lipid-PEG) in the LNP formulation[27,31]. By virtue of using design-of-experiments minimization approaches, these studies have screened large parameter spaces with small, representative LNP libraries and aimed to establish structure-function relationships between the LNP formulation and delivery to the placenta. Although these insights could be applied here with the potential to further improve tropism to the placenta, extrapolating structure-function relationships is complicated, often limiting their broad application for different platform technologies. Alternatively, active targeting strategies have also been used to promote delivery to the placenta, both in the context of LNPs[28] and other drug-delivery technologies[42–45]. In these studies, the nanoparticle surface is functionalized with active targeting moieties such as monoclonal antibodies[28] or peptides[42–45] to promote delivery to a particular cell type of interest in the placenta. For example, groups might be interested in further optimization of the placenta-tropic LNP formulation described in this Protocol by exploring various active targeting strategies to further promote placental tropism.

Besides LNP-mediated nucleic acid delivery to the placenta, there is a large body of literature exploring other technologies for the delivery of various payloads to the placenta. Althouogh LNPs have emerged as the most clinically advanced technology for the non-viral delivery of nucleic acids such as mRNA, other lipid-based[42,45–47] as well as polymeric[43,44,48,49] and inorganic[50] nanoparticle platforms have been explored for delivery to the placenta as well. These delivery technologies might enable more effective delivery of alternative therapeutic payloads such as small-molecule drugs, proteins and antibodies to the placenta than the placenta-tropic LNP described here.

Limitations

Although this Protocol is the first to describe the preparation of placenta-tropic LNPs, it is also associated with several limitations, including some of those described elsewhere that are universal to the preparation of LNPs via microfluidic mixing methods[9,51]. Microfluidic mixing methods offer the potential for a high degree of consistency between batches in terms of physicochemical properties, yet the equipment, consumables and dead volumes associated with preparation can be cost prohibitive for some researchers and settings. Here, we suggest using each NanoAssemblr Ignite cartridge only once, discarding it after use to limit fouling and clogging in the microfluidic channels in an effort to avoid material loss and inconsistent formulation results. However, this further exacerbates the consumables costs associated with completing the steps outlined in this Protocol.

Besides the limitations associated with microfluidic mixing methods, we describe here steps to evaluate LNP biodistribution in pregnant mice by labeling placenta-tropic LNPs with the lipophilic fluorescent dye DiD. This technique offers certain advantages in that the fluorescent dye is relatively cheap, and the steps are simple to implement because the dye is added to the LNP solution after formulation. Alternative lipophilic dyes include DiO (3,3′-dioctadecyloxacarbocyanine perchlorate), DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) and DiR (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide), which each have unique fluorescence spectra. DiO and DiI have blue-to-green peak emission wavelengths that may result in lower signal-to-noise ratios because of mouse tissue-derived autofluorescence; DiR has a far-red peak emission wavelength that could be used to further improve detection of LNP delivery from background autofluorescence. Regardless of the choice of dye, this method is also associated with limitations, including the fact that biodistribution of the LNP itself is assessed rather than biodistribution of the mRNA payload, which may be preferred in some applications[36,51]. However, fluorescently labeled reporter mRNA cargoes are often prohibitively expensive, given the quantity required for in vivo experiments.

Finally, species-specific differences in placental structure might limit the application of this Protocol beyond rodents toward larger animal models of pregnancy[52,53]. Fortunately, rodents (including mice, as described here), guinea pigs, rabbits, non-human primates and humans all have hemochorial placentas, the most invasive placenta type, characterized by the direct exposure of trophoblast-lined chorionic villi to maternal blood[52,53]. However, humans have hemomonochorial placentas—with a single layer of trophoblast present in the villi—whereas rodents have hemotrichorial placentas with three layers of trophoblasts[52,53]. To date, we have used only pregnant mouse models to evaluate the placenta-tropic mRNA LNPs described in this Protocol, and their adaptation for larger animal models, particularly considering their unique placental structure, has yet to be explored.

Experimental design

Synthesis and purification of the C14-494 ionizable lipid (Steps 1–13)

The design and synthesis of increasingly potent ionizable lipids for nucleic acid delivery with limited cellular toxicity has been a central focus for the LNP field over the last several years[54,55]. These ionizable lipids offer distinct advantages over permanently cationic lipids or polymer excipients in that they adopt a positive charge only in acidic environments; they are neutral at physiological pH. In other words, these lipids become protonated or ionized in the acidic compartment of the endosome, enabling fusion with and disruption of the anionic endosomal membrane for nucleic acid delivery to the cell cytosol[6].

Structurally, ionizable lipids generally possess an amine-containing headgroup or core, linker regions and hydrophobic tails. Groups have explored many structural modifications to this general structure, including the number of ionizable amine moieties[7,15,56], the incorporation of biodegradable linker regions[57,58] and the degree of branching[8,59] of the lipid tails. In this Protocol, we describe the synthesis of C14-494: a multi-tailed ionizable lipid with a single piperazine motif and multiple ether and hydroxyl moieties (Fig. 3a). Multi-tailed ionizable lipids offer the potential for improved endosomal escape compared to their two-tailed counterparts because of the increased cross-sectional area of the tail region[54]. Although in the context of delivery to the placenta, it is essential that there is a balance between potency and toxicity.

In pursuit of this balance, we explored a library of lipids containing piperazine motifs—these moieties are found in several FDA-approved small-molecule drugs and used widely in medicinal chemistry[15,29], suggesting their tolerability in the body.

To promote the proportion of products that are fully substituted (five tails), we perform the S_N2 reaction for ionizable lipid synthesis with a molar excess of alkyl epoxide (7:1 ratio of tail:polyamine core) (Supplementary Data 1). Synthesis of the crude lipid product is performed at 80 °C under gentle stirring for 2 d. We describe here crude C14-494 synthesis on the ~1-g scale, although these steps can be adapted for smaller- or larger-scale synthesis as needed.

Preparation of placenta-tropic mRNA LNPs (Steps 14–28)

The placenta-tropic mRNA LNP described in this Protocol is prepared via microfluidic mixing of a lipid mixture in ethanol and an mRNA mixture in citrate buffer. The C14-494 ionizable lipid, DOPE phospholipid, cholesterol and lipid-PEG are combined in ethanol at molar ratios of 35:24:46.5:2.5[29] (Fig. 3b). This particular excipient composition was identified from a large library of LNP formulations, all of which introduced small alterations to the standard 35:16:46.5:2.5 molar ratios that have been previously optimized for mRNA delivery[60]. Similarly, LNPs are prepared by assuming a 10:1 (wt/wt) ratio of ionizable lipid to mRNA. Although siRNA LNPs are traditionally formulated by assuming a 5:1 (wt/wt) ratio, the higher ratio used here was determined through optimization approaches to enable potent delivery of a larger payload such as mRNA[60]. The LNP formulations used by the Pfizer/BioNTech and Moderna COVID-19 mRNA vaccines suggest that weight ratios as high as 20:1 can be used for mRNA delivery, although the impact of potential lipid-mediated immunogenicity should be carefully considered for each application.

For the mRNA mixture, citrate buffer (pH 3.0) is used to promote the charge-based formation of multilamellar lipoplexes between the negatively charged mRNA and the protonated ionizable lipid during microfluidic mixing[6] (Fig. 3c). Through the dialysis process, whereby the pH is increased to 7.4, the ionizable lipid returns to its neutral form and phase separates to form LNPs[6]. Both the microfluidic mixing and dialysis steps are essential for the preparation of mRNA LNPs with the typical multilamellar morphology and amorphous hydrophobic core. The dialysis process also serves to remove free lipid, although unencapsulated mRNA molecules are too large to pass through the 20-kDa pore sizes in the dialysis membrane. Therefore, the Quant-it RiboGreen reagent and RNA assay kit is used to determine the concentration of encapsulated mRNA in the LNP sample. This is accomplished by measuring both the total mRNA concentration in lysed LNP samples and the unencapsulated mRNA concentration in intact LNP samples.

Measuring the concentration of encapsulated mRNA is essential before proceeding to the remaining sections of the procedure. In addition, if equipment access allows, groups may wish to perform additional physicochemical characterization assays, including dynamic light scattering to measure LNP diameter and polydispersity as well as zeta potential measurements to determine LNP surface charge. Detailed procedures for these assays can be found in previously published protocol articles[51], and representative characterization data for the placenta-tropic mRNA LNPs described here can be found in Supplementary Table 1.

Readers are encouraged to use RNase-free materials and supplies and to maintain an RNase-free work surface during LNP formulation, dialysis and characterization. Because RNases are notoriously stable enzymes that can regain functionality after exposure to organic solvents, it is important to limit RNase contamination from the onset by using RNase surface decontaminant as well as RNase-free buffers and consumables. After mRNA LNPs are prepared and stored in an RNase-free microcentrifuge tube (Step 28), it is best for all subsequent handling (i.e., for in vitro and in vivo assays) to be RNase free as well. Procedures for the synthesis and purification of the C14-494 ionizable lipid are not explicitly written to occur in an RNase-free environment for practical considerations, but readers should also make every effort to avoid RNase contamination during these steps.

Evaluating the effect of protein adsorption on in vitro mRNA transfection to placental trophoblasts (Steps 29–40)

Given that mRNA LNPs are immediately exposed to serum proteins after intravenous administration (Fig. 1a), we sought to explore the role of protein adsorption on in vitro mRNA

transfection in placental trophoblasts. We were inspired to pursue this mechanistic work by the strong spleen transfection that we have observed concurrently with placenta tropism[26,29]. Although the understanding of protein corona formation on placenta-tropic LNPs has only recently evolved, groups have more thoroughly investigated protein adsorption on liver-tropic and spleen-tropic LNPs[36,40,61]. It is well understood that traditional LNP formulations will accumulate in the liver and facilitate nucleic acid delivery to hepatocytes after intravenous administration; delivery is achieved via interactions between adsorbed ApoE on the LNP surface and the low-density lipoprotein receptor on hepatocytes[61]. More recently, a study has characterized the protein corona for spleen-tropic LNPs and identified β_2-GPI as the protein with the greatest abundance in the corona[36].

Because strong spleen tropism is observed with the placenta-tropic mRNA LNP described here, we then explored the potential for β_2-GPI to facilitate tropism to the placenta. β_2-GPI plays an important role in modulating coagulating and complement activation for the developing placenta[62,63] (Fig. 1b). In the chorionic villi of the placenta, cytotrophoblast cells fuse to form a multinucleated syncytiotrophoblast layer that forms the barrier between maternal and fetal circulation. During this syncytialization process, the anionic phospholipid phosphatidyl serine (PS) is externalized from the inner leaflet of the cell membrane to the outer leaflet[64,65]. The syncytiotrophoblast layer synthesizes and secretes β_2-GPI, which binds to the externalized PS on trophoblast cells via a highly cationic amino acid region in β_2-GPI[63,66]. Therefore, we evaluated β_2-GPI adsorption on placenta-tropic LNPs, hypothesizing that it could play a role in facilitating mRNA delivery to trophoblasts[29].

Here, we describe steps to prepare protein-coated placenta-tropic LNPs to evaluate the effect of protein adsorption on in vitro luciferase mRNA transfection to placental trophoblasts (Fig. 4a). BeWo b30 cells are seeded in 96-well plates and allowed to adhere overnight; on the following day, LNPs are incubated with increasing amounts of ApoE or β_2-GPI at 37 °C for 15 min. Before transfecting the seeded BeWo b30 cells with LNPs, the culture medium in the 96-well plate is replaced with Opti-MEM medium to eliminate the effects of FBS proteins present in the culture medium. For both uncoated and protein-coated mRNA LNP formulations, BeWo b30 cells are treated with 20 ng of encapsulated mRNA, and luciferase expression is evaluated 24 h later by measuring luminescence on a microplate reader. This Protocol can be readily adapted for other LNP formulations, proteins of interest or in vitro models. For example, readers may wish to fluorescently label the LNP itself or the mRNA payload or encapsulate a fluorescent protein-encoding nucleic acid to assess the effect of protein adsorption on cell-specific uptake and transfection. For these assays, groups may refer to previously published protocols that include detailed descriptions of procedures used to evaluate mRNA LNPs in vitro using flow cytometry and confocal microscopy[51]. Data from similar experiments for the placenta-tropic mRNA LNP formulation described here can be found in our associated research article[29].

In vivo mRNA transfection and LNP biodistribution in pregnant mice (Steps 41–58)

To evaluate mRNA transfection and LNP biodistribution, we first describe the procedure for setting up matings to achieve time-dated pregnant mice. Because gestation is only ~19 d in C57BL6 mice, there are substantial changes in both placental and fetal development across each gestational day. Therefore, we recommend using time-dated pregnant mice in which the exact gestational age is known for assessing mRNA transfection and LNP biodistribution. Here, we use C57BL6 mice, although other strains of time-dated pregnant mice (e.g., BALB/c and CD-1[31,32]) can also be used, which may affect the expected litter size and total gestational period. We provide instructions to check the females for the presence of a vaginal plug after mating (Fig. 5a), although this step is nonessential; the presence of a vaginal plug indicates that mating has occurred but does not guarantee pregnancy. Rather, females with a vaginal plug can be separated or similarly marked to visually confirm pregnancy on or around gestational day 11.

Here, we describe the evaluation of mRNA transfection and LNP biodistribution on gestational day 16 in at least four independent animals (biological replicates), although this Protocol can be readily adapted to assess delivery as a function of gestational age. In our experience and consistent with other protocols[67], clean dissection of the mouse placenta is possible beginning around gestational day 11. We have found that mRNA-induced protein

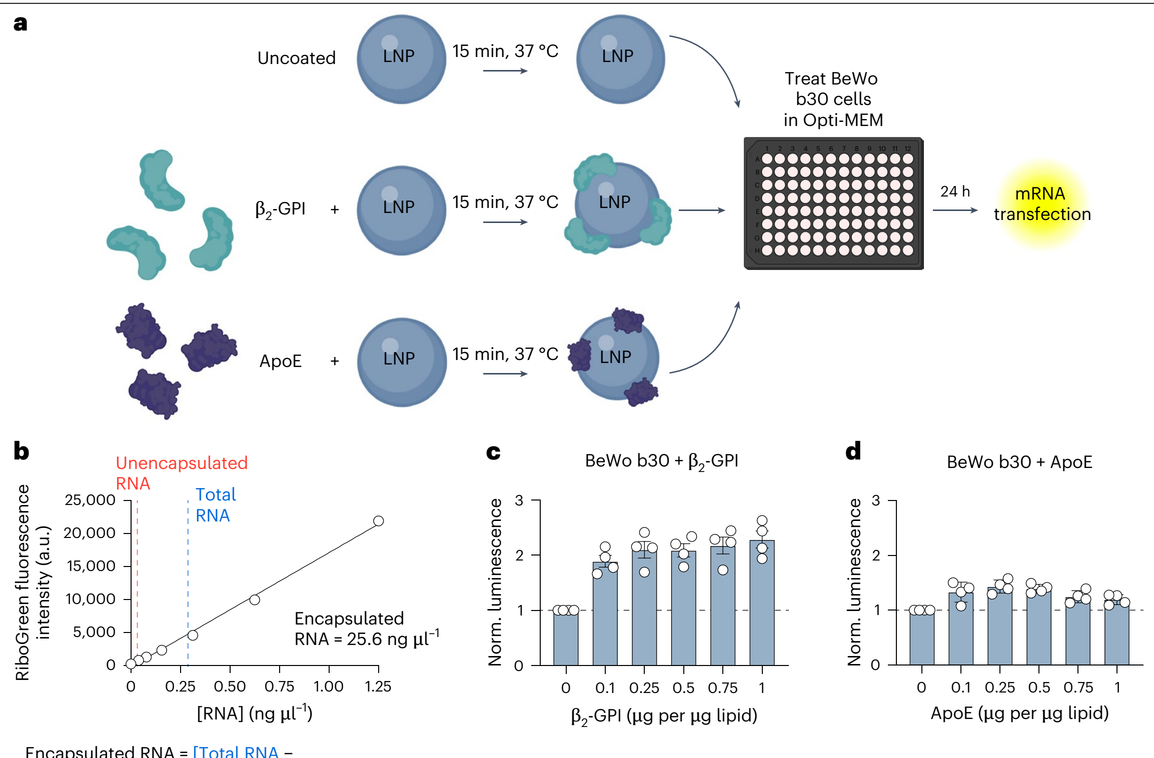


Fig. 4 | Effect of protein adsorption on mRNA transfection to placental trophoblasts. a, Placenta-tropic LNPs encapsulating luciferase mRNA were incubated with proteins (i.e., $β_2$-GPI and ApoE) for 15 min to generate protein-coated LNPs to assess the effect of protein adsorption on mRNA transfection to BeWo b30 placental trophoblasts. Before treatment, culture medium in the well plate was replaced with Opti-MEM reduced serum medium to eliminate the effects of FBS from culture medium on LNP delivery. **b**, Across all amounts of protein tested, cells were treated with 20 ng of encapsulated luciferase mRNA; encapsulated mRNA was estimated by comparison with a standard curve of RNA standard fitted by using univariate least-squares linear regression. **c**,**d**, 24 h after LNP treatment, mRNA transfection to BeWo b30 cells was evaluated across increasing amounts of $β_2$-GPI (**c**) and ApoE (**d**). Normalized (Norm.) luminescence is reported as mean ± s.e.m. (n = 4 biological replicates with 4 technical replicates each). The illustrations in **a** were created in BioRender. Swingle, K. (2026) https://BioRender.com/hb4r031.

production peaks between 6 and 18 h after administration and can easily be detected via IVIS, flow cytometry or immunofluorescence at these time points. If, after dissection, in vivo imaging is the main outcome, mRNA LNPs can be administered to pregnant mice in the morning on gestational day 16, with dissections and in vivo imaging performed in the afternoon. If, after dissection, additional post-processing of the placentas or other tissues (i.e., for flow cytometry[68] or histology) is required, we recommend a 12-h time point, with mRNA LNP administration in the evening on gestational day 15.5, dissections in the morning on gestational day 16, followed by additional post-processing steps. In a related article by our group, we provide an in-depth protocol for the flow cytometric analysis of the murine placenta to assess nanoparticle delivery to placental trophoblasts, endothelial cells and immune cells[68]. For assessing cell-specific delivery of placenta-tropic LNPs encapsulating mRNA constructs encoding fluorescent proteins (e.g., GFP or mCherry mRNA), readers are encouraged to review this article. However, given that many of the intended therapeutic nucleic acid payloads for these placenta-tropic LNPs would probably be secreted proteins (i.e., VEGF, placental growth factor or interleukin-4 mRNA), evaluating transfection on a whole-tissue level by using luciferase mRNA and IVIS might be sufficient.

Expertise needed to implement the Protocol

Researchers with experience in bioengineering, nanotechnology, biomedical sciences, biology, chemistry and pharmaceutical sciences should be able to perform the experiments outlined in

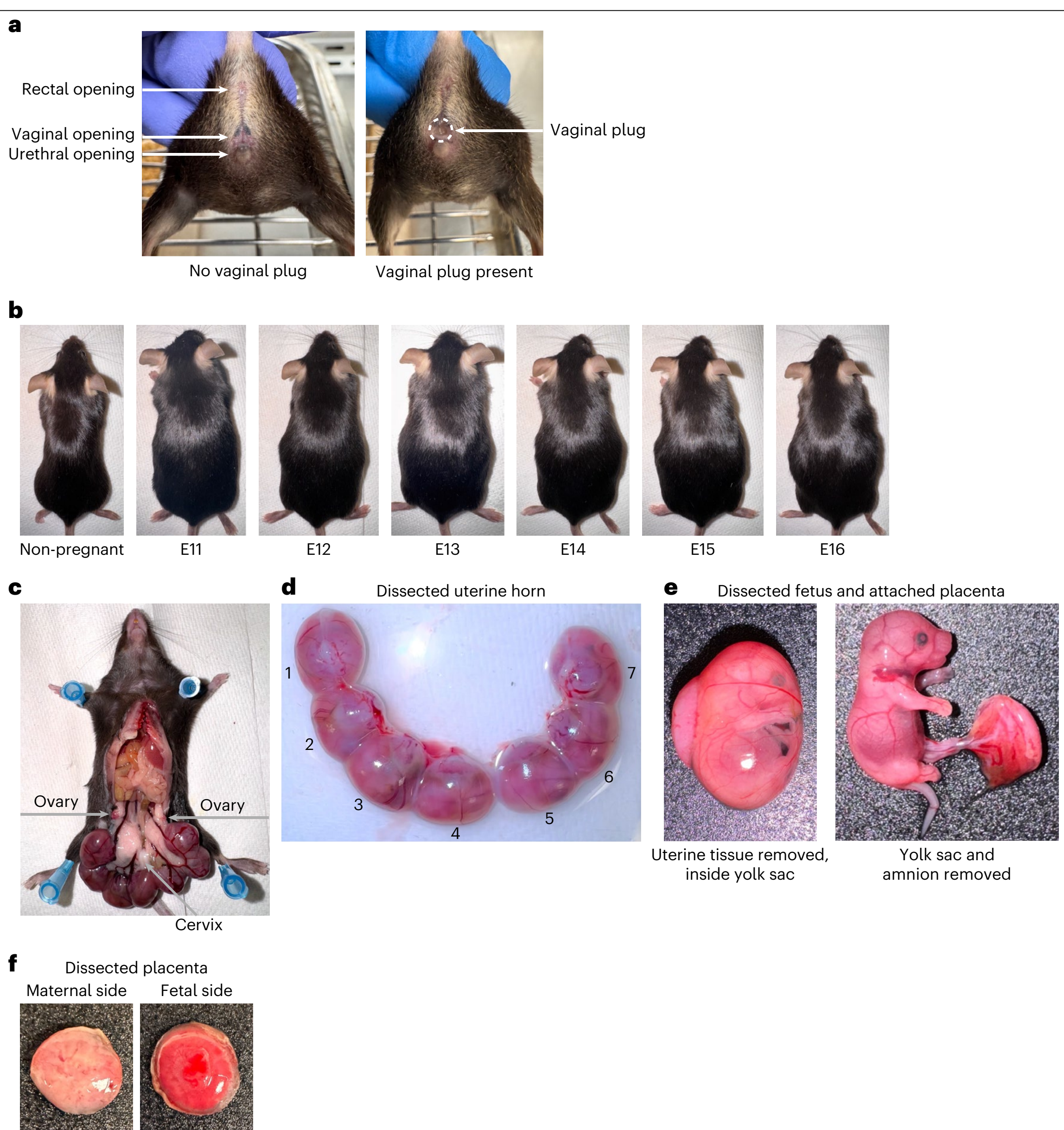


Fig. 5 | Preparation of time-dated pregnancies and dissection of gestational day 16 placentas and fetuses. a, Male and female C57BL6 mice ≥10 weeks of age were housed together overnight to generate time-dated pregnant mice. On the following day (gestational day 0, E0), males and females were separated, whereby females could be lifted by the base of the tail to examine the vaginal opening for the presence of a clear-to-white, mucus-like plug. The presence of a vaginal plug indicates that mating has occurred but does not guarantee pregnancy. **b**, Beginning on or around gestational day 11, pregnancy status can be confirmed visually by the presence of a rounded, pear-shaped abdomen. **c**, After administration of mRNA LNPs, mice were euthanized to enable dissection of the uterine horn and enclosed fetuses and placentas to assess tissue-specific LNP biodistribution and mRNA transfection. The uterine horn can be dissected proximal to the ovaries on both sides of the mouse and at the cervix near the base of the tail. **d**, Excess tissue can be removed from the uterine horn to identify the number of individual yolk sacs that are present—here, seven. **e**, Fetuses and their attached placentas were then dissected from the uterine tissue (left) and subsequently removed from the yolk sac and amnion (right). **f**, Finally, placentas were dissected by cutting the umbilical cord and were subsequently imaged with either the maternal or fetal side facing up by using an IVIS.

this Protocol. In part 1, organic chemistry researchers should be able to perform the synthesis and purification of the C14-494 ionizable lipid if the required chemical fume hoods and equipment are not accessible in a biological lab. Part 4 of this procedure necessitates working

with an animal husbandry facility and access to an IVIS instrument. Researchers should consider working in small teams of two to four people to complete the dissections and IVIS imaging that is described in part 4 of the procedure in a timely manner.

Materials

Biological materials

- C57BL6 mice (male and female, 10–12 weeks old) were purchased from Jackson Laboratory and housed separately in the Clinical Research Building Animal Facility at the University of Pennsylvania. Mice were housed in a vivarium with a 12-h light-dark cycle and were provided with food and water ad libitum. Vivarium temperature and humidity were maintained between 68 °F and 76 °F and 30% and 70%, respectively
  ▲ **CAUTION** Any experiments involving live animals must conform to relevant institutional and national regulations. All animal use was in accordance with the guidelines of and approval from the University of Pennsylvania's Institutional Animal Care and Use Committee.
- BeWo b30 cells[69] (RRID: CVCL_LB83) were provided by D. Huh at the University of Pennsylvania with permission from A. Schwartz at Washington University School of Medicine in St. Louis. The original BeWo cell line is available from the American Type Culture Collection (cat. no. CCL-98), although it lacks the monolayer-forming ability associated with the b30 clone
  ▲ **CAUTION** The cell lines used in research should be regularly checked to ensure that they are authentic and are not infected with mycoplasma. BeWo b30 cells tested negative for mycoplasma at the University of Pennsylvania Cell Center. For authentication purposes, cell morphology was checked at every subculture to ensure that they were free from contamination.

Reagents

- 494 (2-{2-[4-(2-{[2-(2-aminoethoxy)ethyl]amino}ethyl)piperazin-1-yl]ethoxy}ethan-1-amine) (Enamine, cat. no. EN300-1472331)
- Acetonitrile (Millipore Sigma, cat. no. 1000291000)
  ▲ **CAUTION** Acetonitrile is highly flammable and hazardous to human health. Use it in a chemical fume hood and wear appropriate personal protective equipment (PPE) including eye goggles, laboratory coat and gloves. Dispose of acetonitrile appropriately after use.
- Ammonium hydroxide (ThermoFisher Scientific, cat. no. 460801000)
  ▲ **CAUTION** Ammonium hydroxide is corrosive and hazardous to human health. Use it in a chemical fume hood and wear appropriate PPE. Dispose of ammonium hydroxide appropriately after use.
- C14 (1,2-epoxytetradecane) (TCI, cat. no. E0314)
- C14-PEG_{2000} (Avanti Polar Lipids, cat. no. 880151P-1g)
  ▲ **CRITICAL** Divide into ~5-mg aliquots and store under nitrogen at −20 °C to avoid degradation.
- Cholesterol (Millipore Sigma, cat. no. C8667-500MG)
- Citrate buffer, 100 mM, pH 3.0 (Teknova, cat. no. Q2446)
- CO_2 cylinders (supplied by AirGas)
- Dichloromethane (DCM; ThermoFisher Scientific, cat. no. 610050040)
  ▲ **CAUTION** DCM is hazardous to human health and a highly volatile chemical. Use it in a chemical fume hood and wear appropriate PPE including eye goggles, a laboratory coat and gloves. Dispose of DCM appropriately after use.
- $CDCl_3$ (deuterated chloroform) (ThermoFisher Scientific, cat. no. AC166251000)
- D-Luciferin potassium salt (Revvity, cat. no. 122799)

- DOPE (Avanti Polar Lipids, cat. no. 850725P-25mg)
  ▲ **CRITICAL** Divide into ~5-mg aliquots and store under nitrogen at −20 °C to avoid degradation.
- DMEM, 1 l, high glucose (ThermoFisher Scientific, cat. no. 11965084)
- Ethanol (EtOH), 200 proof, 0.125 gallons (ThermoFisher Scientific, cat. no. 07-678-007)
  ▲ **CAUTION** Ethanol is highly flammable and hazardous to human health. Wear appropriate PPE and dispose of ethanol appropriately after use.
- FBS, 500 ml (Corning, cat. no. 35-010-CV)
  ▲ **CRITICAL** Divide into 50-ml aliquots and store at −20 °C to avoid freeze-thaw cycles.
- Luciferase assay system, 1,000 assays (Promega, cat. no. E4550)
- Luciferase mRNA, CleanCap N1-methylpseudouridine (TriLink, cat. no. L-8102-1000)
  ▲ **CRITICAL** Divide into 100-µg aliquots and store at −80 °C to avoid degradation and freeze-thaw cycles.
- Methanol (ThermoFisher Scientific, cat. no. 325740025)
  ▲ **CAUTION** Methanol is highly flammable and hazardous to human health. Use it in a chemical fume hood and wear appropriate PPE. Dispose of methanol appropriately after use.
- N_2 cylinders (supplied by AirGas)
- Opti-MEM reduced serum medium (ThermoFisher Scientific, cat. no. 31985070)
- Penicillin-streptomycin, 10,000 U ml^{-1} (ThermoFisher Scientific, cat. no. 15140122)
- PBS, 10×, RNase free, pH 7.4 (ThermoFisher Scientific, cat. no. AM9625)
- Quant-iT RiboGreen reagent and RNA assay kit (ThermoFisher Scientific, cat. no. R11490)
- Recombinant human ApoE (Sino Biological, cat. no. 10817-H30E)
  ▲ **CRITICAL** Divide into ~20-µl aliquots and store at −20 °C to avoid freeze-thaw cycles.
- Recombinant mouse β_2-GPI (apolipoprotein H) (BioLegend, cat. no. 784906)
  ▲ **CRITICAL** Divide into ~20-µl aliquots and store at −20 °C to avoid freeze-thaw cycles.
- Reporter lysis 5× buffer (Promega, cat. no. E3971)
- RNase AWAY surface decontaminant (ThermoFisher Scientific, cat. no. 7002)
- Trifluoroacetic acid (Millipore Sigma, cat. no. 80457-10ML)
  ▲ **CAUTION** Trifluoroacetic acid is hazardous to human health and a highly volatile and corrosive chemical. Use it in a chemical fume hood and wear appropriate PPE. Dispose of trifluoroacetic acid appropriately after use.
- Triton X-100 (Millipore Sigma, cat. no. X100-5ML)
- Trypan blue solution, 0.4% (vol/vol) (ThermoFisher Scientific, cat. no. 15250061)
- Trypsin-EDTA, 0.25% (vol/vol) (ThermoFisher Scientific, cat. no. 25-200-056)
- Ultrapure water (supplied by the University of Pennsylvania)
- Vybrant DiD solution (ThermoFisher Scientific, cat. no. V22887)

Equipment

- 24-well clear microplates (Corning, cat. no. 3524)
- 96-well black-walled, clear-bottom microplates (Corning, cat. no. 3340)
- Acquity ultra-performance liquid chromatography ethylene bridged hybrid C8 column, 1.7 µm, 2.1 mm × 50 mm (Waters Corporation, cat. no. 186002877)
- Aluminum foil (ThermoFisher Scientific, cat. no. 01-213-100)
- Analytical balance (e.g., Mettler Toledo, LA104E)
- Beaker, 4 l (Corning, cat. no. 1000-4L)
- Biosafety cabinet (e.g., ThermoFisher Scientific, 1300 Series Class II Type A2)
- Cell counter (e.g., Invitrogen, Countess 3 automated cell counter)
- Centrifuge with 15-ml tube adapter
- Chemical fume hoods
- Clamp for rotary evaporator, 19/22 joint (ThermoFisher Scientific, cat. no. CG14504)
- CO_2 euthanasia system
- CombiFlash NextGen 300+ equipped with evaporative light scattering detection (Teledyne Isco)

- Conical tubes, 15 ml (ThermoFisher Scientific, cat. no. 14-959-49B)
- Dissection forceps (e.g., ThermoFisher Scientific, cat. no. 08-875)
- Dissection scissors (e.g., ThermoFisher Scientific, cat. no. 08-951-5)
- Dropper bulbs (ThermoFisher Scientific, cat. no. 14-127-516)
- Freezer (−20 °C and −80 °C)
- Glass vials with caps, 1 dram (ThermoFisher Scientific, cat. no. 14-955-327)
- Glass vials with caps, 20 ml (ThermoFisher Scientific, cat. no. 03-337-7)
- Glass vial with pre-slit screw cap, 2 ml (ThermoFisher Scientific, cat. no. 6AK95W)
- Ice bucket (Corning, cat. no. 432129)
- Ice maker
- Incubator (e.g., ThermoFisher Scientific Heracell VIOS 160i CO_2 incubator)
- Insulin syringes, 0.5 ml (ThermoFisher Scientific, cat. no. 14-841-32)
- Inverted light microscope (e.g., Zeiss Primovert)
- IVIS (e.g., Perkin Elmer Xenogen IVIS-200 imager)
- Living Image software (Revvity)
- Low-fluorescence IVIS mat (Revvity, cat. no. 117839)
- Magnetic stir bar (Millipore Sigma, cat. no. HS120547-10EA)
- Magnetic stirring hot plate (e.g., ThermoFisher Scientific, cat. no. SP88857100)
- Marker, chemical resistant (e.g., ThermoFisher Scientific, cat. no. 22-026-700)
- Marker, water resistant (e.g., Sharpie)
- Mnova software (Mestrelab Research)
- Microcentrifuge tubes, 1.5 ml, RNase free (ThermoFisher Scientific, cat. no. 05-408-129)
- Microcentrifuge tubes, 2 ml, RNase free (ThermoFisher Scientific, cat. no. 02-681-332)
- Microplate reader (e.g., Tecan Infinite 200 Pro)
- Microplate shaker (e.g., ThermoFisher Scientific, cat. no. 88882005)
- Microspatula (ThermoFisher Scientific, cat. no. 21-401-25A)
- Mini centrifuge with a PCR eight-strip tube rotor (e.g., Ohaus, cat. no. 30134157)
- NanoAssemblr Ignite (Cytiva, cat. no. NIN0001)
- NanoAssemblr Ignite NxGen cartridges (Cytiva, cat. no. NIN0061)
- Needles, 25-gauge × 25 mm (ThermoFisher Scientific, cat. no. 14-821-13D)
- NMR spectrometer (Avance Neo Bruker 400-MHz spectrometer at the University of Pennsylvania Department of Chemistry NMR Facility)
- NMR tubes (e.g., ThermoFisher Scientific, cat. no. K8971930050)
- Parafilm (ThermoFisher Scientific, cat. no. 13-374-12)
- Pasteur pipettes, glass (ThermoFisher Scientific, cat. no. 13-67B-20B)
- PCR eight-tube strips with detached domed caps (BrandTech, cat. no. 781327)
- Petri dishes, 150-mm diameter (ThermoFisher Scientific, cat. no. FB0875714)
- Pipette tips, RNase free (e.g., Fisherbrand SureOne)
- Prism (GraphPad)
- RediSep gold silica gel disposable flash columns, 12 g (Teledyne Isco, cat. no. 692203345)
- Refrigerator (4 °C)
- Rotary evaporator (e.g., Buchi Rotovapor R-300 system professional)
- Rotary evaporator vial adapter kit (Millipore Sigma, cat. no. Z684546-1EA)
- Round-bottom disposable glass tubes, for CombiFlash tube racks (ThermoFisher Scientific, cat. no. 14-961-32)
- Round-bottom flask, 50 ml (ThermoFisher Scientific, cat. no. CG150689)
- Sectional block for stirring hot plate, for use with 15-mm vials (ThermoFisher Scientific, cat. no. 01-922-040)
- Serological pipette controller (e.g., Drummond Portable Pipet-Aid)
- Serological pipettes, sterile (ThermoFisher Scientific, cat. no. 13-678-11E)
- Slide-A-Lyzer G3 dialysis cassettes, 20-kDa molecular weight cutoff, 3 ml (ThermoFisher Scientific, cat. no. PIA52976)
- Slide-A-Lyzer 0.5–3-ml cassette float buoys (ThermoFisher Scientific, cat. no. 66430)
- Syringe filters, 0.22 µm, sterile (Genesee Scientific, cat. no. 25-243)

- Syringes with Luer-Lok tips, 1 ml, Becton Dickinson (ThermoFisher Scientific, cat. no. 14-823-30)
- Syringes with Luer-Lok tips, 3 ml, Becton Dickinson (ThermoFisher Scientific, cat. no. 14-823-435)
- Syringes with Luer-Lok tips, 10 ml, Becton Dickinson (ThermoFisher Scientific, cat. no. 14-823-16E)
- Tissue culture flask, 75 cm^2 (ThermoFisher Scientific, cat. no. 07-000-225)
- Ultracentrifugal filter, 100-kDa molecular weight cutoff, 4-ml sample volume (Millipore Sigma, cat. no. UFC810008)
- Ultrapure water-purification system (e.g., Millipore Sigma, Milli-Q EQ 7000)
- Variable-volume pipettes (e.g., Eppendorf Research Plus 0.1–10, 10–100 and 100–1,000 µl)
- Vacuum pump (e.g., Buchi vacuum pump V-300)
- Vortex (e.g., ThermoFisher Scientific, cat. no. 88882011)
- Water bath (e.g., PolyScience, cat. no. WBE10A11B)

Reagent setup

▲ **CRITICAL** Unless otherwise specified, the prepared reagents below can be stored at room temperature (20–25 °C) for several months.

Citrate buffer (10 mM, pH 6)
Dilute 100 mM citrate buffer 10-fold (vol/vol) with ultrapure water.

DMEM supplemented with 10% (vol/vol) FBS and 1% (vol/vol) penicillin-streptomycin
Combine 890 ml of DMEM, 100 ml of FBS and 10 ml of penicillin-streptomycin. The supplemented medium can be stored at 4 °C for ~1 month.

Luciferase assay substrate
Add the thawed luciferase assay buffer to the vial containing the luciferase assay substrate. Mix well to dissolve. Create 10-ml aliquots of the dissolved substrate in 15-ml conical tubes. Aliquots of luciferase assay substrate can be stored at −20 °C protected from light for several months.

Opti-MEM supplemented with 1% (vol/vol) penicillin-streptomycin
Combine 495 ml of Opti-MEM and 5 ml of penicillin-streptomycin. The supplemented medium can be stored at 4 °C for ~1 month.

PBS (1×, pH 7.4)
Dilute RNase-free 10× PBS 10-fold (vol/vol) with ultrapure water.

Reporter lysis buffer (1×)
Dilute the 5× reporter lysis buffer 5-fold (vol/vol) with 1× PBS.

Tris-EDTA (TE) buffer (1×)
Dilute the 20× TE buffer from the Quant-iT RiboGreen reagent and RNA assay kit 20-fold (vol/vol) with ultrapure water.

Triton X-100 (0.1% vol/vol) in TE buffer
In a 50-ml conical tube, add 49.5 ml of 1× TE buffer and 0.5 ml of Triton X-100. Mix well by inverting the tube to dissolve the detergent. Further dilute this solution 10-fold in 1× TE buffer to achieve 0.1% (vol/vol) Triton X-100.

Ultra solution
Prepare 2 l of ultra solution by combining 1,500 ml of DCM, 440 ml of methanol and 60 ml of ammonium hydroxide.

Procedure

Part 1: synthesis and purification of the C14-494 ionizable lipid

● **TIMING** 4 d

1. Add a magnetic stir bar to a 1-dram glass vial.
2. Using Pasteur pipettes, add 2-{2-[4-(2-{[2-(2-aminoethoxy)ethyl]amino}ethyl)piperazin-1-yl]ethoxy}ethan-1-amine (200 mg, 0.659 mmol, 1.0 equivalent) and 1,2-epoxytetradecane (979.8 mg, 4.614 mmol, 7.0 equivalents) to the vial (details on mass calculations are provided in Supplementary Data 1). Fill the vial with EtOH and seal with Parafilm.
   ▲ **CAUTION** EtOH is highly flammable and hazardous to human health. Wear appropriate PPE and dispose of EtOH appropriately after use.
3. Place the sealed vial on a magnetic stirring hot plate, dissolve the reagents with stirring and gradually increase the temperature to 80 °C. Allow the reaction mixture to stir for 48 h.
4. Remove the vial from the stirring hot plate and let it cool to room temperature. Using a Pasteur pipette, transfer the crude product in EtOH into a 50-ml round-bottom flask, leaving behind the magnetic stir bar. Dilute the crude product with 3 ml of DCM, rinsing the 1-dram vial to collect any remaining product before transferring to the round-bottom flask.
   ▲ **CAUTION** DCM is hazardous to human health and a highly volatile chemical. Use it in a chemical fume hood and wear appropriate PPE. Dispose of DCM appropriately after use.
5. Purify the crude product via flash chromatography using a CombiFlash. Load a 12-g RediSep Gold silica gel disposable flash column by inserting the column into the top of the column mount and lowering the injection valve while aligning the bottom of the RediSep column.
6. Set up the flash method, entering the correct 'Column' (Silica 12 g), 'Flow Rate' (7 ml min^{-1}), 'Run Length' (35 min) and 'Sample Name' (C14-494). The gradient of the mobile phase should be 95% DCM and 5% Ultra solution (solvent A, vol/vol) to 80% DCM and 20% Ultra solution (solvent B, vol/vol). Load the collection tube racks with test tubes. Select the correct 'Sample Loading' (Liquid) and indicate the correct collection rack and start tube.
7. Insert a 10-ml Luer-Lok syringe into the top of the column mount. Using a Pasteur pipette, transfer the diluted crude product from the 50-ml round-bottom flask to the 10-ml syringe. Inject the crude product into the column, waiting ~30 s before injecting an additional 3 ml of DCM to wash the sample onto the column. Start the separation.
8. After the separation has finished, analyze the chromatogram and identify the test tube fractions containing product. Because of their hydrophobicity, unreacted lipid tails will elute from the column first, followed by the purified C14-494 product around 20–30 min through the run.
9. To confirm the chemical identity of product in the test tube fractions, prepare each individually for analysis via LC–MS. For each test tube fraction, prepare a 2-ml glass vial with a pre-slit screw cap filled with 995 µl of 200 proof EtOH. Add 5 µl of sample to each glass vial. Acquire mass accuracy LC–MS data by injecting sample into an ultra-performance liquid chromatography ethylene bridged hybrid column with a 2-min wash. The gradient of the mobile phase should be 50% water (1% trifluoroacetic acid) and 50% acetonitrile (1% trifluoroacetic acid) to 100% acetonitrile (1% trifluoracetic acid) over 8 min[59] (all solutions are vol/vol). Analyze LC–MS chromatograms by using Mnova software, confirming the presence of product (calculated molecular weight: 1,365.3 g mol^{-1}).
   ▲ **CAUTION** Acetonitrile is highly flammable and hazardous to human health. Trifluoroacetic acid is hazardous to human health and a highly volatile and corrosive chemical. Use these chemicals in a fume hood and wear appropriate PPE. Dispose of acetonitrile and trifluoroacetic acid appropriately after use.
10. Using a Pasteur pipette to transfer the solutions, pool the test tube fractions containing product into a 20-ml glass scintillation vial.
    ▲ **CRITICAL STEP** Do not fill the scintillation vial with >10 ml of volume, to avoid 'bumping' the solution into the collection trap and losing product. If the volume of the pooled test tube fractions exceeds 10 ml, repeat Steps 10–12 to evaporate the DCM, progressively adding all of the pooled volume to the scintillation vial.

11. Set the water bath temperature of a rotary evaporator to 50 °C. Place the 20-ml scintillation vial onto the rotary evaporator to evaporate the DCM and Ultra solution. Carefully control the vacuum and slowly decrease the pressure; the DCM will begin to evaporate immediately.
12. Slowly decrease the pressure to ~50 mbar to ensure that the methanol and aqueous solutions have completely evaporated; this may take up to 30 min. The C14-494 product should be isolated at the bottom of the 20-ml scintillation vial as a clear-to-yellow viscous oil.
▲ **CRITICAL STEP** The purity of the C14-494 ionizable lipid should be ≥80% because this excipient is critical for the formulation of placenta-tropic LNPs.
◆ **TROUBLESHOOTING**
13. (Optional) The C14-494 ionizable lipid can also be characterized using ^{1}H NMR—combine ~5 mg of isolated product with 1 ml of $CDCl_3$ in a microcentrifuge tube. Vortex well to dissolve and transfer the solution to an NMR tube to obtain the NMR spectrum on an Avance Neo 400-MHz spectrometer. MestReNova software can be used to perform NMR analysis.
■ **PAUSE POINT** The purified C14-494 ionizable lipid can be stored under nitrogen at −20 °C for several months.

Part 2: preparation of placenta-tropic mRNA LNPs

● **TIMING 1 d**

▲ **CRITICAL** In this section, we describe the preparation of placenta-tropic mRNA LNPs[29] via microfluidic mixing using the NanoAssemblr Ignite. Other LNP preparation methods including the pipette mixing and vortex mixing methods are described elsewhere[9], although LNPs formulated by using these methods have demonstrated variable physicochemical properties and in vivo nucleic acid delivery[70,71]. The placenta-tropic mRNA LNP described here is formulated with the C14-494 ionizable lipid, DOPE, cholesterol and C14-PEG_{2000} according to the formulation details presented in Tables 1–3.

▲ **CRITICAL** Throughout all steps of mRNA LNP preparation, materials and supplies should be RNase free to avoid RNase-mediated mRNA degradation. RNase AWAY reagent should be applied evenly on benchtop surfaces and glassware for decontamination and then rinsed away with distilled water.

Table 1 | Formulation details of a 1× batch of placenta-tropic mRNA LNPs

Ionizable lipid:mRNA = 10:1 (wt/wt)		Total lipid:mRNA = 3:1 (vol/vol)	
100	μg of mRNA	450	Lipid mix total volume (μl)
1,000	μg of C14-494	1,350	mRNA total volume (μl)

Table 2 | Preparation of the lipid mixture in EtOH

Lipid	Molar ratio	Concentration (mg ml^{-1})	Volume (μl)	
C14-494	35	40	25.0	–
DOPE	24	5	74.7	
Cholesterol	46.5	5	75.3	
C14-PEG_{2000}	2.5	5	28.2	
–	–	–	+246.8	Add EtOH (μl)
–	–	–	450	Lipid mix total volume (μl)

Table 3 | Preparation of the mRNA mixture in citrate buffer

Component	Concentration	Volume (μl)	
mRNA	1 μg μl^{-1}	100	–
Citrate buffer	10 mM	1,250	
–	–	1,350	mRNA mix total volume (μl)

14. Fill a clean, RNase-free 4-l glass beaker with 2 l of 1× PBS. Add a magnetic stir bar and place on a stirring plate set to 300 r.p.m.
15. To pre-wet the dialysis cassette membrane, label a 3-ml dialysis cassette (20-kDa molecular weight cutoff) by using a water-resistant marker (i.e., Sharpie), insert the top into a float buoy and add to the beaker.
16. Using a Pasteur pipette, weigh 40 mg of the C14-494 ionizable lipid prepared in Step 12 in a tared, RNase-free 1.5-ml microcentrifuge tube on an analytical balance. Add 1 ml of 200 proof EtOH to dissolve the lipid and create a stock solution with a concentration of 40 mg ml^{-1}.
   ▲ **CRITICAL STEP** When creating all lipid stock solutions, ensure that the lipid is completely dissolved in EtOH before use. Check the microcentrifuge tube for evidence of deposited or sedimented lipid on the interior sides, bottom or lid of the tube before use to avoid discrepancies in the actual and calculated concentration of the stock solution.
   ◆ **TROUBLESHOOTING**
17. Using a stainless steel spatula, weigh 5 mg of DOPE in a tared, RNase-free 1.5-ml microcentrifuge tube on an analytical balance. Add 1 ml of 200 proof EtOH to dissolve the lipid and create a stock solution with a concentration of 5 mg ml^{-1}.
18. Repeat Step 17 to create stock solutions of cholesterol and C14-PEG_{2000}.
19. In a separate RNase-free 1.5-ml microcentrifuge tube, use adjustable-volume pipettes with RNase-free plastic tips to prepare the lipid mixture according to the volumes in Table 2. Manually mix the lipid solutions by using a micropipette to create a clear EtOH mixture with a final volume of 450 µl.
   ▲ **CRITICAL STEP** The volumes in Tables 2 and 3 can be scaled as needed to prepare different amounts of mRNA LNPs. The formulation details and volumes provided assume a batch size of 100 µg of mRNA, enough for the in vitro work described in this Protocol or for two to three pregnant mice, assuming a typical 50% yield.
20. In another RNase-free 1.5-ml microcentrifuge tube, use an adjustable-volume pipette with RNase-free plastic tips to prepare the mRNA mixture according to the volumes in Table 3. Mix well to create a clear citrate mixture with a final volume of 1,350 µl. Store the mRNA and lipid mixtures on ice until use.
   ▲ **CRITICAL STEP** Note that the volumes for creating the mRNA mixture provided in Table 3 assume that the stock solution of mRNA has a concentration of 1 µg $µl^{-1}$ (1 mg ml^{-1}). If mRNA is synthesized or purchased in bulk, we recommend creating 100-µg mRNA (100-µl) aliquots in RNase-free 1.5-ml microcentrifuge tubes to avoid potential mRNA degradation caused by freeze-thaw cycles. These mRNA aliquots can be stored at −80 °C for several months. To prepare LNPs, one or more mRNA aliquots can be thawed, and 1,250 µl of 10 mM citrate buffer can be added directly into the 1.5-ml microcentrifuge tube to create the mRNA mixture.
21. Prepare mRNA LNPs via microfluidic mixing using the NanoAssemblr Ignite. Set up a 'Quick Run' by entering the 'Syringe Brand' and 'Size' (C: BD 3 ml; R: BD 1 ml), 'Flow Rate Ratio' (3:1, C:R), 'Total Volume' (1.6 ml), 'Total Flow Rate' (12 ml min^{-1}), 'Start Waste' (0.2 ml) and 'End Waste' (0.05 ml). Insert one Ignite cartridge into the cartridge adapter, ensuring that the cartridge Luer inserts are accessible.
22. Using a 3-ml BD plastic syringe fitted with a 25-gauge × 25-mm needle, load the mRNA mixture from the 1.5-ml microcentrifuge tube into the syringe. Remove the needle, eliminate air bubbles from the syringe and use the plunger to push the liquid to the tip of the syringe without expelling drops of the mRNA mixture.
   ▲ **CRITICAL STEP** Ensure that the 3-ml syringe contains ≥1.2 ml of the total mRNA mixture (1,350 µl).
   ◆ **TROUBLESHOOTING**
23. Insert the Luer-Lok tip of the 3-ml syringe containing the mRNA mixture into the 'C' inlet of the Ignite cartridge.
24. Repeat Step 22 to load the lipid mixture from Step 19 into a 1-ml BD plastic syringe.
   ▲ **CRITICAL STEP** Ensure that the 1-ml syringe contains ≥0.4 ml of the total lipid mixture (450 µl).

25. Insert the Luer-Lok tip of the 1-ml syringe containing the lipid mixture into the 'R' inlet of the Ignite cartridge. Label two 15-ml conical centrifuge tubes with either 'mRNA LNP' or 'Waste' for sample and waste collection, respectively. Insert them uncapped into their respective clips in the NanoAssemblr Ignite. Start the mRNA LNP preparation.
26. After the preparation has finished, remove the conical tube containing 1.6 ml of mRNA LNPs and transfer to the pre-wet dialysis cassette. Dialyze for 2 h at room temperature. Remove and appropriately discard the waste conical tube and cartridge from the NanoAssemblr Ignite.
27. After dialysis has finished, remove mRNA LNPs from the cassette by using a 3-ml plastic syringe fitted with a 25-gauge × 25-mm needle in a biosafety cabinet. Remove the needle from the syringe, fit with a sterile syringe filter (0.22-µm pore size) and sterile-filter the mRNA LNPs into a 2-ml RNase-free microcentrifuge tube.
28. Measure the concentration of encapsulated mRNA by using the Quant-iT RiboGreen reagent and RNA assay kit. It is expected that the mRNA encapsulation efficiency will be >80% and that the encapsulated mRNA concentration will be ~25 ng µl^{-1}.
    - Dilute mRNA LNPs 100-fold. Label one RNase-free 1.5-ml microcentrifuge tube 'Unencapsulated mRNA' and another 'Total mRNA'; add 5 µl of mRNA LNPs to the bottom of each. To the tube labeled 'Unencapsulated mRNA', add 495 µl of 1× TE buffer and to the tube labeled 'Total mRNA' add 495 µl of 0.1% (vol/vol) Triton X-100.
    - Mix well by inverting the closed tubes. By measuring mRNA concentration in both intact (1× TE buffer) and lysed (0.1% Triton X-100) LNP samples, we can calculate the encapsulation efficiency and the concentration of encapsulated mRNA.
    - Prepare the RNA standard curve by using a 2-fold serial dilution according to the volumes in Table 4.
    - Prepare the 200-fold diluted RiboGreen reagent: add 2,985 µl of 1× TE buffer and 15 µl of RiboGreen reagent to a 15-ml conical tube. Both the stock vial and diluted RiboGreen reagent are light sensitive; protect them from light by covering them in aluminum foil.
    - Into a single black-walled, flat-bottom, 96-well microplate, add 100 µl of each of the seven RNA standards in duplicate (14 wells total) and 100 µl of each of the 'Unencapsulated mRNA' and 'Total mRNA' samples in quadruplicate (8 wells total). Add 100 µl of the diluted RiboGreen reagent to all 22 wells.
    - Immediately measure fluorescence intensity (excitation: 480 nm; emission: 520 nm) by using a microplate reader.
    - Calculate mRNA concentration in both the 'Unencapsulated mRNA' and 'Total mRNA' samples by comparison to the RNA standard curve by using linear regression. The concentration of encapsulated mRNA is calculated by subtracting the concentration of 'Unencapsulated mRNA' from 'Total mRNA' and multiplying this difference by 100, the sample dilution factor. The encapsulation efficiency is calculated by using the formula ((1 – 'Unencapsulated mRNA')/'Total mRNA') × 100 (Fig. 4b).

    ■ **PAUSE POINT** Sterile, RNase-free mRNA LNPs can be stored at 4 °C for a few weeks, although we recommend using them within a few days, particularly for in vivo assays.

    ◆ **TROUBLESHOOTING**

Table 4 | RNA standard curve to measure the encapsulated mRNA concentration in LNPs

[Total mRNA] (ng µl^{-1})	Volume of 1× TE (µl)	Volume of mRNA
1.25	592.5	7.5 µl of 100 µg ml^{-1} (100 ng µl^{-1}) ribosomal RNA standard
0.625	300	300 µl from diluted 1.25-ng µl^{-1} tube
0.3125	300	300 µl from diluted 0.625-ng µl^{-1} tube
0.15625	300	300 µl from diluted 0.3125-ng µl^{-1} tube
0.078125	300	300 µl from diluted 0.15625-ng µl^{-1} tube
0.0390625	300	300 µl from diluted 0.078125-ng µl^{-1} tube
0	300	–

Part 3: evaluating the effect of protein adsorption on in vitro mRNA transfection to placental trophoblasts

● TIMING 3 d

29. In this section, we describe the preparation of protein-coated mRNA LNPs to assess the effect of protein adsorption on luciferase mRNA transfection to immortalized BeWo b30 placental trophoblasts. Specifically, the procedure outlines steps to coat placenta-tropic mRNA LNPs in various amounts of β_2-GPI or ApoE protein[29], although these steps can easily be adapted for different proteins of interest. Protein amounts (micrograms of protein per microgram of total lipid in the LNP formulation) were selected on the basis of previous studies[29,36] and calculated by using the measured mRNA concentration, the assumed 10:1 weight ratio of ionizable lipid:mRNA and the molar percentage of ionizable lipid in the LNP formulation (Table 5).
30. Culture BeWo b30 cells in DMEM supplemented with 10% (vol/vol) FBS and 1% (vol/vol) penicillin-streptomycin in a T75 flask with a seeding density of 2×10^6 cells. The cells should reach confluency ($8–10 \times 10^6$ cells) in 3–4 d, enabling subculture approximately two times per week.
31. Once BeWo b30 cells have reached ~80% confluency on the tissue culture–treated surface of the T75 flask, remove the culture medium and add 3 ml of prewarmed 0.25% (vol/vol) trypsin-EDTA by using a serological pipette. To allow for cell detachment, return the flask to the incubator for 5 min. Confirm that the cells have detached from the flask by using a microscope and add 3 ml of pre-warmed DMEM to quench the trypsinization process. Transfer the cell suspension to a 15-ml conical tube and centrifuge at room temperature for 3 min at 500*g*.
32. Remove the trypsin/medium solution above the cell pellet and resuspend the cells in 2 ml of DMEM, mixing well to generate a homogeneous single cell suspension. Transfer 10 µl of the cell suspension to a microcentrifuge tube; add 10 µl of trypan blue solution and mix well. Using a cell counter, determine the concentration of live cells; cell viability should be >85% to use cells for in vitro evaluations. Prepare 6 ml of BeWo b30 cells in DMEM at a concentration of 2×10^5 cells ml^{-1}. Add 100 µl (2×10^4 cells) per well of this diluted cell suspension to 48 wells of a black-walled, clear-bottom, tissue culture–treated, 96-well plate according to the plate layout depicted in Supplementary Fig. 4a. Incubate overnight at 37 °C in 5% CO_2 to allow the cells to adhere to the well plate.

    ▲ **CRITICAL STEP** After trypsinization, centrifugation and resuspension, BeWo b30 cells rapidly settle out of solution. We recommend thoroughly and repeatedly mixing the cell suspension to ensure accurate cell counts and consistent seeding across a well plate.

    ▲ **CRITICAL STEP** It is critical to perform in vitro assays in a clear-bottom well plate because luminescence is typically measured on a microplate reader from the bottom of the well plate.

    ◆ **TROUBLESHOOTING**
33. On the next day, remove DMEM from the well plate and replace with 100 µl per well of pre-warmed Opti-MEM reduced serum medium supplemented with 1% (vol/vol) penicillin-streptomycin. Return the well plate to the incubator while preparing mRNA LNPs.

Table 5 | Preparation of protein-coated placenta-tropic mRNA LNPs

Protein (µg per µg total lipid)	Wells (no.)	Volume of mRNA LNPs (µl)	Volume of protein (µl)	Treatment volume of protein-coated mRNA LNPs (µl)
0	4.5	18	0.00	4.00
0.1	4.5	18	0.56	4.12
0.25	4.5	18	1.39	4.31
0.5	4.5	18	2.78	4.62
0.75	4.5	18	4.17	4.93
1	4.5	18	5.55	5.23

[Placenta-tropic mRNA LNP] = 5 ng μl^{-1}; ionizable lipid:mRNA (wt/wt) = 10; molar percent ionizable lipid = 32.41; [total lipid] (µg μl^{-1}) = 0.154; total lipid in 20-ng mRNA dose (µg) = 0.617.

34. Using the current volume of mRNA LNPs (V_1) and encapsulated mRNA concentration measured in Step 28 (C_1), calculate the volume of sterile 1× PBS to add ($V_2 - V_1$) to achieve a final concentration of 5 ng µl^{-1} (C_2).
35. Prepare 0.5 µg µl^{-1} solutions of β_2-GPI and ApoE by adding sterile 1× PBS to the vials containing dissolved protein. Mix well, prepare 20-µl aliquots per tube and store at −20 °C, avoiding repeated freeze-thaw cycles.
36. In two strips of 0.2-ml PCR tubes, combine mRNA LNPs and increasing amounts of either protein according to the details provided in Table 5. The table provides details to prepare protein-coated mRNA LNPs to treat four wells of 2 × 10^4 cells each with 20 ng of mRNA; in these calculations, we include a slight excess (four and a half wells) to account for possible volume loss in the tube. Manually mix with a micropipette and incubate at 37 °C for 15 min. Use a mini-centrifuge fitted with a PCR strip tube rotor to spin down the mRNA LNPs to the bottom of the PCR tubes for 2–3 s.
37. Treat the cells with protein-coated mRNA LNPs according to the treatment volumes provided in Table 5. For the negative control, treat four wells of cells each with 4 µl of sterile 1× PBS. Incubate for 24 h at 37 °C in 5% CO_2.
38. After incubation, remove Opti-MEM from the well plate and add 50 µl of prepared 1× reporter lysis buffer to each well. Then, add 100 µl of thawed luciferase assay substrate to each well. Place on a microplate shaker at room temperature for 5 min at 300 r.p.m.
39. Measure luminescence signal on a microplate reader with an integration time of 1,000 ms. Anticipated raw luminescence values will range from 10^3 to 10^6.
    ◆ **TROUBLESHOOTING**
40. Analyze luminescence measurements. First, subtract the mean luminescence value of cells treated with 1× PBS from the luminescence values of cells treated with mRNA LNPs. Then, for each well, divide by the mean luminescence value of cells treated with uncoated mRNA LNPs (i.e., LNPs with no protein). These normalized luminescence values should be reported across three to five biological replicates. For statistical analysis, we recommend using nested one-way ANOVAs, which will consider the standard deviation in luminescence signal in both the technical replicates (i.e., individual wells) and the biological replicates (i.e., distinct experiments/well plates).

Part 4: In vivo mRNA transfection and LNP biodistribution in pregnant mice

● **TIMING** 16 d

Gestational day −1

41. Set up matings in the afternoon/evening. House sexually mature, ≥10-week-old male C57BL6 mice individually in cages for ≥1 week after a previous mating. Place one to four sexually mature ≥10-week-old female C57BL6 mice in the cage with the male. By placing females into the male's cage, females are exposed to pheromones secreted in male urine that can induce estrus and result in higher pregnancy rates.
    ▲ **CRITICAL STEP** Using these methods, we have achieved pregnancy rates of 10–50% varying as a function of acclimatization time, age, virginity of the females and the ratio of females to males in a single cage. Fewer females (one or two) for each male often result in higher pregnancy rates.

Gestational day 0

42. Separate males and females the following morning. Mice are most likely to mate overnight, with the greatest likelihood of detecting a vaginal plug the morning after setting up matings. Although the presence of a vaginal plug indicates that mating has occurred, it does not guarantee pregnancy.

Gestational days 11–13

43. Confirm the number of pregnancies visually or by palpating the abdomen to identify fetuses. In our experience, gestational day 11 is the earliest that one can visually identify pregnant mice via the presence of a distended, rounded abdomen.
    ◆ **TROUBLESHOOTING**

Table 6 | Example dose and batch size calculations for the preparation of mRNA LNPs for E16 pregnant mice

Pregnant mice (no.)	Required mRNA LNPs (µg of encapsulated RNA)	mRNA LNPs to prepare, assuming ~50% yield (µg)	Batch size
2–3	24–36	100	1×
4–5	48–60	150	1.5×
6–7	72–84	200	2×

Approximate weight of E15.5–16 C57BL6 pregnant mice = 30 g; mRNA LNP dose (mg of encapsulated mRNA kg body weight^{-1} = 0.4; mRNA LNP dose per mouse = 12 µg; [mRNA LNPs] in 200-µl injection volume = 60 ng µl^{-1}.

Gestational day 14

44. Prepare placenta-tropic luciferase mRNA LNPs as described in Steps 14–28.
▲ **CRITICAL STEP** The 1× batch size for 100 µg of mRNA can be scaled as needed depending on the number of pregnant mice, the desired dose and the weight of the pregnant mice. It is important to note that mouse weight will vary dramatically as a function of gestational age and perhaps less dramatically among mice of the same gestational age as a function of litter size. In Table 6, we provide example dose calculations assuming mRNA LNP administration on gestational day E15.5–16, a dose of 0.4 mg of encapsulated luciferase mRNA per kg body weight and a 200-µl injection volume. In Table 6, we also provide recommended batch sizes as a function of the number of pregnant mice, assuming an ~50% yield for encapsulated mRNA.

Gestational day 15

45. For intravenous administration, the mRNA LNP volume will need to be concentrated 2- to 3-fold (assuming a post-preparation concentration of ~25 ng µl^{-1}) to a final concentration of 60 ng µl^{-1}. To concentrate mRNA LNPs, pre-wet an ultra-centrifugal filter (4-ml sample volume, 100-kDa molecular weight cutoff) by adding 2 ml of sterile 1× PBS in the sample reservoir. Centrifuge the filter at 800*g* for 5 min at room temperature. Discard both the concentrate (in the sample reservoir) and the ultrafiltrate (in the 15-ml centrifuge tube).
46. Add ≤4 ml of mRNA LNP sample to the sample reservoir. Centrifuge at 800*g* for 15 min at room temperature. If necessary, add the remaining sample volume, discard the ultrafiltrate and repeat until the volume in the sample reservoir is less than the total injection volume for all mice.
47. Using a micropipette with an RNase-free tip small enough to reach the bottom (e.g., 100 µl) of the sample reservoir, rinse the interior of the filter membrane with the concentrated mRNA LNP solution. Transfer the sample from the filter reservoir to an RNase-free tube. Measure the volume of concentrated mRNA LNPs by using a micropipette.
48. Measure the concentration of encapsulated mRNA as described above in Step 28. Using the current volume of mRNA LNPs (V_1) and encapsulated mRNA concentration (C_1), calculate the volume of sterile 1× PBS to add ($V_2 - V_1$) to achieve a final concentration of 60 ng µl^{-1} (C_2).
▲ **CRITICAL STEP** After adjusting the concentration to 60 ng µl^{-1}, ensure that the final volume of mRNA LNPs is greater than the total injection volume for all pregnant mice.
49. Add 1% (vol/vol) Vybrant DiD labeling solution to fluorescently label LNPs for biodistribution analysis. Mix well by inverting the closed tube.

Gestational day 15.5–16

50. Load 200 µl of mRNA LNPs in a 0.5-ml insulin syringe, removing air bubbles. We recommend using insulin syringes because they have less dead volume than typically found in the hub of a Luer-Lok needle and syringe. Administer mRNA LNPs intravenously via tail vein or retro-orbital injection. Wait 6–12 h.

Gestational day 16–16.5

51. In a tared 1.5-ml microcentrifuge tube, weigh 25 mg of D-luciferin potassium salt on an analytical balance. Add 1.12 ml of sterile 1× PBS and mix well. Protect the dissolved D-luciferin solution from light by wrapping the tube in aluminum foil.

▲ **CRITICAL STEP** D-luciferin should be administered to mice at a dose of 150 mg kg^{-1} in a 200-µl injection volume. The mass of D-luciferin and volume of 1× PBS provided here assume a cohort of five pregnant mice, each weighing 30 g, with slight excess; however, these details should be adjusted for the specific experiment as needed.

52. Set up the Living Image software on the IVIS. Initialize the system and set up a sequence of images to be taken—first, a fluorescence image to capture DiD-labeled LNP biodistribution (excitation: 646 nm; emission: 663 nm) and second, a bioluminescence image to capture LNP-mediated luciferase mRNA expression. For both images, the 'Exposure Time' should be 'Auto', and the 'Field of View' should be 'C'.
53. Using 0.5-ml insulin syringes, intraperitoneally administer 200 µl of the D-luciferin solution to pregnant mice. Wait 5 min and then euthanize the mice in a CO_2 chamber.

    ▲ **CRITICAL STEP** Because the abdomen of the mouse is significantly distended during pregnancy, perform a modified intraperitoneal injection to avoid piercing the uterine horn or one of the amniotic sacs. One person should scruff the mouse, tenting the abdominal skin by using their other hand. The second person should intraperitoneally administer the D-luciferin solution into the peritoneal cavity at the base of the tented pocket of skin. Alternatively, the mouse can be temporarily anesthetized by using isoflurane, allowing one person to both tent the abdominal skin and peritoneally inject D-luciferin.
54. After cervical dislocation to confirm euthanasia, the abdominal wall should be cut open from the top of the thorax to the bottom of the abdomen, again taking care to tent and lift the skin upward from the cavity to avoid piercing the amniotic sacs in the uterine horn. Gently begin removing the uterine horn from the abdominal cavity, dissecting the uterine horn proximal to the ovaries on both sides. Identify the V-shaped base of the uterine horn—that is, the cervix—to completely cut and remove the intact uterine horn from the mouse. Place the uterine horn in a 150-mm round Petri dish filled with 1× PBS to remove any blood, setting it aside.
55. Dissect and remove the remaining organs of interest from the cavity of the mouse, including the heart and lungs from behind the ribcage, the liver (with the gallbladder removed), the kidneys and the spleen. As they are removed from the mouse, add the dissected organs to a 15-ml conical tube filled with 8 ml of 1× PBS to remove blood. Appropriately discard the mouse carcass.
56. Remove the uterine horn from the Petri dish and begin dissecting the individual fetuses and placentas; we recommend predominantly using forceps to prevent destroying the fragile placental tissue. Both the uterine tissue and amnion membrane will need to be ruptured and removed to isolate the fetus and attached placenta via the umbilical cord. Scissors can be used to cut the umbilical cord, separating the fetus and placenta, as well as for trimming the placental tissue. Each fetus and placenta pair can be placed in a single well of a 24-well plate, partially pre-filled with 1× PBS.
57. Image the dam organs as well as the fetuses and placentas on two separate mats by using an IVIS. To obtain higher-quality IVIS images, remove excess liquid from the tissues by dabbing them on a paper towel or absorbent pad before placing them on the IVIS mats. Ensure that the tissues are far enough apart to avoid signal spillover from one organ to the next, but close enough that they still fit in the 'C' 'Field of View'.

    ▲ **CRITICAL STEP** Placentas can be imaged either maternal (rounded) or fetal (flat, dark red) side up, although we recommend consistently selecting one or the other for the entire experiment.

    ■ **PAUSE POINT** Data can be analyzed in aggregate, after completing all dissections to ensure that the time point after mRNA LNP administration remains as consistent as possible among mice of the same treatment group as well as different cohorts.
58. Use the ROI tool in the Living Image software to analyze luminescence signal from mRNA-mediated luciferase expression and fluorescence signal from DiD-labeled LNPs in IVIS images. Luminescence and fluorescence measurements should be reported for at least four biological replicates with photons s^{-1} as the unit. For statistical analysis, ordinary one-way ANOVAs can be used to compare luminescence or fluorescence measurements between treatment groups in tissues such as the heart, lungs, liver, kidneys and spleen. Given that data will be collected for multiple placentas and fetuses per mouse, we recommend using

nested one-way ANOVAs, which will consider both the standard deviation in luminescence/fluorescence signal in the placentas from one mouse as well as among mice of the same treatment group.
◆ TROUBLESHOOTING

Troubleshooting

Troubleshooting information can be found in Table 7.

Table 7 | Troubleshooting table

Step	Problem	Possible reason	Solution
12	C14-494 lipid purity <80%	Incomplete isolation of product from unreacted alkyl epoxides	Repeat flash chromatography steps and pool only test tube fractions that definitively contain product as analyzed by using LC–MS
16	Lipids do not immediately dissolve in EtOH	Some of the lipid excipients, particularly DOPE, have limited solubility in EtOH at room temperature	Ensuring that the microcentrifuge tube lid is properly sealed to avoid EtOH evaporation, incubate the lipid solutions at 37 °C on a ThermoMixer with gentle shaking at 300 r.p.m. for 5–10 min. Tubes can be vortexed as needed
22	Lipid and/or mRNA syringes do not have sufficient volume for mixing	Loss of ≥50 µl from the total lipid mixture and/or ≥150 µl from the mRNA mixture	Avoid solution loss from the microcentrifuge tube, the hub of the needle and the tip of the syringe during loading
28	mRNA encapsulation efficiency is <80%	Poor microfluidic mixing of mRNA LNPs	Increase the mRNA LNP 'batch size' by 50–100% or increase the 'Start Waste' and 'End Waste' volumes on the NanoAssemblr Ignite instrument
32	BeWo b30 cell viability is <85%	Cells are overconfluent (>80%) in the T75 flask or are no longer growing in a single monolayer—they have begun to grow on top of each other	Subculture cells one to two additional times in a T75 flask at a seeding density of 2×10^6 cells, ensuring that cells are well suspended before adding to the flask. After the cells have adhered in the flask, remove dead cells, replace with fresh culture medium and culture for 2–3 d. Passage cells before they reach 80% confluency. If problems persist, discard cells with bleach and thaw a new vial from cold storage
39	A coefficient of variation >20% is observed among wells of the same treatment group for in vitro luminescence measurements	Inconsistent cell seeding densities or treatment with mRNA LNPs between wells	Use a repeater pipette to rapidly seed cells in the well plate or a multichannel pipette to simultaneously treat multiple wells with mRNA LNPs
43	Pregnancy rates <25%	Mating virgin females, using non-proven stud male breeders	Mate virgin females before 15 weeks of age and track pregnancy rates from stud males, replacing ineffective male breeders as needed. Replace males and females >12 months of age
58	A coefficient of variation >20% is observed for in vivo tissue luminescence and/or fluorescence measurements	Inconsistent intravenous injections of mRNA LNPs or inconsistent times between D-luciferin administration and tissue imaging	Perform in vivo experiments with multiple people—one for each mouse in the treatment group—to perform dissections in parallel and ensure that tissue imaging is performed at the same time point after D-luciferin administration

Timing

Part 1: synthesis and purification of the C14-494 ionizable lipid (4 d)
Steps 1–3, synthesizing crude lipid: 2 d
Steps 4–13, purifying C14-494 via flash chromatography: 1–2 d

Part 2: preparation of placenta-tropic mRNA LNPs (1 d)
Steps 14–27, formulating mRNA LNPs via microfluidic mixing: 3–4 h, including 2 h for dialysis
Step 28, measuring the concentration of encapsulated mRNA: 1–2 h

Part 3: evaluating the effect of protein adsorption on in vitro mRNA transfection to placental trophoblasts (3 d)
Steps 29–32, seeding BeWo b30 cells: 1–2 h and overnight incubation
Steps 33–37, preparing protein-coated LNPs and treating cells: 2–3 h and 24-h incubation
Steps 38–40, measuring luminescence in lysed cells by using luciferase assay substrate: 1–2 h

Part 4: in vivo mRNA transfection and LNP biodistribution in pregnant mice (16 d)
Step 41, set up matings: 1–2 h and wait overnight
Step 42, separate males and females: 1–2 h and wait 11 d
Step 43, check pregnancies: 1–2 h
Steps 44–49, formulate and prepare mRNA LNPs for administration: 1–2 d
Step 50, administer mRNA LNPs via tail vein injection: 1–2 h and wait 6–12 h
Steps 51–58, euthanize and dissect tissues from mice: 2–4 h

Anticipated results

The results from the synthesis and purification of the C14-494 ionizable lipid were as expected, with the product identified in both the NMR and LC–MS spectra with purity >80% (Supplementary Figs. 1 and 2). The purified C14-494 ionizable lipid was used to prepare luciferase mRNA LNPs via microfluidic mixing. After microfluidic mixing, dialysis against 1× PBS for 2 h and filtration with a 0.22-µm pore-size syringe filter, the Quant-iT RiboGreen reagent and RNA assay kit was used to measure the encapsulated mRNA concentration. As expected, the RNA standard curve was linear, with a squared Pearson's correlation coefficient of 0.996, enabling estimation of encapsulated mRNA by using least squares linear regression. The mean encapsulated mRNA concentration for the formulated placenta-tropic LNPs was 25.6 ng μl^{-1} with an encapsulation efficiency of 89% (Fig. 4b and Supplementary Fig. 3).

With an mRNA encapsulation efficiency >80%, LNPs were then used to evaluate the effect of protein adsorption on in vitro luciferase mRNA transfection to BeWo b30 cells. At ~80% confluency, BeWo b30 cells were trypsinized, resuspended in fresh DMEM and counted by using a Countess 3 automated Cell Counter. Because cell viability was >90%, 2×10^4 cells in culture medium were seeded in wells of a 96-well plate (Supplementary Fig. 4a) and allowed to adhere overnight. On the following day, luciferase mRNA LNPs were diluted ~5× with sterile 1× PBS to a concentration of 5 ng μl^{-1} and used to prepare protein-coated LNPs (Table 5). In the 96-well plate, culture medium was replaced with 100 µl of Opti-MEM reduced serum medium before treating cells with 1× PBS (untreated cells), uncoated mRNA LNPs, mRNA LNPs coated in β_2-GPI or mRNA LNPs coated in ApoE. 24 h after treatment, medium was removed from the well plate, and luminescence in each well was evaluated by using the luciferase assay system and a microplate reader. Raw luminescence values from untreated cells were $<1 \times 10^3$, whereas luminescence values from treated cells were $>1 \times 10^5$ (Supplementary Fig. 4b), indicating successful transfection of the BeWo b30 placental trophoblasts. Normalized luminescence was calculated by first subtracting the mean luminescence signal of untreated cells and then dividing by the mean luminescence signal from cells treated with uncoated mRNA LNPs (Supplementary Fig. 4b). This was repeated for a total of $n = 5$ biological replicates (Fig. 4c,d), enabling statistical analysis of the effect of protein adsorption on in vitro mRNA transfection to BeWo b30 placental trophoblasts. Because normalized luminescence data were collected from five independent subcultures/passages of cells each with four wells, statistical analysis was performed by using a nested two-sided, one-way ANOVA to consider the error both among technical replicates and across biological replicates (Supplementary Figs 5 and 6).

Next, to assess in vivo LNP biodistribution and mRNA transfection in pregnant mice, matings were set up between C57BL6 male and female mice. Here, we placed one to two females ≥10 weeks of age into the cage of a singly housed stud male overnight, separating the following day on gestational day 0. Upon separation, we visually inspected the vaginal cavity of females for the presence of a thick, white, mucus-like vaginal plug (Fig. 5a). Pregnant mice were identified visually by the presence of a rounded abdomen starting as early as gestational day 11 and were tracked daily through the time point of the study on gestational day 16 (Fig. 5b). In our experience, vaginal plugs were identified in approximately twice as many females as those that were ultimately identified to be pregnant.

Luciferase mRNA LNPs were prepared as above and concentrated to 104.3 ng μl^{-1} encapsulated mRNA via centrifugation at 800*g* in a 100-kDa filter (Supplementary Fig. 7).

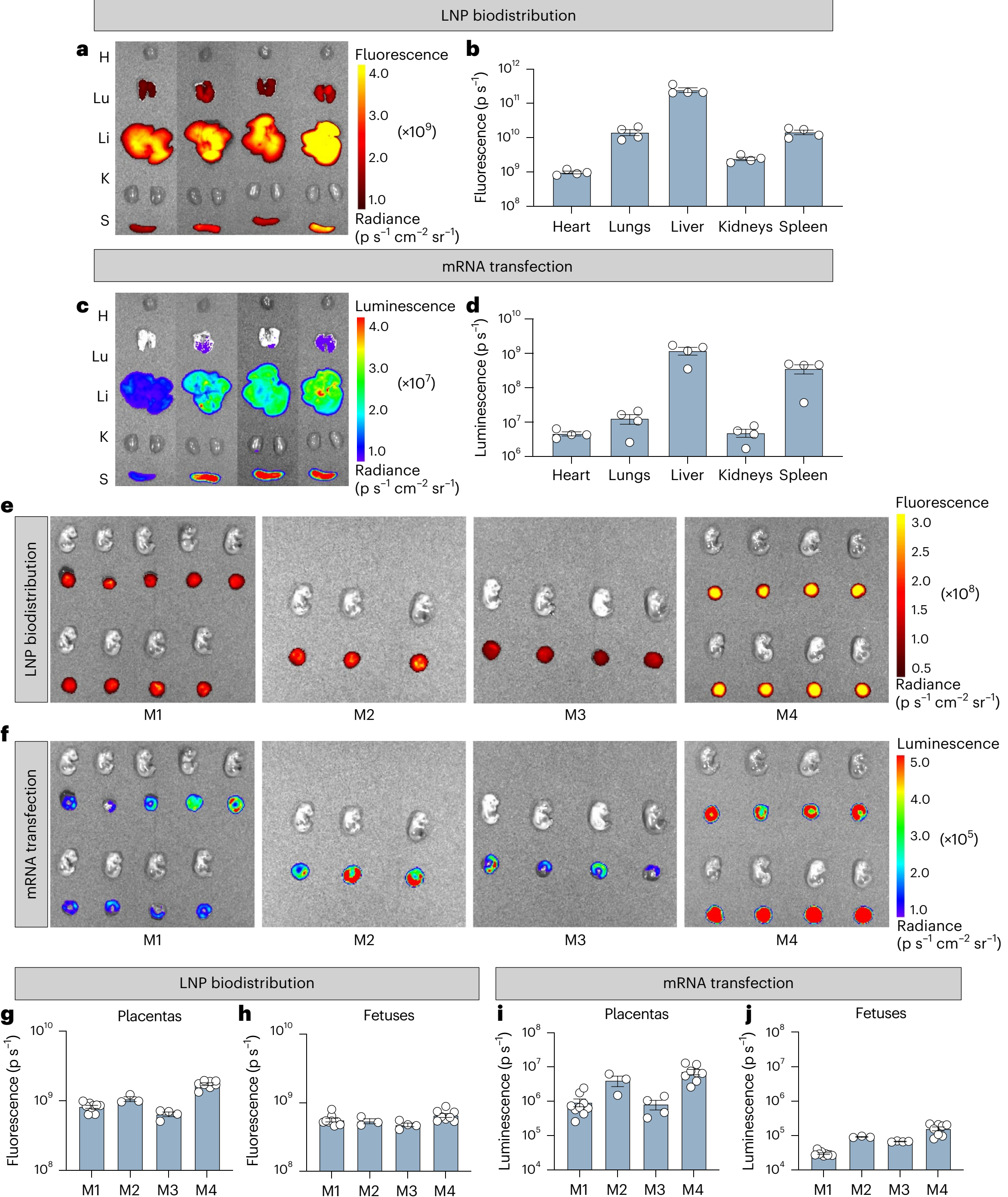


Fig. 6 | Evaluation of LNP biodistribution and mRNA transfection with the C14-494 placenta-tropic LNP in pregnant mice. a–**j**, C14-494 placenta-tropic LNPs were formulated with luciferase mRNA and fluorescently labeled with 1% (vol/vol) DiD. DiD-labeled LNPs were administered intravenously to pregnant mice (gestational day 15.5) at an mRNA dose of 0.4 mg per kg body weight. 12 h later, 150 mg kg^{-1} D-luciferin substrate was administered, tissues (**a** and **c**), placentas and fetuses (**e** and **f**) were dissected, and fluorescence and luminescence imaging was performed by using an IVIS. DiD fluorescence (**b**,**g** and **h**) and luciferase luminescence (**d**,**i** and **j**) were quantified in tissues (**b** and **d**), placentas and fetuses (**g**–**j**) by using the Living Image software. In the heart (H), lungs (Lu), liver (Li), kidneys (K) and spleen (S), fluorescence and luminescence are reported as mean ± s.e.m. (n = 4 biological replicates). In the placentas and fetuses, fluorescence and luminescence are reported as mean ± s.e.m. (n = 4 biological replicates for each mouse (M1–4) with 3–9 placentas or fetuses per mouse).

LNPs were diluted with sterile 1× PBS to 60 ng μl^{-1} and dyed with 8.78 µl (1%, vol/vol) of the lipophilic fluorescent dye DiD. DiD-labeled luciferase mRNA LNPs were administered intravenously to pregnant mice at an mRNA dose of 0.4 µg kg^{-1} via tail vein injection using 0.5-ml insulin syringes. 12 h later, D-luciferin was administered intraperitoneally at a dose of 150 mg kg^{-1} to enable detection of luminescence via an IVIS. 5 min later, gestational age E16 pregnant mice were euthanized by using CO_2, and the abdominal wall was cut open to remove the uterine horn (Fig. 5c). The intact uterine horn was dissected from the cavity proximal to the ovaries (Fig. 5d). For each fetus and its respective placenta, the uterine tissue, yolk sac and amnion were removed, enabling the fetus to be separated from the placenta by cutting the umbilical cord (Fig. 5e). This process was repeated across the entire uterine horn, and the fetuses and their respective placentas were placed with the fetal side facing up (Fig. 5f) on a mat for both fluorescence (LNP biodistribution) and luminescence (mRNA transfection) imaging (Fig. 6e,f).

The remaining tissues (i.e., the heart, lungs, liver, kidneys and spleen) were also dissected from the mouse, rinsed with 1× PBS and similarly placed on a mat for imaging (Fig. 6a,c). DiD fluorescence and luminescence were quantified in all tissues, placentas and fetuses by using the ROI tool on the Living Image software (Fig. 6b,d,g–j). As expected, we observed LNP accumulation and mRNA transfection in the liver, but also potent extrahepatic mRNA transfection in the spleen. There was no evidence of LNP accumulation or mRNA transfection in the fetuses, and the mean luminescence signal in the placentas was 3.27×10^6 p s^{-1}. Together, this Protocol describes the preparation and evaluation of placenta-tropic mRNA LNPs in time-dated pregnant mice to facilitate increased research at the interface of nanomedicine, gene modulation and reproductive health.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The data associated with this Protocol are provided in the article and Supplementary Information. Source data for Figs. 4 and 6 are provided with this paper. Source data are provided with this paper.

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Author contributions

K.L.S. designed and conducted experiments, analyzed the data, wrote the original draft of the manuscript, and contributed to reviewing and editing the manuscript. M.J.M. supervised the experiments and overall study, was responsible for funding acquisition for the experiments, and contributed to reviewing and editing the manuscript.

Competing interests

K.L.S. and M.J.M. have filed a patent application based on this work. M.J.M. is an inventor on a patent related to this work filed by the Trustees of the University of Pennsylvania.

Additional information

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Software and code

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Data collection	i-control for Tecan Infinite 200 PRO microplate reader Perkin Elmer Living Image 4.7.3. for In Vivo Imaging System (IVIS)
Data analysis	Perkin Elmer Living Image 4.7.3. for In Vivo Imaging System (IVIS) GraphPad Prism Version 10.4.2 (534) Mnova Version 16.0.0

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Sample size	All sample sizes had at least n = 4 independent biological replicates. No statistical power methods were used to predetermine sample sizes.
Data exclusions	No data were excluded from analyses.
Replication	For in vitro assays, experiments were performed with n = 5 independent biological replicates each with 4 technical replicates. An independent biological replicate was plates from a unique cell subculture/passage and a technical replicate was considered one well of plated cells. For in vivo assays, experiments were replicated with n = 4 biological replicates (mice). All attempts at replication were successful.
Randomization	Samples were randomly allocated into experimental groups.
Blinding	Investigators were not blinded during outcome assessment or data analysis. Blinding was not relevant to the study as no subjective data collection or scoring was required.

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Methods

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[x]	[ ]	Flow cytometry
[x]	[ ]	MRI-based neuroimaging

Eukaryotic cell lines

Policy information about cell lines and Sex and Gender in Research

Cell line source(s)	BeWo b30 cells were provided by D. Huh at the University of Pennsylvania with permission from A. Schwartz at Washington University School of Medicine in St. Louis. The original BeWo cell line is available from ATCC (cat. no. CCL-98), though it lacks the monolayer-forming ability associated with the b30 clone.

Authentication	The morphology of cells was checked at every subculture/passage to ensure they were free from contamination for authentication purposes.
Mycoplasma contamination	Cells tested negative for mycoplasma at the University of Pennsylvania's Cell Center.
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Laboratory animals	C57BL6 mice (male and female, 10–12 weeks old) were purchased from Jackson Laboratory and housed separately in the Clinical Research Building Animal Facility at the University of Pennsylvania. Mice were housed in a vivarium with a 12 h light-dark cycle and were provided with food and water ad libitum. Vivarium temperature and humidity were maintained between 68 and 76 ºF and 30 and 70%, respectively.
Wild animals	N/A
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