

Lipid Polymer Hybrid Nanomaterials for mRNA Delivery

WEIYU ZHAO,¹ CHENGXIANG ZHANG,¹ BIN LI,¹ XINFU ZHANG,¹ XIAO LUO,¹ CHUNXI ZENG,¹ WENQING LI,¹ MIN GAO,² and YIZHOU DONG^{1,3,4,5,6,7}

¹Division of Pharmaceutics & Pharmaceutical Chemistry, College of Pharmacy, The Ohio State University, 434 Riffe Building, 496 West 12th Avenue, Columbus, OH 43210, USA; ²Liquid Crystal Institute, Kent State University, Kent, OH 44242, USA; ³Department of Biomedical Engineering, The Ohio State University, Columbus, OH 43210, USA; ⁴The Center for Clinical and Translational Science, The Ohio State University, Columbus, OH 43210, USA; ⁵The Comprehensive Cancer Center, The Ohio State University, Columbus, OH 43210, USA; ⁶Dorothy M. Davis Heart & Lung Research Institute, The Ohio State University, Columbus, OH 43210, USA; and ⁷Department of Radiation Oncology, The Ohio State University, Columbus, OH 43210, USA

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Abstract

Introduction—In the past decade, messenger RNA (mRNA) has been extensively explored in a wide variety of biomedical applications. However, efficient delivery of mRNA is still one of the key challenges for its broad applications in the clinic. Recently, lipid polymer hybrid nanoparticles (LPNs) are evolving as a promising class of biomaterials for RNA delivery, which integrate the physicochemical properties of both lipids and polymers. We previously developed an *N*¹,*N*³,*N*⁵-tris(2-aminoethyl)benzene-1,3,5-tricarboxamide (TT) derived lipid-like nanomaterial (TT3-LLN) which was capable of effectively delivering multiple types of mRNA. In order to further improve the delivery efficiency of TT3-LLN, in this study, we focused on studying the effects of incorpor-

ating different polymers on establishing LPNs and aimed to develop an optimized lipid polymer hybrid nanomaterial for efficient mRNA delivery.

Methods—We incorporated a series of biodegradable and biocompatible polymer materials into the formulation of TT3-LLNs to develop LPNs. mRNA delivery efficiency of different LPNs were evaluated and a systematic orthogonal optimization was further carried out.

Results—Our data indicated that PLGA4 (MW 24,000–38,000 g/mol) dramatically increased delivery efficiency of TT3-LLNs in comparison to other polymers. Further optimization identified PLGA4-7 LPNs (PLGA:mRNA = 9:1, mass ratio; TT3:DOPE:Cholesterol:DMG-PEG₂₀₀₀ = 25:25:45:0.75, molar ratio) as a lead formulation, which displayed significantly enhanced delivery of two types of mRNA in three different human cell lines as compared with TT3-LLNs.

Conclusions—Results from this study potentially provide new insights into developing LPNs for mRNA based therapeutics.

Keywords—Lipid polymer hybrid nanoparticles (LPNs), mRNA therapeutics, Lipids, Polymers, Poly (lactic-co-glycolic acid) or PLGA, Orthogonal experiment design.

Address correspondence to Yizhou Dong, Division of Pharmaceutics & Pharmaceutical Chemistry, College of Pharmacy, The Ohio State University, 434 Riffe Building, 496 West 12th Avenue, Columbus, OH 43210, USA. Electronic mail: dong.525@osu.edu

Yizhou Dong is an Associate Professor in the College of Pharmacy at The Ohio State University. He received his B.S. from Peking University, Health Science Center (2002) and M.S. from Shanghai Institute of Organic Chemistry (2005). In 2009, he received his Ph.D. degree in pharmaceutical sciences from University of North Carolina at Chapel Hill under the supervision of Professor K.-H. Lee. From 2010 to 2014, he was a postdoctoral fellow in the laboratory of Professors Robert Langer and Daniel Anderson at MIT. Dr. Dong joined OSU as an Assistant Professor in 2014, and was promoted to Associate Professor in 2018. His research focuses on the design and development of biotechnology platforms for treating genetic disorders, infectious diseases, and cancers. Dr. Dong has authored over fifty papers and patents. Several of his inventions have been licensed and are currently under development as drug candidates for clinical trials. He is the recipient of numerous honors, including Early Career Investigator Award from the Bayer Hemophilia Awards Program, Research Awards from the National PKU Alliance, New Investigator Grant from the AAPS Foundation, Maximizing Investigators' Research Award from the National Institute of General Medical Sciences, and 2017 Ohio State Early Career Innovator of the Year.

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ABBREVIATIONS

mRNA	Messenger RNA
TT	N^1, N^3, N^5 -tris (2-aminoethyl) benzene-1, 3, 5 tricarboxamide
LLNs	Lipid-like nanoparticles
LPNs	Lipid polymer hybrid nanoparticles
DOPE	1,2-dioleoylsn-glycero-3-phospho-ethanolamine
DMG-PEG ₂₀₀₀	1, 2-dimyristoyl-snglycerol, methoxy-polyethylene glycol
PLGA	Poly (lactic- <i>co</i> -glycolic acid)
PLA	Poly (D,L-lactic acid)
HA	Hyaluronic acid

INTRODUCTION

Messenger RNA (mRNA)-based therapeutics has been rapidly developed for a number of biomedical applications, such as vaccines for infectious diseases and cancers, protein replacement therapy, and genome editing.^{12,18,21,22,25,26,35} mRNA-based therapy possesses advantages over other nucleic acid-based approaches due to its endogenous translation process in the cytosol and no potential risk of genome mutagenesis.^{15,28,32} However, the instability and insufficient translatability of mRNA remain a limitation to achieving maximized therapeutic windows.²⁷ Therefore, new types of nanomaterials are needed to improve mRNA delivery and the corresponding protein expression.

Biomaterial-based nanoparticles, as non-viral delivery vehicles, have shown great potential for effective delivery of mRNA.^{10,15,22,29} For example, lipid nanoparticles (LNPs) or lipid-like nanoparticles (LLNs) were reported for a wide variety of mRNA delivery.^{16,21,23,25,36} Meanwhile, polymeric nanoparticles, constructed with biodegradable polymers such as poly(D,L-lactic acid) (PLA), poly lactic-*co*-glycolic acid (PLGA), dextran, and hyaluronic acid (HA), are also widely applied for RNA delivery since polymeric components are able to form stable nanoparticles and controlled release the cargos.^{1,3–6,8,13,14,19,30,33,34} In addition, lipid conjugated poly(b-amino esters) (PBAEs) and poly(glycoamidoamine) were reported for effective delivery of mRNA.^{7,17} Recently, lipid polymer hybrid nanoparticles (LPNs), by integrating the complementary properties of lipid and polymeric nanomaterials, are emerging as a class of nanomaterials for RNA delivery.^{2,11,31,37} Although various combinations of lipids and polymers were applied in establishing LPNs for siRNA delivery, little is known

about the effects of different polymers on lipid based nanomaterials for mRNA delivery.

We previously developed a series of N^1, N^3, N^5 -tris(2-aminoethyl)benzene-1,3,5 tricarboxamide (TT) derived lipid-like nanomaterials for mRNA delivery.²¹ Among these TT-LLNs, TT3 LLN was found as a lead nanomaterial, which demonstrated capability of effectively delivering multiple types of mRNA.^{16,21} With TT3 as a significant lipid derived material for the development of LPNs, herein, we aimed to integrate an appropriate polymer material in establishing LPNs together with TT3 lipids for improving mRNA delivery. Therefore, we studied the effects of incorporating different biodegradable and biocompatible polymer materials into the TT3-LLNs on their delivery efficiency. A lead LPN was identified after initial screenings and an orthogonal optimization of the LPNs. Our findings indicated that PLGA4 (MW 24,000–38,000 g/mol) was a polymer component significantly superior to other polymer materials when formulating TT3-LPNs. Through further orthogonal optimization on the formulation of PLGA4 incorporated TT3-LPNs, we identified a lead LPNs formulation, PLGA4-7 LPNs, which demonstrated significantly enhanced mRNAs delivery efficiency than TT3-LLNs *in vitro*.

METHODS AND MATERIALS

Materials

Firefly luciferase mRNAs (FLuc mRNAs) and enhanced green fluorescent protein mRNA (eGFP mRNAs) were purchased from TriLink Biotechnologies, Inc. (San Diego, CA). DOPE was purchased from Avanti Polar Lipids, Inc (Alabaster, AL). Ribogreen reagent and fetal bovine serum were purchased from Life Technologies (Grand Island, NY). Polymers (Table 1) and other chemicals were purchased from Sigma Aldrich and used without further purification.

Cell Culture

Human hepatoma Hep3B cell line, human embryonic kidney 293T cell line, and human acute monocytic leukemia THP-1 cell line were all purchased from American Type Culture Collection (ATCC, Manassas, VA). Hep3B cells were cultured in Eagle's Minimum Essential Medium (EMEM, Corning Incorporated, Corning, NY); HEK293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Corning Incorporated, Corning, NY); and THP-1 cells were cultured in RPMI-1640 Medium (Corning Incorporated, Corning, NY). All the cells were maintained in

TABLE 1. Properties of polymers applied in establishing LPNs.

Polymers	Properties		
	Hydrophilicity	Molecular weight/viscosity	Other
PLGA1	Hydrophobic	MW 24,000–38,000 g/mol, viscosity 0.32–0.44 dL/g	Ester terminated
PLGA2	Hydrophobic	MW 7000–17,000 g/mol, viscosity 0.16–0.24 dL/g	Ester terminated
PLGA3	Hydrophobic	MW 38,000–54,000 g/mol, viscosity 0.45–0.60 dL/g	Ester terminated
PLGA4	Hydrophobic	MW 24,000–38,000 g/mol, viscosity 0.32–0.44 dL/g	Acid terminated
PLA	Hydrophobic	MW 18,000–28,000 g/mol, viscosity 0.25–0.35 dL/g	Ester terminated
Dextran	Hydrophilic	MW 15,000–25,000 g/mol	–
HA	Hydrophilic	MW 1.5–2.2 million g/mol	–

Note All the information regarding the properties of polymer materials listed in the table are provided by Sigma-Aldrich. Viscosity data are given based on the condition: 0.1% (w/v) in chloroform (25 °C, Ubbelohde) (size 0c glass capillary viscometer).

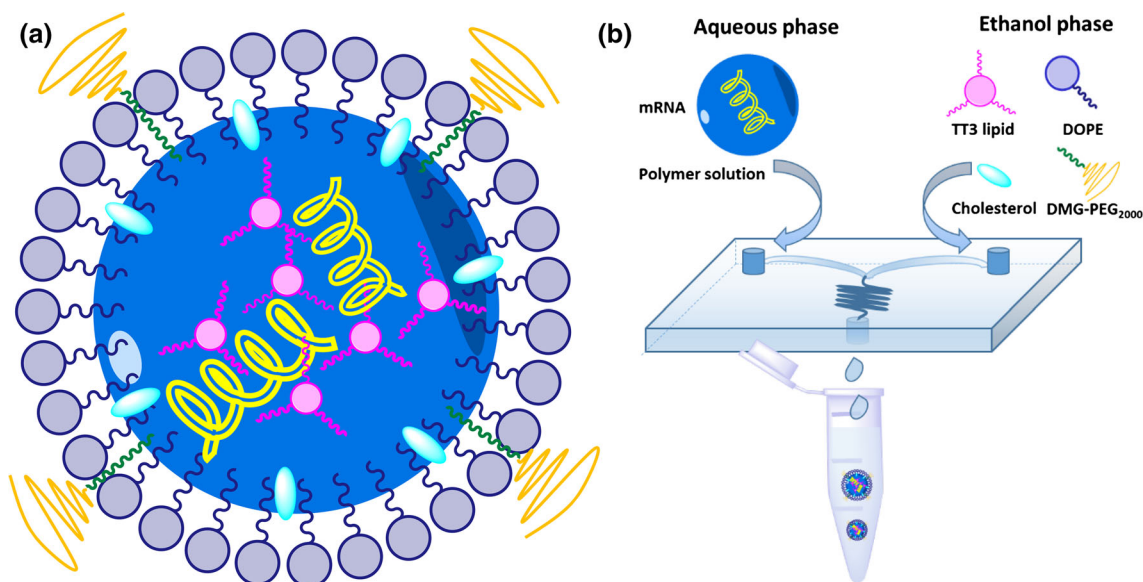


FIGURE 1. Schematic illustrations of (a) structure of LPNs and (b) the process of formulating LPNs through a microfluidic device.

media supplemented with 10% heat inactivated FBS at 37 °C incubator with an atmosphere of 5% CO₂.

Formulation and Characterization of mRNA-Loaded LPNs

To prepare LPNs formulations for the initial screening, TT3 was formulated with DOPE, cholesterol, DMG-PEG₂₀₀₀ at the molar ratio of 20/30/40/0.75 in ethanol phase.²¹ Polymer solutions were prepared by dissolving polymers in acetone (hydrophobic polymers) or citrate acid buffer (hydrophilic polymers). For initial screenings and orthogonal optimization studies, each of the polymer solutions was added respectively to the FLuc mRNA or eGFP mRNA solution in the aqueous phase, and then the aqueous phase was mixed with ethanol phase by pipetting. For cryo-TEM imaging and stability studies, LPNs formulations were obtained by mixing aqueous

and ethanol phases *via* a microfluidic device (Precision NanoSystems, Vancouver, BC, Canada) (Fig. 1).

Particle size and zeta potential of LPNs were measured by dynamic light scattering (DLS) using NanoZS Zetasizer (Malvern, Worcestershire, U.K.) at 25 °C with a scattering angle of 173°. Entrapment efficiency of LPNs was determined using the Ribogreen assay as reported previously.^{20,21}

LPNs Luciferase Expression Assay

Hep3B cells were seeded in white 96-well plates at the density of 2×10^4 cells per well, allowed to attach to plates for overnight, and then treated with FLuc mRNA encapsulated LPNs at the dose of 50 ng mRNA/well. Each sample was tested in triplicate. Culture medium containing LPNs was carefully discarded 6, 12 or 24 h after transfection, followed by addition of a mixture of 50 μL luciferase substrate

(Bright-Glo reagent, Promega, Madison, WI) and 50 μL of serum-free EMEM to each well. Five minutes later, the relative luminescence intensity was measured with the SpectraMax M5 microplate reader (Molecular Devices, LLC., Sunnyvale, CA). Free FLuc mRNA was used as a control.

LPNs eGFP Expression Assay

Hep3B cells were seeded in 24-well plates at the density of 8×10^4 cells per well. After overnight incubation, the cells were treated with eGFP mRNA encapsulated LPNs at the dose of 125 ng mRNA/well. After 24 h of treatment, the cells were collected, washed, and suspended in 400 μL PBS. Relative green fluorescence intensity was then quantified using a BD LSR II flow cytometer (BD Biosciences, San Jose, CA).

Orthogonal Array Experimental Design

In order to identify an optimal composition ratio of the formulation components in PLGA4 LPNs, an orthogonal array study was carried out as previously reported.²¹ Three formulation components (TT3, DOPE and cholesterol) at each three different levels were assigned in the orthogonal experiments with a fixed TT3: mRNA ratio (10:1). A series of eGFP mRNA encapsulated PLGA4-LPNs (PLGA4-1 to PLGA4-9) were prepared according to the orthogonal array design table L9 (3^3) and used to transfect Hep3B cells at the dose of 50 ng mRNA/well. Green fluorescence intensity of Hep3B cells from each formulation groups was quantified 24 h after treatment using a BD LSR II flow cytometer (BD Biosciences, San Jose, CA). The average eGFP intensity (Kn) of each component (factor) at the same molar ratio (level) was calculated and used to depict the impact of the levels and factors on delivery efficiency. The best formulation ratio was predicted and validated by the impact trend curve.

Cryo-Transmission Electron Microscopy (Cryo-TEM) and Stability

Cryo-TEM specimens were prepared by using plunge-freezing in a FEI Vitrobot (Mark IV).^{9,36} Low-dose cryo-TEM imaging was carried out in a FEI Tecnai G2 F20 200 kV microscope equipped with an UltraScan 4 K CCD camera and a Gatan twin-blade anticontaminator.⁹

For stability studies, the freshly prepared PLGA4-7 LPNs were dialyzed in PBS buffer using Slide-A-Lyzer dialysis cassettes (3.5 K MWCO, Life Technologies, Grand Island, NY) and stored at 4 °C. Particle size of PLGA4-7 LPNs were monitored on 0, 1, 3, 5, 7 and

14 days post preparation using a NanoZS Zetasizer (Malvern, Worcestershire, UK) at 25 °C with a scattering angle of 173°.

Dose Dependency and Delivery Efficiency of PLGA4-7 LPNs in Different Cell Lines

For dose dependency study, Hep3B cells were seeded in 24-well plates at the density of 8×10^4 cells per well. After overnight incubation, the cells were treated with eGFP mRNA encapsulated TT3-LLNs or PLGA4-7 LPNs at the dose of 25, 50 and 100 ng mRNA/well, respectively. After 24 h of treatment, the cells were collected, washed, and suspended in 400 μL PBS. Relative green fluorescence intensity was then quantified using a BD LSR II flow cytometer (BD Biosciences, San Jose, CA).

For delivery efficiency study, 293T cells or THP-1 cells were seeded in 24-well plates at the density of 8×10^4 cells per well. After overnight incubation, the cells were treated with eGFP mRNA encapsulated LPNs at the dose of 50 ng mRNA/well. After 24 h of treatment, the cells were collected, washed, and suspended in 400 μL PBS. Green fluorescence intensity was then quantified using a BD LSR II flow cytometer (BD Biosciences, San Jose, CA). All the experiments were carried out in triplicate.

RESULTS

Construction of Lipid Polymer Hybrid Nanoparticles (LPNs)

In order to study the effects of incorporating different polymer materials on the delivery efficiency of the previously reported TT3-LLNs, four types of polymers (PLGA, PLA, dextran and HA, Table 1) were used to develop TT3-derived LPNs (Fig. 1a). Among these polymers, PLGA and PLA are hydrophobic polymers while dextran and HA are hydrophilic polymers. PLGA1 to PLGA4 are four different PLGA polymers with either different molecular weight/viscosity or different terminus (Table 1).

As illustrated in Fig. 1b, TT3, DOPE, cholesterol, and DMG-PEG₂₀₀₀ were mixed in the ethanol phase using the previously optimized formulation ratio (TT3/DOPE/Cholesterol/DMG-PEG₂₀₀₀ = 20/30/40/0.75, molar ratio).²¹ Each of the polymers was incorporated into previously reported TT3-LLN formulation by adding the polymer solution to the mRNA solution in aqueous phase at three different polymer to mRNA mass ratios, which were 9:1 (A), 3:1 (B), and 1:1 (C). TT3-derived LPNs were then afforded by mixing the aqueous phase with the ethanol phase.

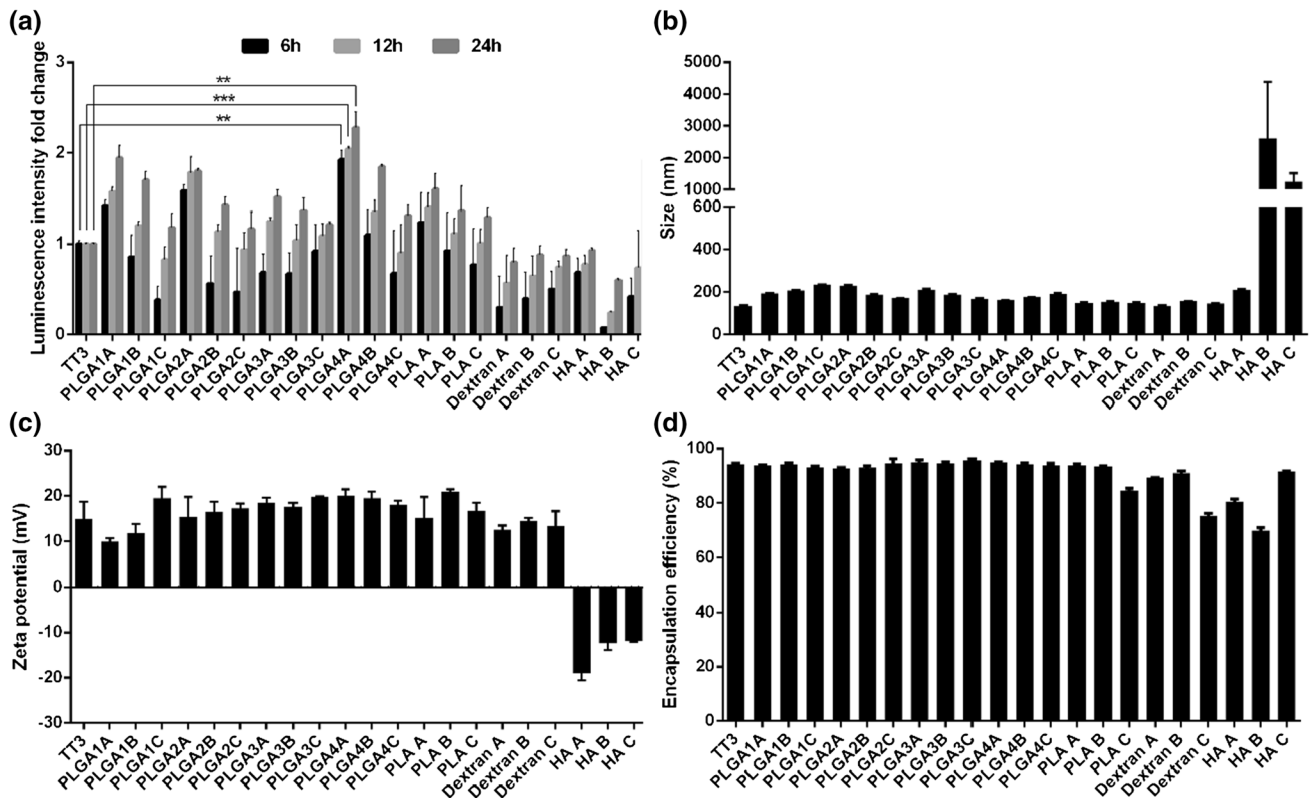


FIGURE 2. Characterizations of LPNs for mRNA delivery using a luciferase assay. (a) Fold changes of luciferase expression levels using different polymer incorporated LPNs as compared to TT3 LLNs; (b) size, (c) zeta potential, and (d) encapsulation efficiency of different FLuc mRNA encapsulated LPNs. Note A, B and C represents the mass ratio of polymer to mRNA equal to 9:1, 3:1 and 1:1, respectively. ($n = 3$; two-tailed t test; $**p < 0.01$; $***p < 0.001$).

Evaluation of LPNs Through a Luciferase Expression Assay

In order to investigate mRNA delivery efficiency of the newly formulated LPNs with different polymers, we utilized a luciferase expression assay in human hepatoma Hep3B cell line for an initial screening. Cells were treated with different LPNs encapsulated with firefly luciferase mRNAs (FLuc mRNAs) and bioluminescence intensity of each group was subsequently quantified at the following time points 6, 12 and 24 h (Fig. 2a). Luciferase expression assay revealed the following trends: (i) PLGA4A LPNs group showed the highest bioluminescence intensity as compared with TT3-LLNs and other LPNs at all time points; (ii) Generally, hydrophobic polymer incorporated LPNs showed better transfection efficiency than TT3-LLNs and hydrophilic polymer incorporated LPNs, especially at prolonged time points (12 and 24 h); (iii) Regarding PLGA and PLA LPNs at 24 h, LPNs in group A (polymer: mRNA = 9:1, mass ratio) generally are better than LPNs in groups B and C (polymer: mRNA = 3:1 and 1:1, mass ratio). Results indicated that the incorporation of polymer material into TT3-LLNs had significant effects on mRNA delivery effi-

ciency. Hydrophobic polymers potentially led to increased mRNA delivery efficiency. Moreover, the 9:1 polymer-to-mRNA mass ratio was better than 3:1 and 1:1 for LPNs development. This ratio was selected as a representative of each LPNs group for further studies.

Meanwhile, the size, zeta potential and encapsulation efficiency of each LPNs were also characterized (Figs. 2b–2d and Supplementary Information Table S1). The size of LPNs were in the range of 100–250 nm, except the HA groups. Most LPNs showed positive zeta potential and within the range of 10–20 mV, while HA-LPNs were negative in zeta potential. For the encapsulation efficiency, hydrophobic polymers LPNs exhibited similar encapsulation efficiency (> 90%) as TT3-LLNs, which were better than most of hydrophilic polymers LPNs (around 80%).

Delivery Efficiency of LPNs Encapsulating eGFP mRNAs

In order to further verify the effects of polymers on the delivery efficiency of TT3-LLNs, another reporter mRNA, enhanced green fluorescent protein mRNA (eGFP mRNA), was used to conduct eGFP expression

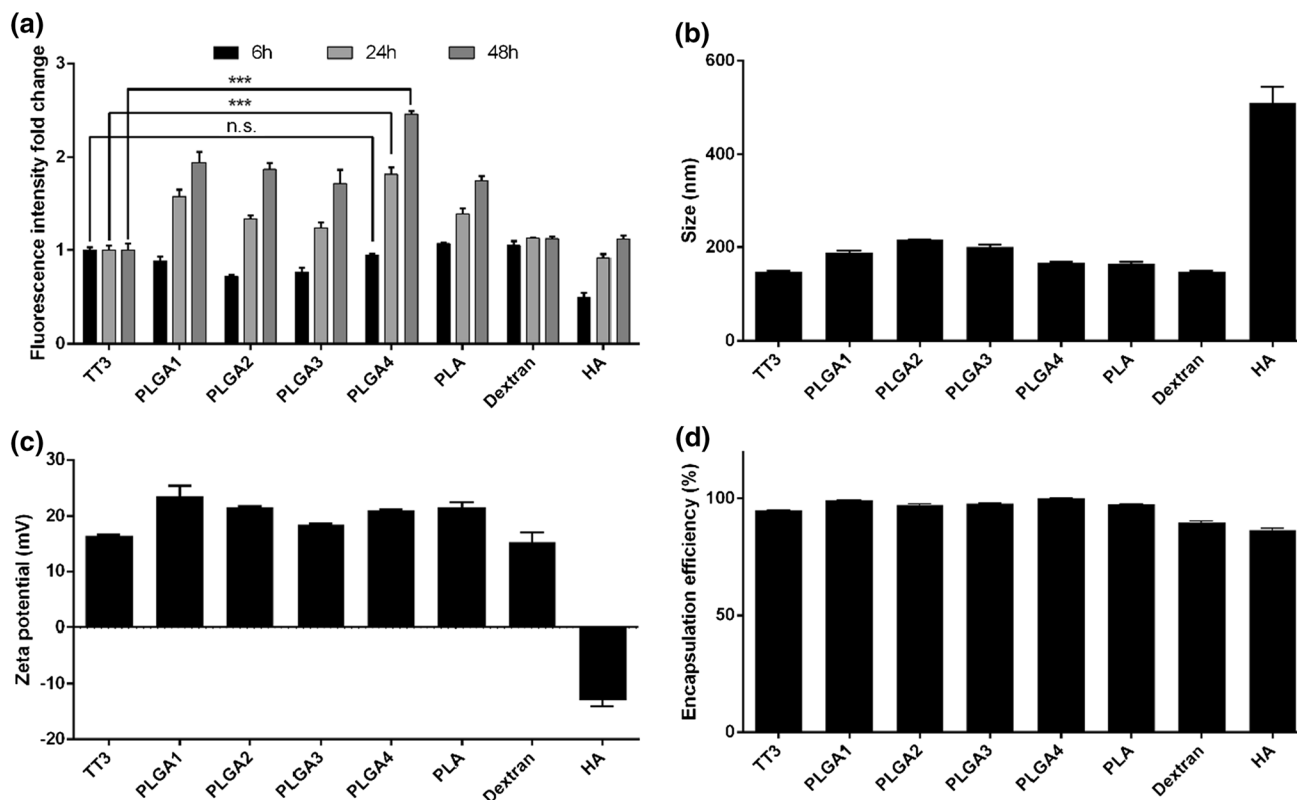


FIGURE 3. Evaluation of LPNs to deliver eGFP mRNA. (a) Fold changes of eGFP expression levels using different polymer incorporated LPNs as compared to TT3 LLNs; (b) size, (c) zeta potential, and (d) encapsulation efficiency of different eGFP encapsulated LPNs. ($n = 3$; two-tailed t -test; $n.s.$ not significant; $***p < 0.001$).

assay in Hep3B cells. In this assay, the 9:1 polymer-to-mRNA mass ratio was selected as a representative of each LPNs group. According to results collected from flow cytometry (Fig. 3a), a similar trend was observed in different LPNs. All hydrophobic polymer LPNs showed prolonged and increased transfection efficiency as compared with TT3-LLNs and hydrophilic polymer LPNs. PLGA4 LPNs group showed the highest eGFP intensity at 24 h and 48 h time points among all the groups. Moreover, all the physicochemical properties of eGFP mRNA encapsulated LPNs were similar to that of FLuc mRNA encapsulated LPNs (Figs. 3b–3d and Supplementary Information Table S2). Based on the *in vitro* screening results from luciferase and eGFP expression assays, PLGA4 LPNs significantly increased the delivery efficiency of TT3-LLNs by over two fold. Therefore, PLGA4 LPNs were selected as the lead LPNs for further studies.

Optimization of PLGA4 LPNs Formulation Through an Orthogonal Experiment Design

In order to identify an optimal molar ratio of the formulation components in the currently selected PLGA4 LPNs, an orthogonal array design was applied

in the optimization as previously reported.²¹ Three out of four formulation components (TT3, DOPE and cholesterol; Fig. 4a) were assigned in the following orthogonal experiments with a fixed TT3: mRNA molar ratio (10:1) and the selected polymer: mRNA mass ratio (9:1). A series of PLGA4 LPNs (PLGA4-1 to PLGA4-9) were prepared according to the orthogonal array design table L9 (3^3) and used to transfect Hep3B cells with eGFP mRNA. Results collected from flow cytometry at 24 h (Fig. 5a) showed that PLGA4-7 LPNs group produced the highest eGFP intensity. Besides, all the LPNs in the orthogonal array showed similar physicochemical properties (Figs. 5b–5d and Supplementary Information Table S3). To further predict the best ratio of these formulation components, an analysis of orthogonal array was carried out. The average eGFP intensity of each component (factor) at the same molar ratio (level) was calculated and used to measure the impact of the levels and factors on delivery efficiency (Figs. 4b–4d). According to the trend analysis results, PLGA4-7 LPNs (TT3/DOPE/Cholesterol/DMG-PEG₂₀₀₀ = 25/25/45/0.75, molar ratio) was predicted as the optimal formulation ratio based on the orthogonal assay and selected as an optimized formulation for further studies.

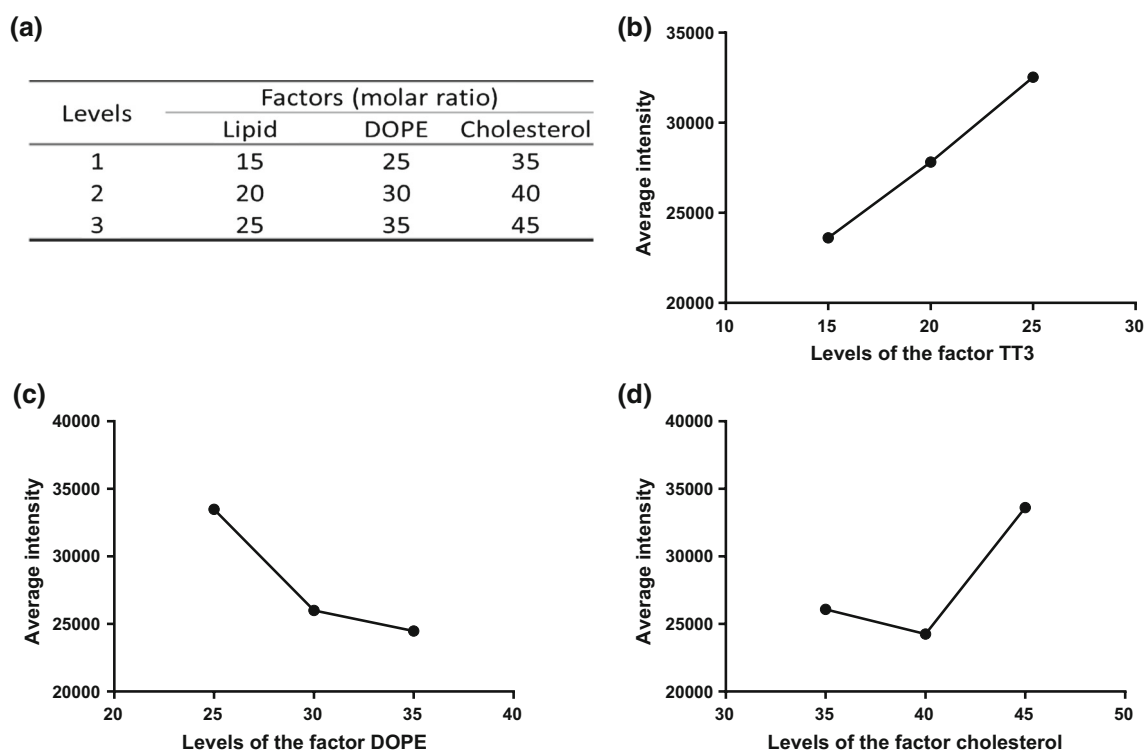


FIGURE 4. An orthogonal array analysis of PLGA4-LPNs. (a) Three levels for each formulation component: TT3, DOPE and cholesterol; the impact trend of TT3 (b), DOPE (c), and cholesterol (d) on delivery efficiency.

We also performed further characterizations on the optimized PLGA4-7 LPNs to ensure optimal physicochemical properties. Based on the Cryo-TEM imaging data, PLGA4-7 LPNs were spherical nanoparticles with the size of around 100 nm in diameter (Fig. 6a). A stability assay further indicated that particle size of PLGA4-7 LPNs remained stable for a minimum of 2 weeks when kept at 4 °C (Fig. 6b).

Dose Dependency and Delivery Efficiency of PLGA4-7 LPNs in Different Cell Lines

To further study the delivery capability of PLGA4-7 LPNs, a dose dependency assay was carried out. Hep3B cells were transfected with PLGA4-7 LPNs at different doses of eGFP mRNA (25, 50 or 100 ng/well). Fluorescence intensity was examined by flow cytometry after 24 h. Results showed that PLGA4-7 LPNs enabled significantly higher eGFP expression level than that of previously reported TT3-LLNs at every dose level (Fig. 7a). While eGFP expression level of TT3-LLNs increased proportionally with dose, eGFP expression of PLGA4-7 LPNs increased with dose as well but not proportionally. At lower doses, PLGA4-7 LPNs demonstrated a more significant fold increase of eGFP expression over TT3-LLNs.

Next, two additional cell lines, 293T and THP-1, were used to examine the delivery efficiency of PLGA4-7 LPNs. We observed significantly increased mRNA delivery efficiency of PLGA4-7 LPNs in both cell lines as compared with TT3-LLNs (Figs. 7b and 7c).

DISCUSSIONS AND CONCLUSIONS

In summary, we incorporated a series of biodegradable and biocompatible polymer materials into TT3-LLNs to develop lipid polymer hybrid nanoparticles (LPNs). Most of hydrophobic polymer incorporated LPNs showed better transfection efficiency than TT3-LLNs and hydrophilic polymer incorporated LPNs, especially at prolonged time points. Through *in vitro* screenings and orthogonal optimization, an optimized LPNs formulation, PLGA4-7 LPNs, was obtained with significantly enhanced mRNA delivery efficiency and prolonged protein expression. In the optimized PLGA4-7 LPNs, polymer material PLGA4 is an acid-terminated PLGA with the molecular weight around 24,000–38,000 g/mol and viscosity of 0.32–0.44 dL/g. When incorporated into TT3-LLNs, PLGA4 showed the highest improvement of mRNA delivery efficiency as com-

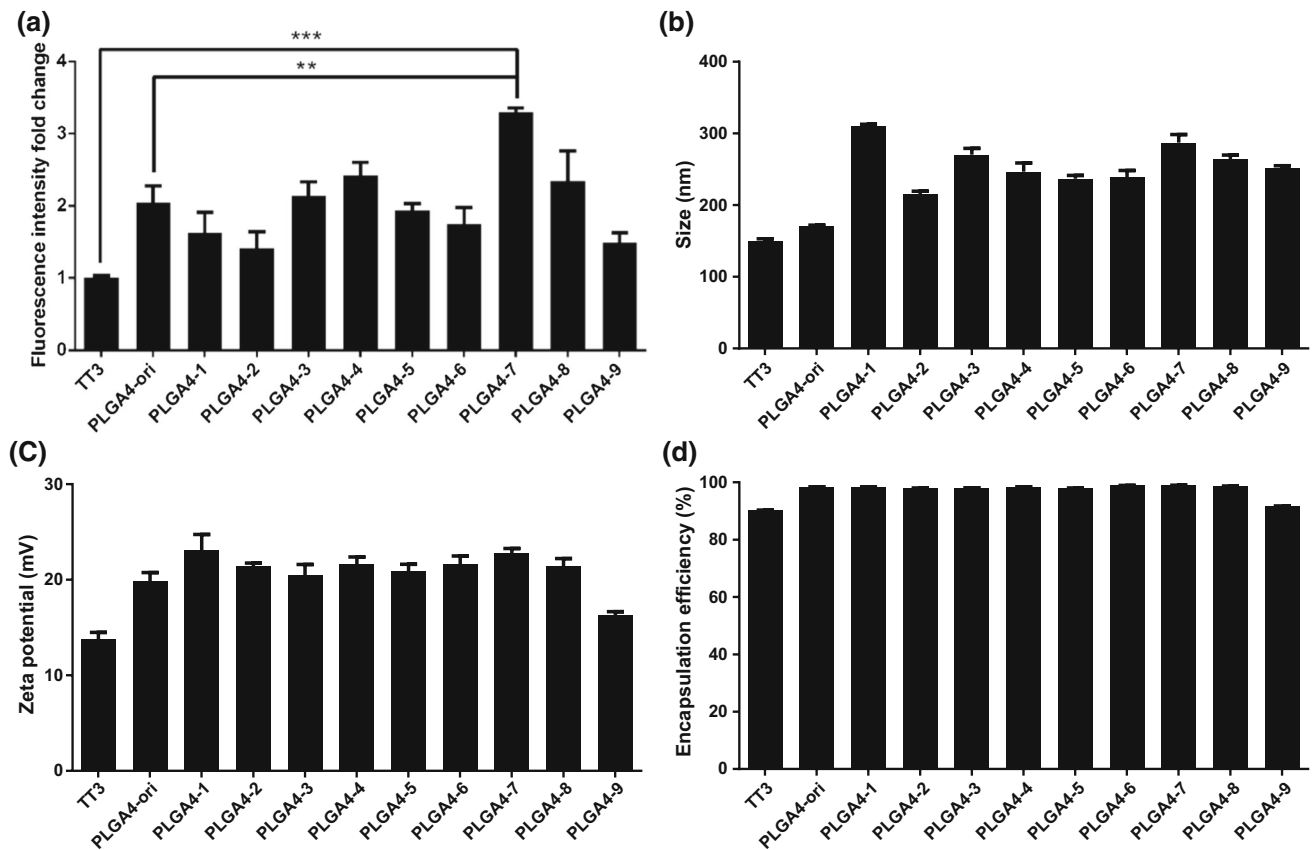


FIGURE 5. An orthogonal array optimization of PLGA4-LPNs. (a) Fold changes of eGFP expression levels of different PLGA4-LPNs in the orthogonal array as compared to TT3 LLNs; (b) size, (c) zeta potential, and (d) encapsulation efficiency of different PLGA4-LPNs. ($n = 3$; two-tailed t -test; $**p < 0.01$; $***p < 0.001$).

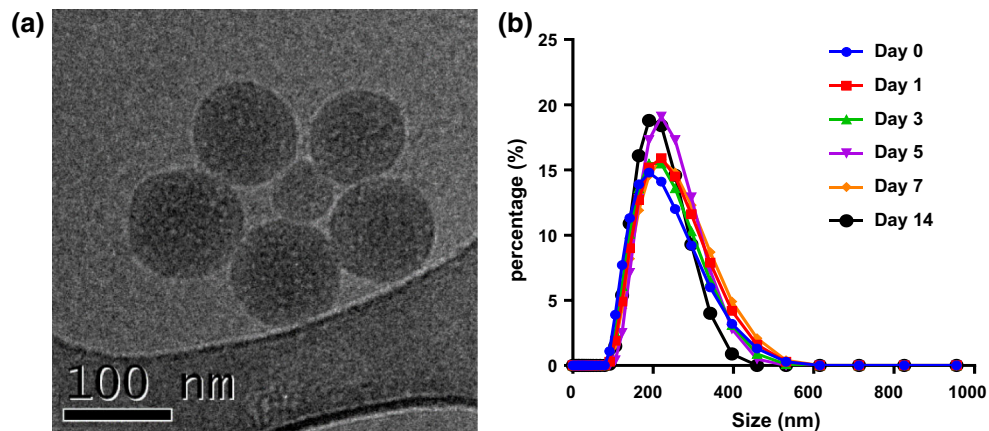


FIGURE 6. Characterizations of PLGA4-7 LPNs. (a) A representative cryo-TEM image of PLGA4-7 LPNs revealing a spherical morphology (Scale bar = 100 nm); (b) Particle size of PLGA4-7 LPNs were stable for at least 2 weeks at 4 °C.

pared with TT3-LLNs and other LPNs tested. After analyzing the results, we found that PLGA4 incorporated LPNs showed slightly enhanced zeta potential

and encapsulation efficiency as compared to TT3 LLNs, which may facilitate mRNA packaging by PLGA4 incorporation.^{2,3,24} Moreover, PLGA4 LPNs

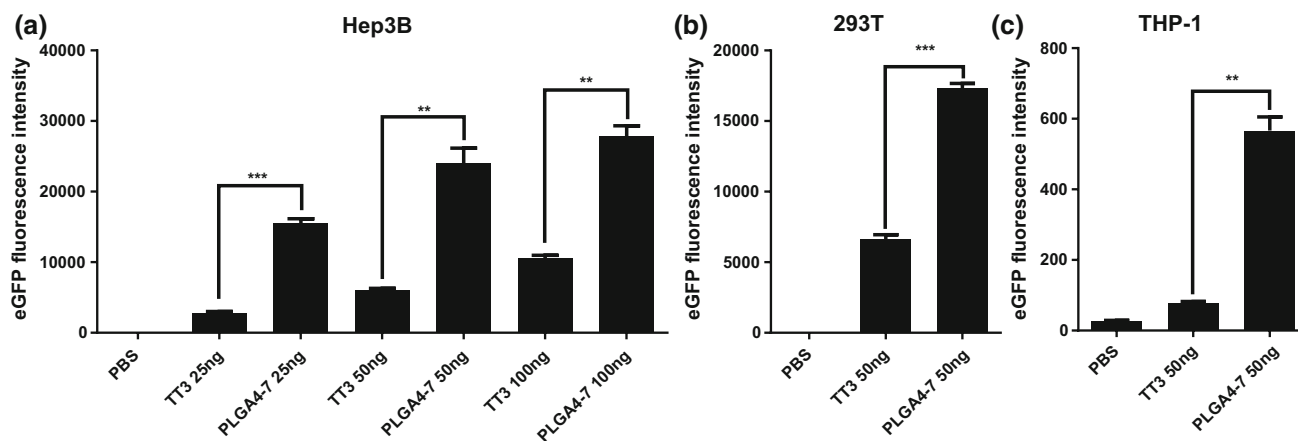


FIGURE 7. (a) Dose dependency of PLGA4-7 LPNs in Hep3B cells. Delivery efficiency of PLGA4-7 LPNs in HEK293T (b) and THP-1 (c) cells. ($n = 3$; two-tailed t -test; ** $p < 0.01$; *** $p < 0.001$).

displayed significantly improved delivery efficiency at prolonged time points, which indicates that PLGA4 polymer material may enable a sustained release of mRNA from nanoparticles.^{3,24} Consequently, these new LPNs formulations merit further study and development for mRNA delivery and potential therapeutic applications.

ELECTRONIC SUPPLEMENTARY MATERIAL

The online version of this article (<https://doi.org/10.1007/s12195-018-0536-9>) contains supplementary material, which is available to authorized users.

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CONFLICT OF INTEREST

W. Z., C. Z., B. L., X. Z., X. L., C. Z., W. L., M. G., and Y. D. declare no conflicts of interest.

ETHICAL STANDARDS

No human or animal studies were carried out by the authors for this article.

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