Optimization of the Linker Length of Mannose-Cholesterol Conjugates for Enhanced mRNA Delivery to Dendritic Cells by Liposomes

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Liposomes (LPs) as commonly used mRNA delivery systems remain to be rationally designed and optimized to ameliorate the antigen expression of mRNA vaccine in dendritic cells (DCs). In this study, we synthesized mannose-cholesterol conjugates (MPn-Chs) by click reaction using different PEG units (PEG100, PEG1000, and PEG2000) as linker molecules. MPn-Chs were fully characterized and subsequently used to prepare DC-targeting liposomes (MPn-LPs) by a thin-film dispersion method. MPn-LPs loaded with mRNA (MPn-LPX) were finally prepared by a simple self-assembly method. MP1000-LPX displayed bigger diameter (about 135 nm) and lower zeta potential (about 40 mV) compared to MPn-LPs. The in vitro transfection experiment on DC2.4 cells demonstrated that the PEG length of mannose derivatives had significant effect on the expression of GFP-encoding mRNA. MP1000-LPX containing MP1000-CH can achieve the highest transfection efficiency (52.09 ± 4.85%), which was significantly superior to the commercial transfection reagent Lipo 3K (11.47 ± 2.31%). The optimal DC-targeting MP1000-LPX showed an average size of 132.93 ± 4.93 nm and zeta potential of 37.93 ± 2.95 mV with nearly spherical shape. Moreover, MP1000-LPX can protect mRNA against degradation in serum with high efficacy. The uptake study indicated that MP1000-LPX enhanced mRNA expression mainly through the over-expressing mannose receptor (CD206) on the surface of DCs. In conclusion, mannose modified LPs might be a potential DC-targeting delivery system for mRNA vaccine after rational design and deserve further study on the in vivo delivery profile and anti-tumor efficacy.

Keywords: mRNA vaccine, dendritic cell targeting liposomes, mannose conjugates, linker length, click reaction

INTRODUCTION

Messenger RNA (mRNA) has recently generated great attention as one of promising therapeutics with the potential for cancer immunotherapy and vaccines because the in vitro-transcribed (IVT) mRNA does not need to enter the nucleus and induces only transient protein expression without the risk of genomic integration compared with the widely investigated DNA (Sahin et al., 2014; Pardi et al., 2018). Some mRNA vaccines have been demonstrated to be effective in the preclinical mouse models of cancer (Kreiter et al., 2015;
Oberli et al., 2017; Sayour et al., 2017). Nevertheless, the anionic character of mRNA does not facilitate its penetration into cells, resulting in low antigen expression and curative effect. It has been shown that the cellular uptake rate of naked mRNA is less than 1 in 10,000 molecules (Sahin et al., 2014). Moreover, mRNA is prone to degradation by RNases present everywhere (Tsui et al., 2002). Thus, sufficiently efficacious delivery system is urgently required to target antigen presentation cells (APCs) and protect mRNA from nuclease degradation, which will be beneficial for the clinical application of more mRNA vaccines (Pardi et al., 2018).

Non-viral vectors such as lipids, lipid-like materials, polymer or hybrid systems are widely studied for delivery of mRNA vaccines, which have low unwanted immune responses in contrast to the viral systems including adenovirus-associated viruses, lentiviruses and the Sendai virus (Giacca and Zacchigna, 2012; Midoux and Pichon, 2015; Hají and Whitehead, 2017). Liposomes (LPs) are the most appealing and commonly used non-viral carriers of mRNA vaccines (Markov et al., 2015; Kranz et al., 2016; Persano et al., 2017; Verbeke et al., 2017). The mRNA loaded LPs namely RNA-LPX for cancer immunotherapy have been in phase I dose-escalation trial (Kranz et al., 2016). RNA-LPX protected mRNA from RNases and the encoded antigen can be efficiently expressed in the specialized APCs, like DCs (Kranz et al., 2016). Furthermore, the antigen-specific T-cell responses were also induced in melanoma patients. However, only 1 in 3 patients showed regression of a suspected metastatic thoracic lymph node lesion. The limited antitumor efficacy of RNA-LPX indicated that the LPs were worthy of being further reformed by functionalization of particles with ligands targeting DCs.

Dendritic cells express several mannose residue-recognizing membrane lectins like CD206 (mannose receptor, MR), CD209 (DC-SIGN) and CD207 (langerin) (Caminschi et al., 2012; Le Moignic et al., 2018). Macrophages also expressed CD206 receptor (Chen et al., 2016; Kim et al., 2017) with the ability to present antigens (Malissen et al., 2014). They can mediate endocytosis of cargos encapsulated in mannose-modified nano-preparations (Li et al., 2013; Chen et al., 2014; Wang C. et al., 2014). Of note, enhanced in vitro anticancer efficacy via mannose modification on the nano-preparations has been widely reported in the literatures (Lai et al., 2018; Le Moignic et al., 2018; Yang et al., 2018). LPs can be easily modified because phospholipids and cholesterol are typically included (Hua and Wu, 2013). These lipophilic molecules can conjugate with various moieties binding to surface receptors of the target cells with high selectivity. Our previous studies demonstrated that folic acid-conjugating LPs can specifically deliver DNA into folate receptor-overexpressing tumor (He Z.Y. et al., 2013; Yang et al., 2016). The targeting molecule folic acid was linked to cholesterol, which efficiently kept its binding specificity to folate receptor (He Z. et al., 2013). Taking into account of these, mannosylated cholesterol derivatives were designed and synthesized to prepare mannosylated LPs to help delivery to DCs in this study.

According to literatures, the length and flexibility of the space between ligand molecules and the surface of particles might be important parameters for efficient recognition of receptors (Engel et al., 2003; Stefanick et al., 2013; Jeong et al., 2014). A short linker may restrict the translational freedom of ligand, while the longer one might bury a large fraction of the conjugated ligand (Stefanick et al., 2013). The optimal linker provides a more effective ligand-receptor interaction (Stefanick et al., 2013; Jeong et al., 2014). Thus, a rational design of the targeting LPs is crucial to enhanced mRNA delivery to DCs.

In our study, MPn-CHs containing different PEG units were firstly synthesized and then used to prepare the MPn-LPs by a typical thin-film dispersion method. The mRNA encapsulating liposomes (MPn-LPX) were constructed by complexing the obtained MPn-LPs and mRNA. The preferable MPn-LPX were picked out according to the in vitro transfection efficiency of GFP-encoding mRNA on DCs. The pharmaceutical properties and preliminary cytotoxicity of the optimal delivery system were also assessed to favor its potential application for mRNA delivery.

MATERIALS AND METHODS

Materials

1,2,3,4,6-Penta-O-acetyl-alpha-D-mannopyranoside was obtained from Jinan Samuel Pharmaceutical Co., Ltd. (Shandong, China). Cholesterol was supplied from Shanghai Yuanju Biology Technology Company (Shanghai, China). Cholesterol-PEG2000-N3 was purchased by Shanghai Ponsure Biotech, Inc (Shanghai, China). 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) was provided by Shanghai A.V.T. Pharmaceutical Co., Ltd. (Shanghai, China). GFP-mRNA was obtained from TriLink (San Diego, CA, United States). DMEM and fetal bovine serum (FBS) were purchased from Gibco. All the other chemical reagents were of analytical grade or better without further purification unless otherwise stated.

Cell Culture

DC2.4 cells were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin with 5% CO2 at 37°C in a humidified atmosphere.

Synthesis and Characterization of MPn-CH

Synthesis of MP100-CH

As shown in Figure 1A, MP100-CH was obtained with similar procedures as described previously (Kim et al., 2012; Nguyen et al., 2016). In brief, Diethylene glycol (5.0 eq), paratoluensulfonyl chloride (TosCl, 1.0 eq) and triethylamine (TEA, 1.1 eq) were dissolved in anhydrous dichloromethane (DCM) and stirred for 24 hours (h) at room temperature (rt). The crude product was purified by silica gel with a mixed solvent system of DCM and methanol to harvest compound 1. 1,2,3,4,6-Penta-O-acetyl-alpha-D-mannopyranoside (1.5 eq), compound 1 (1.0 eq) and BF3-Et2O (1.5 eq) were dissolved in anhydrous DCM. Compound 2 was acquired and purified by column chromatography. Compound 2 (1.0 eq) and sodium
azide (5.0 eq) were added into anhydrous N, N-dimethyl-Formamide (DMF) and stirred suitably for 24 h at 60°C to prepare compound 3. Subsequently, deacetylation of compound 3 was performed in methanol solution (HPLC grade) of sodium methoxide (NaOMe) (10:1, v/v). Then the reaction system was treated with (H++) resin to get product named compound 4. Compound 5 was prepared as described previously (Rull-Barrull et al., 2016). In brief, Cholesterol (1.0 eq) and 3-bromopropyne (2.0 eq) were dissolved in component solvent containing anhydrous ether and anhydrous DMF (1:1, v/v). Sodium hydride (NaH, 5.0 eq) was added mildly and the solution was stirred at rt for 24 h. Finally, compound 4, compound 5 and copper iodide were mixed in equal molar ratio and dissolved in anhydrous DMF. The mixture was reacted for 24 h at rt and concentrated to obtained compound 6 (MP<sub>100</sub>-CH) via column chromatography.

Synthesis of MP<sub>1000</sub>-CH

The synthetic scheme was shown in Figure 1B and specific experimental steps were as follows. Compound 7 was prepared and purified according to our previously reported method (He Z.Y. et al., 2010). PEG<sub>1000</sub> (5.0 eq), propargyl bromide (2.0 eq) and hydrogenated sodium (NaH, 3.0 eq) were dissolved in anhydrous tetrahydrofuran (THF). The mixture was stirred at rt overnight. Compound 8 was purified by column chromatography. Compound 7 (1.2 eq), 8 (1.0 eq), DMAP (0.5 eq), and EDCI (2.0 eq) were dissolved in DCM and stirred at rt for 24 h. Compound 9 was obtained after the crude product was purified by column chromatography. Similar to the synthetic method of compound 6, compound 4 was connected to compound 9, and compound 10 (MP<sub>1000</sub>-CH) was obtained.

Synthesis of MP<sub>2000</sub>-CH

Compound 14 were prepared according to the scheme showed in Figure 1C. Briefly, compound 11 was prepared according to the synthesis method of compound 2 but replace the compound 1 with propargyl alcohol. Subsequently, compound 12 was acquired using the similar synthesis method of compound 4. Compound 14 (MP<sub>2000</sub>-CH) was obtained with the similar synthesis method of compound 10 after column chromatography.

General Characterization of Prepared Compounds

1H-NMR spectra of MP<sub>n</sub>-CH and other prepared compounds dissolved in CDCl<sub>3</sub>, D<sub>2</sub>O or Dimethyl Sulfoxide-D<sub>6</sub> containing TMS were recorded on a Unity Inova-400 (400 MHz) (Varian Inc., Palo Alto, CA, United States). Chemical shifts were analyzed in ppm relative to the residual solvent peaks of TMS. The
mass spectra of various compounds were obtained using a Waters Q-TOF Premier (Milford, MA, United States) equipped with the ion spray source and using N₂ as nebulization gas. In addition, the identity of the conjugate was also verified by Fourier Transform infrared spectroscopy (FTIR) using a Vector 22 spectrometer (Bruker, Ettlingen, Switzerland). The purity and retention time of MPn-CHs and other cholesterol derivatives were evaluated by high performance liquid chromatography (HPLC, Waters, Milford, MA, United States) at 201 nm. The mobile phase, at 1 mL/min flow rate, was composed of 100% chromatographic methanol. The retention time and purity of cholesterol derivatives were summarized in Table 1.

**Preparation and Characterization of MPₙ-LPX**

**Preparation of MPₙ-LPs**

The cationic LPS were prepared using a thin-film dispersion method with some modifications (Wang F. et al., 2018). Briefly, cationic lipid DOTAP, the helper lipid DOPE, CH and MPₙ-CHs at a molar ratio of 50:10:35:3 or 50:10:40:0 (Table 2) were dissolved in a mixture solvent of chloroform/ethanol (1:1, v/v) to prepare MPₙ-LPs and LPS, respectively. The organic solvents were evaporated using a rotary evaporator at 37°C for 2 h. The lipid film was rehydrated with 2 mL RNase-free water at 60°C for 40 min to obtain a suspension with the final lipid concentration of 6 mM. Subsequently, the above suspension was sonicated at 80 W for 3 min and filtered with a 0.22 µm sterilized filter for the following experiment. The coumarin-6 (Cou-6) loaded LPs were acquired using the similar procedure with the addition of Cou-6 into the chloroform/ethanol (1:1 v/v) solvent mixture. The fluorescent intensity of Cou-6 loaded LPs was measured using a thin-film dispersion method with some modifications (Kranz et al., 2016). Briefly, mRNA particles was measured using CyrationTM3 (BioTek Instruments, Inc, United States).

**Preparation of MPₙ-LPX**

In our study, MPₙ-LPX was composed of DOTAP contained LPS and mRNA at N/P ratio of 3, 5 or 7, named MPₙ-LPX NP 3, MPₙ-LPX NP 5 or MPₙ-LPX NP 7, respectively. MPₙ-LPX were prepared according to previous reported methods with some modifications (Kranz et al., 2016). Briefly, mRNA was diluted by water and 1.5 M NaCl followed by adding corresponding MPₙ-LPs diluted with water to reach the desired ratio of N/P with the final concentration of NaCl of 150 mM. The lipid film was sonicated at 80 W for 3 min and filtered with a 0.22 µm sterilized filter for the following experiment. The coumarin-6 (Cou-6) loaded LPs were acquired using the similar procedure with the addition of Cou-6 into the chloroform/ethanol (1:1 v/v) solvent mixture. The fluorescent intensity of Cou-6 loaded particles was measured using CyrationTM3 (BioTek Instruments, Inc, United States).

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**Size and Zeta Potential Measurements**

The average particle size, size distribution (polydispersity index, PDI) and zeta potential of different formulations were recorded by Zetasizer Nano ZS90 (Malvern Instruments, Malvern, United Kingdom). All measurements were carried out using diluted samples at 25°C and were conducted in triplicate.

**Cellular Transfection of MPₙ-LPX**

To optimize the appropriate ratio of N/P, DC2.4 cells in the logarithmic growth period were seeded in 24 well plates at 4 × 10³ cells/well and incubated for 24 h at 37°C, followed by incubation with different N/P of LPX (0.5 µg GFP-mRNA per well) in triplicate from 3 to 7. Before transfection, the culture medium was replaced with 500 µL FBS-free DMEM. Subsequently, LPX were added. After 4 h of incubation, 500 µL complete medium was added, and the cells were incubated for another 20 h. Expression of GFP by DC2.4 cells was visualized using a fluorescence microscope (Olympus Corp., Tokyo, Japan) and transfection efficiency was obtained based on the percentage of GFP positive cells from the live cell population by flow cytometry. Additionally, mean GFP fluorescence intensity of individual cells from GFP positive cells population after transfection was measured using FlowJo software (Li et al., 2017b).

To further investigate the transfection efficiency of MPₙ-LPX, DC2.4 cells in 24 well plates were incubated with MPₙ-LPX with the N/P of 5 following the same procedure described above. Transfection efficiency and mean fluorescence intensity (MFI) of GFP positive DC2.4 cells were evaluated by flow cytometry. In brief, DC2.4 cells were captured via forward scatter (FSC) and side scatter (SSC). Live DC2.4 cells were gated as shown in Region 1 (R1), of which GFP positive cells were selected (R2). Transfection efficiency (% GFP⁺ cells) was auto displayed with R2. MFI of GFP expression in GFP positive cells was acquired using FlowJo software. MFI was calculated after subtraction of background values of untreated DC2.4 cells. To further elaborate the kinetics of mRNA transfection in vitro, transfection efficiency of MP₁₀₀₀-LPX NP 5 on DC2.4 cells from 12 to 72 h has also been studied.

**Table 1** The retention time and purity of cholesterol derivatives was evaluated by HPLC.

| Compound | Peak 1 | Peak 2 | Peak 3 |
|----------|--------|--------|--------|
|          | Time (min) | Purity (%) | Time (min) | Purity (%) | Time (min) | Purity (%) |
| 6        | 9.743   | 97.948 | 10.493  | 1.996    |          |          |
| 9        | 7.14    | 2.9    | 11.557  | 2.204    |          |          |
| 10       | 7.548   | 1.627  | 11.556  | 1.878    |          |          |
| 13       | –       | –      | 10.278  | 8.749    |          |          |
| 14       | 6.907   | 91.532 | 10.896  | 2.321    | 13.796   | 91.251   |

Bold values represents the retention time and purity of each compound.
Characterization of Optimal MP<sub>1000</sub>-LPX

Microscopy Investigation
The appearance and Tyndall effect of MP<sub>1000</sub>-LX were recorded by a digital camera. The morphology of MP<sub>1000</sub>-LX NP 5 was examined by transmission electron microscopy (TEM, H-600, Hitachi, Japan). Briefly, 100 µL of MP<sub>1000</sub>-LX suspension was added onto copper electron microscopy grids. Subsequently, they were negatively stained with 2% phosphotungstic acid for observation.

Gel Electrophoresis Retardation Assay
To evaluate the complexation of mRNA and MP<sub>1000</sub>-LPS, 1 µg free mRNA and MP<sub>1000</sub>-LPX (containing 1 µg mRNA) were diluted with RNase-free water. Then NorthernMax<sup>®</sup> formaldehyde load dye containing ethidium bromide (50 µg/mL) was added and mixed. After incubating the samples for 10 min in 65°C, the samples were loaded into a 1% denaturing formaldehyde agarose gel in precooled MOPS buffer. The gel was run for 20 min at 180 V and analyzed using a molecular imager, ChemiDoc<sup>TM</sup> 219 XRS system (Bio-Rad, United States). RNA Millennium<sup>TM</sup> markers (Ambion) with bands at a range of 0.5–9 kb was included to provide size determination of the mRNA.

Stability Assay
For storage stability experiments, prepared MP<sub>n</sub>-LPX NP 5 were stored at 4°C for 1 and 3 days and another 1 h at rt before particle size and transfection efficiency measurement following the similar procedure described previously (Kranz et al., 2016).

To evaluate the serum stability, 1 µg free mRNA and MP<sub>n</sub>-LPX NP 5 (containing 1 µg mRNA) were incubated in parallel with 150 mM NaCl supplemented with or without FBS at 50% final concentration at 37°C for 2 h, respectively. To release mRNA from LPX, 1 µL of 10% Triton X-100 was added to 10 µL of MP<sub>n</sub>-LPX samples and incubated at rt for 10 min. After mixed with NorthernMax<sup>®</sup> formaldehyde load dye, samples were treated and visualized using the similar process as described in Section “Gel Electrophoresis Retardation Assay.”

Cytotoxicity Assay
To test potential cytotoxicity, DC2.4 cells were treated with MP<sub>1000</sub>-LPX according to the transfection procedure. Cell viability was investigated using an Apoptosis Detection Kit according to the manufacturer’s protocol by flow cytometry. Three independent cytotoxicity assays were performed in duplicate.

### Cellular Uptake of MP-LPX

Cellular uptake study was performed using Cou-6 as previously reported (Xu et al., 2016). DC2.4 cells in the logarithmic growth period were collected and seeded at a density of 8 × 10<sup>5</sup> cells/well in a 24-well plate and incubated for 24 h at 37°C. To screen the appropriate incubation time, DC2.4 cells were treated with LPX (Cou-6, 5 ng/mL) in triplicate by different time from 0.5 h to 6 h. At the end of the study, the cells were collected and washed three times with cold phosphate buffer saline. The MFI of cells was quantified by BD FACS. To further screen the uptake concentration of Cou-6, DC2.4 cells were treated in triplicate by different concentrations of Cou-6 from 2.5 µg/mL to 20 µg/mL for 2 h.

To investigate the MR mediate uptake of MP<sub>1000</sub>-LPX (Cou-6, 10 ng/mL), DC2.4 cells were pre-treated with or without 0.16 mol/L of mannose solution for 30 min followed by incubation with LPX and MP<sub>1000</sub>-LPX at 37°C, respectively. For binding assays, DC2.4 cells were incubated at 4°C for 30 min. Subsequently, LPX and MP<sub>1000</sub>-LPX were added and incubated for 2 h at 4°C.

### Statistical Analysis
The data were presented as mean ± SEM unless otherwise noted. Statistical analysis was performed using Graphpad Prism 5.0. Data of two or multiple groups were analyzed using Student’s t-test or non-parametric one-way ANOVA, respectively. The p-values < 0.05 were considered statistically significant.

### RESULTS

#### Characterization of MP<sub>n</sub>-CH

**Characterization of MP<sub>1000</sub>-CH**

We construct MP<sub>1000</sub>-CH (compound 6) according to reasonable design as shown in Figure 1A. The structure of compounds 1, 2, 3, 4, and 5 were validated in Supplementary Figures S1–S5, respectively. As shown in Figure 2A, the 1H NMR spectra of compounds 4, 5 and 6 were recorded. The single peaks at δ5.33 (s) were attributed to the protons of olefinic bond (−CH<sub>2</sub>−CH = C−) in cholesterol. The single peak at 88.01 (s) came from the protons of olefinic bond (N−CH = C−N) in coupled places. The peaks at δ3.15-3.88 (m) were attributed to the protons from the glycol unit (−O-CH<sub>2</sub>-CH<sub>2</sub>-O−CH<sub>2</sub>−) in PEG chain. These results indicated that MP<sub>1000</sub>-CH has been successfully synthesized. As seen in Figure 2B, the mass spectrum of MP<sub>1000</sub>-CH showed a peak at 740.49 (product + Na<sup>+</sup>), which was consistent with the expected molecular weight of MP<sub>1000</sub>-CH. In addition, compound 6 was further confirmed by FTIR spectroscopy with of the following principal peaks: ν−OH (3700–3400 cm<sup>−1</sup>), ν−CH<sub>3</sub> and ν−CH<sub>2</sub> (2960–2850 cm<sup>−1</sup>), ν−CH<sub>2</sub>−O−CH<sub>2</sub>− (1210–1050 cm<sup>−1</sup>) presence but ν−N = N−N− (2100–2270 cm<sup>−1</sup>) attributed to compound 4, ν−CH (about 3300 cm<sup>−1</sup>) attributed to compound 5 absence in compounds 6 (Figure 2C). Finally, as shown in Figure 2D, the spectrum of HPLC exhibited a characteristics absorption peak of MP<sub>1000</sub>-CH at 9.743 min (201 nm). According to the method of area normalization, the purity of MP<sub>1000</sub>-CH is 97.948%.

#### TABLE 2 | Formulation component and relative molar content used in the manuscript.

|       | LPs | MP<sub>100</sub>-LPs | MP<sub>1000</sub>-LPs | MP<sub>2000</sub>-LPs |
|-------|-----|---------------------|----------------------|----------------------|
| DOTAP | 50  | 50                  | 50                   | 50                   |
| DOPE  | 10  | 10                  | 10                   | 10                   |
| CH    | 40  | 35                  | 35                   | 35                   |
| MP<sub>100</sub>-CH | 0   | 5                   | 0                    | 0                    |
| MP<sub>1000</sub>-CH | 0   | 0                   | 5                    | 0                    |
| MP<sub>2000</sub>-CH | 0   | 0                   | 0                    | 5                    |
Characterization of MP\textsubscript{1000}-CH

To construct MP\textsubscript{1000}-CH (compound 10), compound 4 was jointed to compound 9 via click reaction as shown in Figure 1B. The structure of compounds 8 and 9 were firstly confirmed in Supplementary Figures S6, S7, respectively. The \textsuperscript{1}H NMR spectra of compounds 4, 9 and 10 were recorded in CDCl\textsubscript{3}. The principal proton peaks at 82.42-2.45 (t) attributed to the protons of alkynyl group (-CH) in compounds 9 were disappeared in compounds 10 as in shown Figure 2E. Similar to MP\textsubscript{100}-Chol (presence of single peak at 88.01), the successful synthesis of MP\textsubscript{1000}-Chol has also been validated. The mass spectrum of MP\textsubscript{1000}-Chol showed broad peaks from 1484.8359 to 1830.8468 (Figure 2F) while that of compound 9 were from 1185.8085 to 1480.0358 (Supplementary Figure S8). The increased molecular...
weight coincided with the molecular weight of compounds 4, which also confirmed the structure of compound 10 referring to previously report (Li et al., 2014). The FTIR spectroscopy of compounds 10 (Figure 2G) was similar to compounds 4 but the presence of unique v-CH₂-CO-O- of compounds 10 at around 1740 cm⁻¹. Additionally, the characteristics absorption peak (201 nm) of compounds 10 and compounds 9 were at 13.122 min with the purity of 96.595% (Figure 2H) and 16.692 min with the purity of 94.881%, respectively. Consistent with expectation, the hydrophilicity of compound 10 was increased and the retention time was decreased when compared with compound 9. All of the results of ¹H NMR spectra, mass spectrum, FTIR spectroscopy and HPLC confirmed the successful synthesis of compounds 10.

Characterization of MP₂₀₀₀-CH
Similar to compound 10, compound 14 was acquired according to our designed strategies (Figure 1C) and authenticated via ¹H-NMR (Figure 2I), electrospray ionization mass spectrometry (ESI-MS) (Figure 2J), FTIR (Figure 2K), and HPLC (Figure 2L). The structure of compounds 11 and 12 were firstly confirmed in Supplementary Figures S9, S10, respectively. Of note, the measured molecular weight of compound 12 was 241.07 (product + Na⁺) (Supplementary Figure S11), which was consistent with the expected molecular weight. When compared mass spectrum of compound 14 (Figure 2J) with compound 13 (Supplementary Figure S12), nearly 200 molecular weight were increased. The retention time at 201 nm of compound 14 and compound 13 were at 6.907 min with the purity of 91.532% and 13.662 min with the purity of 91.251% (Figure 2L), respectively.

Particle Size and Zeta Potential Measurement
The size and zeta potential of all formulations in this study were evaluated. There was no statistical difference between the particle size and zeta potential among different MPₙ-LPs or MPₙ LPX formulations. The particle size and zeta potential of all different MPₙ-LPs was about 60 nm (Figure 3A) and 50 mV (Figure 3B), respectively. In addition, the particle size and zeta potential of all different LPX was about 135 nm (Figure 3C) and 40 mV (Figure 3D), respectively. The PDI were all less than 0.3. As was shown, the size of MPₙ-LPX was larger and the zeta potential was lower than the corresponding MPₙ-LPs formulations.

In vitro Transfection of MPₙ-LPX
To investigate the in vitro transfection efficacy of MPₙ-LPX in DC2.4 cells, the appropriate ratio of N/P of LPX was optimized firstly. GFP expression on DC2.4 cells with different treatment were observed and recorded by fluorescence microscope (Supplementary Figures S13A–H). As shown in Supplementary Figure S13I, both LPX NP 7 and LPX NP 5 achieved significant increment in transfection efficiency compared with LPX NP 3. What's more, LPX NP 5 with similar transfection efficiency to LPX NP 7 exhibited dramatically enhanced GFP fluorescence intensity (mean GFP expression level per cell) (Supplementary Figure S13J). By the way, calculation of MFI and GFP positive cells was shown in Supplementary Figure S14. Thus, MPₙ-LPX were prepared by setting the N/P ratio at 5:1. The GFP expression was subsequently observed by fluorescence microscope (Figure 4A). As shown in Figure 4B, MP₁₀₀₀-LPX induced the most GFP positive cells and the
percentage was up to 52%, which was significantly higher than any other groups ($p < 0.001$). However, the MFI of the GFP positive cells of MP$_{1000}$-LPX NP 5 was not the best among these groups (Figure 4C). Taking into account of transfection efficiency and MFI, MP$_{1000}$-LPX with the highest transfection efficiency and moderate MFI were selected for further study in this manuscript.

The kinetics of MP$_{1000}$-LPX NP 5 transfection on DC2.4 cells was studied. As shown in Figures 4D,E, transfection efficiency first increased and then reached a plateau with the increase of the incubation time (from 12 to 72 h) while the MFI of GFP positive cells first increased and then decreased. In summary, the transfection efficiency achieved the maximum at 24 h and MFI was also the strongest at 24 h.

**Further Study on the Optimal MP$_{1000}$-LPX Morphology Examination**

The appearance and morphological studies of MP$_{1000}$-LPX were conducted. The colloidal solution was colorless and transparent (Figure 5A). Overt Tyndall effect of MP$_{1000}$-LPs and MP$_{1000}$-LPX colloidal solution were observed compared with water as was shown in Figure 5B. Representative images of size and
zeta potential of MP\textsubscript{1000}-LP(X) were shown in Figures 5C–F, respectively. As shown in Figure 5G, the morphological of MP\textsubscript{1000}-LPX was observed distinct lipid membrane structure with nearly spherical in shape. Moreover, complete complexation of the mRNA with MP\textsubscript{1000}-LPs was confirmed when the N/P ratio of 3 and 5 (Figure 5H).

**Stability Assessment**

The preliminary storage stability of the MP\textsubscript{1000}-LPX was determined by the size, zeta potential and transfection efficiency. The particle size and zeta potential of MP\textsubscript{1000}-LPX were determined at predetermined time of storage at 4°C. MP\textsubscript{1000}-LPX displayed a little decrease in particle size but not zeta potential (Figure 6A) As shown in Figure 6B, the transfection efficiency of MP\textsubscript{1000}-LPX remained about 50% when stored at 4°C for 3 days. Additionally, MP\textsubscript{1000}-LPX (Supplementary Figure S15A) and MP\textsubscript{2000}-LPX (Supplementary Figure S15B) performed excellent storage stability in the preliminary test.

For serum stability, 5 μL of fresh MP\textsubscript{1000}-LPX were diluted in FBS (1:1, v/v) and incubated for 2 h at 37°C. As shown in Figure 6C, the signal of naked mRNA band in serum was completely disappeared (lane 3) compared to naked mRNA alone (lane 1). MP\textsubscript{1000}-LPX did not dissociate after incubation in 50% serum (lane 5) similar to that incubation in NaCl of 150 mM (lane 4). When the free mRNA or MP\textsubscript{1000}-LPX were treated with Triton X-100, the free mRNA (lane 2) and mRNA dissociated from MP\textsubscript{1000}-LPX (lane 4) were visible in line with mRNA treated without Triton X-100 (lane 1) as shown in Figure 6D. Similarly, MP\textsubscript{1000}-LPX and MP\textsubscript{2000}-LPX exerted good stability in the presence of serum (Supplementary Figure S15C). These results confirmed the adequate protection of the mRNA against degradation.

**Cytotoxicity Assay**

After incubation with indicated formulations for 24 h, cytotoxicity analysis was performed by flow cytometry. Representative figure of each condition was showed in Figure 7A. The percentage of living DC2.4 cells were found to be 86.7 ± 3.6%, 86.4 ± 1.7%, and 90.1 ± 1.2% (Figure 7B) for control (treated with equal volume of medium), MP\textsubscript{1000}-LPX and Lipo 3K group, respectively. None significant percentage difference of living cells, early apoptosis, late apoptosis or necrosis was found among these three groups indicating that MP\textsubscript{1000}-LPX might do no harm to DC2.4 cells. Overall, MP\textsubscript{1000}-LPX NP 5 performed good safety in vitro. Considering the excellent transfection efficacy, MP\textsubscript{1000}-LPX might be one of good candidates for DC-targeting mRNA nanovaccine for in vivo application.

**In vitro Uptake**

In the preliminary uptake experiment, incubation time and concentration of Cou-6 were optimized. According to the results in Supplementary Figures S16A,B, 2 h and 10 ng/mL were selected for future cellular uptake experiment, respectively. To evaluate the potency of MP\textsubscript{1000}-LPX NP 5 on targeted delivery into DC2.4 cells, the fluorescent of Cou-6 internalized by cells was assayed by BD FACS. The results in Figure 7C indicated that intracellular uptake of MP\textsubscript{1000}-LPX was significantly higher than that of LPX at 37 and 4°C, respectively. When pretreated the cells with mannose, no significant difference of Cou-6 in cellular uptake between MP\textsubscript{1000}-LPX and LPX was observed. Moreover, intracellular uptake of LPX and MP\textsubscript{1000}-LPX was significantly lower at 4°C than that at 37°C, indicating that uptake of LPX loaded with mRNA was energy dependent.

**DISCUSSION**

In the presented study, we designed facile and inexpensive approach to prepare mannose-cholesterol conjugates with various linker length as synthetic ligands applied to mRNA nanovaccine. MP\textsubscript{n}-LPs were prepared by a modified thin-film dispersion method. Subsequently, the DCs targeting MP\textsubscript{n}-LPX were prepared by complexing MP\textsubscript{n}-LPs with mRNA. No significant difference in size and zeta potential was observed among MP\textsubscript{n}-LPX comprised MP\textsubscript{n}-CH with different PEG units. The effect of linker length of mannose derivatives in MP\textsubscript{n}-LPX on transfection by DC2.4 cells was investigated. Our results might provide a rational design element of mRNA vaccine.

Linker length of ligand exerted significantly effect on targeting cellular uptake (Engel et al., 2003; Stefanick et al., 2013; Jeong et al., 2014). Thus, a proper linker length of the ligands was essential for effective receptor recognize and binding. For recognition and binding to MR, linker length of mannose should consist of at least two PEG units according to previously report (Jeong et al., 2014). In our study, mannose-cholesterol conjugates with different linker length were designed and constructed by facile strategies utilizing the click reaction. In detail, mannose derivatives have been conjugated to cholesterol derivatives modified with PEG of different lengths (PEG\textsubscript{100}, PEG\textsubscript{1000}, or PEG\textsubscript{2000}). Each target product was fully characterized by \textsuperscript{1}H-NMR, ESI-MS, FTIR to confirm the successful of synthesis. HPLC was used to evaluate the purity and the successful synthesis of the products as previously reported (Shariat et al., 2014). The synthetic strategies designed here offered some overt advantages over the previously reported methods for Man-C\textsubscript{6}-chol (Kawakami et al., 2000) and Man-C\textsubscript{6}-chol (Li et al., 2013), because our target products were easily to synthesis, purify and characterize. What's more, the length of PEG-linkers could be varied with desired length, resulting in many other analogous compounds with MR targeting function.

We constructed MP\textsubscript{n}-LPs with various linker lengths between cholesterol and mannose using different length of PEG linker. All MP\textsubscript{n}-LPs were constructed with the same molar ratio of mannose modified cholesterol. The MP\textsubscript{n}-LPs had similar average size and surface charge despite of the introduction of MP\textsubscript{n}-CHs with different linker lengths of PEG. We then prepared MP\textsubscript{n}-LPX and performed cellular transfection studies in DC2.4 cells with the evaluated parameters of transfection efficiency and MFI of GFP positive cells, respectively. Transfection efficiency and MFI of LPX was preliminary optimized to find optimal ratio of N/P. Our results showed no significant difference in transfection efficiency of LPX NP 5 and LPX NP 7, while both exhibited significantly higher transfection efficiency than LPX NP 3. However, MFI of the GFP positive cells of LPX NP 5.
FIGURE 5 | Characterization of MP_{1000}-LPX. Appearance (A) and Tyndall effect (B) of MP_{1000}-LPX. Size (C) and zeta potential (D) of MP_{1000}-LP were recorded by Zetasizer Nano ZS90, and representative images were showed. Representative images of size (E) and zeta potential (F) of MP_{1000}-LPX. (G) TEM images of the MP_{1000}-LPX NP 5. (H) Agarose gel electrophoresis image of mRNA maker, Free mRNA, MP_{1000}-LPX (N/P 5) and MP_{1000}-LPX (N/P 3).

was significantly higher compared to that of LPX NP 7. Taken the transfection efficiency and MFI of the GFP positive cells into consideration, the ratio of N/P of 5 was selected for future studies. The difference between the percentage of transfected cells and the MFI of transfected cells was consistent with previously reported results (McLenachan et al., 2013; Avci-Adali et al., 2014;
Lee et al., 2015; Li et al., 2017a). Transfection using the same transfection reagent led to similar transfection efficiency but not the MFI (Figures 4D,E) with the increase of the incubation time, which was consistent with previously report (Avci-Adali et al., 2014). In addition, increasing the amount of mRNA can significantly increase the average fluorescence intensity without affecting the transfection efficiency within a certain range (Avci-Adali et al., 2014). Moreover, MFI values showed the strength of the fluorescence intensity. Higher MFI values reflected a higher production of GFP by individual cell but not higher percentage of GFP positive cells.

According to literatures, upon interaction with serum, nanocarriers rapidly absorbed protein and formed a corona (Bertrand et al., 2017; Pan et al., 2017). It was the nanocarrier–corona complex, rather than the nanocarrier, that interacted with biological systems, here with a cell membrane receptor (CD206), which might partially obscure the role of target ligands (Monopoli et al., 2012). To reduce this effect, culture medium without serum was used at initial and complete culture medium with serum were added 4 h after the adding of LPX. As was known, the fate of nano-preparations in serum (mimic the in vivo environment) was very important to predict its potential therapeutic efficacy. Our preliminary test showed that the presence serum significantly affected the transfection of MP1000-LPX. We were still working on this. Hopefully, we would show the data of mRNA delivery in the presence of serum in vitro and expression of the nano-preparations in vivo in our future work.

MP1000-LPX exhibited a higher level of transfection efficiency than LPX, MP100-LPX, MP2000-LPX and positive control Lipo 3K. No size and charge variation were found in the vectors then no inference can be made on the transfection efficiency correlation with size and charge. The excellent transfection efficiency of MP1000LPX was most likely attributed to the appropriate linker length used to conjugate mannose and cholesterol. However, MP1000-LPX exerted the highest transfection efficiency but moderate MFI. The inconsistency of cellular transfection efficiency and MFI observed in the field of targeted mannose modified LPX partially resulted from differences in the types of cell, incubation time, the amount of mRNA, ability of lysosome escape and types of lipid mannose modified (Kim et al., 2012; Li et al., 2013; Avci-Adali et al., 2014; Chen et al., 2015; Wang C. et al., 2015). The best effect of MP1000-LPs conjugates may be related to the above factors. However, most likely it was attributed to the linkers used to conjugate mannose and the other different component of targeting formulations (Kim et al., 2012; Wang N. et al., 2014; Wang C. et al., 2015). There are many other known and unknown factors for ligand receptor affinity beside linker length of ligand. Moreover, the intracellular metabolism of mRNA nanovaccine might also affect the expression of protein encoded by mRNA. Accurately, we cannot declare that the linker length

![FIGURE 6](Figure6.png)
of MP\textsubscript{1000}–LPX is optimal for transfection by DC2.4 cells but selected MP\textsubscript{1000}–LPX as a representative formulation from our result for further investigation.

The pharmaceutical properties including particle size, zeta potential, storage stability and the ability to protect mRNA against serum degradation of optimal MP\textsubscript{1000}–LPX were then characterized systemically. MP\textsubscript{1000}–LPX displayed bigger diameter and lower zeta potential compared to MP\textsubscript{1000}–LPs, indicating the complexation of MP\textsubscript{1000}–LPs with mRNA. We were surprised to find that particle size measurement results of MP\textsubscript{1000}–LPX by Zetasizer Nano ZS90 was much larger than that by TEM although some other researchers also observed the similar phenomenon (Wang K. et al., 2018; Yang et al., 2018). The larger size distribution by Zetasizer than TEM observed in the field of size measurement partially resulted from the interference of the dispersant into the hydrodynamic diameter. Complete complexation of the mRNA with MP\textsubscript{1000}–LPs was also validated according to the results of gel electrophoresis retardation assay. The preliminary storage stability experiment revealed that MP\textsubscript{1000}–LPX could maintain its excellent transfection efficiency at least for 3 days at 4°C, which might benefit from the protection of the mRNA against degradation. It has been reported that triMN-LPR with high zeta potential (about 35 mV) could better target human and murine dendritic cells, result in higher recruitments of DCs to draining lymph nodes, and induced significant antitumor responses (Le Moignic et al., 2018). Additionally, Folate modified cationic LPs loaded with DNMT1 gene with positive charge (>30 mV) exerted excellent \textit{in vitro} targeted genome editing and \textit{in vivo} antitumor effects (He et al., 2018). However, there seemed to be much cationic charge in the MP\textsubscript{1000}–LPX. Further formulation optimization will be done to balance the transfection efficiency and the high positive zeta potential in our future work.

The cellular cytotoxicity and uptake mechanism of MP\textsubscript{1000}–LPX were also evaluated. MP\textsubscript{1000}–LPX presented good safety \textit{in vitro} according to the data of cell apoptosis and might be a safe formulation for \textit{in vivo} application. It has been reported that the presence of free mannose could decrease the uptake of mannose modified preparations (Li et al., 2013; Wang C. et al., 2014). The amount of free mannose in this study was used according to a previously reported literature (Wang C. et al., 2014). Moreover, the experiment design was similar to
previous reports (Li et al., 2013; Wang C. et al., 2014). When DC2.4 cells were pretreated with free mannose as an inhibitor, no significant effect on uptake by LPX was observed, while uptake by MP$_{1000}$-LPX was significantly decreased. This difference in uptake indicated that the enhanced uptake and transfection were mainly through the MR on DC2.4 cells in line with previous reports (Li et al., 2013; Wang C. et al., 2014). Taken together, our results of uptake in vitro confirmed that the enhanced transfection of MP$_{1000}$-LPX occurs mainly via a MR-mediated mechanism and the linker length of mannose exerts a crucial role. Although MP$_{1000}$-LPX exhibited higher level of transfection efficiency through the MR than MP$_{100}$-LPX and MP$_{2000}$-LPX, we could not exclude that other linker length of mannose modified cholesterol would exhibit more effective transfection in DC2.4 cells through MR. Nevertheless, a rational design element was proposed and more detailed future studies will be indispensable to facilitate the progression of mRNA nanovaccine.

**CONCLUSION**

In summary, MP$_n$-CHs with different linker molecules (PEG$_{100}$, PEG$_{1000}$, and PEG$_{2000}$) were successfully synthesized by a simple and cost-efficient method. The DC-targeting LPs complexed with mRNA were self-assembled using MP$_n$-CHs as the targeting lipids. The linker molecules had no effect on the particle size and zeta potential of LPs and mRNA-complexed LPs but significantly affected the transfection efficiency of GFP-encoding mRNA. Unexpectedly, PEG$_{1000}$ rather than PEG$_{100}$ or the commonly used PEG$_{2000}$ as the linker achieved the maximal level of GFP expression. MP$_{1000}$-LPX containing MP$_{1000}$-CH displayed good profiles including small size with nearly spherical shape, good stability in serum and little cytotoxicity, indicating a hopeful DCs-targeting delivery system for mRNA vaccine.

**AUTHOR CONTRIBUTIONS**

XS conceived the project. MF and JW designed the experiments. FW, WX, and XB conducted most of the experiments. WX and QZ further performed and analyzed the transfection and characterization of formulations. FW, ME, and WX drafted the manuscript. LG and SY performed the 1H-NMR and HPLC analysis. QZ and YZ performed the some preliminary experiments. WX, QZ, and AF participated in literature searching. XS and MF finished the manuscript editing. All authors reviewed and approved the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2018.00980/full#supplementary-material

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