International Journal of Pharmaceutics 520 (2017) 216–227

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

# Lipid nanovehicles with adjustable surface properties for overcoming multiple barriers simultaneously in oral administration

Lei Wu, Min Liu, Wei Shan, Yi Cui, Zhirong Zhang, Yuan Huang*

*Key Laboratory of Drug Targeting and Drug Delivery System, Ministry of Education, West China School of Pharmacy, Sichuan University. No. 17, Block 3, Southern Renmin Road, Chengdu 610041, PR China*

ARTICLE INFO

*Article history:*
Received 6 December 2016
Received in revised form 31 January 2017
Accepted 5 February 2017
Available online 6 February 2017

*Keywords:*
Mucus barrier
Intestinal epithelium barrier
Adjustable surface characteristic
Lipid nanoparticles
N-(2-hydroxypropyl) methacrylamide copolymer (pHPMA)
Hydrophobic substitute

ABSTRACT

Lipid nanoparticles (LNs) are widely investigated for oral drug delivery, and for achieving significant advantages in colloidal stability, biocompatibility and scaled-up possibility. However, researchers face challenge of developing methods to improve the ability of LNs in overcoming multiple barriers (i.e., mucus and epithelium barrier) in gastrointestinal (GI) tract because of the contradictory requirement of nanoparticle (NP) surface properties in the two processes. Therefore, we designed novel LNs with adjustable surface properties by coating lipid core with hydrophobic substitutes grafting N-(2-hydroxypropyl) methacrylamide copolymer (pHPMA). In the present study, different substitutes (i.e., monocyclic, polycyclic, and linear segments) were grafted on pHPMA backbone. Screening studies demonstrated that type and grafting degree of substitutes both influenced hydrophilic-hydrophobic properties of NP surface and improved penetration through mucus. When a hydrophilic-hydrophobic balance was achieved, NPs showed elevated mucus permeability compared with bare LNs; this phenomenon subsequently contributed to higher cellular uptake. Moreover, β-sitosterol (SITO)-modified pHPMA-coated (grafting degree: 5%) LNs (5% SITO-LNs) exhibited the highest mucus permeability, transepithelial transport, and *in situ* absorption. Interestingly, even with the highest surface hydrophilicity, 5% SITO-LNs with Caco-2 cells did not show impaired membrane affinity, which was not observed in other groups. Further investigations of mechanism demonstrated that membrane affinity was significantly enhanced by β-SITO-mediated interaction with Niemann-Pick C1-like 1 (NPC1L1) protein on cell membranes. These results proved that hydrophobic substitutes play a critical role in altering hydrophilic-hydrophobic property of particle surface and improving penetration through multiple barriers. β-SITO-induced specific interaction can provide additional benefits to efficiency of oral delivery of LNs.



## 1. Introduction

Lipid nanoparticles (LNs) are widely applied in various drug administration routes, among which oral delivery is most readily accepted (Dong et al., 2012). Owing to desirable colloidal stability, LNs can protect the drugs from extreme pH and enzymatic conditions in the gastrointestinal (GI) tract (Fan et al., 2014; Silva et al., 2012). However, studies did not focus on methods that can improve ability of LNs to simultaneously conquer multiple barriers in GI tract.

As lipophilic carriers and as the result of superior membrane-affinity, LNs have unique advantages in overcoming epithelial barrier (Wu et al., 2015). Stronger hydrophobic interaction with lipid bilayers can enhance uptake of LNs. Unfortunately, LNs exhibit weak penetration into overlaying net-like mucus layer, which can effectively trap and remove foreign particles by constant secretion and turnover as hydrophobic surface of LNs is more prone to interact with lipophilic segments of mucin via hydrophobic force. To enhance mucus diffusion, polyethylene glycol (PEG)-modified LNs were engineered; however, their application is limited by undesirable cellular uptake induced by extremely hydrophilic nanoparticle (NP) surface (Yuan et al., 2013; Yu et al., 2015).

Hence, we adjusted hydrophilic-hydrophobic property of NP surface and created amphiphilic surface for LNs by coating them with hydrophobic substitutes grafting hydrophilic polymers N-(2-hydroxypropyl) methacrylamide copolymer (pHPMA). It was supposed that shielding effect of densely conjugated hydrophilic

* Corresponding author.
*E-mail address:* huangyuan0@163.com (Y. Huang).

http://dx.doi.org/10.1016/j.ijpharm.2017.02.015
0378-5173/

polymers on surface that weakened cellular internalization, would be prevented by amphiphilic coating. Type and grafting degree of hydrophobic substitutes were reported to affect formation and membrane-affinity of NPs, whereas their influence on mucosal transport remains unexplored.

In the present study, a series of amphiphilic pHPMA were synthesized by grafting them with different types of hydrophobic substitutes (i.e., monocyclic, polycyclic, and linear segments). NPs were assembled with pHPMA shell and lipid-based core (*p*-LNs), and core-shell structure was identified. Studies determined influence of type and grafting degree of lipophilic fraction on mucus permeability. Comparisons involved cellular uptake, transepithelial transport, and *in situ* absorption of varying formulations. Intriguingly, β-sitosterol-modified pHPMA-coated (grafting degree: 5%) LNs (5% SITO-LNs) showed the highest mucus permeability and transepithelial transport. 5% SITO-LNs did not exhibit impaired membrane affinity with Caco-2 cells even at high surface hydrophilicity. Mechanistic study was performed to explore the endocytic pathways, and experiments investigated specific interaction between β-SITO and NPC1L1 protein on cell membranes via chemical inhibitors.

## 2. Materials and methods

### 2.1. Materials

Soybean phospholipid was supplied by Taiwei Pharmaceutical Co. (Shanghai, China). β-SITO was purchased from Chengdu Push Bio-technology Co., Ltd. (Chengdu, China). Fluorescein isothiocyanate (FITC) was obtained from Acros Organics. Rhodamine B isothiocyanate (RITC), benzyl methacrylate, ezetimibe, tetradecylamine, dodecanamine, and octylamine were purchased from Huaxia Reagent (Chengdu, China). Octadecylamine and hexadecylamine were purchased from Xiya Reagent (Linyi, China). Mucin from porcine stomach (type II) was gained from Sigma–Aldrich (St. Louis, MO, USA). Coomassie Brilliant Blue (CBB) G-250, *N,N′*-dicyclohexylcarbodiimide was purchased from Aladdin (Shanghai, China). N-3-Aminopropylmethacrylamide hydrochloride (APMA) was obtained from PolySciences. All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA), Alfa Aesar (Ward Hill, USA), and Shanghai Chemical Reagents Co. (Shanghai, China) and were used as received unless otherwise stated.

### 2.2. Synthesis and characterization of various pHPMA derivatives

Monomers of HPMA, *N*-methacryloylglycylglycyl-*p*-nitrophenyl ester (MA-GG-ONp), and N-Methacryloylglycylglycyl-b-sitosterol ester (MA-GG-SITO) were synthesized according to previous reports (Li et al., 2014; Zhou et al., 2014).

For monocyclic hydrophobic group-modified pHPMA, benzyl methacrylate (Bn) was selected as substitute with graft degrees of 1%, 5%, and 10%. Reaction relied on radical polymerization in methanol (Azobisisobutyronitrile (AIBN), 2 wt%; monomer concentration 12.5 wt%). Reaction was carried out in sealed ampoules filled with inert nitrogen gas at 50 °C for 24 h. Copolymer was isolated from reaction solution by precipitation in large volume diethyl ether, followed by dialysis against distilled water for 3 d and freeze-drying, yielding benzyl-modified pHPMA with different graft degrees (1%Bn-pHPMA, 5% Bn-pHPMA, and 10% Bn-pHPMA).

To obtain polycyclic hydrophobic group-modified pHPMA, MA-GG-SITO was polymerized at ratios of 1%, 5%, and 10%. Copolymerization and purification occurred under same conditions as mentioned above (Zhou et al., 2014). Then the target products, SITO-pHPMA of different graft degrees (1% SITO-pHPMA, 5% SITO-pHPMA, and 10%SITO-pHPMA), were obtained.

Linear carbon chains with different lengths were polymerized along with HPMA. First, HPMA and MA-GG-ONp were copolymerized by radical precipitation polymerization in acetone (AIBN, 2 wt%; monomer concentration, 12.5 wt%). The sediment of pHPMA-GG-ONp was dissolved in *N,N*-dimethylformamide followed by addition of aliphatic amine at certain type and ratio. Mixture was stirred at room temperature for 5 h and then loaded in dialysis bag against ethanol/water (1:1 v:v) for 5 d and water for another 3 d. Straight chains of 8–18 carbon atoms were modified onto pHPMA, obtaining C8-, C12-, C14-, C16-, and C18-pHPMA.

The molecular weight (Mw) and polydispersity index (PDI) of pHPMA derivatives were determined by size exclusion chromatography via Superose 200 10/300GL analytical column (Amersham Biosciences, NJ) using fast protein liquid chromatography (AKTA) system (Amersham Biosciences, NJ) (Liu et al., 2016). Graft degree was measured by high-performance liquid chromatography (Agilent 1200 series) and proton nuclear magnetic resonance spectra (Fig. S1).

### 2.3. Preparation and characterization of phospholipid complex-loaded LNs

As advanced lipid-based nanocarriers, LNs were prepared by solvent evaporation and high-pressure homogenization as previously described (Wang et al., 2011). In brief, 60 mg of soybean phospholipids and 100 μg of Dil were dissolved in 10 ml of acetone and gently agitated at 50 °C for 1 h to form clear mixture. Lipid matrix (50 μl) was then added, and solution was stirred for another 5 min. Afterward, solvent was removed via decompressing evaporation, and lipid film was obtained. Lipid film was rehydrated in deionized water (10 ml) and subjected to ultrasonication at 250 W (10 s for 5 times). At last, the pre-emulsion was induced using high-pressure homogenizer for 5 cycles at operating pressure of ~100 MPa, obtaining LNs dispersed in aqueous phase with phospholipid concentration of 6 mg/ml.

To prepare hydrophilic polymer-coated LNs with core-shell structure, LN dispersion rapidly stirred with aqueous medium containing amphiphilic pHPMA derivatives. The exposed lipophilic groups of the core and pHPMA derivatives were combined via hydrophobic interaction, forming LNs with hydrophilic coating (*p*-LNs). Dynamic light-scattering was used to determined size, PDI, and zeta potential of LNs and *p*-LNs via Malvern Zetasize NanoZS90 (Malvern Instruments Ltd., UK). LNs and *p*-LNs were both analyzed by using NanoSight LM10 system (Malvern Instruments Ltd., UK) equipped with video capture and particle tracking software.

### 2.4. Structural determination of NPs

The morphology of NPs was observed using transmission electron microscope (TEM, H-600, Hitachi, Japan). Freshly prepared NPs were placed on a copper grid covered with Formvar film and then dyed with phosphotungstic acid (2%, pH 6.5). After deposition for 2 min, the grid was carefully dabbed with filter paper to remove water on surface, air dried, and then examined at 75 kV (Liu et al., 2016).

Fluorescent resonance energy transfer (FRET) analysis was applied to detect core-shell structure of *p*-LNs (Shan et al., 2015). First, RITC-labeled phospholipid and FITC-labeled pHPMA were synthesized to prepare NPs. In brief, RITC and phospholipid were dissolved in methanol under stirring conditions at the molar ratio of 5:95. After 8 h, solvent was evaporated, and mixture was freeze-dried to obtain RITC-labeled phospholipids. FITC-labeled pHPMA was obtained by conjugating FITC with HPMA-APMA polymer precursor at 5% molar ratio. Prior to the FRET study, LN suspension was purified in deionized water using dialysis bag with 100 kDa molecular weight cutoff to remove free RITC. Then, labeled