1 **Lipid nanovehicles with adjustable surface properties for**
2 **overcoming multiple barriers simultaneously in oral**
3 **administration**

4
5 *Lei Wu, Min Liu, Wei Shan, Yi Cui, Zhirong Zhang and Yuan Huang**

6
7 Key Laboratory of Drug Targeting and Drug Delivery System, Ministry of Education,
8 West China School of Pharmacy, Sichuan University. No. 17, Block 3, Southern
9 Renmin Road, Chengdu 610041, P.R. China

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.**

Keywords: mucus barrier, intestinal epithelium barrier, adjustable surface characteristic, lipid nanoparticles, N-(2-hydroxypropyl) methacrylamide copolymer (pHPMA), hydrophobic substitute

1. Introduction

Lipid nanoparticles (LNs) **are** widely applied in various **drug administration routes**, among which oral delivery is most readily accepted [1]. **Owing** to desirable colloidal stability, LNs **can** protect the **drugs** from **extreme** pH and enzymatic conditions in the gastrointestinal (GI) tract [2, 3]. 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 [4]. **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

1 LNs is more prone to interact with lipophilic segments of mucin via hydrophobic
2 force. To enhance mucus diffusion, polyethylene glycol (PEG)-modified LNs were
3 engineered; however, their application is limited by undesirable cellular uptake
4 induced by extremely hydrophilic nanoparticle (NP) surface [5, 6].

5

6 Hence, we adjusted hydrophilic-hydrophobic property of NP surface and created
7 amphiphilic surface for LNs by coating them with hydrophobic substitutes grafting
8 hydrophilic polymers N-(2-hydroxypropyl) methacrylamide copolymer (pHPMA). It
9 was supposed that shielding effect of densely conjugated hydrophilic polymers on
10 surface that weakened cellular internalization, would be prevented by amphiphilic
11 coating. Type and grafting degree of hydrophobic substitutes were reported to affect
12 formation and membrane-affinity of NPs, whereas their influence on mucosal
13 transport remains unexplored.

14

15 In the present study, a series of amphiphilic pHPMA were synthesized by grafting
16 them with different types of hydrophobic substitutes (i.e., monocyclic, polycyclic, and
17 linear segments). NPs were assembled with pHPMA shell and lipid-based core
18 (p-LNs), and core-shell structure was identified. Studies determined influence of type
19 and grafting degree of lipophilic fraction on mucus permeability. Comparisons
20 involved cellular uptake, transepithelial transport, and *in situ* absorption of varying
21 formulations. Intriguingly, β -sitosterol-modified pHPMA-coated (grafting degree: 5%)
22 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

1 Monomers of HPMA, *N*-methacryloylglycylglycyl-*p*-nitrophenyl ester
2 (MA-GG-ONp), and *N*-Methacryloylglycylglycyl-b-sitosterol ester (MA-GG-SITO)
3 were synthesized according to previous reports [7, 8].

4

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

13

14 To obtain polycyclic hydrophobic group-modified pHPMA, MA-GG-SITO was
15 polymerized at ratios of 1%, 5%, and 10%. Copolymerization and purification
16 occurred under same conditions as mentioned above [8]. Then the target products,
17 SITO-pHPMA of different graft degrees (1% SITO-pHPMA, 5% SITO-pHPMA, and
18 10%SITO-pHPMA), were obtained.

19

20 Linear carbon chains with different lengths were polymerized along with HPMA.
21 First, HPMA and MA-GG-ONp were copolymerized by radical precipitation
22 polymerization in acetone (AIBN, 2 wt%; monomer concentration, 12.5 wt%). The

1 sediment of pHPMA-GG-ONp was dissolved in N,N-dimethylformamide followed by
2 addition of aliphatic amine at certain type and ratio. Mixture was stirred at room
3 temperature for 5 h and then loaded in dialysis bag against ethanol/water (1:1 v:v) for
4 5 d and water for another 3 d. Straight chains of 8-18 carbon atoms were modified
5 onto pHPMA, obtaining C8-, C12-, C14-, C16-, and C18-pHPMA.

6

7 The molecular weight (Mw) and polydispersity index (PDI) of pHPMA derivatives
8 were determined by size exclusion chromatography via Superose 200 10/300GL
9 analytical column (Amersham Biosciences, NJ) using fast protein liquid
10 chromatography (AKTA) system (Amersham Biosciences, NJ) [9]. Graft degree was
11 measured by high-performance liquid chromatography (Agilent 1200 series) and
12 proton nuclear magnetic resonance spectra (Figure S1).

13

14 **2.3. Preparation and characterization of phospholipid complex-loaded LNs**

15 As advanced lipid-based nanocarriers, LNs were prepared by solvent evaporation and
16 high-pressure homogenization as previously described [10]. In brief, 60 mg of
17 soybean phospholipids and 100 µg of Dil were dissolved in 10 ml of acetone and
18 gently agitated at 50 °C for 1 h to form clear mixture. Lipid matrix (50 µl) was then
19 added, and solution was stirred for another 5 min. Afterward, solvent was removed via
20 decompressing evaporation, and lipid film was obtained. Lipid film was rehydrated in
21 deionized water (10 ml) and subjected to ultrasonication at 250 W (10 s for 5 times).
22 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 [9].

Fluorescent resonance energy transfer (FRET) analysis was applied to detect core-shell structure of p-LNs [11]. First, RITC-labeled phospholipid and FITC-labeled pHPMA were synthesized to prepare NPs. In brief, RITC and phospholipid were

1 dissolved in methanol under stirring conditions at the molar ratio of 5:95. After 8 h,
 2 solvent was evaporated, and mixture was freeze-dried to obtain RITC-labeled
 3 phospholipids. FITC-labeled pHPMA was obtained by conjugating FITC with
 4 HPMA-APMA polymer precursor at 5% molar ratio. Prior to the FRET study, LN
 5 suspension was purified in deionized water using dialysis bag with 100 kDa molecular
 6 weight cutoff to remove free RITC. Then, labeled polymers, uncoated LNs, and
 7 p-LNs were dispersed in water at same concentration of fluorescence dye.
 8 Fluorescence intensity was measured by fluorescence spectrophotometer (Shimadzu
 9 RF-5301, Japan) at excitation wavelength of 440 nm; emission spectrum was recorded
 10 from 490 nm to 590 nm. The following formulas were then used to calculate energy
 11 transfer efficiency and average donor-acceptor distance between FRET dye molecules
 12 [12, 13].

$$E = R \frac{R_0^6}{R^6 + R_0^6} = 1 - \frac{F_{DA}}{F_D}$$

$$R = R_0 [(1 - E) - 1]^{1/6}$$

16 where F_{DA} and F_D represent fluorescence intensity of donor alone and with presence
 17 of acceptor, respectively. R_0 is Förster radius, and R is distance between donor and
 18 acceptor.

19

20 **2.5. Determination of hydrophobic microenvironment of NPs**

21 Microenvironmental hydrophobicity of NPs was determined with CBB G-250 dye as
 22 previously reported [14, 15]. With indicator of bathochromic shift ($\Delta\lambda$) of absorption

1 peak of CBB, binding experiments were carried out by varying NP concentration at
2 constant CBB concentration. LNs and p-LNs were determined by the protocol as
3 follows. In brief, 2 ml of NP solution, of different concentrations, was mixed with 2
4 ml of CBB solution (0.1 mM), and mixture was incubated at room temperature for 20
5 min. NPs were replaced by phosphate-buffered saline (PBS) in control group.
6 Absorption spectrum of CBB was monitored within range of 400-800 nm to detect the
7 shift in peak absorption wavelength, using Varioskan Flash Multimode Reader
8 (Thermo Fisher Scientific, USA).

9

10 **2.6. Stability study**

11 To test stability of NPs in oral delivery, PBS (pH 7.4) and 10 mg/ml mucin solution
12 were used as media, respectively. Freshly prepared LNs and p-LNs were incubated in
13 the medium at 37 °C in a shaker. At predetermined time points (0, 3, and 12 h),
14 samples were collected and particle size was measured by dynamic light scattering via
15 Zetasize Nano ZS90.

16

17 **2.7. Mucus penetration and in vivo distribution**

18 A conceptually straightforward method was applied to study *in vitro* mucus
19 permeability of LNs and p-LNs. Model used was Transwell® chamber with 100 µl
20 porcine intestinal mucus covering the filters (thickness of mucus: ~1 mm to 2 mm)
21 [16, 17]. First, 800 µl PBS (pH 7.4) was added in the acceptor compartment, and after
22 15 min of equilibration, 200 µl NPs were slightly and carefully instilled in the donor

1 side. At determined time points (0, 0.5, 1, 2, 3, 4, and 5 h), an aliquot of sample (50 µl)
2 was withdrawn from the acceptor side, followed by the addition of 75 µl dimethyl
3 sulfoxide (DMSO) to destroy NPs. Permeated Dil was measured by Varioskan Flash
4 Multimode Reader (Thermo Fisher Scientific). Papp value was calculated as follows:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A \times C_0}$$

7 where dQ/dt is the flux of Dil from donor compartment to acceptor compartment; C₀
8 is the initial concentration of Dil in donor side, and A is the membrane area (cm²).

10 Viscosity was measured as described previously [18]. A total of 8 ml porcine gastric
11 mucin solution (5% w/w) was freshly prepared and equilibrated for 15 min at room
12 temperature. Then, the sample was added into the sample cup and measured at spindle
13 speed of 10 rpm (Brookfield LVDV-C; spindle: S18). Control used was apparent
14 viscosity of mucin solution. To test the influence of LNs and p-LNs on the viscosity
15 of mucin solution, 5% mucin solution containing LNs and p-LNs was separately
16 prepared and measured.

18 *In vivo* distribution of NPs was studied on rats as previously reported [19, 20]. In brief,
19 50 µl NPs was delivered by oral gavage, and rats were sacrificed after 2 h, followed
20 by the excision of ileum. Isolated intestinal segments were washed with normal saline
21 to remove the NPs in guts. Tissues were sliced longitudinally and clamped between
22 two glass slides sealed with glue. Images were obtained using Olympus microscope

1 and triplicate samples were measured for each group. Blank tissue was prepared to
2 assess background tissue fluorescence levels to ensure that all **captured** images were
3 well above background levels. The area covered was quantified with ImageJ, and the
4 percentage of coverage was calculated.

5

6 **2.8. Cellular uptake, transepithelial transport, and in situ absorption studies**

7 Mucus-secreting HT29-MTX-E12 (E12) cells were cultivated in Dulbecco's Modified
8 Eagle's Medium (Invitrogen) with high glucose, 10% (v/v) fetal bovine serum
9 (Sigma), 1% (v/v) nonessential amino acid, 1% penicillin and streptomycin (100
10 IU/ml), and 1% (v/v) L-glutamine. Culture was maintained at 37 °C and 95% relative
11 humidity with 5% CO₂. E12 cells were seeded onto 96-well plates (Corning, NY,
12 USA) at 1×10^4 cells/well for cytotoxicity and uptake assays. For transport study, the
13 cultured cells were seeded onto the Transwell® chambers consisting of polycarbonate
14 membrane (**pore size: 3 μm; cell growth area: 0.33 cm²**) at 3×10^4 cells/well. Medium
15 on both sides was refreshed every two days, and transepithelial electrical resistant
16 (TEER) values were measured to observe the barrier formation. After **culturing** for 21
17 days, the monolayer was available for further use [21].

18

19 In this part, NPs were prepared with Dil **loading**. First, cell survival study was
20 determined by resazurin survival assay to test cytotoxicity of NPs on E12 cells as
21 **previously described** [22]. After co-incubation with NPs for 4 h, cells were cultured
22 with fresh medium for another 24 h followed by the detection of live cells.

1

2 To explore cellular internalization of NPs and influence of mucus, the mucus layer
3 was removed by pretreatment using N-acetyl-L-cysteine (NAC) [23]. Then, E12 cells
4 with and without mucus were incubated with NPs at the determined concentration for
5 4 h, respectively. To differentiate NPs trapped in mucus from those internalized by
6 cells, mucus was removed by washing the cell monolayers thrice with 200 µl of 3%
7 (v/v) formalin solution in PBS after co-incubation. Cells were then washed twice with
8 ice-cold PBS. Cells washed with PBS alone were used to detect NPs in both mucus
9 and cells. The cell-associated fluorescence concentration was determined with
10 Varioskan Flash Multimode Reader and number of cells was calculated by Alamar
11 Blue assay [24].

12

13 E12 cells seeded onto the Transwell® chambers were used in investigation of
14 transepithelial transport of NPs [9]. Prior to study, media in both apical and
15 basolateral chambers were replaced by prewarmed Hank's balanced salt solution
16 (HBSS) followed by equilibration for 30 min at 37 °C. Then, HBSS in apical
17 chambers was replaced by 200 µl of fresh batch of same solution containing NPs.
18 After 10 h, an aliquot of basolateral medium (100 µl) was withdrawn and DMSO was
19 added to destroy NPs. Then, fluorescence concentration was determined as described
20 above. TEER value of each well was detected before and after the co-incubation to
21 explore the integrity of cell monolayer.

22

1 To better understand the absorption of NPs in the intestinal villi, an *in situ* absorption
2 study was performed in mice [25]. Animals were fasted overnight before experiment
3 with free access to water. The mice were anesthetized by pentobarbital sodium (40
4 mg/kg), and midline laparotomy was performed to expose the ileum. At the
5 proximal-to-pylorus segments, section (2 cm) of small intestine was ligated at both
6 ends. Subsequently, 200 µl NPs was directly administered into the sections. After 2 h
7 of administration, the mice were sacrificed by cervical dislocation. Intestinal section
8 was withdrawn and washed with abundant normal saline, fixed with 4%
9 paraformaldehyde for 2 h, and dehydrated in 30% sucrose at 4 °C overnight. Samples
10 were quickly frozen with liquid nitrogen-cooled optimal cutting temperature
11 (OCT)-compound. The tissue was then sectioned and stained with
12 4',6-diamidino-2-phenylindole (DAPI) (5 µg/ml). Finally, images of intestinal villi
13 were obtained using confocal laser scanning microscope (CLSM).

14

15 All animals received care in compliance with relevant laws and guidelines outlined in
16 the Guide for the Care and Use of Laboratory Animals. All procedures were approved
17 by Sichuan University Animal Care and Use Committee.

18

19 **2.9. Uptake mechanism study**

20 To investigate the mechanism of cellular internalization of LNs and p-LNs, uptake
21 study was performed with different specific endocytic inhibitors [26, 27]. Rottlerin
22 was used as the macropinocytic inhibitor, lovastatin as the caveolae-mediated

1 endocytic inhibitor, chlorpromazine as the clathrin-mediated endocytic inhibitor,
2 methyl- β -Cyclodextrin (M- β -CD) as the disruptor of lipid raft, and NaN₃ as
3 energy-dependent endocytic inhibitor. To explore influence of ezetimibe on cellular
4 uptake, ezetimibe (0.005 mM) was added in ethanol (0.04%) as previously reported
5 [28].

6

7 Caco-2 cells were first pre-incubated for 30 min with inhibitors and then treated with
8 NPs for another 4 h in the presence of inhibitors. The relative amount of uptake was
9 measured as described above. Control was amount of cell uptake without
10 co-incubation with inhibitors.

11

12 Effect of ezetimibe was also studied on everted intestinal rings using previously
13 described method [29]. Male Sprague-Dawley rats (280–320 g) were sacrificed, and
14 segments of rings were cut and weighed (60–90 mg). Then, intestinal segments were
15 immersed in 95% O₂/5% CO₂-saturated pH 7.4 Krebs Henseleit (KH) salts buffered
16 with NaCO₃ at 4 °C. Before uptake study, rings were everted and placed in 24-well
17 plates. After washing rings twice with KH, 1 ml freshly prepared KH containing
18 ezetimibe (0.005 mM) was added, and the co-incubation lasted for 45 min. The
19 medium was removed and the mixture of NPs and ezetimibe was added. The rings
20 were incubated with NPs for 45 min, followed by washing with ice cold KH thrice.
21 Then, solution on tissue surface was adsorbed with filter paper, and rings were
22 weighed, destroyed via co-treatment with 10% NaOH for 5 h, and centrifuged (10000

rpm, 30 min). Fluorescence intensity was measured with Varioskan Flash Multimode Reader.

3. Results and discussion

3.1. Polymer synthesis and characterization

As highly hydrophilic polymers, the multifunctionality of pHPMA over PEG enables the synthesis of amphiphilic polymers by readily grafting with hydrophobic segments.

Radical polymerization was used to synthesize series of pHPMA derivatives conjugated with different types and grafting degrees of hydrophobic substitutes. Grafting degrees were adjusted by manipulating monomers. The characteristics including M_w , PDI, and grafting degree were summarized in Table 1. M_w of pHPMA ranged from 41.04 kDa to 48.56 kDa with PDI of ~ 1.5 . Different grafting degrees of hydrophobic substitutes were obtained by conjugating monocyclic Bn (4.2–16.5 mol %), polycyclic β -SITO (0.94–6.64 wt %), or lauroyl groups (C12, 2.83–18.07 mol %) to the HPMA polymer backbone. Other linear carbon chain-modified pHPMA were also synthesized with 8, 14, 16 and 18 carbons at grafting degree of 9.92%, 10.21%, 10.00%, and 11.46%, respectively.

3.2. Characterization of nanoparticles

Nanoparticles were prepared via two steps. First, uncoated LNs were obtained by high pressure homogenization, and size and zeta potential exhibited ~ 209 nm and -27.8 mV, respectively. Then, the core-shell nanocarriers were assembled with LNs and pHPMA

1 coating. Based on the shielding effect of polymer shell on the negative charge of LNs,
2 the change in zeta potential was used as an indicator of coating efficiency. The
3 adequate coating was expected to result in nearly neutral zeta potential by the robust
4 shielding of negative charge.

5

6 HPMA copolymers were classified by the type of hydrophobic substitutes, and results
7 were used to identify function of grafting degree in determining coating efficiency of
8 polymer shell. First, zeta potential of LNs coated by Bn-pHPMA of different grafting
9 degrees was measured. Only 10% Bn-LNs exhibited the nearly neutral potential (~5
10 mV), implying the best coating efficiency compared to those with lower or higher
11 grafting degrees, which endowed LNs with zeta potential of ~-25 mV and ~-23 mV
12 respectively (Figure S2). Similar grafted amphiphilic polymers were previously
13 investigated in studies of micelles. As reported, the micelles could not be formed with
14 very low grafting degree of hydrophobic group in the polymer precursor [30].
15 Similarly, with lower grafting degree, Bn-pHPMA did not have enough hydrophobic
16 substitutes to adsorb on lipid core via hydrophobic interaction. By contrast, a higher
17 grafting degree provided stronger driving force for micelle formation but possibly
18 induced instability, resulting in larger aggregates as described before [31]. Thus,
19 Bn-pHPMA with higher grafting degree may form micelles by itself instead of
20 interacting with the lipid core during stirring.

21

22 For β -SITO-pHPMA coated LNs, groups with different grafting degrees all exhibited

1 nearly neutral potential around -5 mV (Fig.1A), implying the desirable shielding
2 effect on the negative charge and further achieving coating efficiency. For
3 C12-pHPMA, 10% C12-LNs showed the minimum zeta potential (~-5 mV), which
4 possibly resulted from similar causes as that of Bn-pHPMA groups (Fig.1A). Thus, to
5 obtain desirable coating efficiency, for each hydrophobic segment, different optimal
6 ranges of grafting degrees must be considered. Additionally, formation of p-LNs was
7 hindered by either too low or too high grafting degree.

8

9 The influence of the length of carbon chains on coating efficiency was then evaluated.
10 As shown in Fig.1B, all the NPs had nearly neutral potential around -5 mV regardless
11 of carbon chain length. Succeeding studies investigated different carbon
12 chain-modified pHPMA (grafting degree: 10%), 10% Bn-pHPMA and SITO-pHPMA
13 at varying grafting degrees for their excellent coating efficiency. Additionally, the size
14 of p-LNs did not change significantly compared with bare LNs (Fig.1C); PDI was all
15 around 0.25 (Fig. S3). Both LNs and p-LNs demonstrated Brownian motion in water.
16 Fig.1C showed captured images.

17

18 As highly biocompatible nanovehicles, LNs are attractive carriers for oral drug
19 delivery due to their efficient superior delivery efficiency of poorly water-soluble
20 drugs; their absorption is typical rate-limited dissolution in the GI tract and also
21 formulation-dependent. Thus, mucus penetration and epithelial transport are
22 benefitted by well-performing LNs tailor-designed for mucosal delivery. Hydrophobic

1 dye Dil displayed encapsulation efficiency of over 95% in all formulations.

2

3 3.3. Structural identification of NPs

4 NP appearance was observed via TEM, and visible boundary of the lipid core and
5 polymer shell was visualized as shown in Fig.2A. The core-shell structure was also
6 proven by FRET analysis. The p-LNs prepared with FITC-labeled pHPMA and
7 RITC-labeled phospholipid exhibited strong FRET phenomenon. Then, the energy
8 transfer efficiency and average donor-acceptor distance were calculated as ~11% and
9 3.84 nm, respectively (Fig.2B). Consequently, these two studies revealed core-shell
10 structure of p-LNs. Hydrophobic microenvironment of NPs was tested via
11 co-incubation with CBB G-250 dye. CBB has high water-solubility and a highly
12 hydrophobic domain. Hydrophobicity of microenvironment is reflected by
13 wavelength shift in maximum adsorption of the dye [15]. Wavelength shift
14 continuously rose with increasing NP concentration. At each concentration, all p-LN
15 groups exhibited greater shift in peak absorption wavelength compared with LNs
16 (Fig.2C and 2D), validating the hydrophobic microenvironment induced by pHPMA
17 coating; such result was consequence of hydrophobic interaction between lipid core
18 and hydrophobic segments of polymer shell. Hence, p-LNs were coated with pHPMA
19 and exhibited regular shape via hydrophobic force.

20

21 3.4. Stability study

22 After coating with pHPMA shell, stability of NPs was investigated as zeta potential

1 **became** nearly neutral, and electrostatic repellant among NPs **possibly decreased**. Fig.
2 3A showed the variation in particle size of Bn-LNs and SITO-LNs (1%, 5%, and 10%
3 of grafting degree in pHPMA coating) incubated in PBS (pH 7.4) for 3 h. All p-LNs
4 showed no significant particle growth ($p>0.05$), implying their desirable stability.
5 Also, Fig. 3C demonstrated the similar stability of LNs coated with carbon chains
6 modified pHPMA, suggesting their excellent stability in PBS. Considering the
7 hydrophobic regions of mucin, NPs **bound** with more mucin **with further increase in**
8 hydrophobicity of particle surface, **resulting in** more remarkable increase in size [19].
9 Fig.3B showed variation in particle size of NPs incubated with mucin solution. LNs
10 and Bn-LNs exhibited significant particle growth (~112% to initial, $p<0.05$) after 3h.
11 In contrast, the size of 1% and 5% SITO-LNs showed no obvious change ($p>0.05$),
12 indicating the superior stability. Difference in stability may be related to different
13 hydrophobic interactions **induced** by monocyclic and polycyclic substitutes. Sufficient
14 hydrophobic interaction between pHPMA and the lipid core was very crucial for the
15 maintenance of core-shell nanostructure. For SITO-LNs, sufficient hydrophobic
16 interaction stabilized hydrophilic shell better, which protected the lipid core from
17 binding with hydrophobic groups of mucin. Thus, with less adsorption of mucin,
18 particle growth of SITO-LNs was slighter than that of Bn-LNs.
19
20 Different grafting degrees also played a vital role in **protecting** NPs **from** mucin.
21 **Unlike** 1% and 5% SITO-LNs, 10% SITO-LNs exhibited significant increase in size
22 after co-incubation. **As LN surface had limited** hydrophobic sites, excess β -SITO

1 substitutes possibly “uncombined” during saturation of binding sites in the interlayer;
2 this phenomenon increased possibility of mucin interaction. So the idea was disproved
3 that higher grafting degree would further protect lipid core. Instead, optimal range of
4 grafting degree should be determined to ensure the mucus-inert surface.

5

6 Upon observation of effect on stability by different lengths of carbon chains, samples
7 with longer carbon chains (C14, C16, and C18-LNs) did not exhibit visible change in
8 size because stronger hydrophobicity of long carbon chain groups induced greater
9 hydrophobic force that stabilized hydrophilic shell (Fig.3D).

10

11 Consequently, type and grafting degree of hydrophobic substitutes both influenced the
12 interaction with mucin, affecting the behaviors of NPs during the penetrating through
13 the mucus layer.

14

15 3.5. Mucus penetration and *in vivo* mucosal distribution

16 Interaction between NPs and mucin could reflect the potential of NPs in evading the
17 trapping of mucus. But the permeability through mucus was still worthy to be
18 measured to test the ability of NPs to overcome mucus barrier. Hence, mucus from
19 porcine intestine was taken to simulate the human intestinal condition and Transwell®
20 chambers were utilized to build a 3D model. As shown in Fig.4A, the grafting degree
21 of hydrophobic segments had effect on mucus permeability and 5% SITO-LN showed
22 the highest Papp value of $\sim 13 \times 10^{-5} \text{ cm/s}$ (~ 2.3 -fold to LNs, $p < 0.01$), while $5.57 \times$

1 10^{-5} cm/s and 7.29×10^{-5} cm/s of Papp value were found in 1% and 10% SITO-LN.
2 It indicated that excessively or insufficiently grafted SITO did not favor formation of
3 muco-inert surface in NPs. Compared with the stability study, the superior shielding
4 effect of 5% SITO-pHPMA was significantly magnified when penetrating through
5 mucus. That was because porcine mucus created more severe conditions for NPs, in
6 which 1% SITO-pHPMA could not support enough hydrophobic force and the surface
7 of 10% SITO-LNs showed higher hydrophobicity. Consequently, the grafting degree
8 significantly influenced the hydrophilic-hydrophobic feature of NP surface, and
9 further affected their permeability through mucus.

10

11 In addition, the length of grafted carbon chains also influenced the mucus
12 permeability of p-LNs, among which C16-LNs exhibited the highest Papp value,
13 which was ~1.78-fold higher than that of LNs (Fig.4B, $p < 0.05$). From C8-LNs to
14 C16-LNs, the mucus permeability showed continuous enhancement, which resulted
15 from the increasing hydrophobicity of carbon chains. However, the Papp value of
16 C18-LNs ($\sim 8.77 \times 10^{-5}$ cm/s) was lower than that of C16-LNs ($\sim 9.97 \times 10^{-5}$ cm/s,
17 $p < 0.05$); this observation contradicted our assumption. Considering the amphiphilicity
18 of C18-pHPMA, this phenomenon may be related to emulsification. As reported,
19 when conjugated with carbon chains, vesicles composed of amphiphilic
20 phosphatidylcholine (PCs) derivatives varied in size depending on carbon number
21 [32]. Interestingly, C18 group was distinguishable from other samples of shorter
22 carbon chains. When PC-C18 was mixed with shorter chain groups, the size of

1 vesicles increased sharply, whereas when PC-C18 was replaced by PC-C16 (or
2 PC-C14), only slight increase was observed. Hence, it was supposed that when carbon
3 number reached the critical point, the capability of emulsification of amphiphilic
4 materials did not increase nor decrease, inducing marked growth in particle size.
5 Similar phenomenon may also occur in pHPMA grafted with carbon chains, leading
6 to inferior coating efficiency of C18-pHPMA compared with C16-pHPMA. Therefore,
7 mucus permeability of C18-LNs was lower than that of C16-LNs.

8

9 After exploring influence of grafting degree and length of carbon chains, the influence
10 of different types of hydrophobic substitutes was studied. Fig.4C shows highest
11 mucus permeability ($\sim 13.0 \times 10^{-5} \text{ cm/s}$) in SITO-LNs (5%) followed by C16-LNs
12 ($\sim 9.69 \times 10^{-5} \text{ cm/s}$), whereas Bn-LNs ($\sim 6.26 \times 10^{-5} \text{ cm/s}$) only got a slight enhancement
13 (~ 1.1 -fold, $p < 0.05$) in contrast to LNs ($\sim 5.65 \times 10^{-5} \text{ cm/s}$). The structure of SITO was
14 rather planar and rigid, whereas carbon chains were relatively flexible, which possibly
15 induced distinct hydrophobic interaction as previously described in several researches
16 about micelles. As similarity was observed between micelle formation and the
17 maintenance of core-shell structure, it was supposed that different structures of
18 hydrophobic substitutes probably induced the variation in mucus permeability.

19

20 Difference in mucus permeability was studied by measuring the viscosity of mixture
21 composed of NPs and mucin solution. As reported, viscosity increased upon addition
22 of microemulsion or lipid into mucin solution; this phenomenon resulted from

1 interaction between mucin and lipophilic materials, **whereas** surfactant micelles **did**
2 **not show any influence** [18]. Therefore, the viscosity of 5% mucin solution containing
3 different NPs was measured. As shown in Fig.4D, the viscosity of 5% mucin solution
4 (~180 cP) was **selected** as control and the addition of LNs resulted in ~1.35-fold
5 increase **in** viscosity (~243 cP), which was significantly higher than that of p-LNs
6 ($p<0.01$). **Results** indicated that pHPMA coating successfully shielded lipid core and
7 avoided its interaction with mucin. Further, the type of grafting hydrophobic
8 substitutes on pHPMA affected the viscosity. **The** 5% SITO-LNs group showed the
9 lowest viscosity (~179 cP, $p<0.05$) followed by C16-LNs (~188 cP) and Bn-LNs
10 (~201 cP). Thus, increase **in** viscosity and mucus permeability **are negatively related**,
11 suggesting that the type of grafting hydrophobic segments affected the entanglement
12 with mucin and further the penetration through mucus.

13
14 From *in vivo* study in intestinal mucus, evident difference in uniformity of distribution
15 among NPs tested above was observed. As shown in Fig.4E, relatively narrow
16 distribution existed in LNs group, **whereas** more uniform distribution **was noted** in
17 other p-LNs groups (Bn-LNs, 5%SITO-LNs, and C16-LNs), suggesting that
18 hydrophilic coating enhanced the mobility of NPs via avoiding their **interaction** with
19 mucin. Moreover, SITO-LNs showed the highest tissue coverage of 81.6%, followed
20 by C16-LNs (60.8%) and Bn-LNs (44.7%). Only about 37% of the mucosal surface
21 was **covered** with LNs, which were relatively mucoadhesive compared with p-LNs
22 (Fig.4F). This trend **in** tissue coverage was consistent with that of *in vitro* mucus

diffusion, and broadened distribution on mucosal surface remarkably increased possibility of NPs accessing epithelium [20].

Thereby, amphiphilic pHPMA coating could shield the lipid core and facilitate the mucus permeability of LNs. Maximum elevation of mucus penetration required moderate grafting degree of hydrophobic segments or optimal length of carbon chains. In addition, SITO-LNs showed highest mucus permeability and the most uniform *in vivo* distribution among p-LNs with monocyclic, polycyclic and linear hydrophobic segments.

3.6. Cellular uptake, transepithelial transport and *in situ* absorption

Absorption barrier of epithelium is another challenging obstacle to orally delivered NPs, especially those with highly hydrophilic surface. Hence, the cellular internalization of LNs and p-LNs was then evaluated with mucus secreting cells to explore the influence of different surface characters. Three p-LNs (Bn-LNs, 5% SITO-LNs, and C16-LNs), coated by pHPMA grafted with monocyclic, polycyclic, and linear groups, respectively, showed the highest mucus permeability and were investigated in this part. HT29-MTX-E12 (E12) cell line is sub-clone isolated from HT29 clone, which can mimic the mucosal tissues due to the high mucus production [9]. Thus, the cellular uptake and influence of the overlaying mucus can be both investigated on E12 cells with pre-treating or post-treating procedure.

1 Initially, no significant cytotoxicity was observed for all formulations at phospholipid
2 concentration of 0.3125 mg/ml to 5 mg/ml (Figure S4). Then, the release of loaded
3 dye Dil was studied against the medium of PBS (pH 7.4) and no obvious release was
4 detected within 24 h (Figure S5), confirming the feasibility of tracking the cellular
5 behaviors of NPs by measuring fluorescence of Dil.

6

7 The cellular internalization on E12 cells was first observed by CLSM. As shown in
8 Fig.5A, the SITO-LNs exhibited the strongest fluorescence intensity followed by
9 C16-LNs; Bn-LNs displayed slight enhancement compared with LNs. Further,
10 quantification study verified the results again, showing most significant enhancement
11 in internalization in SITO-LNs (~2-fold higher, $p < 0.01$) than LNs. Since the trend in
12 cellular uptake was in accordance with that of mucus permeability, it was
13 hypothesized that the hindrance of mucus would have vital impact on internalization
14 of NPs. As shown in Fig.5B, the difference between the black bar and red bar
15 represented the NPs trapped in the mucus layer. It was found that NPs trapped in
16 mucus accounted for a considerable proportion in LNs (~38.6%) and Bn-LNs groups
17 (~38.4%), whereas uptake of SITO-LNs and C16-LNs was barely influenced by
18 mucus; results were in accordance with their much higher mucus permeability.
19 Therefore, desirable hydrophilic pHPMA shell successfully enhanced the cellular
20 internalization in the presence of mucus by elevating mucus permeability of NPs.

21

22 Furthermore, to investigate the permeation of NPs across the intestinal epithelium, an

1 *in vitro* transport study was performed on the E12 cell monolayer cultured on a
2 polycarbonate filter. Integrity of E12 cell layer was not destroyed by incubation with
3 NPs (Fig.5C) and the amount of transport (Fig.5D) confirmed **superior** transepithelial
4 permeation **of SITO-LNs** (~1.35-fold **higher than that of** LNs, $p<0.05$). From apical to
5 basolateral side, NPs underwent more authentic and **complex** process compared with
6 cellular uptake. However, cell models and isolated intestinal models are still limited
7 **because of** lack of *in vivo* clearance like enzymatic degradation in the intestinal
8 lumen.

9
10 Therefore, *in situ* adsorption was delivered sequentially to visualize the transport
11 across epithelium in mouse intestine, which was necessary for the effectiveness of
12 orally delivered nano-systems. For LNs and Bn-LNs, most red signals for Dil were
13 located at the epithelium because the mucus layer hindered their access to **internal**
14 villi where capillaries **are found**, suggesting that Bn-HPMA was not available to
15 enhance the transport across epithelium. **By** contrast, successful deep transport into
16 villi (pointed by white arrows) was observed in SITO-LNs and C16-LNs groups, and
17 SITO-LNs exhibited stronger signal (Fig.5E), indicating accessibility to blood
18 circulation. Thus, transepithelial transport of LNs **can be elevated** by altering the
19 hydrophobic-hydrophilic property of NP surface. Thereby, the ability of LNs to
20 conquer the absorption barrier of epithelium was strengthened by the pHPMA coating
21 with proper hydrophobic segments, among which SITO was the best candidate, **as**
22 **proven** by both *in vitro* and *in situ* studies.

1

2 Although the quantitative dominance of p-LNs reaching the epithelium could provoke
3 higher cellular uptake, the hydrophilic surface decreased the membrane affinity. So
4 the cellular internalization with the pre-removal of mucus (blue bar in Fig.5B) was
5 evaluated to test the affinity between NPs and cell membranes. Unmodified LNs
6 showed much higher internalization, and this result was in accordance with our
7 hypothesis that lipophilic surface had superior membrane affinity. Bn-LNs and
8 C16-LNs both showed lower internalization (~74.9% and ~65.7% of LNs respectively)
9 owing to the increased hydrophilicity of particle. However, the uptake amount of
10 SITO-LNs was unexpectedly almost equal to that of LNs. Since SITO-LNs have
11 higher surface hydrophilicity, their uptake mechanism may differ from that of other
12 p-LNs groups, which would be analyzed in-depth in the following contents.

13

14 **3.7. Uptake mechanism study**

15 As shown in preceding studies, 5%SITO-LNs exhibited significantly enhanced mucus
16 permeability through optimal hydrophilic coating, whereas the very hydrophilic
17 surface did not hamper the cellular internalization, which was not observed in other
18 p-LNs groups. Thus, some unique functions may be involved during interaction
19 between 5% SITO-LNs and cell membranes. To test our hypothesis, the cellular
20 uptake on Caco-2 cells was first evaluated to completely eliminate effect of the
21 overlaying mucus. As shown in Fig.6A, significant difference was not observed in
22 uptake between unmodified LNs and 5%SITO-LNs with higher hydrophilic surface,

1 **whereas** Bn-LNs and C16-LNs still **demonstrated lower internalization**.

2

3 In our postulate, grafting SITO **possibly** exposed on the particle surface and interacted
4 with cell membranes. As the exposure of SITO may **be closely related** with grafting
5 degree, the internalization of 1% SITO-LNs and 10% SITO-LNs was then studied;
6 **these groups** had **unique** surface property **compared with** 5% SITO-LNs. For uptake
7 study on E12 cells (Fig.5C), **1% SITO-LNs displayed highest** proportion of NPs
8 trapped in mucus (~41.2%), followed by 10% SITO-LNs (~23.8%). Only ~12.3% of
9 5% SITO-LNs **was** trapped in mucus. The grafting degree dependent phenomenon
10 was in accordance with results of mucus diffusion study.

11

12 In the absence of mucus, uptake continuously rose with increase in grafting degree in
13 both Caco-2 and E12 cells (Fig.6B and 6C), indicating that the surface property
14 differed among three formulations. The uptake of 10% SITO-LNs got strengthened by
15 the pre-removal of mucus (~1.59-fold **elevation**, $p<0.05$), **whereas** enhancement was
16 not observed in 1% SITO-LNs group, suggesting the higher surface hydrophobicity of
17 the former. According to the comprehensive analysis with mucus diffusion study,
18 when the grafting degree was relatively lower, the interaction between SITO and lipid
19 matrix was **insufficient** to stabilize core-shell structure, **exposing** hydrophobic core
20 when struggling against mucin fiber. When **influence** of mucus layer was removed,
21 the hydrophilic surface of 1% SITO-LNs resulted in weaker membrane affinity and
22 lower internalization.

1

2 On the other hand, owing to its significantly higher mucus permeability, surface
3 hydrophilicity of 5% SITO-LNs (or 10% SITO-LNs) was expected to be lower than
4 that of LNs (Fig.4A). However, the amount of uptake on Caco-2 cells was not
5 compromised by enhanced hydrophilicity of surface, and 10% SITO-LNs showed
6 even higher internalization. Thus it was assumed that the cellular uptake not only
7 depended on the surface hydrophobicity, but also on specific mechanisms related to
8 external exposure of SITO.

9

10 To unravel the endocytic mechanism, endocytic pathways were switched off with
11 chemical inhibitors, which was the most reliable and efficient method for exploring
12 the internalization process as reported [33]. Endocytosis of eukaryotic cells consists of
13 several pathways of pinocytosis, including macropinocytosis, lipid rafts,
14 clathrin-dependent, caveolin-dependent and clathrin- (or caveolin-) independent
15 pathways. Thus, chemical inhibitors for each pathway were used respectively and the
16 uptake was then quantified. The ratio of inhibition induced by each inhibitor was
17 calculated as shown in Fig.6D.

18

19 According to previous reports, depletion of adenosine triphosphate (ATP) in the
20 environment hindered the endocytosis, which was an energy-dependent uptake [26].
21 Treatment with NaN_3 can disrupt production of ATP in cells and consequently hinder
22 the internalization of NPs. The significant reduction induced by NaN_3 occurred in

1 both LNs and p-LNs groups, indicating that the energy-dependent endocytosis was
2 **unimpeded** by the hydrophilic coating. Moreover, macropinocytosis pathway was
3 inhibited by rottlerin, which interacted with protein kinase C, and accelerated both
4 actin rearrangement and pinosome closure [34]. All formulations showed no
5 significant reduction of internalization after co-treatment with rottlerin, suggesting
6 that Caco-2 cells did not engulf NPs through macropinocytosis. Similar results were
7 observed in the endocytosis of LNs by Raw264.7 cells as reported previously.

8

9 Both particle size and surface characteristics **s** influenced **d** the mechanism of endocytosis.
10 All p-LNs were relatively mildly affected by the inhibition of NaN₃ (over 80% of
11 initial) compared with LNs (~64% of initial), which may be resulted from the
12 differential hydrophilicity of particle surfaces. More intriguingly, the uptake of
13 SITO-LNs were unaffected by M-β-CD, whereas other formulations all exhibited
14 reduction to varying degrees. Since M-β-CD **depletes** cholesterol and disturbs lipid
15 rafts, β-SITO, with similar structure, may be competitive substrates to cholesterol,
16 diminishing the inhibition effect of M-β-CD and preserving the function of lipid rafts
17 [35]. **Previous reports confirmed** similar influence on cell membranes by β-SITO and
18 cholesterol, and suggested similarity in their functions despite **their slightly different**
19 chemical structures [36]. Further, the caveolin-dependent endocytosis pathway was
20 investigated and SITO-LNs were also the only NPs that **did not show reduced**
21 internalization after co-treatment with lovastatin.

22

1 However, the difference in caveolin/lipid rafts-dependent pathway could not explain
2 the higher uptake of SITO-LNs because exposed β -SITO possibly induced
3 interruption of the inhibition effect, further disturbing the assessment of endocytosis.
4 Afterwards, the clathrin-dependent pathway was switched off by chlorpromazine,
5 which generated its inhibition effect by assembly of clathrin lattices on endosomal
6 membranes and simultaneously preventing coated pit assembly on cell surface [37].
7 Interestingly, only SITO-LNs showed significant reduction (82% of initial amount,
8 $p < 0.01$) after co-incubation with chlorpromazine. Clathrin-dependent pathway is
9 unrelated to endogenous cholesterol, and its involvement was reliable in the
10 SITO-LNs group. Since clathrin is the most vital protein in receptor-mediated
11 endocytosis, it was assumed that receptor-mediated pathway and β -SITO-induced
12 interaction were comparable.

13

14 Per mechanistic study with chemical inhibitors, β -SITO segments were probably
15 exposed on surface of SITO-LNs. And the grafting degree would influence the
16 exposure and further the amount of uptake. To further explore the possibility of
17 specific interaction between β -SITO and cell membrane, the NPC1L1 protein was
18 then studied. NPC1L1 is the putative intestinal sterol transporter, which is highly
19 expressed in the small intestine of mammals and plays a critical role in facilitating
20 absorption of both cholesterol and phytosterols [28, 38]. Ezetimibe was widely used
21 to target and interfere with NPC1L1 to explore the relation between NPC1L1 and
22 sterol transepithelial transport. Hence, ezetimibe was co-incubated with Caco-2 cells

at the **previously described** concentration [28]. As shown in Fig.6E, the internalization of SITO-LNs was reduced by co-treatment with ezetimibe despite of the grafting degree. **Additionally**, no reduction was observed in LNs group, indicating that the inhibition was not non-specific for any particulate. Thereby, SITO-LNs were internalized by Caco-2 cells via NPC1L1 mediated pathway probably because of the participation of SITO segments. To further **investigate** the function of SITO, the inhibition study was conducted on everted intestinal rings of rats because the intestinal tissue could better mimic the physiological conditions and the binding affinity of ezetimibe was enhanced by *in vivo* metabolism. It was concluded from Fig.6F that the inhibition of 10% SITO-LNs was significantly greater (~1.25-fold, $p<0.05$) than that of 1% SITO-LNs, **reflecting** influence of grafting degree on the **endocytic pathway involving NPC1L1**. **This result** was consistent with our hypothesis that **grafting degree affected amount** of SITO exposed outside. Taken together, the grafting β -SITO was multifunctional for maintaining the hydrophobic force between the pHPMA coating and lipid core, and binding to NPC1L1 protein on cell membranes. This specific targeting effect subsequently enhanced the membrane affinity and mediated the cellular uptake. Most importantly, only with the optimal grafting degree could SITO-LNs have desirable exposure of β -SITO on the surface, which was critical **in** maintaining hydrophilic surface **upon penetration of** mucus layer and providing enough β -SITO **upon** reaching the epithelium.

4. Conclusions

1 In this study, a series of amphiphilic pHPMA were synthesized by grafting **them** with
2 different types of hydrophobic substitutes (**i.e.**, monocyclic, polycyclic, and linear
3 segments). The core-shell nanostructure with adjustable surface characteristic was
4 assembled with p-LNs. The formulations with desirable coating efficiency were
5 selected. Amphiphilic pHPMA coating induced higher surface hydrophilicity and
6 facilitated the mucus permeability of LNs. The promotion effect depended on type
7 and grafting degree of hydrophobic substitutes. Transepithelial transport of p-LNs
8 varied with different hydrophobic segments. Most importantly, only 5% SITO-LNs
9 showed both rapid penetration through mucus and improved transepithelial transport.
10 **This result can be attributed** not only to hydrophilic-hydrophobic balance, but also
11 specific interaction between β -sitosterol and NPC1L1 on cell membranes. Our study
12 demonstrated that NP surface properties **play** a vital role in GI transport, and the
13 core-shell nanostructure **can** overcome both diffusion and absorption barrier when
14 hydrophilic-hydrophobic balance of surface was achieved.

17 **ASSOCIATED CONTENT**

18 **Supporting Information**

19 ¹H NMR spectra of hydrophobic substitutes grafted pHPMA; Size, zeta potential
20 and PDI of NPs; Cell viability of E12 cells incubated with NPs; Release of Dil from
21 NPs.

AUTHOR INFORMATION

Corresponding Author

*E-mail: huangyuan0@163.com, Tel.: +86-28-85501617, Fax: +86-28-85501617.

ACKNOWLEDGMENTS

We gratefully acknowledge financial support from the National Science Foundation for Distinguished Young Scholars (81625023) and the Major Research Plan of National Natural Science Foundation of China (81690261).

REFERENCES

- [1] Y. Dong, W.K. Ng, S. Shen, S. Kim, R.B. Tan, Solid lipid nanoparticles: continuous and potential large-scale nanoprecipitation production in static mixers, *Colloids Surf. B. Biointerfaces*, 94 (2012) 68-72.
- [2] T. Fan, C. Chen, H. Guo, J. Xu, J. Zhang, X. Zhu, Y. Yang, Z. Zhou, L. Li, Y. Huang, Design and evaluation of solid lipid nanoparticles modified with peptide ligand for oral delivery of protein drugs, *Eur. J. Pharm. Biopharm.*, 88 (2014) 518-528.
- [3] A.H. Silva, F.B. Filippin-Monteiro, B. Mattei, B.G. Zanetti-Ramos, T.B. Creczynski-Pasa, In vitro biocompatibility of solid lipid nanoparticles, *Sci. Total Environ.*, 432 (2012) 382-388.
- [4] L. Wu, M. Liu, X. Zhu, W. Shan, Y. Huang, Modification Strategies of Lipid-Based Nanocarriers for Mucosal Drug Delivery, *Curr. Pharm. Des.*, 21 (2015)

1 5198-5211.

2 [5] H. Yuan, C.-Y. Chen, G.-h. Chai, Y.-Z. Du, F.-Q. Hu, Improved transport and
3 absorption through gastrointestinal tract by PEGylated solid lipid nanoparticles. *Mol.*
4 *Pharm.* 10 (2013) 1865-1873.

5 [6] T. Yu, K.W. Chan, A. Anonuevo, X. Song, B.S. Schuster, S. Chattopadhyay, Q. Xu,
6 N. Oskolkov, H. Patel, L.M. Ensign, P.C. van Zijl, M.T. McMahon, J. Hanes,
7 Liposome-based mucus-penetrating particles (MPP) for mucosal theranostics:
8 demonstration of diamagnetic chemical exchange saturation transfer (diaCEST)
9 magnetic resonance imaging (MRI), *Nanomed. Nanotechnol. Biol. Med.*, 11 (2015)
10 401-405.

11 [7] L. Li, Q. Yang, Z. Zhou, J. Zhong, Y. Huang, Doxorubicin-loaded, charge
12 reversible, folate modified HPMA copolymer conjugates for active cancer cell
13 targeting, *Biomaterials*, 35 (2014) 5171-5187.

14 [8] Z. Zhou, L. Li, Y. Yang, X. Xu, Y. Huang, Tumor targeting by pH-sensitive,
15 biodegradable, cross-linked N-(2-hydroxypropyl) methacrylamide copolymer micelles,
16 *Biomaterials*, 35 (2014) 6622-6635.

17 [9] M. Liu, J. Zhang, X. Zhu, W. Shan, L. Li, J. Zhong, Z. Zhang, Y. Huang, Efficient
18 mucus permeation and tight junction opening by dissociable “mucus-inert” agent
19 coated trimethyl chitosan nanoparticles for oral insulin delivery, *J. Control. Release*,
20 222 (2016) 67-77.

21 [10] Q. Wang, T. Gong, X. Sun, Z. Zhang, Structural characterization of novel
22 phospholipid lipid nanoparticles for controlled drug delivery, *Colloids Surf. B.*

1 Biointerfaces, 84 (2011) 406-412.

2 [11] W. Shan, X. Zhu, M. Liu, L. Li, J. Zhong, W. Sun, Z. Zhang, Y. Huang,
3 Overcoming the diffusion barrier of mucus and absorption barrier of epithelium by
4 self-assembled nanoparticles for oral delivery of insulin, ACS nano, 9 (2015)
5 2345-2356.

6 [12] J. Lei, L. Wang, J. Zhang, Superbright Multifluorescent Core– Shell Mesoporous
7 Nanospheres as Trackable Transport Carrier for Drug, ACS nano, 5 (2011)
8 3447-3455.

9 [13] P. Batard, J. Szollosi, I. Luescher, J.C. Cerottini, R. MacDonald, P. Romero, Use
10 of phycoerythrin and allophycocyanin for fluorescence resonance energy transfer
11 analyzed by flow cytometry: advantages and limitations, Cytometry, 48 (2002)
12 97-105.

13 [14] T. Akagi, K. Watanabe, H. Kim, M. Akashi, Stabilization of polyion complex
14 nanoparticles composed of poly(amino acid) using hydrophobic interactions,
15 Langmuir, 26 (2010) 2406-2413.

16 [15] X. Zhu, W. Shan, P. Zhang, Y. Jin, S. Guan, T. Fan, Y. Yang, Z. Zhou, Y. Huang,
17 Penetratin derivative-based nanocomplexes for enhanced intestinal insulin delivery,
18 Mol. Pharm., 11 (2013) 317-328.

19 [16] W.M. Saltzman, M.L. Radomsky, K.J. Whaley, R.A. Cone, Antibody diffusion in
20 human cervical mucus, Biophys. J., 66 (1994) 508.

21 [17] S.K. Lai, Y.-Y. Wang, J. Hanes, Mucus-penetrating nanoparticles for drug and
22 gene delivery to mucosal tissues, Adv. Drug Del. Rev., 61 (2009) 158-171.

- 1 [18] J. Zhang, Y. Lv, B. Wang, S. Zhao, M. Tan, G. Lv, X. Ma, Influence of
2 Microemulsion–Mucin Interaction on the Fate of Microemulsions Diffusing through
3 Pig Gastric Mucin Solutions, *Mol. Pharm.*, 12 (2015) 695-705.
- 4 [19] Q. Xu, L.M. Ensign, N.J. Boylan, A. Schon, X. Gong, J.C. Yang, N.W. Lamb, S.
5 Cai, T. Yu, E. Freire, J. Hanes, Impact of Surface Polyethylene Glycol (PEG) Density
6 on Biodegradable Nanoparticle Transport in Mucus ex Vivo and Distribution in Vivo,
7 *ACS nano*, 9 (2015) 9217-9227.
- 8 [20] X. Zhu, J. Wu, W. Shan, Z. Zhou, M. Liu, Y. Huang, Sub - 50 nm Nanoparticles
9 with Biomimetic Surfaces to Sequentially Overcome the Mucosal Diffusion Barrier
10 and the Epithelial Absorption Barrier, *Adv. Funct. Mater.*, (2016).
- 11 [21] I. Behrens, P. Stenberg, P. Artursson, T. Kissel, Transport of lipophilic drug
12 molecules in a new mucus-secreting cell culture model based on HT29-MTX cells,
13 *Pharm. Res.*, 18 (2001) 1138-1145.
- 14 [22] Y. Baglo, L. Hagen, A. Hogset, F. Drablos, M. Otterlei, O.A. Gederaas, Enhanced
15 efficacy of bleomycin in bladder cancer cells by photochemical internalization,
16 *Biomed Res Int*, 2014 (2014) 921296.
- 17 [23] I. Behrens, A.I. Pena, M.J. Alonso, T. Kissel, Comparative uptake studies of
18 bioadhesive and non-bioadhesive nanoparticles in human intestinal cell lines and rats:
19 the effect of mucus on particle adsorption and transport, *Pharm. Res.*, 19 (2002)
20 1185-1193.
- 21 [24] S.D. Sarker, L. Nahar, Y. Kumarasamy, Microtitre plate-based antibacterial assay
22 incorporating resazurin as an indicator of cell growth, and its application in the in

1 vitro antibacterial screening of phytochemicals, *Methods*, 42 (2007) 321-324.

2 [25] C. Chen, X. Zhu, Y. Dou, J. Xu, J. Zhang, T. Fan, J. Du, K. Liu, Y. Deng, L. Zhao,
3 Y. Huang, Exendin-4 Loaded Nanoparticles with a Lipid Shell and Aqueous Core
4 Containing Micelles for Enhanced Intestinal Absorption, *J Biomed Nanotechnol*, 11
5 (2015) 865-876.

6 [26] N.W.S. Kam, Z. Liu, H. Dai, Carbon nanotubes as intracellular transporters for
7 proteins and DNA: an investigation of the uptake mechanism and pathway, *Angew.*
8 *Chem.*, 118 (2006) 591-595.

9 [27] J. Zhang, X. Zhu, Y. Jin, W. Shan, Y. Huang, Mechanism study of cellular uptake
10 and tight junction opening mediated by goblet cell-specific trimethyl chitosan
11 nanoparticles, *Mol. Pharm.*, 11 (2014) 1520-1532.

12 [28] F.J. Field, K. Watt, S.N. Mathur, Ezetimibe interferes with cholesterol trafficking
13 from the plasma membrane to the endoplasmic reticulum in CaCo-2 cells, *J. Lipid*
14 *Res.*, 48 (2007) 1735-1745.

15 [29] P.S. Leppert, J.A. Fix, Use of everted intestinal rings for in vitro examination of
16 oral absorption potential, *J. Pharm. Sci.*, 83 (1994) 976-981.

17 [30] P. Chytil, T. Etrych, C. Konak, M. Sirova, T. Mrkvan, J. Boucek, B. Rihova, K.
18 Ulbrich, New HPMA copolymer-based drug carriers with covalently bound
19 hydrophobic substituents for solid tumour targeting, *J. Control. Release*, 127 (2008)
20 121-130.

21 [31] Y. Wang, C.Y. Ke, C. Weijie Beh, S.Q. Liu, S.H. Goh, Y.Y. Yang, The
22 self-assembly of biodegradable cationic polymer micelles as vectors for gene

transfection, *Biomaterials*, 28 (2007) 5358-5368.

[32] F. Ishii, T. Nii, Properties of various phospholipid mixtures as emulsifiers or dispersing agents in nanoparticle drug carrier preparations, *Colloids Surf. B. Biointerfaces*, 41 (2005) 257-262.

[33] L. Kastl, D. Sasse, V. Wulf, R. Hartmann, J. Mircheski, C. Ranke, S. Carregal-Romero, J.A. Martínez-López, R. Fernández-Chacón, W.J. Parak, Multiple internalization pathways of polyelectrolyte multilayer capsules into mammalian cells, *ACS nano*, 7 (2013) 6605-6618.

[34] R. Zeineddine, J.F. Pundavela, L. Corcoran, E.M. Stewart, D. Do-Ha, M. Bax, G. Guillemín, K.L. Vine, D.M. Hatters, H. Ecroyd, SOD1 protein aggregates stimulate macropinocytosis in neurons to facilitate their propagation, *Mol. Neurodegener.*, 10 (2015) 1-18.

[35] B.J. Nichols, A.K. Kenworthy, R.S. Polishchuk, R. Lodge, T.H. Roberts, K. Hirschberg, R.D. Phair, J. Lippincott-Schwartz, Rapid cycling of lipid raft markers between the cell surface and Golgi complex, *J. Cell. Biol.*, 153 (2001) 529-542.

[36] D.A. Mannock, M.G. Benesch, R.N. Lewis, R.N. McElhaney, A comparative calorimetric and spectroscopic study of the effects of cholesterol and of the plant sterols β -sitosterol and stigmasterol on the thermotropic phase behavior and organization of dipalmitoylphosphatidylcholine bilayer membranes, *Biochim. Biophys. Acta*, 1848 (2015) 1629-1638.

[37] L.-H. Wang, K.G. Rothberg, R. Anderson, Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation, *J. Cell. Biol.*, 123

1 (1993) 1107-1117.

2 [38] J. Chen, Q. Li, Y. Zhang, P. Yang, Y. Zong, S. Qu, Z. Liu, Oleic acid decreases
3 the expression of a cholesterol transport-related protein (NPC1L1) by the induction of
4 endoplasmic reticulum stress in CaCo-2 cells, J. Physiol. Biochem., 67 (2011)
5 153-163.

6

7

8

9

10

11

12

13

14

15

16

17

18

19

20

21

22

- 1 **Table 1** Characteristics of the synthesized pHPMA derivatives grafted with different
 2 hydrophobic groups.

	Grafting degree	Mw (kDa)	Polydispersity index (PDI)
5%Bn-pHPMA	4.20 mol%	45.45	1.63
10%Bn-pHPMA	12.45mol%	41.04	1.54
20%Bn-pHPMA	16.50 mol%	46.43	1.58
1%SITO-pHPMA	0.94 wt%	44.72	1.82
5%SITO-pHPMA	3.30 wt%	46.23	1.58
10%SITO-pHPMA	6.64 wt%	43.89	1.65
10%C8-pHPMA	9.92 mol%	44.08	1.83
5%C12-pHPMA	2.83mol%	46.58	1.70
10% C12-pHPMA	8.23mol%	48.56	1.59
20%C12-pHPMA	18.07mol%	41.50	1.77
10%C14-pHPMA	10.21 mol%	42.96	1.61
10%C16-pHPMA	10.00 mol%	43.67	1.54
10%C18-pHPMA	11.46 mol%	44.07	1.65

- 3 Data are presented as mean $\pm$ SD.

Graphical abstract

Fig.1 (A) Size and zeta potential of LNs and p-LNs coated by cyclic substitutes grafted pHPMA. Error bars represent SD ($n \geq 3$). (B) Size and zeta potential of LNs and p-LNs coated by carbon chains grafted pHPMA. Error bars represent SD ($n \geq 3$). (C) LNs and p-LNs were verified using a NanoSight LM10 system. The particle size (x-axis) versus relative intensity (y-axis) of nanoparticles was generated by LNs and p-LNs (on the left side). Images (on the right side) represented results from nanoparticle tracking analysis (NTA) video capture.

Fig. 2 (A) Transmission electron microscope (TEM) images of LNs and p-LNs. Red dotted lines indicated the lipid core and polymer shell of p-LNs respectively. Scale bars, 100 nm. (B) Emission spectrum of Rho-labelled LNs (red line), FITC-labelled pHPPA (blue line) and FITC-labelled pHPPA coated LNs (black line) excited at 440 nm. (C and D) The shifts of peak absorption wavelength of CBB G-250 dye after incubation with NPs of different concentrations. Error bars represent SD ($n \geq 3$).

Fig.3 Variation in particle size of NPs incubated in PBS (pH 7.4) (A and C) and mucin solution (B and D) after 3 h. Error bars represent SD ($n \geq 3$). * $p < 0.05$, ** $p < 0.01$, versus the initial size.

Fig.4 (A, B and C) Papp values of the NPs permeation across mucus from donor and acceptor compartments. Error bars represent SD ($n \geq 3$). [#] $p < 0.05$. * $p < 0.05$, ** $p < 0.01$, versus LNs. (D) The viscosity (cP) of the mixture containing 5% mucin solution and NPs. Error bars represent SD ($n \geq 3$). [#] $p < 0.05$. * $p < 0.05$, ** $p < 0.01$, versus LNs. Images (E) and surface coverage (F) of NPs on the flattened small intestinal tissue surface after administrated with NPs. Error bars represent SD ($n \geq 3$). ** $p < 0.01$, versus LN. Scale bars, 200 μm.

Fig. 5 (A) Confocal laser scanning microscope (CLSM) images of E12 cell monolayer incubated with LNs and p-LNs. Scale bars, 20 μ m. (B) Relative fluorescence intensity of Dil in E12 cells after incubation with NPs for 4 h. Error bars represent SD ($n \geq 3$). # $p < 0.05$, ## $p < 0.01$. ** $p < 0.01$, versus LNs. Variation in transepithelial electrical resistance (TEER) value after co-incubation with NPs (C) and the relative amount of transport across E12 cells after 10 h (D). Error bars represent SD ($n \geq 3$). * $p < 0.05$, versus LNs. (E) Fluorescence images of sections of mouse intestine after administration of LNs and p-LNs. Scale bars, 30 μ m. White arrows indicated NPs in the deeper villi.

Fig. 6 (A and B) Relative fluorescence intensity of Dil in Caco-2 cells after incubation with NPs for 4 h. Error bars represent SD (n ≥ 3). *p < 0.05, **p < 0.01, versus LNs (A) or 5% SITO-LNs (B). (C) The uptake of NPs on E12 cells. Error bars represent SD (n ≥ 3). #p < 0.05, ##p < 0.01. *p < 0.05, **p < 0.01, versus LNs. (D) The internalization of NPs on Caco-2 cells when pretreated with different chemical inhibitors. Fluorescence intensity in the non-inhibited cells was used as control. Error bars represent SD (n ≥ 3). *p < 0.05, **p < 0.01, versus control. The uptake by Caco-2 cells (E) and intestinal tissues (F) after co-treatment with ezetimibe. Samples without ezetimibe were used as control. Error bars represent SD (n ≥ 3). #p < 0.05, *p < 0.05, **p < 0.01, versus control.