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RESEARCH PAPER

Recombinant High Density Lipoprotein Nanoparticles for Target-Specific Delivery of siRNA

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ABSTRACT

Purpose Regulation of gene expression using small interfering RNA (siRNA) is a promising strategy for treatments of numerous diseases. However, the progress towards broad application of siRNA requires the development of safe and effective vectors that target to specific cells. In this study, we developed a novel recombinant high density lipoprotein (rHDL) vector with high siRNA encapsulation efficiency.

Methods They were prepared by condensing siRNA with various commercial cationic polymers and coating the polyplex with a layer of lipids and apolipoprotein AI (apo AI). The rHDL nanoparticles were used to transfect SMMC-7721 hepatoma cells with stable luciferase expression. The uptake and intracellular trafficing of siRNA were also investigated.

Results Characterization studies revealed these rHDL nanoparticles had similar physical properties as natural HDLs. The various rHDL formulations had high silencing efficiency (more than 70% knockdown) in hepatocytes with minimum cytotoxicity. Moreover, the uptake of rHDL by SMMC-7721 was confirmed to be mediated through the natural HDL uptake pathway.

Conclusions The work described here demonstrated the optimized rHDL nanoparticles may offer a promising tool for siRNA delivery to the liver.

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KEY WORDS cationic polymer · gene silencing · high density lipoprotein · siRNA delivery

ABBREVIATIONS

apo A-I	apolipoprotein A-I
PEI	polyethyleneimine
PLL	poly-L-lysine
RES system	reticuloendothelial system
rHDL	recombinant high density lipoprotein

INTRODUCTION

RNA interference (RNAi) is a natural cellular process that mediates sequence specific gene silencing in both plant and mammalian cells by small interfering RNA (siRNA) molecules (1,2). The siRNA molecules are 21–23 nucleotide (nt) double-strand RNA segments that would suppress gene expression by activating RNA-induced silencing complex (RISC) and subsequently cleaving targeted mRNA(3,4). RNAi has been widely reported to have significant therapeutically potentials. However, the main obstacle of successful RNAi is that siRNA cannot freely penetrate membrane barriers. To transport the intact RNA into the cytoplasm of the target cells, various carriers have been developed, including viral and non-viral delivery systems. The viral vectors have high transfection efficiencies, but safety concerns and high cost limit their applications (5). The non-viral approaches attracted increasing attention in recent years. There were lipid-based and polymer-based systems such as cationic liposomes, polyethyleneimine (PEI), dendrimers etc. (6–10). Some of these vectors had shown promising efficiencies in gene silencing, but the safety and selectivity of the delivery still require significant improvement.

High density lipoproteins (HDLs) are a class of natural molecular assembly structures consisting of a hydrophobic

core surrounded by a monolayer of lipids. The cholesterols and apolipoprotein AI (apo AI) are embedded in the lipid monolayer. These HDL nanoparticles are totally natural and non-immunogenic. They play a key role in the reverse cholesterol transport (RCT) process, in which cholesterols are removed from peripheral cells and transported into the liver for metabolism. Their interactions with the hepatocytes involve two distinctive pathways: selective cholesterol uptake mediated by the scavenger receptor class B Type I (SR-BI), or holo-particle uptake mediated by the mitochondrial β chain of ATP synthase and P2Y13. Based on these functions, recombinant HDL (rHDL) nanoparticles were considered ideal for the delivery of drugs and other materials to the liver. It was reported that rHDL containing cationic lipid DOTAP (1,2-dioleoyl-3-trimethylammonium-propan) could be used for the delivery of DNA and synthetic siRNA to liver (11,12). But the resulted nanoparticles were positively charged with much bigger sizes (>150 nm), which may significantly impair their *in vivo* distribution and uptake behavior. Other reports have showed that cholesterol-conjugated siRNA or DNA molecules could be assembled in rHDL nanoparticles and deliver to cells expressing HDL receptors (13–15). However, siRNA modified with lipophilic moieties was only inserted into the lipid monolayer of rHDL, not encapsulated inside the core(13).

We hypothesize that it would be more desirable to construct recombinant HDL particles that resemble the structure and especially surface properties of natural HDLs. Nanoparticles with positive surface charges may interact with other anionic serum proteins and more easily cleared by the RES system. Gene delivery vectors with negative surface charges were considered more biocompatible. Therefore, anionic lipid based systems were explored (7,16–21). But because of the electrostatic repulsion between DNA molecules and anionic lipids, most studies employed some bridging agents, including metal ions (such as Ca^{2+}, Mg^{2+}, Mn^{2+}), cationic polymers or cationic lipids (22–24).

In this study, we took the cationic polymer/DNA complexation approach but constructed a rHDL nanoparticle with very similar surface properties of a natural HDL. We compared various cationic polymers including protamine, PEIs with three different molecular weights (600 Da, 1,800 Da and 25 kDa) and PLL (15 k–30 kDa). The resulted rHDLs were characterized for their loading efficiencies, sizes and zeta potentials. The optimized rHDL formulation had minimum cytotoxicity, great serum stability, and better siRNA silencing efficiency in the hepatocellular carcinoma cell line SMMC-7721 compared to the cationic polymers by themselves. More importantly, it was demonstrated that the rHDL nanoparticles entered cells via the specific HDL receptor-mediated pathway. We propose that such a system can be used as a promising liver-specific delivery system for siRNA.

MATERIALS AND METHODS

Materials

Branched polyethylenimine (PEI, MW 600 and 1800), linear polyethylenimine (MW 25 K) and sodium cholate were purchased from Alfa Aesar. Protamine sulfate salt and poly-L-lysine (MW 500–2000 and 15000–30000, P7890) were purchased from Sigma-Aldrich. Egg phosphatidylcholine (EPC) and cholesterol were purchased from NOF (Tokyo, Japan). Recombinant human apolipoprotein A-I was expressed in *Escherichia coli* and purified by His-Trap nickel affinity chromatography as described by Ryan *et al.* (25). Other materials were purchased from Sigma-Aldrich unless otherwise stated.

All siRNAs were synthesized by Genepharm Co. (China). The siRNA targeting the luciferase gene (siLuc) consisted of the anti-sense strand 5′–UCGAAGUACUCAGC GUAAGdTdT-3′ and sense strand 5′-CUUACGCUGA GUACUUCGAdTdT-3′. The non-silencing control siRNA (siNS) consisted of anti-sense stand 5′-AUUUGCACAGAU CAGCUGCUCAUUC-3′ and sense strand 5′-UAAACGU GUCUAGUCGACGAGUAAG-3′. For confocal studies, Cy5 dye was labeled at the 5′-end of siLuc siRNA.

Preparation of Recombinant HDL

Several cationic polymers were used for the complexation of siRNA, including protamine sulfate, branched PEI (600 Da and 1,800 Da), linear PEI (25 kDa) and PLL (15 k-30 kDa). One microgram of siRNA was dissolved in 20 μl of Rnase-free water. Various amounts of polymers were also dissolved in 20 μl of Rnase-free water. Then the siRNA solution was added to polymers solutions while vigorous pipetting, resulting in polymer/siRNA complexes with different N/P ratios or weight ratios.

Liposomes with the lipid composition of EPC : cholesterol (20:1, molar ratio), at a total lipid concentration of approximately 100 mM, were prepared using the thin film hydration method. Briefly, chloroform solutions of EPC and cholesterol were mixed, and then the organic solvent was evaporated under nitrogen flow until a thin lipid film was formed. Vacuum desiccation overnight ensured the removal of the residual organic solvent. The film was hydrated in 10 mM Tris buffer (pH8.0) and the liposome suspension was sonicated in an ice bath to obtain a transparent solution. Then 5 μL of cholate solution (100 mg/ml in Tris buffer) and 36 μL of apo A-I protein solution (7 mg/ml) were added to liposome solutions to form mixed micelles containing lipid, cholate and apo AI protein. The polycation/siRNA complex solution was then vortex-mixed with 100 μL of the mixed micelles for 5 min. The final volume was adjusted to 200 μL in 10 mM Tris buffer. The mixture was incubated overnight at 4°C, and then dialyzed against 2 L of Tris buffer, changing 3 times over 2 days.

Agarose Gel Retardation

The siRNA loading efficiency of each rHDL formulation was analyzed using 1% agarose gel electrophoresis. After addition of 6 μl of 6× loading buffer (TAKARA, Japan), the samples were loaded into individual wells of 1% agarose gel and then electrophoresed for 10 min at a voltage of 120 V in 1% TAE buffer solution. The siRNA bands were visualized under a UV transilluminator after stained with a 1:10000 dilution of GelRed dye prepared with Rnase-free water. GelRed was chosen as it was more sensitive than ethidium bromide for staining small RNA.

Particle Size and Zeta Potential Measurements

Fifty microliters of rHDL nanoparticle containing 0.25 μg siRNA were diluted with 1 mL of 10 mM Tris buffer (pH 8.0). The particles size and zeta potential of rHDL formulations were measured by dynamic light scattering with Zetasizer Nano-ZS90 (Malvern, UK).

Transmission Electron Microscopy (TEM)

The rHDL samples were dialyzed against a buffer consisting of 0.125 M ammonium acetate and 2.6 mM ammonium carbonate at pH7.4, and then mixed with a 2% sodium phosphotungstate solution (pH7.4 in distilled water) at 1:1 ratio by volume as described by Forte *et al.* (26). A droplet was placed on a glow-discharged carbon coated 200-mesh grid and sat for 1 min. The excess fluid was removed by touching the grid with filter paper. The rHDL nanoparticles were visualized using JEM-2010 transmission electron microscope linked to a GATAN 794 CCD.

Serum Stability of siRNA

To evaluate their serum stability, rHDL nanoparticles were incubated with RPMI-1640 medium supplemented with 10% and 50% final concentration of fetal bovine serum (FBS) at 37°C. At different time points (0, 12, 24 and 48 h), an aliquot of 30 μl was withdrawn and immediately frozen at −20°C until gel electrophoresis was performed.

To release siRNA from rHDL nanoparticles, one microliter of 20% Triton X-100 (v/v) and 20% heparin (w/v) were added to 30 μl of rHDL samples and incubated at room temperature for 1 h. After added with 6× loading buffer, samples were loaded on a 1% agarose gel and electrophoresed as described above.

Cell Culture

The human hepatocellular carcinoma cell line SMMC-7721 (27), stably expressing luciferase, was grown in RPMI-1640 medium supplemented with 10% FBS and 1% pencillin/streptomycin. Cells were incubated at 37°C in a 5% CO_2 atmosphere.

In Vitro Luciferase Gene Knockdown Studies

SMMC-7721 cells were trypsinized, counted and diluted to a concentration of 1×10^5 cells/ml. Then 0.5 ml of cell suspension was added to each well of a 24-well plate and the cells were cultured for at least 24 h to reach 70–80% confluency. Fifty μl of rHDL sample containing 0.25 μg siRNA was mixed with 450 μl of medium containing 15% FBS. Then the mixture was added to the cells and incubated at 37°C for 12 h, 24 h or 48 h, and then the medium containing rHDLs was removed and replaced with 500 μl of fresh medium containing 10% FBS. Forty-eight hours later, luciferase activities were determined using the Luciferase Assay System from Promega. Briefly, the cells were washed with PBS and incubated with lysis buffer at room temperature for 30 min. Ten μl of cell lysate was mixed with 25 μl substrate and the luminescene was measured by a Berthold™ tube luminometer. The total protein concentrations of the samples were determined by BCA assay (Thermo). The luciferase activity of cells treated with each rHDL formulation was normalized against the total protein concentration. The silencing efficiency was calculated as percent luciferase activity compared to the untreated control.

Polymer/siRNA polyplexes were used as control for assaying silencing efficiency. The polyplexes were diluted with PBS and then incubated with SMMC-7721 cells for 6 h. After removal of complexes, the cells were cultured in fresh medium for a further 42 h. The luciferase knockdown efficiency of polymer/siRNA complexes was calculated as described above.

The rHDL formulations including the non-silencing siRNA were used as control for evaluating the gene silencing effect of rHDL nanoparticles. The rHDL nanoparticles were incubated with SMMC-7721 cells for 48 h. The luciferase knockdown efficiency were determined as described above.

Confocal Microscopy Study of the Cell Uptake and Intracellular Distribution of rHDL Nanoparticles

The rHDL nanoparticle internalization was studied using confocal microscopy. SMMC-7721 cells were seeded into 35 mm glass bottom dishes (MatTek) at a density of 5×10^4 cells/ml and cultured in RPMI 1640 medium containing 15% FBS to reach 50% confluency. The rHDL nanoparticles were prepared with PEI 1800 and Cy5 labeled siRNA. The cells were incubated with rHDL nanoparticles at a siRNA concentration of 200 nM. After 1 h, 2 h and 8 h of

incubation, the medium was aspirated, washed twice with PBS and then incubated with Lysotracker Green at a concentration of 100 μM for 0.5 h. After washed with PBS, the cells were fixed with 4% formaldehyde for 15 min at room temperature. Then the cells were stained with 4′, 6-diamidino- 2-phenylindole (DAPI) for cell nuclei. The fluorescence of the cells was visualized with Leica TCS SP5 confocal microscope.

Competitive Inhibition of Cellular Uptake and Transfection of rHDL

To investigate the uptake mechanism of rHDL, the competition experiments were carried out with natural HDL without siRNA. SMMC-7721 cells were seeded into 24-well plates and cultured to reach 70–80% confluency. For cellular uptake study, rHDL nanoparticles containing Cy5-siRNA were applied to each well at a concentration of 100 nM siRNA. Natural HDL containing 5 times of apo AI proteins were also added. After 6 h of incubation, cells were washed 3 times with PBS, trypsinized and suspended in PBS. The fluorescence intensity was measured using a FACSCalibur flow cytometer (BD Biosciences). The results were expressed as a mean and standard deviation obtained from three samples.

For gene silencing study, rHDL nanoparticles were prepared with siLuc siRNA. The cells were treated as described above. They were added into the cell culture in the presence of natural HDL containing about 5 times of apo AI protein. After 24 h of incubation, the cells were cultured with fresh medium for another 24 h. Then the luciferase knockdown efficiency of rHDL was determined as described above.

Cellular Toxicity of rHDL Nanoparticles

The SMMC-7721 cells were seeded into 96-well plates and cultured for 24 h to assure attachment and 70–80% confluency. After the culture medium was aspirated away, the cells were incubated with rHDL for 24 h or 48 h. The cell viability was assayed using the Cell Counting Kit-8 (CCK-8) according to the manufactory's instructions (Dojindo Laboratories, Tokyo, Japan). Briefly, 10 μl of CCK-8 agent was added to each well and incubated for 0.5 h at 37°C in 5% CO_2. After that, the absorbance was read on a microplate reader at 450 nm with a reference wavelength of 600 nm.. cellular viability was calculated as follow: Cell viability (%) = $\left(OD_{450(sample)}/OD_{450(control)}\right) \times 100$, where $OD_{450(sample)}$ is the absorbance of the transfected cells at 450 nm and $OD_{450(control)}$ is the absorbance of the untreated cells at 450 nm.

Statistical Analysis

The results are presented as the mean ± standard deviation (SD) of at least three independent experiments. One- or two-way ANOVA with Bonferroni's post-test was used to determine statistically significant differences of the means. Statistical differences are presented at probability levels of $*p<0.05$, $**p<0.01$, and $***p<0.001$.

RESULTS

Preparation and Characterization of siRNA-Encapsulated rHDL Nanoparticles

In order to encapsulate the anionic and hydrophilic siRNA inside the rHDL nanoparticles, we attempted to prepare complexes of siRNA with different cationic polymers and then coat them with anionic lipid layers. The overall scheme was shown in Fig. 1. At first, different polymers were used to mix with siRNA at various ratios to form polyplex cores. Subsequently the cationic polyplex core was mixed with anionic micelle, which composed of the apo A-I proteins, lipid and sodium cholate. After the removal of unbound sodium cholate, rHDL nanoparticles were obtained, in which the cationic core were shielded by a layer of neutral lipid, sodium cholate and apo A-I proteins.

The siRNA encapsulation efficiency of rHDL was estimated using 1% agarose gel electrophoresis. If siRNA was totally encapsulated in rHDL, migration of siRNA should be completely retarded. As shown in Fig. 2, almost all siRNA was complexed and there were no siRNA band observable when the mass ratio of protamine/siRNA reached 2:1, the N/P ratios of PEI 600/siRNA reached 20:1, PEI 1800/siRNA reached 20:1, PEI 25k/siRNA reached 20:1, PLL 15k-30k/siRNA reached 20:1, respectively.

Moreover, the polymer/siRNA complex ratio had an impact on the sizes and zeta potentials of rHDL nanoparticles. As shown in Fig. 3, the size of rHDL varied from 20 nm to 200 nm. All the formulations at low polymer/siRNA ratios had small particles sizes of about 30 nm, which were similar to that of rHDL nanoparticles (28). However, the sizes of the rHDLs would increase significantly at high polymer/siRNA ratios. But the values of negative surface charge of rHDLs decreased considerably with the increase in polymer/siRNA ratio. Based on these results, we selected the optimized formulations of rHDL nanoparticles prepared with protamine/siRNA at the mass complex ratio of 2/1, PEI/siRNA at N/P ratio of 20, and PLL/siRNA at N/P ratio of 10, respectively. rHDL nanoparticles prepared at these selected ratios were used in the following studies.

By using the five different cationic polymers commonly available, we prepared five different rHDLs with their physicochemical characterizations summarized in Table I. All these rHDL nanoparticles had negative zeta potentials, indicating that all the cationic polyplexes were encapsulated.

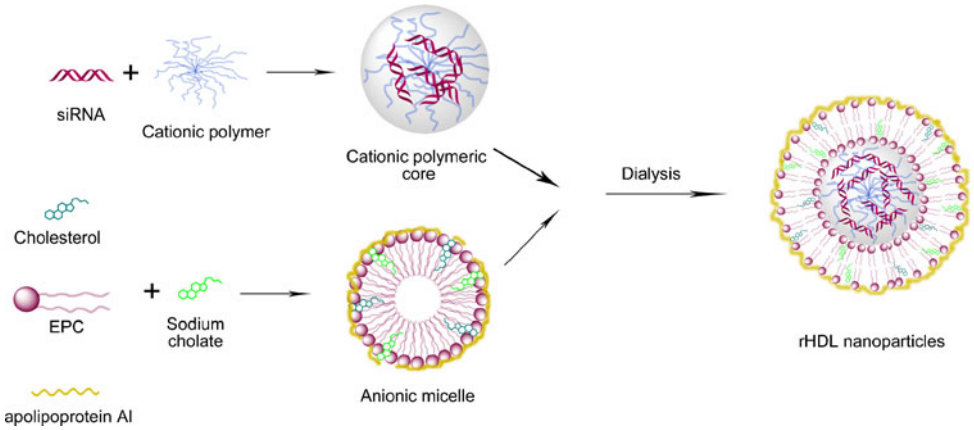

Fig. 1 Schematic depiction of the preparation of rHDL nanoparticles.

It was also observed that the molecular weight of polymers had some effect on the size and zeta potential of rHDL nanoparticles with the same polymer/siRNA N/P ratio. The high molecular weight PEI resulted in larger rHDL particles, but the zeta potential values were slightly lower.

TEM Imaging of rHDL Nanoparticles

The morphology of rHDL nanoparticles was investigated by negative staining TEM, which was intensively used in the studies of rHDL nanoparticles. As shown in Fig. 4, these rHDL nanoparticles were quite small, condensed and

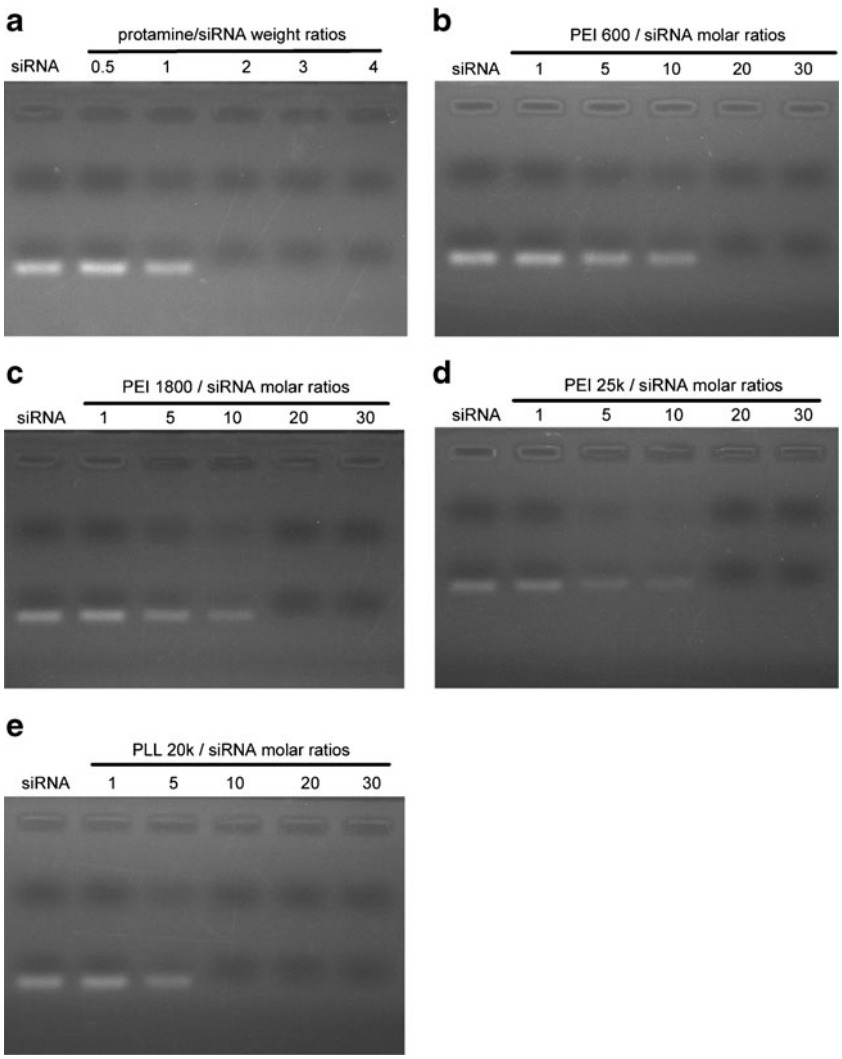

Fig. 2 Electrophoretic mobility assay of rHDL nanoparticles formed with different polymers. (**a**) Protamine was used to prepare the rHDL nanoparticles at various mass ratios of 0.5, 1, 2, 3, 4, respectively; (**b**) PEI 600 was used to prepare the rHDL nanoparticles at various N/P ratios of 1, 5, 10, 20, 30, respectively; (**c**) PEI 1800 was used to prepare the rHDL nanoparticles at various N/P ratios of 1, 5, 10, 20, 30, respectively; (**d**) PEI 25k was used to prepare the rHDL nanoparticles at various N/P ratios of 1, 5, 10, 20, 30, respectively; (**e**) PLL was used to prepare the rHDL nanoparticles at various N/P ratios of 1, 5, 10, 20, 30, respectively. All samples were run on 1% agarose gel and stained with GelRed dye.

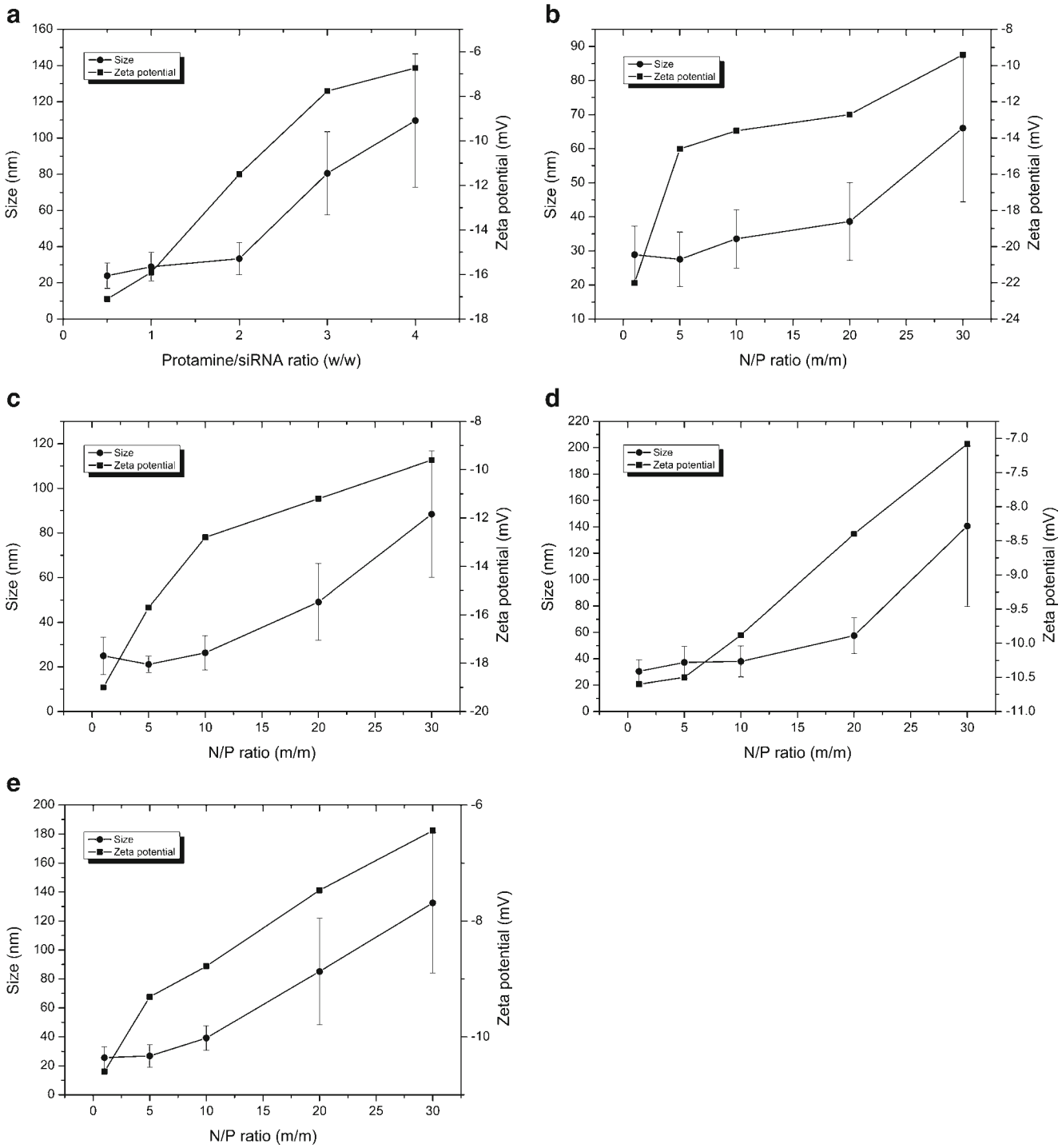

Fig. 3 Effect of different polymer/siRNA ratios on the size and zeta potential of resulted rHDL nanoparticles. (**a**) Protamine, (**b**) PEI 600, (**c**) PEI 1800, (**d**) PEI 25k and (**e**) PLL. Protamine complexed with siRNA at various mass ratios, and other four polymers complexed with siRNA at N/P molar ratios. Bars are the mean ± SD of 3 independent experiments.

spherical. Most of them were close to 30 nm in diameters, which were in reasonable agreement with the results of DLS measurements. The rHDL containing PEI/siRNA however looked smaller under TEM than what was measured by DLS. It might be because they would shrink a little when they were dried and prepared in the specimens. But the nanoparticles were quite uniform and resembled the size and morphology of the natural HDL.

Table I Characterization of rHDL Nanoparticles

	Polycation/ siRNA ratios	Size[a] (nm)	Polydispersity Index[b] (PdI)	Zeta potential[c] (mV)
Portamine	2:1 (w/w)	33.65	0.203	−11.5
PEI 600	20:1 (mol/mol)	38.64	0.238	−21.7
PEI 1800	20:1 (mol/mol)	49.06	0.184	−11.2
PEI 25k	20:1 (mol/mol)	57.63	0.292	−8.4
PLL	10:1(mol/mol)	39.23	0.164	−8.78

[a] Samples were diluted 1:50 with Tris buffer (pH8.0) for DLS measurement

[b] The polydispersity index of rHDL nanoparticles correspond to the square of the normalized standard deviation of an underlying Gaussian size distribution

[c] Samples were diluted 1:50 with Tris buffer (pH8.0) for zeta potential measurement

Serum Stability of siRNA Encapsulated in rHDL

Because the electrostatic interaction between serum components and siRNA delivery vectors could lead to siRNA dissociation and subsequently degradation by serum ribonucleases, the serum stability is an important factor in the designing of siRNA delivery. To examine whether rHDL nanoparticles protected siRNA in serum, samples containing 0.2 μg siRNA were incubated in 10% or 50% FBS medium for various times, respectively. The results demonstrated that the naked siRNA was significantly degraded after 48 h in 10% FBS and completely degraded in 50% FBS for 48 h (Fig. 5). However, in contrast with naked siRNA, the siRNA encapsulated in rHDL nanoparticles showed high stability and remained intact up to 48 h in 10% FBS and slightly degraded in 50% FBS, respectively. The results indicated that rHDL could efficiently protect siRNA from the serum ribonuclease degradation and preserve the structural integrity of siRNA.

In Vitro Investigation of Luciferase Gene Silencing

To investigate whether rHDL nanoparticles were able to transport siRNA into cells and release siRNA to induce gene knockdown, we chose a known anti-luciferase siRNA (siLuc) sequence (29) and a non-silencing control siRNA (siNS) sequence. Human hepatocellular carcinoma cell line SMMC-7721 that was stably transfected with the luciferase gene was incubated with rHDL nanoparticles at a dose of 40 nM siRNA and then analyzed the knockdown efficiency of luciferase expression after 48 h. As shown in Fig. 6, all rHDL formulations that contained the siLuc siRNA had significant efficiency in terms of silencing luciferase expression. And it's demonstrated that the gene knockdown effect improved with the increasing incubation time. From the results, the PEI 1800 formulation had the highest gene silencing efficiency after transfection in 10% FBS medium for 48 h (75.5% of gene knockdown), and was considered to be the optimal formulation. The PLL formulation had the lowest gene silencing effect under the same condition, yet 67% luciferase expression knockdown was observed.

The polymer/siRNA polyplexes by themselves were used as controls. Compared with the rHDL nanoparticles, these polyplexes showed lower gene silencing efficiency. The maximum silencing efficiency was achieved by the high molecular weight polymers (PEI 25k and PLL) after incubation in PBS for 6 h. But all these positively charged polyplexes had no silencing efficiency after transfection in the presence of serum. It's also showed that rHDL nanoparticles containing siNS had no knockdown effect of luciferase expression in 10% FBS for 48 h, indicating that the rHDL structure did not cause the gene expression change in SMMC-7721 cells.

In Vitro Intracellular Trafficking Mechanism of siRNA

To elucidate the siRNA delivery mechanism, the intracellular behavior of rHDL nanoparticles were investigated over time on SMMC-7721 cells using confocal microscopy. For this purpose, siRNA was labeled with Cy5 dye and the acidic organelles including endosomes and early lysosomes were stained with Lysotracker Green. As shown in Fig. 7a, rHDL nanoparticles were taken up slowly by SMMC-7721 cells and Cy5-siRNA was seen bound to the plasma membrane at 1 h. Subsequently, a partial co-localization (yellow dots) of Cy5-siRNA and Lysotracker were found inside the cells after 2 h incubation, indicating that some siRNA was located in the endosome or early lysosome compartments (Fig. 7b). But on the other hand, there were always some red fluorescent signals that were not overlapping with the Lysotracker signals, suggesting that the uptake of siRNA might have other pathways. After 8 h incubation, the intensity of red fluorescence of Cy5-siRNA in cytoplasm substantially increased with rare overlapping with green signals of Lysotracker, indicating that most of the Cy5-siRNA had escaped from the endosome into cytoplasm (Fig. 7c).

Competitive Inhibition of Cellular Uptake and Transfection by rHDL

In order to understand the uptake and internalization mechanism of rHDLs in more details, we designed competitive assay of cellular uptake and gene silencing in which rHDL nanoparticles containing siRNA was co-incubated with native HDL when treating SMMC-7721 cells. The protein concentration of the competing native HDLs was 5 times of the concentration in rHDL nanoparticles. As demonstrated by flow cytometric analysis, the cell associated fluorescence

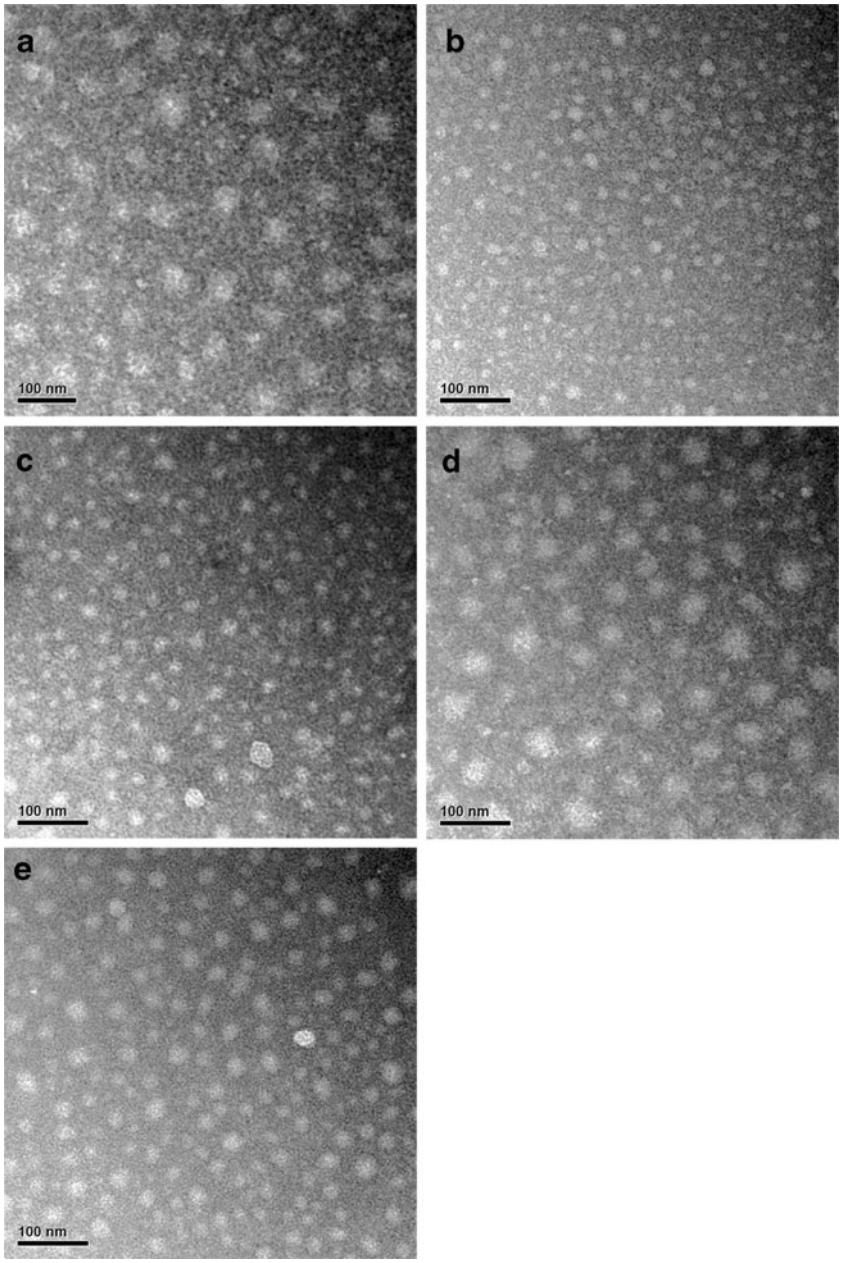

Fig. 4 TEM images of rHDL nanoparticles prepared with different polymers. (**a**) Protamine, (**b**) PEI 600, (**c**) PEI 1800, (**d**) PEI 25k, and (**e**) PLL 15k-30k. Bar=100 nm.

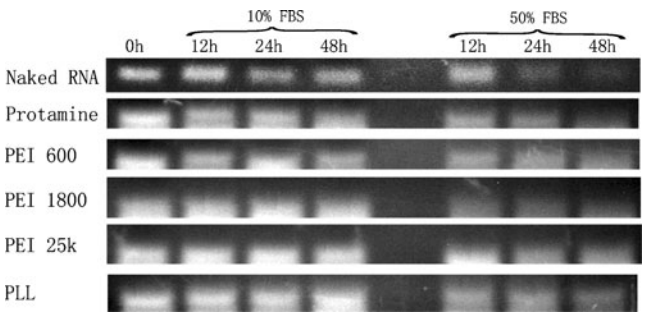

Fig. 5 Stability of siRNA encapsulated in rHDL nanoparticles following incubation with 10% FBS and 50% FBS medium, respectively. The siRNA was released in the presence of Triton X-100 and heparin. All samples were run on 1% agarose gel and stained with GelRed dye.

signals from rHDL nanoparticles significantly decreased when co-incubated with natural HDL (Fig. 8a). Furthermore, It was shown that the gene silencing efficiencies of five siLuc-rHDL nanoparticles also dramatically decreased with the competition by native HDLs (Fig. 8b). In the control experiment, native HDLs had no effect on luciferase expression in SMMC-7721 cells. Therefore, we think the rHDLs were most likely taken up via HDL receptor-mediated pathways.

Cytotoxicity of rHDL Nanoparticles *in Vitro*

To eliminate the possibility that non-specific cytotoxicity resulted in the decrease of luciferase gene expression, the

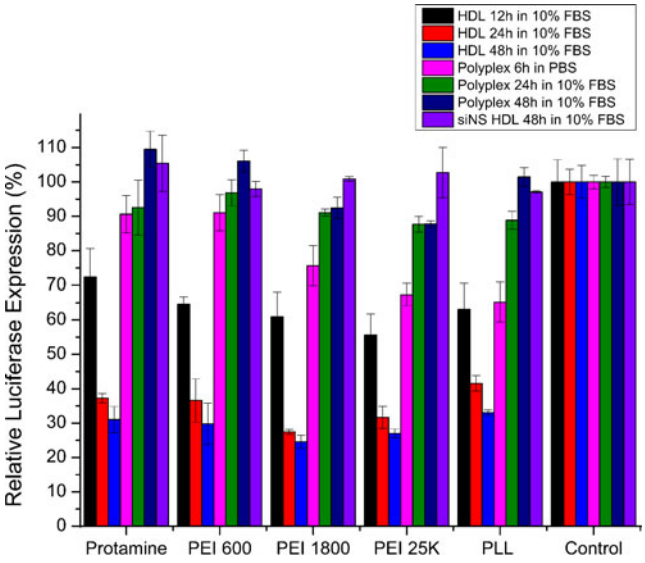

Fig. 6 Knockdown of luciferase expression *in vitro*. SMMC-7721 cells were treated with siLuc siRNA encapsulated in rHDL nanoparticles or in a polymer/siRNA polyplex. And cells were also treated with non-silencing siNS encapsulated in rHDL nanoparticles. Bars are the mean ± SD of 3 independent experiments.

cytotoxicity of rHDL nanoparticles was determined at the working concentration of HDL by CCK-8 assay. The viability of SMMC-7721 cells were expressed as a fraction of viable cells and normalized to that of untreated cells. As shown in Fig. 9, over 90% of cell viability was observed for all rHDL nanoparticles after incubation for 24 h and 48 h, respectively. However, at the same working concentration, five polyplexes all showed high toxicity after incubation in PBS for 6 h. Though polyplexes resulted in low cytotoxicity in the presence of serum, these polyplexes had negligible gene silencing efficiencies.

DISCUSSION

In this study, we developed a new rHDL formulation for siRNA delivery to hepatocyte. The rHDL nanoparticles were constructed under the hypothesis that the electrostatic interaction between cationic polymers and siRNA could drive the formation of dense polyplex cores while the amphiphilic lipids, apo AI proteins and anionic cholate could form a lipid envelope around the cores. This type of rHDL nanoparticles are considered more stable and biocompatible because of the lipid-lipoprotein surface shell.

Polyplexes had been extensively reported to enable effective delivery of siRNA *in vitro*. But they were found to have no significant therapeutic effects *in vivo*. Some reports demonstrated that polymers could delivery siRNA to liver (1–4), but the studies revealed that the accumulation of siRNA in the liver was mainly due to the phagocytic activity of the RES(3,5,6). Such a phenomenon was mainly attributed to the non-specifically interaction between cationic polyplexes and negatively charged biofluid's components (such as serum proteins, enzymes and opsonins). The destination of delivered siRNA in the liver was not target hepatocytes but Kuppfer cells, in which siRNA could be degraded by macrophagocytosis. Therefore, The passive targeting ployplexes were not appropriate for the hepatocyte delivery of siRNA.

To overcome this problem, these polymers were usually modified with a PEG moiety (PEGylation) to prevent the binding of biofluid's components to reduce RES phagocytosis (6–8). These PEGylated polymers displayed low cytotoxicity and extended circulating time, but recently several studies reported that the modified polymers could induce the formation of anti-PEG IgM antibodies and subsequently resulted in a rapid clearance (6,9,10). These

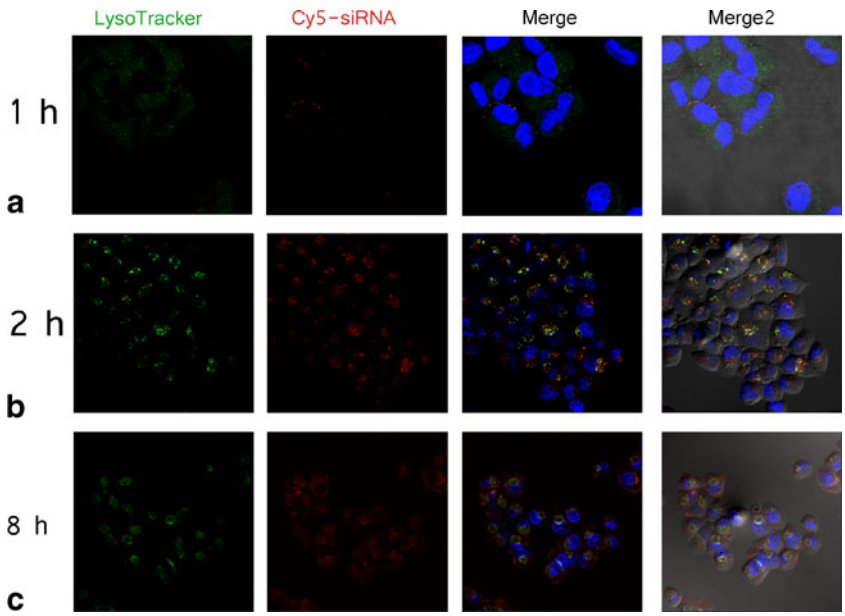

Fig. 7 Confocal microscopy images showing intracellular trafficking of rHDL nanoparticle prepared with PEI 1800. SMMC-7721 cells were incubated with rHDL containing Cy5 labeled siRNA (*red*) for 1 h (**a**), 2 h (**b**) and 8 h (**c**), respectively. After incubation, the cells were stained with Lysotracker Green (*green*). And then the nucleus were stained with DAPI (*blue*) after fixation. Yellow dots indicated co-localization of Cy5-siRNA and Lysotracker, suggesting the complexes were trapped in the lysosomes. Overlay images of blue, green, red and transmitted light channels were shown.

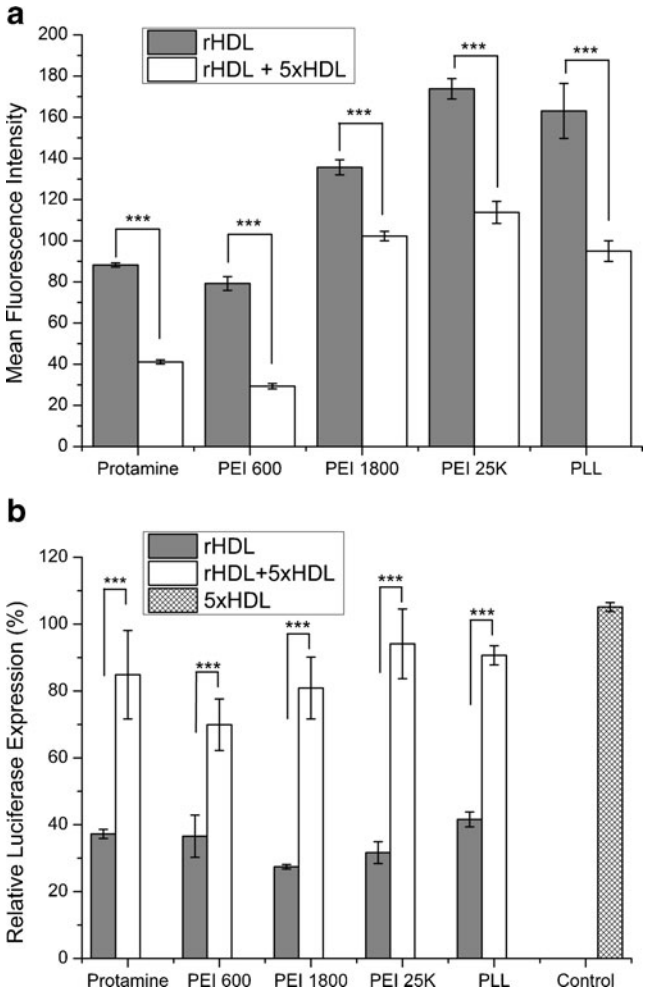

Fig. 8 Competitive inhibition effect of native HDL on the cellular uptake and gene silencing efficiency of rHDL nanoparticles in SMMC-7721 cells. (**a**) Cellular uptake of rHDL nanoparticles in the presence of natural HDL was determined by flow cytometry. (**b**) Gene silencing efficiency of rHDL nanoparticles in the presence of natural HDL was evaluated by luciferase assay. Bars are the mean ± SD of 3 independent experiments. Two-way ANOVA analysis was used for comparison between cells incubated with rHDL and cells incubated with rHDL + natural HDL. ***$p < 0.001$.

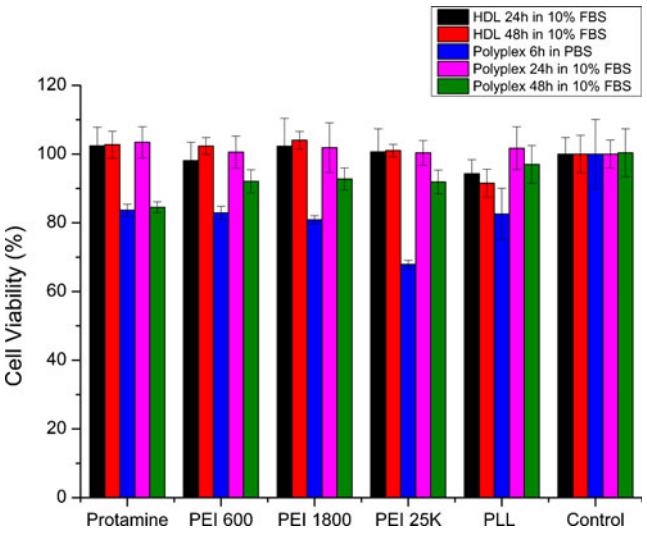

Fig. 9 Cell viability of SMMC-7721 cells after incubation with different rHDL nanoparticles or cationic polyplexes at the working concentration of 40 nM for 24 h and 48 h, respectively. Bars are the mean ± SD of 5 independent experiments.

side effects of PEGylated polymers led us to develop alternative approach to stabilize and enhance the target specificity of polyplexes.

Therefore, the rHDL formulation was designed to reduce cytotoxicity and promote target specificity of polyplexes. Because the biodistribution of rHDL nanoparticles were govern by apo AI proteins embedded in the lipid membrane, the encapsulated polyplexes were protected from the biological environment in the blood circulation and could be delivered to HDL-target tissues (hepatocytes or other steroidogenic organs). The shielded polyplexes could be lower cytotoxicity, lower immunogenicity and more stable compared with unmodified polyplexes. Meanwhile, the cationic polymers were expected to retain their functions to facilitate the cytoplasmic escape of the delivered siRNA cargo.

The formulation optimization studies indicated that the optimized rHDL formulation had the best charge balance between the three components—siRNA, cationic polymers and apo AI proteins. The resulted optimized rHDL nanoparticles closely mimic the size, surface charge and morphology of the natural spherical HDLs while maintaining satisfactory siRNA encapsulation efficiency. The five formulations exhibited sizes in the range of 33.65 nm and 57.63 nm. They were a little larger than native HDLs, but they were still within the size range for the uptake mediated by the HDL receptors (11,12). And the rHDL nanoparticles were similar to those rHDL prepared by the cholate reconstitution method (13).

In the rHDL formulation, the proportion of apo AI protein was of vital importance to stabilize the rHDL nanoparticles. In our preliminary studies that used only lipids but not Apo AI, there was significant aggregations observed after mixing the lipoplexes with lipid/cholate mixed micelles. An appropriate amount of apo AI protein was required to prevent the aggregation and modulate the size and surface charge of rHDL. Low apo AI proportion could not prevent the formation of aggregation, then the increase in apo AI proportion could prominently reduce the size and surface charge of rHDL. But the higher amount of apo AI proteins might repel siRNA from polyplexes after competition for electrostatic binding to polymers. Thus, the proportion of apo AI was optimized to achieve the balance between these parameters. In addition, the optimized formulations showed that the determined alpha-helix contents of rHDL nanoparticles were similar to that of natural HDL, indicating that the apo AI protein in rHDL might preserve its biological properties which were important in the HDL receptor recognition (data not shown) (14,15).

The hepatocellular carcinoma cell line SMMC-7721 was chosen to examine the uptake, trafficking, and knockdown efficiency of the rHDL nanoparticles. SMMC-7721 cells were shown to express high levels of HDL receptors, they had been widely used for the study of rHDL nanoparticles (16,17). Because of the great biocompatibility and very low toxicity, rHDL nanoparticles were incubated with SMMC-7721 cells in the presence of serum for 48 h. The maximum silencing efficiency was achieved with the rHDL formulations containing PEI 1800 and PEI 25k. It could be due to the more efficient endosomolytic activity of PEI compared to protamine and PLL. Interestingly, rHDLs containing PEI 1800 had better knockdown efficiency than that of PEI 25k, it may be due to the smaller sizes of rHDL containing PEI 1800 which could be easier to be recognized and taken up by HDL receptors.

The uptake and internalization mechanism of rHDL was investigated by confocal microscopy and competitive uptake experiment. In confocal images, the cellular endosomes/lysosomes were stained by Lysotracker green. After incubation for 2 h, the co-localization of green signals with red signals of Cy5 labeled siRNA indicated that rHDL nanoparticles were internalized by endocytosis. This result was consisted with the previous reports that demonstrated apo AI proteins in HDL were endocytosed and targeted to lysosome for degradation in hepatocytes (18,19). The successful escape of siRNA from endosome and release into cytoplasm might be mainly attributed to the high buffering capacity of polymers and the "proton sponge effect" (30,31). These polymers could facilitate the influx of protons, lead to osmotic swelling, endosomal membrane disruption, and eventual release of the siRNA into cytoplasm (32). On the other hand, the observation of free Cy5-siRNA in the cytoplasm might suggest that rHDL could be taken up via holo-particle uptake pathway, which was mediated by a complex of different proteins, including the ectopic β-chain of ATP synthase and $P2Y_{13}$ receptors (33–35). In addition, since the cellular uptake and knockdown efficiency of rHDL nanoparticles were shown to be significantly inhibited by the presence of native HDLs, our data strongly indicated that rHDL nanoparticles were taken up by SMMC-7721 via interaction mediated by HDL receptors.

Taking together of all the data of uptake and internalization of rHDL *in vitro*, this special rHDL formulation exhibited biological properties similar to natural HDL. Because the common obstacle for current siRNA delivery strategies is the accumulation in organs of RES systems, the unique properties of the rHDL nanoparticle make it a potential vector for hepatocyte-specific delivery of siRNA. Many researcher have attempted to dose siRNA up to high values, but were often limited by the severe toxicity of the vector. In the case of rHDL formulation, the siRNA dose could be increased without high toxicity. Future studies of gene silencing, biodistribution and toxicology in different animal models would be required for the further development of rHDL nanoparticles to achieve effective and safe delivery of siRNA *in vivo*.

CONCLUSIONS

We have constructed a novel rHDL nanoparticle suitable for siRNA delivery. The rHDL nanoparticles mimic the morphology of natural HDL and had a structure with a siRNA/polymer polyplex core surrounded by a layer consisting of lipid and apo AI proteins. The rHDL formulations had structural similarity to natural HDL and protected siRNA from the RNase degradation in the presence of serum. In comparison to cationic polymer/siRNA polyplexes, the optimized rHDL formulations were shown to achieve much better knockdown efficiency with negligible cytotoxicity in the serum-containing medium in hepatocellular carcinoma cell model. Moreover, the advantage of rHDL formulations is that these nanoparticles were proven to preserve the biological functions similar to natural HDL. Thus, such rHDL nanoparticles could be used as a prospective delivery system for safe and efficient delivery of siRNA *in vitro* as well as *in vivo*.

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