Inhibition of HCV 5′-NTR and Core Expression by a Small Hairpin RNA Delivered by a Histone Gene Carrier, HPhA

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Abstract

siRNA (small interfering RNA) interference represents an exciting new technology that could have therapeutic applications for the treatment of viral infections. However, a major challenge in the use of siRNA as a therapeutic agent is the development of a suitable delivery system. We demonstrated that a new non-viral transgene carrier, recombinant archaeal histone from the hyper-thermophile Pyrococcus horikoshii OT3 (HPhA), can transfect short hairpin RNA (shRNA) expressing plasmids into HL-7702 cells to inhibit the expression of HCV 5’NTR and Core protein and mRNA. Plasmids Psilencirle transfected by HPhA inhibited the expression of HCV 5’-NTR and Core protein and mRNA in HL-7702 cells. The transfection efficiency of HPhA in HL-7702 cells was not affected by 10% fetal calf serum (FCS). HPhA exhibited effects of transfection without apparent toxicity, and with high affinity for DNA. This suggests that HPhA may be useful for RNAi-based gene therapy in vivo.

Key words: RNA interference; hepatitis C virus; small hairpin RNA; gene therapy; HPhA

Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver disease and affects over 170 million individuals worldwide. Persistent HCV infection is a leading cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [1-4]. The current standard of care for genotype 1 HCV, triple therapy of PEG-interferon, ribavirin, and a protease inhibitor, results in sustained virological response rates of 70-75% [2,4]. HCV belongs to the family Flaviviridae, and its genome is a positive-strand 9.6 kb RNA. HCV has a 5′-non-translated region (NTR), a long open reading frame, and a 3′-NTR. An internal ribosome entry site (IRES), containing the 5′-NTR and part of the Core coding region, forms a secondary structure and supports translation initiation of an HCV genome in a cap-independent manner [5]. HCV 5′-NTR and Core are conserved regions in the HCV genome [6-8].

RNAi is the process whereby double-stranded RNA (dsRNA) induces the sequence-specific degradation of homologous messenger RNA (mRNA) [9]. Introduction of siRNA into mammalian cells leads to mRNA degradation with exquisite sequence specificity without activating an interferon response. Thus, RNA interference represents an exciting new tech-
nology that could have therapeutic applications for the treatment of viral infections [10,11]. To date, RNAi has been used effectively to inhibit the replication of several different pathogenic human viruses in culture, including respiratory syncytial virus (RSV), poliovirus, papillomavirus, HIV-1, hepatitis C virus (HCV), and HBV (hepatitis B virus). Unfortunately, small RNAs have not been applied in treating diseases. One of the reasons is that there is no suitable delivery system for effectively transferring siRNA in vivo [10-15]. A major challenge in the use of siRNA (small interfering RNA) as a therapeutic agent is the development of a suitable delivery system. Because the main goal of in vivo delivery is to have active siRNA oligos in the target cells, the stability of siRNA oligos in the extracellular and intracellular environments after systemic administration is the most challenging issue [16].

The first hurdle is the size of the 21-nucleotide double-stranded siRNA oligos: these oligos are relatively small, and thus, are rapidly excreted through urine when administered into the blood stream, even if siRNA molecules remain stable through chemical modifications. Second, the double-stranded siRNA oligos are relatively unstable in the serum environment and they can be degraded by RNase activity within a short period of time.

More recently, DNA expression vectors have been developed to express small hairpin RNAs (shRNA). These vectors employ the type III class of RNA polymerase promoters to drive the expression of siRNA molecules within cells [5,17,18]. DNA is relatively stable, but still can be degraded by DNase and it is difficult to introduce naked plasmids into cells. Therefore, suitable carriers are required for this purpose. Currently, there are two major methods by which siRNAs are delivered into hepatocytes in experimental animals [19,20]. One is the hydrodynamic method, in which siRNA expressing plasmids in a solution that is injected into the blood by the tail vein. This solution causes high pressure in blood circulation so that siRNA expressing plasmids enter into hepatocytes within a short period of time.

Several proteins as non-viral vectors have been studied using positive charge of the protein to bind with DNA to form complexes in cells [25]. Histone is a potential protein because of its excellent DNA-assembling ability. Our idea was to use HPhA which comes from the hyperthermophile Pyrococcus horikoshii OT3 which has the same basic structure of eukaryotic histones. Its molecular weight is smaller than eukaryotic histones, and it has good stability and DNA-assembling ability. In this paper, the aim was to investigate the possibility of using HPhA as carriers for transferring shRNA plasmids into cells to inhibit the expression of HCV 5′-NTR and Core protein.

**Materials and Methods**

**Cells, HCV Replicon and carrier**

HL-7702 cells were maintained at 37°C in an atmosphere of 5% CO₂ in 1640 medium (Invitrogen) containing 20% fetal bovine serum, 200 U/ml of penicillin G, and 200 g/ml of streptomycin. HCV subgenomic replicon (genotype 1a) pCMV/T7 NTRCA-luc consisting of HCV 5′-NTR, part of Core sequences and a luciferase reporter gene, was kept in our laboratory. HphA was kindly provided by the Key Laboratory for Molecular and Engineering of Ministry of Education at Jilin University. Lipofectamine™2000 (1 mg/mL), cationic liposomes were obtained from Invitrogen and used according to the manufacturer’s recommendations.

**shRNA expression constructs**

The GenBank database was searched for unique sequences within HCV to exclude known cellular genes. Target sequences for the siRNAs were determined by using the Ambion web-based criteria, followed by generation of the HCV- specific siRNAs using the SilenCircle™ RNAi Kit (Allele Biotechnology & Pharmaceutical. Inc). Three sites, HCV -72 (5-aaaggtcctgccaggtggtctttcctt-3) and HCV -274 (5-aaaggtcctttggtactgctct-3) in the 5′NTR and HCV-365 (5-aagaaaaaccaacgtacacctaccc-3) in the Core of the common sequences of the HCV 1a. Five DNA inserts were designed to generate shRNA: HCV-72 sense 5′-acaccagcgtctagccatggcgttttgcttgaaaacgccatggctagac gtt-3′; antisense 3′-g tcgcagatctgccaggtcggct-3′; HCV-274 sense 5′-acaccagcgttttgtggtctgctctgctctgttctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtttgtctgtt...
9-nucleotide loop sequence. Scrambled siRNA (con- nected to the 5'-end of the antisense strand, connected by a short spacer (TTG CTT GAA), the reverse complement of the sense strand, and five thymidines as an RNA polymerase III transcriptional stop signal. The resultant plasmids (psh-72, psh-274, psh-365, and psh-1019) were used in the experiments. The plasmids psh-72, psh-274, psh-365, and psh-1019 were named to correspond with their respective targets, HCV-72, HCV-274, HCV-365 and luciferase-1019. The constructs contained the 3’-end of the sense strand and the 5’-end of the antisense strand, connected by a 9-nucleotide loop sequence. Scrambled siRNA (control) cloned into the same vector was used as a negative control in all of the experiments.

Cytotoxicity assays

HL-7702 cells were inoculated onto a 96-well plate at a density of 1×10⁵/mL in 200 µL per well and cultured for 24 h prior to HphA or Lipofectamine™2000 treatment. HphA or Lipofectamine™2000 was added at various concentrations and allowed to incubate for 24 h. HphA or Lipofectamine™2000 dissolved in 40 µL PBS, was added to 160 µL 1640 medium containing 10% fetal bovine serum. The cytotoxicity was measured by the reduction of methylthiazol tetrazolium (MTT; Sigma, USA) observed in mitochondria at 24 h after the initial treatment. The inhibition rate was calculated for each experimental group by comparison with MTT results from non-treated (1640 medium alone) cells [27].

Transfection

5 × 10⁴ HL-7702 cells (500 µL) were plated in 24-well cluster plates for measuring luciferase activity after transfection. The following day, the plasmids pCMV/T7-NTRCΔ-luc, 1 µg, and shRNA expression plasmids (psh-72, psh-274, psh-365, psh-1019 and scrambled control), 1 µg, were simultaneously introduced into HL-7702 cells by 3 μg HphA and 3 μL Lipofectamine™2000 (Invitrogen), respectively.

HphA and Lipofectamine™2000 were dissolved in medium containing 10% FCS or without 10% FCS when transfection. After transfection 6 h, the cell medium was exchanged for fresh medium containing 20% fetal bovine serum [27].

Western Blotting

Cells were harvested using sodium dodecyl sulfate sample buffer in 72 h after transfection. Proteins were subjected to electrophoresis on a 10% polyacrylamide gel and transferred onto a nitrocellulose membrane (Bio-Rad). The membrane was probed with a monoclonal antibody to 5’-NTR (Biodesign International, Saco, ME) or actin (Santa Cruz Biotechnology, Santa Cruz, CA). Proteins were visualized using an enhanced-chemiluminescence detection kit (Amersham Pharmacia, Piscataway, NJ) [11].

Luciferase activity measuring

Cells in 24-well cluster plates were digested by trypsinogen 72 h after transfection. Cells were washed twice with PBS, and then 100 µl cell lysate was added. Cells were digested for 15 sec, then centrifuged for 15 sec. Supernatant was transferred to a new tube and placed at room temperature, then 100 µl supernatant was added to 100 µl luciferase substrate per well in 96-well plates. Luciferase activity was measured by a FLX800 luminometer [11].

DNA binding by HphA

HphA was mixed with plasmids at ratios of 0 : 1, 1.5 : 1, 2.0 : 1, 2.5 : 1, 3.0 : 1, 3.5 : 1, and the mixture of HphA and DNA was electrophoresed for 12 h on an agarose gel (0.7 V/cm) [25].

Intracellular RNA purification and analysis

HL-7702 cells were harvested by trypsin digestion 72 h after transfection and washed three times with phosphate buffered saline (PBS, pH 7.3). Total RNA was isolated from cells using the TRIZOL reagent (Invitrogen). For RT-PCR analysis, RNA was reverse-transcribed at 42°C for 90 min using a commercially available cDNA synthesis kit (Invitrogen). PCR was performed with gene specific primers for HBV mRNA and β-actin: HCV (forward), 5’-TGCACTGAGAACGATACTCTAAC-3’; HCV nt2659-2636 (reverse), 5’-CCGGGAACCTAACGTTCTGTCGCG-3’; β-actin (forward), 5’-AGGGCAAGGT CATCACC-3’; β-actin (reverse), 5’-AGCTGAGA GAATGACCCG-3’; qPCR was performed on the ABI 7300 Sequence Detection System using the SYBR Green kit (Invitrogen). The fold increase was calculated using 2^ΔΔCT [38].

http://www.medsci.org
Statistical analysis

Statistical analyses were performed on SAS software version 8.0 (USA). Data are expressed as mean ± standard deviation (SD). Simple comparisons between groups were carried out with the Student’s t-test. A P-value less than 0.05 was considered statistically significant.

Results

Cytotoxicity assays

In order to evaluate toxicity, the number of surviving HL-7702 cells was monitored after 24 h incubation with various concentrations of either the HPhA or Lipofectamine™2000. No apparent cytotoxicity was observed after treatment with HPhA at lower concentrations (200 μg/mL), whereas incubation with even lower amounts of Lipofectamine™2000 caused considerable cell death (10 μg/mL). This finding suggested that the suppressive activities of HPhA on expression of HCV 5'-NTR and Core were not a result of induced cytotoxicity (Figure 1).

HphA binding DNA

To determine the ability of HphA to bind plasmids, HphA was mixed with plasmids in various ratios, and agarose gel electrophoresis was performed. The results showed that when HphA bound DNA the mixture moved faster in an agarose gel (Figure 2).

![Figure 1. Cytotoxicity of Lipofectamine™2000 or HPhA in HL-7702 cells. The data represent mean±SD (n=4). *P<0.05 vs. the corresponding non-treated control (Student’s t-test).](http://www.medsci.org)

![Figure 2. An agarose gel showing HPhA binding of DNA.](http://www.medsci.org)
Influence of serum on transfection

HCV subgenomic replicon pCMV/T7 NTRCΔ-luc was transfected into HL-7702 cells by Lipofectamine™2000 or HPhA in medium with or without FCS. Luciferase activity was measured 72 h after transfection. They were 57±49, 4468±223, 4950±359 and 4538±268 RFU respectively at Lipofectamine™2000 With FCS (10%), Lipofectamine™2000 Without FCS, HphA With FCS (10%) and HphA Without FCS group in medium. Luciferase activity was lower at Lipofectamine™2000 With FCS (10%) than in the other group in medium (p<0.05). In medium containing 10% FCS, no expression of luciferase was observed when Lipofectamine™2000 was used to transfect plasmids pCMV/T7 NTRCΔ-luc into cells. However, expression of luciferase was clearly shown when HphA was used to transfect.

Specific shRNA introduced by HphA inhibited the expression of HCV 5′-NTR and Core

The plasmid pCMV/T7 NTRCΔ-luc and shRNA expression plasmids (psh-72, psh-274, psh-365, psh-1019 and scrambled control) were simultaneously introduced into HL-7702 cells (co-transfection) by HphA. The results showed that specific shRNA introduced by HphA could obviously inhibit the expression of HCV 5′NTR and Core in medium containing 10% FCS (59%, 59%, 55%) and in medium without FCS (58%, 62%, 54%), p<0.05. The luciferase activity was the same with and without FCS in the medium for the same shRNA group, p>0.05. The inhibition rates were different for the various targeting sites (Table 1).

Table 1. Specific shRNA Introduced by HphA Inhibits the Expression of HCV 5′-NTR and Core Genes

| Luciferase activity (RFU) | Blank | Mock | Psh-72 | Psh-274 | Psh-365 | Psh-1019 | Scrambled |
|--------------------------|-------|------|--------|---------|---------|----------|----------|
| With FCS (10%) in medium | 0     | 6154 | 2095   | 2458    | 3257    | 2982     | 5471     |
|                          | 5224  | 3305 | 1987   | 1926    | 2425    | 2334     | 6234     |
|                          | 6157  | 2608 | 2909   | 731     | 5012    | 5917±441 |          |
|                          | 5845±414 | 2269±424* | 2318±221* | 2536±81* | 2016±856* | 5572±414 |          |
|                          | 59%   | 59%  | 55%    | 64%     |         |          |          |
| Without FCS in medium    | 0     | 5843 | 2178   | 2475    | 2878    | 2309     | 5617     |
|                          | 5709  | 2357 | 2067   | 2390    | 2256    | 6146     |          |
|                          | 5324  | 2987 | 2378   | 2641    | 1668    | 5987     |          |
|                          | 5625±200 | 2507±319* | 2307±160* | 2635±164* | 2077±273* | 5917±199 |          |
|                          | 59%   | 62%  | 54%    | 65%     |         |          |          |

* p<0.05 compared with luciferase activity in the Mock group in the same FCS medium.

Specific shRNA introduced by HphA inhibits the expression of HCV 5′-NTR and Core by Western Blot

Western Blot showed that the expression after inhibition of the HCV 5′-NTR was lower for the shRNA expression plasmids (psh-72, psh-274, psh-365, psh-1019) group than the Mock and scrambled groups (Figure 3).

To characterize the functional effects on mRNA by HCV-specific knock-down, we measured the amounts of HCV mRNA present after exposure to shRNA. Intracellular HCV mRNA levels were analyzed by real time PCR. As shown in Figure 3, the shRNA expression plasmids (psh-72, psh-274, psh-365, psh-1019) reduced the levels of HCV mRNA in all groups. The inhibition rates for plasmids psh-72, psh-274, psh-365, psh-1019 were significantly higher than that for mock and scrambled groups (P<0.05). There were no significant differences between levels in mock and scrambled groups (P=0.81). The psh-72 plasmid yielded the highest inhibition rate (82%) of mRNA levels.

We also studied an HCV replicon Huh-7 cell line kindly provided by the Department of Hepatology of the First Hospital, JiLin University. This replicon contains a full-length genome of an HCV genotype 1a virus. The shRNA expression plasmids (psh-72, psh-274, psh-365, psh-1019) reduced the levels of HCV RNA in all groups. The inhibition rates (%) for plasmids psh-72, psh-274, psh-365, psh-1019 were 85±8.5, 88.5±7.4, 90±9.8, 87±9.5 respectively, which were significantly higher than that for mock and scrambled groups (P<0.05). There were no significant differences between levels in mock and scrambled groups (P=0.83).
Figure 3. The effects of shRNA introduced by HphA on the expression of HCV 5'-NTR and Core.

Figure 4. The effects of shRNA introduced by HphA on the expression of HCV mRNA. *: p<0.05 VS scrambled group.

Discussion

Wilson et al. reported that specific siRNA targeting to NS3 and NS5 of the HCV genome could inhibit replication and expression of HCV replicons. However, after repeated RNAi therapy, they found that resistance to the siRNAs developed, and the siRNAs targeting to the other sites were still active [28]. The sequence analysis of NS3 and NS5 showed that there were mutations in targeting sequences of siRNA. After multiple exposures to RNAi therapy, increasing numbers of mutations have been found. This development of resistance to siRNAs is important, and may suggest that viruses mutate under the pressure of siRNA.

Two methods could prevent resistance due to virus mutation: 1. targeting sites should be chosen in the conservative areas; for example, 5'-NTR and Core region of HCV in our study; 2. the use of several different siRNAs targeting sites simultaneously, could limit the development of escape mutation of viruses. This is also an important advantage of siRNAs. The siRNAs prepared by endonuclease (esiRNAs) has been used to simultaneously target several sites of the viral genome with excellent effects [27].

Histones are the basic component of eukaryotic chromatin. Almost all of DNA binds to histones and folds to form chromatin fibers. In eukaryotes, histones and DNA form nuclear bodies which are the basic structural units of chromatin. The core of the nuclear body is histone, wound tightly by DNA. However, the tight binding can loosen during DNA binding to other proteins such as polymerase [25,29,30].

The amino acid sequence of HphA (60-70 amino acids) is similar to eukaryotic histones, but it is smaller (approximately 100~140 amino acids). HphA is the core structure of eukaryotic histone [31]. The complete genomic sequence of Pyrococcus horikoshii OT3 has been reported. It predicted open reading frames PHS051 and PHS046 [32,33]. KEY Laboratory for Molecular and Engineering of Ministry of Education in Jilin University prepared this recombinant archaeal histone HphA and used it for transfection in NIH 3T3, HEK 293, HL-7702, HepG2 and Cos 7 cell lines in vitro. HphA maintains basic second and third class structure of a eukaryotic histone, its molecular
weight is less (70 amino acids) and it is stable, thermostolerant, and has good DNA-assembling ability [32].

We mixed HphA and siRNA expressing plasmids in various ratios, and then the plasmid DNA-HphA complexes were electrophoresed for 12 hours in an agarose gel [34]. The current result showed that the mixture moved faster in gel. It suggested that HphA bound to plasmids changed the size or charge of the complexes resulting in faster migration.

Cell toxicity was evident with shrinkage and cell debris after transfection by LipofectamineTM2000 and cell death. Cells transfected by HphA were healthy after 20 h, even without replacement of the culture medium. The data suggested that HphA is non-toxic and safe.

The influence of serum on transfection should be considered in vivo, because even if transfection is very successful in vitro, it may not be successful in vivo due to serum effects. Sometimes the effect of transfection is much lower in vivo [35].

Therefore, we investigated the effect of transfection of HphA and LipofectamineTM2000 in the presence of serum [36]. Serum components such as serum albumin, immunoglobulin, fibrin and apoprotein, could bind with liposomes and alter the charge on the surface of liposomes. This could decrease the effectiveness of liposome-mediated transfection [37].

In summary, HphA has good stability and DNA-assembling ability as a non-viral transgene carrier. The transfection mediated by HphA was not affected by FCS. HphA may be useful for RNAi-based gene therapy in vivo, but this will require further study for confirmation.

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Competing Interests

The authors have declared that no competing interest exists.

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