

Cationic α -Helical Peptides for Gene Delivery into Cells

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1. Introduction

Development of nonviral gene transfer techniques has progressed, particularly the use of several kinds of cationic lipids and cationic polymers such as polylysine derivatives, polyethyleneimines, polyamidoamine dendrimers, and so on, which electrostatically form a complex with the negatively charged DNA, which can be taken up by the cells. Furthermore, targeted gene transfer has also been realized by modification of the gene carriers using cell-targeting ligands such as asialoorosomucoid, transferrin, insulin, or galactose.

Recently, novel gene transfer techniques have been reported, in which an amphiphilic α -helical peptide, containing cationic amino acids is used as a gene carrier into cells. Wyman et al. (*1*) employed a peptide, KALA (WEAK-LAKA-LAKA-LAKH-LAKA-LAKA-LKAC-EA), which is derived from the sequence of the N-terminal segment of the HA-2 subunit of the influenza virus hemagglutinin involved in the fusion of the viral envelope with the endosomal membrane. This peptide showed several functions in the transfection process, e.g., condensing DNA and causing an endosome-membrane perturbation, which enables it to deliver the incorporated DNA to the cytosol, which is essential for efficient transfection. Similarly, the authors also found the transfection technique, which is mediated by some amphiphilic α -helical peptides (e.g. Ac-LARL-LARL-LARL-LRAL-LRAL-LRAL-NHCH₃ [4₆] and KLLK-LLLK-LWKK-LLKL-LK [Hel]) as shown in **Table 1 (2–5)**. After that, for the purpose of refining of the peptide structure, we investigated the influence of the peptide chain length on gene transfer ability. As a result, 16 and 17 amino acid residues were sufficient to form aggregates with the DNA,

Table 1
Structures of Amphiphilic α -Helical Peptides

Peptide	Sequence	Chain length	Cationic charge
4 ₆	Ac-LARL-LARL-LARL-LRAL-LRAL-LRAL-NH ₂	24	6
4 ₆ Δ 8	Ac-LARL-LRAL-LRAL-LRAL-NH ₂	16	4
Hel	KLLK-LLLK-LWKK-LLKL-LK	18	7
Hel Δ 1	LLK-LLLK-LWKK-LLKL-LK	17	6

and transfer the DNA into the cells in the deletion series of 4₆ and Hel, respectively (**Table 1; 6**). In addition, the authors succeeded in constructing a multiantennary galactose-modified peptide containing four galactose residues that serve for efficient binding to the asialoglycoprotein receptor on hepatoma cells (7).

This chapter focuses on synthesis of the peptides and a method of gene transfer using them. As is well known, a peptide is readily synthesized because of the development of an automatic peptide synthesis apparatus and reagents for synthesis. From this point of view, it is expected that the gene transfer method mediated by the peptide is easily accepted by many researchers taking part in the gene therapy study.

2. Materials

2.1. Peptide Synthesis

1. Peptide synthesis apparatus (ABI 431A, PE Biosystems).
2. Fmoc-Lys(Boc) preloaded Wang resin, Rink amide resin (100–200 mesh) (Calbiochem-Novabiochem, CA).
3. Fmoc protected amino acids, 2-(1H-benzotriazole-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate (HBTU), *N,N*,*-*diisopropylethylamine, NMP, dichloromethane (DCM), piperidine (Watanabe Chemical, Hiroshima, Japan).
4. Thioanisole, *m*-Cresol, ethandithiol, trifluoroacetic acid (TFA), acetonitrile (Wako Chemicals, Osaka, Japan).
5. High-performance liquid chromatography (HPLC) apparatus (Hitachi L7100 System, Tokyo, Japan).
6. Reverse-phase (RP)-HPLC column (YMC-Pack C4, ϕ 10 \times 150 mm, Kyoto, Japan).
7. Matrix-assisted Laser Desorption Ionization-Time of Flight-Mass Spectra (MALDI TOF-MS) apparatus (Voyager DE STR, PE Biosystems).

2.2. Preparation of Plasmid DNA

1. Plasmid DNA, which contains a luciferase gene and SV40 promoter (PicaGene control vector, PGV-C), was purchased from Toyo Ink (Tokyo, Japan).
2. Plasmid DNA (pCMVluc), containing a luciferase gene under control of cytomegalovirus enhancer/promoter, was prepared by removing the *Bgl*/II and

*Hind*III insert of the plasmid PGV-C (Toyo Ink), and ligating with the *Bgl*II and *Hind*III fragment from the pRc/CMV (Invitrogen), which contains cytomegalovirus promoter.

3. Closed circular plasmid DNA was purified by ultracentrifugation in cesium chloride gradients. The plasmid preparations showed a major band of closed circular DNA and minor amount (<20%) of nicked plasmid.

2.3. Cell Culture and Gene Transfer into Cells

1. COS-7 cells (a monkey kidney cell line, RCB accession no. RCB0539), HeLa cells (a human cervix, RCB accession no. RCB0271) and CHO cells (a Chinese hamster ovary, RCB accession no. RCB0285) (RIKEN Cell Bank, Tsukuba, Japan).
2. HuH-7 cells (a human hepatoma cell line, JCRB accession no. JCRB0403) (Health Science Research Resources Bank, Osaka, Japan).
3. Dulbecco's modified Eagle's medium, RPMI 1640, media supplements, and heat-inactivated fetal calf serum (IWAKI Glass, Chiba, Japan).
4. PicaGene luminescence kit (Toyo Ink).
5. Luminometer (Maltibiolumat LB9505, Berthold, Germany).

3. Methods

3.1. Peptide Synthesis

As shown in **Table 1**, the authors recommend two kinds of peptides, $4_6\Delta 8$ and Hel $\Delta 1$, which were refined from two original α -helical peptides, 4_6 and Hel, respectively (**6**).

3.1.1. Elongation of Peptide Chain on Resin

Peptides can be manually synthesized by the stepwise elongation of Fmoc protected amino acid on Rink amide resin (for 4_6 ; 4-(2',4'-dimethoxyphenyl)-Fmoc-aminomethyl)-phenoxy resin, 0.43 mmol/g resin) or Fmoc-Lys(Boc) preloaded Wang resin (for Hel; Fmoc-Lys(Boc)-*p*-benzoyloxy alcohol resin, 0.58 mmol/g resin) in 0.1 mmol scale as described by Fields et al. (**8**), and in Catalog & Peptide Synthesis Handbook of Calbiochem-Novabiochem. The Fmoc amino acid derivatives used are as follows: Ala, Arg(Pbf), Leu, Lys(Boc), and Trp. The coupling protocol is shown as follows:

1. Wash: 2 mL DCM (3 \times).
2. Wash: 2 mL NMP (3 \times).
3. Wash: 2 mL of 20% piperidine/*N,N*-dimethylformamide (1 \times).
4. Deprotection of Fmoc group: 2 mL 20% piperidine/DMF (15 min).
5. Wash: 2 mL NMP (5 \times).
6. Coupling of amino acid; Fmoc-amino acid (0.3 mmol), HBTU (0.3 mmol), HOBt (0.3 mmol), and *N,N*-diisopropylethylamine (DIEA) (0.6 mmol) in 3 mL NMP:DMF (1:1) (15 min).
7. Wash: 2 mL DMF (3 \times).
8. Kaiser test (**9**): When the coupling is incomplete, the protocol is repeated from **step 6**.

As a matter of course, it is possible to elongate the peptide chain using automatic peptide synthesizer (PE Biosystems, ABI 431A). In this case, the peptides are also synthesized by FastMoc method in 0.1 mmol scale. Even if single coupling of amino are applied for all elongation steps, satisfactory peptides in purity are obtained. In the case of modifying by acetyl group to N-terminal of peptide, 4₆, the Fmoc-protected peptide on resin is treated with 1 mmol acetic anhydride in 2 mL NMP for 20 min. After the resin is washed by DCM and methanol, they are dried *in vacuo*.

3.1.2. Deprotection and Cleavage of Peptide Resin

The protecting groups and the resin are removed with TFA (2.0 mL) in the presence of *m*-cresol (60 μ L), ethanedithiol (180 μ L), and thioanisole (360 μ L), in a round flask. After swirling at room temperature for 60 min, the resin is removed by filtration under reduced pressure and washed by TFA. Twenty milliliters of cold diethylether is added to the filtrate, then, the flask can be cooled with ice to further assist precipitation. Crude peptide is isolated by filtration under reduced pressure and washed by cold diethylether.

The crude peptides can be purified by RP-HPLC on a YMC-Pack C4 column (ϕ 10 \times 250 mm) with a linear gradient of water–acetonitrile containing 0.1% TFA. Peptide 4₆ Δ 8 and Hel Δ 1 are eluted at about 80 and 55% of acetonitrile, respectively. The authors recommend that if possible, the crude peptide be passed through a column of Sephadex G-10 or G-15 (ϕ 10 \times 250 mm) with 10% acetic acid before purification by RP-HPLC to avoid damaging the HPLC column. The peptide fractions are collected, then lyophilized. The yields obtained after purification are about 80 (34 μ mol) and 100 mg (36 μ mol) in the cases of 4₆ Δ 8 (as 4TFA salt) and Hel Δ 1 (as 6TFA salt), respectively. The purified peptides can easily be identified by MALDI-TOF-MS ($[M + H]^+ = 1874.4$ [4₆ Δ 8], 2105.7 [Hel Δ 1]).

3.2. Preparation of Peptide–DNA Complex

Complex of peptide and DNA is prepared by mixing 2.5 μ g plasmid DNA (PGV-C) with the peptides at peptide:DNA charge ratio of 2.0 in serum-free medium. The authors protocol is shown as follows:

1. Prepare the DNA solution: 2.5 μ g plasmid DNA in 250 μ L serum-free culture medium.
2. Prepare the peptide solution: 1.6 mM (as cationic groups) peptide aqueous solution. Practically, 930 μ g 4₆ Δ 8 • 4TFA or 743 μ g Hel Δ 1 • 6TFA are dissolved in 1.0 mL sterilized water.
3. 10 μ L Peptide solution is added to 250 μ L plasmid DNA solution.
4. Stand for 15 min at room temperature.

3.3. Gene Transfer Protocol into Cultured Cells

1. Plate cells in 24-well tissue culture dishes (ϕ 16 mm) at 1×10^5 cells/well and grow overnight in an atmosphere of 5% CO₂ at 37°C.

2. Wash twice with 1 mL serum free medium.
3. The peptide–DNA complex as described above is poured gently to the cells.
4. After incubation for 3 h at 37°C, 1 mL medium containing serum is added.
5. After incubation for 12 h at 37°C, the medium is replaced with 1 mL fresh medium containing serum and the cells are further incubated for 24 h.
6. Harvest of cells and luciferase assays are performed as described in the protocol of PicaGene luminescence kit using a luminometer.
7. The protein concentrations of the cell lysates are measured by Bradford assay using bovine serum albumin as a standard. The light unit values shown in the figures represent the specific luciferase activity (relative light units/mg protein), which is standardized for total protein content of the cell lysate. The measurement of gene transfer efficiency is performed in triplicate.

To date, the authors have tested gene transfer efficiencies of the peptides into several cell lines, such as COS-7, HeLa, CHO, and HuH-7 cells. **Figure 1** shows the results for each cell line. To compare the efficiencies of the peptides to a commercially available gene transfer agent, we employed Lipofectin (Gibco-BRL). As a result, the efficiencies of the peptides were similar to or about 10-fold lower than those of Lipofectin. Except for the case of needing a large amount of expression product, it can be said that the peptides are enough to use as a gene carrier. On the other hand, the peptides showed different efficiencies depending on the cell lines. These peptides can be chosen according to the cell lines.

The authors also evaluated cytotoxic activities of the peptide–DNA complexes, using Alamer Blue™, under the same conditions as those in the transfection procedure. As a result, little cytotoxic activity of the complexes could be observed. However, when the complex is prepared at peptide:DNA charge ratio of 4 and more, considerable cytotoxic activities are observed, which the authors consider to result from membrane perturbation activities originated from the amphiphilic structures of the peptides (3,5).

3.4. Active Targeting of Gene into Hepatoma Cells Using Galactose Modification of Peptide

As described in the Introduction, peptide is easily available because of the development of its synthetic technology. Therefore, this allows design and synthesis of functional gene carrier molecules such as carbohydrate-modified peptide for targeted gene delivery. Furthermore, peptide-based gene carrier enables construction of well-defined molecules, which cannot be achieved by polymer-based molecule. Here is introduced a synthesis of a multiantennary galactose-modified peptide and its application to a human hepatoma cell line.

3.4.1. Preparation of Multiantennary Galactose-Modified Peptide

The synthesis method is described as follows and is summarized in **Fig. 2**.

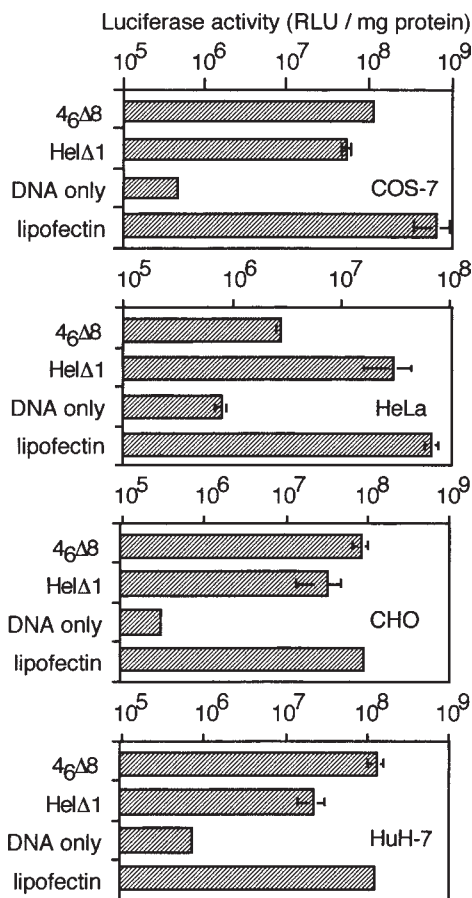


Fig. 1. Gene transfer efficiencies of the peptides into several cell lines.

1. α -Helical peptide portion can be synthesized by ordinary Fmoc solid-phase method on Rink amide resin (*see Subheading 3.1.1.*).
2. After coupling Fmoc-2-(2-aminoethoxy) ethanol as a linker, Fmoc- β Ala-Lys(Fmoc) is coupled twice. Fmoc groups of secondary coupled Fmoc- β Ala-Lys(Fmoc) are removed by piperidine. At this step, the peptide has four amino groups in the molecule.
3. After cleavage from resin and deprotection, the peptide is purified by RP-HPLC as similar condition to the case of peptide 4₆ (*see Subheading 3.1.2.*)
4. The amino groups of the peptide can be modified with lactose by aldimine formation, followed by reduction with NaBH₃CN of the secondary amines as follows. The purified peptide (11 μ mol, 50 mg as 10TFA salt) is mixed with a solution of lactose (530 μ mol, 190 mg as monohydrate) in 500 μ L 10 mM aqueous

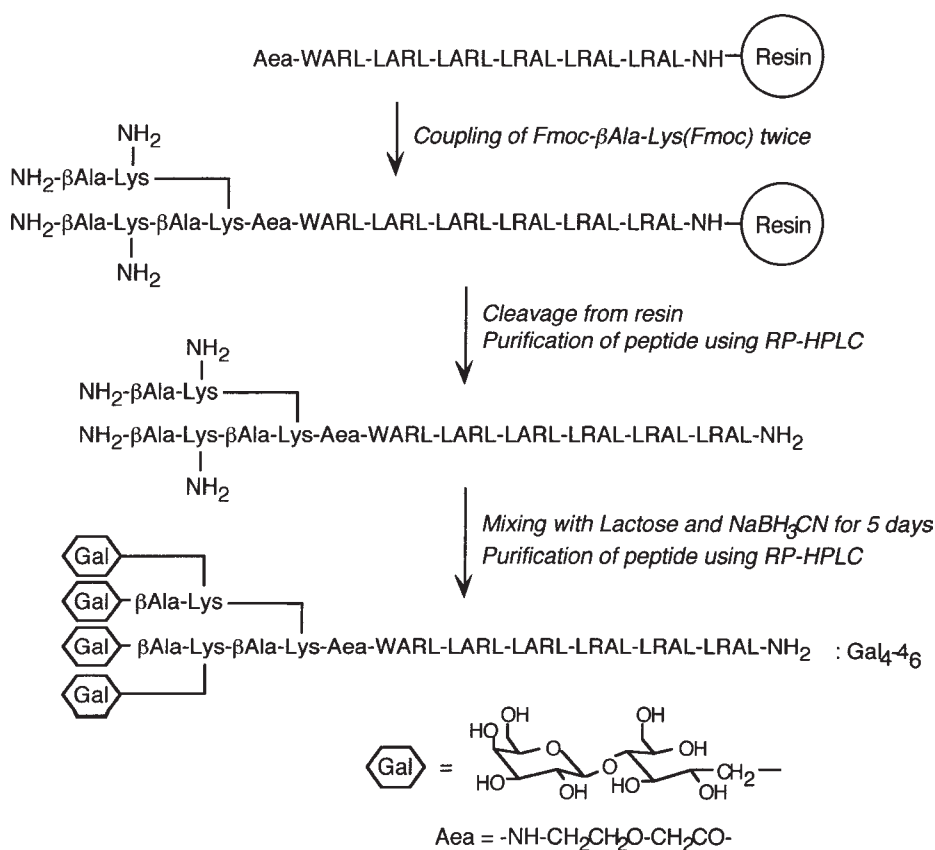


Fig. 2. Outline of synthesis of galactose-modified peptide, Gal₄-4₆.

sodium acetate, pH 5.0, at 37°C, then NaBH₃CN (44 μ mol, 2.8 mg each) is added to the solution at 12-h intervals. After total incubation for 60 h at 37°C, the resultant galactose-modified-peptide is purified by RP-HPLC. Yield is 20 mg (38%).

5. Identification is performed by MALDI-TOF-MS ($[M + H]^+ = 4816.7$). The peptide concentration in solution is determined from UV-absorbance of Trp at 280 nm in a buffer containing 6 M Gu \cdot HCl ($\epsilon = 5500$).

3.4.2. Gene Transfer Using Galactose-Modified Peptide into Hepatoma Cell Line, HuH-7

Protocol for gene transfer into HuH-7 cells, a human hepatoma cell line, is similar to that described in **Subheadings 3.2.** and **3.3.** Complex of peptide and DNA is prepared by mixing 2.5 μ g plasmid DNA (pCMVluc) with Gal₄-4₆ at peptide:DNA charge ratio of 2.0 in serum free medium.

1. Prepare the DNA solution: 2.5 μ g plasmid DNA in 250 μ L serum free RPMI 1640.

2. Prepare the peptide solution: 1.6 mM (as cationic groups) peptide aqueous solution. Practically, 1.5 mg Gal₄-4₆ • 10TFA is dissolved in 1.0 mL sterilized water.
3. 10 μ L peptide solution is added to 250 μ L plasmid DNA solution.
4. Stand for 15 min at room temperature.
5. The peptide-DNA complex as described above is poured gently on to the cells, which are washed twice with 1 mL serum-free RPMI 1640, beforehand. Following procedures are same to **steps 4–7** in **Subheading 3.3**.

As a result of measurement of luciferase activity in HuH-7 cells, the activity of Gal₄-4₆ was 300-fold higher than that of 4₆. In addition, the authors could confirm that the DNA complex of Gal₄-4₆ was internalized into the cells via the asialoglycoprotein receptor (*see Subheading 3.5.*).

This chapter introduced a galactose-modified peptide containing 2-(2-aminoethoxy)ethanol and β Ala as a linker, which allows the galactose residues to be easily recognized by the receptor. However, the authors found that a Gal₄-4₆ derivative without any linker showed a high efficiency into HuH-7 cells, as similar to that of Gal₄-4₆. Insertion of linker into the peptide system is not always necessary for recognition of the galactose residues by the receptor.

3.5. Commentary

In the authors' series of studies, it has become clear that the hydrophobic region on the amphiphilic structure of the peptides plays an important role in binding to the plasmid DNA and formation of aggregates with the DNA. It is likely that the hydrophobic region of the peptides induces stable oligomers with a well-defined number of monomers by self-association with their hydrophobic faces in aqueous solution. As a result, the oligomer would behave like a polycation, which can form aggregates with DNA (**Fig. 3**). Furthermore, the authors indicate that the hydrophobic region is also important for the disruption of the endosomal membrane in the cell, which can transfer the incorporated DNA to cytosol and prevent the degradation of the DNA in the lysosomal vesicles (**3.5**).

In order to clear detail translocation pathway of the DNA in the cell, the authors evaluated effects of several endocytosis inhibitors on the gene transfer efficiencies of peptide 4₆. Treatment with cytochalasin B, which depolymerizes the microfilaments of actin and blocks the uncoated pit-mediated endocytosis (macropinocytotic process) (**10**), reduced to 25% of the original efficiency of 4₆; no effect was observed in the case of treatment with chlorpromazine, an inhibitor of clathrin-dependent, receptor-mediated endocytosis (**11**). Furthermore, *N*-ethylmaleimide (NEM), which inhibits fusion between endosomes at an early stage of the endocytic pathway (**12**), reduced to 50% of the original efficiency, and the microtubule-depolymerizing agent, nocodazole, which interferes with transport from the early to late endosome mediated by endocytic carrier vesicles (**13**), increased the efficiency of the peptide by sixfold. From

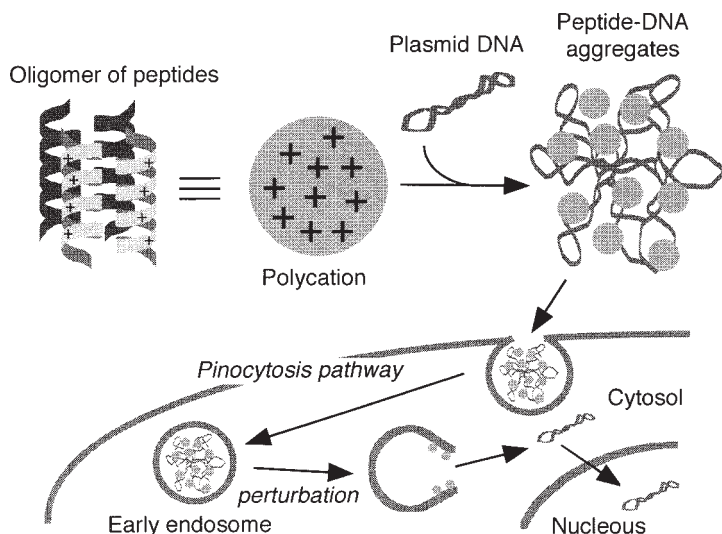


Fig. 3. Formation of peptide–DNA complex and its transfer pathway into cell.

these results, it is reasonable to suppose that the complexes of peptide 4₆ and plasmid DNA were internalized by macropinocytotic process, and a part of the complexes could be translocated from the endosomal compartment to the cytosol during an early endosome step (**Fig. 3**). However, the other part of the complexes would be translocated into degradation compartments such as late endosome and lysosome, where the complex could no longer be translocated to the cytosol. In order to enhance gene transfer efficiency, it will be necessary to consider active translocation to the cytosol of the DNA complex. Because there is now no information for translocation into nucleus of the complex, elucidation of this transfer mechanism in the cell will give a clue to construct a novel gene carrier with higher efficiency.

The authors have also studied the transfer pathway of the DNA complex with Gal₄–4₆. At first, the competitive effects of asialofetuin, which is internalized into hepatoma cells via the asialoglycoprotein receptor-mediated endocytosis, and fetuin, which is not recognized by the receptor, on the transfer efficiencies of the peptides were examined. As a result, the transfer efficiency of Gal₄–4₆ is reduced to 1% of the original efficiency in the presence of the asialofetuin, but no effect was observed in 4₆. However, fetuin showed weak effect compared with asialofetuin.

In addition, the authors evaluated effects of several endocytosis inhibitors on the gene transfer efficiencies of Gal₄–4₆. Treatment with chlorpromazine significantly reduced the efficiency; no significant reduction was observed in the case of treatment with cytochalasin B. This result suggested that the

internalization of Gal₄-4₆ was mediated by the clathrin-dependent, receptor-mediated endocytosis. Furthermore, NEM reduced to 50% of the original efficiency, and nocodazole increased the efficiency by twofold. From these results, DNA complex with the galactose modified peptide, Gal₄-4₆, would be translocated from the endosomal compartment to the cytosol during an early endosome step as in the case of 4₆.

Finally, to apply these galactose-modified peptides for targeted gene delivery in vivo, it is necessary to further examine the cell selectivity using several cell lines, stability in blood, capture by the reticuloendothelial system, and so on. When these points are solved, this delivery system will be one of the powerful tools for gene therapy.

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