Recombinant Mussel Adhesive Protein as a Gene Delivery Material

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ABSTRACT: Efficient target gene delivery into eukaryotic cells is important for biotechnological research and gene therapy. Gene delivery based on proteins, including histones, has recently emerged as a powerful non-viral DNA transfer technique. Here, we investigated the potential use of a recombinant mussel adhesive protein, hybrid fp-151, as a gene delivery material, in view of its similar basic amino acid composition to histone proteins, and cost-effective and high-level production in Escherichia coli. After confirming DNA binding affinity, we transfected mammalian cells (human 293T and mouse NIH/3T3) with foreign genes using hybrid fp-151 as the gene delivery carrier. Hybrid fp-151 displayed comparable transfection efficiency in both mammalian cell lines, compared to the widely used transfection agent, Lipofectamine® 2000. Our results indicate that this mussel adhesive protein may be used as a potential protein-based gene-transfer mediator.

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KEYWORDS: mussel adhesive protein; hybrid fp-151; DNA binding; gene delivery material; transfection; mammalian cells

Introduction

Gene therapy is a promising approach for the treatment of acquired or genetic disorders. A major obstacle in this methodology is inefficient target gene delivery from the outside to the nucleus of cells, since barriers, such as plasma membrane and endogenous nuclease, prevent cells from taking up exogenous genetic information. Thus, successful gene therapy depends on the development of an efficient and non-toxic gene delivery system (Kouraklis, 2000). While viral gene delivery systems have been developed and improved over the decades, safety concerns have limited their use in practical gene delivery (Crystal, 1995). Consequently, alternative non-viral gene delivery systems such as cationic liposomes, cationic proteins, and polymer systems have been investigated (Balicki and Beutler, 2002; Chen et al., 2001; Felgner et al., 1987; Jiang et al., 2007). Cationic liposomes are commercialized non-viral carriers due to their non-immunogenicity and simplicity of large-scale production (Felgner et al., 1987). However, their efficiencies can be relatively low due to inactivation by serum or blood components and loose condensation of DNA associated with decreased uptake into cells (Lesoonwood et al., 1995). To overcome the problems of liposomes, polymer systems or cationic proteins have been introduced (Balicki and Beutler, 1997; Boussif et al., 1995; Choi, 2007; Dai et al., 2003; Fritz et al., 1996; Haberland et al., 2000; Zaitsev et al., 2002).

The cationic protein-based gene delivery system has several advantages, including ease of use in serum- and/or antibiotic-containing medium, the ability to target nucleic acids to specific cell types, no limit on the size or type of target nucleic acid, possibility of modular attachment of targeting ligands, and the potential for cost-effective, large-scale manufacture (Balicki and Beutler, 1997; Chen et al., 1994; Demirhan et al., 1998; Fritz et al., 1996; Zaitsev et al., 1997). A number of histone proteins have been widely analyzed as potential gene delivery materials. Histones display similar or higher transfection efficiency in mammalian cells, compared to the widely used transfection agent, Lipofectamine® (Jung et al., 2008; Puebla et al., 2003). Histones are basic proteins that contain several lysines and arginines. These positively charged amino acids facilitate electrostatic interactions with the negatively charged phosphate backbone of DNA. However, application of eukaryotic histones for gene delivery is limited by low recombinant expression levels and infection risks (Kaouass et al., 2006).

Mussel adhesive proteins (MAPs) are water-resistant bioadhesives (Cha et al., 2008; Dove and Sheridan, 1986;
Grande and Pitman, 1988; Pitman et al., 1989; Waite and Tanzer, 1981) that adhere tightly to substrata using the byssus secreted from the foot, which comprises a bundle of threads. At the end of each thread, there is an adhesion plaque containing a water-resistant adhesive that enables anchorage to wet, solid surfaces (Waite, 1983). Previously, we successfully produced recombinant MAPs with functional adhesion properties in Escherichia coli (Hwang et al., 2004, 2005, 2007a). In particular, mass production of recombinant MAP was possible due to the development of a hybrid fp-151, a fusion of 6 repeated MAP type 1 (fp-1) decapeptides on both termini of type 5 (fp-5) (Hwang et al., 2007a; Kim et al., 2008). Interestingly, we noticed that recombinant MAPs display similar physical properties to basic proteins, such as histones, including significant basic amino acid content and high pI values (Cha et al., 2008; Hwang et al., 2004, 2005, 2007a). In the present study, we investigated the potential use of a recombinant MAP, hybrid fp-151, as a protein-based gene delivery material for mammalian cells.

Materials and Methods

Production and Purification of Hybrid fp-151

Hybrid fp-151 fused with the hexa-histidine affinity ligand (Fig. 1A) was produced in E. coli as described previously (Hwang et al., 2007a). In brief, E. coli cells were cultured in 7 L LB medium supplemented with 50 μg/mL ampicillin (Sigma-Aldrich, St. Louis, MO) within a 10 L bioreactor (KoBiotech, Incheon, Korea) at 37°C and 250 rpm. At optical density (OD 600) of 0.2–0.5, 1 mM (final concentration) isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma) was added to the broth to induce fp-151 expression, and cultured for 8 h at 37°C and 250 rpm. Following centrifugation of culture broth at 18,000g for 10 min at 4°C, cell pellets were stored at ~80°C for further analysis. Harvested cell pellets containing hybrid fp-151 were resuspended in 5 mL lysis buffer (10 mM Tris–Cl, 100 mM sodium phosphate, pH 8.0) per gram wet weight. Samples were lysed with constant cell disruption systems (Constant Systems, Daventry, Northants, UK) at 20 kpsi, lysates centrifuged at about 18,000g for 20 min at 4°C, and the cell debris dissolved in binding buffer (8 M urea, 10 mM Tris–Cl, 100 mM sodium phosphate, pH 8.0) for immobilized metal affinity chromatography (IMAC) purification. IMAC was performed under denaturing conditions using the Acta Prime Puriﬁcation System (Amersham Biosciences, Piscataway, NJ) at room temperature at a rate of 1 mL/min. The affinity purification resin used was 10 mL of Ni-nitrilotriacetate agarose (Qiagen, Valencia, CA) charged with 10 mL of 0.1 M NiSO4. Target fp-151 was eluted with elution buffer (8 M urea, 10 mM Tris–Cl, 100 mM sodium phosphate, pH 4.5), and dialyzed in 5% (v/v) acetic acid buffer overnight at 4°C, using Spectra/Por molecular porous membrane tubing (Spectrum Laboratories, Greensboro, NC). The sample was concentrated by freeze-drying, and finally dissolved in distilled water.

DNA Binding Activity Assay

DNA binding activity was determined with electrophoretic DNA retardation. Hybrid fp-151 (4 mg/mL) was mixed with the plasmid pcDNA/3.1/His/lacZ (133 μg/mL; Invitrogen, Carlsbad, CA) at ratios (w/w) of 0.5:1–8:1, and incubated for 30 min at room temperature. Bovine serum albumin (BSA;
Mammalian Cell Culture and Transfection

Wild-type human 293T (#CRL-11268, ATCC) and mouse NIH/3T3 (#CRL-1658, ATCC) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; HyClone, Logan, UT) supplemented with 10% (v/v) fetal bovine serum (FBS; HyClone) and penicillin/streptomycin (Gibco, Grand Island, NY) at 37°C in a humidified atmosphere of 5% CO2 and 95% air. Initially, 2 × 105 cells were plated onto a 12-well culture plate. At 70–90% confluence, cells were transfected with pcDNA/3.1/His/lacZ (Promega) as a substrate, and absorbance at 420 nm was measured using o-nitrophenyl-beta-D-galactopyranoside (ONPG; Promega) as a substrate, and absorbance at 420 nm was measured using a UV-vis spectrophotometer (Shimadzu, Kyoto, Japan). The fluorescence intensity of EGFP was measured at 488 nm for excitation and 513 nm for emission, using a fluorescence spectrophotometer (Shimadzu). EGFP-expressing fluorescent cells were additionally analyzed by flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ) and examined by fluorescence microscopy (Olympus, Tokyo, Japan). For nuclear staining, transfected cells were fixed with 4% (v/v) paraformaldehyde, and permeabilized with 0.1% (v/v) Triton X-100. Fixed cells were washed with PBS, and immersed in 0.001% (w/v) 4’,6-diamidino-2-phenylindole (DAPI) in PBS for 20 min. Finally, cells were fixed with Lisbeth’s embedding medium (30 mM Tris–Cl, pH 9.5, 70% glycerol, 50 mg/mL N-propyl gallate), and examined by fluorescence microscopy. Transfection with 500 ng plasmid DNA only (in the presence and absence of CaCl2) was performed as the negative control. As a positive control, cells were transfected with 500 ng plasmid DNA using 1 μg of Lipofectamine™ 2000 (Invitrogen), which can be added directly to culture medium even in the absence of calcium ions, hybrid fp-151-mediated transfection was minimal (Fig. 2C and D). The calcium ion concentration used for investigation of optimal binding ratio between hybrid fp-151 and DNA in the medium was 6 mM, previously reported as the optimum for histone H1-mediated transfection (Haberland et al., 2000). A hybrid fp-151:DNA ratio of 32:1 (w/w) displayed the best transfection efficiency in both 293T (Fig. 2A) and NIH/3T3 (Fig. 2B) cells. Therefore, after fixing the hybrid fp-151:DNA ratio to 32:1, we optimized the calcium concentration in the medium. 293T cells showed the highest transfection efficiency with 18 mM Ca2+ (Fig. 2C), while 12 mM Ca2+ was better for NIH/3T3 cells (Fig. 2D).

Results

DNA Binding Assay of Hybrid fp-151

Hybrid fp-151 fused with the hexa-histidine affinity ligand (Fig. 1A) was prepared with ~95% purity using IMAC to eliminate E. coli components with possible negative effects on mammalian cell culture (lane AF, Fig. 1B). We investigated the DNA binding ability of purified hybrid fp-151 using a gel retardation assay. The electrophoretic mobility of DNA on a 1% agarose gel was retarded in the presence of hybrid fp-151 (Fig. 1C), in proportion to the amount of protein. Moreover, the hybrid fp-151/plasmid DNA complex displayed completely retarded migration on the agarose gel at a hybrid fp-151:DNA ratio of 4:1 (w/w). Thus, the minimum ratio for complete binding of hybrid fp-151 with plasmid DNA was estimated as ~4:1 (w/w).

Transfection of Mammalian Cells Using Hybrid fp-151

Based on its confirmed DNA binding ability, we investigated whether hybrid fp-151 can deliver foreign DNA molecules into mammalian cells in the presence of 10% (v/v) FBS. Note that we used two different cell lines (human 293T and mouse NIH/3T3 cells) and two different reporter genes (lacZ encoding beta-galactosidase and egfp encoding EGFP) to prove the validity of hybrid fp-151 as a general gene delivery material. To maximize transient transfection efficiency, we initially optimized the binding ratio (w/w) between hybrid fp-151 and plasmid DNA harboring lacZ gene as the reporter (Fig. 2A and B). In the absence of calcium ions, hybrid fp-151-mediated transfection was minimal (Fig. 2C and D). The calcium ion concentration used for investigation of optimal binding ratio between hybrid fp-151 and DNA in the medium was 6 mM, previously reported as the optimum for histone H1-mediated transfection (Haberland et al., 2000). A hybrid fp-151:DNA ratio of 32:1 (w/w) displayed the best transfection efficiency in both 293T (Fig. 2A) and NIH/3T3 (Fig. 2B) cells. Therefore, after fixing the hybrid fp-151:DNA ratio to 32:1, we optimized the calcium concentration in the medium. 293T cells showed the highest transfection efficiency with 18 mM Ca2+ (Fig. 2C), while 12 mM Ca2+ was better for NIH/3T3 cells (Fig. 2D).
To further evaluate the transfection efficiency of hybrid fp-151, we performed a comparative study with Lipofectamine™ 2000 as a positive control. Lipofectamine™ 2000 is a widely used cationic liposome originally developed for use in serum-containing medium. Hybrid fp-151/DNA mixtures (32:1 (w/w)) were transfected into mammalian cells in the presence of calcium ions (18 mM for 293T and 12 mM for NIH/3T3). Transfection efficiencies using hybrid fp-151 were significantly higher (~3.5-fold) than those with the Lipofectamine-mediated method with lacZ as the reporter gene in both 293T (Fig. 3A) and NIH/3T3 (Fig. 3B) cell lines. To confirm the potency of hybrid fp-151 as the DNA carrier, another reporter gene, egfp, was used for transient transfection. Using hybrid fp-151-mediated transfection, EGFP fluorescence was successfully observed from transfected 293T cells with fluorescence microscopy (right image in Fig. 4A). In the case of egfp reporter gene transfection into 293T cells, we also performed flow cytometric analyses to compare actual transfection efficiencies. The fp-151-mediated transfection showed comparably similar percentages of EGFP-expressing cells to Lipofectamine™ 2000-based method while negative control and only calcium added cases exhibited minimal transfections (Fig. 4B). We found that these flow cytometric results had a somewhat correlation with EGFP expression levels from spectrophotometric analyses (Fig. 4C). While fluorescence in transfected 293T cells using hybrid fp-151 with 18 mM Ca²⁺ was lower (~0.7-fold) than that with the Lipofectamine™ 2000-based method (Fig. 4C), EGFP fluorescence from NIH/3T3 cells transfected using hybrid fp-151 in the presence of 12 mM Ca²⁺ was higher (~1.8-fold) (Fig. 4D). However, when the CaCl₂ concentration in the medium was reduced from 18 mM to 12 mM, EGFP fluorescence in 293T cells transfected using hybrid fp-151 was higher (~1.6-fold) than that with Lipofectamine™ 2000, while β-galactosidase activity was lower (~0.8-fold) (data not shown).
Cytotoxic Assays of Transfected Cell Lines Using Hybrid fp-151

To check the cytotoxicity of hybrid fp-151-mediated transient transfection, we performed a MTT assay (Mosmann, 1983) of lacZ-transfected 293T and NIH/3T3 cells. Measurement of the metabolic activity of viable cells and absorbance at 570 nm in the MTT assay is linearly correlated with the number of live cells. The viability of 293T cells transfected with hybrid fp-151 was lower (~70%),

Figure 3. Comparison of the transfection efficiencies of lacZ reporter gene into human 293T (A) and mouse NIH/3T3 (B) cells under serum-presence condition. NC, DNA only as a negative control; Ca, DNA with 18 mM (293T) or 12 mM (NIH/3T3) CaCl₂; Lipo, DNA with Lipofectamine™ 2000; fp-151, DNA with hybrid fp-151:DNA ratio of 32:1 (w/w) and 18 mM (293T) or 12 mM (NIH/3T3) CaCl₂. For each transfection, 500 ng DNA was used. After 48 h total incubation time, β-galactosidase expression was measured to determine transfection efficiencies. β-galactosidase activity values were normalized based on the value of the case of Lipofectamine™ 2000. Each value and error bar represents the mean of duplicate samples for two independent experiments and its standard deviation. The typical P-value criterion (<0.01) was employed to determine statistical differences.

Figure 4. A: EGFP-expressing fluorescent (green) and DAPI nuclear-stained (blue) cells transfected with Lipofectamine™ 2000 or hybrid fp-151 were observed using fluorescence microscopy (400 x magnification). B: Comparison of the transfection efficiencies of egfp reporter gene into human 293T cells using flow cytometric analyses. Comparison of the expression efficiencies of EGFP reporter in (C) human 293T and (D) mouse NIH/3T3 cells. NC, DNA only as a negative control; Ca, DNA with 18 mM (293T) or 12 mM (NIH/3T3) CaCl₂; Lipo, DNA with Lipofectamine™ 2000; fp-151, DNA with hybrid fp-151:DNA ratio of 32:1 (w/w) and 18 mM (293T) or 12 mM (NIH/3T3) CaCl₂. For each transfection, 500 ng DNA was used. After 48 h total incubation time under serum-presence condition, EGFP expression was measured. Each value and error bar represents the mean of duplicate samples for two independent experiments and its standard deviation. The typical P-value criterion (<0.01) was employed to determine statistical differences. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]
compared to that with Lipofectamine™ 2000 (Fig. 5A), but that of NIH/3T3 cells transfected with hybrid fp-151 was similar to that estimated with Lipofectamine™ 2000 (Fig. 5B).

Discussion

In this study, we performed gene delivery into mammalian cells using the recombinant mussel adhesive protein, hybrid fp-151, a fusion of six repeated fp-1 decapeptides on both termini of fp-5. Several studies have demonstrated that H1-type histones are effective protein-based mediators of transfection (Dai et al., 2003; Fritz et al., 1996; Haberland et al., 2000; Kaouass et al., 2006; Puebla et al., 2003; Zaitsev et al., 1997). Positively charged histone H1 can adhere tightly to the cell membranes of living cells (Bolton and Perry, 1997; Lundberg and Johansson, 2002), and mediate efficient gene transfer in the presence of CaCl₂ and/or chloroquine (Haberland et al., 2000; Kaouass et al., 2006; Puebla et al., 2003; Zaitsev et al., 1997). Efficient gene transfection possibly occurs through DNA condensation, DNase protection, and/or mediation of nuclear import (Fritz et al., 1996; Lucius et al., 2001; Puebla et al., 2003). Analogous to histone protein (~29% basic amino acid percentage and 10.9 pI value; GenBank accession #AY184811), we noticed that ~25% of the total amino acids in hybrid fp-151 are basic (lysine, arginine, histidine), and its pI value is 9.9 (Hwang et al., 2007a). Thus, hybrid fp-151 is positively charged at neutral pH and might facilitate binding to the sugar phosphate backbone of DNA. Additionally, hybrid fp-151 is efficiently expressed in E. coli (Hwang et al., 2007a), while histone proteins are neither economically extracted from eukaryotic organisms nor expressed well in recombinant systems (Kaouass et al., 2006; Puebla et al., 2003). In addition, because hybrid fp-151 is only viscous in very high concentration (~300 mg/mL solubility in water; Hwang et al., 2007a) and we used low concentration (<0.2 mg/mL) in the transfection work, the protein was not viscous and difficult to handle. Therefore, hybrid fp-151 can be a potential economic gene delivery carrier.

Hybrid fp-151 bound to DNA and neutralized DNA mixture at a hybrid fp-151:DNA ratio of around 4:1 (w/w) (Fig. 1B). Interestingly, the hybrid fp-151/DNA mixture left behind in the gel loading well gradually decreased as the hybrid fp-151:DNA ratio increased to >4:1. We surmise that the mixture moved to the opposite direction, since the net charge of hybrid fp-151/DNA mixture shifted to positive. Positive charges in the mixture may aid in targeting the negatively charged mammalian cell surface. Thus, transfection efficiency was gradually increased with the addition of more hybrid fp-151 to DNA (Fig. 2A and B). However, transfection efficiency was not enhanced any further at a hybrid fp-151:DNA ratio of >32:1 (w/w), possibly because the size increase in the mixture has a negative effect on gene transfer (Chen et al., 2001). A comparative study with Lipofectamine™ 2000 was performed to confirm the potential of hybrid fp-151 as a gene delivery carrier. At a hybrid fp-151:DNA ratio of 32:1, the protein displayed superior transfection efficiency to Lipofectamine™ 2000 in 10% FBS in the presence of calcium ion (12–18 mM), further supporting its potential as gene delivery material (Figs. 3 and 4). Optimal calcium ion concentrations differed depending on the cell lines utilized (Fig. 2C and D), indicating that preliminary investigations to determine the optimal concentration are needed for diverse cell lines. We observed significantly improved gene delivery efficiency with both reporter gene transfections in mouse NIH/3T3 cells (Figs. 3B and 4D). However, human 293T cells were somewhat dependent on the calcium ion concentration and/or gene type. It was reported that trend of transfection efficiency can be changed according to reporter genes, cell
lines, and calcium concentrations in medium (Puebla et al., 2003). The transfection efficiency of the hybrid fp-151-based gene delivery system was higher using the lacZ gene with 18 mM CaCl2 (Fig. 3A) or egfp gene with 12 mM CaCl2 (data not shown), compared to Lipofectamine™ 2000, but lower using the lacZ gene with 12 mM CaCl2 (data not shown) or egfp gene with 18 mM CaCl2 (Fig. 4C). In fact, human 293T cells were relatively more sensitive to the culture environment (stress) than mouse NIH/3T3 cells (experimental observation). We suspect that this sensitivity contributes to the selective dependency of gene delivery efficiency on calcium ion and/or gene type.

In the absence of calcium, hybrid fp-151-mediated transfection was barely detected in both cell lines (Fig. 2C and D), consistent with previous reports on the role of calcium ions in histone H1-mediated transfection (Haberland et al., 2000; Zaitsev et al., 2002). Moreover, transfection with reporter plasmids only led to negligible β-galactosidase activity and EGFP fluorescence intensity in the presence or absence of calcium (Figs. 3 and 4), suggesting that foreign gene delivery does not occur solely by DNA-calcium co-precipitation (Zaitsev et al., 2002). Monitoring of the cellular and intracellular fate of the histone H1/DNA mixture revealed that the mixtures were localized within endosome/lysosome vesicles, implying uptake of transfection mixtures via endocytosis (Zaitsev et al., 2002). Thus, it appears that while the uptake of transfection complexes is Ca2+-independent, calcium ions are required to release transfection complexes from the endosome/lysosome compartments (Zaitsev et al., 2002).

To evaluate the cytotoxicity of hybrid fp-151-mediated transfection, the MTT assay was performed using lacZ as a reporter gene with both cell types. At optimal concentrations of hybrid fp-151 (16 μg) and CaCl2 (12 mM), hybrid fp-151-mediated transfection did not exert toxicity in mouse NIH/3T3 cells (Fig. 5B). However, in human 293T cells, hybrid fp-151-mediated transfection with 18 mM CaCl2 led to lower cell viability than that with Lipofectamine™ 2000 (Fig. 5A). The viability of 293T cells transfected with hybrid fp-151 in medium containing lower CaCl2 concentrations (12 mM) was also lower than that of cultures incubated with Lipofectamine™ 2000/DNA complex, even though its reduction was smaller than the case of 18 mM CaCl2 (data not shown). However, the cytotoxicity of hybrid fp-151-mediated transfection was similar at significantly lower CaCl2 concentrations (~6 mM) to that of Lipofectamine™ 2000-based transfection (data not shown). Additionally, higher amount (16 μg) of hybrid fp-151 was used than Lipofectamine™ 2000 (1 μg). Therefore, we surmise that human 293T cells are sensitive at high calcium ion concentrations and relatively high amounts of hybrid fp-151. Developments of a novel mussel adhesive protein with higher DNA affinity and lower requirement for calcium ion are two of the possible solutions for overcoming these problems.

Cell transfection in the presence of serum is very important to evaluate the feasibility of the gene delivery system under in vivo conditions. A major drawback of cationic liposomes, including Lipofectamine, is deactivation in serum. Thus, cationic proteins, such as histones and MAPs, are possible alternatives to liposomes in the presence of serum. To date, histone-based transfection (histonefection) has been widely investigated as a potent non-viral protein-based gene delivery system. However, the use of histones in gene delivery is significantly limited by high production cost and infection risk. Natural extraction from eukaryotic organisms is considerably expensive, and recombinant histones are not well expressed in E. coli (Puebla et al., 2003). In contrast, hybrid fp-151 can be easily produced from E. coli at a low cost, compared to recombinant histone. Moreover, fusion of hybrid fp-151 with a signal peptide, such as nuclear localization signal (NLS) or protein transduction domain (PTD), may reduce usage quantity, improve transfection efficiency, and promote positive effects on in vivo gene delivery. In fact, fusion of hybrid fp-151 with a biologically active peptide has been attempted to introduce new characteristics in hybrid fp-151 (Hwang et al., 2007b). Our study provides a footstone for the development of hybrid fp-151 terminal-derived peptides as useful agents for efficient gene delivery. Further comparative studies with other cell lines and transfection reagents in vitro and in vivo are required. Physicochemical properties of hybrid fp-151/DNA complex will be also carried out in the future.

**Conclusion**

This is the first report demonstrating that recombinant mussel adhesive protein is capable of delivering foreign genetic material into mammalian cells. Hybrid fp-151 displays similar basic amino acid content and high theoretical pI values as histone H1 proteins. Moreover, the hybrid fp-151 exhibits efficient DNA binding ability and higher transfection efficiency (at specific hybrid fp-151/DNA mixed ratios) in mammalian cells, compared to the widely used Lipofectamine-based method. Our findings collectively support the potential use of hybrid fp-151 as an economic protein-based mediator for efficient gene delivery.

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**References**


