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Non-covalent complexes of folic acid and oleic acid conjugated polyethylenimine: An efficient vehicle for antisense oligonucleotide delivery

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Abstract

Polyethylenimine (PEI) was conjugated to oleic acid (PEI-OA) and evaluated as a delivery agent for LOR-2501, an antisense oligonucleotide against ribonucleotide reductase R1 subunit. PEI-OA/LOR-2501 complexes were further coated with folic acid (FA/PEI-OA/LOR-2501) and evaluated in tumor cells. The level of cellular uptake of FA/PEI-OA/LOR-2501 was more than double that of PEI/LOR-2501 complexes, and was not affected by the expression level of folate receptor (FR) on the cell surface. Efficient delivery was seen in several cell lines. Furthermore, pathway specific cellular internalization inhibitors and markers were used to reveal the principal mechanism of cellular uptake. FA/PEI-OA/LOR-2501 significantly induced the downregulation of R1 mRNA and R1 protein. This novel formulation of FA/PEI-OA provides a reliable and highly efficient method for delivery of oligonucleotide and warrants further investigation.

Keywords

Oligonucleotide delivery; Non-covalent; Folic acid; Polyethylenimine (PEI); Oleic acid Pathway

1. Introduction

Delivery of oligodeoxynucleotide into tumor cells remains a critical challenge in cancer therapy [1,2]. Developing oligodeoxynucleotide delivery vectors with high efficiency and low toxicity is imperative [3–5]. Polyethyleneimine (PEI) is a cationic polymer with a high charge density. High molecular weight PEI (PEI 25 kDa) possesses better transfection efficiency than low molecular weight PEI. However, it has higher toxicity. Previous studies have shown that hydrophobic modification of PEI with oleic or stearic acids provides a

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Disclosure

The author reports no conflicts of interest in this work.

stable and efficient system for siRNA or antisense oligonucleotide delivery to cells, it could also reduce the toxicity of PEI [6–9].

Folic acid (FA) is a vitamin with high affinity for the FR [10,11]. It tends to self-associate into hydrogen-bonded planar tetramers which may further stack to form octamers [12–14]. Covalent attachment of FA has been shown to target drugs to FR positive tumor cells [15]. Meanwhile, electrostatic PEI/pDNA complexes with FA have been shown to have excellent transfection efficiency. It has been shown that FA was able to coat the PEI/pDNA complexes electrostatically. It was further suggested that anionic FA/PEI/pDNA complexes were taken up via the FR-mediated pathway in B16-F10 cells [16].

LOR-2501 is a 20-mer phosphorothioate antisense oligonucleotide (ASO) that is complementary to a coding region of the mRNA of RRM1 (R1), a subunit of ribonucleotide reductase, an enzyme associated with drug resistance [17]. In vitro studies have shown that LOR-2501 inhibited the growth of human lung, liver, brain, ovary and breast tumor cells [18–20].

In this study, oleic acid-conjugate PEI, PEI-OA, was synthesized and evaluated as a carrier for LOR-2501. PEI-OA/LOR-2501 complexes were further coated with FA. We demonstrated that FA was able to coat the complexes electrostatically without covalent conjugation, and the coated complexes showed high bioactivity both in FR^+ and FR^- tumor cells. The mechanism of cellular internalization was also investigated.

2. Material and methods

2.1. Materials

Branched PEI (25,000 Da), triethylamine (TEA), oleoyl chloride (OA, 99%) and FA were obtained from Sigma–Aldrich (St. Louis, MO). Anhydrous ethyl ether and dichloromethane (DCM) were purchased from Fisher Scientific (Fairlawn, NJ). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma–Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was obtained from Gibco BRL (Bethesda, MD). 4′,6-Diamidino-2-phenylindole (DAPI) was purchased from Invitrogen Molecular Probes (Eugene, OR). LOR-2501 (5′-CTC TAG CGT CTT AAA GCC GA-3′) and 5′-Cy3-LOR-2501 were obtained from Biomics Biotechnologies (Nantong, Jiangsu).

2.2. Cell culture

HeLa and SK-HEP-1 cell lines were grown and propagated in Dulbecco’s modified Eagle’s medium (DMEM), A549 and KB cell lines were grown and propagated in RPM1640, supplemented with 10% FBS at 37 °C and humidified 5% CO_2.

2.3. Synthesis and characterization of PEI-OA

PEI-OA was synthesized as reported previously [21]. Briefly, 50 mg of PEI was dissolved in 2 ml DCM under N_2 at room temperature. After addition of 2 μl of TEA, the desired oleoyl chloride was dissolved in 2 ml DCM and gradually added to the PEI solution at a fatty acid:ethylenimine ratio of 1:1.5 over 1 h. The solution was stirred for 12 h under N_2. Excess of ethyl ether was added to precipitate and wash (×3) the product, which was then dried

under vacuum overnight at room temperature. The composition of the reaction products was determined by a 300 MHz ^{1}H NMR spectroscope (Bruker 300 AM; Billerica, MA). The proton shifts specific for fatty acids (0.8 ppm; terminal —CH_3) and PEI (2.5–2.8 ppm; —HN—CH_2—CH_2—NH—) were integrated, normalized to the number of protons in each peak, and used to obtain the lipid substitutions on polymers.

2.4. Preparation of FA/PEI-OA/LOR-2501 complexes

Anhydrous ethanol was used to dissolve PEI-OA (5 mg/ml), and 10 μl PEI-OA solution was injected into 90 μl PBS buffer. The mixture solution was vortex mixed for 10 s, sonicated for 20 s, and then LOR-2501 (0.07765 mg/ml) was added into the system, mixed thoroughly. FA was dissolved in phosphate buffer (pH 6.5) at 8.8 mg/ml, and was added into the mixed solution. In this study, we constructed the complexes at various theoretical charge ratios, including FA carboxylate/PEI nitrogen/LOR-2501 phosphate (FA/PEI-OA/LOR-2501) = 0:6:1 (PEI-OA/LOR-2501), 5:6:1 (FA5/PEI-OA/LOR-2501), 10:5:1 (FA10/PEI-OA/LOR-2501), 15:6:1 (FA15/PEI-OA/LOR-2501), 20:6:1 (FA20/PEI-OA/LOR-2501), 25:6:1 (FA25/PEI-OA/LOR-2501), and 30:6:1 (FA30/PEI-OA/LOR-2501).

2.5. Determination of PEI-OA/LOR-2501 and FA/PEI-OA/LOR-2501 complex formation by gel retardation assay

LOR-2501 was combined with PEI-OA to form PEI-OA/LOR-2501 complexes at N/P ratios of 1–10, and FA/PEI-OA/LOR-2501 complexes at FA/PEI-OA/LOR-2501 (C/N/P) ratios of 0:6:1–30:6:1. Two microliter of 6× sample loading buffer (50% glycerol, 1% bromophenol blue, and 1% xylene cyanol FF in Tris-borate EDTA (TAE) buffer) was then added to each sample. The samples were loaded onto 3% agarose gel containing 0.2% mg/ml ethidium bromide. Electrophoresis was run at 120 V for 15 min. The resulting gels were photographed under UV-illumination.

2.6. Size and zeta potential measurement

The size and surface morphology of the FA15/PEI-OA/LOR-2501 complex was investigated by field emission scanning electron microscope (FE-SEM) (JSM-6700F, JEOL, Tokyo, Japan) [22]. Samples were fixed on a brass stub using double-sided adhesive tape and were made electrically conductive by coating with a thin layer of gold. SEM images were taken at 3.0 kV accelerating voltage.

The zeta potential of the complexes was measured on a Zetasizer Nano ZS 90 (Malvern Instruments, Ltd., Malvern, UK). Zeta potential of each complex formulation FA/PEI-OA/LOR-2501 ratios of 0:6:1–30:6:1 was calculated averaging 3 measurements.

2.7. Cytotoxicity assay

Cells were seeded at a density of 1×10^4 cells/well in a 96-well plate and grown for another 24 h. Cells were then washed three times with serum-free media and incubated with PEI-OA with varying ratios or FA/PEI-OA and varying concentrations of FA. Transfection medium was removed after 4 h. Fresh culture medium was then added and the cells were incubated at 37 °C for an additional 20 h. Then, cell viability was analyzed by MTT assay. Briefly, each well was then incubated with 20 μl of MTT solution in culture medium (0.5 mg/ml) for 2 h.

Crystals formed were dissolved by adding 150 μl of DMSO to each well. Optical density was then measured at 570 nm. The results were converted into % viability by using the absorbance from untreated wells as a reference (100%), and expressing the absorbance obtained from the treatment groups as a percentage of the reference value. The results were summarized as mean and SD of 6 replicates for each sample.

2.8. Uptake of FA/PEI-OA/LOR-2501 by different cell Lines

In these experiments, 5′-Cy3-labeled LOR-2501 was formulated in the complexes. The cells were plated on 24-well plates at a density of 1×10^5 cells/well and cultivated in 1 ml of culture medium. In the transfection experiment, after 24 h culture, the medium was removed and washed by PBS three times and replaced with 1 ml of medium (FA free), and each formulation containing 0.1 nmol of LOR-2501 was added to the cells and incubated for 4 h. After transfection, the cells were washed with PBS three times and were fixed with 350 μl 4% formaldehyde solution, LOR-2501-positive cells was determined by fluorescence activated cell sorting (FACS). For this, the samples were evaluated on an EPICS XL flow cytometer (Beckman Coulter Corp., Tokyo, Japan) and the data were analyzed with the Cell Quest software. Each sample was subjected by 3 measurements.

2.9. Treatment with inhibitors for internalization pathways

Cells were seeded in a 24-well plate at a density of 1×10^5 cells/well. After 24 h of incubation, cells were treated with medium, or media containing sucrose (0.2 M), cytochalasin D (cyto D, 2.5 μM), or nystatin (25 μM) for 1 h. Complexes composed PEI, PEI-OA or FA/PEI-OA with Cy3-LOR-2501 were then added and the cells were incubated for 4 h. The cells were then washed twice with PBS and treated with 0.25% trypsin/EDTA at 37 °C for detachment, followed by fixation in 4% formalin. The mean fluorescence intensity of the cells was measured by EPICS XL flow cytometer (Beckman Coulter Corp., Tokyo, Japan). This was followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz, Santa Cruz, CA, USA) for 4 h at 4 °C. Blots were developed on an ECL detection system (GE Healthcare, Waukesha, WI, USA).

2.10. Quantitative analysis of mRNA by RT-PCR

Quantitative analysis of R1 mRNA was performed as reported previously [23]. Briefly, cells were seeded in a 6-well plate for 24 h, then cells were treated with various complexes in serum-free medium. After incubation at 37 °C for 4 h, the medium were removed and the cells were washed twice. Then the fresh medium were added in the plate, cells were incubated for an additional 44 h. Total RNA was extracted from cells by using Trizol reagent (Invitrogen Molecular Probes, Eugene, OR, USA). And the total RNA was transcribed into cDNA using the first-strand cDNA synthesis kit (Invitrogen Molecular Probes, Eugene, OR, USA). The cDNA was then amplified by RT-PCR. GAPDH was used as housekeeping gene to normalize the gene expression levels of each sample. Relative gene expression values were determined by the Pfaffl method.

2.11. Determination of R1 protein expression by Western blot

Western blot was used to determine the effect of LOR-2501 with different carriers on R1 protein expression. Cells, treated with various complexes, were homogenized in radio-immunoprecipitation assay buffer (RIPA Buffer, Sigma, St. Louis, MO, USA) with 1% protease inhibitor cocktail (Sigma, St. Louis, MO, USA) and 2% phenylmethanesulfonyl fluoride (PMSF, Sigma, St. Louis, MO, USA). Protein was quantified by Bradford protein assay using Coomassie brilliant blue G-250 (Bio-rad Laboratories Inc, USA). Then, 30 μg protein samples were loaded onto a 10% SDS-PAGE gel for electrophoresis. Proteins were then transferred to a PVDF membrane. Transferred blots were blocked with 5% BSA in Tris-buffered saline for 2 h and immunoblotted against the primary antibodies, against R1 or GAPDH (Abcam Inc., Cambridge, MA, USA) at 4 °C overnight.

2.12. Confocal microscopy and cellular internalization analysis

Cells were seeded in a glass bottom cell culture dish overnight and treated with Cy3-LOR-2501, PEI/Cy3-LOR-2501, PEI-OA/Cy3-LOR-2501 or FA/PEI-OA/Cy3-LOR-2501 for 4 h at 37 °C. Cellular nuclei were stained with DAPI for 3 min at room temperature. Internalization of free and the complexed Cy3-LOR-2501 were observed on a Zeiss 710 LSMNLO Confocal Microscope (Carl Zeiss; Jena, Germany) [24].

For cellular internalization analysis, cellular markers of clathrin-mediated endocytosis (transferrin-AlexaFluor488, Tf-A488, 0.1 mg/ml, Invitrogen), macropinocytosis (70 kDa FITC-dextran, 5 mg/ml, Sigma–Aldrich), and lipid raft/caveolae-mediated endocytosis (cholera toxin subunit B-AlexaFluor488 conjugate, CT-B-A488 0.005 mg/ml, Invitrogen, Grand Island, NY, USA) were also added into the wells at the same time. After the removal of transfection medium, cells were washed twice with PBS and fixed in 4% formalin. Cellular nuclei were stained with DAPI (Invitrogen, Grand Island, NY, USA) for 3 min at room temperature. Internalization of free or complexed Cy3-LOR-2501, the co-localization of Cy3-LOR-2501 and pathway-specific markers was observed on a Zeiss 710 LSMNLO Confocal Microscope (Carl Zeiss; Jena, Germany) [25].

2.13. Statistical analysis

The data were analyzed for statistical significance by one-way ANOVA. Where indicated, the results were summarized as mean ± SD.

3. Results

3.1. Physicochemical property of LOR-2501 complexes

The zeta-potentials of PEI-OA/LOR-2501 complexes with different N/P ratios and FA/PEI-OA/LOR-2501 complexes with varying FA concentrations are shown in Fig. 1A and B. The PEI-OA/LOR-2501 complexes had a 15.73 ± 0.058 mV zeta-potential at N/P = 6. The addition of FA decreased the zeta-potential of PEI-OA/LOR-2501 complexes in a concentration dependent manner, which reached a bottom at FA25/PEI-OA/LOR-2501 complexes (Fig. 1B). The data showed that increasing the concentration of FA induced a reversal of zeta potential from positive to negative. The particle size and the shape of FA15/PEI-OA/LOR-2501 complexes were observed under SEM, the image was shown in Fig. 1C.

A series of PEI-OA/LOR-2501 complexes with varying N/P ratios and FA/PEI-OA/LOR-2501 with varying FA concentrations were analyzed on a 3% agarose gel. The results (Fig. 2A) were consistent with those of the gel retardation assay where full complexation between LOR-2501 and PEI-OA was detected in all formulations at varying N/P ratios. PEI-OA was able to completely retard LOR-2501 at N/P above 6. The complexes remained intact after adding different concentrations of FA. In fact, FA/PEI-OA complexes completely retarded LOR-2501 at the tested ratios (Fig. 2B).

3.2. Cytotoxicity of PEI-OA and FA/PEI-OA complexes

The cytotoxic effect of the complexes on A549 and HeLa cells was assessed by the MTT assay. Cytotoxicity studies were conducted to explore whether the polymer concentrations used for LOR-2501 delivery had any indirect effect on the uptake due to decrease of cell viability. As shown in Fig. 3, after 24 h of incubation, cell viability was maintained at over 70% across all PEI-OA and FA/PEI-OA complexes.

3.3. Uptake of LOR-2501 complexes

Free LOR-2501, PEI/LOR-2501, PEI-OA/LOR-2501 and FA/PEI-OA/LOR-2501 uptake by FR overexpressing (HeLa, KB) and FR negative (A549, SK-HEP-1) cells was determined in vitro. As shown in Fig. 3, when treated with PEI-OA/LOR-2501 at N/P = 6, the cells exhibited markedly increased mean fluorescence intensity relative to those treated with free LOR-2501. Moreover, FA/PEI-OA/LOR-2501 was shown to have significantly higher transfection efficiency than PEI/LOR-2501 and PEI-OA/LOR-2501. The influence of FA concentrations on cellular uptake by different cells was shown in Fig. 4A. Fig. 4B showed the cellular uptake of the FA/PEI-OA/LOR-2501 complexes at various concentrations of free FA. Free FA (0.01 mM, 0.1 mM and 1 mM) was added before transfection, then the FA/PEI-OA/LOR-2501 complexes were added to the cells. The free FA concentration (1 mM) was much higher than the K_d of FA for the folate receptor, but the complexes were also uptaked by FR^+ cells and FR^- cells. Free FA failed to inhibit the transfection by FA/PEI-OA/LOR-2501 complexes in either FR^+ or FR^- cells.

3.4. Treatment with inhibitors

To further study the uptake pathway of the FA/PEI-OA/LOR-2501 complexes in a quantitative manner, A549 cells were treated with pathway specific inhibitors along with Cy3-LOR-2501 complexes, and analyzed by flow cytometry to determine their mean fluorescence intensity (Fig. 4C). Sucrose is an inhibitor of clathrin-mediated uptake, Cyto D is reported to impede macropinocytosis, and nystatin is known to inhibit caveolae-mediated endocytosis. As shown in Fig. 4C, for FA/PEI-OA/LOR-2501 complexes, when the A549 cells were treated with sucrose, the clathrin-mediated uptake process was inhibited by 80.5% relative to the control without the inhibitor.

3.5. Determination of R1 mRNA expression by RT-PCR

The ability of the complexes to reduce R1 mRNA levels was further examined in HeLa cells and A549 cells. As shown in Fig. 5, the R1 mRNA level was significantly suppressed by PEI-OA/LOR-2501 and FA/PEI-OA/LOR-2501 in both HeLa cells and A549 cells. The R1

mRNA down regulations of FA/PEI-OA/LOR-2501 were 51.70% and 45.57% in HeLa cells and A549 cells, respectively. However, the R1 mRNA inhibition efficiency of naked LOR-2501 was only 7.20% and 4.47% in HeLa cells and A549 cells.

3.6. Determination of R1 protein expression by Western blot

Western blot analysis was conducted to determine the effect of PEI/LOR-2501, PEI-OA/LOR-2501 and FA/PEI-OA/LOR-2501 on R1 protein level of A549 and HeLa cells. As shown in Fig. 6, PEI-OA/LOR-2501 caused decreases in R1 protein levels. Moreover, the FA/PEI-OA/LOR-2501 complexes showed significant down regulation activity. The R1 protein down regulations of FA/PEI-OA/LOR-2501 were 63.60% and 59.95% in A549 and HeLa cells, respectively.

3.7. Confocal microscopy

In order to investigate the uptake of the complexes by HeLa cells, confocal microscopy was employed (Fig. 7). DAPI was used for observation of the cellular nuclei. The results showed extensive internalization of fluorescently labeled FA/PEI-OA/Cy3-LOR-2501 and trafficking to the cytosol.

3.8. Cellular endocytic pathway of LOR-2501 complexes

It is important to understand the cellular uptake and trafficking mechanisms of the oligo complexes to eventually study structure-function relationships between NP design and transfection efficiency. To investigate the uptake of FA/PEI-OA/LOR-2501 NPs by HeLa cells, confocal microscopy was used to study co-localization of FA/PEI-OA/LOR-2501 NPs with Tf-A488 (clathrin-mediated endocytosis maker), FITC-dextran (macropinocytosis marker), and CT-B-A488 (caveolae-mediated endocytosis marker). As shown in Fig. 8, the overlay of Cy3-LOR-2501 (red) and markers (green) was observed in all three endocytic pathways, meaning that the NPs could be taken up by cells mainly via clathrin-mediated endocytosis pathways.

4. Discussion

It has been demonstrated that targeting cancer cells with siRNA or ASO induced apoptosis. However, efficient oligonucleotide delivery remains challenging, which limits the prospect of their clinical translation. Previous study found that inclusion of oleic acid (OA), an unsaturated fatty acid, into the nanoparticles formulation could significantly enhance the delivery efficacy for siRNA [26,27]. Folic acid, a specific ligand, has been shown to target drugs to tumor cells, complexes coated with FA electrostatically could significantly improve the transfection efficiency. In this study, a novel antisense oligonucleotide carrier, PEI-OA was synthesized and then electrostatically coated with FA (FA/PEI-OA) for delivery of LOR-2501. This was based on the previous finding that FA enhanced transfection activity of PEI. PEI-OA exhibited excellent properties as a transfection agent, with cationic charge and DNA affinity (Figs. 1A and 2A). The addition of FA to the PEI-OA/LOR-2501 complexes decreased the zeta-potential of PEI-OA/LOR-2501 complexes (Fig. 1B). On the other hand, the PEI-OA complexes could still form complexes with LOR-2501 after adding different concentrations of FA (Fig. 2B). These results indicated that FA itself could coat the PEI-OA/

LOR-2501. The affinity between FA and PEI-OA polyplexes was likely the result of the negative charge of FA that has formed oligomeric aggregates in solution.

An important question is whether enhanced transfection activities of the FA complexes were the result of high affinity binding to FR. Our results showed that FA-PEI-OA/LOR-2501 had high cellular uptake, both in FR overexpressing (HeLa, KB) and FR negative (A549, SK-HEP-1) cells. FA15/PEI-OA/LOR-2501 complexes were added to the cells in medium containing various concentrations of FA (0.01–1 mM), which had much higher FA contents than those in the complexes and the K_d of FR. These concentration of FA should block the high affinity FR interaction. The delivery efficiency of the complexes was not affected by the presence of excess free FA in either FR^+ cells (KB and HeLa cells) or FR^- cells (A549 and SK-HEP-1 cells). These results suggested that FA in the FA/PEI-OA/LOR-2501 complexes enhanced transfection in an FR-independent manner. This is most probably due to FA tends to self-associate into hydrogen-bonded planar multimers, which are similar to anionic detergent micelles [28]. These aggregates may be disruptive to the endosome membrane in the context of the PEI-OA complexes [29].

Our results on the comparison of cellular pathways between PEI-OA/LOR-2501 and FA-PEI-OA/LOR-2501 showed no significant differences (Figs. 4C and 8). Clathrin-mediated endocytosis was the main pathway used by both FA/PEI-OA/LOR-2501 and PEI-OA/LOR-2501 to enter the cells. On the other hand, clathrin-mediated endocytosis, which is the main pathway for FR endocytosis, was the least entered pathway. Folic acid was non-covalently incorporated and may not promote receptor-mediated uptake of the complexes. It is important to note that FA most likely cannot fit into the FR binding pocket while simultaneously remain associated with the non-covalent complex. This is because upon FR binding, only one carboxyl would remain free, carrying a single negative charge. This is insufficient to mediate electrostatic binding. So this essentially excludes the possibility that FA can be used to target FR outside the context of covalent conjugation. FA in the FA/PEI-OA/LOR-2501 complexes could significantly increase PEI-OA transfection activity in different cell lines, regardless of the presence of FR on the cell surface. FA/PEI-OA/LOR-2501 complexes were not taken up by cells via the FR-mediated pathway.

5. Conclusion

We have developed an effective oligonucleotide delivery system based on FA/PEI-OA/LOR-2501 complexes with electrostatic interactions. The addition of oleoyl chloride to PEI increases delivery efficiency of LOR-2501. FA could coat PEI-OA/LOR-2501 complexes stably and further enhance the delivery efficiency. FA/PEI-OA/LOR-2501 was able to efficiently downregulate R1 protein level in HeLa, A549, KB and SK-HEP-1 cells, regardless of the expression status of FRs. There did not appear to be any limitation of the delivery system based on the type of tumor cell lines. The FA/PEI-OA/LOR-2501 complexes were mainly taken up by cells via the clathrin-mediated endocytosis. The FA/PEI-OA/LOR-2501 complexes warrant further in vivo evaluations.

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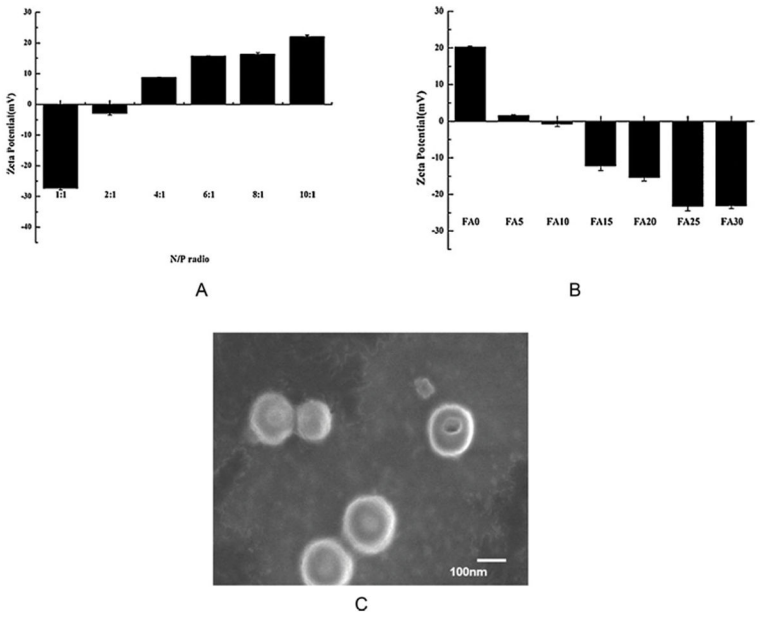

Fig. 1.

Zeta potential and agarose gel analysis of complexes. (A) Zeta potential of PEI-OA/LOR-2501 complexes at varying N/P ratios. (B) Zeta potential of FA/PEI-OA/LOR-2501 complexes at different FA concentrations. Error bars represent standard deviations ($n = 3$) (FA0:FA carboxylate/PEI nitrogen/LOR-2501 phosphate = 0:6:1, FA5–FA30:FA carboxylate/PEI nitrogen/LOR-2501 phosphate = 5:6:1–30:6:1). (C) The SEM image of FA15/PEI-OA/LOR-2501 complexes.

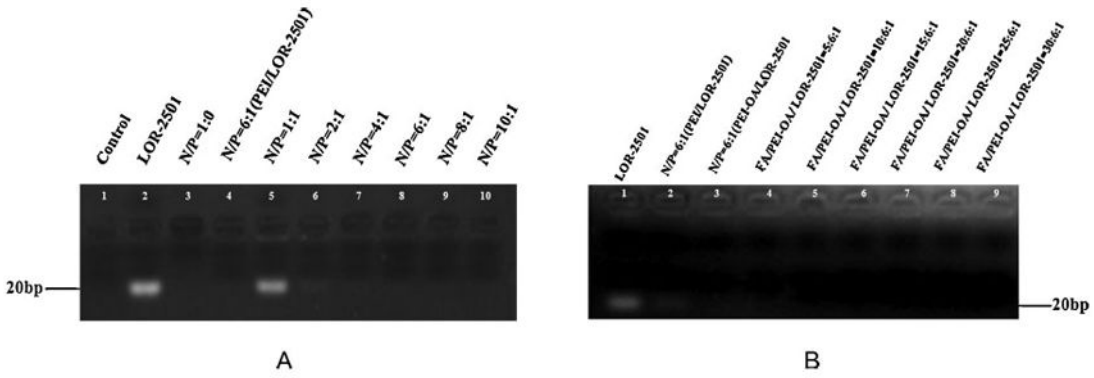

Fig. 2.

Agarose gel analysis of the complexes. (A). PEI-OA/LOR-2501 complexes at varying N/P ratios. (B). FA/PEI-OA/LOR-2501 complexes with different amount of FA.

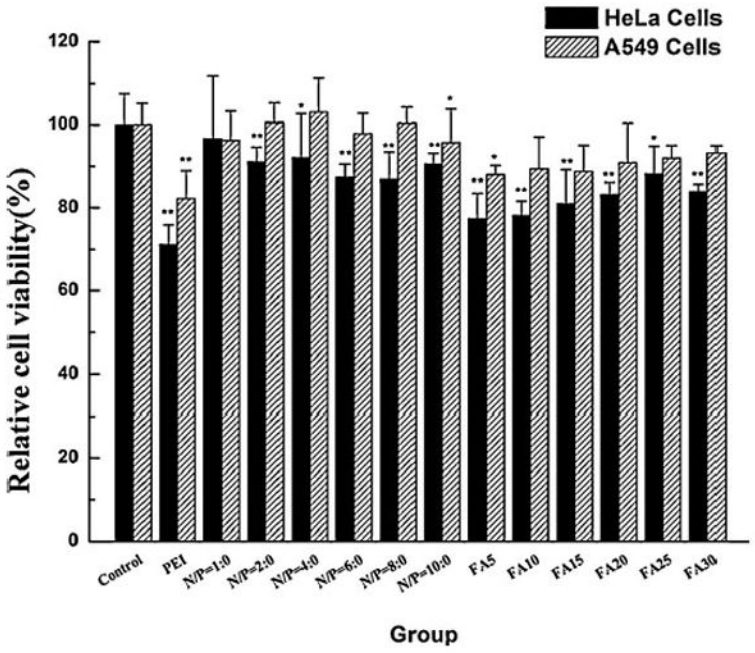

Fig. 3.

Cytotoxicity tests of various complexes on HeLa and A549 cells. Viabilities of cells treated with various complexes were measured by the MTT assay. Cells were incubated with various complexes for 4 h, and cell viability was measured at 20 h after treatment. Data shown are percentages relative to untreated cells. Each bar is the mean (and SD of six experiments). *$P < 0.05$ vs control, **$P < 0.01$ vs control.

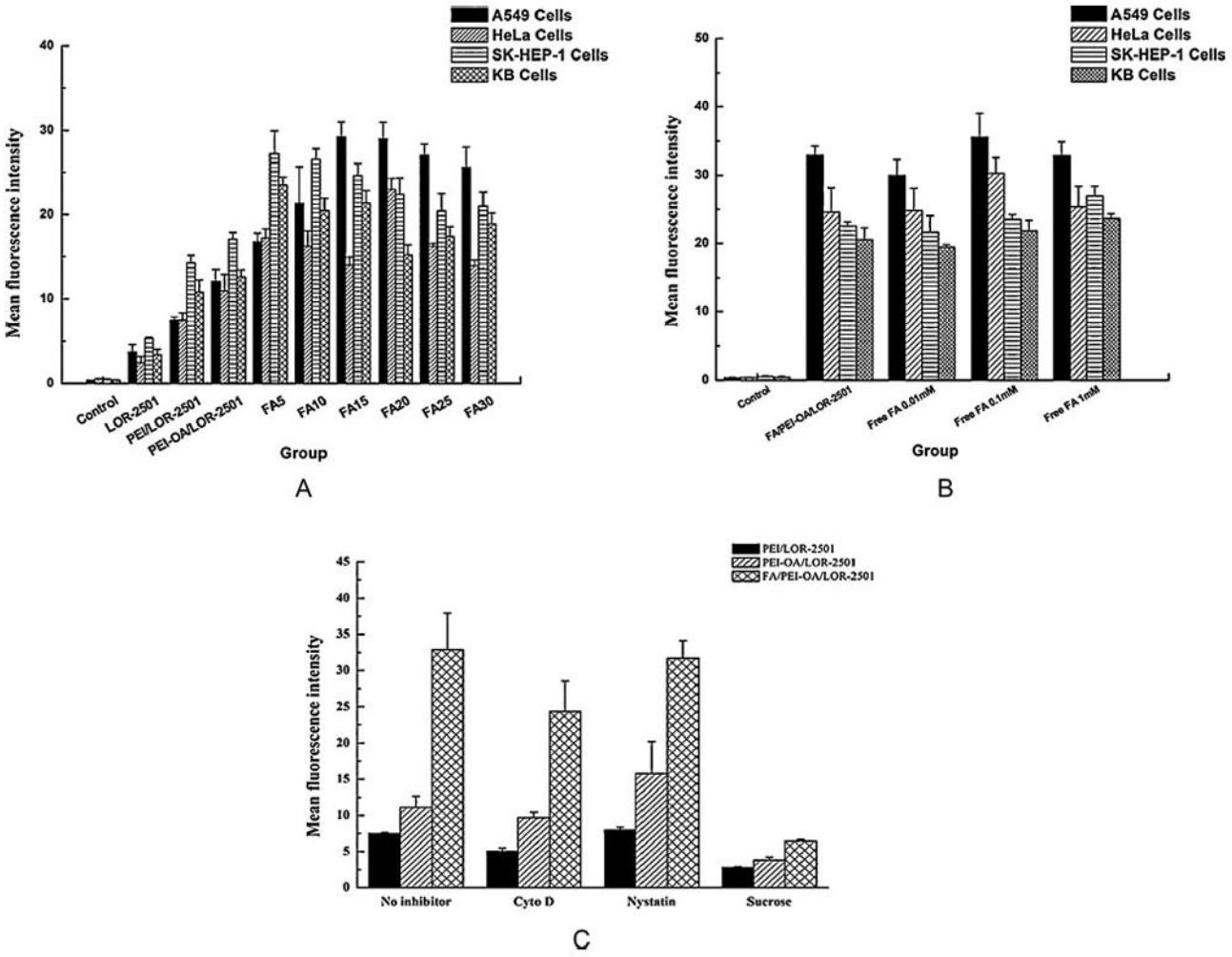

Fig. 4.

Cellular uptake of complexes. (A) Influence of FA concentrations on cellular uptake of LOR-2501 complexes; (B) influence of free FA on cellular uptake. (C) Influence of endocytosis inhibitors on NP cellular uptake. Mean fluorescent intensity of Cy3-LOR-2501 after incubation with various inhibitors. Each bar is the mean of three experiments.

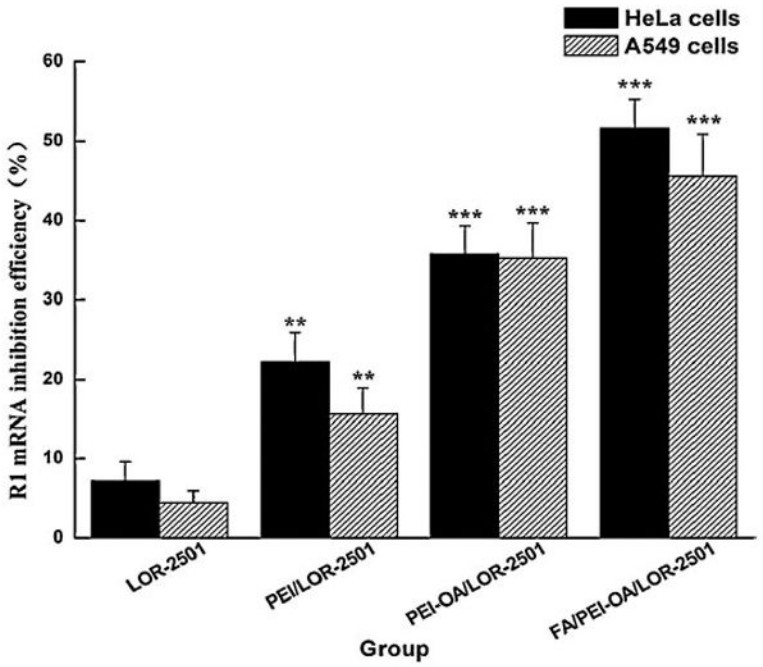

Fig. 5.

Determination of R1 mRNA expression by RT-PCR in HeLa cells and A549 cells. Each bar is the mean of three experiments. $^{**}P < 0.01$ vs control, $^{***}P < 0.001$ vs naked LOR-2501.

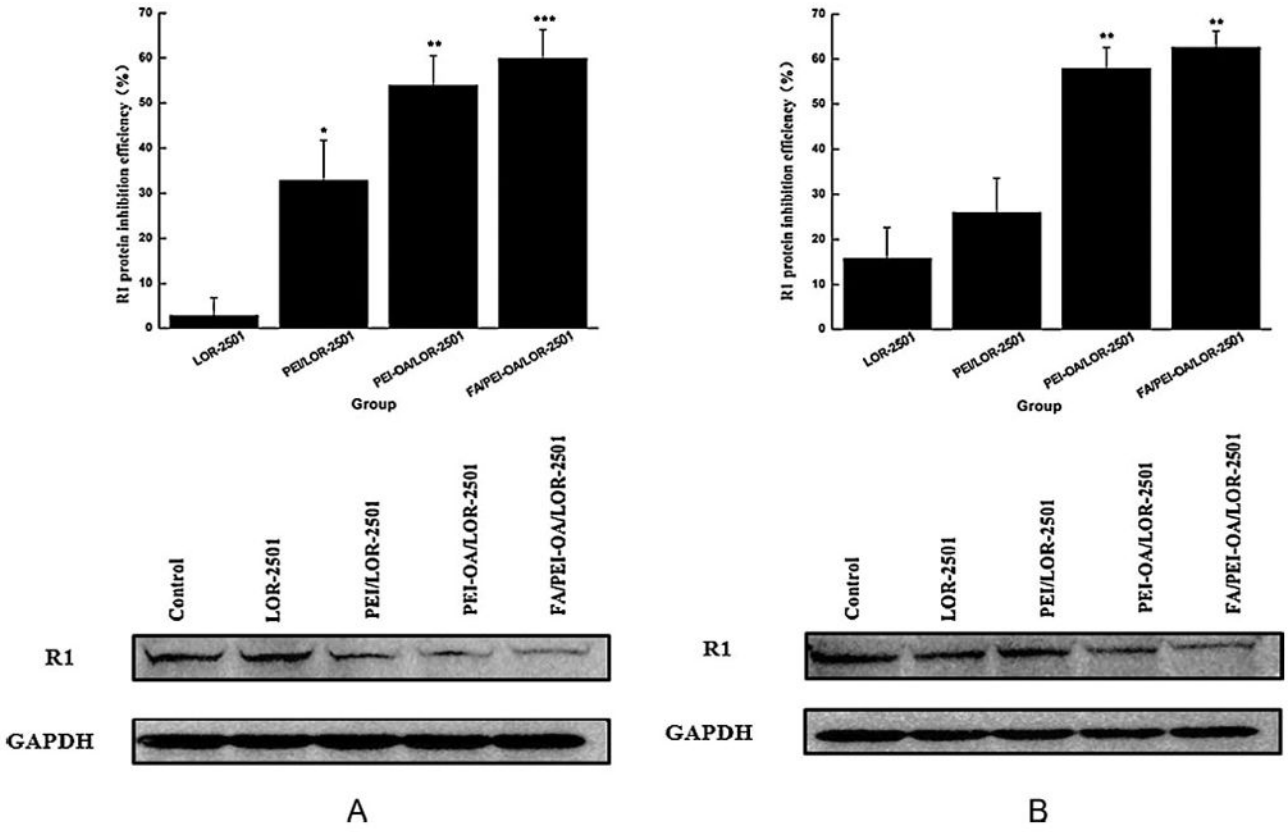

Fig. 6.

Determination of R1 protein expression by Western blot in HeLa cells and A549 cells. (A) HeLa cells (B) A549 cells. Each bar is the mean of three experiments. $*P < 0.05$ vs control, $**P < 0.01$ vs naked LOR-2501.

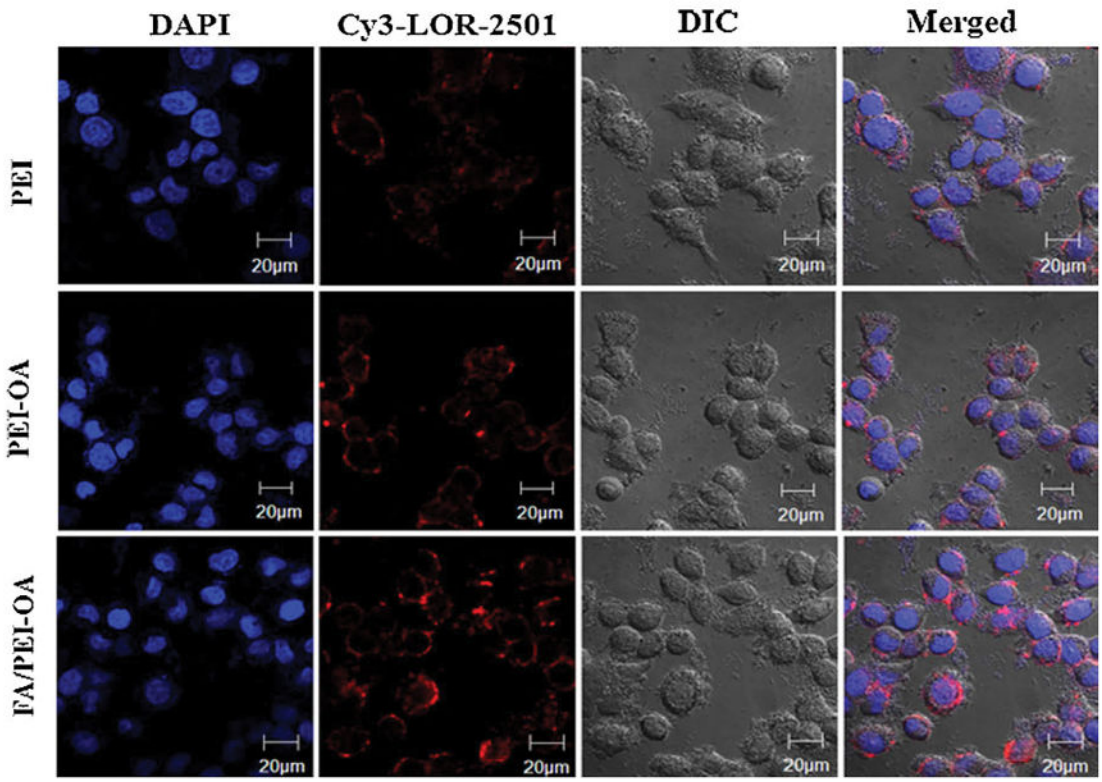

Fig. 7.

Intracellular localization of PEI/LOR-2501, PEI-OA/LOR-2501 and FA/PEI-OA/LOR-2501 complexes. Cy3 fluorescence is shown in red, DAPI nuclear stain is shown in blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

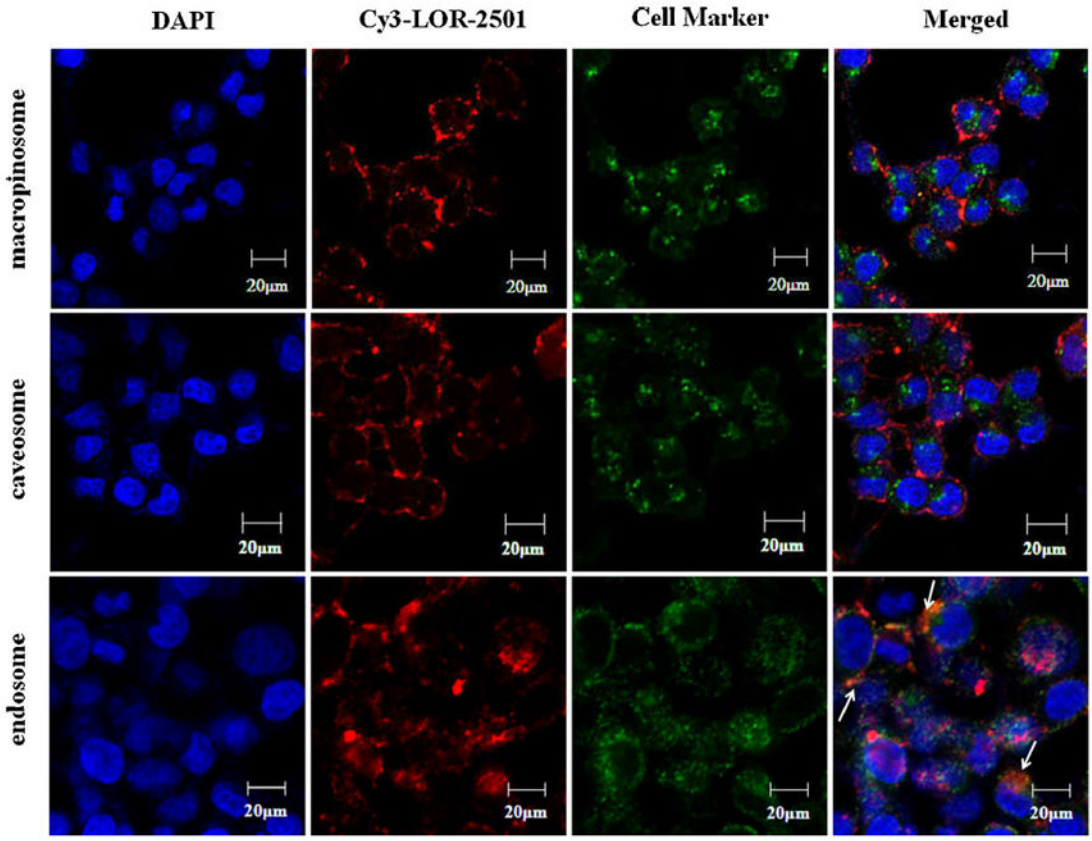

Fig. 8.

Intracellular location of FA/PEI-OA/LOR-2501 complexes in HeLa cells. NPs co-localized with Tf-A488 (endosomes), FITC-dextran (macropinosomes), and cholera toxin B Alexa 488 (caveosomes) separately after 1 h incubation. The Cy3-LOR-2501 is shown in red, the cellular markers are shown in green, and overlay of Cy3-LOR-2501 and markers is shown in yellow. White arrows indicate the co-localization. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)