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Synthesis, characterization and transfection of a novel folate-targeted multipolymeric nanoparticles for gene delivery

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Abstract Novel folate-conjugated biodegradable multipolymeric nanoparticles (NPs) were constructed and evaluated for potential use in gene delivery to human cervical carcinomas Hela cells, which overexpressed folate receptors. Folate-poly(ethylene glycol)-poly(D, L-lactic-*co*-glycolic acid) (PELGA-F) was synthesized and collaborated with poly-L-lysine (PLL) to form polymer-polycationic peptide-DNA (PPD) NPs. Fluorescein sodium and polylysine-condensed DNA (PD) were encapsulated in both PELGA nanoparticles (PELGA-NPs) and folate modified nanoparticles (PELGA-F-NPs), which were prepared by a modified solvent extraction/evaporation method. Effects of the folate conjugation and PLL introduction on the uptake of NPs was qualified by fluorescent invert microscopy and quantified by spectrofluorometric measurement, while effect on the gene expression was measured by X-gal staining and luciferase assay, both using Hela cells as an in vitro model. Results showed that cellular uptake of NPs was enhanced by folate modification, but had no difference after PLL encapsulation. In transfection tests, increased gene expression also confirmed the different functions of folate and PLL introduction. It is feasible that folate-linked multipolymeric NPs should be an efficient targeted carrier for gene delivery.

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1 Introduction

Non-viral delivery systems, including cationic liposomes [1], polypeptides, hydrogel emulsion and nanoparticles (NPs) [2] are attractive gene delivery vehicles, because of safety concerns and easy preparation. Among those systems, biodegradable polymeric NPs with entrapped plasmid DNA have shown the potential for achieving sustained gene expression. Although matrix-type NPs have been formulated for gene delivery using different polymers [3], such as chitosan, gelatin and cyclodextrin, NPs formulated from poly(D, L-lactide-*co*-glycolide) (PLGA) and polylactide (PLA) are of special interest for gene delivery due to their sustained release properties, ability to protect pDNA from degradation [4] as well as biocompatibility and biodegradability approved by FDA [5]. However, intravenously injected particulate drug carriers could be rapidly up taken by mononuclear phagocyte system (MPS), which is the main limitation for drug targeting to other sites in the human body. Therefore, increasing attention has been paid to develop stealth NPs, which can avoid recognition by MPS. One of the major methods for preparation of stealth NPs or long-circulating NPs is to modify the surface with a hydrophilic, flexible and non-ionic polymer, such as poly(ethylene glycol) (PEG). The biodegradable PEG-coated NPs have been found to be important potential therapeutic applications as injectable colloidal systems for the controlled release of drug and site-specific drug delivery [6, 7]. Recently, more and more PEGylated

poly(lactic-*co*-glycolic acid) (PEG-PLGA) has been reported as some hydrophobic and hydrophilic drug carrier [8, 9].

We have synthesized PELGA polymer as a novel NPs material, which is based on the PLGA modified with PEG. This novel biomaterial demonstrated fine blood compatibility [10] according to the International Standard Organization (ISO) and US Pharmacopoeia XXIII recommendations. And then it was used as a gene carrier, combining with cationic peptide poly-L-lysine (PLL), resulting in better protection of plasmid DNA and enhanced transfection on HepG2 and Hela cells [11].

Moreover, the tissue-specific gene expression is another important issue for designing a safe carrier. It was attained by ligand or antibody modification generally. Among various targeting ligands, folate has been widely employed to avoid non-specific attack on normal tissues as well as to increase cellular uptake within target cells, as previously shown by others [8, 9], because folate receptor is vastly overexpressed in several human tumor cells [12]. Until now, folate has covalently attached to a wide array of drug delivery carriers such as liposomes, polymer conjugates and NPs [8, 9, 12].

Folate ligand conjugated to liposomes via PEG-spacer have been used for the delivery of chemotherapeutic, protein agents and DNA to receptor-bearing tumor cells, while stealth NPs modified by folate only focused on the chemical drug.

In present study, copolymer PEG-PLGA was synthesized, conjugated with folate for the purpose of enhancing targeting property, and then was used to formulate NPs with combining PLL to be a novel multipolymeric gene carrier. Distinctive functions of folate and PLL were also discussed.

2 Materials and methods

2.1 Materials

Poly(D, L-lactide-*co*-glycolide) (PLGA, DLLA/GA = 7:3, M_W = 8,000) was supplied by Shandong Institute of Medical Instrument (China), poly(ethyleneglycol)-bis-amine with M_W 2,000 was obtained from Aldrich Chemical Company. Folic acid was from Sangon (China). Dicyclohexylcarbodiimde (DCC), N-hydroxysuccinimide (NHS), poly(L-lysine)-hydrobromide (M_W 25,000) and galactosidase reporter gene staining kit were purchased from Sigma (St Louis, MO). Polyvinyl alcohol (PVA, 88% hydrolyzed, M_W 22,000) was from Acros Organics (USA), F68 (Pluronic F68, namely Poloxamer 188) was from Nanjing Weier Company (injection grade). The plasmid pORF lacZ (3.54 kb) was purchased from Invivogen (USA), and Qiagen Giga Endo-free plasmid purification kit was from Qiagen (CA, USA). Luciferase assay system and plasmid pGL3 was from Promega. Cervical carcinomas cell line Hela was obtained from Shanghai Cell Institute, China Academy of Sciences. Cell culture media RPMI 1640 and RPMI-1640 medium without folic acid were obtained from Gibco Co. (USA). All the other chemicals and reagents used were of the analytical grade obtained commercially.

2.2 Synthesis and purification of PELGA copolymer

PELGA (PEG-PLGA) copolymer was synthesized by amidation of PLGA and PEG. First of all, PLGA (800 mg) was activated by DCC and NHS in dichloromethane (4 ml) with molar ratio of PLGA:DCC:NHS = 1:1.1:1.1, which was stirred under argon overnight at room temperature [8]. And then, the resultant solution was centrifuged to remove the precipitation (dicyclohexylurea, DCU). The activated PLGA was precipitated by ice-cold diethyl ether and dried under vacuum. To a solution of activated PLGA in anhydrous dichloromethane was treated with bisamine-PEG (800 mg) at the molecular ratio of 1:5, and stirred for 24 h under argon protection. For purification, the reaction solution was added into vigorously stirred ethanol dropwise at room temperature, and then dried the precipitate under vacuum. The structure of the product was confirmed by ^{1}H-NMR.

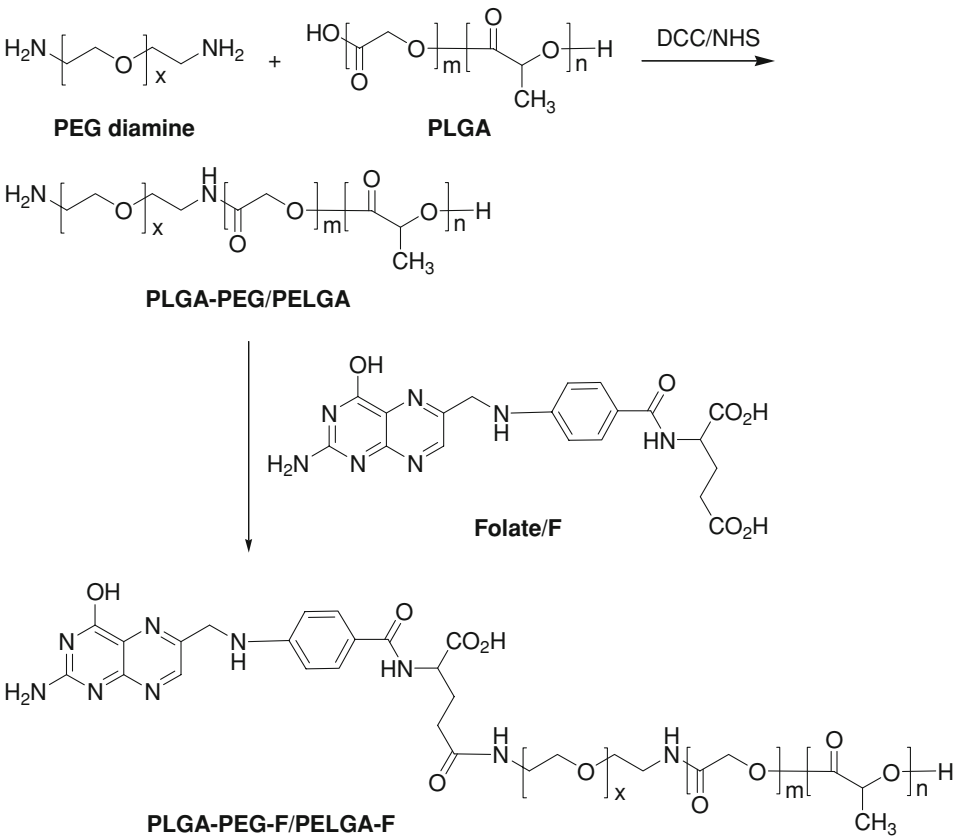
2.3 Preparation of folate-conjugate di-block copolymer

The PELGA copolymer was added to an activated folic acid as described in previous studies with a minor modification [8, 13, 14]. To a solution of the di-block copolymer (400 mg) in dimethysulfoxide (DMSO, 2 ml) was mixed with folic acid (53 mg) and DCC (30 mg). The reaction was performed at room temperature for 24 h and then centrifuged to remove the precipitation. The supernatant was dialyzed in $NaHCO_3$ buffer (pH 8.0) for 3 h and then in water for 5 h. After that, the supernatant was lyophilized to remove water and trace amount of organic solvent. Whole synthetic route was shown in Scheme 1 and the formation of folate-conjugated di-block copolymer, PLGA-PEG-FOL (PELGA-F), was monitored and confirmed by UV and TLC. The conjugation percentage of folate to PELGA was 15.5% on a molar ratio basis, which was calculated by determining the amount of folic acid conjugated in PELGA-F. Serially diluted concentrations of folic acid, which have UV absorption at 365 nm in DMSO, were used to construct a calibration curve.

2.4 Preparation of DNA loaded nanoparticles

PELGA-F-NPs were prepared by traditional double emulsification-solvent evaporation method [11]. In brief,

Scheme 1 Synthetic schemes of PEG-PLGA (PELGA) and PLGA-PEG-FOL (PELGA-F)



PLL-pDNA complex (PD, pDNA: PLL = 1:1, w/w) was prepared by gentle mixing of pDNA (500 μg/ml) in glucose injection (50 μl) with PLL (500 μg/ml) in the same solution (50 μl), and subsequently incubated at room temperature for 30 min. PD was emulsified into 1 ml of PELGA-F solution (10 mg/ml, solvent with acetic acid ethyl ester:acetone = 9:1 (v/v)) by probe sonication for 20 s. Afterwards, 2 ml of F68 glucose injection solution (1%, w/v) was added to the resulting emulsion followed by another 20 s sonication to form the water-in-oil-in-water (w/o/w) emulsion. Finally, this double emulsion was diluted in 8 ml of F68 solution and evaporated at reduced pressure. It allowed solidification of the nanodroplets and evaporation of the organic solvents. Collected NPs were lyophilized and characterized by particle size, morphology and zeta potential.

2.5 Formulation of nanoparticles containing fluorescein

Fluorescein loaded NPs were prepared using similar technique described above, with minor modification [15]. In a typical procedure, to a solution of PELGA or PELGA-F (10 mg) in mixed solvents (ethyl acetate:acetone = 9:1, v/v, 1 ml), an aqueous solution of fluorescein sodium (6 mg/ml) with PD or DNA was added to be inner water phase, while a 0.5% solution of PVA substituted F68 solution to be external water phase. After the solidification of nanodroplets, it was centrifuged at 50,000 rpm for 20 min at 4°C in a MLS50 rotor (Beckman Optima™ MAX-E ultracentrifuge, Beckman Instruments). The pellet was resuspended in distilled water and sonicated for 30 s on an ice bath to disperse any aggregates. This washing step was repeated twice to remove PVA and unencapsulated fluorescein sodium from the formulation. Finally, the NPs were resuspended in 2 ml of distilled water and sonicated for 30 s on an ice bath followed by lyophilization.

2.6 Qualitative and quantitative analysis of nanoparticles uptake by Hela cells

For qualitative and quantitative determination of folate and PLL effect on cellular uptake, Hela cells were seeded in 6-well plates with 1 ml of folate-free RPMI-1640 growth medium at a density of 3×10^4 cells/ml for cell attachment. And then, it was incubated with a suspension of fluorescein NPs (1.6 mg/ml PELGA NPs and PELGA-F NPs with or without PLL, respectively) in folate-free RPMI-1640 growth medium for 4, 8 and 16 h.

In the qualitative measurement, for the purpose of preventing the intracellular fluorescence is due to the dye that released from the NPs, a control sample was performed.

NPs was first incubated in growth medium at 37°C for 16 h, and then centrifuged to get the supernatant solution as control, which is the fluorescence that was released from NPs. At the end of the incubation periods, cells were rinsed three times with cold PBS buffer to remove excessive NPs and/or free dye. Fresh PBS buffer was added to the plates and the cells were viewed and imaged under an Axiovert40 CFL fluorescent inverted microscope (Zeiss, Germany) using FITC filter (Ex (λ) 495 nm, Em (λ) 520 nm). The images were processed using Carl Zeiss LSM software (version 3.99).

For quantitative analysis, performance was almost the same as the one in qualitative test. The only difference was after PBS rinse, cell membrane was permeated with 0.5% Triton X-100 in 0.2 M NaOH solution. The cell-associated fluorescence was then measured in the lysis buffer extract in a HITACHI RF-5301 PC fluorescence spectrofluorophotometer (Ex = 492 nm, Em = 512 nm).

2.7 Cell transfection

Hela cells were cultured in folate-free RPMI-1640 and RPMI-1640 growth medium respectively with 10% fetal bovine serum and streptomycin (100 μg/ml). The cells were seeded at 1×10^5 cells per well in 12-well plates. When it reached 80% confluence, cells were washed twice by PBS. And for each well in a transfection, PELGA-F or PELGA NPs containing 2.5 μg pORF-1acZ or pGL3 plasmid were overlaid and mixed gently. The cells were incubated with NPs from 6 to 24 h at 37°C in a CO_2 incubator. Following incubation, NPs was removed and the cell surfaces were rinsed thoroughly and treated with 2 ml fresh medium. Then the cells were returned to the incubator for 48 h to allow intracellular gene expression.

Estimation of the transfection efficiency by X-gal stain was performed using galactosidase assay [16, 17]. After the incubation, the cells were washed with PBS twice and fixed with 2% formaldehyde and 0.2% glutaraldehyde for 10 min at room temperature. Then the cells were rinsed twice and stained by X-gal (20 mg/ml) according to the manufacturer's instructions. The cells were incubated at 37°C overnight and observed under a microscope. The transfected cells turned into blue after X-gal staining. For each well, five visual fields were chosen randomly. Cells stained blue were counted and the transfection efficiency was calculated as the percentage of the blue cells in each field.

Luciferase gene expression was determined 48 h after transfection by using a commercial luciferase assay kit (Promega, USA) [18]. The luciferase activity was monitored for 15 s in an Lmax II 384 luminometer (Molecular Devices Corporation, USA). The transfection efficiency was expressed as amount of luciferase of cell protein (fg/mg), the concentration of which was measured by BCA Protein Assay Kit (Pierce, USA).

Furthermore, in order to evaluate the role of folate in the cellular uptake of folate-conjugated PELGA-F-NPs, another transfection experiment on Hela cells were treated with 1 mg/ml NPs in full RPMI-1640 containing additional folic acid as inhibitor.

3 Results and discussion

3.1 Measurement and characterization of copolymer

3.1.1 Determination of PELGA

The basic chemical structure of PELGA copolymer is confirmed by ^{1}H NMR (Fig. 1). One of the striking features is the peak at 3.65 ppm (CH_2), corresponding to PEG blocks. Overlapping doublets at 1.55 ppm (CH_3) and multiplets at 5.18–5.23 ppm (CH) are attributed to the D, L-LA repeat units, while the multiplets at 4.65–4.90 ppm (CH_2) correspond to the GA. The high complexity of the peaks resulted from mixed D-lactic, L-lactic, glycolic acid sequences in the polymer backbone.

To obtain the number average molecular weight, the integrals of three peaks: 4.25 of 5.18–5.23 ppm (CH of D, L-LA), 3.14 of 4.65–4.90 ppm (CH_2 of GA) and 21.45 of 3.65 ppm (CH_2 of PEG) were used. Similar results were reported by other laboratories [19].

$$H_2N\,[\underset{\text{PEG}}{CH_2CH_2O}]_x\,[\underset{\text{GA}}{COCH_2O}]_m\,[\underset{\text{LA}}{COCH(CH_3)O_n}]\,H$$

LA/GA number ratio = (area of CH peaks at 5.18–5.23 ppm)/(1/2 area of CH_2 peaks at 4.65–4.90 ppm) = 4.25/(3.14/2) = 7:3.

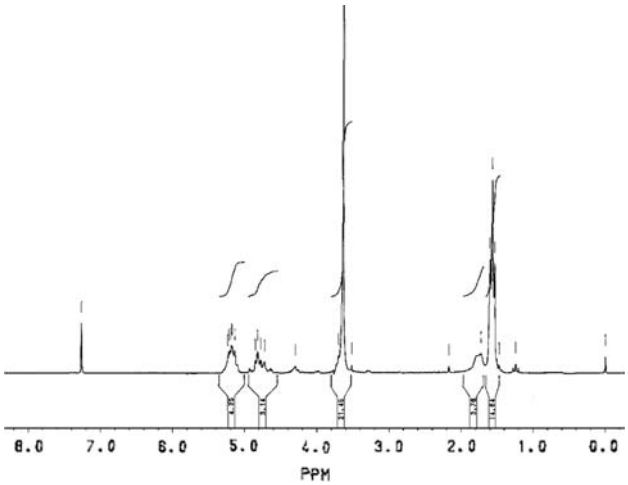


Fig. 1 ^{1}H NMR spectrum of PELGA copolymer

PLGA/PEG number ratio = (10/3 area of CH_2 peaks at 4.65–4.90 ppm)/(1/4 area of CH_2 peaks at 3.65 ppm) = (10/3) × 3.14/[(1/4) × 21.45] = 3:10.

So the number ratio of PEG/PLGA is 1:1 and the M_W of PELGA was about 10,000. In addition, molecular weight distribution of the obtained copolymer was determined by means of GPC. The unimodal mass distribution ($M_W/M_n = 1.23$) excluded the presence of free PEG or PLGA.

3.1.2 Determination of PELGA-F

Thin-layer chromatography (TLC) of the reaction system was carried out on TLC aluminum sheets silica gel 60 F254 (Merck) using the solvent system: ethanol/ammonia (13.5 M)/1-propanol (60:20:20 v/v) [20, 21]. The spots were located under the UV light showed a new spot appearance due to the formation of the novel product PELGA-F.

Folate has UV absorption in alkali solution at 275 and 365 nm (Fig. 2, green line), but neither DCM solution of folate (Fig. 2, black line) nor PELGA (Fig. 2, blue line) can detect the UV absorption (folate can not dissolve in DCM). Here, UV scan chromatogram of the synthetic product emerged a spike peak in both 275 and 325 nm (Fig. 2, red), which must be due to the conjugation of folate to PELGA.

3.2 Physicochemical properties of nanoparticles

Photon correlation spectroscopy (PCS) (Zetasizer Nano ZS90, Malvern instruments Ltd., UK) demonstrated the average diameter of NPs was 155 ± 2.97 nm (Fig. 3), with a very narrow distribution (polyindex = 0.105 ± 0.095). PELGA-NPs with PLL (−43.26 ± 1.73 mV), or without PLL (−45.5 ± 0.66 mV) in HEPES buffer (10 mM, pH 7.4), showed similar negative charge without statistic significance ($P > 0.05$), which suggested that most PLL may be encapsulated in the NPs or the surface oriented PEG has well shielded the charge of PLL. Size of fluorescein loaded NPs was larger, with average size of 246 ± 2.90 nm and polyindex of 0.12 ± 0.013.

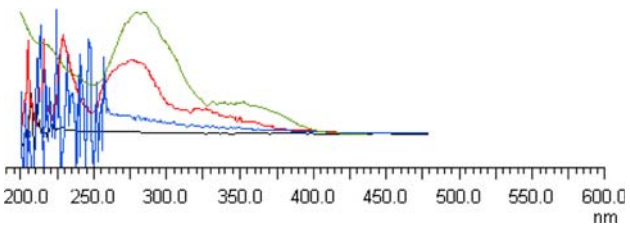


Fig. 2 UV spectrum of folate in alkali solution (*green line*), with two peaks at 275 and 365 nm. UV spectrum of PELGA-F (*red line*, also emerging a spike peaks at 275 and 365 nm), PELGA (*blue line*) and folate (*black line*) dissolved in DCM (from top to bottom)

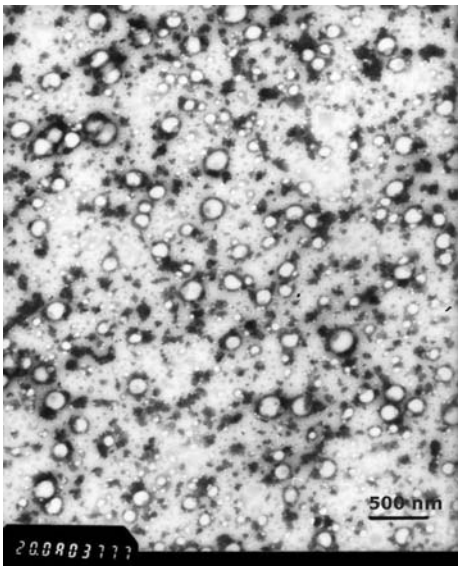


Fig. 3 Electronic transmission microscopy of pDNA loaded PELGA-F NPs (×20,000)

3.3 Cell uptake of nanoparticles

In order to study the cellular uptake of NPs in vitro or in vivo, the use of fluorescent or radioactive NPs is the most common approach found in literature. But fluorescent labeling could avoid exposure to radioactive materials. It makes cellular uptake of NPs readily detectable by fluorescence microscopy [22]. The extent of particle uptake can be determined by flow cytometry, fluorometry or quantitative extraction of the markers from the cells [20, 23]. In this study, preparation of these fluorescent NPs was slightly different from that of pDNA loaded ones, because of the different properties of the encapsulated substances. High encapsulation efficiency of fluorescein was obtained by using PVA as excipient in the external water phase.

Images of fluorescent invert microscopy clearly indicated the internalization of NPs by the Hela cells. It showed that the fluorescence intensity in the PELGA-F-NPs wells (Fig. 4b, d) was significantly higher than PELGA-NPs one (Fig. 4a, c), which confirmed that modification with folate improved the cellular uptake [15]. The cells which were exposed to NPs (PELGA-F-NPs and PELGA-NPs respectively) under fluorescence microscopy also demonstrated increased fluorescence activity in the cells with increased incubation time (Fig. 4a–d). Free dye which was released from the NPs accounted for very weak fluorescence (Fig. 4e, f) indicating that fluorescein detected in the cells was mainly associated with the NPs.

The preliminary study showed that the standard plot had linear relationship ($r^2 = 0.9892$) between fluorescence NPs (0.025–4 ng/ml) and fluorescence intensity (18.45–364.58). So the uptake of NPs (whether conjugated with or

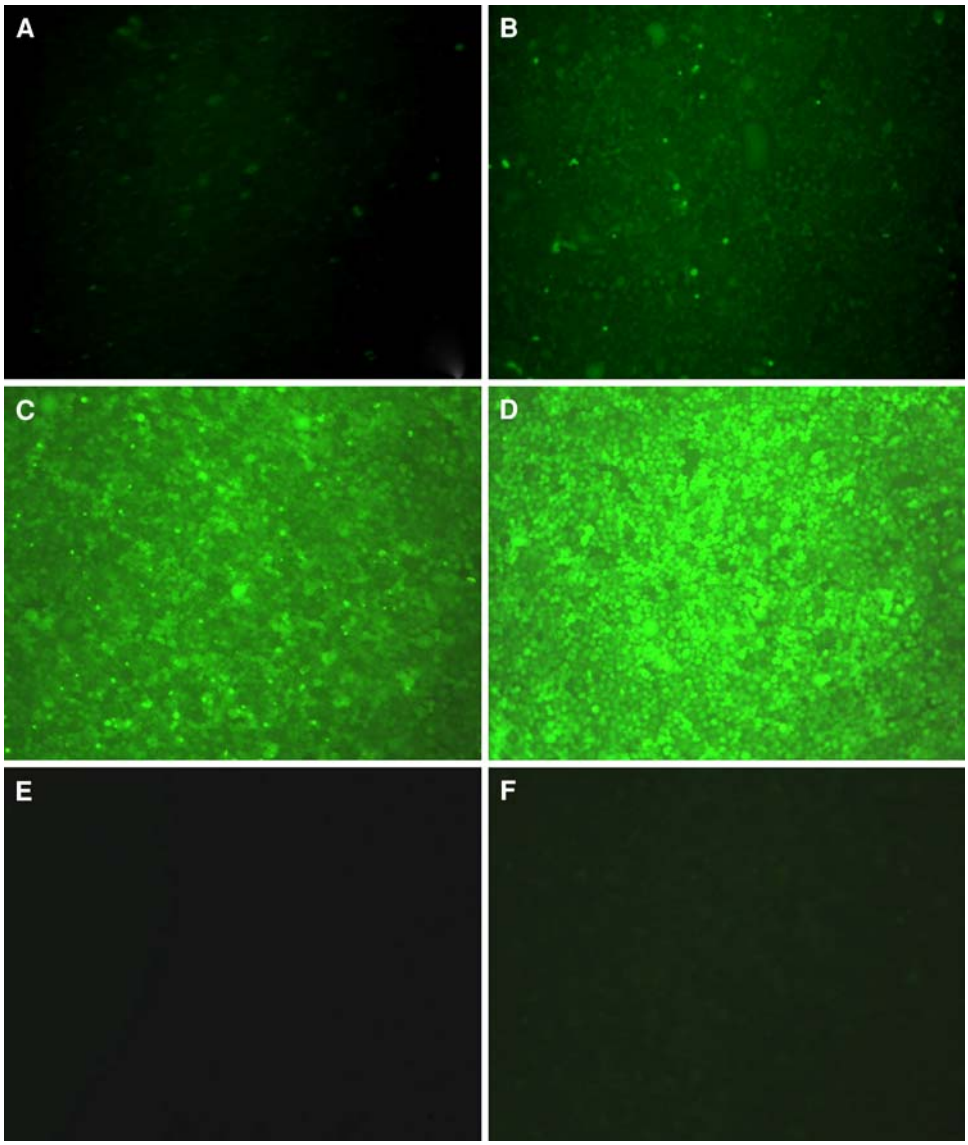


Fig. 4 **a** Fluorescence inverted microscope observation of cellular uptake of NPs by Hela cells. Cells were imaged at 4 h (**a**, **b**) and 16 h (**c**, **d**) from the time of incubation with PELGA-NPs (**a**, **c**) and PELGA-F-NPs (**b**, **d**). The control experiment was performed by incubating cells with fluorescein solution released from PELGA-NPs and PELGA-F-NPs in vitro at 16 h (**e**, **f**). **a**–**f** are magnified equally (×50)

without folate) by Hela cells was depended on the incubation time, as shown in Fig. 5. Furthermore, the association of PELGA-F-NPs with Hela cells was compared with that of PELGA-NPs [24]. At various time intervals, unendocytosed NPs were removed by the rinse with PBS, while endocytosed NPs remained in the cell and were measured using fluorescence photometer. The association of PELGA-F-NPs was found to be gradually higher than that of PELGA-NPs after 4 h incubation, which reached 2-fold higher at 16 h point (Fig. 5). On the other hand, the fluorescence intensity had no statistic difference between PELGA-NPs with or without PLL ($P > 0.05$) for 16 h (Fig. 5), which means PLL showed no effect on cellular uptake. The main reason could be that the surface charge and particle size of NPs with or without PLL (Sect. 3.2) are nearly the same.

3.4 Cellular transfection efficiency of nanoparticles

3.4.1 X-gal staining and luciferase assay

Previous studies with folate-targeted liposomes had indicated that only low molar fractions of folate were required to successfully mediate liposome uptake by folate receptor-bearing cells [14]. So even the conjugation percentage of folate to PELGA was 15.5% (molar ratio), the targeted function of folate in vitro was obvious. Both X-gal staining and luciferase assay proved that PELGA-F-NPs had higher transfection efficiency in Hela cells than NPs without folate, the blue stained transfected cells were visualized by microscope for qualitative cooperation (Figs. 6, 7).

Luciferase expression on Hela cells showed about 1.5- to 2.3-fold higher level for PELGA-F-NPs compared

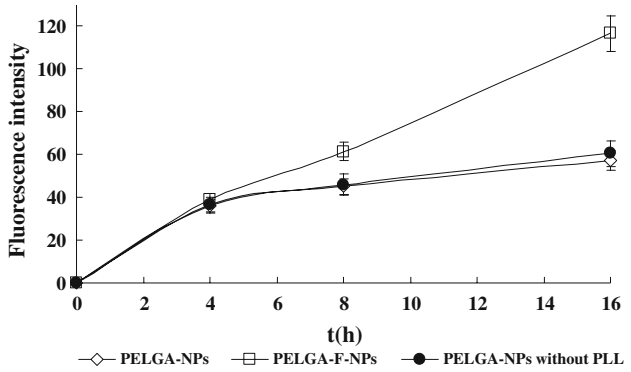


Fig. 5 Quantitative cellular uptake of different fluorescence NPs by Hela cells. Values represent mean ± SEM ($n = 4$)

with the PELGA-NPs before 12 h, and 3.4-fold higher after 24 h ($P < 0.05$, $n = 6$) (Fig. 7a), with the same dose of NPs. However, in the wells that NPs without PLL, protein expression has sharply decreased (Fig. 7a). Furthermore, no statistical difference was found between PELGA-F-NPs without PLL and PELGA-NPs without PLL.

Tumor cell recognition is the first premise of gene delivery in vivo, but there are still a number of intracellular barriers that must be overcome, such as DNase degradation, low pH suffering in lysosome and unclear entry obstacle. Previous study of the Taxol-7-PEG-FA conjugate [25] confirmed that, after a folate–drug conjugate enters cells via the folate receptor pathway, the conjugate could remain trapped within the endosomes if it does not have an efficient release mechanism. Low's group also realized the importance of DNA condensation in nonviral liposomal vectors [26]. Panels of polycations were examined for their abilities to facilitate FA-targeted gene expression. Only the high molecular weight polylysine, acylated polylysine and polyamidoamine dendritic-based polymers yielded high transfection efficiencies while maintaining the FR-specificity. In contrast, complexation of plasmid DNA with small cationic molecules ended up with weakly active FA-targeted liposomal vectors. It is consistent with our result that transfection efficiency has no statistical distinctness between PELGA-F-NPs without PLL and PELGA-NPs without PLL.

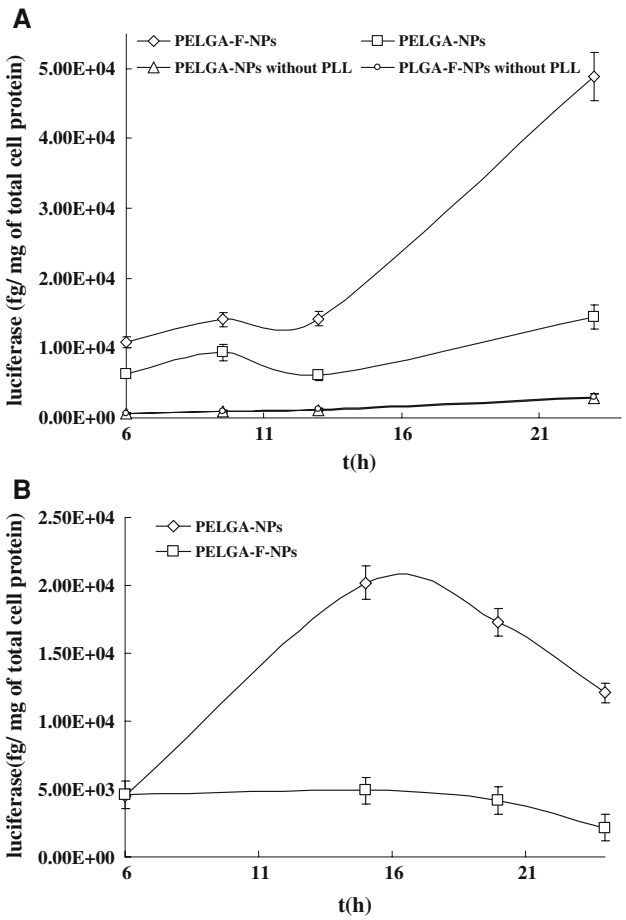


Fig. 7 Luciferase expression of different NPs in distinct medium. **a** Expression of luciferase in folate-free 1640 medium. **b** Expression of luciferase in complete 1640 medium with extra folate

3.4.2 Inhibition of PELGA-F NPs uptake

It has been reported that the binding of the folate grafted liposomes to carcinomas could be competitively inhibited by free folate or by an anti-serum against the folate receptor, demonstrating that the interaction is mediated by the cell surface folate receptor [13, 27]. So the binding competition of PELGA-F-NPs was also studied to evaluate the folate ligand affinity for the folate receptor in Hela cells.

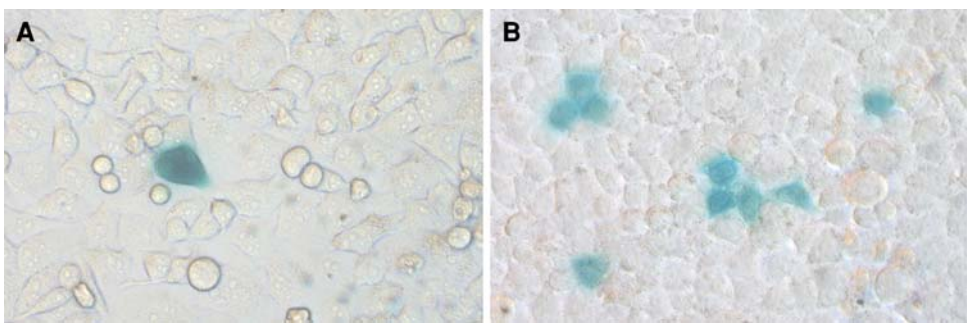


Fig. 6 X-gal staining of PELGA-F-NPs and NPs without folate in Hela cells (the *blue stained cells* were transfected ones): **a** PELGA-NPs group. **b** PELGA-F-NPs group

As shown in Fig. 7b, the luciferase expression of the folate-conjugated PELGA-F-NPs was obviously inhibited by adding excess free folate, while the transfection of PELGA-NPs was higher. These reversed results from Sect. 3.4.1 suggested that, conjugated PELGA-F-NPs must be endocytosed via the folate receptor. Because free folate is a small molecule, it is prone to collaborate with the receptor on the surface of Hela cells.

Moreover, folate is essential cofactors for one carbon transfers in enzyme systems required for DNA synthesis. Hence, acquisition of folate is extremely important for maintaining the viability of both normal and proliferating cells [28]. The different expression efficiency of PELGA-NPs in RPMI-1640 and folate-free RPMI-1640 demonstrated that folate is crucial, not only as a ligand but also as an important nutrition in cell culture.

4 Conclusions

Novel folate-targeted multipolymeric delivery system for gene therapy was successively constructed. Physicochemical properties and in vitro cellular uptake of this delivery system were characterized. In this multipolymeric system, conjugation with folate as a ligand increase the cellular uptake of NPs, while encapsulation of PLL to condense DNA increase transfection efficiency obviously. It confirmed that the condensation and protection of integrity of DNA in nonviral delivery system is a crucial aspect for gene expression in vitro, and high expression of therapeutic gene should overcome both extracellular and intracellular barriers. These novel multipolymeric NPs might be used as promising target carrier for gene delivery.

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References

1. Stuart DD, Allen TM. A new liposomal formulation for antisense oligodeoxynucleotides with small size, high incorporation efficiency and good stability. Biochim Biophys Acta. 2000;1463:219–29. doi:10.1016/S0005-2736(99)00209-6.
2. Prabha S, Zhou WZ, Panyam J, Labhasetwar V. Size-dependency of nanoparticle-mediated gene transfection: studies with fractionated nanoparticles. Int J Pharm. 2002;244:105–15. doi:10.1016/S0378-5173(02)00315-0.
3. Roy K, Mao HQ, Huang SK, Leong KW. Oral gene delivery with chitosan-DNA nanoparticles generates immunological protection in a murine model of peanut allergy. Nat Med. 1999;5:387–91. doi:10.1038/7385.
4. Prabha S, Labhasetwar V. Critical determinants in PLGA/PLA nanoparticle-mediated gene expression. Pharm Res. 2004;21:354–64. doi:10.1023/B:PHAM.0000016250.56402.99.
5. Labhasetwar V, Bonadio J, Goldstein S, Levy RJ. Gene transfection using biodegradable nanospheres: results in tissue culture and a rat osteotomy model. Colloids Surf B Biointerfaces. 1999;16:281–90. doi:10.1016/S0927-7765(99)00079-X.
6. Yang H, Lopina ST, DiPersio LP, et al. Stealth dendrimers for drug delivery: correlation between PEGylation, cytocompatibility, and drug payload. J Mater Sci Mater Med. 2008;19:1991–7. doi:10.1007/s10856-007-3278-0.
7. Huang YZ, Chen JL, Chen XJ, et al. PEGylated synthetic surfactant vesicles (Niosomes): novel carriers for oligonucleotides. J Mater Sci: Mater Med. 2008;19:607–14. doi:10.1007/s10856-007-3193-4.
8. Yoo HS, Park TG. Folate receptor targeted biodegradable polymeric doxorubicin micelles. J Control Release. 2004;96:273–83. doi:10.1016/j.jconrel.2004.02.003.
9. Lee ES, Na K, Bae YH. Polymeric micelle for tumor pH and folate-mediated targeting. J Control Release. 2003;91:103–13. doi:10.1016/S0168-3659(03)00239-6.
10. Duan YR, Nie Y, Gong T, et al. Evaluation of blood compatibility of MeO-PEG-poly(D, L-lactic-co-glycolic acid)-PEG-OMe triblock copolymer. J Appl Polym Sci. 2006;100:1019–23. doi:10.1002/app.22961.
11. Nie Y, Zhang ZR, Duan YR. Combined use of polycationic peptide and biodegradable macromolecular polymer as a novel gene delivery system: a preliminary study. Drug Deliv. 2006;13:441–6. doi:10.1080/10717540600640302.
12. Lu YJ, Low PS. Folate-mediated delivery of macromolecular anticancer therapeutic agents. Adv Drug Deliv Rev. 2002;54:675–93. doi:10.1016/S0169-409X(02)00042-X.
13. Guo W, Lee T, Sudimack JJ, et al. Receptor-specific delivery of liposomes via folate-PEG-chol. J Liposome Res. 2000;10:179–95. doi:10.3109/08982100009029385.
14. Lee RJ, Low PS. Folate-mediated tumor cell targeting of liposome-entrapped doxorubicin in vitro. Biochim Biophys Acta. 1995;1233:134–44. doi:10.1016/0005-2736(94)00235-H.
15. Davda J, Labhasetwar V. Characterization of nanoparticle uptake by endothelial cells. Int J Pharm. 2002;233:51–9. doi:10.1016/S0378-5173(01)00923-1.
16. Sakurai F, Nishioka T, Saito H, et al. Interaction between DNA-cationic liposome complexes and erythrocytes is an important factor in systemic gene transfer via the intravenous route in mice: the role of the neutral helper lipid. Gene Ther. 2001;8:677–86. doi:10.1038/sj.gt.3301460.
17. Armeanu S, Pelisek J, Krausz E. Optimization of nonviral gene transfer of vascular smooth muscle cells in vitro and in vivo. Mol Ther. 2000;1:366–75. doi:10.1006/mthe.2000.0053.
18. Jeong JH, Park TG. Poly(L-lysine)-g-poly(D, L-lactic-co-glycolic acid) micelles for low cytotoxic biodegradable gene delivery carriers. J Control Release. 2002;82:159–66. doi:10.1016/S0168-3659(02)00131-1.
19. He Y, Wei SL. Study on the degradation of polylactide microspheres in vitro. J Peking Univ Health Sci. 2001;33:358–61.
20. Wina KY, Feng SS. Effects of particle size and surface coating on cellular uptake of polymeric nanoparticles for oral delivery of anticancer drugs. Biomaterials. 2005;26:2713–22. doi:10.1016/j.biomaterials.2004.07.050.
21. Kwon GS, Okano T. Polymeric micelle as new drug carriers. Adv Drug Deliv Rev. 1996;16:107–16. doi:10.1016/S0169-409X(96)00401-2.
22. Lu ML, Wu DC, Guo N. Novel functionalized ternary copolymer fluorescent nanoparticles: synthesis, fluorescent characteristics and protein immobilization. J Mater Sci Mater Med. 2009;20:563–72. doi:10.1007/s10856-008-3596-x.
23. Betancourt T, Shah K, Peppas LB. Rhodamine-loaded poly (lactic-co-glycolic acid) nanoparticles for investigation of in vitro

interactions with breast cancer cells. J Mater Sci Mater Med. 2009;20:387–95. doi:10.1007/s10856-008-3594-z.
24. Hattori Y, Maitani Y. Enhanced in vitro DNA transfection efficiency by novel folate-linked nanoparticles in human prostate cancer and oral cancer. J Control Release. 2004;97:173–83. doi:10.1016/j.jconrel.2004.03.007.
25. Lee JW, Lu JY, Low PS, et al. Synthesis and evaluation of taxol-folic acid conjugates as targeted antineoplastics. Bioorg Med Chem. 2002;10:2397–414. doi:10.1016/S0968-0896(02)00019-6.
26. Reddy JA, Dean D, Kennedy MD, et al. Optimization of folate-conjugated liposomal vectors for folate receptor-mediated gene therapy. J Pharm Sci. 1999;88:1112–8. doi:10.1021/js990169e.
27. Bazile D, Prud'homme C, Bassoullet MT, et al. Stealth MePEG-PLA nanoparticles avoid uptake by the mononuclear phagocyte system. J Pharm Sci. 1995;84:493–8. doi:10.1002/jps.2600840420.
28. Reddy JA, Allagadda VM, Leamon CP. Targeting therapeutic and imaging agents to folate receptor positive tumors. Curr Pharm Biotechnol. 2005;6:131–50. doi:10.2174/1389201053642376.