Target-Specific Cellular Uptake of PLGA Nanoparticles Coated with Poly(l-lysine)–Poly(ethylene glycol)–Folate Conjugate

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Poly(l-lactic-co-glycolic acid) (PLGA) nanoparticles with anionic surface charge were surface coated with cationic di-block copolymer, poly(l-lysine)–poly(ethylene glycol)–folute (PLL–PEG–FOL) conjugate, for enhancing their site-specific intracellular delivery against folate receptor overexpressing cancer cells. The PLGA nanoparticles coated with the conjugate were characterized in terms of size, surface charge, and change in surface composition by XPS. By employing the flow cytometry method and confocal image analysis, the extent of cellular uptake was comparatively evaluated under various conditions. PLL–PEG–FOL coated PLGA nanoparticles demonstrated far greater extent of cellular uptake to KB cells, suggesting that they were mainly taken up by folate receptor-mediated endocytosis. The enhanced cellular uptake was also observed even in the presence of serum proteins, possibly due to the densely seeded PEG chains. The PLL–PEG–FOL coated PLGA nanoparticles could be potentially applied for cancer cell targeted delivery of various therapeutic agents.

Introduction

Nanoparticulate drug delivery systems using biodegradable polymeric carriers have attracted increasing attention in recent years. Polymeric nanoparticles, primarily based on biodegradable poly(l-lactic-co-glycolic acid) (PLGA) polymers, have been extensively used for intravenous administration of diverse therapeutic agents including water insoluble anticancer agents such as paclitaxel.1-5 The size and surface charge of PLGA nanoparticles are the most important factors in determining their in vivo pharmacokinetic and bio-distribution behaviors.6-8 When injected in the blood stream, they are rapidly cleared by the reticulo-endothelial system (RES); hence, their pharmacological effect is dramatically reduced.9 Recent reports demonstrate that the rapid RES uptake of PLGA nanoparticles could be significantly reduced by modifying their surface with poly(ethylene glycol) (PEG).10-12 The PEG modified PLGA nanoparticles, prepared mostly by using a di-block copolymer of PLGA-PEG as an additive, prolonged their half-life considerably in the circulation due to the presence of highly mobile and flexible PEG chains on the surface.13 PEG modified PLGA nanoparticles, however, cannot be delivered to specific cells in a target-specific manner. Although PEG decorated nanosized particles, such as liposomes, micelles, and polymeric nanoparticles, are passively targeted to solid tumor tissue by “enhanced permeation and retention” effect, they are further required to be taken up by specific cancer cells for exerting pharmacological effect.14 For enhancing the intracellular delivery capacity of polymeric nanoparticles to specific cells, the most widely utilized approach is tethering cell recognizable targeting ligands, such as monoclonal antibodies, endogenous targeting peptides, and low molecular weight compounds such as folate, onto the surface of the nanoparticles.15-18 One of the widely utilized approaches for targeted drug delivery is based on a receptor-mediated endocytic delivery. The folate receptor on the cell membrane is a potential molecular target for tumor-selective drug delivery. The receptor is highly expressed in a number of epithelial carcinomas, but it occurs in only a limited number of normal cells.20 Conjugation of folate to anticancer drugs, liposomes, and micelles facilitated their cellular uptake by folate receptor-mediated endocytosis.21-23 We have previously shown that polymeric micelles self-assembled from a di-block copolymer of PEG modified PLGA nanoparticles could be potentially applied for cancer cell targeted delivery of various therapeutic agents.

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PLGA–PEG–FOL exhibited far greater extent of intracellular delivery of doxorubicin in a target specific manner.24

This study aimed at designing a convenient cancer-specific drug delivery system for biodegradable PLGA nanoparticles. Negatively charged PLGA nanoparticles were coated with a synthetic di-block copolymer conjugate, poly(l-lysine)–poly(ethylene glycol)–folic acid (PLL–PEG–FOL). It was hypothesized that the positively charged PLL block in the structure of PLL–PEG–FOL conjugate was anchored on the surface of PLGA nanoparticles via ionic interactions, while the PEG block was oriented outside with exposing a folic moity in its distal end. PLGA nanoparticles coated with PLL–PEG–FOL conjugate were formulated under various conditions, and characterized by dynamic light scattering (DLS) and X-ray photoelectron spectroscopy (XPS). Their cellular uptake behaviors were comparatively evaluated by using flow cytometry and confocal microscopy.

Experimental Section

Materials. Poly(l-lysine-co-glycolic acid) (RG502H, Mn = 86 000) having a lactic/glycolic molar ratio of 50/50 was purchased from Boehringer Ingelheim (Ingelheim, Germany). Fluorescein isothiocyanate (FITC), ethylenediamine (C2H6N4), poly-(ε-carboxylic acid)–l-lysine (ε-CBZ-PLL, Mn = 1000), folate, N-hydroxysuccinimide (NHS), and penicillin/streptomycin solution were all obtained from Sigma Chemical Co. (St. Louis, MO). Poly(ethylene glycol) (NH2–PEG-COOH, Mn = 3400) was from Nektar (Huntsville, AL). 1,3-dicyclohexylcarbodiimide (DCC) was supplied from Fluka Chemie (Buchs, Switzerland). Pyridine and dimethyl sulfoxide (DMSO) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Fetal bovine serum (FBS), Roswell Park Memorial Institute 1640 (DMSO) were purchased from Aldrich (Milwaukee, WI). All other chemicals and reagents were of analytical grade.

Preparation of PLL–PEG–FOL Conjugates. PLL–PEG–FOL was synthesized by coupling ε-CBZ-PLL-NH2 to COOH–PEG–FOL. The COOH–PEG–FOL was prepared by conjugating COOH–PEG–NH2 with activated folic acid as described previously.25 Briefly, folate (60 mg) was dissolved in anhydrous dimethyl sulfoxide (DMSO, 15 mL) and activated by dicyclohexyl carbodiimide (DCC) and N-hydroxy sulosuccinimide (NHS) (molar ratio, folate:DCC:NHS = 1:1.25) at room temperature under nitrogen for 30 min. Hetero-functional PEG derivative (COOH–PEG-NH2, 250 mg) dissolved in 2 mL of DMSO was added to the activated folate solution. The folate conjugation was performed under nitrogen at room temperature for 2 h. The resultant solution was filtered through 0.45 μm filter unit (Millipore, Bedford, MA), dialyzed against deionized water (MWCO 1,000), and lyophilized. The carboxylic acid end group of COOH–PEG-FOL (150 mg) was activated to the succinimidy ester by DCC/NHS chemistry and conjugated to the primary amine terminated ε-CBZ-PLL-NH2 (117 mg) in DMSO. After 2 h of reaction, the resulting product was dialyzed against deionized water and freeze-dried. The ε-CBZ groups of folate–PEG–b-poly(ε-carboxylic acid-l-lysine) were reacted with 1 mL of 0.1% Pluronic F127 by homogenization (Powergen 700, Fischer Medicin). PLL–PEG–FOL was purified by dialyzing against excess deionized water (MWCO 3000) and lyophilized. The reaction steps were monitored by using gel permeation chromatography (Waters 510 HPLC pump system, Shodex K-802 column (8.0 × 800 mm), dual UV detector) at 283 and 368 nm and 1H NMR analysis in d6-DMSO (Bruker, DRX 400).

Preparation and Characterization of PLL–PEG–FOL Coated FITC–PLGA Nanoparticles. FITC–PLGA nanoparticles, having negative surface charges, were coated with positively charged PLL–PEG–FOL conjugate via ionic interactions in an aqueous phase. PLL–PEG–FOL coated FITC–PLGA nanoparticles were prepared by a simple mixing method: 50 μg of FITC–PLGA nanoparticles in 50 μL of phosphate buffered saline (PBS, pH 7.4) was mixed with a desired amount of PLL–PEG–FOL (0–50 μg) serially diluted in 50 μL of PBS and left at room temperature for 15 min.

(a) Dynamic Light Scattering (DLS). The size distribution and zeta-potential of PLL–PEG–FOL coated FITC–PLGA nanoparticles were evaluated by using a dynamic light scattering (DLS) instrument (Zeta-Plus, Brookhaven, New York).

(b) X-ray Photoelectron Spectroscopy (XPS). Surface composition of PLL–PEG–FOL coated FITC–PLGA nanoparticles or uncoated FITC–PLGA nanoparticles was investigated by an X-ray photoelectron spectroscopy (XPS, ESCALAB MK II, V. G. Scientific Ltd., U.K.) equipped with a Mg Kα X-ray source at a pass energy of 100 eV. Survey scans were taken over a binding energy range of 1 keV at a penetration depth of about 6 nm. For XPS analysis, PLGA nanoparticles without conjugating FITC were used.

Cell Culture. A human epidermal carcinoma cell line, KB cells (folate receptor overexpressing cell line), and a human lung carcinoma cell line, A549 cells (folate receptor deficiency cell line), were purchased from the Korean Cell Line Bank (Seoul, Korea). KB and A549 cells were maintained in RPMI 1640 medium with or without folate, respectively, at 37 °C in a humidified atmosphere of 5% of CO2. The cell culture media were supplemented with 100 units/mL penicillin, and 100 μg/mL streptomycin and, 10% fetal bovine serum (the only source of folate). The final concentration of folate in the serum-supplemented medium of KB cells was an approximately physiological level. Cells were regularly passaged and reseeded in the absence of antibiotics 24 h before cellular uptake experiments.

Intracellular Delivery of PLGA Nanoparticles. KB and A549 cells were plated in a 6-well plate at a density of 2.5 × 104 cells per well in RPMI medium and incubated for 24 h at 37 °C. The culture medium was replaced with 1 mL of medium with or without free folic acid (1 mM). Cells were incubated with the PLL–PEG–FOL coated FITC–PLGA nanoparticles, which were prepared under various coating conditions, for 3 h at 37 °C. The incubated cells were washed three times with cold PBS and fixed with 1% (w/v) para-formaldehyde solution. The extents of intracellular uptake of FITC–PLGA nanoparticles by the cells were monitored by flow cytometry (FACSscan, Becton Dickinson) using a CELLQUEST software (PharMingen). Intracellular translocation of FITC–PLGA nanoparticles was examined by

value of PLGA nanoparticles by a dynamic light scattering. Under these circumstances, the surface coating of PLL was utilized to coat with a cationic di-block copolymer conjugate, PLL-FOL. The positively charged PLL part of PLL-FOL conjugate and its surface charge of PLGA nanoparticles was fully covered with PLL–PEG–FOL at that weight ratio. The PLGA nanoparticles coated with the conjugate maintained their sizes at 108.7 ± 11.1 nm upon increasing the weight ratio of PLL–PEG–FOL conjugate/PLGA nanoparticle, suggesting that the PLGA nanoparticles after treating with the conjugate did not aggregate severely, although their surface charge changed to become almost neutral. The unchanged sizes of PLGA nanoparticles even after coating the conjugate implies that ionic anchoring of the positively charged conjugate on the negatively charged surface might induce partial shrinkage of the surface layer. The surface charge is an important factor in affecting the aggregation of polymeric nanoparticles. The reason for observing the nonaggregation was due to the presence of PEG chains surrounded around the PLGA nanoparticles. Densely seeded PEG chains on the surface, acting as a protective shield, were likely to prevent interparticular aggregation between the PLGA nanoparticles by a sterically hindered mechanism. A weight ratio of PLL–PEG–FOL conjugate/PLGA nanoparticle at 0.2 (10 µg/mL of PLL–PEG–FOL conjugate for 50 µg of PLGA nanoparticles) was selected as an optimized coating condition for further studies.

To further prove the surface modification, the surface elemental composition of PLGA nanoparticles was determined before and after treating the conjugate by using X-ray photoelectron spectroscopy (XPS). Typical XPS survey scans of uncoated and coated PLGA nanoparticles are illustrated in Figure 3. The peaks at binding energies of 280–305, 396–416, and 528–548 eV were ascribed to the elements of C1s, N1s, and O1s, respectively. The chemical composition percents of carbon, oxygen, and nitrogen on the surface of uncoated PLGA nanoparticles were 75.0%, 25.0%, and 0%, respectively; those on the surface of coated PLGA nanoparticles were 67.3%, 30.5%, and 2.2%, respectively. There were significant changes in chemical composition after the coating of PLL–PEG–FOL conjugate. The nitrogen atom was not detected on method. As shown in Figure 2, the zeta-potential value of PLGA nanoparticles increased from −18.4 ± 1.9 to 0.0 ± 0.1 mV with increasing the weight ratio of PLL–PEG–FOL conjugate/PLGA nanoparticles. The surface charge of PLGA nanoparticles became near 0 mV at a weight ratio of 0.2. This result suggests that the negatively charged surface of PLGA nanoparticles was fully covered with PLL–PEG–FOL at that weight ratio. The PLGA nanoparticles coated with the conjugate maintained their sizes at 108.7 ± 11.1 nm upon increasing the weight ratio of PLL–PEG–FOL conjugate/PLGA nanoparticle, suggesting that the PLGA nanoparticles after treating with the conjugate did not aggregate severely, although their surface charge changed to become almost neutral. The unchanged sizes of PLGA nanoparticles even after coating the conjugate implies that ionic anchoring of the positively charged conjugate on the negatively charged surface might induce partial shrinkage of the surface layer. The surface charge is an important factor in affecting the aggregation of polymeric nanoparticles. The reason for observing the nonaggregation was due to the presence of PEG chains surrounded around the PLGA nanoparticles. Densely seeded PEG chains on the surface, acting as a protective shield, were likely to prevent interparticular aggregation between the PLGA nanoparticles by a sterically hindered mechanism. A weight ratio of PLL–PEG–FOL conjugate/PLGA nanoparticle at 0.2 (10 µg/mL of PLL–PEG–FOL conjugate for 50 µg of PLGA nanoparticles) was selected as an optimized coating condition for further studies.

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the surface of uncoated PLGA nanoparticles but observed on the surface of PLL–PEG–FOL coated PLGA nanoparticles. The detection of 2.2% nitrogen means that the PLL–PEG–FOL conjugate was ionically adsorbed onto the PLGA nanoparticles. Theoretically, when the PLL–PEG–FOL conjugate was fully covered on the surface, 6.8% nitrogen should be detected assuming that the X-ray penetration depth was limited within coated layer. The underestimated nitrogen atomic percent was due to the deeper penetration (6 nm) of X-ray into the PLGA bulk phase.

To evaluate the extent of target-specific cellular uptake of FITC–PLGA nanoparticles coated with PLL–PEG–FOL conjugate, folate receptor overexpressing cancer cells, KB cells, were used. Figure 4 shows the cellular uptakes of FITC conjugated PLGA nanoparticles depending on the weight ratio of PLL–PEG–FOL or PLL–PEG conjugate to FITC–PLGA nanoparticles. The relative extent of cellular uptake was qualitatively analyzed by a flow cytometry method. The flow cytometry measures the fluorescence intensity of each cell and plots it against the number of cells. In the case of the PLL–PEG–FOL coated FITC–PLGA nanoparticles, increasing the weight ratio up to $2 \times 10^{-1}$ shifted fluorescence intensity profiles to the right direction, indicating that the cellular uptake of FITC–PLGA nanoparticles was significantly enhanced due to folate receptor mediated endocytosis. They exhibited no further increase of cellular uptake above the weight ratio of $2 \times 10^{-1}$, coinciding with the observation in Figure 2, which shows that the PLL–PEG–FOL conjugate was fully adsorbed on the surface of FITC–PLGA nanoparticles at that weight ratio. Above the weight ratio of $2 \times 10^{-1}$, uncoated PLL–PEG–FOL conjugate species might remain in the aqueous phase, and they were likely to compete with the PLL–PEG–FOL coated FITC–PLGA nanoparticles for binding to folate receptors in KB cells. This might be the reason for observing the slight decrease of cellular uptake at the higher weight ratio. The extent of cellular uptake for optimally modified FITC–PLGA nanoparticles was about more than 6.7 times greater than that of unmodified FITC–PLGA nanoparticles, when assessing within an arbitrary gate region set (a scale bar range $R$ in Figure 4) in the flow cytometry result. The cellular uptake of PLL–PEG coated FITC–PLGA nanoparticles was very slightly enhanced with raising the weight ratio compared to unmodified PLGA ones. The increase of cellular uptake by coating with the PLL–PEG conjugate was likely caused by the fact that the negative surface zeta potential value ($-18.4 \pm 1.9 \text{ mV}$) of unmodified FITC–

Figure 3. Typical XPS survey scan of the surfaces of PLL–PEG–FOL coated PLGA nanoparticles and uncoated PLGA nanoparticles.

Figure 4. Intracellular uptake extents of PLL–PEG coated PLGA nanoparticles (upper panel) and PLL–PEG–FOL coated PLGA nanoparticles (lower panel) for KB cells as a function of the conjugate/nanoparticle weight ratio; 0–1 (50 µg of PLGA nanoparticles). Control represents the fluorescence emitted from naked KB cells. $R$ represents a gated region [fluorescence intensity (arbitrary unit): $10^2$–$10^4$].

PLGA nanoparticles became neutral after the coating, as shown in Figure 2. The negatively charged FITC–PLGA nanoparticles could interact with negatively charged cell membrane to a lesser extent, thereby limiting their chance of cellular uptake via adsorptive endocytosis. Increasing their surface charge to near neutral, despite the enriched steric PEG chains on the surface, might provide more opportunity for cellular transport. With increasing the weight ratio of PLL–PEG conjugate/FITC–PLGA nanoparticles, however, the extents of cellular uptake for PLL–PEG/FITC–PLGA nanoparticles not having FOL moieties were not increased, as compared to those for PLL–PEG–FOL/FITC–PLGA nanoparticles having FOL moieties. Thus it can be postulated that the greatly improved intracellular uptake of PLL–PEG–FOL coated FITC–
PLGA nanoparticles was caused by a folate receptor-mediated endocytosis process.

To make sure that the intracellular delivery of FITC–PLGA nanoparticles occurred in a folate receptor-mediated specific manner, comparative cellular uptakes of PLL–PEG–FOL coated PLGA nanoparticles were estimated using two different cancer cell lines, KB and A549 cells:

A549 cells are folate receptor deficient cancer cell line. The cellular uptake of FITC–PLGA nanoparticles was exceedingly higher for KB cells than for A549 cells (Figure 5, A and B). This result also suggests that targeted intracellular delivery of PLL–PEG–FOL coated FITC–PLGA nanoparticles took place via a folate receptor-mediated endocytosis mechanism. To further confirm the folate receptor-mediated endocytosis, an excess amount of free folate was added in the medium to see whether it inhibited the cellular uptake within KB cells by competitive binding to folate receptors. As shown in Figure 5 C, the cellular uptake of PLL–PEG–FOL coated PLGA nanoparticles was effectively suppressed in the presence of 1 mM folate in the medium, supporting the role of folate receptor-mediated endocytosis. Figure 5 D shows the cellular uptake of PLL–PEG–FOL/FITC–PLGA nanoparticles in the presence of serum proteins. It can be seen that, although the cellular uptake extent was slightly diminished in the presence of serum proteins, they could be delivered within KB cells to a far greater level than unmodified FITC–PLGA nanoparticles. This was attributed to the protective and shielding effect of PEG corona surrounding the FITC–PLGA nanoparticles. Maxi-

**Figure 5.** Cellular uptake extents of PLGA nanoparticles under various conditions. (A) KB cells (folate receptor positive carcinoma) were treated with plain PLGA nanoparticles (black line) or PLL–PEG–FOL coated PLGA nanoparticles (blue line), or without the nanoparticles (red line) in serum free media containing physiological level of free folate. (B) A549 cells (folate receptor negative carcinoma) incubated in the media containing uncoated PLGA nanoparticles or PLL–PEG–FOL coated PLGA nanoparticles. (C) The effect of free folate on the cellular uptake of PLGA nanoparticles coated with or without PLL–PEG–FOL for KB cells. Cells were incubated in the media with or without 1 mM of free folate. (D) The effect of serum proteins in the media on the cellular uptake of PLGA nanoparticles for KB cells. Cells were treated with uncoated PLGA nanoparticles or PLL–PEG–FOL coated PLGA nanoparticles in the media with or without 10% fetal bovine serum. Control represents the fluorescence emitted from naked KB cells. The weight ratio of conjugate/PLGA nanoparticles was fixed at 2 × 10^−1. R indicates a gated area [fluorescence intensity (arbitrary unit): 10^2–10^4].

**Figure 6.** Confocal images of KB cells treated with (A) uncoated PLGA nanoparticles, (B) PLL–PEG coated PLGA nanoparticles, and (C) PLL–PEG–FOL coated PLGA nanoparticles.
mally coated PLL–PEG–FOL conjugates on the surface were likely to produce densely anchored PEG chains, which could sterically repel negatively charged serum proteins as previously reported. The result reveals that noncovalently adsorbed PLL–PEG–FOL conjugate on the surface was stable enough even in the presence of serum proteins. Charge interactions of surface adsorbed PLL–PEG–FOL conjugate with negatively charged serum proteins could detach the conjugates from the surface, resulting in reducing the cellular uptake extent. There are many examples for reduced cellular uptake of polyelectrolyte complex nanoparticles formed between negatively charged macromolecules (DNA and proteins) and PLL–PEG di-block copolymer in the presence of serum proteins. This was caused by interfering charged serum proteins to the complexes that were primarily formed by multiple charge-to-charge interactions. In the case of PLGA nanoparticles, the cellular uptake was reasonably well maintained after the surface coating of PLL–PEG–FOL conjugate, implying that the PEG seeding density on the surface plays an important role in suppressing the ionic exchanging reaction with serum proteins. Figure 6 represents confocal images of KB cells incubated with unmodified FITC–PLGA, PLL–PEG/FITC–PLGA, and PLL–PEG–FOL/FITC–PLGA nanoparticles. For only PLL–PEG–FOL/FITC–PLGA nanoparticles, green fluorescent and scattered nanoparticles were detected within the cytosol region, as depicted in the panel C. On the other hand, KB cells treated with uncoated and PLL–PEG coated FITC–PLGA nanoparticles (panel A and B) exhibited very weak fluorescent intensities. The confocal image evidently indicates that PLL–PEG–FOL conjugate can improve the cellular uptake of PLGA nanoparticles by folate receptor-mediated intracellular delivery. This visualization result was consistent with those presented in the flow cytometry analysis as shown in Figures 4 and 5. The PLL–PEG–FOL coated PLGA nanoparticles could be potentially applied to target-specific intracellular delivery of various hydrophobic anticancer agents.

Conclusions

A cancer cell targeted nanoparticulate delivery system was developed by using PLL–PEG–FOL conjugate. Negatively charged PLGA nanoparticles were surface anchored with the positively charged PLL part in the conjugate. The PLL–PEG–FOL/PLGA nanoparticles were surrounded with a protective PEG layer while exposing FOL moieties for cell recognition. The enhancement of cellular uptake was gained via a folate receptor-mediated intracellular delivery mechanism, even in the presence of serum proteins. The present formulation could be used as a promising cancer cell specific delivery system for diverse anticancer agents encapsulated within PLGA nanoparticles.

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