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Uptake of folate-conjugated albumin nanoparticles to the SKOV3 cells

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Abstract

The bovine serum albumin nanoparticles (BSANPs) were prepared by a coacervation method and chemical cross-linking with glutaraldehyde. Furthermore, the BSANPs were reacted with the activated folic acid to conjugate folate via amino groups of the BSANPs, to improve their intracellular uptake to target cells. The nanoparticles were apparently spherical with diameters less than 150 nm and their average diameter was 70 nm by a transmission electron microscope (TEM) and a laser light scattering particle analyzer, respectively. An extent of folate conjugation with the BSANPs was 169 μmol/g BSA by spectrophotometric analysis. Cell uptake studies were carried out in SKOV3 cells (human ovarian cancer cell line) using fluorescein isothiocyanate labeled nanoparticles. The extent of BSANPs taken up by the cells was measured with a fluorescence spectrophotometer. The nanoparticles were taken up to the cells and levels of binding and uptake were increased with the time of incubation until 4 h. The levels of folate-conjugated BSANPs were higher than those of BSANPs and saturable. The association of folate-conjugated BSANPs to SKOV3 cells was inhibited by an excess amount of folic acid, suggesting that the binding and/or uptake were mediated by the folate receptor. These results implied that the folate-conjugated BSANPs might be useful as a drug carrier system to deliver drugs into the cells expressing folate receptor.

Keywords: Target; Folate receptor; SKOV3 cells; Albumin nanoparticles

1. Introduction

Site-specific delivery of drug receives a lot of attention because it can reduce drug toxicity and increase therapeutic effects (Allen et al., 1995). Nanoparticles have been considered as effective delivery systems for

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many reasons including: (i) sufficient physical and biological stability that may facilitate drug entrapment and controlled release (Yang et al., 1999; Maia et al., 2000), (ii) good tolerability of the components (Maia et al., 2000), (iii) simplicity of the formulation processing (Oyewumi and Mumper, 2002) and (iv) possibility of scaling up the formulation process (Muller et al., 2000). Therefore, nanoparticles have been extensively employed to deliver drugs, genes, vaccines and diagnostics into specific cells/tissues (Coester et al., 2000; Jenning et al., 2000; Stella et al., 2000).

Among these colloidal systems those based on proteins may be of promising because of their biodegradability, lack of toxicity and antigenicity, stability, shelf life, controllable drug-release properties and high loading capacity for hydrophilic molecules (Rubino et al., 1993).

To solve the problem of site-specific targeting for the colloidal systems, some authors have attempted to increase the tissue specificity of colloidal drug carriers by coupling targeting agents. Among the possible targeting agents, folic acid could be exploited to realize delivering drugs into cancer cells. Folic acid is a low molecular weight (441 Da) vitamin whose receptor is frequently overexpressed in human cancer cells (Weitman et al., 1992; Franklin et al., 1994). This receptor has been identified as a tumor marker, especially in ovarian carcinomas (Campbell et al., 1991), and it is highly restricted in most normal tissues. Folic acid presents advantages as a targeting device to the tumor cells as follows. First, it is stable, inexpensive, and non-immunogenic compared with proteins such as monoclonal antibodies. Second, folic acid binds to the folate receptors at cell surfaces with very high affinity (K_d = ~1 nM) and is internalized by receptor-mediated endocytosis (Kamen and Caston, 1986; Lee and Low, 1994, 1995; Lee and Huang, 1996; Atkinson et al., 2001). The feasibility of tumor-targeting via folate receptors has been reported (Lee and Low, 1995). It has also been shown that folate-conjugated macromolecules can be specifically taken up by the tumor cells (Lee and Low, 1995; Lee and Huang, 1996; Stella et al., 2000; Oyewumi and Mumper, 2003).

In this study, bovine serum albumin nanoparticles (BSANPs) were prepared and conjugated with folic acid via amino groups. Furthermore, uptake of folate-conjugated albumin nanoparticles in SKOV3 cells, human ovarian cancer cells, was examined.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA), folic acid, dicyclohexylcarbodiimide (DCC), fluorescein isothiocyanate (FITC), 2,4,6-trinitrobenzenesulfonic acid (TNBS) and *N*-hydroxysuccinimide were all purchased from Sigma. SKOV3, a human ovarian cancer cell line, was a gift from immunity laboratory of Sichuan University. RPMI-1640 medium without folic acid was obtained from Gibco. All other reagents were of analytical grade.

2.2. Preparation of albumin nanoparticles

The albumin nanoparticles were prepared by a desolvation technique. BSA (10 mg) was dissolved in 1.0 ml of purified water. Under constant stirring desolvation of the 1% BSA solution was achieved by dropwise addition of 6.0 ml of ethanol. After the desolvation process, 25 μl of 0.25% glutaraldehyde solution was added to induce particle crosslinking. The crosslinking process was performed by stirring the suspension over a time period of 24 h (Weber et al., 2000; Langer et al., 2003). The suspension was centrifuged and the pellets were redispersed to the original volume in water.

2.3. Preparation of folate-conjugated albumin nanoparticles

N-Hydroxysuccinimide ester of folic acid (NHS-folate) was prepared according to the method of Lee and Low (1994). Folic acid (10 g dissolved in 200 ml of dry dimethyl sulfoxide plus 5.0 ml of triethylamine) was reacted with *N*-hydroxysuccinimide (5.2 g) in the presence of dicyclohexylcarbodiimide (9.4 g) overnight at room temperature. The by-product, dicyclohexylurea, was removed by filtration. The dimethyl sulfoxide solution was then concentrated under reduced pressure and heating, and NHS-folate was precipitated in diethylether. The product, NHS-folate, was washed several times with anhydrous ether, dried in vacuum, and yielded as yellow powder.

A conjugation of folate to BSANPs was as follows: NHS-folate (50 mg) was dissolved in 1.0 ml of dimethyl sulfoxide and added slowly to the stirring BSANP suspension (2 ml, pH was adjusted to 10 using 1 M carbonate/bicarbonate buffer). After stirring for

45 min at room temperature, the reaction mixture was passed down a Sephadex G-50 column to separate the folate-conjugated BSANPs from unreacted folic acid and other by-products. The folate-conjugated BSANPs eluted in the void fraction. The suspension was centrifuged and the pellets were redispersed to the original volume in water.

2.4. Characterization of nanoparticles

2.4.1. Quantification of amino groups at a surface of BSANPs

The determination of amino groups was performed using the 2,4,6-trinitrobenzenesulfonic acid method (Satake et al., 1960) according to the procedure adapted by Edwards-Levy et al. (1993). This procedure consisted of the incubation of the material with an excess of TNBS and the back titration of the unreacted amount of the reagent.

The BSANPs were washed three times with water by centrifugation at 20,000 × *g* for 20 min, followed by redispersion with 4% sodium bicarbonate solution (pH 8.5). Two milliliters of the BSANP dispersion and 4.0 ml of TNBS solution (4.0 μmol/ml in 4% sodium bicarbonate solution) were added. The reaction mixture was shaken at 400 rpm for 1 h at 40 °C. The samples were centrifuged (30,000 × *g*; 1 h) for separating the nanoparticles from the supernatant. In order to measure an unreacted TNBS, 0.9 ml of the supernatant was added to 0.1 ml of valine water solution (40 μmol/ml) and incubated at 40 °C in the dark for 1 h. Then, 5 ml of HCl (0.5 mmol/L) were added and the absorbance of solution was measured at 410 nm against a blank prepared as described above, but containing 0.1 ml of trichloroacetic acid (1%) instead of valine solution. The content of amino groups on the particle surface was calculated relative to a TNBS reference that was treated in the same manner as described above, using water instead of the BSANP dispersion.

2.4.2. Determination of folate content

The folate-conjugated BSANPs were purified from unreacted products with Sephadex G-50 column and digested by trypsin (0.05 mg/mg BSA). The digesting process was performed under stirring at 37 °C for 2 h. After tryptic hydrolysis of folate-conjugated BSANPs, the extent of folate conjugation was determined by a spectrophotometric analysis at the absorbance of 358 nm (folic acid $\varepsilon = 8643.5\ M^{-1}\ cm^{-1}$).

2.4.3. Determination of the particle size

The size of albumin nanoparticles was determined by Malvern Mastersizer-2000 laser light scattering particle analyzer (Malvern Instruments Ltd., Malvern, UK). The samples were diluted with distilled water and measured at room temperature.

2.4.4. Transmission electron microscopy (TEM)

Size and morphology of nanoparticles were observed using a Hitachi H-600 Electron Microscope (Japan). A carbon-coated 200 mesh copper specimen grid was glow-discharged for 1.5 min. One drop of nanoparticle suspension was deposited on the grid and allowed to stand for 1.5 min. After any excess fluid was removed with filter paper, the grid was later stained with one drop of 2% phosphotungstic acid and allowed to dry in air for 10 min before examination under the electron microscope.

3. Cell culture

Cell uptake studies were performed using SKOV3 cells, a human ovarian cancer cell line. The cells were cultured in T-75 flasks at 37 °C in a humidified atmosphere containing 5% CO_2 using folate-free RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum. The only source of folic acid in the media was due to the presence of the 10% fetal calf serum.

4. Cell uptake studies

BSA was labeled with FITC to investigate uptake of nanoparticles to SKOV3 cancer cells according to the method by Leamon and Low (1991). After the labeling reaction, the BSA-FITC conjugates were purified from unreacted FITC with Sephadex G-50 column and nanoparticles were prepared using the BSA-FITC conjugates following the process described in the preparation of BSANPs.

FITC-labeled folate-conjugated BSANPs or BSANPs suspensions in folate-free RPMI-1640 were added to monolayers of SKOV3 cells (0.90×10^5) grown in 24-well plastic dishes. The cells were then

incubated at 37 °C for various lengths of time. Cell monolayers were thoroughly washed in phosphate-buffered saline (PBS), and lysed in 0.75 ml of lysis buffer (PBS containing 1% Triton X-100). The cell-associated fluorescence was then measured in the lysis buffer extract with a Shimadzu fluorescence spectrophotometer (Japan). To determine the fraction of ineternalized nanoparticles, the cell surface-bound nanoparticles were stripped by two washes with acidic saline (0.9% NaCl adjusted to pH 3 with acetic acid) followed by two washes with PBS. A remaining fluorescence was considered as internalization (Lee and Low, 1994).

To study a role of folate receptor in nanoparticles uptake, SKOV3 cells (1.0×10^5) grown in 33 mm culture dishes were treated with 0.45 mg/ml FITC-labeled folate-conjugated BSANPs or BSANPs in PBS containing different concentrations of free folate. Uptake of FITC-labeled folate-conjugated BSANPs was assayed as outlined above, comparing with the uptake of FITC-labeled BSANPs.

To study cellular uptake of nanoparticles by the fluorescence microscopy, the cells were cultured in 6-well plastic dishes containing 18 mm coverslips for 24 h. The FITC-labeled folate-conjugated BSANPs or BSANPs were added to the cell culture media at a particle concentration of 0.15 mg/ml. After 3 h of incubation at 37 °C, the cells were washed six times with PBS. The coverslips were put on slides coated with buffered mounting medium and viewed by Olympus fluorescence microscopy (Japan).

5. Results and discussion

5.1. Preparation and purification of folate-conjugated BSANP

Folic acids were covalently linked to BSANPs by the method described above. To activate the folate carboxylic groups for coupling with amino groups of the BSANPs, the NHS-ester of folic acid was prepared. Then, the NHS-folate was added to the BSANPs suspension. The reaction of NHS-folate with amino groups of BSANPs led to formation of an amide bond. The folate-conjugated nanoparticles were then purified from unreacted folic acid by a gel chromatography, using bicarbonate buffer as an eluent (Fig. 1). Two

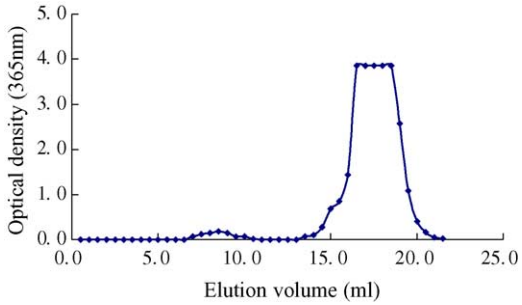

Fig. 1. Gel filtration chromatogram of folate-BSANPs nanoparticles on a sephadex 50 column eluted with 5 mM carbonate/bicarbonate buffer (pH 10).

distinct peaks, the first one corresponding to folate-conjugated BSANPs, which were eluted in excluded volume, and the second one corresponding to unreacted folate, were observed. As shown in Fig. 1, the folate-conjugated BSANPs could be separated from unreacted folate completely.

5.2. Characterization of nanoparticles

5.2.1. Quantification of amino groups of albumin nanoparticles

The TNBS method was used to determine the number of amino groups of the BSANPs. The procedure included the incubation of the BSANPs with an excess of TNBS and the back titration of the unreacted amount of the TNBS. The number of amino groups of folate-conjugated BSANPs was also measured using the same method. Our results showed that the number of amino groups of BSANPs and folate-conjugated BSANPs was 598 μmol/g BSA and 434 μmol/g BSA, respectively.

5.2.2. Determination of folate content

To evaluate the extent of folate conjugation of folate-conjugated BSANPs, the spectrophotometric analysis was performed after tryptic hydrolysis of the folate-conjugated BSANPs. It was found that about 169 μmol/g BSA folate were linked to the amino groups of BSANPs. The estimation of the folate content of the folate-conjugated BSANPs was approximately consistent with the decrease of the number of amino groups of BSANPs by the conjugation of folic acid.

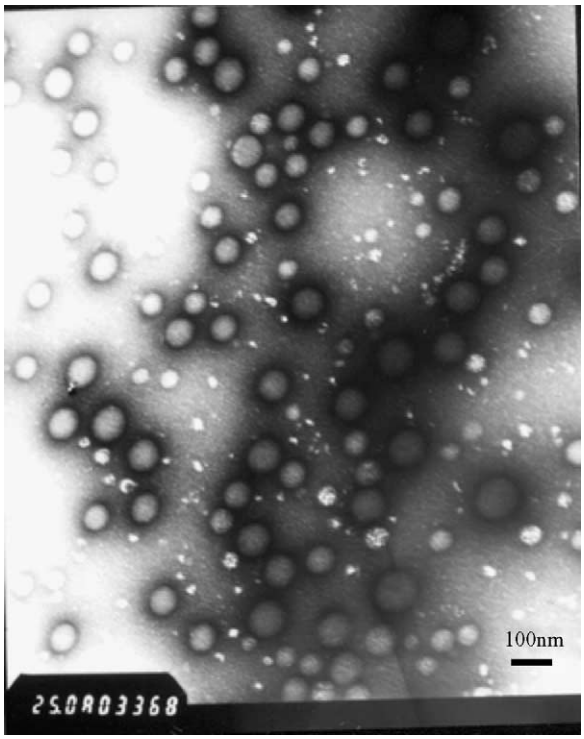

Fig. 2. Transmission electron micrograph (TEM) showing the size and morphology of folate-conjugated BSANPs.

5.2.3. Determination of particle size and transmission electron microscopy (TEM)

Fig. 2 showed the TEM micrograph of folate-conjugated BSANPs. The nanoparticles were apparently spherical with diameters less than 150 nm (Fig. 2). Results obtained from laser light scattering measurements of folate-conjugated BSANPs (Fig. 3) gave a similar trend as TEM results. The average diameter was about 70 nm and it was slightly larger than that before folate-conjugation (65 nm).

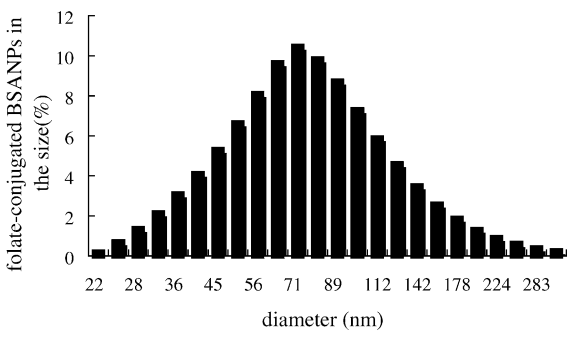

Fig. 3. Size distribution of folate-conjugated BSANPs according to laser light scattering measurment.

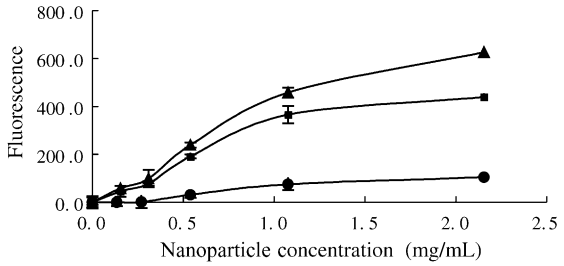

Fig. 4. Concentration dependence of nanoparticle uptake by SKOV3 cells. SKOV3 cells were incubated with different concentrations of FITC-labeled nanoparticles for 4 h at 37 °C. (▲) Cells treated with FITC-labeled folate-conjugated BSANPs and washed with PBS. (■) Cells treated with FITC-labeled folate-cojugated BSANPs and washed with pH 3 saline. (●) Cells treated with FITC-labeled BSANPs and washed with PBS. Error bars represent standard deviations ($n = 3$).

5.3. Concentration dependence of nanoparticle uptake

SKOV3 cells were incubated with different concentrations of FITC-labeled folate-conjugated BSANPs or BSANPs for 4 h at 37 °C. To distinguish surface bound nanoparticles, SKOV3 cells were washed with either cold PBS to remove unassociated nanoparticles or acidic saline to strip bound but not internalized nanoparticles. The cells were then lysed and assayed for residual cell fluorescence using the protocol described above. As shown in Fig. 4, the intensity of fluorescence would be enhanced with increasing the concentration of FITC-labeled folate-conjugated BSANPs in the incubating system. The level of uptake gradually reached a platform, suggesting that the uptake of FITC-labeled folate-BSANPs could be saturated. Therefore, we speculated that FITC-labeled folate-conjugated BSANPs were taken up by SKOV3 cells via folate receptor. Furthermore, the association of FITC-labeled folate-conjugated BSANPs with the SKOV3 cells was significantly higher than that of FITC-labeled BSANPs (6–43 folds at different concentration points).

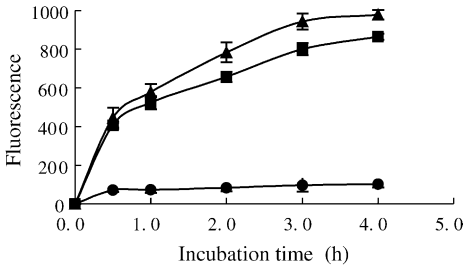

Fig. 5. Time course of nanoparticle uptake by SKOV3 cells. SKOV3 cells were incubated with FITC-labeled nanoparticles (0.45 mg/ml) at 37 °C for different lengths of time. (▲) Cells treated with FITC-labeled folate-conjugated BSANPs and washed with PBS. (■) Cells treated with FITC-labeled folate-cojugated BSANPs and washed with pH 3 saline. (●) Cells treated with FITC-labeled BSANPs and washed with PBS. Error bars represent standard deviations ($n = 3$).

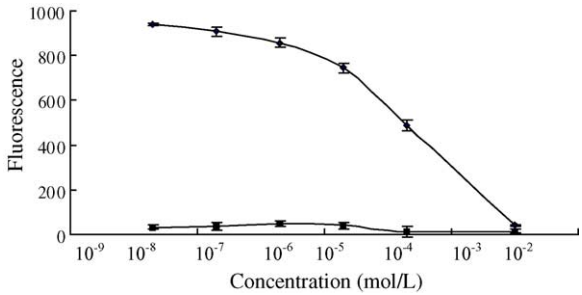

Fig. 6. Inhibition of FITC-labeled folate-conjugated BSANP uptake by free folic acid. SKOV3 cells were incubated for 4 h at the concentration of 0.45 mg/ml in the presence of various concentrations of free folic acid at 37 °C. (▲) Cells treated with FITC-labeled folate-conjugated BSANPs and washed with PBS. (●) Cells treated with FITC-labeled BSANPs and washed with PBS. Error bars represent one standard deviation ($n = 3$).

5.4. Time course of folate-conjugated nanoparticle uptake by SKOV3 cells

In order to study time course of nanoparticle uptake by receptor-bearing cells, SKOV3 cell monolayers were incubated with 0.45 mg/ml FITC-labeled nanoparticles for various lengths of time, washed extensively, and evaluated for cell-associated fluorescence by fluorescence spectrophotometer following cell lysis in 1% Triton X-100 (Fig. 5). Uptake of FITC-labeled folate-conjugated BSANPs increased as the time went on, but the uptake rate gradually declined after the first 0.5 h of incubation. This gradual decline in the rate of internalization appeared to derive from saturation of the uptake system rather than depletion of folate-conjugated BSANPs, since folate-conjugated BSANPs concentration in the medium did not measurably decline over the course of the incubation.

5.5. Inhibition of folate-conjugated BSANP uptake

In order to evaluate a role of folate in the cellular uptake of folate-conjugated BSANPs, SKOV3 cells were treated with 0.45 mg/ml nanoparticles in PBS containing folic acid. As shown in Fig. 6, the uptake of the FITC-labeled folate-conjugated BSANPs was inhibited by excess folate, but the FITC-labeled BSANP uptake was not influenced by the addition of folate. The observations that free folate inhibited folate-conjugated BSANPs association suggested that the folate-conjugated BSANPs might be endocytosed via the folate receptor. Although free folate had a K_d of 0.01–1 nmol/L for the folate receptor (Kamen et al., 1988), our results showed that 200 μmol/L folate was required to reduce cellular folate-conjugated BSANP uptake by 50%. Therefore, folate-conjugated BSANPs exhibited a much higher affinity for the cells than free folate. We believe the reason for this enhanced avidity is that folate-conjugated BSANPs form the multivalent interactions with the folate receptors of SKOV3 cells. The fact that folate receptors normally exist in large clusters (Kamen et al., 1988) and that one folate-conjugated BSANP has many folate molecules on its surface allows for multivalent interactions. One folate-conjugated BSANP might occupy many receptors due to the multivalency of the folate-conjugated BSANP construct. As folate concentration in the serum seldom exceeded 20 nmol/L, folate-conjugated BSANP binding should not be compromised in vivo.

5.6. Observation of uptake of nanoparticles to the cell by fluorescence microscopy

The uptake of the folate-conjugated BSANPs and BSANPs by SKOV3 cells was visualized using fluorescence microscopy (Fig. 7). The absence of cell-associated FITC fluorescence in the control sample in contrast to the intense cell-associated fluorescence in the sample containing folate-conjugated BSANPs revealed the dependence of nanoparticle recognition and uptake on the conjugation of folate.

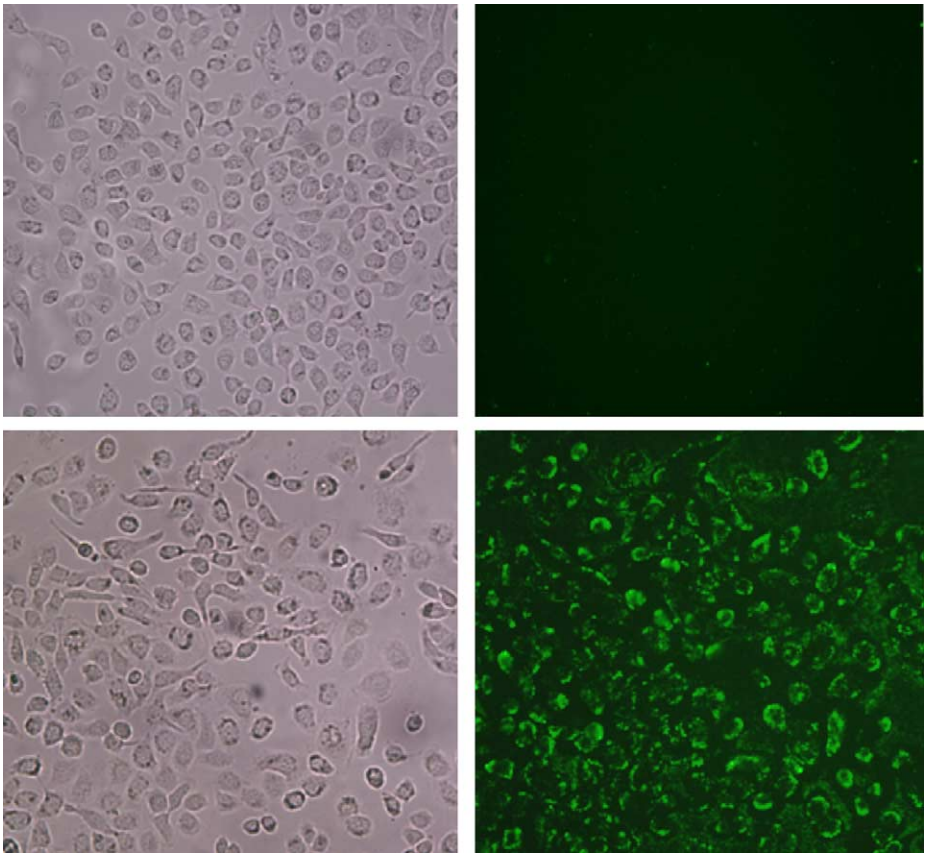

Fig. 7. Effect of folate derivatization on nanoparticle uptake by cultured SKOV3 cells. SKOV3 cell monolayers were incubated with FITC-labeled nanoparticles (0.15 mg/ml) for 3 h at 37 °C and viewed by either phase contrast (left panels) or fluorescence microscopy (right panels). The upper two panels show the same field of cells following treatment with FITC-labeled BSANPs lacking folate (control), whereas the lower two panels display a field of cells treated with FITC-labeled folate-conjugated BSANPs. Magnification is ×200.

6. Conclusions

In this study, the folate-conjugated BSANPs were prepared and characterized. Furthermore, folate-conjugated BSANPs were taken up to human ovarian cancer cells (SKOV3). The level of uptake of folate-conjugated BSANPs increased with a saturation manner. The uptake of folate-conjugated BSANPs to SKOV3 cells could be inhibited by folic acid, suggesting that the internalization could be mediated by the folate receptor.

The folate-conjugated BSANPs represent a new potential delivery system for compounds with anticancer activity because folate receptor is frequently overexpressed in the malignant cells. Moreover, the encapsulation of anticancer drugs into folate-conjugated BSANPs may increase a targetability of the drugs to the tumor cells; protect the drug from in vivo degradation and reduce side effects and toxicities of the drugs. Further studies are in progress to test this new drug delivery system in vivo.

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