# Cellular Uptake Study of Biodegradable Nanoparticles in Vascular Smooth Muscle Cells

**Hearan Suh,[1] Byeongmoon Jeong,[1] Feng Liu,[1] and Sung Wan Kim[1,2]**

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## INTRODUCTION

The use of polymeric nanoparticles has been shown to be promising in cancer chemotherapy (1,2), intracellular viral and bacterial infection (3,4) and many other pathological states (5,6) due to their high internalization into the cells compared to larger micron size particles. In fact, nanoparticles can significantly affect the cellular pharmacokinetic profiles of drugs by altering the cellular uptake and residence time of the drug.

For the quantitative evaluation of cellular uptake, the particles are labeled with fluorescent dyes (7,8) or radioisotopes (9,10). The nanoparticles labeled with fluorescent dyes are frequently used to avoid the problems of handling radioactive materials. Assaying is simple using flow cytometry, and internalization can be visualized using confocal fluorescence microscopy. However, there is a lack of appropriate molecular probes that form stable bonds between the polymer and dye, and that produce high fluorescence output per molecule.

In this study, highly fluorescent nanoparticles were prepared using the biodegradable polymer, poly(ethylene oxide)-poly(lactide/glycolide)-polylysine-fluorescein isothiocyanate (PEO-PLGA-PLL-FITC). PLL was adopted as a spacer between PEO-PLGA polymer and FITC molecules. The multiple labeling of a polymer with FITC via formation of stable thiourea bonds between FITC and the ε-amine group of PLL can greatly enhance the fluorescence intensity of nanoparticles, thus making it possible to perform kinetic studies of cellular uptake and retention.

## MATERIALS AND METHODS

### Materials

Poly(ethylene glycol monomethyl ether) (MW = 5,000) and poly-L-lysine (PLL) (MW = 4,000) were purchased from Aldrich (Milwaukee, WI), FITC from Pierce (Rockford, IL), and poly(vinyl alcohol) (PVA) (MW = 25,000, 98.5% mole hydrolyzed) from Polyscience (Warrington, PA). D,L-lactide (DLLA) (Aldrich) and glycolide (GA) (Polyscience) were recrystallized in ethyl acetate and sublimed, respectively, before use. All other solvents and chemicals were of analytical grade and used without further purification.

### Polymer Synthesis

PEO-PLGA polymer (DLLA:GA = 3:1 molar ratio) was synthesized by the ring-opening polymerization of lactide and glycolide in the presence of monomethoxy PEO using stannous octoate as a catalyst (11). The obtained polymer was characterized for its molecular weight, copolymer composition, and glass transition temperature ($T_g$) by $^1$H-NMR (200 MHz, IBM), and differential scanning calorimetry (DSC, Perkin Elmer). For fluorescence labeling, the PEO-PLGA polymer was first activated with 4-nitrophenyl chloroformate (4-NPC), and subsequently PLL was linked to the activated carboxyl group of the polymer via its N-terminal amine group. Finally, FITCs were conjugated to the ε-amine groups of PLL by formation of a thiourea bond.

PEO-PLGA polymer (2 g) was dissolved in toluene/DMSO mixture (3:1) and toluene was evaporated by boiling at 110°C. After cooling the solution to room temperature, 4-NPC (4 mg) and triethylamine (TEA, 2 mg) were added to the solution and stirred for 2 hours, and PLL (120 mg) was then coupled to the activated polymer (PEO-PLGA) by reaction for 2 hours at room temperature. The product was precipitated in ethyl ether, purified by washing with water and allowed to react with 150 mg of FITC in DMSO for 8 hours with protection from light. The conjugation of FITC to the polymer was quantified spectrophotometrically at 450 nm. Using the FITC-conjugated polymer, nanoparticles (~150 nm in diameter) were prepared by emulsion-solvent evaporation methods (12).

### Confocal Fluorescence Microscopy

The bovine arterial smooth muscle cells were seeded on cover glass (24 × 24 mm) and grown for 48 hours. Then, cells were incubated with FITC-conjugated polymeric nanoparticles for 4 hours at 37°C, washed three times with cold PBS and fixed with 3.7% formaldehyde solution for 30 min at room temperature. A coverglass containing fixed cells was placed, cell side down, on the droplet of mounting medium (2 mg paraphenylenediamine/ 1 ml of 1:1 mixture of PBS and glycerol) on a microscope slide, and the edges of the coverglass were sealed to the slide with Cytoseal™. The cells were observed using a laser scanning confocal imaging system (Bio-Rad MRC 600) consisting of a Zeiss Axioplan microscope and a Krypton/Argon laser. A plan-apo objective lens (× 60, numerical aperture 1.4, oil) and BHS block filter were employed to collect FITC fluorescence images.

### Cellular Uptake Study (Flow Cytometry)

The bovine smooth muscle cells were seeded in T-25 flask and incubated at 37°C in a humidified atmosphere containing 5% $CO_2$. At 48 hours, the cell medium was replaced with nanoparticle-containing medium, and the cells were incubated further for the indicated period of time either at 37°C or 4°C. At the end of the incubation time, cells were washed three times with cold PBS, harvested by trypsin treatment and suspended in PBS ($1 \times 10^6$ cells/0.5 ml). The cell-associated nanoparticle content was immediately assayed by flow cytometry (FACScan,

[1] Department of Pharmaceutics and Pharmaceutical Chemistry / CCCD, University of Utah, Salt Lake City, Utah 84112.
[2] To whom correspondence should be addressed.



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Becton Dickinson) interfaced with a computer and calibrated with FITC-labeled beads (CaliBRITE™, Becton Dickinson). Signals for forward, 90-degree light scatter, and fluorescence at 530 ± 15 nm were collected from 10,000 cells with a 90 degree light scatter as a master signal.

## RESULTS

### PEO-PLGA-PLL-FITC Polymer

$^1$H-NMR spectra of PEO-PLGA copolymers are shown in Fig.1 with their respective chemical structures. The peaks at 1.55 and 5.20 ppm were assigned to a methine proton and methyl protons of DLLA, respectively. Also, the methylene protons of PEO and GA appeared at 3.65 and 4.80 ppm, respectively. The number average molecular weight of PEO-PLGA calculated by end group analysis (13) was approximately 103,000 from the composition of copolymer and the molecular weight of PEO (5,000). The $T_g$ of the polymer was determined as 46°C using DSC. Spectrophotometric analysis showed that about 16 FITC molecules were conjugated to each polymer molecule. In vitro stability tests were performed at 37°C and revealed no dissociation of FITC from the polymeric particles during the experimental period (7 days) both at pH 7.4 and pH 5.0, indicating a stable linkage of FITC to the polymer in neutral and acidic environments.

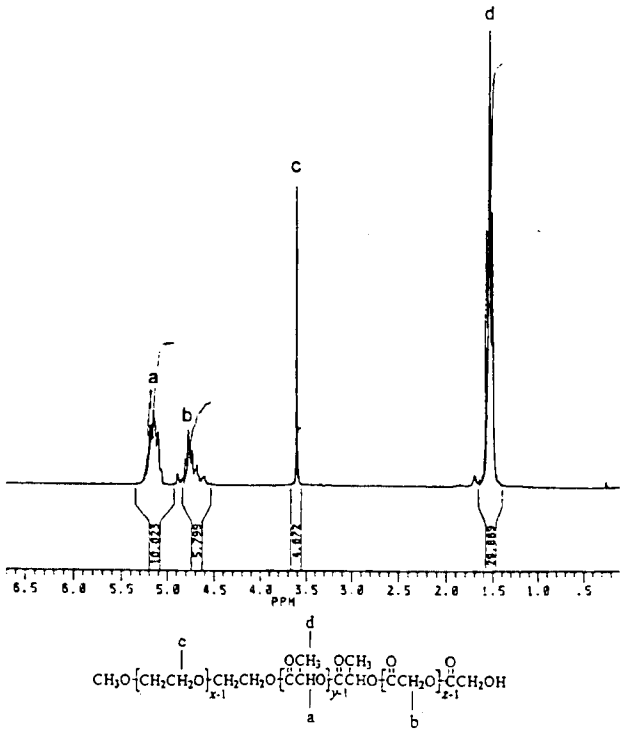


**Fig. 1.** Proton NMR spectra of PEO-PLGA (5,000–98,000) copolymer in $CDCl_3$ and chemical structures. The number average molecular weight of the polymer was calculated using following equations: (1). (3 Y)/4 (112) + 2 = $A_{1.55}/A_{3.65}$, (2). (2Z)/4(112) + 2 = $A_{4.80}/A_{3.65}$, (3). M.W. = 5,000 + 72Y + 58Z. $A_{1.55}$, $A_{3.65}$ and $A_{4.80}$ are integration values at 1.55, 3.65 and 4.80 ppm, respectively.

### Confocal Microscopy

Confocal microscopy can give sharp two- or three-dimensional images that are essentially undisturbed by scattering and blurring from light in the out-of focus areas. Therefore, it can be applied to observation deep inside the materials by focusing on a selected plane inside a specimen. This microscopic technique was employed to visualize the internalization of fluorescent nanoparticles by vascular smooth muscle cells in this study.

Fig. 2B represents a confocal image of a cell incubated with FITC-conjugated nanoparticles. The spherical fluorescent particles are seen as bright spots concentrated in discrete areas surrounding the nucleus.

For subcellular trafficking of nanoparticles, the lysosomal compartment was marked using a lysosomotrophic dye, neutral red, which readily diffuses across cellular membranes at neutral pH, but becomes membrane impermeable at low pH, therefore, concentrating in the lysosomes. Using the two channel operation mode of the confocal microscope (Bio-Rad MRC 600), the fluorescence images of FITC (Fig. 3A) and neutral red (Fig. 3B) were simultaneously collected using K1 and K2 block filters. Superposition of the two images (Fig. 3C) collected from both channels shows that many organelles emit both FITC and neutral red fluorescence which indicates lysosomotropism of the nanoparticles.

### Flow Cytometry

In this study flow cytometry was used for the rapid measurement of cellular uptake of fluorescent particles. As shown in Fig. 4, the cellular level of nanoparticles progressively accumulated with incubation time at 37°C and did not reach saturation until 12 hours of incubation. When the cells were subsequently incubated in nanoparticle-free medium, following 4 hours of incubation with nanoparticle-containing medium, the cellular level of FITC remained high. After a 12 hour post-incubation period about 60% of the particles still resided in the cells. These polymers/nanoparticles were regurgitated from the cells and the kinetics of this regurgitation was reflected by the retention curve in Fig. 4. Alternatively, when cells were incubated at 4°C, cellular uptake was decreased to about 10% as compared with that in 37°C incubation, which probably represents cell-surface bound nanoparticles without being internalized. The cell-surface binding of nanoparticles at 4°C was confirmed using confocal microscopy (data not shown). The 90% reduction of cellular level by low temperature, a general metabolic inhibitor, indicates that the cellular uptake of nanoparticles is an energy-dependent process. It is concluded from the cellular trafficking study, progressive cellular uptake profile, and uptake inhibition at low temperature that the pinocytosis is a major uptake mechanism for the nanoparticles.

## DISCUSSION

The fluorescein labeled particles are frequently used to provide both visual and quantitative evaluation of cellular uptake and to avoid the problems associated with the handling of radioactive materials. For the cellular uptake study of nanoparticles, fluorescent markers have been either loaded into the particles during the particle preparation process or adsorbed onto the surface after particle formation. However, the loading of fluorescent markers into nanoparticles leads to leakage of

free fluorescent molecules (7), and alternatively, a simple adsorption or noncovalent binding of fluorescent molecules onto the surface of preformed particles (8) can cause relatively easy dissociation (1,14). This can mislead the accurate evaluation of cellular uptake of nanoparticles. On the other hand, covalent linking of fluorescent probes to the polymer forms a stable compound both in extracellular and intracellular (intralysosomal) pH and, therefore, a thorough determination of nanoparticle cellular uptake and trafficking is possible. In this study, the conjugation of FITC molecules to the biodegradable PEO-PLGA polymer via a PLL spacer formed a stable thiourea bond between the isothiocyanate group of FITC and ε-amine group of PLL. This served as a stable molecular probe for the in vitro, cellular uptake, kinetic study.

Studies with polymeric nanoparticles in non-phagocytic cells often show very poor internalization via pinocytosis (15). Therefore, the use of proper molecular probes that can provide sufficient sensitivity for the quantitative analysis of cellular uptake is essential. In this study, the fluorescence output per particle is greatly enhanced by increasing the number of marker molecules that are linked to the polymer using PLL as a spacer molecule.

Consequently, the use of PEO-PLGA-PLL-FITC polymeric nanoparticles enabled quantitative cellular uptake and trafficking studies.

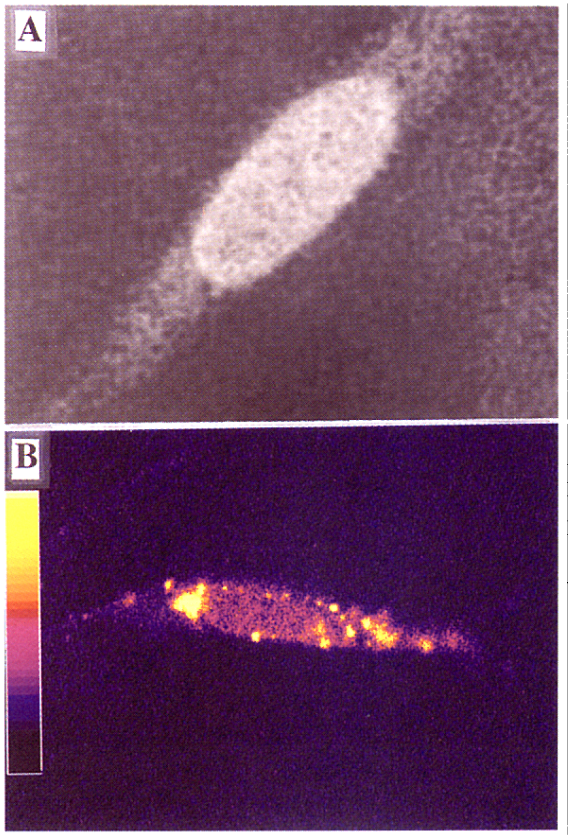


**Fig. 2.** Confocal fluorescence images of bovine smooth muscle cells (A; negative control cell, B; a cell after incubation with FITC-conjugated polymeric nanoparticles at 37°C for 4 hours). The relative fluorescence intensity is indicated by color grid: yellow; high fluorescence, black; low fluorescence.

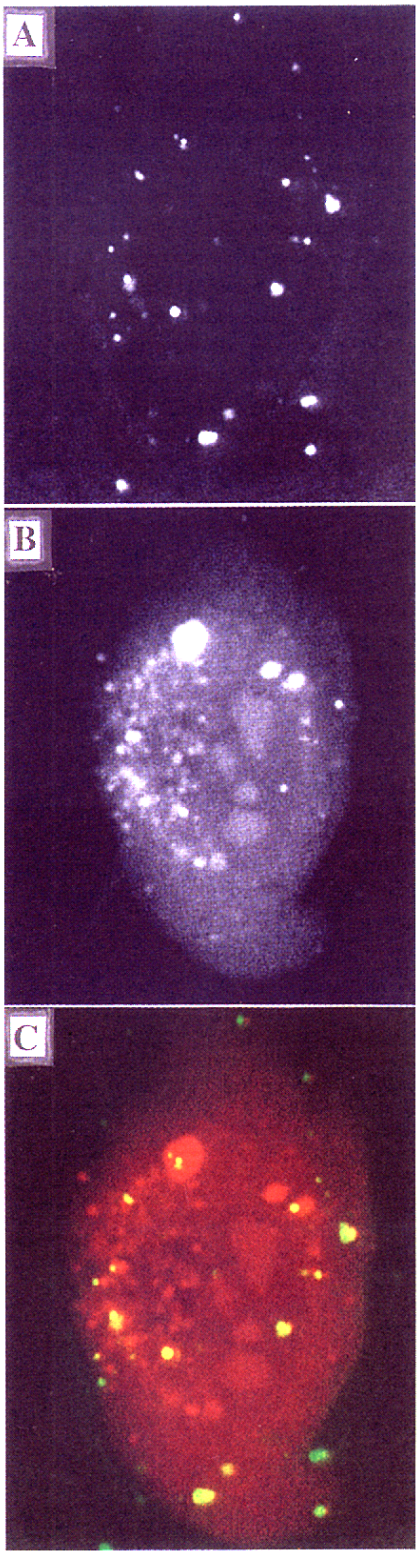


**Fig. 3.** Confocal fluorescence images of smooth muscle cells incubated with FITC-conjugated nanoparticles (320 μg/ml) for 4 hrs at 37°C and subsequently with neutral red dye (20 μM) for 30 min. The left image (A) was collected from FITC channel, and the center image (B) was collected from neutral red channel simultaneously. The superposition of two images (C) shows that many organelles emit both FITC and neutral red fluorescence, indicating lysosomotropism of nanoparticles.

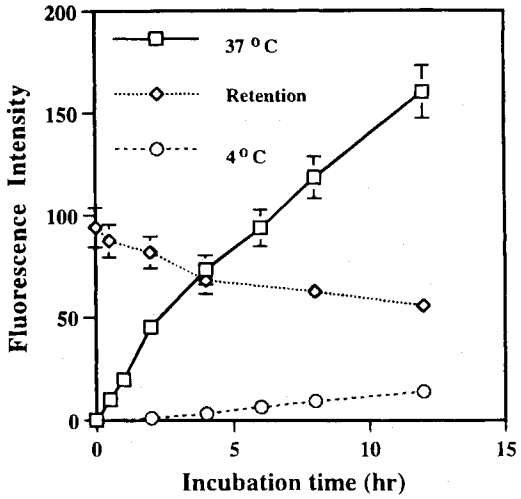


**Fig. 4.** The uptake and retention profiles of nanoparticles in bovine smooth muscle cells. The cells were incubated with FITC-conjugated nanoparticles for the indicated times at 37°C or 4°C. Retention profile was obtained by measuring the cell-associated fluorescence after replacing the medium with nanoparticle-free medium followed by incubation for the indicated time periods. Each data point represents the mean (± SD) of three separate determinations.

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