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Cellular-uptake Behavior of Polymer Nanoparticles into Consideration of Biosafety

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Abstract: Nanoparticles have tremendous potential in cancer prevention, detection and augmenting existing treatments. They can target tumors, carry imaging capability to document the presence of tumors, sense pathophysiological defects in tumor cells, deliver therapeutic genes or drugs based on the tumor characteristics, respond to external triggers to release an appropriate agent, document the tumor response, and identify the residual tumor cells. Nanoparticles < 30 nanometers in diameter show unexpected and unique properties. Furthermore, particles < 5 nanometers in size can easily penetrate cells as well as living tissues and organs. This study evaluated the safety of nano materials in a living body and the relationship between the living tissue and synthetic nano materials by examining the *in-vitro* cytotoxicity of poly(lactic-*co*-glycolic) acid (PLGA) nano-spheres and fluorescein isothiocynate(FITC)-labeled dendrimers as polymer nanoparticles. PLGA was chosen because it has been used extensively for biodegradable nanoparticles on account of its outstanding bio-compatibility and its acceptance as an FDA approved material. The dendrimer was chosen because it can carry a molecule that recognizes cancer cells, a therapeutic agent that can kill those cells, and a molecule that recognizes the signals of cell death. Cytotoxicity in L929 mouse fibroblasts was monitored using MTT assay. Microscopic observations were also carried out to observe cell growth. All assays yielded meaningful results and the PLGA nanoparticles showed less cytotoxicity than the dendrimer. These nanoparticles ranged in size from 10 to 100 nm according to microscopy and spectroscopic methods.

Keywords: PLGA, dendrimer, cytotoxicity, nano particle safety.

Introduction

The era of molecular medicine is providing new insights into the diagnosis, treatment and prevention of cancer. Exciting advances of technology over the last decade have heralded a new discipline called nanotechnology, which will also have a major impact on clinical cancer diagnosis and treatment.[1]

In vitro results have shown that magnetically guided delivery customized nanocarriers to tumor cells can allow more precise targeting, while boosting the cellular uptake of photodynamic therapy drugs contained inside them. Magnetically guided drug delivery can allow the use of a lower drug concentration to deliver a therapeutic dose, which can significantly reduce the amount of drugs for photodynamic therapy that accumulate in normal tissue. Nanoparticles are generally internalized into cells through fluid phase endocytosis,[2,3] receptor-mediated endocytosis[4] or phagocytosis.[5]

However, nanotechnology applications have not been marketed long enough to allay the claims regarding risks to human health or the environment. Small nanoparticles can enter the human body through pores and accumulate in cells. The health effects of such nanoparticles in humans is

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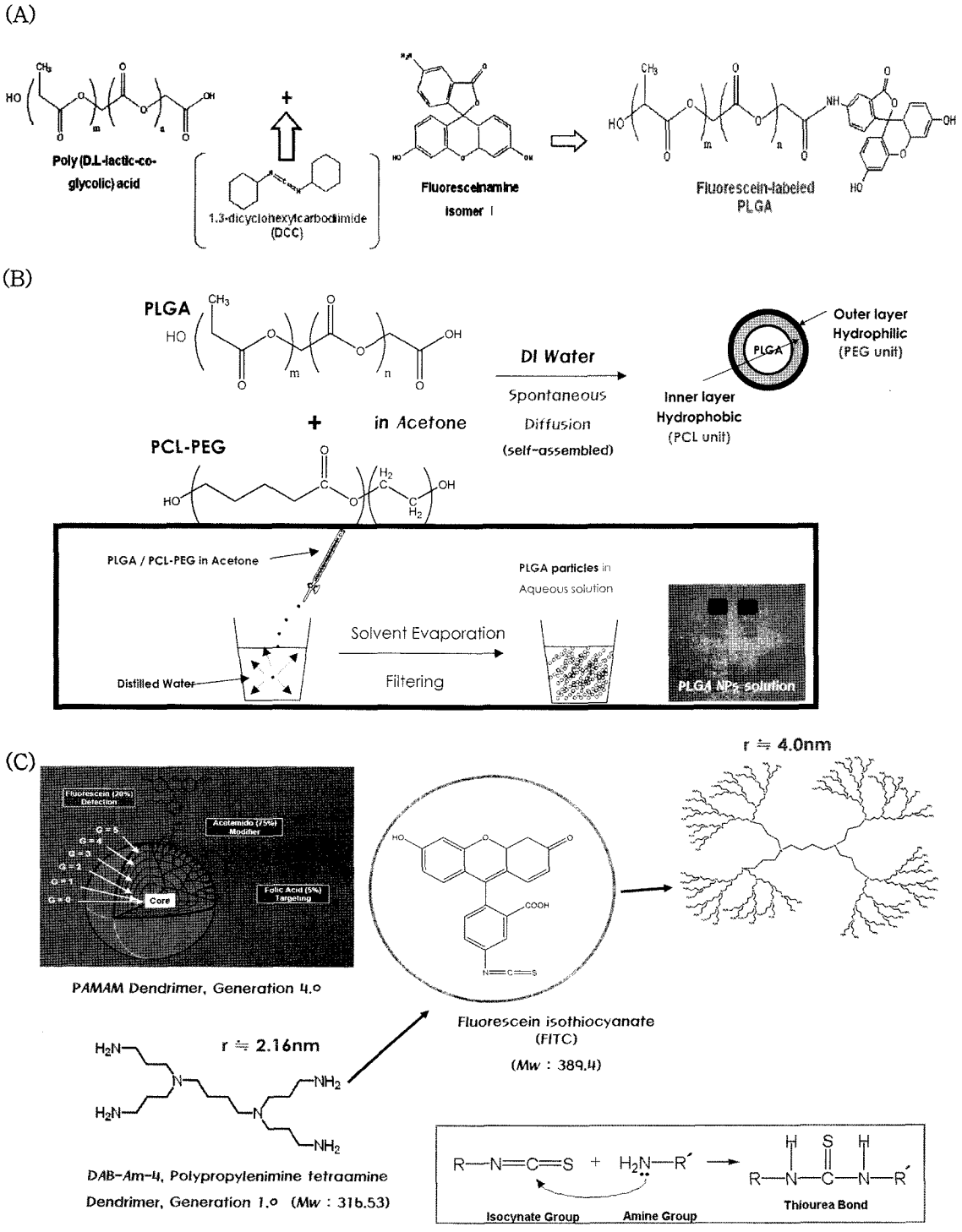


Figure 1. Preparation of the nanoparticles. (A) Reaction scheme for the fluorescein amine labeled PLGA, (B) Preparation method of the PLGA nanoparticles by solvent evaporation, and (C) Reaction scheme for the FITC-labeled dendrimer.

still unknown.[6-8]

Therefore, in order to apply nanotechnology for a clinical trial, it is essential to evaluate the safety of nano materials and determine the relationship between living tissue and artificial nano materials.[9]

This study examined the *in-vitro* cytotoxicity of poly(lactic-*co*-glycolic) acid (PLGA) nano-spheres and dendrimers as polymeric nanoparticles in order to predict the *in vivo* safety of nanoparticles after being injected into a living body. PLGA, as a large sized particle (about 100 nm), was

chosen because it has been used extensively as a biodegradable material and has good biocompatibility. PLGA nanoparticles were prepared using the solvent evaporation method.[10-16] The dendrimer, as a small sized particle (about 10 nm), was chosen because of own structure and diameter at the nano level in addition to it being a molecular carrier and a therapeutic agent for cancer treatment, as well as a sensors for the signal transduction of cells.[17-21]

Experimental

Materials and Reagents. DAB-Am-4 (polypropylenimine tetraamine dendrimer, Generation 1.0), DAB-Am-64 (polypropylenimine hexadecaamine dendrimer, Generation 5.0), PAMAM dendrimer (Generation 4.0), fluorescein isothiocyanate (FITC), fluorescein amine (Isomer I), 1, 3-dicyclohexyl-carbodiimide (DCC), hexamethylenediamine, thiazolyl blue tetrazolium bromide (MTT), PKH26 red fluorescent cell linker mini kit and dimethylsulfoxide (DMSO) were purchased from Aldrich Chemical Co. (Milwaukee, MO, USA). Poly(D,L-lactic-*co*-glycolic acid) (PLGA) (LA: GA= 70:30, M_w=170,000) was kindly supplied by Metabiomed Co., Ltd. (Okchon, Korea). Poly ε-caprolactone-polyethylene glycol diblock copolymers (PCL-PEG=1.8:1 and 1:1) were obtained from Amore Pacific Research Center (Yongin, Korea). Acetone, dichloromethane, ethanol and methanol were purchased from SamChen Chemicals Co. (Seoul, Korea) and used without further purification.

Conjugation of PLGA with Fluorescine Amine/Preparation of PLGA Nanoparticles. As shown in Figure 1(A), the carboxylic acid end group of PLGA was activated using 1,3-dicyclohexylcarbodiimide. Fluorescein amine-labeled PLGA was also prepared by conjugating fluorescein amine (Isomer I) to the DCC-activated carboxylic terminal group in PLGA.

0.1 g of fluorescein amine-labeled PLGA and 0.1 g of the two types of PCL-PEG diblock coplolymers were dissolved in 25 mL of acetone. 2 mL of the above mixture solution was added slowly and dispersed into 20 mL of distilled water without stirring until a blue pale solution was obtained. A rotary evaporator was used to remove the organic solvent from the manufactured emulsion. Finally, PLGA nanoparticles were obtained by filtering the concentrated solution through a nano filter (0.45 μm in average diameter).

The particle size distribution of the prepared PLGA nanoparticles was determined by dynamic laser light scattering (DLS) and a particle analyzer (Mastersizer, Malyern, UK) with Image J 1.33u software (Wayne Rasband National Institutes of Health, USA). The shape of the nanoparticles produced were examined by energy-filtering transmission electron microscopy (FETEM; JSM 7000F, JEOL, Japan).

Conjugation of Dendrimer with Fluorescein Isothiocyanate (FITC). As shown in Figure 1(B) and 1(C), a solution of FITC in methanol was slowly added to the dendrimer solution in phosphate-buffered saline (PBS) (dendrimer:FITC in molar ratio, 1:1.2) at room temperature and incubated for 24 h in the dark with constant stirring. The unreacted FITC was separated using a dialysis tube (molecular weight cutoff 1,350, Medicell International Ltd., London, UK) until no free FITC could be detected by thin-layer chromatography (mobile phase; chloroform, methanol and ammonia (5:4:1)) in dialyzed solution.[23] The introduction of FITC to the dendrimer was verified by spectrofluorimetry (Fluroskan, Ascent FL, Model 374, Finland, excitation wavelength 475 nm, emission wavelength 583 nm). Solution stability tests of FITC-PAMAM dendrimers at 37 °C in PBS (pH 7.4) showed no additional dissociation of FITC from the conjugated dendrimer over a period of 5 days, which confirmed the stable covalent bond between FITC and the dendrimer.

***In vitro* Test.**

Cytotoxicity Test: L929 mouse fibroblasts cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) containing 10% (v/v) fetal bovine serum, 1% antibiotics (penicillin-streptomycin) at 36.5 °C and 5% CO_2 to assess the dose- and time-dependent cytotoxicity of the dendrimers and PLGA nanoparticles. The culture medium was changed on alternate days. The number of cells was determined using trypan blue exclusion analyses.

Two other parts of the cultures were used for the cytotoxicity test. In one part, 1.67×10^5 cells were seeded into individual wells of 6-well tissue culture plates. After cells seeding, the cells were incubated with predetermined amounts of the dendrimers and PLGA nanoparticles for 48 h or 96 h.

In another part, the cells were seeded into 6-well plates and cultured without the nanoparticles. Predetermined amounts of the dendrimers and PLGAs were added to the proliferating L929 cells and incubated for 4 h. Subsequently, the changes in morphology and detachment behaviors of the L929 cells from the dish were observed by optical microscopy (DIPHOTO-MD, Nikon, Tokyo, Japan).

***Fluorescene Analysis* - Cell Membrane Staining - PKH26 Labeling and Preparation of Sample:** The cell agglomerates treated with trypsin-EDTA were washed several times with a serum free medium, and aliquots were removed after centrifugation at 400 g for 5 min. The precipitated cells were suspended again with 1 mL of a Diluent C solution. The suspended cell solution (1 mL) was added to a PKH26 dye/Diluent C solution (4 μL in 996 μL of Diluents C) and incubated for 5 min at room temperature. 2 mL of FBS was then added to the cell suspension and incubated for an additional 1 min. 4 mL of DMEM 1640 was added to the above cell mixture suspension and centrifuged at 400 g for 10 min. After centrifugation, the stained cell was separated from the staining solution.

L929 mouse fibroblasts cells were cultured in 6-well cell culture plates with DMEM containing 10% (v/v) FBS, 1%

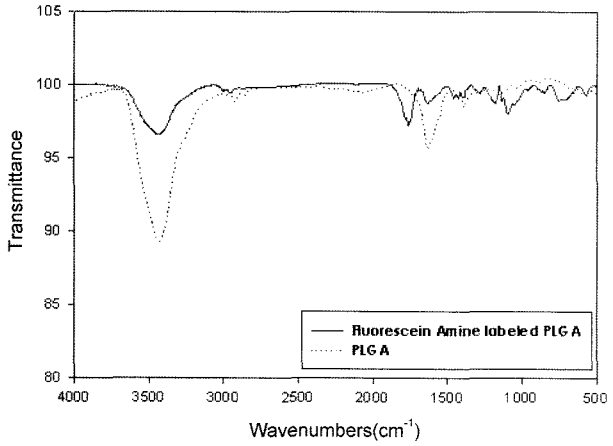


Figure 2. FT-IR spectra of the PLGA and fluorescein amine labeled PLGA.

antibiotics (penicillin) at 36.5 °C in an atmosphere containing 5% CO_2. A predetermined amount of fluorescent labeled dendrimers or PLGA nanoparticles were added to the above 6-well culture plate and cultured for 48 h. The cells were then washed with PBS, and 3 mL/well of 10% formalin solution was then added to the wells. After 4 h, the formalin solution was removed and the cells were washed with distilled water. The fixed cells were dehydrated in a graded series of ethanol (50%, 70%, and 100% for 20 min each). The ethanol solution was removed and the cells were dried 24 h. The intracellular translocation of the dendrimers and PLGAs was examined by confocal laser scanning microscopy (LSM 510, Carl Zeiss, Germany).

Cell Viability Test: The cell viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT- formazan) method in all experiments and repeated 4 times. The cultured cells were resuspended in the culture medium and seeded (500 μL/well) in a 96 well at 2×10^4 cells/well. The plate was incubated for 48 h, at 36.5 °C in a humidified atmosphere containing 5% CO_2. After proliferation, the MTT solution (25 μL) was added to each well. After additional 4 h incubation, the supernatant was removed, and 100 μL of DMSO and 12.5 μL of a glycine buffer (pH 10.5) were added to all the wells and mixed thoroughly to confirm the solvation of dark blue crystals. The suspended cell solution (100 μL) was then transferred to an ELISA microplate, and the optical density (OD) was measured at 540 nm using the spectroscopic method reported elsewhere.[24]

The relative cell viability was calculated by comparing the optical absorbance of the MTT suspended cell solution with the untreated cell solution used as the control.

Results and Discussion

Preparation of Fluorescine Amine-labeled PLGA Nanoparticle. Figure 2 shows the FT-IR (Nicolet 6700, Thermo

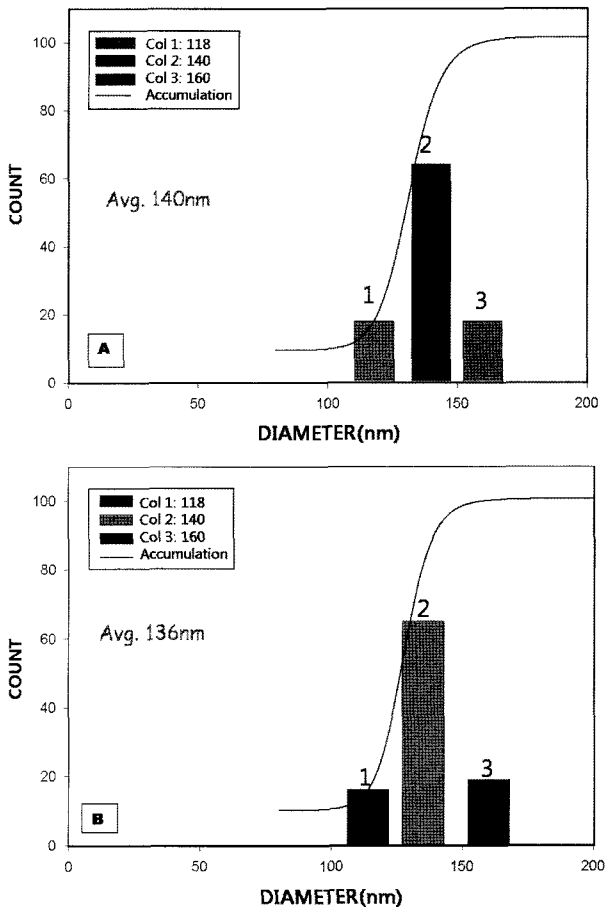
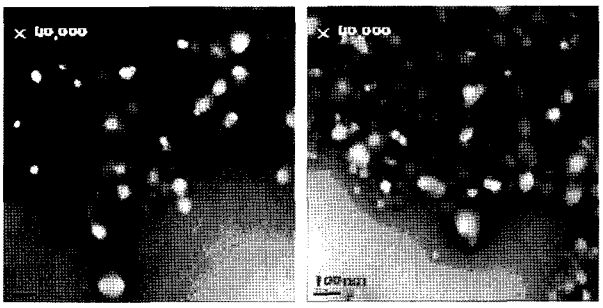


Figure 3. Size distribution of PLGA emulsion : (a) PCL-PEG = 1.8 : 1(mean diameter; 140 nm) and (b) PCL-PEG = 1 : 1(mean diameter; 136 nm).



(a) (b)

Figure 4. FE-SEM images of PLGA nanoparticles : (a) PCL-PEG = 1.8 : 1 and (b) PCL-PEG = 1 : 1.

Fischer Scientific Inc., USA) spectrum of PLGA and fluoresceine amine labeled PLGA. The introduction of fluorescein amine to PLGA was confirmed by the appearance of IR absorbance peaks at 1730 and 1640 cm^{-1} corresponding to the elastic vibrational molecular motion of C=O and C=C bonds in the secondary amine group.

The PLGA nanoparticles were prepared using a solvent

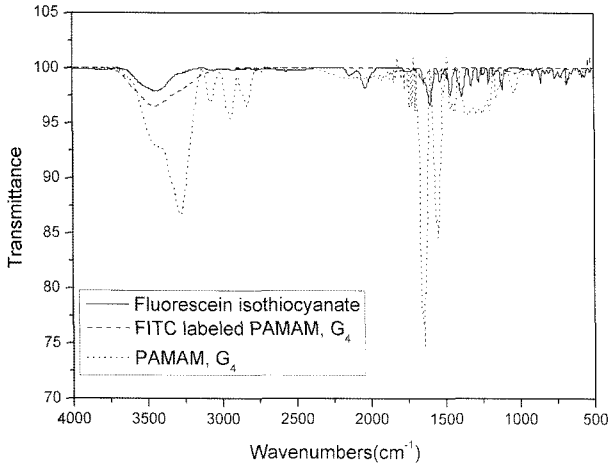


Figure 5. FT-IR spectra of PAMAM dendrimer, FITC and FITC labeled PAMAM dendrimer.

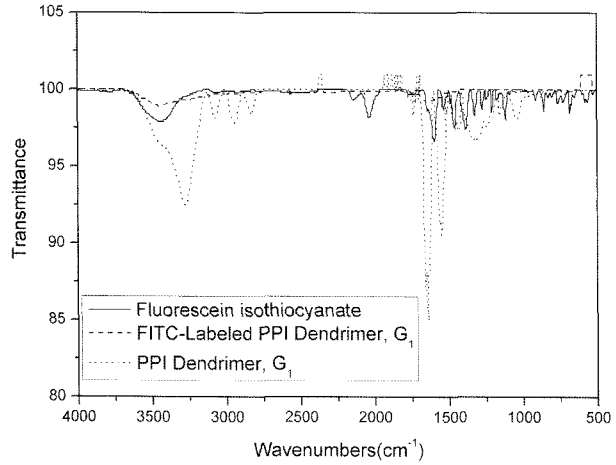


Figure 6. FT-IR spectra of the PPI dendrimer, FITC and FITC labeled PPI dendrimer.

evaporation method. The particle size distribution resulting from different conditions was observed by dynamic laser light scattering and a particle analyser.

The self-assembled nanoparticles were prepared using solvent evaporation method with PLGA and PCL-a PEG diblock copolymer. The nanoparticles were diffused spontaneously in the liquid state using water soluble acetone, which works as a co-solvent of the two materials. As shown in Figure 3, suitable PLGA nano particles could be obtained under these conditions and their shape was confirmed by FE-SEM (Figure 4).

Preparation of FITC-labeled Dendrimer. Figure 5 shows a FT-IR spectrum of the FITC-labeled PAMAM dendrimer. The weak absorbance band at 3250~3400 cm^{-1} of NH_2 and the disappearance of the absorbance band at 2000-2280 cm^{-1} of N=C=S indicates the formation of a thiourea bond that could be confirmed by the absorption peaks at 1150-1450 cm^{-1}. The band at 1150-1450 cm^{-1} was assigned to the reaction between the primary amine of the dendrimer surface and the isothiocyanate group of FITC. The theoretical particle size of PAMAM (G_4) was 4 nm.[17,18] This PAMAM core-shell architecture shows a linear increase in diameter with increasing generation.[19]

Figure 6 shows a FT-IR spectrum of another FITC-labeled PPI dendrimer as DAB-Am-4 and DAB-Am-64. The disappearance of the absorbance band at 2000-2280 cm^{-1} of N=C=S, the appearance of a weak absorbance band at 3250-3400 cm^{-1} of NH_2, the absorbance band at the thiocarbonyl group connected to a nitrogen atom (C=S) (1090-1563 cm^{-1}) and a thiourea bond (1150-1450 cm^{-1}) indicate the covalent conjugation of FITC to the dendrimer.

Theoretical particle sizes of DAB-Am-4 (G_1) and DAB-Am-64 (G_5) was 1.5 and 5.3 nm, respectively.[22]

***In vitro* Test.**

Cytotoxicity Test: There was no change in cell morphology observed after the addition of a low concentration (up to 2.8×10^{-5} M and 18 μg/mL, dendrimers and PLGA respec-

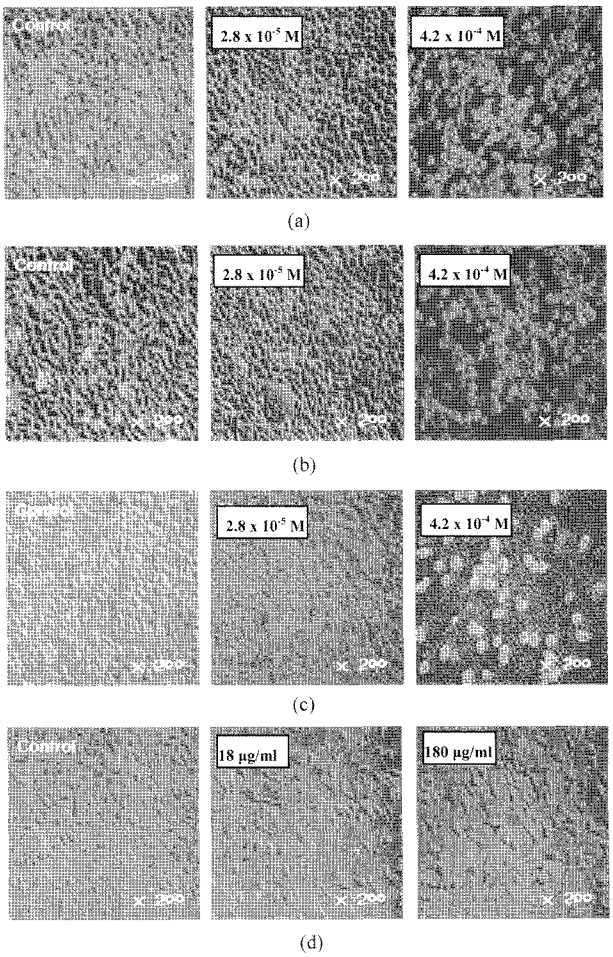


Figure 7. Optical microscopy observations for the cytotoxicity evaluation in L929 mouse fibroblasts after 2 days culture. (a) PPI dendrimer (G_1), (b) PPI dendrimer(G_4), (c) PAMAM dendrimer (G_5), and (d) PLGA nanoparticles with different concentration.

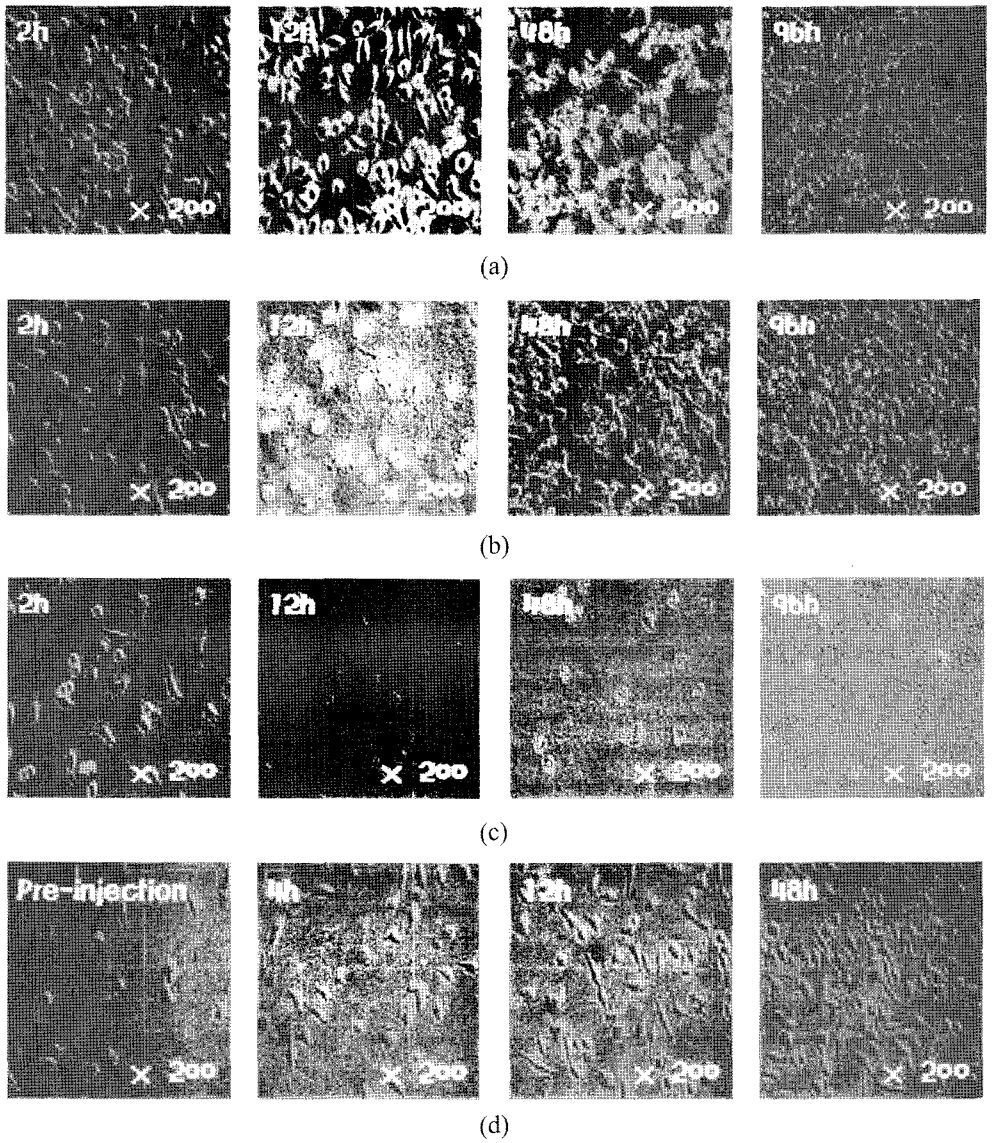


Figure 8. Optical microscopy observations for the cytotoxicity evaluation in L929 mouse fibroblasts with the same concentration of nanoparticles. (a) PPI dendrimer (G_1), (b) PPI dendrimer (G_4), (c) PAMAM dendrimer (G_5), and (d) PLGA nanoparticles after different culture time.

tively) of the nanoparticles solution and control solution within 4 days culturing at same concentration (Figure 7). However, the addition of a high concentration (above 2.8×10^{-5} M and 18 μg/mL, dendrimers and PLGA respectively) caused an increase in the incidence of cell apoptosis compared with the control.

In the case of PLGA nanoparticles, despite their large diameter (about 140 nm), no or little damage could be detected (up to 180 μg/mL) compared with the control cells. This means that the size of the nanoparticles has a lesser effect on cytotoxicity than their concentration.

The cytotoxicity dependence on the type of polymer nanoparticles, and substantial changes in cell morphology were detected by optical microscopy after culturing with the different particles (at the higher concentration of 2.8×10^{-4} M and 180 μg/mL for dendrimers and PLGA respectively) for 2 (only the case of PLGA) or 4 days. Such dependencies at relatively high concentrations of nanoparticles were more prominent after the longer culturing time. Although the L929 mouse fibroblasts show a large, spindle-shaped morphology and a good adherent cell growth behavior under general culture conditions, the fibroblasts showed altered morphology after 48 h culture with the dendrimer but not with the PLGA nanoparticle (Figure 8). This means that the biosafety of small nanoparticles (approximately 10 nm in diameter) was not confirmed at concentrations > 10^{-4} M *in vitro*. However, in the case of large nanoparticles (approximately 140 nm in diameter) a confluent monolayer of fibro-

blasts was observed and the biosafety was confirmed after 48 h culture despite the high concentration (over 180 μg/mL).

The following cellular behaviors were often observed using a high concentration (>2.8×10^{-4}) of dendrimer and 180 μg/mL of a PLGA nanoparticle solution: debris, morphological changes (due to cell lysis), a loss of spindle shape and detachment from the culture plate. This means that a large concentration of nano particles affects the cell functions in an *in vitro* environment, and there is a critical concentration for biosafety despite the different diameter and characteristics of the nanoparticles. Therefore, care must be taken when applying various nanoparticles to a living body under the biosafety considerations.

Fluorescene Analysis: The permeation of nanoparticles through the cell membrane and the cell uptake behavior was examined by confocal laser scanning microscopy. The nanoparticles were found to be associated with the cell membranes and many particles were internalized into the cell through the cell membrane (Figure 9).

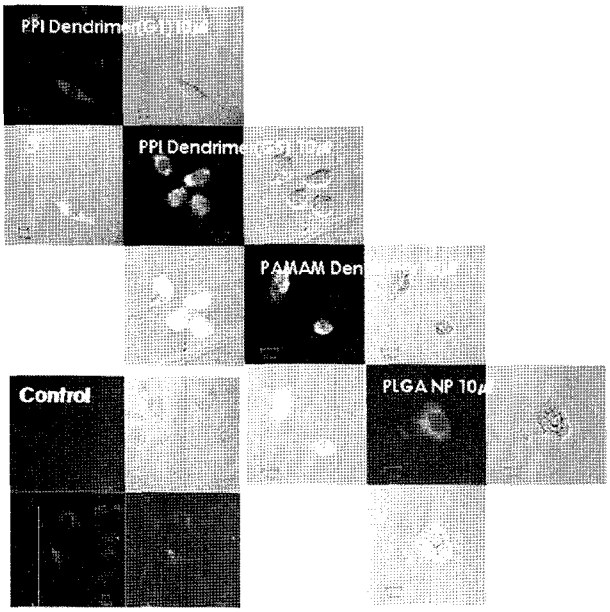


Figure 9. Intracellular translocation of nanoparticles by the living cell *in vitro*.

In an *in vivo* environment, the possible mechanisms for particles to pass through the gastrointestinal (and other physiological) barriers could be (1) **paracellular passage**-particles "kneading" between the intestinal epithelial cells due to their extremely small size (<50 nm); (2) **endocytotic uptake**-particles absorbed by intestinal enterocytes through endocytosis (particles size <500 nm); and (3) **lymphatic uptake**-particles adsorbed by M cells of the Peyer's patches (particle size <5 μm).[25-27]

Using large PLGA nanoparticles, cell uptake due to the endocytotic uptake mechanism could be detected and the fluorescence emission intensity was relatively weak compared with the small dendrimer nanoparticles. However, the small particle sized dendrimer influenced the cell uptake behaviors significantly due to the paracellular passage mechanism (through cell wall in this study), even though the concentration was below the critical level (Figure 10). Laser scanning confocal microscopy revealed internalized nanoparticles throughout the inside of the cell surrounding the nucleus. The probability of finding nanoparticles in the nucleus was examined through the unexpected DNA expression behavior by PR-PCR and DNA analysis.

From these results, it was found that the dendrimer nanoparticle showed relatively high cytotoxicity, due to easy permeation into the cytoplasm as a result of the small particle size.

Cell Viability Test: All measurements (n=4, in each group) were carried out at least in triplicate and are expressed as the mean ± standard deviation. The results were analyzed by analysis of variance (ANOVA) test. Values of $p<0.05$ were considered statistically significant.

The cellular uptake behavior of nanoparticles was investigated using the FITC-dendrimer and PLGA nanoparticles.

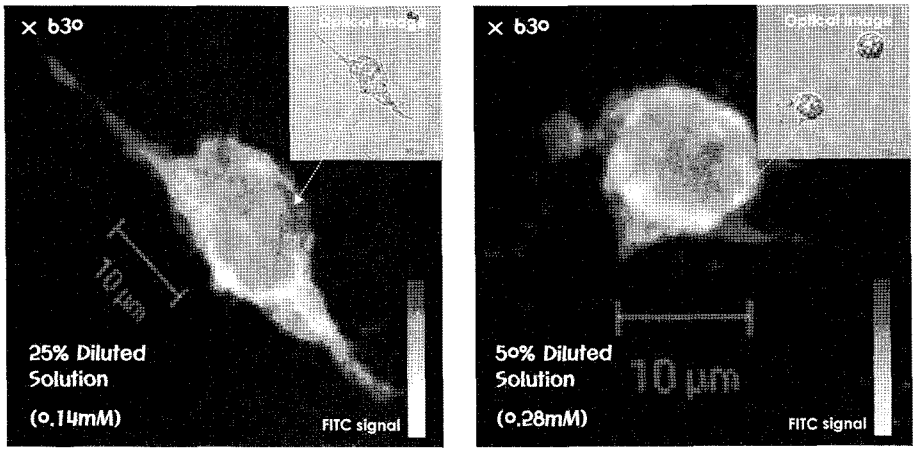


Figure 10. Fluorescence emission behavior of the FITC labeled PPI dendrimer (G_5) in the inner side of the cell.

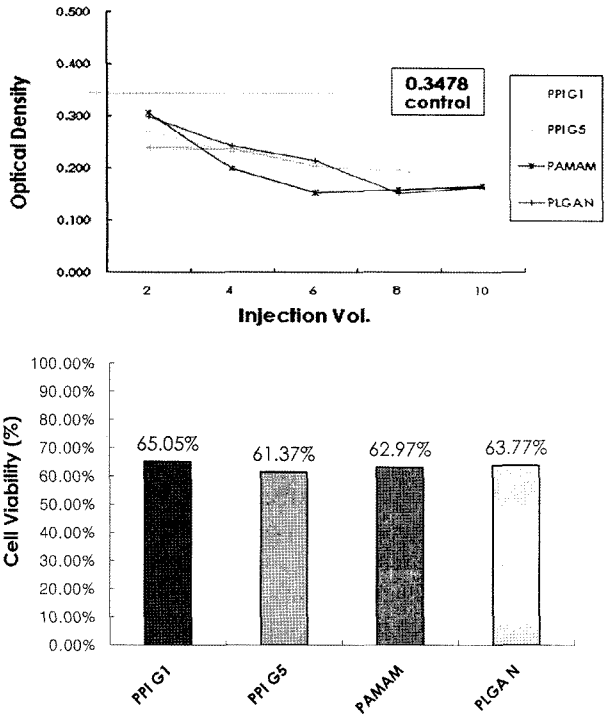


Figure 11. Optical density and calculated relative cell viability of the dendrimers and PLGA nanoparticles using the MTT Assay.

The influence of each nanoparticle on the response between the cell viability and cellular uptake was monitored and compared using their intracellular fluorescent activity, and is expressed relative to the activity of the corresponding control groups. The cell viability of the PPI dendrimers G_1 and G_5 was 65%, 61% respectively, and that of the PAMAM dendrimer G_4 and the PLGA nanoparticles was 63% and 64% respectively (Figure 11).

In the case of the dendrimer nanoparticles addition, the cell viability was similar to the case of the PLGA particles. On the other hand, the morphological changes in the cultured cells were different from those observed with the PLGA nanoparticles.

In this study, hydrophobic PLGA nanoparticles were coated with hydrophilic PCL-PEG moieties. Therefore, the synthesized PLGA nano particles had a significantly higher cellular uptake than hydrophobic nanoparticles, such as polystyrene and unmodified PLGA. These results were similar to the previous results in that the vitamin E TPGS(Vitamin E succinated polyethylene glycol 1000; water soluble vitamin E) coated PLGA and the PVA-coated PLGA nanoparticles have advantages in favor of cellular uptake.[28]

Conclusions

Polymer nanoparticles were prepared using a phase inversion emulsion method. The *in vitro* biocompatibility and cellular uptake of the fluorescein-labeled PLGA nanoparticles and dendrimer were demonstrated. The safety of these nanoparticles safety and the relationship between the cells and nanoparticles were examined using biological assays and microscopy and fluoro spectroscopic methods. The PLGA and dendrimer nanoparticles did not show significant toxicity, and these particles exhibited moderate biocompatibility and similar cell viability despite small particles becoming easily internalized into cell through the cell wall compared with large particles.

These results show that nanoparticles of biodegradable polymers of sufficiently small size and with the appropriate surface modification for applications to oral drug delivery of anticancer agents as well as other therapeutic agents may have great potential considering biosafety. The *in-vivo* tissue distribution in rats to determine the safety of these nanoparticles is currently under investigation.

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