Characterization of nanoparticle uptake by endothelial cells

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Abstract

Endothelium is an important target for drug or gene therapy because of its important role in the biological system. In this paper, we have characterized nanoparticle uptake by endothelial cells in cell culture. Nanoparticles were formulated using poly DL-lactide-co-glycolide polymer containing bovine serum albumin as a model protein and 6-coumarin as a fluorescent marker. It was observed that the cellular uptake of nanoparticles depends on the time of incubation and the concentration of nanoparticles in the medium. The uptake of nanoparticles was rapid with confocal microscopy demonstrating their localization mostly in the cytoplasm. The mitogenic study demonstrated biocompatability of nanoparticles with the cells. The study thus demonstrates that nanoparticles could be used for localizing therapeutic agents or gene into endothelial cells. Nanoparticles localized in the endothelium could provide prolonged drug effects because of their sustained release characterics, and also could protect the encapsulated agent from enzymatic degradation. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Biodegradable polymer; Poly DL-lactide-co-glycolide; Sustained release; Vascular disorders; Localized drug delivery; Restenosis

1. Introduction

Endothelium is an important target for drug or gene therapy because it is involved in a number of normal and pathophysiologic conditions such as angiogenesis, atherosclerosis, tumor growth, myocardial infarction, limb and cardiac ischemia, restenosis, etc. (Nabel, 1991; Dass and Su, 2000; Martin and Murray, 2000; Parikh and Edelman, 2000). Various therapeutic approaches have been investigated to modify the endothelium to counteract the disease conditions (Nabel, 1995). Vascular endothelial cells are a particularly important target for functional genes because of their large population and contiguity with the blood stream (Yao et al., 1991). Different systems such as drug-conjugates and immunoliposomes have been studied to actively target therapeutic agents to the endothelium (Scherpereel et al., 2001; Stahn et al., 2001). In this paper, we have characterized the uptake of biodegradable nanoparticles by endothelial cells in cell culture, and have proposed strategies for endothelial delivery of nanoparticles in vivo.

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2. Materials and methods

2.1. Materials

Poly (DL-lactide-co-glycolide) (PLGA, MW 143 900, copolymer ratio 50:50) was purchased from Birmingham Polymers, Inc. (Birmingham, AL). Bovine serum albumin (BSA, Fraction V), polyvinyl alcohol (PVA, average MW 30 000–70 000), heparin sodium salt (Grade I-A from porcine intestinal mucosa), Triton-X 100 were purchased from Sigma Chemical Co. (St. Louis, MO). 6-Coumarin was obtained from Polyscience, Inc. (Warrington, PA). Fetal bovine serum (FBS, non heat-inactivated), $1 \times$ trypsin–EDTA, and penicillin–streptomycin solution were purchased from Gibco Laboratories (Lenexa, KS). Endothelial cell growth supplement (ECGS) was obtained from Collaborative Biomedical Products (Bedford, MA). All organic solvents were of HPLC grade. Tissue culture plates and flasks, and all salts used in the preparation of buffer solutions were purchased from Fisher Scientific (Pittsburgh, PA).

2.2. Formulation of nanoparticles containing BSA and 6-coumarin

Nanoparticles were prepared using a water-in-oil-in-water (w/o/w) emulsion solvent evaporation technique. In a typical procedure, 90 mg PLGA was dissolved in 3 ml of chloroform containing 50 μg of 6-coumarin (stock solution 0.5 mg/ml in chloroform). A 2% solution of PVA was prepared in cold distilled water, saturated with 25 μl of chloroform, and centrifuged at 1000 rpm for 5 min and then filtered through a 0.22 μm hydrophilic polysulfonic membrane syringe filter (25 mm Nalgene® filter unit, Nalge Co, Rochester, NY) to remove any undissolved PVA. An aqueous solution of BSA (300 μl, 10% w/v) was added, in two portions, to the PLGA solution with vortexing for 1 min after each addition. It was then placed on an ice bath for 5 min and emulsified using a microtip probe sonicator set at 55 W of energy output (XL 2015 Sonicator® ultrasonic processor, Misonix, Inc, Farmingdale, NY) for 30 s, to obtain a primary water-in-oil

emulsion. The primary emulsion was then added in two portions to 12 ml of the PVA solution with intermittent vortexing to obtain the multiple w/o/w emulsion. The emulsion was placed on an ice bath for 5 min and then sonicated for 2 min. The w/o/w emulsion was stirred overnight on a magnetic stir plate to allow the evaporation of chloroform and formation of the nanoparticles. The suspension of nanoparticles was stirred in a vacuum desiccator placed on the magnetic stir plate for an additional hour to ensure complete removal of the organic solvent. The suspension was transferred into Ultra-Clear™ centrifuge tubes and centrifuged at 27 000 rpm ($\sim 110\,000 \times g$ using SW28 rotor) for 20 min at 4 °C in an ultracentrifuge (Beckman Optima™ LE-80K, Beckman Instruments, Inc, Palo Alto, CA). The pellet was resuspended in distilled water and sonicated for 30 s on an ice bath to disperse any aggregates. Ultracentrifugation was repeated two more times at 25 000 rpm ($\sim 85\,000 \times g$) for 20 min each. This washing step was meant to remove PVA and unencapsulated BSA from the formulation. The supernatant and the washings were stored to determine the amount of BSA that was not entrapped in the nanoparticles. From the total amount added and the amount not entrapped into nanoparticles, the protein loading in the nanoparticles was determined. After the last centrifugation, the nanoparticles were resuspended in 7 ml of distilled water and sonicated as above for 30 s on an ice bath. The nanoparticles were then centrifuged at 1000 rpm for 10 min at 4 °C to remove any large aggregates. The supernatant was collected and frozen at -70 °C for 45 min and subsequently lyophilized for 2 days (VirTis Co, Inc, Freeze Dryer, Gardiner, NY). The lyophilized nanoparticles were stored desiccated at 4 °C.

2.3. Particle size analysis and zeta potential

The diameter of the nanoparticles was obtained with photon correlation spectroscopy (PCS) using quasi elastic light scattering equipment (Zeta-Plus™ zeta potential analyzer, Brookhaven Instruments Corp., Holtsville, NY) and ZetaPlus™ particle sizing software (version 2.07). PCS gives

the hydrodynamic diameter, which is the particle diameter plus the thickness of the surrounding solvent double layer. To measure particle size, a dilute suspension (~ 200 µg/ml) of nanoparticles was prepared in distilled water and sonicated on an ice bath for 30 s. The sample was filled in a cuvette and subjected to particle size analysis $3 \times$ in the ZetaPlus™ for 5 min each. Following particle size analysis of the nanoparticles, the electrode was fitted inside the cuvette and five measurements of zeta (ζ) potential were recorded in the ZetaPlus™.

2.4. Cell culture

The human umbilical vein endothelial cell (HUVEC) line and Ham's F-12K medium were obtained from American Type Culture Collection (ATCC, Manassas, VA). HUVECs obtained at passage 13 were cultured in T-75 tissue culture flasks in Ham's F-12 K medium (Kaighn's modification of Ham's F-12 containing 2 mM L-glutamine and 1500 mg $NaHCO_3$ per l) supplemented with 10% FBS, heparin (100 µg/ml), ECGS (40 µg/ml) and 1% penicillin–streptomycin solution. The cells were passaged in a split ratio of 1:2 or 1:3. All experiments were performed with cells between passages 16 and 20.

2.5. Seeding of HUVE cells for nanoparticle uptake study

Growth medium was aspirated from the T-75 flask and the cell monolayer was washed with sterile phosphate buffered saline (PBS; 154 mM, pH 7.4). After aspirating the PBS, 1.5 ml of trypsin–EDTA was added to the flask. The flask was placed in the incubator (Napco® water-jacketed CO_2 incubator, model series 6000, Precision Scientific, Winchester, VA) at 37 °C for 10 min to allow cell detachment. Fresh growth medium (20 ml) was added to the flask. The cells were flushed with a 10-ml pipette several times to ensure that all the cells were in suspension and the cell suspension was transferred to a 50-ml Eppendorf tube. The cells were centrifuged at 1500 rpm (4 °C) for 5 min in order to pellet the cells. The supernatant was discarded and the cells were re-suspended in 10 ml of growth medium. A cell count was performed with 50 µl of the cell suspension using a hemacytometer (0.1 mm deep, Bright-Line® improved Neubauer, Hausser Scientific, Horsham, PA). To study the cellular uptake of nanoparticles, HUVECs were seeded in a 24-well plate at a density of 30 000 cells/well in complete growth medium and allowed to grow for 2 days, with medium changed once.

2.6. Quantitative analysis of nanoparticle uptake

To determine the effect of concentration of nanoparticles on uptake, a stock suspension of nanoparticles (2 mg/ml) in complete growth medium was prepared and sonicated for 10 min over a water bath sonicator (Model 8852, Cole-Palmer Instrument Co, Chicago, IL). Different dilutions of nanoparticles from the stock in the growth medium in the concentration range of 10–300 µg/ml were prepared. The medium in the wells was replaced with the suspension of nanoparticles and incubated for 1 h. In a separate experiment, to study the effect of incubation time on nanoparticle uptake, the medium was replaced with 1 ml per well of a 50 µg/ml suspension of nanoparticles in complete growth medium and the plate was incubated for 30 min, 1 and 2 h. At the end of the incubation period, the nanoparticle suspension was removed from the wells and the cell monolayers were rinsed three times with cold PBS. The cells were solubilized in 1% triton-X 100 and 20 µl of the cell lysate from each well was used to determine the total cell protein content using the Pierce BCA protein assay (Rockford, IL). A standard curve was obtained with BSA solution. The remainder of the cell lysates were lyophilized and used for the analysis of 6-coumarin content as described herein.

2.7. Extraction and HPLC analysis of 6-coumarin

The lyophilized cell lysates were reconstituted in 500 µl of methanol and incubated at 37 °C for 18 h with shaking to extract the fluorescent dye from the nanoparticles. The extraction time and the conditions were optimized. The samples were

centrifuged at 14 000 rpm for 10 min at 4 °C and 400 μl of the supernatants were collected for HPLC analysis. A standard curve for nanoparticles was constructed by suspending different weight concentrations of nanoparticles (8–40 μg/ml) in 1% Triton-X 100 followed by lyophilization and extraction of 6-coumarin in methanol. The standard was run at the same time and treated in a similar way as samples from the cell culture experiments. The 6-coumarin levels in the extracts were analyzed by ion-pair chromatography. A 20 μl of each sample was injected in the system. The HPLC system (Shimadzu Scientific Instrument, Inc, Columbia, MD) consisted of a system controller (Model SCL-10A), pump (Model LC-10AT), auto-injector (Model SIL-10A) and a fluorescence detector (Model RF-10AXL; $Ex(\lambda)$ 450 nm/ $Em(\lambda)$ 490 nm). A Nova-Pak® C-8 column (2×150 mm^2) with 4 μm packing (Waters, Milford, MA) was used. The separations were achieved using acetonitrile:water:1-heptane sulfonic acid sodium salt (65:35:0.005 M) as the mobile phase. The uptake of nanoparticles by HUVECs was calculated from the standard curve and expressed as the amount of nanoparticles (μg) taken up per mg cell protein. The efficiency of nanoparticle uptake was calculated from the total amount of nanoparticles added in the well and the amount detected in the cells.

2.8. Nanoparticle uptake by HUVE cells: confocal microscopy

HUVECs were seeded in Bioptech® tissue culture dishes (Bioptechs, Inc, Butler, PA) in 1 ml of complete growth medium at a density of 30 000 cells/ml. The plates were incubated overnight to allow cell attachment. The cells were then incubated with a suspension of 6-coumarin nanoparticles (300 μg/ml) in growth medium for 30 min, 1 and 2 h. To determine that the intracellular fluorescence is not due to uptake of the dye that might have been released from the nanoparticles, a sample of nanoparticles was suspended in growth medium (600 μg/ml, 10 ml) and dialyzed against plain growth medium (10 ml) for 2 h using a dialysis bag (Molecular weight cut-off of 3500; Fisher Scientific). The control uptake experiment was performed by incubating HUVECs with this solution of 6-coumarin that was released from nanoparticles in growth medium in vitro. Cells incubated with plain growth medium served as zero-hour controls. At the end of the incubation periods, the cell monolayers were rinsed three times with HEPES buffer to remove excess nanoparticles and/or free dye. Fresh HEPES buffer was added to the plates and the cells were viewed and imaged under a confocal laser scanning microscope (Carl Zeiss LSM 410, Goettingen, Germany) using FITC filter ($Ex(\lambda)$ 495 nm, $Em(\lambda)$ 520 nm). The images were processed using Carl Zeiss LSM software (version 3.99).

2.9. Seeding of cells for mitogenic assay and biocompatibility of nanoparticles

Biocompatibility of nanoparticles with endothelial cells was tested using a mitogenic assay. Cells were seeded into a 96 well tissue culture plate at a cell density of 5000 cells per well. The plate was incubated at 37 °C in humidified 5% CO_2 overnight to allow the cells to attach to the bottom of the wells. The medium from each well was replaced with 100 μl of fresh medium and to each wells 50 μl of nanoparticle suspension of different concentrations prepared in complete medium was added so that the nanoparticle concentration ranged from 0 to 500 μg/ml. The plate was incubated as above, and the assay was carried out at the end of 48 h, using the Cell Titer 96® AQ$_{ueous}$ Cell Proliferation Assay Kit (Promega, Madison, WI). This is a colorimetric method that determines the number of metabolically active cells. In brief, a tetrazolium compound (Owen's reagent), in the presence of an electron-coupling agent, is bioreduced by dehydrogenase enzymes in metabolically active cells, to a soluble formazan, the absorbance of which can be measured directly from the plate at 490 nm. The reagents were mixed and added to the wells (20 μl per well) and the plate was incubated for 1 h as earlier. The reaction was stopped at the end of the incubation period with a 10% solution of SDS (25 μl per well). The samples from each well were transferred to microcentrifuge tubes, spun at 15 000 rpm at 4 °C for 5 min to settle nanoparticles, the

supernatants (125 μl) were transferred to a separate 96 well plate, and the absorbance (OD) of the formazan was measured at 490 nm in a MicroKinetics® plate reader (Model BT 2000, Bio-Tek Instruments, Fisher Biotech). The experiment was performed in quadruplicate wells for each nanoparticle concentration.

3. Results and discussion

The nanoparticle size ranged from 277 to 372 nm with the mean hydrodynamic diameter of 321 nm and polydispersity of 0.132, suggesting uniformity of the particle size distribution (Fig. 1). The ζ potential of nanoparticles was -10.37 mV. The BSA loading in the particles was 21% w/w (70% entrapment efficiency) and the dye loading was 0.53 μg/mg nanoparticles, suggesting that almost all the dye added to the formulation got entrapped into nanoparticles. In the previous studies, we have shown that less than 0.6% of the incorporated dye leaches out from the nanoparticles over 48 h under in vitro sink conditions (Desai et al., 1997). Therefore, this formulation of nanoparticles is suited for the cellular studies to qualitate the uptake and also to observe the cells for uptake by fluorescence microscopy (Desai et al., 1996, 1997). The standard plot for the nanoparticles containing 6-coumarin dye was linear in the concentration range of 0–50 μg/ml or 0–1 μg quantity ($R^2 = 0.9917$) with sensitivity of detection as low as ~ 5 ng nanoparticles. The commercially available polystyrene latex nanoparticles, which are commonly used for the cellular uptake study, have different physical properties than PLGA-nanoparticles in terms of their hydrophobicity and surface charge (Florence et al.,
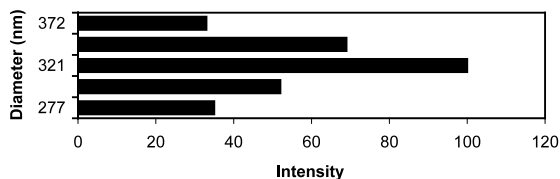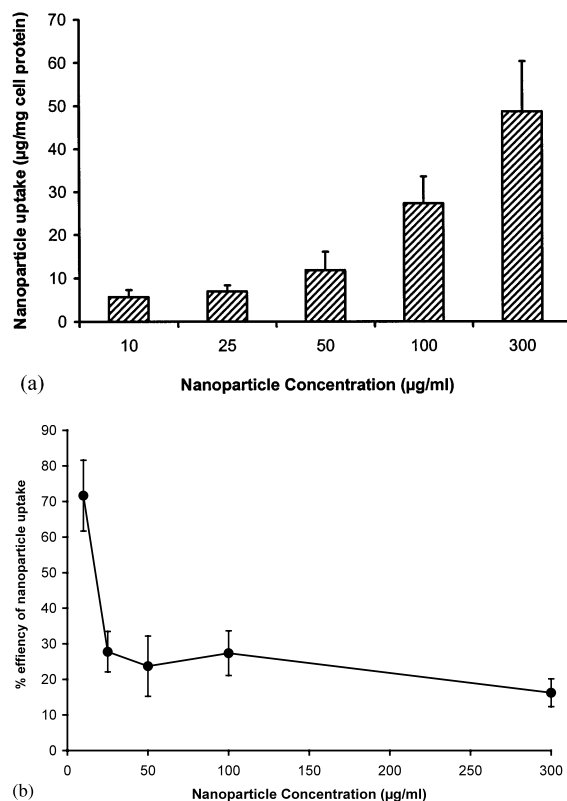
(a)

(b)

Fig. 2. (a) The effect of nanoparticle concentration on uptake of nanoparticles by HUVECs. (b) The efficiency of uptake of nanoparticles by HUVECs with concentration of nanoparticles in the medium. Values represent mean $\pm$ SEM ($n = 4$).

1995; Hussain, 2001). Therefore, the results obtained from these particles can be significantly different from the PLGA nanoparticles.

The uptake of nanoparticles by HUVECs was dependent on the concentration of the nanoparticles in the medium. The uptake increased with increase in the concentration, showing almost a first order kinetics (Fig. 2a). The efficiency of nanoparticle uptake was greater at a lower nanoparticle concentration, which was then reduced at higher concentrations, suggesting that the cells were reaching their saturation capacity (Fig. 2b). The nanoparticle uptake was also dependent on the incubation time. The uptake was seen as early as at 30 min, which increased gradually with the incubation time (Fig. 3a). Confocal microscopy of the cells exposed to nanoparticles demonstrated increased fluorescence activity in

Fig. 1. The particle size distribution of 6-coumarin PLGA nanoparticles obtained by quasi-light scattering.

the cells with increase in the time of incubation (Fig. 3b). Serial *z*-sections of the cells, each 1 μm in thickness, demonstrated fluorescence activity in all the sections between 10 and 25 μm from the surface of the cells indicating that the nanoparticles were internalized by the cells and not simply bound to their surface (Figure not shown). Nanoparticles were mostly seen localized in the cytoplasm (Fig. 3B–E). It was observed that free dye released from the nanoparticles accounted for only about 3% of the total uptake of the dye indicating that 6-coumarin detected in the cells was mainly associated with the nanoparticles (Fig. 3A–F). Quantitative analysis of the uptake and also the confocal microscopy studies confirmed the above results.

Nanoparticles have been investigated for the delivery of different types of therapeutic agents including proteins, peptides, and DNA (Davis, 1997; Labhasetwar et al., 1997, 1999). In addition to providing sustained release, nanoparticles can protect the encapsulated agent from enzymatic degradation (Hedley et al., 1998). Since PLGA is a biodegradable and biocompatible polymer, it is well tolerated by the body and cells (Mooney et al., 1997). As evident from the mitogenic assay, our study also demonstrates that nanoparticles in

the concentration range of 62.5–500 μg/ml have no adverse effect on the cell viability (Fig. 4). This is important because most of the cationic polymers and lipids, which are commonly used for gene transfection have toxic effect on cells at higher concentrations (Putnam et al., 2001).

In general, two strategies could be used for localizing nanoparticles into endothelial cells: ex vivo and direct in vivo administration. In ex vivo method, endothelial cells from the host could be removed, cultured and expanded in the laboratory, and incubated with drug-loaded nanoparticles. The cells with internalized nanoparticles could then be reimplanted into the host. Other investigators have demonstrated the feasibility of such an approach. Nabel et al. have used genetically engineered endothelial cells expressing β-galactosidase gene and successfully seeded them on the denuded arterial wall in a porcine model (Nabel et al., 1989). Endothelial cells could also be seeded onto stainless steel stents in vitro, which can be then implanted in vivo into the blood vessels to prevent thrombus formation and also to increase the biocompatibility of the stents (Scott et al., 1995; Eton et al., 1999). Similarly, vascular grafts could be seeded with endothelial cells containing drug-loaded nanoparticles to increase their
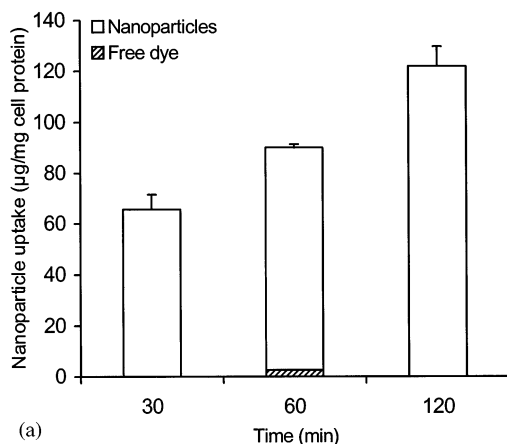
Fig. 3. a: Effect of time of incubation on uptake of nanoparticles by HUVECs. Values represent mean ± SEM (*n* = 4). b: Confocal fluorescence microscopy: time-dependent uptake of nanoparticles by HUVECs. Cells were imaged at 0 (A), 30 (B), 60 (C), and 120 (D) min from the time of incubation. The control experiment was performed by incubating cells with 6-coumarin solution released from nanoparticles in vitro in 2 h (F). Intracellular fluorescence was insignificant (F) compared to that of cells incubated with nanoparticles (E). (A) to (D) are magnified equally. Bar in (D): 50 μm. (E) and (F) are magnified equally. Bar in (F): 25 μm. The pictures were taken at the midsection of the cells.
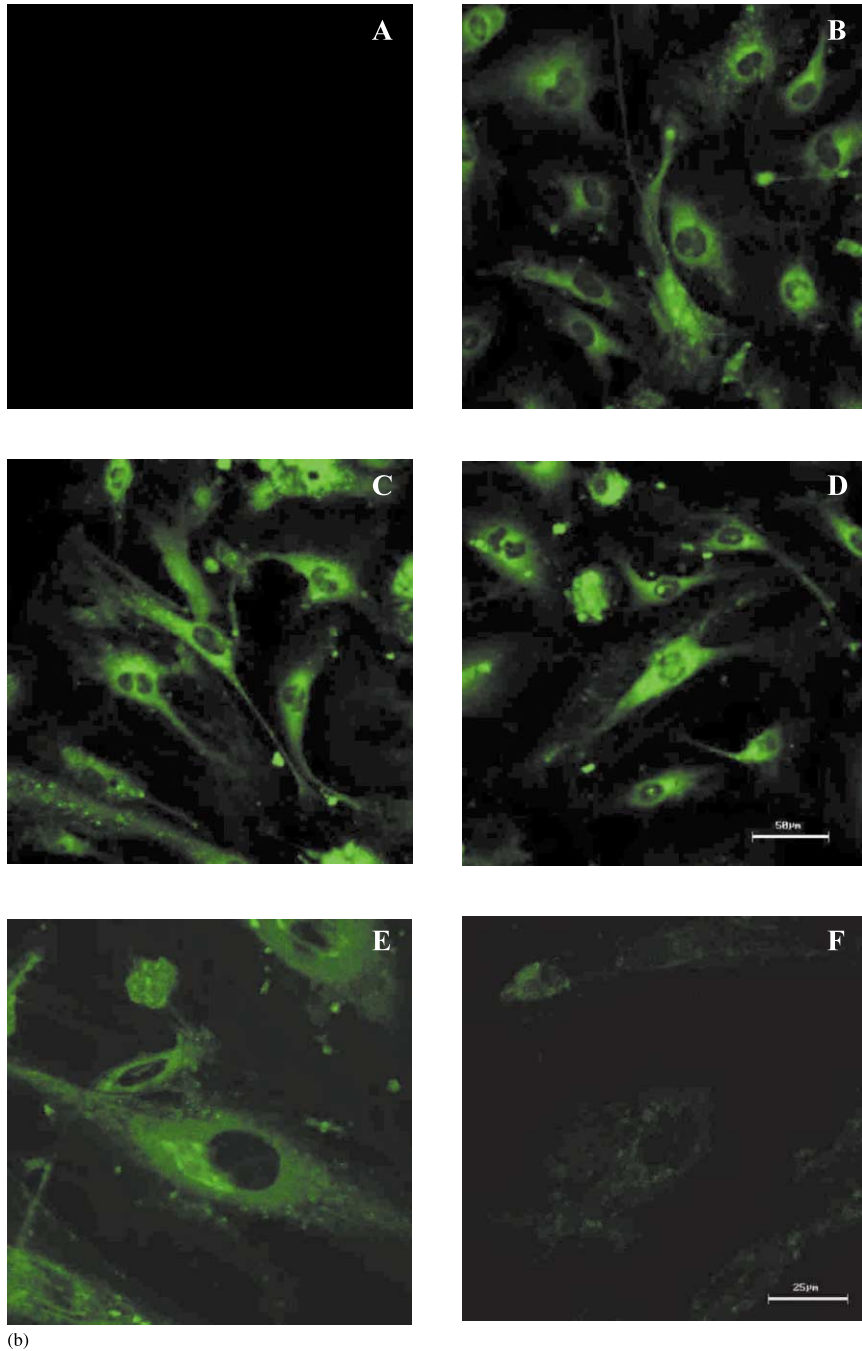
Fig. 3. (*Continued*)

long-term patency (Consigny, 2000; Ratcliffe, 2000; Teebken et al., 2000). The slow release of drug from the nanoparticles could provide local and prolonged therapeutic effect (Tanner et al., 1997).

Direct intravascular administration of nanoparticles to localize nanoparticles into the endothelium could be less efficacious because the particles could be taken up rapidly by the circulating monocytes for clearance by the reticuloendothelial cells (Fernandez Urrusuno et al., 1996). The other alternative could be to infuse the drug-loaded nanoparticles selectively in the diseased tissue. PLGA nanoparticles can be easily suspended in normal saline and infused using infusion catheters in an accessible selected tissue. The advantage of nanoparticles is that there is no risk of arterial occlusion because of their smaller size as against larger size microparticles. In our prior studies, we have shown localized uptake of nanoparticles in the coronary artery in pig studies (Labhasetwar et al., 1998) and in carotid artery in rat studies (Guzman et al., 1996) using catheter-based techniques. This strategy was shown to be useful in providing higher drug levels in the target artery for a prolonged period of time (Guzman et al., 1996). The sustained localized drug effect seems to be necessary to prevent restenosis, a major problem occurring following balloon angioplasty. The catheter-based technique has been used by others to achieve localized vascular gene delivery using viral vectors and DNA–lipid complexes (Ohno et al., 1994; Stephan et al., 1996).
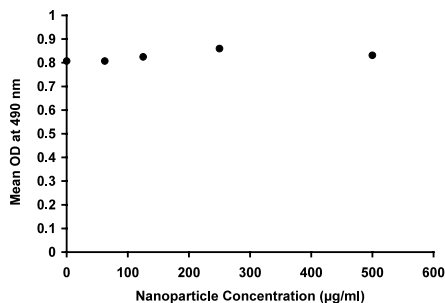

Fig. 4. Effect of nanoparticle concentration on cell viability using mitogenic assay. Each data point represents the mean of four samples.

Although we have not studied the mechanism of uptake of nanoparticles in this paper, our previous studies have shown in other cell lines (e.g. Caco-2) that the particle uptake depends on their size, with nanoparticles showing relatively greater efficacy of uptake than microparticles (Desai et al., 1997). The uptake has been shown to depend on the temperature of incubation, with relatively lower uptake at 4 °C than at 37 °C, suggesting that the uptake is an energy dependent process (Qaddoumi et al., 2000). The uptake of nanoparticles seems to be mediated by endocytosis (Qaddoumi et al., 2000). Further investigation on the mechanism of nanoparticle uptake, and the kinetics of drug uptake and retention in the endothelial cells using nanoparticles as compared to a free drug in vivo will be useful to establish the efficacy of nanoparticles for various therapeutic applications.

4. Conclusions

In this study, we have shown a rapid uptake of nanoparticles by endothelial cells. The uptake depends on the concentration of nanoparticles, and the particles were seen to localize mainly in the cytoplasm. Nanoparticles are biocompatible with the cells, as they did not affect the cell viability in a 48 h mitogenic assay. Nanoparticles could be used to target drugs or genes to the endothelium to achieve sustained therapeutic effect.

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