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Intracellular uptake and release of poly(ethyleneimine)-*co*-poly(methyl methacrylate) nanoparticle/pDNA complexes for gene delivery

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Abstract

Our previous studies demonstrated that cationic nanoparticles composed of well-defined poly(methyl methacrylate) (PMMA) cores surrounded by a hairly poly(ethyleneimine) (PEI) shells have comparative advantages over the PEI system for gene delivery. In this study, we focused on the intracellular uptake and release of PEI–PMMA nanoparticle/pDNA complexes. The behavior of the nanoparticle/pDNA complexes in recipient cells was monitored by using confocal laser scanning microscopy. We found that the nanoparticle/pDNA complexes were internalized very effectively by endocytosis. In the recipient cells the nanoparticles were found localized in the cytoplasm. At the same time, the pDNA carried by the nanoparticles successfully detached from the nanoparticles and localized in the nucleus of the HeLa cells.

Keywords: Cationic PEI–PMMA nanoparticles; Nanoparticle/pDNA complexes; Intracellular behavior; Confocal laser scanning microscopy

In the treatment of genetic diseases, especially single-gene disorders, e.g. haemophilia, thalassemia and cystic fibrosis, gene therapy is a strategy that holds the most promises. Due to the fact that naked DNA can hardly penetrate the membrane of most cells, carriers or vectors are required to deliver the exogenous gene(s) into the target cells and to be more precise, the nuclei of the target cells.

Among the many types of carriers, the viral vectors appear to be most effective since it is the natural behavior of viruses to infect cells. However, undesirable effects, including major immune reactions and cancer induction, resulting in death of recipient patients have been found to accompany these viral gene delivery systems. In the different non-viral systems, the use of cationic polymers have the best potentials and been widely investigated in transfection experiments (Curiel et al., 1991; Bielinska et al., 1996; Shah et al., 2000; Kunath et al., 2003). The poly(ethyleneimine) (PEI), a positively charged synthetic polymer composed of rich amine groups, is one of the most extensively tested cationic polymers (Godbey et al., 1999a). PEI polymers are able to condense DNA into compact particles and protect DNA from enzymatic degradation. It has a high but variable transfection efficiency that depends on several factors such as the chemical structure (linear or branched), the molecular weight, complex formation (the molar ratio of PEI nitrogen to DNA phosphate) and the size of the PEI/DNA complex (Wightman et al., 2001; Choosakoonkriang et al., 2003; Kichler, 2004). The size of the PEI/DNA complex is in fact a critical parameter for successful gene delivery. It has been shown that PEI/DNA complexes of larger sizes (>100 nm) have significantly higher transfection efficiency than the smaller ones (40 nm) (Ogris et al., 1998). However, the size of PEI/DNA complexes can vary from 50 nm to a few hundred nanometers in diameter, all depending on the complex formation protocol. Many different factors, such as the ionic strength in the medium, the concentrations of PEI and DNA, the ratio of different solutions, the speed of mixing, even the order of addition of reagents (Boussif et al., 1996; Tang and Szoka, 1997; Dunlap et al., 1997; Goula et al., 1998) will affect the size of the resultant PEI–DNA complexes, rendering it almost impossible to obtain complexes with consistent size made from different labs.

A novel nanoparticles that consist of a biocompatible core of poly(methyl methacrylate) (PMMA) surrounded by a cationic

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poly(ethyleneimine) (PEI) shell were synthesized based on our previously developed method (Li et al., 2002) and used as carriers to transfer gene into mammalian cells. Unlike the loose and toroidal structure of PEI/DNA complexes, the PEI–PMMA nanoparticles are monodispersed, spherical and tight in structure and with a narrow size range of 100–200 nm when complexes are formed with pDNA. Our previous studies have demonstrated that the cationic PEI–PMMA nanoparticles have comparative advantages over the PEI system for gene delivery (Zhu et al., 2005). The advantages include higher transfection efficiency and lower cytotoxicity. In this study, we focused on the intracellular uptake and release of PEI–PMMA nanoparticle/DNA complexes. The nanoparticles and pDNA were labeled with fluorescent imaging agents respectively and the behavior of the nanoparticle/pDNA complexes in recipient cells was monitored by confocal laser scanning microscopy. The possible mechanism of PEI–PMMA nanoparticles delivering pDNA into cell has also been discussed.

Fluorescein isothiocyanate (FITC) and paraformaldehyde were obtained from Sigma. Label IT®TM-Rhodamine Labeling Kit was purchased from Mirus. pGL3-Control plasmid was from Promega. The human HeLa cell line was purchased from the American Type Culture Collection (ATCC). Fetal bovine serum (FBS) was obtained from Sigma. Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco. Antifaded reagent, diazabicyclooctan, was obtained from Merck. All buffer materials were obtained from Sigma.

PEI–PMMA core-shell nanoparticles were prepared as our previously described (Li et al., 2002). PEI–PMMA nanoparticles were diluted with double-distilled water resulting in concentrations of 1.0 mg/ml. Corresponding to a nitrogen–phosphorous (N/P) ratio requested (ranging from 5:1 up to 20:1), the 360 μg/ml of DNA solution was pipetted into different amounts of the PEI–PMMA nanoparticle suspension, mixed by pipetting up and down. The complexes were incubated for 20 min at ambient temperature before use.

The diameters of the nanoparticle/pDNA complexes and their zeta-potentials were determined with a Malvern Zetasizer 3000HS (Malvern, UK) in a 1.0 mM NaCl aqueous solution. The morphologies of nanoparticles were observed with a JEM 100 CX transmission electron microscope (TEM) with a voltage of 100 kV. The sample was negatively stained with 2% phosphotungstic acid for 30 min and dried at room temperature. The morphologies of nanoparticles were also observed with a scanning electron microscopy (SEM, JEOL, JSM-5400, Japan) at 25 kV. The sample was coated with gold/palladium using an Ion Sputter (JEOL, JFC-1100) at 20 mA for 4 min.

The PEI–PMMA nanoparticles were labeled with fluorescein isothiocyanate (FITC) using the modified method described in literatures previously for labeling of PEI (Gautam et al., 2001). PEI–PMMA nanoparticles (20.0 mg/mL) were mixed with FITC (2.0 mg/mL) in borate buffer (0.1 M, pH 8.5) and incubated at room temperature on a shaker for 2 h. the unbound FITC was removed by centrifugation. pGL3-Control plasmid was labeled with rhodamine by using the Label IT®TM-Rhodamine Labeling Kit following the protocol provided by Mirus Inc. The rhodamine-labeled plasmid DNA/FITC-labeled PEI–PMMA nanoparticle complexes (N/P = 5) were prepared as the PEI–PMMA nanoparticle/pDNA complexes described above.

HeLa cells were maintained in Dulbecco's modified essential medium (DMEM) supplemented with 10% defined fetal bovine serum (FBS) and supplemented with 100 mg/ml penicillin G and 100 mg/ml streptomycin sulfate. Cells were cultured in a culture incubator at 37 °C and in a humidified atmosphere containing 5% CO_2. The cells were plated in 150 cm^2 cell culture flasks and subcultured before reaching confluency using a 0.1% trypsin solution in EDTA. The culture medium was changed every 2 days.

HeLa cells (1×10^5 cells/well) were cultured in a six-well culture dish for 24 h in 2 ml of DMEM medium supplemented with 10% fetal bovine serum (FBS) (10% FBS-DMEM) under a humidified atmosphere of 5% CO_2 in air. Cells had been washed twice with PBS solution; 0.2 mL (0.1 mg/mL) of FITC-labeled PEI–PMMA nanoparticles, rhodamine-labeled plasmid DNA/FITC-labeled PEI–PMMA nanoparticle complexes (N/P = 5), FITC-labeled PEI or rhodamine-labeled plasmid DNA with 0.8 mL of fresh DMEM medium without FBS was added. The cells were then incubated at 37 °C for 1 h, followed by removal of the medium and washing the cells twice with PBS solution. Then 10% FBS-DMEM was added for another 2 or 4 h incubation.

The cellular entry of rhodamine-labeled plasmid DNA/FITC-labeled PEI–PMMA nanoparticle complexes were monitored by confocal laser scan microscopy (CLSM) (Zeiss, LSM510). First, the cells treated with samples were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. After a wash with PBS, the coverslips were mounted on glass microscope slides by using a drop of antifade solution, diazabicyclooctan (2.5% w/v). Then the samples were observed under CLSM with an argon laser (488 nm excitation) to induce the green fluorescence of FITC and their emission was observed using a band filter (515–565 nm). Red fluorescence was induced by the 568 nm excitation and detected as 575–640 nm wavelengths.

The positive charges carried by the complexed DNA and DNA compaction into nanostructures are essential for the interaction of the DNA complexes with the cell membrane and cellular uptake. The zeta potential and average size of nanoparticle/pDNA complex were assessed and shown in Table 1. When DNA was complexed onto the nanoparticles, the average size of the nanoparticle/pDNA complexes was 133 nm (at a PEI nitrogen to DNA phosphate [N/P] ratio of 5:1) and 139 nm (at an N/P ratio of 20:1), respectively. The corresponding average

Table 1
Particle size and zeta potential of PEI–PMMA nanoparticles and nanoparticle/DNA complexes

Samples	N/P ratio[a]	Particle size ± S.D. (nm)	Zeta potential ± S.D. (mV)
PEI–PMMA nanoparticles	0	172 ± 7	50.3 ± 2.6
DNA/nanoparticles complex-1	5:1	133 ± 14	21.4 ± 5.9
DNA/nanoparticles complex-2	20:1	139 ± 9	33.7 ± 8.1

[a] N/P ratio is the ratio of PEI nitrogen to DNA phosphate.

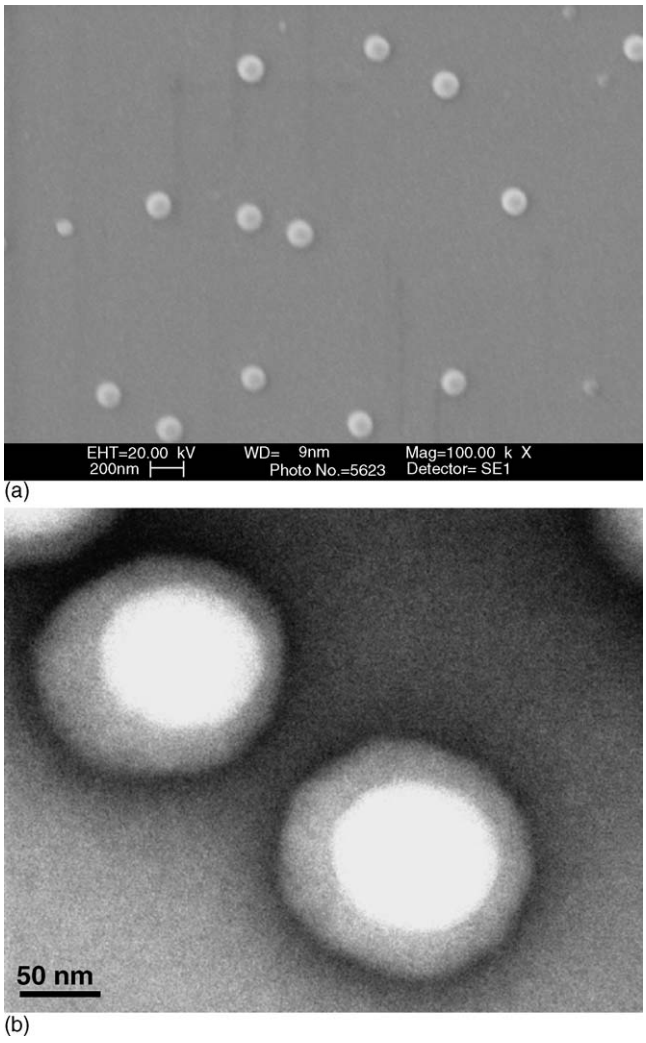

Fig. 1. The images of PEI–PMMA nanoparticles observed by (a) SEM and (b) TEM.

zeta potential was 21.4 and 33.7 mV, respectively. Compared with the average diameter and zeta potential of PEI–PMMA nanoparticles, the smaller size and lower zeta potential of the nanoparticle/pDNA complexes indicated that electrostatic interactions of the positively charged amine groups of the PEI shell with the negatively charged phosphate groups of DNA have occurred, resulting in condensation of DNA into nano-sized particles. After binding of the DNA, the zeta potential of the nanoparticle/pDNA complexes remained net positive. The net positive charge on the complex surface will facilitate adherence to the negatively charged cell membrane and thus will induce and increase cellular uptake (Godbey et al., 1999b). Furthermore, between the N/P = 5 and the N/P = 20 samples, there was no significant difference in the average particle size measured. We believe that this is mostly due to the presence of the hard PMMA core providing the mechanical strength of the complexes, rendering the consistent particle size and with a narrow range of size distribution. It was also confirmed by SEM and TEM images that the PEI–PMMA nanoparticles were monodispersed, spherical and tight in structure (Fig. 1a) and a well-defined core-shell structure (Fig. 1b) with the PMMA core in white about 103 nm in diameter and the PEI shell layer in grey about 30 nm thick. Comparing with the use of the PEI polymers along as the DNA complexing agent, the PEI–PMMA nanoparticles offer a much more stable carrier with the consistent size of the complexes.

The carrier for gene transfer entering into targeted cells efficiently is the first step in successful gene delivery. Thus, cellular entry of the fluorescent agent labeled PEI–PMMA nanoparticles and PEI polymers was investigated by CLSM. Both the fluorescence signal emitted from FITC-labeled PEI–PMMA nanoparticles and FITC-labeled PEI was observed in HeLa cell (Fig. 2). The green signal was localized in the cytoplasm of HeLa cell and the cells kept in the normal state, after treating with FITC-labeled PEI–PMMA nanoparticles, as shown in Fig. 2a. However, the HeLa cells with the green fluorescence turned into round when the cells were incubated with FITC-labeled PEI at the same level, as shown in Fig. 2b. It may be suggested that the nanoparticles have a lower cytotoxicity than the PEI polymers and this may also contribute significantly to the higher transfection efficiency and the higher level of reporter gene expression reported by our previous work (Zhu et al., 2005).

In order to understand the possible mechanism of the PEI–PMMA nanoparticles carrying the exogenous DNA into the cell, we labeled, respectively, the plasmid DNA carrying the firefly luciferase reporter gene (pGL3-Control), with the fluorescent dye rhodamine and the PEI–PMMA nanoparticles with FITC before allowing them to form complexes. Then the double labeled complex was administrated to the HeLa cells in culture and monitored with CLSM with an argon ion laser (488 nm) and a He–Ne laser (543 nm). The fluorescence signal emitted from FITC-labeled nanoparticles was green and emission from the rhodamine-labeled plasmid DNA was red. The overall emission from the nanoparticle/pDNA complexes was yellow (the combination of green overlapped red fluorescence). By 2 h posttransfection, yellow, green and red fluorescence from PEI–PMMA nanoparticle/pDNA complexes were all detected in HeLa cells. It suggested that many nanoparticle/pDNA complexes were internalized in target cells. Whereas no red fluorescence was detected from cells treated with naked rhodamine-labeled plasmid DNA (no figure showed here). This confirmed that the nanoparticle/pDNA complexes had a high affinity for the cultured cells due to the net positive surface charge of the complexes that allowed them to attach onto the negatively charged cell membrane, but negatively charged naked DNA was hard to attach to the cultured cells for the electrostatic repulsion with each other. The results (Fig. 3) showed that the distribution of both the nanoparticles (Fig. 3b) and the carried plasmid DNA (Fig. 3c) was different. The yellow represented the colocalization of nanoparticles and plasmid DNA, which localized in cytoplasm and the regions near but not in the nucleus of the cells. The red represented the localization of pDNA, which concentrated in the nucleus region. It was noted that the yellow and green fluorescence appeared in cell as patches and the size of these patches with several micrometer in diameter was much larger than that of nanoparticle/pDNA complexes (about 130 ± 10 nm) (Fig. 3a and b). It may suggest that the nanoparticle/pDNA complexes entered into cell not by diffusion, but multiple of them had been enclosed in vesicles and then endocytosed by cells, resulting in the large aggregations of

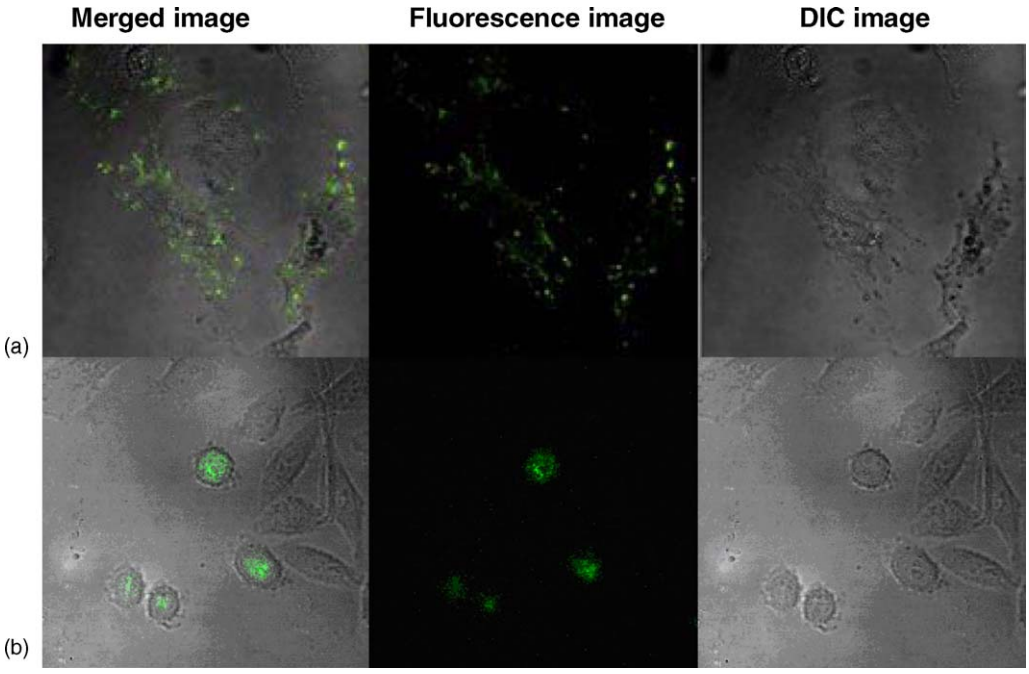

Fig. 2. CLSM images of intracellular uptake of (a) FITC-labeled PEI–PMMA nanoparticles and (b) FITC-labeled PEI.

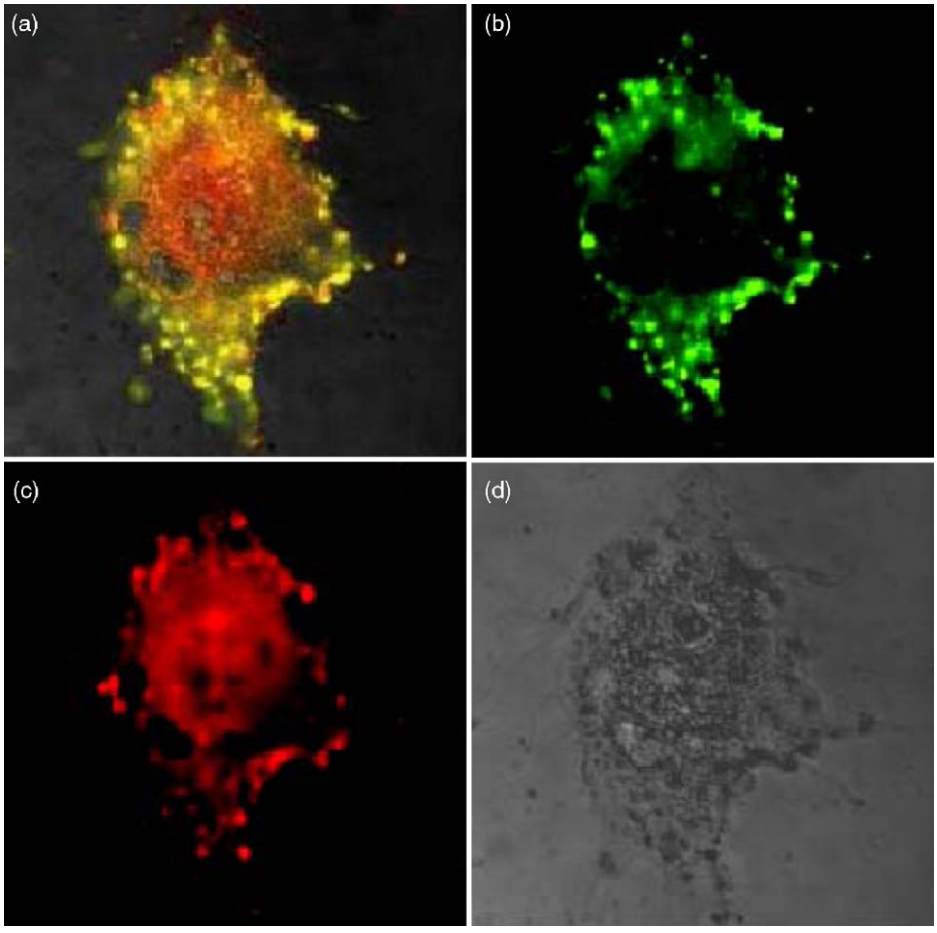

Fig. 3. CLSM images showing the interacellular distribution of plasmid DNA in HeLa cells after transfection by PEI–PMMA nanoparticles. HeLa cells were incubated with the complex of FITC-labeled PEI–PMMA nanoparticles and rhodamin-labeled plasmid DNA (N/P ratio of 5/1) at 37 °C for 1 h and posttansfection for 2 h. The focal plane of the sample was set on the middle of nuclei. (a) The merged CLSM image; (b) green fluorescence indicated the localization of FITC-labeled PEI–PMMA nanoparticles; (c) red fluorescence, that of rhodamin-labeled plasmid DNA; (d) differential interference contrast image.

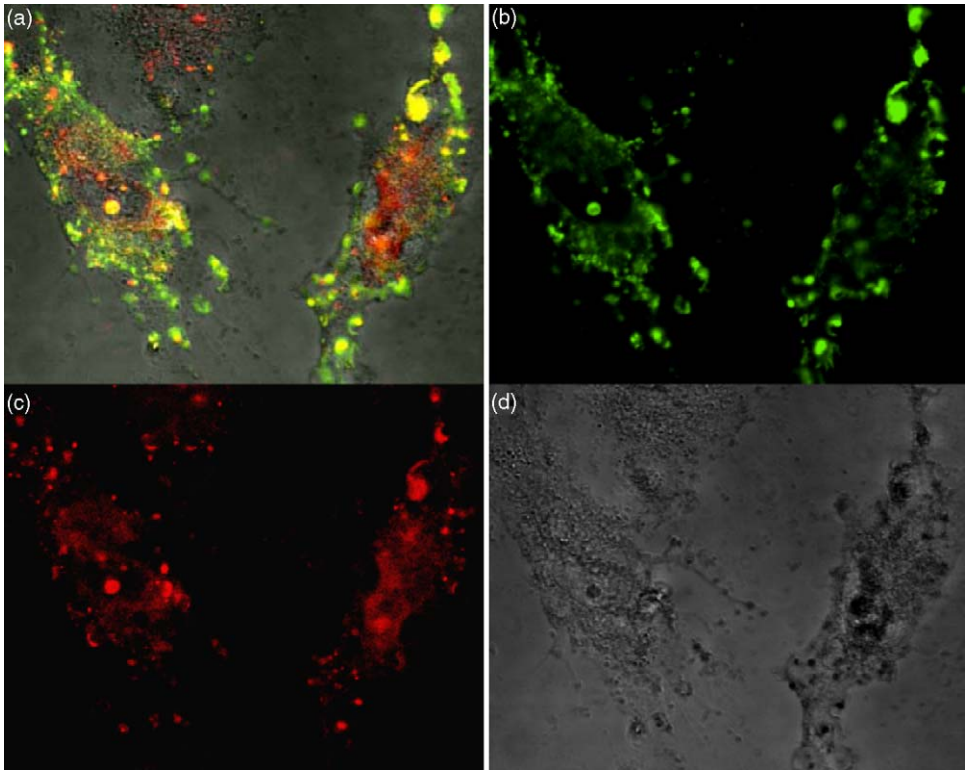

Fig. 4. CLSM images showing the interacellular distribution of plasmid DNA in HeLa cells after posttansfection for 4 h. The focal plane of the sample was set on the middle of nuclei. (a) The merged CLSM image; (b) green fluorescence indicated the localization of FITC-labeled PEI–PMMA nanoparticles; (c) red fluorescence, that of rhodaminlabeled plasmid DNA; (d) differential interference contrast image.

fluorescent signals in the cytoplasmic region of the cell. These results were consistent with those obtained by Godbey (Godbey et al., 1999b). The signal emitted from the nucleus region was strong red (Fig. 3c), indicating that the nanoparticle/pDNA complexes broke through the endosomes successfully and plasmid DNA was released from the nanoparticles then transported into the nucleus. After 4 h posttransfection, the number of the discrete green clumps decreased and the green fluorescence was noted more homogeneous in cytoplasmic regions with constant microscope setting (Fig. 4b). The decrease in number was likely caused by disruption of endocytotic vesicles and then nanoparticles and DNA complexes aggregated in the endosomes released and freely dispersed in the cytoplasm. In endosomes pH is around 5 and is lower than the physiological pH of around 7.4. On the other hand, when pH changes from 7 to 5, PEI, a 'proton sponge', will protonate and increase its hydrodynamic volume up to 20–45% (Suh et al., 1994). What may have happened is that the protonation process causes the excessive amount of protons will also trigger the passive influx of chloride ions into the endosome, resulting in osmotic bursting of the endosomes (Duncan et al., 1979) and at the same time the detachment of the negatively charged DNA from the nanoparticles complexes. The plasmid DNA, now being released from the nanoparticles, will no longer be enclosed in the endosomes and therefore, is available for transport into the nucleus. However, we have no evidence as to how the exogenous DNA was transported into the nucleus. Compared with the treated cells with posttransfection for 2 h, after 4 h posttransfection, the yellow fluorescence represents the localization of nanoparticle/DNA complexes weakened instead of uncombined green and red fluorescence in cells (Fig. 4a). It indicated that more plasmid DNA released from the complexes and the release was time-dependent. The plasmid DNA carried by or released from the complexes (Fig. 4c) was present in cells and tended to concentrate in the nucleus regions. The evidence of the subcellular distribution of nanoparticle/DNA complexes may suggest the internalization and release of plsmid DNA carried by PEI–PMMA nanoparticles was an endosomal–lysosomal route.

The present work shows evidence that the PEI–PMMA nanoparticles internalized and released carried plasmid DNA into HeLa cells very efficiently. DNA molecules carried by the nanoparticles were specifically delivered into the nucleus of the target cells. The PEI–PMMA nanoparticles maintaining the stable spherical structure, the monodispersed size distribution, tighter structure and the proper net positive surface charge after forming complexes with plasmid DNA, did not obviously disorder the cultured cells observed by confocal laser scanning microscopy. On the basis of these preliminary results, these core-shell nanoparticles may have application as efficient non-viral DNA carriers for gene delivery. It can also be utilized for further modification such as attaching antibodies, proteins, or small molecules recognized by a specified cell type on the particle surface to greatly enhance the transfection efficiency.

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