

Transfection Ability and Intracellular DNA Pathway of Nanostructured Gene-Delivery Systems

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ABSTRACT

Considerable efforts have been devoted to the design of structured materials with functional properties. Polyelectrolyte multilayer films are now a well-established nanostructured concept with numerous potential applications, in particular as biomaterial coatings. This technique allows the preparation of nanostructured architectures exhibiting specific properties for cell-activation control and local drug delivery. In this study, we used a multilayered system made of poly-(L-lysine)/hyaluronic acid (PLL/HA) as a reservoir for active DNA complexes with nonviral gene-delivery vectors, PLL, β -cyclodextrin (CD), and PLL-CD. When embedded into the multilayered films, the transfection efficiencies of the DNA complexes and the cell viability were improved. The highest transfection efficiency was obtained with the PLL-CD/plasmid DNA (pDNA) complexes. We found that this high transfection efficiency was related to an efficient internalization of the complexes in the cell cytoplasm and selected nuclei domains through a nonendocytotic pathway. For the first time, we report the intracellular pathway of the pDNA in complexes incorporated into the multilayered system.

In recent years, considerable efforts have been devoted to the design and controlled fabrication of structured materials

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with functional properties. The layer-by-layer (LBL) buildup of polyelectrolyte multilayers (PEM) films using oppositely charged polyelectrolytes offers new opportunities for the preparation of functionalized biomaterial coatings.^{1,2} This technique allows the preparation of supramolecular nano-architectures exhibiting specific properties for cell activation and development of local drug-delivery systems.^{3,4} Peptides, protein drugs or DNA, chemically bound to polyelectrolytes, adsorbed or embedded in PEM films, have been shown to retain their biological activities.⁵⁻¹¹

During the past decade, gene therapy has also become a worldwide research focus and has been advanced considerably. The main objective in gene therapy is constructing an efficient gene-delivery system able to transfer the therapeutic DNA to the targeted tissues and cells. However, although a great deal of effort has been expended, the development of

an efficient and safe gene-delivery system remains the main challenge for gene therapy.

The vectors in gene therapy can be divided into viral and nonviral vector systems. So far, viral systems have been used most often but the issues of viral immunogenicity, difficulties of large scale industrial production, and possible random genomic integration remain as problems.¹² Therefore, non-viral delivery systems are increasingly investigated as alternative vectors. These systems include complexes of cationic lipids, polymers, or peptides with plasmid DNA. Controlled delivery of DNA from coating, hydrogel, tissue engineering scaffolds and nanoparticles, has also been developed.^{13,14} An ideal gene-delivery vector should be efficient, nonimmunogenic, stable, and nontoxic. Although nonviral vectors represent attractive alternative to viral vectors for transfection *in vitro* and *in vivo*, they still suffer from relatively low efficiencies.^{15,16}

Multilayers containing pDNA are of great interest for applications in sensing,¹⁷ diagnostic,¹⁸ electronics,¹⁹ and gene-delivery.^{20–22} Although the incorporation of pDNA into the multilayered films was first reported in 1993 by Lvov et al.,²³ substantive investigation indicated that this method has a significant impact on the development of gene therapies. In the past few years, it has also been shown that PEMs are efficient to deliver pDNA. For instance, Lynn et al. found that multilayered films composed of naked pDNA and a degradable polyamine are able to efficiently release pDNA from the surfaces of model substrates under physiological conditions and subsequent *in vitro* transfection of adherent cells.^{21,22} They suggested that the films present DNA molecules in a condensed form, which could improve the internalization of DNA by cells.²² Moreover, our group studied the use of PEM as vectors for polymer-precomplexed DNA,²⁴ and β -cyclodextrin-mediated DNA delivery to perform multiple and time-scheduled cell transfection.¹⁰ Jessel et al. realized the sequential induction of nuclear and cytoplasmic expression products of two different pDNA embedded in PEM. Nevertheless, until now, there has been no report available that shows the intracellular pathway of pDNA in the multilayer system, which is one of the most critical steps in cell transfection. Therefore, to fill in this blank we, unprecedentedly, investigated in this paper the intracellular pathway of the pDNA in complexes in the multilayer system.

Our previous work showed that PEM films onto which cationic CD/pDNA complexes are adsorbed can act as an efficient gene-delivery tool to transfect cells.¹⁰ Moreover, cationic CD derivatives are incorporated into polymer and dendrimer vectors, CDs were shown to increase the cell viability and transfection efficiency.^{25,26} Thus, we chose poly(L-lysine) (PLL), a widely used nonviral gene-delivery vector,²⁷ to incorporate CD. Accordingly, PLL-CD was synthesized at a Lysine and cyclodextrin molar ratio of 2:1 and included as a complex with pDNA in a multilayer system made of the multilayered film PLL/HA. The transfection efficiency and the pDNA intracellular pathway with this PEM were investigated and compared with the same PEM in which pDNA has been complexed with PLL or CD, instead of PLL-

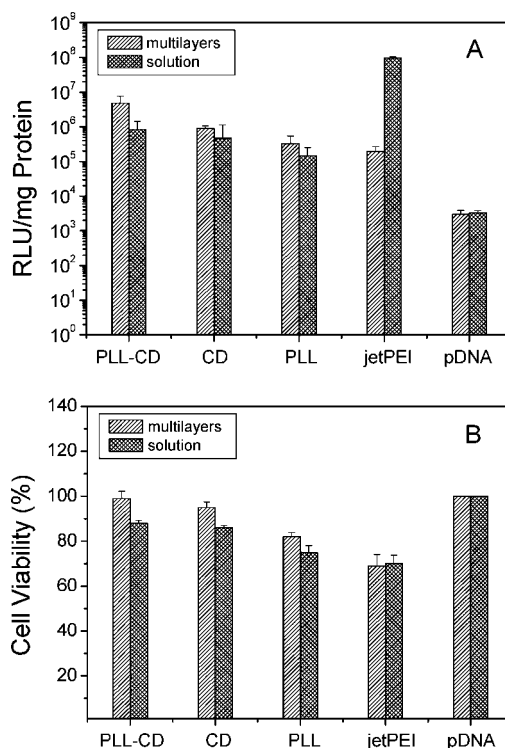


Figure 1. Transfection efficiency (A) and cytotoxicity (B) of the complexes of pDNA with PLL, CD and PLL-CD at a N/P ratio of 3 for *HeLa* cells grown on (PLL-HA)₅-(complexes)-(PLL-HA)₅ films or in the absence of multilayers for 24 h. Cells were lysed for luciferase activity quantification. Gene expression determined from the luciferase assay was expressed as RLU/mg of protein. JetPEI/pDNA complex and naked pDNA were used as controls.

CD. Comparison with complexes of pDNA with PLL, CD, or PLL-CD in solution was also performed. The multilayer systems showed much higher transfection efficiencies than the complexes in solution, and the best transfection efficiency was obtained with PLL-CD in the PEM. Using the bis-intercalator YOYO-1 to label the pDNA and an endocytosis marker FM 4-64,²⁸ we found using confocal microscopy that the enhanced efficiencies with the multilayers could be due to the pDNA intracellular pathway that differs from endocytosis.

The polyelectrolyte multilayer gene-transfer system we studied was based on pDNA in complexes with PLL, CD, and PLL-CD. The buildup of (PLL-HA)₅-pDNA complexes-(PLL-HA)₅ multilayered films was first monitored by quartz crystal microbalance (QCM). The PLL/pDNA and PLL-CD/pDNA complexes were prepared at N/P = 3, a ratio that is used commonly with PLL for transfection.^{29,30} The CD/pDNA complexes were prepared at a CD and pDNA molar ratio of 3, where the amount of CD in complexes with pDNA was the same as that of conjugated CD in PLL-CD/pDNA complexes. The observed increase in $-\Delta f/\nu$ with the number of deposited layers (data not shown) suggested that this multilayer system exhibits a regular film deposition.

In the next step, transfection experiments in the presence of serum were performed with *HeLa* cells by using the pCMV-Luc plasmid encoding the luciferase gene (Figure 1). To check the incidence of the multilayer systems on the

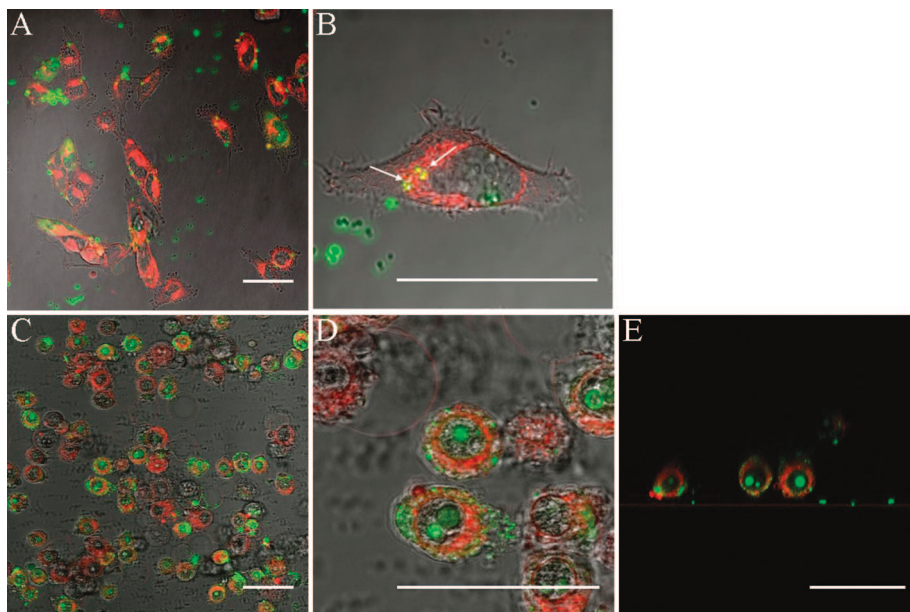


Figure 2. Internalization of the complexes of pDNA with PLL-CD in *HeLa* cells grown on the surface of (PLL-HA)₅-PLL-CD/pDNA complexes—(PLL-HA)₅ multilayered films (C–E) or in the absence of multilayers (A and B). Panels A–D correspond to *x*, *y* sections; Panel E corresponds to *x*, *z* sections. The concentration of pDNA was 60 μM, and the N/P ratio was 3. The images correspond to the overlay of light transmission and fluorescence confocal images and were taken at 24 h post transfection. Yellow spots (arrows) correspond to areas where the complexes and FM4-64 are colocalized. Red and green spots correspond to areas containing only FM4-64 and YOYO-1 labeled complexes, respectively. Images are representative of more than 90% of the observed cells (*n* = 3 specimens). Scale bars = 40 μm.

transfection efficiency of the pDNA complexed with nonviral gene-delivery vectors (PLL, CD, and PLL-CD), we compared the luciferase gene expression of the complexes in solution with those embedded in the multilayers.

In solution, the transfection efficiencies of the complexes of pDNA with PLL-CD, PLL, and CD were lower than that of jetPEI and significantly higher than that of naked pDNA, in line with the literature (Figure 1A). When embedded into the multilayers, the efficiencies of these complexes were increased dramatically and were much higher than that of naked pDNA, even higher than that of jetPEI. Within the tested multilayer systems, the highest efficiency was observed with PLL-CD/DNA complexes, which shows 5- and 14-fold higher transfection efficiency as pDNA complexed with CD and PLL, respectively. Furthermore, the cell viabilities were enhanced significantly when the pDNA complexes with PLL-CD, PLL, and CD were included in the multilayer systems (Figure 1 B). This increase in cell viability may contribute to their improved transfection efficiencies.

To further understand the differences in transfection efficiency, the intracellular trafficking of the complexes of pDNA with PLL-CD, PLL, and CD in solution and embedded into the multilayered system was monitored by confocal microscopy (Figures 2–4). We labeled the pDNA and the endosomes with YOYO-1 and FM4-64, a marker of membrane endocytosis, respectively.³¹ These two dyes can be followed simultaneously because the FM4-64 red fluorescence is easily distinguished from the YOYO-1 green fluorescence. In this respect, colocalization of FM4-64 and pDNA complexes will yield yellow spots. The intracellular pathway of the complexes was followed 24 h post transfection of *HeLa* cells.

First, the PLL-CD/pDNA complexes in solution were perceived mainly close to the cell surface (Figure 2A and B). Only a limited number of complexes entered in cells. The complexes entering into the cells provided perinuclear yellow spots that indicated internalization through endocytosis with an accumulation of the complexes in late endosomes and lysosomes (Figure 2B). In sharp contrast to the solution, numerous green spots but no yellow spots were observed in the cytoplasm and the nucleus when PLL-CD/pDNA complexes were delivered from multilayers (Figure 2C–E). The absence of yellow spots and perinuclear accumulation of the complexes seemed to indicate that the PLL-CD/pDNA complexes delivered from the multilayer system are not internalized by endocytosis. We suggest that the efficient internalization by the nonendocytic intracellular pathway contributes to the higher transfection efficiency when the complexes were delivered from the multilayer system. This unknown intracellular pathway could facilitate the pDNA in complexes to escape from late endosomes and lysosomes, where pDNA is degraded.³²

To further confirm these observations above, we also monitored the intracellular trafficking of the complexes of pDNA with PLL and CD in both conditions (Figures 3 and 4). The PLL/pDNA complexes in solution were perceived mainly as large aggregates close to the cell surface (Figure 3A and B). Only a limited number of complexes entered in cells. The complexes entering into the cells were of smaller size and gave yellow spots, suggesting that the complexes were internalized by endocytosis (Figure 3B). Both the limited number of PLL/pDNA complexes that are internalized and their probable accumulation into lysosomes are fully consistent with the observed low transfection efficiency. In

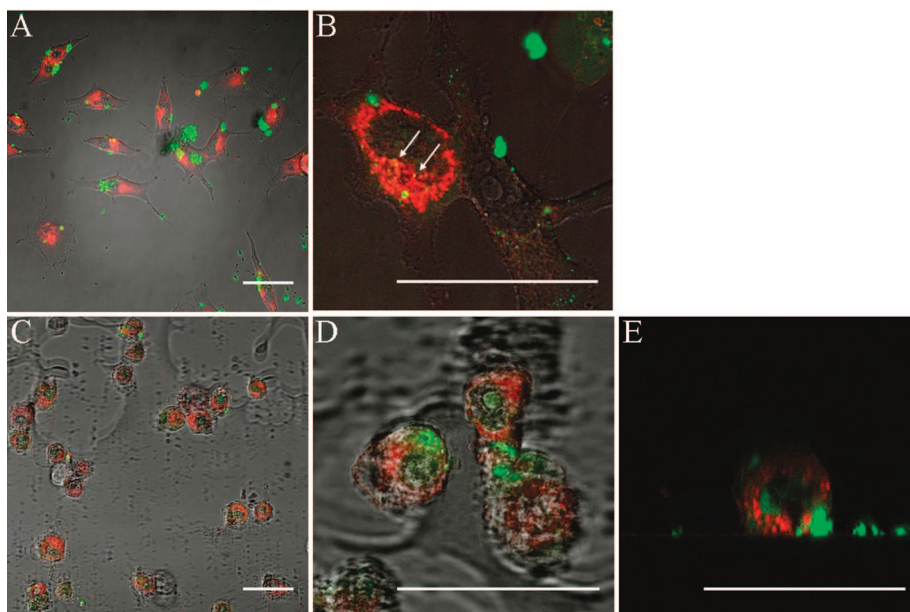


Figure 3. Internalization of the complexes of pDNA with PLL in *HeLa* cells grown on the surface of (PLL-HA)₅-PLL/pDNA complexes—(PLL-HA)₅ multilayered films (C–E) or in the absence of multilayers (A and B). Panels A–D correspond to *x*, *y* sections; Panel E corresponds to *x*, *z* sections. Experimental conditions were the same as those in Figure 2.

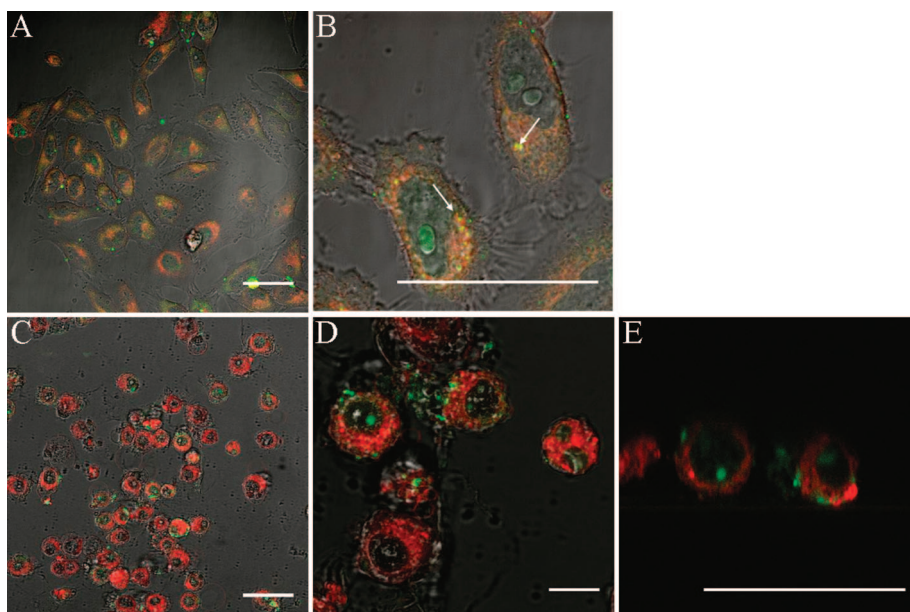


Figure 4. Internalization of the complexes of pDNA with CD in *HeLa* cells grown on the surface of (PLL-HA)₅-CD/pDNA complexes—(PLL-HA)₅ multilayered films (C–E) or in the absence of multilayers (A and B). Panels A–D correspond to *x*, *y* sections; Panel E corresponds to *x*, *z* sections. Experimental conditions were the same as those in Figure 2.

the case of the CD/pDNA complexes, a significant number of small yellow spots could be observed when they were in solution, indicating an internalization by endocytosis in this case also (Figure 4B). This conclusion was further substantiated by the mainly perinuclear localization of the complexes, in line with an accumulation of the complexes in the late endosomes and lysosomes.^{31,33}

When the PLL/pDNA and CD/pDNA complexes were released from the multilayers, as the PLL-CD/pDNA complexes, no yellow spot could be perceived, indicating that the complexes are not internalized by endocytosis either.

Moreover, the complexes of pDNA with PLL and CD were observed as numerous green spots in the cytoplasm and the nucleus (Figure 3C–E and 4C–E), suggesting that their intracellular trafficking is more efficient than that solution, which gives rise to their higher transfection efficiency.

Taken together, our data suggest that the higher transfection efficiency of the nonviral gene-delivery vectors (PLL-CD, PLL, and CD)/pDNA complexes delivered from the multilayer system than in solution may be related to their efficient internalization in the cytoplasm and the nuclei of the transfected cells. Their internalization clearly differs from

endocytosis by several lines of evidence. First, no colocalization with the endocytosis marker FM4-64 was observed in any transfected cell. Second, no perinuclear accumulation in late endosomes and lysosomes could be observed with the complexes delivered from the multilayers. Third, the complexes were found in a large number of cell nuclei, indicating that the complexes delivered from the multilayers easily cross the nuclear membranes and accumulate in selected regions of the nuclei. A comparison with the same complexes added in solution strongly suggests that escape from late endosomes and lysosomes as well as degradation in the lysosomes may constitute the main bottlenecks for efficient transfection in this case. In addition, complexes embedded in multilayers exhibited low cytotoxicity, indicating that multilayer systems are highly suited for in vivo applications.

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Supporting Information Available: Experimental procedures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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