Sustained release of complexed DNA from films: Study of bioactivity and intracellular tracking[a)]

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Sustained DNA delivery from polymeric films provides a means for localized and prolonged gene therapy. However, in the case of bioactive molecules such as plasmid DNA (pDNA), there are limitations on the achievable release profiles as well as on the maintenance of bioactivity over time. In this report, the authors have investigated the bioactivity of the released DNA (naked and complexed with lipofectamine) from polymeric films using *in vitro* cell transfection of COS-7 cell lines. The polymeric system consists of a biodegradable semicrystalline polymer such as poly(ε-caprolactone) (PCL) with or without blended gelatin. Sustained release of lipoplexes and of pDNA is shown over several days. However, lipoplexes released from pure PCL films show no transfection on day 18, whereas lipoplexes released from PCL-gelatin films continue to transfect cells on day 18 of release. Confocal studies were used to determine the reasons for this difference in transfection efficiency, and it is proposed that association of the lipoplex with gelatin confers protection from degradation in the cytoplasm. The results also showed that the bioactivity of released lipoplexes was superior to that of the naked pDNA. For both naked pDNA and the lipoplexes, the presence of gelatin helped to maintain the bioactivity over several days. © *2010 American Vacuum Society.* [DOI: 10.1116/1.3493692]

I. INTRODUCTION

Most of the research in DNA delivery is focused on developing new carriers that can match the transfection rates exhibited by viral vectors. However, another aspect of DNA delivery, that of localizing and sustaining its release, has not received as much attention. This feature is also critical as it enhances the availability of the pDNA at the site of action and sustains its availability over time, so that the frequency of injections may be reduced.

The advantages of such sustained release polymeric gene delivery systems may be summarized.[1,2]

- Sustained delivery of plasmid DNA at the tissue site offers a source for continual gene uptake, thereby prolonging the gene expression.
- There are increased amounts of plasmid DNA (pDNA) uptake.
- Site specific delivery can be achieved by simple implantation or direct injection.
- Repeated administration is not necessary.

Although in recent years sustained release systems have been successfully employed to deliver proteins and macromolecules, successful development of sustained release pDNA systems has not been reported. Existing studies concentrate mainly on microsphere formulations and not on implantable films or coatings. Furthermore, detailed investigations of the bioactivity of the release pDNA have not received much attention. Sustaining the release of the gene vector over several days or months, with prolonged and continuous DNA delivery to tissues, will enhance gene expression.[3]

Earlier, we reported the sustained delivery of complexed and naked pDNA from poly(ε-caprolactone) (PCL) polymer matrices.[4,5] The work was mainly focused on the control of pDNA and complexed DNA release, as well as on a comparison of lipoplex and polyplex release. In brief, the findings of that study are summarized as follows.

1. There was a substantial burst release of naked plasmid DNA from all films studied.
2. When lipoplexes are released from PCL, there is no burst, but release is slow and sustained over 1 month.
3. Addition of gelatin to the PCL matrix increases the release rate overall of lipoplexes, and sustains it for 1 month.
4. When transfection efficiency of the released lipoplexes is

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evaluated, it was found that the efficiency drops over time. Without gelatin, lipoplexes released from PCL films do not transfect at all even on day 5. Addition of gelatin to the matrix allows for sustained bioactivity up to day 19.

This article explores the possible reasons for changes in transfection efficiency by monitoring the bioactivity and intracellular tracking of the lipoplexes released from polymer films.

PCL is a semicrystalline, biocompatible, and biodegradable polyester with a low melting point (57 °C) and a low glass transition temperature. It degrades slowly relative to the poly(lactide)/poly(lactide-co-glycolide) (PLG) polymers. The amorphous portion of PCL is rubbery at room temperature, and this characteristic contributes to the very high permeability of PCL for many therapeutic drugs. PCL exhibits certain desirable characteristics for drug-delivery applications, including biodegradability, biocompatibility, and commercial availability.[2,6–8] PCL is known to degrade *in vitro* and *in vivo* by a hydrolytic mechanism, although the rate of degradation is slow. One study reported that degradation of semicrystalline polyester (PCL) in aqueous media occurs in two steps: The first step starts with water diffusion into the amorphous regions, and the second step starts with the hydrolytic degradation of the crystalline domains after most of the amorphous regions degraded.[9–12] Other authors reported that PCL is also degraded by a number of microorganisms and enzymes such as lipases.[9]

Gelatin is a natural polymer, derived from collagen, and is commonly used for pharmaceutical and medical applications because of its biodegradability and biocompatibility in physiological environments.[13–17] In our study, blends of gelatin with PCL appeared to give the best release profile and bioactivity retention of the formulations studied.

Other polymer matrices have been explored for the delivery of plasmid DNA. However, DNA encapsulation efficiency is very low with poly(lactic-glycolic acid (PLGA), and DNA damage has been observed as result of PLGA degradation and degradation products.[18] Howard *et al.* observed a sustained release profile of the poly(ethylenimine)/DNA complex from PLGA microparticles, but a significant initial burst was found.[19] Hydrogels have been used to increase encapsulation efficiency, but the release rates are too high to be of much practical use as a sustained release system for long periods. For example, Sacks *et al.* observed a substantial 50% burst effect of the lipofectamine-pDNA complex on day 1 from collagen films.[20]

Rives *et al.* fabricated layered porous PLG scaffolds for the *in vivo* plasmid DNA delivery. They observed that the incorporation efficiency of plasmid DNA significantly increased with layering and that the transgene expression was sustained over a period of 1–2 weeks with a peak in expression levels around day 4 or 5, and then there is a decline.[21] But a large initial burst effect was observed within the first 3 days.[21] In our earlier work, we found sustained release of complexed pDNA (as lipoplex) over almost a month using gelatin in a blend with PCL.[5] Gelatin extends the duration of release of bioactive complexed pDNA, but transfection efficiency decreased around day 19. In this report, we explore the characterization of the released complex pDNA to provide some insight into the bioactivity preservation with gelatin blends and the decline in bioactivity around day 19.

II. EXPERIMENT

A. Materials

The dual vector pEGFP*Luc* (EGFP is enhanced green fluorescent protein), encoding a fusion protein of EGFP and luciferase from the firefly *Photinus pyralis*, was purchased from Clontech (supercoiled, 6.4 kb). It was propagated according to the standard method using a giga filter kit from Qiagen and was conditioned in an autoclaved TE buffer. Lipofectamine (LPF) was purchased from Invitro Life Technologies. Sodium oleate salt was purchased from Sigma Chemicals; it was dissolved in water at a concentration of 1 mg/ml. Ethidium bromide was purchased from Sigma Chemicals. It was diluted in water at a concentration of 80 μg/ml. Polycaprolactone (Mn 80 000; polydispersity index 1.4) and gelatin [type A, medium bloom: 175, Mn 40 000 (low molecular (LMW)) and type A; high bloom: 300, Mn 50 000–100 000 (high molecular weight (HMW))] were purchased from Sigma Chemicals as well. Chloroform (GR grade) was purchased from Tedia. The films were cast with the Automatic Film applicator AG-2150, which was purchased from BKY Gardner. Dulbecco's minimum essential medium (DMEM) and penicillin-streptomycin were purchased from Invitrogen. A Luciferase Reporter Assay Kit was purchased from BD Clontech. A Micro BCA TM Protein Assay Reagent Kit was purchased from Pierce Biotechnology, Rockford, IL. Oregon Green® 488 gelatin was purchased from Molecular Probes. 4′,6-diamidino-2-phenylindole (DAPI, λ_{ex}=345 nm, λ_{em}=460 nm) was purchased from Invitrogen. Propidium iodide (PI, λ_{ex}=546 nm, λ_{em}=575–640 nm) was purchased from Aldrich.

B. Preparation of lipofectamine-pDNA complexes

pDNA was diluted to a working range of 28 μg in 100 μl (280 μg/ml). Lipofectamine was diluted using a serum-free medium (SFM: DMEM without the addition of fetal bovine serum (FBS). Different LPF/DNA (w/w) ratios were used to optimize the lipoplexes for the film loading part, and a LPF/DNA ratio of 8 (w/w) was selected as a standard for the complex preparation for the film loading part as higher transfection efficiency was observed at this (w/w) ratio (data not shown here). Lipofectamine was added to the pDNA such that the lipofectamine was eight times the weight of pDNA. The complexes were vortexed vigorously, and then they were incubated at room temperature for 30 min. No agglomeration was noted in the sample preparation.

C. Preparation of gelatin solution

Gelatin solution (10% w/v) was prepared in water (w/v) and autoclaved.

D. Incorporation of complex into the PCL films

A 6.6% (w/v) PCL solution was made by dissolving the polymer in chloroform. Then, 300 μl of PCL solution was mixed with 100 μl of SFM containing LPF/DNA or pDNA, and the mixture was vortexed at 2200 rpm and was cast as a film. Gelatin-containing films were prepared by adding a gelatin solution (to obtain a gelatin content of 10% w/w in the final film) to SFM containing LPF/DNA or pDNA.

All films were dried in the laminar hood for 3 days to enable the evaporation of the solvent. The specific concentrations selected here are based solely on the quality of the emulsion obtained. The choice of chloroform is mandated by pDNA activity retention in chloroform compared to other organic solvents. To determine this effect of solvent on bioactivity, we incubated the lipoplex with 300 μl of different solvents (acetone, chloroform) for 1 h and then carried out the transfection studies. Lipoplexes prepared with fresh SFMs were used as a control.

E. Fluorescence imaging of pDNA, lipoplex, and gelatin onto polymer films

The spatial distribution of pDNA, lipoplexes, and gelatin in the polymer films was determined by a confocal fluorescence microscope (Leica TCS SP5) equipped with a UV laser (405 nm) to induce the blue fluorescence of DAPI with 345 nm excitation wavelength and emission wavelength at 460 nm. Green fluorescence of Oregon Green 488-tagged gelatin was induced by the 488 nm laser (488 nm excitation and detected at 515–565 nm wavelengths). The images were taken by a sequential scan and were later processed and analyzed by LAS AF software. Polymeric films with and without fluorescent labeled gelatin (Oregon Green® 488 Gelatin) were prepared as described above by the emulsion technique for the incorporation of pDNA and lipoplexes. However, prior to the formation of lipoplexes, DNA was labeled with blue-fluorescent dye, DAPI. For confocal imaging, dry polymer films were washed with phosphate buffered saline (PBS) and then imaged immediately on a Leica confocal fluorescence microscope.

F. Bioactivity studies

The pDNA and lipoplex were released from the polymer films, and the released amounts were quantified using ethidium bromide (EtBr), as described in our previous report.[5] The released pDNA/lipoplexes were collected at predetermined intervals, and a volume containing 0.5 μg of DNA (pDNA/lipoplex) was then used for transfection studies.

Samples were air dried in the laminar hood for a few hours and resuspended in SFM (0.5 ml). COS-7 cells were seeded in 24-well tissue culture grade plates at 2×10^4 cells per well and were grown overnight to approximately 80% confluence under normal growth conditions. On the following day, the cell culture medium was removed, and SFM containing the released pDNA/lipoplexes was added to the cells. The cells were incubated with pDNA/complexes at 37 °C in a 5% CO_2 incubator for 5 h. The medium was replaced with 0.5 ml of 2X medium and was left overnight; 0.5 ml of 1X medium was added on the following day. Luciferase activity was determined using a Luciferase assay kit (BD Sciences) after 48 h of transfection. The total protein was assayed using a Micro BCA protein assay (Pierce, Rockford, IL). The activity was normalized to the total protein content.

G. Particle size measurement

Particle size measurement of the LPF/pDNA complexes was performed by photon correlation spectroscopy (PCS) using Zeta Sizer NS (Malvern Instruments Ltd., Malvern, U.K.). Samples were diluted in PBS and measured in glass cuvettes at 25 °C and a scattering angle of 90°.

H. Cellular tracking of pDNA/lipoplexes by confocal fluorescence microscopy

COS-7 cells (1×10^5 cells/well) were cultured in a six-well culture dish for 24 h in 1 ml of the DMEM medium supplemented with 10% FBS (10% FBS DMEM) and were grown overnight to approximately 80% under normal growth conditions under a humidified atmosphere of 5% CO_2 in air. On the following day, the medium was removed and the air dried released samples were treated with fresh SFM (0.5 ml). This was added to the cells. The cellular entry of PI labeled pDNA/complexes was monitored by a confocal fluorescence microscope (Leica TCS SP5). First, the cells treated with samples were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. After washing twice 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 confocal laser scanning microscopy with an argon laser (488 nm excitation) to induce the green fluorescence of Oregon green, and their emission was observed using a band filter (515–565 nm). Red fluorescence was induced by the 546 nm excitation and detected at 575–640 nm wavelengths. However, prior to loading the lipoplexes into the polymer films, plasmid DNA was labeled with PI (λ_{ex}=546 nm, λ_{em}=575–640 nm). PI (a red dye) is more contrasting against gelatin (labeled green) inside the cell. So, it was used to label the lipoplex/pDNA. In this case, DAPI was used to stain the nucleus of the cell so as to differentiate between the nucleus and the lipoplex/pDNA entering the cell. Plasmid DNA was mixed with PI at a 1:1 weight ratio of pDNA to PI in the TE buffer (10 mM tris-HCl; 1 mM ethylenediamine tetra-acetic acid; pH 7.4). The solution was incubated for 1 h at room temperature in the dark, and the mixture was dialyzed to remove the free PI.

I. Statistical analysis

All the experiments were performed in triplicates, and all the data were presented as mean ± standard deviations. Analysis of variance has been done to indicate the statistical significance of the observations. Differences were considered statistically significant at $p < 0.05$.

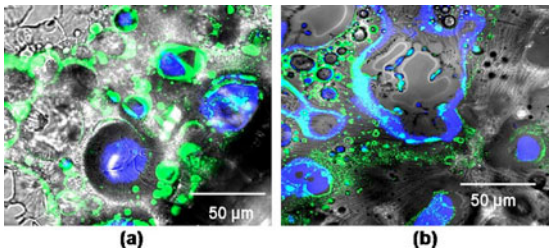

FIG. 1. (Color online) Confocal images of (a) PCL film with labeled pDNA and labeled gelatin and (b) PCL film with lipoplex prepared with labeled DNA and labeled gelatin.

III. RESULTS AND DISCUSSION

A. Fluorescence imaging studies of PCL/gelatin film

When a bioactive molecule is incorporated in a polymer film, it may be either dispersed or dissolved in the matrix. Both pDNA and lipoplex, when incorporated in PCL, are expected to be dispersed in the hydrophobic PCL matrix as both of them are considered fairly hydrophilic relative to PCL. However, because of the partial hydrophobicity (due to the fatty acid chain), lipoplexes dissolve more in the hydrophobic PCL matrix compared to pDNA. When gelatin is blended with PCL, the distribution of pDNA or the lipoplex may be substantially different. First, to characterize the distribution of pDNA, lipoplexes, and gelatin in the PCL film, fluorescence imaging studies were performed by confocal fluorescence microscopy. Plasmid DNA was labeled with DAPI, a blue-fluorescent nucleic acid stain, which preferentially stains dsDNA, and lipoplex was prepared with DAPI labeled pDNA.

Figures 1(a) and 1(b) show the pDNA and lipoplex distribution in a PCL film with fluorescent labeled gelatin (Oregon Green® 488 Gelatin). We could see some regions of overlap between pDNA/lipoplex and gelatin phases, indicating some degree of miscibility between pDNA/lipoplex and gelatin. So, it is clear that both pDNA and lipoplex tend to associate with gelatin rather than with the continuous matrix (PCL). In other words, immiscibility in the PCL regions of the films indicates the preferential partitioning of pDNA and lipoplex in gelatin (as compared to PCL).

B. Bioactivity studies

Transfection experiment was carried out to evaluate the bioactivity of complexed DNA (lipoplex) using different solvents. Figure 2 shows the effect of solvents on the bioactivity of lipoplexes. Transfection efficiency was much better with chloroform when compared with the other solvent (acetone). Since the chloroform seems to affect the complexed DNA (lipoplex) much less than acetone, it was decided to use chloroform as a solvent for the film's formulations.

The bioactivity of the released DNA was determined by *in vitro* cell transfection efficiency using COS-7 cell lines. Transfection efficiency of released DNA (pDNA/complexed DNA) from polymer films was observed by an inverted phase contrast microscope. The transfection is quantified by using enzyme (luciferase) activity, normalized using the total protein content of cell extracts. Transfection efficiency of released DNA from polymer films was studied at all the time points for the release study.

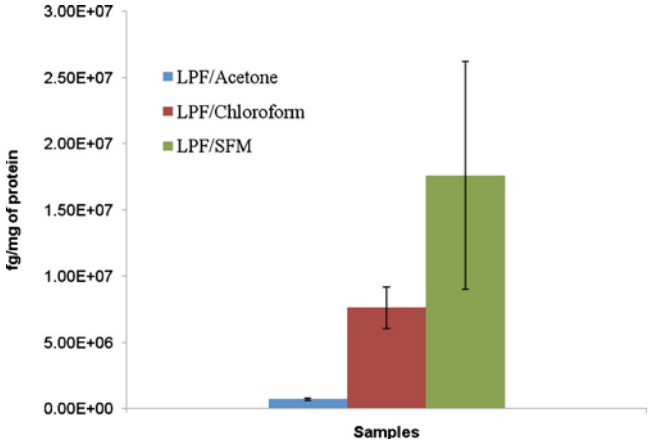

FIG. 2. (Color online) Effect of solvents on bioactivity of lipoplex.

Figure 3 shows the comparative transfection efficiency of pDNA and lipoplex released from PCL films with gelatin on day 5. Transfection efficiency of the released pDNA is much lower than lipoplexes as expected. This is mainly due to the repulsion of the negatively charged DNA (−78 mV) and the negatively charged cell membrane and to the consequent lesser ability to penetrate the cell membrane, whereas the complex carries a net positive charge. In addition, the lower mobility of pDNA in the cytoplasm may also contribute to a lower observed transfection efficiency assessed after 24 h of incubation. Thus, lower transfection efficiency is mainly due to the large size and the negative charge density of pDNA.[22,23]

Figure 4 shows the comparison of transfection efficiency of released lipoplexes from PCL films with lipoplexes (free) incubated in PBS. Clearly, incubating lipoplexes in a buffer over 20 days does not alter the transfection efficiency,

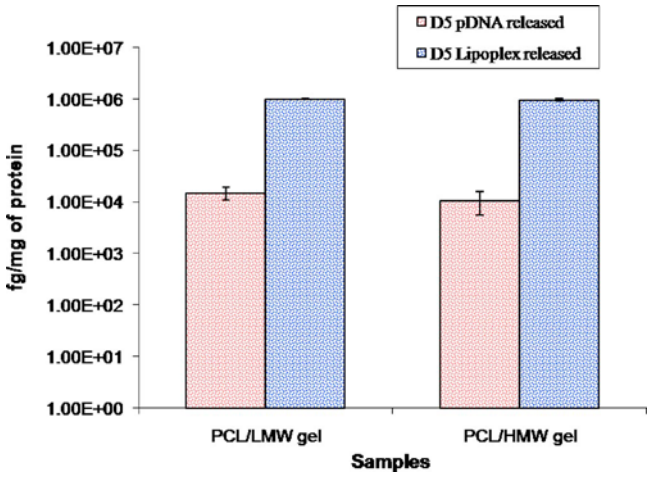

FIG. 3. (Color online) Transfection of released pDNA vs lipoplexes from PCL/gelatin films.

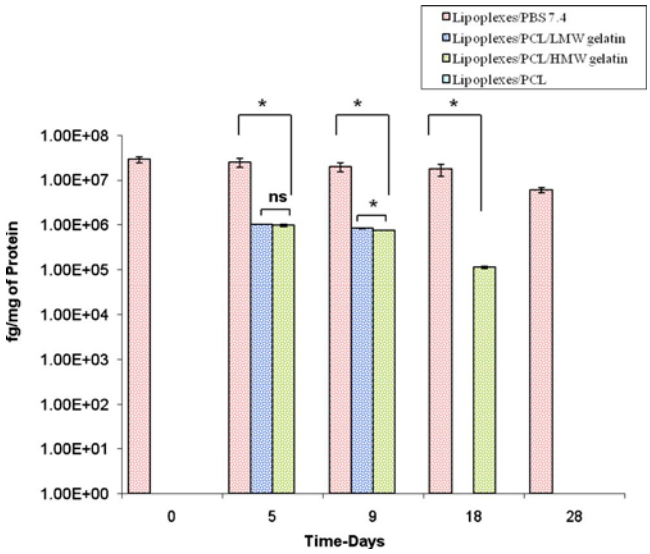

FIG. 4. (Color online) Comparison of transfection of released lipoplexes from films with lipoplexes incubated in PBS.

whereas lipoplexes released from polymer films show a drop over time. In particular, lipoplexes released from PCL films show no transfection at all, even on day 5. Second the addition of either low-MW or high-MW gelatin substantially increases the transfection efficiency compared to lipoplexes released from PCL films (Table I). We discuss the reasons for this further below based on the details of intracellular trafficking. When we compare the high- and low-MW gelatin, it appears that by day 18, only the film with high-MW gelatin continues to release a bioactive lipoplex. In general, however, the transfection efficiency decreases over time of residence in all the polymer films.

Various possibilities can be postulated for such decreased transfection efficiency, e.g., reduced DNA release, reduced pDNA transfection capability, and degradation of DNA. In our previously reported work,[4,5] we found that there is an adequate amount of lipoplexes released from the matrices under study. So, we can rule out the possibility of reduced release from the matrix. As far as transfection capabilities are concerned, we have observed reasonable transfection in the early release time points, which shows that lipoplexes have retained their transfection capability. The third possibility, lysosomal degradation of the lipoplex, seems to be the more likely explanation as there is reduction in transfection efficiency with time. What causes this increased degradation in the cytoplasm is not clear to us at present

In order to further explore the reasons for the differences in the transfection efficiencies of the released lipoplexes, the extent of decomplexation of the lipoplex (Table II), as well as the particle size of the released lipoplexes, was determined. From the particle size data (Table III), it is clear that one of the reasons for decreased transfection with the time of release is aggregation of the released complexes (compared to the lipoplex in the buffer), which reduces nuclear penetration. The size of the released lipoplexes from PCL film increases to 590 nm, while those released from PCL/LMW gelatin films increases to about 700 nm and to about 710 nm from PCL/HMW gelatin films by day 18. In contrast, lipoplexes incubated in a buffer for the same length of time show an increase in size from about 60 nm on day 0 to about 175 nm on day 18 (Table III). Although there is a possibility of detecting gelatin molecules, which could be present in the sample, the size distribution was obtained in a single peak, and thus we can confirm that the lipoplexes fall in the same size range.

So, one of the factors for reduced transfection of the released lipoplexes is increased size. However, this factor does not explain why the released lipoplexes from PCL/gelatin films continue to show some transfection while those released from PCL films (which are actually smaller in size) do not transfect at all. This difference must be related to the intracellular fate of these particles or to the decomplexation of the lipoplexes.

Considering the degree of decomplexation, however, we find that (Table II) released lipoplexes from PCL in fact show complete retention of the complexed form. Thus, neither aggregation nor decomplexation levels explain why

TABLE I. Amount of transfection (fg/mg of protein) of released DNA from different polymer films.

	Transfection amount (fg/mg of protein)			
	Lipoplex			pDNA
Samples	Day 5	Day 9	Day 18	Day 5
PCL	⋯	⋯	⋯	⋯
PCL/LMW gelatin	$1.00\times10^{+06}$	$8.35\times10^{+05}$	⋯	$1.50\times10^{+04}$
PCL/HMW gelatin	$9.75\times10^{+05}$	$7.32\times10^{+05}$	$1.13\times10^{+05}$	$1.07\times10^{+04}$

TABLE II. Percentage (%) of complexed form of released lipoplexes at each time point.

	Complexed form (%)		
Samples	Day 5	Day 9	Day 18
PCL/lipoplex	100	100	100
PCL/LMW gelatin/lipoplex	79	85	90
PCL/HMW gelatin/lipoplex	72	80	86

TABLE III. Particle size of lipoplexes (lipoplexes incubated in PBS vs released from polymer films).

	Particle size (nm)			
Samples	Day 0	Day 5	Day 9	Day 18
Lipoplex (incubated in PBS)	60 ± 6	120 ± 9	160 ± 5	180 ± 8
PCL/lipoplex	⋯	440 ± 11	530 ± 9	590 ± 13
PCL/LMW gelatin/lipoplex	⋯	460 ± 12	515 ± 8	700 ± 6
PCL/HMW gelatin/lipoplex	⋯	420 ± 9	530 ± 7	710 ± 10

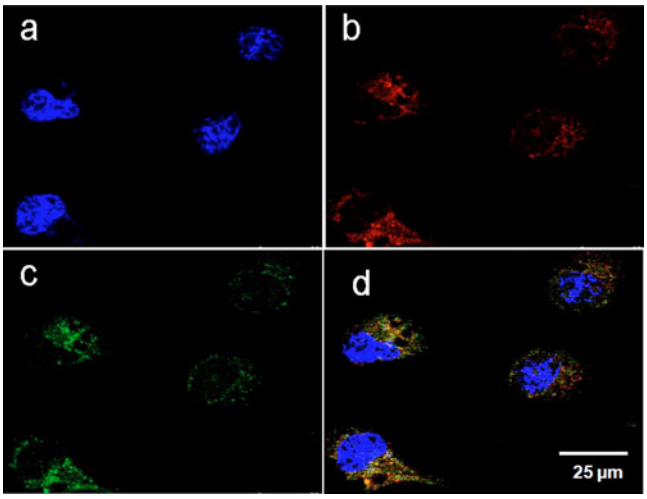

FIG. 5. (Color online) Intracellular distributions of released lipoplexes and gelatin at day 5 from PCL/gelatin film (2 h after transfection). (a) Blue fluorescence indicates the nucleus. (b) Red fluorescence indicates the localization of lipoplexes. (c) Green fluorescence indicates the localization of gelatin. (d) Merged image of all fluorescence.

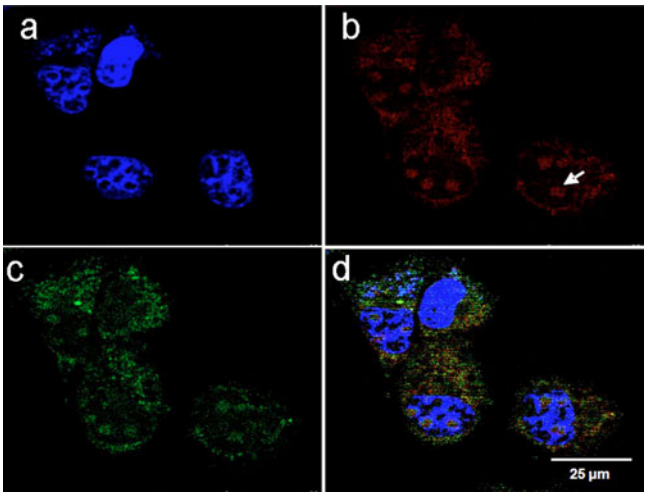

FIG. 6. (Color online) Intracellular distributions of released lipoplexes and gelatin at day 5 from PCL/gelatin film (24 h after transfection). (a) Blue fluorescence indicates the nucleus. (b) Red fluorescence indicates the localization of lipoplexes (white arrow indicates the presence of red fluorescence in the nucleus). (c) Green fluorescence indicates the localization of gelatin. (d) Merged image of all fluorescence.

gelatin-containing films release more bioactive lipoplexes. For this, we need to examine the details of the intracellular trafficking of these lipoplexes.

C. Cellular tracking of pDNA/lipoplexes by confocal fluorescence microscopy

In order to determine the reasons for the different levels of transfection observed from different film types, a study was undertaken to determine the intracellular localization of pDNA and lipoplexes at different time points. The cell nucleus was labeled with DAPI staining (blue color). Confocal laser spectroscopy was used to track the lipoplex and/or naked pDNA.

Figures 5 and 6 show the intracellular distribution of day 5 released lipoplexes and gelatin, taken 2 and 24 h after transfection, respectively. Figure 5 clearly shows the red fluorescence (PI labeled pDNA within the lipoplex) in the cell cytoplasm [Fig. 5(b)] as well as the green fluorescence of gelatin in the cell cytoplasm [Fig. 5(c)]. Thus, lipoplexes and gelatin are both internalized (in an associated manner) through the cell membrane, and after 2 h of incubation with COS-7 cells, lipoplexes and gelatin are present at or near the region of the cell nucleus. The merged image [Fig. 5(d)] shows the colocalization of lipoplex and gelatin in the cytoplasm. It is noteworthy that inside the cytoplasm, the gelatin is always associated closely with the lipoplex, with few unassociated complexes to be found elsewhere. The possibility of "cross-talk" between the gelatin and lipoplex fluorescence has been eliminated by sequential scanning at different laser wavelengths.

Figure 6 shows the cellular distribution of particles 24 h after transfection. We now see red and green fluorescences within the cell nucleus (a white arrow indicates the presence of red fluorescence), indicating that these gelatin-associated particles can still enter the nucleus. *In vitro* size measurement of the released lipoplexes on day 5 shows particles of around 460 nm. Since the nuclear pores allow free diffusion entry only for small particles ($\leq$50 nm), this unexpected entry of the larger particles ($\sim$460 nm) into the nucleus may occur either because the particles inside the cytoplasm are in fact much smaller than 460 nm or because such particles may have entered during the cell division when the nuclear membrane temporarily breaks down.[24,25] We cannot also rule out disassociation of the lipoplex prior to nuclear entry.

In contrast, let us examine the fate of lipoplexes released from PCL films (no gelatin). For lipoplexes released from a PCL matrix, Figs. 7 and 8 show the red fluorescence (PI

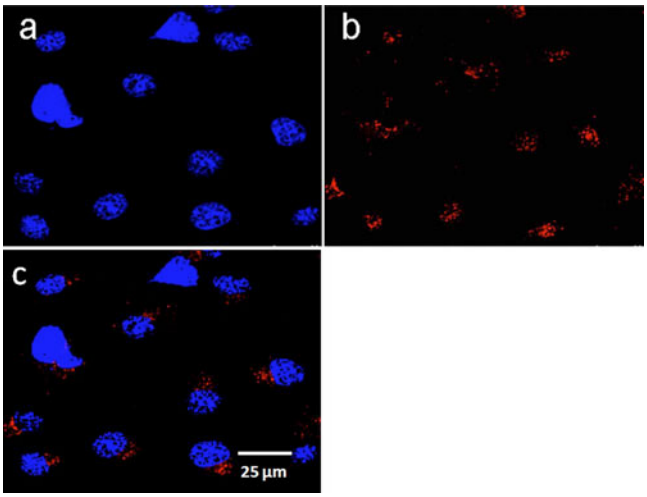

FIG. 7. (Color online) Intracellular distributions of released lipoplexes from PCL at day 5 (2 h after transfection). (a) Blue fluorescence indicates the nucleus. (b) Red fluorescence indicates the localization of lipoplexes. (c) Merged image of all fluorescence.

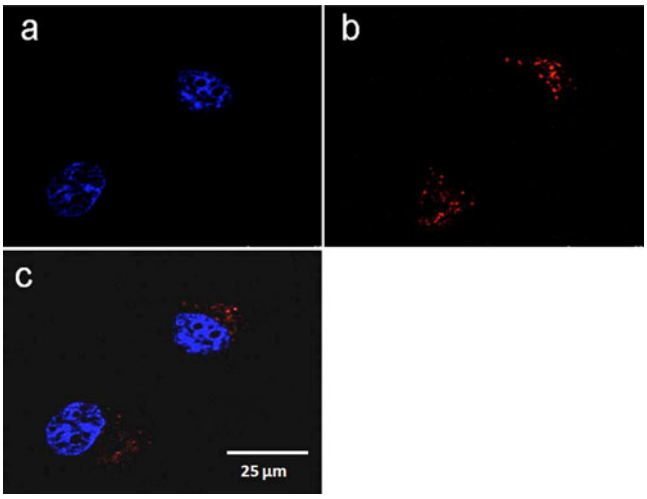

FIG. 8. (Color online) Intracellular distributions of released lipoplexes from PCL at day 5 (24 h after transfection). (a) Blue fluorescence indicates the nucleus. (b) Red fluorescence indicates the localization of lipoplexes. (c) Merged image of all fluorescence.

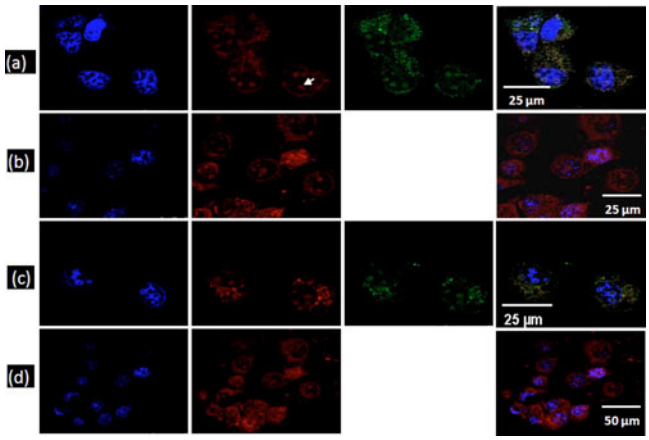

FIG. 10. (Color online) Comparison of intracellular distributions of released lipoplexes with lipoplexes incubated in buffer. (a) Released lipoplexes PCL/gelatin film at day 5. (b) Lipoplexes incubated in buffer at day 5. (c) Released lipoplexes PCL/gelatin film at day 9. (d) Lipoplexes incubated in buffer at day 9 (24 h after transfection).

labeled DNA) in the cell cytoplasm and near the nuclear membrane, but the fluorescence does not reach into the nucleus, even 24 h after transfection.

Figure 9 makes a side-by-side comparison of lipoplexes released from PCL and from PCL/gelatin films on day 5. Figure 9(a) shows that some of the lipoplexes released from PCL/gelatin films do in fact enter the nucleus, whereas this is not the case for the lipoplexes released from PCL [Fig. 9(b)]. It should also be noted that on a relative basis, there are many more lipoplexes (with gelatin) inside the cytoplasm compared to lipoplexes from PCL films.

Similar observations have been made for day 9 and day 18 (data not shown). From the intracellular trafficking pictures, it appears that gelatin-associated lipoplexes enter the cytoplasm in larger numbers and some of them penetrate the nucleus to show transfection. In contrast, fewer of the lipoplexes released from PCL (without any gelatin association) appear to be in the cytoplasm after 24 h of incubation, and none appear to penetrate the nucleus.

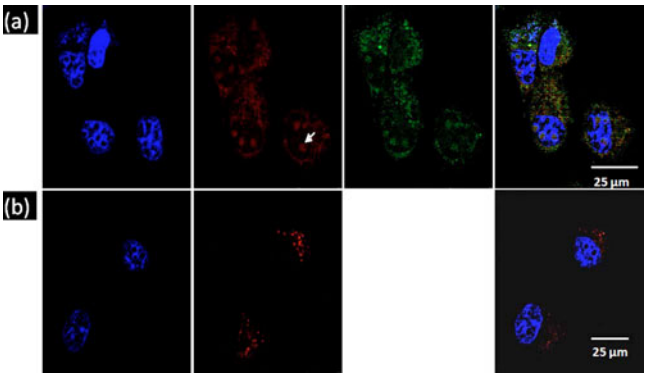

FIG. 9. (Color online) Comparison of intracellular distributions of released lipoplexes at day 5 from (a) PCL/gelatin film (white arrow indicates the presence of red fluorescence in the nucleus), (b) PCL film (24 h after transfection).

Thus gelatin-associated lipoplexes appear to survive in the cytoplasm long enough to allow for some to enter the nucleus. This protection afforded by gelatin has been previously observed in gelatin particles with incorporated plasmid DNA.[26] In that study, smaller gelatin nanoparticles entered the cells by nonspecific endocytosis and then survived endosomal attack in the cytoplasm, as observed by the visualization of the tagged particles in confocal microscopy. The reason for this protection is not entirely clear, although it is likely that the endosomal pH of 5–5.5 is not low enough to degrade gelatin (the measured pH of our gelatin solutions is around 4.7), and the lipoplexes are most likely coated with a layer of gelatin.

There is a size effect on transfection efficiency. In general, lower sizes allow for greater transfection. In order to illustrate what happens with lipoplexes simply incubated in a buffer, we show the images in Fig. 10. Such incubated lipoplexes grow in size upon incubation, but only to about 170 nm after 18 days. It is clear that much larger numbers of lipoplexes are present in the case of the incubated lipoplexes (compared to the released lipoplexes) inside the cytoplasm, and more also enter the nucleus [Figs. 10(b) and 10(d)]. This difference in behavior is due substantially to a size effect, as explained above, as the lipoplexes incubated in a buffer do not increase in size over 18 days as much as the released lipoplexes do (Table III). However, sizes are roughly comparable for lipoplexes released from PCL and from PCL/gelatin, so size is not the reason for the observed differences in the bioactivity of the released lipoplexes from these films. Clearly, there is protection conferred by the associated gelatin.

The possibility of autofluorescence of lipofuscin (oxidative stress product within lysosomes) was ruled out as the confocal images of the control cells treated with lipofectamine (same amount as lipoplex) did not show any fluorescence (data not shown here). The reason could be the

amount of lipofuscin (oxidative stress product within lysosomes) is not enough to produce the autofluorescence.

To summarize, we observed the following.

(i) Released lipoplexes from PCL films do not show any transfection on days 5, 9, and 18. These lipoplexes are larger in size compared to lipoplexes incubated for similar lengths of time in buffer and are also fully complexed (Table II). However, confocal studies show very few lipoplexes inside the cytoplasm and none at all in the nucleus. We believe that this is most likely due to endosomal/lysosomal degradation of the larger particles.

(ii) When lipoplexes are released from PCL-gelatin films, they could be associated with gelatin. In the cytoplasm, this association is clearly seen using confocal microscopy. Such lipoplexes appear to be in the cytoplasm in larger numbers, with some of them reaching the nucleus in spite of their large size. Thus, gelatin association of the lipoplex appears to protect them from endosomal/lysosomal degradation. At this point, we are not sure of the mechanism for this protection, and further studies are planned to address this.

(iii) In general, larger-sized lipoplexes find it more difficult to penetrate the nucleus. This inference is drawn from comparing the higher transfection of incubated lipoplexes (which increase modestly in size to about 170 nm) with that of the released lipoplexes (which increase in size to about 700 nm).

(iv) Although it is possible that the increased size may decrease nuclear penetration, the size effect does not explain why gelatin-associated particles appear to transfect even on day 18 while PCL-released lipoplexes do not (they are similar in size and hence must be able to enter the cell in roughly equal measure). It is the cytoplasmic fate of these lipoplexes that appears to be different, as explained above.

It should be mentioned here that we have chosen to use serum-free media for release and for transfection studies. It is acknowledged that the presence of serum proteins would influence the transfection efficiency through protein binding to pDNA,[27–29] although other authors have not reported any effect on the lipoplex uptake.[29] Nevertheless, to isolate the effect of the carrier film on the bioactivity of released pDNA or lipoplex, it is more illustrative to assess this in serum-free media as the serum proteins may confound the assessment of bioactivity. This was the main focus of this work.

The site of injection may also play a role: For example, release in subcutaneous tissue may not involve much interaction with serum proteins. So, to understand the differences in bioactivity induced by the encapsulating polymer, serum-free media are a good starting point. Future studies will address the effects of serum.

IV. CONCLUSIONS

Our group has reported earlier that both naked (pDNA) and complexed DNA with cationic agents (lipofectamine) can be successfully incorporated into slowly degrading polymer (PCL) films and can be released from these films in a sustained manner. However, the lipoplexes released from different films show differing levels of bioactivity, as assessed by transfection efficiency in COS-7 cells. In this study, we present possible reasons for the observed differences in transfection levels. Based on the confocal studies, we show that lipoplexes released from PCL films are able to enter the cells but unable to transfect due to degradation in the endosomes/lysosomes. On the contrary, association of these lipoplexes with gelatin enables them to survive this degradation enough to show transfection up to day 18. However, increased size of lipoplexes in general leads to reduced transfection due to reduced nuclear penetration. Further studies are underway to pinpoint the reasons for the protective effect conferred by gelatin association.

It should be noted that the *in vivo* situation is likely to be favorable than the *in vitro* environment, as released pDNA or the lipoplex may in fact transfect cells soon after release and may be less aggregated than in the *in vitro* case. On the other hand, serum proteins may play a significant role if the pDNA or lipoplex is meant to exert its action by circulation in blood.

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