Abstract: This report investigates the comparative in vitro controlled release and transfection efficiencies of pDNA-lipofectamine complex (lipoplex) and pDNA-poly(ethylene imine) complex (polyplex), from a biodegradable polycaprolactone (PCL) film. The effect of molecular weight of gelatin used as a porogen on in vitro release and transfection efficiency was also studied. A sustained release profile was obtained for naked pDNA and lipoplex from polymeric films for a month, while the release of polyplexes (PEI/DNA) is simply a burst at day 5, with little or no release thereafter. The release of polyplexes from PCL films is retarded due to interaction between the polyplexes and the polymer. A high burst release was seen for naked pDNA which was suppressed in the presence of gelatin. The extent of suppression of the burst effect by gelatin increased with its molecular weight. For complexed pDNA (lipoplex), the release was slow, but could be accelerated using gelatin; again the acceleration in release is dependant on the molecular weight of the gelatin used. The addition of gelatin as a porogen has no effect on the release of polyplexes from PCL films. The bioactivity of released plasmid DNA and complexes was studied by in vitro transfection using COS-7 cells. Transfection was observed from released lipoplex samples till day 9 from PCL film with lower MW gelatin and till day 18 in the case of PCL films with higher MW gelatin. The results also showed that the bioactivity of released lipoplexes was superior to that of the naked pDNA.

Keywords: lipoplex; polyplex; PCL film; gelatin; gene transfection; sustained release

INTRODUCTION

The delivery of plasmid DNA encoding a therapeutic protein or tissue inductive proteins presents a powerful alternative to the delivery of the protein itself. The inherent chemical stability of DNA facilitates the application of traditional methods established for the controlled release of proteins toward achieving controlled gene delivery.

The success of therapeutic gene delivery, however, requires a system that both facilitates cellular uptake of DNA and maintains a prolonged period of gene expression. Although viral vectors present high transfection efficiency and sustained expression, the risks and limitations associated with their safety has placed an increasing attention on the exploration of nonviral methods for gene delivery. Nonviral vectors also have important disadvantages, including their low efficacy and transient gene expression. However, a problem common to both vectors is the need for repeated administration of the vector with pDNA. To avoid this, we reported on one method for sustained delivery of the complexed and naked pDNA in an earlier paper. The earlier report focused on pDNA complexed with lipofectamine (lipoplex). In this article, we report on the release of polyplex (pDNA complexed with poly(ethylene imine), or PEI, also known as polyplex) from polycaprolactone (PCL) films, and the subsequent transfection efficiencies. We evaluated a hydrophilic additive (gelatin) and its effects on the release.

PCL is biodegradable polyester with a low melting point (62°C) and a low glass transition temperature. PCL is rubbery at room temperature, which contributes to the very high permeability of PCL for many therapeutic drugs. The rate of release of proteins from PCL microspheres has been reported to be rather slow, and the release is diffu-
sion controlled. Gelatin is a natural polymer that is derived from collagen, and has been extensively used for pharmaceutical and medical applications, because of its biodegradability and biocompatibility. Gelatin is also used as a porogen material to fabricate porous PLA scaffolds for tissue engineering. Recently, various biodegradable polymers have been explored for the delivery of plasmid DNA. DNA encapsulation efficiency is very low with PLGA, and DNA damage has been observed as a result of PLGA degradation. Howard et al. show more of a sustained release from PLGA microparticles, but there is also a significant initial burst effect for PEI/DNA complex. Hydro gels have been used to increase encapsulation efficiency, but the release is relatively rapid, thus making it difficult to sustain release for long periods. Sacks et al. found a substantial 50% burst effect of lipofectamine-pDNA complex on day 1 from collagen films.

Except for our earlier work, we extend the study of the sustained release of complexed pDNA to include pDNA complexed with PEI (polyplex), and compare its release characteristics to that of lipofectamine-complexed pDNA (lipoplex); we use gelatin as a release-modifier, as reported earlier for lipoplex release.

To be specific, plasmid DNA, both “naked” and complexed with PEI, was loaded into a slow degrading polymer (PCL), and the release of the polyplex was evaluated and compared with the release of pDNA and of lipoplex pDNA. The observed differences are then rationalized on the basis of specific polymer-DNA interactions, and confirmed using FTIR. We extended the studies to include the effect of different types of gelatin (higher molecular weight and lower molecular weight gelatin) on the release of pDNA and complexed DNA.

MATERIALS AND METHODS

Reagents

pDNA Dual vector pEGFPLuc was purchased from Clontech (supercoiled, 6.4 kb) encoding a fusion protein of enhanced green fluorescent protein (EGFP) and luciferase from the firefly Photinus pyralis. It was propagated according to the standard method using a giga filter kit from Qia-tech. Branched poly-ethylenimine, PEI ($M_w$ 25,000) was provided by Sigma. Lipofectamine (LPF) was provided by Invitrogen Life Technologies. Sodium oleate salt was provided by Sigma Chemicals. It was dissolved in water, pH 7.4 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. Poly caprolactone ($M_n$ 80,000, PDI 1.4), Poly (ethylene-co-vinyl acetate) (40% of vinyl acetate by weight, $M_w$ 100,000), and gelatin [type A, medium bloom: 175, ($M_L$ 40,000) (LMW) (pl 7–9) and type A, high bloom: 300, ($M_L$ 50,000–100,000) (HMW) (pl 7–9)] were purchased from Sigma Chemicals as well. Chloroform was purchased from Tedia, in GR grade. The films were cast with the Automatic Film applicator AG-2150 purchased from BKY Gardner. Dulbecco’s Minimum Essential Medium (DMEM), Penicillin, and Streptomycin were supplied by Invitrogen. Luciferase Reporter Assay Kit was provided by BD Clonetech. Micro BCA TM Protein Assay Reagent kit was provided by Pierce Biotechnology, Rockford, IL.

Preparation of Lipofectamine-pDNA Complexes

pDNA was diluted to a working range of 28 µg in 100 µL (280 µg/mL). LPF was diluted using serum free medium (SFM: DMEM without the addition of fetal bovine serum). LPF was added to the pDNA such that the lipofectamine was eight times the weight of pDNA. The complexes were vortexed vigorously, and then incubated at room temperature for 30 min. No agglomeration was noted in the sample preparation.

Preparation of PEI-pDNA Complexes

pDNA was diluted to a working range of 28 µg in 100 µL (280 µg/mL). PEI was diluted using serum free medium (SFM: DMEM without the addition of fetal bovine serum). PEI was added to the pDNA such that the PEI was four times the weight of pDNA. The complexes were vortexed vigorously, and then incubated at room temperature for 30 min. Some agglomeration was noticed during incubation; this was broken down by pipetting and vortexing before the complexes were mixed with the polymer solution.

Preparation of Gelatin

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

Incorporation of Complex Into the Films

A 6.6% (w/v) PCL solution was made by dissolving the polymer in chloroform. About 300 µL of this solution was mixed with 100 µL of PEI/DNA or LPF/DNA or pDNA in SFM. The ratio between the pDNA/PEI-DNA/LPF-DNA in SFM and polymer is 1:3. PCL solution was added to the SFM, vortexed at 2200 rpm, and cast as a film. Gelatin films were made with the addition of 10% gelatin (w/w) (HMW/LMW) of the polymer.

All the films were dried in the laminar hood for 3 days, for 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 when compared with solvents such as dichloromethane or acetone.

SEM Study

The morphology of the films was analyzed by field emission scanning electron microscopy (JEOL JSM-6340). The dried film samples were cut in to small pieces and mounted...
on an aluminum tape using a double sided carbon tape. The samples were coated with platinum using a Fine Auto Coater (JEOL JSM-6340). The coated samples were examined using an electron acceleration voltage of around 5 kV.

Quantification of the Released pDNA/LPF-pDNA/PEI-pDNA Complexes

The naked pDNA concentration was determined using Ethidium Bromide (EtBr) with $E_x = 485$ nm and $E_m = 590$ nm. The measurement was done on a Bio-Tek, FLX 800—multi detection micro plate reader. A standard curve was constructed with different concentrations of pEGFP\textit{Luc} (pDNA) in the presence and absence of sodium oleate. The DNA concentrations were determined from unknown samples by comparison to the standard curve. The naked pDNA loaded film samples were collected and quantified directly. In the case of films loaded with complexed pDNA, the pDNA had to be disassociated from LPF/PEI before quantification. Sodium oleate and heparin sulphate was used for the dissociation of lipoplexes and polyplexes, respectively. The amount of sodium oleate and heparin sulphate added was about 10 times higher than the LPF and PEI amounts, respectively. The samples were incubated at 37°C for 4 h for lipoplexes and for 1 h for polyplexes. To the decomplexed samples, about 60 μL of ethidium bromide (80 μg/mL) were added and mixed well and the fluorescence intensity was measured. Appropriate blank solutions of PCL with sodium oleate/heparin sulphate at different intervals were used to get the final value. Another determination of free pDNA (without the decomplexing step) was also carried out on the released lipoplex samples, to quantify the amount of uncomplexed and complexed pDNA at each time point.

Polymer Degradation Study

Size exclusion chromatography from Agilent Technologies 1100 Series was used for the degradation study of all the polymer films before and after the immersion in water. A PLgel 5 μm-mixed C column was used with the mobile phase chloroform. The flow rate was set at 1 mL/min and about 100 μL of sample was injected. A separate set of films were prepared and collected at different intervals for the GPC studies. Wet films were dried before dissolving in the mobile phase.

In Vitro Release Study

The films were placed in a 12 well cell culture plates. About 0.8 mL autoclaved water was used for the in vitro studies. Plates were incubated at 37°C at different time points ($t = 5, 9, 18, 23, 30$ days). At every time point the buffer was removed and replaced with the fresh buffer. About 1% antibiotic (ampicillin) was used to avoid contamination.

In Vitro Bioactivity

The released samples of pDNA/ lipoplexes/polyplexes from the polymer films were air dried in the laminar hood for a few hours. Cos-7 cells were seeded in a 24 well tissue culture grade plates at $2 \times 10^5$ cells per well and grown overnight to ~80% confluence under normal growth conditions. On the following day, the medium was removed and the air dried samples were treated with SFM (0.5 mL). This was added to the cells. The cells were incubated with complexes at 37°C in a 5% CO$_2$ incubator for 5 h unless otherwise stated. Luciferase activity was determined after 48 h transfection using Luciferase assay kit (BD Sciences). The total protein was assayed by micro BCA protein assay (Pierce, Rockford, IL). The activity was normalized to the total protein content.

From our experience, we know that ~1 μg of pDNA in complexed form must be available to provide detectable transfection. Hence, the time points were dictated by the amount of pDNA released in that time interval: if the released amount was sufficient, we proceeded with transfection studies. Thus, the time intervals for different formulations are not exactly the same.

Particle Size Measurement

Particle size measurement of LPF/pDNA complexes were performed by photon correlation spectroscopy and electrophoretic mobility, respectively on a zeta sizer NS (Malvern Instruments) instrument bearing integrated size and zeta module, equipped with a 10-miliWatt helium neon laser at a wavelength of 633 nm and the Malvern PCS software. Samples were measured in glass cuvettes at 25°C at a fixed scattering angle of 90°. Samples were diluted in PBS, which was filtered through a 0.2 μm membrane.

RESULTS

Surface Morphology of Polymer Films

The thickness of all the dried films was around 80 μm, measured using a micrometer. Gelatin acts as a porogen and leaches out of the film, it leaves pores behind. The presence of pores is visible in the SEM pictures (Figure 1).

Degradation Studies of Polymer Films by Size Exclusion Chromatography

A slight drop of molecular weight from day 23 to day 30 is noted in samples with high molecular weight gelatin. No significant molecular weight drop was observed in films with 0% gelatin/LMW gelatin (Figure 2).

Naked DNA Release

The release profile of pDNA from PCL films is shown in Figure 3. The release profile shows a biphasic behavior, a burst phase followed by diffusion-controlled phase. The
release is dominated by the burst release (50% by day 5) of undissolved pDNA.4 The films loaded with low molecular weight and high molecular weight gelatin release 62% and 45% of pDNA at the end of 30 days.

Figure 1. FESEM images of polymer films surface on day 23; (a) PCL film alone, (b) PCL film with LMW gelatin (10% w/w), and (c) PCL film with HMW gelatin (10% w/w).

Figure 2. Degradation profile of PCL films.

Figure 3. Effect of gelatin on the release % of pDNA from PCL films.

Lipoplex Release

The release profile of lipoplexes from PCL films is shown in Figure 4. In contrast to the pDNA loaded films, no burst effect is observed with the lipoplex loaded samples.4 The addition of gelatin increases the release of lipoplexes. High
molecular weight gelatin shows the highest 5-day release of about 60% as compared with low molecular weight (10%) and films with no gelatin (3%).

**Polyplex Release**

The release profile of polyplexes from PCL films were shown in Figure 5. A very slow release is observed for films in the absence of gelatin. The addition of gelatin, in this case, suppresses the release of the polyplexes. The reason for this will be explored further in the Discussion section.

**Lipoplex Versus Polyplex Release**

A comparison of lipoplex and polyplexes release from PCL films in the absence of gelatin is showed in Figure 6. Clearly, the release of the polyplexes is considerably slower than that of the lipoplexes, for reasons to be discussed below.

**FT-IR Analysis**

The FTIR analysis (Figures 7 and 8) show a shift in the N—H vibration peak of the PEI (Figure 9), from 1595 cm$^{-1}$ to about 1548 cm$^{-1}$. No such peak shift is seen for the LPF (Figure 10) peaks in the lipoplex.

**In Vitro Bioactivity of Released DNA**

The released samples were used for transfection studies in COS-7 cells. This is done to test the bioactivity of the released samples.

The amount of polyplexes released from polyplexes/PCL films was low. Hence, transfection was not performed with polyplexes. The transfection results for the pDNA and lipoplexes released from the polymer films are shown graphically in Figures 9 and 10.

In the case of PCL films, lipoplexes loaded with LMW gelatin were studied for transfection on day 5 and 9. In addition the transfection studies were extended till day 18 for HMW gelatin/lipoplexes films.
A comparison of transfection results between naked DNA and lipoplexes (day 5, LMW/HMW gelatin) is shown in Figure 10. The lipoplexes show better transfection rates when compared with naked pDNA as expected. In addition a slight increase in transfection efficiency for lipoplexes in HMW gelatin films was noted.

As a positive control, we have evaluated the transfection efficiency of lipoplexes which have been incubated in buffer for the same lengths of time as the released lipoplexes from the films. A comparison of transfection results between the lipoplexes (incubated in PBS for days 5, 9, 18, 28) and the lipoplexes released from polymeric films is shown in Figure 13. It is shown that the lipoplexes released from polymeric matrices are not as efficient as the control lipoplexes.

**DISCUSSION**

**pDNA Release**

The biphasic release of naked pDNA from PCL films is dominated by the lack of dissolution of pDNA. The addition of gelatin suppresses the burst release of pDNA from PCL films. As gelatin is added, more of the pDNA partitions into the gelatin, as the solubility is higher in gelatin, thus suppressing the burst effect. The burst effect suppression is enhanced with the higher molecular weight gelatin. This is due to the slower partitioning of the gelatin phase, as the molecular weight of gelatin increases. In addition to the solubilization effect of gelatin, it is also possible that the positive charge of gelatin interacts with the negatively charged pDNA, suppressing the burst and retarding the release of pDNA from PCL films.

**Lipoplex Release**

The burst effect is suppressed for films loaded with lipoplexes. This is due to the lipophilic nature of lipoplexes, which results in the uniform dissolution of the complexes in a hydrophobic matrix.

The addition of gelatin increases the release of lipoplexes from PCL films. High molecular weight gelatin shows the highest 5-day release of about 60% when compared with low molecular weight (10%) and films with no gelatin (3%) (Figure 4). The addition of a hydrophilic polymer gelatin reduces the overall amount of hydrophobic polymer available for lipoplexes to dissolve, and this might lead to some lipoplex being ejected from the dissolved phase, leading to a noticeable burst effect. The magnitude of the burst release increases with the increase in the molecular weight of gelatin. In addition, the rate of release increases around day 23 for both the lower molecular weight and higher molecular gelatin samples. The GPC data (Figure 2) does not show any substantial differences in degradation among the three samples, around day 23. Thus the higher earlier release obtained with gelatin addition may be due to a combination of pore creation [Figure 1(b,c)] and lipoplex “precipitation.” The reason for the substantially higher burst seen for the higher molecular weight is not clear at present and it will be explored in future studies.

**Polyplex Release**

In the case of polyplexes, there is no appreciable burst effect (Figure 5). As is the case with the lipoplex, the condensation of DNA with PEI has prevented the large burst
effect seen in the case of free pDNA. The burst effect observed for pDNA could be ascribed to differential solubility: pDNA is hydrophilic and does not dissolve appreciably in the hydrophobic polymer (PCL) matrix; additionally, there may be some repulsion between the negatively-charged pDNA and the slightly-negatively charged PCL. In the case of polyplexes and the lipoplexes, firstly the complexed pDNA is more hydrophobic and hence dissolves more in the matrix. This suppresses the burst effect. The additional “incubation period” (where there is no detectable release of the complexed pDNA) could be caused by polymer-pDNA complex interactions: the excess positive charge on the complex (due to excess molar amounts of PEI) could interact with the negatively charged PCL, in the same way that the PEI interacts with the pDNA. However, this explanation is not satisfactory, as lipoplex release should also be similarly affected, but is not (Figure 6). Thus the more valid explanation is that there is hydrogen bond formation between C=O groups of PCL and N/C0/C0H groups of PEI, as explained below (Section 4.4), an interaction not seen for lipoplex in PCL. The effect of gelatin (Figure 5) is further suppressing the release implies additional interaction between the gelatin and PEI complexes; this is contrary to what is seen with lipoplexes (Figure 4), where gelatin addition in fact increases early release.

**TABLE I. The % Complexed Form of Lipoplexes and Polyplexes From PCL Films**

<table>
<thead>
<tr>
<th>Samples</th>
<th>% Complexed Form</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 5</td>
</tr>
<tr>
<td>PCL/lipoplex</td>
<td>100</td>
</tr>
<tr>
<td>PCL/LMW gelatin/lipoplex</td>
<td>70%</td>
</tr>
<tr>
<td>PCL/HMW gelatin/lipoplex</td>
<td>72%</td>
</tr>
<tr>
<td>PCL/polyplex</td>
<td>100</td>
</tr>
<tr>
<td>PCL/LMW gelatin/polyplex</td>
<td>–</td>
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<tr>
<td>PCL/HMW gelatin/polyplex</td>
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</table>

**Figure 11.** Transfection of released lipoplexes/time interval from PCL films (LMW vs. HMW gelatin films). Statistical significance was performed using one-way analysis of variance (ANOVA): *p < 0.05, ns = no significant difference; p > 0.05.

**Figure 12.** Comparison of transfection of released pDNA versus lipoplexes from PCL/gelatin films.

**Polyplex Versus Lipoplex Release**

The main functional groups in the ethylenimine unit of PEI’s are —CH2— and —NH— (Figure 7). The IR spectra of PEI show a peak at 1453 cm⁻¹ corresponding to in plane bending of CH2. The peaks for the bending vibrations of the N—H group and the stretching vibration of the C—N groups of PEI can be seen at 1595 and 1114 cm⁻¹, respectively. The C=O stretching band of PCL lies close to 1720 cm⁻¹. In the case of the lipofectamine, the ester portion of DOSPA gives rise to a carbonyl stretching band at 1738 cm⁻¹ (Figure 8). Overall, the IR spectra of PEI, LPF, and PCL show peaks that are characteristic of their main functional groups. However, there are distinct differences between the IR spectra PEI/PCL and that of PEI or PCL alone.

In the case of PEI/PCL, the position of the peak for NH of PEI is shifted from 1595 to 1548 cm⁻¹. This is due to the hydrogen bond formation between C=O groups of PCL and N—H groups of PEI, as explained below (Section 4.4), an interaction not seen for lipoplex in PCL. The effect of gelatin (Figure 5) is further suppressing the release implies additional interaction between the gelatin and PEI complexes; this is contrary to what is seen with lipoplexes (Figure 4), where gelatin addition in fact increases early release.

**Figure 13.** Comparison of lipoplexes released from PCL films with lipoplexes incubated at different time points.
complex was studied. The role of the molecular weight of the additive, gelatin was also evaluated. For pDNA, the release is dominated by lack of solubility of the pDNA in the PCL matrix, and burst release was observed. Addition of gelatin allows some of the pDNA to dissolve into the gelatin phase, hence suppressing the burst. The burst effect suppression is enhanced with higher molecular weight gelatin. This is due to the slower partitioning of the pDNA from the higher molecular weight gelatin phase. For lipoplexes, there is no burst effect, but the rate of release from the PCL is also too low for practical use; however, addition of the hydrophilic gelatin enhances the release of lipoplexes. The addition of HMW gelatin led to the transfection of lipoplexes till day 18. Released lipoplexes from gelatin loaded films were substantially in the complexed form and were able to transfect the cells. However, the extent of transfection decreases with time.

In the case of polyplexes, the release is dominated by the charge interaction/hydrogen bonding between the PCL molecules and positively charged PEI. The addition of gelatin hinders the release even more.

Our future studies will concentrate more on the release of complexed DNA for an extended period, evaluating the long term bioactivity of released DNA and understanding the mechanistic pathway governing the observed released pattern of complexed DNA and factors which affect the transfection efficiency.

REFERENCES


