In Vitro Release of Complexed pDNA from Biodegradable Polymer Films

Y. Ramgopal,[1] S. S. Venkatraman,[1] W. T. Godbey[2]

[1]*School of Materials Science and Engineering, Nanyang Technological University, Nanyang Avenue, Singapore 637819*
[2]*Department of Chemical and Biomolecular Engineering, Tulane University, New Orleans, Louisiana 70118*

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ABSTRACT: The controlled delivery of low-molecular weight drugs and proteins from biodegradable polymers has received considerable attention. However, controlled release studies of pDNA from such polymers have not been reported to date. In this study, a plasmid DNA was complexed with the cationic polymer called polyethylenimine (PEI). This gene vector has been shown to be very effective in transfecting cells. The complexed DNA were then incorporated into different types of poly-lactic-*co*-glycolic acid (PLGA) film; PLGA 53/47 (M_w 90 kDa), 50/50 (M_w 11 kDa, end group is lauryl ester) and 75/25 (M_w 120 kDa). Their release profiles from a buffer solution were studied. An initial (small) burst release of PEI-DNA from film was observed in PLGA 53/47 and 50/50, followed by a plateau phase and finally a rapid erosion-controlled release. For PLGA 50/50, the rapid release started after 14 days; erosion-controlled release for PLGA 53/47 started after 9 days; for PLGA 75/25, the release rate was governed by an initial burst release (10%) followed by a slow release controlled by diffusion. No obvious erosion-controlled release rate was observed for this polymer up to 27 days. Thus, the controlled release of complexed DNA follows the general features exhibited by lower- M_w drugs. This is of significance in designing gene vector matrices that offer the promise of more lasting gene therapy compared with particulate formulations. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 108: 659–664, 2008

Key words: biodegradable; biomaterials; pDNA; hydrophilic polymer; non-viral gene vector

INTRODUCTION

Biodegradable materials are being increasingly used for the sustained release of drug in treating many diseases. A number of drug-releasing polymeric products have been approved by FDA. A recent example of a product that uses drug release from a biodegradable polymer is the Gliadel® wafer which was implanted in the brain to treat brain tumor.[1] Controlling the release of small molecule drugs, and even of protein drugs, from polymers may now be considered a mature technology. However, there has been very little work reported on controlled release of DNA from polymers. The potential of DNA delivery is for the treatment of many inherited genetic diseases as well as for cancer. Most of the work on DNA carriers has centered on particle incorporation.[2–4] In this sort of approach, DNA is typically encapsulated or dispersed in a biodegradable matrix particle, and then exposed to cells to assess the transfection capabilities. DNA complexed with cationic polymers such as polyethylenimine (PEI) show reduced sizes in the 50- to 70-nm range. In addition, these condensed DNA entities have been shown to protect DNA against degradation by DNase[5] and also to be very effective in transfecting many types of cell. It is less immunogenic than a viral vector and has a high-buffering capacity which enhances endosomal escape.[6,7] Furthermore, it can also be linked with a targeting ligand which bind to a specific cell type.[8]

Different types of biomaterials, natural and synthetic, have been utilized for the controlled release of drugs and proteins. Hydrophilic polymers such as collagen and alginate can form hydrogels in water and thus release active component through diffusion only.[9] However, the diffusional rate from such swollen systems tend to be high. Because of this limitation, synthetic polymers such as poly-lactic-*co*-glycolic acid (PLGA) have become an attractive alternative. The reason is that the release profile can be controlled by diffusion and the erosion of the polymer.[10,11] Furthermore, PLGA has been proven to be biocompatible and safe to use in many medical applications.[12,13] It degrades into natural products such as lactic acid and glycolic acid. PLGA are available in different molecular weights, and different lactic/glycolic acid ratios. All these have a significant effect on the polymer degradation rate and this is considered one of the major advantages of PLGA.

In spite of considerable effort, sustained release of DNA from such polymer matrices has not been demonstrated to date; such a release is desirable to overcome the problems associated with transience of current non-viral gene delivery techniques. In other

Correspondence to: S. S. Venkatraman (assubbu@ntu.edu.sg).

words, current non-viral DNA carriers have to be injected periodically to sustain the effect of gene therapy, which is clearly undesirable from the patient and cost viewpoints.

There are generally three approaches in somatic gene therapy; *ex vivo*, localized release, and systemic administration. To achieve systemic delivery, many researchers focus their studies on the release of drug or plasmid DNA from PLGA microspheres or nanoparticles.[3,14–16] Such particles have to satisfy many requirements, including long blood lifetimes, low immunogenicity, good cell membrane penetration, withstand endosomes in the cytoplasm, and effective nuclear membrane penetration. Because of these demanding requirements, systemically administered particle-based DNA transfection rates have been low. Localized administration of genes has some advantages: less potential for immunogenicity, no need for long blood lifetimes, and better chances of reaching the target cells. Moreover, proven complexed DNA can be directly administered to the site, rather than via dispersion/encapsulation in particles. The issue that has been difficult to deal with is the controlled release of such complexes from devices/films. Not only must one control the release of the DNA (for chronic treatment), but also ensure that the DNA is released as intact complex capable of transfection. The other issue with non-viral gene carriers is the transience of the treatment. These particulate DNA carriers have to be injected periodically to obtain clinical benefits, as the DNA is released quickly from the particles, and thus the transfection is transient. Sustained release could overcome this problem to some degree, reducing the frequency of administration.

We have attempted to address this issue here. In this work, plasmid DNA was complexed with PEI and loaded into three types of PLGA film. These polymers have different molecular weights, different ratio of lactic acid to glycolic acid, and different end groups. The objective of this work was to study the release rate of the DNA from the films into a phosphate buffer solution, as well as to assess the nature of the DNA that is released.

MATERIALS AND METHODS

Reagents

pDNA Dual vector pEGFP*Luc* was purchased from Clontech (Palo Alto, CA) (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 Qiagen (Hilden, Germany) and conditioned in autoclaved TE buffer. Branched PEI (25 kDa) and heparin salt was provided by Sigma Chemicals (Sigma Aldrich, Steinheim, Germany). Heparin was dissolved in PBS buffer, pH 7.4 at a concentration of 1 mg/mL. Ethidium bromide (Etbr) was purchased from Sigma. It was diluted in water at a concentration of 80 μg/mL. PLGA 50/50 (M_w 11 kDa; I.V 0.22) was supplied by Absorbable Polymer Technologies (Pelham, AL), PLGA 53/47 (M_w 90 kDa; I.V 0.88) and PLGA 75/25 (M_w 120 kDa; I.V 0.9) were purchased from Purac (Purac Biochem, Holland). Dichloromethane (DCM) was purchased from Tedia (USA), in GR grade. The films were cast with the Automatic Film applicator AG-2150 purchased from BKY Gardner (USA).

Preparation of polyethylenimine-pDNA complexes

PEI was prepared at a concentration of 32 mg/mL in PBS, pH 7.4. The solution was filtered through a 0.2-μm filter. The pDNA/PEI complexes were prepared with a 1 : 4 w/w in PBS pH 7.4. The complexes were mixed together by gentle shaking and incubated at room temperature for 30 min.

Entrapment of DNA in polymer film

Solutions of 25% (w/v) of 53/47, 25% (w/v) of 75/25, and 60% (w/v) of 50/50, in DCM were used in the study. The DNA solutions were added to the polymer solutions, with a mixing ratio (v/v) [of DNA solution to polymer solution] of 1 : 5. About 1 mL of the aqueous DNA solution (either naked DNA or PEI-DNA) and 5 mL of polymeric solution were mixed together. A medium sized magnet was used to stir the mixture vigorously for about 10 min before it was cast into a thin film on a clean glass plate using a film applicator. All the films were dried in the fume hood after casting for 5 h. Then they were further dried in a vacuum oven at 25°C for 5 days.

Study on the water retention on films (Karl Fischer Test)

The residual water content of films after vacuum drying for 5 days was analyzed using Karl Fischer (Metrohm Titrator, Switzerland). A piece of the film was cut and weighed in a 10-mL glass bottle. NMP (1 mL) was poured into the bottle to dissolve the film for about 1 h.

SEM study

The morphology of the films was analyzed by Scanning Electron Microscopy (SEM) (Jeol JSM 5310, Japan) (Day 8). A part of the above-prepared samples was used for the study. The samples were cut into small pieces and mounted on an aluminum tape using a double-sided carbon tape. The samples were coated with gold using a sputter coater (SPI-Mod-

ule). The coated samples were examined using an electron acceleration voltage of around 10 kV.

Quantification of the released pDNA/PEI-pDNA complexes

DNA concentration was determined using Etbr with Ex = 500 nm, Em = 550 nm, Slit width = 10 mm. The measurement was done on a Shimadazu spectroflorometric system (Columbia, MD). A standard curve was constructed with different concentrations of pEGFP*luc* in the presence of heparin salt. The DNA concentrations were determined from unknown samples by comparison to the standard curve. The naked DNA-loaded film samples are collected and quantified directly. In the case of films loaded with condensed DNA, the DNA had to be disassociated from PEI prior to quantification. Heparin salt was used for the dissociation. Heparin salt was added as 10 times higher than the PEI amount. About 200 μL of sample was added to 40 μL (1 mg/mL) of heparin salt. PBS buffer was added to bring up the final volume of 1100 μL. The samples were incubated at 37°C for an hour. From this, 900 μL was used for the quantification study. To the 900 μL of sample, about 100 μL of Etbr (80 μg/mL) was added and mixed well. The fluorescence intensity was measured. Appropriate blank solutions with PLGA and heparin salt, at different intervals were used to get the final value.

Polymer degradation study

Gel permeation chromatography (GPC) from Agilent Technologies 1100 Series (Santa Clara, CA) was used for the degradation study of all the polymer films before and after the immersion in PBS. A PLgel 5 μm-mixed C column was used with the mobile phase DCM/THF (20 : 80). The flow rate was set at 1 mL/min and about 100 μL of sample was injected. Wet films were first dried in a freeze drier before dissolving in the mobile phase.

Release study in buffer

The films were cut into three pieces, measuring 3 cm by 3 cm each. The cut films were placed in a 10 mL PBS pH 7.4 buffer solutions in 60-mL glass bottles. The bottles were sealed and incubated at 37°C. The samples were collected at different intervals. At every interval, 1.1 mL of the sample was drawn and was replenished with the fresh PBS pH 7.4 buffer.

RESULTS

Film appearance and water content

The thickness of all the dried films was around 50 μm, measured using a micrometer. The appearance of PLGA 53/47 and 75/25 film was quite rough, whereas PLGA 50/50 film was smooth. After the immersion in PBS for 1 week, both PLGA 53/47 and 75/25 films appeared to have many pores on the surface (Fig. 1). However, PLGA 50/50 film crumbled after 1 week in buffer [Fig. 1(c)]. The reason is that the glass transition temperature of PLGA 50/50 was about 25°C and the study temperature was 37°C, as shown in Table I.

The water content in the film after drying in vacuum oven for 5 days was analyzed. The average value was about 0.26% w/w.

Degradation

The degradation rate of polymers in PBS is shown in Figure 2. There was a significant drop in average molecular weight (M_w) for PLGA 53/47; 56% reduction after 1 week and 90% after 2 weeks. PLGA 75/25 had slower degradation rate compared with PLGA 53/47, where it had only about 60% drop after 2 weeks. M_w of PLGA 50/50 dropped slowly below 2 weeks but thereafter the rate increased, and there was about 60% reduction on Week 3.

DNA release

The release profile of DNA from different polymer films is shown in Figures 3–5.

The release profile of PLGA 53/47 (Fig. 3) has two phases: burst phase and an erosion-controlled phase. After a small burst release on Day 1 (3%), the release rate also slowed down to Day 9. The burst release is due to the presence of undissolved PEI-DNA molecules in the polymer matrix, more specifically on its surface. A diffusion controlled phase is absent and hence no release is noted from Days 3 to 10. Beyond Day 10, the release rate increased rapidly due to the erosion of PLGA 53/47. This also coincided with the degradation profile of this polymer where its M_w dropped by 90% on Day 14. The amount of PEI-DNA released on Day 27 was about 55%.

The other copolymer, PLGA 50/50 has its end groups capped with a lauryl ester, but in relative composition, is similar to PLGA 53/47. The release profile observed (Fig. 4) was similar to that of PLGA 53/47. It was noticed that after an initial burst release on Day 1 (10%) the release of PEI-DNA slowed down significantly. Beyond Day 14, the release rate started to increase rapidly. This coincided with the onset of a significant drop in M_w for this polymer in degradation. However, unexpectedly, the measured PEI-DNA Day 27 (30%) was lower than the value on Day 14 (60%). This is attributed to DNA denaturation, see later.

For PLGA 75/25, the release rate was governed by an initial burst release (10%) followed by a slow

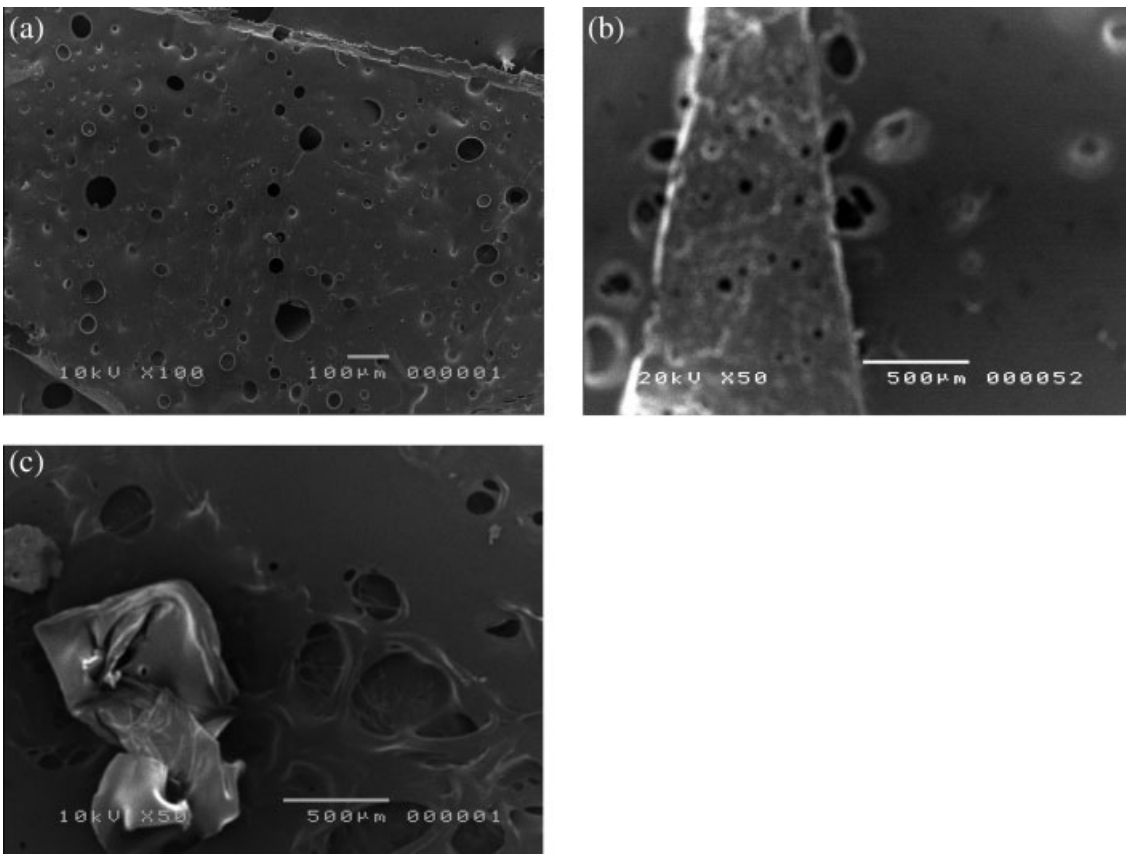

Figure 1 SEM pictures of PLGA films after immersion in buffer. (a) PLGA 53/47, (b) PLGA 75/25, and (c) PLGA 50/50.

release controlled by diffusion (Fig. 5). No obvious erosion-controlled release rate was observed for this polymer up to 27 days. This was quite different from the other two polymers although we did observe a significant drop in M_w on Day 21 (70%). We believe the reason for the difference between PLGA 75/25 and the other two, is that there is a specific polymer-PEI interaction in both PLGA 50/50 and PLGA 53/47, and the extent of this interaction is much less in LGA 75/25. The nature of this interaction is currently being defined in our laboratory.

DISCUSSION

In general, the release profiles of the complexed pDNA from the PLGA copolymers reflected the effects of both diffusion and degradation. Clearly, for the PLGAs with lower lactide contents (PLGA 53/47 and PLGA 50/50), the starting molecular weights were lower. We have shown in a previous study,[17,18] that the onset of substantial mass loss in these degrading polymers occurs when the overall M_W reaches about 10–20 kDa (the number is not fixed, as it depends also on MWD). This stage is reached within about 3 weeks for the PLGA 53/47 and the PLGA 50/50, but not for the PLGA 75/25. Thus, the onset of substantial mass loss is coincident with the onset of degradation-controlled release of the complexed pDNA, similar to observations for

TABLE I
Chemical Properties of Polymer Films

	Average molecular weight (Dalton)	Glass transition temperature (°C)
PLGA 50/50	1.1×10^4	25
PLGA 53/47	9×10^4	40
PLGA 75/25	1.2×10^5	42

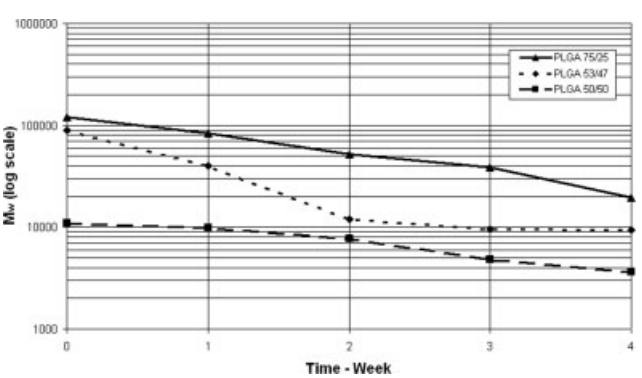

Figure 2 Degradation rate of different polymers in phosphate buffer solution at 37°C.

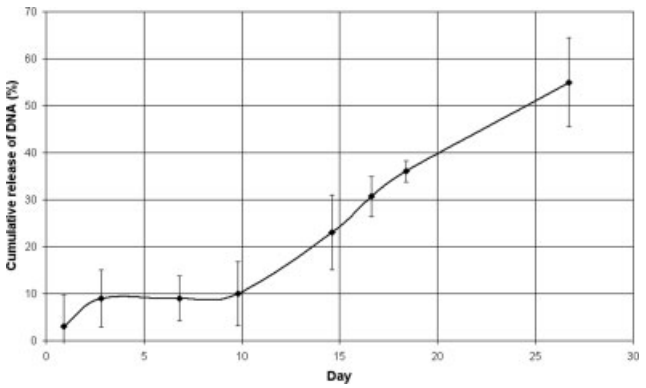

Figure 3 Release profile of DNA from PLGA 53/47 film during immersion in PBS at 37°C.

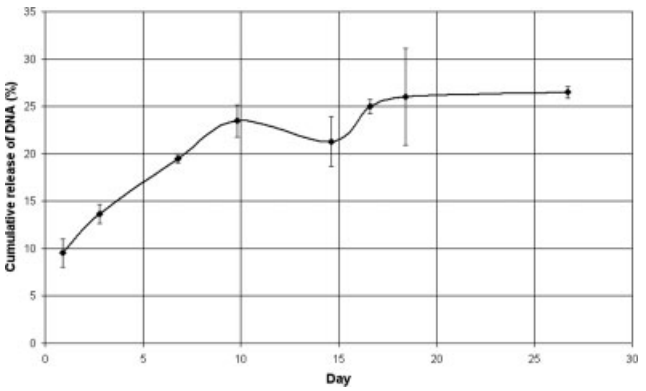

Figure 5 Release profile of DNA from PLGA 75/25 film during immersion in PBS at 37°C.

lower-MW drugs. The reason is that the leaching out of soluble products sets up a network of water-filled connected pores. Subsequent pDNA release is through water-filled pores rather than through the intact polymer matrix.

It has been known that PLGA of different M_w and of different lactic to glycolic acid ratios exhibit vastly different degradation rates. In this study, only amorphous polymer was selected since such polymers have faster degradation rate, allowing for a reasonable time period of study, and for quantifiable amounts of DNA to be released.

The anomalous downturn in the released amounts of DNA for the PLGA 50/50 is explained as follows: the M_w of PLGA 53/47 was comparable to PLGA 75/25 but that of PLGA 50/50 was about 10 times lower. Because of the overall lower MW for the PLGA 50/50 polymer, the end-group concentration is relatively higher at any given time point. As degradation proceeded for this polymer, carboxylic end groups were generated, and when these are formed in large enough numbers, the pH drops significantly. In the initial stage (up to Day 18), the drop is relatively small, from pH 7.4 to 6.2. After Day 18, sufficiently small oligomers with carboxylic groups have been formed to leach out into solution, and this drastically affects the pH. This accounts for the measured low value of the heparinized DNA after Day 18. The pH drop is much less for the other polymers, presumably on account of a more gradual decrease in M_w and higher starting M_w. The pH effect in measurement of pDNA is elaborated further in Quantification Issues section, later.

There are very few studies that we can compare our results to, for the release of pDNA from polymer matrices. In a study done by Howard et al.,[15] rapid release of PEI-DNA (85%) from microspheres (PLGA 50/50, M_w 14 kDa) was observed after 4 days. The diameter of their microspheres was about 25 times smaller than our film thickness and the resulting higher surface-to-volume ratio may be the reason behind the quicker onset of degradation-controlled release in their case.

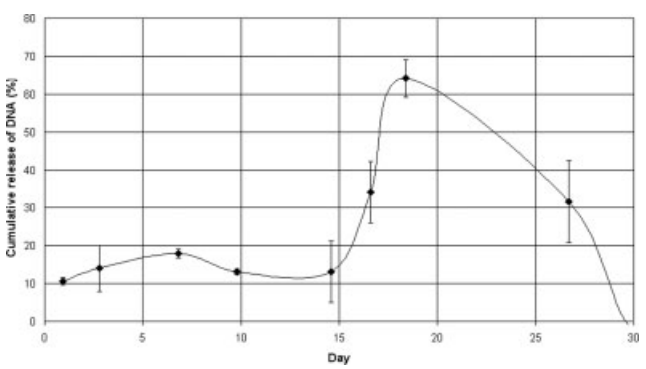

Figure 4 Release profile of DNA from PLGA 50/50 film during immersion in PBS at 37°C.

Quantification issues

Without the use of heparin salt, the detected DNA amounts were small, thus confirming that the released DNA was mostly in the complexed form. The heparin salt treatment is essential in decomplexing the DNA for detection.

In the measurement of PEI-DNA, it was found that dissolved oligomers (lactic and glycolic acid) interfered with the fluorescence intensity measurement. Therefore, EtBr and heparin salt were added in access. Heparin salt was used to separate PEI-DNA, and EtBr was used to quantify the amount of DNA based on a predetermined calibration curve. The DNA amount was determined by deducting the value of the corresponding value for the blank film. It should be noted that the pH also affects the quantification of DNA. For example, in the case of PLGA 50/50 films, the pH in the release medium dropped from 6.5 (Day 18) to 2.9 (Day 27). At this pH, the DNA strand is opened up and although EtBr can

still bind to it, its fluorescence intensity value will drop. In another separate study (data not shown), it was found that there was about 70% drop in the measured DNA values at pH 3. This was also noted by other researchers.[19] As a result, the measured value of PEI-DNA for PLGA 50/50 on Day 27 was significantly lower. The actual value for the released amount is probably much higher. The pH of PLGA 53/47 and 75/25 on Day 27 dropped only slightly to 6.9 and 7.1, respectively.

Miscellaneous

Some agglomeration of PEI-DNA was observed during the condensation process. However, this PEI-DNA pellet was broken into fine particles after being mixed a few times using a pipette.

CONCLUSIONS

The release profiles of PEI-DNA in three different types of biodegradable polymer film (PLGA) in a phosphate buffer solution were studied. PLGA 53/47 and 50/50 exhibited an initial burst release, followed by a plateau phase and then a rapid erosion-controlled release. The results suggest that both PLGA 50/50 and PLGA 53/47 can sustain the release of the PEI-pDNA complex, but at the later stages of release, it is likely that the PEI-DNA is affected adversely by lowered pH. The third polymer, PLGA 75/25 did not show any erosion-controlled release, but did exhibit slower, diffusion-based kinetics. Clearly, the released PEI-DNA will be less affected after release from PLGA 75/25, as the degree of degradation at 4 weeks is low, and consequently the effect of lowered pH on the complex is also minimal.

This study has demonstrated that the release of condensed DNA could be controlled by polymer type and composition; no significantly large burst effects are observed, thus ensuring a prolonged release of genetic material for sustained transfection. Further studies of the bioactivity of the released pDNA complexes are in progress.

References

1. Brem, H.; Gabikian, P. J Controlled Release 2001, 74, 63.
2. Wang, D.; Robinson, D. R.; Kwon, G. S.; Samuel, J. J Controlled Release 1999, 57, 9.
3. Tinsley-Brown, A. M.; Fretwell, R.; Dowsett, A. B.; Davis, S. L.; Farrar, G. H. J Controlled Release 2000, 66, 229.
4. Perez, C.; Sanchez, A.; Putnam, D.; Ting, D.; Langer, R.; Alonso, M. J. J Controlled Release 2001, 75, 211.
5. Godbey, W. T.; Barry, M. A.; Saggau, P.; Wu, K. K.; Mikos, A. G. Int J Pharm 1999, 78, 231.
6. Kunath, K.; Von Harpe, A.; Fischer, D.; Petersen, H.; Bickel, U.; Voigt, K.; Kissel, T. J Controlled Release 2003, 89, 113.
7. Hong, J. W.; Park, J. H.; Huh, K. M.; Chung, H. S.; Kwon, I. C.; Jeong, S. Y. J Controlled Release 2004, 99, 67.
8. Kunath, K.; Von Harpe, A.; Fischer, D.; Kissel, T. J Controlled Release 2003, 88, 159.
9. Gombotz, W. R.; Petit, D. K. Bioconjugate Chem 1995, 6, 332.
10. De Jong, S. J.; Arias, E. R.; Rijkers, D. T. S.; Van Nostrum, C. F.; Kettenes-van den Bosch, J. J.; Hennink, W. E. Polymer 2001, 42, 2795.
11. Anderson, J. M.; Shive, M. S. Adv Drug Delivery Rev 1998, 28, 5.
12. Visscher, G. E.; Robison, R. L.; Maulding, H. V.; Fong, J. W.; Pearson, J. E.; Argentieri, G. J. J Biomed Mater Res 1986, 20, 667.
13. Menei, P.; Daniel, V.; Montero-Menei, C.; Brouillard, M. Biomaterials 1993, 14, 470.
14. Panyam, J.; Labhasetwar, V. Adv Drug Delivery Rev 2003, 55, 329.
15. Howard, K. A.; Li, X. W.; Somavarapu, S.; Singh, J.; Green, N.; Atuah, K. N.; Ozsoy, Y.; Seymour, L. W.; Alpar, H. O. Biochim Biophys Acta 2004, 1674, 149.
16. Kasturi, S. P.; Sachaphibulkij, K.; Roy, K. Biomaterials 2005, 26, 6375.
17. Frank, A.; Rath, S. K.; Venkatraman, S. S. J Controlled Release 2005, 102, 333.
18. Frank, A.; Venkatraman, S. S.; Rath, S. K.; Gan, L. H. J Appl Polym Sci 2006, 102, 3111.
19. Vardevanyan, P. O.; Antonyan, A. P.; Parsadanyan, M. A.; Davtyan, H. G.; Karapetyan, A. T. Exp Mol Med 2003, 35, 527.