Polymer(Korea), Vol. 29, No. 1, pp 69-73, 2005

DNA가 봉입된 Poly(*D,L*-lactic-*co*-glycolic acid) 미립구의 제조 및 시험관내 방출

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(2004년 10월 14일 접수, 2004년 11월 30일 채택)

Preparation and In Vitro Release of DNA-Loaded Poly(*D,L*-lactic-*co*-glycolic acid) Microspheres

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(Received October 14, 2004;accepted November 30, 2004)

초록 : 비바이러스성 유전자 전달체의 주요 단점인 낮은 transfection 효율에 기인한 반복투여 등을 극복하기 위하여 poly (*D,L*-lactide-*co*-glycolide)를 이용하여 DNA가 봉입된 미립구를 제조하였다. pDNA 그 자체 또는 여러 비율의 키토산/pDNA 복합체를 사용하여 봉입하였고, 그 결과 44%(pDNA 그 자체), 5%(0.7:1 키토산/pDNA 복합체), 그리고 8% (1:1 키토산/pDNA 복합체)의 봉입효율을 나타내었다. 주사전자현미경(SEM)을 통해 본 표면구조에서는 미립구 제조 직후에서는 매우 매끈한 구형을 보이다가 제조 후 41일 경에는 찌그러진 다공성의 구조를 보였는데 이는 미립구 제조에 사용한 poly(*D,L*-lactic-*co*-glycolic acid)(PLGA) 고분자의 분해에 의한 것으로 생각된다. 시험관내 방출실험에서는 0.7:1 키토산/pDNA 복합체를 사용한 미립구에서 47%의 pDNA가 26일만에 방출된데 반해, pDNA 그 자체 혹은 1:1 키토산/pDNA 복합체를 사용한 미립구에서는 각각 15% 혹은 32%의 pDNA 방출을 나타내었다.

Abstract: To overcome the main disadvantages of non-viral gene delivery systems such as repeated administration due to the low transfection efficiency, poly(*D,L*-lactide-*co*-glycolide) was applied to encapsulate pDNA in its microsphere formulation. Free pDNA or various ratios (w/w) of chitosan/pDNA complexes was used for encapsulation, with the resulting encapsulation efficiency of 44%, 5%, and 8% for free pDNA, 0.7:1 and 1:1 ratios, respectively. Scanning electron micrographs of poly(*D,L*-lactic-*co*-glycolic acid) (PLGA) microspheres encapsulating pDNA or chitosan-condensed pDNA revealed a smooth spherical shape immediately after microsphere preparation and a collapsed porous shape in 41 days due to the degradation of PLGA. In vitro release profile showed that the 0.7:1 (w/w) ratio formulation exerted 47% release in 26 days, whereas free pDNA or 1:1 (w/w) ratio formulation did only 15% or 32%, respectively.

Keywords: microsphere, PLGA, DNA, chitosan.

1. Introduction

Gene therapy has already captured the attention of the public and the biomedical research community. Actually, human gene therapy is successful or under going clinic studies for the treatment of adenosine deaminase (ADA) deficiency,[1] haemophilia,[2] various cancers,[3] etc. However, recent studies indicate that development of efficient and safe delivery vehicles remain a crucial barrier to the ultimate success of gene therapy.[4]

The administration of naked DNA, though simple and safe, has shown to be rather inefficient due to its anionic charge, large size and the presence of nuclease in the blood stream that might degrade the DNA before reaching the target site. The use of gene carriers, or gene vectors, has been proposed to circumvent these problems.[5,6] There are two classes of gene carrier systems; virus-based and non-viral gene carrier systems. Viral gene delivery system is currently the most efficient way to transfer genes to the target cells, though it has big disadvantages such as toxicity and difficulty in scale up.[7,8] Non-viral gene delivery system, especially based on polycationic polymers, has recently generated high interest due to the easiness to use and low toxicity.[9]

Poly(*D,L*-lactic-*co*-glycolic acid) (PLGA) is a biodegradable and biocompatible polymer and has widely been investigated for the delivery of therapeutic agents ranging from conventional small molecules to macromolecules.[10,11] Encapsulation of pDNA into PLGA microspheres is expected to protect pDNA from nuclease attack, enabling sustained release of pDNA possible for the extended period of time.

Chitosan is a biodegradable polysaccharide which is extracted

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from crustacean shells. It has been shown to be non-toxic in a range of toxicity tests, both in experimental animals and humans, and it has been widely used for pharmaceutical research.[12,13] Positively charged chitosan has also been shown to effectively condense DNA and protect DNA from nuclease degradation.[12,14] Furthermore, chitosan may actually be endocytosed into the cell.[15] Uptake of DNA/polymer complexes into cells is obviously a very important step for successful gene transfer in vitro and in vivo.[16] Introduction of chitosan prior to microsphere preparation is expected not only to condense DNA but also to affect release pattern of pDNA from PLGA microspheres.

Among various cytokines, interleukin-12 (IL-12) is known to possess the most potent anticancer activity by stimulating the T-cell and/ or natural killer (NK) cell's immune response.[17,18] Based on its biological activities, IL-12 has been examined for its capacity to induce anticancer effects, and therapeutic activity has been observed in various murine tumor models.[19-23] Therefore, encapsulation of p2CMVmIL-12 (IL-12 encoding gene) into PLGA microspheres is expected to reflect long-lasting anticancer effect with the protection of pDNA from nuclease attack. This article presents the result of preparation and in vitro release of p2CMV mIL-12-loaded PLGA microsphere as a sustained release formulation of non-viral gene delivery systems.

2. Experimental

2.1 Materials

pCMV SPORT-βgal (~7.9 kb) was purchased from Gibco BRL (Grand Island, NY) and p2CMVmIL-12, containing the coding sequences for the p35 and p40 subunits of murine IL-12 vector, was kindly provided by Prof. Sung Wan Kim at the University of Utah (Salt Lake City, UT). pDNA was prepared with QIAGEN® Plasmid Mega Kit (QIAGEN, USA). poly(*D,L*-lactide-*co*-glycolide) (PLGA, 50:50 lactide:glycolide, m.w. 40000~75000) and polyvinyl alcohol (PVA, m.w. 30000~70000) were purchased from Sigma Chemical (St Louis, MO). Chitosan (low molecular weight 50000~190000) and dichloromethane were purchased from Aldrich Chemical (Milwaukee, WI). PicoGreen reagent was purchased from Molecular Probes (Eugene, OR). All other reagents were of analytical grade.

2.2 Plasmid DNA

pCMV·SPORT-βgal and p2CMVmIL-12 were used in this study as a reporter gene and therapeutic gene, respectively. After the transformed *E. coli* were grown in 10 mL of LB broth with 100 μg/mL ampicillin, 4 mL of LB broth was moved into 2000 mL of LB broth with 100 μg/mL ampicillin. Plasmid DNA was extracted from transformed *E. coli* by using QIAGEN® Plasmid Mega Kit. The concentration and purity of the DNA were determined at 260 and 280 nm, respectively, by UV spectrophotometry (Ultraspec® 2000, Pharmacia Biotech).

2.3 Gel Retardation Assay

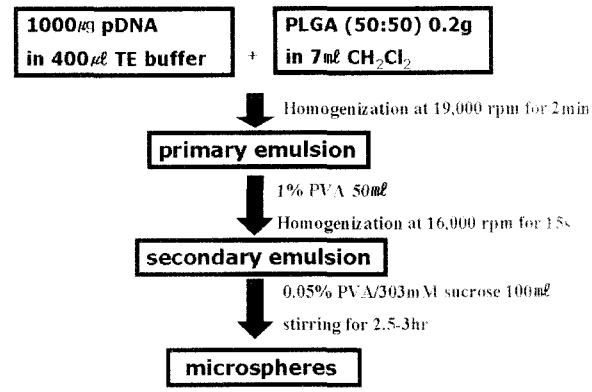
Various amounts of chitosan were mixed with the fixed amount of plasmid DNA and incubated for 30 min at room temperature to ensure the formation of the complex. The mixture was electrophoresed on 1% (w/v) agarose gel for 90 min at 85 V. Gel was stained with ethidium bromide (0.5 μg/mL) for 10 min and destained with distilled water for 5 min, following illumination on UV illuminator to show the location of the DNA in various complex formations.

2.4 Preparation of Microsphere

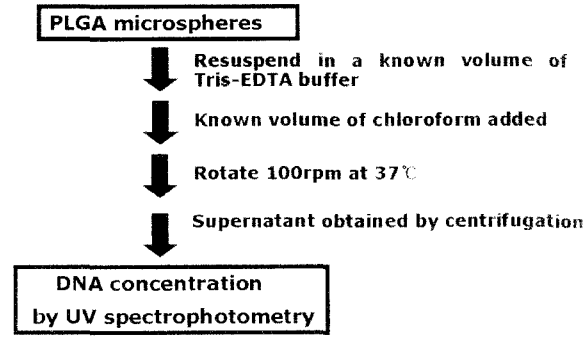
PLGA microspheres encapsulating free pDNA or chitosan-condensed pDNA were prepared by the double emulsion solvent-evaporation method.[24,25] Briefly, oil phase was prepared by dissolving 200 mg of PLGA in 7 mL of dichloromethane and the water phase was prepared by dissolving 1000 μg of free pDNA or chitosan-condensed pDNA in 400 μL TE buffer. Initial water phase was added into oil phase and homogenized at 19000 rpm for 2 min (ULTRA TURRAX®, T25 Basic). Primary emulsion was poured into 50 mL of 1% PVA and homogenized at 16000 rpm for 15 s to form a secondary emulsion. Secondary emulsion was again poured into 100 mL of 0.05% PVA/303 mM sucrose solution and stirred for 2.5~3 hr to evaporate residual dichloromethane. Microspheres collected by centrifugation were washed 2-3 times with distilled water and lyophilized and stored at –20 ℃ (Scheme 1).

2.5 Scanning Electron Microscopy (SEM)

Freeze-dried microspheres were coated with palladium prior to observation and the size and surface morphology of microspheres was examined by a JSM-5410 Scanning Microscope (JEOL, vol-



Scheme 1. Microsphere preparation by the double emulsion solvent-evaporation method.



Scheme 2. Extraction of pDNA from PLGA microsphere.

tage : 20 kV).

2.6 Encapsulation Efficiency of DNA into Microsphere

Ten mg of PLGA microspheres were resuspended in a known volume (500 μL) of Tris-EDTA buffer (pH 8.0). A known volume (500 μL) of chloroform was added to the suspension to solubilize the microspheres. The mixture was then placed in a shaking incubator (Vision Scientific Co., Korea) with rotating speed of 100 rpm at 37 ℃ for 3.5 hr to facilitate the extraction of DNA from the organic phase into the aqueous supernatant. Samples were centrifuged at 14000 rpm, 4 ℃ for 5 min and 100 μL of the supernatant was removed into an Eppendorf tube.[24,25] DNA concentrations were determined by UV spectrophotometry (Scheme 2). Encapsulation efficiency (%) was calculated using the following formula:

$$\text{Encapsulation efficiency (\%)} = (B/A) \times 100$$

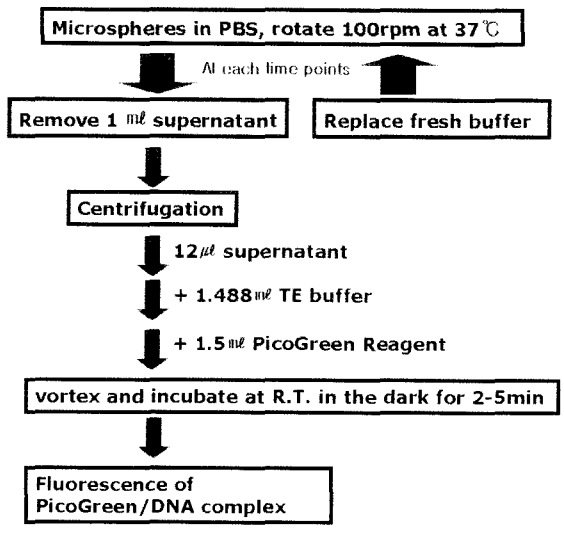
,where A is initial amount of pDNA used for preparing PLGA microsphere and B is amount of pDNA extracted from PLGA microsphere.

2.7 pDNA Quantitation by PicoGreen

The concentration of pDNA which is released from PLGA microspheres was measured by fluorescence of PicoGreen/DNA complex. Briefly, 15 mg of microspheres were resuspended in 10 ml phosphate buffered saline (pH 7.4). Samples were placed in a shaking incubator with a rotating speed of 100 rpm at 37 ℃ and 1 mL of the supernatant was removed and replaced with fresh buffer at each time points. The supernatant was centrifuged at 14000 rpm, 4 ℃ for 5 min and the sample obtained by this step was used for measuring the fluorescence intensity. An aqueous working solution of the PicoGreen reagent was prepared by making a 200-fold dilution of the concentrated dimethylsulfoxide solution in TE buffer. And, the 1.5 mL PicoGreen working solution was added to each sample. The tubes were capped, vortexed and incubated at room temperature in the dark for 2~5 min. After incubation, samples were excited at 480 nm and fluorescence was measured at 520 nm using spectrofluorometer (SFM25, Kontron) (Scheme 3).[24,26] Cumulative release (%) was calculated using the following formula:

$$\text{Cumulative Release(\%)} = (B/A) \times 100$$

, where A is the amount of pDNA encapsulated in 15 mg of microsphere and B is cumulative amount of pDNA in 1 mL supernatant at each time points.



Scheme 3. pDNA quantitation by PicoGreen fluorescence probe.

3. Results and Discussion

3.1 Complex Formation of pDNA/Chitosan

Degree of complex formation of pDNA with chitosan was elucidated by gel retardation assay, where it showed that the electrophoretic mobility of pDNA was retarded as the amount of chitosan increased, indicating that chitosan forms a complex with pDNA. As the complete retardation of the electrophoretic mobility was achieved at the 0.8:1 (w/w) chitosan/pDNA ratio, this formulation together with 0.6:1 ratio complex were selected for use throughout the experiment (Figure 1).

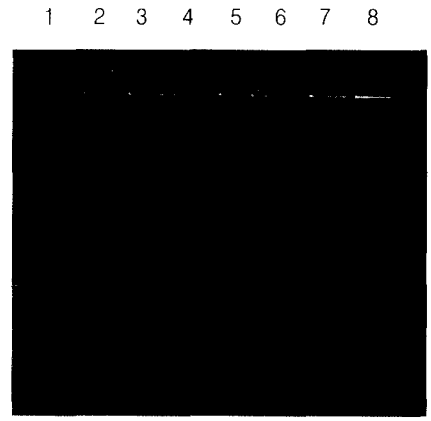


Figure 1. Gel retardation assay. Lane 1) DNA marker, Lane 2) pDNA only, Lanes 3~8) 0.2:1, 0.3:1, 0.5:1, 0.6:1, 0.8:1 and 1.2:1 (chitosan/pDNA, w/w ratio) complexes, respectively.

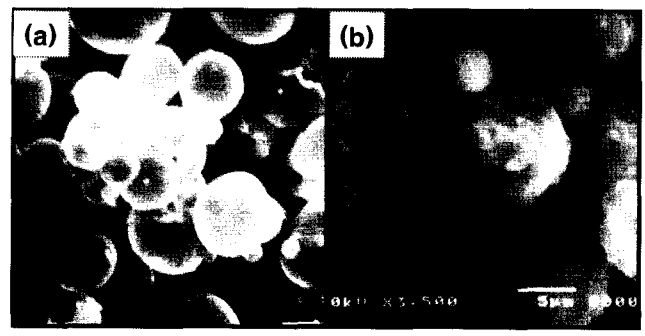


Figure 2. Scanning electron micrographs of microsphere. (a) immediately after preparation and (b) 41days after pDNA release.

3.2 Surface Morphology by SEM

The surface morphology of PLGA microspheres encapsulating pDNA or chitosan-condensed pDNA was observed by scanning electron micrographs as shown in Figure 2. It appears that the surface of PLGA microspheres has a smooth spherical shape immediately after the preparation (Figure 2(a)). As the pDNA is released by degradation of poly(*D,L*-lactide-*co*-gylcolide) in PBS (pH 7.4) at 37 ℃, the inner structure of microspheres reveals collapsed and porous shapes with the reduction of size (Figure 2(b)).

3.3 Encapsulation Efficiency and In Vitro Release

Table 1 shows the encapsulation efficiency of pDNA in various microsphere formulations. Free pDNA resulted in the highest encapsulation efficiency of 44%, whereas chitosan-condensed pDNA showed only 5~8% encapsulation efficiency. This is probably related with the fact that free pDNA is very soluble in aqueous phase (such as in PBS) but the chitosan-condensed pDNA is not so and sometimes it remains as a precipitated form which might have resulted in low encapsulation efficiency.

In vitro release experiments were carried out in phosphate buffered saline (pH 7.4) at 37 ℃. Introduction of chitosan-condensed pDNA into PLGA microsphere exhibited different release profile compared to that of free pDNA, where the former shows a faster release pattern than the latter (Figure 3). Possible explanation for this could be that the chitosan-condensed pDNA seems to be localized outside of the microsphere due to the localized positive (from the chitosan) or negative (pDNA) charges. So the release of chitosan-condensed pDNA from the PLGA microspheres is much faster and more complete than the free pDNA. The pDNA release profile, as shown in Figure 3, indicates that 15%, 47% and 32% of pDNA is released from PLGA microspheres in 26 days with free pDNA, chitosan-condensed pDNA of 0.7:1 and 1:1 w/w chitosan/pDNA formulations, respectively. In general, the release of drug molecules (including pDNA) from PLGA microspheres is known to occur by two mechanisms. First mechanism is the diffusion of drug molecules through aqueous pores or channels formed during or after microsphere preparation.[7] Burst effect observed from PLGA microspheres with free pDNA and chitosan-condensed pDNA, as seen in Figure 3, is probably due to the diffusion of surface-embedded pDNA. Second mechanism involves the degradation and solubilization of the PLGA matrix.[7] The release of pDNA following the burst seems to be due to this degradation and solubilization mechanism as the continuous release of pDNA is seen after wards.

Table 1. Encapsulation Efficiency of pDNA into PLGA Microspheres

Chitosan/pDNA (w/w ratio)	Encapsulation efficiency (%)
Free pDNA	44
0.7:1	5
1.0:1	8

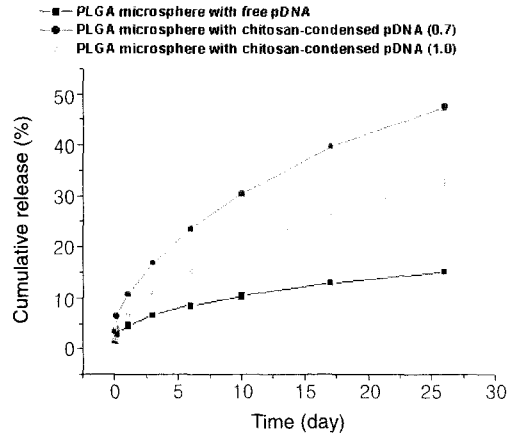


Figure 3. In vitro release of pDNA from PLGA microspheres.

4. Conclusions

The main barrier to the success of human gene therapy using non-viral gene delivery systems lies in the fact that the transfection efficiency is relatively low compared pDNA-encapsulating microsphere formulation was exploited using poly(*D,L*-lactide-*co*-glycolide) and chitosan. Therefore, pDNA could be protected from nuclease degradation and a sustained release of pDNA was also maintained for better transfection efficiency.

Cationic property of chitosan causes pDNA to condense (Figure 1) and degradation property of PLGA causes pDNA to be released slowly (Figure 2). Chitosan-condensed pDNA does not seem to affect the surface morphology of PLGA microspheres compared with free pDNA (Figure 2). However, encapsulation efficiency of free pDNA into PLGA microsphere is higher than that of chitosan-condensed pDNA, presumably due to the aqueous solubility. Introduction of chitosan for pDNA pre-condensation prior to microsphere preparation makes the release of pDNA faster owing to the decreased hydrodynamic volume of pDNA owing to the condensation (Figure 3).

In conclusion, encapsulation of pDNA into the PLGA microspheres was successful and the result from the in vitro release might be useful in designing an efficient microsphere formulations for the delivery of pDNA in the future.

Acknowledgment : This research was supported by Sookmyung Women's University Research Grants (2003).

References

1. A. Aiuti, F. Ficara, F. Cattaneo, C. Bordignon, and M. G. Roncarolo, *Curr. Opin. Allergy. Clin. Immunol.*, **3**, 461 (2003).
2. L. B. Couto, *Semin. Thromb. Hemost.*, **30**, 161 (2004).
3. B. Sangro, G. Mazzolini, J. Ruiz, M. Herraiz, J. Quiroga, I. Herrero, A. Benito, J. Larrache, J. Pueyo, J. C. Subtil, C. Olague, J. Sola, B. Sadaba, C. Lacasa, I. Melero, C. Qian, and J. Prieto, *J. Clin Oncol.*, **22**, 1389 (2004).
4. K. W. Leong, H. Q. Mao, V. L. Truong-Le, K. Roy, S. M. Walsh, and J. T. August, *J. Control. Rel.*, **53**, 183 (1998).
5. M. Nakanishim, *Crit. Rev. Ther. Drug Carr. Syst.*, **12**, 263 (1995).

6. P. Felgner, *Adv. Drug Deliv. Rev.*, **5**, 807 (1990).
7. H. Temin, *Hum. Gene Ther.*, **1**, 111 (1990).
8. A. Kabanov and V. Kabanov, *Bioconjug. Chem.*, **6**, 7 (1995).
9. T. Merdan, J. Kopecek, and T. Kissel, *Adv. Drug. Deliv. Rev.*, **54**, 715 (2002).
10. G. Khang, J. M. Rhee, J. K. Jeong, J. S. Lee, M. S. Kim, S. H. Cho, and H. B. Lee, *Macromol. Res.*, **11**, 207 (2003).
11. G. Khang, S. A. Seo, H. S. Choi, J. M. Rhee, and H. B. Lee, *Macromol. Res.*, **10**, 246 (2002).
12. S. K. Liao, C. C. Hung, and M. F. Lin, *Macromol. Res.*, **12**, 466 (2004).
13. Y. Kato, H. Onishi, and Y. Machida, *Macromol. Res.*, **11**, 382 (2003).
14. K. Y. Lee, I. C. Kwon, Y. H. Kim, W. H. Jo, and S. Y. Jeong, *J. Control. Rel.*, **51**, 213 (1998).
15. F. C. MacLaughlin, R. J. Mumper, J. Wang, J. M. Tagliaferri, I. Gill, M. Hinchcliffe, and A. P. Rolland, *J. Control. Rel.*, **56**, 259 (1998).
16. W. Zauner, N. A. Farrow, and A. M. R. Haines, *J. Control. Rel.*, **71**, 39 (2001).
17. R. I. Mahato, M. Lee, S. Han, A. Maheshwari, and S. W. Kim, *Mol. Ther.*, **4**, 130 (2001).
18. J. W. Yockman, A. Maheshwari, S. Han, and S. W. Kim, *J. Control. Rel.*, **87**, 177 (2003).
19. M. J. Brunda, *J. Leukocyte Biol.*, **55**, 280 (1994).
20. M. J. Brunda, L. Luistro, P. R. Warrier, R. B. Wright, B. R. Hubbard, M. Murphy, and S. F. Wolf, *J. Exp. Med.*, **178**, 1223 (1993).
21. Y. Noguchi, E. C. Richards, Y. T. Chen, and L. J. Old, *Proc. Natl. Acad. Sci.*, **92**, 2219 (1995).
22. T. Tada, S. Ohzeki, K. Utsumi, H. Takiuchi, M. Muramatsu, X. F. Li, J. Shimizu, H. Fujiwara, and T. Hamaoka, *J. Immunol.*, **146**, 1077 (1991).
23. D. J. Verbik, W. W. Stinson, M. J. Brunda, A. Kessinger, and S. S. Joshi, *Clin. Exp. Metastasis*, **14**, 219 (1996).
24. S. P. Barman, L. Lunsford, P. Chambers, and M. L. Hedley, *J. Control. Rel.*, **69**, 337 (2000).
25. T. Hao, U. McKeever, and M. L. Hedley, *J. Control. Rel.*, **69**, 249 (2000).
26. V. L. Singer, L. J. Jones, S. T. Yue, and R. P. Haugland, *Anal. Biochem.*, **249**, 228 (1997).