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Research paper

Direct plasmid DNA encapsulation within PLGA nanospheres by single oil-in-water emulsion method

Hyejung Mok, Tae Gwan Park *

Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon, Republic of Korea

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Abstract

Plasmid DNA was encapsulated within poly(D,L-lactic-*co*-glycolic acid) (PLGA) nanospheres by using polyethylene glycol (PEG) assisted solubilization technique of plasmid DNA in organic solvents. Plasmid DNA was solubilized in an organic solvent mixture composed of 80% methylene chloride and 20% DMSO by producing PEG/DNA nano-complexes having an average diameter less than 100 nm. DNA could be solubilized in the organic solvent mixture to a greater extent with increasing the weight ratio of PEG/DNA. PLGA nanospheres encapsulating DNA were successfully prepared by the single O/W emulsion method. They exhibited greater loading efficiency and better structural integrity, compared to those prepared by the W/O/W double emulsion method. Plasmid DNA could be successfully delivered to macrophage cells to express an exogenous gene. This new formulation enabled high loading of intact plasmid DNA within PLGA nanospheres useful for DNA vaccines.

Keywords: Plasmid DNA; Nanospheres; Gene delivery; Biodegradable; PLGA

1. Introduction

Poly(D,L-lactic-*co*-glycolic acid) (PLGA) has been known for many years as biodegradable and biocompatible polymer for encapsulating a wide range of bioactive agents including proteins and peptides. PLGA microspheres were prepared by various formulation methods to release an active ingredient in a sustained manner over a prolonged period. Recently, PLGA microspheres have also been utilized for sustained release of various genetic drugs including plasmid DNA, oligonucleotides, and adenovirus [1–6]. It was also previously reported that PLGA microparticles improved delivery of DNA to antigen presenting cells (APC) by efficient trafficking through local lymphoid tissue and uptake by dendritic cells [7]. For encapsulating plasmid DNA within PLGA microspheres, a water-in-oil-in-water (W/O/W) double emulsion/solvent evaporation method has been popularly used for a long time. Since plasmid DNA is a highly charged macromolecule, DNA should be first dissolved in a small volume of an aqueous phase, emulsified in an oil phase, and then re-emulsified in an aqueous phase under high shear stress conditions to prepare PLGA microspheres. During the formulation process, however, it was often observed that encapsulated plasmid DNA was structurally damaged under the harsh conditions with ultimately losing its biological activity after release. In addition, polymer degradation elicits an acidic microenvironment inside eroding PLGA microspheres, which additionally degrades the structural integrity of DNA during the release period.

Based on the same double emulsion and solvent evaporation method, plasmid DNA can also be encapsulated within PLGA nanospheres having a few hundred nanometer size. PLGA nanospheres encapsulating DNA can be efficiently internalized into desired cells by endocytosis with concomitantly expressing an exogenous gene [8–10]. The DNA encapsulating PLGA nanospheres are particularly

* Corresponding author. Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Republic of Korea. Tel.: +82 42 869 2621; fax: +82 42 869 2610.
E-mail address: tgpark@kaist.ac.kr (T.G. Park).

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attractive for DNA vaccination. In contrast to PLGA microspheres that are mainly administered at a local tissue site for sustained release of naked plasmid in the vicinity, PLGA nanospheres can be injected systemically to target specific immuno-modulating cell types such as macrophages and dendritic cells. DNA encapsulating PLGA nanospheres would be an ideal system for efficient intracellular delivery of plasmid DNA for gene expression. However, the fabrication of PLGA nanospheres containing plasmid DNA requires far more harsh processing conditions than that of PLGA microspheres in order to produce much smaller nanoparticulates. Ultra-sonication or high speed homogenization formulation step is often required, which elicits severe DNA instability problems during the DNA encapsulation process. Shear stress exerted during the formulation process can cause severe degradation and fragmentation of DNA [11]. For example, encapsulation of plasmid DNA within PLGA nanospheres by using the double emulsion and solvent evaporation method showed a low DNA loading efficiency of about 20% [12]. To increase the loading amount of DNA in PLGA nanospheres, DNA was complexed with cationic amphiphile molecules such as surfactant and lipids via hydrophobic ion pairing (HIP) and the DNA/amphiphile nano-complexes were encapsulated within PLGA nanospheres [13–15]. In this manner, DNA structural stability was also preserved during the encapsulation. Nevertheless, DNA release profiles from the resultant PLGA nanospheres were often uncontrollable mainly due to the limited diffusion of large nano-complex particles from the PLGA nanospheres. As another approach, a modified double emulsion and solvent evaporation method based on a cryogenic technique was also suggested to protect DNA from shear stress and maintain DNA stability [12].

In this study, a new formulation method for encapsulating intact plasmid DNA within PLGA nanospheres was presented. Plasmid DNA was solubilized in a nanoscale complex in a mixture of organic solvents composed of methylene chloride and dimethylsulfoxide (DMSO) (80/20 (v/v) %) by using PEG as a DNA condensing agent. Previously, we have shown that DNA could be solubilized in selected organic solvents such as DMSO and methanol by forming nano-complexes with PEG [16]. In this work, PEG/DNA nano-complexes were directly encapsulated within PLGA nanospheres by dispersing them in a more volatile and hydrophobic solvent medium composed of methylene chloride and DMSO based on the single O/W emulsion and solvent evaporation method. PEG/DNA nano-complexes in the mixed organic solvent, such as solubility and effective diameter, were characterized as a function of PEG/DNA weight ratio. The structural integrity of DNA, loading efficiency, and cellular uptake and gene delivery efficiencies for DNA encapsulating PLGA nanospheres prepared by the O/W emulsion method were compared to those prepared by the W/O/W double emulsion method.

2. Materials and methods

2.1. Materials

Polyethylene glycol (Mw 3350) was obtained from Sigma (St. Louis, MO). Dialysis membrane (Mw cut-off, 50000) was purchased from Spectrum (Houston, TX). Salmon sperm DNA from Stratagene (La Jolla, CA) was used after dialysis for desalting. Plasmid DNA (pEGFP-C1) was extracted from transformed *Escherichia coli* by a standard alkaline lysis technique and purified by a DNA-purification column which was purchased from Qiagen (Valencia, CA). The purity and concentration of plasmid DNA were determined by measuring the absorbance ratio at 260/280 nm using a spectrophotometer (UV-1601, Shimadzu, Japan). Poly(D,L-lactic-*co*-glycolic acid) (PLGA) having a lactic/glycolic molar ratio of 50/50 with Mw of 10000 was purchased from Wako Pure Chemical Industries (Tokyo, Japan). Cell culture media and materials were products of Gibco BRL (Grand Island, NY).

2.2. Methods

2.2.1. DNA solubilization in organic solvent and characterization

Salt-free salmon sperm DNA or GFP plasmid DNA (1 mg) was first mixed with polyethylene glycol (PEG) in deionized water with increasing the weight ratio of PEG/DNA from 1 to 40 and then lyophilized. The dried PEG/DNA mixture was solubilized at 0.2 mg/ml of DNA concentration in 5 ml of an organic solvent mixture composed of methylene chloride and DMSO (80/20 (v/v) %). To show the degree of solubilization of DNA, transmittance value of the mixed organic solvent containing the PEG/DNA nano-complexes was measured at 400 nm using a UV spectrophotometer. The size of PEG/DNA complexes in a mixture of organic solvent at various PEG/DNA ratios was measured by a dynamic light scattering instrument (Zeta plus, Brookhaven, New York) equipped with a He–Ne laser at a wavelength of 632.2 nm at 0.2 mg/ml of DNA concentration. To measure the amount of dissolved DNA in the organic phase, the solution containing dispersed PEG/DNA nano-complexes was centrifuged and the supernatant was dialyzed in deionized water. The amount of DNA was measured at 260 nm using a UV spectrophotometer. GFP plasmid DNA in water and PEG/DNA nano-complexes in organic solvent mixture were observed by TEM (transmission electron microscopy). One drop of sample solution was deposited onto a formvar/carbon support grid with 300 mesh and dried for 2 min. The specimen on the grid was stained for 1 min with one drop of 2% (w/v) uranyl acetate solution. Negatively stained samples were analyzed using Zeiss Omega 912 TEM (Carl Zeiss, Oberkochen, Germany).

2.2.2. Effect of homogenization on DNA stability in water and organic solvent

For stability test of naked DNA, 100 μg of DNA was dissolved in 20 ml of deionized water and the solution was homogenized at 5000 rpm for a pre-determined time. PEG/DNA nano-complexes in a mixture of organic solvent composed of methylene chloride and DMSO (80/20 (v/v) %) were also homogenized under the same condition. To examine the structural integrity of plasmid DNA, homogenized DNA samples were loaded in agarose gel after the solvent was eliminated by evaporation. Electrophoresis was carried out with a current of 100 V for 20 min and DNA was stained with EtBr (ethidium bromide).

2.2.3. Preparation of PLGA nanospheres

For encapsulating plasmid DNA into PLGA nanospheres by the single O/W emulsion and solvent evaporation method, a mixed solvent (2.5 ml) of methylene chloride and DMSO (80/20 (v/v) %) containing 50 mg of PLGA and 0.5 mg of lyophilized PEG/DNA mixture (PEG/DNA weight ratio = 15/1) was slowly added into 50 ml of 0.11% (w/v) aqueous Pluronic F127 solution pre-saturated with methylene chloride. The O/W emulsion solution was homogenized at 5000 rpm for 5 min by using a probe-type homogenizer (Fisher Scientific PowerGen 700). The solution was then placed under a magnetic stirring condition for 4 h to evaporate methylene chloride. The hardened PLGA nanospheres were dialyzed for 3 h and stored at −20 °C. For the PLGA nanospheres prepared by the W/O/W emulsion method, 0.25 ml of deionized water containing 0.5 mg GFP plasmid was added to the organic solvent mixture (methylene chloride/DMSO = 80/20 (v/v) %) solution and homogenized for 1 min at 5000 rpm. Then the primary emulsion solution was added to 50 ml of 0.1% (w/v) aqueous Pluronic F127 solution pre-saturated with methylene chloride, homogenized for 4 min at 5000 rpm, and purified similarly.

2.2.4. Characterization of PLGA nanospheres

The size of PLGA nanospheres was analyzed by a dynamic light scattering instrument. Surface morphology of nanospheres was visualized by scanning electron microscopy (SEM, Philips 535 M). The nanospheres were mounted on a brass stub using a double-sided adhesive tape and vacuum coated with a thin layer of gold particles. To measure the amount of plasmid DNA loaded within the nanospheres, the solution was centrifuged at 14,000 rpm for 30 min and the pellet was dissolved in DMSO. After dialysis in deionized water to remove DMSO, the loading amount and the structural integrity of plasmid DNA were determined by UV spectrophotometer and agarose gel electrophoresis, respectively.

2.2.5. Release profile of plasmid DNA from PLGA nanospheres

In vitro releases of plasmid DNA from PLGA nanospheres were performed in phosphate buffered saline (PBS, pH 7.4) solution. Dried PLGA nanospheres (1 mg) were resuspended in 1 ml of PBS solution and incubated at 37 °C. At a pre-determined time, the supernatant was collected after centrifugation to determine the amount of released plasmid DNA by the Picogreen assay (Molecular Probe, Eugene, OR).

2.2.6. Intracellular uptake of PLGA nanospheres

Raw264.1 macrophage cells were seeded on a six-well plate at a cell density of 1×10^6 cells/well containing RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate. Next day, the culture medium was replaced with the fresh medium containing 1.3 mg of DNA containing PLGA nanospheres. After 1 h, cells were washed with PBS solution and incubated for 2 days in the culture medium. Optical image of Raw264.1 cells engulfing PLGA nanospheres by phagocytosis was visualized by fluorescence microscopy (Nikon Eclipse TE300, USA). Total DNA was isolated from the cells incubated with or without PLGA nanospheres using a DNeasy tissue kit (Qiagen, CA). GFP plasmid in cells was examined by polymerase chain reaction (PCR) performed with GFP primers (forward sequence: 5′-TGGTGAGCAAGGGCGAGG AG-3′, reverse sequence: 5′-GGGGGTGTTCTGCTGGT AGT-3′) and β-actin primers (forward sequence: 5′-TGG CACCACACCTTCTACAATGAGC-3′, reverse sequence: 5′-GCACAGCTTCTCCTTAATGTCACGC-3′) (Bioneer, Daejeon, Korea) using rTaq polymerase (Takara, Japan), according to the manufacturer's instruction.

3. Results and discussion

In our previous study, DNA was solubilized in selected organic solvents such as DMSO and methanol by forming nano-complexes by using PEG as a DNA condensing additive [16]. DNA was complexed with PEG to form nanoscale particulates, presumably through hydrogen bonding between DNA and PEG in the polar organic solvent. In this study, DNA was solubilized in a more hydrophobic organic solvent mixture composed of methylene chloride/DMSO (80/20 (v/v) %) to prepare PLGA nanospheres. The organic phase containing PEG/DNA nano-complexes prepared with different PEG/DNA weight ratios was used to measure its transmittance value by UV spectrophotometer. As shown in Fig. 1a, the transmittance value increases as raising the weight ratio of PEG/DNA and reaches near 100% at a weight ratio of 15. The resultant organic phase was completely transparent without showing any precipitates. This suggests that in the mixed methylene chloride/DMSO solvent phase, DNA was also complexed with PEG to form much smaller nanoscale particles than the wavelength of irradiated UV light, which did not scatter the UV light at all. In this sense, it can be regarded that DNA was solubilized in the organic solvent mixture, although DNA was actually present in a condensed and dispersed state as a nanoparticulate. As a matter of fact,

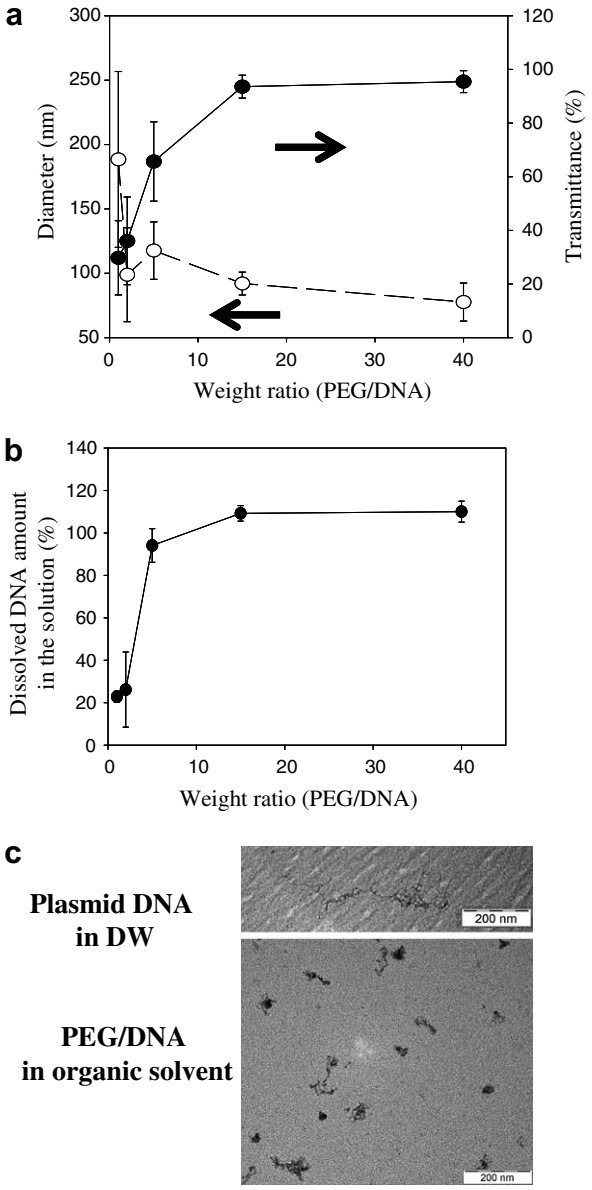

Fig. 1. (a) Ttransmittance of organic solvent mixture (methylene chloride/DMSO = 80/20 (v/v) %) with PEG/salmon sperm DNA complexes at 0.2 mg/ml of DNA concentration and the size of the complexes at various PEG/salmon sperm DNA weight ratios. (b) Dissolved salmon sperm DNA amount in the solution at various PEG/DNA weight ratios. (c) TEM image of plasmid DNA in water and PEG/plasmid DNA complexes in the organic solvent mixture.

the size of PEG/DNA nano-complexes in the solvent mixture as determined by DLS decreased from 188.5 ± 68.2 to 77.7 ± 14.8 nm with increasing weight ratio of PEG/DNA from 1 to 40, directly indicating that DNA was non-covalently interacted with PEG to form nanoscale complexes in the organic phase. It was previously reported that hydrogen bonding of DNA occurred with nonionic polymers, poly(vinyl alcohol) (PVA) or poly(vinyl pyrrolidone) (PVP), to form nanoparticulate complexes in aqueous solution [17–19]. Thus it is conceivable that an ether bond of PEG interacts with N–H of DNA by hydrogen bonding in the organic phase to form DNA/PEG nano-complexes.

The amount of solubilized DNA in organic solvent mixture (methylene chloride/DMSO = 80/20 (v/v) %) was also determined as shown in Fig. 1b. It can be seen that DNA was solubilized almost 100% at a PEG/DNA weight ratio of 15. PEG molecular weight did not significantly affect the solubility of DNA in the organic solvent, but the PEG/DNA weight ratio was more important (data not shown). Fig. 1c shows TEM pictures of PEG/DNA nano-complexes in the organic solvent and naked DNA in water. It can be seen that DNA was greatly condensed below 100 nm in the organic phase, but naked plasmid DNA in water phase has a relaxed and stretched form with a contour length of about 1 μm. When the PEG/DNA nano-complexes formed in the organic phase were transferred in the aqueous phase, DNA regained its original stretched form probably by breaking up hydrogen bonding between DNA and PEG.

The structural integrity of plasmid DNA during a high shear stress condition was examined as shown in Fig. 2. Naked plasmid DNA in aqueous phase and PEG/DNA nano-complexes in organic solvent were homogenized for pre-determined periods, integrity of plasmid DNA was visualized by gel electrophoresis. While naked plasmid DNA in the water phase was massively degraded without showing a supercoiled form after 10 min homogenization, PEG/DNA nano-complexes in the organic phase fully maintained supercoiled, nicked circular, and linear forms under the same homogenization condition. This reveals that plasmid DNA in a state of nano-complex could be survived without any structural damage against harsh formulation processes involved during the preparation of PLGA nanospheres.

PLGA nanospheres encapsulating plasmid DNA were prepared by both a single O/W and a double W/O/W emulsion and solvent evaporation method. The average size of PLGA nanospheres prepared by the single emulsion and the double emulsion methods was 507.8 ± 170.2 nm and

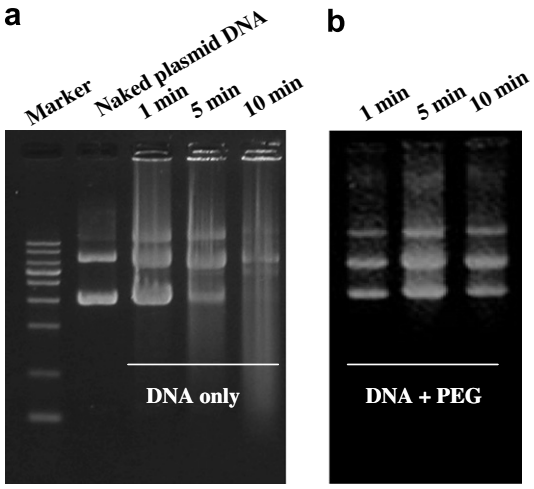

Fig. 2. Effect of homogenization on DNA integrity. (a) naked plasmid DNA in water and (b) PEG/plasmid DNA complexes in the organic solvent mixture were homogenized for predetermined time.

447.7 ± 26.6 nm, respectively. Fig. 3 shows SEM pictures and DLS results for the two PLGA nanospheres. Both of them appear to have similar diameters and morphological characters. However, loading efficiencies and structural integrities of DNA encapsulated within the two nanospheres were quite different. The DNA loading efficiency within O/W and W/O/W PLGA nanospheres were 62.8 ± 8.2% and 21.2 ± 3.8%, respectively. The loading amount of DNA for the O/W PLGA nanospheres was 6.2 ± 0.8 μg DNA per mg PLGA nanospheres. The DNA loading efficiency of O/W PLGA nanospheres was about three-fold higher than that of W/O/W PLGA nanospheres. This was because PEG/DNA nano-complexes were more effectively and stably encapsulated within PLGA nanospheres without undergoing DNA fragmentation during the homogenization procedure. It is obvious that DNA in a collapsed form is more resistant to the shear-induced fragmentation. Fig. 4a shows structural integrities of DNA extracted from the two PLGA nanospheres. DNA from the O/W PLGA nanospheres was intact, whereas that from the W/O/W PLGA nanospheres was completely degraded. Thus the loading efficiency of DNA for the W/O/W PLGA nanospheres was greatly decreased possibly due to rapid elimination of small molecular weight DNA fragments during the encapsulation process. In contrast, plasmid DNA extracted from O/W PLGA nanospheres maintained its intact conformational structures, nicked circular and supercoiled forms, although the relative fractional ratio between the two forms altered to some extent after encapsulation. This suggests that the O/W PLGA nanospheres encapsulated an intact form of plasmid DNA even under the high shear stress condition, while the W/O/W PLGA nanospheres contained severely degraded DNA fragments. Accordingly, the DNA loading efficiency of the W/O/W PLGA nanospheres (21.2 ± 3.8%) was for degraded DNA molecules, not for intact DNA. Fig. 4b shows in vitro release profile of intact plasmid DNA from the O/W PLGA nanospheres. The W/O/W nanospheres were not subjected to the release experiment because DNA encapsulated within them was chemically degraded. The O/W PLGA nanospheres exhibited an initial burst of 46.3% at 1 h and a subsequent slow release up to 24 h. The initial burst profile, followed by the sustained release, might result from the non-uniform distribution of PEG/DNA nano-complexes within PLGA nanospheres having a heterogeneous size distribution. The rapid release of DNA within 24 h also suggests that hydrophilic PEG/DNA nano-complexes entrapped within PLGA nanospheres were preferentially hydrated and swollen upon incubating in aqueous medium and then quickly released out by osmotic rupture of surrounding PLGA polymer phase. GFP plasmid DNA encapsulating PLGA nanospheres were incubated with macrophage Raw 264.1 cells to show the extent of cellular uptake and gene expression. They were readily taken up by macrophage cells without any surface modifications. It can be observed that many PLGA nanospheres are present in the intracellular area (Fig. 4c). The cells certainly expressed an exogenous GFP gene as shown in the RT-PCR result of Fig. 4d. However, the GFP expression level in the macrophage cells was very low to quantify, compared to those transfected with commercially available polyplexes and lipoplexes. This might be due to the fact that DNA loaded PLGA nanospheres could not efficiently escape from the endosome and lysosome compartments after cellular uptake. It will be desirable to incorporate fusogenic peptides in the PLGA nanospheres to enhance the gene expression efficiency.

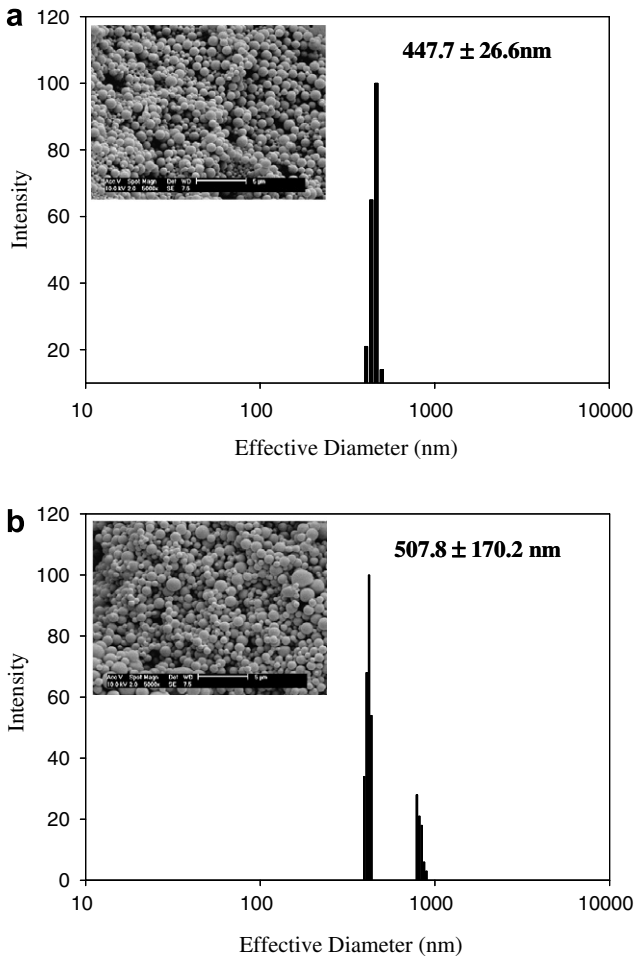

Fig. 3. Morphology and diameter of PLGA nanospheres formulated by (a) double emulsion method and by (b) single emulsion method as observed by scanning electron microscope and dynamic light scattering, respectively.

This study mainly aims to efficiently and stably encapsulate intact plasmid DNA within PLGA nanospheres by a single O/W emulsion method. The formation of PEG/DNA nano-complexes in a mixed organic solvent was used as a novel formulation strategy to achieve the above goal. Since an intact plasmid DNA was released out within 24 h, the current DNA encapsulating PLGA nanospheres would be a suitable delivery system for DNA vaccines. Recently, naked DNA vaccines have received much attention to overcome many of the limitations of conventional antigenic vaccines, such as time and cost for production

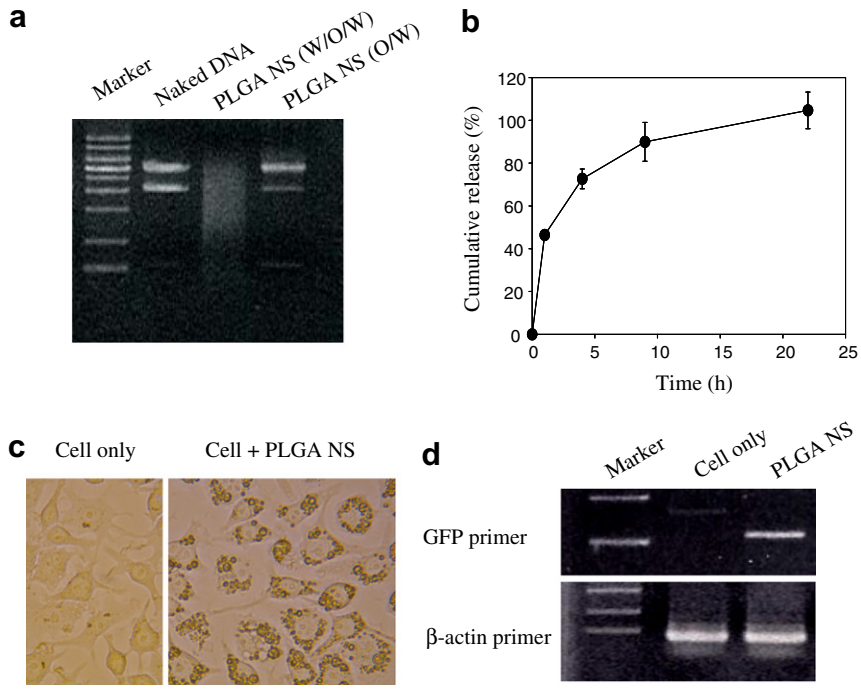

Fig. 4. (a) Integrity of plasmid DNA within PLGA nanospheres was examined in gel electrophoresis. (b) In vitro release profiles of plasmid DNA loaded PLGA nanospheres. (c) Optical image of Raw264.1 cells engulfing PLGA nanospheres (PLGA NS) by phagocytosis. The cells were completely washed to remove surface bound PLGA nanospheres. (d) Expression of GFP plasmid DNA to macrophage cells by PLGA nanospheres was confirmed by PCR with using GFP primer and β-actin primer.

[20–22]. Micro- or nanoparticles have been investigated as potential carriers for DNA vaccines. However, the encapsulation of DNA within micro- or nanoparticles by conventional methods results in degradation during formulation. Although PLGA nanospheres formulated in this study could not exhibit a long-term sustained release of plasmid DNA, they could be readily internalized within cells for eliciting fast immune responses. Thus plasmid DNA encapsulated PLGA nanospheres could be effectively used as mucosal or prime-boosting vaccines [23,24]. Nonviral cationic polymeric vectors have also been used for DNA vaccine delivery to escape significant degradation during formulation and enhance gene delivery efficiency [25–27]. In spite of good gene delivery efficiency, cationic polymers have innate problems, such as serious cytotoxicity for in vivo administration [28]. To compensate these problems, biocompatible and biodegradable PLGA nanospheres encapsulating intact plasmid DNA, introduced in the current work, would be used as a much safer vehicle for antigen presentation in immuno-modulating cells such as macrophages and dendritic cells.

In conclusion, PEG-assisted DNA solubilization in organic solvent enabled the encapsulation of intact plasmid DNA within PLGA nanospheres by a single O/W emulsion formulation. This method improves both the maintenance of DNA structural integrity during formulation and loading efficiency within PLGA nanospheres. This new formulation strategy can be applied to a wide range of gene delivery systems including DNA vaccines.

Acknowledgements

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