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Rapid communication

A modified process for preparing cationic polylactide-*co*-glycolide microparticles with adsorbed DNA

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Abstract

We have previously shown that cationic polylactide-*co*-glycolide (PLG) microparticles can be effectively used to adsorb DNA and generate potent immune responses in vivo. We now describe a modified and easier process containing a single lyophilization step to prepare these cationic PLG microparticles with adsorbed DNA. Cationic PLG microparticle formulations with adsorbed DNA were prepared using a modified solvent evaporation technique. Formulations with a fixed CTAB content and DNA load were prepared. The loading efficiency and 24 h DNA release was evaluated for each formulation and compared to the earlier method of preparation. Select formulations were tested in vivo. The modified cationic PLG microparticle preparation method with a single lyophilization step, showed comparable physico-chemical behaviour to the two lyophilization steps process and induced comparable immune. The modified process with a single lyophilization step is a more practical process and can be utlized to prepare cationic PLG microparticles with adsorbed DNA on a large scale.

Keywords: PLG microparticles; Lyophilization; Double emulsion; Cationic surface

1. Introduction

DNA vaccines have been shown to induce potent humoral and cellular responses with various bacterial plasmids encoding a variety of antigens (Ulmer et al., 1993; Gurunathan et al., 2000; Rice et al., 2000; Prudhomme et al., 2002; Lemieux, 2002). These studies established the need for more potent delivery systems for plasmids to induce higher in vivo transfection efficiency and higher immune responses with reduced doses of plasmid. Approaches used to improve the potency of DNA vaccines include, vector modification to enhance antigen expression (Zur Megede et al., 2000), physical delivery methods (Selby et al., 2000; Ng and Liu, 2002), and the use of vaccine adjuvants (Ulmer et al., 1999). We previously described the development of cationic polylactide-*co*-glycolide (PLG) microparticles with adsorbed plasmids as a novel delivery system for DNA vaccines (Singh et al., 2000). This approach utilized the positive charge of cetyltrimethylammonium bromide (CTAB) to make a cationic PLG/CTAB microparticle, which efficiently adsorbed negatively charged plasmid DNA onto its surface. This micropartice formulations allowed efficient delivery of DNA into antigen presenting cells and induce strong immune response against the antigen encoded by the plasmid.

Biodegradable PLG polymer was selected as a polymer of choice to form the microparticles based on its safety profile and long use in humans (Okada and Toguichi, 1995). Although we have previously described the preparation and characterization of cationic PLG microparticles using a double emulsion process with two lyophilization steps (Singh et al., 2000), we subsequently modified the process to allow a single lyophilization step and more adaptable for scale up. We report here that the concentration of surfactant, and its effect on plasmid adsorption and release is an important parameter in the modified process

Abbreviations: TE, Tris–EDTA buffer; SDS, sodium dodecyl sulphate; CTAB, cetyltrimethylammonium bromide; PLG, polylactide-*co*-glycolide; LYO, lyophilization

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and is of importance to allow the preparation of uniform and reproducible microparticles.

In our earlier publications, we reported the use of a fixed concentration of the cationic surfactant (0.5%, w/w) for the preparation of PLG/CTAB microparticles, but most of the CTAB was washed away during the microparticle preparation process (Singh et al., 2000; Briones et al., 2001). In this current paper, we describe the use of a fixed low CTAB concentration (0.015%, w/v) in the microparticle preparation process and describe its impact on adsorption efficiency and release of DNA. In addition, we describe the in vivo performance of PLG microparticles prepared using the single lyophilization process, with a fixed concentration of CTAB. Importantly, this work demonstrates that cationic PLG microparticles can be prepared by alternative simplified process, which is easier to scale up than the original process, and crucially, that the potency of the microparticles for DNA vaccine delivery is not impaired by preparation by the novel method.

2. Materials and methods

2.1. Materials

Polylactide-*co*-glycolide was obtained from Boehringer Ingelheim, USA. CTAB and other reagents were obtained from Sigma Chemical Co., St. Louis, USA and used as shipped. The HIV-1 pCMVkm p55 gag plasmid was made at Chiron and has been previously described (Zur Megede et al., 2000). U96-Nunc Maxisorp plates (Nalgene Nunc International, Rochester, NY), Goat anti-Mouse IgG-HRP conjugate (Caltag Laboratories, Burlingame, CA), and TMB Microwell Peroxidase Substrate System (Kirkegaard & Perry Laboratories, Gaithersburg, MD) were used for the ELISA.

2.2. The preparation and characterization of PLG/CTAB microparticles

The standard two step lyophilized PLG/CTAB microparticles were prepared using a solvent evaporation technique as described previously (Singh et al., 2000; Briones et al., 2001; O'Hagan et al., 2001). For preparing PLG/CTAB/DNA microparticles using the modified single lyo process, 10 ml of a 6% (w/v) polymer solution in Methylene chloride was emulsified with 1 ml of Tris–EDTA (1× TE) buffer at high speed using an IKA homogenizer for 3 min. The primary emulsion was then added to 50 ml of distilled water containing CTAB (0.015%, w/v) and further emulsified for 15 min under high shear. This resulted in the formation of a w/o/w emulsion, which was then stirred on a magnetic stirrer at 6000 rpm for 12 h at room temperature, to allow the methylene chloride to evaporate. The resulting microparticle suspension (15 mg/ml) was then directly used for adsorption of plasmid DNA, formulation stabilizers (cryoprotectant sugars within an isotonic range) were added, and the microparticles were lyophilized in vials. Fig. 1 illustrates the two methods for comparison.

The plasmid was adsorbed at a fixed load of 4% (w/w) for both the microparticle preparation processes, single and dual lyo. The amount of adsorbed DNA was determined by hydrolysis of the PLG microparticles, followed by measurement of absorbance at $A_{260\,nm}$. The size distribution of the microparti-

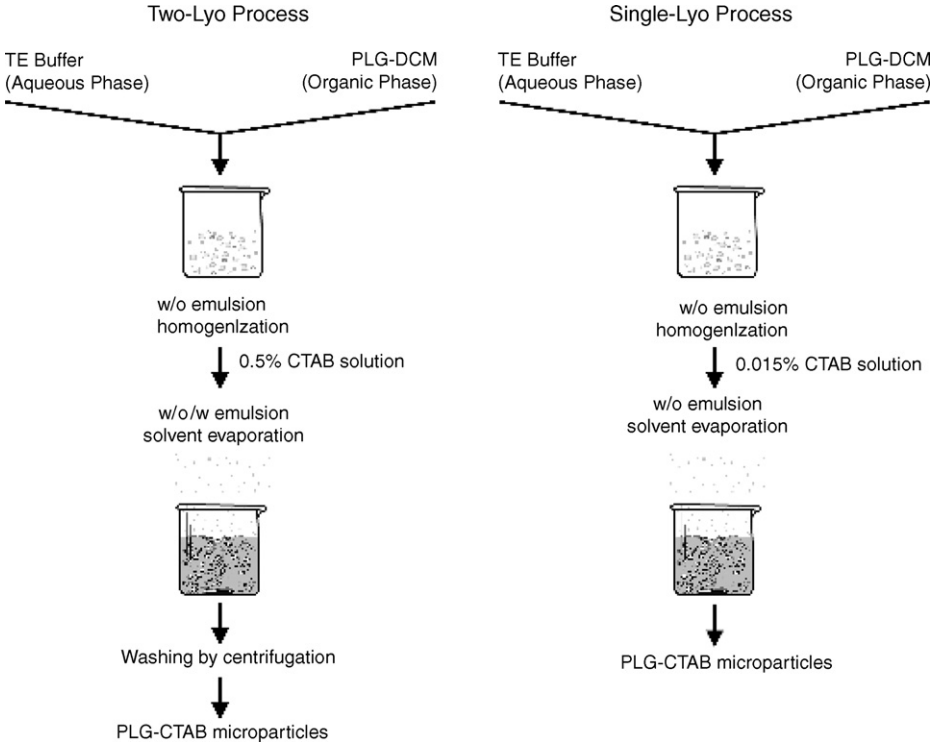

Fig. 1. Comparison of the single-lyo and two-lyo process to prepare charged cationic microparticles with adsorbed DNA.

cles preparations was measured using a particle size analyzer (Malvern Instruments, Malvern, UK).

2.3. SEM analysis of PLG/CTAB/DNA formulations

Scanning electron microscopy (SEM) analysis of the two microparticle formulations prepared with the different processes was undertaken with a Hitachi S-5000 (UC Berkeley Electron Microscope Laboratory, Berkeley, CA). A dilute suspension of the particles was dried onto an adhesive, conductive surface and coated with a 5 nm layer of platinum followed by a 20 nm protective carbon overcoat. A field-emission source was accelerated to 10 keV and the electron beam was focused on to the coated particles to permit spatial resolution of a few nanometers. Various fields within the same stub were monitored and recorded.

2.4. Determination of CTAB content in the microparticles and it's distribution

Since the previously described colorimetric CTAB detection method (Singh et al., 2000) was not sufficiently sensitive to determine the low levels of CTAB used in the new microparticle preparation process, we developed a more sensitive HPLC approach. The total amount of CTAB in PLG/CTAB microparticles was estimated after hydrolysis by HPLC. Briefly, 10 mg of PLG/CTAB particles were dissolved in 1 ml 1N NH_4OH–1% Sodium dodecyl sulphate (SDS) solution overnight at room temperature and 100 μl of this was injected on an IonPac NS1 (10 μM) 4 mm × 250 mm column (Dionex, USA) with an IonPac NG1 guard column (4 mm × 35 mm). The column was run at room temperature with a water–acetonitrile mixture containing 2 mM nanafluoropentanionic acid, using a Waters Alliance System (Waters, USA) at a flow rate of 1 ml/min, and an Alltech 2000 Evaporative Light Scattering detector (Altech Inc. USA) using 2.8 l/min ultra high purity 5.0 nitrogen gas and drift tube temperature at 93 °C.

A standard curve was generated by dissolving 5 mg of polymer in 1 ml of 1N NH_4OH–1% SDS with varying amounts of CTAB in 1× TE and from it unknown samples were calculated. The limit of detection (LOD) of this assay was around 500 ng of CTAB. The assay had a linear range from 0.5 to 500 μg. The extraction efficiency of CTAB from the PLG microparticles by hydrolysis was >99%. The amount of CTAB bound to the microparticles and the amount released over time was calculated by re-suspending 10 mg of freeze dried microparticles in 1 ml of PBS and separating the pellet from the supernatant after 1 h at room temperature.

2.5. DNA adsorption efficiency and release rate

The adsorption efficiency of DNA was estimated for both the batches of PLG/CTAB microparticles prepared by the different processes. The adsorption efficiency was estimated by taking a 1 ml aliquot of the PLG/CTAB/DNA suspension and centrifuging it for 1 min at 1000 rpm on a table top centrifuge. The resulting supernatant was evaluated for amount of unadsorbed DNA by a spectrophotometer at $A_{260\,nm}$.

The in vitro release of DNA was estimated by incubating 10 mg of freeze dried PLG/CTAB/DNA microparticles in PBS at room temperature and estimating the released DNA by measuring the supernatant at $A_{260\,nm}$. Samples were collected at time 1 h (0.04 days) and days 1, 3, 7, 14 and 21.

2.6. Evaluation of immune responses to PLG/CTAB/DNA micropaticles

Groups of 10 females Balb/C mice aged 6–8 weeks and weighing about 20–25 g were immunized with a 1 and 10 μg dose of DNA adsorbed to microparticles prepared by the different processes at days 0 and 28. Control animals ($n = 10$) were also injected with soluble DNA alone at days 0 and 28. A 100 μl of the formulations in saline was injected by the TA route in the two hind legs (50 μl per site) of each animal. Mice were bled on day 42 through the retro-orbital plexus and the sera were separated. HIV-1 p55 gag specific serum IgG titers were quantified by ELISA, as previously described and were compared to immunization with the same dose of naked DNA (Singh et al., 2000; Briones et al., 2001).

3. Results and discussion

3.1. Characterization of PLG/CTAB microparticles

Cationic microparticles were prepared with a mean size of around 1 μm (size distribution of 1.2–1.9 μm). The scanning electron micrographs showed the particles to be spherical in shape, with a smooth outer surface obtained from both the processes (data not shown). The physical characteristics of the two preparations in suspension were undistinguishable.

To allow adsorption of DNA onto the microparticles, it is assumed that the CTAB needs to be present on the microparticle surface. Following microparticle preparation using a high concentration (0.5%, w/v) in the external phase requires a subsequent washing step to remove CTAB not bound to the microparticles. In this process the total amount of residual CTAB was estimated to be around 1% (w/w) to the PLG. To avoid the removal of the excess CTAB from the final suspension, we modified the process using a fixed CTAB concentration (0.015%, w/v) solution in the external phase allowing for immediate addition of DNA for adsorption to this suspension.

The concentration of CTAB (0.015%, w/v) was picked based on the amount of CTAB that would remain in the final PLG suspension (1%, w/w). Adding higher amounts of CTAB led to a decrease in DNA release as shown in earlier studies (Briones et al., 2001). This process required only a single lyophilization step, leading to a more practical way of making these cationic PLG/DNA microparticles. We also determined the CTAB distribution between microparticles and supernatant for both the batches of microparticles. The data exhibited that CTAB remained predominantly bound to the PLG surface (>90%) for the two lyo process and for the modified single lyo process only about 30% of the total CTAB was associated to the PLG surface (Table 1).

Table 1
Characterization of PLG/CTAB/DNA formulation prepared from the two different methods (single lyo process and the two lyo process)

Formulation	Theoretical load (%)	Actual load (%)	Loading efficiency (%)	Size (μm) pre-adsorption	Total residual surfactant concentration (%)	CTAB adsorbed to the PLG (%)
LG-CTAB-pCMVp55 gag DNA (single lyo process)	4.0	3.88	97	1.45	0.96	0.32
PLG-CTAB-pCMVp55 gag DNA (two lyo process)	4.0	3.98	99	1.86	0.88	0.84

Values represent a mean of two measurements.

3.2. Release rate of DNA

Fig. 2 shows the release rates over 21 days for DNA adsorbed to PLG microparticles prepared with the two methods (two step and single step lyophilization process). Both the 1 h release (0.04 days) and the release upto day 21 did not show statistical differences between the two formulations ($p < 0.05$). It is clear that the in vitro DNA release rate is not significantly altered by the two methods during the full observation period (4 weeks). Furthermore the quality of DNA from the two formulations (percent supercoiled versus open circular form) remained unaltered as seen on a 1% agrose gel (data not shown).

3.3. In vivo evaluation in small animals

Table 2 summarizes the in vivo immunogenicty of PLG/CTAB/DNA formulations prepared using the two methods (single lyo process and the two step lyo process) and their comparison with naked DNA at the same dose. Both of the formulation processes produced microparticles that induced higher responses than naked DNA alone and were comparable to each other in potency. This finding illustrates that the new single-lyo process which is more efficient and less time consuming can be used to prepare PLG/CTAB/DNA formulations.

Overall, we believe that this new method using a single lyophilization is a more practical method and we have been able to scale this process using aseptic processing to a 1.5 kg batch size (data not shown). Also the inclusion of the internal aqueous phase (TE buffer) aids in uniform distribution of the primary emulsion that is formed before addition of the CTAB solution. This step has been retained in the modified process for consistency and for comparing historical in vivo data.

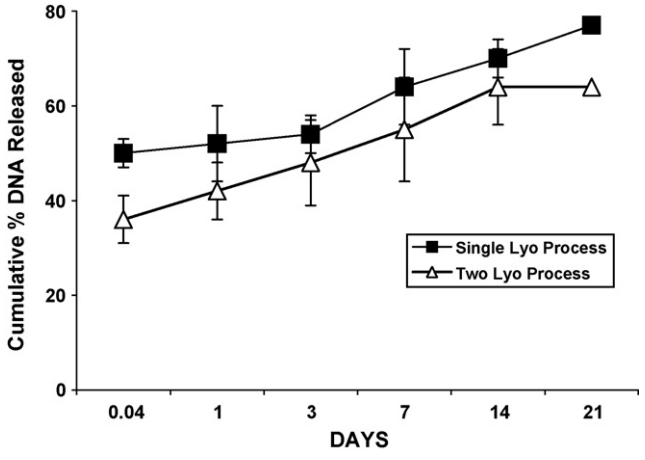

Fig. 2. In vitro release of DNA up to day 21 from PLG/CTAB/DNA prepared by the two different methods (single lyo process and the two lyo process). Mean ± S.E. ($n = 3$) is shown for each time point.

This novel approach of presenting DNA on cationic PLG microparticles has a number of advantages over an alternative approachs, involving microencapsulation of DNA. Our approach is simple, scaleable and robust, allows efficient adsorption and release of intact DNA, and induces significantly enhanced responses in comparison to naked DNA (Singh et al., 2000; Briones et al., 2001; O'Hagan et al., 2001). The approach of presenting antigens on the surface of PLG microparticles has also been used recently to induce potent immune responses against protein antigens (Kazzaz et al., 2000). Hence, surface presentation of antigens represents a novel way to use PLG microparticles as an effective vaccine delivery system. Recent studies have indicated that the microparticles are effective for the induction of enhanced immune responses largely as a consequence of the delivery of the adsorbed DNA into antigen presenting cells (Denis-Mize et al., 2000).

We also showed that the in vivo performance of PLG/DNA microparticles was not impaired by this preparation method or amount of CTAB adsorbed to the PLG surface. The accumulated evidence from the current and previous studies firmly establish PLG/CTAB microparticles as a robust and reliable means to deliver DNA for enhanced immune responses in a variety of species, including non-human primates (O'Hagan et al., 2001). Prototype PLG/DNA formulations prepared using the above single lyo process are now undergoing clinical evaluation.

Table 2
Serum IgG p55 gag titers for mice immunized with PLG/CTAB/DNA microparticles prepared using the two methods (single lyo process and the two lyo process)

Formulation	Dose (μg)	Serum P55 gag 2wp2 titers (GMT) ± S.E.
Naked DNA	1	367 ± 128
PLG/CTAB/DNA (single lyo process)	1	5710 ± 3831
PLG/CTAB/DNA (two lyo process)	1	4261 ± 1980
Naked DNA	10	2185 ± 1240
PLG/CTAB/DNA (single lyo process)	10	19679 ± 4163
PLG/CTAB/DNA (two lyo process)	10	17406 ± 2124

Antibody responses are shown as geometric mean titers ± S.E. ($n = 10$) at day 42. The titers from the two methods are not significantly different from one another and they are both significantly higher than naked DNA ($p < 0.05$).

4. Conclusions

Overall, the current studies have shown that PLG/CTAB microparticles can be prepared with a fixed CTAB concentration that does not require a removal step for excess CTAB. The in vivo performance of the two methods was not significantly different from each other, and they were both significantly better than naked DNA. This modified process is more easy to scale up for clinical evaluation as the CTAB removal step is very complex and inefficient for aseptic manufacturing.

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