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Stability and release characteristics of poly(D,L-lactide-co-glycolide) encapsulated CaPi-DNA coprecipitation

Yaping Li [a,b,*], Manfred Ogris [a], Jaroslave Pelisek [a], Wolfgang Röedl [a]

[a] *Center of Drug Research, Department of Pharmacy, Ludwig-Maximilians-University, Butenandtstr. 5-13, D-81377 Munich, Germany*
[b] *Institute of Materia Medica, Shanghai Institute for Biological Science, Chinese Academy of Sciences, 555 Zu Chong Zhi Road, Shanghai 201203, China*

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Abstract

The aims of this work were to determine the stability of DNA-calcium-phosphate coprecipitation (CaPi-DNA) against various conditions during double emulsification microencapsulation and assess the release and physicochemical characteristics of poly(D,L-lactide-co-glycolide) (PLGA) microparticles loading CaPi-DNA. CaPi-DNA prepared at pH 6.5 showed a good stability with over 60% CaPi-DNA remained after emulsification, but no more than 40% at pH 8.0. Polyvinyl alcohol (PVA, 1–5%) could make over 80% CaPi-DNA (pH 7.0) preserved after homogenization. The dichloromethane (DCM), mixture of DCM and ethyl acetate, ether and *n*-hexane (1:1) exhibited neglectable influence on CaPi-DNA under homogenization. PLGA had influenced on CaPi-DNA without any additional stabilizer, in particular, PLGA (75:25, 4%, w/v) demonstrated a profound damage with only about 10% of the original CaPi-DNA remained. PLGA microparticles loading CaPi-DNA were spherical in shape with size range of 2.0–5.0 μm, and entrapment efficiency 30–50%. CaPi-DNA was found to increase the stability of pDNA in PLGA microparticles without losing its structure integrity. The release of CaPi-DNA from microparticles showed a low burst release (<7.5%) within 24 h and following sustained release process. The amount of cumulated CaPi-DNA release over 30 days was: 17.6% for PLGA (lactide:glycolide = 50:50), 27.3% for PLGA (65:35) and 44.8% for PLGA (75:25) microparticles, respectively. The encapsulation of CaPi-DNA in microparticles could significantly protect CaPi-DNA from degradation of nuclease with average over 80% of total DNA recovery. These results suggested that the encapsulation of CaPi-DNA in PLGA microparticles could improve stability of pDNA.

Keywords: pDNA; Calcium phosphate coprecipitation; Stability; Microparticles; Poly(lactide-co-glycolide)

1. Introduction

Gene therapy is becoming a promising approach for the treatment of disease with the more understanding of the genetic basis of disease. Although delivery systems of viral origin, such as adenovirus and retrovirus, can efficiently introduce genes, their immunogenicity, toxicity, limits on the size of the plasmid to be incorporated and lack of tissue specificity restrict their use for only ex vivo gene therapy (Van de Wetering et al., 1998; Tagawa et al., 2002). Non-viral delivery systems, for example, polycations

* Corresponding author. Tel.:+86-21-5080-6820; fax: +86-21-5080-7088.
E-mail address: ypli@mail.shcnc.ac.cn (Y. Li).

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(cationic lipids, polylysine and polyethyleneimine) and particulate carriers (liposomes, nanoparticles and microspheres) have been increasingly proposed as alternatives to viral vector because of their potential advantages, such as tissue-specific targeting, relative ease of large-scale production, non-immunogenic and relative safety (Kircheis et al., 2001; Cemazar et al., 2002; Hasegawa et al., 2002). Unfortunately, these non-viral delivery systems lack efficiency of gene transfer of viruses. Therefore, design and manufacturing of safe and effective gene delivery system have been a challenge for researchers interested in gene therapy.

Plasmid DNA (pDNA) has shown great potential utility not only as gene replacement therapy and therapeutic application, but also as a new vaccination approach (Jones et al., 1997; Hedley et al., 1998; Middaugh et al., 1998). However, free pDNA, without any delivery system, is highly susceptible to nuclease degradation. In order to deliver pDNA efficiently, in past few years, much attention had focused on biodegradable microparticles of poly(D,L-lactide-co-glycolide) (PLGA) because the rate and duration of release of entrapped drugs could be controlled by varying the molecular weight or ratio of lactide and glycolide of PLGA, and PLGA microparticles could protect pDNA from nuclease degradation, facilitate uptake of pDNA targeted to phagocytic cells such as macrophages and increase tissue specificity due to localized delivery (Jeong and Park, 2001; Walter et al., 2001; McKeever et al., 2002; Vandervoort and Ludwig, 2002; Walter and Merkle, 2002). Nevertheless, these investigations also showed that stability of pDNA and the properties of microparticles depend mainly on processing parameters; such as emulsification energies, high interfacial tension at the dichloromethane–water interphase, organic solution, polymer molecular weight and concentration, phase viscosities and stabilizers, because pDNA possesses some unique features, namely, its supercoiled form that is more efficient in gene expression than the relaxed form is susceptible to convert to the linear and open circular forms during the microencapsulation and result in a significant reduction in gene express (Luo et al., 1999; Wang et al., 1999). Recently, some effective approaches for encapsulating pDNA within PLGA microparticles, such as the spray-drying and the inversion/solvent diffusion techniques, have been investigated (Walter et al., 1999; Hirosue et al., 2001). Unfortunately, these authors discovered that the encapsulated pDNA was degraded during the encapsulation or toxic excipients needed to be replaced. A cryopreparation method has been proposed to prevent the exposure of the pDNA to shear forces created during the homogenization process resulting in the inactivation of the pDNA (Ando et al., 1999). However, the stabilization of the pDNA in a long-term release process requires further investigation. Another approach is the encapsulation of pDNA previously complexed with poly(L-lysine) (PLL) to improve its encapsulation and release in the supercoiled form (Capan et al., 1999a,b; Seemann et al., 2002). Whereas, limitations of this approach were its low encapsulation efficiency and large amounts of open circle pDNA occurred. Thus, the encapsulation of pDNA within PLGA microspheres makes it imperative to find some new approaches to prepare stabile pDNA formulation.

We are interested in exploring a new delivery system that DNA-calcium-phosphate coprecipitation (CaPi-DNA) is incorporated into PLGA microparticles because CaPi-DNA is a commonly used method for gene transfer, and its usefulness in biomedical research and in biotechnological applications for the generation of highly productive recombinant cell lines has been largely demonstrated (Batard et al., 2001). The aim of this study is to examine the influences of various preparation parameters during double microencapsulation on the stability of CaPi-DNA, prepare PLGA microparticles loading CaPi-DNA, and determine their physicochemical characteristics and in vitro release of CaPi-DNA from microparticles.

2. Materials and methods

2.1. Materials

PLGA including PLGA 50:50 (lactide:glycolide, M_w = 40–75 kDa), PLGA 65:35 (M_w = 40–75 kDa), PLGA 75:25 (M_w = 90–126 kDa), polyvinylpyrrolidone (PVP, M_w = 40 kDa) and dichloromethane (DCM) were purchased from Sigma (St. Louis, MO). pGShIL-2tet coding for human interleukin-2 (IL-2, supercoiled, 4.3 kb) was used and described in Schreiber et al. (1999). Polyvinyl alcohol (PVA,

M_w = 16 kDa, 98% hydrolyzed) was obtained from Fluka (Switzerland). Pico Green® reagent was purchased from Molecular Probes (Eugene, OR, USA). All other chemicals were obtained commercially as analytical grade reagent.

2.2. Preparation and analysis of CaPi-DNA

CaPi-DNA was prepared by a slight modification as described previously (Yang and Yang, 1997). Briefly, 100 μl pDNA solution containing 120 μg pDNA was mixed with 100 μl of 2.0 M $CaCl_2$. This solution was slowly added to 1 ml of 2× HBS (containing 280 mM NaCl, 10 mM KCl, 1.5 mM $Na_2HPO_4{\cdot}2H_2O$, 12 mM dextrose, 50 mM HEPES) at various pH with gentle shaking. At the end of titration, 800 μl of sterilized, deionized water was added to give a final volume of 2 ml. The mixture was incubated at room temperature for 30 min, during which time fine CaPi-DNA complex were formed, and the resulting solution was translucent in appearance. Experiments were performed to investigated whether CaPi form a complex with pDNA by determining the amount of free DNA in supernatant after ultracentrifuge at 25 000 × g for 20 min. The CaPi-DNA was prepared immediately prior to the experiment.

Integrity of CaPi-DNA was checked by 0.7% agarose gel electrophoresis. Gels were electrophoresed for 90 min at 110 V/cm in a Tris–acetate–EDTA buffer system (pH 8.0) and CaPi-DNA was visualized using ethidium bromide staining. Pico Green® analysis for CaPi-DNA was performed in 96-well plates with standard fluorescein wavelengths excitation 480 nm and emission 520 nm according to the manufacture's instructions using an automated plate reader (Tecan, Spectrafluor Plus, Austria). The ratio of remained to original CaPi-DNA was quantitated densitometrically using a Kodak Scanner (Kodak digital science, electrophoresis documentation and analysis system 120).

2.3. Stability of CaPi-DNA

The stability of CaPi-DNA against various conditions during the double emulsification microencapsulation was investigated. First, in order to examine the influence of emulsification process on CaPi-DNA that were prepared at various pH, 2 ml of CaPi-DNA solution containing 120 μg pDNA was homogenized at 7500 rpm (PT1300D, Polytron, Germany) for 15 s in an ice bath. In addition, the CaPi-DNA solution that was prepared at pH 7.0 (2× HBS) was homogenized at 7500 rpm for 5, 15 and 25 s, respectively. Then, the ratio of remained to original CaPi-DNA was determined as described above. Second, the protection of PVA and PVP on CaPi-DNA was investigated, 2 ml of CaPi-DNA solution with different concentrations of PVA or PVP was homogenized at 7500 rpm for 15 s in an ice bath, and the remained CaPi-DNA was determined. Third, the influence of organic solutions on CaPi-DNA was checked up, 0.6 ml of CaPi-DNA solution was emulsified in 2 ml of organic solutions at 7500 rpm for 15 s in an ice bath. Then, the emulsion was centrifuged at 5000 rpm for 10 min (4 °C, Eppendorf, Germany) to break emulsion and separate aqueous phase from oil phase. The CaPi-DNA was extracted from the aqueous phase and agarose gel electrophoresis was used for determining the integrity of DNA. Finally, the influence of PLGA on CaPi-DNA was investigated, 0.6 ml of CaPi-DNA solution was emulsified in 2 ml of DCM containing different concentrations or types of PLGA at 7500 rpm for 15 s in an ice bath. Then, the emulsion was centrifuged at 5000 rpm for 10 min (4 °C, Eppendorf, Germany) and CaPi-DNA from the aqueous phase was measured.

2.4. Preparation of CaPi-DNA microparticles

PLGA microparticles loading CaPi-DNA were prepared by a water–oil–water emulsion solvent evaporation method. Briefly, 0.6 ml of CaPi-DNA solution containing 36 μg pDNA was emulsified in 2 ml of DCM containing PLGA by homogenization at 7500 rpm in an ice bath for 5 s (PT1300D, Polytron, Germany) to form primary emulsion (W/O). Thereafter, the first emulsion was poured into 10 ml of the PVA aqueous solution (3.0%, w/v) and homogenized at 7500 rpm in an ice bath for 5 s (PT1300D, Polytron, Germany). The double emulsion (W/O/W) was diluted in 20 ml PVA solution (0.5%, w/v) under moderate magnetic stirring. The magnetic stirring was maintained at ambient temperature for 2 h to allow solidification of the microparticles, and DCM was eliminated by evaporation under reduced pressure (Rotavapor R-114, Büchi, Switzerland) at 37 °C.

Finally, the microparticles were collected by centrifugation at 15 000 × *g* for 15 min (Rc5c, Sorvall Instruments, Germany) and washed three times.

2.5. Entrapment efficiency and physicochemical characteristics of CaPi-DNA microparticles

The amount of CaPi-DNA encapsulated in microparticles was determined by suspending 10 mg of microparticles in 1 ml of 10 mM Tris–EDTA buffer (pH 7.4), then, adding 2 ml of chloroform and rotating end-over-end for 60 min, following centrifugation at 5000 rpm for 10 min (4 °C, Eppendorf, Germany). The aqueous layer containing the CaPi-DNA was transferred to a fresh tube. The amount of the extracted CaPi-DNA was calculated by Pico Green® assay as described above. The integrity of DNA was checked by agarose gel electrophoresis after the other portion was precipitated by the addition of EtOH as described previously (Seemann et al., 2002).

The morphology was examined by scanning electron microscopy (SEM, XL40, Philip) after palladium-gold coating of the microparticles sample on an aluminum stub.

Size, size distribution and ζ potential of microparticles were measured by laser light scattering following their resuspension in 10 mM NaCl (pH 7.2) using a Zetasizer 3000 HS (Malvern Instruments, UK). Calculation of particles sizes was based on Mie's theory accounting for the optical properties of the polymer (Thomasin et al., 1996). The average particle size was expressed as the volume mean diameter n_{md} (μm).

2.6. In vitro release experiment

The release of CaPi-DNA from microparticles was studied by suspending the microparticles (20 mg) in 1.5 ml of Tris–EDTA buffer (pH 7.4) preserved with 0.02% sodium azide at 37 °C under horizontal shaking (300 rpm, Thermomixer, Eppendorf, Germany). At predetermined time intervals, the suspension of microparticles was centrifuged at 15 000 × *g* for 10 min (5402, Eppendorf, Germany) and the supernatant collected for further DNA analysis. The microparticles were resuspended in the same volume fresh medium and incubated again under the same conditions. The amount of CaPi-DNA released in each time interval was determined by the Pico Green® assay.

2.7. DNase I digestion study

In order to check up the protection of microparticles on CaPi-DNA, CaPi-DNA microparticles (10 mg) were suspended with 5 μg of DNase I in 10 mM Tris–HCl buffer containing 10 mM $MgSO_4$ (pH 8.0) for 30 min at 37 °C. After digestion, microparticles were rinsed three times with 200 μl of fresh Tris–EDTA buffer. For extraction of CaPi-DNA, fresh Tris–EDTA buffer was added to suspend microparticles, and chloroform was used to solubilize the microparticles. The mixture was rotated end-over-end for 60 min at ambient temperature. The samples were centrifuged at 5000 rpm for 10 min and supernantant was removed and analyzed by the Pico Green® assay.

3. Results and discussion

3.1. Preparation and analysis of CaPi-DNA

CaPi-DNA solution could be prepared by mixing pDNA, $CaCl_2$ solution and HBS. Preliminary studies showed that an opaque CaPi-DNA solution, even a visible precipitation could easily produce when concentration of $CaCl_2$ was higher than 2 M or pH of HBS over 8.0. The size of CaPi-DNA rapidly increased with increasing pH of HBS (Table 1). Pico Green® dye exclusion proved to be a useful indicator of CaPi-DNA complex, even if a visible precipitation of CaPi-DNA occurred with the emitted fluorescence decreasing, however, its fluorescence intensity was much higher than baseline fluorescence. CaPi-DNA showed that about 75% of the original fluorescence intensity of pDNA was preserved when CaPi-DNA was prepared at pH 7.0. This indicates that CaPi-DNA is not a particle with a compact shell that could fully prevent the interaction of pDNA and Pico Green® dye. In addition, preliminary experiment showed that CaPi-DNA could be decomplexed when pH of CaPi-DNA solution was lower than 4.0 by addition of HCl, and no free pDNA occurred in supernatant after CaPi-DNA solution (pH $\geq$ 7.0) was separated by ultracentrifuge (not published data). The integrity of CaPi-DNA was checked by 0.7% agarose gel electrophoresis, and showed a slight lag compared with free pDNA (see latter Fig. 2). This difference could result from a slower movement during electrophore-

Table 1
Characteristics of CaPi-DNA coprecipitation

	pH	Particle size[a] (nm)	Homogenized time (s)	Remained CaPi-DNA (%)
Free pDNA	7.0	–	15	29.1 ± 1.4[b]
CaPi-DNA	6.5	184.5 ± 29.6	15	62.9 ± 1.9
	7.0	274.3 ± 40.7	5	64.7 ± 1.5
			15	56.1 ± 1.6
			25	45.4 ± 1.2
	7.4	1790.9 ± 230.5	15	39.2 ± 2.4
	8.0	8434.3 ± 521.4	15	38.3 ± 2.9

[a] Particle size was determined by Zetasizer 3000HS before homogenization ($n = 3$).
[b] Remained dsDNA was compared with original free pDNA ($n = 3$).

sis due to pDNA was condensed by CaPi to form a particle compared with free pDNA.

3.2. Stability of CaPi-DNA

The stability of CaPi-DNA showed that the process of homogenization produced different influence on CaPi-DNA prepared at various pH (Table 1). The CaPi-DNA formed at low pH could significantly resist damage from the process of homogenization. For example, CaPi-DNA prepared at pH $\leq$ 7.0 showed a good stability. If pH $>$ 7.0, then, the stability of CaPi-DNA decreased, for instance, when pH was 8.0, about 65% of CaPi-DNA was damaged under the same time and speed of homogenization. The result could result from different size of CaPi-DNA. CaPi-DNA prepared at high pH was much larger in size and could be more easily damaged by shear stress. In addition, CaPi-DNA prepared at pH 7.0, the extent of damage increased with increasing time of homogenization. The result could be explained due to more damage opportunities with longer time of homogenization.

The protection of CaPi-DNA by PVA was shown in Fig. 1. When CaPi-DNA solution (pH 7.0) contained 1, 3 and 5% PVA, after homogenization, average 83.5, 95.3 and 98.1% of the original CaPi-DNA were preserved, respectively. A great deal of foam occurred when CaPi-DNA solution was homogenized with PVA, this could form a layer of membrane around the particles of CaPi-DNA, therefore, indirectly reducing the damage from the shear stress of homogenization. However, PVP showed a weak protective effect on CaPi-DNA, and no obvious foam occurred during homogenization of CaPi-DNA solution with PVP.

During the microencapsulation of CaPi-DNA, organic solution had to be used because of hydrophobic property of PLGA. Our results indicated that CaPi-DNA without any stabilizer was slightly influenced by DCM, mixture of DCM and ethyl acetate, ether or hexane (1:1) after homogenization (Fig. 2). However, when organic solution was mixture of DCM and acetone or ethanol (1:1), almost no CaPi-DNA could be observed after water phase was separated from organic phase by centrifuge and was checked by agarose gel electrophoresis (Fig. 2). This difference could result from that acetone and ethanol are miscible with water and could cause precipitation of CaPi-DNA.

The different concentrations and types of PLGA showed different influence on CaPi-DNA (Fig. 3). The CaPi-DNA was remained about 80 and 65% when PLGA (75:25) concentrations were 0.5 and 1% (w/v), respectively. To polymers (50:50 and 65:35), the remained CaPi-DNA reduced slowly with polymer concentration increasing from 0.5 to 4.0% (w/v). As to PLGA (75:25), an interesting result occurred,

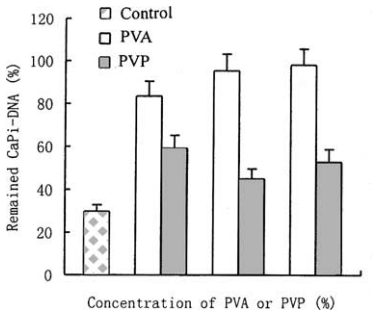

Fig. 1. Stability of CaPi-DNA during homogenization in the presence of PVA and PVP. Data were given as mean ± S.D. for $n = 3$.

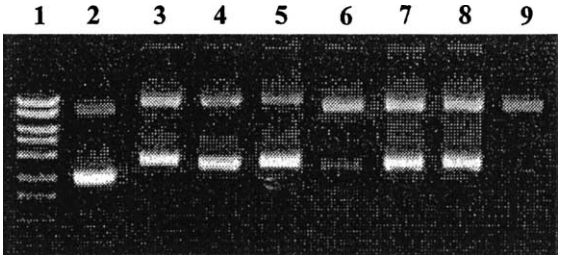

Fig. 2. Stability of CaPi-DNA (pH 7.0) during homogenization (15 s) in the presence of organic solvents. lane 1, DNA ladder; lane 2, pDNA control; lane 3, CaPi-DNA control; lane 4, CaPi-DNA emulsified in DCM; lane 5, CaPi-DNA emulsified in DCM:ethyl acetate (1:1); lane 6, CaPi-DNA emulsified in DCM:EtOH (1:1); lane 7, CaPi-DNA emulsified in DCM:ether (1:1); lane 8, CaPi-DNA emulsified in DCM:*n*-hexane (1:1); lane 9, CaPi-DNA emulsified in DCM:acetone (1:1).

CaPi-DNA demonstrated a rapid reduction with its concentration increasing from 1 to 4% (w/v), in particular, the CaPi-DNA was remained only about 10% with its concentration 4% (w/v). This difference could result from the drop of pH in the aqueous phase containing CaPi-DNA after emulsification. To three polymers, after emulsification, drops of pH in the aqueous phase were obvious with polymer concentration, and pH in the aqueous phase was observed which reached values as low as 5.2 (50:50), 4.7 (65:35) and 3.9 (75:25), respectively, when polymer concentration was 4.0% (w/v).

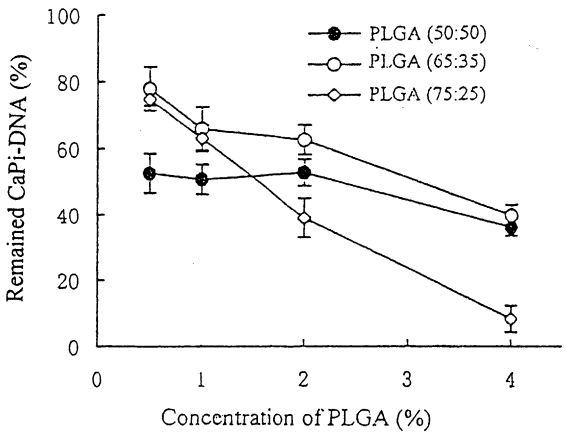

Fig. 3. Stability of CaPi-DNA during homogenization in the presence of PLGA. Data were given as mean ± S.D. for $n = 3$.

3.3. Entrapment efficiency and physicochemical characteristics of CaPi-DNA microparticles

So far, several techniques have already been developed to prepare microparticles loading pDNA (Walter et al., 1999; Hirosue et al., 2001). However, the double emulsion technique was considered to be one of the most appropriate methods because pDNA is soluble in water. In this paper, we selected the double emulsion method to prepare CaPi-DNA microparticles. According to the above result of preparation and stability of CaPi-DNA, in order to abstain the formation of a large size or cluster of CaPi-DNA, and decrease the influence on stability of CaPi-DNA from various factors during the microencapsulation, we selected CaPi-DNA that was prepared at pH 7.0 with the size 274 ± 40.7 nm (Table 1, Fig. 4a) for following study. The organic solution was DCM, PVA (3%, w/v) was contained in CaPi-DNA solution and the time of homogenization 5 s at 7500 rpm every time.

Microparticles were observed by scanning electron microscopy (Fig. 4b–d). From these micrographs, it can be seen that microparticles loading CaPi-DNA are spherical in shape with a particle size range of 2.0–5.0 μm. The microparticles loading CaPi-DNA exhibited a different surface charge with different concentrations and types of PLGA (Table 2). The PLGA (50:50) microparticles showed a change of ζ potential from about −8.0 to −16 mV with increasing concentration from 0.5 to 4.0% (w/v). To PLGA (65:35) microparticles, their surface charge changed from about −0.6 mV (0.5%, w/v) to −10 mV at 4.0% (w/v). However, the PLGA (75:25) microparticles demonstrated a relatively low negative surface charge with its concentration no more than 4.0% (w/v).

CaPi-DNA was incorporated in PLGA microparticles with different encapsulation efficiency by double emulsion technique (Table 2). PLGA microparticles (50:50 and 65:35) showed an increase of the encapsulation efficiency with PLGA concentration, and the highest encapsulation efficiency could access about 50% when the concentration of PLGA (50:50) was 4% (w/v). However, PLGA microparticles (75:25) demonstrated the average encapsulation efficiency to be no more than 40%.

Fig. 5 showed that a large amount of CaPi-DNA was remained after CaPi-DNA was extracted from microparticles loading CaPi-DNA. As seen from lane

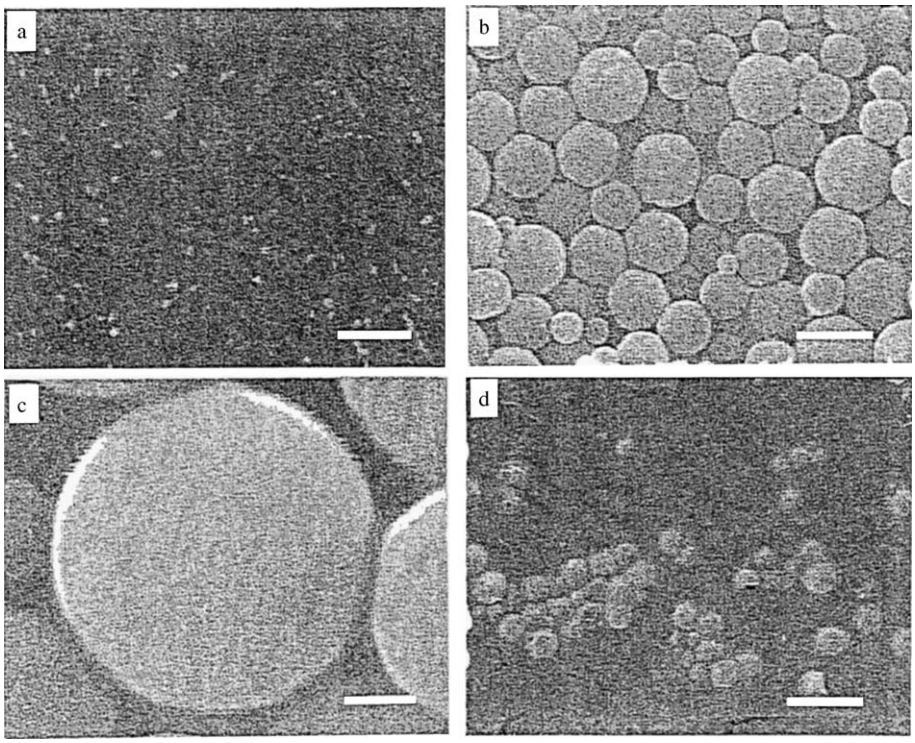

Fig. 4. Scanning electron micrographs of CaPi-DNA and typical microparticles from different PLGA type polymer. (a) CaPi-DNA (bar = 1 μm); (b) PLGA (50:50) (bar = 5 μm); (c) PLGA (65:25) (bar = 1 μm); (d) PLGA (75:25) (bar = 10 μm).

4–6, linearized and open circle pDNA also presented after encapsulation, however, a significant amount of CaPi-DNA could be observed when compared with the encapsulated free pDNA. The supercoiled DNA was hardly observed after free pDNA was extracted from microparticles loading free pDNA (Fig. 5; lane 7), which meant that CaPi-DNA could increase the stability of pDNA in PLGA microparticles.

Table 2
Entrapment efficiency and physicochemical characteristics of CaPi-DNA microparticles

	Lactide:glycolide	PLGA (%)	Entrapment efficiency	Particle size (μm)	Zeta-potential (mV)	Protection from DNase I
CaPi-DNA	50: 50	0.5	38.9 ± 3.3	2.6 ± 0.3	−8.1 ± 1.1	81.9 ± 2.6
		1.0	44.8 ± 2.6	3.5 ± 1.1	−9.7 ± 3.1	80.1 ± 3.9
		2.0	46.3 ± 4.6	3.6 ± 0.8	−13.9 ± 2.6	84.1 ± 6.3
		4.0	50.3 ± 4.4	2.7 ± 0.6	−15.9 ± 3.3	91.6 ± 5.5
	65: 35	0.5	36.2 ± 3.1	2.2 ± 0.2	−0.6 ± 0.2	83.3 ± 4.1
		1.0	42.2 ± 2.3	2.4 ± 0.3	−1.0 ± 0.7	89.7 ± 4.0
		2.0	31.2 ± 4.5	3.0 ± 0.9	−1.5 ± 1.2	86.6 ± 3.9
		4.0	45.1 ± 2.3	2.7 ± 0.5	−10.1 ± 3.1	92.5 ± 3.8
	75: 25	0.5	34.2 ± 2.2	3.4 ± 0.6	−1.8 ± 1.6	90.7 ± 5.6
		1.0	36.3 ± 1.4	2.7 ± 0.3	−1.3 ± 0.8	84.2 ± 3.3
		2.0	30.8 ± 2.3	3.7 ± 0.4	−2.7 ± 0.5	83.4 ± 2.4
		4.0	39.6 ± 3.2	4.5 ± 0.5	−5.1 ± 1.3	88.6 ± 5.1

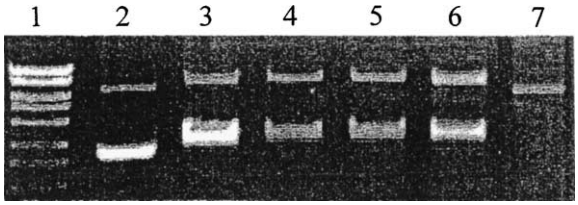

Fig. 5. Agarose (0.7%) gel electrophoresis of CaPi-DNA extracted from PLGA microparticles (1%, w/v). Lane 1, DNA ladder; lane 2, pDNA control; lane 3, CaPi-DNA control; lane 4, CaPi-DNA extracted from PLGA (50:50) microparticles; lane 5, CaPi-DNA extracted from PLGA (65:35) microparticles; lane 6, CaPi-DNA extracted from PLGA (75:25) microparticles; lane 7, pDNA extracted from PLGA (50:50) microparticles.

3.4. In vitro release experiment

In general, the release of drugs from PLGA microparticles mainly has two mechanisms, namely, diffusion through channels formed during microparticle preparation, and release following polymer degradation or solubilization. The in vitro release profiles of CaPi-DNA were obtained by representing the percentage of CaPi-DNA release with respect to the amount of CaPi-DNA encapsulation (Fig. 6). To three kinds of CaPi-DNA microparticles, their release within 1 day showed slight differences, an initial burst release, 1.2% of CaPi-DNA was released from PLGA (75:25) microparticles, however, only negligible amounts of CaPi-DNA were detected, 0.31% for PLGA (50:50) and 0.65% for PLGA (65:35) microparticles, respectively. After 1 day, CaPi-DNA release profiles of three microparticles displayed a sustained release. The amount of cumulated CaPi-DNA release over 30 days was: 17.6% for PLGA (50:50), 27.3% for PLGA (65:35) and 44.8% for PLGA (75:25) microparticles, respectively. For three microparticles loading CaPi-DNA, the difference of CaPi-DNA release in the first phase could be from (i) different amounts adsorbed onto the wall of microparticles that would be immediately released during the initial stage, and (ii) different size of channels on the surface of PLGA microparticles resulting in a different release of CaPi-DNA during the first phase. After the first phase, their sustained release could result from diffusion of CaPi-DNA through PLGA channels as well as the erosion of the polymer. CaPi-DNA encapsulated in PLGA microparticles showed a low burst release and provided a constant slow release rate over time.

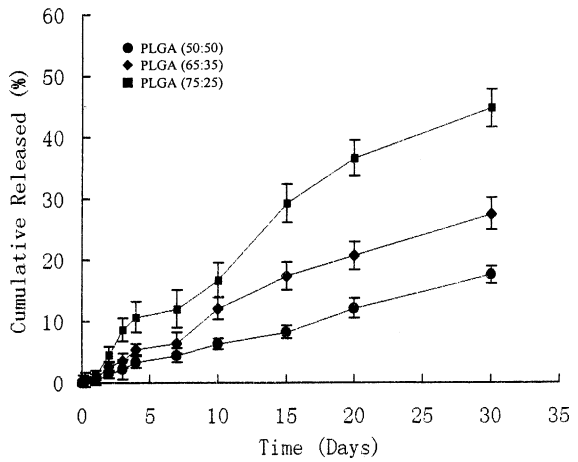

Fig. 6. Release of CaPi-DNA from microparticles prepared using different PLGA (1%, w/v). Data were given as mean ± S.D. for $n = 3$.

3.5. Protection from DNase I

In order to test whether PLGA microparticles could protect encapsulated CaPi-DNA from nuclease digestion, three PLGA microparticles formulations loading CaPi-DNA were exposed to DNase I. The result of PLGA microparticles protecting CaPi-DNA against the endonuclease activity of DNase I was shown in Table 2. PLGA microparticles demonstrated a profound effect on protecting CaPi-DNA from enzymatic degradation. After enzyme treatment, the protection from enzyme degradation with three PLGA microparticles was similar with average over 80% of total DNA recovery, and it was not an obvious relation to concentration of PLGA during microparticles prepared. On the other hand, it had been reported that free pDNA was highly susceptible to DNase I, and was completely digested within 30 min (Capan et al., 1999). Incubation of CaPi-DNA with DNase I also resulted in a degradation profile that was similar to that observed for solution of free pDNA (data not shown).

4. Conclusion

The process of emulsification showed different influence on stability of CaPi-DNA prepared at various pH. The addition of PVA could obviously resist

from the damage of the process of homogenization. DCM showed a neglectable influence on the stability of CaPi-DNA. PLGA microparticles loading CaPi-DNA exhibited spherical in shape with size range of 2.0–5.0 μm, and entrapment efficiency 30–50%. CaPi-DNA was found to increase the stability of pDNA in PLGA microparticles without losing its structure integrity. The CaPi-DNA encapsulated in PLGA microparticles showed low burst release and provided a constant slow release rate over time. The encapsulation of CaPi-DNA in microparticles could significantly protect CaPi-DNA from degradation of DNase I. These results suggested that the encapsulation of CaPi-DNA in PLGA microparticles could improve stability of pDNA. Further studies would focus on optimization of entrapment efficiency, and evaluating in vitro and in vivo transfection efficiency of this delivery system.

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