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Cationic supported lipid bilayers for antigen presentation

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Abstract

Polystyrene sulfate (PSS) particles (301 nm mean diameter) were covered with single cationic dioctadecyldimethylammonium bromide (DDA) bilayers and used for antigen adsorption and presentation. The antigen was a mixture of purified 18/14 *Taenia crassiceps* proteins (18/14-*Tcra*). Firstly, the DDA/PSS assembly was characterized at 1 mM NaCl and 5×10^9 PSS particles/mL over a range of DDA concentrations (0.001–1 mM) by means of dynamic light scattering for particle sizing and zeta-potential analysis. 0.01 mM DDA is enough to produce homodisperse and cationic bilayer-covered particles. Secondly, under these experimental conditions, 18/14-*Tcra* adsorption isotherms onto biomimetic particles or aluminium hydroxide ($Al(OH)_3$) yield limiting adsorption of 0.36 and 1.32 mg protein/mg biomimetic particles or $Al(OH)_3$, respectively. Finally, in mice, superior humoral and cellular immunoresponse from serum IgG and footpad swelling was obtained for antigen/biomimetic particles in comparison to conventional $Al(OH)_3$. Cationic bilayer-covered particles are a novel, highly organized and, possibly, general immunoadjuvant for antigen presentation and subunit vaccine design.

Keywords: Polymeric particles; Cationic lipid; Dioctadecyldimethylammonium bromide; Bilayer and protein adsorption; Antigen presentation; *Taenia crassiceps* antigens

1. Introduction

Particles are finding a large variety of biomedical and pharmaceutical applications since their size scale can be similar to that of biological molecules (e.g., proteins, DNA) and structures (e.g., viruses and bacteria). They are currently being used in imaging, gene and drug delivery, and vaccines (O'Hagan et al., 2004; El-Sayed et al., 2005). Particulate systems are naturally targeted to antigen presenting cells (APC) so that particles deliver antigens to APC more efficiently than soluble antigen (Kovacsovics-Bankowski et al., 1993; Vidard et al., 1996). Recently, positively charged particles with diameters of 500 nm and below were shown to be optimal for dendritic cells uptake (Foged et al., 2005).

On the other hand, cationic lipids electrostatically combine with a vast variety of negatively charged biomolecules or biological structures (Carmona-Ribeiro, 2006). Silica (Moura and Carmona-Ribeiro, 2003), latex (Carmona-Ribeiro and Midmore, 1992; Carmona-Ribeiro, 2001) or hydrophobic drug particles (Pacheco and Carmona-Ribeiro, 2003; Lincopan and Carmona-Ribeiro, 2006) have been coated with cationic lipids with optimal bilayer deposition on particles achieved by coalescence of bilayer fragments at an adequate ionic strength (Moura and Carmona-Ribeiro, 2003; Pereira et al., 2004).

Here the optimal bilayer coverage of polystyrene sulfate (PSS) nanoparticles with a cationic dioctadecyldimethylammonium bromide (DDA) bilayer finds an important application adsorbing and successfully presenting a mixture of purified 18/14 *Taenia crassiceps* proteins (18/14-*Tcra*) (Espíndola et al., 2005) to the immunological system.

2. Experimental section

Dioctadecyldimethylammonium bromide (DDA) 99.9% pure was obtained from Sigma–Aldrich (St Louis, MO, USA). Polystyrene sulfate (PSS) particles (Lot 10-66-58), nominal

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Table 1
Physical properties of particles, DDA dispersion, DDA-covered particles at maximal coverage, proteins and proteins/DDA-covered particles at 1 mM NaCl

Sample[a]	DDA (mM)	Ag (μg/mL)	Mean diameter (nm)	Zeta-potential (mV)	Polydispersity index
PSS[b]	–	–	301 ± 2	−60 ± 1	0.064 ± 0.020
DDA	2.00	–	81 ± 1	45 ± 2	0.230 ± 0.006
PSS/DDA	0.01	–	309 ± 2	48 ± 2	0.040 ± 0.010
18/14-*Tcra*	–	25	310 ± 5	−52 ± 1	0.214 ± 0.030
DDA/18/14-*Tcra*	0.01	25	295 ± 3	6 ± 6	0.167 ± 0.023
PSS/DDA/18/14-*Tcra*	0.01	25	328 ± 3	11 ± 8	0.060 ± 0.020
$Al(OH)_3$	–	–	883 ± 29	28 ± 3	0.381 ± 0.013
$Al(OH)_3$/18/14-*Tcra*	–	25	9574 ± 2361	−23 ± 1	0.525 ± 0.030

The $Al(OH)_3$ and PSS were tested at final concentration of 0.05 and 0.075 mg/mL, respectively.
[a] Dispersions in 1 mM NaCl.
[b] The particle number density was 5×10^9 particles/mL; PSS particle mean diameter from transmission electron microscopy, given by the supplier, is 301 ± 2 nm.

mean diameter of 301 ± 2%, 188, 944 $cm^2\ g^{-1}$ specific surface area, surface charge density of 1.68 μC cm^{-2} and −60 ± 1 mV mean zeta-potential (Table 1) were purchased from Interfacial Dynamics Corporation (Portland, OR, USA) and a stock suspension containing 2×10^{10} particles/mL was prepared in 1 mM NaCl, which is an adequate ionic strength to assemble DDA BF as a single bilayer onto particles (Pereira et al., 2004). Purified fractions of native 18- and 14-kDa *T. crassiceps* proteins (18/14-*Tcra*) were prepared as previously described (Espíndola et al., 2005) and diluted 1:20 in 0.15 M NaCl to obtain a stock solution at 1.4 mg/mL. Bovine serum albumin (BSA) was purchased from Sigma–Aldrich, prepared as a 1 mg/mL stock solution in 1 mM NaCl and stored in a freezer in 1 mL aliquots for quick use. Protein concentration was determined by the Bio-Rad Protein microassay, based on the method of Bradford (Bradford, 1976) (reagent cat# 500-0006, Bio-Rad, Hercules, CA, USA), using a standard curve (5–35 μg/mL) of BSA. Aluminium hydroxide adjuvant was obtained from Merial do Brasil (Merial Ltda.). NaCl and all other reagents were of an analytical grade. Water was Milli-Q quality. BALB/c female mice 8–12-week old were purchased from the University of São Paulo, São Paulo, Brazil.

Small DDA bilayer fragments (BF), 81 ± 1 nm mean diameter and 45 ± 2 mV mean zeta-potential (Table 1), were prepared by sonication with titanium macrotip probe in 1 mM NaCl Milli-Q water solution at ca. 2.0 mM DDA as previously described (Carmona-Ribeiro, 1992). Analytical concentrations of DDA or NaCl, approximately 1 mM, were determined by halide microtitration (Schales and Schales, 1941).

PSS particles (2×10^{10}/mL), and DDA lipid BF (2.0 mM) were always prepared in 1 mM NaCl and diluted to the final desired concentration using this same salt solution. First, to obtain monodisperse lipid-covered nanospheres, PSS, at 5×10^9 particles/mL final concentration, and oppositely charged DDA BF solutions ranging from 0.01 μM to 1 mM, interacted for 1 h/25 °C. DDA final concentration for producing the assemblies was selected as 10 μM at 5×10^9 PSS particles per mL since this concentration is the one required to cover each nanosphere with a DDA bilayer. In fact, experimentally it is shown in Section 3 (Table 1, Fig. 1) that from this concentration cationic monodisperse particles are indeed obtained. In a second experimental step, the stock 18/14-*Tcra* solution (1.4 mg/mL) was used to obtain final protein concentrations ranging from 5 to 50 μg/mL after addition to the PSS/DDA mixture, for 1 h/25 °C interaction. Thereafter, sizes, zeta-potentials, and polydispersities were determined. Details on particle number densities, DDA concentrations and/or protein concentrations are in figure captions. Considering the PSS total area, the selected DDA concentration (10 μM) was precisely sufficient to produce bilayer-covered nanospheres and DDA is not expected to be found free in dispersion (Carmona-Ribeiro and Midmore, 1992; Carmona-Ribeiro,

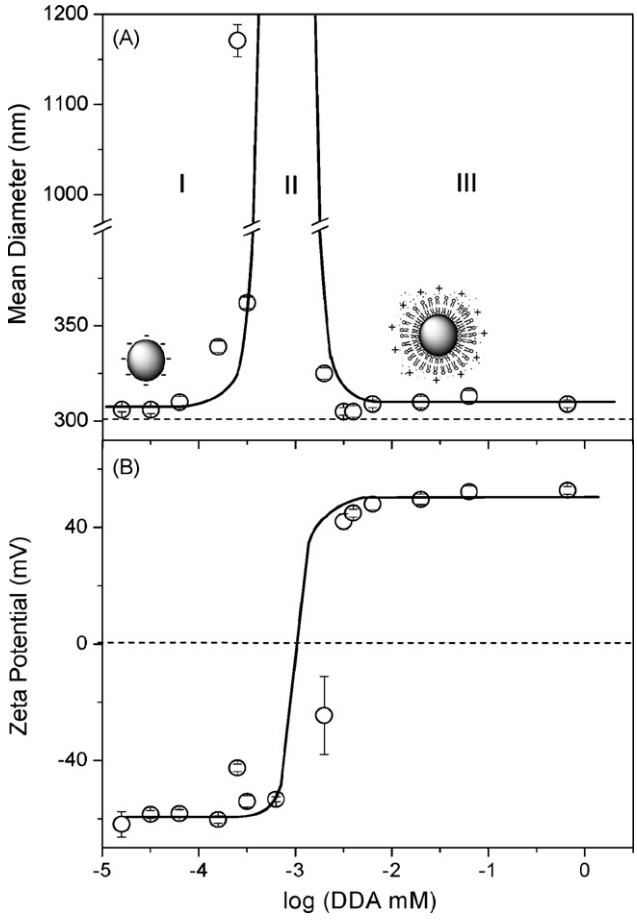


Fig. 1. Effect of DDA concentration on mean z-average diameter (A) and zeta-potential (B) of PSS particles at 5×10^9 particles/mL, 25 °C, in 1 mM NaCl. Bare particle diameter is 301 ± 2 nm. Regions I, II and III define particle charge, which is negative, zero and positive.

2001). Particle size (mean diameter D_z), size distribution, polydispersity and zeta-potential (ζ) in the presence or absence of PSS, DDA or proteins were determined using the ZetaPlus-ZetaPotential Analyzer (Brookhaven Instruments Corporation, Holtsville, NY), which was equipped with a 677 nm laser and dynamic light scattering (PCS) at 90° for particle sizing. Mean diameters were obtained by fitting data to log-normal size distributions which do not discriminate between one, two, or more different populations and considers always all scattering particles as belonging to one single Gaussian population. On the other hand, for the size distribution data, fitting was performed by the apparatus software using the non-negatively constrained least squares (NNLS) algorithm, which is a model independent technique allowing to achieve multimodal distributions (Grabowski and Morrison, 1983). ζ was determined from electrophoretic mobility μ in 1 mM NaCl and the Smoluchowski's equation: $\zeta = \mu\eta/\varepsilon$, where η is the medium viscosity and ε the medium dielectric constant.

Adsorption isotherms on PSS/DDA particles were obtained by mixing 0.05 mL of the stock PSS solution (2×10^{10} particle/mL) with 0.01 mL DDA BF (0.2 mM) and 0.14 mL of the appropriate 18/14-*Tcra* protein dilution in 1 mM NaCl. Final concentration of 18/14-*Tcra* in the assay ranged from 0 to 72 μg/mL, respectively, at fixed concentration of 5×10^9 PSS particle/mL and 0.01 mM DDA BF. After 1 h interaction at 25 °C, a clear supernatant was obtained by centrifugation at 15,000 rpm for 1 h. The concentration of protein in the supernatant was determined by Bradford microassay using a standard curve prepared from 5 to 35 μg/mL BSA. A microplate reader equipped with a 595 nm filter (Ultramark, Model 550 Bio-Rad, Hercules, CA, USA) was used for absorbance measurement. The amount of adsorbed protein was determined by the difference between the total protein added and the amount of protein recovered in the supernatant. Adsorption isotherms on $Al(OH)_3$ were prepared as above and the final concentration of 18/14-*Tcra* in assay ranged from 0 to 123 μg/mL, at fixed concentration of 0.05 mg/mL $Al(OH)_3$. The adsorptive capacity for the adsorption of 18/14-*Tcra* by PSS/DDA or $Al(OH)_3$ was calculated from maximal adsorption and expressed as maximal amount adsorbed in mg per mg adjuvant.

Five groups of eight female mice were challenged subcutaneously (s.c.) in the abdomen at two separate sites. Total volume injected in each site was 0.2 mL. The dispersion injected contained either: (a) 10 μg antigens in 1 mM NaCl; or (b) 10 μg antigens in 1 mM NaCl and 0.05 mg/mL $Al(OH)_3$; or (c) 10 μg antigens in 1 mM NaCl and 5×10^9 PSS/DDA particles/mL; or (d) 5 μg of antigens in 1 mM NaCl and 5×10^9 PSS/DDA particles/mL.

For delayed-type hypersensitivity (DH) evaluation, the footpad swelling test was carried out essentially as described previously (Katz et al., 1994; Tsuruta et al., 1997). On the fifth day post subcutaneous immunization, mice pretreated with 18/14-*Tcra* alone, PSS/DDA18/14-*Tcra*, $Al(OH)_3$/18/14-*Tcra* or PSS/DDA alone were challenged in the left hind footpad with a total elicitation dose of 5 or 30 18/14-*Tcra* μg proteins in 50 μL 1 mM NaCl. Footpad swelling was measured 24 h later with a Mitutoyo engineering micrometer. Depending on the age of the animals, the thickness of uninjected hind footpad varied from 1.60 to 1.70 mm. Percentage footpad swelling (%fs) is calculated according to the formula below with results expressed as %fs ± standard error of the mean (S.E.M.):

$$\%\text{fs} = 100 \times \frac{\text{left hind footpad thickness} - \text{right hind footpad thickness}}{\text{mean thickness uninjected left hind footpad}}$$

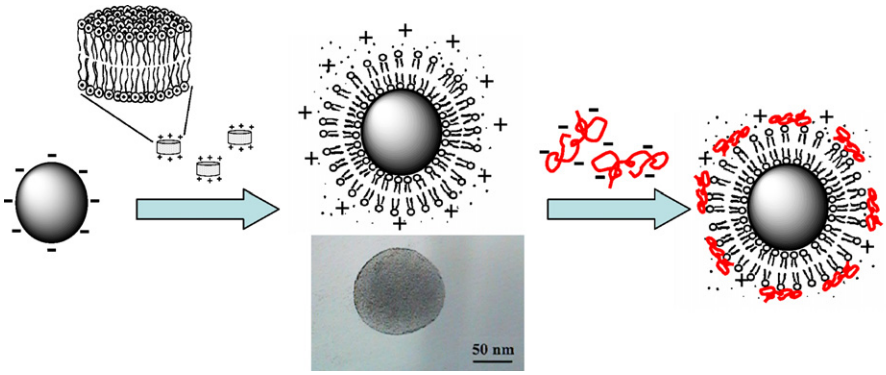
For evaluation of humoral response, the same groups previously immunized and challenged with 5 μg antigen (Ag) above were bled through the ophthalmic plexus in days 15 and 24 after immunization. The sera obtained were analyzed by ELISA using 18/14-*Tcra* antigen. Each well of 96-well ELISA polystyrene high binding plates (Costar Corning Inc., Cambridge, MA) was coated with 100 μL of antigen (0.1 μg/mL) in 0.5 M carbonate–bicarbonate buffer (pH 9.6) for 18 h in a humidified chamber at 4 °C. The wells were blocked for 1 h with 5% milk in PBS containing 0.05% Tween 20 (PBS/T), and then incubated for 1 h with serum samples diluted 1:2000, for IgG antibody quantitation. Goat anti-mouse IgG peroxidase-conjugate (Sigma) were added and plates were incubated for 1 h. After each incubation step, the plates were washed using an automatic washer, with four cycles of PBS/T. *Ortho*-phenylenediamine (1 mg/mL) (Sigma) and H_2O_2 (1 μL/mL) diluted in 0.2 M citrate buffer (pH 5.0) were added (in the dark) as chromogenic substrate and plates were incubated for 10 min. The reactions were stopped by adding 100 μL of 2 M H_2SO_4. Color intensity was quantified using an ELISA plate reader (Diagnostics Pasteur, Strasburg-Schiltigheim, France) at 492 nm. All incubations were carried out at 37 °C.

ANOVA one-way multiple comparison tests and the Kruskal–Wallis non-parametric test were used when needed. A P value of ≤ 0.05 was considered significant.

3. Results and discussion

3.1. Assembly of cationic lipid bilayer from bilayer fragments onto oppositely charged polymeric particles

The model particulate adjuvant was obtained in a few steps as shown in Scheme 1. In the first step, at 1 mM NaCl, the polystyrene sulfate (PSS) polymeric particles were covered by oppositely charged DDA bilayer fragments (BF). This procedure was previously shown to result in one bilayer coverage surrounding each particle, from determination of adsorption isotherm, electron transmission microscopy of positively stained bilayer-covered particles, increase in the mean particle diameter of 8–10 nm, reversal of particle zeta-potential from negative to positive and improved colloid stability at and above bilayer coverage (Carmona-Ribeiro and Midmore, 1992; Pereira et al., 2004; Carmona-Ribeiro, 2006). Apparently, 1 mM ionic strength provided by 1 mM NaCl was effective in inducing an hydrophobically driven auto-associative process of adjacent bilayer fragments deposited onto the polymeric particles that did not take place in water or ionic strengths smaller than 1 mM (Pereira et al., 2004). The adsorption of DDA bilayer



Scheme 1.

fragments on particles changes size and sign of particle surface charge (Fig. 1). At charge neutralization, there is maximal interparticle aggregation and minimal colloid stability. Further increasing DDA concentration stabilizes the system at sizes and zeta-potentials consistent with DDA bilayer adsorption (Fig. 1A and B). Table 1 shows sizes and zeta-potentials for PSS particles, DDA BF dispersions, DDA-covered particles, proteins, PSS/DDA/protein assemblies and $Al(OH)_3$, commonly used as vaccines adjuvant. The large polydispersity of $Al(OH)_3$ both in absence or in the presence of the antigens contrasts with the low polydispersity of the PSS/DDA/18/14-*Tcra* assemblies. One should notice that the zeta-potential for DDA BF is the same as the one for PSS/DDA (Table 1). In addition, PSS particles diameter of 301 nm increases to 309 nm in the presence of 10 μM DDA indicating deposition of one single, 4 nm thick DDA bilayer on particles (Table 1). This reinforces the zeta-potential suggestion that the DDA bilayer indeed covered the PSS particles. Adsorption isotherms for DDA onto 301 nm PSS particles have previously shown a limiting adsorption consistent with bilayer deposition (Carmona-Ribeiro and Midmore, 1992; Carmona-Ribeiro, 2001) which could be visualized from transmission electron microscopy after labelling the adsorbed DDA bilayer on particles with 1% ammonium molybdate to stain the DDA layer on particles and create contrast for observation under transmission electron microscopy (Petri and Carmona-Ribeiro, 2007) (Scheme 1).

3.2. Antigen presentation by cationic, lipid-covered polymeric particles

Antigen adsorption to the PSS/DDA assembly did not disturb the excellent order of the particulate which acquires a mean diameter of 328 nm, a zeta-potential of 11 mV and a polydispersity index close to the one usually exhibited by latex particles, namely, 0.060 (Table 1). This was clear from particle size distributions for PSS, PSS/DDA and PSS/DDA/proteins at three different proteins concentrations: 5, 25 and 50 μg/mL (Fig. 2). The PSS/DDA system at 5×10^9 can accommodates well at most 25 μg/mL antigens as seen from the comparison between size distributions obtained at 25 (Fig. 2D) and 50 μg/mL (Fig. 2E). Above this concentration, further protein addition apparently aggregates and disrupts the high order, low size and polydispersity of the particulate. Curiously, there is protein–protein aggregation when the purified antigen is dispersed in 1 mM NaCl water solution at the water pH of 6.3. This aggregation does not occur in the presence of the DDA/PSS biomimetic particles over a range of antigen concentrations (0–25 μg/mL) as shown in Fig. 2C and D at submaximal protein adsorption on the particulate. At 50 μg/mL adsorbed proteins on biomimetic particles, protein-induced particulate aggregation substantially increases mean particle size and reduces zeta-potential values (Fig. 2E).

Protein adsorption on particulates was quantitatively described from adsorption isotherms of 18/14-*Tcra* antigens onto PSS/DDA cationic particles (Fig. 3A), or onto aluminium hydroxide (Fig. 3B). Both adsorption isotherms were obtained at 1 mM NaCl. Over a range of low protein concentrations, there is a linear dependence of protein adsorption on protein amount added. However, further increasing protein amount added leads to a limiting adsorbed amount and particulate saturation with the protein. This behaviour was similar for PSS/DDA and $Al(OH)_3$. Maximal adsorption of 18/14-*Tcra* antigens onto PSS/DDA and aluminium hydroxide adjuvants was 0.36 and 1.32 mg adsorbed antigen per mg of adjuvant, respectively. Anyway, the main requirement for use of adjuvant systems, namely, 100% antigen adsorption at final concentration of adjuvant was fulfilled for both carriers.

The superior humoral response elicited by PSS/DDA/protein as compared to the one elicited by $Al(OH)_3$/protein at 5 and 10 μg protein is depicted from Fig. 4. Regarding delayed-type hypersensitivity (DH), a marker of cellular immunoresponse, the PSS/DDA adjuvant doubled the immunological response when compared to $Al(OH)_3$ adjuvant (Table 2). Therefore, the high degree of organization achieved on PSS/DDA particles could be advantageously used to efficiently present the antigens to the immunological system. Furthermore, as latex particles are available over a broad range of particle sizes, the present work opens new frontiers in the investigation on adjuvant efficacy as a function of particle size and provides a myriad of possibilities for entirely novel and controlled gene, drug, vaccine and therapeutical formulations.

At the root of the improved adjuvant effect was the highly organized but minute amount of cationic lipid adsorbed onto the polymeric particles, which acts as adequate support for presentation of the oppositely charged antigen. Considering 0.6 nm^2

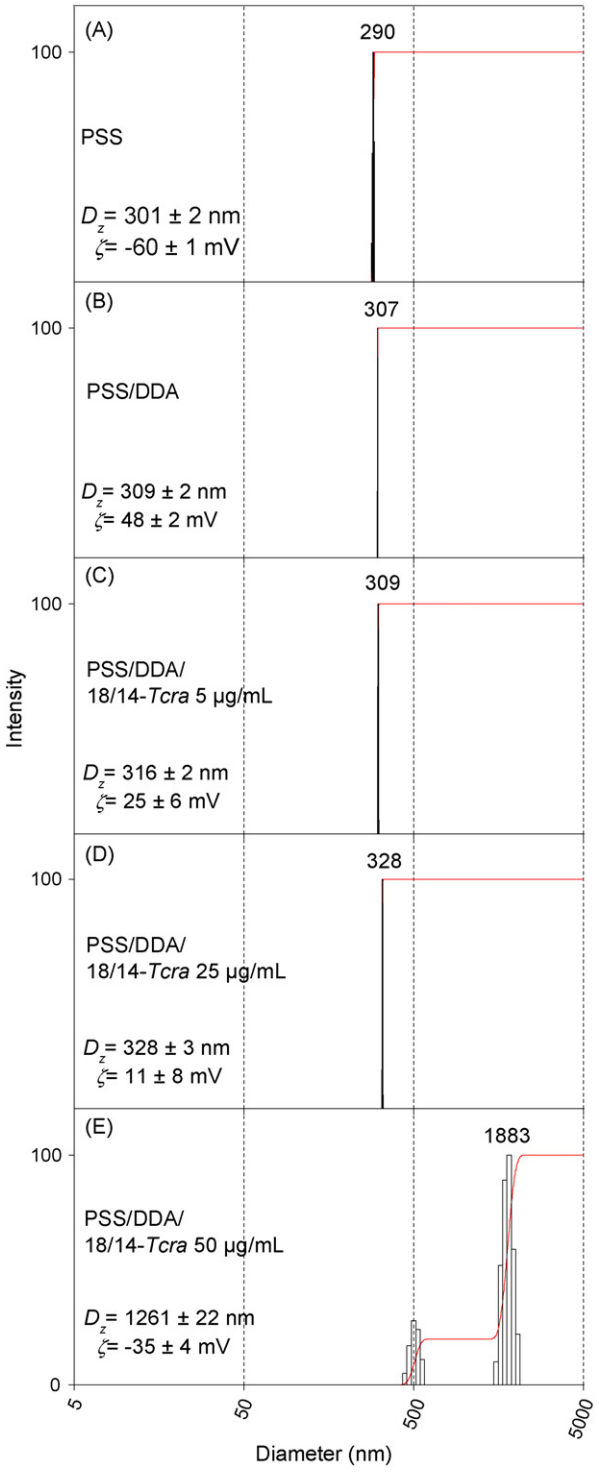


Fig. 2. Particle size distribution and zeta-potential (ζ) for different dispersions in 1 mM NaCl. In (A), 5×10^9 particles/mL PSS particles. In (B), PSS/DDA cationic particles (5×10^9 particles/mL; 0.01 mM DDA); PSS/DDA/18/14-*Tcra* at 25 °C and 5 μg/mL (C), 25 μg/mL (D) and 50 μg/mL (E) after 1 h PSS/DDA interaction with protein. On the left in each subfigure, mean zeta-potential and diameter ± S.E. are quoted.

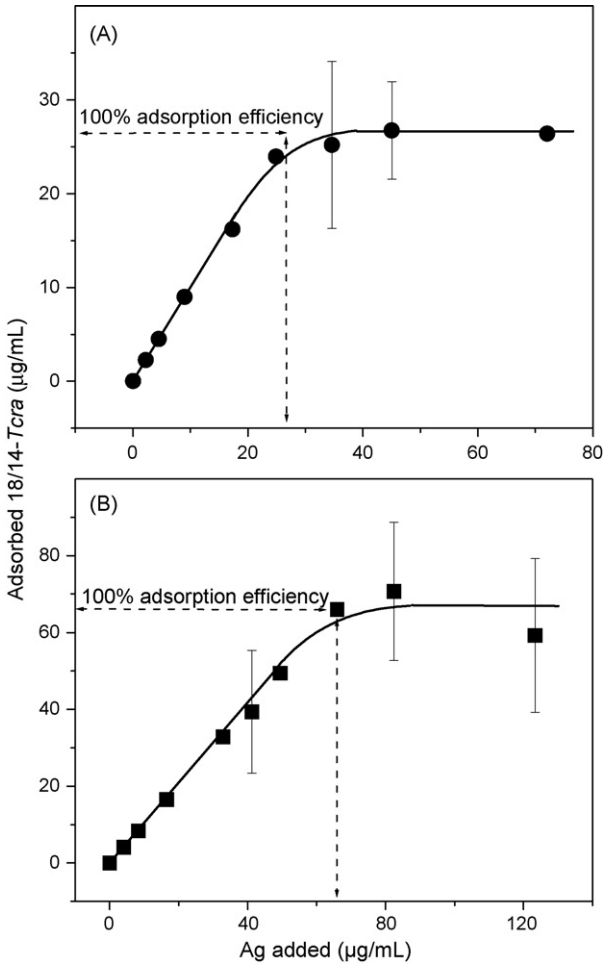


Fig. 3. Adsorption isotherms of 18/14-*Tcra* antigens on PSS/DDA cationic particles (A), or aluminium hydroxide (B) at 1 mM NaCl, 25 °C, 1 h interaction time between particles and proteins. Concentrations of 18/14-*Tcra* antigens (0–72 or 0–123 μg/mL) were used to prepare the adsorption isotherms. The final PSS particle concentration is 5×10^9 particles/mL (or 0.075 mg/mL) at 0.01 mM DDA (A). Final aluminium hydroxide concentration is 0.05 mg/mL (B). One should notice that in (A) there is 100% antigen adsorption efficiency up to 27 μg/mL of antigen added. In (B), there is 100% antigen adsorption efficiency up to 66 μg/mL of antigen added. Maximal adsorption of 18/14-*Tcra* antigens onto PSS/DDA (A) and aluminium hydroxide (B) adjuvants was 0.36 and 1.32 mg adsorbed antigen per mg of adjuvant, respectively.

Table 2
Percentage footpad swelling (%fs) associated to delayed-type hypersensitivity reaction to 18/14-*Tcra* supported on DDA-covered PSS nanospheres

Sensitization[a]	Elicitation 18/14-*Tcra* (μg)	
	5 (% footpad swelling ± S.E.M.)	30 (% footpad swelling ± S.E.M.)
PSS/DDA	–	5 ± 5
18/14-*Tcra* (10 μg)	5 ± 3	12 ± 6
$Al(OH)_3$/18/14-*Tcra* (10 μg)	15 ± 4	17 ± 3
PSS/DDA/18/14-*Tcra* (5 μg)	27 ± 5[b]	28 ± 4[b,c]
PSS/DDA/18/14-*Tcra* (10 μg)	33 ± 5[b,c]	40 ± 9[b,c]

[a] PSS at final concentration of 5×10^9 particle/mL (0.075 mg/mL); DDA at final concentration of 0.01 mM; $Al(OH)_3$ at final concentration of 0.05 mg/mL.
[b] $P < 0.05$, compared to animals that were 18/14-*Tcra*-pretreated and received the same elicitation dose in 1 mM NaCl.
[c] $P < 0.05$, compared to animals that were $Al(OH)_3$/18/14-*Tcra*-pretreated and received the same elicitation dose in 1 mM NaCl.

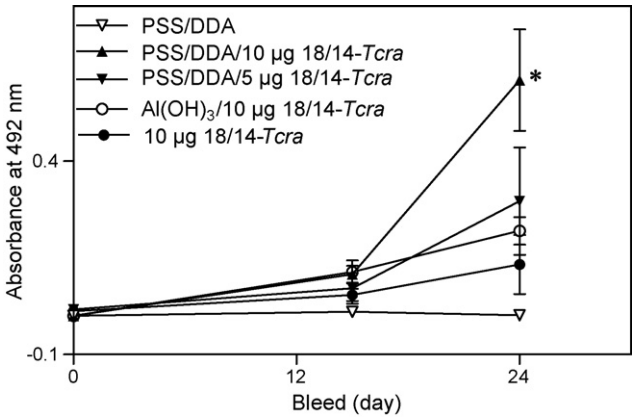


Fig. 4. Absorbance at 492 nm as a function of number of days after challenging mice with 0.4 mL 1 mM NaCl solution containing 10 μg of 18/14-*Tcra* antigens (filled circles), 0.02 mg aluminium hydroxide and 10 μg of 18/14-*Tcra* antigens (open circles), 2×10^9 PSS/DDA particles (inverted open triangles) and 2×10^9 PSS/DDA particles carrying 10 μg of 18/14-*Tcra* antigens (filled triangles). Mice were alternatively challenged with 0.2 mL of 1×10^9 PSS/DDA particles carrying 5 μg of 18/14-*Tcra* antigens (filled inverted triangles). Animals were immunized subcutaneously on the abdomen, at two separate sites and bled through the ophthalmic plexus at days 15, and 24. The sera obtained were analyzed by ELISA using 18/14-*Tcra* antigen for quantitation of IgG antibodies. Each value is the average ± S.E. of five individual values. One should notice that 2×10^9 PSS/DDA particles is equivalent to 0.03 mg.

per DDA molecule as the mean DDA area per molecule at the air–water interface (Carmona-Ribeiro, 1992, 2001), bilayer coverage of total surface area on 5×10^9 particles would require 10 μmol DDA, a prediction perfectly in agreement with experimental data in Fig. 1. At this minute amount, the usual toxicity of the cationic lipids is not important. DDA has been often used as immunoadjuvant at large and toxic DDA concentrations (>1 mM) (Katz et al., 1994; Tsuruta et al., 1997; Davidsen et al., 2005). The DDA concentration used in this work to provide a thin bilayer coverage on each PSS particle, namely, 0.01 mM DDA represents a tiny amount, so that toxic DDA effects associated to high DDA doses are avoided.

4. Conclusions

One of us pioneered the description of bilayer-covered polymeric particles in 1992 (Carmona-Ribeiro and Midmore, 1992) and the PSS/DDA system is not new. The novelty in this work is the specific application of the PSS/DDA system for antigen presentation. The uniqueness of the bilayer-covered particles as compared to other cationic particles rely on antigen preservation and avoidance of protein denaturation which usually takes place via the hydrophobic interaction with the adjuvant, as often reported for bare, hydrophobic polymeric particles. Therefore, although antigen or DNA presentation by particles has been widely studied, structure and antigenicity of proteins can be affected, so that the generated immunoresponse will not suit the native protein structure. Upon coating the hydrophobic particles with a sufficiently hydrated bilayer membrane will probably preserve protein structure avoiding exposure of protein hydrophobic moieties and protein denaturation. The main point of the present work is the *in vivo* effectiveness of PSS/DDA/18/14-*Tcra* for eliciting humoral and delayed-type hipersensitivity responses of the immunological system, which represent the main requirement for vaccines effectiveness. Regarding the effect of ionic strength on colloid stability of PSS/DDA, colloid stability of the bilayer-covered particles has already been described (Tsuruta et al., 1995) and pH effects are not expected to be relevant since DDA has a quaternary ammonium group, which does not change charge as a function of pH. The adjuvant effectiveness in a vaccine seldom depends on adjuvant colloid stability. The classical $Al(OH)_3$ adjuvant is very unstable nevertheless widely used in vaccines just because many vaccines are designed for local instead of systemic administration. The key point is activation of specialized local cells and this requirement was fulfilled by the PSS/DDA/18/14-*Tcra* prospective vaccine.

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