



Latex–protein complexes from an acute phase recombinant antigen of *Toxoplasma gondii* for the diagnosis of recently acquired toxoplasmosis

Leandro E. Peretti^a, Verónica D.G. Gonzalez^a, Iván S. Marcipar^b, Luis M. Gugliotta^{a,*}

^a INTEC (Universidad Nacional del Litoral and CONICET), Güemes 3450, Santa Fe 3000, Argentina

^b Laboratorio de Tecnología Inmunológica, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Santa Fe 3000, Argentina



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ABSTRACT

The synthesis and characterization of latex–protein complexes (LPC), from the acute phase recombinant antigen P35 (P35Ag) of *Toxoplasma gondii* and “core-shell” carboxylated or polystyrene (PS) latexes (of different sizes and charge densities) are considered, with the aim of producing immunoagglutination reagents able to detect recently acquired toxoplasmosis. Physical adsorption (PA) and chemical coupling (CC) of P35Ag onto latex particles at different pH were investigated. Greater amounts of adsorbed protein were obtained on PS latexes than on carboxylated latexes, indicating that hydrophobic forces govern the interactions between the protein and the particle surface. In the CC experiments, the highest amount of bound protein was obtained at pH 6, near the isoelectric point of the protein ($\text{IP} = 6.27$). At this pH, it decreased both the repulsion between particle surface and protein, and the repulsion between neighboring molecules. The LPC were characterized and the antigenicity of the P35Ag protein coupled on the particles surface was evaluated by Enzyme-Linked ImmunoSorbent Assay (ELISA). Results from ELISA showed that the P35Ag coupled to the latex particles surface was not affected during the particles sensitization by PA and CC and the produced LPC were able to recognize specific anti-P35Ag antibodies present in the acute phase of the disease.

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1. Introduction

In the diagnosis field, serological tests are useful to detect the presence of specific antibodies (Ab) generated for the immune system as a response to the attack of an infectious agent. The antigen–antibody reaction is used to detect the presence of both antigens (Ag) and Ab. The purpose of any diagnostic test is to amplify the Ag–Ab reaction so that it can be more easily detected. Some methods usually employed for such detection are Enzyme-Linked ImmunoSorbent Assay (ELISA), Indirect ImmunoFluorescence (IIF), Direct Agglutination Test (DAT), Indirect HemAgglutination (IHA) and Latex Agglutination Test (LAT).

The LAT has been successfully used for the detection of various analytes [1–10]. In the development of a LAT, monodisperse latex particles are sensitized with Ag (or Ab) of interest, so that the presence of specific Ab (or Ag) cause the Ag–Ab reaction thus producing immunoagglutination. LAT is a fast and cheap detection method, which results particularly useful for screening purposes.

Latex particles can be of polystyrene (PS), in which case sensitization occurs only by physical adsorption (PA) of proteins, or they can be mainly of PS but with external functional groups, such as aldehyde, acetal, amine, carboxyl and chloromethyl [11]. In this case sensitization is performed by PA and/or chemical coupling (CC) of proteins onto the surface functional groups of the polymer particles. The sensitization products are the so-called latex–protein complexes (LPC).

The LPC obtained by PA are susceptible to partial desorption or denaturalization of the adsorbed proteins, implying the variation of their properties along the time [12], and consequently their limited applicability in immunodiagnosis. In contrast, CC produces higher quality LPC, which result more stable and maintain their immunoreactivity, thus exhibiting improved properties in the agglutination test [2].

For the CC of proteins, hydrophilic latexes with uniform particle size and external functional groups are used. Such characteristics increase the stability of the LPC, allow a homogeneous distribution of the proteins on the particle surface and prevent non-specific interactions [13]. Also, the chemical binding of proteins is strongly affected by the medium conditions, such as ionic strength and pH [14].

Functionalized latexes are typically produced by emulsion copolymerization. The “seeded” process comprises a two stage

* Corresponding author. Tel.: +54 342 455 8450/+54 342 455 8451; fax: +54 342 455 0944.

E-mail address: lgug@intec.unl.edu.ar (L.M. Gugliotta).

polymerization and particles exhibiting “core–shell” morphologies are usually obtained. First, PS particles are synthesized by emulsion homopolymerization of styrene (St). Then, PS particles are used as “seed” in a copolymerization of two or more comonomers. In this case, new polymer particles are not generated along the polymerization, and the final particle diameters are the result of the simple growth of the original seed [4,11,15–17].

Toxoplasmosis is a worldwide disease caused by the parasite *Toxoplasma gondii*, which seriously affects immunocompromised patients and pregnant women. Toxoplasmosis acquired during the gestation period, mainly in the first trimester, can cause abortion or induce malformations, hydrocephalus, macro or microcephaly and eye damage [18]. Therefore, the diagnosis of recently acquired infection in pregnant women is of great importance.

Diagnosis of *T. gondii* infection is usually based on the detection of specific Ab. There are immunodiagnostic kits commercially available for toxoplasmosis based on LAT but, as far as the authors are aware, they use homogenate of the parasite which includes a complex mixture of largely undefined antigens and therefore it is difficult to standardize a rigorous testing methodology. These reagents normally contain varying amounts of Ag which affect the specificity and reproducibility of the test results [19]. Moreover, they are not able to differentiate between the phase (acute or chronic) of the disease. The use of recombinant proteins covalently attached to latex particles could avoid these disadvantages and provide more reliable and stable reagents. Also, when compared to the method of obtaining a particular native Ag, recombinant Ag are easily produced in highly purified forms and large amounts, and can be readily standardized for diagnostic assays [20].

Several recombinant *T. gondii* proteins have been expressed and tested as Ag for the detection of anti-*T. gondii* Ab [21–29]. Preliminary studies on various proteins (SAG1, SAG2, GRA7, P35) allow us to define that P35Ag, which has been proposed for detecting Ab mainly generated during the acute phase of the infection [30–33], was the best choice for our purposes.

The ultimate goal is to obtain reagents for a LAT capable to detect the presence of acute phase Ab of toxoplasmosis disease. In this work, the novel P35Ag recombinant protein of *T. gondii* was produced, which results from a modification of the native P35 protein, and would be a useful serologic marker to differentiate between recently acquired infection and that acquired in the more distant past; and employed for sensitizing different polymer particles. To this effect, PS latex particles with controlled size were first synthesized. Then, “core–shell” latexes with external carboxyl groups were obtained by emulsion copolymerization of St and methacrylic acid (MAA) onto the PS particles. After their colloidal characterization, latex particles were sensitized with P35Ag by (i) PA onto the PS and carboxylated latexes; and (ii) CC with the functional groups of carboxylated latexes. The effect of particle size and functional groups density on the amount of bound protein was evaluated. Then, ELISA assays were performed to evaluate the antigenicity of the P35Ag coupled on the particles surface and to determine if the sensitization process affected the protein conformation.

2. Experimental

2.1. Materials

Technical grade St (Petrobras Energía S.A., Argentina) and MAA (Merk, purity >99%) monomers were used in polymerization reactions. The St monomer was vacuum distilled. The employed emulsifier was sodium dihexylsulfosuccinate (Aerosol MA-80), the initiator was potassium persulfate ($K_2S_2O_8$, Mallinckrodt, purity >99%) and the buffer was sodium bicarbonate ($NaHCO_3$, Cicarelli). Solutions of HCl (Cicarelli) and NaOH (Cicarelli) were used for

conductometric titrations and solutions of KBr (Cicarelli) were used for the evaluation of critical coagulation concentrations (c.c.c.).

The employed Ag of *T. gondii* was the novel recombinant protein P35Ag, which is a region of 100 amino acids from the N-term of the native P35 protein. Reagents used for protein production and purification were: Luria–Bertani (LB) medium, ampicillin (USB), isopropyl-β-D-thiogalactopyranoside (IPTG, Promega), phosphate buffered saline (PBS), NaH_2PO_4 (Cicarelli), $NaCl$, imidazole (Sigma), acrylamide/bisacrylamide (Promega), 2-amino-2-hydroxymethyl-propane-1,3-diol (TRIS) buffer and Coomassie brilliant blue (Sigma).

Phosphate (Cicarelli) buffer and borate (Anedra) buffer were used for the synthesis of LPC. A water solution of *N*-*N*-(3-dimethylamine propyl)*N'*-ethyl carbodiimide (EDC, Fluka) was prepared shortly before its use in the sensitization process by CC. The emulsifier employed for protein desorption was 4-(1,1,3,3-tetramethylbutyl)phenyl-polyethylene glycol (Triton X-100, Sigma).

The concentrations of dissolved protein were determined through the copper reduction/bicinchoninic acid (BCA) method [34]. BCA was from Pierce Reagents.

Serum samples from *T. gondii*-infected patients (and from non infected persons) were obtained from the Central Laboratory of the Province of Santa Fe (Argentina). The serum samples were analyzed and classified by reference techniques. IIF was used to detect immunoglobulin G (IgG) and ELISA-IgM-DS (Radim) to detect immunoglobulin M (IgM). Serum samples were classified as follows: (i) “chronic” sera are positive for IgG Ab and negative for IgM Ab, (ii) “acute” sera are positive for both IgG and IgM Ab, and (iii) “negative” sera are negative for both IgG and IgM Ab. For each group, control sera were prepared by mixing various sera with similar titers.

Polystyrene microplates (Costar) were used in ELISA. To this assay, the carbonate buffer (Sigma), a solution of polysorbate (Tween, Croda International PLC) in PBS and a solution of skinned milk (Molico) were used. Peroxidase-conjugated goat anti-human IgG, Fc_γ (Zymed), tetramethyl benzidine (Zymed) in H_2O_2 (Sigma), and a solution of H_2SO_4 (Cicarelli) were used.

Doubly deionized and distilled water (DDI) was used throughout the work.

2.2. Synthesis of functionalized latex particles

A 1-L jacketed glass reactor fitted with a stainless steel stirrer and a thermostatic bath was used. Monodisperse particles with carboxyl functionalities were synthesized by a two step emulsion polymerization process. In the first step, 2 monodisperse PS latexes (PS I and PS II) were synthesized by emulsion polymerization in presence of different concentrations of MA-80, with the aim of obtaining latexes with different particle sizes. To this effect, St/MA-80 mass ratios of 26.2 and 30.8 were, respectively, employed for PS I and PS II, with a St/ H_2O ratio of 0.42 in both cases. A MA-80 concentration below its critical micelle concentration (CMC) was used for producing PS II latex, but the MA-80 concentration was higher than the CMC for synthesizing the PS I latex, thus ensuring micelles formation. Other reaction conditions were taken from [35]. Finally, the unreacted St and $K_2S_2O_8$ were eliminated by serum replacement.

In the second step, the cleaned PS latexes were used as seeds for producing carboxyl latex particles, which were synthesized by emulsifier-free emulsion copolymerization of St and MAA, following the batch strategy reported in [17]. Four latexes were obtained, (PS-MAA I and PS-MAA II from PS II; PS-MAA III and PS-MAA IV from PS I). The monomers ratio was varied to control the carboxyl groups density (δ_{COOH}). In one case (PS-MAA II), a second load of initiator solution was injected after 4 h of reaction, to increase the

final conversion and the final surface density of SO_4^{\ominus} ($\delta_{\text{SO}_4}^{\ominus}$). Finally, the unreacted comonomers and $\text{K}_2\text{S}_2\text{O}_8$ were eliminated by serum replacement to purify the functionalized particles.

2.3. Characterization of polymer latexes

For all latexes, the monomer conversion (x) was determined by gravimetry as the ratio of the produced polymer to the total charged monomers. The mean (intensity-based) particle diameters (D_{DLS}) were measured by Dynamic Light Scattering (DLS, Brookhaven Instruments Inc.) at 90°. The particle size distribution (PSD) was determined by Scanning Electron Microscopy (SEM, JEOL-JSM 35C) on representative samples, and the polydispersity index (PI_{SEM}) was calculated from the number PSD.

The shell thickness was calculated from the difference between the functionalized latexes mean diameters, obtained by DLS, and those obtained for their seeds.

For surface characterization, the total surface charge density (σ) and the functional groups density ($\delta_{\text{SO}_4}^{\ominus}$, δ_{COOH}) were measured by conductometric titration employing an automatic titrator (KEM, model At-510). For the conductivity measurements, the final samples were diluted with DDI water under magnetic agitation and HCl solution was added to produce the complete protonation of the accessible acidic groups (corresponding to the sulfate groups from the initiator and to the carboxyl groups from the MAA units). The titrating agent was a NaOH solution.

Flexible polymer chains at the latex surface (hairy layer, h) extend into solution or collapse on the surface as a function of the electrolyte concentration. Particle diameter was measured by DLS varying the concentration of electrolyte (KBr) from 0 mM to 100 mM in order to determine the thickness of h [36]. In all cases, the maximum diameters were measured at 0 mM (where surface polymer chains are extended), while the minimum diameters were determined at higher KBr concentrations, where polymer chains are completely collapsed on the particle surface.

The effect of the medium ionic strength on the colloidal stability was determined through the c.c.c., by direct visualization and by DLS at two different pHs (6 and 8), when known concentrations of a KBr solution were added.

Electrophoretic mobility (μ_e) and Z Potential (ζ) were measured for all latexes with a Zetasizer Nano (Malvern Instruments), by taking the average of at least 10 measurements. The influence of pH on these electrokinetic variables was studied by employing phosphate buffer (pH 5, 6 and 7).

2.4. Production, purification and characterization of the recombinant protein P35Ag

Escherichia coli BL21 (DE3) cells bearing the plasmidic construction pET-32a/P35Ag (i.e., DNA sequences which contain the genetic information to express the recombinant protein P35Ag), were grown overnight under agitation, in LB medium, supplemented with ampicillin at 37 °C. Protein expression was induced with IPTG for 3 h. Cells were washed with PBS buffer, centrifuged, and resuspended in 50 mM NaH_2PO_4 (pH 8), 300 mM NaCl, and 20 mM imidazole buffer. Cells were lysed by sonication in an ice bath, employing a Sonics VibraCell Sonicator (Sonics & Materials, Inc.), and centrifuged 30 min at 13,000 rpm. The protein present in the supernatant was purified by Ni-nitrilotriacetic acid column (GE) nickel affinity chromatography. Supernatants were first applied to the columns, and then washed with the same buffer and eluted into different fractions, using the mentioned buffers plus 50, 100, and 250 mM imidazole, consecutively. The purity and identity of the purified protein were analyzed by 15% polyacrylamide gel electrophoresis (PAGE), and stained with Coomassie brilliant blue [37].

The P35Ag has a molar mass of 29.7 kDa, a theoretical isoelectric point (IP) of 5.94 (both calculated from the ExPasy Program, <http://web.expasy.org/protparam/>) [38], and an experimental IP (IP_{exp}) of 6.27 determined by isoelectrofocusing analyses. Based on a model created on the server I-TASSER (<http://zhanglab.cmb.med.umich.edu/I-TASSER/>) [39], square mean radius (R_g) was calculated for P35Ag using routine g_gyrate of the free software Gromacs V.4.0.7, and it was $R_g = 2.88 \text{ nm}$.

2.5. Synthesis of latex–P35Ag complexes

LPC were obtained by both PA and CC. In the PA experiments, PS latexes PS I and PS II and carboxylated latexes PS-MAA I, PS-MAA II, PS-MAA III and PS-MAA IV (0.2 m² of latex surface) were mixed with purified P35Ag at a concentration of 0.6 mg/mL (C°_{P35Ag}). The reaction was carried out at low ionic strength (0.002 M) in phosphate buffer (pH 6). The mixture was maintained under stirring along 5 h at room temperature. After incubation, LPC were centrifuged during 30 min at 15,000 rpm. The sensitized particles were resuspended in borate buffer (pH 8) and kept at 4 °C. The adsorbed protein was determined from the difference between the total added protein (C°_{P35Ag}) and the protein remaining in solution ($C_{\text{P35Ag,sol}}$).

In the CC experiments, carboxylated latexes PS-MAA I, PS-MAA II, PS-MAA III and PS-MAA IV (0.2 m² of latex surface) were mixed with increasing concentrations of recombinant protein ($C^{\circ}_{\text{P35Ag}} = 0.3, 0.6 \text{ and } 0.9 \text{ mg/mL}$) in the presence of the EDC activator. The activation of carboxyl groups was performed simultaneously to the CC to minimize the hydrolysis of the acilurea intermediate. Incubation was carried out in 0.002 M phosphate buffer (pH 5, 6 or 7) under stirring along 5 h at room temperature. The resulting LPC were first isolated from the solution by ultracentrifugation during 30 min at 15,000 rpm, and then resuspended in Triton X-100 1% (v/v) for 24 h, to desorb proteins not covalently attached to the particles. Once again the LPC were isolated from the solution by ultracentrifugation, resuspended in 0.002 M borate buffer (pH 8) and kept at 4 °C. The total-linked protein (i.e., both physically adsorbed and covalently bound) and the covalently coupled protein (i.e., the protein that remains on the particle surface after desorption with Triton X-100) was determined through a mass balance.

A centrifuge Sorvall RC-5B (Du Pont Instruments) and an UV-vis spectrophotometer Lambda 25 (PerkinElmer) were used for protein separation and quantification.

2.6. Characterization of the latex–P35Ag complexes

The mean particle diameters of the LPC were measured by DLS at 90°. Additionally, LPC were measured by multi-angle dynamic light scattering (MDLS) from 50° to 120° to analyze the possible PSD broadening and/or particle agglomeration during sensitization from the change of D_{DLS} with the detection angle. For MDLS measurements, particle concentration was adjusted for each angle in order to obtain a counting rate of about 2×10^5 counts/s for ensuring a regime of simple light scattering. Measurement time ranged between 100 and 200 s [40].

The c.c.c. of the LPC was measured at pH 8 by direct visualization and by DLS at 90°; and it was compared to the c.c.c. of the unsensitized latexes. Also, μ_e and ζ were measured at pH 8.

2.7. Protein antigenicity evaluation by ELISA

ELISA is a widely used technique for detecting the presence of a specific analyte, and in this case it was employed to analyze if P35Ag retains its capability for detecting Ab anti-P35Ag after sensitization of latex particles. To this effect, polystyrene microplates

were sensitized with 500 ng/well of P35Ag (in carbonate buffer, pH 9.6) or about 20 µg of each LPC (in borate buffer, pH 8) per well, and they were incubated over night at 4°C. Then, they were washed thrice with 0.05% (v/v) Tween in PBS, and the free polystyrene surfaces were blocked with 5% (w/v) skimmed milk in PBS for 1 h at 37°C. Microplates thus sensitized were incubated with a 1:100 dilution of the serum samples in 1% (w/v) skimmed milk in PBS. After three washes with 0.01% Tween in PBS, microplates were incubated with a 1:2000 dilution of the peroxidase-conjugated goat anti-human IgG, Fc_γ in 1% skimmed milk in PBS. The colorimetric reaction was developed using 100 µL of tetramethyl benzidine in H₂O₂ and stopped with 100 µL of H₂SO₄.

ELISA results were recorded as optical density (OD) at 450 nm (ELx808 Absorbance Microplate Reader, BioTek Instruments). All serum samples were evaluated by duplicate and the mean OD of these simultaneous determinations was calculated.

3. Results and discussion

3.1. Synthesis and characterization of polymer latexes

Main characteristics of PS latexes are shown in Table 1. As expected, PS particles with different sizes were obtained by varying the MA-80 concentration. For the synthesis of the PS I latex, the MA-80 concentration was higher than for PS II latex, and it was also higher than the emulsifier CMC. In contrast, PS II latex was synthesized employing a MA-80 concentration below its CMC, and particles with higher D_{DLS} than PS I particles were obtained. Latexes PS I and PS II were cleaned and concentrated at about 7% solids content. Latex PS I showed certain instability at such concentration, probably due to its small size and low values of σ and h . For this reason, it was used throughout the work at lower solids content.

Carboxylated latexes with different surface charge densities were obtained by varying the MAA/St ratio in the polymerization recipe. The higher the MAA/St ratio the higher the surface carboxyl groups density and for this reason PS-MAA II and PS-MAA III latexes exhibited higher δ_{COOH} than PS-MAA I and PS-MAA IV latexes, respectively, which importantly affected the latex stability. In the synthesis of PS-MAA II latex a pulse of initiator was added after 4 h of reaction, and for this reason it exhibited a higher $\delta_{SO_4^-}$ than latex PS-MAA I. Notice that some values of σ (161.7 µC/cm² and 171.1 µC/cm²) resulted high, maybe by the rough nature of the particles surface, which suggests that the particles have a larger area than the nominal one. However, similar values of σ have also been reported for latexes with acetal and carboxyl functionalities [11], and for carboxylated latexes [41] with mean particle diameters of around 200 nm. In all cases, final latex particles were quasi-monodisperse in size ($1.007 \leq PI_{SEM} \leq 1.029$) and suitable for their use in immunodiagnostic.

As expected h was higher for carboxylated latexes than for PS latexes and it also increased with the MAA/St ratio. This is because chains protruding from the particle surface that exhibit carboxylic-groups ends are longer than chains protruding from the PS particles with sulfate-groups ends. The same phenomenon occurs in the particles with higher δ_{COOH} (PS-MAA II and PS-MAA III).

Fig. 1 shows the μ_e measurements of the 6 latexes as function of the pH. In all cases the mobility increased with pH and decreased with the density of surface groups. The pH affects the ionization of surface functional groups. At higher pH the electric charge on the particle surface was increased thus augmenting μ_e . Notice that even though both surface charge density and particle size affect μ_e and ζ the electric effect was prevalent in this case.

The mobility of PS particles was greater than that for carboxylated particles, indicating that the PS systems are more

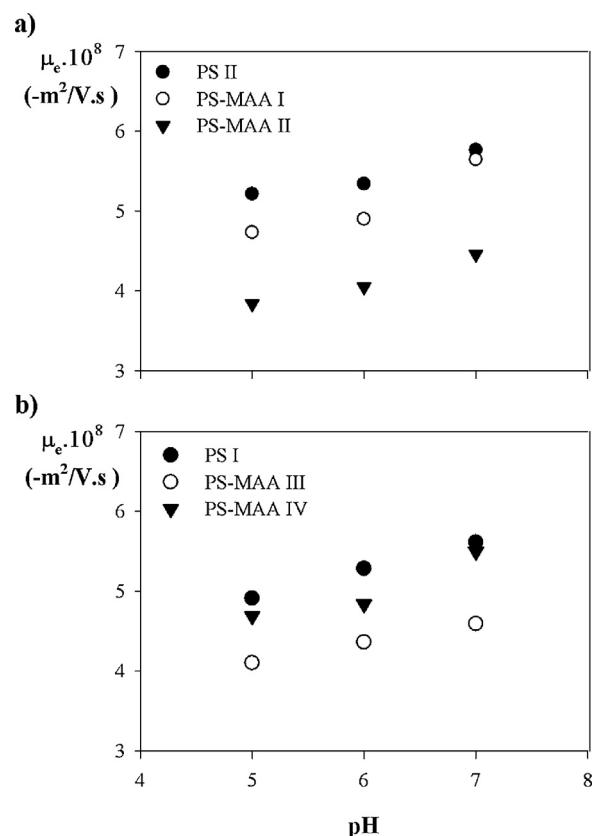


Fig. 1. Electrophoretic mobility of PS and carboxylated latexes as a function of pH. (a) Latex PS II and carboxylated latexes obtained from PS II; (b) latex PS I and carboxylated latexes obtained from PS I.

electrostatically stable than the PS-MAA systems. Electrophoretic mobility should be interpreted on the basis of the particles effective charge, which is different from σ obtained by conductometric titration. In this case it was observed that latex with higher σ exhibited lower μ_e . Similar behavior was described for liposomes exhibiting different charge by Haro-Pérez et al. [42], who observed that the effective charge decreased as the number of surface charged groups increased, due to a possible condensation of ions on the particle surface, which would affect the dissociation degree of ionizable groups. Moreover, Suzawa and Shirahama [43] observed a similar behavior when studying the behavior of the electric double layer of a hydrophobic PS homopolymer and a hydrophilic copolymer of St/2-hydroxyethyl methacrylate (HEMA). They observed that ζ was greater for the PS latex than for the P(St/HEMA) latex. In this last case, a hydrated polymer layer exists on the latex surface, and this layer shifts the position of the slipping plane away from the latex surface.

With respect to the experiments varying the ionic strength of the medium, it was observed that the higher the density of surface groups and the hairy layer thickness, the greater the c.c.c. High values of c.c.c. (>2000 mM) for the carboxylated latexes PS-MAA II and PS-MAA III could be explained by the combination of two different mechanisms: the electrostatic interaction given by the charged carboxyl groups, and the steric effect due to the hydrophilic chains protruding from the particles surface. The combined effect of both mechanisms is known as electrosteric stabilization [11,44–46]. Also, functionalized latexes presented greater c.c.c. at pH 8 than at pH 6. At pH 8, the carboxyl groups were more ionized (and the hydrophilic chains were more extended), thus increasing the repulsion between particles.

Table 1

Main reagents and final latex characteristics.

	PS I	PS II	PS-MAA I	PS-MAA II ^a	PS-MAA III	PS-MAA IV
MA-80/CMC	1.05	0.91	—	—	—	—
MAA/St	0	0	0.13	0.25	0.15	0.08
x (%)	100	98	70	86	78	95
D _{DLS} (nm) at 90°	134	300	340	354	193	180
Shell-thickness (nm)	—	—	20	27	29.5	23
P _I _{SEM}	1.010	1.007	1.021	1.027	1.017	1.029
σ (μC/cm ²)	17.0	25.0	78.5	161.7	171.1	72.9
δ _{SO₄} (mEq/cm ²)	1.8 × 10 ⁻⁷	2.6 × 10 ⁻⁷	2.4 × 10 ⁻⁷	3.8 × 10 ⁻⁷	3.5 × 10 ⁻⁷	4.1 × 10 ⁻⁷
δ _{COOH} (mEq/cm ²)	—	—	5.7 × 10 ⁻⁷	13 × 10 ⁻⁷	14 × 10 ⁻⁷	3.5 × 10 ⁻⁷
h (nm) ^b	5.33	5.58	8.60	11.50	11.18	7.75
−μ _e × 10 ⁸ (m ² /Vs) ^c	5.28	5.34	4.90	4.05	4.36	4.84
−ζ (mV) ^c	69.50	69.30	62.90	52.00	56.10	62.00
c.c.c _{vis} (mM KBr)						
pH 6	50	200	350	2000	>2000	300
pH 8	50	200	650	>2000	>2000	600
c.c.c _{DLS} (mM KBr)						
pH 6	50	50	200	1700	>2000	100
pH 8	50	100	250	>2000	>2000	200

^a A pulse of initiator was added after 4 h of reaction.^b Determined at pH 6.

3.2. Production of latex–P35Ag complexes

In PA experiments, the effect of particle size and surface charge density on the amount of adsorbed protein was considered. The adsorption experiments were carried out at C_{P35Ag} = 0.6 mg/mL and pH 6 (i.e. close to the IP of the protein). Under these conditions, intermolecular and intramolecular repulsions are diminished, thus maximizing protein adsorption. The obtained results are shown in Fig. 2.

No significant effect of particle size on the amount of adsorbed protein per unit area was observed for PS latexes (PS I, D_{DLS} = 134 nm; PS II, D_{DLS} = 300 nm, which produced the PA I and PA II LPC, respectively). Also, as the δ_{COOH} groups were increased (PS-MAA II and PS-MAA III, with respect to PS-MAA I and PS-MAA IV, respectively), the amount of physically adsorbed protein on the carboxylated latexes was decreased (compare PA IV and PA V, produced from PS-MAA II and PS-MAA III, respectively, with PA III and PA VI, produced from PS-MAA I and PS-MAA IV, respectively). This would indicate that hydrophobic forces governed the interactions between P35Ag and the particles surface, and for this reason physical adsorption was maximal for PS latexes (Fig. 2).

The CC of P35Ag onto “core–shell” carboxylated particles was carried out at 3 different pHs (5, 6 and 7). Figs. 3 and 4 show the

surface densities of covalently coupled protein ($\Gamma_{P35Ag,cov}$) onto the carboxylated latexes as a function of pH and C_{P35Ag}.

In all cases, the amount of total-linked protein increased with the concentration of added protein and the ratio between the total-linked protein and the total added protein decreased when increasing the amount of added protein. For example, for latex PS-MAA I at pH 6, these ratios were 0.90 for C_{P35Ag} = 0.3 mg/mL, 0.75 for C_{P35Ag} = 0.6 mg/mL, and 0.68 for C_{P35Ag} = 0.9 mg/mL. Similar results were observed for the 4 employed latexes at the 3 studied pHs, and they could be due to the saturation of the particle surface by the P35Ag protein.

In general, for a given C_{P35Ag} the greatest amount of bound protein was obtained at pH 6, close to the P35Ag IP. For example, for latex PS-MAA II, $\Gamma_{P35Ag,cov} = 4.4 \text{ mg/m}^2$ for C_{P35Ag} = 0.9 mg/mL (Fig. 3b). At this pH, the repulsion between the particle surface and the protein was decreased, and the repulsion between neighboring molecules was also reduced.

At pH 5 the latex surface has a negative charge, while the recombinant protein has a positive charge. Under these conditions, the convergence of proteins toward the particle surface is favored by attractive electrostatic interactions. However, there is a repulsion effect between adjacent protein molecules, because they have the same electric charge with each other. At pH 7 the protein is negatively charged, the repulsion between neighboring molecules is maintained, but it is added the electrostatic repulsion between protein molecules and particle surface, that also presents negative charge. Continuing with the previous example of latex PS-MAA II, for C_{P35Ag} = 0.9 mg/mL, $\Gamma_{P35Ag,cov} = 3.6 \text{ mg/m}^2$ at pH 5 and $\Gamma_{P35Ag,cov} = 3.3 \text{ mg/m}^2$ at pH 7 (Fig. 3b).

Regarding the effect of the density of surface functional groups on the amount of bound protein, it was observed a greater amount of protein covalently bound to PS-MAA II (Fig. 3b) and PS-MAA III (Fig. 4a), the latexes with higher δ_{COOH}, than to the other carboxylated latexes. This result could be explained by the increased amount of available functional groups in these latexes, allowing that a greater number of protein molecules react with such groups.

Also, the high fractions of chemically-bound proteins may render unnecessary the final operation of physical desorption previous to the LPC employment in immunoagglutination assays. The effect of the emulsifier on the desorption process was previously analyzed in [10], where the anionic emulsifier sodium dodecyl sulfate (SDS) was used instead of the non-ionic Triton X-100, here also employed, for the desorption step and similar results were obtained.

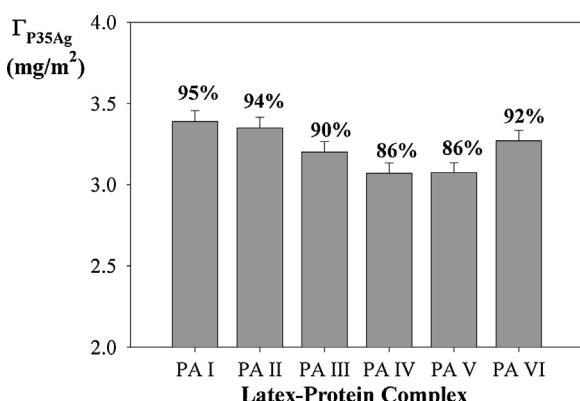


Fig. 2. Physical adsorption of P35Ag onto PS latexes (PA I and PA II obtained from PS I and PS II, respectively) and carboxylated latexes (PA III to PA VI obtained from PS-MAA I to PS-MAA IV, respectively). The percentages on the bars indicate the fractions of adsorbed protein with respect to C_{P35Ag} and error bars indicate the standard deviations for n = 3.

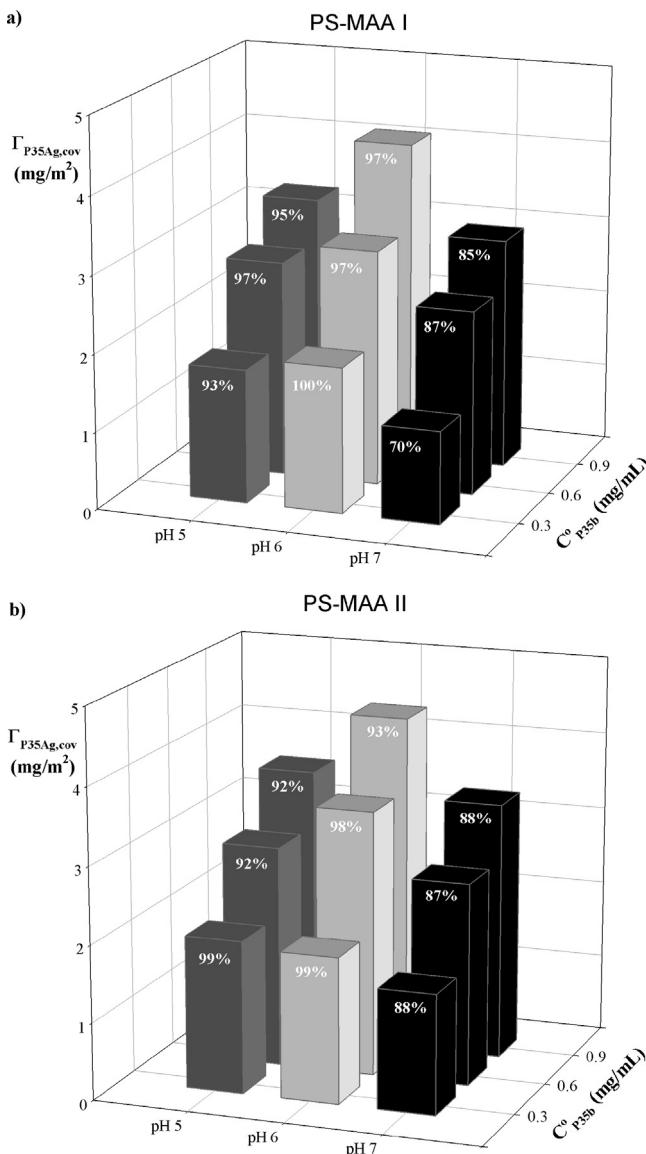


Fig. 3. Chemical coupling of P35Ag onto the carboxylated latexes PS-MAA I (a) and PS-MAA II (b) as a function of the initial protein concentration and the pH. The percentages on the bars indicate the fractions of covalently coupled protein respect to the total linked protein after desorption with Triton X-100. (For $n=3$, an average error of 5% was obtained in the amount of covalently coupled protein.)

With the aim of determining whether the protein coupled to the particles remains stable in time, LPC were analyzed after 6 months of storage. To this effect, they were centrifuged and the amount of protein in the supernatant was quantified. The LPC obtained by PA from the latexes of PS (i.e. PA I and PA II), experienced a significant loss of P35Ag, with desorption percentages of 19% and 21%, respectively. However, in the case of the LPC obtained by CC from PS-MAA I to PS-MAA IV (i.e. CC I to CC IV) at a $C^0_{P35Ag} = 0.6 \text{ mg/mL}$, the desorption of P35Ag was less than 7% in all cases.

3.3. Characterization of the latex–P35Ag complexes

Main results of the characterization of 14 LPC (2 obtained by PA and 12 obtained by CC, all at pH 6) are presented in Table 2. In general, lower values of c.c.c., μ_e and ζ were obtained for LPC with greater Γ_{P35Ag} , indicating a reduced colloidal stability. LPC stability is also strongly affected by the characteristics of the unmodified latexes. Thus, the LPC obtained from latexes PS-MAA II and

Table 2
Final characteristics of the LPC obtained at pH 6.

	PA I	PA II	CC I	CC II	CC III	CC IV
$\Gamma_{P35Ag} (\text{mg/m}^2)$	3.4	3.3	1.9	3.0	1.7	2.7
% UC	–	–	100	97	98	94
D_{BLS} at 90° (nm)	218	330	370	413	420	349
$-\mu_e \times 10^8 (\text{m}^2/\text{V s})$	2.67	3.28	2.8	2.4	2.08	2.52
$-\zeta (\text{mV})$	37.6	46.2	39.1	33.4	31.9	27.6
c.c.c. (mM BrK)	300	600	600	400	>1000	1000
c.c.c. (mM BrK)	100	200	200	100	>1000	>1000

PA I and PA II obtained from latex PS-MAA I, II, III and IV obtained from latex PS-MAA I, II, III, IV, respectively, in CC experiments.

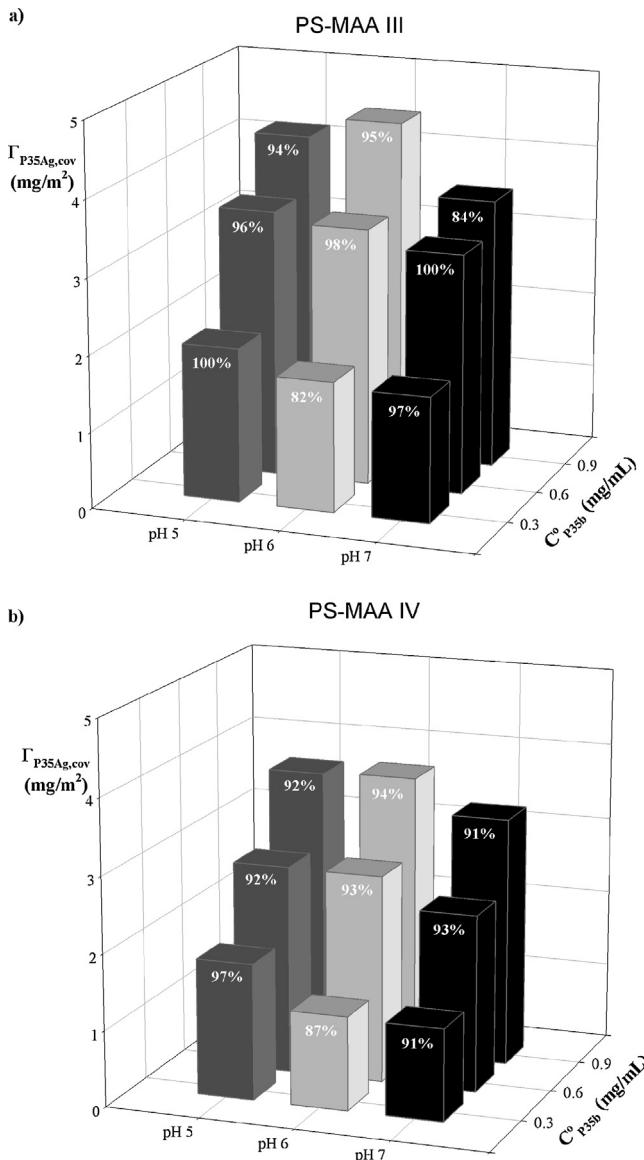


Fig. 4. Chemical coupling of P35Ag onto the carboxylated latexes PS-MAA III (a) and PS-MAA IV (b) as a function of the initial protein concentration and the pH. The percentages on the bars indicate the fractions of covalently coupled protein respect to the total linked protein after desorption with Triton X-100. (For $n=3$, an average error of 5% was obtained in the amount of covalently coupled protein.)

PS-MAA III (with exhibited greater δ_{COOH} and h) showed higher values of c.c.c. In addition, the greater the Γ_{P35Ag} , the larger the mean-average particle diameter (measured by DLS).

The increment in D_{DLS} with respect to the unsensitized latexes observed for greater Γ_{P35Ag} could not only be due to the protein linkage, but also to some polydispersity increment that importantly augment D_{DLS} , and is more likely to occur at higher protein concentrations.

Fig. 5a shows that μ_e drastically decreased when the carboxylated latexes PS-MAA III and PS-MAA IV were sensitized by CC at pH 6 with increasing amounts of P35Ag. (Even though not shown, similar results were observed for PS-MAA I and PS-MAA II). These results are consistent with those reported by Ortega-Vinuesa et al. [47] for carboxylated latexes sensitized by PA and CC with IgG (in comparison with unsensitized latexes).

Fig. 5b shows the mean diameters (D_{DLS}) measured by MDLS for PS-MAA II and the respective LPC (CC II) obtained at pH 6 for the three amounts of bound P35Ag here considered, at detection angles

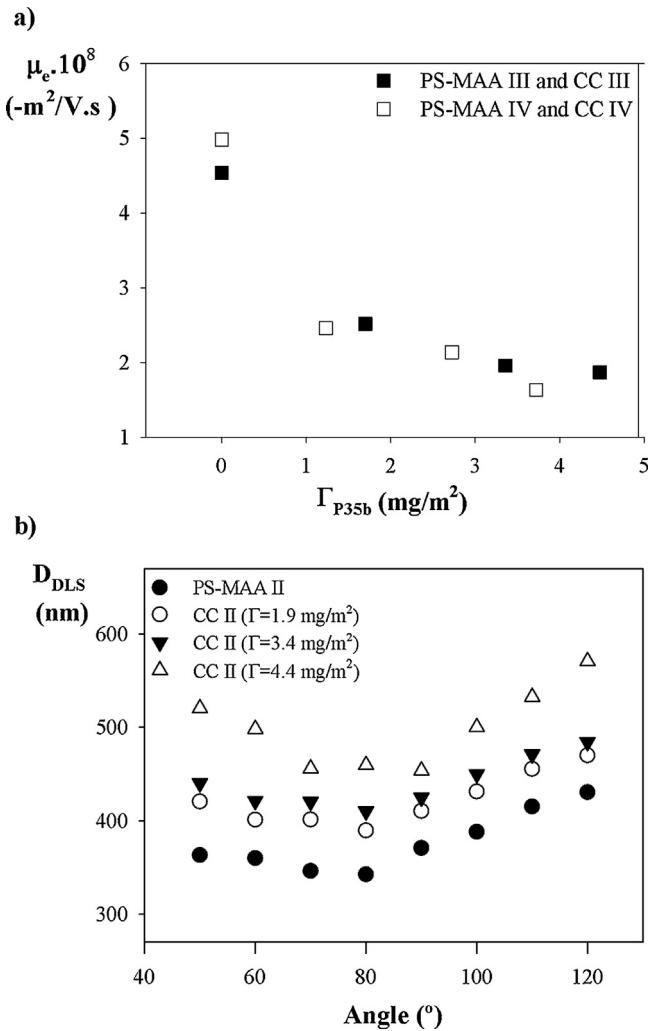


Fig. 5. (a) Electrophoretic mobilities of the carboxylated latexes PS-MAA III, PS-MAA IV and their LPC CC III and CC IV determined at pH 8 as a function of the amount of bound protein. (b) Mean diameters measured by MDLS ($50\text{--}120^\circ$) for the PS-MAA II latex and three LPC obtained from it at pH 6 (CC II) and at three different protein concentrations.

between 50° and 120° at regular intervals of 10° . Some agreement between the variation (with the detection angle) of D_{DLS} for the unsensitized latexes and for the LPC was observed, which would indicate the absence of particle agglomeration along the sensitization process. However, it was also observed that the greater the amount of bound protein, the larger the diameter change along the MDLS measurements, and the higher the polydispersity of the particle size distribution. Even though not shown, a PSD broadening was observed from the DLS measurements at 90° when increasing the bound protein.

Finally, the same behavior previously described was observed for all other latexes (and LPC) here considered.

3.4. Protein antigenicity evaluation by ELISA

As previously indicated, the main objective of evaluating the LPC by ELISA was to study if P35Ag retained its antigenic properties after the particles sensitization. The following LPC were employed to sensitize the polystyrene plate: PA I, PA II, CC I, CC II, CC III and CC IV, all exhibiting a similar density of bound protein Γ_{P35Ag} ($3.0\text{--}3.7 \text{ mg/m}^2$). Main results shown in Fig. 6 compare the antigenic profile of the pure protein to those obtained with the six different LPC.

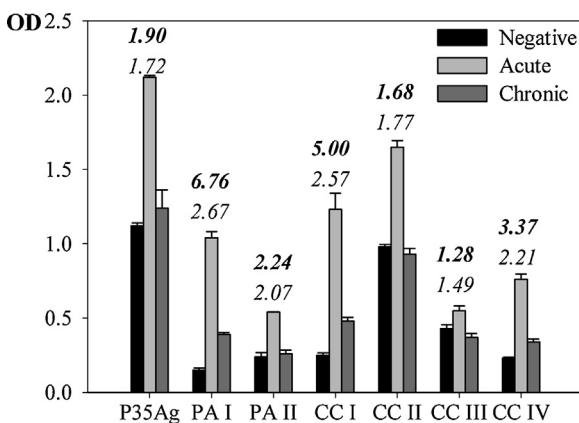


Fig. 6. ELISA results obtained using the protein P35Ag and 6 different LPC produced by physical adsorption and chemical coupling (with similar Γ_{P35Ag} between 3.0 and 3.7 mg/m²). Numbers on the bars indicate the ratio OD_{ac}/OD_{neg} (bold) and the ratio OD_{ac}/OD_{ch} (nonbold). Error bars represent the range of duplicate samples.

One way of analyzing the ELISA results is evaluating the relationship between the OD obtained when the sample was an acute serum, and the OD obtained when the sample was a negative or a chronic serum. In all cases, the OD obtained with the acute serum (OD_{ac}) was greater than that produced by the negative (OD_{neg}) and the chronic (OD_{ch}) sera, thus indicating that the Ab anti-P35Ag generated in the acute phase of the disease have been differentially recognized. The best differentiation between acute sera respect to chronic and negative sera was obtained with the complexes PA I and CC I, which exhibited OD_{ac}/OD_{neg} ratios of 6.76 and 5.00, respectively; and OD_{ac}/OD_{ch} ratios of 2.67 and 2.57, respectively. Considering that during the latex sensitization process, proteins could suffer partial denaturation or other unwanted changes that would affect their characteristics, the assays confirmed that the P35Ag was not affected during the sensitization, and consequently the produced LPC are able to recognize specific Ab anti-P35Ag.

Finally notice that the lower antigenicity was obtained with LPC CC II and CC III, which exhibit higher values of h in their unsensitized latexes (PS MAA II and PS MAA III, respectively). This result could be explained by the fact that ELISA was performed under conditions of high ionic strength, where the chains of the hairy layer were collapsed, thus probably interfering with the antigenicity of the protein bound to the latex.

4. Conclusions

The acute phase recombinant protein of *Toxoplasma gondii* P35 was expressed and purified; and it was used to produce LPC through PA and CC onto PS particles and “core–shell” carboxylated particles. Then, the LPC were tested with negative, acute and chronic sera by means of ELISA assays.

Two PS latexes and four carboxylated latexes were synthesized and their characteristics (D_{DLS} , PlSEM, σ , $\delta_{SO_4}^=$, δ_{COOH} , μ_e , ζ and c.c.c.) showed that they are useful as reagents for producing immunoagglutination kits. Carboxylated latexes PS-MAA II and PS-MAA III exhibited higher values of σ , δ_{COOH} , and c.c.c. than PS-MAA I and PS-MAA IV.

Greater amounts of P35Ag were physically adsorbed on PS latexes than on carboxylated latexes, indicating that hydrophobic forces govern the interactions between the protein and the particle surface.

The amount of P35Ag covalently bound onto carboxylated latexes was higher at pH 6 (close to the IP), where both the repulsion between particle surface and protein, and the repulsion between neighboring macromolecules were reduced. The high fractions of

covalently bound P35Ag would make unnecessary the process of physical desorption previous to the employment of the LPC in immunoagglutination assays. Also, the fraction of total-linked and covalently bound P35Ag (with respect to the initially added protein) decreased with the amount of added P35Ag, probably due to the saturation of the particle surface.

Lower values of c.c.c., μ_e and ζ (and reduced colloidal stability) were in general obtained for LPC with greater Γ_{P35Ag} . The LPC stability was also strongly affected by the characteristics of the unsensitized latexes (i.e., CC II and CC III showed higher values of c.c.c. as their respective unsensitized latexes PS-MAA II and PS-MAA III).

Confirming previous reports, the P35Ag used as sensitizing antigen was useful to differentiate acute sera, with respect to negative and chronic sera [30,32,33]. By comparing the performance of this single antigen with the same antigen bounded to different latexes, we could determine that the P35Ag was not affected during the sensitization by PA and CC; and consequently the produced LPC would be able to recognize specific Ab anti-P35Ag. Finally, PA I and CC I were the best complexes in the sense of improving the differentiation between acute, negative and chronic sera by ELISA assay.

However, as IA and ELISA are clearly different assays, the LPC which showed better performance in the present study, it would probably not be the most useful for IA.

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