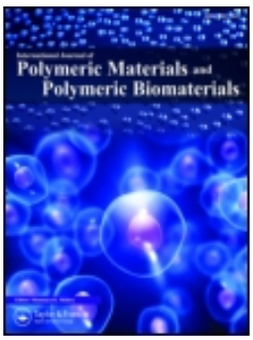

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Synthesis and characterization of latex-protein complexes from different antigens of *Toxoplasma gondii* for immunoagglutination assays

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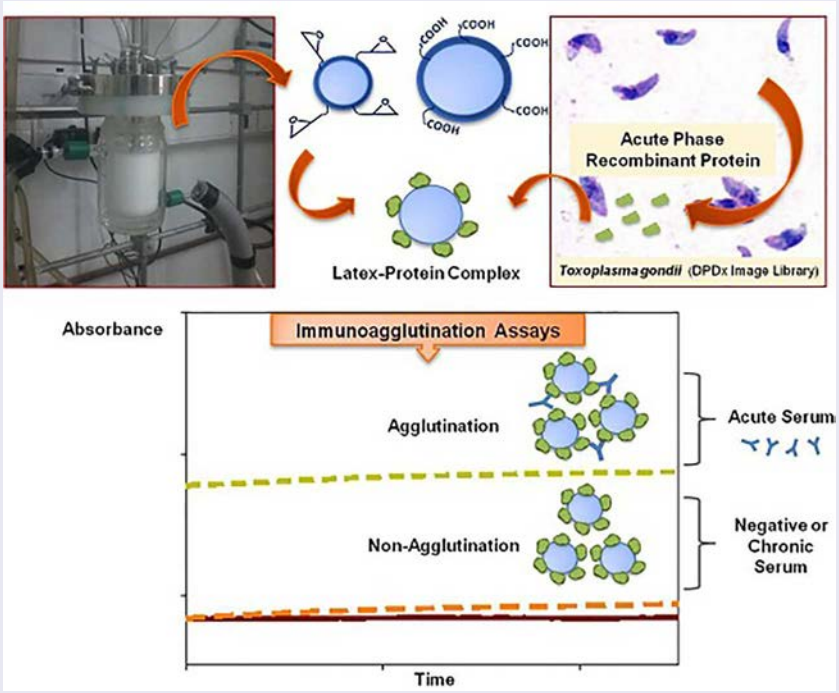
ABSTRACT

The acute phase recombinant protein of *Toxoplasma gondii* P22Ag was expressed and purified and the homogenate of the parasite was obtained from an infected mouse. These antigens were used to produce latex-protein complexes (LPC) through physical adsorption and chemical coupling onto different latexes, with the aim of producing immunoagglutination (IA) reagents able to detect recently acquired toxoplasmosis. Polystyrene and "core-shell" latexes where employed, exhibiting varied particle size, functionality (carboxyl or epoxy), and charge density. In sensitization experiments for producing LPC, the recombinant protein showed better coupling efficiency onto the particles surface than the homogenate and this could be explained by the complex mixture of the homogenate, which includes a large number of proteins of different molecular mass, isoelectric points, and hydrophobicity. The synthesized LPC were employed in IA assays. To this effect, the agglutination reaction was followed by measuring the changes in the optical absorbance by turbidimetry. Experiments against control sera were performed to evaluate the performance of various LPC and it was observed that the IA test based on P22Ag and the carboxylated latex of 350 nm of particle diameter allowed a good discrimination between acute sera and chronic/negative ones. The proposed test is cheap, rapid, and easy to implement.

GRAPHICAL ABSTRACT



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1. Introduction

Toxoplasmosis is a protozoan disease caused by the intracellular parasite *Toxoplasma gondii*, which infects both animals and human. It is widespread throughout the world, affecting more than one third of the world population [1]. In immunocompetent individuals the disease runs smoothly, usually

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asymptomatic and self-limiting. However, serious complications arise when the infection occurs in immunocompromised patients or pregnant women.

The parasite can be transmitted via transplacental severely affecting the fetus, since different manifestations such as premature birth, permanent neurological damage, hydrocephalus, visual impairment, and spontaneous abortion could take place [2,3]. Probability of transmission from mother to fetus increases with pregnancy time, being 1% in first six weeks to 80% by the third trimester. However the most severe consequences are associated to infection during the first weeks of pregnancy [4,5].

Acute infection in pregnant women is usually asymptomatic and can be detected by serological tests. The employed diagnostic tests are often expensive, require highly skilled professionals and often produce conflicting results, so more than one test is generally performed. Main tests are enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence (IIF), direct agglutination test (DAT), Sabin-Feldman reaction (SF), indirect hemagglutination (IHA), and latex agglutination test (LAT). LAT is a fast and cheap detection method, which results particularly useful for screening purposes and has been successfully used for the detection of various analytes [6–8]. It consists in latex particles of a certain size that have bound biomolecules such as antigens (Ag) of *T. gondii*, which can be recognized by specific antibodies (Ab) present in the analyzed sample. If it contains specific Ab the biorecognition of the sensitized particles occurs and the agglutination reaction takes place. As far as we are aware, LAT kits commercially available for toxoplasmosis only use homogenate of the parasite, which includes a complex mixture of largely undefined Ag. Under this condition, it is difficult to standardize a rigorous testing methodology, thus affecting the specificity and reproducibility of the test results [9]. Moreover, commercial LAT do not provide any information about the moment in which infection has occurred (i.e, whether it is a recent or a remote infection).

The use of recombinant proteins for latex particles sensitization could avoid these disadvantages and provide more reliable and stable reagents. Several recombinant *T. gondii* proteins have been expressed and tested as Ag for the detection of anti-*T. gondii* Ab [10–17]. In particular, the surface Ag P22 has been proposed for detecting Ab mainly generated during the acute phase of the infection [18–20]. Thus, if Ag specially chosen for their selective reactivity were used to sensitize latex particles, immunoagglutination (IA) reagents able to determine whether the infection is acute or happened in the distant past could be obtained.

The ultimate goal of the present study is to detect early, economical and safe way acute phase Ab anti-*T. gondii* by developing an IA reagent useful for screening studies. First, the synthesis of monodisperse latex particles of polystyrene (PS) was conducted to control particles size (in the range of 100–1100 nm). Core-shell particles were then synthesized with carboxyl or epoxy functionalities on PS latex seeds to control the external chemical functionality and charge density of the particles. Then, the *T. gondii* P22Ag recombinant protein and the *T. gondii* homogenate from peritoneal exudate of infected mouse were obtained and employed for sensitizing polymer particles. Latex protein complexes (LPC) were produced by both physical adsorption (PA) onto PS latexes and chemical coupling (CC) with the external functional groups of carboxylated or epoxylated latexes. The effect of particle size, type of functional groups and charge density on the amount of bound protein was evaluated. LPC were carefully characterized and employed in IA assays against control (negative, acute, or chronic for toxoplasmosis) sera to evaluate their ability to discriminate acute sera from negative and chronic ones.

2. Experimental

2.1. Materials

Technical grade styrene (St; Petrobras Energía S.A., Argentina), methacrylic acid, (MAA; Merck, purity >99%), and glycidyl methacrylate (GMA; Aldrich, purity >97%) monomers were used in polymerization reactions. The St monomer was vacuum distilled. For dispersion polymerization, polyvinylpyrrolidone (PVP; Sigma-Aldrich) was employed as steric stabilizer and ethyl alcohol (Anedra)/doubly deionized and distilled water as dispersion medium. The employed initiators were azobisisobutyronitrile (AIBN; Molekula, purity 99.2%) or potassium persulfate ($K_2S_2O_8$, Mallinckrodt, purity >99%) and the buffer was sodium bicarbonate ($NaHCO_3$, Cicarelli). Other reactives used were HCl, NaOH, sodium thiosulfate ($Na_2S_2O_3$), sodium carbonate (Na_2CO_3), and potassium bromide (KBr), all from Cicarelli. PS and core-shell particles previously synthesized in a two stages procedure were employed [21]. In the first stage, the so called PS I and PS II polystyrene particles were synthesized by emulsion polymerization of St, in the presence of sodium dihexylsulfosuccinate (Aerosol MA-80) as emulsifier. In the second stage, PS I and PS II were employed as seeds in emulsifier-free emulsion copolymerizations of St and MAA, where MAA provided the carboxyl functionality of the PS-MAA particles with core-shell morphology, and named as PS-MAA I, PS-MAA II, PS-MAA III, and PS-MAA IV. Table 1 resumes the principal characteristics of the latexes previously synthesized.

The employed Ag of *T. gondii* were the recombinant protein P22Ag and the homogenate of the parasite, which is a mixture of native proteins prepared from tachyzoites. Reagents used for protein production and purification of recombinant Ag 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), 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 onto carboxylated latexes. 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 [22]. BCA was from Pierce Reagents.

Mouse peritoneal exudate infected with tachyzoites of *T. gondii* was donated by the Central Laboratory of the Province of Santa Fe (Argentina).

Table 1. Main characteristics of previously synthesized latexes.

	PS I	PS II	PS-MAA I	PS-MAA II	PS-MAA III	PS-MAA IV
D_{DLS} (nm)	134	300	340	354	193	180
PI_{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
$\delta^{=}_{SO4}$ (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)[a]	5.33	5.58	8.60	11.50	11.18	7.75
$-\mu_e^g \times 10^8$ (m^2/V.s)	5.28	5.34	4.90	4.05	4.36	4.84
-ζ (mV)	69.50	69.30	62.90	52.00	56.10	62.00
c.c.c. $_{DLS}$ (mM KBr)						
pH 6	50	50	200	1700	>2000	100
pH 8	50	100	250	>2000	>2000	200

D_{DLS} = average particle diameter by dynamic light scattering; PI_{SEM} = polydispersity index by scanning electron microscopy; **σ** = surface charge density; $\boldsymbol{\delta^{=}_{SO4}}$ = sulfate group density; $\boldsymbol{\delta_{COOH}}$ = carboxyl group density; ***h*** = hairy layer thickness; $-\mu_e \times \mathbf{10^8}$ = electrophoretic mobility; **-ζ** = zeta potential; ***c.c.c.*$_{DLS}$** = critical coagulation concentrations by dynamic light scattering. [a]Determined at pH 6.

Serum samples from *T. gondii*-infected patients (and from non infected persons) were obtained from the same laboratory. The serum samples were analyzed and classified by reference techniques. IIF was used to detect immunoglobulin G (IgG), ELISA-IgM-DS (Radim) to detect immunoglobulin M (IgM) and IgG avidity ELISA (Biomerieux) was performed when detection of IgM was positive. Serum samples were classified as follows: (a) Chronic sera are positive for IgG Ab and negative for IgM Ab; (b) acute sera are positive for both IgG and IgM Ab, and low IgG avidity; and (c) negative sera are negative for both IgG and IgM Ab.

A saline solution of bovine serum albumin (BSA), glycine, and polyethylene glycol 8000 (PEG), all from Sigma was used for the formulation of the IA assays reaction medium.

2.2. Synthesis of other functionalized latex particles

A 200 mL jacketed glass reactor fitted with a stainless steel stirrer and a thermostatic bath was used. Monodisperse particles with carboxyl or epoxy functionalities were synthesized by a two steps emulsion polymerization process. In the first step, monodisperse PS latex (PS III) was synthesized by dispersion polymerization. First, the reactor was loaded with the steric stabilizer PVP dissolved in the dispersion medium ethyl alcohol/water and the St monomer, thus forming a single homogeneous phase. The temperature was fixed at 70°C and mechanical agitation with bubbling N_2 started. After 30 minutes, the initiator AIBN dissolved in a fraction of the medium (ethyl alcohol/water) was added, and the reaction temperature was maintained at 70°C for 10 h. During polymerization, when the growing polymer chains reach a critical size they become insoluble and precipitate out of the medium to form monodisperse polymer particles of about 1000 nm of diameter. After finishing the reaction, the unreacted St and other reactants were eliminated by serum replacement. In the second step, the cleaned PS latex was used as a seed for producing the carboxyl latex PS-MAA V, which was synthesized by emulsifier-free emulsion copolymerization of St and MAA, following the batch strategy reported by Gonzalez et al. [23]. Also, the latex PS II was used as a seed for producing latex particles with epoxy functionality, which were synthesized by emulsifier-free batch emulsion copolymerization of St and GMA (PS-GMA I and PS-GMA II), where the monomers ratio was varied to control the epoxy groups density (δ_{C2H3O}). To increase both the final conversion and the surface density of $SO_4^{=}$ ($\delta^{=}_{SO4}$) a second load of initiator was injected after 4 h of reaction. In one case (PS-GMA II), a second load of monomers solution was also injected after 3 h of reaction to increase δ_{C2H3O}. Finally, the unreacted comonomers and $K_2S_2O_8$ were eliminated by serum replacement to purify the functionalized particles.

2.3. Characterization of polymer latexes

For all latexes synthesized in this work, 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 a detection angle of 90°. The δ_{C2H3O} was determined by potentiometric titration [24–26] employing an automatic titrator (KEM, model At-510). This assay is based on a redox reaction between the epoxy groups and sodium thiosulfate ($Na_2S_2O_3$), leading to the production of OH^- proportionally to the amount of oxirane rings. The titrating agent was a HCl solution. The polydispersity index (PI_{SEM}) was calculated from the number particle size distribution (PSD) determined by Scanning Electron Microscopy (SEM; JEOL-JSM 35C) on representative samples. Also, particle diameter was measured by DLS varying the concentration of electrolyte (KBr) in order to determine the thickness of the hairy layer (*h*). The total surface charge density (σ) and the sulfate and carboxyl functional groups density ($\delta^{=}_{SO4}$, δ_{COOH}) were measured by conductometric titration. The critical coagulation concentration (*c.c.c.*) was determined by DLS at two different pHs (6 and 8), when known concentrations of a KBr solution was added. The electrophoretic mobility (μ_e) and the zeta potential (ζ) were determined with a Zetasizer Nano (Malvern Instruments), by taking the average of at least 10 measurements [21].

2.4. Production and purification of the different antigens

Escherichia coli BL21 (DE3) cells bearing the plasmidic construction pET-32a/P22 (i.e., DNA sequences that contain the genetic information to express the recombinant protein P22Ag), 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 0.020 M 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 [27].

P22Ag molar mass (MM = 35.3 kDa) and theoretical isoelectric point (IP = 5.86) were calculated from the ExPasy Program (http://web.expasy.org/protparam/) [28]. Those properties were also experimentally determined. Thus, MM measured by PAGE was 34 kDa and IP measured by isoelectrofocusing was 6.1. Based on models created on the server I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) [29], square mean radius was calculated using the routine g_gyrate of the free software Gromacs V.4.0.7, resulting Rg = 2.83 nm.

To obtain *T. gondii* homogenate, peritoneal exudate of infected mouse was first observed by light microscopy for the presence of *T. gondii* tachyzoites. Then it was centrifuged 15 min at 2,000 rpm and parasites were redispersed in saline solution. This procedure was repeated three times. Cells were lysed by sonication in an ice bath and centrifuged 15 min at 2,000 rpm again. Soluble proteins were quantified by fluorometry (Qubit 1.0 Fluorometer). It was observed by PAGE that homogenate MM ranged between 10 and 95 kDa, with the main mass fraction (45%) belonging to the 30–55 kDa range.

2.5. Synthesis of latex-protein complexes

LPC were obtained by both PA and CC of P22Ag and homogenate onto the different latex particles exhibiting variable size, chemical functionality and charge density.

In the PA experiments, PS I, PS II, and PS III latexes (0.2 m^2 of latex surface) were mixed with each protein (P22Ag and homogenate) at a concentration of $C^\circ = 0.6$ mg/mL. The reaction was carried out at low ionic strength (0.002 M) in phosphate buffer (pH 6) and the mixture was maintained under stirring along 5 h at room temperature. After incubation, LPC were centrifuged during 30 min at 15,000 rpm and the sensitized particles were resuspended in borate buffer (pH 8) and kept at 4°C. The adsorbed protein was determined from the difference between C° and the protein remaining in solution (C_{sol}).

In the CC experiments onto carboxylated latexes, PS-MAA I, PS-MAA II, PS-MAA III, PS-MAA IV, and PS-MAA V (0.2 m^2 of latex surface) were mixed with P22Ag at increase concentrations ($C^\circ = 0.3$–0.9 mg/mL) or with the homogenate at $C^\circ_{Hom} = 0.6$ 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 in phosphate buffer (pH 6) was carried out under stirring along 5 h and at room temperature.

In the CC experiments onto epoxylated latexes, where no prior activation of the functional groups is required and the reaction occurs spontaneously, PS-GMA I (0.2 m^2 of latex surface) were mixed with P22Ag at increase concentrations ($C^\circ_{P22Ag} = 0.3$–0.9 mg/mL) carrying out the incubation along 96 h in phosphate buffer (pH 7), under stirring and at room temperature [30].

In all CC experiments, 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) were determined through a mass balance.

A refrigerated centrifuge Neofuge 18 R (Heal Force) and an UV–vis spectrophotometer Lambda 25 (Perkin Elmer) were used for protein separation and quantification, respectively.

2.6. Characterization of the latex-protein complexes

The average particle diameters of the LPC were measured by DLS at 90°. The effect of the medium ionic strength on the colloidal stability of the LPC was determined through the *c.c.c.*, by DLS at pH 8, when known concentrations of a KBr solution were added, and it was compared to the *c.c.c.* of the unsensitized latexes. The μ_e and the ζ of the LPC were measured at pH 8, by taking the average of at least 10 measurements.

2.7. Immunoagglutination assays

Before the agglutination test latex-protein complexes were conditioned. To this effect, complexes were first centrifuged and then redispersed in a low ionic strength (20 mM) saline solution of BSA at pH 8, and in the presence of glycine and PEG. BSA was used as blocking agent of hydrophobic surface zones, and glycine was added with the aim of being linked to the free carboxyl or epoxy groups present on the particles surface [31], thus reducing the reactions with such groups that could give nonspecific agglutinations during the immunoassays. In previous works, it was found that PEG promotes Ag-Ab reaction due to it allows a better exposure of the Ag epitopes bound to the particle surface, to be recognized by Ab present in the serum [8,32].

To carry out the immunoassays, 950 µL of a dispersion containing the investigated LPC (at a known concentration) were mixed with 50 µL of serum. The agglutination reaction was detected by turbidimetry, measuring the changes in the optical absorbance (A) at 570 nm, after 5 min of reaction. The increment in A (ΔA) was determined by subtracting the A of a blank (the complex without serum) to the A measured for the (complex + serum) sample. The colloidal stability of the complexes, in the same dispersion medium where immunological reactions take place, was previously evaluated by measuring the time evolution of A. The absence of changes in A during the period of analysis was indicative of colloidal stability.

Table 2. Main characteristics of the latexes obtained by dispersion polymerization (PS III) and seeded emulsion copolymerization of St/MAA (PS-MAA V) and St/GMA (PS-GMA I and II).

	PS III	PS-MAA V	PS-GMA I	PS-GMA II
Conversion (*x*, %)	99	99	69	80
D_{DLS} (nm)	1043	1134	362	519
PI_{SEM}	1.003	1.005	1.016	1.069
σ (μC/cm^2)	—	153.8	59.3	44.4
$\delta^{=}_{SO4}$ (mEq/cm^2)	—	7.4×10^{-7}	6.1×10^{-7}	4.6×10^{-7}
δ_{COOH} (mEq/cm^2)	—	8.6×10^{-7}	—	—
δ_{C2H3O} (mEq/cm^2)[a]	—	—	0.93×10^{-7}	1.75×10^{-7}
h (nm)[b]	6.58	14.18	13.10	12.20
$-\mu_e \times 10^8$ (m^2/V.s)[b]	3.82	3.06	3.82	3.41
-ζ (mV)[b]	49.10	39.30	48.80	43.90
c.c.c. $_{DLS}$ (mM KBr)				
pH 6	100	100	200	250
pH 8	100	200	200	250

Other variables as in Table 1.[a]Epoxy group density. [b]Determined at pH 6.

3. Results and discussion

3.1. Synthesis and characterization of functionalized latex particles

Main characteristics of the synthesized latexes are shown in Table 2.

Dispersion polymerizations were carried out varying the dispersion medium composition, the amount of stabilizer (PVP), initiator (AIBN), and monomer (St) to obtain stable latexes [33]. It was found that the ethyl alcohol/water mixture in a ratio 2.2:1 was the most suitable to obtain stable particles with sizes close to 1000 nm (PS III). Under the studied experimental conditions, changes in PVP, AIBN, and St concentrations had not significant effect on particle size and latex stability.

Synthesis of the carboxylated latex PS-MAA V was based on the recipes reported by Gonzalez et al. [23], with the addition of the following two ingredients: MA-80 emulsifier and $NaHCO_3$, to provide better stability to the particles under an alkaline pH. Notice that the employed MA-80 concentration was below the critical micellar concentration, to avoid the presence of micelles and the production of new particles under secondary nucleation. The final conversion in PS-MAA V reached about 99% with a low growth of D_{DLS} respect to the employed PS III seed (Table 2). Thus, the incorporation of functional groups and the increment in the charge density was achieved without significantly changing the average diameter. Furthermore, the polydispersity of the carboxylated latex was similar to that of the PS latex, which is an indication that new particles were not formed.

Latexes with epoxy functionality (PS-GMA I and PS-GMA II) reached the maximum conversion value at approximately 4 h of reaction. The final D_{DLS} obtained for latex PS-GMA I was about 20% higher than the particle size of the base latex seed PS II ($D_{DLS} = 300$ nm) achieving the objective of incorporating epoxy functional groups with a polydispersity lower than 1.02. However, the final D_{DLS} and the PI_{SEM} of latex PS-GMA II were higher than expected, and for this reason it would not be suitable for IA purposes.

3.2. Production of latex-protein complexes

Main results of PA and CC experiments are shown in Figure 1. Figure 1a shows the amount of P22Ag or homogenate adsorbed onto the three PS latexes; and Figure 1b shows the surface densities of P22Ag covalently coupled onto the five carboxylated latexes PS-MAA I to V and the epoxylated latex PS-GMA I.

In PA experiments, the effect of particle size on the amount of adsorbed protein was considered. The adsorption experiments were carried out at $C^\circ = 0.6$ mg/mL and pH 6 (i.e., close to the IP of P22Ag). Under these conditions, intermolecular and intramolecular repulsions are diminished, thus maximizing protein adsorption (Figure 1a and Table 3).

Higher amounts of adsorbed protein were obtained for the recombinant protein than for the homogenate. As previously observed by Garcia et al. [34] when comparing the PA of various Ag of *Trypanosoma cruzi* (etiologic agent of Chagas disease), low amounts of adsorbed protein were obtained when the homogenate of the parasite was used, and this is probably because homogenate includes a complex mixture of largely undefined Ag of different MM, IP and affinity for the particles surface.

For the latexes PS I and PS II, the values of Γ_{P22Ag} and Γ_{Hom} were similar, despite the difference in particle sizes. However, when the latex of larger particle size PS III was employed, a

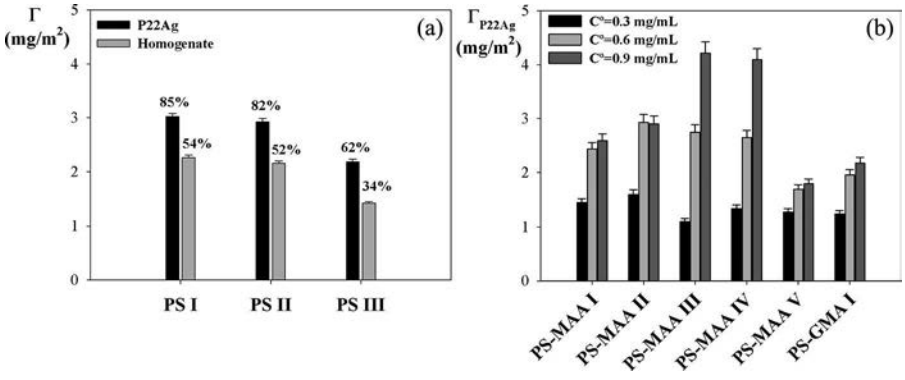

Figure 1. (a) Physical adsorption of P22Ag and homogenate of *T. gondii* onto PS I ($D_{DLS} = 134$ nm), PS II ($D_{DLS} = 300$ nm) and PS III ($D_{DLS} = 1043$ nm). The percentages on the bars indicate the fractions of adsorbed protein with respect to C°, and error bars indicate the standard deviations for three replicates; (b) Chemical coupling of P22Ag after desorption with Triton X-100 onto the latexes with carboxyl (PS-MAA) and epoxy (PS-GMA) groups at three concentrations of initial protein $C^\circ_{P22Ag} = 0.3$–0.9 mg/mL.

Table 3. Final characteristics of the LPC obtained by PA onto PS latexes at pH 6 (PS I-P22Ag, PS I-Homogenate, PS II-P22Ag, PS II-Homogenate, PS III-P22Ag, and PS III-Homogenate).

Antigenic protein	P22Ag			Homogenate		
Latex	PS I	PS II	PS III	PS I	PS II	PS III
Γ (mg/m^2)	3.0	2.9	1.9	2.3	2.2	1.1
D_{DLS} at 90° (nm)	322	463	1212	388	460	1254
-$\mu_e \times 10^8$ (m^2/V.s)[a]	2.6	3.1	2.1	2.3	3.2	2.0
-ζ (mV)[a]	36.5	42	28.5	29.2	43.5	27.8
c.c.c.$_{DLS}$ (mM KBr)[a]	100	200	200	200	400	200

[a]Determined at pH 8.

Table 5. Final characteristics of the LPC obtained by CC of the homogenate of *T. gondii* onto four carboxylated latexes at pH 6; effect of the protein density Γ_{Hom}.

	PS-MAA I-Hom	PS-MAA II-Hom	PS-MAA III-Hom	PS-MAA IV-Hom
Γ_{Hom} (mg/m^2)	1.3	1.3	1.6	1.2
% CC	58	57	62	72
D_{DLS} a 90° (nm)	456	442	412	291
-$\mu_e \times 10^8$ (m^2/V.s)[a]	2.6	2.4	2.4	2.6
-ζ (mV)[a]	36.0	32.7	32.0	35.5
c.c.c.$_{DLS}$ (mM KBr)[a]	500	>1000	>1000	300

[a]Determined at pH 8.

clear decrease in Γ was observed for both Ag. Notice that experiments were carried out by keeping fixed the total surface area of particles (0.2 m^2; i.e., the number of particles was different in each case). Thus, the smaller the number of particles, the lower the amount of adsorbed protein.

The CC of P22Ag onto the core-shell carboxylated particles PS-MAA I to V (Figure 1b) was carried out at pH 6 and increasing values of C°_{P22Ag} (0.3–0.9 mg/mL).The percentage of covalent coupled protein with respect to the total linked one is shown in the second row of Table 4.

The effect of both particle size and surface charge density was analyzed. It was observed that higher values of bound protein were achieved for latexes of smaller diameters (PS-MAA III and PS-MAA IV). For such latexes and $C^{\circ}_{P22Ag} = 0.9$ mg/mL, $\Gamma_{P22Ag,\ cov}$ were 4.26 mg/m^2, and 4.09 mg/m^2, respectively (Table 4). Also, the lowest $\Gamma_{P22Ag,\ cov}$ values were observed for the latexes exhibiting the largest particle diameter (PS-MAA V). Moreover, in the case of latexes PS-MAA I, II, and V a *plateau* in the amount of bound protein was observed. However, in the case of smaller latexes PS-MAA III and IV, $\Gamma_{P22Ag,\ cov}$ increased when increasing C°_{P22Ag}, without reaching the previously mentioned *plateau*. A possible explanation for this behavior is that proteins could be better distributed over smaller particles (less steric hindrance). Notice that, as in PA experiments, a lower amount of bound protein was observed for large particles, which involve a reduced number of particles.

With respect to the effect of surface charge density, when analyzing latexes with similar particle size and different δ_{COOH} (PS-MAA I vs. PS-MAA II and PS-MAA III vs. PS-MAA IV) it was observed that higher linked protein values were obtained for PS-MAA II and PS-MAA III for P22Ag. These results can be explained by the greater δ_{COOH} on such latexes that permit a greater linkage of proteins molecules.

Even though not shown, the fraction of covalently bound (f_c) protein with respect to the initially added protein for the 15 produced LPC from carboxylated particles was calculated. For latexes PS-MAA I, II and V, f_c importantly decreased when increasing C°_{P22Ag} (from 0.3 to 0.9 mg/mL). When the concentration of the added protein was low, a high percentage of protein was linked to the latex particles; and when increasing the added protein, higher Ag fractions remained in solution. This could be due to the saturation of the particle surface by the protein. In contrast, for latexes PS-MAA III and IV (of lower particle diameters), the particles surface was probably unsaturated and f_c remains practically constant despite increasing C°_{P22Ag}.

The CC of homogenate was carried out onto the core-shell carboxylated particles PS-MAA I to IV, at pH 6 and a protein concentration $C^{\circ}_{Hom} = 0.6$ mg/mL, and the results are shown in the upper part of Table 5. Notice that when the homogenate of *T. gondii* was used to sensitize the latex particles, lower values of covalently bound protein were obtained with respect to the recombinant Ag (compare Tables 4 and 5). Also, it was observed that after the desorption step with Triton-X100, the percentage of remaining protein to the total linked protein was greater for P22Ag than for the homogenate, with values above 79% for P22Ag (Table 4) and below 72% for the homogenate (Table 5). This could be due to the proteins heterogeneity present in the homogenate, which exhibit different MM, IP, and affinity for the particles surface.

The CC of P22Ag onto core-shell epoxylated particles was carried out at pH 7 and increasing values of C°_{P22Ag} (0.3–0.9 mg/mL) onto PS-GMA I and at $C^{\circ}_{P22Ag} = 0.6$ mg/mL onto PS-GMA II (Figure 1b and Table 6). According to Dornan and Mani [30] the reaction between a protein and the epoxy groups occurs at pH between 7 and 9. For this reason, CC experiments onto epoxylated latexes were carried out at pH 7, which is close to the IP of the protein. It can be seen that Γ_{P22Ag} increases with C°_{P22Ag} without reaching a *plateau*, thus indicating that the particles surface would not be saturated by proteins. Moreover, it was observed that Γ_{P22Ag} was similar for both epoxylated latexes when $C^{\circ}_{P22Ag} = 0.6$ mg/mL (columns

Table 4. Final characteristics of the LPC obtained by CC of P22Ag onto carboxylated latexes at pH 6; effect of the protein density Γ_{P22Ag}.

	PS-MAA I- P22Ag			PS-MAA II-P22Ag			PS-MAA III-P22Ag			PS-MAA IV-P22Ag			PS-MAA V-P22Ag		
Γ_{P22Ag}	1.5	2.4	2.6	1.6	2.9	2.9	1.1	2.7	4.2	1.3	2.6	4.1	1.3	1.7	1.8
% CC	97	85	88	98	97	88	83	93	96	81	92	95	97	85	79
D_{DLS} a 90°	538	534	606	401	421	485	280	329	425	199	311	322	1253	1281	1297
-$\mu_e \times 10^8$[a]	4.0	3.7	3.5	3.7	3.2	3.3	4.1	3.3	2.6	4.2	3.7	2.9	2.2	2.0	2.0
-ζ[a]	50.1	48.5	45	46.5	43	42.7	50.8	46	32.8	53.8	48.2	36.8	29.0	27.7	27.5
c.c.c.$_{DLS}$[a]	100	200	200	>1000	800	700	600	600	400	400	100	400	100	200	200

Units: Γ_{P22Ag} (mg/m^2), D_{DLS} (nm), -$\mu_e \times 10^8$ (m^2/V.s), -ζ (mV), c.c.c. (mM KBr).
[a]Determined at pH 8.

Table 6. Final characteristics of the LPC obtained by CC of P22Ag onto epoxylated latexes at pH 7; effect of the protein density Γ_{P22Ag}.

	PS-GMA I-P22Ag			PS-GMA II-P22Ag
Γ_{P22Ag} (mg/m^2)	1.0	2.0	2.2	2.1
% CC	84	88	87	90
D_{DLS} a 90° (nm)	366	387	398	599
$-\mu_e \times 10^8$ (m^2/V.s)[a]	3.0	2.7	2.6	2.4
-ζ (mV)[a]	40.3	34.5	33.9	31.2
$c.c.c._{DLS}$ (mM KBr)[a]	300	500	500	400

[a]Determined at pH 8.

3 and 5 of Table 6), indicating that the differences in δ_{C2H3O} were not enough to affect the coupling process. Also, the amount of bound protein Γ_{P22Ag} was lower than that obtained for equivalent carboxylated latexes, which could be due to the lower density of functional groups of PS-GMA I and PS-GMA II latexes, with respect to those of carboxylated ones (Tables 1 and 2).

3.3. Characterization of the latex-protein complexes

Main characterization results of the LPC obtained by PA and CC from the antigenic protein P22Ag and the homogenate of *T. gondii* are presented in Tables 3–6.

It must be noticed that the increment in D_{DLS} with respect to the unsensitized latexes (see values of Tables 1 and 2), mainly observed for greater Γ values, could not be only due to the protein linkage, but also to some polydispersity increment that importantly augment D_{DLS}, and that it is more likely to occur at higher protein concentrations.

Also, μ_e and ζ resulted drastically decreased when latexes were sensitized by PA and/or CC in all cases. Thus, the higher the amount of bound protein, the lower μ_e and **ζ** (Tables 3–6). These results are in agree with those reported by Ortega-Vinuesa et al. [35], where the μ_e of unsensitized carboxylated latexes were compared to those sensitized with IgG by PA and CC. These authors emphasized that when a polymer particle is covered by macromolecules, its surface becomes irregular, moving the position of the slipping plane away from the latex surface and causing a decrease in both μ_e and **ζ**.

Moreover, LPC stability was strongly affected by the characteristics of the unsensitized latexes. Thus, the LPC obtained from PS-MAA II and III (which exhibited greater δ_{COOH} and *h*) showed higher *c.c.c.* values.

Notice that LPC obtained by PA of the different Ag onto latexes PS I, PS II, and PS III (Table 3), and LPC produced by CC of P22Ag onto epoxylated latexes PS-GMA I and II (Table 6) showed *c.c.c.* values higher than those obtained for unsensitized latexes (Tables 1 and 2). The *c.c.c.* for LPC were measured at pH 8, which is higher than the IP of the employed proteins, which are negatively charged under such conditions. This possibly explains the increase in the *c.c.c.* values observed for LPC obtained from PS and epoxylated latexes with respect to the unsensitized ones. Similarly, Ortega-Vinuesa and Bastos-Gonzalez [36] reported a case where *c.c.c.* was determined at different pHs for LPC obtained by adsorption of BSA onto particles with sulfonate functionality. At pH 4.8, corresponding to BSA IP, the *c.c.c.* decreased when increasing the amount of adsorbed BSA. However, at pH 7, where the protein is negatively charged, the *c.c.c.* increased when increasing the amount of adsorbed BSA onto the particles surface, due to the contribution of the protein charges at this pH.

Furthermore, in LPC obtained from the carboxylated latexes PS-MAA I, II, III, and V (Table 4), the *c.c.c.* values were generally lower than those observed in the unsensitized latexes. In these cases, the electric charge of the complexes could be lower due to the carboxyl groups, which were primarily responsible for the electrical charge of the unsensitized latexes, decreased as a consequence of their covalently reaction with proteins.

Finally, the reported *c.c.c.* values indicate that complexes would remain stable under conditions at which IA test normally takes place.

3.4. Immunoagglutination assays from control sera

The LPC obtained from P22Ag and the homogenate of *T. gondii* by PA and CC were employed for IA assays. Figure 2 shows the ΔA produced after the addition of control sera (negative, acute or chronic) for the different LPC-P22Ag.

The best results in the sense of maximizing the discrimination of acute sera from negative and chronic ones were obtained with complexes PS-MAA II-P22Ag and PS-MAA

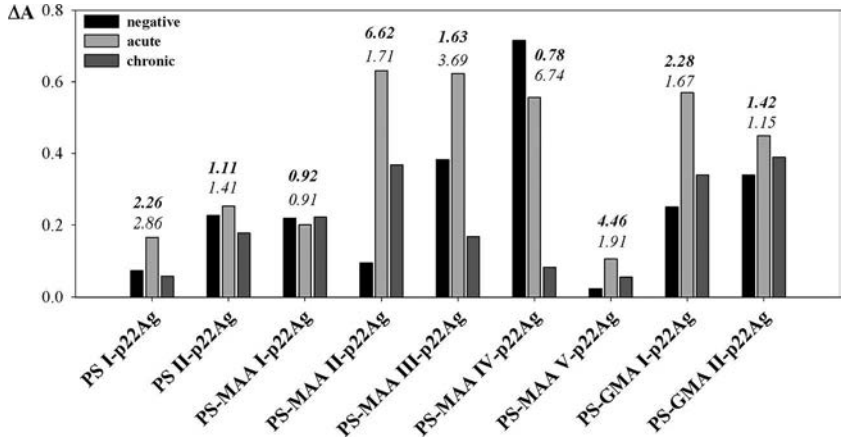

Figure 2. Results of the IA assay with control sera for nine different LPC obtained from CC and PA of P22Ag (Γ_{P22Ag} = 1.6–3.0 mg/mL). The absorbance change (ΔA) after mixing with negative, acute, and chronic sera are represented for the different LPC. Numbers on the bars indicate the ratio $\Delta A_{ac}/\Delta A_{neg}$(bold) and the ratio $\Delta A_{ac}/\Delta A_{ch}$ (nonbold).

III-P22Ag, where unsensitized latexes showed higher values of σ and *c.c.c.* The values for the ΔA ratios were $\Delta A_{ac}/\Delta A_{neg} = 6.62$ and $\Delta A_{ac}/\Delta A_{ch} = 1.71$ for the LPC PS-MAA II-P22Ag and $\Delta A_{ac}/\Delta A_{neg} = 1.63$ and $\Delta A_{ac}/\Delta A_{ch} = 3.69$ for the LPC PS-MAA III-P22Ag. Furthermore, PS-MAA II-P22Ag, produced from the latex with particle size of about 350 nm, provided better results than that obtained from the smaller one. Notice that PS-MAA III-P22Ag showed good discrimination between acute and chronic sera, although the negative serum showed a high ΔA, which could be due to nonspecific agglutination.

Although the ΔA ratios observed for PS-MAA V-P22Ag (obtained from the latex of larger particle size), showed good discrimination of acute sera, the signal of A resulted too low for all sera. This could be due to the smaller amount of bound protein to this latex (compared to other LPC) or because of the experimental conditions here tested were not particularly optimized for this particle size. Regarding the LPC derived from epoxylated latexes, only PS-GMA I-P22Ag achieved the discrimination of acute serum from negative and chronic ones.

Some complexes would be unsuitable as IA reagents due to the large ΔA value against negative serum (PS-MAA I-P22Ag, PS-MAA IV-P22Ag, and PS-GMA II-P22Ag). This could be explained by the low *c.c.c.* values for such LPC (Tables 3, 4, and 6) and/or due to possible serum interference. It should be noticed that when a LPC is mixed with a serum sample whose composition is very complex, nonspecific agglutination may occur that invalidate the test.

Finally and even though not shown, the behaviour observed for the LPC obtained from the homogenate of *T. gondii* was poorer than that produced from the recombinant protein. This was expected because the homogenate is a total parasite lysate that includes nonspecific Ag of the acute phase of the disease. Therefore, latex-homogenate complexes are not suitable reagents for IA assays according to the purposes of this study.

4. Conclusions

Carboxylated and epoxylated latexes were synthesized by seeded emulsion copolymerization of St and MAA or GMA, respectively, to obtain "core-shell" particles with different size (180–1150 nm), chemical functionality and charge densitiy. Their main characteristics (D_{DLS}, IP, *h*, σ, $\delta_{SO4}^{=}$, δ_{COOH}, δ_{C2H3O}, *c.c.c.*, μ_e, and ζ) showed that they may be useful for producing IA reagents.

Both PS and "core-shell" functionalized latexes were employed to produce LPC by PA or CC of the acute recombinant protein P22Ag and the homogenate of *T. gondii*.

In sensitization experiments Γ_{P22Ag} was higher than Γ_{Hom} and this could be due to the homogenate includes a complex mixture of largely undefined Ag of different MM and affinity for the latex particles surface.

The amount of bound protein onto latexes with surface carboxyl groups was higher than that obtained for the epoxylated latexes, which could be due to the lower density of functional groups of the epoxylated latexes with respect to the carboxylated ones.

The LPC stability was strongly affected by the characteristics of the unsensitized latexes. PS-MAA II-P22Ag and PS-MAA III-P22Ag were more stable than the other LPC because of the higher δ_{COOH} of their respective unsensitized latexes PS-MAA II and III. Measurements of *c.c.c.* were carried out at pH 8, where free carboxyl groups are ionized, improving LPC stability. It was also observed that the *c.c.c.* of LPC obtained from PS and epoxylated latexes was higher than that of unsensitized latexes. This is because at pH 8 the antigenic proteins present negative charge, thus increasing the repulsion between particles and the *c.c.c.* values.

Finally, IA assays against control sera were performed to determine which of the latex-P22Ag or latex-Hom complexes exhibited better results in the sense of discriminating acute sera from negative and chronic ones. It was found that PS-MAA II-P22Ag is a promising alternative to discriminate Ab generated in the acute phase of toxoplasmosis. The test is cheap, rapid, and easy to implement, but its results should be confirmed by reference techniques.

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