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Synthesis of latex-antigen complexes from single and multiepitope recombinant proteins. Application in immunoagglutination assays for the diagnosis of *Trypanosoma cruzi* infection

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ABSTRACT

The physical adsorption and the chemical coupling of recombinant proteins of *Trypanosoma cruzi* onto polystyrene and core-shell carboxylated particles were respectively investigated with the ultimate aim of producing latex-protein complexes to be used in an immunoagglutination assay able to detect the Chagas disease. To this effect, two single proteins (RP1 and RP5) and a multiepitope protein derived from three antigenic peptides (CP2) were evaluated, and sensitizations were carried out at different pHs. The maximum physical adsorption was produced at pHs close to the protein isoelectric point (i.e., pH 6 for RP5 and pH 5 for RP1 and CP2). High fractions of antigens were chemically bound to the carboxyl groups, and the highest surface density of linked protein was also observed at pHs close to the protein isoelectric point. The three latex-protein complexes obtained by covalent coupling at such pHs were tested with sera from a panel of 16 infected and 16 non-infected patients. In the immunoagglutination assays, the latex-CP2 complex produced the best discrimination between positive and negative sera.

1. Introduction

Polymer colloids are useful for developing diagnostic reagents that are often used in the clinical evaluation of a great variety of analytes. In particular, latex-protein complexes are widely used for the macroscopic detection of antigen-antibody reactions. Main advantages of this method are its rapidness, simplicity, and convenient determination by direct visualization or by instrumental methods [1–10].

Latex-protein complexes can be produced by physically or chemically coupling the proteins to the surface of the polymer particles. The latex-protein complexes obtained by physical adsorption have a limited applicability in immunodiagnosis due to the partial desorption suffered by the proteins, as well as to their reduced immunological capability originated by structural rearrangements. In contrast, the covalent coupling of proteins to the surface functional groups of the polymer particles provides improved properties to the immunoagglutination complex (e.g., prevents the desorption of the proteins and maintains their native conformation) [2,11].

Carboxylated polystyrene (PS) latexes have been the basis of several immunoassay kits [12–17]. Ortega-Vinuesa et al. [12] synthesized latex-protein complexes by chemical and physical coupling of anti-C reactive protein polyclonal IgG onto carboxylated latexes, and observed a better immunological response of the chemically-coupled complexes. Lee et al. [13] investigated the chemical coupling of bovine serum albumin (BSA) and anti-human IgG onto a core-shell carboxylated latex and verified that the sensitivity of the agglutination test depends on the temperature and the mass of the bound antigen. Menshikova et al. [14] investigated the physical and the chemical coupling of BSA onto a carboxylated latex of polystyrene-*graft*-polyvinylpyrrolidone (PS-*g*-PVP). The pyrrolidone groups present on the external surface stabilized the particles and interacted with BSA. High levels of chemical binding and physical adsorption onto the surface of the PS-*g*-PVP particles were obtained [14].

The chemical linkage of proteins is strongly affected by the medium (ionic strength, pH and buffer) [18]. In principle, the amine groups of proteins and peptides can directly be coupled onto the carboxyl groups at the polymer particles surface. Since such reactions are too slow, it is convenient to transform the

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carboxyl groups into the faster acylureas by addition of an activator such as the water-soluble N-N-(3-dimethylamine propyl) N′-ethyl carbodiimide (EDC) [12].

The Chagas disease is caused by infection with the parasite *Trypanosoma cruzi*, and it is mostly diagnosed when specific antibodies against *T. cruzi* antigens are detected in patiens blood. Total homogenate of the parasite at the epimastigote stage provides antigens for serological tests, that exhibit an appropriate sensitivity to detect low antibodies levels. However, the homogenate includes a complex mixture of largely undefined antigens; and therefore not only specificity problems appear but also difficulties to standardize a rigorous testing methodology [19–21]. The current trend is the use of recombinant proteins as sensitizing elements. Typically, the proteins can be synthesized from DNA sequences engineered to encode peptide fragments where the specific regions responsible of cross-reactivity have been excised [19,22–24]. Through this method, large amounts of proteins can be obtained in a highly purified form. Due to the low sensitivity observed when single recombinant peptides are used to detect antibodies, several authors have suggested the use of a peptide mixture, which would reach a sensitivity similar to the sum of the contributions from the individual antigens [25–29]. Alternatively, chimeric or multiepitope proteins able to express several antigenic determinants have also been proposed to enhance sensitivity [22,30,31].

Our previous works [15–17] aimed at synthesizing and sensitizing carboxylated latexes for producing latex-antigenic protein complexes to be employed in immunoagglutination tests for the detection of Chagas disease. Core-shell latexes with external carboxyl groups were first synthesized through batch or semibatch emulsion copolymerizations of styrene and methacrylic acid onto monodisperse PS seeds [15]. Then, the single recombinant protein Ag36 of *T. cruzi* was coupled onto two base carboxylated latexes of similar particle diameters and different charge densities [16]. The final density of covalently-bound Ag36 protein was 2.44 mg/m^2, around 80% of the total linked Ag36 was chemically-coupled, and the maximum chemical coupling was observed at pH 5 (i.e., close to the protein isoelectric point: Ip). The PS and core-shell carboxylated latexes were respectively sensitized by physical adsorption and by covalent coupling, with three different proteins of *T. cruzi*: a homogenate of *T. cruzi*, and two recombinant proteins (Ag36 and CP1) [17]. With the homogenate, 30–60% of the total linked protein was chemically-coupled. With the recombinant proteins, around 90% of the linked proteins were chemically coupled, with a maximum coupling at pH 5 (i.e., close to the Ip). The latex-protein complexes produced in [16,17] were not tested in immunoagglutination assays.

Only other two publications have investigated the development of immunoagglutination latexes for the detection of Chagas disease [32,33]. In both cases, the complexes were obtained by physical adsorption of homogenates of *T. cruzi* onto PS latex particles, with the above-described problems associated to the employment of total homogenate. Again, the use of recombinant proteins as sensitizing elements would be desirable to improve specificity and standardization of the diagnostic reagent.

In this work, PS and core-shell carboxylated latexes are sensitized by physical adsorption and covalent coupling of three different recombinant proteins of *T. cruzi* (two single and one multiepitope, with the aim of producing immunodiagnosis kits for detecting the Chagas disease. The produced latex-protein complexes are then tested with sera from a panel of 16 infected and 16 non-infected different patients, to assess whether the complexes are capable of discriminating between positive and negative sera. The immunoagglutination reaction was monitored by measuring absorbance changes (at 570 nm).

Table 1
Characteristics of the employed base latexes.

	PS latex	Carboxylated latex (C2)
Particle morphology	Homogeneous	Core-shell[a]
$\bar{D}_{DLS}$[b] (nm) by DLS at 90°	300	418
PDI by SEM	1.06	1.05
s (nm) by DLS at 90°	–	35
h (nm) by DLS at 90°	4	11
σ[c] (μC/cm^2)	14.9	78.4
$\delta_{SO_4^=}$[c] (mEq/cm^2) $\times 10^7$	1.54	1.76
δ_{COOH}[c] (mEq/cm^2) $\times 10^7$	–	7.95
c.c.c.[d] (mM KBr) by Visual Observation	250	450
c.c.c.[d] (mM KBr) by DLS at 90°	75	200

[a] PS core/PS-MAA shell.
[b] Average particle diameter.
[c] Measured by conductimetric titration.
[d] Measured at pH 6.

2. Experimental

2.1. Materials and methods

Two base latexes were employed (see Table 1). The uniform PS latex was synthesized through an unseeded emulsifier-free emulsion polymerization of styrene (St), according to the recipe and the procedure described in [15]. The C2 carboxylated latex was synthesized through a semibatch copolymerization of St and methacrylic acid (MAA) onto a uniform PS latex seed, thus producing particles with a core-shell morphology. Most of the polymer molecules contained sulfate groups at their chain ends, corresponding to remnants of the persulfate initiator that was employed for the latex syntheses. After the end of polymerizations, the unreacted comonomers and initiator were eliminated by serum replacement. The average particle diameter and the polydispersity index (PDI) of the PSD were determined by dynamic light scattering (DLS) at 90° and scanning electron microscopy (SEM), respectively. The shell-thickness (s) of latex C2 particles and the hairy-layer thickness (h) were also determined by DLS at 90°. The total-charge surface density (σ), and the surface density of functional groups ($\delta_{SO_4^=}$ and δ_{COOH}) were determined by conductimetric titration. The critical coagulation concentration (*c.c.c.*) was determined at pH 6, by direct visual observation and by DLS at 90°.

The employed recombinant antigens of *T. cruzi* were the two single proteins RP1 and RP5 and the multiepitope protein CP2, built up as the tandem expression of 3 highly antigenic peptides (RP1, RP2 and RP5) [34]. The proteins were purified by nickel affinity chromatography; and their purities were determined by 15% polyacrylamide gel electrophoresis (PAGE), stained with Coomassie brilliant blue. The main characteristics of the proteins are presented in Table 2.

The following buffers were used. The 0.1 M glycine buffer (pH 3) contained $C_2H_5NO_2$ (Sigma) and HCl. The 0.1 M acetate buffer (pH 5) contained $CH_3COONa.3H_2O$ (Cicarelli) and CH_3COOH (Anedra). The 0.1 M phosphate buffer (pH 6 and pH 7) contained NaH_2PO_4 (Cicarelli) and Na_2HPO_4 (Cicarelli). The 0.002 M borate buffer (pH 8) contained $Na_2B_4O_7{\cdot}10H_2O$ (Anedra) and HCl. The

Table 2
Characteristics of the employed recombinant proteins.

	RP1	RP5	CP2
Number of epitopes	1	1	3
Molar mass (kDa)[a]	27.9	26.2	40.8
Isoelectric point: Ip	5.4[a]/6.2[b]	6.1[a]/6.3[b]	5.4[a]/5.8[b]

[a] Calculated through ExPasy Program (ExPasy Proteomic Service, http://www.expasy.org/tools/protparam.html).
[b] Measured by isoelectric focusing.

Table 3
Physical adsorption of proteins onto PS latex particles: recipes and surface densities. (Experimental results are reported as mean value ± standard deviation for $n = 2$.)

		Sample			
		Blank	RP1	RP5	CP2
	Common Recipe				
	PS Latex (mL)	0.500[a]	0.500[a]	0.290[b]	0.500[a]
	Buffer sol.[c] (mL)	0.030	0.030	0.020	0.030
	H_2O (mL)	0.870	0.594	0.248	0.608
	Prot. sol. (mL)	–	0.276[d]	0.442[e]	0.262[f]
	C^o_{Prot}[g] (mg/mL)	–	0.9	0.8	0.9
Experiment					
A (pH 3)	$\Gamma_{Prot,ad}$[h] (mg/m^2)	–	5.27 ± 0.13	2.56 ± 0.06	2.20 ± 0.05
B (pH 5)	$\Gamma_{Prot,ad}$[h] (mg/m^2)	–	5.40 ± 0.14	–	3.95 ± 0.10
C (pH 6)	$\Gamma_{Prot,ad}$[h] (mg/m^2)	–	–	3.45 ± 0.09	–
D (pH 7)	$\Gamma_{Prot,ad}$[h] (mg/m^2)	–	5.16 ± 0.13	–	3.15 ± 0.08
E (pH 9)	$\Gamma_{Prot,ad}$[h] (mg/m^2)	–	5.35 ± 0.13	3.27 ± 0.08	3.00 ± 0.08

[a] Or total surface area = 0.20 m^2.
[b] Or total surface area = 0.15 m^2.
[c] Containing a 0.1 M solution of the appropriate buffer.
[d] 4.56 mg/mL.
[e] 1.81 mg/mL.
[f] 4.8 mg/mL.
[g] Initial protein concentration.
[h] Amount of adsorbed protein per unit area of particle surface (Prot = RP1, RP5 or CP2).

0.1 M carbonate buffer (pH 9) contained Na_2CO_3 (Cicarelli) and $NaHCO_3$ (Cicarelli). A 15 mg/mL water solution of EDC (Fluka) was prepared shortly before its use. The emulsifiers were 4-(1,1,3,3-tetramethylbutyl)phenyl-polyethylene glycol (Triton X-100) from Sigma and sodium dodecyl sulfate (SDS) from Cicarelli.

The concentrations of dissolved protein were determined through the copper reduction/bicinchoninic acid (BCA) method [12]. In this method, the protein reduces copper (II) into copper (I) in alkaline conditions, and copper (I) generates a (soluble and intensely-colored) complex with BCA. The BCA was from Pierce Reagents (US).

Serum samples from *T. cruzi*-infected patients were obtained from the Regional Hospital of Reconquista (Santa Fe, Argentina). The *T. cruzi* infection status of the patients was determined by using two different conventional tests, namely enzyme-linked immunosorbent assay (Chagatest ELISA) and indirect hemagglutination (Chagatest HAI), both from Wiener Lab (Argentina). Negative serum samples were obtained from non-infected blood donors from the same hospital. A pool of 16 positive and 16 negative sera was selected. Large volumes of a "positive control serum" (i.e., with a high antibody titer able to produce a strong immunoassay response) as well as a "negative control serum" were also available.

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

2.2. Physical adsorption

The passive adsorption experiments on PS particles were carried out as follows (see Table 3): (1) antigenic proteins were added to the latex samples in 1.5 mL microcentrifuge tubes; (2) the mixtures were gently shaken during 5 h at room temperature; (3) the latex-protein complexes were isolated from the solution by ultracentrifugation during 30 min at 10,000 rpm (or 7200 *g*); (4) the concentration of dissolved protein was quantified by the BCA method; and (5) the latex-protein complexes were redispersed in the borate buffer (pH 8). The amount of adsorbed protein was determined from the difference between the total added protein $\left(C^o_{Prot}\right)$ and the protein remaining in solution ($C_{Prot,\,sol}$). The buffers used in the adsorption were glycine at pH 3, acetate at pH 5, phosphate at pH 6 and pH 7, and carbonate at pH 9 (Experiments A–D). The

Table 4
Covalent coupling of the single RP1 onto carboxylated latex particles: recipes and surface densities. (Experimental results are reported as mean value ± standard deviation for $n = 2$.)

		Sample No.			
		1	2	3	4
	Recipe[a]				
	Carboxylated Latex (mL)	0.580[b]	0.580[b]	0.580[b]	0.580[b]
	EDC sol.[c] (mL)	0.110	0.110	0.110	0.110
	Buffer sol.[d] (mL)	0.030	0.030	0.030	0.030
	H_2O (mL)	0.680	0.512	0.344	0.176
	RP1 sol.[e] (mL)	–	0.168	0.336	0.504
	C^o_{RP1} (mg/mL)	–	0.3	0.6	0.9
Experiment					
1 (pH 5)	$\Gamma_{RP1,\,cov}$[f] (mg/m^2)	–	2.10 ± 0.12	2.96 ± 0.16	3.77 ± 0.21
2 (pH 7)	$\Gamma_{RP1,\,cov}$[f] (mg/m^2)	–	1.70 ± 0.09	2.52 ± 0.14	3.27 ± 0.18

[a] For the desorption process, 1.4 mL of Triton X-100 (1%) were used.
[b] Or total surface area = 0.2 m^2.
[c] 15 mg/mL.
[d] Containing a 0.1 M solution of the phosphate buffer.
[e] 2.5 mg/mL.
[f] Amount of covalently-bound RP1 per unit area of particle surface.

Table 5
Covalent coupling of the single RP5 onto carboxylated latex particles: recipes and surface densities. (Experimental results are reported as mean value ± standard deviation for $n = 2$.)

		Sample No.			
		1	2	3	4
	Recipe[a]				
	Carboxylated Latex (mL)	0.390[b]	0.390[b]	0.390[b]	0.390[b]
	EDC sol.[c] (mL)	0.082	0.082	0.082	0.082
	Buffer sol.[d] (mL)	0.020	0.020	0.020	0.020
	H_2O (mL)	0.508	0.342	0.177	0.011
	RP5 sol.[e] (mL)	–	0.166	0.331	0.497
	C^o_{RP5} (mg/mL)	–	0.3	0.6	0.9
Experiment					
3 (pH 5)	$\Gamma_{RP5,\,cov}$[f] (mg/m^2)	–	1.75 ± 0.10	2.74 ± 0.15	3.47 ± 0.19
4 (pH 7)	$\Gamma_{RP5,\,cov}$[f] (mg/m^2)	–	1.36 ± 0.07	2.87 ± 0.14	3.62 ± 0.20

[a] For the desorption process, 1.4 mL of Triton X-100 (1%) were used.
[b] Total surface area = 0.12 m^2.
[c] 15 mg/mL.
[d] Containing a 0.1 M solution of the phosphate buffer.
[e] 1.81 mg/mL.
[f] Amount of covalently-bound RP5 per unit area of particle surface.

final ionic strength was 0.002 M. For each experiment, a blank of buffer without protein was prepared.

2.3. Covalent coupling

The covalent coupling experiments were carried out as follows: (1) antigenic proteins of increasing concentrations were added to the C2 carboxylated latex samples in the presence of EDC activator and in microcentrifuge tubes of 1.5 mL; (2) the mixtures were gently shaken during 5 h at room temperature; (3) the latex-protein complexes were isolated from the solution by ultracentrifugation during 30 min at 10,000 rpm (or 7200 *g*); and (4) the protein remaining in solution was quantified via the BCA method. The total linked protein (i.e., both physically-adsorbed and covalently-bound) was determined from the difference between the total added protein and that remaining in solution. The buffer used in the chemical coupling was phosphate at pH 5 and pH 7. The final ionic strength was 0.002 M. For each experiment, a blank of buffer without protein was prepared.

The carboxyl activation and the covalent coupling reactions were carried out in parallel to minimize hydrolysis of the acylurea intermediate. To these effects, the protein was simply mixed with the carboxylated latex and the EDC activator. The concentration of EDC was 100-fold greater than the concentration of carboxyl groups in order to ensure complete transformation of all carboxyl groups into acylurea, and to produce a recommended surface density of 10 mg EDC/m^2 latex [35].

After reactions, the physically adsorbed protein was desorbed by employing Triton X-100 emulsifier, as follows: (a) the latex-protein complexes were redispersed in 1.4 mL of emulsifier solution at 1%; (b) the mixtures were left for 24 h under gentle agitation at room temperature; (c) the dispersions were centrifuged; (d) the latex-protein complexes were redispersed in borate buffer (pH 8); and (e) the concentration of desorbed protein was determined by the BCA method. The surface density of covalently-bound protein ($\Gamma_{RP1,\,cov}$, $\Gamma_{RP5,\,cov}$, and $\Gamma_{CP2,\,cov}$) was calculated on the basis of the total-linked protein (Γ_{Prot}) and the desorbed protein that is present in solution ($C_{Prot,\,sol}$). Finally, to analyze the effect of the emulsifier on the desorption process SDS was employed instead of Triton X-100 for the experiment with CP2.

Experiments 1 and 2 involved the single protein RP1 at 2 different pHs (Table 4). Experiments 3 and 4 involved the single protein RP5 at 2 pHs (Table 5). Experiments 5 and 6 involved the multiepitope protein CP2 at 2 pHs (Table 6).

2.4. Immunoagglutination assays

The latex-protein complexes were conditioned before the agglutination test. To this effect, complexes were first centrifuged and then redispersed in a (low ionic strength) saline solution of bovine serum albumin (BSA) at pH 8, and in the presence of a blocking agent (B). The BSA is an inert protein that was added with the aim of being adsorbed onto the hydrophobic surface zones of the latex-protein particles, thus avoiding unspecific reactions. The blocking agent B was added with the aim of being linked to the free carboxyl groups present on the particles surface, thus reducing the reactions with such groups that could give non-specific agglutinations during the immunoassays.

To carry out the immunoassays, 950 μL of the dispersion containing the investigated latex-protein complex (at a known concentration) were mixed with 50 μL of serum. Turbidimetry was used to detect the agglutination reaction by 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 absorbance of a blank (the complex without serum) to the absorbance measured for the (complex + serum) sample. The "colloidal stability" of the complex, 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".

During the immunoassay, the values the ΔA were used to build the ROC (receiver operating characteristic) curve through the graphic software MedCald. A ROC curve is a graphical plot of the sensitivity vs (100 − specificity), for a binary classifier system (e.g., "true positive" or "false positive") as its discrimination threshold (e.g., the cut-off) is varied. The ROC curve has been extensively used for medical test evaluations and allows the estimation of the cut-off, the sensitivity and the specificity of an immunoassay [36,37]. From the resulting cut-off value, the relative optical distribution (ΔA/cut-off) was plotted by means of a scatter computer graphic software (Graf Pad Prism, Version 2.00).

3. Results and discussion

3.1. Physical adsorption

For the employed recombinant proteins, the resulting surface densities of antigenic proteins adsorbed onto the particles ($\Gamma_{RP1,ad}$, $\Gamma_{RP5,ad}$ and $\Gamma_{CP2,ad}$) are presented in Table 3. In all cases, the

Table 6
Covalent coupling of the multiepitopoe CP2 onto carboxylated latex particles: recipes and surface densities. (Experimental results are reported as mean value ± standard deviation for $n = 2$.)

		Sample No.			
		1	2	3	4
	Recipe[a]				
	Carboxylated Latex (mL)	0.580[b]	0.580[b]	0.580[b]	0.580[b]
	EDC sol.[c] (mL)	0.110	0.110	0.110	0.110
	Buffer sol.[d] (mL)	0.030	0.030	0.030	0.030
	H_2O (mL)	0.680	0.593	0.505	0.418
	CP2 sol.[e] (mL)	–	0.087	0.175	0.262
	C^o_{CP2} (mg/mL)	–	0.3	0.6	0.9
Experiment					
5 (pH 5)	$\Gamma_{CP2,\,cov}$[f] (mg/m^2)	–	1.78 ± 0.10	2.81 ± 0.15	3.27 ± 0.18
6 (pH 7)	$\Gamma_{CP2,\,cov}$[f] (mg/m^2)	–	1.50 ± 0.08	1.98 ± 0.11	2.65 ± 0.15

[a] For the desorption process, 1.4 mL of Triton X-100 (1%) were used.
[b] Or total surface area = 0.2 m^2.
[c] 15 mg/mL.
[d] Containing a 0.1 M solution of the phosphate buffer.
[e] 4.8 mg/mL.
[f] Amount of covalently-bound CP2 per unit area of particle surface.

maximum adsorption was observed at pH close to the Ip (pH 6 for RP5 and pH 5 for RP1 and CP2).

The observed behavior for RP5 and CP2 proteins can be explained as follows. Close to the Ip, the protein has a zero net charge, both the intra- and intermolecular repulsions decrease, and a maximum amount of adsorbed protein would be expected. At pH values different from the Ip, the protein becomes charged and its electrostatic charge may affect the adsorption process. At pH 3, the protein has a predominantly positive charge, which increases the intermolecular repulsions. Moreover, the aminoacid present in the reaction buffer (glycine) could interfere in the adsorption process by competition with the protein, yielding a low amount of adsorbed protein. In contrast, at pH 9 the protein becomes negatively charged, thus increasing the electrostatic repulsions between the protein molecules, and between the protein and the particle surface, which reduce the protein adsorption.

Only slight changes in the amount of adsorbed protein with pH were observed for RP1 (Table 3). According to a theoretical study [38], this could be related to its three-dimensional structure in solution, which presents a pattern of three alpha-helices connected by two loops, with interacting charges groups within the molecule. From such study, the outside of RP1 is practically neutral and therefore is unaffected by changes in the pH. This result is consistent with the Ip values reported in Table 2. In fact, the experimental Ip obtained by isoelectric focusing for RP1 is greater than its theoretical value. This would suggest that the charged groups might interact within the molecule, and therefore fewer protons are needed to neutralize the negative charge of the protein. In contrast, for CP2 and RP5 the experimental and theoretical Ip are more similar, and therefore the negative charge of the molecules would be more exposed than in RP1. Consequently, CP2 and RP5 exhibit a higher variation in their net charge with pH, and therefore the amount of adsorbed protein is more affected by pH changes.

In summary, the highest adsorptions occurred at the Ip values via a hydrophobic interaction mechanism, as justified by reduced intra- and intermolecular electrostatic forces [39,40], which increases the stability of the adsorbed protein and avoids the protein denaturation [41,42].

3.2. Covalent coupling

The results corresponding to the covalent coupling of recombinant proteins RP1, RP5 and CP2, are presented in Tables 4 (Exps 1 and 2), 5 (Exps 3 and 4) and 6 (Exps 5 and 6), respectively. In Fig. 1, the surface densities of total-linked and chemically-coupled protein are represented as a function of the initial protein concentration $\left(C^o_{Prot}\right)$. Both the total-linked and the chemically-coupled protein increase with C^o_{Prot}. The highest density of covalently-linked protein occurred at pH 5 for RP1 and CP2, and at pH 7 for RP5 (close to their corresponding Ip). The covalently-bound protein represents more than 90% of the total-linked protein [3.77 ± 0.21 mg/m^2 (90%) for RP1 at pH 5, 3.62 ± 0.20 mg/m^2 (92%) for RP5 at pH 7, and 3.27 ± 0.18 mg/m^2 (92%) for CP2 at pH 5]. Notice that, as in the physical adsorption experiments, the greatest variation in the amount of bound protein with pH was observed for CP2. Even though not shown for space reasons, similar covalently-bound results were observed (at pH 5) when the final redisolution operations (to induce protein desorption) were carried out with the anionic emulsifier SDS instead of the non-ionic Triton X-100 (91% covalently-linked CP2 with SDS and 92% covalently-linked CP2 with Triton X-100).

For the three recombinant proteins, relatively high fractions of chemically-bound proteins were observed (see Fig. 1). The amount of covalently-linked protein obtained at pH 5 was higher for the single recombinant proteins (RP1 and RP5) than for the multiepitope recombinant protein (CP2). This may be due to the smaller sizes of the single proteins (27.9 kDa for RP1 and 26.2 kDa for RP5) with respect to the multiepitope protein (40.8 kDa), which enable their better distribution and arrangement on the particles surface. In addition, the high levels of covalently bound proteins observed in all cases turn unnecessary the desorption process, thus decreasing the risk of protein denaturation (and loss of biological activity) caused by the use of the emulsifier.

In contrast, at pH 7 no trend was observed. When the medium pH is greater than the protein Ip, all proteins become negatively charged, increasing the electrostatic repulsions between the protein molecules, and between the proteins and the particle surface, and therefore the covalent coupling becomes more difficult. In general, when environmental conditions hinder the approach of proteins to the particle surface, the covalent coupling occurs in a lesser extent [43].

In all cases, the amount of total-linked protein increases with the concentration of the added protein, exhibiting a low dependence with pH, and reaching the highest values at the Ip. Nevertheless, the fraction of total linked (f_T) and covalently-bound (f_C) protein with respect to the added protein both decreased when increasing C^o_{Prot} (from 0.3 to 0.9 mg/mL), as it is shown in Fig. 2. Notice that, when the concentration of the added protein was low, a high percentage of protein was linked to the latex particles; and when the added protein concentration was increased, a higher protein

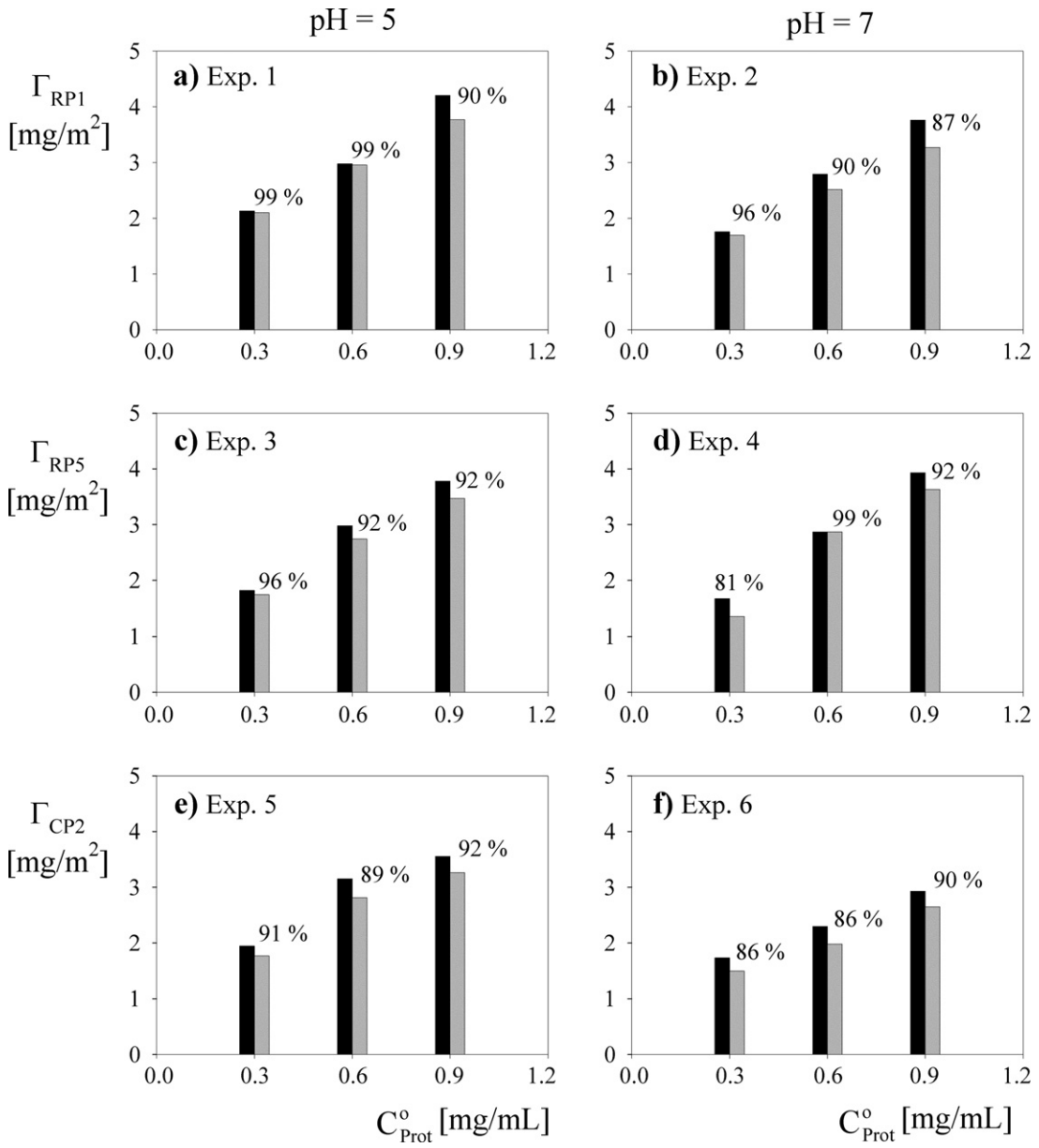

Fig. 1. Exps 1–6: chemical coupling of RP1 (a and b), RP5 (c and d), and CP2 (e and f) onto the carboxylated latex particles at pH 5 (a,c,e) and pH 7 (b,d,f) vs. the initial protein concentration. The surface density of total linked protein (black bar) is compared with the covalently-linked protein (grey bar), after a desorption operation with Triton X-100. The percentages indicate the fractions of covalently-bond protein.

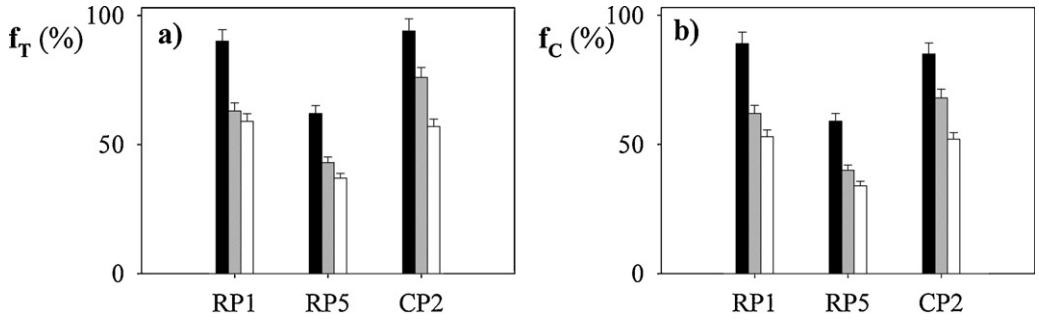

Fig. 2. Percentages of total linked protein (a) and covalently-coupled protein (b), with respect to the initially added protein, for the different latex-protein complexes: $C^o_{Prot} = 0.3$ mg/mL (black bar), $C^o_{Prot} = 0.6$ mg/mL (grey bar), and $C^o_{Prot} = 0.9$ mg/mL (white bar). (Error bars indicate the standard deviations for $n = 3$.)

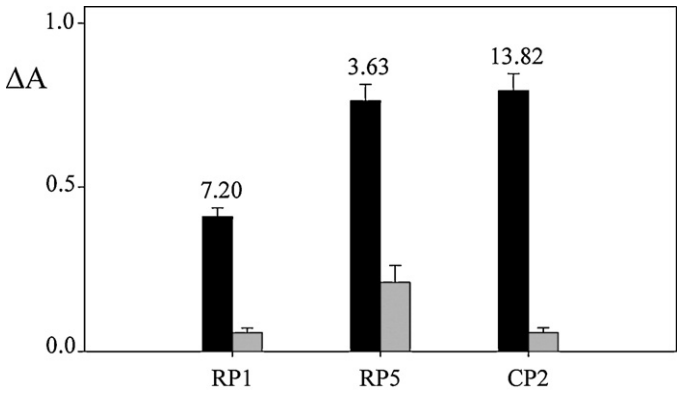

Fig. 3. Results of the immunoagglutination assay with control sera. Absorbance change of the different latex-protein complexes, after mixing with serum from infected patients (black bar) and serum from non-infected patients (grey bar). (Numbers above the black bars indicate the ratio between positive and negative sera $\Delta A(+)/\Delta A(-)$, and error bars indicate the standard deviations for $n = 3$.).

fraction remained in solution. This could be due to the saturation of the particle surface by the protein.

3.3. Immunoagglutination assays

The latex-protein complexes obtained by covalent coupling at pH 5 (Tables 4–6; Exps 1, 3, and 5; Sample No. 3) were employed for the immunoassays. Fig. 3 shows the absorbance change (ΔA) of the three latex-protein complexes, produced after the addition of control sera from infected and non-infected patients. Even though some changes in A are produced in the presence of serum from non-infected patients, higher ΔA values are observed when the latex-protein complex is mixed with serum from infected patients, thus showing an adequate behavior for the detection of Chagas disease. When analyzing the behavior of a chimera vs. single recombinant proteins, for a similar concentration of bound protein, the CP2 discrimination efficiency between positive and negative control sera was remarkably higher ($\Delta A(+)/\Delta A(-) = 13.82$) than those of the RP1 and RP5 ($\Delta A(+)/\Delta A(-) = 7.20$ and $\Delta A(+)/\Delta A(-) = 3.63$, respectively). This last result is attributed to the fact that CP2 is a chimera that includes three antigenic determinants in the structure of the combined protein, thus allowing an increase in the assay sensitivity.

When the anionic emulsifier SDS was used instead of Triton X-100 for the protein desorption process, similar amount of covalent bound proteins were obtained. However, the SDS acts by breaking non-covalent bonds in proteins, thus causing the loose of their native conformation. This is because SDS binds to nonpolar regions of the polypeptide providing a negative charge and inducing electrostatic repulsions. Due to protein denaturation, the complexes obtained by using SDS are unable to differentiate between sera from infected and non-infected patients, and for this reason they are not suitable for application in immunoagglutination tests [44].

Finally, the ability of the C2-CP2 complex for detecting *T. cruzi* infection was tested on the basis of a panel of 16 negative sera and 16 positive sera previously typified (Table 6; Exp 5; Sample No. 3), and its performance was evaluated through a ROC curve. The sensitivity and specificity for the whole sera panel were 92% and 84%, respectively (Fig. 4a). The mean value of ΔA for positive sera was greater than the cut-off value (mean ± standard deviation = 1.709 ± 0.771; $n = 16$), and most positive serum samples showed a noticeable reactivity (Fig. 4b). Also, most of the negative serum samples rendered ΔA values lower than the cut-off (mean ± standard deviation = 0.685 ± 0.345; $n = 16$). These results show that the C2-CP2 complex allows a good efficiency for discriminating between positive and negative sera, and therefore it is adequate for the diagnosis of the Chagas disease.

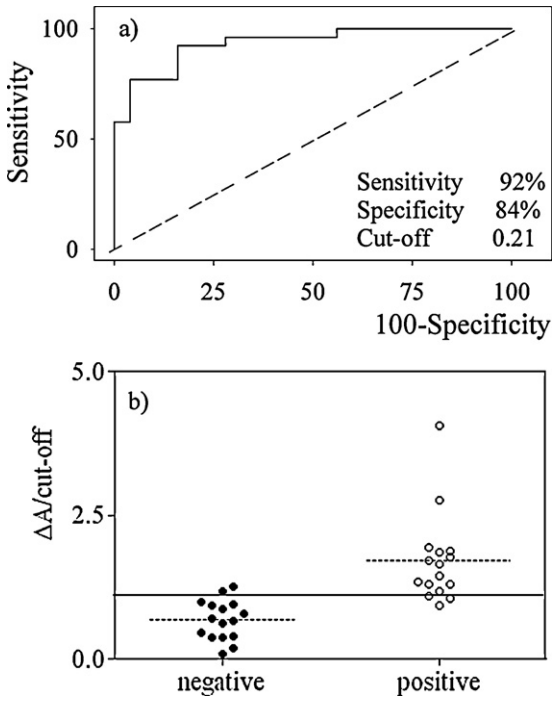

Fig. 4. Results of the immunoagglutination assay for the C2-CP2 complex, with a panel of 16 positive and 16 negative sera. (a) ROC curve; (b) relative optical distribution (ΔA/cut-off). The dashed lines show the relative mean values for each assay and the continuous line indicates the relative cut-off value (ΔA/cut-off = 1).

4. Conclusions

Three recombinant proteins (two single: RP1 and RP5, and one multiepitope: CP2) were used to synthesize latex-protein complexes through physical adsorption onto PS particles and chemical coupling onto core-shell carboxylated particles. Then, they were tested with sera from infected and non-infected patients. The following was observed:

(1) For the three recombinant proteins, the maximum physical adsorption onto the PS latex particles was produced close to the protein Ip. For RP1, a lower variation in the amount of the adsorbed protein with pH was observed, which could be attributed to its conformation in solution where most of the negative charge of the protein is not exposed to the medium.
(2) With the employed recombinant antigens, relatively high fractions of chemically-bound proteins were observed. With the low molar mass RP1 and RP5, the fraction of chemically-bound protein with respect to the total linked protein at pH close to the Ip was 99% for $C^o_{Prot} = 0.6$ mg/mL. With the larger CP2, the fraction of chemically-bound protein was 89%, for $C^o_{Prot} = 0.6$ mg/mL. The high fractions of chemically-bound proteins may render unnecessary the final operation of physical desorption previous to the complexes employment in immunoagglutination assays. The highest surface densities were observed at pH values close to the proteins Ip. Also, the fraction of total-linked and covalently-bound protein (with respect to the initially added protein) decreased with the amount of added protein, probably due to the saturation of the particle surface.
(3) The three different latex-protein complexes produced by covalent coupling allowed an adequate discrimination between

infected and non-infected sera, but the latex-CP2 complex clearly showed the highest sensitivity. This is because CP2 is a multiepitope protein able to be recognized by a greater number of antibodies present in the sera from patients infected with either acute or chronic stages of the disease. When the C2-CP2 complex was tested with a panel of 16 positive and 16 negative sera, sensitivity and specificity values of 92% and 84% were respectively obtained.

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