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Latex of immunodiagnosis for detecting the Chagas disease: II. Chemical coupling of antigen Ag36 onto carboxylated latexes

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Abstract A novel immunodiagnosis reagent for detecting the Chagas Disease was developed, by chemical coupling of antigen Ag36 of *Trypanosoma cruzi* onto two (carboxylated and core-shell) latexes. The coupling reactions involved the use of a carbodiimide intermediate. Bovine serum albumin (BSA) was used as a model protein for determining the appropriate conditions for its physical and chemical coupling. BSA showed an increased adsorption onto the base carboxylated latexes, with respect to a PS latex without carboxyl groups. The chemical bonding experiments only involved the carboxylated latexes. With BSA, the final density of covalently bound protein was 2.30 mg/m^2. In addition, around 55% of the total linked protein was chemically coupled, and the reaction was little affected by the pH. With Ag36, the final density of covalently bound protein was 2.44 mg/m^2, around 80% of the total linked protein was chemically coupled, and the chemical coupling was maximum at pH = 5 (i.e., close to the isoelectric point).

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Introduction

Immunoassay or immunodiagnosis latexes are useful for detecting pregnancies, rheumatoid arthritis, toxoplasmosis, etc. The tests involve specific reactions between an antigen of a human fluid and an antibody of the immunoassay latex, or viceversa. The results of immunodiagnosis tests are visualized by particle agglutination.

In the first part of this series [1], two core-shell latexes with external carboxyl groups were synthesized through emulsion copolymerizations of styrene and methacrylic acid onto monodisperse polystyrene seeds. The present article describes the sensitization of such latexes with antigen Ag36 (a recombinant protein of *Trypanosoma cruzi*), with the aim of producing an immunodiagnosis kit for detecting the Chagas disease. As far as the authors are aware, this is the first article where Ag36 is used with such an aim.

Functionalized latexes are basic materials for the chemical coupling of antibodies or antigens onto their surface. Ideally, such latexes should present uniform distributions of their particle sizes and of their functional group density. The hydrophilic nature of functional groups increases the latex stability and it is convenient for preventing nonspecific latex-protein interactions [2].

Biomolecules are attached onto the latex particles either by simple physical adsorption and/or by covalent coupling. The latex-protein complexes obtained by physical adsorption are considered of inferior quality, for the potential desorption of the protein, and for the potential denaturalization of the adsorbed protein [3]. In a chemical coupling reaction, the physical adsorption of proteins onto the hydrophobic surface fraction of the particles is inevitable [4]. However, the adsorbed protein can be desorbed with an emulsifier in a final cleaning operation [5].

Several publications have reported on the production of immunoassay tests prepared with base carboxylated latexes. Bastos-González et al. [6] bound the IgG antibody onto a base carboxylated latex. In relation with the binding of IgG onto base carboxylated latexes, Ortega-Vinuesa et al. [7] observed better immunological responses in the chemically coupled complexes compared with the physically coupled complexes. Lee et al. [8] investigated the chemical coupling of bovine serum albumin (BSA) and IgG onto a core-shell carboxylated latex; observing that the sensitivity of the agglutination test depended on the temperature and on the mass of bound antigen. Menshikova et al. [9] investigated the physical and chemical coupling of BSA onto a (monodisperse and carboxylated) polystyrene latex.

The chemical linkage of proteins is strongly affected by the medium. For example, the ionic strength can either increase or decrease the total bound protein. Also, the reaction is sensitive to the pH and to the buffer [10]. Proteins and peptides can be coupled onto carboxylated latexes by direct reaction between an amine of the formers and a carboxyl of the latter. However, the relatively low reactivity of carboxyl groups makes it convenient to include an activation reaction that transforms carboxyl groups into acilureas. To this effect, the water-soluble *N*-*N*-(3-dimethylamine propyl) *N*′-ethyl carbodiimide (EDC) is employed. Figure 1 illustrates the resulting activation-coupling process.

Only a small amount of antigen Ag36 was available for our work. For that reason, BSA was used as a model protein for the adsorption and chemical coupling experiments (both proteins exhibit similar isoelectric points, and therefore similar behaviors regarding electrostatic interactions were to be expected).

Experimental work

Three base latexes were employed (Table 1). Polystyrene (PS) latex S2 was synthesized in an emulsifier-free and unseeded emulsion polymerization of styrene (St) [1].

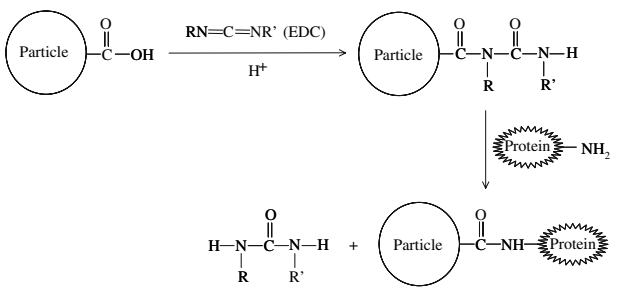

Fig. 1 Activation of the carboxylated latex with EDC and covalent protein coupling

Table 1 Characteristics of the three base latexes

	Latex S2	Latex C1	Latex C2
Particles	PS	Core-shell[a]	Core-shell[a]
% Solids	2.63	0.78	1.06
$\bar{D}_{DLS}$ at 90° (nm)[b]	347.1	403.6	417.7
σ (μC/cm^2)[c]	0.279	1.623	3.694
$\delta_{SO_4^=}$ (mEq/cm^2)[d]	0.289×10^{-8}	0.043×10^{-8}	0.536×10^{-8}
δ_{COOH} (mEq/cm^2)[e]	–	1.682×10^{-8}	3.828×10^{-8}

[a] With a PS core and a PS-MAA shell

[b] Average particle diameter determined by dynamic light scattering

[c] Total charge of surface density

[d] Surface density of sulphate groups

[e] Surface density of carboxyl groups

Latexes C1 and C2 were synthesized by copolymerizing St and methacrylic acid onto monodisperse PS latex seeds [1]. Latex C2 exhibited a higher concentration of external carboxyl groups than latex C1 (Table 1). All the three latexes contained sulphate groups at the polymer chain ends, that correspond to the remnants of the persulphate initiator employed in their syntheses.

The BSA was from Sigma (Cat. No. A4503), and the Ag36 antigen was from Wiener Laboratory (Argentina). Native BSA is a heart-shaped molecule of dimensions $8 \times 8 \times 3$ nm [11]; molar mass 67,000 D; and isoelectric point Ip = 4.7 [12]. Ag36 is a recombinant protein of *T. cruzi*; of molar mass 26,000 D; and Ip $\cong$ 5 [13].

The phosphate buffer was a 5 mM solution of KH_2PO_4 (Cicarelli, Argentina); and the pH was adjusted

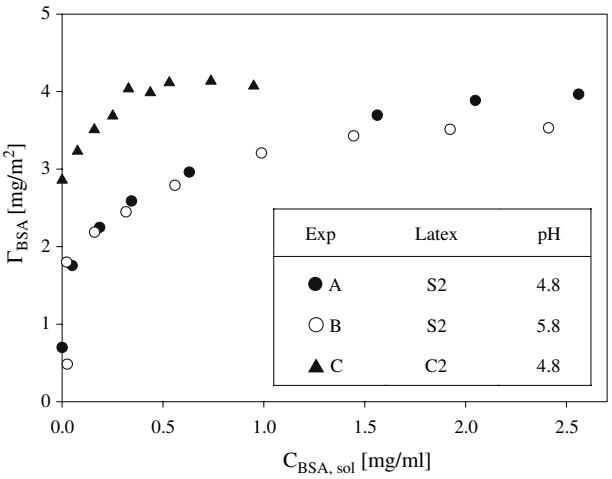

Fig. 2 Physical adsorption experiments of BSA onto PS latex S2 (Exps. A and B), and onto carboxylated latex C2 (Exp. C). The mass of adsorbed protein per unit surface area (Γ_{BSA}) is represented versus the concentration of nonadsorbed protein that remained in solution at equilibrium ($C_{BSA,sol}$)

Table 2 Physical adsorption of BSA onto latexes S2 and C2

Experiment		Sample No.								
		1	2	3	4	5	6	7	8	9
A (pH = 4.8)	Latex S2 (mL)	0.70[a]	0.70	0.70	0.70	0.70	0.70	0.70	0.70	0.70
	Buffer sol. (mL)	1.26	1.18	1.10	1.02	0.90	0.70	0.50	0.30	0.10
	BSA sol. (mL)[b]	0.04	0.12	0.20	0.28	0.40	0.60	0.80	1.00	1.20
	C^0_{BSA} (mg/mL)[c]	0.1	0.3	0.5	0.7	1.0	1.5	2.0	2.5	3.0
	Γ_{BSA} (mg/m^2)	0.70	1.75	2.25	2.59	2.96	–	3.69	3.88	3.97
	BSA molec./part.						–			13,500
B (pH = 5.8)	Latex S2 (mL)	0.70[a]	0.70	0.70	0.70	0.70	0.70	0.70	0.70	0.70
	Buffer sol. (mL)	1.26	1.18	1.10	1.02	0.90	0.70	0.50	0.30	0.10
	BSA sol. (mL)[b]	0.04	0.12	0.20	0.28	0.40	0.60	0.80	1.00	1.20
	C^0_{BSA} (mg/mL)	0.1	0.3	0.5	0.7	1.0	1.5	2.0	2.5	3.0
	Γ_{BSA} (mg/m^2)	0.48	1.80	2.18	2.45	2.79	3.20	3.43	3.51	3.53
	BSA molec./part.									12,000
C (pH = 4.8)	Latex C2 (mL)	1.00[d]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	Buffer sol. (mL)	0.84	0.76	0.68	0.60	0.52	0.44	0.36	0.20	0.04
	BSA sol. (mL)[e]	0.16	0.24	0.32	0.40	0.48	0.56	0.64	0.80	0.96
	C^0_{BSA} (mg/mL)	0.2	0.3	0.4	0.5	0.6	0.7	0.8	1.0	1.2
	Γ_{BSA} (mg/m^2)	2.86	3.23	3.51	3.69	4.04	3.98	4.11	4.14	4.08
	BSA molec./part.								20,500	

[a] Or 0.30 m^2
[b] 5 mg/mL
[c] Initial protein concentration
[d] or 0.14 m^2
[e] 2.5 mg/mL

Table 3 Covalent coupling of BSA onto the carboxylated latex C2. Recipes and results[a]

Experiment No.		Sample No.		
		1	2	3
1 (pH = 4.8)	Latex C2 (mL)	1.00[b]	1.00	1.00
	EDC sol. (mL)[c]	0.10	0.10	0.10
	Buffer sol. (mL)	0.58	0.34	0.10
	BSA sol. (mL)[d]	0.32	0.56	0.80
	C^0_{BSA} (mg/mL)	0.4	0.7	1.0
	Initial COOH:BSA	4.64:1	2.64:1	1.86:1
	$\Gamma_{BSA,\ cov}$ (mg/m^2)	1.79	2.13	2.27
	Initial COOH: coupled BSA			11.3:1
	BSA molec./part.			11,200
2 (pH = 5.8)	Latex C2 (mL)	1.00[b]	1.00	1.00
	EDC sol. (mL)[c]	0.10	0.10	0.10
	Buffer sol. (mL)	0.58	0.34	0.10
	BSA sol. (mL)[d]	0.32	0.56	0.80
	C^0_{BSA} (mg/mL)	0.4	0.7	1.0
	Initial COOH:BSA	4.64:1	2.64:1	1.86:1
	$\Gamma_{BSA,\ cov}$ (mg/m^2)	1.57	2.07	2.30
	Initial COOH: coupled BSA			11.1:1
	BSA molec./part.			11,300

[a] For the desorption process, 2 mL of SDS (1% in weight) were used
[b] or 0.14 m^2
[c] 15 mg/mL
[d] 2.5 mg/mL

Table 4 Covalent coupling of Ag36 onto the carboxylated latexes C1 and C2. Recipes and results[a]

Experiment No.		Sample No. 1	Sample No. 2
3 (pH = 5)	Ag36 sol. (mL)[b]	0.074	0.148
	Latex C1 (mL)	1.000[c]	1.000
	EDC sol. (mL)[d]	0.075	0.075
	Buffer sol. (mL)	0.251	0.177
	C^0_{Ag36} (mg/mL)	0.4	0.8
	Initial COOH:total Ag36	0.88:1	0.44:1
	$\Gamma_{Ag36,\ cov}$ (mg/m^2)	1.70	2.10
	Initial COOH: coupled Ag36		2.1:1
	Ag36 molec./part.		25,000
4 (pH = 7)	Ag36 sol. (mL)[b]	0.074	0.148
	Latex C1 (mL)	1.000[c]	1.000
	EDC sol. (mL)[c]	0.075	0.075
	Buffer sol. (mL)	0.251	0.177
	C^0_{Ag36} (mg/mL)	0.4	0.8
	Initial COOH:total Ag36	0.88:1	0.44:1
	$\Gamma_{Ag36,\ cov}$ (mg/m^2)	1.14	1.35
	Initial COOH:coupled Ag36		3.2:1
	Ag36 molec./part.		16,000
5 (pH = 5)	Ag36 sol. (mL)[b]	0.074	0.148
	Latex C2 (mL)	1.000[e]	1.00
	EDC sol. (mL)[d]	0.100	0.100
	Buffer sol. (mL)	0.226	0.152
	C^0_{Ag36} (mg/mL)	0.4	0.8
	Initial COOH:total Ag36	2.58:1	1.29:1
	$\Gamma_{Ag36,\ cov}$ (mg/m^2)	1.38	2.44
	Initial COOH:coupled Ag36		4.1:1
	Ag36 molec./part.		31,000
6 (pH = 7)	Ag36 sol. (mL)[b]	0.074	0.148
	Latex C2 (mL)	1.000[e]	1.000
	EDC sol. (mL)[d]	0.100	0.100
	Buffer sol. (mL)	0.226	0.152
	C^0_{Ag36} (mg/mL)	0.4	0.8
	Initial COOH:total Ag36	2.58:1	1.29:1
	$\Gamma_{Ag36,\ cov}$ (mg/m^2)	0.69	1.67
	Initial COOH:coupled Ag36		5.9:1
	Ag36 molec./part.		21,200

[a] For the desorption process, 1.4 mL of Triton X-100 (1% in weight) were used

[b] 7.58 mg/mL

[c] or 0.11 m^2

[d] 15 mg/mL

[e] or 0.14 m^2

with a KOH solution. The emulsifiers were sodium dodecil sulphate (SDS from Mallinckrodt); and 4-(1,1,3,3-tetramethylbutyl)phenyl-polyethylene glycol (Triton X-100 from Sigma). The anionic SDS was expected not only to exhibit a higher desorption power than Triton X-100, but also an increased tendency to altering the protein. The EDC carbodiimide was from Fluka, and a 15 mg/mL water solution was prepared shortly before its use.

The protein that remained in solution was determined either by UV absorbance at 280 nm with a Perkin Elmer Lambda 40 UV–Vis spectrophotometer, or through the copper reduction/bicinchoninic acid (BCA) reaction method [7]. The BCA was from Pierce Reagents. In the BCA method, the protein reduces copper (II) into copper (I) in alkaline conditions, and the latter ion produces a (soluble and intensely-colored) complex with BCA.

The centrifuge was a Sorvall RC-5B from Du Pont Instruments.

Physical adsorption of BSA

BSA was physically adsorbed onto latexes S2 and C2 (Table 2). Experiments A and B involved the PS latex S2 at two different pHs. Experiment C involved carboxylated

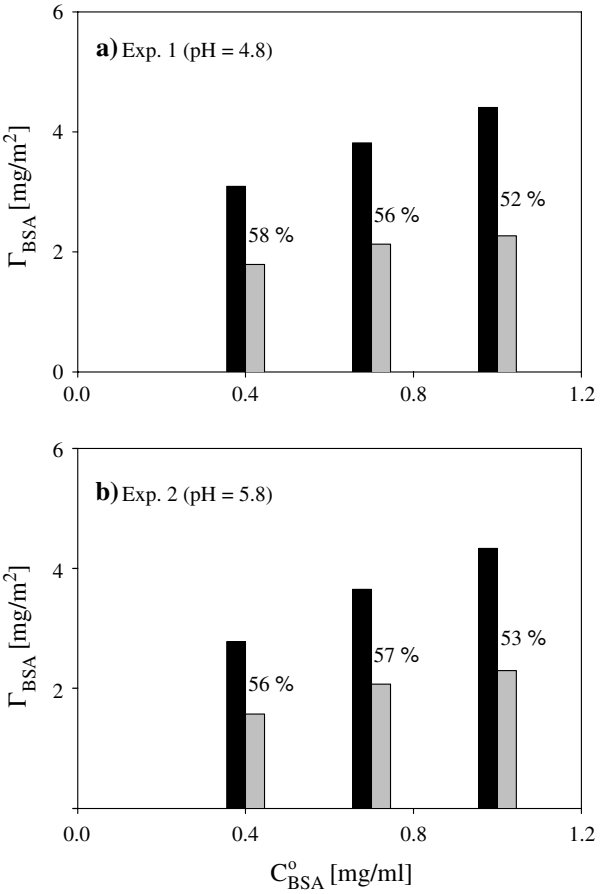

Fig. 3 Chemical coupling of BSA onto Latex C2 (Exps. 1 and 2). The adsorbed BSA is represented versus the initial protein concentration, at two pH values. The total linked protein (in black) is compared with the covalently linked protein (in grey), after the desorption operation with an emulsifier. The percentages indicate the fraction of covalently bonded protein with respect to the total linked protein

latex C2 at pH = 4.8 (i.e. close to the protein Ip). The aim of these experiments was to produce surface densities in the range 3–5 mg BSA/m^2 [14–17].

In each experiment, nine samples were prepared, containing varying amounts of latex, BSA solution, and buffer solution (Table 2). The adsorptions were carried out in 2 mL microcentrifuge tubes. Also, blank samples without protein were prepared as references for the UV determinations. The experiments were as follows: (1) BSA was physically adsorbed for 5 h at room temperature and under gentle agitation; (2) the latex-protein complexes were separated from the solution by ultracentrifugation at 15,000 rpm for 30 min; and (3) the protein remaining in solution was quantified by UV absorbance.

Figure 2 presents the adsorption isotherms. The adsorbed protein (Γ_{BSA}, in mg/m^2) is represented versus the concentration of protein remaining in solution ($C_{BSA,\ sol}$, in mg/mL). Table 2 exhibits the final adsorbed protein. The isotherms show relatively high initial slopes and well-defined plateaus. The initial slopes indicate a good protein-latex affinity. The final plateaus were previously observed in BSA adsorptions onto different (charged and uncharged) PS latexes [14–17].

In Experiments A and B with the PS latex, the maximum adsorption occurred in Exp. A at pH = 4.8 (close to the protein Ip). The value of 3.97 mg BSA/m^2 corresponded to around 13,500 BSA molecules/particle (Table 2). The high adsorption at the protein Ip was explained by the reduced intra- and intermolecular electrostatic forces [18, 19]; that are also convenient for increasing the stability of the adsorbed protein, and for avoiding the protein denaturalization (that would increase the average surface area per protein molecule) [14, 20].

In Exp. C, the final plateau was obtained with a relatively low protein remaining in solution. The value of 4.14 mg/m^2 corresponded to around 20,500 BSA molecules/particle (Table 2). The increased adsorption onto the carboxylated latexes compared with the noncarboxylated latex was explained by Shirahama and Suzawa [18] by an increased hydrogen bonding between latex and protein.

Covalent coupling of BSA and Ag36

In Exps. 1 and 2, BSA was chemically coupled onto latex C2 at two pHs and at three protein concentrations (Table 3). In Exps. 3–6, Ag36 was coupled onto latexes C1 and C2 at two pHs and at two protein concentrations (Table 4). The reagent concentrations and experimental conditions were based on those of the adsorption experiments with BSA (Table 2). The reactions were at room temperature and under gentle agitation. The total reaction times were 5 h. After the reactions, the physically adsorbed protein was desorbed in a final washing operation with an emulsifier. To this effect, SDS was employed in Exps. 1 and 2 with BSA, and Triton X-100 was employed in Exps. 3–6 with Ag36.

The activation and sensitization reactions were carried out in parallel, to minimize the hydrolysis of the acilurea intermediary. To this effect, the protein was mixed with the carboxylated latex and the EDC activator, in a simple step. The carbodiimide was added in 100-fold excess with respect to the carboxyl groups. This was to ensure complete transformation of the carboxyl groups into acilurea, and to produce a recommended nominal surface density of 10 mg EDC/m^2 latex [21]. The ratios between the initial carboxyl groups and the total added protein were larger than one in Exps. 1, 2, 5, and 6; but lower than one in Exps. 3 and 4 (Tables 3 and 4). The protein remaining in solution was determined by UV absorbance in Exps. 1 and 2, and by the BCA method in Exps. 3–6. In all the

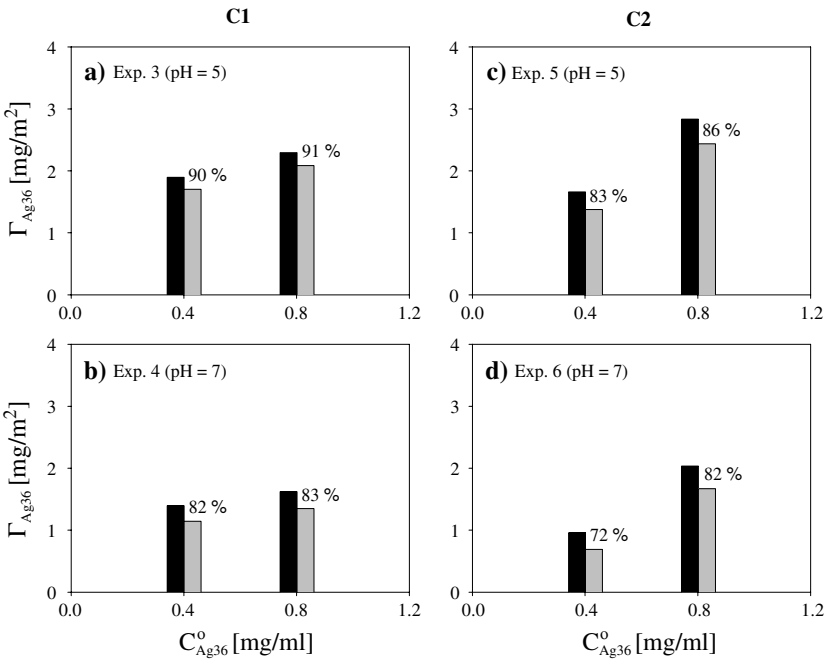

Fig. 4 Chemical coupling of Ag36 onto latexes C1 and C2 at 2 pH values (Exps. 3–6) versus the initial protein concentration. The total linked protein (in black) is compared with the covalently linked protein (in grey), after the desorption operation with an emulsifier. The percentages indicate the fraction of covalently bonded protein with respect to the total linked protein

experiments, blank solutions without protein were prepared.

After the reactions, the samples were centrifuged for 30 min at 15,000 rpm. Then, the precipitated latex-protein complexes were separated from the supernatant solution, and the dissolved protein was quantified. The total linked protein (i.e., the physically-adsorbed and covalently-linked protein) was determined from the difference between the total added protein and the protein remaining in solution. Then, the physically adsorbed protein was redissolved as follows: (a) the latex-protein complexes were redispersed in 1.4 or 2 mL of the emulsifier solutions (1% in weight); (b) the mixtures were left for 24 h under gentle agitation and at room temperature; (c) the dispersions were centrifuged; and (d) the concentration of the redissolved protein in the supernatant solutions was determined. The covalently-bound protein ($\Gamma_{BSA,\,cov}$ and $\Gamma_{Ag36,\,cov}$) was obtained from the difference between the total-linked and the redissolved protein.

The results of Exps. 1 and 2 are in Table 3 and in Fig. 3. Figure 3 presents the total-linked and chemically coupled BSA versus the initial protein concentration (C^0_{BSA}). The fraction of chemically coupled protein falls with increasing values of C^0_{BSA}, and shows a small dependence with pH. In Sample No. 3, each latex particle contained around 11,000 covalently bound molecules, and the ratio between initial COOH groups and covalently bound protein molecules was around 11 (Table 3). This large excess of carboxyl groups suggests the possibility of two or more chemical linkages per BSA molecule, and/or that a fraction of the unreacted acilurea or carboxyl groups could have remained occluded by the bound protein.

The results of Exps. 3–6 with Ag36 are in Table 4 and Fig. 4. As expected, the total bound protein increased with the initial protein concentration (C^0_{Ag36}). For both latexes, the covalently linked protein was highest at pH = 5 (i.e., close to the Ip of the protein). For initial protein concentrations of 0.8 mg/mL, the maximum bound protein occurred with latex C2, possibly due to its higher density of carboxyl groups. The chemically bound protein (2.44 mg/m^2) was highest in Sample No. 2, Exp. 5. In this case, the ratio between initial COOH groups and covalently bound Ag36 molecules was approximately four, and the final complex contained around 31,000 chemically bound Ag36 molecules/particle. This value surpasses that of Sample No. 3, Exp. 2 with BSA, possibly due to the smaller size of Ag36 molecules. Even though not shown for space reasons, similar results were observed when the final redissolution operations of Exps. 3–6 were carried out with SDS rather than with Triton X-100.

Conclusions

As observed in previous publications [14–17], the adsorption isotherms of BSA show well-defined plateaus at high

initial protein concentrations. The BSA adsorption was highest at pH = 4.8 (i.e., close to the protein Ip). Also, the adsorption onto carboxylated latex C2 was higher than onto PS latex S2, possibly due to the increased hydrogen bonding between BSA and the carboxyl groups of latex C2. In the chemical coupling experiments with BSA, around 55% of the total-linked protein was chemically bound, and the reactions showed a small dependence with the pH.

In the chemical coupling experiments with Ag36, the fraction of chemically bound protein with respect to the total linked protein varied between 72% and 91%, and this last maximum value occurred at the protein Ip. These high percentages of chemical coupling may render unnecessary the final redissolution operation with an emulsifier, thus reducing the risk of protein denaturation. The maximum amount of chemically bound Ag36 (2.44 mg/m^2) was observed in Sample No. 2, Exp. 5 (carried out at pH = 5 with latex C2). This latex-protein complex exhibited around 31,000 Ag36 molecules/particle, and a fraction of chemical coupling with respect to the total linked protein of 86%.

At present, the Ag36-latex complexes are being evaluated with antibodies of the Chagas disease, but such work will be the subject of a future communication.

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