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J.A. Molina-Bolívar
F. Galisteo-González
M. Quesada-Pérez
R. Hidalgo-Álvarez

Agglutination kinetics of $F(ab')_2$ coated polymer colloids

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J.A. Molina-Bolívar · F. Galisteo-González
M. Quesada-Pérez
R. Hidalgo-Álvarez (✉)
Grupo de Física de Fluidos y Biocoloides
Departmento de Física Áplicada
Facultad de Ciencias
Universidad de Granada
18071 Granada
Spain
e-mail: rhidalgo@goliat.ugr.es

Abstract In a previous publication we have described a simple quantitative model describing the agglutination process due to antigen–antibody interactions in IgG–latex immunoassays. To achieve colloidal stability, however, some additives had to be used. In this paper we study the immunoreactivity of $F(ab')_2$ antibody physical and chemically adsorbed onto sulphonate polystyrene and chloromethylstyrene particles, respectively. This kind of latex immunoassay does not show stability problems under physiological conditions. The influence on the agglutination kinetics of the protein coating level has been now studied for the whole coverage range. Also the influence of coupling procedure, storage time and ionic strength of the reaction medium have been investigated.

Key words Particle-enhanced immunoassay – aggregation kinetics – covalent coupling – physical adsorption – reactivity of $F(ab')_2$

Introduction

The latex agglutination immunoassay is one of the most important methods in immunology based on reactions at the solid–liquid interface [1–3]. Latex microspheres are used as carriers of antibodies (or antigens) to detect complementary antigens (or antibodies) present in biological samples.

Recently, the latex agglutination immunoassay has been widely studied, favored by the recent advances in colloid science that have made possible to produce uniform latex particles with control of the most important characteristics such as particle size, surface charge density and functionality. In this way, many kinds of functionalized latexes with reactive surface groups suitable for covalent protein immobilization have been described [4–7].

This immobilization may then be achieved either by classical physical adsorption or by covalent coupling. The latter might have, in principle, several advantages such as minimal protein denaturation on the surface, no desorption of antibodies, minimal non-specific interactions and a uniform coating procedure [8].

One of the basic requirements of the protein–latex complex for its applications in the field of clinical diagnostics is the immunoreactivity of the protein molecules after adsorption. It is important that antibodies adsorb retaining a maximum of their biologic activity. The active sites of the molecule must be directed towards the solution to facilitate the reaction with the antigen [9].

Specific knowledge about the kinetics of the antigen–antibody reaction at the solid–liquid interface is useful for optimizing a solid-phase assay technique. Moreover, it is necessary when choosing an appropriate mathematical expression for fitting a standard curve to empirical data [10, 11]. There exists a special interest on the possibility of finding theoretical models for the kinetics of different solid-phase methods like the enzyme-linked immuno-sorbent assay (ELISA) or latex agglutination

immunoassay [12]. Parameters as the average number of active antibody sites and equilibrium constant are useful to improve the sensitivity and selectivity of latex agglutination immunoassays.

In a previous paper [13] we reported on the application of a simple quantitative model for the aggregation kinetics of IgG-coated latex particles. Since this kind of antibody–latex complex is colloidally unstable under physiological conditions, two approaches were then employed to attain stability, namely acetylation of the IgG molecule prior to physical adsorption is sulphonate latex (electrostatic stabilization) and the use of Tween 20 in the case of covalent adsorption to chloromethylstyrene latex (steric stabilization).

In this paper we present the application of the model to a better-characterized system by using $F(ab')_2$ fragments instead of the whole IgG. With this variety of the antibody, complexes are colloidally stable under physiological conditions, thus avoiding the use of additives and their possible influence on agglutination kinetics. Moreover, a wider range of coverages may be studied (up to full surface coating), whereas in the IgG case only up to half coverage could be addressed. Apart from revising the influence of protein coverage and coupling procedure on the agglutination kinetics for this well-characterized system, new contributions respect to the influence of ionic strength and storage time are presented.

Experimental

Material

All chemicals used were of analytical quality. Water was purified by reverse osmosis followed by percolation through charcoal and a mixed bed of ion-exchange resins. The pH was controlled using phosphate buffer.

A polystyrene latex (*P*1) without functional groups able to bind protein covalently was synthesized by means of an emulsion copolymerization without emulsifier and with sodium styrene sulfonate as co-monomer. Styrene and sodium styrene sulfonate were purchased from Merck. Styrene was distilled under reduced pressure (10 mm Hg) at 40 °C and stored at −5 °C until required.

The chloromethylstyrene latex used in this work (*C*1) was kindly provided by J. Sarobe and J. Forcada (Chemical Engineering Group, University of Basque Country, Spain. Synthesis details are described elsewhere [14].

Both latexes were cleaned by serum replacement until the electrical conductivity of the liquid was below 2 μS/cm. The particle size distribution (PSD) was obtained by transmission electron microscopy (TEM) using a H-7000 FA Hitachi microscope on representative samples of more than 500 particles (automatically analyzed with Bolero software, AQ Systems). Surface charged groups were determined by conductimetric titration, and chloride surface groups by hydrolysis with glycine/NaOH, acidification with HNO_3, and subsequent titration of free Cl^- with $AgNO_3$. The main characteristics of the latexes are shown in Table 1.

C reactive protein (CRP), anti-CRP $F(ab')_2$ fragments and monomeric BSA were kindly supplied by Biokit, SA (Barcelona). The $F(ab')_2$ fragments were obtained by pepsin digestion of polyclonal rabbit IgG, and purified by affinity chromatography to remove undigested IgG. Purity was checked by sodium dodecyl sulphate polyacrilamide gel electrophoresis (SDS-PAGE) and the molecular weight found to be 102 kDa. No IgG contamination was detected.

Lyophilized monomeric bovine serum albumin (M-BSA), separated from the oligomers by exclusion chromatography, was dialysed before use. All proteins were stored at −30 °C.

Adsorption

The protein concentration in solution was determined before and after adsorption at pH 7.2 by direct UV spectrophotometry at 280 nm (Spectronic 601, Milton Roy). The total polymer area added was 0.4 m^2 and the ionic strength of the medium 2 mM. The sample was gently shaken in a thermostatically controlled with at 35 °C for 5 h. After incubation the samples were separated from the supernatant by high-speed centrifugation and the supernatant filtered using a polyvinyldene-difluoride filter (Millipore, pore diameter 0.1 μm) before the remaining protein concentration was measured. Such a filter has an extremely low affinity for protein adsorption and thus the filtration step does not interfere with the calculation of adsorbed protein.

Agglutination tests

The immunoreactivity of the sensitized latexes coated with aCRP-$F(ab')_2$ was measured by monitoring the immunoaggregation reaction with human CRP in a nephelometer. We have used a non-commercial apparatus with

Table 1 Main characteristics of the latexes

Latex	*D* [nm]	P.D.I.	σ [μC/cm^2]	[Cl^-] [μmol/g pol.]
*P*1	196 ± 10	1.005	−4.2 ± 0.6	0
*C*1	204 ± 8	1.0045	−3.1 ± 0.4	121 ± 10

a 10 mW He/Ne laser ($\lambda = 632.8$ nm) and a rectangular flow cell with 2 mm path length. The scattered light was measured at an angle of 10° for 5 min. The experiments were performed in glycine-buffered saline (GBS–BSA) containing 0.1 M glycine, 0.17 M NaCl, 1 mg/ml NaN_3 used as a preservative, and 1 mg/ml M-BSA to cover patches of latex surface free of aCRP-F(ab′)$_2$ in order to avoid bridging coagulation of the complexes. The pH was adjusted to 8 with NaOH.

The injection into the nephelometer cell was performed by simultaneous stopped flow mixing of two dispersions: one made up of 1100 μl reaction buffer and 50 μl CRP at different concentrations, and the other of 1100 μl reaction buffer and 50 μl sensitized latex. The final particle concentration for $P1$ and $C1$ latexes was ca. 1.85×10^{10} and 1.98×10^{10} part/ml, respectively.

Results and discussion

According to the model previously cited [13], if we use the nephelometer to monitor the immunoagglutination, the initial slope of the variation of scattered light with time is given by

$$v_0 = (\mathrm{d}I/\mathrm{d}t)_0 = (C/4)[(N_0 n)^2 - (A + K^{-1} - \Delta^{1/2})^2] \,, \quad (1)$$

where K is the chemical equilibrium constant, N_0 is the initial concentration of latex particles, A is the initial concentration of antigen in the bulk solution, n is the total number of immunologically active antibody sites per particle and

$$\Delta = (N_0 n + A + K^{-1})^2 - 4AN_0 n \quad (2)$$

and

$$C = 2k^* i(Q, 0) n^{-2} \sin(Qd_0)/Qd_0 \quad (3)$$

is a proportionality constant for every experiment. Q is the modulus of the scattering vector, $i(Q, 0)$ is the initial intensity and d_0 is the distance between the centers of monomers forming a dimer. Thus, v_0 depends on three fitting parameters, namely $N_0 n$, K^{-1} and C.

This model, originally applied for IgG–latex complexes, has been now extended to F(ab′)$_2$ fragments–latex complexes to avoid colloidal stability problems, and at the same time to avoid the presence of additives which can affect the immunological reaction kinetics.

Influence of protein coverage on the immunoagglutination kinetic

Figure 1 shows the value of v_0 obtained as a function of CRP concentration for three $C1$–F(ab′)$_2$ conjugates with 0.9, 2.1 and 3 mg/m^2 of protein coating level (data points). There is a big difference in the agglutination rates of the lowest protein coverage and the intermediate value (2.1 mg/m^2), while an increase over this value practically does not influence the kinetics. It seems to indicate that, over a certain value, the immunological agglutination rates do not necessary increase with an increasing number of immobilized antibody molecules, namely, sites with which antigens interact. This extreme was not observed in the case of covalently bound IgG [13], since coverages higher than half-coating could not be employed due to stability problems.

The application of the kinetic model is represented by the curves in the figure. The three fitting parameters $P_1 = C/4$, $P_2 = N_0 n$ and $P_3 = K^{-1}$, as well as the estimates of the percentage of F(ab′)$_2$ active sites, are shown in Table 2. In all cases this percentage value is extremely low, increasing with a decrease in the level of protein coating. Similar percentage values of active antibody sites

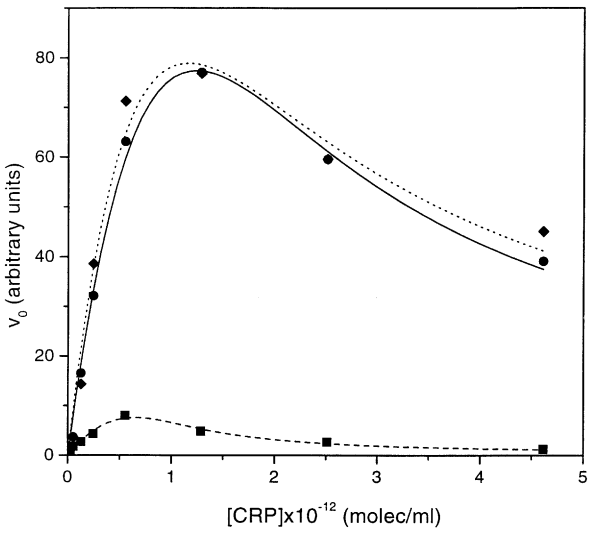

Fig. 1 v_0 as a function of CRP concentration for three $C1$-F(ab′)$_2$ immunolatexes: ■, 0.9 mg/m^2; ●, 2.1 mg/m^2; ◆, 3 mg/m^2. The lines correspond to the theoretical curves

Table 2 Fitting parameters and percentage of active F(ab′)$_2$ on latex particles for the three $C1$-F(ab′)$_2$ immunolatexes used

Protein coverage [mg/m^2]	$C/4 \times 10^{24}$	$N_0 n \times 10^{-12}$	$K^{-1} \times 10^{-11}$ [molecules^{-1}ml]	Active F(ab′)$_2$ [%]
0.9	7.4 ± 2.3	1.01 ± 0.20	1.5 ± 0.4	3.7 ± 0.5
2.1	44 ± 14	1.33 ± 0.21	5.7 ± 0.9	2.1 ± 0.4
3.0	44 ± 17	1.37 ± 0.40	5.2 ± 1.0	1.5 ± 0.4

were obtained by Von Schulthess et al. [15] using a different model. It suggests that upon adsorption most of the antibody molecules loose their ability to bind antigens, probably as a consequence of unfavourable orientation and/or steric hindrance of the antigen binding sites at the sorbent surface, reducing antigen accessibility. Although changes in the protein structure upon adsorption are likely to occur, they do not seem to play a major role, since they are usually favoured in the case of low coverage [16, 17].

The fitting parameters P_3, i.e. the reciprocal of the chemical equilibrium constant K, depends on the protein coating degree as the aggregation rates do (Table 2). The values for the two complexes with high coverage are very similar, but rather different of the lowest coating case. It implies that with an increase in the number of antibodies bound to a latex particle, the chemical equilibrium constant decreases, probably because a too densely packed protein layer is disadvantageous for the efficiency of the immunological reaction. Similar results were obtained by Kitano et al. by direct visual observation of the agglutination process [18].

Influence of the protein immobilization mechanism

Figure 2 shows the value of v_0 as a function of CRP concentration obtained for immunolatexes with anti-CRP $F(ab')_2$ molecules passively adsorbed on *P*1 latex and covalently bound to *C*1 latex, in both cases with the same antibody type and coverage (2.1 mg/m^2). This was, a comparison between both adsorption procedures may be suitable once we have observed the influence of coating level. The percentage of active antibody sites (Table 3) is lower when the $F(ab')_2$ molecules are physically adsorbed than when they are chemically bound. Upon protein adsorption some degree of denaturation may occur, changing its conformation toward an immunologically less active state [19, 20]. Although it has been reasoned previously that these structural rearrangements do not seem to play a decisive role, their influence should be present in this case where we are comparing similar protein coverages but different coupling procedures. Since covalent linking is a slower process than physical adsorption [21], in principle the same coating level should afford more or less the same surface molecular orientation and steric hindrance. This result is in line with the idea that protein rearrangements may be reduced when particles with functional groups are employed for the covalent union instead of physical adsorption [10]. It represents an advantage of the covalent coupling of proteins onto polymer surfaces.

Influence of ionic strength

There are thought to be at least two distinct stages in the immunoagglutination reaction [22]. The first stage is a primary reaction involving binding of antigen to antibody. The antigen (CRP) is polyvalent while the antibody ($F(ab')_2$) is bivalent. The second stage involves the formation of antigen–antibody binding between different latex particles and the subsequent agglutination. The kinetics of the immunoagglutination reaction is influenced by the ionic species and strength in the reaction environment, as well as the pH [23]. It is possible that these factors affect both stages of the reaction to different degrees.

The use of reaction buffers at high ionic strength has been proposed to stabilize latex-antibody systems by

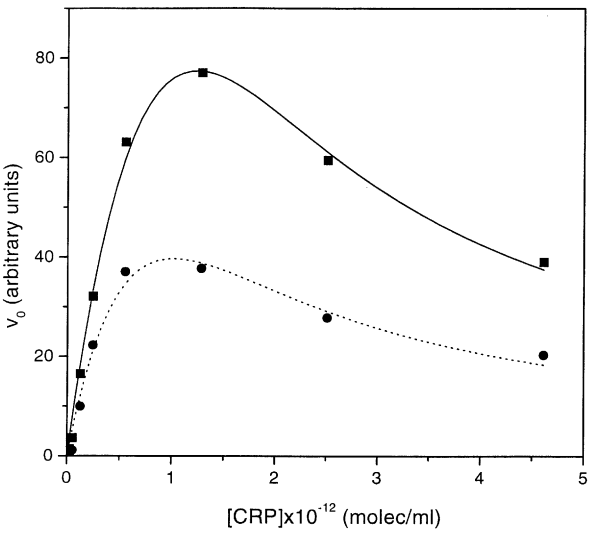

Fig. 2 v_0 as a function of CRP concentration: ■, $F(ab')_2$ chemically bound to *C*1 latex; ●, $F(ab')_2$ physically adsorbed to *P*1 latex. Both conjugates with 2.1 mg/m^2 of protein coverage. The lines correspond to the theoretical curves

Table 3 Fitting parameters and percentage of active $F(ab')_2$ on latex particles for *C*1-$F(ab')_2$ and *P*1-$F(ab')_2$ conjugates with similar protein coating level (2.1 mg/m^2)

System	$C/4\times10^{24}$	$N_0 n\times10^{-12}$	$K^{-1}\times10^{-11}$ [molecules^{-1} ml]	Active $F(ab')_2$ [%]
*C*1 latex	44 ± 14	1.33 ± 0.21	5.7 ± 0.9	2.1 ± 0.4
*P*1 latex	57 ± 22	0.8 ± 0.4	6.0 ± 1.6	1.5 ± 0.3

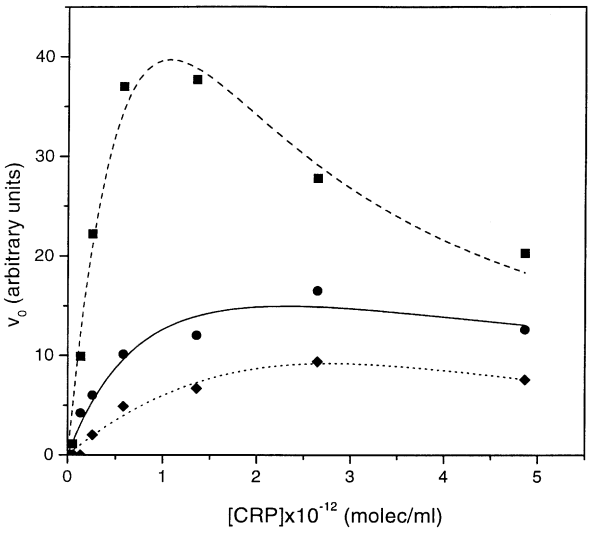

Fig. 3 v_0 as a function of CRP concentration for a *P*1-F(ab′)$_2$ immunolatex with a protein coverage of 2.1 mg/m^2 in different reaction buffers: ■, 170 mM NaCl; ●, 800 mM NaCl; ◆, 300 mM $Mg(NO_3)_2$ plus 170 mM NaCl. The lines correspond to the theoretical curves

hydration forces [24, 25]. To test the influence of these buffers on the immunoagglutination kinetics, the reactivity of anti-CRP F(ab′)$_2$ molecules adsorbed onto the *P*1 latex was determined using three different reaction buffers at pH 8: 170 mM NaCl (buffer A), 800 mM NaCl (buffer B), 300 mM $Mg(NO_3)_2$ plus 170 mM NaCl (buffer C).

Figure 3 shows the value of v_0 obtained as a function of CRP concentration for the F(ab′)$_2$-*P*1 immunolatex in those reaction buffers. It can be seen that the rates were strongly affected by the ionic strength. The results for buffer B and C cannot be fitted adequately by the proposed kinetic model. When curves are fitted to the experimental data points, the obtained adjusted parameters fall out of logical values (even negative).

The decrease in the initial agglutination rates with increasing ionic strength is not attributable to the electrostatic double-layer repulsion of antibody-coated particles

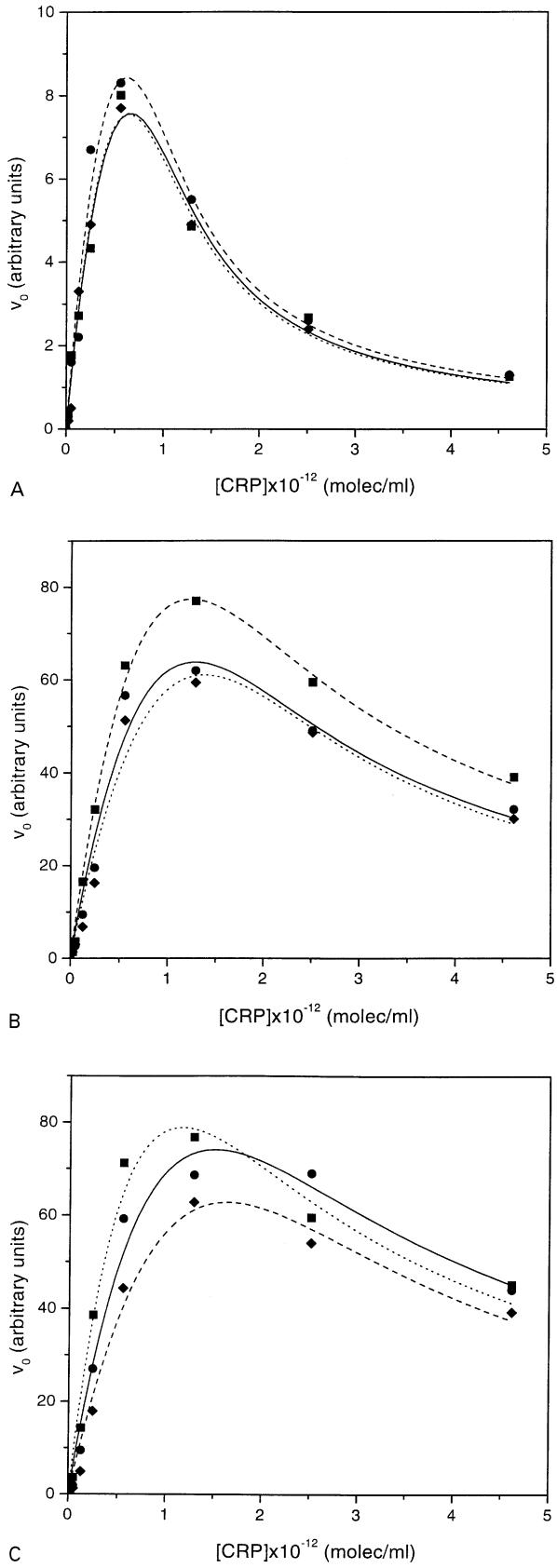

Fig. 4 (A) v_0 as a function of CRP concentration for a *C*1-F(ab′)$_2$ immunolatex with a protein coverage of 0.9 mg/m^2 after different storage periods: ■, sample freshly prepared; ●, 21 days ago; ◆, 4 months ago. The lines correspond to the theoretical curves. (B) v_0 as a function of CRP concentration for a *C*1-F(ab′)$_2$ immunolatex with a protein coverage of 2.1 mg/m^2 after different storage periods: ■, sample freshly prepared; ●, 21 days ago; ◆, 4 months ago. The lines correspond to the theoretical curves. (C) v_0 as a function of CRP concentration for a *C*1-F(ab′)$_2$ immunolatex with a protein coverage of 3 mg/m^2 after different storage periods: ■, sample freshly prepared; ●, 21 days ago; ◆, 4 months ago. The lines correspond to the theoretical curves

because the repulsion decreases with increasing the ionic strength. Moreover, for this high ionic strength the double-layer is screened. The inhibition of the agglutination by desorbed antibodies is not a dominant factor since desorption of protein from the surface was tested with ionic strength up to 3 M during one week and no desorption was observed. A possible explanation for this decrease may be offered if we observed that the complex is colloidally stabilized by hydration forces in the buffers with high ionic strength. It is well established that water molecules bind strongly and hydrated cations to protein surfaces. A simplified explanation of the hydration forces is that they represent the opposition exerted by water molecules surrounding adsorbed cations to being removed, thus imposing an energy barrier towards close approximation between particles [26–28]. Hydration forces hinder detection of the antigen, probably because the hydration of the $F(ab')_2$ molecules decreases the antigen chances of access.

Influence of storage time

Since latex agglutination tests must be stored during long periods of time before use, it is important to check the possible reduction of immunoreactivity with time. In fact, this reduction was observed in the case of physically adsorbed IgG systems, and the measured desorption of antibody molecules proposed to explain it [29]. In principle, the immunoreactivity of the covalent linked antibodies should then be maintained better than when physically adsorbed due to the decrease in desorption. We have studied the response of different conjugates with $F(ab')_2$ molecules covalently bound which have been stored for 21 days and 4 months (similar conditions than in reaction buffer A but without NaCl at 5 °C). The results are compared with freshly prepared samples in Fig. 4A–C for 0.9, 2.1 and 3 mg/m^2 protein coating levels, respectively. The corresponding parameters of the fitings are shown in Table 4. For the lowest coverage (0.9 mg/m^2) the percentage of active $F(ab')_2$ does not change with storage time. However for the two other immunolatexes (2.1 and 3 mg/m^2) the percentage of active antibody increases with the storage time. Previous studies [21, 30] relating the extension of covalent binding of proteins to chloroactivated latexes report that not all of the protein is covalently bound, but a fraction remains as physically adsorbed depending on the number of chloromethyl groups and the protein coverage. The initial steps of adsorption isotherms indicate that all the adsorbed protein at low coverage becomes covalently bound. With more protein in solution, the fraction of covalent binding decreases until a constant value (ca. 50–60% covalency) [21]. According to these studies, it is possible that the increase in the percentage of active $F(ab')_2$ with storage time may be due to the desorption of (part of) the physically adsorbed protein fraction, thus reducing the steric impediments between antibody molecules. For the lowest protein coverage, however, desorption is not significant, and the percentage of active $F(ab')_2$ is constant.

Table 4 Fitting parameters and percentage of active $F(ab')_2$ on latex particles for the three *C*1-$F(ab')_2$ immunolatexes after different storage periods

Protein coverage and storage period [mg/m^2]	$C/4\times10^{24}$	$N_0n\times10^{-12}$	$K^{-1}\times10^{-11}$ [molecules^{-1}ml]	Active $F(ab')_2$ [%]
0.9-freshly	7.4 ± 2.3	1.01 ± 0.20	1.5 ± 0.4	3.7 + 0.5
0.9-21 days	10 ± 4	0.93 ± 0.20	1.5 ± 0.5	3.4 ± 0.4
0.9-4 months	8 ± 2	0.97 ± 0.15	1.5 ± 0.4	3.5 ± 0.4
2.1-freshly	44 ± 14	1.33 ± 0.21	5.7 ± 0.9	2.1 ± 0.4
2.1-21 days	29 ± 9	1.5 ± 0.5	5 ± 2	2.3 ± 0.4
2.1-4 months	20 ± 6	1.8 ± 0.6	5 ± 2	2.7 ± 0.3
3-freshly	44 ± 17	1.4 ± 0.4	5 ± 1	1.5 ± 0.4
3-21 days	37 ± 12	1.4 ± 0.6	8 ± 3	1.5 ± 0.3
3-4 months	17 ± 5	1.9 ± 0.6	6.8 ± 2.4	2.0 ± 0.4

Table 5 Fitting parameters and percentage of active $F(ab')_2$ on latex particles for a *P*1-$F(ab')_2$ conjugate with 2.1 mg/m^2 different storage periods

Protein coverage and storage period [mg/m^2]	$C/4\times10^{24}$	$N_0n\times10^{-12}$	$K^{-1}\times10^{-11}$ [molecules^{-1}ml]	Active $F(ab')_2$ [%]
2.1-freshly	57 ± 22	0.8 ± 0.4	6.0 ± 1.6	1.5 ± 0.3
2.1-21 days	31 ± 9	1.2 ± 0.4	4.8 ± 1.3	2.2 ± 0.5
2.1-4 months	18 ± 1	1.5 ± 0.4	4.9 ± 1.7	2.7 ± 0.6

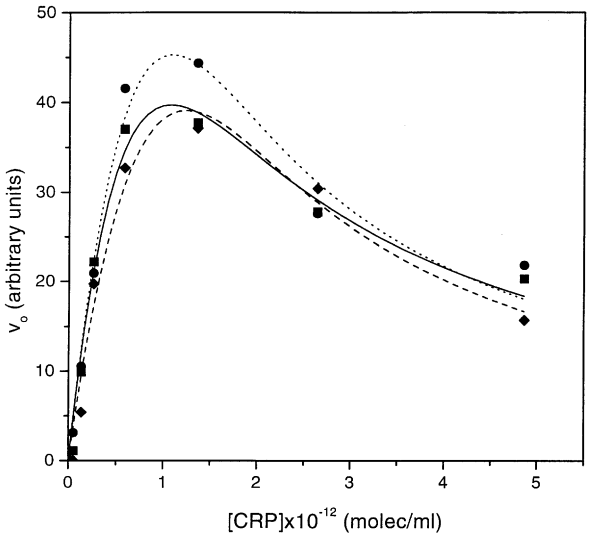

Fig. 5 v_0 as a function of CRP concentration for a *P*1-F(ab′)$_2$ immunolatex with a protein coverage of 2.1 mg/m^2 after different storage periods: ■, sample freshly prepared; ●, 21 days ago; ◆, 4 months ago. The lines correspond to the theoretical curves

Figure 5 shows the influence of storage time on the immunoagglutination kinetics of a *P*1-F(ab′)$_2$ conjugate with 2.1 mg/m^2, i.e. all protein physically adsorbed. The fitting parameters are shown in Table 5. As in the latter case, the percentage of active F(ab′)$_2$ increases with the storage time. The increase after 4 months amounts 1.2%, whereas that obtained for the covalent conjugate with the same protein coating level (Table 4) is 0.6%. In accordance with the previous reasoning, this difference could be ascribed to the higher amount of protein desorption that can occur in the case of only physically adsorbed antibodies.

Conclusions

We have used a previously proposed simple kinetic model to study the agglutination kinetics of latex immunoassays. To this end, F(ab′)$_2$ has been employed instead of the whole IgG to avoid colloidal stability problems and, thereby, the use of additives. According to this kinetic model the percentage of active antibody on polymer carriers is extermely low (maximum found 3.7% for F(ab′)$_2$, 5% for IgG [13]). This percentage decreases with increasing the degree of protein coverage, probably because unfavorable orientation and/or steric impediments of the antigen binding sites in a too densely packed protein layer.

For a similar coating level, this percentage depends on the mode of adsorption of the antibody molecules to the latex surface, being higher in the case of covalent coupling. The immunoagglutination kinetics (sensitivity) depends on the reaction buffer, decreasing with increasing the ionic strength of the medium. The storage time provokes an increase in the percentage of active F(ab′)$_2$ probably because of protein desorption of (part of) the physically adsorbed fraction.

All this information is helpful to search the optimum conditions under which both the sensitivity and selectivity of latex agglutination tests may be enhanced.

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