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Covalent coupling of antibodies to aldehyde groups on polymer carriers

J. M. PEULA, R. HIDALGO-ALVAREZ
Grupo de Física de Fluidos y Biocoloides, Dpto. de Física Aplicada, Universidad de Granada, Granada, 18071 Spain

R. SANTOS, J. FORCADA
Grupo de Ingeniería Química, Dpto. de Química Aplicada Facultad de Químicas, Universidad del Pais Vasco-EHU Apd. 1072, San Sebastián, 20080 Spain

F. J. DE LAS NIEVES
Departamento de Física Aplicada, Facultad de Ciencias Experimentales, Universidad de Almería, Almería, 04120 Spain

The aim of the present work is to prepare and characterize a functionalized latex with acetal groups on the surface and to obtain the covalent coupling of an a-CRP IgG protein. The acetal latex was synthesized by means of a core-shell emulsion polymerization in a batch reactor. The core was a seed of polystyrene and the shell was obtained by terpolymerization of styrene, methacrylic acid and methacryloylacetaldehyde di(n.methyl)acetal. The latex was characterized by TEM and conductimetric and potentiometric titration, in order to obtain the particle size distribution and the amount of carboxyl and acetal groups on the surface, respectively. Several latex-protein particles with the IgG physically or chemically bound to the surface were obtained by modifying the incubation conditions. In the covalent coupling experiments of the IgG, the protein physically adsorbed was removed by redispersion of the complexes in the presence of a non-ionic surfactant (Tween 20). The latex-protein complexes were characterized from the electrokinetic point of view with the aim to determine the isoelectric point of the complexes and to detect any difference in the electric state of the protein when these molecules are physically or chemically coupled to the surface. The final part of this work was to study the immunoreactivity of several latex-IgG complexes at several experimental conditions. By measuring the change in the turbidity after the addition of CRP antigen into the dispersion, it was possible to compare the immunoreactivity results when the protein is physically or chemically bound to the surface, and to study the effect of the presence of a surfactant in the reaction medium.

1. Introduction

The study of the covalent coupling of antibodies to polymer surfaces is of great interest because it enables us to gain a much better understanding of the immobilization of proteins on artificial surfaces. From the point of view of its application in the development of new immunodiagnostic tests, the covalent bound of protein to the particle surface presents the advantage of eliminating the later desorption of the proteins, linking the molecules in such way that the active sites are directed toward the solution, improving the specific character of the test, etc. Most of the works about covalent bound of protein have been conducted with carboxylated latexes and requires some chemical reaction [1–3]. Recently some authors [4, 5] have indicated that the use of aldehyde groups on the surface could simplify the covalent bound of the protein due to the direct reaction between the aldehyde groups of the particle surface and the amine groups of the protein molecules. The preparation of aldehyde latex, however, is only known from the last five years and the aldehyde groups tend to decompose with time losing the capacity to bound the proteins. As suggested by Kapmeyer *et al.* [6, 7], another possibility is to produce latex particles with acetal groups on the surface. These groups can be transformed to aldehyde groups at the time to produce the covalent coupling of the proteins, by moving the medium to acid pH.

Once the latex has been prepared and the protein coupled, the next step is to characterize the formed latex-protein particles and to check their colloid stability and immunoreactivity. However, there is not much work which has made the adsorption of the proteins and has completed the characterization of the latex-protein complexes.

In the present work, we have focused our attention on the preparation of a functionalized latex with

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acetal groups on the surface and the later covalent coupling of an a-CRP (C-reactive protein) IgG protein. Some experiments on the physical and chemical adsorption of the IgG onto acetal polystyrene beads have been performed under different experimental conditions. Several latex-protein particles were obtained with the protein physically or chemically bound to the surface. These biointerfaces were characterized from the electrokinetic point of view in order to determine their isoelectric point and to detect any difference in the electric state of the protein when this molecule is physically or chemically coupled. To complete the characterization of the latex-protein complexes we have estimated their colloid stability at two pH and at physiological ionic strength. The final aspect of this work was to measure the immunoreactivity of the IgG-latex complexes by optical methods at several experimental conditions and to compare the results when the protein is physically or chemically bound.

2. Materials and methods

2.1. Synthesis of the functionalized latex

The functionalized latex (called LKM-1) with acetal groups on its surface was synthesized by means of a core-shell emulsion polymerization in a batch reactor. The core was a seed of polystyrene and the shell was obtained by terpolymerization of styrene (St), methacrylic acid (MAA) and methacryloylacetaldehyde di (n-methyl) acetal (MAAMA), following the slightly modified procedure reported by Kapmeyer *et al.* [6].

The monomers St and MAA and all other materials were used as received. Potassium persulfate, sodium dihydrogen phosphate and sodium hydrogen phosphate were used as initiator and buffers, respectively. The surfactant was sodium lauryl sulfate. Deionized water was used throughout. The acetal monomer MAAMA is not a commercial one and its synthesis was carried out using amine acetaldehyde dimethyl acetal, methacryloyl chloride and potassium carbonate as buffer, the reaction medium was chloroform, previously dried with $CaCl_2$.

The polymerization was carried out in a litre glass reactor fitted with a reflux condenser, stainless-steel stirrer, sampling device, nitrogen inlet and feed inlet tubes. The seed was prepared at 90 °C by means of batch emulsion homopolymerization of St. After polymerization the seed was kept overnight at 90 °C to decompose the initiator. The volume average diameter of the seed used was 103 nm, calculated from its particle size distribution (PSD). The polydispersity index (PDI) of the PSD of the seed was 1.017.

The synthesis of the latex LKM-1 was carried out using 40 g of the seed and 752 g of the water. The initiator concentration was 3.2 wt% of the total monomer and the weight ratio of the termonomers was 4/12/3 (MAAMA/St/MAA), respectively. The emulsifier concentration was calculated to cover 35% of the particle surface. The charge sequence of the reactor was: the seed, the emulsifier and buffer solutions, the MAA monomer neutralized with NaOH, the St and finally the MAAMA. The reaction mixture was kept for 1 h at room temperature in order to swell the seed particles with the termonomers. Once the reaction temperature (70 °C) was reached, the initiator aqueous solution was charged into the reactor to start the polymerization. The stirring rate was 200 rpm and the reaction time 5 h. The final latex was removed from the reactor and the polymerization was quenched with hydroquinone. The conversion of the latex was 60%, determined gravimetrically, and the final pH was 7.

The particle size distribution of the seed and final latex were obtained by transmission electron microscopy (TEM) on representative samples of more than 1000 particles. The latex samples were diluted in an aqueous solution of PTA (phosphotungstic acid) (negative staining) and dried by means of a UV light source in order to harden the particles and to avoid film formation while drying, and particle deformation and shrinkage by electron beam. The volume average diameter of the latex particles, 112 nm, was calculated from the PSD. The PDI was 1.02.

The surface charge density of the latex, due to the carboxyl groups provided by the MAA monomer, was $(10.1 \pm 0.3)\ \mu C/cm^2$ as determined by conductimetric titration. The content of aldehyde surface groups (after acid cleavage of the acetals and titration with hydroxylamine) was $2.36\ 10^{-7}$ meq/cm^2. The final amount of carboxyl and acetal groups are similar to those obtained by Kapmeyer *et al.* [6, 7], although we are using a different monomer to provide the acetal groups.

3. Protein adsorption experiments

The protein IgG a-CRP (IgG anti-C-reactive protein), rabbit polyclonal was kindly donated by Biokit SA (Spain). The IgG a-CRP sample was purified from CRP immunized rabbit serum by ammonium sulfate fractionation followed by anion exchange chromatography. The purified rabbit IgG was stored at − 20 °C. The purity of the IgG preparation was ascertained by immunoelectrophoresis [8].

The physical and chemical adsorption of the IgG were performed at different pHs. For the covalent coupling of the protein the acetal latex was activated by adding HCl to reach pH 2. The amount of adsorbed IgG on the LKM-1 latex, was determined by using the depletion method described in [8]. In order to be sure about the no disturbance of the surfactant during the determination of the amount of protein in solution, we have employed two different methods for those measurements. The usual way was to measure the optical absorbance at 280 nm. The results were compared with those obtained by using a Protein Assay Reagent BCA (Pierce, USA). In this second method we have performed a calibration curve in the presence of the surfactant (Tween 20), which will be the condition for our measurements. The results obtained by both methods were in very good agreement and, therefore, we can continue by using the simplest: optical absorbance. Thus, the amount of IgG covalently bound to the latex surface by the aldehyde

groups was determined in the presence of Tween 20 to remove the protein physically adsorbed [9, 10].

The electrophoretic mobility of the IgG-latex complexes were measured with a Zeta-Sizer IV (Malvern Instruments, UK) by calculating the average of four measurements at the stationary level in a cylindrical cell. In these measurements the latex particle concentration was 0.03 mg/ml. The particle size of the complexes was also measured with the same device in order to check the colloid stability of these type of particles in specific conditions.

The immunoreactivity of the particles sensitized with different amounts of IgG a-CRP was measured by the changes in the turbidity of the dispersion after several reactions by a spectrometer (Spectronic 601, Milton Roy). 950 µl of the dispersion containing the latex-protein complex was mixed with 50 µl of a solution containing different concentrations of CRP ranging from 0.25 to 40 µg/ml and diluted with saline solution containing 1 mg/ml of BSA (Bovine Serum Albumin) and an ionic strength of 150 mM. The dispersion was stirred and after several minutes the increments in the optical absorbance were measured at a wavelength of 570 nm.

4. Results and discussion

Fig. 1 shows the physical adsorption isotherms of the IgG on the acetal latex at pH 5 and 7, and an ionic strength of 2 mM. The physics adsorption occurs because the latex LKM-1 has not been treated at low pH and, therefore, the surface acetal groups have not been modified to produce the covalent coupling. Both isotherms are of high affinity and display a plateau of around 4.3 mg IgG/m^2, at pH 5 and 7. The similar behaviour showed by the IgG at both pH is a consequence of the polyclonal character of the adsorbed protein, which means a wide range of isoelectric points (i.e.p.). The amount of adsorbed protein is slightly lower as compared with sulfate [11] and sulfonate latexes [8]. This result could be due to the most hydrophilic character of the acetal latex in comparison with the previous one. Similar conclusions were obtained during the characterization of aldehyde latexes [12]. In Fig. 1 we show the amount of protein which remains on the latex surface after the redispersion of the latex–protein complexes at pH 8. The desorption of IgG is significant and the final amount of IgG is around 3.7 mg/m^2, which is the same for both pH.

Once the position of the plateau has been determined, we have studied the maximum adsorption of the protein as a function of the pH. In Fig. 2 we can see that the maximum adsorption appears at pH between 5 and 7, which indicates that the i.e.p. of the complex will be into this pH range. The maximum adsorption found at pH between 5 and 7 confirms our previous results for the adsorption isotherms. To estimate the desorption of IgG, all the latex–protein complexes were redispersed at pH 8. The desorption is maximum at pH around 6 and decreases at pH far from the i.e.p., which indicates the influence of the electrostatic forces in the physics adsorption at basic

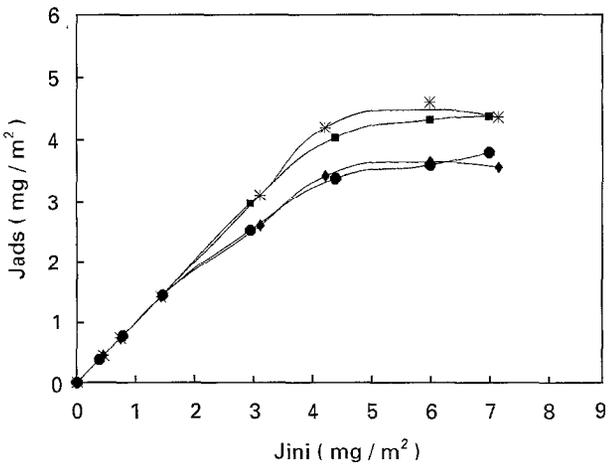

Figure 1 Adsorption isotherms of a-CRP IgG onto acetal latex (ionic strength of 2 mM) and adsorbed protein after the redispersion at pH 8: pH 5 (*) and after desorption (◆); pH 7 (■) and after desorption (●).

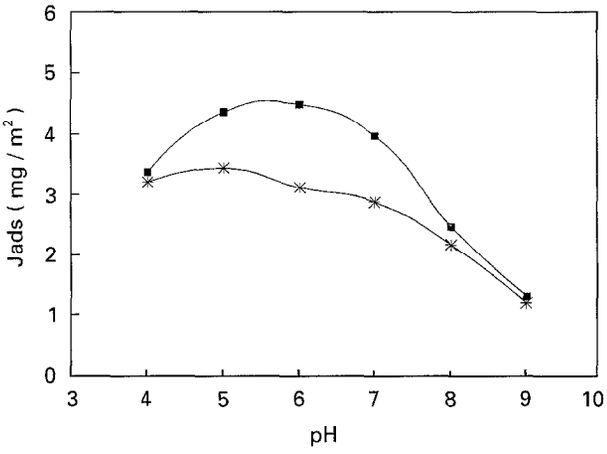

Figure 2 Maximum adsorption of a-CRP IgG onto acetal latex versus the pH (ionic strength of 2 mM) (■) and after redispersion at pH 8 (*).

pH, when both the surface and the protein are negatively charged. From the experiments shown in Figs 1 and 2 we can obtain several latex–protein complexes with different amounts of IgG on the surface, and their immunoreactivities will be compared with those displayed by the complexes with IgG covalently coupled to the surface.

The covalent bound of the IgG to the latex surface takes place at pH 2 by rapid mixture of the latex and the protein. After incubation for 30 min, the pH of the dispersion is moved to pH 5 or 7. At pH 2 the acetal groups change to aldehyde groups and it is possible for the direct reaction with the amine groups of the protein molecules to form a covalent complex. The process is shown in Fig. 3. The imine bound is unstable because it has the tendency to hydrolize and give again amine and aldehyde groups. The imine bound is reduced by the addition of sodium borohydride ($NaBH_4$) [6, 13].

The reaction between the latex and the protein was also assayed by putting the latex at pH 2 during 30 min, in order to change the acetal to aldehyde groups in a first step. As a second step the protein was

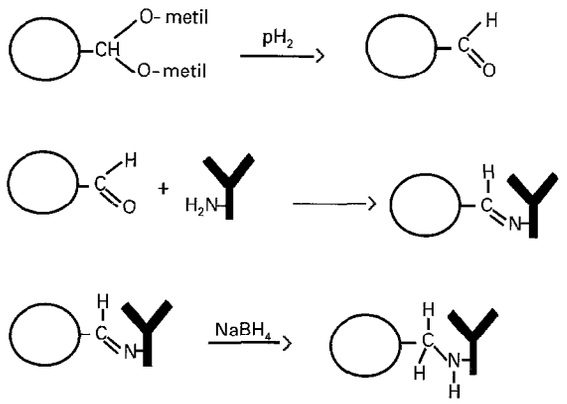

Figure 3 Scheme of the process of activation of the acetal latex and the covalent coupling of the protein.

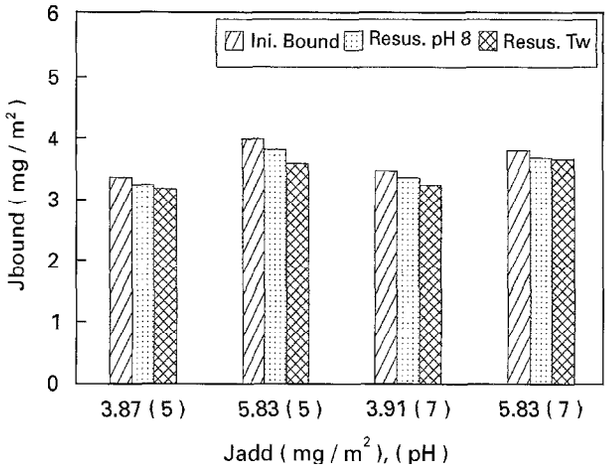

Figure 4 Amount of a-CRP IgG covalently coupled to the acetal latex versus the amount of protein added in solution: initially coupled protein (first column); after redispersion at pH 8 (second column); and after redispersion in the presence of Tween 20 (third column).

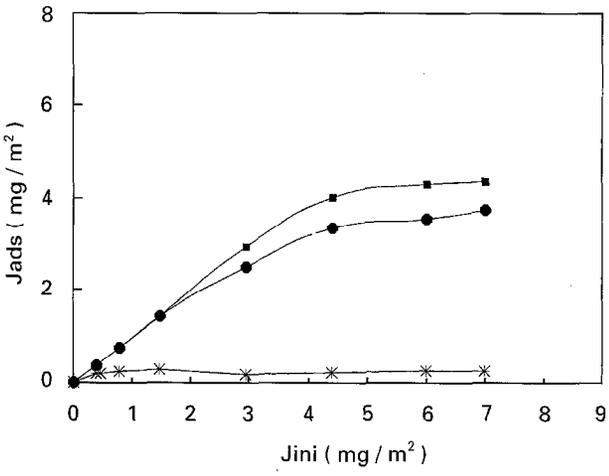

Figure 5 Adsorption isotherms of a-CRP IgG at pH 5 (■) and pH 7 (●) and protein remaining on the latex surface after redispersion in the presence of Tween 20 (∗).

added to the suspension. In this way the amount of coupled protein is around 10% of that found in the previous one, when the latex is activated at the same time that the protein is in the medium. The reason for that behaviour could be the following: at pH 2 the carboxyl groups are protonated and favour the approach of the protein molecules to the acetal surface groups, while at neutral pH (after the activation of the aldehyde groups) there is a repulsion force between the carboxyl groups and the protein molecules, which reduces the covalent bound of the protein to the particle surface.

Fig. 4 shows the amount of IgG covalently coupled to the latex particles as a function of the amount of protein added into the solution. For each experiment we also show the amount of protein which remains on the surface after the redispersion at pH 8 or at the presence of a surfactant (Tween 20). As shown in Figs 1 and 2, the redispersion at pH 8 produces a significant desorption of the IgG physically adsorbed, while in the case of the previously described covalence procedure the desorption is negligible. The resuspension in the presence of a no-ionic surfactant in solution is because the surfactant molecules compete with the protein molecules for the surface and can desorb the majority of the protein physically adsorbed. Fig. 5 shows the comparison of one adsorption isotherm from Fig. 1 (physics adsorption), the amount of IgG that remains on the surface after the redispersion at pH 8 and after the redispersion in the presence of Tween 20. As can be observed all the protein physically adsorbed is practically desorbed after this treatment. In this way we can remove the protein physically adsorbed and detect the final amount of protein which is covalently coupled.

For all the experiments shown in Fig. 4 (and others repeated at the same conditions) the final amount of protein on the polymeric surface is greater than 85% of the total protein initially adsorbed, which means that the majority of the protein molecules are covalently coupled to the latex surface and cannot be removed. Once we have several complexes with different amounts of proteins physically or chemically bound on the surface, the next step is to characterize the sensitized latexes and check their immunoreactivity.

Fig. 6 shows the electrophoretic mobilities of several latex–protein complexes with the protein physically or chemically adsorbed. For comparison we also show the mobility of the bare latex. The complexes with 3.85 or 3.69 mg/m^2 of IgG physically and chemically adsorbed, respectively, display almost identical electrokinetic behaviour with the i.e.p. around pH 4.5. When the amount of protein on the surface is lower, 1.46 mg/m^2, the electrophoretic mobility is greater at neutral or basic pH and the i.e.p. is shifted to lower pH. Thus, the mode in which the protein is (covalently or physically) adsorbed on the particle surface does not influence the net charge of the complex, which could indicate that the conformation is similar in both cases and the number of active sites of the protein directed toward the solution is almost the same.

The electrokinetic results for the complexes with a significant amount of IgG physically or chemically adsorbed seem to indicate that the latex–protein particles will be colloidally stable at neutral or basic pH, while they will be unstable at pH 5 or lower, i.e., near the i.e.p. of the complex. In order to check the stability,

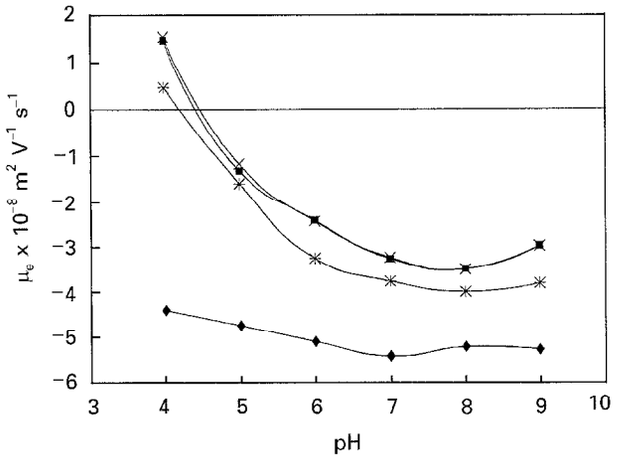

Figure 6 Electrophoretic mobilities of several latex–protein complexes: (◆) bare latex; (∗) 1.46 mg/m^2 of IgG physically adsorbed; (■) 3.85 mg/m^2 of IgG physically adsorbed; and (×) 3.69 mg/m^2 of IgG chemically adsorbed.

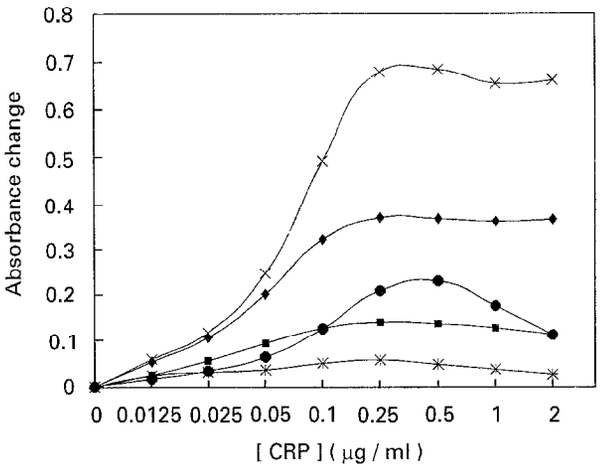

Figure 7 Optical absorbance change versus the amount of CRP in solution, for two latex–protein complexes with physics adsorption and different particle concentrations (N): (∗) 1.46 mg/m^2 (N = 10^{11} part/ml); (●) 1.46 mg/m^2 (N = 2.57 10^{11} part/ml); (■) 3.85 mg/m^2 (N = 10^{11} part/ml); (◆) 3.85 mg/m^2 (N = 2.57 10^{11} part/ml); (×) 3.85 mg/m^2 (N = 5 10^{11} part/ml).

we have measured the particle size of the coverage particles at pH 7 and an electrolyte concentration of 150 mM NaCl. We also show some experiments made at different particle concentration in order to see if the increase in the probability of collision between the particles could modify the stability of the samples. At pH 5 all the samples gave a particle diameter higher than 500 nm, which increased with time. Table I shows the particle size of several latex–protein complexes with the IgG physically or chemically bound to the surface. These sizes have to be compared with the diameter of the bare latex (112 nm). It is possible to observe that the particle size is very similar in all cases and it was constant with time, which confirms our previous expectations about the stability of the latex–protein particles at pH 7 and obtained by different adsorption ways. These complexes were unstable at pH 5, near their i.e.p.

The last part of this work is to detect the immunoreactivity of the complexes and to compare the response of the complexes with the IgG covalently or physically coupled. In the experiments of immunoreactivity we have also checked the effect of the presence of surfactant in the solution and the particle concentration. Fig. 7 shows the changes in the optical absorbance versus the CRP concentration in solution for two complexes of different degree of coverage (1.46 and 3.85 mg/m^2) and several particle concentrations. These changes were obtained after 5 min of reaction with the CRP. The latex with a higher degree of coverage displays a better response which increases as the particle number does. In these cases the change in the absorbance does not show the typical bell curve of the immunoprecipitin reaction and gives a plateau when the CRP concentration is higher than 0.25 μg/ml. This form of the curve could mean a less specific character of the immunological reaction, which seems to saturate the signal changes.

Fig. 8 shows the change in the optical absorbance versus the CRP concentration for a complex obtained by covalent coupling of the IgG (3.32 mg/m^2). In Fig. 8 we show the response after 5 or 10 min of the reaction with the CRP and a particle concentration of 1.58 10^{11} part/ml. The response is similar to that previously shown in Fig. 7 for a similar particle number and a slightly higher amount of protein on the surface (3.85 against 3.32 mg/m^2). After 10 min of reaction the signal increases but continues appearing in the plateau at higher concentrations of CRP. In Fig. 8 we also show the changes in the optical absorbance when the

TABLE I Partical size of several latex–protein complexes obtained by physical adsorption or covalent bound of the IgG, and for several particle concentrations

JIgG bound (mg/m^2)	Bound type	Particle concentration (part/ml)	Particle size (nm)
1.46	Physics	1.0×10^{11}	133
1.46	Physics	2.6×10^{11}	154
3.85	Physics	1.0×10^{11}	148
3.85	Physics	2.6×10^{11}	152
3.85	Physics	5.0×10^{11}	157
3.69	Covalent	1.7×10^{11}	144
3.69	Covalent	2.6×10^{11}	179
3.67 (with Tw)	Covalent	1.7×10^{11}	151
3.26 (with Tw)	Covalent	2.6×10^{11}	152
3.60 (with Tw)	Covalent	5.0×10^{11}	131

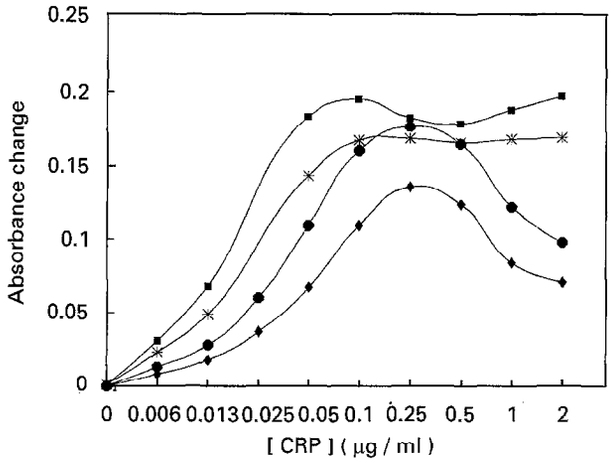

Figure 8 Optical absorbance change versus the amount of CRP in solution, for a complex redispersed without Tween 20 (3.32 mg/m^2: (∗) after 5 min, and (■) after 10 min) and with Tween 20 (3.01 mg/m^2: (◆) after 5 min, and (●) after 10 min).

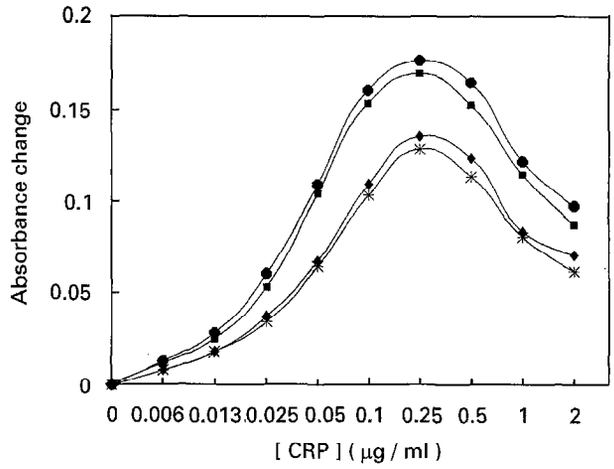

Figure 9 Optical absorbance change versus the amount of CRP in solution, for a complex redispersed in the presence of Tween 20: after 5 min, with (∗) and without (◆) Tween in the reaction medium; after 10 min, with (■) and without (●) Tween in the reaction medium.

complexes are resuspended in the presence of surfactant (Tween 20) in order to remove the protein physically adsorbed. In this case the coverage degree diminished to 3.01 mg/m^2 and the response after 5 min is lower than before, although now the curve shows the typical bell form of the immunoprecipitin reaction, which could mean a larger sensitivity for the antigen–antibody reaction. After 10 min the response is higher but with the same form of the curve. For these results the change in the absorbance is lower than without resuspension in the presence of surfactant because there is less IgG on the surface (3.01 mg of protein in comparison with 3.32 mg/m^2).

We have also checked the effect of the presence of surfactant in the reaction medium. Fig. 9 shows the results of immunoreactivity when the latex–protein complexes were redispersed in the presence of Tween 20 to remove the protein physically adsorbed, before the immunological reaction. After the cleaning of the complexes to eliminate the desorbed protein and the excess of surfactant in the solution, the reaction took place with and without surfactant in the medium. In this case the presence or not of surfactant does not influence the final results because the effect of Tween 20 was produced during the first redispersion when the protein physically adsorbed was removed by the surfactant. The results were practically the same after 5 and 10 min of reaction. Thus, if we redisperse the complexes in the presence of surfactant, its later addition to the solution does not modify the results about immunoreactivity.

Fig. 10 shows the effect of the presence of a surfactant when the immunological reaction takes place with and without Tween 20 in the medium. In this case the latex–protein particles were not in contact with the surfactant before the immunological reaction. When the surfactant is in the reaction medium, at low CRP concentrations the slopes of the curves are lower, which means a low sensitivity. As a simple explanation, this result could be due to a lower amount of protein on the surface, but the diminution from 3.32 to 3.01 mg/m^2 in the amount of protein is too small to

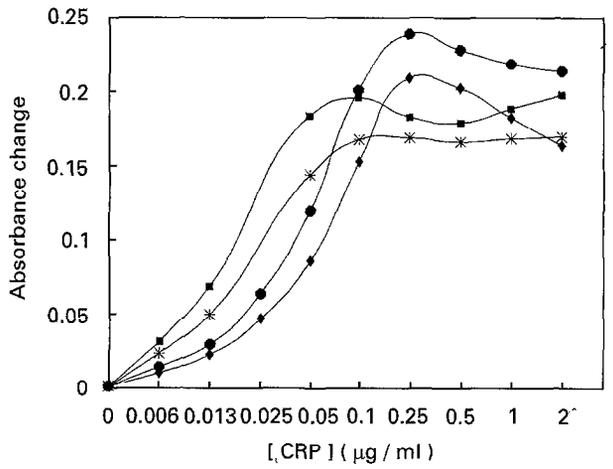

Figure 10 Optical absorbance change versus the amount of CRP in solution, for a complex non-redispersed with Tween 20: after 5 min, with (◆) and without (∗) Tween in the reaction medium; after 10 min, with (●) and without (■) Tween in the reaction medium.

explain this change in the optical absorbance. The presence of free IgG and surfactant molecules could be the reason for that behaviour. On the one hand, some of the added CRP molecules could react with the free IgG molecules in solution, which are produced by the desorption from the particles because of the presence of Tween 20. This reaction between the proteins in solution does not produce changes in the optical absorbance which only takes into account the reaction between the CRP molecules and the IgG molecules linked to the particles. On the other hand, the surfactant could diminish the antigen–antibody reaction because the surfactant molecules could screen the specific sites of the antibodies adsorbed on the particle surface [14].

However, in Fig. 10 we can also see that for the two times assayed (5 and 10 min), the absorbance change is higher in the range of the maximum signal and seems to show a decrease in the absorbance at larger CRP concentrations. This result could indicate that the protein desorbed for the presence of the surfactant

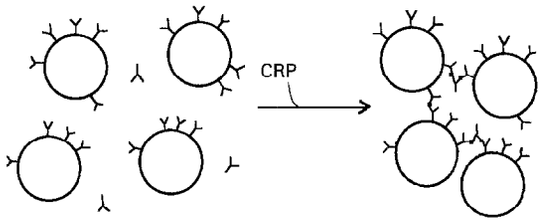

Figure 11 Scheme of the bridging effect by the IgG molecules desorbed from the surface and saturated by antigen.

would participate in the immunological reaction improving the final signal: the protein in solution is now surrounded by a great number of antigen molecules which react with the antibodies before the reaction with the IgG of the particle surface. The high concentration of antigen makes possible the usual immunological reaction but, furthermore, the IgG saturated by antigen can also act like a bridging agent to produce the aggregation of the particles, as we show in the scheme of Fig. 11.

In short, the preparation of acetal latex has permitted to adsorption a significant amount of proteins in a covalent way. This reaction takes place in a simple way by changing the pH of the suspension to pH 2 and no preactivation is necessary. The latex–protein complexes so obtained show a good immunological response which is not disturbed by the presence of surfactant in the reaction medium. Besides, only the complexes with the protein covalently coupled show immunological curves that follow the typical form of the immunoprecipitin reaction.

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