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Acetal-functionalized polymer particles useful for immunoassays. III: Preparation of latex-protein complexes and their applications

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Monodisperse polymer colloids with dimethyl and diethyl acetal functionalities synthesized by a two-step emulsion polymerization process were chosen as the polymeric support to carry out covalent coupling with the antibody IgG anti C-reactive protein, and to test the utility of the latex-protein complexes formed in immunoassays with the specific CRP antigen. More than the 80% of the initially linked protein was covalently coupled in all of the latexes. The agglutination reaction was followed by turbidimetry. With the aim of analyzing the effect of some of the variables of the immunological reaction, the reaction time, the particle concentration and the coverage degree of protein in the complexes were varied.

1. Introduction

Polymeric colloids synthesized by emulsion polymerization are currently finding new applications in the biomedical field. By controlling the experimental conditions of the emulsion polymerization process, one can obtain colloidal systems with particle sizes, monodispersities and specific surface characteristics required for their use in biomedical applications. Moreover, colloidal systems comprise small polymeric particles suspended in an aqueous medium, having a high surface area. For all of these reasons, polymeric particles are being used as carriers of biomolecules, such as proteins, enzymes, etc. Among the variety of applications of supported biomolecules on the surface of polymeric particles are immunodiagnosis tests [1–15] and the identification, quantification and separation of cells [16–21].

In the first paper of this series [22] several core-shell type monodisperse latex particles with surface acetal groups were synthesized by means of a two-step emulsion polymerization in a batch reactor. The effect on the amount of functionalized groups of the thickness of the shell, the pH of the reaction medium and the weight ratio of the termonomers in the shell, were analyzed. The results showed that when the reaction medium was acid, the amount of carboxyl surface groups was higher than in neutral medium. By increasing the thickness of the shell, the amounts of carboxyl and aldehyde surface groups were increased. The weight ratio of the termonomers used to prepare the shell affected the amounts of COOH and CHO groups when increasing the content of styrene in the recipe with respect to the acetal monomer. Otherwise, the changes were not significant. The latex particles synthesized were found to be useful in the covalent bonding of IgG protein leading to latex-protein complexes with immunological response.

In the second part of this series [23], and by using the results mentioned above, the surface characterization of the acetal functionalized polymer particles was extended with the aim of analyzing the long-term stability of these groups, the hydrophilic character of the latex surface and the colloidal stability of the latexes synthesized. The acetal- functionalized latexes were characterized by transmission electron microscopy (TEM) and conductimetric titration, in order to obtain the particle size distribution and the amount of carboxyl and acetal groups on the surface, respectively. The chemical stability of the functionalized surface groups during storage was studied. The hydrophilic character of the surface of the polymer particles was determined by means of non-ionic emulsifier titration. The colloidal stability of the latexes was studied by measuring the critical coagulation concentration (CCC) against KBr electrolyte, and the existence of a hairy layer on the surface of the latex particles was analyzed by measuring the hydrodynamic particle diameter at several electrolyte concentrations. The surface functionalized groups remained stable for two years. The relative hydrophilic character and the colloidal stability were affected by the pH of the medium. On the other hand, the higher the surface charge, the larger the thickness of the hairy layer.

This work is devoted to the application of acetal-functionalized latex particles in immunoassays and is the third part of the series.

An immunoassay is based on the specific reaction between an antigen and an antibody. When antigens or antibodies are bonded to the surface of the latex particles,

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the immunological reaction is amplified due to the agglutination of the particles. By means of the immunoassay, depending on the type of antigen or antibody used, a great variety of diseases can be detected.

The fixation between the biomolecules and the surface of the polymeric particles can be physical or chemical. Singer and Plotz [24] were pioneers in the fixation of proteins to polymeric surfaces, working on the physical adsorption of proteins on polystyrene particles. The latex-protein complexes obtained in this way can provoke unspecified reactions due to the hydrophobic character of the polystyrene particles adsorbing proteins in an unspecified way on free parts of the surface. Moreover, the complexes have low stability and can give unspecified agglutinations, resulting in false diagnosis. Some authors [2, 19] gave some hydrophilic character to the surface of the latex particles in order to eliminate the unspecified interactions. On the other hand, the addition of an emulsifier can prevent these interactions, but the emulsifier desorbs the proteins that are physically adsorbed on the surface of the particles [9, 10, 25]. As a consequence of this desorption the immunological capacity of the complexes decreases.

Therefore, the latex-protein complexes obtained by physical adsorption have a limited applicability in immunodiagnosis due to the partial desorption of the protein. The desorption of proteins that are on the surface of the particles does not occur by simple dilution. Rather, proteins can be removed from the surface by increasing the ionic strength of the medium [26] or by using emulsifiers. The ionic strength has a pronounced effect on the adsorption of proteins onto charged polymeric surfaces due to the electrostatic interactions. By increasing the ionic strength, the electrostatic forces between the protein and the polymer surface decrease, causing the desorption of the protein. The use of emulsifiers prevents unspecific interactions and it is necessary to clean the automatic clinical apparatus and to eliminate bubbles.

On the other hand, the physical adsorption process provokes a high decrease in the immunological capacity of the adsorbed biomolecules due to the structural rearrangements they suffer during the adsorption process [26].

Nowadays, a new type of polymeric particles with surface functional groups, able to react with the functional groups of the proteins resulting in a chemical bonding, has been developed. The covalent bonding between proteins and surface functional groups of the polymeric support prevents the partial desorption of the bonded protein and maintains the native conformation of the protein [27]. The functional groups mainly used to produce covalent bonding with proteins are: aldehyde [20, 21, 28–34], amino [35], carboxylic acid [16, 36, 37], hydroxyl [38], chloromethyl [39–41] and acetal [9–11, 22, 23, 42].

In this work, latexes with acetal functionality were chosen as the polymeric support to carry out the covalent bond with a protein, the IgG a-CRP (antibody, IgG anti-C reactive protein). These latexes have carboxylic surface groups in addition to the acetal ones. Due to the presence of these two different functional groups the latexes can be used to bond by two different mechanisms. The first, by means of the carboxylic acid surface groups uses the carbodiimide method [37]. The second, used in this work, transforms the surface acetal groups to aldehydes by acidification [10]. The aldehyde groups thus formed react with the proteins directly, and without preactivation producing imines. These imines have an unstable double bond that could suffer hydrolysis with the risk of even removing of the protein from the latex surface. To avoid this, the double bonds are reduced to single bonds by means of a reducing agent [33].

During the process of covalent coupling the adsorption or physical bonding of proteins is also produced. It is therefore necessary to carry out a desorption process to remove the adsorbed proteins and to ensure that the proteins that are on the surface of the polymeric particles are covalently bonded. The addition of an emulsifier to the medium in which the latex-protein complexes are dispersed will force the desorption of the physically bonded protein [9, 10].

Once the latex-protein complexes are obtained it is possible to carry out the immunoassays. The immunoassays consist of the addition of specific antigens or antibodies to the medium in which the corresponding late-antibody or latex-antigen, is dispersed. The antigens and antibodies react specifically causing the agglutination of the complexes. The degree of agglutination of the complexes is determined as a function of the concentration of antigen or antibody added.

The reaction of agglutination of the complexes that occurs during the immunoassay is followed and determined by optical methods. Hence the polymeric particles must be monodisperse. Moreover, the monodispersity ensures the uniform distribution of proteins on the surface of the polymeric carrier. Therefore, monodispersity is an essential factor in this immunological application [43].

The agglutination reaction produced during the immunoassay can be detected by different optical methods: the visual method and those based on instrumental techniques. The visual method [1–5] is the simplest and it is also cheaper because it does not require special equipment. The immunoassays are made on plates or in tubes and the agglutination is observed visually. These tests are subjective, and they are therefore not quantitative. Among the instrumental techniques generally used to detect the agglutination produced during an immunoassay there are: turbidimetry [1, 6–9], nephelometry [10–12], particle recount [13], and light scattering [14, 15]. By using an appropriate apparatus, an accurate and sensitive assay is achieved, which can also be automated. The sensitivity and accuracy of the immunoassay depends on the method used to detect the agglutination.

In this work the reaction of agglutination was followed by turbidimetry, i.e., by measuring the changes in the turbidity or optical absorbance by using a spectrophotometer. This technique was chosen because it gives high sensitivity and it needs small amounts of sample.

2. Materials and methods

The dimethyl and diethyl acetal-functionalized core-shell latex particles were synthesized by a two-step

emulsion polymerization in a batch reactor. The polymerization reaction conditions for the cores and for the core-shell polymer particles, and the polymeric and colloidal characterization of the synthesized latexes, were those reported previously [22, 23].

The latex-protein complexes were prepared using the core-shell latexes with acetal functionality as polymeric carriers. The coupled protein was the antibody IgG a-CRP rabbit polyclonal, kindly donated by the Grupo de Física de Fluidos y Biocoloides of the University of Granada (Spain). This polyclonal antibody has the isoelectrical point over a wide range of pH (6–8).

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 $-20\,^{\circ}C$ until used. The concentration of protein was determined by measuring the optical absorbance at 280 nm (Spectronic Genesys 5 UV spectrophotometer).

To obtain the latex-protein complexes, the chosen latexes were previously cleaned by means of serum replacement. The latexes were LS2, LS3 and LKMBl, all of them with dimethyl acetal functionality and the latex LD2, having diethyl acetal functionality. In the preparation of the latex-protein complexes, for each of these latexes, the concentration of added protein was varied. The remaining conditions and experimental variables (amount of sensitized latex, pH of sensitization, activation time, sensitization and reduction) were held constant.

The experimental procedure followed to prepare the latex-protein complexes coupled covalently consisted of several steps [44]. Firstly, the amounts of IgG a-CRP of known concentration were added to the latex samples ($0.4\,m^2$ of latex and water to complete a total volume of 4 ml) in glass tubes. For each latex, a blank without protein was prepared. After this, the acetal groups were activated. This step consisted of the transformation of acetal group into aldehyde. The activation took place during 30 min by acidificating the medium (pH 2) with HCI (Merck).

Once the acetal groups were converted into aldehyde, the sensitization step or protein coupling was carried out. The pH was adjusted to 5 by adding acetate buffer (Merck). The experiments of sensitization were made at pH 5 because under these conditions the approach of the protein to the surface of the latex particle is favored. This approach is due to the attractive electrostatic interactions. At pH 5, the latex surface has negative charge, while the IgG a-CRP has a positive charge at this pH due to its isoelectric point. The conditions in the medium must be just right to allow the protein to approach the surface of the polymeric particles. If they are not, covalent coupling would not be possible or would take place at a very low level. According to Konings *et al.* [45] if there is electrostatic repulsion between the protein and the polymeric surface, it is difficult to produce the covalent bonding of the antibody because it will not penetrate the electric double layer of the particle. However, if the electrostatic interaction is attractive, the covalent coupling will be favored.

The coupling was carried out under longitudinal agitation in a thermostatic bath (shaker bath, Grant SS–40 D) at $25\,^{\circ}C$ for 2 h.

During the sensitization step the passive adsorption together with the covalent coupling of the IgG a-CRP are produced on the surface of the polymer particles. The covalent coupling takes place through the chemical reaction between the surface aldehyde groups of the latex particles and the amino groups of lisine, one of the aminoacid which constitutes the protein. As mentioned previously, from this reaction an imine is obtained. Imines have an easily hydrolizable and unstable double bond that again gives amino and aldehyde groups. To avoid this hydrolysis, the double bonds were reduced to single bonds by adding a reducing agent, sodium boron hydride (BH_4Na, Sigma), at a level of $20\,mg/m^2$. The reduction step was carried out for one hour at room temperature.

The sensitized latexes were centrifuged (centrifuge Centrikon H-401 Kontrom Hermle) at 17 000 rpm for 30 min at $10\,^{\circ}C$. The latex-protein complexes were separated from the supernatant liquid, which were filtered through 100 nm pore size filters (Nucleopore). By measuring the optical absorbance at 280 nm, in the supernatants of the complexes and in the supenatants of the corresponding blanks, the amounts of protein not coupled to the latex surface were quantified. From the material balance, the amount of protein initially linked (both physically and covalently) to the surface of the polymeric particles was determined. The amount of coupled protein is the amount of the added protein minus the non-coupled protein determined in the supernatant.

The complexes were redispersed ultrasonically, in a solution of GBS at pH 8.2. This solution contains 0.1 M glycine buffer (Sigma), 0.04 mg/ml of sodium azide (NaN_3, Sigma) and 0.17 M NaCl (Panreac). The glycine buffer acts as a blocking agent [27]. The amino group of the glycine can react with the aldehyde groups of the surface which do not react with the amino groups of the protein. The presence of free aldehyde groups at the surface of the particles could give non-specific agglutinations during the immunoassays. The addition of NaCl to the resuspension medium increases the ionic strength of the medium. At high ionic strength, the desorption of the linked protein by electrostatic interactions is favored due to the charge shielding. This desorption process is also favored by the pH of the medium. At pH 8.2, both protein and latex surface (due to the COOH groups) are negatively charged and there is a repulsion between them. Moreover, the pH of the medium contributes to the colloidal stability of the charged latex-protein complexes.

The desorption process of non-coupled protein carried out in the presence of an emulsifier has been studied. Bale Oenick and Warshawsky [39] used sodium lauryl sulfate in complexes with proteins covalently coupled to aldehyde groups. Kapmeyer *et al.* [10] used Tween 20 (poly(oxyethylene (20) sorbitan monolaurate) in complexes with covalently coupled proteins to latexes with acetal functionality. In a previous work [9] we have verified the efficiency of the emulsifier Tween 20 to desorb non-coupled protein.

To study the effect of the presence of emulsifier in the desorption step of the physically adsorbed protein from the surface of the sensitized latexes that takes place during the covalence stage, Tween 20 emulsifier (Merck)

was added (1%) to some of the resuspended latexes. After 24 h, both the latexes resuspended in Tween 20 and the latexes without Tween 20, were centrifuged at 17 000 rpm for 30 min, to separate the complexes and the supernatant. The supernatants were filtered and the optical absorbances of the supenatants and the corresponding blanks were measured at 280 nm to determine the amount of covalently coupled protein by applying a material balance that takes into account the initially (physically and chemically) coupled protein and the desorbed protein.

Finally, the complexes were resuspended in a GB solution (0.4 mg/ml of sodium azide and glycine buffer 0.1 M, pH 8) for storage and subsequent use in the immunoassays.

To carry out the immunoassays, specific antigen CRP was used. This antigen is considered as an indicator of inflammatory processes [10, 12].

The latex-protein complexes were prepared by centrifugation and redispersion in a saline solution of bovine serum albumin (BSA, Sigma) at pH 8. This solution contains 1 mg/ml of BSA, 0.1% w/w of sodium azide and NaCl to adjust the ionic strength to 150 MM (the physiological ionic strength) in a buffered medium at pH 8 with borate buffer (Merck). BSA is an inert protein which is added with the objective of being adsorbed on the hydrophobic surface zones of the complexes and, in this way, unspecific reactions are avoided.

The immunoassays consisted of the mixing of 950 µl of the former suspension containing the latex-IgG a-CRP complex at a known concentration and 50 µl of the specific CRP antigen at different concentrations. The agglutination reaction was detected by turbidimetry, by measuring the changes in the optical absorbance at 570 nm in the spectrophotometer after 5 and 10 min of reaction. The increment in absorbance was determined by subtraction of the absorbance of a blank (the complex redispersed in the former solution but without antigen) to the measured absorbance. By measuring the absorbance of the blank, the colloidal stability of the complexes in the resuspension medium, in which the immunological reaction takes place, is proven.

To carry out the immunoassays, LKMB1–IgG a-CRP and LD2–IgG a-CRP complexes with dimethyl and diethyl acetal functionality, respectively, were chosen.

3. Results and discussion

Figs 1 to 4 show the amounts of protein initially linked to the surface of the latexes and the amount of protein which remains coupled after the desorption process, in the presence and absence of Tween 20, against the added protein to the different latexes used in the preparation of the latex-protein complexes. In all of the latexes, except LKMB1, the desorption induced on redispersion of the complexes by increasing the ionic strength in the absence of Tween 20, was practically negligible, since the amount of protein that remains on the surface of the latex particles after this process has practically that initially added. The latex LKMB1 showed a higher desorption (Fig. 3). This latex has a high surface charge density

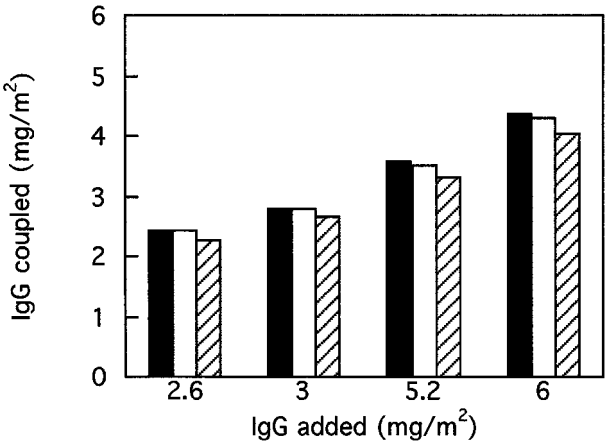

Figure 1 Amount of IgG a-CRP coupled to the latex LS2 versus the concentration of added protein. (■) initially linked protein; (□) protein that remains on the latex surface after the desorption without Tween 20; (▨) protein that remains on the latex surface after the desorption in the presence of Tween 20.

(71.34 µC/cm^2), higher than that of the other latexes (LS2, 16.21 µC/cm^2, LS3, 11.97 µC/cm^2, LD2, 10.42 µC/cm^2). Its high surface charge density favors the coupling of proteins by means of electrostatic interactions during the sensitization stage, and as the ionic strength increases, these interactions disappear causing the desorption of the protein. In the desorption process in the presence of Tween 20, in all of the cases, the amount of desorbed protein was higher than that in the absence of emulsifier, although the difference is very slight.

Fig. 5 shows that the final amount of protein on the surface of the particles, after desorption with Tween 20, was higher than 80% with respect to the Initially linked protein for the latex LKMB1 and 90% for the rest of the latexes. This means that the majority of the initially linked protein on the surface of the latex particles is covalently coupled.

Figs 1 to 4 show the relationship between the added and the linked IgG a-CRP protein. In all of the cases, by

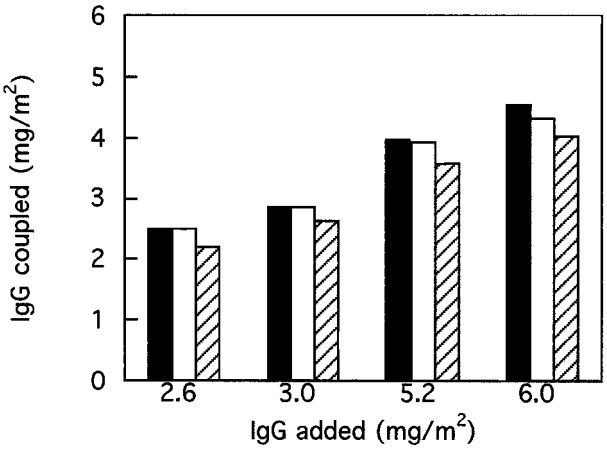

Figure 2 Amount of IgG a-CRP coupled to the latex LS3 versus the concentration of added protein. (■) initially linked protein; (□) protein that remains on the latex surface after the desorption without Tween 20; (▨) protein that remains on the latex surface after the desorption in the presence of Tween 20.

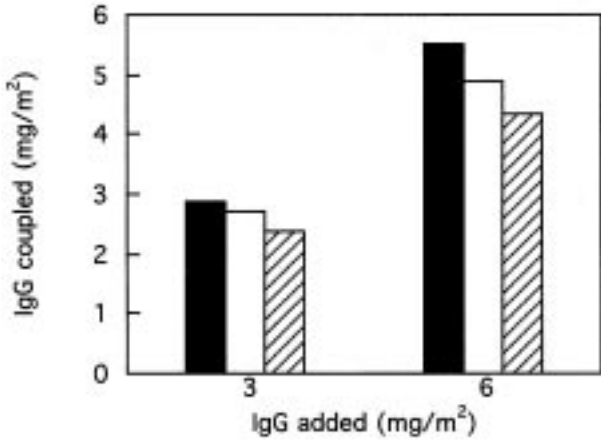

Figure 3 Amount of IgG a-CRP coupled to the latex LKMBI versus the concentration of added protein. (■) initially linked protein; (□) protein that remains on the latex surface after the desorption without Tween 20; (▨) protein that remains on the latex surface after the desorption in the presence of Tween 20.

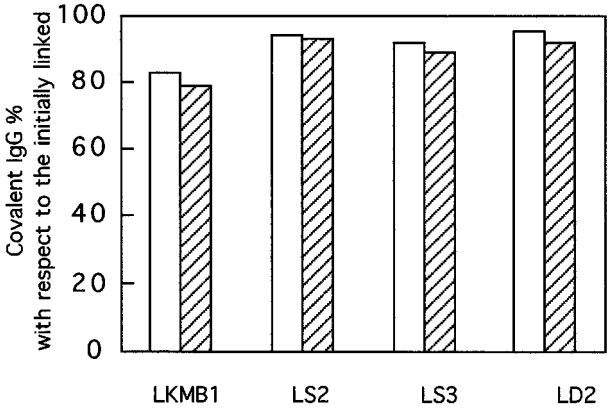

Figure 5 Covalently coupled protein (after desorption with Tween 20) with respect to the initially linked protein, for the different latexes: (□) 3 mg/m^2, and (▨) 6 mg/m^2 of added IgG a-CRP.

increasing the concentration of added protein, the amount of initially linked protein increased, and since the desorption was very low, the amount of covalently coupled protein also increased. Nevertheless, the amount of protein initially linked with respect to that added decreased when the latter increased as is shown in Fig. 6. In this figure the amount of initially linked protein with respect to that added (3 and 6 mg/m^2) for the different latexes is shown. All of the latexes, except for the latex LKMB1, showed a similar behavior. When the concentration of the IgG a-CRP added was low (3 mg/m^2), almost all of the protein was linked to the latex particles. However when the added concentration was higher (6 mg/m^2), not all of the protein was linked to the particles, part of it remained in the solution. This can be due to the fact that this concentration would be above the proteinic saturation at the surface of the particles. In the case of the latex LKMB1, when the concentration of IgG a-CRP was 6 mg/m^2, the amount of linked protein was similar to that obtained when 3 mg/m^2 was added. This latex has the highest surface charge density which favors the electrostatic interactions between the polymeric surface and the proteins. This fact was verified previously by the higher desorption found in this latex compared to the other latexes.

Fig. 7 confirms the trend observed in Fig. 6; there was a higher amount of coupled protein when the concentration of added IgG a-CRP was lower. On the other hand, there was no dependence between the amount of functional surface groups and the amount of protein covalently coupled. Latex LS3 had a lower amount of aldehyde groups ($1.13\,10^{-7}$ mEq/cm^2) than latex LS2 ($1.86\,10^{-7}$ mEq/cm^2). However, it was able to bond similar amounts of protein. Moreover, there is a relationship between the charge and the bonded protein. Latex LKMB1 presented differences compared to LS2 and the other latexes, this latex having the highest surface charge density and an amount of aldehyde groups ($1.81\,10^{-7}$ mEq/cm^2) similar to that of latex LS2. The difference is due to the amount of desorbed protein, higher in the latex LKMB1. Therefore, there was a dependence between the covalently coupled protein, the

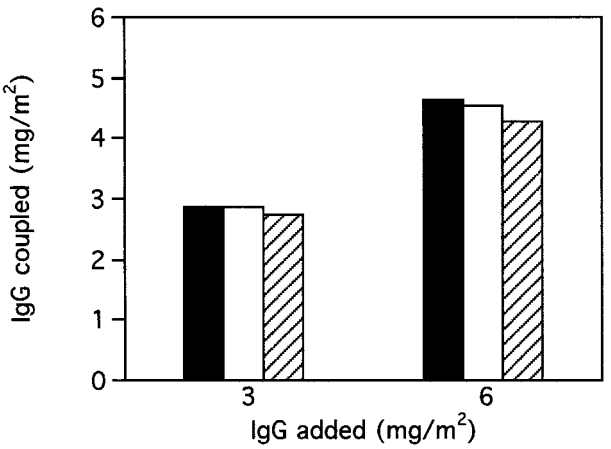

Figure 4 Amount of IgG a-CRP coupled to the latex LD2 versus the concentration of added protein. (■) initially linked protein; (□) protein that remains on the latex surface after the desorption without Tween 20; (▨) protein that remains on the latex surface after the desorption in the presence of Tween 20.

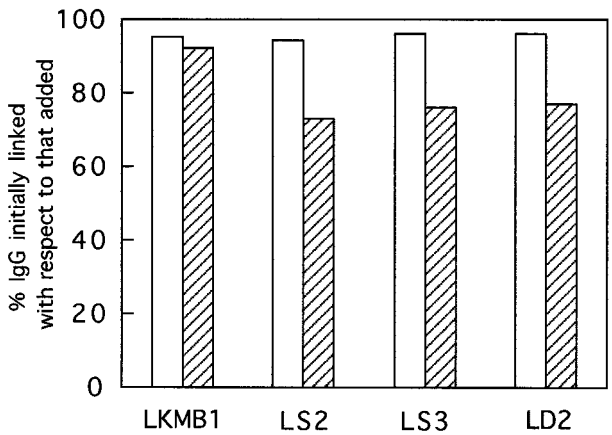

Figure 6 Amount of IgG a-CRP initially linked with respect to that added, for the different latexes: (□) 3 mg/m^2, and (▨) 6 mg/m^2 of added IgG a-CRP.

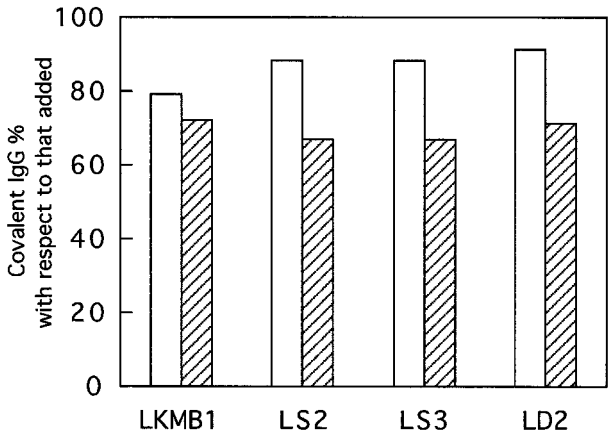

Figure 7 Amount of protein covalently coupled with respect to the amount added for the different latexes: (□) 3 mg/m^2 and (▨) 6 mg/m^2 of added IgG a-CRP.

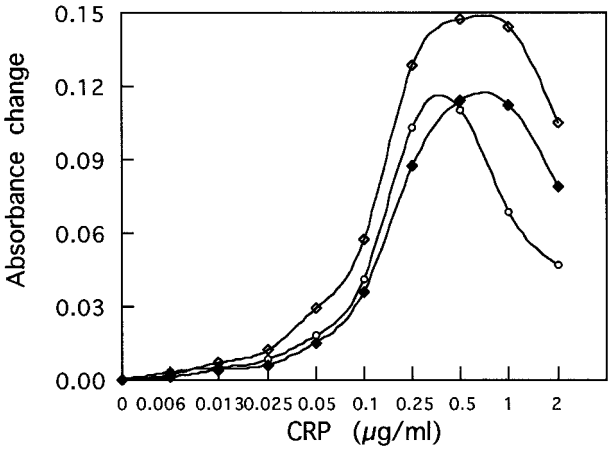

Figure 8 Immunological response of the complex LKMB1-protein versus the concentration of CRP. Effect of the particle concentration: 1.6 10^{11} particles/ml (○) after 10 min, and 2.4 10^{11} particles/ml (◆) after 5 min, and (◇) after 10 min.

added one and the surface charge density of the latex. By increasing the amount of added protein, that of covalently coupled protein decreased. When the surface charge density was high, the amount of covalently coupled protein also decreased.

To determine if the latexes were stable during the process of covalency, the diameters of the latex-protein complexes were measured by light scattering, a constant size being found during the reaction.

As reported in our previous work [9], the immunoassays carried out with complexes that had been treated with Tween 20 in the desorption stage, gave immunological response with the typical bell of immunoprecipitin. Hence, this type of complex was used in the following immunoassays. Moreover, these complexes were not affected by the use or not of emulsifier in the reaction medium, therefore the immunoassays were carried out without emulsifier.

Fig. 8 shows the immunological response of the LKMB1-protein complexes after 5 and 10 min of reaction with different concentrations of CRP. The complex had 2.38 mg/m^2 of coupled IgG a-CRP. The effect of the concentration of particles used in the immunoassays was studied using 1.6 10^{11} and 2.4 10^{11} particles/ml. By increasing the concentration of particles, the immunological response increased. In these complexes the absorbance of the blanks remained constant during the immunoassays. Therefore, the complexes can be considered colloidally stable under the conditions of the immunological reaction. In Fig. 8, the effect of the reaction time is also shown. On increasing the reaction time, the immunological response increased.

To study the dependence of the immunological response with the coverage degree of the IgG a-CRP in the complexes, immunoassays with two different LKMB1-protein complexes, having 2.38 and 4.34 mg/m^2 of coupled protein, respectively, were carried out. These immunoassays are shown in Fig. 9. On increasing the surface coverage of the IgG on the polymeric particles, the response increased. It was also observed that the higher the reaction time, the higher the immunological response.

Fig. 10 shows the immunoassays carried out with the LD2-protein complex, having 2.73 mg/m^2 coupled

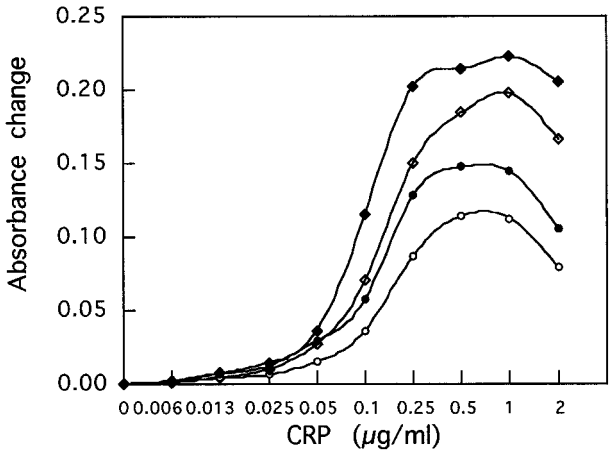

Figure 9 Immunological response of the complex LKMBI-protein versus the the concentration of CRP. Effect of the coverage degree: 2.38 mg/m^2 (○) after 5 min, and (●) after 10 min; and 4.34 mg/m^2 (◇) after 5 min, (◆) after 10 min.

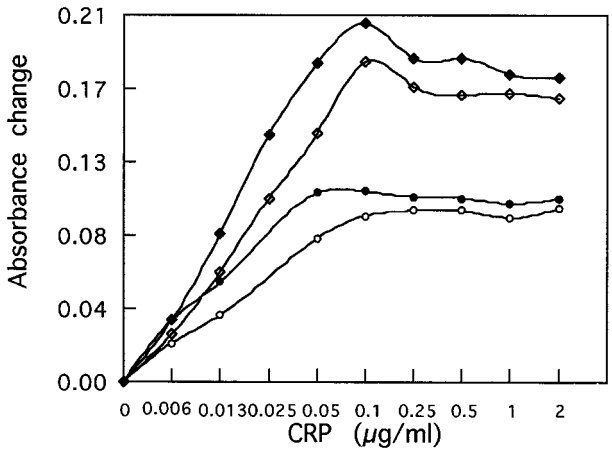

Figure 10 Immunological response of the complex LD2-protein versus the concentration of CRP. Effect of the particle concentration: 1.6 10^{11} particles/ml (○) after 5 minutes, and (●) after 10 min; and 2.4 10^{11} particles/ml, (◇) after 5 min, and (◆) after 10 min.

protein. The results were similar to those obtained with the LKMB1 complexes. By increasing the concentration of particles and the reaction time, the response increased. These complexes did not have a well defined bell type curve at the end. At high concentrations of CRP, this would be a sign of colloidal instability. However, the blank maintained a constant absorbance value indicating that the complexes were stable.

4. Conclusions

The utility of latexes with acetal functionality to be used in tests of immunodiagnosis has been demonstrated.

The IgG a-CRP antibody was coupled covalently to the surface of the polymer particles. This reaction takes place, without preactivation, by changing the pH of the suspension. During the covalent coupling there was also a physical adsorption of protein on the particle surface. To remove the physically adsorbed protein, Tween 20 emulsifier was used together with an increment of the ionic strength. The desorption was higher in the latex with highest surface charge density. The amount of coupled protein depended on the concentration of that added. More than 80% of the initially linked protein was covalently coupled in all of the latexes.

Once the latex-protein complexes were prepared, the immunoassays were carried out with the specific antigen CRP. The agglutination reaction was followed by turbidimetry measuring the changes in optical absorbance. Some of the variables of the immunological reactions were analyzed. These variables were: the reaction time, the particle concentration and the surface coverage of protein in the complexes. The higher the reaction time, the particle concentration and the coverage degree, the higher the immunological response.

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