Functionalized Monodisperse Particles with Chloromethyl Groups for the Covalent Coupling of Proteins

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ABSTRACT: The functionalized core−shell monodisperse latex particles with surface chloromethyl groups were synthesized by means of a two-step emulsion polymerization process in a batch reactor at two reaction temperatures. In a first step, the core was synthesized by means of a batch emulsion polymerization of styrene (St), and in the second step, the shell was formed by batch emulsion copolymerization of St and (chloromethyl)styrene (CMS) using the seed obtained previously. The latexes were characterized by TEM and conductopotentiometric titrations in order to obtain the particle size distribution and the amount of the different surface groups, respectively. Covalent binding of protein to chloromethyl groups was studied using lysozyme at two pHs, 7 and 11. Two different methods were used to determine the amount of protein covalently bound or physically adsorbed: desorption with surfactants, and chloride ions release upon chemical binding.

Introduction

The attachment of biological molecules through covalent coupling to a solid support is a common procedure in various fields such as affinity chromatography, peptide or oligonucleotide synthesis and fermentation technology, in vitro sensing, etc. Polymer latexes, usually with antibodies or antigens attached to their surfaces, are often used as elements of diagnostic tests. Selected examples are given below including detection of antibodies against HIV,[1] hepatitis,[1,2] salmonella, and trichinella,[3] etc.

Binding of proteins to latex particles can be accomplished by physical adsorption or covalent coupling. A large variety of latexes are described with functional surface groups suitable for covalent immobilization of protein and macromolecules. Some of these groups must be activated prior to protein immobilization (e.g., hydroxyl groups with cyanogen bromide and carboxyl groups with carbodiimide[4,5]). Other groups could be used for protein immobilization without additional activation (e.g., aldehyde,[6−8] succinimide,[9] and vinylbenzyl chloride[10−11]). The potential to improve reagent shelf life and more defined orientation of bound ligand are the main advantages of covalent attachment.

In a standard technique of immunoassay, washing buffers containing Tween 20 or other surfactants are used to remove loosely bound proteins from the device surface and to avoid the nonspecific adsorption of proteins such as the first and the second antibodies, etc. on the surface.[12−14] Since there is a possibility that adsorbed proteins may be desorbed by surfactants,[15,16] it is advisable to use covalent coupling of the antibodies.

The synthesis of polymer particles with surface chloromethyl groups and by different reaction conditions has been investigated previously. Thus, homopolymers or copolymers, even terpolymers of (chloromethyl)styrene and other monomers (styrene, vinylnaphthalene, acrylic acid, 2-hydroxyethyl acrylate, etc.), were synthesized by emulsion polymerization[17,18] or by dispersion polymerization.[19,20]

Although little has been published, some authors have investigated the side reactions that the chloromethyl group could undergo in the aqueous phase: hydrolysis of the chloromethyl group into a hydroxymethyl group and cross-linking reaction by radical transfer to the polymer chain.[17]

In the present work we describe the synthesis and characterization of polystyrene particles with chloromethyl groups. We have used these latexes to study the covalent immobilization of the model protein lysozyme, monitoring the extent to which the protein is bonded to the latex particles.

Experimental Section

Materials. The monomer St was distilled under reduced pressure. (Chloromethyl)styrene (CMS) was purified by washing with a 0.1% sodium hydroxide solution or was used as received. Both monomers were stored at −18 °C until used. All the other materials were used as received. Potassium persulfate (Fluka) was used as initiator in the polymerizations of the polystyrene seeds. Potassium persulfate (Fluka) and sodium disulfite (Panreac) were used as the components of the redox initiator system in the polymerization of the shells. Sodium dihexyl sulfosuccinate (Aerosol MA-80, Cyanamid) and sodium hydrogen carbonate (Merck) were used as surfactant and buffer, respectively. Double deionized (DDI) water was used throughout the work.

Lysozyme (Sigma) was dialyzed prior to use in order to remove salt impurities (mainly Cl^-, which can affect the determination of the covalently bound quantity). The buffers used were phosphate pH 7 and borate pH 11 (both 2 mM ionic strength).

Sodium dodecyl sulfate (SDS) and Tween 20 (Poly(oxyethylene(20)) Sorbitan Monolaurate) were purchased from Merck and used without further purification.

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Table 1. Recipes Used To Obtain the Seeds (S1, S2, S3) and Shells (JX1, JX2, JX3)

	S1	S2	S3	JX1	JX2	JX3
seed (150 g)				S1	S2	S3
T (°C)	90	90	90	80	60	60
amt of St (g)	526	526	526	9.2	9.2	0
amt of CMS (g)				9.2	9.2	18.4
amt of DDI water (g)	1250	1250	1250	30	30	30
amt of Aerosol MA80 (g)	16.94	16.94	16.94			
amt of $NaHCO_3$ (g)	2	2	2			
amt of $Na_2S_2O_5$ (g)				0.224	0.224	0.224
amt of $K_2S_2O_8$ (g)	2	2	2	0.329	0.329	0.329
rpm	250	250	250	200	200	200
reacn time (h)	24	24	24	7	4	4

Table 2. Results of the Reactions Carried Out To Obtain the Seeds and Shells

	S1	S2	S3	JX1	JX2	JX3
d_v (nm)	209	185	185	227	201	204
PDI	1.0052	1.0064	1.0042	1.0044	1.0032	1.0045
$[Cl^-]$ (μmol/g pol)				21	59	121
σ_0 (μC/cm^2)	nd	nd	nd	−2.7	−3.0	−3.1
conversion	total	total	total	total	total	Total
final pH	7.5	7.5	7.5	1.8	2.2	2

Polymerizations. Emulsion polymerizations were carried out in a 1 or 2 L thermostated reactor fitted with a reflux condenser, stainless steel stirrer, sampling device, and nitrogen inlet tube.

All of the core−shell polymer particles were obtained by a two-step emulsion polymerization process. In the first step, the core of the polymer particles (the seed used in the second step) was prepared at 90 °C by batch emulsion homopolymerization of St using the recipe given in Table 1. After polymerization, the seed was kept overnight at 90 °C to decompose the initiator.

The seeded batch emulsion copolymerization of St and CMS were carried out to put the shell onto the polystyrene core synthesized in the first step, using the recipes given in Table 1 as well. Two different reaction temperatures were used, 60 and 80 °C.

The weight ratio of the comonomers was 1/1 in runs JX1and JX2, and in run JX3 the shell formation was carried out by emulsion homopolymerization of CMS, maintaining the same monomer/water weight ratio. The redox initiator concentration was 3% of the total monomer. The seed obtained in the first step was not cleaned; hence no emulsifier was used in the polymerization of the shells. The seeds used as the core of the latexes JX1, JX2, and JX3 were S1, S2, and S3, respectively. The weight of polymer in the seed was 45 g in all the reactions carried out to synthesize the shells.

DDI water, buffer solutions, and seed were charged into the reactor and purged with nitrogen for 20 min. Monomers were added, and the system was stirred and purged with N_2 for 1 h at 30 °C to swell the seed particles with the comonomers. Once the reaction temperature was reached, the aqueous solution of the redox initiator was then added to the reaction mixture to start the polymerization. The stirring rate was 200 rpm and the reaction time 4 h, except for the JX1 reaction (7 h). The final latex was removed from the reactor, and the polymerization was quenched with hydroquinone.

Latex Characterizations. The overall conversions of the reactions at the end of the polymerizations were determined gravimetrically. The final pHs were measured.

The particle size distribution (PSD) of the seeds and the final latexes were obtained by transmission electron microscopy (TEM, H-7000 FA Hitachi) on representative samples of more than 500 particles (automatically analyzed with software Bolero, AQ systems). Number (d_n), weight (d_w), volume (d_v), and surface (d_s) average diameters and the polydispersity index (PDI) were calculated from the PSD.[21]

The amount of surface groups was determined by conductometric and potentiometric titrations. The latexes were cleaned by means of a serum replacement cell before titration. Due to the existence of two types of surface groups, sulfate and sulfonate surface groups provided by the redox initiator (charged ones) and chloromethyl groups from the functionalized comonomer, each of them has to be titrated in a different way.

The sulfate and sulfonate surface groups were titrated with NaOH. In this case, to improve the quantification of the titration, the following cleaning process of the latexes was performed. First, they were cleaned with DDI water in a serum replacement cell. Then a dilute solution of HNO_3 was passed through the cell for 12−24 h, maintaining the pH around 3−3.5, to protonate the surface groups. Finally, DDI water was passed until the conductivity of the DDI water was achieved. The titrations were carried out at 30 °C under a nitrogen atmosphere. All the titrations were carried out at least twice.

Determination of active surface groups of the latex particles is based on the nucleophilic attack of the deprotoned glycine on the chloromethyl groups. The latex was reacted with 0.24 M glycine and 0.182 M NaOH. The reaction was carried out at 35 °C. To include the possible hydrolysis of chloromethyl groups by temperature, a blank without glycine/NaOH is determined for each data point. The chloride ions released were determined, as a function of time, after acidification with concentrated HNO_3 by using a chloride-selective electrode to locate the end point of the automatic titration with $AgNO_3$.

Protein Adsorption and Desorption Experiments. The protein concentration in solution was determined, before and after adsorption, 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. Incubation was carried out in a thermostatic bath where the sample was gently agitated at 35 °C for 5 h. After incubation, the samples were separated from the supernatant by high-speed centrifugation and the supernatant was filtered by using a poly(vinylidene difluoride) filter (Millipore, pore diameter 0.1 μm) before measuring the remaining protein concentration. Such a filter has an extremely low affinity for protein adsorption, so that the filtration step does not interfere with the calculation of the adsorbed protein amount.

To obtain the extent of covalent protein binding to the surface chloride groups, the sensitized latex particles are redispersed (after centrifugation) in 3 mL of 1% SDS, 0.2 M Tris, pH 11 at 25 °C during 12 days.

This method desorbs all physically adsorbed protein. The amount of lysozyme covalently bound to the latex surface was determined by using the copper reduction/bicinchoninic acid reaction (BCA method, Pierce Reagents).

Results and Discussion

Polymerizations. The overall conversions of the polystyrene seeds were 100% within the experimental error. Table 2 shows the calculated volume average diameter (nm) and the polydispersity indices of the synthesized seeds. All polystyrene latexes used as seeds were monodisperse (PDI index less than 1.007). The final pH of the seeds was 7.5.

In the same table the volume average diameters (nm) of the core−shell latexes, the PDI indices, the amount of chloromethyl surface groups per gram of polymer (μmol/g pol), and the surface charge density (σ_0, μmol/cm^2) of the latexes due to the sulfate and sulfonate groups provided by the redox initiator are shown. All reactions achieved complete conversion and the final pH of the latexes was in the range of 1.8−2.2.

The results showed that all of the latexes have a monodispersity in accordance with the requirements of the application of this kind of particle in immunoassays.[22] Since optical monitoring of the aggregation is based on the increase of the average size of the

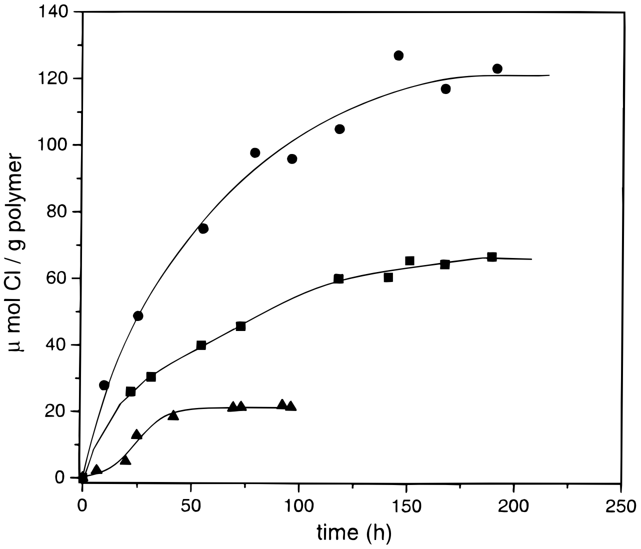

Figure 1. Time dependence of chloride ion release in the reaction of latex with 0.24 M glycine and 0.182 M NaOH: (▲) JX1; (■) JX2; (●) JX3.

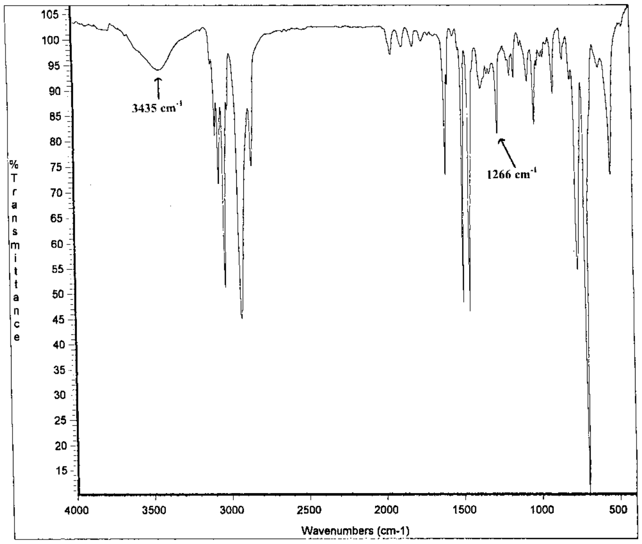

Figure 2. FTIR spectrum of JX2 latex.

aggregates with time, a polydisperse sample would not allow us to distinguish between aggregates and particles.

In the synthesis of JX1 and JX2 latexes, the amount of CMS monomer in the recipe was the same, 9.2 g, and in the case of the JX3 latex, the amount of CMS was doubled (18.4 g). This fact had a direct effect on the amount of chloromethyl surface groups obtained.

The surface charge density of all latexes was calculated using the amount of sulfate and sulfonate groups on the surface. The surface charge densities obtained are similar in all cases because they were provided by the same amount of redox initiator in the recipes.

Surface Chloromethyl Groups. Figure 1 shows the time dependence of chloride ions release when latexes were allowed to react with glycinate. As can be seen, after a certain time a plateau value is reached. Since glycinate cannot dissolve in the organic phase of the latex particle, the reaction involves only the surface of the latex particle.[10]

The plateau values for the three latexes (used for covalent binding) are shown in Table 2. The differences in surface chloromethyl groups can be explained by the effect of the added amount of CMS monomer in the recipe and the temperature of synthesis.

As expected from the difference in the amount of CMS monomer added in the second step of the polymerization reaction for latexes JX2 and JX3 (9.2 and 18.4 g, respectively), while keeping constant the rest of parameters, a similar difference is observed for the chloromethyl groups detected on the surface (59 and 121 μmol/g pol, respectively).

The influence of synthesis temperature on the amount of active chloromethyl surface groups is clearly depicted for latexes JX1 and JX2. Both were prepared with similar amounts of CMS monomer, but different temperatures (Table 1). As can be seen in Table 2, with an increase in synthesis temperature a decrease in chloromethyl groups at the surface is observed. The most plausible explanation for this effect is the low chemical stability of the chloromethyl group with temperature. Hydrolysis of the chloromethyl functionality group into hydroxymethyl, rendering a free chloride ion, is proposed as responsible for the loss of active groups.[17] The presence of the hydroxymethyl function was directly observed by IR spectroscopy of the JX2 dry sample (Figure 2): a wide band at 3435 cm^{-1} (O−H) and a sharp one at 1266 cm^{-1} (C−O).

Chemical Stability of the Chloromethyl Group. To further study the influence of temperature on these groups once attached at the surface, the presence of free chloride ions has been determined after incubation of the latexes at two different temperatures (50 and 80 °C) and in the refrigerator (4 °C) as a function of time.

Parts a and b of Figure 3 show the effect of high temperature (50 and 80 °C) for the three latexes. As expected from the low chemical stability of these groups, hydrolysis increases with increasing temperature. It is noteworthy that at 80 °C the amount of chloride ions released from the particles is much higher than the surface amount detected. Moreover, a clear plateau is not reached within the time of the experiment, suggesting that inner chloromethyl groups are being crosslinked onto the polymer.[23,24]

The effect of the conservation temperature (4 °C) is summarized in Table 3. The three latexes have been measured for surface chloromethyl groups (glycine method) after a long period of time to compare with the results obtained directly after the synthesis. As can be seen, even at a low temperature of 4 °C, some hydrolysis occurs.

Protein Binding. A major problem when working with active groups capable of coupling protein covalently is the elucidation of the amount of chemically or physically bound protein. To ensure a separation between these two ways of adsorption, different methods have been applied over a standard polystyrene (PS) latex (without functional groups able to bind protein covalently) fully covered with protein. A reliable method should desorb 100% of the protein from this surface, since no covalent coupling can take place.

Traditional treatment with Tween 20[24,25] did not fully desorb the protein from the surface (42% desorption with 0.5% Tween 20). Different methods involving several surfactants and conditions were also tried. Only the treatment with 1% SDS, 0.2 M Tris at pH 11 over 12 days was able to recover practically all of the adsorbed protein from the PS surface (98%). Moreover, this surfactant is preferred because of minimum interaction with the protein detection techniques.

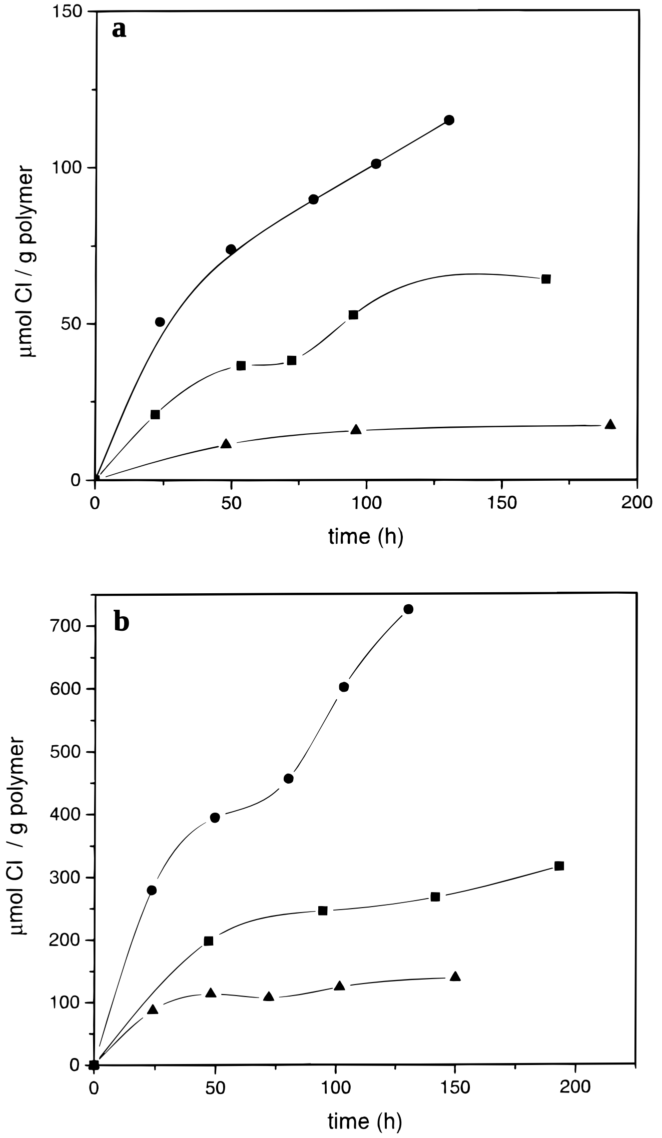

Figure 3. Time dependence of chloride ions release at different temperatures: (▲) JX1; (■) JX2; (●) JX3; (a) 50 °C, (b) 80 °C.

Table 3. Surface Chloromethyl Groups of the Latexes Conserved at 4 °C

	[Cl$^-$] (μmol/g pol.)	% lost
JX1 (19 months)	15.2	28
JX2 (13 months)	47	20
JX3 (7.5 months)	100	17

Another method to show the existence of covalency is the determination of free chloride ions after protein adsorption and comparison with a blank. In Figure 4 the evolution of chloride ion release is presented as a function of sensitization time for protein-saturated conditions. The most remarkable point in this figure is the slow kinetic of the amino−chloromethyl reaction at the interface. Although physical adsorption achieves saturation after some minutes of sensitization,[26] covalent binding is quite slower, needing some hours to be completed (5−6 h). This means that the first contact between the protein and surface is always physical, while chemical linking develops later.

Figure 5 shows the adsorption of lysozyme on the three latexes at pH 7. The first remarkable point is the existence of a maximum in the adsorbed amount before reaching a plateau value. Similar experiments with chloroactivated particles have been carried out in other studies,[27,28] but using different proteins (F(ab′)$_2$ fragment and BSA).

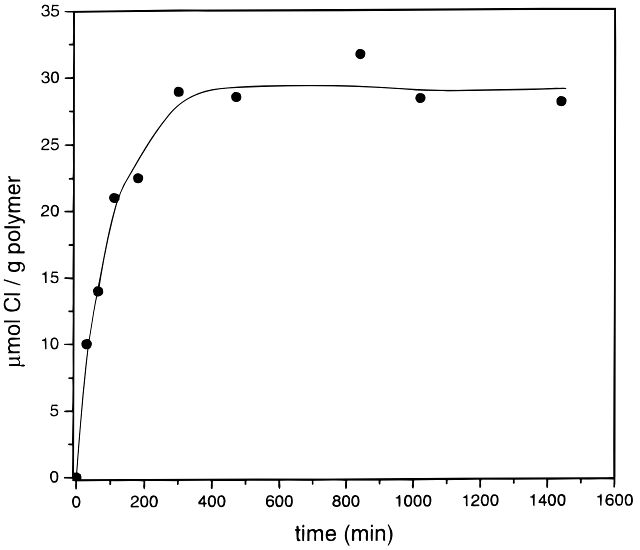

Figure 4. Release of chloride ions as a function of time after adsorption of lysozyme (3.3 mg/m^2) onto JX2 latex at pH 11.

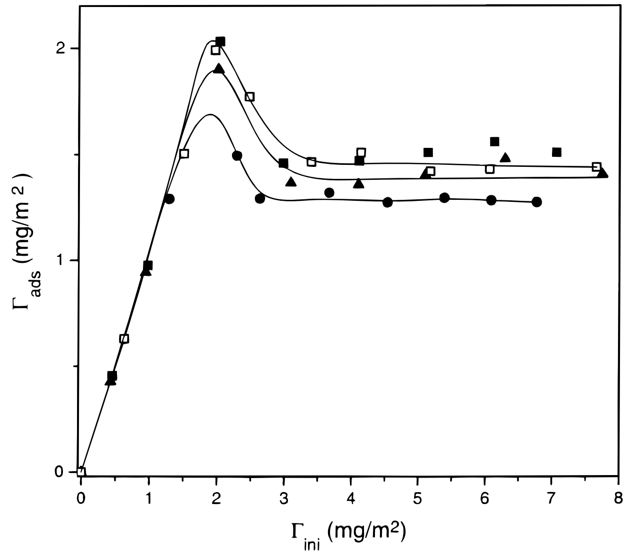

Figure 5. Adsorption isotherm of lysozyme on latex at pH 7: (▲) JX1; (■) and (□) JX2; (●) JX3.

In those cases, the maximum does not appear, suggesting lysozyme to be responsible for this behavior. Since phosphate ions from the buffer could be adsorbed on the protein surface due to charge contrast, the isotherm for the JX2 latex was repeated without buffer, adjusting the pH to 7.2 prior to adsorption. After the adsorption, the pH did not change significantly (7.36). The results are shown in Figure 5 (open symbols), and as observed, the presence of phosphate does not affect the process. Although no explanation is clearly depicted, aggregation of lysozyme molecules at the surface under certain experimental conditions, or changes in the orientation depending on the surface concentration, might be suggested as responsible.

The initial steps of the adsorption isotherms are similar for the three latexes, with high affinity of the protein for the surface. The plateau value, nevertheless, shows a maximum as a function of the amount of

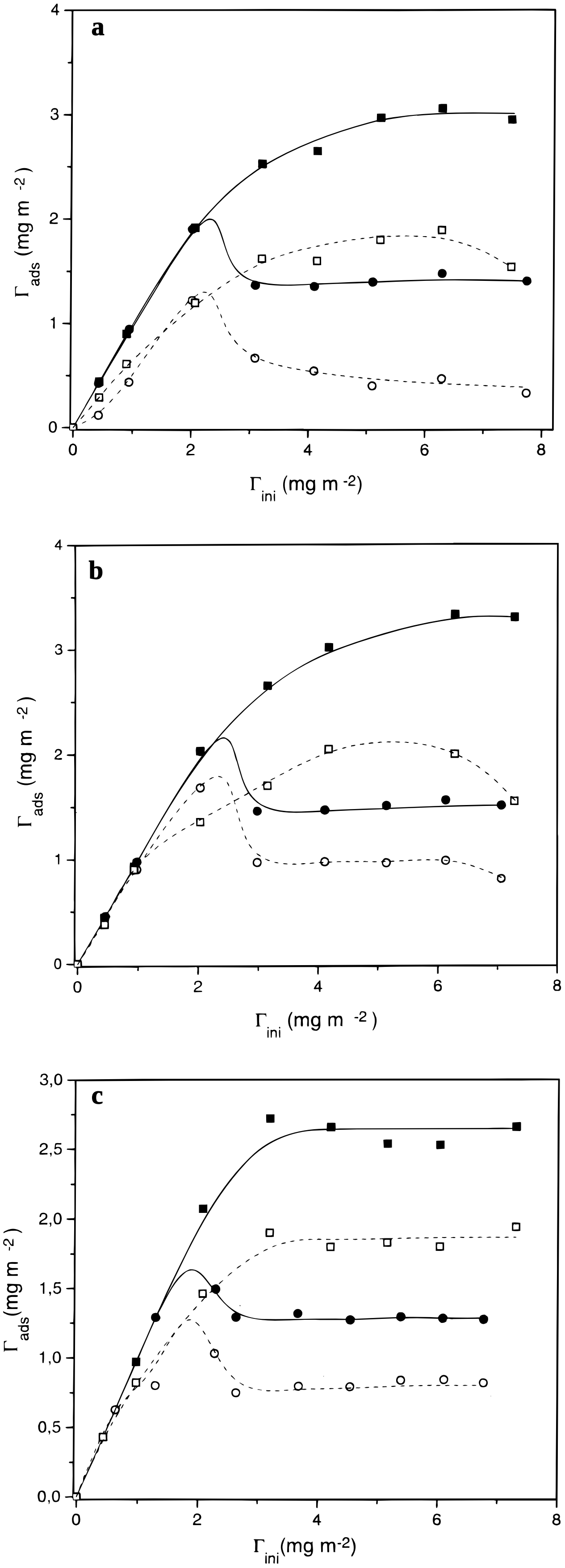

Figure 6. Adsorption isotherms of lysozyme on the three latexes: (a) JX1; (b) JX2; (c) JX3; (circles) pH 7; (squares) pH 11; (closed symbols) total adsorption; (open symbols) covalent binding.

Table 4. Total and Covalent Adsorption in the Plateau Region

	pH 7			pH 11		
	total[a] (mg/m^2)	covalent (mg/m^2)	% covalency (mg/m^2)	total (mg/m^2)	covalent (mg/m^2)	% covalency (mg/m^2)
JX1	1.4	0.47−0.66	35−50	3	1.8	60
JX2	1.51	0.97	64	3.3	2	61
JX3	1.27	0.84	66	2.66	1.84	70

[a] Physically adsorbed plus covalently coupled protein.

chloromethyl groups. The one with 59 μmol/g pol (JX2) presents higher adsorption than the other two. In the case of the latex with 121 μmol/g pol, synthesized with double the amount of CMS monomer in the shell, a more hydrophilic character of the surface is expected. As a consequence, attractive surface−protein interaction may diminish, and less protein adsorption may take place (assuming that the main force in protein adsorption is the hydrophobic interaction[29,30]). In the case of the latex JX1, with only 21 μmol/g pol, it was synthesized with the same amount of CMS monomer as but a higher temperature (80 °C) than JX2. The difference in chloromethyl groups between these two latexes arises then from the hydrolysis of this active group to hydroxymethyl, a group that can be expected to be much more hydrophilic (more polarized). Once again, the more hydrophilic character of this surface may respond for the reported decrease in adsorption.

At pH 11, adsorption of lysozyme is quite enhanced for the three latexes, as inferred from Figures 6a−c. The proximity of this pH to the iep of the protein (11.1) shows that adsorption is favored when the net electrostatic charge is reduced and even preferred to a situation of opposite charge between surface and protein, as at pH 7 (as commonly observed for any protein, since hydrophobic interaction is the main force governing this process[31,32]).

Comparison between the total adsorbed amount and the covalent fraction of the adsorption is presented for the three latexes in Figures 6a−c at pH 7 and 11. In the initial steps of the isotherms, a clear difference is observed for the three latexes. While in the case of the JX2 latex (59 μmol/g pol) adsorption is 100% covalent up to 1 mg/m^2, this value decrease to only 0.6 mg/m^2 for the latex JX3 (121 μmol/g pol). Latex JX1 (21 μmol/g pol) does not show 100% covalent coupling at any studied conditions.

If, as stated before, physical contact occurs prior to chemical linking, a sufficiently high number of chloromethyl groups are needed to ensure that covalent binding can take place. This can explain that initial adsorption in the JX1 latex is not 100%, even when there should be between 4 and 7 chloromethyl groups per lysozyme molecule, depending on the orientation of the protein molecule.

Data of total and covalent adsorption in the plateau region are resumed in Table 4. It can be observed that, although plateau values are somewhat different depending on latex type, the percentages of covalency are practically identical for all of them (around 60−70%, except for JX1 at pH 7). Observing these data, one can conclude that latex JX2, with an intermediate surface density of chloromethyl groups, is the most adequate for the design of covalent immunoaglutination tests.

Conclusions

Monodisperse core–shell latex particles with chloromethyl functionality were synthesized by a two-step emulsion polymerization process. Once the core was formed by batch emulsion polymerization of St at 90 °C, the shell was added by batch emulsion copolimerization of St and CMS or homopolymerization of CMS in a second step. All reactions achieved complete conversion.

The thermal stability of the chloromethyl function on these latexes is strongly dependent on temperature. The hydrolysis rate increases with increasing temperature. Even at the storage temperature (4 °C), some hydrolysis occurs. After long periods of time (between 7 and 19 months for the case studied), approximately 20% of the surface chloromethyl groups disappear.

Covalent binding of protein to the functional groups has been shown using two methods: first, desorbing physically adsorbed protein by SDS treatment and, second, detecting the release of chloride ions upon the reaction between amino protein and chloromethyl groups. The latter technique also shows that the process of chemical linking is rather slow compared to the physical adsorption.

The initial steps of the adsorption isotherms indicate that, if a certain surface density of functional groups exists, all the adsorbed protein becomes covalently bound. With more adsorbed protein at the surface, the covalent extent decreases to a more or less constant value between 60 and 70% independent of the type of latex and pH.

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