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Adsorption of monoclonal IgG on polystyrene microspheres

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Abstract: In this paper the adsorption of a monoclonal antibody IgG-1 isotype against HBsAg onto positively and negatively charged polystyrene beads has been studied. To determine the role played by electrostatic forces in the adsorption process different pH values were used. It was confirmed that the affinity of adsorption isotherms depends on the electrostatic interaction between protein and polymer surface. The maximum adsorption amount is located around the i.e.p. of the dissolved protein, and decreases markedly as pH moves away. Thus, the major driving force for adsorption of monoclonal antibodies on polystyrene beads comes from the hydrophobic interaction between the antibody molecules and the adsorbent surface. Desorption of preadsorbed IgG molecules by increasing ionic strength has shown that the positively charged polystyrene is also more hydrophobic in character than the negatively charged one. Finally, electrokinetic experiments have determined that the electric double layer (e.d.l.) of monoclonal antibody changes as the consequence of adsorbing on charged polymer surfaces.

Key words: Protein adsorption – monoclonal IgG – polystyrene microspheres – immunodiagnostic test

Introduction

Investigation of the mechanisms involved in the adsorption process of immunoglobulin-G molecules (IgG) onto colloidal polymeric substrates is of considerable interest in the field of medical diagnosis, as the latex-IgG system is widely used for a macroscopic detection of an antigen-antibody reaction. Main advantages of this method are rapidity, low cost, simplicity, and easy determination by direct visual control or by analysis with an optical reading device (spectrophotometer, nephelometer, etc.).

Application of this process for diagnostic test systems was first reported by Singer and Plotz [1]. The basis for this reactive is the physical adsorption of IgG molecules on monodisperse latex particles and the colloidal stabilization of the complex so formed. In this way, the presence of an antigen recognized by the antibody will produce an immunological reaction with the subsequent aggregation of the particles. It is evident that the attached antibody molecule will determine the specific immunoreaction, and there are two basis kinds of IgG that can be used for this purpose depending on the way of obtaining it: polyclonal IgG, which is separated as the immunological fraction from the serum of a sensitized animal; or monoclonal IgG, obtained from only one T-cell type artificially reproduced in ascites. The first kind is less specific as it is a mixture of a multitude of slightly different IgG molecules, but is cheaper and recognizes different antigen epitopes. Monoclonal IgG, nevertheless, is a very homogeneous sample, and then it is the more adequate for a basic investigation in the mechanism involved in this process. In some previous papers [2–4], we have studied the adsorption of polyclonal IgG on polystyrene microspheres of different surface charge density and sign, and now we present here the results obtained in the adsorption of monoclonal IgG on cationic and anionic latex, trying thus to complete our investigation into the physical basis of protein adsorption.

Experimental

Materials

All the chemicals used in this study were of analytical grade and were used without further purification. Water used in all experiments was double distilled and deionized with a Milli-Q Water Purification System (Millipore).

Negatively charged polystyrene beads for this study were from Rhône Poulenc (Estapor K030), with a particle average diameter of 297 ± 7 nm, and a surface charge density of 6.9 ± 0.2 $\mu C/cm^2$. Positively charged polystyrene beads were prepared using the initiator ADMBA (azo-*N*,*N'*-dimethylene-isobutyramidine hydrochloride) as previously described [5]. In this case, the particle size and surface charge density were 231 ± 4 nm and 20.4 ± 0.5 $\mu C/cm^2$ respectively. In all cases, cleaning of latex samples was carried out by ion-exchange, and surface charge densities were determined by conductimetric and potentiometric titrations. Charged surface groups on the anionic and cationic polystyrene beads were sulphate and amidine, and they behaved as strong acid and weak basic groups, respectively.

Monoclonal antibody was kindly donated by Biokit, S.A. (Spain). The sample used in this study is an IgG-1 isotope directed against HBsAg (Hepatitis B virus surface antigen). It was purifed from mouse ascitic liquid by ammonium sulphate precipitation followed by anion exchange chromatography.

Methods

The purity of the IgG preparation was ascertained by immunoelectrophoresis using the method described by Grabar and Williams [6] and gel filtration. The gel filtration was performed in an FPLC (fast protein liquid chromatography) system using a Superose 12 HR 10×30 column (Pharmacia) equilibrated in 13 mM phosphate buffer pH 7.2, 0.14 M NaCl, containing 0.1% sodium azide as a preservative. The chromatogram showed one predominant peak with 95% of the total protein.

The isoelectric point (IEP) of monoclonal IgG molecules was determined by isoelectric focusing (IEF) performed on the Phast System equipment (Pharmacia) using a Phast Gel IEF polyacrylamide media (Pharmacia) that covers the pH range 3–9. The gels were silver stained using a modification of the method of Heukeshoven and Dernick [7]. The IEP values obtained for this monoclonal IgG are in the range 5.2–5.5.

IgG adsorption was performed for 2 h at (20 ± 1) °C by adding 1 ml of a 20 mg/ml latex suspension over 9 ml of 0 to 1250 μg/ml IgG solutions in different buffers. These were acetate at pH 4 and 5, phosphate at pH 6 and 7 and borate at pH 8, 9 and 10 for the adsorption experiments with the negatively charged polystyrene, and acetate at pH 4 and 5, bis-tris at pH 6, 7, tris at pH 8–9, and AMP (2-Amino-2-Methyl-1-Propanol) at pH 10, with the positively charged polystyrene beads. The salt concentrations were calculated to get a final concentration of 2 mM. The IgG solutions and the mixtures were gently shaken during and after the addition of latex suspension. The aggregation of latex particles after sensitization was ascertained using a dark field optical microscope. The sensitized particles were centrifuged at 39 000xg for 1 h and the supernatants filtered using a Nucleopore polycarbonate filter (pore size 0.2 μm) before measuring the protein concentration before and after adsorption was determined by direct UV spectrophotometry at 280 nm, using a Spectronic 601 spectrophotometer.

Electrophoretic mobilities were obtained with a Zeta-Sizer IIc of Malvern Instrument, by taking the average of four measurements at the stationary level in a cylindrical cell. In these measurements, the latex particle concentration was 0.05 mg ml^{-1}. In all cases desorption of IgG molecules from sensitized particles by dilution was never observed.

The electrophoretic titration curve of the monoclonal IgG sample was obtained by two-dimensional isoelectric focussing (2D-IEF) measurements with a Phast System equipment (Pharmacia) using the same Phast Gel as in normal IEF. The gel was stained with Coomassie Blue. This method permits information to be obtained on the electrostatic state of a protein as a function of pH, as electrophoretic displacement is proportional to the charge.

The experiments of desorption caused by an increase of ionic strength were carried out by resuspending 10 mg of centrifuged sensitized latex particles in 2.5 ml of buffer. After 2 h at 20 °C, the suspensions were centrifuged again and the protein concentrations measured in the supernatant.

Results and discussions

Stability of protein in solution

The conformational stability of a protein is determined by intramolecular factors and solvent interaction (hydration of interfacial groups). Solubility, nevertheless, is determined primarily by intermolecular effects (protein-protein), but protein molecules are solvated, so that hydration effects are also involved in changes in solubility. The energy of the hydration interaction will depend on the groups placed in the interfacial zone of the protein, and then solubility and conformational stability are closely related. Solubility is a good index of denaturation, and undergoes a minimum in the neighborhood of the isoelectric pH. From the adsorption point of view the solubility of a protein is of major importance, as the method for determining adsorbed protein amount is based on the difference between the initial and the supernatant concentration. If protein molecules denature in the process, they would form aggregates and precipitate in the centrifugation step. This amount of protein should be quantified as adsorbed, and it could be a cause of error.

With this aim we have tried to determine the stability in solution of the monoclonal IgG at different experimental conditions, measuring the optical density (O.D.) at 280 and 320 nm. The latter is indicative of protein denaturation, and only in the case of pH 5 and low ionic strength (2 mM) did the O.D. at 320 nm increase significantly, even using acetate buffer system for stabilizing the protein

Adsorption kinetics

Adsorption of proteins on hydrophobic surfaces is usually an irreversible process and it occurs very rapidly; nevertheless, the experimental conditions (temperature, ionic strength, and pH, mainly) could alter the adsorption rate [9]. For that reason, we first performed some IgG adsorption experiments at different incubation pH and times.The plateau value of IgG adsorption on polystyrene microspheres was reached after 3 min, and it was independent of incubation pH. According to these adsorption kinetics, we established the optimum time to perform the experiments as 2 h, since increasing time favors the instability of IgG in solution.

Electrostatic forces between protein and latex

The major driving force for adsorption of proteins onto polymer surfaces is the dehydration of hydrophobic side groups [8–15], which is almost completely due to the entropy increase in water that is released from contact with hydrophobic components. The electrostatic forces, however, at low ionic strength can play a certain role in protein adsorption on polymer-solution interfaces [2, 3]. For that reason, we have studied the adsorption of monoclonal IgG molecules onto latices with different signs of surface charge as an attempt to investigate the influence of the electrostatic forces in protein adsorption at low ionic strength.

The initial slopes in the adsorption isotherms give information about the affinity between protein and latex surface. With this aim, we have studied adsorption isotherms on the system varying the possible electrostatic interactions between the components.

The first step has been the adsorption of IgG Mab-1 on both latices with adsorption at pH 7 (Fig. 1), where protein molecules have a net negative charge. We can see the differences in affinity between the protein and the polymer surface when electrostatic forces influence the adsorption process. Effectively, the initial adsorption values for anionic latex do not coincide with the total adsorption line, showing that electrostatic repulsion between negative charges makes the approach of protein to the surface difficult. Similar results were found by Elgersma et al. [15] with the adsorption of BSA on positively and negatively charged polystyrene latices.

A symmetrical behavior can be seen when adsorption isotherms are studied at pH 4 (Fig. 2); now the initial adsorption slope for cationic latex does not coincide with the total adsorption line, emphasizing the fact that electrostatic forces between protein and latex surface play a certain role in the adsorption mechanism of monoclonal antibodies on polymer beads.

Also, we have studied the monoclonal IgG adsorption on both latices, cationic and anionic, under experimental conditions in which the protein molecule has no net charge in its surface, that

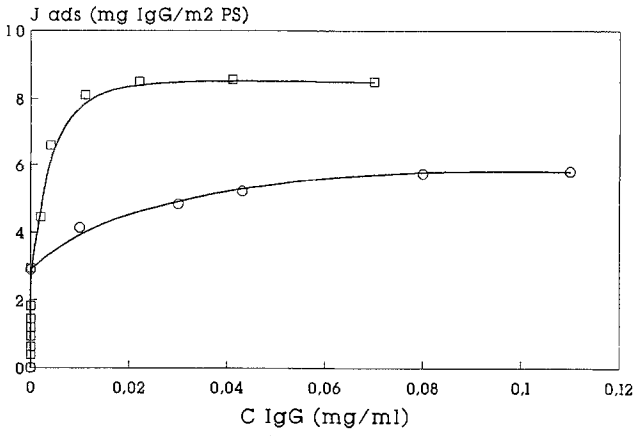

Fig. 1. Adsorption isotherms of IgG Mab-1 on cationic (□) and anionic (○) latex (pH 7, 2 mM)

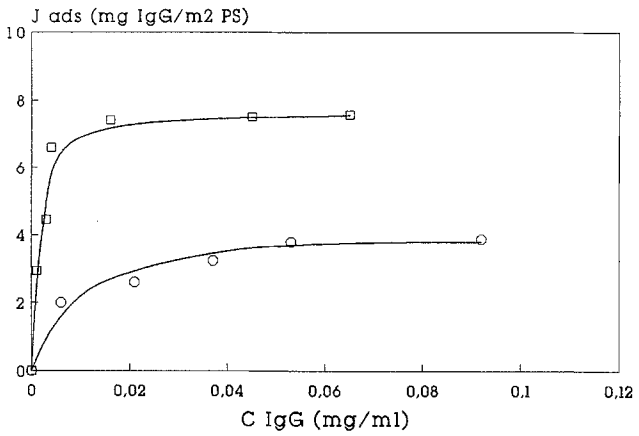

Fig. 3. Adsorption isotherms of IgG Mab-1 on cationic (□) and anionic (○) latex (pH 5.5, 2 mM)

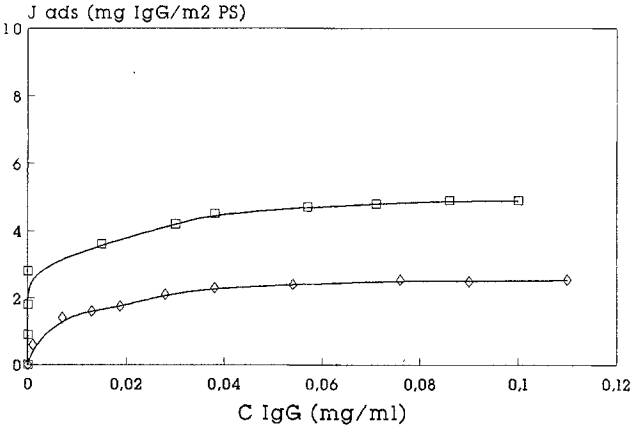

Fig. 2. Adsorption isotherms of IgG Mab-1 on cationic (□) and anionic (○) latex (pH 4, 2 mM)

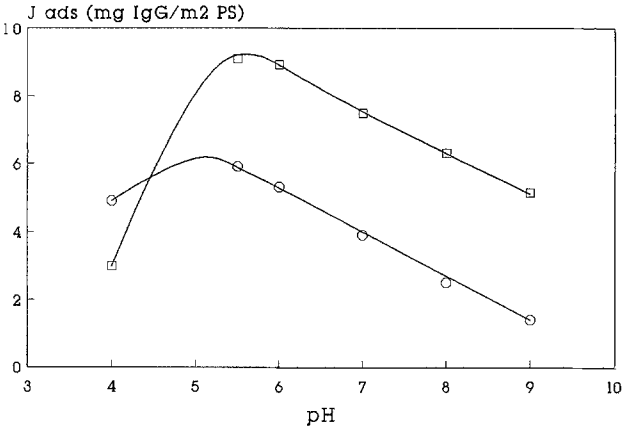

Fig. 4. Maximum adsorption of IgG Mab-1 on cationic (□) and anionic (○) latex as a function of pH

is, the pH of zero charge (i.e.p. $\approx$ 5.5). Figure 3 shows the adsorption isotherms of the IgG Mab-1 on both latices at pH 5.5 and ionic strength 2×10^{-3}. As can be seen, affinity between protein and latex surface is larger in the case of cationic particles, accommodating more molecules per area. If we assume that at the i.e.p. of the protein electrostatic forces should have little influence in the adsorption process, we can conclude that cationic latex is more hydrophobic than the anionic one (in the case of amidine and sulfate groups, respectively). These results are in agreement with those obtained in the adsorption of surfactants on cationic and anionic latices, which were described in a previous paper [16].

Electrostatic forces between protein molecules and conformational stability

Figure 4 shows the adsorption plateau values of monoclonal antibody Mab-1 on cationic and anionic latex as a function of pH, and we can see that maximum values occur in the neighborhood of the i.e.p. of the protein.

As the surface charge groups in both latices are supposed to not change practically in this pH range, an explanation of the fact that more protein is adsorbed on a polymeric surface when there is no electrostatic (interaction than in the case of an attractive one) should come from the inter- and/or intramolecular interactions of the protein. Elgersma et al. [17] propose that the

change in the conformational stability of protein molecules as the net charge increases should be responsible for the structural variations that the protein suffers in the adsorption process, and also the large area per molecule (intramolecular factors). Bagchi and Birnbaum [8], however, also propose that electrostatic repulsion between adjacent protein molecules is responsible for the decrease in the adsorbed amount as pH moves away from the i.e.p. of the protein (intermolecular factors).

In Fig. 4, we can see that this decrease in the adsorbed amount of the protein as pH moves away from the i.e.p. occurs on both latices. There is, however, a large difference between the adsorbed protein amounts which seems to indicate that the extra-adsorption on a cationic latex (over a theoretical monolayer) is due to a stronger electrostatic attraction between the negatively charged monoclonal antibody molecules and the cationic polystyrene surface.

Hydrophobic versus electrostatic forces

In an attempt to distinguish more effectively between hydrophobic and electrostatic interaction in the adsorption of antibodies on polymeric substrates, we have studied the process for both kind of latices as a function of pH under saturation conditions (Fig. 5). The IgG-latex complexes so formed have been then resuspended in a high ionic strength solution (0.5) with the aim of diminishing electrostatic interaction, and that way all protein molecules adsorbed by electrostatic effects should be removed from the latex surface. In order to be sure that desorption is not produced only by dilution effect, in a separate experiment we have resuspended the complexes in the same buffer solution used for adsorption. As can be seen in Fig. 6, where we present the results obtained in the case of cationic latex at pH 7, there is practically no desorption of monoclonal antibody by dilution of the complexes.

Figure 5 shows that maximum desorption occurs at pH values close to the i.e.p. of the protein, just as maximum initial adsorption. With a cationic latex, where adsorption and desorption are very high, there must be more electrostatic interaction. The initial adsorption values onto this latex suggests even the possibility of electrostatic interaction between protein molecules at-

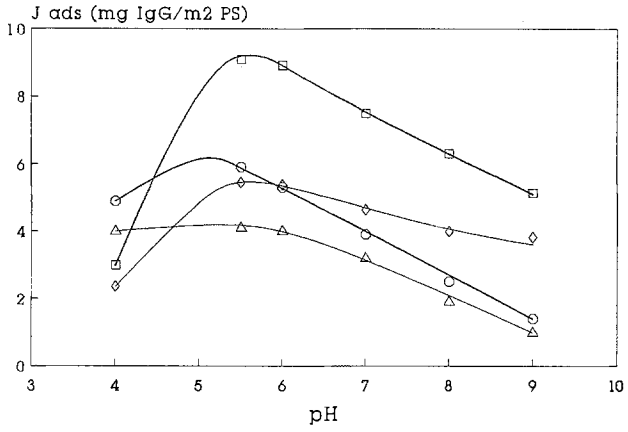

Fig. 5. Maximum adsorption of IgG Mab-1 on cationic (□) and anionic (○) latex and desorption with 500 mM solution, cationic (◇) and anionic (△)

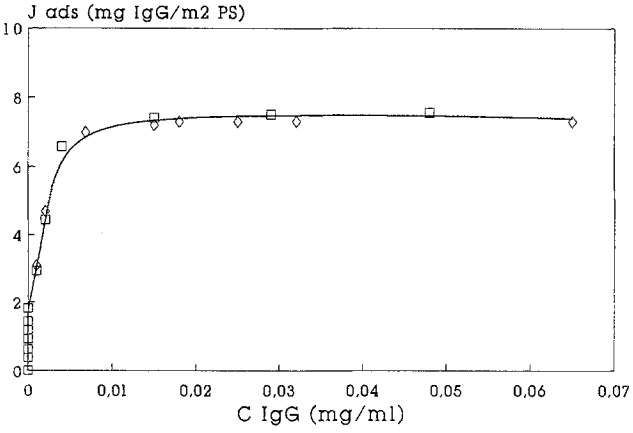

Fig. 6. Desorption of IgG Mab-1 from cationic latex by dilution at pH 7.2 mM. Adsorption values (□) and final values (◇)

tached to the surface and protein molecules in solution, as a compact monolayer of IgG molecules in a flat surface is assumed to be about 7 mg IgG m^{-2} [8, 10]. Differences between adsorbed protein amounts in both latices are minimized after desorption with a high ionic strength solution, which implies that there must be more electrostatic interaction on the cationic than the anionic latex (σ_0 values are $+20.4\ \mu C\,cm^{-2}$ and $-6.9\ \mu C\,cm^{-2}$, respectively). It must be noted that the final adsorbed amount remains larger on cationic latex than that on anionic latex, except at pH 4. This result seems to indicate that the cationic polystyrene surface is also more hydrophobic in character than the anionic sample,

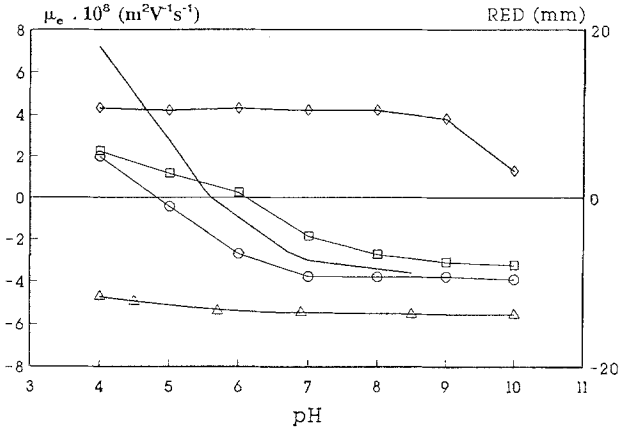

Fig. 7. Mobility of bare latex (cationic ◇ and anionic △) and covered latex particles (cationic □ and anionic ○) (left Y axis) together with the relative electrophoretic displacement (RED right Y axis) of IgG Mab-1 as obtained by 2D-IEF (straight line) as a function of pH

although according to their surface charge densities the opposite would be expected. To confirm this result the contact angles of the anionic and cationic polystyrene air-aqueous solution interfaces were measured. The values found by means of the conventional method of measuring of contact angles were 89° and 91°, respectively. More details of these experiments can be found elsewhere [18]. Also, the adsorption of a non-ionic surfactant (Triton X-100) on both latices revealed that the hydrophobic character of their interfaces is rather different. The areas per molecule of non-ionic surfactant were 33 $Å^2$ and 50 $Å^2$ on the cationic and anionic latex, respectively.

Electrokinetic characterization

In an attempt to relate the electric double layer (e.d.l) structure of monoclonal IgG molecules, bare latex and the complexes formed by both systems after adsorption, we have performed some electrokinetic experiments to determine the change in electrical properties as a consequence of the adsorption process. Figure 7 shows the electrophoretic mobility of bare and covered latex particles for cationic and anionic latex. In this figure we can see that, although a latex particle is fully covered with protein molecules, electric surface properties of the complex depends on the particle used, as cationic and anionic complexes have a different and symmetric behavior respect to the protein in solution. The fact that the point of zero charge (p.z.c.) on these complexes is displaced from the i.e.p. of the IgG Mab-1 depending on the latex surface charge implies that these surface groups play an important role in the final e.d.l. structure of the sensitized particle, and as a consequence in the colloidal stability of the complex so formed. As can be seen, when polymeric substrate is cationic, p.z.c. of the complex is displaced to upper values of pH, while if anionic, the displacement is to lower values of pH. This means that, depending on the i.e.p. of the protein to be attached to a surface, the kind of latex must be chosen to get stability in particular pH conditions.

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