Immobilization of immunoglobulins on silica surfaces

Kinetics of immobilization and influence of ionic strength

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The kinetics of, and the influence of ionic strength on, the immobilization of rabbit immunoglobulin G (IgG) on different types of well-characterized silica surfaces were investigated. Adsorptive immobilization was compared with covalent attachment via thiol–disulphide exchange reactions. The amount of immobilized IgG on five different types of silica surfaces as a function of IgG concentration, at two different ionic strengths, was determined. The IgG–solid-surface interaction involved different types of interaction forces, depending on the surface chemistry of the solid surface. The solid-surface chemistry is an important parameter determining the immobilized amount of IgG. When conditions for covalent attachment of IgG to the surfaces were fulfilled, the IgG showed high affinity and the immobilized amount of IgG showed a fast saturation. Changes in ionic strength showed no significant influence on the kinetics of immobilization on these surfaces. The amount of covalently attached IgG was partially ionic-strength-dependent, indicating that adsorptive interactions were involved. The results are of fundamental interest for the development of new immunosensors based on surface-concentration-measuring devices.

The mechanism of IgG immobilization on solid surfaces is of fundamental interest in such applications as analytical solid-phase assays and affinity chromatography. To obtain optimum performance in these applications the immobilized amount of IgG and its orientation, conformation and stability should be controlled. This is of particular importance in the case of new immunosensors based on surface-concentration-measuring devices (see Jönsson *et al.*, 1985b), where usually only a small surface area is measured and maximum activity of the IgG molecules at the measuring site is required.

In the preceding paper (Jönsson *et al.*, 1985b) we reported on the stability of IgG immobilized on different types of silica surfaces. The chemical attachment was effected by a thiol–disulphide exchange reaction. The advantage of this technique is that the degree of covalent bonds can be controlled and optimum stability and activity of the immobilized molecules achieved. Stability of im-

mobilized IgG is required in environments used, for example, to regenerate a sensor or an affinity column. The chemically attached IgG, as compared with physically adsorbed only, was found to be stable in such environments.

The literature pertaining to the interaction of proteins with solid surfaces is extensive but somewhat confusing. Experimental adsorption isotherms of proteins on various surfaces often conflict, and minor changes such as pH and ionic strength may result in major differences in the measured isotherms. It is therefore of extreme importance to define the experimental conditions carefully and to use well-defined solid surfaces and highly purified protein preparations. Several parameters, such as pH, ionic strength and the surface chemistry of the solid surface, have been shown to influence the amount of immobilized protein at a solid surface. These parameters can also be expected to influence the orientation and conformation and thereby the biological activity of the protein.

In the case of IgG the influence of the solid-surface chemistry (Baszkin & Lyman, 1980) and

Abbreviation used: SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate.

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pH (Oreskes & Singer, 1961; Bagchi & Birnbaum, 1981) has been studied before. We now report here on the influence of ionic strength on the immobilized amount of IgG on different types of well-characterized silica surfaces.

The immobilization was brought about by physical adsorption and by a chemical attachment by use of the thiol–disulphide exchange reaction. The results were evaluated by an optical technique, ellipsometry, which directly measures the amount of immobilized IgG. With this technique it is also possible to follow the kinetics of the immobilization reaction. The influence of ionic strength on the kinetics of immobilization to three types of surfaces was studied.

Materials and methods

The surface pretreatment of the silicon slides, their chemical modifications and surface characterization, the IgG and its chemical modification and the ellipsometric technique as well as the handling of the slides during the experiments are described in the preceding paper (Jönsson *et al.*, 1985b).

The influence of ionic strength on the amount of immobilized IgG was tested in 10 mM-sodium phosphate buffer, pH 7.5 (P buffer), and 0.1 M-sodium phosphate buffer, pH 7.5, containing 0.1 M-NaCl (PP buffer).

IgG and SPDP-modified IgG were first incubated for 16–24 h at different concentrations together with hydrophilic, control, amino, thiol and methyl slides in either P buffer or PP buffer. Then the slides were rinsed in the same buffers and measured in the ellipsometer. The immobilized amount of IgG was calculated as the difference between blank slides that were kept in buffer only and the slides with immobilized IgG. This procedure is justified in view of the reproducible surface chemistry of the slides (Jönsson *et al.*, 1985b).

The experimental set-up for the study of kinetics of immobilization has been described before (Jönsson *et al.*, 1982). In the experiments stirring was performed during the first minute of the experiment and 1000 ml of buffer was used in the rinsing procedure.

Results and discussion

Kinetics of immobilization

The kinetics of the immobilization were followed (Fig. 1). The experiments are representative for at least three different experiments on each type of surface. The results are given as the angular change in the ellipsometric parameter Δ . This is necessary since no theory exists for translating the change in Δ ($\delta\Delta$) at early stages of protein interac-

Fig. 1. Kinetics of IgG and SPDP-modified IgG interactions with hydrophilic, methyl and thiol slides at two different ionic strengths

(a) P buffer; (b) PP buffer. $\blacktriangle$, SPDP-modified IgG on thiol slides; $\bullet$, IgG on methyl slides; $\blacksquare$, IgG on hydrophilic slides. White arrows indicate injection of SPDP-modified IgG and IgG respectively to a concentration of 100 $\mu\text{g}/\text{ml}$ of protein in the cuvette. Black arrows indicate a rinsing procedure whereby 1000 ml of protein-free buffer continuously passed through the cuvette. Numbers indicate amount of immobilized protein (ng/mm^2) after the rinsing.

tion with solid surfaces to amount of immobilized protein. As shown by Jönsson *et al.* (1985a), $\delta\Delta$ is proportional to amount of material per surface area. Although the exact relationship at early stages of interaction is not known, Fig. 1 shows in a qualitative way the amount of immobilized proteins as a function of time. The translation of $\delta\Delta$ into amount of immobilized protein after rinsing is indicated in Fig. 1.

The kinetics showed a fast saturation on the methyl and thiol slides whereas a somewhat slower saturation was found for hydrophilic slides. The influence of ionic strength was also much more pronounced on hydrophilic slides as compared with

methyl and thiol ones. Also, in parallel experiments it was shown that the time to reach saturation for methyl and thiol slides was essentially the same for the concentration range 20–300 $\mu\text{g/ml}$. Below 20 $\mu\text{g/ml}$ this time was concentration-dependent. The results at these low concentration levels are, however, difficult to interpret, owing to non-specific adsorption of IgG on the cuvette walls and the air/liquid interface.

The covalent immobilization of SPDP-modified IgG may thus be accomplished within 1–2 h, and no increased amount of IgG on the surface is found after longer incubation periods.

The cuvettes were rinsed 12–13 h after the addition of protein. The concept of reversibility upon dilution for adsorbed protein molecules was discussed by van der Scheer (1978). Most experiments in the literature point towards irreversibility or partial reversibility, depending on the surface chemistry of the solid surface. Our results show practically no desorption of immobilized IgG upon rinsing. The difference in kinetics at the initial phase, when the proteins are immobilized, and the desorption phase suggests immobilization via multipoint attachment to the solid surfaces (Jennissen, 1980).

Influence of ionic strength

Fig. 2 shows the amount of immobilized IgG on five different types of silica surfaces as a function of IgG concentration at two different ionic strengths. In Fig. 3 the same experiments were repeated with SPDP-modified IgG. Three separate experimental runs with two different batches of IgG were performed. Minor quantitative differences, $\pm 15\%$, exist in the adsorbed or bound amount of IgG between separate runs. The qualitative behaviour was, however, the same in all experiments. Figs. 2 and 3 show mean values from three separate slides of each type of surface modification in one experimental run. The maximum deviations from these values in this run were within the limits of the symbols.

One important parameter influencing protein–solid-surface interactions is the surface tension of the solid surface. The contact-angle data reported in Table 2 in Jönsson *et al* (1985b) clearly show that each type of surface modification performed can be distinguished by the solid surface tension. The data also indicate that each type of surface modification is reproducible for separate modification runs. Thus conclusions concerning the protein–solid-surface interactions for each type of surface modification may be related to differences in solid surface tension, which in turn is related to the solid-surface chemistry (Zisman, 1964).

The influence of ionic strength on immobilized amounts of IgG and SPDP-modified IgG should

Fig. 2. Immobilized amount of IgG as a function of IgG concentration at two different ionic strengths. Hydrophilic (○), control (□), amino (▲), thiol (△) and methyl (●) slides were first incubated for 16–24 h with IgG at the indicated concentrations in (a) P buffer and (b) PP buffer. Secondly, the slides were rinsed in the respective buffer and measured in the ellipsometer. The amount of immobilized IgG was calculated from the difference in ellipsometric parameters for these slides as compared with slides incubated in protein-free solutions.

be most pronounced for those surfaces expected to carry a net charge at the pH used in the experiments. The point of zero charge has been estimated to lie between pH 1 and 4 for silica (Tadros & Lyklema, 1968), and the hydrophilic and control slides therefore carry a net negative charge.

The contact-angle data for hydrophilic slides show a dominating polar contribution to the solid surface tension. The critical surface tension agrees

Fig. 3. Immobilized amount of SPDP-modified IgG as a function of IgG concentration at two different ionic strengths. For explanations see Fig. 2 legend. (a) P buffer; (b) PP buffer.

very well with that reported for hydrated-oxide-covered surfaces (Bernett & Zisman, 1968). The data indicate a surface structure consisting of one or several layers of adsorbed water similar to that reported for silica gel (Scott, 1980).

The data for control slides, however, show considerably decreased polar and increased dispersive contributions to the solid surface tension as compared with hydrophilic slides. No appreciable conversion of surface silanols into siloxanes occurs at the temperatures used in the preparation of these slides (Boehm, 1966). Accordingly no chemical modification of the control slides as com-

pared with hydrophilic ones is expected. The difference may thus be attributed to partial loss of adsorbed water and/or adsorption of unknown impurities to the slides. Hydrophilic surfaces are known to adsorb rapidly impurities, such as hydrocarbons, in air (Raider *et al.*, 1975). This was elucidated in parallel experiments where hydrophilic slides, after drying in N_2 gas, were kept in dust-free air. In this case the slides, with respect to the solid surface tension, gradually convert into control slides and within 3–4 days they are indistinguishable from the control slides.

In conclusion, the hydrophilic slides may, at neutral pH, be considered as negatively charged and hydrated surfaces. The control slides possess adsorbed impurities, increasing the possibilities for non-polar interactions and in part screening the charged groups at the silica surface.

Our results are consistent with this view concerning hydrophilic and control slides. Both types of IgG tested showed a distinct decrease in immobilized amount at the high ionic strength as compared with the low ionic strength. Electrostatic interactions are thus predominant at these surfaces.

The amino slides, because of the amino groups introduced at the surfaces by the silanization, are expected to carry a positive charge. It is furthermore expected that remaining hydroxy groups at the silica surface will be covered by an 'umbrella' of silane molecules (Boehm, 1966), thereby making them unavailable for direct interaction with proteins. The contact-angle data for these surfaces as compared with the control slides indicate a more-polar surface that may be attributed to the amino groups, but dispersive components from the carbon chain of the silane are also present. The structure of the amino silane at the silica surface is discussed in Jönsson *et al.* (1985b).

Our contact-angle data from the amino slides agree reasonably well with those found in the literature. However, as pointed out by Sacher (1980), it is difficult to compare numerical results from different laboratories, since the solid surface tension may depend on both the silanization technique used and the post-silanization treatment. In our case, if the silanized surfaces were kept in ethanol at room temperature, no time-dependent (within 3 weeks) change in contact-angle data could be detected.

Figs. 2 and 3 show only a slight influence of ionic strength on the amino slides. The increased amount of immobilized protein at low ionic strength as compared with high ionic strength would seem to indicate, however, that electrostatic interactions are involved.

The contact-angle data for the methyl slides are consistent with a closely packed layer of methyl

groups at the surface (Zisman, 1964). Hydrophobic interaction is therefore expected to be involved in the interaction of proteins with this surface. Hydrophobic interaction, in contrast with electrostatic, is known to be stabilized by high concentrations of certain salts, e.g. NaCl in the 1M range (Hofstee, 1976, 1979; Srinivasan & Ruckenstein, 1980). Figs. 2 and 3 do not reveal any major influence of ionic strength on the immobilized amount of protein on methyl slides. Approximately the same amounts of IgG and SPDP-modified IgG were adsorbed when parallel experiments with 1M-NaCl were carried out (results not shown). These results strongly suggest that non-electrostatic forces are predominant at these surfaces.

It is also interesting to compare Figs. 2 and 3 for the thiol slides. In Fig. 3 conditions for covalent binding of the IgG via thiol-disulphide exchange reactions are fulfilled. Considerably higher amounts of SPDP-modified IgG are immobilized on these surfaces as compared with IgG. A slight influence of ionic strength was also detected. In addition to the proposed covalent binding in Fig. 3, other interaction forces may be involved. This assumption is supported by the fact that somewhat higher amounts of IgG were immobilized on the thiol slides at high ionic strength (Fig. 2b) as compared with low ionic strength (Fig. 2a). The findings were similar with SPDP-modified IgG. The assumption was further supported by parallel experiments, performed as described in Jönsson *et al.* (1985b), in which the stability of immobilized SPDP-modified IgG was tested in 0.1M-dithiothreitol. This treatment is expected to reduce the disulphide bonds between the surface and the IgG, thereby completely desorbing the molecules if no other interaction forces were involved. However, only about 50% of the immobilized IgG could be desorbed under these conditions. This decrease in immobilized amount of IgG may also be due to reduction of interchain disulphide bridges, thereby fragmenting the IgG molecule.

Adsorption isotherms are obtained when the immobilized amount of IgG is in equilibrium with a certain concentration of IgG in solution. The curves in Figs. 2 and 3 can therefore not be called adsorption isotherms, since the immobilized amount of IgG measured is that remaining after rinsing.

The kinetics of immobilization (Fig. 1) show that no major changes in bound amounts are seen on lowering the concentration of IgG in the surrounding solution. This fact may justify a comparison of our data with those found in the literature concerning adsorption isotherms of IgG.

The existence of 'steps' in the adsorption isotherm for IgG has been reported in several studies (Oreskes & Singer, 1961; Nyilas *et al.*, 1974; Fair &

Jamieson, 1980). As discussed by Fair & Jamieson (1980), the steps suggest two different modes of adsorption, one operating at low, the other at higher, protein concentrations. It was furthermore suggested that the lower plateau phenomenon is the result of a random independent adsorption mode, whereas the upper plateau is due to a co-operative mode of adsorption.

The amount of IgG bound to the various surfaces used in our investigations levels off at IgG concentrations around 100 µg/ml. A levelling off, corresponding to the first plateau, has been reported for this concentration range and various surfaces by several workers (Lee & Kim, 1974; Pesce *et al.*, 1977; Watkins & Robertson, 1977; Fair & Jamieson, 1980; Bagchi & Birnbaum, 1981). However, Cantarero *et al.* (1980) reported a levelling off at IgG concentrations around 1 µg/ml. When comparing our findings of bound amounts of IgG at the first plateau in the adsorption isotherm, it is important to bear in mind that this amount depends on many parameters, such as surface chemistry, ionic strength, types of ions, pH and temperature.

Watkins & Robertson (1977) obtained an adsorbed amount of IgG of about 2–2.5 ng/mm² in 10mM-phosphate-buffered 0.85% NaCl, pH 7.4, at the first plateau, on surfaces comparable with the methylated ones used in our investigation. They used the total internal reflection fluorescence technique. Using u.v.-spectrophotometry Bagchi & Birnbaum (1981) measured an adsorbed amount of about 7 ng/mm² on negatively charged latex particles in 2mM-KNO₃, pH 7.8. Fair & Jamieson (1980) report an adsorbed amount of roughly 5 ng/mm² on polystyrene latex, at ionic strength 0.005 and pH 7.4. Grant & Dehl (1980) obtained values around 3–4 ng/mm², in 0.1M-phosphate buffer, pH 7.4, of adsorbed IgG on hydrophilic and hydrophobic silica respectively. These values were obtained by ellipsometry.

Cantarero *et al.* (1980) found amounts of radio-labelled IgG adsorbed on polystyrene to be in the range 2–3 ng/mm² in 0.1M-carbonate buffer, pH 9.6. Using radiolabelled IgG Lee & Kim (1974) measured adsorbed amounts in the range 8–47 ng/mm² for various polymer surfaces. Similar high amounts of ¹²⁵I-labelled IgG on polymer films were also found by Baszkin & Lyman (1980).

Lensen *et al.* (1984) monitored the adsorption of IgG to polystyrene latex by radioactivity measurements or u.v. spectroscopy. Their results showed that ¹²⁵I-labelled IgG was adsorbed in higher amount as compared with native IgG. Great care should therefore be taken in interpreting adsorption results involving radiolabelled molecules. The value at the plateau for native IgG was around 4 ng/mm².

These studies elucidate the variations in the amounts of bound IgG reported in the literature. Our results, however, are within the range 2–7 ng/mm² obtained in most studies. The lower limit in this range of bound amount at the first plateau corresponds approximately to a theoretical monolayer of side-on adsorbed IgG (Baszkin & Lyman, 1980; Cantarero *et al.*, 1980; Fair & Jamieson, 1980). End-on adsorbed IgG corresponds to a bound amount of 14–19 ng/mm² (Baszkin & Lyman, 1980; Fair & Jamieson, 1980). A continuous transition, depending on such parameters as pH and ionic strength, of side-on to end-on adsorbed IgG in the adsorption isotherm may in part explain the discrepancies in bound amounts of IgG obtained in different studies. The situation is further complicated by the possibility of unfolding of the adsorbed IgG (Walton & Koltisko, 1981). This unfolding may cause intermolecular interactions and multilayer adsorption. In principle, the refractive index and thickness of the IgG layer can be determined by ellipsometry. It is, however, in the case of silicon covered by spontaneously formed silica not possible to determine these parameters of the IgG layer by ellipsometry with good accuracy (Jönsson *et al.*, 1985a). Therefore our measurements do not give any information about the orientation and structure of the bound IgG.

Conclusions

Covalent and adsorptive immobilization of IgG on a series of well-characterized silica surfaces, mainly differing in surface chemistry, were studied.

When conditions for covalent binding of IgG were fulfilled, the IgG showed a high affinity. This was shown by the increased amount of immobilized IgG as compared with other types of surfaces and also from the fast kinetics of interaction with the surface. Together with the results given in the preceding paper (Jönsson *et al.*, 1985b), this suggests immobilization via thiol–disulphide exchange reactions. However, the experiments performed at two different ionic strengths also suggest that adsorptive interaction forces are involved.

The interaction of IgG with solid surfaces was found to be complex. Several interaction forces, such as electrostatic and non-polar, are involved. The solid-surface chemistry was found to be an important parameter determining the amount of immobilized IgG and presumably also the orientation and conformation of the molecules. It is therefore concluded that extended knowledge of the fundamental properties of IgG immobilization on solid surfaces is required. This is of special import-

ance in designing new immunosensors based on surface-concentration-measuring techniques.

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