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Chapter 21

Orientation of Adsorbed Antibodies: *In Situ* Monitoring by QCM and Random Sequential Adsorption Modeling

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Antibodies (IgGs) are widely used for diagnostic assays, for which they are in certain cases immobilized by adsorption on hydrophobic substrates. Antigen recognition efficiency will depend on the orientation of the adsorbed IgG molecules. The aim of the present study was to investigate the binding-ability of a range of IgG isotypes from rat and mouse, all directed against the same antigen, using quartz crystal microbalance. The results allow identifying some isotypes which adsorb in higher amount and which provide a better bound antigen to adsorbed IgG ratio. This ratio was found to remain rather constant with the adsorbed IgG amount. Random sequential adsorption (RSA) modeling was used to simulate IgG adsorption. In the chosen modeling conditions, it is shown that even if adsorption in flat orientation is more favorable, a high proportion of IgG molecules adsorb in end-on orientation when surface coverage increases, owing to the low surface area spaces left between IgG molecules already adsorbed in flat orientation. The apparent discrepancy between experimental data collected by QCM and the output of RSA modeling may be attributed to variations in the water content of the adsorbed layer, to steric hindrance and multivalency effects upon antigen binding, or to the role of albumin molecules used to prevent non specific adsorption of the antigen.

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Introduction

Antibodies, which are proteins made of two identical light chains and two identical heavy chains, recognize specific antigens through interaction with their variable parts, located at two extremities of their three-lobular structure (*1*). Owing to this property, they are essential for the design of many diagnostic tools. In particular, the very widespread enzyme-linked immunosorbent assays (ELISA) rest on the adsorption of antibodies at the surface of hydrophobic polystyrene plates. The ability of antibodies to bind antigens after adsorption is related to the accessibility of their variable parts. Therefore, a better response is expected if the antibodies are adsorbed through their constant part, in an end-on orientation, rather than in side-on or, even worse, flat orientation (*2*).

The forces governing antibodies adsorption have been investigated, and hydrophobic as well as electrostatic interactions have been identified as playing a major role (*3*). On hydrophobic surfaces, it was actually shown that the affinity of antibodies for the surface was not much affected by pH and ionic strength, pointing to the weak contribution of electrostatic interactions, which were however shown to affect the maximum adsorbed amount. On hydrophilic surfaces, adsorption was strongly reduced in conditions of electrostatic repulsion (*4*).

Studying the orientation of adsorbed antibodies is highly challenging. In a first approach, if it is considered that adsorbed proteins tend to form a monolayer at the interface, the adsorbed amount may give an indication on orientation, since less molecules will fit in the monolayer in flat compared to end-on orientation. Based on close-packed monolayers, an adsorbed mass of 2.0 and 3.7 mg/m^2 was indeed estimated for flat and end-on adsorbed antibodies, respectively (*4*). Using neutron reflectivity, Petrash and colleagues showed that a human γ-globulin adsorbed on a hydrophobic self-assembled monolayer formed a dense layer at close contact with the substrate, on top of which a second, less dense layer was found. This was attributed to the combination of molecules lying down on the substrate and of end-on adsorbed antibodies (*5*).

To probe the orientation of adsorbed antibodies in relation with their use for diagnostic assays, it is actually more convenient (although more indirect) to measure their antigen binding ability. From theoretical considerations, it may be expected that antibodies adsorb mainly in flat orientation on hydrophobic substrates to maximize hydrophobic interactions with the surface. However, experimental evidences clearly show that antigen recognition occurs, thus that part of the adsorbed antibodies have their variable parts pointing towards the solution, but in an extent that is higly variable from one antibody to another and that is difficult to predict (*3*). When a low bound antigen to adsorbed antibody ratio is observed, it is actually difficult to know whether this is due to a low amount of antibodies adsorbed in end-on orientation, with the variable parts pointing towards the solution, or to steric hindrance between adjacent recognition sites which may occur at high surface coverage, as suggested in some studies (*6*). Using quartz crystal microbalance (QCM) to measure the adsorption from different concentrations of an anti-streptavidin antibody, followed by streptavidin binding,

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Wiseman and colleagues suggested that antibodies adsorb in flat orientation at low surface coverage, when enough space is available at the interface to do so, while they tend to adsorb in end-on orientation at high surface coverage *ie* when adsorption occurs from a more concentrated solution (*2*).

Given the difficulty to predict the behavior, and in particular the orientation, of antibodies upon adsorption, attempts to maximize the sensitivity of ELISA assays are usually empirical. Factors known to affect the antigen binding efficiency include concentration of antibody solution, nature of blocking agent used (albumin, casein, surfactants or combination thereof), order and duration of adsorption and washing steps. Strategies can be used to immobilize antibodies in end-on orientation, through coupling of the constant part to the substrate (*3*, *7*). For example, protein A or protein G are known receptors for the Fc fragment of antibodies, and can be preadsorbed or linked to the substrate to further direct the orientation of antibodies. Owing to the perspectives opened by the possibility to produce chimeric antibodies, an alternative approach could consist in selecting an antibody with a constant part that would be particularly suitable for adsorption in end-on position, then coupling the desired variable part to this constant part. For this reason, it is interesting to evaluate the antigen-binding efficiency of antibodies with different constant parts, ie from different species or different isotypes (*1*).

The aim of this work is to study the orientation of adsorbed γ-immunoglobulins (IgGs). A range of IgG isotypes from rat and mouse directed against the same antigen, 2,4-dinitrophenol (DNP), was selected. Their adsorption as well as their antigen-binding ability were probed using quartz crystal microbalance (QCM). The results are then interpreted in the light of random sequential adsorption modeling.

Materials and Methods

Materials

IgGs used in this study originate from either rat or mouse, and are from different isotypes as detailed in Table I. These monoclonal antibodies are all directed against 2,4-dinitrophenol (DNP). They were purchased from IMEX (Brussels, Belgium) under the reference listed in Table I, and received as 1 mg/ml solutions in phosphate buffer supplemented with 0.1 % sodium azide.

DNP coupled to albumin (DNP-alb; 30 to 40 DNP groups per albumin molecule; Sigma-Aldrich) was used as the antigen in QCM experiments. The molar mass of DNP alone is indeed too small to allow its detection using QCM. Bovine serum albumin (BSA; fraction V, ≥96 %; Sigma-Aldrich) was used as the blocking agent.

All solutions (IgG, DNP-alb and BSA) were prepared in phosphate buffer saline (PBS). The composition of PBS, prepared using ultrapure water (PurelabUltra, Elga), was as follows: 0.2 g/l KCl (Sigma-Aldrich), 8 g/l NaCl (Sigma-Aldrich), 0.88 g/l KH_2PO_4 (Merck), 1.28 g/L $Na_2HPO_4.12\ H_2O$ (VEL).

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NaN_3 (Sigma-Aldrich) at 2 g/l was added to prevent biological contamination and pH was adjusted to 7.4 using 1 M NaOH. PBS was filtered before use (0.2 µm sterile syringe filter, VWR). The concentration of the IgG solutions was 4, 10 and 20 µg/ml, BSA blocking solution was prepared at a concentration of 10 mg/ml, and DNP-alb solution had a concentration of 20 µg/ml.

Table I. IgGs used in the present work

Species	*Isotype*	*IMEX reference*
Mouse	IgG 1κ	MA-DNP-1
	IgG 2aκ	MA-DNP-2
	IgG 2bκ	MA-DNP-3
	IgG 3κ	MA-DNP-4
Rat	IgG 1κ	LO-DNP-1
	IgG 1κ	LO-DNP-2
	IgG 2aκ	LO-DNP-16
	IgG 2bκ	LO-DNP-57

For QCM experiments, standard gold-coated crystals (QSX 301 from Q-Sense) were used. Their surface was modified by deposition of a thin polystyrene layer. This was done by spin-coating of a 0.5 % (w:w) polystyrene (M_w = 230,000 g/mol; Aldrich) solution in toluene (VWR). Spin-coating was performed under a nitrogen atmosphere on a WS-400B-6NPP-Lite spin-coater from Laurell, using the following parameters: volume of solution = 100 µl, speed = 2,000 rpm, acceleration = 20,000 rpm/s, time = 20 s. The obtained polystyrene-coated sensors were then heated for 1 h in an oven at 80°C to remove traces of solvent.

QCM Experiments

IgG adsorption, followed by blocking step with BSA then DNP-alb recognition were monitored *in situ* using quartz crystal microbalance (QCM). The system used was a Q-Sense E4 (Biolin Scientific, Sweden), equipped with four QFM 401 flow modules. The flow was ensured by a peristaltic pump (Ismatec, Germany); it was set at 10 µl/min throughout the whole experiment. The temperature was fixed at 20°C. Experiments were performed using the successive steps described in Table II. To reduce the amount of IgG solution needed, a closed-loop circuit was used after 1 h of IgG adsorption, until the end of that step.

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Table II. Steps used in QCM experiments

Step	*Solution used*	*Typical duration*
Establishment of a baseline	PBS	2 h
IgG adsorption	IgG (4, 10 or 20 µg/ml)	14 h
Rinsing	PBS	90 min
Blocking	BSA (10 mg/ml)	90 min
Rinsing	PBS	90 min
Antigen binding	DNP-alb (20 µg/ml)	90 min
Rinsing	PBS	90 min

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The mass deposited on the crystal surface after each deposition step (IgG, BSA, DNP-alb) was calculated on the basis of the shift of frequency (Δf) recorded between the baseline established before that step in PBS, and the one established during the subsequent rinsing step in PBS. Although this means that the adsorbed mass is then the one of the molecules remaining at the interface after rinsing (and not the one accumulated at the interface in equilibrium with the protein solution), this is necessary to avoid effects related to the density and viscosity of the solutions interfering with the results. Δf was then converted into an increment of adsorbed mass Δm using Sauerbrey equation:

$$\Delta m = - C. \Delta f/n$$

in which C is the mass sensitivity constant, related to the quartz crystal (here, $C = 17.7$ ng.cm^{-2}.Hz^{-1}) and n is the overtone used for the measurement. Note that Sauerbrey equation only holds if the characteristics of the adsorbed layer can be assimilated to the one of the quartz crystal, *ie* if the layer is rigid and homogeneous. In the present case, the dissipation shifts recorded upon IgG adsorption were always lower than 2.10^{-6}, and the ΔD/Δf ratio was in the range of 5.10^{-8} or lower, where ΔD is the recorded shift of energy dissipation. In such conditions, it is considered that the Sauerbrey equation gives a good estimation of the adsorbed mass (*8*). Dissipation values will therefore not be further exploited. The obtained Δm (in ng/cm^2) were further converted into molar surface concentrations (in pmol/cm^2), using a molar mass of 146 kDa and 71.4 kDa for IgGs and DNP-alb, respectively. The calculated values must however be interpreted with caution given the fact that not only the adsorbed molecules but also water coupled to the adsorbed layer are probed by QCM (*9*).

Random Sequential Adsorption Modeling

Random sequential adsorption (RSA) modeling was developed by Schaaf, Talbot and colleagues (*10–12*). It consists in considering the filling of an interface by objects using the following hypotheses: (i) objects arrive sequentially at the interface, in a randomly chosen position; (ii) once deposited, an object cannot

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move from its initial position, (iii) objects cannot overlap. This approach can be used to simulate protein adsorption, if one considers that proteins are not deformable, that they only adsorb in the form of a monolayer (three-dimensional aggregates are not formed), that they cannot diffuse in the lateral plane and that their adsorption is irreversible. This latter assumption is not too far from reality on hydrophobic substrates (*13*).

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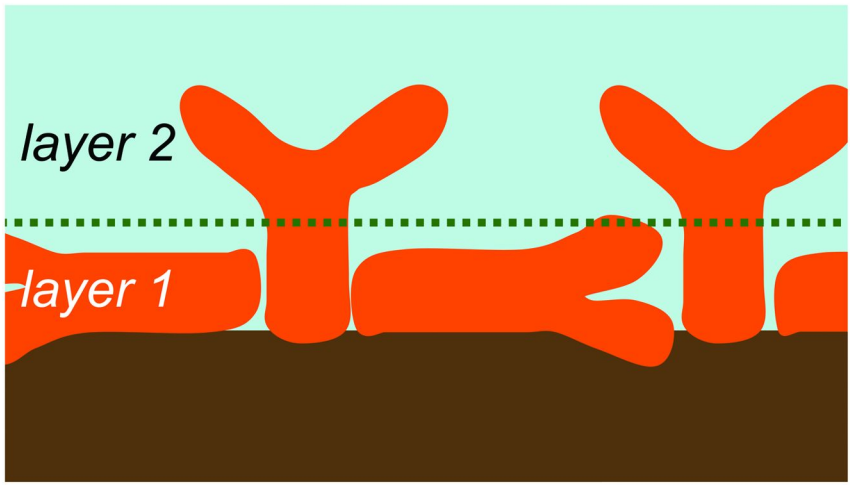

Figure 1. Schematic representation of the two-layer system chosen for RSA modeling.

RSA modeling was performed using the Matlab environment (The Mathworks Inc.). The approach was first validated for the deposition of hard spheres (not shown here), by comparing the obtained results with the ones reported in the literature (*10*). The approach was then extended to the case of IgGs. Given the anisotropy of the adsorbed object in that case, new hypotheses were made. The shape of IgGs was simplified to a Y-shaped object, with arms of a width of 4 nm and a length of 7 nm, in agreement with the known dimensions of IgG molecules (*1*). An angle of 90° was imposed between the two arms bearing the variable parts. Only two possible orientations were considered for adsorption: end-on with the constant part in contact with the substrate or flat, which correspond respectively to the minimum (10 nm^2) and maximum (84 nm^2) occupied surface area at the interface. The orientation was randomly chosen (end-on or flat with equal probability) at each deposition of a particle in a randomly chosen location. The interfacial area was divided in two layers, as depicted in Figure 1. Layer 1, at close vicinity of the substrate surface, can accommodate IgG molecules in

flat orientation, as well as the stalk of IgGs standing in end-on position. As in the original RSA model, no overlap of molecules is allowed within this layer. A second layer (layer 2 on Figure 1) was designed to accommodate the upper part of end-on-oriented IgGs (area considered for the projection of this upper part = 40 nm^2). While no overlapping is allowed within layer 2, it is however allowed to overlap molecules between layer 1 and 2. This means that the adsorption of end-on IgGs is allowed in small spaces left between flat IgGs. Finally, given the fact that adsorption in flat orientation is *a priori* energetically more favorable on hydrophobic substrates, after being assigned to a given location, IgGs adsorbed in end-on orientation were allowed to lie down if enough surface area was available at this location.

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Results and Discussion

Monitoring the Typical Steps of an ELISA Assay Using QCM

The Δf measured within the course of typical QCM experiments are presented in Figure 2, for an IgG 2aκ from rat (LO-DNP-16) adsorbed from solutions at three different concentrations (4, 10 and 20 μg/ml). For each experimental curve, the three main steps of the experiment are clearly visible, *ie* a significant negative Δf, corresponding to an increase of mass coupled to the sensor, is recorded after IgG adsorption (t ~ 15h), BSA blocking (t ~18 h), and DNP-alb binding (t ~ 20 h). The increase in adsorbed IgG amount with the concentration of IgG in solution is also evident, Δf being of the order of 15, 30 and 45 Hz for IgG solutions at 4, 10 and 20 μg/ml, respectively. Further decrease of the measured frequency upon blocking of the free surface sites using BSA is inversely related to the adsorbed IgG amount. Finally, the amount of bound DNP-alb seems to be directly related to the IgG adsorbed amount.

The results obtained by QCM for the 8 different IgGs tested, adsorbed from solutions at three different concentrations, are summarized in Figure 3. For most IgGs, the trend observed in Figure 2 is found again, *ie* the adsorbed amount increases with the concentration in solution (Figure 3a). For the IgG 2aκ from mouse, however, saturation seems to be already reached at a concentration of 10 μg/ml. This antibody is also the one that adsorbs with the highest amount (~8 pmol/cm^2) in the concentration range tested. On the contrary, IgG 2bκ from rat adsorbs only in very small amounts, and the amount of adsorbed IgG 3κ from mouse is only slightly higher. To give an point of comparison, an adsorbed amount of 6 pmol/cm^2 corresponds to 8.76 mg/m^2. This is roughly twice the value expected for a closely-packed layer of end-on oriented IgGs (*4*). It should however be kept in mind that water coupled to the adsorbed layer contributes to the frequency shift recorded by QCM (*9*).

The absolute amount of antigen bound to the preadsorbed IgGs (Figure 3b) is the highest for IgG 1κ and 2aκ from rat. As a general trend, this absolute amount increases with the concentration of the solutions used for IgG adsorption. The relation between the adsorbed IgG amount and the corresponding recognized

antigen amount is better visualized in Figure 3c, which shows detected DNP-alb/adsorbed IgG molar ratios. This ratio shows values in the range of 0.5 to 1, the highest ratios being recorded for IgG 3κ from mouse and IgG 1κ and 2aκ from rat. Note that the high value reported for IgG2bκ from rat must be ignored since it results from the very low and uncertain amounts of adsorbed IgGs for this isotype. At this stage, it seems thus clear that a significant fraction of the adsorbed IgGs have their variable parts available for binding. In principle, each IgG molecule could bind with two DNP molecules. The maximum value achievable for the antigen/IgG ratio is thus two. It should be recalled here that DNP is coupled to albumin, with 30-40 DNP molecules per albumin molecule. It is thus possible for a single detected DNP-alb molecule to bind to several binding sites of IgGs. A antigen/IgG ratio of 0.5 is compatible with a layer in which half of the IgGs would be in flat orientation (no binding), and the other half in end-on orientation, with one binding site occupied (or two binding sites occupied by the same DNP-alb molecule). Of course, many other combinations of orientations can be invoked to explain this ratio, including mixtures of flat, side-on, and end-on orientation, with zero, one or two sites occupied per IgG molecule, possibly by the same DNP-alb molecule.

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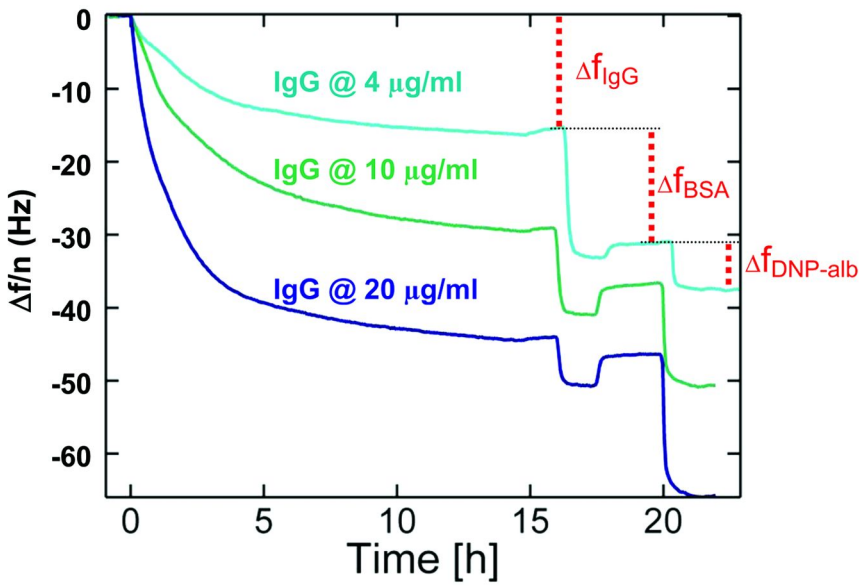

Figure 2. Results of QCM experiments performed with an IgG 2aκ from rat. The IgG concentration in solution is indicated above each recorded curve. The results are shown for the 11th overtone. Δf_{IgG}, Δf_{BSA} and $\Delta f_{DNP\text{-}alb}$ refer to the shift of frequency measured for IgG adsorption, BSA blocking and DNP-alb binding steps, respectively.

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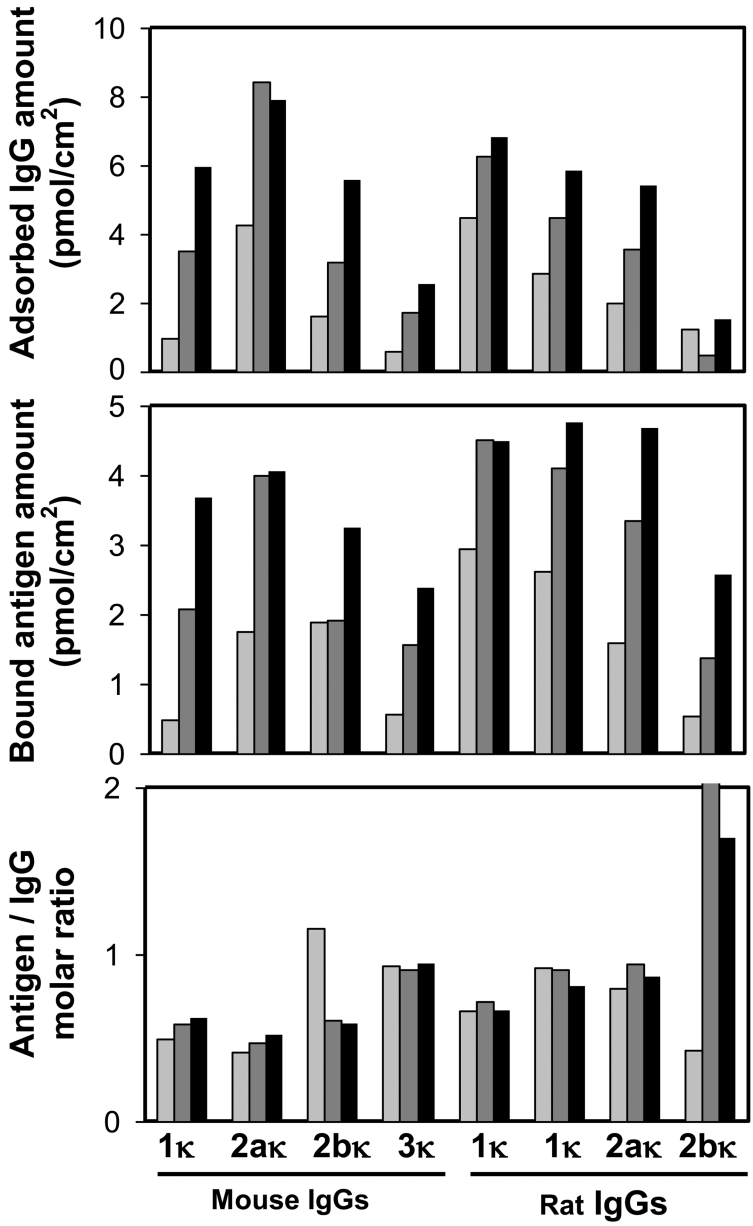

Figure 3. Summary of QCM results obtained with the 8 different IgGs used in this study: (a) adsorbed IgG amount, (b) detected antigen binding, (c) bound antigen to adsorbed IgG ratio. Three different concentrations of IgGs in solution were used: light grey = 4 µg/ml, grey = 10 µg/ml, black = 20 µg/ml.

Figure 4 shows the evolution of Δf recorded after bringing the antigen in contact with the adsorbed IgG layer, as a function of the Δf recorded upon IgG adsorption. Two main trends are observed. On the one hand, there is a roughly linear behaviour for IgGs of a given species. This implies that, whatever the adsorbed amount, the proportion of available sites remains approximately constant. This can also be observed in Figure 3c, where the antigen / IgG ratio remains rather constant with the concentration of the IgG solution. This is in contradiction with the results of Wiseman and colleagues (*2*), which indicated that antibodies shifted from flat to end-on orientation when surface coverage increased. On the other hand, the overall orientation of antibodies within the adsorbed layer seems to be related to the species from which the IgGs originate. The constant part of rat IgGs would thus be the more suitable for binding in an appropriate orientation for further recognition by the antigen.

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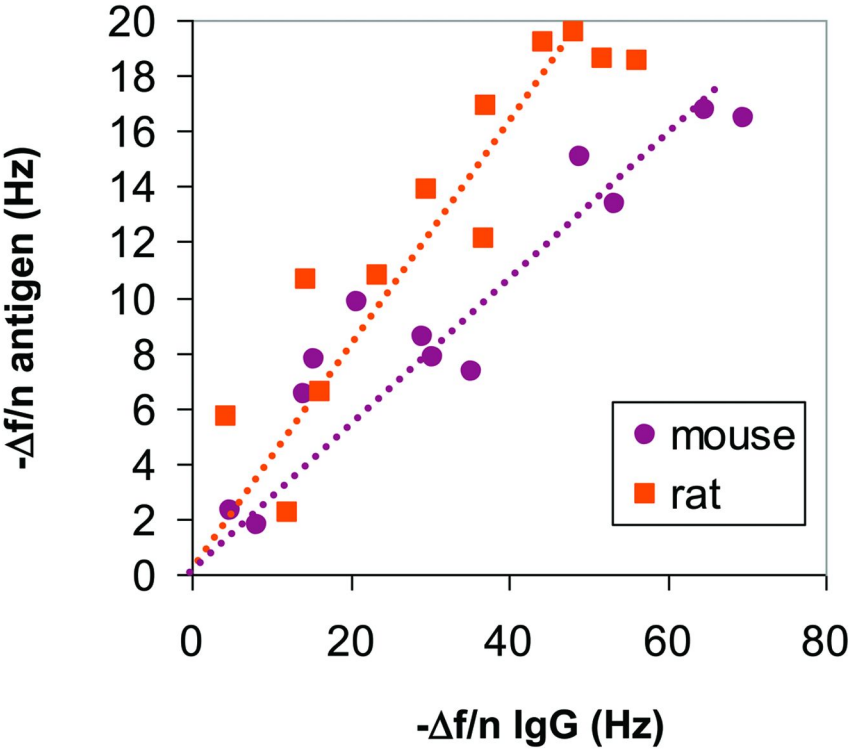

Figure 4. Evolution of Δf recorded by QCM upon antigen binding as a function of Δf recorded for IgG adsorption in the same experiment. The results are reported for the 8 different IgGs tested, adsorbed from three different concentrations each (closed circles for mouse IgGs and closed squares for rat IgGs). The broken lines are visual guides highlighting correlations.

BSA is commonly used to block surface sites remaining available after IgG adsorption, in order to prevent unspecific binding of the antigen to the surface. This is illustrated in Figure 5, in which the Δf recorded upon BSA adsorption is plotted as a function of Δf recorded upon the previously performed IgG adsorption step. A clear inverse correlation is observed, showing that when the adsorbed IgG amount increases, less BSA molecules can be further added to the adsorbed layer. At the highest IgG adsorbed amounts (Δf ~70 Hz), a Δf close to zero is actually observed for the BSA adsorption step. It should be kept in mind that exchanges between the adsorbed IgG layer and BSA molecules brought in solution may occur, resulting in no net increase (or even a decrease since the molar mass of albumin is lower than the one of IgGs) of mass detected by QCM. On polystyrene, protein adsorption is however generally found to be rather irreversible, and such exchanges are not favored (*13*). More particularly, Elgersma et al. (*14*) studied the effect of BSA addition in the solution on a layer of IgGs previously adsorbed on polystyrene. At pH 7, the adsorbed amount, as estimated from reflectometry results, was shown not to be affected, suggesting the absence of exchange between these proteins.

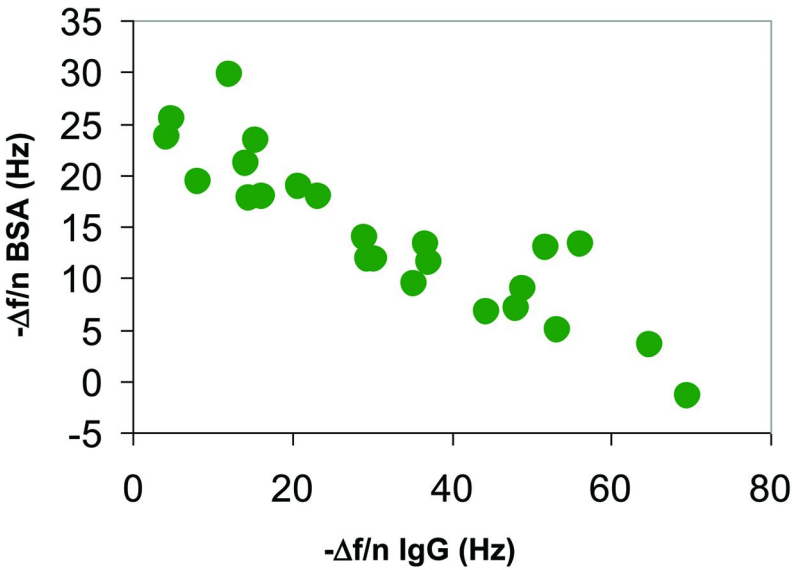

Figure 5. Evolution of Δf recorded after the BSA blocking step as a function of Δf recorded upon IgG adsorption in the same experiment. The results are reported for the 8 different IgGs tested, adsorbed from three different concentrations each.

IgGs Orientation Simulated Using RSA Modeling

RSA modeling was performed to tentatively clarify the evolution of IgGs orientation within adsorbed layers upon increasing the surface coverage. Figure 6 shows snapshots of the progressively filled surface, together with the computed end-on / flat orientation ratio at each depicted stage of the process.

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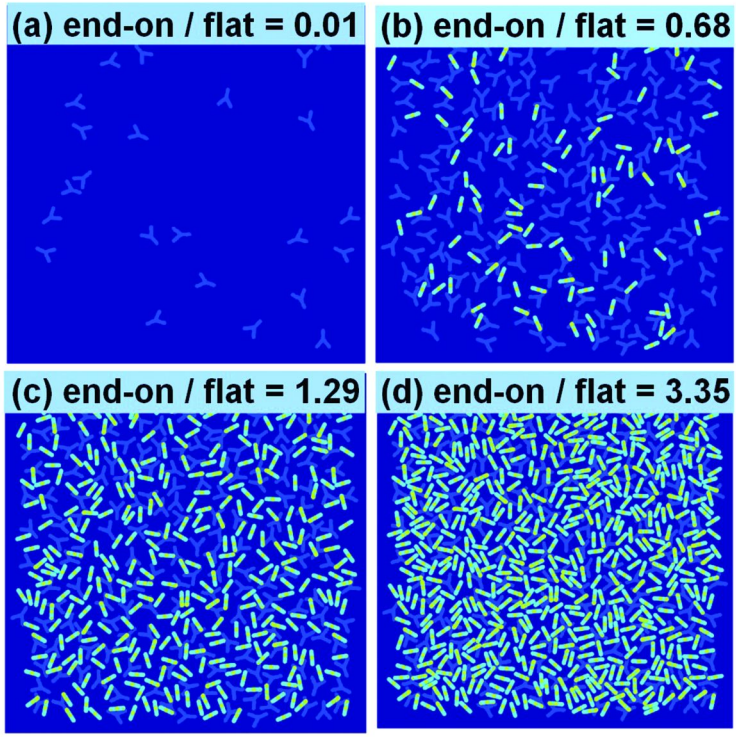

Figure 6. Snapshots (from a to d) illustrating the progressive coverage of the surface with IgG molecules adsorbed in flat orientation (medium blue) or in end-on orientation (light blue). The yellow areas refer to overlapping between molecular parts present in the first and the second layers defined for RSA modeling (see text for details).The end-on / flat orientation ratio corresponding to each depicted stage of the process is given in inset. (see color insert)

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At the beginning of the filling process, since the probability is high that enough surface area is available for end-on adsorbed IgGs to shift to flat orientation, IgG molecules are mostly adsorbed in flat orientation. When the surface is progressively filled, this probability is reduced, and IgGs adsorbed in end-on orientation start to stay in this orientation, which increases the end-on / flat orientation ratio. Finally, in the last stages of the surface filling procedure, the only spaces available at the interface become too small to accommodate IgGs in flat orientation, and only end-on oriented IgGs continue filling the surface, which causes a high increase of the end-on / flat orientation ratio.

This is further illustrated in Figure 7, which summarizes the whole virtual adsorption process. Up to a total adsorbed amount of 1.4 pmol/cm^2, the amount of IgGs adsorbed in flat orientation is higher than the one of end-on adsorbed IgGs. At higher adsorbed amounts, the amount of IgG molecules in flat orientation

does not increase anymore, while there is a sharp increase of adsorption in end-on orientation. The maximum achievable adsorbed amount is of about 3.3 pmol/cm^2, which corresponds to 4.8 mg/m^2. At this surface concentration, the end-on / flat ratio reaches a value of 3.38. A surface concentration of 2 mg/cm^2 is expected for a closed-packed layer of flat IgGs, while values between 2.6 and 5.5 are expected for end-on adsorbed IgGs, depending on the contraction of their Fab fragments (*4*). The value obtained here by RSA simulation seems thus reasonable, since close-packing cannot be achieved when random adsorption is considered, but on the other hand, the combination of flat and end-on adsorbed molecules increases the adsorbed amount.

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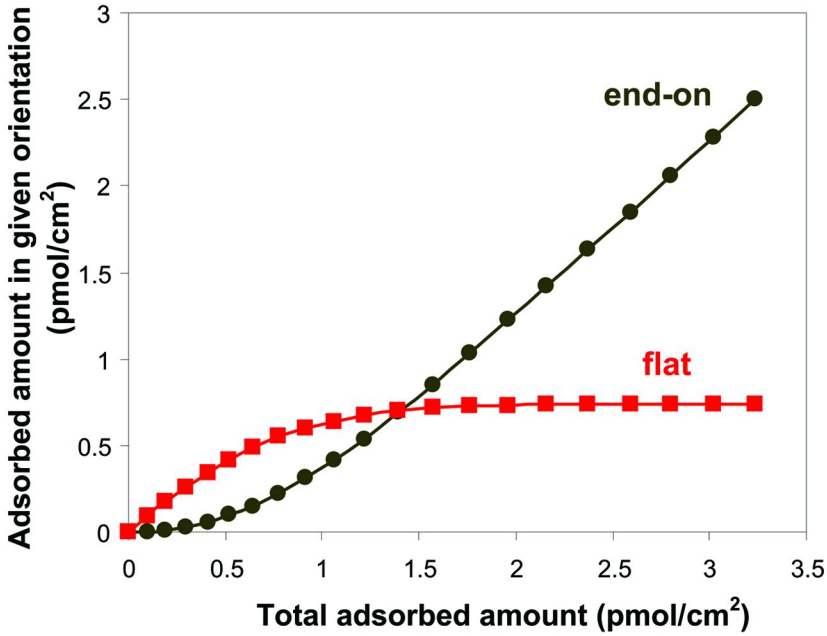

Figure 7. Results of RSA modeling : evolution of the adsorbed amount of IgG molecules in a given orientation (closed circles: end-on, closed squares: flat) as a function of the total adsorbed amount.

Experimental Observations versus Modeling Outputs

Besides allowing differences in behavior related to IgGs origin and isotype to be observed, our QCM results show that the bound antigen to adsorbed IgG ratio is in the range of 0.5 to 1. We observed however that this ratio does not vary much with the adsorbed amount, *ie* it remains quite constant when the adsorbed IgG amount increases.

RSA modeling was used to highlight the fact that although IgG adsorption in flat orientation is *a priori* energetically more favorable on hydrophobic substrates, a significant amount of end-on adsorbed IgG can be found in the adsorbed layer. This is due to the much lower surface area occupied by the latter compared to the former. After the surface is filled to a certain level by IgG molecules adsorbed in flat orientation, the low surface area of the left interfacial spaces does not allow much more adsorption in that orientation, but many more IgG molecules can accommodate in these left spaces in end-on position. At high level of surface coverage, a majority of the adsorbed IgG molecules are thus in end-on position.

In the present modeling approach, we only considered two orientations *ie* flat and end-on. This constitutes of course a very simplified view. Moreover, we only considered end-on adsorption with the two variable parts of IgG molecules standing upwards. Actually, if one of the variable parts is used for adsorption, very similar results would be obtained, but only one binding site would then be available for antigens instead of two. It would still lead to the conclusion that the amount of binding sites pointing outwards is increasing with surface coverage.

We can first compare the adsorbed amount predicted by RSA modeling to the values computed from QCM experiments with the range of antibodies studied here. The values extracted from QCM data for the highest IgG concentration in solution are for most of the IgGs studied roughly two times higher than the value of 3.3 pmol/cm^2 predicted by RSA modeling. The value obtained from modeling depends of course on the shape and dimensions chosen to simulate IgG molecules. However, the most important difference certainly arises from the presence of coupled water in the adsorbed layer, which is probed by QCM together with the adsorbed IgGs (*9*). Water could thus account for about half of the detected mass.

In the work of Wiseman et al. (*2*), for the adsorption of a monoclonal anti-streptavidin human IgG1 on a model hydrophobic substrate, the dissipation shift recorded by QCM increased exponentially when the IgG adsorbed amount increased. This may suggest a higher water content in the adsorbed layer at high surface occupancy, which could result from the more important presence of end-on-oriented molecules. The progressive increase of IgG molecules adsorbed in end-on position with surface coverage predicted here by RSA modeling fits thus well with the conclusions of the study of Wiseman et al. (*2*). On the contrary, our QCM data show a quite constant bound antigen to adsorbed IgG ratio with increasing adsorbed amount. There might be different tentative explanations to this discrepancy.

First, the increased presence of coupled water in the adsorbed layer with surface coverage may lead to an overestimation of the IgG adsorbed amount. Consequently, the antigen to antibody ratio measured when higher IgG concentrations are used for adsorption would be decreased. In the present study, however, ΔD values were always very low, and were roughly linearly correlated to Δf values (results not shown), indicating that the water content of the adsorbed layer remains quite constant whatever the surface coverage.

Second, the antigen used in the present study (DNP) is coupled to albumin to allow its detection by QCM. This may lead, on the one hand, to steric hindrance in reason of the size of albumin. Albumin shape can be assimilated to an equilateral triangle with sides of ~8 nm and a thickness of ~3 nm (*15*). On the other hand, 30 to

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40 DNP molecules are coupled to each albumin molecule, allowing multivalency effects. The detection of one albumin molecule may actually account for multiple binding events. These two effects will be amplified at high IgG surface coverage, due to the proximity of variable parts of different IgG molecules in that case. This would cause the bound antigen to adsorbed IgG ratio to remain relatively constant with the IgG adsorbed amount, despite the higher proportion of IgGs adsorbed in end-on orientation at higher surface coverage.

A third explanation to the relatively constant antigen binding level with IgG adsorbed amount could reside in the role played by albumin used for the blocking step applied between IgG adsorption and antigen binding. Our results indicate that the lower the IgG adsorbed amount, the higher the albumin adsorbed amount upon blocking. The adsorbed albumin molecules may actually modify the orientation of previously adsorbed antibodies. Albumin is a soft protein (*16*), and tends to denature in contact with hydrophobic surfaces. Upon relaxation at the interface, it may provoke a change of orientation of adsorbed IgGs, which would cause a better availability of the variable parts. Similarly, it was shown that the availability of cell-binding domains of adhesion proteins was increased by adsorbing them together with albumin (*17*, *18*). In these latter studies, albumin was however brought in solution simultaneously to the investigated proteins. It is difficult to predict if albumin may play a similar role when it is brought at the interface after IgG adsorption has already taken place. If this was the case, even though RSA modeling predicts adsorption in flat orientation at low surface coverage, orientation could change to end-on after albumin adsorption, thereby increasing recognition by the antigen.

Conclusion

The adsorption of 8 different IgG isotypes from rat and mouse, all directed against DNP, and their antigen binding efficiency was monitored using QCM. The results show that the adsorbed IgG amount increases with the IgG concentration in solution, and that the maximum adsorbed amount differs from one isotype to another, the highest adsorbed amount being recorded for IgG 2aκ from mouse. A blocking step, consisting of BSA adsorption, was applied before antigen binding. The QCM data show an inverse correlation between the IgG and BSA adsorbed amounts, in line with the fact that albumin molecules are expected to fill the empty spaces remaining between IgG adsorbed molecules at low IgG adsorbed amount. Antigen binding was then shown to be the most efficient for rat antibodies, which points to their better orientation upon adsorption. For a given IgG, the ratio of bound antigen to adsorbed IgG was relatively constant for different IgG adsorbed amounts, which could mean that the fraction of IgG molecules oriented with their variable parts pointing upwards remains quite constant whathever the adsorbed amount.

RSA modeling was performed to tentatively clarify the evolution of IgG molecule orientation upon increasing the surface coverage. The results of modeling show that the fraction of IgG molecules adsorbed in end-on orientation increases with the surface coverage, owing to the fact that, when the surface

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ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

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coverage becomes higher, the only spaces available at the interface are too small to accommodate IgGs in flat orientation, and only end-on oriented IgGs continue filling the interface. The apparent discrepancy between the experimental results collected using QCM and the theoretical results obtained through RSA modeling could be explained by a variable content in coupled water in the adsorbed layers depending on surface coverage, by steric hindrance and multivalency effects upon antigen binding, or by the role of albumin molecules used to prevent non specific adsorption of the antigen.

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