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Research paper

# Influence of pH and ionic strength on IgG adsorption to vials

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ABSTRACT

It was the aim of this work to investigate the influence of the formulation parameters pH and ionic strength on the adsorption of IgG to borosilicate glass vials. The charge characteristics of IgG and glass surface were determined by isoelectric focusing and electrokinetic measurements. It could be shown that IgG adsorption highly depends on formulation pH and ionic strength. The amount of IgG adsorbed results from an interplay of attractive and repulsive electrostatic interactions between protein molecules and the glass surface as well as among adsorbed protein molecules. The pH value where the ion uptake in the adsorption boundary was minimal coincided well with the pH of maximum adsorption. At pH 4, the presence of $Na_2SO_4$ gave rise to a stronger increase in adsorption than NaCl at equal ionic strength, whereas we observed no differences at e.g. pH 7.2 and 8.6. In summary, it can be stated that IgG adsorption on borosilicate glass is to a large extent mediated by electrostatic interactions. Other driving forces like hydrophobic interactions or surface-induced structural alterations contribute to a much lesser extent.



## 1. Introduction

Protein adsorption is a ubiquitous phenomenon in nature. In the context of protein drug formulation, it plays an important role e.g. upon contact of the protein molecules with the air–liquid interface, with processing material interfaces like filters or tubings as well as with the primary packaging material. It is important to understand the mechanisms of protein adsorption to the different materials surfaces in order to take the appropriate measures to cope with adverse effects. In this study, we focus on the primary packaging material and analyzed the interaction of an IgG model protein with glass type I vials. Typically, a surface contributes to protein adsorption mainly through an energy gain from dehydration, as well as through an ion transfer and an overlap of its electrical field with that of the proteins [1]. Hydrophobic dehydration often exceeds the strength of electrostatic effects [2]. Electrostatic interactions themselves highly depend on the charge properties of both protein and surface. Charges are to a great extent influenced by factors such as pH and ionic strength. Hence, the protein formulation is of great importance. In adsorption studies of IgG and other proteins, pH and ionic strength [3–6], as well as sorbent surface properties, such as electrical charge density and hydrophobicity [7,8], have often been used as variables to get information on the driving forces and the adsorption mechanism. With the example of IgG adsorption on hydrophilic and hydrophobic silica, pH and ionic strength were described to affect the adsorption kinetics, the equilibrium adsorbed amount, as well as the degree of adsorption irreversibility [9]. Shifts in pH and ionic strength have also been described to affect the structural state of a protein [8,10,11], which, in addition, may lead to differences in the protein adsorption characteristics.

It is a common rule that protein adsorption on hydrophilic surfaces is for the most part governed by charge–charge interactions. Adsorption should increase through electrostatic attraction in case protein and surface carry a different (net) charge, whereas adsorption becomes reduced with increasing charge of the same sign [12]. In the latter case, or if either one of the "partners" holds a pronounced charge preponderance, a strong electrostatic potential is formed in the interfacial region, which is energetically unfavorable [2]. For judging the electrical state of a protein and a sorbent surface, the zeta potential ($\zeta$) is of utmost importance. For proteins, especially the amino acid composition of the molecule surface is determinative, whereas for glass, it is the chemical composition of the outermost surface layer. In a protein formulation, decisive parameters for $\zeta$ are ionic strength ($I$), viscosity, temperature, and pH. The concentration of protons has the highest influence on $\zeta$ due to their small dimensions and their high charge density [13]. Neutral salts like NaCl decrease $\zeta$ through double-layer compression [14]. Explicitly, the Debye length ($\kappa^{-1}$) depends on the factor $1/\sqrt{I}$.

Within the scope of this work, zeta potentials were calculated for borosilicate glass and IgG1 from electrophoretic mobility data [15,16]. Generally, two basic approximation models relate the measured electrophoretic mobility ($\mu_e$) to the zeta potential. In

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our case, glass particles exhibit relatively large dimensions compared to $\kappa^{-1}$. Accordingly, the Helmholtz–Smoluchowski equation was used to compute the zeta potential [17]. For IgG1, the ratio of protein radius and electrical double-layer thickness was largely decreased due to the small protein dimensions. In the case of a thick double layer, with regard to particle diameter, the zeta potential is determined by applying the Hueckel approximation. Finally, the electrokinetic charge density ($\sigma_e$) can be derived from the zeta potential. The value of $\sigma_e$ gives a measure of the amount of electric charges per surface area. For symmetric electrolytes, in which the absolute values of the signed units of charge, $z_i$, are the same for all ions present, the electrokinetic charge density on the surface of particles can be calculated [18]. The objective of measuring $\sigma_e$ is its use for adsorption-specific mathematical calculations. For example, the amount of low molecular weight ions can be determined, which is integrated in the adsorption boundary and neutralizes unfavorable charge accumulation [15,19].

Besides their simple contribution to ionic strength, salts can affect the stability of proteins in the formulation. In general, salts can have either stabilizing or destabilizing effects on proteins [20], insofar as protein solubility and stability are affected by the preferential exclusion of the respective salt type [21].

It was the aim of this study to investigate the influence of the most important formulation parameters on the adsorption behavior of IgG1, mainly on borosilicate glass. Moreover, from the response of the IgG1 to the particular adsorption conditions, basic knowledge on the adsorption mechanism and the driving forces of adsorption should be gained. Special attention was paid to the impact of pH and ionic strength. In a typical IgG formulation of rather high concentration, the total amount of adsorbed protein is not relevant with respect to total loss. However the adsorption mechanism of this highly relevant class of molecules was in the focus. Since the charge properties of the protein appeared to be a decisive property governing the adsorbed amount, a monoclonal IgG1 with distinct isoforms was compared with pooled human IgG (h-IgG) with a broader distribution in charge characteristics. Based on the results of an initial isotherm study, a concentration of 1 mg/ml protein assured surface saturation conditions (data not shown). For the IgG1, 2 mg/ml and for the h-IgG, which was limited in availability, 1 mg/ml were selected for the studies.

## 2. Materials and methods

### 2.1. Materials

#### 2.1.1. Protein formulation and chemicals

For adsorption experiments, a 2 mg/ml solution of IgG1 (MW ≈ 152 kDa) in 10 mM phosphate buffer plus 145 mM NaCl (pH 7.2) was used, which was kindly provided by Merck Serono GmbH (Darmstadt, Germany). For ionic strength adjustment, the solution was dialyzed against a 10 mM phosphate buffer solution ($NaH_2PO_4$ and $Na_2HPO_4$ from Merck Chemicals, Darmstadt, Germany) without NaCl, using Vivaflow® 50 tangential flow filtration cartridges (Sartorius-Stedim Biotech, Goettingen, Germany) equipped with a 30 kDa PES membrane. The IgG1 concentration was determined by UV spectroscopy. Variable ionic strengths were adjusted by the addition of NaCl or $Na_2SO_4$ (Merck Chemicals, Darmstadt, Germany), followed by pH adjustment (1 M NaOH or HCl (Sigma–Aldrich, Munich, Germany)). Finally, adequate amounts of salt were added to the solutions to retain the appropriate ionic strength. IgG from human serum (h-IgG) (Sigma–Aldrich, Munich, Germany) as a salt-free lyophilized powder was dissolved in the respective buffer solution to yield the formulations corresponding to the IgG1 and quantified by UV spectroscopy. All protein solutions were filtered through a 0.2 μm PES filter (Pall GmbH, Dreieich, Germany) before use. Ultrapure water (0.055 μS/cm) for all applications was obtained from a Purelab Plus UV/UF system (ELGA LabWater, Celle, Germany).

#### 2.1.2. Vials and closure systems

Fiolax® 2R borosilicate glass vials were kindly provided by SCHOTT AG (Mainz, Germany). Vials were washed in a vial washing machine FAW 500 (Bausch & Stroebel, Ilshofen, Germany) with ultrapure water and heat sterilized at 250 °C for 1 h before use. Resin CZ® 2 ml plastic vials (Daikyo Seiko, Ltd., Japan), made upon a cyclic polyolefin (COP), were washed in the same way but dried at 80 °C for 1 h. After filling, the vials were closed with FluroTec® stoppers and sealed with Flip-Off® seals (West Pharmaceutical Services, Eschweiler, Germany).

#### 2.1.3. Glass powder

A glass powder was prepared from the glass vials so that the zeta potential of the glass could be measured by dynamic laser light scattering (DLS) measurements. Glass vials were shattered and the flinders, apart from the vial neck, were milled in a Pulverisette® 5 laboratory planetary mill (Fritsch GmbH, Idar-Oberstein, Germany) for 8 h. The particle fraction ⩽45 μm was collected and washed three times by suspending and centrifuging in water. The particles were dried at 90 °C and heat sterilized at 250 °C for 1 h (primary fraction). For electrokinetic measurements, we obtained non-sedimenting glass particles by suspending the primary fraction in water, followed by sedimentation providing secondary fraction in the supernatant.

### 2.2. Methods

#### 2.2.1. Isoelectric focusing

Isoelectric focusing (IEF) was performed on a Multiphor II™ electrophoresis system combined with an EPS 3501 XL power supply and a MultiTemp III thermostatic circulator (GE Healthcare Europe GmbH, Freiburg, Germany). IEF gels were precast Servalyt® Precotes® Wide Range pH 3–10 as well as Range pH 6–9 gels (Serva Electrophoresis GmbH, Heidelberg, Germany). Serva Liquid Mix IEF Marker 3–10 was used as protein standard. The protein sample concentration was 2 mg/ml in a 10 mM NaCl solution. Final gel staining was accomplished using the Serva Violet 17 staining kit.

#### 2.2.2. Electrophoretic mobility and hydrodynamic diameter measurements by DLS

DLS was applied to investigate the electrophoretic mobility of both glass particles and IgG1 molecules. Zeta potentials and surface charge densities were derived from this data. Measurements were performed on a Zetasizer Nano ZS (Malvern Instruments GmbH, Herrenberg, Germany) exclusively in "monomodal mode" (50 V const.). The IgG1 concentration was 6 mg/ml in 10 mM NaCl (concentrated as described in Section 2.1.1), since a concentration higher than 2 mg/ml was necessary for adequate results. The pH adjustment of the samples was computer controlled by a Malvern MPT-2 Autotitrator. The samples were stirred in polypropylene (PP) tubes and titrated with 0.1 M NaOH and 0.1 M HCl. The hydrodynamic diameter ($d_h$) of IgG1 was measured in non-invasive backscatter mode at 173° and was taken as the mean from 3 series of 10 measurements.

#### 2.2.3. UV spectroscopy

UV spectroscopy for protein concentration measurements was performed on a temperature-controlled Agilent 8453 UV/VIS spectrophotometer (Agilent Technologies GmbH, Boeblingen, Germany) at 25 °C, $\lambda$ = 280 nm using quartz cuvettes and applying an $e_{1\%}$ of 1.40 for antibodies [22].

*2.2.4. Adsorption process*

The preprocessed glass vials were filled with 3.5 ml (COP vials 2.5 ml) of 2 mg/ml IgG1 solution or 1 mg/ml h-IgG solution, closed, and incubated for 24 h in a water bath at 25 °C with slow horizontal movement (25 rpm). The vials were emptied using a syringe with an injection needle and rinsed in four steps with buffer solution correlating with the respective formulation. For desorption of adsorbed protein, the glass vials were filled with 3.5 ml (COP vials 2.5 ml) PBS buffer pH 7.2 containing 0.05% SDS (Sigma–Aldrich, Munich, Germany), sealed and stored at 25 °C overnight.

*2.2.5. Size-exclusion HPLC*

Desorbed protein samples were quantified via size-exclusion HPLC on an Agilent 1100 (Agilent Technologies GmbH, Boeblingen, Germany) equipped with a Tosoh TSKgel G3000SWXL and a TSKgel SWXL guardcolumn (Tosoh Bioscience GmbH, Stuttgart, Germany). The desorption buffer (see Section 2.2.4) was used as mobile phase. The protein fluorescence signal was recorded at $\lambda_{ex}/\lambda_{em}$ 280 nm/334 nm. In each HPLC batch run, a 10-point IgG calibration from of 0.1 to 10.0 μg/ml was included. LOD and LOQ were 0.048 μg/ml and 0.130 μg/ml, respectively. In glass vial adsorption studies, the determined protein concentrations typically ranged between 0.2 μg/ml and 4 μg/ml. This reflects an adsorption of 0.01–0.2% of the initial 2 mg/ml. The method was cross validated against total organic carbon analysis after acid hydrolysis of the adsorbed protein.

## 3. Results and discussion

As described before, protein adsorption on charged surfaces is governed by electrostatic interactions. Proteins consist of a multiplicity of dissociable groups, and the overall charge of a protein molecule results from an overlay of all dissociation equilibria. For glass, the surface charge primarily arises from the dissociation of surface-exposed ≡Si—OH groups. For proteins, it is mainly the dissociation of amino acid residues that accounts for charges on the surface. Furthermore, the surface potential is altered by the attachment of electrolytes, e.g. salt ions, with or without a specific surface affinity. The effect of surface-attached ions or charged molecules is associated with compensation resp. screening of "free" charges, which similarly affects the protein and the glass surface.

*3.1. Charge characterization of IgG and glass surface*

Initially, the charge state of glass and of both IgGs was characterized according to pH and ionic strength. The region of the isoelectric pH as well as the number of discrete isoforms of both IgG types, the monoclonal IgG1 and the human IgG from pooled serum was determined with isoelectric focusing. As shown in Fig. 1, the IgG1 exhibits 6–7 characteristic isoforms in the range from pH 7.62 to 8.16. Different isoforms originate from micro-heterogeneities of C-terminal lysines, from deamidation within several Asn – Gly deamidation sites, as well as from differences in the glycosylation pattern. For the human IgG, no characteristic isoforms, but a broader distribution, are apparent and the pI range reaches from approx. pH 7.0 to 8.6.

Electrokinetic mobility measurements were accomplished as a function of pH for IgG1, h-IgG, as well as the borosilicate glass particles. Both colloids, the glass particles and the protein molecules, were treated as spheres with a homogeneously distributed surface charge. Zeta potentials were calculated from electrokinetic mobility data and are shown in Fig. 2a and b.

For both IgGs, typical protein titration curves are obtained (Fig. 2a). The mean pI of IgG1 and human IgG, as the intercept with the *x*-axis at zero mV, was determined at 8.33 and 8.28, respectively, which corresponds with the results from isoelectric focusing. The isoelectric points of the materials investigated are within the spectrum of pH 5.8 [3] to 9.0 [23], described in literature. Concerning the average charge status of all isoforms, there is hardly any difference between both IgG samples, as indicated by the identical curve shape. The results for IgG1 were verified by comparison with the theoretical charge, which was calculated from the protein sequence using the IEP module of the European Molecular Biology Open Software Suite (EMBOSS) [24] (Fig. 2a). Although the theoretical calculation can only be considered a rough approximation, it coincides well with the curve shape derived from electrophoretic mobility measurements. Minor deviations can be observed, like a flattening of the titration curves at the low and high pH end. This phenomenon is not exceptional and occurs at pH values far from the pI, where molecules are considerably charged [25]. The theoretically calculated pI is located at 8.69. Since the measured pI does not seriously deviate from the theoretical value, it can be concluded that the zeta potential or the protein net charge in solution is not dramatically changed by preferential adsorption of a certain ion type from the protein solution.

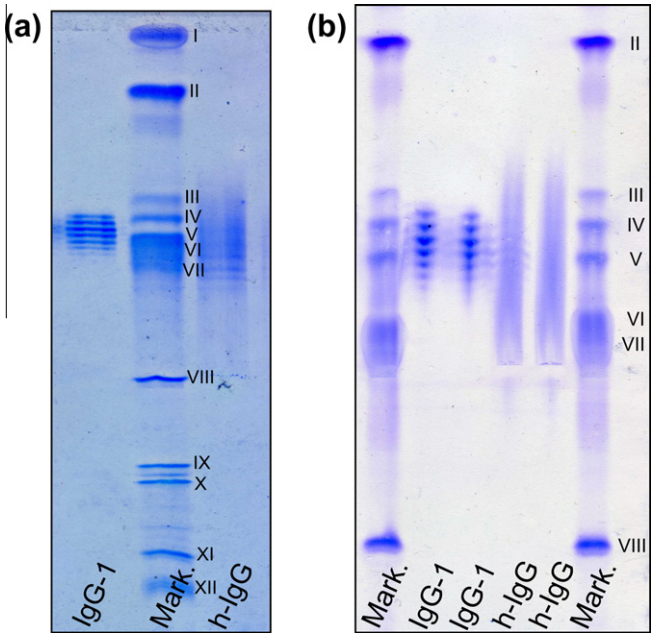


**Fig. 1.** Isoelectric focusing gels for the determination of the pI of IgG1 and h-IgG in a broad range (a) pH 3–10 and a narrow range (b) pH 6–9; marker bands can be assigned to the pI values: (I) 10.7; (II) 9.5; (III) 8.3; (IV) 8.0; (V) 7.8; (VI) 7.4; (VII) 6.9; (VIII) 6.0; (IX) 5.3; (X) 5.2; (XI) 4.5; (XII) 4.2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

As expected, the glass surface is highly negatively charged in the whole alkaline pH region with zeta potential values down to −70 mV (Fig. 2b). This high value does not appreciably vary in the range between pH 12 and 7. Within this range, surface-exposed ≡Si—OH groups are largely dissociated. With further decreasing pH, the surface potential becomes less negative through increased protonation and the glass surface finally reaches its point of zero charge (PZC) at 2.28. According to literature, the PZC of silica is approx. 2 [6,17,26], but may vary in the range from pH < 1 to 5.5, depending on the ions present in the liquid phase [26]. It is rather likely that borosilicate glass, which contains additional components, like e.g. aluminum oxide and boron oxide, shows a PZC different from pure silica. Barz et al. mentioned a PZC of borosilicate glass in the range between pH 1.7–2.0 [27]. Fig. 2b also reveals a

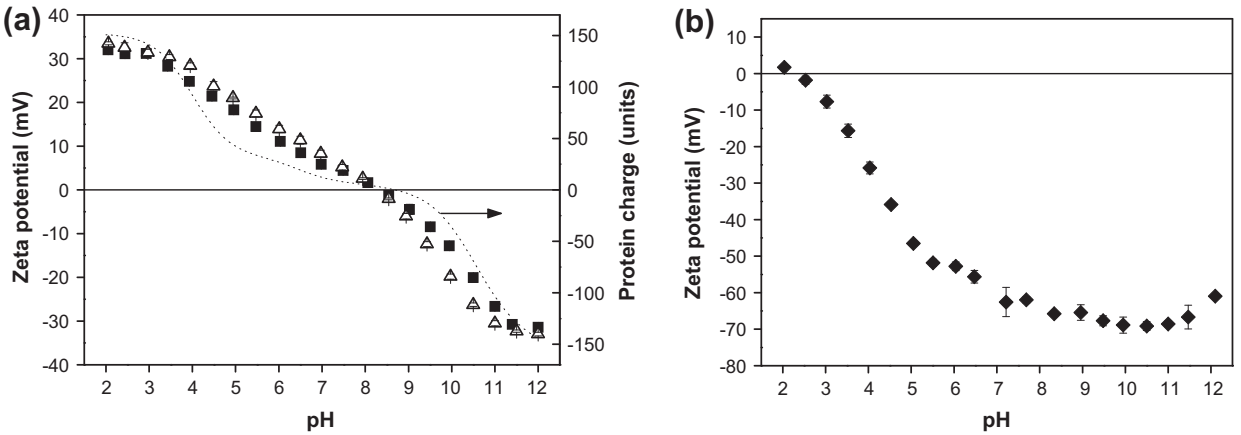


**Fig. 2.** Zeta potential as a function of pH, determined by electrophoretic mobility measurements in 10 mM NaCl solution ($n$ = 3); (a) zeta potential of IgG1 (■) and h-IgG (△) as well as IgG1 theoretical charge distribution determined from the primary sequence (dotted line); the arrow indicates the corresponding axis; (b) zeta potential of washed and heat sterilized (250 °C, 1 h) borosilicate glass particles (♦).

certain drift of the zeta potential to less negative values above pH 11, which again was presumably attributed to an increase in the ionic strength through the titrant solution. Such a phenomenon could not be observed in the acidic threshold region. In the following, the zeta potential is simplified and referred to as "charge" of the respective material.

### 3.2. Impact of the formulation pH on IgG adsorption

#### 3.2.1. The adsorbed quantity as a function of pH

The influence of pH on the adsorption of IgG on borosilicate glass vials was investigated in the following. Adsorption plateau values as a function of pH ($\Gamma_{pl}$(pH)) were determined in the range from pH 2 to 12. According to adsorption isotherms (data not shown), plateau values ($\Gamma_{pl}$) for adsorption were assumed for incubation at 1 mg/ml. They are typically reached after approx. 5 h, and the time for incubation was consequently set uniformly to 24 h. In Fig. 3, the $\Gamma_{pl}$ values are plotted against the formulation pH. For both IgG1 and h-IgG, a bell-shaped dependency is observed, which is common for protein adsorption on solid surfaces [5,28,29]. For monoclonal IgG1, the highest surface concentration is found at approx. pH 4–5. Unambiguously, the maximum is not placed at the pI, the point at which one may expect, e.g., the lowest protein solubility and the least inter-protein repulsion. From pH 6 on, adsorption steadily decreases toward zero with increasing pH. The most pronounced drop occurs in the upper alkaline pH region. $\Gamma_{pl}$(pH) also decreases from pH values below 4 toward pH 2, albeit not to the same extent.

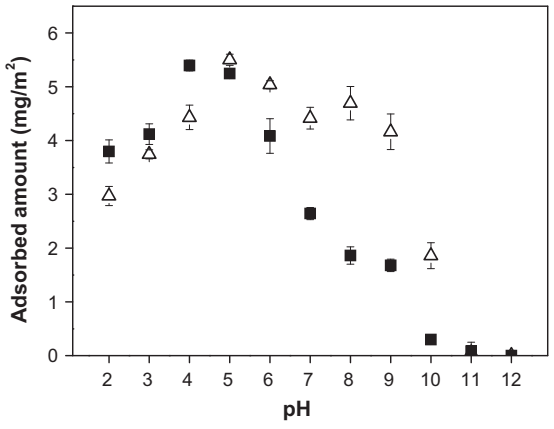


**Fig. 3.** Adsorption profiles of monoclonal IgG1 (■) and pooled human IgG (△) on borosilicate glass depending on pH; incubation for 24 h, 2.0 mg/ml IgG1 (1.0 mg/ml h-IgG) including 10 mM phosphate buffer, 170 mM total ionic strength ($n$ = 3).

The adsorbed amounts of h-IgG compare well with those of IgG1. The pH of maximum adsorption for the h-IgG is located in the same pH region, although it may be slightly shifted toward a higher pH value. We cannot tell whether the maxima are different as measurements were performed at pH intervals of approx. one pH unit and the actual maximum may have been missed. However, $\Gamma_{pl}$(pH) values do not show the same continuous behavior as was observed for IgG1. A second local maximum occurs at the pI (7.0–8.6). A pronounced drop of $\Gamma_{pl}$ for pH ≈ 9 as well as a decrease of $\Gamma_{pl}$ toward zero for pH values above pH 9 becomes obvious for both the pooled h-IgG and the monoclonal IgG1. Reasons accounting for the observed differences must be related to intrinsic protein properties. As stated above, the two species differ in their electrostatic properties. It is assumed that the broadening of the bell-shaped adsorption characteristic is due to the wider range of the pIs of h-IgG, as compared with the discrete, but rather similar isoforms of IgG1.

Protein molecules as well as the borosilicate glass surface in aqueous solution are present in a charged state. The electrostatic interactions can be classified into interactions between protein and surface, as well as between protein molecules on the surface in adsorbed state. As depicted in Fig. 2a, the net charge of protein molecules increases with increasing pH values above the pI, and with it, the repulsive forces between the molecules. Accordingly, for both IgG1 and h-IgG, the adsorbed quantities substantially decrease at pH > pI. Similar results were described by Zhu et al., who found decreasing protein adsorption with increasing charge difference of the same sign, i.e. increasing repulsion [30]. However, in our case, adsorption does occur to a high extent above the pI region. It was described that protein adsorption occurs spontaneously although the surface and the sorbent bear the same charge [5,31,32]. This can be enabled by the incorporation of ions in the inner region of the adsorbed layer to avoid extremely high charge density in the contact region [7]. Furthermore, the spatial charge distribution on the surfaces of the protein molecules can have an influence as well. The IgG1 crystal structure model revealed that charges are not uniformly distributed on the surface. Thus, even if the net charge of the molecules and the glass surface is of the same sign, e.g. for pH above the pI of the protein, still patches of an opposite charge excess can exist, leading to unhesitant adsorption through electrostatic attraction.

Furthermore, a contribution of other adsorption mechanisms than electrostatic interactions has to be taken into consideration. Structural rearrangements, which outweigh an unfavorable contribution of electrostatic repulsion, have been described to allow adsorption at pH values above the pI [7]. Last but not least, protein adsorption on glass is to a certain extent driven by dehydration reactions (hydrophobic interactions) since the glass vial surface is not completely hydrophilic. But at least for high pH values greater or equal than 11, the circumstances become highly unfavorable and adsorption vanishes almost completely.

For IgG1, the $\Gamma_{pI}(\mathrm{pH})_{max}$ is located away from the pI of the antibody and shifted toward lower pH values (pH 4–5). Below pH 8.6, both the glass and IgG1 carry different charge signs, and the differences in zeta potential initially increase with decreasing pH. The pronounced negative charge of the glass in this pH region is largely preserved. Accordingly, the net electrostatic attraction toward the sorbent surface increases. But with an increasing net charge of IgG, the intermolecular repulsion forces increase as well. Both aspects have to be taken into account, and the combination is decisive for the resulting adsorbed amount. After passing an optimum in electrostatic conditions, circumstances become more adverse with decreasing pH, since the amount of negative charges on glass decreases and thus the electrostatic attraction toward the glass. Additionally, the electrostatic repulsion among adsorbed protein molecules further increases. Below the PZC of borosilicate glass (pH 2.3) when glass becomes net positively charged, electrostatic attraction toward the surface turns into repulsion, which leads to a further decrease in adsorption.

In several cases, $\Gamma_{pI}(\mathrm{pH})_{max}$ has been found at pH values away from the protein pI, e.g. for HSA on colloidal $TiO_2$ [16] or BSA on negatively charged polyethersulfone membrane [32]. According to the authors, increased electrostatic attraction forces between the oppositely charged protein and sorbent surface below the pI led to increased adsorption. Consequently, electrostatic interactions between the proteins and the surface outweigh the influence of intermolecular repulsion. Furthermore, according to literature, an increased irreversibility of protein adsorption can occur in the pH range below the protein pI because of the local prevalent beneficial electrostatic interactions [33]. In contrast, various researchers have reported maximum adsorption at the protein pI, for several IgGs on hydrophilic and hydrophobic silica surface [9], lattices [28], or polystyrene microspheres [22]. This incident seems to be independent of protein and surface type, or adsorption conditions. An increased protein rigidity at the pI and hence a smaller surface area occupied are given as an explanation for maximum adsorption at the pI [9,22,34]. Lower $\Gamma_{pI}(\mathrm{pH})$ values than the maximum $\Gamma_{pI}(\mathrm{pH})_{max}$ have been attributed to the progressive structural deformation of the adsorbed protein molecules [31]. It was also discussed that the amount of adsorbed protein may be dominated by lateral repulsion rather than by attraction between the proteins and the surface. This would cause decreased adsorption at pH values other than at pI, regardless of increasing electrostatic attraction toward the sorbent surface [22,35]. Considering the situation of both IgG1 and h-IgG on borosilicate glass, electrostatic attraction between surface and protein increases below the pI, which appears to have a decisive impact on adsorption. Typically, the IgG structure is not or only marginally affected in the pH range from 4 to 9 (data not shown). Furthermore, the hydrodynamic diameter of the monomer is not dramatically affected in the pH range investigated (see Section 3.5). In conclusion, the results point to the fact that adsorption of IgG1 on borosilicate glass is to a great extent driven by electrostatic interactions.

### 3.2.2. Electrostatic interactions within the adsorption interface

Upon adsorption, the electrical fields of the solvated protein and solvated glass surface overlap. Hence, charges on both surfaces redistribute, and a charge transfer between protein molecules, the glass surface, and the bulk solution occurs [15]. As a result, a new charge equilibrium within the protein–glass complex is formed. The pH-dependent electrophoretic characterization of this complex can be helpful for interpreting the location of $\Gamma_{pI}(\mathrm{pH})_{max}$. In this regard, Haynes, Norde, and coworkers emphasized the importance of the electrostatic state after adsorption. They postulated that the pH of maximum adsorption is affected by the charge of the sorbent surface and the protein [2,12]. In particular, maximum affinity for adsorption is observed when the charge of opposite sign of the protein molecule exactly compensates for the charge on the surface. Besides, low molecular weight ions are additionally involved in the adsorption process. They are located on the surface of both the glass and the protein molecules and influence the zeta potential. This ion balance may be reorganized upon the adsorption process [2].

Initially, zeta potentials of the IgG1-surrounded glass particles were determined via electrokinetic mobility measurements by applying the Smoluchowski model. Although IgG1 adsorption strongly depends on pH, it was assumed that the glass particles were fully covered with IgG between pH 4 and 7. Fig. 4 depicts $\zeta$ of the IgG1-coated glass particles. As expected, $\zeta$ of the IgG1-covered glass particles becomes more negative with increasing pH. The isoelectric point of the complex, however, does not coincide with the PZC of the blank glass (pH 2.3) or with that of the pure IgG1 (pH 8.3), but is located in between at pH 5.8. This indicates that the positively charged groups of IgG1 are, at least in part, compensated by the negatively charged groups of the glass surface [19]. It has been found by means of electrokinetic measurements that $\Gamma_{pI}(\mathrm{pH})_{max}$ normally coincides rather with the isoelectric point of the sorbent–protein complex [19,29] than with the isoelectric point of the protein [3]. Our electrokinetic measurements were performed in solutions of NaCl at 10 mM ionic strength.

The electric charges of the protein and the sorbent surface will hardly ever match exactly. This implicates growing forces of repulsion. Since a very low dielectric permittivity prevails in the protein–surface contact zone, this state is energetically unfavorable. Therefore, low molecular weight ions are transferred from the surrounding liquid to the adsorption layer to prevent accumulation of charges in the contact region [2]. However, this ion transfer also includes a chemical effect which itself has turned out to be energetically unfavorable and which hinders spontaneous protein adsorption [36]. Hence, maximum affinity of proteins to the respective surface type is observed when no further ion incorporation is required [2]. The participation of low molecular weight ions

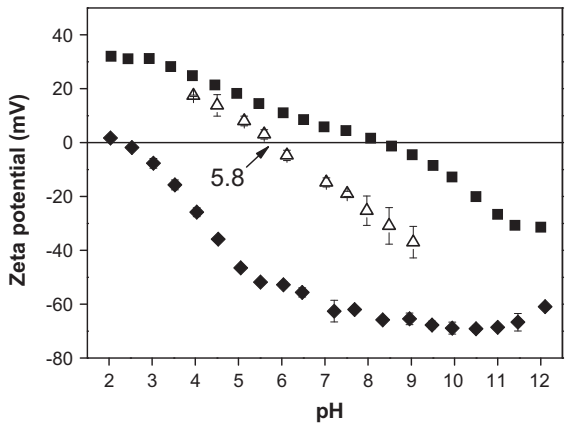


**Fig. 4.** Zeta potential of IgG1-surrounded glass particles at variable pH between 4 and 9 (△); for comparison, zeta potentials of the free IgG1 (■) and free borosilicate glass particles in a protein-free solution (♦) from pH 2 to 12 are shown ($n = 3$).

of the solution can be determined by comparing $\sigma_{ek}$ before and after protein adsorption. $\sigma_{ek}$ is obtained from $\zeta$ according to the theory of diffuse double layers by Eq. (1) [17]:

$$\sigma_{ek} = -\sqrt{8\varepsilon_0\varepsilon cRT}\sinh(zF\zeta/2RT) \quad (1)$$

where $\varepsilon$ is the relative dielectric permittivity of the medium, $\varepsilon_0$ the vacuum permittivity, $c$ the concentration of the symmetric $z$–$z$ electrolyte, $F$ the Faraday constant, $R$ the gas constant, and $T$ the absolute temperature. In our case, $\sigma_{ek}$ was calculated from $\zeta$ measured in 10 mM NaCl. The extent of incorporated ions was estimated by applying Eq. (2) [12,19]. Because of the overall electric charge neutrality, the charge transfer $\Delta_{ads}\sigma_{ek}$ between the adsorbed layer and the surrounding liquid is given by:

$$\Delta_{ads}\sigma_{ek} = \sigma_{ek}(\text{IgG/glass}) - \sigma_{ek}(\text{glass}) - \sigma_{ek}(\text{IgG}) \cdot \Gamma \cdot A \quad (2)$$

$\sigma_{ek}$ equals the electrokinetic charge density at the slipping layer of the IgG–glass complex, the glass, and the IgG, respectively. The term $\Gamma{\cdot}A$ is the proportion of the cumulated surface of adsorbed protein molecules to the corresponding glass surface. $\Gamma$ is the mass of protein adsorbed per surface area glass. $A$ equals the surface area per unit mass and is defined by the term:

$$A = \frac{4r_h^2\pi \cdot N_A}{M_w} \quad (3)$$

For IgG1, the surface area of one molecule ($4r_h^2\pi$) is approx. 335 nm$^2$, assuming a globular molecule shape and a mean hydrodynamic radius ($r_h$) of 5.16 nm (see Section 3.5). $N_A$ equals Avogadro's number, and $M_w$ equals the molecular weight of IgG1. As an approximation, $\Gamma_{pI}$(pH) values, measured at $I$ = 40 mM (see below), were used for $\Gamma$ in the calculations. In the plot of $\Delta_{ads}\sigma_{ek}$ against the formulation pH (Fig. 5), the contribution of ions to the adsorption process by means of either an uptake in or a release from the adsorption region of IgG1 on the borosilicate glass surface is depicted. Zero-crossing indicates the energetically favorable state with the least participation of countervailing ions. The intersection at pH 6.1 is in accordance with the pI of the glass–protein complex (5.8) and the pH of $\Gamma_{pI}(\text{pH})_{max}$ determined at low ionic strength. The data indicate that below pH 6, the negative charge of the glass surface is overcompensated by the positive charges of the IgG1 molecules. Therefore, an incorporation of additional negative charges is required to achieve electric neutrality. Above pH 6, positively charged ions are necessary to compensate for the excess of negative charges on part of the borosilicate glass surface, which had not been sufficiently balanced by adsorbed IgG1 molecules.

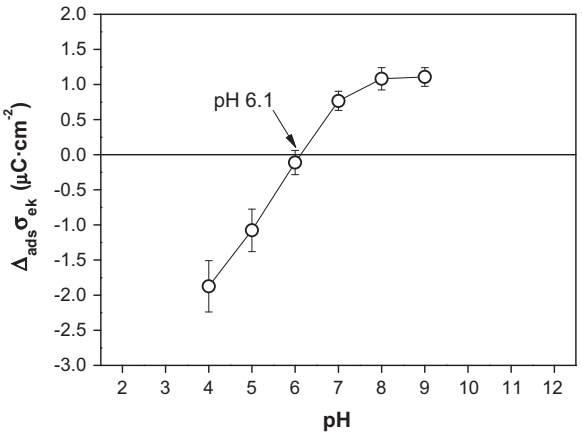


**Fig. 5.** Charge transfer ($\Delta_{ads}\sigma_{ek}$) between the contact region of the adsorbed layer and the surrounding liquid ($n$ = 3); $\Delta_{ads}\sigma_{ek}$ approximated according to Eq. (2) ($\sigma_{ek}$ from electrokinetic mobility measurements in 10 mM NaCl; $\Gamma$ taken from the pH adsorption profile at $I$ = 40 mM).

### 3.3. Impact of the formulation ionic strength on IgG adsorption

In order to further gauge the contribution of electrostatic interactions from IgG1 and the borosilicate glass surface on the adsorbed amount, adsorption experiments with protein solutions of pH 4.0, pH 7.2, and pH 8.6 containing variable concentrations of NaCl were performed. As shown in Fig. 6a, the ionic strength has a different impact on the adsorption behavior of IgG1 depending on the pH. At pH 7.2 and 8.6, an increasing NaCl concentration leads to a continuous decrease in the adsorbed amount of IgG1. Slight attraction of the marginally positively charged IgG molecules, or rather of positively charged patches, prevails toward the highly negatively charged glass surface. Besides ubiquitous dispersion forces and dehydration phenomena, there is only marginal electrostatic interaction among adsorbed proteins. Considering the impact of ionic charge shielding, mainly surface–protein attractive forces are diminished, giving rise to a steady decrease in adsorption. This decrease is more pronounced at the pI where the system responds more sensitive to changes in charge interaction. In contrast, at pH 4.0, a steady increase in adsorption with increasing ionic strength is observed. As opposed to the situation above, attraction toward the surface is assumed to be increased at this pH since the positive protein net charge increased and the negative charges of glass is only decreased to a minor degree. However, with increasing protein net charge, the electrostatic repulsion between the protein molecules continues to gain in importance. Its diminution by ionic charge shielding therefore leads to a denser packaging of IgG on the surface, associated with increasing adsorbed quantities.

In order to further corroborate the above theory, adsorption experiments were conducted with COP plastic vials. As opposed to glass, the polymer surface has a low surface free energy and is only marginally charged due to the lack of ionizable groups. Although minor specific ion adsorption due to non-electrostatic interaction, e.g. van der Waals interactions, may lead to the formation of an electrical double layer causing a measurable zeta potential [14], electrostatic forces between the surface and the protein were assumed to be missing. Instead, the molecule attachment should be mainly governed by hydrophobic interactions and dispersion forces. But charges still play a major role in protein–protein interactions, thereby affecting the adsorbed amount through intermolecular electrostatic repulsion forces. Thus, by varying the ionic strength in the protein solution, the impact of intermolecular charge–charge interactions on IgG1 adsorption can be evaluated. In Fig. 6b, hardly any impact of the increasing salt concentration is observed at both pH 7.2 and 8.6, and the adsorbed amount remains almost constant. This confirms the insignificance of intermolecular electrostatic interactions in the area of the pI. At pH 4.0, the high net protein charge causes high intermolecular electrostatic repulsion. At low ionic strength, these repulsive forces have the ability to greatly reduce the adsorbed amount. At the same time, charge shielding leads to a marked increase in adsorption with increasing ionic strength at pH 4.0. Despite the lack of strong electrostatic attraction toward the surface, the increase is even stronger than for the glass surface through the impact of hydrophobic interactions. It has to be mentioned that a logarithmic relationship was found between ionic strength and the adsorbed quantity of IgG1, regardless of the kind of sorbent surface.

With the effect of ionic strength on protein adsorption in mind, in addition to the $\Gamma_{pI}$(pH) at $I$ = 170 mM (Fig. 3), the shape of $\Gamma_{pI}$(pH) as well as the location of $\Gamma_{pI}(\text{pH})_{max}$ was investigated at $I$ = 40 mM (Fig. 7). For lower ionic strength, $\Gamma_{pI}(\text{pH})_{max}$ is shifted toward the protein pI (higher pH values) with retention of the overall curve shape. Between pH 2 and 5, where IgG1 has a high net charge, the adsorbed amounts at $I$ = 40 mM are below the respective values determined for $I$ = 170 mM. No change in the adsorbed

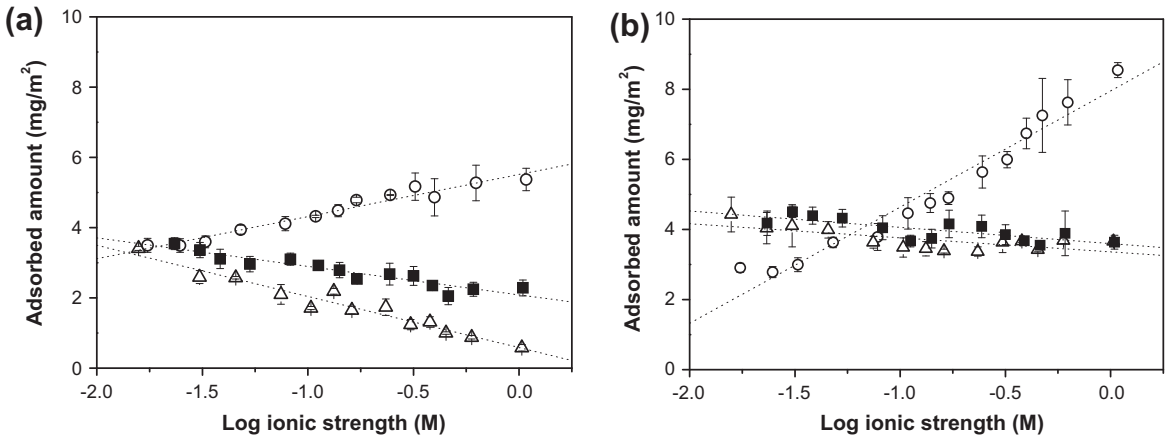


**Fig. 6.** Adsorption of IgG1 on (a) borosilicate glass and (b) COP plastic surface as a function of ionic strength, adjusted with variable amounts of NaCl at three different pH values 7.2 (■), 4.0 (○) and 8.6 (△); protein solution contained 2 mg/ml IgG1 and 10 mM phosphate buffer ($n = 3$); dotted lines approximate a linear curve progression.

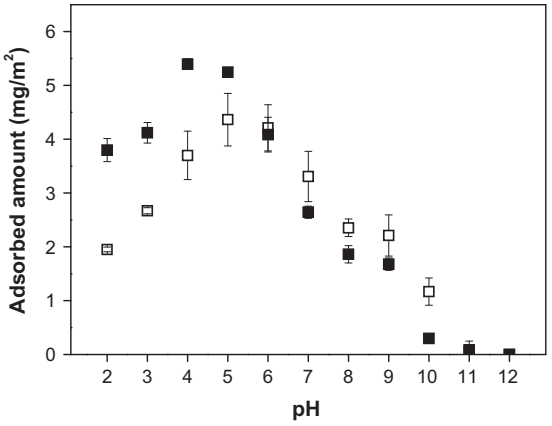


**Fig. 7.** Adsorption profile of IgG1 on borosilicate glass depending on the formulation pH; adsorption was investigated at two different ionic strengths of 170 mM (■) and 40 mM (□); incubation for 24 h, 2.0 mg/ml IgG1 including 10 mM phosphate buffer ($n = 3$).

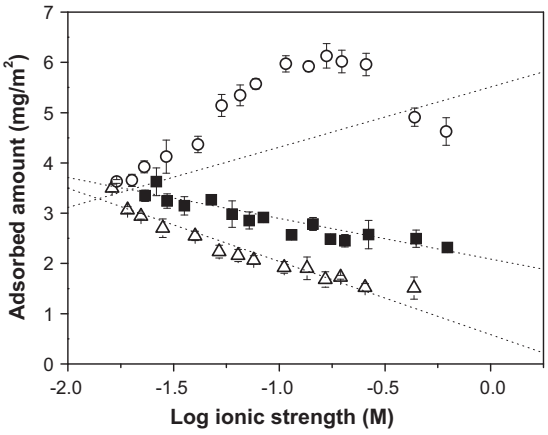


**Fig. 8.** Adsorption of IgG1 on borosilicate glass depending on ionic strength, adjusted with variable amounts $Na_2SO_4$ at pH 7.2 (■), pH 4.0 (○) and pH 8.6 (△); incubation for 24 h, 2.0 mg/ml IgG1 including 10 mM phosphate buffer ($n = 3$); dotted lines correspond to the curve progression derived from the use of NaCl (Fig. 7a).

protein mass is observed at pH 6 as this is the minimum of ion uptake according to the electrokinetic calculations. Above pH 6, where the net charge of IgG1 is low, the influence of electrostatic interaction between the protein and the glass surface gains in importance since the glass surface becomes increasingly negatively charged. For pH values above pH 8.6, where both protein and glass surface carry net negative charges, a reduction in the electrostatic repulsive forces at higher ionic strengths was expected to increase adsorption. However, this was not the case.

Similar results have been described by Buijs et al., who studied IgG adsorption on hydrophilic silica [9]. By increasing ionic strength, they also observed a shift of $\Gamma_{pl}(\mathrm{pH})_{max}$ toward a lower pH. Furthermore, adsorption increased at low pH values and decreased toward high pH values of the incubation medium. Also, Xu et al. found a shift of the adsorption maximum of an IgG1 on hydrophilic silica. $\Gamma_{pl}(\mathrm{pH})_{max}$ was shifted from the pI at low ionic strength toward a lower pH when ionic strength during incubation was increased [33].

### 3.4. Influence of the salt type

As mentioned before, not only the ionic strength itself but also the salt type used may affect the adsorption of proteins. Therefore, adsorption experiments were also performed using $Na_2SO_4$ instead of NaCl for ionic strength adjustment (Fig. 8). Adsorption profiles at pH 7.2 and pH 8.6 are almost identical to those obtained by using NaCl. At pH 4.0, increasing ionic strength with $Na_2SO_4$ leads to a stronger increase in adsorption. Besides, the linear dependency of the adsorbed IgG1 quantity on the logarithm of $I$ no longer applies. Above an ionic strength of approx. 0.2 M, the excessive increase stops and equilibrium adsorption decreases.

At pH 4.0, where the compensation of interfacial charges is a limiting factor for adsorption, predominantly negatively charged ions are incorporated in the adsorption region. The incorporation of divalent ions like $SO_4^{2-}$ is preferred because of the higher valency and higher polarizability compared to the monovalent ion types [31]. The high charge screening capacity of $SO_4^{2-}$ contributes to increasing IgG1 adsorption due to a higher density of the protein layer. At a pH above 6.1, predominantly cations are incorporated in the boundary layer and consequently, no particular effect of $Na_2SO_4$ can be observed. Solely charge–charge interactions cannot sufficiently explain the decrease in adsorbed amounts at pH 4.0. Possibly, the salts differently affect intramolecular forces in the protein molecules [37]. Another explanation refers to the effect of preferential exclusion of salts. In this regard, it is well known from the Hofmeister series, together with extensive investigations by Arakawa and Timasheff [20], that $Na_2SO_4$ is a stronger protein precipitant than NaCl, and also shows a stronger preferential

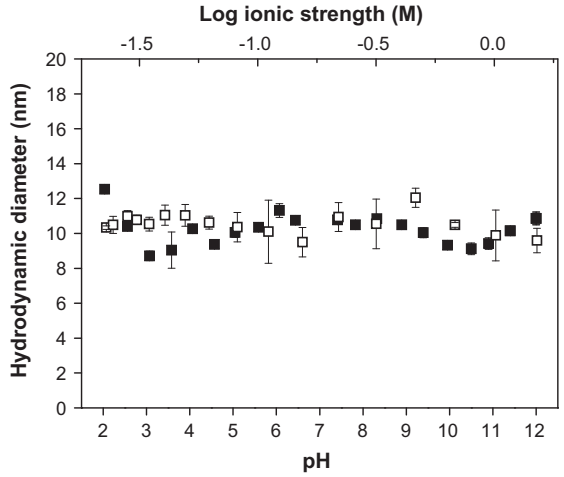


**Fig. 9.** Hydrodynamic diameter of IgG1 monomers as a function of pH at $I$ = 170 mM (■) and as a function of ionic strength at pH 7.2 (□) ($n$ = 3) (Fig. 6a).

hydration and therefore stabilization. Influences of stabilizing salts on protein adsorption are only sparsely described in literature. Adsorption of firefly luciferase on borosilicate glass surface, for instance, was studied by Suelter and DeLuca [38], who described decreasing adsorption with increasing concentrations of $(NH_4)_2SO_4$. The decrease in adsorbed amount could be explained by the increased protein stability in the presence of $Na_2SO_4$ resulting in less conformational change by adsorption and less irreversibility [39]. Even more likely, $SO_4^{2-}$ ions are preferentially excluded from the protein surface resulting in an unfavorable surplus of intermolecular repulsive charges. This leads to a less compact protein layer, and hence, to decreasing adsorption.

### 3.5. IgG1 monomer size as a function of pH and ionic strength

Changes in protein conformation induced by pH changes may alter the space requirement of the molecules and consequently the adsorbed protein quantities [28]. Furthermore, it has been described that higher ionic strength may lead to an increased hydrodynamic diameter by a growing hydration shell [11]. In order to judge the importance of this effect, the IgG1 hydrodynamic diameter was investigated as a function of the formulation pH in the range from pH 2 to 12 and as a function of the ionic strength between 23 mM and 1660 mM. The absolute values of $d_h$ depicted in Fig. 9 are very well in line with the findings of Jøssang et al. (11.0–11.4 nm) [40] or Bagchi and Birnbaum (11.0–12.5 nm) [28] for IgG. Neither a clear trend of $d_h$ with variable pH nor a minimum at the pI [28] could be observed. Thus, an influence of the monomer dimensions on adsorption can be ruled out for this study.

## 4. Conclusions

It was shown that the adsorption of IgG on the surface of borosilicate glass vials is a function of the formulation pH and ionic strength and depends to some degree on the salt type added. In general, the adsorption process is to a large extent mediated by electrostatic interactions. During adsorption, attractive or repulsive forces between protein and glass surface as well as repulsive forces between the adsorbed IgG molecules on the surface interact. The magnitude of each factor varies independently by changing pH or ionic strength, respectively. The resulting charge of both sorbent surface and protein, together with the charge screening by ions present on both entities, is of fundamental importance. The interplay of the arising forces became apparent, for example, from the shift of the pH of maximum adsorption, when the ionic strength was altered. Furthermore, increasing NaCl concentrations can result in either an increase or a decrease in the adsorbed amount of protein, depending on whether protein–surface or intermolecular electrostatic interactions are most pronounced and primarily screened. However, the adsorption of IgG on glass must be, at least in part, mediated by other forces than electrostatic interactions. Especially in the area of the protein pI, hydrophobic interactions or surface-induced structural changes can occur. For pH values below the protein pI, electrostatic interactions are of utmost importance with a residual contribution of other interaction forces still likely. Electrokinetic measurements on IgG1 and glass particles allowed a deeper insight into the prevailing electrostatic interactions. This includes the uptake of low molecular weight ions into the adsorption boundary layer during the adsorption process. Thus, the study provides a better insight into the mechanisms of protein adsorption to primary packaging materials. In addition, it provides the basis for a formulation adjustment in order to cope with this problem without the need of adding surfactants, which is often a cosmetic approach and might induce other problems such as oxidation of the protein.

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