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RESEARCH PAPER

The Role of Polysorbate 80 and HPβCD at the Air-Water Interface of IgG Solutions

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ABSTRACT

Purpose To test the hypothesis of surface displacement as the underlying mechanism for IgG stabilization by polysorbates and HPβCD against surface-induced aggregation.

Methods Adsorption/desorption-kinetics of IgG-polysorbate 80/-HPβCD were monitored. Maximum bubble pressure method was used for processes within seconds from surface formation. Profile analysis tensiometry was applied over long periods and to assess surface rheologic properties. Additionally, the kinetics of adsorption, desorption and surface displacement was followed by a double-capillary setup of the profile analysis tensiometer, allowing drop bulk exchange.

Results Weak surface activity for HPβCD *vs*. much higher surface activity for polysorbate 80 was shown. Protein-displacement when exceeding a polysorbate 80 concentration close to the CMC and a lack of protein displacement for HPβCD was observed. The drop bulk exchange experiments show IgG displacement by polysorbate 80 independent of the adsorption order. In contrast, HPβCD coexists with IgG at the air-water interface when the surface layer is built from a mixed IgG-HPβCD-solution. Incorporation of HPβCD in a pre-formed IgG-surface-layer does not occur.

Conclusions The results confirm surface displacement as the stabilization mechanism of polysorbate 80, but refute the frequently held opinion, that HPβCD stabilizes proteins against aggregation at the air-water interface in a manner comparable to non-ionic surfactants.

KEY WORDS antibody · drop profile analysis · hydroxypropyl-beta-cyclodextrin (HPβCD) · polysorbate · surface dilational rheology

ABBREVIATIONS

BSA	bovine serum albumin
CDs	cyclodextrins
CMC	critical micellar concentration
HPβCD	hydroxypropyl-β-cyclodextrin
IgG	immunoglobulin G
mAbs	monoclonal antibodies
MBPM	maximum bubble pressure method
Rh-GCSF	recombinant human granulocyte colony-stimulating factor

INTRODUCTION

Polysorbates as well as cyclodextrins (CDs) are valuable excipients for the prevention of surface-induced protein aggregation, as encountered for example during agitation of liquid protein formulations associated with exposure of the protein to the air-water interface (1–7). While polysorbates are well established stabilizers already present in many marketed formulations, they suffer from a number of shortcomings, such as in some cases an increased tendency of

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protein oxidation and aggregation upon quiescent storage at elevated temperature (2,3,8). In contrast, the stabilizing potential of the CD-derivative hydroxypropyl-β-cyclodextrin (HPβCD) against surface-induced aggregation is not accompanied by protein oxidation or compromised by aggregation during storage (6). Furthermore, HPβCD possesses a favorable toxicological profile as excipient for parenteral administration, considering that doses as high as 8–16 g/day are administered to patients in approved parenteral products (9). Therefore HPβCD can be considered a valuable alternative to non-ionic surfactants.

From a mechanistic point of view, the stabilizing effect of polysorbates has been extensively studied, and most studies link protein stabilization against surface-induced aggregation to the competition between protein and surfactant at the air-water interface (2,3,8,10–13). For example, the adsorption of polysorbate 80 in the presence of recombinant Factor VIII (280 kDa) was studied using a Wilhelmy Plate tensiometer, where the steady state interfacial behavior was shown to be entirely governed by surfactant adsorption (13). Another study investigated the rheological, structural and mechanical properties of mixed adsorption layers of bovine serum albumin (BSA) and polysorbate 80 (14). The study confirmed competitive adsorption between BSA and polysorbate 80, with almost complete displacement of the protein at high polysorbate 80 concentrations. However, to the best of our knowledge no detailed studies are available for monoclonal antibodies (mAbs) in the presence of polysorbates, with mAbs currently being by far the most widespread class of therapeutic proteins (15). Polysorbate 80 was frequently reported to stabilize monoclonal antibodies (16,17) and stabilization by competition at the air-water interface was implicitly assumed, however never directly demonstrated.

In comparison to polysorbates, very little is known so far about the stabilizing mechanism of HPβCD against surface-induced aggregation. Two possible mechanisms are discussed in the literature. In the first, the ability of CDs to bind to proteins and incorporate hydrophobic protein residues in their interior cavity is held accountable for their stabilizing effect (9,18). However, for the model proteins IgG and rh-GCSF, direct binding to CD-derivatives in bulk solution as a reason for aggregation inhibition was rendered unlikely by previous studies (6,20).

The second possible stabilization mechanism points to the surface activity of some CD-derivatives (19), thereby potentially being able to compete with proteins at the air-water interface similar to non-ionic surfactants (1,6,7,19). For example, the surface activity of HPβCD was reported to strongly depend on the degree of substitution. (7,21,22), with surface tension values between 69 mN/m and 52 mN/m reported for degrees of substitution ranging from 2.5 to 11.3. This surface activity was held by Charman *et al.* as the reason for the effectiveness of HPβCD in reducing interfacially-induced precipitation of porcine growth hormone with a mechanism analogous to that of polysorbate 20 (1). In another study, the proposed relationship between the interfacial stabilization of rh-GH by HPβCD and surface activity of HPβCD was substantiated by correlating the increasing degrees of substitution of HPβCD (that translate into increasing surface activity) to reduced amounts of aggregates in vortexed rh-GH formulations (7). Finally, a study measuring the surface-tensions of pure HPβCD and IgG-solutions as well as mixed IgG-HPβCD solutions using a Wilhelmy-plate tensiometer confirmed that both IgG and HPβCD are surface-active (6), and thus there is a high likelihood for competition at the air-water interface, though the ability of HPβCD to displace IgG at the interface was not shown.

It can be concluded that it is of high interest to study in detail the surface characteristics of IgG-polysorbate and of IgG-HPβCD solutions in order to get more insight into the stabilizing mechanisms of both excipients. In this study, the hypothesis of a competitive-displacement as the most likely mechanism of aggregation inhibition at the air-water interface is tested. To this end, surface tension measurements by maximum bubble pressure method for solutions of polysorbate 80, HPβCD, IgG and mixtures of IgG-stabilizer were performed to investigate the surface adsorption at short time scales. Moreover, surface tension measurements using drop profile analysis were performed to investigate surface adsorption at equilibrium. Additional drop profile analysis studies were performed using a special double-capillary-setup, which allows exchange of the bulk sample solution, thus shedding more light on the surface-displacement mechanisms and adsorption/desorption-kinetics at the air-water-interface. Concurrently, surface rheological properties were determined by surface dilational rheology in order to verify actual surface layer composition. In all the experiments, the adsorption behavior of polysorbate 80 with/without IgG was compared to that of HPβCD with/without IgG, and mechanistic conclusions on the stabilization principles of both excipients were drawn.

MATERIALS AND METHODS

Materials

A monoclonal antibody (mAb) of the IgG class, that was also used in a previous stability study (6), was kindly donated by Roche Diagnostics GmbH, Penzberg, Germany. The IgG bulk material provided for this work was formulated in a 20 mM histidine buffer at pH 5.8. Bulk concentration was 2.4 mg/ml. Protein solutions were filtered through Acrodisc® 0.2 μm PVDF syringe filter units (Pall GmbH, Dreieich, Germany) prior to usage in all solutions. The total

molecular weight of this particular antibody is 146.3 kDa as determined by MALDI mass spectrometry. HPβCD (pharmaceutical grade, average molecular weight 1400 $gmol^{-1}$) was kindly donated from Wacker Chemie AG, Burghausen, Germany. Polysorbate 80 (average molecular weight 1312 $g.mol^{-1}$) was kindly donated from Croda Inc. (Edison, NJ, USA) in super-refined quality and used as received. Histidine was from Merck KGaA, Darmstadt, Germany.

Methods

Preparation of Dilutions

All dilutions of the IgG were carried out into histidine buffer at a concentration of 20 mM and a pH of 5.8. Mixed IgG-HPβCD and mixed IgG-polysorbate 80 solutions were prepared from stock solutions of the respective excipients in 20 mM histidine buffer. Solutions were prepared with Milli-Q deionised water and the glassware used for preparation of the solutions was cleaned with concentrated sulphuric acid.

Maximum Bubble Pressure Measurements

The dynamic surface tension of solutions of polysorbate 80, HPβCD or mAb alone as well as of mixed solutions of the mAb with either polysorbate 80 or HPβCD at short adsorption times was measured using the maximum bubble pressure technique. The basic principle of this analytical technique is the determination of the maximum pressure of a bubble that is growing at the end of a thin steel capillary (inner diameter 0.25 mm) which is immersed into the solution under investigation. The calculation of the surface tension using the maximum bubble pressure method is based on the Laplace equation:

$$\gamma = \frac{(P - P_h) \cdot r}{2}$$

Here P is the maximum bubble pressure, P_h the hydrostatic pressure of the liquid and r the capillary radius. By determining the surface tension at different life times of the bubble, the dynamic surface tension is obtained. The advantage of the method over other methods for the determination of the dynamic surface tension is the possibility to measure already after a few milliseconds of surface age. The instrument used for these studies was the BPA-1P (Sinterface Technologies, Berlin, Germany).

Drop Profile Analysis and Dilational Shear Rheology

Drop profile analysis was employed for detailed characterization of the dynamic surface tension of surface layers of pure excipients or IgG and also of mixed IgG-HPβCD as well as of IgG-polysorbate 80 solutions. The instrument used for these investigations was a Profile Analysis Tensiometer (PAT 1, Sinterface Technologies, Berlin, Germany). Some single capillary-measurements with polysorbate and IgG/polysorbate-samples were performed using PAT 2P (Sinterface Technologies, Berlin, Germany) which operates in analogous mode as PAT 1.

As indicated in Fig. 1, the basic principle of drop profile analysis is that the coordinates of the shape of a pendant drop of the studied solutions are recorded by a video camera and compared to its theoretical profile which can be calculated from the Gauss-Laplace equation. Thereby the dynamic surface tension, as the only free variable in the theory, can be obtained (14). There is a balance of capillary and gravitational forces; whereas the surface tension acts to form a spherical drop, gravity acts oppositely giving the drop a prolonged shape.

Double-Capillary Experiments

A special setup of the drop profile analysis instrument (PAT 1), using 2 concentric capillaries (Fig. 2), allows the exchange of the droplet bulk without changing its volume. While measuring the surface tension using the CCD camera, the internal capillary can (slowly) pump fresh liquid into the drop while the outer capillary drains an equivalent amount of the fluid, thus maintaining a constant drop volume (24–26).

For drop bulk exchange experiments, a droplet with a 13 mm^3 volume was first formed by the outer capillary. At specific time points, new solution was pumped into the drop via the inner capillary. Parameters for bulk exchange were $\Delta V = 0.067$ mm^3 and $\Delta t = 0.1$ s in which ΔV describes the exchanged volume per pulse and Δt the time between two pulses. For all exchange experiments, the drop was flushed in total with 2000 mm^3 new solution, which represents more than 150 fold of the actual drop volume. Duration of the exchange process was volume controlled and varied between 6500 and 7500 s. Different parameters may have an influence on exchange effectiveness (27), therefore the setup of the aforementioned parameters for exchange-procedure was initially adjusted by flushing a 13 mm^3 droplet of C_{12}DMPO (10^{-4} M) with pure water.

The same setup was used to determine dilational rheological properties of the surface layers. For this purpose harmonic area oscillations of the drop at low frequency (0.01, 0.02, 0.04, 0.1, and 0.2 Hz) were performed by the dosing system, with droplet size oscillation from 12 mm^3 to 14 mm^3. The corresponding response of the surface-tension is measured and the elastic as well as the viscous contributions can be determined separately. Low frequencies of the oscillations are important in order to maintain the Laplacian shape of the drop (28).

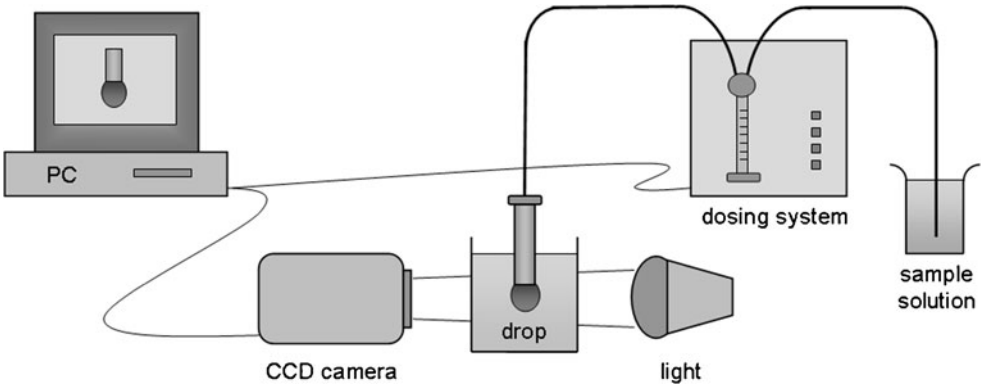

Fig. 1 Schematic representation of main components of the drop profile tensiometer PAT 1 (Sinterface Technologies, Berlin, Germany) for drop profile analysis with video image and profile coordinates. Taken from (23) and printed with permission.

RESULTS

Maximum Bubble Pressure Experiments at Short Adsorption Time Scales

Measurements using the maximum bubble pressure method (MBPM) were performed to evaluate the surfactant and protein adsorption directly after the formation of the air-water interface. Results in Fig. 3 show the dynamic surface tension of HPβCD, polysorbate 80 and the IgG solutions in histidine buffer at concentrations that were previously used in an earlier study demonstrating the effectiveness of the excipients against surface-induced aggregation (6). It can be seen that polysorbate 80 alone lowers the surface tension much faster and to a higher extent as compared to HPβCD. Even at the first value that was recorded (33 ms) the surface tension of the polysorbate 80 solution (65.04 mN/m) is already substantially lower than the surface tension of the pure histidine buffer (72.6–73.4 mN/m). This is an indication that the de novo surface is very rapidly occupied by polysorbate 80 when employed at this concentration (3×10^{-5} M=0.004%).

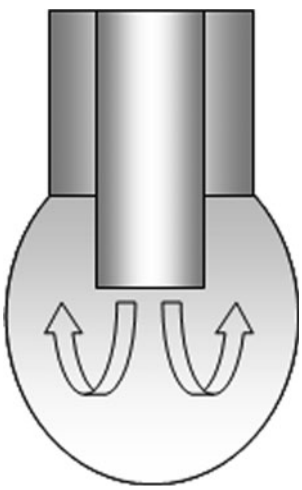

Fig. 2 Double capillary setup for drop bulk exchange at PAT-1 instrument.

By contrast, HPβCD only leads to a very slight decrease of surface tension during the experiment, which is probably due to its lower surface activity. Interestingly, in the mixed IgG-stabilizer solutions, the adsorption of polysorbate 80 exhibits a lag phase of about 1 s before a measurable decay of the surface tension is observed. In contrast, the IgG-HPβCD solution does not show a significant reduction in the surface tension directly after the formation of a new air-water interface.

Surface Tensiometry by Drop Profile Analysis

In order to gain a better understanding of the adsorption behavior of IgG-polysorbate 80 and IgG-HPβCD drop profile analysis was chosen as a different experimental approach. The basic idea was to investigate more diluted

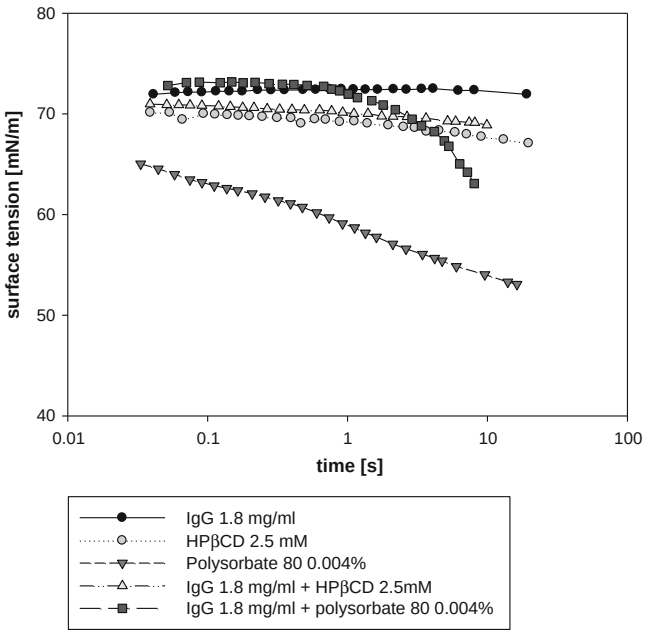

Fig. 3 Dynamic surface tension of solutions of polysorbate 80, HPβCD and the IgG as well as their respective mixtures as determined by the maximum bubble pressure technique. Note that x-axis is in logarithmic time scale.

solutions as compared to the actual formulations' concentrations used earlier (6), in order to create conditions under which the adsorption processes and possible competition mechanisms occur at a slower time scale, which can then actually be followed in detail. By making the adsorption behavior "visible" at lower concentrations, it was expected to obtain conclusions which also apply on the actual formulations by extrapolating to higher concentrations and hence faster adsorption rates.

Before studying the mixtures of HPβCD or polysorbate 80 with IgG, every component was investigated separately, as seen in Fig. 4a–c. Equilibration at the air-water interface is rather slow, however all equilibrium surface tensions that were observed in this experiment lie in the same range as the values that were determined earlier by different experimental methods (7,21,22,29). Figure 4a shows the adsorption profile of different concentrations of polysorbate 80 in histidine buffered solution. Polysorbate 80 shows a strong surface activity, with a significant decrease in surface tension with increasing concentrations. At concentrations above 1×10^{-5} M the surface tension slightly increases again, which refers to the critical micellar concentration (CMC) value of the system. The CMC-values for polysorbate 80 that are reported in literature vary significantly due to the chemically heterogeneous nature of polysorbate 80. The concentration of 1×10^{-5} M determined for the present system is well in the (lower) range of reported values (12,14,30,31). Meanwhile, the adsorption kinetics of increasing concentrations of HPβCD are shown in Fig. 4b. The data confirm that HPβCD possesses (comparably weak) surface activity as evidenced by the drop in surface tension with increasing concentrations. Contrary to polysorbate 80, HPβCD does not show a CMC in the studied range (up to 7.5 M≈1%w/v), in accordance with previous studies, which reported that HPβCD does not show a CMC at concentrations up to 7% (32).

In Fig. 4c, the dynamic surface tension of the pure IgG at different concentrations is shown. It can be seen that at the lowest investigated concentration (1×10^{-8} M) a long induction period (approximately 80,000 s, which corresponds to 22 h) precedes measurable adsorption to the air-water interface. In contrast, lysozyme in comparable concentration showed a relatively short induction period of about 10,000 s as determined by the same method at comparable concentrations (33). One possible reason for the difference in induction period could be size of both proteins (146 kDa for IgG *vs.* 14.3 kDa for Lysozyme). Because of its large molecular weight, the diffusion of IgG to the subsurface from which adsorption to the air-water-interface takes place might occur rather slowly (34). It can be speculated that also the different degree of charge repulsions between the protein molecules could influence the diffusion time to the subsurface. In addition, the induction period also depends on the structural stability of the investigated molecule, because unfolding of the adsorbed proteins at the interface contributes to the surface pressure. More flexible, non-globular proteins such as β-casein partially unfold faster and therefore show shorter induction periods (33,35). It is also worth noting, that the observed adsorption profile shows differences to the published adsorption profile of another IgG (34). Whereas for the IgG investigated in the current study the equilibrium surface tension reaches a steady value of about 53 mN/m beginning at concentrations of 1×10^{-7} M, the published results reveal a saturation of the interface at concentrations as high as 2×10^{-5} M also at a surface tension of about 53 mN/m. However, it has to be taken into account that IgG adsorption was followed for different time periods in the two studies which renders comparison of the surface tension values difficult.

For the analysis of the mixed solutions of the IgG with polysorbate 80 or HPβCD, a constant IgG-concentration of 1×10^{-6} M was chosen. This concentration was considered as a compromise between a reasonable time to achieve equilibrium conditions (80,000 s) and an initial adsorption that is slow enough to allow mechanistic observations without interference from multilayer protein adsorption. The equilibrium surface tension of the IgG in the absence of any excipients is indicated by a straight line in Fig. 5, and the equilibrium surface tension of the pure polysorbate 80-solution and the pure HPβCD-solution at different concentrations are also included into Fig. 5 for comparison. As observable from Fig. 5a, at low surfactant concentrations, the surface tension of the polysorbate 80-IgG mixture is lower than that of the pure surfactant solution. However, the values of the mixture more or less match the value of the pure IgG solution (about 53 mN/m). Increasing polysorbate 80 concentrations from 1×10^{-7} M to 1×10^{-6} M does not lower the surface tension of the mixture. These findings indicate the dominating contribution of the IgG to the composition of the adsorption layer of the mixture in this concentration range. When the polysorbate 80 concentration is further increased, the surface tension of the mixed solution drops to a value that is very close to that of the pure polysorbate 80 solution and significantly below that of the pure IgG solution, which strongly suggests that beginning from a concentration of 1×10^{-5} M, polysorbate 80 dominates the surface layer of the IgG-polysorbate 80 mixture, and this concentration is close to the CMC of the pure polysorbate 80 solution as discussed above.

For the mixed IgG-HPβCD solution, a very different surface-tension isotherm than for the IgG-polysorbate 80 system is obtained, as shown Fig. 5b. No matter how high the concentration of HPβCD, the surface tension of the mixture changes only slightly. Moreover, the surface tension

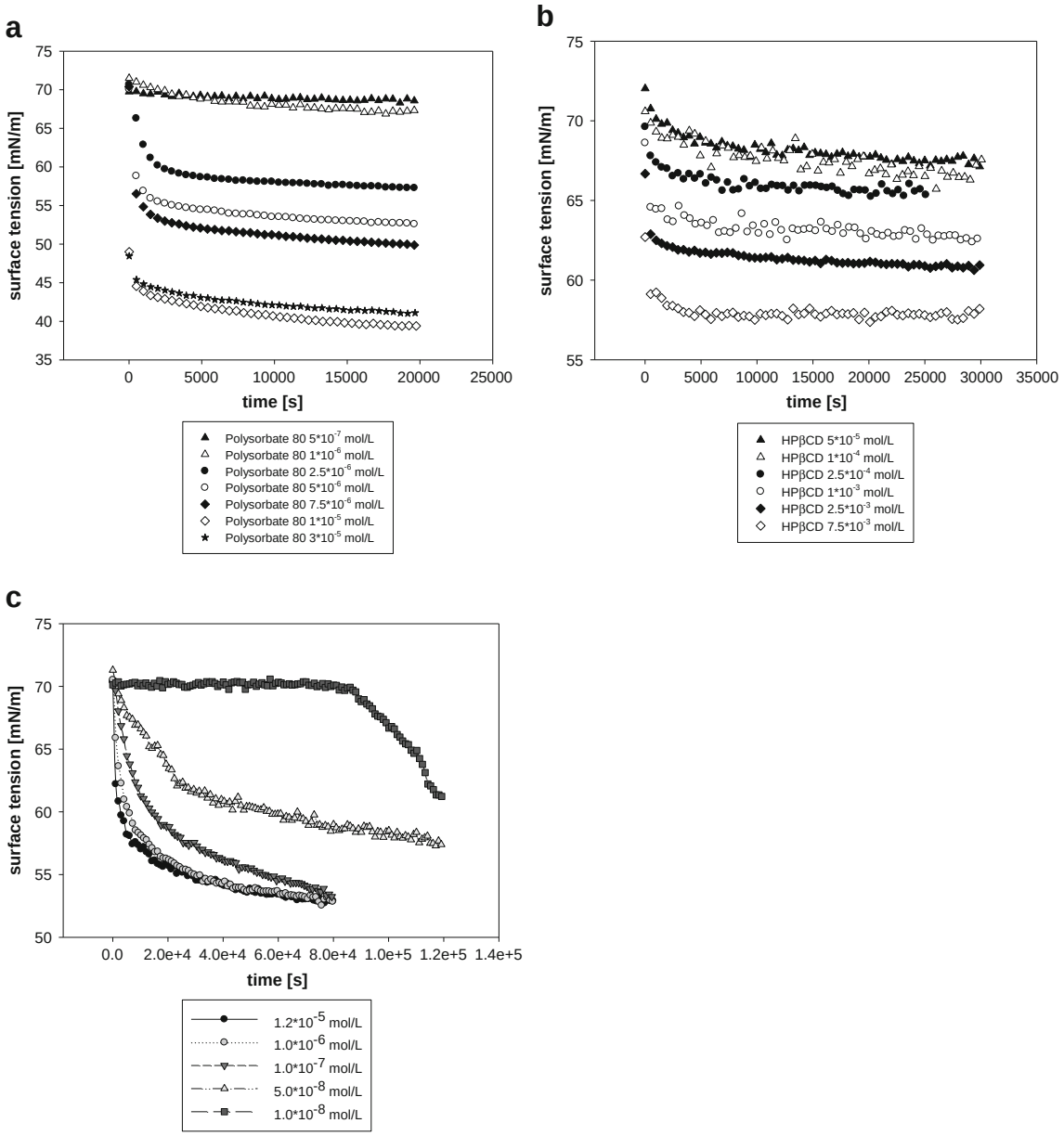

Fig. 4 Dynamic surface tension of increasing concentrations of polysorbate 80 (**a**), HPβCD (**b**) and IgG (**c**) as determined by drop profile analysis.

of the mixed IgG-HPβCD solutions is higher than the surface-tension of the pure IgG, even at the lowest HPβCD-concentrations.

Double Capillary Experiments

The behavior of polysorbate 80, HPβCD, and IgG was further analyzed by PAT-measurements using the double-capillary-setup, with drop bulk-exchange during surface tension measurement. PAT experiments with the double-capillary-setup were performed as single- and double-exchange studies. In the single-exchange studies, the droplet bulk was exchanged with new solution once, while in the double-exchange studies, two different solutions are consecutively exchanged with the droplet bulk. The IgG concentration was kept constant at 1×10^{-6} M. Polysorbate 80 was measured at 1×10^{-5} M and 2.5×10^{-5} M; HPβCD at 2×10^{-4} M and 1×10^{-3} M. Results for both excipient concentrations provided the same conclusions, therefore only the results of one dataset are shown.

Single Exchange Studies. Single exchange experiments allow drawing conclusions about the reversibility of adsorption for each single component as well as the excipient-IgG-mixtures. The timeline for the measurements is shown in Fig. 6.

As seen in Fig. 7a and b, the surface tension of pure IgG solution showed a rapid initial reduction followed by a slower

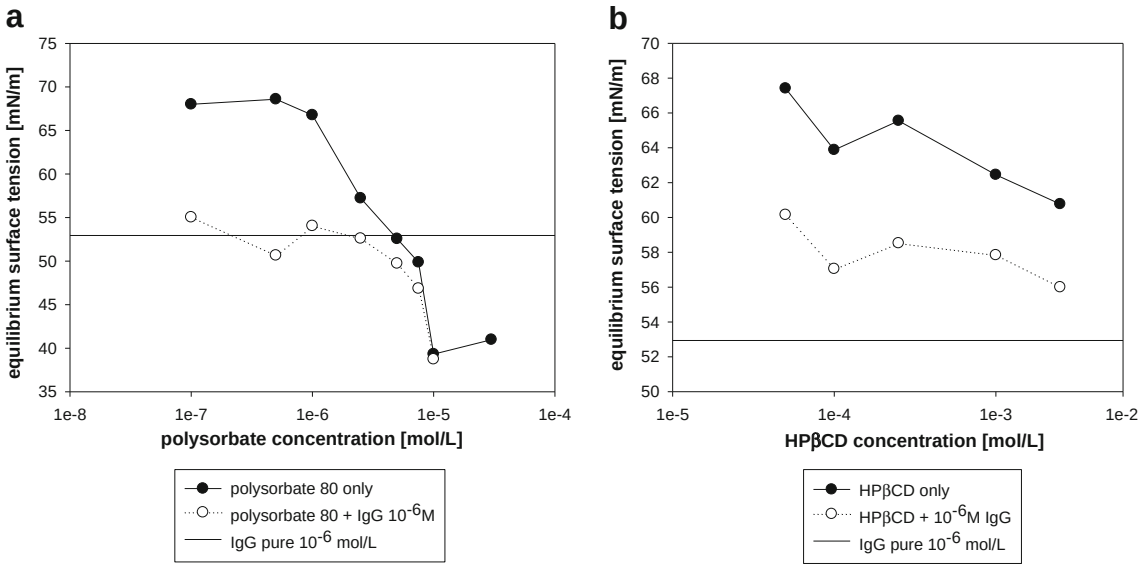

Fig. 5 Surface tension isotherms of pure polysorbate 80 solutions and IgG-polysorbate 80 mixtures (**a**) as well as surface tension isotherms of pure HPβCD-solutions and IgG-HPβCD mixtures (**b**) as determined by drop profile tensiometry. The solid straight line indicates the surface tension of a 1×10^{-6} M IgG-solution.

reduction till 5000 s, where the bulk was exchanged against histidine buffer. This bi-phasic reduction in surface tension probably reflects the diffusion of the protein to the surface in the first phase followed by a slower diffusion of the protein to the already occupied surface and/or possible protein unfolding or interfacial rearrangements at the interface. The bulk exchange with pure histidine buffer did not affect the IgG adsorption process, which is demonstrated by the unmodified monotonous decrease of the surface tension. This continuous reduction of the surface tension despite the depletion of the protein from the bulk might be due to slow conformational changes and unfolding of the already adsorbed protein at the interface, leading to exposure of its hydrophobic residues.

The surface tension of pure polysorbate 80 solutions is shown in Fig. 7a. Polysorbate 80 turns out to be a relatively "sticky" surfactant, so that even after flushing the drop with 2000 mm^3 histidine buffer (>150 fold droplet volume), the surface tension reaches a plateau value which is lower than that of the pure buffer, indicating the presence of traces of polysorbate at the surface. Interestingly, the mixture of IgG and polysorbate 80 exhibits a very similar behavior as compared to the pure polysorbate solution. For the mixture the drop in dynamic surface tension is much steeper than in the case of pure IgG and identical to that of the pure polysorbate. After exchanging the droplet bulk with buffer, the surface tension increases similar to the pure polysorbate and does not maintain the monotonous reduction as in the case of pure IgG.

Contrary to polysorbate 80, adsorption of the pure HPβCD solution was completely reversible (Fig. 7b). After

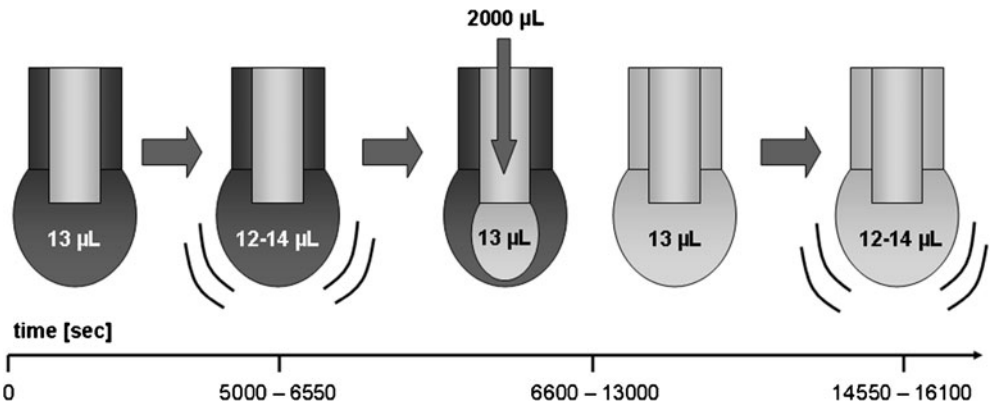

Fig. 6 Timeline for single-exchange experiments. Sample solution is illustrated by black color and pure histidine buffer by light grey color. Surface tension of the sample solution is measured for 5000 s. From 5000 s to 6550 s, the first oscillation was performed to measure the rheological properties of the surface. Afterwards, the droplet bulk was replaced by pure histidine buffer between 6600 and 13000 s, and finally rheological properties of the surface were measured again between 14550–16100 s.

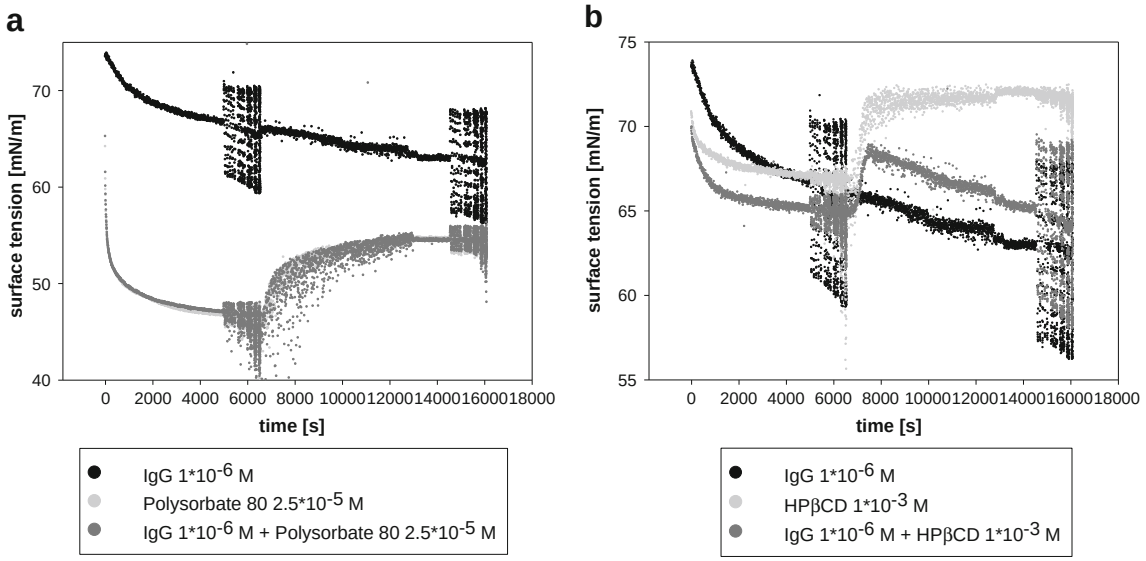

Fig. 7 Dynamic surface tension of (**a**) IgG (black), polysorbate 80 (light grey), and their mixtures (dark gray), (**b**) IgG (black), HPβCD (light grey), and their mixtures (dark gray) during drop bulk exchange with pure histidine buffer. N.B.: In (**a**) the graphs of polysorbate 80 (light grey) and the mixture of IgG and polysorbate 80 (dark grey) nearly superimpose.

bulk exchange, surface tension of the pure buffer is recovered rather rapidly (within approximately 1000 s). However, the IgG-HPβCD-mixture behaves differently from either the pure IgG or HPβCD solutions. Before bulk exchange, the reduction in the dynamic surface tension is relatively steeper at the beginning compared to pure IgG or HPβCD. After buffer exchange, an increase of surface tension similar to pure HPβCD solution is observed and lasts approximately 1000 s, nearly the same period required to re-establish the surface tension of the histidine buffer in case of the pure HPβCD solution. After that, a strong kink occurs and surface tension starts to decrease analogous to the pure IgG solution. Hence it can be speculated that HPβCD was washed out by buffer exchange while IgG remains on the surface. This points out to the concomitant presence of both components in the surface layer before starting the exchange process.

The drop oscillations used to measure surface rheological properties are visible in the surface tension curves as large fluctuations. Large amplitudes are characteristic of less flexible molecules, such as protein (24), which form a very thin "membrane" on the surface that is compressed and expanded during the oscillation process. Components with lower surface adsorption energy compensate changes in surface area by fast adsorption/desorption processes. Therefore a surface layer covered by surfactant results in low amplitudes during oscillation. Samples containing HPβCD, polysorbate 80, and IgG-polysorbate 80-mixture show low magnitude of surface tension changes during oscillation. This further confirms the absence of protein in the surface layer for mixed IgG/polysorbate 80 solutions. In contrast, the amplitude of IgG-HPβCD-mixtures at the first oscillation before buffer exchange shows a medium amplitude between pure IgG and HPβCD, and increases significantly in the second oscillation after buffer exchange. These observations additionally illustrate that the 2 components coexist at the interface before washing, and that HPβCD but not the protein is washed out during buffer exchange.

The above qualitative assessment was quantified by evaluating the surface rheology at five different oscillation frequencies: 0.01, 0.02, 0.04, 0.1, and 0.2 Hz. Results for surface elasticity and viscosity are illustrated in Figs. 8 and 9. The protein exhibits the highest elasticity values, while HPβCD has the lowest value and polysorbate 80 is somewhere in between. Comparable to the results of surface tension measurements, the mixture of IgG/polysorbate 80 exhibits the same rheological properties as the pure polysorbate 80 solution before and after buffer exchange. In contrast, the IgG-HPβCD-mixture exhibits values between the pure IgG- and HPβCD-solutions during the first oscillation (Figs. 8a and 9a) whereas after buffer exchange, the rheological properties are quite similar to those of the pure IgG.

Double Exchange Studies. To draw further conclusions about surface-displacement of IgG by excipients, sequential adsorption experiments were performed in double-exchange studies, with the timeline shown in Fig. 10.

Figure 11a shows that upon exchange of the bulk IgG with polysorbate 80, the latter rapidly displaces the IgG from surface and reduces the surface tension dramatically reaching values similar to pure polysorbate 80 (c.f. Fig. 7a). The second exchange against buffer resembles the results of the single exchange experiments for IgG/Polysorbate mixture (Fig. 7a), which indicates a rather complete protein replacement from the surface

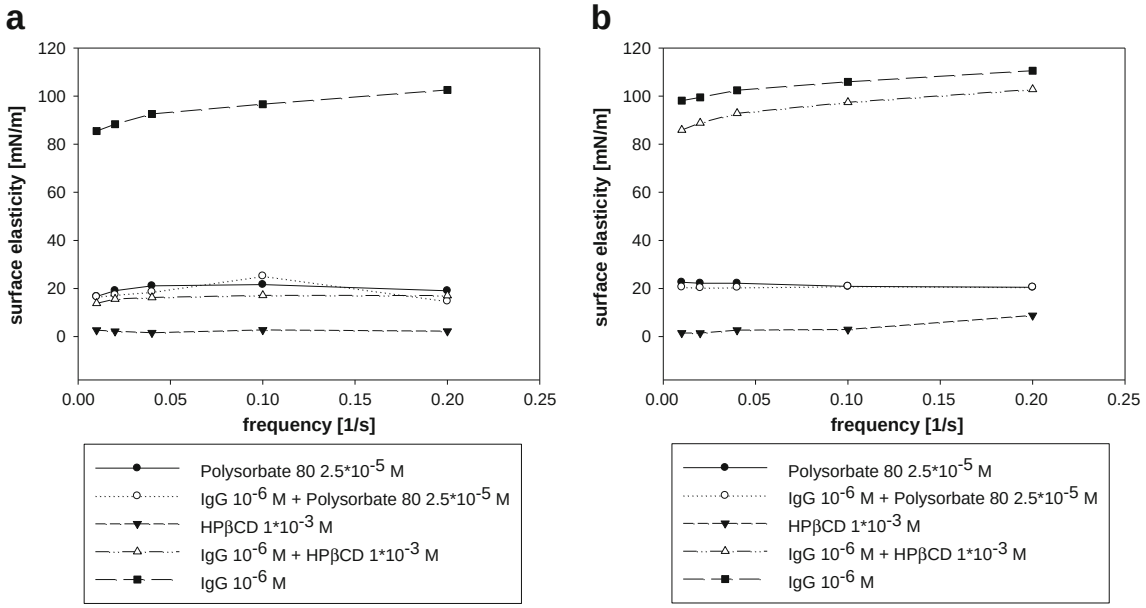

Fig. 8 Surface elasticity of polysorbate 80 2.5×10^{-5} M, HPβCD 1×10^{-3} M, IgG 1×10^{-6} M, and respective mixtures before (**a**) and after (**b**) buffer exchange.

layer. The low oscillation amplitudes during the second and third oscillation further confirm the absence of protein on the surface.

On the other hand, HPβCD seems not to affect the adsorption process of IgG (Fig. 11b), where the drop bulk exchange of IgG against HPβCD results in similar surface tension values as seen for exchange of IgG against pure histidine buffer (Fig. 7). The second washing with pure buffer did not result in a rapid and short increase in surface tension as seen for the pre-mixed IgG/HPβCD-solutions (Fig. 7b). Furthermore the amplitudes of the second and third oscillations (Fig. 11b) did not decrease in intensity, indicating that an inclusion of HPβCD into an already-adsorbed protein layer on the surface probably did not take place.

Information about surface rheology from the drop oscillations, before and after drop-bulk-exchange, corroborate the above results, where flushing the IgG droplet with polysorbate 80 leads to a clear reduction of surface elasticity and

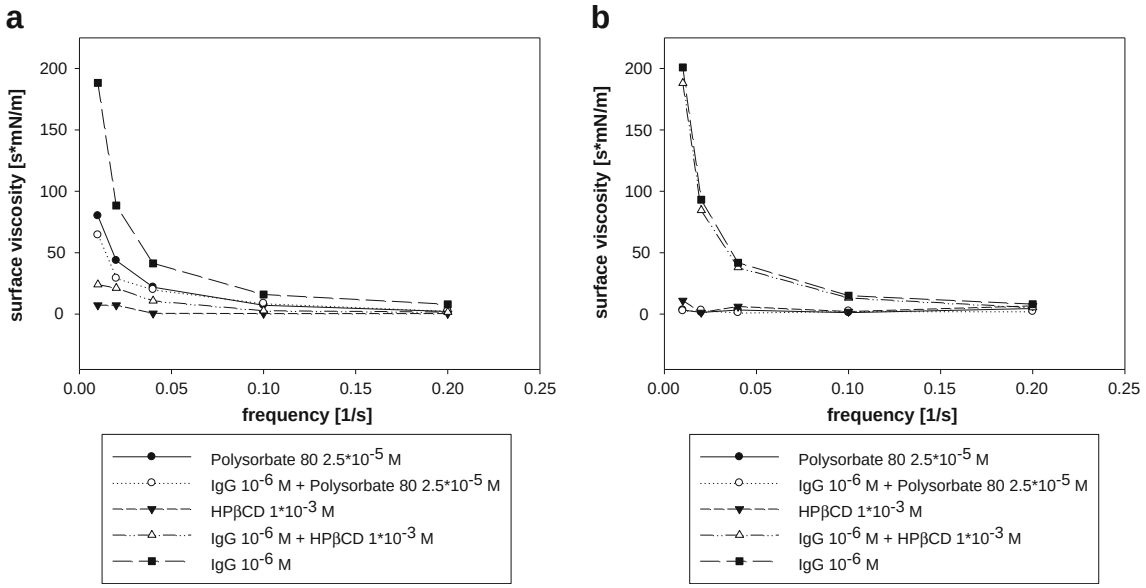

Fig. 9 Surface viscosity of polysorbate 80 2.5×10^{-5} M, HPβCD 1×10^{-3} M, IgG 1×10^{-6} M, and respective mixtures before (**a**) and after (**b**) buffer exchange.

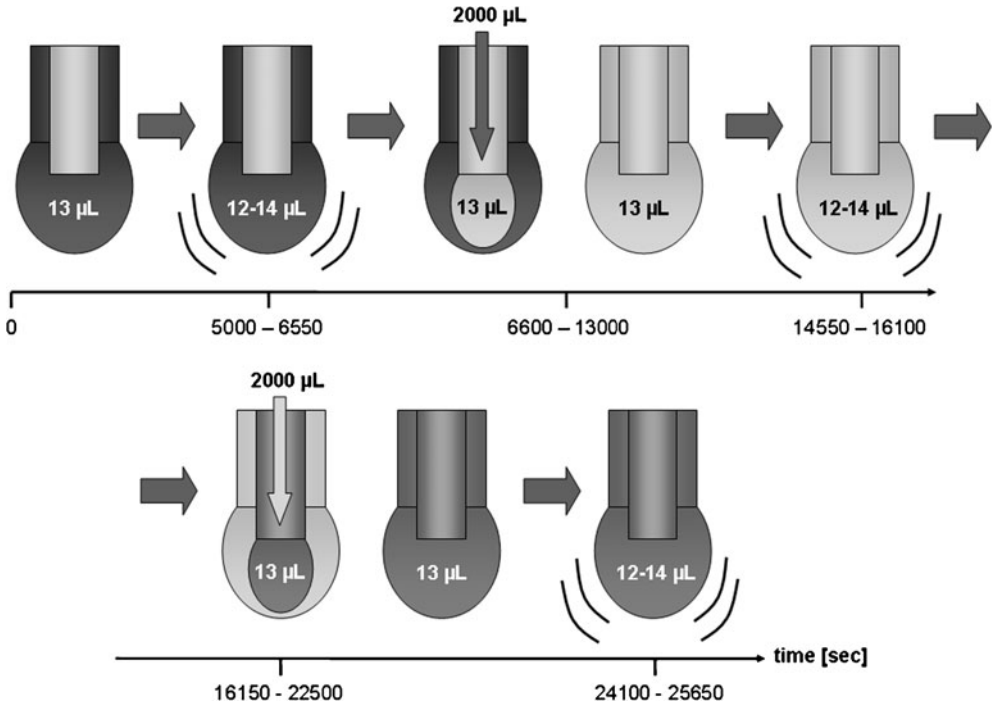

Fig. 10 Timeline for double exchange experiments. IgG solution is illustrated by black color, excipient solution by light grey, and pure histidine buffer by dark grey color. Pure IgG solution was used to build the surface layer at the beginning. After the first oscillation (5000–6550 s), polysorbate 80- or HPβCD-solution was pumped into the drop. Oscillations were performed again (14550–16100 s) and droplet bulk was exchanged afterwards with histidine-buffer. Measurement was finished at 25650 s after the third oscillation.

viscosity (Figs. 12a and 13a—second oscillation). Hence a displacement of IgG from the air-water interface by polysorbate 80 could be proven also after sequential adsorption. In contrast, pumping HPβCD into the IgG droplet does not reduce elasticity, or change viscosity (Figs. 12b and 13b, respectively). These results confirm that cyclodextrin did not displace the protein from surface, and that surface elasticity and viscosity are always determined by IgG once the IgG has adsorbed to the interface.

DISCUSSION

This work tests the validity of the theory of surface displacement as the underlying mechanism for the observed

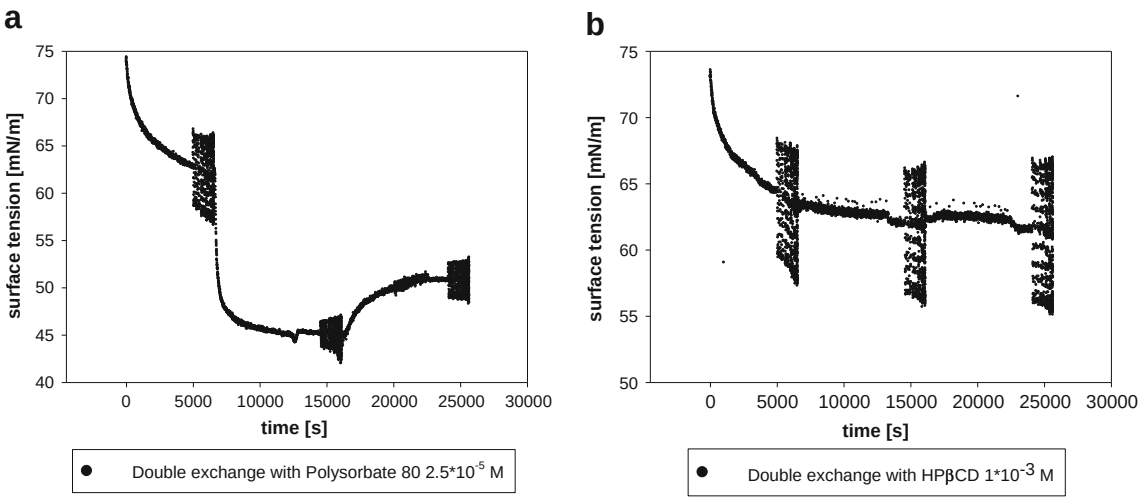

Fig. 11 Dynamic surface tension curves for double exchange studies using polysorbate 80 2.5×10^{-5} M (**a**) or HPβCD 1.0×10^{-3} M (**b**).

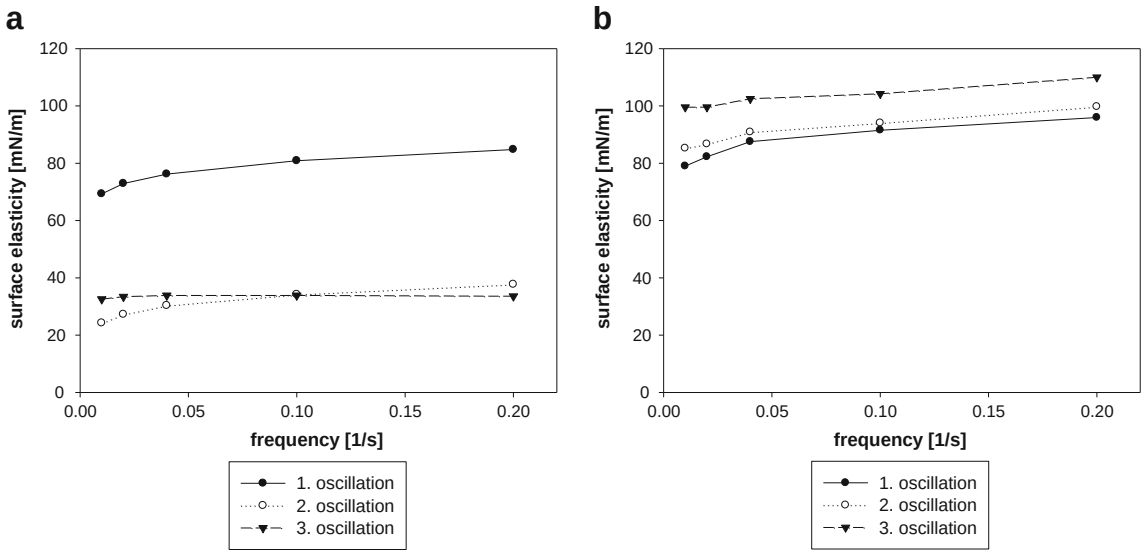

Fig. 12 Surface elasticity of first, second, and third oscillation for double exchange experiments. First oscillation was performed with pure IgG 1×10^{-6} M solution, second oscillation after drop bulk exchange against Polysorbate 80 2.5×10^{-5} M (**a**) or HPβCD 1×10^{-3} M (**b**), and third oscillation after subsequent drop bulk exchange against pure buffer.

stabilization effect of polysorbate 80 and HPβCD against surface-induced aggregation of mAbs. To this end, several methods were used to monitor the surface tension at very short time periods directly after the formation of a new interface (the maximum bubble pressure method, MBPM), as well as for long time periods until reaching equilibrium (drop profile analysis tensiometry). Additionally, a special setup of the drop profile tensiometry applying a concentric capillary system (double-capillary setup) was used to follow dynamics of adsorption/desorption and displacement of the different components upon exchange of the droplet bulk. Surface rheological measurements using the double-capillary setup provided additional information about the elastic and viscous behavior of the surface layer.

In the literature discussing aggregation at the air-water interface, it is sometimes assumed that during agitation processes a constant "renewal" of the air-water interface takes place (4,16,36,37). In this context, renewal refers to

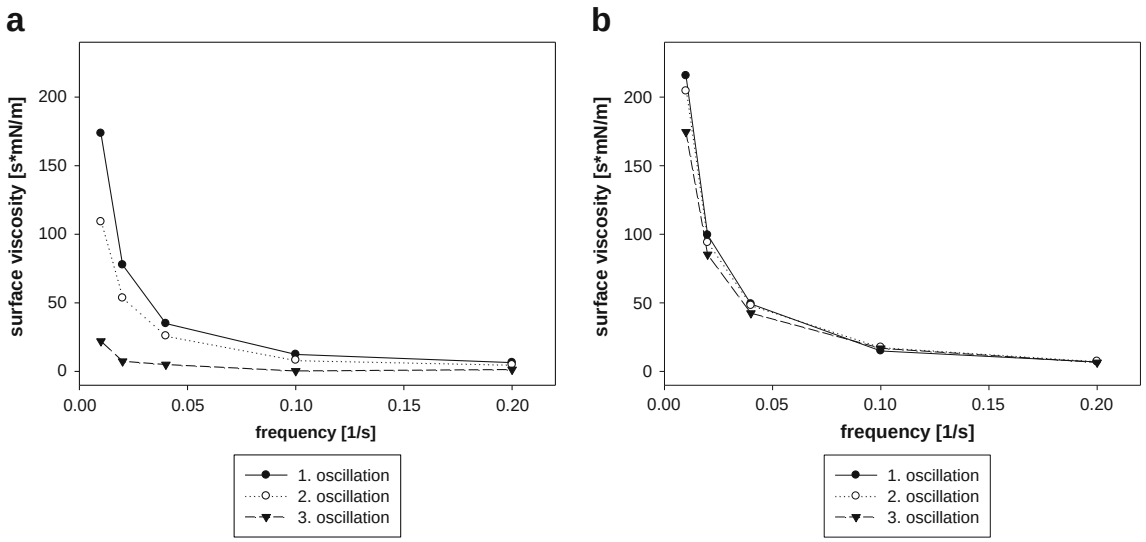

Fig. 13 Surface viscosity of first, second, and third oscillation for double exchange experiments. First oscillation was performed with pure IgG 1×10^{-6} M solution, second oscillation after drop bulk exchange against Polysorbate 80 2.5×10^{-5} M (**a**) or HPβCD 1×10^{-3} M (**b**), and third oscillation after subsequent drop bulk exchange against pure buffer.

mechanical destruction of the surface and subsequent formation of a fresh surface. Accordingly, MBPM was chosen to monitor the surface tension directly after surface formation. MBPM can measure surface tension over a surface lifetime ranging from few milliseconds to several seconds (38–43). It is thus a valuable tool to monitor the adsorption of polysorbate 80 and HPβCD to newly formed interfaces in the presence and absence of IgG. Results in Fig. 3 show that polysorbate 80 adsorbs within a few milliseconds to a newly formed interface, much faster than IgG and HPβCD. Interestingly, this rapid adsorption is slightly delayed (on the order of 1 s) in the presence of IgG in the solution, while the mixture of IgG and HPβCD does not show any significant changes. Hence rapid adsorption of HPβCD does not explain stabilization of the IgG by HPβCD.

Contrary to the MBPM, the drop profile analysis tensiometry was used to monitor dynamic changes in the surface tension over long time periods (sometimes up to 80,000 s) until reaching equilibrium. This technique has several advantages over ring tensiometry, including the fact that it is a contactless method, i.e. no further interface (e.g. the platinum-water interface in the Wilhelmy-plate instruments) is introduced into the investigated system, leading to more accurate results (44). As described earlier, the experiments using drop profile analysis tensiometry were carried at lower protein concentrations than the MBPM concentrations. Therefore, care has to be taken when relating these results to processes in more highly concentrated protein formulations.

Measurements with the drop profile analysis tensiometry confirm the surface activity of all three components, where the surface tension decreases with increasing concentration, with the equilibrium surface activity of polysorbate 80>IgG>HPβCD. Mixtures of IgG with increasing concentrations of polysorbate 80 show a constant surface tension similar to that of pure IgG below a concentration of 1×10^{-5} M of polysorbate, but this surface tension decreases dramatically above this concentration approaching the surface tension value of pure polysorbate 80. This is an indication that above this concentration, the surfactant displaces IgG form the interface. Such a behavior is however not seen for HPβCD, despite the relatively high concentrations used (2 orders of magnitude higher than polysorbate).

Single-exchange experiments using the double-capillary-setup of the profile analysis tensiometry and the associated surface rheological measurements show that exchanging the bulk of the droplet containing single components with buffer leads to rapid desorption of HPβCD and polysorbate 80 (though it is not complete in case of the later), while IgG remains bound to the surface. This observed irreversibility of adsorption of IgG is probably due to large adsorption energy as already shown for several proteins (24,25,45). In the meantime, IgG-polysorbate behaves nearly the same as pure polysorbate before and after buffer exchange, indicating that at this concentration (2.5×10^{-5} M) polysorbate did replace IgG at the surface as already seen in the aforementioned equilibrium measurements. In contrast, the mixture of IgG and HPβCD showed a rapid washing out of the latter, while IgG remained at the surface, indicating that both components coexisted at the interface.

These results were corroborated by the double exchange experiments, which showed that addition of polysorbate 80 to a solution of IgG leads to rapid displacement of the latter from the surface (Fig. 11a). On the other hand, addition of HPβCD to a solution of IgG showed that the former was not only unable of displacing IgG from the surface, but also probably excluded from the surface.

The above results provide direct evidence that polysorbate 80 displaces IgG from surface in both simultaneous and sequential adsorption. This characteristic behavior of non-ionic surfactants is well known and was already shown elsewhere (14,24,25,46). The mechanism of protein replacement by ionic and non-ionic surfactants after sequential adsorption was explained by the orogenic displacement model (47). Another explanation for protein displacement after subsequent buffer exchange describes adsorption of surfactant molecules onto the protein via hydrophobic interaction (25), which leads to a hydrophilisation of the protein. Despite high protein surface adsorption energy, hydrophilic protein/surfactant complexes possess lower surface activity and lead to a protein displacement from the air-water interface.

However, HPβCD exhibits a rather different behavior. The single exchange experiments could show that, for a pre-mixed IgG-HPβCD-solution, the protein and the cyclodextrin coexist in the surface layer. Meanwhile, the double exchange experiments showed that an integration of HPβCD in an already-adsorbed IgG-layer did not occur. In contrast, formation of a protein/polysaccharide layer after sequential adsorption was shown for a β-lactoglobulin/pectin-system (48). However, HPβCD did not show similar behavior.

Results of the current study provide supporting evidence for the surface displacement theory as a mechanism for the observed stabilizing effect of polysorbate 80 against the surface induced aggregation of IgG. In concentrations $>1\times 10^{-5}$ M, which are in accordance with the concentrations used previously (6), polysorbate 80 displaces IgG from the interface. Meanwhile, despite the fact that we used the same HPβCD concentrations as those which elicited a protein stabilizing effect in a previous study (6), our observations refute the surface displacement theory as the underlying mechanism for the protein stabilization observed for HPβCD. Probably a different mechanism takes place at the interface, namely an interaction between HPβCD and the partially unfolded protein, preventing further protein unfolding or aggregation. The investigation of this hypothesis is currently underway.

CONCLUSIONS

In this study, the mechanism of stabilization of polysorbate 80 and HPβCD against surface-induced IgG-aggregation was investigated. Accordingly, the surface tension of IgG in the absence and presence of polysorbate 80 or HPβCD was monitored using the maximum bubble pressure method, drop profile analysis tensiometry with different concentrations, double-capillary drop profile analysis tensiometry with single and double bulk exchange, as well as surface dilation rheometry. Results show that polysorbate 80 displaces IgG from the surface, with this effect starting very rapidly after the formation of new surface (after approximately 1 s) as shown by the maximum bubble pressure method. Additionally, using different concentration of polysorbate 80 showed that this replacement takes place when the concentration of the later exceeds 1×10^{-5} M. Drop bulk exchange experiments showed that this replacement takes place from a comixture of polysorbate and IgG, or even upon addition of polysorbate to a preformed surface film of IgG. Meanwhile, HPβCD could not displace IgG despite its surface activity. Mixtures of IgG and HPβCD coexisted at the interface. Single exchange experiments showed that HPβCD was rapidly washed from the interface leaving the protein film at the surface, while the double exchange experiments showed that HPβCD was excluded from a preformed IgG film. These results support the theory of surface displacement as the underlying mechanism for the stabilization effect of polysorbate 80, but refute the frequently held opinion that HPβCD stabilizes proteins against aggregation at the air-water interface in a manner comparable to non-ionic surfactants.

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