Antibody adsorption on the surface of water studied by neutron reflection

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Abstract
Surface and interfacial adsorption of antibody molecules could cause structural unfolding and desorbed molecules could trigger solution aggregation, resulting in the compromise of physical stability. Although antibody adsorption is important and its relevance to many mechanistic processes has been proposed, few techniques can offer direct structural information about antibody adsorption under different conditions. The main aim of this study was to demonstrate the power of neutron reflection to unravel the amount and structural conformation of the adsorbed antibody layers at the air/water interface with and without surfactant, using a monoclonal antibody ‘COE-3’ as the model. By selecting isotopic contrasts from different ratios of H\textsubscript{2}O and D\textsubscript{2}O, the adsorbed amount, thickness and extent of the immersion of the antibody layer could be determined unambiguously. Upon mixing with the commonly-used non-ionic surfactant Polysorbate 80 (Twee80), the surfactant in the mixed layer could be distinguished from antibody by using both hydrogenated and deuterated surfactants. Neutron reflection measurements from the co-adsorbed layers in null reflecting water revealed that, although the surfactant started to remove antibody from the surface at 1/10 critical micelle concentration (CMC) of the surfactant, complete removal was not achieved until above 1/10 CMC. The neutron study also revealed that antibody molecules retained their globular structure when either adsorbed by themselves or co-adsorbed with the surfactant under the conditions studied.

Keywords: Antibody; co-adsorption; mAb; neutron reflection; surface adsorption; structural unfolding; self-assembly; surfactant

Introduction
Monoclonal antibodies (mAbs) represent an increasingly important class of therapeutic drugs in today’s pharmaceutical pipeline. High concentration liquid formulations are a prerequisite for low volume subcutaneous injections to meet the desired clinically efficacious dose. It is during the optimization of such formulations that careful attention must be paid to mitigating the formation of aggregates and particulates, which may arise via several destabilization processes. Mechanisms leading to protein aggregation in bulk solution in the context of formulation have been widely reviewed. Briefly, they include covalent changes such as oxidation and deamination, and physical changes arising from protein-protein interactions and surface adsorption-desorption. The latter is encountered by mAbs exposed to air/liquid, solid/liquid and silicone oil/liquid interfaces as present during filling (pumping) and storage in primary packaging, such as glass vials with rubber stoppers and plastic/glass prefilled syringes. Aggregation leading to particulate formation may be accelerated during manufacturing processes, in particular, fill-finish activities wherein the mAb is exposed to interfacial stresses. Particulate limits are set by the pharmacopeias: for each unit dose referred to here, there must be less than 6000 particles > 10 \(\mu\text{m}\) and less than 600 particles > 25 \(\mu\text{m}\) (USP <787> and EP 2.9.19). The thorough characterization of the size distribution and nature of particulates remains an area of importance in the industry, and will inform our understanding of the potential capacity of protein particulates to elicit immunogenic responses.

In the biopharmaceutical industry, there is sufficient empirical data to link surface adsorption-desorption effects to particulate formation, although the underlying molecular origins remain sparsely defined. Early work used simple agitation to simulate high shear rates for different surface roughness conditions, as could be encountered during fill-finish. More recent work by Shieh and Patel investigated how air/water surface pressure measurements could predict mAb aggregation as a consequence of interfacial adsorption-desorption; this, when applied as a screening tool, would benefit the assessment of mAb behavior upon filling into primary packaging or dilution into an intravenous bag and giving set. An elegant experimental approach using a

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custom dilatational interfacial rheometer with simultaneous pressure and bubble shape measurements, has further enabled an understanding of aging of the adsorbed mAb film and consequent generation of aggregates.\textsuperscript{14}

Characterizing the precise molecular nature of adsorbed mAbs at an interface, however, requires state-of-the-art analytical techniques and data interpretation. Quartz crystal microbalance and total internal reflection fluorescence are useful benchtop methods, but lack the resolution to distinguish between individual adsorbed protein layers and the corresponding molecular orientation relative to the surface.\textsuperscript{15,16} This becomes limiting when the intention is to define molecular changes to mAbs that undergo adsorption to an interface followed by partial unfolding revealing hydrophobic (core) residues. Consideration must then be given to pathways involving desorption, protein-protein self-assembly (oligomers), and the formation of soluble aggregates that nucleate sub-visible and visible particles. In this study, we apply neutron reflection to the problem of understanding mAb adsorption to an interface (reviewed in ref. 17). Neutron reflection has enabled molecular models of protein adsorption to be constructed,\textsuperscript{18} has been used to determine the orientation of antibodies non-specifically adsorbed at the solid/liquid interface,\textsuperscript{19} and has yielded the structural characterization of membrane proteins in bilayer models resting on a water-filled layer.\textsuperscript{20}

Current approaches to mitigate chemical and physical instabilities require the addition of buffers and excipients to mAb formulations.\textsuperscript{21} Regarding protein adsorption and aggregation as discussed above, the industry generally uses non-ionic surfactants such as Polysorbate 20 and 80 (Tween\textsuperscript{\textregistered} 20 and 80) to control shake-induced aggregation as would be experienced during drug product transportation, for instance. In general, polysorbates compete with the protein for an interface and adsorb to exposed hydrophobic patches on the protein surface. During formulation development, Tweens around 0.02–0.05% (w/v) are often found to minimize particulate formation during shaking studies designed to expose the protein to air/liquid interfaces and accelerate aggregation through surface adsorption/desorption. Low concentrations of Tween 20 (0.0025%, below the critical micelle concentration (CMC) of ca. 0.01%) may also confer some prevention of IgG1 aggregation when shaken.\textsuperscript{22} A fundamental understanding of the competition between polysorbate and mAb at the air/water interface is directly relevant to our understanding of the behavior of drug substance (formulated mAb containing surfactant) in prefilled syringes. For example, Polysorbate 20, when added at concentrations above or below its CMC, attenuates mAb particle formation in agitated syringes harboring an air bubble; this action was further correlated with polysorbate-mediated inhibition of ‘gelation’ of the adsorbed mAb interfacial film.\textsuperscript{23}

The main aim of this study was to demonstrate the power of neutron reflection to unravel the amount and structural conformation of the adsorbed antibody layers at the air/water interface with and without surfactant. With the help of isotopic contrasts achieved by varying the ratio of H\textsubscript{2}O and D\textsubscript{2}O, we show that the antibody molecules in the adsorbed surface layers could be well represented by a uniform layer or Gaussian layer distributions, indicating no unfolding leading to the formation of polymer-like distributions along the surface normal direction. With the combined use of deuterated and hydrogenous Polysorbate 80 (Tween 80) surfactants (denoted as d-Surf and h-Surf, respectively), we have measured the surface composition from co-adsorbed surfactant and antibody, with the concentration of antibody fixed at 50 ppm, showing the concentration range above which mAb adsorption is inhibited. This study has demonstrated the potential of neutron reflection in helping to investigate how mixing of surfactant or addition of any other excipient affects the amount and structural conformation of an antibody adsorbed at the air/water interface.

**Neutron reflection and isotopic contrast variation**

A schematic representation of the relationship between the optical geometry of the neutron beam setup and the surface adsorbed antibody layer is shown in Fig. 1a. Note that the actual beam incidence angles are much lower than what is schematically depicted here. Specular neutron reflectivity, \( R \), is usually plotted as a function of momentum transfer or wave vector, \( \kappa \), perpendicular to the reflecting surface where

\[
\kappa = \frac{4\pi \sin \theta}{\lambda}
\]

where \( \theta \) is the beam incidence angle and \( \lambda \) the wavelength. \( R \) is mostly characterized by a broadly decaying shape when plotted against \( \kappa \),\textsuperscript{24} but, as is often observed in optical scattering interference patterns, also occur in neutron scattering. Following the Bragg law of \( n\lambda = 2\pi\sin \theta \) (where \( \tau \) denotes film thickness and \( n \) is an integer), \( \kappa \) is inversely proportional to \( \tau \). Because antibody molecules are largely globular and their dimensions

![Figure 1](image-url)
are much greater than typical surfactants, the thicker antibody layer adsorbed tends to make the reflectivity decay faster with the interference minimum occurring at the lower $\kappa$, implying that the measurable reflectivity signal is largely captured over the lower $\kappa$ range as well.25

As solvent molecules can also become mixed with adsorbed antibody layers, the change in layer composition, which is determined by resolution of the scattering length density (SLD) along the surface normal direction, is more appropriately linked to $R$. Because of the difference in the scattering length between hydrogen and deuterium, isotopic substitution can be used to change the SLD if the antibody layer is mixed with water. In this work, neutron reflectivity profiles were first measured from antibody adsorption at the air/ null reflecting water (NRW) interface, where NRW consists of 8.1% $D_2O$ and 91.9% $H_2O$ by volume, with SLD = 0. Because the water is invisible under this isotopic contrast, the specular reflectivity only contains the information about the adsorbed protein layer, allowing a precise determination of its interfacial adsorbed amount and the thickness.26 It is possible that the antibody layer is neither fully immersed nor fully afloat. When the immersed part of the layer is contrast matched to water, it becomes invisible and only the remaining part of the layer exposed to the air is seen by neutron reflection. This condition is met when the SLD is equal to $2.58 \times 10^{-6}$ $A^{-2}$ (close to the equal ratio of $H_2O$ and $D_2O$, termed CM2.58). When measured in $D_2O$, the signal contains information about the entire interfacial layer including the association of water. Although the structural interpretation in this case is more complex, its combined analysis with the other 2 profiles measured in NRW and CM2.58 offers significant benefit to highlight the adsorbed antibody layer differently. Thus, the isotopic contrast of water shown as the faint background within the model protein layers, as depicted in Fig. 1, could be varied by adjusting the ratio of $H_2O$ to $D_2O$ in a given neutron reflection measurement.

Under the co-adsorption of surfactant and antibody (Fig. 1b), it is possible to resolve the different adsorbed amount from the 2 surface active species because the use of d- and h-Surf under a given surfactant concentration gives us 2 different reflectivity profiles. Changes of scattering length for surfactant or water produce different SLDs for a given interfacial structure so that different reflectivity profiles can be produced. These reflectivity profiles together enable us to determine the composition of an interfacial system and in this work this technical feature is explored to determine the co-adsorption region of the antibody-surfactant system with increasing surfactant concentration.

The reflectivity profiles were analyzed using Motofit,27 which uses an optical matrix formalism as described by Born and Wolf29 to fit Abeles layer models to the interfacial structure. Briefly, the fitting process consists of a procedure where an interfacial model is first assumed and the reflectivity is then calculated by fitting the structural parameters of the interface to the measured one. The structural parameters are then refined in a repetitive process to achieve the best fit. To account for the structural changes along the surface normal direction, the interfacial layer is often divided in a series of sublayers, each of them being described by thickness ($\tau_i$), scattering length density ($\rho_i$) and roughness, where appropriate.

For the antibody adsorption systems, a uniform layer model was often appropriate, where the surface adsorbed amount or surface concentration ($\Gamma_p$, measured in mg/m$^2$) of the antibody can be expressed as

$$\Gamma_p = \frac{\rho_p \tau_p MW}{6.023 \Sigma b_p}$$

(2)

where $MW$ is the molecular weight of the antibody (in g/mol), $\Sigma b_p$ is its scattering length (in $A$), $\tau_p$ the thickness obtained from the fit (in $A$) and $\rho_p$, the scattering length density (in $A^{-2}$), respectively.26 The constant of 6.023 is related to the conversion of the Avogadro’s number ($N_A$) and the unit difference between Angstrom ($A$) and meter (m). The area per molecule ($A_p$, in $A^2$) can be obtained using:

$$A_p = \frac{MW}{6.023 \Gamma_p}$$

(3)

For the measurements in $D_2O$, the immersed part of the antibody layer must be fitted by taking into account the contributions from the solvent for space filling as well, with the total of the antibody volume fraction ($\rho_p$) and solvent volume fraction ($\rho_w$) being equal to unity. To ensure the SLD contributions consistent to the interfacial composition, we used the following equations:29,30

$$\rho = \rho_p \phi_p + \rho_w \phi_w$$

(4)

In the case of the antibody-surfactant systems in NRW, a single-layer model involving scattering length density ($\rho$) and thickness ($\tau$), was used to solve the following simultaneous equations involving d-Surf and h-Surf:

$$(\rho \tau)_d = 6.023 / MW (\Gamma_{sd} b_{sd} + \Gamma_p b_p) = \frac{b_{sd}}{A_{sd}} + \frac{b_p}{A_p}$$

(5)

$$(\rho \tau)_h = 6.023 / MW (\Gamma_{sh} b_{sh} + \Gamma_p b_p) = \frac{b_{sh}}{A_{sh}} + \frac{b_p}{A_p}$$

(6)

where it was assumed that $\Gamma_{sd} = \Gamma_{sh}$ or $A_{sd} = A_{sh}$ at a given surfactant concentration. Solving these equations allowed us to calculate precisely the surface concentrations of surfactant and antibody in the mixed interfacial layer.

Results

Surface tension measurements

The surface tension changes of both hydrogenated and ethoxy-late head deuterated Polysorbate 80 (denoted as h-Surf and d-Surf) were first measured, with the dynamic tension profiles measured for h-Surf at representative concentrations shown in Fig. 2a. It can be seen that, while the surface tension decreases with increasing surfactant concentration, the time dependent change after the initial period occurs very slowly. As the concentration increases, the fast initial surface tension reduction
becomes more pronounced, but the second stage of relaxation takes much longer. Even after the first 8000 seconds (over some 2 hr), the true equilibration might still have not been reached. For example, at the highest h-Surf concentration of 0.3 mM studied, the surface tension decreased steadily from 4000 to 8000 seconds and the net change was about 2 mN/m. The change during this slow process reflected minor structural rearrangements relating to the adjustment of the adsorbed layer structures. As the ethoxylate head groups were produced via polymerization, they are composed of a range of sizes that may have subtle differences in surface activity.

For convenience, we have, however, taken the surface tension readings at 8000 seconds as the equilibrated values. Fig. 2b compares these values measured from the 2 differently labeled surfactants. It can be seen that, although the surfactants were made separately, the equilibrated values overlap well over the experimental errors. The continuous line was drawn to highlight the kink that is very close to 0.012 mM, the widely cited CMC of this surfactant.\textsuperscript{31,32} Note that the surface tension continues to fall, although the rate of decrease slows down substantially. This is very typical of polymer-like surfactants with very low CMCs, implying that, as the total surfactant concentration increases above their CMCs, additional monomers become available to reduce the surface tension further.

The surface tension of the mAb alone (denoted as COE-3) was then measured. It was found that the adsorption of the mAb had little influence on surface tension reduction when its concentration was varied from 10 to 500 ppm (0.5 mg/ml) (data not shown). Given that each measurement of surface tension needed about 15 ml of the sample and 50 ml for the subsequent neutron reflection, we chose to fix the mAb concentration at 50 ppm throughout this study to minimize the amount of mAb used. Fig. 2c shows that the presence of 50 ppm of COE-3 did not measurably alter the dynamic surface tension over the entire surfactant concentration range, and even the equilibrated surface tension readings at the 8000 second interval show an almost exact overlap with the data measured without the mAb (Fig. 2d). This outcome reveals that the presence of the mAb did not influence the surface tension much, but, as shown below, neutron reflection revealed the co-adsorption of the mAb molecules over a wide range of surfactant concentrations studied.

**Antibody adsorption on the surface of water**

At the air/NRW interface, the adsorbed antibody layer is the only component that contributes to the specular neutron reflection as the water surface is made invisible to neutrons. The reflectivity measured thus offers useful information about the adsorbed layer thickness and composition without any complication arising from the solvent. Fig. 3 shows a set of reflectivity profiles measured at several representative antibody concentrations, plotted as $\log [R]$ versus $\log[\kappa]$ for better visualization. Because most surface changes tend to occur over the first 30–40 min, neutron reflectivity measurements were taken after the first hour of surface equilibration (mainly during the 2–4 hour period to avoid further complications relating to possible surface sample aging). It can be seen from Fig. 3 that an increase in the bulk antibody concentration can steadily increase the level of the reflectivity profile, but its shape appears not to change much, implying that, while the surface adsorbed amount increases, the thickness of the antibody adsorbed layer remains almost constant.
As outlined previously, the most common approach for the analysis of measured neutron reflectivity data are to adopt the model fitting based on the optical matrix formula.27,28 The continuous lines shown in Fig. 3 represent the best uniform layer fitting to each of the reflectivity profiles measured under different antibody concentrations, with the structural parameters listed in Table 1. It can be seen from Fig. 3 that the fits generated from the uniform layer model do reproduce the measured reflectivity profiles well within the experimental error, adding confidence to the structural parameters obtained from the analysis.

The changes of layer thickness and adsorbed amount obtained from the best uniform layer fitting are shown in Fig. 4. Over the concentration range studied (2 to 100 ppm), the surface adsorbed amount shows a steady increase from 1 to 2.5 mg/m², but the thickness of the adsorbed layers changes very little. Layer thicknesses could be well fitted within 45 to 55 Å over the concentration range studied. The errors as shown in Table 1 after the thickness values indicated the range of the values that could give acceptable fits to the measured reflectivity under each antibody concentration. This structural feature clearly signifies the retaining of globular framework of the Fab (fragment antigen-binding) and Fc (fragment crystallisation) segments at the interface, as any unfolding of the globular structure would lead to polymer-like structural inhomogeneity along the surface normal direction that must be taken into account by a multilayer model.33

In contrast to the analysis using the optical matrix based layer fitting as described above, a more direct analysis method is based on the kinematic approach where the neutron reflectivity R measured from NRW can be related to the layer structural parameters from the following Equation:24

\[
h_{pp}(\kappa) = \frac{R_0^2}{16\pi \beta_b^2} = \frac{1}{A^2} \exp \left( -\frac{\kappa^2 \sigma^2}{8} \right) \quad (7)
\]

\[
\ln[h_{pp}(\kappa)] = -2\ln A - \frac{\kappa^2 \sigma^2}{8} \quad (8)
\]

where \(h_{pp}(\kappa)\) is termed antibody’s self-partial structure factor, and by assuming that the adsorbed layer takes the Gaussian distribution the layer thickness \(\sigma\), defined as the full width at the height of 1/e, and area per molecule \(A\), the same as previously defined, can be obtained from the linear plotting of \(\ln[h_{pp}(\kappa)]\) vs. \(\kappa^2\) as shown in Equ. (8). In contrast to the values of \(r\) based on the uniform layer model, the corresponding \(\sigma\) values should be smaller by about 10%.

Fig. 5 shows the plots of \(\ln[h_{pp}(\kappa)]\) vs. \(\kappa^2\) converted from reflectivity profiles as shown in Fig. 2, but the new plots enabled us to interpret the data by fitting the straight lines using Equs 8. It can be seen from Fig. 5 that the measured data appear to become more scattered, and this is certainly the case for the 2 lower concentrations, but the best linear fits give the layer thicknesses and adsorbed amount directly. As shown in Table 1, the values in brackets from the kinematic approach broadly match those from the optical matrix model fitting well.
surface adsorbed amount, the range of variations falls in \( \pm 0.2 \text{ mg/m}^2 \). In contrast, the layer thickness from the low concentration of 5 ppm of COE-3 has large experimental error, and, as a result, there appears to be some inconsistency, but such a discrepancy is well within the experimental error range. As the mAb concentration increases, better consistency is observed, that is, the values in \( \sigma \) from the Gaussian model are slightly lower than those from the corresponding uniform layer fits. This exercise shows that consistent structural parameters could be obtained about adsorbed amount and layer thickness in spite of different data analysis approaches adopted.

It should be noted that like other proteins, COE-3 has labile hydrogen atoms associated with polar groups such as -NH\(_2\), -OH and -NH\(_2\), which will undertake H/D exchanges with the bulk solvent. As most labile hydrogens can freely access water, the exchanges tend to be complete. Thus, the exact scattering length \( (\Sigma b) \) and scattering length density (SLD or \( \rho \)) of the antibody in a given mixture of H\(_2\)O and D\(_2\)O, such as NRW can be calculated and the values are listed in Table 2. From these values, the surface adsorbed amount and the equivalent area per molecule were calculated from Equ. (2–3) by taking into account the appropriate H/D exchanges.

When the SLD of the solvent increases from 0 to 6.35 \( \times 10^{-6} \text{ Å}^{-2} \), that for the antibody increases from 2.05 to 3.36 \( \times 10^{-6} \text{ Å}^{-2} \) (Table 2). At SLD = 2.58 \( \times 10^{-6} \text{ Å}^{-2} \), the 2 SLD values become identical, implying that when the antibody is immersed in water under this contrast, it becomes invisible to the specular neutron reflection. The reflectivity in this solvent contrast can, however, inform about the part of the layer that stays out of the water surface. Figure 6 shows the reflectivity measured at 50 ppm of the antibody concentration together with the best uniform layer fit, giving a thickness of 12 ± 2 Å and SLD of 0.7 \( \times 10^{-6} \text{ Å}^{-2} \). The SLD gives the same volume fraction of antibody as that calculated from the SLD value obtained in NRW, consistent with the uniform layer structure. For comparison, the parallel measurements in NRW and D\(_2\)O under the same antibody concentration of 50 ppm are also plotted in Fig. 6. As already described above, the reflectivity profile from NRW could be well represented by a uniform layer of \( \tau = 50 \pm 5 \text{ Å and SLD = (0.56 ± 0.03)} \times 10^{-6} \text{ Å}^{-2} \), giving \( \phi_v = 0.27 \pm 0.05 \). The fitting to the corresponding D\(_2\)O profile must reflect the structural constraints obtained from the NRW and CM2.58 (contrast matched to mAb) profiles, consisting of a top layer of 12 Å on the air side and a bottom layer of 38 Å immersed in water. The corresponding SLDs for the 2 layers on the surface of D\(_2\)O are 0.9 and 5.6 \( \times 10^{-6} \text{ Å}^{-2} \), and the acceptable fitting led to the same thickness and SLD for the top layer as converted from the 2 other water contrasts, but the bottom layer was represented by the best fitted thickness of 35 ± 3 Å and SLD of 5.85 \( \times 10^{-6} \text{ Å}^{-2} \). The fitting from the D\(_2\)O contrast shows a highly consistent outcome that reinforces the overall structural features, but the minor discrepancies from the immersed bottom layer might be caused by a slightly lower amount of antibody adsorbed from the D\(_2\)O profile.

**Co-adsorption of antibody and surfactant**

It is widely considered that the addition of non-ionic surfactants in mAb solutions can prevent the antibody molecules from undergoing surface adsorption, thereby minimizing surface-induced structural unfolding. While this is widely

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**Table 2.** The scattering lengths \( (\Sigma b) \), scattering length densities \( (\rho) \), volumes and molecular weights (MW) of antibody COE-3 and hydrogenous and deuterated Tween 80 surfactants (h-Surf and d-Surf) used for the model fitting.

| Component | Contrast | \( \Sigma b \) [Å\(^2\)] | SLD [Å\(^{-2}\)] | \( V \) [Å\(^3\)] | MW [gmol\(^{-1}\)] |
|-----------|----------|------------------|-----------------|----------------|------------------|
| COE-3     | NRW      | 35227            | 2.05            | 171740         | 144754           |
|           | CM2.58   | 44309            | 2.58            | 171740         | 144754           |
|           | D\(_2\)O | 57648            | 3.36            |                |                  |
| d-Surf    | NRW      | 949              | 5.13            | 1850           | 1310             |
|           | D\(_2\)O | 988              | 5.28            |                |                  |
| h-Surf    | NRW      | 116              | 0.63            | 1850           | 1310             |
|           | D\(_2\)O | 155              | 0.84            |                |                  |
accepted, there is a lack of direct experimental evidence to demonstrate that surfactant adsorption can indeed help 'protect' an antibody from undergoing surface and interfacial processes associated with structural changes. For a specific surfactant, it is also important to know when and how it dominates surface adsorption in a given mixture and if there is a concentration range over which co-adsorption occurs. The aim of the following neutron reflection experiment was to reveal that the respective surface composition of a mixture of surfactant and antibody can be determined from the appropriate use of isotopic contrasts.

As evident from Table 2, the h-Surf has a scattering length of $1.16 \times 10^{-3}$ Å, while with 20 deuterated ethoxylate units, the d-Surf has a scattering length of $9.49 \times 10^{-3}$ Å, giving their respective SLD of 0.63 and 5.13 $\times 10^{-6}$ Å$^{-2}$ (in NRW). This means that while a strong neutron reflectivity will be detected from the d-Surf over most of the concentration range studied, the signal from the h-Surf will be weak and difficult to detect. However, the signal from specular neutron reflection is dictated by the adsorbed amount and following Equ (2), the adsorbed amount $\Gamma$ is proportional to layer thickness $\times$ SLD, i.e., $\tau \rho$. This works well for the adsorption of the antibody alone; as the adsorbed amount increases, the level of reflectivity rises and so is the product of $\tau \rho$, although it is $\rho$ that increases with $\tau$ varying little in this case.

At a fixed antibody concentration, similar changes are expected when a surfactant is added. Following the relations as shown in Equ (5) and (6), the product of $\tau \rho$ will respond to the co-adsorption from both surfactant and antibody with 2 noticeable effects. Note that the actual amount of antibody adsorbed varies with surfactant adsorption because it is a competitive process. When surfactant adsorption starts to become dominant, the product of $\tau \rho$ will deviate from that of the antibody alone due to the different adsorbed amount, and the actual signal difference was also linked to $b_{ad}$ and $b_{sh}$ (the scattering length for d-Surf and h-Surf, respectively). The products of $\tau \rho$ measured from co-adsorption of h-Surf and d-Surf with the concentration of antibody fixed at 50 ppm are shown in Fig. 7. As before, the solution pH was fixed at 5.5 with His buffer and the same ionic strength fixed at 25 mM. Over the lowest surfactant concentration range from 1/1000 to 1/100 CMC, the products of $\tau \rho$ remain the same as that of the antibody alone, showing little co-adsorption of the surfactant. As the surfactant concentration increases from 1/100 to 1/50 CMC, $(\tau \rho)_{ad}$ begins to increase while $(\tau \rho)_{sh}$ begins to decrease, indicating the early co-adsorption of the surfactant. From 1/50 to 1/20 CMC, a noticeable decline is observed from $(\tau \rho)_{sh}$ while $(\tau \rho)_{ad}$ rises sharply, indicating that surfactant adsorption is becoming dominant. From 1/10 CMC onwards, $(\tau \rho)_{sh}$ undergoes a further slight decline before plateau but $(\tau \rho)_{ad}$ keeps rising sharply; the difference reflects the contributing effects from $b_{ad}$ and $b_{sh}$. Thus, without solving the combined Equs (5) and (6), Fig. 7 clearly shows how surface composition varies with increasing surfactant concentration, with COE-3 completely removed from the surface at surfactant concentrations above 1/10 CMC. It should be noted that the product of $\tau \rho$ was measured over a narrow low momentum transfer range. Thus, the reflectivity data could be measured relatively quickly.

To further exemplify the changes in surface composition and layer thickness as a result of surfactant co-adsorption, Fig. 8 shows 3 sets of reflectivity profiles measured at surfactant concentrations of 1/100, 1/20 and 1/2 CMC using both d-Surf and h-Surf in NRW at pH 5.5 and ionic strength 25 mM. Unlike the reflectivity profiles corresponding to the extractions of $\tau \rho$ shown in Fig. 7, the reflectivity profiles shown in Fig. 8 were measured across the full $\kappa$ range so that small differences between the different labels could be revealed. At 1/100 CMC of surfactant (Fig. 8c), COE-3 adsorption is dominant and surfactant co-adsorption is minor due to its very low concentration. Nevertheless, the difference between the 2 reflectivity profiles is already obvious. Data analysis revealed that while the total layer thickness remained at $\tau = 48 \pm 4$ Å, the values for $A_p = 17000 \pm 3000$ Å$^2$ ($\Gamma = 1.4 \pm 0.2$ mg·m$^{-2}$) and $A_s = 2500 \pm 400$ Å$^2$ indicated the early desorption of COE-3 from the surface even at this very low surfactant concentration. At 1/50 CMC of surfactant, the total layer thickness still remained at $\tau = 48 \pm 4$ Å, but $A_p = 19000 \pm 3000$ Å$^2$ and $A_s = 1600 \pm 400$ Å$^2$ indicated further desorption of COE-3 (Section 3, Supporting Information). At 1/20 CMC of surfactant (Fig. 8b), the gap between the 2 reflectivity profiles becomes wider, indicating the more dominant contribution from the adsorbed surfactant. The total layer thickness was slightly reduced to $\tau = 44 \pm 4$ Å with a concomitant fall in $A_p = 35000 \pm 4000$ Å$^2$ ($\Gamma = 0.7 \pm 0.3$ mg·m$^{-2}$) and $A_s = 300 \pm 30$ Å$^2$. This trend continues on further increase in surfactant concentration to 1/10 CMC, with the total layer thickness remaining at $\tau = 44 \pm 4$ Å, but $A_p = 53000 \pm 4000$ Å$^2$ ($\Gamma = 0.4 \pm 0.3$ mg·m$^{-2}$) and $A_s = 260 \pm 30$ Å$^2$. At a surfactant concentration of 1/5 CMC, the total layer thickness declines to 35 Å and $A_s$ falls into the error margin, with $A_s = 170 \pm 15$ Å$^2$. The decline of the layer thickness is consistent with the desorption of COE-3 from the surface.
Thus, from a surfactant concentration of 1/2 CMC onwards (Fig. 8a), the layer thickness remains almost constant at 35 ± 3 Å and $A_0 = 150 \pm 15$ Å. Hence, although at the very low surfactant concentration of 1/100 CMC the surfactant begins to desorb COE-3, the detailed analysis of these reflectivity pairs (including those in Supporting Information) depicts a clear picture of progressive replacement of COE-3 from the surface by the surfactant over a wide surfactant concentration range. Complete replacement could well occur when surfactant concentration is above 1/10 CMC.

**Discussion**

Our study demonstrates that neutron reflection in combination with deuterium labeling is an effective method for revealing the amount and physical state of antibody adsorbed at the air/water interface with and without surfactant co-adsorption. From a nearly constant layer thickness and uniform layer model (or Gaussian distribution), it can be inferred that the adsorbed COE-3 molecules retained their globular structure with no indication of unfolding. All mAb layer thicknesses were around $50 \pm 5$ Å under the conditions studied. These values are close to the dimensions of Fab and Fc, showing that Fab and Fc fragments are rather well aligned in the adsorbed layers and that they do not stack up with fragments above or below each other. As shown in Fig. 4, the increase in bulk mAb concentration leads to the rise of surface packing density. The slight thickness increase indicates the orientation adjustment in response to the increasing packing density.

Subsequent studies of COE-3 co-adsorption with Polysorbate 80 revealed that the surfactant at 1/100 CMC started to desorb the mAb molecules, but that co-adsorption was retained over a wide surfactant concentration range and complete desorption of the mAb did not occur until the surfactant concentration reached 1/10 CMC. Under the solution conditions studied, there was no sign of surfactant-induced surface unfolding of the mAb molecules.

Surface tension measurements revealed that COE-3 alone did not reduce surface tension, even at concentrations up to 0.5 mg/ml (500 ppm), nor did COE-3 much influence the surface tension when mixed with Polysorbate 80. However, the neutron reflection measurements indicate that COE-3 alone adsorbed steadily over the concentration range studied (Fig. 4) and, more importantly, co-adsorption also occurred over a wide Polysorbate 80 concentration range. Thus, COE-3 is not only surface active, but its surface adsorbed amount is quite high. Even at the lowest concentration of 2 ppm studied, its adsorbed amount is about 1 mg/m² and by 50 ppm, the adsorbed amount increases to 2 mg/m². Fig. 4 indicates a clear trend wherein the amount of surface adsorbed COE-3 increases notably with its solution concentration, while the concomitant change in the layer thickness is much less pronounced.

In contrast, the layer thicknesses over the co-adsorption region remained relatively constant (Fig. 8 and the related text describing about the surfactant concentration range for co-adsorption to occur), again suggesting the dominant influence of the mAb present at the surface. Once mAb molecules are desorbed from the surface, the layer thicknesses decreases markedly, consistent with the typical surfactant layers adsorbed.

In summary, neutron reflection could play an active role in unravelling the surface and interfacial composition and structure for different antibodies, different interfaces and different solution conditions, particularly when another surface active...
ingredient is involved. The current work forms a useful basis for further neutron reflection experiments investigating mAb adsorption at concentrations well above 100 ppm and the effects of surfactant co-adsorption. As illustrated in Fig. 4, surface adsorption tends to plateau above 100 ppm, but any changes as a result of mAb instability and interaction with surfactant can be unravelled by neutron reflection with appropriate contrast variations.

**Materials and methods**

Hydrogenous Polysorbate (Tween®) 80 surfactant (denoted as h-Surf) was purchased from Sigma-Aldrich and was used as supplied. Its critical micellar aggregation in water was found to be 0.012 mM from surface tension measurements, consistent with the value reported from literature. The deuterated Polysorbate 80 (20 ethoxylate groups deuterated only, denoted as d-Surf) was synthesized by reacting the sorbitan ester, sorbitan monooleate with deuterated ethylene oxide following the standard procedures. Its CMC was also checked by surface tension measurements and the comparison with h-Surf is given in Fig. 1b.

The antibody, denoted as COE-3, was expressed in Chinese hamster ovary cells and purified using industry-standard methods. The solution behavior of COE-3 under different mAb concentrations and ionic strengths has been studied by Roberts et al. It was a full length IgG1 with sequence molecular weight equal to 144.8 kDa and supplied as a stock solution of 46.4 mg/ml in histidine (‘His buffer’ composed of histidine and histidine hydrochloride) at pH 6 with an ionic strength of 25 mM; 7 % w/v sucrose was also added to stabilize the antibody. The stock sample was stored under −80 °C, and, when needed, it was thawed and diluted directly into histidine buffer at pH 5.5, also at the ionic strength of 25 mM. It was diluted into subphases of different ratios of H2O and D2O. As the concentrations studied in this work were very low, typically below 1 mg/ml (1000 ppm), its dilutions into D2O meant that the levels of mixing of H2O were very low, with the exact amount noted and taken into account during neutron data analysis. The exact sequences of the light and heavy chains are given under Section 2.3 of the Supporting Information, allowing the relevant physical properties such as scattering lengths to be calculated under different solvent isotopic contrasts.

D2O (99% D), histidine and histidine hydrochloride were purchased from Sigma-Aldrich and also used as supplied. H2O was processed using an Elgastop PURELAB water purification system. Scattering length and SLD for basic elements are given in Table S1I from which the scattering length and SLD for H2O, D2O and any different ratios of them can be calculated. These values for the surfactants and antibody are given in Table 2.

**Surface tension measurements**

Surface tension measurements were made using a Krüss K11 tensiometer. The du Noiyy ring was freshly flamed before each surface tension measurement. The solution dishes and other glassware used were freshly cleaned by soaking them in dilute Decon solution, followed by copious water rinsing. All the measurements were performed at the room temperature of 22 ± 2 °C. The surface tension was recorded by raising the sample dish up to touch the ring and once in contact, the balance automatically adjusted the height so that the maximal pulling force was achieved. Each measurement was followed for up to 2–3 hour to monitor the time-dependent change. All the experiments were repeated at least 3 times, to ensure the reproducibility of the measurements.

**Neutron reflectivity measurements**

Neutron reflectivity measurements were performed using both the SURF reflectometer at ISIS Neutron Facility, Rutherford Appleton Laboratory, STFC, UK and the FIGARO reflectometer at the Institut Laue-Langevin (Grenoble, France). The neutron optical system in SURF provided the neutron wavelength range, typically between 0.5 and 7 Å. With the help of a supermirror setup, neutron reflectivity can be measured at the 3 incidence angles of 0.35, 0.8 and 1.5°, covering a momentum transfer range (k) from about 0.01 to 0.5 Å. In contrast, the FIGARO instrument gave the neutron wavelength range between 2 and 30 Å and the data could be acquired at 2 incident angles of 0.62° and 3.8°, giving a momentum transfer range from about 0.005 to 0.4 Å⁻¹. Both instruments were calibrated by taking the reflection measurements from a clean D2O surface. All the measurements were performed at the room temperature of 20 ± 3 °C.

Key structural constants needed for undertaking the model analysis using the above equations are listed in Table 2, with further information about the elementary scattering lengths and means to calculate the scattering lengths and SLDs of the surfactants (h-Surf and d-Surf) and COE-3 under different water contrasts given in Sections 1 and 2 of the Supporting Information.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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