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Interfacial Adsorption of a Monoclonal Antibody and its Fab and Fc Fragments at the Oil/Water Interface

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ToC:

Abstract

The physical stability of a monoclonal antibody (mAb) solution for injection in a pre-filled syringe may in part depend on its behaviour at the silicone oil/water interface. Here, the adsorption of a mAb (termed COE-3) and its fragment antigen-binding (Fab) and crystallisable (Fc) at the oil/water interface were measured using neutron reflection. A 1.4 ± 0.1 μm hexadecane oil film was formed on a sapphire block by a Spin-Freeze-Thaw process in order to retain its integrity upon contact with the protein solutions. Measurements revealed that adsorbed COE-3, its Fab and Fc retained their globular structure, forming layers that did not penetrate substantially into the oil phase. COE-3 and Fc were found to adsorb flat-on to the interface, with a denser 45 and 42 Å inner layer, respectively, in contact with the oil and a more diffuse 17-21 Å outer layer caused by fragments adsorbing in a tilted manner. In contrast, Fab fragments formed a uniform 60 Å monolayer. Monolayers were formed under all conditions studied (10-200 ppm, using three isotopic contrasts), though changes in packing density across the COE-3 and Fc layers were observed. COE-3 had a higher affinity to the interface than either of its constituent fragments, while Fab had a lower interfacial affinity consistent with its higher net surface charge. This study extends the application of high resolution neutron reflection measurements to the study of protein adsorption at the oil/water interface using an experimental set-up mimicking the protein drug product in a siliconized pre-filled syringe.

Keywords:

Pre-filled syringe, mAb, antibody adsorption, oil-water interface, structural unfolding, biopharmaceuticals, self-assembly, neutron reflection

1. Introduction

Monoclonal antibodies (mAbs) are an important class of therapeutics, with 12 new approvals by the U.S. Food and Drug Administration in 2018 and 62 more in late-stage clinical trials.¹ The immunoglobulin-1 (IgG1) isotype is a common scaffold for mAb therapeutics, comprised of multiple regions including two Fab (fragment antigen-binding), one Fc (fragment crystallisable) and a hinge region. MAb are bioengineered through sequence modifications to Fab and Fc to optimize blocking of a receptor target, modulate cell effector function and enhance interactions such as neonatal transport receptor binding. However, these sequence modifications influence local structural arrangements and protein surface characteristics, changing protein-protein and protein-surface interactions which may lead to physical instability such as aggregation.

For chronic diseases, improved patient compliance may be best achieved by sub-cutaneous (s/c) administration of high concentration solutions.² In this scenario, mAbs can be presented simply in pre-filled syringes, which facilitates convenience, sterility and safety,^{3,4} and allows for further— development towards home-administration.^{5,6} For a mAb of ~145 kDa, concentrated solutions of ≥ 100 mg/ml are typically required to deliver the therapeutic dose in a ~1 ml volume for s/c injection.^{7,8} Pre-filled syringes are often coated with the silicone oil polydimethylsiloxysilane (PDMS) to lubricate the plunger and allow free movement,⁹ especially important for dealing with high frictions arising from the highly viscous solutions under high mAb concentrations.^{2,7} This oil film is typically a few μm thick and presents a large hydrophobic surface to which mAbs may adsorb or break loose and form suspended oil droplets in the mAb solution, particularly with shake-stress as may be encountered during packaging, transport and storage.¹⁰ Vials coated with PDMS are also used to minimise protein-surface interactions and reduce aggregate formation during lyophilisation (freeze-drying) of mAb products.¹¹

MAbs, like most proteins, are amphiphilic, and tend to adsorb to accessible surfaces and interfaces, a process which can potentially cause structural rearrangements. It has been reported that mAb solutions in contact with silicone oil experience denaturation and aggregate formation.^{12–14} This process causes losses in therapeutic efficacy, reduces the effective doses of drugs administered, can result in adverse immune reactions^{15–17} and may breach particulate guidelines set by pharmacopeias.^{18–20} The exact mechanism for this destabilisation is not currently known but most hypotheses relate to the adsorption of mAbs to the oil surface.¹² Though there is not yet any direct structural evidence of mAb adsorption causing denaturation,

it has been suggested that mAb adsorption promotes the formation of oil droplets under agitation providing nucleating sites for aggregation¹⁰ and both droplet formation by emulsification and protein aggregate formation are heavily associated with mAb adsorption-desorption processes at the silicone/water interface, the oil effectively catalysing protein structural damage.^{14,15}

Literature investigating the adsorbed structure of mAbs adsorbed at fluid/fluid interfaces is relatively sparse, despite the importance of this topic. This is likely due to the lack of suitable techniques sensitive to such small scales at buried, complex interfaces. Neutron reflection (NR) with contrast variation has proven to be a powerful technique for investigating protein adsorption at air/water and solid/water interfaces,^{21–24} but until relatively recently techniques allowing measurement at the oil/water interface were complex, involving condensation of a thin layer of volatile oil onto the liquid surface.^{25,26} The recently developed Spin-Freeze-Thaw approach²⁵ makes it far easier to form a uniform oil film on the optically flat substrate, providing a more convenient route to exploit the power of NR at the oil/water interface.

Though no studies have yet investigated the adsorption of mAbs at fluid oil/water interfaces, the conformation of mAbs adsorbed at hydrophobic interfaces has been studied using the Quartz Crystal Microbalance with Dissipation (QCM-D).^{27,28} However, these studies have used thin ($< 5\text{Å}$) silane surfaces, which are chemically bound to silica and do not mimic the fluid properties of the oil phase. Using such a thin layer would not allow, for example, partial penetration of globular mAb molecules into the oil, or their large-scale structural unfolding, resulting in hydrophobic residues mixing with the oil. Developing a stable oil/water interface that can facilitate NR is essential for structural measurements of the mAb adsorption by exploiting its structural sensitivity. This technical capability will enable us to study how mAbs adsorb at different oil/water interfaces and develop strategies to prevent mAb adsorption and improve mAb drug stability.

As the first effort of developing the experimental capability of NR for studying mAb adsorption at the oil/water interface, we have used hexadecane ($\text{C}_{16}\text{H}_{34}$) as the oil phase, partly because the process for creating the oil film to facilitate NR measurements is established and partly because deuterated hexadecane ($\text{C}_{16}\text{D}_{34}$) is readily available, essential for manipulating isotopic contrasts (described in detail in the Methods Section). Several previous studies of surfactant adsorption^{29,30} and one study of adsorption of Bovine Serum Albumin (BSA)³¹ using the same Spin-Freeze-Thaw approach to form the oil/water interface for neutron reflection have been reported, providing the technical basis for us to investigate mAb

adsorption. This work also builds on our previous investigations of adsorption of mAbs at the air/water^{21,24} and silica/water^{22,23} interfaces. Together these studies will provide an overview of the challenges posed by mAb adsorption throughout production, fill-finish, packaging and application, and offer investigative methods to find solutions combating these challenges.

2. Experimental Methods

2.1 Materials

COE-3, provided by AstraZeneca plc, is a full-length human IgG1 with a molecular weight (MW) of 145560 Da (non-glycosylated/modified). Its Fab fragments have a MW of 47291 Da each and its Fc fragment has a MW of 49958 Da. COE-3 has an extinction coefficient of 1.43 mL/mg/cm at 280 nm and an isoelectric point of 8.44. AstraZeneca provided the sample at 46.4 mg/ml in 25 mM histidine/histidine hydrochloride ('His buffer'), 7% w/v sucrose, pH 6.0 (Batch SP12-423). COE-3 was cleaved into Fab and Fc fragments by papain digestion. The fragments were then separated by cation exchange chromatography, since the Fc does not bind protein A resin. The samples were exchanged into phosphate buffered saline (PBS) and, concentrated via ultrafiltration to 50.4 mg/ml for Fab (Batch SP17-121) and 47.74 mg/ml for Fc (Batch SP17-122), as measured by UV absorbance at 280 nm using extinction coefficients of 1.43 and 1.36 mL/mg/cm for Fc and Fab, respectively. Samples were stored at -80°C, and thawed and diluted directly into pH 5.5, 25 mM His buffer of the desired isotopic contrasts when required.

Deuterated oil is crucial to manipulate isotopic contrasts and reduce neutron attenuation but deuterated PDMS was not available, hexadecane was instead used as model oil. Hexadecane (C₁₆H₃₄) is an alkane with a convenient freezing point (18 °C). Hydrogenated n-hexadecane was purchased from Merck Sigma-Aldrich (Poole, UK). Deuterated n-hexadecane (>98% D) was purchased from Cambridge Isotopes (Tewksbury, MA, US). Before use, hexadecane was purified by passing through a column filled with powdered alumina (activated, neutral, Brockmann I grade) 3 times. Two sapphire (Al₂O₃) blocks, dimensions 7cm × 5cm × 2cm, were used in this work and their large surfaces were polished to a 4 Å roughness (PI-KEM Ltd, Tamworth, UK), as measured by NR. His buffer components, alumina powder and D₂O (>99% D) were purchased from Merck Sigma-Aldrich (Poole, UK). Purified H₂O was generated at 18.2 MΩ.cm using a Millipore UHQ system (Merck-Millipore, Watford, UK).

2.2 Neutron Reflection (NR)

Specular NR is a powerful depth profiling technique for determining the layer thickness and composition of interfacial films and molecular layers. Such structures are investigated by measuring and modelling reflectivity (R) as a function of momentum transfer perpendicular to the surface (Q):

$$Q = \frac{4\pi \sin \theta}{\lambda} \quad (1)$$

where θ is the incident angle of the neutron beam and λ is the de-Broglie wavelength of the incident neutron.³² Like optical reflection experiments, neutron reflectivity can be modelled as a wave phenomenon. Thus, interference effects are produced by layers with structures of similar scales to the neutron wavelength. Continuing the optical reflection analogy, Scattering Length Density (SLD) would be analogous to the refractive index of an optical medium. SLD is the sum of the scattering lengths of the atoms per unit volume in a material. As optical reflection is sensitive to changes in refractive index, NR is sensitive to changes in SLD. Across a boundary with change in SLD, the total internal reflection occurs below a critical Q value, Q_c , known as the critical edge:³²

$$Q_c = \sqrt{16\pi\Delta SLD} \quad (2)$$

Fig 1. Schematic illustration of the oil/water neutron reflection setup, with a zoomed-in section showing the experimental geometry at the interface.

Hydrogen and deuterium (^2H , D) have scattering lengths of $-3.74 \times 10^{-5} \text{ \AA}$ and $6.67 \times 10^{-5} \text{ \AA}$, respectively. By substituting hydrogen for deuterium in an interfacial material or a solvent, the SLD across the interface can be varied, allowing different components of the interface to be

highlighted or masked, known as contrast variation. This can be used to change the SLD of the oil, by mixing hydrogenated and deuterated oil, or of the aqueous solvent, by mixing H₂O and D₂O. Currently, expressing deuterated proteins is prohibitively expensive, though technically feasible.³³ The practical alternative is to highlight the protein layer by carefully selecting the SLDs of the solvents.

Hydrogen's scattering length results in neutrons being incoherently scattered out of the beam path, causing attenuation of the neutron beam. This presents a complication in NR at the oil/water interface, as most oils of interest contain significant quantities of hydrogen, and thus a neutron beam directed through a bulk oil phase will have a large reduction in signal. To mitigate this, the amount of hydrogen in the beam path must be minimised, which can be achieved by using a thinner oil layer and deuterating the oil.

The Spin-Freeze-Thaw technique developed by Zarbakhsh et al.^{25,34} allows a thin, smooth and stable oil film to be created. Briefly, the oil is spin coated onto a solid, neutron-transparent substrate to form a smooth oil film which is then cooled to freeze the oil, keeping it stable whilst the neutron block is assembled with the liquid cell. The mAb solution is subsequently injected into the solid-liquid cell, and the oil is then allowed to thaw. This produces a thin, homogenous oil film with a thickness between 1 and 5 μm , depending on the exact spin-coating parameters adopted. Using parameters that produce a 1-2 μm film minimises neutron attenuation while still simulating a bulk oil phase. Figure 1 shows a diagram of the neutron reflection setup with a portion of the interface zoomed in.

Deuteration of the oil reduces beam attenuation but can also be used to minimise the reflection between the solid substrate and oil. Sapphire (Al₂O₃) has a high SLD ($5.65 \times 10^{-6} \text{ \AA}^{-2}$). Contrast matching the oil layer to sapphire has a dual benefit, giving a larger contrast with a typical protein layer (SLD $\approx 2.4 \times 10^{-6} \text{ \AA}^{-2}$) than other typical substrates such as silicon (SLD $2.07 \times 10^{-6} \text{ \AA}^{-2}$), and allowing more highly deuterated contrast matched (CM) oil, further reducing beam attenuation (84% d-hexadecane by volume for oil CM to sapphire, compared to 65% for oil CM to silicon).³¹

The total reflectivity of the sapphire/oil/water interface is given by the thick-film approximation:

$$R_{tot} = \frac{R_1 + R_2 A(\lambda) + 2R_1 R_2 A(\lambda)}{1 - R_1 R_2 A(\lambda)} \quad (3)$$

where R_1 is the reflectivity of the sapphire/oil interface, R_2 is the reflectivity of the oil/aqueous solution interface, and $A(\lambda)$ is the wavelength-dependent attenuation of the neutron beam through the oil layer.^{26,34} Contrast matching the oil layer to the sapphire substrate minimises R_1 , permitting the assumption that $R_1 \ll R_2$. This allows us to simplify equation 3 to:

$$R_2 \approx \frac{R_{tot} - R_1}{A(\lambda)} \approx \frac{R_{tot}}{A(\lambda)} \quad (4)$$

To determine R_2 , $A(\lambda)$ must be calculated which is given by:

$$A(\lambda) = e^{(-\chi(\lambda) \cdot L)} = e^{\left(\frac{-2\chi(\lambda)d}{\sin \theta}\right)} \quad (5)$$

where $\chi(\lambda)$ is the attenuation per unit length of the oil, L is the path length through the oil, d is the oil layer thickness and θ is the incident angle of the beam.²⁵ $\chi(\lambda)$ was calculated by measuring the intensity of a neutron beam straight through an oil filled cuvette of known thickness, further information can be found in the Supporting Information (Figure S2). d was calculated using the “double critical-edge method” (see Figure S3).²⁵ By using an extremely low incident angle ($\theta = 0.025^\circ$), a reflection was taken from the sapphire/D₂O interface at the critical edge ($Q = 0.0055 \text{ \AA}^{-1}$) and from the same interface with an oil layer of known SLD and $\chi(\lambda)$. With a known $\chi(\lambda)$, d can then be found by:

$$\frac{I_1}{I_0} = \frac{I_0 A(\lambda)}{I_0} = A(\lambda) \quad (6a) \quad d = -\frac{\sin \theta \ln A(\lambda)}{2\chi(\lambda)} \quad (6b)$$

with the parameters as defined above. Using these parameters, the reflectivity data can be corrected for the attenuation through the oil using equation 5.

NR measurements were taken at the INTER reflectometer at the ISIS Neutron Facility, Rutherford Appleton Laboratory, Didcot, UK. INTER has a wavelength range of 1.5-16 $\text{\AA}$.³⁵ Using a neutron supermirror to allow the oil/water interface to remain horizontal, measurements were taken at a single angle of $\theta = 1.4^\circ$, giving a momentum transfer range of 0.019 - 0.205 $\text{\AA}^{-1}$. A resolution of $\delta Q/Q \approx 3\%$ was used. As Q -resolution was less important due to the broad nature of features in the data, collimating slits were kept at maximum opening to minimise potential scaling effects due to off-specular scattering. All measurements were carried out at the controlled room temperature of $20 \pm 3^\circ \text{C}$.

The protein film was modelled as a series of layers, each with a thickness and SLD. The SLD of a protein layer in a given contrast SLD_c is dependent on the component fractions of protein, solvent and oil:

$$SLD_c = \varphi_{oil}SLD_{oil} + \varphi_{prot}SLD_{prot}^c + \varphi_{sol}SLD_{sol} \quad (7)$$

where φ_x is the volume fraction of component x , and SLD_x is the SLD of component x . SLD_{prot}^c , the SLD of the protein, is not a fixed constant, but changes with SLD_{sol} , due to the exchange of labile hydrogens with deuterium in the solvent. With multiple aqueous contrasts (SLD_{sol} values), this effectively provides a set of simultaneous equations that can be solved once a best SLD distribution is found or used as constrained fitting parameters in more substantial fitting techniques. A table of the SLD values used for the oil, solvents and proteins can be found in the Supporting Information (Table S1).

The volume fraction of mAb can then be used to determine the adsorbed mass per unit area, Γ , and area per molecule APM :

$$\Gamma = \rho_{prot} T \varphi_{prot} \quad (8a) \quad APM = \frac{MW}{\Gamma N_A} \quad (8b)$$

where ρ_{prot} is the density of the protein, in this case given by Fischer *et al.*'s molecular weight dependent formula,³⁶ T is the fitted layer thickness, N_A is Avogadro's constant and MW is the protein's molecular weight.

Fig. 2a, b and c. Schematic visualizations of mAb adsorbed at the interface in the three aqueous isotopic contrasts used in the NR experiments

The three aqueous contrasts used were: CM Sapphire buffer (Figure 2a, $SLD = 5.65 \times 10^{-6} \text{ \AA}^{-2}$, 11% H_2O , 89% D_2O) which measures only the protein layer and provides an accurate adsorbed mass; CM Protein buffer (Figure 2b, $SLD = 2.58 \times 10^{-6} \text{ \AA}^{-2}$, 55% H_2O , 45% D_2O) which

measures only protein penetration into the oil phase since the solvent matches the protein SLD,; H₂O buffer (Figure 2c, SLD = $-0.56 \times 10^{-6} \text{ \AA}^{-2}$) which measures the full structure of the mAb at the interface.

NR data analysis was performed using RasCAL,³⁷ the software modelling neutron reflectivity based on the optical matrix formulism by a Born and Wolf.^{38,39} Initial analysis was completed by fitting to the minimum χ^2 . Further analysis was then performed using RasCAL's built-in Bayesian Model Selection capability with a Bayesian Markov chain Monte Carlo algorithm (DRAM),⁴⁰ assuming a Gaussian distribution for each parameter as a starting value. Bayesian Model Selection uses Bayesian statistical methods to calculate a likelihood distribution for each parameter, which allows the uncertainty in each parameter and the model and the uniqueness of the solution to be estimated based on how the model replicates the data. This allows a more robust analysis with comparisons between different models and provides a better understanding of the uncertainty in the model and the best-fit parameters.

3. Results and Discussion

3.1 Oil Thickness and Attenuation

The best-fit parameters for the oil attenuation measurements are shown in Figure S2 and Table S2 in the Supporting Information. From multiple thickness measurements the oil film was found to have an average thickness of $1.4 \pm 0.1 \text{ }\mu\text{m}$, demonstrating that the thickness was well controlled. A single thickness measurement was then made with a 200 ppm COE-3 solution to ensure that protein adsorption did not cause any detectable detachment of the oil from the interface and resulted in the thinning of the oil layer. This measurement found a thickness of $1.3 \pm 0.1 \text{ }\mu\text{m}$, demonstrating that the film thickness was not significantly adsorption-dependent, and that separate thickness measurements would not be required for each individual protein adsorption measurement.

3.2 Protein Penetration into Oil Phase

Fig 3. Reflectivity profiles of COE-3, its Fc and Fab, at 200 ppm in CM protein buffer compared to buffer alone. The simulated profile represents a volume fraction of BSA in the oil phase of 6% with a thickness of 75 Å.³⁰

Figure 3 shows that the reflectivity profiles for the whole mAb (COE-3), its Fab and Fc at a concentration of 200 ppm in CM protein buffer overlap entirely with profile of the CM protein buffer alone, within experimental error. The absence of a shift in the reflectivity profile suggests little or no penetration of COE-3 or its fragments into the oil phase. For comparative purposes, Figure 3 also shows a simulated reflectivity profile of an adsorbed BSA layer with a thickness of 75 Å and a 6% volume fraction in the hexadecane phase, as observed by Campana *et al.*³¹

Using a fitting process in which the protein is allowed to protrude across the interface,²¹ yielded models in which the proteins retained their globular structure while penetrating the oil layer by only 1-4 Å (Table S2). This layer thickness is consistently lower than the experimental errors and implies that the NR measurements were not sufficiently resolved to unambiguously define this minimal extent of protein penetration into the oil phase. This is in contrast to the behaviour of COE-3 at the air/water interface, wherein COE-3 protruded ~10 Å into the air,²¹ likely due to the absence of viscosity for air compared to hexadecane. The data are also in contrast to the observed partial unfolding of BSA at the hexadecane/water interface, forming a 75 Å mixed protein/oil layer.³¹ The nature of the adsorbed BSA layer can be attributed to its amphipathic α -helices and hydrophobic ‘pockets’ which predispose BSA to partial unfolding at the oil/water interface and impart an emulsifying behaviour.^{40,41}

We have previously shown that the net surface charge of proteins also affects their interfacial behaviour, wherein higher Fc adsorption at the air/water interface, as compared to Fab, was linked to its lower surface charge.²¹ This is relevant because the NR measurements by

Campana *et al.* were made near the isoelectric point of BSA (pI 4.8), reducing electrostatic repulsion from the hydrophobic oil layer. In contrast, here the measurements were made at pH 5.5, below the isoelectric points of COE-3, its Fab and Fc (pI 8.44, 9.64 and 6.36, respectively) which therefore all had net positive surface charge.

3.3 Reflectivity data fitted to a one-layer model

Fig 4. Reflectivity data fitted to a one-layer model measured from 3 isotopic contrasts (left), corresponding SLD profiles (middle) and schematics (right) for 50 ppm COE-3 (a), and its Fc (b) and Fab (c) fragments. The shaded areas in the fits and SLD plots represent the 95% confidence intervals for the model.

Figure 4 shows the simultaneous fits to the reflectivity profiles, their corresponding SLD profiles and schematics providing physical interpretation for COE-3, Fc and Fab, at 50 ppm, by modelling the adsorbed proteins as one-layer. The fits follow the measured reflectivity profiles well under the 3 isotopic contrasts for COE-3, Fc and Fab, and confirm the adequacy of the model, along with the χ^2 values shown in Table S4. Parallel measurements with one-layer fits at 10 and 200 ppm together with those from 50 ppm COE-3 are compared in Figure S4.

Fig 5. Thicknesses for each protein layer obtained from the one-layer model.

Figure 5 shows the comparison of the fitted one-layer thicknesses for COE-3, Fc and Fab at each concentration measured, with other relevant parameters from the analysis given in Table S4. The confidence bound errors were calculated from RasCAL's parameter estimation capability, as described in the Experimental Methods section. To a good approximation, an Fc is a thick torus in shape, with a diameter of 70 Å and a short axis of 40 Å, while a Fab is a thick, elliptical torus with an indented ring, with a wider diameter of 80 Å, a short diameter of 45 Å and a height of 45 Å.²¹ The layer thicknesses in Figure 5 are consistently higher than the shortest axes of the mAb fragments, but lower than the longest axis. In a one-layer model, this can be explained by the fragments adsorbing in a tilted manner, rather than entirely flat or side-on. In the absence of strong charge effect and hydrophobicity, we would expect COE-3 and its fragments to adsorb onto the interface with as much contact area as possible. In this case we would expect the layer thickness to be the protein's shortest axial thickness. The tilting may arise from a combination of factors, including steric overlaps, local electrostatic repulsion or hydrophobic effects. These interactions constrict molecules in the possible orientations they could adopt. With the exception of COE-3, the layer thicknesses are relatively consistent across

concentrations (see Figure 5 and Table S4), so this tilting does not appear to be affected by increased protein density much, showing less influence from packing density or steric interference.

Fig 6. Reflectivity data fitted to a two-layer model (cf. Fig. 5) measured from 3 isotopic contrasts (left), corresponding SLD profiles (middle) and schematics (right). The shaded areas in the fits and SLD plots represent the 95% confidence interval for the model.

It is also possible that the deviation in layer thickness from the shortest axial lengths of the globular fragments arose from their structural rearrangements, instead of tilting. If such structural rearrangement did occur, it would imply that the mAb fragments were structurally very flexible and that the thickness of the layers could be smaller than the shortest axial lengths when the packing density was low but become larger as the packing density rises. This was however not observed from Figure 4. Structural flexibility could be associated with unfolding, but this is unlikely as unfolding would promote the mixing of buried hydrophobic peptide chains with oil, which does not corroborate with the low oil-protein mixing demonstrated earlier. The low volume fraction achieved even at the highest concentrations studied (Table S4) further demonstrates that none of the mAb fragments was unfolded. Unfolded polypeptides usually form a thinner but more densely packed layer, with the volume fraction of the densest region often well above 0.4^{43,44}.

The full COE-3 molecule is approximately 140 Å in length. From the measured thicknesses we can assume that the full COE-3 adsorbs largely flat-on at the oil/water interface, with all 3 fragments in direct contact with the oil. This structural feature is consistent with our previous observed COE-3 conformation adsorbed at the air/water²¹ and hydrophilic silica/water interfaces.²² In their QCM-D study of how several mAbs adsorbed to interfaces coated with hydrophobic silanes, Wiseman and Frank also concluded that monolayers of globular mAbs had been formed at the surface.²⁷

3.4 Reflectivity data fitted to a two-layer model

Although a one-layer model fits all the measured reflectivity profiles adequately, their thicknesses are greater than the shortest dimensions of Fc, Fab and the whole mAb. Since Fc and Fab are not rigid, the actual layer thicknesses would be smaller than the shortest axial lengths if minor deformation is considered. It can then be assumed that the inner region in direct contact with the oil is densely packed and, due to different tilting, the outer region on the aqueous side is loosely packed. Further analysis by a two-layer model fitting could provide insight into the uneven distributions within the adsorbed molecular films. Figure 6 shows some of examples of two-layer fits to the measured reflectivity profiles measured under 3 isotopic contrasts, with corresponding SLD distributions and schematics providing physical interpretation of COE-3 at 200 and 50 ppm, and Fc at 50 ppm. Similar two-layer fits to the reflectivity profiles measured for other concentrations of COE-3 and Fc are shown in Figure S5, with all structural parameters listed in Table S5. On the basis of simultaneous fitting using RasCAL and the parameters measuring the consistency of the fits, the two-layer model

improved the quality of the COE-3 and Fc fits significantly. However, fitting a second layer to Fab did not noticeably improve the quality of the fits, indicating that the adsorbed Fab fragments formed a uniform layer with little change in packing density or tilting along the surface normal direction.

Both COE-3 and Fc were best fitted with an inner, thicker layer of higher protein fraction in contact with the oil phase, and an outer, thinner layer of low protein fraction protruding into the aqueous solution. For example, for COE-3 at a concentration of 50 ppm the inner layer had a thickness of 46 ± 2 Å, containing 0.29 ± 0.01 protein in fraction, while the outer layer had a thickness of 17 ± 3 Å, containing 0.12 ± 0.03 protein in fraction. For Fc, the inner layer is very similar in thickness to the fragment's short axis of 43 Å containing 0.19 ± 0.01 protein while the outer layer had a thickness of 21 ± 4 Å, containing 0.09 ± 0.01 protein. These fitted structural parameters further affirm the interpretation that the inner layer is comprised of fragments adsorbed flat-on to the surface and that the outer layer is composed of protruding parts of tilted fragments. The large uncertainty in the thickness of the second layer reflects the relatively low sensitivity due to the low volume fraction, but this layer is likely not to be well defined due to the distribution in protein tilting. For Fc, the volume fraction of both the inner and outer layers increases with concentration, and there is a sufficient sensitivity to justify the fitting of the existence of the second layer even at low concentrations, suggesting that this conformation is likely to be caused by charge interactions associated with how charge groups are distributed on Fc surface.

At 10 ppm, the thickness for each layer is the same within error as the corresponding value observed from 50 ppm. This is the case for both Fc and COE-3. The combined thickness of the two-layer model for COE-3 approximates that for the Fab fragment (~ 62 Å compared to ~ 60 Å), while the inner layer is similar in thickness to that modelled for the Fc fragment (46 Å vs 43 Å). While it may be assumed that these two layers represent an inner Fc + Fab and an outer Fab-only, the combined thickness of the two-layer modelled for Fc is very close to that of COE-3, indicating that Fc is likely to be present in the outer diffuse layer as well. The tilting of the Fc presumably arises from a combined influence of structural constraints associated with bonding and steric effects and local charge interactions such as electrostatic repulsion.

At 200 ppm the layer thickness for both Fc and COE-3 increases, though the extent of increase for Fc is far less. The thickness increase of COE-3 appears to be caused by an increase in inner layer thickness, largely associated with increased protein fraction causing changes in the preferred orientation of the fragments. Interestingly, a similar two-layer structure was also

adopted by COE-3 at the silica/water interface,²² with similar thicknesses (45 Å inner layer, 20 Å outer layer), though the protein volume fractions were consistently higher, suggesting a higher affinity for hydrophilic than hydrophobic interfaces.

As indicated previously, the confidence bounds and errors in parameters for these two-layer model fits are higher than those from the one-layer fits, especially in the outer diffuse layer. Adding another layer greatly increases the number of possible states and hence the uncertainty in the final model. For example, adding a 3rd layer on the aqueous side at COE-3 concentrations of 50 and 200 ppm slightly improved the fitting but the volume fraction of protein in the 3rd layer was only around 0.01. As the 3rd layer does not represent a significant fraction of the adsorbed structure, the two-layer model balances the accurate representation of the physical state of the adsorbed layer and fitting uncertainty.

3.5 Protein Adsorbed Mass and Effect of Concentration

Fig 7. The adsorbed mass of protein per unit area in mg/m² (a) and nmol/m² (b) as a function of concentration for COE-3, its Fc and Fab, calculated from one-layer fits for the Fab and two-layer fits for COE-3 and Fc. Masses assume a H₂O solvent. Note the logarithmic concentration scale.

Figure 7 shows that the adsorbed quantities of COE-3, its Fc and Fab, increased with increasing solution concentration. Within experimental error, the trend of concentration-dependent increase for COE-3, its Fc and Fab are broadly similar. The results are largely independent of the fitted model, since the sapphire-matched aqueous contrast is extremely sensitive to the adsorbed mass. COE-3 had a higher adsorbed mass per unit area than either of its constituent fragments but measured as moles per unit area, COE-3 adsorbed the least and appeared driven more by Fab than the Fc adsorption, at least up to 50 ppm (Figure 7b). The high adsorption of the Fc, measured in moles per unit area, can be attributed to its significantly

lower pI (6.36) and thereby weaker net surface charge and electrostatic repulsion from the hydrophobic oil layer.

These results can be compared with the work by Li et al. studying the adsorption of COE-3, Fc and Fab at the air/water interface²¹. The concentration ranges in both experiments overlap at 10 and 50 ppm, allowing some comparison of adsorbed mass and concentration effect in this range. Adsorption at both interfaces shows a steady increase with bulk concentration and the adsorbed mass per unit area at a given solution concentration occurs in the order COE-3, Fc and then Fab. The same trend indicates similar interfacial nature, consistent with the notion that air is hydrophobic. However, there are differences in the adsorbed masses and concentration-dependent behaviour. COE-3 and to a lesser extent, Fab, show a much weaker concentration-dependent effect at the oil/water interface, while the effect of concentration on Fc adsorption, which is almost negligible at the air/water interface, increases significantly at the oil/water interface. Furthermore, at the air/water interface, Fab dominates the concentration dependence of the mAb adsorption, while at the oil/water interface, this effect was weaker and apparent only below 50 ppm. These differences may be caused by the lower penetrability of the oil layer, restricting the possible conformations that could be adopted by COE-3 and its fragments at high concentrations. This highlights the importance of investigating the oil/water interface separate from the air/water interface, as interfacial behaviours can be markedly different.

From equation 8 we can calculate the area per molecule (APM) for each protein, shown in Figure S6. Projections of the human mAb 1HZH, which has a similar sequence to COE-3 (75% sequence identity in Fab and almost identical in Fc), give estimated footprints of 4600 Å² for the Fc fragment and 4000 Å² for the Fab fragment when adsorbed with the maximum footprint.²¹ COE-3's limiting area can be estimated as the sum of 2 Fab fragments and 1 Fc fragment, with a total of 16600 Å². It can be seen that Fab does not reach this limiting area even at 200ppm, while Fc and COE-3 exceed their respective limiting values significantly, providing some explanation as to the observed tilting within the adsorbed layer.

4. [SummaryConclusions](#)

Despite extensive studies investigating the adsorbed structure of mAbs at air/water and solid/water interfaces,^{21,23,45} their adsorption at oil/water interfaces remains largely unexplored. This work describes the interfacial structures of a mAb and its Fab and Fc, adsorbed at the hexadecane/water interface, measured by NR. Interfacial tension measurements showed that the adsorbed protein layer was at equilibrium before NR measurements began and remained stable over the 2 h acquisition period. The thickness and attenuation of the hexadecane oil layer

spin-coated onto a sapphire block was measured to allow attenuation-correction of further reflectivity measurements. The adsorbed structures of the proteins at the oil/water interface were then measured at 3 bulk concentrations (10-200 ppm) in 25 mM His buffer, pH 5.5. None of the proteins was found to penetrate into the oil phase within the sensitivity of the NR measurements ($< 4 \text{ \AA}$). Instead, the NR data described a protein monolayer at the interface that could be fitted using one- and two-layer models due to the adsorbed protein molecules adopting differing orientations ('tilts') relative to the surface as a function of protein concentration and type (mAb, Fc and Fab). MAb molecules adsorbed 'side-on' at the interface (both the Fc and Fab of the mAb were in contact with the oil), modelled as an inner, dense layer, $45 \text{ \AA}$ thick, and an outer layer, $\sim 20 \text{ \AA}$ thick, representing the fraction of mAb domains tilted further away from the surface as a consequence of closer packing or protein-protein electrostatic repulsion. Similarly, the Fc adsorbed as an inner, dense layer, $42 \text{ \AA}$ thick, consistent with a side-on orientation, and an outer layer, $\sim 16 \text{ \AA}$ thick. The Fab also adsorbed as a layer whose thickness was a little greater than its shortest axial length but, in contrast to the Fc, the data were best fitted as one-layer (rather than a two-layer) model. This indicated that the Fab adopted a different packing preference to the Fc which may reflect a stronger lateral electrostatic repulsion within the adsorbed layer on account of its high pI (9.64). The Fc was found to have the highest affinity (measured as moles per unit area) to the hydrophobic oil surface, with the lower affinity of the mAb weakly linked to that of the Fab. COE-3 was found to adsorb in a similar structure to that found in previous studies at the silica/water interface²², but at lower volume fractions, suggesting a higher affinity for hydrophilic than hydrophobic surfaces. These NR results were corroborated by interfacial tension measurements; the higher affinity of the Fc reflecting its significantly lower pI (6.36) and reduced electrostatic repulsion against the hydrophobic oil layer. This study establishes a hexadecane/water interface which provides a model of the silicone oil/water interface experienced by mAbs in solution filled into siliconized syringes. We show that the behaviour of the mAb and its constituent fragments at the oil/water interface is distinct from that reported previously at air/water and SiO_2 /water interfaces.²⁰⁻²³ Our work thus paves the way for future studies [directly utilizing PDMS oil to measuring the exact interfacial adsorption of the mAb and its constituent fragments and examining the effects](#) of ionic strength, pH, surfactant co-adsorption, etc., which will advance our understanding of interfacial effects during mAb fill-finish processes and injection device selection.

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Supporting Information

The Supporting Information including key structural parameters required for neutron data analysis, characterisation of transmission through the oil film, additional information from reflectivity modelling, estimation of the extent of protein mixing into the oil and interfacial tension for Fab, Fc and the whole COE-3 is available free of charge on the ACS Publications webpage.

Author contributions

JRL, CFW, JRPW, CK and SMB conceived the project. SR, ZL, MC, FU and JRL designed and performed the experiments. SR, ZL, XH, HG, MC, JRPW and JRL contributed to neutron reflection measurements, data analysis and interpretation. SR, ZL, CK, CFW and JRL wrote the manuscript. All authors reviewed the manuscript and approved its submission.

References

- (1) Kaplon, H.; Reichert, J. M. Antibodies to Watch in 2019. *mAbs* **2019**, *11* (2), 219–238. <https://doi.org/10.1080/19420862.2018.1556465>.
- (2) Garidel, P.; Kuhn, A. B.; Schäfer, L. V.; Karow-Zwick, A. R.; Blech, M. High-Concentration Protein Formulations: How High Is High? *European Journal of Pharmaceutics and Biopharmaceutics* **2017**, *119*, 353–360. <https://doi.org/10.1016/j.ejpb.2017.06.029>.
- (3) Makwana, S.; Basu, B.; Makasana, Y.; Dharamsi, A. Prefilled Syringes: An Innovation in Parenteral Packaging. *Int J Pharm Investig* **2011**, *1* (4), 200–206. <https://doi.org/10.4103/2230-973X.93004>.
- (4) Sacha, G.; Rogers, J. A.; Miller, R. L. Pre-Filled Syringes: A Review of the History, Manufacturing and Challenges. *Pharmaceutical Development and Technology* **2015**, *20* (1), 1–11. <https://doi.org/10.3109/10837450.2014.982825>.
- (5) Leveque, D. Subcutaneous Administration of Anticancer Agents. *Anticancer Research* **2014**, *34* (4), 1579–1586.
- (6) Condino, A. A.; Fidanza, S.; Hoffenberg, E. J. A Home Infliximab Infusion Program. *Journal of Pediatric Gastroenterology and Nutrition* **2005**, *40* (1), 67.
- (7) Shire, S. J.; Shahrokh, Z.; Liu, J. Challenges in the Development of High Protein Concentration Formulations. *Journal of Pharmaceutical Sciences* **2004**, *93* (6), 1390–1402. <https://doi.org/10.1002/jps.20079>.

- (8) Narasimhan, C.; Mach, H.; Shameem, M. High-Dose Monoclonal Antibodies via the Subcutaneous Route: Challenges and Technical Solutions, an Industry Perspective. *Therapeutic Delivery* **2012**, *3* (7), 889–900. <https://doi.org/10.4155/tde.12.68>.
- (9) Badkar, A.; Wolf, A.; Bohack, L.; Kolhe, P. Development of Biotechnology Products in Pre-Filled Syringes: Technical Considerations and Approaches. *AAPS PharmSciTech* **2011**, *12* (2), 564–572. <https://doi.org/10.1208/s12249-011-9617-y>.
- (10) Shah, M.; Rattray, Z.; Day, K.; Uddin, S.; Curtis, R.; van der Walle, C. F.; Pluen, A. Evaluation of Aggregate and Silicone-Oil Counts in Pre-Filled Siliconized Syringes: An Orthogonal Study Characterising the Entire Subvisible Size Range. *International Journal of Pharmaceutics* **2017**, *519* (1), 58–66. <https://doi.org/10.1016/j.ijpharm.2017.01.015>.
- (11) Abdul-Fattah, A. M.; Oeschger, R.; Roehl, H.; Bauer Dauphin, I.; Worgull, M.; Kallmeyer, G.; Mahler, H.-C. Investigating Factors Leading to Fogging of Glass Vials in Lyophilized Drug Products. *European Journal of Pharmaceutics and Biopharmaceutics* **2013**, *85* (2), 314–326. <https://doi.org/10.1016/j.ejpb.2013.06.007>.
- (12) Basu, P.; Krishnan, S.; Thirumangalathu, R.; Randolph, T. W.; Carpenter, J. F. IgG1 Aggregation and Particle Formation Induced by Silicone-Water Interfaces on Siliconized Borosilicate Glass Beads: A Model for Siliconized Primary Containers. *Journal of Pharmaceutical Sciences* **2013**, *102* (3), 852–865. <https://doi.org/10.1002/jps.23434>.
- (13) Gerhardt, A.; McGraw, N. R.; Schwartz, D. K.; Bee, J. S.; Carpenter, J. F.; Randolph, T. W. Protein Aggregation and Particle Formation in Prefilled Glass Syringes. *Journal of Pharmaceutical Sciences* **2014**, *103* (6), 1601–1612. <https://doi.org/10.1002/jps.23973>.
- (14) Thirumangalathu, R.; Krishnan, S.; Ricci, M. S.; Brems, D. N.; Randolph, T. W.; Carpenter, J. F. Silicone Oil- and Agitation-Induced Aggregation of a Monoclonal Antibody in Aqueous Solution. *Journal of Pharmaceutical Sciences* **2009**, *98* (9), 3167–3181. <https://doi.org/10.1002/jps.21719>.
- (15) Uchino, T.; Miyazaki, Y.; Yamazaki, T.; Kagawa, Y. Immunogenicity of Protein Aggregates of a Monoclonal Antibody Generated by Forced Shaking Stress with Siliconized and Nonsiliconized Syringes in BALB/c Mice. *Journal of Pharmacy and Pharmacology* **2017**, *69* (10), 1341–1351. <https://doi.org/10.1111/jphp.12765>.
- (16) Carpenter, J. F.; Randolph, T. W.; Jiskoot, W.; Crommelin, D. J. A.; Middaugh, C. R.; Winter, G.; Fan, Y.-X.; Kirshner, S.; Verthelyi, D.; Kozlowski, S.; [Clouse, K.A.; Swann, P.G.; Rosenberg, A.; Cherney, B., et al.](#) Overlooking Subvisible Particles in Therapeutic Protein Products: Gaps That May Compromise Product Quality. *Journal of Pharmaceutical Sciences* **2009**, *98* (4), 1201–1205. <https://doi.org/10.1002/jps.21530>.
- (17) Rosenberg, A. S. Effects of Protein Aggregates: An Immunologic Perspective. *AAPS J* **2006**, *8* (3), E501–507. <https://doi.org/10.1208/aapsj080359>.
- (18) Langille, S. E. Particulate Matter in Injectable Drug Products. *PDA Journal of Pharmaceutical Science and Technology* **2013**, *67* (3), 186–200. <https://doi.org/10.5731/pdajpst.2013.00922>.
- (19) United States Pharmacopeial Convention, Inc. <788> Particulate Matter in Injections. In *The United States Pharmacopeia 41 and the National Formulary 36*; United States Pharmacopeial Convention, Inc.: Rockville, MD, 2018.
- (20) United States Pharmacopeial Convention, Inc. <787> Subvisible Particulate Matter in Therapeutic Protein Injections. In *The United States Pharmacopeia 41 and the National Formulary 36*; United States Pharmacopeial Convention, Inc.: Rockville, MD, 2018.
- (21) Li, Z.Y.; Li, R.H.; Smith, C.; Pan, F.; Campana, M.; Webster, J. R. P.; van der Walle, C. F.; Uddin, S.; Bishop, S. M.; Narwal, R.; Warwicker, J.; Lu, J.R. Neutron Reflection Study of Surface Adsorption of Fc, Fab, and the Whole MAb. *ACS Appl. Mater. Interfaces* **2017**, *9* (27), 23202–23211. <https://doi.org/10.1021/acsami.7b06131>.

- (22) Li, Z.; Pan, F.; Li, R.; Pambou, E.; Hu, X.; Ruane, S.; Ciumac, D.; Li, P.; Welbourn, R. J. L.; Webster, J. R. P.; Bishop, S.M.; Narwal, R.; van der Walle, C.F.; Lu, J.R. Coadsorption of a Monoclonal Antibody and Nonionic Surfactant at the SiO₂/Water Interface. *ACS Appl. Mater. Interfaces* **2018**. <https://doi.org/10.1021/acsami.8b16832>.
- (23) Pan, F.; Li, Z.; Leyshon, T.; Rouse, D.; Li, R.; Smith, C.; Campana, M.; Webster, J. R. P.; Bishop, S. M.; Narwal, R.; van der Walle, C.F.; Warwicker, J.; Lu, J.R. Interfacial Adsorption of Monoclonal Antibody COE-3 at the Solid/Water Interface. *ACS Appl. Mater. Interfaces* **2018**, *10* (1), 1306–1316. <https://doi.org/10.1021/acsami.7b13332>.
- (24) Smith, C.; Li, Z.; Holman, R.; Pan, F.; Campbell, R. A.; Campana, M.; Li, P.; Webster, J. R. P.; Bishop, S.; Narwal, R.; Uddin, S.; van der Walle, C.F.; Lu, J.R. Antibody Adsorption on the Surface of Water Studied by Neutron Reflection. *MAbs* **2017**, *9* (3), 466–475. <https://doi.org/10.1080/19420862.2016.1276141>.
- (25) Zarbakhsh, A.; Bowers, J.; Webster, J. R. P. A New Approach for Measuring Neutron Reflection from a Liquid/Liquid Interface. *Meas. Sci. Technol.* **1999**, *10* (8), 738. <https://doi.org/10.1088/0957-0233/10/8/309>.
- (26) Clifton, B. J.; Cosgrove, T.; Richardson, R. M.; Zarbakhsh, A.; Webster, J. R. P. The Structure of Block Copolymers at the Fluid/Fluid Interface. *Physica B: Condensed Matter* **1998**, *248* (1), 289–296. [https://doi.org/10.1016/S0921-4526\(98\)00252-X](https://doi.org/10.1016/S0921-4526(98)00252-X).
- (27) Wiseman, M. E.; Frank, C. W. Antibody Adsorption and Orientation on Hydrophobic Surfaces. *Langmuir* **2012**, *28* (3), 1765–1774. <https://doi.org/10.1021/la203095p>.
- (28) Oom, A.; Poggi, M.; Wikström, J.; Sukumar, M. Surface Interactions of Monoclonal Antibodies Characterized by Quartz Crystal Microbalance with Dissipation: Impact of Hydrophobicity and Protein Self-Interactions. *Journal of Pharmaceutical Sciences* **2012**, *101* (2), 519–529. <https://doi.org/10.1002/jps.22771>.
- (29) Zarbakhsh, A.; Querol, A.; Bowers, J.; Webster, J. R. Structural Studies of Amphiphiles Adsorbed at Liquid–Liquid Interfaces Using Neutron Reflectometry. *Faraday Discussions* **2005**, *129* (0), 155–167. <https://doi.org/10.1039/B404732J>.
- (30) Zarbakhsh, A.; Querol, A.; Bowers, J.; Yaseen, M.; Lu, J. R.; Webster, J. R. P. Neutron Reflection from the Liquid–Liquid Interface: Adsorption of Hexadecylphosphorylcholine to the Hexadecane–Aqueous Solution Interface. *Langmuir* **2005**, *21* (25), 11704–11709. <https://doi.org/10.1021/la0518086>.
- (31) Campana, M.; Hosking, S. L.; Petkov, J. T.; Tucker, I. M.; Webster, J. R. P.; Zarbakhsh, A.; Lu, J. R. Adsorption of Bovine Serum Albumin (BSA) at the Oil/Water Interface: A Neutron Reflection Study. *Langmuir* **2015**, *31* (20), 5614–5622. <https://doi.org/10.1021/acs.langmuir.5b00646>.
- (32) Sivia, D. S. *Elementary Scattering Theory: For X-Ray and Neutron Users.*; Oxford University Press.
- (33) Cowsill, B. J.; Zhao, X.; Waigh, T. A.; Eapen, S.; Davies, R.; Laux, V.; Haertlein, M.; Forsyth, V. T.; Lu, J. R. Interfacial Structure of Immobilized Antibodies and Perdeuterated HSA in Model Pregnancy Tests Measured with Neutron Reflectivity. *Langmuir* **2014**, *30* (20), 5880–5887. <https://doi.org/10.1021/la4036166>.
- (34) Bowers, J.; Zarbakhsh, A.; Webster, J. R. P.; Hutchings, L. R.; Richards, R. W. Neutron Reflectivity Studies at Liquid–Liquid Interfaces: Methodology and Analysis. *Langmuir* **2001**, *17* (1), 140–145. <https://doi.org/10.1021/la001119o>.
- (35) Webster, J.; Holt, S.; Dalglish, R. INTER the Chemical Interfaces Reflectometer on Target Station 2 at ISIS. *Physica B: Condensed Matter* **2006**, *385–386*, 1164–1166. <https://doi.org/10.1016/j.physb.2006.05.400>.
- (36) Fischer, H.; Polikarpov, I.; Craievich, A. F. Average Protein Density Is a Molecular-Weight-Dependent Function. *Protein Sci* **2004**, *13* (10), 2825–2828. <https://doi.org/10.1110/ps.04688204>.
- (37) Hughes, A. V. *RasCAL: Reflectivity Calculations Software*; 2011.

- (38) Lu, J. R.; Thomas, R. K. Neutron Reflection from Wet Interfaces. *Journal of the Chemical Society, Faraday Transactions* **1998**, 94 (8), 995–1018.
- (39) Heavens, O. S. *Optical Properties of Thin Solid Films*; Courier Corporation, 1991.
- (40) Haario, H.; Laine, M.; Mira, A.; Saksman, E. DRAM: Efficient Adaptive MCMC. *Stat Comput* **2006**, 16 (4), 339–354. <https://doi.org/10.1007/s11222-006-9438-0>.
- (41) Carter, D. C.; Ho, J. X. Structure of Serum Albumin. In *Advances in Protein Chemistry*; Anfinsen, C. B., Edsall, J. T., Richards, F. M., Eisenberg, D. S., Eds.; Lipoproteins, Apolipoproteins, and Lipases; Academic Press, 1994; Vol. 45, pp 153–203. [https://doi.org/10.1016/S0065-3233\(08\)60640-3](https://doi.org/10.1016/S0065-3233(08)60640-3).
- (42) Tang, C.-H.; Shen, L. Role of Conformational Flexibility in the Emulsifying Properties of Bovine Serum Albumin. *J. Agric. Food Chem.* **2013**, 61 (12), 3097–3110. <https://doi.org/10.1021/jf305471k>.
- (43) Lu, J. R.; Su, T. J.; Thirtle, P. N.; Thomas, R. K.; Rennie, A. R.; Cubitt, R. The Denaturation of Lysozyme Layers Adsorbed at the Hydrophobic Solid/Liquid Surface Studied by Neutron Reflection. *Journal of Colloid and Interface Science* **1998**, 206 (1), 212–223. <https://doi.org/10.1006/jcis.1998.5680>.
- (44) Fragneto, G.; Su, T. J.; Lu, J. R.; Thomas, R. K.; Rennie, A. R. Adsorption of Proteins from Aqueous Solutions on Hydrophobic Surfaces Studied by Neutron Reflection. *Physical Chemistry Chemical Physics* **2000**, 2 (22), 5214–5221. <https://doi.org/10.1039/b004221h>.
- (45) Kalonia, C. K.; Heinrich, F.; Curtis, J. E.; Raman, S.; Miller, M. A.; Hudson, S. D. Protein Adsorption and Layer Formation at the Stainless Steel-Solution Interface Mediates Shear-Induced Particle Formation for an IgG1 Monoclonal Antibody. *Mol. Pharm.* **2018**, 15 (3), 1319–1331. <https://doi.org/10.1021/acs.molpharmaceut.7b01127>.
- (46) Huh, C.; Mason, S. G. A Rigorous Theory of Ring Tensiometry. *Colloid & Polymer Sci* **1975**, 253 (7), 566–580. <https://doi.org/10.1007/BF01753960>.
- (47) Goebel, A.; Lunkenheimer, K. Interfacial Tension of the Water/ *n* -Alkane Interface. *Langmuir* **1997**, 13 (2), 369–372. <https://doi.org/10.1021/la960800g>.
- (48) Langevin, D. Capillary-Wave Techniques for the Measurement of Surface Tension and Surface Viscoelasticity. *Colloids and Surfaces* **1990**, 43 (2), 121–131. [https://doi.org/10.1016/0166-6622\(90\)80284-B](https://doi.org/10.1016/0166-6622(90)80284-B).

Interfacial Adsorption of a Monoclonal Antibody and its Fab and Fc Fragments at the Oil/Water Interface

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S.1 Fitting Parameters

Table S1 shows the parameters used for the fitting of neutron reflectivity data for each protein in the three aqueous contrasts used. Protein densities and resulting adsorbed masses are given in H₂O.

Component	Contrast buffer	SL ($\times 10^{-4}$ Å)	SLD ($\times 10^{-6}$ Å ⁻²)	V (Å ³)	MW* (g mol ⁻¹)	Density (g cm ⁻³)
COE-03	CM Sapphire	5526	3.16	175139	146,478	
	CM Protein	4440	2.58		145,499	
	H ₂ O	3336	1.91		144,505	1.37
Fc	CM Sapphire	1855	3.05	60852	50,608	
	CM Protein	1502	2.52		50,326	
	H ₂ O	1143	1.88		49,958	1.36
Fab	CM Sapphire	1835	3.21	57173	47,955	
	CM Protein	1468	2.61		47,852	
	H ₂ O	1096	1.92		47,291	1.37

Table S1. The fitting parameters used for each contrast and protein. *non-glycosylated

S.2 “Straight-Through” Method

Fig S1. The direct neutron beam passing through an empty and oil-filled cuvette respectively (a and b). Plot of $\chi(\lambda)$ for oil CM to sapphire ($\text{SLD } 5.65 \times 10^{-6} \text{ \AA}^{-2}$) and CM 3.15 oil ($\text{SLD } 3.15 \times 10^{-6} \text{ \AA}^{-2}$) and best fitted polynomials (c).

The “straight-through” method is used measure to $\chi(\lambda)$, the attenuation per unit length of the oil. The neutron beam is directed straight through a cuvette of known thickness, in this case 1 mm, with no reflection. The intensity of the resulting beam is measured as a function of λ . This intensity function is then normalised using the intensity through an empty cuvette, eliminating all factors except the oil attenuation $A(\lambda)$, which can be used to find $\chi(\lambda)$ via equation 5 in the main text. $\chi(\lambda)$ was then fitted with a 3rd order polynomial for data analysis purposes, the best fit values are shown in Table S2. This method was used to determine $\chi(\lambda)$ for oil CM to sapphire ($\text{SLD } 5.65 \times 10^{-6} \text{ \AA}^{-2}$) and CM 3.15 oil ($3.15 \times 10^{-6} \text{ \AA}^{-2}$).

Oil	SLD ($\text{\AA}^{-2}$)	A (m^{-4})	B (m^{-3})	C (m^{-2})
CM Sapphire	5.65×10^{-6}	-0.16	23.99	82.03
CM 3.15	3.15×10^{-6}	-0.64	54.35	92.97

Table S2. Values for the polynomial fits of $\chi(\lambda)$ in the format $\chi(\lambda) = A\lambda^3 + B\lambda^2 + C\lambda$.

S.3 “Double Critical-Edge” Thickness Measurements

Fig S2. Schematic of the thickness of the layers determined by the “Double Critical-Edge” method. a) shows the reflection from the substrate, b) shows the reflection with oil contrast matched to the sapphire layer, c) shows an exaggerated example of the additional refraction effect caused by a non-contrast matched oil, and d) shows reflectivity data demonstrating this double critical edge effect.

Figure S2 shows how the double critical edge method was used to determine the attenuation of the layer, which, with a known $\chi(\lambda)$, can be used to determine the layer thickness. Figure S2a shows the total internal reflection from the substrate, below the critical angle, this measurement is used to give an absolute scaling value for effect. Figure S2b shows that when an oil film contrast matched to sapphire is added, the total internal reflection occurs from the oil/water interface, with the beam passing through the oil layer. If oil with a lower SLD is added, as shown in Figure S2c, there is a slight refractive effect, decreasing the effective angle and therefore increasing the path length through the oil. However, as the change in neutron refractive index of materials is extremely low relative to, for example, photons, this change in angle is extremely small, and therefore the resulting change in the path length is negligible.

The measurements used to determine the thickness, with an example displayed in Figure S2d, used CM 3.15 (SLD $3.15 \times 10^{-6} \text{ \AA}^{-2}$) oil, with a higher proportion of h-hexadecane than oil CM to sapphire, and therefore greater $\chi(\lambda)$. This gives a larger attenuation, increasing the difference between the un-attenuated and attenuated critical edges (and therefore the signal-to-error ratio), allowing more accurate measurements of the thickness.

S.4 Modelling Globular Penetration

Table S3 demonstrates the best fitted thicknesses modelled for globular protein penetrating into the oil interface. As shown in Figure S3, this model treats the volume fraction of the protein as consistent in both oil and water layers, and varies the overall protein layer thickness and the depth of the oil-hydrated layer. More complex models allowing variation of volume fraction or three-phase oil-water-protein mixing were also used, and found similar results. At lower concentrations, the penetration into the interface appears to increase. This appears counterintuitive, as one might expect the amount of protein mixing to increase with concentration. However, this is due to the increased uncertainty in the penetration, as a smaller amount of total protein in the layer means that the same depth of penetration into the oil would result in lower total oil-protein mixing, and thus a smaller measurable effect on the H₂O and CM protein buffers. The measurements with the highest adsorbed masses, which show little penetration, are therefore the most sensitive.

Concentration (ppm)	COE-3 Penetration (Å)	Fab Penetration (Å)	Fc Penetration (Å)
10	0.5 ± 2	3 ± 4.5	2 ± 3.5
50	0.5 ± 1.5	3.5 ± 4.5	2 ± 3
200	0.5 ± 0.5	0.5 ± 2.5	0.5 ± 1.5

Table S3. Fitted parameters for protein penetration into the oil assuming adsorption from globular Fc, Fab and COE-3.

Fig S3. Schematic of the parameters of the globular penetration model, where $X+Y = 100$.

Fig S4. Fits to measured reflectivity profiles and SLD profiles for COE-3, Fab and Fc using a one-layer model.

S.5 Fitted Reflectivity Data

Protein	Concentration (ppm)	Layer Thickness (Å)	ϕ_{prot}	Γ (mg/ml)	χ^2
COE-3	10	54 $\pm$ 1	0.22 $\pm$ 0.01	1.61 $\pm$ 0.06	9.56
	50	54 $\pm$ 2	0.28 $\pm$ 0.02	2.06 $\pm$ 0.05	9.24
	200	60 $\pm$ 2	0.29 $\pm$ 0.02	2.40 $\pm$ 0.06	11.94
Fab	10	57 $\pm$ 2	0.10 $\pm$ 0.01	0.73 $\pm$ 0.07	6.34
	50	59 $\pm$ 1	0.10 $\pm$ 0.01	0.78 $\pm$ 0.06	5.75
	200	59 $\pm$ 2	0.20 $\pm$ 0.01	1.58 $\pm$ 0.08	8.41
Fc	10	50 $\pm$ 1	0.16 $\pm$ 0.01	1.12 $\pm$ 0.06	6.33
	50	50 $\pm$ 1	0.19 $\pm$ 0.01	1.29 $\pm$ 0.04	6.52
	200	52 $\pm$ 2	0.28 $\pm$ 0.01	2.01 $\pm$ 0.09	9.16

Table S4. Fitted parameters and total adsorbed mass for the one-layer model with their respective errors and the goodness of fit in χ^2 . Note that the proportional error in the adsorbed mass is in some cases lower than the results of simple error propagation. This is as the Bayesian analysis accounts for the fact that layer thickness and volume fraction (and their corresponding errors) are anti-correlated, resulting in a more consistent mass value.

Fig S5. Fits to the reflectivity data, and SLD profiles for COE-3 and Fc using a two-layer model.

Protein	Concentration (ppm)	Inner Layer				Outer Layer				χ^2
		Thickness (Å)		φ_{prot}	Thickness (Å)		Φ_{prot}			
COE-3	10	46	± 3	0.22 ± 0.01	20	± 6	0.12 ± 0.03	8.36		
	50	46	± 2	0.29 ± 0.01	17	± 3	0.12 ± 0.03	8.84		
	200	53	± 2	0.30 ± 0.01	20	± 4	0.12 ± 0.03	11.08		
Fc	10	44	± 2	0.16 ± 0.01	20	± 4	0.08 ± 0.01	5.29		
	50	43	± 2	0.19 ± 0.01	21	± 4	0.09 ± 0.01	5.89		
	200	44	± 3	0.27 ± 0.01	16	± 4	0.18 ± 0.04	7.32		

Table S5. Fitted parameters for the two-layer model with their respective errors and goodness of fit.

S.5 Area per Molecule

Figure S6 shows the calculated Area Per Molecule (APM) for COE-3 and its Fab and Fc fragments plotted against concentration. These values assume a one-layer coverage.

Fig S6. Area per molecule for COE-3, Fab and Fc plotted against concentration.

S.6 Oil/Water Interfacial Tensiometry

Surface tension measurements were taken using a Krüss K11 surface tensiometer, adopting the Du Noüy ring method in a “pull” configuration. The protein solution is prepared in a clean borosilicate vessel. Then a platinum ring connected to a force meter is lowered into the solution. Purified hexadecane is then pipetted carefully onto the surface, creating an oil/water interface. The ring is then pulled into the interface, and the force measured. At the oil/water interface, the surface tension γ is given by the equation:

$$\gamma = \frac{F}{4\pi R} f \quad (S1)$$

where R is the radius of the ring, f is the Huh and Mason correction factor⁴⁶ and F is the maximum pulling force acting on the ring at equilibrium. All measurements were taken at $21 \pm 1^\circ\text{C}$.

Fig S7. Interfacial tension measurements for COE-3 and its fragments at a) 10ppm and b) 200ppm concentration. All measurements taken in pH 5.5 25mM ionic strength Histidine buffer.

Figure S7 shows oil/water interfacial tension measurements for COE-3 and its fragments at 10 ppm (Figure S7a) and 200 ppm (Figure S7b). Points are calculated by the average from 10 moving measurements, with the error calculated as the standard deviation of these 10 points. The first 10 measurements have been excluded. Measurements without protein are in excellent agreement with published values for interfacial tension of the purified hexadecane/water interface (55.2 mN/m)⁴⁷. None of these measurements results in interfacial tensions below 30

mN/m. Low interfacial tension values can result in large-scale roughness due to capillary waves, thermal disruptions of the interfacial boundary, but at these relatively high interfacial tension values, this effect should be relatively insignificant⁴⁸.

For COE-3 and Fc at 200 ppm, the interfacial tension reached equilibrium rather quickly (within ~30 min) and only slightly changed thereafter. This suggests that there were no significant long-term changes in protein structure, which would usually result in a notable decrease in surface tension, due to protein structural rearrangements producing a greater contact with the oil. The measurement for the Fc at 10ppm appears to show a slight long term decrease in interfacial tension but the difference is very small over the two hour period (~ 1.5 mN/m) suggesting relatively small, if any, structural rearrangements. Similarly, for 10 ppm Fab and COE-3 there was little change in surface tension after 30 min. These tensiometry data inform us that, for the NR measurements, allowing the interfaces to equilibrate for > 30 min minimized the risk of significant changes occurring at the interface during the 2 hr NR measurement period.

Consistent with the NR data of the adsorbed amount in nmol/m² (Figure 7), adsorption of Fc lowered the interfacial tension by the greatest amount. While the interfacial tensions for Fab and COE-3 were similar, the adsorbed amount of Fab in nmol/m² was higher than that of the whole mAb.