Interfacial Adsorption of Monoclonal Antibody: a Combined Study of Spectroscopic Ellipsometry and Neutron Reflection

A thesis submitted to

The University of Manchester

For the degree of

Doctor of Philosophy

In the Faculty of Science and Engineering

2017

Zongyi Li

School of Physics and Astronomy
# List of Contents

<table>
<thead>
<tr>
<th>List of Contents</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Contents</td>
<td>2</td>
</tr>
<tr>
<td>List of Tables</td>
<td>6</td>
</tr>
<tr>
<td>List of Figures</td>
<td>8</td>
</tr>
<tr>
<td>Abstract</td>
<td>14</td>
</tr>
<tr>
<td>Declaration</td>
<td>15</td>
</tr>
<tr>
<td>Copyright Statement</td>
<td>16</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>17</td>
</tr>
<tr>
<td>List of Publications</td>
<td>18</td>
</tr>
</tbody>
</table>

## Chapter 1: Introduction

1.1 Scientific Background and Motivation 21  
1.2 Outline of Thesis 22  
1.3 Surfactant 23  
  1.3.1 Definition and Classification 23  
  1.3.2 Surfactants Surface Adsorption 24  
  1.3.3 Micellization 25  
  1.3.4 Tween 80 26  
1.4 Antibody 27  
  1.4.1 Protein Structure 27  
  1.4.2 Antibody Structure 29  
  1.4.3 Monoclonal Antibodies (Mabs) 31  
1.5 References 32

## Chapter 2: Experimental Techniques and Theoretical Background

2.1 Spectroscopic Ellipsometry 35  
  2.1.1 Introduction 35  
  2.1.2 Light Polarization 35
2.1.3 Polarization Light Reflection on a Single Interface 36
2.1.4 Thin Film Interference 38
2.1.5 Spectroscopic Ellipsometry Measurement 42
2.1.6 Data Analysis 44
2.2 Neutron Reflection 47
   2.2.1 Introduction 47
   2.2.2 Neutron Properties and Advantages 47
   2.2.3 One-dimensional Elastic Scattering 49
   2.2.4 Elastic Scattering from an Assembly of Atoms 52
   2.2.5 Neutron Reflection Theory 56
   2.2.6 Measurement and Data Analysis 63
2.3 References 68

Chapter 3: Neutron Reflection Study of Surface Adsorption of Fc, Fab and the Whole mAb 71
   • Abstract 73
1. Introduction 74
2. Materials and Experimental Methods 76
   2.1 Materials 76
   2.2 Neutron Reflection Measurements 77
3. Results and Discussion 79
   3.1 NR Measurements and Data Analysis 79
   3.2 Time and Concentration Dependent Adsorption 82
   3.3 Effect of Solution pH 83
   3.4 Changes in Layer Thickness and Extent of Immersion in Water 85
4. Conclusions 93
   • References 95
   • Supporting Information 98

Chapter 4: Antibody Adsorption on the Surface of Water Studied by Neutron Reflection 105
   • Abstract 107
1. Introduction 108
2. Neutron Reflection and Isotopic Contrast Variation 110
3. Results 113
   3.1 Surface Tension Measurements 113
   3.2 Antibody Adsorption on the Surface of Water 115
   3.3 Co-adsorption of Antibody and Surfactant 121
4. Discussion 126
Chapter 5: Interfacial Adsorption of Monoclonal Antibody COE-3 at the Solid/Water Interface  137

- Abstract  139
1. Introduction  140
2. Materials and Experimental Methods  142
   2.1 Materials  142
   2.2 Adsorption Model  144
   2.3 Model Parameters  147
3. Results and Discussion  148
   3.1 Dynamic Adsorption  148
   3.2 Effect of mAb Concentration  153
   3.3 Effect of Solution pH  154
   3.4 Effect of Solution Ionic Strength  156
   3.5 Structural Conformation of the Adsorbed mAb Molecules  158
   3.6 Comparison with Adsorption at the Air/Water Interface  159
   3.7 Comparison with the Adsorption of other mAb Molecules  160
   3.8 Limitations of the Simulation Approach  161
4. Conclusion  162
   - References  164
   - Supporting Information  167

Chapter 6: Adsorption of Monoclonal Antibody COE-3 and Nonionic Surfactant at the SiO\textsubscript{2}/water Interface  180

- Abstract  182
1. Introduction  183
2. Materials and Experimental Methods  185
   2.1 Materials  185
   2.2 Spectroscopic Ellipsometry (SE)  186
   2.3 Neutron Reflection (NR)  188
3. Results and Discussion  189
   3.1 Adsorption of COE-3 at the Bare SiO\textsubscript{2}/Water Interface  189
   3.2 Adsorption of Non-ionic Surfactant at the SiO\textsubscript{2}/Water Interface  193
3.3 Co-adsorption of COE-3 with Tween80 (20EO) and C\textsubscript{12}E\textsubscript{5} 193
3.4 Co-adsorption of COE-3 with Tween80 (7EO) 195
3.5 NR Data Analysis to Unravel the Co-adsorbed Layer Structure 198

4. Conclusion 203
   • References 205
   • Supporting Information 208

Chapter 7: Summary and Future Work 212
   7.1 Concluding Remarks and Discussion 213
   7.2 Future Work 215
   7.3 References 218

Total word count (Inc. figures and tables): 46377
List of Tables

Chapter 2: Experimental Techniques and Theoretical Background

Table 2.1 The scattering length parameters for hydrogen and deuterium. 51

Chapter 3: Neutron Reflection Study of Surface Adsorption of Fc, Fab and the Whole mAb

Table 1 The scattering length (SL), scattering length density (SLD, ρ), volume (V) and molecular weight (MW) of Fc, Fab and mAb (COE-3) used for the model fitting to neutron reflectivity. 78

Table 2 Structural parameters obtained from the best fits to NR profiles measured under 3 solvent contrasts from adsorption of Fab, Fc and mAb (COE-3) at 50 ppm and pH 5.5. 90

Table S1 The sequences of the light and heavy chains in Fab and Fc. 98

Table S2 Key parameters used for H₂O and D₂O. 99

Table S3 Amino acid sequence comparison between Fc of COE-03 and Fc of 1HZH. 99

Table S4 Amino acid sequence comparison between Fab of COE-3 and Fab of 1HZH. 100

Chapter 4: Antibody Adsorption on the Surface of Water Studied by Neutron Reflection

Table 1 Structural parameters obtained from the best uniform layer fits to the adsorbed COE-3 layers on the surface of NRW water at pH 5.5. 118

Table 2 The scattering lengths (Σb), scattering length densities (ρ), 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. 129

Table S1 Scattering lengths (SL) of the key elements that comprise proteins, water and surfactants. 133

Table S2 Key parameters used for H₂O and D₂O. 133

Table S3 Key parameters for Polysorbate 80 in hydrogenated (h-Surf) and deuterated (d-Surf) forms under different water contrasts used. 134
Table S4  Key structural parameters obtained from the best fits to the reflectivity profiles measured using d-Surf and h-Surf with the concentration of COE-3 fixed at 50 ppm and the ionic strength fixed at 25 mM.

Chapter 5: Interfacial Adsorption of Monoclonal Antibody COE-3 at the Solid/Water Interface

Table 1  Effective radii used for COE-3 for varying buffer parameters: different buffer pH at ionic strength (I) 25 mM, and different buffer ionic strength at pH 6.

Table 2  Structural parameters obtained from best uniform layer fits to the NR profiles shown in Figure 3(b), relating to Equ. 10-13.

Table S1A  The sequences of the light and heavy chains in Fab and Fc.

Table S1B  Key parameters used for D₂O and COE-3.

Table S2A  Amino acid sequence comparison between Fc of COE-03 and Fc of 1HZH.

Table S2B  Amino acid sequence comparing between COE-3 Fab and Fab of 1HZH.

Chapter 6: Adsorption of Monoclonal Antibody COE-3 and Nonionic Surfactant at the SiO₂/water Interface

Table 1  The fitting parameters for the neutron reflection profiles in Fig 1.

Table S1  The scattering length (SL), scattering length density (SLD,ρ), volume (V) and molecular weight (MW) of COE-3 and Tween80 7EO used for the model fitting to neutron reflectivity.

Table S2  Structural parameters obtained from the best fits to NR profiles measured under 4 contrasts from adsorption COE-3 at 0.01mg/ml in His 25mM buffer, pH 5.5.
## List of Figures

<table>
<thead>
<tr>
<th>Chapter 1: Introduction</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Figure 1.1</strong></td>
<td>Schematic of the general classification based on the head group net charge.</td>
</tr>
<tr>
<td><strong>Figure 1.2</strong></td>
<td>(a) A typical interfacial adsorption isotherm measured from surface tension experiments. (b) Surface excess deduced by the Gibbs equation as a function of surfactant concentration.</td>
</tr>
<tr>
<td><strong>Figure 1.3</strong></td>
<td>A simple schematic demonstrates the structure of a typical spherical micelle.</td>
</tr>
<tr>
<td><strong>Figure 1.4</strong></td>
<td>Molecule structure of Tween® 80.</td>
</tr>
<tr>
<td><strong>Figure 1.5</strong></td>
<td>Molecular structures of the 20 standard alpha-amino acids</td>
</tr>
<tr>
<td><strong>Figure 1.6</strong></td>
<td>Diagrams of simplified secondary structures of polypeptides: (a) right-handed α-helix and (b) β-sheet.</td>
</tr>
<tr>
<td><strong>Figure 1.7</strong></td>
<td>Simplified schematic of a typical IgG antibody and its fragments produced by enzymes.</td>
</tr>
<tr>
<td><strong>Figure 1.8</strong></td>
<td>Schematic representation of IgG domains in threedimension.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 2: Experimental Techniques and Theoretical Background</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Figure 2.1</strong></td>
<td>Linear, circular or elliptical polarisation of light.</td>
</tr>
<tr>
<td><strong>Figure 2.2</strong></td>
<td>Different states of polarisation.</td>
</tr>
<tr>
<td><strong>Figure 2.3</strong></td>
<td>Reflection and refraction on a single interface.</td>
</tr>
<tr>
<td><strong>Figure 2.4</strong></td>
<td>A polarised light reflected from a sample surface.</td>
</tr>
<tr>
<td><strong>Figure 2.5</strong></td>
<td>The light is reflected on the top and bottom boundaries of a single thin film.</td>
</tr>
<tr>
<td><strong>Figure 2.6</strong></td>
<td>Multiple reflections of light within a single thin film.</td>
</tr>
<tr>
<td><strong>Figure 2.7</strong></td>
<td>M2000 series spectroscopic ellipsometer, J.A. Woollam Co. Inc.</td>
</tr>
<tr>
<td><strong>Figure 2.8</strong></td>
<td>Schematic diagram of the Ellipsometer shows its main components.</td>
</tr>
<tr>
<td><strong>Figure 2.9</strong></td>
<td>The flowchart describing the common procedure of ellipsometry data analysis.</td>
</tr>
<tr>
<td><strong>Figure 2.10</strong></td>
<td>A particle scattered elastically by a fixed particle P in one-dimension.</td>
</tr>
<tr>
<td><strong>Figure 2.11</strong></td>
<td>A particle scattered elastically by a fixed particle J in an assembly of atoms.</td>
</tr>
</tbody>
</table>
Figure 2.12  Schematic shows the specular reflection geometry.

Figure 2.13  A beam of neutrons incident perpendicularly to a thin slab of material.

Figure 2.14  Neutrons wave propagates through and not through a thin slab of material.

Figure 2.15  Schematic layout of the INTER Reflectometer.

Figure 2.16  Spectra of time-of-flight collected by monitor 1, 2 and reflection detector in the measurement with run number 118751.

Figure 2.17  Spectra of wavelength collected by monitor 1, 2 and reflection detector in the measurement 118751.

Figure 2.18  Reflectivity present in Q space for the measurement 118751 before normalisation.

Figure 2.19  The transmission measurement for measurement 118751.

Figure 2.20  The final reflectivity obtained by data reduction.

Chapter 3: Neutron Reflection Study of Surface Adsorption of Fc, Fab and the Whole mAb

Figure 1  Schematic representation of the optical geometry of neutron beam reflected from the mAb layer adsorbed on the surface of water.

Figure 2  Neutron reflectivity profiles measured from 2 representative concentrations of 12.5 and 50 ppm on the surface of NRW at pH 5.5 (His buffer, ionic strength = 25 mM): (A) Fc; (B) Fab; (C) mAb COE-3 at 20-22 °C.

Figure 3  Adsorbed amount plotted against time measured at concentrations ranging from 2 to 100 ppm with the solution pH fixed at 5.5: (A) Fc; (B) Fab; (C) mAb COE-3.

Figure 4  The equilibrated amount of adsorption in mg/m² (A) and nmol/m² (B) taken from the last point of each set of measurements in Figure 3 plotted against concentration for Fc (▲), Fab (♦) and the whole mAb COE-3 (●).

Figure 5  Adsorbed amount plotted against time measured at pH 5.5, 7, 8 and 8.8 with the concentration fixed at 50 ppm: (A) Fc; (B) Fab; (C) mAb COE-3.

Figure 6  Equilibrated amount of adsorption measured in mg/m² (A) and nmol/m² (B), taken from the last point of each set of measurements from Figure 5 plotted against pH for Fc (▲), Fab (♦) and mAb COE-3 (●).

Figure 7  Changes in layer thickness under the equilibrated adsorption taken from the last point of each set of concentration dependent adsorption measurements as
shown in Figure 2, plotted against the concentration of Fc (blue), Fab (green) and mAb COE-3 (red) at pH 5.5.

**Figure 8** Neutron reflectivity profiles (left) measured at 50 ppm of Fc (A), Fab (B) and mAb (COE-3, C) on the surface of water under NRW, CM2.58 and D₂O at pH 5.5 (His buffer, ionic strength=25 mM) and schematic representations (right) of the surface adsorbed Fc (D), Fab (E) and mAb (F) layers.

**Figure S1** Charge distributions as a function of pH for Fab and Fc and one-third of the whole COE-3 (1/3 contribution from 1 Fc and 2 Fabs).

**Figure S2** Projections of the 3D Fc crystalline structure with estimated width and height marked.

**Figure S3** Projections of the 3D crystalline structure of Fab (1HZH) with estimated width and height marked.

**Figure S4** Projections of the 3D crystalline structure of the whole antibody1HZH with estimated width and height marked.

### Chapter 4: Antibody Adsorption on the Surface of Water Studied by Neutron Reflection

**Figure 1** Schematic representation of (a) the optical geometry of the incoming and exiting neutron beam with respect to the adsorbed antibody layer and (b) the co-adsorption of antibody and surfactant.

**Figure 2** Surface tension profiles measured from (a) h-Surf over a range of surfactant concentration plotted against time (plots from top to bottom represent separately: 0.0001, 0.0003, 0.001, 0.003, 0.01, 0.03, 0.1 and 0.3 mM), (b) both h-Surf and d-Surf taking the readings at 8000 seconds, the longest time measured, (c) the same plots as in (a) but containing 50 ppm of COE-3 and (d) h-Surf with and without 50 ppm of COE-3, taking the readings at 8000 seconds.

**Figure 3** Neutron reflectivity profiles measured on the surface of null reflecting water (NRW) from COE-3 adsorption at 4 representative concentrations of 5, 25, 50 and 100 ppm under the His buffer of pH 5.5 (25 mM ionic strength).

**Figure 4** Changes in the amount (○) and layer thickness (▲) from COE-3 adsorption plotted as a function of COE-3 solution concentration.

**Figure 5** Plots of ln[hpp(κ)] versus κ² converted from reflectivity profiles measured at 5, 25, 50 and 100 ppm at pH 5.5 and an ionic strength of 25 mM (His buffer).

**Figure 6** Reflectivity profiles measured from the adsorption of 50 ppm COE-3 on the surface of NRW, CM2.58 (contrast matched to mAb) and D₂O at pH 5.5 (His buffer, ionic strength of 25 mM).

---

**List of Figures**
Figure 7  Plots of thickness × SLD (τp/10^6 Å²) versus the concentration of surfactant [expressed as the fraction of CMC] for both h-Surf and d-Surf, with the concentration of COE-3 fixed at 50 ppm.

Figure 8  Neutron reflectivity profiles measured from the co-adsorption of COE-3 and surfactant from NRW at pH 5.5 (His buffer, ionic strength 25 mM) with the COE-3 concentration fixed at 50 ppm but with varying concentrations of d-Surf and h-Surf at 1/2 CMC (a, top); at 1/20 CMC (b, middle) and 1/100 CMC (c, bottom).

Figure S1  The molecular structure of a normal Tween 80.

Chapter 5: Interfacial Adsorption of Monoclonal Antibody COE-3 at the Solid/Water Interface

Figure 1  Ellipsometric scans of the amplitude component Ψ (a) and the phase difference Δ (b) against wavelength measured at the SiO₂/water interface for 0.02 mg/ml COE-3 in 25 mM His buffer, pH 5.5; after 1, 5 and 30 min of adsorption.

Figure 2  (a) SE measurements of surface adsorbed amount (Γ) of COE-3 plotted against time at the SiO₂/water interface, in 25 mM His buffer, pH 5.5, 20-21°C, at concentrations as indicated. (b) comparison of the adsorbed amount of COE-3 at 0.02 mg/ml bulk concentration plotted against time and measured by SE and NR. (c) NR profiles measured at the SiO₂/D₂O interface after 10, 30 and 60 min COE-3 adsorption from 0.02 mg/ml bulk concentration, buffer as in (a).

Figure 3  (a) Surface adsorbed amount (Γ) measured by SE after 60 min adsorption and by NR after 3-4 hr adsorption; (b) NR profiles measured at the SiO₂/D₂O interface for COE-3 adsorption from 0.02, 0.05 and 0.2 mg/ml bulk concentration, buffer as in Fig. 2(a).

Figure 4  Surface adsorbed amount (Γ) plotted against time measured by SE at the SiO₂/water interface for COE-3 at (a) 0.02mg/ml and (b) 0.2mg/ml, in 25 mM His buffer, 20-21°C and pH as indicated.

Figure 5  Surface adsorbed amount of COE-03 measured at 60 min plotted against concentration for pH 5.5, 7 and 9 (a), and against pH with concentrations of 0.02 and 0.2 mg/ml (b), in 25 mM His buffer, 20-21°C, measured by SE and NR as indicated.

Figure 6  Surface adsorbed amount plotted against time measured by SE at the SiO₂/water interface to show the effect of His buffer ionic strength (as indicated) for COE-3 concentrations of (a) 0.02 mg/ml and (b) 0.2 mg/ml, pH 6, 20-21°C
Figure 7 Schematic representation of COE-3 adsorbed at the SiO2/water interface as measured in the concentration range of 0.02-0.2 mg/ml at pH 5.

Figure S1 Charge distribution as a function of pH for Fab and Fc and the whole COE-3 (1/3 contribution from 1 Fc and 2 Fabs) as calculated from the heavy chain sequences only.

Figure S2 Ellipsometric scans of the amplitude component Ψ (a) and the phase difference Δ (b) against wavelength measured at the SiO2/water interface after 5 min of adsorption under COE-3 concentrations of 0.005, 0.02 and 0.1 mg/ml in 25 mM His buffer, pH 5.5.

Figure S3 The change in surface tension when 500 ppm COE-3 was added to histidine buffer 25mM at pH 6.

Figure S4 Gibbs distribution plots.

Figure S5 The protein-protein interaction map.

Chapter 6: Adsorption of Monoclonal Antibody COE-3 and Nonionic Surfactant at the SiO2/water Interface

Figure 1 Ellipsometric scans of the amplitude component Ψ (a) and the phase difference Δ (b) against wavelength measured at the SiO2/water interface for 0.01 mg/ml COE-3 in 25 mM His buffer, pH 5.5; after 1, 5 and 30 min of adsorption.

Figure 2 Surface saturated adsorbed amount measured by SE and by NR plotted against concentration.

Figure 3 NR profiles measured at the SiO2/D2O interface for COE-3 adsorption at 0, 0.01, 0.02, 0.05 and 0.2 mg/ml bulk concentration in 25mM His buffer, pH 5.5.

Figure 4 Interface adsorbed amount (Γ) plotted against time measured by SE at the SiO2/water interface for COE-3 adsorption with and without surfactants in 25 mM His buffer: (a) 0.01mg/ml COE-3 and 0.02% (w/w) Tween80 20EO at pH 5.5; (b) 0.1mg/ml COE-3 and 0.02% (w/w) Tween80 20EO at pH 5.5; (c) 0.01mg/ml COE-3 and 10CMC C12E5 at pH 7.

Figure 5 Surface adsorbed amount (Γ) plotted against time measured by SE (Left) at the SiO2/water interface for COE-3 (a) 0.01mg/ml and (b) 0.1mg/ml with and without 0.02%(w/w) Tween80 7EO in 25 mM His buffer, pH 5.5. NR profiles (right) measured at the SiO2 surface for COE-3 adsorption at 0.01mg/ml mixed with 0.02%(w/w) protonated and deuterated Tween80 7EO in (c) D2O and (d) contrast matching COE-3 water, all under pH 5.5 (25mM His buffer).

Figure 6 (a) NR profiles measured at the SiO2 surface for COE-3 adsorption at 0.01mg/ml mixed with 0.02%(w/w) protonated and deuterated Tween80 7EO in D2O and
contrast matching COE-3 water under pH 5.5 (25mM His buffer); the continuous lines denote the best fits of which the scattering length density plotted against the distance in (b).

**Figure 7** Schematic showed the adsorption of (a) COE-3 alone and (b) the structure of the compound layer of COE-3 and Tween80 7EO.

**Figure 8** The bar chart showed the adsorbed amounts for each component (COE-3, Tween80 7EO head group and tail group) in each layer calculated from the fitting of NR measurements in Figure 8.

**Figure S1** The molecular structure of a normal Tween 80.

**Figure S2** SE measurements of the surface adsorbed amount of COE-3 plotted against time at the SiO2/water interface, in 25 mM His buffer, pH 5.5, at concentrations as indicated.

**Figure S3** NR profiles measured at SiO2/D2O interface with and without 0.02% (w/w) protonated Tween80 7EO as bulk concentration.

**Figure S4** NR profiles measured at SiO2/D2O interface for the compound layer of COE-3 and Tween80 7EO formed by the injection of the mixture (blue circles) or injection in sequence (red triangles). The other NR profiles measured for the formed compound layer after a rinse of buffer (black squares) and the second injection of 0.01mg/ml COE-3 (magenta diamonds).

**Figure S5** Surface adsorbed amount (Γ) plotted against time measured by SE at the SiO2/water interface for COE-3 at 0.01mg/ml with 0.02%(w/w) Tween80 7EO in 25 mM His buffer, pH 5.5. The bar chart showed the adsorbed amounts for each component (COE-3, Tween80 7EO head group and tail group) calculated from the fitting of NR measurements in Figure 8.
Abstract

Zongyi Li
The University of Manchester
Doctor of Philosophy
2017

Interfacial Adsorption of Monoclonal Antibody: a Combined Study of Spectroscopic Ellipsometry and Neutron Reflection

Therapeutic monoclonal antibodies (mAbs) are a rapidly growing class of biopharmaceutical products that are used in treating serious diseases such as cancers, autoimmune diseases and Alzheimer's disease. The interfacial adsorption of mAb is a crucial issue in product manufacturing, shipping and storage, as the process can trigger antibody aggregation and loss of bioactivity. A non-ionic surfactant, Tween 80 (Polysorbate 80), is commonly used to prevent interfacial adsorption and stabilise products. In work presented in this thesis, the interfacial adsorption behaviour of a mAb, COE-3, was studied at air/water interface and SiO$_2$/water interface, in the presence and absence of Tween 80.

The influence of fragment crystallisation (Fc) and antigen-binding fragment (Fab) on the intact mAb adsorption at the air/water interface were investigated by Neutron Reflection (NR). By taking advantage of isotopic contrast variation, the thickness, adsorbed amount, molecular orientation and immersion of the adsorbed layers could be determined. The results indicated that Fab adsorption was slower and had lower adsorbed amount than Fc, but could dominate the time and concentration dependency of adsorption from the intact mAb. Moreover, change of buffer pH has little effect on its adsorption at the air/water interface. Tween 80 with 20 ethoxylates (Tween 80 20EO) can start to remove antibody from the water surface at the surfactant concentration of 1/100 CMC. The complete replacement of COE-3 was achieved when the surfactant concentration reached 1/10 CMC. The adsorbed Fc, Fab and the intact COE-3 all retained their globular structures on the water surface, in the presence and absence of Tween 80 20EO.

The adsorption dynamics of COE-3 at SiO$_2$/water interface was studied using Spectroscopic Ellipsometry (SE) under various buffer conditions (pH and ionic strength). A simulation based on the DLVO theory helped interpret the data, indicating that electrostatic interaction played an essential role in mAb adsorption on the SiO$_2$ surface. The equilibrated adsorption layer was characterised by NR, implying the adsorbed COE-3 molecules adopted a ‘flat-on’ position with the short axes of their segments perpendicular to the interface. Tween 80 20EO and Tween 80 7EO could not prevent COE-3 adsorption under the conditions studied. However, Tween 80 7EO can form a well-defined surfactant bilayer on the adsorbed antibody layer via self-assembly. The surfactant bilayer formed regardless of the mode of mixing, i.e., from a mixed mAb-surfactant solution or from the binding of surfactant to pre-adsorbed mAb layer.

This work has demonstrated the power of the combined approach to unravel the detailed interfacial molecular processes and will benefit the future search for more effective surfactants to alleviate mAb adsorption.
Declaration

The author hereby declares that no portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.
Copyright Statement

i. The author of this thesis (including any appendices and/or schedules to this thesis) owns certain copyright or related rights in it (the “Copyright”) and s/he has given The University of Manchester certain rights to use such Copyright, including for administrative purposes.

ii. Copies of this thesis, either in full or in extracts and whether in hard or electronic copy, may be made only in accordance with the Copyright, Designs and Patents Act 1988 (as amended) and regulations issued under it or, where appropriate, in accordance with licensing agreements which the University has from time to time. This page must form part of any such copies made.

iii. The ownership of certain Copyright, patents, designs, trademarks and other intellectual property (the “Intellectual Property”) and any reproductions of copyright works in the thesis, for example graphs and tables (“Reproductions”), which may be described in this thesis, may not be owned by the author and may be owned by third parties. Such Intellectual Property and Reproductions cannot and must not be made available for use without the prior written permission of the owner(s) of the relevant Intellectual Property and/or Reproductions.

iv. Further information on the conditions under which disclosure, publication and commercialisation of this thesis, the Copyright and any Intellectual Property and/or Reproductions described in it may take place is available in the University IP Policy (see http://documents.manchester.ac.uk/DocuInfo.aspx?DocID=24420), in any relevant Thesis restriction declarations deposited in the University Library, The University Library’s regulations (see  http://www.library.manchester.ac.uk/about/regulations/), and in The University’s policy on Presentation of Theses.
Acknowledgements

First and foremost, I would like to express my sincere gratitude to my supervisor, Prof. Jian Lu, for his guidance, advice and help over my PhD. He started my path in research and made me a researcher I am now.

A big thank you to Dr Fang Pan, who shared her invaluable experience with me, not only in research but also in life.

I would like to thank Dr Mario Campana for the knowledge of neutron scattering he passed on to me and his guidance during the days and nights we spent together in ISIS and ILL.

I would also like to thank Dr Marcus Swann and Dr Paul Coffey, who gave me great help on the DPI and constructive suggestions on my project.

I acknowledge the help and support from all the members of the biological physics group: Elias, Zhiming, Ruiheng, Jing, Charlie, Xuzhi, Daniela, Sean, Haoning, Henry, Jamie and Dr Thomas Waigh.

Thanks to all the neutron instrument scientists at ISIS and ILL. Without their assistance and support, the work I have done is impossible.

I wish to thank the studentship support from the University of Manchester via an Overseas Research Scholarship (ORS) award and a physics research merit award.

Thanks for the help and encouragement from all my friends. I am very grateful for the beautiful moments we had together and the happiness they brought to my life.

Finally, I want to thank my parents for the fantastic life given to me. Thanks to them for always supporting my work and decisions, and for believing in my ability.
List of Publications

Papers in Preparation


Published Papers


6. E. Pambou, X Hu, Z. Li, M. Campana, A. Hughes, P. Li, J.R.P. Webster, G. Bell, J.R. Lu, Structural features of reconstituted wheat wax films upon interaction with non-ionic surfactant C12E6 Langmuir, Published online, DOI:10.1021/acs.langmuir.8b00143 (2018).


Chapter 1

Introduction

1.1 Scientific Background and Motivation 21
1.2 Outline of Thesis 22
1.3 Surfactant 23
1.4 Antibody 27
1.5 References 32
1.1 Scientific Background and Motivation

Currently, therapeutic monoclonal antibody (mAb) research is a fast developing area in the pharmaceutical industry. MAbs are pure antibodies generated by identical clonal immune cells that have the same specific binding ability to a unique antigen.\(^1\) Due to their predefined specificity, great versatility and high efficacy, they are used to treat a broad range of serious diseases such as cancers, autoimmune diseases and Alzheimer's diseases.\(^2\) Their multiple modes of action include incapacitation of the target, host complimented cytotoxicity, and directed drug delivery as antibody-drug conjugates.\(^3\)

However, a major issue that must be considered before these treatments become widely adopted is their stabilization during product manufacturing, shipping and storage. A key factor related to product stabilization is the interfacial adsorption of mAbs, which can trigger antibody aggregation and loss of bioactivity, and even possible occurrence of unexpected immunogenic reactions.\(^4\) In addition, interfacial adsorption of mAbs on the inner wall of tools or containers such as syringe, needle and infusion tube may lower the actual drug concentration below the desired dose.\(^4,5\)

Nonionic surfactant Tween 80 was approved by the US Food and Drug Administration (FDA). It is commonly used in the pharmaceutical industry as an excipient to stabilize active components and prevent their interfacial adsorption. It is reported that more than 70% of the marketed formulations of monoclonal antibody products contain Tween 80 (Polysorbate 80) or Tween 20 (Polysorbate 20).\(^6\)

In this project, the interfacial adsorption of a monoclonal antibody, COE-3, is investigated at air/water and SiO\(_2\)/water interfaces by Spectroscopic Ellipsometry and Neutron Reflection. One aim is to obtain an understanding of how buffer conditions and non-ionic surfactant affect the interfacial adsorption of mAbs. The influence of fragment crystallisation (Fc) and antigen-binding fragment (Fab) on the adsorption of intact mAb were also studied at the air/liquid interface. The mechanisms behind these phenomena were interpreted.

Furthermore, this work has demonstrated a way to study protein interfacial adsorption and the protein-surfactant co-adsorption using a combined approach of Spectroscopic Ellipsometry and Neutron Reflection.
1.2 Outline of Thesis

This thesis is submitted in the journal format, of which chapters 3-6 were written in the format of publication in a peer-reviewed journal. It also contains an introduction, methods and a final summary presented in the form of Chapter 1, Chapter 2 and Chapter 7. Chapters 3-6 are self-contained papers; some texts about scientific background and methods may be repeated. Some variables have different names and symbols in different papers, but they are obvious.

Chapter 1 provides the framework and scientific background throughout the thesis. This part also offers the general concepts of antibody and surfactant, the main materials used in this project.

Chapter 2 describes the basic theory and operation of Spectroscopy Ellipsometry (SE) and Neutron Reflection (NR). It contains the explanation of necessary formulas and data processing methods. SE and NR are the main techniques used in this project, probing the information of thin films adsorbed on the interface.

Chapter 3 discusses the interfacial adsorption of a monoclonal antibody, COE-3, at the air/liquid interface. The discussion includes the adsorption behaviour of the intact antibody molecule and its fragments (Fc and Fab). The work in this chapter was published in the journal of ACS Applied Materials & Interfaces in June 2017.7

Chapter 4 presents the results obtained from the co-adsorption of COE-3 and a non-ionic surfactant Tween 80. The work was published in the journal of mAbs in April 2017.8

Chapter 5 discusses the interfacial adsorption of intact COE-3 molecules at the SiO₂/liquid interface under different buffer conditions. A simulation based on the DLVO theory was applied to help interpret the adsorption phenomenon. The work in this chapter was submitted to the journal of ACS Applied Materials & Interfaces in August 2017.

Chapter 6 is a study of the influence of Tween 80 on COE-3 adsorption at the SiO₂/liquid interface. Two Tween 80 samples with different numbers of ethylene oxide groups were used in this study. The work in this chapter has been written and ready to submit to Langmuir.
Chapter 7 will summarise the thesis and offer ideas for future work.

The candidate designed and performed experiments, analysed data for the papers in Chapter 3 and Chapter 6 as the first author. Co-authors have contributed by assisting with experiments and providing advice. For the article presented in Chapter 4, the candidate undertook neutron experiments with Mr Charles Smith, and made contributions to data analysis, data interpretation and presentation as the second author. For the publication of Chapter 5, the candidate made equal contributions with Dr Fang Pan as joint first authors. Mr Thomas Leyshon and Mr Dominic Rouse made the contribution to the simulation work. The candidate undertook the neutron reflection experiments and a part of ellipsometry measurements and analysed and presented the results.

1.3 Surfactant

1.3.1 Definition and Classification

Surfactant means surface active agent, which refers to a type of compound that can lower the surface tension or interfacial tension. A surfactant molecule contains two distinct regions, a hydrophilic region and a hydrophobic region, so that surfactants are amphiphilic. The hydrophobic region, also called the tail group, is typically a hydrocarbon group. The hydrocarbon group is oil-soluble but not water-soluble. The hydrocarbon group can be branched, linear or aromatic, and its chain length, size and structure influence the whole surfactant hydrophobicity. The hydrophilic region is conveniently called head group, consisting of a polar group making the surfactant water-soluble. The polar groups are multifarious and can either just consist of several atoms or have a very complex structure. Hence, the head groups extensively influence the surfactant interfacial behaviour and define the surfactant classification as shown in Figure 1.1.
A general classification based on the net charge of surfactant head group includes ionic, non-ionic and zwitterionic surfactants. The head group of an ionic surfactant carries a net charge, which is either positive in a cationic surfactant or negative in an anionic surfactant. An non-ionic surfactant has a non-charged head group. The head group of a zwitterionic surfactant contains both positively charged and negatively charged centres within the same molecule, resulting in an overall net charge neutral. As is well-known, a weak electrolyte group such as amine carries a pH-dependent net charge. Therefore, the net charges of the surfactants containing these weak electrolyte groups can vary with the pH of the ambient solution.

\section*{1.3.2 Surfactants Surface Adsorption}

Amphiphilicity is the most important property of surfactants. It gives rise to complex interfacial and solution behaviours and endows surfactants with potential applications in industry and commerce\textsuperscript{10}.

Whether in a polar or non-polar solvent, there is always one part (head or tail) of the surfactant molecule disfavoured in bulk. Therefore, the molecules energetically orientate and organise themselves to minimise the system free energy until reaching a thermal equilibrium.

At an interface between a polar solvent and a non-polar solvent such as water and air, the surfactant molecule spontaneously orientates itself so that the head group stays in water while the tail group extends into the air. Therefore, the interface becomes the energetically favoured place for surfactants, which allows both the head group and tail group to stay in their preferred surroundings. Also, the surface tension and the system free energy are dramatically reduced in this process. As a result, more and more
surfactant molecules are adsorbed and aggregate on the interface, forming an oriented monolayer. However, there is a threshold of adsorbed surfactant amount, when the surface is saturated, and the adsorption reaches a dynamic equilibrium which means that the number of monomers coming to the surface equals to the monomers leaving the surface. The saturated adsorption amount of surfactant at this equilibrium is quantified by the surface excess, $\Gamma$, which is defined as the difference between the molecular amount present in the interfacial region and that present in the bulk phase. The relationship linking the surface excess, surface tension and surfactant concentration is described by the Gibbs Adsorption Isotherm.

![Interfacial Tension vs Concentration](image1)

**Figure 1.2** (a) A typical interfacial adsorption isotherm measured from surface tension experiments. (b) Surface excess deduced by the Gibbs equation as a function of surfactant concentration. The diagrams were generated after the Eastoe’s book.

As shown in Figure 1.2, the surface tension decreases gradually with the increasing surface excess within region A for low concentrations; at region B, surface excess approaches an upper limit while the surface curve has a rapid and steady decay; after the system reaches point C, both surface excess and surface tension become stable: the surfactant surface adsorption becomes saturated with concentration. The concentration at point C is called the critical micelle concentration (CMC).
1.3.3 Micellization

Apart from interfacial adsorption, micellization is another strategy for reducing the system free energy. Micellization starts to occur at the CMC, at which point surface and interfacial adsorption are saturated with concentration as discussed before. In other words, the surfactant can no longer reduce the system free energy by surface adsorption beyond the CMC so that it has to find another strategy. Instead of pointing the tail group to air, the surfactant monomers aggregate to form micelles, sequestering the hydrophobic tail groups in the micelle centre surrounded by the head groups so that the contact between water and tail groups is minimized. Figure 1.3 shows the structure of a typical spherical micelle.

![Figure 1.3](image)

**Figure 1.3** A simple schematic demonstrates the structure of a typical spherical micelle.

When the surfactant concentration is about the CMC, the additional surfactant monomers form micelles, keeping the concentration of the free monomers in bulk stable. Along with the monolayer adsorbed on the surface, the system reaches an equilibrium of these three phases (micelles, surface monolayer and free monomers).

The value of the CMC is determined by the structure and chemical properties of the surfactant molecule such as the length and size of the hydrocarbon chain and the size and charge of the head groups. Moreover, many ambient factors can influence the CMC of a surfactant, such as a temperature, pH, addition of salts and type of counterions.\(^\text{12}\)

The geometry of micelles can be spherical, ellipsoidal, cylindrical, vesicles, bilayers and even more complex. The different sizes and shapes of micelles and their forming mechanism are governed by the intrinsic structure of the surfactant molecule and environmental factors such as sample concentration, temperature, pH and solution ionic strength. As one of the key structure factor, the molecular geometry was studied by abundant experimental and theoretical efforts and reviewed by Mitchell and Ninham\(^\text{13}\), Oliver\(^\text{14}\), and Israelachvili\(^\text{15}\).
1.3.4 Tween 80

Tween® 80 is the trade name of polyoxyethylene sorbitan monooleate, which is also called Polysorbate 80. It is one of the polysorbate-type nonionic surfactants formed by the ethoxylation of sorbitan. Its structure is shown in Figure 1.4. A sorbitan ring is attached by ethylene oxide polymers at its 3 hydroxyl groups. The numbers of repeat ethylene oxide group (EO) are uncertain at each position, the total number within the whole molecule is about 20 for the standard Polysorbate 80. Moreover, the degree of ethoxylation groups can be varied. For example, Tween 80 (7EO) used in this project contains seven ethylene oxide groups in its head group. Therefore, Tween 80 (20EO) and Tween 80 (7EO) have the same tail group but different size head group of polyoxyethylene to have different interfacial properties.

Figure 1.4  Molecule structure of Tween® 80.

Tween 80 is widely used in the food, cosmetics and pharmacy. In pharmacy, it is often added to the formulation of biotherapeutic products as an excipient, to stabilize active components and prevent their surface adsorption. It was reported that over 70% of the marketed formulations of monoclonal antibody products contain Tween 80 or Tween 20 (Polyoxyethylene sorbitan monolaurate).

1.4 Antibody

The antibody is a type of global protein, which can bind antigens and help mediate immune systems via several mechanisms. To understand the main features of typical antibodies, we discuss proteins first.
1.4.1 Protein Structure

As one of the basic building blocks of life, proteins perform a diverse range of functions such as catalysing biochemical reactions, cell signalling and immune responses. Despite their multifarious roles in an animal body, all the natural proteins are made up of only twenty standard alpha-amino acids as shown in Figure 1.5. Each alpha-amino acid contains an amine group, a carboxyl group and a specific side chain which are all linked to the first (alpha) carbon. The amino acids link with each other to form the proteins by a covalent bond called peptide linkage.

![Figure 1.5 Molecular structures of the 20 standard alpha-amino acids.](image)

The enormous variety of protein structures results from the sequences built from amino acids. The structures of proteins are divided into four levels: primary, secondary, tertiary and, sometimes, quaternary.

The primary structure is defined as the linear sequence of amino acids in the protein, which is governed by the nucleotide sequence of genes. Secondary structure refers to that of the polypeptide, which constitutes the amino acids bends and twists to give

---

Chapter 1  
- 28 -
the more compact structure. The folding results from the hydrogen bonds formed between the C=O and N-H groups of nearby amino acids. The two most common secondary structures are α-helix and β-sheet, shown in Figure 1.6.

![Diagram of simplified secondary structures of polypeptides](image)

**Figure 1.6** Diagrams of simplified secondary structures of polypeptides: (a) right-handed α-helix and (b) β-sheet. The pictures are taken from Waigh’s book.21

In natural proteins, the backbone chain of the α-helix structure is always right-handed, and the helical pitch shown in the figure is 3.6 amino acid groups. Since $360^\circ / 3.6$ equals $100^\circ$, there is a $100^\circ$ displacement between each amino acid and its predecessor along the chain. The hydrogen bond formed between two adjacent strands also stabilise another secondary structure called β-sheet. The two strands are different segments of polypeptide and can be arranged parallel or antiparallel adjacent with each other. The polypeptide can further fold or refold into more complex three-dimensional conformations called the protein tertiary stucture.22 The intramolecular interactions in the tertiary structure refer to covalent bonds like disulphide linkages and a range of non-covalent interactions such as van der Waals force, electrostatic force, hydrogen bonding and hydrophobic effect. The quaternary often refers to a superstucture describing the assemble of serveral polypeptide chains.

### 1.4.2 Antibody Structure

The antibody, as a “Y” shaped immunoglobulin (IgG), is composed of two identical light(L) polypeptide chains and two identical heavy(H) polypeptide chains. The chains
are linked together by disulphide linkages and non-covalent interactions. Since antibodies can react specifically with a vast array of antigens, they have an enormous versatility in amino acid sequence and property. However, it is known that each light or heavy chain contains a region that is constant in different types of antibodies, called the constant (c) region. The rest of chain is called variable (v) region, showing high variability. Figure 1.7 shows a simplified schematic of the structure of a typical IgG antibody, in which the blue part is the variable region while the rest is the constant region.

Each arm of the “Y” shape is called a Fab fragment, which contains the variable region so that it can bind to the antigen. Its name of Fab hence means the fragment of antigen binding. On the other hand, the remaining portion called the Fc fragment cannot usually bind to antigen but only to cell receptors, Fc means “fragment crystallizable” because this fragment tends to be crystallised at low temperature. The enzyme papain can hydrolyse peptide bonds in IgG and produce two Fab and one Fc fragments while the enzyme pepsin cleaves at different sites to form one F(ab’)2 fragment.

Figure 1.7  Simplified schematic of a typical IgG antibody and its fragments produced by enzymes. The picture is adapted from Elgert’s book.23

Further fine structure of the antibody can be discussed using the concept of the immunoglobulin domain. The immunoglobulin domain is a kind of protein domain which consists of two beta sheets forming a "sandwich" shape. The two beta sheets are held together by a disulphide bridge between conserved cysteines near the domain centre.24 Figure 1.8 shows the antibody on the domain level, indicating each Fab consists of 4 domains (VH, VL, CH1 and CL) while Fc contains two CH2 and two CH3 domains. Each heavy chain has three constant domains labelled CH1, CH2 and CH3.
Even in the variable region, the variability of amino acid sequence is only localised in several particular sections called hypervariable regions or complementarity-determining regions (CDRs). The rest portion is framework residues, controlling the position of CDRs to ensure the CDRs are exposed on the surface of the antibody. Therefore, CDRs are exposed to the environment and can bind to the antigen specifically because of its specific shape and ionic properties.

**Figure 1.8** Schematic representation of IgG domains in three-dimension. The picture is adapted from Elgert’s book.²³,²⁵

### 1.4.3 Monoclonal Antibodies (Mabs)

Monoclonal antibodies (Mabs) are pure antibodies which have single antigenic determinant specificities.²³ Considering there are $10^{16}$ antibody molecules with an enormous diversity in one millilitre of standard serum²³, it is impossible to collect or purify Mabs from the natural living sample for research and other applications. Therefore, producing Mabs from cultured identical B cells is the current feasible method. Because normal antibody-secreting B cells are end cells of a differentiation series, they generally cannot be immortalised in cell culture. Thus the techniques of hybridoma have been invented and developed to solve this issue. By cell hybridization, a clone cell, hybridoma, can be generated from one antibody-secreting cell and a myeloma cell. The hybridoma cell inherits the advantages from both original cells, and this is the power of specific antibody production and immortalisation in cell culture.
1.5 Reference

Chapter 2

Experimental Techniques and Theoretical Background

2.1 Spectroscopic Ellipsometry 35

2.2 Neutron Reflection 47

2.3 References 68
2.1 Spectroscopic Ellipsometry

2.1.1 Introduction

Spectroscopic Ellipsometry is a powerful optical technique. It can characterise material surfaces and thin films by measuring the changes in the polarisation state of the light reflected from the sample surface.\(^1\),\(^2\) It has been widely used in material science and surface science for semiconductors\(^3\)–\(^5\), optical coatings\(^6\),\(^7\) and polymers\(^8\),\(^9\), from basic research to industrial applications. It has a high thickness sensitivity within angstrom and has a capacity of quantifying monolayer surface coverage.\(^1\) Additionally, ellipsometry measurement is nondestructive and contactless. Hence, many studies in surface biology have been tackled with Spectroscopic Ellipsometry, especially for the topics studying the interfacial adsorption behaviour of biological molecules such as DNA\(^10\),\(^11\) antibodies\(^12\),\(^13\), toxin molecules\(^14\) and surfactants\(^15\),\(^16\). Furthermore, its remarkable data accumulation speed enables this technique to investigate the kinetics of adsorption processes.

2.1.2 Light Polarization

Light is a transverse electromagnetic wave, of which both the electric field and magnetic field vibrate in directions perpendicular to the wave propagation direction\(^17\). The polarisation of an electromagnetic wave is defined as its E-field vector direction. If the E-field vector direction is random or the trace of the E-field vector has no periodicity, this light is unpolarized. For a linearly polarised light, its E-field vector always oscillates within a plane. The trace of the E-field vector of an elliptically or circular polarised light is an ellipse or circle.

![Figure 2.1 Linear, circular or elliptical polarisation of light. Produced from J.A.Woollam website\(^29\).](image)

The polarisation state can be better understood when we only consider the pure polarisation states which are shown in Figure 2.1. In each of them, the E-field vector...
of the light can be decomposed into two orthogonal components which are along x and y-axis separately. Moreover, the two components, wave 1 and wave 2, are coherent sinusoidal waves with the same frequency. The x-y plane is perpendicular to the light propagation direction.

Figure 2.2 shows the linear polarisation that only happens when the two coherent sinusoidal waves are in phase or antiphase; otherwise, the phase difference between the two sinusoidal waves results in elliptical polarisation. A special case of elliptical polarisation is called circular polarisation, in which wave 1 and wave 2 are out of phase by 90° but have the same amplitudes.

![Figure 2.2 Different states of polarisation](image)

### 2.1.3 Polarization Light Reflection on a Single Interface

When light is passed through the interface between two different optical media, it is split into a reflected part and a refracted part as shown in Figure 2.3. The law of reflection states that light is reflected at an angle equal to the angle of incidence. Snell's law indicates the relationship between the refraction angle and the refractive index as Equation 2.1.

![Figure 2.3 Reflection and refraction on a single interface.](image)
\[ n_i \sin \theta_i = n_t \sin \theta_t \]  

(2.1),

where \( \theta_i \) and \( \theta_t \) are the incident angle and refraction angle; the refractive indexes of the media the light travelled from and transmitted in are \( n_i \) and \( n_t \). Snell’s law can be derived either by Fermat’s principle using a geometric method or by Maxwell equations with boundary conditions.\(^\text{20}\)

To find a visual interpretation of the reflection and refraction phenomena, we can consider that light wiggles the charges on the interface when it has struck the media boundary\(^\text{21}\) since light is an electromagnetic wave. The moving charges emit radiation, and all the radiation are in phase only in the direction at \( \theta_r = \theta_i \). Thus, the constructively superimposed radiation is the reflected ray. In any other direction, the radiations are overall destructive so that the beam is non-existent. Similarly, the refracted beam is the constructive interference between the incident radiation and the radiations from the wiggling charges on the surface. The vibration direction of the charges depends on the E-field vector direction which is the polarisation direction of the incoming light. Hence, the reflection and refraction of light are polarisation related.

As we discussed in Section 2.1.2, a linear polarised E-field vector is decomposed into two orthogonal components. Here, we define the x and y-axes as p- and s- plane as shown in Figure 2.4, which are parallel and perpendicular to the plane of incidence respectively. For each component, the boundary conditions are different and result in different solutions to Maxwell’s equations. For a single interface, the reflection and transmission coefficients are described by Fresnel equations\(^\text{22}\):

\[
    t_p^{\text{Fresnel}} = \frac{E_{tp}}{E_{ip}} = \frac{n_t \cos \theta_i - n_i \cos \theta_t}{n_t \cos \theta_i + n_i \cos \theta_t} 
\]

(2.2),

\[
    t_p^{\text{Fresnel}} = \frac{E_{tp}}{E_{ip}} = \frac{2n_i \cos \theta_i}{n_t \cos \theta_i + n_i \cos \theta_t} 
\]

(2.3),

Figure 2.4 A polarised light reflected from a sample surface.
\[ r_{s}^{\text{Fresnel}} = \frac{E_{rs}}{E_{ls}} = \frac{n_{i} \cos \theta_{i} - n_{t} \cos \theta_{t}}{n_{i} \cos \theta_{i} + n_{t} \cos \theta_{t}} \]  
(2.4),

\[ t_{s}^{\text{Fresnel}} = \frac{E_{ts}}{E_{ls}} = \frac{2n_{i} \cos \theta_{i}}{n_{i} \cos \theta_{i} + n_{t} \cos \theta_{t}} \]  
(2.5),

where E is the wave amplitude, and the i, r and t subscripts indicate incident, reflected and transmitted; p and s in the subscripts stand for the p and s polarisation; \( \theta_{i} \) and \( \theta_{t} \) are the incident angle and refraction angle; the refractive indexes of the media the light travelled from and transmitted in are \( n_{i} \) and \( n_{t} \).

The reflectivity \( R \) of a single interface is then expressed as the square of the Fresnel reflection coefficient. The transmissivity can then be deduced according to the conservation of energy.\(^{23}\)

\[ R_{s} = |r_{s}^{\text{Fresnel}}|^{2} \]  
(2.6)

\[ T_{s} = 1 - R_{s} \]  
(2.7)

\[ R_{p} = |r_{p}^{\text{Fresnel}}|^{2} \]  
(2.8)

\[ T_{p} = 1 - R_{p} \]  
(2.9)

For unpolarized light that contains an equal amount of s and p polarisation, the reflectivity is:

\[ R_{\text{unpolarized}} = \frac{(R_{s} + R_{p})}{2} \]  
(2.10)

### 2.1.4 Thin Film Interference

Ellipsometry is a technique for thin film study. Figure 2.5 shows how the light is reflected at angle \( \theta_{i} \) by a single thin film on a substrate. The refractive indexes of the media the thin film and the substrate are \( n_{0}, n_{1} \) and \( n_{2} \), respectively, and d stands for the thickness of the film. As shown, the light is reflected twice by the top and bottom boundaries of the thin film. The interference of these two reflected waves results in the total reflected light.
The superposition of the two light waves can be expressed with the difference in their phase. The optical path difference (OPD) between these two reflected waves is

$$\text{OPD} = n_1 (AB + BC) - n_0 AD = 2n_1 \left( \frac{d}{\cos \theta_1} \right) - 2n_0 d \tan \theta_1 \sin \theta_i \quad (2.11)$$

According to Snell’s law in Equation 2.1, Equation 2.11 is rewritten into:

$$\text{OPD} = 2n_1 \left( \frac{d}{\cos \theta_1} \right) - 2n_1 d \tan \theta_1 \sin \theta_1 = \frac{2n_1 d}{\cos \theta_1} \left( 1 - \sin^2 \theta_1 \right) = 2n_1 d \cos \theta_1 \quad (2.12)$$

Then the half optical phase difference ($\beta$) can be deduced as:

$$\beta = \frac{2\pi \text{OPD}}{\lambda} = \frac{2\pi n_1 d \cos \theta_1}{\lambda} \quad (2.13)$$

It should be noted that a phase shift of $\pi$ needs to be added to the phase change when the light comes from material 1 and is then reflected at the boundary of material 2, and the refractive index of material 1 is less than the refractive index of material 2.23
In reality, the light beam is reflected by the top and bottom boundaries of the thin film several times as shown in Figure 2.6. The total reflected wave \( r_{\text{tot}} \) is the superposition of all the waves emitted out from the film, which are labelled as wave 1, wave 2, etc. The amplitude change upon each reflection and refraction follows the Fresnel reflection and transmission coefficients discussed in Section 2.1.3 and labelled with red letters in Figure 2.6. The Fresnel reflection and transmission coefficients are different for \( s \) and \( p \) polarisations. The phase changes due to the optical path difference are expressed by the factor of \( e^{-i\beta} \), in which \( \beta \) is the half optical phase difference given in Equation 2.13. Here, we assume the refractive indexes \( n_0 < n_1 < n_2 \), and an extra phase change \( \pi \) must be included every time the light strikes on interfaces from bulk to the film or from the film to substrate. The minus signs before the expressions of wave 1 and wave 3 only refer to their wave E-field vector orientations. As shown, the expressions of the waves except for wave 1 are composed of a geometric progression with the factor of \( r_{12}r_{10}e^{-i2\beta} \). Therefore, the total reflected wave \( (r_{\text{single-tot}}) \) for a single film can be summed numerically:

\[
r_{\text{single-tot}} = -r_{01} + \frac{t_{01}t_{10}r_{12}e^{-i2\beta}(1-(r_{12}r_{10}e^{-i\beta})^N)}{1+r_{12}r_{10}e^{-i2\beta}}
\]  \( \text{(2.14)} \)

where the Fresnel reflection coefficients \( r_{01}, r_{12} \) describe the reflection at refractive index changing from \( n_0 \) to \( n_1 \) and \( n_1 \) to \( n_2 \); Fresnel transmission coefficients \( t_{01}, t_{10} \) describe the light propagation from one medium to another changing refractive index from \( n_0 \) to \( n_1 \) and \( n_1 \) to \( n_0 \); \( N \) is the number of terms in this geometric progression.

In this case, \( N \) is positive infinity so that the term of \( (r_{12}r_{10}e^{-i2\beta})^N \) can be treated as 0, since the Fresnel coefficients are always less than 1. From the Fresnel equations 2.2-2.5, we can deduce that \( r_{10} \) equals to \(-r_{01}\) and \( t_{01}t_{10} = 1 - r_{01}^2 \). Thus, Equation 2.14 is rewritten as:

\[
r_{\text{single-tot}} = -r_{01}r_{01}r_{12}r_{10}e^{-i2\beta} + (1-r_{01}^2)r_{12}e^{-i2\beta} = \frac{r_{10}+r_{12}e^{-i2\beta}}{1+r_{12}r_{10}e^{-i2\beta}}
\]  \( \text{(2.15)} \)

The reflectivity is then expressed:

\[
R_{\text{single-tot}} = |r_{\text{single-tot}}|^2 = r_{\text{single-tot}}^* \cdot r_{\text{single-tot}} = \frac{r_{10}^2 + r_{12}^2 + 2r_{10}r_{12}\cos2\beta}{1+r_{10}^2r_{12}^2+2r_{10}r_{12}\cos2\beta}
\]  \( \text{(2.16)} \)

where \( |r|^2=r\cdot r^* \) was applied to a complex number \( r \), and \( r^* \) is the complex conjugate of \( r \).
For a multiple thin film system, the total reflection coefficient can be deduced using the formula of the single thin film in sequence. However, the calculation becomes complicated and tedious with the increasing number of layers. The Abeles matrix approach was developed based on Equations 2.15-2.16 and can be applied to simplify this issue. The derivations and applications of this approach were introduced in detail by Abeles, Parratt, and Byrnes. In brief, a matrix containing the Fresnel coefficients is used to describe the optical property of each thin film. For example, the n-th thin film between the interface n and n+1 is described by Matrix n ($M_n$) in Equation 2.17:

$$M_n = \begin{bmatrix} e^{i\beta_n} & r_{n+1} e^{i\beta_n} \\ r_{n+1} e^{-i\beta_n} & e^{-i\beta_n} \end{bmatrix}$$

(2.17),

where $r_{n+1}$ stands for Fresnel reflection coefficient of the (n+1)-th interface and $\beta$ is the half optical phase difference of the n-th film. If the number of layers in the multiple thin-film system is m, the matrix describing the whole system is:

$$M = [M_0] \cdot [M_1] \cdot [M_2] \cdot [M_3] \cdots [M_m]$$

(2.18),

where $M_0$ is the matrix for the interface between the bulk material the light came from and the first layer. $\beta_0$ equals to 0 in the matrix $M_0$.

Thus the total reflectivity is given by:

$$R_{tot} = \frac{M_{21} M_{21}^*}{M_{11} M_{11}^*}$$

(2.19),

where $M_{11}$, $M_{21}$ is the elements of matrix $M$.

In summary, for a multiple-film system containing a finite number of layers, the total wave reflection coefficient and total light reflectivity can be expressed by a function of the films’ thickness, refractive index and the light wavelength. The substrate and bulk material are assumed to have fixed refractive indices and infinite thickness. Note that the reflectivity is dependent on the thin film interference, which is strongly related to the wave phase change in the material. Because the phase difference depends linear on the layer thickness, the technique of Ellipsometry is sensitive to films down to sub-nano level.
2.1.5 Spectroscopic Ellipsometry Measurement

In this work, a Woollam Spectroscopic Ellipsometer (J.A. Woollam Co. Inc) (Figure 2.7) was used to perform the ellipsometry measurements over a wavelength range from 200 to 600 nm.

Figure 2.7 M2000 series spectroscopic ellipsometer, J.A. Woollam Co. Inc. Photo produced from CompleteEASE™ Data Analysis Manual²⁷.

Figure 2.8 Schematic diagram of the Ellipsometer shows its main components.

As shown in Figure 2.8, a light source produced unpolarised light covering the required wavelength range. After passing the polariser, the light becomes linear polarised. The polariser axis is between the p- and s- planes of the sample surface, and the passing wave can be decomposed into p- and s- components and written as:

\[
E_i = \begin{pmatrix} E_p^i \\ E_s^i \end{pmatrix} = \begin{pmatrix} |E_p^i| e^{i\delta_p^i} \\ |E_s^i| e^{i\delta_s^i} \end{pmatrix} \tag{2.20}
\]

where \(E\) stands for the E-field wave function and \(|E|\) is the E-field vector amplitude; \(\delta\) indicates the wave phase of each wave; p and s mean p- and s- polarisations; i indicates the wave is the original incident wave.
Similarly, the reflected light wave also can be written in this form. \( r \) means reflection instead of incident “i” in Equation 2.20.

\[
E_r = \begin{pmatrix} E_p^r \\ E_s^r \end{pmatrix} = \begin{pmatrix} |E_p^r| e^{i\delta_p^r} \\ |E_s^r| e^{i\delta_s^r} \end{pmatrix}
\]  

(2.21)

In the ellipsometry measurements, two parameters \( \Delta \) and \( \Psi \) are defined to describe the alterations in the light polarisation state due to reflection. The former is related to the wave phase changes in \( p \) and \( s \) polarisation while the latter gives the information of the wave amplitude differences in each polarisation state.

\[
\Delta = (\delta_p^r - \delta_s^r) - (\delta_p^i - \delta_s^i)
\]  

(2.22)

\[
tan \psi = \frac{|E_p^r|/|E_p^i|}{|E_s^r|/|E_s^i|}
\]  

(2.23)

The reflectivity properties of a sample are given by the reflection coefficients \( r_p, r_s \), which link to the sample film thickness and refractive index as discussed before. The reflection coefficient is a complex ratio between the reflected electric field wave \( E_r \) and the incident field wave \( E_i \). As their definition, the reflection coefficients of \( p \) and \( s \) polarisations are expressed as:

\[
r_s = \frac{E_s^r}{E_s^i} = \frac{|E_s^i|}{|E_s^i|} e^{i(\delta_s^r - \delta_s^i)}
\]  

(2.24)

\[
r_p = \frac{E_p^r}{E_p^i} = \frac{|E_p^i|}{|E_p^i|} e^{i(\delta_p^r - \delta_p^i)}
\]  

(2.25)

By combining Equations 2.20-2.25, the core equation of Ellipsometry is obtained:

\[
\rho = tan \psi \cdot e^{i\Delta} = \frac{r_p}{r_s}
\]  

(2.26)

After the reflected light passes through a rotating analyser, the detector converts the light intensity into an electronic signal in real time. The monochromator allows the light waves with different wavelengths to be analysed individually. Using a Fourier analysis method\(^{2,28}\), the intensity waveform obtained from the measurement can be written as:

\[
I(t) = I_0(1 + \alpha \cos 2\phi + \eta \sin 2\phi)
\]  

(2.27)

where \( I \) is the intensity signal function against time \( t \), \( I_0 \) is the average intensity measured over a period, \( \phi \) is called instantaneous azimuth of the analyser transmission.
axis which equals to $2\pi t$, $\omega$ is the rotating frequency of the analyser, and $\alpha$ and $\eta$ are the Fourier coefficients, given as:

$$\alpha = \frac{\tan^2 \psi - \tan^2 P}{\tan^2 \psi + \tan^2 P}$$

(2.28)

$$\eta = \frac{2 \tan \psi \tan P \cos \Delta}{\tan^2 \psi + \tan^2 P}$$

(2.29)

where $P$ is the polarisation azimuth indicating the ellipse longer axis orientation, and $P$ can be measured by the maximum and minimum intensity of light passed through the analyser. Therefore, the ellipsometry parameters $\Delta$ and $\Psi$ are rewritten with $\alpha$ and $\eta$:

$$\tan \psi = \tan \left( P \sqrt{\frac{1+\alpha}{1-\alpha}} \right)$$

(2.30)

$$\cos \Delta = \frac{\eta}{\sqrt{1-\alpha^2}}$$

(2.31)

Hence, the ellipsometry parameters $\Delta$ and $\Psi$ can be deduced from the measured raw data ($I(t)$) by Equations 2.27-2.31.

### 2.1.6 Data Analysis

The ellipsometry parameters $\Delta$ and $\Psi$ are measured in experiments so that $\rho$ is obtained as a function of wavelength by Equation 2.26. $\rho$ is the ratio between the reflection coefficients ($r_p$, $r_s$) which is a function of film’s optical constants and thicknesses as stated in Section 2.1.3-2.1.4. The function $\rho$ is complicated and there are too many variables involved. Thus, the equation is hard to be solved numerically and a regression analysis is required.
As shown in Figure 2.9, after a measurement is completed, the sample structure is described by a model, in which a series of thin layers with parameters such as thickness and optical constant are stacked in sequence. By applying the formulas discussed in Section 2.1.3-2.1.4, the ellipsometry parameters $\Delta$ and $\Psi$ are obtained by calculation. For the unknown parameters of these layers, an estimate is made for the preliminary calculation. The differences between the calculated $\Delta$ and $\Psi$ and the measured results are quantified by Mean Squared Error (MSE).

\[
MSE = \frac{1}{2N-M} \sum_{i=1}^{N} \left( \frac{(\psi_i^{mod} - \psi_i^{exp})^2}{s_{\psi_i}^{exp}} + \left( \frac{\Delta_i^{mod} - \Delta_i^{exp}}{s_{\Delta_i}^{exp}} \right)^2 \right)
\]  

(2.32),

where $N$ is the number of $(\Psi,\Delta)$ pairs measured, or in other words, $N$ equals to the number of wavelengths applied in the measurement; $M$ is the number of variables used in the simulated sample structure model; $S$ is the standard deviation of the i-th pair of $(\Psi,\Delta)$; And the superscripts ‘mod’ and ‘exp’ indicate whether the value is calculated in the simulation or measured in the experiment.

By variation of the unknown parameter values until the minimum MSE is reached, the best fitting result can be obtained in the regression process. If the best fitting result is still far away from the measured data or is not reasonable, the model needs to be improved by adding, deleting layers or changing fitting range.

It is worth noting that the refractive index discussed in this chapter and used in the data analysis process is a complex number:

\[
\tilde{n} = n + ik
\]  

(2.33),
where \( n \) is the index and \( k \) is the extinction coefficient. The extinction coefficient \( k \) is relative to the loss of light energy in the material. Light loses intensity following Beer’s law when it propagates in an absorbing material.

\[
I(x) = I_0 e^{-i\gamma x}
\]  
(2.34),

where \( I \) is the light intensity and \( I_0 \) is the original light intensity, \( x \) is the distance the light propagates in the material and \( \gamma \) is the absorption coefficient given by:

\[
\gamma = \frac{4\pi k}{\lambda}
\]  
(2.35),

where \( \lambda \) is the light wavelength. For all the materials used in this project, the light adsorption is negligible for thin films and was not considered.

The index \( n \) is the ratio between the light speed \( c \) in a vacuum and the light speed \((v)\) in the material.

\[
n = \frac{c}{v}
\]  
(2.36)

The index \( n \) can vary for different wavelengths. The dispersion relationship is often described using the Cauchy equation for a transparent and homogeneous sample.

\[
n(\lambda) = A + \frac{B}{\lambda^2} + \frac{C}{\lambda^4}
\]  
(2.37)

Where \( \lambda \) is the wavelength; \( A, B \) and \( C \) are the Cauchy coefficients which are related on the material properties.

Once a reasonable optical model was achieved, the film mass density (expressed in milligrams per meter squared) can be obtained by De Feijter’s formula\(^{30}\):

\[
\Gamma = \frac{(n-n_0)\cdot d}{(dn/dc)}
\]  
(2.38),

where \( \Gamma \) is the mass density; \( n \) and \( n_0 \) are the refractive indexes of the sample and the ambient environment of the sample. \( dn/dc \) is the refractive index increment of the sample.
2.2 Neutron Reflection

2.2.1 Introduction

Neutron is a subatomic particle, constituting the nuclei of atoms together with protons. It was predicted in the theoretical work of Ernest Rutherford in 1920 and discovered in 1932 via a series of experiments undertaken by James Chadwick. Neutron scattering, the scattering of the free neutron by matter, was exploited as a technique to investigate materials in the 1940s by crystallographers. Since then, neutron scattering has been well-developed as a powerful experimental method and widely used in the fields of condensed matter, physical chemistry, material science and biology. Neutron scattering techniques can be divided into different branches of methods such as single crystal and powder diffraction, small angle neutron scattering, neutron reflection, Quasi-elastic scattering and so on. The classification is based on the different experimental setups, and each of them can provide specific structure information of materials for various research topics.

In this project, the method of neutron reflection has been undertaken as the main technique to study thin film systems on different interfaces. Neutron reflection, in which the free neutrons reflected from the flat interface of interest, can give information on the adsorbed layer such as thickness, adsorbed amount and components. In this section, the basic aspects of this technique are discussed. It should be stated first that the scattering related in this project is all elastic scattering, in which the neutron energy never changes by the scattering.

2.2.2 Neutron Properties and Advantages

Neutron Properties

In neutron scattering experiments, the neutron behaves as an elementary particle. Therefore, it is not necessary to consider its inner structure on quark level. A free neutron has zero of charge, a mass \( m_n \) about \( 1.67495 \times 10^{-27} \) kg, and a lifetime \( t_n \) 886±1 seconds. As a moving object described in classical physics, the momentum \( p \) and energy \( E \) for a free neutron with speed \( v \) is expressed in Equation 2.39 and 2.40.

\[
p = m_n v
\]  

(2.39)
Due to the wave-particle duality, the neutron also can be described as a wave with frequency \( f \) and wavelength \( \lambda \). Thus the energy \( E \) of the particle wave is given in Equation 2.41, and the wavelength \( \lambda \) is expressed through De Broglie relationship in Equation 2.42:

\[
E = \frac{1}{2} m_n v^2 = \frac{p^2}{2m_n} \tag{2.40}
\]

Thus the energy \( E \) of the particle wave is given in Equation 2.41, and the wavelength \( \lambda \) is expressed through De Broglie relationship in Equation 2.42:

\[
E = hf \tag{2.41}
\]

\[
\lambda = \frac{h}{p} = \frac{h}{m_n v} \tag{2.42}
\]

where \( h \) is Planck’s constant.

The neutron has a spin of \( \frac{1}{2} \) and a magnetic moment which is associated with its polarisation properties and magnetic properties. These properties should be considered when the experiment makes use of polarised neutrons, or the experiment is related to magnetic scattering, but this is not in the case in this project.

**Neutron Advantages**

As a powerful probe, neutron scattering has significant advantages over other radiation methods due to its properties. Because the neutron is uncharged, it interacts with the nucleus of the matter rather than the electrons. The nucleus occupies a tiny volume of atoms, making it easy for neutrons to penetrate into the interior structure of the material interested. Moreover, the high penetrating ability makes it possible to use complex sample environments.

In other techniques such as X-ray, electron or optical microscopy, only the surface layer can be studied because electrons and light (electromagnetic waves) would be absorbed or scattered away within a quite short distance by interacting with electrons.\(^{34}\)

In addition, the scattering power of X-ray increases in proportion to the electron density which is strongly related to the atomic numbers of elements. Therefore, X-rays cannot be sensitive to light atoms in the presence of heavy atoms, and it is hard to distinguish neighbouring elements in the periodic table. However, it is not a problem for neutrons because the elements have substantially different neutron scattering cross sections.

Furthermore, isotopes of the same element have substantially different neutron cross sections.\(^{32,33}\) Therefore, isotopic substitution can be used to highlight or mask a
selected component of the investigated material in the scattering while keeping the chemistry unchanged. This method is called contrast control. By simultaneous analysing a set of experimental results measured on the same structure but different isotopic compositions, the ambiguity of the model structure can be reduced.\textsuperscript{35,36}

### 2.2.3 One-dimensional Elastic Scattering

**Scattering Length**

Scattering length and scattering cross section are the most basic concepts in the scattering theory. The concepts are discussed briefly below, in the simplest condition of the one-dimensional elastic scattering of a fixed particle.

As shown in Figure 2.10, a neutron travelling in the direction of x with an initial wave vector $k_i$ is scattered with a fixed particle p and leave with the final wave vector $k_f$. According to the wave-particle duality stated in Section 2.2.2, the incident neutron is described by a plane wave function $\psi_i$ (Equation 2.43). The probability density of measuring this particle is the squared modulus of the wave function $|\psi_i|^2$.

$$\psi_i = e^{ik_i x} \quad (2.43),$$

where $k$ is the wave number which equals to $2\pi/\lambda$, and $\lambda$ is the wavelength of the neutron.

The wave function of the outgoing neutron is a spherical wave as in Equation 2.44.

$$\psi_f = \frac{f(\theta, \lambda)}{r} e^{ik_f x} \quad (2.44),$$

where $k_f$ is the wave number, modulus equaling with $k_i$ in the case of elastic scattering; $r$ is the radial distance from the fixed particle P. The probability density $|\psi_f|^2$ should fall with the distance according to the inverse-square law so that the wave function has
a coefficient of 1/r. The coefficient $f(\theta, \lambda)$ is related to the possible distribution in direction-space and wavelength-space.

The scattering is independent of outgoing direction and particle wavelength. Equation 2.44 can be altered to Equation 2.45:

$$\psi_f = \frac{-b}{r} e^{ikfx}$$  \hspace{1cm} (2.45),

where the constant $b$ is called the scattering length. The minus sign before $b$ is a matter of convention to ensure that the values of $b$ for the most elements are positive. A negative value of $b$ only relates to the sign of the wave function, which indicates whether there is a $180^\circ$ phase change of the particle wave after scattering.

The magnitude of the scattering length $b$ refers to the strength of the scattering and can be experimentally measured for elements. It is found that the scattering length of one element has a relationship with its spin relative to the scattered neutron. An neutron has a spin of $\frac{1}{2}$. Hence, for a nucleus with non-zero spin, the combined spin states give two values of $b$ ($b^+$ and $b^-$), since their spins are either parallel or antiparallel. According to the arithmetic of quantum mechanical spin, the average and standard deviation of scattering length for each element can be obtained using Equation 2.46, 2.47 and 2.48.

$$\langle b \rangle = p^+b^+ + p^-b^-$$  \hspace{1cm} (2.46)

$$\Delta b = \sqrt{\langle b^2 \rangle - \langle b \rangle^2}$$  \hspace{1cm} (2.47)

$$\langle b^2 \rangle = p^+(b^+)^2 + p^-(b^-)^2$$  \hspace{1cm} (2.48)

where $\langle b \rangle$ is the average scattering length, $p^+$ and $p^-$ are possibility coefficients for the two combined spin states related to $b^+$ and $b^-$. $\Delta b$ is the standard deviation of scattering length and $\langle b^2 \rangle$ is the average of the square of scattering length. For instance, the parameter values of hydrogen and deuterium are listed in Table 2.1.
<table>
<thead>
<tr>
<th></th>
<th>Hydrogen</th>
<th>Deuterium</th>
</tr>
</thead>
<tbody>
<tr>
<td>$b^+$</td>
<td>$1.085 \times 10^{-14} m$</td>
<td>$0.953 \times 10^{-14} m$</td>
</tr>
<tr>
<td>$b^-$</td>
<td>$-4.750 \times 10^{-14} m$</td>
<td>$0.098 \times 10^{-14} m$</td>
</tr>
<tr>
<td>$p^+$</td>
<td>$3/4$</td>
<td>$2/3$</td>
</tr>
<tr>
<td>$p^-$</td>
<td>$1/4$</td>
<td>$1/3$</td>
</tr>
<tr>
<td>$\langle b \rangle$</td>
<td>$-0.374 \times 10^{-14} m$</td>
<td>$0.668 \times 10^{-14} m$</td>
</tr>
<tr>
<td>$\Delta b$</td>
<td>$2.527 \times 10^{-14} m$</td>
<td>$0.403 \times 10^{-14} m$</td>
</tr>
<tr>
<td>$\langle b^2 \rangle$</td>
<td>$6.524 \times 10^{-28} m^2$</td>
<td>$0.608 \times 10^{-28} m^2$</td>
</tr>
</tbody>
</table>

**Table 2.1** The scattering length parameters for hydrogen and deuterium.
Cross Section

The scattering length can be measured through its link to the scattering cross section. The total cross section \( \sigma_{\text{tot}} \) is defined below, as the scattering ratio total on all the directions per unit area of the sample:

\[
\sigma_{\text{tot}} = \frac{\text{number of neutrons scattered in all directions per unit time}}{\text{number of neutrons incident on unit area of the sample per unit time}} \tag{2.49}
\]

Obviously, \( \sigma_{\text{tot}} \) has the same unit as the area by dimensional analysis, for which reason it is called cross section. In a simpler expression, the cross section \( \sigma_{\text{tot}} \) is the area of the classical target for an incoming particle beam, and the beam has a transverse section of the unit area.\(^{32} \)

In the case we discussed in this section, Equation 2.50 can be deduced from Equations 2.43-2.45 and 2.49:

\[
\sigma_{\text{tot}} = \frac{\left| \psi_f \right|^2 \cdot dV_{\text{outgoing}}}{\left| \psi_i \right|^2 \cdot dV_{\text{incident}}} = \frac{\left| \psi_f \right|^2 \cdot S \cdot v \cdot dt}{\left| \psi_i \right|^2 \cdot v \cdot dt \cdot dS} = \frac{b^2/r^2 \cdot 4\pi}{1} = 4\pi b^2 \tag{2.50},
\]

where \( dV, dS \) and \( dt \) stand for unit volume, unit area and unit time; \( S \) is the total superficial area with a radius of \( r \); \( v \) is the speed of the neutron.

The differential cross section is defined by \( d\sigma/d\Omega \), where \( \Omega \) is the solid angle. Solid angle represents an area of the spherical shell fragment generated by a radius \( r \):

\[
\Omega = \frac{\text{area}}{r^2} \tag{2.51}
\]

Therefore, the differential cross section is obtained from Equation 2.50:

\[
\frac{d\sigma}{d\Omega} = \frac{f_{\text{tot}} d\sigma}{f_{\text{tot}} d\Omega} = \frac{\sigma_{\text{tot}}}{4\pi} = b^2 \tag{2.52}
\]

2.2.4 Elastic Scattering from an Assembly of Atoms

Wave-functions Superposition

A more complicated condition is discussed below, in which neutrons are elastically scattered from an assembly of atoms. We assume the sample contains a number of atoms, which are the same element but isotopes of non-zero spin. The scattering from each atom individually has been described in Section 2.2.3. Therefore, the total
scattering can be described as the sum of scattering events from all the atoms. It should be noted that the sum is a superposition of wave functions.

Figure 2.11  A particle scattered elastically by a fixed particle J in an Assembly of atoms

As shown in Figure 2.11, an incident neutron beam scattered with particles P and J. The particle J was at position \( R_j \) and the particle P was at the original point \( R_o=0 \). Thus the scattered particle wave from nucleus P is already presented in Equation 2.45.

At a position \( r \), the phase difference \( \Delta \varphi \) between the wave scattered by the nucleus P and nucleus J can be separated into two parts:

\[
\Delta \varphi_1 = k_i (R_j - R_o) = R_j \cdot k_i \\
\Delta \varphi_2 = k_f (r - R_j) 
\]

(2.53),

(2.54),

where \( k_i \) and \( k_r \) are the wave vector of the incident and outgoing particle wave; \( \Delta \varphi_1 \) stands for the phase difference between the two particles before the scattering while \( \Delta \varphi_2 \) is the phase difference after the scattering.

Therefore, the scattered particle wave from nucleus J is:

\[
\psi_f' = e^{i\Delta \varphi_1} \frac{-b_j}{|r-R_j|} e^{i\Delta \varphi_2} = \frac{-b_j}{|r-R_j|} e^{i(\Delta \varphi_1 + \Delta \varphi_2)} = \frac{-b_j}{|r-R_j|} e^{i|k_f \cdot r|} \cdot e^{iR_j(k_i-k_f)} 
\]

(2.55)

As known, the wave-vector transfer \( Q \) is \((k_i-k_f)\), enabling Equation 2.55 to be rewritten as:

\[
\psi_f' = \frac{-b_j}{|r-R_j|} e^{i|k_f \cdot r|} \cdot e^{iR_j Q} 
\]

(2.56)

Equation 2.56 is the general form of the scattered wave function for any nucleus in the sample. It equals to Equation 2.45 when the nucleus is at the original point as \( R_j \) and
\( \mathbf{R}_0 \) equals to 0. Hence, it enables us to calculate the total scattered wave by adding up the waves from all N nucleus in the sample.

\[
\psi_f^{\text{tot}} = \sum_{j=1}^{N} \frac{-b_j}{|\mathbf{r} - \mathbf{R}_j|} e^{ik_f \mathbf{r} \cdot \mathbf{e}^{i\mathbf{R}_j \cdot \mathbf{Q}}} = e^{ik_f \mathbf{r} \cdot \mathbf{e}^{i\mathbf{R}_j \cdot \mathbf{Q}}} \sum_{j=1}^{N} \frac{-b_j}{|\mathbf{r} - \mathbf{R}_j|}
\]

(2.57)

We ignore any multiple scattering of the neutron due to its weak interactio. Moreover, the size of the sample measured is relatively tiny compared with the distance to the detector so that we can make a good approximation as Equation 2.58:

\[
|r - \mathbf{R}_j| = |\mathbf{r}| = r
\]

(2.58)

Thus, Equation 2.57 can be simplified to Equation 2.59:

\[
\psi_f^{\text{tot}} = \frac{-e^{ik_f r}}{r} \sum_{j=1}^{N} (b_j \cdot e^{i\mathbf{R}_j \cdot \mathbf{Q}})
\]

(2.59)

According to Equation 2.49-2.52, the differential cross section for the scattering is:

\[
\frac{d\sigma}{d\Omega_{\text{tot}}} = \left| \sum_{j=1}^{N} (b_j \cdot e^{i\mathbf{R}_j \cdot \mathbf{Q}}) \right|^2
\]

(2.60)

With the relationship of \(|z|^2 = z \cdot z^*\) for a complex number \(z\), where \(z^*\) is the complex conjugate of \(z\), Equation 2.60 is rewritten as a double summation:

\[
\frac{d\sigma}{d\Omega_{\text{tot}}} = \sum_{j'=1}^{N} \sum_{j=1}^{N} (b_j \cdot b_{j'} e^{i\mathbf{Q} \cdot (\mathbf{R}_j - \mathbf{R}_{j'})})
\]

(2.61)

**Coherent and Incoherent Scattering**

If we assume the spin states of the nuclei in this sample are distributed randomly and uncorrelated with each other, the quantity \(b_j b_{j'}\) in Equation 2.61 is statistically equal to and can be replaced by its average value \(< b_j b_{j'}>\). For the condition of \(j=j'\), the \(< b_j b_{j'}>\) and \(d\sigma/d\Omega\) is:

\[
\langle b_j \cdot b_{j'} \rangle = \langle b_j^2 \rangle = \langle b^2 \rangle
\]

(2.62)

\[
\frac{d\sigma}{d\Omega_{j=j'}} = \sum_{j'=j}^{j} \langle b_j \cdot b_{j'} \rangle e^{i\mathbf{Q} (\mathbf{R}_j - \mathbf{R}_{j'})} = \sum_j \langle b_j^2 \rangle
\]

(2.63)

On the other hand, when \(j\) is not equal to \(j'\) so that \(j,j'\) refer to nuclei on different locations, the \(< b_j b_{j'}>\) and \(d\sigma/d\Omega\) should be represented as:

\[
\langle b_j \cdot b_{j'} \rangle = \langle b_j \rangle \cdot \langle b_{j'} \rangle = \langle b \rangle^2
\]

(2.64)
\[
\frac{da}{d\Omega_{j\neq j'}} = \sum_{j,j'}^\pi \langle b_j \cdot b_{j'} \rangle \exp(iQ(R_j - R_{j'})) = \sum_{j,j'}^\pi \langle b_j \rangle^2 \exp(iQ(R_j - R_{j'})) \tag{2.65}
\]

To obtain the equation of total \(d\sigma/d\Omega\), we can simply add Equation 2.64 and 2.65 together as:

\[
\frac{da}{d\Omega} = \sum_{j,j'}^\pi \langle b_j \rangle^2 \exp(iQ(R_j - R_{j'})) + \sum_j (\langle b^2 \rangle - \langle b \rangle^2) \tag{2.66}
\]

The final expression can be presented in Equation 2.67 by further simplification, inserting an additional term for \(j=j'\) in the first summation term in Equation 2.66 and subtracting the same term from the second summation term:

\[
\frac{da}{d\Omega} = \sum_{j,j'} (\langle b_j \rangle^2 \exp(iQ(R_j - R_{j'})) + \sum_j (\langle b^2 \rangle - \langle b \rangle^2) \tag{2.67}
\]

where the terms of \(\langle b \rangle\), \(\langle b^2 \rangle\) and \(\langle b \rangle^2\) have been discussed in Section 2.2.3.

The first term in Equation 2.67, containing the part of \(\exp(iQ(R_j - R_{j'}))\), is related to the spatial distribution of atoms in the material. Hence, this term presents the scattering raised from the interference of scattering waves from different nuclei. The scattering of this part is called coherent scattering and can provide information about the sample structure interested. The second term in Equation 2.67 has no interference contributions and only related to the deviations of the scattering lengths. The scattering presented by this part is called incoherent scattering and merely resulted in a constant background to the scattering signal.

The cross section for coherent and incoherent scattering can be deduced from Equation 2.67 and presented separately in Equation 2.68 and 2.69.

\[
\sigma_{coh} = 4\pi\langle b \rangle^2 = 4\pi b_{coh}^2 \tag{2.68}
\]

\[
\sigma_{incoh} = 4\pi(\langle b^2 \rangle - \langle b \rangle^2) = 4\pi b_{incoh}^2 \tag{2.69}
\]

For instance, we can figure out the coherent and incoherent cross section for hydrogen and deuterium using the values in Table 2.1. For hydrogen, the \(\sigma_{incoh}\) is 80.3 barns, and \(\sigma_{coh}\) is 1.8 barns; on the other hand, \(\sigma_{incoh}\) and \(\sigma_{coh}\) are 2.1 barns and 5.6 barns for deuterium.\(^{32}\) Barn is an area unit used for the cross section which equals to \(10^{-28}\) m\(^2\). It is evident that the incoherent scattering of hydrogen is much more prominent, and that is the reason H\(_2\)O always results in a higher background than D\(_2\)O as ambient in neutron reflection experiments. In the experiments of small neutron angle scattering, we have...
to limit the path length for the samples in H$_2$O ambient to reduce the background and then count for a long time as a trade-off.

Moreover, if the nuclei number density $N$ of the sample is known, the scattering length density (SLD) is:

$$S LD = N b_{coh}$$  (2.70)

For a sample containing different nuclei, the SLD is:

$$S LD = \sum N_i (b_{coh})_i$$  (2.71)

2.2.5 Neutron Reflection Theory

Briefly, the reflectivity is measured via experiment as the ratio between reflected neutron intensity and the incident neutron intensity. On the other hand, a neutron can be treated as a particle and wave so that it is an analogue of light which is an electromagnetic wave. Therefore, neutron reflection should follow the optical reflection theory discussed in Section 2.1 with a few modifications. Similarly to the optical reflectivity, neutron reflectivity can be derived theoretically as a function of neutron wavelength, neutron grazing angle and the scattering length density of the thin film material studied.

From Optical Reflection to Neutron Reflection

![Figure 2.12 Schematic shows the specular reflection geometry.](image)

The wavevector transfer $Q$ is used to gather and present the two variables, neutron wavelength and glancing angle.
As shown in Figure 2.12, a neutron particle wave with wave vector $k_i$ impinges on the interface at a grazing angle of $\theta_i$. In the case of elastic specular reflection, the wave bounces back at a reflection grazing angle $\theta_f$ equal to $\theta_i$, and the final wave vector $k_f$ has a modulus as $k_i$. According to its definition, the wave-vector transfer $Q$ is equal to $k_i - k_f$ and is shown in the figure by vector subtraction. Using geometry method, the modulus of $Q$ is:

$$Q = |Q| = 2 \sin \theta_i |k_i| = \frac{4\pi \sin \theta_i}{\lambda}$$

(2.72),

where $\lambda$ is the neutron wavelength.

Benefit from the wave-vector transfer $Q$, the neutron wavelength and glancing angle are gathered into one variable. Hence, neutron reflectivity can be presented as a profile of $Q$. To yield the reflectivity profile via experiments, measurements are undertaken at different angles with a monochromic neutron beam (monochromatic mode), or at a certain angle with a neutron beam covering a range of wavelengths (Time-of-Flight mode).

Snell’s law stated in Equation 2.1 can be rewritten with the grazing angle to describe neutron wave refraction.

$$n_i \cos \theta_i = n_t \cos \theta_t$$

(2.73),

where $n_i$ and $n_t$ are the neutron refractive indexes of the materials on each side of the interface. As an extension of the optical refractive index, the neutron refractive index has the relationship below:

$$n_i k_i = n_t k_t$$

(2.74)

For convenience, we define refractive index $n$ as $n_t/n_i$, and then Equation 2.73 becomes:

$$\cos \theta_i = n \cos \theta_t$$

(2.75).

When total reflection occurs, the grazing angle should be below a value, which is called critical angle. When the grazing angle is right of the critical angle, the grazing refraction angle $\theta_t$ equals 0. Therefore, we obtain the relationship from Equation 2.75 as:

$$\cos \theta_c = n$$

(2.76)
Again, when the grazing angle is larger than the critical angle, the reflection and transmission coefficients are described by Fresnel equations as discussed in Section 2.1.3. Notice that sine should be used here instead of cosine in Equation 2.2-2.5 since the angles we used for neutron reflection are all grazing angles rather than the normal incident or reflected angle. Hence, we can write the reflection coefficients for a single interface as:

\[ r_p^{\text{Fresnel}} = \frac{n_t \sin \theta_i - n_i \sin \theta_t}{n_t \sin \theta_i + n_i \sin \theta_t} \]  
(2.77)

\[ r_s^{\text{Fresnel}} = \frac{n_i \sin \theta_i - n_t \sin \theta_t}{n_i \sin \theta_i + n_t \sin \theta_t} \]  
(2.78)

By substituting Equation 2.73 into Equations 2.77 and 2.78, the equations are rewritten as:

\[ r_p^{\text{Fresnel}} = \frac{\cos \theta_i \sin \theta_i - \cos \theta_i \sin \theta_t}{\cos \theta_i \sin \theta_i + \cos \theta_i \sin \theta_t} = \frac{\sin 2\theta_i - \sin 2\theta_t}{\sin 2\theta_i + \sin 2\theta_t} = \frac{\cos(\theta_i + \theta_t) \sin(\theta_i - \theta_t)}{\cos(\theta_i - \theta_t) \sin(\theta_i + \theta_t)} = \frac{\tan(\theta_i - \theta_t)}{\tan(\theta_i + \theta_t)} \]  
(2.79)

\[ r_s^{\text{Fresnel}} = \frac{\cos \theta_i \sin \theta_i - \cos \theta_i \sin \theta_t}{\cos \theta_i \sin \theta_i + \cos \theta_i \sin \theta_t} = \frac{\sin(\theta_i - \theta_t)}{\sin(\theta_i + \theta_t)} \]  
(2.80)

For most materials, the neutron refractive index is close to 1. It is the reason that the neutron reflection experiments are always undertaken at a small grazing angle. In this case, both \( \theta_i - \theta_t \) and \( \theta_i + \theta_t \) are tiny angles, in which case the tiny angle approximation can be applied:

\[ \theta = \sin \theta = \tan \theta \]  
(2.81)

As a consequence, \( r_s \) equals to \( r_p \) for a small grazing angle. Thus we will only use \( r_s \) as the reflection coefficient of neutron reflection from now on. To facilitate the derivation, the reflection coefficient can be expressed in another form as:

\[ r = \frac{1-\mu}{1+\mu}, \quad \mu = \frac{n_t \sin \theta_t}{n_i \sin \theta_i} \]  
(2.82)

By using the relationship shown in Equation 2.75, the factor \( \mu \) is represented as:

\[ \mu = \frac{n \sin \theta_t}{\sin \theta_i} \sqrt{1 - \cos^2 \theta_i} = \frac{n}{\sin \theta_i} \sqrt{1 - \left(\frac{\cos \theta_i}{n}\right)^2} = \frac{n}{\sin \theta_i} \sqrt{1 - \left(1 - \frac{\sin^2 \theta_i}{n^2}\right)} \]  
(2.83)

For further simplification, the factor \( \mu \) is expressed with Q according to the Equation 2.72:

---

Chapter 2 - 58 -
\[ \mu = \sqrt{1 - \left( \frac{1 - n^2}{\sin^2 \vartheta} \right)} = \sqrt{1 - \frac{16\pi^2(1-n^2)}{\lambda^2 Q^2}} \approx 1 - \frac{8\pi^2(1-n^2)}{\lambda^2 Q^2} \] (2.84)

Here, we used the binomial expansion for the square root, which is:

\[ (1 - x)^{\frac{1}{2}} = 1 - \frac{x}{2} - \frac{x^2}{8} - \frac{3x^2}{48} - \cdots \] (2.85)

The higher-order terms can be omitted when \( x \) is a small value. In Equation 2.84, this approximation was applied due to \( n \) is close to 1, and it becomes more accurate as \( Q \) tend to positive infinite.

Substituting \( \mu \) back into Equation 2.82, the reflection coefficient becomes a function of \( Q \) and neutron refractive index \( n \). With the approximation of \( 1 + \mu \approx 2 \), the function is expressed as:

\[ r = \frac{4\pi^2(1-n^2)}{\lambda^2 Q^2} \] (2.86)

Moreover, the reflectivity of a single interface is:

\[ R = |r|^2 = \frac{16\pi^4(1-n^2)^2}{\lambda^4 Q^4} \] (2.87)

Again, the neutron reflection on a single thin film, which related to the thin film interference, is described in the light reflection part of Section 2.1.4. As shown in Section 2.1.4, Equation 2.15-2.16 are presented here again to state the total reflection coefficient \( r \) and reflectivity \( R \) for a single thin film neutron reflection:

\[ r_{\text{single-tot}} = \frac{r_{10} + r_{12}e^{-i2\beta}}{1 + r_{12}r_{10}e^{-i2\beta}} \] (2.88),

\[ R_{\text{single-tot}} = |r_{\text{single-tot}}|^2 = \frac{r_{10}^2 + r_{12}^2 + 2r_{10}r_{12} \cos 2\beta}{1 + r_{12}r_{10}^2 + 2r_{12}r_{10} \cos 2\beta} \] (2.89),

where \( \beta \) is the half optical phase difference, and \( r_{ij} \) is the Fresnel reflection coefficient of the interface between material i and material j.

\[ \beta = \frac{2\pi n d \sin \vartheta}{\lambda} \] (2.90),

where \( n \) and \( d \) are the thin film neutron refractive index and thickness; \( \lambda \) is the neutron wavelength; \( \vartheta \) is the grazing refraction angle in the film.

For the condition of multiple film system reflection, the Abeles matrix method discussed in Section 2.1.4 enables the total neutron reflectivity to be solved.
**Neutron Refractive Index**

So far we have discussed the method of deducing reflectivity as a function of wavevector transfer $Q$ and neutron refractive index $n$ for a given sample. Since $Q$ is a known parameter in neutron reflection experiments, the only issue left is finding the link between neutron refractive index and the property of the sample material. This section will discuss the derivation of the neutron refractive index in terms of neutron wavelength and the scattering length density of the sample. This derivation is based on Fermi-Huygens theory\textsuperscript{32,38–40}.

![Figure 2.13](image.png)

**Figure 2.13** A beam of neutrons incident perpendicularly to a thin slab of material.

Figure 2.13 shows a beam of neutrons incident in the direction of $z$-axis and striking perpendicularly a thin slab of thickness $t$. As discussed in Section 2.2.3, the incident neutrons can be described as a plane wave:

$$\psi = \exp(ikz)$$  \hspace{1cm} (2.91),

where $k$ is the neutron wave vector.

The neutrons scattered by the nuclei individually in the material, and the scattered wave raised from the scattering is described by the spherical wave:

$$\psi_{sc} = \frac{-b}{r} e^{ikr}$$  \hspace{1cm} (2.92)

According to Huygens’s principle, the coherent superposition of all the scattered waves in the forward direction generates the refraction. Therefore, we can first consider the scattered wave function of an annulus with radius $a$ in the plane. The volume of this annulus can be expressed as:

$$V = 2\pi a \cdot da \cdot t$$  \hspace{1cm} (2.93)

Where $da$ stands for the differential of radius $a$ so that it is the width of the annulus.
Therefore, the number of nuclei in the annulus is:

\[ \text{Num}_{\text{nuclei}} = 2\pi a \cdot da \cdot t \cdot N \]  

(2.94), where \( N \) is the number of nuclei per unit volume, so-called nuclei number density.

Then the amplitude of the total wave function emitted from the annulus to the point \( P \) can be obtained by summation of the coherent scattering amplitude:

\[ |\psi_{sc-annulus}| = -\frac{b}{r} e^{ikr} \cdot 2\pi a \cdot da \cdot t \cdot N \]  

(2.95), where \( b \) is the mean coherent scattering length of the nuclei in the plate.

Because we assume the plate is sufficiently thin, we do not need to consider the difference between the waves scattered from the front and back of the plate here. The total scattered wave amplitude from the whole plate to the point \( P \) can be obtained from Equation 2.95 by integration. Since \( r^2 = a^2 + z^2 \), it is evident that \( ada = rdr \).

\[ |\psi_{sc-plate}| = -\int_z^\infty be^{ikr} \cdot 2\pi t N \cdot r dr = -2\pi Nbt \cdot \frac{i}{k} e^{ikz} \]  

(2.96)

The whole wave arriving at the point \( P \) is the sum of the initial and scattered wave:

\[ \psi' = \exp(ikz) - 2\pi Nbt \cdot \frac{i}{k} e^{ikz} = \exp(ikz) \left( 1 - \frac{2\pi Nbt i}{k} \right) \]  

(2.97)

Figure 2.14 Neutrons wave propagates through vacuum (bottom) and through a thin slab of material (top).

On the other hand, we can consider this problem in terms of neutron refractive index. According to Equation 2.74, the wave vector of neutrons tends to \( nk \) in the thin slab. As shown in Figure 2.14, the initial neutron wave 2 propagated in vacuum can be expressed by Equation 2.91. The wave 1 describes the neutron wave passing through the thin slab. \( P \) is a point behind the slab and on the neutron propagation path. It is evident that the phase difference \( \Delta \phi \) between wave 1 and wave 2 at point \( P \) is:
\[ \Delta \phi = nkt - kt = (n - 1)kt \] (2.98)

Hence, the wave function of wave 1 can be expressed based on wave 2 as:

\[ \psi' = \exp(ikz) \cdot \exp(i\Delta \phi) = \exp(ikz) \cdot \exp(i(n - 1)kt) \] (2.99)

If \( k(n - 1)t \ll 1 \), it can be rewritten as:

\[ \psi' = \exp(ikz) \cdot \exp(i(n - 1)kt) \approx \exp(ikz) \cdot (1 + i(n - 1)kt) \] (2.100)

By combing Equation 2.97 and 2.100, we obtain:

\[ (1 + i(n - 1)kt) = 1 - \frac{2\pi Nb ti}{k} \] (2.101)
\[ n = 1 - \frac{2\pi Nb}{k^2} = 1 - \frac{\lambda^2 Nb}{2\pi} \] (2.102)

For small \( (\lambda^2 Nb/2\pi) \ll 1 \),

\[ n^2 \approx 1 - \frac{\lambda^2 Nb}{\pi} \] (2.103)

It is a simplified derivation, in which case neutron absorption and multiple scattering are not considered. In addition, we made some approximations during the process. There is another more complete derivation based on wave energy theory and Fermi pseudopotential.\(^{32,41}\) The conclusions of these two derivations are consistent with each other when the refractive index is close to 1.

Once the link between the neutron refractive index and the scattering length density is found, the reflectivity and other conclusions obtained in Section 2.2.5 can be represented:

\[ n = \cos \vartheta_c \approx 1 - \frac{\vartheta_c^2}{2} \rightarrow \vartheta_c = \lambda \sqrt{\frac{Nb}{\pi}} \] (2.104),
\[ Q_c = \frac{4\pi \sin \vartheta_c}{\lambda} = \sqrt{16\pi Nb} \] (2.105),
\[ R_{\text{single-interface}} = \frac{16\pi^2(1 - n^2)^2}{\lambda^4} = \frac{16\pi^2(Nb)^2}{Q^4} \] (2.106),
\[ R_{\text{single-film}} = \frac{r_{10}^2 + r_{12}^2 + 2r_{10}r_{12}\cos 2\beta}{1 + r_{10}^2 r_{12}^2 + 2r_{12}r_{10}\cos 2\beta} \] (2.107),
\[ R_{\text{single-film}} = \frac{16\pi^2}{Q^4} [((Nb_1 - Nb_2)^2 + (Nb_3 - Nb_2)^2 + 2(Nb_1 - Nb_2)(Nb_2 - Nb_3) \cos(Qd)] \] (2.108),
where $\vartheta_c$ and $Q_c$ are the critical angle and critical value of $Q$; $b_1$, $b_2$ and $b_3$ are mean coherent scattering length of the media neutrons incident, the thin film and the media neutrons refracted in; $d$ is the thickness of the thin film.

### 2.2.6 Measurement and Data Analysis

**Measurement**

As Cubitt and Fragneto stated in their book, the neutron reflection experimental setup is featured by a radiation source, a wavelength selector or chopper, a system of collimation, the sample and a detection system.$^{41}$

Nowadays, two main mechanisms are used to produce neutron beams for neutron scattering experiments. One is a nuclear reactor source, in which case a continuous neutron beam is generated from nuclear fission, and the other one is a spallation source. In this project, the spallation source was used for the experiments undertaken on two time-of-flight reflectometer, SURF and INTER, at Rutherford Appleton Laboratory (RAL). The spallation source produces neutron pulses by the collisions of accelerated high energy particles with a heavy atom target. The energy of neutrons generated by either reactor source or spallation source is much greater than what is required by the experiments. Therefore, the target is surrounded by several moderators, in which the high-energy neutrons are multiple scattered, losing energy until they reach thermal equilibrium. The energy level of slow neutrons emitted from the moderators can be controlled by the moderator temperature. Then, the thermal neutrons pass through collimators to reach each beamline instrument. A series of wavelength selectors or choppers can be set up on the path to allow only neutrons with a certain time-of-flight window pass, preventing overlap between each pulse.

Figure 2.15 shows the schematic layout of a typical neutron reflectometer, INTER at RAL. Monitor 1 and 2 can record the number of neutrons incident in real time while a reflection detector is used to count the neutrons reflected by the sample. Four adjustable slits on the neutron flight path define the illuminated area on the sample and keep a required resolution. The sample stage can adjust the height and tilt angle to align the sample at a required angle. Moreover, the tilt angle controls the grazing incident angle which is related to the wave vector transfer $Q$. 

*Chapter 2* - 63 -
Figure 2.15 Schematic layout of the INTER reflectometer. Photo produced from RAL website.

Data Reduction

Neutron reflectivity profile is measured as a function of wave vector transfer $Q$, via dividing the reflected intensity by the incident intensity. The raw data collected by the monitor and reflection detector need to undergo a correction process to obtain the final reflectivity. This process is called “data reduction”. In this part, we discuss this process using a reflection data measured at the SiO$_2$/D$_2$O interface by SURF, RAL.

Figure 2.16 Spectra of time-of-flight collected by monitor 1 (black), 2 (red) and reflection detector (green) in the measurement with run number 118751.

In the time-of-flight neutron reflection, the neutron velocity is obtained from its travelling time in a certain distance. In the case of the spallation source, the distance is from the target to respective reflection detectors or monitors, and the start time $t_0$ is defined by the pulse. During the measurement, the number of neutrons passing the reflection detector (or monitor) is recorded in a real time. The total detection time of one pulse is divided into many ‘time bins’$^{43}$ with a small width, and the neutron count refers to the sum of neutrons in each ‘time bin’. The width of the ‘time bins’ affects the time-of-flight resolution which decides the wavelength resolution. Thus time-of-
flight spectra can be obtained by plotting the counts against the travelling time as shown in Figure 2.16. The sharp drops of each spectrum are caused by the time-of-flight window selection by the choppers and wavelength selectors. Note that the edges of each spectrum are not aligned, and the signal levels are different. The reason is that the neutrons spend different time reaching each detector so that the pulse spreads out in time for the longer travelling distance. Also, the detecting efficiency of each detector is different.

By using the De Broglie relationship stated in Equation 2.42, the time spectra are converted into a spectrum in terms of the unit of neutron wavelength as Figure 2.17.

![Figure 2.17](image)

**Figure 2.17** Spectra of wavelength collected by monitor 1, 2 and reflection detector in the measurement 118751.

Since the grazing incident angle is known for each measurement, the spectra can be further converted into units of wave vector Q by Equation 2.72. Following this, reflectivity profile against Q is obtained by dividing the reflection detector signal to monitor signal and then times an instrument factor. The instrument factor is involved because the detector efficiency and solid angle coverage are different between the monitor and reflection detector. The factor should be fixed during the experiment and be measured in an instrument calibration process.
The reflectivity shown in Figure 2.18 has to be further corrected by a ‘transmission’ run because there is a loss of neutrons in the flight path between the monitor and reflection detector. The transmission measurement shown in Figure 2.19 was undertaken using same slits setting with the reflection run. The neutron beam passed through an identical flight path and went directly into the reflection detector without reflection on the sample. Identical flight path means the distances neutron travelling in air and any other materials should be approximately equal in both reflection run and transmission run. In the case of the measurement 118751 which is a measurement at the SiO$_2$/D$_2$O interface, the neutron beam should pass through an identical silicon block to obtain the transmission.

To obtain data covering a required wide $Q$ range, the measurements are taken at different grazing incident angles on the same sample. After the data correction process applied on each of the measurements, the profiles of each measurement can be stitched together to obtain the final analyzable reflectivity as shown in Figure 2.20.
Data Fitting

The data fitting process is the same as the approach described in Section of 2.1.6 for Ellipsometry. Firstly, a model of the interface is constructed as a system containing multiple homogeneous thin films which are parallelly stacked in sequence. Each of the layers is characterised by the parameters of layer thickness and scattering length density. By the arithmetic discussed in Section 2.2.5, the reflectivity can be calculated as a function of the wave vector transfer Q. An estimation of the unknown parameters needs to be made for the preliminary calculation. A constant background is added to each data point of the simulation, and the instrumental resolution must also be included. After then the difference between the calculated reflectivity profile and the measured result is quantified by $\chi^2$:

$$\chi^2 = \sum_{i=1}^{N} \left( \frac{R_i^f - R_i^m}{s_{R_i}} \right)^2 \quad (2.109),$$

where $N$ is the number of data points; $S$ is the standard deviation of the i-th data point in the measured reflectivity; The superscripts ‘f’ and ‘m’ indicate the fitted reflectivity and measured reflectivity.

By variation of the unknown parameter values, the best fitting result with a minimum $\chi^2$ can be reached in the regression process. If the optimum fitted result is still far away from the measured profile visually or the fitted model is unpractical, the model needs to be improved by adding, deleting layers or changing fitting ranges. Once a reasonable model is achieved, the film mass density can be calculated from scattering length density.

Figure 2.20 The final reflectivity obtained by data reduction.
2.4 Reference


doi:10.1117/3.784938.ch1
doi:10.1117/3.855480.ch5
doi:10.1117/3.784938.ch3
doi:10.1002/col.5080120310


42. ISIS. INTER Science Case. https://www.isis.stfc.ac.uk/Pages/inter-science-case6738.pdf#search=inter [Accessed July. 2017]

43. L.Clifton, C.Kinane, M.Skoda, P. L. Reflectivity Practical Worksheet. in ISIS Neutron Training Course
Chapter 3

- Abstract 73

1. Introduction 74

2. Materials and Experimental Methods 76

3. Results and Discussion 79

4. Conclusions 93

- References 95

- Supporting Information 98
Neutron Reflection Study of Surface Adsorption of Fc, Fab and the Whole mAb

Zongyi Li [1], Ruiheng Li [1], Charles Smith [1], Fang Pan [1], Mario Campana [2], John R P Webster [2], Christopher F van der Walle [3], Shahid Uddin [3], Steve M Bishop [4], Rojaramani Narwal [4], Jim Warwicker [5], Jian Ren Lu* [1]

[1] Biological Physics Laboratory, School of Physics and Astronomy, University of Manchester, Oxford Road, Schuster Building, Manchester M13 9PL, UK.

[2] ISIS Neutron Facility, STFC, Chilton, Didcot OX11 0QZ, UK.


[4] Formulation Sciences, MedImmune LLC, Gaithersburg, MD 20878, USA.

[5] Manchester Institute of Biotechnology, School of Chemistry, University of Manchester, 131 Princess Street, Manchester M1 7DN, UK.

*Corresponding author: Jian R Lu
(email: j.lu@manchester.ac.uk; tel: +44 161 2003926)

DOI: 10.1021/acsami.7b06131

Keywords:

mAb, surface adsorption, co-adsorption, antibody, structural unfolding, self-assembly, globular stability, neutron reflection
Abstract

Characterizing the influence of fragment crystallization (Fc) and antigen-binding fragment (Fab) on monoclonal antibody (mAb) adsorption at the air/water interface is an important step to understanding liquid mAb drug product stability during manufacture, shipping and storage. Here, neutron reflection is used to study the air/water adsorption of a mAb and its Fc and Fab fragments. By varying the isotopic contrast, the adsorbed amount, thickness, orientation and immersion of the adsorbed layers could be determined unambiguously. Whilst Fc adsorption reached saturation within the hour, its surface adsorbed amount showed little variation with bulk concentration. In contrast, Fab adsorption was slower, and the adsorbed amount was concentration dependent. The much higher Fc adsorption, compared to Fab, was linked to its lower surface charge. Time and concentration dependence of mAb adsorption was dominated by Fab behavior although both Fab and Fc behaviors contributed to the amount of mAb adsorbed. Changing the pH from 5.5 to 8.8 did not much perturb the adsorbed amount of Fc, Fab or mAb. However, a small decrease in adsorption was observed for the Fc over pH 8-8.8 and vice versa for the Fab and mAb, consistent with a dominant Fab behavior. As bulk concentration increased from 5 to 50 ppm, the thicknesses of the Fc layers were almost constant at 40 Å, while Fab and mAb layers increased from 45 Å to 50 Å. These results imply that the adsorbed mAb, Fc and Fab all retained their globular structures and were oriented with their short axial lengths perpendicular to the interface.
Monoclonal antibody (mAb) therapies are fast becoming effective treatments for many diseases worldwide. Many therapeutic mAbs are of the IgG1 isotype and (like isotypes IgG2, 3 and 4) comprised of a fragment crystallization (Fc) and two antigen-binding fragments (Fabs). The Fc comprises constant domains \( C_{\text{H} \gamma 3} \) and \( C_{\text{H} \gamma 2} \) for each of the two heavy chains, linked via a hinge to \( C_{\text{H} \gamma 1} \) and a variable domain (V\( _\text{H} \)), which together with the light chain \( C_{\text{L}\kappa/\lambda} \) and V\( _\text{L} \) domains comprise each Fab; Asn297 on \( C_{\text{H} \gamma 2} \) harbors the biantennary N-glycan comprising a core structure of two \( \beta-N\)-acetylglucosamine (GlcNAc) and three mannose (Man) residues, partially fucosylated as in the predominant G0f: GlcNAc\(_2\)Man\(_3\)GlcNAc\(_2\)Fuc. Like many other protein molecules, mAbs are amphiphilic and capable of adsorbing and desorbing from the air/water interface. This interfacial behavior is relevant to manufacturing processes where a liquid mAb product may be exposed to air during fill-finish into a container closure system, or during shipping/storage of vials filled with the liquid drug product. It is also relevant to the intravenous administration of an mAb at very low concentrations via a giving set, where significant adsorption to the infusion bag, air/water interface and tubing may result in a lower than the desired dose. One could expect to quantify the loss of mAb in this scenario using a facile UV-vis spectroscopy method, though characterizing the nature of the adsorbed layer(s) has proven more complex. Supplementary surface tension measurements (which are well established, relatively straightforward and usefully employed in this field) could next be used to generate surface pressure data but these provide little insight to the structural implications for the adsorbed protein. A number of reports on mAb adsorption at the interface have employed either fluorescently- or non-labelled protein with spectroscopic and microbalance techniques. Thus, a body of evidence is emerging and is reasonably in agreement with regard to the extent of mAb adsorption at interfaces under the various surface and buffer conditions. In addition to quantifying the adsorbed layer, infrared spectroscopy has been used to probe mAb unfolding at the interface. Similarly, measurement of hydrophobicity of the mAb layer adsorbed at the air/liquid interface via Nile Red fluorescence provides a simple tool to infer the kinetics of adsorption and unfolding.

The adsorption-unfolding-desorption sequence of events is generally regarded to increase the propensity of protein aggregation through partially folded intermediates;
a recent report proposes that electrostatic interactions remain dominant.\textsuperscript{12} The relationship between mAb adsorption and the generation of particulates has also been demonstrated through competition with increasing polysorbate 20 concentrations, in agitated siliconized syringes.\textsuperscript{13} To more precisely capture the change in alignment, reorientation and conformation of adsorbed protein molecules as a function of surface fraction it is necessary to probe the interface using neutron reflection (NR).\textsuperscript{14} Such detailed data is well established for several surface active proteins encompassing a variety of structural motifs.\textsuperscript{15,16} Thus, it is expected that NR will play an increasingly important role in determining mAb surface active behavior at the level of the individual domains and thereby help mitigate the risk of adsorption induced unfolding and aggregation.

Prior work by our group in this area set out to study mAb adsorption at the air/liquid interface in the presence of polysorbate 80, which is widely added to mAb drug substances.\textsuperscript{17} Comparing Fc and Fab adsorption under similar conditions will lead to an understanding of how these fragments affect the behavior of the whole mAb. As a first step towards this goal, Fc and Fab domains were obtained by digestion and repurification of an IgG1 termed ‘COE-3’. The propensity of each domain versus the intact mAb to undergo reorientation and unfolding at the interface can then be related to the physicochemical properties of the two bulk phases which have a distinct physicochemical nature. Through the NR study, the amount and structural conformation of the adsorbed fragment and antibody layers at the air/water interface can be determined with the help of isotopic contrasts, achieved by varying the ratio of H\textsubscript{2}O and D\textsubscript{2}O. We show that adsorbed mAb is most accurately represented by a uniform layer, indicating no unfolding or the formation of polymer-like distributions along the surface normal direction. However, Fc and Fab behave differently and their respective contributions to the interfacial behavior of the mAb are quite different and explored herein. Because Fc is relatively more hydrophobic, it prefers to stay out of the surface of water. This study has demonstrated the power of NR in helping to investigate how the constituent fragments affect the amount and structural conformation of adsorbed mAb molecules.
2. Materials and Experimental Methods

2.1 Materials

The mAb used in this study, COE-3, was expressed in Chinese hamster ovary cells and purified using industry-standard methods by MedImmune. Roberts et al. have previously studied its solution behavior under a range of mAb concentrations. COE-3 is a full length human IgG1κ with molecular weight equal to 145,560 Da (assuming average glycans), isoelectric point (pI) of 8.44 and extinction coefficient at 280 nm of 1.43 (mLmg⁻¹cm⁻¹). It was supplied as a stock solution of 46.4 mg/ml in ‘His buffer’ (25 mM histidine/histidine hydrochloride, 7 % w/v sucrose pH 6.0, Batch No: SP12-423). Fc and Fab fractions were produced by papain digestion of COE-3 with exactly the same batch number, followed by protein A capture of Fc (Fab flow-through) and ultrafiltration into phosphate buffered saline (PBS), supplied as stock solutions at 55.6 mg/ml (BN: SP14-220, MW = 52,806 Da, pI = 6.36) and 45.0 mg/ml (BN: SP14-219, MW = 47,450 Da, pI = 9.64), respectively. The respective extinction coefficients at 280 nm were 1.36 for Fc and 1.43 for Fab (mLmg⁻¹cm⁻¹). Purity of the Fc and Fab fractions determined by high-performance size exclusion chromatography was 99.2 and 99.7 %, respectively.

The stock samples were stored under -80 °C and when needed, each was thawed and diluted directly into histidine buffer at pH 5.5, also at the ionic strength of 25 mM. Samples were diluted into subphases of different ratios of H₂O and D₂O under the same ionic strength and pH as specified. Phosphate buffers (sodium salts) were used to prepare buffer solutions at the other pHs (7, 8 and 8.8) studied in this work, again with the total ionic strength fixed at 25 mM. As the concentrations studied in this work were very low, typically below 1 mg/ml (1000 ppm), sample dilutions into D₂O introduced very low levels of H₂O, with the exact amount noted and taken into account during neutron data analysis. The translated sequences of the heavy and light chains are given in Table S1 of the Supporting Information, allowing the relevant physical properties such as scattering lengths (SL) and scattering length densities (SLD, ρ) to be calculated under different solvent isotopic contrasts (Table 1).

D₂O (99% D), histidine and histidine hydrochloride were purchased from Sigma-Aldrich and also used as supplied. H₂O was processed using an Elgastat PURELAB
water purification system. SL and SLD for H\textsubscript{2}O, D\textsubscript{2}O are given in Table S2 from which any different ratios of them can be calculated.

### 2.2 Neutron Reflection Measurements

NR measurements were carried out using the SURF reflectometer at ISIS Neutron Facility, Rutherford Appleton Laboratory, STFC, UK. The neutron optical system in SURF provides the neutron wavelength range typically between 0.5 and 6.9 Å. With the help of a supermirror setup, neutron reflectivity can be measured at the three incidence angles of 0.35, 0.8 and 1.5°, covering a momentum transfer range (\(\kappa\)) from about 0.01 to 0.5 Å. The reflectivity from a clean air/D\textsubscript{2}O surface was first measured as calibration. All the measurements were carried out 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 1, with further information about the elementary scattering lengths and means to calculate the SLs and SLDs of Fab, Fc and COE-3 under different water contrasts given. SL or SLD varies in response to the exact ratio of H\textsubscript{2}O and D\textsubscript{2}O as shown in Table 1. This arises from the exchanges of labile Hs on the amide bond linkages and polar and charged side chains with Ds from the solvent.
<table>
<thead>
<tr>
<th>Component</th>
<th>Contrast</th>
<th>SL/10^{-5} Å</th>
<th>SLD/10^{-6} Å^{-2}</th>
<th>V/ Å^{3}</th>
<th>MW/ gmol^{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>COE-03</td>
<td>NRW</td>
<td>35227</td>
<td>2.05</td>
<td>171740</td>
<td>145021</td>
</tr>
<tr>
<td></td>
<td>CM2.58</td>
<td>44345</td>
<td>2.58</td>
<td></td>
<td>145912</td>
</tr>
<tr>
<td></td>
<td>D2O</td>
<td>57648</td>
<td>3.36</td>
<td></td>
<td>147213</td>
</tr>
<tr>
<td>Fc</td>
<td>NRW</td>
<td>12014</td>
<td>2.01</td>
<td>59664</td>
<td>50033</td>
</tr>
<tr>
<td></td>
<td>CM2.58</td>
<td>15022</td>
<td>2.52</td>
<td></td>
<td>50326</td>
</tr>
<tr>
<td></td>
<td>D2O</td>
<td>19403</td>
<td>3.25</td>
<td></td>
<td>50753</td>
</tr>
<tr>
<td>Fab</td>
<td>NRW</td>
<td>11607</td>
<td>2.07</td>
<td>56038</td>
<td>47552</td>
</tr>
<tr>
<td></td>
<td>CM2.58</td>
<td>14661</td>
<td>2.61</td>
<td></td>
<td>47852</td>
</tr>
<tr>
<td></td>
<td>D2O</td>
<td>19123</td>
<td>3.41</td>
<td></td>
<td>48289</td>
</tr>
</tbody>
</table>

Table 1. The scattering length (SL), scattering length density (SLD, ρ), volume (V) and molecular weight (MW) of Fc, Fab and mAb (COE-3) used for the model fitting to neutron reflectivity. The exact sequences for the light and heavy chains in Fab and Fc are given in Table S1.
3. Results and Discussion

3.1 NR Measurements and Data Analysis

The relationship between the optical geometry of the neutron beam and the surface adsorbed mAb layer is schematically shown in Figure 1. Neutron reflectivity, $R$, is often plotted as a function of momentum transfer, $\kappa$, perpendicular to the reflecting surface:

$$\kappa = \frac{4\pi \sin \theta}{\lambda}$$

where $\theta$ is the beam incidence angle and $\lambda$ the wavelength. $R$ usually decays fast against $\kappa$ above the critical point.$^{19}$ As often observed in optical systems, interferences also occur in neutron reflection and scattering, with peak positions occurring by following $\kappa = 2n\pi/\tau$ (where $\tau$ denotes film thickness and $n$ is an integer). Hence, the larger the layer thickness, the lower $\kappa$ at which interference occurs.

![Figure 1](image)

**Figure 1.** Schematic representation of the optical geometry of neutron beam reflected from the mAb layer adsorbed on the surface of water.

Each mAb molecule is comprised of an Fc and 2 Fabs, with each of them having the shortest dimensions ranging from 40 to 50 Å.$^{20-22}$ Importantly, the surfaces of the Fc and Fab bear different charges and polar groups. Thus, they tend to show amphiphilic characteristics. As discussed above, it is useful to establish how Fc, Fab and mAb molecules adsorb individually. Since these molecules are much larger than typical surfactants and lipids that have been extensively studied by NR, the measurable reflectivity tends to fall over the low $\kappa$ range, mostly below 0.1 Å$^{-1}$.24
One of the main advantages offered by NR is that hydrogen and deuterium have different scattering lengths and opposite in sign. As a result, the scattering lengths (SL) for H\textsubscript{2}O and D\textsubscript{2}O are also opposite in sign. By mixing H\textsubscript{2}O and D\textsubscript{2}O, the scattering length density (SLD) of the aqueous subphase can thus be fine-tuned to cancel out (match) or highlight different parts of the system. As a result, the sensitivity to the interfacially adsorbed layer can be strongly enhanced. When 8.1\% D\textsubscript{2}O and 91.9\% H\textsubscript{2}O (by volume) are mixed, the SLD for the water mixture is zero. This mixed water is termed null reflecting water (NRW) as at the air/NRW interface no specular reflection occurs. Upon adsorption of mAbs or their fragments at this interface, specular reflection arises entirely from the adsorbed layer. Hence, the specular reflectivity only contains information about the adsorbed mAb layer in NRW, providing an accurate determination of the adsorbed amount and layer thickness.\textsuperscript{25}

Figure 2A shows the exemplar reflectivity profiles measured at 12.5 and 50 ppm (12.5 and 50 \(\mu\)g/ml) of Fc under pH 5.5 from the surface of NRW. The difference between the reflectivity profiles is very small, showing that an increase in bulk Fc concentration is only associated to a small increase in adsorbed amount. To avoid the possible time dependent effect, these reflectivity profiles were measured 3-5 hr after the surfaces were equilibrated.

The reflectivity profiles were analyzed using Motofit\textsuperscript{26}, and the algorithm was based on the optical matrix formalism as described by Born and Wolf.\textsuperscript{27} The fitting process started with the simulation of an interfacial model from which the reflectivity was calculated. The calculated reflectivity was then compared with the measured one and the process was iterated until the best fit was obtained from the \(\chi^2\) values from the fitting and visual assessment. The adsorbed layer may need to be divided into a series of sublayers to account for the structural changes along the surface normal direction.
and each of the sublayers is described by thickness ($\tau_i$), scattering length density ($\rho_i$) and roughness, where appropriate. A uniform layer model is often the easiest starting point for protein adsorption, where the volume fraction ($\phi_p$) in the layer can be expressed as:

$$\phi_p = \frac{\rho}{\rho_p}$$  \hspace{1cm} (2),

where $\rho$ and $\rho_p$ are the SLDs of the protein layer. The area per protein molecule ($A_p$) can be obtained using:

$$A_p = \frac{V_p}{\tau_p \phi_p}$$  \hspace{1cm} (3),

where $V_p$ is the volume of the protein and $\tau_p$ is the thickness obtained from the fit (in Å). The surface adsorbed amount or surface concentration ($\Gamma_p$, measured in mg/m$^2$) can be expressed as

$$\Gamma_p = \frac{MW}{6.023A_p}$$  \hspace{1cm} (4),

where MW is the protein molecular weight (in g/mol) and $A_p$ is in Å. ²⁵

Using the above approach, the best fits, shown as continuous lines through the measured reflectivity profiles in Figure 2A, led to $\Gamma_p = 1.3$ and 1.7 mg/m$^2$ from the Fc adsorption at the two concentrations, with thickness constant at around 40±2 Å. For comparison, Figure 2B shows the two reflectivity profiles measured from Fab adsorption under the same concentrations. The two reflectivity profiles differ both in their intensity and slope in this case, showing very different adsorbed amount and layer thickness. The best uniform layer fits led to $\Gamma_p = 0.5$ and 1.1 mg/m$^2$ from Fab adsorption at the two concentrations, with thicknesses at 45±2 and 50±2 Å, respectively. Unlike Fc, Fab adsorption is clearly concentration-dependent.

Figure 2C shows the two reflectivity profiles measured for the mAb under the same concentrations. Like Fab adsorption, the two reflectivity profiles differ both in intensity and slope, also showing the clear effect of mAb concentration. The best uniform layer fits revealed that $\Gamma_p = 1.0$ and 2.0 mg/m$^2$ at the two concentrations, with thicknesses at 45 and 50±2 Å, respectively. Thus, whilst changes in concentration affect layer thickness and the adsorbed amount in a manner similar to the Fab adsorption, the actual mass of mAb adsorbed almost doubled.
3.2 Time and Concentration Dependent Adsorption

From the reflectivity analysis approach as described above, the time-dependent adsorption processes were also examined, with the results shown in Figure 3A for Fc, Figure 3B for Fab and Figure 3C for mAb. As each reflectivity measurement requires a minimum of 40-60 min to achieve the statistically acceptable quality, any dynamic process faster than 1 hr would not be resolvable. It can be seen from Figure 3A that Fc can adsorb fast and tends to equilibrate within the first 1-2 hr as the adsorbed amount increases very slightly after this period. In contrast, Fab adsorption is clearly slower and the dynamic process occurs over the period of several hr. Over the low concentration range below 12.5 ppm, the dynamic process lasted over 10 hr. Over the intermediate concentration range of 25-50 ppm studied, the time-dependent adsorption occurred over the first 6-8 hr. At the highest concentration of 100 ppm studied, it reached equilibration after the first 2-3 hr. The main feature of the dynamic adsorption for mAb (Figure 3C) is broadly similar to that as described from Fab adsorption, that is, the adsorption tended to equilibrate faster with higher concentration, showing the dominant influence of the two Fabs to the behavior of the mAb.

The concentration-dependent adsorption is summarized in Figure 4, where the adsorbed amount was taken from the last points of the time-dependent data sets as shown in Figures 3A, 3B and 3C. Over the concentration range studied (below 100 ppm), the mass adsorbed from Fc (Figure 4A) is clearly higher than that obtained from Fab, but the Fc adsorbed amount shows a reluctant increase with increasing bulk concentration whilst that of the Fab shows a fast increase. This rate of concentration-
dependent increase is clearly inherited by the mAb and more interestingly, the amount of mAb adsorbed is also influenced by Fc: its adsorbed amount is greater than that of Fab but lower than that of Fc. However, the rate of concentration-dependent rise is similar to that of Fab. This leads the mass adsorbed amount for mAb to cross that of Fc at 15 ppm. Above this concentration, the mAb displays an adsorption above Fc and Fab. This means that the mAb combines the different features of its fragments to reach a newly balanced amphiphilicity that bears the distinct influences from its constituent fragments.

Figure 4. The equilibrated amount of adsorption in mg/m$^2$ (A) and nmol/m$^2$ (B) taken from the last point of each set of measurements in Figure 3 plotted against concentration for Fc (▲), Fab () and the whole mAb COE-3 (●).

Figure 4B shows that when the adsorbed amount is presented in nmol/m$^2$, the Fab and the mAb have almost identical adsorption curves against bulk concentration whilst the molar adsorbed amount of Fc is 2-3 times greater, again showing that Fc is significantly more hydrophobic.

3.3 Effect of Solution pH

The results as shown above were measured at pH 5.5. As the isoelectric points for Fc, Fab and mAb vary between pH 7 and 10 (Figure S1), pH shift affects their charges differently. To examine how surface adsorption is affected by solution pH, changes in adsorbed amount were also examined at several other pH values up to 8.8. The time-dependent change of the adsorbed mass of Fc is shown in Figure 5A, that for Fab in Figure 5B and that for mAb in Figure 5C. It can be seen from Figure 5 that whilst Fc adsorption tends to equilibrate within the first 2-3 hr Fab adsorption shows a steady rise with time, even after the first 10 hr. In contrast, mAb adsorption again bears features from the constituent fragments. At pH 5.5, there is no apparent time effect after the first 1 hr. The time-dependent adsorption becomes slightly more pronounced at pH 7 and 8 but the dynamic process becomes rather less obvious again at pH 8.8.
Taken together, these time-dependent variations are clearly less significant than what occurs in Fab adsorption but more substantial than the dynamic changes observed from Fc adsorption.

Figure 5. Adsorbed amount plotted against time measured at pH 5.5, 7, 8 and 8.8 with the concentration fixed at 50 ppm: (A) Fc; (B) Fab; (C) mAb COE-3.

Figure 6A shows the equilibrium adsorbed mass for Fc, Fab and mAb again by taking the last adsorbed amount measured at each time-dependent pH change in Figures 5A, 5B and 5C. It can be seen from Figure 6A that the equilibrium adsorbed mass from Fc is greater than that of Fab whereas that of the mAb is the highest under the fixed concentration of 50 ppm. From pH 5.5 to 8, the trend of pH-dependent change is small. From pH 8 to 8.8, Fc shows a trend of decrease in adsorption whilst Fab shows a rather sharp increase. The exact mechanistic process is unclear but the trend for the mAb is again more influenced by Fab adsorption, possibly because of the presence of 2 Fabs in each mAb. The pH dependent process together with the highest mass adsorption from the mAb shows the balanced amphiphilicity arising from both Fc and Fab segments.

When plotted in terms of nmol/m² for surface adsorption (Figure 6B), Fc shows the highest adsorption whilst the adsorbed amount for Fab and mAb is almost identical. In spite of changes in the relative orders of the adsorbed amount when plotted in mass and molar surface concentration, the pH effects remain the same.
Figure 6. Equilibrated amount of adsorption measured in mg/m$^2$ (A) and nmol/m$^2$ (B), taken from the last point of each set of measurements from Figure 5 plotted against pH for Fc (▲), Fab (●) and mAb COE-3 (●).

3.4 Changes in Layer Thickness and Extent of Immersion in Water

Figure 7 shows the thicknesses measured from equilibrated layers adsorbed under representative concentrations of Fc, Fab and mAb, at pH 5.5. The results clearly show very small changes in layer thickness despite large Fc, Fab and mAb concentration variations. Under the conditions studied, the adsorbed Fc layers remained constant at 40±2 Å whilst the thicknesses of Fab and mAb layers were 45 Å at 5 and 12.5 ppm, then slightly increasing to 50 Å at the two high concentrations of 25 and 50 ppm. This suggests possible adjustment of structural conformations in response to the increase of surface packing density with bulk solution concentration.

Table S1 shows the translated sequences of heavy and light chains of COE-3. Sequence alignment as shown in Table S3 reveals the complete identity between the COE-3 Fc and the Fc reported for a human IgG1 in the Protein Data Bank (PDB ID 1HZH) $^{20}$ and 96% similarity to another human Fc (PDB ID 1H3T). $^{21}$ In contrast, alignment of the COE-3 Fab sequence with that of the 1HZH Fab as shown in Table S4 reveals 74.5% similarity $^{20}$ and with that of another Fab reported by Tamada et al. with PDB ID 3X3G revealed 55% similarity only; $^{22}$ the differences reflect the nature of the variable (V$_{H}$/V$_{L}$) domains which are nevertheless folded consistently in the IgG superfamily. Figure S2 depicts the projections of the crystalline structure of Fc. It is approximately in a doughnut shape, with the outer diameter of 70 Å and height of 40 Å. In contrast, Figure S3 depicts the projections of the crystalline structure of Fab. It is approximately in a disc shape but still has a large ‘dent’ in the center, with the outer diameter of 80 Å and height of 45 Å. Figure S4 illustrates the projections of the whole mAb in which the fragments are packed with their short axial lengths aligned perpendicular to the substrate surface, resulting in a height of ca 45 Å and a width of
ca 100 Å. The thicknesses measured from NR are close to the short dimensions of Fab and Fc, suggesting that both Fab and Fc retain their globular structures, and that they do not suffer from any measurable deformation either. An increase in bulk concentration led to increased surface packing density but the Fc layers did not show any measurable change in thickness, although the Fab layers did increase from 45 to 50 Å arising from possible adjustment in structural conformation. Figure S2 shows that the charges on Fc are rather uniformly distributed. It can, however, be seen from Figure S3 that the surface viewed from the ‘left side’ bears distinctly more charged groups than that from the ‘right side’. The presence of unevenly balanced charge distributions could help the tilting of the Fab once the surface is sufficiently packed. The tilting of surface adsorbed lysozyme molecules has been previously observed by NR studies. Its transition of surface conformational orientations with rising packing density is also thought to be affected by the uneven charge distribution on the molecular surface.

![Figure 7](image.png)

**Figure 7.** Changes in layer thickness under the equilibrated adsorption taken from the last point of each set of concentration dependent adsorption measurements as shown in Figure 2, plotted against the concentration of Fc (blue), Fab (green) and mAb COE-3 (red) at pH 5.5.

In the case of the mAb, similar thicknesses to those of the Fab layers were observed. This implies that Fab and Fc are well aligned in the monolayer and that there is no stacking below or above each other. In contrast, bovine serum albumin (BSA) is a rod-shaped molecule that is loosely linked from 3 consecutive main domains. NR revealed the formation of layers of about 30 Å thick, close to its short axial length and indicative of the sideways-on adsorption. Increase in bulk concentration could lead to thickness increase to some 40 Å before a sublayer was formed under the main surface layer. Such structural transition is indicative of the structural flexibility of BSA as the 3 main domains are loosely linked up. This feature of structural transition is very different
from that of mAb and its segments. On the other hand, NR revealed that surface adsorption of lactoferrin caused structural unfolding from the exposure of the protein molecules to the unsymmetrical energetic stretching across the interface forming two distinct regions, with a top dense layer of 15-20 Å on the air side and a bottom diffuse layer of some 50 Å into the aqueous subphase. In contrast, small angle neutron scattering revealed that lactoferrin held its dimeric globular framework in aqueous solution, with the distance between the centers of the dimer varying in response to the ionic strength.

The pH-dependent thickness changes were also assessed and similar results were obtained for Fc, Fab and mAb, suggesting that pH variation did not cause measurable differences within the conditions studied. As shown from Figure 6B, increase in pH from 5.5 to 7 led to little change in their adsorption. Further pH increase from 8 to 8.8 led to modest decline in Fc adsorption but Fab adsorption showed a clear rise. The adsorption of mAb was dominated by the Fabs, modulated by Fc, resulting in a slight increase. Figure S1 depicts how the net charge on each fragment varies with increasing pH. Fc bears the lowest net charge over the pH range studied and this may explain why it adsorbs most on the molar basis as shown in Figure 6B. In contrast, Fab has the highest net charge and this fits to its persistent lowest adsorption. The mAb adopts an intermediate position, also consistent with its net charge distribution across the pH range studied. An absence of thickness change indicates that the structural conformations adopted by Fc, Fab and mAb molecules on the surface of water did not alter whilst their surface charges varied, implying their robustness against pH. In contrast to the pH-dependent changes as observed from surface adsorption of lysozyme and BSA, \(^{28,29}\) the relative changes in adsorbed amount and layer thickness from mAb and its segments are small in spite of the large decrease in their net charges with rising pH (Figure S1).

As already outlined, the reflectivity profiles measured from NRW reveal the area per molecule (\(A_p\)) and layer thickness from which the globular state and layer packing can be inferred. As shown in Table 2, \(A_p\) can be estimated from the uniform layer modelling, giving 5100 Å\(^2\) for Fc against its limiting value of 4500 Å\(^2\), and 6800 Å\(^2\) for Fab against its limiting value of 4000 Å\(^2\) as shown from the projections in Figure S2 and S3. The differences between them reveal the greater extent of layer packing from Fc due to its greater surface activity. Note that this only reflected the extent of layer packing at 50 ppm. In contrast, \(A_p\) was found to be about 12000 Å\(^2\) for the mAb
and is the same as the limiting area estimated from the lateral packing of 1 Fc and 2 Fabs, assuming that each adopts its own limiting area as described above. This reveals a closer packing for the mAb layer compared to its constituent Fc and Fab layers whilst also taking into account the projection as schematically shown in Figure S4.

The surface adsorbed layers of Fc, Fab and mAb can be fully immersed, fully afloat or somewhere intermediate. Parallel measurements in solvents with non-zero contrasts could be informative of the extent of immersion of the layer in water. As mentioned earlier, the exact isotopic contrast of the water as solvent can be changed by varying the ratio of H$_2$O and D$_2$O. When contrast matched to 2.58×10$^{-6}$ Å$^{-2}$ (CM2.58), the SLD of water exactly matched that of the mAb. Thus, only the mAb region that stays out of water is visible to NR and the remaining region immersed in water is indistinguishable from the solvent. The reflectivity measured from CM2.58 thus determines the thickness of the region above water. When measured from D$_2$O, both regions are visible but the total layer thickness and volume fraction must remain the same as measured from NRW. Changes in the ratio of H$_2$O and D$_2$O strongly influence the shape of the reflectivity profiles, as exemplified in Figure 8 from the adsorption of 50 ppm Fc, Fab and mAb under the 3 water contrasts. The large differences offer us confidence in revealing the extent of layer immersion in water through simultaneous model fitting.

In D$_2$O, the immersed part of a protein layer must be fitted by taking into account the contribution from the solvent for space filling as well, with the total of protein volume fraction ($\phi_p$) and solvent volume fraction ($\phi_w$) being equal to unity. The SLD contributions are related to the interfacial composition via the following equation$^{31,32}$:

$$\rho_2 = \rho_p\phi_p + \rho_w\phi_w$$  \hspace{1cm} (5)

By undertaking simultaneous fitting to the reflectivity profiles measured under NRW, CM2.58 and D$_2$O, we can not only determine the extent of protein layer immersion into water but also confirm the total protein layer thickness and volume fraction as obtained from NRW alone previously.

Figure 8 shows the best fits to the 3 sets of the 3 reflectivity profiles measured at 50 ppm and pH 5.5 for Fab, Fc and mAb, with the matching structural parameters listed in Table 2. All 3 reflectivity profiles measured in CM2.58 could be fitted to 10-15Å, implying partial immersion into water. In the case of Fc adsorption, the D$_2$O profile
could be fitted to an upper layer of 15±2 Å in air and a lower layer of 25±2 Å in water and the total thickness of 40±2 Å, consistent with the value obtained from the fitting to the NRW profile. In addition, the volume fraction of the Fc in CM2.58 and D$_2$O was kept the same within experimental errors. Therefore, the only parameters that varied were the upper and lower layer thicknesses and the matching SLDs, with the total thickness and volume fraction kept the same as those obtained from the fitting to the NRW profile. For the adsorption of Fab, the thickness of the lower layer was fitted into 40±2 Å and this together with the upper layer of 10±2 Å makes the total of 50±2 Å, also consistent with that from the fitting of the NRW profile. In contrast, the best fitting to the 3 reflectivity profiles measured for mAb produced similar structural parameters as obtained from Fab adsorption, suggesting an inter-domain flexibility during surface adsorption.

The outcome of the data analysis is also shown as schematic drawings in Figure 8, where it can be seen that both Fc and Fab fragments are partially immersed. The total Fc layer is thinner than that of the Fab layer, but the mAb layer is maintained under similar thickness to that of the Fab layer, showing that all the fragments are also packed in parallel in the mAb layer. As also evident from Table 2, Fc shows preferable adsorption out of the surface of water because of its relatively more hydrophobic nature.

Both lysozyme and BSA layers were found to have the portion of some 10-15 Å exposed to air, with the exact extent being dependent on solution conditions. Thus, this feature of 10-15 Å of the top region being exposed to air seems to be rather common for the adsorbed protein layers. This feature is also evident from Table 2 for all adsorbed layers studied here; as the Fc is less charged and more hydrophobic its top layer of 15 Å in air occupies a greater fraction, although this effect does not appear in the mAb layer.

The concentration range of Fab, Fc and mAb studied in this work was relatively low due to the limited amount of samples available. However, the nature of surface adsorption of proteins is such that they tend to reach saturation at rather low bulk concentrations. As evident from Table 2, the average volume fraction of mAb or its fragments packed in the layer at 50 ppm reached some 0.3. At such a high packing density, it is likely that physical interactions occur within the adsorbed layer, causing structural deformation or entanglement, with different implications on their ability to desorb. This is an aspect that will be pursued in future work.
These results present some biopharmaceutical considerations: i) modelling the net surface charge during the design and engineering of a mAb candidate provides information that can be correlated to adsorption behavior; ii) alteration of the formulation buffer pH, particularly over the mildly acidic range commonly used for mAbs, does not have significant impact on adsorption behavior; iii) adsorption at relatively high surface packing densities is not necessarily associated with extensive conformational unfolding, though the effect of packing on desorption needs further investigation; iv) high surface packing densities exist even at low bulk concentrations and may impact the dose administered via a giving set containing very low mAb concentrations.
Figure 8. Neutron reflectivity profiles (left) measured at 50 ppm of Fc (A), Fab (B) and mAb (COE-3, C) on the surface of water under NRW, CM2.58 and D_2O at pH 5.5 (His buffer, ionic strength = 25 mM) and schematic representations (right) of the surface adsorbed Fc (D), Fab (E) and mAb (F) layers.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Contrast</th>
<th>Thickness/Å</th>
<th>SLD×10⁶/Å²</th>
<th>Volume fraction</th>
<th>Area per molecule/Å²</th>
<th>Adsorbed amount/mgm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fc 50ppm, pH 5.5</td>
<td>NRW</td>
<td>40</td>
<td>0.583</td>
<td>0.29</td>
<td>5100</td>
<td>1.61</td>
</tr>
<tr>
<td></td>
<td>CM 2.58</td>
<td>15</td>
<td>0.79</td>
<td>0.31</td>
<td></td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>D₂O layer 1/2</td>
<td>15/25</td>
<td>1/5.47</td>
<td>0.31/0.27</td>
<td></td>
<td>0.65/0.96</td>
</tr>
<tr>
<td>Fab 50ppm, pH 5.5</td>
<td>NRW</td>
<td>50</td>
<td>0.340</td>
<td>0.16</td>
<td>6800</td>
<td>1.16</td>
</tr>
<tr>
<td></td>
<td>CM 2.58</td>
<td>10</td>
<td>0.415</td>
<td>0.16</td>
<td></td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>D₂O layer 1/2</td>
<td>10/40</td>
<td>0.55/5.83</td>
<td>0.16/0.16</td>
<td></td>
<td>0.23/0.93</td>
</tr>
<tr>
<td>COE-3 50ppm, pH 5.5</td>
<td>NRW</td>
<td>50</td>
<td>0.59</td>
<td>0.29</td>
<td>12000</td>
<td>2.01</td>
</tr>
<tr>
<td></td>
<td>CM 2.58</td>
<td>10</td>
<td>0.85</td>
<td>0.33</td>
<td></td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>D₂O layer 1/2</td>
<td>10/40</td>
<td>1.1/5.5</td>
<td>0.33/0.27</td>
<td></td>
<td>0.46/1.55</td>
</tr>
</tbody>
</table>

**Table 2.** Structural parameters obtained from the best fits to NR profiles measured under 3 solvent contrasts from adsorption of Fab, Fc and mAb (COE-3) at 50 ppm and pH 5.5
4. Conclusions

NR has been used in this work to study the surface adsorption of a mAb and its constituent Fab and Fc through parallel studies under different water contrasts to help highlight interfacial structural resolution. The results have revealed that Fc adsorbed fastest and the dynamic process tended to equilibrate over the first 1hr, with the highest adsorbed amount in nmol/m$^2$ observed. In contrast, Fab adsorbed slowest and the dynamic process was concentration-dependent with the lower concentration showing the longer equilibration time. Its adsorbed amount in nmol/m$^2$ was the lowest. The dynamic process and the adsorbed amount of the mAb were dominated by the two Fabs, with evident influence from Fc. The different behavior appears to be well correlated to the net charges they bear: Fc carries a much lower net surface charge than Fab and is thus far more surface active. As the bulk concentration increased, Fc layers remained at about 40 Å; Fab and mAb layers were about 45-50 Å thick, showing that all adsorbed layers adopted the conformations with their short axial dimensions perpendicular to the water surface. The slight thickness increase in the case of Fab and mAb was indicative of conformational adjustment with increasing concentration. The net charge on Fab, Fc and mAb varied as pH increased from 5.5 to 8.8, but these charge differences did not appear to cause any major changes in adsorbed amount, although there was a small and opposite trend of adsorption between Fab and Fc from pH 8 to 8.8. The layer thicknesses remained little changed, showing that mAb and its constituent fragments remained globular and held similar conformations, even though they carried different charges. Although the concentration range studies were low, the average surface volume fraction from the adsorbed layer at 50 ppm reached 0.3, which is quite high for protein systems. Under such a high surface packing density, molecules can easily become deformed and entangled. Future work will examine how desorption is affected by different surface packing density.

Supporting Information

The Supporting Information including sequences for the light and heavy chains of the mAb studied here and comparisons against the one with crystalline structures of its fragments is available free of charge on the ACS Publications webpage.
Acknowledgements

We thank funding support from MedImmune Ltd, neutron beam times awarded to undertake this work at ISIS Neutron Facility, Chilton, Didcot, under the support of STFC. ZL acknowledges studentship support from University of Manchester via an Overseas Research Scholarship (ORS) award and a physics research merit award. CS acknowledges a joint PhD studentship from STFC and Unilever.
References


Support Information

Table S1. The antibody molecule is comprised of 2 identical light and heavy polypeptide chains.

The non-modified sequence of the Light Chain:
DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAAASS
LQSGVPSRFSGSGTDTTLTISSLQPEDFATYYCQYSYSTPLTFGGGSKEIK
RTVAAPSVFIFPSDEQLKSGTASVCLLNNFYPREAKVQWKVDNALQSNGS
QESVTEQDSDKSTYSLSSTLCLSKADYEKHKVYACEVTGHQGLSSPVTKSFNR
GEC

The non-modified sequence of the Heavy Chain:
QVNLRESGGGLVQPGGSLRLSCAASGFTFGSYAMSWVRQAPGKGLEWVSAI
SGSGSTYYADSVKGRFTISRDNSKNSLYLQMNLRAEDTAVYVCARRSIYG
GNYYFDYWGRGTLYVSSASTKGSVPFSAPSSKSTSGTAALGCLVKDYFP
EPVTWSWNGALTGVHTFAVLSGSRLSSVTVPSSSLGTQTYICNVNH
KPSNTKVDKKEPKSCDKTHTCPACPPELLGGSVPFLFPPKPKDTELMSRTYP
EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTPREEQYNSTYRVVSVLT
VLHQDWLNGKEYKCKVSNKALPAPIEKTISAKGQPREPQVYTLPPSRDELTV
KNQVSLTCLVKGFLPSDIAVEWESNGQPENNYKTTPVLDSDGSFYYSLKTV
VDKSRWQQGKVSCVMHEALHNHYTQKSLSPGK

The sequences were entered into the Biomolecular Scattering Length Density Calculator (http://psldc.isis.rl.ac.uk/Psldc/) and the influence of the isotopic contrast of water was set accordingly to obtain the respective scattering lengths (SL) and scattering length density (SLD) under NRW, CM 2.58 and D$_2$O, with the results shown in Table 1.

Following papain digest, each non-modified Fab will be composed of the Heavy Chain sequence in red text and the whole of the Light Chain; each non-modified Fc will be composed of the Heavy Chain sequence in blue, existing as a dimer. The N-glycan at Asn (underlined) is predominantly G0f [2].
Table S2. Key parameters used for H2O and D2O

<table>
<thead>
<tr>
<th></th>
<th>Molecular structure</th>
<th>SL×10^5 Å²</th>
<th>Volume Å³</th>
<th>SLD×10^6 Å⁻²</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O</td>
<td>-1.68</td>
<td>30</td>
<td>-0.56</td>
<td></td>
</tr>
<tr>
<td>D2O</td>
<td>19.14</td>
<td>30</td>
<td>6.35</td>
<td></td>
</tr>
</tbody>
</table>

Tables S3. Amino acid sequence comparison between Fc of COE-03 and Fc of 1HZH.

<table>
<thead>
<tr>
<th>No. of amino acid residues</th>
<th>1HZH Fc</th>
<th>COE-3 Fc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>30</td>
</tr>
</tbody>
</table>

Table S4. Amino acid sequence comparison between Fab of COE-3 and Fab of 1HZH.

<table>
<thead>
<tr>
<th>No. of amino acid residues</th>
<th>Heavy Chain</th>
<th>Light Chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1HZH Fab</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1COE Fab</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Similarity</td>
<td>74.49%</td>
<td></td>
</tr>
</tbody>
</table>

**Figure S1.** Charge distributions as a function of pH for Fab and Fc and one third of the whole COE-3 (1/3 contribution from 1 Fc and 2 Fabs). These charge distributions were calculated with a Debye-Hückel model for pKa changes due to charge-charge interactions, applied at an ionic strength of 0.025 M to mAb, Fc and Fab models previously generated for COE-3 (Roberts et al Mol Pharm 2014 11:2475).

Note that these charge variations against pH as calculated from the constituent amino acids are different from the measured IP values from real experimental measurements due to the ignorance of charges on sugar groups and associations with ions from bulk solution.
Figure S2. Projections of the 3D Fc crystalline structure with estimated width and height marked: (a) Front view; (b) Rear view; (c) Rightside view; (d) Leftside view; (e) Top view; (f) Bottom view. Blue color marks basic amino acid groups and red color marks acidic amino acid groups.

Fc of 1HZH:
**Figure S3.** Projections of the 3D crystalline structure of Fab (1HZH) with estimated width and height marked: (a) Front view; (b) Rear view; (c) Rightside view; (d) Leftside view; (e) Top view; (f) Bottom view. Blue color marks basic amino acid groups and red color marks acidic amino acid groups.

Fab of 1HZH:
**Figure S4.** Projections of the 3D crystalline structure of the whole antibody1HZH with estimated width and height marked: (a) Front view; (b) Rear view; (c) Rightside view; (d) Leftside view; (e) Top view; (f) Bottom view. Blue color marks basic amino acid groups and red color marks acidic amino acid groups.
Chapter 4

- Abstract 107

1. Introduction 108

2. Neutron Reflection and Isotopic Contrast Variation 110

3. Results 113

4. Discussion 126

5. Materials and Methods 127

- References 130

- Supporting Information 133
Antibody adsorption on the surface of water studied by neutron reflection

Charles Smith[1], Zongyi Li[1], Robert Holman[1], Fang Pan[1], Richard A Campbell[2], Mario Campana[3], John R P Webster[3], Steve Bishop[5], Rojaramani Narwal[5], Shahid Uddin[4], Christopher F van der Walle[4], Jian R Lu[1]

[1] Biological Physics Laboratory, School of Physics and Astronomy, University of Manchester, Oxford Road, Schuster Building, Manchester M13 9PL, UK.


[3] ISIS Neutron Facility, STFC, Chilton, Didcot OX11 0QZ, UK.


[5] Formulation Sciences, MedImmune LLC, Gaithersburg, MD 20878, USA.

Corresponding author: Jian R Lu
(email: j.lu@manchester.ac.uk; tel: +44 161 2003926)

mAbs, 2017, 9 (3), pp 466-475
DOI: 10.1080/19420862.2016.1276141

Keywords:
Antibody, co-adsorption, mAbs, neutron reflection, surface adsorption, structural unfolding, self-assembly, surfactant
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 is to demonstrate the power of neutron reflection (NR) 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 H₂O and D₂O, 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 (Tween 80), the exact amount of surfactant in the mixed layer could also be determined by using both hydrogenated and deuterated surfactants. Our data revealed that although the surfactant started to remove antibody from the surface at 1/100 CMC (critical micelle concentration) of the surfactant, complete removal was not achieved until above 1/10 CMC. Neutron reflection data also revealed that antibody molecules retained their globular structure when either adsorbed by themselves or co-adsorbed with the surfactant under the conditions studied.
1. Introduction

Monoclonal antibodies (mAbs) represent an increasingly important class of therapeutic drugs in today’s pharmaceutical pipeline [1]. High concentration liquid formulations are a prerequisite for low volume subcutaneous injections in order 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 a number of destabilization processes [2]. Mechanisms leading to protein aggregation in bulk solution in the context of formulation have been widely reviewed [3]. Briefly, they include covalent changes such as oxidation and deamination, and physical changes arising from protein-protein interactions and surface adsorption-desorption [4, 5]. 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 [6,7]. Aggregation leading to particulate formation may be accelerated during manufacturing processes, in particular, fill-finish activities wherein the mAb is exposed to interfacial stresses [8]. Particulate limits are set by the pharmacopeias: for each unit dose referred to here, there must be less than 6000 particles > 10 μm and less than 600 particles > 25 μm (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 [9-11].

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 utilized simple agitation to simulate high shear rates for different surface roughness conditions, as could be encountered during fill-finish [12]. 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 [13]. An elegant experimental approach using a 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 [14].
However, characterizing the precise molecular nature of adsorbed mAbs at an interface 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 [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 then being given to pathways involving desorption, protein-protein self-assembly (oligomers), the formation of soluble aggregates which 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 [17]). Neutron reflection has enabled molecular models of protein adsorption to be constructed [18], has been used to determine the orientation of antibodies non-specifically adsorbed at the solid/liquid interface [19], and has yielded the structural characterization of membrane proteins in bilayer models resting on a water-filled layer [20].

Current approaches to mitigate chemical and physical instabilities require the addition of buffers and excipients to mAb formulations [21]. In regard of protein adsorption and aggregation as discussed above, the industry generally employs non-ionic surfactants such as Polysorbate 20 and 80 (Tween® 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 [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 to polysorbate-mediated inhibition of ‘gelation’ of the adsorbed mAb interfacial film [23].
The main aim of this study is 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$_2$O and D$_2$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. 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.

2. Neutron Reflection and Isotopic Contrast Variation

Figure 1a provides a schematic representation of the relationship between the optical geometry of the neutron beam setup and the surface adsorbed antibody layer. 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

$$\kappa = \frac{4\pi \sin \theta}{\lambda}$$  \hspace{1cm} (1)$$

where $\theta$ is the beam incidence angle and $\lambda$ the wavelength. $R$ is mostly characterized by a broadly decaying shape when plotted against $\kappa$ [24], but as 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 $\tau$. As antibody molecules are largely globular and their dimensions 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 (SL) 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/NRW interface, where NRW stands for null reflecting water consisting of 8.1% D$_2$O and 91.9% H$_2$O 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 $2.58 \times 10^{-6}$ Å$^{-2}$ (close to the equal ratio of H$_2$O and D$_2$O, termed CM2.58). When measured in D$_2$O, 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 two 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 Figure 1 could be varied by adjusting the ratio of H$_2$O to D$_2$O in a given neutron reflection measurement.

Under the co-adsorption of surfactant and antibody (Figure 1b), it is possible to resolve the different adsorbed amount from the two surface active species because the use of d- and h-Surf under a given surfactant concentration gives us 2 different reflectivity profiles. Changes of SL 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.
Figure 1. Schematic representation of (a) the optical geometry of the incoming and exiting neutron beam with respect to the adsorbed antibody layer and (b) the co-adsorption of antibody and surfactant where surfactant molecules could be hydrogenated (h-Surf) and ethoxylate head deuterated (d-Surf) in the case of polysorbate (Tween®) 80.

The reflectivity profiles were analysed using Motofit [27], which uses an optical matrix formalism as described by Born and Wolf [28] 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 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:

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

where $MW$ is the molecular weight of the antibody (in g/mol), $\sum b_p$ is its scattering length (in Å), $\tau_p$ the thickness obtained from the fit (in Å) and $\rho_p$ the scattering length density (in Å$^{-2}$), 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 (Å) and metre (m). The area per molecule ($A_p$) can be obtained using:

$$A_p = \frac{1}{\Gamma_p N_A}$$
For the measurements in D$_2$O, 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 ($\phi_p$) and solvent volume fraction ($\phi_w$) being equal to unity. To ensure the SLD contributions consistent to the interfacial composition we used the following equations [29, 30]:

$$\rho_2 = \rho_p \phi_p + \rho_w \phi_w$$  \hspace{1cm} (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:

$$(\rho \tau)_d = N_A (\Gamma_{sd} b_{sd} + \Gamma_p b_p) = \frac{b_{sd}}{A_{sd}} + \frac{b_p}{A_p}$$  \hspace{1cm} (5)

$$(\rho \tau)_h = N_A (\Gamma_{sh} b_{sh} + \Gamma_p b_p) = \frac{b_{sh}}{A_{sh}} + \frac{b_p}{A_p}$$  \hspace{1cm} (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.

3. Results

3.1 Surface Tension Measurements

The surface tension changes of both hydrogenated and ethoxylate 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 Figure 2a. It can be seen that whilst 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 polymerisation, they are comprised of a range of sizes which may have subtle differences in surface activity.

For convenience, we have however taken the surface tension readings at 8000 seconds as the equilibrated values. Figure 2b compares these values measured from the two differently labelled 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 µM, the widely cited CMC (critical micellar concentration) of this surfactant [31, 32]. Note that the surface tension continues to fall albeit that the rate of decrease slows down substantially. This is very typical of this type 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 have chosen to fix the mAb concentration at 50 ppm throughout this study to minimize the amount of mAb used. Figure 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 (Figure 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.
Figure 2. Surface tension profiles measured from (a) h-Surf over a range of surfactant concentration plotted against time (plots from top to bottom represent separately: 0.0001, 0.0003, 0.001, 0.003, 0.01, 0.03, 0.1 and 0.3 mM), (b) both h-Surf and d-Surf taking the readings at 8000 seconds, the longest time measured, (c) the same plots as in (a) but containing 50 ppm of COE-3 and (d) h-Surf with and without 50 ppm of COE-3, taking the readings at 8000 seconds. The continuous lines in (b) and (d) were drawn to indicate the occurrence of the CMC around 0.012 µM.

3.2 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. Figure 3 shows a set of reflectivity profiles measured at several representative antibody concentrations, plotted as log [R] versus log[κ] for better visualization. As 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 ageing). It can be seen from Figure 3 that 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 whilst 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 is to adopt the model fitting based on the optical matrix formula [27,28]. The continuous lines shown in Figure 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 Figure 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.

Figure 3. Neutron reflectivity profiles measured on the surface of null reflecting water (NRW) from COE-3 adsorption at 4 representative concentrations of 5, 25, 50 and 100 ppm under the His buffer of pH 5.5 (25 mM ionic strength). The continuous lines denote the best uniform layer fits with thickness and SLD given in Table 1. The scatter of the data indicates the statistical errors in each reflectivity profile measured.

Figure 4 shows the changes of layer thickness and adsorbed amount obtained from the best uniform layer fitting. Over the concentration range studied from 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].

Figure 4. Changes in the amount (○) and layer thickness (▲) from COE-3 adsorption plotted as a function of COE-3 solution concentration. The 3 data points measured at 48 and 50 ppm indicate the error range of the data.

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]:

$$h_{pp}(\kappa) = \frac{R\kappa^2}{16\pi^2b_p^2} = \frac{1}{A^2}\exp\left(-\frac{\kappa^2\sigma^2}{8}\right)$$

(7)

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

(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 $\ln[h_{pp}(\kappa)]$ versus $\kappa^2$ as shown in Equ. (8). In contrast to the values of $\tau$ based on the uniform layer model, the corresponding $\sigma$ values should be smaller by about 10%.
Table 1. Structural parameters obtained from the best uniform layer fits to the adsorbed COE-3 layers on the surface of NRW water at pH 5.5. The numbers in brackets were obtained from the kinematic approach using Equ (8) by assuming Gaussian distribution. Note the 3 repeats at 50 ppm were to check the reproducibility of the measurements from independently prepared solutions.

<table>
<thead>
<tr>
<th>[COE-3]/ppm</th>
<th>τ (σ) ±4/Å</th>
<th>ρ±0.02/10^{-6}Å^{-2}</th>
<th>Γ±0.15/mgm^{-2}</th>
<th>Α±200/Å²</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>45</td>
<td>0.31</td>
<td>0.95</td>
<td>25200</td>
</tr>
<tr>
<td>5</td>
<td>45 (48)</td>
<td>0.40</td>
<td>1.23 (0.98)</td>
<td>19600 (24500)</td>
</tr>
<tr>
<td>10</td>
<td>45</td>
<td>0.45</td>
<td>1.38</td>
<td>17400</td>
</tr>
<tr>
<td>25</td>
<td>50 (48)</td>
<td>0.51</td>
<td>1.74 (1.38)</td>
<td>13800 (17400)</td>
</tr>
<tr>
<td>50</td>
<td>50 (47)</td>
<td>0.56</td>
<td>1.91 (2.10)</td>
<td>12600 (11500)</td>
</tr>
<tr>
<td>50</td>
<td>53</td>
<td>0.54</td>
<td>1.95</td>
<td>12300</td>
</tr>
<tr>
<td>50</td>
<td>48</td>
<td>0.57</td>
<td>1.86</td>
<td>12870</td>
</tr>
<tr>
<td>100</td>
<td>55 (52)</td>
<td>0.65</td>
<td>2.45 (2.66)</td>
<td>9800 (9060)</td>
</tr>
</tbody>
</table>
Figure 5 shows the plots of \( \ln[h_{pp}(\kappa)] \) versus \( \kappa^2 \) converted from reflectivity profiles as shown in Figure 2, but the new plots enable us to interpret the data by fitting the straight lines using Equ 8. It can be seen from Figure 5 that the measured data appear to become more scattered and this is certainly the case for the two 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. For the 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 suffers from 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.

![Figure 5](image)

**Figure 5.** Plots of \( \ln[h_{pp}(\kappa)] \) versus \( \kappa^2 \) converted from reflectivity profiles measured at 5, 25, 50 and 100 ppm at pH 5.5 and an ionic strength of 25 mM (His buffer). The straight lines denote the best fits using Equ 8 with the values of \( \sigma, \lambda \) and \( \Gamma \) listed in brackets in Table 2. The extent of scatter indicates the statistical errors in each data set.

It should be noted that like other proteins, COE-3 has labile hydrogen atoms associated with polar groups such as \(-\text{NH}_2\), \(-\text{OH}\) and \(-\text{NH}_-\), 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.

Table 2 shows that when the SLD of the solvent increases from 0 to \(6.35 \times 10^{-6} \text{Å}^2\) that for the antibody increases from \(2.05 \times 3.36 \times 10^{-6} \text{Å}^2\). At \(SLD = 2.58 \times 10^{-6} \text{Å}^2\), the two 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 [34]. Figure 6 shows the reflectivity measured at 50 ppm of the antibody concentration together with the best uniform layer fit, giving a thickness of \(12 \pm 2 \text{Å}\) 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 Figure 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 = \((0.56 \pm 0.03) \times 10^{-6} \text{Å}^2\), giving \(\varphi_p = 0.27 \pm 0.05\). 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 two other water contrasts, but the bottom layer was represented by the best fitted thickness of 35 \(\pm 3 \text{Å}\) 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.
Figure 6. Reflectivity profiles measured from the adsorption of 50 ppm COE-3 on the surface of NRW, CM2.58 (contrast matched to mAb) and D$_2$O at pH 5.5 (His buffer, ionic strength of 25 mM). The continuous line through the NRW profile represents the uniform layer with $\tau = 50$ Å and $\rho = 0.56 \times 10^{-6}$ Å$^2$; part of the layer that stays out of the surface of the CM2.58 contrast water was measurable, with $\tau = 12$ Å and $\rho = 0.71 \times 10^{-6}$ Å$^2$, showing the same volume fraction as that from the NRW profile; the D$_2$O profile was fitted with $\tau_1 = 12$ Å and $\rho_1 = 0.90 \times 10^{-6}$ Å$^2$ for the surface layer exposed to air and $\tau_2 = 35$ Å and $\rho_2 = 5.85 \times 10^{-6}$ Å$^2$ for the remaining layer immersed in water, apart from the slightly higher SLD for the immersed layer the fitting was entirely consistent with the other two contrasts.

3.3 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 minimising surface-induced structural unfolding. Whilst this is widely 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 is 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 whilst 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 whilst 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 $\tau \rho$, though 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 two noticeable effects. Note that the actual amount of antibody adsorbed varies with surfactant adsorption as 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 $b_{sd}$ and $b_{sh}$ (the scattering length for d-Surf and h-Surf, respectively). Figure 7 shows the products of $\tau \rho$ measured from co-adsorption of h-Surf and d-Surf with the concentration of antibody fixed at 50 ppm. 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, $(\tau \rho)_{sd}$ 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 $(\tau \rho)_{sh}$ whilst $(\tau \rho)_{sd}$ 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 $(\tau \rho)_{sd}$ keeps rising sharply; the difference reflects the contributing effects from $b_{sd}$ and $b_{sh}$. Thus, without solving the combined Equs (5) and (6), Figure 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.
Figure 7. Plots of thickness × SLD ($\tau\rho/10^6$ Å²) versus the concentration of surfactant [expressed as the fraction of CMC] for both h-Surf and d-Surf, with the concentration of COE-3 fixed at 50 ppm. The product from the binary mixture of d-Surf and COE-3 is marked in blue diamonds ((τρ)$_{sd}$) and that from the mixture of h-Surf and COE-3 in red squares ((τρ)$_{sh}$). The τρ data from d-Surf alone are shown in green triangles.
Figure 8. Neutron reflectivity profiles measured from the co-adsorption of COE-3 and surfactant from NRW at pH 5.5 (His buffer, ionic strength 25 mM) with the COE-3 concentration fixed at 50 ppm but with varying concentrations of d-Surf and h-Surf at 1/2 CMC (a, top); at 1/20 CMC (b, middle) and 1/100 CMC (c, bottom).
To further exemplify the changes in surface composition and layer thickness as a result of surfactant co-adsorption, Figure 8 shows three 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 Figure 7, the reflectivity profiles shown in Figure 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 (Figure 8c), COE-3 adsorption is dominant and surfactant co-adsorption is minor due to its very low concentration. Nevertheless, the difference between the two reflectivity profiles is already obvious. Data analysis revealed that whilst the total layer thickness remained at \( \tau = 48 \pm 4 \text{ Å} \), the values for \( A_p = 17000 \pm 3000 \text{ Å}^2 \) and \( A_s = 2500 \pm 400 \text{ Å}^2 \) 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 \( \tau = 48 \pm 4 \text{ Å} \) but \( A_p = 19000 \pm 3000 \text{ Å}^2 \) and \( A_s = 1600 \pm 400 \text{ Å}^2 \) indicated further desorption of COE-3 (Section 3, Supporting Information). At 1/20 CMC of surfactant (Figure 8b), the gap between the two reflectivity profiles becomes wider, indicating the more dominant contribution from the adsorbed surfactant. The total layer thickness was slightly reduced to \( \tau = 44 \pm 4 \text{ Å} \) with a concomitant fall in \( A_p = 35000 \pm 4000 \text{ Å}^2 \) (\( \Gamma = 0.7 \pm 0.3 \text{ mg.m}^{-2} \)) and \( A_s = 300 \pm 30 \text{ Å}^2 \). This trend continues on further increase in surfactant concentration to 1/10 CMC, with the total layer thickness remaining at \( \tau = 44 \pm 4 \text{ Å} \) but \( A_p = 53000 \pm 4000 \text{ Å}^2 \) (\( \Gamma = 0.4 \pm 0.3 \text{ mg.m}^{-2} \)) and \( A_s = 260 \pm 30 \text{ Å}^2 \). At a surfactant concentration of 1/5 CMC, the total layer thickness declines to 35 Å and \( A_p \) falls into the error margin, with \( A_s = 170 \pm 15 \text{ Å}^2 \). 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 (Figure 8a), the layer thickness remains almost constant at 35 ± 3 Å and \( A_s = 150 \pm 15 \text{ Å}^2 \). 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.
4. Discussion

The results described above demonstrate that neutron reflection in combination with deuterium labelling 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 near 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.

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 1/5 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 (Figure 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. Figure 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 (Figure 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.

5. Materials and Methods

5.1 Materials

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 µM from surface tension measurements, consistent with the value reported from literature [31, 32]. The deuterated Polysorbate 80 (20 ethoxylate groups deuterated only, denoted as d-Surf) was synthesised by reacting the sorbitan ester, sorbitan monooleate with deuterated ethylene oxide following the standard procedures [35]. Its CMC was also checked by surface tension measurements and the comparison with h-Surf is given in Figure 1b.

The antibody, denoted as COE-3, was expressed in Chinese hamster ovary cells and purified using industry-standard methods. The solution behaviour of COE-3 under different mAb concentrations and ionic strengths has been studied by Roberts et al. [36]. 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 stabilise 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 Support 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 Elgastat PURELAB water purification system. SL and SLD for basic elements are given in Table SI1 from which the SL 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.

5.2 Surface Tension Measurements

Surface tension measurements were made using a Krüss K11 tensiometer. The du Noüy 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 carried out 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 three times, to ensure the reproducibility of the measurements.

5.3 Neutron Reflectivity Measurements

Neutron reflection measurements were carried out 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 provide the neutron wavelength range typically between 0.5 and 7 Å. Neutron reflectivity can be measured at the three incidence angles of 0.35, 0.8 and 1.5°, covering a momentum transfer range (kz) 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 two 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 carried out 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 (SLs) and scattering length
densities (SLDs) of the surfactants (h-Surf and d-Surf) and COE-3 under different water contrasts given in Sections 1 and 2 of the Support Information.

<table>
<thead>
<tr>
<th>Component</th>
<th>Contrast</th>
<th>$\Sigma_b/10^5$ Å</th>
<th>SLD$/10^{-6}$ Å$^{-2}$</th>
<th>V/Å$^3$</th>
<th>MW/gmol$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>COE-3</td>
<td>NRW</td>
<td>35227</td>
<td>2.05</td>
<td>171740</td>
<td>144754</td>
</tr>
<tr>
<td></td>
<td>CM2.58</td>
<td>44309</td>
<td>2.58</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D2O</td>
<td>57648</td>
<td>3.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>d-Surf</td>
<td>NRW</td>
<td>949</td>
<td>5.13</td>
<td>1850</td>
<td>1310</td>
</tr>
<tr>
<td></td>
<td>D2O</td>
<td>988</td>
<td>5.28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>h-Surf</td>
<td>NRW</td>
<td>116</td>
<td>0.63</td>
<td>1850</td>
<td>1310</td>
</tr>
<tr>
<td></td>
<td>D2O</td>
<td>155</td>
<td>0.84</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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.

Acknowledgements

RH and FP were supported by funding from MedImmune Ltd. We thank neutron beam times awarded to undertake this work at the Institut Laue Langevin, Grenoble and ISIS Neutron Facility, Chilton, Didcot, under the support of STFC.
References


[35] Tucker IM, Petkov JT, Penfold J, Thomas RK, Li PX, Cox AR, Hedges N,

Support Information

1. Scattering lengths (SL) of the key elements that comprise proteins, water and surfactants

Table S1 SLs for the key elements

<table>
<thead>
<tr>
<th>Elements</th>
<th>SL×10^5/Å</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>6.65</td>
</tr>
<tr>
<td>H</td>
<td>-3.74</td>
</tr>
<tr>
<td>D</td>
<td>6.67</td>
</tr>
<tr>
<td>O</td>
<td>5.80</td>
</tr>
<tr>
<td>N</td>
<td>9.37</td>
</tr>
<tr>
<td>S</td>
<td>2.80</td>
</tr>
</tbody>
</table>

The data were taken from the Special Feature section of neutron scattering lengths and cross sections of the elements and their isotopes in *Neutron News*, Vol. 3, No. 3, 1992, pp. 29-37.

2. Molecular structures used to calculate scattering lengths (SLDs) and estimate molecular volumes

2.1 Key parameters of Water

Table S2 Key parameters used for H2O and D2O

<table>
<thead>
<tr>
<th>Molecular structure</th>
<th>SL×10^5/Å</th>
<th>Volume/Å^3</th>
<th>SLD×10^6/Å^-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O</td>
<td>-1.68</td>
<td>30</td>
<td>-0.56</td>
</tr>
<tr>
<td>D2O</td>
<td>19.14</td>
<td>30</td>
<td>6.35</td>
</tr>
</tbody>
</table>
2.2 Tween 80 surfactants

**Figure S1** The molecular structure of a normal Tween 80 surfactant (shown below in the usual hydrogenated form):

![Molecular structure of Tween 80 surfactant](image)

Its chemical formula is: C64H124O26.

There are 3 OH groups whose Hs can fully exchange with the labile hydrogens in the solvent. The exact molecular scattering lengths and scattering length densities depend on the exact ratios of H2O and D2O. These exchanges also occur in the case of the deuterated surfactant with the hydrogen atoms on its 20 units of ethoxylates replaced by deuteriums. The exact SL and SLD values under these conditions are listed in the following:

**Table S3**: Key parameters for Polysorbate 80 in hydrogenated (h-Surf) and deuterated (d-Surf) forms under different water contrasts used.

<table>
<thead>
<tr>
<th>Isotopic labelling</th>
<th>Solvent Contrast</th>
<th>SL×10^5/Å</th>
<th>Volume Å^3</th>
<th>SLD×10^6/Å^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>h-Surf</td>
<td>NRW</td>
<td>116</td>
<td>1850</td>
<td>0.63</td>
</tr>
<tr>
<td>h-Surf</td>
<td>D2O</td>
<td>155</td>
<td></td>
<td>0.84</td>
</tr>
<tr>
<td>d-Surf</td>
<td>NRW</td>
<td>949</td>
<td></td>
<td>5.13</td>
</tr>
<tr>
<td>d-Surf</td>
<td>D2O</td>
<td>988</td>
<td></td>
<td>5.28</td>
</tr>
</tbody>
</table>
2.3 Sequences, scattering lengths and scattering length densities of COE-3 under different water contrasts

The antibody molecule is comprised of 2 identical light and heavy polypeptide chains.

The sequence of the light chain:

DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASS
LOSGVPSRFSGSGTDTDFTLTISSLQPEDFATYYCQSYSTPLTFGGGSVKVEIK
RTVAAPSFIIFPSDEQLKSGTASVCLNNFYPEAKVIKVDNALQSGNS
QESVTEQDSKSTYLSSTLTLKDYKHEKTVACEVTHQGLSSPVTCSFNRE
GEC

The sequence of the heavy chain:

QVNLRESGGGLVQPGGLRLSCAASGFTFGSYAMSWVRQAPGKGLEWVSAI
SGSGGTYYADSVKGRFTISRDNSKNSLYLQMNSLRAEDTAVYCCARRSIYGG
GNYYFDYWGRGTLVTSSASTKGPSFPLAPSSKSTSGTAALGCLVKDYFP
EPVTVSWNSGALTSGVHTFPAVLQSGLYSLSSVTVPSLSLGTQTYICN
KPSNTKVDKVEPKSCDKTHTCPACPAPELLGGPSVFLFPKPKDTLMISRT
EVTCVVVDVSHEPVEKFNWVDGVEVHNAKTPEEQYNSRYVVSVT
VLHQDLNGKEYKKCVSNKAPIESKAKGQPREPQVTVLPPRDEL
KGNQLTECLVKGFPYPSIAVEWESNQPPENNYKTTPPVLDSDGSLFLSYK
LDKSRWQGNNVFSCSVNMHEALHNHYTQKSLSPGK

The combined sequence was entered into the Biomolecular Scattering Length Density Calculator (http://psldc.isis.ri.ac.uk/psldc/) and the influence of the isotopic contrast of water was set accordingly to obtain the respective SLs and SLDs under NRW, CM 2.58 and D$_2$O, with the results shown in Table 2.
### 3. Analysis of reflectivity pairs measured under d-Surf and h-Surf in NRW at different surfactant concentrations

Each pair of reflectivity profiles under a given surfactant concentration was analysed using Equ (5-6) and the key structural parameters are given in Table S4 below.

**Table S4** Key structural parameters obtained from the best fits to the reflectivity profiles measured using d-Surf and h-Surf with the concentration of COE-3 fixed at 50 ppm and the ionic strength fixed at 25 mM.

<table>
<thead>
<tr>
<th>Surf Conc/CMC</th>
<th>$\rho_d/\rho_h \times 10^6$/Å$^{-2}$</th>
<th>$\tau$/Å</th>
<th>$A_p$ Å$^2$</th>
<th>$A_p$/Å$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.56</td>
<td>50±4</td>
<td>13500±3000</td>
<td></td>
</tr>
<tr>
<td>1/100</td>
<td>0.51/0.42</td>
<td>48±4</td>
<td>2500±400</td>
<td>17000±3000</td>
</tr>
<tr>
<td>1/50</td>
<td>0.53/0.39</td>
<td>48±4</td>
<td>1600±400</td>
<td>19000±3000</td>
</tr>
<tr>
<td>1/20</td>
<td>0.98/0.32</td>
<td>44±4</td>
<td>300±30</td>
<td>35000±4000</td>
</tr>
<tr>
<td>1/10</td>
<td>1.05/0.26</td>
<td>44±4</td>
<td>260±30</td>
<td>53000±4000</td>
</tr>
<tr>
<td>1/5</td>
<td>1.83/0.30</td>
<td>35±3</td>
<td>170±15</td>
<td>80000±10000</td>
</tr>
<tr>
<td>1/2</td>
<td>1.93/0.26</td>
<td>35±3</td>
<td>150±15</td>
<td>-</td>
</tr>
</tbody>
</table>

CMC was found to be $1.2 \times 10^{-5}$ M.
Chapter 5

- Abstract 139

1. Introduction 140

2. Materials and Experimental Methods 142

3. Results and Discussion 148

4. Conclusion 162

- References 164

- Supporting Information 167
Interfacial Adsorption of Monoclonal Antibody COE-3 at the Solid/water Interface

Fang Pan¹, Zongyi Li¹, Thomas Leyshon¹, Dominic Rouse¹, Ruiheng Li¹, Charles Smith¹, Mario Campana², John R P Webster², Steve Bishop⁴, Rojaramani Narwal⁴, Christopher F van der Walle³, Jim Warwicker⁵, Jian R Lu¹

¹Biological Physics Laboratory, School of Physics and Astronomy, University of Manchester, Oxford Road, Schuster Building, Manchester M13 9PL, UK.

²ISIS Neutron Facility, STFC, Chilton, Didcot OX11 0QZ, UK.

³Formulation Sciences, MedImmune Ltd, Sir Aaron Klug Building, Granta Park, Cambridge CB21 6GH, UK

⁴Formulation Sciences, MedImmune LLC, Gaithersburg, MD 20878, USA.

⁵School of Chemistry, University of Manchester, Oxford Road, Chemistry Building, Manchester M13 9PL, UK.

Corresponding author: Jian R Lu
(email: j.lu@manchester.ac.uk; Tel: +44 161 2003926)

FP and ZL made equal contributions

Keywords:
mAbs, interfacial adsorption, antibody, structural unfolding, self-assembly, neutron reflection
Abstract

Spectroscopic ellipsometry (SE) and neutron reflection (NR) data for the adsorption of a monoclonal antibody (mAb, termed COE-3, pI 8.44) at the bare SiO$_2$/water interface are here compared to simulations based on DLVO theory. COE-3 adsorption was characterized by an initial rapid increase in the surface adsorbed amount ($\Gamma$) followed by a plateau. Only the initial rate of increase in $\Gamma$ was strongly correlated to the bulk concentration (0.002-0.2 mg/ml), with $\Gamma$ at plateau being 2-2.2 mg/m$^2$ (pH 5.5). Simulations captured COE-3 adsorption at equilibrium most accurately, where repulsion experienced by molecules within the adsorbed plane matched the adsorption flux. Increasing buffer pH from 5.5 to 9 increased $\Gamma$ at equilibrium to ~3 mg/m$^2$ (0.02 mg/ml COE-3), revealing a dominant role for lateral repulsion between adsorbed mAb molecules. In contrast, increasing the buffer ionic strength (pH 6) reduced $\Gamma$, which was captured by simulations accounting for electrostatic screening by ions, in addition to mAb/SiO$_2$ attractive forces and lateral repulsion. NR data at the same bulk concentrations corroborated the SE data, albeit with slightly higher $\Gamma$ due to longer adsorption times for data acquisition, e.g. at pH 9, $\Gamma$ was 3.6 mg/m$^2$ (0.02 mg/ml COE-3), equivalent to a relatively high volume fraction of 0.5. An absorbed monolayer of thickness of 50-52 Å was consistently determined by NR, corresponding to the short axial lengths of the Fab and Fc, and implying minimal structural perturbation. Thus, simulations enabled a mechanistic interpretation of the experimental data of mAb adsorption at the SiO$_2$/water interface.
1. Introduction

The development of monoclonal antibodies (mAbs) in the 1970’s has given rise to a novel class of biotherapeutics.\(^1\) Over the past 10 years, mAbs have become the dominant recombinant therapeutic proteins used in the clinic whilst many more are in the different stages of clinical trials.\(^2\,^3\) The basic molecular structure of a mAb is its ‘Y-shape’, comprised of 1 fragment crystallization (Fc) and 2 fragment antigen-binding arms (Fabs). The two constant domains (C\(_{H\gamma}3\) and C\(_{H\gamma}2\)) of each heavy chain form the Fc, and the C\(_{H\gamma}1\) and variable domain (V\(_H\)) together with the light chain C\(_L\kappa/\lambda\) and V\(_L\) domains form each Fab. Several crystalline structures of Fc, Fab and whole mAb from various origins (different species, recombinant or bioengineered) have been reported over the past few decades.\(^4\,^6\)

Sequence modifications based on recombinant and biochemical technologies can afford mAbs with new biological functions but also affect their secondary and tertiary structures, thereby imposing direct consequences on their physical stability.\(^7\,^8\) The surfaces of Fc and Fab segments are distributed with different amino acid groups that are polar, apolar and charged. As these groups are often distributed unevenly, mAb molecules are inherently amphiphilic, meaning that they can adsorb onto different interfaces and also become desorbed. Protein adsorption is a spontaneous process and depending on the exact nature of the interface, protein and solution conditions, adsorbed molecules can strongly interact with a particular substrate and between themselves within the adsorbed layers, resulting in structural deformation and unfolding of the protein.\(^9\) Although the exact mechanisms remain unclear, it is widely accepted that adsorption and desorption processes cause structural changes to proteins in solution and thereby promote aggregation.\(^10\) These molecular processes occur during mAb production, purification and storage in aqueous solution. Structurally deformed or partially unfolded mAb molecules can promote aggregation and sub-visible particulates, which must be characterized for injection products in accordance with the pharmacopoeias (e.g. USP <787> Subvisible Particulate Matter in Therapeutic Protein Injections). Thus, it is important to study surface and interfacial adsorption to understand the impact that sequence modifications may have.

Many mAbs such as COE-3 studied here are based on the immunoglobulin-1 (IgG1) isotype. Like all other candidate mAb therapeutics, COE-3 harbors substantial sequence modifications in the variable and constant domains of the Fab (Tables S1 and \(\text{Chapter 5} \quad - \quad 140 \quad -\))
S2). While the interfacial properties of various mAbs have been reported, relating adsorption behavior to a specific mAb sequence is lacking in the literature. This study therefore makes an important contribution to the literature by reporting experimental and molecular modelling data together. We have studied the adsorption of COE-3 at the hydrophilic silicon oxide (SiO$_2$)/water interface, which is particularly relevant since the SiO$_2$ surface is a good model of glass which is widely used to make vials and syringes for mAb products.

Protein adsorption at the solid/water interface has been studied by a number of groups using techniques such as ellipsometry, fluorescence and quartz microbalance.$^{11-16}$ These reports provide a useful consensus about the typical ranges observed for the amount of protein adsorbed and its physical state, under well-defined surface and solution conditions. Although these studies have demonstrated the relevance of adsorption and desorption processes to structural perturbation and solution aggregation,$^{10-16}$ little insight about mAbs within the adsorbed layers have been reported. Neutron reflection (NR) is a powerful tool able to reveal the thickness and composition of multiple adsorbed protein layers.$^{17-21}$ A key value of NR lies in its high depth resolution and therefore capability to determine the thickness and composition of the adsorbed layer(s) from which the structural conformation of the adsorbed molecules can be inferred.$^{22-24}$ NR data together with the measured globular structures (e.g from X-ray crystal diffraction) of proteins enables us to determine the probable conformational orientations of the adsorbed protein molecules at a given interface, therefore also implying the extent of structural deformation and unfolding.$^{22}$

However, each NR experiment generally requires a minimum of 30 min to acquire sufficient data for statistical analysis, making NR an impractical technique to follow a fast, dynamic adsorption process. In contrast, spectroscopic ellipsometry (SE) requires only seconds for each experiment and while it is highly sensitive to the adsorbed amount at a surface it cannot resolve the depth of adsorbed layer(s). Thus, coupling the excellent depth resolution that NR brings to dynamic adsorption monitored by SE, facilitates comparison of these combined data sets to computer simulations of the equivalent protein adsorption event. This approach yields a mechanistic insight at the molecular level to a protein’s surface and interfacial behaviour, that has to date not been fully realised. Building on our recent studies on the adsorption of COE-3 and its constituent Fab and Fc at the air/water interface,$^{23,24}$ here we use NR and SE to characterize COE-3 adsorption at the bare SiO$_2$/water interface, comparing the data to
computer simulations and so enabling a mechanistic interpretation of the adsorption process.

2. Materials and Experimental Methods

2.1 Materials

MAb. COE-3, a human IgG1κ of molecular weight (MW) 144,750, isoelectric point (pI) 8.44 and extinction coefficient 1.43 ml.mg⁻¹.cm⁻¹ (280 nm), was expressed in Chinese hamster ovary cells and purified using industry-standard methods. It was supplied as a stock solution of 46.4 mg/ml in ‘His buffer’ (25 mM histidine/histidine hydrochloride, 7 % w/v sucrose, pH 6.0), batch no. SP12-423, and stored at -80 °C until solution preparation. The thawed solution was diluted directly into histidine buffer at a given pH, maintaining an ionic strength of 25 mM; H₂O buffers for a range of pH and COE-3 concentrations were used for SE measurements, with D₂O buffers at the equivalent pH were prepared for NR measurements. Despite the original stock solution being in H₂O, the large dilutions made into D₂O resulted in only very low percentages of H₂O, the exact amounts being taken into account during neutron data analysis. Table S1A gives the sequences of the light and heavy chains of COE-3 from which scattering length (SL) and scattering length density (SLD, ρ) in D₂O can be calculated. Note that the solution behavior of COE-3 has been studied by Roberts et al. The pH dependent changes to the net charges on the Fab, Fc and whole mAb were modelled without taking into account any charge contribution from the glycosylated groups (Figure S1).

Solvents and buffer. D₂O (99% D), histidine and histidine hydrochloride were purchased from Sigma-Aldrich and used as supplied. H₂O was processed using an Elgastat PURELAB water purification system. SL and SLD for D₂O are also given in Table S1B.

Spectroscopic ellipsometry (SE). Interfacial adsorption was determined using a Woollam spectroscopic ellipsometer (J.A. Woollam Co. Inc). The SE measurements were performed over a wavelength range between 200 and 600 nm. A special liquid cell with fused quartz windows was constructed to enable the SE measurements at the solid/liquid interface with the incident light beam at 70°. It
facilitated the confinement of a <111> silicon wafer cut (2cm×2cm) in the central bottom of the liquid cell and thus enabled the reflection of incoming and existing light from its surface. Following the optical alignment in air, the thickness of the native oxide layer was first determined. The surface oxide layer was usually 12±3 Å thick. Its precise value was obtained by fitting an optically transparent model with prefixed refractive index by assuming that the oxide layer contains no voids or defects and that its wavelength dispersion follows the Cauchy equation. The subsequent SE measurement in water would offer similar layer thickness within the error range, as this consistency indicated good window alignment. The cleaning of the liquid cell and regeneration of the oxide surface were undertaken by using 5% dilution of neutral Decon solution as described for neutron blocking cleaning below.

The experimental data were analyzed using the software developed by J.A. Woollam Co. Inc. SE measured changes in the polarization state of light reflected from the surface of the sample from which information about layer thickness and refractive index could be revealed through the simultaneous analysis of two ellipsometric angles \( \Psi \) and \( \Delta \), where \( \Psi \) denotes the change in amplitude and \( \Delta \) denotes the phase of polarisation of the light after reflection in two components, the plane of reflection (p-plane), and that perpendicular to it (s-plane). The sample ellipticity, \( \rho_e \), is defined as the ratio of the Fresnel coefficients of the p and s planes (R_p and R_s) and is expressed as: \(^{26,27} \)

\[
\rho_e = \frac{R_p}{R_s} = \tan \Psi e^{i\Delta}
\]  

(1).

The refractive index \( n_f \) and the corresponding film thickness \( \tau_f \) were subsequently calculated by the software using Equ. 1. The surface excess \( \Gamma \) (mg/m\(^2\)) of the sample is finally calculated from \( n_f \) and \( \tau_f \) (in Å) through Equ. 2:

\[
\Gamma = \frac{\tau_f (n_f - n_o)}{(dn/dc)}
\]  

(2),

where \( n_o \) is the refractive index of the buffer, \( dn/dc \) stands for the change of refractive index against solution concentration and a value of 0.18 ml/g was used in this work.

**Neutron reflection (NR).** Neutron reflectivity measurements were made on the SURF reflectometer at the ISIS Neutron Facility, STFC near Oxford, UK. Measurements
were made using a single detector at fixed angles ($\theta$) of 0.5, 0.8 and 1.8° and for neutron wavelengths ($\lambda$) in the range 0.5 - 6 Å to provide a momentum transfer (Q) range of 0.012 - 0.5 Å$^{-1}$. The absolute reflectivity (R) was calibrated with respect to the 100% reflectivity below the critical edge at the silicon/D$_2$O interface, and the background determined from the reflectivity at the limit of high Q (>0.25 Å$^{-1}$). The <111> silicon block used in NR measurements had approximate dimensions of 1.2 x 4.0 x 6.0 cm$^3$. One of its large surfaces was polished by Crystran Ltd, Poole, UK. Before use, it was immersed in a Piranha solution (in a volume ratio of 6:1 for H$_2$SO$_4$ (98%) to H$_2$O$_2$ (35%)) around 90 °C for 1 min to enhance its surface hydrophilicity. After being taken out and cooled down, it was washed with plenty of distilled water before it was placed in a 5% (w/w) solution of Decon 90 (Decon Ltd, UK) for 5 min, followed by subsequent rinsing with a large amount of ultra-high quality water (UHQ). As shown previously, the cleaning process ensured the creation of a reproducible silicon oxide layer of thickness 12 ± 3 Å. The same Decon washing was also used to help remove adsorbed mAb molecules and regenerate the silicon oxide surface between adsorption measurements. It has been shown previously that the reproducible surface properties could be reconfirmed by identical adsorption of lysozyme at 1 mg/ml, pH 7. The liquid trough used was processed from Perspex attached with flow system. It was clamped against the cleaned surface of the block. Solutions of buffer and mAb at different concentrations were introduced to the measuring surface from the flow system.

### 2.2 Adsorption Model

The DLVO (Derjaguin, Landau, Verwey and Overbeek) theory states that the total Gibbs potential of interaction, when the interacting bodies are a distance $z$ apart, is given by:

$$G_{i2j}(z) = G_{i2j}^{lw}(z) + G_{i2j}^{da}(z) + G_{i2j}^{el}(z)$$

(3)

where the subscript $i2j$ refers to a body $i$ interacting with a body $j$ through a liquid medium 2, and each term defines the contribution from one of the three forces involved: The Lifshitz-van der Waals (LW), the donor-acceptor (DA) and the electrostatic (EL). In the model we consider two different bodies interacting with the COE-3 in solution: the silica wafer and the adsorbed layer of COE-3. Following standard practice, we treat the silica wafer as a semi-infinite plane located in the x-y plane at $z = 0$ and
the COE-3 in solution as hard spheres of fixed radius, $R$.\textsuperscript{32} The adsorbed COE-3 layer is modelled as a fractional semi-infinite plane (FSIP) which evolves towards a true semi-infinite plane as the wafer reaches saturation. In each time interval in the model, a number of COE-3 molecules adsorb onto the silica wafer. This is represented as becoming trapped in the minimum of the Gibbs potential (at $z = a$) after which the protein-protein Gibbs potential is added to the minimum, treated as a FSIP interacting with a hard sphere. Therefore, as more proteins become adsorbed the shape and depth of the minimum changes dramatically, and the change is heavily dependent on whether the protein-protein interactions are repulsive or attractive. Furthermore, the interactions caused by the adsorbed COE-3 layer were added to the minimum symmetrically, beginning from half the width of the well. Physically this corresponds to the FSIP of absorbed proteins forming in the $x$-$y$ plane within a layer of thickness equal to the width of the well. Hence, by letting a subscript 1 denote the silica wafer and a subscript 3 denote COE-3, the Gibbs potential experienced by an adsorbing protein when $N$ proteins are adsorbed is given by:

$$G_N(z) = G_{123}(z) + \frac{1}{N_{\text{max}}} \sum_{n=1}^{N} \begin{cases} G_{323} \left( \frac{L_{n-1}}{2} + (z - a_{n-1}) \right), & \text{if } z \geq a_{n-1} \\ G_{323} \left( \frac{L_{n-1}}{2} - (z - a_{n-1}) \right), & \text{if } z \leq a_{n-1} \end{cases}$$

where $N_{\text{max}}$ is the number of proteins needed to saturate the silica wafer, and $a_n (L_n)$ is the position of the minimum of the distribution (width of the well) when $n$ proteins have adsorbed. The width of the minimum was defined as the size of the well in the initial distribution, $G_{123}(z)$ at a height equal to the root mean square (r.m.s) energy above the minimum in the current distribution, $G_N(z)$. In this way we assume that the interactions experienced by a protein in the FSIP with the remainder of the FSIP cancels, but that when a protein is displaced from the FSIP (and so is attempting to desorb) this force no longer cancels in the $z$-direction and the protein experiences the potential in full. The specific forms of each term of Equ. 4 and exemplar Gibbs distributions (Figure S4) can be found in Section S2A, but it should be noted that the LW and DA interactions are strongly influenced by the surface tensions between the two interacting bodies and the liquid medium. The EL interactions are instead influenced by the surface potentials of the bodies.

All properties of the adsorption process are calculated from the Gibbs distribution. The flux of adsorbing proteins when the FSIP has $N$ proteins of a possible $N_{\text{max}}$ is given by:
\[ F_N = \frac{D C}{\int_{h}^{h} \exp\left(\frac{f_N(z)}{k_B T}\right) \, dz} \]  
\[ (5), \]

where \( D \) is the diffusion coefficient, \( C \) is the concentration of the proteins in the buffer, \( h \) is the maximum height of the protein above the wafer (taken as 1 cm) and \( T \) is the temperature of the solution. The denominator of Equ. 5 can be interpreted as the resistance experienced by the proteins due to the Gibbs potential. 33

The desorption rate is dependent on two quantities. The first is the desorption attempt frequency, defined as the frequency at which an adsorbed protein collides with the wall of the minimum furthest from the silica surface. It is assumed that all adsorbed proteins have an energy equal to the r.m.s. energy above the minimum of the potential well, therefore the escape attempt frequency is:

\[ \nu_N = \frac{2L_N}{\nu_{rms}} \]  
\[ (6), \]

where \( \nu_{rms} \) is the r.m.s. velocity. The second quantity is the probability that a desorption attempt is successful. This is the probability of the protein having sufficient kinetic energy to overcome the barrier needed to escape from the potential well at temperature \( T \), denoted as \( P_N \) and calculated from the Maxwell-Boltzmann distribution. Hence, the total change in the number of adsorbed proteins in a time interval \( \Delta t \), if there were \( N \) adsorbed proteins at the beginning of the interval, is:

\[ \Delta N = \frac{F_N}{m} A \left( 1 - \frac{N}{N_{\text{max}}} \right) \Delta t - \nu_N P_N N \Delta t \]  
\[ (7), \]

where \( A \) is the area of the silica wafer, \((1 - N/N_{\text{max}})\) is the fractional area of the silica wafer available for adsorption and \( m \) is the mass of each COE-3 molecule. In Equ. 7 the first term is the number of adsorbing proteins and the second term is the number of desorbing proteins. Due to the number of proteins involved, Equ. 4 is in practice only computed for certain values of completion of the FSIP, rather than for each individual protein. After calculating \( N(t) \) iteratively using Equ. 7, the surface adsorbed amount is calculated via:

\[ \Gamma(t) = N(t)m/A \]  
\[ (8). \]
2.3 Model Parameters

When COE-3 was added from low concentration (25 mM) histidine buffer, the measured change in the total surface tension was almost zero (see Figure S3). Hence we conclude that the total surface tension must be similar to that of water, at 72.8 mN/m, and that the buffer can be modelled as water when at low COE-3 concentrations. Further restrictions on the individual components of the surface tension (that is, the Lifshitz-van der Waals, $\gamma_{lw}^3$ and donor, $\gamma_d^3$ and acceptor, $\gamma_a^3$ components) could be enforced by assuming that protein-protein interactions are repulsive, which avoids aggregation once in solution, and by requiring a minimum to form in the initial Gibbs distribution, permitting adsorption. From the remaining valid parameters (illustrated in Figure S5) the set in which the interactions gave sufficient strength in protein-protein repulsion to replicate the experimental data were $\gamma_{lw}^3 = 24.35$ mN/m, $\gamma_d^3 = 31.05$ mN/m and $\gamma_a^3 = 18.90$ mN/m. A more detailed discussion of this choice can be found in Section S2B. The surface tension components of water are $\gamma_{lw}^2 = 21.80$ mN/m, $\gamma_d^2 = 25.50$ mN/m and $\gamma_a^2 = 25.50$ mN/m and those of silica are $\gamma_{lw}^1 = 39.00$ mN/m, $\gamma_d^1 = 4.10$ mN/m and $\gamma_a^1 = 0.80$ mN/m, which are well documented.31

We assumed COE-3 to be a hard sphere protein, for which we took the radius of COE-3 to be the average of its Fab and Fc constituents, which results in a radius of 56 Å. For simplicity we assumed that the radius of COE-3 in solution was not affected by buffer pH and ionic strength, but that the effective area occupied by each molecule when adsorbed was. In doing this we account for conformational changes in the lateral COE-3 repulsion within the adsorbed layer. Table 1 states the effective radii which were calculated from the experimental data for the equilibrated values of the 0.2 mg/ml adsorption curves of Figure 4(a) and Figure 6(a). The high concentration curves were chosen as these gave a more accurate description of how equilibrated values depend on pH and ionic strength, because mAb concentration was high enough that influence from further increase was negligible.
### Tables 1

Effective radii used for COE-3 for varying buffer parameters: different buffer pH at ionic strength (I) 25 mM, and different buffer ionic strength at pH 6.

<table>
<thead>
<tr>
<th>pH</th>
<th>Effective radius (Å) at I = 25 mM</th>
<th>Ionic strength (mM) at pH 6</th>
<th>Effective radius (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>59.5</td>
<td>5</td>
<td>56.5</td>
</tr>
<tr>
<td>5.5</td>
<td>58.9</td>
<td>13.3</td>
<td>57.5</td>
</tr>
<tr>
<td>6.0</td>
<td>58.3</td>
<td>25</td>
<td>58.3</td>
</tr>
<tr>
<td>7.0</td>
<td>53.5</td>
<td>50</td>
<td>61.0</td>
</tr>
<tr>
<td>8.0</td>
<td>49.4</td>
<td>100</td>
<td>73.0</td>
</tr>
<tr>
<td>9.0</td>
<td>49.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 3. Results and Discussion

#### 3.1. Dynamic Adsorption

Prior to monitoring COE-3 adsorption it was necessary to characterize the silicon substrate surface, which bears a native oxide layer whose thickness was determined by SE. If pores were absent, the refractive index would correspond to the 100% silicon oxide. For all the wafer cuts used, the native oxide layers were found to be 12-14 Å thick, without any sign of porous defects or requirement to incorporate a parameter for roughness during data fitting. The smooth oxide layer was also confirmed by NR measurements in D$_2$O.

Subsequent SE measurements were made to investigate the time-dependent adsorption of COE-3 at pH 5.5 and ionic strength 25 mM, 20-21 °C. An exemplar set of SE measurements is shown in Figure 1, where the amplitude component $\Psi$ is shown in Figure 1(a) and the phase difference $\Delta$ is shown in Figure 1(b). Because each pair of $\Psi$ and $\Delta$ could be measured in 10-15 s, SE is ideal for following the dynamic adsorption process. As evident from Figure 1, whilst changes from $\Psi$ were not so obvious, clear differences were observed in $\Delta$. These SE measurements were undertaken with COE-3 at 0.02 mg/ml in bulk solution, with similar measurements made at other concentrations under the same solution conditions. An exemplar set of concentration effects is shown in Figure S2, measured after 5 min of adsorption. Again, changes in $\Psi$ are small but differences in $\Delta$ are obvious, similar to the situation as described in Figure 1.
The amount of protein adsorption was obtained from data fitting following the procedure as described in the Materials and Experimental Method and the wavelength ($\lambda$) dependence of the refractive index $n$ is taken into account using the Cauchy dispersion equation:

$$n(\lambda) = A - \frac{B}{\lambda^2}$$

(9),

where $A$ is typically 1.45 and $B$ is taken to be 0.003 $\mu$m$^2$. The time-dependent adsorption as obtained from the above described data analysis process is shown in Figure 2(a) where it can be seen that the rate of adsorption increases with the mAb concentration; at 2×$10^{-3}$ mg/ml (2 ppm), very little adsorption is detected over the first 60 min, while at 5×$10^{-3}$ mg/ml, adsorption increased steadily in a linear fashion over the first 40 min with a plateau at around 2 mgm$^{-2}$. The initial rate of adsorption further increased with increasing concentration to 1×$10^{-2}$ and 2×$10^{-2}$ mg/ml, reaching a plateau (saturation) after the first 20 and 10 min, respectively. At and above 5×$10^{-2}$ mg/ml, the adsorption profiles overlapped, with saturation being reached within the first minute.

**Figure 1.** Ellipsometric scans of the amplitude component $\Psi$ (a) and the phase difference $\Delta$ (b) against wavelength measured at the SiO$_2$/water interface for 0.02 mg/ml COE-3 in 25 mM His buffer, pH 5.5; after 1, 5 and 30 min of adsorption. The continuous lines show the uniform layer fits.
At the concentration of 0.02 mg/ml (20 ppm), the adsorbed amount of COE-3 at the bare silicon oxide/water interface was calculated to be 2.2 mg/m² (Figure 2b). From Equ. 12 and with the molecular weight (MW) of 144,750 from Table S1B, this is equivalent to an area per molecule of 11,000 Å². COE-3 is comprised of 1 Fc and 2 Fabs, and the dimensions of the Fc and Fab from the crystalline structure are 65×70×40 and 85×45×45 Å³, respectively. On the assumption that the globular fold is maintained and COE-3 adsorbed ‘sideways-on’ with the shortest axes projected perpendicular to the interface and without any domains stacking, then the Fc would occupy a limiting area of about 4600 Å² and each Fab about 4000 Å², reaching a value of 12600 Å². As this theoretical adsorbed area is very close to the limiting values estimated, the mAb fragments within the adsorbed layer must be very closely packed.

In a separate study, we have examined the adsorption of COE-3 on surface of water using neutron reflection. It was found that the adsorbed mAb occupied an area per molecule of 12000 Å² at the mAb concentration of 0.1 mg/ml under same solution pH and ionic strength. In addition, mAb molecules formed a uniform layer thickness of some 50 Å. At these relatively high concentrations, the similarity in adsorption between these two interfaces implies that the limiting area was dictated by the horizontal mAb-mAb packing and that the saturated adsorption was little affected by the different substrates (air versus SiO₂).

In contrast, below 5×10⁻² mg/ml, the two substrates affected mAb adsorption very differently. Whilst little adsorption was detected at 2×10⁻³ mg/ml at the SiO₂/water interface, the equilibrated adsorbed amount was about 0.9 mgm⁻² on the surface of water. Furthermore, at 5×10⁻³ mg/ml the saturated adsorption tended to 2 mgm⁻² at the SiO₂/water interface but 1-1.2 mgm⁻² at the air/water interface. Between 5×10⁻³ and 5×10⁻² mg/ml, COE-3 adsorption at the air/water interface slowly increased to saturation, whilst adsorption at the SiO₂/water interface had already reached saturation (albeit after 60 min). This implies two different concentration dependent adsorption processes, most likely reflecting the weak negative charge of the SiO₂ surface that is electrostatically attractive to the positively charged mAb at weakly acidic pH.

Figure 2(c) shows exemplar NR profiles measured at 10, 30 and 60 min upon the contact of mAb solution with the substrate surface. In this case, each NR run took 8-10 min to complete. As it can be seen from Figure 2(c), the difference between the reflectivity profiles measured over the dynamic adsorption period is very small. For a
uniformly adsorbed layer under water, the volume fraction of mAb ($\phi_p$) and its surface excess ($\Gamma_p$, in mg/m$^2$) within the layer can be calculated on the basis of the following equations:21

$$\phi_p = \frac{\rho - \rho_w}{\rho_p - \rho_w}$$

(10),

$$\Gamma_p = 0.1\phi_p \tau \rho_p'$$

(11),

where $\rho$ is the best fitted layer SLD, $\rho_w$ and $\rho_p$ are the SLD for water and mAb, respectively, $\tau$ (in Å) is the layer thickness and $\rho_p'$ is the density of the mAb and is equivalent to $\frac{MW}{V}$, where MW is in g/mol and $V$, its molecular volume, is in g/cm$^3$. The area per molecule (A, in Å$^2$) can be obtained using:

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

(12),

where the constant of 6.023 is related to the conversion of the Avogadro’s number and the unit difference between Angstrom (Å) and meter (m). Equ. 4 specifies that the antibody layer is filled by the solvent, with the total of the antibody volume fraction ($\phi_p$) and solvent volume fraction ($\phi_w$) being equal to unity. To ensure the SLD contributions consistent to the interfacial composition the following equations must be conserved:19-22

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

(13).

The best uniform layer fits as shown in Figure 2(c) led to the pairs of $\rho$ and $\tau$ for the 3 different sets of reflectivity data measured under the specified time points from which $\Gamma_p$ could be obtained from Equ. 3 and 4. The surface adsorbed amount from these short NR profiles is shown in Figure 2(b) where it can be seen that both SE and NR data are in good agreement within the experimental errors.
Figure 2. (a) SE measurements of surface adsorbed amount ($\Gamma$) of COE-3 plotted against time at the SiO$_2$/water interface, in 25 mM His buffer, pH 5.5, 20-21°C, at concentrations as indicated. Plotted lines are simulations produced at the same concentrations with the exception of 2x10$^{-3}$ mg/ml for which an effective concentration of 2x10$^{-4}$ mg/ml was used; (b) comparison of the adsorbed amount of COE-3 at 0.02 mg/ml bulk concentration plotted against time and measured by SE and NR; (c) NR profiles measured at the SiO$_2$/D$_2$O interface after 10, 30 and 60 min COE-3 adsorption from 0.02 mg/ml bulk concentration, buffer as in (a). The continuous lines denote the best uniform fits as described in the text. For clarity, the reflectivity profiles at 30 and 60 min were divided by 10 and 100.

Figure 3. (a) Surface adsorbed amount ($\Gamma$) measured by SE after 60 min adsorption and by NR after 3-4 hr adsorption; (b) NR profiles measured at the SiO$_2$/D$_2$O interface for COE-3 adsorption from 0.02, 0.05 and 0.2 mg/ml bulk concentration, buffer as in Fig. 2(a). The continuous lines denote the best uniform layer fits with thicknesses of 50±2 Å and adsorbed amount plotted in (a). For clarity, the reflectivity profiles measured at 0.05 and 0.2 mg/ml were divided by 10 and 100.
3.2. Effect of mAb Concentration

From Figure 2(a) it is clear that after 60 min the adsorbed amount of COE-3 reached a plateau for concentrations > $5\times10^{-3}$ mg/ml. The adsorbed amount at 60 min or above can therefore be considered the equilibrated value at a given concentration; this value being 2 mg/m$^2$ for all concentrations $\geq 5\times10^{-3}$ mg/ml.

In the simulations, an effective concentration that was an order of magnitude smaller than the experimental protein concentration of $2\times10^{-3}$ mg/ml resulted in a significantly better fitting to the measured adsorption data. This indicates that in solutions with very low protein concentrations, a relatively large proportion of the COE-3 would have remained attached to the sides of its storage container. It is therefore likely that the effective concentration of COE-3 used in the experiment was nearer $2\times10^{-4}$ mg/ml, rather than $2\times10^{-3}$ mg/ml. Other than the lowest concentration, the simulations show a good match to the equilibrated adsorbed amounts. Since only concentration was varied, all curves were produced with the same effective radius. Therefore, in the model the differences in the equilibrated values are due to repulsion experienced by solution COE-3 with the FSIP of adsorbed COE-3 becoming as strong as the adsorption flux. The simple model does not capture the full effect of the initial adsorption process. At lower adsorbed amounts the number of adsorbed proteins will not be great enough to justify the assumption of formation of a FSIP. This would likely result in an overestimation of the repulsive protein-protein interactions, causing initial adsorption to be reduced relative to the experimental values. This discrepancy was observed in the initial adsorption region corresponding to the medium and high COE-3 concentrations studied and is more visible in Figure 4(a).

NR measurements of COE-3 adsorption from 0.02, 0.05 and 0.2 mg/ml bulk concentration reveal very little difference in layer thickness and composition (Figure 3b). A uniform layer model was sufficient to represent the main features, with $\Gamma$ of 2.4, 2.5 and 2.65 mg/m$^2$, respectively (Table 2). As evident from Figure 3(a), the NR

<table>
<thead>
<tr>
<th>Conc (mg/ml)</th>
<th>$\tau \pm 3$ /Å</th>
<th>SLD$\pm0.02$ /$10^{-6}$ Å$^-2$</th>
<th>$\phi_p$ $\pm 0.03$</th>
<th>$A_p \pm 200$ /Å$^2$</th>
<th>$\Gamma \pm 0.1$ /mgm$^-2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02</td>
<td>50</td>
<td>5.28</td>
<td>0.34</td>
<td>9900</td>
<td>2.4</td>
</tr>
<tr>
<td>0.05</td>
<td>51</td>
<td>5.23</td>
<td>0.35</td>
<td>9600</td>
<td>2.5</td>
</tr>
<tr>
<td>0.2</td>
<td>52</td>
<td>5.19</td>
<td>0.37</td>
<td>9100</td>
<td>2.65</td>
</tr>
</tbody>
</table>

Table 2: Structural parameters obtained from best uniform layer fits to the NR profiles shown in Figure 3(b), relating to Equ. 10-13.
values are slightly higher than their respective values measured by SE, which probably arise from the different adsorption times: NR data were collected 3-4 hr after COE-3 was introduced to the substrate surface, with SE data collected 1 hr after.

3.3. Effect of Solution pH

COE-3 adsorption profiles at pH values from 5 to 9, measured by SE, showed an initial fast adsorption phase followed by plateau (Figure 4(a) and 4(b)). A clear change in the absorbed amount as a function of pH was observed for both concentrations tested, this being around 2, 2.5 and 3 mg/ml for pH < 7, 7 and > 7, respectively. The ten-fold increase in concentration caused a greater rate of adsorption, as observed above (Figure 2(a)), with only a small increase in the absorbed amount at plateau. Saturation of adsorbed COE-3 had therefore been reached even at 0.02 mg/ml (after ~50 min), with the small increase seen at 0.2 mg/ml most likely representing a slow relaxation process during which the adsorbed COE-3 molecules became rearranged (but not necessarily structurally deformed) with the layer.

The continuous lines shown in Figure 4 denote the best fitted curves using the same model as described above by taking into account the pH dependent charge changes on COE-3 and the surface. It can again be seen from Figure 4 that whilst the fits can represent the final adsorbed amount well, they tend to underestimate the adsorbed amount over the initial adsorption processes. This situation is more evident from Figure 4(a). As already explained, this discrepancy was caused by the overestimation of the interactions associated with inadequate assumption of the FSIP model. But at the high COE-3 concentration relating to Figure 4(b), the dynamic adsorption occurred much faster and the surface was more quickly filled with more COE-3 molecules, making the FSIP model more applicable with respect to the experimental time scale undertaken by SE and resulting in better model fitting.
Figure 4. Surface adsorbed amount ($\Gamma$) plotted against time measured by SE at the SiO$_2$/water interface for COE-3 at (a) 0.02mg/ml and (b) 0.2mg/ml, in 25 mM His buffer, 20-21°C and pH as indicated. The solid lines represent the simulated curves.

From these SE data, the adsorbed amount of COE-3 can be seen to have reached an equilibrium after 60 min. Figure 5(a) shows the equilibrated adsorption plotted versus COE-3 concentration at pH 5.5, 7 and 9, whilst Figure 5(b) shows the equilibrated adsorption plotted versus solution pH at COE-3 concentrations of 0.02 and 0.2 mg/ml. It is apparent that the SiO$_2$ surface becomes saturated at bulk concentrations ≤ 0.01 mg/ml independent of pH, with the effect of pH seen as a change in the saturated amount absorbed. This pH trend is more clearly seen from Figure 5(b) which shows a greater range of pH values; the higher amount absorbed at equilibrium follows the shift from mildly acidic to basic conditions at low and high concentrations.

Over the pH range studied, the bare SiO$_2$ surface bears weak negative charges and its charge density rises with pH above pH 7-8. In contrast, COE-3 carries net positive charge at pH ≤ 8 (pI 8.44).$^{25}$ As the polypeptide sequences for both light and heavy chains are known (Table S1A), the pH dependent charges for Fab, Fc and the whole COE-3 can be calculated (Figure S1), and shown to be quite different. For the whole COE-3, the decrease in net charge with increasing pH corresponds to a steady rise in the amount adsorbed. This is most obvious at pH 9 where the highest surface adsorbed amount is reached: 3.6 mg/m$^2$ which is equivalent to an area per molecule of 6700 Å$^2$ (Figure 6b).
Figure 5. Surface adsorbed amount of COE-03 measured at 60 min plotted against concentration for pH 5.5, 7 and 9 (a), and against pH with concentrations of 0.02 and 0.2 mg/ml (b), in 25 mM His buffer, 20-21 °C, measured by SE and NR as indicated.

As the bare SiO₂ surface and COE-3 molecules are of opposite charges, electrostatic attraction is the main driving force for initiating interfacial adsorption. However, as more mAb molecules are adsorbed, lateral repulsion within the adsorbed layer occurs, constraining the maximal amount of COE-3 molecules that can be adsorbed. Given the relationship between COE-3 net charge and pH, lateral repulsion emerges as the key mechanism by which COE-3 adsorption at equilibrium is limited. This is consistent with previous observations for other proteins. These results were also obtained in the simulations showing that the dominant influence to equilibrated values is the effective area of adsorbed COE-3, with adjustments caused by the pH induced changes to electrostatic interactions involving adsorbing COE-3 molecules as indicated by changes in equilibrated values in Figures 4(a) and 4(b).

3.4. Effect of Solution Ionic Strength

Figure 6 shows the time-dependent adsorption of COE-3 at the SiO₂/water interface measured by SE for different buffer ionic strengths and mAb concentrations. The profiles in general reflect those acquired at various pH, being characterized by an initial fast adsorption phase and subsequent slow relaxation process at the plateau, reached between 2-15 min dependent on COE-3 concentration. The buffer ionic strength had little effect on the initial absorption phase for a given concentration but clearly changed the amount of COE-3 absorbed at equilibrium. At both high and low COE-3 concentrations, the adsorption profiles at 5 and 13 mM ionic strength overlap and a small drop in $\Gamma$ is observed at 25 mM, following which much larger falls in $\Gamma$ occur. The COE-3 bulk concentration is clearly the main driving force during the initial fast adsorption process. Over the plateau region, the dominant role is played by electrostatic interactions which are screened out at both the substrate and mAb surfaces as ionic...
strength increases, i.e. both the attractive interactions between the mAb molecules and substrate surface, and the lateral repulsion between mAb molecules, are reduced.

In the model, ionic strength variations change the electrostatic screening length, the potency of ion binding and lateral repulsion in the surface layer. As ionic strength is increased we find that the decrease in the electrostatic screening length causes the equilibrated value to decrease, but this ceases to change appreciably beyond a relatively low ionic strength ($\gtrsim 25$ mM). If ion binding to the COE-3 and SiO$_2$ surfaces is also included, then the decrease in the equilibrated values instead diverges, but not sufficiently to fit the experimental data. This shortcoming is exaggerated at higher protein concentrations, for which there are a decreased number of ions for each protein molecule in the solution. Therefore, ion binding takes a prominent role only in low COE-3 concentration adsorption processes, and changes of the electrostatic screening length take noticeable effect only at low ionic strength. These effects decrease the electrostatic interaction which is repulsive from the FSIP but attractive to the silica. The interaction with the silica is inherently stronger, therefore overall this results in a decrease in the equilibrated values. The final effect of a greater ionic strength is to increase the lateral repulsion in the adsorbed layer, and so increase the effective area occupied per adsorbed COE-3 molecule. Only the inclusion of all three effects gives a good fit to the experimental data, as seen in Figure 6(a) where the initial adsorption dynamics was underestimated for the same reason as in Figures 2(a) and 4(a).

![Figure 6](image.png)

**Figure 6.** Surface adsorbed amount plotted against time measured by SE at the SiO$_2$/water interface to show the effect of His buffer ionic strength (as indicated) for COE-3 concentrations of (a) 0.02 mg/ml and (b) 0.2 mg/ml, pH 6, 20-21°C. Solid lines represent simulations produced using the model described in the text.
3.5. Structural Conformation of the Adsorbed mAb Molecules

As described in Table 2, the thickness (τ) and surface excess (Γ) of the adsorbed COE-3 layers for bulk concentrations 0.002-0.2 mg/ml, pH 5.5, did not appreciably change, being 50-52±3 Å and 2.2-2.6 mg/m², respectively. Given that both Fab and Fc are globular and their short axial lengths are 40-50 Å, these results indicate that the absorbed Fab and Fc were packed as monolayers under these solution conditions. Figure 7 schematically depicts how Fab and Fc segments may adopt their conformational orientations, resulting in a layer of 50 Å thick. Since each mAb is comprised of 1 Fc and 2 Fabs, the packing of these fragments into a monolayer suggests a high degree of structural flexibility.

Figure 7. Schematic representation of COE-3 adsorbed at the SiO2/water interface as measured in the concentration range of 0.02-0.2 mg/ml at pH 5 where magenta denotes Fc and red denotes Fab segments.

As discussed above, from the SE data (Figures 2(b)), 2.2 mg/m² COE-3 was absorbed at equilibrium at the higher bulk concentrations studied, which is equivalent to an area per molecule (Aₚ) of 11000 Å². For a cross-sectional area of ~4600 Å² for Fc and ~4000 Å² for each Fab, the limiting area per mAb under this type of conformational orientation as depicted in Figure 7 is consistent with the experimental value suggesting that the adsorbed layer was rather densely packed. It should however be noted that as the cross-sectional area was only estimated from the two dimensional lengths based on the crystalline structures of Fc and Fab there must be additional unoccupied space between the segments within the adsorbed layer. The parallel NR measurements at 0.02 mg/ml COE-3 led to a higher Γ of 2.4 mg/m², equivalent to Aₛ = 10000 Å² (Table 2 and Figure 3a)), as a result of the longer time for adsorption, showing that more mAb molecules could still be packed into the monolayer under the experimental conditions. It is interesting that this monolayer model is retained under either higher COE-3
concentrations or solution pH, albeit with further increased packing density, e.g. the volume fractions (\( \phi_p \)) within the adsorbed layer is ~0.3 at pH 5.5 but ~0.4-0.5 at pH 9 for bulk concentrations of 0.02-0.2 mg/ml (Figure 5(b)). As \( \phi_p \) reaches 0.5, mAb/mAb lateral interactions may incur structural deformation, although the absence of any significant change in layer thickness suggests this is minimal under the conditions studied. Nevertheless, further experiments testing the limit of lateral packing in the monolayer could be warranted in order to reveal whether or not short range attractions between neighboring mAb molecules also limit their desorption. Since adsorption-induced denaturation is commonly anticipated to occur during industrial process steps, these data highlight that such an event must require either higher bulk concentrations, longer adsorption times or agitation, that is, the early adsorption of a mAb molecule at the solid/water interface is not necessarily associated with gross structural deformation.

3.6. Comparison with Adsorption at the Air/water Interface

Adsorption of COE-3 on the surface of water has also been undertaken recently under similar solution conditions using a combined study of NR and surface tension.\(^{23,24}\) The most noticeable feature was that COE-3 surface adsorption caused only a small decrease in surface tension at bulk concentrations up to 1 mg/ml. This effect could be linked to a high globular stability which is also revealed in the data acquired here and reflected in Figure 7: the layer thicknesses remaining consistent with the short axial lengths of the Fc and Fab, up to volume fractions (\( \phi_p \)) of 0.5. Although no direct comparison between SE and surface tension measurements can be made, the central time-dependent pattern appears, that is, a fast initial rise in COE-3 adsorption at the interface followed by a slow relaxation process (representing rearrangement of packing between molecules) with the adsorbed amount tending towards plateau at equilibrium. The slow relaxation process is particularly noticeable at the air/water interface and conversely COE-3 tended to adsorb faster and reached a higher adsorbed amount at the solid/water interface. The latter can be accounted for by favorable electrostatic attraction between mAb molecules and the oppositely charged SiO\(_2\) surface, as modelled in the simulations. The most noticeable difference in COE-3 adsorption behavior at the air/water and solid/water interfaces was its dependency on the buffer pH; the former changing little while the latter showed a steady rise on moving from mildly acidic to alkaline buffer. This difference can be related to the dominant role played by electrostatic forces between the mAb surface charge and SiO\(_2\) substrate. In
the context of industrial formulation and fill-finish, changing buffer pH may offer a route to modulating surface adsorption, at least at very low bulk concentrations of mAb and assuming such a requirement was identified as necessary during developability assessment.

3.7. Comparison with the Adsorption of other mAb Molecules

Using the same techniques and almost the same solution conditions we have previously studied the SiO$_2$/water interfacial adsorption of two other IgG1 mAbs.$^{20,21}$ Whilst the exact sequences of these other two mAbs are publicly unavailable, Table S2 compares the sequences between COE-3 and a human derived mAb, showing little dissimilarity over the Fc but around 50% dissimilarity over the Fab. Zhao et al.$^{20}$ showed that the surface adsorption of monoclonal anti-human prostate specific antigen (anti-hPSA) at pH 7 increased steadily with bulk concentration from 0.002 to 0.02 mg/ml; $\Gamma$ approaching saturation at 3 mg/m$^2$ compared to 2.5 mg/m$^2$ obtained for COE-3 at 0.02 mg/ml here. However, $\Gamma$ for anti-hPSA increases to 3.6 mg/m$^2$ as the bulk concentration reaches 0.05 mg/ml, while $\Gamma$ for COE-3 reaches a plateau at bulk concentrations above 0.01 mg/ml, which indicates a greater structural stability for COE-3 molecules within the adsorbed monolayer. The adsorption profile of COE-3 at the SiO$_2$/water interface is more consistent with that of a mAb to the $\alpha$-binding unit of human chorionic gonadotrophin (anti-$\alpha$-hCG), which also plateaued at 0.01 mg/ml for $\Gamma$ approaching 3 mg/m.$^{2,21}$ These three studies together show that in spite of some differences in their time and concentration-dependent processes, the main features of surface and interfacial adsorption are similar and the final equilibrated adsorbed amounts are generally around 2-3.5 mg/m$^2$, at these concentrations and buffer conditions tested. The lower saturated adsorbed amount for COE-3 may arise from a more robust globular stability and different charge characteristics. The hypothetical scenario wherein a therapeutic protein is sequestered by interfacial adsorption during i.v. administration from low bulk concentrations using a giving set therefore needs consideration; this is demonstrated by simulation of COE-3 adsorption from $2\times10^{-3}$ mg/ml being more accurately modelled from $2\times10^{-4}$ mg/ml (Figure 2(a)).

The adsorption of mAb molecules onto a support substrate could in principle form different conformational orientations, namely, head-on, tail-on, side-on and flat-on. In reality, it is widely thought that they may adopt a combination of these configurations.
under a given set of surface and solution conditions. By these designations, the NR measurements at the SiO\textsubscript{2}/water interface have revealed the predominantly flat-on conformation in which all 3 fragments (Fc and Fab) lie parallel to the substrate surface. This basic conformational orientation leads to direct contacts of all fragments with the substrate and between themselves as the packing density goes up. These features are similar for the three mAbs studied, i.e. they all adopt predominantly flat-on conformations with the short axes of Fc and Fab segments lying perpendicular to the SiO\textsubscript{2} surface. To gain an indirect understanding of desorption events for individual molecules during an overall adsorption process, it will be necessary in future work to explore how mAb/mAb and mAb/surface contacts affect the extent of structural deformation as a function of packing density.

### 3.8. Limitations of the Simulation Approach

The simulation based on the DLVO theory relies on the input of the measured data to tune parameters such as the effective radii. This is in contrast to the phenomenological models such as random sequential adsorption (RSA) which calculate the jamming limits of geometries randomly placed onto a planar surface, with the restriction that no overlapping can occur. The jamming limit in each case identifies the maximum fractional coverage that the adsorbing geometry can achieve, which for identical hard disks is 0.547.\textsuperscript{37} This jamming limit is taken into consideration by the effective radii.

Many models are constructed by assuming that adsorption onto the surface is retarded by the Gibbs potential. Often, this manifests itself in the model by means of a retardation factor, which damps the adsorption rate by a constant amount.\textsuperscript{38} In these types of model the retardation factor and an effective protein radius are adjusted until each dataset can be best fitted. The DLVO approach provides a further level of insight by allowing us to identify how each component of the Gibbs potential contributes to an adsorption process. However, this additional insight beyond the retardation factor requires far more than two input values, many of which have to be estimated.

The assumption of hard sphere proteins is one that is only justified for simplicity. Computer simulations into model globular proteins often composed of many connected hard spheres, aid in the discussion on the effect of conformational changes on adsorption processes.\textsuperscript{39} However, these simulations are extremely difficult to apply to specific protein forms, yet this approach has been carried out using Monte Carlo
simulations with some success. A further related assumption made in this work is that COE-3 has the same charge distribution across its surface, which neglects the fact that Fc and Fab have different charges, size and shape. Thus, these issues must be taken into consideration if the adsorption process must be simulated with accuracy. Given these assumptions and the estimate of the surface tension components of COE-3 made, our model have aimed to aid the experimental data interpretation and understand how protein adsorption is affected by changes to solution properties.

4. Conclusion

NR measurements revealed that adsorption of COE-3 at the SiO₂/water interface led to the formation of a monolayer with mAb molecules adsorbed flat-on. The depth sensitivity from NR together with the fast dynamic SE measurements revealed that free surface space was initially rapidly occupied by mAb molecules and subsequent adsorption towards equilibrium was not accompanied so much by structural perturbation as by tighter packing rearrangement, especially if accompanied by increasing pH or simply time. Simulations conducted as described and using full amino acid sequence information enabled the rationalization of the equilibrium adsorption data, most notably the short and long range forces dominating buffer and pH effects as a function of bulk concentration. Modelling the initial adsorption phase remains challenging since the sparsity of mAb molecules at the surface does not accurately constitute a FSIP as assumed, thereby overestimating the lateral repulsion. In comparison with previously published data for other mAbs adsorbed at the SiO₂/water interface, the characteristic profile with respect to time, pH and concentration are largely in agreement, with at equilibrium appearing to vary within a defined range according to the individual mAb conformational stability and pI.

Supporting Information

The Supporting Information including sequences for the light and heavy chains of the mAb studied here and comparisons against the one with crystalline structures of its fragments, the charge variations of Fab, Fc and the whole COE-3 against pH and the key considerations adopted in the setup of the simulation model under the DLVO framework is available free of charge on the ACS Publications webpage.
Acknowledgements

We thank funding support from MedImmune Ltd, neutron beam times awarded to undertake this work at ISIS Neutron Facility, Chilton, Didcot, under the support of STFC. ZL acknowledges studentship support from University of Manchester via an Overseas Research Scholarship (ORS) award and a physics research merit award. CS acknowledges a joint PhD studentship from STFC, Unilever and University of Manchester. We also thank EPSRC for support under EP/F062966/1.
References


Support Information

Section S1: Surface and interfacial measurements of mAb adsorption and physical properties of mAb

Figure S1. Charge distribution as a function of pH for Fab and Fc and the whole COE-3 (1/3 contribution from 1 Fc and 2 Fabs) as calculated from the heavy chain sequences only.

These charge variations against pH as calculated from the constituent amino acids are different from the measured IP values from real experimental measurements due to the ignorance of charges on sugar groups and associations with ions from bulk solution.
Figure S2. Ellipsometric scans of the amplitude component Ψ (a) and the phase difference Δ (b) against wavelength measured at the SiO₂/water interface after 5 min of adsorption under COE-3 concentrations of 0.005, 0.02 and 0.1 mg/ml in 25 mM His buffer, pH 5.5. The continuous lines were best uniform layer fits with the adsorbed amount plotted in Figure 2(a).
**Table S1.** Key sequence information for COE-3

**Table S1A.** The antibody molecule is comprised of 2 identical light and heavy polypeptide chains.

The sequence of the Light Chain:

DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLIIIYAAASS
LQSGVPSRFSGSGTDTTLTISSLQPEDFATYYCQQSYSTPLTFGGGSKVEIK
RTVAAPSVFIFPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNS
QESVTEQDKSTYSLSSTLKSADYEKHKVYACEVTHQGLSPVTKSFNR
GEC

The sequence of the Heavy Chain:

QVNLRESGGGLVQPGGLRLSCAASGFTFGSYAMSWVRQAPGKGLEWVSAI
SGGGSTYADSVKGRFTISRDSNKNSSLYLQMNSLRAEDTAYYCARRSIYG
GNYYFYWGRGLTNTISSASTKGPSVFPLAPSSKSTSGGTAAALGCLVKDYFP
EPVTVSWSNGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNH
KPSNTPKVDKKVPEKSKCDKTHTCPCTPAPELLGGSVFLFPKPKDTLMISETP
EVTCVVVDVSHEDPEVFKNWYVDGEVHNAKTPREEQYNSTYRVVSVT
VLHQQDWNKLYKCKVSNKLPAIETLSKAKQPREPQVYTLPPSRDELT
KNQVSLTCLVKGFYPSIDAVESWESNGQPENNYKTTPVLDSDGSSFLYSLKT
VDKSRWQQQGNYFCSVCSVMHEALHNHYTQKSLSPGK

The two constant domains (C_{H\gamma3} and C_{H\gamma2} in blue) of each heavy chain form the Fc, which is then dimeric. The C_{H\gamma1} and variable domain (V_{H}) of the heavy chain, both in red text, together with the light chain (C_{L\kappa/\lambda} and V_{L} domains) form each of the two Fab arms.

The sequences were entered into the Biomolecular Scattering Length Density Calculator (http://psldc.isis.rl.ac.uk/Psldc/) and the influence of the isotopic contrast of water was set accordingly to obtain the respective scattering lengths (SL) and scattering length density (SLD) under D_{2}O, with the results shown below.
Table S1B. Key parameters used for D₂O and COE-3

<table>
<thead>
<tr>
<th>Molecular structure</th>
<th>SL×10⁵/Å</th>
<th>Volume/Å³</th>
<th>SLD×10⁶/Å⁻²</th>
</tr>
</thead>
<tbody>
<tr>
<td>D₂O</td>
<td>19.14</td>
<td>30</td>
<td>6.35</td>
</tr>
<tr>
<td>COE-3</td>
<td>57648</td>
<td>171740</td>
<td>3.36</td>
</tr>
</tbody>
</table>

The molecular weight of COE-3 is approximately 144,750 in H₂O.
Tables S2A. Amino acid sequence comparison between Fc of COE-03 and Fc of 1HZH (Protein Data Bank ID code, reference below).

The 1HZH Fc sequence was from Saphire et al.\textsuperscript{S1} at the end of Support Information.
Table S2B. Amino acid sequence comparing between COE-3 Fab and Fab of 1HZH.

The 1HZH Fab sequence was from Sapphire et al\textsuperscript{SI} at the end of Support Information.
**Figure S3.** The change in surface tension when 500 ppm COE-3 was added to histidine buffer 25mM at pH 6.

The surface tension shows little change from 72.8 mN/m, suggesting COE-3 has a surface tension similar to water, especially over the duration of an adsorption experiment.
Section S2A: DLVO theory

The specific forms of the Gibbs potentials of the LW and DA forces between a hard sphere of radius $R$ and a semi-infinite plane are

\[ G_{12}^{lw}(z) = 2\pi l_0^2 R G_{12}^{lw} || /z \]  
(S1),

and

\[ G_{12}^{da}(z) = 2\pi \chi R G_{12}^{da} || \exp\left[\chi (l_0 - z)\right] \]  
(S2),

where $G_{12}^{x||}$ are the Gibbs potentials for a force $x$ between two semi-infinite planes separated by the equilibrium distance $l_0 = 0.157$ nm.\textsuperscript{S2} The remaining parts of the equations convert this interaction to be between a semi-infinite plane and a hard sphere a distance $z$ away.\textsuperscript{S3} In Equ. S2, $\chi$ is the DA interaction decay length whose precise value varies but can be taken to be 1 nm.\textsuperscript{S2} As indicated in the main text, $G_{12}^{x||}$ are functions of the surface tensions of the silica (1), liquid medium (2) and COE-3 (3) when the force $x$ is LW or DA. The general forms are given by the Dupré equations, which are

\[ G_{123}^{x||} = \gamma_{13}^x - \gamma_{12}^x - \gamma_{32}^x \]  
(S3),

for silica - COE-3 interactions, and

\[ G_{323}^{x||} = -2\gamma_{23}^x \]  
(S4),

for COE-3 - COE-3 interactions, where $\gamma_{ij}^x$ are functions of the individual materials surface tensions, with a form dependent on the interaction type.\textsuperscript{S4} The total surface tension, $Y$ of a material $i$ can be decomposed into three components. These are the LW component, $\gamma_i^{lw}$, and the donor and acceptor components, $\gamma_i^d$ and $\gamma_i^a$ respectively, which sum as

\[ Y_i = \gamma_i^{lw} + 2 \sqrt{\gamma_i^{da} \gamma_i^{aa}} \]  
(S5).

The combination rules for LW and DA interactions are

\[ \gamma_{ij}^{lw} = \left( \sqrt{\gamma_i^{lw}} - \sqrt{\gamma_j^{lw}} \right)^2 \]  
(S6),
and

\[ \gamma_{ij}^{\text{dn}} = 2 \left( \sqrt{\gamma_i^d} - \sqrt{\gamma_j^d} \right) \left( \sqrt{\gamma_i^a} - \sqrt{\gamma_j^a} \right) \]  

(S7),

respectively.\(^{82}\) Finally, the EL contribution depends instead on the surface potentials of the two interacting bodies, \(\psi_i\) and \(\psi_j\) via

\[ G_{12}^i(z) = 4\pi \varepsilon_0 \varepsilon_r \varepsilon_0 \psi_i \psi_j \ln[1 + \exp(-\kappa z)] \]  

(S8),

where \(\varepsilon_0\) (\(\varepsilon_r\)) is the vacuum (relative) permittivity, and

\[ \kappa = \left( \frac{2000e^2N_A I}{\varepsilon_0 \varepsilon_r k_B T} \right)^{0.5} \]  

(S9),

is the inverse of the Debye screening length in m\(^{-1}\) where \(k_B\) is the Boltzmann constant, \(e\) is the electron charge, \(N_A\) is Avogadro’s number, \(T\) is the solution temperature and \(I\) is the ionic strength of the liquid medium in molars.\(^{83, 85}\) The surface potential for amphoteric surfaces such as silica with isoelectric point \(pH_0\) and ionization constant \(\Delta pK\) is found from

\[ \frac{1}{\alpha} \sinh \left( \frac{\gamma}{2} \right) = \frac{\delta \sinh(2.303\Delta pH - \gamma)}{1 + \delta \cosh(2.303\Delta pH - \gamma)} \]  

(S10),

where \(\delta = 2 \times 10^{-\Delta pK/2}\), \(\Delta pH = pH_0 - pH\) where \(pH\) is that of the liquid medium and \(\alpha = \frac{N_s}{4N_A}\) where \(N_s\) is the number of sites on silica, commonly taken as 8 nm\(^{-2}\) and \(\gamma = \frac{\psi_3 e}{k_B T}\) is the dimensionless potential.\(^{83, 86}\) The presence of the amphoteric surface modifies the pH of the solution in its immediate vicinity to

\[ pH_s = pH + \frac{\gamma}{2.303} \]  

(S11),

which is the pH that the adsorbed protein layer resides within.\(^{83}\) The surface potential of the protein, \(\psi_3\) is often taken to be equal to its zeta potential, \(\zeta\). This is a measurement of the potential due to the charge of the protein as well as any buffer ions within the slipping plane. The relation between zeta potential and surface potential is approximately

\[ Q = 2 \frac{e R^2 \kappa}{\lambda_0} \sinh \left( \frac{\zeta}{2} \right) \left[ 1 + \frac{2}{(\kappa R) \cosh^2(\zeta/4)} + \frac{8 \log \left( \cosh^2(\zeta/4) \right)}{(\kappa R)^2 \sinh(\zeta/2)} \right]^{0.5} \]  

(S12),
where $\tilde{\zeta} = \frac{\zeta e}{k_B T}$ is the dimensionless zeta potential and $l_B = \frac{e^2}{4\pi\varepsilon_0 \varepsilon_r k_B T}$ is the Bjerrum length. As indicated in the main text Equ. S12 is for a monovalent buffer but was used for simplicity. Figure 4 shows example plots of silica - protein (a), and protein - protein (b) interactions. Note that in our model we assume that the proteins cannot become bound in the infinite well, as the barrier preventing this is so large.

![Figure S4. Gibbs distribution plots.](image)

(a) Example plot of Gibbs interaction between a protein in solution and the semi-infinite silica plane. Proteins cannot absorb into the infinite well at zero distance from the plane, but can adsorb into the finite minimum. (b) Example plot of Gibbs interaction between the protein FSIP and a protein in solution. Here the proteins are repulsive, with no minimum forming.
Section S2B: Protein-protein interaction map

The interactions between proteins can either be repulsive, attractive or long-range attraction with short-range repulsion (type-2 attraction). This depends on the surface tensions of the protein in the solution. The LW component is always negative but the DA can be either negative or positive.\textsuperscript{S2} When DA is positive this produces overall attraction. The sign of the DA force is decided by the sign of the factor $G_{||123}^{sd}$ and therefore entirely by the surface tensions. So, the condition separating attraction from repulsion (and type-2 attraction), is

$$G_{||123}^{sd} = -2 \gamma_{23}^{sd} = -4 \left( \sqrt{\gamma_3^d} - \sqrt{\gamma_3^a} \right) \left( \sqrt{\gamma_2^d} - \sqrt{\gamma_2^a} \right) \begin{cases} > 0, \text{ for repulsion} \\ < 0, \text{ for attraction} \end{cases} \quad (S13),$$

If $G_{||123}^{sd} > 0$, interactions are only completely repulsive if an additional condition is met, this being $|G_{323}| > |G_{||123}^{sd}|$. If this is not satisfied then at large distances the proteins will exhibit type-2 attraction, only repelling when close. Therefore, the second condition for repulsion is

$$\left| \sqrt{\gamma_3^d} - \sqrt{\gamma_2^d} \right| > \frac{\left( \sqrt{\gamma_2^w} - \sqrt{\gamma_3^w} \right)^2}{2 \sqrt{\gamma_2^d} - \sqrt{\gamma_3^d}} \quad (S14),$$

The LW Gibbs potential energy component is always negative. Ignoring electrostatic contributions (as these do not depend on surface tension), this implies that the DA component between the silica and protein must be positive in order to produce a minimum in the initial Gibbs distribution. The sign of this interaction between the silica layer and COE-3 is determined by $G_{||123}^{sd}$, which leads to the adsorption condition

$$\sqrt{\gamma_3^d} > -\sqrt{\gamma_3^a} \sqrt{\gamma_2^d - \gamma_2^a} + 2 \sqrt{\gamma_2^d - \gamma_1^d} \sqrt{\gamma_3^d - \gamma_1^d} \quad (S15),$$

Figure S5 below shows the protein-protein interaction map used to determine suitable surface tension parameters.
Figure S5. The protein-protein interaction map. The lower green contour represents equation S15, above which a minimum exists in the initial distribution. The higher green contour indicates where $\gamma^{lw}_3$ is equal to zero. Therefore, between these boundaries lies the region in which adsorption onto the silica layer can occur. The horizontal (vertical) black lines represent when $\gamma^d_3 (\gamma^a_3)$ and $\gamma^d_2 (\gamma^a_2)$ are equal. Hence these lines separate the map into regions of $G^{dal}_323$ greater (top left/bottom right) and less than (top right/ bottom left) zero. An example contour with $\gamma^{lw}_3 = 40$ mN/m is plotted in the dotted blue. The red contours represent equation S14, above this line in the top left region repulsion exists, whilst repulsion exists below this in the bottom right region.

By increasing the $\gamma^{lw}_3$ component, the depth of the minimum is increased, and by having a $G^{dal}_323$ value closer to the horizontal or vertical black lines the strength of the repulsion is decreased. The values for the surface tension of COE-3 were chosen such that sufficient repulsion (filling of the potential well) was exhibited to recover the experimental data. This only occurred for the surface tension values stated in the main paper because the depth of the minimum and strength of the repulsion are both extremely sensitive.
References S


Chapter 6

- Abstract 182

1. Introduction 183

2. Materials and Experimental Methods 185

3. Results and Discussion 189

4. Conclusions 203

- References 205

- Supporting Information 208
Co-adsorption of Monoclonal Antibody COE-3 and Nonionic Surfactant at the SiO$_2$/water Interface

Zongyi Li$^1$, Fang Pan$^1$, Ruiheng Li$^1$, Elias Pambou$^1$, Daniela Ciumac$^1$, Peixun Li$^2$, Mario Campana$^2$, John R P Webster$^2$, Steve Bishop$^4$, Rojaramani Narwal$^4$, Christopher F van der Walle$^3$, Jian R Lu$^1$

$^1$Biological Physics Laboratory, School of Physics and Astronomy, University of Manchester, Oxford Road, Schuster Building, Manchester M13 9PL, UK.

$^2$ISIS Neutron Facility, STFC, Chilton, Didcot OX11 0QZ, UK.

$^3$Formulation Sciences, MedImmune Ltd, Sir Aaron Klug Building, Granta Park, Cambridge CB21 6GH, UK

$^4$Formulation Sciences, MedImmune LLC, Gaithersburg, MD 20878, USA.

Corresponding author: Jian R Lu
(email: j.lu@manchester.ac.uk; Tel: +44 161 2003926)

ZL and FP made equal contributions

Keywords:
mAbs, Tween 80, interfacial adsorption, antibody, structural unfolding, self-assembly, neutron reflection
Abstract

Therapeutic monoclonal antibodies (mAbs) are becoming increasingly attractive in treating diseases, but before widespread use, these delicate molecules must be stabilized against structural deformation and unfolding caused by adsorption, desorption and aggregation. Nonionic surfactants such as Tween 80 have been widely used to attempt to prevent their interfacial adsorption and retain their bioactivity. In this work, we have examined the interfacial adsorption from an mAb called COE-3 and non-ionic surfactant at the bare SiO$_2$/water interface by using spectroscopic ellipsometry (SE) to follow dynamic adsorption and neutron reflection (NR) for interfacial structure and composition. Results revealed that nonionic Tween 80 with 20 ethoxylates (Tween 80 20EO) and C12E5 had little affinity to COE-3 or the interface under the conditions studied. They thus can’t prevent COE-3 adsorption. In contrast, Tween 80 with 7 ethoxylates (Tween 80 7EO) could co-adsorb, but it could not influence the dynamic process and the equilibrated amount of COE-3. Once the mAb layer tended to plateau and underwent structural adjustments the surfactant started to bind to the mAb layer. Thus, surfactant only started to bind to the well-formed mAb layer and then form a well-defined surfactant bilayer via self-assembly. It was found that the same surfactant bilayer could also be formed from the sequential process of initial mAb adsorption followed by surfactant co-adsorption. The interfacial layer is comprised of an inner mAb layer of some 70 Å thick and an outer surfactant layer of some 60 Å thick with distinct transitional regions across the mAb-surfactant and surfactant-bulk water boundaries. Once formed, such interfacial layers were very robust. They could work as a protective shell against further mAb adsorption and desorption, thereby enhancing the structural stability of the mAb molecules. Thus, the combined dynamic and structural examinations from SE and NR have unravelled the direct molecular interactions at the SiO$_2$/water interface, and further study could help search for more effective surface active reagents for mAb stabilization.
1. Introduction

Monoclonal antibodies (mAbs) are fast becoming important therapeutic treatments to major diseases such as immune compromises and cancers. Before these treatments are widely adopted, however, technical hurdles must be overcome in product package and storage so that mAbs are well protected and stabilized. An attractive route that has been explored and further developed over the recent years is to formulate mAbs in the concentrated aqueous solution ready for clinical administration [1-3]. However, mAb molecules can undergo adsorption and desorption, and this molecular process can elicit structural damage, triggering the formation of mAb aggregates and precipitates. This cascade of molecular processes causes loss of bioactivity [4-10] and imposes a great deal of uncertainty in the treatment outcome.

Addition of nonionic surfactants into mAb formulations has been widely thought to inhibit protein adsorption. Many studies including our work have already shown that nonionic surfactants can prevent protein adsorption on the surface of water [11,12] when their concentrations are sufficiently high, but it remains unclear if they can also stop mAb adsorption at the solid/water interface. The aim of this study is to investigate how mAb and nonionic surfactant co-adsorb and if it is possible to prevent mAb adsorption at the solid/water interface.

In the much broader context, adsorption of proteins has both fundamental and practical significance [13]. Whilst there are many cases where protein adsorption is undesired, there are also other cases where the opposite is true. An example of the former is the fouling of proteins to porous membranes used in protein filtration which can cause membrane blockage and halt the process. In contrast, immobilization of extracellular membrane proteins onto implants can improve their integration with local tissue, thereby enhancing their chances of successful deployment and function.

Although the co-adsorption of protein and surfactant can be similarly studied as in the case of protein adsorption[4-12], the difficulty often lies in the ability to distinguish adsorbed protein from surfactant and water in the adsorbed layer. Techniques such as fluorescence measurement, dual polarisation interferometry and SE can provide useful indications relating to the amount of materials adsorbed. In contrast, NR together with deuterium labelling can reveal the thickness and composition of adsorbed protein with and without surfactant [4, 11,13-17]. NR data together with the globular structures of
proteins allow us to determine how interfacial adsorption and surfactant binding affect conformational orientations of the adsorbed protein molecules from which the extent of structural deformation and unfolding can be inferred.

A protein molecule is folded from one or more polypeptide chain(s), often with a well-defined 3D structure. Interfacial adsorption can cause structural deformation or unfolding, resulting in the loss of its bioactivity. Due to relative structural simplicity, lysozyme, human serum albumin (HSA) and bovine serum albumin (BSA) have been used to examine their adsorption features with and without surfactants[13, 18, 19]. Ionic surfactants such as sodium dodecyl sulphate (SDS) and dodecyl trimethyl ammonium bromide (C_{12}TAB) can bind to pre-adsorbed proteins, causing the deterioration of the globular framework. They are thus unsuitable for mAb stabilization.

In contrast, nonionic surfactants are mild to proteins, but little has been explored as to if and how they can prevent protein adsorption. A mAb is comprised of 1 fragment crystallization (Fc) and 2 fragment antigen-binding arms (Fabs). The Fc is formed from the two constant domains (C_{\gamma3} and C_{\gamma2}) of each heavy chain, and each Fab is formed from the C_{\gamma1} and variable domain (V_{H}) together with the light chain C_{\kappa/\lambda} and V_{L} domains. Because of larger sizes [20, 21], mAb would have a greater area of contact with the substrate upon interfacial adsorption. Nonionic surfactants would have to be more surface active to provide the desired effect.

In this work, we have chosen both C_{12}E_{5} and Tween 80 with different ethoxylate head group sizes to examine how they could co-adsorb when mixed with COE-3 and if any of them could prevent COE-3 adsorption. Because of their different sizes and hydrophobicity, they could interact with COE-3 differently, thereby offering the potential to mediate COE-3 adsorption or co-adsorption. COE-3 is of the immunoglobulin-1 (IgG1) isotype. Its sequence is known, and it allows substantial sequence modifications in the variable and constant domains of the Fab [11, 17]. Its solution properties and surface and interfacial adsorption have been extensively studied [11, 17, 22]. This study of its co-adsorption with representative nonionic surfactants at the solid/water interface represents an important step to unravel the molecular basis of molecular interactions.

Thus, by combining the capabilities of following dynamic process from SE and structural determination from NR, this work aims to unravel how COE-3 adsorb in the
presence of a group of nonionic surfactants. As COE-3 represents a base mAb structure from which a series of functional mAbs has been engineered, the adsorption of COE-3 and its constituent Fab and Fc at the air/water interface and solid/water interface has been studied [11, 17, 23]. The SiO$_2$ surface is a good model of glass as it is widely used to make vials and syringes for the storage of mAb products. The co-adsorption at the bare SiO$_2$/water interface has been used to mimic the main features at the glass/water interface.

2. Materials and Experimental Methods

2.1 Materials

**COE-3** It is a mAb of human IgG1$\kappa$ type with a molecular weight (MW) of 144,750 and isoelectric point (pI) of 8.44 [22]. Its stock was supplied as a solution of 46.4 mg/ml in ‘His buffer’ (25 mM histidine/histidine hydrochloride, 7 % w/v sucrose, pH 6.0, batch no. SP12-423). The mAb was expressed in Chinese hamster ovary cells and purified using industry-standard methods. The stock solution is stored at -80 °C and when solution preparation is needed it was thawed and then diluted directly into histidine buffer at a given pH, maintaining an ionic strength of 25 mM; H$_2$O buffers for a range of pH and mAb concentrations needed for SE measurements, with D$_2$O and contrast matched to antibody (CMAb) buffers at the equivalent pH were prepared for NR measurements. Despite the original stock solution being in H$_2$O, the large dilutions made into CMAb and D$_2$O resulted in only very low changes in the scattering length density (SLD) of the solvents, but the exact amounts were taken into account during neutron data analysis. As the sequences of the light and heavy chains of COE-3 are known [11, 17], its scattering length (SL) and scattering length density (SLD, $\rho$) in CM2.58 (or CMAb) and D$_2$O are listed in Table S1.

**Surfactants** Pentaethylene glycol monododecyl ether (C$_{12}$E$_5$, from Sigma, 99% pure) is a popular model nonionic surfactant and was used as received. The molecular structure of the hydrogenated Tween 80 is schematically shown in Figure S1. It has 20 ethoxylates (denoted as Tween 80 20EO) and is widely studied for its capacity to stabilise protein biotherapeutics. Following the recent work by Tucker et al on the examination of the impact of the acyl chain length and head group size of this group
of surfactants on their interaction with hydrophobin [24], we have synthesised Tween 80 with 7 ethoxylates, with the head group hydrogenated (HTween 80 7EO) and deuterated (DTween 80 7EO). The respective SLs and SLDs for these surfactants are also shown in Table S1. Griffin [25] has developed a method for estimating the amphiphilicity of non-ionic surfactants via the so-called hydrophilic-lipophilic balance (HLB) as follows:

\[
HLB = 20 \frac{M_h}{MW}
\]  

(1),

where \(M_h\) is the molecular mass of the hydrophilic head of the molecule, and \(MW\) is the mass of the entire molecule, giving a result between 0 and 20 with 0 corresponding to a completely hydrophobic molecule and 20 corresponding to a completely hydrophilic one. From equation (1) the HLB values were estimated to be 11.7 for C12E5, 9.9 for Tween 80 7EO and 14.3 for Tween 80 20EO.

**Silicon wafers and blocks** Silicon wafers (<111> orientations, minimal doping) with 10 cm diameter and one side optically flat were purchased from Compart Technology, UK. They were cut into 2cm × 2cm squares to fit the solid/liquid cell specially built for spectroscopic ellipsometry measurements. The large faces of silicon blocks (5cm × 8cm × 1.2cm), also <111> orientations) were polished into optically flat by Crystran Ltd (Poole, UK). The optically flat silicon surfaces bear an ultrathin native oxide layer. Before use, all the wafer and block surfaces were carefully rinsed with dilute Decon (neutral, containing 3-5% the concentrated stock supplied by Decon UK) and then plenty of pure water before drying to ensure good they were thoroughly clean.

**Buffers.** Pure H\(_2\)O was processed from an Elgastat PURELAB purification system. D\(_2\)O (99% D), histidine and histidine hydrochloride were purchased from Sigma-Aldrich and used as supplied.

**2.2 Spectroscopic Ellipsometry (SE)**

SE measures the variation of the light polarisation state from the reflection at the sample surface or interface. Two optical parameters \(\Delta\) and \(\Psi\) are used to describe this variation, with \(\Delta\) showing the wave phase changes in \(p\) and \(s\) polarisations, and \(\Psi\) showing the wave amplitude differences. The sample ellipticity, \(\rho\), is expressed as the ratio of the Fresnel reflection coefficients of \(p\) and \(s\) polarizations , \(r_p\) and \(r_s\), respectively [15, 26]:
\[ \rho = \tan \psi \cdot e^{i\Delta} = \frac{r_p}{r_s} \quad (2). \]

The experimental data were analyzed using the software developed by J.A. Woollam Co. Inc. Data analysis starts with the assumed interfacial structure model incorporating the sample layer from which \( \rho, \Delta \) and \( \Psi \) can be calculated theoretically from equation (2). The fitting to the experimental data via a regression process leads to the thickness and refractive index when the MSE (mean squared errors) between measured and simulated data is minimal.

The dispersion relationship of sample refractive index is described by the Cauchy equation for a transparent and homogeneous material:

\[ n(\lambda) = A + \frac{B}{\lambda^2} \quad (3), \]

where \( \lambda \) is the wavelength; \( A \) and \( B \) are the Cauchy coefficients which were taken as 1.45 and 0.003, respectively, in this work. The surface excess \( \Gamma \) (mg/m\(^2\)) of the sample was calculated from refractive index \( n \) and sample thickness \( \tau \) by De Feijter’s formula [27]:

\[ \Gamma = \frac{\tau(n-n_0)}{(dn/dc)} \quad (4), \]

where \( n \) and \( n_0 \) are the refractive indices of the sample and the ambient environment; \( dn/dc \) is the refractive index increment of the sample which was assumed to be 0.18 ml/g in this work.

A common technical challenge with the study of ultrathin films (<50-100Å) by SE lies in the difficulty in decoupling layer thickness from refractive index, but the product always ensures good consistency and reliability. In checking the thicknesses of the native oxide layers on silicon wafer cuts and polished neutron blocks, it was assumed that the refractive indices of the oxide layers were the same as that of the pure SiO\(_2\), with no pores or defects. The SE measurements led to consistent oxide layer thicknesses 13±2Å. SE measurements at the solid/water interface were performed using a specially designed liquid cell with a pair of quartz windows to allow light entering and exiting from the reflecting interface. The windows were aligned to be perpendicular to the incoming and exiting light beams. A silicon wafer cut (2cm × 2cm) was confined at the centre of the liquid cell bottom and thus enabled the light beam entering and becoming reflected at 70° to the surface normal. A useful check of
the window alignment was the consistent thickness of the native oxide layer at the solid/water interface. The amount lysozyme adsorbed at 1 mg/ml and pH 7 could help check the consistent hydrophilicity of the wafer surfaces after cleaning [28].

2.3 Neutron Reflection (NR)

Neutron reflection measurements were undertaken on the SURF and INTER reflectometers at ISIS Neutron Facility, Rutherford Appleton Laboratory, UK. The polished and freshly cleaned face of the silicon block was clamped against a purposely-built liquid cell. The setup was then mounted on the sample stage, and alignment was made to ensure the neutron beam to be specularly reflected from the solid/liquid interface and collected by the detector. The illuminated area at the solid/liquid interface was defined by a series of slits, and a monitor was in front of sample stage to count the incoming neutrons. The reflectivity was obtained as the ratio between the number of incoming neutrons and that of reflected neutrons, calibrated to the 100% reflectivity below the critical edge at the SiO$_2$/D$_2$O interface.

The data were analysed using the software named Motofit, which could calculate the reflectivity profile from a simulation model based on neutron reflection theory and the Abeles optical matrix method as described in our previous work [29, 30]. The simulation model contained a series of thin layers with adjustable parameters such as layer thickness $\tau_i$ and average scattering length density ($SLD_i$ or $\rho_i$). The difference between a measured profile and simulated profile is quantified by $\chi^2$ in the least-squares method. The optimal fit was found when $\chi^2$ tended to the minimum. The $\rho_i$ of each layer can be expressed as:

$$\rho_i = \phi_i b_i + \phi_h b_h + \phi_t b_t + \phi_w b_w$$  \hspace{1cm} (5)

Where $\phi$ stands for the volume fraction of the antibody ($a$), surfactant head group ($h$), surfactant tail group ($t$) and water as solvent ($w$); $b$ is the scattering length density of each component. The sum of volume fractions should always be unity.

For the adsorbed layer containing antibody only, the values of $\phi_t$ and $\phi_h$ are zero. Therefore, the volume fraction of antibody $\phi_a$ was calculated straightforward with known values of $b_a$ and $b_w$ as listed in the Table S1. For the layer involving 2 or more sample materials, volume fractions can be determined by solving equation (5) from a set of parallel experiments measured under different isotopic contrasts. Importantly,
the reflectivity profiles measured from each isotopic contrast were fitted again based on the obtained volume fractions to optimize the fits, with the results given in Table S2. Structure parameters did vary between parallel experiments, but based on the sensitivity and tolerance fitted their average values and deviations were also presented in Table S2.

The adsorbed mass $\Gamma_i$ of each component can be estimated from

$$\Gamma_i = \frac{\tau \phi_i M_w}{V N_A}$$

(6).

where $V$ and $M_w$ are molecular volume and molecular weight; $N_A$ is the Avogadro constant; $\phi_i$ denotes the volume fraction of the component in the layer.

3. Results and Discussion

3.1 Adsorption of COE-3 at the Bare SiO$_2$-water Interface

SE measurements were first made to determine the thickness of the native oxide layer on the surface of each wafer to ensure the surface to be clean. The oxide layer could be well fitted into 13±2Å from all wafer cuts used, suggesting good consistency between the surfaces used. Similar SE measurements at the air/solid interface were also undertaken for the large polished faces of the silicon blocks before neutron reflection. On each surface, SE measurements were undertaken at the middle and 2 side points 1 cm away from the edge. The thicknesses were very consistent with the values obtained from the wafer cuts, suggesting close oxide structures. Subsequent measurements were made at the solid/water interface using a purposely-built SE liquid cell for the wafer cuts. Similar oxide thicknesses were obtained and this process ensured good window alignment before proceeding to the study of COE-3 adsorption. Figure 1 shows a set of $\Psi$ (Figure 1(a)) and $\Delta$ (Figure 1(b)) measured from the SiO$_2$/water interface at 1, 5 and 30 min. The water phase was the aqueous His buffer at pH 5.5 with the ionic strength at 25 mM. The continuous lines represent the best fits to the respective measured data, resulting in the oxide layer thickness of 13±1Å.

In the plots as shown in Figure 1, the changes as detected are small in $\Psi$ are small, but larger or more obvious changes are shown in $\Delta$. In the analysis of the thicknesses of the oxide layers, it was assumed that the layers were free from defects with their
refractive index the same as that of the amorphous SiO$_2$. The Cauchy equation as depicted in equation (3) was used to describe the wavelength dependent change in refractive index.

![Figure 1](image.png)

**Figure 1.** Ellipsometric scans of the amplitude component $\Psi$ (a) and the phase difference $\Delta$ (b) against wavelength measured at the SiO$_2$/water interface for 0.01 mg/ml COE-3 in 25 mM His buffer, pH 5.5; after 1, 5 and 30 min of adsorption. The continuous lines show the uniform layer fits of the SiO$_2$ layer of 13±1Å.

Following the determination of the thickness of the SiO$_2$ layer, COE-3 adsorption was undertaken at a range of concentrations starting from the lowest of 0.01 mg/ml (10 ppm), also at pH 5.5. Similar to what is shown in Figure 1, each set of measurements of $\Psi$ and $\Delta$ could be made over some 5 seconds (dependent on the number of points measured and the wavelength range) and plots of changes in $\Psi$ and $\Delta$ were obtained against time at each COE-3 concentration. Fitting to each pair of $\Psi$ and $\Delta$ led to the layer’s refractive index and thickness from which the adsorbed amount $\Gamma$ could be calculated from the De Feijter’s formula as shown in equation (4). In the analysis of these $\Psi$ and $\Delta$ pairs, the refractive index and thickness of the SiO$_2$ layer were fixed. To avoid any irrational variations arising from the coupling of the refractive index and thickness of the mAb layer, $A$ was fixed at 1.45 in equation (3). This means the matching thickness was nominal and would not have any bearing on the change of the real mAb layer thickness. However, the treatment of fixing $A$ was for simplicity; it did not affect the determination of $\Gamma$ from equation (4). Figure S1 shows the time-dependent changes of $\Gamma$ at the 4 concentrations of 10, 50, 100 and 200 ppm. It took about 15 min for the adsorption to reach equilibration at 10 ppm. In contrast, the initial dynamic process occurred much faster for the 3 higher mAb concentrations, and it only
took about 2 min for the adsorbed amount to plateau. Figure 2 shows the equilibrated adsorbed amount plotted versus the COE-3 concentration. \( \Gamma \) is just about 2.5 mg/m\(^2\) at 10 ppm. As mAb concentration increases, the adsorbed amount shows a small but measurable rise. At the highest concentration of 200 ppm, \( \Gamma \) reaches 2.6 mg/m\(^2\). These values and changes are consistent with the data from our previous studies [23].

![Figure 2](image1.png)

**Figure 2.** Surface saturated adsorbed amount measured by SE and by NR plotted against concentration.

NR measurements were made at the SiO\(_2\)/water interface under similar conditions. Instead of H\(_2\)O, D\(_2\)O was used to improve the isotopic contrast and signal. Other than this, other conditions were kept the same as used in SE measurements. Figure 3 shows reflectivity profiles measured under a set of COE-3 concentrations with that measured from the SiO\(_2\)/D\(_2\)O interface as a control. For clarity, the reflectivity profiles were called down by plotting them with 0.2\(^n\) offsets from the highest concentration to make better separation. The difference in shape between that measured at 10 ppm and the control (buffer) clearly indicates COE-3 adsorption.

![Figure 3](image2.png)

**Figure 3.** NR profiles measured at the SiO\(_2\)/D\(_2\)O interface for COE-3 adsorption at 0, 0.01, 0.02, 0.05 and 0.2 mg/ml bulk concentration in 25mM His buffer, pH 5.5; the continuous lines denote the best fits for each profile with the fitted parameters showed in Table 1. For clarity, the profiles were plotted with 0.2\(^n\) offsets to separate them from each other.
Unlike SE fitting, the modelling to the reflectivity measured at the SiO$_2$/D$_2$O interface led to the independent determination of layer thickness and scattering length density (SLD or $\rho$) equivalent to $n$ in SE. The oxide layer was also found to be $13\pm1$ Å and free of defects. Subsequent analysis of the structure of the adsorbed COE-3 layer was undertaken by fixing the oxide layer structure and assuming that COE-3 was adsorbed onto the oxide surface. It was found that the main shapes of the measured reflectivity profiles could be represented by assuming the formation of a uniform layer from COE-3 adsorption. However, the further improvement was achieved by assuming a dense inner layer together with a diffuse outer layer into the aqueous phase. The consistency between the fitted (continuous lines) and measured data as shown in Figure 3 adds confidence in the quality of the model analysis adopted, showing the good representation of the distribution of the adsorbed COE-3 layers by the 2-layer model. The key structural parameters obtained from the 2-layer model fits are listed in Table 1.

<table>
<thead>
<tr>
<th>$\text{Con.} , \text{mgml}^{-1}$</th>
<th>$\text{Layer}$</th>
<th>$S$</th>
<th>$\tau$</th>
<th>$\phi$</th>
<th>$\Gamma_{\text{mol}}$</th>
<th>$\Gamma_{\text{mass}}$</th>
<th>$\tau_{\text{total}}$</th>
<th>$\Gamma_{\text{total}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>Inner</td>
<td>5.22</td>
<td>45</td>
<td>0.35</td>
<td>1.53</td>
<td>2.26</td>
<td>65</td>
<td>2.48</td>
</tr>
<tr>
<td></td>
<td>Outer</td>
<td>6.02</td>
<td>20</td>
<td>0.08</td>
<td>0.15</td>
<td>0.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.02</td>
<td>Inner</td>
<td>5.21</td>
<td>45</td>
<td>0.36</td>
<td>1.56</td>
<td>2.30</td>
<td>65</td>
<td>2.55</td>
</tr>
<tr>
<td></td>
<td>Outer</td>
<td>6.00</td>
<td>20</td>
<td>0.09</td>
<td>0.17</td>
<td>0.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>Inner</td>
<td>5.21</td>
<td>45</td>
<td>0.36</td>
<td>1.56</td>
<td>2.30</td>
<td>65</td>
<td>2.55</td>
</tr>
<tr>
<td></td>
<td>Outer</td>
<td>6.00</td>
<td>20</td>
<td>0.09</td>
<td>0.17</td>
<td>0.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>Inner</td>
<td>5.19</td>
<td>45</td>
<td>0.37</td>
<td>1.59</td>
<td>2.35</td>
<td>65</td>
<td>2.59</td>
</tr>
<tr>
<td></td>
<td>Outer</td>
<td>6.00</td>
<td>20</td>
<td>0.09</td>
<td>0.17</td>
<td>0.25</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. The best fitted parameters to the neutron reflection profiles as shown in Figure 3 by modelling the COE-3 adsorbed layer using a 2-layer model, with the adsorbed amount ($\Gamma$) calculated from equation (6).

It can be seen from Table 1 that the main features obtained from the 2-layer model analysis are similar in that the main layer is about 45 Å and the diffuse outer layer is about 20 Å. The only difference is the small but measurable change in the SLD of the main layer. As explained previously, the NR measurements were undertaken 1-2 hr after the solutions were in contact with the SiO$_2$ surface and the data were thus representative of the equilibrated adsorption. The adsorbed amount was also plotted...
in Figure 2 where it can be seen that the SE and NR data are highly consistent with experimental errors.

3.2 Adsorption of Nonionic Surfactants at the SiO$_2$/water interface

Adsorption of non-ionic surfactants at the SiO$_2$/water interface has been previously studied by a number of groups by different techniques including ellipsometry and NR [31, 32]. Nonionics such as C$_{12}$E$_5$ and C$_{12}$E$_6$ can adsorb onto the interface, but the exact amount is highly dependent on solution pH, ionic strength and even surface treatment. Under the buffer conditions used in this work, C$_{12}$E$_5$ showed very weak adsorption, and this was possibly caused by the relatively high ionic strength compared to other work reported previously. However, there was no sign of adsorption from Tween 80 with 7 and 20 ethylates over a wide concentration range well above their CMCs. Figure S3 shows the NR data with and without Tween 80 7EO (hydrogenated, denoted as HTween 80 7EO). The concentration of 0.02% (w/w) is some 10 times the CMC of the surfactant. Reducing the ionic strength could lead to nonionic surfactant adsorption, but the amount adsorbed from Tween 80 20EO (denoted as Tween 80 20EO) was low due to its high hydrophilicity. In the buffers considered for therapeutic mAb stabilisation, the ionic strength is usually even higher than what has been used in this work.

3.3 Co-adsorption of COE-3 and Tween 80 20EO and C$_{12}$E$_5$

The co-adsorption of COE-3 and Tween 80 20EO at the bare SiO$_2$/water interface was first undertaken using the mixed solution containing 0.01 mg/ml (10 ppm) COE-3 and 0.02% (w/w) Tween 80 20EO (also 10 times above its CMC), with the ionic strength of His buffer kept at 25 mM. It can be seen from Figure 4(a) that the time-dependent adsorption is comprised of two distinct stages. The adsorbed amount increases fast within the first 12-13 min and then tends to plateau with the equilibrated amount of adsorption around 2.5 mg/m$^2$.

The adsorption of COE-3 alone at the same concentration and solution conditions is also shown for comparison in Figure 4(a). The adsorption profile is the same as that measured from the mixture, showing no co-adsorption from Tween 80 20EO. As also shown in Figure 4(a), when the solution of 0.01 mg/ml COE-3 was replaced by that of Tween 80 20EO at 0.02% (w/w) after it has plateaued, the replacement did not alter
the adsorbed amount, indicating that whilst the surfactant could not remove any pre-adsorbed mAb it cannot co-adsorb either.

Similar measurements were carried out at the high COE-3 concentration of 0.1 mg/ml (100 ppm). As shown in Figure 4(b), the basic features remain the same, that is, the adsorption tends to saturation after the initial dynamic process. In addition, the saturated adsorbed amount remains the same at 2.5 mg/m². However, the dynamic process leading to the equilibrated adsorption occurred much faster. Furthermore, the dynamic adsorption from COE-3 alone at the same concentration is very close to that obtained from the mixture, again showing little sign of co-adsorption from the surfactant. The replacement of the mAb solution by that of the surfactant did not alter the trend of adsorption, again indicating the absence of influence to pre-adsorbed COE-3 layer. The slight decrease of the COE-3 adsorption curve after reaching the plateau might be caused by the instability of the measuring liquid cell setup.

As non-ionic surfactant C₁₂E₅ is widely used as a model surfactant, it has also been used in this work to verify the main features observed from Tween 80 20EO. As evident from Figure 4(c), the main features of the dynamic adsorption are the same, but the dynamic process leading to the equilibration is slower, and the equilibrium adsorbed amount tends to 3.4±0.2 mg/m². These differences arose from the higher solution pH. As the pH is higher and closer to the isoelectric point of the mAb, the adsorbed amount increases. However, the close similarity between the two adsorption profiles as shown in Figure 4(c) confirms little co-adsorption from C₁₂E₅ either.

Figure 4. Interface adsorbed amount (Γ) plotted against time measured by SE at the SiO₂/water interface for COE-3 adsorption with and without surfactants in 25 mM His buffer: (a) a mixed solution of 0.01mg/ml COE-3 and 0.02% (w/w) Tween80 20EO at pH 5.5; (b) a mixed solution of 0.1mg/ml COE-3 and 0.02% (w/w) Tween80 20EO at pH 5.5; (c) a mixed solution of 0.01mg/ml COE-3 and 10CMC C₁₂E₅ at pH 7. The red diamonds present the adsorption process of COE-3 alone followed by blue diamonds for the injection of surfactants, while the black circles present the data measured from mixtures of COE-3 and surfactants.
3.4 Co-adsorption of COE-3 with Tween 80 7EO

The lack of co-adsorption from the non-ionic surfactants as described above might arise from their rather high hydrophilicity. To test this hypothesis, we have examined the co-adsorption of COE-3 and Tween 80 7EO at pH 5.5, again with the total ionic strength of the Tris buffer fixed at 25 mM. As before, the concentration of the surfactant was fixed at 0.02% (w/w) and that for the mAb was fixed at 0.01 mg/ml (Figure 5(a)) and 0.1 mg/ml (Figure 5(b)). As evident from Figure 5(a) and 5(b), the initial dynamic adsorption process is now much longer, and it takes some 50 min before tending to equilibration. The equilibrium adsorbed amount is about 8 mg/m². These differences clearly mark the impact from Tween 80 7EO, suggesting co-adsorption from the surfactant. However, the equilibrium adsorbed amount is little influenced by the change in mAb concentration.

To gain further insight into the dynamic adsorption process, the adsorption from COE-3 alone under the same solution condition is also shown in Figure 5(a) and 5(b), followed by the replacement of the COE-3 solution by that of the surfactant and the measurement procedures were kept the same as described for the other surfactants. It can be seen from Figure 5(a) and 5(b) that the very initial dynamic adsorption processes remain almost identical at respective mAb concentrations with and without surfactant; this is well demonstrated by the slow dynamic process at the low mAb concentration and the fast dynamic process at the high mAb concentration. Once the surfactant is introduced the co-adsorption starts and the dynamic process proceeds almost parallel to that measured from the mixed solutions, implying co-adsorption would only occur after the mAb layers have been well formed. Note that in spite of different dynamic adsorption processes from the two different mAb concentrations the equilibrated amount of adsorption is almost the same. The two sets of surfactant dynamic adsorption processes are almost parallel to each other as well, consistent with the same surfactant concentration studied.

It typically takes 0.5 - 1 hr to acquire neutron reflection data with sufficient statistical quality in a typical measurement. Thus, NR is inappropriate for following dynamic adsorption process. However, it can help determine the final equilibrated amount of adsorption under different adsorption conditions. As an example, we show in Figure 5(c) and 5(d) the combination of reflectivity profiles measured under co-adsorption but with characteristic NR features. As NR signal is sensitive to H/D substitution, the NR
measurements have been made using the surfactants with hydrogenated and deuterated ethoxylates (denoted as HTween 80 7EO and DTween 80 7EO), respectively, in D$_2$O (Figure 5(c)) and in the water bulk with its contrast matched to antibody (CMAb) (Figure 5(d)). The NR profile from COE-3 adsorption alone is also shown for comparison. Its difference from the measurements in the presence of deuterated and hydrogenated surfactants confirms co-adsorption.

A distinct feature as evident from Figure 5(c) is the occurrence of sharp interference fringes caused by surfactant co-adsorption. This is in contrast to the weak and broad interference as displayed from COE-3 adsorption alone. To a good approximation, the dimension of the layer $d$ is inversely proportional to momentum transfer, $\kappa$, where

$$d = \frac{n\pi}{\kappa}$$  \hspace{1cm} (7)

with $n$ being the integer. From this simple relation, we can estimate the thickness of the adsorbed COE-3 layer to be about 63 Å from the position of the fringe around 0.05 Å$^{-1}$ and that of the mixed layer upon surfactant adsorption about 143 Å from the positions of the primary peaks around 0.022 Å$^{-1}$. It is interesting to note that in spite of very different shape from the COE-3 adsorbed profile the two surfactant co-adsorbed profiles under d- and h-labelling are remarkably similar. The almost identical interference fringes suggest that the layers have same structural features in terms of the amount and composition relating to the distributions of mAb and surfactants. The second and third interference fridges reveal the dimensions of domain thicknesses within the co-adsorbed layers, but their interpretation requires the support of more detailed data analysis as will be described later. The different amplitude between the two profiles containing the two differently labelled surfactants reveals the different contributions of deuterated and hydrogenated ethoxylates to neutron reflectivity.

On the other hand, shape differences between these reflectivity profiles become less obvious once the measurements are made in CMAb, as evident from Figure 5(d). Under this contrast, mAb is invisible. The weak reflectivity measured from mAb alone largely arises from the native SiO$_2$ layer present at the solid/CMAb interface. In contrast, the two reflectivity profiles from deuterated and hydrogenated surfactants are distinctly different, showing the sensitivity to the surfactant distributions under these isotopic contrast conditions. The primary fringes as observed in D$_2$O disappear here, but the secondary and third fringes are clearly displayed through their exact dimensions.
within the adsorbed layers need to be determined again with the support of more systematic data analysis.

Although NR is unsuitable for following the dynamic adsorption process, its high sensitivity can be used to help determine if different combinations of co-adsorption could influence the final amount and layer structure. Figure S4 compares the reflectivity profiles measured from mixed COE-3 and HTween 80 7EO. The reflectivity was measured 1-2 hr after the injection of the mixed solution into the measuring liquid cell to allow equilibration of co-adsorption. All the solution conditions were kept the same as described for SE measurements relating to Figure 5(a). Measurements after buffer rinsing and subsequent injection of COE-3 also at 0.01 mg/ml are also shown for comparison. NR measurement following sequential injection, i.e., the replacement of the initial COE-3 solution after its first 40 min adsorption as depicted in Figure 5(a) followed by surfactant co-adsorption, is also shown in Figure S4. These NE profiles in D₂O are identical within experimental error, again confirming the initial mAb adsorption, followed by Tween 80 7EO coadsorption after the mAb layer has been well formed.

Figure 5. Surface adsorbed amount (Γ) plotted against time measured by SE (Left) at the SiO₂/water interface for COE-3 (a) 0.01mg/ml and (b) 0.1mg/ml with and without 0.02%(w/w) Tween80 7EO in 25 mM His buffer, pH 5.5. The red diamonds present the adsorption process of COE-3 alone followed by blue diamonds for the injection of surfactants, while the black circles present the adsorption from the mixture of COE-3 and surfactant. NR profiles (right) measured at the same interface for COE-3 adsorption at 0.01mg/ml mixed with 0.02% (w/w) protonated and deuterated Tween80 7EO in (c) D₂O and (d) contrast matching COE-3 water, all under pH 5.5 (25mM His buffer).
The results would imply that in spite of pre-mixing, mAb and surfactant do not appear to have any affinity towards each other in bulk solution. Furthermore, surfactants have little affinity to the surface, as in the case of surfactant adsorption alone. This suggests that during the early dynamic mAb adsorption process, the surfactant has no affinity to the adsorbed mAb either, implying that the adsorbed mAb molecules must retain their globular structure. As the mAb adsorption tends to plateau, structural adjustments must also occur within the adsorbed layer, resulting in structural rearrangements. It is clear from both SE and NR measurements that Tween 80 7EO would only bind or co-adsorb after structural rearrangements have happened.

3.5 NR Data Analysis to Unravel the Co-adsorbed Layer Structure

The equilibrated co-adsorption from the mixed solution of 0.01 mg/ml COE-3 and 0.02% (w/w) Tween 80 7EO at pH 5.5 has been measured by NR under 4 isotopic contrasts using deuterated and hydrogenated surfactants in CMAb and D₂O. For easy comparison, these 4 reflectivity profiles have been replotted in Figure 8(a). Any models that could generate the fits to replicate the 4 measured reflectivity profiles must also satisfy the SE measurement in terms of the total adsorbed amount. As buffer rinsing does not remove any adsorbed mAb or alter the adsorbed layer structure, we can assume that the total amount of mAb co-adsorbed remains the same as that of the COE-3 by itself. Hence, the difference arises entirely from surfactant co-adsorption. As surfactant only starts to co-adsorb after the mAb layer is well formed and underwent structural rearrangements, it can be further assumed that the inner region of the adsorbed layer is predominantly occupied by mAb molecules and the subsequent thickening is primarily caused by surfactant binding in the outer region. Change in SLD (scattering length density) in the inner mAb layer away from that adsorbed from the mAb itself would indicate association or insertion of surfactant molecules into it.

It was found that whilst a 2-layer model could replicate most of the main features of the measured reflectivity profiles in terms of the general shapes and positions of interference fringes, there was lack of precise consistency. When adjustments were made at the interface between the inner mAb layer and outer surfactant layer and that between the surfactant layer and bulk water, the fits were significantly improved. Figure 6(b) shows the 4 layer fits with the matching calculated reflectivity profiles plotted as continuous lines in Figure 6(a). It should be noted that the fitting process was undertaken by following the least-squared minimization process using the set of
equations as derived from equations (5) and (6). Under each isotopic contrast, SLD in each layer is coupled to the volume fraction of mAb and surfactant. The simultaneous fitting ensured that the thickness and composition of each layer were consistent. However, minor deviations in SLD and thickness between contrasts in each layer were allowed for fitting optimization by achieving the minimal mean-square errors. The SLDs for mAb, the head and tail of deuterated and hydrogenated surfactants are given in Table S1. The best fitted values under each contrast and the average values are given in Table S2.

![Figure 6.](image)

The optimal fitting indicates the slight thickening of the inner mAb layer (layer 1). It is slightly thicker than that obtained from the adsorbed mAb by itself. As already explained, the COE-3 layer alone formed under the same solution conditions was fitted into a monolayer of 50-55 Å from our previous work, but it could also fit into a 2-layer model with a total thickness of 60-65 Å as schematically shown in Figure 7(a), comprised of an inner dense layer of 40 Å with the volume fraction ($\phi_{COE-3}$) of mAb of 0.35 and an outer layer of 25 with $\phi_{COE-3} = 0.08$ (Table 1). In the case of binding from Tween 80 7EO, the COE-3 was fitted into a uniform layer of 70 Å with $\phi_{COE-3} = 0.25$ (Table S2). The difference may indicate the stretching associated with surfactant binding. Whilst the fitting indicated clearing surfactant binding into the mAb layer. However, the difference in SLD in this layer between the deuterated and hydrogenated surfactants is very small, as evident from Figure 6(b), showing the amount of surfactant bound is very low. Furthermore, the model analysis revealed the association of
surfactant head groups mainly with this layer whilst the association of surfactant tails is negligible within the experimental error. The second layer (layer 2) is in average about 12-13 Å thick and contains surfactant head groups mainly. In contrast, the third layer (Layer 3) is about 36-38 Å thick and largely contains surfactant tails. The fourth layer (layer 4) is about 17-18 Å thick and largely contains the head groups pointing towards the bulk water. Thus, once the co-adsorption starts on the outer surface of the mAb layer, the surfactant molecules self-assemble into a sandwiched bilayer. Figure 7(b) depicts a schematic representation of the co-adsorbed interface as determined from NR studies.

![Figure 7](image)

**Figure 7.** Schematic showed the adsorption of (a) COE-3 alone and (b) the structure of the compound layer of COE-3 and Tween80 7EO.

In addition to the follow-up of different dynamic processes under different adsorption sequences and conditions, SE determines the adsorbed amount accurately. Whilst NR can’t offer dynamic information, it does provide an accurate determination of the total adsorbed amount for the equilibrated layer. Figure S5 shows the comparison of the total adsorbed amount as determined from both SE and NR, but it also highlights the main technical features of the two techniques: whilst NR is incapable of following dynamic adsorption, it can determine the total amount of COE-3, surfactant heads and tails, thus complementing the SE measurements. It is interesting to note that the mass ratio between surfactant head and tail is close to the theoretical value with ±10% errors. The small variation reflected the good consistency of the model fitting.

More importantly, NR measurements made under different isotopic contrasts can together help determine the distributions of COE-3, surfactant heads and tails across the interface, as illustrated in Figure 8. The bar charts as shown in Figure 8 indicate the amount of COE-3, surfactant heads and tails distributed in each layer, with the total amount consistent with the SE analysis.
Thus, although the quantitative analysis as depicted above reveals little amount of surfactant chains associated with the mAb layer (layer 1) and the transitional layer (layer 2), the formation of the surfactant bilayer on top of the COE-3 layer must be initiated by the affinity of the surfactant to the mAb layer. The importance of this association process is illustrated by the lack of similar affinity from the two other non-ionic surfactants Tween 80 20EO and C_{12}E_{5} due to their relatively greater hydrophilicity and weaker hydrophobicity.

Interfacial adsorption of proteins has been studied extensively as the process underlines many technological applications [13]. Protein adsorption at the bare SiO_{2}/water interface has been used as the model interface by our own and many other groups to ascertain how different proteins adsorb and desorb with and without surfactants. This is because silicon surfaces can be readily polished reproducibly with optically flat surface structure. The polished silicon surface bears a native oxide layer that offers surface properties similar to glass. In the context of this work, glass containers are amongst popular storage devices for future protein therapeutics. Hence, fundamental work on the adsorption of mAbs with and without surfactants is of direct relevance.

We have previously studied protein adsorption at the solid/water interfaces under different surface and solution conditions. Ionic surfactants such as anionic sodium dodecyl sulphate (SDS) and cationic dodecyl trimethyl ammonium bromide (C_{12}TAB) bind with proteins through electrostatic interaction [18,19]. Depending on the exact molar ratio and solution environment (pH and ionic strength) surfactant molecules may cause structural unfolding to protein molecules once bound electrostatically. These interactions often lead to the formation of complexes and even insoluble precipitates, thus complicating the intended studies. On the other hand, non-ionic surfactants such as C_{12}E_{5} usually do not show affinity to protein molecules. At the air/water interface, C_{12}E_{5} can replace proteins such as lysozyme and albumins because of their different surface activities. Once lysozyme and albumins are adsorbed at the bare SiO_{2}/water interface, C_{12}E_{5} can’t co-adsorb due to its low affinity associated with its high hydrophilicity. The co-adsorption of COE-3 and non-ionic surfactants such as Tween 80 20EO is very similar to the behaviour of the lysozyme-C_{12}E_{5} system on the surface of water [33]. But their co-adsorption at the bare SiO_{2}/water interface would also be similar as studied for BSA-C_{12}E_{5} system [18] in that the presence of non-ionic C_{12}E_{5} and Tween 80 20EO can’t affect the adsorption of protein molecules, irrespective of the sequence of adsorption. Thus, the lack of influence of non-ionic surfactants on mAb
adsorption is very similar to other protein-nonionic surfactant systems previously studied. The results together show that mAb adsorption at the bare SiO$_2$/water interface is not affected by the presence of Tween 80 20EO or C$_{12}$E$_5$ due to their high hydrophilicity.

In their recent studies of the interfacial interaction between hydrophobin and non-ionic surfactants, Tucker et al. have explored the effects of head group size and alkyl chain length [24] using neutron reflection from the layers adsorbed on the surface of water. Hydrophobin is a small globular protein with a distinct hydrophobic and flexible surface domain that displays high surface physical activity and affinity towards surfactants through hydrophobic interactions. They showed that whilst Tween 80 20EO displays weak affinity toward hydrophobin, a decrease in the ethoxylate size in the head group could increase the affinity of the non-ionic surfactant towards the protein.

In this study, we have shown that whilst the presence of Tween 80 7EO can’t stop COE-3 adsorbing at the bare SiO$_2$/water interface, it can start to bind to the adsorbed COE-3 layer once it becomes packed and structurally adjusted. This suggests that the reduced ethoxylate head makes the surfactant more hydrophobic. Although it still shows no affinity to Abs molecules at the early stage, it can become associated to mAb molecules with hydrophobic patches exposed during subsequent structural adjustments at the later stage. NR measurements revealed that the amount of Tween 80 7EO directly associated with the COE-3 layer is very low, but it was enough to initiate further binding and self-assembly of a surfactant bilayer on top of the COE-3 layer.

Figure 8. The bar chart showed the adsorbed amounts for each component (COE-3, Tween80 7EO head group and tail group) in each layer calculated from the fitting of NR measurements in Fig. 6.
Just like other non-ionic surfactants, Tween 80 7EO can’t prevent COE-3 adsorption at the bare SiO$_2$/water interface, but the surfactant bilayer formed is very robust and it can stop further mAb adsorption. It should be considered as a better alternative to Tween 80 20EO to alleviate mAb adsorption under different interface and solution conditions.

4. Conclusion

Although Tween 80 20 EO is widely used to stabilise mAb formulations, little work has been done to unravel its working mechanism and support its role in preventing mAb adsorption. Using the bare SiO$_2$/water interface as a model, our combined SE and NR study has demonstrated that common non-ionic surfactants such as Tween 80 20EO and C$_{12}$E$_5$ can’t prevent COE adsorption. The use of Tween 80 7EO still can’t stop mAb adsorption, but its binding to the well-formed mAb layer and the subsequent formation of the robust surfactant bilayer prevents further mAb adsorption and the associated processes leading to structural degradation and mAb instability. This work reports how SE and NR can be used to help reveal how mAb and surfactant interact and co-adsorb at the solid/water interface dynamically and structurally. Further studies could shed more light on the implication to other mAbs and will also help search for more effective surface active species to improve the stability of mAb formulations.

Supporting Information

The Supporting Information including dynamic adsorption from COE-3, the additional neutron reflection measurements on the binding of Tween 80 7EO into adsorbed CIE-3 layers and structural parameters obtained from the combined neutron data analysis is available free of charge on the ACS Publications webpage.

Acknowledgements

We thank funding support from MedImmune Ltd, neutron beam times awarded to undertake this work at ISIS Neutron Facility, Chilton, Didcot, under the support of STFC. ZL acknowledges studentship support from University of Manchester via an
Overseas Research Scholarship (ORS) award and a physics research merit award. We also thank the support from a Marie Curie Fellowship ITN grant (grant number 608184) under SNAL (Small nano-objects for alteration of lipid-bilayers) and EPSRC for support under EP/F062966/1.
References


[28] Su, T.J.; Lu, J.R.; Thomas, R.K.; Cui, Z.F.; Penfold, J. The Effect of pH on the Adsorption of Lysozyme at the Hydrophilic Silicon Oxide-Water Interface, a Neutron


Support Information

**Figure S1.** The molecular structure of a normal Tween 80 surfactant (shown in the usual hydrogenated form): with the total values of n equal to about 20.

**Figure S2.** SE measurements of the surface adsorbed amount of COE-3 plotted against time at the SiO$_2$/water interface, in 25 mM His buffer, pH 5.5, at concentrations as indicated.
Figure S3. NR profiles measured at the SiO$_2$/D$_2$O interface with and without 0.02% (w/w) protonated Tween80 7EO as bulk concentration. The almost identical reflectivity profiles measured indicate little surfactant adsorption at the interface.

Figure S4. NR profiles measured at the SiO$_2$/D$_2$O interface for the layer of COE-3 and Tween80 7EO formed by the injection of the mixture (blue circles) or injection in sequence (red triangles). The other NR profiles measured for the formed mixed layer after a rinse of buffer (black squares) and the second injection of 0.01mg/ml COE-3 (magenta diamonds) are also shown for comparison. No difference between them indicates the high robustness of the layers once formed.
Figure S5. Surface adsorbed amount (Γ) plotted against time measured by SE at the SiO$_2$/water interface for COE-3 at 0.01mg/ml with 0.02% (w/w) Tween80 7EO in 25 mM His buffer, pH 5.5. The bar chart showed the adsorbed amounts for each component (COE-3, Tween80 7EO head group and tail group) calculated from the fitting of NR measurements in Figure 6.

Table S1. The scattering length (SL), scattering length density (SLD, ρ), volume (V) and molecular weight (MW) of COE-3 and Tween80 7EO used for the model fitting to neutron reflectivity.
(a) Best fitted parameters under individual contrasts

<table>
<thead>
<tr>
<th>Contras</th>
<th>SLD $\times 10^6$ Å$^{-2}$</th>
<th>$\tau$ Å</th>
<th>$\phi$ COE-3</th>
<th>$\phi$ head</th>
<th>$\phi$ tail</th>
<th>$\phi$ water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Layer 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D$_2$O-H</td>
<td>5.39</td>
<td>70</td>
<td>0.25</td>
<td>0.04</td>
<td>0.003</td>
<td>0.71</td>
</tr>
<tr>
<td>D$_2$O-D</td>
<td>5.52</td>
<td>70</td>
<td>0.25</td>
<td>0.04</td>
<td>0.003</td>
<td>0.71</td>
</tr>
<tr>
<td>2.58-H</td>
<td>2.53</td>
<td>70</td>
<td>0.25</td>
<td>0.02</td>
<td>0.003</td>
<td>0.73</td>
</tr>
<tr>
<td>2.58-D</td>
<td>2.63</td>
<td>70</td>
<td>0.25</td>
<td>0.02</td>
<td>0.003</td>
<td>0.73</td>
</tr>
<tr>
<td>Layer 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D$_2$O-H</td>
<td>3.77</td>
<td>13</td>
<td>0</td>
<td>0.41</td>
<td>0.08</td>
<td>0.51</td>
</tr>
<tr>
<td>D$_2$O-D</td>
<td>6.44</td>
<td>15.1</td>
<td>0</td>
<td>0.40</td>
<td>0.06</td>
<td>0.54</td>
</tr>
<tr>
<td>2.58-H</td>
<td>1.73</td>
<td>13</td>
<td>0</td>
<td>0.42</td>
<td>0.09</td>
<td>0.49</td>
</tr>
<tr>
<td>2.58-D</td>
<td>4.18</td>
<td>11</td>
<td>0</td>
<td>0.38</td>
<td>0.09</td>
<td>0.53</td>
</tr>
<tr>
<td>Layer 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D$_2$O-H</td>
<td>1.18</td>
<td>35</td>
<td>0</td>
<td>0.23</td>
<td>0.68</td>
<td>0.10</td>
</tr>
<tr>
<td>D$_2$O-D</td>
<td>2.79</td>
<td>38.5</td>
<td>0</td>
<td>0.25</td>
<td>0.65</td>
<td>0.10</td>
</tr>
<tr>
<td>2.58-H</td>
<td>0.63</td>
<td>37</td>
<td>0</td>
<td>0.25</td>
<td>0.72</td>
<td>0.05</td>
</tr>
<tr>
<td>2.58-D</td>
<td>1.81</td>
<td>37</td>
<td>0</td>
<td>0.19</td>
<td>0.75</td>
<td>0.07</td>
</tr>
<tr>
<td>Layer 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D$_2$O-H</td>
<td>5.10</td>
<td>17.8</td>
<td>0</td>
<td>0.17</td>
<td>0.05</td>
<td>0.78</td>
</tr>
<tr>
<td>D$_2$O-D</td>
<td>6.13</td>
<td>17.9</td>
<td>0</td>
<td>0.15</td>
<td>0.05</td>
<td>0.80</td>
</tr>
<tr>
<td>2.58-H</td>
<td>2.28</td>
<td>17.6</td>
<td>0</td>
<td>0.15</td>
<td>0.03</td>
<td>0.83</td>
</tr>
<tr>
<td>2.58-D</td>
<td>3.21</td>
<td>17.8</td>
<td>0</td>
<td>0.15</td>
<td>0.03</td>
<td>0.82</td>
</tr>
</tbody>
</table>

(b) Summarised best fitted parameters for each of the sublayers

<table>
<thead>
<tr>
<th>Layer</th>
<th>$\tau$/Å</th>
<th>$\phi$ COE-3</th>
<th>$\Gamma$ COE-3</th>
<th>$\phi$ head</th>
<th>$\Gamma$ head</th>
<th>$\phi$ tail</th>
<th>$\Gamma$ tail</th>
</tr>
</thead>
<tbody>
<tr>
<td>Layer 1</td>
<td>70±3</td>
<td>0.25±0.03</td>
<td>2.44±0.15</td>
<td>0.23±0.08</td>
<td>0.003±0.01</td>
<td>0.23±0.15</td>
<td>0.003±0.01</td>
</tr>
<tr>
<td>Layer 2</td>
<td>13±1</td>
<td>0</td>
<td>0.41±0.05</td>
<td>0.67±0.02</td>
<td>0.08±0.02</td>
<td>0.11±0.03</td>
<td></td>
</tr>
<tr>
<td>Layer 3</td>
<td>37±2</td>
<td>0</td>
<td>0.23±0.04</td>
<td>1.08±0.10</td>
<td>0.70±0.05</td>
<td>2.66±0.2</td>
<td></td>
</tr>
<tr>
<td>Layer 4</td>
<td>18±1</td>
<td>0</td>
<td>0.15±0.03</td>
<td>0.35±0.02</td>
<td>0.04±0.01</td>
<td>0.07±0.02</td>
<td></td>
</tr>
<tr>
<td>Sum</td>
<td>137±5</td>
<td>2.44±0.15</td>
<td>2.33±0.22</td>
<td>2.86±0.15</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table S2. Structural parameters obtained from the best fits to the adsorbed mAb layers in NR profiles measured under 4 contrasts from co-adsorption from the mixed solution of COE-3 at 0.01mg/ml and Tween 80 7EO at 0.02% w/w in His 25mM buffer, pH 5.5. (a) Best fitted parameters under individual contrasts and (b) Summarised best fitted parameters for each of the sublayers. H/D indicate head hydrogenated and head deuterated Tween 80 7EO used.
Chapter 7

Summary and Future Work

7.1 Concluding Remarks and Discussion 213

7.2 Future Work 215

7.3 References 218
7. 1 Concluding Remarks and Discussion

Currently, mAb therapy is the largest and fastest-developing area in the pharmaceutical industry.\(^1\) MAbs, like many other protein molecules, are amphiphilic. They can adsorb onto different interfaces, and some of them will then become desorbed. The adsorption-desorption process is linked to mAb aggregation. A widely accepted pathway is that adsorption can cause structural damage. Once desorbed, the structurally damaged mAbs can promote aggregate formation, and in some cases, cause precipitation. As already explained, structural damage and the subsequent cascade of aggregation is associated with loss of activity, thus complicating the efficacy of medical treatments. Thus, understanding antibody interfacial adsorption is of great significance in the design, formulation, production and application of mAbs.

In this project, the adsorption behaviour of mAb “COE-3” was investigated at the air/liquid and liquid/solid interfaces by Ellipsometry and Neutron Reflection. The results were obtained under various concentrations and buffer conditions. Also, a series of experiments undertaken revealed the influence on the mAb adsorption process in the presence of a non-ionic surfactant, Tween 80.

Firstly, the dynamic adsorption processes of COE-3 and its fragments were observed at the air/liquid interface in 25mM His buffer at pH 5.5. The sample bulk concentration range studied was from 2ppm to 100ppm. The results indicated that the Fc fragment adsorbed faster than Fab and the intact antibody, and had the highest adsorbed amount in nmol/m\(^2\). In contrast, the Fab fragment adsorbed slowest, but its equilibrated adsorption amount showed a stronger concentration dependence than Fc in the concentration range studied. In other words, Fc nearly reached saturated adsorption at the lowest concentration measured, but Fab did not, even at the concentration of 100ppm. These observations agree broadly with the net charges. Fc has a much lower net surface charge than Fab so that it is more favourable for it to be at the interface. By comparing the adsorption between Fc, Fab and the intact antibody, it was found that the adsorption of COE-3 was dominated by its two Fabs, with an evident influence from Fc. The equilibrated adsorbed amount of the Fab pair (two Fab) and the whole antibody were similar in nmol/m\(^2\) at each concentration. Moreover, the thickness of the adsorbed layer was 40 Å for Fc and 45-50 Å for Fab and mAb, and this trend remained little changed with increased bulk concentration. Against the molecular structure of COE-3, all adsorbed layers adopted the conformations with their short
axial dimensions perpendicular to the water surface. Also, the mAb and its fragments remained globular and not unfolded when adsorbed at the air/liquid interface. Buffer pH variation from 5.5 to 8.8 had little effect on the adsorption process.

The competitive adsorption from 50ppm COE-3 and non-ionic surfactant Tween 80 (20EO) was studied in 25mM His buffer at pH 5.5. Tween 80 (20EO) started to desorb the mAb COE-3 at 1/100 CMC and completely removed it from the air/liquid interface at 1/10 CMC. Thus the co-adsorption was retained over a wide surfactant range. The thickness of the co-adsorbed layer was almost constant, indicating the mAb molecules retained their globular structure without unfolding.

Next, COE-3 adsorption at the SiO$_2$/liquid interface was studied by ellipsometry and neutron reflection with various buffer conditions, with pH varying from 5 to 9, and the ionic strength from 5mM to 100mM. Independent of pH and ionic strength, the COE-3 adsorption at the SiO$_2$/liquid interface was so fast that the adsorbed amount reached a similar plateau in the first hour for all concentrations studied. Although at a certain buffer condition, the equilibrated adsorption amount was similar for all concentrations beyond 5ppm, the adsorption dynamics showed a strong relationship with the concentration. In contrast, the buffer pH and ionic strength had little influence on the adsorption dynamics but can determine the equilibrated adsorption amount. More specifically, the equilibrated adsorption amount increased with pH or with decreasing ionic strength.

The DLVO theory was applied to help interpret the observations. The results indicated a two-step process driven largely by electrostatic interaction. In the first step, the antibody molecules adsorbed on the bare SiO$_2$ surface through a favourable electrostatic attraction. Then, as more molecules adsorbed, the electrostatic repulsion between the molecules in bulk and the adsorbed layer started to play a dominant role, stopping further adsorption. The electrostatic interaction was affected by the ambient pH and ionic strength because the net charge of COE-3 varies with pH and the ions can play a role of electrostatic screening. Over the concentration range studied, neutron reflection experiments revealed that the COE-3 adopted a flat-on orientation on the interface at pH5.5, resulting in a layer with a thickness of some 50 Å. It also indicated that the adsorption-induced denaturation of antibody molecules did not occur at the conditions studied.
Tween 80 (20EO) has been shown not to influence the adsorption behaviour of COE-3 at the SiO\textsubscript{2}/water interface, and this conclusion was not affected by the mode of adsorption: the same results were achieved from mixed solution of Tween 80 (20EO) and COE-3 and from sequential adsorption starting from COE-3 followed by surfactant co-adsorption. Although Tween 80 (7EO) also cannot desorb or prevent antibody adsorption, it can self-assemble onto the adsorbed antibody layer and form a surfactant bilayer. Moreover, the surfactant bilayer can work as a protective shell stopping further antibody adsorption.

Overall, COE-3 molecules can adsorb at both air/water and SiO\textsubscript{2}/water interfaces. The adsorption dynamics is related to antibody bulk concentration which determines the adsorption flux. The difference between these two interface adsorption processes arises from the electrostatic interaction, which played a significant role at the SiO\textsubscript{2}/water interface but less so at the air/water interface. Hence, electrostatic attraction enables the adsorption on the SiO\textsubscript{2}/water interface to reach equilibrium in a relatively short time. The electrostatic interaction is related to the buffer conditions such as pH and ionic strength. Therefore, the dependency of adsorbed amount on buffer pH and ionic strength was observed in the case of the SiO\textsubscript{2}/water interface but not the air/water interface. On the other hand, due to the electrostatic interaction at the SiO\textsubscript{2}/water interface, the adsorption reaches saturation at a relatively low concentration. Hence, the equilibrated adsorption amount seems to have a relatively weak relationship with concentration in the range studied. Furthermore, Tween 80 (20EO) can compete with COE-3 in the adsorption on the water surface by its liquid surface activity. However, its surface adsorption ability is so weak at the SiO\textsubscript{2}/water interface that it can neither adsorb alone nor affect COE-3 adsorption.

**7.2 Future Work**

This project has so far focused on COE-3 adsorption behaviour at air/liquid and SiO\textsubscript{2}/liquid interfaces, in the absence and presence of a non-ionic surfactant Tween 80. Different lines of work can be planned in future to improve the understanding of mAbs interfacial adsorption by using mAbs with different Fab modifications and different non-ionic surfactants.
At the air/liquid interface, the co-adsorption of Tween 80 (20EO) with COE-3 fragments (Fc, Fab) is interesting to investigate. From the work described in this thesis, Fc has a stronger ability to adsorb at the air/liquid interface, but the behaviour of intact antibody is dominated by Fab. However, it is unclear what the contribution is made by each fragment to the competitive adsorption. By investigating their competitive adsorption against selected non-ionic surfactants such as Tween 80, we could gain insight about which fragment is more easily removed.

At the SiO$_2$/liquid interface, the influences of buffer conditions (pH and ionic strength) on the COE-3 desorption with Tween 80 (20EO) have not been studied. Although Tween 80 (20EO) cannot desorb or affect the adsorption of COE-3 in the 25mM His buffer at pH 5, it could reveal its influence at other buffer conditions, particularly at low pH or under larger ionic strength in which the electrostatic interactions are weakened. Furthermore, adsorption and desorption behaviour of Fc and Fab fragments can be studied as well to provide more insight, either in the presence or absence of Tween 80.

In Penfold and Tucker’s work, the structures formed by hydrophobin and polyethylene sorbitan monostearate surfactants were investigated at the air/liquid interface. In the thesis work, Tween 80 (7EO) formed a surfactant bilayer on the top of adsorbed antibody layer at the SiO$_2$/liquid interface. It is thus fascinating to explore the co-adsorption between COE-3 or its fragments and Tween 80 containing different numbers of EO groups. Other proteins such as hydrophobin and HSA can also be studied as control groups, from which we can see whether the Tween 80 (7EOs) bilayer formation is triggered by a particular fragment of COE-3 or by the general protein properties. Moreover, it is stated that pH and ionic strength have a remarkable influence on the adsorption of COE-3 on SiO$_2$ surface. Hence it can be expected that pH and ionic strength may affect the co-adsorption of COE-3 and Tween 80 (7EO).

Additionally, Couston et al. reported that mAb showed different adsorption and desorption on bare SiO$_2$ and hydrophobized silica surfaces. Similar experiments can be designed for COE-3 and its fragments. The hydrophobicity of the surface is controlled using different silanes. It is of great interest to compare the results obtained from the SiO$_2$ surface, hydrophobized silica surface and air/liquid interface because hydrophobized silica surface is hydrophobic as at the air/liquid interface, but it is also a solid/liquid interface. In the work of Nabok et al., protein A was pre-coated on the
SiO$_2$ surface and used to control the orientation of adsorbed antibody molecules so that it is possible to investigate whether the Tween 80 (7EOs) bilayer formation has a relationship with the orientation of adsorbed antibody. The SiO$_2$ surface can also be pre-coated with polymer, oil and metal to mimic the inner wall of different tools or containers involved in mAbs therapy, such as syringe, needle and infusion tube.$^1$

We can also turn our interest towards finding another surface active material instead of non-ionic surfactants to reduce mAbs adsorption. Peptide surfactant is one of the choices. In a series of recent studies$^{6-9}$, short peptides had been designed with surfactant-like property and used to stabilise proteins. Peptide surfactants have great biocompatibility and are less likely to denature or unfold mAbs. The co-adsorption of peptide surfactant and mAb has great potential in the future.
7.3 References


