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The structural orientation of antibody layers bound to engineered biosensor surfaces

Anton P. Le Brun [a,1], Stephen A. Holt [b,1], Deepan S.H. Shah [c], Charles F. Majkrzak [d], Jeremy H. Lakey [a,*]

[a] Institute for Cell and Molecular Biosciences, The Medical School, Newcastle University, Framlington Place, Newcastle upon Tyne NE2 4HH, United Kingdom
[b] ISIS Neutron Facility, STFC Rutherford Appleton Laboratory, Harwell Science and Innovation Campus, Didcot, Oxfordshire OX11 0QX, United Kingdom
[c] Orla Protein Technologies Ltd, Biosciences Centre, International Centre for Life, Times Square, Newcastle upon Tyne NE1 4EP, United Kingdom
[d] NIST Centre for Neutron Research, National Institute for Standards and Technology, Gaithersburg, MD 20899, USA

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ABSTRACT

This paper describes a membrane protein array that binds immunoglobulin G at its constant regions whilst leaving the variable regions free to bind antigen. The scaffold of the array is the transmembrane domain of outer membrane protein A (tOmpA) from *Escherichia coli* engineered to assemble as an oriented monolayer on gold surfaces *via* a single cysteine residue. Other protein domains can be fused to the N and C termini of the scaffold. In this study we use circularly permuted ctOmpA fused to two Z domains of *Staphylococcus aureus* protein A (ZZctOmpA) to create the immunoglobulin G-binding array. The solution structure of the engineered proteins was assessed by circular dichroism spectroscopy. Assembly of the array, attachment of antibodies and antigen binding were measured using surface plasmon resonance and neutron reflection. Compared to mouse IgG2, polyclonal IgG from rabbit bound very strongly to ZZctOmpA and the dissociation of the immunoglobulin was slow enough to allow neutron reflection studies of the assembled layer with antigen. Using both magnetic and isotopic contrasts a complete layer by layer model was defined which revealed that the 223 Å high layer contains antibodies in an upright orientation.

1. Introduction

The biosensor market is driven by the need to identify and/or quantify biomolecules in complex samples in a rapid, reliable and economic way. The molecular recognition step is provided by biological molecules in many guises including: proteins, nucleic acids or even microorganisms. Proteins such as enzymes, antibodies, ion channels and receptors are polymers that can have highly specific functions and affinities for analytes and make ideal materials for biosensors. The transducer that converts the recognition event to an output can take the form of a conductimetric [1], dielectrimetric [2], optical [3], colourimetric [4], acoustic [5] or surface plasmonic [6] measurement. The ideal output is an electrical signal presented on a digital display. However, the marriage of the sensing component of a biosensor to a transducer is one of the biggest technical challenges in biosensor development [7]. The biological component usually needs to be immobilised to a surface and the most commonly used test in medical diagnosis is the immunoassay, which uses specific antibodies to bind the target molecule. Our goal is to assemble, on transducer surfaces, antibodies at the highest possible density and correctly orientated for antigen binding.

We have previously described the formation of dense oriented monolayers on gold surfaces using outer membrane proteins from bacteria [8,9]. These can be engineered to display selected protein sequences in a highly ordered manner [10]. One such group of proteins are the antibody-binding domains from the surface of infectious bacteria such as *Staphylococcus* and *Streptococci* [11]. One protein in particular *Staphylococcus aureus* protein A (SpA) has five immunoglobulin binding domains: E, D, A, B and C [12]. The high resolution structures of the B domain [13] and the B domain bound to IgG [14] have been solved. The B domain binds the Fc region of IgG, leaving the antibodies free to bind antigen [15]. Two mutations (A1V and G29A) have being introduced into the B domain to create what is termed the Z domain [16], A1V creates a convenient DNA cloning site and G29A removes a hydroxylamine-sensitive site [16].

For surface assembly we use the β-barrel transmembrane domain (residues 1 to 171) of outer membrane protein A (tOmpA) [17,18]. In ctOmpA this is circularly permuted in extracellular loop 1, has 6xHis tag at the N-terminus and a cysteine on the 'periplasmic' side to

* Corresponding author. Fax: +44 191 222 7424.
E-mail address: j.h.lakey@ncl.ac.uk (J.H. Lakey).
[1] Present address: Bragg Institute, Australian Nuclear Science and Technology Organisation, Locked Bag 2001, Kirrawee DC, NSW 2232, Australia.

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allow binding to gold [8,10]. This acts as a scaffold to which we can fuse motifs for different biosensor applications [10] such as the two Z domains which were fused to the N-terminus of ctOmpA creating the protein ZZctOmpA. The assembly of the sensing layer consists of a protein immobilisation step followed by the addition of a thiol-containing amphiphile which completes the self-assembled monolayer [8]. This amphiphile has been shown to stabilise the bound protein [8,9] and in this work has a poly(ethylglycol) head group which reduces non-specific binding to the remaining surface [19].

The self-assembly of biosensors in this way is simple but there are few methods to assess the quality of the layer in terms of surface coverage and thickness. In previous studies we have shown that neutron reflection can be used to probe this class of thin organic/protein layers [20–22]. In particular we showed that the increase in thickness upon antibody binding to ZZctOmpA could be easily detected [21]. Here we combine surface plasmon resonance (SPR) and neutron reflection to probe more deeply the assembly of the protein array, antigen binding capacity and the structural orientation of the bound antibody/antigen layer. The result is a clear, layer by layer, model which provides an unusually clear picture of an engineered biosensor surface.

2. Materials and methods

2.1. Materials

General reagents were either from Melford Laboratories (Ipswich, Suffolk, UK) or from Sigma–Aldrich (Poole, Dorset, UK) unless other wise stated. Antibodies and human serum albumin were from Sigma–Aldrich. Chromatography columns were from GE Healthcare (Chalfont Saint Giles, Buckinghamshire, UK). ThioPEG was purchased from Prochimia Surfaces (Sopot, Poland).

2.2. Molecular biology

The pORLA9 plasmid contains the gene encoding for the circularly permuted ctOmpA [23] such that the N and C termini occur in external loop 1. The Z domain genes were amplified by PCR from plasmid pEZZ [24] and cloned into pORLA9 between the histidine affinity tag and the N-terminus of ctOmpA thus creating (His_6-)ZctOmpA and (His_6-)ZZctOmpA.

2.3. Protein expression and purification

The ctOmpA, ZctOmpA and ZZctOmpA proteins were expressed as inclusion bodies in *E. coli* BL21(DE3) cells grown in LB media induced by the addition of IPTG at 1 mM when the cells were at OD_{600} of 0.6–0.7 in flask cultures shaken at 180 rpm and 37 °C. After 3 h induction the cells were harvested by centrifugation at 6000×*g* for 10 min. The cells were disrupted by incubation overnight at −20 °C in a detergent solution of Bugbuster (Novagen, Nottingham, UK) (using 10 mL for every gram of wet cell pellet) which was supplemented with DNase, RNase and lysozyme. The inclusion bodies were isolated by centrifugation at 12 000×*g* for 20 min and washed three times by homogenisation in a 1 in 10 dilution of Bugbuster in deionised water followed by centrifugation at 12 000×*g* for 20 min. The proteins were then purified from inclusion bodies as previously described [25]. Pure protein fractions were concentrated to a volume of 1 mL using a Vivaspin 10 000 molecular weight cut-off spin concentrator. The proteins were refolded by slowly diluting the protein plus urea 1:10 into refold buffer (20 mM Tris–HCl pH 8.0, 1% (w/v) OG, 0.1 mM EDTA and 1 mM DTT) and left to incubate for 48 h at 37 °C to allow for complete refolding. Residual urea was removed by buffer exchange using a PD10 column equilibrated with refold buffer with 1 mM TCEP replacing DTT.

2.4. Circular dichroism

Circular Dichroism spectroscopy was carried out using 0.02 cm pathlength demountable cuvettes in a Jasco J-810 spectropolarimeter. The protein concentration was determined by protein absorbance at 280 nm and circular dichroism was scanned from 250 nm to 185 nm ten times, averaged and a buffer blank was subtracted. The circular dichroism was expressed as $\Delta\epsilon$ and imported into the CDSSTR website for secondary structure content analysis [26].

2.5. Preparations of proteins and filling molecules for surface deposition

Before deposition on gold, TCEP reducing agent was added to the protein and the filling molecules at a final concentration of 1 mM and incubated at room temperature for >10 min. The filling molecule, 1-mercaptoundec-11-yltriethylene glycol (thioPEG), diluted in a solution of 1% (w/v) OG, 20 mM Tris–HCl pH 8.0 and 1 mM TCEP was heated to 50 °C before deposition to ensure complete dissolution.

2.6. Surface plasmon resonance

Surface Plasmon resonance (SPR) experiments used Biacore X and 2000 instruments and Au sensor chips. The gold surface of the chip was cleaned by incubation for 15 min in an acid piranha solution (70% (v/v) concentrated sulphuric acid 30% (v/v) hydrogen peroxide) by incubation for 15 min. The surface was then cleaned with 1% (v/v) Hellmanex (Hellma UK, Southend on Sea, Essex, UK) and rinsed with deionised water. The chip surface was passivated by 1% (v/v) BME solution in ethanol for 10 min followed by washing with 1% (w/v) SDS and distilled water [8,27]. The running buffer was phosphate buffered saline (PBS, 20 mM sodium phosphate pH 7.6, 137 mM NaCl and 2.7 mM KCl). The protein was injected in a typical volume of 50 μL at a flow rate of 5 μL min^{-1} followed by injections of 1% (w/v) SDS to remove non-specifically bound protein before the filling molecule, thioPEG, was deposited. Three times 50 μL of 0.25 mg mL^{-1} thioPEG in a solution of 1% OG at a rate of 5 μL min^{-1} was used for each deposition, with a wash of 1% (w/v) SDS between each deposition to remove non-specifically bound thioPEG. Antibodies (IgG) and antigen (human serum albumin, HSA) were diluted in PBS and injected into the flow cell at a rate of 2 μL min^{-1}. The regeneration buffer was 100 mM glycine pH 2.0.

The BIAcore 2000 generated kinetic data to measure the equilibrium dissociation constants for ZZctOmpA arrays and immunoglobulins, 20 μL of IgG at various concentrations were flowed over a ZZctOmpA array at 10 μL min^{-1}. Flow rates of 5, 10 and 20 μL min^{-1} did not alter the association rate (Supplementary Fig. S1). IgG dissociation rate from the array, in a flow rate of 10 μL min^{-1} buffer, was measured for 20 min before regeneration. All data was corrected by subtracting appropriate blanks. After IgG injection is completed the decrease in response can be considered purely a dissociation event and the dissociation rate constant is calculated using Eq. (1) [28]:

$$Ln(R_0/R_n) = k_{off}(t_n - t_0) \quad (1)$$

Where R_0 is the response at time zero, t_0, R_n is the response at time t_n and k_{off} is the dissociation rate constant. The gradient of a plot of $Ln(R_0/R_n)$ versus $(t_n - t_0)$ is the dissociation rate constant. During the IgG injection phase the change in response is a function of both the IgG association and dissociation from the ZZctOmpA array and equation (2) is used to calculate the association rate constant, k_{on}:

$$dR/dt = (k_{on}CR_{max}) - \left\{\left[(k_{on}C) + k_{off}\right]R\right\} \quad (2)$$

Where dR/dt is the relative change in response per second, R is the response at any given time, C is the concentration of IgG and R_{max} is the maximum response. The gradient for a plot of dR/dt versus R for each concentration measured is calculated and then the gradient of a second plot of slope versus IgG concentration is the association rate constant. The equilibrium dissociation constant, K_d is thus:

$$K_d = k_{off}/k_{on} \quad (3)$$

Experiments were measured in triplicate and the standard error of the mean (SEM) is quoted as the error values.

2.7. Neutron reflection

Polarised reflection experiments were carried out on the CRISP reflectometer at ISIS, Rutherford Appleton Laboratory, UK or on the NG-1 reflectometer at the NIST Centre for Neutron Research, Maryland, USA. Both reflectometers were used in polarised mode. The surfaces were made at NIST (NG-1) or by Microsystems and Nanotechnologies, Lisbon, Portugal (CRISP). The silicon substrate was coated with an 80 to 140 Å layer of μ-metal (an alloy of 75% iron, 15% nickel with traces of copper and molybdenum) and then with a 150 to 200 Å layer of gold by either magnetron sputtering (NG-1) or ion beam deposition (CRISP). The substrates were 50/100 mm in diameter and 5/10 mm thick for NG-1/CRISP. Once removed from vacuum the surfaces were washed with >18 MΩ water, followed in order by ethanol (AnalaR grade), 1% (v/v) BME in ethanol, 1% (w/v) SDS, water and ethanol again and dried under a stream of nitrogen gas. The protein array was assembled by placing gold surface in a glass Petri-dish with lid and pouring the protein solution on to cover the whole gold surface (7–9 mL at a concentration of 0.25 mg mL^{-1}). After 1 h at room temperature the protein solution was removed and the surface washed with 1% (w/v) SDS and then >18 MΩ water. The original protein solution was returned to the surface and this cycle was repeated three times to gain maximum surface coverage. Then thioPEG was added to cover the surface and the lid replaced. After 1 h the surface was once again washed with 1% SDS and water and this cycle was also repeated three times. The surface was then mounted into its cell and placed on the beamline where all subsequent additions of antibody, antigen, change of buffer etc were carried out *via* the cell's inlet and outlet values using a syringe and ×10 excess of the cell's void volume.

In reflectivity experiments the intensity of the reflected neutrons with respect to the intensity of the incident neutrons (reflectivity) is measured as a function of momentum transfer, Q which is defined as