Oriented Self-Assembling Protein Monolayers for Antibody Capture on Gold Surfaces

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The immunoassay is a powerful tool in diagnostics; antibody technology provides exquisitely specific capture of biological markers for disease. In the last 15 years there has been a drive to transfer the benefits of the immunoassay onto the surfaces of advanced electronic biosensors. Traditional methods of antibody immobilisation such as adsorption and chemical coupling have some disadvantages that are magnified in the arena of ultrasensitive miniaturised electronic detection of antibody-antigen interaction *(Table 1)*. In order to address some of these problems we have developed a method for creating oriented, stable, self-assembled monolayers of protein using engineered bacterial outer membrane proteins (omp) as scaffolds for fusion proteins [1,2]. The core technology requires the fusion of a protein of interest to a scaffold protein with self assembling properties. The fusion protein is assembled in a monolayer by a simple 'apply-and-wash' process. Gaps between the proteins are filled in with filler molecules such as PEG-thioalkanes, leaving only the protein of interest exposed *(Figure 1)*. This method overcomes many of the problems associated with traditional methods of protein application to surfaces. The scaffold protein is highly stable with a melting temperature of 88°C. It is resistant to protease digestion and remains intact in SDS and extremes of pH. This basic technology was used to create a set of proteins for antibody immobilisation.

Table 1. Comparison of antibody immobilisation technologies.

Property	Adsorption	Chemical Coupling	Orla Monolayer
Antibody orientation	No control	No control or only partial control	Always in the correct orientation
Non-specific binding	High Background	Low to medium	Non specific interactions are minimal
Reproducibility	Poor reproducibility	Reproducible	Self assembly is highly reproducible
Complexity of manufacture	Simple	Involves complex chemistries	Simple assembly from aqueous solution
Antibody density on surface	Poor control	Controllable	Controllable
Scale up for manufacture	Scale up problematic	Scale up problematic	Easily scaled
Antibody functionality	Poor (~1-5% of immobilised antibody available for antigen)	Reduced (25-50% available for antigen binding)	Excellent (80-100% available for antigen)
Proximity of antibody-antigen reaction to the surface	Not controlled. Usually distant from surface.	Large variations dependent upon chemistry and capture layers.	The antigen binding site is ~20 nm from surface.

Self-assembling proteins for antibody capture

A set of IgG-binding proteins *(Table 2)* was generated by fusing tandem repeats of IgG-binding domains from Staphylococcus aureus protein A (SPA) [3], Streptococcus spp. Protein G (SPG) [4] and/or Peptostreptococcus Protein L (PPL) [5] to the Orla scaffold - an engineered variant of the E.coli outer membrane protein OmpA. SPA and SPG bind to the Fc region of IgG whereas PPL binds to the κ-light chain of the variable domain. These proteins bind antibody in an orientation where the antigen-binding sites are exposed *(Figure 2)*. The dimensions shown in *Figure 2* were measured by polarised neutron reflection [6]. The total height of the capture layer and the antibody was close to 190 Å suggesting that the antibody is not completely upright as depicted but is partially tilted towards the surface. Consequently, the distance between the antigen binding site and the gold surface is only ~20 nm and this is a great advantage for most types of biosensor.

Table 2. The IgG-binding protein set.

Protein	Structure	Amino acids	Molecular Weight (kDa)
Orla18	SPA-Omp	401	34.5
Orla85	SPG-Omp	404	44.0
Orla86	PPL-Omp	459	44.5
Orla87	SPG-SPA-Omp	462	50.6
Orla88	PPL-SPA-Omp	553	51.1
Orla89	PPL-SPG-Omp	474	60.6

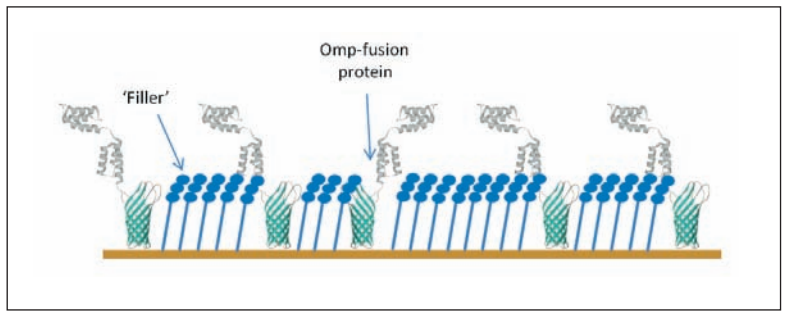

Figure 1. Schematic diagram of protein monolayer formed using Orla technology.

Analysis of antibody-binding proteins by surface plasmon resonance

The IgG-binding properties of these proteins were examined using surface plasmon resonance (Biacore 2000 instrument, GE Healthcare). The binding of a rabbit polyclonal antibody and two of the common subclasses of mouse monoclonal antibodies were tested *(Figure 3)*. It is apparent from these data that the different constructs provide a complex set of binding characteristics that may be exploited for different applications. In many cases the binding is extremely stable and only reversed by the acid regeneration.

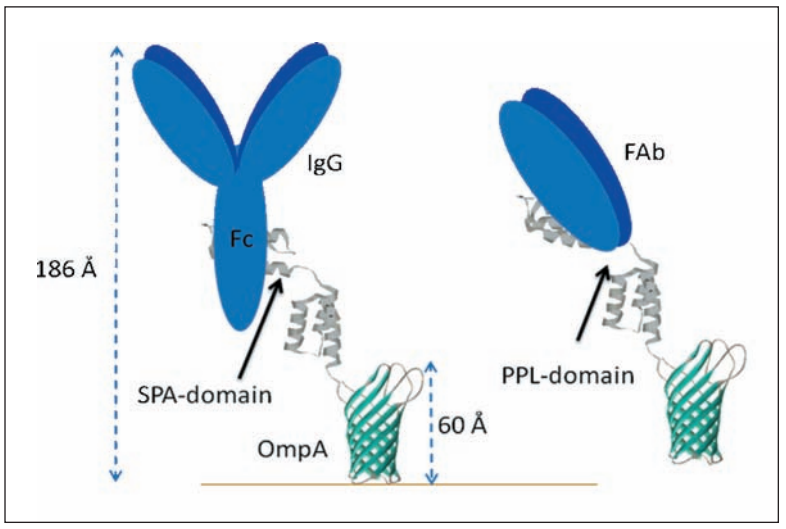

Figure 2. Schematic representations of IgG-binding domains fused to OmpA with bound antibody or Fab fragment. The dimensions shown were measured by polarised neutron reflection [6]. The total height of the capture layer and the antibody was measured at close to 190 Å suggesting that the antibody is not completely upright as depicted but is partially tilted towards the surface. Consequently, the distance between the antigen binding site and the gold surface is only ~20 nm and this is a great advantage for most types of biosensor.

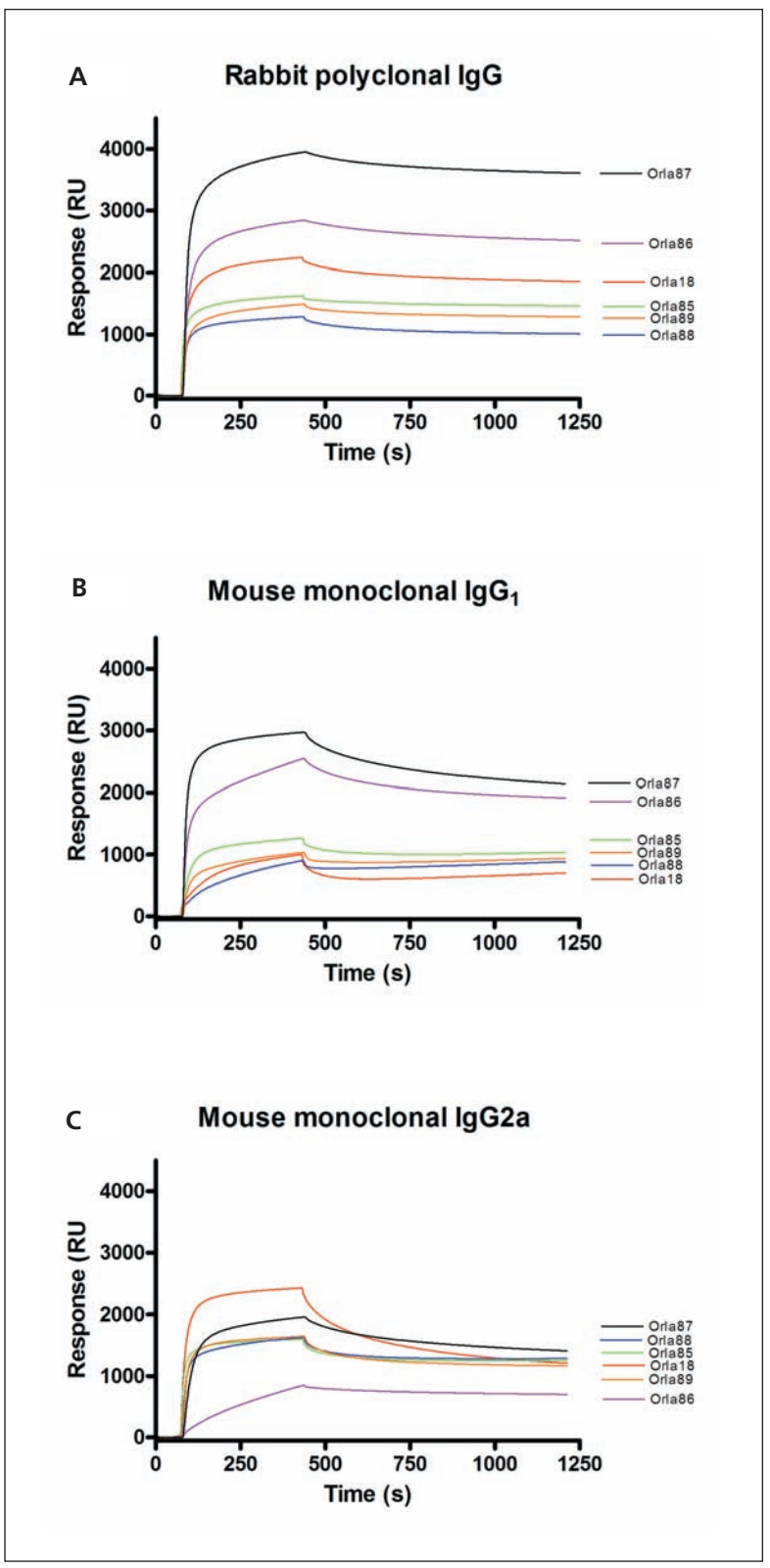

Figure 3. Sensorgrams showing binding of rabbit polyclonal IgG (A), mouse monoclonal IgG_1 (B) or mouse monoclonal IgG_{2a} (C) analysed by surface plasmon resonance. Protein-PEG thioalkane monolayers were assembled in situ on a Biacore Au chip. Antibody was diluted in tris-buffered saline, 0.05% Tween 20 (TBS-T) at 30 µg mL^{-1} at a flow rate of 5 µL min^{-1}. Antibody injection was started at 80s for 6 min until 440s. Then TBS-T was flowed over the surface for approx 13 min. Surfaces were regenerated with 100 mM HCl for 3 min prior to next antibody injection.

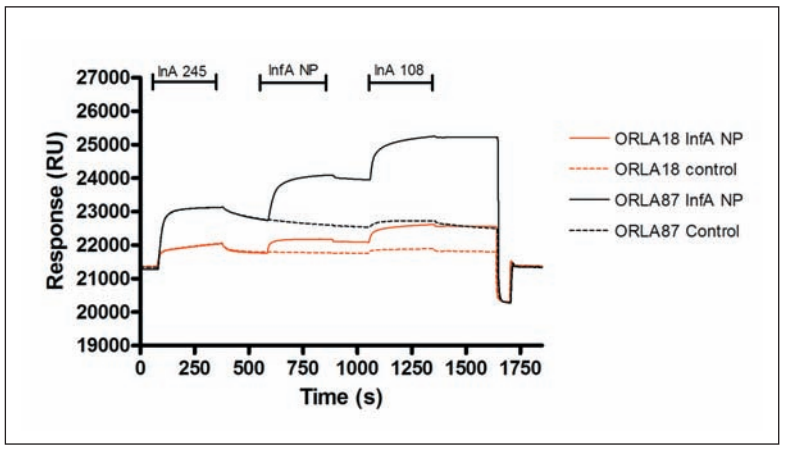

Figure 4: Sensorgram of sequential binding of antibody, antigen and secondary antibody. The antigen was Influenza A nucleoprotein (InfA NP) at 10 µg mL^{-1} in TBS-T. Antibodies InA 245 (mouse mAb IgG_{2b}) and InA 108 (mouse mAb IgG_1) were obtained from Hytest, Finland, and were diluted to 30 µg mL^{-1} in TBS-T. ORLA18 surface was pre-assembled in Fc_1 and ORLA87 surface in Fc_2 both with PEG-thioalkane filler. TBS-T washes were carried out between injections. A control run without antigen was also carried out. The surfaces were regenerated with 100 mM HCl after each cycle. The flow rate was 5 µL min^{-1} throughout.

In a second experiment (on a Biacore X-100 machine) the binding of antibody was followed by the binding of antigen and a secondary antibody that binds at a site different from the primary antibody i.e. to mimic a typical 'sandwich' immunoassay *(Figure 4)*. The molar ratio of antigen to antibody was calculated *(Table 3)*. A change of +1 RU was taken to represent 1 pg/mm^2 of protein [7]. The molecular weight of InA245 is 150 kDa and that of the NP protein is 57 kDa. The molar ratio of 2.1 shows that there are two molecules of antigen binding for every one of antibody i.e. 100% of the antibody binding sites are available for antigen binding. This compares to 25-50% for amine coupled antibody and 1-5% for adsorbed antibody; clearly demonstrating the advantage of correctly oriented antibody. The sandwich assay was carried out using a range of antigen concentrations. A plot of the change in signal upon secondary antibody addition is shown in *Figure 5*. These data show the usefulness of the antibody capture monolayer in 'on-chip' immunoassay applications.

Table 3. Molar ratio of antigen to antibody.

	ORLA18		ORLA87	
	Relative Response	nmol/mm^2	Relative Response	nmol/mm^2
InA 245 1° Antibody	416.6	2.8 x 10^{-6}	1479.2	1 x 10^{-5}
Antigen InfA NP	327.1	5.8 x 10^{-6}	1202.3	2.1 x 10^{-5}
Molar ratio Ag:1°Ab	2.1		2.1	

Surface stability and longevity

The stability of ORLA18 surface over 30 cycles of antibody injection and regeneration was tested by SPR on Biacore *(Figure 6)*. There was a net loss of binding signal response of 10% over 30 cycles. The quantity of antibody bound at the last cycle was greatly in excess of that required for antigen detection.

The longevity of the assembled surface was tested using surface acoustic wave (SAW) technology. ORLA18 protein surfaces with a PEG-thioalkane filler were assembled on the gold surface of SAW chips (Japan Radio Co. Ltd) and the binding of IgG analysed over 6 cycles. The chips were regenerated with 100 mM HCl and dried in nitrogen and stored in airtight containers at 4°C. The binding of IgG was analysed again after 144 days (~20 weeks) of storage. The average phase angle change on day 1 was -52.79° (± 2.33) whereas that on Day 144 was -48.42° (± 2.25) indicating that the surfaces are very stable.

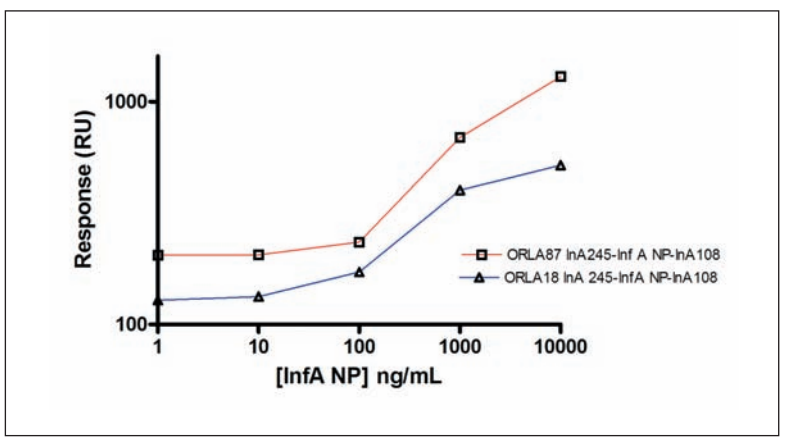

Figure 5. Sensorgram showing the concentration of InfA NP Vs the response obtained after secondary antibody injection on Biacore. The lower detection limit was 10-100 ng/mL on ORLA18 and 100 ng/mL on ORLA87.

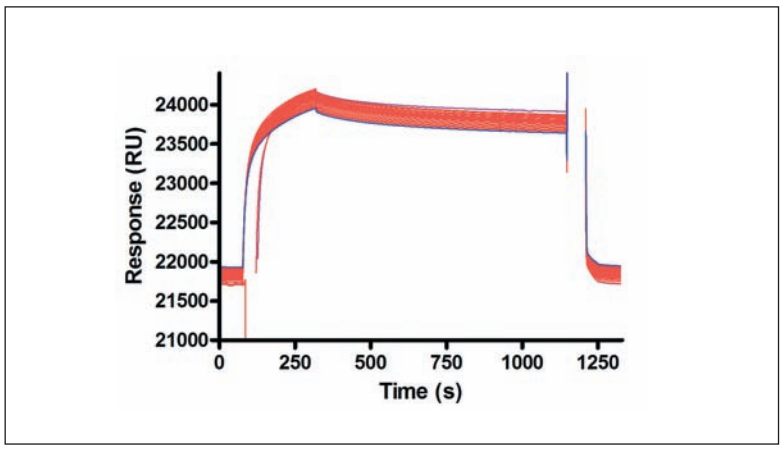

Figure 6. Injection of 30 µg mL^{-1} rabbit polyclonal antibody and regeneration with 100 mM HCl over 30 cycles. The first cycle is shown in purple and the last in blue. Antibody injection was from 60-360 seconds with a 15 min wash with TBS-T buffer prior to regeneration. The flow rate was 5 µL min^{-1} throughout.

Acknowlegements

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Summary and Outlook

The transfer of an immunoassay onto a biosensor chip device can be greatly facilitated by the use of these types of antibody binding monolayers. The surfaces are stable and amenable to long-term storage.

The self-assembly from aqueous buffer using a simple 'apply and wash' method is highly manufacturable and protein production is also easily scaled up. The technology is amenable to microcontact, or dip-pen lithography for the generation of antibody-binding arrays.

Beyond the examples presented above, the technology has been applied to the design and manufacture of highly specialised surfaces for cell culture. It is possible to create fusions with a huge variety of proteins and peptides e.g. the fusion of single chain Fv domains of antibodies to create highly specific capture molecules with self-assembling properties.

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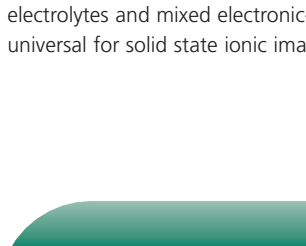
Partnership Delivering Optimum Solutions in Freeze Drying Technology



Linkam Scientific Instruments and freeze drying technology, Biopharma Technology Limited, celebrate ten years of partnership in supplying solutions to the pharmaceutical industry.

Biopharma Technology Limited (BTL) was founded in 1997 to develop freeze drying solutions to multiple materials in the world of pharmaceuticals. Freeze drying, also known as 'lyophilisation,' is a method of processing a liquid product into a dry solid product. In 2001, Director of Research at BTL, Dr Kevin Ward, came across Linkam and their range of temperature controlled stages. Freeze drying had often been a conducted in a hit and miss process without clear knowledge of the critical temperatures needed to produce the best product. The ability to provide accurate thermal characterisation right down to -196°C took the guesswork out of the process quickly and simply.

The two companies went on to develop a number of instruments, the latest being the Lyostat 3 freeze drying microscope. The combination of BTL's applications knowledge and Linkam's instrumentation expertise brings a real added-value product to the freeze drying market place.

A typical experiment will look at the temperature at which a product undergoes collapse as this is the single most important characteristic for almost all freeze drying formulations. The Lyostat3 system uses polarised light microscopy to capture images of the sample's structural changes as temperature increases, information that is vital for successful freeze drying. This data is invaluable when developing a freeze drying cycle from scratch or in helping to identify the small adaptations in a freeze drying cycle that can transform the process operating costs. The advantages of a tailored production process are obvious; research has shown that a 1°C increase in product temperature can result in as much as a 13% increase in primary drying rate, demonstrating that thermal accuracy can transform process operating efficiency and costs. The collaboration has also brought benefits to BTL through being able to provide quality pictures to illustrate the process at varying precisely controlled temperatures and pressures. For Linkam, it has been the benefit of being at the leading edge of an exciting and growing market. Freeze drying is growing. Whereas it used to be solely the domain of big pharma companies, it is now the smaller, specialty businesses in the fields such as biotherapeutics and biopharmaceutics that are using the BTL technology to study bigger, more complex molecules.

Circle no. 198

High-Content Cellular Analysis System

GE Healthcare's IN Cell Analyser 6000 is a high-performance, laser-based, confocal imaging platform designed for the most demanding high-content cellular assays and screens including live cell assays, 3D imaging, co-localisation studies and imaging of assays with low signal. In contrast to conventional fixed aperture confocal systems, the IN Cell Analyzer 6000 has a proprietary optical system, which features a variable, iris-like aperture, which mimics the action of the eye. The fully adjustable aperture is intuitively controlled via the user interface, and can be adjusted to be used in; open mode to maximise speed; full confocal mode to maximise image quality and background rejection; or any setting in between to optimise the imaging performance of a specific biology for each channel of an assay. Incorporating the latest generation scientific CMOS camera, which delivers a 4x larger field of view with 5◊ less noise than traditional CCD cameras, the IN Cell Analyser 6000 delivers significantly increased sensitivity and reduced exposure times to maximise throughput in screening settings.

Circle no. 199

Electrochemical Strain Microscopy for Energy Storage Research

Asylum Research has announced the new Electrochemical Strain Microscopy (ESM) imaging technique for its Cypher™ and MFP-3D™ AFMs. Developed by Oak Ridge National Laboratory (ORNL) and Asylum Research, ESM is an innovative scanning probe microscopy (SPM) technique capable of probing electrochemical reactivity and ionic flows in solids on the sub-ten-nanometer level. ESM is the first technique that measures ionic currents directly, providing a new tool for mapping electrochemical phenomena on the nanoscale. The capability to probe electrochemical processes and ionic transport in solids is invaluable for a broad range of applications for energy generation and storage ranging from batteries to fuel cells. ESM has the potential to aid in these advances with two major improvements over other conventional technologies: (a) the resolution to probe nanometer-scale volumes and (b) the inherent ability to decouple ionic from electronic currents with (c) imaging capability extended to a broad range of spectroscopy techniques reminiscent of conventional electrochemical tools.

Roger Proksch, President of Asylum Research, commented: "Progress in energy storage and conversion will be greatly facilitated by the ability to study batteries and fuel cells at the level of several nanometers. ESM provides functional imaging of electrochemical phenomena in volumes millions to a billion times smaller than conventional current-based electrochemical techniques. This new technique opens the pathway to understanding energy technology and ionic devices on the level of individual grains and defects, thus bridging macroscopic functionalities and atomistic mechanisms. This in turn will lead to improved energy storage solutions – batteries with extremely high energy densities and long lifetimes and fuel cells with very high energy densities and efficiencies."Traditionally, scanning probe microscopy techniques allowed measurement of electronic currents and short- and long-range forces," added Sergei Kalinin, Senior Research Staff Member in the Center for Nanophase Materials Sciences at ORNL and co-inventor (with Nina Balke and Stephen Jesse) of ESM. "ESM extends this capability to measure ionic currents, and has already been demonstrated for a variety of Li-ion cathode, anode, and electrolyte materials, as well as oxygen electrolytes and mixed electronic-ionic conductors. The ubiquitous presence of concentration-molar volume coupling in electrochemical systems suggests that this technique is in fact universal for solid state ionic imaging – from batteries and solid state to memristive electronics."

Circle no. 200