



Use of Orla technology to generate self-assembled monolayers of IgG-binding domains of Staphylococcal protein A.

Application Note
OPT/031106/DS

- Simple generation of fusion
- Self assembly
- Correctly oriented monolayer
- Functional protein surface
- Minimised non-specific binding

Summary

This application note describes the application of Orla Protein Technologies' unique 'surface biology' platform to generate functional protein monolayers on an analytical surface. The IgG-binding domains of *Staphylococcus aureus* Protein A (SPA) were fused to a 'scaffold' protein with inherent self assembly and gold-binding properties. The protein function was tested on an SPR platform (Biacore). The ORLA-SPA monolayer demonstrated a stable, high capacity of IgG binding and very low non-specific binding compared to a native protein A surface. In a second experiment ORLA18 immobilised by amine coupling showed much poorer binding capacity and signal:noise ratio than the monolayer.

Introduction

A need exists for technology that allows the direct integration of materials and physical devices with biological systems. This interaction is best performed by a truly biomimetic interface. Nature uses proteins and lipids to provide this function, but *in vitro*, proteins are difficult to immobilise in a functional way. Orla Protein Technologies provides unique expertise for addressing these challenges. The Orla self-assembly technology has the potential to revolutionise the use of immobilised proteins in biosciences and extend their application into other technologies.

The ability to control the density, orientation and functionality of surface immobilised proteins is critical in their application. Traditional methods of protein immobilisation are based upon physical adsorption or complex chemical attachment and both of these methods present many problems that can be alleviated by Orla's proprietary surface assembly platform (see Table 1).

<i>Existing Surface Chemistries</i>	<i>Orla Platform</i>
Poor Orientation	Protein orientation is controllable
High Background	Non specific interactions are minimal
Poor reproducibility	Self assembly is highly reproducible
Reliant on Physical adsorption or complex Chemistry	Simple assembly from aqueous solution
Scale up problematic	Easily scaled
Limited Control	Exquisite control of surface assembly
Reduced Functionality	Proteins retain functionality
Lacking Quality Assurance methods	All surfaces and proteins fully characterised and quality controlled

Table 1

The basic technology involves the fusion of the protein of interest with a proprietary, inherently self-assembling scaffold protein. The purified fusion protein in aqueous buffer is applied to the surface where the scaffold attaches covalently in the correct orientation. A simple wash step is used to remove non-covalently attached protein, leaving behind a precisely oriented monolayer. The spaces between the proteins in the monolayer are then covered with a 'filler' molecule that is also covalently attached



and oriented (Figure 1). The filler molecule stabilises the scaffold protein and masks it so that only the protein of interest is exposed at the surface. This simple principle can be used to produce surfaces with exquisitely controlled properties. Most protein molecules or peptides may be fused to the scaffold e.g. single chain antibody fragments, enzymes, proteins that bind to analytes, proteins that promote cell adhesion or differentiation etc. Since the protein is presented correctly oriented as a monolayer, the density of the functional protein on the surface can be controlled by applying it at higher or lower concentration. The upper limit of density is dependent upon the width of the fusion protein. Mixtures of proteins can be applied to give multi-functional surfaces and proteins may be laid down in patterns to produce surfaces with regional functionality, protein arrays and even gradients. The properties of the surface not covered by protein can also be controlled by choice of head group on the filler molecules. Thus, the surface can be manipulated to have minimal non-specific binding. The advantages of this approach in a large variety of applications are self-evident.

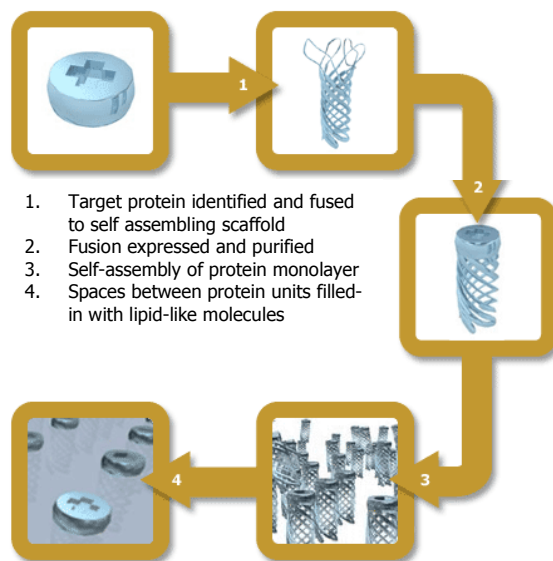


Figure 1

In this work, we describe in detail how Orla technology is applied in practice. The IgG-binding Z domain was used as a model target protein.

Methods and Results

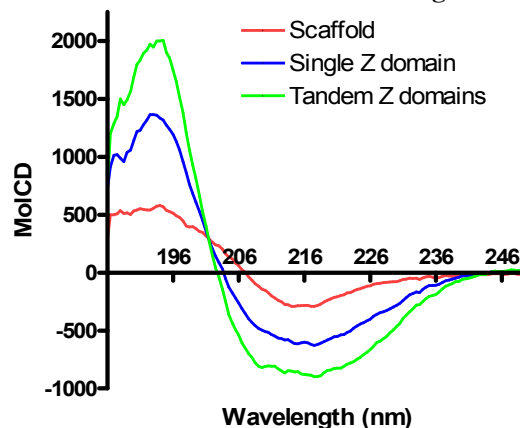
Generation of fusions

Z-domains were PCR amplified and cloned into the scaffold coding sequence such that the Z domains would be fused at the N-terminus of the scaffold. Two fusions were generated: ORLA17, containing a single Z domain; and ORLA18, containing two Z domains in tandem.

Purification and Refolding

ORLA17 and 18 as well as ORLA9 (scaffold protein alone) were expressed as insoluble inclusion bodies in *E. coli*. The inclusions were purified and solubilised in 8M urea. The ORLA proteins were purified by Ni-affinity chromatography utilising a 6xHis tag on the scaffold protein. A polishing step using ion-exchange chromatography was carried out to yield protein purified to >98% homogeneity. This protein was refolded by 1/100 dilution into a urea-free detergent buffer. Correct refolding was confirmed by circular dichroism spectroscopy. The scaffold has a β -barrel structure whilst the Z domains are predominantly α -helical. The spectrograph confirms that ORLA18 has the largest α -helix signal whilst ORLA17 is intermediate between ORLA9 and -18 as expected (Figure 2).

Figure 2





Formation of Self-assembled monolayers

The formation of the monolayer is carried out in two stages:

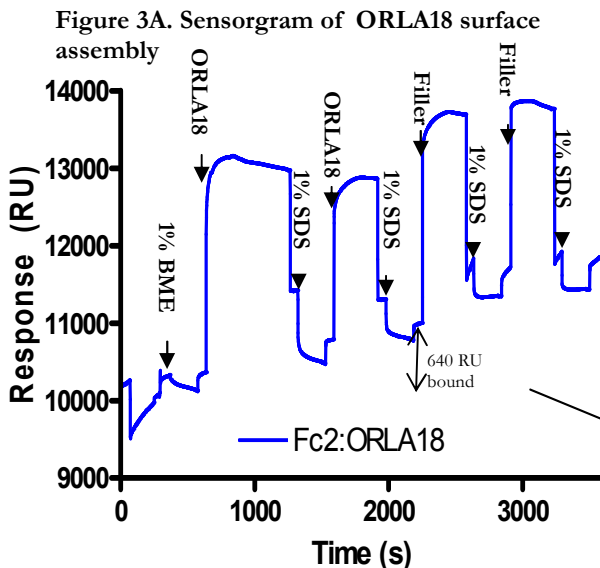
Stage 1. Protein monolayer formation

1. Gold surface passivation by β -mercaptoethanol.
2. Protein application followed by SDS wash to remove protein that is not covalently bound and is incorrectly oriented.
3. Repeat steps 1 and 2 to gain greater protein coverage

Stage 2. Filling-in the gaps

1. Application of filler molecule.
2. SDS wash to remove unbound material that is not part of the monolayer.
3. Repeat steps 1 and 2 to complete the surface.

The formation of such surfaces can be monitored by carrying out the assembly *in situ* in a surface plasmon resonance (SPR) system. The results of such an experiment carried out using ORLA18 are shown in Figure 3A. In the experiment shown, a Biacore Au chip was Pirhana cleaned, docked in a Biacore X machine and primed with TBS (Tris-Buffered Saline pH7.5). TBS was the wash buffer throughout. The flow rate was $5\mu\text{L min}^{-1}$ and the temperature was maintained at 25°C . Details of analyte applications are given in Table 2.



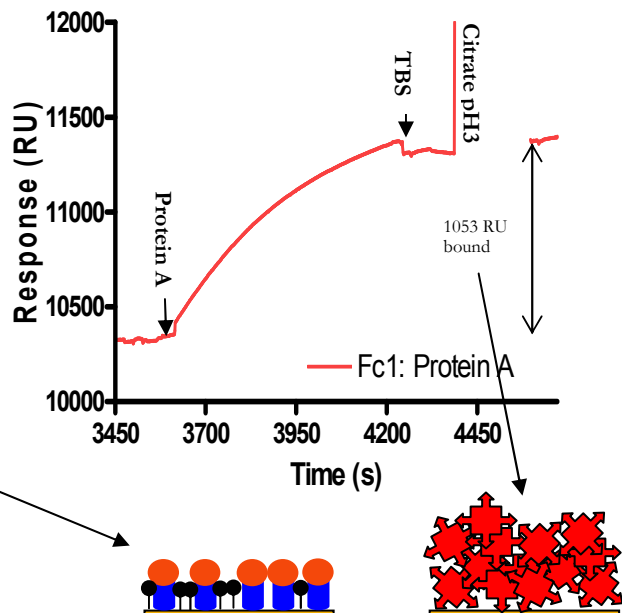
RU stands for ‘resonance units’ where a 1° angle change corresponds to 10 000 RU. The filler was an oligo-ethyleneglycolated thioalkane.

Table 2.

Active Flow Cell	Injected analyte	Vol (ml)	Contact Time (min)
2	1% β ME	15	3
2	ORLA18 ($5\mu\text{M}$)	50	10
2	1% SDS	15	3
2	ORLA18 ($5\mu\text{M}$)	35	7
2	1% SDS	15	3
2	Filler solution	35	7
2	1% SDS	15	3
2	Filler solution	25	5
2	1% SDS	15	3

To provide a comparison, on the same chip, native protein A was immobilised by adsorption on the surface of flow cell 1 as shown in Figure 3B. $50\mu\text{L}$ of recombinant *Staphylococcus aureus* protein A (SPA, Sigma Cat No. P7837) diluted to $5\mu\text{M}$ in TBS was injected into FC1. This was followed by a wash with pH3 citrate buffer. Note that the ORLA18 surface has a monolayer of protein and filler compared to the disordered nature of the native Protein A surface (see cartoons).

Figure 3B. Sensorgram of Protein A application





In a second experiment, ORLA18 and SPA were immobilised on a Biacore CM5 chip using amine coupling chemistry. The greater surface area on the CM5 chip resulted in substantially greater amounts of immobilised protein (data not shown).

IgG binding properties of the surfaces.

In the first experiment, mouse IgG (Sigma Cat. I5381) was diluted in TBS to a final concentration of 30 mg mL⁻¹. This was injected over both the flow cells on the Au chip (Figure 4). Flow path was Fc1-Fc2. To regenerate the surface, the IgG was eluted with citrate buffer at pH 3.0. In the first two injections 15 µL of IgG was injected at a flow rate of 2 µL min⁻¹ giving a contact time of 7.5 min.

For the third injection 30 µL of IgG was injected at a flow rate of 5 µL min⁻¹ with a reduced contact time of 6 min. Adsorbed SPA binds IgG very poorly compared to the ORLA18 surface.

The ORLA18 surface gives consistently high signal:noise ratios i.e. very low non-specific binding.

The binding capacity of the ORLA18 surface does not diminish significantly after repeated cycles although the contact time has an effect on how much antibody binds to the surface. The surface is highly stable. The adsorbed SPA surface shows signs of deterioration after 3 cycles as indicated by the negative change in RU after wash.

In the second experiment mouse IgG was injected over both flow cells of the CM5 chip (same concentration, volume and flow rate as for the Au chip).

For comparison of different types of surface the critical parameters are: (i) the signal:noise ratios, obtained by dividing the total bound IgG (in RU) by the remainder bound after the pH3 elution; and (ii) The IgG bound per unit of immobilised capture protein i.e. specific IgG bound (total minus remainder after pH3 wash) divided by the amount of capture protein (e.g. SPA or ORLA18) immobilised on the surface.

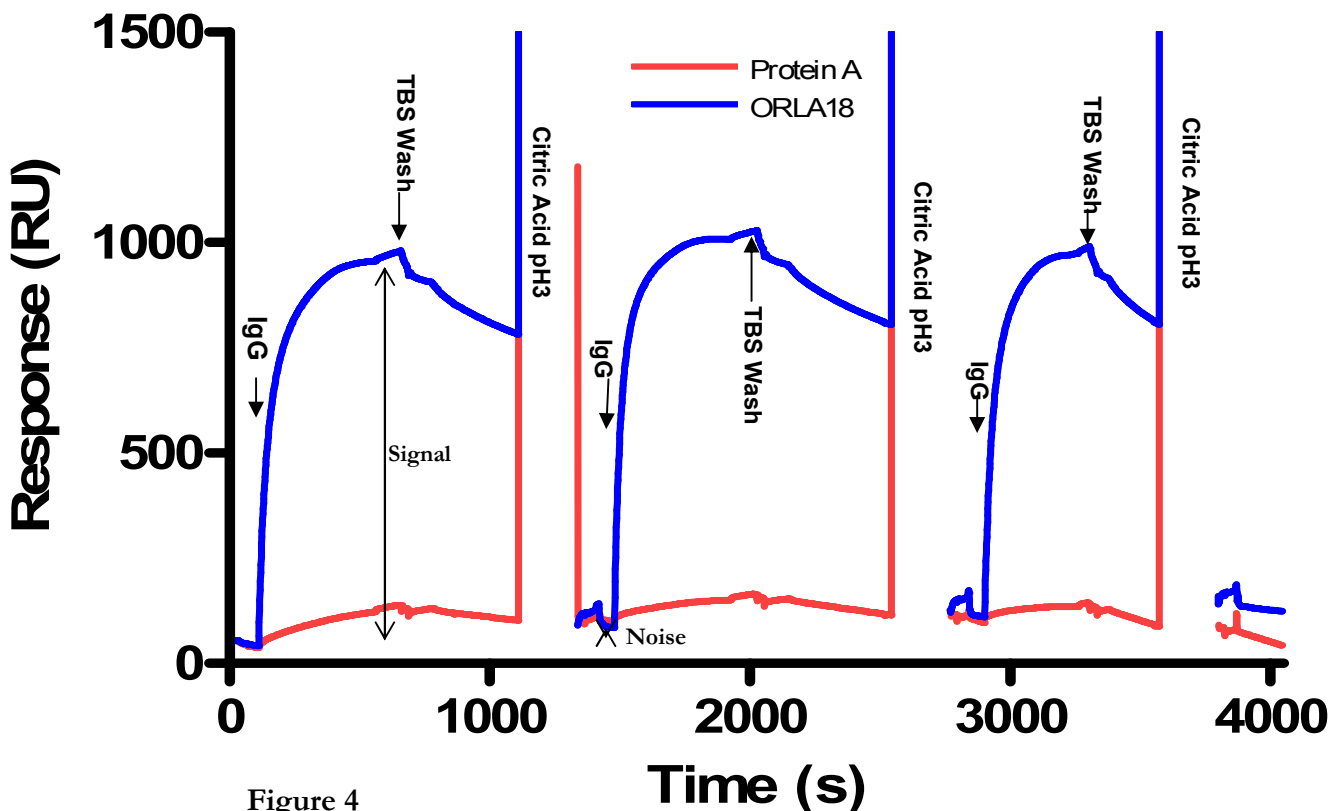


Figure 4



These parameters for all four types of surface are summarised in Table 3 below.

Table 3.

	Signal: Noise	IgG per unit protein
ORLA18 Au monolayer	47	1.5
ORLA18 CM5 amine coupled	34	0.5
Protein A Au adsorbed	2.5	0.06
Protein A CM5 amine coupled	26	1.6

The adsorbed SPA surface shows the poorest performance for both criteria. The ORLA18 self-assembled monolayer has binding capacity comparable to that of amine coupled SPA but has much better signal to noise ratio; most likely because there is minimal exposure of non-specific regions of the molecule in the monolayer. It is interesting that the amine coupled ORLA18 shows comparatively poor binding capacity compared to ORLA18 in a monolayer. The protein in the monolayer is all correctly oriented with the SPA domains exposed. In the amine coupled surface, many of the SPA domains on ORLA18 would be occluded because of the non-selective nature of chemical coupling.

Conclusions

This study demonstrated the superiority of a precisely oriented self-assembled monolayer of a combination of protein and filler compared with a relatively disordered layer of adsorbed protein or chemically coupled protein on the surface of an analytical device.

This exemplifies the applicability of Orla surface technology to an analytical platform. The inherent properties of the combined protein-filler monolayer in terms of stability and low non-specific binding are very attractive for use in analytical devices. The Orla method of generating these surfaces is highly scalable and may be incorporated into a manufacturing process. It can be used to generate mixed surfaces with a variety of fusion proteins with different functions incorporated in the same surface. The technology may also be used to produce functional protein arrays. Virtually any peptide sequence may be incorporated into the system for a variety of analytical applications.

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