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Use of Orla technology to generate self-assembled monolayers for the detection of specific antibody.

Application Note
OPT/041106/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 FLAG epitope was fused to a 'scaffold' protein with inherent self assembly and gold-binding properties. On an SPR platform (Biacore), the ORLA-FLAG monolayer was able to detect an anti-FLAG antibody at a concentration of 10 pmol in 30μL in both simple saline (TBS) and complex mixtures (Fetal Bovine Serum, FBS) spiked with the anti-FLAG antibody. The Orla monolayer showed better sensitivity and lower non-specific binding compared to a surface with protein immobilised by amine coupling.

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).

Existing Surface Chemistries	***Orla Platform***
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.

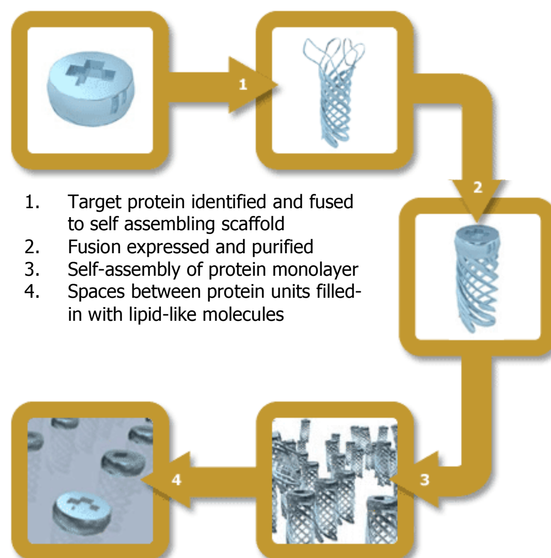


Figure 1

One such application in diagnostics is the detection of circulating antibodies that may be markers for certain types of disease (e.g. microbial infection, autoimmune diseases etc.) where rapid detection, possibly at the point of care, would greatly enhance diagnosis and treatment of the patient.

In this work, we describe in detail how Orla surface technology may be applied for detection of antibody in solution. We have employed the FLAG epitope as a model system to detect anti-FLAG antibody in solution using surface plasmon resonance as the detection platform.

Methods and Results

Generation of fusions

Complementary oligonucleotides encoding the hydrophilic octapeptide DYKDDDDK (FLAG-epitope) were annealed and ligated in-frame into the gene encoding the scaffold protein (an engineered OmpA: ORLA0) such that the expressed protein would have a FLAG epitope on one of the external loops (Figure 2). This protein was named ORLA5.

Figure 2

Purification and Refolding

Both ORLA0 and ORLA5 were expressed as inclusion bodies in *E. coli*. The inclusions were isolated and solubilised in 8M urea. The proteins were purified by Ni-affinity chromatography followed by ion exchange. Pure protein was refolded by 1/100 dilution in a urea-free refolding buffer.

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.

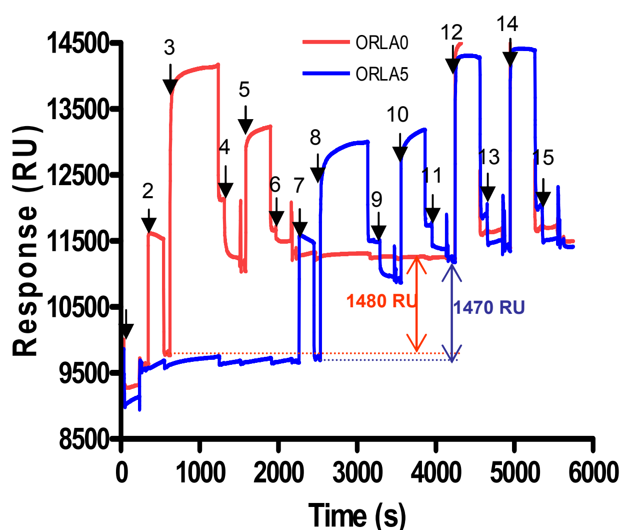
The formation of such surfaces can be monitored by carrying out the assembly *in situ* in a surface plasmon resonance (SPR) system. The results of such an experiment are shown in Figure 3A. In the experiment shown, a Biacore Au chip was Pirhana cleaned, docked in a Biacore X machine and primed with TBS (Tris-Buffered Saline pH7.5). TBS was the wash buffer throughout. The flow rate was 5μL min^{-1} and the temperature was maintained at 25°C. Details of sample applications are given in Table 2. In the data, RU stands for resonance units where a 1° angle change corresponds to 10 000 RU. The filler was an oligo-ethyleneglycolated thioalkane. The sensorgram for the surface assembly is shown in Figure 3.

Step	Active Flow Cell	Injected analyte	Vol (ml)	Contact Time (min)
1	1,2	1%SDS	15	3
2	1	1% βME	15	3
3	1	ORLA0 (9μM)	50	10
4	1	1% SDS	15	3
5	1	ORLA0 (9μM)	25	5
6	1	1% SDS	15	3
7	2	1% βME	15	3
8	2	ORLA5 (9μM)	50	10
9	2	1% SDS	15	3
10	2	ORLA5 (9μM)	25	5
11	2	1% SDS	15	3
12	1,2	Filler Solution	25	5
13	1,2	1% SDS	15	3
14	1,2	Filler solution	25	5
15	1,2	1% SDS	15	3

Table 2.

Protein remaining bound on the surface after SDS washes is indicated by the two-headed arrows. Similar quantities of both protein were immobilised in the monolayer.

Figure 3. See Table 2 and text for details



Formation of amine coupled surfaces

A Biacore CM5 chip was docked in a Biacore X machine and primed with TBS. Protein immobilisation was carried out sequentially in each flow cell by using the amine coupling kit (Biacore). The surfaces were activated by NHS/EDC and protein in pH5 acetate buffer was injected (50 μL of 9 μM solution). 8781 RU of ORLA0 was immobilised in FC1 and 5500 RU of ORLA18 in FC2. There is substantially more protein immobilised on these surfaces because the CM5 chip has a greater surface area and the protein is not in a monolayer.

Capture of anti-FLAG antibody

Anti-FLAG alkaline phosphatase conjugate was diluted in TBS or Fetal Bovine Serum (FBS) to 10 μg mL^{-1} and 1 μg mL^{-1} concentrations. 30μL was injected over the surfaces, starting with the lowest concentration first. Sample sensorgrams are shown in Figure 4 and 5. The data is summarised in Table 3.

Table 3. The numbers refer to the change in response seen after antibody injection and TBS wash in resonance units (RU). n/d = not done.

	Ab 10 μg mL^{-1} in TBS	Ab 1 μg mL^{-1} in TBS	Ab 10 μg mL^{-1} in FBS	Ab 1 μg mL^{-1} in FBS
ORLA0 Monolayer	60	32	370	350
ORLA5 Monolayer	1800	300	2044	574
ORLA0 amine coupled	28	25	390	n/d
ORLA5 amine coupled	689	80	1554	n/d

There is a significant decrease in response between ORLA5 monolayer surface and amine coupled surface for all solutions of antibody. The protein in the monolayer is all correctly oriented with the FLAG epitopes exposed. In the amine coupled surface, many of the FLAG epitopes would be occluded because there is a lysine residue within the epitope that could be amine coupled to the CM5 chip thereby occluding the binding site. This highlights the advantage of oriented monolayers over non-selective chemical coupling.

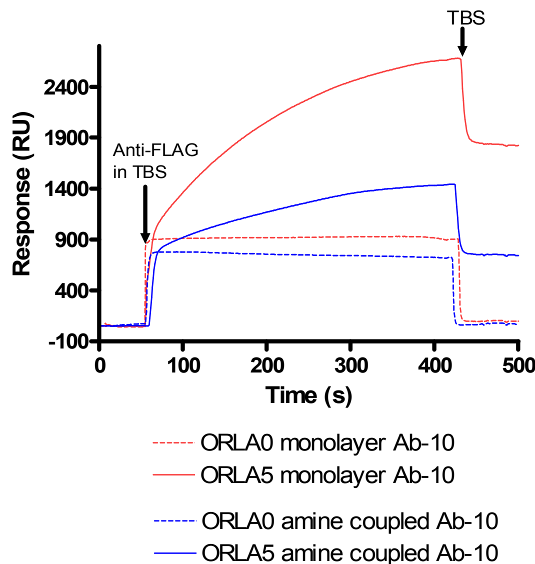


Figure 4: Sensorgram showing injection of 10 μg mL^{-1} of Anti-FLAG antibody in TBS buffer over the monolayer or amine coupled surfaces. The data from the different experiments were normalised and plotted.

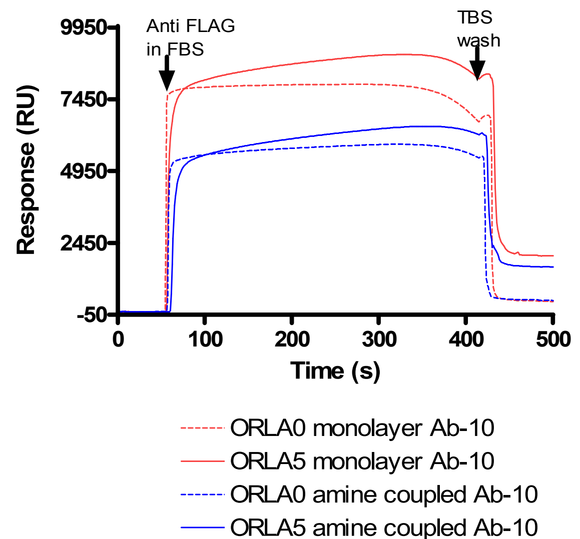


Figure 5. Sensorgram showing injection of 10 μg mL^{-1} of Anti-FLAG antibody in FBS.

The results obtained from fetal bovine serum spiked with antibody suggest that ORLA surfaces could have an application in rapid diagnostics. We are able to detect 1 μg mL^{-1} of antibody in FBS. In the 30 μL that were injected there were 30 ng of the antibody which equates to around 10 pmol of anti-FLAG antibody per injection. On both types of surface, there was a significant amount of non-specific binding from the FBS. However, the monolayer had the better signal to noise ratio.

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 chemically coupled protein on the surface of an analytical device. This exemplifies the applicability of Orla surface technology to diagnostics. The results indicate that Orla technology is applicable for the diagnostic detection of circulating antibodies. The inherent properties of the combined protein-filler monolayer in terms of stability and low non-specific binding are very attractive for use in such applications. The large background from FBS is a peculiar property of SPR. Other detection technologies might eliminate such 'noise', making the Orla technology even more attractive.

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 diagnostic applications.
Current work is focusing on incorporating the CDR regions of antibodies into the Orla scaffold in the scF v (single chain Fv) format. This will enable the generation of capture molecules for a wide range of analytes in many different areas e.g. diagnostics, environment monitoring, in-process analysis etc.

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