

# A manufacturable surface-biology platform for nano applications

## Cell culture, analyte detection, diagnostics sensors

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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 immobilize in a functional way. The ability to control the density, orientation, and functionality of surface-immobilized proteins is critical in their application. Traditional methods of protein immobilization are based upon physical adsorption or complex chemical attachment, both of which may present challenges that may be met by Orla Protein Technologies' surface self-assembly platform (*Table 1*).

Orla technology enables the automatic immobilization, from aqueous solution, of functional proteins on a surface in such a manner that they are correctly oriented in a monolayer, with no additional chemistry required to effect their covalent attachment to the surface<sup>1</sup>. In practice, it is only necessary to introduce the protein solution to a primed surface, and the proteins immobilize and orient themselves. This technology is designed to reproducibly present precisely oriented molecules (e.g., enzymes, receptors, cell-adhesion molecules) as single layers on surfaces by self-assembly.

Applicability of Orla's technology platform ranges from simple protein immobilization to complex biomimetic surfaces. The company has developed approaches to solve the problem of the attachment of proteins to surfaces in an ordered and functional manner, in a scalable and reproducible single-step process in aqueous solution. This technology finds application in protein biochips, cell culture, bioseparation systems, medical devices, and nanoscale diagnostics. Throughout the process, Orla conducts quality control, applying a number of physical analytical techniques to characterize the proteins and surfaces.

The self-assembling protein layer technology was developed jointly by the research groups of Jeremy Lakey at the University of Newcastle upon Tyne (UK) and Professor Horst Vogel of the École Polytechnique Fédérale de Lausanne (Lausanne, Switzerland). The combination of robust bacterial proteins and thiolipid creates a

**Table 1. Comparison of Orla Protein Technology's platform with traditional protein-immobilization technologies**

Existing surface chemistries	Orla platform
Little control over orientation	Protein orientation is controllable
High background	Minimized nonspecific interaction
Problems with reproducibility	Reproducible self-assembly
Reliance on physical adsorption or complex chemistry	Simplified assembly from aqueous solution
Low surface density	High density, controllable
Challenges in scale-up	Designed for scalability
Limited control	Control of surface assembly
Reduced protein functionality	Proteins retain functionality
May lack quality-assurance methods	Surfaces and proteins fully characterized

## METHODS

durable biomimetic layer that can be engineered to create a wide variety of surfaces.

### Core technology

The basic technology involves the fusion of the protein of interest with a proprietary, inherently self-assembling scaffold protein. Orla has constructed vectors to enable a variety of fusions to be generated (N-terminal, C-terminal, central), increasing the flexibility of the technology and providing tools to tackle recalcitrant proteins.

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 noncovalently attached protein, leaving behind a precisely oriented monolayer. The spaces between the proteins in the monolayer are then covered with a “filler” molecule

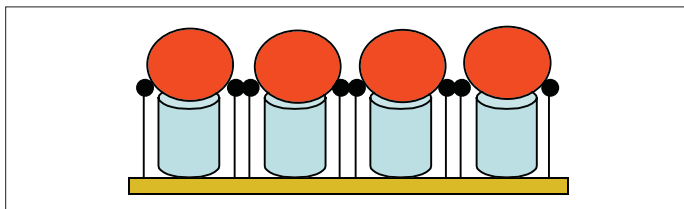


Figure 1. Orla core technology. Scaffold protein is shown in blue, and the fused protein of interest (e.g., cell-adhesion protein, enzyme, or single-chain antibody molecule) is shown in red. Filler molecules shown in black.

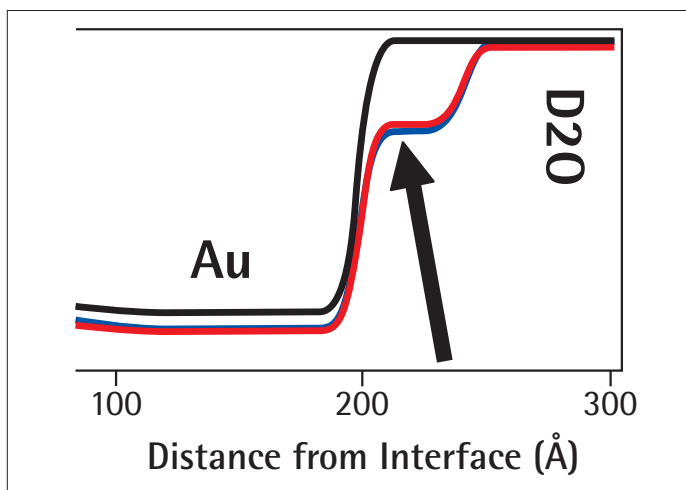


Figure 2. Synchrotron experiment. Scaffold protein was applied in aqueous buffer to a 60 cm<sup>2</sup> gold surface and washed off after 10 min. The distance from the gold surface to the top of the monolayer was measured to be 43 Å (arrow).

that is also covalently attached and oriented (Figure 1). The filler molecule stabilizes the scaffold protein and masks it such that only the protein of interest is exposed at the surface.

This simple principle can be used to produce surfaces with finely controlled properties. Any protein molecule or peptide 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 multifunctional 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 nonspecific binding. The advantages of this approach in a large variety of applications are self-evident.

The nature of the self-assembled surfaces produced using Orla's

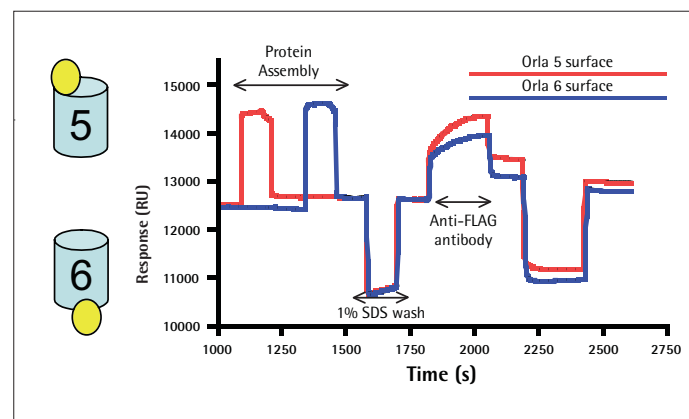
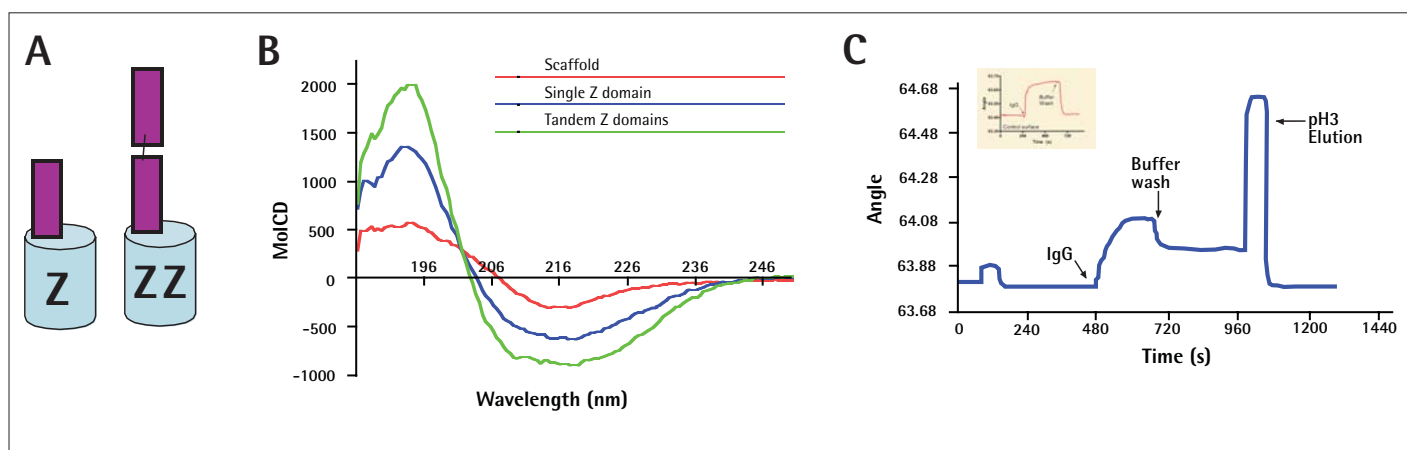


Figure 3. SPR experiment demonstrating the correct orientation of protein in the monolayer. Protein ORLA 5 has a FLAG epitope (yellow circle) fused to the part of the scaffold (blue) predicted to be distal to the substrate. ORLA6 has the same epitope fused to the side of the scaffold that is designed to bind to the substrate. Self-assembled monolayers of the proteins were generated in separate flow cells on a Biacore (Uppsala, Sweden) Au chip. After an SDS wash to remove non-covalently bound material, an anti-FLAG antibody was applied to both channels. Note that the surface was not assembled with filler molecules. The antibody was able to bind only to the ORLA5 surface, whereas the binding on the ORLA6 surface was no greater than that bound to a FLAG-free scaffold control surface (not shown). The anti-FLAG antibody was able to bind to both ORLA5 and ORLA6 in a Western blot experiment (not shown).



**Figure 4.** Immobilization of Staphylococcal protein A immunoglobulin-binding Z domains onto an SPR surface via the Orla scaffold. (A) Constructs with either one (Z) or two (ZZ) immunoglobulin-binding domains (purple) fused to the scaffold (blue). (B) CD spectra of the scaffold and the two fusion proteins. Note that the scaffold has very little alpha-helix signal, whereas the ZZ construct has a strong alpha helix signal as expected. The Z construct has an intermediate signal between that of the scaffold and the ZZ construct. (C) SPR experiment showing IgG binding to a self-assembled monolayer of the ZZ fusion. The monolayer was assembled ex situ on a Spreeta™ gold chip (Texas Instruments, Dallas, Texas) with filler molecules containing a hydrophilic head group. Stable binding of IgG to the ZZ monolayer was achieved. The surface could be regenerated using a low pH wash and used again at least 5 times with no reduction in IgG binding capacity (data not shown). No IgG binding was observed on a control surface made only with scaffold protein (without Z domains) and hydrophilic filler (inset).

technology has been investigated. Crystal structure of the scaffold protein indicated a height of 45 Å. Neutron-scattering experiments gave a height measurement of ~43 Å for a self-assembled monolayer of scaffold protein (Figure 2). This correlates closely with the predicted height for the protein, indicating that the protein is indeed assembled on the surface as a monolayer.

To investigate the orientation of the scaffold after self-assembly, scaffold protein was engineered to contain the FLAG epitope, a commonly used tag that binds to anti-FLAG antibodies with high affinity. Two versions of the FLAG-tagged protein were constructed: one with the FLAG sequence cloned in the region of the scaffold exposed at the external surface of a correctly oriented monolayer, the other with the FLAG sequence cloned in the region of the scaffold that binds to the substratum and thus expected to be inaccessible to antibody if the protein be correctly oriented. Both proteins were used to form self-assembled monolayers, and anti-FLAG antibody binding was investigated using surface plasmon resonance (Figure 3). Results indicate the anti-FLAG antibody is able to bind to the scaffold monolayer only when the FLAG epitope is present on the top of the scaffold protein, whereas it is unable to access a FLAG epitope at the bottom of the scaffold, clearly demonstrating that the molecules in

the monolayer are oriented as predicted.

The utility of Orla technology to produce analytical surfaces was demonstrated by generating fusions of the immunoglobulin-binding Z domains from staphylococcal protein A with the Orla scaffold (Figure 4A). The fusion protein was expressed as insoluble inclusion bodies and refolded after purification. CD spectroscopy was used to confirm that the protein was correctly refolded (Figure 4B). IgG was able to bind to self-assembled monolayers of the fusion containing two protein A Z-domains (Figure 4C). Note that nonspecific binding to the control surface is near zero and that the monolayer is stable to repeated cycles of regeneration.

## Application areas

### Cell culture

The control of cell behavior in cell culture is hampered by the requirement of many cells to attach to extracellular matrix components or feeder cells in order to attach to a surface and grow. The majority of such surface treatments are animal-sourced, and most contain various contaminants in the form of growth factors. There is a need to move away from the current crude surface treatments to finely defined, well-characterized, animal-free surface treatments.

## METHODS

Orla modified surfaces have been tested with several cell types, including 3T3 fibroblasts and PC12 neural cells. For each cell type, Orla modified surfaces have promoted attachment and growth of cells beyond that observed with unmodified surfaces (*Figure 5*). Current work focuses on manufacturing surfaces for cell culture that not only promote attachment and growth but also modify cell behavior and direct the differentiation of certain types of cells and tissues. The ability to create patterns and gradients of protein across surfaces offers plenty of scope in this area.

### Analytical devices

Orla has also combined the surface biology platform with a novel analytical platform for high-throughput proteomics developed by commercial partner, Cambridge Consultants Ltd. (Cambridge, UK, and Boston, Massachusetts). The technology offers substantial potential improvements over current systems: A conventional SPR instrument involves the reflection of light at a thin gold film to which ligand molecules are attached. Binding of molecules (e.g., analytes), in an applied solution, to ligands (e.g., Orla proteins) attached to the surface of the film, gives rise to changes in refractive index and, thus, the intensity of the reflected light. Unlike conventional intensity-based SPR instruments, the basis of the new system is the interferometric

measurement of phase, rather than intensity changes in the reflected light caused by the SPR effect. The spatial variation of this phase change is measured using a new design of interferometer that is inherently stable and can be of monolithic construction, resulting in very low-phase noise ( $\sim 10^{-6}$  RIU at 1.5Hz), and reductions in temperature-, strain-, and vibration-sensitivity, thus enabling the high throughput of sample arrays. This phase-based technique is better suited to the measurement of multiple small binding sites than are current intensity-based methods. The technique enables the highly parallel measurement of multiple small binding sites using high-density arrays, speeding up analysis by a significant factor. The patented design is expected to enable high-density arrays with greater than  $10^3$  parallel measurements, label-free detection, real-time capability for kinetics, high sensitivity, high stability, and a compatibility with flow-through operation.

Traditionally, surface plasmon resonance has proven a technique difficult to multiplex. The current technique allows imaging of binding events on a 3.5 mm x 6 mm area on a 30 nm-thick gold surface. The ability to multiplex increases the usefulness of SPR in clinical laboratory medicine by allowing spatial and temporal imaging of arrays. As opposed to repeated runs on a conventional system, a potential clinical application is to flow a sample solution laden with antibodies past an array of engineered proteins. By image analysis of the binding events

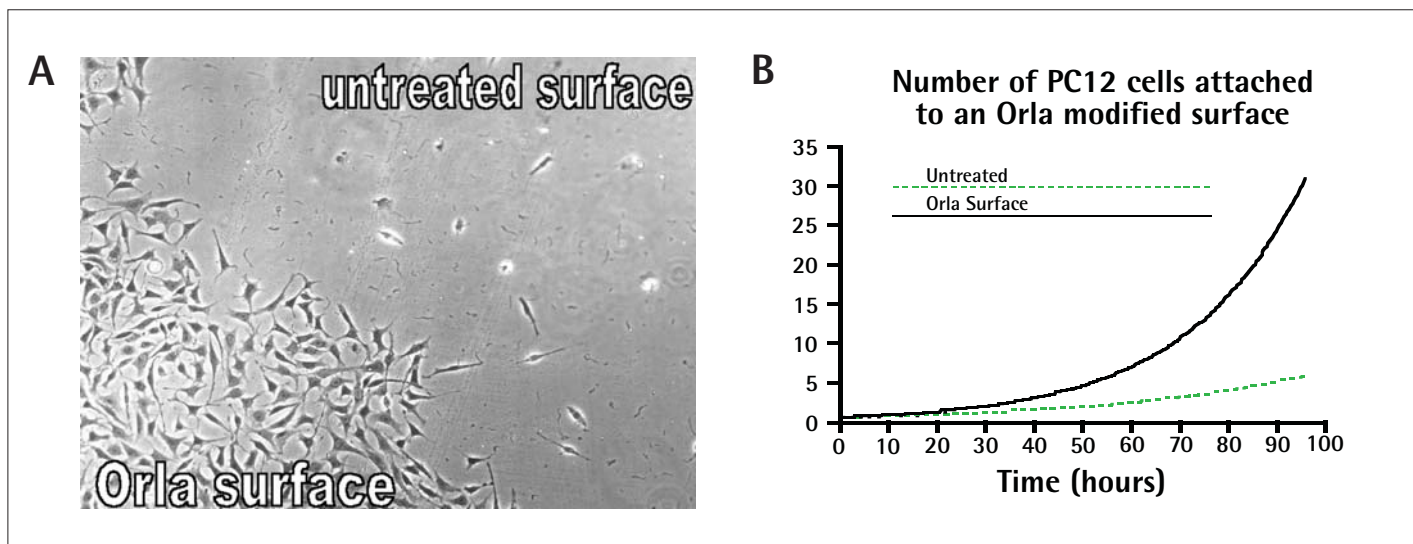


Figure 5. Surfaces for cell culture. (A) Fibroblasts attached to a spot of self-assembled monolayer presenting a cell-adhesion motif. Cells adhere and grow preferentially in the modified spot and avoid the untreated area around the spot. (B) The neuronal PC12 cell line also showed preferential binding to a surface with a self-assembled monolayer of a combination of protein constructs containing three different cell-adhesion motifs, compared to an untreated surface.

over the array, the detection of a particular target antibody is possible.

The combination of Orla technology and the novel SPR technology was tested on a prototype instrument. Self-assembled monolayers of a scaffold containing the influenza virus HA epitope were prepared on two wells of the gold chip. Both wells were treated with an anti-HA antibody (Figure 6A). In the second experiment, one well was treated with anti-HA antibody and the other with a nonspecific antibody (Figure 6A). Binding was only observed with the specific anti-HA antibody. The result is the ability to discern quantitatively that the protein contains the antibody binding site.

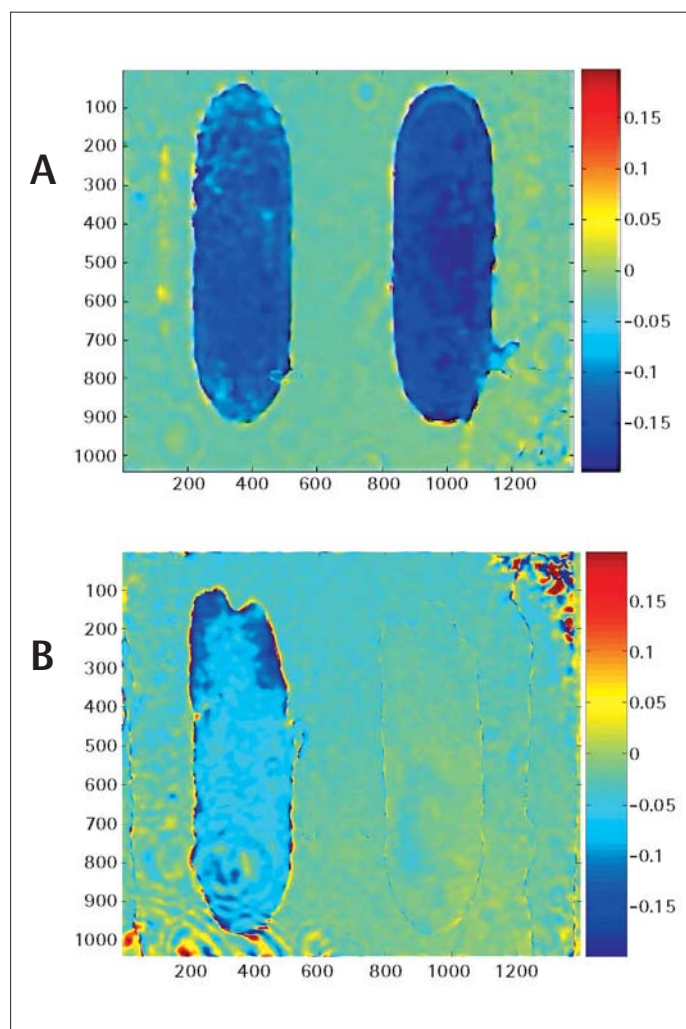
The combination of Orla surface assembly technology and CCL SPR technology offers the potential to create high-throughput diagnostic tools applicable in a wide range of areas. Orla is currently commercializing the technology in the area of autoimmune diagnostic biochips. Such biochips at the point of healthcare intervention can provide a tool to enable physicians to diagnose and provide appropriate therapy at an early stage and for monitoring patients' responses to therapy.

## Conclusion

Beyond these presented example applications of Orla's protein immobilization technology, potential applications for the technology are wide-ranging. In cell culture applications, Orla currently has a variety of surfaces in testing. The technology shows promise for the design of highly specialized surfaces for directing the growth, development, and behavior of cells, especially in the area of stem cell growth and differentiation. In addition, Orla is developing arrayed platforms for specific uses in the diagnostics, biomedical, biodefense, and food- and water-safety markets. The utility of Orla technology has been demonstrated in generating surfaces for rapid, accurate detection of microbial pathogens, and there is further potential in the development of surfaces for detection of viruses and bacterial toxins. Inherent flexibility of the technology allows it to be applied to surfaces for several different modes of detection and incorporation into medical devices and implants.

## REFERENCE

1. Terrettaz S, Ulrich W-P, Vogel H, Hong Q, Dover LG, and Lakey JH. Stable self-assembly of a protein engineering scaffold on gold surfaces. *Protein Sci* 11(8), 1917-1925 (2002).



**Figure 6.** SPR using Orla surfaces on the CCL instrument. The image area contains two oval wells and a control region in the remaining area, achieved by a PDMS mask. Binding events appear blue after image analysis; dissociation events appear red. Orla scaffold containing the HA epitope was assembled in both wells. (A) Anti-HA antibody applied to both wells. (B) Anti-HA antibody applied to left-hand well and a nonspecific antibody applied to the right-hand well. Note that both antibodies were at a lower concentration than that in (A).