

Self-assembling layers created by membrane proteins on gold

D.S. Shah*, M.B. Thomas†, S. Phillips*, D.A. Cisneros‡, A.P. Le Brun†, S.A. Holt§ and J.H. Lakey†¹

*Orla Protein Technologies Ltd, Nanotechnology Centre, Newcastle upon Tyne NE1 7RU, U.K., †Institute for Cell and Molecular Biosciences, University of Newcastle upon Tyne, Framlington Place, Newcastle upon Tyne NE2 4HH, U.K., ‡Biotechnology Center, Dresden University of Technology, Tatzberg 49, 01307 Dresden, Germany, and §ISIS, Rutherford Appleton Laboratory, Chilton, Didcot OX11 0QX, U.K.

Abstract

Membrane systems are based on several types of organization. First, amphiphilic lipids are able to create monolayer and bilayer structures which may be flat, vesicular or micellar. Into these structures membrane proteins can be inserted which use the membrane to provide signals for lateral and orientational organization. Furthermore, the proteins are the product of highly specific self-assembly otherwise known as folding, which mostly places individual atoms at precise places in three dimensions. These structures all have dimensions in the nanoscale, except for the size of membrane planes which may extend for millimetres in large liposomes or centimetres on planar surfaces such as monolayers at the air/water interface. Membrane systems can be assembled on to surfaces to create supported bilayers and these have uses in biosensors and in electrical measurements using modified ion channels. The supported systems also allow for measurements using spectroscopy, surface plasmon resonance and atomic force microscopy. By combining the roles of lipids and proteins, highly ordered and specific structures can be self-assembled in aqueous solution at the nanoscale.

Introduction

Biochemistry is by its very nature a nanoscale science. The major players proteins, nucleic acids and membranes all operate on length scales of nanometres and above. Thus bionanotechnology is in fact a meeting between biomolecules and new technologies that have enabled us to manufacture and measure at the nanoscale. The benefits of nanotechnology, other than benefits of scale, result largely from the novel behaviour of materials at these dimensions.

To benefit from the combination of biochemistry and nanotechnology there is a need to create a link that is either physical or virtual. By virtual we mean in this context an ability to communicate between the biological and physical elements without a tangible link. In this respect the addition of a latex bead to a biomolecule for manipulation by optical tweezers is a physical link but the laser trapping of the bead is a virtual link. In most cases where we wish to exploit biomolecules we will require some form of physical link, e.g. labelling of quantum dots, protein arrays, cell culture scaffolds. In this respect the more precise the nanoscale application, the more precise the linkage should be and the exact placing of a defined number of molecules is to be expected to be a normal requirement.

The electronics revolution, which has been driven by microtechnology since the 1950s, kept its momentum by an ever decreasing size of the processors that could be manu-

factured. Thus Moore's law was obeyed by reducing the size of the smallest features that could be manufactured [1]. Manufacture was by externally applied guidance, such as photolithography, applied to large substrates and was termed 'top-down'. Beyond the feature-size limits imposed by the minimum wavelength of light we can now use electron-beam methods to go to angstrom-sized structures but these are increasingly expensive approaches. Bottom-up methods, where we move from small molecules to increasingly larger and more complicated structures, are required if we wish to reduce costs, increase productivity and ensure robustness. The heart of the bottom-up revolution is the concept of self-assembly, which implies that the individual small-molecule components possess the ability to create a structure of greatly reduced entropy with levels of organization much above the individual molecule level [2].

Self-assembling systems

Amphipathic molecules, those that have both water soluble and non-polar regions, are the stars of the self-assembly world, most of us has carried out nanotechnology within our very early childhood years by playing with foam in the bath or by blowing bubbles using detergent solutions. Here the free energy change that accompanies the hiding of polar headgroups and water away from air in the soap film is enough to stabilize structures of great complexity [2]. In aqueous solution, several groups have shown how this simple structural device can create liposome-like structures whose architecture can be fine-tuned by modifications such as charge and covalent bonds [3]. On surfaces, the technology

Key words: atomic force microscopy (AFM), Fourier-transform infrared (FTIR), impedance spectroscopy, neutron reflection, outer membrane protein F (OmpF), thiolipid.

Abbreviations used: AFM, atomic force microscopy; FTIR, Fourier-transform infrared; OmpF, outer membrane protein F; SLD, scattering length densities.

¹To whom correspondence should be addressed (email j.h.lakey@ncl.ac.uk).

of self-assembling monolayers originally [4] uses covalent attachment to surfaces and hydrophobic self-assembly to make highly ordered interfaces from randomly dissolved single molecules. This has been coupled with a top-down approach called microcontact printing to create patterned self-assembling structures [5].

Higher orders of self-assembly are possible by introducing the self-organizational properties of DNA, the usefulness of which relies on the specific recognition and binding of complementary sequences. The four-base code means that the number of unique combinations of binding partner for a sequence n bases long is n^4 , although with sequences longer than 20 residues the ability to discriminate a single base change becomes more problematic especially at low temperatures. This approach has been used widely to easily create complex complementary nanostructures based on synthesized oligonucleotides [6].

Proteins as self-assembling structures in nanotechnology

Proteins have three strong contributions to make to bionanotechnology: they are capable of the most complex self-assembly known, they can precisely recognize and respond to a huge range of targets and, finally, they have a diverse range of intrinsic catalytic activities.

The self-assembly of proteins provides a palette of nanoscale molecules in large amounts, with good quality control and extremely high complexity. It is arguable whether the self-assembly process starts with transcription/translation but it is clear that by possessing the right untranslated regions the nucleic acids direct their own part of the assembly process. The self-assembly continues after translation with protein folding, a true and, probably, the most complex self-assembly step known [7]. First demonstrated in the laboratory by Anfinsen et al. [8], the self-assembly involved in protein folding requires only the information contained within the protein sequence. In the cell, assistant protein molecules called chaperonins guide proteins through the various stages of protein folding helping them avoid such fates as misfolding or aggregation. This is achieved by protecting hydrophobic areas of the cell until folding hides them or by increasing the rate of protein folding by catalysing the isomerization of prolyl bonds or the formation of disulfide links between cysteine residues. These are usually found in some of the proteins destined to leave the cell and be secreted into the surrounding medium. Anfinsen et al. [8] and many others since have shown that the process of protein folding can also be achieved *in vitro*. Normally this consists of holding the protein in a denatured state by addition of high concentrations of urea or guanidine chloride before dilution (~10-fold for most proteins) allows the folding to begin. After this step a folded protein, usually monomeric, is achieved in which the individual atoms and amino acid residues occupy unique positions. Admittedly some regions of proteins are flexible and some entire proteins even display all the properties of being unfolded, but this flexibility is

often closely linked to function and often these regions bind specific target molecules [9,10].

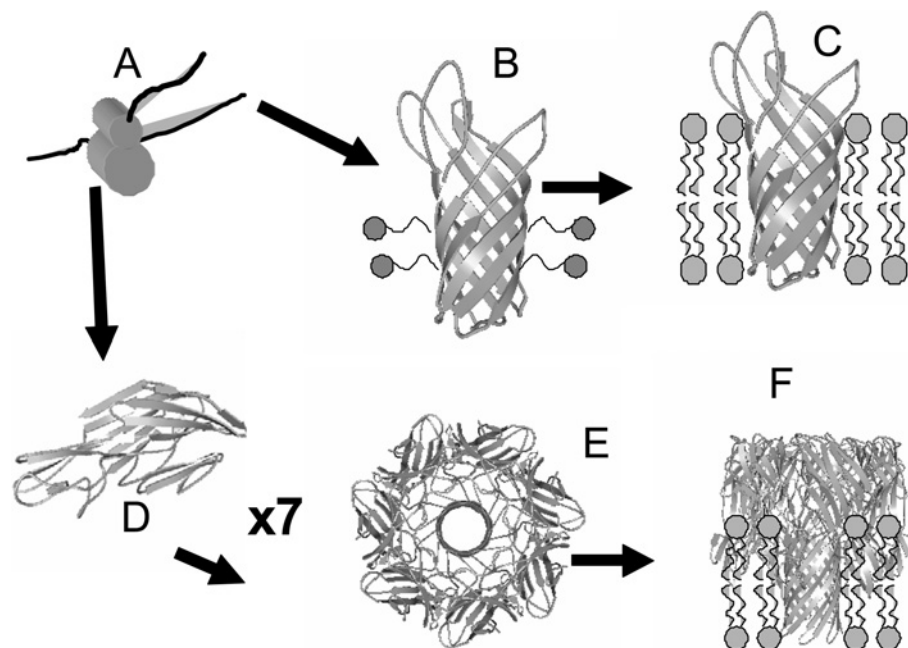
Thus the proteins provide a way to create reproducible nanoscale structures of high complexity and with recombinant methods of gene expression these can be made in large amounts. However, in general, it is not simple to create your own protein machine from scratch due to the 'protein folding problem'. This is due to the fact that we do not, apart from a small number of exceptions, know the relationship between amino acid sequence and complex tertiary structure. Thus we can exploit simple rules to make small domain proteins [11] but in general the larger structures and the enzymes we use are those that have evolved naturally. These proteins discovered in the natural world possess the properties that we seek and by transferring the genes to a suitable host we can start to use them for nanotechnology [12–15]. Here the subject is membrane proteins and this is interesting to nanotechnology because we have yet one more level of self-assembly available to us if we use this group of molecules. As shown in Figure 1 we can add two-dimensional oriented assembly to the list if we use membrane proteins. This in fact exploits the properties of amphiphiles to create the large self-assembled and ordered structures discussed above but this time to control and order whole protein molecules. Another advantage of using a membrane system is that the proteins reside in an electrically insulating layer which can be used to separate two aqueous compartments [16–18]. The unique relationship between the protein and the lipid layer means that it is always precisely positioned and in some cases the protein can be 100% oriented as well.

Self-assembly of membrane systems on surfaces

Membrane systems can be assembled on to surfaces in a variety of ways to allow the ordering effects of the amphiphile systems to be applied to solid devices [19]. The simplest method is to add liposomes to surfaces such as silicon oxide where they form bilayers. The surface layer of water on the oxide layer ensures that the bilayer is stable. Any proteins reconstituted into the vesicles can thus be arranged in a lipidic medium. A larger water reservoir is made possible by tethered lipid bilayers in which a lipid is attached to the surface via a hydrophilic linker such as poly(ethylene glycol). These lipids do not fill the lower leaflet of the bilayer but as their name implies tethers a freely fluid bilayer to the surface [20]. We used this method to make an ion-channel-based biosensor which included the *Escherichia coli* OmpF (outer membrane protein F) porin [21]. This was used to measure the binding of the colicin toxin by surface plasmon resonance and impedance spectroscopy, which measured the increase in bilayer resistance when the ion channel in the OmpF protein was blocked by the toxin. Subsequently we investigated whether it is possible to adhere the OmpF protein directly to the gold surface via the thiol group on a cysteine residue in a periplasmic turn [14]. This enabled the protein to bind at densities close to half that of a two-dimensional crystal and

Figure 1 | Routes to self-assembly of membrane protein systems

After translation on the ribosome (A), we can obtain two main groups of membrane proteins for use in nanotechnology. (B) Integral membrane proteins (here OmpA from the outer membrane of *E. coli* bacteria [27]) can be purified from the host membrane or refolded from insoluble inclusion bodies [28,29]; in either case the purified form is stabilized by detergent micelles as shown here. The protein can then be reconstituted into lipid bilayers (C) by removal of the detergent in the presence of free lipid. Membrane-active toxins are secreted mainly by bacterial hosts into the surrounding medium as water-soluble precursors, which later insert into the membrane without detergent. In this case the monomer protein (LukF; PDB: 1LKF) [30] (D) heptamerizes (E) to form a transmembrane ion channel (F) (α -haemolysin; PDB: 7AHL) [31] which has been engineered in many ways to show the usefulness of protein-based nanopores.



the rest of the surface to be completed with a monolayer of thiolipid lacking a long tether, followed by free phospholipid which adhered to the hydrophobic surface. The protein retained activity by binding the colicin toxin and grazing incidence FTIR (Fourier-transform infrared) measurements confirmed the retention of β -structure.

This approach has been extended to the monomeric porin OmpA, which due to its β -structure is a very useful protein engineering scaffold. The core protein transmembrane region is easily refolded from bacterial inclusion bodies and the surface loops can be replaced by anything from short peptides to larger protein domains. It can be assembled as the OmpF was previously (Figure 2) [14] by use of an inserted cysteine mutation. Insertion of peptide sequences that code for cell attachment shows that layers can be made for cell culture applications (Figure 2), while inserted epitopes can make surfaces useful for antibody detection. Larger domains assembled in this way can functionalize surfaces for enzyme assays.

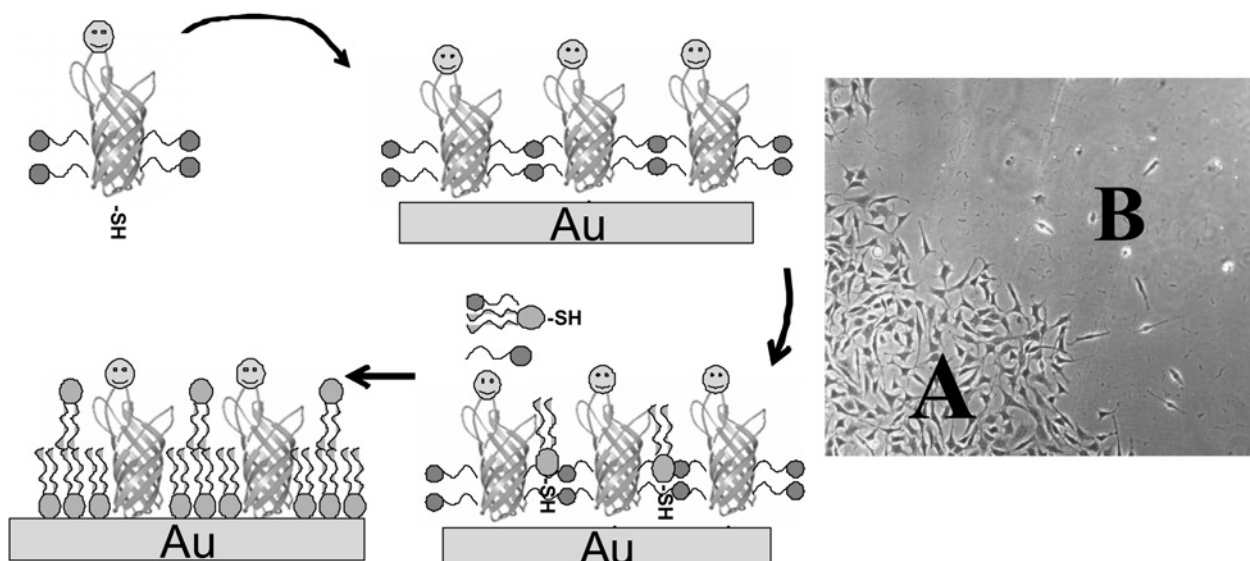
The structure of the immobilized porin on gold was recently confirmed as native by the use of AFM (atomic force microscopy) [22]. Two clear results were achieved; for the first time high resolution was achieved on proteins not packed in a two-dimensional crystal and this was made

possible by the compensatory rigid packed thiolipid without which it was not possible to image the proteins at high resolution. Providing proteins can be fixed to the gold this appears to be a useful method for imaging membrane proteins. It is very interesting that it could be achieved with only the lower thiolipid monolayer present, leaving a bare hydrophobic surface [23]. The height of the monolayer is sufficient because outer membrane proteins are shorter than cytoplasmic versions and need less hydrophobic thickness for their stability. The hydrophobic surface may attract mobile material, such as residual detergents, that we do not see by AFM but in principle there appears to be no reason why it should not allow the high quality of imaging that we observe. The water/methylene group interface may simply consist of ordered water as in the classical clathrate models of water structure at such surfaces or in view of recent neutron reflection data it may simply consist of decreased water density [24]. The protein appears not to be affected since it resembles closely the structure of OmpF from two-dimensional crystals and X-ray crystallography.

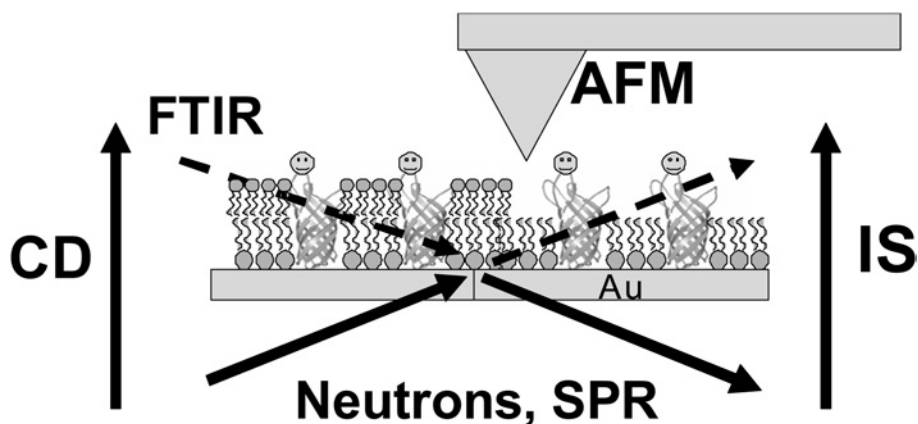
The immobilization of the proteins on to gold enables them to be stable enough for study by a range of biophysical techniques (Figure 3). In addition to AFM, FTIR and impedance spectroscopy mentioned above, we have recently

Figure 2 | Stages in the assembly of an OmpA self-assembling layer

The OmpA gene is engineered to include the new protein sequence, expressed as bacterial inclusion bodies then refolded in a detergent mixture, and it contains a cysteine mutation which provides the thiol or -SH group which anchors the protein to the gold. After assembly of the protein from detergent solution, thiolipid in detergent is added, which forms a dense layer wherever gold is exposed between the proteins. Finally, a top layer of free lipid is added to complete the surface. In the picture, fibroblast cells are shown to adhere preferentially to an area (A) containing an OmpA protein which has an inserted RDGS sequence compared with the area of lipid filler (B).

**Figure 3 | A summary of the methods used to investigate the structure of immobilized protein layers on gold**

IS (impedance spectroscopy) [14,32,33], CD [25,34], ATR (attenuated total internal reflectance)-FTIR spectroscopy [35] might be used from the lower side (as opposed to grazing incidence FTIR which must be used from the top and needs a dry sample) but a very thin gold layer is needed. Other methods include AFM [22] and SPR (surface plasmon resonance) [14]. Neutron reflectivity is achieved by guiding the neutron beam through the silicon substrate [26,36].



employed CD [25]. The advantage of this method is that a full liquid layer is retained, whereas the grazing incidence FTIR is carried out on dry samples which may be denaturing. However, the method is at the limits of sensitivity of current machines and our published results use an α -helical protein [25]. Nevertheless, we were able to see an unfolding and folding cycle in the presence of the urea denaturant. We have

also used neutron reflection to measure the distribution of protein, lipid and water in these layers in the z-axis [26]. This method uses the different neutron 'refractive indices' called SLD (scattering length densities) of the components to build up a layer-by-layer model of the substrate (silicon), gold, protein, lipid and water. By exchanging the water phase for a range of water and $^2\text{H}_2\text{O}$ mixtures, the contrast with the SLD

of the sample can be tuned to enhance the differentiation of the layers. Recently we have employed the two polarization states of neutrons to enable two independent sets to be obtained from one sample; this will significantly increase the ability to effectively model multilayer systems.

In summary, membrane protein monolayers on gold provide easily assembled, precisely arranged structures that can be used for both biotechnology and fundamental research.

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