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Anodized alumina
High throughput screening
Cell culture
Microbiology
Label-free imaging

Porous aluminum oxide (PAO) is a ceramic formed by an anodization process of pure aluminum that enables the controllable assembly of exceptionally dense and regular nanopores in a planar membrane. As a consequence, PAO has a high porosity, nanopores with high aspect ratio, biocompatibility and the potential for high sensitivity imaging and diverse surface modifications. These properties have made this unusual material attractive to a disparate set of applications. This review examines how the structure and properties of PAO connect with its present and potential uses within research and biotechnology. The role of PAO is covered in areas including microbiology, mammalian cell culture, sensitive detection methods, microarrays and other molecular assays, and in creating new nanostructures with further uses within biology.

1. Introduction
Nanoporous membranes can have unusual or extreme properties, which have attracted intense interest from nanotechnologists and material scientists, and which have also resulted in applications in quite diverse sectors of biotechnology. Such materials can have an exceptionally high surface area — at least 2 to 3 orders of magnitude greater than planar surfaces. A high surface area has major advantages in chemical catalysis, bioreactors and displaying arrays of detector molecules. Nanopores have other attractive properties: narrow pores with high aspect ratios (pore diameter vs pore length) (Bolton et al., 2011) can act as selective portals in a way that mimics the way pores in cells sense or take up specific compounds and can be effective supports for chemical purifications and separations. Also, highly ordered nanoscale surfaces can greatly enhance some optical and physical detection methods (e.g. optical wave guides, surfaced enhanced Raman spectroscopy). Finally, those nanoporous materials
that do exist can be used as templates to create other nanoscale materials with further useful properties.

The variety and ingenuity of fabricated nanoporous membranes is growing. These materials include fibrous meshes such as nanocellulose and other organic polymer filters, some of which are produced by processes taken from the textile industry such as electrospinning (Poineau et al., 2011). Self-ordering block copolymers have also been used to fabricate nanoporous membranes (Bolton et al., 2011). Other attractive types include nanoporous materials of silica/glass and a range of metal foams and metal oxides. The latter group includes oxides capable of self-assembling into nanoporous structures under anodizing conditions and can be formed from metals such as titanium, tin and aluminum oxides. The nanoporous forms of aluminum oxide have long attracted the attention of materials scientists and electrochemists. Porous aluminum oxide (PAO; or anodic aluminum oxide (AAO) or porous anodic alumina (PAA)) is now finding many uses at the intersection between nanotechnology and biotechnology. In part, the widespread application of PAO in biotechnology is related to the controllability of fabrication. Remarkably well-ordered, consistent and regular arrays of pores with a high aspect ratio can be formed by self-assembly when an appropriate voltage is applied to aluminum. The resulting PAO has high aspect ratios, with deep and narrow pores, that are often beautifully ordered, i.e. features that polymeric membranes or other manufacturing processes struggle to match. Moreover, aluminum oxide is inert and biocompatible. Aluminum is one of the lowest valency metals not commonly found in life, the oxide is highly insoluble and, unlike many other aluminum salts, non-toxic which results in excellent biocompatibility. PAO has a low background with respect to many detection techniques, including fluorescence microscopy. Additionally, aluminum oxide does not change volume greatly with temperature changes or wetting, is compatible with a wide range of solvents and is exceptionally thermostable (some forms withstand temperatures in excess of 1000 °C). The manufacture of nanoporous metal oxides is not recent, and the availability of commercial forms over the last few decades may have contributed to the wide range of ingenious applications. To date over 500 patents have been filed on the manufacture or use of anodic alumina (source – European Patent Office, searches for anodic alumina and anodic aluminum oxide).

2. Origins and fabrication of PAO

An industrial process for the production of pure aluminum has only existed for around 150 years (Edwards et al. 1930). Fifty years before that time the metallic element was known (and indeed sometimes prized above gold) but was not widely accessible. Aluminum is an interesting metal for its lightness, abundance and anticorrosion properties. Resistance to corrosion is primarily due to passivation, the formation of an inert and protective oxide layer on the surface. This native oxide layer can be made more substantial by anodization, an electrochemical oxidative process, which creates resistant non-porous surfaces when performed using neutral electrolytes (Keller et al., 1953). Both the lightness and corrosion resistance were drivers for the introduction of this material to protect components in seaplane manufacture (Lee, 2010). Over the past century PAO has been introduced by premolding the aluminum metal (Hans Deville (1856)), Henri Deville develops the first industrial method for aluminum production (Keller et al., 1995). The limited number of known self-ordering conditions puts restrictions on the available membrane formats. More flexibility is introduced by preforming the aluminum film with an imprint stamp (Masuda et al., 1997). During subsequent anodization, the imprint pattern is reproduced in the oxide, resulting in exceptionally ordered arrays (Fig. 1). However, the maximum aspect ratio of well-ordered pores that can be obtained after the imprinting of deviating patterns remains limited (Asoh et al., 2001).

In recent years it has been shown that self-ordering regimes also can be created at high anodization voltages (Lee et al., 2006). Anodization outside the operation window of mild anodization can result in the PAO, as acid anions are incorporated into the oxide during anodization (Thompson and Wood, 1981). This can be expected to have significant effects on the mechanical properties of the PAO. It appears unlikely that anions that contaminate PAO will be available as nutrients for cell growth. However, there will also be effects on the charge and chemical reactivity of PAO that may be relevant to cell attachment and functional modification of the alumina. Other parameters that are important during anodization are temperature (Aerts et al., 2010; O’Sullivan and Wood, 1970) and bath agitation.

A major advance, originating from the aluminum manufacturer Alcan, was to progressively reduce the anodizing voltage during manufacture (Furneaux et al., 1989). This results in detachment of the oxide film from the underlying aluminum, yielding a porous membrane. During detachment, the barrier layer in contact with the aluminum plate thins, so that for a few microns of depth individual pores are narrowed and subdivided (Figs. 1 and 2). This results in the most widely available commercial form of PAO, which has clear asymmetry between the two sides. Properties of commercially available formats are displayed in Table 1. These formats are mainly sold for the purposes of filtration (e.g. Whatman/General Electric Anotop and Anodisk filters, Table 2) and mammalian tissue culture (e.g. Nunc Cell Culture multowell plates).

Under specific anodization conditions, prolonged oxidation results in self-organization of the pores, thereby creating highly ordered structures (Masuda and Fukuda, 1995). This has led to the development of a two-step oxidation process, where a sacrificial oxidation layer is formed until the oxidation reaches a steady state. Removal of the oxide layer leaves the underlying aluminum imprinted with the periodic structure, from which a well-ordered porous structure is directly formed during the subsequent oxidation step (Masuda and Satoh, 1996). PAO formed under these self-ordering conditions has a porosity of about 10% (Nielsch et al., 2002) and has defect-free areas up to several microns in size. The requirement for a constant voltage does not allow lift-off using progressive voltage reduction. Therefore, well-ordered PAO is usually manufactured from thin aluminum films. After anodization, the substrate is etched, e.g. with an aqueous CuCl₂–HCl solution. Alternatively, etching can be performed with saturated HgCl₂ followed by 5% phosphoric acid. These processes remove the remainder of the aluminum and open the pores to produce a symmetrical membrane.

The limited number of known self-ordering conditions puts restrictions on the available membrane formats. More flexibility is introduced by preforming the aluminum film with an imprint stamp (Masuda et al., 1997). During subsequent anodization, the imprinted pattern is reproduced in the oxide, resulting in exceptionally ordered arrays (Fig. 1). However, the maximum aspect ratio of well-ordered pores that can be obtained after the imprinting of deviating patterns remains limited (Asoh et al., 2001).

Table 1

Table 1: Key developments in PAO manufacturing.

<table>
<thead>
<tr>
<th>Year</th>
<th>Advance</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>1827</td>
<td>Frederic Wohler isolated pure aluminum metal (Hans Ørsted partially credited 1925)</td>
<td>Edwards et al. (1930)</td>
</tr>
<tr>
<td>1856</td>
<td>Henri Deville develops the first industrial method for aluminum production</td>
<td>Deville (1859)</td>
</tr>
<tr>
<td>1923</td>
<td>Anodization of aluminum for the protection of seaplane components from corrosion.</td>
<td>Lee (2010)</td>
</tr>
<tr>
<td>1953</td>
<td>Effect of acid electrolyte and voltage on controlling pore size described</td>
<td>Keller et al. (1953)</td>
</tr>
<tr>
<td>1989</td>
<td>Publication of controlled manufacturing process resulting in porous membrane</td>
<td>Furneaux et al. (1989)</td>
</tr>
<tr>
<td>1999</td>
<td>Improved versions (pore regularity, new processes)</td>
<td>Lee (2010)</td>
</tr>
<tr>
<td>2011</td>
<td>driven by nanotechnology and semiconductor industries</td>
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breakdown of the oxide film (Ono et al., 2004), but when an oxide layer of >400 nm was preformed, anodization at higher voltages proved to be stable. This hard anodization process yielded well-ordered structures in a relatively wide operation regime. The elevated voltage also results in an increased anodization rate (50 μm/h compared to 2–6 μm/h for mild anodization). On the other hand, the porosity obtained was reduced to only 3.3%.

The availability of different anodization regimes has led to the fabrication of more advanced structures (Lee, 2010). By alternating mild and hard anodization, an oxide with an alternating pore diameter could be obtained (Lee, 2010). The formation of serrated pores was also demonstrated in another advanced fabrication method (Li et al., 2010). Gradients of different diameter nanopores over distances up to a centimeter were obtained using asymmetric voltages (Kant et al., 2010). By combining anodic oxidation with selective aluminum etching, very thin PAO membranes that were supported by unetched aluminum were obtained, these membranes showed enhanced flow rates during filtration (Thormann et al., 2007). Finally, composites between PAO and other materials and surface chemistry modifications that complement the properties of the base material are also emerging (e.g. Choi et al., 2010; Lee et al., 2002; Pera et al., 2010).

Whereas much empirical knowledge exists on the formation of PAO, the mechanism of pore formation is still not fully understood. Pore formation has been explained by the combination of electrochemical oxidation with field-assisted oxide dissolution (Hoar and Mott, 1959; O’Sullivan and Wood, 1970), as well as the direct injection of Al3+ ions into the solution (Siejka and Ortega, 1977). To describe the ordering phenomena, models that are based on mechanical stress formation (Jessensky et al., 1998; Li et al., 1998) and, more recently, on equal field strength were introduced (Su and Zhou, 2008). Alternatively, pore formation has also been related to oxygen evolution (and atmospheric pressure) during anodization, something not adequately explained by other theories of PAO formation (Yang et al. 2009; Zhu et al., 2008).

A drawback of PAO is that it is relatively brittle: it is a strong material relative to its mass but thin sections with high porosity can be vulnerable to breakage. Care must be taken to support or reinforce large sheets. PAO can be cut using a laser or precise high-pressure water jet and sterilized by plasma, dry heat, irradiation or solvents. A side effect of PAO manufacture, sometimes seen on commercial PAO, is a phenomenon called “hot spots” which are intense points of fluorescence on an otherwise low background material. In other contexts, such as gold or silver coated PAO, deliberately created hot spots may be a useful image enhancement technique (Qiu et al., 2010).

The intention of the remainder of this review is to examine the role of PAO membranes within biotechnology (Fig. 3 gives key examples),

<table>
<thead>
<tr>
<th>Property</th>
<th>Range</th>
</tr>
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<tbody>
<tr>
<td>Pore size</td>
<td>20–200 nm</td>
</tr>
<tr>
<td>Thickness</td>
<td>50–100 μm</td>
</tr>
<tr>
<td>Porosity</td>
<td>25–50%</td>
</tr>
<tr>
<td>Pore density</td>
<td>102–1015 cm−2</td>
</tr>
<tr>
<td>Size</td>
<td>13, 25 or 45 mm diameter circles</td>
</tr>
</tbody>
</table>
pays particular attention to the exploitation of this material for cell culture and imaging over the last two decades.

3. PAO in microbiology

3.1. Counting and cell identification

Counting microorganisms, stained for greater contrast, on the surface of a microporous membrane by microscopy is a common activity. Relatively rapidly after the widespread availability of commercial asymmetric PAO membranes, these were adapted for bacteriological counting (Jones et al., 1989; Williamson and Palframan, 1989) using acridine orange or ethidium bromide as fluorogenic dyes with counting by fluorescence microscopy. Improved sensitivity over flexible polymer membranes was reported, attributed to a low auto-fluorescence background of the alumina and the ease of focus on the microorganisms filtered onto the rigid, flat upper surface of the PAO. Subsequent work on plankton supported these conclusions and suggested applications in presenting microbial samples for scanning electron microscopy (McKenzie et al., 1992). Additionally, fluorescence in situ hybridization has been performed successfully on PAO (Ramsing et al., 1996). The low background properties were also used to capture microorganisms for fluorogenic Gram-staining from cerebrospinal fluid (Durtschi et al., 2005a) with >10-fold sensitivity compared to polycarbonate membranes. This technique increased the contrast obtained by fluorogenic staining on PAO by blackening the alumina with nickel nanoparticles (Durtschi et al., 2005b). Coating the PAO surface with patterned silver nanocap arrays is also known to enhance fluorescence microscopy of cells (Qiu et al., 2010). This is believed to work by transferring plasmon resonance energy from the silver nanocaps to the nearby fluorescent molecules.

Non-fluorescence sensing methods (covered more fully in Section 6) have also been used for the quantification of microorganisms. Infrared spectroscopy has been used to quantify bacterial spores, taking advantage of the good IR imaging properties of gold-coated PAO and also the even distribution of spores filtered onto the surface (Schiza et al., 2005).

In the above assays the pore size of all forms of PAO is adequate to act as a sterile filter and therefore capture all cells for counting. The precision to which the pore size can be fine-tuned has also been used for selective capture of bacteriophage phi29. By engineering a precise pore diameter of 40 nm Moon et al. (2009) managed to capture intact viral particles and array them within the pores of the PAO, while viral capsids lacking DNA could be passaged through the alumina membrane. This technique has applications in analysis of viral assembly, screening for variants in assembly and the possibility of arraying phi29 packaging nanomotors on PAO as part of a hybrid bio-nano structured device.

3.2. Growth and microcolony imaging of microorganisms on PAO

The methods described in the previous section are all performed on microorganisms captured without any attempt to culture them on the PAO surface. Subsequently, microcolony growth and imaging on PAO has been developed. This effort has been primarily directed at rapid antibiotic sensitivity testing of bacterial pathogens and in studies of microbial population heterogeneity, particularly in response to the imposition and recovery from stress.

To date PAO has been shown to culture any organism tested that can be grown in a Petri dish on agar. Tests on bacterial growth indicate that growth on PAO supplied with the appropriate nutrients from beneath is as rapid as in liquid medium (den Besten et al., 2007, 2010; den Hertog et al., 2010; Ingham et al., 2005, 2008b). Additionally, many microorganisms that cannot be grown on conventional growth media can be cultured on PAO. One such group includes organisms for which agar is either impractical or toxic. An example is the growth of the Archaeal extremophile Sulfolobus sulfotaricus at 80 °C and pH<4 on PAO, conditions under which agar cannot remain gelled, but which are not a problem when using aluminum oxide as a matrix. Also, porous culture supports can be used to grow microbes in close communication with their natural habitat. This results in higher culturability for a variety of reasons, including the presence of an appropriate level and type of nutrients, and the presence of microbially secreted products such as siderophores and quorum sensing molecules that influence the growth of other species (Ferrari et al., 2005; Kaerberlein et al., 2002). This type of technique also provides the ability to image microcolonies, enabling the detection of very slow growing organisms or ones which will not form visible macroscopic colonies. PAO has formed at least a component of porous culture systems that have been used to screen for new species of soil and river bacteria (Ferrari et al., 2005; Ingham et al., 2007).

One group of applications is microcolony based antibiotic susceptibility tests based upon culture of microbial isolates on PAO with and without antibiotics, staining with fluorogenic dyes and image analysis have been devised. Such tests have been applied to rapidly growing pathogens such as the Enterobacteriaceae and methicillin resistant Staphylococcus aureus, producing results within hours (Ingham et al., 2006; Tsou et al., 2010). Greater value is added for slow growing organisms, with susceptibility testing for Mycobacterium tuberculosis reduced from over 10 days to 3 days (Ingham et al., 2008a).

An interesting alternative for M. tuberculosis is real-time monitoring of growing microcolonies, allowing the effect of the addition or removal of drugs during culture to be assessed (den Hertog et al., 2010).

Fig. 3. Key applications and developments of PAO in biotechnology. Important advances in green/red/blue and white biotech and in related areas.
A second area of using microcolony culture and imaging (Durtschi et al., 2005c) on PAO is in investigating phenotypic heterogeneity. The ability to analyze large numbers of cells has facilitated quantification of subpopulations. As with antibiotic susceptibility testing, culture on PAO that can be moved between different Petri dishes allows stresses to be imposed and removed. Culture under extreme conditions where growth is marginal and minimal has been used to assess responses to high salt (den Besten et al., 2007), low temperatures (den Besten et al., 2010) and low pH (Ingham et al., 2008b).

PAO has also been used to investigate the communication and competition between different strains of the swarming bacterium Proteus mirabilis (Budding et al., 2009). In this case, the selectivity of permeation of signaling molecules though the controlled pores was an important issue. The controllability of permeation through PAO pores, via modifying size and/or surface chemistry, are exploitable features and will be further discussed in Sections 4 and 5.

3.3. High throughput microbiology

A logical extension of microbial culture on unstructured PAO is to subdivide different areas of the surface to create discrete growth compartments with a PAO base (Fig. 4). Given the small size of microcolonies that can be effectively imaged the sample density can be extremely high. In conjunction with other components, such as an imaging and cell recovery platform, this allows high throughput. Printed polymers allow medium to low sample density (Ingham et al., 2005) while microengineering processes can be used to create culture chips with an unprecedented number of discrete culture areas: 1 million in an 8 × 36 mm area (Ingham et al., 2007). This is part of a trend within microbiology, of accelerating miniaturization of culture formats that is a similar to Moore’s Law that describes an exponential growth in computing power (Gefen and Balaban, 2008). Uses of culture chips to date have included viable counting, using the grid pattern of the microwells to facilitate software recognition of microcolonies. High throughput screenings using fluorogenic substrates to detect enzymatic activities of interest have been completed (Ingham et al., 2007). The porous base has permitted culture on near-natural substrates (sediment, river water, fecal material) as the growth media. As with other culture systems that use natural materials separated from the samples (Ferrari et al., 2005; Kaeb erlein et al., 2002) this system permits the culture of microbes that have not been successfully grown in defined media. Such a strategy, combined with phenotyping, has value in isolation of new microbes and in the creation of metagenomics libraries.

A high-density microbial culture format requires complementary technologies for full functionality. A high throughput contact printing method has been developed using an array of thousands of polydimethylsiloxane (PDMS) pins to print microorganisms within 40 × 40 μm wells (Fig. 4). The PDMS stamp is targeted and deployed using a microscope, imaging through the transparent PDMS (Ingham et al., 2010). This technique can print thousands of microcolonies on planar PAO and hundreds on culture chips, the main limitation for the latter being the precision to which the PDMS stamp can be aligned with the target wells. An advantage of a physical stamp is that it can be used to print replicas in multiple culture chips; this may be useful in the storage of strain libraries or in screening processes. It is likely that non-contact printing of bacteria (Zheng et al. 2011) is also possible within wells of a culture chip. Additionally, low cost microscopy and imaging supports this type of approach (Albeau et al., 2008; den Hertog et al., 2010; London et al., 2010).

4. Eukaryotic cell culture

The culture of mammalian cells is also possible on PAO. This is commonly performed in co-culture systems where two cell types are separated by a PAO membrane. Commercial tissue culture inserts generally fit from 6 to 96 well microtiter plates. One advantage is, as for bacteria, clarity of imaging by fluorescence microscopy compared to more autofluorescent microporous membranes. Additionally, the precision to which the nanopores can be engineered allows large molecular complexes or subcellular aggregates to be excluded with greater confidence than for less consistent membranes. A relevant example of cell culture is that used to study communication between malarially infected and uninfected human blood cell populations separated by a 20 nm diameter pore PAO membrane (Schlozen et al., 2009). Apart from separation of the two cell populations (for which purpose other porous membranes would suffice) the precise and fine pore size had two functions: (a) to exclude the transfer of hemozoin crystals (a method of disposal of otherwise toxic hem by the malaria parasite) from the infected to uninfected cell populations and (b) to exclude transfer of major histocompatibility complex class II exosomes which could have otherwise mediated antigen transfer between the two cell populations.

Part of the attraction of PAO is in creating scaffolds for the development of cultured cells in a desired direction. This may be simply through alterations in texture (pore size, spacing) or more sophisticated surface functionalizations that recruit specific cell types to precise locations or induce particular types of development and growth.

Engineered cell platforms intended for screening purposes and based around PAO have been developed. Broadly, the approach of subdividing the material into microwells with PAO at the base has been implemented for mammalian culture, as for microbiology. For example, microwells have been created using a polyethylene glycol (PEG) hydrogel (Yu et al., 2009). This was achieved by silanating the PAO surface followed by photolithography using a shadow mask to polymerize the PEG in the desired pattern. The detection method (impedence) used to read out changes in cell growth with anticancer drugs is described in Section 5.

Beyond simple cell growth, i.e. single cell types or cocultures separated by a PAO membrane, lies tissue engineering: the engineered manufacture of tissues and organs for research and transplantation. Cells grown on an appropriate 3D scaffold and supplied with nutrients and growth factors behave very differently to those grown by
conventional cell culture methods on planar plastics or glass. Non-planar scaffolds are often far better at encouraging cell growth that more realistically mimics what is happening within the human body. Alumina was first suggested as a suitable transplantation material for hip replacements (Hamadouche et al., 2002). Nanoporous alumina allows fine-tuning of cellular responses by altering surface properties and the selective delivery of small compounds and larger aggregates of biomolecules (Schlozen et al., 2009). Organic polymers have a significant role in tissue engineering too; biodegradability and the ability to mimic the mechanical properties of the tissue are attractive features. However, nanoporous ceramics and other inorganic supports can be less inflammatory (Poinern et al., 2011). Bone cell (osteoblast) culture has been achieved on PAO — indeed PAO appears as an excellent scaffold that is close to the porous nature of the surfaces that exist in the body. While aluminum ions have been reported to have negative effects on some types of bone development, this does not seem to be a significant difficulty in this situation (Poinern et al., 2011). PAO that is more flexible than that used in bone culture, to allow molding of the shape of the tissue to body surfaces, is also being investigated for culture of skin tissue for subsequent transplantation (Parkinson et al., 2009). For artificial tissue engineering the PAO may not necessarily be introduced into the human body, although this is possible in some scenarios. PAO is also used in creating implantable drug delivery systems with low inflammation potential (Gultepe et al., 2010). If phosphoric acid is used in the anodization process then the remaining phosphate incorporated into the alumina surface may improve biomineralization. Immunosuppressive drugs have been delivered in vivo at appropriate doses using cardiovascular implants of drug-loaded PAO in rabbits, in addition to supportive in vitro studies on drug release from this material (Wieneke et al., 2003).

Some tissue transplantation may require selective permeability — for example to permit low molecular weight compounds to reach the cell but to protect the newly grafted tissue from the host immune system. PAOs with controlled permeability due to functionalization with polyethylene oxide have been created for the immunosolation of tissue transplants (Lee et al., 2011). Such modified membranes exclude antibodies but permit access of insulin and glucose and therefore may be suitable for the encapsulation of pancreatic islet cells for treatment of type I diabetes.

In addition to the use of PAO as a direct scaffold for cell, it is possible to create structures using PAO as a template; such nanowire arrays have significant uses in tissue engineering. These are discussed in Section 7 (Indirect uses — PAO as the basis for fabricating other nanostructures).

5. Surface modification and functionalization of PAO

Coupling other molecules to an inert aluminum oxide surface may seem like a somewhat quixotic aim, but it has enormous potential in using the high surface area productively (Chen et al., 2010; Javid et al., 2006; Lee et al., 2002; Mateo et al., 2011; Milka et al., 2000; Matalib et al., 2009; Szczepanski et al., 2006; Wang et al., 2011). Surface modification allows fine-tuning of the basic properties of alumina, e.g. hydrophobicity, reflectivity and surface charge, but also membrane selectivity, anti fouling resistance and the recruitment of biomolecules in specific and potentially high-throughput assays. Examples are given in Table 3.

Aluminum oxide surfaces can be modified in several ways, which are usually based on polymer adsorption or monolayer formation. The adsorption of poly-L-lysine to PAO results in positively charged surfaces that can bind DNA fragments by electrostatic interactions. This has been exploited for the fabrication of a FRET based sensor with single pore resolution (Masumoto et al., 2004). Similarly, the adsorption of poly(diallyldimethylammonium chloride) can facilitate gold nanoparticle binding (Ko and Tsukruk, 2008). In situ polymer formation on PAO has been demonstrated by plasma polymerization (Losic et al., 2008). This yields an amine-terminated surface, but also reduces the effective pore diameter.

The advantages of monolayer formation are that it is usually based on self-assembly, resulting in full and uniform surface coverage, and that it does not notably change the structural properties of the substrate. Several classes of compounds are known to form monolayers on aluminum oxide, including carboxylic acids, organosilanes and phosphonic acids (Fig. 5). The adsorption of n-alkanoic acids on PAO is possible, but as this adsorption process is reversible, the resulting monolayers were found to be unstable under aqueous conditions (Chang and Suen, 2006). Most reports, however, deal with organosilanes. This is remarkable, since the packing density and stability of organosilane monolayers is known to be limited on alumina in comparison to other substrates. (Liakos et al., 2004; Oberg et al., 2001). The deposition of silica prior to monolayer formation is suggested to improve monolayer stability, but complicates the modification procedure (Szczepanski et al., 2006). Phosphonic acids, on the other hand, do form stable monolayers on alumina (e.g. Liakos et al., 2004; Yildirim et al., 2010), but there is only one example of the adsorption of these compounds onto PAO (Koutsoubas et al., 2008), possibly due to the limited commercial availability of functional phosphonic acids. Finally, it was recently shown that chemisorption of terminal alkynes onto PAO also results in the formation of stable monolayers (ter Maat et al., 2011).

Modification of PAO with organosilanes has been applied to change the surface charge (Chen et al., 2010), to create a super-hydrophobic material (Park et al., 2010) and to reduce membrane fouling (Popat et al., 2004; Yeu et al., 2005). In a more advanced study, a preformed monolayer is used as the initiator for the controlled growth of polymer brushes. Subsequent functionalization with a nitriloacetate-copper complex results in a membrane that can be used for affinity separation of histidine-tagged proteins (Sun et al., 2006). An interesting development is the layered surface modification reported by Jani et al. (2009, 2010), in which anodization and organosilane monolayer formation are repeated. This process allows one to further tune the transport properties of the membrane.

By coupling (functionalized) inorganic nanoparticles to the PAO surface, new properties may also be introduced to the substrate. This coupling can be facilitated by modification of the PAO with a polymer or organic monolayer (Ko and Tsukruk, 2008), but can also be performed directly onto the PAO. (Thormann et al., 2007). Coupling of nanoparticles to the PAO is often performed with the aim of Raman enhancement (see Section 6), but also for the introduction of fluorogenic detection reagents. A modified PAO membrane was created by covalently coupling fluorophore-loaded silica nanoparticles to the PAO (Fig. 6). As the fluorophore (pHrodo, Invitrogen) displays pH-dependent fluorescence, the resulting surfaces can be used for screening of strains of acid-producing bacteria. An exciting and commercially viable set of applications for functionalized surfaces is the recruitment of biomolecules for specific assays — potentially massively parallel with a large number of binding sites (e.g. immobilized antibodies or oligonucleotides) for different targets (Wu et al., 2004). An elegant example is the use of PAO to create silica nanotubes that are able to display functional antibodies capable of distinguishing between drug enantiomers. Such a system can selectively bind one enantiomer. An extension of this idea was to reduce the affinity of the antibody, without removing selectivity, using DMSO as a co-solvent. This allows a PAO surface to be used as a chromatography system — effectively billions of nano-chromatography columns in parallel, for the separation of high value biomolecules (Lee et al., 2002). Nucleic acid microarrays, displaying oligonucleotides for mRNA or the capture of single nucleotide polymorphisms (generally amplified as PCR products) have also been developed (Wu et al., 2004). These display rapid kinetics compared to planar arrays, with active pumping of the target molecules through a 3D array of detector molecules. This has been extended to peptide display arrays used in
protein kinase assays (Hilhorst et al., 2009) and in multivalent carbohydrate arrays (Pera et al., 2010). One of the advantages of PAO arrays is that information on binding kinetics (e.g. kinase interacting with a peptide substrate and how kinase inhibitors affect such an interaction) can be obtained, something that is potentially valuable to drug screening operations (Vivanco et al., 2010).

Moreover, surface functionalization may also allow the addition of cell-specific detection reagents and cell-capture agents. This may allow the recruitment, culture and detection of specific types of bacteria. These types of assays are likely to have further roles in the screening for microorganisms for industrial applications, but also in the detection of infections in medical diagnostics.

6. Physical detection technologies

As described in Sections 3 and 4, PAO has a low background for fluorescence measurements, which can be advantageous for cell imaging studies. However, there are other imaging technologies that are actively enhanced by PAO or by structures templated by PAO. Sensitive optical detection is possible on nanoporous substrates. One method is to apply the PAO as an optical waveguide, for example by stacking a metal-supported PAO substrate to a prism (‘Kretschmann configuration’). This allows the monitoring of exceptionally subtle changes in the refractive index due to adsorption of e.g. molecules or proteins inside the pores. (Lau et al., 2004). This sensitivity can be attributed to the well-ordered pore arrays (resulting in distinct waveguide modes) and to the high surface area of the PAO. Such composites of PAO/Al have proven competitive with surface plasmon resonance (SPR) in terms of sensitivity (Yamaguchi et al., 2009). What is more, it is also possible to extract spectral information on the adsorbed compounds from these measurements (Hotta et al., 2010).

An alternative waveguide-based method using PAO was recently shown to detect small concentrations of pesticide (Trivinho-Strixino et al., 2010).

Thin PAO substrates have also been used as surface plasmon resonance (SPR) sensors, using the same Kretschmann configuration (Fig. 5).

Table 3
Biomolecules and biofunctional agents immobilized on PAO.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Category</th>
<th>Direct PAO modification</th>
<th>Secondary modification</th>
<th>Application</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alliinase</td>
<td>Enzyme</td>
<td>Silane</td>
<td>Sugar-lectin</td>
<td>Bioreactor</td>
<td>Milka et al. (2000)</td>
</tr>
<tr>
<td>Phenol isothiocyanate</td>
<td>Fluorophore</td>
<td>Silane</td>
<td>Direct adsorption</td>
<td>Polychlorobiphenol sensor</td>
<td>Wang et al. (2011)</td>
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* POP, proof of principle.

Fig. 5. Surface modification of PAO. Examples of monolayer types that can be formed on PAO, resulting from the adsorption of: A. Carboxylic acids, B. phosphonic acids, and C. organosilanes.

Fig. 6. PAO culture chip with intrinsic nanodetection reagent. A. Scanning electron microscopy of PAO with up to 200 nm pores and customizable spherical 80 nm nanoparticles loaded with pH sensitive reporter dye. The particles have been colored blue-green to increase their visibility in this image. B. Coupling chemistry used to attach nanoparticles to the PAO functionalized (F) with an alkyne surface chemistry. C. Increase in fluorescence of nanoparticle (N) by decreasing pH.
Arrays (Choi et al., 2010). Sputtering a well-ordered PAO substrate (Koutsioubas et al., 2008). In an alternative approach, the ordered structure of PAO was used as a template for the condensation of nanocrystalline gold, which allowed the monitoring of antibody–antigen interactions with localized SPR (Kim et al., 2008).

Raman spectroscopy is a detection method that is very suitable for aqueous solutions. PAO is highly compatible with this technique, as it has a low background in the visible and near-infrared range. In addition, it can be used as a substrate for surface enhanced Raman spectroscopy (SERS), which is an exceptionally sensitive, label-less and potentially real-time variant (Moskovits et al. 2011). The surface-enhancement effect is induced by strongly localized electromagnetic fields (‘hot spots’) that can be obtained by excitation of nanostructured metals. Therefore, early studies used PAO as a sacrificial template to form ordered metal nanostructures (Schierhorn et al., 2006; Wang et al., 2006). In addition, SERS-active substrates have been obtained by adsorption of silver and gold nanoparticles onto PAO (Ji et al., 2009; Ko and Tsukruk, 2008; Ko et al., 2009; Lu et al., 2009). These surfaces show increased sensitivity compared to planar surfaces due to the higher surface area, but also due to the optical waveguide effect. The latter was estimated to give rise to an additional enhancement of about three orders of magnitude (Ko et al., 2009), and corresponding substrates were used to detect concentrations of explosives to a sensitivity of one part in $10^{12}$ (Ko et al., 2009).

Other approaches exploit the specific nanostructural features of PAO to create SERS substrates. The highly ordered and organized PAO was lined with a metal to obtain a surface with homogeneous SERS activity, the advantages of these methods are their simplicity and scalability.

In addition to optical methods, there are also PAO-based detection methods that rely on electrical conductance through the nanopores. Because of the large surface to volume ratio of PAO, the ionic conductance through the pores is sensitive to the interaction of ions with the walls. An electrical biomolecule sensor has been made, by depositing gold as electrodes on both faces of the PAO. Binding of DNA or RNA to a complementary nucleic acid sequence (or a neutral nucleic acid analog) immobilized in the pores leads to a decrease in ionic flux through the pores (Wang and Smirnov, 2009). The effect is far larger in 20 nm pores compared to larger diameters. This method has been used to detect single nucleotide polymorphisms, while a related method was capable of assaying the effect of an anti-cancer drug on mammalian cells arrayed in nanowells on PAO (Yu et al., 2009).

PAO supports also show advantages for mass spectroscopy. Typically, conventional matrix-assisted laser desorption/ionization mass spectroscopy (MALDI-MS) uses an organic matrix to transfer the energy for desorption to the target biomolecules. MALDI-MS has been developed into a key method for biomolecule analysis, but it has some shortcomings that relate to the use of a matrix (e.g. significant background in the low mass range). This has led to the development of matrix-free LDI methods on nanotextured surfaces, including PAO. It was found that effective desorption/ionization requires coating the PAO with a metal (Okuno et al., 2005). Other parameters, such as the porosity and the thickness of the PAO and metal coating, also influence the signal intensity (Nayak and Knapp, 2007; Wada et al., 2007). Absorption of the laser irradiation by the thin metal coating on the thermally isolating PAO results in high local temperatures and metal melting (Wada et al., 2007). Energy transfer to the analyte then results in its desorption/ionization. PAO-based LDI has been shown to give comparable performance to MALDI, but with lower backgrounds in the low mass range. (Nayak and Knapp, 2007). In addition, the samples are also compatible with desorption electrospray ionization (DESI), a complementary ionization mode (Nayak et al., 2008).

In summary, the enhancement of a number of imaging and detection technologies due to the PAO structure offers the opportunity for imaging methods to enter common usage, some of which may otherwise be too specialized or limited to enter the market place. Techniques that are already finding a place in routine diagnostics, such as MALDI-TOF, may also benefit from sample carriers that include PAO in their manufacture.

7. Indirect uses — PAO as the basis for fabricating other nanostructures

The structure of PAO and the physical and chemical properties of aluminum oxide are qualities that can be separated. PAO is capable of acting as a structural template for other nanofabrication processes. Broadly there are three possibilities: (a) an inverse structure is made using PAO as a scaffold — effectively a nanowire or nanotube; (b) a copy or at least a similar structure of the PAO structure is made in another material; (c) the pores are used to create another structure that is neither a copy nor the complement of PAO, such as a nanoparticle.

Inverse structures can be fabricated from polymers, metals or semiconductors. The result is an array of nanowires or nanotubes contained within the PAO pores or liberated as free structures. The literature on the use of PAO as a scaffold is extensive, and several dedicated reviews on this subject are available (e.g. Al-Kaysi et al., 2009; Sarkar et al. 2007). Therefore, we only present a selection of examples. One application of nanowires is the culture of mammalian cells (Grimm et al., 2010). Nickel wires grown in PAO by electrodeposition can be aligned in a magnetic field to create surfaces for the culture of mammalian cells. Externally imposed magnetic fields can be used to exert precise forces on the cells (sometimes after internalization of a nanowire). This allows creation of unique textures and patterns (coupled with surface modification (Johansson et al., 2010)). Biodegradable scaffolds for cell and tissue engineering can also be produced using PAO as a template. For example, poly(e-caprolactone) supports for stem cell research, neuronal cell regeneration and bone tissue engineering have been created by extrusion of the polymer through PAO. Sodium hydroxide is then used to destroy the PAO and liberate arrays of nanowires with an average diameter of 200 nm (Bechara et al. 2010, Porter et al. 2009). These permit the growth and development of cell types that are difficult to achieve on other surfaces. An interesting aspect of this method is the possibility to incorporate active biomolecules into such a nanowire array; for example agents designed to promote the differentiation of particular cell lines.

Silica nanotubes can be fabricated within PAO pores then individually released by reactive ion etching to create silica nano test tubes (Buyukserin and Martin, 2010). The etching process can also close the nano test tubes to create capsules. The potential for surface functionalization (e.g. antibody coating as a targeting mechanism), low levels of cytotoxicity and rapid dispersal make these attractive as a drug delivery system.

At least partial copies of the well structure of PAO are manufacturable using the alumina as a mask to selectively etch another material, generally resulting in an array of nanowells that reflects the structure of the mask (Kang et al., 2005). The PAO mask is simply placed on top of a planar piece the material to be etched (e.g. glass, carbon, polymers,
metals) and argon plasma used to selectively remove material to create nanowells with a similar pattern to the mask. Porous silicon nanowell arrays have been made in this way and applied to matrix-free laser desorption mass spectroscopy of proteins and other biomolecules (Gulbakan et al., 2010). Light emitting diodes have even been created in semiconductors textured with nanowells using a PAO template (Soh et al., 2010). This process can be thought of as a simple method of fabricating textured nanosurfaces. However, it has also been pointed out (Kang et al., 2005) that when combined with nanospheres that may vary in surface properties, and which occupy individual nanowells, the nanowell system can store chemical information that can be read out by confocal microscopy or an array of optical detectors.

By evaporating aluminum onto PAO and melting of the metal, inverse aluminum structures have been obtained. However, the diameter of the obtained aluminum features was bigger than that of the original PAO template, which was attributed to the penetration of the aluminum into Al2O3 layer at the surface. This metal:metal oxide composite confers improved mechanical properties, which allows the templated structure to be used as a mold for nanoimprint lithography (Lee et al., 2005). The PAO itself can also be used for imprinting purposes. Hot embossing, where a (supported) PAO template is pressed onto a polymer film at elevated temperature, has been reported for polystyrene and cyclic olefin copolymer films (Koponen et al., 2007; Lee et al., 2008). This resulted in inversion of the PAO pattern into the polymer, yielding an array of polymer nanopillars. Similar structures were obtained using UV nanoimprint lithography, in which a PAO template is pressed onto a polymer precursor, which is subsequently cured with UV light to produce the nanostructured polymer (Bessonov et al., 2010). Finally, injection molding of polyvinyl chloride into PAO templates has been demonstrated, yielding a patterned polymer with shallow features (Koponen et al., 2007).

Other types of nanoparticles can be created using PAO, as with nano test tubes with applications in drug delivery. Uniform, nearly perfectly spherical polymer nanoparticles can be created by using PAO to emulsify monomers, which are photopolymerized after passaging through the membrane, the pore size being used to control the final size of the nanoparticle (Yanagashita et al., 2009). Particle size was slightly larger than the pore size and nearly linearly related to the pore diameter. Biodegradable polymers can also be created from materials such as chitosan (a polymer of N-acetylglucosamine and glucosamine). The method of creation is to passage the monomers through PAO so a rapid increase in pH is achieved, allowing highly controlled polymerization (Guo et al., 2010). Small molecules can be incorporated into chitosan during this process. Finally, particles related to quantum dots can be grown within the nanopolores of alumina (Zelenksi et al., 1997).

8. Future prospects

Diverse and inventive minds have seized upon the properties of PAO and exploited this material in biotechnology applications as wide-ranging as molecular separations, cell imaging, high throughput screening, sensors of environmental hazards, drug delivery, tissue engineering and single molecule imaging (Fig. 3). The structural properties of PAO have been transferred to other materials, as inverse structures (nanotubes and nanowires) or by replicating the pore pattern by using the PAO as a shadow mask. Improvements in the basic structure of PAO have resulted in more regular pores. Alterations in the surface properties of PAO have been achieved by chemical functionalization. Functionalization has allowed fine-tuning of basic surface charge and hydrophobicity, but also the specific capture of biomolecules, facilitating highly multiplexed assays.

Despite intense interest, we still do not completely understand the self-ordering process that anodization brings to aluminum. Therefore, it is necessary to investigate this phenomenon more completely, which should, in turn, unlock further advances in controlling and extending the range of PAO variants available. In terms of manufacture, it is likely that the biotechnology and nanotechnology applications are sufficient to drive more widespread commercial PAO formats. For the material to be widely usable within biotechnology, further decreases in cost and improvements in durability are critical to enable PAO to be used disposables or scaled-up processes. Here, the bottom line may be the cost of high purity aluminum.

Surface chemistry, to date, generally functionalizes PAO in limited ways. Several chemistries exist but generally the same modification is made across a piece of this material. Where different biomolecules are recruited to different areas (e.g. in fabricating a microarray with different biomolecules in different spots) then this is usually done by printing and a secondary conjugation. In the future we may expect higher resolution and more versatile surface chemistries.

Two trends in detection methods are supportive of multiplexed assays on PAO. The first is related to the advantages that the material brings in terms of optical imaging methods (SERS, optical wave guides). If these sophisticated and powerful methods can be brought to the marketplace then this opens up powerful types of reagent free imaging. Additionally, advances in simple and cheap illumination methods, particularly LEDs (Albeau et al., 2008), and the emergence of new detection reagents (nanoparticles) should allow fluorescence-based detection methods to increase in power. Trends in imaging, such as low cost digital data capture, USB microscopes, direct detection of cell autofluorescence using CCD chips (London et al., 2010) can be expected to support wider usage. The decrease in LED costs and cost-per-pixel of digital data capture supports the increasing miniaturization of microbial culture with low cost and point-of-testing and/or online read-out methods, a goal that has been described as telemicrobiology (Scheid et al., 2007).

Within microbiology there is some competition between molecular and culture based approaches. However, the failure of molecular techniques to displace traditional culture methods indicates that both approaches are necessary. One logical, but not entirely easy, step would be assays that integrate elements from both technology streams. This would allow a combination of phenotyping and genotyping, or identification of a target organism combined with an analysis of whether it is viable and what it is capable of (e.g. pathogenicity or carcogenicity). This goal could be achieved on PAO systems by capturing organisms based around their surface properties (e.g. an antibody) followed by culture; or the reverse — culture then lysis and molecular analysis.

Materials can have a major impact on biotechnology. For example, relatively simple and inexpensive plastics have transformed many aspects including cell growth and high throughput screening through disposables, such as the multiwell plate and the disposable Petri dish. We are only just learning to leverage the potential of nanoporous materials in biology, but it is our expectation that such materials, including PAO, will have a significant impact too.

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References


Ingham CJ, Ingham et al. / Biotechnology Advances 30 (2012) 1089–1099

Durtschi JD, Erali M, Bromley LK, Herrmann MG, Petti CA, Smith RE, et al. Increased De

Chang CS, Suen SY. Modifi

Hamadouche M, Boutin P, Daussange J, Bolander ME, Sedel L. Alumina-on-alumina total


