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Stimuli-Responsive Platforms for Integrated Multifunctional Intelligent Systems

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This paper presents a comprehensive outline of designing diversified stimuli-responsive platforms, putting emphasis on the mechanistic variation of responsiveness. Representative examples and experimental results are given on the development of integrated multifunctional biosensors based on stimuli-responsive lipid bilayers for the (spatial and temporal) control of protein-surface interactions, an area that is expected to benefit largely diagnostic microarrays, biomaterials, regenerative medicine and drug delivery. The experimental results indicate that important limiting factors for the function and application of the stimuli-responsive platforms are the degree of change and the rate at which the change occurs. In many cases the understanding of the underlying mechanisms is still limited and needs to be extended, so that improved, rational design of sophisticated systems can be accessed.

1. Introduction

Integrated multifunctional intelligent systems are, by definition, flexible and adaptive structured assemblies that have sentience and responsiveness to changing environments (Motornov et al., 2010). The high impact growth of the field, rapidly matured due to synergistic interdisciplinary efforts across sectors of universities, government and industry, yields drug delivery devices, sensors, thin films and colloidal dispersions that use stimuli-responsive natural, hybrid or synthetic particles at the micro- or nano-scales (Zhang et al., 2012). The 'responsive behaviour' follows a simple concept, quite common in living systems: external stimuli cause changes in the properties of the particles, i.e., in dimensions, structure, or interactions, and lead to their re-arrangement or to changes in their physicochemical state (Hirst et al., 2008). Yet, the realization of this concept, i.e., the manipulation of complex and dynamic molecular interactions to generate a self-modulated spectrum of tuneable properties (Jarvis et al., 2011) and functions (Seong et al., 2011), is challenging and resource-intensive.

Nature relies on complex hierarchical structures that are optimized in vivo (via gene activation, transcriptional control and hormonal function) to achieve the mechanical and physicochemical performance that each sub-system requires for optimizing the whole superstructure. Seen in isolation, the change of each sub-system function is triggered by electrical current, ionic strength, phase transitions, channel switching, temperature fluctuations, concentration and potential gradients, the occurrence and intensity of which lead to differentiated responses and superior adaptivity. The simulation, however, of the underlying entropy may be neither accurate nor easy (Siontorou and Batzias, 2013). Consider, for example, a bacterial cell functioning in vivo and a microbial biosensor using the bacterial cell as its biological recognition element: transport of molecules is affected by diffusion, rather than bulk flow; movement is resisted by viscosity, not inertia; the energy of thermal fluctuation is large enough to perturb the cell's motion; cells move through the sample rather than pushing the sample towards the cell in a sensor format.

To overcome these barriers, two approaches may be followed: pre-assembly morphology engineering and post-assembly transformations. While the former entails complicated and time-consuming synthesis (Ponzoni et al., 2012), the latter is more fit for applications in intelligent systems, broadening the range of responses and allowing for better control of initiation and propagation processes (Mendes, 2008). This is

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particularly important for targeted drug delivery (Rigogliuso, 2012) and clinical diagnostics (Yoshida and Lahann, 2008), where dynamic platforms are significant for controlling molecular and cellular interactions. To achieve a macroscopically observable change in platform properties, two fundamental design challenges need to be addressed simultaneously: (i) molecular switching structures have to be designed and constructed and (ii) the ordered organization of the building units must ensure the desired switching. If small molecules are employed as building units, careful design must not only address the ability to undergo conformational transition between different physicochemical states in response to an external stimulus, but also ensure high degrees of alignment among the molecular units. In this context, the ability of certain molecular motifs to support spontaneous self-assembly in well-ordered mono- bi- and multi-molecular films plays a pivotal role (Weiss, 2008). Amphiphiles, like lipids and surfactants, express a natural tendency to organise spontaneously into thermodynamically favoured fluid-like architectures, continuously switching states and conformations in response to exogenous changes (Weber et al., 2012).
Responsivity and multiple platform functionality have been studied herein on lipidic ordered configurations that provide an easy way to the dynamic control over structural reorganization and functionalization. This paper presents a comprehensive outline of designing diversified stimuli-responsive platforms, putting emphasis on the mechanistic variation of responsiveness, an area that is expected to benefit largely diagnostic microarrays, biomaterials, regenerative medicine and drug delivery.

2. The bilayer lipid platforms

Lipid membranes are two-dimensional fluid nano-structures where two, preferably, lipid layers are held together by non-covalent hydrophobic interactions of amphipathic molecules. The films have a thickness of about 5 nm, varying with the lipid tail length. Pure lipid bilayers spanning an aperture that separates two electrolyte solutions (freely suspended BLMs) exhibit a resistance of 100 MΩ cm^2 allowing for a small (on the order of few picoamperes) transmembrane ion current (Venkatanarayanan and Spain, 2014). For analytical applications, various biological moieties (e.g., enzymes, ion carriers, receptors, DNA, etc.) are incorporated into the membranes to add functionality (Siontorou and Batzias, 2013). Although sensor's sensitivity and selectivity towards a given analyte are endowed by the biological moiety (dependent on its affinity for the target), differentiation and speed of response rests on the lipid film: the thinner the lipid film, the more quickly it re-organises and the higher the degrees of elasticity it exhibits. Needless to say that ultrathin films make the use of even highly expensive molecular switches (as DNA) economically possible (Ampelli et al., 2014).
A wide range of materials have been used, employing a variety of techniques for guiding the self-assembly process (Figure 1). The hydrophilic/hydrophobic characteristics of the lipid molecules allow them to spontaneously form organized structures on supports, given that adequate hydration is provided. Self-assembly through vesicle fusion (Rawle et al., 2011) is a simple technique in which unilamellar lipid vesicles rupture upon contact with a surface and form a supported lipid membrane covering the interface (Figure 1a(i)). Vesicle fusion occurs spontaneously on some hydrophilic substrates (e.g. silicon oxide and silicon nitride) to form planar bilayers (Figure 1a(ii)-(iii)): on hydrophobic surfaces (Figure 1a (ii)), e.g. pre-formed thiol-alkyl monolayers, lipid monolayers are formed instead of bilayers from lipid vesicles; more complex tethers (Figure 1a(iii)), providing additional aqueous space under the self-assembled lipid layer such as hydrophilic spacers with covalently bound lipids, can be used to drive liposome fusion. The construction of Langmuir-Blodgett films (Figure 1b) include three stages (Simon et al., 2007): (i) a motorised stage is used to move the substrate between an aqueous and a gas phase, and the resultant lipid monolayer is held at a defined tension at the interface, which controls the packing density; (ii) vertical reinsertion of the lipid monolayer formed through the interface deposits a second monolayer on top, formulating a bilayer; (iii) If the substrate is taken through the interface horizontally the same result is achieved, but it is referred to as Langmuir-Schäfer deposition or sometimes as 'tip-dip' when capillaries are used. Detergent dialysis and painting (Figure 1c) are, also, commonly applied (Weber et al., 2012). The former uses micelles of lipids mixed with detergents (Figure 1c(i)): the detergent is continuously removed from the micelles by dialysis leading to decomposition of the micelles and the formation of a planar lipid bilayer at the interface. For painting (Figure 1c(ii)), a drop of organic solvent containing dissolved lipids is added to a surface in an aqueous phase; the amphiphilic lipids will align at the solvent interface and when the solvent is extracted, the lipids at the interface fuse to form a bilayer at the substrate–aqueous solution interface. Among these techniques, the most efficient in producing nature-relevant and biocompatible lipid films have been the bilayer spanning apertures between two aqueous compartments (Figure 1b), the so called freely-suspended planar bilayer lipid membrane or simply BLM. Although these bilayer platforms have been a very successful laboratory approach, there is one major drawback that still needs to be solved: instability manifested in the collapse of the bilayer structure at voltage-induced stress, increased

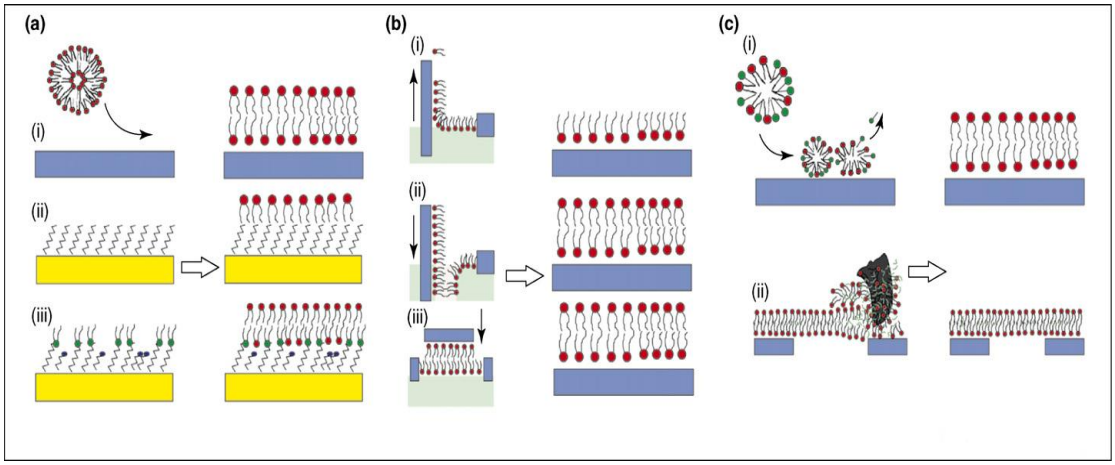

Figure 1: Methods used to assemble lipid membranes on sensor substrates. (a) Vesicle fusion: (i) on hydrophilic support; (iii) on hydrophobic support; (iii) on tethers. (b) Langmuir-Blodgett techniques: vertical monolayer formation (i) and vertical zipping (ii); (iii) (horizontal) Langmuir-Schäfer deposition. (c-i) Detergent dialysis. (c-ii) Painting and solvent extraction

protein loading or mechanical shock (Siontorou and Batzias, 2013). As a result, there has been an increasing emphasis in recent years on developing membrane platforms that will enhance mechanical robustness, a property that influences significantly a set of design criteria for membrane platforms (Schwarzboeck et al., 2012).

3. Design of lipid platforms: pre- assembly control and post-assembly manipulation

The strategy developed for supporting platform design and assembly, is built on a dialectic interplay between nature and nanotechnology that involves three methodological stages: (i) the conversion or translation of platform characteristics (surface level) to physicochemical parameters and variables (deeper level) for pre-assembly control, (ii) the post-assembly manipulation of these parameters at the physicochemical level to optimize structures and functions, and (iii) their conversion to surface level architecture. This approach has been implemented herein in producing Langmuir-Blodgett phosphatodylcholine bilayers with enhanced operational stability.

In order to identify the important physicochemical parameters that correlate to the stability specification for stage (i), membrane instability has to be defined. Lipid membranes can undergo two basic kinds of instability (Liu et al., 2014): (a) rupture, leading to formation of pores or/and fragmentation of the membrane and (b) buckling, resulting in bending or folding of the membranes. The rupture is usually due to growth of local disturbances of the membrane thickness (surface waves, holes and cracks, etc.). The membrane buckling can be induced by decreasing the membrane tension (e.g., by applying a compressing force to the membrane edges) as well as by different effects due to membrane asymmetry. The general conditions for stability of a system, particularly a membranous one, are given by the thermodynamics: the membrane is stable when its free energy has a minimum value in the space of the independent thermodynamic variables. This means that any infinitesimal change of the independent parameters (pressure, temperature, electrical potential, surface tension, etc.) should lead to an increase of the free energy of the system. However, in many cases, as during protein function or flip-flop, the membrane can be unstable with respect to some of the thermodynamic parameters, but the rate of change of the membrane state is so small that during the characteristic time-scale of the experiment, this membrane behaves as stable; that is the case of ‘meta-stable’ membranes, a state considered critical to responsivity and intelligence. Notwithstanding, membrane instability can be brought about by a variety of reasons (Hua et al., 2013), including osmotic pressure difference, hydrodynamic instability or dielectric breakdown that will alter the surface tension of the lipid film leading initially to molecular re-arrangement and finally to membrane discontinuity. While the former does not alter substantially the functionality of the membrane, the latter is irreversible and results in terminal rupture. The organization of the lipid films into the bilayer structure during membrane formation is critical to the film tolerance towards rupture, as the viscoelasticity is built-in and defined during the process of reconstitution (Siontorou et al., 2010).

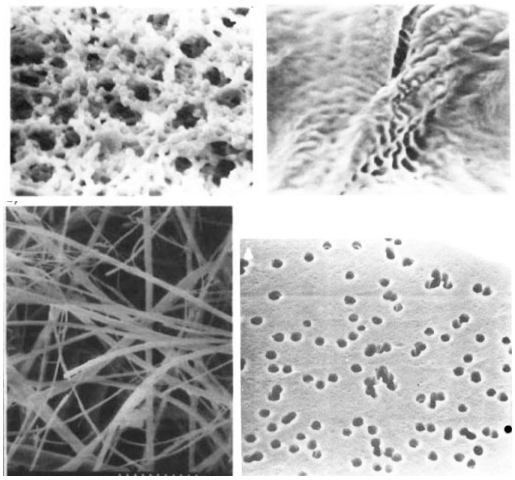

Figure 2: Scanning electron micrographs of the filters used as displays for lipid self-assembly: (top left) cellulose ester filter; (top right) PTFE filter; (bottom left) glass fibre filter; (bottom right) polycarbonate. All SEMs were observed at 45 ^{o} (1cm=1x10^{-6} m)

Thus, applying stage (ii), membrane stability is defined by (i) septum properties that provide the edge mechanical support and govern the solvent-film equilibrium at the annulus and (ii) the geometry of the lipid film, determined by the membrane diameter to thickness ratio (*d/t*), limited by the aperture thickness to diameter ratio (t_a/d_a). Ideal (theoretical) stability is achieved at small and thick apertures: considering a 50 Å bilayer thickness, a small diameter aperture favours substantially the film geometry whereas the thickness of the platform helps maintaining a bulky annulus, i.e., stability requires a high t_a/d_a at a low *d/t*. On the other hand, extremely small aperture diameter values will prohibit ion flow through the bilayer, whereas extremely thick platforms cannot stabilize the bilayer format as it favours multilayer structures.

The reduction of the membrane film size can be easily achieved with the use of microporous filters as self-assembly displays (Lautscham et al., 2014). The microporous filters selected are shown in Figure 2. The cellulose ester filters are composed of tightly woven fibres which have no preferred orientation. These fibres are approximately 1 μm thick; the interstitial spaces formed by the apposition of these polymer strands provide apertures across which lipid films could form. These apertures, for a filter of nominal pore of 0.45 μm, range from 2 to 5 μm in approximate diameter and are highly irregular in shape. Similarly, the polytetrafluoroethyle (PTFE) filters and the glass microfibre filters have apertures which are irregular in shape and vary greatly in size. The polycarbonate filters have a highly uniform series of holes separated by smooth, nonporous polymer. It is known that membrane formation is optimized by the relative smoothness and circular regularity of the support aperture. This condition appears to be met in the polycarbonate filters. However, multiple hole formation may comprise up to 30 % of the total aperture area. This leads to irregularly shaped apertures unfavourable for supporting lipid self-assembly. In addition, the two faces of the polycarbonate filters are asymmetric in that one side may he characterized by incomplete pore formation. These incomplete pores are manifested as wells of undetermined depth in the 15 μm thick filter. Lipid solution trapped in these wells will be electrically inactive and will provide a sink for the lipid-soluble membrane stimulants added to the surrounding solution.

All alternatives displays have been bench tested for stage (iii) and exhibited good characteristics as regards easiness of membrane formation (> 95 % of the attempts were successful in all cases). Typical values for the specific resistance of the stabilized films were about 10^7 Ω cm^2. The dielectric breakdown voltage of these membranes was about 350 mV. Measurements of membrane capacitance were used to show whether a single lipid membrane occupies the total area of a filter paper, or whether an undefined number of micro-films cover only the apertures of the filter. The capacitance of the self-assembled bilayers was found to be about 0.7 nF (polycarbonate filters) and 0.85 nF (glass microfibre filters), indicating that the total lipid membrane area during the formation of a filter-supported film would approximately span the aperture, yet only those areas in the pores of the filters would express responsivity. The lipid configuration thus resembles a network of micro-films that cover the whole available space (0.32 mm aperture) increasing manifold membrane responsivity and multi-functionality.

The responsivity of the membranes has been tested using abrupt pH and voltage alterations. The in situ pH dropping from normal to acidic (from 5.5 to 3.0) resulted in the appearance of transient signals of μs duration indicative of transient pore formation and ion flux through the bilayer. The thicker the display, the

higher the ion current measured, ranging from 6 pA for cellulose ester supports to 37 pA for glass microfibres. Similar results have been obtained for changes at the alkali scale (from 8.0 to 5.5), although the ion currents were larger. The local hydronium activity at the surface of a bilayer platform may be different than that of bulk solution and depends on the surface potential of membranes and ultimately on the surface charge density. The response of the platforms to voltage step alterations were similar, although such stimuli forces the whole membrane to re-organise quickly over a transmembrane potential gradient; not surprising glass microfibre displays exhibited the higher and the more reproducible responses.

4. Concluding remarks

Stimuli-responsive lipid bilayer platforms have been presented herein in an attempt to demonstrate both, (i) easy pre- and post-assembly control, provided knowledge of the thermodynamic parameters, and (ii) multi-responsivity, provided knowledge of the kinetic parameters, without the need of molecular engineering. Lipid bilayers are excellent hosts for molecular switches with response times that assure real-time monitoring of, e.g., an interacting protein adsorbing state to a non-interactive protein-repellent state or vice versa. Thereby, effective chemical or physical stimuli can be selected based on the information of interactions between the protein and the platform, such as the type and strength of interactions, which are readily measured. The results of this study indicate, also, that important limiting factors for the function and application of the stimuli-responsive platforms are the degree of change and the rate at which the change occurs. In many cases the understanding of the underlying mechanisms is still limited and needs to be extended, so that improved, rational design of sophisticated systems can be accessed.

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