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Liposomes and Lipid Bilayers in Biosensors

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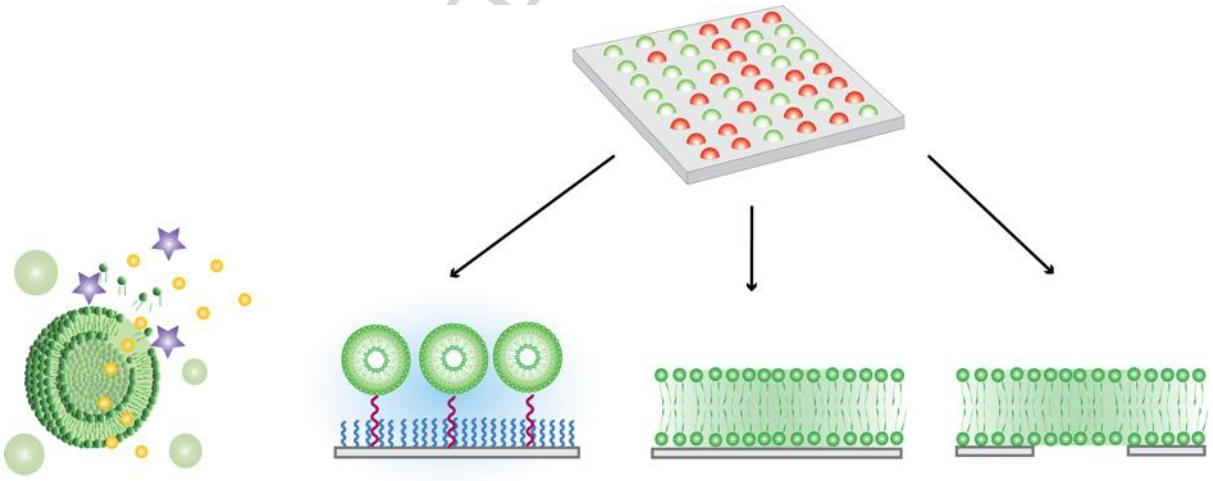
Abstract

Biosensors for the rapid, specific, and sensitive detection of analytes play a vital role in healthcare, drug discovery, the maintenance of food safety, and environmental monitoring. Although a number of sensing concepts and devices have been developed, many longstanding challenges to obtain inexpensive, easy-to-use, and reliable sensor platforms remain largely unmet. Nanomaterials offer exciting possibilities for enhancing the assay sensitivity and detection limits down to a single-molecule resolution. In this review, we present an overview of liposomes and lipid bilayers in biosensing applications. Lipid assemblies in the form of spherical liposomes or two-dimensional planar membranes have been widely used in the design of biosensing assays; in particular, we highlight a number of recent promising developments of biosensors based on liposomes in suspension, liposome arrays, and lipid bilayers arrays. Assay sensitivity and specificity are discussed, advantages and drawbacks are reviewed, and possible further developments are outlined.



Graphical abstract

Keywords: Liposome, Lipid Bilayer, Biosensor, Array, Biomarker

Contents

1. Introduction

Innovative analytical devices that enable fast, specific, and sensitive detection of compounds continue to be developed with importance in health, food and environmental monitoring. In the medical field, diagnostics are fundamental in the effective prevention or treatment of diseases, and there is a growing research and a substantial expansion in the capacity to identify molecular markers linked to diseases. Enzyme-linked immunosorbent assay (ELISA) remains the current gold standard in research and clinical diagnostics due to its high specificity, standard configuration, and ease of readability [1,2]. The ELISA principle has been adapted to improve the analytical performance of the assay, but practical challenges still exist. Its inherent limitations include the need for large amount of analyte, the many incubation and washing steps required, and it is slow and expensive. Alternative approaches towards simple, cost-effective, easy-to-use, reliable, specific, and sensitive assays are therefore essential and remain as challenges to be solved.

Biosensors are analytical devices used to detect the presence and concentration of a wide spectrum of analytes, including ions, proteins, nucleic acids, drugs, cells, among others [3-7]. Conceptually, biosensors contain three main components: 1) a recognition element (bioreceptor) to specifically interact with or to capture a target analyte; 2) a signal transducer to transform an analyte-receptor binding event into a measurable signal; and 3) a reader to output a result. Converting biological information into an easily read signal is challenging, and the overall performance of sensors is often determined by the interfaces between the sensing element and the biological sample at the nanometer scale.

Nanomaterials offer exciting possibilities for enhancing assay sensitivity; they include carbon nanotubes [8], gold and silver nanoparticles [9], and quantum dots [10]. Lipid assemblies in the form of spherical liposomes or two-dimensional planar membranes have played a key role in the design of biosensing assays [11-13]. Due to their similar components

and structures with cell membranes, they have been frequently used in protein-membrane interaction studies, in membrane protein biosensing, and in drug discovery research [14-16]. Lipid bilayer surfaces can be modified with bioreceptors (e.g., protein, peptide, nucleic acid, antibody, biotin, polymer) to perform recognition functions with a wide range of analytes. This, together with the intrinsic non-fouling nature of phosphatidylcholine bilayers makes lipid membranes excellent candidates in the establishment of a biosensing platform.

Biological samples such as blood, saliva, or urine are rich in physiological information. Therefore, biosensors that can monitor multiple analytes simultaneously will be beneficial for accurate analysis of one's physiological state. Parallel analysis of analytes (multiplexing) requires the integration of a large number of recognition elements on a surface that can be assembled in a geometrically defined pattern – an array. Accordingly, a number of biosensing strategies relying on liposome array and lipid bilayer array have been proposed.

In this review, we highlight and discuss selected recent and promising examples of liposomes and lipid bilayers in biosensing applications; in particular, biosensors based on liposomes in suspension, liposome arrays, and lipid bilayers arrays (Fig. 1). The fabrication processes employed to produce such sensors are outlined, and successful applications towards high-throughput detection and screening are illustrated. The advantages and limitations are reviewed and possible further developments are discussed.

2. Liposomes as biosensors

Liposomes are supramolecular assemblies of lipids, which self-associate to enclose an aqueous compartment surrounded by a lipid bilayer membrane. Several approaches have been developed for liposome formation; conventional methods include thin-film hydration, solvent injection, reverse-phase evaporation, sonication, and membrane extrusion, as well as novel methods using microfluidic technology [17,18]. Readers are referred to excellent reviews that

cover detailed aspects of liposome preparation and their physicochemical properties [17-20]. Liposomes are typically 20 nm to 10 μm in diameter with the phospholipid bilayer being 4-5 nm thick. In biosensing applications, liposomes are excellent signal transducers because they have a large surface area and a relatively large encapsulation volume, hence they can encapsulate large amounts of hydrophilic and hydrophobic signaling molecules across a wide spectrum of sensing modalities in the core or bilayer, respectively, and can thereby amplify the signal intensity. This allows the conversion of a one-to-one biological binding event into a one-to-many signal output occurrences, and in turn, considerably lower detection limits for analytes are observed. Signaling molecules (e.g., fluorescent and chemiluminescent markers, enzymes) can be encapsulated in liposomes via passive or active loading, with improved loading efficiency in the latter case [18]. In particular, their amphiphilic nature allows the incorporation of water-insoluble molecules, which would be otherwise challenging to retain their activities in aqueous environment. Once encapsulated, triggers including pH, enzymes, temperature, and light enable the regulation of the release of the signal markers from the liposomes, of which the intensity of the markers is modulated in a ratiometric fashion in direct proportion with analyte concentration. In addition to signal transducers, liposomes can also serve as recognition elements, for example for the detection of toxins and enzymes (lipases). This part of the review focuses on biosensors based on liposomes in suspension. Sensors based on tethered liposomes will be discussed in Section 3.

2.1. Liposomes as signal transducers

A wide range of signal markers have been encapsulated in liposomes without the need for chemical modification, e.g., fluorescent and chemiluminescent markers, chelates, enzymes, and nucleic acids [21-23]. Among them, enzymes are particularly interesting for biosensing applications. Enzymes are powerful catalysts that can convert substrates into

products (in biosensing application, products = signal markers) in a highly specific and selective manner. Enzymatic reactions can occur in aqueous environments, at relatively low temperatures, and typically at neutral or slightly acidic or alkaline pH. In addition, liposomes have been shown to preserve enzyme activities against unfolding and denaturation and to concentrate embedded enzymes [24,25]. The broad applicability and power of liposome-enzyme based approach is illustrated by discussion of selected examples.

Chi et al. developed a liposome-enzyme based biosensor for pesticide detection [26,27]. Enzyme acetylcholinesterase (AChE) was encapsulated within liposomes, and porins were embedded with the lipid membranes to control the selective transport of acetylcholine substrate into the liposomes. In the absence of analytes, acetylthiocholine chloride (substrates) can freely diffuse into the liposome cavities via porin channels and be hydrolyzed into thiocholine by active AChE, and the reaction can be followed electrochemically. Dichlorvos, an organophosphate pesticide, inhibits AChE activity leading to a decrease in the generation of thiocholine (Fig. 2a), and a limit of detection (LoD) of 0.68 μg/L of dichlorvos was achieved using this platform. In this case, liposomes with low enzyme permeability and high substrate permeability are prerequisites for their use as signal transducers.

In another example, Yang et al. developed a liposome-enzyme sensor for the detection of disease biomarkers, thrombin or C-reactive proteins (CRP) [28]. In this method, liposomes are used to noncovalently entrap amyloglucosidase or invertase for signal transduction and signal amplification. The liposome surfaces are coated with aptamers or antibodies and, in the presence of analytes, form a sandwich with aptamer- or antibody-functionalized magnetic beads. Triton X-100 is used to lyse the liposomes to release the enzymes, which can subsequently catalyze glucose generation for a final readout by a personal glucose meter (PGM) (Fig. 2b). With this method, thrombin and CRP can be quantitatively detected by a PGM within 1.5 h with a high detection limit of 1.8 and 0.30 nM, respectively, values which

are well-correlated with the LoD of the traditional ELISA method. PGM has been demonstrated as one of the most successful examples of patient self-testing due to their low price, compact size, easy operation, and reliable quantitative results. By varying the antibody or aptamer pairs according to the analytes of interest, this approach might be further extended to portable quantitative detection of a variety of disease biomarkers. In another approach, instead of using enzymes to generate glucose, an alternative strategy was demonstrated by Lin et al. [29] by directly releasing encapsulated glucose from liposomes in response to a target. With a similar principle as [28], treating the liposomes with surfactant released the encapsulated glucose in proportion to target concentration, which can be quantified by a glucose meter.

Among the fluorescent markers as encapsulants, self-quenching dyes (e.g., carboxyfluorescein) and fluorescence resonance energy transfer (FRET) agents are most widely used. Similar to liposome-enzyme based biosensors, lysis of liposomes is usually required to induce leakage of the fluorescent markers and to output a signal or to enhance the fluorescence intensity. In the FRET strategy, donor fluorophores can be inserted into the bilayer of liposomes. As the analyte-acceptor fluorophores interact with the liposomes, energy transfer takes place from donors to acceptors, and the modulation of fluorescence intensity is correlated with analyte quantification. In liposome sensors, the fluorescent strategy was successfully used to detect a range of analytes, such as endocrine disrupting chemicals [30], phospholipase A_2 [31], adenosine [32], *Cronobacter sakazakii* [33], chloramphenicol [34], mycotoxins zearalenone and aflatoxin B1 [35], phosphate [36], and neomycin [21]. To overcome the drawbacks of low loading dye efficiency and poor control of the amount of encapsulated dyes, Kim et al. demonstrated liposome labeling with rhodamine 6G (R6G) through the formation of H-aggregates driven by Coulombic interactions between R6G dyes and the phospholipids [21]. Liposomes are surface modified

with phosphatidylinositol 4,5-bisphosphate (PIP_2), a lipid component of cellular membranes, and its specific interaction with neomycin, an aminoglycosidic antibiotic, leads to the displacement of R6G dyes from the surface of the liposomes, inducing fluorescence recovery of R6G as a sensory signal. This assay has an LoD of 2.3 nM and represents one of the most sensitive aminoglycosidic antibiotic detection systems reported among liposome-based sensors. While fluorescent markers are a versatile strategy, it is inappropriate for human diagnostic applications since human serum albumin can interfere with the dyes used.

Polydiacetylene (PDA) is a unique conjugated polymer that exhibits color change upon conformational change of the conjugated backbone of the polymer. It provides chromogenic and fluorogenic responses – chromatic transition from blue (650 nm) to red (540 nm) and non-fluorescent to fluorescent (530 nm) in response to external stimulation [37-40]. These unique characteristics and the ability to manipulate them with a range of recognition elements make PDA liposomes ideal as signal transducers in biosensing. Recent examples of PDA liposome-based sensors are highlighted in Table 1; this includes sensors for ions [41-44], surfactants [45,46], gas [47], proteins [48,49], antibiotic [50], bacteria [51-53], and viruses [54,55]. For bacteria detection, most of the previously reported assays focus on the conjugation of sensors with receptors that can specifically bind to the bacterial strain itself. Mooney and Kim demonstrated an alternative route for sensing bacteria with high selectivity and sensitivity, where amine-functionalized PDA liposomes were used to detect bacteria indirectly based on the specific interaction of released surfactin (bacterial cyclic lipopeptides) and PDA liposomes [51]. Using this approach, they were able to distinguish *Bacillus subtilis* NCIB3610 (a surfactin-producing strain) with *Bacillus subtilis* SSB466 (a surfactin-less strain).

2.2. Liposomes as recognition elements

In a different case than the one presented in Section 2.1, liposomes can be used directly as recognition elements for the detection of analytes. Phospholipase A_2 (PLA_2) has been known to be upregulated in inflammatory diseases, and is a suitable biomarker for cancer, acute pancreatitis, and rheumatoid arthritis [56-59]. Numerous analytical methods have been developed to monitor PLA_2 activity [60,61]. However, many of these methods require the use of unnatural PLA_2 substrates that may alter enzyme kinetics. By taking advantage of the fact that phospholipids are natural PLA_2 substrates, Stevens et al. reported a liposome-based sensor for the detection of PLA_2 [31]. In this system, a helix-loop-helix polypeptide was encapsulated in liposomes. In the presence of biomarkers, PLA_2 cleaved liposomes and released encapsulated peptides, which caused aggregation of nanoparticles that can be readily detected through colorimetric responses [31]. This system is further translated into a lateral flow assay format, where the aggregation of nanoparticles was triggered by streptavidin-biotin binding events (Fig. 2c) [62-64]. A limit of detection of 1 nM of PLA_2 in human serum can be achieved and the assay can be completed within 20 minutes without expensive machine.

Liposomes can also be directly used for the detection of toxins secreted by bacteria. In this concept, liposomes encapsulate carboxyfluorescein dye, and exotoxins from *Staphylococcus aureus* and *Pseudomonas aeruginosa* destabilize liposomes and trigger encapsulated dye. By varying the lipid compositions, Thet et al. showed that the liposome sensors can be modified to respond to toxins from S*taphylococcus aureus* only, *Pseudomonas aeruginosa* only, and both S*taphylococcus aureus* and *Pseudomonas aeruginosa* [65]. Sensitivity of the liposome sensors to 40 clinically derived strains of S*taphylococcus aureus* and *Pseudomonas aeruginosa* was demonstrated.

3. Liposome array biosensors

This part of the review discusses recent advances in liposome-based arrays for membrane protein-related biosensing purposes. In contrast to the prior outlined concepts, surface adherent patterns aim at high-throughput sensing ideally employing heterogeneous array formats.

Liposome-based arrays for biosensing are an approach that holds promise for fast throughput multiplexed membrane protein analysis. However, despite the early successes starting around 10 years ago, the field seems to have stopped making tremendous progress in the past 5 years. The initial concepts to assemble homogenous or heterogeneous (single) liposome arrays were discussed in our prior review [12]. Herein, we focus on subsequent developments, highlighting the most exciting advances.

While microcontact printing and photolithography-based approaches have dominated the early (indirectly) creation of liposome arrays, alternatives have been explored, including laser-induced forward transfer [66], the use of chitosan-modified breath figure pore arrays [67], robotic printing of azide reactive (modified) liposomes [68,69], focus ion beam to drill sub-micron sized holes into glass for peptide-mediated single liposome immobilization [70], or micro-transfer molding of liposome-containing hydrogels [71]. Selective recognition elements to immobilized liposomes on surfaces in an array format are a key aspect towards the creation of heterogeneous arrays. While the early approaches focused on the interaction of complementary DNA strands [72,73], the Kros group illustrated that peptide coiled-coil binding could also serve this purpose [74]. In an attempt to shorten the liposome arraying time, Sugawara and coworkers reported a reusable array by employing a cleavable disulfide-based linkage between the liposomes and the substrate [75].

Multilayered liposome arrays have the potential to overcome low loading efficiencies for sensing and (drug) delivery applications. However, the numbers of reported examples are

limited [76-80] with even fewer examples considering liposome multilayers in an array format. An interesting example was reported by the Ravoo group employing microcontact printing to provide the chemical contrast for the initial liposome patterning [81]. The 3D liposome structure was then assembled using cyclodextrin-modified liposomes containing biotin displaying host molecules, which were connected into multilayers via streptavidin. Similarly, Sakamoto et al. aimed at the detection of undecapeptide substance P (SP), a neuropeptide, using $F_{ab'}$ fragment of anti-SP modified liposomes and gramicidin-aided fluorescence enhancement [82]. The modified liposomes and SP were mixed in solution and allowed to interact prior to the liposome catching using an array modified with anti-SP. Sub-pg mL^{-1} SP was detected in (very) diluted serum in shorter times and with better detection limit compared to a commercial competitive enzyme immunoassay.

PDA [83], which undergoes colorimetric transition due to external stimuli (temperature, pH, pressure etc.), has been considered in the context of liposome-based microarrays as a sensory element for probe-target detection. Seo et al. fabricated PDA liposome arrays for the detection of influenza A virus M1 antibody and the Influenza A H1N2 virus itself [55]. They not only achieved a detection limit (2^{-2} HAU) comparable to commercial kits when non-polymerizable lipids were added to the PDA liposomes, they also concluded that PDA sensory signal is mainly related to the steric repulsion and not the binding strength between probe–target complexes. In an alternative approach, a PDA liposome array was employed for the detection of aminoglycosidic antibiotics [84]. The concept was based on mimicking the interaction of neomycin with PIP_2 phospholipids (Fig. 3ai) and a detection limit of 61 ppb for neomycin was identified. Further, comparing different aminoglycosidic antibiotics, neomycin yielded the highest fluorescent signal, likely due to its higher charge density and molecular weight leading to stronger interactions with the PDA–PIP_2 liposomes and greater induced stress, respectively (Fig. 3aii). In an attempt to enhance the sensitivity, Lee and coworker

proposed a 3D networked PDA sensor system using PDA vesicles immobilized in a 3D carbon nanotube pillar network [85]. The authors found a more sensitive and better response upon exposure of the 3D sensor system to different concentration of cyclodextrins in comparison to a 2D sensor system.

Giant unilamellar vesicles (GUV) [86,87] are widely used to assess and visualize membrane-processes. In this context, Kang et al. reported an interesting approach based on microcontact printing to assemble arrays of membrane protein containing GUVs [88]. Upon stamping of proteoliposomes (small unilamellar vesicles (SUV) containing proteins) using a agarose hydrogel stamp and subsequent electro-formation (Fig. 3bi), over 30 copies of the same GUV arrays could be fabricated with the same stamp and the need for additional membrane protein reconstitution was avoided. This concept was exemplified by employing aquaporin Z (AqpZ) SUV for the creation of a GUV array (Fig. 3bii). Further, the preserved function of AqpZ was confirmed by encapsulating 100 mM glucose and replacing the environmental solution with 100 mM glucose plus 50 mM NaCl solution (Fig. 3biii). Compared to the control liposomes, the size of AqpZ-containing GUVs decreased much faster indicating that the channel retained its bioactivity and facilitated water out-flux from the GUVs.

Due to their size, GUVs are also envisioned as cell mimics or as artificial cells on a microchip when put in a microarray format. While this challenge has not been accomplished yet, efforts towards this goal are promising. For instance, GUV array formation in the presence of the GFP synthesis system has been shown [89]; lipid bilayer-lipid bilayer contact [90] or streptavidin-biotin linkages [91] have been used to mimic tight cell junctions employing patterned GUVs; and the application of a short-time pulse voltage [92] or an array of through-holes [93] was considered to release GUVs from the surface. Further, the

reversible binding of GUVs to a lysosome array was suggested as a new tool to separate non-encapsulated reagents from loaded GUVs [94].

Exosomes, cell derived vesicles, have recently attracted considerable interest due to the high level of information they carry relevant for diagnosis and treatment of a variety of medical issues. Detection of exosomes however, is a complex challenge because of their small size, their inherent heterogeneity, their rather low abundancy, as well as the complexity of their environment as recently reviewed by Kastelowitz and Yin [95]. Concepts based on microfluidics [96] or capturing exosomes in microarray format would be very beneficial for their multiplexed analysis. Jorgensen et al. have realized the latter concept using antigenic capturing of exosomes for multiplexed phenotyping (Fig. 3ci) [97]. Employing an array of 21 different cellular surface antigens, the authors were able to phenotype plasma-derived exosomes from 7 healthy donors (Fig. 3cii). Although this study focused on healthy individuals, the same concept should be applicable to use exosomes as diagnostic markers when detectable in a sensitive and high-throughput manner without the need for purification and/or enrichment. In a follow up publication, they successfully extended this concept to up to 60 printed capturing antibodies to detect and phenotype exosomes from plasma [98].

4. Planar lipid bilayer array biosensors

Planar lipid bilayers are formed with a similar architecture to liposomes but are typically confined to surfaces. They represent an attractive alternative to liposomes in biosensor technology in a number of cases. Supported lipid bilayers (SLBs), i.e. planar lipid bilayer structures sitting on a solid support, have attracted considerable interest when probing membrane-related biomolecular interactions due to their compatibility with surface-sensitive techniques including label-free optical biosensors, electrochemical sensors, or with fluorescence-based imaging techniques. Planar bilayers spanning over micro- [99,100] or

nanoscopic [101-104] apertures, herein referred as suspended lipid bilayers (SusLBs), further offer the possibility of permitting access the membrane from both sides of the membrane facilitating the measurement of molecular and ionic transport across membranes. In this part of the review, we focus on the development of experimental strategies allowing for assay multiplexing using planar lipid bilayers.

Planar lipid bilayers can be formed by taking advantage of the surfactant properties of the amphiphilic lipids making up the membrane. For example, methods involving the painting of lipid solutions across apertures have been proposed. Solvent-based deposition however has the disadvantage that solvent residue may affect structure and function of the membrane proteins or its mechanical properties. Bilayer deposition can also be achieved solvent-free using Langmuir-based techniques [105] relying on sequentially spreading lipid monolayers at an air-water to construct the bilayer. Alternatively, planar membranes can be obtained by substrate-induced vesicle fusion [106]. Indeed, small unilamellar lipid vesicles (SUVs) have been shown to spontaneously rupture and spread when in contact with a solid support leading to the formation of a planar bilayer. The advantage of this method lies in its simplicity and reproducibility as well as in the good quality of the obtained bilayer, which exhibits low numbers of defects [103]. The vesicle rupture process is however highly dependent on the interaction between the substrate and the vesicle and on the vesicle's mechanical properties, limiting the use of this technique to certain substrates (glass, mica or silica or indium tin oxide have commonly been used) and to vesicles of selected composition, although the vesicle's fusogenecity can be improved by osmotic shock [107,108] or using peptides inducing membrane rupture [109]. A coating can also be applied to a substrate which would not induce SLB formation otherwise, leading to the formation of so-called tethered-SLBs, if bilayer and surface are connected by a molecular tether [110,111]. Soft polymeric tethers can offer the additional advantage of increasing the distance between the

substrate and the membrane, thereby limiting the interaction between bulky transmembrane protein domain and the surface, avoiding their denaturation [112].

4.1. Arrays of supported lipid bilayers

SLBs have been used in a variety of biosensing applications. These include the investigation of the interactions between soluble ligands and membrane proteins, the study of membrane-binding proteins, or the detection of interactions mediated by (glyco)lipids in a fluid and native-like environment. Because of this potential in biosensing applications, efforts have been devoted to the development of highly parallelized microfluidic platforms or of bilayer patterning methods towards assay multiplexing.

Microfluidic systems capable of handling sub-microliter volumes of fluids reliably and efficiently have attracted considerable interest in biosensing technology during the last decade. Indeed, they promise to lead to the development of comprehensive point-of-care lab-on-a-chip devices capable of performing bioanalytical procedures rapidly, accurately, with low sample consumption [113]. Accordingly, microfluidic systems, typically consisting of separated parallel channels have been among the first approaches proposed to multiplex SLB-based bioanalytical assays offering the advantage of creating SLB microcompartments that can be individually addressed with aqueous solutions to perform multiplexed biomolecular binding assays on supported lipid bilayers [114-116]. Additional multiplexing capability of such devices was demonstrated by creating bilayer patches of different compositions within the channels [117] or using a cross-patterning approach by sequentially applying channels perpendicular to each other [107,108].

A device consisting of parallel channels was used starting in the years 2000 in the context of probing interactions mediated by membrane-embedded glycolipids [115,116,118] or to gain fundamental biophysical understanding of multivalent interactions [119]. In the last

5 years, the device was further implemented in conjunction with new types of signal transduction. Indeed, Cremer et al. proposed to use SLBs containing pH sensitive fluorescent lipids [120] to probe the binding of charged analytes to SLB without labeling the analyte itself. For signal transduction, this approach therefore relies on the change in fluorescent signal resulting from the change of the local interfacial pH upon biomolecule [121] or Cu^{2+} ion binding [122].

The success of protein and DNA microarray technology encountered over the last decade [123,124] has stimulated the development of strategies to create microarrays of membrane patches towards highly multiplexed investigation of biomolecular interactions involving membrane proteins or (glyco-)lipids. Over the last two decades a number of methods to pattern supported lipid bilayer were proposed yielding mostly bilayer arrays of a single composition [125]. These approaches include, among other, microcontact printing of vesicle solutions, the use of patterned substrates or the use of deep ultraviolet lithography to degrade locally a bilayer through UV illumination as reviewed in [12,126]. Naturally, a number of methods have been proposed in the last five to ten years to further develop such approaches towards the preparation multicompositional arrays. Furukawa et al., for example, take advantage of lipid self-spreading on a patterned surface to create parallel SLB lines on SiO_2 patterns corralled by gold, after loading the lipid mixture in a reservoir connected to the lines and gentle hydration [127] (Fig. 4a). Majd et al. presented a method to create a heterogeneous array based on microcontact printing. In this approach topographically patterned hydrogels are manually loaded with membrane preparations, which are then stamped onto the substrate [128,129] (Fig. 4b). With this method multiple copies of an array can be easily produced with minimal sample consumption. Stamped arrays were used to probe the interaction between a protein (annexin V) or an anti-inflammatory drug

(nimesulide) with membranes of different compositions [129]. Arrays of membrane proteins were also generated [128].

To increase array reproducibility, throughput and multiplexing capability, efforts have been directed at adapting commercially available array printers, so called spotters, to create membrane arrays. Such devices allow for the automation of the patterning process and rely on delivering small amounts of vesicle suspension to pre-defined location on the surface. However, since very small volumes are typically deposited (typically nanoliters), evaporation becomes important leading to difficulties considering the fragile nature of the membranes and in particular their lack of air-stability. Spotting membranes therefore requires either the development of methods to stabilize the bilayer or the printing process to be carried out in aqueous solution or in highly humid environments to avoid drying of the small spotted volume. Spotting of membrane arrays containing G protein coupled membrane receptor (GPCR) [130,131] or ganglioside for toxin detection [132] onto surfaces coated with γ–aminopropylsilane (GAPS) was reported by Lahiri et al. as early as 2002 although bilayer formation on such GAPS layers and membranes stability has been a matter of debate [133]. Spotting on cholesteryl-poly(ethylene glycol) (PEG) chains has been proposed more recently as an alternative to produce membrane bilayer arrays while ensuring air-stability and preserving membrane fluidity [134]. Such glycan microarrays obtained by vesicle fusion on a PEG-cholesteryl surface were used to produce fluid spots of mannose moieties of controlled concentration. These arrays were used to study the multivalent binding of bacteria to mannose and to reveal new a adhesion mechanism [135]. As an alternative to the use of surface-coatings to stabilize the membrane, the bilayer membrane structure can be preserved by spotting in a high humidity environment [136] or even in a liquid. In 2009, Binkert et al. [137], for example, prevent lipid-drying by multiple spotting into arrays of microwells using a contact-mode printer (Fig. 5a). In this case, a microwell array consisting of >1000 PDMS

wells/cm^2 deposited on an optical waveguide for highly sensitive fluorescence-based read-out, was coated with SLBs. In this case, evaporation was further limited by adding glycerol, a now volatile compound. In this configuration, the array spot size was decreased to 100 µm while the distance between the wells was 200 µm. Direct dispensing through a thin water film was also reported. In 2011, Kaufmann et al. [138] used a non-contact printer to deposit vesicles directly on a hydrophilic surface through a thin liquid interface (Fig. 5b). In 2013, Yamada et al. produced prepatterned surfaces where exposed glass patches corralled by cross-linked polymeric 1,2-bis(10,12-tricosadiynoyl)-sn-glycero-3-phosphocholine (DiynePC) are first filled with an aqueous solution containing agarose and trehalose, followed by printing vesicle suspensions into the droplet (Fig. 5c) [139]. In both cases, the lipid bilayer never leaves the aqueous environment. Prepatterning, albeit experimentally much more cumbersome, allows for the creation of smaller membrane patches. Most recently, efforts have been put into finding solutions to the production of stable and storable membrane microarrays, which would greatly facilitate membrane array commercialization. In this context, the disaccharide trehalose has been proposed as a promising printing compound as it has been shown to protect lipid membranes to a large extent against dehydration [140]. Most interestingly, the use of trehalose in the vesicle spotting buffer offers the possibility of creating vesicle that can be vitrified for storage in dehydrated state. These droplets will upon rehydration and rinsing with buffer, lead to the creation of SLB patches of good quality [141,142] (Fig. 5d). In proof-of-principle experiments, the label-free investigation of individual binding responses of cholera toxin to gangliosides was carried out using rehydrated microarrays and imaging surface plasmon resonance [141]. Generally, such an approach which increases the storability of membrane-based microarrays promises to facilitate the implementation of such platform in a variety of biosensing applications.

As an alternative to adapting commercial printers research efforts have been directed at designing microfluidic devices capable of depositing supported lipid bilayers while overcoming the limitations of spotting small volumes. The continuous flow microspotter developed by Conboy et al. for example, is a microfluidic device made of PDMS and consisting of a series of inlet of outlet wells connected by an inlet and outlet channel (Fig. 6a) [143]. When the printing head is in contact with the substrate, a continuous channel is formed, allowing for a flow of vesicle solution over the substrate. This leads to the formation of up to 48 well-delimited membrane patches 400 µm × 400 µm with a pitch of 875 µm [144]. This device was used to created membrane microarrays in the context of probing protein-ligand [145] or small molecule -membrane interactions [146]. These arrays were, for example, used in conjunction with counter-propagating second harmonic generation imaging to probe the interaction between the anesthetic drug tetracaine and membranes of different composition. After biorecognition, the bilayers could be stabilized through crosslinking, making the arrays stable in vacuum and thereby allowing for subsequent investigation with MALDI-TOF mass spectrometry [145]. In 2013, Jesorka et al. further presented a microfluidic toolbox to draw two-dimensional supported lipid membrane networks. Their multifunctional pipette, a hydrodynamically confined flow device, allows for the creation of complex patterns of supported lipid bilayers by locally dispensing small lipid vesicles. This method therefore offers unprecedented possibilities to draw complex membrane networks, including membrane arrays to study membrane-related processes [147] (Fig. 6b).

The size of the membrane patches can be scaled down using nanopatterning methods such as dip-pen nanolithography (DPN), a technique based on the use of an atomic force microscope (AFM) tip to create patterns on a surface by depositing molecular inks on the substrate [148]. The creation of heterogeneous multilayer lipid nanopatterns with characteristic sizes in the 100 nm range [149] and their use to probe biomolecular interactions

[150] was first reported by Lenhert et al about 10 years ago. Large scale arrays can be obtained using a two-dimensional cantilever array consisting of 55 000 tips covering 1 cm^2, making this method promising in the context of high density arrays. In the past 5 years, moving towards biosensing applications, lipid-DPN was used to generate allergen arrays by stamping the allergen 2,4-dinitrophenyl[1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[6-[(2,4 dinitrophenyl)amino] hexanoyl] (DNP) together with the phospholipid 1,2-dioleoyl-sn-glycero- 3-phosphocholine (DOPC). The allergen efficiently captured allergen-specific IgE antibodies [151]. Lipid-DPN printing of membrane patches on graphene and subsequent detection of biotin-streptavidin interactions was also reported reently, opening the way of bio-functionalize those surfaces for biosensing applications [152].

4.2. Arrays of suspended lipid bilayers

While SLBs are extremely valuable in the context of probing membrane-mediated biomolecular interactions, their implementation in the context of studying transport processes across the membrane remains limited due to the inherent presence of a surface under the membrane. SusLBs have therefore been proposed as an alternative. Experimental setups based on SusLB make it possible to study membrane-protein mediated transport across membranes, in particular ion transport via ion-channel. This transport is often activated by an external stimulus, ligand-binding in the case of ligand-gated ion channels which are switched on and off by biomolecular interactions. Because channel dysfunction is a major cause of disease (e.g., epilepsy, diabetes, hyperthesion and other cardiac conditions [153]) but also because many therapeutic agents target ion channels either by interfering with the ligand-gating process or through a channel-blocking action, these membrane proteins are a common target of biosensors. In addition to this, attempts have been made to engineer relatively simple ion-channels (e.g., the self-inserting peptide gramicidin or staphylococcal α-

haemolysin) as sensory elements in electrochemical biosensors. Here, the general idea is to chemically modify ion-channels in such a way that binding of the analyte of interest results in a change in pore conductivity. In such an assay, the molecular recognition element (i.e. the engineered ion-channel) becomes therefore responsible both for the specific detection of the target and for signal amplification, based on inducing large changes in the total ion flux by a single molecule detectable by electric read-out [154,155]. Finally, SusLB make it possible to study transmembrane receptors which need to be accessible from both sides. This is the case, for example when studying GPCRs, a major target in drug discovery. GPRC functional studies indeed require that the receptor is accessible both from the extracellular side, where it becomes activated upon ligand binding, and from the intracellular side, where it interacts with the G-protein complex. As detailed below, experimental setups based on microfluidics and integrated lab-on-a-chip devices have been proposed towards SusLB assay multiplexing.

A first type of setups is typically based on the solvent-based deposition of a bilayer on top of micron-sized aperture contained with a well connected to microfluidic channels. Assay multiplexing is achieved by parallelizing the wells [156-160], although electric read-out is often addressed separately for each well, due to the rather complex electronics needed for parallelization of electrical measurements making scaling a complex task. Several groups have nevertheless demonstrated parallel ion-current monitoring. Towards highly multiplexed assays, Takeuchi et al. showed that such a setup can be scaled to a 96-well plate [161] (although the current is detected by sequencially moving the recording electrode), a configuration which was used to detect the current through reconstituted gramicidin pores [161] but also to detect DNA translocation though profiling of the blocked current [162].

As an alternative, membranes have been spanned over an aperture using droplet interface bilayers (DIB) which are formed, for example, at the contact of two aqueous drops in an oil solution carrying lipid monolayers at their interface [163] or at the contact between a

monolayer at the air-oil interface and a lipid-coated aqueous droplet. Membrane proteins can be incorporated into the bilayer and the ionic current across the pores or channels can be measured by embedding electrodes within the droplets or aqueous solution [164]. The fluorescence-based detection using fluorescence detection in response to an ion molecular flux represents an alternative. In 2012, Castell et al. for example, use an array of droplets in contact with a hydrogel-supported monolayer to probe calcium transport through alpha-hemolysin using a calcium-sensitive fluorescent dye [165]. Using chips with multiple cavities [164], or even setups relying on 96 well-plates [166] experiments can be performed in parallel. Nevertheless, electrical read is often performed in a sequential manner using movable electrodes with some exceptions [167,168]. An exception is however, for example, the automated chip presented recently by Takeuchi et al. that allows for recording in 16 channels [167].

In proof-of-principle experiments the incorporation of channels such as gramicidin, alpha-hemolysin or alamethicin and the resulting ion-conductance are reported [166]. In the example by Syeda et al. based on a two-droplet system, one droplet is filled with a cell-free in vitro transcription and translation mixture, leading to the production and insertion of a viral potassium channel. Channel blockers are then added to the other droplet allowing for the rapid screening of their influence on the pore activity [164].

5. Conclusions and future perspectives

We have illustrated a number of promising examples of liposomes and lipid bilayers in biosensing applications. Liposomes offer advantageous physical and chemical properties as biosensors, including their large internal cavity for encapsulation of signal markers, high surface area, and the ability to conjugate liposome surfaces with a range of recognition elements that allows the detection of a wide variety of biomolecular analytes. Planar lipid

bilayers are of interest when probing membrane-related biomolecular interactions and for monitoring the measurement of molecular and ionic transport across membranes. While there is an abundance of developed concepts, there are many interesting challenges ahead before the full potential of liposome- and lipid bilayer-based sensors can be realized into a biochip that is applicable for the detection of analytes in complex physiological media (human serum or human whole blood). These include improving the stability of liposomes and lipid bilayers, minimizing leakage of encapsulated markers, and preventing liposomes against fusion. To date, sugars have been used to preserve liposome integrity and to protect membranes from solute leakage during drying [169]. Beyond materials development work, we believe that the use of computer simulations will be an important tool for understanding phenomena and optimizing sensor performances, in particular for shortening the detection period. Currently, the assay time is limited by changes in membrane permeability e.g., to release signaling molecules. The advent of multiplex technology complements an ongoing shift towards personalized medicine. It is highly likely that multiplex assays will continue to garner popularity, and overcoming the challenges of the aspects mentioned above will enable the development of liposome- and lipid bilayer-based biosensors for rapid, precise, and convenient point-of-care clinical diagnosis.

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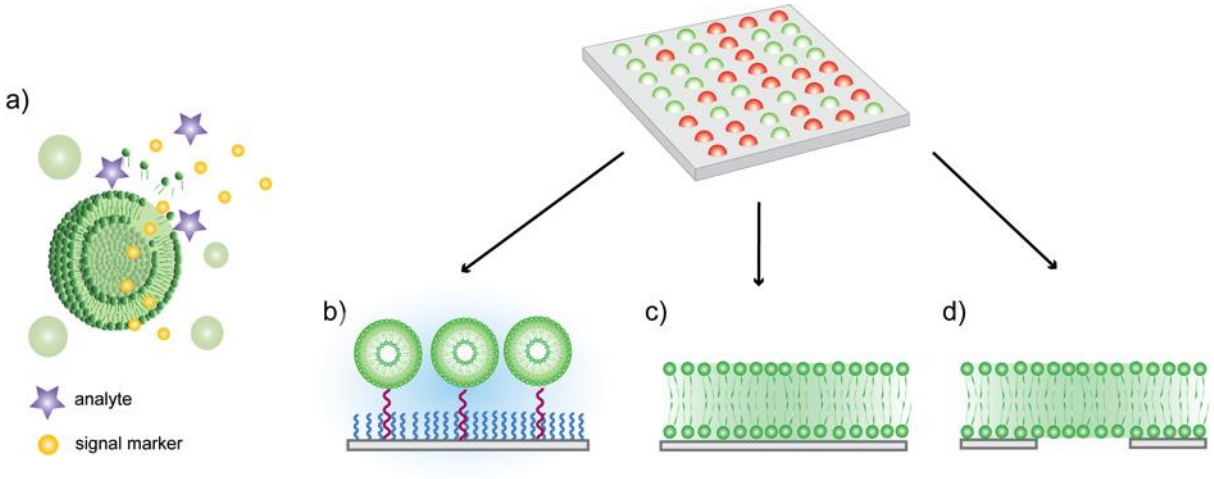

Fig. 1. Liposomes and lipid bilayers in biosensors. a) Biosensors based on liposomes in suspension, b) liposome arrays, c) supported lipid bilayers arrays, and d) suspended lipid bilayer arrays.

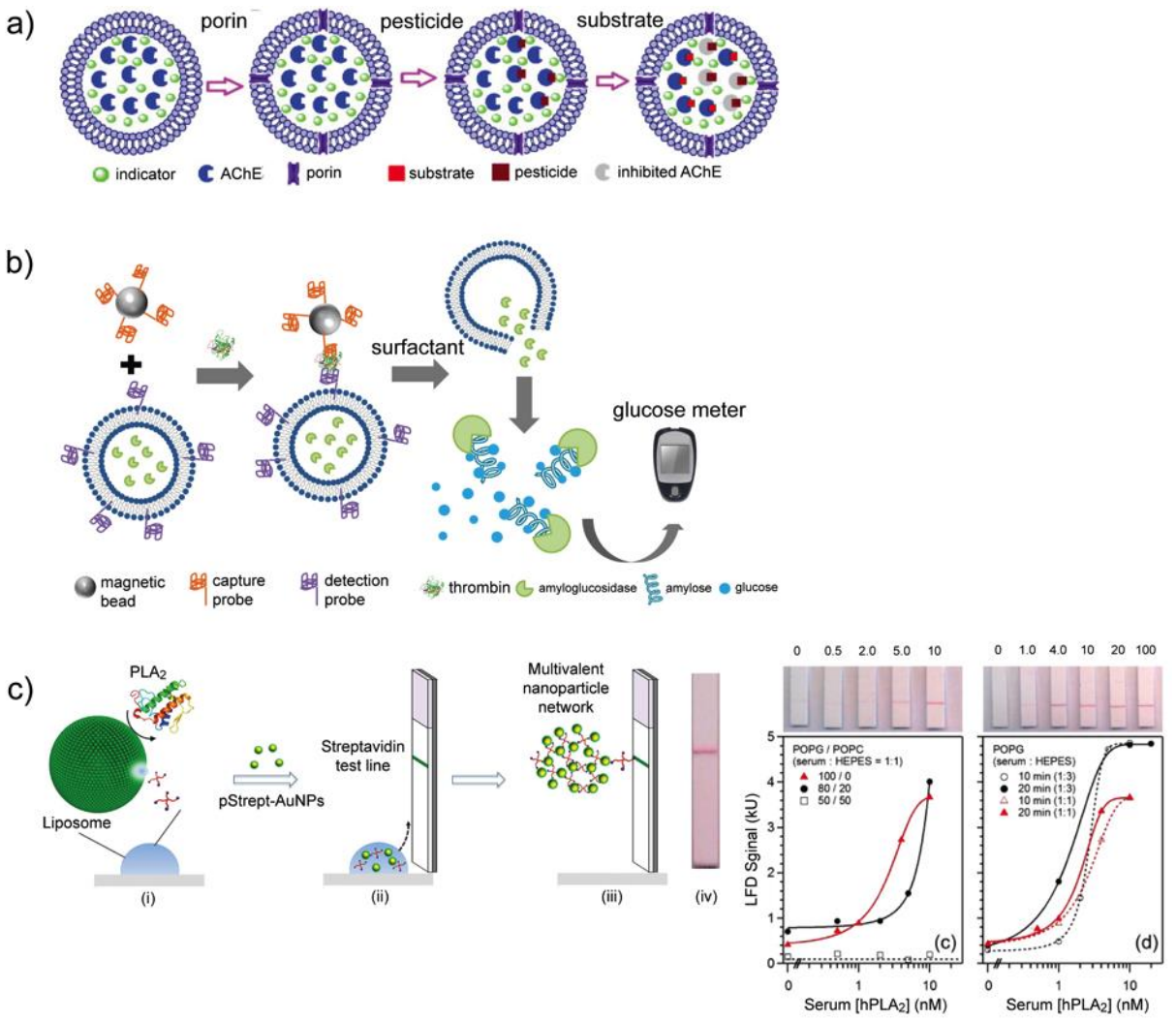

Fig. 2. a) A liposome-enzyme based biosensor for pesticide detection. Acetylcholinesterase (AChE) is entrapped within the liposomes and porins are embedded into the lipid membrane to allow for free substrates and pesticide (analytes) to pass through the membranes. The pesticide inhibits AChE activity, leading to a decrease in the generation of products. Pesticide detection and the inhibition reaction is monitored electrochemically. Reprinted with permission from [26]. b) A liposome-enzyme based biosensor for thrombin detection. Capture aptamer or antibody (capture probe) functionalized magnetic beads are first introduced into a sample solution to capture the target protein, followed by the addition of detection aptamer or antibody (detection probe) functionalized liposomes. Capture probe and

detection probe bind with the protein to form a “sandwich” structure. After washing, Triton X-100 (surfactant) is added to lyse the liposomes leading to release of a large amount of amyloglucosidase encapsulated in the liposomes, which catalyzes amylose to produce a large amount of glucose that can be quantitatively monitored by a personal glucose meter. Reprinted with permission from [28]. c) Liposome sensors for the detection of phospholipase A_2 (PLA_2). Liposomes encapsulating biotinylated poymer linkers are mixed with a sample solution and streptavidin-coated gold nanoparticles. A lateral flow strip is introduced and the solution mixture runs up the nitrocellulose membrane by capillary forces. In the presence of PLA_2, the enzyme cleaves liposome and releases PEG linker. If the liposome is cleaved, the linker will adhere streptavidin-coated gold nanoparticles to the test line and form multivalent nanoparticle networks, which can be read with the naked eye due to the localized surface plasmon resonance (LSPR) effect of the gold nanoparticles. Adapted with permission from [62].

Table 1. Examples of polydiacetylene-based sensors for the detection of various classes of analytes

Classification of analytes	Targets	Ref.
Ions	Zn^{2+}	[41]
	Cu^{2+}	[42]
	Pb^{2+}	[43]
	Hg^{2+}	[44]
Surfactants	Anionic surfactants	[45]
	Cationic surfactants	[46]
Gas	CO_2	[47]
Proteins	Phosphinothricin acetyltransferase (PAT)	[48]
	β-glucuronidase	[49]
Antibiotic	Neomycin	[50]
Bacteria	*Bacillus subtilis*	[51]
	Salmonella	[52]
	Escherichia coli	[53]
Viruses	H5 influenza virus	[54]
	Influenza A virus	[55]

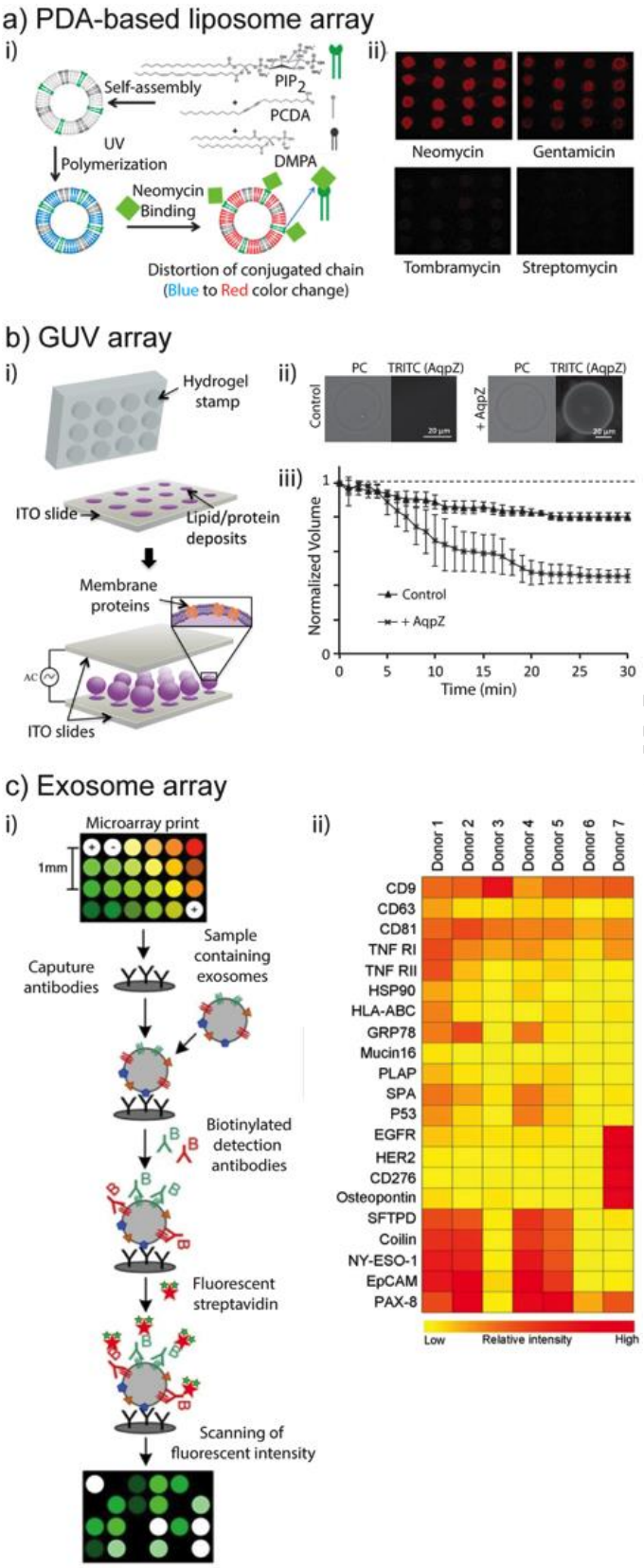

Fig. 3. Liposome arrays: a) i) Cartoon showing the sensing principle for neomycin based on PDA-liposomes containing PIP_2 as a specific receptor for neomycin. ii) Fluorescence microscopy images of PDA-PIP_2 liposome microarrays upon exposure to different

aminoglycosidic antibiotics showing the highest intensity for neomycin. Reprinted with permission from [84]. b) Schematic illustration of the GUV array fabrication process based on the printing of (protein-containing) SUV followed by electro-formation and localized fusion. ii) Protein free (left) and AqpZ-containing (right) GUV after exposure to primary and fluorescent secondary antibodies against AqpZ. (PC: phase contrast). iii) The time dependent volume changes of AqpZ-containing and control vesicles that caused water out-flux from the GUV due to an osmolality difference inside and outside of the GUVs. Reprinted with permission from [88]. c) Cartoon illustrating the exosome sensing approach based on a custom-made protein array. The immobilized exosomes are detected using biotinylated antibodies against CD9, CD63 and CD81. Finally, fluorescently labeled streptavidin is employed visualize the array. ii) Overview over the phenotyping of exosomes in plasma of 7 healthy donors. Reprinted with permission from [97].

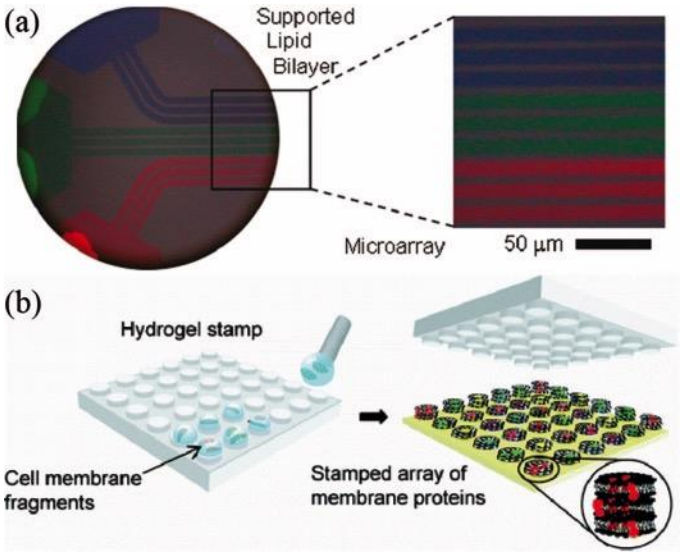

Fig. 4. (a) Bilayer array formation by lipid spreading. Each lipid reservoir is connected to 3 SiO_2 lanes corralled by gold. Reprinted with permission from [127]. (b) Bilayer array formation upon stamping with a hydrogel stamp. Different SUV, proteoliposome or membrane preparations are loaded to the pillars. The membranes are then stamped leading to the formation of bilayers or multibilayers. Reprinted with permission from [128].

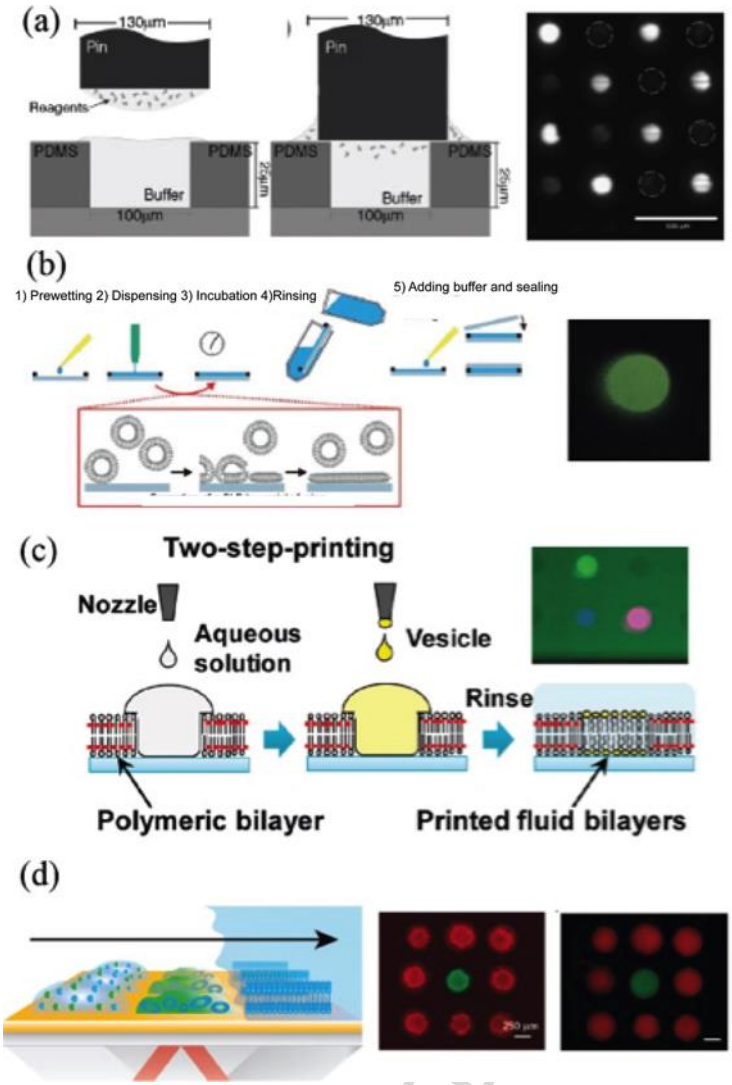

Fig. 5. (a) Spotting into nanowells using a contact printer together with a microarray reader image of alternating spots of DOPC (control) and DOPC/DOPS (9:1) bilayers after incubation with annexin V. Reprinted with permission from [137]. (b) Spotting into a liquid film using a non-contact printer together with a fluorescence image of a fluorescently labelled bilqyer spot. Reprinted with permission from [138]. (c) Spotting into droplets onto a pre-patterned surface. The array image shows the deposition of multiple fluorescent-labelled bilayers in the corrals. Reprinted with permission from [139]. (d) Spotting small unilamellar in trihalose and formation of a supported lipid bilayer after dehydration. Array of two fluorescent bilayers before (middle) and after hydration (right). Reprinted with permission from [141,142].

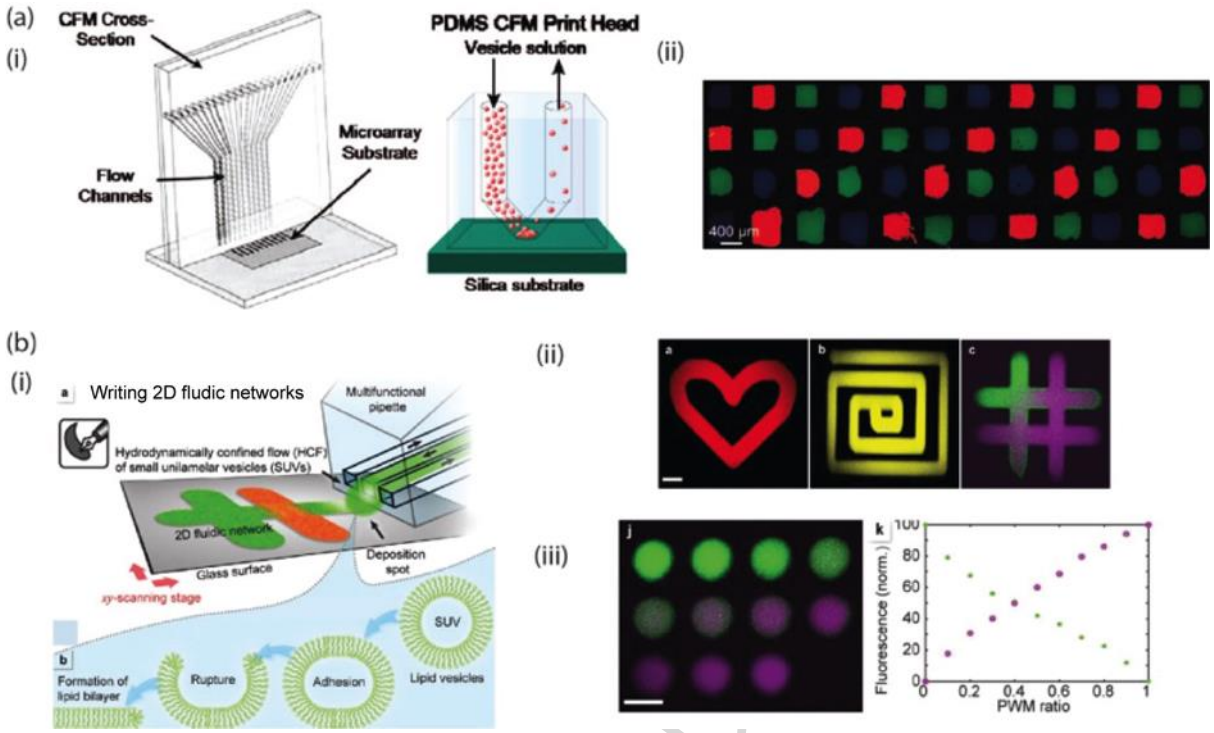

Fig. 6. Microfluidic printing of supported lipid bilayers. (a) (i) Continuous flow microspotter (ii) together with an example of the produced spots (fluorescently-labelled SLBs). Reprinted with permission from [144]. (b) (i) Micropipiette-based approach to print SLBs in complex patterns. (ii) Complex patterns consisting of one or several types of fluorescent lipids. (iii) Spots of lipid mixtures formed of gradually changing composition. Reprinted with permission from [147].

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Liposomes and Lipid Bilayers in Biosensors

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Highlights

- Biosensors play a vital role in health, food and environmental monitoring
- Lipid assemblies in the form of spherical liposomes or 2D planar membranes have been widely used in the design of biosensing assays
- Liposomes have large internal cavity for encapsulation of signal markers and high surface area for conjugation of recognition elements
- Planar lipid bilayers are of interest for probing membrane-related biomolecular interactions
- Parallel analysis of analytes (multiplexing) requires the integration of recognition elements in an array