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Biomolecular Sensing with Colorimetric Vesicles

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Abstract This chapter summarizes recent studies employing colorimetric vesicle-based systems for biomolecular sensing. Vesicular aggregates exhibit an important advantage as a biological sensing platform in that they mimic the cell membrane—the site of molecular docking, ligand–receptor binding, and other important processes that can be exploited as a means of signal generation. Particularly attractive for sensing applications is the use of colour changes visible to the naked eye or detected spectroscopically as the signal transduction mechanism.

Vesicle assemblies comprising polydiacetylene (PDA)—a chromatic polymer that undergoes blue–red transformations in response to varied biological analytes and processes—are the primary focus of this chapter. We discuss the features of PDA that make it a promising constituent in biosensing platforms, in particular its self-assembly properties, the rigid framework allowing incorporation of varied lipid constituents, and the chromatic transformations induced by reactions with biological analytes.

Recent studies depicting distinct vesicle assemblies are summarized. Vesicles comprising chemically modified PDA, in which receptor units are attached to the polymer-surface head groups, have been employed for detection of chemical and biological toxins, viruses, and bacteria. Mixed vesicles in which lipid bilayer domains are incorporated within the PDA matrix have also been extensively used as colorimetric biomimetic membrane platforms for studying diverse membrane processes and cell-surface phenomena. The PDA-embedded lipid bilayers further facilitate anchoring of varied molecular markers and recognition modules.

Keywords Biomimetic sensors · Chromatic polymers · Colour biosensors · Polydiacetylene · Vesicle biosensors

Abbreviations

CR	Colorimetric response
DMPC	Dimyristoylphosphatidylcholine
ELISA	Enzyme-linked immunosorbent assay
PDA	Polydiacetylene

1 Introduction

Development of chemical approaches for the detection and screening of biological molecules is an important and highly active field of research because of the scientific and practical significance of biosensors. Numerous bioanalytical technologies have been developed for the identification and study of biological compounds [1–5]. The utilization of *colour* as the transduction mechanism in biosensor technologies is particularly attractive. Besides the obvious advantage of identification of colour changes with the naked eye, colorimetric transitions can also be easily recorded using conventional apparatuses such as spectrophotometers or ELISA plate readers. An additional feature of colour detection is the possibility of coupling the colorimetric sensor with existing optical technologies, such as optical fibres, optical signal processing, and others. Examples of colour-based sensor technologies include monosaccharide detection by boronic acid derivatives [6], colour sensors based on active interference filters constructed from silicon-compatible materials [7], vapochromic detection of organic compounds using optical fibres [8], optical thin-film waveguide ion sensors [9], flow-through cell detectors based on colour-changing organic compounds [10], colorimetric toxin detection [11], and other molecular dye applications [12–15].

This chapter focuses on *vesicle-based* colorimetric sensor systems. We limit discussion here to a somewhat narrow definition of vesicles: aggregates that self-assemble in aqueous solutions and contain organized amphipathic molecules, generally lipids but also polymers. This definition precludes broad areas of sensing applications using other types of molecular aggregates, such as nanoparticles and quantum dots [16], which are beyond the scope of this chapter. However, we do refer to systems in which organized lipid and/or polymeric structures were combined with other chemical entities for creation of sensor assemblies.

Vesicular particles have been employed in diverse applications in biological research, mostly due to their relative ease of preparation and variability in composition. In addition, vesicles are often perceived as closely mimicking the cell membrane, thus functioning as convenient biomimetic platforms.

These properties have promoted the use of vesicles in biosensor assemblies. In this chapter we summarize recent developments in chromatic vesicle-based biosensor applications. We particularly emphasize the contribution of *chromatic polydiacetylene-based vesicles* as a vehicle for the detection and analysis of biological analytes.

2 Vesicle-Based Chromatic Sensors

Vesicular particles can be useful sensing platforms due to their defined molecular organizations, ease of preparation, and overall robustness and stability. Nevertheless, actual biosensor devices and applications employing vesicles are rare, mostly due to the necessity for maintaining vesicles in aqueous solutions (rather than in solid morphologies), their insufficient homogeneity, lack of complete control of their composition and organization, and inadequate means for incorporation of signal-generating modules in vesicles. In addition, introducing *specificity* into vesicle-based sensors, for example through incorporation of biological recognition elements, is challenging; one has to accomplish efficient insertion of the desired receptor molecule into the vesicle bilayer without adversely impacting the biological properties and molecular recognition capabilities of the receptor, while in addition retaining vesicle stability.

Several studies have presented innovative applications of vesicles in sensors and biological assays. Fluorescence-doped vesicles have often been used in neurobiology assays [17], or for studying the pH effect of excitory proteins [18]. Oxygen sensors based on phospholipid vesicles doped with fluorescent markers have been reported [19]. Vesicles have been used as components in immunosensors [20–22] and for other biochemical applications [23–25]. Other applications of vesicle-based sensors include taste sensors [26], molecular recognition [27], and transport through thermo-responsive substances [28].

The use of *colorimetric* vesicles in sensor platforms has been particularly challenging because of the additional requirement for the inclusion of optical reporter molecules within the vesicular aggregates. The optical probe merocyanine 540 was incorporated within unilamellar vesicles, and changes in its absorption spectra were used to monitor bilayer interactions of surfactant molecules [29]. An immunoassay based on liposome migration, optically detected by employing vesicles encapsulating a coloured dye, was also described [30, 31]. That work underlined one of the drawbacks of colour-based biosensing techniques—the limited capability of the human eye to differentiate between mixed colours and shades. This limitation is generally remedied by the use of spectroscopy or even digital scanning, facilitating better discrimination of real signals.

3 Colorimetric Polydiacetylene-Based Sensors

3.1 Polydiacetylene Vesicles

Conjugated polydiacetylene (PDA) is a remarkable polymeric system which exhibits unique chromatic properties. PDA is formed through 1,4 addition of aligned diacetylenic monomers, initiated by ultraviolet (UV) irradiation [32] (Fig. 1). The resulting polymer is intensely blue to the eye, due to electronic

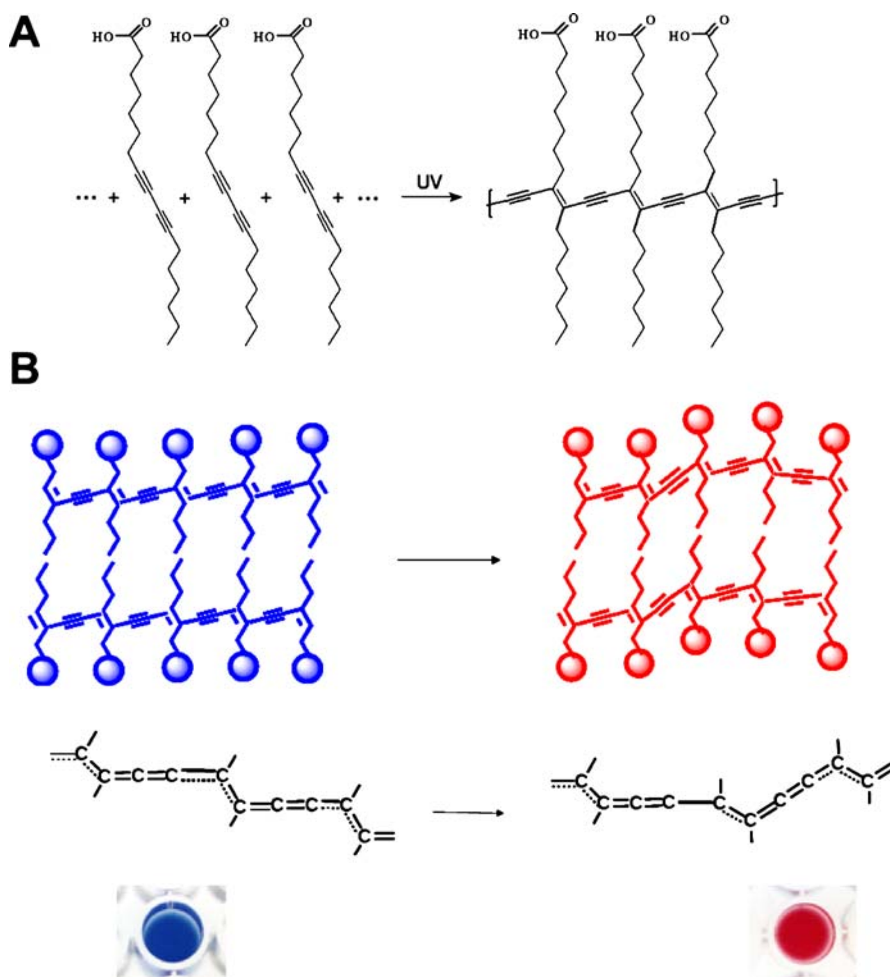

Fig. 1 Structural features of polydiacetylene. **a** Creation of the polymerized backbone from the diynoic acid monomers; **b** induction of the blue–red colour transitions

delocalization within the conjugated framework, giving rise to absorption at around 650 nm in the visible region of the electromagnetic spectrum. Importantly, PDA can undergo rapid blue–red colorimetric transitions due to a variety of external perturbations, such as temperature changes [33–36], pH, and surface pressure [37]. The molecular mechanism corresponding to the colour change is believed to be an irreversible stress-induced structural transition of the conjugated backbone of the polymer [38, 39]. This externally induced conformational transformation of the PDA backbone essentially shortens the effective conjugation network, giving rise to an appearance of the polymer as a *red* substance. The structural transition of the backbone is believed to be primarily affected by surface perturbations to the pendant side chains of the PDA assemblies [36, 39]. The colorimetric transformations of supramolecular PDA assemblies are *irreversible*; however, there have been reports describing the introduction of colour-change reversibility via chemical modification of the polymer head groups, thus altering the molecular packing and topochemical transformations within the polymer modules [40, 41].

The lipidomimetic structural features of PDA, i.e. hydrophobic tail (terminated by a methyl group) and hydrophilic head group (carboxylate), result in the formation of biomimetic membrane assemblies, such as monolayers at the air/water interface and vesicular aggregates in aqueous solutions. These organizations have facilitated utilization of the unique optical properties of PDA for varied biological sensing applications. In a sense, PDA assemblies could combine the role of both the cytoskeleton, through stabilization of various natural proteins and other molecules in the biomimetic polymer layers, as well as mimicking the lipid scaffolding of the cellular membrane [32]. Indeed, the proliferation of biological applications utilizing PDA assemblies is based upon the ability to induce structural modifications, and thus colorimetric transitions, within the polymeric PDA framework through perturbations induced by binding of biological analytes onto the surface of the vesicle bilayer [42, 43]. Such interfacial disruption could occur through biological recognition processes, which correspondingly affect the pendant side chains of PDA, leading to the colorimetric transitions observed [40, 42–48].

Bilayer configurations are not the only self-assembled structures utilized for exploiting the chromatic properties of PDA for biosensing applications. PDA bolaamphiphiles (containing polar head groups at both sides) were shown to form stable vesicles [49]. Interestingly, these vesicles exhibited dramatic colorimetric responses to varied external stimuli, suggesting that both head-group and side-chain organization within PDA systems affect the conjugated framework and contribute to the structural and colorimetric transformations. Intriguing "dendrimer scaffolds" that might facilitate construction of colorimetric PDA vesicle sensors were recently described [50]. In such systems polyamidoamine (PAMAM) dendrimers were employed to display the PDA moieties, putatively amplifying the signals induced by varied stimuli [50]. Similarly, PDA covalently attached to silica nanocomposites exhibited

very high colorimetric sensitivity, stability, and reversibility pointing to potentially superior properties for practical biosensing applications [51].

Many reports have appeared in recent years depicting biosensing applications of PDA vesicles [47, 50, 52–55]. Most such applications involved chemical modification of the PDA head groups [53, 56, 57] for incorporation of functional and biomolecular recognition units at the PDA surface (Fig. 2). A generic example of this concept has been the utilization of diacetylene monomers displaying biotin in vesicle frameworks [58]. The high affinity between soluble streptavidin and the PDA–biotin complex gave rise to the blue–red transition, accompanied by vesicle aggregation due to the multimeric interactions involving streptavidin and four biotin units.

Experiments depicted in the literature have demonstrated diverse avenues for colorimetric detection of proteins, nucleic acids, and whole microorganisms through covalent binding of specific receptor units to the PDA vesicle framework. Detection of influenza virus was achieved through attachment of sialic acid residues to the surface of PDA vesicles [52, 54, 59–61]. Colour sensing of DNA strains through PDA functionalized with oligonucleotides was also illustrated [62, 63]. The design utilized the structural/chromatic transformations of PDA as a vehicle for amplification of the oligonucleotide recognition signal. An original "bacterial fingerprinting" approach has relied on vesicles constructed from different ratios of diacetylene monomers functionalized with indole or phenol units [64]. The different affinities of bacterially expressed lipopolysaccharide (LPS) to the functionalized liposomes facilitated identification of distinct fingerprints for bacterial species through a combination of vesicles having different compositions.

Immobilization of colorimetric PDA vesicles onto or within various solid supports was demonstrated, enhancing the potential of PDA systems as practical biosensing solutions. PDA vesicles have been immobilized on solid

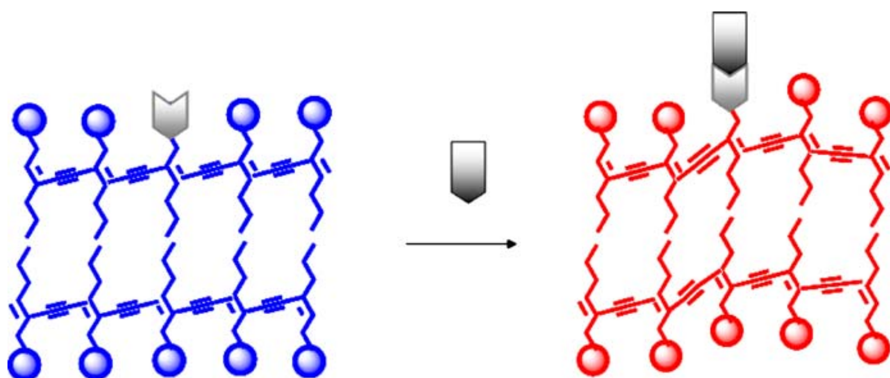

Fig. 2 Colorimetric detection of molecular recognition using modified PDA. Introducing recognition units through chemical modification of the PDA head group

surfaces while still retaining their colorimetric properties [65, 66]. Sol–gel matrixes were also employed as hosts for PDA vesicles displaying surface recognition groups [60]. The enhanced stability of sol–gel materials, their optical transparency, and feasibility of analyte diffusion within the porous substance point to powerful advantages for immobilization of the PDA vesicles and construction of viable biosensors.

3.2 Lipid/PDA Vesicles

An important development enhancing the applicability of the colorimetric PDA technology for biological and pharmaceutical sensing systems has been the construction of *mixed lipid/PDA vesicles*, comprising both the polymer and phospholipids and/or other constituents of the cell membrane. The advantages of such mixed assemblies stem from the observation that the PDA framework can act as "scaffolding" for the stabilization of additional lipophilic dyes and/or recognition elements that can be incorporated into the vesicles (rather than covalently attached to the polymer). In particular, the inclusion of additional molecular components into the vesicles creates a "modular" molecular architecture facilitating diverse biomolecular recognition capabilities, without the need to resort to often cumbersome and technically difficult synthetic manipulation of the PDA framework.

Early examples of lipid/PDA vesicle biosensor systems were the incorporation of gangliosides into the PDA vesicle framework, exploiting the affinity between the ganglioside head group and cholera toxin for viral detection [55, 67–70]. Another example of the implementation of novel sensing schemes using lipid/PDA vesicles is the incorporation of cholesterol moieties as a "bait" for colorimetric sensing of pore-forming toxins produced by bacteria [71]. That scheme has been further expanded, employing fluorescently labelled lipid insertion into the vesicles for bacterial sensing [72].

A particularly important feature of lipid/PDA vesicle systems is the feasibility of incorporating a significant concentration of lipid constituents within the PDA matrix—up to 50% (mole ratio). This architecture is designed to better mimic the cell surface and is radically different from PDA systems containing smaller quantities of lipid "dopants", both in structure as well as functionality. Essentially, such mixed vesicles comprise distinct lipid domains embedded within the polymer framework that still retains its structural and chromatic properties [43, 44, 48, 73]. Figure 3 depicts a schematic description of lipid/PDA vesicles. Previous studies indicated that the lipids and PDA most likely form interspersed microscopic phases within the vesicles [48]. The phospholipids incorporated within the PDA matrix adopt a bilayer structure, the dominant lipid organization within cellular membranes. Published data further point to the contribution of changes in fluidity within the lipid domains in inducing the blue–red transitions [43, 48].

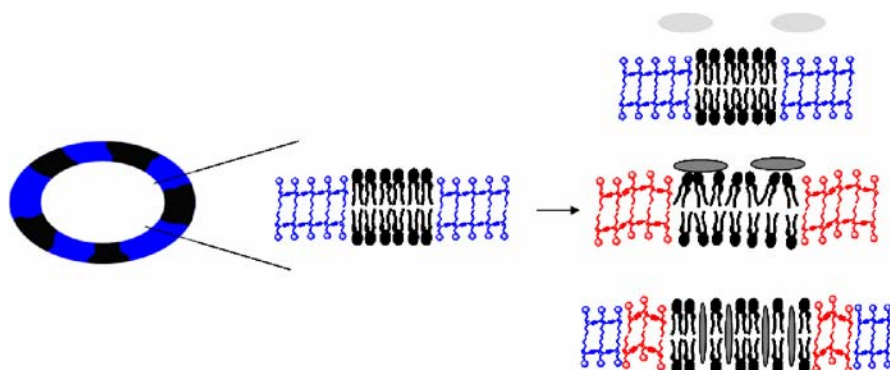

Fig. 3 Colorimetric sensing with lipid/PDA vesicles. Structural/colorimetric transformations of PDA (*blue*) induced by molecules (*grey ovals*) interacting with the lipid bilayer domains (*black*)

The observation that the lipid components in the mixed vesicles form distinct bilayer domains is significant in the context of biosensing applications; such vesicles could then closely mimic the membrane surface of a cell. Enhancing the utilization of lipid/PDA vesicles for biological applications has been the capability of incorporating within the vesicles varied synthetic and natural phospholipids, glycolipids, lipopolysaccharides, cholesterol, or total membrane extracts, essentially mimicking the lipid compositions of different membranes and cellular systems [74].

Utilization of lipid/PDA vesicles for biosensing applications has been based upon the observation that numerous biological analytes primarily interacting with the *lipid* domains can still give rise to the blue–red transformations of the polymer. This phenomenon means that PDA in the mixed vesicles essentially constitutes a reporter module for lipophilic or membrane-active molecules. In that regard, the generic affinity of varied biological molecules, drug compounds, viruses, and microorganisms to lipid assemblies could make lipid/PDA vesicles a powerful biosensing platform. In the subsections below we summarize several biosensor applications of the system.

The mechanisms accounting for the induction of colorimetric transformations in lipid/PDA vesicle systems by lipid-bond biomolecules have not been fully elucidated; however, several studies have shed light on the factors contributing to the blue–red changes [44, 48]. Specifically, previous studies determined that the PDA framework in the mixed lipid/PDA vesicles retains its conjugated backbone structure, accounting for the initial blue colour of the vesicles. The externally induced colorimetric transformations are a consequence of structural and dynamical perturbations within the lipid domains which affect the PDA through the lipid/polymer interfaces [43, 44, 48, 73].

Molecular events occurring and affecting the vesicle *surface* are the principal biological phenomena which trigger the colour transitions. Indeed,

correlation was detected between the extent of lipid bilayer surface perturbation induced by membrane-active compounds and the degree of colorimetric transformations [42]. This interpretation is based on the observation that molecules that preferably interact with and disrupt the lipid head group region were shown to induce more pronounced colour transitions, while deeper penetration into the hydrophobic lipid core generally gave rise to more moderate blue–red transformations [42].

The colorimetric transitions of the lipid/PDA vesicles can also be *quantified*, making the system a useful bioanalytical tool. Enumeration of the blue–red transformations is carried out through computing the relative intensities of the "blue" and "red" components, respectively, in the visible spectra of the solutions. The parameter commonly used for quantifying the colour change was denoted %CR, i.e. percentage colorimetric response, defined as [75]:

$$\mathrm{CR} = \frac{\mathrm{PB}_0 - \mathrm{PB}_\mathrm{I}}{\mathrm{PB}_0} \cdot 100\% ,$$

where $\mathrm{PB} = \frac{A_\mathrm{blue}}{A_\mathrm{blue}+A_\mathrm{red}}$, and A is the absorbance of either the blue component in the UV–Vis spectrum (peak at 640 nm) or the red component (550 nm). (Note: blue and red refer to the visual appearance of the material, not its actual absorbance.) PB_0 is the red/blue ratio of the control blue sample, while PB_I is the value obtained after colour change occurred. In principle, a higher CR value indicates a greater reddish appearance of the solution, compared to the blue control sample (the initial blue solution yields, by definition, a zero %CR).

3.2.1 Colorimetric Detection of Enzymatic Catalysis

An important property of lipid/PDA vesicles is the feasibility of incorporation of varied lipid species. This versatility facilitates the application of the colorimetric assay for detection and analysis of lipophilic enzyme hydrolysis [44, 76] (Fig. 4). Figure 4a schematically depicts a colorimetric enzyme screening experiment. Vesicles containing different lipid molecules were exposed to lipophilic enzymes; a colorimetric reaction occurred only when the enzyme recognized its lipid substrate embedded within the vesicles. The colorimetric response has been ascribed to binding and cleavage of the lipid substrate by the enzyme that overall induce structural perturbation of the polymer and the corresponding colorimetric transformation.

Representative data presented in Fig. 4b clearly demonstrate that the colour transitions induced by particular enzymes are directly related to the lipid composition of the vesicles. For example, the enzyme phospholipase A2 gave rise to a strong blue–red transition when the PDA matrix contained phosphatidylcholine, but this enzyme did not induce colorimetric transitions

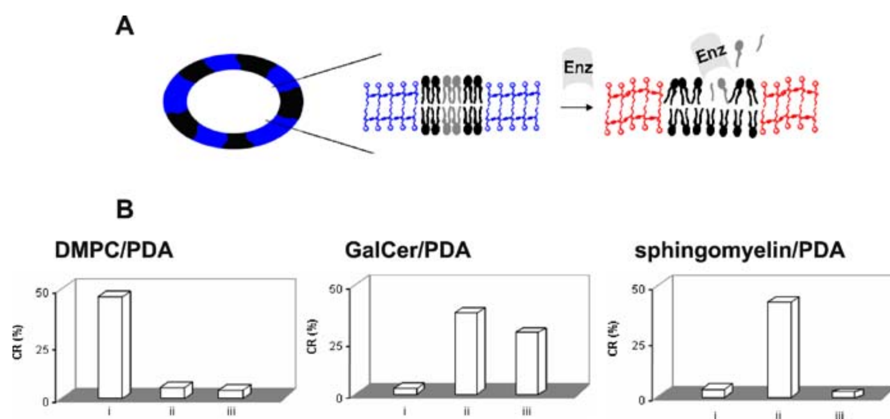

Fig. 4 Colorimetric detection of lipophilic enzyme catalysis. **a** Schematic description of the experiment in which the blue–red colour transitions are induced by enzymatic hydrolysis of vesicle-embedded lipid molecules. **b** Colorimetric response induced by different enzymes following mixing with lipid/PDA vesicles having the indicated compositions: *i*—phospholipase A2; *ii*—sphingomyelinase; *iii*—galactocerebrosidase

when other types of lipids were present within the vesicles (Fig. 4b, bars i). Another demonstration of the specificity of the assay was the absence of significant colorimetric transitions induced in pure PDA vesicles (where no lipid molecules were incorporated) [76]. In general, Fig. 4 demonstrates that the lipid/PDA vesicle assay can constitute a useful platform for screening enzymes and molecules with putative enzymatic properties. Importantly, the strategy based on the colorimetric vesicles does not require additional chemical reagents or post-cleavage chemical analysis. The "one-step" approach can be easily applied, for example, for identifying enzyme inhibitors through simply monitoring colour changes of the vesicle suspensions in standard multiwell plates.

3.2.2
Colorimetric Detection of Peptide–Membrane Interactions

Interactions between peptides and lipid membranes play major roles in numerous physiological processes, such as signalling, formation of ion channels, cytolysis, and cellular recognition. Furthermore, membrane permeation plays a crucial role in determining the activity of antimicrobial peptides [73]. Several reports have demonstrated that lipid/PDA vesicles undergo colour changes upon binding of antimicrobial peptides [42, 73, 77–80]. Moreover, studies have shown that important biophysical parameters, such as the degree of penetration of the peptides into lipid bilayers and mechanisms of peptide–lipid binding, affect the extent and dynamics of the colorimetric transitions. Figure 5 depicts an example of colour changes induced by different pep-

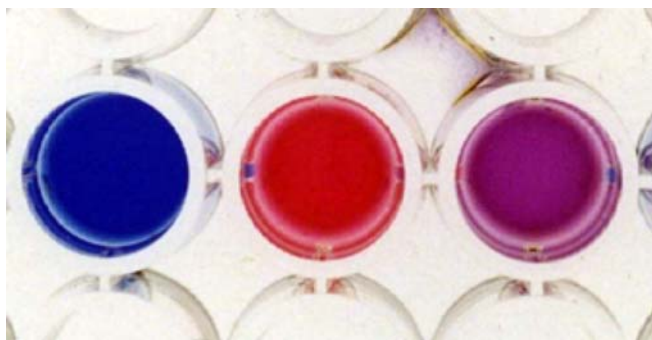

Fig. 5 Colour transitions induced by membrane-associated antimicrobial peptides. *Left*: control vesicle solution; *center*: cryptdin-4 added; *right*: melittin added. Peptide concentrations were 0.1 mM

tides (at identical concentrations) in lipid/PDA vesicle solutions. The distinct colours are indicative of the purported mode of membrane interaction of the peptides: stronger interfacial association of the positively charged beta-sheet structured cryptdin-4 yielded a more pronounced red colour ascribed to surface perturbations induced by the peptide [79, 80], while melittin, a helical antibacterial peptide that inserts into lipid bilayers, is expected to give rise to a more moderate colour change, as is indeed observed in Fig. 5.

Investigations employing the lipid/PDA vesicle assay for screening and analysis of antimicrobial peptides have been reported, including evaluation of the contribution of specific lipid molecules in the bilayer to peptide adsorption and penetration [77, 78, 80, 81], comparative study of the contributions of specific residues within antimicrobial peptide sequences to their membrane interactions [42, 73, 79], and membrane binding of pre-fibrillar assemblies and its significance to amyloid toxicity [82]. The observation of rapid colorimetric transitions induced by antimicrobial peptides opens the way for using lipid/PDA vesicles as a useful bioanalytical tool. The assay could be applied as a vehicle for rapid colorimetric screening of bilayer interactions and membrane binding of antimicrobial compounds, or the absence of such interactions.

3.2.3
Colorimetric Vesicles for Pharmaceutical Screening

A significant part of pharmaceutical research and development efforts entails the evaluation of interactions and penetration of tested substances and formulations through hydrophobic physiological barriers, such as the blood–brain barrier (BBB) [83–85]. In that regard, lipid/PDA vesicles might become a useful platform for evaluation of membrane interactions of pharmaceutical materials [86]. That work demonstrated colorimetric screening of a large

number of common pharmaceutical compounds by lipid/PDA vesicle solutions. The important feature of the vesicle assay apparent in that study was the observation that the tested molecules could be distinguished according to the *extent* of colorimetric response elicited following their addition to DMPC/PDA vesicles (Fig. 6). Specifically, the analysis showed that the concentration ranges in which the blue–red transformations were induced varied significantly among the molecules. Roughly, three groupings could be distinguished: compounds inducing colour changes at *micromolar* ranges, substances affecting transitions at *millimolar* concentrations, and those that did not affect the chromatic vesicles even at much higher concentrations (Fig. 6).

The different colorimetric dose–response curves in Fig. 6 correspond to the types of interactions of the pharmaceutical compounds with the lipid/PDA vesicles. Specifically, molecules that preferably aggregate at the *lipid/water interface* (rather than penetrate or cross through the lipid barrier) give rise to pronounced surface perturbations, thus inducing colour changes even at very low, micromolar concentrations (curve i, Fig. 6). Another grouping encompasses compounds that tend to *insert into the bilayer*. Such interaction would give rise to smaller surface perturbations, leading to higher, millimolar concentrations inducing the blue–red transitions (curve ii, Fig. 6). A third group of compounds distinguished by the colorimetric vesicle assay are molecules that *do not bind or interact with lipid membranes*, thus not inducing noticeable colorimetric transitions (curve iii, Fig. 6).

The phospholipid/PDA vesicle assay exhibits important practical advantages for application as a generic tool for drug screening. The vesicle solutions can be placed and stored for long periods in conventional 96-well (or 384-well) plates. The colorimetric transitions are induced within a very short time (seconds) after mixing the reagents. The colorimetric technique is robust and easy to apply, and data for large compound libraries can be obtained in a few

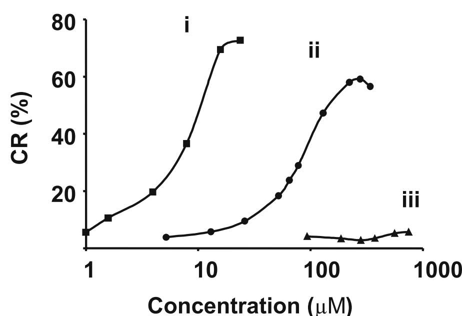

Fig. 6 Colorimetric screening of pharmaceutical compounds. Representative colorimetric dose–response curves induced by pharmaceutical compounds: *i*—imipramine; *ii*—lidocaine; *iii*—theophylline

minutes. The lipid/PDA vesicle assay can thus become a useful tool for predicting membrane interactions and bilayer permeation at early stages of drug development and profiling.

3.2.4
Bacterial Sensing with Lipid/PDA Vesicles

The observation that colour changes could be induced within lipid/PDA vesicles by interactions with amphiphilic and membrane-associated molecules opens the way for other sensing applications. An intriguing recent avenue has been the utilization of lipid/PDA vesicles as a vehicle for bacterial detection [87]. In that new sensing approach, microorganisms are detected through the blue–red change induced in lipid/PDA vesicles by the *amphiphilic and membrane-active molecules* they secrete to their environment [88–90]. Figure 7a depicts the schematic arrangement of the bacterial sensor, in which phospholipid/PDA vesicles were embedded in agar scaffolding containing bacterial-growth medium [87]. The *agar matrix* serves as an amplification vehicle. It facilitates bacterial multiplication, thereby promoting the release of secreted substances by the bacteria. Essentially, molecules released by bacteria that proliferate on the agar surface diffuse through the semi-porous agar substrate and induce chromatic changes in the agar-embedded vesicles, thus reporting on the bacterial presence.

Figure 7b depicts a representative scanned image of a DMPC/PDA/agar plate showing the colour transitions induced by bacteria (*Salmonella typhimurium*). The picture in Fig. 7b clearly shows that red hollows form

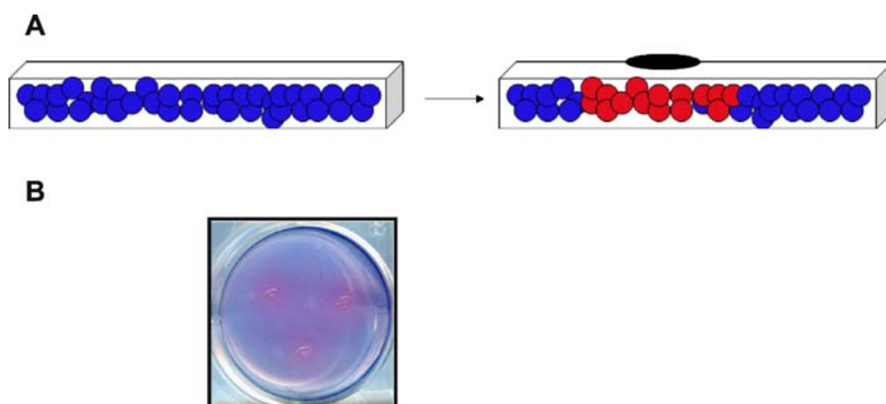

Fig. 7 Bacterial sensing with lipid/PDA vesicles. **a** Schematic description of the lipid/PDA vesicles (*blue circles*) embedded in an agar matrix (*white box*). Bacterial proliferation and colony formation (*black oval*) results in the blue–red transformation of the vesicles. **b** An example of the colour transformations induced by bacterial colonies (*S. typhimurium*) on a DMPC/PDA/agar plate

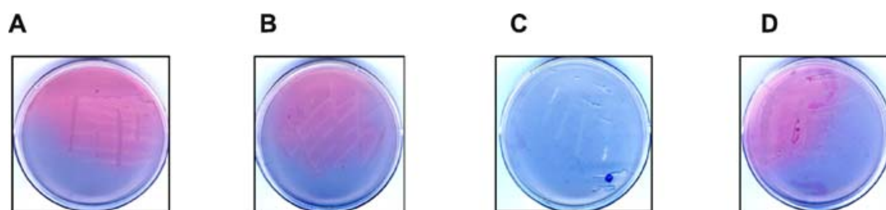

Fig. 8 Colorimetric screening of bacterial resistance. Scanned images of DMPC/PDA/agar plates further containing antibiotic substances onto which different bacterial strains were streaked. **a** *E. coli K-12 C600* streaked on DMPC/PDA/agar *not containing* antibiotics; **b** *E. coli C600 pMRInv* streaked on DMPC/PDA/agar *not containing* antibiotics; **c** *E. coli K-12 C600* streaked on DMPC/PDA/agar that contained kanamycin; **d** *E. coli C600 pMRInv* streaked on DMPC/PDA/agar containing kanamycin

around the bacterial colonies following incubation (note that the apparent "doublets" in Fig. 7b are due to the reflection of the scanner light). The blue–red transformation of the matrix was directly related to bacterial proliferation; *each* colony was surrounded by an area in which the blue agar matrix changed colour to red, while the remaining agar matrix stayed blue. The dispersion of red regions under and around the bacterial colonies indicates that the colour transitions were due to diffusion of substances released by the bacteria into the surrounding matrix.

Figure 8 demonstrates an application of the lipid/PDA/agar assay for evaluation of antibiotic resistance of bacterial strains [87]. In the experiment shown in Fig. 8, *Escherichia coli* strains exhibiting different antibiotic resistance were streaked onto plates containing lipid/PDA/agar matrix that further incorporated antibiotic compounds. The striking colour transitions shown in Fig. 8 appeared only in plates in which the genotype of the streaked bacteria exhibited resistance to the antibiotic included within the matrix. For example, a red colour (corresponding to transformed vesicles) appeared in a plate containing kanamycin/DMPC/PDA/agar onto which the kanamycin-resistant *E. coli C600 pMRInv* strain [91, 92] was streaked (Fig. 8d), while no colour change occurred when *E. coli K-12 C600* strain was streaked—a bacterium that cannot grow on kanamycin-containing substrates [91, 92] (Fig. 8c).

3.3 Lipid/PDA Vesicles Incorporating Recognition Elements

Incorporation of natural and artificial receptors within lipid/PDA assemblies (Fig. 9) has been a particularly important development in the utilization of the chromatic vesicles for colorimetric detection of biological analytes. The design of new systems for rapid detection of interfacial biomolecular interactions has to fulfil two main objectives. First, the chemical construct should

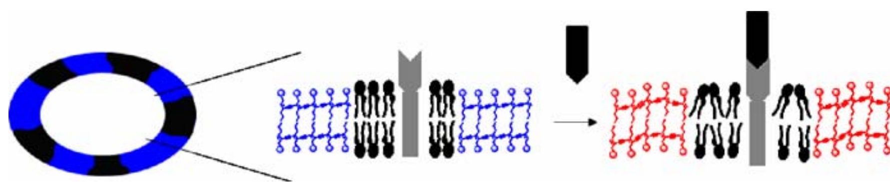

Fig. 9 Colorimetric molecular recognition using lipid/PDA vesicles. Recognition elements (*grey*) are embedded within the lipid moieties, and the colorimetric transformations are induced following ligand–receptor binding at the vesicle surface

allow physical access and binding between the receptor and the ligand in an aqueous solution. The second requirement is that the ligand–receptor interactions could be reported through easily detected chemical or physical transformations within the system. Lipid/PDA vesicles embedding recognition elements adhere to the above requirements. In such vesicles the phospholipid framework is exploited as an anchoring platform for receptors containing hydrophobic moieties, overall facilitating display of the recognition elements at the vesicle surface.

Colorimetric detection of ligand–receptor interactions through *physical incorporation* of receptors within lipid/PDA vesicles presents important advantages over *chemical attachment* of recognition units to the PDA itself, discussed above. First, chemical derivatization of PDA can be technically demanding, and the organic synthesis procedures limit the scope of this approach. Furthermore, attaching additional chemical units onto the diacetylene monomers often disrupts the organization and self-assembly of the monomers and adversely affects polymerization. Consequently, the abundance of recognition modules in previously reported derivatized PDA vesicles is low [61, 67]. Such limitations are generally not encountered in *lipid*/PDA vesicles incorporating recognition elements. No chemical modification of the diacetylene monomers is needed because the lipid moieties constitute the scaffolding modules for anchoring the receptor modules. In addition, a higher number of receptors can be incorporated in the vesicles because of the high mole ratio—almost 50%—of the lipids in the mixed lipid/PDA vesicles [43, 93]. Another noteworthy feature of the lipid/PDA system as a vehicle for receptor display is the generic nature of this approach; in principle, attachment of appropriate lipophilic residues is the only precondition for displaying any receptor unit at the vesicle surface.

Incorporation of biological receptor modules in PDA-based vesicles can be combined with other scaffolding systems for the creation of versatile sensing modules. For example, sol–gel assemblies comprising phospholipid/PDA vesicles that further contained immunoglobulins were shown to respond to the presence of specific antigens through visible blue–red changes [93]. Below we describe several sensor systems utilizing receptor/lipid/PDA vesicles for specific molecular recognition.

3.3.1 Ion Discrimination by Ionophore/Lipid/PDA Vesicles

An early study reported the construction of an ion sensor through incorporation of *ionophores* in phospholipid/PDA vesicles [94] (Fig. 10). Selective colorimetric response to ions depended on the different affinities between the soluble ions and vesicle-embedded ionophores. The mechanism of colorimetric changes in this vesicle system has been ascribed to the structural transformations and mobility of the ionophores within the lipid bilayers, induced by ion binding [94].

Figure 10b depicts representative colour changes observed in DMPC/PDA vesicles incorporating different ionophores. The ionic selectivity demonstrated in Fig. 10b is consistent with the established binding affinities of these particular ionophores. Valinomycin, for example, binds potassium ions with much higher affinity compared to sodium cations [95]. On the other hand, the more pronounced blue–red colour change within the monensin/DMPC/PDA vesicles is induced by addition of *sodium* cations to the solution, reflecting the higher affinity of monensin to Na^+ ions. Another important observation in the ionophore/phospholipid/PDA system was the capability of the system

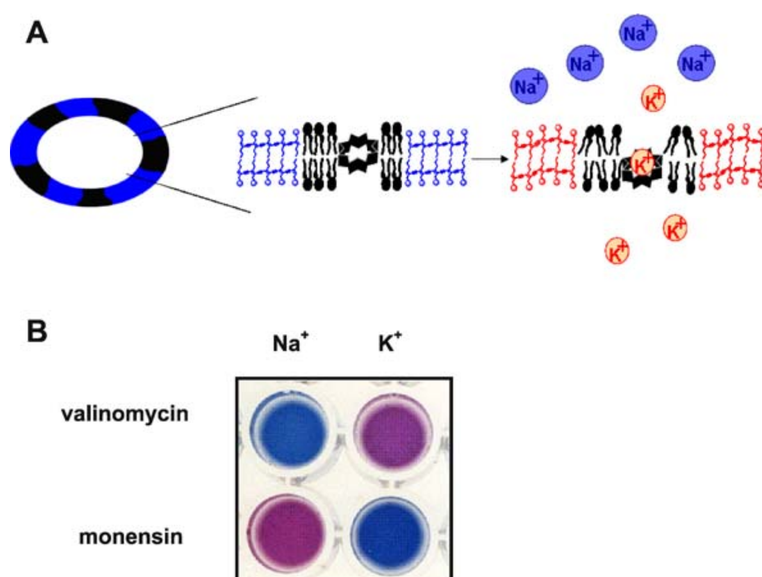

Fig. 10 Ion sensing by ionophore/lipid/PDA vesicles. **a** Schematic description of colorimetric ion sensing by lipid/PDA vesicles embedding ionophores. The ionophore (macrocyclic unit) is incorporated in the lipid domains (*black*); ion binding to the ionophore gives rise to the blue–red transformation of the PDA scaffold. **b** Representative colour transitions induced by ion–ionophore binding. *Top row*: valinomycin incorporated in vesicles; *bottom row*: monensin

to *distinguish* among ions in a mixture, depending on the specific ionophore incorporated within the vesicles [94]. Overall, the assay might be applied to rapid determination of physiological ionic species, investigation of intra- and extra-cellular ion concentrations, and evaluation of the performance and selectivity of putative ion-binding compounds and metal-binding peptides.

3.3.2 Antibody Detection by Epitope/Lipid/PDA Vesicles

An innovative approach designed to accomplish biomolecular recognition using lipid/PDA vesicles has been introduced through implanting *epitopes* within the phospholipid scaffolding [97]. The vesicle system is schematically shown in Fig. 11a. Vesicular particles of PDA and phospholipids further incorporated peptide epitopes covalently attached to the N-termini of hydrophobic amino acid sequences. This configuration facilitated display of the epitope at the surface of the phospholipid bilayer that essentially served for anchoring the hydrophobic peptide moiety.

The design shown in Fig. 11a assures that specific antibody–epitope interactions at the vesicle surface would affect structural perturbations of the PDA and consequent colorimetric transformations. Figure 11b depicts representative colorimetric data recorded with the epitope/lipid/PDA assemblies. The hydrophobic sequence employed for anchoring the epitope was an Ala-Leu re-

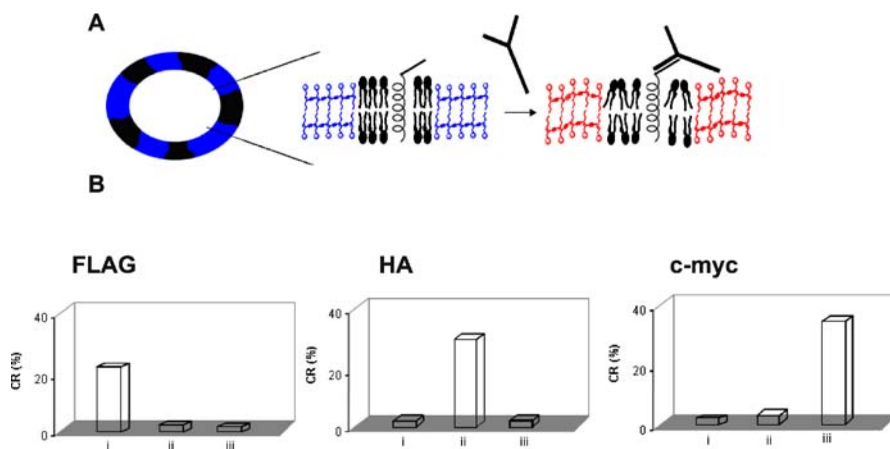

Fig. 11 Colorimetric detection of antibodies using epitope/lipid/PDA vesicles. **a** Schematic description of the antibody-induced colour transitions. The epitope is attached to a helical peptide sequence embedded within the lipid bilayer; binding of the specific antibody gives rise to the blue–red transition of the polymer. **b** Colorimetric response (%CR) induced by specific antibody–epitope binding; each graph summarizes colorimetric data recorded for a specific epitope/lipid/PDA assembly: *i*—anti-FLAG antibody added; *ii*—anti-HA antibody added; *iii*—anti-c-myc antibody added

peat, flanked by Lys residues designed for anchoring the hydrophobic sequence at both sides of the bilayer [98, 99]. Epitopes that were displayed include the c-myc epitope [100], the FLAG epitope (amino acid sequence DYKD-DDDK [101]), which is widely used in epitope tagging experiments, and the HA epitope (YPYDVPDYA), derived from the human influenza virus haemagglutinin protein [102]. The colorimetric data in Fig. 11b clearly demonstrate that pronounced colorimetric transitions occurred *only* when the displayed epitopes were recognized by their specific antibodies. The colour changes induced by *non-specific* interactions between the antibodies and the lipid–polymer particles were considerably smaller than the real signal.

Figure 11 confirms the correlation between colorimetric response and the occurrence of specific epitope–antibody interactions at the vesicle interface. In particular, binding between the antibodies, which are relatively big macromolecules, and the epitopes displayed at the vesicle surface is expected to result in significant surface-induced perturbations of the pendant side chains of the polymer, giving rise to the structural transformation and observed colour changes within the PDA matrix.

3.3.3 Artificial Receptors Embedded in Lipid/PDA Vesicles

Indirect induction of chromatic signals within the polymer matrix by specific ligand–receptor binding can be further extended to include *synthetic* receptor systems. A generic approach for high-sensitivity and specific detection of catecholamine ligands through implanting synthetic hosts within phospholipid/PDA vesicles has been recently described [103]. The system is depicted schematically in Fig. 12, showing a representative artificial receptor designed to recognize catecholamines with high specificity embedded in a phospholipid/PDA vesicle; complexation of the catecholamine ligand triggers the blue–red transformation.

The schematic structure in Fig. 12a shows the phospholipid-flanked cavity which is open to the aqueous solution, available for binding the catecholamine guests. Figure 12b depicts an image of a receptor/lipid/PDA vesicle solution before and after addition of two catecholamine ligands. The colour response of the vesicles clearly echoes the specific binding between the catecholamine ligand (noradrenaline) and the synthetic host designed to bind the molecule [104]. Published data further demonstrated that *chromatic selectivity* is achieved in these vesicle systems; for example, the inclusion of noradrenaline-binding host in DMPC/PDA vesicles could discriminate between noradrenaline and adrenaline—a highly similar ligand—in a solution mixture of the two compounds.

Protein sensors based on synthetic receptors embedded within lipid/PDA vesicles have also been recently demonstrated [106]. Protein sensing by artificial molecules is a challenging endeavour, especially if the recognition

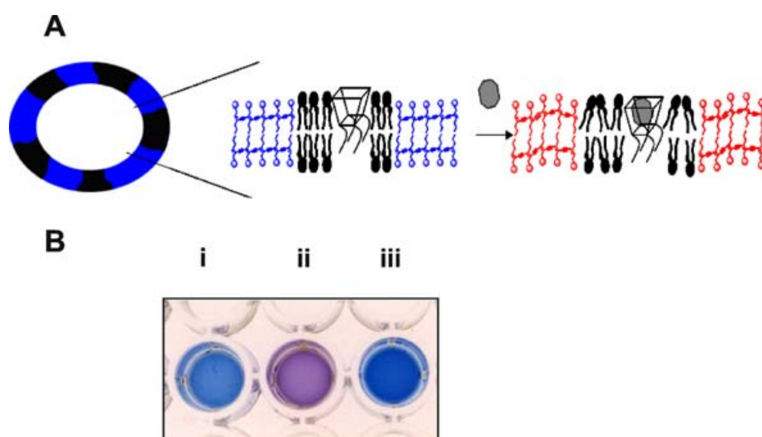

Fig. 12 Colorimetric detection of ligands through vesicle-embedded receptors. **a** Schematic description of the experiment: ligand–receptor recognition between soluble ligand and embedded receptor gives rise to the colorimetric transformation of PDA. **b** Scanned image of lipid/PDA vesicles incorporating a synthetic host designed to specifically bind noradrenaline [104]: *i*—control vesicle (no ligand added); *ii*—noradrenaline added; *iii*—adrenaline added

event is desired to be coupled to a simple quantifiable readout. The innovative scheme for colorimetric determination and fingerprinting of proteins through electrostatic interactions with vesicle-embedded calixarene derivatives is depicted in Fig. 13. Specifically, the hydrophobic calixarene hosts were incorporated within the lipid bilayers, while their charged moieties bound soluble proteins through multivalent electrostatic interactions with charged protein surfaces [105].

Figure 13b presents UV–Vis spectra of DMPC/PDA vesicle solutions, and the effect of protein–host interactions. It shows that addition of pepsin to lipid/PDA vesicles not containing additional receptors did not give rise to a noticeable colour transformation (the solution remained blue, Fig. 13b, ii) due to the fact that both pepsin (pI = 1) and the PDA surface display a negative charge in the pH conditions employed in the experiment. However, adding pepsin to DMPC/PDA vesicles to which a positively charged calixarene was pre-added gave rise to a distinct blue-to-purple colour change, clearly reflected in the visible spectrum of the solution mixture (Fig. 13b, iii).

The differences in colour changes induced after addition of proteins to lipid/PDA vesicles containing the calixarene hosts can also be quantified according to the %CR formula shown above. Specifically, the *net colorimetric response* (ΔCR) can be calculated for each protein and vesicle-embedded receptor [106]. ΔCR corresponds to the difference between the colour response induced by a tested protein added to lipid/PDA vesicles containing the calixarene receptor and vesicles that did not include the receptor. Interestingly, the set of ΔCR values obtained for different proteins makes possible *protein*

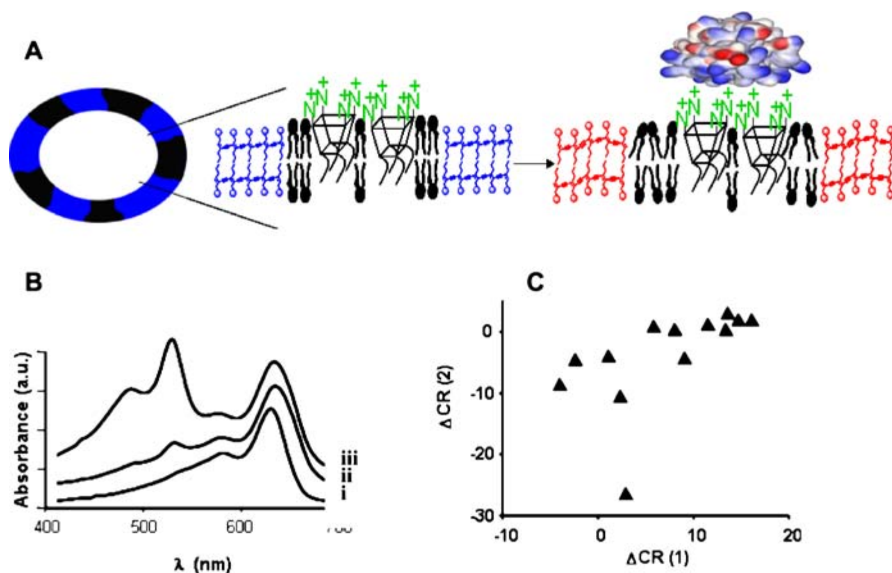

Fig. 13 Protein fingerprinting by charged receptors incorporated within lipid/PDA vesicles. **a** Schematic description of a lipid/PDA vesicle incorporating a synthetic host which binds a protein molecule through electrostatic attraction. **b** Visible spectra depicting protein sensing by the receptor/lipid/PDA vesicles: *i*—control vesicles (no protein added); *ii*—pepsin (a negatively charged protein, pI = 1.0) added to lipid/PDA vesicles *not containing* the receptor; *iii*— pepsin added to lipid/PDA vesicles that also incorporate a positively charged synthetic host. **c** "Protein fingerprinting": each triangle corresponds to a specific protein; *x* and *y* axes represent the changes in %CR recorded after embedding receptors **1** and **2**, respectively, within the vesicles prior to protein addition [106]

fingerprinting (Fig. 13c). Specifically, proteins can be distinguished, in principle, by a combination of net colorimetric effects recorded by using *different* vesicle-embedded receptors.

The protein fingerprinting concept is shown in Fig. 13c [105]. Each data point in the two-dimensional graph represents a protein for which ΔCR values were recorded by using positively-charged calixarene (*x* axis) or negatively-charged calixarene (*y* axis). The dispersion of protein data points is particularly large for either acidic or basic proteins, which is an expected outcome since the platform relies on *electrostatic interactions* between the proteins and the calixarene hosts. Specifically, when *negative* proteins were added to vesicles containing the positively charged calixarene host **1**, more pronounced colour changes were recorded (positive ΔCR) due to the enhanced binding of the negative proteins to the vesicles. On the other hand, when *positive* proteins were added to lipid/PDA vesicles containing negative hosts, *negative* ΔCR values were obtained due to binding of proteins to the receptor rather than the negative PDA framework [105]. Overall, the distribution map in Fig. 13c indicates that, in principle, the construction of

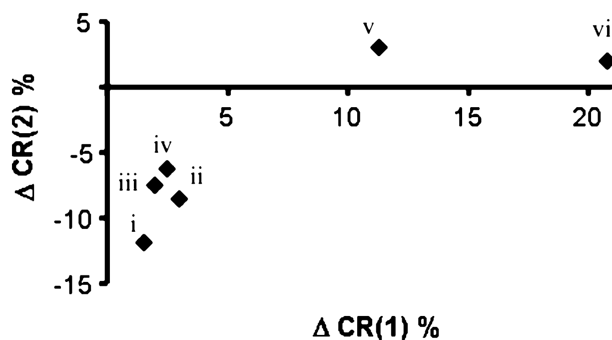

Fig. 14 Colorimetric fingerprinting of small molecules by receptor/lipid/PDA vesicles. Net colorimetric responses (ΔCR) recorded for several biological and pharmaceutical molecules added to lipid/PDA vesicles containing the positively charged receptor **1** and the negatively charged receptor **2**: *i*—amitriptyline (pI = 9.4); *ii*—maprotiline (10.5); *iii*—promethazine (9.1); *iv*—nortriptyline (9.7); *v*—nicotinamide adenine dinucleotide (NADH, 4.8); *vi*—adenosine triphosphate (ATP, 4.2)

a sufficiently broad colorimetric protein database would allow identification of proteins by combining information on their molecular weights with the colorimetric assay.

The concept of colorimetric fingerprinting based upon electrostatic affinity between vesicle-embedded receptors and charged species in aqueous solutions can be broadened to also include detection of small biological analytes and not only proteins. Figure 14 depicts the net colorimetric response graph of small biomolecules and pharmaceutical compounds following their addition to lipid/PDA vesicles incorporating a positively charged calixarene (**1**) and a negatively charged host (**2**) [105]. Similar to the situation encountered for proteins (Fig. 13), the insertion of a positively charged host into the vesicles gave rise to more pronounced colorimetric transformations when adding *negative* analytes to the vesicles, and lesser colour changes when *positive* compounds were added.

4
Conclusions

This chapter discussed biosensor platforms and applications employing colorimetric vesicles as the signal-generating module. Most vesicle systems presented are based on polydiacetylene (PDA)—a polymer that self-assembles in water into vesicle bilayers, and that further exhibits dramatic blue–red transformations. Important features of the vesicle arrangements that were emphasized include their generic nature, simplicity of signal generation, recording, and analysis, and diversity of biological processes and analytes that can be

studied. Indeed, one of the attractive aspects of many of the colorimetric vesicle systems discussed as biosensing platforms is the "one step" characteristic: generation of the colorimetric signals does not require complex procedures or initiation of a cascade of chemical reactions, but is rather a "mix and observe" process.

PDA vesicles that further contain embedded lipids point to promising avenues for research applications and practical utilization. Such vesicle systems facilitate detection of varied biological events, including enzyme hydrolysis, peptide–membrane interactions, and drug permeation through lipid barriers. Lipid/PDA vesicles were also shown to constitute useful scaffolding for implanting receptor modules, thus facilitating detection and analysis of varied ligand–receptor interactions. Overall, the use of visible colour changes as the thrust of biosensing platforms most likely would continue to generate significant interest in further development and practical applications.

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