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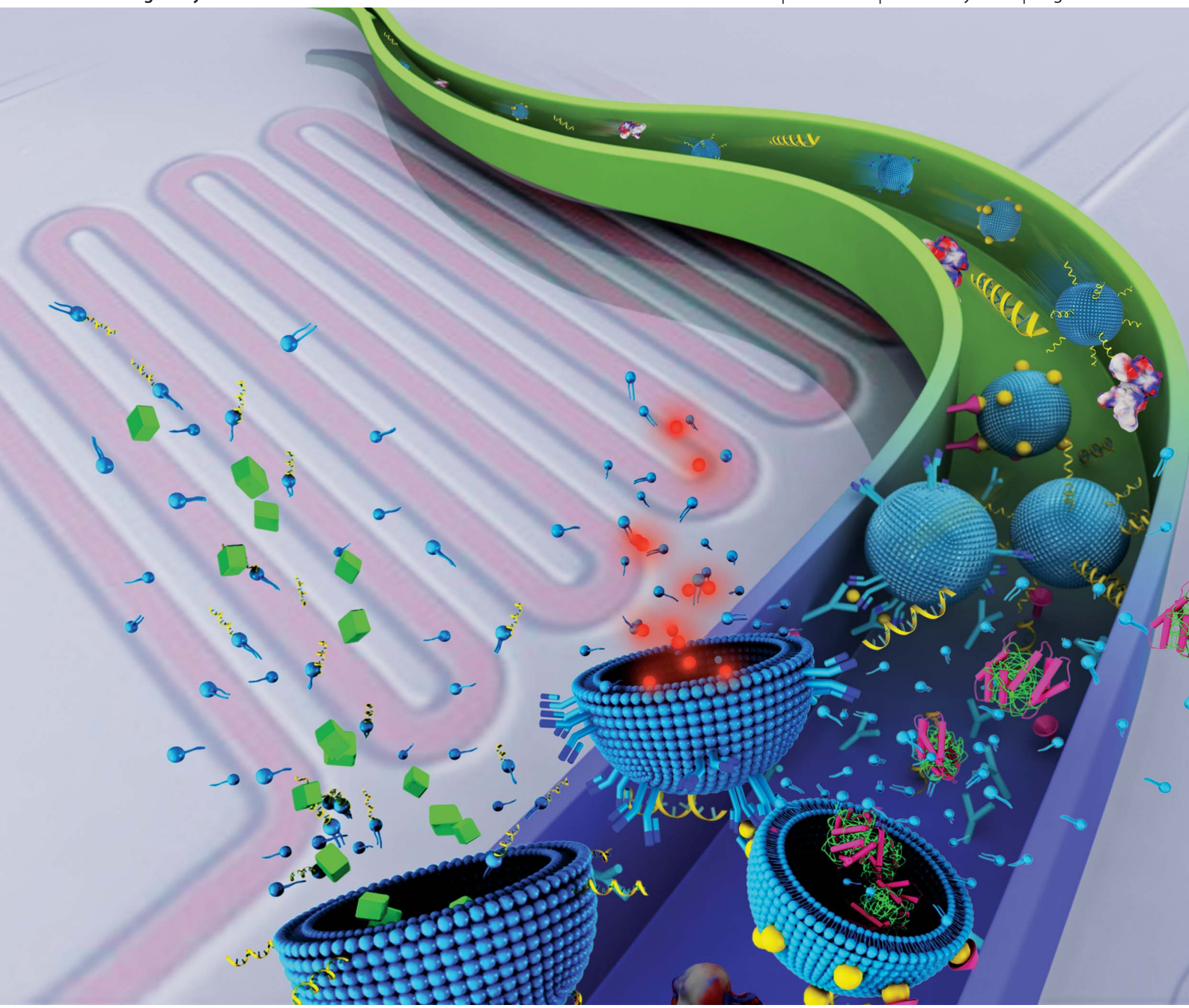

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CRITICAL REVIEW

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Liposomes have been widely applied as carriers of molecules in the fields of nanodevices, drug delivery and gene delivery, as well as a mimic for cell membranes. In the biosensor field, liposomes find special application in signal amplification, due to their excellent carrier properties for encapsulation of signal marker compounds across a wide spectrum of sensing modalities. Additionally, the ability to modify the liposome surface structure to perform recognition functions with a wide range of types of analytes is an important aspect of their use in bioanalysis. This article provides an extensive review of the utility of liposomes in the fields of biosensor and bioanalysis with a focus on these two aspects. The aspects related to the translation of liposomes into commercially viable devices for biosensing are also discussed, and the hurdles to more widespread deployment in point of care systems are addressed.

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1 Introduction

Liposomes are artificial microscopic vesicles composed of a lipid bilayer with the hydrophobic chains of the lipids forming the bilayer, and the polar headgroups of the lipids orienting towards the extravesicular solution and inner cavity.[1] Liposomes are usually classified into small unilamellar vesicles (SUV), large unilamellar vesicles (LUV), multilamellar vesicles (MUV), oligolamellar vesicles (OUV) and multivesicular vesicles (MVV).[2,3] Both water soluble and water-insoluble agents can be encapsulated into the inner cavity or incorporated into the bilayer membrane of the liposome, respectively. Membrane permeability can be greatly reduced by addition of cholesterol to the bilayer membrane of phospholipid.[4–6] In addition, phospholipids, as the main component of liposomes, have distinct advantages over synthetic materials including lack of toxicity, biodegradability and biocompatibility. Consequently, liposomes are utilized as versatile carriers in the fields of medical,[7] therapeutic,[8] and analytical applications.[2,3]

Liposomes were discovered by Bangham *et al.*[9] and were investigated initially as applied drug carriers by Gregoriadis.[10–12] There are several advantages of using liposomes as drug delivery carriers, including targeting *via* surface functionalization,[13] long-circulation behaviour, cellular internalization,[14] and protection of labile drugs. Liposomes have been explored as drug delivery carriers to deliver a wide range of drug types, including anti-cancer, anti-parasite, anti-bacterial drugs, hormones, enzymes, detoxicated reagents, gene drug and immunoactivators to name several. Liposomes have shown an advantage in the therapy of tumors,[15–17] and many liposomal anti-cancer drugs have been successfully commercialized as products including Doxil. In a similar vein to their application as a carrier for drugs, liposomes can act as a carrier for a wide range of signaling molecules. Such encapsulation has allowed a

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natural progression towards the analytical application of liposomes in biosensors and bioanalysis, the principal subject of this review.

1.1 Application of liposomes to biosensors

Biosensors are self-contained integrated devices capable of providing specific quantitative or semiquantitative analytical information. They usually possess a biological recognition element (biochemical receptor) that is retained in direct spatial contact with a transduction element.[18] A common working process for a biosensor is one in which analytes are recognized by biorecognition elements, and the recognition event activates a signal through transducers in the biosensor (Fig. 1).[19] As an important and necessary component of a biosensor, the transducer is usually required to increase the sensitivity of the biosensor *via* signal amplification.[20,21]

Liposomes have attracted great attention for application in the biosensor field over a number of decades as a means to amplify the signal.[2,3] Liposomes can encapsulate various signal markers including dyes,[22] enzymes,[23] salts,[24] chelates,[25] DNA,[26–28] electrochemical[29–31] and chemiluminescent markers.[32] Consequently, liposomes are an excellent candidate component for biosensors to transduce and amplify signals (Fig. 2).[2,3]

In addition, liposomes can also serve as cell membrane models[33–36] because liposomes have the similar components and structure as cell membranes. Therefore, liposomes can provide a simple platform to simplify the investigated system in the research of related interactions and physiological phenomena with cells.[37–39] The relatively low cost of lipids and successful experience from commercialization of liposomal medicines also are important advantages to promote the steps of application and commercialization of liposome-based biosensors.

1.2 Surface modification of liposomes

Liposome surface-modification[40,41] has been developed for improving specificity towards tissues or for reduced recognition in the bloodstream. Antibody-tagged immunoliposomes, and long-circulating liposomes grafted with a protective polymer, are two important strategies employed for liposomal drug products to achieve these respective outcomes.[15] Immunoliposomes can increase the accumulation of liposomal drugs in the desired tissues and organs,[42] while PEG-coated long-circulating liposomes can prevent the interaction between liposomes with opsonizing proteins, resulting in a prolonged circulatory time in blood. The technology for surface modification of liposomes ensures a variety of biorecognition elements can be conjugated to the surface of liposomes,[43,44] including peptide, protein, enzyme, antigen, biotin, avidin and DNA segments.[40,41]

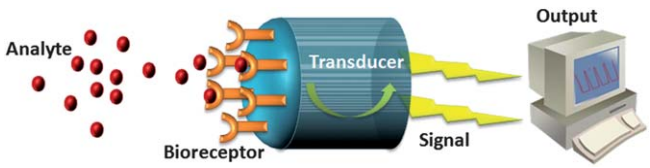

Fig. 1 A schematic diagram of components and structure of a generic biosensor.

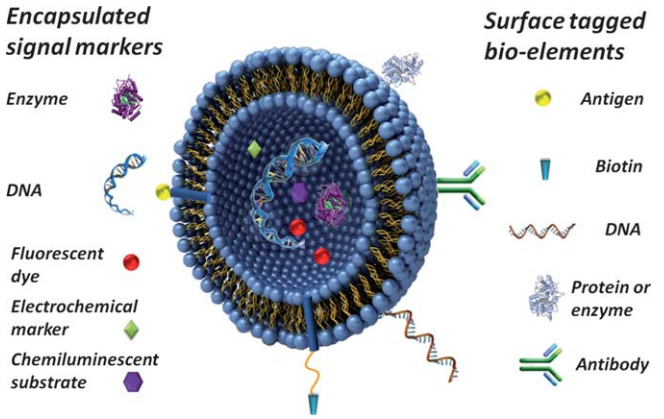

Fig. 2 Structural model, encapsulated materials and surface modification of a liposome.

1.3 Release of encapsulated substances from liposomes

Controlled release of liposome-encapsulated molecules has been developed as a means to enhance the delivery efficiency of liposomal drug delivery systems. Several strategies have been utilized to control the release of encapsulated drug from liposomes, including phase transitions, ultrasound and lysis. For the lysis strategy, surfactant (or detergent) and natural lytic agents, such as complement,[45] mellitin[46] or membrane-permeabilizing antimicrobial peptides (AMPs),[47,48] have been reported. Furthermore, sensitive liposomes have been developed to control the release of encapsulated molecules in response to specific stimuli or to a change in the environment.[49] Various types of responsive liposomes have been reported, including pH sensitive liposomes,[50,51] thermosensitive liposomes,[52] photosensitive liposomes,[53–55] enzyme sensitive liposomes and magnetic liposomes.[56] The responsive liposomes can be destabilized and quickly release encapsulated drugs at the target site depending on the external stimuli, or might exploit the differences between the normal environment and lesion region, such as the low pH or reductive environment in tumors. The aforementioned properties of responsive liposomes consequently signal their potential use in biosensor systems, as encapsulation of a signaling molecule within the liposome might be of use in signal amplification (described below), and the need to rapidly release the encapsulated signaling molecule in response to a selective stimulus is key to their potential application in rapid biosensing technologies.

2 Using liposomes in biosensors

In the broadest sense liposomes have been used as an integral sensing component in a number of different ways. A wide range of signal markers have been encapsulated in liposomes for amplification purposes, including enzymes, fluorescent dyes, electrochemical and chemiluminescent markers. The ability to conjugate the bilayer lipid with a variety of biorecognition elements also ensures that liposomes are able to recognize various analytes for transduction of the signal. The general schematic for how a liposome might be deployed in a simple

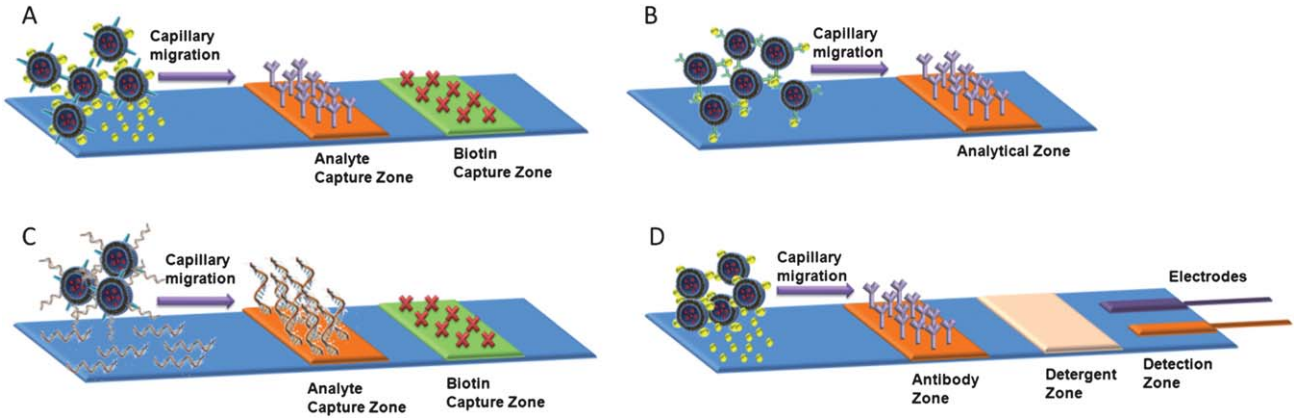

Fig. 3 Schematic strip-based liposome assays. (A) Basic competitive liposome immunoassay in strip with BC zone; (B) basic sandwich liposome immunoassay in strip; (C) basic competitive liposome DNA hybridization assay and (D) basic competitive liposome immunoassay in electrochemical strip-based device with detergent zone.

biosensor is illustrated in Fig. 3. A number of different types of liposome-based assays have been reported using liposomes as a signal amplifier, including liposome immunoassay (LIA), liposome immunolysis assay (LILA), liposome immunosorbent assay (LISA), flow-injection liposome immunoanalysis (FILIA) and cytolysin-mediated liposome immunoassay (CyMLIA), *etc.*

Liposomes have also been combined with other analytical techniques such as micro-cantilever,[57] surface plasmon resonance (SPR)[58] and quartz crystal microbalance (QCM),[59] to quantitatively or semi-quantitatively detect analytes.

Liposomes have also attracted much attention in cytologic research because liposomes can be a useful simplified cell membrane model. In this context liposome-based biosensors have been utilized to monitor the simulated physiological processes of cells and to detect the interaction between bioreceptor and ligand based on liposomal cell model.[60] Furthermore, the liposomal bilayer membrane has also been widely used as a supporting film to coat electrodes, substrates, and novel metal films (Au, Ag) in biosensors.[61]

Each of these different modes of application of liposomes in sensors are described in more detail below.

3 Using liposomes to amplify the signal in biosensors

Increased sensitivity and lower limit of detection for biosensors have been pursued through various strategies in bioanalysis and clinical diagnosis. Signal amplification is one of most efficient strategies to realize this goal in a biosensor. The ability to amplify a one-to-one biological binding event into a one-to-many signal provides the opportunity for physicochemical amplification of the signal by orders of magnitude. In this sense, liposomes have potential to act as excellent signal amplifiers when the ability to bind and/or encapsulate multiple signaling entities to a single liposome is combined with traditional assays. Consequently, low detection limits are often achieved in liposome-based biosensors or assays for analytes including hormones, viruses, bacteria, DNA/RNA segments, pesticides, tumor markers, proteins, antibodies and some drugs. Many different formats have employed liposomes as the amplifying component; these are introduced below in context with the class of signaling molecule incorporated into the liposomes to amplify the output signal.

3.1 Classes of signaling molecules encapsulated in liposomes

The different classes of encapsulated signaling molecules for liposome-based amplification strategies in biosensors are described below. The signaling molecules and output signals are summarized in Table 1.

3.1.1 Colorimetric signaling. Colorimetry is a simple method that has been widely applied in biochemical analysis and sensors.[62] There are several advantages of colorimetry, including low cost, simple instrumentation (or in the case of visual detection, no instrumentation) compared with other methods, and a qualitative or semi-qualitative test can be conducted often by the naked eye. However, the disadvantage of colorimetry is that it generally has low sensitivity. Therefore, dye-encapsulated liposomes have been employed as amplifiers of the signal to increase the sensitivity of colorimetric-based biosensors. The liposome-based assays using color as the signaling output are summarized in Table 2.

The strip-based liposome immunoassay is a common and efficient analytical technique for point of care diagnosis based on competitive and sandwich immunoassays, with colorimetric detection.[63] For a simple competitive immunoassay, a basic plastic nitrocellulose strip device (Fig. 3A) is reported in which anti-analyte antibodies are immobilized on the analyte capture (AC) zone of the strip. Capillary action draws the analyte and analyte-tagged, dye-encapsulated liposomes through the AC zone, enabling a competitive binding environment where the analyte and liposomes compete to bind to the antibody. The optical density is measured in the AC zone, and is inversely proportional to the analyte concentration in the sample. Various antigens, haptens, and low-weight molecules have been detected using a competitive liposome immunoassay.

Strip-based liposome sandwich immunoassays have also been developed to detect antigens, antibodies, proteins and

Table 1 Summary of signaling and signal markers used in liposome-based biosensors[a]

Classification of signal markers	Signal marker	Type of signal	Ref.
Dyes	SRB	Color density	64
	SRB	Fluorescence	118
	Methyl blue	Color density	73
	Arrenazo III (Mg II)	Color change	235
	Calain	Fluorescence	22
	Carboxylfluorescein	Fluorescence	119
	(2′,7′;Bis(caboxyethyl)-4-carboxylfluorescein	Fluorescence	102
	Eosin-Y	ECL	236
Enzyme ⟨substrate⟩	ALP ⟨ascorbic acid-2phosphate⟩	Color density	137
	HRP (inside) ⟨*P*-fluorophenal⟩	Electronic signal	72 and 132
	HRP (outside) ⟨Luminol⟩	CL	141
	PQQ (and GDH) ⟨2,6-dichlorophenal indophenol⟩	Color density	83
	Acetykcholunesterase ⟨acetylthiochline⟩	Fluorescence	17
	GOD ⟨glucose⟩	Electronic signal	237
	G-6-P-dh ⟨NAD^+⟩	Color density	109
	LDH ⟨NADH⟩	Color change	238
Salt or chelates	$K_4Fe(CN)_6$	Electronic signal	239
	Ascorbic acid	Electronic signal	240
	$Ru(NH_3)_6Cl_3$	Electronic signal	136
	$EuCl_3$	Fluorescence	85
	Tb/citrate	Fluorescence	12
	$Ru(bpy)_3^{2+}$	ECL	241
	Europium ion chelate	Ratio of fluor signal and background	106
Substrate	Luminol	CL	138
Nucleic acid	DNA (for secondary signal amplification)	Fluorescence	128

[a] Where SRB = sulforhodamine B; ALP = alkaline phosphatise; HRP = horseradish peroxidise; PQQ = pyrroloquinoline quinone; GDH = glucose dehydrogenase; GOD = glucose oxidase; G-6-P-dh = glucose-6-phosphate dehydrase; LDH = L-lactate dehydrogenase; $(Ru(bpy)_3^{2+}$ = tris(2,2′-bipyridyl)ruthenium; CL = chemiluminescence; ECL = electrochemiluminescence.

large bio-macromolecules. In a common liposome strip-device with sandwich immunoassay format (Fig. 3B), anti-analyte antibodies are also immobilized on the AC zone of the strip, and capillary action draws the analyte and the antibody-tagged liposomes containing the dye in sequence or together through the AC zone. The analyte and liposomes form sandwich immune complexes with the immobilized antibody, and the optical density in the AC zone is directly proportional to the concentration of analyte present in the sample.

Durst *et al.* developed a series of strip-based liposome-enabled biosensors to detect alachlor,[64] allergenic peanut protein Ara h1,[65,66] polychlorinated-biphenyls (PCB),[67] biotin,[68] *botulinum toxin*,[69] *cholera toxin*,[17,70] *Escherichia coli O157:H7*[71,72] and *salmonella*[73] based on immune competitive or sandwich immunoassays. Alachlor and PCB were quantified based on a derivational competitive immunoassay in a strip format, and were detected at low levels, 1 ppb and 0.4 nmol respectively. Detection of *botulinum toxin* and *cholera toxin* were conducted using a basic sandwich liposome immunoassay in strip-based devices, and the detection limit for each toxin was 15 pg mL^{-1} and 10 fg mL^{-1} respectively.

A derivative sandwich immunoassay with more precise results was reported by Ho, also using a strip-based device. A new biotin capture (BC) zone was combined into the plastic nitrocellulose strip device which was not present in the basic strip-based sandwich liposome immunoassay. Antibody + biotin dual-tagged liposomes containing encapsulated dye were captured in the AC zone and BC zone in sequence, providing a positive control for the strip device. The derivational strip-based sandwich liposome immunoassay was used to detect *Salmonella*[31,73] and *Escherichia coli O157:H7*[74] at levels as low as 1680 and 2500 cells, respectively.

Strip-based liposome biosensors have also been developed to detect DNA/mRNA, virus and bacterium based on the DNA/RNA hybridization assay. Rule *et al.* used liposome-based nitrocellulose strip biosensors to detect DNA segments with a specific sequence through complementary DNA sandwich hybridization.[75,76] In this assay, one end of a nitrocellulose strip with an analyte capture zone containing immobilized capture DNA segments was placed into a test tube with a mixed solution of sample DNA and oligonucleotide-tagged liposomes containing dye. During the capillary migration of the mixed solution, the target DNA segment and surface-hybridized liposomes were captured to form a sandwich-structured DNA hybridization complex in the AC zone. The color intensity of the AC zone was directly proportional to the amount of target DNA in the sample. A low detection limit of 200 amol for the target DNA was obtained in optimized conditions.

Polymerase chain reaction (PCR) or nucleic acid sequence-based amplification (NASBA) has been combined with DNA/RNA hybridization assays to increase the sensitivity of the strip-based liposome-enabled biosensor. Prior to the test, the mRNA of the virus in the sample was extracted, purified and finally amplified using NASBA.[77] Then a similar liposome DNA/RNA hybridization assay was carried out to quantify the mRNA using a reflectometer in the strip-based membrane biosensor. High sensitivity and low detection limit to bacterial and virus mRNA were realized due to the usage of NASBA. Specifically, *Escherichia coli*, viable *B. anthracis spores*[78,79] and *Dengue serotypes*[80] were detected down to 40 *E. coli* cfu mL^{-1}, 1 fmol target mRNA and 10 plaque forming units pfu mL^{-1}, respectively.

Chang *et al.* also reported a membrane-strip-based lateral-flow (LF) assay which, when combined with a reverse transcriptase polymerase chain reaction (RT-PCR), enabled the detection of a small number (ten) of viable *Mycobacterium* (M.) *avium* subsp. *paratuberculosis* (MAP) cells in fecal samples.[81] A wide detection range from 10^1 to 10^6 MAP cells was achieved in the strip-based liposome biosensor.

A strip-based liposome biosensor based on the DNA/RNA competitive hybridization assay was also developed for the detection of *Cryptosporidium parvum*.[82] The DNA/RNA competitive hybridization assay combined with NASBA was carried out

Table 2 Summary of liposome-based biosensors that utilise color/colorimetric signaling[a]

Analyte (in matrix)	Liposome composition (lytic agent)	Assay format (analytic technique)	Marker (signal)	Detection range (detection limit)	Ref.
Alachlor	DPPC, chol	Competitive (strip assay)	SRB	5–10 ng mL^{-1}	64
Allergenic peanut protein Ara h1	DPPC, DPPG, chol, DPPE-biotin, DPPE-ATA	Competitive (strip assay)	SRB	1–10 μg mL^{-1}	65 and 66
Alachlor; polychlorinated-biphenyls	DPPC, DPPG, chol, DPPE-biotin, DPPE-analyte	Competitive (strip assay)	SRB	(1 ppb; 0.4 nmol)	67
Biotin	DPPC, DPPG, chol, DPPE-biotin	Competitive (strip assay)	SRB	(6 ng mL^{-1})	68
IgE	DPPC, chol, DPPG, PE-MCC	Competitive (strip assay)	SRB	1.85–500 ng mL^{-1}	242
Botulinum toxin	DPPC, DPPG, chol, DPPE-ATA	Sandwich (strip assay)	SRB	1 to 1 × 10^6 pg mL^{-1} (15 pg mL^{-1})	69
Cholera toxin (seafood)	DPPC, DPPG, chol, GM1	Sandwich (strip assay)	SRB	0.1–30 pg mL^{-1} (10 fg mL^{-1})	70
Escherichia coli O157:H7	DPPC, DPPG, chol, MMCC-DHPE	Sandwich (strip assay)	SRB	(1 × 10^4 CFU mL^{-1}; 2500 cells)	71
Salmonella	DPPC, DPPG, chol, biotin-X-DHPE, DPPE	Sandwich (strip assay)	Methyl blue and SRB	(1680 cells; 1 × 10^2 CFU mL^{-1})	73
DNA	DPPC, chol, DPPE-DNA; DPPC, DPPG, chol, DPPE-DNA	Sandwich hybridization (strip assay)	SRB	(200 fmol; 200 amol)	75 and 76
Viable *B. anthracis spores*	DPPC, DPPG, chol, DPPE-DNA	Sandwich hybridization (strip assay)	SRB	1.5–100 fmol; (1 μmol mL)	78 and 79
Dengue serotypes	DPPC, DPPG, chol, DPPE-DNA	Sandwich hybridization (strip assay)	SRB	10 plaque forming units pfu mL^{-1}	80
MAP (in fecal)	DPPC, DPPG, chol	Sandwich hybridization (strip assay)	SRB	1 to 1 × 10^6 cells	81
Cryptosporidium parvum	DPPC, DPPG, chol, DPPE-biotin, MMCC-DHPE	Competitive hybridization (strip assay)	SRB	(80 fmol)	82
DNA	Egg PC, maleimine PE, chol, (Tween 20)	Sandwich hybridization	DCPIP	10–500 fmol (62 fmol)	83
MMP-9	Peptide–lipid, POPC	Destabilization	HRP	(10 nM)	104
PLA_2	DOPC	Destabilization	Peptide (wavelength)	700 pM to 7 nM	105
Amine derivatives	Peptide–lipid	Activate enzyme	NADH	10 nM (maximum)	238

[a] Where MAP = *Mycobacterium avium* subsp. *Paratuberculosis*; MMP-9 = matrix metalloproteinases 9; PLA_2 = phospholipase A2; SRB = sulforhodamine B; HRP = horseradish peroxidise; DCPIP = 2,6-dichlorophenol indophenol.

in a strip-based device with two zones (AC zone and BC zone) (Fig. 3C). In the strip-based liposome DNA/RNA hybridization assay, immobilized DNA, complementary to the reporter probe in the AC zone, and amplicons of *Cryptosporidium parvum* RNA, competitively hybridize with reporter DNA and biotin dual-tagged, dye-entrapping liposomes. If the target RNA is present at the hybridization step, the liposomes are captured on the BC zone. Otherwise, the liposomes bind to the AC zone. The detection limit of the assay was determined to be 80 fmol amplicon per assay. Liposomes can encapsulate other probes that could activate or catalyze a substrate and then indirectly generate a color density signal. Meyerhoff *et al.* reported a DNA sandwich hybridization assay using DNA-tagged liposomes containing pyrroloquinoline quinone (PQQ) to detect specific DNA sequences in a microtiter plate.[83] PQQ was used for its ability to activate an apo-enzyme glucose dehydrogenase (GDH). In the presence of glucose and DCPIP (2,6-dichlorophenol indophenol), activated GDH could reduce DCPIP and yield an optical color change from blue to colorless. Arbitrary target DNA sequences could be detected using the assay at a low detection limit of 62 fmol.

3.1.2 Fluorescence. Fluorescent dyes are widely used as encapsulated agents in liposome-based biosensors for signal amplification, as summarized in Table 3. Homogeneous liposome immunoassays are desired for convenience (no separating or washing step required) and potential commercial value. Fluorescence enhancement from self-quenching dyes (calcein and carboxyfluorescein (CF) *etc.*) and fluorescence resonance energy transfer (FRET)[84] are efficient strategies to realize homogeneous immunoassays. For the fluorescence enhancement strategy, lysis and destabilization of liposomes using a cytolytic reagent is usually utilized to enhance fluorescence intensity, through the leakage of encapsulated self-quenching dyes from liposomes for homogeneous liposome immunoassays.

Complement, melittin[85] and phospholipase[86–88] are widely used as lytic reagents to lyse liposomes in homogeneous cytolysin-mediated liposome immunoassay (CyMLIA). A wide range

Table 3 Summary of liposome-based biosensors using fluorescence the signaling output[a]

Analytic technique	Analyte (in matrix)	Liposome composition	Assay format (lytic agent)	Marker	Detection range (detection limit)	Ref.
Homogeneous	Mycotoxin T-2	DMPC, chol, DCP, Ag-PE	(Complement)	CF	1×10^{-8} to 1×10^{-4} M (2 ng)	89
	Antibody	DPPC, chol, DCP, Ag-PE	(Complement)	CF	—	90 and 130
	Atrazine	Lecithin, chol, decetyl, atrazine-DMPE	(Complement)	Calcein or SR 101	(0.13 ng mL^{-1})	45
	Carbofuran	PC, PE	Competitive (CPCF)	CF (Tb/citrate)	10 pg to 10 ng	91
	Ferritin (in serum)	DPPC, chol, DPPE, DTP-DPPE	Sandwich (complement)	CF	10–2000 μg L^{-1}	99
	Antibody	MPB-DPPE, DPPC, chol, DCP	(Complement)	CF	—	93
	Archaebacteria	MPB-DPPE, DPPC, chol, DCP	(Complement)	CF	—	96
	Insulin	DPPC, chol	Competitive (phospholipase C)	Calcein	(8 μIU mL^{-1})	86
	Enrofloxacin	NERO-DPPE, DPPC, chol, DCP	Competitive (complement)	SRB	5–20 ng mL^{-1} (1 ng g^{-1})	98
Energy transfer	Biotin	BDMPE, DMPC, chol	Competitive	Eu III	0.1–100 μM	97
Destabilization	Avidin	PC, PE, DNP-PE, biotin-PE, MBP-PE	—	BCECF	10–1000 ng mL^{-1}	102
	GHRP; somatostatin	PC, chol, PE	—	BCECF	0.5–50 ng mL^{-1} (0.2; 0.3 ng mL^{-1})	243
	Paraoxon	Egg PC	—	AChE	(6.7×10^{-10} M)	103
	Theophylline	DOPC, DOPE, theophylline-PE, MPB-PE DOPS	(Antibody)	Calcein	—	92
LISA	hiFN-γ; interleukin-2	DPPC, DPPG, chol, biotin-X-DHPE	Sandwich (Triton-100)	CF	6–2400 × 10^{-12} M; 0–100 IU mL^{-1} (3IU mL^{-1})	110 and 111
	Cholera toxin	DPPC, DPPG, chol, GM1, ganglioside	Sandwich (OG)	SRB	0.43–500 ng mL^{-1} (0.34 ng mL^{-1})	112
	DNA	DPPC, DPPG, chol, *N*-glutaryl-DPPE	Sandwich hybridization (Tween-20)	SRB	5 nmol mL^{-1} to 50 μmol mL^{-1} (1.7 nmol mL^{-1})	113
	RNA/DNA	DPPC, DPPG, chol, chol-oligonocleotides	Sandwich hybridization (OG)	SRB	(0.11 ng mL^{-1})	114
	α-Thrombin	DPPC, DPPG, chol, chol-TEG-aptamer	Sandwich (OG)	SRB	(2.35 ng mL^{-1})	244
	Feline calicivirus	DPPC, DPPG, chol, *N*-glutaryl-DPPE, DPPE-SRB	Sandwich (OG)	SRB	1.6×10^5 PFU mL^{-1}	125
FILIA	Alchlor (in water)	DPPE-alachlor	Competitive (OG)	SRB	(2 ppm)	118
	Imazethapy	DPPC, DPPG, chol, DPPE-analyte	Competitive (OG)	SRB or CF	(0.1 ppm)	119
	Theophylline (in serum)	DMPC, DCP, chol, Ag-PE	Competitive (OG)	CF	25–400 ng mL^{-1} (39 nM)	121
	Fumonisin B1	DPPC, DPPG, chol, 2DPPE-analyte	Competitive (OG)	SRB	1–1000 ng mL^{-1} (1 ng mL^{-1})	123
	Cholera toxin	DPPC, DPPG, chol, GM1	Sandwich (OG)	SRB	0.1–10 fg mL^{-1} (0.066 fg mL^{-1})	124
	Insulin	DPPC, DPPG, chol, DPPE-acetylthioacetate	Sandwich (OG)	SRB	10 pM–10 nM (136 attomole)	126
	Escherichia coli O157:H7	DPPC, DPPG, chol, DPPE	Sandwich (OG)	SRB	6×10^3 to 6×10^7 cells mL^{-1} (360 cells mL^{-1})	127
Secondary amplification	PSA	DPPC, chol, DPPE	Sandwich (Triton-100)	DNA/ FITC-probe	0.1 fg mL^{-1} to 0.1 ng mL^{-1} (0.08 fg mL^{-1})	128
	B. anthrcis	DPPC, DPPG, chol, DPPE	Sandwich (OG)	Fluorescein/ SRB	4.1–5000 ng mL^{-1} (4.1 ng mL^{-1})	129

[a] Where LISA = liposome immunosorbent assay; FILIA = flow-injection liposome immunoanalysis; GFRP = growth-hormone-related peptides; hiFN-γ = human interferon-γ; PSA = prostate specific antigen; OG = *n*-octyl-*b*-D-glucopyranoside; CF = carboxylfluorescein; AChE = acetylcholinesterase; CPCF = 5-(2-2-dimethyl-2,3-dihydro-benzofuran-7-yloxy)-pentanoic acid; BCECF = 2′7′-bis(carboxyethyl)-4 or 5-carboxyfluorescein.

of different analytes could be detected using these lytic reagents based on competitive and non-competitive CyMLIAs, such as antigens,[89,90] antibody, herbicide,[45] pesticide,[12,91] haptens,[92] proteins,[93–95] bacteria,[86,96] biotin[97] and drugs.[98]

In CyMLIA, using complement as the lytic reagent, complement proteins bind to antibodies in a specific order for lysis of liposomes with the antibodies in competitive or non-competitive homogeneous liposome immunoassays. In the competitive format, antigens and antigen-tagged liposomes competitively bind to antibodies in a specific order, and the bound liposomes with antibodies are lysed by complement proteins later to release fluorescent dyes. In the non-competitive format, antigens and antibodies bind in sequence to the antibody-tagged liposomes containing dye, to form a sandwich immune complex on the liposome surface, with the liposomes in the sandwich immune complex being subsequently lysed by complement proteins.[99]

In CyMLIA using melittin as the lytic reagent, the competitive format is common because bound melittin is unable to lyse liposomes. In competitive CyMLIA, analytes and melittin–analyte competitively bind with antibodies, and the free melittin–analyte conjugate will lyse the liposomes and release the dye, enabling quantitation of the analytes.

Destabilization of liposomes, as opposed to lysis, is an alternative strategy which uses self-quenching or pH sensitive dyes for liposome-based bioanalysis. For the destabilization strategy, the interaction between analytes and liposomes induces a change of environment or leakage of dye,[22,100] which results in a change in fluorescence intensity. This strategy was again successfully utilized to detect a wide range of analytes including IgG,[101] *Herpes Simplex Virus* (HSV), DNP-glycine, dichoryos,[102] paraoxon,[103] MMP9,[104] PLA2,[105] avidin, neurokinin A and substance P.

The FRET strategy has also been utilized to develop homogeneous liposome immunoassays. For the FRET strategy, the fluorescent donor is inserted into the bilayer of liposomes.[97,106] When the receptor-conjugated-analyte recognizes and contacts the liposomes, energy transfer from donors in the liposomes to receptors can take place, and the fluorescence enhancement of receptors was therefore utilized to quantify the analyte. In addition, homogeneous liposome immunoassays were also realized through color density using enzyme encapsulated in liposomes to catalyze substrates.[107–109] For example, in CyMLIA a glucose 6-phosphate (G6P)-encapsulated liposome has been utilized, where the color change can be realized by the change from nicotinamide ademine dinucleotide (NAD^+) into NADH which is catalysed by released G6P.

Liposome immunosorbent assay (LISA) is an important and common assay, which combines liposomes as a signal amplifier and enzyme linked immunosorbent assay (ELISA). Many derivative formats were developed based on competitive or sandwich formats using LISA. In the basic competitive LISA (Fig. 4A), analyte-tagged liposomes containing fluorescent dye compete with the analyte to bind to the immobilized antibody on the substrate. The encapsulated dye in the bound liposomes is then released by detergent. The intensity of fluorescent signal is then inversely proportional to the concentration of the analyte. In the basic sandwich LISA (Fig. 4B), antibody is first immobilized on the substrate, then analyte and secondary antibody-tagged liposomes were bound in sequence to the substrate to form sandwich immune complexes. Finally, the liposomes were lysed to quantify the analyte in a proportional relationship. Several derivative sandwich LISA assays have been developed based on the interaction between biotin and avidin. Higher sensitivity and lower detection limit were both achieved in the LISA approach compared with traditional ELISA due to the signal amplification provided by the dye-encapsulated liposomes. Human interferon-γ,[110,111] *cholera toxin*[112] and *botulinum antitoxin* were detected using the LISA approach.

Viral DNA and mRNA were detected using fluorescent dye encapsulated in liposomes as the signal amplifier, based on DNA/RNA hybridization assay as described above for colorimetric detection (Fig. 5). A derivative sandwich DNA hybridization assay based on the interaction of biotin and streptavidin was performed to detect the DNA sequence. Low detection

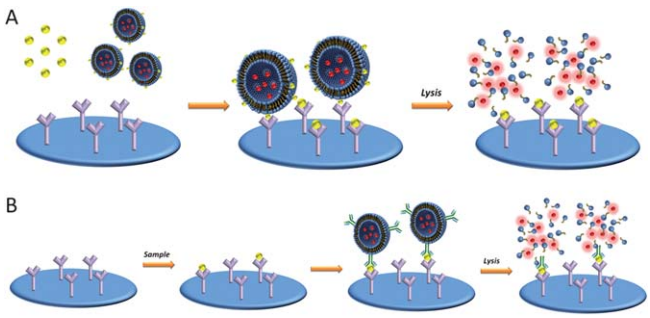

Fig. 4 Schematic of basic LISAs. (A) Competitive format; (B) sandwich format.

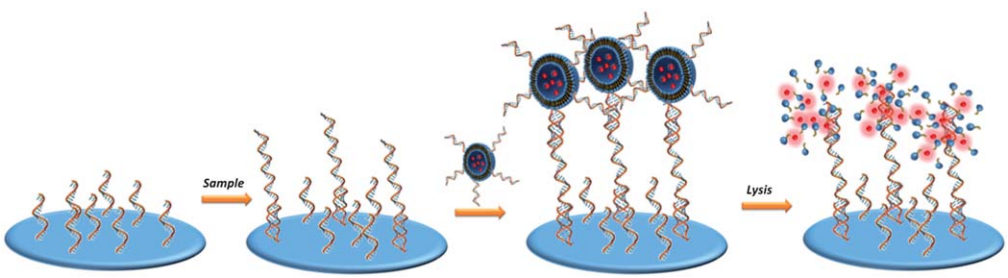

Fig. 5 Schematic of a basic sandwich liposome DNA hybridization assay.

limits were achieved in the biosensor for a synthetic DNA concentration down to 1.7 pmol L^{-1}.[113] A more sensitive assay was achieved or the detection of atxA gene of *Bacillus anthracis* as 1.1×10^{-7} mg mL^{-1} through combination with NASBA.[114]

Flow-injection analysis (FIA) is a simple but elegant analytical technique, which has been widely used in biochemical analysis.[115] In a FIA system, a discrete liquid sample is injected into a liquid carrier biochemical analysis stream, wherein the sample undergoes on-line biochemical reaction during transport to a flow-through detector. Flow-injection liposome immunoanalysis (FILIA) has been developed based on the combination of the FIA technique and liposomal signal amplification.[116] FILIA has several advantages including high sensitivity, short testing period, recycling and the use of an automated instrument. A short testing period is required in the FILIA system because the washing step is conducted automatically, and an incubation stage is not required.[117]

In competitive FILIA, antibodies are usually immobilized onto a solid phase in the immunoreactor. Analyte-tagged liposomes containing the dye and the sample are injected in sequence or together into the immunoreactor, and competitive binding to the immobilized antibody occurs. Detergent is injected to release fluorescent dye from the liposomes for signal generation after the unbound sample and liposomes are washed away with flow buffer or water. The fluorescent signal is measured, and is inversely proportional to the amount of analyte in the sample. An individual test is performed in 10 to 30 min, and the immunoreactor of FILIA is recycled by de-binding the immune complexes using methanol–water mixed solvents.

The herbicide alachlor,[118] imazethapy,[119] hormone 17-b-estradio,[120] theophylline[121] and fumonisin B1[122,123] could all be detected by competitive FILIA, and low detection limits of 2 ppb were obtained for alachlor in water, 0.1 ppb for imazethapyr, and 1 ng mL^{-1} for fumonisin B1.

Sandwich FILIA is usually performed in a similar instrument based on different testing process compared with the competitive FILIA. During a sandwich FILIA, sample and antibody-tagged liposomes are injected and captured by the immobilized antibody to form sandwich immune complexes in the immunoreactor, then the encapsulated dye is released through injection of detergent to detect the amount of analyte in sample. Detection of *cholera toxin*,[124] feline calicivirus (FCV),[125] insulin[126] and *Escherichia coli O157:H7*[127] have been reported using sandwich FILIA, with low detection limits of 6.6×10^{-17} g mL^{-1}, 136 attomole, and 360 cells per mL, respectively.

Secondary amplification of the signal has also been developed to increase the sensitivity of biosensors based on various strategies. Chu *et al.* developed the rolling circle amplification (RCA) immunoassay strategy through encapsulating DNA with a special sequence for secondary signal amplification.[128] In the RCA strategy, a special DNA segment as the primary probe was used to initiate secondary amplification of signal. The released DNA segments initiate RCA to generate a long tandem repeated sequence after which a sandwich LISA was carried out. The RCA products were hybridized with FITC-labeled probes and captured by microbeads to quantify prostate-specific antigen (PSA), and PSA was detected at concentrations as low as 0.08 fg mL^{-1} based on this strategy.

Baeumner and Edwards developed other secondary signal amplification strategies to detect *B. anthracis*, where encapsulated DNA segments are the analyte in LISA to perform secondary signal amplification based on the DNA hybridization assay in a microtiter plate.[129] Liposomes encapsulating fluorescein-labelled DNA were used as the primary probe amplifier in the first sandwich LISA in a microtiter plate well. Then the released fluorescein-labeled DNA was injected into the second microtiter plate well, and a sandwich DNA hybridization assay was performed using anti-fluorescein antibody-tagged liposomes containing RSB as signal amplifier. *B. anthracis* as low as 4.1 ng mL^{-1} was detected based on this strategy.

3.1.3 Electrochemistry. Electrochemistry is a sensitive, fast and convenient analytical technique widely used in the sensor field. There are several advantages of electrochemical detection. First, the electronic signal is a stable and sensitive signal that can be rapidly and easily detected. Secondly, the electrochemical devices are readily miniaturized for development of portable sensors, without the need for larger detectors. Therefore, liposome-based electrochemical biosensors based on electrochemical markers encapsulated in liposomes as a signal amplifier (Table 4), have attracted great attention in biochemical analysis.

Electronic markers encapsulated in liposomes as signal amplifiers were successful utilized in strip-type biosensors and FILIA devices. Baumner and Lee developed strip-based electrochemical biosensors with a thick film electrode printer. Two zones (antibody zone as the competition zone and signal generation zone as detergent zone) were used in the strip-based electrochemical biosensors. Hapten-tagged liposomes containing ascorbic acid were incubated with the sample, and capillary action used to migrate the solution across the antibody zone and signal generation zone (Fig. 3D). The released ascorbic acid then acts as the electrochemical marker and is detected by a graphite electrode. Atrazine[130] and terbutylazine at concentrations as low as 1 mg L^{-1} were detected in tap water by the biosensor. Lee reported a strip-based competitive biosensor for the detection of theophylline.[131] In the biosensor, three zones were used; a sample loading zone containing a theophylline–melittin conjugate, an antibody zone with anti-theophylline antibody, and a signal generation zone with hexacyanoferrate(II)-loaded liposomes. The sample and theophylline–melittin competitively bound to the antibody zone after passing the sample loading zone, then unbound theophylline–melittin migrated into the signal generation zone and released the encapsulated hexacyanoferrate(II) for generation of electronic signals. The concentration of analytes was related to current, and a range of 10–20 μg mL^{-1} of analyte was detected.

Durst and Wu developed a competitive FILIA assay using the electrochemical technique to detect theophylline[132] and alachlor.[30] In this assay, peroxidase was encapsulated in liposomes for generation of fluoride ions from the organofluorine substrate, and the fluoride ions were measured by ion-selective electrode for quantification of theophylline. A wide range of detection for theophylline from 0.2 to 4000 ng mL^{-1} was achieved by the FILIA.

Table 4 Summary of liposome-based biosensors utilizing electrochemical or chemiluminescent signaling markers[a]

Analytic technique	Analyte (in matrix)	Liposome composition (lytic agent)	Assay format	Marker	Detection range (detection limit)	Ref.
Strip assay (electronic)	Atrazine; terbutulazine	DPPC, DPPG, chol, DPPE (Triton-100)	Competitive	AA	1 ng mL^{-1}	240
	Theophylline	PC (Tween-20)	Competitive	Potassium hexacyanoferrate II	10–20 μg mL^{-1}	131
FILIA (electronic)	Theophylline	Ab-liposome (OG)	Competitive	HRP/fluoride ion	0.2 ng mL^{-1}	132
	Alachlor	DPPC, DPPG, chol, Ag-DPPE (Triton-100)	Competitive	($K_2Fe(CN)_6$)	0.5 ng	30
LIA (electronic)	*Cholera toxin*	DPPC, DPPG, chol, GM1 (Triton-100)	Sandwich	Potassium ferrocyanide	0.01–100 000 pg mL^{-1} (0.1 fg mL^{-1})	134
	CEA (in saliva or serum)	DPPC, DPPG, chol, DPPE-aceylthioacetate (Triton X)	Sandwich	Ferrocene	5–500 000 pg mL^{-1} (1 pg mL^{-1})	133
	Nucleic acid	DOPC, DOPE, DSPE-PEG-biotin	Sandwich hybridization	Ca^{2+}	0.2 nM	29
	Escherichia coli O157	DPPC, DPPG, chol, PE-MCC (Triton-100)	Sandwich hybridization	$Ru(NH_3)_6Cl_3$	1 to 1 × 10^6 fmol	136
	PSA	PC, chol, PE (Triton-100)	Sandwich	AA; phosphate	0.01–100 ng mL^{-1} (0.0007 ng mL^{-1})	137
	NSE; proGRP	DPPC, chol, DPPG (Triton-100)	Sandwich	AA and uric acid	50–1000 pg mL^{-1}; 5–100 ng mL^{-1}	245
LIA (ECL)	Hemagglutinin molecule	DPPC, SPDP-SPPE, chol (Triton-100)	Competitive	Ru II complex	0.03–2 pg mL^{-1}	140
	Tobramycin; dinitrophenyl; Oligonucleotide	DPPC, DPPG; DPPE; DSPE-PEG (2000)-biotin (DOC)	Competitive	Luminol	0.96 μM; 0.58 μM; 18 μM.	32
	Cholera toxin	DMPC, PE, GM1	Sandwich	HRP (outside)	1 pg mL^{-1} to 1 ng mL^{-1} (0.8 pg mL^{-1})	141
	PSA	DPPC, PE. (Triton-100)	Sandwich	HRP (luminol)	0.74 pg mL^{-1} to 0/74 μg mL^{-1} (0.7 pg mL^{-1})	142
	HCRP	DSPC, DSPG, chol, DSPE-PEG-biotin (Triton-100)	Sandwich	$Ru(bpy)_3^{2+}$	100 ng mL^{-1} to 10 μg mL^{-1}	25
	NT-proBNP	DPPC, DPPE, chol (Triton X)	Sandwich	$Ru(bpy)_3^{2+}$	0.01–500 ng mL^{-1} (0.77 pg mL^{-1})	139

[a] Where LISA = liposome immunosorbent assay; FILIA = flow-injection liposome immunoanalysis; ECL = electrochemiluminescence; CEA = carcinoembryonic antigen; NSE = neuron-specific enolase; proGRP = pro-gastrin-releasing peptide; HCRP = human C-reactive protein; NT-proBNP = N-terminal pro-brain natriuretic peptide; PSA = prostate specific antigen; OG = *n*-octyl-*b*-D-glucopyranoside; AA = Ascorbic acid; HRP = horseradish peroxidise.

Alachlor was detected using ferrocyanide as the electrochemical marker in FILIA. In addition, electrochemical LISA has also been used for the detection of neuron-specific enolase, progastrin-releasing peptide, carcinoembryonic antigen, *cholera toxin* and insulin based on combination of traditional LISAs and electrochemical technique. Ascorbic acid, uric acid, ferrocene carboxylic acid, potassium ferrocyanide and ferrocene were used as the electrochemical markers for detection of neuron-specific enolase at a concentration as low as 50 pg mL^{-1}, progastrin-releasing peptide at 5 ng mL^{-1}, carcinoembryonic antigen at 5 × 10^{-7} g mL^{-1},[133] *cholera toxin* at 1 × 10^{-16} g (ref. 134) and insulin at 10 pg mL^{-1},[135] respectively. Nucleic acid sequences and *Escherichia coli O157* based on DNA hybridization assays[29,136] were also detected using liposome-encapsulated electrochemical markers.

Secondary electrochemical signal amplification was also reported by Chu *et al.* to detect human prostate specific antigen (PSA) through encapsulation of alkaline phosphatase (ALP) in liposomes and relying on sandwich LISA.[137] In this strategy, ALP was utilized as the signal marker for electrochemical signal amplification, because ALP can hydrolyze ascorbic acid 2-phosphate (AA-p) to generate ascorbic acid, and yield a reduced amount of silver ion to deposit on the surface of the electrode of the biosensor. Linear sweep voltammetry (LSV) was then used to detect the amount of the deposited silver for quantification of the analyte. A detection limit as low as 0.007 ng mL^{-1} for PSA was detected using this approach.

3.1.4 Chemiluminescence (CL). Chemiluminescent immunoassay (CLIA) has been widely used and successfully commercialized in clinical diagnosis. There are two main types of CLIA, enzyme chemiluminescent immunoassay (ECLIA) and electrogenerated chemiluminescent immunoassay. Enzyme and Ru^{2+} chelate are utilized to generate chemiluminescence through catalyzing CL substrate and electronic excitation in ECLIA and electrogenerated CLIA, respectively. Liposomes provide an opportunity for amplification of CL signals in CLIA through encapsulation of enzyme, CL substrate[138] and Ru^{2+}

chelate,[139] or embedding enzymes onto the surface of liposomes. Liposome-based CLIA was successfully utilized to increase the sensitivity of the CL sensor and to detect trace analytes (Table 4).

Bard and Zhan developed a liposome-based sandwich CLIA to detect human C-reactive protein (CRP) through magnetic separation.[25] In the CLIA, antibody-labelled magnetic beads, relying on the interaction between avidin and biotin, are used to remove the unbound components. $Ru(bpy)_3^{2+}$ as the CL marker was encapsulated in antibody-tagged liposomes for generation and amplification of CL in a liposome-based sandwich CLIA. The detection range for CRP between 100 ng mL^{-1} to 10 μg mL^{-1} was detected by the biosensor. Liposome-based competitive CLIA was also developed using Ru(II) complex encapsulated in liposomes to detect hemagglutinin molecules of the influenza virus and Legionella antigen in a microtiter plate format[140] and strip-based biosensors, respectively. Hemagglutinin molecules of the influenza virus and Legionella antigen were detected at concentrations as low as 2×10^{-9} g mL^{-1} and 2×10^{-12} g mL^{-1} respectively.

Liposome-based sandwich ECLIA was also developed to detect *cholera toxin* by Jiang *et al.*[141] In the ECLIA, horseradish peroxidase (HRP) and monosialotetrahexosyl ganglioside (GM1) dual-tagged liposomes were utilized to form sandwich immune complexes comprising an antibody–*cholera toxin*–liposome structure, and yielded the CL signal through HRP catalyzing a chemiluminescent substrate. A low detection limit down to 0.8 pg mL^{-1} was achieved for *cholera toxin* in the biosensor. Similar liposome-based sandwich ECLIA was also developed to detect prostate-specific antigen (PSA) based on the encapsulation of HRP into liposomes, and a wide range of PSA from 0.74 pg mL^{-1} to 0.74 μg mL^{-1} was detected by the ECLIA.[142] In addition, the strategy to generate CL through encapsulation of CL substrate into liposomes, was also developed to detect specific antigen–antibody reactions and nucleotide hybridization.[32]

3.1.5 Other liposome amplification assays. An additional approach to using liposomes to amplify signal in biosensors aside from the aforementioned encapsulation approach is the liposome-based turbidimetric assay (LTA) strategy. Turbidimetric assay (TA) is a widely used immunoassay in clinical diagnosis. TA detects analytes based on turbidimetric change from immune recognition between antibodies and analytes. Nanoparticles such as polymeric latex or metallic particles are usually utilized to increase the sensitivity of TA because recognition of antibody/antigen-labeled particles and analytes in the sample induces them to aggregate into large particles which more effectively scatter light, providing the enhanced turbidity. Liposomes, being particles in the nano- to micro-scale, are also useful in this regard, and hence were used to develop the liposome turbidimetric assay.[143,144] DNA sequences and proteins such as CRP were detected by LTA based on immune recognition and DNA hybridization,[145] respectively.

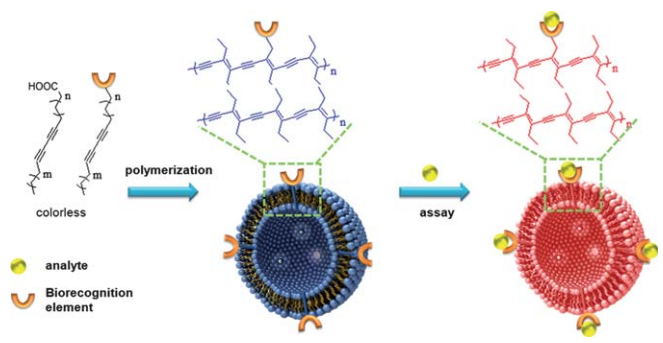

Fig. 6 Schematic showing the structure and assay mechanism for PDA-liposomes.

3.2 Responsive liposomes for signal amplification in biosensors

Obviously from the above extensive discussion, marker encapsulation is an important amplification strategy, however, liposomes may also be utilized to respond to the environment and provide amplification that is not dependent on release of an encapsulated entity. For example, liposomes prepared using polydiacetylene (PDA) lipids[146] can respond to the interaction between analyte and liposome through a change of color. Liposomes can also be used to quantify analytes when coupled to other special analytical techniques including surface plasmon resonance (SPR) and quartz crystal microbalance (QCM). This section will review the construction of non-marker-encapsulated liposome systems as assays or biosensors.

3.2.1 Biosensors based on liposomes with polydiacetylene (PDA) lipids. Polydiacetylene (PDA) is an important optical material due to its property of changing color in response to environmental stimuli such as heating, solvent changes, exposure to vapors and changes in pH. The color change is rapid, and can be observed as a color shift by the naked eye. Therefore, PDA is an excellent material for a sensor, and has been employed in liposome-based biosensors through the usage of PDA either included as part of the phospholipid fatty acid chains, or in a second molecule that occupies the bilayer and still engages in polymerization within the bilayer.[147] In the PDA liposome biosensor, either adsorption or binding of analytes to biorecognition elements conjugated to the PDA liposome can result in a color change,[148] usually within a few minutes (Fig. 6).

Various analytes, including proteins, viruses, bacteria and antibodies, were detected using PDA liposome-based biosensors. Jelinek *et al.* developed a PDA liposome-based biosensor to detect antibody, protein and bacteria based on biorecognition element-embedded PDA liposomes.[149–152] Li *et al.* focused on the development of biosensors to detect *Escherichia coli* relying on functionalized PDA-glycolipid liposomes.[153–155] In this biosensor, the interaction between the glycolipid and *Escherichia coli* affects PDA disposition in the liposome, and a color change from blue to red on binding of the *Escherichia coli* results. Park *et al.* developed a PDA liposome-based colorimetric biosensor for detection of streptavidin, based on aggregation of PDA liposomes. In this biosensor, the streptavidin as analyte causes the aggregation of biotinylated PDA liposomes, and stimulates the color change.[156,157] The strategy was also utilized to detect nucleic acids based on the interaction of nucleic acids and positively charged PDA liposomes.[158]

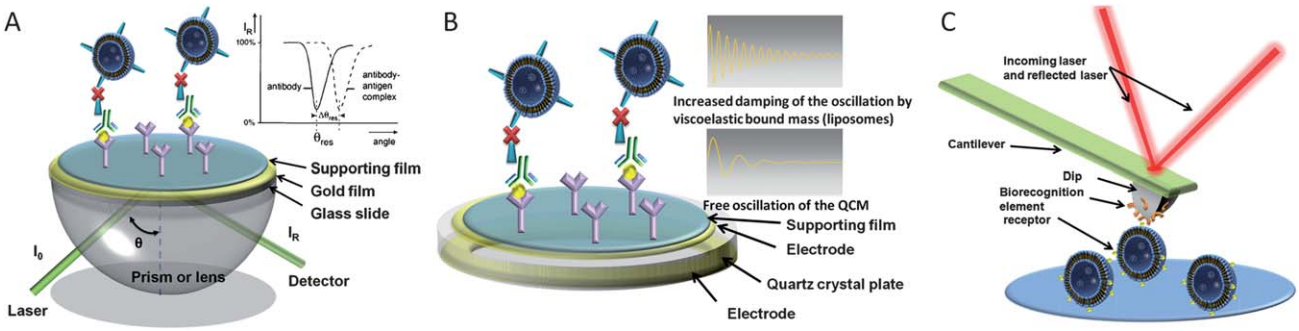

Fig. 7 Schematic of the basic liposome-based SPR, QCM and micro-cantilever biosensors. (A) A sandwich immunoassay using liposomes to increase the sensitivity in SPR sensor; (B) a sandwich immunoassay using liposomes to increase the sensitivity in QCM and (C) the mechanism of detection using a micro-cantilever to detect the interaction between bio-macromolecules based on liposomes as a cell-model.

A fluorescence resonance energy transfer (FRET) biosensor was also developed using a fluorescent dye-encapsulated, biotinylated PDA liposomes to detect streptavidin.[159,160] In the FRET PDA liposome-based biosensor, the fluorescence of a dye is quenched in unbound PDA liposomes due to FRET from dyes to PDA; fluorescence is enhanced when streptavidin binds to the biotinylated PDA liposome. PDA liposome-based biosensors have also been utilized to detect other analytes, including autoantibodies,[161] influenza virus,[162,163] human serum albumin,[164] carbonic anhydrase, *Escherichia coli*,[165,166] bacterial pore-forming toxin,[167] thrombin,[168] melamine[169] and other pathogenic agents.[170]

The PDA liposome biosensor has advantages including rapid output, high sensitivity and visualization by the naked eye. Disadvantages include a lack of specificity, qualitative analysis and expensive cost from complicated organic synthesis. Therefore the commercial application of PDA liposomes has not yet been realized. Nevertheless, the PDA liposome has still opened up a new approach to construct liposome-based biosensors.

3.2.2 Using liposomes in combination with a surface plasmon resonance (SPR) sensor. SPR spectroscopy has been an important characterization technique which has been applied to a variety of chemical and biological sensing.[171,172] A total reflection occurs when a beam of monochromatic light with certain incident angle (SPR angle) irradiates a prism[173] which is coated with a thin metal film (usually noble metal). Surface plasmon, which is the collective oscillations of free electrons in a metal film, can be excited to resonate with light. In the process, dissipation of optical energy in the metal film accompanies the transfer of optical energy into the surface plasmon (Fig. 7A). The SPR angle is usually determined by the coupling medium, refractive index, and thickness of adsorbed layers of analyte under special conditions. Here, the reflection index of the gold film affects the SPR angle, which is proportional to the amount of bound molecules on the surface of the gold film. The SPR sensor is extremely sensitive, and can detect bound trace amount of molecules on the surface of the gold film. Liposomes have a similar bilayer membrane structure to cells, and can be surface modified with bio-receptors. Therefore, liposomes as a supporting film are used in the SPR sensor to investigate the interaction among biomolecules and cells (this aspect will be discussed later). SPR sensors are also utilized to quantify analyte through the use of liposomes. Wink *et al.* developed a liposome sandwich immunoassay to detect interferon-γ in an SPR biosensor.[174] In this assay, a basic sandwich immunoassay was carried out. Antibodies were immobilized on a polystyrene layer which covered the gold film on a hemicylindrical lens. Then the sample and biotinylated secondary antibodies were added in sequence to form sandwich immune complexes. The biotinylated liposomes were bound to the biotinylated sandwich immune complex through bridging of streptavidin for amplification of the shift in SPR (Fig. 7A).

3.2.3 Using liposomes in combination with the quartz crystal microbalance (QCM). QCM is also a sensitive and highly efficient surface-specific technique to investigate the interaction among biomolecules and cells or to quantify analytes. In QCM, the frequency of oscillation of the piezoelectric quartz-crystal is dependent on the mass of adsorbed material on the electrode surface, given by the Sauerbrey equation.[175] QCM is widely used in bioanalysis for detection down to the nanogram level. Liposome-based assays can be combined with QCM to increase the sensitivity for detection of analytes (Fig. 7B).

Willner *et al.* developed a liposome-based QCM biosensor for quantitative analysis based on a sandwich immunoassay.[59,176,177] In this QCM biosensor, a sandwich immunoassay was firstly carried out to form biotinylated sandwich immune complexes through binding analytes and secondary antibody. Next the HRP-tagged biotinylated liposomes were bound to the sandwich immune complexes through bridging of streptavidin. HRPs on liposomes were used to catalyze 4-chloronaphthol in the presence of H_2O_2 to form the insoluble product, which deposited on the Au electrode of quartz crystal. The insoluble deposit was measured by QCM, and was proportional to the amount of analyte.

The strategy of using HRP-tagged liposomes is also suitable to detect DNA/mRNA based on sandwich DNA hybridization. Anti-dinitrophenyl antibody (DNP-Ab) as low as 1×10^{-11} g mL^{-1}, DNA as low as 6.5×10^{-13} M and *cholera toxin* (CT) as low as 1.0×10^{-13} M were detected using QCM biosensors. Competitive LISA was also reported to detect antigen by QCM.[178] In a competitive LISA, the incubated mixed solution with

immunoliposomes and analyte were injected into the QCM for binding of the liposomes with immobilized hapten-BSA on the gold surface of crystal. The bound liposomes resulted in frequency changes which were used to quantify the amount of analyte. The detection range for a derivative of 2-phenyl-oxazolone from 10^{-5} to 10^{-8} M was achieved in the biosensor.

3.2.4 Using liposomes in combination with other analytical techniques. The micro-cantilever technique has been applied in the burgeoning biological analysis field. In bioanalysis based on the micro-cantilever, the tip of the micro-cantilever is normally modified with a biorecognition element. The functional micro-cantilever is then used to recognize the matching analyte, and the interaction between micro-cantilever tip and analytes is measured through laser-beam deflection technique or piezoelectric scanners. Liposomes, as carriers of biorecognition elements or as a cell membrane model are usually used to investigate the related interaction with cell membranes in the micro-cantilever device (Fig. 7C).

There are a number of different ways to use liposomes in the micro-cantilever device for the purpose of modeling cells and cellular interactions. Firstly, the interaction between the antigen immobilized on the tip of the cantilever and liposomes immobilized on a substrate is measured in the presence of other components such as calcium.[57] Alternatively, in a novel variation of the microcantilever approach, the recognition element, such as a capture antigen (for example C2A domain of synaptotagminM I) is immobilized on the *upper surface* of the micro-cantilever[179] and interaction with liposomes has been shown to lead to cantilever deflection, in a similar manner to a microbalance.

4 Using liposomes in biosensors for point of care diagnosis and commercialization

Liposomes offer obvious advantages in bioanalysis, such as signal amplification and high sensitivity. Commercialized liposome diagnostic products have been pursued based on proof of concept of the many liposome-based assays and sensors described in detail above. As a potential tool for point of care diagnosis, liposome-based microfluidic chips and strip systems based on the combination of biochip technique and liposomal signal amplification have good commercial prospects in point of care diagnosis. However, several problems still limit the development of commercialized liposome diagnostic products, such as leakage of probe molecules and poor stability. Solutions to these problems, and the development of suitable liposome systems for commercial applications are necessary for commercialization of liposome in the biosensing field. In this final section, the progress towards commercialization of liposome-based diagnostic chip and related techniques is discussed.

4.1 Increasing the stability of liposomes

Stability is a critical feature in the commercialization of liposome-based biosensors because commercial liposomal diagnostic reagent kits or devices are normally required to be stored longer than 1 year. Strategies have been developed to avoid fusion of liposomes in suspension because unilamellar liposomes tend to fuse into large liposomes in suspension. In addition, the rehydration technique is another way to realize the long storage of liposomes, and has also been developed for liposome-based point of care devices.

Steric and electrostatic repulsion are the common strategies to avoid the fusion of liposomes. In the steric repulsion strategy, hydrophilic polymer chains such as PEG are usually tagged on the surface of the liposome to sterically hinder the fusion of liposomes.[180] For the electrostatic repulsion strategy, liposomes are modified with a charged surface which results in the repulsion. For example, Zhang and Granick reported an electrostatic repulsion strategy to stabilize liposomes through using charged nanoparticles.[181] The simple strategy of mixing phospholipid liposomes with carboxyl-modified polystyrene (PS) latex was utilized to produce particle-stabilized liposomes, which repel each other and do not fuse. The particle-stabilized liposome suspension remained stable for 50 days, without leakage of encapsulated dye probes. Polymerized liposomes such as PDA liposomes have high stability because the structure of liposomes is fixed through polymerization.

Carbohydrates are often utilized to protect liposomes during lyophilization.[182] Durst *et al.* used trehalose and sucrose to protect liposomes during dehydration, and immobilized the dehydrated liposomes on a nitrocellulose strip-based sensor for point of care detection of biotin.[68] In the strip-based sensor, 70 and 80 percent of the liposomes were recovered after a cycle of dehydration and rehydration. The dehydrated liposomes on the strips can remain stable for at least 1 year when stored in vacuum-sealed plastic bags at 4 °C.

4.2 Immobilization and array of liposomes

Immobilization and array of liposomes are an important process for development of liposome-based chip biosensor. Strategies were utilized to immobilize liposomes on substrates through various interactions such as covalent binding,[183–187] recognition between biotin and streptavidin,[188–191] electrostatic interaction,[192,193] dehydration[194] and adsorption.[195] Liposomes were also immobilized on various substrates of biosensors such as gel particles, polymeric microtiter plate, metallic electrode and capillary electrophoresis media. In the immobilization process, the substrate was first chemically or biologically modified, and then liposomes were bound to the substrate through various interactions.

Covalent interactions are the most stable binding interaction, and have been widely utilized to immobilize liposomes. Covalent binding between carboxyl and amide groups is one of the main strategies used for covalent interaction to immobilize liposomes onto substrates. In this strategy, the substrate is modified with carboxyl groups through self-assembly monolayer (SAM) with carboxyl group or surface carboxylation, then *N*-hydroxysuccinimide (NHS) is utilized to activate the carboxyl groups on the substrate. The amino group is then introduced as an active group present on the liposome surface. The activated carboxyl group on the substrate covalently binds with the amide

group of liposomes when the substrate is immersed into the liposome dispersion.

A similar binding strategy was utilized to immobilize liposomes based on the interaction of avidin and biotin. The avidin-modified substrate recognizes the biotinylated liposome. Other strategies based on sandwich biotin–avidin structure can also be used to immobilize liposomes where a biotinylated substrate immobilizes biotinylated liposomes through bridging by streptavidin. Electrostatic interaction has also been utilized to immobilize liposomes through opposite charge attraction between the substrate and liposomes. Specific examples of these cases are described below.

Arrays of liposomes were developed as another important technical approach for application of liposomes in biochips based on immobilized liposomes. Arrays of liposomes usually require surface patterning for immobilization of liposomes only on functional micro-zones of the surface patterned substrate. Electron-beam lithography[196–198] and micro-contact printing are usually utilized to prepare chemical or bio-element functional pattern on substrates. The surface-patterned substrate could be modified and then used to capture liposomes relying on immobilized liposome strategy such as interaction of avidin and biotin,[199] electrostatic interaction[192] and complementary DNA hybridization.[200–203] For example, in a liposome-array strategy based on DNA hybridization, capture DNA segments were immobilized on regular functional micro-zones, and then DNA-tagged liposomes were hybridized with immobilized complementary DNA to form a regular array of liposomes.[204,205]

Interaction between biotin and avidin is another common strategy to array liposomes where streptavidin is directly or indirectly applied on the micro-zone of patterned substrate for preparation of arrays of biotinylated liposomes. Vogel *et al.* utilized the micro-contact printing technique to immobilize biotinylated bovine serum albumin (BSA) with matrix array on the substrate,[199] then biotinylated liposomes were immobilized on the matrix through bridging with streptavidin.

4.3 Using liposomes in a microfluidic chip

There are several obvious advantages associated with using biochips in bioanalysis and diagnosis of diseases, including convenience, portability and point of care diagnosis. The microfluidic chip technique has been combined with liposomal signal amplification to develop convenient, quick and highly sensitive liposome-based biosensors.[206] The liposome-based microfluidic chip was usually designed based on traditional liposome-based assays and miniaturized through advanced manufacturing techniques.[125,207]

Detection of nucleic acids is an important application for liposome-based microfluidic chips, and was developed based on complementary DNA hybridization and signal amplification of marker-encapsulated liposomes. Baeumner *et al.* developed liposome-based microfluidic chips for the detection of DNA/mRNA by using fluorescent or electrochemical markers encapsulated in liposomes to amplify signal. For the basic liposome-based microfluidic chip, micro-channels of polymer with various structures were covered on glass substrate to form the fluid system of the microfluidic chip.[208–213] Magnetic beads with capture DNA segments were utilized to bind targeting DNA/mRNA segments for separation of unbound DNA segments through magnetic field.[211–213] During the testing process, the targeting DNA segments and report DNA-tagged liposomes with encapsulated marker were captured and washed in sequence to form sandwich DNA hybridization complexes on magnetic beads. The detergent was then flowed past the immobilized liposomes to release the encapsulated markers in liposomes to quantify targeting DNA.

Charge-coupled-device (CCD) camera[209] and interdigitated ultramicroelectrode array[208,210] have been utilized to measure fluorescent signals and electronic signals in microfluidic chip devices, respectively. The advantages of the liposome-based microfluidic chip is the shortening of detection time to only 20 min, and significantly lower limit of detection down to pmol mL^{-1} or fmol mL^{-1} levels when using NASBA. For example, a low concentration of *Dengue fever virus* down to 10 pmol L^{-1} was detected using the liposome-based microfluidic chip through sandwich DNA hybridization assay.[209] Such benefits have allowed the application of liposome-based microfluidic chips for the precise, sensitive, point of care clinical diagnosis, and this could promote commercialized progress of liposome-based biosensors for point of care diagnosis.

5 Using the liposomes as cell membrane model in biosensor

Cellular research is a complicated subject in the field of biology because many biochemical reactions occur within cells. Hence, the development of a simple cell model to simplify the research system is significant, especially, for the cell membrane research. Liposomes have similar components (phospholipids) and membrane structures (bilayer membrane of phospholipids) as cell membranes, and physiological processes can be realized in liposomes such as the construction of transmembrane channels through embedding membrane proteins in liposome membrane cell membrane model for screening of drugs, simulation of apoptosis in cells and recognition of virus to host cell. The wide range of signaling strategies described above, as well as coupling to surface specific techniques such as QCM and SPR, have been reported for the application of liposomes as a sensor of model cellular interactions. In the sense of liposomes acting as a biosensor in research rather than diagnosis we briefly discuss the role of liposome biosensors in this context as the final section of this review.

Liposomes containing probe molecules have been utilized as cell membrane models in the screening of drugs,[214,215] investigation of the interaction between protein and cell membrane, simulation apoptosis of cells,[216] among other functions. Noda *et al.* investigated the interactions between protein and cell membranes through leakage of the electrochemical marker as $K_4[Fe(CN)_6]$ from liposomes.[217–219] In the measurement process, a clear leakage measured as a current in the nA range was observed by the microsensor when carbonic anhydrase from bovine (CAB) interacted with the liposomes. A similar method was utilized to detect haemolytic bacteria using liposomes as a

cell model, based on the damaging action of haemolytic bacteria to cell membranes.[220] A probe molecule, 2,6-dichlorophenolindophenolsodium salt (DCPIP) was encapsulated in various liposomes including SUVs, MLVs, and LUVs, to detect *Staph aureus*. The detected range for *Staph aureus* was from 5×10^5 to 2×10^7 cfu mL^{-1}.

Liposomes were also utilized as cell membrane models to simulate the invasion process of viruses into normal cells. Bilek *et al.* utilized capillary electrophoresis to detect the amount of virus and monitor the process of viral infection based on protein-functional liposomes.[39,60,221,222] In this research, the interaction of virus with a liposome-anchored receptor and the process of viral attachment to host cells were simulated and monitored. Sánchez-Martín *et al.* investigated the effect of virus structure on cells using liposomes as cell model. Fluorescence anisotropy probes in liposomes were utilized to monitor the dynamic interaction between *E1 (145–162)* sequence of *hepatitis GB virus C* and liposomes. It was indicated that the peptide *E1 (145–162)* preferentially interacted with the lipid surface without penetrating inside the bilayer.[223]

Membrane proteins also play an important role in the physiological action of cells. Liposomes can provide a platform for research into membrane proteins, because the structure and function of membrane proteins can be integrally maintained while embedding the membrane protein into the bilayer of the liposome.[224] Sensitive analytical techniques including SPR, QCM[225,226] and micro-cantilever,[57] have been widely utilized to investigate the interaction between cell and biological ligands due to their high sensitivity to recognition or adsorption. For example, in an SPR sensor, membrane protein or bio-receptor embedded into liposomes form a mimicking membrane, which was immobilized on an SPR sensor. The interaction of biomolecule to bio-receptor on liposomes can be measured through the change of SPR angle, and the interaction also can be dynamically measured in the SPR sensor.[58,227–231] A much easier and common method is using a bilayer membrane of liposomes as the supporting film, into which is embedded the bio-receptor, to investigate the interaction between bioreceptor and biological ligand. The supporting film strategy was also utilized in other analytical systems for detection of interaction between biomolecules.

From these reports, it has been clearly demonstrated that the use of liposomes as a cell membrane model can provide a research biosensor platform for simplifying cytological research and the study of interaction between bio-elements.

6 Summary and outlook for the use of liposomes in biosensors

Liposomes offer much utility in biosensor field due to their large internal volume of cavity, high surface area and ability of conjugate bilayer liposome with a variety of biorecognition elements. Low limit of detection for the analyte of interest down to pmol mL^{-1} or fmol mL^{-1} levels has been achieved using liposome-based biosensors through liposomal signal amplification in optimized conditions. A wide variety of analytes are able to be detected due to the possibility to conjugate bilayer lipids with biorecognition elements. Furthermore, simple preparation techniques, low cost and successful commercialization experience in medicine are also obvious advantages for the commercialization of liposome-based biosensors. Although there are several disadvantages of liposomes such as low stability (leakage, fusion and lipid oxidation) and generally low encapsulation rates, liposomes still provide an effective signal amplifier in bioanalysis, and as cell membrane model are valuable in cytological research.

6.1 Overcoming the disadvantages currently limiting liposome-based amplification strategies

Overcoming the disadvantages of liposomes will be an important development direction for liposome-based biosensors.

Improvement of stability under various conditions is a major issue requiring further attention, particularly rehydration efficiency to enable dry sensor forms to be developed. There is little known about strategies for enabling the dehydration of liposomes on surfaces that would be a necessary step towards a strip-based liposome point of care device. However, there is much literature on the lyophilisation/freezedrying of liposomes for pharmaceutical applications.[182] The use of cryoprotectants is a major tool for enabling liposomes to be rehydrated intact after dehydration. The cryoprotectant (often a sugar) provides a scaffold/water substitute inhibiting lipid fusion and preserving the integrity of individual particles. This is critical in pharmaceutical applications for the safe intravenous administration of liposomes.[182] The knowledge built up in this field therefore is likely to provide clues to enable the analogous drying of liposomes onto sensor substrates in development of sensor systems.[68]

Minimizing leakage of signaling compounds is also a major challenge to be overcome in development of liposomes in biosensors; in the drug delivery field there have been a limited number of reports of synthetic liposomes such as niosomes[232] and polymersomes[233,234] where leakage of encapsulated drug molecules is apparently reduced compared to phospholipid-based liposomes. However this strategy has not to our knowledge been employed in the biosensor field as yet.

Liposomes as the transducer element in the biosensor are expected to generate the signal quickly during triggering and therefore future attention will also be focused on efficient, fast release methods for signaling agents to shorten the testing period. Currently, the release time in some instances is limited by changes in membrane permeability, rather than complete destruction of the liposome carrier. Methods to destroy the liposome structure, or to facilitate a rapid transformation to a different assembly such as a micelle are likely to yield faster triggering systems based on liposome carriers.

The ultimate solution to these aspects will enable development of highly sensitive, liposome-based biochips for rapid, precise, and convenient point of care clinical diagnosis.

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