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Review

Liposomes in analyses

Katie A. Edwards, Antje J. Baeumner*

Department of Biological and Environmental Engineering, Cornell University, 318 Riley-Robb Hall, Ithaca, NY 14850-8101, USA

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Abstract

The use of liposomes as analytical and bioanalytical reagents has been shown to be successful of in a variety of different applications that will be reviewed here. Due to their high surface area, large internal volume, and ability to conjugate bilayer lipids with a variety of biorecognition elements liposomes have been used in homogenous and heterogeneous assays, providing signal amplification both as intact or lysed vesicles. This review covers the discussion of their application in recent liposome-based immunoassay publications and includes the growing number of other non-immunoassay applications as an evidence of their immense versatility. In this article, a general background about liposomes is given first that extends past the use of liposomes as analytical tools. The main discussion is then divided by the manner in which liposomes are utilized as signaling reagents for the assays. Where available, the detection limits for common analytes that have been assayed using multiple liposome-based detection systems are presented. The advantages of using liposomes in terms of sensitivity versus other techniques are also discussed.

Keywords: Liposome; Detection; Amplification; Review; Analysis

Contents

1. General background on liposomes 1421
2. Liposomes in analyses: general background and assay formats 1422
3. Assays relying on liposome encapsulation volume and bilayer composition 1423
   3.1. Detection based on intact liposomes 1423
   3.2. Detection based on lysed liposomes 1424
      3.2.1. Fluorescence detection 1425
      3.2.2. Electrochemical detection 1427
4. Assays relying on liposome size and bilayer composition 1427
5. Comparison of liposomes to other signal enhancement methods 1428
6. Future directions 1428
   References 1429

1. General background on liposomes

Liposomes are highly versatile structures for research, therapeutic, and analytical applications. They are composed of a lipid bilayer with the hydrophobic chains of the lipids forming the bilayer and the polar headgroups of the lipids oriented towards the extravesicular solution and inner cavity (Fig. 1).

Phospholipids with different polar headgroups functionalized for conjugation or to reduce liposome aggregation and hydrophobic regions of different chain length and saturation are used to modify the properties of the resulting liposomes. Cholesterol is often included with membrane phospholipids to reduce the membrane permeability towards encapsulated materials. Their structure is similar to that of cells and thus can be used as a more easily characterized vessel for studying interactions between membrane lipids and biomolecules such as DNA [1] and proteins [2]; permeability of ions [3,4] and drugs [5]; and elucidating the mechanism of action of pesticides [6] and

* Corresponding author. Tel.: +1 607 255 5433; fax: +1 607 255 4080.
E-mail address: ajb23@cornell.edu (A.J. Baeumner).

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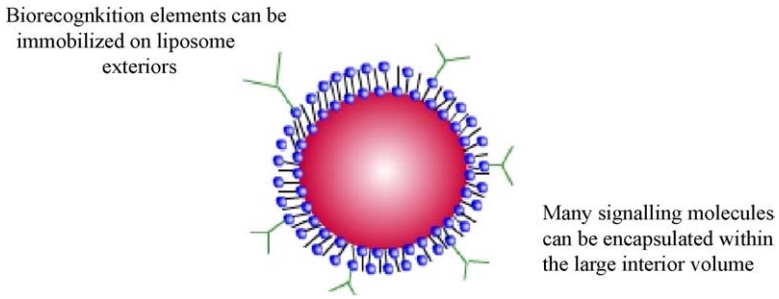

Fig. 1. Diagram of a general liposome structure. Lipids form a bilayer entrapping an aqueous core. Biorecognition elements can be tagged to the outside membrane and highly water-soluble marker molecules can be entrapped in the inner volume.

antibiotics on target organisms [7,8]. Liposomes have been used as models in several recent studies for estimating the partitioning of drugs into cells by surface plasmon resonance [9,10] and chromatography [11–13].

Molecules can be associated with liposomes in several ways, including encapsulation within the aqueous inner cavity, partitioning within the lipid tails of the bilayer [14], and covalent and electrostatic interactions with the polar head-groups of the lipids.

The surface of liposomes can be modified through the choice of lipids to allow conjugation to a variety of biorecognition elements. Typical functionalized lipids include those with an amino group, such as phosphatidylethanolamine (PE) [15]; a carboxy-group, such as in *N*-glutaryl-PE [16–18]; a maleimide group, such as in maleimidomethyl cyclohexane-carboxamide (MCC)-PE [19] or maleimidophenyl butyramide (MPB)-PE [20,21]; a protected disulfide group, such as pyridyldithio propionate (PDP)-PE [22]; and a hydroxyl group, using cholesterol [23] or polyethylene glycol [24] based entities. Common methods for conjugation of biorecognition elements to liposomes have relied on heterobifunctional cross-linking agents, such as succinimidyl-4-(*N*-maleididomethyl)-cyclohexane-1-carboxylate (SMCC) [25,26], *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) [27,28], 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)[17,29], and *N*-4-(*p*-maleimidophenyl)butyrate (MPB) [30], or non-covalent interactions such as those provided by the biotin-streptavidin interaction [31,32], or protein A/G mediated association [26,33]. The reaction chosen depends on the functional groups available on the biorecognition element and lipid bilayer; the desired orientation of the biorecognition element; and the effects of the functionalized lipid and reaction conditions on liposome stability [17,34,35]. Such modification permits the liposomes to be targeted towards specific cell types and target organs thus reducing the toxicity associated with non-localized therapies [36]. Peptides [37,38], lectins [39,40], antibodies [41,42], and folate [43,44] are frequently used to allow targeting of liposomes to desired cell types. A thorough discussion on liposome targetting is available in a recent review article by Forssen and Willis [45].

A wide variety of hydrophilic molecules can be encapsulated within the inner cavity, including enzymes [46,47], DNA [48–50], vaccines [51], fluorescent dyes [52–56], electrochemical [57] and chemiluminescent [58,59] markers, and some pharmaceutical compounds. The bilayer structure can prolong the longevity of the encapsulated molecules by shielding them from destructive entities within the body. For example, the activity of the pesticide target enzyme acetylcholinesterase was found to be retained when encapsulated in liposomes despite the presence of proteolytic enzymes in the surrounding media [47]; liposome-encapsulated RNA was observed to be protected from RNase present in the external solution [60]; and the oxidation of heme groups present in hemoglobin was minimized when encapsulated within liposomes [61]. The encapsulation of drugs within liposomes allows for their delayed release which is beneficial for reducing toxic effects and maximizing the therapeutic index [62,63]. Such benefits have allowed the therapeutic application of liposomes to be realized [64]. Several pharmaceutical compounds using liposomes as a drug-delivery system are currently approved by the FDA, including doxorubicin, daunorubicin, amphotericin B, morphine, and cytarabine [65,66]. These drugs are used for the treatment of refractory ovarian and breast cancers, Karposi's sarcoma, fungal infections, management of post-surgical pain, and neoplastic and lymphomatous meningitis, respectively [65]. Some drugs are also associated with the lipid bilayer through electrostatic interactions [67]. A number of other pharmaceuticals are in various phases of clinical trials, as reviewed in a recent article [68].

In addition, the sequestration of various molecules within liposomal cavities has been used for a variety of unique applications: DNA has been encapsulated into liposomes for use as an internal control for real-time PCR [69]; reagents have been entrapped in liposomes to allow for internal DNA transcription [60] and replication [70]; hemoglobin-based blood substitutes have been encapsulated within liposomes to enhance their stability and clinical utility [71]; and upon fusion, the contents of liposomes containing different reactants have been shown to mix, yielding a chemical transformation [72].

In some cases, a lysis step yielding release of contents is required in order to maximize the benefits of molecule-encapsulating liposomes. Means of disrupting the lipid bilayer will be discussed in more detail in Section 3.2.

2. Liposomes in analyses: general background and assay formats

Liposomes offer much utility as analytical reagents due to their high surface area, large internal volume, and ability to conjugate bilayer lipids with a variety of biorecognition elements.

Supported planar bilayers formed upon liposome fusion for the study of molecular interactions are beyond the scope of this article, but have been extensively reviewed elsewhere [73,74]. While excellent reviews of the uses of liposomes in immunoassays are available in the literature [75–77], this review extends the discussion of the use of liposomes to more recent liposome-based immunoassay publications and to other non-immunoassay applications. Their versatility is evidenced by the ways liposomes have been used as reagents in various analytical assays. The following discussion is divided by the manner in which liposomes are utilized as signaling reagents for the assays.

Liposomes have been used in a variety of homogeneous and heterogeneous assay formats. Homogeneous assays are carried out in a single vessel without a separation step while heterogeneous formats require a separation step of bound and free materials [76]. The latter are commonly employed using microtiter plates or membranes to which a biorecognition element has been immobilized. Since the separation step in heterogeneous assays removes unbound materials, the detection of the remaining bound materials can be non-selective. Two main heterogeneous assay formats have been utilized: direct detection through sandwich-hybridization and indirect detection through competitive binding (Fig. 2). Common examples include the microparticle enzyme immunoassay (MEIA), where analyte molecules are bound between particle-immobilized antibodies and antibodies labeled with enzymes [78]; and competitive ELISAs where target molecules compete with enzyme-labeled analyte for an immobilized antibody, respectively. In non-competitive binding assays, the signal obtained is directly proportional to the analyte concentration which leads to more intuitive data analysis. However, sandwich complex formation requires that the analyte possesses multiple recognizable sites and is not suitable for small-molecule analytes. Competitive assays yield a signal that is indirectly proportional to the analyte molecule concentration.

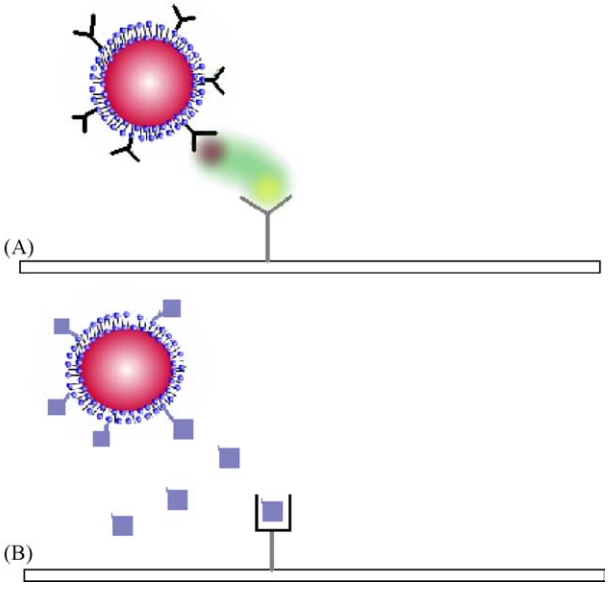

Fig. 2. Heterogeneous assay formats. (A) Sandwich-hybridization assay format: liposomes tagged with a biorecognition element bind to one site on the target molecule and form a sandwich complex with a second biorecognition element immobilized onto a surface. (B) Competitive assay format: analyte-tagged liposomes compete with free analyte molecules for binding to an immobilized recognition element.

In contrast, the detection in homogeneous formats is selective, meaning that the binding event itself changes the properties of the label. Common examples include the enzyme-multiplied immunoassay technique (EMIT) and the fluorescence polarization immunoassay (FPIA). In the EMIT, analyte-tagged enzyme competes with the free analyte and binding of the tagged enzyme by an analyte-specific antibody inhibits enzyme activity [79]. In the FPIA, a fluorescein-labeled analyte competes with the analyte for an analyte-specific antibody. The rotation of antibody-bound fluorescein-labeled analyte is slower than that of free fluorescein-labeled analyte; thus, it can emit plane-polarized light whereas the latter rotates too rapidly to do so [80]. Homogeneous assays are desirable since they typically minimize sample handling by the analyst and provide for faster reaction kinetics. However, the omission of a separation mechanism leads to potential interference from components present in the assay medium. In addition, such assays are typically more difficult to develop since a binding-selective signaling entity must be identified. The general format of both heterogeneous and homogeneous assays has been described in more detail in a recent review article [81].

3. Assays relying on liposome encapsulation volume and bilayer composition

Labels for nucleic acid diagnostics and immunoassays ideally yield stable, rapid, sensitive and inexpensive analytical assays [82]. They can generally be grouped into three broad categories: individual labels, such as quantum dots, fluorescent or radioactive tags; multiple labels, such as branched DNA, dendrimers, or latex beads; and labels which actively generate signaling molecules, such as enzymes. Liposomes fall into the multiple label category since hundreds to hundreds of thousands of signaling molecules (depending on liposome and encapsulant molecule concentration) can be encapsulated within the internal cavity of the liposomes, thus serving to elicit significant signal enhancement. The relatively large internal volume and ability to modify the surface of the bilayer with various biorecognition elements has made liposomes quite useful in optical and electrochemical biosensors, and flow-injection analysis systems [53–57,101,120–127]. Their surfaces have been modified with a variety of moieties including small molecules, antibodies [83], nucleic acids [19,84], enzymes [15–85] and also with generic avidin [86] or streptavidin [87,88] tags for facile conjugation to biotinylated ligands. Unlike enzyme-based assays, the signal enhancement provided by encapsulated molecules, such as fluorescent dyes, is not time-dependent.

3.1. Detection based on intact liposomes

Durst and colleagues have introduced a heterogeneous competitive assay, termed the liposome immunocompetition (LIC) format, and applied it to the detection of the herbicide alachlor [89], biotin [90], aflatoxin B1 [91], and PCBs [92]. In these

assays, analyte-tagged liposomes competed with the analyte for a limited number of antibody binding sites available on a nitrocellulose membrane. The liposomes also contained a biotinylated lipid to permit their capture at an anti-biotin zone which was located above the anti-analyte antibody zone on the membrane. The sample and analyte-tagged liposome mixture was permitted to migrate up the membrane by capillary action yielding a signal at the antibody zone which was inversely proportional to the concentration of analyte in the sample, while the signal at the anti-biotin capture zone was directly proportional to the analyte concentration. Oligonucleotides have also been detected using liposomes in a competitive assay format [93]. In this assay, NASBA amplified RNA from the target organism *Cryptosporidium parvum* was incubated with liposomes tagged with both biotin and a reporter probe DNA oligonucleotide sequence that was complementary to a segment of the RNA. A membrane with zones containing an immobilized capture probe DNA oligonucleotide sequence that was complementary to the liposome-conjugated reporter probe and immobilized antibiotin was then added and the liposome mixture was permitted to migrate by capillary action. If target was present, hybridization to the sequence on the liposomes would occur, thus decreasing the extent of the liposome hybridization to the immobilized capture probe sequence in an inverse manner to the concentration of target present in the sample. Liposomes not captured at the capture probe zone would bind at the second zone due to the biotin-streptavidin interaction. If target was not present, then the dye-encapsulating liposomes would hybridize at the oligonucleotide zone. The assay allowed for both direct detection at the anti-biotin zone and inverse detection at the oligonucleotide zone. A detection limit of 80 fmol of synthetic DNA oligonucleotide per assay was reported.

A direct detection heterogeneous format based on sandwich hybridization also utilized an antibody to the target immobilized onto a nitrocellulose membrane. Dye-containing liposomes conjugated to an antibody, which was directed at another site on the target, were mixed with the sample solution. Upon migration up the membrane strip, the liposomes would complete a sandwich complex with target in the sample and the immobilized antibody. This format has been used successfully for the detection of cholera toxin at concentrations as low as 10 fg/mL [94], *Escherichia coli* at 10^4 CFU/mL [95], and botulinum toxin at concentrations as low as 15 pg/mL [96]. The sandwich-hybridization format has been adapted for the detection of nucleic acid sequences by immobilizing a short oligonucleotide sequence complementary to the target onto a nitrocellulose membrane and using dye-encapsulating liposomes tagged with another short oligonucleotide which was complementary to another portion of the target nucleic acid [84,19]. This format has been used for the detection of astrovirus [97], *Bacillus anthracis* [56], Dengue virus [54,55], and *E. coli* [53].

The nucleic acid sandwich-hybridization format utilizing liposomes has been adapted for use in microfluidic devices [52,98]. In one format, superparamagnetic beads coupled with a capture probe via a streptavidin-biotin linkage were introduced into a polydimethylsiloxane (PDMS) fabricated device and captured by a magnet [98]. In another format, the capture probe was immobilized using a disulfide linkage onto a glass slide, onto which a PDMS fabricated device was placed [52]. In both cases, the sample was introduced, followed by liposomes with another sequence complementary to a different region on the target. Both systems offered a significant reduction in reagent usage and time over the membrane immobilized formats. A detection limit of 0.4 nM and 10 pM was reported for a synthetic DNA target based on *C. parvum* [52] and Dengue virus [98], respectively. Both systems relied on the fluorescence stemming from intact liposomes sandwich-hybridized within the channels, though a lower limit of detection might be realized if the liposomes were lysed.

In a competitive format known as the liposome immunoaggregation assay (LIA), analyte-tagged liposomes (also incorporating biotin) competed with analyte in the sample for available antibody in the solution phase [92]. Due to the multivalent nature of the liposomes, aggregation with anti-analyte antibodies occurred in an inverse manner to the concentration of analyte in the sample. When permitted to migrate up a nitrocellulose membrane, aggregated liposomes were unable to flow above the aggregation zone near the base of the membrane. Free liposomes bound at an anti-biotin zone located above the aggregation zone, thus providing a signal that was directly proportional to the concentration of analyte in the sample. PCBs [92] have been detected using the LIA at concentrations as low as 2.6 pmol and using the LIC assay as low as 360 pmol. The lower limit of detection in the LIA format was attributed to the increased time allowed for liposome aggregation, which approached equilibrium conditions, versus the transient time where the liposomes were permitted to pass the competition zone by capillary action in the LIC format [92]. The LIA format has also been used for the detection of potato glycoalkaloids at concentrations as low as 0.11 ppm [99] and alachlor as low as 1 ppb [100].

3.2. Detection based on lysed liposomes

The assays described in the previous section relied on the optical detection of intact liposomes; however, a significantly lower limit of detection for the analytes of interest could be expected by inducing lysis to release the entrapped contents. Also, transduction principles could be expanded to electrochemical detection that require direct interaction with the entrapped marker molecules. The sensitivity afforded by liposome lysis has been exemplified in a variety of microplate, flow-injection and microfluidic systems. While liposomes can encapsulate compounds leading to visible or fluorescence detection, they offer a unique feature when fluorescent dyes are considered. The fluorescence from some dyes, such as calcein [116,117], fluorescein [101], sulforhodamine 101 [102], and sulforhodamine B (SRB) [19], is quenched to a large degree when encapsulated at high concentrations within liposomes. This phenomenon is attributed to the formation of non-fluorescent aggregates (static quenching), collisional quenching, and energy transfer from monomers to non-fluorescent dimmers [102,103]. Upon lysis, dilution of the entrapped fluorophores reverses this quenching phenomenon leading to a significant signal enhancement.

The release of liposomal contents can be accomplished through several means, including chemical-based lysis using the addition of solvents or surfactants [104,105]; enzymatic lysis through the addition of phospholipase C (PLC) [106] or trypsin [107]; addition of natural cell lysis agents from bee or snake venom (mellitin) [108]; and the initiation of the complement system by binding of an antibody to the liposomes [109]. Surfactants or solvents can be used in heterogeneous assay formats since a separation step is required so that only the bound liposomes are lysed. The complement system is composed of roughly 30 serum proteins which bind in an ordered fashion to antibodies directed against foreign cells [110]. It serves as an initial defense mechanism by the host against infection. Initiation of this cascade results in lysis of antibody-associated liposomes, though some non-specific lysis of liposomes has been reported [111]. Mellitin is a 26-amino acid peptide which induces the formation of transient pores in lipid membranes [108]. Mellitin-analyte conjugates are often used in competitive assays with free analyte for an available antibody. The lytic properties of the peptide-analyte conjugate are negated when this complex binds to an antigen-specific antibody, provided that the antigen is relatively small in size. These two methods are amenable to use in homogeneous assays since liposome lysis is hindered by a specific antibody in the case of mellitin or enhanced by a liposome-bound antibody in the case of complement, thus all liposomes in the assay are not specifically lysed. A summary of both the complement and mellitin-based immunoassays found in the literature through 1997 can be found in the review article by Rongen et al. [76].

3.2.1. Fluorescence detection

Liposomes have been used in several homogeneous assay formats utilizing visible spectrophotometry for detection. A biosensor for theophylline that made use of liposomes encapsulating enzymes was reported by Canova-Davis et al. The release of encapsulated enzymes allows for a secondary amplification in that each enzyme is capable of facilitating the conversion of numerous substrate molecules into quantifiable entities. In this competitive indirect assay, theophylline-tagged liposomes encapsulated a solution containing glucose-6-phosphate dehydrogenase [112]. Anti-theophylline antibody present in the assay bound competitively to sample theophylline or theophylline-tagged liposomes. Binding of the specific antibody to the liposomes initiated liposome lysis by complement. The release of the entrapped enzyme oxidized glucose-6-phosphate and formed NADH from NAD^+ that was present within the assay. NADH could then reduce the dye 2,6-dichlorophenolindophenol which resulted in a color change from blue to colorless. The authors reported a linear range for theophylline from 2.5 to 40 μg/mL and a cross-reactivity with caffeine of 2.5% [112]. Liposomes encapsulating sulforhodamine B tagged with Fab' fragments were used in a homogeneous sandwich assay for the detection of albumin [113]. The Fab' fragments were unable to elicit complement-induced liposome lysis, but lysis was induced upon the sandwich formation between liposomes, albumin, and an intact anti-albumin antibody. The absorbance at 560 nm was proportional to the degree of liposome lysis and the amount of albumin in the sample.

A rapid colorimetric assay in a homogeneous format for the antibodies present in patients with systemic lupus erythematosus (SLE) was developed [114]. Antibodies are directed against double-stranded DNA in this autoimmune disease, though they also exhibit some cross-reactivity to various phospholipids. For example, the phospholipid cardiolipin inhibits the action of the SLE antibodies [115]. Arsenazo III-encapsulating liposomes were prepared with cardiolipin in their lipid bilayers. Upon lysis, liposomes encapsulating this red dye were found to form a blue complex with magnesium ions present in the external solution [114]. In the presence of serum from healthy patients, the introduction of magnesium ions destabilized the lipid bilayer and resulted in the formation of a blue complex between the released dye and cations. However, in SLE patients, the anti-dsDNA antibody bound to the cardiolipin incorporated bilayer and prevented the magnesium ion-induced destabilization. Consequently in anti-dsDNA-positive samples, the red color of the encapsulated Arsenazo III dye was retained. The liposome-based assay showed comparable specificity to a standard immunofluorescence assay and slightly lower sensitivity than another standard assay for a different antibody involved in SLE [114].

Several homogeneous assays using fluorescence detection have also been reported. Liposomes encapsulating carboxyfluorescein with an anti-ferritin antibody on their surface were used in a sandwich assay for ferritin. The addition of a second antibody to a different region of the target formed a sandwich complex. Complement triggered the lysis of the lipid bilayer and release of carboxyfluorescein which was then detected [111]. Calcein-encapsulating liposomes were used in a homogeneous competitive assay between sample gentamicin and phospholipase C-conjugated gentamicin for binding to a gentamicin antibody [106]. The lytic properties of the PLC conjugate were inhibited by the gentamicin antibody yielding a reduction in the liposome-released fluorescence. Liposomes composed of dioleoylphosphatidylethanolamine and encapsulating calcein have been used for the detection of herpes simplex virus (HSV) [116] and dinitrophenyl (DNP) [117]. In these assays, the dioleoylphosphatidylethanolamine did not form stable liposomes without the incorporation of an anti-HSV glycoprotein or DNP-labeled phosphatidylethanolamine into the bilayer. In the presence of either an immobilized antibody to the target in the case of DNP or the HSV antigen in the HSV assay, the liposomes become destabilized and release their fluorescent marker. Upon binding to the target, the formerly freely diffusible tags on the liposomes which were necessary for stability became localized on the bilayer. The destabilization was believed to be a function of the formation of regions rich in dioleoylphosphatidylethanolamine which could no longer support a stable bilayer structure. In another homogeneous competitive assay, a cytolytic polypeptide was conjugated to atrazine which competed with samples containing atrazine for the available anti-atrazine antibody present in a microwell plate. The cytolytic potential of the analyte-tagged polypeptide was inhibited when it was bound to the anti-analyte antibody which resulted in a decrease in the extent of lysis upon the addition of terbium ion-encapsulating liposomes. The fluorescence was read upon the addition of dipicolinic acid which formed a highly fluorescent

complex with terbium ions released from the lysed liposomes. The fluorescent signal was directly proportional to the amount of analyte present in the samples and the assay could be used to detect down to 10 pg of analyte per microwell [118].

Liposomes have been used extensively in heterogeneous formats similar to the enzyme-linked immunosorbant assay (ELISA.) In an ELISA assay for interferon-γ, anti-interferon-γ was immobilized into microwells, the antigen was then added, followed by a biotinylated antibody specific to another region on the antigen. Lastly, an anti-biotin conjugated enzyme and appropriate substrate were added. Variations using liposomes (LISAs) included the addition of streptavidin and biotinylated dye-encapsulating liposomes in lieu of the anti-biotin conjugated enzyme [87], and in the simplest form, streptavidinylated dye-encapsulating liposomes in lieu of the anti-biotin conjugated enzyme [88]. In both of the LISAs, Triton X-100 was used to lyse bound liposomes [87,88]. A similar detection limit was observed between the ELISA and the biotinylated-liposome ISA (3 IU/mL, ∼166.7 pg/mL), but the linear dynamic range was wider with the latter [87]. The streptavidinylated-liposome ISA had a lower detection limit (1 IU/mL, ∼55.6 pg/mL), but had a more limited range of detection [88]. In another microplate format, liposomes labeled with gangliosides and a membrane-incorporated rhodamine dye were used in a sandwich assay format for the detection of cholera, botulinum, and tetanus toxins [119]. In this assay, samples were added to microwells in which antibodies specific to the analyte of interest were immobilized. After washing to remove unbound components, tagged liposomes were added. Unbound liposomes were removed by washing. The bound liposomes were lysed with a detergent to reverse the self-quenching of the membrane-immobilized dye. Between 1.9×10^4 and 2.2×10^4 molecules of membrane-immobilized dye were reported to be present per liposome, yielding a minimum detectable concentration of 1.2×10^9 M for botulinum and tetanus toxins, and 1.5×10^9 M for cholera toxin [119]. The advantage of the strip assay format for the same analytes described earlier is the reduction in sample manipulations required and the significantly reduced detection time of only 20 min, versus several hours for the microplate format (though up to 96-samples can be processed simultaneously in the latter).

Liposomes were reported for use in flow-injection analysis systems as early as 1988 [101] and have been used since for the detection of theophylline [120,121], estrogens [122], fumonisin B1 [123], alachlor [124,125], imazethapyr [126,127], and *E. coli* [128]. In these assays, antibodies to the analyte of interest were immobilized within a capillary column or onto glass beads. The sample was permitted to pass over the immobilized antibody, followed by the introduction of liposomes incorporating either a lipid-conjugate of the analyte for the competitive assay format or conjugated to another antibody to the target analyte for the sandwich-complex format. In the sandwich assay, the amount of the bound antibody-target-liposome complex formed was proportional to the concentration of target in the sample. Lysis of bound liposomes was achieved by the addition of a detergent solution and the resulting fluorescence was directly proportional to the concentration of target. In the competitive assay format, the analyte-tagged liposomes competed with the target present in the sample for the available antibody binding sites. The amount of encapsulant released due to lysis of the bound liposomes was inversely proportional to the concentration of target in the initial sample. Another FIA method utilized liposome-encapsulated horseradish peroxidase (HRP) to generate fluoride ions from *p*-fluorophenol and yielded a detection limit for theophylline of 0.2 ng/mL [120].

Liposomes encapsulating carboxyfluorescein have been immobilized in sol–gel films. Sol–gel films are widely used as solid supports for sensors due to their mechanical and chemical stability, ease of preparation, and utility for fluorescence assays, but suffer from leakage of hydrophilic small molecules such as fluorescent dyes [129]. Fluorescent dyes are typically covalently linked to the sol–gel support or conjugated to larger molecules such as dextran to minimize their leakage from sol–gel matrices. However, such modifications often adversely affect the fluorescent properties of the dye. In this paper, the authors described the use of a liposome-entrapped sol–gel as a pH sensor based upon the exquisite pH sensitivity of carboxyfluorescein. They found that the resulting sensor was significantly more stable to photobleaching, more resistant to dye leakage, and required no more response time than an equivalent sensor made with free carboxyfluorescein. Sol–gel entrapped liposomes prepared with the fluorescent lipid 7-nitro-2,1,3-benzodiazol-4-yl-dipalmitoylphosphatidylethanolamine (NBD-PE) as part of the lipid bilayer were used as a pH sensor [130]. As the pH changed, structural perturbations of the membrane due to changes in ionization resulted in an increase or decrease in self-quenching of this membrane immobilized dye. Liposomes encapsulating the hydrophobic fluorescent dye tris(1,10-phenanthroline)ruthenium chloride, which was strongly quenched by molecular oxygen, were used as signaling components in an oxygen sensor [131]. In this sensor, dye-encapsulating liposomes were mixed with glucose oxidase and glucose which yields hydrogen peroxide and gluconic acid in the presence of oxygen. The fluorescence of the dye increased as the reaction progressed; thus, the liposomes were found to provide a quantitative means for kinetic measurements of oxygen generation.

Ruthenium-encapsulating liposomes have been used in an immunoassay relying on electrochemiluminescence (ECL) detection. In this assay, an anti-Legionella antibody was immobilized onto the surface of liposomes which were permitted to migrate up a nitrocellulose membrane with immobilized antigen. The nitrocellulose membrane was placed in direct contact with a glass fiber membrane housing electrodes and a dried detergent. Liposomes that did not bind to the nitrocellulose-immobilized antigen traveled towards the glass fiber membrane and were lysed by the detergent. The ruthenium released was in proportion to the amount of unbound liposomes which was also proportional to the amount of antigen present in the sample [58]. In another chemiluminescence assay, human serum albumin (HSA)-labeled liposomes loaded with Eosin-Y competed with sample HSA for an anti-HSA antibody immobilized onto a glass bead. The supernatant from this assay was then subjected to capillary electrophoresis with chemiluminescence detection yielding an increased signal with increasing

sample HSA. When compared to fluorophore-labeled HSA, the liposome-based assay was reported to be five times more sensitive [59].

3.2.2. Electrochemical detection

Aside from fluorescent markers and enzymes, liposomes can be used to encapsulate a variety of electrochemical markers. One of the early reports in this regard discussed the encapsulation of potassium ferrohexacyanide at $\sim 10^4$ molecules per liposome [57]. Liposomes encapsulating ascorbic acid have been used in a competitive assay format for the pesticide atrazine using both lateral and horizontal flow formats and amperometric detection following Triton X-100 induced lysis [132]. A similar format was reported using liposomes encapsulating potassium hexacyanoferrate(II) for signaling reagents in a competitive assay format for the bronchodilator theophylline [133]. In this assay, anti-theophylline antibody was immobilized onto a nitrocellulose membrane which was placed in direct contact with a disposable thick-film electrode. Theophylline conjugated to the liposome-lysis agent melittin competed for theophylline present in the sample at the antibody competition zone. Unbound mellitin-conjugated theophylline migrated up the membrane to the signal generation zone where the electrochemical liposomes were located. As the concentration of theophylline increased, the amount of unbound conjugate increased which resulted in a higher current at the signaling zone.

In another assay, liposomes encapsulating potassium ferrohexacyanide were immobilized onto an electrode and used to assess the presence of pore-forming toxins. When present, the pore forming toxins would increase the permeability of the bilayer and allow leakage of the encapsulated marker. Using cyclic voltammetry, a linear relationship between the concentration of pore-forming toxin and current was observed [134]. While the assay was not specific to the identity of the pore forming toxin, this liposome-based biosensor could be used as a measure of agents responsible for cell lysis.

One homogeneous competitive theophylline biosensor used theophylline-tagged liposomes that encapsulated the enzyme horseradish peroxidase [135]. Sample theophylline and theophylline-tagged liposomes competed for antibody sites. Binding to the antibody initiated the activation of complement which lysed the liposomes and resulted in the release of the entrapped horseradish peroxidase. The released HRP catalyzed the conversion of NADH to NAD^+ and the corresponding depletion of oxygen was monitored by an oxygen electrode. The authors reported a detection limit for theophylline of 0.72 ng/mL [135].

4. Assays relying on liposome size and bilayer composition

The following papers describe using liposomes purely for their comparatively large size and bilayer composition to generate analytical signals. Measurements from quartz-crystal microbalance (QCM) are commonly employed. QCMs are piezoelectric quartz-crystal transducers which exhibit a decrease in frequency upon binding of materials onto their surface [136]. The change in frequency is directly related to the mass of the materials bound and can extend into the nanogram range [137]. Surface-plasmon resonance (SPR) relies on a change in refractive index to gain information about the binding of materials onto surfaces, i.e. materials bound to the metal surface of the transducer induce a change in the resonant angle which is a function of the altered refractive index [138]. Other techniques include chronocoulometry and faradaic impedance spectroscopy.

In a recent paper, oligonucleotide-tagged liposomes were used as a means for amplifying the sandwich hybridization of a DNA target using chronocoulometry, faradaic impedance spectroscopy, and QCM measurements [139]. In this approach, the target DNA was permitted to hybridize to a complementary DNA probe immobilized onto a gold electrode. Liposomes tagged with another complementary sequence were added and the change in mass or negative charge was recorded. The sensitivity of this approach was reported to be 1 pM target DNA. In another approach, the same immobilized probe and DNA target was used, but instead of a liposome-tagged probe, a biotinylated probe was used. Avidin was then added to the mixture, followed by biotinylated liposomes. Through an excess of avidin, additional biotinylated liposomes were able to bind to the first liposome which was linked to the biotinylated probe. This multiple liposome approach allowed for significant amplification, yielding a detection limit of 0.1 pM [139].

Antibody-tagged liposomes were used in a competitive assay format for the small-molecule target 2-phenyloxazolone [140]. In this assay, liposomes were incubated with the target, and were then introduced onto a quartz-crystal surface to which analyte-conjugated BSA had been immobilized. The resulting frequency change was inversely proportional to the concentration of analyte in the sample and permitted the detection of 10 nM target. Using the same system in a microtiter well format utilizing lysis and quantification of released encapsulated dye by fluorescence, the detection of 1 nM target was realized. Dual antibody-tagged liposomes have been used for the detection of C-reactive protein though their aggregation upon interaction with the target and subsequent detection by turbidimetry [141].

Another system utilized a small-molecule antigen (dinitrophenyl) immobilized onto an electrode where, if present, an antibody to the analyte could bind. A biotinylated anti-antibody was then added followed by avidin. Liposomes conjugated to both horseradish-peroxidase (HRP) and biotin were then added to form a sandwich complex linked by avidin. HRP then catalyzed the hydrogen peroxide-mediated conversion of 4-chloro-1-napthol to an insoluble product which precipitated onto the electrode surface. This precipitation insulated the electrode and yielded an increase in the resistance resulting in the ability to detect specific antibody concentrations as low as 10 pg/mL [142]. The liposomes in this case were used as an enzyme carrying reagent and simultaneously provided signal amplification through changing of the electrode properties through their large size and negative charge. A simplified form of this assay using ganglioside-labeled liposomes in a sandwich format with immobilized antibody was also reported to detect cholera toxin at a concentration of 0.1 pM using impedance measurements [143]. Using the same system and relying on the

liposome-induced mass change through QCM measurements, these authors reported the detection of 1.2 pM cholera toxin [143]. A similar approach was reported with a DNA probe immobilized onto the electrode. In the presence of target, a sandwich hybridization complex formed between an added biotinylated probe and the immobilized probe. The addition of avidin and biotinylated-HRP liposomes yielded a sensor capable of detecting as low as 0.65 pM target DNA [142].

One of the disadvantages to surface-plasmon resonance detection is the inability to detect small molecule analytes at reasonable concentrations due to their insignificant effect on the refractive index of the evanescent-field layer. In an attempt to overcome this difficulty, liposomes have been used in a sandwich-hybridization format for interferon-γ using SPR detection [144]. In this assay, the antigen was permitted to bind to an antibody specific to the antigen immobilized into the wells of a microtiter plate. A second biotinylated antibody specific to the antigen was added followed by the addition of avidin and biotinylated liposomes. Unbound materials were removed following each addition. The SPR signal was a function of the number of liposomes bound to the plate and yielded a detection limit of 100 pg/mL. By contrast, the same system without liposomal amplification had a detection limit for interferon-γ of only 1 μg/mL [144].

5. Comparison of liposomes to other signal enhancement methods

The advantage of liposomes as signal amplification tool has been pointed out by all researchers integrating these multi-label systems into the analytical assay. However, encapsulation efficiency, steric hindrance of the binding events due to the large size of the vesicles and their multivalency make a theoretical calculation of signal amplification in comparison to single labels more difficult. This section will review the available literature on experimental data comparing liposome attached and encapsulated molecules to more conventional singly-tagged biorecognition elements.

An early review article on the use of liposomes in immuoassays compared LIA, radioimmunoassay (RIA), and enzyme immunoassay (EIA) formats for the analysis of theophylline and showed comparable detection limits, lower health hazards, and significantly less time required for the liposome-based format [75]. The signals that result from typical immunoassays such as ELISAs are enhanced by increasing the enzyme concentration which results in a larger amount of converted substrate. This has been done by conjugating multiple enzyme molecules to an antigen-specific antibody or to streptavidin which can then be linked to a biotinylated antigen-specific antibody [145]. Jones et al. reported on the use of liposomes surface-tagged with both biotin and HRP in a competitive assay for biotin in a microwell format. These authors compared the signal of biotin-enzyme-tagged liposomes to a biotin-tagged enzyme and reported a 100 times higher signal with the liposomes at low immobilized antibody concentrations [15]. In a subsequent report, the same group reported that five times less immobilized antibody was required and reported a 10-fold lower detection limit for biotin using dually tagged liposomes versus the biotin-labeled enzyme [85]. In a report comparing the chemiluminescence from an HRP-tagged antibody versus lysed antibody-tagged liposomes encapsulating HRP, the liposome-based system yielded a 125 times greater signal per antibody [146]. In a non-competitive sandwich-immunoassay for D-dimer comparing enzyme-tagged antibody to antibody and enzyme-tagged liposomes, the format using liposomes exhibited a nine times lower detection limit [147]. The same group later reported on a sandwich-immunoassay for the same analyte using fluorophore and antibody-tagged liposomes versus a fluorophore labeled antibody. In this report, the liposomes yielded a 120-fold lower detection limit [148]. When compared to the use of an antibody-tagged with fluorophore, dye-encapsulating liposomes were reported to yield a 1000-fold increase in sensitivity in a sandwich-hybridization FIA system [149]. A 10-fold increase in sensitivity was observed when analyte-tagged liposomes were used in lieu of an analyte-tagged fluorophore in a competitive assay using a planar waveguide immunosensor [150].

From these reports, it has been clearly demonstrated that the use of liposomes as signal-enhancing reagents can lower the limit of detection when compared to singly tagged molecules. However, in most cases, the enhancement was not as significant as would be expected given the high internal capacity of liposomes. This has been attributed to two main factors: steric hindrance and multivalency. The relatively large diameter of liposomes has been suggested as a hindrance towards allowing multiple liposomes to bind to adjacent antigens [147,148]. Liposomes also have many biorecognition elements on their surfaces, thus one liposome can theoretically bind to several targets. While the signal enhancement stemming from a singly-tagged fluorophore is one to one on a molar basis, a single liposome can bind to multiple antigens thus the relationship is not direct [148]. This multivalency was believed to be responsible for the higher association constant of antibody-labeled liposomes versus fluorophore-labeled antibodies [15,148,151]. The low amplification noted in this case was attributed to the multivalent nature of the liposomes allowing a single liposome to occupy multiple antibody binding sites and steric hindrance which limited the number of liposomes that could bind. In addition, the size of biorecognition element-liposome conjugates may be limited by mass action due to their significantly lower diffusion coefficients.

6. Future directions

This review was intended to elucidate the variety of ways in which liposomes have been used to date as analytical reagents. These methods included relying on the substantial mass and charge difference that a tagged liposome could provide and the large number of signaling molecules that can be released to provide a signal. While many variations were presented, further study will likely yield even more options for using liposomes in analysis including furthering the use of chemiluminescent molecules and quantum dots as encapsulants. The use of chemiluminescent molecules in conjunction with liposome-based assays is limited to only a few reports [58,59]. No references were found which compared liposome-encapsulated

markers by both fluorescence and chemiluminescence detection, though this might be an interesting future study. Chemiluminescence is advantageous due to its higher sensitivity, wider dynamic range, and inexpensive instrumentation versus fluorescence. Cha et al. have reported on the encapsulation of quantum dots within ~3 μm polyamine homopolymer vesicles [152]. Quantum dots have a broad absorption spectra with high molar absorptivity which allows for the used of a common light source, yet narrow emission spectra with high quantum yield which permits sensitive, multi-analyte detection [153]. In one recent report, quantum dots were reported to have a 20 times brighter signal, were 100 times more stable towards photobleaching, and had a spectral linewidth that was 1/3 that of rhodamine dye [154]. Encapsulation of such molecules would be especially useful if liposomes were to be employed in a multiplex form of analysis. Further study into maximizing the encapsulation efficiency of such molecules as well as catalytic entities such as enzymes will likely broaden the applicability of liposomes for use in analytical systems.

References

[1] M. Angelova, I. Tsoneva, Chem. Phys. Lipids 101 (1999) 123–137.
[2] A. Fischer, T. Oberholzer, P. Luisi, Biochim. Biophys. Acta 1467 (2000) 177–188.
[3] A. Bangham, M. Standish, J. Watkins, J. Mol. Biol. 13 (1965) 238–252.
[4] N. Toyran, F. Severcan, Chem. Phys. Lipids 123 (2003) 165–176.
[5] M. Romanowski, X. Zhu, V. Ramaswami, A. Misicka, A. Lipkowski, V. Hruby, D. O'Brien, Biochim. Biophys. Acta 1329 (1997) 245–258.
[6] W. Braguini, S. Cadena, E. Carnieri, M. Rocha, M. de Oliveira, Toxicol. Lett. 152 (2004) 191–202.
[7] D. Trombetta, S. Arena, A. Tomaino, B. Tita, G. Bisignano, A. Pasquale, A. Saija, Il Farmaco 56 (2001) 447–449.
[8] A. Grancelli, A. Morros, M. Cabañas, O. Domènech, S. Merino, J. Vazquez, M. Montero, M. Viñas, J. Hernández-Borrell, Langmuir 18 (2002) 9177–9182.
[9] Y. Abdiche, D. Myszka, Anal. Biochem. 328 (2004) 233–243.
[10] E. Danelian, A. Karlen, R. Karlsson, S. Winiwarter, A. Hansson, S. Lofas, H. Lennernas, M. Hamalainen, J. Med. Chem. 43 (2000) 2083–2086.
[11] F. Beigi, Q. Yang, P. Lundahl, J. Chromatogr. A 704 (1995) 315–321.
[12] G. Caldwell, J. Masucci, M. Evangelisto, R. White, J. Chromatogr. A 800 (1998) 161–169.
[13] P. Lundahl, C. Zeng, C. Hagglund, I. Gottschalk, E. Greijer, J. Chromatogr. B 720 (1999) 103–120.
[14] H. Schott, D. Von Cunow, H. Langhals, Biochim. Biophys. Acta 1110 (1992) 151–157.
[15] M. Jones, P. Kilpatrick, R. Carbonell, Biotechnol. Prog. 9 (1993) 242–258.
[16] V. Weissig, J. Lasch, A. Klibanov, V. Torchilin, FEBS Lett. 202 (1986) 86–90.
[17] V. Kung, C. Redemann, Biochim. Biophys. Acta 862 (1986) 435–439.
[18] K. Maruyama, S. Kennel, L. Huang, Proc. Natl. Acad. Sci. U.S.A. 87 (1990) 5744–5748.
[19] G. Rule, R. Montagna, R. Durst, Anal. Biochem. 244 (1997) 260–269.
[20] R. Bredehorst, F. Ligler, A. Kusterbeck, E. Chang, B. Gaber, C. Vogelt, Biochemistry 25 (1986) 5693–5698.
[21] S. Katoh, M. Kishimura, K. Tomioka, Colloids Surf. A 109 (1996) 195–200.
[22] C. Hansen, G. Yao, E. Moase, S. Zalipsky, T. Allen, Biochim. Biophys. Acta 1239 (1995) 133–144.
[23] T. Carroll, A. Davison, A. Jones, J. Med. Chem. 29 (1986) 1821–1826.
[24] S. Zalipsky, in: D. Lasic, F. Martin (Eds.), Stealth Liposomes, CRC Press, Boca Raton, FL, 1995, pp. 93–102.
[25] J. Ho, R. Durst, Anal. Chim. Acta 414 (2000) 51–60.
[26] C. Chen, A. Baeumner, R. Durst, Talanta 67 (2005) 205–211.
[27] J. Barbet, P. Machet, L. Leserman, J. Supramol. Struct. Cell Biochem. 16 (1981) 243–258.
[28] F.J. Martin, T.D. Heath, R.R.C. New, in: R.R.C. New (Ed.), Liposomes, A Practical Approach, IRL Press at Oxford University Press, Oxford, 1992, pp. 163–182.
[29] N. Zhang, P. Ping, G. Huang, W. Xu, Int. J. Pharm. 294 (2005) 247–259.
[30] C. Hansen, G. Yao, E. Moase, S. Zalipsky, T. Allen, Biochim. Biophys. Acta 1239 (1995) 133–144.
[31] A. Baeumner, J. Pretz, S. Fang, Anal. Chem. 76 (2004) 888–894.
[32] A. Plant, M. Brizgys, L. Locascio-Brown, R. Durst, Anal. Biochem. 176 (1989) 420–426.
[33] L.D. Leserman, J. Babet, F. Kourilsky, J.N. Weinstein, Nature 288 (1980) 602–604.
[34] R. Bredehorst, F. Ligler, A. Kusterbeck, E. Chang, B. Gaber, C. Vogel, Biochemistry 25 (1986) 5693–5698.
[35] J. Zhu, F. Yan, Z. Guo, R. Marchant, J. Colloid Interface Sci. 289 (2005) 542–550.
[36] V. Torchilin, Eur. J. Pharm. Sci. 11 (2000) S81–S91.
[37] B. Lestini, S. Sagnella, Z. Xu, M. Shive, N. Richter, J. Jayaseharan, A. Case, K. Kottke-Marchant, J. Anderson, R. Marchant, J. Control. Release 78 (2002) 235–247.
[38] J. Moreira, C. Hansen, R. Gaspar, T. Allen, Biochim. Biophys. Acta 1514 (2001) 303–317.
[39] M. Clark, H. Blair, L. Liang, R. Brey, D. Brayden, B. Hirst, Vaccine 20 (2001) 208–217.
[40] T. Minko, Adv. Drug Deliv. Rev. 56 (2004) 491–509.
[41] E. Moase, W. Qi, T. Ishida, Z. Gabos, B. Longenecker, G. Zimmerman, L. Ding, M. Krantz, T. Allen, Biochim. Biophys. Acta 1510 (2001) 43–55.
[42] T. Ishida, M. Kirchmeier, E. Moase, S. Zalipsky, T. Allen, Biochim. Biophys. Acta 1515 (2001) 144–158.
[43] S. Wang, P. Low, J. Control. Release 53 (1998) 39–48.
[44] M. Turk, D. Waters, P. Low, Cancer Lett. 213 (2004) 165–172.
[45] E. Forssen, M. Willis, Adv. Drug Deliv. Rev. 29 (1998) 249–271.
[46] P. Walde, S. Ichikawa, Biomol. Eng. 18 (2001) 143–177.
[47] B. Chaize, J. Colletier, M. Winterhalter, D. Fournier, Artif. Cells Blood Substit. Immobil. Biotechnol. 32 (2004) 67–75.
[48] S. Semple, S. Klimuk, T. Harasym, N. Dos Santos, S. Ansell, K. Wong, N. Maurer, H. Stark, P. Cullis, M. Hope, P. Scherrer, Biochim. Biophys. Acta 1510 (2001) 152–166.
[49] P. Monnard, T. Oberholzer, P. Luisi, Biochim. Biophys. Acta 1329 (1997) 39–50.
[50] A. Bailey, S. Sullivan, Biochim. Biophys. Acta 1468 (2000) 239–252.
[51] G. Gregoriadis, B. McCormack, M. Obrenovic, R. Saffie, B. Zadi, Y. Perrie, Methods 19 (1999) 156–162.
[52] M. Esch, L. Locascio, M. Tarlov, R. Durst, Anal. Chem. 73 (2001) 2952–2958.
[53] A. Baeumner, R. Cohen, V. Miksic, J. Min, Biosens. Bioelectron. 18 (4) (2003) 405–413.
[54] A. Baeumner, N. Schlesinger, N. Slutzki, J. Romano, E. Lee, R. Montagna, Anal. Chem. 74 (6) (2002) 1442–1448.
[55] N. Zaytseva, R. Montagna, E. Lee, A. Baeumner, Anal. Bioanal. Chem. 380 (2004) 46–53.
[56] H. Hartley, A. Baeumner, Anal. Bioanal. Chem. 376 (2003) 319–327.
[57] R. Kannuck, J. Bellama, R. Durst, Anal. Chem. 60 (1988) 142–147.
[58] C. Yoon, J. Cho, H. Oh, M. Kim, C. Lee, J. Choi, S. Paek, Biosens. Bioelectron. 19 (2003) 289–296.

[59] K. Tsukagoshi, H. Akasaka, Y. Okumura, R. Fukaya, M. Otsuka, K. Fujiwara, H. Umehara, R. Maeda, R. Nakamija, Anal. Sci. 16 (2000) 121–124.
[60] K. Tsumoto, S. Nomura, Y. Nakatani, K. Yoshikawa, Langmuir 17 (2001) 7225–7228.
[61] H. Sakai, K. Tomiyama, K. Sou, S. Takeoka, E. Tsuchida, Bioconjug. Chem. 11 (2000) 425–432.
[62] A. Wagner, K. Vorauer-Uhl, H. Katinger, Eur. J. Pharm. Biopharm. 54 (2002) 213–219.
[63] A. Sharma, U. Sharma, Int. J. Pharm. 154 (1997) 123–140.
[64] A. Ulrich, Biosci. Rep. 22 (2002) 129–150.
[65] T. Allen, P. Cullis, Science 303 (2004) 1818–1822.
[66] M. Rios, Pharm. Technol. 28 (2004) 16–17.
[67] N. Deo, T. Somasundaran, P. Somasundaran, Colloids Surf. B 34 (2004) 155–159.
[68] D. Felnerova, J. Viret, R. Gluck, C. Moser, Curr. Opin. Biotechnol. 15 (2004) 518–529.
[69] E. Berg, K. Skaug, J. Microb. Methods 55 (2003) 303–309.
[70] T. Oberholzer, M. Albrizio, P. Luisi, Chem. Biol. 2 (1995) 677–682.
[71] D. Arifin, A. Palmer, Biotechnol. Prog. 19 (2003) 1798–1811.
[72] D. Chiu, C. Wilson, F. Ryttsen, A. Stromberg, C. Farre, A. Karlsson, S. Nordholm, A. Gaggar, B. Modi, A. Moscho, R. Garza-Lopez, O. Orwar, R. Zare, Science 283 (1999) 1892–1895.
[73] J.T. Groves, M.L. Dustin, J. Immunol. Methods 278 (2003) 19–32.
[74] M. Trojanowicz, Fresenius J. Anal. Chem. 371 (2001) 246–260.
[75] D. Monroe, J. Liposome Res. 1 (1989) 339–377.
[76] H. Rongen, A. Bult, W. Bennekom, J. Immunol. Methods 204 (1997) 105–133.
[77] D. Litzinger, L. Huang, Biochim. Biophys. Acta 1113 (1992) 201.
[78] Abbott Laboratories Immunoassay Detection Technologies (Chapter 2). Available at: http://www.abbottdiagnostics.com/Science/pdf/learning_immunoassay_02.pdf. Accessed 4/9/2005.
[79] M. Vogl, M. Parisch, C. Klein, M. Muller, Clin. Biochem. 29 (1996) 389–391.
[80] W. Dandliker, R. Kelly, J. Dandliker, W. Farquhar, J. Levin, Immunochemistry 10 (1973) 219–227.
[81] A. Baeumner, Anal. Bioanal. Chem. 377 (2003) 434–445.
[82] L. Kricka, Clin. Chem. 45 (1999) 453–458.
[83] A. Robinson, J. Creeth, M. Jones, Biochim. Biophys. Acta 1369 (1998) 278–286.
[84] G. Rule, R. Montagna, R. Durst, Clin. Chem. 42 (1996) 1206–1209.
[85] M. Jones, P. Kilpatrick, R. Carbonell, Biotechnol. Prog. 10 (1994) 174–186.
[86] A. Plant, M. Brizgys, L. Locascio-Brown, R. Durst, Anal. Biochem. 176 (1989) 420–426.
[87] H. Rongen, H. van der Horst, G. Hugenholtz, A. Bult, W. Bennekom, P. van der Meide, Anal. Chim. Acta 287 (1994) 191.
[88] H. Rongen, T. van Nierop, H. van der Horst, R. Rombouts, P. van der Meide, A. Bult, W. Bennekom, Anal. Chim. Acta 306 (1995) 333–341.
[89] S. Siebert, S. Reeves, R. Durst, Anal. Chim. Acta 282 (1993) 297–305.
[90] D. Martorell, S. Siebert, R. Durst, Anal. Biochem. 271 (1999) 177–185.
[91] J. Ho, R. Wauchope, Anal. Chem. 74 (2002) 1493–1496.
[92] M. Roberts, R. Durst, Anal. Chem. 67 (1995) 482–491.
[93] M. Esch, A. Baeumner, R. Durst, Anal. Chem. 73 (2001) 3162–3167.
[94] S. Ahn-Yoon, T. DeCory, A. Baeumner, R. Durst, Anal. Chem. 75 (2003) 2256–2261.
[95] S. Park, R. Durst, Anal. Biochem. 280 (2000) 151–158.
[96] S. Ahn-Yoon, T. DeCory, A. Baeumner, R. Durst, Anal. Bioanal. Chem. 378 (2004) 68–75.
[97] J. Tai, M. Ewert, G. Belliot, R. Glass, S. Monroe, J. Virol. Method 110 (2003) 119–127.
[98] S. Kwakye, A. Baeumner, Anal. Bioanal. Chem. 376 (2003) 1062–1068.
[99] P. Glorio-Paulet, R. Durst, J. Agric. Food. Chem. 48 (2000) 1678–1683.
[100] S. Siebert, S. Reeves, M. Roberts, R. Durst, Anal. Chim. Acta 311 (1995) 309–318.
[101] L. Locascio-Brown, A. Plant, R. Durst, Anal. Chem. 60 (1988) 792–797.
[102] A. Plant, Photochem. Photobiol. 44 (1986) 453–459.
[103] R. Chen, J. Knutson, Anal. Biochem. 172 (1988) 61–77.
[104] I. Jääskeläinen, A. Urtti, J. Pharm. Biomed. Anal. 12 (1994) 977–982.
[105] J. Lasch, Biochim. Biophys. Acta 1241 (1995) 269–292.
[106] C. Kim, K. Park, J. Immunol. Methods 170 (1994) 225–231.
[107] L. Huang, D. Liu, Anal. Biochem. 202 (1992) 1–5.
[108] S. Rex, G. Schwarz, Biochemistry 37 (1998) 2336–2345.
[109] C. Alving, S. Kinsky, J. Haxby, C. Kinsky, Biochemistry 8 (1969) 1582–1587.
[110] J. Szebeni, L. Baranyi, S. Savay, J. Milosevits, M. Bodo, R. Bunger, C. Alving, Methods Enzymol. 373 (2003) 136–154.
[111] Y. Ishimori, K. Rokugawa, Clin. Chem. 39 (1993) 1439–1443.
[112] E. Canova-Davis, C. Redemann, Y. Vollmer, V. Kung, Clin. Chem. 32 (1986) 1687–1691.
[113] S. Frost, J. Chakraborty, G. Firth, J. Immunol. Methods 194 (1996) 105–111.
[114] A. Janoff, S. Green, A. Weiner, J. Seibold, G. Weissman, M. Ostro, Clin. Chem. 29 (1983) 1587–1592.
[115] E. Lafer, J. Rauch, C. Andrejewski, J. Exp. Med. 153 (1981) 897–909.
[116] R. Ho, B. Rouse, L. Huang, J. Biol. Chem. 262 (1987) 13979–13984.
[117] R. Ho, L. Huang, J. Immunol. 134 (1985) 4035–4040.
[118] M. Bacigalupo, A. Ius, R. Longhi, G. Meroni, Talanta 61 (2003) 539–545.
[119] A. Singh, S. Harrison, J. Schoeniger, Anal. Chem. 72 (2000) 6019–6024.
[120] T. Wu, R. Durst, Mikrochim. Acta 1 (1990) 187–195.
[121] L. Locasio-Brown, A. Plant, R. Chesler, M. Kroll, M. Ruddel, R. Durst, Clin. Chem. 39 (1993) 386–391.
[122] L. Locasio-Brown, S. Choquette, Talanta 40 (1993) 1899–1904.
[123] J. Ho, R. Durst, Anal. Chim. Acta 414 (2000) 61–69.
[124] G. Rule, D. Palmer, S. Reeves, R. Durst, Anal. Proc. 31 (1994) 339–340.
[125] S. Reeves, G. Rule, M. Roberts, A. Edwards, R. Durst, Talanta 41 (1994) 1747–1753.
[126] M. Lee, R. Durst, J. Agric. Food Chem. 44 (1996) 4032–4036.
[127] M. Lee, R. Durst, R. Wong, Talanta 46 (1998) 851–859.
[128] J. Ho, H. Hsu, M. Huang, Anal. Biochem. 330 (2004) 342–349.
[129] T. Nguyen, K. McNamara, Z. Rosenzweig, Anal. Chim. Acta 400 (1999) 45–54.
[130] T. Besanger, Y. Zhang, J. Brennan, J. Phys. Chem. 106 (2002) 10535–10542.
[131] K. McNamara, Z. Rosenzweig, Anal. Chem. 70 (1998) 4853–4859.
[132] A. Baümner, R. Schmid, Biosens. Bioelectron. 13 (1998) 519–529.
[133] K. Lee, T. Kim, M. Shin, W. Lee, J. Park, Anal. Chim. Acta 380 (1999) 17–26.
[134] D. Xu, Q. Cheng, JACS Commun. 124 (2002) 14314–14315.
[135] M. Haga, S. Sugawara, H. Itagaki, Anal. Biochem. 118 (1981) 286–293.
[136] A. Janshoff, H. Galla, C. Steinem, Angew. Chem. 39 (2000) 4004–4032.
[137] K. Marx, Biomacromolecules 4 (2003) 1100–1120.
[138] M. Cooper, Anal. Bioanal. Chem. 377 (2003) 834–842.
[139] F. Patolsky, A. Lichtenstein, I. Willner, J. Am. Chem. Soc. 123 (2001) 5194–5205.
[140] E. Kobatake, K. Yun, Y. Yanagida, T. Haruyama, K. Keinanen, M. Aizawa, Sens. Actuators B 65 (2000) 42–45.
[141] T. Ueno, S. Tanaka, M. Umeda, Adv. Drug Deliv. Rev. 24 (1997) 293–299.
[142] L. Alfonta, A. Singh, I. Willner, Anal. Chem. 73 (2001) 91–102.
[143] L. Alfonta, I. Willner, D. Throckmorton, A. Singh, Anal. Chem. 73 (2001) 5287–5295.
[144] T. Wink, S. van Zuilen, A. Bult, W. van Bennekom, Anal. Chem. 70 (1998) 827–832.
[145] D. Bates, Trends Biotechnol. 5 (1987) 204–209.
[146] T. Suita, T. Kamidate, Anal. Sci. 15 (1999) 349–352.

[147] A. Singh, P. Kilpatrick, R. Carbonell, Biotechnol. Prog. 11 (1995) 333–341.
[148] A. Singh, P. Kilpatrick, R. Carbonell, Biotechnol. Prog. 12 (1996) 272–280.
[149] M. Lee, R. Durst, R. Wong, Anal. Chim. Acta 354 (1997) 23–28.
[150] S. Choquette, L. Locascio-Brown, R. Durst, Anal. Chem. 64 (1992) 55–60.
[151] L. Locascio-Brown, A. Plant, V. Horvath, R. Durst, Anal. Chem. 62 (1990) 2587–2593.
[152] J. Cha, H. Birkedal, H. Euliss, M. Bartl, M. Wong, T. Deming, G. Stucky, J. Am. Chem. Soc. 125 (2003) 8285–8289.
[153] R. Bailey, A. Smith, S. Nie, Physica E 25 (2004) 1–12.
[154] W. Chan, S. Nie, Science 281 (1998) 2016–2018.