Biomedical Applications: Liposomes and Supported Lipid Bilayers for Diagnostics, Theranostics, Imaging, Vaccine Formulation, and Tissue Engineering

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1 Diagnostic Applications

Clinical diagnostic strategies mainly focus on the detection and quantification of biotargets (e.g., lipids, nucleic acids, and proteins) from circulation or other bodily fluids [1]. Developing reliable, inexpensive, and robust diagnostic assays at the point-of-care has revitalized existing platforms by paving the way for more individualized monitoring and increased accessibility to diagnostics in remote and resource-limited settings. Despite the utility and broad audience of DNA microarray-based diagnostic technologies [2], developing protein arrays has still obstacles due to their fragile nature, limiting their three-dimensional (3-D) structure and functionality outside their native milieu. The need for novel analytical concepts is hence inevitable. In the next sections, we provide multiple liposome and SLB-based systems that are seeking to address these challenges in the realm of diagnostics.

1.1 Immunoassay Approaches

In a vast majority of immunoassays for diagnostic applications, liposomes are employed as signal amplification element, creating larger surface for detection.

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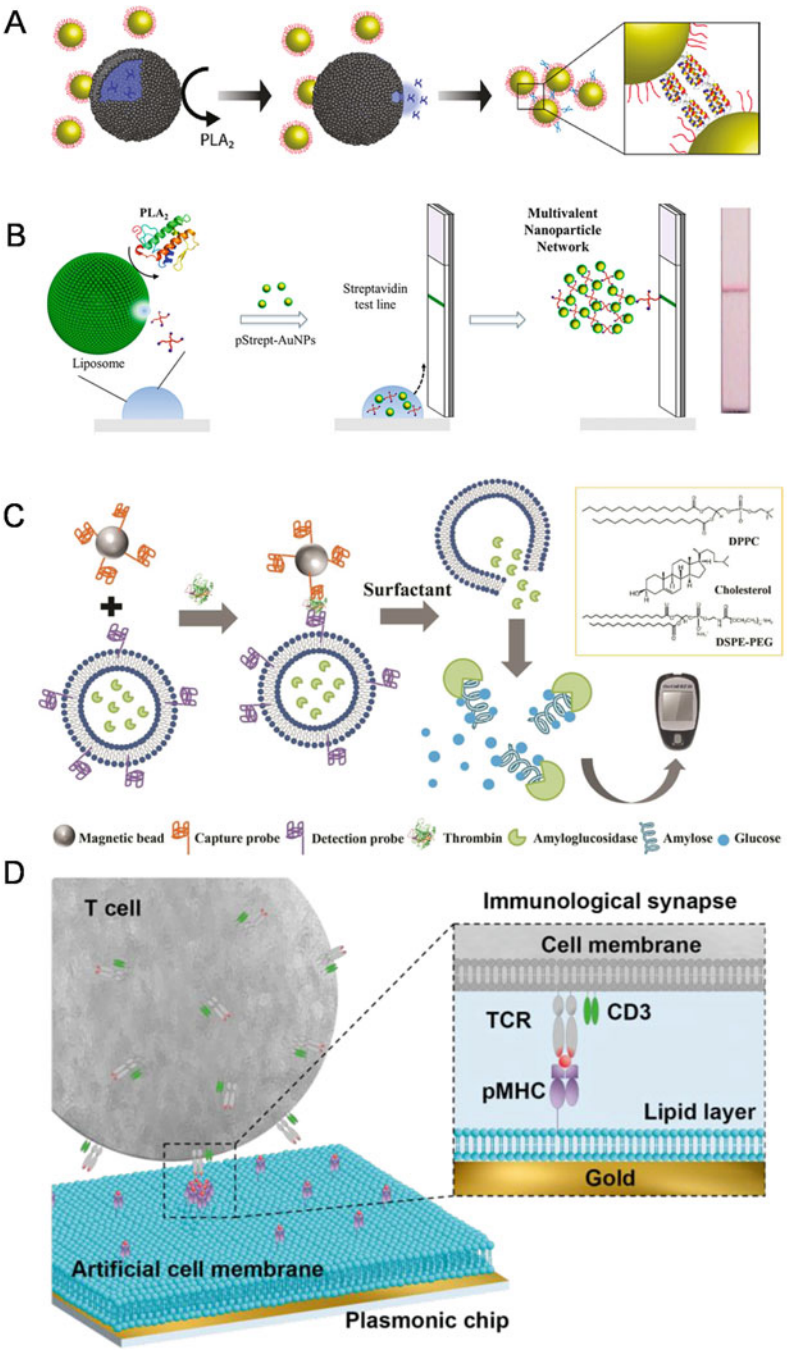

Fig. 1 Immunoassay approaches. (**a**) Schematic illustration for colorimetric detection of PLA_2. Briefly, gold nanoparticles are modified with a synthetic polypeptide whereas liposomes encapsulate a bifunctional complementary polypeptide, which interacts with the peptide on gold

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They can also be assigned as a recognition element for biotarget detection. As an example, phospholipase-A_2 (PLA_2), a biomarker for cancer, sepsis, rheumatoid arthritis, inflammatory bowel diseases, acute coronary syndrome, and asthma [3, 4], was targeted on many analytical assays, utilizing unnatural PLA_2 substrates [5]. However, their unnatural substrates may alter enzyme kinetics. As lipids are natural PLA_2 substrates, a liposome-stemmed detection strategy was developed by encapsulating a helix-loop-helix polypeptide in liposomes (Fig. 1a) [6]. In the presence of biomarkers, PLA_2 chopped the liposomes, and thus, the encapsulated peptides were released, leading to the aggregation of gold nanoparticles. Through biotinylation of polypeptides/polymers in liposomes, this strategy was also adapted into a lateral flow assay format and the aggregation of nanoparticles was subsequently elicited through streptavidin-biotin conjugation (Fig. 1b) [6, 7, 10]. Without the need for sophisticated instrument, a detection limit of 1 nM PLA_2 was achieved in human serum within 10 minutes.

Besides, liposomes could be facilitated for the detection of bacterial toxins such as exotoxins from *Staphylococcus aureus* and *Pseudomonas aeruginosa*, which were designed to rupture the liposomes and enable the release of carboxyfluorescein from them [11]. By specific tuning of lipid composition, the specificity was adapted to the toxins from *Staphylococcus aureus* only, *Pseudomonas aeruginosa* only, and both organisms. Such a broad range versatility provides not only specificity but also multiplexing that responses to distinct analytes on the same sensor. As another liposome-leakage assay, researchers integrated an ELISA-inspired strategy with lab-on-a-chip platform for virus detection using liposome tagging and ion-release impedance spectroscopy [12]. The interdigitated electrodes were modified with anti-gp120 antibodies to capture HIV particles. Ion-encapsulating dipalmitoylphosphatidylcholine (DPPC) liposomes tagged with same antibodies were used as virus labelling and the impedance signal changed after Triton X treatment for ion release. Via measuring impedance alterations on the sensor, viral load is determined with a detection limit of 6.7×10^{11} virus/mL when ~1000 liposomes/μL was introduced. This proof-of-concept work could be improved via the use of larger liposomes to reach higher sensitivity for clinical diagnostics of HIV.

Similarly, a liposome-enzyme sensor was developed to detect disease biomarkers, i.e., thrombin or C-reactive proteins (CRP) (Fig. 1c) [8]. Liposomes encapsulat-

Fig. 1 (continued) nanoparticles. By application of PLA_2, liposomes release the complementary polypeptide and lead to nanoparticle aggregation through a four-helix bundle. Reprinted with permission. (Copyright 2011, American Chemical Society [6].) (**b**) Similar mechanism i adapted to a lateral flow assay. Here, biotinylated PEG linkers loaded liposomes are cleaved with PLA_2, therefore releasing PEG linker. The biotinylated PEG linkers and polystreptavidin-coated gold nanoparticles (pStrept-AuNPs) form multivalent nanoparticle networks, generating a second signal line (positive signal). (Reprinted with permission. Copyright 2015, American Chemical Society [7].) (**c**) Further adaptation of this strategy is implemented into a glucose meter. (Copyright 2016, American Chemical Society [8].) (**d**) Schematic illustration depicts the biomimetic assay strategy on a plasmonic chip. Briefly, a planar lipid bilayer represents tumor-specific pMHC receptors on the surface. T cells interact with the lipid bilayer through their receptors (TCR). (Reprinted with permission. Copyright 2018, American Chemical Society [9])

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ing amyloglucosidase or invertase were used for signal transduction, and liposome surfaces were modified with either antibodies or aptamers to detect thrombin or CRP via the conjugation of modified magnetic beads. Likewise, Triton X-100 lysed the liposomes to release the enzymes for subsequent catalysis of glucose. A personal glucose meter was used as a readout system and the lowest detection limits were 1.8 and 0.30 nM. Such a device holds great potential for self-testing even at home-settings due to inexpensive reagents/tools, portability, and ease of operation.

Moreover, an SLB platform was developed to analyze two-dimensional affinity analysis of T cell receptor (TCR)-peptide-major histocompatibility complex (pMHC), expressed on tumor or antigen-presenting cells (Fig. 1d) [9]. In the experimental design, a chemical linker (alkanethiol SAM), SLB scaffold, and hybrid bilayer membrane (HBM: a lipid layer tethered with ODT) were evaluated with capture of T cells through anti-CD3 antibodies and pMHC. SLB and HBM systems were further tested with T cells expressing different receptor molecules such as the wild-type (WT) TCR isolated from a melanoma patient, a high-affinity (DMβ) and a low-affinity (V49I) receptors designed *in silico*. Cell concentrations and binding kinetics were employed as criteria for selection of the best interactions. Briefly, compared to the WT cells, the DMβ variant reached to the highest binding signal cells for all cell concentrations, indicating a higher number of cells attaching to the sensor. Besides, the engineered V49I variant has very low affinity for the detection of T cells, and T1φ cells (null-TCR: negative control) did not attach to the sensor. Both SLB and HBM scaffold provided similar results for the capture of T cells via those receptors. Also, this 2-D strategy provided more reliable kinetic measurements comparable to the flow cytometer. The 3-D analyses of these receptors mostly failed, indicating low sensitivity. Therefore, this work confirmed the significance of a SLB (biomimetic) microenvironment. Since the system was versatile, it could be applied to the screening of multiple cell types and benchmark new surface marker candidates for cancer diagnostics.

1.2 Micro- and Nano-Arrays

Liposomes and SLBs denote ultimate opportunities over conventional sensing systems by confining 3-D cell-mimicking matrix that permits protein flexibility and movement, therefore avoiding direct contact of proteins to the surface. Considering their implementation into diagnostic approaches requires specialized surface chemistry strategies, particularly sensor passivation via peptide layers [13], self-assembled monolayers [14], SLB immobilization [15], and poly(ethylene glycol) (PEG)-based polymeric matrices [16] in order to make the platform being inert against non-specific liposome or protein binding [17]. Through this strategy, diagnostic assays provide more specific detection, which is crucial for medical use. Besides, liposomes are functionalized through biotin-avidin [18], antibody-antigen [19], histidine linkages [20], and disulfide binding [21] for detection purposes. Despite providing stable and selective conjugations, these

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functionalization strategies are limited for multiplexing sensing, minimizing their broad use in diagnostics. To overcome this challenge, oligonucleotides provide a wide versatility to develop array format for assay multiplexing. For instance, a hybrid metallic structure (Au/SiO_2) was employed as a sensor surface, enabling patterning, functionalization, and surface passivation (Fig. 2a) [22]. Biotinylated bovine serum albumin (BSA-biotin) was then immobilized on the gold region, whereas the SLB was decorated on the SiO_2 area as a non-fouling agent. Streptavidin and biotin-terminated oligonucleotides were captured on the BSA-biotin structures, allowing tethering spots for the DNA-tagged liposome. For further manipulation on multiplexing idea, Molecular Assembly Patterning by Lift-off (MAPL) technique was developed to create homogenous arrays by decorating the sensor surface with PLL-g-PEG (PLL-g-PEG/PEG-biotin) for detection and PLL-g-PEG for low-fouling (Fig. 2b) [23]. Therefore, sequentially introducing streptavidin and biotin-terminated single-stranded oligonucleotides (ssDNA) enabled the detection of liposomes with the complementary ssDNA[16]. By altering the sequence and integrating microfluidic device, heterogeneous array structures were also developed. Similarly, larger membrane proteins, such as G-protein-coupled receptors (GPCRs), were also integrated into liposomes to detect specific ligand detection [25].

Furthermore, nano-patterning strategies were adapted to the liposome and lipid membrane-based homogeneous and heterogeneous arrays by employing photolithography [26], soft lithography [27], robotic-arraying (spotting) [28], dip-pen nanolithography (DPN) [29], and colloidal lithography [30]. As an example, lipids were used as an ink for the DPN technique to create functional micro/nanopatterning on different substrates in high-throughput manner (Fig. 2c) [24]. Researchers combined the scanning probe and optical imaging techniques and analyzed lipid microdomain formation on the self-assembled monolayers on the gold surface. Also, graphene was used as a substrate for the direct writing of tailored phospholipid membranes using DPN [29]. In contrast to the commonly used SiO_2 substrates, phospholipids exhibit higher mobility on graphene substrates, leading to stable and well-spread uniform membranes with multiplexing capability. Combining with microfluidics, SLBs could allow non-covalently immobilization of different functional groups on graphene for clinical diagnostics in the future.

1.3 Naked-Eye Detection

In addition to the array systems, a naked-eye detection strategy was achieved using a bidentate aptamer-functionalized polydiacetylene (PDA) liposome (Fig. 3a) [31]. Briefly, PDA forms a self-signaling sensor that undergoes colorimetric conversion on the course of external stimuli such as pH, temperature, and pressure [33]. In this study, three distinct types of PDA liposome were synthesized with different combination of aptamers. For instance, the type I includes only the BOCK aptamer to bind to the fibrinogen-recognition exosite of thrombin, whereas

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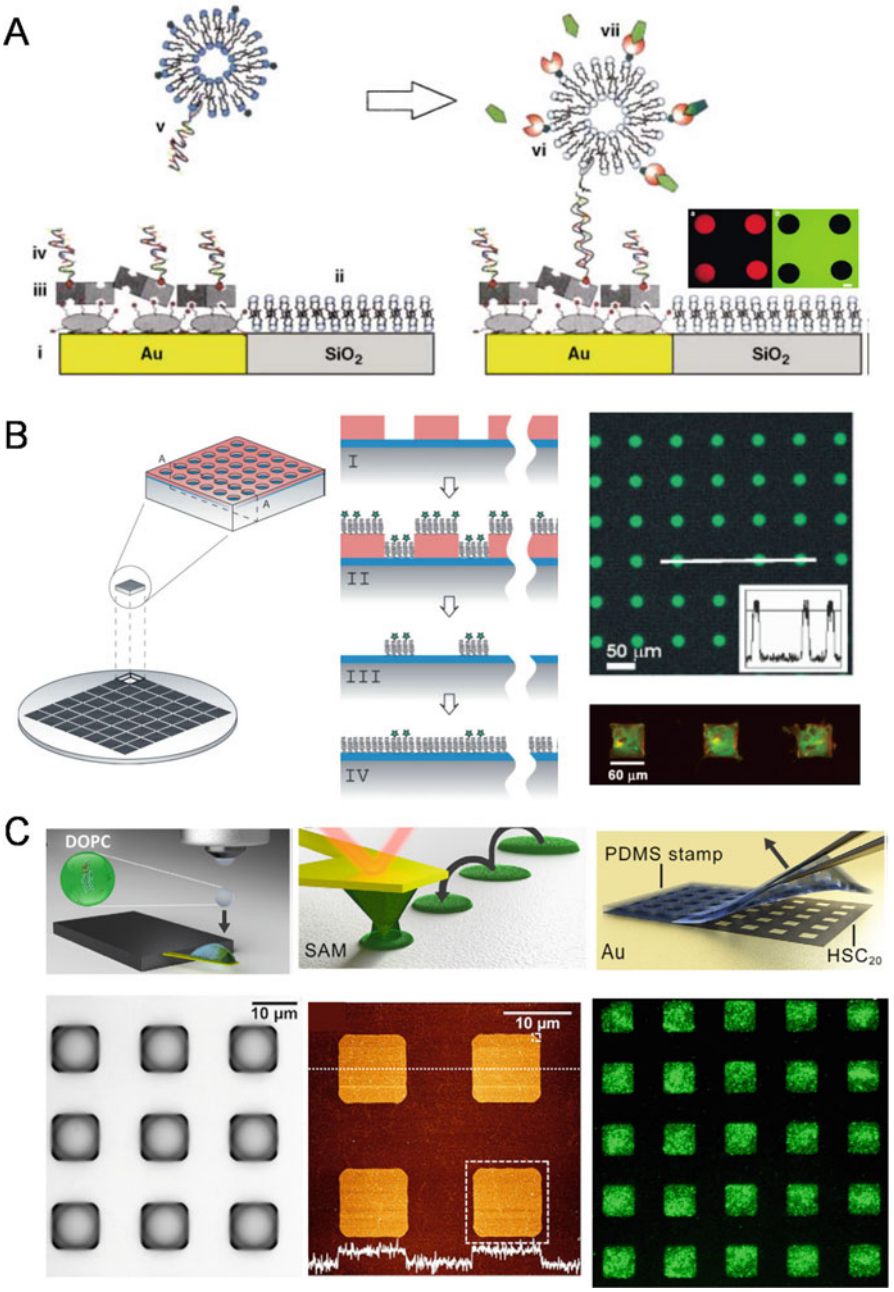

Fig. 2 Micro-arrays formation and dip-pen lithography techniques for liposomes and lipid bilayers. (a) The illustration depicts the substrate-directed surface modification strategy enabling patterning, functionalization, and surface passivation. Through streptavidin and biotin-terminated oligonucleotides, liposomes are captured on the surface, indicating a fluorescent signal (inset).

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the type II incorporates only the TASSET aptamer, targeting the heparin-binding exosite. The type III sensor utilizes both aptamers to evaluate the combined effect. While applying thrombin solution, the type III exhibited a clear color transition from blue to red, and resulted in higher colorimetric response. This strategy was able to detect different proteins from serum with a detection of ~0.5 μM within 15 minutes, therefore indicating an alternative strategy for point-of-care diagnostics, even at resource-constrained settings. However, detecting biotargets with lower concentrations or numbers such as viruses and circulating tumor cells (one in a billion cell population) requires further improvements in sensitivity and specificity, and the use of conjugated polymer materials with multidentate aptamers may hold great potential to achieve this challenge at the same time providing inexpensive and easy-to-use naked-eye detection from complex biofluids.

As another example, benefiting from PDA color conversion, influenza A virus M1 antibody and the influenza A H1N2 virus were detected with a detection limit of 2^{-2} hemagglutinin units (HAU) comparable to the commercial kits (Fig. 3b) [32]. As another example, a PDA liposome array was developed to detect aminoglycosidic antibiotics through the interactions of neomycin with PIP2 phospholipids [34]. This array system provided a detection limit of 61 ppb for neomycin. Further comparisons with other aminoglycosidic antibiotics, i.e., gentamicin, streptomycin, and tobramycin, showed that the system was more specific to neomycin due to its higher charge density and molecular weight leading to stronger interactions and greater induced stress. By hybridizing this strategy with nanomaterials such as 3-D carbon nanotube pillar network, the sensitivity was amplified compared to the 2-D stemmed sensors [35].

Fig. 2 (continued) (Reprinted with permission. Copyright 2003, WILEY-VCH Verlag GmbH & Co. [22].) (**b**) Molecular Assembly Patterning by Lift-off (MAPL) technique is demonstrated. Homogenous arrays are imaged on confocal images. Further, cells are patterned on the surface. Reprinted with permission. Copyright 2004, WILEY-VCH Verlag GmbH & Co. [23]. (**c**) Schematic illustration demonstrates the writing process. The lipids used in this study are 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), fluorescently labelled Liss Rhodamine-PE, biotinylated Biotinyl Cap PE, and negatively charged DOPA. (Copyright 2013, Springer Nature Group [24])

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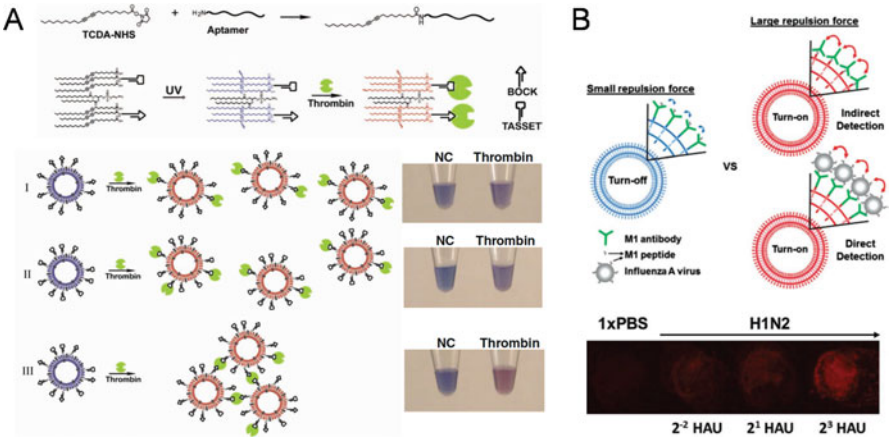

Fig. 3 Naked-eye detection via liposome-based systems. (**a**) Schematic demonstrates the strategy of protein detection on PDA sensors containing three different sets of DNA aptamers (BOCK, TASSET, and combination). Color changes of PDA sensors after interaction with thrombin is imaged (inset photos). Despite minimal color transition on the type I and type II sensors, the type III sensor presents a distinct color change that is detectable by the naked eye. (Reprinted with permission. Copyright 2010, WILEY-VCH Verlag GmbH & Co. [31].) (**b**) The illustration depicts the target size effects on the signaling of liposome assay. Fluorescent microscope images of the assay are demonstrated in the presence of H1N2 virus. Reprinted with permission. (Copyright 2013, WILEY-VCH Verlag GmbH & Co.[32])

2 Theranostic and Imaging Approaches

For a long time, liposomes and self-assembled lipid vesicles have been facilitated to deliver encapsulated drug molecules for cancer treatment, and also to label the cancer milieu as an imaging agent. As demonstrated in the recent reports [36], hybrid constructs offer great opportunities to project new theranostic nanoscale delivery systems by combining synergistically therapeutic and imaging functions. Chemical composition, surface modification, large surface area, and flexibility on the ligand types promote these structures to be compatible with the biological milieu in vitro and in vivo. Earlier, cationic magnetoliposomes (iron oxide nanoparticle-containing liposomes) have been mostly employed for the gene delivery such as plasmid DNA into cells, consequently allowing the isolation of the transfected cells using a magnetic field [37]. Also, those magnetoliposomes have been used to induce hyperthermia under a magnetic field [38]. In these days, theranostic strategies are shifted to create multifunctional vehicles combining imaging and therapy on the same structure.

In the therapeutic delivery regard, drug encapsulation within nanocarriers that specifically target malignant cells offers to diminish potential side effects of conventional chemotherapy and to allow delivery of the unique drug combinations projected for personalized medicine (Fig. 4) [39]. As an example, porous

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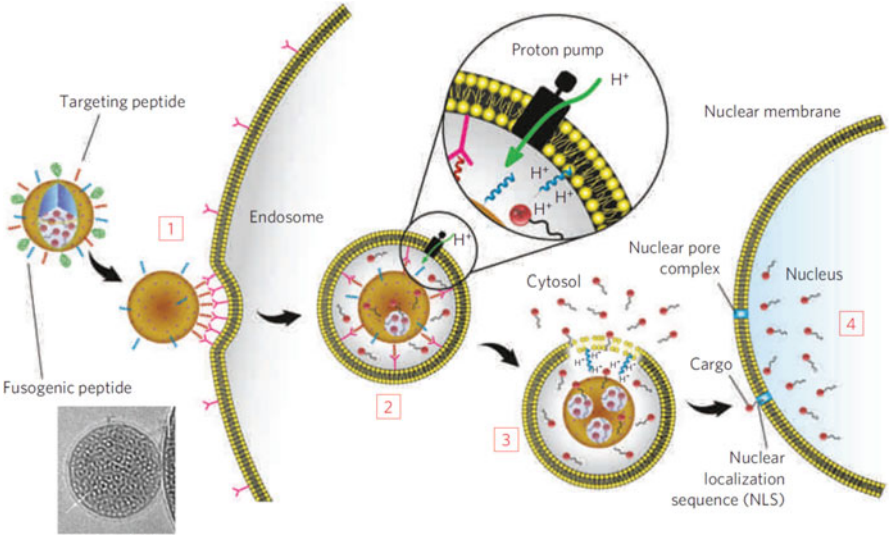

Fig. 4 Schematic diagram showing multivalent binding and internalization of targeted protocells. Briefly, the protocells (1) bind to cellular membrane through specific receptors, (2) are internalized via receptor-mediated endocytosis, and (3) release the cargo into the cytosol. (4) Cargos carrying an NLS unit are transported through the nuclear pore complex. The cryogenic Transmission Electron Microscope (TEM) image of the protocell is demonstrated in the inset figure. The image depicts the nanoporous core and the SLB (∼4 nm thick). Reprinted with permission. (Copyright 2011, Springer Nature Group [39])

nanoparticle-supported lipid bilayers (i.e., protocells) were developed through synergistically combination of liposomes and nanoporous particles. These protocells were programmed with a target peptide that binds to human hepatocellular carcinoma, and this structure presented a 10^4-fold greater affinity and specificity for human hepatocellular carcinoma comparing to the other cells, such as hepatocytes, endothelial cells, and immune cells. Versatility feature of these protocells allowed to load multiple combinations of therapeutic (drugs, small interfering RNA, and toxins) and diagnostic (quantum dots) agents, as well as enabled endosomal escape and nuclear accumulation of selected cargos. Moreover, combining the high-surface-area nanoporous core with the supported lipid bilayers enabled a single protocell loaded with a drug cocktail to kill drug-resistant human hepatocellular carcinoma cells with a 10^6-fold improvement compared to the liposomal systems. In the future, this unique feature of protocells, i.e., the integration of two or more types of ligands on the protocell surface, could potentially target to surface receptor(s) on the target cells, and subsequently promote intracellular delivery of therapeutics for cancers, where cell-specific receptors are not normally endocytosed.

Furthermore, contrast agents encapsulated in liposomes have been employed for experimental diagnostic imaging of liver, spleen, brain, cardiovascular system, tumors, inflammation, and infections [40, 41]. For instance, the combined delivery

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and magnetic resonance imaging (MRI) of doxorubicin-containing liposomes have been successfully used for cancer models such as Kaposi's sarcoma [42]. In this study, MRI was utilized to track the liposome tissue distribution and monitor drug delivery and release. Various MRI agents (ProHance (Gd(HPDO3A)-(H_2O)) or manganese sulfate ($MnSO_4$)) and doxorubicin were encapsulated in temperature-sensitive liposomes, and this allowed non-invasive and real-time imaging of drug release during hyperthermia application [43, 44]. Paramagnetic gadolinium (Gd) liposomes enriched with phosphatidylserine (PS) were also designed to image atherosclerotic plaques, and MRI imaging on ApoE(−/−) mouse model revealed rapid and considerable image enhancement of the aortic wall after injection [45]. By integrating an anti-inflammatory drug, Gd-loaded liposomes were employed as both imaging and therapy agent for atherosclerosis. In another study, the liposomal formulation of glucocorticoids (L-PLP) was applied intravenously into a rabbit model of atherosclerosis [46]. Noteworthy anti-inflammatory effects were achieved as early as 2 days and continued up to 7 days after a single dose administration.

3 Vaccines

Vaccination is one of the most successful interventions in public health approaches to control infectious diseases and cancer, leading to a significant societal and economic burden on mankind throughout history [47]. Despite the large use of conventional vaccine formulations through live-attenuated or inactivated/killed microorganisms, new generations of vaccines have emerged: subunit peptide-based vaccines and DNA-based vaccines. They (i) promise to evoke a potent and specific immunological response; (ii) can be mass-producible within a short period of time, and (iii) remove the risk of attenuated organisms evolving into a more virulent form. Regrettably, from a formulation perspective, modern peptide- and DNA-based vaccines are prone to enzymatic cleavage; are not good candidate for the absorption through oral route; and are less-reactogenic that rarely stimulate a strong immune response [48].

Later, the researchers have discovered better alternatives through inspiration of drug delivery systems (liposomes, nanoparticles, and microspheres), which produce a sustained and potent immune response [49]. In particular, liposomes provide innovative aspects to the field due to their natural, biodegradable, nontoxic, and nonimmunogenic characteristics [50]. Besides, they hold multiple advantages over the other systems and enhance bioavailability and therapeutic window with the respect to easy manipulation of their size, flexibility in composition, alternative surface charges, high resistance to enzymatic degradation, and increased absorption rates into cellular membranes. Liposomes also provides multiple formulation strategies: (i) encapsulation in their hydrophilic core, (ii) entrapment into the liposomal bilayer, (iii) absorption on their surface, (iv) attachment to their membrane by surface modification, (v) electrostatically or polymeric attachment to the liposomal membrane, and (vi) direct coupling to their surface (Fig. 5a) [51].

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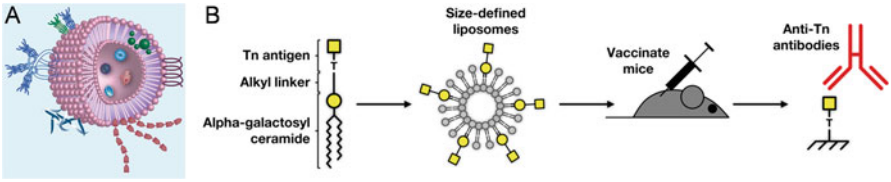

Fig. 5 Vaccine strategies. (**a**) Liposomal entities are employed in vaccine formulation via different strategies. (Copyright 2012, Future Medicine [51].) (**b**) Altered liposome sizes induce specific, high-affinity antibodies against the carbohydrate antigen with characteristics of T cell-dependent immunity, which is evaluated on a microarray scan. (Reprinted with permission. Reprinted with permission. Copyright 2018, American Chemical Society [52])

Liposome-formulated vaccines are capable of inducing both humoral and cellular immune responses owing to the liposomal antigens [53]. For instance, synthetic human MUC1 peptides as therapeutic cancer vaccines have been formulated via various ways (encapsulation, attachment, or both) that lead to strong antigen-specific T-responses [54]. As another example, liposomes improved immune response even with Antennapedia homeodomain fused to a poorly immunogenic cytotoxic T-lymphocyte (CTL) epitope due to their significant protection [55]. As another strategy, peptide vaccines and CTL epitopes encapsulated in liposomes were delivered to dendritic cells (DCs) for improving the immune response to antigenic peptides [56, 57].

In a recent study, a two-component formulation via the facile conjugation of carbohydrate antigens to α-galactosylceramide (α-GalCer) was prescribed to yield fully synthetic vaccine candidates for potential preventive and therapeutic cancer research (Fig. 5b) [52]. By changing liposome size, the researcher achieved to induce specific, high-affinity antibodies against the carbohydrate antigen with characteristics of T cell-dependent immunity. Interestingly, varying in liposome size affected the glycan antibody responses (either a cellular or a humoral immune response). Moreover, this glycolipid vaccine platform offered strong and accurate anti-glycan antibody responses in vivo without the need for an external adjuvant. In summary, the liposomal integration to vaccine candidates creates a unique feature to improve our understanding in the vaccine realm and provide promising strategies by fine-tuning immunological response.

4 Tissue Engineering Applications

Tissue engineering is an emerging interdisciplinary field dedicated to develop functional biological substitutes for the regeneration of injured or diseased tissues [58]. General treatments of patients with tissue degeneration involve the application of mechanical devices to replace tissue functions and tissue/organ transplantations (autograft or allograft). Although these procedures have been lifesaving for many

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patients, they present major problems, such as infection, donor site morbidity, and rejection [59, 60]. Alternatively, the field of tissue engineering aims to regenerate and maintain normal functions of tissues by culturing desired type of cells into natural physiological conditions—like scaffolds with bioactive agents [61]. For successful tissue engineering applications, the properties and microenvironment of desired cell source must be studied well, and appropriate scaffold which can mimic and model natural microenvironment of that specific cell type must be designed to stimulate the normal function of the desired tissue, accordingly.

Cells create unique microenvironment a dynamic repertoire of mechanical, chemical, and physical signatures that enables multiple functionality, such as adhesion, expansion, differentiation, and apoptosis [62]. These factors constitute various types of extracellular matrix (ECM) macromolecules, signaling factors, cytokines, growth factors, and cell adhesion molecules [63]. When designing new cell substrates to control cellular behavior, natural microenvironment of the cell type of interest, cell-matrix interactions, and cell-cell interactions must be considered. SLBs provide unique capability by (i) mimicking cell matrix through the integration of Gly-Pro-Hyp [64], Arg-Gly-Asp [65], Arg-Gly-Asp-Ser, Leu-Asp-Val, Arg-Glu-Asp-Val [66] sequences, collagen type I [67], collagen type I and III [68], collagen type IV [69], fibronectin [70], laminin [65, 71], and (ii) creating cell-cell integration via transmembrane proteins such as Epithelial (E)-cadherin [72], N-cadherin [73, 74]; neuronal adhesion promoters, such as Ephrin A5 [75, 76]; epidermal growth factor [77]. In this section, we will specifically mention the utilization of SLBs in the realm of tissue engineering to demonstrate their wide impact on complex cellular processes, such as cellular differentiation, functionality, and adhesion (Fig. 6).

4.1 Cellular Differentiation and Functionality Approaches

SLBs are cell membrane mimicked substrates with adjustable physicochemical and mechanical properties. Zwitterionic groups in the head groups of lipids in SLBs resist protein adsorption, therefore minimizing non-specific cellular adhesion [78]. However, ligands can be incorporated in the SLBs to allow desired cellular interactions. In addition, the mobility of the ligands can be altered by the alkyl composition of base lipids in SLBs, which are characterized by the melting temperature. In physiological temperatures, if lipids in SLBs are low melting temperature lipids, they present mobile ligands (e.g., DOPC); whereas if lipids in SLBs are high melting temperature lipids, they present immobile ligands (e.g., DPPC) [79]. SLB roughness, compliance, and fluidity can be changed by stacking bilayers in SLBs, which influence cellular attachment and morphology [80–82]. These developments in the SLB design have provoked further research about the effects of SLB characteristics on cellular differentiation. Evans et al. modified a SLB membrane with recombinant N-cadherin and investigate the behavior of primary adult periosteum-derived multipotent cells (PDCs) on these SLBs in order to explain the effect of cell-cell interactions in differentiation and

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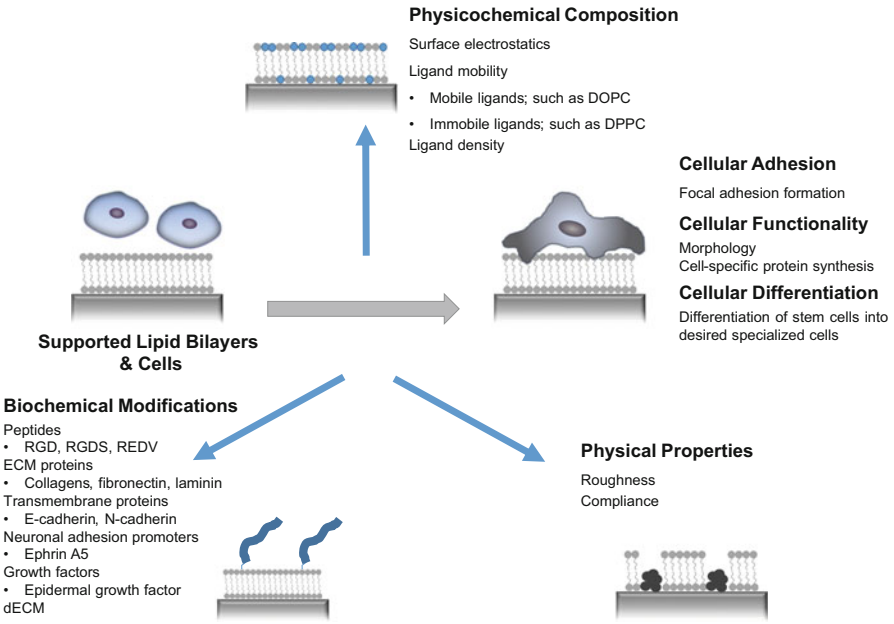

Fig. 6 Summary of SLB characteristics on cellular behavior. Tissue engineering-stemmed approaches have largely benefited from SLB structures to analyze (i) physiochemical composition, (ii) cellular adhesion, (iii) cellular functionality, and (iv) cellular differentiation by changing biochemical compositions and physical properties

de novo tissue growth. N-cadherin-functionalized SLBs were found to induce cell aggregate formation when PDSs were seeded at high density. In addition, a downregulation of cell-cell adhesion proteins (N-cadherin and ZO-1), matricellular protein (periostin), and early osteochondral and adipogenic markers was found with the upregulation of hyaluronic acid, presenting the transition of multipotent cells into a pre-condensation state on functionalized SLBs. On the other hand, at low seeding densities, PDCs acquired rounded morphology on functionalized SLBs and PDCs cultured on non-modified SLBs showed no attachment, but upregulation of osteochondral lineage markers [83]. Another study by Lee et al. investigated the regulation of adhesion and differentiation of neural stem/progenitor cells (NSPCs) on SLBs with adsorbed polyelectrolyte multilayers (PEM) without addition of serum or growth factors. Short-term cell culture studies resulted that SLB-PEM system enhanced neuronal differentiation and functional axonal growth [84].

Koçer et al. reported that human mesenchymal stem cell (hMSC) adhesion and osteogenic differentiation can be regulated by the ligand density and mobility of RGD ligand-functionalized SLBs. In this study both fluid DOPC (low melting temperature)- and non-fluid DPPC (low melting temperature)-based SLBs were modified with RGD ligand linked to different amounts of biotin-conjugated 2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE: 0.01, 0.50, and 1.00 mol%)

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and used to show the effect of ligand mobility of SLBs on hMSC adhesion. Upon the CellProfiler software quantifications, the increase in the RGD ligand density was found to increase cell density on both SLBs; however cell density on RGD DOPC SLBs was higher in all groups when compared to RGD DPPC SLBs. Since the ligands can freely move and rearrange on RGD functionalized DOPC SLBs, dynamic interactions are more likely to occur between integrin receptors of cells and these mobile ligands, which provided enhanced hMSCs attachment. Osteogenic differentiation on SLBs was evaluated by using Alkaline phosphatase (ALP) activity and higher ALP activity was found with RGD functionalized DOPC SLBs (increased with increasing ligand density), which also supported increased cell spreading [79].

Another SLB study on stem cell differentiation reported that SLBs with low fluidity provided the formation of focal adhesions and FAK phosphorylation, which then activated the MEK/ERK signaling pathway and subsequently enhanced neuronal differentiation of neural stem cells. In addition to the differentiation, better morphology and adhesion of neural stem cells were shown on low fluidity SLBs. These effects of SLBs fluidity on stem cell behavior may be correlated with the dynamic ECM and stem cell interactions and could be promising for the development of novel tissue engineering strategies in the future [85].

4.2 Cellular Adhesion Feature

Due to the surface electrostatics and lateral fluidity of SLBs, they are protein- and cell-resistant substrates. In order to provide cellular adhesion, SLBs are functionalized with some specific molecules, generally with peptides or proteins. Using electrostatics is an alternative and a simple method to enhance cellular behavior on SLBs. Introducing charged lipids to SLBs allows cell attachment by reducing the fluidity. This was achieved by the addition of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) into the 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) SLBs. Rat embryonic cortical neuron attachment was better on positively charged DOTAP-SLBs than on the POPC-SLBs, showing that the charge of SLBs had a promotive effect on cellular adhesion [86].

A similar study was reported by Choi et al., in which they used plasma activated gold substrates, instead of glass. Different ratios of POPC and DOTAP lipid vesicles were deposited on gold surfaces and primary neuronal cells were cultured on these SLBs for 10 days. PDL coating was taken as control, where viability and morphology of cells on SLBs were similar to the control group. No cytotoxic effect was recorded causing from DOTAP; moreover only POPC layer on gold substrates inhibited cellular attachment [87].

Besides, ferritin-supported lipid bilayers with different surface charges were prepared to investigate the behavior of retinal microvascular endothelial cells (ECs) by mixing positively charged 1-palmitoyl-2-oleoyl-*sn-glycero*-3-ethylphosphocholine

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(POEPC) and zwitterionic POPC with different ratios. Cellular adhesion and proliferation were found to be increased in all groups with ferritin, when compared to bare SLBs (POPC/POEPC, 95:5 ratio) and control glass. Among all ferritin-supported substrates, the membranes of POPC/POEPC (75:25 ratio) resulted in the highest ECs adhesion [5].

Lipid membranes on solid supports maintain their fluid environment, which make the reactions in these systems difficult to control [88]. Ensuring the unique properties of membrane constituents and enabling a better control, patterned surfaces have attracted the interest [76, 89]. Studies showed that micrometer-sized patterned, ligand-containing SLBs help the structural reorganization of cellular components, in addition to increasing the size of receptor clusters [90, 91]. Moulick and coworkers recently explored that EA5-Fc modified and patterned SLBs promoted cellular adhesion and growth. Gold grids in the range of 0.5–2.5 μm with 200 nm wide barriers were prepared for the patterning of SLBs, and to enhance cell adhesion, Fc-EA5 was tethered on POPC-SLBs [76].

When designing scaffolds to enhance cell attachment, biochemical signals originating from the extracellular matrix of cells can be mimicked or manipulated by using surface modifications of SLBs. Modifications can be applied by using cell-type-specific ECM components, such as peptides or proteins. However, in cellular attachment processes, cells can also recognize the mechanical properties of their extracellular matrix through focal adhesions [82]. Polymer-tethered lipid bilayers are used to investigate the effect of mechanical matrix properties on cellular behavior. Multi-lipid cell substrate's compliance can be adjusted by increasing the number of lipid bilayers in the stack, which increases the substrate fluidity. The stacking affects the mechanical properties of substrate, so the adhesive ligand binding properties remain unchanged [80, 81]. Minner et al. reported that bilayer stacking has increased membrane roughness and fluidity. They also investigated the relation between the number of bilayers in the stack of laminin-functionalized lipid bilayers and the behavior of mouse 3T3 fibroblasts. Cell culture on these laminin-functionalized single, double, and quadruple bilayer membranes showed different cytoskeletal organization of cells, such as crescent, triangle, spindle, and dendritic [81].

Decellularized extracellular matrix (dECM) holds great impact on the fields of tissue engineering and regenerative medicine, as it provides structural and biological cues to cells, with eliminated inflammatory response [92–94]. Vafaei et al. designed a study by combining dECM and SLBs to produce a hybrid biomimetic platform for the human hepatocyte Huh 7.5 cells. Mouse adipose tissue was decellularized and covalently attached to the surface of SLBs (80% DOPC and 20% DP-NGPE). Bright field images showed that a limited amount of cells were attached to the bare SLBs and they did not spread over 24 h. On the other hand, cells on dECM-functionalized SLBs showed a nice attachment and spreading. Quantification of images indicated that cell number and their projector area are two times higher in dECM-SLBs, when compared to bare SLBs. However, the projected area of cells on dECM-glass substrates was a little bit higher than on dECM-SLBs. Even though the

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cellular attachment and proliferation features are similar on dECM-SLBs and other control platforms, dECM-SLBs are more promising platforms, which can mimic ECM-oriented responses [95].

5 Conclusions

Owing to biophysical and chemical versatility of liposomes and SLBs, they have been facilitated in broad biomedical applications spanning from clinical diagnostics, immunoassays, array formations, vaccine formulations, theranostics, and labelling approaches to tissue engineering. Multiplexing through array systems would potentially expend our current knowledge in lipid-derived systems, accelerating their use in clinical applications in the near future.

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