Liposomes: Technologies and Analytical Applications

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Abstract

Liposomes are structurally and functionally some of the most versatile supramolecular assemblies in existence. Since the beginning of active research on lipid vesicles in 1965, the field has progressed enormously and applications are well established in several areas, such as drug and gene delivery. In the analytical sciences, liposomes serve a dual purpose: Either they are analytes, typically in quality-assessment procedures of liposome preparations, or they are functional components in a variety of new analytical systems. Liposome immunoassays, for example, benefit greatly from the amplification provided by encapsulated markers, and nanotube-interconnected liposome networks have emerged as ultrasmall-scale analytical devices. This review provides information about new developments in some of the most actively researched liposome-related topics.

1. INTRODUCTION

Since the first observation of phospholipid vesicles (liposomes) in 1965 by Bangham and colleagues, who effectively determined the character of lipid membrane–enclosed volumes and their connection to biological cells (1), liposome-related topics have become commonplace in the literature (see, for example, References 2–4).

The nature of lipid vesicles as well as their relevance in biological environments have been extensively studied (5–7), and applications have extended into many fields. Today, liposome-derived technologies are established as one of the cornerstones of bionanotechnology (8). The unique versatility of lipid vesicles with respect to composition, size variety, and capacity for embedding and encapsulating materials has led to applications in chemical and biochemical analytics and even to industrial-scale applications in drug delivery, cosmetics, food technology, and proteomics (9). Liposomes are commonly utilized as precursors in the fabrication of suspended bilayers (10, 11), and recently, nanotubes-conjugated liposome networks have emerged as novel biomimetic chemical reactor systems with capabilities for single-molecule analysis (12, 13). In this review, we give an overview of the fields of liposome research, technologies, and applications, particularly in analytical chemistry, covering fundamentals and advances in preparation and characterization and new techniques in areas such as chromatography and biosensors.

2. LIPOSOMES

2.1. Phospholipid Membrane Vesicle (Liposome) Characteristics and Properties

Liposomes are spherical soft-matter particles consisting of one or more bilayer membrane(s), and are most commonly composed of phospholipids encapsulating a volume of aqueous medium. The aqueous medium is typically the same as that in which the liposomes are suspended, but each can often be individually exchanged, for instance by microinjection or dialysis. Liposomes are readily prepared in the laboratory (5, 14).

In liposome formation, dissolved lipid molecules, consisting of a hydrophilic headgroup and a hydrophobic tail, self-assemble into bimolecular lipid leaflets upon decreasing their solubility in the surrounding medium. Whereas ordinary amphiphiles have critical micelle concentrations (CMCs) of 10^{-2}–10^{-4} M, the CMC of bilayer forming lipids is four to five orders of magnitude smaller, meaning that the water solubility of these materials is extremely low. The lipid's headgroups are exposed to the aqueous phase and the hydrophilic hydrocarbon moieties are forced to face each other in the bilayer. This free energy–driven process is recognized as one of the most powerful mechanisms in bottom-up engineering (15).

Liposomes as analogs of natural membranes are generally assembled by spontaneous self-organization from pure lipids or lipid mixtures. **Figure 1** schematically depicts the fundamental assembly process, in this case leading to a unilamellar vesicle.

The low water solubility of lipid molecules greatly affects the dynamics of lipid exchange between the bilayer and the surrounding medium. Residence times for

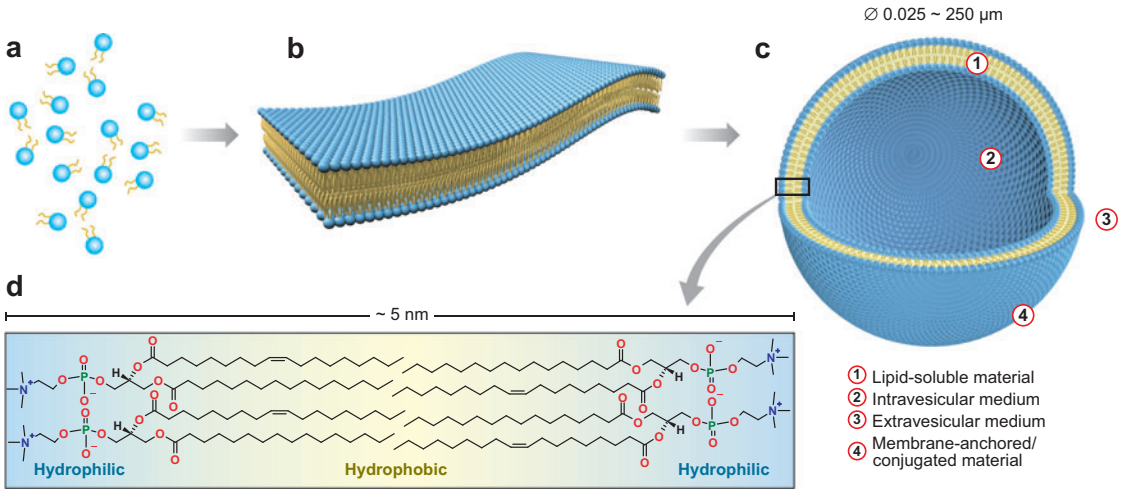

Figure 1

Schematic illustration of the fundamental self-assembly process from individual phospholipid molecules (*a*) to bilayer membrane leaflets (*b*), followed by transformation into liposomes (*c*). A single bilayer is typically ~5 nm thick and consists of neatly arranged individual lipid molecules with their hydrophobic tails facing each other and their hydrophilic headgroups facing toward the internal and external aqueous medium (*d*). Apart from the structural characteristics of the lipid molecules themselves, the properties and functionality of liposomes are largely defined by their size and the composition of the four distinct regions highlighted in panel *c*. Various natural and synthetic lipid molecules are available for the preparation of bilayer membranes and liposomes (140).

phospholipids are on the order of 10^4 s, compared to 10^{-4} s of typical micelle-forming amphiphiles (16).

The most commonly utilized lipids are phospholipids, in particular the charge-neutral phosphatidylcholine and the negatively charged phosphatidic acid, phosphatidylglycerol, phosphatidylserine, and phosphatidylethanolamine, each of which has a different combination of fatty acid chains in the hydrophobic region of the molecule. Stearylamine can be employed when cationic liposomes are needed. Apart from charge, the nature of the fatty acid residues in each lipid molecule, particularly the number of double bonds in the chain, is responsible for fundamental bilayer properties such as phase behavior and elasticity.

To obtain liposome preparations, crude or purified plant or animal lipid extracts are often employed. Plant and cyanobacteria lipid extracts contain mostly phosphoglycerides and glycosylglycerides, whereas extracts from erythrocytes or liver cells are mainly composed of phosphoglycerides, sphingolipids, and sterols. Unpurified extracts contain other hydrophobic molecules, such as membrane protein material as contaminants, and are therefore of limited use (17, 18).

A highly specific group of phospholipids are synthetic lipids with designed functionalities. To this group belong lipids that are headgroup modified for coupling reactions (19), chelating metal ions (20), and even multifunctional lipids (21).

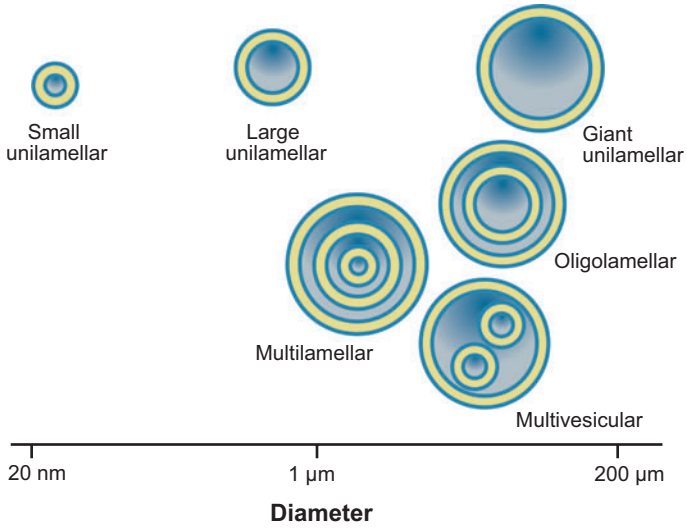

Figure 2

Schematic representation of the commonly applied classification scheme for liposomes. Small unilamellar vesicles (~0.02 μm to ~0.2 μm), large unilamellar vesicles (~0.2 μm to ~1 μm), and giant unilamellar vesicles (>1 μm) are the three most important groups for analytical applications. Multilamellar vesicles are frequently used in pharmaceutical and cosmetic applications (56). Multivesicular vesicles are giant vesicles encapsulating smaller liposomes and have been used in nanoreactor assemblies (141) and as drug delivery tools (vesosomes) (142). The drawings are not to scale.

Embedding functional regions such as reactive double bonds into the hydrophobic tail section has led to the creation of classes of polymerizeable lipids that function as cross-linking elements and, under UV or radical polymerization conditions, lead to membranes and vesicles of enhanced stability (22–24).

Liposomes are, from a morphological perspective, most frequently classified by their size and number of membrane bilayers (lamellae) (**Figure 2**).

Unilamellar vesicles are of special interest in research, mostly due to their well-characterized membrane properties and facile preparation. They are divided into three size types: small, large, and giant. Controlled processes for formation of oligolamellar vesicles are rare, although an example of a polymer-induced transformation of unilamellar to bilamellar vesicles has been reported (25). Multilamellar vesicles often show physical properties and behavior that are very different from the unilamellar species, and they are commonly used for industrial applications such as drug delivery. They are also employed as lipid reservoirs in the process of nanotube-vesicle network (NVN) fabrication (26).

Liposomes are generally not considered to be at thermodynamic equilibrium because curvature energy is being confined in the vesicles as they are produced. The curvature free energy, also known as the bending energy or curvature elastic energy of the liposome, is determined by the bending rigidity and curvature of the membrane and is responsible for the large variety of shapes that liposomes can take. Three

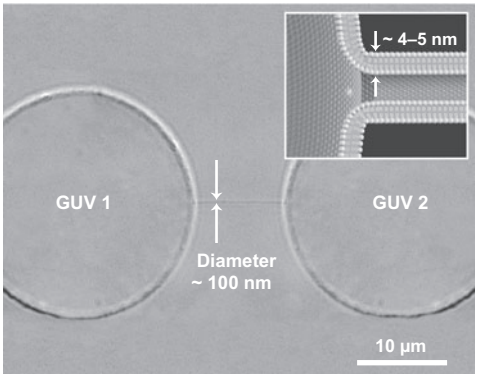

Figure 3

Differential interference contrast micrograph of a phosphatidylcholine lipotube suspended between two giant unilamellar vesicles (GUVs). The tube is only stable in the suspended state and will retract and merge with the connected GUV when cut off and released on one end. *Inset*: A detailed schematic view of the high-curvature region where the nanotube is connected to the vesicle.

models have been developed to describe these dependencies: the spontaneous curvature model (27), the bilayer couple model (28), and the area-difference elasticity model (29). Many other physical aspects of liposome structure and dynamics of formation were described in a recent overview (30).

2.2. Lipotubes (Lipid-Membrane Nanotubes)

An important feature of the liposome is the fluidity of its membrane, which allows for incidents of membrane deformation without disrupting bilayer integrity. A practical consequence of this property is the formation of lipotubes (lipid membrane nanotubes) when a point load is applied to a vesicle (31). Lipotube formation involves first-order shape transitions, wherein the obtained shape represents a minimum in surface free energy, i.e., the surface-to-volume ratio of the tubular structure is optimized.

Lipotubes are flexible membrane conduits with a diameter of ~100 nm and a variable length of up to several hundred micrometers. The diameter of the structure depends largely on the lipid composition, which determines membrane tension and curvature. Transport of biopolymer material and detection of single molecules in lipotubes have been demonstrated in the context of NVN technology, as discussed below. **Figure 3** shows a lipotube tethered from a phosphatidyl choline liposome and suspended between two giant unilamellar vesicles (GUVs), as used for single-molecule transport experiments (32).

2.3. Vesicular Gels

Vesicular phospholipid gels (VPGs) are semisolid, aqueous phospholipid dispersions wherein the lipid phase consists of liposomes (33). They can be viewed as a hydrogel

variant, but, unlike other hydrogels, tightly packed vesicles rather than macromolecular constituents cause the gel-like rheological behavior. In VPGs, the vesicles are so densely packed that the aqueous volume between the vesicles is reduced to a minimum, giving rise to steric interactions between neighboring vesicles and thus to a semisolid consistency. VPGs are distinctly different from liposome gels, wherein a hydrophilic polymer forms an embedding matrix.

VPGs are capable of storing and releasing drugs in a controlled manner over extended periods of time and may serve as depot implants for controlled release. When VPGs are exposed to excess aqueous medium, they are readily transformed into small unilamellar vesicle (SUV) dispersions, where the SUVs display high encapsulation efficiency, irrespective of the molecular weight, charge, or membrane permeability of the loaded material (34).

3. LIPOSOME PREPARATION AND ANALYTICAL CHARACTERIZATION

The beneficial properties of liposomes as drug carriers were first recognized in the 1970s and 1980s, and the number of their biopharmaceutical applications has increased rapidly (35). The need for liposomal drug carrier formulations in particular has stimulated research on liposome-preparation procedures. At least four major and several minor methodologies are in use today, including methods based on dry lipid films or emulsions and methods involving the use of micelle-forming detergents or the principles of solvent injection. Some commonly used procedures (**Figure 4**) are outlined in the following section.

The specificity, homogeneity, and laboratory-scale availability of liposomes containing natural or synthetic lipids has had an immediate impact on studies of diverse cellular and biophysical phenomena. A multitude of methods exists for the

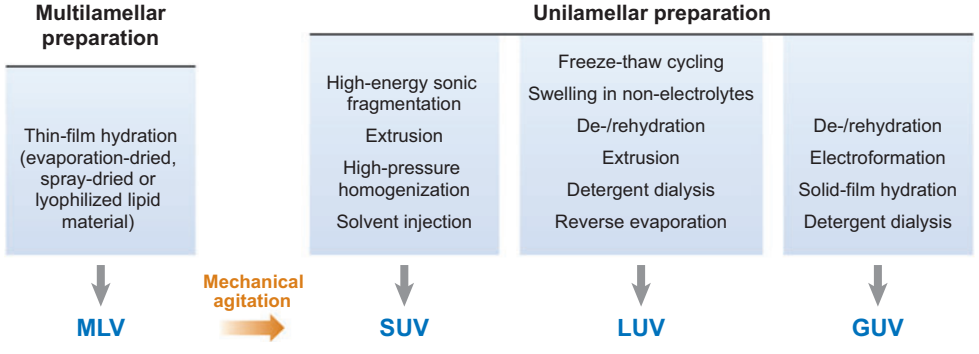

Figure 4

Common preparation techniques for different types of liposomes, categorized by lamellarity and size range. Multilamellar vesicles (MLVs) can be transformed into unilamellar vesicles by various means of mechanical treatment (5). Abbreviations: GUV, giant unilamellar vesicle; LUV, large unilamellar vesicle; SUV, small unilamellar vesicle.

preparation of liposomes of different size and lamellarity. Reviews and books have been published describing the protocols in great detail (7, 9, 14, 36).

Several fabrication methods start from a dry film that is subsequently hydrated with an aqueous solution containing the substance to be encapsulated (37); others are based on the reverse-evaporation technique (38). Using the film-hydration method, (mostly) multilamellar vesicles (MLVs) are formed with high encapsulation efficiency, but also with great variations in vesicle size, size distribution, and lamellarity. Some approaches replace a water-insoluble organic solvent that contains the dissolved lipids with an aqueous solution containing the substance to be encapsulated (39). This rapid solvent exchange is a novel strategy featuring direct transfer of the lipid mixture from the organic solvent to an aqueous buffer. The method is very fast and provides considerable entrapment efficiency. The solvent-injection method (40) and variants (41) involve the very slow injection of, typically, an ethanolic solution of lipids into an aqueous phase, leading to the formation of unilamellar vesicles. Liposomes obtained in this manner display a well-defined size distribution and a high encapsulation efficiency; residual ethanol is then removed by repeated dialysis steps against distilled water. Another methodology of liposome formation is founded on addition of detergent (42) or chaotropic ions (43) to the aqueous/organic mixture of lipids, and the subsequent removal of these reagents by gel filtration or dialysis.

New procedures have been reported, including a coacervation (phase-separation) technique based on water mixing with lipid solutions in different soluble alcohols (44) and microfluid-directed liposome formation (45).

3.1. Multilamellar Vesicle Preparation

The preparation of MLVs involves a simple and robust film-hydration technique (46). Lipid solution is initially dried, either by using an evaporator or by spray drying/lyophilization (47) for larger-scale preparations. Alternatively, this step can be performed at very low pressure for a few hours in the presence of a neutral dessicant. For subsequent MLV formation, the sample is mechanically agitated in the presence of a hydration medium such as buffer. MLVs in the micrometer-size range are spontaneously formed when the film is exposed to an excess volume of aqueous buffer.

For transformation into unilamellar vesicles, MLVs can be dispersed by various mechanical methods, providing means for easy scale-up, large capacity, and fast processing (up to hundreds of liters per hour). Disadvantages are possible sample degradation and residual lipid particles of different size ranges.

3.2. Extrusion

Extrusion is a technique suitable for generating unilamellar liposomes of well-defined size (48). When MLVs are forced through narrow-pore membrane filters under pressure, membrane rupture and resealing occur and encapsulated content leaks out. Therefore, extrusion is performed in the presence of medium containing the final load concentration, and external solute is removed only after formation is complete (49).

The hydrated vesicles are initially subjected to several cycles of a freeze-thaw procedure and are subsequently forced through double-stacked polycarbonate membranes of decreasing pore size (e.g., sequentially 200, 100, and 50 nm) at elevated temperatures. The vesicles are commonly extruded five to ten times through each double-stacked membrane until the desired size is reached.

On-chip extrusion of micrometer-sized vesicles and extraordinarily long lipotubes through fabricated surface apertures has recently been reported (50). This method is a novel fabrication approach based on a combination of top-down and bottom-up processes.

3.3. Sonication

Sonication is a simple method for reducing the size of liposomes (51). The common laboratory method involves treating hydrated vesicles for several minutes with a titanium-tipped probe sonicator in a temperature-controlled environment. The vesicle preparations are subsequently passed through a 0.45-μm membrane filter to remove titanium particles that have detached from the tip surface during the procedure.

3.4. Freeze Drying of Monophase Solutions

Stability-influencing effects such as aggregation, fusion, and phospholipid hydrolysis are mainly due to the aqueous environment, as liposome preparations obtained by traditional methods are buffered suspensions. To improve this situation, a strategy for preparing proliposomes was developed (52). This method involves the coating of dry carrier powders with phospholipids that, upon hydration, form liposomes of a broad size distribution. A similar novel procedure for the preparation of large unilamellar vesicles (LUVs) of narrow size distribution is based on the initial formation of a uniphase solution of lipids and water-soluble support materials, such as sucrose in a *t*-butanol/H_2O cosolvent system, which is then lyophilized to remove the solvents (53). Upon the addition of water, the lyophilized product rapidly forms homogenous submicrometer-sized vesicles.

3.5. Hydration, Dehydration, and Swelling

Hydration/dehydration methods are based on the swelling of dried lipid films upon exposure to an aqueous medium. Because of the presence of buffer salts within the dehydrated film, an osmotic pressure gradient forces water between the individual bilayers, and the lamellae separate to form liposomes. This procedure is frequently used to obtain mixtures of multilamellar and giant unilamellar liposomes (54, 55). A critical factor that affects the yield of formation is the degree of bilayer separation, which is influenced by temperature, lipid composition, and the ionic composition of the surrounding medium. Inclusion of negatively charged lipids is known to enhance separation of the lamellae. A relatively low-ionic-strength buffer (10–50 mM) and the absence of multivalent ions that interlink charged lipids are essential. The process

is comparatively fast (minutes compared to hours or days with other protocols), and unilamellar vesicles are produced in high yield.

The most commonly proposed mechanism for liposome formation from a dehydrated film is the detachment (56) of membrane sheets, which eventually separate and close upon themselves, remaining connected to the film via membrane tethers. Mechanical agitation is needed to detach the liposomes from the film.

3.6. Electroformation

There is no available method to exclusively produce GUVs, although the electroformation method yields high numbers of unilamellar vesicles (57, 58). Some lipid mixtures are very difficult to swell into vesicles. Electroformation, wherein an alternating electric field is applied while swelling the lipid film (57), is an efficient means of overcoming this problem. The field is believed to cause fluctuations in the bilayers, thus inducing detachment of lamellae and formation of vesicles. Lipid-film thickness, peak-to-peak voltage, duration of treatment, and frequency influence the process. The method is limited to low ionic-strength buffers, but a method to increase the ionic strength while preserving osmolality has also been reported (59).

Electroformation in combination with spincoating is a recent addition to the methodology (60). Very uniform lipid films are generated over a large substrate area, and film thickness can be controlled by spin speed, lipid concentration, and solvent viscosity. Films are typically 25–50 μm thick, which is reported to be the optimum thickness for electroformation. GUVs obtained by this method are considerably (two to five times) larger than those formed with standard techniques, and even lipids that do not readily form liposomes can be successfully assembled.

3.7. Ink-Jet Injection into an Aqueous Phase

By utilizing ink-jet technology, it is possible to prepare and suspend SUVs directly in one step. In this method, an ink-jet cartridge injects droplets of lipid solution in a water-miscible solvent with high reproducibility into an aqueous volume. Vesicle formation proceeds via a nucleation (micellization) and growth mechanism. The well-controlled distribution of monodisperse droplets leads to a stable level of supersaturation, which determines the number of nuclei and thus the final vesicle size. Routinely, monodisperse vesicle dispersions with high reproducibility of the mean particle diameter can be prepared for a given ink-jet cartridge type and amphiphile concentration. Volumes of 1–1.5 mL/min^{-1} are possible to obtain (61).

3.8. Chemical Analysis and Characterization of Liposome Preparations

Five important aspects of liposome analysis have been pointed out in a recent review (62): lamellarity determination, size determination, quantitative lipid analysis, encapsulant determination, and characterization of liposomes with respect to manufacturing (quality assurance). A detailed description of today's most commonly used

methods and of novel techniques for the treatment of these aspects, including advantages and limitations, is provided therein.

Particle mean size and size distribution, measured with flow field–flow fractionation coupled to multiangle light scattering (63) or other detection methods (64, 65), and polydispersity are the most important parameters to consider when describing a liposome or any other colloidal dispersion. For liposomes, size distribution is closely related to the characteristics of the lipid bilayer, including spontaneous curvature, the Helfrich elastic moduli, or even molecular parameters such as the extended length of a surfactant chain or the area per headgroup (66, 67). Even though size distribution is normally measured by dynamic light scattering, accurate values for liposome suspensions cannot be reliably obtained from light or other scattering methods. Transmission electron microscopy at cryogenic temperature (cryo-TEM), freeze-fracture TEM, and quasi-elastic light scattering are complementary methods to determine mean radii, polydispersity, and size distribution (68).

A powerful method is gel exclusion chromatography, in which the hydrodynamic radius can be accurately determined. Commercial columns can separate liposomes in the size range of 30–300 nm. Other columns can separate SUVs from micellar contaminants. However, these columns with their colloidal polymer particles are prone to clogging; there is a risk of electrostatic interactions of positively charged packing material with the (sometimes) slightly negatively charged medium. In addition, high salt content can cause sample precipitation.

The lamellarity of liposomes is determined by electron microscopy or by spectroscopic techniques. Nuclear magnetic resonance (P-NMR or F-NMR) spectroscopy is applied with or without the addition of a paramagnetic agent that shifts or bleaches the signal of the observed nuclei.

Encapsulation efficiency is commonly measured by encapsulating a hydrophilic marker (i.e., radioactive sugar, ion, fluorescent dye), sometimes using single-molecule detection (69). Electron spin resonance methods allow for the measurement of the internal volume of preformed vesicles. The surface potential is accessible via the zeta potential, and osmolality can be determined by vapor pressure osmometry. Phase transition and phase separations are measured by fluorescence-based pH indicators, NMR, fluorescence methods, Raman spectroscopy, or electron spin resonance (6).

Limiting factors for some applications are physical (70), chemical, and biological liposome stability (71). Especially in clinical applications of liposome preparations, shelf-life stability is of importance, and the lifetime of chromatography and sensor/assay components is also influenced by vesicle instability. Physical stability includes size stability and the ratio of lipid to encapsulated or membrane-bound agent; it is often improved by low temperature storage.

Chemical instability arises primarily from hydrolysis and oxidation of lipid molecules, but also from digestion by degrading enzymes. Hydrolysis removes the fatty acid residues, and oxidation is an influential factor in the presence of unsaturated lipids.

Biological stability of liposomes is one of the most problematic issues and is generally rather limited. For example, depending on the type of lipids present in a

formulation, leakage, aggregation, and binding interactions with other solution constituents are common. Fluorescent markers such as carboxyfluorescein or pyrene have been used to investigate membrane fluidity and permeability in terms of solute leakage from liposomes by optical methods (72).

4. LIPOSOME APPLICATIONS IN ANALYTICAL SCIENCES

The beneficial structural and functional characteristics of liposomes have led to intense and widespread use within the analytical sciences. These characteristics include biocompatibility, a flexible membrane that has affinity to accommodate lipophilic or polymeric molecules, a self-assembly mechanism of membrane formation that is flexible enough to allow for embedding of material within the membrane, and the ability to conjugate a large variety of functional species directly to single lipid molecules (19). Probably most importantly, the secluded internal volume can hold encapsulated material, such as water-soluble marker molecules, which may be released under well-defined conditions.

Several very recent reviews have systematically surveyed the area of analytical chemistry with respect to the role of liposomes in analytical applications (73–75). In the following section, we briefly discuss important applications. Liposomes can either function as analytes, i.e., the target of the analytical process, or can be used as a tool, component, or device in analytical applications. A recent comprehensive review (62) is entirely dedicated to the analytical options available to investigate liposomes (see Sections 3.8 and 5.5).

Interest in liposome technologies and applications for analytical purposes is constantly growing, and four major areas of activity can be identified: liquid chromatography (LC), capillary electrophoresis (CE), immunoassays, and biosensors. Moreover, applications of liposomes in single-molecule spectroscopy, imaging, and cell biology are emerging, and liposome-nanotube networks have proven to be flexible, reconfigurable nanobioreactor systems (26).

4.1. Liposome-Affinity Chromatography and Capillary Electrophoresis

LC and electrophoresis applications are some of the most important methods in the analytical sciences and are central to product analytics in the pharmaceutical and biotechnology industries. To study the interactions between certain analytes and phospholipid membranes, liposomes have been utilized in liquid chromatographic and capillary electromigration techniques. The close structural resemblance of liposomes to natural cell membranes makes them suitable tools to exploit specific interactions. Several reviews summarize the latest progress in these separation sciences (74–76).

There are various well-known ways to immobilize phospholipids and liposomes onto LC columns. Immobilized artificial membranes are composed of propylaminosilica particles onto which phospholipids are covalently linked. Stationary phases containing immobilized liposomes can be prepared sterically or dynamically or by using

hydrophobic ligands, covalent binding, or the avidin-biotin technique. A growing area of interest is the development of column gels with larger pore sizes to allow the immobilization of larger liposomes, as an increase in external liposome surface area leads to a better approximation of natural membranes. Only liposomes containing relatively simple phospholipids have been used for immobilization so far; an extension to more complex compositions is one of the goals of further development.

Vesicles have also been employed as coated stationary phases and pseudostationary phases in various electrokinetic chromatography (EKC) applications (75). Most applications so far have focused on the development of analytical techniques and on the characterization of vesicle phases. In addition, the use of vesicles in EKC has the potential to greatly improve separation; to determine the lipophilic or hydrophobic character of drugs and other analytes; to monitor liposome preparations in the pharmaceutical industry; and to aid the modeling of interactions between biological membranes and membraneophilic material, such as hormones and proteins. Of particular interest is the use of liposomes as models for biological membranes and lipoprotein particles and the potential to modify the vesicle composition to mimic natural biological nanoparticles. Many methods in affinity CE may be applied to the analysis of these interactions for quantitative determination of binding constants and binding sites.

In contrast to applications where liposomes are used as an analytical tool in electrophoresis, which is still uncommon, CE has already become a powerful and established technique for the characterization of vesicle preparations, yielding information about properties such as size, polydispersity, surface charge, permeability, homogeneity, rigidity, and composition (76). However, the mechanism of vesicle electromigration is not yet fully understood and better models are needed.

In summary, there are as yet no firmly established separation techniques utilizing phospholipids and liposomes. Until now, researchers have focused upon developing techniques, characterizing the vesicular phases employed, and evaluating the separation capabilities towards specific types of analytes. Future studies in this challenging field, presumably aided by advances in nanotechnology as well as micro- and nanofluidics, can be expected to successfully identify areas of opportunity for separations on immobilized lipid phases (phospholipids, liposomes, and proteoliposomes).

4.2. Liposome-Based Immunoassays and Biosensors

Two other areas in analytics where liposomes have been very successfully applied are immunoassays and biosensors (77). Liposomes can enclose a large number of fluorescence-marker molecules and amplify the fluorescence signal. One of the success factors in the sensor applications area is the absence of pronounced stability problems. Liposomes remain stable for weeks or months when handled under controlled conditions and stored under nitrogen at low temperature. Moreover, due to the large available membrane surface area, they can accommodate a great number of receptor molecules.

Recent reviews give an overview of applications of liposomes in homogeneous and heterogeneous immunoassays, including methods to conjugate liposomes to antigens or antibodies, and discuss applications of flow-injection liposome immunoassays as well as liposome immunosensors (73, 78). Several typical examples are described in detail therein.

The beneficial amplification properties of liposomes have become apparent in a number of biosensor applications, for example for the determination of pH and oxygen or the detection of bacteria, alkaloids, and other agents (79), and they employ a broad range of detection systems such as microgravimetry and optical, electrochemical, and densitometric methods.

4.3. Liposomes for In Vivo Imaging Applications

Scintigraphic techniques based on various radiolabels, in particular ^{99m}Tc, are useful tools for the noninvasive analysis of the in vivo behavior of liposomes. Using these techniques, quantitative information regarding the in vivo movement, distribution, and fate of the liposomes becomes readily available.

Several techniques for labeling liposomes have been developed, the most promising of which are the afterloading methods. One successful afterloading method for the labeling of liposomes with ^{99m}Tc is based on preformed liposomes loaded with a reducing agent. A lipophilic chelator, hexamethylpropyleneamine oxime (HMPAO), is applied to the label, which is then mixed with the liposomes. After entering the membrane, the lipophilic complex is converted into a hydrophilic form, causing the label to be released into the internal volume. A second afterloading method, which also yields liposomes with high radiochemical purity and good stability, is based on the fixation of the radiolabel by a lipid-chelator conjugate in the lipid bilayer.

Another promising bioimaging concept that does not rely on radionuclides is the use of magnetoliposomes for in vivo magnetic resonance imaging. This method was recently employed to obtain direct evidence of the stealthiness of poly(ethylene) glycol (PEG)-ylated magnetic fluid–loaded liposomes (80).

4.4. Liposome Reactors and Networks

Surfactant nanotube-vesicle networks represent some of the smallest and most structurally flexible devices known for performing controlled chemistry (12). Our group has developed GUV-lipotube networks as a means of transporting reactive material between containers, for initiation and control of chemical reactions in ultrasmall volumes, and as analytical devices with a resolution down to the single-molecule level. Fabrication, functionalization, and analytical capabilities of these systems have been reviewed recently (26); here, we briefly discuss aspects of their application in the area of ultrasmall-scale chemical analysis.

4.4.1. Network formation and modification. A self-organization methodology to control geometry, dimensionality, topology, and functionality in surfactant

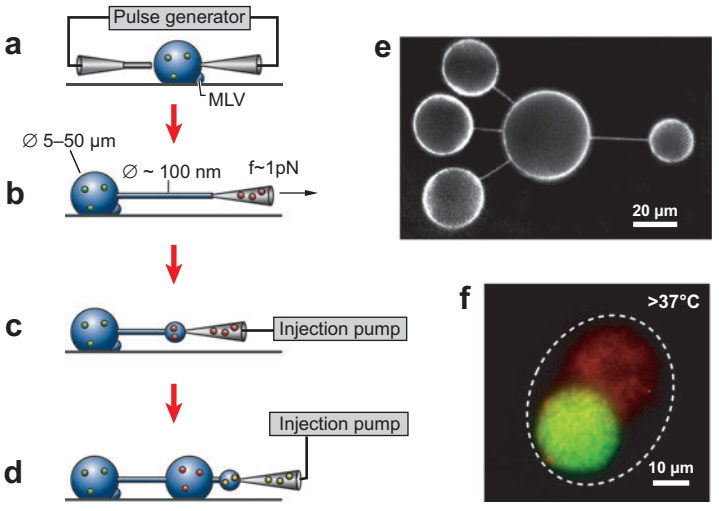

Figure 5

Schematic drawings showing micropipette-assisted formation and internal functionalization of networks of vesicles and nanotubes. (*a*) A micropipette is inserted into a unilamellar vesicle by means of electroinjection. (*b*) The pipette is then pulled away, forming a lipid nanotube. (*c*) A new vesicle is formed by injecting buffer into the nanotube orifice at the tip of the pipette. Repeating this procedure forms networks of vesicles. (*d*) By exchanging the solution in the pipette during the network formation, the interior contents of the vesicles can be differentiated. (*e*) Micrograph of a five-vesicle network with a central container and four daughter containers connected by single lipotubes. The membrane is stained with a fluorescent dye to enhance visibility, and the multilamellar reservoir was removed from the structure. (*f*) Micrograph of a giant unilamellar vesicle with two individually internalized hydrogel compartments as a means of interior functionalization. Each hydrogel compartment encloses fluorescent nanoparticles.

membranes for nanoscale soft-matter device fabrication is the foundation of the network concept (81) (**Figure 5**). Unconventional yet highly effective fabrication routes yield three-dimensional functionalized liposomal nanodevices. In the last few years, our group, as well as other groups, has extended the scope and contributed to the development of novel functionalization, subcompartmentalization, and content control methods, for example by using macromolecular hydrogel–forming (82) or phase-separation systems (83).

4.4.2. Transport phenomena in container networks. In order to take full advantage of lipid NVNs as systems for performing chemical operations, controlled means of material transport through the nanotubes are of central importance. Three fundamental mechanisms have been established so far. The first, Marangoni transport, is based on membrane tension gradients and utilizes the dynamic and fluid character of the bilayer membrane. The second is electrophoretic transport, and the third is based on diffusion, a very effective means of transport over short distances. In **Figure 6*a***, a two-container network for diffusive transport is shown. **Figure 6*b-c*** displays a network with a small particle entrapped in the interconnecting

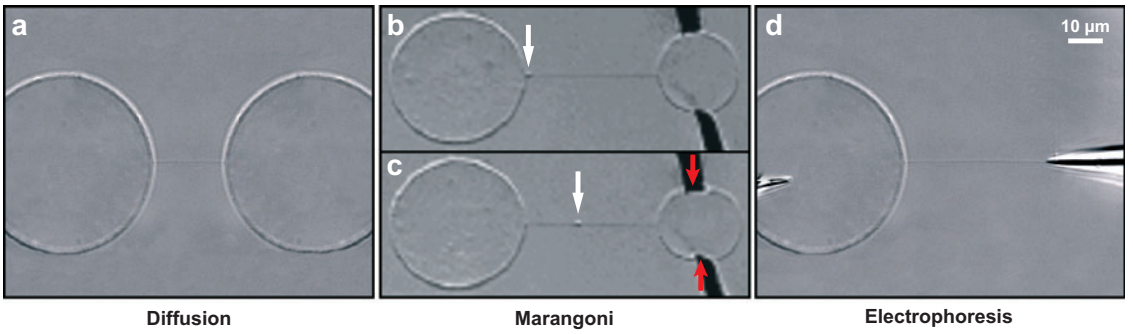

Figure 6

Photomicrographs of giant unilamellar vesicles (GUVs) connected by a lipid nanotube. Three different approaches to performing nanotube-mediated transport are shown. (*a*) Diffusion-driven transport. Using diffusion as a way of transport means that there is no flow of fluid in the system; instead, chemical potentials strive to eliminate concentration gradients across the nanotube. (*b*, *c*) Tension-driven lipid flow induces solvent Marangoni flow inside the nanotube and concomitant intratubular fluid transport. Carbon fibers are used to increase the tension in the vesicle to the right (*red arrows*), thereby establishing a tension gradient across the nanotube. This leads to a lipid flow and a concomitant fluid flow inside the nanotube (illustrated by the particle indicated by the white arrow). (*d*) Electrophoretic transport. A GUV is connected to a pipette via a lipid nanotube. By applying an electric field across the lipid nanotube, charged species such as DNA can be made to move through the nanotube.

tube, utilized to demonstrate the membrane-coupled transport upon the mechanical creation of a tension gradient. The particle, indicated by a white arrow, travels along the tube in the direction of the gradient, as the membrane is moved to reduce tension at its source. **Figure *6d*** shows the basic setup of an electrophoresis experiment, with two electrode-pipettes penetrating, on one side, a vesicle and, on the other side, the opening of a suspended lipid nanotube.

4.4.3. Liposome-nanotube networks mimicking exocytosis. Small, lipid nanotube–connected vesicles contained within larger GUVs have been used to mimic and analyze the membrane mechanics in a cell during exocytosis, a fundamental cellular process (84, 85). In particular, the dynamics of pore opening was successfully studied using this system. **Figure 7** outlines the experimental setup. A microinjection pipette is electro-inserted into the interior of a unilamellar vesicle, then pushed out through the opposing wall and finally pulled back into the interior. Spontaneous formation of a lipid nanotube and of an artificial exocytosis vesicle occurs at the tip of the micropipette. Fluid injection at a constant flow rate results in growth of the newly formed vesicle with a simultaneous shortening of the nanotube until vesicle opening, the final stage of exocytosis, takes place. After releasing the contents of a vesicle in this system, a new vesicle is formed at the pipette tip through the attached nanotube, and the experiment can be repeated in several consecutive cycles. Highly quantitative and temporal measurements of released catechol have been carried out by amperometry using a carbon fiber electrode pair, as shown in **Figure 7**. These

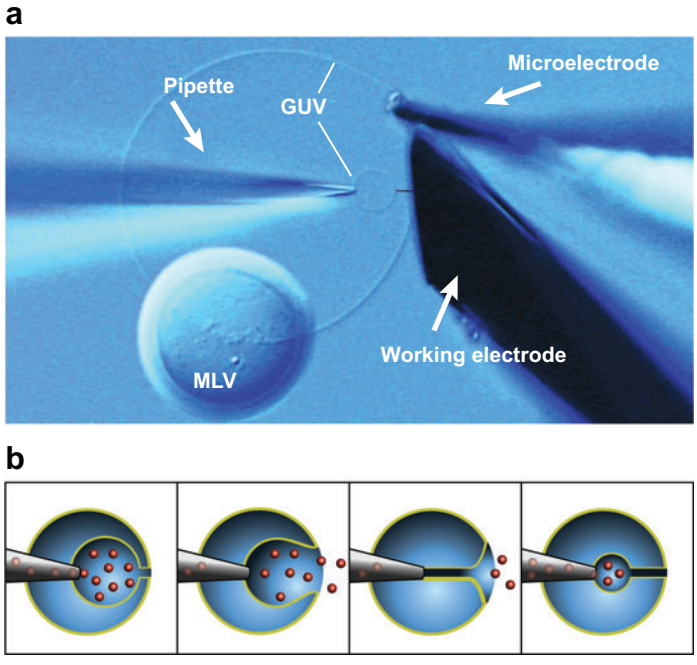

Figure 7

Vesicle-nanotube network used for modeling exocytosis. (*a*) A microphotograph showing a small pipette-attached giant unilamellar vesicle (GUV) inside a larger surface-attached GUV. A small dark line represents the lipid nanotube, which is difficult to observe under a light microscope. The vesicles are linked via a lipid nanotube and a multilamellar vesicle (MLV) is attached as a membrane reservoir. Also shown is a carbon fiber microelectrode used for electroinjection of the pipette into the vesicle and a carbon fiber working electrode used for measuring electrochemically active substances released from the exocytosis event. (*b*) Schematics illustrating the event of nanotube-mediated merging of the two vesicles and subsequent release of material from the small pipette-attached vesicle to the outside of the large vesicle. To repeat the process, a new vesicle is generated at the pipette tip. Reprinted with permission (18).

measurements have provided evidence that membrane mechanics can account for the temporal aspects of the final stage of vesicle opening in exocytosis, and that lipid flow is important in regulating release via the fusion pore.

4.4.4. Chemical reactions in complex nanotube-vesicle networks. For certain enzyme-catalyzed reactions, strong confinement leads to unusual phenomena such as oscillatory behavior in product formation or enhanced enzyme stability (86). Thus, size and dimensionality of a reactor are important properties that can shape the dynamic properties of a chemical reaction. NVNs can be used to conveniently study the dynamics of closely confined chemical reactions (87).

Concentration of reactants, mixing, and relevant time scales are controlled by injection protocols and geometric factors of the networks (e.g., length, diameter of the

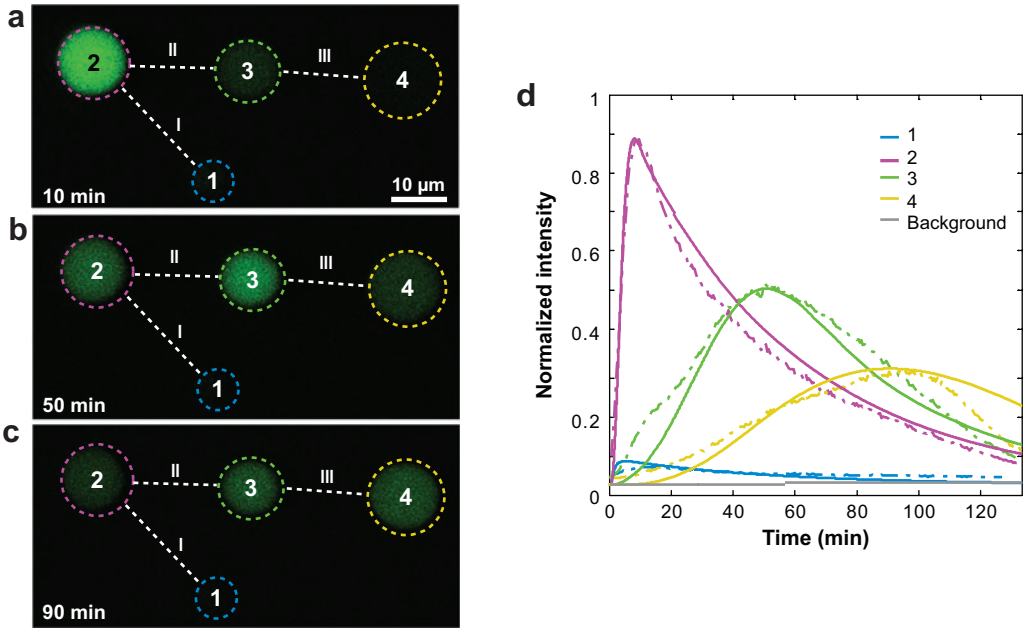

Figure 8

(*a–c*) Fluorescence microscopy images showing product formation from a 3,6,–fluorescein diphosphate–alkaline phosphatase reaction in a linear network. (*d*) The normalized fluorescence intensities of the corresponding measurements for each vesicle in the network versus time. Initially, the enzyme-filled vesicle is vesicle 1; the other vesicles are filled with a nonfluorescent substrate that becomes fluorescent when converted to product. The dashed lines in panel *d* represent the theoretical fit to the experimentally measured product formation. Fluorescence images were digitally edited to improve quality.

nanotubes, diameter of the vesicles, size of nanotube junctions). The system also offers smooth transitions from three-dimensional space (vesicles and large inner-diameter nanotubes) to one-dimensional space (small inner-diameter nanotubes). Container size and geometry, connectivity, and topology can be controlled in the networks, and polymers can be included to create crowded environments (88).

Experiments have been performed to explore enzyme-catalyzed reactions in solitary vesicles as well as in GUVs, and enzyme-catalyzed reaction-diffusion systems were studied theoretically and experimentally in different network geometries (13, 89). It could be shown that the transition from a compact geometry (sphere) to a structured geometry (several spheres connected by nanoconduits) induces an ordinary enzyme-catalyzed reaction to display wave-like or front-propagating properties (**Figure 8**). In the network depicted, an enzyme is introduced into one node and diffuses down its concentration gradient into neighboring nodes through nanotubes. The chemical potential and the directionality of enzyme diffusion can be exactly controlled. The temporal pattern of front propagation as well as the rate of reaction depends upon the network geometry.

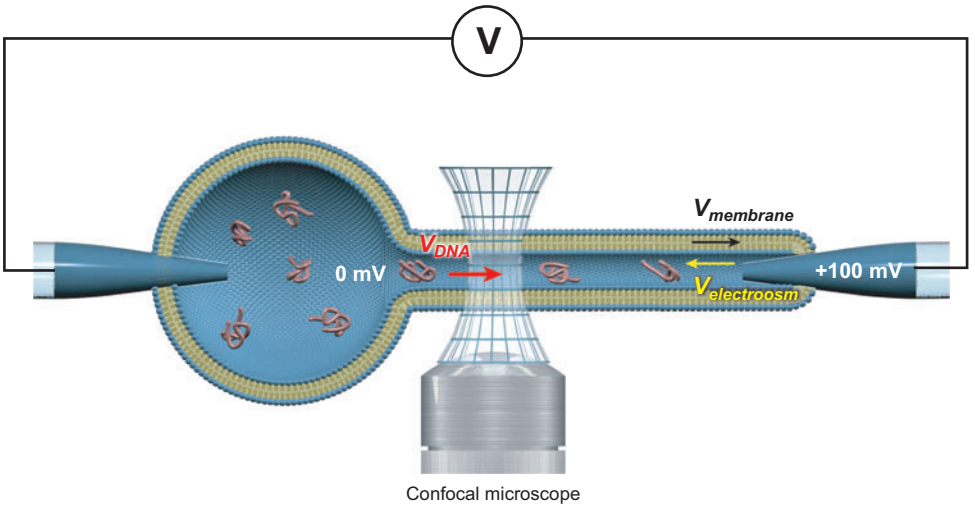

Figure 9

Schematic depicting the geometry of the nanotube-vesicle network system and confocal fluorescence detection used in the single-molecule transport and detection experiments. The nanotube is aligned across the confocal excitation/detection spot of the confocal microscopy setup and an electric potential is applied between the electrodes, with the nanotube-coupled electrode having positive potential. The fluorescence signal from the DNA molecules is detected as the molecules pass the excitation/detection spot. V_{DNA} is the velocity of the DNA molecules, V_{membrane} is the velocity of the membrane, and $V_{\text{electroosm}}$ is the velocity of the electroosmotic flow. Because the latter two cancel each other out, no net liquid flow occurs in the nanotube.

4.4.5. Single-molecule detection in nanotube-vesicle networks. The recent considerable progress in single-molecule detection techniques has opened up a new era of biological and biophysical research. In single-molecule studies, results are not obscured by the ensemble-averaged measurements inherent to classical biochemical investigations (90). The electrophoretic transport technique, in conjunction with ultrasmall electrodes operating in the millivolt range, confocal microscopy, and single-molecule fluorescence detection, has been used by Tokarz et al. to transport and detect large (5.4–166 kbp) double-stranded DNA molecules with a detection efficiency close to unity (32). The size dependence of the DNA conformation inside the nanotube can be elucidated from the fluorescence bursts originating from the individual DNA molecules as they are detected during intratubular transport (**Figure 9**).

Three parameters were extracted from the electropherograms: the maximum fluorescence signal detected from each DNA molecule (peak intensity I_p), the duration of the peak (transit time Δt), and the integrated fluorescence intensity (peak area A_I). The average peak area increases linearly with increasing DNA size, which is consistent with a strong confinement inside the nanotube because it ensures that the entire molecule passes through the detection volume. Therefore, the system is capable of single-file transport and counting of DNA molecules. The average peak intensity is observed to increase linearly with DNA size as well. The transit time

exhibits a linear dependence on DNA size, too, but in this case with an intercept with the ordinate axis at $t_0 = 20.0$ ms. This corresponds to the transit time for a small DNA molecule across the finite detection volume (0.8 μm in diameter), yielding a velocity of 40 μm/s towards the positive electrode. From these parameters it is possible to characterize the electrophoretic system and to interrogate the conformation of the DNA molecules during transport.

A theoretical description of semiflexible polymers, such as DNA, in soft tubes has been proposed (91). It predicts that small polymers will be squeezed by the tube into an elongated shape that can be described by the blob model (92), but if a polymer is large enough it will deform the tube and retain a globule-like conformation. The coil is spherical in shape, but compacted compared with the unconfined polymer coil because of forces arising from the wall of the expanded tube.

5. LIPOSOME TECHNOLOGIES

The progress arising from basic liposome research, namely the ability to (*a*) prepare liposomes of controlled size distribution; (*b*) control liposome permeability; (*c*) achieve high biocompatibility by polymer-modified "stealth" liposomes; (*d*) control temperature and pH sensitivity by an accordingly defined lipid composition; and (*e*) control liposome aggregation and fusion has almost immediately led to technological solutions for industrial applications of liposomes, most notably in a medical and pharmaceutical context (93). Steric stabilization by surface-grafted hydrophilic molecules has beneficially influenced the pharmacokinetics and bioavailability of liposomes and their contents (94). Remote-loading methods have allowed stable liposome encapsulation at high drug-to-lipid ratios with considerable practical impact. Numerous technologies and methodologies are presently known and have been extensively reviewed in a new series of books (9). Novel applications are constantly being developed, following closely each new step in progress in basic lipid research and in analytical and physicochemical sciences of liposomes. In this section, we discuss liposome drug and gene delivery, transdermal delivery, and GUV proteomics applications.

5.1. Liposome-Based Drug and Macromolecule Delivery

Liposomes and other types of vesicles such as virosomes, niosomes, transfersomes, proteosomes, archaeosomes, and phospholipid-alkylresorcinol liposomes (PLARosomes) are commonly applied as substance-delivery tools (95). The liposomal shell can enclose or bind many different classes of substances; liposomes are therefore successfully utilized as therapeutic agents for the delivery of antibacterial, antiviral, and anticancer drugs, as well as hormones, enzymes, and nucleotides (96–98).

The objective of using liposomes as drug carriers is to achieve a high localization of active compounds at disease sites such as tumors or inflammations. In addition, the liposome-encapsulated or -associated drug should become available to the target cells. In this respect, the liposome differs from many other controlled-release strategies, in which drug release occurs either in plasma or directly at the site of

administration. Liposomes have many superior features, particularly biocompatibility, biodegradability, low toxicity, and structural variability. However, certain problems limit manufacture and development (11): Apart from factors that complicate all liposome applications, such as stability and undesirable size distribution, there are several other critical factors of importance in industrial applications, such as low entrapment efficiency, irreproducibility of preparation conditions, sterility in the context of clinical use, upscaling difficulties, and short circulation half-lives. Conventional liposomes in particular, which are prepared from neutral phospholipids, are comparatively unstable structures. They also have low load capabilities and tend to leak the internalized substances through their boundary. Cholesterol is often added to the formulations with the aim to stabilize these structures (99, 100). Stealth liposomes, also known as sterically stabilized or PEG-ylated liposomes, are phospholipid vesicles modified by incorporation of hydrophilic polymers, such as PEG, in the bilayer (80, 96, 101). The resulting coated liposome surface provides stabilization enhancement and additional protection of the encapsulated substances. The circulation half-life in vivo as compared to conventional substance delivery liposomes is enhanced.

Active liposomes are vesicles containing substances that provide a selective, controlled release of the liposome content. When one of two main controlled-release mechanisms is activated in vivo, a change in the liposome structure or in the liposome reactivity promotes release of entrapped material from its interior. One mechanism of targeted release is based on the development of an affinity reaction. Another, the triggered-release mechanism, involves incorporating an element into the liposome; this promotes structural changes in the bilayer membrane as a consequence of an external stimulus, for example pH (102), temperature variation (103), or light irradiation (104). These triggered responses involve loss of membrane integrity and in situ liberation of the entrapped compound. Use of stealth liposomes is essential in both release principles.

Lipoplexes, used in gene delivery, are liposomes containing charged phospholipids. These liposomes provide covalent interactions with oppositely charged macromolecules such as DNA, RNA, and proteins, and allow for internalization of high-molecular-weight substances.

In addition, liposomes currently have widespread applications in the cosmetics industry (for transdermal substance delivery) and as food ingredients (105–107).

5.2. Lipoplexes (Gene Delivery)

Progress in identification of functional genes has stimulated the application of new and modern medical procedures such as gene vaccination and gene therapy (108, 109). Although gene therapy as a treatment for certain severe medical conditions continues to hold great promise, progress in developing effective clinical protocols has been rather slow. Problems such as upscaling, batch reproducibility, and other industrial parameters remain to be addressed (110). Nevertheless, in the last ten years progress has been considerable (111, 112), and a large step forward has been made from the initial cell culture studies performed in the 1990s.

The ideal liposomes for therapeutic gene delivery will encapsulate plasmid DNA with high efficiencies, shield the DNA from enzymatic degradation, and feature a narrow size distribution of ~100 nm or less in diameter to be able to access extravascular regions. Only very recently have there been significant advances in the formulation of plasmid DNA into relatively small, stable plasmid DNA–containing lipidic particles or liposomes that protect plasmid DNA from enzymatic degradation. For example, the addition of polycationic polymers such as polylysine to plasmid DNA, prior to or during the addition of cationic liposomes, results in the formation of sub-100-nm particles (113).

The interaction of cationic liposomes with anionic DNA is based on the electrostatic attraction between the positive charges of the vesicle and the negative charges of the phosphate backbone of the DNA. Upon mixing of the two species, neutralization of the negative charge of the DNA results in condensation of the lipid with the DNA. Still, the structure of the lipid/DNA complex depends on several factors, mainly the chemical composition of both lipid and buffer and the lipid-to-plasmid ratio. Additionally, the formation of lipoplexes depends not only on the method of liposome preparation (114), but also on the procedure used to obtain lipid/DNA complexes. Audouy and Hoekstra (115) and Segura and Shea (116) have reviewed aspects of the successful in vivo use of cationic lipid–based gene delivery systems to enhance our understanding of fundamental and structural parameters that govern transfection efficiency. This includes lipid/DNA complex formation (117), bioavailability and -stability, complex target-membrane interactions and translocation, and gene integration into the nucleus (118).

5.3. Transdermal Delivery Applications

In order to make more pharmaceutics and cosmetics bioavailable via the transdermal route, novel substance-delivery systems are actively being developed. Vesicular systems are steadily gaining importance (119, 120), complementing other dermatological methods such as iontophoresis and microneedles.

Liposomes improve penetration of agents into the skin, but not permeation into the lower epidermal layers. Interestingly, most experts working in the field of active dispersions seem to agree that liposomes do not penetrate as intact vesicles into or permeate through the skin. Liposomes are believed to be deformed and transformed into fragments (121), and are merely considered to be a convenient means of working with phospholipids. Therefore, size, shape, and lamellarity of liposomes are not critically relevant for dermatological and cosmetic formulations.

5.4. Giant Liposome–Based Proteomics

A range of methods has been developed for reconstitution of membrane proteins in unilamellar vesicles (122–124) as well as in GUVs that can be used to build liposome networks (125). Reconstitution in GUVs includes fusion of proteoliposomes generated by detergent-mediated reconstitution or insertion of proteins into preformed GUVs via peptide-induced fusion (125–128). This approach has certain

limitations. Proteins have to be removed from their native environment, usually by application of detergents, where it is often not possible to ensure that all the proteins are reconstituted uniformly in their functional orientation. Furthermore, as the isolation procedures can be quite harsh, protein activity may be reduced or completely lost. The most beneficial approach with respect to these problems is the formation of NVNs directly from a native cell membrane. Simplified access to the cell membrane can be obtained by exploiting the natural reactions of biological cells to particular means of stress. Upon chemical or mechanical stress, cells can form unilamellar micrometer-sized protrusions (plasma membrane–derived vesicles). If there is a local defect in membrane-cytoskeleton attachment, a protrusion is extruded through inflation of the detached membrane by intracellular fluid flow while the overall cell volume stays nearly constant. These cell-derived membrane structures are compatible with the micromanipulation procedures and tools used for manipulation and modification of GUVs, and serve as precursors for NVN formation.

In order to utilize plasma membrane–derived vesicles for hybrid structures combining the cellular plasma membrane with vesicle networks, they must be induced to form. Formation can be triggered in various ways, for instance chemically by applying a combination of dithiothreitol and formaldehyde. Membrane-derived vesicles can be used to build surface-adhered networks of plasma membrane vesicles, with typical vesicle diameters of 5–10 μm and tube lengths of several tens of micrometers, using the electroinjection technique developed for NVN fabrication. Native membrane composition, orientation, and function are fully preserved.

Figure 10*a* is a schematic representation of an adherent cell with a protruding cell-derived vesicle, connected via a nanotube to a daughter vesicle that is still attached to the micropipette. The cell-derived vesicle is used as a membrane source for formation of a daughter vesicle. Organelles and cytoskeletal structures remain largely in the adherent cell, whereas the membrane protrusion most likely encloses low-molecular-weight cytosolic components. The initial protrusion and the subsequently formed daughter vesicle have membrane proteins embedded in the membrane that are properly oriented and fully functional. Using this method, networks of complex connectivity entirely derived from plasma membrane material can be constructed. However, larger structures such as organelles, if present in the mother protrusion, cannot always pass through the nanotubes because of size constraints. The presence of membrane glycoproteins in the network boundary has recently been verified, in this case by a selective dye (WGA-Alexa 488) with affinity to sialic acid and N-acetylglucosaminyl residues (129).

5.5. Analytical Procedures for Quality Control in Technological Applications

The use of liposome-based drug delivery systems, just like small-scale lipid preparations for research purposes, requires determination of relevant chemical and physical characteristics, such a size distribution and composition, encapsulation efficiency, and chemical stability (73, 130).

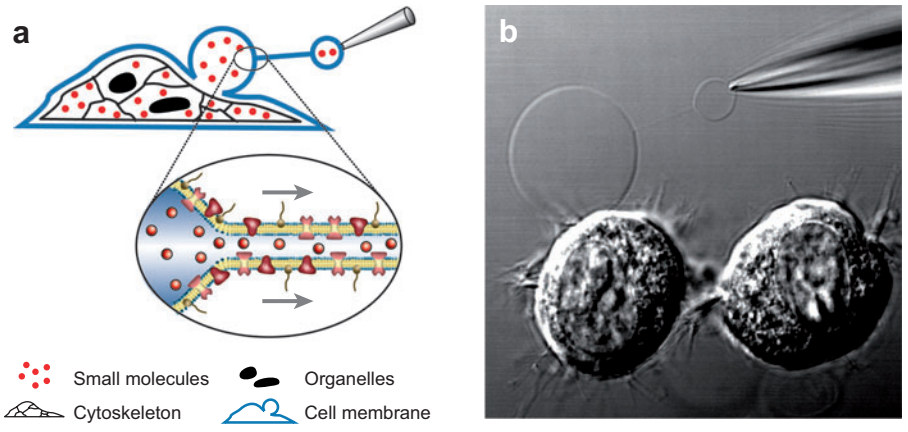

Figure 10

(*a*) Schematic showing the principle of cell–nanotube-vesicle networks. (*b*) Simple two-container network constructed using lipid directly from a cultured NG 108-15 cell.

Today, photon correlation spectroscopy is widely employed to obtain mean particle size and polydispersity of liposomal delivery formulations. Electron paramagnetic resonance imaging methods (131) are commonly employed to investigate the influence of liposome size on transport kinetics and bioavailability of active substances. The most commonly applied method to determine the encapsulation efficiency of loaded liposomes involves removal of the nonencapsulated drug. This is achieved by size exclusion chromatography (132), dialysis, filtration, or centrifugation. Proton NMR spectroscopy is an alternative technique that allows the determination of liposomal content without the need for physical separation of entrapped and nonentrapped material (133).

An important stability-related measurement is the determination of the pH of aqueous liposomal preparations, as nonphysiological pH-values promote lipid hydrolysis, in some cases catalyzed by the loaded drug material. Lipid hydrolysis products can be quantitatively analyzed by high-performance thin-layer chromatography with densitometric detection. The optimal pH for the encapsulated material should ideally be compatible with that for the phospholipids, which is in practice often difficult to achieve. Another important stability parameter, namely oxidation damage to the polyunsaturated fatty acid residues in lipids molecules, can be judged from the presence of conjugated dienes and hydroperoxides, alkyl peroxides, aldehydes, and ketones, which can be detected and quantified by UV/visible spectroscopy, either directly or after derivatization (134, 135). Another method is to determine the change in concentration of unsaturated fatty acids by differential scanning calorimetry (136).

Analytical methods applied to the study of lipoplexes include electron microscopy, calorimetry, and scattering methods to characterize the liposomes. The interaction between DNA and cationic liposomes is determined using the PicoGreen® dye exclusion assay (137). Lipoplexes in liquid formulation are known not to have a very long

shelf-life; aggregation and loss of transfection efficiency occur rather rapidly (138, 139).

6. SUMMARY AND OUTLOOK

Liposomes are currently being successfully applied in many areas of chemistry, medicine, and biotechnology, often in larger batch sizes, as in drug delivery or transdermal drug application at low toxicity levels.

Analytical applications of liposomes are predominant in the areas of liposome-affinity chromatography, sensors, and immunoassays, which largely benefit from signal enhancement due to the liposomes' capacity to hold comparatively large amounts of internalized marker compounds.

Liposomal delivery systems are established as effective vehicles to incorporate active compounds into compartmentalized structures, such as living cells and foods. This technological area has a variety of analytical methods for quality control associated to monitor their features and to determine their active components and ingredients. However, analytical procedures commonly measure only the total drug concentration and do not distinguish between free and encapsulated material. Vesicular phospholipid gels are, in some areas, a potential remedy to the problem, as the amount of noninternalized material in the gel is exceptionally low.

Progress is also apparent in single-molecule spectroscopy and in liposome-based imaging. Emerging technologies are micro- and nanoreactor concepts based on liposome-encapsulated, lipotube-interconnected volumes. NVNs allow for controlled transport of material down to the single-molecule level by diffusion or field-driven transport. Hybrid structures of biological cells and giant liposomes directly derived from cells open pathways to nondestructive mobilization and analysis of membrane proteins.

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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