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Preparation of Vesicles (Liposomes)

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1. WHAT ARE VESICLES (LIPOSOMES)?

1.1. Introduction

Vesicles—more precisely "*normal* vesicles"—are a particular type of polymolecular aggregate (polymolecular assembly) of certain amphipathic molecules, formed in aqueous solution. A vesicle is composed of one or more closed shells (which are usually 4–5 nm thick) that entrap a small volume of the aqueous solution in which the vesicle is formed. Vesicles are often spherical (under osmotically balanced conditions) and can have diameters between about 20 nm and more than 0.1 mm. If an analogous type of aggregate is formed in a water-immiscible, apolar solvent, the aggregate is called a *reversed vesicle* (see Section 4). In the following, the term "vesicle" or "lipid vesicle" always stands for a normal type of vesicle and *not* for a reversed vesicle.

For the sake of simplicity, a so-called *uni*lamellar vesicle is first considered. It is a closed lamella with an aqueous interior. The lamella is composed of amphipathic molecules, compounds that comprise at least two opposing parts, a hydrophilic part (which is soluble in water) and a hydrophobic part (which is not soluble in water but is soluble in an organic solvent that is not miscible with water, in this context also called "oil"). Amphipathic molecules have a "sympathy" as well as an "antipathy" for water. Because of the mixed affinities within the same chemical structure, amphipathic molecules are also called *amphiphiles* (meaning "both loving," water as well as oil). They are *surfactants*, which stands for "surface active agents" and means that they accumulate at the surface of liquids or solids. The accumulation of surfactant molecules on the surface of water (at the water-air interface) leads to a reduction in the surface tension of water, as a result of an alteration of the hydrogen bonds between the interfacial water molecules.

The aqueous solution in which vesicles form is present outside of the vesicles as well as inside. Figure 1 is a schematic representation of a unilamellar spherical vesicle formed by the amphiphile POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) in water at room temperature. The vesicle drawn in Figure 1 is assumed to have an outer diameter of 100 nm. The bilayer has a thickness of only about 4 nm, which corresponds in a first approximation to about two times the length of an extended POPC molecule.

Lipid vesicles in the size range of 0.1 μm (=100 nm) can be visualized by electron microscopy, for example, by the freeze-fracture technique [1, 2], or by cryofixation [2, 3] (see Fig. 2A and B). For vesicles in the micrometer range, light microscopy can be applied [4] (see Fig. 2C).

Based on simple geometric considerations, one can calculate the approximate number of lipid molecules present in a particular defined vesicle. In the case of the unilamellar vesicle shown in Figure 1 (outer diameter 100 nm), about 8.1×10^4 POPC molecules form the shell of one vesicle, and all of these molecules are held together by noncovalent bonds. The single lamella of the giant vesicle shown in Figure 2C (diameter $\sim$ 60 μm) contains about 2.6×10^{10} POPC molecules. The shell is a molecular bilayer with an arrangement of the POPC molecules in such a way that the *hydrophobic* ("water-hating") acyl chains are in the interior of the bilayer and the *hydrophilic* ("water-loving") polar head groups are on the two outer sites of the bilayer, in direct contact with either the trapped water inside the vesicle or with the bulk water in which the vesicle is dispersed. Since the bilayer shell in a sphere is necessarily curved, the number of amphiphiles constituting the inner layer is expected to be smaller than the number of amphiphiles present in the outer layer. For the 100-nm vesicle of Figure 1, the calculated number of POPC molecules is 3.74×10^4 in the inner layer and 4.36×10^4 in the outer layer, assuming a mean head group area of one POPC molecule of 0.72 nm^2 [5]

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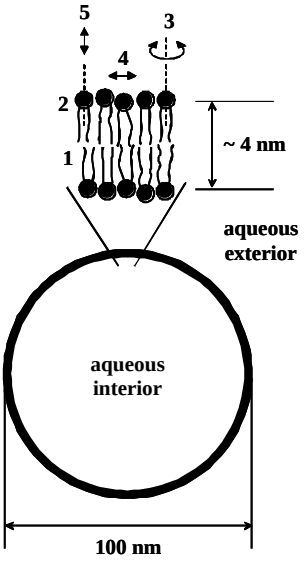

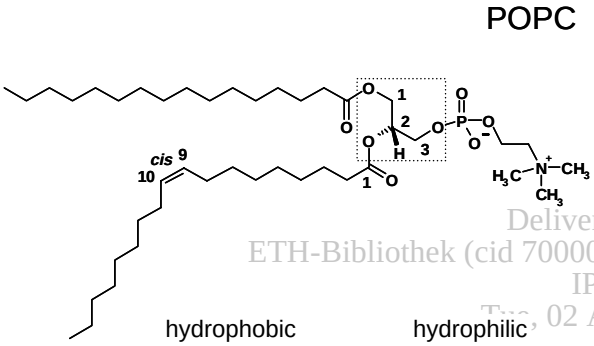

Figure 1. Schematic representation of the cross section through a particular unilamellar, spherical vesicle that has an assumed outer diameter of 100 nm and is formed in water from the surfactant POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine). POPC constituting the single, closed lamellar shell of the vesicle is represented as a filled circle to which two tails are connected. The tails stand for the two hydrophobic (water-insoluble) chains of POPC, and the filled circle symbolizes the hydrophilic (water-soluble) phosphocholine head group. POPC in the vesicle bilayer is present in the fluid-disordered state, above T_m (see Section 2.3). The chemical structure of POPC, which is a naturally occurring glycerophospholipid, is also shown. The glycerol moiety with its stereospecifically numbered carbon atoms is localized inside the dotted rectangle. The important molecular motions above T_m are indicated: (1) conformational transitions in the hydrophobic tails; (2) conformational transitions in the head group; (3) rotational diffusion about the axis perpendicular to the surface of the bilayer; (4) lateral diffusion within the bilayer plane; (5) vertical vibrations, out of the bilayer plane; and (not shown) collective undulations of the membrane. See text for nomenclature details and [392] for a detailed description of the vesicle membrane dynamics.
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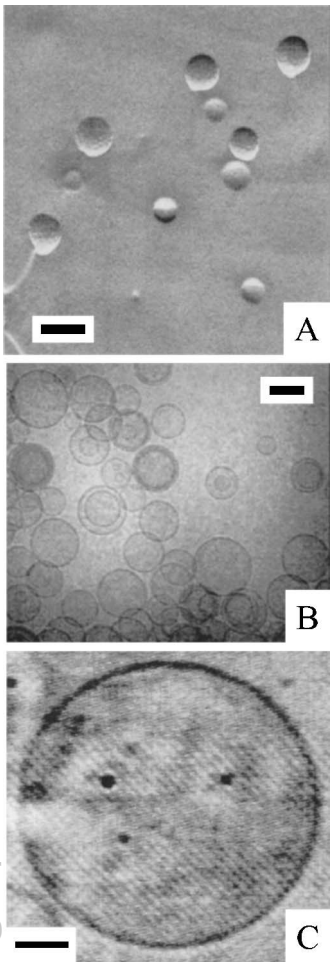

Figure 2. Electron and light microscopic visualization of vesicles prepared from POPC in water at ~ 25 °C. (A and B) Transmission electron micrographs of a suspension containing LUVs (FAT-VET$_{100}$), prepared by the extrusion technique (see Section 3.8 and Fig. 3). The samples were analyzed by the freeze-fracture technique (A) and by cryo-fixation (B). The length of the bars corresponds to 100 nm. (C) Light micrograph (differential interference contrast mode) of a single GUV prepared by the electroformation method (see Section 3.3). Length of the bar: 10 μm. The electron micrographs were taken by M. Müller, E. Wehrli, and N. Berclaz, Service Laboratory for Electron Microscopy, in the Department of Biology at the ETH Zürich. The light micrograph was taken by R. Wick at the Department of Materials Science at the ETH Zürich.

and a bilayer thickness of 3.7 nm [6]. This is certainly a rough estimation, and the real situation in a curved bilayer is always asymmetric with different packing conditions (and mean surfactant head group areas) in the inner and outer layers [7, 8]. Whether vesicles are thermodynamically stable or not (see Sections 2 and 6) depends critically on whether the chains are flexible enough to accommodate these asymmetric constraints [7].

The mean head group area of a surfactant, abbreviated a_0, corresponds to the area that is occupied on average by the polar head group packed within the vesicle's bilayer as a consequence of the actual molecular size and the interaction between the neighboring surfactant molecules. A simple geometric model that, in addition to a_0, takes into account the overall geometric shape of the surfactant as a critical packing parameter (or "shape factor"), p $(=v/(a_0 l_c))$, has been developed [7–10] and successfully applied in a useful simple theory toward an understanding of molecular self-assembling systems at large [11]; v is the volume of the hydrophobic portion of the molecule and l_c is the critical

length of the hydrophobic tails, effectively the maximum extent to which the chains can be stretched out. According to this simple theory, lipid bilayers and vesicles can be prepared if p for a particular amphiphile has a value between 0.5 and 1.0 (conditions under which the surfactants will pack into flexible, curved, or planar bilayers).

1.2. Terminology

There are a large number of amphiphiles that form vesicles. The most intensively investigated are certain lipids present in biological membranes, glycerophospholipids, lipids that contain a glycerol-3-phosphate unit. Actually, the geometric structure of vesicles as *spherulites*—which is the term originally used [12]—containing one (or more) concentric bilayer shell(s) was elaborated for the first time with vesicle preparations made from (mixtures of) naturally occurring glycerophospholipids [12, 13]. This is why it has been proposed to call these aggregates *lipid vesicles* [14] or *liposomes* (actually meaning "fat bodies") [15, 16]. Since the type of aggregate shown in Figures 1 and 2 has not so much to do with a "fat body," it is in principle more appropriate to use the term *lipid vesicle* or just *vesicle* instead of *liposome* [17]. In any case, all of the terms *liposome, lipid vesicle,* and *vesicle* are used here for the same type of polymolecular aggregate. Sometimes, however, one also finds the term *synthetic vesicles* [18, 19], referring to vesicles formed by synthetic, often charged, nonnatural surfactants. Others use the term *vesicle* exclusively for a closed *unilamellar* (not multilamellar) aggregate of amphiphiles [20, 21], such as the one shown schematically in Figure 1. Other names appearing in the literature are *niosomes* (vesicles prepared from nonionic surfactants) [22, 23], *polymer vesicles* or *polymersomes* (vesicles prepared from polymeric surfactants) [24–26], and so on. Table 1 summarizes different terms to describe a particular type of vesicle. Whenever one uses one of these terms, one should specify how it is used and how it is actually defined, to avoid any possible confusion or misunderstanding.

1.3. Nomenclature and Chemical Structures of Vesicle-Forming Glycerophospholipids

Although the basic principles of vesicle formation are for all types of vesicle-forming amphiphiles in the end the same—independently of whether they are charged, neutral, or polymeric—the descriptions in Sections 2 and 3 focus on just one particular group of phospholipids, the so-called phosphatidylcholines (PCs). POPC (see Fig. 1) is a particular PC, namely 1-palmitoyl-2-oleoyl-phosphatidylcholine, more precisely, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (also called 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphorylcholine). Since the nomenclature of glycerophospholipids (such as POPC) is often not so well known to those not working with this particular type of biological molecule, a short overview of the main nomenclature rule is given next. The nomenclature of lipids outlined and used here has been proposed by the International Union of Pure and Applied Chemistry–International Union of Biochemistry (IUPAC-IUB) [27, 28].

POPC is a chiral phospholipid with one chiral center at that carbon atom that is localized in the middle of the glycerol moiety; POPC belongs to the *glycerophospholipids*, the quantitatively most important structural group within the class of phospholipids. Glycerophospholipids have a *glycerol* backbone to which a *phosphate* group is bound through a phosphoric acid ester linkage to one of the glycerol hydroxyl groups. To designate the configuration of this glycerol derivative, the carbon atoms of the glycerol moiety are *numbered stereospecifically* (indicated in the chemical name as prefix *-sn-*). If the glycerol backbone is written in a Fischer projection (see, for example, [29]) in such a way that the three carbon atoms are arranged vertically and the hydroxyl group connected to the central carbon atom is pointing to the left, then the carbon atom on top is C-1, the carbon atom in the middle is C-2 (this is the actual chiral center common to all glycerophospholipids), and the carbon atom at the bottom is C-3. With this convention, the chemical structure of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine is defined. It is the naturally occurring form of the two possible enantiomers. The mirror image of POPC is 2-oleoyl-3-palmitoyl-*sn*-glycero-1-phosphocholine. In the Cahn-Ingold-Prelog nomenclature system (also called the R/S convention) [29], the configuration of C-2 in POPC is R. (Another shorthand description of POPC sometimes used is PamOleGro*P*Cho or PamOlePtdCho [27, 28]; Pam stands for palmitoyl, Ole for oleoyl, Gro for glycerol, *P* for phosphate, Cho for choline, and Ptd for phosphatidyl).

All naturally occurring diacylglycerophospholipids that have a zwitterionic phosphocholine head group are also called 3-*sn*-phosphatidylcholines, or just *phosphatidylcholines*. In the biochemical and biophysical literature, phosphatidylcholine is also called *lecithin* (or L-α-lecithin because of its stereochemical relationship to the naturally occurring L-α-glycerol-phosphate). Therefore, POPC is a lecithin. The name *lecithin* implies that egg yolk (*lekithos* in Greek) contains large amounts of phosphatidylcholines. According to the IUPAC-IUB, the use of the term *lecithin* is permitted but not recommended [27]. It is indeed better to avoid using this term since it may have another meaning in the food technology literature. The International Lecithin and Phospholipid Society (ILPS) of the American Oil Chemists Society (AOCS) defines *lecithin* as a mixture of lipids obtained from animal, vegetable, or microbial sources, which includes PCs but also contains a variety of other substances, such as sphingosylphospholipids, triglycerides, fatty acids, and glycolipids [30].

Egg yolk is one of the cheapest commercial sources for the isolation of phosphatidylcholines; the other one is soybeans. Phosphatidylcholines from egg yolk contain a number of chemically different phosphatidylcholines. All of these PCs have, however, the same glycerol backbone and the same polar head group (phosphocholine). They only differ in the acyl chains esterified with the glycerol hydroxyl groups at C-1 and C-2 (see Table 2) [31].

Many studies on the preparation and characterization of lipid vesicles have been carried out with egg yolk PC. On average, in position C-1 in egg yolk PC is often palmitic acid, and in position C-2, oleic acid [31]. POPC is therefore a representative PC molecule for egg yolk PCs. In contrast to egg yolk PCs, however, POPC has a well-defined chemical

Table 1. List of some of the terms used to describe a particular type of vesicle.

Term	Meaning and use of the term in the literature
Algosome	Vesicle prepared on the basis of 1-*O*-alkylglycerol [485].
Archaesome	Vesicles prepared from archaebacterial, bolaamphiphilic lipids [359, 486].
Bilosome	Vesicle prepared from a particular mixture of non-ionic surfactants (1-monopalmitoyl-glycerol), cholesterol, dihexadecylphosphate (5:4:1 molar ratio), and bile salt (particularly deoxycholate) [487].
Catanionic vesicle	Vesicle prepared from a mixture of a cationic and an anionic surfactant [59, 488].
Cerasome	Vesicle with a silicate framework on its surface [366, 489].
Ethosome	Vesicle that contains in the final preparation a considerable amount of ethanol (prepared by a particular method described in Section 3.20) [229–231].
Fluorosome	SUV containing a fluorescent dye embedded in its bilayer to monitor the entry of molecules into the bilayer [490–492].
Hemosome	Hemoglobin-containing vesicle [493].
Immunoliposome	Vesicle as a drug delivery system that contains on the external surface antibodies or antibody fragments as specific recognition sites for the antigen present on the target cells [69, 373, 494].
Lipid vesicle	Vesicle prepared from amphiphilic lipids [31, 69].
Liposome	Vesicle prepared from amphiphilic lipids [31, 69].
Magnetoliposome	Vesicle containing magnetic nanoparticles (e.g., magnetite Fe_3O_4) [495–498].
Marinosome	Vesicle based on a natural marine lipid extract composed of phospholipids (PCs and phosphatidylethanolamines) containing a high amount (~65%) polyunsaturated aycl chains [499].
Niosome	Vesicle prepared from non-ionic surfactants [23, 500]. In some cases, at room temperature polyhedral niosomes exist, which transform into spherical niosomes upon heating, cholesterol addition, or sonication [501–503].
Novasome	Oligo- or multilamellar vesicle prepared by a particular technology that involves the addition of vesicle-forming surfactants in the liquid state (at high temperature) to an aqueous solution (Section 3.10) [185].
Phospholipid vesicle	Vesicle prepared from (amphiphilic) phospholipids [71].
PLARosome	Phospholipid-alkylresocinol liposome: phospholipid vesicle containing resorcinolic lipids or their derivatives [504].
Polymer vesicle	Vesicle prepared from polymeric amphiphiles, such as di- or triblock copolymers [24, 26].
Polymerized vesicle	Vesicle prepared from polymerizable amphiphiles that were (partially) polymerized after vesicle formation [33, 352, 353, 505].
Polymersome	Vesicle prepared from polymeric amphiphiles, such as di- or triblock copolymers [24, 26].
Proliposomes	A preparation that upon mixing with an aqueous solution results in the formation of vesicles. The preparation contains vesicle-forming amphiphiles and an alcohol (ethanol, glycerol, or propyleneglycol) (see Section 3.19). Dry (ethanol-free) granular preparations of vesicle-forming amphiphiles, which upon hydration lead to vesicle formation, are also called proliposomes (Section 3.19) [506].
Proniosomes	A dry, granular product containing mainly (but not exclusively) non-ionic surfactants which, upon the addition of water, disperses to form MLVs [507].
Reversed vesicle	Inverted vesicle formed in a water-immiscible, apolar solvent in the presence of a small amount of water (Section 4) [508].
Spherulite	Onion-like vesicle prepared with spherulite technology, which involves the use of shear forces (Section 3.11).
Sphingosome	Vesicle prepared on the basis of sphingolipids present in human skin [69, 509].
Stealth liposome	Sterically stabilized vesicle, achieved through the use of co-amphiphiles that have PEG (poly(ethyleneglycol))-containing hydrophilic head groups [510–512]. Stealth liposomes are not so easily detected and removed by the body's immune system (they are long-circulating in the blood). The name stems from an analogy to the American "Stealth bomber" aircraft, which is not easily detected by radar. Alternatively to PEG, polysaccharides have also been used [373].
Synthetic vesicle	Vesicle prepared from synthetic surfactants (surfactant mixtures) that are not present in biological membranes. The surfactants usually have a single hydrophobic tail [17, 488].
Toposome	Vesicle that has a surface that is site-selectively (toposelectively) modified in a stable manner at specific and deliberate locations (e.g., through chemical modifications or chemical functionalizations) [513].
Transfersome	Ultradeformable ethanol-containing mixed lipid/detergent vesicle claimed to transfer water-soluble molecules across human skin (Section 3.28) [275, 276].
Ufasome	Vesicle prepared from unsaturated fatty acid/soap mixtures [330].
Vesicle	General term to describe any type of hollow, surfactant-based aggregate composed of one or more shells. In the biological literature, the term *vesicle* is used for a particular small, membrane-bounded, spherical organelle in the cytoplasm of an eukaryotic cell [97].
Virosome	Vesicle containing viral proteins and viral membranes, reconstituted from viral envelopes, the shells that surround the virus [69, 514–516].

Note: In this chapter, all of the terms listed in the table are called vesicles (or lipid vesicles), independent on the chemical structure of the amphiphiles (surfactants) constituting the vesicle shell(s).

structure. For more basic studies, POPC may be more suited than the egg yolk PC mixture. For applications, however, the cheaper egg yolk PCs may be advantageous.

Although lipid vesicles prepared from egg yolk PCs are similar in many respects to vesicles prepared from POPC, the properties of POPC vesicles at a particular fixed temperature may be very different from those of the chemically related DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) vesicles, for example. The reason for this is outlined in Section 2.

Table 2. Main approximate fatty acid content in egg yolk and soybean PCs (see [31]).

		Relative abundance in phospholipids					
		Egg yolk			Soybeans		
Fatty acid (trivial name)	Abbreviation	Total (%)	at *sn*-1 (%)	at *sn*-2 (%)	Total (%)	at *sn*-1 (%)	at *sn*-2 (%)
Hexadecanoic acid (palmitic acid)	C 16:0	~35	~69	~2	~17	~34	
Octadecanoic acid (stearic acid)	C 18:0	~14	~26	~1		~8	
Cis-9-octadecenoic acid (oleic acid)	C 18:1*c*9	~27	~5	~49	~23	~30	~16
Cis, cis-9,12-octadecadienoic acid (linoleic acid)	18:2*c*9*c*12				~48	~24	~71
Cis, cis-6,9-octadecadienoic acid	18:2*c*6*c*9	~6		~11			
All *cis*-9,12,15-octadecatrienoic acid	18:3*c*9*c*12*c*15				~9	~4	~13
All *cis*-5,8,11,14,17-eicosapentaenoic acid	20:5*c*5*c*8*c*11*c*14*c*17	~4		~7			
All *cis*-4,7,10,13,16,19-docosahexaenoic acid	22:6*c*4*c*7*c*10*c*13*c*16*c*19	~13		~25			

Note: The abbreviation 16:0, for example, indicates that the linear fatty acid has 16 carbon atoms without any double bonds; 18:1*c*9 indicates that the linear fatty acid is composed of 18 carbon atoms with one *cis* double bond in position 9,10 (starting at position 9), where the carboxy C atom is carbon number 1.

1.4. There Are Not Only Unilamellar Vesicles

Vesicles are not only classified by the chemical structure of the molecules constituting the vesicle shell(s) as reported in Table 1, but also according to their size, lamellarity and morphology, and method of preparation (see Table 3). Small unilamellar vesicles (SUVs) have one lamella and diameters of less than about 50 nm. So-called large unilamellar vesicles (LUVs) have one lamella and diameters between about 50 nm and about 500 nm (see Fig. 1 and Fig. 2A and B). Giant vesicles (GVs) can be observed by light microscopy and have diameters of more than about 0.5–1 μm (Fig. 2C). Oligolamellar vesicles (OLVs) have a few and multilamellar vesicles (MLVs) have many concentrically arranged lamellae. Multivesicular vesicles (MVVs) contain nonconcentrically arranged vesicles within a larger vesicle.

As described in detail in Section 3, the preparation of vesicle suspensions generally involves the use of a particular technique, a particular preparation method. Depending on the technique applied, the vesicle suspensions are characterized by a certain degree of homogeneity, a certain mean size and mean lamellarity, and a certain trapped volume. The trapped volume is the aqueous volume that is encapsulated by the lipid vesicles, expressed as microliters of aqueous solution per micromole of lipid (=liters/mol). A trapped volume of 1 μl/μmol means that in a vesicle suspension containing 1 μmol lipid, for example, only 1 μl of the aqueous solution is trapped by the vesicles. The spherical unilamellar POPC vesicle shown in Figure 1 has a trapped volume of about 3 μl/μmol, as calculated based on simple geometric considerations. This means that in a vesicle suspension prepared from 10 mM POPC (=7.6 g/liter), only 30 μl out of 1 ml is trapped by the vesicles (3 volume %). The total lipid-water interfacial area in this vesicle suspension is 4.3×10^3 m^2!

Since, in many cases, the vesicle suspensions prepared by one particular technique are not further characterized with respect to mean size, size distribution, and lamellarity, the vesicles are just named according to the method used. Examples include REVs (reversed-phase evaporation vesicles, vesicles prepared by the so-called reversed-phase evaporation method), VETs (vesicles prepared by the so-called extrusion technique), etc.; see some entries in Tables 1 and 3.

2. VESICLES AND THE LIQUID CRYSTALLINE STATES OF SURFACTANTS

2.1. Introduction

Since in most cases, lipid vesicles can be considered as dispersions of a liquid crystalline state of a vesicle-forming surfactant, it is useful to give a short introduction to some of the liquid crystalline phases of amphiphilic molecules, particularly focusing on the so-called L_α- and L_β- or L'_β-phases, which are considered to be the relevant thermodynamic equilibrium states of most glycerophospholipids under the conditions in which vesicle formation is observed. For a recent excellent general review on surfactant liquid crystals, see [32].

Table 3. List of some of the abbreviations often used for a particular type of ve sicle.

Abbreviation	Meaning of the abbreviation	Characteristics
DRV	Dehydrated-rehydrated vesicle (or dried-reconstituted vesicle)	Vesicle prepared by the dehydration-rehydration method (Section 3.7) [31]
EIV	Ethanol-injected vesicle	Vesicle prepared by the ethanol injection method (Section 3.18)
FATMLV	Vesicle prepared by repeatedly freezing and thawing a MLV suspension	Equilibration and homogenization procedure (Section 3.6) [133]
v FPV	Vesicle prepared with a French press	Unilamellar vesicle or OLV prepared with a French press for vesicle size homogenization (Section 3.8) [72]
GUV	Giant unilamellar vesicle	Unilamellar vesicle with a diameter larger than about 500 nm
GV	Giant vesicle	Vesicle with a diameter larger than about 500 nm
IFV	Interdigitation-fusion vesicle	Vesicle prepared by the interdigitation-fusion method (Section 3.21) [232, 517]
LUV	Large unilamellar vesicle	Unilamellar vesicle with a diameter between about 50 nm and 500 nm
LUVET	Large unilamellar Vesicle prepared by the extrusion technique	Vesicles prepared by the extrusion technique are usually large and mainly unilamellar (Section 3.8)
MLV	Multilamellar vesicle	The vesicle contains several concentrically arranged lamellae osmotically stressed after formation because of an exclusion of solute molecules during their formation [76]
MVL	Multivesicular liposome	A large vesicle that contains internal, nonconcentrically arranged vesicular compartments; also called MVV [211, 212]
MVV	Multivesicular vesicle	A large vesicle that contains internal, nonconcentrically arranged vesicular compartments; also called MVL [210]
OLV	Oligolamellar vesicle	The vesicle contains a few concentrically arranged lamellae
REV	Reversed-phase evaporation vesicle	Vesicle prepared by the reversed-phase evaporation technique (Section 3.14)
RSE vesicle	Rapid solvent exchange vesicle	Vesicle prepared by the rapid solvent exchange method [238]
SPLV	Stable plurilamellar vesicle	Similar to MLV but not osmotically stressed after its formation [76]
SUV	Small (or sonicated) unilamellar vesicle	Unilamellar vesicle with a diameter of less than about 50 nm, as typically obtained by sonicating MLVs (Section 3.5)
ULV	Unilamellar vesicle	Vesicle with only one lamella
UV	Unilamellar vesicle	Vesicle with only one lamella
VET	Vesicle prepared by the extrusion technique	Vesicles prepared by the extrusion technique are usually large and mainly unilamellar (Section 3.8)

Note: The abbreviations are based on the size and morphology, the lamellarity, or method of preparation.

2.2. Lamellar Phase and "Gel Phase"

A liquid crystalline state (also called the "mesophase") of a substance is a state between a pure crystal (characterized by a high order of rigid molecules) and a pure liquid (characterized by rapid molecular motions of disordered molecules) [32]. There are dozens of different liquid crystalline states, all characterized by a different degree of molecular mobility and order. Liquid crystals can be produced either by heating a particular crystalline solid—called a "thermotropic liquid crystal"—or by dissolution of particular substances in a solvent—called a "lyotropic liquid crystal." Many surfactants in water form lyotropic liquid crystalline phases, such as the lamellar phase (L_{α}), the so-called gel phase (L_{β} or L'_{β}), the normal or reversed hexagonal phase (H_I or H_{II}), or one or more of the known cubic phases (Ia3d, Pn3m, Im3m). The type of phase formed depends on the chemical structure of the surfactant used and on the experimental conditions (such as concentration and temperature, or the presence of other compounds).

In the L_{α}-phase (also called the liquid-analogue [33] or liquid-disordered state [34, 35]), the surfactant molecules are arranged in bilayers, frequently extending over large distances (1 μm or more) [32]. The hydrophobic chains are rather disordered, with a lot of *gauche* conformations in the saturated hydrocarbon parts of the hydrophobic chains, making the bilayers fluid, characterized by fast lateral and rotational diffusions of the surfactant molecules, similar to a liquid. Comparable molecular motions are also present in the liquid-disordered state of vesicles. In the case of SUVs prepared from POPC, for example, the lateral diffusion coefficient seems to be on the order of $3–4 \times 10^{-8}$ cm^2/s, as determined between 5 °C and 35 °C [36]. The rotational correlation time is on the order of 10^{-9} to 2×10^{-8} s [37].

The L_{β} (or L'_{β}-)-phase of surfactant molecules (also called solid-analogue [33] or solid-ordered state [34, 35]) closely resembles the L_{α}-phase in the sense that the surfactant molecules are also arranged in bilayers. The viscosity is very high, however. This is a consequence of the rigidity of the individual surfactant molecules which are mostly present with all-*trans* conformations in the saturated hydrocarbon parts of the hydrophobic chains. The motion of the molecules is rather restricted, similar to the molecules in a crystal. To specify the relative arrangement of the lipid molecules, the gel phase may be abbreviated as L'_{β} or L_{β},

depending on whether the alkyl chains are tilted (P'_β) or not titled (L_β) with respect to the normal of the lipid bilayer. If it is not known whether the chains are tilted, L_β is often used as a general abbreviation.

The phase behavior of a number of phosphatidylcholines [38, 39] and a number of other lipids and lipid mixtures [38, 40] has been determined and reviewed.

2.3. Main Phase Transition Temperature T_m of Glycerophospholipids

In the case of conventional glycerophospholipids, either DPPC or POPC, the P'_β-phase is formed at thermodynamic equilibrium at temperatures at least 5–10° below a lipid specific temperature called the main phase transition temperature (or lamellar chain melting temperature) (T_m). T_m is also called the lamellar gel-to-liquid crystalline phase transition temperature and can be determined, for example, as the endothermic peak maximum in heating scans of differential scanning calorimetry (DSC) measurements [41–43]. Above T_m the lipids are in the L_α-phase.

Between the L_β- (or P'_β-) phase and the L_α-phase, an intermediate gel phase, called the ripple phase (abbreviated as P'_β) is often observed at high water content in the case of PCs [44]. This particular lipid phase takes its name from the fact that in freeze-fracture electron micrographs, a "ripple" structure can be seen if the lipid dispersion is rapidly frozen from the particular temperature interval in which the ripple phase is formed [44–47]. The transition from the "gel phase" to the "ripple phase" is called *pretransition*.

With respect to certain practical aspects in the methods for lipid vesicle preparations described in Section 3, the T_m value of the lipid (or the lipid mixtures) used is important to know. In the case of dilute POPC-water systems, for example, T_m is around −3 °C [48, 49]. If the water content is decreased below ~10 wt%, T_m increases above 0 °C, until it reaches a value of 68 °C in the anhydrous system [49].

A list of different T_m values for a number of dilute aqueous phosphatidylcholine systems (MLVs) is given in Table 4. For a more detailed list of T_m values, including other glycerophospholipids, see [38, 40, 50]. Please note that in the case of phospholipids with charged head groups, the T_m values depend on the degree of protonation and may depend considerably on the chemical nature of the counter-ions present [51]. Furthermore, measurements carried out with SUVs give values about 4–5° lower than the T_m values obtained from MLVs [43, 52, 53].

Table 4. Main $P_{\beta'}$-L_α phase transition temperature (T_m) values of dilute aqueous dispersions of certain common bilayer-forming phosphatidylcholines, data taken from [39] and [518] (for soybean PCs).

Phosphatidylcholine	T_m (°C)
DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine), 14:0/14:0	23.6 ± 1.5
DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine), 16:0/16:0	41.3 ± 1.8
DSPC (1,2-distearoyl-*sn*-glycero-3-phosphocholine), 16:0/16:0	54.5 ± 1.5
POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), 16:0/18:1*c*9	−2.5 ± 2.4
SOPC (1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), 18:0/18:1*c*9	6.9 ± 2.9
DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine), 18:1*c*9/18:1*c*9	−18.3 ± 3.6
Egg yolk PCs (see Table 2)	−5.8 ± 6.5
Soybean PCs (see Table 2)	−15 ± 5
Hydrogenated soybean PCs	51–52

Note: 16:0/18:1*c*9, for example, indicates that the linear acyl chain at *sn*-1 has 16 carbon atoms without any double bonds; the linear acyl chain at *sn*-2 has 18 carbon atoms with one *cis* double bond in position 9,10 (see also Table 2).

3. METHODS FOR PREPARING NORMAL VESICLES

3.1. Introduction

The thermodynamic equilibrium state of glycerophospholipids (and many other bilayer-forming amphiphiles) in water (or in a particular aqueous solution) is—probably under most experimental conditions—a stacked bilayer arrangement of the surfactant molecules, either as L_α-phase (above T_m) or as L_β-, P'_β- (or $P_{\beta'}$)-phase (below T_m) in equilibrium with excess aqueous phase (see Section 2).

Upon dispersing in an aqueous solution, vesicles generally form from an amphiphile (or a mixture of amphiphiles) that forms a lamellar L_α-phase at thermodynamic equilibrium. Depending on how the dispersion is actually prepared (in other words, which method or technique is applied), the vesicles formed by the dispersed amphiphiles are either very heterogeneous or rather homogeneous and are mainly small (below about 50 nm), mainly large (between about 50 nm and 500 nm), or mainly very large (above about 500 nm). It all depends on the lipid (or lipid mixture) used, on the aqueous solution, and particularly on the preparation method.

In the following, the principles of some of the best known and widely used methods for the preparation of lipid vesicle suspensions—mainly on a laboratory scale of a few milliliters up to about 100 ml—will be described. For each method, a different vesicle preparation with different typical general characteristics is obtained.

It is important to point out once more that in most of the cases the resulting vesicle suspension is not at thermodynamic equilibrium, but represents only a metastable, kinetically trapped state. The equilibrium phases are L_α, $P_{\beta'}$, L_β or L'_β, as discussed in Section 2. A particular vesicle dispersion prepared is therefore physically (with respect to vesicle size and lamellarity) not indefinitely stable; it may slowly transform into the thermodynamically most stable state (stacked bilayers), as a result of a so-called aging process [54, 55]. This aging may occur either through the fusion of vesicles or because of an exchange of amphiphiles that are not aggregated (free, monomeric surfactant) [55]. This latter process—called Ostwald ripening (in analogy to the corresponding process occurring in emulsion systems)—is expected to be particularly relevant in the case of synthetic short-chain amphiphiles with a high monomer solubility (10^{-8} M). In the case of DPPC (and probably also POPC), the monomer solubility is on the order of 10^{-10} M [56], which means that aging through an Ostwald ripening process is less likely in these cases.

Although often only metastable, vesicle suspensions may be stable for a prolonged period of time, for example, for days, weeks, or months, provided that the vesicle-forming amphiphiles are chemically stable during this period of time [57, 58].

In the presence of other amphiphilic molecules (cosurfactants), the situation may change, particularly if the cosurfactant tends to form micellar aggregates, characterized by a packing parameter $p \sim 1/3$ (relatively large head group cross-sectional area, a_0). In this case mixed micelles may exist at thermodynamic equilibrium (in equilibrium with cosurfactant monomers), if the micelle-forming surfactant is present to a large extent. Such mixed micellar systems are used as a starting solution in the case of the so-called detergent depletion method described in Section 3.27. Furthermore, there are also known cases where there appear to be thermodynamically stable vesicles (particularly composed of mixtures of surfactants) [59, 60] (see Section 6).

The presence of cholesterol (or other sterols)—molecules that are water insoluble and do not form vesicles alone—may also influence the properties of lipid vesicles, depending on the amount of cholesterol present [61, 62]. In the case of DPPC, for example, up to 33 mol% cholesterol, the T_m value of hydrated bilayers changes only slightly [63]. With increasing cholesterol concentration, however, the phase transition temperature is completely eliminated at 50 mol% (1:1 molar ratio of DPPC to cholesterol). The fluidity of the bilayer membrane is thereby changed, resulting in an increase in the fluidity below T_m of the PC and a decrease in fluidity above T_m, a state of the membranes that is intermediate between solid-ordered and liquid-disordered (see Section 2.3). This state is called liquid-ordered [34].

For all of the methods outlined in the following, more detailed descriptions can be found in the original literature cited. Furthermore, most of the generally known methods have already been summarized before—more or less extensively—in review articles or books about lipid vesicles (liposomes) [14, 31, 64–75].

Most of the methods can be roughly divided into two groups:

(i) Methods that are based on the simple swelling of initially dried, preorganized lipids and the mechanical dispersion and mechanical manipulation of the dispersed bilayers (Sections 3.2–3.12).

(ii) Methods that involve the use of (a) a cosolvent in which the lipids are soluble (Sections 3.14–3.26 and 3.30), (b) an additional non-bilayer-forming "helper amphiphile," a coamphiphile (Sections 3.27 and 3.30), or (c) certain ions that influence the initial aggregational state of the lipids (Sections 3.13 and 3.29). All three type of molecules control the assembly process of the bilayer-forming amphiphile in a particular way during the vesicle preparation process, and all three types of molecules may at the end be difficult to remove completely from the final vesicle suspensions.

3.2. MLVs, GUVs, or Myelin Figures Formed by Thin Lipid Film Hydration

One of the easiest ways to prepare a vesicle suspension is to disperse a dried lipid film in an aqueous solution [13, 76, 77]. The vesicle-forming and swellable [78] amphiphile is first dissolved in an organic solvent in which the amphiphile is soluble (usually chloroform). This solution is then placed inside a round-bottomed flask, and the solvent is completely removed by rotatory evaporation under reduced pressure, followed by high-vacuum drying overnight. The remaining amphiphiles form a dry thin film that is oriented in such a way as to separate hydrophilic and hydrophobic regions from each other [31]. If an aqueous solution is added to this film at a temperature above the main phase transition temperature T_m (see Section 2.3), the head groups of the dry lipids become hydrated and hydrated bilayers form. The hydration and swelling process is usually speeded up by gentle or vigorous shaking (using a vortex mixer), thereby dispersing the bilayers in the aqueous solution, resulting in the formation of mainly MLVs, which are very heterogenous with respect to size and lamellarity. Lipid film thickness and extent of shaking have an influence on the properties of the resulting vesicle suspension. The interlamellar repeat distance in equilibrated, completely hydrated PC bilayers above T_m is around 6.5 nm [79, 80]. This means that in a MLV formed from POPC as example, the aqueous space between two neighboring lipid lamellae is about 2.5 nm thick (taking into account a bilayer thickness of 4 nm). On average, a MLV may be composed of up to 10 bilayers [81].

The formation of closed bilayers (vesicles), in contrast to open bilayers, can be easily understood on the simple basis that interactions between the hydrophobic chains of the amphiphiles and the water molecules—as would be the case in open bilayers—are energetically unfavorable and therefore rather unlikely.

The preparation of MLVs by the dispersal of a dried lipid film (also called hand-shaken MLVs [31]) is often a first step in the preparation of more defined vesicle suspensions (see, for example, Sections 3.5 and 3.8). With respect to the equilibration of water-soluble molecules between the bulk aqueous medium and the inner aqueous compartments of MLVs, ionic species may be unevenly distributed [76]. A more even distribution can be achieved by applying freeze/thaw cycles (see Section 3.6).

The experimental conditions under which a dried lipid film is hydrated affect the resulting lipid aggregates obtained. In the case of phospholipid mixtures containing 90 mol% PC and 10 mol% of a negatively charged phospholipid (egg yolk phosphatidylglycerol, bovine brain phosphatidylserine, or bovine heart cardiolipin), the dried lipid film prepared inside a test tube can first be prehydrated at 45 °C with water-saturated nitrogen gas for 15–25 min. Afterward, an aqueous solution containing water-soluble molecules to be entrapped (e.g., 100 mM KCl and 1 mM $CaCl_2$) is gently added, and the tube is sealed under argon and incubated at 37 °C for 10–15 h. During this incubation, the lipid film is completely stripped from the glass surface and forms vesicular aggregates as a kind of white, floating precipitate in the aqueous solution. The analysis of this

precipitate indicated the presence of many mainly unilamellar giant vesicles (not MLVs) with diameters on the order of tens of micrometers up to more than 300 μm [82]. In addition, much smaller vesicles, large multilamellar vesicles as well as myelin figures (see below) and undispersed lipid material, could also be observed [82]. In the case of egg PC alone (no negatively charged phospholipids present), no giant unilamellar vesicles (GUVs) formed under the experimental procedure used [83]. It therefore seems that electrostatic repulsions between the charged head groups facilitate the formation of unilamellar membranes by opposing the intrinsic adhesive force between the membranes [83]. If divalent cations (1–30 mM Ca^{2+} or Mg^{2+}) are present, giant vesicle formation is also observed with zwitterionic phospholipids alone (POPC), with the use of a procedure almost identical to the one just described [84]. Divalent cations seem to promote giant unilamellar vesicle formation in the case of POPC due to a binding of the ions to the free phosphate oxygen of the lipid head group, which is known to alter the mean head group conformation [85] and the fluidity of the lipid bilayer [86], and which makes a zwitterionic PC molecule positively charged overall [87].

In an independent study and with a different experimental procedure, the formation of giant vesicles from PCs (DOPC or soybean PCs) in the presence of Mg^{2+} (<10 mM) has also been observed [87].

The general role of ions (including buffer ions)—as well as dissolved gas molecules—in vesicle formation and in physical chemistry at large is an open question [88] (see Section 6).

The preparation of MLVs by thin lipid film hydration generally involves the use of round-bottomed flasks and gentle shaking. If flat-bottomed flasks are used instead and if additionally the lipid swelling process occurs undisturbed (no shaking) above T_m of the lipids [89] over a period of several hours, the vesicles formed are no longer mainly multilamellar but rather mainly unilamellar, with diameters between 0.5 and 10 μm [90] or even at 300 μm [91]. These are GUVs.

The preparation of GUVs by simple hydration of certain amphiphiles deposited from an organic solution (e.g., a chloroform-methanol mixture) on a flat surface can be considerably improved to yield a higher fraction of unilamellar vesicles by using a roughened flat disk of Teflon (polytetrafluoroethylene) [92]. The vesicles formed (e.g., from DMPC after a slow swelling at a temperature of 30–35 °C) can be harvested by gentle pipette aspiration for further individual investigation and micromanipulation [92, 93]. The sizes of the vesicles thus formed are in the range of 20–40 μm [93, 94].

The slow swelling of dried phosphatidylcholines with the addition of water had already been observed and investigated by light microscopy in the middle and at the end of the nineteenth century by Rudolf Virchow [95] and Otto Lehmann [96]. The elongated, tubular structures that are observed to grow from a deposit of PC molecules (e.g., egg yolk PCs) with the addition of water under undisturbed conditions (no shaking) are called myelin figures, as named by Virchow [95] while making these observations with a (PC-containing) lipid extract from myelin, the isolating lipid sheath surrounding the elongated portion of nerve cells [97]. Myelin figures are structures dozens of micrometers long and a few micrometers thick. In the case of egg yolk PCs and 25 °C, water addition leads to the formation of myelin figures that have a diameter of about 20 μm and a length of several hundred micrometers [98]. Myelin figures are cylindrical, rodlike structures composed of many lamellae formed by the amphiphiles, stacked coaxially around the rod axis, with water between all of the bilayers [98, 99].

The conventional solid surface on which the lipid film is initially dried—glass, as described above—has been replaced by a support made from microcrystals (such as zeolite X with a crystallite size of 400 nm) [100]. Using very dilute mixtures of egg yolk PC and the positively charged amphiphile hexadecyltrimethylammonium bromide, the swelling in water (or 5 mM NaCl) of the very thin dried film deposited on zeolite X led neither to the formation of MLVs nor to GUVs or myelin figures, but to uniform SUVs with diameters around 22 nm [100]. In general, the size distribution can be controlled to some extent by the topography of the surface upon which the phospholipid film is deposited [101].

3.3. GUVs Prepared by the Electroformation Method

If the swelling of PCs (or other phospholipids) in water (or in an aqueous solution with low ionic strength) is carried out in a controlled way by applying an alternating electric field, instead of myelin figures, GUVs form, with diameters typically between 5 and 200 μm, depending on the chemical structure of the lipids used, on the lipid composition, on the swelling medium, and on the external electric field parameters [102–104]. The vesicles are often formed under a light microscope in a specially designed cell on platinum wires that are positioned at a certain fixed distance [103, 104]. This so-called electroformation method has been proved to be rather powerful for the investigation (including microinjection [105, 106]) of individual GUVs of defined size by light microscopy [103–110].

The mechanism of GUV electroformation in an alternating electric field is not fully understood. It is likely, however, that the electroosmotic motion of the water molecules is responsible for a controlled swelling and separation of the bilayers deposited on the platinum wires, leading to the formation of unilamellar vesicles on or close to the metal wires [103, 109]. The individual giant vesicles formed seem to be connected to the platinum wires through thin lipid bilayer tubes (so-called tethers [111, 112]) and possibly myelin-like protrusions [113].

In comparison with other methods for the preparation of giant unilamellar PC vesicles, the electroformation method offers several advantages with respect to reproducibility and later vesicle manipulations [114].

3.4. MLVs Prepared by Hydration of Spray-Dried Lipids

Instead of the preparation of a thin lipid film first, followed by hydration (Sections 3.2 and 3.3), the lipids can first be spray-dried and then hydrated [115]. With respect to the reproducibility and mass production of MLVs, the spray-drying method has several advantageous over the thin-film dispersion method, although it seems that lipids with low T_m

(e.g., egg yolk PCs) cannot be used, because of adhesion to the containers in which the vesicles are prepared [115].

In the spray-drying method, the vesicle-forming amphiphiles are first dissolved in chloroform or methylene chloride (in which, advantageously, mannitol is dispersed) and then spray-dried with a commercial spray-dryer. Vesicle formation is observed after hydration of the spray-dried lipids and vortexing above T_m of the amphiphile used. The presence of the mannitol prevents adhesion of the (saturated) lipids to the reaction containers and leads to a better hydration behavior. (The role of sugars in surfactant assembly at large has hardly been touched on.)

The vesicles prepared are rather heterogeneous with respect to size and lamellarity. One of the homogenization procedures described below may follow the spray-drying and hydration step.

3.5. SUVs (and Possibly LUVs) Prepared by Sonication (and Storage)

The treatment of a MLV suspension with ultrasound at a temperature at least about 5 °C above T_m leads to a homogenization of the vesicles by reducing the size of most of the vesicles to probably the smallest possible diameter (about 20 nm in the case of egg yolk PC mixtures [10, 116] or POPC), due to simple molecular packing considerations. The vesicles thus prepared are unilamellar and called SUVs, an abbreviation that stands for sonicated unilamellar vesicles or small unilamellar vesicles. If the sonication is performed below T_m, structural defects within the bilayers are observed that result in an increased bilayer permeability [117, 118].

So-called probe sonication (the insertion into the vesicle suspension of a metal rod that releases ultrasound waves from the tip of the rod) [119–121] is more efficient than bath sonication [31, 122]. In both cases, however, the sizes of the vesicles (20–50 nm usually) in the sonicated suspension depend on the sonication time [122], on the lipid composition, and on whether other compounds (such as cholesterol) are present [14, 119, 123]. Furthermore, the vesicle suspension may not be free from larger MLVs, and a separation step, for example, size exclusion chromatography (using, for example, sepharose 4B) or high-speed centrifugation, is usually necessary [119, 121, 124]. A centrifugation step may also be used in the case of probe sonication to remove the possibly released metal particles from the tip of the metal rod [125].

The ultrasound used in the treatment of MLVs consists of pressure waves with frequencies around 20 kHz, and the ultrasound propagation gives rise to periodic changes in local pressure and temperature [126]. Therefore, the heat generated during sonication has to be controlled and compensated for by cooling, otherwise the vesicle-forming lipids may undergo a partial chemical degradation [125, 127]. Furthermore, it has been reported that nonvesicular aggregates may be generated as a result of the high-energy input into the system, and the ultrasound treatment may lead to a drastic decrease in the surface tension of sonicated MLV suspensions due to the accelerated movement of lipid molecules form the vesicles to the water-air interface [128]. This process is usually much slower in the case of vesicles that have been prepared under milder conditions, such as by extrusion (Section 3.8) [128].

Based on this latter observation, more uniform and reproducible SUV preparations may be obtained by annealing at elevated temperatures (50 °C in the case of DMPC:1,2-dimyristoyl-*sn*-glycero-3-phosphoserine vesicles), followed by a removal of possibly present large aggregates by centrifugation at $100{,}000 \times g$ (30 min at 37 °C in the particular case) [129].

If carefully prepared SUVs from the saturated DPPC at a concentration in the range of 20–120 mM (in a 20 mM piperazine-N',N''-bis(2-ethanesulfonic acid) buffer, pH 7.4, containing 10 mM NaCl) are kept at a temperature considerably lower than the T_m of DPPC (which is around 41 °C; see Table 4), then the vesicles fuse into uniform LUVs with a diameter of about 70 nm (after 7 days at 4 °C) or 95 nm (after 3–4 weeks at 4 °C) [130]. At higher temperatures (21 °C) but still below T_m, the fusion process is slower [131], whereas at 50 °C (above T_m), no appreciable fusion occurs over a period of at least 5 days [130]. A similar fusion of SUVs below T_m is observed for DSPC, resulting in vesicles of about 60- and 100-nm diameter [132], with an apparently higher fusion rate [130].

The implications of these experimental observations are twofold: (a) LUVs of about 70 or 100 nm can be prepared from saturated PCs by simple storage of SUVs below T_m. (b) SUVs should not be stored below T_m, if one likes to keep the vesicle sizes small; otherwise the vesicles may fuse to form larger vesicles.

In general, SUVs are often prepared in a first step to fuse them in a second step to LUVs by using another methodology, for example, in the case of the cochleate-cylinder method (see Section 3.13) or in the case of the interdigitation-fusion method (see Section 3.21).

3.6. MLVs and MVVs (and Possibly LUVs) Prepared by Repetitive Freezing and Thawing

A MLV suspension—as prepared by the thin-film dispersion method—is put, for example, inside a thick-walled test tube and repetitively (3–6 or, better, 10 times) completely frozen in liquid nitrogen (at −195 °C) and then thawed in a water bath kept at a temperature above T_m. In this way, the vesicle suspension undergoes a sort of equilibration procedure caused by the water crystals and transient rigidification of the lipid molecules. It is a kind of homogenization process, and it has been reported that the population of MVVs may increase [133] and that the amount of very small vesicles tends to decrease [134, 135], depending on the lipid used and on the salt content [136]. It may also be that freeze-thaw cycles lead to a fragmentation of large MLVs into large or small unilamellar vesicles [136, 137]; it all very much depends on the experimental conditions, such as type and concentration of lipid (or lipid mixture) and salt content [136, 138].

A further effect of freezing and thawing of vesicle suspensions is the removal of dissolved gas [88], which has not yet been explored. It is known that removal of dissolved gas strongly affects hydrophobic interactions and colloidal stability, as well as the structure of water [88, 139].

Freeze-thaw cycles have been applied to a series of phospholipid vesicular suspensions, particularly focusing on phospholipids with unsaturated acyl chains (DOPC) and DOPC/DOPA mixtures (DOPA is the abbreviation for the negatively charged 1,2-dioleoly-*sn*-glycero-3-phosphate) [136]. It has been shown that freeze-thaw cycles can be applied successfully to the preparation of mainly LUVs starting from MLVs, depending on the experimental conditions [136]. With the use of a 0.1 M Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer of pH 8.0 containing 0.1 M KCl and 5 mM EDTA, 10 freeze-thaw cycles of aqueous suspensions of DOPC (or POPC) MLVs, for example, led to suspensions that contained about 98% unilamellar vesicles with diameters below 200 nm. The remaining 2% were multilamellar. The vesicles obtained by repetitively freezing and thawing MLVs are often abbreviated as FATMLVs [140]. In the case of DOPC/DOPA (80:20 mol%), almost no MLVs could be detected after the freeze-thaw procedure; the vesicles again had diameters below 200 nm after 10 freeze-thaw cycles [136]. After 50 cycles, the vesicle diameters decreased to below 100 nm [136]. In the case of saturated PCs (DMPC or DPPC), MLV suspensions remained essentially multilamellar, even after 10 freeze-thaw cycles under the conditions used [132].

Fusion of SUVs into larger vesicles upon repetitive freezing and thawing [31, 136, 140, 141] seems to occur efficiently only in pure water (without added salt) [136]. Furthermore, freeze-thaw cycles may not only affect the mean vesicle size and lamellarity, but also the entrapment efficiency for water-soluble molecules [142–145]. In some cases, the trapping efficiency may be dramatically increased [142]. Furthermore, the composition of the aqueous solution may generally influence the freeze-thaw behavior of the vesicles; all of the freeze-thaw effects depend on the experimental conditions [136, 138].

Depending on the phospholipid(s) used, freezing and thawing of SUVs may lead to the formation of giant MLVs with diameters ranging from 10 to 60 μm in the presence of salt (30–500 mM KCl, in the case of phospholipid mixtures containing egg yolk phosphatidylethanolamine and bovine brain phosphatidylserine, 7:3, mol/mol, at pH 7.0, for example) [146].

Although the process is not completely understood, freeze-thaw cycles often precede a further downsizing of the vesicles, for example, by extrusion (Section 3.8).

3.7. MLVs Prepared by Dehydration and Rehydration

The dehydration of a vesicle suspension followed by a controlled rehydration at a temperature above T_m leads to the fusion of small vesicles present in the suspension, resulting finally in the formation mainly of MLVs [147–149]. This procedure is based on the instability of dried SUVs and a lipid lamellae formation (SUV fusion) during the water removal. The resulting vesicle suspensions are referred to as dehydration-rehydration vesicles or dried-reconstituted vesicles (DRVs) [31]. DRVs are often prepared to obtain high entrapment efficiencies (high encapsulation yields) for water-soluble enzymes and other water-soluble molecules. The entrapment efficiency is defined as the percentage amount of the encapsulated molecule in relation to the total amount of molecules present during the vesicle formation and entrapment process.

In a typical, originally described preparation of DRVs [148], SUVs of the appropriate lipid composition are first prepared in distilled water by probe sonication. After centrifugation to remove large vesicles and titanium particles released from the sonicator probe (see Section 3.5), the SUV suspension is mixed with an equal volume of an aqueous solution of the compounds to be trapped. After rapid freezing of the mixture, it is lyophilized with the use of a commercial freeze-dryer at reduced pressure. After freeze-drying, the preparation is rehydrated with a volume of distilled water equivalent to one-tenth of the total volume of the initial SUV suspension. The use of a small volume necessarily has a positive effect on the entrapment yield. The rehydration is usually aided by light vortexing and equilibration for 30 min. Nonentrapped molecules are removed by centrifugation or dialysis after an appropriate dilution of the vesicle preparation [148].

Since DRVs are generally not uniform with respect to size and lamellarity, a second downsizing process often follows a DRV preparation, such as extrusion (Section 3.8) or microfluidization (Section 3.9).

The essential process in the preparation of DRVs is the *disintegration* of the initially prepared SUVs during the dehydration step, which finally results in high entrapment yields of the DRVs. If the experimental conditions during dehydration (during freeze-drying) are such that the vesicles remain largely intact, the rehydration will not lead to exceptionally high entrapment yields of DRVs. One particular such case is the use of lyoprotectants, chemicals that protect the lipid vesicles against drying stress (also called cryoprotectants [150]), such as trehalose [151], glucose [152], sucrose [153], or maltose [154], used typically at concentrations of 10% (wt/wt) [153]. For a particular lyoprotectant, the lyoprotection effect is very much dependent on the type of vesicle-forming lipid used, on the bilayer composition, on the size of the vesicles, on the temperature at which the vesicles are kept before rehydration, and on the freezing rate [153, 155, 156]. Lyoprotectants are thought to prevent the increase in the T_m of the lipid during dehydration (see Section 2.3), to bind water molecules and to interact with the polar head groups of the lipids. In the latter case, water molecules around the head groups would be replaced by the lyoprotectants, thereby protecting the vesicles from aggregation and fusion during the freeze-drying process [157, 158]. In the particular case of POPC, the presence of sucrose or sorbitol results in a value of T_m in an almost dry state of less than 6 °C, in comparison with about 60 °C in the absence of lyoprotectant [159] (see Section 2.3).

Lyoprotectants are only effective in protecting the lipid vesicles if the proper concentrations of the added lyoprotectants are used. If, for example, the potentially effective lyoprotectant sucrose is used at a concentration below the one known to preserve the stability of vesicles during dehydration [157], DRVs can be prepared from SUVs, which are characterized by high entrapment yields and a size no greater than that of the initially prepared SUVs, but also not as large as the size obtained in the absence of sucrose: 90–200 nm in the presence of low sucrose concentrations

(<150 mM, with an optimal entrapment efficiency at a molar ratio of sucrose to lipid of 1), in comparison with about 1–6 μm in the absence of sucrose [160].

3.8. LUVs Prepared by the Extrusion Technique

This method is one of the most popular for the reproducible preparation of rather homogeneous vesicle suspensions containing LUVs, often with a mean diameter of about 70 nm or 100 nm [161–163]. The principle of the method is the following (see Fig. 3): a MLV suspension is passed under moderate pressure repetitively (usually 10 times) at a temperature at least 5 °C above the T_m [164, 165] through the pores of track-etched polycarbonate membranes. The pores are almost cylindrical, and vesicles (unilamellar or multilamellar) that are larger than the mean pore diameter are reduced in size and lamellarity during the passage through the pores, resulting in a final mean vesicle size that corresponds in a first approximation to the mean size of the pores. Vesicles smaller than the pore diameters pass through the pores without significant size change. Usually a MLV suspension is first passed 10 times through polycarbonate membranes with a relatively large mean pore diameter of 400 nm, followed by a passage 10 times through membranes with 200-nm pores and, finally, 100-nm pores. If desired, membranes with 50-nm pores or even 30-nm pores can be used for final extrusions. The corresponding vesicle suspensions obtained are abbreviated as VET_{400}, VET_{200}, VET_{100}, VET_{50}, or VET_{30}. VET stands for "vesicles prepared by the extrusion technique." The subscript indicates the pore size of the membranes used for final filtrations. The mean vesicle diameter of VET_{200} is usually less than 200 nm because of the presence of vesicles considerably smaller than 200 nm in the original vesicle suspension [166, 167], unless the very small vesicles are first eliminated by freeze-thaw cycles [162, 163] (see Section 3.6). If freezing-thawing is used as a homogenization method before extrusion, the resulting vesicles are often abbreviated as FAT-VET (e.g., FAT-VET_{100}: repetitively frozen and thawed vesicles that have been repetitively extruded through polycarbonate membranes with mean pore diameters of 100 nm for final extrusions).

In the early stages of the development of the extrusion technique, freeze-thaw cycles were not performed [166, 168, 169], and, depending on the application, it may even be better to avoid it, for example, in the case of the preparation of vesicles containing entrapped enzymes that may be inactivated during freeze/thaw cycles [75].

VET_{100}- or FAT-VET_{100} suspensions are generally rather monodisperse, containing vesicles with a mean diameter close to 100 nm. The mean vesicle diameter of VET_{50} or VET_{30} is generally larger than 50 nm or 30 nm, respectively. This observation can be explained on the basis of the possible mechanism by which the vesicles transform during the passage through the pores [170–172]: most likely, spherical vesicles change their shape to cylindrical structures within the pores. Because of a velocity profile inside the pores—with a low velocity close to the pore wall and a high velocity toward the center of the pores—the cylinders pearl off ellipsoidal vesicles, which then relax in size and transform into spheres upon leaving the pores. Figure 3 shows a schematic drawing of the extrusion method (Fig. 3A) together with an electron micrograph of a top view of a section of a 100-nm polycarbonate membrane (Fig. 3B).

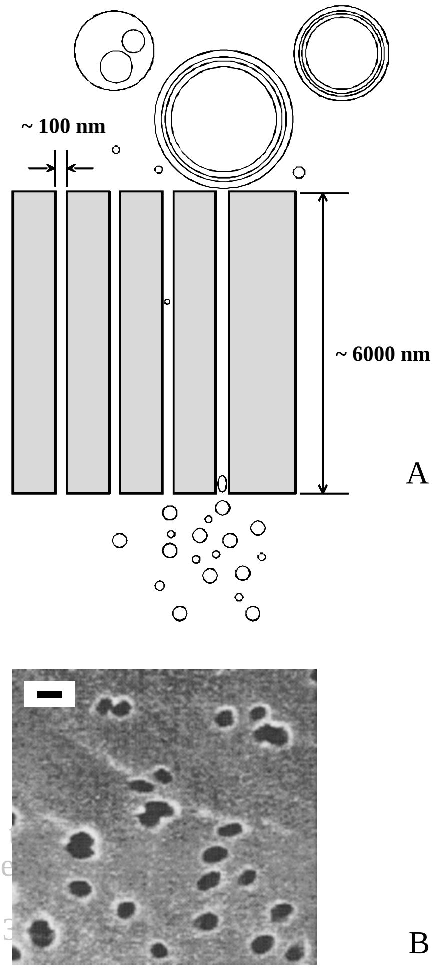

Figure 3. Illustration of the extrusion technique, which is the most popular technique for the reproducible preparation of sub-micrometer-sized unilamellar lipid vesicles from a heterogeneous multilamellar vesicle suspension (see Section 3.8). (A) Schematic representation showing (more or less) cylindrical pores with a mean diameter of 100 nm (as an example), spanning a polycarbonate membrane of about 6 μm thickness; MLVs; a MVV; and LUVs before and LUVs after repetitive extrusions (passages through the pores). (B) Transmission electron micrograph of a commercial polycarbonate membrane (Nucleopore) with pores that have a nominal mean diameter of 100 nm. Length of the bar: 100 nm. The electron micrograph was taken by E. Wehrli, Service Laboratory for Electron Microscopy, at the Department of Biology at the ETH Zürich.

There are several commercially available devices that have been developed for the preparation of extruded vesicle suspensions [173] with volumes between 0.2 ml and 1.0 ml (e.g., LiposoFast [174]), between 1.0 ml and 10 ml (called the Extruder [163]), between 100 ml and 800 ml (the Extruder), or between 200 ml and 1 l (e.g., the Maximator [175]). A comparison between lipid vesicles prepared with the different devices (and the same set of lipids) has been performed [173], and it has been found—with the exception of FAT-VET_{50}—that the vesicles prepared with the LiposoFast were smaller than the vesicles prepared with the Maximator,

most likely because of higher flow rates and pressure drops. In the case of FAT-VET$_{50}$, the vesicles had the same mean diameter (around 50–60 nm) [173].

Since the vesicles prepared by the extrusion techniques are large (LUVs as defined in Table 3) and mainly unilamellar, extruded vesicle suspensions are often abbreviated as LUVET (e.g., LUVET$_{100}$, meaning large unilamellar vesicles prepared by the extrusion technique, using for final extrusions polycarbonate membranes with 100-nm pore diameters) [161]. FAT-VET$_{200}$ and particularly FAT-VET$_{400}$ suspensions usually contain a considerable amount of oligolamellar vesicles [162].

The extrusion technique can be applied as final down sizing and homogenization in principle for any type of vesicle suspension, MLVs, DRVs (Section 3.7), or REVs (Section 3.14), resulting in MLV-VET, DRV-VET, or REV-VET suspensions, respectively.

With a so-called French press cell—a device that does not contain any polycarbonate membranes at all but is often applied for the disintegration of biological cells—a vesicle suspension can be "extruded" through a small orifice at high pressure, resulting in the formation of unilamellar or oligolamellar vesicles with diameters between about 30 and 80 nm, depending on the pressure [31, 66, 71, 176, 177]. These vesicles are referred to as vesicles prepared by the French press (FPVs) [72]. The mechanism of the change in vesicle size in a French press is very different from the actual extrusion technique using polycarbonate membranes [66]. In the French press, shear forces seem to play a particular role [66] (see also Section 3.11).

3.9. LUVs and OLVs Prepared by the Treatment of a MLV Suspension with a Microfluidizer

Vesicle suspensions containing a mixture of mainly large MLVs can be downsized at a temperature above T_m with a high-pressure homogenizer (a microfluidizer) [178–181]. The resulting vesicles usually have a relatively narrow size distribution centered around a value between about 50 and 300 nm [179–181]. The mean size of the vesicles obtained (LUVs and OLVs) depends on the experimental conditions, such as homogenization pressure [182], number of cycles [181–183], ionic strength [181], lipid concentration [183], and lipid composition [182]. Relatively high lipid concentrations can be used, and a large-scale production is possible [181, 184]. To achieve high entrapment yields, a microfluidization step can be performed on vesicles first prepared by the dehydration-rehydration method, which itself results in high entrapment yields [180] (see Section 3.7).

3.10. Preparation of Oligolamellar and Multilamellar Novasomes

Novasomes, also called novasome vesicles [185], can be manufactured from a variety of amphiphiles (including PCs and many nonphospholipid surfactants) by a unique and cheap process on a laboratory scale (a few milliliters) or for industrial bulk applications.

The bilayer-forming amphiphile (including any bilayer-modulating molecules to be added, such as cholesterol) is first heated above the melting temperature (above about 70 °C in the case of POPC; see Section 2.3) to give a liquid. This liquid is then injected at high velocity (10–50 m/s) through small channels with 1–3-mm diameters (or through a needle) with turbulent mixing into excess aqueous phase and immediately cooled to room temperature. The rapid injection leads to the formation of tiny droplets of the amphiphiles that are quickly converted into small aggregates. The subsequent cooling under conditions of continued turbulence then leads to the formation of vesicles (the Novasomes) within milliseconds [185]. Novasomes are OLVs or MLVs with diameters typically between 200 nm and 1 μm, depending on the experimental conditions. The size of the vesicles is controlled by the chemical structure of the amphiphile used and by the hydrodynamic shearing during the fusion of the small aggregates during the cooling process. Furthermore, the diameter of the exit channel plays a role [185].

3.11. Preparation of Multilamellar Spherulites

Spherulites (also called onions or multilamellar spherulites) are relatively uniform, densely packed MLVs that are prepared by shearing of a lyotropic lamellar phase under controlled conditions [186, 187]. The MLVs formed show very little polydispersity and can have mean diameters somewhere within 100 nm and 20 μm [188] or within 200 nm and 50 μm [187], depending on the formulation (i.e., the chemical structure of the amphiphile and possible additives) and the shear rate.

The starting lamellar phase is concentrated and composed of a stack of bilayers of the amphiphiles, separated by water layers. This lamellar phase can be composed of a large number of different amphiphiles or mixtures of amphiphiles [189–192], including soybean PCs [193]. During the shearing, which is performed in specially prepared Couette cells [186], the lamellar phase reorganizes into spherical or polyhedral microdomains [186], which can be dispersed in an excess amount of aqueous phase. The process is called spherulite technology [186, 187, 189]. Spherulites can be considered droplets of dispersed lamellar phase [188, 193], and the technology allows the entrapment of a variety of different water-soluble compounds at high yields, including enzymes [194], nucleic acids [195], and metal ions for nanoparticle synthesis [187, 190].

The formation of spherulites is a direct consequence of global packing constraints [196].

3.12. MLVs Prepared by the Bubble Method

This method is based on the bubbling of an inert gas (nitrogen) over several hours through a coarse dispersion of (initially) nonhydrated lipid particles (containing phospholipids or non-ionic surfactants) and results in vesicle preparations with a clearly non-uniform size distribution [197]. The actual bubbling unit consists of a round-bottomed flask with three necks, one used for a water-cooled reflux, one for a thermometer, and one for the gas supply. A continuous stream of gas bubbles is generated at the bottom of the flask. A coarse vesicle dispersion is first formed usually with a

homogenizer, and the gas bubbling is carried out at a temperature above the T_m of the lipids (actually at 80 °C in the case of a mixture of hydrogenated soybean PCs and dicetylphosphate (10:1, mol:mol). The T_m of hydrogenated soybean PCs is about 51–52 °C (see Table 4). Depending on the experimental conditions, the resulting vesicles have a mean diameter between 200 and 500 nm [197].

3.13. LUVs and GUVs Prepared by the Cochleate Cylinder Method

A so-called cochleate cylinder is a precipitate of a cylindrical, cigar-like phospholipid aggregate that looks like a snail with a spiral shell (its Greek name is *cochleate* [198]).

In the standard procedure, cochleate cylinders form upon stepwise addition of Ca^{2+} to SUVs prepared by bath sonication from phospholipid mixtures containing negatively charged amphiphiles, such as phosphatidylserine (or phosphatidylglycerol) [31, 199].

Cochleate cylinders are rolled bilayers that do not contain an interlamellar aqueous space. The divalent cation brings the negatively charged lipids into close contact and excludes water. A majority of the lipid present must therefore be negatively charged [198], and an excess of Ca^{2+} with respect to the negative charges present has to be added.

Instead of adding Ca^{2+} to the SUVs prepared, the vesicle suspension can also be dialyzed against an aqueous solution that contains the required amount of Ca^{2+} ions [198]. Furthermore, instead of SUVs, unilamellar vesicles prepared by the detergent-depletion method (see Section 3.27), with D-glucopyranoside as a detergent, can be applied [198].

Once cochleate cylinders are prepared, LUVs are formed under the appropriate conditions upon removal of the Ca^{2+} ions by the complexing agent EDTA (ethylenediaminetetraacetate) at a controlled pH of around 7.4. Experimentally, the cochleate cylinders are first separated from the bulk Ca^{2+}-containing solution by centrifugation to form a tight pellet, which is further used and made free from lipid-bound Ca^{2+} by one of three different procedures, with the use of EDTA (direct EDTA addition, rotatory dialysis, or agarose plug diffusion [198]). EDTA binds Ca^{2+}, which leads to an unrolling of the bilayers and then to a formation of (not very uniform) unilamellar vesicles with diameters in the range of below 1 μm to about 10 μm, depending on the experimental conditions used, such as the Ca^{2+} complexation procedure (see above) [198]: while the direct EDTA addition results in vesicle sizes below 1 μm, rotatory dialysis yields vesicles with an average size of 0.5–1 μm, and the agarose plug diffusion method gives vesicles below 1 μm or 5–10 μm, depending on the procedure.

3.14. LUVs, OLVs, MLVs, and SPLVs Prepared by the Reversed-Phase Evaporation Technique

For vesicles prepared by the so-called reversed-phase evaporation technique, cosolvents are used [31, 200]. The bilayer-forming amphiphile (e.g., POPC) is first dissolved in a water-immiscible organic solvent of low boiling point (e.g., diethylether, isopropylether, or mixtures of these ethers with chloroform or methanol). This solution is then mixed with an aqueous solution in which the final vesicles are to be formed. After vortexing and brief sonication in a bath-type sonifier, a reverse emulsion (a so-called water-in-oil emulsion, abbreviated w/o emulsion) is formed in which aqueous droplets are stabilized for a certain period of time by the amphiphiles at the interface between the droplets and the bulk organic solution. The organic solvent is then removed under reduced pressure at a temperature above T_m. During the solvent removal, the reversed emulsion collapses and is transferred into bilayered vesicles. The resulting vesicles (abbreviated REVs) are often unilamellar or oligolamellar vesicles [200], and the size is usually not very uniform, ranging from about 200 nm to 1 μm [200] or even more. Therefore, a homogenization step, such as extrusion (Section 3.8), often follows REV preparation [169, 201].

Vesicles prepared by the reversed-phase evaporation technique are useful for obtaining high encapsulation yields for water-soluble molecules (similar to the DRV preparation described in Section 3.7).

In a modification of the originally developed REV method, the experimental conditions are altered in such a way that the phospholipid/water ratio in the water-in-oil emulsion is changed; and the vesicles formed are no longer mainly uni- or oligolamellar, but mainly MLVs with sizes above 600 nm, thereby allowing higher encapsulation yields for water-soluble compounds [202–204].

With the aim of applying the principles of the REV method to large scale productions, the original method has been modified with the use of the nontoxic and cheap supercritical fluid CO_2 [205]. CO_2 has a critical temperature of 31 °C and a critical pressure of 73.8 bar [205], above which CO_2 exists as liquid. For the preparation of DPPC vesicles, the temperature was set at 60 °C (well above T_m; see Table 4) and the pressure was kept at 200 bar [205]. The resulting suspension contains mainly unilamellar vesicles with diameters between ~0.1 and 1.2 μm and high trapping efficiencies [205].

Another procedure that is rather similar to the originally developed preparation of REVs has been described; it resulted in the formation of stable MLVs (SPLVs, stable plurilamellar vesicles [76], to distinguish them from the MLVs obtained by the thin-film method, as described in Section 3.2). In the case of SPLVs, a dry phospholipid film is first dissolved in diethyl ether. After the addition of a buffer solution, the two-phase mixture formed is emulsified with a sonication bath, during which a gentle stream of nitrogen gas is passed over the mixture, until the ether is almost completely evaporated. The resulting mass ("cake") is resuspended in buffer solution, followed by a pelleting by centrifugation and washing with buffer [76].

3.15. LUVs Prepared From W/O- and W/O/W-Emulsions

This multistep procedure for the formation of vesicles [206, 207] is very similar to the reversed-phase evaporation method (Section 3.14). It starts with the preparation of a w/o emulsion containing the water-soluble molecules to be entrapped in the final vesicle preparation. The emulsion is formed from soybean PCs, cholesterol, and benzene

(or methylene chloride). This w/o emulsion is then emulsified with another aqueous phase to form a water-in-oil-in-water emulsion (w/o/w emulsion), paralleled by evaporation of the solvent molecules, which is speeded up by mechanical agitation and a stream of nitrogen gas [207]. The mainly unilamellar vesicles thus obtained have a reported mean diameter of about 400 nm [207]. The size of the vesicles (apparently always between 50 and 500 nm) depends on the experimental conditions, such as the intensity of the second emulsification step (to form the w/o/w emulsion) [206] and the chemical nature of the organic solvent used [207]. With increasing boiling point of the solvent, the mean size of the vesicles tends to decrease [207].

3.16. MLVs, GUVs, and MVVs Prepared by the Solvent-Spherule (W/O/W Emulsion) Method or DepoFoam Technology

This particular method is called the solvent-spherule method by its inventors [208], since solvent spherules (surfactant-stabilized o/w emulsion droplets) are the starting system from which the oil (the solvent) is removed in a particular way, resulting in the formation of micrometer-sized MLVs [208]. The method is conceptually similar to the methods described in Sections 3.14 and 3.15.

The vesicle-forming amphiphiles (necessarily containing a small amount of negatively charged surfactants) are first dissolved in a 1:1 (v/v) mixture of chloroform and diethyl ether. This lipid solution is placed under the surface of an aqueous solution (5% glucose) with a glass capillary pipette. After agitation for about 1 min, surfactant-stabilized solvent spherules (droplets) form in the aqueous phase (w/o/w emulsion). The volatile solvent mixture is then removed in a particular way by careful dropwise addition to a flask to which a stream of nitrogen gas is added. The flask is kept at 37 °C and gently swirled. The average size of the MLVs formed is affected by the lipid concentration and the size of the lipid spherules formed by mechanical agitation.

In a modification of the method, which involves the additional use of triolein and a certain complex way of mixing, evaporation and centrifugation steps, GUVs in the 5–10-μm size range can be prepared, depending on the experimental conditions (strength and duration of the initial vortexing to form the o/w emulsion droplets) [31, 209].

In a further variation of the method, multivesicular vesicles (MVVs), with sizes between about 5 and 30 μm, can be prepared by a similar stepwise procedure (which involves pelleting by centrifugation), using a particular lipid mixture and, as solvents chloroform, diethyl ether and triolein [31, 210]. The technology for the preparation of this type of MVV is known as DepoFoam technology [211].

DepoFoam technology is a double emulsification process that has been developed based on initial observations [210] of the formation of MVVs. The vesicles formed are multivesicular vesicles (also called multivesicular liposomes), micrometer-sized vesicles that contain internal, nonconcentrically arranged compartments. The internal packing is comparable to the way gas bubbles are packed in a gas-liquid foam [211, 212]. The contacts between the compartments exhibit a tetrahedral coordination.

The first step in the formation of DepoFoam MVVs is the preparation of a w/o emulsion by dissolving a mixture of vesicle-forming amphiphiles (e.g., phospholipids) containing at least one neutral lipid (e.g., triolein) in one or more volatile, water-immiscible organic solvents (e.g., chloroform) and the addition of an aqueous solution containing water-soluble molecules to be entrapped in the final vesicles formed. In a second step, the w/o emulsion is mixed with a second aqueous solution, followed by mechanical mixing to yield solvent spherules suspended in the second aqueous phase (a w/o/w emulsion [212]). The organic solvent is then removed from the spherules by evaporation at reduced pressure or by passing a stream of nitrogen gas over or through the suspension [211]. The properties of the MVVs formed (such as captured volume) depend on the experimental conditions, such as molar fraction of the neutral lipid [210].

The presence of a neutral lipid like triolein is important since it allows a particular type of compartmentation [211]. Triolein acts as a hydrophobic space filler at bilayer intersection points and stabilizes these junctions. Furthermore, triolein is also present as oil droplets dispersed in the encapsulated aqueous space [211].

3.17. GVs Prepared from an Organic/Aqueous Two-Phase System

Giant vesicles—claimed to be unilamellar [213]—can be prepared rapidly by first dissolving the vesicle-forming amphiphiles in a chloroform-methanol solution in a round-bottomed flask, and then adding carefully along the flask walls an aqueous solution (water or buffer) that may also contain water-soluble molecules to be entrapped. After removal of the organic solvent in a rotatory evaporator under reduced pressure and at elevated temperature (40 °C), a suspension is obtained that contains many giant vesicles with diameters up to 50 μm, which can be removed for further investigation [213, 214].

3.18. SUVs and LUVs Prepared by the Ethanol Injection Method

This method is a rather simple one that uses ethanol as a cosolvent and does not require homogenization devices [31, 215, 216]. The bilayer-forming amphiphile (e.g., POPC or another PC) is first dissolved at a certain concentration in ethanol (or methanol [217]). A transparent solution is obtained. If a small amount of this ethanolic (or methanolic) lipid solution is now rapidly added at a temperature above the T_m to an aqueous solution, the formation of vesicles is observed. The reason for vesicle formation is the miscibility of ethanol (or methanol) with water and the migration of alcohol molecules—originally surrounding the lipid molecules—away from the lipids into the bulk solution. Depending on the experimental conditions (e.g., lipid concentration in the alcohol, speed of adding the alcoholic lipid solution, final concentration in the aqueous suspension, and stirring rate), the vesicles formed are more or less homogeneous with respect to size and lamellarity. The most important factor seems to be the concentration of the lipid in the alcohol injected into the buffer solution.

Vesicles prepared from DMPC (at 35 °C) or DPPC (at 55 °C), for example, were mainly unilamellar vesicles with diameters between about 30 nm and about 120 nm if the PC concentration in the ethanolic solution was varied between about 3 mM and about 40 mM (with an ethanol concentration in the buffer after the addition of 2.5–7.5%, v/v) [210]. Similar results were obtained with a mixture of soybean phospholipids [218].

In the case of POPC injected as a methanolic solution, POPC concentrations in methanol up to 25 mM result in vesicles with diameters between 40 and 70 nm [217].

The ethanol (or methanol) present in the final vesicle suspension may be removed almost completely by dialysis if required [216].

One of the limitations of the method is the limited solubility of the phospholipids in the alcohol (e.g., 40 mM soybean PC in ethanol [218]). This necessarily results in relatively dilute vesicle suspensions (a few millimolar). Furthermore, whereas entrapment yields for ethanol-soluble substances are high [218], the encapsulation efficiency for water-soluble compounds is low [218], unless a sophisticated cross-flow injection technique is used [219], which also allows an upscaling to at least several 100 ml [220].

The ethanol injection method has also been combined with high-speed homogenization [221, 222], thereby allowing the preparation of uniform vesicles with a diameter of 170–200 nm on an industrial scale [222].

In a further modification of the method, an ethanolic lipid solution is not injected into an aqueous solution, but water is poured into a concentrated lipid-ethanol solution, followed by the removal of the ethanol in an evaporator and the addition of water [223]. The particular lipid mixture contained a defined amount of soybean PC, cholesterol, β-sitosterol β-D-glucoside, and oleic acid, and the resulting polydisperse vesicles had mean diameters between about 150 nm and 1.3 μm, depending on the experimental conditions [223].

3.19. ULVs and OLVs Prepared by the Proliposome Method

This method is related to the ethanol injection method described in Section 3.18 in the sense that ethanol is also used as a cosolvent. An initial mixture (called proliposome mixture [224]) containing vesicle-forming amphiphiles (egg PCs [225], soybean PCs, or hydrogenated soybean PCs [224]), ethanol (or glycerol or propyleneglycol), and water is converted into vesicles by a dilution step [224–226]. It is a method that seems to be particularly applicable to the bulk production of lipid vesicles.

The vesicles (liposomes) only form after water addition since the proliposome mixture does not contain enough water to trigger vesicle formation. The proliposome mixture is probably built up of extended hydrated lipid bilayers that are separated by an ethanol-rich hydrophilic medium [224].

The encapsulation efficiencies for water-soluble or bilayer soluble compounds are rather high [224, 226]. The vesicles formed by the proliposome method may be predominantly unilamellar or oligolamellar vesicles with a broad size distribution between 20 nm and about 400 nm [218]. The mean size and lamellarity of the vesicles obtained seem, however, to depend on the actual experimental conditions. In the case of vesicles formed from a "proliposome mixture" containing egg yolk PC:ethanol:water at a ratio of 100:80:20 (w/w/w), the mean size varied between 100 nm and 1.2 μm, and most vesicles were oligo- or multilamellar [225].

In a modification of the actual proliposome method, a large-scale production of lipid vesicles could be achieved by diluting about 10–20 times—with an aqueous phase using a dynamic mixing device above T_m of the lipids—a water-miscible solvent mixture composed of *N*-methylpyrrolidone and *tert*-butyl alcohol (1:4, v/v) containing POPC:1,2-dioleoyl-*sn*-glycero-3-phosphoserine (7:3, mol/mol) and a drug to be entrapped [227]. The resulting mean size of the mainly unilamellar vesicles formed after the dilution varied with the composition of the aqueous phase between about 50 and 150 nm [227].

Please note that the term "proliposome" has also been used for particular dry granular phospholipid preparations which, upon dispersion in water, result in the formation of MLVs [31, 228]. These preparations do not contain ethanol at all.

3.20. Preparation of Multilamellar Ethosomes

With a third method in which ethanol plays an important role, so-called ethosomes can be prepared. Ethosomes are lipid vesicles that contain in the final preparation a considerable amount of ethanol. Ethosomes are prepared by first dissolving a phospholipid (such as soybean PC mixtures) in ethanol. Water is then slowly added in a fine stream with constant mixing to a specially prepared container, followed by an equilibration of the system at 30 °C. The final vesicle preparation contains 2% (w/w) soybean PCs and 30% (w/w) ethanol and seems to be particularly useful in pharmaceutical applications for drug transport across the skin (transdermal drug delivery) [229–231]. The vesicles formed are mainly MLVs and are apparently relatively monodisperse, with a mean reported diameter of about 150 nm [229]. The size of the vesicles seems to increase with decreasing ethanol concentration [229]. At 20% (w/w) ethanol, the mean ethosome diameter is around 190 nm; at 45% (w/w) it is around 100 nm [229]. The mean vesicle size is also dependent on the lipid concentration. At 30% (w/w) ethanol, the vesicles' mean diameter varies from about 120 nm to about 250 nm on going from 0.5% (w/w) to 4% (w/w) soybean PCs [229].

In transdermal drug delivery applications, the ethanol present in an ethosome preparation may act as a skin permeation enhancer because of the interaction with the lipid layers of the skin's horny layer (stratum corneum), thereby allowing the passage of drugs across the skin.

3.21. GVs Prepared by the Interdigitation-Fusion Method

A fourth method that uses ethanol is based on the fact that under certain conditions certain glycerophospholipids are known to form bilayers that have interpenetrated (interdigitated) hydrophobic chains. This means that the methyl groups localized at the end of the hydrophobic chains of a monolayer in a bilayer are in contact with the methylene groups of the hydrophobic chains of the other monolayer

and vice versa. Such interdigitated structures (interpenetrated lamellar sheets) are formed upon the addition of ethanol to SUVs prepared from specific saturated PCs (e.g., DPPC), at a temperature below T_m. When the temperature is increased above T_m, the interdigitated lamellar sheets fuse and transform into vesicles that are mainly unilamellar and have diameters above 1 μm (called IFVs, vesicles prepared by the interdigitated fusion method [232]).

The trapped volume of the IFVs depends on the chemical structure of the lipid, the concentration of ethanol used to induce interdigitation fusion, and the size of the precursor SUVs [232].

3.22. ULVs and MLVs Prepared by the Coacervation Technique

The starting system in this method of vesicle preparation is a mixture of naturally occurring egg yolk phospholipids (including about 81% PCs), an alcohol in which the phospholipids are soluble (methanol, ethanol, *n*-propanol, or 2-propanol), and water [233]. Under appropriate conditions, phase separation is observed in this three-component system, corresponding to a region in the phase diagram that is related to a so-called coacervation. *Coacervate* is an old term used in colloid chemistry [234]. It refers to a system in which an amphiphile-rich aqueous phase is in equilibrium with an amphiphile-poor aqueous phase. It seems that coacervates actually correspond to the so-called sponge phase (the L_3-phase), a disordered version of the bilayered bicontinuous cubic phase [235].

After the initial coacervation system is dialyzed against water, vesicles form that seem to be either relatively homogeneous and unilamellar vesicles (in the case of methanol or propanol) or mainly MLVs (in the case of ethanol), ranging in size from about 100 nm to 1 μm, depending on the experimental conditions used [233].

3.23. Vesicles Prepared by the Supercritical Liposome Method

With the use of a specially designed, technically rather complex apparatus, vesicles with an average size of about 200 nm can be prepared by mixing at low pressure an aqueous solution with supercritical CO_2 (kept at high pressure (25 MPa) and 60 °C) containing the vesicle-forming amphiphile (POPC:cholesterol, 7:3, mol/mol). The mean vesicle sizes vary with the experimental conditions, such as geometric dimensions of the important parts of the apparatus [236].

3.24. Vesicles Prepared by the Ether Injection Method

Vesicles can be prepared by slowly injecting (at 0.2 ml/ml) a diethyl ether/phospholipid solution into an aqueous phase that has been warmed to a temperature (55 °C) above the boiling point of diethyl ether [31, 237]. The diethyl ether vaporizes upon contact with the aqueous phase, and the dispersed lipids form preferentially (but not entirely) unilamellar vesicles. These vesicles can then be sized down by extrusion (see Section 3.8) or simple Millipore filtration. In the latter case, the reported vesicle diameters are in the range of 100 to 300 nm [237].

3.25. OLVs Prepared by the Rapid Solvent Exchange Method

The rapid solvent exchange (RSE) method has been specifically designed for the preparation of vesicles from phospholipid-cholesterol mixtures containing high amounts of cholesterol [238]. The method is based on a rapid transfer of the vesicle-forming lipids from an organic solvent to an aqueous buffer solution in which the vesicles are meant to be formed. This rapid solvent exchange avoids the transient formation of solid lipid mixtures, which often demix (phase separate) and result in inhomogeneous vesicle preparations.

The lipids and the membrane soluble additives (i.e., cholesterol) are first dissolved in a solvent that is not miscible with water (e.g., chloroform or methylene chloride). This lipid solution is then added to an aqueous solution above the T_m of the lipids in a particular manner at reduced pressure with a specially designed apparatus in such a way that the solvent is rapidly (within 1 min) and almost completely evaporated, as a result of pressure changes during the injection process.

Vesicles prepared by the RSE strategy from POPC, for example, are oligolamellar with an expected lamellar repeat distance of 6.5 nm (see Section 3.2) and an entrapped volume of about 4.5 liters/mol [238].

3.26. Vesicles Prepared from an Initial O/W Emulsion

In this simple method [239], an o/w emulsion is first formed by bath sonication from a vesicle-forming amphiphile (egg yolk PC and cholesterol), an aqueous solution (containing the water-soluble molecules to be entrapped), and *n*-decane. This o/w emulsion is then transferred to a second aqueous solution, which gives two separated phases, an upper organic phase and a lower aqueous phase. The two-phase system is centrifuged at 3500 $\times$ *g* for 10 min, resulting in the movement of the amphiphiles from the organic phase into the aqueous phase and, as a consequence, the formation of vesicles in the lower aqueous phase, which is separated from the upper phase. The resulting vesicles, which may contain small amounts of *n*-decane in the bilayer, have a diameter in the range of 50–200 nm and are characterized by relatively high encapsulation yields [239].

3.27. ULVs Prepared by the Detergent-Depletion Method

If a bilayer-forming lipid is mixed in an aqueous solution with a micelle-forming surfactant (often called detergent, from the Latin word *detergere*, meaning to wipe off or to clean) under such conditions that the detergent molecules "dominate," mixed detergent/lipid micelles are formed [240–243]. These aggregates are composed of bilayer-forming amphiphiles as well as micelle-forming amphiphiles and are disc-like, sheet-like, or cylindrical structures.

In the detergent-depletion method (also called the detergent dialysis or detergent removal method), the starting system from which the vesicles are formed is mixed detergent/lipid micelles. The micelle-forming detergent molecules (with their large a_0; see Section 1.1) are expected to be distributed in the mixed-micelle aggregate in such a way that they particularly occupy the highly curved edges of the aggregates [31]. The micelle-forming surfactant is also present in a relatively high amount in the bulk phase as nonaggregated, monomeric detergent, at a concentration corresponding in a first approximation to a value a bit lower than the detergent's cmc, the critical concentration for micelle formation determined separately under comparable conditions.

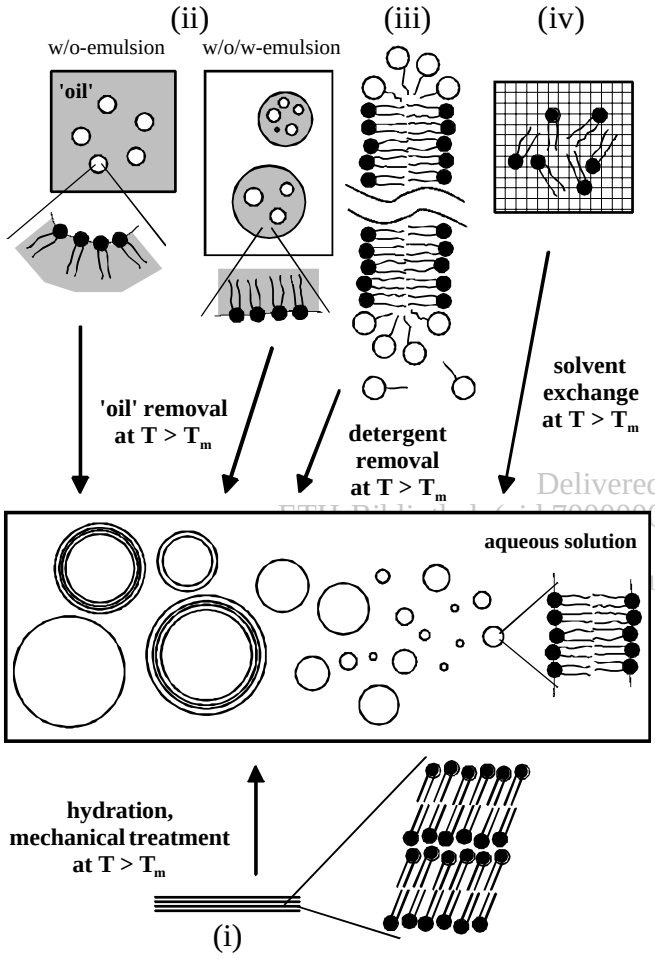

Figure 4. Simplified schematic representation of the principal pathways for the preparation of (normal) lipid vesicles in the case of "conventional amphiphiles" (surfactants that do not form a true vesicle phase at thermodynamic equilibrium). The pathways involve, as starting state of the amphiphiles, (i) preorganized dry lipids, which are hydrated and (possibly) mechanically manipulated above the T_m of the lipids; (ii) preorganized lipids in w/o emulsions (or w/o mircoemulsions or reversed micelles) or w/o/w emulsions prepared in a volatile solvent that is removed during the vesicle preparation procedure above T_m of the lipids; (iii) preorganized lipids in the presence of micelle-forming detergents (mixed detergent/lipid micelles) existing in dynamic equilibrium with free detergent monomers that are removed during the vesicle preparation procedure above the T_m of the lipids; or (iv) lipids dissolved in a solvent that is miscible with water and is exchanged with water during the vesicle preparation procedure above the T_m of the lipids. Once a vesicle suspension is formed, the mean vesicle size and size distribution can always be altered by mechanical treatments above T_m.

The amount of monomeric detergent in the mixed micellar system is important, as it is this nonaggregated amphiphile that is removed from the solution during the detergent removal process, which finally leads to the formation of vesicles [31, 66, 69, 71, 244–247]. The principle of the detergent-depletion method is the following: mixed detergent/lipid vesicles, present in rapid equilibrium with detergent monomers, are put into a dialysis bag or another dialysis device [31] at a temperature above T_m of the lipid used [248]. The dialysis membrane is characterized by a permeability for the monomers, whereas the much larger mixed micelles cannot pass the membrane. Then, at a temperature above T_m [248], the dialysis device is put in contact with a buffer solution in which the mixed micelles were formed. Since the monomers can pass the dialysis membrane, the amount of monomers in the solution inside the dialysis device continuously and slowly decreases, and detergent monomers move from the mixed micellar aggregate into the bulk solution. This process continues until the amount of detergents in the micellar aggregates is so low that mixed micelles can no longer exist, and extended mixed bilayer fragments (sheets) and finally mixed lipid/detergent vesicles form. Extensive dialysis leads to the formation of vesicles that are almost (but not necessarily completely [249]) free from detergent molecules. These vesicle suspensions are often to a large extent unilamellar and have a narrow size distribution. The mean size depends on the experimental conditions, such as type of detergent used, initial lipid and detergent concentrations in the mixed micellar solution, and speed of detergent removal [247, 250–252].

Table 5 lists detergent molecules that are often used for the preparation of vesicles by the detergent-depletion method, together with characteristic size ranges of the mainly unilamellar vesicles formed.

In the case of a system containing egg yolk PC and the bile salt sodium taurochenodeoxycholate (which aggregates itself stepwise into a particular type of unconventional, small micelles [253–255]), the mixed micelle-mixed vesicle transformation process—initiated by a rapid dilution process—has been investigated by time-resolved static and dynamic light-scattering measurements [256]. The scattering data analysis indicates that the key kinetic steps during vesicle formation are the rapid appearance of disc-like intermediate micelles, followed by growth of these micelles and closure of the large discs formed into vesicles [256].

In addition to detergent removal through dialysis, gel permeation chromatography [256, 257] (which is based on the partitioning of detergent monomers into the pores of a swollen gel matrix) or so-called Bio Beads [246, 247, 258, 259] (which bind detergent monomers) can also be applied.

In the case of saturated PCs like DMPC and DPPC and octyl-β-D-glucopyranoside, the originally developed detergent-dialysis method has been modified slightly because of the relatively high T_m value of these lipids (see Table 4) [248]. The important modification is a slow dilution step before the actual dialysis procedure [248], resulting in mainly unilamellar vesicles with a mean diameter of 98 nm

Table 5. Some of the detergents most often used for the preparation of vesicles by the detergent depletion method (Section 3.27) and approximate mean sizes of the vesicles formed in the case of egg yolk PCs.

Detergent	cmc at 25 °C (mM)	Method for removing the detergent	Reported approximate vesicle diameters (nm)	Refs.
Sodium cholate	~11	Gel permeation chromatography	30	[257]
		Dialysis	60	[244]
			70	[252]
			60–80	[245]
			80–100	[251]
			50–150	[519]
Sodium glycocholate	~10	Dilution and dialysis	30–100	[143]
Sodium deoxycholate	~4	Dialysis	150	[252]
Sodium chenodeoxycholate	~5	Dialysis	160	[252]
n-Octyl-β-D-glucopyranoside	~23	Dialysis	180	[245, 524]
			230	[525]
		Bio Beads	300–500	[246]
			250	[526]
$C_{12}EO_8$ (*n*-dodecyl octaethylenglycol monoether)	~0.08–0.09	Bio Beads	60–90	[247]
			25–80	[527]
			120	[526]
CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate)	~5–10	Dialysis	380	[519]

Note: The size of the vesicles may very much depend on the experimental conditions (see text). In particular, the resulting sizes may also depend on the presence of bilayer soluble substances (e.g., cholesterol [245, 246]) or in particular cases (cholate) on the presence of divalent cations (e.g., Ca^{2+}) [519]. The approximative cmc values given in the table are taken for the bile salts from [520]; for $C_{12}E_8$ from [521, 522]; for CHAPS from [522, 523]; and for *n*-octyl-β-D-glucopyranoside from [522].

(DMPC) and 94 nm (DPPC) under the corresponding conditions used [248].

It is quite generally possible to first simply dilute the mixed micellar system, followed by dialysis to completely remove the detergent [260]. If the detergent is not completely removed, the vesicle preparation by simple dilution will always contain detergent molecules, even if the dilution is 100- or 200-fold [250].

In a particular case [261], SUVs with a mean diameter of 23 nm were first prepared by sonication from egg yolk PC at a concentration of 20 mM. The detergent sodium deoxycholate was then added to give an aqueous mixture at a ratio of deoxycholate to PC of 1:2 (mol/mol). This mixture contained vesicles that were considerably larger than the SUVs used, because of the uptake of the detergent molecules. Deoxycholate was then removed to about 96–98% first by gel filtration and then almost completely by a second gel filtration. The final preparation—containing less than one deoxycholate molecule per PC molecule—were dispersed unilamellar vesicles with a mean diameter of 100 nm [261]. The same mean vesicle sizes were also obtained if instead of SUVs a dry egg yolk PC film was treated with deoxycholate, followed by bulk sonication and detergent removal [261].

Large-scale production of vesicles by detergent dialysis is possible (e.g., [262]), and commercial devices are available under the trade names Liposomat and Mini Lipoprep.

The detergent depletion method is the method of choice for the reconstitution of water-insoluble membrane-associated proteins, which in a first step are extracted from the biological membrane by a mild detergent that does not lead to an irreversible protein denaturation [258, 263]. The membrane protein-containing mixed detergent/lipid micelles are then converted to membrane protein-containing vesicles by one of the detergent removal techniques described above.

3.28. (Mixed) Vesicles Prepared by Mixing Bilayer-Forming and Micelle-Forming Amphiphiles

As mentioned in Section 3.27, mixed lipid/detergent vesicles form transiently during detergent removal from detergent/lipid micelles. Such mixed vesicles can also be prepared by adding to preformed lipid vesicles (prepared by any method) above T_m an appropriate amount of a particular detergent [264, 265] or by simply diluting a mixed detergent/lipid micellar solution [266]. Examples of detergents that have been used include sodium cholate [267], *n*-octyl-β-D-glucopyranoside [268], or 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) [269]. The size of the resulting mixed lipid/detergent vesicles depends on the experimental conditions, such as the size (and lamellarity) of the initial vesicles, the chemical structure of the lipid, the lipid concentration, the chemical structure of the detergent, and the detergent concentration.

Phosphatidylcholines like POPC, SOPC, DMPC, DPPC, or the mixtures extracted from egg yolk or soybeans are the amphiphiles whose mixed lipid/detergent vesicle (bilayer) formation has been studied most extensively. These PCs all have two long hydrophobic acyl chains containing 14 to 18 or 22 carbon atoms (see Tables 2 and 4).

PCs with two short acyl chains, containing fewer than 10 carbon atoms, do not form bilayers (vesicles) in dilute aqueous solution, but rather micelles (as long as the concentration is kept above the cmc) [270, 271]. While a very short-chain PC (1,2-dibutanoyl-*sn*-glycero-3-phosphocholine) forms more or less spherical micelles, an increase in the chain length up to eight carbon atoms leads to the formation of extended, elongated micellar structures. All of these short-chain PCs are detergents in the sense of the term used here (Section 3.27) [272], and unilamellar vesicles composed of long-chain PCs and short-chain PCs can easily be prepared, for example, by the addition of a micellar solution of 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine (final concentration 5 mM) to a DPPC dispersion (final concentration 20 mM) in an aqueous solution [273]. The resulting vesicles are rather stable and have mean diameters below as well as above 1 μm [274], depending on the experimental conditions, such as the ratio of the short-chain PC to the long-chain PC and cholesterol content [274].

One particular type of mixed lipid/detergent vesicular system (also containing ethanol)—not prepared by detergent dialysis, but by simple detergent and lipid mixing—are the so-called Transfersomes, vesicles that are claimed to be able to transfer water-soluble drugs through the skin to the blood circulation (transdermal drug delivery), particularly through the outermost physical barrier of the skin, the horny layer, called the stratum corneum. Transfersomes seem to be ultradeformable and may squeeze through the stratum corneum pores, which have diameters only one-tenth of the size of the Transfersomes. In this way, encapsulated water-soluble drugs may be transported across the skin [231]. Transfersomes are prepared by mixing an ethanolic solution of soybean PCs with (for example) sodium cholate to yield a suspension typically containing 8.7% (w/w) soybean PCs and 1.3% (w/w) cholate as well as approximately 8.5% (v/v) ethanol [275, 276]. For a reduction in vesicle size to about 500 nm, the suspension was treated by sonication, freeze-thaw cycles, and processing with a homogenizer.

In the case of the particular non-ionic industrial amphiphile Brij 30 (*n*-dodecyl tetraethylene glycol ether, abbreviated as $C_{12}E_4$ or $C_{12}EO_4$, also called Laureth-4), vesicles have been prepared by simple dilution from so-called hydrotrope solutions, which contained in addition to $C_{12}EO_4$ the micelle-forming surfactant sodium xylene sulfonate [277]. Through a detailed elaboration and analysis of the three-component phase diagram containing $C_{12}EO_4$, sodium xylene sulfonate, and water, the conditions under which vesicles form have first been established. Appropriate starting conditions were then chosen, and a corresponding dilution resulted finally in the desired vesicular system, which contained not only the vesicle-forming amphiphile, but also the hydrotrope (as constituent of the aqueous phase) [277].

3.29. Vesicles Prepared from Lipids in Chaotropic Ion Solutions

This method is related to the detergent-depletion method described in Section 3.27. Chaotropic substances (e.g., urea, guanidinium hydrochloride, potassium thiocyanate, trichloroacetate, or trichlorobromate) are known to disturb (break) the structure of water, and lipids are soluble in chaotropic solutions [278]. Solutions of trichloroacetate, for example, dissolve PCs as micellar solution [279], through a binding of the ions to the head groups, which leads to an increase in a_0 (see Section 6). If a SUV suspension prepared from phospholipids in buffer solution by probe sonication is mixed with an aqueous solution containing sodium trichloroacetate, a micellar solution is first obtained (at about 1–3 M sodium trichloroacetate in the case of 0.1–0.5 mM phospholipids). This solution is then extensively dialyzed against a buffer solution to remove all of the chaotropic ions, resulting in the formation of uni- or oligolamellar vesicles with diameters between 10 and 20 μm, although many small vesicles are also present [278]. If freeze-thaw cycles are used, the amount of chaotropic ions needed to generate giant vesicles is lowered considerably [278].

3.30. GVs Prepared from a W/O Emulsion with the Help of a Detergent

This method involves the use of nonphospholipid amphiphiles in a four-step procedure [280]. First, a w/o emulsion is formed by homogenization of an aqueous solution, *n*-hexane and a mixture of soybean phospholipids and the non-ionic surfactant Span 80 (sorbitan monooleate). Second, *n*-hexane is removed by rotatory evaporation from the emulsion under reduced pressure, resulting in the formation of a water-in-lipid mixture. Third, this mixture is mixed with an aqueous solution containing a water-soluble detergent (such as sodium dodecyl sulfate, hexadecytrimethylammonium bromide, or Tween 80 (POE (20) sorbitan monooleate, which is a modified sorbitan monooleate that contains on average a total of 20 hydrophilic oxyethylene units). Fourth, the highly water-soluble detergent is removed by dialysis, leading to the formation of micrometer-sized vesicles.

4. PREPARATION OF REVERSED VESICLES

There seems to be a kind of symmetry among most self-organized surfactant aggregates with regard to the distribution of the hydrophilic and the hydrophobic parts of the surfactants in the aggregate [281–283]. There are (normal) micelles (L_1-phase) and reversed micelles (L_2-phase), a (normal) hexagonal phase (H_I) and a reversed hexagonal phase (H_{II}), and (normal) emulsions (oil-in-water, o/w) and reversed emulsions (water-in-oil, w/o) [235]. In the case of vesicular aggregates, the existence of reversed vesicles has been demonstrated [283], for example, for a system containing the non-ionic surfactant tetraethyleneglycol dodecyl ether (*n*-dodecyl tetraethyleneglycol monoether, abbreviated $R_{12}EO_4$, $C_{12}EO_4$, or $C_{12}E_4$), the solvent *n*-dodecane, and water [281, 282]. These reversed vesicles form first upon the preparation of a mixture composed of 1 wt% water and 99 wt% of a *n*-dodecane solution which contains 2.5 wt% $C_{12}EO_4$ [281]. After equilibration at 25 °C, a two-phase system forms that is composed of a lamellar liquid crystalline

phase that is in equilibrium with an excess *n*-dodecane phase. When these two phases are mixed by hand-shaking, a heterogeneous dispersion of mainly multilamellar reversed vesicles forms with diameters of the reversed vesicles from less than 1 μm to 10–20 μm [281]. Strong vortexing leads to a reduction in size below 200 nm [282]. Without water, no reversed vesicles form.

A reversed vesicle formed in an oil (in a water-immiscible, apolar solvent) is composed of an oily core and one or several closed "reversed" surfactant bilayer shells. The reversed bilayers are organized in such a way that the hydrated hydrophilic head groups of the amphiphiles are facing toward the center of the reversed bilayer, while the hydrocarbon chains are in contact with the oil [281].

The particular type of reversed vesicles formed from $R_{12}EO_4$, water, and *n*-dodecane seem to be rather unstable and convert back into the thermodynamically stable two-phase system within hours or days [281]. Considerably more stable reversed vesicles can be obtained from a mixture of the non-ionic surfactants sucrose monoalkanoate (which is, to at least 95%, a monoester containing 10 wt% tetradecanoyl, 40 wt% hexadecanoyl, and 50 wt% octadecanoyl chains [283]), hexaethyleneglycol hexadecyl ether (abbreviated $R_{16}EO_6$, $C_{16}EO_6$, or $C_{16}E_6$), *n*-decane, and water [284]. The two surfactants are mixed at a weight ratio of sucrose monoalkanoate to $C_{16}EO_6$ of 85:15, and vesicle formation is observed at typically 3 wt% surfactant in *n*-decane with a typical weight ratio of surfactant to water of 6 [283]. Treatment of the vesicle suspension with a probe sonicator at 30 °C leads to a reduction in the reversed vesicle size to about 150–300 nm, depending on the experimental conditions, such as the water-to-surfactant ratio and the surfactant concentration [284].

The formation by vortexing of micrometer-sized reversed vesicles (and reversed myelin figures) has also been observed in *m*-xylene at a surfactant-to-water weight ratio around 0.8 for a number of polyethyleneglycol oleoyl ethers (abbreviated $R_{18:1}EO_x$ or $C_{18:1}EO_x$, with mean values of x varying between 10 and 55) [285]. Furthermore, it seems that some phospholipids can also form reversed vesicles under particular conditions, as reported in the case of a system containing soybean phosphatidylethanolamine and triolein, saturated with water through vapor equilibration [286]. The particles formed—which are most likely reversed vesicles—have diameters in the range of 500 nm to 1.7 μm [286].

5. CHARACTERIZATIONS AND APPLICATIONS OF VESICLES

5.1. Characterizations of Vesicles

There are a number of typical properties that characterize a vesicle suspension, particularly the mean vesicle size, the vesicle size distribution, the mean number of lamellae per vesicle, and the chemical and physical stability [287]. All of these properties depend on (i) the chemical structure of the amphiphiles (or mixtures of amphiphiles) from which the vesicles are formed; (ii) the solution in which the vesicles are formed (e.g., salt and buffer content in the case of (normal) vesicles and aqueous solutions); and (iii) the method of vesicle preparation (see Section 3). A minimal characterization of a vesicle suspension prepared is always needed. In many cases, a routine size measurement is appropriate, particularly by photon correlation spectroscopy (PCS), also called dynamic light scattering or quasi-elastic light scattering. PCS provides information about the homogeneity of a vesicle suspension, and it is relatively straightforward to find out whether the vesicles in a preparation are relatively monodisperse or whether they are polydisperse. For monodisperse and spherical vesicles, the mean hydrodynamic diameter of the vesicles can be determined relatively easily. In the case of polydisperse vesicle preparations and/or nonspherical vesicles, the interpretation of the scattering curves is more delicate, and it is recommended to apply additional methods as well, such as electron microscopy. A most probable vesicle size can be obtained by cryotransmission electron microscopy (cryo-TEM), although this method is limited to sizes below about 250 nm, and the sizes and morphologies obtained have to be taken with caution [288–290]. The use of different independent methods is always recommended.

Table 6 lists a number of methods that are used for characterizing vesicle suspensions. In many cases, fluorescently labeled amphiphiles are used in the vesicle membranes at a concentration of 1–2 mol%. These amphiphiles contain fluorescent groups, and the behavior of these molecules is then taken as a measure for the behavior of the bulk vesicle-forming amphiphiles, for example, for the determination of the lateral diffusion coefficient of the amphiphiles. Since the fluorescent groups are sometimes rather bulky, the kinetic constants obtained with these labeled molecules may be different from the actual membrane-forming lipids [291, 292]. The same is true for nitroxide-labeled amphiphiles used for ESR (electron spin resonance) measurements [292] (see Table 6).

For the detection of the internal aqueous volume in (normal) vesicles—usually expressed as microliter of trapped aqueous solution per micromole of amphiphile (μl/μmol)—dye molecules are often used, which can be easily quantified spectroscopically. Meaningful results, however, can only be obtained if the dye molecules do not interact with the vesicle membrane (no incorporation inside the membrane, no adsorption to the membrane).

In the case of permeability measurements, the use of dye molecules is convenient, and the release of vesicle-trapped molecules into the external medium can easily be quantified. In this case, however, one should always be aware that the permeability measured is the permeability of the particular dye molecules used under the particular experimental conditions. In this respect, NMR methods that are based on the use of paramagnetic shift reagents are better. The external addition of the shift reagents allows a distinction between the particular solute molecules present inside the vesicles and the solute molecules present outside of the vesicles, presuming that the shift reagent does not permeate the membranes. This has first to be proved. The permeability may very much depend on the experimental conditions, such as the presence of certain buffer species [293].

The list in Table 6 is certainly not complete. However, it gives a few hints about the general principles and problems of the vesicle characterization and lists some of the methods and techniques used. Depending on the vesicle preparation, a particular characterization may be more useful than

Table 6. Some of the principles and analytical methods used for characterizing the properties of vesicles (see also [66, 287]).

Property	Methods used and comments	Ref.
Mean vesicle size and size distribution	*PCS* Analysis of the time dependence of intensity fluctuations in scattered laser light due to the Brownian motion of the vesicles, which is related to the mean hydrodynamic radius (R_h) of the vesicles. If the vesicle suspension is very polydisperse and/or contains non-spherical vesicles (e.g., caused by an osmotic imbalance between the inside and the outside of the vesicles), the size analysis is rather complex and difficult	[2, 31, 528–531]
	SLS Average scattering intensities are measured as a function of the scattering angle and the vesicle concentration, allowing the determination of the mean radius of gyration (R_g) of the vesicles	[532–534]
	FFEM Replicas of fractured vesicles are analyzed. The vesicles are fractured at low temperature (−100 °C) under conditions where the water is in an uncharacterized (amorphous), "glassy" solid state. Nonequatorial fracturing leads to replicas that do not represent the "true" vesicle size	[1, 2, 136, 162, 300, 535]
	Cryo-TEM The vesicles are directly observed at low temperature (−170 °C) after rapid freezing (~10^6 °C/s)—under conditions where the water is in an amorphous solid state—to represent the state of the vesicles at the temperature from which the sample is cooled. The observed diameters of the vesicles correspond to the "true" vesicle diameters. Only applicable for vesicles with sizes below ~250–500 nm. Not free of "artefacts" ("artifacts"), i.e., formation of microstructures due to specimen preparation, electron optics, or radiolytic effects [288–290]	[2, 3, 536]
	LM Only for GVs	[4, 66, 89, 91, 536]
	Size exclusion chromatography Conventional and high-performance liquid chromatography (HPLC) separation based on the principle that the partitioning behavior of vesicles in the pores of a solid column matrix depends on the size of the vesicles	[66, 386, 537]
	NMR Size determinations possible from ^{31}P-NMR spectra of phospholipid vesicles that have sizes below 1 μm with the help of a comparison with simulated spectra. For spectra simulation, the dynamics of the vesicles and the phospholipids in the vesicle membranes are considered	[136]
	AFM The vesicles have to be deposited on a solid surface, which may lead to vesicle deformations and vesicle aggregations	[538, 539]
	SANS Measurements in D_2O that may influence the vesicle size *via* altered head group interactions. Relative complex analysis involving fitting of experimental data	[540, 541]
Bilayer thickness	*X-ray* Based on the interaction of X-rays with the electrons of the amphiphiles in the vesicles	[80, 300, 542]
	Cryo-TEM	[543]
	SANS Measurements in D_2O. Bilayer thickness determinations possible in the gel, in the ripple, and in the lamellar phase of the vesicle-forming surfactants (see Section 2)	[540, 541, 544–546]
Vesicle shape and morphology	*LM* Only for GVs. Detection of MVVs	[547–552]
	FFEM Detection of MVVs	
	Negative staining EM Heavy metal ions have to be added to the vesicle dispersion, which always alters the vesicles. Furthermore, the vesicles are observed in a dry state	[12]

continued

Table 6. Continued

Property	Methods used and comments	Ref.
	Cryo-TEM Only for vesicles with sizes below ~250–500 nm	[553]
Lamellarity	*Cryo-TEM* Only for vesicles with sizes below ~250–500 nm	[3, 293]
	Negative Staining EM Addition of heavy metals (osmium tetroxide) needed. The number of lamellae detected may not represent the "true" number of lamellae	[12, 31]
	NMR ^{31}P-NMR in the case of phospholipid vesicles. Externally added membrane-impermeable Mn^{2+} influences the ^{31}P-NMR signal of the phospholipids in the outermost monolayers by broadening the resonance beyond detection. The resonances of the inner phospholipids are unaffected. Paramagnetic shift reagents may also be used (e.g., Pr^{3+} and Eu^{3+})	[161, 162, 293, 554]
	Fluorescence quenching An appropriate amphiphilic fluorescent probe molecule is used in the vesicle preparation, and the fluorescence of the amphiphiles in the outermost layers is quenched by adding to the vesicles a membrane-impermeable reagent	[555]
	SAXS Based on the interaction of X-rays with the electrons of the amphiphiles in the vesicles	[556]
	Chemical modification Functional groups present in the hydrophilic head groups of the amphiphiles in the external layer(s) of the vesicle and exposed to the bulk aqueous solution are chemically modified by externally added reagents that do not permeate the membranes and therefore do not react with the amphiphiles that are present in the inner layer(s) of the vesicles with their head groups exposed to the trapped aqueous space. The chemically modified amphiphiles are then quantified	[31]
Lipid domains in the membranes (rafts)	*Two-photon fluorescence microscopy* Use of membrane soluble fluorescent probes. Only for GVs	[557]
	NMR ^{31}P-, ^{13}C-, or ^{2}H-NMR	[558, 559]
	Confocal fluorescence microscopy Distinction between solid-ordered and fluid-disordered domains in vesicle membranes made with two fluorescent probes that have different affinities for the two domains. Only for GVs	[560]
Phase transition temperature (T_m)	*DSC* A vesicle sample and an inert reference are heated independently to maintain an identical temperature in each. In endothermic solid-ordered/liquid-ordered phase transitions, heat is required in excess in the vesicle sample over the heat required to maintain the same temperature in the reference	[43, 300, 540]
	Fluorescence Fluorescence polarization measurements of a membrane soluble fluorescent probe (e.g., 1,6-diphenylhexatriene, DPH)	[31, 561, 562]
	CD Induced circular dichroism below the T_m of an achiral probe (1,6-diphenylhexatriene, DPH) embedded inside the vesicle membrane constituted by chiral amphiphiles. No CD signal above T_m	[563]
Mobility of the amphiphiles in the vesicle membranes	*NMR* ^{1}H-, ^{2}H-, ^{13}C-, ^{31}P-NMR	[37, 564–567]
	ESR Use of spin-labeled amphiphiles	[568]
	Quasi-elastic neutron scattering	[569, 570]

continued

Table 6. Continued

Property	Methods used and comments	Ref.
	Fluorescence Use of fluorescent amphiphiles	[571]
	Fluorescence correlation spectroscopy Analysis of the translational diffusion of a fluorescent probe. Only for GVs	[560]
	Fluorescence recovery after photobleaching Lateral diffusion of fluorescently labeled amphiphiles determined by first bleaching the lipids with an intense laser pulse, and then analyzing the fluorescence recovery kinetics in the bleached area, due to the diffusion of unbleached molecules into the previously bleached area	[37]
Molecular conformation of the amphiphiles in the vesicle membranes	*NMR* ^{13}C- or ^{2}H-NMR using labeled phospholipids	[300, 559, 564, 565, 572]
	FT-IR and *Raman* Determination of the equilibrium conformational characteristics of the amphiphiles	[37, 300, 573]
	CD For vesicles composed of chiral amphiphiles	[563]
Membrane fluidity and order	*Fluorescence* Use of fluorescent membrane probes. Measurements of static or time-resolved fluorescence anisotropy	[37]
	NMR Use of partially and specifically deuterated amphiphiles	[300, 564]
	ESR Use of amphiphiles that have a nitroxide radical. The ESR spectrum of these amphiphiles is sensitive to the motions of the molecules	[37]
Transmembrane lipid diffusion (flip-flop)	*ESR* Use of spin-labeled amphiphiles (nitroxide radical)	[292, 574]
	Fluorescence Use of fluorescently labeled amphiphiles	[292]
Surface charge	*Zeta potential measurements, microelectrophoresis* Problematic, since the method is derived from classical theories of the double layer that do not include specific ion effects (see Section 6). Limited to particular background salt conditions (usually NaCl)	
Internal volume and entrapment (encapsulation) yield	*Use of dye molecules* The dye molecules (often fluorescent) are water-soluble and should not interact with the vesicle membrane. The dye molecules are entrapped during vesicle preparation, and the amount of entrapped dye molecules is determined quantitatively either after separation of the nonentrapped molecules from the vesicles or by addition to the vesicles of a membrane-impermeable reagent, which leads to a complete quenching of the fluorescence of the externally present dyes (e.g., calcein with quencher Co^{2+}) [575]. Simple dilution of the externally present dye may also be possible [577]	[31, 66, 575–577]
	NMR ^{17}O-NMR of the water oxygen. Addition of the membrane-impermeable paramagnetic shift reagent $DyCl_3$ to the vesicles below T_m leads to a shift in the ^{17}O resonance corresponding to the external water. The internal water peak remains the same. Above T_m, only one peak is observed because of rapid water equilibration	[554]
Membrane permeability	*Use of dyes or radioactively labeled molecules* Determination of the release of dye molecules entrapped inside the aqueous interior of the vesicles as a function of time under particular storage conditions	[31, 578]
	NMR Use of ^{1}H- or ^{17}O-NMR and an externally added membrane-impermeable paramagnetic shift reagent (Mn^{2+}, Pr^{3+}) to distinguish between internal and external permeants	[579–582]

continued

Table 6. Continued

Property	Methods used and comments	Ref.
	Use of ion-selective electrodes The ions present outside of the vesicles can be detected	[583]
Chemical stability	*Thin-layer chromatography* Analysis of possible degradation product due to hydrolysis and oxidation reactions	[72]
	High-performance liquid chromatography Analysis of possible degradation product due to hydrolysis and oxidation reactions	[72]
Physical stability	*Turbidity, PCS*, *FFEM*, *Cryo-TEM*, and others Analysis of possible size changes during storage due to vesicle aggregation and fusion	[66, 69, 584]

Abbreviations: AFM, atomic force microscopy; CD, circular dichroism; cryo-TEM, cryo-transmission electron microscopy (also called cryo-fixation); DSC, differential scanning calorimetry; EM, electron microscopy; ESR, electron spin resonance (also called electron paramagnetic resonance, EPR); FFEM, freeze-fracture electron microscopy; FT-IR, Fourier transformed infrared; LM, light microscopy; NMR, nuclear magnetic resonance; PCS, photon correlation spectroscopy (also called dynamic light scattering (DLS) or quasi-elastic light scattering (QELS)); SANS, small-angle neutron scattering; SAXS, small-angle X-ray scattering; SLS, static (or classical) light scattering.

another, and certain methods may not be applied at all (e.g., ^{31}P-NMR obviously cannot be used for the determination of the lamellarity of vesicles that are not composed of phosphorous-containing amphiphiles).

5.2. Applications of Vesicles

Lipid vesicles are used successfully in many different fields as interesting and versatile submicrometer- or micrometer-sized compartment systems [69, 294–297]. This wide applicability of vesicles and the broad interest in vesicles can be understood at least on the basis of the following four reasons:

(i) Lipid vesicles can be considered membrane or biomimetic systems [298, 299], since the molecular arrangement of conventional vesicle-forming amphiphiles in a vesicle is a (more or less curved) bilayer, like the lipid matrix in biological cell membranes [97, 300] or in the outer coat of certain viruses [97].
(ii) Vesicles prepared from amphilphiles present in biological systems allow applications as biocompatible and biodegradable systems.
(iii) Water-soluble as well as certain water-insoluble molecules can be entrapped inside the aqueous or hydrophobic domains of the vesicles, allowing the use of vesicles as carrier systems and nanometer- or micrometer-sized reaction compartments.
(iv) Vesicles can be prepared not only from the conventional PC type of bilayer-forming amphiphiles, but also from a large number of different nonphospholipid surfactants (or mixtures of surfactants), allowing the preparation of application-tailored and specifically designed and functionalized systems.

Among the more than 30,000 known surface active compounds [301], a large number of surfactants and surfactant mixtures (including many nonphospholipid amphiphiles) have been reported to form vesicles. The basic principles that lead to the formation of vesicles are the same for all, that is, the requirements of (i) an effective packing parameter $p\ (=v/a_0 l)_{\text{eff}} \approx 1$ (see Section 1.1), (ii) chain flexibility ($T > T_m$), and (iii) sufficiently low amphiphile concentration (global packing constraints) [7]. Examples of vesicle-forming amphiphiles include

- cationic di-*n*-alkyldimethylammonium ions [302, 303] (such as di-*n*-dodecyldimethylammonium bromide (DDAB) [304] and other di-*n*-dodecyldimethylammonium halides [305] and di-*n*-octadecyldimethylammonium chloride [306–308] or bromide [308, 309] or other counter-ions [310]);
- the cationic oleyldimethylaminoxide [311];
- anionic phospholipids (such as egg yolk phosphatidic acid mixtures and ox brain phosphatidylserine mixtures [312, 313]);
- anionic di-*n*-alkylphosphates [314–316] (e.g., di-*n*-dodecylphosphate [317], di-*n*-hexadecylphosphate [318–322], and various di-polyprenylphosphates [323, 324]);
- anionic linear and branched monoalkylphosphates (such as different polyprenylphosphates [325], *n*-dodecylphosphate [326], 6-propylnonylphosphate [327], 4-butyloctylphosphate [327], and 2-pentylheptylphosphate [327]);
- anionic tridecyl-6-benzene sulfonate in the presence of salt (sodium chloride) [328];
- anionic fatty acid/soap mixtures (e.g., *n*-octanoic acid/*n*-octanoate [329], *n*-decanoic acid/*n*-decanoate [329–331], oleic acid/oleate [332–334]);
- anionic surfactant/alcohol mixtures (such as sodium *n*-dodecylsulfate/*n*-dodecanol [329, 335] and sodium *n*-decanoate/*n*-decanol [336], or sodium oleate/*n*-octanol [337], which can form a highly viscous phase of densely packed vesicles);
- mixtures of cationic and anionic surfactants, so-called catanionic mixtures (such as *n*-hexadecyltrimethylammonium tosylate/sodium *n*-dodecylbenzenesulfonate [20, 59], *n*-hexadecyltrimethylammonium bromide/sodium *n*-octylsulfate [338–342], and sodium *n*-dodecylsulfate and di-*n*-dodecyldimethylammonium bromide [343, 344]);
- ganglioside GM3 [345, 346];
- phosphatidylnucleosides (such as 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocytidine [347, 348]);
- diblock copolymers [24] (such as the ethylene oxide (EO)/butylene oxide (BO) diblock copolymers EO_6BO_{11}, EO_7BO_{12}, $EO_{11}BO_{11}$, $EO_{14}BO_{10}$, and $EO_{19}BO_{11}$ [349]);

- triblock copolymers (such as the ethylene oxide (EO)/propylene oxide(PO)/ethylene oxide (EO) triblock copolymer $EO_5PO_{68}EO_5$ (called Pluronic L121) [350] and the polymerizable poly(2-methyloxazoline)/ poly(dimethylsiloxane)/ poly(2-ethyloxazoline) triblock copolymers [351]);
- polymerizable amphiphiles [33, 352, 353];
- perfluorated surfactants [354] (such as short-chain perfluorophosphocholines [355] and perfluoroalkyl PCs [356]);
- bolaamphiphiles, which are membrane-spanning amphiphiles with two hydrophilic head groups at the two ends of the molecule and actually form vesicles containing not bilayered but monolayered shells [357–360];
- gemini surfactants that contain two hydrophobic tails and two hydrophilic head groups linked together with a short linker [361, 362];
- industrial, not very well defined surfactant/cosurfactant mixtures (such as *N*-methyl-*N*-alkanoyl-glucamine/ octanol or oleic acid mixtures [363]);
- calixarene-containing [364] or cryptand-containing amphiphiles [365];
- fullerene-containing amphiphiles with two hydrophobic chains and two charged head groups [366];
- double-chain amphiphiles with a polar alkoxysilyl head group, allowing the preparation of a kind of organic-inorganic hybrid vesicle, called cerasomes [367];
- triple-chain amphiphiles containing three hydrophobic chains and two charged polar head groups [365, 368];

and many more (e.g., [33, 358, 369]).

Finally, complex vesicle-based surfactant aggregates can be prepared (e.g., large vesicles composed of one or more types of amphiphiles may contain vesicles of another type of amphiphile), based on principles that include the specific molecular recognition between different types of vesicles initially prepared by one of the established methods described in Section 3 [370–372].

It is rather obvious that one consequence of the fact that vesicle formation is observed from so many different classes of amphiphiles (or mixtures of amphiphiles) is the very broad range of applications in very different fields. Vesicles are applied—or investigated for potential applications—at least in the following areas:

- in pharmacology and medicine [69, 73, 296, 297, 373–378] as parenteral or topical drug delivery systems [297, 379–381], in the treatment of infectious diseases, in anticancer therapy, as gene delivery systems, as immunoadjuvants, and as diagnostics;
- in immunoassays [382–385];
- in chromatographic separations using immobilized vesicles [386, 387];
- in cosmetics as formulations for water and nutrient delivery to the skin [388–391];
- in a variety of biophysical investigation of biological membrane components, including the reconstitution and use of membrane-soluble proteins [4, 69, 258, 263, 392];
- in research on membrane-soluble ion channels [393–399];
- in research related to the question of the origin and evolution of life, as models for the precursor structures of the first cells [400];
- in research aimed at constructing artificial (or minimal) cells [401, 402], for example, for potential biotechnological applications [402];
- in food technology and nutrition as carrier systems for food additives and ingredients and for the control of certain food processes (e.g., cheese ripening) [403–405];
- in agrochemistry [406];
- as nanometer- or micrometer-sized bioreactors containing catalytically active enzymes [75, 407–411];
- in nanoparticle technology [190, 299, 412–414], for example, for the preparation of semiconductor particles [412];
- in catalytic processes as simple models for enzymes [415–420] or simple models of other protein functionalities (e.g., as catalysts for the unfolding [421] or folding [387, 422] of proteins);
- in biosensor developments [423–425], particularly for the controlled preparation of bilayers adsorbed to solid surfaces [426–429];
- in the extraction of heavy metal ions with the help of functionalized, metal-sorbing vesicles [430];
- as supramolecular, nanostructured polymeric materials (as polymerized vesicles) [431–433];
- in biomineralization [434–436];
- as templates for the synthesis of inorganic mesoporous materials [437, 438] or biomaterials [436] and in the preparation of hollow polymer capsules [356, 439–441];
- as templates for modifying the distribution of reaction products, for example, in reactions that lead to products that are only sparingly soluble in the absence, but soluble in the presence, of vesicles [442, 443];
- as a medium for the preparation of size-defined polymer particles from monomers that are soluble in the vesicle shell [444];
- as supramolecular, self-assembly-based devices [33, 298, 299, 358, 445], for example, for the conversion of light energy into chemical energy (artificial photosynthesis) [446, 447], for signaling and switching [448, 449], for the construction of molecular wires [450], and for a number of different redox processes [418];

and many more (e.g., [295, 299]).

One illustration of vesicles loaded with water-soluble molecules is shown in Figure 5. A cryo-transmission electron micrograph of POPC vesicles containing the protein ferritin is shown. The vesicles were prepared in the presence of ferritin, and the nonentrapped protein molecules were separated from the vesicles by size exclusion chromatography after vesicle preparation [451, 452]. Since this particular protein has a dense iron core with a size of about 8 nm, it is visible by electron microscopy, and the actual number of protein molecules per individual vesicle can be directly counted. This type of loaded vesicles can be used, for example, in basic studies on vesicle transformation processes [451, 452]. If the vesicles contain catalytically active proteins (enzymes), they may be used in drug delivery or as small bioreactors [75, 410, 411].

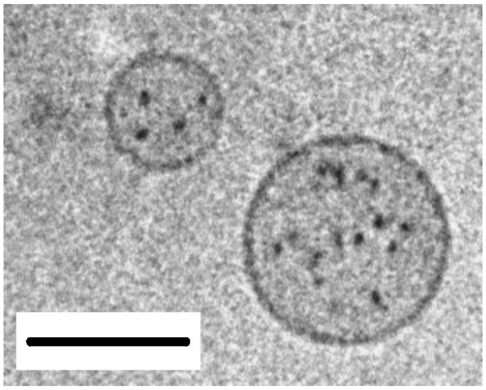

Figure 5. Cryo-transmission electron micrograph of two unilamellar vesicles that have been prepared from POPC by the reversed-evaporation technique (see Section 3.14) loaded with the iron storage protein ferritin, followed by extrusion (REV-VET_{100}). Nonentrapped ferritin molecules were removed by size-exclusion chromatography. Each black spot inside the vesicles represents one iron core (the core of an individual protein). The length of the bar corresponds to 100 nm. The electron micrograph was taken by M. Müller and N. Berclaz, Service Laboratory for Electron Microscopy, at the Department of Biology at the ETH Zürich. See [451, 452] for experimental details and for an application of ferritin-containing vesicles in the investigation of vesicle transformation processes.

Conceptually, an interesting principle of vesicle applications is vesicle transformation (at the site of application) to another type of surfactant assembly as a result of temperature changes that lead to changes in the surfactant packing parameter *p* (see Section 1.1). One particular example is the transformation of a 1-monoolein MLV suspension (L_α-phase) into a 1-monoolein bicontinuous cubic phase [9, 453–456].

There is no doubt that the number of applications will increase with increasing molecular complexity of the vesicular systems. Vesicles—and other surfactant assemblies (e.g., hexagonal and cubic phases)—will be applied more and more in all fields related to nanoscience and nanotechnology. The title of a recent publication in the field of vesicle drug delivery is an example of one of the directions in which vesicle research and application may go: "Biotinylated Stealth Magnetoliposomes" [457], a particular vesicle preparation that combines steric stabilization (Stealth) with molecular recognition (biotin) and magnetic nanoparticle properties. In other words, it is likely that the complexity of the vesicles prepared and investigated will increase to make them functional, possibly by combining the principles of supramolecular chemistry and surfactant self-assembly, to prepare nanometer- or micrometer-sized synthetic systems that may carry out some of the functions biological systems do rather efficiently [458, 459].

6. CONCLUDING REMARKS

There are many different methodologies that have been described in the literature for the preparation of lipid vesicles. Some (but not all), and certainly the most prominent and well known, are mentioned in this review, focusing on normal lipid vesicles (Section 3), although reversed vesicles are briefly mentioned as well (Section 4).

As outlined in Sections 1 and 2, the use of a number of different terms in the field of vesicles (and surfactant assemblies at large) is often rather confusing, and confusion even exists over the general use of the term "surfactant." It is worth pointing out that a reduction in the surface tension of water by surfactants can be achieved not only with classical micelle-forming, single-chain amphiphiles (such as sodium *n*-dodecyl sulfate, SDS) [460], but also with typical vesicle-forming double-chain phospholipids (such as SOPC or DPPC). Certainly, the kinetics and extent of adsorption of a particular surfactant at the water-air interface very much depend on the precise experimental conditions [461]. In the case of DPPC, for example, the equilibrium surface tension and rate of monolayer formation at the water-air interface depend on the temperature [462]. Below T_m, in the solid-ordered state of the molecules (see Section 2), the decrease in the surface tension of water by DPPC is considerably lower than that above T_m [462]. However, there is no doubt that glycerophospholipids like SOPC, POPC, or DPPC are surfactants in the sense of the definition of this term given in Section 1.1.

Since in dilute aqueous solution many of the bilayer-forming surfactants known (at least the most studied phospholipids) at thermodynamic equilibrium do not form a true vesicle phase (with a defined vesicle size, size distribution, and lamellarity), but under the appropriate conditions rather form a lamellar phase (L_α, L_β, L'_β or P'_β) with extended stacked bilayers, the preparation of vesicle dispersions necessarily involves the use of a particular method, a particular technology.

Depending on the experimental conditions, as mentioned in Section 3, depending on the chemical structure of the amphiphile or mixtures of amphiphiles used, the amphiphile concentration, the substances that may be encapsulated in the vesicle's aqueous interior or in the vesicle membrane, etc., there may be one particular method that is more advantageous in comparison with others.

Some of the methods described in Section 3 involve the use of organic solvents. This may be a problem for certain applications. Some methods can be scaled up to a bulk preparation [287], some methods involve mechanical treatments that may cause an inactivation of sensitive molecules one may like to entrap [75]; some methods are cheaper than others; some methods lead to very small vesicles (SUVs); other methods lead mainly to MLVs or LUVs or GUVs, or even MVVs; some methods are fast; some methods are relatively time consuming, etc.

There is no "best method." The choice of a certain method that may be useful very much depends on the particular problem one is trying to solve [67]. There are, at least in the case of the conventional type of phospholipids (or in the case of lipid mixtures containing phospholipids, particularly PC), a few general findings that are often valid; but one should be aware of the existence of possible exceptions:

- The hydration of a dried thin lipid film above the T_m of the lipids usually leads to the formation of MLVs if the film is dispersed vigorously (vortexing or hand-shaking) (see Section 3.2).
- The hydration of a dried thin lipid film above the T_m of the lipids usually leads to the formation of GUVs if

the film is undisturbed while being hydrated slowly (see Section 3.2).

- The electroformation method can be used for the preparation of GUVs with diameters between about 10 and 50 μm (or more) in the case of certain lipids (and lipid mixtures) and buffers with low ionic strength (Section 3.3).
- Relatively homogeneous preparations of SUVs with diameters around 50 nm or below can often be obtained by probe sonication (Section 3.5), although degradation of the surfactants and consequences of the possibly present metal particles (which may be a source of nucleation for vesicle transformation processes) have to be taken into account.
- Rather homogeneous preparations of LUVs with diameters around 50 or 100 nm can be obtained by the extrusion technique as FAT-VET$_{50}$ or FAT-VET$_{100}$ (Section 3.8, Fig. 3).
- The dehydration-rehydration method usually results in high encapsulation yields (Section 3.7).
- Freeze-thaw cycles may lead to vesicle size homogenization and solute equilibration between the external bulk solution and the trapped aqueous solution (Section 3.6).
- The detergent-depletion method often results in rather uniform vesicles with sizes below 100 nm, although the possibility of an incomplete removal of the detergent should be considered (Section 3.27, Table 5).
- The use of volatile cosolvents (oils) during vesicle preparation is often based on the principle that either w/o or w/o/w emulsions are formed from which the solvent is removed, limiting the solvents to all those that have a boiling point considerably lower than the boiling point of water (Sections 3.14–3.17, 3.24, and 3.25).

The physicochemical properties (such as chemical and physical stability, membrane permeability) of the vesicles formed very much depend on the surfactant (or surfactant mixtures) used. Depending on the surfactant, the characteristic properties may be very different from those of the conventional PC type of vesicles. Furthermore, the whole equilibrium phase behavior may be different, and cases are known where even true vesicle phases seem to form "spontaneously" [7, 463–465] and seem to exist at thermodynamic equilibrium [7, 59, 60, 463]. In these cases, vesicles of a certain size and lamellarity just form by mixing, independent of the method of preparation. The stability of the vesicles is understood in the case of the so-called catanionic vesicles (which are composed of a mixture of positively and negatively charged amphiphiles) on the basis of a most likely uneven distribution of the amphiphiles in the two bilayers. This allows the required differences in curvature and molecular packing in the two halves of a bilayer [60, 466], similar to the case of lipid vesicles prepared from mixtures of long-chain and short-chain PCs (Section 3.28) [273, 274]. With respect to the spontaneity in the formation of this type of vesicles, it has been argued, however, that shear forces present during the preparation (mixing of solutions) play an important role in vesicle formation [467].

An interesting case of unilamellar vesicles as thermodynamic equilibrium state has been described in the case of certain ionized phospholipids (e.g., 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol, DMPG) [468–472]. Under the experimental conditions used, unilamellar DMPG vesicles apparently only form at the critical temperature (T^*) of 31.6 °C [471], which is different from T_m. Above T^* MLVs are formed; below T^* the phospholipids arrange into a sponge phase [471]. Further investigations are needed to fully understand this critical phenomenon and to clarify whether this unilamellar vesicle formation is a particular case or whether it can be more generally observed.

With the exception of a few cases—such as the detergent depletion method (Section 3.27) (e.g., [256])—the general mechanism of vesicle formation is not yet completely understood in its details, although general principles have been elaborated [7, 69, 473–477].

From a more practical point of view, and by looking at all of the methods described in Section 3, it is evident that the vesicles often form from a preorganized state of the lipids. This preorganization may be

(i) Lamellar sheets present in a dry film deposited on a solid surface (Sections 3.2 and 3.3) or in the ethanolic pro-liposome state (Section 3.19).
(ii) W/o or w/o/w emulsion droplets (Sections 3.14, 3.15, and 3.30).
(iii) Mixed micelles in the case of the detergent-depletion method (Section 3.27) or micelles in the case of chaotropic ion solutions (Section 3.29).

In a few cases, the vesicle formation is triggered as a result of a direct contact of a nonorganized state of the lipids with an aqueous environment, as in the case of the Novasome technology (Section 3.10), the ethanol injection method (Section 3.18), or the ether injection method (Section 3.24). A simple summarizing schematic representation of some of the different pathways for the formation of (normal) lipid vesicles is given in Figure 4. It is expected that more methods for the preparation of vesicles will be developed, although the general pathways may remain the same. The range of pre-organized starting systems from which vesicles can be formed may be expanded, and more will probably soon be understood for the vesicle formation at large.

(Normal) vesicles are just a particular state of aggregation of surfactants (or surfactant mixtures) in an aqueous solution that are topologically closed with an internal aqueous space. Vesicles can be unilamellar or multilamellar, and the general principles of surfactant assembly have been outlined [7–11]. As mentioned above, vesicles sometimes appear to be thermodynamically stable, sometimes not. Like all self-assembled amphiphile aggregates—micelles, microemulsions, cubic phases, even biological membranes, etc.—the formation of vesicles depends on only a few things: (i) local curvature, (ii) global packing constraints (including interaggregate interactions), and (iii) flexibility of the hydrophobic chain(s). All of the methods described essentially depend on satisfying these criteria.

Physicochemical conditions of inside and outside of vesicles often differ greatly. This is a consequence of the closed topology. This fact, together with adverse packing conditions, can often result in a stable state of so-called supra-self-assembly (e.g., surfactant micelles existing inside surfactant *vesicles*) [478–480].

The basic principles that underlie self-assembly of vesicles are quite general and are firmly based in thermodynamics and statistical mechanics [7, 9, 10, 481]. The requirements for "designing" a vesicle are conceptually simple: a packing parameter close to unity (which means effectively a double-chain surfactant or mixed single-chain surfactants), flexible hydrophobic chains (a temperature above T_m), and control of inter- and intraaggregate interactions. Local and global packings are the key principles to consider.

Vesicles, however, are a very small part of a much larger class of self-assembled surfactant aggregates that include cubic phases, which are usually bicontinuous structures of zero average curvature. "Bicontinuous" means that both the aqueous and "oily" parts of the structure are continuously connected over the whole system. Cubic phases or bicontinuous structures in general are ubiquitous in biology for directing biochemical traffic [11]. Likewise, hexagonal phases and microtubules are close to lamellar (and vesicular) phases in a phase diagram [11].

Although these things are known and are even beginning to be understood, quantitative predictions remain a problem. This can be traced to the fact that the underlying theory of molecular forces that underpins physical chemistry and colloid and surface science is flawed [482, 483]. Previous theories cannot deal with specific ion effects (so-called Hofmeister series), dissolved gas, and other solutes that change the water structure. There is rapid development in this area at the moment, which is likely to provide predictability in vesicle design [482–484], which is certainly what one is aiming for.

GLOSSARY

Amphiphile A molecule that comprises at least two opposing parts, a *solvophilic* (for example "hydrophilic", meaning water-loving) and a *solvophobic* (for example "hydrophobic", meaning water-hating). Amphiphiles are surfactants.

Detergent A surfactant that in dilute aqueous solution forms micelles, spherical or non-spherical aggregates that contain in the interior of the aggregate the hydrophobic part of the surfactant and on the surface the hydrophilic part of the surfactant.

Glycerophospholipid A particular phospholipid that contains a glycerol backbone to which a phosphate group is bound.

Lamellar phase, L_α A particular liquid crystalline equilibrium state of surfactant molecules, also called liquid-disordered state. In an aqueous environment, the surfactant molecules are arranged in layers in which the hydrophobic part of a surfactant is in the interior of the layer and the hydrophilic part on the two surfaces of the layer, exposed to the aqueous environment.

Liposome Vesicle prepared from amphiphilic lipids.

Phase transition temperature, T_m Characteristic temperature (also called lamellar chain melting temperature) of surfactants that form a lamellar phase. Above the phase transition temperature, the surfactant molecules are in a liquid-disordered state, below in a solid-ordered state.

Phosphatidylcholine, PC A particular glycerophospholipid that contains a choline group in the hydrophilic part, bound to the phosphate.

Phospholipid A surfactant that is present in some of the biological membranes and contains at least one phosphate group.

Reversed vesicle Inverted vesicle formed in a water-immiscible, apolar solvent in the presence of a small amount of water.

Surfactant A molecule that is surface active, meaning that it accumulates at the surface of liquids or solids. Surfactants are amphiphiles.

Vesicle General term to describe any type of hollow, surfactant-based aggregate composed of one or more shells. In the biological literature, the term *vesicle* is used for a particular small, membrane-bounded, spherical organelle in the cytoplasm of an eukaryotic cell.

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