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Giant Vesicles: Preparations and Applications

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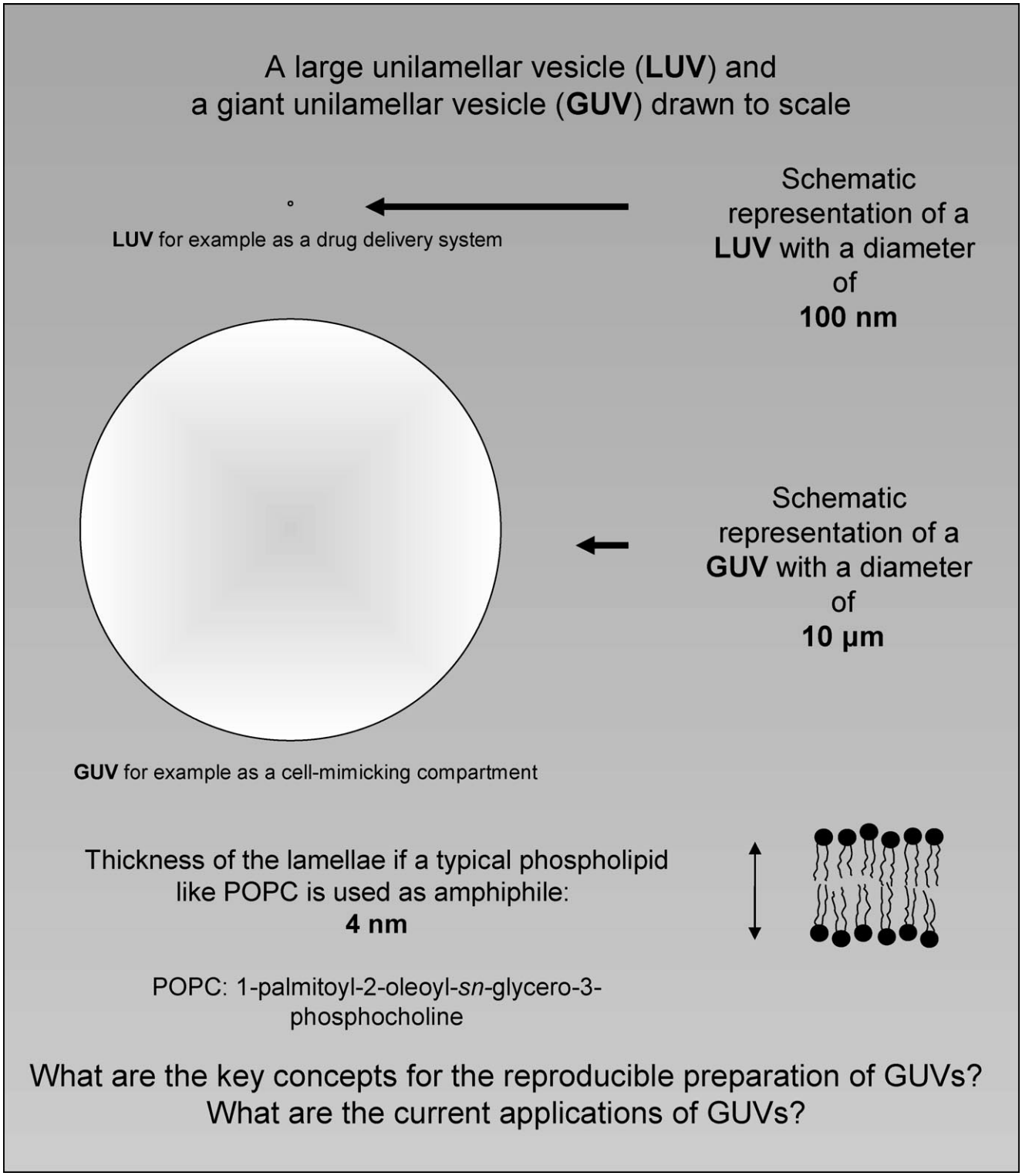

There is considerable interest in preparing cell-sized giant unilamellar vesicles from natural or nonnatural amphiphiles because a giant vesicle membrane resembles the self-closed lipid matrix of the plasma membrane of all biological cells. Currently, giant vesicles are applied to investigate certain aspects of biomembranes. Examples include lateral lipid heterogeneities, membrane budding and fission, activities of reconstituted membrane proteins, or membrane permeabilization caused by added chemical compounds. One of the challenging applications of giant vesicles include gene expressions inside the vesicles with the ultimate goal of constructing a dynamic artificial cell-like system that is endowed with all those essential features of living cells that distinguish them from the nonliving form of matter. Although this goal still seems to be far away and currently difficult to reach, it is expected that progress in this and other fields of giant vesicle research strongly depend on whether reliable methods for the reproducible preparation of giant vesicles are available. The key concepts of currently known methods for preparing giant unilamellar vesicles are summarized, and advantages and disadvantages of the main methods are compared and critically discussed.

1. Introduction

The term "vesicle" (from the Latin *vesicula, -ae*, small bladder) is used here to designate a particular type of compartment, formed in vitro in an aqueous medium. The interior of a vesicle is a small aqueous volume and the boundary of each vesicle is constituted by one (or a few) thin layers, also called membranes, composed of amphiphilic molecules. The amphiphiles contain hydrophilic and hydrophobic (that is, lipophilic) parts and are often bilayer-forming low-molar-mass compounds (phospholipids or synthetic surfactants) or amphiphilic block copolymers. The amphiphiles in a vesicle membrane are arranged in such a way that the hydrophilic parts are in contact with the aqueous medium, while the hydrophobic parts associate to form the interior in each layer. In the case of *unilamellar vesicles* composed of conventional biomembrane *phospholipids*, which have one hydrophilic head group and two lipophilic chains, the vesicle membrane is built from one single bilayer with the head groups of the lipids of the inner layer facing towards the interior of the vesicles and the head groups of the outer layer being in contact with the external aqueous medium.[1] If the amphiphiles are *amphiphilic diblock copolymers* composed of a hydrophilic block and a hydrophobic block, the arrangement is basically the same as in the case of phospholipids.[2] However, the physicochemical properties of the two types of bilayers might be quite different, for example, bilayer thickness, bending elasticity, permeability.[3] Furthermore, it might be that in the case of vesicles formed from block copolymers an apolar organic solvent is needed to stabilize and fluidize the hydrophobic membrane core.

Vesicles in which the membranes are constituted from lipids present in biological membranes (particularly phospholipids such as POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) are often called *lipid vesicles*[4,5] or more frequently *liposomes*,[6,7] although they are not at all "fat bodies", which one might expect from the name. To distinguish vesicles prepared in vitro from vesicles existing in vivo, that is, the small, membrane-bounded, spherical organelles in the cytoplasm of eukaryotic cells,[8] the term *artificial vesicles*[9] is sometimes also used instead of liposomes or vesicles. Finally, vesicles prepared from synthetic surfactants, which are chemically different from naturally occurring amphiphilic lipids, are also called *(synthetic) surfactant vesicles*,[10] while vesicles made from polymers are referred to as *polymersomes*.[2,3] In the following, we will use the general term *vesicle* to include all types of vesicles prepared in vitro. To a first approximation, the general principles of vesicle formation as well as the general properties of vesicles are independent of the type of vesicle-forming amphiphile used.

In this review, we will focus on vesicles that have diameters in the range of about 1–100 μm, the size range of most biological cells.[11] These types of vesicles are called *giant vesicles*[12] due to their remarkably large, that is, "giant", size. We further limit our review to giant vesicles with a relatively uniform size and with a shell that is composed of only one or a few bilayers. Giant vesicles are usually investigated by light microscopy. The limited resolution of light microscopy, however, does not allow to readily distinguish unilamellar from oligolamellar vesicles, except if unilamellarity is confirmed by corresponding bending elasticity measurements,[13] or if the lamellarity is determined with a fluorescence quenching assay using a fluorescent membrane probe and a water soluble quencher.[14]

Depending on the experimental conditions, such as the osmotic pressure difference between the vesicle's interior and exterior, giant vesicles can be spherical or nonspherical.[15] Table 1, lists selected recent examples of amphiphiles from which the formation of giant vesicles has been reported. Although their size is often larger than 1 μm, multilamellar vesicles (MLVs) with their onion-like structures are *not* considered here.

2. Simple Geometric Considerations

A giant unilamellar vesicle (GUV) resembles the basic compartment structure of all biological cells, in the sense that the vesi-

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cle membrane is a mimic of the self-closed lipid matrix of the plasma membrane. To illustrate the geometric properties, a spherical unilamellar giant vesicle with a diameter of 50 μm and a membrane thickness of 4 nm is first considered; 4 nm is the approximate thickness of a hydrated POPC bilayer.[16] It is astounding that a membrane that is only 4 nm thin can separate the inside of such a large object from the external aqueous solution. If extrapolated to sizes with which humans are more familiar, the relationship between vesicle diameter and membrane thickness becomes more obvious. Taking instead of a 50 μm giant vesicle a balloon with a diameter of 50 m, this balloon would have a skin with a thickness of only 4 mm.

If constituted from POPC, one single 10 μm sized unilamellar vesicle is composed of about 8.7×10^8 POPC molecules, with a calculated internal aqueous volume of 5.22×10^{-13} L (=522 fL). In the case of a 50 μm unilamellar POPC vesicle, as many as 2.2×10^{10} POPC molecules constitute the membrane of one single vesicle, enclosing an internal aqueous volume of 6.54×10^{-11} L (≈65 pL). If compared with conventional large unilamellar vesicles (LUVs) with diameters of about 100 nm or less, as used for drug delivery applications,[17] the internal volume of giant vesicles is extremely large, because the vesicle volume scales with the third power of the radius. This means that a tenfold increase in vesicle diameter corresponds to a 1000-fold increase in entrapped volume. To further illustrate this, let us consider a POPC LUV with a diameter of 100 nm. The membrane of this LUV is composed of 8.1×10^4 POPC molecules, and the corresponding calculated internal volume of one single vesicle is only 4.16×10^{-19} L (≈0.0004 fL). One of the consequences of the size difference between LUVs and GUVs is clearly emphasized with the following practical example. If the vesicle's internal aqueous solution contains a water soluble compound, for example, a fluorescent dye molecule or a protein, at an assumed concentration of 20 μM, there are about 6.3×10^6 molecules enclosed in a single 10 μm GUV, as compared to an average of five molecules inside a 100 nm LUV. It is therefore expected that stochastic fluctuations in the number of entrapped molecules inside LUVs play an important role, particularly if LUVs are compared with giant vesicles on the basis of chemical reactions inside the vesicles.[18]

Due to their large size, giant vesicles can be observed by optical microscopy, allowing for example a real time monitoring

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of morphological changes of individual vesicles and of chemical and enzymatic reactions occurring on the surface of or within a single vesicle, see later in Section 4, Table 2.

In the following, we will first describe the key concepts for the formation of giant vesicles with a short discussion of the advantages and disadvantages of the different methods, followed by a description of some of the current applications.

3. Methods for Preparing Giant Vesicles

Historically, one of the first methods for the preparation of giant vesicles was described by Reeves and Dowben in 1969.[19] It was a controlled hydration of a thin dry film of egg yolk phosphatidylcholines (egg PC) deposited from 0.5 mL of a 5 μM chloroform/methanol (1:2, *v/v*) solution on the glass surface on the bottom of a flat-bottomed 2 L Erlenmeyer flask. Before hydration, the lipid film was carefully dried by passing nitrogen gas through the flask, "taking care not to disturb or agitate the solution".[19] Hydration of the film was initiated by circulating water-saturated nitrogen through the flask for 15 min or more, followed by addition of an aqueous solution, which was "gently poured down the side of the flask".[19] The lipid film was allowed to swell for two hours or more, and this led to the formation of giant thin-walled vesicles. "Even slight agitation during the swelling period tended to diminish the yield of thin-walled vesicles".[19]

Table 1. Selected examples of bilayer-forming amphiphiles (lipids and diblock copolymers) from which giant vesicles could be formed successfully. The methods used for giant vesicle formation are indicated.

Method[a]	Amphiphile[b]	Remarks	Lit.
1a	Egg PC/egg PG (9:1) Egg PC/egg PA (9:1) Egg PC/bovine brain PS (9:1) Egg PC/bovine heart CL (9:1) Egg PE/egg PG (9:1)	Importance of the presence of negatively charged lipids and prehydration with water saturated nitrogen for giant vesicle formation in physiological salt solutions.	[27]
	Egg PC/DOPG		[35]
	DOPC	Presence of glucose, mannose or fructose in the dry lipid film; hydration with deionized water and saline buffer (up to 0.1 M NaCl).	[26]
		Basic study of the hydration process. No vesicle formation from DPPC at room temperature, that is, below T_m of DPPC.	[28]
	DOPC/DOPE-PEG$_{2000}$ (98:2)	Vesicle formation in aqueous solutions containing up to 2 M salt.	[30]
	POPC, DOPS, POPG, Asolectin POPC/cholesterol (9:1 and 8:2) POPC/POPG (1:1 and 9:1) POPC/DOPS (9:1) POPC/DPPE-PEG$_{2000}$ (95:5)	Hydration of "hybrid films" containing lipids and agarose in physiological buffer solution.	[33]
	PEO-*b*-PBO ($E_{16}B_{22}$, $M_w = 2300$)	Use of patterned surfaces. Spontaneous formation of controlled-size vesicles.	[34]
1b	Egg SM/DOPC/cholesterol		[142, 143]
	Cerebrosides/POPC/cholesterol		[144]
	Egg PC Egg PC/POPE (1:1) Egg PC/DOPS (8:2 and 9:1) Asolectin POPC	Surface-attached giant vesicles are filled with water-soluble molecules during formation in a flow chamber; conditions of high ionic strength (up to 2 M NaCl).	[48a]
	Egg PC/egg PA	Prepared in microfluidic channels.	[49]
	DOPC	Preparation of polysaccharide-coated giant vesicles.	[145]
	DOPC	Lipid deposition from a w/o emulsion.	[40]
	DOPC/POPS	Too many negatively charged lipids do not favor the formation of giant vesicles.	[42]
	DMPC/cholesterol (7:3) DPPC/cholesterol (7:3)		[146]
	SOPC POPC Soybean PC	Lipid deposition from preformed SUV and LUV. Giant vesicle formation in aqueous solutions up to 0.25 M NaCl possible.	[39]
	Native erythrocyte membranes	Lipid deposition from preformed SUV and LUV. Giant vesicle formation in physiological salt solution possible.	[147]
	POPC POPC/cholesterol DOPC	Basic study on the kinetics of vesicle formation.	[38]

The detailed description in the paper of Reeves and Dowben[19] was important because experimental details for a successful preparation of defined giant vesicles are essential. If the hydration of a dry film of egg PC is carried out under simultaneous shaking of the flask containing the deposited lipids, most of the lipids will arrange into multilamellar vesicles (MLVs, often with sizes above 1 μm)[20] and only a few GUVs will coincidentally form. Once present as MLVs, there will be no conversion into GUVs because this process would involve the energetically unfavourable peeling off of the different layers of the onion-like MLV structure and the arrangement into one large self-closed bilayer constituting a GUV. If external energy is put into the system, MLVs will be broken down to vesicles that are much smaller in size, usually below 0.5 μm. So-called sonicated (or small) unilamellar vesicles (SUVs, typically with diameters below 50 nm) form if the external energy is sonication;[5,21] LUVs (with diameters often around 100 nm) can be obtained by forcing MLVs through polycarbonate membranes containing cylindrical pores with typically 100 nm diameter (so-called extrusion process).[5,22]

GUVs, as well as LUVs and SUVs are aggregates that are usually not at a "true" thermodynamic equilibrium, but rather in a kinetically trapped state.[23] The

Table 1. (Continued)

Method[a]	Amphiphile[b]	Remarks	Lit.
2a	DOPC Egg PC	Mineral oil as solvent.	[57]
	POPC Egg PC/cholesterol (8:2) Egg PC/DOPS (8:2) PABu-PAM	Dodecane as solvent.	[56]
	DPPE DOPC DMPC DPPC	Oleic acid as solvent.	[65]
	POPC/DPPC/cholesterol (3:3:4) DOPC	Preparation of giant vesicles with asymmetric bilayers. Mineral oil as solvent.	[57]
	PEO-*b*-PBD PEG-*b*-PA444	Toluene as solvent.	[64]
	DOPC/POPS	Too many negatively charged lipids do not favor the formation of giant vesicles.	[42]
	Asolectin	Stable vesicles only at elevated surfactant concentration. Dodecane, tetradecane, hexadecane or olive oil as solvent.	[148]
2b	Egg PC/cholesterol/SA (5:5:1)	"Lipid-coated ice droplet hydration method". Hexane as solvent.	[68]
3	DMPC DPPC POPC DOPC DSPC DPPS DPPC/DPPS (10:1)	Mixture of toluene and chloroform as solvent.	[69b]
	PBA-*b*-PAA	Mixture of tetrahydrofuran and toluene as solvent.	[69a]
	PEG_{5000}-*b*-PLA_{5000}	Mixture of toluene and chloroform as solvent.	[69c]
4	Oleic acid/oleate (1:1)	Giant vesicles did not form from phospholipids like POPC.	[67]
	Egg PC		[74]
5	DPhPC	Simultaneous formation and loading of GUVs by microfluidic jetting.	[77]
		Continuous generation of size controlled giant unilamellar vesicles from a microfluidic T junction.	[78]
6	COS-g-PCL		[81]
7	Egg PC		[82]
8	Egg PC DPPC		[84]

[a] See Figure 1 and text. [b] Abbreviations used: CL, cardiolipin; PA, phosphatidic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PS, phosphatidylserine; SM, sphingomyelin; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DPhPC, 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; SOPC, 1-stearoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DOPE-PEG_{2000}, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000]; DPPE-PEG_{2000}, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000]; SA, stearylamine; PEO, poly(ethylene oxide); PBO, poly(butylene oxide); PBD, poly(butadiene); PEG, poly(ethylene glycol); PA444, a "side-on nematic polymer";[64] PABu-PAM, diblock copolymer composed of a polybutylacrylate block and a polyacrylamide block; PBA, poly(*n*-butylacrylate); PAA, poly(acrylic acid); PLA, poly(lactic acid); COS-g-PCL, graft copolymer of chitooligosaccharide (COS) as backbone and polycaprolactone (PCL) as branches.

energy barrier often is so high that the "true" thermodynamic equilibrium of the system can not be reached easily. Once formed, the vesicles might remain as a kinetically stable system for an extended period of time, which can be hours, days or even weeks. This is the reason why the way how the vesicles are formed, that is, the applied preparation procedure, is of great importance. In other words, it is not enough to use for the formation of giant unilamellar vesicles an amphiphile whose chemical structure is prone to self-assemble into a flat bilayer in aqueous solution.[24] It is equally important that the transition into closed bilayers occurs in a defined and to some extent controlled manner.

If a GUV preparation is started with amphiphiles deposited on a flat solid surface, as in the method originally described by Reeves and Dowben,[19] the lipids are already preorganized to some degree in stacks of dry bilayer sheets. Addition of water leads first primarily to a hydration of the hydrophilic head groups and then to a continuous swelling of the bilayers, that is, more and more water molecules penetrate between the bilayers, into the interlamellar space. If this swelling is disturbed mechanically by shaking, hydrated bilayer fragments will be released from the surface into the bulk aqueous solution where they self-close, yielding a heterogeneous population of mainly multilamellar vesicles, which on average are much smaller than the desired giant vesicles. Self-closing occurs as a direct consequence of the elimination of energetically unfavourable interactions between the lipophilic interior of an open bilayer and excess water. Multilamellar giant vesicles as well as elongated, tubular vesicles might also form from a deposit containing too many lipid layers, even if the hydration is carried out in a controlled, undisturbed way.[25] Therefore, not only the hydration conditions, but also the amount and uniformity of the deposited bilayers are important for the formation of unilamellar giant vesicles with the method described by Reeves and Dowben.[19]

In addition to the controlled hydration method originally described by Reeves and Dowben,[19] there are at least seven other principle ways to obtain relatively homogeneous and mainly uni- or oligolamellar giant vesicles (see Figure 1). In the following, the essential features and selected basic aspects of all main methods are briefly summarized. Some of these meth-

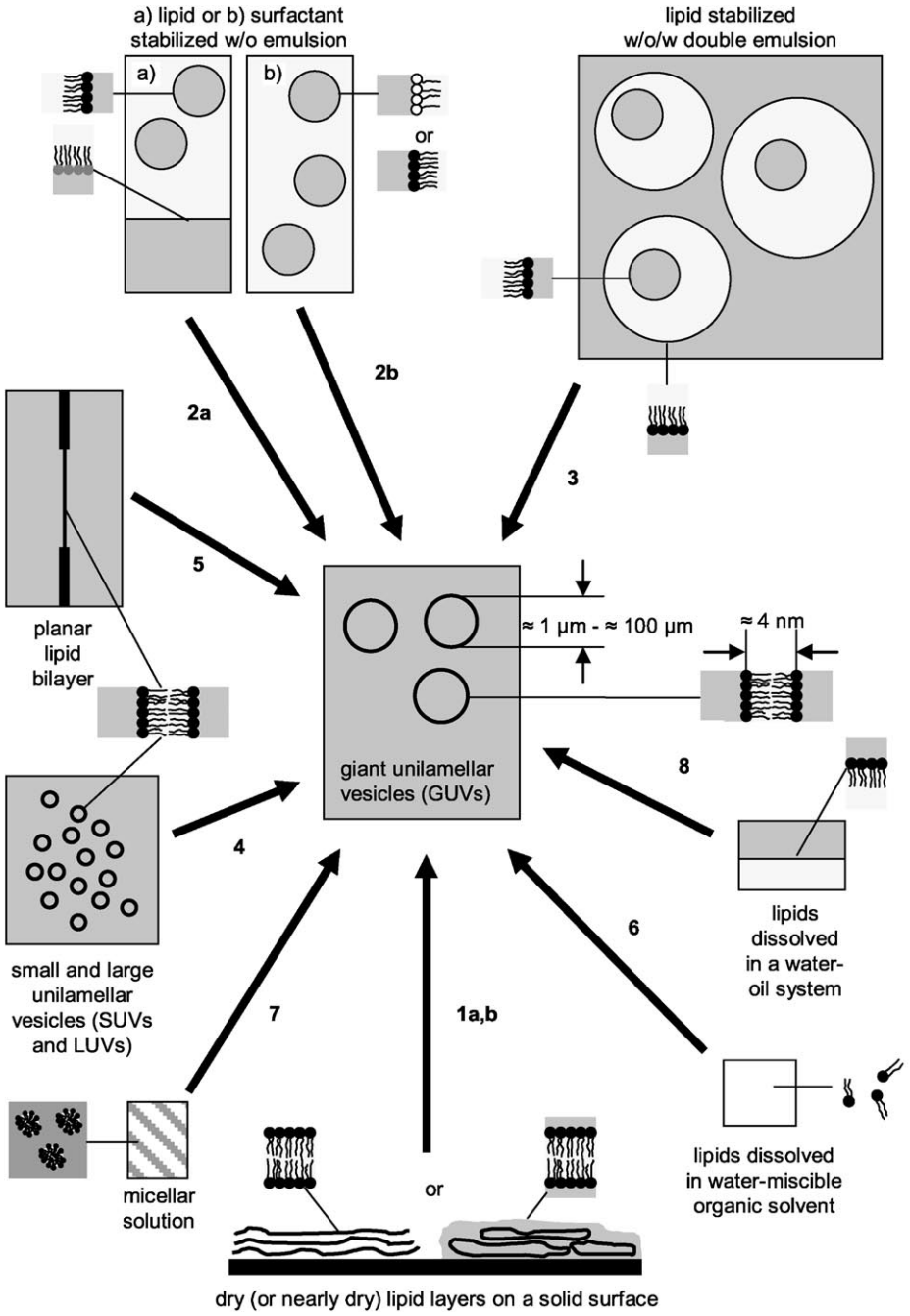

Figure 1. Schematic representation of the key concepts of the main methods for the formation of giant unilamellar vesicles (GUVs). The methods are the following. Method **1:** Controlled hydration of dried (or nearly dried) films of lipids deposited on a solid surface. The hydration is carried out under undisturbed conditions, either in the absence of any applied electric field (method **1a**: spontaneous swelling or gentle hydration), or in the presence of an applied electric field (method **1b**: electroformation or electroswelling), see also Figure 2. Method **2:** Transformation of a lipid-stabilized w/o emulsion (method **2a**) or a surfactant-stabilized w/o emulsion (method **2b**) into giant vesicles, see also Figure 3. Method **3:** Transformation of a lipid stabilized w/o/w double emulsion into giant vesicles. Method **4:** Giant vesicles formed by the fusion of small unilamellar vesicles (SUVs) or large unilamellar vesicles (LUVs). Method **5:** Giant vesicles formed by jet-blowing onto an initially planar lipid bilayer kept between two aqueous solutions. Method **6:** Giant vesicles formed from lipids dissolved in an organic solvent that is miscible with water. Method **7:** Giant vesicles formed from micellized bilayer-forming lipids. Method **8:** Giant vesicles formed from bilayer-forming lipids that are initially present in a w/o two-phase system. For each method, the starting states are schematically indicated in which the lipids are preorganized to some extent. A "lipid" is considered here as a bilayer-forming amphiphilic molecule, like corresponding phospholipids that occur in biological membranes. The lipid molecule is represented with one hydrophilic head (black or grey circles) and two lipophilic chains. The Figure applies also for the preparation of giant vesicles from bilayer-forming amphiphiles that are structurally very different from phospholipids, that is, bilayer-forming mixtures of appropriate single chain amphiphiles or bilayer-forming amphiphilic diblock (or triblock) copolymers. Giant vesicles from amphiphilic triblock copolymers that tend to form monolayers by self-assembling in a stretched conformation[141] can be prepared by adopting the schemes in which the lipid analogues are preorganized in bilayers. The term "surfactant" is used here for an amphiphilic molecule which itself does not form bilayers in aqueous solution, but rather helps in stabilizing w/o emulsion droplets (amphiphile with white head group). The range of typical giant vesicle sizes is indicated (from ≈1 μm–100 μm). The approximate bilayer thickness (4 nm) is given for a bilayer of POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine).

ods are currently more often applied than others; some methods appear to be more convenient than others for certain selected applications (see later). Some methods have only recently been developed and need further exploration with respect to their possible wider applicability.

3.1. Giant vesicles formed by the lipid film hydration method (method 1)

The principle of the method is the one just described (Figure 2). It consists of the controlled hydration of (dry) lipids deposited on a solid surface, mainly glass, as originally developed by Reeves and Dowben.[19] This method is also known as spontaneous swelling, natural swelling or gentle hydration method (method **1 a**).[26] The method is usually particularly successful for the preparation of GUVs from samples containing charged lipids.[27] Furthermore, it is important to note that the hydration has to be carried out in the liquid-disordered state of the bilayers,[28] that is, at a temperature above T_m, corresponding to the so-called solid-ordered (s_o)/liquid-disordered (l_d) or gel/liquid-crystalline main phase transition temperature.[5] A recent AFM study reported on a correlation between physical state of the lipid layers and ability to generate giant vesicles:[29] hydration of smooth, flat layers in the l_d phase resulted in the successful formation of giant vesicles, while hydration in the s_o phase did not yield giant vesicles.[29] Formation of giant vesicles *in physiological salt solutions* was shown to be possible from mixed lipid films composed of PC or PE and 10–20 mol% of a charged lipid (e.g., PG, PS, PA, CL, for abbreviations, see Table 1).[27] In these studies, hydration of the dried film was first carried out by using water-saturated nitrogen gas ("prehydration"), followed by hydration with the desired aqueous solution, similar to the descriptions given by Reeves and Dowben.[19] The importance and necessity of the "prehydration" step was emphasized if vesicle formation was carried out at high ionic strength.[27] Similarly, giant vesicle formation with the gentle hydration method (method **1 a**) is possible from lipid mixtures containing pegylated lipids, that is, lipids modified with the hydrophilic polymer poly(ethylene glycol), PEG, which is covalently attached to the hydrophilic head group of the lipid.[30] After integration of PEG-conjugated lipids into the bilayer, the polymer chains protruding from the membrane surface into the surrounding medium increase the repulsive force between the membranes; this promotes the formation of giant vesicles and confers a higher stability to the resulting vesicles. Vesicle formation was possible in aqueous solutions containing up to 2 M salt (NaCl or $CaCl_2$).[26,30]

During the last forty years several modifications and improvements to the original spontaneous swelling method have been published. It was shown that giant vesicle formation by the gentle hydration method (method **1 a**) is not only possible from mixtures containing a certain amount of charged lipids, but also from neat zwitterionic phosphatidylcholines (DOPC) if the dry lipid film contains nonelectrolytic monosaccharides (glucose, mannose or fructose).[27] The presence of these neutral molecules increases the spacing between the lipid layers during the swelling process due to an enhanced water movement toward the interlamellar space, as a result of osmotic pressure differences.[27] It has been suggested that a similar osmotic effect plays a role in the observed formation of giant DOPC vesicles in deionized water or in the presence of buffer ions if $MgCl_2$ is added to the hydration medium.[31]

Karlsson et al.[32] first prepared soybean PC SUVs that were dehydrated on a borosilicate cover slip at 4 °C, followed by a careful rehydration at room temperature. In another study, the hydration of *partially dried* "hybrid films" composed of lipids (e.g., POPC or POPC/POPG (1:1)) and agarose was studied, and it was found that giant vesicle formation is possible in solutions of physiological ionic strength.[33]

Recently, the use of patterned surfaces prepared by photolithography and adsorbed amphiphilic diblock copolymers (PEO-*b*-PBO) resulted in the formation of giant polymersomes with a narrow size distribution.[34] It seems that the spatially separated deposition of the block copolymers controlled the size of the vesicles obtained upon hydration due to a limitation of the amount of deposited polymers present.

Encapsulation of macromolecules during formation of giant vesicles by lipid film hydration does not generally occur efficiently (see below). It was shown, however, that the encapsulation yield can be increased if "polymeric coencapsulants" acting as macromolecular crowding agents are added to the aqueous solution containing the macromolecules to be encapsulated, for example, 10 wt% PEG_{2000}.[35]

In 1986, Angelova and Dimitrov published a study concerning the effect of externally applied electric fields on the hydration of lipids deposited from an organic solution on a conductive glass surface (indium tin oxide (ITO) coated glass), or on platinum wires.[36] Depending on the experimental conditions, that is, type, composition and amount of lipids used, applied electric field parameters, hydration medium, rather homogeneous giant unilamellar vesicles can

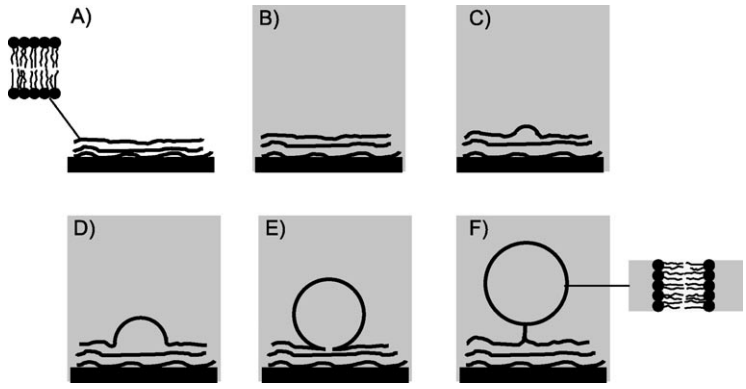

Figure 2. Representation of the formation of giant vesicles by controlled hydration of a film of bilayer-forming lipids deposited onto a solid surface (method **1**), adopted from Shimanouchi et al.[38] Application of an external electric field (method **1 b**) leads to a better control of the hydration process. In the absence of an electric field, the method is called gentle hydration, natural swelling or spontaneous swelling (**1 a**), see also Reeves and Dowben,[19] Angelova,[36b] Mertins et al.[40]

be obtained in aqueous solution or buffer solutions of low ionic strength. The method is known as electroformation or electroswelling method (method **1 b**).[36b,37,38] In some recent modifications of the electroformation method, the lipids were not deposited from an organic solution but from an aqueous suspension containing preformed SUVs or LUVs.[39] In this latter case, the lipid film was not completely dried after deposition, as schematically emphasized in Figure 1 on the right hand side of the drawing, which illustrates the possible starting states of method **1**. Adopting this modification of the original "electroformation recipe" and adjusting the applied voltage of the electric field, it was possible to prepare giant vesicles in aqueous solutions containing as much as 250 mM NaCl.[39] Similarly, lipid deposition was also made from a water-in-oil (w/o) emulsion system consisting of a volatile water-immiscible organic solvent (chloroform) in the continuous oil phase.[40] The oil was removed after deposition, leaving a hydrated mass of lipids on the electrode before complete hydration with excess water was initiated in the presence of an applied electric field.[40] Incorporating a water-soluble substance (chitosan) in the disperse phase of the precursor w/o emulsion and applying the electroformation method to the deposited reversed-phase film allows the preparation of giant vesicles doped with water-soluble compounds.[40]

With the standard electroformation method,[36,41] unilamellar giant vesicles do not form if the lipid film contains too many charged lipids.[42] Mixtures of DOPC/DOPS (8:2), for example, did not yield giant vesicles with the electroformation method (method **1 b**), while with the gentle hydration method (method **1 a**), for which the presence of charged lipids is beneficial,[28] vesicle formation was observed under otherwise identical conditions.[42]

Giant vesicles prepared by electroformation usually exhibit long, thin protrusions that can be stable for many hours.[43] This means that electroformed vesicles remain connected to the residual lipid film on the electrode on which they are formed (Figure 2). This might be an undesired property or a big advantage, particularly if individual vesicles shall be punctured with a microneedle (see below). The electrode provides a back pressure and the connected vesicles do not move away easily during micromanipulation.

There are some concerns about a possible partial lipid oxidation during electroformation, particularly if polyunsaturated lipids are deposited on the electrode, for example, 1,2-dilinolenoyl-*sn*-glycero-3-phosphocholine (di-C18:3 PC).[44] Addressing this potential drawback of the method, it was reported by Okumura et al.,[45] that direct contact of the lipids with the electrodes can be avoided. Hydration of egg PC deposited on a glass tube that is mounted parallel *between* two platinum wires under optimized conditions leads to giant vesicle formation after application of an external electric field.[45]

The electroformation method (**1 b**) was also applied to lipids (DMPC) deposited on micropatterned ITO glass surfaces.[46] The average size of the vesicles obtained was controlled to some extent by the size of the micropatterned features. In another study, egg PC giant vesicles were formed by electroformation from a lipid film deposited on a patterned—natural or chemically modified—silicon substrate.[47] With a change of the chemical composition and microstructure of silicon, it was possible to influence to some extent the physicochemical properties of the vesicles obtained.[47]

In a modification of the original electroformation method (**1 b**), giant vesicles were formed on ITO electrodes assembled in a flow chamber by using solutions of glycerol as hydration medium, which was subsequently replaced inside and outside of the vesicles, by flowing an aqueous solution of high ionic strength (up to 2 M KCl) through the chamber.[48a] The vesicles remained attached to the ITO electrodes even after exchange of the bulk solution. It seems that the presence of glycerol in the initial solution was essential for the observed exchange of the intravesicular solutions.[48b] Interestingly, macromolecules could be entrapped inside the giant vesicles by growing them in a solution containing the macromolecules, followed by solution replacement outside the vesicles.[48a] It seems that this loading of the vesicles occurred through tubules connecting the vesicles to the lipid film on the surface of the electrode,[48b] see above.

Kuribayashi et al.[49] presented an on-chip method for the preparation of giant vesicles by electroformation in microfluidic channels. The exchange of the external solution was reported to be easily possible after vesicle formation.[49]

With some reported exceptions,[35,48] the spontaneous swelling (**1 a**) and electroformation (**1 b**) methods generally do not allow efficient encapsulation of large water-soluble molecules (e.g., enzymes) or charged compounds during the formation process because the molecules to be encapsulated have to somehow move below the outermost layer of the deposited lipid film; this is difficult due to slow transbilayer movements of large or charged molecules. Encapsulation is, however, possible after vesicle formation by microinjection,[50–53] puncturing individual vesicles with microneedles. Because the vesicles are already filled with water, the volume that can be injected without vesicle rupture is, however, comparatively small (up to a few pL),[50,51] and this limits the concentration of entrapped molecules. Large concentrations of entrapped molecules can therefore not be achieved. Microinjections are conveniently carried out with GUVs electroformed on a platinum wire rather than on ITO-coated glass.

Method **1 b** is currently the most widely used method for preparing giant vesicles for different research applications, such as the visualization and analysis of lateral bilayer heterogeneities (lipid rafts) in mixed lipid membranes containing fluorescently labeled lipids, see Table 2. Because electroformed giant vesicles remain attached to the support on which they are formed, some of the physicochemical properties of these vesicles are expected to be different if compared to free vesicles in suspension. Therefore, one might wish to detach electroformed vesicles from the electrode and place them into an equiosmolar medium. For the visualization of detached vesicles by light microscopy, giant vesicles are often prepared by electroformation in a sucrose solution before they are transferred into excess glucose solution.[14,54,55] This leads at the same time 1) to a higher contrast between the vesicle's interior and the external solution, and 2) to a movement of the vesicles to the

bottom of the observation chamber due to the density differences between the entrapped sucrose solution and the outer glucose solution.

3.2. Giant vesicles formed from a lipid-stabilized (method 2a) or from a surfactant-stabilized (method 2b) w/o emulsion

Currently, there are two main procedures for giant vesicle formation that start from a similar initial state, a w/o emulsion. The two methods differ conceptually in the actual procedures leading to the formation of the desired giant vesicles. Therefore, the two procedures are described separately.

*The w/o emulsion transfer method **2a*** (Figure 3 A). If a (stable) w/o emulsion can be prepared from an oil and a bilayer-forming lipid, this w/o emulsion can be used as a starting system for the preparation of giant vesicles.[56,57] The w/o emulsion is poured onto a two-phase system consisting of a lower aqueous phase and an upper oil phase (for example, dodecane,[56] mineral oil,[57] or squalene[56]) containing a bilayer-forming lipid (for example, POPC or egg PC/cholesterol (8:2)[56]). If the two-phase system is first preincubated for a while *prior* to the addition of the w/o emulsion, the interfacial region between the two phases will become saturated with lipids, and form a monolayer in which the hydrophilic head groups of the lipids are in contact with the lower aqueous phase and the lipophilic chains protrude into the upper oil phase containing excess lipid. Because water is heavier than the oil used, the water droplets of the upper w/o emulsion tend to move from the w/o emulsion toward the oil/water interface and finally through it into the lower aqueous phase where giant vesicles are formed. If spontaneous water droplet migration does not occur, or only slowly proceeds, centrifugal forces can be applied to force the water droplets to move from the upper w/o emulsion through the interface into the lower aqueous solution.[56] Water droplet migration can also be promoted by preparing the w/o emulsion from a sucrose solution and by using an equiosmotic glucose solution as lower aqueous phase.[58,59] A direct observation of the transfer of a water droplet from a w/o emulsion to the aqueous phase was conducted by guiding the droplet across the w/o interface by using micromanipulation techniques.[60]

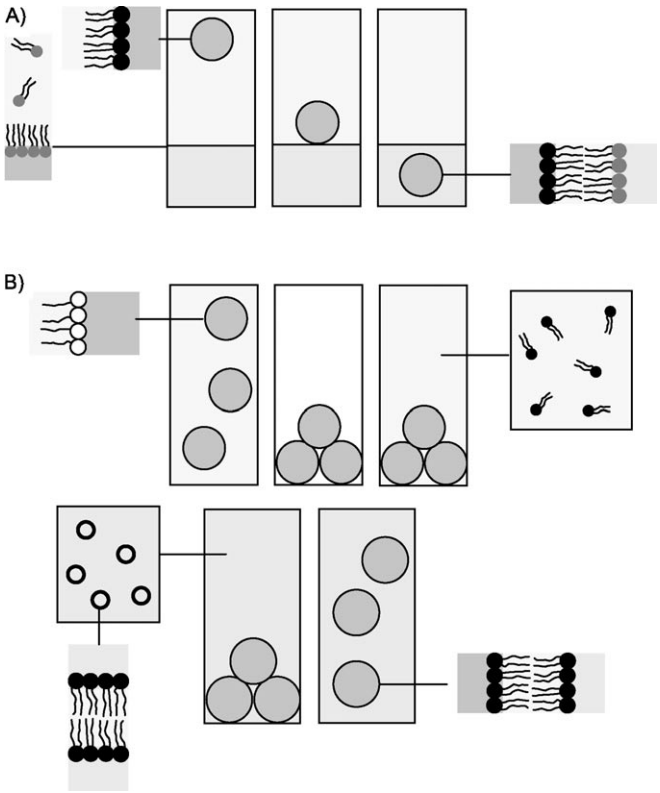

Figure 3. Representation of the key steps in the preparation of giant vesicles from an initial w/o emulsion (method **2**). A) An initial lipid-stabilized w/o emulsion is brought into contact with a less dense aqueous phase; this leads to vesicle formation as the lipid-coated water droplets migrate through the lipid-saturated o/w interface (method **2a**), see Pautot et al.[56,61] B) Starting from an initial surfactant-stabilized w/o emulsion from which the surfactant is replaced with lipids, while keeping the dispersed water droplets in a frozen state, and the oil is replaced with an aqueous solution (method **2b**), see Sugiura et al.[68a]

The preorganization of the lipids present in the final giant vesicles prepared by the w/o emulsion-transfer method (method **2a**) occurs 1) at the surface of the water droplets of the w/o emulsion (expected to constitute mainly the inner monolayer of the final giant vesicle membrane); and 2) at the interface between the lower aqueous phase and the upper w/o emulsion (expected to constitute the outer layer of the vesicle membrane), Figure 3 A. Overall, method **2a** involves the assembly of a second layer of lipids on a lipid-coated water droplet. Based on this building principle, the preparation of giant vesicles with a (transient) asymmetric transbilayer lipid distribution seems to be possible,[58] similar to what has been shown previously for sub-micrometer-sized vesicles,[61] see also Whittenton et al.[62] It is likely that the membrane of the final giant vesicles contains some oil originating from the w/o emulsion. The size of the water droplets of the initial w/o emulsion determines the size of the giant vesicles if the droplet size does not change significantly during the water droplet migration. In this review, method **2a** is called "w/o emulsion transfer method", originally described as "inverted-emulsion method" by Pautot et al.[56] Even millimeter-sized giant vesicles were prepared by the w/o emulsion transfer method,[63] although in this case the water droplets did not constitute the disperse phase of an emulsion, but were rather added to the supernatant oil phase from a syringe.[63] The method was not only applied for the preparation of giant vesicles from low molar mass lipids, but also from amphiphilic diblock copolymers.[56,64]

In another approach, again starting with a lipid-stabilized w/o emulsion, Tan et al.[65] reported that giant vesicles can be formed from an initial w/o emulsion stabilized with a phospholipid (DMPC, DPPC, DOPC or DPPE) by simple addition of this w/o emulsion to an ethanol/water mixture in a microfluidic device. In this case, the oil was oleic acid and the ethanol content in the final vesicle suspension reached values as high as 30 wt%.[65] It is likely that the membrane of the vesicles formed contains substantial amounts of oleic acid and oleate because it is well known that mixtures of the neutral and the

ionized forms of fatty acids form bilayers, that is, small or even giant vesicles.[66,67]

The lipid-coated ice droplet hydration method ***2b*** (Figure 3B). More recently, surfactant-stabilized w/o emulsions generated by microchannel emulsification were used as starting system to prepare uniform giant vesicles.[68] The key idea behind this method is the same as in method **2a**, to preserve the water droplet size during the preparation. In the case of method **2b**, however, the water droplets of the w/o emulsion are effectively stabilized with an appropriate surfactant mixture (Span 80 and dodecylamine)—and not with bilayer-forming lipids. The reason for this is the difficulty in obtaining stable w/o emulsions from bilayer-forming lipids, such as POPC. In contrast to method **2a**, here, the formation of a stable w/o emulsion is essential for an efficient preparation of giant vesicles, as outlined in the following.

The water droplets of the emulsion are frozen by transferring the emulsion system into liquid nitrogen. Thereby, the oil phase (hexane) remains in a liquid state. After 1) replacement of the surfactant mixture with the bilayer-forming lipids under conditions at which the water droplets remain frozen; and 2) replacement of the oil with an aqueous suspension containing small vesicles made from the lipids of which the final vesicles will be constituted (hydration step), the formation of homogeneous and stable giant vesicles can be achieved in a highly reproducible way.[68] The size of the initial water droplets of the w/o emulsion directly correlates with the size of the giant vesicles obtained. Although the vesicle membrane might not be strictly unilamellar but rather might contain a few layers, possibly with small amounts of oil, this lipid-coated ice droplet hydration method (**2b**) allows the preparation of giant vesicles with high entrapment yields.[68] The water soluble molecules originally present inside the water droplets of the surfactant-stabilized w/o emulsion are almost fully recovered inside the giant vesicles obtained. Leakage during the preparation can be minimized by adjusting the experimental conditions. With this method, the assembly of the lipids occurs on the frozen droplets during the surfactant replacement and hydration steps. The preparation of giant vesicle suspensions in which the aqueous pool inside the vesicles is different from the external bulk solution is straightforward with this method. Overall, method **2b** involves 1) the replacement of surfactant molecules which originally stabilize the water droplets in the w/o emulsion with lipid molecules, and 2) the exchange of the oil of the initial w/o emulsion with an external aqueous solution.

3.3. Giant vesicles formed from a lipid-stabilized w/o/w double emulsion (method 3)

Microfluidic techniques allow production of not only defined w/o emulsions but also defined w/o/w double emulsions. Such lipid stabilized w/o/w double emulsions can be used as starting systems for the preparation of giant vesicles.[69] In this case, the oil is a volatile organic solvent which is not miscible with water (e.g., a 2:1 mixture of toluene and chloroform).[69b,c] In a lipid-stabilized w/o/w double emulsion an inner water droplet is localized within an oil droplet which again is in an outer bulk aqueous phase, see Figure 1. Each internal water droplet is coated with lipid molecules (for example, DMPC or POPC[69b] or a diblock copolymer[69a,c]) in such a way that the hydrophilic head groups face towards the aqueous inner part of the droplet and the lipophilic part towards the oil droplet, which itself is coated with lipid molecules with the hydrophilic head groups facing towards the external aqueous bulk phase.

Removal of the volatile oil leads to the formation of giant vesicles in which the lipids originally covering the innermost water droplets of the w/o/w double emulsion are expected to mainly constitute the inner monolayer of the giant vesicle bilayer. The lipids adsorbed to the surface of the oil droplet of the w/o/w double emulsion, localized in the interfacial region which is in contact with the bulk aqueous phase, will mainly constitute the outer monolayer of the giant vesicle bilayer. Because the size of the initial water droplet is much smaller than the size of the surrounding oil droplet, there are more lipids present on the oil droplet than on the inner water droplet. Hence, a considerable lipid rearrangement needs to take place during vesicle formation, possibly leading to lipid aggregates within the vesicle membrane. Furthermore, complete removal of the oil might not be easy. The method, however, allows the efficient encapsulation of large amounts of water soluble molecules, originally present in the inner water droplets of the w/o/w double emulsion.[69b,c] With this method **3**, the final size of the giant vesicles is controlled by the size of the inner water droplet of the w/o/w double emulsion. Conceptually, the preorganization of the lipids occurs on the water and oil droplets in a w/o/w double emulsion. Overall, method **3** involves the removal of a volatile oil from a w/o/w double emulsion, which is prepared in a controlled way by microfluidic techniques.

3.4. Giant vesicles formed by fusion of small vesicles (method 4)

The fusion of vesicles is generally difficult because existing closed bilayers have to be opened up into an energetically unfavourable state, and many small vesicles need to fuse to form one giant vesicle. In general, vesicle fusion can occur with vesicles composed of particular lipids under specific conditions, for example, with vesicles containing negatively charged lipids in the presence of divalent cations,[70] with mixtures of vesicles containing oppositely charged lipids,[71] upon addition of fusogenic peptides or other small molecules,[72] upon addition of PEG,[73] etc.

Taking into account the geometric considerations made above for POPC vesicles, one can calculate that roughly 10700 LUVs with a diameter of 100 nm need to fuse ($1.07\times10^4\times8.1\times10^4$ POPC/$LUV_{100\,nm}$) to deliver the lipids needed to form a GUV with a diameter of 10 μm (8.7×10^8 POPC/$GUV_{10\,\mu m}$). Furthermore, the internal volume of one $GUV_{10\,\mu m}$ (522 fL) is so much larger than the volume of 1.07×10^4 $LUV_{100\,nm}$ ($1.07\times10^4\times4\times10^{-4}$ fL = 4.28 fL) that more than 99% (≈518 fL) of the internal aqueous volume of the giant vesicle formed need to originate from the external aqueous medium through a water uptake during the vesicle fusion and size equilibration processes.

During these steps, vesicle leakage might also occur, that is, molecules originally present in the LUVs might not end up quantitatively inside the giant vesicle.

There are only a few reports on the preparation of giant vesicles through the fusion of LUVs. In one case, fusion was induced by applying freezing-thawing cycles in a concentrated solution of electrolyte (KCl or RbCl).[74] In another particular case (fatty acid/soap mixtures), fusion of LUVs into GUVs occurred through interactions of the LUVs with hydrocarbons present on a glass surface.[67]

3.5. Giant vesicles formed from an initially planar bilayer (method 5)

A recently developed and not yet fully explored method for the preparation of giant vesicles uses planar lipid bilayers as starting system (Figure 1). The planar bilayer is first formed between two aqueous solutions in a double-well chamber.[75] Vesicle formation is then induced by detaching fragments of the planar bilayer by jet-blowing an aqueous solution onto the bilayer.[76,77] Giant vesicle formation depends on the precise way in which the aqueous solution is blown onto the bilayer. The method seems to be useful as high-throughput microfluidic system for giant vesicle formation using an inkjet printer device. Small amounts of organic solvent, which is used for the preparation of the planar bilayer, are expected to be present in the vesicle membrane.

Recently, the general concept of the method was applied and elegantly extended for the high-throughput production of hundreds of giant vesicles per minute by a microfluidic process.[78] In this case, the planar lipid bilayer was first formed at a microfluidic T-junction by bringing an aqueous solution in contact with an oil (hexadecane) containing bilayer-forming lipids (1,2-diphytanoyl-*sn*-glycero-3-phosphocholine). After sweeping off the oil with another aqueous solution, a planar bilayer was obtained which was then used as starting system to obtain the final unilamellar giant vesicles. Vesicle formation was induced by two forces, 1) a flow of aqueous solution onto the lipid bilayer at a microfluidic T-junction, and 2) by shear forces induced by in-built mechanical flow resistance in the cross-flow channel.[78]

3.6. Giant vesicles formed from lipids dissolved in a water-miscible solvent (method 6)

The principle of this method is related to the so-called "ethanol-injection method"[5,79] and the method for preparing so-called "ethosomes",[5,80] yielding in both cases vesicles with average sizes typically below 1 μm. Gao et al.[81] showed that giant vesicles form if water is added dropwise under vigorous stirring to a dioxane solution containing the graft copolymer COS-*g*-PCL;[81] COS stands for chitooligosaccharide, which constitutes the backbone, and PCL stands for polycaprolactones, which form the branches of the graft copolymer. Water was added to the COS–*g*-PCL solution until a water content of maximally 50 wt% was reached, yielding stable giant vesicles. Although the mechanism of giant vesicle formation is not known, it seems that small vesicles are initially formed by self-assembly at low water content, followed by a fusion of these vesicles.[81] Whether the method can be applied to other lipid or polymer systems remains to be explored. Furthermore, a considerable amount of dioxane ($\geq$30 wt%) remains in the final vesicle preparation,[81] similar to the "ethosome" system mentioned above (30 wt% ethanol).[5,80]

3.7. Giant vesicles formed from a micellar lipid solution (method 7)

Bilayer-forming lipids do not form micelles in aqueous solutions in which they self-assemble into bilayers (vesicles).[24] However, if the aqueous solution contains enough micelle-forming surfactants or chaotropic ions, the bilayer structure is disrupted and micellization of bilayer-forming lipids is observed. Related to this, it was reported that egg PC giant vesicles can be obtained from a micellar solution of egg PC in water containing high concentrations of chaotropic ions, for example, trichloroacetate, if the ions that promote the formation of micelles are removed.[82] During removal of the chaotropic ions, the lipid micelles transform into lipid bilayers, to a large extent yield giant vesicles, in addition to small vesicles.[82] The reason for the formation of so many giant vesicles is unclear. Method **7** is related to the "detergent depletion method" known for the preparation of submicrometer-sized vesicles.[5,83]

3.8. Method 8

Somewhat related to method **3**, which consists of the removal of a volatile oil from a w/o/w double emulsion, is the procedure described by Moscho et al.[84–86] (method **8**). Here, the starting state is an oil/water two-phase system. The oil phase (a mixture of chloroform and methanol) containing the lipids (egg PC or DPPC) is heavier than the aqueous solution and, therefore, forms the lower phase. The lipids accumulate at the oil/water interface and arrange in such a way that the hydrophilic head groups are in contact with the upper aqueous phase, while the hydrophobic tails are immersed in the oil phase (Figure 1). Evaporation of the oil leads to the formation of giant vesicles which are, however, rather heterogeneous in size.[84] Giant vesicle formation is believed to occur during the evaporation of the oil as it passes through the oil/water interface; this induces the rupture of the lipid monolayer into fragments and transforms these fragments into closed bilayers, that is, small and mainly giant vesicles.[84]

4. Applications of Giant Vesicles

Due to their cell-mimicking characteristics, GUVs currently are intensively studied in different areas of biomimetic chemistry, biomembrane physics and in the field of artificial cell synthesis (Table 2). In many studies that focus on mimicking biological membranes, for example by reconstituting membrane components in giant vesicle bilayers, the preparation of giant vesicles with thin unilamellar walls and a defined size and shape is often required.

Table 2. Selected examples of applications of giant vesicles. The methods used for giant vesicle formation are indicated.

Application	Method[a]	Lit.
Investigation of the mechanical properties of entire giant vesicles or of giant vesicle membranes	**1 b**	[13, 87]
Imaging and investigation of the lateral lipid heterogeneity in the vesicle membrane as model system of the lipid domain formation in biological membranes	**1 b** **1 a**	[143, 144, 149–154] [155]
Study of the lipid membrane dynamics	**1 b**	[156]
Study of lipid order and membrane fluidity	**1 b**	[157]
Investigation of vesicle growth and shape changes	**1 b** **1 a**	[92, 146, 158, 159] [15a]
Imaging and investigation of membrane fusion of vesicles as bio-membrane model system	**1 a** **1 b**	[105] [72a, 160]
Study of membrane budding and fission	**1 a** **1 b**	[94, 106, 155, 161, 162] [142, 150, 160, 163]
Investigation of the permeabilization of vesicle membranes by pulsed electric fields	**1 b**	[164]
Investigation of the effect of millimeter electromagnetic waves on the size and shape of the vesicles	**1 b**	[165]
Study of the effect of oxidative stress on the vesicles	**1 b**	[166]
Study of protein (peptide) lipid bilayer interactions	**1 a** **1 b**	[107, 108, 167–169] [52, 54, 156, 163, 170–174]
Study of the interaction of virus-like particles with lipid bilayers for understanding virus-membrane interactions	**1 b**	[175]
Use of giant vesicles as biomimetic system of the mitochondrial cristae formation	**1 b**	[176]
Investigation of giant vesicles with a reconstituted cytoskeleton	**1 b**	[125]
Use of giant vesicles containing polymers as mimic of the cytoplasm	**1 a**	[177]
Use of giant vesicles for studying osmotic effects to mimic the processes occurring during the dehydration of cells	**1 a**	[178]
Investigation of membrane proteins reconstituted within vesicle membranes	**1 a** **1 b**	[14, 179–181] [127, 182, 183]
Use of giant vesicles as model systems for investigating certain steps of the exocytosis process	**1 a**	[184]
Construction of networks of giant vesicles which are connected through lipid tubes	**1 a**	[185]
Study of giant vesicles as microreactors	**8**	[85, 86]
Study of single molecule reactions inside giant vesicles	**8**	[186]
Study of the effect of lipid oxidation on giant vesicle shape and bilayer permeability	**1 b**	[187]
Study of polymer-induced permeabilization of giant vesicles	**1 a**	[188]
Investigation of the effect of enzymes acting on the lipids (mainly phospholipases) on vesicle size and morphology, including budding and fission	**1 b**	[50, 189–192]
Study of gene expression inside individual vesicles	**1 a** **2 a** **5**	[116] [119] [78]
Study of pH-responsive polymersomes	**3**	[193]
Study of the thermoresponsiveness of poly(NIPAM) containing giant vesicles; NIPAM, N-isopropyl-acrylamide	**1 b**	[194]

[a] See Figure 1 and text.

One of the most obvious applications of giant vesicles is their use as simple model systems for studying certain physicochemical properties of biological membranes. Examples include mechanical properties of the entire vesicle[87] or of the membrane,[13] lipid domain formation, lipid dynamics, membrane growth,[88–91] budding,[92,93] fission[94–96] and membrane fusion.[98–100]

A further application of giant vesicles is in the field of membrane protein research. The prerequisite for such studies is that the membrane protein of interest can be reconstituted in a giant vesicle membrane. Classical procedures allow the reconstitution of membrane proteins in submicrometer-sized vesicle membranes, typically by the detergent depletion method.[101] However, this method is not directly applicable to the formation of giant vesicles. Girard et al.[14] described a procedure for the successful reconstitution of the sarcoplasmic reticulum Ca^{II}-ATPase and the H^+-pump bacteriorhodopsin in giant vesicle membranes. The protein was first reconstituted in submicrometer-sized vesicles according to the classical procedure.[101] In a second step, the suspension containing the preformed vesicles with the integrated membrane proteins was partially dried over an electrode, followed by a controlled hydration in an electric field (electroformation, method **1 b**). The giant vesicles thus obtained contained the membrane proteins in their active forms.[14] A similar approach was adopted for reconstituting a mechanosensitive channel protein (MscL), a membrane-anchored oligopeptide binding protein (OppA) and an ion-linked transporter protein (LacS).[102]

There are several advantages of using giant vesicles instead of submicrometer-sized vesicles (SUVs or LUVs), for example in studies of the effect of antimicrobial substances (for example, antimicrobial peptides) on phospholipid membranes.[103,104] If a suspension of LUVs is used, only average observations are possible. Reactions occurring within or on the surface of single vesicles can not be measured with LUVs. Studies with LUVs and GUVs yield complementary information.[52] By using giant vesicles (prepared with method **2 a**) it was for example possible to study details of vesicle membrane fusion[105] and fission;[106] this provided unique insights into the two processes. Furthermore, the use of giant vesicles (again prepared with method **2 a**) allowed a direct visualization and quantitative analysis of the pore formation in phospholipid membranes by the peptide magainin-2.[107,108] Other applications are listed in Table 2,

which is by no means a complete tabulation of all types of applications and examples.

Perhaps one of the most fascinating and challenging fields of application of giant vesicles is the research that aims at preparing systems that have at least some of the basic features of living cells. Depending on how the term "artificial cell" is defined and used,[109] the synthesis of a system that has the essential structural and dynamic properties to differentiate it from nonliving systems, is (currently) the ultimate goal of synthetic biology.[110,111] Whether this will ever be achieved depends on the criteria set for the system to "become" a living system, that is, on whether one agrees on a particular definition of "living" and of "life" at large. In any case, giant vesicles are used as compartment structure and filled with various compounds to allow chemical reactions to occur inside the vesicles for controlling the maintenance of the vesicle structure and for its growth and reproduction. Luisi and co-workers[112–114] suggested preparation of a so-called "semisynthetic minimal cell" by entrapment of enzymes, genes, ribosomes, tRNA and small molecules within a giant vesicle or even within LUVs. Currently, the expression of proteins inside vesicles is the state-of-the-art in this field of research. From a theoretical point of view, this is of relevance, because the activity of a synthesized protein might lead to an increased complexity of the dynamics of the system, that is, controlled exchange of solutes between the vesicles interior and the environment.[115]

The first clear demonstration of a functional protein expressed inside giant vesicles (prepared by the natural swelling method, method **1a**) was provided by Nomura et al.[116] Several interesting examples of biomolecular reactions compartmentalized within multilamellar giant vesicles (prepared by rehydrating freeze-dried vesicles, a simple method that is similar to the procedure of methods **1a** and **b**) have been provided by the group of Yomo, as in the case of a two-step genetic cascade inside giant vesicles,[117] or the self-encoded replication of RNA.[118] Giant vesicles prepared by the w/o emulsion transfer method (method **2a**) were utilized by Noireaux and Libchaber[115] in order to build a cell-like compartment that was able to produce the green fluorescent protein for around four days, at the same time expressing α-hemolysine for permeabilizing the vesicle membrane. This was certainly an elegant and important piece of work.

In a recent report, Saito et al.[119] entrapped a plasmid coding for green fluorescent protein (GFP) along with the transcription-translation kit inside giant vesicles, which were prepared by the w/o emulsion transfer method (method **2a**). The real-time monitoring of protein expression revealed that the population of giant vesicles was heterogeneous in terms of yield and reaction kinetics. Therefore, further detailed analysis of the mechanisms of (co)entrapment of macromolecules inside such compartments is required. Notice that theoretically no strong fluctuations in reaction yields should be observed for such large compartments, because the number of molecules entrapped in the individual GUVs is expected to be high (the order of magnitude of fluctuations scales as $1/\sqrt{N}$, in which N is the average number of molecules present in a certain volume). The effect of fluctuations should be evident only for compartments with sizes considerably smaller than 1 μm and for solutes at low concentrations, see the considerations made above in section 2. However, the heterogeneity in solute entrapment has also been observed in giant vesicles (and in similarly sized w/o emulsion droplets),[120] but so far no attention has been devoted to understanding why such heterogeneity exists. In a recently reported work by Dominak and Keating,[121] the individual encapsulation efficiency (internal/external concentration) of giant vesicles prepared by the gentle hydration method (method **1a**) showed large fluctuations around the expected concentration.

In addition to the efforts for expressing proteins inside giant vesicles, transforming them to some extent into cell-like structures, a similar research line consists in reconstituting parts of the cytoskeleton and membrane proteins in giant vesicles, as outlined in the following. This research does not often explicitly refer to the construction of "artificial cells", but is rather aimed at clarifying some details of molecular mechanisms in a controlled biomimetic environment. However, this type of work could contribute significantly to the long-term goal of synthesizing a cell from its molecular components. The proteins required to reconstitute the cytoskeleton or parts of it are isolated and encapsulated within giant vesicles, and therefore the transcription–translation steps can be by-passed.

As stated in a recent review,[122] "we have been much better at taking cells apart than putting them together". Modern reconstitution methods try to address and change this situation. Among the most recent contributions, the encapsulation of F-actin and meromyosin in giant vesicles (formed by starting from a w/o emulsion, method **2a**) was reported, resulting in the formation of actin filaments.[123] Similarly, it was shown that actin filaments can be formed[124] and anchored[125] in the inner layer of giant vesicles prepared by electroformation on ITO electrodes (method **1b**). It was also shown that an actin cortex can be reconstituted inside giant vesicles prepared by the w/o emulsion transfer method (method **2a**).[126] Streicher et al.[127] reported on the successful reconstitution of the transmembrane protein $\alpha_{IIb}\beta_3$ integrin into giant vesicle membranes by applying the electroformation method (method **1b**). In another work, giant vesicles gave rise to membrane tubes and to complex tubular networks when kinesin molecules were attached to the vesicle membrane by means of polystyrene beads.[128,129] Elongated shapes were obtained upon polymerization of microtubules[130,131] and actin filaments[132] inside giant vesicles.

Due to their large size, giant vesicles are not suitable for drug delivery applications, but they might find applications in the field of biosensors. Here, the research is still in an early stage, but there are some interesting reports, as in the case of grafting liposomes to DNA microchips,[133] or to microwells[134] to form a "liposome-microchip". This approach can be useful for developing liposome-based immunoassays.[134] Giant vesicles might act as biosensors to detect permeabilizing toxins by revealing the presence of bacteria producing lysins, that is, substances causing membrane lysis;[136,137] it was proposed that liposomes could be used to detect several classes of xenobiotics.[138] These latter types of applications require the production of large numbers of similar—if not identical—giant

vesicles. This is in general not possible, but the innovative reports on the production of giant vesicles by direct or indirect means of microfluidic devices[68,78] might rapidly change our attitude of thinking about giant vesicle applications in the near future.

5. How to Choose the Preparation Method

It is not easy to provide a general answer to what is the best method for the preparation of giant vesicles, because it depends very much on their use. Some of the advantages and disadvantages of each method are listed in Table 3. It might be convenient to summarize some features of giant vesicles prepared by the most widely used methods, that is, natural swelling (method **1a**), electroformation (method **1b**) and the w/o emulsion transfer method (method **2a**). Actually, the latter has only recently been discovered,[56,61] but has several interesting advantages. In general, all methods are highly dependent on the conditions used (type of lipids, type of buffer, and presence of special solutes), but some principle features can be attributed to each method. Concerning the requirement of equipment, the natural swelling method (method **1a**) is the simplest method and does not require any special equipment, as in the case of the w/o emulsion methods (methods **2a** and **b**)—at least in its simpler implementation. Vice versa, the electroformation method (method **1b**) requires electrodes that are ideally built in an observation chamber. All methods are very dependent on the lipid used. A wide variety of lipid compositions have been tested with the natural swelling method (method **1a**) and with electroformation (method **1b**), see Table 1, however, for the more recently developed w/o emulsion methods (methods **2a** and **b**) a systematic study is still missing. Traces of oil might be present in the giant vesicle membranes prepared by methods **2**, which involves the simul-

Table 3. Some of the advantages and disadvantages of the different methods for giant vesicle preparation.

Method[a]	Advantages	Disadvantages
1a	Simple; no need of special equipment; mild conditions; physiological conditions can be used; use of charged lipids possible	Giant vesicle formation difficult to control (highly dependent on experimental conditions during hydration and dependent on lipid film thickness); difficult if only uncharged lipids are used; low encapsulation efficiency
1b	Control of hydration process by adjusting electric field parameters; high reproducibility; giant vesicles formed are uniform, unilamellar and spherical; the vesicles are attached to the electrode which facilitates microinjection	Special equipment required (electrodes built in an observation chamber and an AC or DC device); no giant vesicle formation if the lipid deposit contains too many charged lipids; low yield of vesicles; low encapsulation efficiency; possible oxidation of polyunsaturated lipids
2a	No need of special equipment; possibility of forming vesicles with asymmetric bilayers; control of vesicle size by droplet size in the w/o emulsion; high encapsulation efficiency; high yield of vesicles	Possible presence of oil in the vesicle membrane; size range only up to a few micrometers; initial w/o emulsion might be heterogeneous
2b	Uniform initial w/o emulsion can be prepared with high reproducibility and uniform droplet size; control of vesicle size by droplet size in the w/o emulsion; high encapsulation yield; high yield of vesicles	Special equipment required (microfluidics); possible presence of oil in the vesicle membrane; vesicles often oligolamellar
3	Uniform initial w/o/w emulsion can be prepared; control of vesicle size by water droplet size in the w/o/w emulsion; high encapsulation efficiency; high throughput	Special equipment and expertise required (microfluidics); operating parameters are very critical; specific requirements for lipids and solvent used; aggregate formation from excess lipids of the outer interface of the initial w/o/w emulsion; possible presence of organic solvent in the vesicle membrane; vesicles might not be unilamellar
4	No need for special equipment; no organic solvent	Limited to particular lipids and conditions; low encapsulation efficiency; no control over size and lamellarity
5	Yields highly monodisperse giant vesicles; control over vesicle size by flow conditions (pulse rate, pulse voltage, pulse volume, cross-flow velocity, solution viscosity); high encapsulation yield; high throughput	Sophisticated assembly of microfluidic chambers/channels and precise control of flow velocity needed; possible presence of oil in the vesicle membrane
6	Simple; no special equipment needed	Limited to certain lipid systems; lack of direct control over vesicle size; formation of polydisperse vesicles; low encapsulation efficiency; exchange of external medium needed after vesicle formation; method not well established
7	Simple; no special equipment needed	Limited to certain lipid systems; giant and smaller vesicles formed; no direct control over vesicle size; method not well established
8	Simple; fast; no special equipment required; high flexibility in terms of possible lipids and buffer solutions that can be used	Lack of size control; yields mainly multilamellar vesicles, organic solvent might not be removed completely; low encapsulation efficiency

[a] See Figure 1 and text.

taneous presence of three components (oil, aqueous solution and lipids), whereas in the lipid film hydration methods (methods **1a** and **b**), only two components are involved (aqueous solution and lipids). The presence of oil, for example an alkane, might significantly affect the physical properties of the lipid bilayers and of embedded membrane proteins. Therefore, methods **2a** and **b** might not be the methods to choose for investigating the physical properties of the lipids in the bilayer or properties of reconstituted membrane proteins. However, methods **2a** and **b** are very useful for experiments in which giant vesicles are used as reaction compartments, as microreactors or as cell-mimicking systems because methods **2a** and **b** provide high encapsulation yields. Furthermore, the presence of small amounts of oil in the vesicle membrane might not have a big influence on the reactions occurring inside the "large" aqueous pool.

The natural swelling method (method **1a**) generally produces a heterogeneous population of vesicles; this can be advantageous when specifically studying vesicle diversity.[35,121] If carefully controlling the experimental conditions, the electroformation (method **1b**) allows for very reproducible formation of giant vesicles on the electrodes,[139] while differences between individual vesicle preparations generated by natural swelling (method **1a**) and the w/o emulsion methods (methods **2a, 2b**) are very probable. Another advantage of the electroformation (method **1b**) is the fact that the vesicles are "immobilized" on the electrode; this allows for long term observations and manipulations, including microinjections, of individual giant vesicles. This is clearly impossible for free-floating giant vesicles as obtained by the natural swelling (method **1a**) or by the w/o emulsion methods (methods **2a, b**), unless a micropipette is used with which the vesicles are kept in a desired position.[85]

The electroformation method (method **1b**) is somewhat disadvantageous in terms of giant vesicle yield. While only a few dozens of GUVs are formed in a typical electroformation experiment, millions of giant vesicles are produced per milliliter suspension applying other methods. The use of microfluidic devices to generate and study giant vesicles will improve the ability to observe and manipulate free giant vesicles, because they are "channeled" in a confined space rather than floating in the bulk fluid.

Finally, the w/o droplet methods (methods **2a** and **b**) are the methods of choice if high entrapment yield is desired. The water droplets of the initial w/o emulsion containing 100% of the solutes to be entrapped do not loose excessive amounts of solutes while passing from the oil phase into the aqueous environment. As a consequence, the environment of the loaded vesicles is virtually free of nonentrapped solutes. Vice versa, for studying confined reactions in giant vesicles prepared by the natural swelling method (method **1a**), a purification step is required for separating filled vesicles from nonentrapped solutes. With certain notable exceptions (see above), the entrapment yield in the case of electroformed GUVs is usually low, but the vesicles can be punctured with a microneedle to inject a small volume of an aqueous solution (see Section 3.1). Extensive dilution inside the vesicles, however, should be taken into account.

6. Summary and Outlook

The electroformation method of Angelova and Dimitrov[36] (method **1b**) is currently the most widely used method for the preparation of giant vesicles. It allows for the reproducible formation of relatively monodispersed and mainly unilamellar vesicles from a number of amphiphiles or mixtures of amphiphiles. The various applications, of which some are listed in Table 2, clearly demonstrate the usefulness of this method—for instance in cases in which a few giant vesicles are analyzed and the focus is on the overall mechanical properties of the entire vesicles or on the vesicle membrane, including physical properties, lipid distributions, lipid dynamics and membrane protein reconstitutions.

If one is interested in applying giant vesicles as bioreactor systems in which the focus mainly is on the vesicle contents, then a method which yields vesicles from an initial w/o emulsion (methods **2a** and **b**)[56,68] currently appears to be the method of choice. In addition to achieving very high entrapment yields, these two methods allow one to prepare suspensions containing a large number of giant vesicles, not only a few. With further progress in the field of microfluidics and the microchip technology,[140] it is expected that microfluidic-based methods, which allow a controlled and high throughput production of giant vesicles, will further be developed, leading most likely to more applications of giant vesicles as bioreactors and compartment structures for the preparation of cell-like systems.

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