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Review

Enzymes inside lipid vesicles: preparation, reactivity and applications☆

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Abstract

There are a number of methods that can be used for the preparation of enzyme-containing lipid vesicles (liposomes) which are lipid dispersions that contain water-soluble enzymes in the trapped aqueous space. This has been shown by many investigations carried out with a variety of enzymes. A review of these studies is given and some of the main results are summarized. With respect to the vesicle-forming amphiphiles used, most preparations are based on phosphatidylcholine, either the natural mixtures obtained from soybean or egg yolk, or chemically defined compounds, such as DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) or POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine). Charged enzyme-containing lipid vesicles are often prepared by adding a certain amount of a negatively charged amphiphile (typically dicetylphosphate) or a positively charged lipid (usually stearylamine). The presence of charges in the vesicle membrane may lead to an adsorption of the enzyme onto the interior

Abbreviations: Bz-Arg-pNA, benzoyl-L-Arg-*p*-nitroanilide; DDV, vesicles prepared by the detergent dialysis method; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)]; DNP, dinitrophenyl; DODAB, dioctadecyldimethylammonium bromide; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)]; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPE-PEG2000, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol)^{2000}]; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)]; DPPS, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoserine; DRV, vesicles prepared by the dehydration–rehydration method; DRV-MFV, vesicles prepared by microfluidization of DRV; DRV-VET$_{100}$, vesicles prepared by using the dehydration–rehydration method first and then extruding through polycarbonate membranes with a mean pore diameter of 100 nm used in the last extrusion step; DSPE-PEG2000, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol)^{2000}]; EE, entrapment efficiency = (amount of enzyme entrapped in the vesicles)/(total amount of enzyme) × 100 (%); FAT-VET$_{200}$, vesicles prepared by freeze–thaw cycles, followed by repeated extrusions through polycarbonate membranes with a mean pore diameter of 200 nm used in the last extrusion step; G_{M1}, monosialoganglioside; GUV, giant unilamellar vesicles; IFV, vesicles prepared by the so-called 'interdigitated-fusion method'; LUV, large unilamellar vesicles; lyso PC, lysophosphatidylcholine; lyso PE, lysophosphatidylethanolamine; lyso PI, lysophosphatidylinositol; MFV, vesicles prepared by using an homogenizer and the Microfluidizer™; MLS, multilamellar spherulites prepared by shearing a lamellar phase; MLV, multilamellar vesicles; MLV-FAT, vesicles prepared by repetitive freezing and thawing a MLV suspension; MLV-MFV, vesicles prepared from MLV by using the Microfluidizer™; MVV, multivesicular vesicles; PA, phosphatidic acid; PC, phosphatidylcholine; PDMS, poly(dimethylsiloxane); PE, phosphatidylethanolamine; PE-DNP, *N*-dinitrophenylaminocaproyldipalmitoylphosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PMOXA, poly(2-methyloxazoline); POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; PS, phosphatidylserine; REV, vesicles prepared by the reverse-phase evaporation method; REV-VET, REV that have been extruded through polycarbonate membranes of a defined pore size; SM, sphingomyelin; SOPC, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; Suc-Ala-Ala-Pro-Phe-pNA: succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide; SUV, small (sonicated) unilamellar vesicles as obtained by sonifying MLV; T_m, main lamellar chain-melting phase transition temperature (also called 'lamellar gel-liquid crystalline phase transition temperature'); VEI, vesicles prepared by the ethanol injection method; VET$_{100}$, vesicles prepared by the extrusion method (without freezing–thawing cycles) using for final extrusions polycarbonate membranes with mean pore diameters of 100 nm; VPL, vesicles prepared by the pro-liposome method; Z-Phe-Val-Arg-pNA, benzyloxycarbonyl-L-Phe-L-Val-L-Arg-*p*-nitroanilide.

☆ Unless otherwise stated, all concentration ratios are given as molar ratios.

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or exterior site of the vesicle bilayers. If (i) the high enzyme encapsulation efficiencies; (ii) avoidance of the use of organic solvents during the entrapment procedure; (iii) relatively monodisperse spherical vesicles of about 100 nm diameter; and (iv) a high degree of unilamellarity are required, then the use of the so-called 'dehydration–rehydration method', followed by the 'extrusion technique' has shown to be superior over other procedures. In addition to many investigations in the field of cheese production—there are several studies on the (potential) medical and biomedical applications of enzyme-containing lipid vesicles (e.g. in the enzyme-replacement therapy or for immunoassays)—including a few in vivo studies. In many cases, the enzyme molecules are expected to be released from the vesicles at the target site, and the vesicles in these cases serve as the carrier system. For (potential) medical applications as enzyme carriers in the blood circulation, the preparation of sterically stabilized lipid vesicles has proven to be advantageous. Regarding the use of enzyme-containing vesicles as submicrometer-sized nanoreactors, substrates are added to the bulk phase. Upon permeation across the vesicle bilayer(s), the trapped enzymes inside the vesicles catalyze the conversion of the substrate molecules into products. Using physical (e.g. microwave irradiation) or chemical methods (e.g. addition of micelle-forming amphiphiles at sublytic concentration), the bilayer permeability can be controlled to a certain extent. A detailed molecular understanding of these (usually) submicrometer-sized bioreactor systems is still not there. There are only a few approaches towards a deeper understanding and modeling of the catalytic activity of the entrapped enzyme molecules upon externally added substrates. Using micrometer-sized vesicles (so-called 'giant vesicles') as simple models for the lipidic matrix of biological cells, enzyme molecules can be microinjected inside individual target vesicles, and the corresponding enzymatic reaction can be monitored by fluorescence microscopy using appropriate fluorogenic substrate molecules.

Keywords: Vesicles; Liposomes; Drug delivery; Microreactor; Nanoreactor; Bilayer; Membrane; Enzyme; Phosphatidylcholine; Phospholipids

1. Introduction

Lipid vesicles (also called *liposomes* [1] or simply *vesicles*) are polymolecular aggregates formed in aqueous solution on the dispersion of certain bilayer-forming amphiphilic molecules. Under osmotically balanced conditions, the vesicles are spherical in shape and contain one or more (concentric) lamellae that are composed of the amphiphiles. These shells are curved and self-closed molecular bilayers in which the hydrophobic part of the amphiphiles forms the hydrophobic interior of the bilayer and the hydrophilic part (the polar head group) is in contact with the aqueous phase. The interior of the lipid vesicles is an aqueous core, the chemical composition of which corresponds in a first approximation to the chemical composition of the aqueous solution in which the vesicles are prepared. Depending on the method of preparation, lipid vesicles can be multi-, oligo- or unilamellar, containing many, a few, or one bilayer shell(s), respectively. The diameter of the lipid vesicles may vary between about 20 nm and a few hundred micrometers, see Fig. 1 and Table 1.

The spheroidal shape of lipid vesicles with their bimolecular arrangement of the amphiphiles is known since the pioneering work of Bangham et al. [2,3], who originally called these aggregates *spherulites* [2]. As such aggregates form particularly from certain phospholipids, specially the long chain phosphatidylcholines present in biological membranes (such as POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine;[2] or the mixtures of 3-*sn*-phosphatidylcholines extracted from

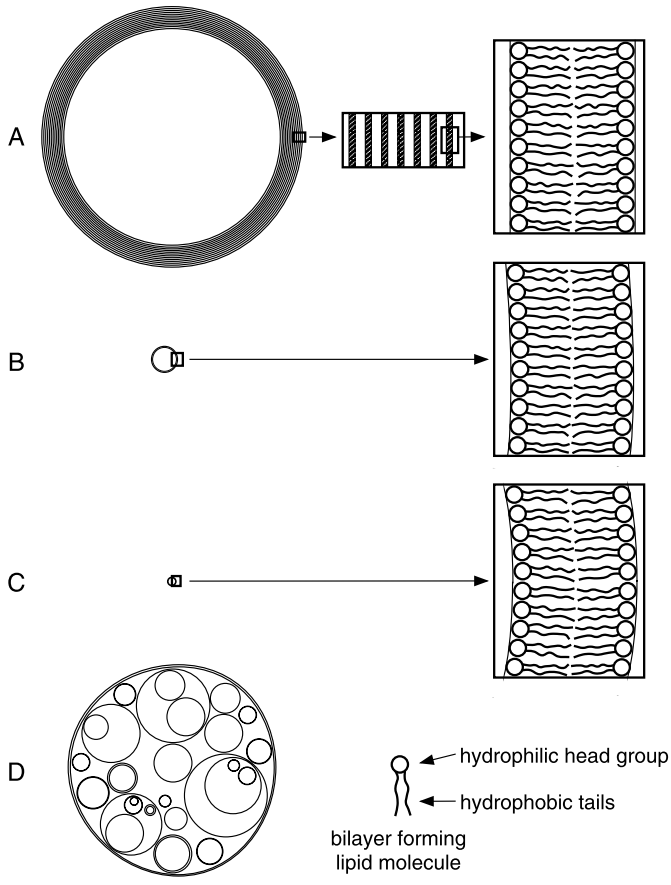

Fig. 1. Schematic representation of different types of lipid vesicles. (A), multilamellar vesicles, MLV (diameter typically 1 μm); (B), large unilamellar vesicles, LUV (diameter 100 nm); (C), small (sonicated) unilamellar vesicles, SUV (diameter ~30 nm); (D), multivesicular vesicles, MVV.

[2] As recommended by the International Union of Pure and Applied Chemistry-International Union of Biochemistry (IUPAC-IUB), for the nomenclature of phospholipids, the stereospecific numbering system (*sn*) is used, see Ref. [4].

Table 1
Calculated approximate aggregation numbers and calculated trapped volumes of typical SUV, LUV, GUV and MLV prepared from POPC

Lipid vesicle type (outer diameter)	Calculated mean number of POPC molecules per vesicle	Calculated internal volume (μl water/μmole lipid)
SUV (20 nm)	2.4×10^3	0.26
LUV (100 nm)	8.1×10^4	2.9
LUV (200 nm)	3.4×10^5	6.7
GUV (1 μm)	8.7×10^6	36
GUV (2 μm)	3.5×10^7	72
GUV (20 μm)	3.5×10^9	720
MLV (1 μm, 10 layers)	7.2×10^7	2.5
MLV (2 μm, 10 layers)	3.2×10^8	6.1

For the calculations made, a constant mean head group area of 0.72 nm^2 and a bilayer thickness of 3.7 nm [98] were used; these values are rather similar to the corresponding values determined for the egg PC [99]. The values for MLV were calculated by taking into account an interlamellar spacing of 6 nm, see also Ref. [100].

egg yolk), these aggregates are also called *phospholipid vesicles* [5] which has been simplified in the past to *lipid vesicles* [6], *artificial vesicles* [7] or just *vesicles* [8,9]. *Vesicles* is used in this review as a general term and includes all types of (often spherical) single or multi-compartment closed bilayer structures, regardless of their chemical composition [8,10], i.e. regardless of whether the bilayer-forming amphiphiles are naturally occurring or completely synthetic [11].[3]

Lipid vesicles are generally not a thermodynamically stable state of amphiphiles and do not form 'spontaneously' (without input of external energy); they are only kinetically stable, kinetically trapped systems [16–20]. In other words, the physical properties of lipid vesicles may very much depend on how and under which conditions lipid vesicles of a certain amphiphile (or of a mixture of amphiphiles) are prepared. To make it clear, the mean size, the lamellarity and the physical stability of the vesicles not only depend on the chemical structure of the amphiphiles used, but in general particularly on the method of vesicle preparation. Physical instabilities of lipid vesicle systems involve vesicle aggregation and fusion (possibly leading to vesicle precipitation and flat bilayer formation) [21,22].

In the following, we will first critically summarize the results obtained for the encapsulation of enzymes in lipid vesicles by using a variety of different vesicle preparation methods. These methods include the most important general methods known today for the preparation of lipid vesicles, irrespective of whether the vesicles are prepared with entrapped compounds or not. The studies summarized in this review are strictly limited to the vesicle encapsulation of water-soluble enzymatic active proteins (enzymes), although there are a number of other papers which deal with the entrapment of other not enzymatically active water-soluble proteins [23–26]. The general principles are of course the same.

In Section 2, (potential) applications of enzyme-containing lipid vesicles will be listed and discussed, and in Section 3 studies on enzymatic reactions inside lipid vesicles will be summarized. Concluding remarks and an outlook will be added at the end of this review.

2. Preparation of enzyme-containing lipid vesicles

Probably the first paper on the entrapment of enzymes in lipid vesicles was by Sessa and Weissmann in 1970 [27]. It is a report on the preparation of lysozyme-containing lipid vesicles, and the influence of varying the lipid composition, in particular the overall charge of the lipid vesicles was investigated. In all the experiments reported therein, mixtures of egg phosphatidylcholines (**1**) were used as the main vesicle forming amphiphile, in combination with cholesterol (**2**)—which alters the organization and mechanical properties of the lipid bilayers [28]—and the anionic dicetylphosphate (**3**, dihexadecylphosphate) or the cationic stearylamine (**4**), with the aim of preventing vesicle fusion [29] (see Fig. 2).

The phosphatidylcholines from egg yolk are amphiphiles, all of which have the same zwitterionic head group (phosphocholine) linked to glycerol through the oxygen atom at *sn*-3 of glycerol. Each of the two hydroxyl groups at *sn*-1 and *sn*-2 is esterified with a fatty acid, see **1** in Fig. 2. An approximate main acyl chain composition for egg yolk PC is given in the legend to Fig. 2, in comparison with the main acyl chain composition of soybean PC. While the acyl chain at *sn*-1 in soybean PC is often C 16:0 ($\sim$34%) or C

[3] The term *liposome* was originally used in the literature before 1950 to designate 'fat bodies', e.g. colloidal aggregates of triglycerides [12], aggregates which are very different from the lipid vesicles described in this review. The usage of the term *liposomes* for the description of *lipid vesicles* was proposed in 1968 [13,14]. As *lipid vesicles* are often single-bilayer hollow spheres — which have nothing in common with 'fat bodies' — the use of the term *liposome* is in principle not appropriate [12]. Some authors use the term *vesicle* exclusively for a closed *single*-bilayer aggregate of amphiphiles [15,16].

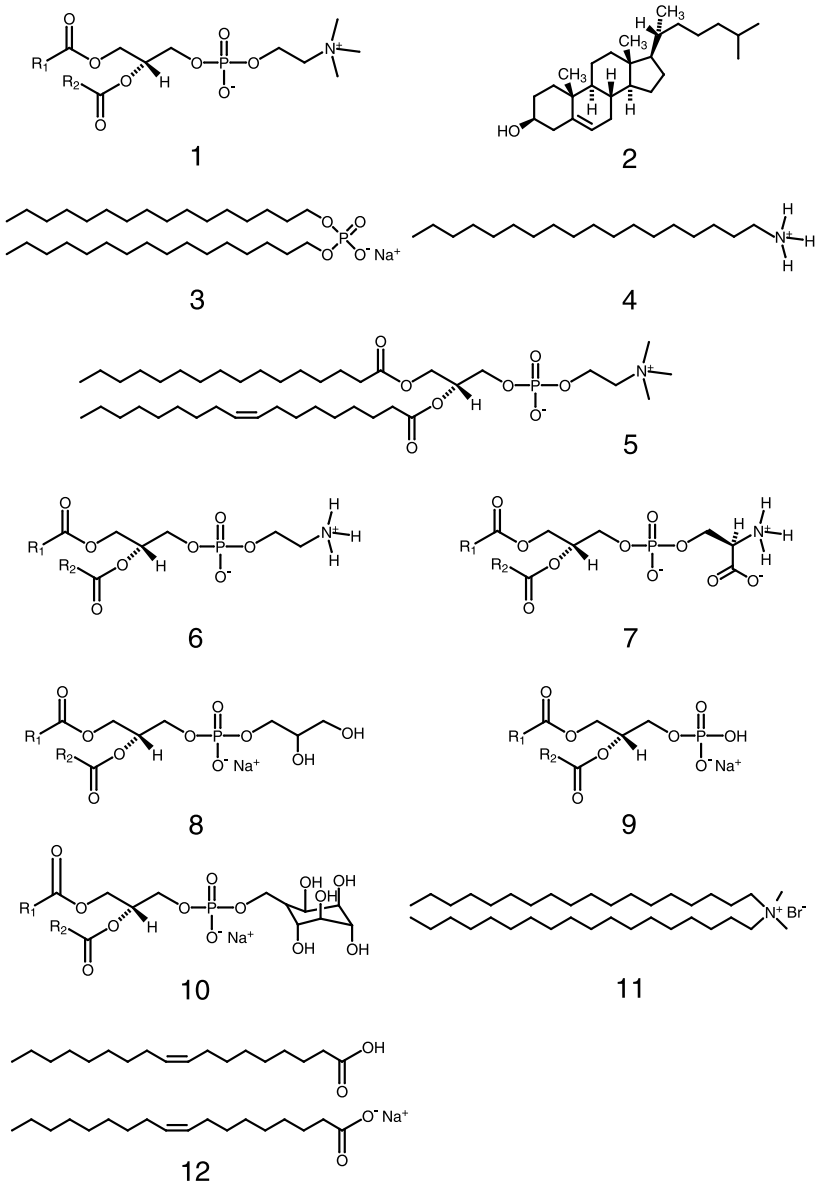

Fig. 2. Chemical structures of phosphatidylcholine (PC, **1**); cholesterol (**2**); sodium dicetylphosphate (sodium dihexadecylphosphate, **3**); protonated form of stearylamine (octadecylamine, **4**); POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, **5**); phosphatidylethanolamine (PE, **6**); phosphatidylserine (PS, **7**); phosphatidylglycerol sodium salt (PG, **8**); phosphatidic acid mono sodium salt (PA, **9**); phosphatidylinositol sodium salt (PI, **10**); dioctadecyldimethylammonium bromide (DODAB, **11**); oleic acid–sodium oleate (**12**). The acyl chain composition in **1** and **6**–**10** varies depending on the source of the phospholipid. Egg yolk PC contains ~35% palmitoyl- (hexadecanoyl-, C 16:0), ~27% oleoyl- (*cis*-9-octadecenoyl-, C 18:1), ~14% stearoyl- (octadecanoyl-, C 18:0), and ~13% all *cis*-4,7,10,13,16,19-docosahexaenoyl- (C 22:6) chains. Soybean PC contains ~48% linoleoyl- (*cis*,*cis*-9,12-octadecadienoyl-, C 18:2), ~23% oleoyl- and ~17% palmitoyl chains. [30] Please note that the numerical abbreviation given in parenthesis indicates the number of carbon chain (first number) and the number of double bonds (second number).

18:1 (~30%) and at *sn*-2 C 18:2 (~72%), in the case of egg PC the acyl chain at *sn*-1 is often C 16:0 (~69%) and at *sn*-2 C 18:1 (~49%) [30]. Therefore, POPC-with C 16:0 at *sn*-1 and C 18:1 at *sn*-2 (**5** in Fig. 2) is often used in basic lipid vesicle research, to reduce the number of chemical species present, as a model compound with certain properties rather similar to the properties of the entire egg PC mixture. Further, it has

been pointed out that natural egg PC often contains negatively charged impurities [31,32].[4]

Since this initial report on lysozyme encapsulation, there has been surprisingly little variation in the chemical composition of the vesicles prepared for the entrapment of enzymes in lipid vesicles. In addition to the zwitterionic egg PC (**1** in Fig. 2 [34–39]) or POPC (**5** [40–44]), DMPC [29,36,45–48], DPPC [49–58], DSPC [59], DOPC [36], soybean PC [60–64] or PE (**6** [80]) have been used. As negatively charged amphiphile, in addition to dicetylphosphate (**3** [29,45,47,65–70]), PS (**7** [45,71], PG (**8** [36,37,40,72,73]), PA (**9** [34,35,50] and PI (**10** [46,49,52,58,59,74]) have been used. As positively charged amphiphile, in addition to stearylamine (**4** [34–36,53–55,74–79]), PE (**10** [80]) and DODAB (**11** [46]) have been used. A mixture of soybean phospholipids, called 'asolectin'[5] and characterized by an overall negative charge has also been applied [7]. More recently, enzyme-containing lipid vesicles have been prepared from lipid mixtures containing a head group with poly(ethylene glycol) chains in order to prolong the life time of the vesicles in the blood circulation: DPPE-PEG2000 [81–83] and DSPE-PEG2000 [84]. These so-called 'stealth®' liposomes[6] are sterically stabilized and difficult to eliminate by the body's immune system [85–89].

So far, in only a few cases, enzymes inside vesicles containing 'non-classical' amphiphiles have been studied, e.g. the case of oleic acid–oleate vesicles (**12** in Fig. 2; [90–92]) or the case of vesicles from particular triblock copolymers [93].

The basic principles for the preparation of enzyme-containing lipid vesicles in general do not so depend much on the chemical structure of the amphiphiles used. One only has to make sure that all type of mechanical treatments possibly used during vesicle preparation, such as lipid dispersion, sonication or filtration through polycarbonate membranes (so-called 'extrusion', see below) have to be carried out at 5–10 °C or more above the *main lamellar chain-melting phase transition temperature* (T_m).[7] Below T_m, the saturated hydrophobic chains exist predominately in a rigid,

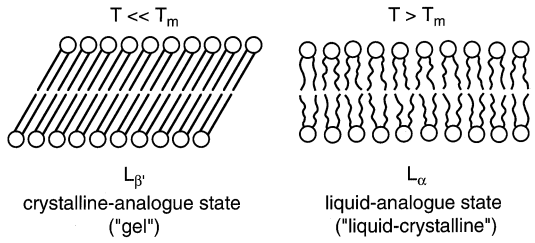

Fig. 3. Schematic representation of the lipid arrangement in a planar bilayer below and above the *main lamellar chain-melting phase transition temperature* (T_m). For more details on the complex nature of the lipid phases and phase transitions, please see Refs. [94–97]. All mechanical treatments of lipid vesicles have to be carried out above T_m, in the fluid state of the membranes.

Table 2
A list of T_m values for a selected number of amphiphiles that have been used for the preparation of enzyme-containing lipid vesicles, see also [101]

Amphiphile	T_m (°C)	References
Egg PC	-5.8 ± 6.5	[96]
Soybean PC	-15 ± 5	[103]
DOPC	-18.3 ± 3.6	[96]
POPC	-2.5 ± 2.4	[96]
SOPC	6.9 ± 2.9	[96]
DMPC	23.6 ± 1.5	[96]
DPPC	41.3 ± 1.8	[96]
DSPC	54.5 ± 1.5	[96]
Egg PA (pH 7–7.5)	~14–19	[101]
DMPA		
DMPA (pH 0)	45	[103]
$DMPA^{0.5-}$ (pH 3–4)	55	
$DMPA^{-}$ (pH 8)	50	
$DMPA^{2-}$ (pH 13)	28	
DPPS		
$DPPS^{+}$ (pH 0)	68.5	[103]
$DPPS^{-}$ (pH 3–4)	61.5	
$DPPS^{-}$ (pH 8)	54	
$DPPS^{2-}$ (pH 13)	32	
DMPG		
DMPG (pH 0)	42	[103]
$DMPG^{-}$(pH 8)	24	
$DMPG^{-}$(pH 13)	24	
Bovine brain PS (pH 7–7.5)	~5–15	[101]
Oleic acid–potassium oleate	11	[104]
DODAB	54	[11]

Please note that the presence of cholesterol changes the phase behavior of the individual lipids [28,94,95]. Further, the use of lipid mixtures may lead to a complex phase behavior [94,102].

[4] Often in biochemical literature, 'lecithin' is used as a trivial name for any type of 3-*sn*-phosphatidylcholine that is accepted, but not recommended, by IUPAC-IUB [4]. In contrast, however, the International Lecithin and Phospholipid Society (ILPS) which is part of the American Oil Chemists Society (AOCS) defines 'lecithin' as a 'mixture of glycerophospholipids obtained from animal, vegetable, or microbial sources, containing a variety of substances, such as sphingosylphospholipids, triglycerides, fatty acids, and glycolipids' [33]. From this different use of the same term it is clear that one must always specify what is meant by the term 'lecithin' if one wishes to use it at all.

[5] The phospholipid composition in asolectin has been reported to be 28% PC, 25.6% PE, 14.4% PI, 8.8% lyso PC, 6.7% cardiolipin, 3.7% lyso PE, 3.6% lyso PI, 3% PG, 2.9% PS, and 3.3% unidentified lipids, see Ref. [7].

[6] 'Stealth® liposomes' received their name from the fact that these type of sterically stabilized lipid vesicles are not so well detectable by the immune system, just like the American Stealth bomber which cannot be detected easily by the radar system owing to the particular shape and surface coating of the aircraft.

[7] T_m is also called the *lamellar gel-to-liquid crystalline phase transition temperature*, see for example Ref. [94].

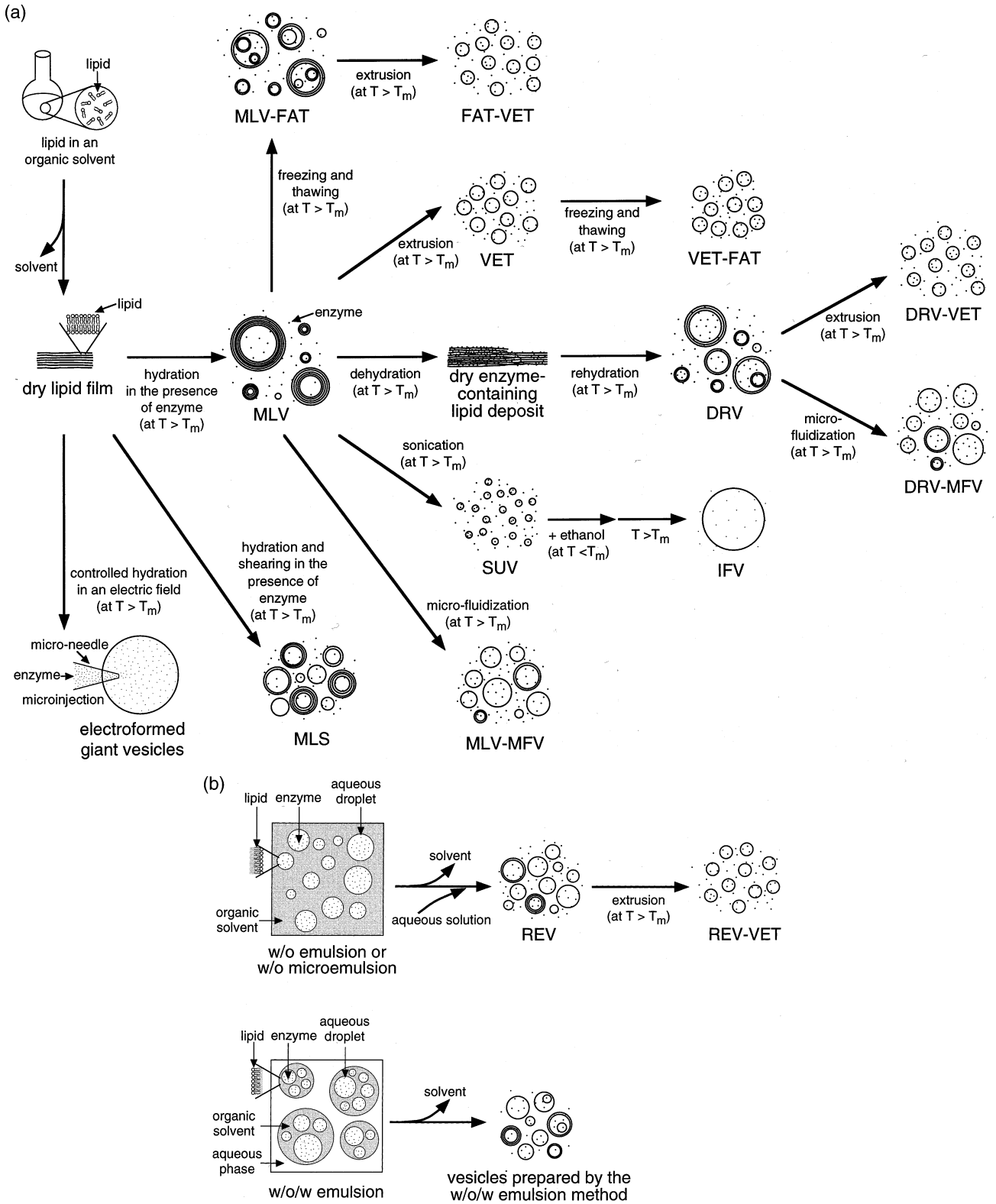

Fig. 4. Schematic summary of the methods that can be used for the preparation of enzyme-containing lipid vesicles: (a) methods which basically start from a dry lipid film; (b) methods which use emulsions (microemulsions) containing a water-immiscible solvent as starting system; (c) methods which involve the use of micelle-forming detergents; (d) methods which are based on mixing a nonaqueous lipid solution with an aqueous solution. See text and list of abbreviations for details.

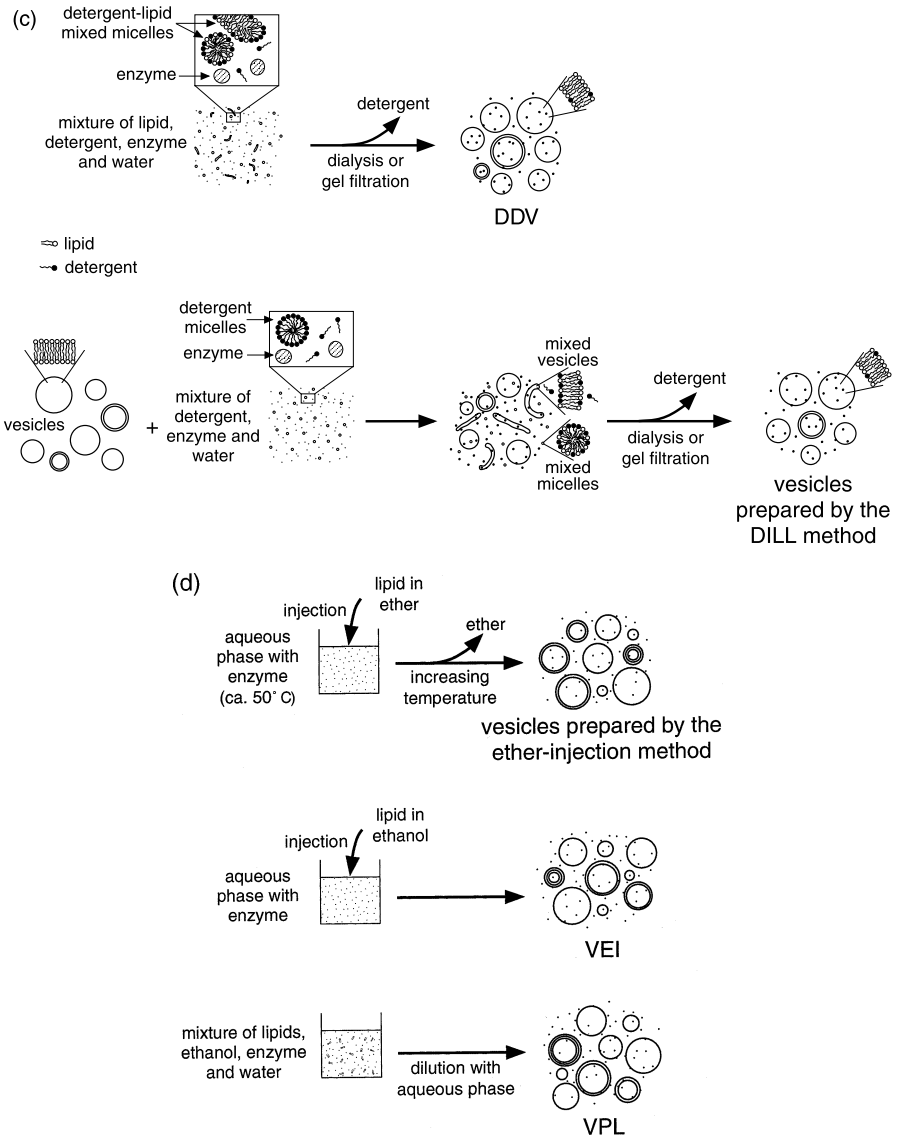

Fig. 4. (*Continued*)

extended all *trans* conformation, similar to their crystalline state. Above T_m, the chains are rather disordered with a lot of *gauche* conformations in the hydrocarbon chains, making the bilayer fluid (mechanically treatable), characterized by increased lateral and rotational lipid diffusion rather similar to a liquid [94], see Fig. 3. It is this fluid state of the lipids in biological membranes which represents a fluid matrix for membrane proteins.

A series of T_m values for amphiphiles that have been used for the preparation of enzyme-containing lipid vesicles are given in Table 2. These values have been obtained experimentally, for example by differential scanning calorimetry (DSC), and a large number of these T_m values have been tabulated [96,101,103]. Please note that the T_m values depend to some extend on the experimental conditions, in particular if the amphiphiles used have polar head groups that can undergo protonation–deprotonation (e.g. PA) [94–97]. The presence of cholesterol can lead to a loss in T_m (loss in cooperativity of the transition), as cholesterol interacts with the membrane lipids in a complex manner [28], thereby disturbing the intermolecular interactions, resulting—depending on the cholesterol content—e.g. in an inhibition of the formation of the crystalline-analog state in the case of DPPC [95], or in the formation of cholesterol-rich and lipid-rich domains [28,94]. Further, T_m values also depend to some extend on the curvature of the bilayer [94,96]. In analogy to the case of cholesterol, the use of lipid mixtures may result in the formation of domains within the bilayers which are enriched with one of the lipids, resulting typically in multiple peaks in DSC measurements [94,102].

Table 3
Selected examples of basic studies of the entrapment of enzymes in liposomes

Enzyme	Type of investigation, observations	References
Alkaline phosphatase	Development of the method to prepare REV, vesicles which are characterized by a relatively large internal aqueous space (egg PG–egg PC–cholesterol, 1:4:5; 0.2–1 μm)	[73]
	Entrapment in vesicles composed of egg PC–cholesterol (2:1, DDV) yielding about 150 enzyme molecules per vesicle	[114]
	Development of the dehydration–rehydration method for encapsulating macromolecules in POPC vesicles (DRV). An increase in the POPC concentration led to lower entrapment yields. A substantial amount of the enzyme seems to adhere to the surface of the vesicles	[44]
	First report on the entrapment of enzymes in spherulites™ (MLS) composed of phospholipon 90–sodium oleate (4:1, weight ratio)	[64]
δ-Aminolevulinate dehydratase	Entrapment experiments using three different methods (SUV, 'millipore-filtered MLV' (diameter <1.2 μm), 'ether-injection method'). Indication that at least a part of the enzyme is localized inside the vesicles	[115]
α-Amylase	Basic study on the influence of vesicles and protein size on the entrapment efficiency. Large proteins cannot be entrapped efficiently inside small vesicles	[26]
	Discussion of entrapment vs. binding of the enzyme in the case of soybean PC vesicles	[116]
Asparaginase	Entrapment in a variety of phospholipid vesicles (DRV), mainly containing egg PC or saturated PCs as main lipid component. Entrapment yields of almost 100% could be achieved	[36]
Chymotrypsin	Investigation of the influence of ionic strength, net charge and enzyme concentration on entrapment yield using the 'pro-liposome method' (VPL) [117,118]. (Apparent) entrapment yields as high as 96%, resulting largely from large amounts of surface adsorbed enzymes, probably based on hydrophobic interactions	[119]
Elastase	Entrapment in vesicles composed of egg PC–cholesterol–dicetylphosphate (80:20:5 or 120:30:7.5 or 160:40:10) showed entrapment yield dependency on the method of vesicle encapsulation (REV and briefly sonicated MLV)	[68]
β-Galactosidase	Yield of entrapment in soybean phosphatidylcholine–cholesterol vesicles (REV or DRV) decreased with an increasing cholesterol content. The enzyme was localized inside as well as on the surface of the vesicles	[60]
Lysozyme	One of the first detailed study on the entrapment of an enzyme within lipid vesicles prepared by the short sonication of MLV with the aim of constructing a model of organelles (lysosomes)	[27]
	Basic study on the influence of vesicles and protein size on the entrapment efficiency. Large proteins cannot be entrapped efficiently inside small vesicles	[26]
	Entrapment in soybean phosphatidylcholine vesicles (DRV) had highest yield at a pH close to the isoelectric point of the enzyme	[120]
Neutrase®	Entrapment yield depended on the charge of the lipid vesicles—highest yields with positively ($\sim$21–34%) and negatively ($\sim$22–27%) charged vesicles, and then with neutral vesicles ($\sim$16–21%), using REV	[35]
	Entrapment yield (up to $\sim$65%) depended on the lipid concentration and on the number of freezing–thawing cycles in the case of MLV-FAT	[45]
Pepsin A	Entrapment in soybean phosphatidylcholine vesicles (DRV) had highest yield at a pH close to the isoelectric point of the enzyme	[120]
Peroxidase	Basic study on the influence of vesicles and protein size on the entrapment efficiency. Large proteins cannot be entrapped efficiently inside small vesicles	[26]
	Entrapment in lipid vesicles (egg PC–DMPG–cholesterol, 26.4:6.6:33; VET_{1000}, VET_{400}, or VET_{100}) yielded between 62 (VET_{100}) and 1182 (VET_{1000}) enzyme molecules per vesicle	[37,72]
Proteinase K	Encapsulation inside preformed POPC vesicles (FAT-VET_{100}) on the addition of the detergent sodium cholate (DILL method). Uptake efficiency investigated as a function of the total cholate concentration. Maximal uptake around bilayer saturation with cholate	[121]
Rulactine	Dependence of the entrapment yield on the type of lipid vesicle prepared: MLV ($\sim$7.5–9%); SUV ($\sim$1–3%); REV ($\sim$5.5–7.5%)	[66]

For earlier reviews, see Refs. [112,113].

2.1. Methods used for the entrapment of enzymes in lipid vesicles

Fig. 4 is a highly schematic summary of all the major methods that have been used so far in the preparation of lipid vesicles, including the methods that have been applied so far in the preparation of vesicles containing entrapped water-soluble enzymes on a laboratory scale (usually a few milliliters). These methods will be commented briefly in the following sections. Details are given in many general review articles and in books published earlier on lipid vesicles [6,30,105–111] and in the original papers referred in this article.

Depending on the preparations, the aqueous vesicle suspensions formed are either more or less homogenous (rather monodisperse) or heterogenous (polydisperse) with respect to lipid vesicle size and lamellarity. In any case, the enzyme molecules to be encapsulated in the vesicles are always only to a certain extend trapped by the vesicles. Depending on the method used and depending on the lipid concentration, the entrapment efficiency can be relatively low (see below). Therefore,

in all the methods described in the following, the nonentrapped enzyme molecules have to be separated from the enzyme-containing vesicles in a last step of the preparation procedure. This is usually carried out either by size-exclusion chromatography (e.g. using sepharose 4B), by centrifugation or by dialysis. If this separation step is not carried out carefully, some of the enzyme molecules will still be in the bulk aqueous solution, which may falsify the results obtained from investigations using these enzyme-containing vesicle dispersions, possibly leading to incorrect interpretations.

Table 3 is a collection of a few basic entrapment studies that have been performed by using different enzymes and different vesicle preparations. In the following, results obtained from a number of investigations that involved the encapsulation of water-soluble enzymes in lipid vesicles are listed according to the method of preparation used.

The enzyme encapsulation yield which has been often determined in these investigations is usually expressed as the so-called 'entrapment efficiency (EE)'. The EE is defined as the percentual amount of enzyme entrapped in the vesicles in relation to the total amount of enzyme present during the vesicle formation and entrapment procedure.

EE (%)
= {(amount of enzyme entrapped in the lipid vesicles) /(total amount of enzyme used)} × 100

As indicated below and in Table 3, the EE value determined depends on the method and in particular on the concentration (and chemical nature) of the vesicle-forming amphiphiles used.

2.1.1. Multilamellar vesicles prepared by dry lipid film dispersion (MLV)

The dispersion of a dry lipid film in an enzyme-containing aqueous solution leads to the formation of a relative polydisperse vesicle suspension with mainly rather large, multilamellar vesicles (MLV). It has been argued that these types of vesicles—in the absence of any enzyme molecules—may on average be composed of up to 10 bilayers [100].

With this method, the vesicle-forming amphiphiles are first dissolved in an organic solvent (often chloroform) and the solvent is completely removed by rotatory evaporation and high vacuum drying in a round bottom flask. In this way, a thin, dry lipid film forms to which an aqueous solution containing the enzyme to be entrapped is added. Vigorous shaking with the help of a Vortex mixer[8] above T_m of the lipids leads to a dispersion of the lipid multilayers in the aqueous solution, which results in the formation of a heterogenous population of vesicles. The preparation is not incredibly reproducible as the resulting vesicle size, size distribution and lamellarity very much depends on the quality of the lipid film and on how the film is dispersed (e.g. extend of shaking, and lipid film thickness). Since most of these vesicles are multilamellar, water-soluble enzymes can be localized not only in the central core, but also in the aqueous inter-lamellar spaces, resulting therefore in relatively high encapsulation efficiency.

In many cases (see below) the MLV-formation is a first step in the preparation of more defined, submicrometer-sized vesicles (see for example VET).

A number of studies on enzyme-containing MLV have been carried out, e.g. with the following enzymes (or enzyme preparations): Neutrase®[9] [34,45,67,122,123], Rulactine [66], trypsin [69], urease [124], β-galactosidase [126], Corolase®[10] PN [67], D-amino-acid oxidase [39], β-glucuronidase [57], butyrylcholine esterase [125], superoxide dismutase and catalase [127]. The polydispersity of the vesicles prepared is clearly evident from the diameters of the enzyme-containing MLV reported earlier, e.g. 0.2–1.3 μm (urease and egg PC–cholesterol–dicetylphosphate, 26:6:1 [124]), 1–6 μm (β-galactosidase and egg PC–cholesterol–dicetylphosphate, 7:2:1 [126]), 0.5–5 μm (Rulactine and egg PC–cholesterol–dicetylphosphate, 5:5:1 [66]), 0.25–5 μm (Neutrase® in PC–cholesterol, 100:55.3, weight ratio [122]). The reported enzyme entrapment efficiency for MLV varies in most cases from below 5% [34,39,122] to about 15% [123]. In the case of δ-aminolevulinate dehydratase, EE values of ~25–40% (egg PC–egg PA, 15:2.12, weight ratio) have been reported [115], and in the case of butyrylcholine esterase ~30% (the soybean PC Phospholipon® 100) [125].[11]

In some preparations, the authors have treated the enzyme-containing MLV preparations briefly with a mild bath sonication in order to eliminate very large vesicles present, e.g. in the case of superoxide dismutase [77], β-galactosidase [126], elastase [68], glucose oxidase [70] or *Citrobacter* sp. phosphatase [129].

2.1.2. Vesicles prepared by repetitive freezing and thawing a MLV suspension (MLV-FAT)

Upon repetitively freezing a MLV suspension in liquid nitrogen (at −195 °C) and thawing at a temperature above T_m, the vesicle suspension may undergo certain physico-chemical changes thereby often equilibrating the

[8] A Vortex mixer usually operates with up to ~3000 rpm. The energy input for dispersing the lipid film in the enzyme-containing aqueous medium possibly influences the resulting vesicle dispersion and the EE value, and in some cases, mild conditions for a slow lipid film dispersion have been used successfully [82].

[9] From Novozymes at http://www.novozymes.com.

[10] From Röhm Enzyme GmbH (ABITEC group) at http://www.roehmenzyme.com.

[11] Phospholipon® 100 and Phospholipon® 90 are native PCs isolated from soybeans by Nattermann Phospholipid GmbH (Cologne, Germany). The corresponding PC contents are ≥95% and 93 ± 3%, respectively, with the main impurities being lyso PC [128].

vesicles aqueous interior and the external bulk aqueous phase [130], resulting in an increased entrapment yield [130–133] and possibly leading to the formation of an increased population of multivesicular vesicles (MVV) [134] and to an elimination—through fusion processes—of very small vesicles possibly present, depending on the lipid used and depending on the salt content [131,132]. The freezing–thawing procedure may also lead to the fragmentation of MLV into smaller vesicles in the presence of electrolytes, as demonstrated in the case of vesicle suspensions prepared from DOPC [135]. The fragmentation process, however, seems to be inhibited in the presence of 50% cholesterol [135]. Typically, freezing–thawing cycles are repeated 3–6 times.

This method has been used for example in the case of Neutrase® [45] yielding polydisperse vesicles with a mean diameter of about 1 μm. The reported entrapment yields were 3–45% (DMPC–cholesterol, 3:2) or 5–70% (DMPC–DPPS–cholesterol, 4:2:4), depending on the number of freezing–thawing cycles and on the lipid concentration [45].

2.1.3. Unilamellar vesicles obtained by sonifying a MLV dispersion (SUV)

The prolonged treatment of MLV with a probe type or bath type sonicator[12] above T_m under an inert atmosphere (usually nitrogen or argon) leads to the defragmentation of MLV into small (sonicated) unilamellar vesicles (SUV) of diameters usually below 50 nm [10,136]. The size of the SUV depends mainly on the sonication conditions and on the vesicle membrane composition (e.g. cholesterol content) [136,137].

This method involves harsh conditions (sonication) that may lead to an inactivation of the enzymes to be entrapped. Further, the sonication treatment is not highly reproducible and it generally does not lead to a complete elimination of MLV. In enzyme entrapment experiments using Rulactine, the reported diameter of the SUV (egg PC–cholesterol–dicetylphosphate, 150:82.5:22.5, weight ratio) obtained was 50–200 nm [66]. In the case of luciferase and negatively charged soybean phospholipid vesicles, bath sonication resulted in vesicles with a mean diameter of $\sim$55 nm [138].

The reported enzyme entrapment efficiencies are usually low: 1% (Neutrase® and egg PC or egg PC–egg PA, 9:1 [34]); 1–3.6% (Rulactine or Corolase® and egg PC–cholesterol–dicetylphosphate, 5:5:1 [66]); 1.5% (elastase and egg PC–cholesterol–dicetylphosphate, 80:20:1 [68]); 2.1% (elastase and egg PC, cholesterol–dicetylphosphate, 120:30:7.5 [68]); 3% (glucose oxidase and egg PC–cholesterol–stearylamine, 86:13:0.2 [38]); $\sim$11–13% (δ-aminolevulinate dehydratase and egg PC–egg PA (15:2.12, weight ratio [115]). In the case of glucose oxidase, the presence of large amount of stearylamine leads to a large increase in the (apparent) entrapment efficiency, up to 45 ± 5% (egg PC–cholesterol–steaylamine, 69:28:3) [38]. Other unexpected high EE values have been reported for superoxide dismutase: 25–40% (DPPC–cholesterol–stearylamine, 7:1:2) [79].

2.1.4. Vesicles prepared by the dehydration–rehydration method (DRV)

Dehydrating preformed vesicles followed by a controlled rehydration above T_m leads to the fusion of small preformed vesicles, resulting in multilamellar vesicles with high entrapment yields [139,140].

This method is a relatively mild procedure and has been used very often in the past for the entrapment of enzymes, particularly for applications in food technology (see below). In order to achieve high entrapment yields, enzyme-free SUV are typically prepared first in distilled water and then mixed with an aqueous solution containing the enzyme to be entrapped. The use of MLV as an initial vesicle preparation seems to result in lower encapsulation efficiency [139]. The thus obtained vesicle suspension is then dried, either by freeze drying [139] or by drying under vacuum without freezing or under a stream of nitrogen at slightly elevated temperatures [139–141]. After rehydration, the new vesicles formed—abbreviated as DRV—are considerably larger than the initially present SUV and multilamellar, and now contain to a large extend entrapped enzyme molecules, depending on the experimental conditions used, such as freezing procedure, initial vesicle properties, lipid composition and aqueous solution used.

This method has been used, e.g. in the case of Neutrase® [34,62,142], cyprosin [143], lysozyme [120], pepsin [120], trypsin [69], chymosin [61], asparaginase [36,74], β-galactosidase [60,65], alkaline phosphatase [44]. The enzyme-containing vesicles obtained usually have been found to be rather polydisperse: e.g. 200–400 nm [34]; 0.2–3.5 μm [69]; $\sim 500 \pm 120$ nm; $\sim 700 \pm 240$ nm; $\sim 1.25 \pm 0.25$ μm, and depending on the lipid composition [36], 2 μm [65]; or $\sim$1250 nm [74].

The reported entrapment efficiencies were in most cases between about 15 and 45%: e.g. $\sim$11–24% (lysozyme or pepsin and soybean PC [120]); $\sim$18% (β-galactosidase and egg PC [65]); $\sim$21% (Neutrase® and soybean PC [62]); 22% (Neutrase® and egg PC–cholesterol, 1:1 [144]), $\sim$24% (β-galactosidase and soybean PC–cholesterol, 1:3 or 1:4 [60]); 24–30% (Neutrase® and egg PC–cholesterol–DL-tocopherol, 2:1:0.011, weight ratio [142]; 26% (Neutrase® and egg PC–egg PA, 9:1 [34]); 28.0 ± 5.3% (asparaginase and DMPC–cholesterol, 4:1 [36]); $\sim$28% (Neutrase® and soybean PC [63]); $\sim$29–32% (β-galactosidase in soybean PC–cholesterol, 1:1 or 1:2 [60]); 31.2 ± 0.4% (asparaginase and egg PC–cholesterol, 1:1 [36]); 33.6 ± 5.5%

[12] For the preparation of SUV, low frequency ultrasound is used in the range of $\sim$20 kHz.

(Neutrase® and egg PC [34]); ~34% (Neutrase® and egg PC: stearylamine, 9:1 [34] or β-galactosidase and soybean PC [60]); 37.3 ± 9.2% (asparaginase and egg PC–cholesterol, 4:1 [36]); 38.3 ± 5.4 (asparaginase and DMPC–DOPC, 7:3 [36]); 39.0 ± 2.8% (asparaginase and egg PC [36]); 44.5 ± 3.5% (asparaginase and DMPC [36]).

In some cases very high EE values have been obtained: 68–70% (asparaginase and egg PC–cholesterol–dicetylphosphate, 7:2:1 or 7:1:2 [36]); 72.5 ± 7.2% (asparaginase and egg PC–cholesterol–stearylamine, 7:2:0.25 [74]); 84.5 ± 7.8% (asparaginase and DMPC–DMPG–stearylamine, 7:2:1 [36]); 86.7 ± 6.8% (asparaginase and DMPC–cholesterol–stearylamine, 7:2:0.5 [36]); 90–100% (asparaginase and egg PC–cholesterol–stearylamine, 7:2:0.5 or 7:2:0.125 [36]).

In a few cases, the reported EE values were below 15%: 3–10% (alkaline phosphatase and POPC [44]); ~12–14% (chymosin and soybean PC [61,63]; or cyprosin and soybean PC [63,143]; or trypsin and egg PC–cholesterol–dicetylphosphate, 5:5:1 [69]).

In a more recent modification of the method, preformed vesicles have been mixed with a sucrose solution containing the solute to be entrapped. Depending on the experimental conditions, vesicles with high entrapment yields and diameters between 90 and 200 nm have been obtained after dehydration and subsequent controlled hydration [145].

In the case of freeze-dried and rehydrated MLV containing a protease from *Mucor miehei*, an EE value of 30% could be reached [146].

2.1.5. Vesicles prepared by the extrusion method (VET)

A controlled reduction in size and lamellarity of MLV can be achieved by using track-etch polycarbonate filters which contain almost cylindrical pores of a defined size [147,148].[13]

The enzyme-containing MLV suspension is passed under moderate pressure repetitively (usually 10 times) through these filters above T_m which leads to a mechanical transformation of the large vesicles into smaller ones [148,151]. The whole process is called as the 'extrusion technique' and the corresponding vesicles are called vesicles prepared by the extrusion technique (VET). Usually, all the extrusion is started with filters containing relatively large pores (mean diameter, e.g. 400 nm), followed by a filtration through smaller pores (200 nm and often finally 100 nm). The corresponding vesicle preparation is abbreviated as VET_{100}, where '100' indicates the mean pore diameter (in nm) used for the final extrusions. VET_{100} are mainly unilamellar and rather monodisperse with a mean diameter of often ~100 nm [148].[14] The reported vesicle diameters in the case of enzyme-containing vesicles were: 85 ± 39–142 ± 23 nm (depending on the lipid composition, asparaginase [36]); 96 ± 4.6 nm (glucose oxidase [58]); 97–108 nm (depending on the lipid composition, peroxidase–glucose oxidase [52]); ~100–110 nm (chloroperoxidase or peroxidase together with glucose oxidase [49]); 112–143 nm (depending on the lipid composition, glucose oxidase–peroxidase or peroxidase [46]); 158–180 nm (asparaginase [74]); and 161 ± 28 nm (peroxidase [37]).

In the case of VET_{200}, VET_{400}, or VET_{1000}, the vesicles are characterized by a lower degree of unilamellarity and a lower degree of monodispersity [148]. In these cases, the mean vesicle diameter is often lower than the mean pore diameter, which is because of the fact that the small vesicles present originally, which was probably smaller than the pore diameter, passed the pores without altering their size. Some of the reported vesicle diameters in the case of VET_{200} are: ~100 nm (Q_β replicase/MDV-1 RNA [92]); 189 nm (glucose-6-phosphate dehydrogenase [40]); ~200 nm (glucose-6-phosphate dehydrogenase [153]); 250 nm (β-lactamase [93]); and 255 ± 150 nm (asparaginase [36]). In the case of VET_{400} and VET_{1000} they are 255 ± 64 nm (peroxidase [37]) and 386 ± 103 nm (peroxidase [37]), respectively.

The reported entrapment yields varied in the case of VET_{100} between ~4% (glucose oxidase–chloroperoxidase and DPPC–PI, 8:17 [49]), 12% (peroxidase and egg PC–DMPG–cholesterol, 26.4:6.6:33 [37]), 15% (polynucleotide phosphorylase and oleic acid–oleate [90]) and ~24% (glucose oxidase–peroxidase and DPPC–PI, 8:17 [49]); and in the case of VET_{200} between 18% (glucose-6-phosphate dehydrogenase and POPG–POPC–cholesterol, 4:76:20, weight ratio [40]).

In the case of VET_{400}, the only reported enzyme entrapment efficiency was 17% (peroxidase and egg PC–DMPG–cholesterol, 26.4:6.6:33 [37]).

For VET_{1000}, relatively high entrapment efficiencies were published: ~27% (peroxidase and egg PC–DMPG–cholesterol–*N*-hydroxysuccinimide ester of palmitic acid, 18:4:16:2 [72]) or 37% (peroxidase and egg PC–DMPG–cholesterol, 26.4:6.6:33 [37]).

Generally, the sizing-down of vesicles by extrusion is highly reproducible and involves relatively mild operations. It can be applied for small vesicle samples from 0.2 to 1.0 ml using the 'LiposoFast™' device (a Mini-Extruder) [154,155], or from 1.0 to 10 ml using 'The Extruder™' [134,156] as well as for large volumes (100

[13] Track-etch membranes are prepared in a complex process, which involves the exposure of the polycarbonate membrane to charged particles in a nuclear reactor yielding sensitized tracks that are then, etched [149]. The pores formed are not perfectly cylindrical but are slightly barrel-shaped, being of the order of 10% greater in diameter in the centre of the membrane than at the edges [150].

[14] To some extent, the vesicle size also depends on the extrusion pressure applied [150,152].

or 800 ml using the 'Extruder™' [156] or 250–1000 ml using the 'Maximator®' [157,158]). It has even been shown that it is possible to prepare VET in the scale of liters for clinical use [159–161].

2.1.6. Vesicles prepared by applying the extrusion technique to repetitively frozen and thawed vesicle dispersions (FAT-VET)

In order to homogenize and equilibrate an enzyme-containing MLV suspension before extrusion, repetitive freezing–thawing cycles are often carried out. The resulting vesicle suspension is usually characterized with a higher entrapment yield and a more homogenous size distribution [134], and the vesicles are abbreviated as FAT-VET_{100} or FAT-VET_{200}, respectively, depending on the pore size of the filters used in the last extrusion.

The following diameters of the enzyme-containing FAT-VET have been reported. FAT-VET_{100}: ~ 100 nm (trypsin and POPC [162]); ~ 125 nm (α-chymotrypsin and POPC [43]); and ~ 160 nm (α-chymotrypsin and POPC [42]). FAT-VET_{200}: ~ 150 nm (β-lactamase and POPC or polymer-stabilized POPC [163]); 192 ± 2 nm (Neutrase® and DMPC–DPPS–cholesterol, 4:2:4 [45]); 197 ± 2 nm (Neutrase® and DMPC–dihexadecylphosphate–cholesterol, 4:2:4 [45]); and 201 ± 4 nm (Neutrase® and DMPC–cholesterol, 3:2 [45]). FAT-VET_{400}: ~ 150 nm (phosphorylase and POPC [164]).

In the case of glucose oxidase-containing FAT-VET_{100}, the reported entrapment yield was only 0.7% (POPC [41]). For trypsin and POPC vesicles (FAT-VET_{100}), the entrapment yield was between 1.0 and 1.6% [162].

Sometimes, the order of the freezing–thawing cycles and the extrusion is reversed, resulting in VET-FAT, e.g. VET_{200}-FAT [165]. In this case, the extruded egg PC-based vesicles (VET_{200}, reported diameter between 230 and 250 nm) were pelleted first by ultracentrifugation and then dispersed in an aqueous solution containing the enzyme (t-plasminogen activator), followed by five freezing–thawing cycles to encapsulate the enzyme (EE: 17%). The mean vesicle diameter thereby increased to 530 nm [165].

2.1.7. Vesicles prepared by applying the extrusion technique to a DRV suspension (DRV-VET)

The extrusion technique may also be used conveniently in combination with the dehydration–rehydration method. In this case, a DRV suspension is extruded accordingly, resulting in a relatively monodisperse vesicle preparation and a high entrapment yield.

In the case of DRV-VET_{100}, the entrapment efficiencies that have been reported in the literature for asparaginase were $\sim 37\%$ (egg PC–cholesterol–bovine liver PI or bovine brain G_{M1}, 10:5:1 [74]) or $\sim 38\%$ (DMPC or egg PC–cholesterol–stearylamine, 7:2:0.25 [36,74]).

For DRV-VET_{200}, an EE of $51 \pm 2\%$ (asparaginase and DMPC–cholesterol–stearylamine, 7:2:0.25 [36]) has been reported.

The entrapment of superoxide dismutase in DRV-VET_{100} or DRV-VET_{200} (egg PC–cholesterol–DSPE-PEG2000, 1.85:1:0.15) resulted in vesicles with diameters of 110 ± 60 nm (EE 7%) and 200 ± 20 nm (EE 20%), respectively [84].

An encapsulation efficiency of 80–90% has been reported in the case of recombinant OPAA (an organophosphate hydrolyzing enzyme) in the case of sterically stabilized DRV-VET_{200} prepared from POPC–cholesterol–DPPE-PEG2000 (60:40:5.4) [82] or DRV-VET_{100} prepared from POPC–cholesterol–DPPE-PEG2000 (90:60:9) [83].

2.1.8. Vesicles prepared by microfluidization of a DRV suspension (DRV-MFV)

Upon treating DRV with a Microfluidizer™ (a high pressure homogenizer),[15] the originally multilamellar DRV transform into smaller vesicles with a mean diameter of around 100–200 nm (depending on the experimental conditions and on the number of microfluidization cycles), called microfluidized dehydration–rehydration vesicles, abbreviated as DRV-MFV [141,167,168]. The retention of the entrapped solute depends on different parameters, e.g. on the number of microfluidization cycles [141,167].

The microfluidization procedure can be applied not only in the case of DRV but also for MLV. Further, instead of a microfluidizer, other type of high-pressure homogenizers could be used in principle, see Ref. [169].

2.1.9. Vesicles prepared by the reverse-phase evaporation method (REV)

REV is the abbreviation of 'reverse-phase evaporation vesicles' and means that these vesicles are prepared by the so-called 'reverse-phase evaporation method' [73,170].

In this procedure, a water-immiscible organic solvent of low boiling point is used in which the vesicle-forming amphiphiles are soluble, e.g. diethyl ether, isopropyl ether, or mixtures of these ethers with chloroform or methanol [73]. The amphiphiles are first dissolved in the solvent—possibly with the help of chloroform or methanol if the solubility is too low. On adding an aqueous enzyme solution (volume ratio of organic phase to aqueous phase typically 3:1), the system is vortexed and briefly sonified in a bath-type sonicator until a relatively stable (reverse) emulsion is formed. The structure of this emulsion is not known but it can be assumed in a first approximation that enzyme-containing water droplets are more or less homogenously dispersed in the organic solvent. The amphiphiles form the interfacial layers between the water droplets and the solvent in such

[15] In a microfluidizer (from Microfluidics™ Inc. [166]), the downsizing of MLV occurs as a result of reuniting a split stream of vesicles under high pressure.

a way that the hydrophilic head groups face the aqueous microphase and the hydrophobic parts are in contact with the organic solvent, just like in the case of enzyme-containing reverse micelles or water-in-oil microemulsions [171–174].

On the removal of solvent under reduced pressure, the reverse emulsion transforms into an aqueous vesicle dispersion which contains a considerable amount of the originally present enzymes trapped inside the vesicles. The entrapment efficiency of REV is usually rather high, although one has to consider that the use of an organic solvent may be harmful for the enzyme. Further, the complete removal of the solvent may be a problem, and the REV formed is not very homogenous with respect to size and lamellarity.

The reported entrapment efficiencies ranged from below 10% to more than 30%; most entrapment yields were between 10 and 30%: 4.7–7.5% (Rulactine and egg PC–cholesterol–dietylphosphate, 5:5:1 [66]); 7.6–27.0% (depending on the buffer concentration, β-galactosidase and egg PC–cholesterol–dicetyphosphate, 5:5:1 [75]); $10.0 \pm 8.6\%$ (Neutrase® and egg PC [34]); 10.3% (β-galactosidase and soybean PC–cholesterol, 1:4 [60]); 12.6% (β-galactosidase and e.g. PC–cholesterol, 1:1 [75]); 14% (Neutrase® in egg PC–egg PA, 9:1 [34]; or Corolase® PN in egg PC–cholesterol–dicetylphosphate, 5:5:1 [67]); 15.8% (β-galactosidase and soybean PC–cholesterol, 1:3 [60]); 16–21% (Neutrase® and egg PC–cholesterol, 1:1 [35]); 17.3% (β-galactosidase and soybean PC–cholesterol, 1:2 [60]); 18.6% (β-galactosidase and egg PC–cholesterol–stearylamine, 5:5:1 [75]); $\sim 20\%$ (β-glucosidase and DMPC–cholesterol–dicetylphosphate, 7:2:1 [29]); 21–34% (Neutrase® and egg PC–cholesterol–stearylamine, 5:5:1 [35]); 21.1% (β-galactosidase and soybean PC–cholesterol, 1:1 [60]); ~ 22–28% (β-galactosidase and egg PC–cholesterol–dicetylphosphate, 5:5:1 [65,75]); 22–27% (Neutrase® and egg PC–cholesterol–egg PA, 5:5:1 [35]); ~ 24–29% (glucose oxidase and DPPC–PI, 83:17 [49,52]); 27.4% (elastase and egg PC–cholesterol–dicetylphosphate, 80:20:5 [68]); $\sim 30\%$ (Neutrase® and egg PC–cholesterol–dicetylphosphate, 5:5:1 [123]; or catalase and DPPC–cholesterol–stearylamine, 14:7:4 [76]); 31.8% (β-galactosidase and soybean PC [60]); and 34% (alkaline phosphatase and egg PG–egg PC–cholesterol, 1:4:5 [73]).

Enzyme-containing REV can also be extruded through polycarbonate membranes to reduce the size of the REV down to about 200 or 100 nm [175,176]. The corresponding vesicles would be abbreviated as REV-VET_{200} or REV-VET_{100}.

2.1.10. Vesicles prepared by the detergent dialysis method (DDV)

Mixing an aqueous dispersion of a bilayer-forming lipid (e.g. egg PC) with an aqueous (micellar) solution of a micelle-forming detergent (e.g. sodium cholate or *n*-octyl-β-D-glucopyranoside) under appropriate conditions (e.g. excess of detergent over lipid) results in the formation of mixed detergent–lipid micelles which are in equilibrium with nonmicellized (monomeric) detergent molecules. On a controlled and continuous removal of the detergent by dialysis [177–180] (or gel filtration [181,182]), the mixed detergent–lipid micelles transform into mixed detergent–lipid vesicles and finally into almost detergent–free lipid vesicles. The resulting vesicles are abbreviated as DDV, which stands for 'detergent dialyzed vesicles'. If the aqueous solutions contain an enzyme, it may be at least partly entrapped in the vesicles. The vesicles formed are mainly unilamellar and their size depends critically on the experimental conditions, such as lipid concentration, dialysis speed or initial detergent–lipid ratio [183–185], see also Ref. [186].

Commercially available equipments for the preparation of DDV are known under the name Mini Lipoprep (0.5–1.0 ml) or Liposomat (3–50 ml) [187].

Carbonic anhydrase has been entrapped in DDV prepared from DPPC–cholesterol–stearylamine (6:3:1 or 5:3:2) using sodium cholate as the detergent with an initial lipid–detergent molar ratio of 0.5, resulting in vesicles with a mean diameter of 61 ± 18 and 29 ± 11 nm, respectively [53,56,78].

In a study with trypsin, sodium deoxycholate has been used as the detergent (initial lipid–detergent molar ratio 0.6) with egg PC–lyso PC–SM–PE (72.5:4.6:3.8:19.1, weight ratio), yielding vesicles with a diameter of 125 ± 30 nm [80].

Alkaline phosphatase has been encapsulated in DDV composed of egg PC–cholesterol (2:1) by using *n*-octyl-β-D-glucopyranoside (initial detergent–PC–cholesterol molar ratio 22:2:1) yielding vesicles with a mean diameter of ~ 200 nm [145].

The entrapment of ascorbate oxidase in DDV containing DPPC–cholesterol (7:3) using sodium cholate (initial detergent–DPPC–cholesterol molar ratio 20:7:3) has given vesicles with a mean diameter of 52 nm [51].

Not much is known about the enzyme encapsulation efficiencies in the case of DDV. In the case of vesicles prepared from egg PC–cholesterol (2:1), and alkaline phosphatase, it has been reported that about 150 enzyme molecules were present per vesicle [114].

Using a variation of the DDV method [188], glucose oxidase or urate oxidase have been entrapped in mixed egg PC–deoxycholate vesicles yielding vesicles with diameters between 200 and 260 nm [189].

The method of vesicle preparation using detergents has proven to be particularly useful not for the encapsulation of water-soluble enzymes, but rather for the reconstitution of membrane-bound enzymes, see Ref. [190].

2.1.11. Vesicles prepared by the ethanol injection method (VEI)

The injection of a small amount of an ethanolic solution of a bilayer-forming amphiphile (usually a PC) into an aqueous solution leads to the rapid formation of vesicles as the amphiphiles are exposed to water. The vesicles thus formed are abbreviated as VEI, 'vesicles prepared by the ethanol injection method' [191,192]. Instead of ethanol, methanol—which is not only more polar but also poisonous—in principle can also be used. Both alcohols are completely miscible with water. Unless specially removed (e.g. by dialysis), the alcohol remains in the vesicle preparation. If the aqueous solution contains enzyme molecules, they will be entrapped to some extent in the vesicles formed. Depending on the experimental conditions (e.g. lipid concentration, speed of adding the alcoholic solution, and stirring rate), VEI are more or less homogenous with respect to size and lamellarity [111].

Without any indication of the obtained entrapment yields, the ethanol injection method has been applied in the case of superoxide dismutase using DSPC–cholesterol–stearylamine (14:7:4), yielding vesicles of 250 ± 50 nm diameter [193].

2.1.12. Vesicles prepared by the pro-liposome method (VPL)

In the so-called 'pro-liposome method', an initial mixture containing vesicle-forming amphiphiles, ethanol and water is converted into vesicles by a simple dilution step [117,118,194–196]. The resulting vesicles are abbreviated as VPL, which stands for 'vesicles prepared by the pro-liposome method'. If enzymes are present in the aqueous phase, they are partially entrapped in the vesicles thus formed.

In the case of α-chymotrypsin, using Pro-Lipo® 3080S (or Pro-Lipo® 3045S),[16] the vesicles obtained were rather polydisperse ranging from 100 nm to 2 μm with a mean diameter of 800 nm (or 725 nm) [119]. The reported entrapment yields were 17–76%, depending on the ionic strength and the enzyme concentration [119].

The encapsulation of a neutral proteinase from *Bacillus subtilis* or an acid proteinase from *Aspergillus oryzae* in VPL by using Pro-Lipo® VPF 012[17] resulted in an entrapment efficiency of 32–36% [198].

2.1.13. Multilamellar spherulites (MLS)

MLS stands for (concentric) multilamellar spherulites (or just spherulites™), prepared by the so-called 'spherulite technology' [199]. This technology involves the application of a controlled shear [199] to an aqueous lamellar phase of amphiphiles, followed by a dilution of the polyhedral type of structures thus obtained [200], resulting finally in the formation of spherical vesicles. MLS have typically diameters between 0.1 and 10–20 μm and have a dense multilamellar structure [64,201].

The lamellar phases that have been used so far for the preparation of MLS can be composed, e.g.: (a) of a mixture of soybean PC (Phospholipon® 90[11]), the polyoxyethylene alcohol C12E5, a fatty acid ($H–(CH_2)_{n-1}–COOH$, $n = 2…12$) and an aqueous phase [201]; (b) of a mixture of the nonionic surfactant Genamin TO20[18] and an aqueous phase [202]; (c) of a mixture of soybean PC–cholesterol–C12E4:water (43:4:3:50, w/w) [203]; (d) of a mixture of potassium oleate–cholesterol sulfate–mannitol oleate (Montanide 80)[19]–soybean lecithin (S100)[20]–water (5:1.5:3.5:40:50, w/w) [205]; or (e) of a mixture of soybean PC (Phospholipon 90)–DOPE–macrogol oleate (Simulsol 2599[19])–aqueous phase (45:1:20:35, w/w) [205].

If the aqueous phase contains an enzyme, it will be partly entrapped in the MLS formed. The spherulite technology has been applied in the case of alkaline phosphatase using soybean PC (Phospholipon 90)–sodium oleate–aqueous alkaline phosphatase solution (40:50:10, w/w) [64] as starting lamellar phase. The reported encapsulation efficiency was as high as 70–95% [64].

2.1.14. Other possible methods for preparing enzyme-containing lipid vesicles

There are in principle a variety of alternative methods that could be used—but have not yet been extensively applied—for the encapsulation of enzymes in lipid vesicles or for the reduction of the size and lamellarity of heterogenous enzyme-containing large MLV. Some of these methods are mentioned in the following; however, to the best of our knowledge, so far, none of these methods have been applied extensively for enzyme entrapment purposes.

One promising method seems to be the so-called 'detergent-induced loading of lipid vesicles', abbreviated as DILL [164,206,207], which is directly related to the DDV method. In the case of the DILL, preformed lipid vesicles are treated with an aqueous solution con-

[16] Pro-Lipo® 3045S and Pro-Lipo® 3080S are the two commercial PC products (available from Lucas Meyer The Lecithin People [197]) which contain negatively charged lipids.

[17] Pro-Lipo® VPF 012 is a commercial PC product (available from Lucas Meyer The Lecithin People [197]) which contains a considerable amount of negatively charged lipids [198].

[18] The chemical formula of the fatty amine mixture Genamin TO20 is given as $(R\text{-}N[(CH_2CH_2O)_x\text{-}H)][(CH_2CH_2O)_y\text{-}H])$, with $x + y = 2$, and R in most of the molecules having 17–18 carbon atoms [202]. Genamin TO20 is a product from Hoechst AG, see http://www.aventis.com.

[19] Montanide 80 and Simulsol 2599 are commercial products obtained from SEPPIC (Paris, France).

[20] The lecithin (S 100) used was a product from Lipoid GmbH (Ludwigshafen, Germany), [204], and contained ~94% (w/w) PC and ~3% (w/w) lyso PC.

taining the enzyme to be entrapped and a micelle-forming detergent (typically sodium cholate or *n*-octyl-β-D-glucopyranoside) at an intermediate detergent concentration. Under appropriate conditions, the preformed vesicles are partly destroyed by the micelle-forming detergent resulting in an equilibration of the enzymes over the whole volume. The enzyme-loaded vesicles contain the micelle-forming detergent, which is in equilibrium with the monomeric, nonassociated detergent molecules in the bulk in the membrane. For a successful application of the DILL method, it is of course required that the presence of the micelle-forming detergent does not lead to an irreversible enzyme denaturation. Further, the final vesicles prepared contain the micelle-forming surfactant, which may be difficult to completely eliminate, if required. This method has already been used in the case of a luciferase extract from the firefly lantern using asolectin vesicles (SUV) and cholate [7], or in the case of proteinase K using POPC vesicles (FAT-VET$_{100}$) and cholate [121]. In the latter case, an EE value of $\sim$10% has been obtained (30 mM POPC, 12.5 mM cholate).

Another method for the lipid vesicle preparation is based on the so-called 'coacervation (phase separation) technique' which involves the use of large amounts of alcohols (methanol, ethanol, *n*-propanol or 2-propanol) and results in the formation of apparently relatively homogenous vesicles with sizes which depend on the conditions used (e.g. type of alcohol) [208].

The preparation of the so-called 'interdigitation-fusion vesicles' (abbreviated as IFV) also involves the use of an alcohol (ethanol) which is added below T_m to the preformed SUV prepared from specific saturated phospholipids (e.g. DPPC). This ethanol addition leads to the formation of lamellar sheets which are characterized by interpenetrated (interdigitated) hydrophobic chains of the lipids. On increasing the temperature above T_m, the interdigitated sheets fuse and transform into vesicles which are mainly unilamellar and larger than 1 μm (the so-called 'giant unilamellar vesicles', GUV) [209,210]. A modified method for the preparation of IFV has been used in the case of streptokinase and DPPC, resulting in an EE of 25–55% and vesicle sizes of 1–3 μm [211].

Recently, a so-called 'supercritical liposome method' has been developed resulting in the formation of vesicles with entrapment efficiencies of up to about 20% [212]. In this method, the lipids (POPC–cholesterol, 7:3) are first dissolved under pressure in (non-toxic) supercritical CO_2 (or in mixtures of supercritical CO_2 and ethanol at 60 °C). After mixing with an aqueous solution at low pressure, lipid vesicles with diameters of about 200 nm are formed. For all the procedures, a specially designed apparatus has been used [212].

If the so-called 'ether-injection method' (see Ref. [213]) is applied for the encapsulation of enzymes, an ethereal solution of a bilayer-forming amphiphile is injected through a fine needle into an equal volume of an enzyme-containing aqueous solution at about 50 °C. The increased temperature leads to the evaporation of the ether while the vesicles are formed. In the case of δ-aminolevulinate dehydratase and egg PC, the use of this method resulted in a drastic inhibition of the enzyme, owing to the required use of elevated temperatures [115].

A novel technique for the preparation of lipid vesicles is called the 'rapid solvent exchange method', a very fast method using a specifically designed equipment resulting apparently in vesicles with high entrapment efficiencies within 1 min of operation [214]. The solvent used has to be water-immiscible (e.g. CH_2Cl_2 or $CHCl_3$), and a solution of a vesicle-forming lipid in this solvent is added to an aqueous solution in a particular way such that the solvent is rapidly and almost completely removed. One could imagine that a subsequent homogenization of the vesicle size thus obtained could be achieved by using the extrusion technique as described above.

Vesicle dispersions have also been obtained by applying a specific emulsification technique, which again involved the use of water-immiscible organic solvents (e.g. diethyl ether, CH_2Cl_2, $CHCl_3$, *n*-hexane or benzene) [215]. In this case, however, the formation of vesicles is preceded by the transient formation of water-in-oil (w/o) and water-in-oil-in-water (w/o/w) emulsions. After solvent removal, the vesicles formed had diameters between 50 and 500 nm. This method is an extension of the method for the preparation of REV and may be applied for the entrapment of enzymes if the solvents used are not inhibitory.

Finally, a newer methodology for the preparation of lipid vesicles is mentioned which does not include the use of organic solvents, high shear forces (like in the case of VET or MLS) and micelle-forming amphiphiles. This method is called as the 'bubble method' [216] as it is based on bubbling gas through a coarse dispersion of nonhydrated lipids (a dispersion which is composed of nonhydrated lipid particles), resulting in the formation of relatively monodisperse vesicles of $\sim$80–220 nm diameter after prolonged exposure to the bubbling process ($\sim$30 h) [216].

2.2. General considerations

As listed above, there are a number of methods which have been used for the encapsulation of water-soluble enzymes in lipid vesicles and there are several other lipid vesicle preparation techniques that have apparently not yet found their applications in the preparation of enzyme-containing vesicles. If one tries to summarize all the studies described above, one can draw the following two general conclusions:

1. The involvement of the highly reproducible extrusion technique, particularly with polycarbonate membranes containing pores with a mean diameter of 100 nm (or less) results in relatively homogenous and mainly unilamellar vesicles (VET) with a mean vesicle diameter of about 100 nm (or less). In addition to the high degree of reproducibility of the preparation, one advantage of the extrusion technique is certainly the fact that no organic solvents are directly involved. A nonaqueous solvent may be used only in the preparation of the initial lipid film. One possible disadvantage is the shear force present when the vesicles are squeezed through the approximately cylindrical pores. This may inactivate the enzyme.
2. High and extraordinarily high enzyme-encapsulation efficiencies have often been reported for vesicle preparations which involved the use of the dehydration–rehydration step (DRV).

Regarding this second point, it is important to mention again that the EE values quite generally depend on the concentration of the vesicle-forming amphiphiles during the encapsulation step, see for example the case of Neutrase® and DMPC-cholesterol containing vesicles [45]. Therefore, it can be understood that a dehydration and a controlled rehydration of the vesicle suspension results in high EE values as highly concentrated suspensions are transiently obtained, favoring efficient encapsulation.

Further, the encapsulation yield at a fixed lipid concentration may also be dependent on the enzyme concentration and on the ionic strength [119] and molarity of the buffer used [75].

In comparison with VET, DRV are generally not unilamellar and not monodisperse, which may be an advantage or a disadvantage, depending on the type of application on the enzyme-containing vesicles. One should also consider that the dehydration–rehydration cycle may inactivate the enzyme. It certainly depends on the type of applications and on the cost of the enzyme, whether achieving high encapsulation efficiencies is important or not. In the case of drug delivery, e.g. high EE values are usually desired [212].

The determination of 'true' EE values is not that trivial as it seems at first sight, and it is likely that many reported values are overestimations owing to the incomplete separation of nonentrapped enzymes from the enzyme-containing vesicles.

One should also pay attention to the fact that some of the enzyme molecules may bind to the lipid bilayer (e.g. lysozyme in the case of DPPG vesicles, VET_{200} [217]) resulting in an increased (apparent) EE value. This may be an important aspect that is relevant to medical applications of enzyme-containing vesicles [112].

There are indeed numerous reports, which indicate that the adsorption of the enzyme molecules onto the vesicle bilayer, has occurred. In the case of β-galactosidase and egg PC-based vesicles (REV [75]), or β-glucuronidase and DPPC-based vesicles (MLV [57]), the entrapment yield has shown a dependency on the charge of the vesicles (on the lipid composition). In experiments with glucose oxidase (SUV), the presence of a positive charge—stearylamine—was essential for the 'entrapment' of the enzyme [38]. The use of neutral lipids (DPPC–cholesterol, 7:3, DDV) did not result in the entrapment of carbonic anhydrase, instead cationic vesicles (DPPC–cholesterol–stearylamine, 6:3:1 or 5:3:2, DDV) resulted in 'enzyme entrapment' [55,56], which was most likely because of electrostatic interactions between the enzyme molecules and the vesicles, resulting in a significant amount of enzyme molecules bound to the external vesicle surface.

In the case of D-amino acid oxidase, the lipid composition also had a significant effect on the entrapment yield (MLV): egg PC (1.3%); egg PC–cholesterol, 1:1 (11%); egg PC–dicetylphosphate, 1:1 (5.4%); and egg PC–stearylamine, 1:0.2 (22%) [39].

The superoxide dismutase-containing vesicles (DPPC–cholesterol–stearylamine, 14:7:4, REV [76]) or ascorbate oxidase-containing vesicles (DPPC–cholesterol, 7:3, SUV [51]) that have been prepared most likely also contained enzyme molecules that were bound to the outer surface of the vesicles. Similarly, in the case of δ-aminolevulinate dehydratase, the enzyme may have been partially bound to the external surface of the negatively charged vesicles (SUV) [115], and luciferase may have been adsorbed onto glycolipid-containing vesicles [218]. Further, it has been discussed that alkaline phosphatase bound to DRV prepared from POPC [44]. In the case of ascorbate oxidase, the interaction of the enzyme with the vesicles has led to a change in the vesicle size [51].

The interaction of the enzyme molecules with the vesicle membrane may also depend on the composition of the aqueous solution in which the vesicles are prepared. It has been reported that α-chymotrypsin, e.g. interacts with egg PC vesicles at high salt concentrations (>0.8 M NaCl) [219].

From all these considerations on (possible) interactions of water-soluble enzymes with the vesicle membrane it is clear that this aspect is important to be taken into account if one likes to understand the behavior of the (truly) entrapped enzymes.

The interaction of enzyme molecules with the lipid vesicle membrane may also have different physicochemical consequences (see Ref. [217]). It has, e.g. been reported that lysozyme induces a size and temperature dependent flocculation of egg PC vesicles [31,220], or an increase in the bilayer permeability for the entrapped fluorescent dye molecules in the case of egg PC

or bovine brain PS vesicles [221]. The same has been observed in the case of DPPG vesicles [217]. Similarly, a lysozyme-induced increase in the bilayer permeability (against Na^+) of negatively charged vesicles (SUV prepared from PS at low ionic strength) has been found in the case of lysozyme [5].

Furthermore, it has been reported that trypsin strongly interacts with negatively charged vesicles at neutral pH [222] and with vesicles prepared from DOPE–oleic acid (2:1, SUV). In the latter case, this interaction has led to a destabilization of the vesicles (lysis) [223]. In another study, it has been seen that the presence of β-glucuronidase in the case of positively charged vesicles might lead to vesicle aggregation and precipitation [57]. In the case of native (not heat-inactivated) glyceraldehyde-3-phosphate dehydrogenase for example, addition of the enzyme to vesicles prepared from egg PC–egg PA (9:1, VEI) led to vesicle fusion, even in the absence of Ca^{2+} [224]. Ca^{2+} is well known to induce aggregation and fusion of negatively charged vesicles.

Although there are no extensive systematic studies on the dependency of entrapment efficiency and possible enzyme activity loss under comparative conditions, a few studies have clearly shown that EE values may vary considerably with the encapsulation method used. Elastase entrapment resulted in higher yields in REV in comparison with MLV: 27.4% versus 1.5–2.9% [68]. In the case of β-galactosidase, the encapsulation in REV surprisingly has shown to be more efficient than in DRV: 27.6% versus 18.3% [65]. Generally, it is more difficult to entrap large enzymes into small vesicles (SUV) than it is for small enzymes, and the probability of a given protein of a certain size being trapped inside vesicles has been calculated [26].

In a few reports, the problem of enzyme inactivation during vesicle preparation has been addressed, sometimes the enzyme is completely inactivated during the encapsulation procedure (e.g. δ-aminolaevulinate in the case of the 'ether-injection method' [115]). A partial inactivation has been found for β-galactosidase and egg PC-containing vesicles [65]; the inactivation was more severe in the case of REV than DRV which most likely is because of the organic solvent used in the preparation of REV, a general problem pointed out by others [225].

3. Applications of enzyme-containing lipid vesicles

There are two main areas of applications (or potential applications) of enzyme-containing lipid vesicles: (i) in the medical or biomedical field,[21] particularly for the enzyme-replacement therapy (see Table 4); or (ii) in the cheese ripening process (acceleration of the process and control over flavor development), see Table 5.

For both of these types of (potential) applications, the lipid vesicles are just carriers for the enzymes, containers which protect the enzymes from getting in immediate contact with the medium to which they are added, the blood circulation in certain applications in (i) or milk in (ii). In the case of the enzyme-containing lipid vesicle assisted cheese ripening process, the entrapped enzyme is gradually released, allowing catalyzing degradation and modification reactions in the cheese matrix during the ripening period [267]. In the (potential) medical applications, the vesicles are used as the drug delivery system, the drug being the entrapped enzyme. In most cases, the vesicles carry enzyme molecules with the aim of replacing- or supporting-endogenous enzymes in the treatment of particular diseases (enzyme-replacement therapy). The entrapped enzyme molecules have to be released at a particular site in the body where they are needed.

In another medical application, enzyme-containing lipid vesicles have been used for the treatment of myocardial infarction (see Fig. 5) [229]. In this case, the entrapped enzyme—t-plasminogen activator—has to be released at the site of the thrombus in order to catalyze its dissolution.

Again, in all the type of applications mentioned so far, the enzyme must be liberated from the vesicles before it can catalyze a particular reaction occurring outside of the vesicles. Alternatively, the entrapped enzyme molecules may not be released from the vesicles, but rather catalyze reactions inside the vesicles. Obviously, in this case, the substrate has to penetrate through the lipid bilayers to meet the enzyme in the internal aqueous space(s).

One interesting case discussed and investigated is the recombinant phosphotriesterase [81] entrapped inside sterically stabilized liposomes with the aim of catalyzing the hydrolysis of toxic organophosphates. Parathion, an organophosporous pesticide, which is one of the most widely used insecticides, is metabolized to its active metabolite paraoxon, which is responsible for most of its toxicity (inhibition of acetylcholinesterase) [81]. The phosphotriesterase-containing vesicle system has been used to antagonize the toxic effect of paraoxon by hydrolyzing it to the less toxic 4-nitrophenol and diethylphosphate. As shown in Fig. 6, paraoxon penetrates across the bilayers into the interior of the vesicles, where the phosphotriesterase catalyzes the hydrolysis reaction. Similarly, using organophosphoric acid anhydrase (OPAA)-containing vesicles, the degradation of externally added diisopropylfluorophosphate occurred inside the vesicles [82].

[21] Biomedicine is medicine based on the application of the principles of the natural sciences particularly biology and biochemistry (Merriam-Webster Online at http://www.eb.co.uk).

Table 4
Selected examples for the (potential) medical and biomedical applications of enzyme-containing lipid vesicles

Enzyme	Investigation, potential application	References
Alkaline phosphatase	Sensitive assay for the Herpes simplex virus using target-sensitive immunoliposomes, see Fig. 8	[232]
Amyloglucosidase	First proposal to use enzyme-containing lipid vesicles in the enzyme-replacement therapy	[233]
	Treatment of a patient with Pompe's disease (enzyme-replacement therapy)	[234]
Asparaginase	Antitumor activity; treatment of lymphoblastic leukemia	[36,74,235–237], see also [238]
Butyrylcholinesterase	Use of enzyme-containing vesicles as toxin scavengers, the target toxin being paraoxon which inhibits the entrapped enzyme	[125]
Chloride peroxidase	Antibacterial activity against the oral bacterium *Streptococcus gordonii*	[49,52]
Dextranase (3.2.1.11)	Development of a model system for a lysosomal storage disease in rat (enzyme replacement therapy)	[239]
DNA photolyase	Repair of DNA damage in UV-B-irradiated skin	[240]
DNA—(apurinic or apyrimidinic site) lyase	Repair of UV-induced DNA damage, treatment of skin cancer	[241–248], see also [238]
β-Fructofuranosidase	Investigation of the possible therapeutic application of enzyme-containing lipid vesicles; coencapsulation of albumin and cross-linking of albumin and the enzyme by externally added glutaraldehyde in order to improve enzyme stability	[249]
β-Galactosidase	In vitro evaluation of the use of enzyme-containing lipid vesicles for the treatment of cultured feline G_{M1} gangliosidosis fibroblasts (enzyme replacement therapy)	[126]
	Use of lipid vesicle trapped enzymes as marker molecules in the investigation of the effect of the complement system on the damage of the vesicles	[250]
Glucocerebroside:β-glucosidase	Treatment of a patient with Gaucher's disease (enzyme-replacement therapy)	[251]
Glucose oxidase	Development of a sensitive immunoassay	[252]
Glucose oxidase–insulin	In vitro evaluation of an insulin delivery system	[253]
	Intragastric administration of glucose oxidase-and insulin-containing lipid vesicles led to a reduction of the glucose levels in the blood of rats	[254]
Glucose-6-phosphate-dehydrogenase	Use of enzyme-containing lipid vesicles in a homogenous enzyme immunoassay	[40]
	Automated immunoassay system for the determination of the total complement activity. Lipid vesicles containing entrapped glucose-6-phosphate dehydrogenase are destroyed upon interaction of the surface adsorbed antibody with the complement, resulting in a release of the enzyme which reacts with externally present substrate (D-glucose-6-phosphate/NAD^+), see Fig. 9	[153]
	Use of lipid vesicle trapped enzymes as marker molecules in the investigation of the effect of the complement system on the damage of the vesicles	[250]
β-Glucuronidase	In vivo investigation (mice) of enzyme-containing lipid vesicles as possible vesicles in the enzyme replacement therapy	[57]
Hexokinase	Use of lipid vesicle trapped enzymes as marker molecules in the investigation of the effect of the complement system on the damage of the vesicles	[250]
OPAA (an organophosphate hydrolyzing enzyme—an anhydrolase—of esterase type A) from *Alteromonas* strain JD6	Hydrolysis of diisopropylphosphate, an acetylcholinesterase inhibitor (similar to the nerve agents soman and sarin). In vivo (mice) studies	[82,83]
Phosphotriesterase	Destruction of toxic organophosphates, see Fig. 6	[81]
Peroxidase	In vitro demonstration of the uptake of immunglobulin-coated lipid vesicles containing entrapped peroxidase by phagocytes lacking endogenous peroxidase	[255]
	Preparation of liposomes containing covalently bound immunoglobulins and entrapped peroxidase for the use in immunoassays	[72,256,257]

Table 4 (*Continued*)

Enzyme	Investigation, potential application	References
Peroxidase + glucose oxidase	Antibacterial activity against the oral bacterium *Streptococcus gordonii*	[52,49]
Streptokinase (Kabikinase® [258])	Investigation of the thrombolytic activity of enzyme-containing lipid vesicles in rabbit	[211]
Superoxide dismutase	Treatment of inflammation	[193,127]
	Prevention of liver necrosis in rats caused by superoxide anions	[77]
	Salvaging distal flap necrosis	[259]
	Pretreatment of rats with superoxide dismutase-containing lipid vesicles decreased the damage of endogenous superoxide dismutase by ultraviolet radiation	[260]
	In vitro treatment of endothelial cells	[79]
	Investigation of the life time and organ specificity of enzyme-containing lipid vesicles after injection into rabbits [261] or rats [262]	[261,262], see also [263]
	Treatment of rats with adjuvant arthritis. The lipid vesicle administration occurred intravenously [84] or subcutaneously [264].	[84,264]
Superoxide dismutase + catalase	Treatment of periodontal inflammation in beagle dogs	[127]
	Treatment of rats with intravenously injected lipid vesicles containing both enzymes for the protection against oxygen toxicity	[76]
t-Plasminogen activator (Actilyse® [265])	Use of lipid vesicle trapped enzymes for the thrombolytic treatment in rabbits showed a higher therapeutic effect of the enzyme (potential treatment of myocardial infarction), see Fig. 5	[165]
Tyrosinase	Investigation of the possible application of tyrosine-entrapped lipid vesicles in the treatment of Parkinson's disease (low levels of dopamine are responsible for the observed motion disorders): the enzyme catalyzed the oxidation of L-tyrosine to L-DOPA (L-3,4-dihydroxyphenylalanine), a precursor of dopamine	[266]

See also Refs. [226–230] for reviews on the medical application of enzyme-containing lipid vesicles, and Ref. [231] for the use of lipid vesicles in immunoassays.

The principles of some other medical or biomedical applications of enzyme-containing vesicles are illustrated in Figs. 7–10 and described in the respective figure legends.

In an—so far unsuccessful—approach, enzyme-containing vesicles have been investigated as possible microreactor system for the decontamination of industrial wastes [129]. The principle is shown in Fig. 11. The lipid vesicles contain entrapped phosphatase that catalyzes the hydrolysis of externally added organic phosphates for which the lipid vesicle has to be permeable. Upon intravesicular hydrolysis, inorganic phosphate is formed which—after interacting with the corresponding metal ions M^{2+} (like Cd^{2+} or UO_2^{2+}) present in the system—should lead to the formation of insoluble metal phosphate complexes $MHPO_4$. This way, the metal ions could be removed from the system. A prerequisite for the functioning of this principle is the lipid vesicle permeability towards the inorganic phosphate formed inside the vesicles, to reach the externally present metal ions. Further, the metal ions should not interact with the lipid bilayers to avoid unwanted side reactions with the microreactor system [129].

4. Enzymatic reactions occurring inside lipid vesicles

In one of the applications (or potential applications) of enzyme-containing vesicles described above, an enzyme-catalyzed degradation of organophosphorous compounds appears to occur inside the vesicles, see Fig. 6 [81,82]. However, regarding detailed basic studies on the enzyme-catalyzed reactions occurring inside lipid vesicles, there are only a few reports known, see Table 6. One reason for this is certainly the often low permeability of the vesicle bilayers for the externally added substrate molecules. Some of the particular substrates that have been used so far include the following: urea [189] in the case of urease; CO_2 [55] or *p*-nitrophenylacetate [53,54,56] in the case of carbonic anhydrase; D-amino acids [39] in the case of D-amino-acid oxidase; Bz-Tyr-pNA [42,43] in the case of α-chymotrypsin; Bz-Arg-pNA [162] and Z-Phe-Val-Arg-pNA [162] in the case of trypsin; glucose [38,41,58,59,189] in the case of glucose oxidase; and ADP [48,90,121,285] in the case of polynucleotide phosphorylase.

For a vesicle system composed of vesicles containing α-chymotrypsin and trypsin, the situation is schemati-

Table 5
Selected examples for the (potential) nonmedical application of enzyme-containing lipid vesicles

Enzyme	Investigation, (potential) application	References
Chymosin	Accelerated cheese ripening	[61]
Chymosin + neutrase® or cyprosin	Accelerated cheese ripening	[63]
Corolase® PN (alkaline protease)	Accelerated cheese ripening	[67]
Cyprosin	Accelerated cheese ripening	[143]
DNase I/DNA	Putative precellular system in the field of the origin of life	[50,272]
Flavourzyme® (from Novozymes)	Influence of the cheese ripening process	[273]
β-Galactosidase	Possible food additive for the treatment of lactose intolerance	[60]
	Possible food additive for the treatment of lactose intolerance. The encapsulated enzyme is added to milk without hydrolyzing lactose to glucose and galactose during storage at 4 °C for up to 20 days. The enzyme should only be active during the digestion process, after enzyme release from the lipid vesicles	[75,65]
Glucose oxidase	Entrapment of the enzyme in lipid vesicles (PC–dicetylphosphate–cholesterol, 7:2:1, SUV), followed by a polymer coating of the vesicles with poly(1,4-pyridinium diethylene salt). The lipids could be removed after polymerization, leaving a highly permeable enzyme entrapped polymer capsule	[70]
	Immobilization of enzyme-containing lipid vesicles on an H_2O_2-sensing electrode for the possible analytical application for the determination of glucose (field of biosensors)	[58,59,274]
Luciferase, crude extract [7] or purified [138]	Use of enzyme-containing vesicles and ATP-containing vesicles as new luminescence assay for vesicle fusion, see Fig. 15	[7,138]
Neutrase®	Acceleration of cheese ripening, improved flavor development	[122,35]
	Accelerated cheese ripening	[34,45,62,123,142, 143,275]
Neutrase® + phospholipase C	Accelerated cheese ripening	[276]
Phosphatase from *Citrobacter* sp.	Investigation of the possible application of enzyme-containing lipid vesicles for the decontamination of metal-laden industrial wastes, see Fig. 11	[129]
Polynucleotide phosphorylase	Model for the anabolic activity of early cells within the field of the origin of life. Model of a primitive cellular system	[48]
	Protocell model system in the field of the origin of life	[90]
Proteinase (neutral) from *Bacillus subtilis* or Proteinase (acid) from *Aspergillus oryzae*	Accelerated cheese ripening	[197]
Q_β replicase/MDV-1 RNA	Experimental approach towards a minimal cell (research on the origin of the first cell)	[92]
Rulactine	Acceleration of cheese ripening, improved flavor development	[66]
SP446 (a serine protease from Novozymes)	Influence of the cheese ripening process	[273]
Trypsin	Accelerated cheese ripening	[69]
	Use of trypsin-containing vesicles and substrate-containing vesicles as a new membrane fusion assay	[277]
Enzymatic extract from *Lactobacillus helveticus*	Influence of the cheese ripening process	[144]

See also Refs. [225,269–271] for reviews on the application in accelerated cheese ripening.

cally illustrated in Fig. 12. In the case of α-chymotrypsin and Bz-Tyr-pNA, the POPC vesicle system (FAT-VET_{100}) has been studied in detail, and it has been shown by dynamic modeling that it is possible to obtain—with certain assumptions made—the permeability coefficient of the substrate for the POPC bilayer directly [43]. One of the assumptions made was the monodispersity and unilamellarity of the vesicles, which is a first approximation. Further, it has been assumed that the enzyme inside the vesicles follows the Michaelis–Menten kinetics identical to the behavior outside of the vesicles; see Ref. [43] for details.

Table 7 lists a number of studies in which enzyme-catalyzed reactions occurring inside lipid vesicles have been used but not studied in great detail.

If one likes to use enzyme-containing lipid vesicles as nano- or microreactors in which the entrapped enzyme catalyzes a particular reaction inside the vesicles at least the four following requirements have to be fulfilled: (i) acceptable enzyme stability; (ii) sufficient vesicle stability; (iii) low leakage of the enzyme from the vesicles; and (iv) sufficient permeability of the vesicle bilayer for the enzyme's substrate(s). Regarding the last point, several attempts have already been carried out. Microwave irradiation has been used to increase the permeability of carbonic anhydrase-containing vesicles (DPPC–cholesterol–stearylamine, 5:3:2) against *p*-nitrophenylacetate [78]. In the case of ascorbate oxidase-containing vesicles (DPPC–cholesterol, 7:3), the use of microwave radiation apparently decreased the bilayer permeability for ascorbate [282]. The application of low frequency, low amplitude magnetic fields led to an increase in the permeability of vesicles composed of DPPC–cholesterol–stearylamine (6:3:1 or 5:3:2) against *p*-nitrophenylacetate [53,54]. The use of a membrane channel protein, porin OmpF extracted from the outer membrane of *E. coli*, allowed the permeation of ampicillin in the case of β-lactamase-containing polymerized vesicles made from nonnatural polymeric amphiphiles [93], see Fig. 13. The same observation has been made in a similar system composed of POPC vesicles or POPC vesicles containing an in situ prepared hydrophobic polymer as the membrane stabilizer [163]. The membrane bound protein seems to allow selectively the passage across the bilayer of molecules which have relative molar masses of ~ 400 or less. An increase in

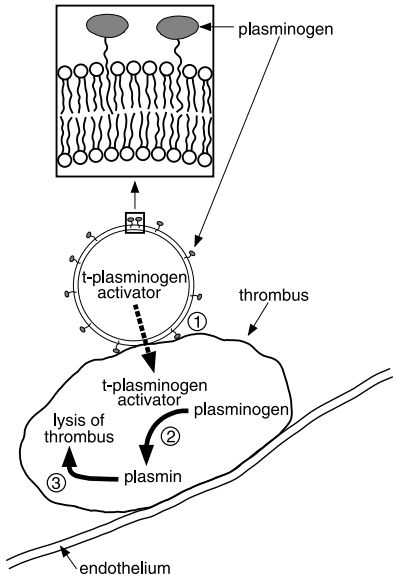

Fig. 5. Schematic representation of the proposed concept for the treatment of myocardial infarction by enzyme-containing lipid vesicles. Myocardial infarction is caused by a thrombus formed on the endothelium in a myocardial blood vessel, leading to a hindrance in the blood flow. Lipid vesicles contain the entrapped enzyme t-plasminogen activator and a surface-bound plasminogen which has a binding affinity to the fibrin clots (the thrombus). When the lipid vesicles bind to the fibrin rich blood clot (1), the t-plasminogen activator has to be released into the thrombus where plasminogen is converted into plasmin (2), which then leads to a lysis of the thrombus (3). See Ref. [229] for details.

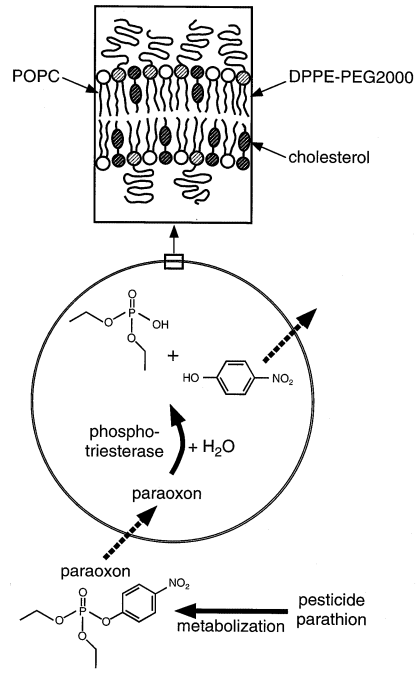

Fig. 6. Schematic representation of the detoxification of paraoxon inside phosphotriesterase-containing, sterically stabilized lipid vesicles composed of POPC–cholesterol–DPPE-PEG2000 (6:4:0.54). Sterically stabilized liposomes show a prolonged circulation time after being administered into the blood stream [85–89] (adopted from Ref. [81] and modified, see also Ref. [82] for a similar approach).

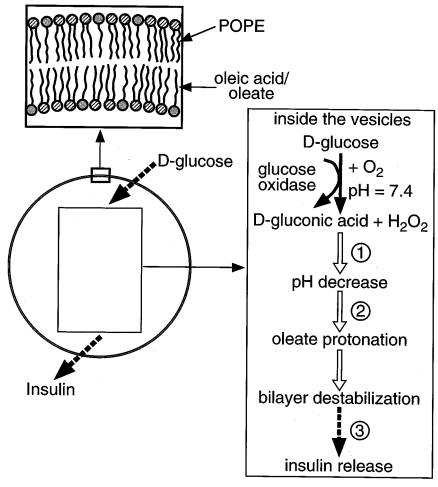

Fig. 7. Schematic representation of a glucose oxidase-containing pH-sensitive vesicle system for potential use as an insulin delivery system. Glucose oxidase and insulin are coencapsulated inside vesicles (POPE–oleic acid, 7:3). The oxidation of externally added glucose inside the vesicles leads to a decrease in pH (1), resulting in a destabilization of the vesicles (2) and release of insulin into the external medium (3). See Ref. [253] for details.

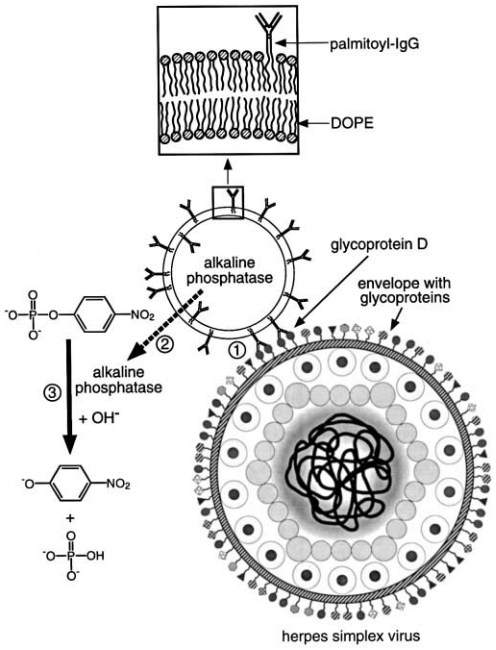

Fig. 8. Schematic representation of the target-sensitive lipid vesicles containing alkaline phosphatase in the vesicle interior and an immunoglobulin (anti-HSV glycoprotein D) on the bilayer surface that specifically interacts with the herpes simplex virus (HSV). On interacting with HSV (1), the vesicles (DOPE–palmitoyl-IgG mixtures) are destabilized and the entrapped enzyme is released into the external medium (2) where it catalyzes the hydrolysis of the substrate *p*-nitrophenyl phosphate (3), yielding in addition to phosphate the spectrophotometrically easily detectable *p*-nitrophenolate. See Ref. [232] for experimental details.

the glucose permeability of vesicles (egg PC based and stearylamine-containing) with entrapped glucose oxidase could apparently be achieved by the addition of insulin [38]. The addition of proteins may quite generally have an influence on the physical properties of lipid vesicles, depending on whether the added proteins adsorb onto the bilayers or not.

The use of micelle-forming detergents to induce a membrane permeabilization at sublytic detergent concentrations has been demonstrated by: (a) using sodium cholate in the case of glucose-1-phosphate and phosphorylase [164], ADP in the case of polynucleotide phosphorylase [121,285], or glucose in the case of glucose oxidase-containing POPC vesicles [41]; or (b) by using triton X-100 in the case of 4-methylumbelliferyl-β-D-glactoside in the case of β-galactosidase and soybean PC vesicles [284]. Further, the presence of deoxycholate in the vesicle preparation has probably led to an increase in the permeability of glucose oxidase- (or urate oxidase-) containing egg PC vesicles against glucose (or uric acid) [189].

In another approach, a change in the lipid composition has been used to change the permeability properties, e.g. the permeability of glucose oxidase-containing lipid vesicles (based on DMPC, DPPC, or DSPC) against glucose [59,274]; or the permeability of Mn^{2+} dependent DNase I and DNA-containing PC-based lipid vesicles against Mn^{2+} (the vesicles were only permeable for Mn^{2+} ions in the presence of egg PA) [50].

An enzymatic modification of vesicle bilayer membranes by added cholesterol oxidase has been reported in the case of PC-based vesicles (FAT-VET$_{100}$) [288]. The vesicles originally contained cholesterol which was then oxidized to cholest-4-en-3-one by the action of the enzyme which led to an increase in the leakage of the entrapped marker molecule carboxyfluorescein (increased permeability) [288]. This principle, however, has not yet been applied to enzyme-containing vesicle systems.

Finally, a variation in temperature has been used to increase the permeability of glucose oxidase–peroxidase-containing DMPC-based vesicles against glucose (or the substrate analog methylglucoside). The highest permeability has been observed around the main phase transition temperature T_m [46].

A quite different approach for the delivery of substrate molecules to vesicle-trapped enzymes is vesicle fusion [7,138,279]. In this case two vesicle dispersions are first prepared, the one containing the enzyme, and

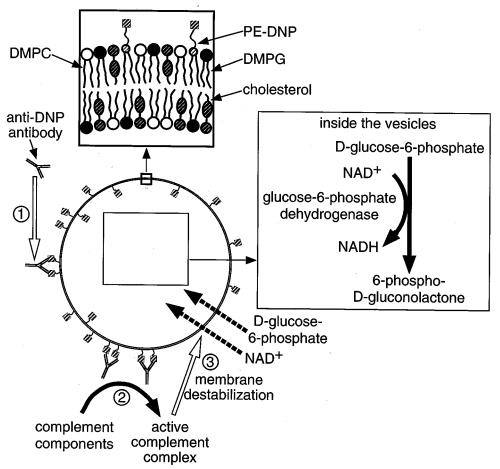

Fig. 9. Schematic representation of the principle of an enzyme-containing lipid vesicle immunoassay for the determination of the complement activity in human serum. The sample and the two reagents (reagents 1 and 2) are used. The sample contains the complement activity (a number of serum proteins). Reagent 1 contains DNP-labeled lipid vesicles (DMPC–DMPG–cholesterol–PE-DNP, 72:8:80:0.8) with encapsulated glucose-6-phosphate-dehydrogenase. Reagent 2 contains anti-DNP antibody and the substrate–coenzyme system (D-glucose-6-phosphate–NAD^+). After mixing the two reagents, the anti-DNP antibody interacts with DNP localized on the outer vesicle surface (1). After the addition of the sample, the complement system interacts with the anti-DNP antibody (2) leading to a destruction of the lipid vesicles (3), thereby making the vesicle membrane permeable for NAD^+, D-glucose-6-phosphate and possibly the entrapped enzyme, resulting in a conversion of NAD^+ to NADH which can be monitored at 340 nm. Details are given in Ref. [153].

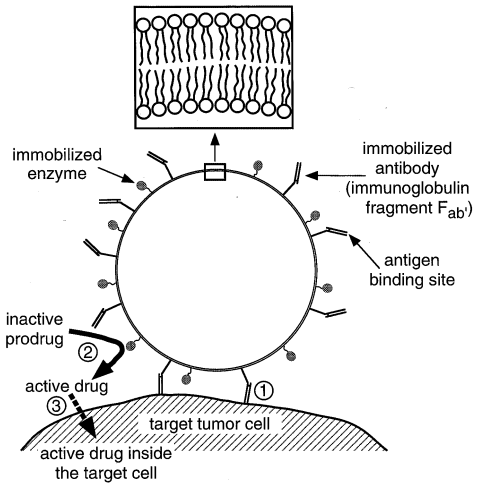

Fig. 10. Schematic representation of the principle of ADEPT (antibody-directed enzyme prodrug therapy) by using the so-called 'immuno-enzymosomes'. The immuno-enzymosomes contain on the outer surface a covalently bound immunoglobulin fragment ($F_{ab'}$), and an enzyme. After the specific binding of $F_{ab'}$ to the corresponding antibody on the surface of the target tumor cell (1), a prodrug is added which is then transformed into the active drug, catalyzed by the vesicle-bound enzyme (2). The drug can then be taken up by the cell (3). For details, see Ref. [268].

the other containing the substrate. On mixing these two type of vesicles, fusion may occur, depending on the composition of the lipids in the vesicle membrane and depending on the ion content, e.g. negatively charged

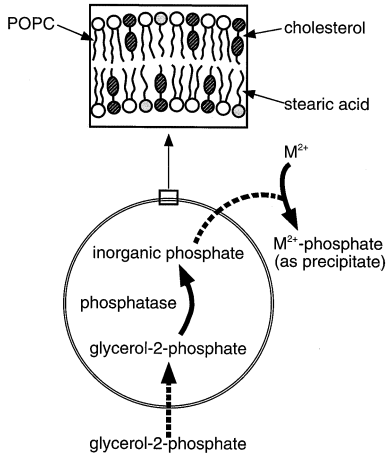

Fig. 11. Illustration of the idea and problems in a possible nonmedical use of enzyme-containing lipid vesicles for the decontamination of metal-laden industrial wastes, see the experimental approach in Ref. [129]. Lipid vesicles contain a phosphatase, and the external phase contains metal ions M^{2+} (e.g. Cd^{2+} or UO_2^{2+}) and an organic phosphate (glycerol-2-phosphate). Glycerol-2-phosphate permeates across the lipid vesicle bilayer and is hydrolyzed inside the vesicles to glycerol and inorganic phosphate. Phosphate forms an insoluble complex with M^{2+} which leads to the elimination of metal ions from the system. This enzyme-containing vesicle system works only if the inorganic phosphate ions can diffuse from the vesicle interior into the external aqueous phase, and if the metal ions do not interact with the lipid vesicle membrane. See Ref. [129] for details on the experiments.

lipids—PA or PS—and divalent ions (Ca^{2+} or Mg^{2+}), see the case of trypsin [277], a luciferase extract from the firefly lantern [7] or purified luciferase [137] (see Fig. 15), glucose oxidase [189] or urate oxidase [189].

The fusion between chymotrypsin-containing POPC vesicles (FAT-VET_{50}) and POPC vesicles containing the chymotrypsin substrate Suc-Ala-Ala-Pro-Phe-pNA could be achieved by the addition of partially denatured cytochrome c (in the presence of 0.5 M guanidinium hydrochloride) [283].

As briefly outlined in the following, there are at least three systems known in which a lipid vesicle fusion is triggered by the addition of enzyme molecules which act specifically on components of the vesicle membrane. So far, however, none of these principles have been applied for enzyme-containing vesicles.

1. The fusion of certain lipid vesicles can be triggered by the action of added phospholipase C from *Bacillus cereus*, e.g. in the case of vesicles composed of egg PC–egg PE–cholesterol, 2:1:1 (VET_{100}) [289–291]; or in the case of phospholipase C from *Clostridium perfringens* and (PE-free) vesicles composed of egg PC–cholesterol, 1:1 to 4:1 (SUV) [292]. The partial hydrolysis of the PC molecules to the membrane destabilizing 1,2-diacylglycerol (and phosphocholine) led to vesicle fusion.
2. It also seems that the action of the added phospholipase D (from *Streptomyces chromofuscus*) on PC in vesicles composed of egg PC–egg PE–egg PA, 20:50:30 or 15:35:50 (REV) can lead to vesicle fusion in the presence of Ca^{2+} as a consequence of the formation of PA from PC and PE [293].
3. In a specifically designed vesicle system, fusion could also be initiated through the addition of a hydrolytic enzyme, but in this case not through a phosphoric diester hydrolase, but through an endopeptidase (leukocyte elastase or proteinase K) [294]. The vesicles contained *N*-acetyl-Ala-Ala-DOPE – *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*trimethylammonium methyl sulfate (called 'DOTAP')–DOPE, 15:15:70 (FAT-VET_{100} or SUV) and the enzymes catalyzed the hydrolysis of the amide bond in the dipeptidic-PE derivative, yielding DOPE and *N*-acetyl-Ala-Ala-OH and resulting in vesicle fusion [294]. The sensitivity and selectivity of these functionalized vesicles could be increased by using tetrapeptide derivative *N*-methoxy-succinyl-Ala-Ala-Pro-Val-DOPE instead of the dipeptide head group [295].

First, entirely theoretical studies on the reactivity of enzyme-containing vesicles have also been published, see Table 8.

There are a couple of reports on enzymes which have been entrapped inside cell-sized giant vesicles and then investigated, see Table 9. In most of these cases, the enzyme has been microinjected inside previously pre-

Table 6
Selected examples for the basic experimental investigation of enzyme-catalyzed reactions occurring inside lipid vesicles

Enzyme	Type of study, remarks	References
D-Amino acid oxidase	Investigation of the activity of the entrapped enzyme against externally added D-amino acids (egg PC-based MLV). The amino acid permeability depended on the lipid composition, on the temperature and on the chemical nature of the amino acid	[39]
Ascorbate oxidase	Entrapped (and partially adsorbed enzyme) was active against externally added ascorbate and O_2 using DDV prepared from DPPC–cholesterol (7:3)	[51,281,282]
Carbonic anhydrase	The entrapped enzyme mainly adsorbed onto the positively charged lipid bilayer (DPPC–cholesterol–stearylamine 6:3:1 or 5:3:2, DDV). Activity measured against externally added CO_2	[55]
	Low frequency, low amplitude magnetic fields increase the permeability of enzyme-containing lipid vesicles against *p*-nitrophenylacetate (DPPC–cholesterol–stearylamine, 6:3:1 or 5:3:2, DDV, with diameters of 61 ± 18 nm, or 29 ± 11 nm, respectively). The enzyme was partially localized on the external vesicle surface, particularly when the ratio of DPPC–cholesterol–stearylamine was 6:3:1	[53,54,56]
α-Chymotrypsin	Activity and stability measurements of the enzyme entrapped inside POPC vesicles (FAT-VET_{100}) against externally added Bz-Tyr-pNA. No activity against the larger substrate Suc-Ala-Ala-Pro-Phe-pNA or casein. Inhibition of externally present enzyme by an inhibitor protein, see Fig. 12	[42]
	Detailed kinetic measurements and kinetic analysis (by dynamic modeling) of chymotrypsin-containing POPC vesicles ($\sim$87 enzyme molecules per vesicle with a diameter of $\sim$125 nm; FAT-VET_{100}) after external addition of the substrate Bz-Tyr-pNA. The modeling yielded directly the permeability coefficient for the substrate	[43]
	Fusion of chymotrypsin-containing vesicles (POPC, FAT-VET_{50}) with vesicles containing the substrate Suc-Ala-Ala-Pro-Phe-pNA (POPC, FAT-VET_{50}) by addition of partially denatured cytochrome c	[283]
DNase I/DNA	Entrapment of the enzyme together with the substrate (DNA) in DPPC (or egg PC)–egg PA (2:1) lipid vesicles (REV). Activation of the enzyme after external addition of Mn^{2+} which could permeate across the vesicle bilayer. The presence of the nonbilayer lipid PA was essential for the cation permeation	[50,272]
β-Galactosidase	Investigation of the change in the permeability of enzyme-containing vesicles against externally added substrate (4-methylumbelliferyl-β-D-galactoside) by triton X-100 under sublytic concentrations (soybean PC, VET_{200})	[284]
β-Glucosidase	Entrapment of the enzyme inside the lipid vesicles (DMPC–cholesterol–dicetylphosphate, 7:2:1 or 7:4:1, REV) led to a stabilization of the enzyme against inhibition by externally added Cu^{2+} ions, as measured with *p*-nitrophenyl-β-D-glucopyranoside as substrate	[29,47]
Glucose oxidase	A few measurements on the activity of entrapped glucose oxidase after external addition of glucose in the presence of variable amounts of added insulin which seems to permeabilize the vesicle bilayers. Egg PC-based vesicles (SUV)	[38]
	Activity measurements in egg PC based enzyme-containing vesicles which had deoxycholate in the membrane against externally added glucose. The enzyme-catalyzed reaction was also initiated by Ca^{2+}-mediated fusion of enzyme-containing vesicles with substrate-containing vesicles	[189]
	Activity measurements of enzyme-containing vesicles against externally added glucose (POPC, FAT-VET_{100}). The presence of sublytic concentrations of triton X-100 or cholate led to an increased glucose permeability and rate of glucose oxidation inside the vesicles. Problems of partial enzyme release	[41]

Table 6 (*Continued*)

Enzyme	Type of study, remarks	References
	Characterization of glucose oxidase-containing lipid vesicles prepared from DPPC–PI (9:1, weight ratio, VET_{100}) after external addition of glucose	[58]
	Activity measurements of the entrapped enzyme after external addition of glucose (DMPC or DPPC or DSPC–wheat germ PI, 9:1, weight ratio, REV)	[59]
Glucose oxidase in combination with peroxidase or lactoperoxidase	Investigation of the activity of the two enzyme containing anionic (DMPC–PI, 1:0.088 or 1:0.97, VET_{100}) or cationic lipid vesicles (DMPC–cholesterol–DODAB, 1:0.45:0.16, VET_{100}) against externally added D-glucose. Highest activity (H_2O_2 production) observed at T_m. The enzymes per se increased the permeability of the bilayer membrane in the case of anionic vesicles	[46]
OPAA	Activity measurements of OPAA-containing lipid vesicles composed of POPC–cholesterol–DPPE-PEG2000 (60:40:5.4 or 90:60:9, VET_{100}) after external addition of diisopropylfluorophosphate by using a fluoride sensitive electrode for the detection of the fluoride ions formed, see also Fig. 6	[82,83]
Polynucleotide phosphorylase	Experimental demonstration that the enzyme inside DMPC vesicles (DRV-VET_{200}) was active against externally added ADP	[48]
	Activity measurements of enzyme-containing oleic acid–oleate vesicles (VET_{100}) upon external addition of ADP, yielding lipid vesicle trapped poly A; simultaneous oleic anhydride hydrolysis resulting in a self-reproducing vesicle system	[90]
	Entrapment of the enzyme in POPC vesicles (FAT-VET_{100} or FAT-VET_{400}). Determination of the poly-A formation inside the vesicles after external addition of ADP in the presence of sublytic concentrations of sodium cholate	[285,121]
Trypsin	Activity and stability measurements of the enzyme entrapped inside POPC vesicles (FAT-VET_{100}) against externally added Bz-Arg-pNA and Z-Phe-Val-Arg-pNA. Inhibition of externally present enzyme by an inhibitor protein. The vesicles were $\sim$100 nm in size and each vesicle contained $\sim$20 enzyme molecules, see Fig. 12	[162]
Tyrosinase	Kinetic investigations against different externally added mono- and diphenols (MLV prepared from egg PC in the absence or presence of up to 8 mol% cholesterol)	[286]
Urease	Kinetic investigation towards externally added urea. Increased stability of the entrapped enzyme. The vesicles were prepared from egg PC–cholesterol–dicetylphosphate, 26:6:1, MLV)	[124]
Urate oxidase	Activity measurements in egg PC based enzyme-containing vesicles which had deoxycholate in the membrane against externally added uric acid. The enzyme-catalyzed reaction was also initiated by Ca^{2+}-mediated fusion of enzyme-containing vesicles with substrate-containing vesicles	[189]

See also Refs. [267,278–280].

pared vesicles with the help of a microneedle and an appropriate microinjection device, a method which is well known in cell biology [305,306] and applicable to giant vesicles [303,307,308].

In one case, so-called 'reverse vesicles' have been used as microcompartment system for the entrapment of an enzyme, polyphenol oxidase (tyrosinase) [309]. The enzyme has been incorporated in reverse vesicles formed from the nonionic surfactant tetra(ethylene glycol) dodecyl ether ($R_{12}EO_4$, 2.5%, w/w) in *n*-dodecane in the presence of water (1%, w/w) as a new approach in the study of enzymes in organic solvents for the potential application in the bioconversion of water-insoluble substrates. The reverse vesicles formed were oligolamellar with sizes between <1 and 10–15 μm. A schematic representation of a reverse vesicle is shown in Fig. 16. One or more concentric water shells are separated by layers of amphiphiles in a water-immiscible organic solvent. While the polar parts of the amphiphiles are localized in the water domains of the

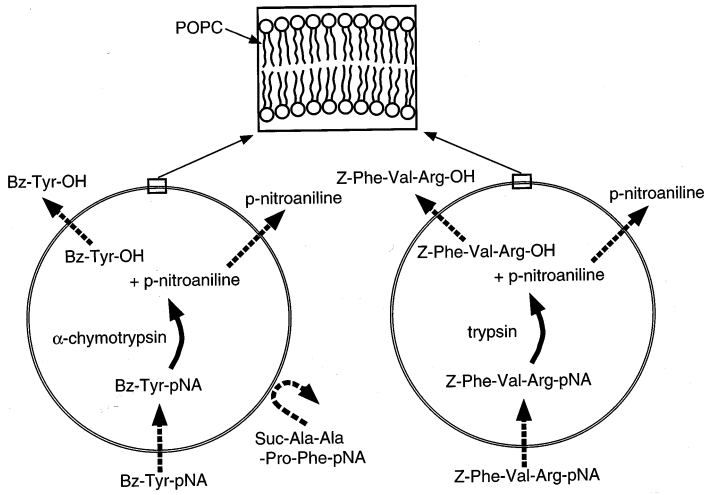

Fig. 12. Schematic representation of an enzyme-containing vesicle system which has been prepared by mixing two type of vesicle dispersions, 'vesicles A' and 'vesicles B'. 'Vesicles A' contained the entrapped α-chymotrypsin and 'vesicles B' the entrapped trypsin. After the external addition of a substrate mixture of Bz-Tyr-pNA, Suc-Ala-Ala-Pro-Phe-pNA and Z-Phe-Val-Arg-pNA, the substrates are hydrolyzed selectively; the α-chymotrypsin substrate Bz-Tyr-pNA is cleaved, whereas the other α-chymotrypsin substrate Suc-Ala-Ala-Pro-Phe-pNA is not cleaved, as it cannot permeate across vesicle's bilayer (so-called bilayer permeability-based substrate selectivity of an enzyme in lipid vesicles [42]). The trypsin substrate Z-Phe-Val-Arg-pNA is hydrolyzed by the entrapped trypsin. Enzyme molecules possibly present outside the vesicles have been inhibited by a trypsin/α-chymotrypsin inhibitor protein which did not permeate inside the vesicles [42,43,162].

system, the hydrophobic tails are in contact with the organic solvent. In these type of systems, the water content is low, which could be but does not need to be a problem, e.g. enzyme inactivation, low overall enzyme content.

5. Concluding remarks and outlook

As outlined in detail above, there are a large number of methods known for the preparation of enzyme-containing lipid vesicles. Depending on the enzyme used and depending on the application, the chemical nature of the vesicle-forming amphiphile and the preparation method can be selected to prepare vesicles of a particular size (or size range) and degree of lamellarity.[22] The extrusion technique in combination with a preceding dehydration–rehydration step—resulting in DRV-VET—is certainly a way that may satisfy in many cases the appropriate needs.

If one critically reviews all the investigations carried out so far, one is tempted to say that a careful analysis of the enzyme-containing vesicles has not often been carried out. The following questions did not yet receive enough attention: Is the enzyme localized inside the vesicles or mainly adsorbed onto the vesicle bilayer? Are there enzyme molecules present outside of the vesicles? Are the enzyme molecules partially inactivated during the encapsulation procedure? Are the enzyme-containing vesicles stable with respect to size and lamellarity? or Do enzyme molecules leak out from the vesicles upon storage? Most of these questions can be approached experimentally.

There is in principle no single vesicle preparation (enzyme-encapsulation) method, no standard lipid or lipid mixture that can be recommended to be generally useful in all the cases. Optimal conditions depend on the enzyme and on the type of application.

With respect to the reported enzyme encapsulation efficiencies, one has to pay attention to the fact that the EE values depend largely on the lipid concentration used during the encapsulation step.

The quantitative analysis of experimental data on the kinetics of reactions catalyzed by enzyme-containing vesicles (see Ref. [43]), and the entire theoretical treatment of enzyme-containing lipid vesicles (see Table 8), certainly need more efforts. This will hopefully lead to a better understanding of enzyme-containing vesicles as nano- or microreactor systems, and hopefully to an individual optimization of the systems by design on a molecular level, e.g. optimization of stability and substrate permeability control.

In this review, we have limited ourselves to an overview on studies on lipid vesicles with entrapped water-soluble enzymes. At the end, we would like to mention two related fields of research which may be of considerable interest: (i) lipid vesicles with covalently bound enzymes for medical and bioanalytical applications; and (ii) lipid vesicle-mediated refolding of enzymes.

[22] Please note that the determination of the mean vesicle size, size distribution and lamellarity is not trivial, particularly if the vesicles are polydisperse [30].

In the case of (i), it has been reported that lipid vesicles have been prepared which contained covalently bound enzyme molecules (β-glucuronidase) on the external surface of lipid vesicles together with an antigen (specific for certain antibodies on the surface of target tumor cells) for an antibody-directed enzyme prodrug therapy (abbreviated as ADEPT). These functionalized lipid vesicle systems have been called 'immuno-enymosomes' [268,314] and are aimed to be active at the site of the tumor, where the immobilized enzyme catalyzes the transformation of an added inactive pro-drug into the active drug which then enters the tumor cell, see Fig. 10.

In another approach, bifunctional vesicles have been prepared which contained immobilized peroxidase and an antigen (e.g. biotin) for an immunosorbent assay to detect solid surface immobilized antibodies (e.g. anti-biotin antibody) [315–318]. This principle has been extended to oligonucleotide-DNA sensing [318].

Regarding point (ii), it has been demonstrated that lipid vesicles (or detergent micelles) may assist the refolding of unfolded enzymes (or proteins in general) [319]. The refolding of heat-inactivated T4 endonuclease V for example seems to be facilitated by the presence of anionic lipid vesicles composed of PC, PE, oleic acid, and cholesteryl hemisuccinate (2:2:1:5) [243]. Further, it has been shown that the refolding of carbonic anhydrase [320] or denatured–reduced lysozyme [321] is assisted by POPC vesicles. All these observations are a consequence of the interaction of the proteins (enzymes) with the vesicle membrane, and it is likely that this type of (relatively unspecific) interactions may be of some advantage—or disadvantage—in other vesicular systems. The (empty) vesicles act here as a kind of catalyst. It has for example been reported that the trypsin-catalyzed hydrolysis of insulin is more efficient in the presence of soybean PC vesicles (particularly in the case of the small VET_{50}) than in the absence of

Table 7
Selected examples for miscellaneous studies of the activity of enzyme-containing lipid vesicles

Enzyme	Type of study, remarks	References
Alkaline phosphatase	Determination of the activity of the entrapped enzyme (phospholipon 90–sodium oleate, 4:1, weight ratio, MLS) against externally added *p*-nitrophenylphosphate	[64]
Butyrylcholinesterase	Demonstration of the activity (and inhibition) of the enzyme inside the lipid vesicles (soybean PC, MLV) against externally added substrate butyrylthiocholine (or inhibitor paraoxon)	[125]
Carbonic anhydrase	Coencapsulation of the enzyme with the fluorescent pH indicator pyranine at high pH allowed the determination of small amounts of enzymes added at low pH externally to the lipid vesicles (made from asolectin, a soybean phospholipid mixture, VET_{100}) and it further allowed the determination of CO_2 flux across the membrane, see Fig. 14	[287]
Catalase	Detection of the activity of entrapped enzyme after external addition of H_2O_2 (DPPC–cholesterol–stearylamine, 14:7:4, REV)	[76]
DNA polymerase/DNA	Polymerase chain reaction detected to occur inside POPC–PS vesicles (9:1, weight ratio, FAT-VET_{400}) in the presence of entrapped nucleotide triphosphates	[71]
Glucose oxidase	Coencapsulation of the enzyme with insulin in pH-sensitive lipid vesicles (POPE–oleic acid, 7:3, REV). Externally added D-glucose permeated into the vesicles, leading to the formation of gluconic acid which in turn led to a decrease in pH, resulting in a vesicle destabilization and therefore to an insulin release (see Fig. 7)	[253]
β-Lactamase	Enzyme entrapment inside POPC or polymer-stabilized POPC-based vesicles (FAT-VET_{200} [163]), or inside polymerized PMOXA-PDMS–PMOXA-triblock copolymer vesicles (VET_{200}, mean diameter 250 nm [93]) containing in the vesicle membrane the channel protein porin OmpF. The enzyme substrate (the antibiotic ampicillin) could permeate across the porin-containing membrane, although in the case of the triblock copolymer vesicles it was 10 nm thick [93] membrane, see Fig. 13	[93,163]
Lipase from *Chromobacterium viscosum*	Investigation of the enzyme activity inside oleic acid–oleate vesicles (VET_{100}) towards ethyl oleate originally present in enzyme-free vesicles as an approach towards vesicle reproduction	[91]
Phosphorylase	Enzyme entrapped inside POPC vesicles (FAT-VET_{400}, diameter: ~150 nm) as models for protocells in the field of the origin of biological cells. Addition of cholate allowed the passage of substrate across the membrane	[164]
Q_β replicase/MDV-1 RNA	RNA-template (MDV-1 RNA) replication catalyzed by the entrapped enzyme in the presence of entrapped substrates (ATP, CTP, GTP, UTP). Vesicles made from oleic acid + oleate (VET_{200}); simultaneous oleic anhydride hydrolysis resulting in a self-reproducing vesicle system	[92]
Trypsin	The bilayer membrane of the vesicles (diameter: 125 ± 30 nm; composed of egg PC–lyso PC: SM–PE, 72.5:4.6:3.8:19.1 weight ratio, DDV) allowed the permeation of the substrate (N_α-benzoyl-DL-arginine-4-nitroanilide) from the exterior medium to the enzyme localized in the interior of the vesicles	[80]
	Fusion of trypsin-containing vesicles with fluorogenic substrate-containing vesicles in the presence of Ca^{2+} (egg PC–bovine brain PS, 1:1, SUV)	[277]

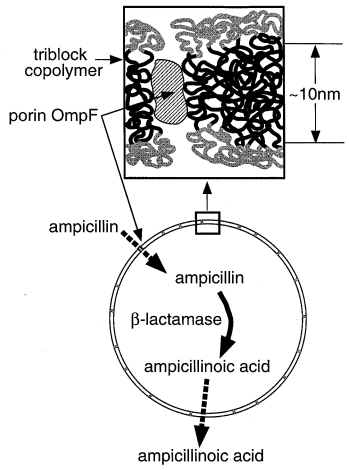

Fig. 13. Schematic representation of a β-lactamase-containing nanoreactor prepared from an amphiphilic triblock copolymer PMOXA–PDMS–PMOXA and the membrane channel protein porin OmpF, adopted from Ref. [93].

vesicles, which is most likely a consequence of electrostatic interactions between insulin (not trypsin) and the vesicles [222,322].

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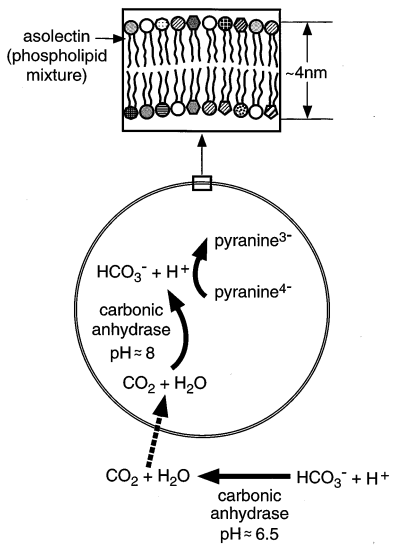

Fig. 14. Schematic representation of an analytical vesicle system containing coencapsulated carbonic anhydrase and the fluorescent pH indicator pyranine (8-hydroxypyrene-1,3,6-trisulfonate, $pK_a \sim 7$) for determining either the externally present carbonic anhydrase or the CO_2 permeability across the vesicle bilayer. Externally present pyranine was quenched by *p*-xylene bispyridinium dibromide. For experimental details, see Ref. [287].

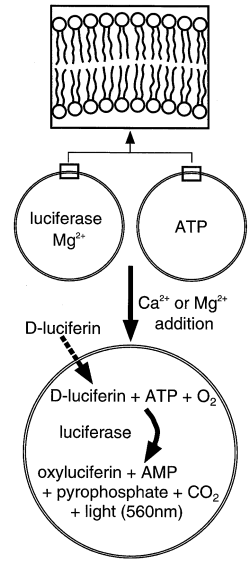

Fig. 15. Schematic representation of the principles used in a vesicle fusion assay which uses two populations of vesicles prepared initially, enzyme-containing vesicles and substrate-containing vesicles. The enzyme applied in the case shown is the ATP-hydrolyzing enzyme luciferase and the corresponding substrate ATP. D-Luciferin was added to the vesicles as it was shown to permeate across the bilayers easily. Upon vesicle fusion, the enzyme-catalyzed reaction takes place, which can be monitored spectrophotometrically, although a quantitative determination of the fusion extent is rather difficult, see Refs. [7,138] for details.

Appendix A

Enzymes mentioned in this review (with EC numbers—if available—and alternative names).

Alkaline phosphatase (3.1.3.1)
D-Amino-acid oxidase (1.4.3.3)
δ-Aminolevulinate dehydratase (4.2.1.24; 5′-aminolevulinic acid dehydratase; porphobilinogen synthase)
α-Amylase (3.2.1.1)
Amyloglucosidase (3.2.1.3; glucan 1,4-α-glucosidase)
Ascorbate oxidase (1.10.3.3; L-ascorbate oxidase)
Asparaginase (3.5.1.1; L-asparagine amidohydrolase)
Butyrylcholine esterase (3.1.1.8; cholinesterase; nonspecific cholinesterase)
Carbonic anhydrase (4.2.1.1, carbonate dehydratase)
Catalase (1.11.1.6)
Chloroperoxidase (1.11.1.10, chloride peroxidase)
Cholesterol esterase (1.1.3.6)
Chymosin (3.4.23.4; rennin)
α-Chymotrypsin (3.4.21.1; chymotrypsin A, chymotrypsin)
Corolase® PN (endopeptidase)
Cyprosin (3.4.23; aspartic endopeptidase from cardoon flowers, *Cynara cardunculus* L.)
Dextranase (3.2.1.11; 1.6-α-D-glucan 6-glucanohydrolase)

Table 8
Theoretical investigations of enzymatic reactions occurring inside lipid vesicles

Enzyme	Investigation	References
Urease	Development of a kinetic model which predicted the concentration and pH changes inside enzyme-containing vesicles upon external addition of urea leading to the formation of CO_2 and NH_3	[296]
General enzyme	Development of a mathematical model for the description of the kinetic behavior of enzyme-loaded lipid vesicles containing a substrate transporter	[297,280]

DNA photolyase (4.1.99.3; deoxyribocyclobutadipyrimidine pyrimidine lyase)
DNA-(apurinic or apyrimidinic site) lyase (4.2.99.18; phage T4 UV endonuclease, T4 endonuclease V)
DNA polymerase (2.7.7.7)
DNase I (3.1.21.1)
Elastase (3.4.21.36; pancreatic elastase; or 3.4.21.37; leukocyte elastase)
Flavourzyme® (a mixture of fungal exopeptidases and endopeptidases form *Aspergillus oryzae*)
β-Fructofuranosidase (3.2.1.26; invertase)
β-Galactosidase (3.2.1.23; lactase)
β-Glucosidase (3.2.1.21; β-D-glucosidase)
Glucose oxidase (1.1.3.4)
Glucose-6-phosphate 1-dehydrogenase (1.1.1.49; NADP-dependent glucose 6-phosphate dehydrogenase)
β-Glucuronidase (3.2.1.31)
Glyceraldehyde-3-phosphate dehydrogenase (1.2.1.12)
Hexokinase (2.7.1.1)
β-Lactamase (3.2.5.6; penicillinase)
Lipase from *Chromobacterium viscosum* (3.1.1)
Luciferase (1.13.12.7; firefly luciferase; *Photinus*-luciferin 4-monooxygenase (ATP-hyrolyzing))
Lysozyme (3.2.1.17)
Neutrase® (3.4.24.28; Bacillolysin, a neutral *Bacillus subtilis* endopeptidase)
OPAA (3.1.82; an organophosphorus acid anhydrase; diisopropylfluorophosphatase from *Alteromonas* strain JD6)
Pepsin A (3.4.23.1; pepsin)
Peroxidase (1.11.1.7; lactoperoxidase; horse radish peroxidase)
Phosphatase from *Citrobacter* sp. (3.1.3)
Phospholipase A_2 (3.1.1.4)
Phospholipase C (3.1.4.3)
Phospholipase D (3.1.4.4)
Phosphorylase (2.4.1.1; 1,4-α-D-glucan:phosphate α-D-glucosyltransferase)
Phosphotriesterase (3.1.8, a phosphoric triester hydrolase)
t-Plasminogen activator (3.4.21.68, tissue plasminogen activator, Actilyse®)
Polynucleotide phosphorylase (2.7.7.8, polyribonucleotide nucleotidyltransferase)
Proteinase K (3.4.21.64; endopeptidase K)
Proteinase (neutral) from *Bacillus subtilis* (3.4)
Proteinase (acid) from *Aspergillus oryzae* (3.4)
Proteinase from *Mucor miehei* (3.4)
Q_β Replicase/MDV-1 RNA (2.7.7.48; RNA-directed RNA polymerase)

Table 9
Examples for the investigation of enzymes in individual micrometer-sized (giant) lipid vesicles

Enzyme	Investigation	References
Alkaline phosphatase	Microinjection into giant POPC vesicles	[299]
	Observation of the enzyme-catalyzed hydrolysis of fluorescein diphosphate inside giant POPC vesicles	[300]
Deoxyribonuclease I	Indication for enzyme migration from outside of giant POPC vesicles to the vesicles interior by some unknown process	[299]
sn-Glycerol-3-phosphate-acyltransferase	Microinjection of the membrane enzyme into giant vesicles composed of POPC–palmitoyl-coenzyme A (9:1, weight ratio) in the presence of glycerol-3-phosphate. Observation of the morphological changes	[301]
Phospholipase D	Microinjection inside giant POPC vesicles and observation of the chemical transformation of fluorescently labeled PC derivative into POPA, without destabilizing the spherical vesicle structure	[302]
Phospholipase A_2	Microinjection inside giant POPC vesicles and light microscopic observation of the vesicle transformation as the reaction products (lyso PC and oleic acid–oleate) destabilized the vesicle bilayer (continuous vesicle size decrease)	[303]
Ribonuclease A	Indication for enzyme migration from outside of giant POPC vesicles to the vesicles interior	[299]
Sphingomyelinase	Microinjection into sphingomyelin-containing SOPC vesicles. Observation of morphological changes in the vesicles (budding)	[304]

See also Ref. [298].

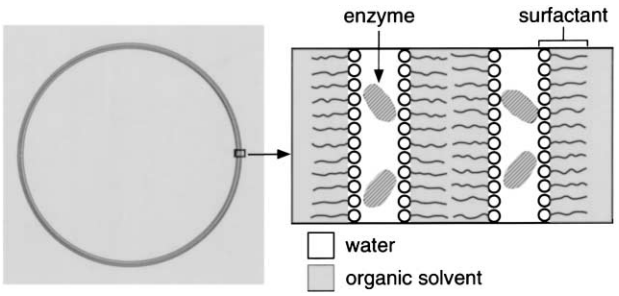

Fig. 16. Schematic representation of an enzyme-containing 'reverse vesicle' system, adopted from Ref. [309]. For basic physico-chemical studies on reverse vesicles, see Refs. [310–313].

Ribonuclease A (3.1.27.5; RNase A, RNase I, pancreatic ribonuclease)
Rulactine (3.4.24; a metalloproteinase from *Micrococcus caseolyticus*)
Sn-glycerol-3-phosphate *O*-acyltransferase (2.3.1.15)
SP446 (a serine protease from Novozymes)
Sphingomyelinase (3.1.4.12; sphingomyelin phosphodiesterase)
Streptokinase (3.4.99.0; Kabikinase®) from streptococcal bacteria
Superoxide dismutase (1.15.1.1)
Trypsin (3.4.21.4)
Tyrosinase (1.14.18.1; monophenol oxidase, polyphenol oxidase, catechol oxidase)
Urate oxidase (1.7.3.3; uric acid oxidase, uricase)
Urease (3.5.1.5; urea amidohydrolase)

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