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Topical Review

Liposome-entrapped Polymerases as Models for Microscale/Nanoscale Bioreactors

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Introduction

The first enzyme-containing lipid vesicles were developed soon after the pioneering work of Bangham et al. (1964, 1965) that outlined the physical properties of these lipid structures in an aqueous medium. Such systems have been envisioned as drug-delivery vehicles for gene therapy or enzyme-replacement therapy and as bioreactors, which could ultimately serve as microcompartments for in vitro selection of, e.g., catalytic RNA (ribozymes), or as blueprints for an artificial minimal cell.

Amphiphile vesicles with encapsulated enzymes, such as polymerases (Gao & Huang, 1993) endonucleases (Yarosh, 2001), or nucleic acid materials (Nabel et al., 1993) have been investigated as delivery systems. In this case, active catalysts are encapsulated into the vesicular structures, and then delivered to a target site by molecular recognition between targeting agents embedded in the vesicle bilayers and receptors on cell membranes. The vesicle contents are then released into the cytoplasm after fusion between the cell membrane and the vesicles, or after endocytosis of the vesicles and subsequent fusion of lysosomal sacs with the pH-sensitive vesicles (Legendre & Szoka Jr., 1992; Hafez & Cullis, 2000).

Amphiphile vesicles with entrapped enzymes have also been studied as potential submicrometer-sized bioreactors (*see* Walde & Ichikawa, 2001 for review). In this case, the vesicles serve as a small compartment where the enzyme catalysis takes place. Substrate molecules are externally added to the bulk medium and must cross amphiphile bilayers before they can be converted into products by the entrapped enzyme. Microsize vesicle-based bioreactors could be developed that combine both the properties of biological and nanotechnological systems. Such systems would represent a kind of artificial minimal cell (Pohorille & Deamer, 2002), and could be engineered for a specific task related to therapeutic and diagnostic applications. Unlike genetically modified cells that require stringent conditions to survive and can be damaged during genetic manipulations, these liposome-based bioreactors would only be composed of the desired genetic material or/and metabolic activity, thereby overcoming safety and ethical issues that are relevant in medical and environmental applications.

A functional micro- or submicroscale bioreactor (*see* Fig. 1) should possess a specific set of chemical and physical properties, and it is worth listing them here to provide a foundation for later discussion. First, the catalytic species should be efficiently entrapped within the bioreactor compartment whose size does not exceed the micrometer range. The decisive parameter is not the amount of entrapped catalytic species in g/l or mol/l but rather the entrapped activity. That is, enzyme molecules, along with primers and templates, have to be simultaneously captured within a single aqueous microenvironment to be integrated in a functional catalytic assembly. Second, the boundaries of the reactor must allow for selective diffusion of substrate molecules and products while retaining the catalytic species components and protecting them from degradation. This can be achieved in different ways: passive diffusion, carrier-mediated passive transport, channels and pumps. Third, bioreactor boundaries must be stable enough to withstand alterations of the external conditions (pH, temperature, ionic strength changes), as well as allow for a prolonged use in their intended capacity. Fourth, the system (bioreactor boundaries, catalytic species, byproducts) must be innocuous to allow their use in a living system.

Amphiphile vesicles with encapsulated polymerases can be custom-designed to possess the essential

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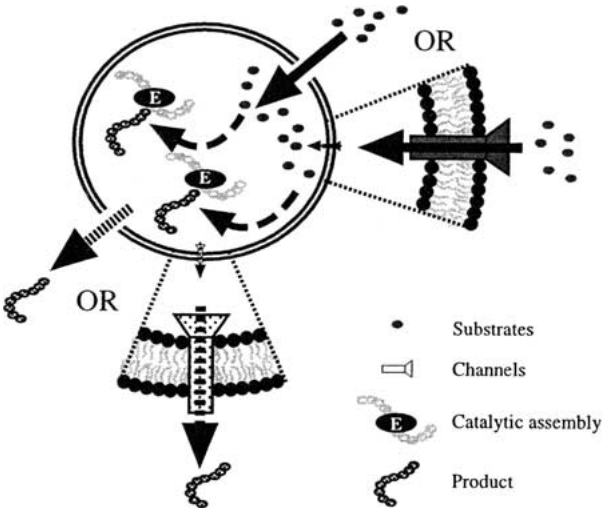

Fig. 1. Schematic representation of a bioreactor. A vesicle-based bioreactor depicted here would be composed of one or several functional catalytic assemblies, such as a polymerase and its template, encapsulated within the vesicle bilayers. The uptake of substrates and the release of the products could occur either by passive diffusion across the bilayers using membrane-packing defects or by passive transport (along concentration gradients) mediated by channels embedded in the reactor boundaries. In both cases, the selectivity of the exchange mechanism between the reaction "chamber" and the external medium would need to be so stringent that no component of the catalytic assembly could be accidentally released, causing loss of activity.

characteristics of a functional bioreactor. An encapsulated RNA transcription represents a crucial step in the development of RNA-selection bioreactors or artificial minimal cells. Because vesicles entrap only a few template molecules on average, using such a system would permit a simplified RNA selection. The overall number of RNAs to select from would be similar to that used in a common selection process in an aqueous solution (Beaudry & Joyce, 1992; Wright & Joyce, 1997; Schultes & Bartel, 2000), but these RNA fragments would be distributed in small pools within individual liposomes. Thus, a single ribozyme could be recovered and amplified with higher specificity. On the other hand, RNA transcription is a crucial metabolic step towards the design of an artificial minimal cell capable of sustained "metabolism". The relative instability of RNA molecules limits the use of an encapsulated pool of mRNA to very short time periods. In contrast, an in situ transcription would ensure a continuous supply of mRNA for prolonged protein synthesis within an artificial cell (Oberholzer, Nierhaus & Luisi, 1999b; Yu et al., 2001), and also allow for a controlled activity.

This review will address issues directly related to the design of liposome/polymerase-based bioreactors with an emphasis on compartmentation of catalytic assembly, steady transmembrane supply of nucleotides across the membrane bilayers, as well as the effect of the compartmentation on the enzymatic activity.

Amphiphile Vesicles and Liposomes

Bangham and co-workers (Bangham et al., 1965) first established that phospholipids dispersed in an aqueous medium spontaneously to form spherical structures, also called vesicles or liposomes, having dimensions in the range of bacterial cells. These structures can also be formed by dispersing simpler amphiphile molecules, such as fatty acid derivatives (Gebicki & Hicks, 1973, 1976; Hargreaves & Deamer, 1978).

Amphiphile vesicles are composed of a small aqueous volume surrounded by shells that are composed of closed molecular bilayers in which the nonpolar moiety of the amphiphile molecules forms the hydrophobic interior of the bilayer and the hydrophilic part (the headgroup) is in contact with the aqueous phases. They can be multilamellar (Multi-Lamellar Vesicles, MLVs), oligolamellar (OLVs), or unilamellar (UVs), i.e., the internal water core is surrounded by several concentric amphiphile bilayers or a single bilayer. Depending on their size, which can range between 20 nm and tens of micrometers in diameter (Luisi & Walde, 2000), unilamellar vesicles are further categorized as small unilamellar vesicles (SUV, less than 50 nm in diameter), large unilamellar vesicles (LUV, less than 1 μm in diameter) or giant unilamellar vesicles (GUV, 1 μm to 100 μm in diameter). Unilamellar vesicular structures with a 200-nm and 10-μm diameter encapsulate an aqueous volume of 4.2×10^{-18} l and 4.2×10^{-12} l, respectively. The aqueous volume of the vesicles usually presents a chemical composition that approximately corresponds to that of the aqueous solution in which the vesicles were prepared. That is, these spontaneously-forming amphiphile structures can be used to capture hydrophilic solutes, such as enzymes and nucleic acids (Mannino, Allebach & Strohl, 1979; Jay & Gilbert, 1987; Monnard, Oberholzer & Luisi, 1997; Apel, Mautner & Deamer, 2002).

Compartmentation

The first experimental step towards the design of an enzymatic system entrapped in amphiphile vesicles is to obtain an efficient encapsulation of the functional catalytic species. That is, the encapsulation procedure does not cause the enzymes to denature, and all the components of the catalytic assembly, for example, the enzyme and its template in the case of RNA transcription, must be trapped in a single aqueous volume in order to be integrated in a functional assembly.

Table 1. Comparison of encapsulation efficiency of DNA templates

Lipid kind (concentration in mM[#])	Liposome preparation method	Homogenization of size with polycarbonate filters (diameter of pores in nm)	Solute type	Encapsulation efficiency (% of initial material)	Reference
POPC (160 mM)	Reverse phase evaporation	None (unextruded)	369 bp DNA	25	(Monnard et al., 1997)
	Dehydration/ rehydration	None (unextruded)		43	
	Freeze/thaw	None (unextruded)		54	
		400	Linearized plasmid (3368 bp)	27	
		100		9	
		50		5	
POPC/DDAB 99/1* (160 mM)	Freeze/thaw	400		50	
		100		50	
		50		17	
DMPC[†] (160 mM)	Dehydration/ rehydration[@]	None (unextruded)	Circular plasmid (4800 bp)	12	(Monnard & Deamer, unpublished results)
		800		11	
		400		8	
		200		5	

[#]: These concentrations refer to the lipid concentrations during the encapsulation step.
*: This is the molar ratio of POPC to DDAB.
[†]: The plasmid encapsulation was performed in the presence of 50 μg/ml BSA.
[@]: The dehydration/rehydration method was used here because of the sensitivity of the T7 RNA polymerase to repeated freeze/thaw cycles.

Vesicle encapsulation efficiency is directly related to the kind of amphiphiles, the vesicle overall lamellarity and size, consequently the preparation method (Monnard et al., 1997), and the external factors such as ionic strength of the medium (Monnard et al., 1999), and pH (Chakrabarti, 1994; Chakrabarti et al., 1992).

The first liposomes formed by hydrating a lipid film are typically multilamellar, and their encapsulation efficiency is not ideal because solutes tend to be excluded from the aqueous liposome interior (Gruner et al., 1985). To improve the overall capture efficiency and achieve $[\text{solute}]_{\text{inside}} \geq [\text{solute}]_{\text{outside}}$, new techniques were developed, such as freeze/thaw (Pick, 1981), dialysis (Zumbuehl & Weder, 1981), reverse evaporation (Szoka & Papahadjopoulos, 1978), and dehydration/rehydration (Deamer & Barchfeld, 1982; Shew & Deamer, 1985). They differ in so far as the physical vesicle characteristics broadly vary, even after size homogenization of vesicles using extrusion through polycarbonate filters (Hope et al., 1985). When the encapsulation efficiency of three common methods was compared ((Monnard et al., 1997); *see* also Table 1) under standardized conditions, i.e., using the same concentrations of guest molecules (DNA templates, tRNA, and ATP) and equally extruded liposomes, the total encapsulation in freeze/thawed vesicles was up to twice that of dehydration/rehydration or reverse-evaporation vesicles. As the average liposome size decreases, less material was encapsulated. However, as discussed below (*see* section Entrapped Polymerase Activity), a higher encapsulation of templates and presumably enzymes with unextruded liposomes does not always result in enhanced enzymatic activity.

The use of mixed vesicles composed of cationic and zwitterionic amphiphiles also increases the encapsulation efficiency of nucleic acid material (*see* Table 1). However, nucleic acids and cationic liposomes will often form complexes that may protect the nucleic acids to some extent from degradation by endonucleases without enclosing them within a liposome (Behr et al., 1989; Dan, 1998; Rädler et al., 1997; Sternberg, Sorgi & Huang, 1994). In some cases, the electronic transitions of double-stranded DNA are affected by encapsulation in cationic intact liposomes, a fact that clearly indicates that the lipid molecules interact with the solute molecules (Akao et al., 1996; Monnard et al., 1997). These structures are not suitable to the design of a bioreactor with

entrapped polymerase activity, as surface interactions may jeopardize the compartment integrity or/and prevent an efficient integration of each component in the catalytic assembly.

Methods that require the use of low temperature (Pick, 1981; Oberholzer, Albrizio & Luisi, 1995a; Monnard et al., 1997), pH-variations, or the adjunction of detergents (Schurtenberger et al., 1984) or organic solvents (Szoka & Papahadjopoulos, 1978) can directly affect the activity of an enzyme. For instance, Oberholzer et al. (1995a) added the DNA polymerase enzyme to their system during the last freeze/thaw cycle to prevent its inactivation.

The choice of an encapsulation method is therefore a difficult compromise between efficiency and preservation of the catalytic activity. The dehydration/rehydration method has proven to be both effective and innocuous to most polymerase enzymes (Chakrabarti et al., 1994; Monnard & Deamer, unpublished results). This method is based on the simultaneous dehydration of a sample containing empty vesicles and all components of the polymerase assembly (enzyme, template, and primers), which results in the formation of stacked lipid bilayers with intercalated solutes. Solute molecules are captured upon rehydration when the lipid bilayers reseal into vesicles. Interestingly, lipid bilayers during the drying step will preserve the functionality of enzymes, such as reverse-transcriptases, which, in the absence of lipids, completely lose their activity upon drying. In some cases, giant vesicles produced by rehydrating a lipid film with a polymerase reaction mixture were also successfully used (Tsumoto et al., 2001; Yu et al., 2001) even though this method is not optimal in terms of encapsulation efficiency. Here, the large internal volume (4.2×10^{-12} l for 10-μm GUV to be compared with 4.2×10^{-18} l for a 200-nm extruded LUV) of these liposomes, consequently the larger pool of substrate molecules, seems to allow for a prolonged catalytic activity. These methods are therefore commonly applied to encapsulate enzymes and their cofactors into amphiphile vesicles.

Permeability

A steady supply of substrate molecules across the bioreactor boundaries must be achieved to develop usable bioreactors. Moreover, these membranes must be selectively permeable to allow passage of the substrates and, when needed, reaction products, whereas they prevent the release of components essential for the catalytic activity.

As recent studies of cellular lipid membranes have clearly shown, the main role of the lipid moiety in the cell membrane is to provide a permeability barrier to free diffusion of polar and ionic solutes. However, amphiphile bilayers are only a semi-permeable barrier between the internal water core and the surrounding aqueous medium. Their permeability to a specific solute will depend on the physical characteristics of both solute and the amphiphile vesicles (Kanehisa & Tsong, 1978; Rosenquist, Gabran & Rydhag, 1981; Deamer & Bramhall, 1986; Langner & Hui, 1993; Chakrabarti et al., 1994; Paula et al., 1996). In general, two kinds of passive diffusion across amphiphile bilayers can be observed: Solutes with relatively high partition coefficients permeate by dissolving in the bilayer, then diffusing across, while ionic solutes such as protons, sodium and potassium ions apparently use transient defects in the bilayer to cross the permeability barrier (Paula et al., 1996). The frequency and number of transient defects is clearly dependent on the gel-fluid phase transition (Mouritsen, Jorgenseb & Honger, 1995), and the length of the hydrocarbon chains. That is, the permeability is inversely proportional to the length and the degree of unsaturation of the hydrocarbon chains. An incubation of vesicles at or near the phase transition temperature (the temperature at which the number of defects at the boundaries between gel- and liquid-crystalline-state domains is the highest) will therefore allow for an enhanced passive diffusion to take place (Monnard & Deamer, 2001).

The lamellarity (Shoemaker & Vanderlick, 2002) and size of the vesicles also determine the amount of substrates permeating across the membranes. The average diameter of vesicles decreases, for instance, by extruding them (Hope et al., 1985), as does the average number of concentric lamellae around the internal aqueous core (Monnard et al., 1999). Consequently, the total membrane surface area in direct contact with solutes added in the external medium will increase, which directly influences the number of substrate molecules diffusing across the membranes into the internal aqueous volume.

The intrinsic permeability of lipid bilayers can also be modulated by varying the membrane composition. For instance, the addition of fatty acids (*see* Fig. 2) or detergents at sublytic concentrations (Oberholzer et al., 1999a; Treyer, Walde & Oberholzer, 2002) to phosphatidylcholine (PC) bilayers tranforms bilayer characteristics. In Fig. 2, the effect of myristic acid (MA) on the permeability of ATP is shown. There is an optimum composition with a molar ratio of dimyristoyl-sn-glycerol-3-phosphocholine bilayers (C14:0, DMPC) to myristoic acid (MA) of 89:11. With mixed liposomes, the ATP permeation rate is 10 times higher than that into pure DMPC vesicles.

The permeability of PC liposomes to large charged substrates such as NTPs significantly increases upon addition of sublytic concentrations of cholate. However, these mixed bilayers are selective only under well-defined molar ratios of cholate to PC, and conditions can be found that allow permeation of

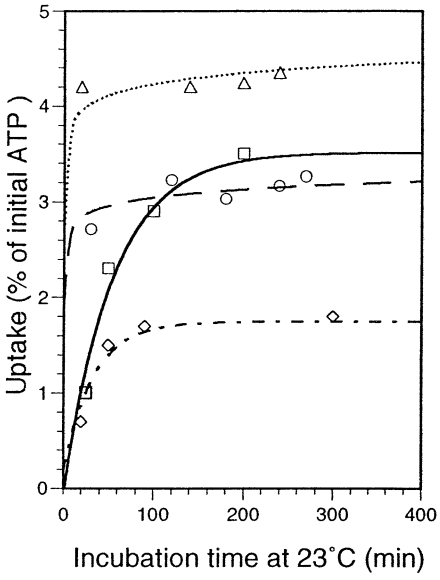

Fig. 2. Permeability of DMPC/MA compared to pure DMPC. The permeability of pure DMPC (*squares* and *continuous line*), mixed DMPC/MA 95:5 (*circles* and *dashed line*), 89:11 (*triangles* and *dotted line*) and 80:20 (*diamonds* and *dot-dashed line*) to ATP was measured at 23.3°C, the lipid phase-transition temperature.

macromolecules, such as DNase I (Oberholzer et al., 1999a) or even hemoglobin (Schubert et al., 1991). In addition, small alterations of this molar ratio, such as dilution of the liposome suspension, will cause detergent molecules to be released from the mixed bilayers. That is, the permeation of substrate could be stalled upon slight dilution. Finally, because of the sensitivity of certain polymerases to detergents, these detergent-phospholipid mixed bilayers may not be suitable for vesicle-bound polymerases.

In studies focusing on the permeability of nucleotides, Chakrabarti et al. (1994) established that PC bilayer permeability to larger ionic molecules, such as ADP, has three distinct regimes. If the lipid chains were shorter than 12 carbons in length, no selective permeability could be observed. Bilayers composed of 16-, 18-carbon chains were relatively impermeable, and could maintain concentration gradients of ADP for hours to days. On the other hand, DMPC bilayers were sufficiently permeable so that passive diffusion could provide substrates, NDPs, for an entrapped polymerase. In our experiments (Monnard & Deamer, 2001 and unpublished observations), we extended the results to NTPs, and took advantage of the increased defect frequency at T_m. DMPC-vesicle suspensions were incubated according to temperature cycles between the optimal permeability temperature (23.3°C) and that for an optimal T7 RNA polymerase activity (37°C). This permitted an increase of the NTP flow by 100-fold compared to a situation where the incubation would have taken place at 37°C. At the same time, it was established that DMPC bilayers at their phase-transition temperature exhibit a surprisingly high barrier to solutes larger than NTPs. Even the shortest oligomer, a dimer, remained encapsulated with no detectable release over periods of many hours. For this reason DMPC is often chosen for the experimental model systems to be described later in this review.

Even though the selective permeability of NTPs across DMPC bilayers has been established, it remains to be shown whether these permeability rates could sustain the catalytic activity of entrapped enzymes with relatively high turnover rates. As discussed in the next paragraphs, experiments with polymerases have demonstrated the effectiveness of the passive diffusion across bilayers as a substrate supply mechanism for entrapped enzyme in laboratory bioreactor models, albeit the synthesis rates were lower than those of free enzymes.

Entrapped Polymerase Activity

Reaction of polymerase entrapped in liposomes have been carried out under various conditions. Two kinds of amphiphiles have been used to form the liposome bilayers, fatty acid and PC derivatives. The former membranes tend to be structurally less stable in the presence of divalent cations required by polymerases as cofactors (Monnard et al., 2002). However, this typical instability is counterbalanced by the noticeable permeability increase towards the mononucleotide substrates (Walde et al., 1994). On the other hand, long-chained PC bilayers support both high concentrations of ionic solutes and large temperature variations. In this case, this stability comes at a price: The permeability of ionic substrates drops significantly. These factors have therefore determined the bioreactor design.

Template-mediated or random enzymatic nucleic acid synthesis in vesicles (*see* Fig. 3) can be classified as total- and partial-encapsulation experiments (see Table 2). In the total encapsulation experiments, the catalytic assembly and the substrates were entrapped simultaneously (Oberholzer et al., 1995a, 1995b; Tsumoto et al., 2001; Yu et al., 2001). These systems are therefore not truly effective bioreactors because the synthesis only proceeds as long as the encapsulated substrate supply lasts. In the partial-encapsulation experiments (Chakrabarti et al., 1994; Walde et al., 1994; Monnard & Deamer, unpublished observations; Treyer et al., 2002), the catalytic assembly was first encapsulated alone, and nucleic acid synthesis can therefore only start after substrate addition in the external medium. In this case, the reaction should proceed as long as the enzyme remains active. Moreover, a steady supply of substrates should allow for an enzymatic activity comparable to that observed with free enzyme (Nasseau et al., 2001).

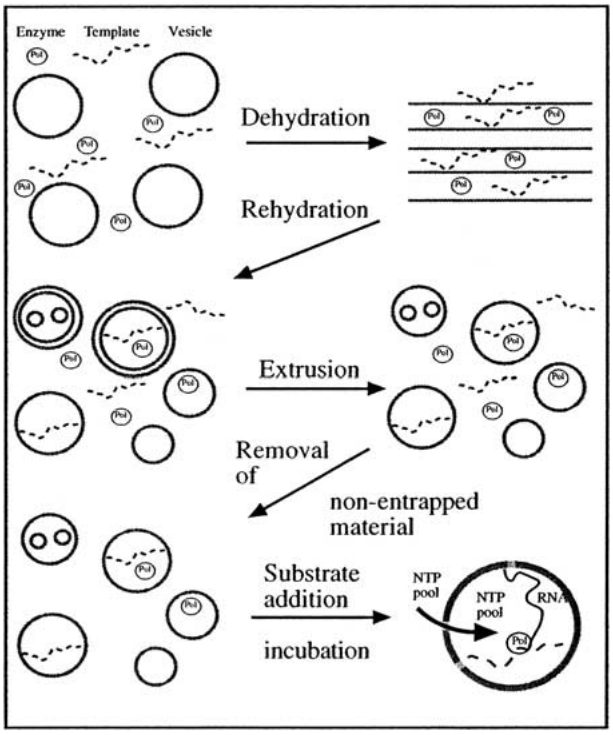

Fig. 3. Schematic representation of a liposome-bound polymerase experiment. Empty liposomes are prepared by rehydrating a dry lipid film, and mixed with a transcription reaction mixture (the enzyme, its template, and its metal catalyst) without NTPs. The catalytic assemblies are entrapped according to the dehydration/rehydration method. The encapsulation is random, therefore three populations of liposomes are obtained: empty vesicles, those containing one of the system components, and those with the complete system. To increase the amount of substrates diffusing across the bilayers, the suspension can be extruded through polycarbonate filters. This procedure reduces the number of concentric bilayers and increases the bilayer area directly in contact with the external medium and thus the amount of substrate diffusing in the reaction compartment in a given time period. External reaction is prevented by removing the non-entrapped enzymes and templates after enzymatic digestion. Substrates, NTPs, are added externally, and begin to permeate through transient bilayer defects, so that the encapsulated reaction proceeds.

We will first consider the details of total-encapsulation experiments. Three different enzymes were investigated: Qβ replicase (Oberholzer et al., 1995b), T7 RNA polymerase (Tsumoto et al., 2001; Yu et al., 2001), and DNA polymerase (Oberholzer et al., 1995a). Both Qβ replicase and T7 RNA polymerase are magnesium dependent, and synthesize an RNA fragment from a template (RNA template for Qβ replicase and DNA template for T7 RNA polymerase) using NTPs as substrates. DNA polymerase is the double-stranded DNA template-dependent enzyme used for PCR reactions, which requires two primers to amplify a DNA sequence. The types of lipid used in all these experiments have a low phase transition temperature (<16°C). Consequently the lipids were already in a liquid-crystalline state at the temperature range used for the incubation, and membrane defects needed for the passive diffusion of large charged molecules were rare. Consequently, a low permeability of the bilayer membranes to NTPs and dNTPs was expected. Thus, substrate molecules and all components of the catalytic assemblies (3 for the Qβ replicase and T7 RNA polymerase, 5 for the PCR) had to be captured simultaneously in a single vesicle. In all cases, products were formed in low yields. Oberholzer et al. (1995a) calculated that their PCR reaction yielded approximately 10 double-stranded 369 bp fragments due to the fact that only 8000 substrate molecules were entrapped. Low yields were also expected because only 0.1% of all 1-palmitoyl, 2-oleyl-*sn*-glycero-3-phosphocholine (POPC, C16:0/C18: 1 Δ9) liposomes could contain all four macromolecular components (template, two primers, and enzyme). Tsumoto et al. (2001) demonstrated that total-encapsulation reactions, such as the T7 RNA polymerase-mediated RNA transcription, could also proceed within GUVs. In this case, the nucleotide supply was significantly increased, allowing for more RNA transcripts to be synthesized.

This approach to the substrate supply is constrained by the physical characteristics of amphiphile bilayers, which determine the maximal size of vesicles. Interestingly, the authors were able to single out one liposome by manipulating it with laser tweezers, a technique which could apply to liposome-assisted RNA selection. Finally, Yu et al. (2001) presented the most convincing evidence that liposomes may be soon used as microscale/nanoscale bioreactors or compartments for an artificial cell. A coupled T7 RNA polymerase-mediated transcription and ribosome-mediated translation of a mutant green fluorescent protein was carried out within GUVs. That is, a functional ribosomal machinery (S-30 extract containing two ribosomal subunits, elongation factors, 20 amino acids, their tRNA, and their aminoacyl-tRNA synthetases) was successfully encapsulated with the T7 RNA polymerase system (7 components). Even though only a few liposomes contained all components of the coupled systems, fluorescent proteins were detected in their internal aqueous core.

Within the limitations of these systems, these results are enlightening in several ways for the development of lipid-based bioreactors. First and foremost, complex catalytic systems, such as coupled transcription-translation machinery, could be entrapped simultaneously within lipid-bound microscale compartments (4.2×10^{-12} l in volume), whereas a DNA amplification with 9 different components was entrapped in a single vesicular nanoscale volume as small as 4.2×10^{-18} l. Second, because the compartmentalization does not drastically inhibit the activity of these catalytic assemblies, the sequence-specific DNA amplification or RNA transcription followed by mRNA translation could proceed. Fi-

Table 2. Overview of the experimental protocols

Experiment type	Reference	Enzyme	Amphiphile	Compounds present during the enzyme encapsulation			
				Template	Metal cofactor	Primers	Substrates
Total-encapsulation	(Oberholzer et al., 1995b)	Qβ replicase	Oleic acid	1	1	–*	4
	(Tsumoto et al., 2001)	T7 RNA polymerase	DOPC/DOPG†	1	1	–*	4
	(Oberholzer et al., 1995a)	DNA polymerase	POPC	1	1	2	4
	(Yu et al., 2001)	T7 RNA polymerase	Egg PC Cholesterol DSPE-PEG5000#	1	1	–*	4
Partial-encapsulation	(Walde et al., 1994)	PNPase	Oleic acid	–**	1	–*	Added later
	(Chakrabarti et al., 1994)	PNPase	DMPC	–**	1	–*	Added later
	(Treyer et al., 2002)	PNPase	POPC/cholate	–**	1	–*	Added later
	(Monnard & Deamer, unpublished results)	T7 RNA polymerase	DMPC	1	1	–*	Added later

†: Dioleyl-*sn*-glycero-3-phosphocholine/Dioleyl-*sn*-phosphoglycerol.
$: In this case, the transcription and translation systems are simultaneously encapsulated.
#: Distearoyl-*sn*-glycero-3-phosphoethanolamine-poly(ethylene glycol).
*: no primers required.
**: no template required.
Added later: Substrates were added to the external medium once the liposomes were formed, extruded, and the non-encapsulated template/enzyme molecules were inactivated and/or removed.

nally, lipid vesicles with encapsulated enzymatic reactions can be subjected to broad environmental changes, for instance, temperature variation, without collapsing. However, these systems lack one essential characteristic of bioreactors in that they cannot truly interact with their surrounding medium. It follows that reactions will proceed only for a limited period of time.

Partial-encapsulation experiments directly address the issue of substrate exchanges between the compartmented systems and their surrounding. Two polymerase enzymes have been studied so far: polynucleotide phosphorylase (PNPase), which in the presence of an NDP excess will incorporate the nucleotides into an RNA of random sequence; and T7 RNA polymerase, a DNA template-mediated RNA transcription enzyme. Both enzymes require metal cofactors such as magnesium, or manganese for optimal reaction rates. The PNPase-mediated RNA synthesis has been carried out in a number of different amphiphile vesicles, DMPC (Chakrabarti et al., 1994), POPC (Treyer et al., 2002), two phospholipids, and oleic acid (C18:1 Δ9) (Walde et al., 1994), an unsaturated fatty acid. In every experiment, ADP was the substrate. RNA in the form of poly(A) was produced after 1–5 days of incubation, and remained within the amphiphile-bound compartment. Note the length difference between the hydrocarbon chains, which clearly underlines the permeability behavior of each type of lipid. Using POPC having one hydrocarbon chain of the oleic acid length precluded the reaction because of extremely low substrate permeation rates unless these bilayers were permeabilized by the addition of cholate, a mild detergent, at sublytic concentration (Treyer et al., 2002).

The reaction rate with the DMPC-encapsulated enzyme was determined to be approximately 20% of that with PNPase in an aqueous buffer, showing that the bilayers were still a substantial barrier to substrate permeation. After a 10- to 15-day incubation, fatty acid bilayers also proved to be sensitive to disruptive interactions between poly(A) fragments and the membranes (Walde et al., 1994; unpublished observations by Monnard, Apel and Deamer). Nonetheless, these systems demonstrated that ADP could permeate across both lipid bilayers at rates sufficient to support polymer synthesis by PNPase, and that passive diffusion of substrate under certain conditions may be fast enough to allow template-directed enzymatic amplification of encapsulated genetic material.

This approach was further developed by designing a system capable of relatively high rates of substrate permeation without using detergents. Because of their stability and relatively high permeability, DMPC liposomes were chosen to encapsulate a template-directed T7 RNA polymerase (Monnard & Deamer, unpublished results). Even though DMPC

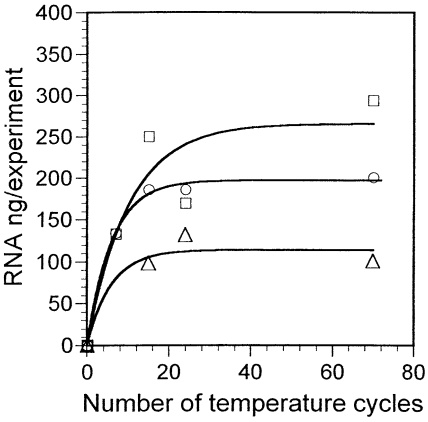

Fig. 4. RNA formation in relation to the liposome average size. RNA polymerization within 400-nm (*squares*) and 800-nm (*circles*) extruded and unextruded (*triangles*) liposomes was assayed by fluorescence (RiboGreen™ assay) at various times during 70 temperature cycles. With 200-nm extruded liposomes, the fluorescence yield was only significant after 70 incubation cycles, and is not shown here. T7 RNA polymerase and its template, circular plasmid pTRI-Xef, were encapsulated.

liposomes are more permeable to NTPs than the POPC vesicles, their permeability coefficient is in the range of 10^{-11} cm sec^{-1} at 37°C, a rate that permits less than one NTP per second to enter a given vesicle. To increase permeability, the reactions were cycled between 23.3°C and 37°C. Under these conditions the vesicles undergo a fluid–gel state phase transition at 23.3°C, a temperature at which they are orders of magnitude more permeable than in the fluid phase. As a result, substrate uptake by a vesicle increases to approximately 1500 molecules during a 5-min incubation at 23.3°C, followed by a 1-min incubation at 37°C to induce optimal T7 RNA polymerase activity. Long RNA fragments were polymerized within the liposomes (*see* Fig. 4). Oligonucleotides with a sequence corresponding to the DNA template could be annealed to the RNA product (1020 nt downstream from the T7 promoter), and the RNA reverse-transcription was initiated.

This experiment further established that the amount of product was clearly dependent on the vesicle size and lamellarity (*see* Fig. 4, 400-nm ≥ 800-nm ≫ unextruded ≈ 200-nm extruded liposomes). These observations were consistent with the physical characteristics of liposome preparations. The unextruded liposomes trapped larger amounts of both enzyme and template, but were multilamellar structures in which substrate molecules had to cross multiple bilayers to reach the entrapped enzyme/template complexes. It is also likely that unextruded liposomes allow for a better template trapping between the bilayers (*see* Table 1), but not a simultaneous entrapment in the central water core. In contrast, 200-nm extruded vesicles were unilamellar and more permeable, but encapsulated only a few enzyme/template complexes, reducing the number of functional catalytic assemblies, and consequently, the product yield.

In addition to the findings made by investigation of total-encapsulation systems, these partial-encapsulation systems demonstrated that passive diffusion of substrates, as large as nucleotides, provided enough substrate for gene amplification to take place in laboratory bioreactor models. Furthermore, the localization of the products inside the vesicles, where they remain effectively protected from degradation by proteases and nucleases, underlines the selective permeability of simple lipid bilayers. Membrane-bounded compartments, such as the amphiphile vesicles described here, allow accumulation of polymeric products, which in turn can further interact and produce more complex systems, such as a coupled RNA transcription and subsequent translation of proteins with amino acids and nucleotides permeating across bilayer membranes.

Conclusion

Existing studies demonstrate that liposome-bound catalytic assemblies may soon function as microscale/nanoscale bioreactors with possible applications in RNA selection, or as artificial minimal cells with medical and analytical applications. They also show that liposome-entrapped transcription represents an essential first experimental step to using the RNA transcripts in protein synthesis, as shown by Yu et al. (2001).

In particular, encapsulation procedures, such as dehydration/rehydration or film rehydration, permit simultaneous trapping of all components of relatively complex catalytic systems, which remain active in the vesicular compartment as small as 10^{-18} l. Furthermore, the membrane boundaries of such vesicles can protect the encapsulated polymerases and templates from agents such as proteases and nucleases even under extreme conditions.

Another interesting aspect of amphiphile vesicles is their selective permeability, which permits the passive diffusion of substrates and prevents the release of an encapsulated catalytic assembly, and thereby leads to accumulation of polymeric products. This product accumulation could promote additional reactions that lead to the increased metabolic complexity needed to create a viable artificial cell.

However, the vesicle-based model systems described here also demonstrate the limitations of membrane-encapsulated reactions that must be overcome before developing an efficient bioreactor. First, in a complex catalytic assembly, individual encapsulation yield of each component is difficult to predict accurately because their physical characteristics, which usually vary broadly, significantly affect their trapping. In addition, as shown by the T7 RNA

polymerase partial-encapsulation experiments, the final activity of complex catalytic assemblies is not solely related to the individual encapsulation yield of its compounds. As system complexity increases, the need for truly quantitative encapsulation procedures will be more acute.

Furthermore, these liposome-based polymerases yield low amounts of RNA/DNA products because they rely on passive diffusion as the sole source of large substrate molecules. To improve the exchange between the compartment and its surrounding medium and ensure a steady supply of nutrient/energy, it is necessary to develop new membrane compositions, or add transmembrane protein channels, or encapsulate molecular systems that synthesize NTP substrates within the liposomes from smaller, more permeable precursor molecules (*see* Walde et al., 1994, for a comparison of AMP and ATP permeation).

Studies by several groups have shown that the insertion of natural or synthetic channels within vesicle bilayers is possible (Steinem et al., 1999; Bezrukov, Kullman & Winterhalter, 2000; Meier, Nardin & Winterhalter, 2000). Varying the number of channels within the bilayers may even permit entrapped enzymatic activity comparable to that of a free enzyme (Nasseau et al., 2001). These channels could be selected such as not to jeopardize the integrity of the encapsulated catalytic assembly, and allow for the passage of monomers and small polymeric products. For instance, α-hemolysin, a natural transmembrane channel, retains double-stranded DNA while allowing translocation of single-stranded polynucleotides (Kasianowicz et al., 1996). The incorporation of channels will also permit the use of new boundary materials because it should obviate the requirement for bilayer defects to promote substrate uptake. Polymerizable amphiphiles (Nardin et al., 2000) can form the bilayers, which can be polymerized after entrapment of the catalytic assembly and insertion of channels, thereby adding long-term stability to the compartment boundaries.

The internal synthesis of substrates from small, more permeable precursor molecules could also be implemented to increase the substrate availability. Bioreactors might then require an internal energy transduction system. For instance, an NTP enzymatic synthesis from NDP, or NMPs would presuppose a large supply of ATP cofactors produced by an ATP-synthase coupled to an artificial photosynthetic system inserted in the bioreactor bilayers. Such a system has already been implemented (Pitard et al., 1996; Steinberg-Yfrach et al., 1998), but not encapsulated within liposomes yet.

Many individual components needed for the design of an efficient bioreactor or an artificial minimal cell have already been developed. The challenge is to integrate them into a single construct and ensure their simultaneous, coupled function in a controlled manner. This integration will lead to systems capable of a wide range of tasks and adaptation to a variety of environments.

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References

Akao, T., Fukumoto, T., Ihara, H., Ito, A. 1996. Conformational change in DNA induced by cationic bilayer membranes. *FEBS Lett.* **391:**215–218

Apel, C.L., Mautner, M.N., Deamer, D.W. 2002. Self-assembled vesicles of monocarboxylic acids and alcohols: Conditions for stability and for encapsulation of biopolymers. *Biochim. Biophys. Acta* **1559:**1–9

Bangham, A.D., Horne, R.W. 1964. Negative staining of phospholipids and their structural modification by surface-active agents as observed in the electron microscope. *J. Mol. Biol.* **8:**660–668

Bangham, A.D., Standish, M.M., Miller, N. 1965. Diffusion of univalent ions across the lamellae of swollen phospholipids. *J. Mol. Biol.* **13:**238–252

Beaudry, A.A., Joyce, G.F. 1992. Directed Evolution of an RNA Enzyme. *Science* **257:**635–641

Behr, J.-P., Demeneix, B., Loeffler, J.-P., Perz-Mutul, J. 1989. Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA. *Proc. Natl. Acad. Sci. USA* **86:**6982–6986

Bezrukov, S.M., Kullman, L., Winterhalter, M. 2000. Probing sugar translocation through maltoporin at the single channel level. *FEBS Lett.* **476:**224–228

Chakrabarti, A.C. 1994. Permeability of membranes to amino acids and modified amino acids: Mechanisms involved in translocation. *Amino Acids* **6:**213–229

Chakrabarti, A.C., Breaker, R.R., Joyce, G.F., Deamer, D.W. 1994. Production of RNA by polymerase protein encapsulated within phospholipid vesicles. *J. Mol. Evol.* **39:**555–559

Chakrabarti, A.C., Clark-Lewis, I., Harrigan, P.R., Cullis, P.R. 1992. Uptake of basic amino acids and peptides into liposomes in response to transmembrane pH gradient. *Biophys. J.* **61:**228–234

Dan, N. 1998. The structure of DNA complexes with cationic liposome-cylindrical or flat bilayers? **1369:**34–38

Deamer, D.W., Barchfeld, G.L. 1982. Encapsulation of macromolecules by lipid vesicles under simulated prebiotic conditions. *J. Mol. Evol.* **18:**203–206

Deamer, D.W., Bramhall, J. 1986. Permeability of lipid bilayers to water and ionic solutes. *Chem. Phys. Lipids* **40:**167–188

Gao, X., Huang, L. 1993. Cytoplasmic expression of a reporter gene by co-delivery of T7 RNA polymerase and T7 promoter sequence with cationic liposomes. *Nucleic Acid Res.* **21:**2867–2872

Gebicki, J.M., Hicks, M. 1973. Ufasomes are stable particles surrounded by unsaturated fatty acid membranes. *Nature* **243:**232–234

Gebicki, J.M., Hicks, M. 1976. Preparation and properties of vesicles enclosed by fatty acid membranes. *Chem. Phys. Lipids* **16:**142–60

Gruner, S.M., Lenk, R.P., Janoff, A.S., Ostro, M.J. 1985. Novel multilayered vesicles: comparison of physical characteristics of multilamellar liposomes and stable plurilamellar vesicles. *Biochemistry* **24:**2833–2842

Hafez, I.M., Cullis, P.R. 2000. Cholesteryl hemisuccinate exhibits pH sensitive polymorphic phase behavior. *Biochim. Biophys. Acta* **1463:**107–114

Hargreaves, W.R., Deamer, D.W. 1978. Liposomes from ionic, single-chain amphiphiles. *Biochemistry* **17:**3759–3768

Hope, M.J., Bally, M.B., Webb, G., Cullis, P.R. 1985. Production of large vesicles by a rapid extrusion procedure. Characterisation of the size distribution, trapped volume and ability to maintain a membrane potential. *Biochim. Biophys. Acta* **812:**55–65

Jay, D.G., Gilbert, W. 1987. Basic protein enhances the incorporation of DNA into lipid vesicles: Model for the formation of primordial cells. *Proc. Natl. Acad. Sci. USA* **84:**1978–1980

Kanehisa, M.I., Tsong, T.Y. 1978. Cluster model of lipid phase transition with application to passive permeation of molecules and structure relaxations in lipid bilayers. *J. Am. Chem. Soc.* **100:**424–432

Kasianowicz, J.J., Brandin, E., Branton, D., Deamer, D.W. 1996. Characterization of individual polynucleotide molecules using a membrane channel. *Proc. Natl. Acad. Sci. USA* **93:**13770–13773

Langner, M., Hui, S.W. 1993. Dithionite penetration through phospholipid bilayers as a measure of defects in lipid molecular packing. *Chem. Phys. Lipids* **65:**23–30

Legendre, J.-Y., Szoka Jr., F.C. 1992. Delivery of plasmid DNA into mammalian cell lines using pH-sensitive liposomes: Comparison with cationic liposomes. *Pharm. Res.* **9:**1235–1242

Luisi, P.L., Walde, P. 2000. Giant vesicles. John Wiley and Sons, Chichester, England

Mannino, R.J., Allebach, E.S., Strohl, W.A. 1979. Encapsulation of high molecular weight DNA in large unilamellar phospholipid vesicles. Dependance on the size of the DNA. *FEBS Lett.* **101:**229–232

Meier, W., Nardin, C., Winterhalter, M. 2000. Reconstitution of channel proteins in (polymerized) ABA triblock copolymer membranes. *Angew. Chemie-Internat. Ed.* **39:**4599–4602

Monnard, P.-A., Apel, C.L., Kanavarioti, A., Deamer, D.W. 2002. Influence of ionic solutes on self-assembly and polymerization processes related to early forms of life: Implications for a prebiotic aqueous medium. *Astrobiology* **2:**139–152

Monnard, P.-A., Berclaz, N., Conde-Frieboes, K., Oberholzer, T. 1999. Decreased solute entrapment in POPC liposomes prepared by freeze/thaw in the presence of physiological amounts of monovalent salts. *Langmuir* **15:**7504–7509

Monnard, P.-A., Deamer, D.W. 2001. Nutrient uptake by protocells: a liposome model system. *Orig. Life Evol. Biosphere* **31:**147–155

Monnard, P.-A., Oberholzer, T., Luisi, P.L. 1997 Encapsulation of polynucleotides in liposomes. *Biochim. Biophys. Acta* **1329:**39–50

Mouritsen, O.G., Jorgenseb, K., Monger, T. 1995. Permeability of Lipid Bilayers Near the Phase Transition. *In:* Permeability and Stability of lipid bilayers. E.A. Disalvo and S.A. Simon, editors. pp. 137–160. CRC Press, Boca Raton

Nabel, G.J., Nagel, E.G., Yangh, Z.-Y., Fox, B.A., Plautz, G.E., Gao, X., Huang, L., Shu, S., Gordon, D., Chang, A.E. 1993. Direct gene transfer with DNA-liposome complexes in melanoma: Expression, biologic activity, and lack of toxicity in humans. *Proc. Natl. Acad. Sci. USA* **90:**11307–11311

Nardin, C., Thoeni, S., Widmer, J., Winterhalter, M., Meier, W. 2000. Nanoreactors based on (polymerized) ABA-triblock copolymer vesicles. *Chemical Communications*:1433–1434

Nasseau, M., Boublik, Y., Meier, W., Winterhalter, M., Fournier, D. 2001. Substrate-permeable encapsulation of enzymes maintains effective activity, stabilizes against denaturation, and protects against proteolytic degradation. *Biotechnol. Bioengin.* **75:**615–618

Oberholzer, T., Albrizio, M., Luisi, P.L. 1995a. Polymerase chain reaction in liposomes. *Chemistry & Biology* **2:**677–682

Oberholzer, T., Meyer, E., Amato, I., Lustig, A., Monnard, P.-A. 1999a. Enzymatic reactions in liposomes using the detergent-induced liposome loading method. *Biochim. Biophys. Acta* **1416:**57–68

Oberholzer, T., Nierhaus, K.H., Luisi, P.L. 1999b. Protein expression in liposomes. *Biochem. Biophys. Res. Commun.* **261:**238–241

Oberholzer, T., Wick, R., Luisi, P.L., Biebricher, C.K. 1995b. Enzymatic RNA replication in self-reproducing vesicles: an approach to a minimal cell. *Biochim. Biophys. Res. Commun.* **207:**250–257

Paula, S., Volkov, A.G., Van Hoek, A.N., Haines, T.H., Deamer, D.W. 1996. Permeation of proton, potassium ions, and small polar molecules through phospholipid bilayers as a function of membrane thickness. *Biophys. J.* **70:**339–348

Pick, U. 1981. Liposomes with a large trapping capacity prepared by freezing and thawing of sonicated phospholipid mixtures. *Arch. Biochem. Biophys.* **212:**186–194

Pitard, B., Richard, P., Dunach, M., Girault, G., Rigaud, J.L. 1996. ATP synthesis by the F0F1 ATP synthase from thermophilic Bacillus PS3 reconstituted unto liposomes with bacteriorhodopsin. 1. Factors defining the optimal reconstitution of ATP synthases with bacteriorhodopsin. *Euro. J. Biochem.* **235:**769–778

Pohorille, A., Deamer, D.W. 2002. Artificial cells: Prospects for biotechnology. *Trends Biotechn.* **20:**123–128

Rädler, J.O., Koltover, I., Salditt, T., Safinya, C.R. 1997. Structure of DNA-cationic liposome complexes: DNA intercalation in multilamellar membranes in distinct interhelical packing regimes. *Science* **275:**810–814

Rosenquist, K., Gabran, T., Rydhag, L. 1981. Studies of permeability across bilayers of lecithin. *In:* 11th Scandinavian Symposium on Lipids. pp. 85–89. Lipidforum, Scand. Forum Lipid Res. & Technol., Bergen, Norway.

Schubert, R., Wolburg, H., Schmidt, K.-H., Roth, H.J. 1991. Loading of preformed liposomes with high trapping efficiency by detergent-induced formation of transient membrane holes. *Chem. Phys. Lipids* **58:**121–129

Schultes, E.A., Bartel, D.P. 2000. One sequence, two ribozymes: Implications for the emergence of new ribozyme folds. *Science* **289:**448–452

Schurtenberger, P., Mazer, N., Waldvogel, S., Känzig, W. 1984. Preparation of monodisperse vesicles with variable size by dilution of mixed micellar solutions of bile salt and phosphatidylcholine. *Biochim. Biophys. Acta* **775:**111–114

Shew, R.L., Deamer, D.W. 1985. A novel method for encapsulation of macromolecules in liposomes. *Biochim. Biophys. Acta* **816:**1–8

Shoemaker, S.D., Vanderlick, T.K. 2002. Stress-induced leakage from phophoslipid vesicles: Effect of membrane composition. *Ind. Eng. Chem. Res.* **41:**324–329

Steinberg-Yfrach, G., Rigaud, J.-L., Durantini, E.N., Moore, A.L., Gust, D., Moore, T.A. 1998. Light-driven production of ATP catalyzed by F_0F_1-ATP synthase in an artificial photosynthetic membrane. *Nature* **392:**479–482

Steinem, C., Janshoff, A., Vollmer, M.S., Gharidi, M.R. 1999. Reversible photoisomerization of self-organized cylindrical peptide assemblies at air-water and solid interfaces. *Langmuir* **15:**3956–3964

Sternberg, B., Sorgi, F.L., Huang, L. 1994. New structures in complex formation between DNA and cationic liposomes visualized by freeze-fracture electron microscopy. *FEBS Lett.* **356:**361–366

Szoka, F., Papahadjopoulos, D. 1978. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proc. Natl. Acad. Sci. USA* **75:**4194–4198

Treyer, M., Walde, P., Oberholzer, T. 2002. Permeability enhancement of lipid vesicles to nucleotides by use of sodium cholate: Basic studies and application to an enzyme-catalyzed reaction occurring inside the vesicles. *Langmuir* **18:**1043–1050

Tsumoto, K., Nomura, S.M., Nakatani, Y., Yoshikawa, K. 2001. Giant liposome as a biochemical reactor: Transcription of DNA and transportation by laser tweezers. *Langmuir* **17:**7225–7228

Walde, P., Goto, A., Monnard, P.-A., Wessicken, M., Luisi, P.L. 1994. Oparin's reaction revisited: Enzymatic synthesis of poly(adenyl acid) micelles and self-reproducing vesicles. *J. Am. Chem. Soc.* **116:**7541–7547

Walde, P., Ichikawa, S. 2001. Review. Enzyme inside lipid vesicles: preparation, reactivity and applications. *Biomol. Engineering* **18:**143–177

Wright, M.C., Joyce, G.F. 1997. Continuous in vitro evolution of catalytic function. *Science* **276:**614–617

Yarosh, D.B. 2001. Liposomes in investigative dermatology. *Photodermatol. Photoimmunol. Photomed.* **17:**203–212

Yu, W., Sato, K., Wakabayashi, M., Nakaishi, T., Ko-Mitamura, E.P., Shima, Y., Urabe, I., Yomo, T. 2001. Synthesis of functional protein in liposome. *J. Biosci. Bioeng.* **92:**590–593

Zumbuehl, O., Weder, H.G. 1981. Liposomes of controllable size in the range of 40 to 180 nm by defined dialysis of lipid/detergent mixed micelles. *Biochim. Biophys. Acta* **640:**252–262