The Molecular Design of Enzyme-Loaded Liposomes

M. C. Annesini
Dipartimento di Ingegneria Chimica,
dei Materiali, delle Materie Prime e Metallurgia
via Eudossiana 18 – 00184 Roma – Italy
E-mail: mariacri@pc_mcan.ing.uniroma1.it

Review
Received: March 2, 1997
Accepted: September 19, 1997

Liposomes can be used to entrap the enzymes and to obtain a catalytic complex useful for biotechnological and biomedical applications. Activity and properties of this complex depend on the properties of the enzyme itself and of the liposomial structure; therefore a deep knowledge of enzyme and lipid bilayer biophysics can allow us to design the liposome formulation rationally. In this review, we first report about the main physico-chemical properties of liposomes, then we show how these properties affect the kinetics of the processes catalyzed by enzyme loaded liposomes; finally, we review some interesting application of such a system.

Key words
Liposome – phospholipid vesicles – enzyme – kinetics

Introduction

Liposomes, i.e. vesicles composed of one or more lipid bilayers surrounding and aqueous environment, are among the most interesting supramolecular structures (see Figure 1) for biological, biomedical and biotechnological researches.

The main liposome characteristic is the resemblance of the lipid bilayers to the living cell membranes: therefore, they are useful model systems for basic biological studies[1], for studying intracellular transport phenomena[2] and understanding how biological membranes recognize and respond to the extracellular signals[1], for studying physical interactions of environmental factors with cell membranes[3,4]. Furthermore they can interact with the cellular membranes – by adsorption, fusion, endocytosis or lipid excange – and they can be used to introduce foreign materials, like genetic materials or drugs, into the cells[5–7].

Even if the most popular uses of liposomes are in the cosmetic field[8,9], with a market of about one billion dollars per year, there is no doubt that, prospectively, the most important use of liposomes is in pharmacology and medicine, predominantly as drug delivery systems, both for topical and systemic applications[10–12]. In the first case,

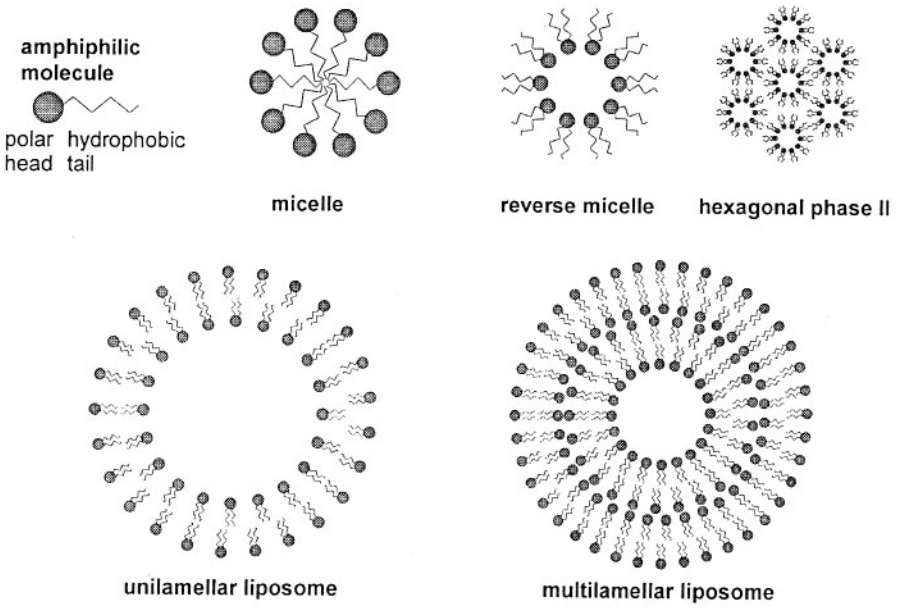


Fig. 1 – *Supramolecular structures formed by amphiphilic compounds in water (micelles, hexagonal phase II, unilamellar or multilamellar liposomes) or in organic solvent (reverse micelles). In hexagonal phase II, cylindrical micelles are packed in an hexagonal array.*

liposomes may be more successful in penetrating the horny layer of the skin than conventional vehicles[13,14]; in systemic applications, the main goals are to protect drugs loaded into the liposomes from being diluted or degraded in the blood, to reduce the toxic side effects by targeting the highly toxic molecules to the specific diseased cells or organs, to obtain a prolonged release of their content and reduce peaks in the drug dose. In this context, it is worth noting that in the last few years three liposomal formulations were approved for clinical use in Europe or in U.S. (Ambisome and DaunoXome by NeXstar Pharmaceuticals and Doxil by Sequus Pharmaceuticals), whereas some other products are now in Phase II or Phase III of clinical studies.

Strictly related to this field is the use of liposome to deliver genetic materials into the cells in gene therapy[15,16] and the liposome-based vaccines, where liposomes enhance the immunogenic response to a poorly immunogenic or non-immunogenic protein[17].

Finally, many interesting non-medical applications of liposomes are emerging in different areas where promising laboratory results have been obtained: in analytical chemistry as chromatographic supports[18,19], in food industry for flavour, vitamin or other agent microencapulsation[20], in agriculture as sustained release system, in environmental engineering for heavy metal decontamination[21–23].

The spread of liposome technology into various sciences and applications is documented by a very large number of scientific and technological papers published every year and the interested readers are referred to the recent textbooks on this subject[24–26].

In this review, no attempt has been made to discuss and refer to all the experimental and theoretical works carried out on liposome science and technology, but only a specific aspect is considered, i.e. the enzyme entrapment in liposome and the activity of the lipo-protein complex obtained in this way. In fact, both hydrophilic and hydrophobic proteins can be entrapped in liposomes by different, but quite simple and mild procedures that, usually, do not result in protein denaturation (dispersion of dry lipid film in the aqueous media containing the proteins, followed by freezing and thawing and extrusion; detergent removal from solutions containing detergent-lipid and detergent-proteins mixed micelles, etc.). On the contrary, enzymes that require the bilayer structure for their activity can be reconstituted in liposomes and exploit their full activity, whereas hydrophilic enzymes that are usually located in the liposome aqueous core can be protected against inhibitory and denaturating compounds. In this way liposomes may be considered as innovative tool to immobilize the enzymes that can exploit their activity in different ways. In particular, four different scenarios can be considered, as reported in Figure 2:

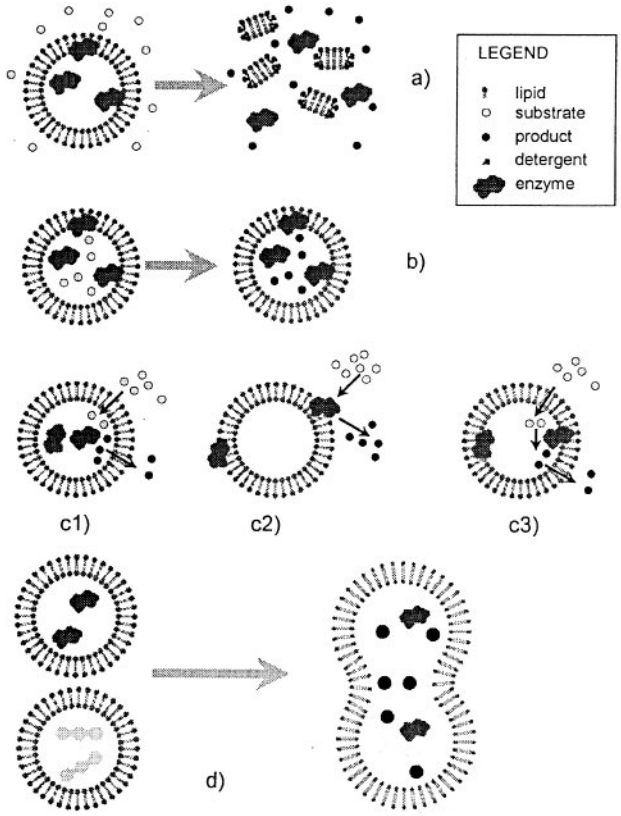


Fig. 2 – *Enzymatic reaction with enzyme-loaded liposomes: a) reaction after liposome disruption and enzyme release; b) reaction of the substrate entrapped with the enzyme inside the liposomes; c) reaction of the substrate present in the external solution catalyzed by the enzyme loaded in liposomes; d) reaction mediated by liposome fusion.*

a) the liposome is only used to protect the enzyme until an external stimulus triggers the liposome structure disruption and the enzyme release in the external environment; the enzyme activity towards the substrates present in the external medium is therefore cryptic until the enzyme is released. An interesting example of such a system is the use of temperature- an pH-sensitive liposomes to deliver drugs to target tissues[27].

b) the enzyme and the substrate are entrapped inside the same liposome under conditions of low enzyme activity; the enzymatic reaction occurs only under proper environmental conditions. This experimental setup has been used, for example, by *Oberholzer* et al. in their studies on prebiotic systems[28,29].

c) the substrate present in the aqueous solution is converted by the enzyme entrapped in the liposomial structure. We can distinguish between different configurations: the hydrophilic enzyme is located in the aqueous core of liposome and the

reaction occurs through the substrate diffusion across the liposome membrane (c1 in the figure) or the enzyme is partially embedded in the bilayer and the reaction occurs directly with the substrate in the external solution (c2 in the figure) or is mediated by the substrate permeation across the membrane (c3 in the figure). These systems are by far the most extensively studied and we report on them later in this review.

d) two different liposome populations, the first one containing the enzyme and the second one the substrate, fuse and the mixing of the aqueous contents allows the enzymatic reaction to occur. Many cellular processes occur according to such a schema and therefore we treat this subject in a specific paragraph.

Whereas the idea of enzyme immobilization in liposomes seems very promising, really no much attention has been devoted till now to enzyme-liposome catalytic systems (see also the short review by *Walde*[30]). The reason for this may be related to the shortcomings of liposomial systems: the relative low stability, the low trapping efficiency, the need for a purification step to remove the unentrapped enzyme, the poor membrane permeability to the substrate as well as the high cost. The aim of this review is to underline that liposomes, apart from being biocompatible and non toxic, are also extremely versatile and their structure can be modified in order to obtain widely different properties. In particular, activity and properties of the enzyme-liposome complex depend both on the properties of the enzyme itself and of the liposomial structure and on their interactions; therefore, a deep knowledge of enzyme and lipid bilayer biophysics can allow us to design the liposome formulation rationally. Such a rational approach will not only result in process optimization, but also allows us to obtain catalytic system with unique properties and of great utility in biotechnological and medical applications.

According to these lines, we first analyse the physico-chemical properties that mainly affect the catalytic behaviour of enzyme-loaded liposomes and, in particular, the lipid bilayer permeability; then the theoretical kinetic model for enzyme/liposome catalysed processes is reported; finally we review some interesting application suggested in the literature for enzyme-loaded liposomes.

Physico-chemical properties of liposomes

The term liposome is used to describe both unilamellar vesicles with a single lipid bilayer enclosing an aqueous core (small unilamellar vesicles (SUV) with a diameter of 200–500 A diameter or large unilamellar vesicles (LUV) with a diameter of 0.1–10 μm) and multilamellar vesicle (MLV) with an onion-like structure, organized as closed concentric membranes. Even if fundamental questions about the spontaneity of liposome formation and thermodynamic stability are still open, it is generally accepted that liposomes are not to be considered as structures in thermodynamic equilibrium but in metastable "kinetic traps"[31]. Anyhow, these structures result from self-aggregation without covalent bonds of lipid molecules. Self-aggregation thermodynamics is, therefore, the suitable theoretical framework to study the vesiculation process[32,33].

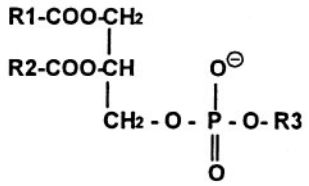
A variety of lipids and lipid mixtures can be used to prepare liposomes (see Table 1); the most frequently used are phospholipids – consisting of two fatty acid chains attached by a glycerol backbone to a phosphate-bearing head – and, among these, phosphatidylcholines or lecithins.

It is worth noting that some phospholipids may also spontaneously arrange into non-bilayer structures such as micelles or hexagonal phases. In order to predict the shape of the structure that lipids tend to form upon hydration, *Israelachvili*[34] suggests the use of a simple packing parameter $\mathscr{P} = v/a_0 l$ where v is the volume of hydrocarbon chains, l is the critical chain length and a_0 the optimal head area. Whereas lecithins, with $\mathscr{P} = \sim 1$, pack best into bilayer structure, lipids with relative small polar head, as phosphatidylethanolamine, with $\mathscr{P} = >1$ tend to pack into inverse structure (invertead hexagonal phase H_{II} – see Figure 1). Phosphatidylethanolamine alone does not form bilayers and liposomes, but it can be added to lipid cocktail to form liposomes; and just for its tendency to form hexagonal phase, it plays an important role in liposome fusion[35,36].

Phase transition. Lipid bilayers undergo several thermotropic phase transitions, by far the most important of which is the gel-to-(liquid crystalline) transition. In this transition, also referred to as chain melting transition, the lipid fatty-acid side chains change from the solid all-trans state to the one with considerable trans-gauche isomerization, with an increase in the surface area and a shortening of the bilayer thickness. In the gell state rotation about the carbon bound is severely restricted, whereas in the liquid-crystalline state molecules achieve a high-degree of rotational freedom[37].

Phase transition can be monitored by various experimental techniques, as spectroscopic measurements of probe motion and differential scanning calorimetry. While spectroscopic measurements give qualitative information about membrane fluidity, differential scanning calorimetry gives also quantitative data about the transition from gel to liquid crystalline state. In planar lipid bilayer the transition is associated with a narrow peak of the heat capacity, which suggests a strong endothermic first order phase transition; nevertheless no discontinuities in the physical properties have been detected: this behaviour is usu-

Table 1 – *Phospholipids commonly used for liposome preparation*



R1, R2 fatty acid hydrocarbon chains
R3 polar head

Fatty acids, R1COOH, R2COOH

saturated		unsaturated	
miristic	$CH_3(CH_2)_{12}COOH$	palmitoleic	$CH_3(CH_2)_5CH{=}CH(CH_2)_7COOH$
palmitic	$CH_3(CH_2)_{14}COOH$	oleic	$CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$
stearic	$CH_3(CH_2)_{16}COOH$	linoleic	$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$

Polar heads, R3

name	abbr	structure	charge (ph=7)	*P*
phopshatidic acid	PA	-H	negative	>1
phopshatidylcholine	PC	$-CH_2CH_2N^+CH_3$	0	~1
phopshatidylethnolamine	PE	$-CH_2CH_2N^+H_3$	0	>1
phopshatidylserine	PS	$-CH_2CH_2N^+H_3COO^-$	negative	~1
phopshatidylglycerol	PG	$-CH_2CHOHCH_2OH$	negative	
phopshatidylinositol	PI	$-HC_6H_5(OH)_5$	negative	~1
cradiolipin	CL	$-CH_2CHOHCH_2$		>1

ally explained in terms of a high, but finite cooperativity, even if the thermodynamic character of gel-to liquid crystalline transition has not yet been resolved and the question whether or not such a transition is truly first order is still open[38–41].

A schematic representation of the thermogram of lipid phase transition is reported in Figure 3. From the curve $H = H(T)$, the fraction of lipids in fluid phase, θ, is obtained as $\theta = H(T)/\Delta H_{cal}$, where ΔH_{cal} is the enthalpy change associated with the phase transition. The phase transition temperature, T_m, is defined as the temperature corresponding to $\theta = 1/2$. The sharpness of the phase transition is usually described referring to a simple model that considers a two-state process (gel) $\Leftrightarrow$ (fluid), with an equilibrium constant $K = \theta/(1-\theta)$. According to this approach, the van't Hoff enthalpy change of the process can be evaluated as:

$$\frac{\Delta H_{vh}}{RT^2} = \left(\frac{\partial \ln K}{\partial T}\right) = \frac{1-\theta}{\theta}\left(\frac{\partial \theta}{\partial T}\right) \qquad (1)$$

Therefore, θ evaluation from calorimetric curve allows us to obtain ΔH_{vh}, that is usually greater than ΔH_{cal}. The ratio $c = \Delta H_{vh}/\Delta H_{cal}$ is usually referred to as the size of cooperative unit

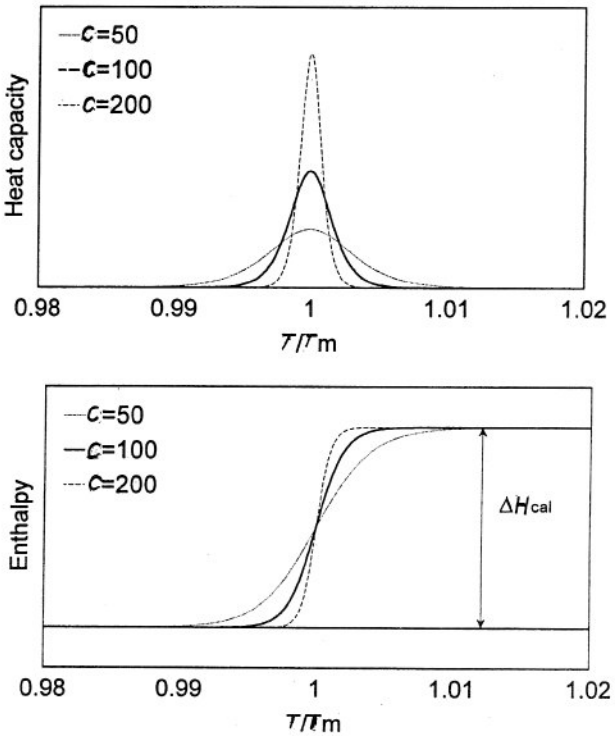


Fig. 3 – *Schematic plots of the thermograms of lipid phase transition for different values of the size of cooperative units.*

and represents the sharpness of the transition. A large size of cooperative unit is characteristic of a very sharp transition, whereas smaller values result in a broader thermal transition (Fig. 3). Values of c from 50 to 300 have been observed with phopshatidylcholine liposomes[42,43].

From a microscopic point of view, in the phase transition region, the domains of lipid molecules in the gel and fluid phase coexist dynamically and the two regions are bounded by an interfacial region. Since the consequent heterogeneity is dynamic, the pattern of clusters changes in time, but there is a well-defined average domain size and measure of the interfacial regions. A picture of the bilayer state in the transition region can be obtained from molecular simulation models[40]. In a simple way, *Freirie* and *Biltonen*[44,45] suggest evaluating the average gel and liquid crystalline cluster sizes from statistical thermodynamic consideration:

$$<L_g> = \frac{q}{1-q} \qquad <L_f> = \frac{z}{1-z}$$

where q and z are the residual partition function defined with respect to the gel and the fluid state respectively. The partition functions can be obtained from calorimetric data as:

$$\ln q = \int_{T_1}^{T} \frac{\Delta H}{RT^2} dT \qquad \ln z = \int_{T_1}^{T} \left(\frac{\Delta H}{RT^2} - \frac{\Delta H_{cal}}{RT^2} \right) dT$$

where T_1 and T_2 are temperatures above and below the transition temperature respectively.

Different lipids show different phase transition behaviour. Saturated phospholipids undergo sharp gel-to-liquid crystalline phase transition, with transition temperature and transition enthalpy that increase with increasing chain length[37] (see Figure 4). The presence of double bonds, mainly cis double bond, reduces the melting temperature and highly unsaturated species undergo a broad, ill-defined phase transition. Egg PC, a natural phospholipid mixture with about 40% of unsaturated lipids, does not show any distinguishable phase transition above 0 °C; as partial hydrogenation increases phopsholipid saturation, the phase transition becomes more marked and the transition temperature increases (see Figure 5)[46].

Finally it is worth noting that transition behaviour also depends on the liposome size. Small unilamellar vesicles usually show a much broader transition than that observed with multilamellar vesicles, with lower transition temperature and enthalpy[38,42]: transition temperature of dipalmitoylphopshatidylcholine liposomes changes from 41.3 °C for MLV to 37 °C for SUV (diameter < 50 nm), whereas transition temperature range varies from 0.1 °C to 3.5 °C and the enthalpy change varies from 8 to 5.5 kcal mole^{-1}. (33.49 to 23.02 J·mole^{-1})

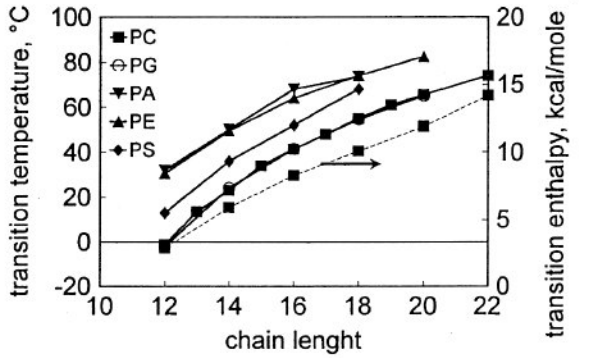


Fig. 4 - *Transition temperatures and enthalpy change of different phospholipids at pH=7 (abbreviations as in Table 1).*

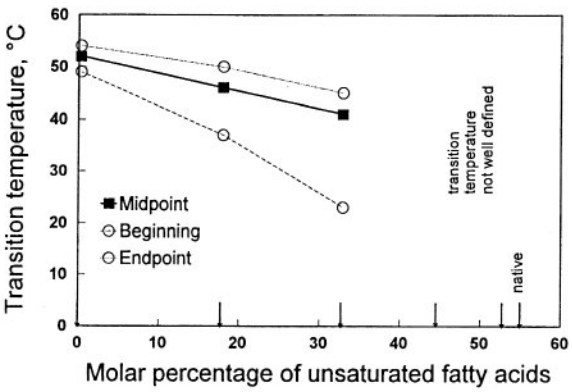


Fig. 5 – *Transition temperature of eggPC at different hydrogenation degree (the arrows refer to the different hydrogenation degree tested). Data from Ref. 46.*

The thermotropic behaviour of mixed lipid films is, of course, quite different from that of pure component and it is usually described with reference to a phase diagram where the melting and freezing temperatures are plotted as a function of mixture composition. These diagrams show that for two lipids with the same head group and differing only slightly in the chain length mixing is close to ideal, whereas more dissimilar lipids often show partial miscibility in the gel state[41,47].

Liposome permeability. The permeability of lipid membranes is the key factor that controls the overall liposome behaviour, both as drug delivery system and as enzymatic biorector. Therefore, we analyse this property in details.

Liposome permeability differs by several order of magnitude for various types of substances (see Table 2), so that, in a practical sense, lipid bilayers are permeable to water and small non polar molecules and impermeable to ions and high molecular weight compounds. Such a behaviour allows us to retain enzymes or hydrophilic compounds inside the liposomes whereas small non polar substrate can diffuse through the bilayer and react inside the vesicle. At the same time, high permeability barrier provided by the bilayer

Table 2 – *Permeabilities (cm/s) for the transport of various solutes across lipid bilayers ([a] Ref. 115; [b] Ref. 26; [c] Ref. 66 – containing 5% dicetylphosphate; [d] gel state; [e] fluid state, [f] depending on protein size)*

Permeant	eggPC[a]	eggPC[b]	DMPC	DPPC
water	$2 \cdot 10^{-4}$	$2 \cdot 10^{-4}$	$2 \cdot 10^{-5\,b,d}$ $6 \cdot 10^{-4\,b,e}$	$4{,}7 \cdot 10^{-6\,b,d}$ $6.3 \cdot 10^{-4\,b,e}$
H^+/OH^-	10^{-4}–10^{-8}			
Na^+	10^{-14}	10^{-12}–10^{-14}		
K^+				
Cl^-	10^{-11}	$4 \cdot 10^{-10}$ $7.6 \cdot 10^{-11}$		
ethanol		10^{-6}		
glucose		$6.8 \cdot 10^{-12}$	$10^{-11\,c,e}$	$5 \cdot 10^{-12}$–$7 \cdot 10^{-13\,c,d}$ 1–$7 \cdot 10^{-10\,c,e}$
sucrose		10^{-12}		
glycerol	$5.4 \cdot 10^{-6}$			
urea	$4 \cdot 10^{-6}$			
dextrane		10^{-13}		
proteins		10^{-9}–$10^{-14\,f}$		

for ions or high molecular weight compounds can be used to protect the enzyme from inhibitor or denaturating compounds (see, for example, the use of liposome to protect the enzyme against protease digestion[48]). In details, non-electrolyte permeation can be described by the classical solution-diffusion model, with the diffusion of the permeant calculated using the free-volume theory[49–51]. The lipid bilayer has a very low permeability for ions and most polar molecules. Anion permeability (10^{-10} cm s^{-1}) is significantly greater than cation permeability (10^{-12} cm s^{-1})[52] and permeability to proton and hydroxide is orders of magnitude greater than to other monovalent ions[53–55].

Glycine, serine and lysine permeability of eggPC liposomes are about to $0.5 \cdot 10^{-11}$ cm s^{-1} whereas tryptophan and phenylalanine (hydrophobic aminoacids) permeabilities are $41 \cdot 10^{-11}$ and $25 \cdot 10^{-11}$ cm s^{-1}, respectively[56]. This behaviour seems strictly relatead to differences in their partition coefficient between organic and water phases.

In order to design enzyme-loaded liposomes as biocatalysts, it is worth considering that a change in the bilayer composition of vesicles greatly afects the permeation rate. For example, permeability through liposomal membranes increases gradually with decreasing chain length[57]; *Chakrabarti* et al.[58] claim that membranes become approximately ten times more permeable to ions for every two carbons subtracted from the phospholipid chains, so that bilayers composed of 18 carbon lipids can maintain ionic gradients for days, whereas the 10 carbon lipids form bilayer so permeable that ion gradients cannot be maintained. Furthermore, high cholesterol concentrations, which are used to stabilize the liposome structure, reduce the bilayer permeability. A similar behaviour has been observed for several sterols[59]. Finally, small molecules (nystatin, gramicidin A, etc.) are able to form pores across the bilayer, increasing the liposome permeability of ionic species[60].

The rate of solute permeation across lipid membranes normally increases with increasing temperature, being strongly affected by the chain melting phase transition. On the basis of classical experiments of *Papaphadjopoulos* et al.[61] who have discovered the anomalous peak of passive permeability of lipid bilayer to cations near the phase transition, several theoretical and experimental works have been carried out about the liposome permeability near the phase transition[62,63]. Even if the major part of these works deals with small cation flux, the anomalous permeability has also been confirmed experimentally for some different solutes. A sharp increase in phospholipid bilayer permeability when temperature raises above the melting temperature has been observed by *Sada* et al[64,65]: permeability of hydrophobic solutes (salicylic acid, salicylamide and n-propanol) increases 6 to 10-fold, whereas permeability of hydrophilic solute (pyroxidine) increases only 2-fold; alanine permeability in DMPC/dicetylphosphate (10:1) liposomes raises from 10^{-11} cm s^{-1} below T_m to 10^{-10}–10^{-9} cm s^{-1} above the transition temperature. Glucose permeability across DMPC and DPPC membrane increases significantly when the lipids undergo the gel to liquid crystalline phase transition (*Bresselers* et al[66]); in more recent studies, *Francis* et al.[67] and *Clerc* and *Thompson*[68] have found a maximum of glucose permeability in the transition region for DPPC-phopshatidylinositol and DMPC-DPPC liposomes respectively. *Chakarabarti* et al.[58] observed a marked increase in the release rate of encapsulated ADP – a large organic ion – from DMPC vesicles at the phase transition temperature (23 °C).

To describe the temperature dependence of liposome permeability near the phase transition temperature, a permeability model can be developed on the fundamental assumption that the bulk and cluster regions have their own characteristic permeability, which are both considerably less than the permeability of the interface[62]. Therefore, the permeability of a molecule across the membrane is given by:

$$P = A_f P_f + A_g P_g + A_i P_i \qquad (4)$$

where A_f, A_g and A_i are the fractional areas occupied by the fluid clusters, the gel clusters and the interface and P_f, P_g and P_i the corresponding permeabilities. In a narrow temperature range in the transition region, temperature dependence of P_f,

P_g and P_i may be neglected, whereas A_f, A_g and A_i depend strongly on temperature. If the fractional areas are evaluated from the model suggested by *Frerie* and *Biltonen*[44] ($A_f = \theta\,(1–A_i)$, $A_g = (1–\theta)(1–A_i)$ and $A_i = 4\theta\,(<L_f>^{1/2}+1)/(<L_f>^{1/2}+2)^2$), the permeability vs. temperature trend shows a maximum near the transition temperature, as reported in Fig. 6.

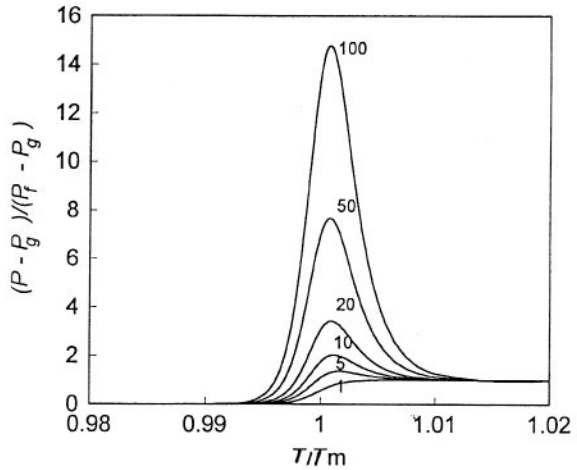


Fig. 6 – *Lipid bilayer permeability in the transition temperature region for different values of the ratio* $(P_i–P_g) / (P_f–P_g)$

Influence of other factors on the liposome properties

The physico-chemical properties of the liposomes are sensitive to the additives that are present in the lipid cocktail (cholesterol) or that partition between the external solution and the membrane (detergent, anaesthetic...) as well as to the ions present in the external medium (H^+, Ca^{2+}). Therefore, a judicious choice of the lipid cocktail and medium composition may be determinant to rational design of a liposomial system. Nevertheless, few systematic and quantitative studies have been carried out on this subject.

Cholesterol. Cholesterol acts as the main lipid rigidifier in natural membranes and it is the component more often added to the lipid cocktail to improve liposome stability. The effect of cholesterol is twice: below the transition temperature, it increases the fluidity of the gel state by disrupting the all-trans crystalline packing of solid phospholipid bilayers, whereas, above the transition temperature, cholesterol reduces the motion of hydrocarbon chains of the fluid lipids. From a macroscopic point of view, the effect of cholesterol is to broaden the main phase transition, which is completely removed at high cholesterol concentration (30–40% mole)[62].

According to the ability of cholesterol to enhance the mechanical coherence of the membrane, high cholesterol concentration suppresses the membrane permeability. On the contrary, at low concentration, the permeability of the system increases with the cholesterol content, for all temperature in the transition region[69].

Detergent. Detergent addition to preformed liposomes results in a complete liposome solubilization, with the release of the liposome content. Many Authors[70–72] agree that the process occurs in three subsequent steps, as determined from turbidity, NMR or electron microscopy measurements. In the first step, detergent is incorporated into the bilayer, until liposome saturation, corresponding to a critical detergent to lipid ratio ($\mathcal{R}^{sat}$); in the second step liposome and mixed detergent/lipid micelles coexist; in the third step, when the detergent to lipid ratio reaches a second critical value ($\mathcal{R}^{sol}$), all lipids are present as mixed micelles (see Figure 7).

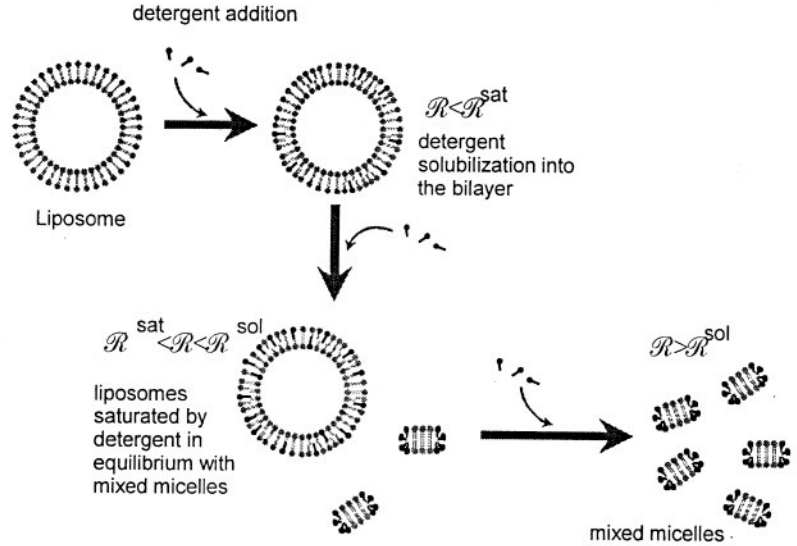


Fig. 7 – *Liposome solubilization by detergent addition.*

Dealing with the liposome properties, we are mainly interested in the effect of a detergent at a detergent to lipid ratio lower than R^{sat}, when the vesicular structure is preserved. In this region, it is usually assumed that detergent intercalates interstitially in the outer lipid layer of the liposome membrane and induces change in the phase transition and permeability[73]. In particular, even if some inconsistencies appear in the experimental observation regarding the detergent-induced leakage, roughly speaking it is possible to affirm that detergents enhance the leakage of entrapped solute from liposomes through a permeability enhancement or the formation of transient holes[74–76].

Electrolytes. The most relevant effects related to the addition of electrolytes to preformed liposomes are their binding to counterions and, consequently, the reduction of net liposome charge. Divalent cations, in the millimolar concentration range, trigger aggregation and fusion of negatively charged liposomes. The fusogenic capability decreases in the sequence $Ca^{2+} > Ba^{2+} > Sr^{2+}$ whereas Mg^{2+} induces massive aggregation of PS liposomes whithout mixing or release of their aqueous content.

pH. Even if it has been reported that pH changes induce structural and dynamic changes also in neutral membrane[77], the most relevant effects are observed with charged lipids. In fact, the pH strongly affects the ionization state of the phospholipid polar head and therefore the phase transition of ionic liposomes[37]. For example, melting temperature of MLV of DPPA rises from ~62 °C at pH < 3.5 (when the phosphoric acid moiety is unionized) to about 70 °C at pH 4–9 (when the polar head has a net negative charge) but decreases faster at pH > 9 up to a value of about 45 °C at pH=11[42].

Using lipids with pH-sensitive head group it is possible to obtain pH-sensitive liposomes that are destabilized and release their content at acidic pH. To this aim, liposomes are preparead using PE as the main component and some stabilizing compounds with pK between 4 and 5. These compounds (e.g. fatty acids, N-palmitoyl homocystein, etc) are charged at neutral pH and stabilize the PE bilayer; at low pH the uncharged species is unable to stabilize the bilayer and liposome fusion and content leakage occur[27,78–80]. These liposomes may be useful to deliver drugs to tissues that, in pathological conditions, have a pH lower than that of the normal ones or to enhance the delivery of liposomal content to endosomes. In a similar way, cationic, pH-sensitive liposomes may be obtainead to mediate DNA transfer into cells[81].

Lipid-protein interaction

Dealing with enzyme-loaded liposomes, a subject of great interest is the interaction proteins/lipid bilayers, since it is well known that lipids affect the properties of membrane enzymes and, on turn, proteins affect the fluidity, permeability and thermotropic phase transition of the phospholipid bilayer.

Recent studies reveal the complexity of this field: questions like protein partitioning, their location into the bilayer, the effects of protein insertion on the bilayer structure and how the partitioning affects the secondary structure of the protein itself are still open[82].

Some rule of thumb may be derived from the classical study of *Papahadjopoulos* et al.[83], recently reexamined by *Lo* and *Rahman*[84]: on the basis of conventional differential scanning calorimetry experiments, the proteins can be grouped into three classes, according to the type of interaction and penetration into the bilayer: 1) proteins that adsorb at the bilayer interface, either by electrostatic or specific interactions; 2) proteins that are adsorbed onto and partially embedded into the bilayer, with deformation of the bilayer; 3) proteins that penetrate (or at least a part of proteins that penetrates) into the bilayer core.

Dealing specifically with enzyme loaded liposomes, the main problem is to determine the amount of enzyme entrapped and its location in the vesicular structure in order to understand the catalytic behaviour of the whole system. Immunological and freeze-fracture techniques have been used to show that ascorbic acid oxidase is at least partially embedded in DPPC-cholesterol liposomes and some of the enzyme molecules are protruding towards the external solution[85]. In this case, lipid-protein interactions result also in a size reduction of liposomes produced by detergent controlled dialysis.

Of course, protein and lipid bilayer electrical charges play an important role both in protein entrapment and location, even if *Lo* and *Rahman*[84] stress that a combination of electrostatic and hydrophobic interactions always occurs. Since pH affects the protein and lipid charge, significative effects of pH have been observed both on lipid-protein interactions[86] and on the enzyme-liposome activity[87]. It has been observed that carbonic anhydrase, which have a net negative charge at pH 6.7, is not entrapped in neutral liposomes, but only in cationic ones (containing stearylamine). In this case, the net liposome charge, which depends on stearylamine concentration, seems to affect also the enzyme location in the vesicle: kinetic experiments, carried out with proteolytic enzyme that digests the enzyme protruding outside the vesicle or with inhibitors in the external solution, show that carbonic anhydrase is present both inside and on the surface of the vesicle at low stearylamine content, whereas the enzyme is only inside the vesicles at high stearylamine content[88].

Enzyme-loaded liposomes as microreactors

As reported in Figure 2, different scenarios are possible for enzyme-liposome systems: in this paragraph we refer only to the most extensively studied cases, where the enzyme is bound to the liposomial structure and the substrate is free in the external solution. We report about both the theoretical model and the experimental works carried out on such systems to clarify the advantages and the shortcomings of enzyme immobilization in liposomes and to show the possibility to improve the efficiency of the system with a proper control of the liposome properties. To simplify, we take into consideration two typical situations: the enzyme is at least partially embedded in the lipid membrane and protruding towards the external solution (schema c2 in Figure 2), or the enzyme is confined in the aqueous core (schema c1 in Figure 2). In the first case the overall kinetics depends mainly on the effect of the lipid environment on the enzymatic activity; in the second one the overall kinetics is determined by the interactions between the substrate diffusion and the enzymatic kinetics. Of course, the real system may be more complex: in many cases the presence of the enzyme both in the aqueous core and on the liposome surface has been detected. In such a case, the kinetic behaviour results from all the phenomena considered separately in the following and may be more difficult to describe and to control. Finally we show the advantages to realize cofactor-requiring enzymatic kinetics in liposomes.

Enzyme embedded in the lipid bilayer

It has been well known that most of the membrane enzymes require phopsholipids to exploit their activity and the enzyme reconstitution in liposomes is a promising tool not only for fundamental biological and biophysical studies but also for biotechnological applications. For example, *Sada* et al.[89] suggest producing prostaglandins (PG), a carboxilic acid with significant pharmaceutical potential, by means of liposomized PG-synthetase and show that the liposomization almost doubles prostaglandin yields with respect to that obtained by the free enzyme.

A deeper study has been developed on the reaction characteristics of sarcosine dehydrogenase – an enzyme useful for clinical assays – reconstituted in MLV and SUV of different lipidic composition[90]. Reconstitution in DMPC liposomes results in an enzyme activity 25-fold higher than that of the free enzyme. The magnitude of activity enhancement by DPPC was less than that by DMPC: since the experiments are carried out at 37 °C, this behaviour is probably due to the reduced flexibility of the enzyme in the tighter packing of the lipid bilayer in the gel phase of DPPC. In fact, the dependence of the activity of sarcosine dehydrogenase in DMPC liposomes on temperature shows a break in the Arrehenius plot at temperature corresponding to the main phase transition and suggests that the activity is sensitive to the physical state of the bilayer.

This behaviour is common to most of the membrane enzymes. Two causes can be registered: 1) the enzyme activity is related to the active site conformation, which, in turn, is affected by the state of the lipid membrane 2) the equilibrium position of the membrane proteins depends on the lipid bilayer fluidity and, for an enzyme partially embedded in the bilayer with the binding site near the hydrocarbon-water interface, a small change in lipid microviscosity causes vertical displacements and modulates the enzyme activity especially towards hydrophilic substrates.

An interesting model, developed by *Shinitzky*[91] to study the activity of enzymes in natural membranes, might be applied also to enzyme reconstituted in liposomes.

Enzyme entrapped in the liposome aqueous core. Several theoretical and experimental studies have been carried out with hydrophilic enzymes entrapped in liposomes. In this case it is reasonable to assume that a homogeneous enzyme solution is entrapped in the aqueous core of the vesicles, even if sometimes there is experimental evidence of a partial adsorption of the enzyme on the bilayer. As clearly pointed out by *Chakarabarti* et al.[58], such a system does work if the bilayer is sufficiently impermeable to maintain macromolecules within the encapsulated microenvironment but is still permeable enough to allow the passage of substrate to react inside the vesicle.

The theoretical models for these systems, recently reviewed by *Cioci* and *Lavecchia*[92], are the classical models for heterogeneous chemical reactors. In such a system the overall enzymatic process can be splitted into four steps:

a) substrate diffusion in the external boundary layer near the external liposome surface;

b) substrate permeation across the liposome membrane

c) substrate diffusion in the aqueous phase inside the liposome

d) enzymatic reaction inside the liposome

The first two steps are in series with respect to the other two, parallel to each other.

The analysis of the characteristic times for the elementary steps allows us to recognize which steps really affect the overall process rate and must be considered in the theoretical model. As reported in Table 3, characteristic times of diffusion through the external boundary layer and in the internal solution depend on d^2 and therefore can be usually neglected, as a consequence of the small liposome diameter. Therefore, a simplified model can be developed accounting only for the substrate permeation across the membrane and the enzymatic reaction inside the liposome. In this case, at steady state the flow rate of the substrate through the liposome membrane must be equal to

Table 3 – *Characteristic times for the elementary steps involved in the enzymatic reaction in liposomes*

elementary step	characteristic time
external diffusion	$\tau_e = d/(6k_C) = d^2/(12\mathscr{D}_f)$ [a]
membrane diffusion	$\tau_P = d/(6P)$
internal diffusion	$\tau_D = d^2/(4\mathscr{D}_f)$
enzymatic reaction	$\tau_R = K_M/(V_{max})$

a) evaluated from $k_C\, d/\mathscr{D}_f = 2$

the substrate consumption rate due to the enzymatic reaction inside the liposomes. Assuming a Michaelis-Menten kinetics $r = V_M \gamma_S / (K_M + \gamma_S)$, we have:

$$\xi(\sigma_b - \sigma) = \frac{\sigma}{1+\sigma} \tag{5}$$

where σ is the dimensionless substrate concentration ($= \gamma_S / K_M$) inside the liposomes, $\sigma_b = \gamma_{Sb} / K_M$ is the dimensionless substrate concentration in the bulk solution and $\xi = \tau_R / \tau_P$ is the ratio between reaction and membrane permeation characteristic times. Eq.(14) shows clearly that the substrate concentration inside the liposome depends on the ratio between the permeation and the reaction times. Small ξ values correspond to low bilayer permeability or to high enzyme concentration inside the liposomes; in this case $\sigma \ll \sigma_b$ and strong diffusional limitations occur. On the contrary, high ξ values correspond to $\sigma = \sigma_b$ and to a reaction controlled process. For any ξ value, for high external substrate concentration the reaction rate tends to v_M whereas in the linear range ($\sigma \ll 1$) the first order kinetic constant is given by $\xi / (1+\xi)\, \tau_R$. Nevertheless, the apparent relation between the reaction rate and the external substrate concentration cannot be expressed by a Michaelis Menten equation with modified values of V_M and K_M; so the analysis of the experimental data on the Lineweaver-Burk plot is incorrect.

The above results can be summarized in the plot of the efficiency factor, η vs. ξ reported in Fig. 8. It is worth noting that the efficiency factor, defined as the ratio between the observed reaction rate and the reaction rate corresponding to an internal substrate concentration equal to the external one, has also the physical meaning of the ratio between the reaction rate obtained with the enzyme entrapped in liposomes and that obtained with the enzyme released from liposome after vesicle disruption. High reaction rates can be obtained with high the efficiency factor, i.e. with a high substrate permeability across the lipid bilayer. Therefore, all the previous analysis on the lipid bilayer permeability can be useful to optimize the catalytic efficiency of enzyme-liposome complex.

In particular, we consider here the effects of detergent addition and temperature. Since detergent addition at sublytic concentration enhances the bilayer permeability, detergent may be used to modulate the activity of enzyme-loaded liposomes. According to this idea, *Ambartsumian* et al[93] have shown that the activity of liposomes loaded with urate oxidase or glucose oxidase increases with the increase of the amount of sodium deoxycholate incorporated in the liposome membrane (a mixed film lipid-detergent is used to prepare liposome). More recently, we have also showed that the addition of Triton X-100, always at sublytic detergent concentration, to preformed soya PC liposomes loadead with β-galactosidase enhances the liposomial complex activity towards a synthetic substrate (4-methilumbelliferil-galactoside)[94].

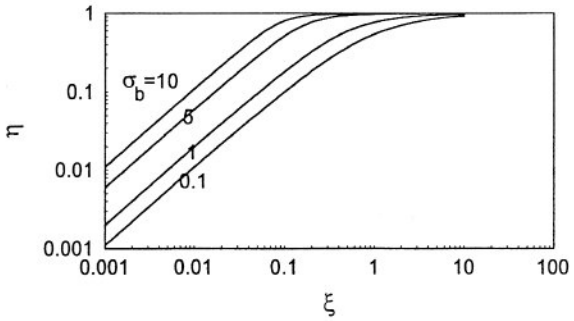


Fig. 8 – *Efficiency factor of the enzymatic reaction in liposomes for different substrate concentration in the bulk solution.*

The effect of the temperature on the overall process kinetics is more complex. In fact the temperature affects both the enzymatic reaction rate (in a small temperature range, it is usual to assume that V_M increases increasing temperature, whereas K_M is almost constant) and the membrane permeability, mainly near the transition temperature. To describe such a behaviour we first evaluate ξ vs T:

$$\xi = \xi_m \frac{P(T)/P(T_m)}{V_M(T)/V_M(T_M)} \tag{6}$$

where ξ_m is the ξ value at the transition temperature T_m. The ratio between the permeabilities at T and T_m can be obtained from the model reported in the previous paragraph, whereas the ratio between the maximal rate can be obtained from Arrhenius equation. A typical trend is reported in Fig. 9. Out of the transition temperature range, the efficiency factor decreases with increasing temperature, as a consequence of the higher activation energy of the chemical reaction with respect to the activation energy of the permeation process; in the transition range, the sharp increase of the permeability results in a peak also in the efficiency factor. This behaviour suggests the possibility to realize a very special catalytic system that is active only in a narrow temperature range. The temperature range can be chosen with a proper selection of the phospholipid used in liposome preparation[95,96].

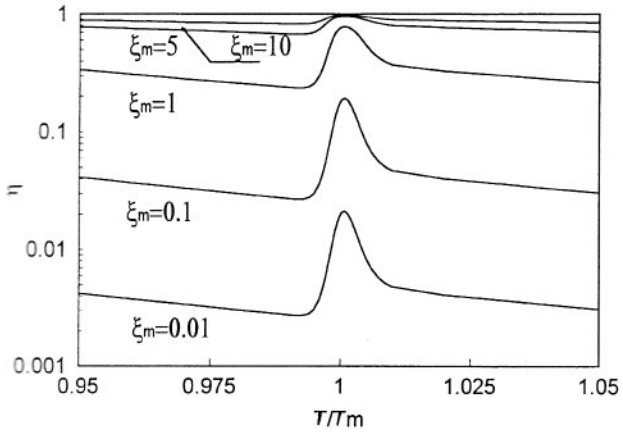


Fig. 9 – *Efficiency factor of the enzymatic reaction in liposomes in the transition temperature region (σ = 1; c = 100; $\Delta H_{cal}/RT_m$ = 10; $(P_i - P_g)/(P_f - P_g)$ = 50).*

Multienzymes cofactor-requiring in liposomes. An interesting application of liposomes is to microencapsulate multienzyme cofactor-requiring systems. In fact, to exploit their activity, important enzymes require cofactors, like NAD, that are oxidised or reduced during the reaction; consequently, a proper cofactor regenerating system is required to obtain a continuous process. Often, the cofactor regenerating system is an enzymatic system itself, so we have to deal with a multienzyme-cofactor system. In general there is no problem in entrapping high concentrations of different enzymes within microcapsules; if we can also retain the cofactors inside microcapsules with a membrane impermeable to cofactors (both in the oxidate and reduced form) but permeable to substrates, a very efficient system can be obtained (see Figure 10). Lipid membrane, permeable to lipophilic molecules, but with negligible permeability to hydrophilic ones, seems suitable to obtain such systems that have been evocatively named "artificial cells". Several studies in this field have been carried out by *Chang*[97–99], using different microcapsules. He showed, in particular that the lipid-polyamide membrane can retain NAD in the free form which is receicled inside the vesicles by the multienzyme systems[98].

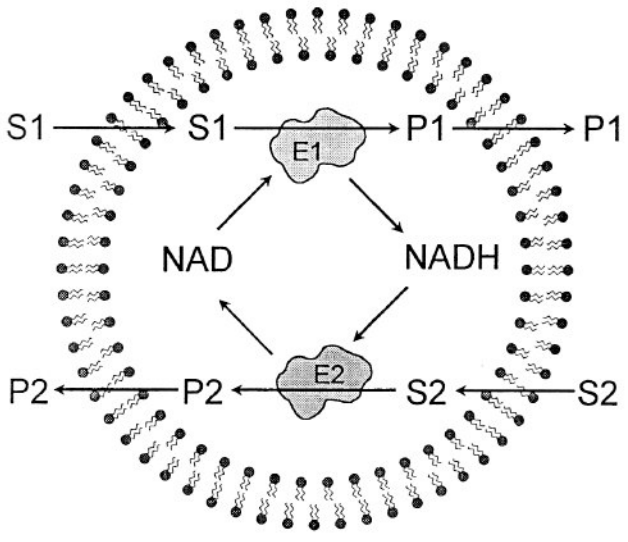


Fig. 10 – *Scheme of cofactor-requiring enzymatic reaction in liposomes.*

In order to clarify the behaviour of such a system, it is useful to consider a simple theoretical model which assumes, for both enzymatic reactions, a simple bimolecular reaction kinetics (similar results can be obtained considering more complex kinetic equations):

$$r_k = \frac{V_{M,k}}{K_{S_k} K_{C_k}} \gamma_{Ck} \gamma_{Sk} \qquad k = 1,2 \tag{7}$$

where γ_C and γ_S refer to the cofactor and the substrate concentration inside the vesicle respectively and the subscript k refers to the different enzymatic reactions. The above equation is derived from Michaelis Menten equation with the hypothesis that $\gamma_C << K_C$ and $\gamma_S << K_S$. Accounting for the substrate permeation rates through the liposome membrane, the main substrate (k = 1) consumption rate can be expressed as a function of the bulk substrate concentration $\gamma_{S,b1}$:

$$r_1 = \gamma_{S,b1} \frac{\alpha}{\alpha \tau_{p1} + \tau_{r1}} \tag{8}$$

where the characteristic time τ_{R1} is defined as $K_{c1} K_{s1} / (V_{M,k} \gamma_{C0})$ with γ_{C0} the total cofactor concentration and α is the fraction of cofactor in the form to react with the first substrate (referring to Figure 10, the fraction of cofactor in the oxidised form NAD^+).

Liposomes with high surface area and high cofactor and enzyme concentration allow us to obtain low τ_{R1} and τ_{p1} value; furthemore, high α values can be obtained incresing the cofactor regeneration rate, i.e. increasing the second substrate concentration.

Fusion mediated enzymatic reaction in liposomes

Enzymatic reaction in liposomes can be carried out according to the scheme c1 or c3 in Figure 2, only if the substrate is able to cross the lipid membrane and to react inside the vesicle. Such a process, therefore, cannot be used to transform high molecular weight substrate with hydrolytic enzymes. In this case, it should be useful to consider the process scheme reported in Fig. 2d: two different populations of vesicles – entrapping the enzyme or the substrate, respectively – fuse allowing the interaction of substrate and enzyme and the conversion of the substrate. Even if only two experimental works report on some results obtained with fusion-mediated process in lipo-

somes[93,100], in our opinion this reaction scheme is very interesting mainly as biomimetic model to study cellular biochemical processes occurring in a membrane bounded system. For example, degradation of extracellular materials by lysosomes occurs through the fusion of endocytic vesicle containing the substrate with the lyposomes containing the digestive enzymes[1].

Two point should be considered to realize such a process: how should the fusogenic lipid mixture be choosen and how to trigger the liposome fusion. In the simplest approach a mixture containing negative charged phosphatidylserine can be used and the fusion can be triggered by Ca^{2+} addition; other fusogenic lipids (e.g. phopshatidylethanolamine) may be added to enhance the fusion rate.

To develop a kinetic model, the fusion-mediated enzymatic process in liposomes may be split in three steps: 1) reversible vesicle aggregation resulting in a close apposition of the vesicle surface, 2) irreversible fusion of aggregated vesicles resulting in mixing of the aqueous content and membrane lipids, 3) enzymatic reaction inside the fused vesicle.

Accounting for the high enzyme and substrate concentration in the vesicles, the aggregation and fusion rate are likely to be the rate limiting steps and that the enzymatic reaction may be regarded as an istantaneous reaction. Therefore, the overall reaction kinetics simplifies into the aggregation-fusion kinetics. On this subject, several studies have been reported in the literature[101,102], based on mass-action kinetic model that results in a large set of differential equations to be solved numerically. In a previous work[103], we have suggested a simplified model based on the idea of the formation of clusters composed of vesicles joined at the aggregation or fusion sites. Collisions of clusters result in the formation of aggregation sites, which can in turn break down (reversible aggregation) or be transformed into fusion sites (fusion). The model predicts the number of aggregation and fusion sites as a function of time and the amount of substrate converted into the reaction product by means of probabilistic considerations.

To enlighten the behaviour of such a system, let us consider the simplest, but an interesting condition, where the liposome aggregation is the rate limiting step and all the liposomes in a cluster fuse together istantaneously to form a single vesicle. In this case the number of vesicles decreases with time according to a second-order kinetics and therefore:

$$\frac{n}{n_0} = \frac{1}{1 + k n_0 t} \tag{9}$$

where n_0 is the initial number of liposomes in the system.

At time t, when n vesicles are present in the system, the vesicle size distribution is given by:

$$n(x) = \frac{n}{(x-1)!}\left(\frac{1-n/n_0}{n/n_0}\right)^{x-1} \exp\left(-\frac{1-n/n_0}{n/n_0}\right) \tag{10}$$

where x is the number of primary liposomes fused together to form a vesicle. Since in a vesicle made up on x liposomes the probability to find i ($< x$) substrate-loaded liposomes is given by:

$$p(i,x) = \binom{x}{i} y^i (1-y)^{x-i} \tag{11}$$

where y and $1–y$ are the fraction of substrate-loaded and enzyme-loaded liposomes in the system. Therefore, the amount of product at time t is given by:

$$Q = Q_0 \sum_{x=2}^{\infty} n(x) \sum_{i=1}^{x-1} i p(i,x) \tag{12}$$

where Q_0 is the amount of product obtained by the complete conversion of the substrate present in a primary liposome. Figure 11 reports on some typical trends of the amount of converted substrate vs. time. The plot shows the effect of the ratio between substrate- and enzyme-loaded vesicles, with a given number of vesicles n_0. Initially, higher substrate consumption rates are predicted for y = 0.5, whereas, of course, the ultimate substrate consumption increases as y increases.

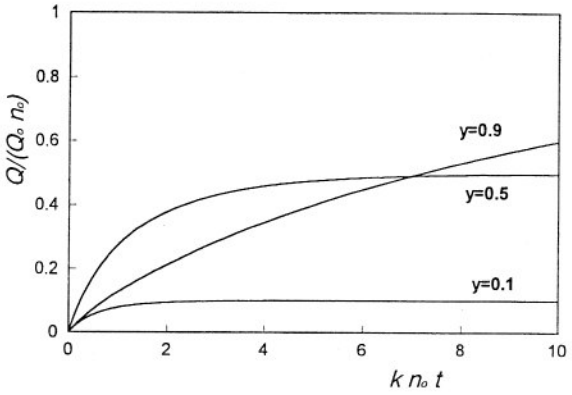


Fig. 11 – *Kinetics of substrate consumption in a fusion-mediated enzymatic process in liposomes.*

Applications of enzyme-loaded liposomes

To conclude this review, let us consider some applications of enzyme-loaded liposomes. In order to emphasize the versatility of such liposomal systems, we report about their applications in quite different fields.

Food industry. Liposomes of natural lipids (egg PC or soya PC) are an ideal system to microencapsulate additives in food industry. Three applications of enzyme-loaded liposomes seem very interesting to accelerate cheese ripening, to improve flavour of low-fat cheeses and to remove lactose from milk.

Several attempts have been carried out to accelerate cheese ripening with enzymes, due to the significant costs of maturation time for as long as one year[20,104]. Several problems must be overcome: the enzyme distribution, the enzyme lost with the whey, the bitter taste which results from early proteolysis. Protease encapsulated in liposomes appears suitable to halve the cheese ripening reducing the enzyme lost and protecting the enzyme against adverse conditions; furthermore, vesicles can be designed for timed release to coincide with a particular event in cheese aging[105]. A wide range of flavours can be generated by multiple enzyme pathways by adding lipid microcapsules to low-fat cheese to enhance its taste. For example, methanethiol can be generated rapid and extensively by encapsulating methionine and methioninase in milkfat-coated microcapsules.

Finally, an interesting application of enzyme-loaded liposomes in food industry is the addition of β-galactosidase entrapped in liposomes to milk for lactose intollerant people[106,107]. Lactose reduction in milk by free β-galactosidase results in an undesirable flavour change in milk and, on the other hand, β-galactosidase addition during meal time is adequate to alleviate lactose maldigestion. Therefore the idea is to add to milk liposome-protected enzyme so that lactose hydrolysis does not occur before consumption, but only after ingestion, in gastrointestinal conditions.

Enzyme-loaded liposomes in biomedicine

Enzyme use as therapeutic agents is very interesting for the treatment of genetic storage diseases, in thrombolytic therapy, in anti-cancer therapy, etc. Nevertheless, the application in everyday clinical use is limited by the rapid clearance from the blood, the toxic and immune response, the difficulty to obtain high enzyme concentrations in the desired region. These drawbacks can be eliminated by appropriate delivery systems. Among these, liposomes have many ideal characteristics: they are biocompatible, they undergo rapid biodegradation, their aqueous core minimizes the risk of protein denaturation, their structural versatility allows to manipulate their fate in vivo.

To date, many enzymes have been encapsulated in liposomes for biomedical uses[108], including asparaginase (anticancer therapy), glucosidase and galactosidase (inherited lysosomal disease), tissue plasminogen activator (myocardial infarction therapy), urease (blood detoxification).

Different studies deal with catalase and superoxide dismutase, two enzymes that scavenge the body for toxic oxygen species (superoxide O_2^- and hydroperoxide H_2O_2) according to the following reactions:

$$\text{superoxide dismutase:} \quad 2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$$

$$\text{catalase:} \quad 2H_2O_2 \rightarrow O_2 + 2H_2O$$

There is a growing evidence that these reactive oxygen species play an important role in tissue damage in a number of pathological conditions (chronic self-sustaining inflammation, radioinduced inflammation, ischemia-reperfusion syndrome). The pharmacological use of antioxidant enzymes is limited by their short life time in vivo and difficult to access to intracellular sites where O_2^- and H_2O_2 are produced. This fact makes liposomes an obvious choice for enzyme delivery and the results obtained both in vitro and in vivo encourage the efforts in this direction[109–111].

Many efforts are devoted to realize drug delivery at the target site, avoiding the absorption of high systemic doses and, consequently, the related undesired side effects. To this aim, liposomes with vector moieties able to recognize the target tissue and binding to it can be designed: the drug enzyme should remain within the liposomes during the transport to the target site; then, after liposome binding to the target, it should be released, or delivered intracellularly or it should work locally, entrapped in liposomes. In a recent paper *Storm* et al.[112] suggest two of such systems, for thrombolitic enzymes and prodrug activating enzymes. In the first case, a tissue plasminogen activator, tPA, that converts plasminogen into plasmin, that, in turn, lyses the thrombi, is loaded in liposomes containg a moiety (gluplasminogen) with affinity for fibrin clots: upon reaching the clot, encapsulated tPA is released for achieving a local lytic effect. In the second, enzymes capable of locally converting relatively non-toxic prodrugs into active cytotoxic agents can be loaded into immunoliposomes directed towards cancer cells. The enzyme bearing immunoliposomes (immuno-enzymosomes) are first allowed to bind to the tumor cells; then a prodrug is given which is activated by the cell-bound immuno-enzymosomes in the proximity of the target cell.

Liposomial biosensors. A promising application of enzyme-loaded liposomes is to realize biosensors where an analyte is enzymatically converted to a product which an appropriate detector is sensitive to. In such a system, the liposomal membrane protects the enzyme from the adverse environmental conditions and controls the substrate availability.

Among these, glucose biosensors have taken a prominent role for their use as monitoring devices for diabetic patients. In these sensors and electrode usually measures H_2O_2 produced from the reactions:

$$D\text{-glucose} + O_2 + H_2O \rightarrow D\text{-gluconic acid} + H_2O_2$$

$$H_2O_2 \rightarrow 2H^+ + 2e^- + O_2$$

the first of which is an enzymatic reaction catalysed by glucose oxidase. The linear range of response to glucose concentration is limited to the Michaelis Menten constant of the enzyme and usually does not allow to measure glucose in undiluted blood of diabetic patients. The linear range may be extended if the enzyme experiences a lower local glucose concentration while maintaining sufficient oxygen for the reaction. To this aim, lipid membrane with high oxygen and low glucose permeability and completely biocompatible appears as the ideal diffusion barrier (Figure 12). *Rosenberg* et al.[113] show that using liposomally entrapped glucose oxidase a linear response can be obtained up to 30–40 mmol · dm^{-3} glucose concentration, as required for clinical use. Since the rate limiting step is the glucose diffusion through the liposome membrane, a proper choice of the lipid composition is critical to achieve an efficient system. In particular, the width of the linear range increases in the series DMPC < DPPC < DSPC, corresponding to the decrease in bilayer permeability. Furthermore, the electrode response is sensitive to the mesomorphic state of the bilayer and largely increases as the temperature approaches the gel to liquid crystalline transition temperature. Of course, in this system a wide linear response range is achieved by a high glucose diffusion barrier at the expence of the electrode response loss. To obtain sensors suitable for clinical use, it is necessary to improve the electrode response, its stability and reproducibility. To increase the electrode response to the level required to assure a high signal/noise ratio, one would have to increase the level of immobilized enzyme, i.e. to increase the quantity of liposome immobilized on the electrode membrane and/or to increase the liposomal enzyme concentration. Good results have been obtained with polylysine and DPPC/PI liposomes that lead to multilayers of liposomes immobilized on the electrode membrane; the loss of electrode response to glucose over time can be avoided by pre-coating the electrode with lipids[114].

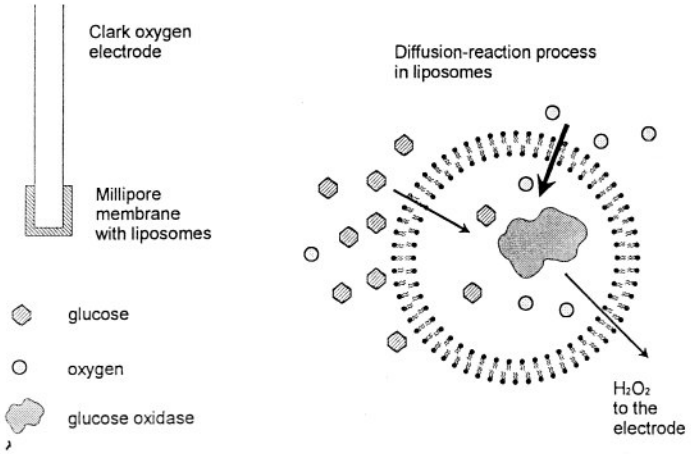


Fig. 12 – *Scheme of a liposomal biosensor for glucose monitoring.*

List of symbols

A — fractional area
a_0 — optimal area of the molecule polar head
c — size of cooperative unit
d — liposome diameter
$\mathscr{D}$ — molecular diffusivity of the substrate in water
E — activation energy
H — entalphy
ΔH — phase transition enthalpy change
k — kinetic constant of liposome aggregation
K — equilibrium constant gel ⇔ fluid transition
k_c — mass transfer coefficient in the external film
K_M — Michaelis constant
l — critical length of the molecule hydrocarbon chain
L — cluster size
n — vesicle number
p — probability
P — permeability
$\mathscr{P}$ — packing parameter
q — residual partition function defined by (3)
Q — amount of product obtained from substrate conversion
r — reaction rate
R — gas constant
$\mathscr{R}$ — detergent to lipid ratio
t — time
T — temperature
v — volume of the molecule hydrocarbon chain
V_M — maximal rate of the enzymatic reaction
y — fraction of substrate-loaded liposomes
z — residual partition function defined by (3)

Greek symbols

α — fraction of cofactor in oxidised form
γ_C — cofactor concentration
γ_S — substrate concentration
θ — fraction of lipids in the fluid phase
η — efficiency factor
ξ — ratio of reaction and permeation characteristic times
σ — dimensionless substrate concentration
τ — characteristic time (see Table 3)

Subscript

b — bulk solution
cal — determined from calorimetric measurements
D — internal diffusion
e — external diffusion
f — fluid phase
g — gel phase
i — interface
m — melting
R — reaction
P — permeation
vh — determined from van't Hoff equation (eq. 1)

Superscript

sat — saturation
sol — complete solubilization

References

1. *Mossa G., Annesini M.C.* and *Di Marzio L.*, Liposomes as biomimetic bioreactors. In Barenholz, Y. and Lasic, D.D. (Ed.) Handbook of Nonmedical applications of liposomes, Vol. III, pp. 273–286, CRC Press, Boca Raton, 1996.
2. *Pregel M.J., Jullien L.* and *Lehn J.M.*, Angew. Chem. Int. Ed. Engl. **31** (1992) 1637
3. *Bhatia R., Viswanathan P.N.* and *Kakkar, P.*, J. Microencapsulation, **8** (1991) 349
4. *Ramundo Orlando A., Mossa G.* and *D'Inzeo G.*, Bioelectromagnetics, **32** (1994) 826
5. *Foldvari M., Faulkner G.T., Mezei C.* and *Mezei M.*, Cells & Materials, **2** (1992) 67
6. *Heath T.D.*, Methods in Enzymology, **149** (1987) 135
7. *Ostro M.J.*, Sci. Am. **257** (1987) 102
8. *Roding J.* and *Ghyczy M.*, SOFW, (1991) 372
9. *Strauss G.*, J. Soc. Cosmet. Chem. **40** (1989) 51
10. *Gregoriadis, G.*, TIBTECH, **13** (1995) 527
11. *Sato T.* and *Sunamoto J.*, Prog. Lipid. Res, **31** (1992) 345
12. *Lasic D.D.*, Liposomes in drug delivery. In Rosoff, M. (Ed.) Vesicles, pp. 447–476, Marcell Dekker, N.Y. 1996.
13. *Schmidt M.H.* and *Korting H.C.*, Critical Reviews in Therapeutic Drug Carrier System, **11** (1994) 97
14. *Margalit, R.*, Vesicles as topical drug delivery systems. In Rosoff, M.(Ed.) Vesicles, pp. 527–560, Marcell Dekker, N.Y. 1996.
15. *Lasic D.D.* and *Templeton, N.S.*, Advanced Drug Delivery Reviews, **20** (1996) 221
16. *Smith J.G., Walzem R.L.* and *German J.B.*, Biochim. Biophys. Acta, **1154** (1993) 327
17. *Alving C.R.*, J. of Immunological Methods, **140** (1991) 1
18. *Lundhal P.* and *Yang Q.*, J. Chromatography, **544** (1991) 283
19. *Powers J.D., Kilpatrick P.K.* and *Carbonell R.G.*, Biotechnol. Bioeng. **33** (1989) 173
20. *Kirby C.*, Food Sci & Tech. **5** (1991) 74
21. *Annesini M.C., Cioci F., Lavecchia R.* and *Marrelli L.*, Annali di Chimica, **85** (1995) 683
22. *Vanzanten J.H., Chang D.S.W., Stanish I.* and *Monbouquette H.G.*, J Membrane Sci, **99** (1995) 49
23. *Walsh A.J.* and *Monbouquette H.G.*, J. Membrane Sci, **84** (1993) 107
24. *Barenholz Y.* and *Lasic D.D.*, Handbook of Nonmedical applications of liposomes, CRC Press, Boca Raton, 1996.
25. *Gregoriadis G.*, Liposomes Technology, CRC Press, Boca Raton, 1993.
26. *Lasic D.D.*, Liposomes: from physics to applications, Elsevier, Amsterdam, 1993.
27. *Ozer A.Y., Farivar M.* and *Hincal A.A.*, Eur. J. Pharm. Biopharm. **39** (1993) 97
28. *Oberholzer T., Wick R., Luisi P.L.* and *Biebricher C.K.*, Biochem. Biophys. Res. Comm. **207** (1995) 250
29. *Oberholzer T., Albrizio M.* and *Luisi P.L.*, Chem Biol. **2** (1995) 677
30. *Walde P.*, Current Opinion in Colloid & Interface Science, **1** (1996) 638
31. *Lasic D.D.*, J. Colloid Interface Sci. **140** (1990) 302
32. *Israelachvili J.N., Mitchell D.J.* and *Ninham B.W.*, J. Chem. Soc. Faraday Trans. 2, **72** (1976) 15225
33. *Israelachvili J.N., Marcelja S.* and *Horn R.G.*, Q. Rev. Biophys. **13** (1980) 121
34. *Israelachvili J.N.*, Intermolecular and surface force, Academic Press, Londra, 1989.
35. *Epand R.M.*, Biochem. Cell Biology, **68** (1990) 17
36. *Epand R.M.* Chem. Phys. Lipids, **81** (1996) 101
37. *Cevc G.*, Phospholipid bilayers. Physical principles and models, John Wiley & Sons, N.Y. 1987.
38. *Biltonen R.L.* and *Lichtenberg D.*, Chemistry and Physics of Lipids, **64** (1993) 129
39. *Lee A.G.*, Biochim. Biophys. Acta, **472** (1977) 237
40. *Mouritsen O.G.*, Chem. Phys. Lipids, **57** (1991) 179
41. *Mabrey-Gaud S.*, Differential scanning calorimetry of liposomes. In Knight, C.G.(Ed.) Liposomes: from physical structure to therapeutics applications, pp. 105–138, Elsevier, Amsterdam, 1981
42. *Fendler J.H.*, Membrane mimetic chemistry, Wiley-Interscience, N.Y. 1982, pp. 113–183
43. *Hinz H.J.* and *Sturtevant J.M.*, J. Biological Chemistry, **247** (1972) 6071
44. *Freire E.* and *Biltonen R.L.*, Biochim. Biophys. Acta, **514** (1978) 54
45. *Biltonen R.L.* J. Chem. Thermodynamics, **22** (1990) 1
46. *Lang J., Vigo-Pelfrey, C.* and *Martin F.*, Chem. Phys. Lipids, **53** (1990) 91
47. *Lee A.G.*, Biochim. Biophys. Acta, **472** (1977) 237
48. *Nema S.* and *Avis K.E.*, PDA J. Pharm. Sci. technol, **50** (1996) 213
49. *Lieb W.R.* and *Stein W.D.*, Nature, **224** (1969) 240
50. *Walter A.* and *Gutknecht J.*, J Membrane Biol, **90** (1986) 207
51. *Stein W.D.*, Transport and diffusion across cell membranes, Academic Press, N.Y. 1986
52. *Deamer D.W.* and *Bramhall J.*, Chem. Phys. Lipids, **40** (1986) 167
53. *Deamer D.W.* and *Nichols J.W.*, Proc. Natl. Acad. Sci. **77** (1980) 165
54. *Miller J.R.*, Bioelctrochem. & Bioenergetics, **19** (1988) 359
55. *Deamer D.W.* and *Volkov A.G.*, Proton permeability of lipid bilayers. In Disalvo E.A. and Simon S.A. (Ed.) Permeability and stability of lipid bilayer, CRC Press, Boca Raton, 1997.
56. *Chakrabarti A.C.*, Amino Acids, **6** (1994) 213
57. *Blok M.C., Van der Neut-kok E.C.M., Van Deenen L.L.M.*, and *De Gier, J.*, Biochim. Biophys. Acta, **406** (1975) 187

58. *Chakrabarti A.C., Breaker R.R., Joyce G.F.* and *Deamer D.W.*, J Mol Evol, **39** (1994) 555

59. *Pugh E.L., Bittman R., Fugler L.* and *Kates M.*, Chem. Phys. Lipids, **50** (1989) 43

60. *Watanabe S.* and *Seno M.*, J Membrane Sci, **44** (1989) 253

61. *Papahadjopoulos D., Jacobson K., Nir, S.* and *Isac, T.*, Biochim. Biophys. Acta, **311** (1973) 330

62. *Mouritsen O.G., Jorgensen K.* and *Honger T.*, Permeability of lipid bilayers near phase transition. In Disalvo E.A. and Simon S.A. (Ed.) Permeability and stability of lipid bialyer, CRC Press, Boca Raton, 1997

63. *Cruzeiro-Hansson L.* and *Mouritsen O.G.*, Biochim. Biophys. Acta, **944** (1988) 63

64. *Sada E., Katoh S., Terashima M., Yamana S., Ueyama N.* and *Nagaya, M.*, Biotechnol. Bioeng. **28** (1986) 1

65. *Sada E., Katoh S., Terashima M., Kawahara H.* and *Katoh M.* J. Pharm. Sci. **79** (1990) 232

66. *Bresselers G.J.M., Goderis H.L.* and *Tobbak P.P.*, Biochim. Biophys. Acta, **772** (1984) 374

67. *Francis S.E., Lyle I.G.* and *Jones M.N.*, Biochim. Biophys. Acta, **1062** (1991) 117

68. *Clerc S.G.* and *Thompson T.E.*, Biophys. J, **68** (1995) 2333

69. *Corvera E., Mouritsen O.G., Singer M.A.* and *Zasadzinski J.A.* Biochim. Biophys. Acta, **1107** (1992) 261

70. *Inoue T., Yamahata T.* and *Shimozawa R.* J. Colloid Interface Sci. **149** (1991) 345

71. *Almog S., Litman B.J., Wimley W., Cohen J., Wachtel E.J., Barenholz, Y., Ben-Shaul, A.* and *Lichtenberg, D.*, Biochemistry, **29** (1990) 4582

72. *Lasch J.*, Biochim. Biophys. Acta, **1241** (1995) 269

73. *Inoue T.*, Interaction of surfactants with phospholipid vesicles. In Rosoff, M.(Ed.) Vesicles, pp. 151–195, Marcell Dekker, N.Y. 1996

74. *Schubert R., Beyer K., Wolburg H.* and *Schmidt K.H.*, Biochemistry, **25** (1986) 5263

75. *Paternostre M.T., Roux M.* and *Rigaud J.L.*, Biochemistry, **27** (1988) 2668

76. *Lasch J., Hoffman J., Omelyanenko W.G., Klibanov A.A., Torchilin, V.P., Binder H.* and *Gawrisch K.*, Biochim. Biophys. Acta, **1022** (1990) 171

77. *Massari S., Folena E., Ambrosin V., Schiavo G.* and *Colonna R.* Biochim. Biophys. Acta, **1067** (1991) 131

78. *Yatvin M.B., Tegmo-Larsson I.M.* and *Dennis W.H.* Methods Enzymol. **149** (1987) 77

79. *Huang L., Connor J.* and *Wang C.Y.* Methods Enzymol. **149** (1987) 88

80. *Duzgunes N., Straubinger R.M., Baldwin P.A.* and *Papahadjopoulos D.*, pH-sensitive liposomes. Introduction of foreign substances into cells. In Wilschut J., and Hoechstra D., (Ed.) pp. 713–725, Marcel Dekker, 1990.

81. *Budker V., Gurevich V., Hagstorm J.E., Bortzov F.* and *Wolff J.A.*, Nature Biotechnology, **14** (1996) 760

82. *White S.H.* and *Wimley W.C.*, Current Opinion in Struct. Biology, **4** (1994) 79

83. *Papahadjopoulos D., Moscarello, M., Eylar E.H.* and *Isac, T.*, Biochim. Biophys. Acta, **401** (1975) 317

84. *Lo Y.L.* and *Rahman Y.E.*, J. Pharm. Sci **84** (1995) 805

85. *Dini L., Di Giulio, A., Pavan A., Ravagnan G.* and *Mossa, G.* Biochim. Biophys. Acta, **1062** (1991) 108

86. *Bergers J.J., Vingerhoeds M.H., van Bloois L., Herron J.N., Janssen L.H., Fischer M.J.* and *Crommelin D.J.* Biochemistry, **32** (1993) 4641

87. *Cho Y., Ko T.S., Cha S.H.* and *Sok D.E.*, Neurochem Res, **20** (1995) 681

88. *Annesini M.C., Di Marzio L., Finazzi-Agrò, A., Serafino A.L.* and *Mossa G.*, Biochem. Molec. Biol. Intern. **32** (1994) 87

89. *Sada E., Katoh S., Terashima M., Kheirolomoom A.* and *Sawai H.* J Chem Eng Japan, **20** (1987) 531

90. *Kheirolomoom A., Katoh S., Sada E.* and *Yoshida K.* Biotechnol. Bioeng. **37** (1991) 809

91. *Shinitzky M.* Membrane fluidity and cellular functions. In Shinitzky M. (Ed.) Physiology of membrane fluidity, pp. 1–52, CRC Press, Boca Raton, 1984

92. *Cioci F.* and *Lavecchia R.*, Enzyme-loaded liposomes as microreactors. In Barenholz Y. and Lasic D.D. (Ed.) Handbook of Nonmedical applications of liposomes, Vol. III, pp. 287–315, CRC Press, Boca Raton, 1996

93. *Ambartsumian T.G., Adamian S.Y., Petrosian L.S.* and *Simonian A.L.*, Biol. Mem. **5** (1992) 1878

94. *Annesini M.C., Braguglia C.M., Memoli A., Palermiti L.* and *Di Sario S.*, Biotechnol. Bioeng. **55** (1997) 261

95. *Savini S.G.*, Dept. Chemical Engineering, University of Roma "La Sapienza", Thesis, 1996

96. *Braguglia C.M.*, Dept. Chemical Engineering, University of Roma "La Sapienza", Ph. D. Thesis, 1996

97. *Chang T.M.*, Biomater Artif Cells Immobilization Biotechnol, **20** (1992) 1121

98. *Chang T.M.*, Artif Organs, **16** (1992) 8

99. *Chang T.M., Bourget L.* and *Lister C.L.*, J. Artif Cells Blood Substit Immobil Biotechnol, **23** (1995) 1

100. *Hoekstra D., Yaron A., Carmel A.* and *Scherpof, G.*, FEBS Letters, **106** (1979) 176

101. *Bentz J.* and *Nir S.J.* Chem. Soc. Faraday Trans. **77** (1981) 1249

102. *Bentz J., Nir S.* and *Wilschut J.*, Colloids and Surfaces, **6** (1983) 333

103. *Annesini M.C., Di Marzio L., Trazza C.* and *Mossa G.*, J. Liposome Research, **3** (1993) 687

104. *Kirby C.*, Chemistry in Britain, (1990) 847

105. *Koide K.* and *Karel M.*, Int J Food Sci & Technol, **22** (1987) 703

106. *Chawan C.B., Penmetsa P.K., Veeramachaneni R.* and *Rao D.R.*, J Food Biochem, **16** (1993) 349

107. *Rao D.R., Chawan C.B.* and *Veeramachaneni R.*, J Food Biochem, **18** (1995) 239

108. *Torchilin V.P.*, Adv. Drug Delivery Rev. **1** (1987) 41

109. *Rotilio G.*, Enzymes and enzyme cofactors as therapeutics agents: a challenge for modern biotechnology. In Alberghina L., Frontali L., and Sensi, P. (Ed.) Proc. 6th European Congress on Biotechnology, Elsevier 1994, 763–769

110. *Marcocci L., Carri M.T., Battistoni A.* and *Rotilio G.*, Bioengineering of superoxide dismutase and related enzymes: basic and clinical aspects. In Verna R., Blumenthal R., and Frati L. (Ed.) pp. 11–28, Raven Press, New York, 1989.

111. *Baker R.R., Czopf L., Jilling T., Freeman B.A., Kirk K.L.* and *Matalon S.*, Am J Physiol, **263** (1992) 1585

112. *Storm G., Koppenhagen F., Heeremans A., Vingerhoeds M., Woodle M.C.* and *Crommelin D.J.A.*, J Controlled Release, **36** (1995) 19

113. *Rosenberg M.F., Jones M.N.* and *Pankaj M.V.*, Biochim. Biophys. Acta, **1115** (1991) 157

114. *Taylor M.A., Jones M.N., Vadgama P.M.* and *Higson S.P.J.* Biosensors & Bioelectronics, **20** (1995) 251

115. *Gennis R.B.* Biomembranes. Molecular structure and function, Springer-Verlag, New York, 1989